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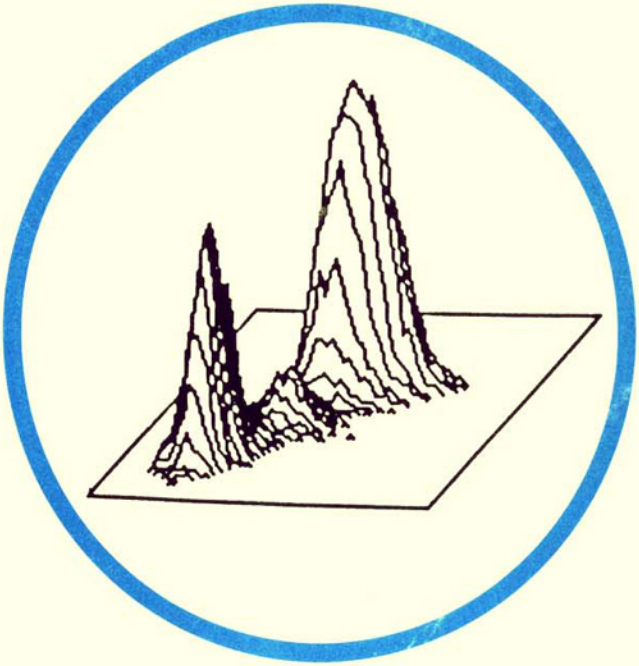
P.T. SHARPE



Methods of cell separation

LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

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R.H. BURDON—*Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow*
P.H. van KNIPPENBERG—*Department of Biochemistry, University of Leiden, Leiden*

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METHODS OF CELL SEPARATION

PAUL T SHARPE
Department of Cell and Structural Biology,
University of Manchester,
Manchester M13 9PL, U.K.

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Abbreviations

TCA	trichloroacetic acid
PBS	phosphate buffered saline
FCS	foetal calf serum
g	gravity
BSA	bovine serum albumin
TLCCD	thin-layer countercurrent distribution
PEG	poly(ethyleneglycol)
HBSS	Hanks buffered saline solution
RBC	red blood cells
MN	mononuclear cells
PMN	polymorphonuclear cells
Plt	platelets
PEO	polyethylene oxide
EDTA	ethylenediaminetetracetic acid
PEG-pal	*poly(ethyleneglycol) covalently coupled to palmitic acid*
SmIg	surface immunoglobulin
ANAE	α-naphthyl acetate esterase
NK	natural killer
TRITC	tetramethylrhodamine thiocyanate
FITC	fluorescene isotiocyanate
Con A	concanavalin A
Tris	tris(hydroxymethyl)adminomethane
HSA	human serum albumin
CNBr	cyanogen bromide
DMEM	Dulbecco's modified Eagle medium
RIA	radio-immunoassay
CRBC	chicken red blood cells
SRBC	sheep red blood cells

TH-HSA-NaN3	tris buffered Hanks salt solution containing 0.2% human serum albumin and 0.02% HaN3
TSH	thyroid stimulating hormone
TRH	thyroid releasing hormone
DNP	2,4-dinitrophenyl
CHO	Chinese hamster ovary
i.d.	inside diameter
o.d.	outside diameter
SPDP	succinimidyl-propiono-dipthiopyridine
DTT	dithiothreitol
rpm	revolutions per minute
BrdU	bromodeoxyuridine

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CHAPTER 1

Introduction

Defining 'cell separation' is more difficult than it might appear. Whereas separation of soluble molecules can be defined as the 'purification of a particular molecule to homogeneity from a mixture of molecules', it is difficult to view cells in terms of 'homogeneous populations'. Can a population of more than one cell ever be considered 'homogeneous?' I think the answer is probably no, and so cell populations can never be considered 'pure' as can perhaps a separated protein. The degree of purity obtained is in many respects a function of the assay used to identify the cells of interest. For example, T-lymphocytes can be separated from whole blood with a high degree of 'purity', perhaps 90%. The T-lymphocyte population obtained can be considered therefore to be 90% pure. However the T-lymphocyte population certainly consists of many different T cells subsets and is not therefore, strickly speaking a pure population of cells.

I state the above point merely to emphasise the unique nature of cell separation which cannot be paralleled with other separation techniques. There is no definitive technique that can produce a pure population of a particular cell type as perhaps techniques such as FPLC can with proteins. Cell separation must, I feel, be viewed in the most general of terms where, 'a population of cells is depleted of cells which share particular characteristics'. This therefore encompasses all levels of cell separation from the separation of red cells

from lymphocytes to the separation of T cell subsets.

There are three terms which need clarifying in their application to cell separation, these are 'recovery,' 'viability' and 'purity'. For reasons discussed above I feel purity is not a particularly apt term for cell separation. *Purity* refers to the 'percentage of cells in the separated population that are detected as having certain desired characteristics'. For example the percentage of haemoglobin containing cells in a population of red cells separated from lymphocytes.

Recovery can be defined in two different ways. Mostly, recovery refers to the total number of cells recovered at the end of the separation (number of separated cells) as a percentage of the total number of cells before separation. This measurement is important in assessing whether cells have been lost during the procedure. More important, but much more rarely measured, is the recovery of the particular cells of interest from the total number of these cells in the original, unseparated population. This data is important because it gives an estimate of the number of the cells of *interest* that have been lost. This is not only important from the point of optimising a particular separation but also because if losses are observed, it is possible that the lost cells may be different in some way to the recovered cells.

The most satisfactory definition of *viability* is the 'proportion of separated cells that remain unchanged throughout the separation process'. Unfortunately this definition can rarely be applied since unless several specific functional assays can be carried out on separated cells, it is difficult to determine whether cells have been affected by the separation process. Viability is most usually expressed as the proportion of cells that remain 'alive' or 'viable'. This viability is commonly assayed by dye exclusion techniques where cells are incubated with a dilute solution of dye which only enters dead (nonviable) cells (Chapter 2). It is important that the viability of cells in all separated fractions be measured.

Maintenance of cell viability is the most important feature of any cell separation procedure. There is little point in separating a cell population with a high degree of 'purity' if the majority of the cells are dead or have changed in perhaps less drastic ways. Viability is

best maintained when cells are separated in conditions that are closest to those from where the cells were isolated. This usually means either some form of growth medium or a balanced solution of salts, possibly including other constituents such as serum etc. The separation medium should not contain any constituents that are likely to change the cells. For all the methods described here suitable media are used that minimise the likelihood of changing cell characteristics.

Changes to cells can also be caused by physical stresses such as prolonged exposure to high *g* forces (centrifugation) and the passage through solid matrices (affinity chromatography). Whatever the conditions in which isolated cells are maintained while being separated, changes will occur with time and it is thus imperative to keep the separation time to a minimum.

All the above points should be considered when selecting a separation procedure but it should be stressed that all cells are different and it is therefore impossible to make general sweeping statements about each method. This is particularly examplified by affinity chromatography separation of cells (Chapter 8). This method has proved very successful for separation of various blood cell populations but few non-blood cell populations have been successfully separated with this procedure. The basic problem is non-specific absorbtion and entrapment of cells in the chromatography matrix.

Although aspects such as recovery and viability are important when considering a separation method there are two further important decisions that have to be made; the mode of separation, i.e. on what basis are the cells to be separated, and the cost. All cell separation methods can be considered to consist of two parts, distinguishing cells which are different, and physically separating these cells of interest from the rest. Excluding separation on the basis of DNA content, there are three criteria that can be used to distinguish cells from each other. These are size and/or density, surface properties and the presence or absence of specific surface molecules. Distinguishing cells which differ in size or density and their separation are part of the same process, which is based on the rate of

sedimentation. For separation based on specific surface molecules (affinity separation), cells that have different surface characteristics can easily be distinguished, for example by binding of surface specific antibodies. The differences between the variety of techniques available is how the different cells e.g. the cells with bound surface antibody, are separated from the rest.

The major decision therefore is to determine the criterion upon which the cells are to be separated. The selection of a separation procedure must initially be based on the suspected difference in cell character, i.e. are the required cells of a different size or density from the rest? Do the required cells have different surface properties from the rest, or is a specific marker molecule available such as an antibody which can distinguish the required cells? If none of these characteristics are known then a decision must be made between methods that will separate on size/density differences and methods that will separate on the basis on non-specific surface differences i.e. differences in unknown surface molecules. It is generally accepted that differences in cell function are usually reflected by differences in surface properties and much less usually by differences in size and density, especially density. There is however no simple, inexpensive method for separating cells with unspecified differences in surface properties. Of the two methods available, phase partition and electrophoresis, phase partition is the cheapest and most sensitive but is more complex and has many more experimental variables than electrophoresis. On the other hand, electrophoresis of cells requires large, and expensive apparatus.

There are three major methods for separating cells on the basis of size or density: density gradient centrifugation, unit gravity sedimentation and centrifugal elutriation. Density gradient centrifugation is simple and cheap and for these reasons is the most commonly used of all separation methods. Historically, the biggest problem with this method has been the compatibility of the gradient medium with cells. With the introduction of Percoll and more recently Nycodenz, problems of compatibility have been drastically reduced. Density gradient centrifugation is however a rather insensitive separation

method and this fact has led to the development of the more sensitive methods such as unit gravity sedimentation, and more especially, centrifugal elutriation. These two methods are more sensitive than density gradient centrifugation but as with the majority of separation methods, increasing sensitivity is unfortunately accompanied by increasing complexity of operation and cost.

The degree of separation (purity) required clearly depends on the limitations of the studies to be carried out on the separated cells. Often an enrichment of a particular cell type is adequate, but more often a high degree of cell purity is required. Balanced-against the requirement of purity is the percentage recovery and viability of the separated cells. Sacrifices can sometimes be made to obtain a cell population most suitable for the particular study in question e.g. if a large amount of material is available, cell recovery may be sacrificed in order to produce a highly pure, viable cell population.

I have attempted in this volume to describe the cell separation methods currently available from a practical point of view. The very nature of cells means that widely different separation conditions are often used for different cells with the same separation method, and it is therefore impossible to present an experimental protocol that is applicable to all cells. Where possible I have detailed general experimental protocols but these are not intended as methods to be followed directly for all cells but more to illustrate what is involved in each method. Since I could not provide experimental conditions for separating cells from different sources I have chosen selected examples for each method and provided the detailed experimental conditions that have been used to separate these cells. For all methods one example I have chosen is the fractionation of lymphocytes because most people have at the very least a basic knowledge of lymphocyte cell types and fortuitously, lymphocytes in some form have been fractionated by all the cell separation methods. In some cases the methods have largely been developed around the separation of lymphocytes. Where applicable I have also included details for separating other cell types but for the most part these are restricted to mammalian cells. I would strongly advice anyone wishing to in-

vestigate the separation of a particular cell population to check the literature of references to published work and also to consult the General References listed on page 269.

The easiest way of obtaining current references is to contact directly the manufacturers of the particular separation equipment being used since these will usually provide extensive reference lists. For example Beckman produce a regularly updated list of cell separations conducted via centrifugal elutriation.

In producing this book I have had to rely heavily on contributions from many people and I would like to express my thanks to all concerned. I am especially indebted to the colleagues listed below for providing me with seemingly endless information and helpful discussions.

I would also like to acknowledge the many people who were very generous in providing reprints etc. and allowing me to reproduce published figures, protocols and details of their work. Thank you all.

Chapter 3 – Centrifugation – Jim Smillie, Nycomed.
Chapter 8 – Affinity chromatography – Dr Pauline Dobson. Department of Human Metabolism and Clinical Biochemistry Medical School, Hallamshire Hospital, Sheffield.
Chapter 9 – Flow Sorting – John Lawry. Department of Virology, Medical School, Hallamshire Hospital, Sheffield.

CHAPTER 2

Methods of cell counting and assaying cell viability

Whatever method is being used to separate cells, the number of cells recovered in each fraction needs to be counted, and their viability assessed. The total number of cells used and the total recovered after separation will also usually need to be counted. An essential prerequisite to any cell separation procedure therefore, is a method for counting cells. There are a variety of direct and indirect methods for counting cells, several of which are outlined below.

2.1. Direct counting methods

2.1.1. Haemocytometer

The haemocytometer, or more accurately the 'cell counting chamber' is perhaps the simplest method of counting cells. The apparatus consists of a specially made microscope slide which is thicker than normal with a precisely ground surface that when covered with a precision ground coverslip contains a precise volume of liquid (Fig. 2.1). Scribed onto the surface are a series of lines that form a grid as shown in Fig. 2.2. The grid (Neubauer type) usually consists of 9 large squares each 1 × 1 mm holding a volume of 0.1 mm^3. Each large square is subdivided into 16 smaller squares. The 16 squares of the large centre square are further divided into 16 smaller squares.

With the coverslip in place, Newtons rings are visible and cells in suspension are drawn under the coverslip by capillary action. Contained within each large square of the grid is 0.1 mm^3 of cell suspension. By viewing the slide under a standard light microscope both the lines and the cells within the grid can be seen. The number of squares in which cells are counted depends upon the accuracy required and the number of cells. If few cells are available (eg. 50 – 200 total under all squares) cells in all squares should be counted. The total cells/ml is then calculated by multiplying $10^4/9$. If the cell suspension is at a higher density eg. around 10^6 cells/ml, it is not usually necessary to count all 9 large squares since there will be 100 cells per large square. An accurate count can be obtained by counting cells in the four corner squares. The total cells/ml is then calculated by multiplying by $10^4/4$. If large numbers of different cell suspensions require counting the quickest method is to count the cells in the central square and multiply by 10^4. There are two identical counting chambers on opposite sides of most slides, both of which can be used with the same

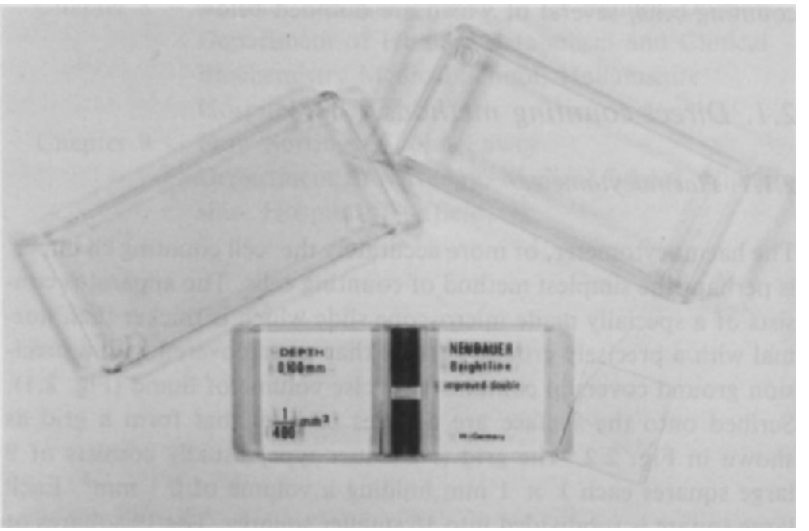


Fig. 2.1. Haemocytometer slide and cover slips. (Courtesy of Shermond Scientific).

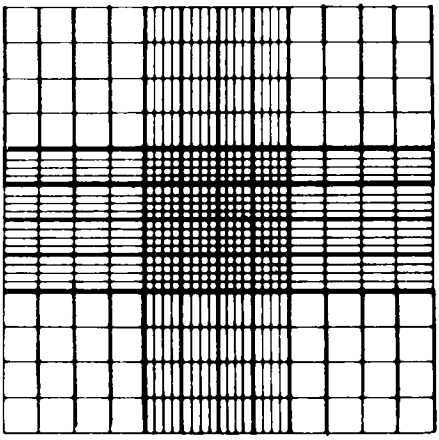


Fig. 2.2. Haemocytometer grid. Improved Neubauer type. (Courtesy of Shermond Scientific).

cell sample to obtain an accurate count or alternatively two different samples can be counted. Cells touching the outer lines of the total area to be counted should only be counted on two of the four lines.

Because of the errors involved in counting small numbers of cells an accurate figure can only be obtained when 50 – 200 cells are counted per large single square. 200 cells/large square is about the maximum number than can be easily counted. Thus when counting cells on a haemocytometer, dilution of the original suspension may have to be carried out in order to obtain a cell density of about $5 \times 10^5 - 2 \times 10^6$ cells/ml. The number of cells counted can then be multiplied by the appropriate dilution factor. The average error in counting is around 10%.

The advantages of haemocytometer counting are that it is simple, direct and cheap. The main disadvantages are that it is laborious and time consuming and since cell suspensions often have to be diluted, errors in diluting will be multiplied in the final count.

2.1.2. Electronic cell counting

The best known of the electronic cell counters is the Coulter counter, the latest model (ZM) of which is shown in Fig. 2.3. The principle of electronic counting is that cells suspended in an electrolyte are drawn through a small aperture (100 μm diameter), through which an electric current is flowing, by means of a vacuum (Fig. 2.4). Each cell displaces electrolyte in the aperture and produces a change in electrical impedance which is detected as a voltage pulse. The height of each pulse is equal to the volume of the cell producing it. The volume of cell suspension drawn into the aperture and counted is accurately controlled to $\pm$ 0.5% in 0.5 ml by a series of electrical contacts on a column of mercury operated on a syphon system. The cell generated pulses are amplified, measured, counted and converted into figures of cell number and size. Several thousand cells per second can be counted and sized.

Upper and lower limits of cell diameter (0.4 – 800 μm) to be counted can be set such that only cells of a certain size are counted.

Fig. 2.3. Coulter Counter Model ZM with Channelyzer. Left, counting aperture; centre, module for setting cell size range and displaying cell count; right, Channelyzer. (Courtesy of Coulter Electronics).

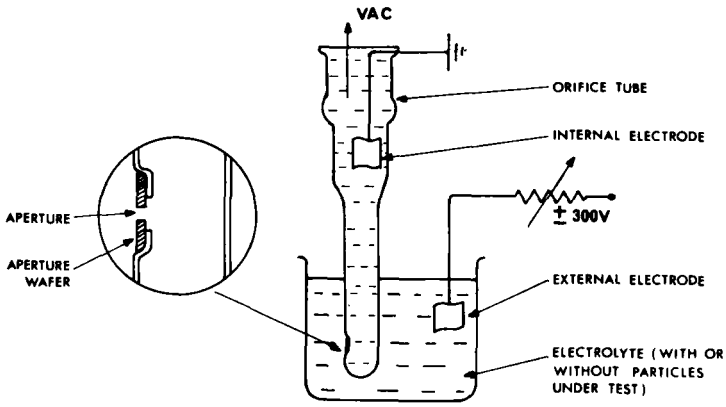


Fig. 2.4. Detail of Coulter Counter aperture arrangement. Cells are drawn in through the aperture via a vacuum and pass between the internal and external electrodes. (Courtesy of Coulter Electronics.)

On the Coulter Model ZM, after calibration with latex particles of defined size, the counter is programmed such that size limits can easily be set and displayed.

Cells are suspended in an electrolyte against which the counter has been calibrated, usually isotonic saline (0.9% NaCl). The minimum volume in which the cells can be suspended is about 10 ml, which when placed in a small vial, easily covers the aperture and allows duplicate counts to be taken. The volume of cell suspension counted can be set at 200, 500 or 2000 μl. The number of cells passing through the aperture in this volume is displayed. A small screen focussed on the aperture allows any blockage to be quickly seen which can then be removed by brushing the aperture.

Small numbers of cells can be counted if necessary. Suspensions of 2000 cells/ml will produce counts of 1000 when 500 μl is counted. However the errors when counting such low numbers are greater than when counting above 10000 counts/500 μl although even with low cell numbers the errors are generally less than 5%. To obtain a true count the cells must be counted in the same electrolyte solution as the counter was calibrated. It is not always convienient to resus-

pend cells after separation and often an aliquot from a fraction is taken, diluted in saline and counted. Any constituents of the cell suspension medium other than saline will affect the background count obtained which may need subtracting from the total count.

Electronic counters are the best method available for cell counting. Large numbers of cell samples containing relatively few cells can be counted accurately and quickly.

2.1.3. Electronic cell sizing

Electronic cell counters can also be used to obtain a size distribution of the cells counted. This is achieved by analysing the height of the pulses produced as cells pass through the aperture. Coulter produce a 'Channelyzer' which is a unit that attaches to the counter and analyses the pulse height. The Channelizer can be coupled to a small computer which converts the pulse height analysis into values of cell size and volume. Coulter also market special software (Accucomp) which enables various different parameters to be measured.

As a cheaper alternative to the Channelizer, it is possible to connect the output of the counter directly to a small computer and with suitable software the peak height can be analysed and converted into values of cell size.

2.2. Indirect cell counting methods

Indirect methods are those where the actual number of cells are not counted, rather an assay is used that is proportional to cell number. All these methods suffer from the same drawback in that the assay used may not show equal proportionality with all the different cell types separated. For example, if total cell protein is used, the number of cells in the suspension to be separated will be proportional to total protein content but when separated some populations may contain more protein than others.

2.2.1. Turbidity

Measurement of turbidity is a simple, albeit rather insensitive method of estimating cell number. A suspension of cells will apparently absorb light due to scattering and the amount absorbed can be used as a measure of cell number. The wavelengths at which absorbance is measured is usually between 550 – 610 nm. Serial dilutions of the cell suspension containing a known number of cells are made and the absorbance read to produce a standard curve. The absorbance can then be used as a measure of the number of cells in a suspension. Generally, a linear relationship is obtained between absorbance and cell number although it is often only linear for a narrow range of cell number. Hence, dilutions have to be made to ensure the linear range is used. Low cell numbers cannot be measured, for example, an increase of 1 absorbance unit is obtained per 10^9 cells/ml suspension of *E. coli.* in a 1 cm cuvette at 610 nm. The amount of light absorbed is dependent on cell size. Smaller cells absorb proportionally less than larger cells. Hence, if the separated cell fractions have different sizes absorbance will not be proportional to cell number in each case.

2.2.2. Protein content

The amount of protein per cell can be used as a measure of cell number. If serial dilutions of a cell suspension containing a known number of cells are made and the total protein content of each measured, this can be used as a measure of cell number. Within the constraints of the particular assay used a linear relationship between cell number and protein content is obtained and it is simply a matter of ensuring the cell suspension assayed is always within the linear range of the assay.

There are numerous assays of total cell protein that can be used. For large numbers of cell fractions the simplest and quickest method is to measure absorbance at 280 nm of a lysed aliquot of cells. Lysis may be produced by sonication or more effectively by addition of

detergent. The majority of detergents absorb strongly at 280 nm and will produce a high background. One detergent that does not absorb at 280 nm is Empigen BB which is available from Albright and Wilson Ltd. A final concentration of 0.1% Empigen is sufficient to lyse most cells.

Care should be taken when using any protein assay on separated cell suspensions since components of the separation medium are likely to interfere with the assay. Removal of any such contaminants may require extensive washing. Similarily, when serum or other proteins are included in the separation medium these may produce such a high background that the cell protein cannot be read. Again extensive washing may be necessary to remove contaminating proteins.

2.2.3. Assays involving radioactive labels

A very sensitive method of measuring cell number is to label cells before separation with a radioactive chemical which cells will incorporate. By using labels with high specific activity, cell numbers as low as 100 cells can be counted. The choice of labelled compound is important to ensure that all cells incorporate the same amount.

DNA can be labelled with ^{14}C or ^{3}H thymidine but this is not recommended since in order to ensure that all cells are equally labelled they must be incubated with the label for the time exactly corresponding to a single growth cycle otherwise only the proportion of cells that have undergone DNA synthesis will be labelled.

Protein can be labelled with radiolabelled amino acids if all cells can be assumed to be synthesising protein at the same rate. This method has been successfully used to count small numbers of fractionated human bone cells (Sharpe et al. 1985). An aliquot of fractionated cells separated from a labelled cell population is removed and the protein precipitated with cold 10% (w/v) TCA by centrifugation at 1000 × g for 15 min. To ensure precipitation of all protein, 1% albumin can be added to act as a carrier. The precipitate is washed with further TCA, solubilised in 0.1 M NaOH and scintillation fluid added for radiolabel counting. The limitations of this method

are that all cells need to be actively synthesising protein and problems occur when assessing radioactivity in alkaline extracts due to chemiluminesence.

Perhaps the best method of radiolabel counting is to use chromate labelling (^{51}Cr). Walter and Krob (1983) have described a method of labelling red blood cells with chromate to enable analysis of separated subpopulations. ^{51}Cr (sodium chromate) is diluted in buffer such that 10 μCi are contained in 100 μl. 1 – 2 ml of red cells in plasma are incubated at room temperature with 10 – 20 μCi ^{51}Cr/ml for 30 min. A few microlitres of ascorbate solution at 500 mg/ml are added to stop the reaction and the labelled cells washed three times with buffer. After separation the cells are lysed and the radioactivity counted on a liquid scintillation counter. Not all the cells in the suspension need be labelled, a small aliquot of cells from the whole population can be removed, labelled, remixed with the excess of unlabelled cells and thereby used as markers for the unlabelled cells. In this way only a small proportion of the separated cells are labelled. The reaction conditions for labelling may need to be varied depending on the cell type used.

2.3. Cell viability

2.3.1. Dye exclusion

The most commonly used method of determining cell viability is dye exclusion. An aqueous solution of dye is added to a small aliquot of cells and after a few minutes the cells are viewed under a light microscope. Dead cells are permeable to the dye and thus become coloured, whereas viable cells do not take up the dye. By counting the number of dyed and non-dyed cells, the percentage of viable cells present can be calculated.

Numerous dyes have been used to stain non-viable cells but the two most used are trypan blue and erythrosine B. Stock solutions of these dyes are made up in isotonic buffer containing 0.05% (w/v) methyl

p-hydroxybenzoate as a preservative at about 4 mg/ml. These solutions can be stored indefinitely at room temperature. Addition of an equal volume of dye solution (final concentration 0.2 mg/ml) to the cell suspension for 5 min is sufficient to stain non-viable cells.

The degree to which cells take up the dye is pH dependent. Maximum uptake of trypan blue occurs at pH 7.5 and erythrosine B at 7.0. A major difference between these two dyes is that trypan blue has a greater affinity for protein in solution than for protein in non-viable cells whereas erythrosine B has a greater affinity for protein in non-viable cells than in solution. This fact should be considered when assaying viability of cells in suspensions containing protein.

2.3.2. Clonogenic Assay

This method can only be used for cells that will grow in culture on a solid surface and is most commonly used for microorganisms. Basically cells in each fraction after separation are counted and diluted such that around 100 cells in suspension can be plated out onto some form of solid medium. After a few days incubation at a suitable temperature small colonies of cells become visible, each colony being formed from each viable cell. Viability is expressed as the number of cells that formed colonies as a percentage of the total plated out.

A similar method can sometimes be employed to assay the viability of cells separated after being grown in tissue culture. Cells should be resuspended in suitable culture medium after separation and around 50 – 100 cells plated into sterile tissue culture-grade dishes. After a suitable period of incubation small colonies should be visible. Only viable cells will form colonies although factors other than viability may affect the number of colonies produced. Inclusion of 0.17% agar in the culture medium will prevent intermixing of colonies. Certain cells will form colonies when not attached to plastic. These can be grown suspended in soft agar. Details of this method can be found in Volume 8 of this series by R.L.P. Adams entitled 'Cell culture for biochemists' (1980).

The problem with plating methods is that the whole separation process must be carried out under sterile conditions.

2.3.3. Functional assays

Specific methods of measuring cell viability can often be carried out with cells that have an easily measurable function. For example the viability of separated human monocytes can be assayed by trypan blue staining or alternatively the phagocytic ability of the monocytes can be assayed as a measure of viability. This is a relatively simple assay to conduct and will give a measure of the functional viability of the monocytes (Walter et al., 1980). Care should be taken however where possible subpopulations of cells are separated that might be of the same phenotype but functionally different.

Centrifugation

3.1. Introduction

Centrifugation is the general name given to separation methods which involve rotation about a fixed axis to produce a centrifugal (g) force. This centrifugal force forces cells down through a liquid medium and the rate of falling or sedimentation varies principally according to cell density and size. Thus cells of different density or size sediment at different rates and at some point will be physically separate from each other.

The sedimentation of cells can be described by Stokes equation for the settling of a sphere in a gravitational field.

$$v = \frac{d^2 (\varrho p - \varrho L) \times g}{18\, n}$$

where v = sedimentation rate or velocity of the sphere; d = diameter of the sphere; ϱp = particle density; ϱL = liquid density; n = viscosity of liquid medium; g = gravitational force.

From Stokes equation it can be seen that:

1. The rate of particle sedimentation is proportional to the particle size.
2. The sedimentation rate is proportional to the difference in density between the particle and the liquid.

3. The sedimentation rate is zero when the particle density is the same as the liquid density.
4. The sedimentation rate decreases as the liquid viscosity increases.
5. The sedimentation rate increases as the gravitational force increases.

The sedimentation rate of any particle in a gradient medium can be expressed as a sedimentation coefficient, S which is expressed in Svedbergs (one Svedberg = 10^{-13} s).

$$S = \frac{1}{w^2 r} \frac{dr}{dt}$$

where, r = distance between the particle and the centre of rotation (cms).

w = the rotor speed (rads/s).

$\frac{dr}{dt}$ = rate of particle movement (cms./s) from Stokes equation.

Values of sedimentation coefficients for cells are of little use because cells are rarely truely spherical and interactions between cell surfaces and the medium can occur producing anomolous sedimentation rates.

There are two basic types of centrifugation for cell separation, differential pelleting and density gradient. In differential pelleting a homogeneous medium is used and since the difference in density between cells and medium is small ($\varrho p - \varrho L$) cells separate principally on the basis of size (d). In density gradient centrifugation, as the name suggests, a heterogeneous medium is used which increases in density down the centrifuge tube. Density gradient centrifugation can be subdivided into two principle types, rate zonal and isopycnic. The main difference between these two is that in isopycnic a high density gradient is used and cells are separated solely on differences in density. In rate zonal a lower density gradient is used and cells are principally separated on size differences.

3.2. Differential pelleting

If particles that have a higher density than water are mixed with water in a tube and left standing, they will be forced by gravity to sediment and will eventually sediment to form a pellet at the bottom of the tube. Particles of different densities or size will sediment at different rates, the densest/largest sedimenting the fastest followed by less dense/smaller. The rate at which all particles sediment can be increased by increasing the gravitational force. This is the basic principle of differential centrifugation or pelleting. Cells are mixed in a homogeneous, low density medium, usually an isotonic buffer or growth medium, and spun at a relatively low speed producing about $1000 \times g$ or less.

Since the liquid medium used is homogeneous with respect to density only two fractions can be obtained, a cell pellet at the bottom of the tube and cells in the supernatant. The number of cells in the pellet for any given g force is dependent on the time of centrifugation. If allowed to spin for long enough all the cells will collect in the pellet. The cell separation obtained is therefore dependent on time. At any one time the distribution of cells between the pellet and the supernatant will be different. The optimum time is that when the cells required are present largely in either the pellet or the supernatant.

Differential centrifugation separates cells principally on the basis of size differences although differences in density can also be involved. In terms of the sedimentation velocity of cells in medium with a density of 1.0 g/ml, size variations can give rise to about a 9 – 16 fold variation in terminal velocity whereas density differences give rise to only about a 2 fold difference (from Stokes equation).

A major problem with differential pelleting is that as shown in Fig. 3.1, the force required to pellet larger cells is also sufficient enough to pellet a proportion of smaller cells. In a single step therefore it is only usually possible to obtain a pure population of the smallest cells since these will remain largely uncontaminated in the supernatant after all the larger cells have pelleted. As shown in Fig. 3.1 a percentage of the smaller cells will also be pelleted and hence the yield of

pure cells in the supernatant is often low. On the other hand, high yields of large cells can be obtained in the pellet but these are contaminated with smaller cells. A purer separation of large cells from the pellet can be obtained by resuspending and recentrifuging them. In this way the purity of larger cells is increased although this is at the expense of the yield, which is reduced.

The main advantages of differential pelleting are that it is rapid, taking perhaps only minutes, the *g* forces are low (and hence bench centrifuges can be used) and the separation medium is simple and cheap.

Differential pelleting is most commonly used for 'harvesting' cells either in the laboratory or on an industrial scale. Cells growing in liquid medium or in the presence of other cells are centrifuged in their growth medium. A pellet of cells is obtained with the majority of contaminants remaining in the supernatant.

An example of the use of differential pelleting is the separation of amoebae of the cellular slime mould *Dictyostelium discoideum* from *E. coli* off which the amoebae feed. Most of the bacteria remain undigested and in order to obtain a pure population of amoebae the contaminating bacteria must be removed. Differential pelleting is the easiest and most effective method of doing this. The growth medium containing amoebae, *E. coli* and the various soluble constituents is centrifuged for 10 min at 1000 × *g* in a bench centrifuge. A pellet

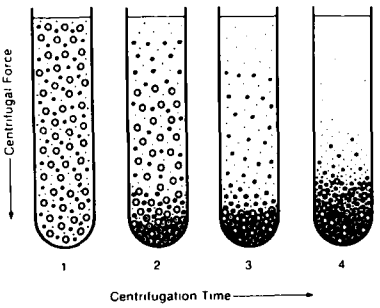


Fig. 3.1. Principle of differential centrifugation. From Griffith (1979). Beckman Instruments – with permission.

is obtained with a cloudy supernatant which contains mostly *E. coli* with a few amoebae. This supernatant is discarded and the pellet containing amoebae and contaminating *E. coli* is resuspended in 50 mM phosphate buffer pH 6.5 and recentrifuged. A pellet is again obtained with a much less cloudy supernatant. The pellet this time contains much fewer contaminating *E. coli*. Further washings can be carried out until a pellet is obtained free of *E. coli*. The yield of amoebae obtained in each pellet is however reduced each washing.

In this example differential pelleting is the ideal method because there are only two cell types present which differ greatly in size (*E. coli* 0.5 μm; *D. discoideum* 10 μm diameter). Although all the other methods described in the following chapters could separate the two cell types, differential pelleting is the method of choice because it is the simplest, quickest and cheapest.

Differential pelleting is often used as a first step in a separation procedure involving several different cell types. For example, rat liver consists of at least four major cell types, parenchymal cells (hepatocytes), Kupffer cells (macrophages), endothelial cells and stellite (fat storing) cells. The first step in the separation of these cell types is to separate the parenchymal cells that make up the bulk of the liver (65%) from the other (non-parenchymal) cells. The average volume of parenchymal cells is around 10 × greater than the average volume of the non-parenchymal cells and can thus be separated by differential pelleting. A suspension of liver cells produced by collagenase digestion is spun at 50 × g for 15s at 4°C. The pellet formed contains the bulk of the parenchymal cells which can be washed to yield a pure suspension of hepatocytes. The supernatant is enriched in non-parenchymal cells but contaminated with some parenchymal cells, particularly dead cells. The majority of contaminating parenchymal cells can be removed by repeated centrifugation at 600 × g for 4 min at 4°C. This produces a supernatant containing a pure suspension of viable non-parenchymal cells. The different cell types that constitute the suspension can be fractionated using other techniques, particularly density gradient centrifugation. The yield of non-parenchymal cells obtained is however low due mainly to the

repeated centrifugation steps. About 25% of the total non-parenchymal cells can be recovered but these are depleted of Kupffer cells which appear to be selectively lost, probably as a result of cosedimentation of cell aggregates with the parenchymal cells (Boyum et al. 1983).

3.3. Density gradient centrifugation

In density gradient centrifugation the homogeneous liquid medium used in differential pelleting is replaced by a solution which increases in density down the tube. Using a gradient of density has several significant advantages over a homogeneous solution all of which result in increased resolution. Because the density of the solution increases down the tube, convection currents that may be set up during centrifugation are minimised. Cells can, if required, be layered on top of the gradient as a tight band allowing cells of the same density/size to sediment together as a tight band. If, as in many cases the density gradient is also a gradient of viscosity, increasing drag will slow cells at the front of the band relative to those at the back and so will make the band narrower. There are two types of density gradient centrifugation, rate-zonal and isopycnic.

3.3.1. Rate-zonal centrifugation

Rate-zonal centrifugation is basically the same as differential pelleting except that the cells are centrifuged through a density gradient (Fig. 3.2). The density of medium increases down the tube but at no point does the density exceed that of the cells to be separated. Centrifugal force is applied which results in cells of different size sedimenting at different rates in the same way as differential pelleting. The difference with rate-zonal is that because of the density gradient, cells of similar size all sediment together as a tight band. Cells of different size all produce tight bands at different places down the gradient depending on their rate of sedimentation. Rate-

zonal centrifugation can therefore be used to separate more than two cell populations.

Because the density of the cells is greater than the density at any position in the gradient, if centrifuged for long enough, all the cells will form a pellet. Rate-zonal centrifugation is therefore time dependent.

3.3.2. Isopycnic centrifugation

In isopycnic or equilibrium centrifugation, the density of the gradient at its highest point is greater than the density of the densest cells to be separated. The result of this is that no matter how long the cells are centrifuged, cells will never sediment to the bottom of the tube. Cells of a particular density sediment until they reach the point where their density is the same as that of the gradient, i.e. their equilibrium position. Cells cannot sediment further because the medium below them is denser and so they accumulate as a band (Fig. 3.3).

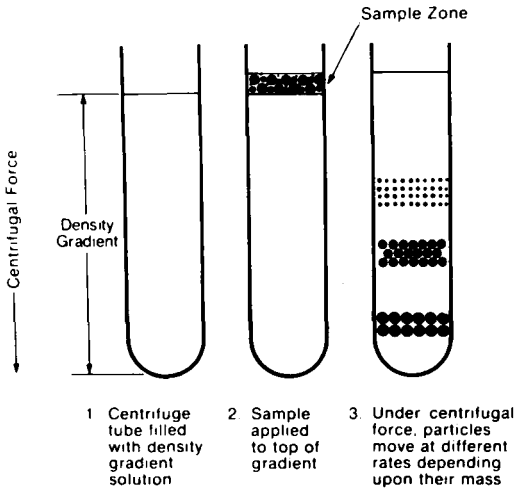


Fig. 3.2. Principle of rate zonal centrifugation for cell separation. From Griffith (1979). Beckman Instruments – with permission.

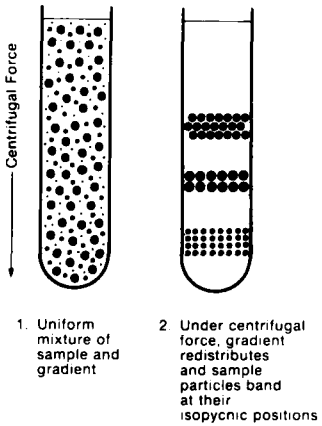


Fig. 3.3. Principle of isopycnic centrifugation for cell separation. Cells can also be layered on top of a preformed gradient. From Griffith (1979). Beckman Instruments – with permission.

Isopycnic centrifugation separates cells purely on the basis of differences in density. Differences in cell size merely effects the rate at which cells reach their equilibrium positions. As long as the gradient is stable and the cells are not affected by the centrifugation, they can be centrifuged for prolonged periods without affecting the separation. An important point to note is that the equilibrium density (i.e. the banding position in the gradient) of any particular cell type may vary when different density gradient media are used. This is because cells may become more or less hydrated depending on the medium used, which effectively alters their banding densities. Isopycnic centrifugation can be performed by layering cells onto an already formed gradient (as rate-zonal) or alternatively they can be mixed with certain gradient materials which form a gradient during centrifugation and the cells reach their isopycnic position as the gradient forms. The latter method is not recommended for the majority of cells since centrifugation for several hours at high *g* forces is required for gradients to self-form. This method can be used for more stable cells such as bacteria and for viruses.

3.3.3. Gradient materials

Listed in Table 3.1 are the properties of an ideal density gradient material which apply to both isopycnic and rate-zonal centrifugation. No currently available medium satisfies all these criteria but most satisfy the major ones which are;

1. The medium must be inert and non-toxic to cells.
2. The compound constituting the medium should be soluble in a wide range of buffers to produce the range of densities required and also provide an environment compatible with the cells.
3. The medium must be easily removed from the cells and not interfere with any assays that need to be carried out.

Because of their strong ionic nature, salts of heavy metals such as cesium chloride which are commonly used to separate soluble materials such as DNA, cannot be used for cell separation. The most

TABLE 3.1
Properties of an ideal density gradient medium

The ideal density gradient solute should satisfy the following criteria:

1. It should be sufficiently soluble in aqueous media to produce the range of densities required.
2. It should not form solutions of high viscosity in the desired density range.
3. It should be neither hyperosmotic nor hypoosmotic when the particles to be separated are osmotically sensitive.
4. Solutions of the gradient solute should be adjustable to the pH and ionic strengths that are compatible with the particles being separated.
5. It should not affect the biological activity of the sample.
6. It should be non-toxic and not metabolised by cells.
7. It should not interfere with assay procedures.
8. It should not react with the tubes, caps or rotors of the centrifuge.
9. Solutions of the gradient solute should exhibit a property that can be conveniently used as a measure of concentration or gradient density.
10. It should be easily removed from the purified product.
11. It should not absorb in the Ultra Violet or visible range of the spectrum.
12. It should be autoclavable.
13. It should not interfere with radioisotopes.
14. It should be of a reasonable cost.

commonly used materials for density gradient centrifugation are described below.

3.3.3.1. Sucrose. You may often read that sucrose is an ideal density gradient material, particularly for rate-zonal centrifugation. This is indeed true for separation of many biological materials but is certainly not true for cells. The main reason for this is the high osmolarity of sucrose solutions at densities greater than 1.03 g/ml (Fig. 3.4). Most mammalian cells have an osmotic strength of around 290 mOsm and thus any unbuffered sucrose solution above 1.15 g/ml (about 35% w/v) is hypertonic. If the solution is buffered then this osmolarity is reached at even lower sucrose concentrations. This single property outweighs all the advantages sucrose might have for cell separation, namely low cost, inertness, stability, high solubility and non-toxic nature.

3.3.3.2. Ficoll. Ficoll (Ficoll 400), is a synthetic high molecular weight polymer (MW 400 000) made by copolymerisation of sucrose and epichlorohydrin and is manufactured especially for use in cell separation by Pharmacia. Ficoll solutions can only be produced to give a narrow density range between 1.00 – 1.17 g/ml. Solutions

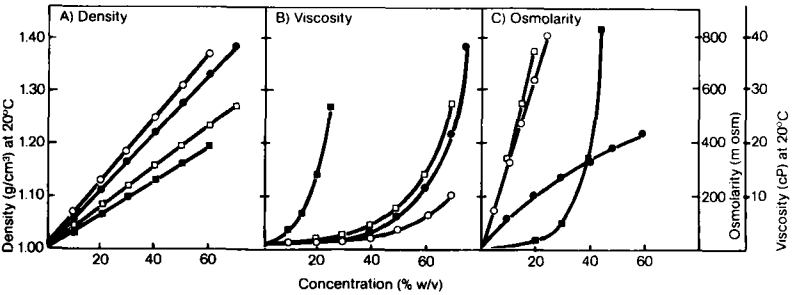


Fig. 3.4. A comparison of the physico-chemical properties of different density gradient media. Relationships are shown between concentration (% w/v) of gradient solute and (A) density, (B) viscosity and (C) osmolarity. Nycodenz (●——●), Sodium metrizoate (□——□), Ficoll (■——■) and sucrose (○——○). Courtesy of Nycomed Ltd.

below 20% (w/v) (1.07 g/ml) are osmotically inert but at concentrations above this there is a sharp increase in osmotic strength (Fig. 3.4).

Ficoll solutions are very viscous and diffuse slowly, so continuous gradients cannot be produced by diffusion. Although the high viscosity is not ideal for cell separation, the gradients are very stable. Ficoll is non-toxic to cells and in some instances may exert a protective influence on cells in common with other high molecular weight sugar polymers such as dextran. Ficoll in aqueous solution is autoclavable but may caramelise if phosphate is present e.g. as phosphate buffer. Alternatively Ficoll solutions can be sterilised by filtration or UV irradiation. In common with sucrose, Ficoll solutions need to be preformed into a gradient, they do not self-generate a gradient on standing (self-forming gradients 3.3.6.5). The gradients produced can be analysed by refractive index measurement (3.3.7). Ficoll does not interfere with most biological assays although some quenching of radiolabel scintillation counting can occur.

Removal of Ficoll from separated cells is best achieved by repeated washings with a suitable buffer. Ficoll cannot be removed by dialysis. The major disadvantage of Ficoll is its high cost.

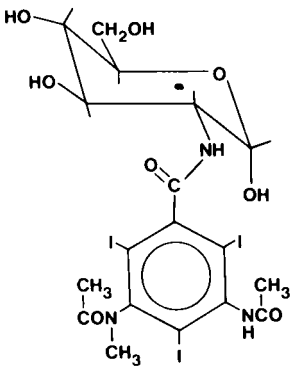


Fig. 3.5. Structure of Metrizamide.

Ficoll has proved particularly useful for separating lymphocytes from whole blood in the form of Ficoll-Paque (Pharmacia) and Lymphoprep (Nycomed).

3.3.3.3. Iodinated media (Metrizamide and Nycodenz). The use of aqueous solutions of low molecular weight iodinated compounds based on a benzene ring structure as density gradient media is the most recent innovation in density gradient centrifugation of cells. The two compounds that have been used most often are Metrizamide and Nycodenz, both of which are produced by Nycomed.

Metrizamide (2-(3-acetamido-5-*N*-methylacetamido-2,4,6,-triiodobenzamido)-2-deoxy-D-glucose) is a tri-iodinated derivative of benzoic acid (Fig. 3.5) with a molecular weight of 789. The 2-amino-2-deoxy-D-glucose group which replaces the carboxyl group of the benzoic acid, is responsible for the high solubility in aqueous solutions and the non-toxic nature.

Nycodenz, (*N,N*-bis (2,3-dihydroxypropyl)-5-[*N*-(2,3-dihydroxypropyl) acetamido]-2,4,6,-triiodo-isophthalamide) is similarly a tri-iodinated derivative of benzoic acid (Fig. 3.6) which has three hydrophilic groups substituted alternately with the iodine. These groups are responsible for the high water solubility. The basic functional differences between Metrizamide and Nycodenz are that Metrizamide is sensitive to temperatures above 55°C due to its glucose group (and cannot therefore be autoclaved) and also interferes with several assays for protein and nucleic acids. Nycodenz

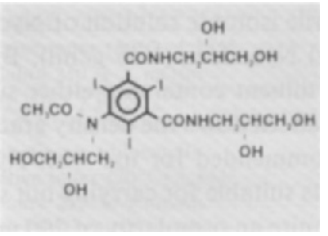


Fig. 3.6. Structure of Nycodenz.

exhibits neither of these properties and for general cell separation purposes has replaced Metrizamide. Sodium Metrizoate is marketed as a specialist density gradient medium for the separation of lymphocytes from whole blood. The following discussion of iodinated density gradient media is confined to Nycodenz since this is the material most suitable for a wide range of cells. The majority of the general points also apply to Metrizamide.

For any given concentration, solutions of Nycodenz are denser than those of most other media. Effectively this means that a lower concentration of solute is required for any particular density. This minimises the possibility of cells becoming dehydrated as can often happen in very concentrated solutions.

Although Nycodenz solutions are relatively viscous, over the density range normally used i.e. up to 1.4 g/ml, the viscosity does not seriously affect the rate at which particles reach their isopycnic positions.

Solutions of Nycodenz show a linear relationship between concentration and osmolarity (Fig. 3.4). The gradient of osmolarity of Nycodenz is shallower than that of sucrose but at concentrations below 35% (w/v) Nycodenz solutions have a higher osmolarity than Ficoll solutions (Fig. 3.4). However because Nycodenz is denser than Ficoll for any given concentration, the osmolarities of solutions of similar density are comparable.

One of the problems of density gradient centrifugation is that as solution density increases so does the osmolarity. The osmolarity of a gradient of Nycodenz (and also Percoll, 3.3.3.4) can be controlled by using an isotonic buffer as a solvent and altering the ratio of buffer: Nycodenz. A sterile isotonic solution of Nycodenz is sold which contains 27.6% (w/v) Nycodenz (1.15 g/ml). By diluting this solution with a buffered diluent containing either sucrose or NaCl, the osmolarity can be balanced down the density gradient. The particular diluent solutions recommended for use with Nycodenz are to produce isotonic gradients suitable for carrying out separations of mammalian cells which require an osmolarity of 290 mOsm. The compositions and properties of these isotonic gradients are shown in Tables

3.2 and 3.3. This same principle can be used to produce isotonic gradients of different osmolarities.

Preformed gradients of Nycodenz used for cell separation can be prepared by any of the methods described in 3.3.6. However Nycodenz also has the property of being able to generate its own gradient (self-forming). Self-forming gradients are discussed in detail in 3.3.6.5, but are simply gradients that are formed by centrifuging the solution of gradient material. Cells can be layered on top of the self-formed gradients or alternatively for some isopycnic separations can be mixed with the Nycodenz solution and the mixture centrifuged.

TABLE 3.2
Compositions of isotonic Nycodenz® solutions

Buffered medium	5 mmol/l Tris/HCl, pH 7.5, containing 3 mmol/l KCl and 0.3 mmol/l $CaNa_2$ EDTA Refractive index (20°C) 1.3331 Osmolality 19 ± 1 mOsm
Nycodenz® in isotonic solution	27.6 g solic Nycodenz® dissolved in the above medium and made up to 100 ml with that medium Refractive index (20°C) 1.3784 Osmolality 290 ± 1 mOsm Density (20°C) 1.15 g/ml
Diluent solutions	0.75 g NaCl or 7.45 g sucrose dissolved in the buffered medium and made up to 100 ml with medium
	NaCl diluent (diluent A) Refractive index (20°C) 1.3345 Osmolality 250 ± 1 mOsm Density (20°C) 1.003 g/ml
	Sucrose diluent (diluent B) Refractive index (20°C) 1.3446 Osmolality 251 ± 1 mOsm Density (20°C) 1.027 g/ml

TABLE 3.3
Properties of isotonic gradients

Nycodenz® – NaCl gradients				
% (w/v) Nycodenz®	27.6	18.4	13.8	9.2
Dilution ratio Nycodenz® : Diluent A[a]	1:0	2:1	1:1	1:2
Observed osmolality (mOsm)	291	295	291	283
Density (g/ml) 20°C	1.146	1.098	1.075	1.050
Refractive index 20°C	1.3784	1.3633	1.3563	1.3490
Nycodenz® – sucrose gradients				
% (w/v) Nycodenz®	27.6	18.4	13.8	9.2
Dilution ratio Nycodenz® : Diluent B[a]	1:0	2:1	1:1	1:2
Observed osmolality (mOsm)	290	295	290	280
Density (g/ml) 20°C	1.146	1.105	1.086	1.066
Refractive index 20°C	1.3784	1.3669	1.3613	1.3553

[a] Composition of diluents A and B as described in Table 2.

The cells reach their isopycnic positions while the gradient is forming. The main advantage of this method is that large numbers of cells can be separated on the same gradient.

Although Nycodenz does not interfere with most biochemical assays, it does interfere with Folin-phenol and Microbiuret assays for protein, and the anthrone assay for polysaccharides. Nycodenz also produces quenching of radiolabel scintillation. The amount of quenching is dependent on the particular isotope and scintillant used. Little quenching occurs in the commonly used PPO/POPOP/toluene/Triton based scintillants when counting ^{3}H, ^{14}C and ^{32}P. Nycodenz does show strong absorbance in the UV end of the spectrum which means it must be throughly washed from cells if protein content measured by optical density at 280 nm is to be used as a measure of cell number (2.2.2).

Nycodenz, like most gradient materials can be removed from cells by repeated washings. It can also be removed by ultrafiltration or dialysis although these methods are not recommended for cells since they are likely to severely impair viability. Nycodenz solutions are heat stable and are therefore easily sterilised by autoclaving. Stability to autoclaving is enhanced by inclusion of millimolar concentrations of Tris and CaNa EDTA. Solutions are very resistant to bacterial degradation. Nycodenz is non-toxic and not metabolised by mammalian cells.

3.3.3.4. Percoll. Percoll is produced by Pharmacia specifically for centrifugation purposes. Percoll is a suspension of colloidal silica particles, diameter 15 – 30 nm. The colloidal particles are coated with polyvinyl-pyrrolidine (PVP) which minimises their interaction with biological material and stabilises them. The osmotic strength of Percoll is very low (Fig. 3.4) and changes little with density. Percoll solutions supplied by Pharmacia are stable to autoclaving but cannot be autoclaved once sucrose or salts have been added. Percoll is precipitated at low pH and destabilises at high ionic strengths.

Since its introduction as the first purpose-made product for density gradient centrifugation in 1977, Percoll has been used extensively for cell separation by isopycnic centrifugation. The Percoll reference list issued in 1985 by Pharmacia contains over 500 entries of which the majority are separation of cells. Gradients of Percoll readily self-form when centrifuged for very short periods e.g. 30 min at 20 000 $\times$ *g*. Iso-osmotic solutions can be produced in a similar way to Nycodenz. For mammalian cells, 9 parts Percoll are diluted with 1 part 1.5 M NaCl or $\times$ 10 concentrated culture medium. This produces a solution with an osmotic strength of 280 – 320 mOsm. The density of the stock solution can be calculated from:

$$V_x = V_o \left(\frac{\varrho_o - \varrho_1}{\varrho_i - \varrho_x} \right) \text{ thus } \varrho_i = \frac{V_{o\varrho v} + V_{x\varrho x}}{V_x + V_o}$$

V_x = vol. of diluting medium (ml)
V_o = vol. of Percoll (ml)
ϱ_o = density of Percoll (1.130 ± 0.005) g/ml
ϱ_x = density of 1.5 M NaCl (= 1.058 g/ml).
ϱ_i = density of stock iso-osmotic solution produced (g/ml)

Thus for a stock solution of Percoll in iso-osmotic saline the density is 1.123 g/ml and stock solutions can be diluted to lower densities by diluting with the appropriate buffer. The following formula can be used to calculate the volumes required to obtain a solution of desired density:

$$V_y = V_i \frac{(\varrho_i - \varrho)}{(\varrho - \varrho_y)}$$

V_y = vol. of diluting medium (ml)
V_i = vol. of stock iso-osmotic Percoll (ml)
ϱ_i = density of stock solution g/ml
ϱ_y = density of diluting medium g/ml
ϱ = density of diluted solution produced g/ml

Percoll solutions do not interfere with the majority of enzyme assays or seriously quench scintillation counting. They do however interfere with the Folin-Ciocalteau protein assay and several nucleic acid and polysaccharide assays. Percoll absorbs strongly in UV light and absorbance at 280 nm cannot therefore be used directly as a measure of cell protein without prior washing of the cells. Contaminating Percoll can be removed from separated cells by repeated washing.

The major disadvantage of Percoll for cell separation is its particulate nature. Percoll particles are easily phagocytosed by capable cells such as monocytes. When phagocytosis occurs, separations based on an increase in cell density due to ingestion of particles can result, producing artificial bands.

3.3.4. Cell viability and recovery

A critical discussion of cell viability after separation in different gradient media is difficult since viability is very much a function of the particular cells being separated and generalisations can therefore be misleading.

Because of the problems of osmotic strength with sucrose solutions, sucrose will not generally maintain high cell viabilities. Ficoll, Nycodenz and Percoll, when used correctly, appear from a general review of the literature to produce separated cells with reasonably high viabilities of at least 70%. Clearly if cells band in a region of the gradient that is non-isosmotic for those cells, the viability may be severely reduced.

The effect of the centrifugal force on cell viability is highly variable. As a general rule the shorter the time cells spend being centrifuged the higher the viability.

Recovery of separated cells from gradients refers to the percentage of cells obtained at the end of the whole separation procedure, after washings etc. and is less a function of cell type and more of the methods used to remove cells from the gradient and to remove contaminating gradient material. Recovery of cells directly from a gradient may be reduced if the viscosity of the gradient solution is high. The main factor in obtaining high cell recoveries is the efficiency of removal of the gradient material from cells. If repeated washings are necessary, then cells will be progressively lost. It can therefore be advantageous to select an assay method for the separated cells that is not affected by the presence of the gradient material.

3.3.5. Gradient shapes

There are two forms of density gradient, continuous and discontinuous (step-gradients). Both these terms are self-explanatory. A continuous gradient is one where there is a continuous increase in density down the tube. A discontinuous gradient is where the gradient consists of a series of solutions of different density layered on

top of each other with a sharp transition in density (a step) between each.

Thew density gradients used for isopycnic centrifugation can be either continuous or discontinuous but for rate-zonal centrifugation, discontinuous gradients are not often used since artifactual bands of cells can collect at the interfaces.

3.3.5.1. Continuous gradients. There are an infinite number of possible shapes of a continuous gradient that can be made if the right equipment is available. The choice of gradient shape used is entirely dependent on the particular cells that require separating. Linear gradients i.e. those where the density increases in a linear fashion down the tube (Fig. 3.7) are usually adequate for most rate-zonal separations and can be used for isopycnic separations. In general, a gra-

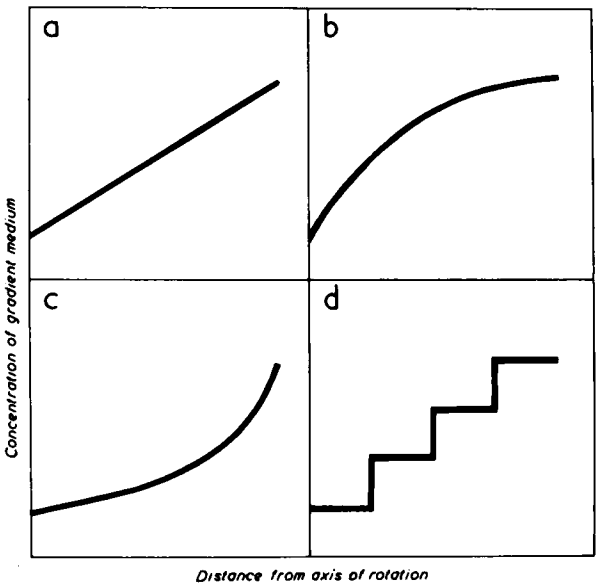


Fig. 3.7. Gradient shapes; (a) linear gradient; (b) exponential convex gradient; (c) exponential concave gradient; (d) discontinuous (step) gradient. From Hames (1984), IRL Press – with permission.

dient with a steep slope will produce better resolution than a more gentle slope because tighter bands are formed due to increasing viscosity. However in isopycnic centrifugation, a balance has to be struck between a steep and shallow gradient since although a steep gradient produces tighter bands, a shallow gradient produces bands that are further apart. Linear gradients are commonly used if the densities or sizes of the cells are evenly distributed.

Increased resolution can often be obtained, particularly where there is an uneven distribution of cell sizes and/or densities, by using an exponential (either concave or convex) gradient (Fig. 3.7). Here the steepness of the gradient increases either at the top (convex) or the bottom (concave) of the tube. Convex gradients are used when the medium at the top of the gradient needs to be dense enough to enable cells to be layered but needs to be less steep lower down in order to effect separation. Alternatively, if the density at the top of the gradient need not be high to support the cells, a concave gradient which has a steep increase in density and viscosity near the bottom of the tube is useful for preventing cells from pelleting. Concave gradients are particularly useful in rate-zonal centrifugation since fast sedimenting cells will slow down rapidly as they reach the higher viscosity at the bottom of the tube while slower sedimentating cells will continue to separate higher up the tube, thus increasing resolution.

Self-forming gradients are always of the continuous type but can be either linear or exponential depending on the medium and the method of centrifugation (3.3.6.5).

3.3.5.2. Isokinetic gradients. Isokinetic gradients are a specialised type of continuous gradient where at a constant rotor speed a cell will sediment at a constant velocity. Thus the increase in velocity that normally occurs as a cell moves further from the centre of rotation (due to the increase in centrifugal force) is exactly balanced by an increase in density and viscosity of the gradient. The distance travelled by a cell in an isokinetic gradient is proportional to the time of centrifugation. Isokinetic gradients can be produced from any gradient

material provided the relationship between concentration and viscosity is known. For cell separation the majority of work conducted has involved the used of isokinetic Ficoll gradients. Linear Ficoll gradients between 2.7% (w/w) at the top, 13.7 cm from the centre of rotation to 18.5% at the bottom, 26.0 cm from the centre of rotation are isokinetic at 4°C for cells with densities between 1.06 – 1.12 g/ml (Pretlow 1971, Pretlow and Williams 1973). An isokinetic gradient will be different for another gradient material, cells of different density and size and for different distances from the centre of rotation (i.e. type of rotor used). The production and use of isokinetic gradients has been extensively discussed in several papers by Pretlow e.g. Pretlow (1971). The principle use of isokinetic gradients is in determining the conditions for separating cells of known size or density.

3.3.5.3. Discontinuous gradients. The use of discontinuous gradients is largely limited to isopycnic centrifugation. The gradients are prepared by carefully layering solutions of different density on top of each other in the centrifuge tube. The cells are layered on top of the gradient and centrifuged. The cells will sediment until a layer of density is reached which is greater than the density of the cells, whereupon they cannot sediment further and therefore collect to form a band at the interface between the two densities. Usually only a few layers or steps in density are used and thus if the sample contains cells of several different densities, only a relatively crude fractionation of each can be obtained. These gradients are commonly used where only a few cell types are present and an interface is provided for each.

3.3.6. Formation of gradients

3.3.6.1. Diffusion. This is the simplest method of forming gradients. Layers of medium solution at different densities are manually layered on top of each other and either used in this form (discontinuous) or allowed to stand for a period of time for diffusion to occur to produce a continuous gradient.

Fig. 3.8 shows two easy methods of producing discontinuous gradients manually. In (a) a syringe fitted with a large-bore needle is used to introduce known volumes of solutions of medium. The least dense solution is added first followed by more dense solutions which, when added, slowly displace the less dense solution. This is repeated to produce a discontinuous gradient with the required number of steps. Another easy method is to add the most dense solution first by slowly allowing it to drain down the side of the tube as shown in Fig. 3.8 (b). The solutions must drain down the side of the tube because dropping one on top of the other will produce mixing. Once a discontinuous gradient has been produced it can be used as such or may be used to form a continuous gradient by diffusion.

Diffusion can be allowed to occur either with the tube vertical or, if the gradient forms slowly, the tube can be sealed and laid horizontally which speeds up the rate of diffusion. A 4-step discontinuous gradient of Nycodenz for example will form a smooth gradient in about 45 min when layered in a 1 cm diameter tube and laid horizontally, compared with 18 – 24 h if allowed to stand in the vertical position. The longer the gradient is left to form, the less steep the gradient will become as diffusion proceeds. Gradients can only realistically be formed by diffusion when the molecular weight of the

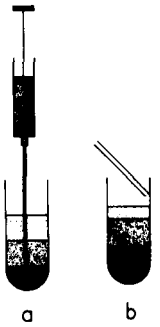


Fig. 3.8. Preparation of density gradient by diffusion methods; (a) underlayering; (b) overlayering. From Hames (1984), IRL Press – with permission.

gradient material is less than 1000. Gradients prepared from high molecular weight media diffuse too slowly, hence Ficoll gradients cannot be made by diffusion.

3.3.6.2. Linear gradient maker. A simple linear gradient maker of a type that is widely used is shown in Fig. 3.9. Such makers are commercially available either specifically for producing density gradients for centrifugation or for other purposes such as producing gradient gels for electrophoresis. It consists of two transparent

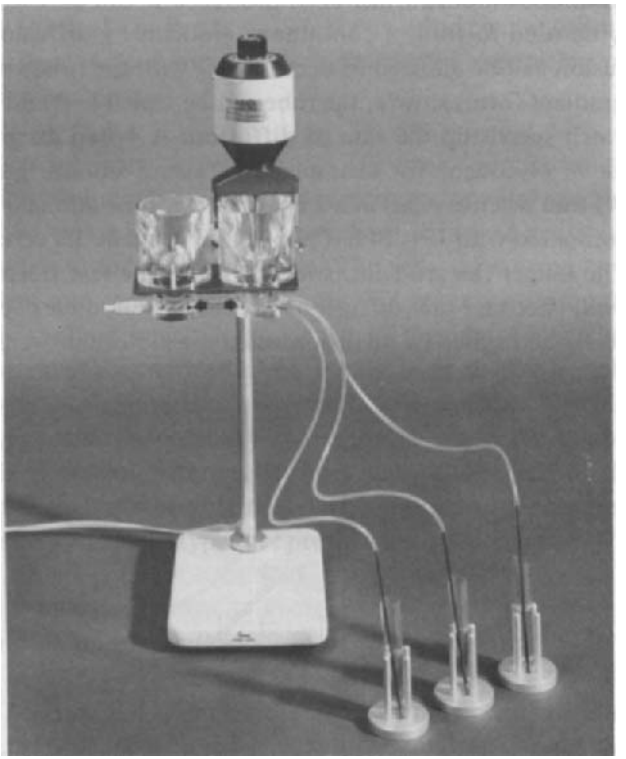


Fig. 3.9. Linear gradient maker. Kontron Instruments; supplied by Fisons Scientific (MSE).

vessels of equal cross-sectional area joined by a connecting channel which can be opened and closed by means of a stop-cock. One chamber has an exit which leads to the centrifuge tube via a length of flexible tubing.

Producing a linear gradient with such a maker is relatively simple. The less dense of the two solutions of gradient medium is placed in the reservoir chamber, not connected to the tube. The more dense solution is placed in the mixing chamber connected to the centrifuge tube via narrow bore plastic tubing. The more dense solution is continuously stirred and the connection between the two chambers opened. Dense medium in the mixing chamber is pumped via a peristaltic pump (or gravity fed) down the side of the centrifuge tube. As the dense solution leaves its chamber it is automatically replaced by an equal volume of less dense solution by hydrostatic pressure. The less dense solution is immediately mixed with the more dense solution thus slightly lowering the overall density of the medium in the mixing chamber which is flowing into the tube. A linear gradient of medium thus fills the tube.

A variation of this method is to reverse the positions of the solutions i.e. the less dense solution in the mixing chamber and the more dense in the reservoir. In this way the solution pumped into the tube first is the least dense and must therefore be directed to the bottom of the tube. The solution entering the tube gradually increases in density and displaces less dense solutions at the bottom.

There are two important practical points that must be noted when using a linear gradient maker. First, the volume of solution in each chamber must be inversely proportional to the densities of each so that equal weights of each are used. If not, when the two chambers are connected together, the more dense solution will immediately flow backward and mix with the less dense solution in the reservoir. Secondly, the volumes used in the chambers must be carefully controlled to allow for the small volume of medium that is always retained by the gradient maker and outlet tubing. It is necessary to always retain a few millilitres of solution to prevent air bubbles from entering the centrifuge tube with the last few drops of medium.

The concentration of gradient solution leaving the mixing chamber at a given time can be calculated from the following formula:

$$C_t = C_m + (C_r - C_m)\frac{V_t}{2V_o}$$

where C_t = concentration of gradient being delivered at time t. C_m = initial concentration of medium in mixing chamber. C_r = initial concentration of gradient in reservoir. V_t = volume of gradient poured at time t. V_o = original volume of liquid in each chamber.

3.3.6.3. Exponential gradient makers. Concave and convex gradients can easily be prepared using a simple gradient maker as

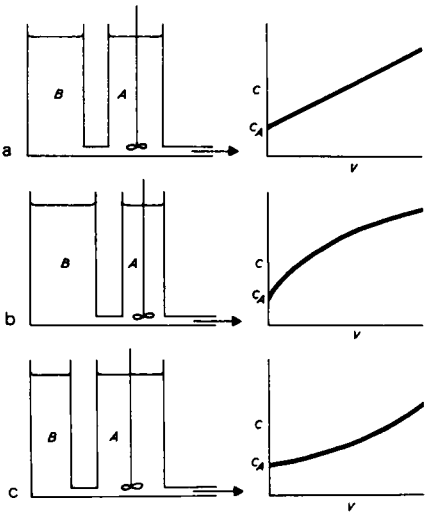


Fig. 3.10. Preparation of exponential density gradients using a simple gradient maker. (a) Linear gradients are produced when the cross-sectional areas of the reservoir and mixing chambers are the same. (b) Convex exponential gradients are produced when the area of the mixing chamber is smaller than that of the reservoir. (c) Concave exponential gradients are produced when the area of the mixing chamber is greater than that of the reservoir. Re-drawn from Ridge (1978), and Hames (1984).

described above by having chambers of different cross-sectional area. As shown in Fig. 3.10 when the cross-sectional area of the mixing chamber is reduced, convex gradients are formed whereas concave gradients are formed when the cross-sectional area of the reservoir chamber is reduced. The volume of the medium in the mixing chamber is kept constant while the gradient is poured. The degree of non-linearity depends upon the relative volume in the mixing chamber compared with the final volume of the gradient, thus:

$$C_t = C_r - (C_r - C_m)_e - \frac{V_t}{V_m}$$

where C_t = concentration of gradient leaving the mixing chamber at time t. C_r = concentration of gradient in reservoir. V_t = volume of gradient solution already drawn from mixing chamber at time t. V_m = volume of mixing chamber.

Since the exact shape of the exponential gradient is dependent on the volume of the mixing chamber, many exponential gradient makers have a mixing chamber where the volume can be varied.

If very complex gradients are needed, with combinations of linear and exponential regions, programmable gradient makers can be used to make these.

3.3.6.4. Freeze-thawing. Another simple method of preparing non-linear gradients is to freeze and then thaw the gradient medium in the centrifuge tube. The shape of the gradient produced depends on the medium and the number of freeze–thaw cycles. Fig. 3.11 shows the shape of Nycodenz gradients produced by freeze-thawing. This method cannot be used for producing isotonic gradients and will not always form reproducible gradients.

3.3.6.5. Self-forming gradients. Of the gradient media discussed, only Percoll and Nycodenz (Metrizamide) are able to self-form density gradients. Self-forming simply means that if a homogeneous solution of gradient medium is centrifuged, a density gradient is formed.

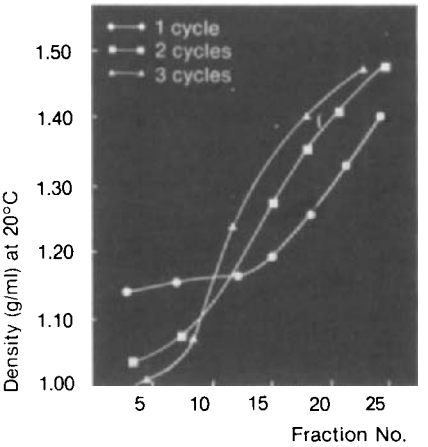


Fig. 3.11. Shapes of Nycodenz density gradients produced by 1, 2 and 3 freeze-thaw cycles. Courtesy of Nycomed Ltd.

Such self-forming gradients can be used in the same way as preformed gradients with the cells being layered on top or alternatively for isopycnic methods the cells can be mixed with the gradient medium which, when centrifuged, produces a gradient in situ with the cells banding at their isopycnic positions.

Centrifugation speed, temperature, time and concentration of gradient medium all influence the shape of the self-formed gradient. The gradients formed by Percoll and Nycodenz are somewhat different.

1. Percoll

When a solution of Percoll in 0.15 M saline or 0.25 M sucrose is centrifuged above 10 000 × g, the Percoll particles start to sediment. Since the particles are of different sizes they sediment at different rates, creating a very smooth gradient. The gradient forms isometrically around the starting density and becomes progressively steeper with time (Fig. 3.12). The shape of the gradient is linearly related to the total g force and time of centrifugation. The gradients are not stable and will continuously change during high speed cen-

trifugation. The S-shaped gradients produced by high speed centrifugation are particularly useful for separating cells with similar densities since the flat region occupying most of the gradient has a narrow density range.

Percoll solutions in 0.25 M sucrose are more viscous than solutions in saline or culture medium and thus require 2 – 3 times longer to form a gradient. A minimum of 10 000 × *g* should be used to self-generate gradients of Percoll in 0.15 M saline and 25 000 × *g* for solutions in 0.25 M sucrose in angle head rotors. The time of centrifugation depends on the gradient shape required. Fig. 3.12 shows the effect of time on the shape of Percoll gradients. Alternatively stable gradients can be formed by centrifuging at 30 000 × *g* for 30 min.

It is not practicable to use swing-out rotors for self-generation of gradients. Gradients form most quickly in vertical and shallow angle rotors.

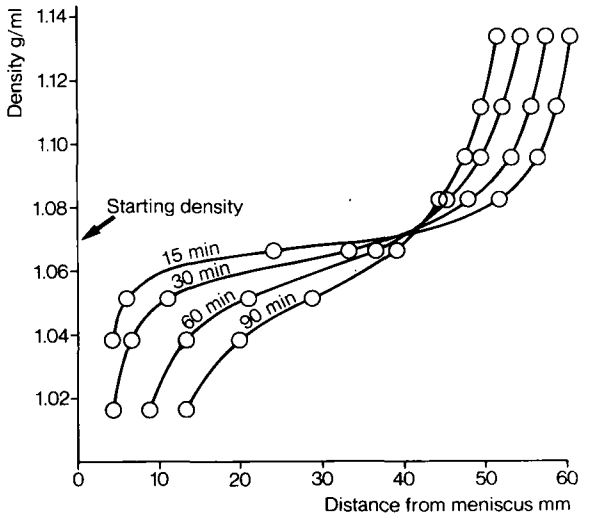


Fig. 3.12. S-shaped percoll density gradients produced under a constant *g* force with different times of centrifugation. Courtesy of Pharmacia.

2. Nycodenz

Self-generated gradients of Nycodenz are less S-shaped than Percoll gradients. Fig. 3.13 shows a summary of gradients formed under a variety of different conditions. Increasing centrifugation speed or

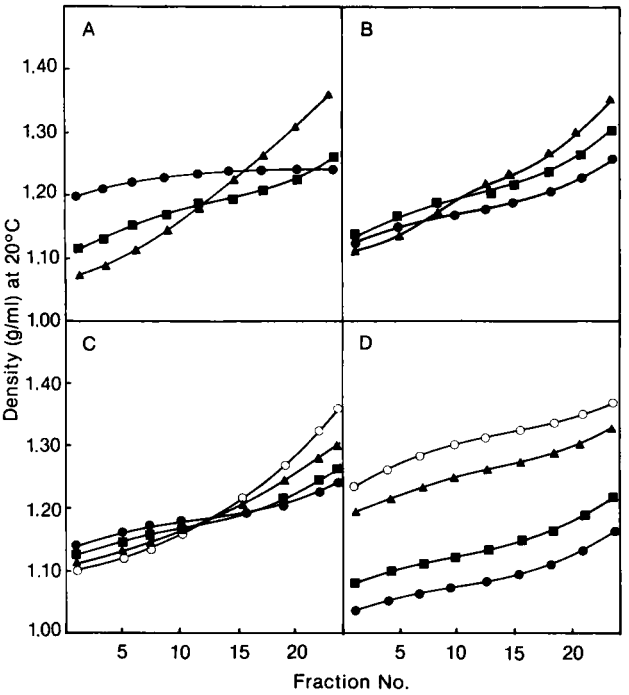


Fig. 3.13. The formation of gradients in solutions of Nycodenz. MSE 10 × 10 ml aluminium rotor.

A. Effect of increased speed of centrifugation. 40% (w/v) solution of Nycodenz centrifuged at 5°C for 24 h at 15 000 rpm (●——●), 30 000 rpm (■——■) and 45 000 rpm (▲——▲).

B. Effect of increasing temperature. 40% (w/v) solution of Nycodenz centrifuged at 30 000 rpm for 24 h at 5°C (●——●), 15°C (■——■) or 25°C (▲——▲).

C. Effect of increasing time of centrifugation. 40% (w/v) solution of Nycondenz centrifuged at 5°C and 30 000 rpm for 16 h (●——●), 25 h (■——■), 48 h (▲——▲) and 75 h (○——○).

D. Effect of initial concentration of Nycodenz. Solutions of Nycodenz centrifuged at 30 000 rpm for 24 h at 5°C at initial concentrations (w/v) of 18% (●——●), 28% (■——■), 49% (▲——▲) and 59% (○——○). Courtesy of Nycomed Ltd.

temperature increases the steepness of the gradient. A gradient will form after 16 h when centrifuged at 63 000 × *g* although this will not reach equilibrium until 75 h centrifugation. The initial concentration of Nycodenz used does not affect the shape of the gradient produced, the curves are merely displaced.

For isopycnic centrifugation using a performed gradient it is important that the gradient shape is identical to the predicted equilibrium gradient under the centrifugation conditions used, otherwise the preformed gradient will be unstable because the gradient solute will redistribute during centrifugation. The time the initial gradient slope at its midpoint is stable, t (h), is given by:

$$t = K(r_b - r_t)^2$$

where r_t = distance (cm) of the top of the gradient from the axis of rotation, r_b = distance (cm) of the bottom of the gradient from the axis of rotation K = constant inversely proportional to the diffusion coefficient of the gradient solute.

The time required to produce an equilibrium gradient depends on the size of gradient solute and length of gradient. Shorter gradients can be formed much quicker than longer ones. The effective length of the gradient decreased with decreasing angle of the tube to the vertical. Thus gradients will form the quickest in vertical rotors where the effective length of the gradient is the diameter of the tube.

The density profiles of preformed gradients can be calculated from the following equations. Alternatively a computer program is available (Rickwood 1984).

1. Slope of gradient

$$\text{Slope} = \frac{dh}{dr} = \frac{w^2 r}{\beta_o} = \frac{1.1 \times 10^{-2} N^2 r}{\beta_o}$$

where w = angular velocity (rad/sec), r = distance from axis of rotation (cm), β = density gradient proportionality constant for gradient solute in gradient solvent, N = rotor speed.

The slope can be calculated for any particular gradient solute, see Ifft et al. (1970). This equation predicts the slope of the gradient along the middle three-quarters of its length.

2. Density range of gradient

$$G_2 - G_1 = \frac{W^2}{2\beta_o}(r_2^2 - r_1^2) = \frac{1.1 \times 10^{-2} \times N^2}{2\beta_o}(r_2^2 - r_1^2)$$

where G_1 and G_2 are the densities (g/cm) at points r_1 and r_2 from the axis of rotation.

3.3.7. Measurement of density gradients

Once a density gradient has been produced it is usual to measure the density profile along the length of the gradient. By far the easiest method of measuring the density of medium from different parts of the gradient is to use refractive index measurement. Once a gradient has been fractionated the refractive index of a small amount of medium from different parts of the gradient, particularly where cells band, can be measured. The apparatus used to measure refractive index is cheap and simple to use. All density media mentioned show a linear relationship between refractive index and density (concentration). This relationship is given by:

$$e = an - b$$

where e = density, n = refractive index a and b = coefficients.

The coefficients a and b are different for each medium and depend on temperature. The coefficients at 20°C for Ficoll are, $a = 2.381$, $b = 2.175$ and for Nycodenz, $a = 3.242$, $b = 3.323$. (Rickwood 1984).

It is important for accurate measurement that when using this method allowance is made for the presence of solutes such as buffers, EDTA etc. in the gradient. When these are present the following equation should be used:

$$n \text{ corrected} = n \text{ observed} - (n \text{ buffer} - n \text{ water})$$

The density of gradient media can be measured directly by accurately weighing known volumes of liquid using a pycnometer but this method is very time consuming.

Percoll density gradients can be measured by refractive index but it is more usual to use special marker beads which are produced for this purpose by Pharmacia. Beads of known density are colour coded and are centrifuged in a blank gradient at the same time as the centrifugation of a gradient containing cells. After centrifugation the distance travelled by each bead is measured and used to plot a density profile for the gradient. These beads can only be used with Percoll and the distance travelled can be affected by other constituents in the gradient, buffers, medium etc.

3.3.8. Loading cells onto a gradient

When cells require loading by layering on top of the gradient for rate-zonal centrifugation they must be layered in as narrow a band as possible. Layering cells in a narrow band increases resolution since tighter bands are formed in the gradient. Cells are best suspended in the same buffer as the gradient solvent. The cell suspension must have a lower density than the top of the gradient otherwise it will immediately start to sediment. One problem that this creates is that the cell sample contains a lower concentration of gradient medium than the top of the gradient and so diffusion of gradient medium into the cell sample zone can occur. This results in an increase in density in the cell sample zone adjacent to the gradient and therefore the sample begins to sink into the gradient. The edges of the sinking zone sink faster and cause the formation of droplets which descend through the gradient (droplet sedimentation). The outcome of droplet sedimentation is a loss of resolution due to effective widening of the cell sample zone. Droplet sedimentation is less of a problem with high molecular weight media such as Ficoll, because these diffuse very slowly. In practice the best way to minimise the effects of

droplet sedimentation is to begin centrifugation immediately after layering the cells so allowing little time for diffusion to occur.

The number of cells and volume of the suspension layered onto the gradient is important. The minimum number is solely determined by the sensitivity of the assay procedure to be used. The maximum number is dependent on the slope of the gradient. A given slope can only tolerate a certain concentration of cells in a zone before the zone becomes unstable and broadens. Increasing the gradient slope can allow larger numbers of cells to be added but the constituent increase in viscosity results in a narrowing of the bands, which effectively increases cell concentration in the zone.

The volume of cell suspension that can be layered onto a gradient is a function of the cross-sectional area of the centrifuge tube. As a general rule, for tubes of 1.0 – 1.6 cm diameter, the cell suspension volume should not exceed 0.5 ml. With larger tubes of 2.5 cm diameter, the volume should not exceed 2.0 ml. The greater the volume of cell suspension layered, the broader the band formed and the less the resolution.

Much larger numbers of cells can be used in isopycnic centrifugation where cells are mixed with the gradient medium and the gradient allowed to form in situ. The number of cells used on preformed isopycnic gradients can be increased by mixing them with the gradient medium in the gradient maker as the gradient is poured.

The exact method employed to layer cells onto a gradient is a matter of personal choice. In principle any method which allows cells to fall gently onto the top of the gradient without disturbing it can be used. One of the simplest methods is to use a Pasteur pipette or automatic pipetter, the cells are sucked up into the pipette and gently allowed to flow down the side of the tube onto the gradient.

An alternative method of loading cells onto a gradient is the flotation method. The cells are mixed in a dense solution of gradient medium and layered under the density gradient. When centrifuged, the cells float up to their isopycnic positions.

3.3.9. Fractionation of gradients

The removal of cells from a gradient after centrifugation, requires considerable care. There is little point in taking great care during gradient preparation and sample loading only to disturb the bands of cells by mishandling. When removing the tubes from the centrifuge rotor be sure to handle them carefully so that the gradient is not disturbed. The formation of convection currents that could disturb the gradient should be avoided by keeping the tubes at the same temperature as the rotor. Any pellet of cells should not be disturbed during fractionation.

The length of tubing used to connect the tube to the collecting vessel should be kept as short and as narrow as possible to prevent excessive mixing occurring in the tubing.

3.3.9.1. Direct unloading. The easiest way of directly unloading a gradient is to pierce the bottom of the tube with a sharp needle and allow the gradient to drip into suitable collection vessels but this method cannot be used when a pellet of cells is present. The main problem with this method is that it is difficult to accurately control the rate of flow of medium out of the tube since it is not always possible to produce exactly the same size hole. The flow can be controlled by attaching a syringe to the top of the tube via a bung with a needle through it and gently forcing medium out of the tube.

Commercial tube piercing apparatus is available which can be an improvement. The apparatus holds the tube tightly, allowing a small hollow needle to be screwed into the bottom of the tube.

The outflow is connected to a drop counter or peristaltic pump and into a suitable collecting vessel. Problems that can be encountered with this method are, variation in drop size and mixing caused by the peristaltic pump, both resulting in loss of resolution. It should also be remembered that centrifuge tubes are ruined by piercing a hole in the bottom!

Gradients can be unloaded directly from the top by passing a long narrow bone needle through the centre of the gradient to the bottom

of the tube and pumping out the gradient via a peristaltic pump. The major problem with this method is that it is difficult to recover the high density fractions that are close to a cell pellet and since denser medium is pumped out in advance of less dense, inversion of the gradient may occur in the needle or tubing before reaching the collecting vessel.

3.3.9.2. Displacement unloading. Upward displacement is probably the best method of unloading gradients although it does require some special apparatus. The principle is to displace the gradient upward out of the tube by progressively adding a dense solution to the bottom of the tube. The dense solution gradually forces the gradient upward. Dense sucrose solutions (> 55% w/w) can be used as displacement media but such solutions are viscous and difficult to handle. Maxidens is a water-immiscible organic liquid with a density of 1.9 g/ml and is marketed by Nycomed especially for displacement unloading of gradients. Maxidens is inert, non-viscous and does not react with gradient media.

The displacement medium can either be introduced via a narrow bore needle passing through the centre of the gradient to the bottom of the tube or by puncturing the base of the tube. The displaced gradient medium is recovered as it leaves the top of the tube. Gradient unloaders for upward displacement are commercially produced by several companies. The one shown in Fig. 3.14 is produced by Nycomed. The hollow needle is filled with unloading medium and gently pushed through the gradient to the bottom of the tube. The top of the centrifuge tube is sealed onto a cap which has an outlet tube feeding into a collecting vessel. As the unloading medium fills the tube from the bottom, the gradient is slowly displaced upward through the outlet and is collected. The flow of unloading medium is kept constant, usually by use of a peristaltic pump. Fractions of equal volume can be collected by using a fraction collector. It is possible to displace gradients upward by gravity with the flow being controlled by using a calibrated burette, keeping the meniscus of the unloading solution above the top of the gradient. The volume enter-

ing the tube from the burette is equal to the volume of gradient medium displaced.

3.3.9.3. Direct recovery of cells. If the cells required have banded such that the bands are clearly visible they can be recovered directly without having to unload the gradient. The easiest way of doing this is to insert a Pasteur pipette or syringe fitted with a long needle and slowly withdraw the cells from the band. Another method is to use soft-walled tubes and insert a syringe directly into the bands of cells through the side of the tube.

The gradient unloader produced by Nycomed shown in Fig. 3.14 can also be used to recover cells directly from a band. The outlet tube, which for upward displacement is flush with the seal, can be lowered into the gradient. This tube is outside (concentric with) the tube containing the unloading medium. The outlet tube is lowered to just above the required cell band and as the unloading medium flows into the tube the cells are pushed upward into the outlet tube.

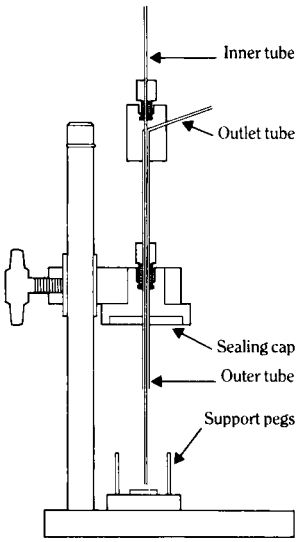


Fig. 3.14. Nycodenz gradient unloader. Courtesy of Nycomed Ltd.

3.3.10. Duration of centrifugation

For rate-zonal separations the duration of centrifugation is crucial since if too long all the cells will pellet to the bottom of the tube. The optimum time is best determined by trial and error but there are several formulae that can be used to predict the centrifugation time (e.g. Griffith 1983) although all require some previous knowledge of cell size or density which is not always available. A point worth considering is that the time of centrifugation can be greatly reduced by using gradients that are short in length. Short gradients may not always produce adequate separation but can be useful in trial experiments to determine the best conditions for centrifugation, especially when considering the expense of gradient media.

For isopycnic separation the duration of centrifugation is less critical than for rate-zonal since cells remain at their equilibrium positions. However it is still advantageous to keep the time to a mimimum to maintain viability. Again trial and error is probably the best way to obtain optimal conditions although the time for cells to reach equilibrium in a particular gradient can be predicted and computer programs are available to assist the calculations (Hames 1984, Rickwood 1984).

3.3.11. Centrifuges and rotors

There are many different types of centrifuges and rotors on the market and deciding which is best suited for a particular cell separation can be confusing. For separation of cells the issue of type of rotor and centrifuge is much simpler than for separation of soluble material since the main criterion with cells is maintainance of viability and one of the best ways of ensuring this is to use low g forces. Hence most cell separations can be conducted using low speed centrifuges and in some cases, a bench-top centrifuge will suffice. The only occasions where very high speeds are required is for the self-generation of gradients.

The basic differences between the three main types of rotor, swing-

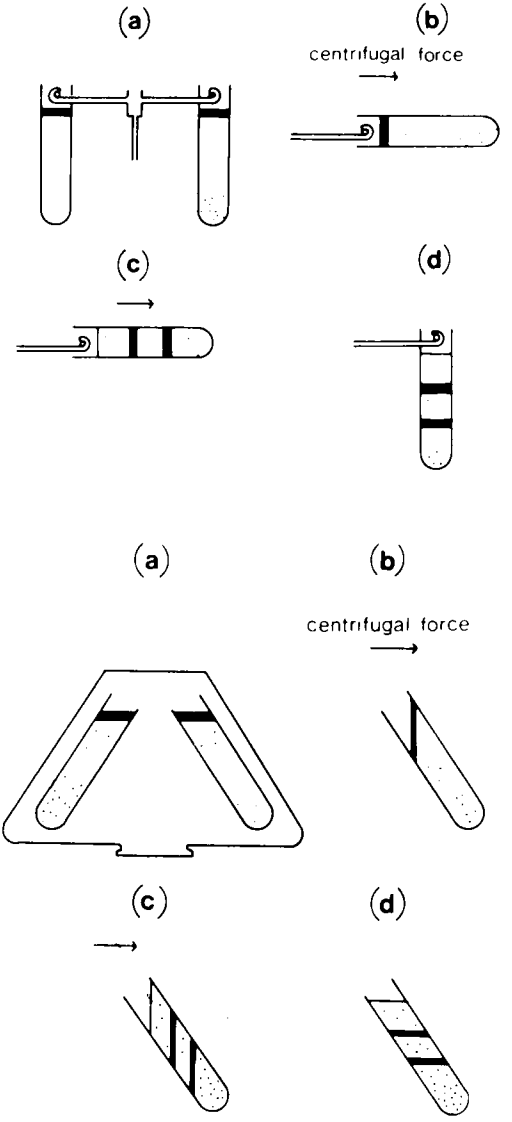


Fig. 3.15. Basic types of centrifuge rotor. (A) Swing-out rotor, (B) Angle rotor, Courtesy of Beckman Instruments.

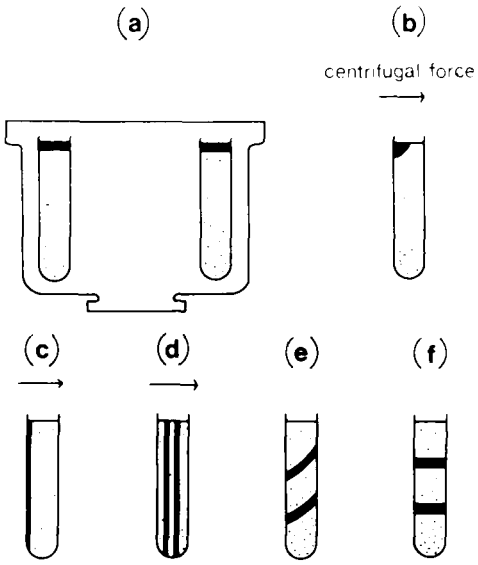


Fig. 3.15. (C) Vertical rotor. Courtesy of Beckman Instruments.

out, fixed angle and vertical are shown in Fig. 3.15. For differential pelleting of cells a bench-top centrifuge is usually the equipment of choice since the medium is low density and most cells will pellet quickly at low *g* forces. Although many bench-top centrifuges have swing-out rotors, fixed angle are more efficient for differential pelleting.

For rate-zonal centrifugation fixed angle rotors are not suitable because cells hitting the sides of the tube slide down the sides and finish in a position they should not be in. Swing-out rotors produce excellent rate-zonal separations. Vertical rotors are not very suitable for rate-zoned separations because the short path length (i.e. width of tube) does not give sufficient distance down the gradient to achieve good separation of cell bands.

In isopycnic centrifugation the effects of cells sliding down the tube walls are less significant since all the cells must eventually reach

their equilibrium positions. It is possible to use either swing-out, fixed angle or vertical rotors. However fixed angle and vertical rotors have several advantages over swing-out rotors. With both fixed angle and vertical rotors the effective pathlength of cells is shorter than in swing-out. The shorter the pathlength, the shorter the time of centrifugation needed for all cells to band at their equilibrium positions. Vertical rotors have the shortest pathlengths and are the rotors of choice for most isopycnic separations, particularly where the cells are labile and rapid separation is required.

A larger band area is formed in both fixed angle and vertical rotors as a result of reorientation of the gradients, which increases the capacity i.e. number of cells that can be accomodated by the gradient. For self-forming gradients, fixed angle and vertical rotors produce increased resolution over swing-out rotors for the same rotor speed.

3.3.11.1. Zonal rotors. Zonal rotors are now produced by all the large manufacturers, Beckman, MSE, Sorvall etc. There are several

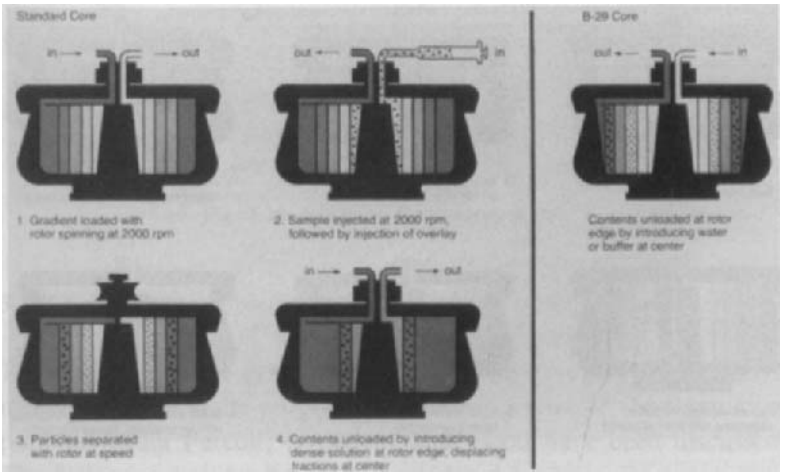


Fig. 3.16. Zonal rotor (Beckman). Cells are loaded and unloaded while the rotor is spinning.

different types of zonal rotor but the aim of all of them is to enable large numbers of cells to be separated by density gradient centrifugation. The principle of zonal rotors is essentially the same as vertical rotors except that larger volumes of gradient medium are used and there are facilities for loading and unloading while the rotor is spinning.

Zonal rotors are either of the batch type or continuous flow type. Batch-type rotors are more extensively used and several different types are available. The basic difference between the various batch-type rotors is in how cells are loaded and unloaded. Cells can be loaded and unloaded while the rotor is spinning (Fig. 3.16), can be loaded and unloaded while the rotor is stationary (Fig. 3.17), or can be loaded while the rotor is spinning and unloaded when it is stationary (Fig. 3.18). Each of these rotor types can be used for rate-zonal or isopycnic separations. Fig. 3.19 shows the principle of continuous rotors.

Zonal rotors have been discussed in detail in a previous volume of this series by Hinton and Dobrota (1980). Further details can also be obtained from the manufacturers and from Graham (1984).

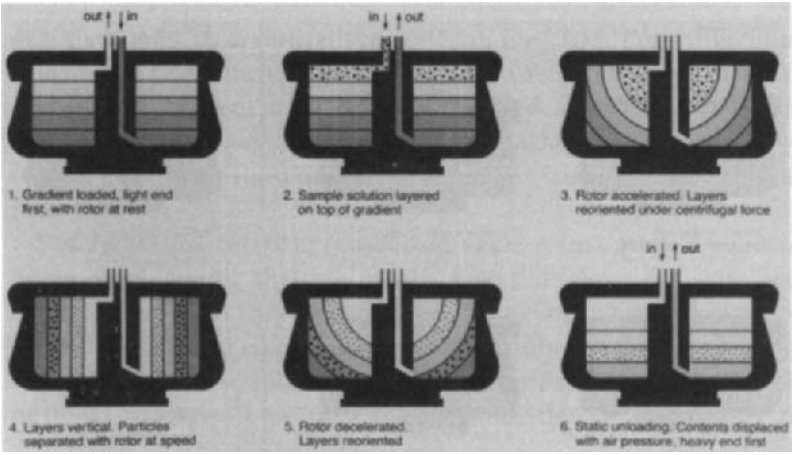


Fig. 3.17. Zonal rotor (Beckman). Cells are loaded and unloaded with the rotor stationary.

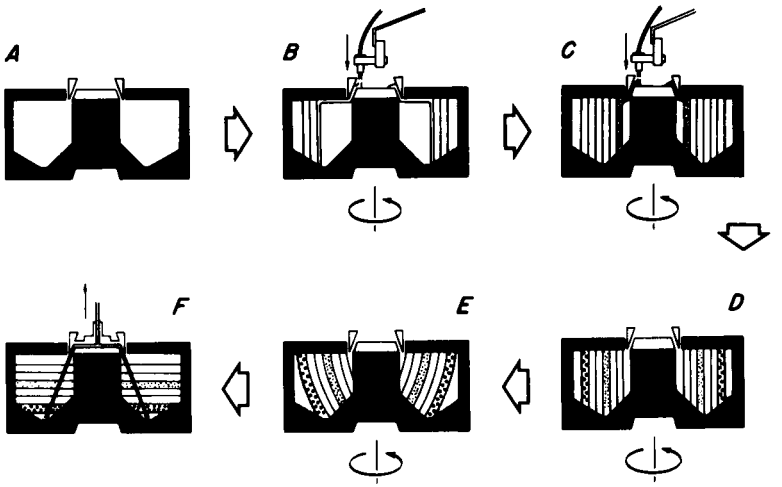


Fig. 3.18. Zonal rotor (Sorvall TZ-28). Cells are loaded while the rotor is spinning and unloaded with the rotor stationary. Courtesy of Du Pont.

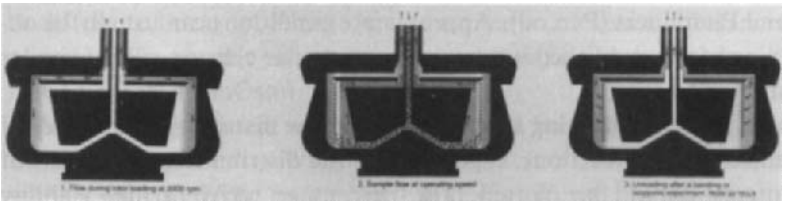


Fig. 3.19. Beckman Continuous Rotor.

3.3.12. Generalised procedure for selecting a density gradient

The selection of the gradient medium is the first step. All gradient media serve the same purpose and the properties of the three most popular media Percoll, Nycodenz and Ficoll have been discussed. The following points should be considered.

1. A method of distinguishing the required cells from all other cells

present should be selected together with a method of counting and determining viability.

2. Decide whether separation is to be on the basis of size (rate-zonal) or density (isopycnic). Density differences between cell types are usually small and rarely reflect cell function. On the other hand differences in size can be large and often reflect different functions. Unless there is good evidence that the cells of interest differ significantly in density from the rest of the population, rate-zonal is the first method to try. A critical discussion of the merits and disadvantages of both these methods can be found in Pretlow and Pretlow (1982).

3. Once a medium and method of centrifugation have been selected, the only real way to obtain the optimal conditions is by trial and error. Linear, continuous gradients are probably the easiest to start with. For rate-zonal centrifugation the most important variable is time, and again trial and error is the only real way to find the optimum time for a particular gradient. A large number of different cell types have been separated by density gradient centrifugation and extensive references lists are available from both Nycomed (Nycodenz) and Pharmacia (Percoll). Approximate conditions can usually be obtained from published work, either on similar cells or cells of similar size or density.

4. When conducting trial separations, the distribution and sizes of all cells in the fractions, together with the distribution of the cells of interest should be plotted. The percentage recovery and viability should also be determined since these may be important in selecting the conditions.

A couple of simple general points to be remembered that apply to all density gradient cell separations.

1. Always equilibrate the rotor and centrifuge chamber to the required temperature. Most centrifuge chambers will equilibrate within 30 min but a rotor may require 2 – 3 hr.

2. Always accelerate and decelerate as slowly as possible to avoid disturbing the gradients. This usually means decelerating without a brake.

3.4. Density gradient cell separations

3.4.1. Nycodenz

1. Isopycnic separation of mammalian cells on continuous iso-osmotic gradients.

Preparation of gradients:

Solution 1. 3 mM KCl, 1.15 mM EDTA, 5 mM Tris/HCl, pH 7.6.

Solution 2. 27.6 g solid Nycodenz dissolved in solution 1 and made up to 100 ml with that solution. Refractive index at 20°C = 1.3784; osmolarity = 290 mOsm; density at 20°C = 1.15 g/ml.

Solution 2 is diluted with either 0.75% NaCl (diluent A) or 7.45% sucrose (diluent B) both dissolved in solution 1 to give final concentrations of 4.6, 9.2, 13.8 and 18.4% (w/v). The osmolarities of A and B are 251 and 248 mOsm respectively. All solutions can be resterilised by autoclaving at 120°C for 20 min. Preformed gradients are produced by underlaying 2 ml of each of the required Nycodenz solutions in 14 ml tubes. Continuous gradients are formed from these by sealing the tops of the tubes and laying them horizontally for 45 min to diffuse to linearity. When sucrose is used as an osmotic balancer, a more stable osmotic profile (290 ± 10 mOsm) is obtained than with NaCl (290 ± 15 mOsm).

A variety of different mammalian cells can be centrifuged on these iso-osmotic gradients. Whole human blood, undiluted or diluted with 3 vol. of 0.9% (w/v) NaCl and centrifuged for 60 min. at around 5000 × *g* in a swing-out rotor produces two bands of red blood cells at 1.102 and 1.124 g/ml (Ford and Rickwood 1983).

Human leucocytes centrifuged under the same conditions on 4.6 – 18.4% Nycodenz gradients produce two or three bands between 1.057 and 1.081 g/ml (Ford and Rickwood 1983).

2. Separation of human blood monocytes on hypertonic gradients.

Monocytes have a lower average density than lymphocytes, but the densities overlap, which makes efficient separation difficult. A novel method of separating these cells was devised by Boyum (1983). The

rationale behind this method is that if lymphocytes and monocytes are both placed in a hypertonic medium, they will expel water and shrink. As a result of cell shrinkage the density increases and the cells sediment faster in density gradients. Lymphocytes expel more water than monocytes when in the same hypertonic medium and thus move further in the density gradient than monocytes, producing an effective separation of the two. This method may prove useful for the separation of other cell types where there is a considerable overlap in density.

Two solutions of Nycodenz with the same density but one with high and the other low osmolarity are prepared. By mixing these two solutions in appropriate proportions, a chosen osmolarity can be obtained.

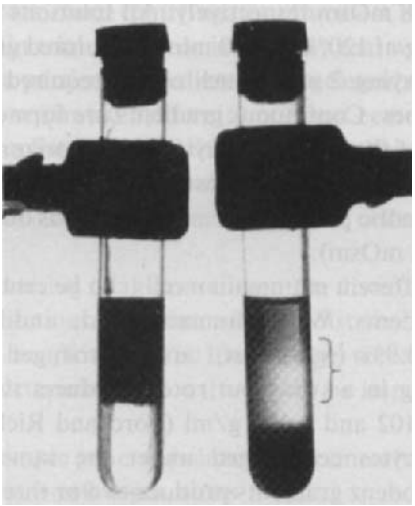


Fig. 3.20. Separation of human monocytes on a hypertonic Nycodenz density gradient (Nycodenz – Monocytes). Left, 1.5 – 6 ml leucocyte-rich blood layered onto 3 ml of Nycodenz – NaCl, density 1.068 g/ml, osmolarity 335 mOsm. Right, after centrifugation for 15 min, at 600 *g* the clear plasma is removed down to 3 – 4 mm above the interface. The remaining plasma together with slightly more than half of the Nycodenz (indicated by the brace) are collected. From Boyum (1983), Blackwell Scientific Publications – with permission.

In the experiments of Boyum (1983), 1.5 ml of leucocyte-rich blood was layered over 3 ml of Nycodenz/NaCl solution in 12 ml siliconised tubes and centrifuged for 15 min at 600 × *g*. Density increments of 0.003 – 4 g/ml were tested systematically in the 1.061 – 1.096 g/ml density range with 4 – 5 different osmolarities. No distinct band formed at the interface between the plasma and the Nycodenz solution but the Nycodenz solution had a greyish tone, mostly caused by non-settled platelets (Fig. 3.20). The clear plasma was removed down to 3 – 4 mm above the interface and the remaining plasma together with slightly more than half the volume of the Nycodenz solution were collected and the cells examined. Erythrocytes and granulocytes sedimented to the bottom and the cells in the upper half of the Nycodenz were almost exclusively lymphocytes and monocytes. Results obtained with Nycodenz of five dif-

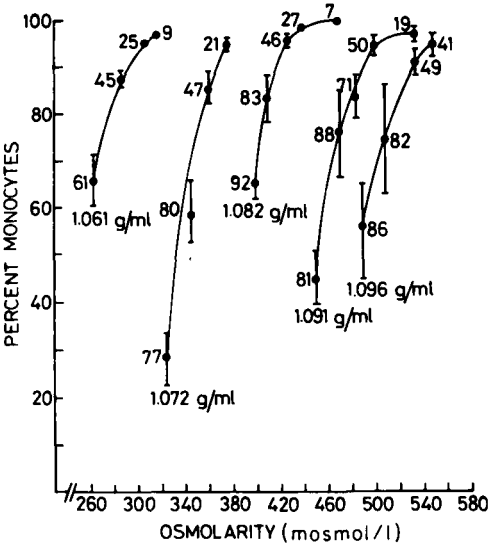


Fig. 3.21. Effect of Nycodenz density and osmolarity on the separation of monocytes from leucocyte-rich human blood. Nycodenz/NaCl solutions with densities of 1.062, 1.068 and 1.078 g/ml and different osmolarities. Mean values (± SE) for 5 experiments of percentage of the total number of monocytes recovered. Boyum (1983), Blackwell Scientific Publications – with permission.

ferent densities are shown in Fig. 3.21. Increasing the osmolarity increases the purity of the monocytes up to 97%. This trend was evident with all the Nycodenz densities used although with increased density higher osmolarities were required. The viability of monocytes purified was around 95% when tested by phagocytic capacity and less than 1% stained with trypan blue. However as Fig. 3.21 shows, the recovery of monocytes was greatly reduced with increasing osmolarity.

On the basis of these results, Nycomed now produce a preparation of Nycodenz specifically for the separation of monocytes, Nycodenz-Monocytes.

3.4.2. Percoll

1. Discontinuous isopycnic separation.

Granulocytes can be purified from human blood by a 2-step discontinuous Percoll gradient (Hjorth et al. 1981). 4 ml of isotonic Percoll with a density of 1.10 g/ml is overlayed with 4 ml of a 1.077 g/ml Percoll solution creating a 2-step gradient. 4 ml of human blood are then layered onto the gradient and centrifuged for 20 min at 350 × *g*. Lymphocytes and monocytes band at the interface be-

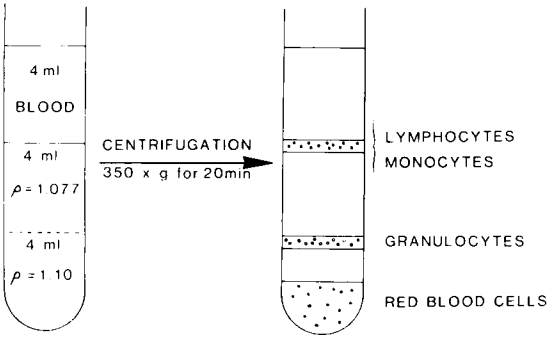


Fig. 3.22. Isopycnic separation of lymphocytes and granulocytes on a discontinuous Percoll gradient. Hjorth et al., (1981).

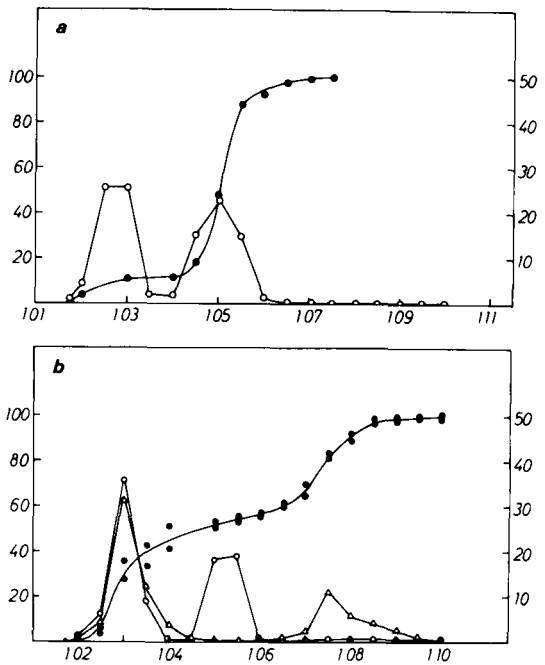


Fig. 3.23. Separation of various rat liver cells on Percoll gradients (a) ^{125}I-labelled heat-denatured albumin (9.8 μCi; 10 mg) was injected i.v into a rat 30 min before being sacrificed. 'Non-parenchymal cells' were prepared, diluted to a concentration of 4 × 10^6 cells/ml. The cells were layered on preformed gradients of Percoll (1.03 – 1.10 g/ml). The sample volume was 15 ml, on 80 ml of gradient solution. Centrifugation was performed at 8000 *g* for 60 min in a swing-out rotor. Radioactivity was measured in each fraction and the percentage of the total amount recovered was plotted vs density (○——○). Accumulated number of cells was also plotted vs (●——●); (b) ^{125}I-labelled, heat-denatured albumin was injected into a rat as described above. The 'parenchymal cell fraction' was prepared and diluted to a final concentration of 2 × 10^6 cells/ml in Eagle's MEM. After separation of the cells as in (a), the radioactivity was measured in each fraction and the percentage of the total amount recovered was plotted vs density (○——○). Accumulated number of cells was plotted vs (●——●). In an identical experiment [^{125}I]asialoceruloplasmin (21 μCi; 10 mg) was injected and the recovery of ^{125}I in this experiment is also given (△——△). Abscissa: density (g/ml); ordinate: (left) accumulated cell counts (%); (right) radioactivity (%). Pertoft et al. (1977), Academic Press – with permission.

tween the blood and the 1.077 g/ml Percoll, the granulocytes band between the two Percoll solutions and the red cells pellet to the bottom of the tube (Fig. 3.22). Occasionally, contamination of the granulocytes with red cells occurs, which is probably due to aggregation with neutrophils (Segal et al. 1980).

2. Continuous isopycnic separation.

A continuous gradient of 1.03 – 1.10 g/ml Percoll self-formed by centrifugation at 20 000 × *g* for 10 min has been used to separate hepatocytes and non-hepatocytes (Kupffer cells) from liver (Pertoft et al. 1977).

15 ml of cell suspension is layered onto an 80 ml preformed gradient and centrifuged at 800 × *g* for 30 min in a swing-out rotor at 4°C. The gradient is fractionated and the cells in each fraction counted and assayed for function and viability. Cells are localised in three areas of the gradient. Non-viable cells accumulate on top of the gradient, phagocytic, non-parenchymal cells (Kupffer cells) between 1.04 – 1.06 g/ml and parenchymal cells between 1.07 – 1.09 g/ml (Fig. 3.23).

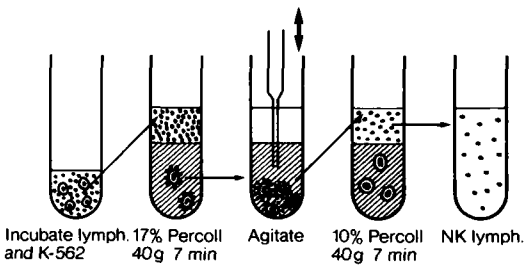


Fig. 3.24. Separation of natural killer cells from lymphocytes. K-562 cells are incubated with lymphocytes and the cell suspension layered onto a 17% solution of Percoll. After centrifugation for 7 min at 40 *g* the large rosettes formed between the K-562 cells and natural killer cells pass through the Percoll and are separated from the remaining lymphocytes. After agitation the natural killer cells are separated from the K-562 cells by centrifugation through a 10% solution of Percoll where the K-562 cells pass through the Percoll layer. From Saksela et al (1979), Munksgaard – with permission.

3. Rate-zonal separation.

An elegant method of separating natural killer cells from a lymphocyte preparation based on rate-zonal centrifugation in Percoll has been described by Saksela et al. (1979). K-562 is a cell line that contains a specific marker for natural killer cells. When incubated with lymphocytes, K-562 cells from rosettes with the natural killer cells. The cell suspension is layered onto a 17% solution of Percoll and centrifuged for 7 min at 40 × *g*. Only the large K-562 – natural killer cell rosettes pass through the Percoll in this time, leaving all other lymphocytes behind. The rosettes are then disrupted by agitation and layered onto a 10% solution of Percoll and recentrifuged for 7 min at 40 × *g*. The K-562 cell pass through the Percoll leaving purified, functional, natural killer cells (Fig. 3.24).

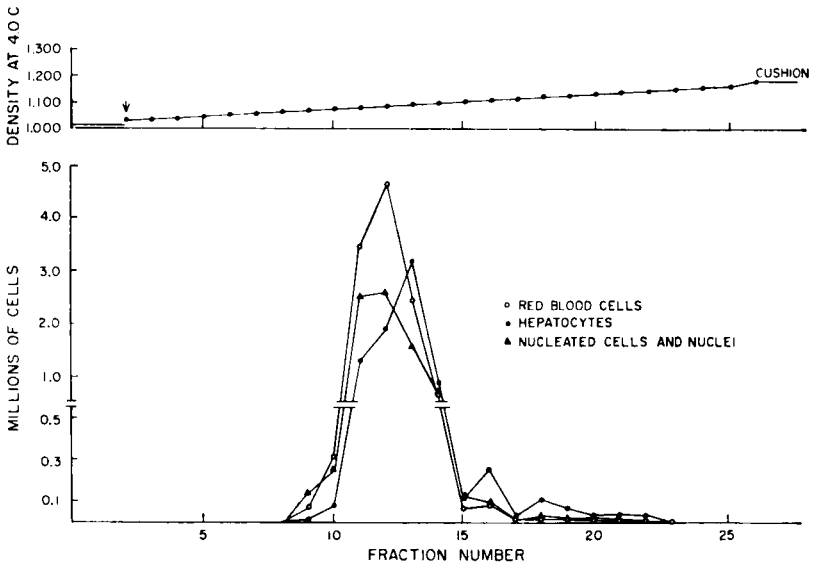


Fig. 3.25. Separation of hepatocytes from other liver cells by isopycnic centrifugation (90 min at 950 g) on an isokinetic Ficoll gradient. From Pretlow and Williams (1973), Academic Press – with permission.

3.4.3. Ficoll

Separation of hepatocytes.

Pretlow and Williams (1973) have compared the separation of hepatocytes from other liver cells by isopycnic and rate-zonal centrifugation in isokinetic Ficoll gradients. Isopycnic centrifugation was carried out in Ficoll gradients that varied linearly from 4.1% at the top (14.9 cm from the centre of rotation) to 43% at the bottom (26 cm from the centre of rotation). 7 ml of cell suspension containing $1.0-3.0 \times 10^7$ cells were layered onto the gradient and centrifuged for 90 min at $950 \times g$. The gradient was fractionated into 3 ml fractions and cells in each fraction counted and assayed, Fig. 3.25 shows the resulting distribution.

Rate-zonal centrifugation was carried out on Ficoll gradients that varied linearly from 2.4% at the top (13.7 cm from the centre of rota-

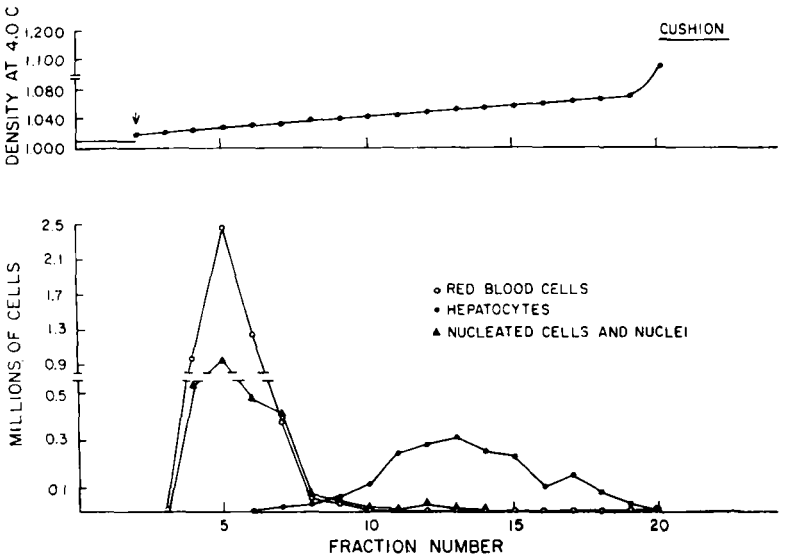


Fig. 3.26. Separation of hepatocytes from other liver cells by rate zonal centrifugation (15 min at 97 g) on an isokinetic Ficoll gradient. From Pretlow and Williams (1973), Academic Press – with permission.

tion) to 18.5% at the bottom (26 cm from the centre of rotation). Cells were layered as per isopycnic separation (above) and centrifuged for 15 min at 97 $\times$ g at 4°C. The gradient was fractionated into 4 ml fractions. The resulting distribution is shown in Fig. 3.26. It is clear from these results that in this particular example, rate-zonal centrifugation produces a much better fractionation than isopycnic.

CHAPTER 4

Unit gravity sedimentation

4.1. Introduction

Separation of cells using the centrifugation methods described in the previous chapter is the most widely used method for separating cells of different size or density. Isopycnic centrifugation is a particularly powerful method for separating cells of different densities. The majority of cells do not, however have major differences in density but do vary considerably in their size. For example, the densities of most mammalian cells lie in the narrow range 1.01 – 1.005 g/ml. The sizes of these cells however varies between 2.5 – 15 μm diameter. Methods have therefore been sought that separate cells purely on the basis of size and that overcome the difficulties and limitations of rate-zonal centrifugation. One such method is unit gravity sedimentation (velocity sedimentation at 1 $\times$ g). This method separates cells on the basis of size differences by utilising the fact that cells in a medium of low density sediment principally on the basis of differences in size, and density has little effect. The principle of unit gravity sedimentation is essentially the same as rate-zonal centrifugation except that sedimentation is allowed to proceed under the influence of the earths gravity (1g), in a very shallow gradient.

The sedimentation velocity of a spherical cell falling through a fluid under the influence of the earths gravity, from Stokes Law is;

$$S = \frac{2(\varrho - \varrho 1)gr^2}{9n}$$

where, n = coefficient of viscosity, ϱ and $\varrho 1$ = densities of cell and fluid medium, g = acceleration due to gravity and r = radius of cell.

It can be seen that where the difference in density between the cells and the gradient is small ($\varrho - \varrho 1$), sedimentation is roughly proportional to cells size (r) at constant gravity. In unit gravity sedimentation, a very shallow density gradient is used so that sedimentation (and hence separation) is related to differences in cell size. Density can however have an effect when the densities of cells differ markedly.

4.2. STAPUT apparatus

Several different types of apparatus have been developed for unit gravity cell separations. In each of the different forms of apparatus, cells start as a narrow band near the top of a shallow gradient of fluid through which they sediment. The function of the gradient is to prevent convection and mixing of the different layers of separated cells during unloading. All the different types appear to produce very similar cell separations. The simplest type of apparatus, the 'STAPUT', has been described by Miller and Phillips (1969) and is shown diagramatically in Fig. 4.1. The apparatus consists of a gradient making device connected to a sedimentation chamber. The sedimentation chamber is constructed from transparent plastic as a cylindrical U or V shaped cavity. An outlet (G) is cut into the bottom of the chamber through which fluid enters and leaves. The outlet is connected to a three-way valve (F). On both the inlet and drain sides of the valve are some form of flow regulators such as peristalic pumps. The inlet side is connected to gradient making vessels. The first is a small vessel (C) which is used to introduce the cell suspension into the chamber. This vessel is connected to two other larger vessels which are used to produce the gradient in a similar way to that

already described for producing density gradients for centrifugation.

A version of the STAPUT apparatus named 'Peri-put' is commercially produced by Biosep and has been developed by Sheeler and Doolittle (1980) (Fig. 4.2). A feature of the Peri-put is that the gradient can be unloaded from the top by pumping a cushion solution in through the lower part which displays the gradient. A series of collection parts also improves recovery of cell bands.

The following procedure is described for conducting a separation in a 500 – 600 ml capacity STAPUT sedimentation chamber with a gradient of 1 – 2% BSA where each mm in the cylindrical region contains 11.4 ml of fluid with a gradient of 1 – 2% BSA (Miller 1976; Miller and Phillips 1969).

1. The apparatus should be set up in a cold room (4°C) with gradient making vessels of internal diameter 7 cm and sample loading vessel 2.2 cm.

2. Fill all the connecting tubing on the chamber side of C with

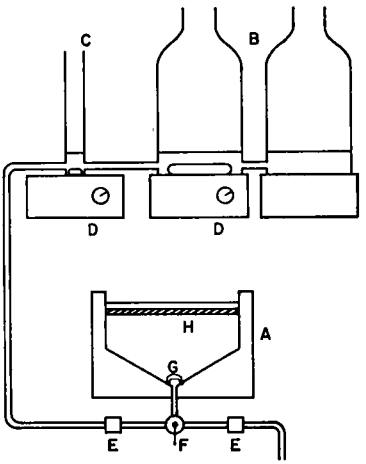


Fig. 4.1. 'STAPUT' type unit gravity sedimentation apparatus. A, sedimentation chamber; H, shallow density gradient; G, baffle and outlet; F, three-way valve; E, pumps; B, gradient maker; D, stirrers; C, vessel for introducing cell suspension. Miller and Phillips (1969), Alan R. Liss – with permission.

suitable isotonic buffer (in this case PBS), making sure no air bubbles are trapped.

3. Centre the baffle (G) inside the cone of the chamber.

4. Clamp all the tubing between the three gradient chambers and load 300 ml of 1% BSA (4°C) into the left hand vessel of B and 300 ml of 2% BSA (4°C) into the right hand vessel.

5. Introduce 30 ml of PBS into the chamber via the small vessel C. This is to prevent disturbance of the cell band by erratic movements of the rising fluid meniscus as the chamber is filling.

6. Load 20 ml washed cells in 0.2% BSA (PBS) into the chamber via C ensuring that the tubing is clamped the instant the buffer chamber empties.

7. Thoroughly rinse the vessel C with 0.2% PBS in buffer making sure that no air bubbles enter the tubing.

8. Fill C with 0.35% BSA in buffer to the same level as the larger vessels B and set the flow rate for 2 – 3 ml/min.

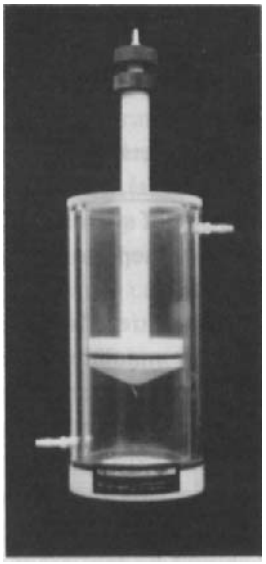


Fig. 4.2. Peri-put unit-gravity sedimentation apparatus.

9. Remove all clamps and the gradient will load itself.

10. Once the cells have been lifted off the bottom the flow rate can be increased to a rate just below that which disturbs the cell band. Loading should be as rapid as possible (10 – 15 min) with the time between cell loading and gradient loading being under 8 min.

11. A steep gradient of 0.35 – 1.0% BSA followed by a shallower gradient up to 2% BSA will be produced.

12. Allow the cells to sediment for between 2 – 4 h (depending on the particular cell type).

13. Start to unload the chamber through the bottom with a flow rate of around 30 ml/min discarding the volume in the cone.

14. Collect the remainder of the gradient in equal sized fractions of around 15 ml.

The above method is for producing a non-linear gradient of BSA. A linear gradient can be produced with the same set up, where the sedimentation chamber C is used as a subsiduary mixing vessel for the 1 – 2% BSA from B after the cells have been loaded. The advantages of using such a non-linear gradient are discussed below.

4.2.1. The Gradient

Gradient materials that have been used are buffered solutions of BSA (1 – 2%), Ficoll (2 – 4%), FCS (3 – 30%) and sucrose (0.5 – 3%). With the exception of sucrose, gradients of all these solutions appear to produce similar separations and preserve cell viability.

Low densities of gradient solutions are used which are well below the densities of the cells. For mammalian cells, gradients between 1.004 – 1.009 g/ml are adequate. Since the gradients are so shallow there is no problem in maintaining osmolarity through the gradient.

Cell viability is best maintained by conducting the separations at 4°C. Care must be taken to ensure the gradient is fully equilibrated at 4°C, otherwise resolution will be lost as a result of convection. Similarly, the chamber should be free from all vibrations that may disturb the gradient.

Two types of gradient have been described for use with the STAPUT apparatus (Miller and Phillips 1969). These are non-linear or 'buffered step' gradients which are produced as described above, or simple continuous gradients can also be used. The buffered step gradient is where there is a sharp increase in gradient density immediately below the cell band which thereafter gradually increases. The advantages of using this shape gradient are that it allows the chamber to be loaded quickly since the steep increase in density prevents disturbance of the cell band during loading and it also reduces the effect of cell 'streaming'.

4.2.2. Cell number and streaming

The single most important factor in achieving cell separation with unit gravity sedimentation is the concentration of cells in the starting band. As with density gradient centrifugation, resolution is greatly improved when the cells are layered as a narrow band onto the gradient. The width of each separated cell band will increase with time of sedimentation due to diffusion, and hence, in theory, maximum resolution will be obtained with cells layered in as narrow a band as possible. However, when concentrated cell suspensions are layered as a narrow band a phenomenon called 'streaming' often occurs, where hundreds of uncontrolled streams of single cells fall rapidly from the cell band. Such streams can be up to 1 cm long and severely impair resolution. Streaming appears to be due to a difference in density and discontinuity in viscosity between cell sample and gradient (Tulp and Bont 1975). There appears to be a critical cell concentration at which streaming occurs which is different for different cell types. The streaming limit is inversely related to the cell volume; thus larger cells have a lower streaming limit than smaller cells. This phenomenon severely restricts the total number of cells that can be loaded since they must be loaded as a narrow band i.e. a small volume. Separation chambers with large volumes can be used to overcome this because they have greater cross-sectional area upon which cells can be layered. However little is gained by this because the volume of gra-

dient material and number of fractions obtained is also increased with larger vessels.

Streaming can be reduced by raising the viscosity of the cell suspension medium with polyethylene oxide providing the density of the cell sample does not exceed the density at the top of the gradient (Bont et al. 1979). This approach may not however decrease the streaming limit for all types of cells (Miller 1976).

Streaming may also be reduced by using a buffered step gradient. The steep gradient immediately below the cell layer reduces the streaming effect allowing higher cell concentrations to be used. The streaming limit for erythrocytes in the STAPUT apparatus with a linear gradient is about 5×10^6 cells/ml. With a buffered step gradient the limit increases to 1.5×10^7 cells/ml. (Miller 1976).

4.2.3. Sedimentation Velocity

There is some debate concerning whether useful values of sedimentation velocity can be calculated from sedimentation at unit gravity (Catsimpoolas and Griffith 1977). The major argument is that factors other than cell size that can affect sedimentation velocity, such as cell shape, surface roughness and membrane deformability will do so to a much greater extent because sedimentation is slow. It has also further been shown that bands of separated cells obtained are considerably broader than would be expected from simple diffusion. Miller and Phillips (1969) have measured the sedimentation velocity of sheep erythrocytes in a buffered step gradient and obtained values that agree well with calculated values. Such agreement may not however be obtainable for all cells.

Sedimentation velocity can be measured by following the method and calculations described by Miller (1976). Once a unit gravity sedimentation has been carried out, and the number of cells in each fraction counted, the distribution obtained needs to be plotted. One common way to plot the results has been to plot cell number against sedimentation velocity. In view of the doubts raised regarding the accuracy of sedimentation velocity values, it is probably better to plot cell number against fraction number.

4.2.4. Separation of mouse spleen cells using the STAPUT apparatus. (Phillips and Miller 1970)

A buffered step gradient of FCS, 5 – 15% immediately below the cells thereafter increasing gradually to 30% was used. 1.0×10^8 cells in 20 ml 3% FCS (in PBS), were loaded and allowed to sediment for 3.5 h at 4°C. The chamber was unloaded and the number of cells in each 15 ml fraction counted and plotted (Fig. 4.3). The volume

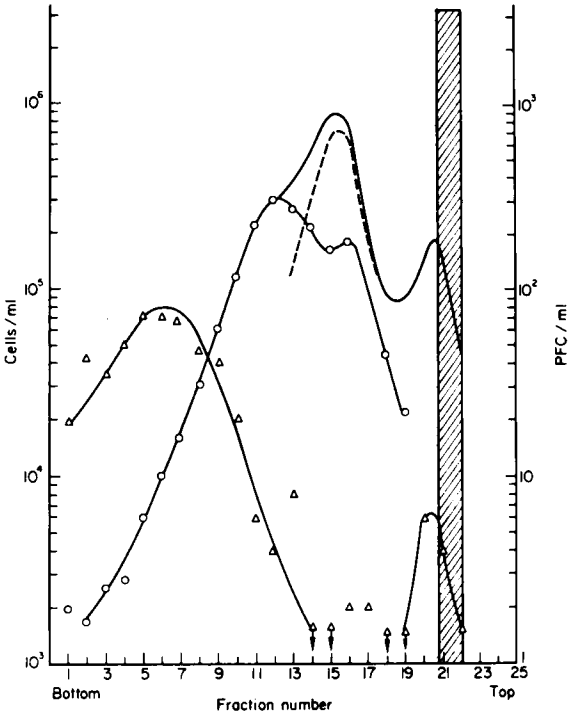


Fig. 4.3. Sedimentation profile of mouse spleen cells 4 days after immunisation with sheep erythrocytes (SRBC) on a FCS gradient in a STAPUT apparatus. The hatched bar is the original position of the cell band. Fractions are numbered in order of collection from the bottom of the chamber. (○——○) nucleated cells/ml; (——) total cell count; (– – –) erythrocytes/ml; (△——△) numbers of 19S-PFC cells/ml (cells producing anti-SRBC antibody). Philips and Miller (1970), Blackwell Scientific Publications – with permission.

distributions of cells from four fractions were analysed using a Coulter counter with channalyser (Fig. 4.4). The cell distribution obtained (solid line) is broader than would be expected if only a single cell population were present, which sediment to produce a Gaussian cell distribution. Cells from each of the fractions were assayed for various markers and revealed several different cell populations. The distributions of nucleated cells, erythrocytes, and antibody producing cells are indicated. Erythrocytes sedimented as a narrow band with a rate of 2.0 mm/h. Two bands of nucleated cells at 1.9 and 2.9 mm/h and a small peak of cells directly beneath the starting band were detected.

Upon microscopic observation, not all the cells counted by the Coulter counter were intact, viable cells. No cells were visible in fractions just below the starting band and this peak would appear to be cell debris. Although cells were visible in the peak of slow sedimen-

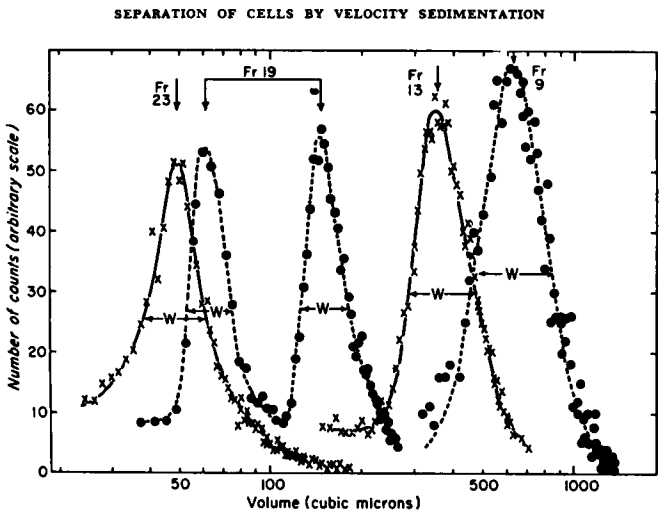


Fig. 4.4. Volume distributions for intact cells from fractions 9, 13, 19 and 23 of Fig. 4.3 obtained using a Coulter Counter and Channalyser. W = full width at half height of each peak. Philips and Miller (1970), Blackwell Scientific Publications – with permission.

ting nucleated cells (1.9 mm/h), these did not incorporate vital dyes, nor has any function yet been ascribed to them, and it would appear that these are damaged cells. The most striking feature of this cell separation is that antibody producing cells are exclusively located in the low number fractions where cells have high sedimentation velocities.

As can be seen from Fig. 4.4, cells isolated from different fractions have different volumes. The largest cells are detected in high numbered fractions (rapid sedimenting). Two cell peaks with different volumes are seen in fractions 18 – 20. These correspond to a high-sedimentation velocity tail of the erythrocyte peak at 2.0 mm/h and a low sedimentation velocity tail of the nucleated cell peak at 3.0 mm/h.

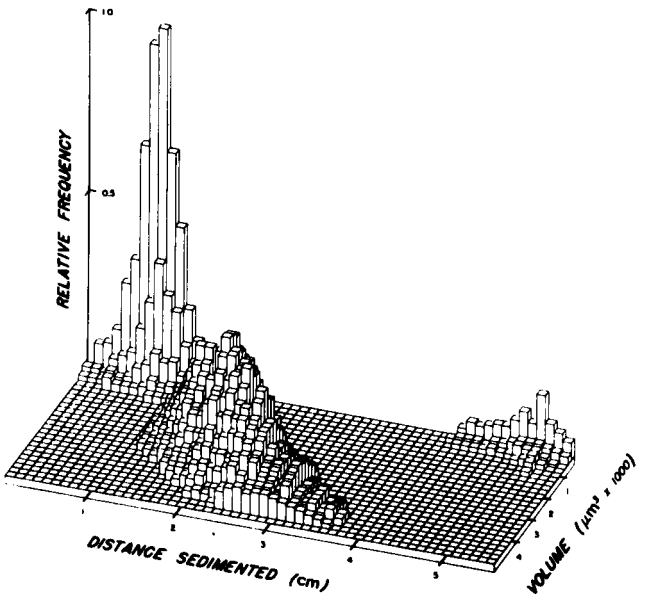


Fig. 4.5. Perspective plot of the separation of teratocarcinoma tumour cells into three size-density classes. Unit-gravity sedimentation on a linear sucrose gradient in a Peri-put apparatus. Sheeler and Doolittle (1980), with permission.

4.2.5. Fractionation of teratocarcinoma tumour cells using the Peri-put apparatus (Sheeler and Doolittle, 1980)

A 1 – 3% (w/w) linear sucrose gradient in HBSS has proved effective for fractionating teratocarcinoma cells grown in 129/J mice on a Peri-put apparatus. Results obtained from a 90 min separation are shown in Fig. 4.5. Three cell populations can be obtained, although the relative numbers of cells in each population varied from tumour to tumour, which probably reflects the age of the tumour. Both the size, volume and density of the cells in each population were measured. The three cell populations have different densities, with two of the populations having a similar size.

4.3. Other types of apparatus

Three alternative types of apparatus have been used to separate cells by unit gravity sedimentation all of which are commercially available. One utilises an apparatus produced for electrophoresis, the Buchler Poly-Prep 200, the other two, the LACS and the Celsep are specially produced for unit *g* cell separation. One of the advantages of these apparatus is that the cells are loaded directly onto the gradient thereby avoiding any problems of cells sticking to the chamber during upward loading.

4.3.1. Buchler Poly-Prep 200. (Buchner Instruments)

The main advantages of this method are that the apparatus provides rapid thermal equilibrium, small volumes of solution can be used (100 – 150 ml), fractions can be collected without mixing and the apparatus has a dual role since it can also be used for cell electrophoresis.

Fig. 4.6 shows a diagram of the apparatus. Details of the setting-up procedure can be obtained from Catsimpoolas et al., (1978) and will not be described here. The arrangement of gradient solutions of

BSA in the column are as follows; 80 ml of 3% BSA forms the bottom solution which acts as a cushion for the density gradient and also serves as a chase solution during elution. 100 ml of gradient consisting of 1 – 2% BSA/PBS giving a density range of 1.0099 – 1.0123 at 4°C is pumped into the column at a rate of 1 ml/min. 10 ml of cell suspension at 5×10^6 cells/ml in 0.5% BSA are layered on top of the gradient immediately followed by 10 ml of PBS. An advantage of being able to load cells on top of the gradient is that it avoids problems caused by large cells attaching to the baffle used in the STAPUT apparatus.

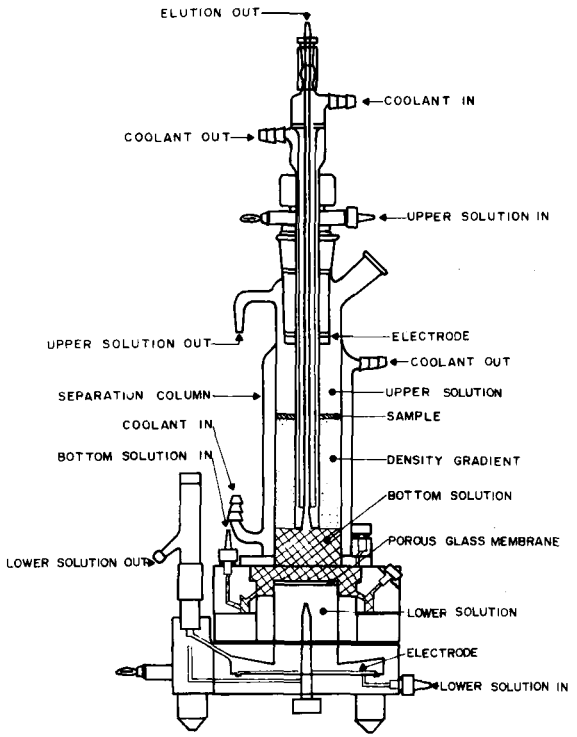


Fig. 4.6. Buchler Poly-Prep 200 unit-gravity/electrophoresis apparatus. Academic Press – with permission.

After the desired time of sedimentation, the bottom solution is pumped out at 1.0 ml/min through the bottom solution inport while simultaneously the gradient is pumped out through the elution capillary and collected as 1.5 ml fractions.

A fractionation of human blood cells by this method is shown in Fig. 4.7. The open circles represent white cells (mononuclear MN and polymorphonuclear PMN), the closed circles are erythrocytes (RBC) and platelets (Plt). Polymorphonuclear cells are clearly separated from mononuclear cells (monocytes and lymphocytes) although erythrocytes are not separated from mononuclear cells. An enriched platelet fraction, relatively free of other cells is also obtained.

4.3.2. LACS cell separator. (De Koningh BV)

The principle aim in construction of the LACS separator has been to increase the numbers of cells that can be separated and decrease the time required for separation. The gradient is poured into a long rec-

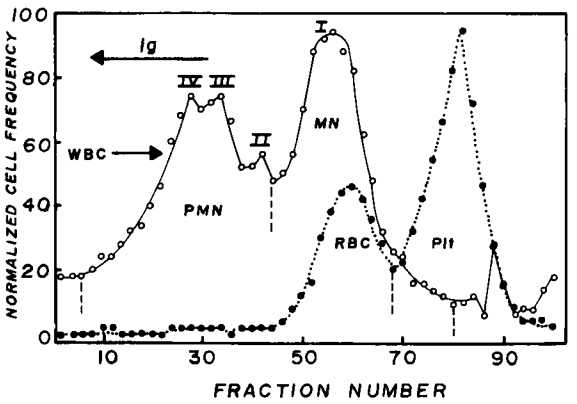


Fig. 4.7. Separation of human blood cells at unit gravity on BSA gradient in a Buchler Poly-Prep apparatus. (○——○) white blood cells (WBC), (●——●) erythrocytes (RBC) and platelets (plt). MN = mononuclear cells, PMN = polymorphonuclear cells. Each distribution has been normalised for peak height by computer. Catsimpoolas et al. (1978), Academic Press – with permission.

tangular perspex cylinder of inner dimension, 3 × 5 × 60 cm. (Fig. 4.8). Cells are layered on top of the gradient and the cylinder is slowly rotated to the horizontal position and the cells allowed to sediment (Fig. 4.9). After sedimentation, the cylinder is rotated back to the vertical position and the gradient unloaded into fractions.

Allowing sedimentation to take place in the horizontal position decreases the thickness of the gradient layers and cell layer by a factor of 20. The accompanying increase in surface area results in rapid formation of a continuous gradient and sedimentation of cells. A LACS separator is shown in Fig. 4.8 and its operation shown diagrammatically in Fig. 4.9. Described below is an example of the

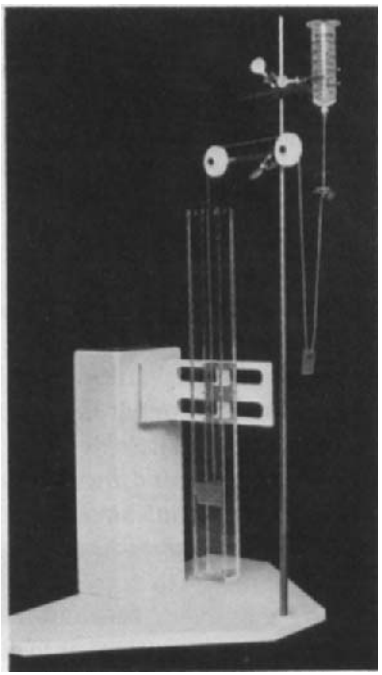


Fig. 4.8. LACS unit-gravity cell separator. (De Koningh BV).

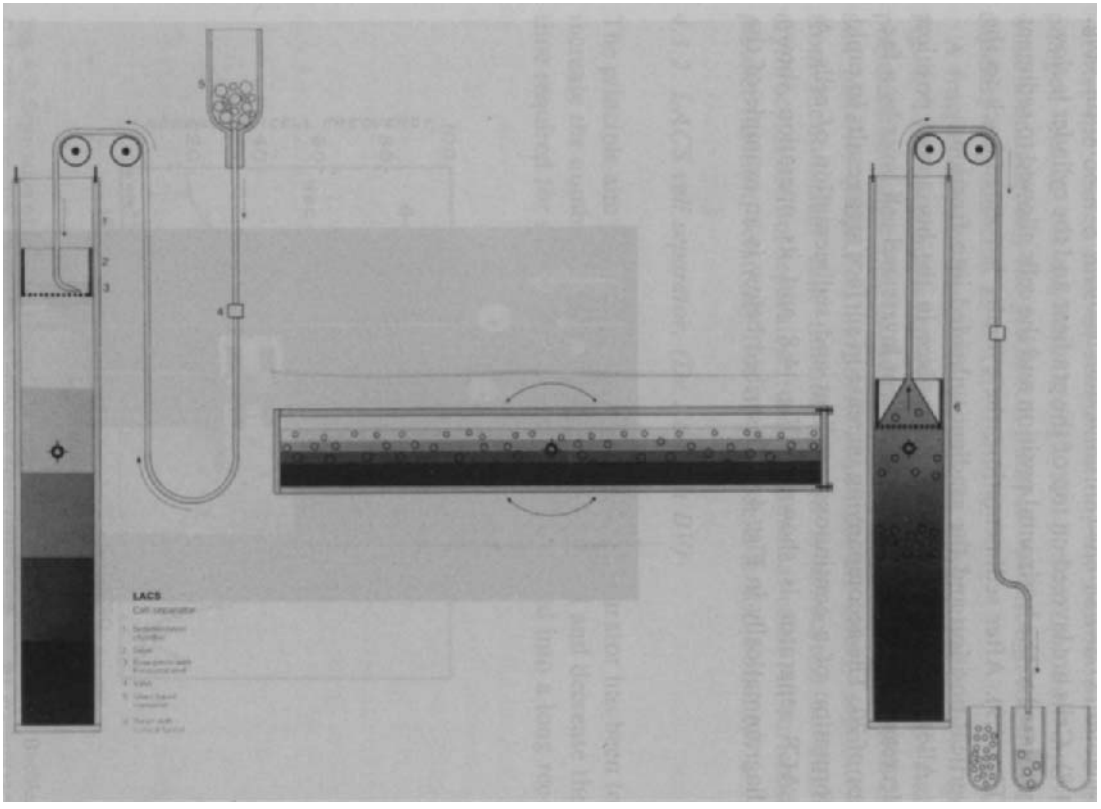


Fig. 4.9. Principle of the LACS cell separator. 1. Sedimentation chamber. 2. Sieve. 3. Flow piece with horizontal end. 4. Valve. 5. Glass liquid container. 6. Sieve with conical funnel.

use of this apparatus to separate human lymphocytes and monocytes, taken from the work of Bont et al. (1979).

4.3.2.1. Separation of human monocytes and lymphocytes on a LACS separator. Twelve, 30 ml solutions of differing densities are prepared by dilution of two stock solutions:

Solution 1. 2.5% (w/v) Ficoll MW. 70 000, 0.05% (w/v) polyethylene oxide (PEO) MW. 600 000, 2% (v/v) FCS in RPMI 1640 medium (GIbco) and buffered to pH 7.2 with 10 mM HEPES.

Solution 2. 7.5% (w/v) Ficoll, 2% FCS in RPMI 1640 buffered with 10 mM HEPES to pH 7.2.

Both solutions also contained 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml fungizone and are 300 mOsm.

30 ml of solution 1 is introduced to the separator as layer 1 (separator in the vertical position). Layer 2, layered immediately above layer 1 comprises 30 ml removed from a mixture of 15 ml solution 1 and 150 ml solution 2. The remaining 135 ml of this solution is diluted with a further 15 ml solution 1 and again 30 ml is removed and layered as layer 3. This is repeated another 8 times to give 11 layers of gradient. The 12th layer is produced from 30 ml of Solution 1.

Mononuclear leucocytes isolated from defibrinated blood are suspended at a density of 10^7 cells/ml in PBS containing 0.2% (w/v) PEO. 20 ml of cell suspension is layered onto the gradient (2×10^8 cells) and immediately overlayered with 20 ml PBS. The sedimentation chamber is closed at the top and turned to the horizontal position by means of either an electric motor or a manual hauling device. This turning does not disturb the gradient and once in the horizontal position, a continuous gradient is formed within 30 min.

The cells are allowed to sediment for 2.5 h. at 4°C and the chamber returned to the vertical position. By inverting the chamber back to the vertical position the distance between cells in different parts of the gradient is increased by a factor of 20. The gradient is fractionated, and the number of cells in each fraction counted. In this particular example the gradient is fractionated into 5 fractions.

Cells from each fraction are harvested by centrifugation at 800 g for 15 min. washed twice in cold EMEM + 1% FCS, resuspended in EMEM + 10% FCS and used for counting and functional studies. Fig. 4.10 shows a summary of the results obtained. The total recovery of lymphocytes in this example is 88% and the total recovery of monocytes is 78%. 60% of the lymphocytes obtained after fractionation are recovered in the 12 – 36 mm/h fraction. 48% of the monocytes are recovered in the 50 – 62 mm/h fraction with a purity of approximately 85%.

4.3.3. Celsep apparatus. (DuPont and Wescor)

The principle of the Celsep apparatus is similar to the LACS and is essentially the same as the 'muffin' type apparatus described by Miller (1979). The apparatus is comprised of a cylindrical chamber mounted on a tilting assembly (Fig. 4.11). The chamber is formed by two plastic plates which are clamped to a cylindrical section of 23 cm diameter. The volume of the chamber is 1000 ml. The tilting

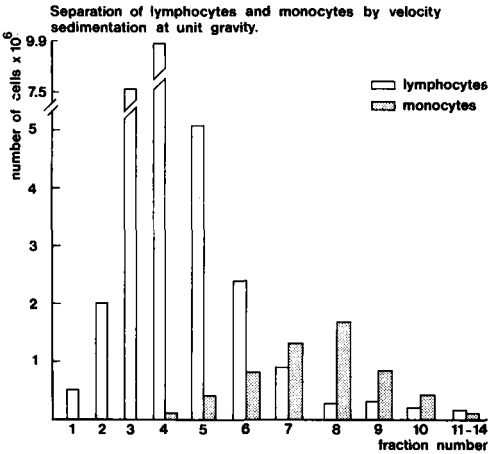


Fig. 4.10. Separation of lymphocytes and monocytes by unit *g* sedimentation. Bont et al. (1979).

assembly is motor driven and tilts the chamber from the horizontal separation position through an angle of 30° to the filling and unloading position. Fig. 4.12 shows the separation procedure. A gradient maker produces the gradient which is pumped (low density first) into the chamber through the lower port, with the chamber tilted, at a rate of 35 – 45 ml/min. When tilted, a funnel is formed around the lower port which allows rapid loading without mixing. The gradient is underlayered with 150 – 180 ml of a dense cushion of gradient material (e.g. 10 – 20% Ficoll) which prevents rapidly sedimenting cells from sticking to the bottom of the chamber. The chamber is completely filled with gradient. Approximately 25 – 35 ml of cell suspension is introduced through the upper port (15 – 20 ml/min) by withdrawing some of the cushion through the lower port and overlayered with a buffer solution (65 – 75 ml) to prevent cells being in contact with the top of the chamber. The chamber thus con-

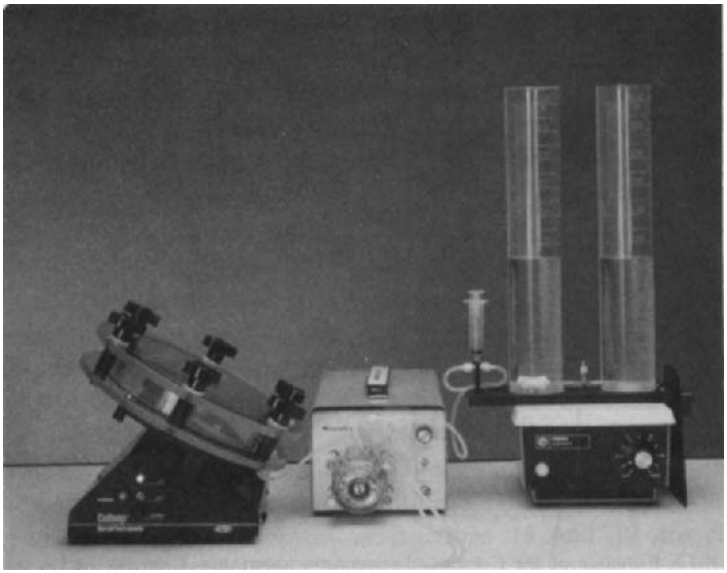


Fig. 4.11. CELSEP with gravity separation apparatus. Courtesy of Du Point.

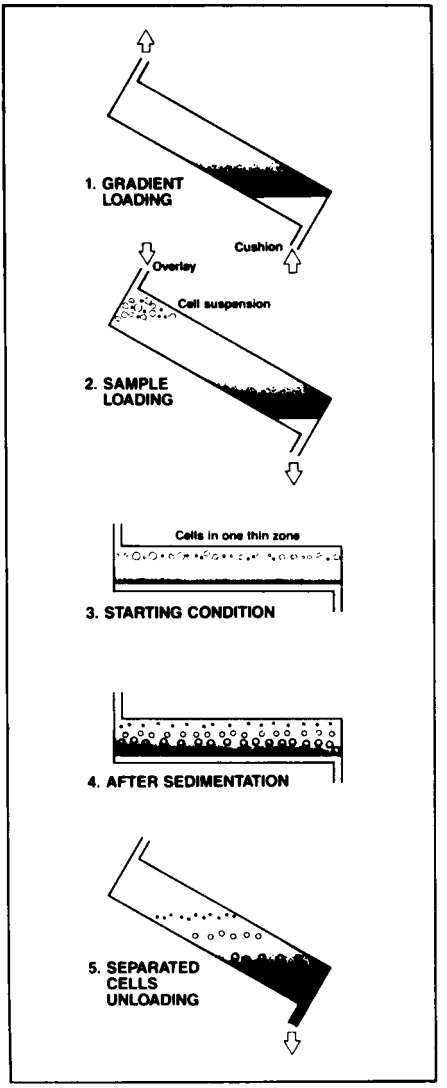


Fig. 4.12. Principle of the Celsep cell separation apparatus. Courtesy of Du Pont.

tains about 820 – 845 ml of gradient, 25 – 50 ml of cushion, 25 – 53 ml cell suspension and 65 – 75 ml of buffer overlay. The chamber is completely filled and slowly tilted back into the horizontal position. After the required period of sedimentation (1 – 2 hours) the chamber is tilted and the gradient unloaded through the lower port at 35 – 40 ml/min. The number and volume of fractions depends on the number and proximity of cell bands obtained. Fractions of 25 – 50 ml are usually adequate giving 12 – 36 fractions.

The apparatus can be sterilised and the separation procedure can be carried out in a tissue culture hood if required. The separation chamber cannot be autoclaved and gas sterilisation is recommended.

The temperature must be fairly well-controlled to prevent convection currents etc.

4.3.3.1. Separation of human monocytes and lymphocytes. (Wells 1982). This particular example of the use of the Celsep to separate human monocytes and lymphocytes is chosen so that a comparison with the LACS separator can be made.

The gradient is prepared from two Ficoll solutions, 2% and 4% (w/w) in Hanks balanced salt solution, containing 1% serum, one in each of the gradient making vessels. The cushion consists of 10% Ficoll. Mononuclear cells are prepared by the Ficoll-Hypaque centrifugation method and resuspended in 50 ml of 1% Ficoll in HBSS containing 1% human serum at a density of $1 - 1.2 \times 10^6$ cells/ml and loaded onto the gradient. Sedimentation is allowed to proceed for 2 h at room temperature before unloading into 30 ml fractions and the number of cells in each fraction is counted. Monocytes are identified by non-specific esterase staining and morphology. Lymphoid cell and basophils identified by morphology and Giemsa staining.

The results obtained by Wells (1982) are shown in Fig. 4.13. The mean recovery of all cells was 83% with more than 98% being viable (trypan blue). The monocytes in fractions 18 and 19 are contaminated with basophils and in fractions 15 – 17 appear as doubles held together by platelets. The purity of monocytes obtained in the

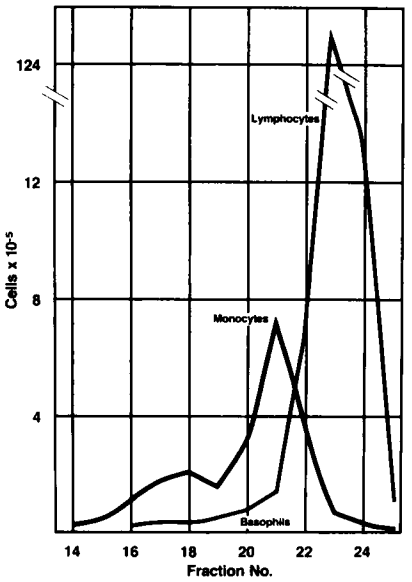


Fig. 4.13. Separation of human monocytes and lymphocytes on a Ficoll gradient in a Celsep apparatus. Wells (1982). Compare with Fig. 4.10, which shows a similar separation with the LACS apparatus.

peak fraction (20), was 69% which represents a recovery of 28%. No loss in resolution was observed when twice the number of cells were loaded in 75 ml.

Qualitatively these results are similar to those obtained with the LACS separator although the LACS separator produces a monocyte fraction of slightly higher purity and yield. This is however only one example and the general consensus is that both types of apparatus produce very similar separations in the same time period with the same total volume of gradient.

Centrifugal elutriation

5.1. Introduction

The concept of separating cells by centrifugal elutriation was devised in the late 1940s by P.E. Lindahl who designed a 'counter-streaming centrifuge' (Lindahl 1948). The principle of this apparatus and its modern day counterpart the Beckman JE-6B Elutriator Rotor, are essentially the same. Cells are separated according to their rate of sedimentation in a gravitational field where the liquid containing the cells is made to flow against the gravitational force. The liquid used is of uniform density and flows toward the centre of rotation through a conical shaped chamber, the apex of which points away from the centre of rotation. Cells are thus subjected to two opposing forces within the separation chamber, a centrifugal force generated by the spinning rotor and the counterflow of the fluid in the opposite direction. Each cell migrates to a zone in the chamber where its sedimentation velocity is exactly balanced by the flow rate of the fluid in the opposite direction. Since a uniform, low density medium is used, sedimentation rate is proportional to cell size.

Cells of different size tend to accumulate in discrete sections of the chamber. Smaller cells accumulate nearer the centre of rotation, larger cells further from the centre (Fig. 5.1). Because the geometry of the chamber produces a gradient of flow rates from one end to the other, cells with a wide range of different sedimentation rates can be

held in suspension at one time. Cells of a particular size that have accumulated in a particular area of the chamber can be eluted (elutriated) by either increasing the flow rate of the liquid or decreasing the centrifugal force (rotor speed).

The major advantage of centrifugal elutriation for separating cells over most other methods is that the fluid in which separation takes place i.e. the fluid pumped into the chamber as the counterflow, can contain virtually any constituents. Thus fluid can be used which is totally compatible with the cells i.e. buffered salt solutions, culture medium etc.

Based on the separations obtained by Lindahl and colleagues, Beckman produced a series of elutriator rotors from the mid 1960s resulting in the version currently available, the JE-6B, designed for use in the Beckman J-6B centrifuges.

5.2. Beckman JE-6B elutriator system

There are four basic parts to the elutriator system, a rotor which houses the separation chamber, a flow assembly, a stroboscope assembly and a modified centrifuge door. The door contains a small window which allows the operator to see the elutriator chamber. A fixed image of the revolving chamber is obtained by synchronising the stroboscope flash rate with the rotor speed. Banding of cells in the chamber can be seen through the window enabling the operator to control the elution of cells from the chamber.

Fig. 5.1. Principle of centrifugal elutriation. Courtesy of Beckman.

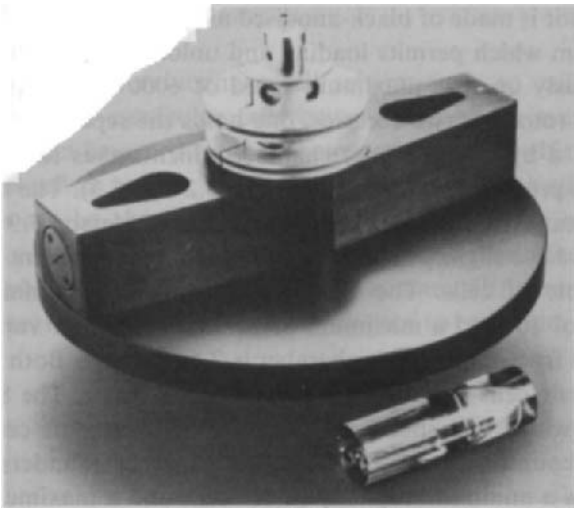


Fig. 5.2. Beckman JE-6B elutriator rotor.

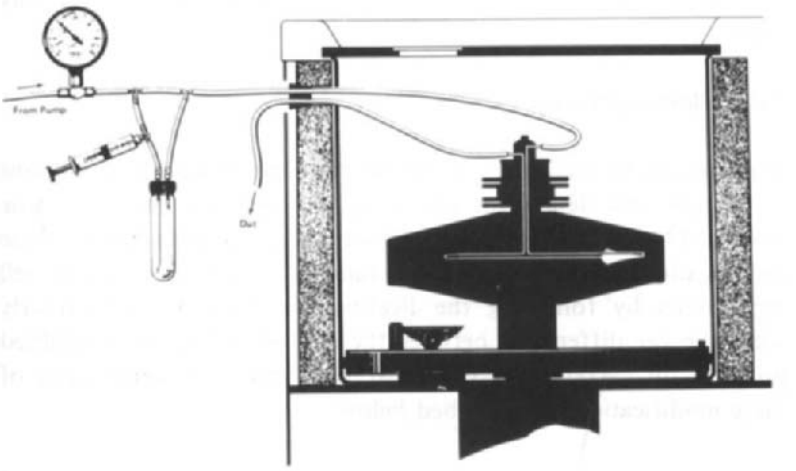


Fig. 5.3. Set-up of elutriator rotor system as described by Beckman.

The rotor is made of black-anodised aluminium and has a rotating seal system which permits loading and unloading of cells and fluid continuously up to a maximum speed of 6000 rpm (Figs. 5.2 and 5.3). The rotor has two recesses, one holds the separation chamber, the other a bypass (dummy) chamber which serves to balance the rotor and provide the fluit outlet (Figs. 5.2 and 5.3). The separation chamber can either be a standard 4.2 ml or a Sanderson 5.9 ml. These chambers have slightly different shapes and have different capacities for number of cells. The standard chamber has a minimum cell capacity of 10^7 and a maximum of 10^9. Cell diameter variation per elutriated fraction with this chamber is $2.5-5.0$ μm. Both chambers can separate cells ranging from $5-50$ μm diameter. The Sanderson chamber which was specifically designed to overcome certain problems encountered with the standard chamber (Sanderson et al. 1976), has a minimum capacity of 10^5 cells and a maximum of 10^7. The cell diameter variation is $1.5-2.5$ μm making this chamber useful for separating cells with small differences in size.

The flow system consists of a pump (usually a peristaltic model – not supplied), a gauge to monitor any back pressure in the rotor, a mixing chamber, a syringe for adding the cells and two three-way valves.

5.2.1. Basic operation

Reported in the literature are a number of significant modifications in the practical details for operating the elutriator rotor to those described by Beckman in their original setting-up instructions. These modifications have arisen out of failures to obtain adequate cell separations by following the Beckman instructions, particularly when the size differences between the cells are small. A generalised protocol for carrying out cell separations which includes many of these modifications is described below.

5.2.2. Calibration of the rotor

The first stage in conducting a cell separation is to calibrate the elutriator for the particular cells being used. As an initial rough guide, Beckman have produced a nomogram (Fig. 5.4). If the size of the smallest cells is known, approximate conditions for beginning the separation can be obtained from the nomogram. These conditions are values for flow rate and rotor speed that will retain the smallest cells. It is obvious from the nomogram that many different combinations of flow rate and rotor speed can be used to retain the cells. The

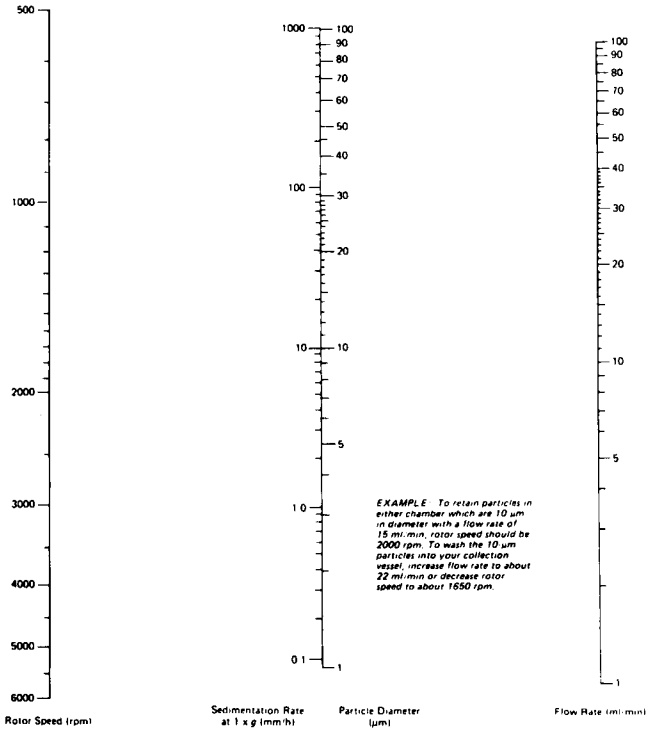


Fig. 5.4. Nomogram for estimating approximate conditions required to retain cells of a particular size in the elutriator separation chamber. Courtesy of Beckman.

actual conditions chosen will depend on the method to be used to elutriate the cells i.e. increase in flow rate or decrease in rotor speed. In general, it appears that increasing the flow rate is the most efficient method of elution largely because the flow rate can be changed much more accurately than can the rotor speed. Both the J-21 and the J2-21 centrifuges tend to overshoot when the speed is increased slightly. This is particularly evident in centrifuges that are several years old. More accurate control of rotor speed adjustment can be obtained by replacing the standard speed adjustment potentiometer with a ten-turn potentiometer, although overshoot may still occur.

The most reliable method is to operate at constant rotor speed and elutriate the cells by small increments in flow rate. Ideally the initial rotor speed should be as low as possible while permitting an initial flow rate that will retain the smallest cells and can be sufficiently increased to elutriate the largest cells. Most cell separations can be achieved by using rotor speeds between 1500 – 3000 rpm, the majority being at 2000 rpm.

When elutriating by increasing flow rate it is important that the pump be accurately calibrated. Beckman recommend a particular make of peristaltic pump but there are several reports suggesting that this particular pump is not reliable. The pump used can be modified if required by adding a ten-turn resistor and/or voltmeter across the pump motor to produce more accurate control and measurement of pump speed. A further modification is the inclusion of a flow integrator which eliminates flow pulsing due to the rollers on the pump head (Sumner and Lodola 1983).

5.2.3. Position of the pump

Fig. 5.3 shows the arrangement of the elutriator for cell separation as described by Beckman. A large number of elutriator users have found that the position of the pump as shown in Fig. 5.3 is not ideal (e.g. Worthington and Nakeff 1981). Better results are obtained by positioning the pump on the outflow side of the chamber, thus drawing fluid instead of pushing it. One of the reasons for this may be

that less turbulance is produced in the chamber by positioning the pump on the outflow.

5.2.4. Preparation of the separation chamber

In order to remove all traces of cellular and particulate debris from the separation chamber before use it can be soaked for several hours in KOH and then throughly rinsed in distilled water. Once assembled in the rotor, the chamber and the entire flow system should be flushed with about 200 – 300 ml of distilled water followed by 200 ml of elutriation buffer. If required the whole elutriator system can be sterilised by passing 500 ml of 70% ethanol or 500 ml hydrogen peroxide through the system.

5.2.5. Cell loading

The rotor is set spinning at the required speed and fluid pumped at an initial flow rate to retain all the cells. Cells suspended in fluid are introduced from the syringe into the mixing chamber where they are further mixed with fluid (elutriation buffer) and allowed to flow into the chamber in the rotor. The direction of fluid flow is controlled by the two three-way taps. Once all the cells are in the chamber the mixing chamber is by-passed and fluid continuously flows directly into the separation chamber (Fig. 5.3).

If the pump is used on the inflow end of the chamber, cells can be injected on either side of the pump. Where particularly fragile cells are being used, it is better to load after the pump so that they do not have to pass through the rollers on the pump head which may cause cell damage.

Assuming the correct starting conditions have been chosen, all the cells will remain in the chamber. By viewing the chamber through the window, bands of cells should be visible in the chamber which can gradually be elutriated by increasing the flow rate.

5.2.6. Cell numbers

The number of cells that can be loaded into a chamber at any one time depends on the type of chamber and also to some extent on the size range of the cells. The Sanderson chamber is particular useful for smaller cell numbers and has a capacity between $10^5 - 10^7$ cells. The standard chamber has a larger capacity between $10^7 - 10^9$ cells. The maximum number of cells per chamber is limited by the ability of cells of different sizes to form discrete bands in the chamber. When many cells are present the adjacent bands may overlay. The precise numbers depend on the number of bands that form and on the size of the cells, larger cells will tend to form broader bands. When smaller numbers of cells are used problems can sometimes occur even when these numbers are within the minimum range of the chamber. Turbulance in the chamber can occur as the buffer flows through with small cell numbers. Any such turbulance is 'buffered' with larger cell numbers by the cell bulk. When turbulance does occur resolution is reduced since discrete bands of cells cannot form. One way of avoiding turbulance with small numbers of cells is to increase the viscosity of the elutriation buffer by inclusion of high molecular weight molecules such as Ficoll, dextran or BSA in the fluid.

A reduction in cell recovery can also occur with low cell numbers. With loads of $6 \times 10^5 - 2 \times 10^7$ cells typical recoveries are between 80 – 90%. With numbers below 6×10^5 recovery can drop to as low as 40% in some cases (Meistrich 1983).

Elutriation of each cell population from the chamber requires about 100 ml of fluid. Thus elutriated cells of a particular size will be distributed in fractions totalling 100 ml. With high cell numbers, cells are elutriated out of the chamber at lower flow rates than those for the same cells loaded in smaller numbers. This is due to the packed volume of the cells themselves.

5.2.7. Temperature

The temperature of the fluid (elutriation medium) used in elutriator separations is a major factor in obtaining cell separations. The optimum temperature for any particular separation is dependent on the type of cells. Most work with isolated cells is conducted at 4°C and many successful elutriator separations have been achieved at this temperature. Table 5.1, taken from work by Worthington and Nakeff (1981) shows the percentage recovery of megakaryocytes after elutriation, as a function of temperature. The maximum recovery was obtained at 12°C although this was not significantly greater than at 4°C. Significantly lower recoveries were however obtained at higher temperatures. In contrast, Sanderson and Bird (1977) and Zahlten et al. (1978) reported that separation of certain cell types cannot be achieved at 4°C but separation could be achieved at room temperature. The fractionation of mouse spleen cells was

TABLE 5.1
Recovery of megakaryocytes by centrifugal elutriation as a function of temperature, flow rate and chelation. Grune and Stratton – with permission.

Conditions	Percent Loaded Megakaryocytes in Chamber	
Temperature (°C)		
4°	71 ± 6*	(n = 3)
12°	77 ± 3	(3)
22°	50 ± 4	(3)
37°	63 ± 1	(3)
Flow rate (12°C)		
10 ml/min	67 ± 8	(4)
20 ml/min	65 ± 13	(3)
Chelation (12°C. 20 ml/min)		
3 mM EDTA	60 ± 3	(3)
3 mM EGTA + 1 mM Mg^{++}	72 ± 8	(3)
1,3 mM citrate	59 ± 7	(3)
PBS alone	65 ± 13	(3)

* Mean + 1SE.

found to be virtually impossible at 4°C due to changes induced in the physical properties of the cell membranes (Sanderson and Bird 1977). Another problem encountered with separations of cells at 4°C has been aggregation of cells, (Zahlten et al. 1978), although depending upon the cell type, aggregation may be just as likely to occur at room temperature. An advantage of keeping cells at lower temperatures is that it can often prevent changes occurring (e.g. cell division) in the cells during the time required for the separation to be achieved, usually 2 – 3 hours. If no published conditions are available for a particular cell type to be separated, it is advisable to conduct several elutriator separations at different temperatures to obtain the optimum conditions to achieve the separation and recovery required.

Since temperature clearly has pronounced effects on separation, it is essential to accurately control the temperature of the elutriation buffer and the rotor. The best way of ensuring that a constant temperature is maintained is to conduct the separations in a constant temperature room, e.g. a cold room etc.. This is of course not always convenient and large centrifuges are rarely located in such rooms. Running the elutriator at room temperature is clearly no problem as long as the refrigeration unit of the centrifuge is switched off. Operating at lower temperatures presents more of a problem. The fluid reservoir, most of the tubing and mixing chamber can usually be maintained at a constant temperature either by immersion in ice or by insulation. The rotor can easily be equilibrated to the required temperature before used. It is however more difficult to maintain the rotor, and more importantly the separation chamber at the correct temperature while spinning in the centrifuge. One problem can be caused by the refrigeration unit of the J2-21 because warm air from the refrigeration motor passes the rotor inlet and outlet lines, producing changes in temperature of the elutriation buffer. These changes in temperature have been shown to produce anomalous fractions (Sumner and Lodola 1983). Temperatures around 10 – 18°C are easier to maintain than 4°C since the refrigeration unit can often be switched off without too much of an effect on the temperature of the elutriation fluid passing through the chamber.

Where cells with large size differences are being separated, small fluctuations in temperature will not greatly affect separation. The control of temperature is more important where the size differences are small, i.e. at the limits of the resolving power of the apparatus.

5.2.8. Fractionation and monitoring of cell size

It is advisable to monitor continuously the sizes of cells elutriated from the rotor during a separation. The easiest way to do this is to remove an aliquot from each fraction being collected, count the cells and determine their size distribution using an electronic cell counter, coupled to some form of size analyser. Alternatively microscopic methods of determining cell number and size can be used (Chapter 2).

One problem that can be encountered, particularly where small numbers of cells are being separated, is that the fractions elutriated from the chamber are diluted in large volumes of elutriation fluid. Removal of an aliquot may not contain enough cells for counting and sizing. It is therefore often necessary to centrifuge the cells in each fraction and resuspend in a smaller volume. This can be considerably facilitated by fractionating the elutriated cells directly into large centrifuge tubes.

The number of cells leaving the chamber can also be continuously monitored by having a flow cell in the outflow tubing which is observed with a low power microscope or by optical density measured at 660 nm.

5.3. Examples of cell separations

There are now a considerable number of examples of the separation of a variety of different cell populations using elutriation. A complete list of current publications is available from Beckman. Detailed below are selected examples that illustrate the variety of different conditions that can be used to achieve cell separation.

5.3.1. Separation of endothelial and Kupffer cells from rat liver. (Zahlten et al. 1978)

A suspension of non-parenchymal cells in Geys balanced salt solution, purified by density gradient centrifugation using Metrizamide, at $4-5 \times 10^7$ cells/ml is placed in the syringe and connected via the three-way tap to the elutriator system. The suspension is slowly allowed to flow into the mixing chamber avoiding any increase in interline pressure above 5 psi. The cell suspension is kept evenly suspended by a rotating stirring bar. Cells are allowed to flow into the separation chamber at 20 ml/min with the rotor spinning at 2500 rpm. Two areas of cells should be visible in the chamber, separated by a sharp border. The smallest cells are elutriated at this flow rate with a total volume of 250 ml over a 12.5 min period. This fraction contains 95% pure endothelial cells. The flow rate is increased to 42 ml/min. and the rest of the cells collected in a total volume of 150 ml for 3 – 4 min. This fraction contains 85 – 90% pure Kupffer cells. The viability of the cells is unaffected by the separation procedure.

5.3.2. Separation of human monocytes and lymphocytes. (Yasaka et al., 1981)

Mononuclear cells separated from whole blood by density gradient centrifugation of Ficoll-Hypaque are suspended at $1-1.5 \times 10^7$ cells/ml in sterile PBS with 5% FCS containing 100 U/ml penicillin, 100 μg/ml streptomycin and 0.01% EDTA (PBS-EDTA). The elutriator system is sterilised with 500 ml 70% ethanol and flushed with 500 ml sterile distilled water and 400 ml sterile PBS-EDTA. PBS-EDTA is used as the elutriation buffer. The flow rate is set at 10 ml/min, and the rotor spun at 1950 rpm at 18°C. 10 – 25 ml of cell suspension is injected into the sample mixing chamber and allowed to flow into the separation chamber. The cells are elutriated continuously with an initial flow rate of 10 ml/min followed by 0.5 ml/min increments every 8 min up to a maximum of 14 ml/min. The cells are collected in 13 fractions of 50 ml each. The large monocytes

are recovered in the final fractions by maintaining a flow rate of 14 ml/min as the rotor is stopped. The size and number of cells in each fraction are measured with a Coulter Counter and Channalyser and are shown in Fig. 5.5. The viability of cells in each fraction is over 90% determined by trypan blue exclusion.

Two peaks of mononuclear cells are obtained. Lymphocytes are isolated in fractions 1 – 6 with flow rates 10 – 12.5 ml/min. 2% contamination of these fractions with monocytes is observed. Fractions

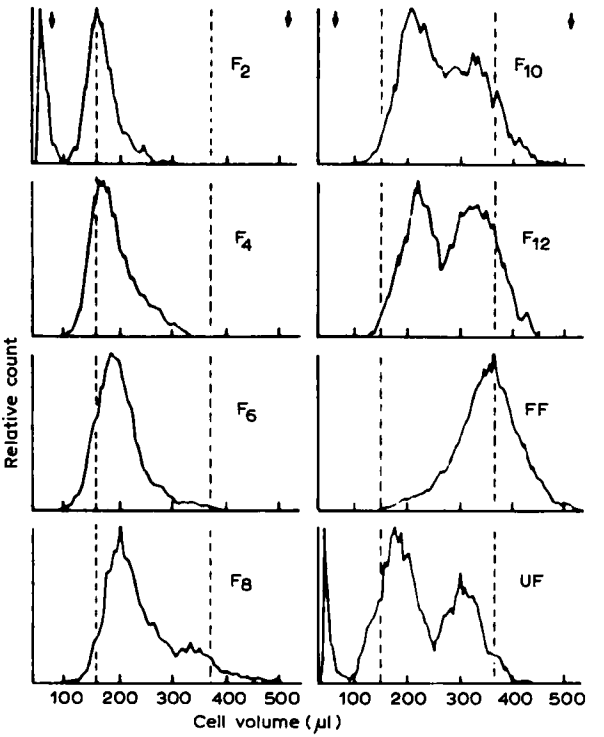


Fig. 5.5. Separation of human monocytes and lymphocytes by centrifugal elutriation. Left arrow shows the volume calibration of 4.88 μm diameter polystyrene beads and right arrow shows calibration with 10.08 μm diameter beads. F = fractions, FF = final fraction and UF = unfractionated cells i.e. as loaded. Yasaka et al., (1981), American Association of Immunologists – with permission.

8 – 12 contain two distinct cell populations, lymphocytes and monocytes. Marked variability in the volume of these monocytes has been noted with blood from different donors. Cells elutriated in the final fraction contain 96% pure monocytes with only 4% contamination with lymphocytes. Two monocyte subsets thus appear to have been separated, small monocytes, with a volume of 332 μm^3 in fractions 8 – 12 representing 25% of the total monocytes and heavily contaminated with lymphocytes and large monocytes with a volume of 380 μm^3, almost free of lymphocytes in the final elutriated fractions. Various assays of monocyte function have been conducted on these two subsets and both are found to possess similar functional characteristics but with quantitatively different activities.

5.4. Separation of cells with different densities

Elutriation as discussed so far represents a powerful method for separating cells of different size. The same technique can also be used to separate cells with different densities by elutriating cells from the chamber with media of different densities. In an extension of the work described above on the separation of monocytes and lymphocytes, different subpopulations of monocytes have been isolated on the basis of having different densities by elutriation with media of increasing density.

5.4.1. Isolation of monocyte subsets. (Figdor et al., 1982)

In this example the single fluid reservoir of the normal elutriator system is replaced with two reservoirs (in this case two plastic bags) with a capacity of 2 litres, and placed at a height of 180 cm above the rotor. Elutriation is carried out at a constant 18 ml/min and pressure of 180 cm resulting in a decrease in flow rate of 1%/h, which eliminates the need for a pump. Each of the reservoirs contains a different medium. Reservoir 1 contains a normal elutriation buffer (CCE), consisting of PBS with 0.14% BSA, 100 U/ml penicillin and

50 μg/ml streptomycin. Reservoir 2 contains CCE buffer plus 12.5% Percoll. Both reservoirs are connected to a mixing chamber via valves. The centrifuge is cooled to 0°C and the media also cooled to the same temperature by passage through a spiral in alcohol at 0°C. The temperature of medium entering the rotor is 4°C and 10°C leaving.

The cell sample (buffy coat from human blood) is introduced at 3200 rpm to remove thrombocytes and most of the erythrocytes. The first cell fraction collected at 3050 rpm contains erythrocytes and 40% lymphocytes. Fractions 2 – 3 elutriated at 2900 and 2750 rpm contain lymphocytes. At this point the rotor speed is kept constant and cells elutriated by stepwise increase in elutriation medium density. Monocyte fractions are isolated by flow ratios of CCE/CCE-Percoll (ml/min) 15:3, 12:6, 9:9, 6:12, 3:15, 0:18. This represents increases of medium density of 0.0027 g/ml. Fraction 4 contains few cells. Fraction 5 contains 85% monocytes (19% of total monocytes), Fractions 6 – 7 contain 88 – 96% monocytes (24 – 30% of total). Fraction 8 is contaminated with granulocytes and contains 11% monocytes. Fraction 9 is mainly granulocytes. The viability of monocytes in all fractions is around 99% and the same size distribution is obtained in all fractions.

5.4.2. Separation of T and B cells. (Griffith 1978)

In this example separation of T and B cells is achieved by increasing the density of the flow medium by four step-wise additions of albumin solutions of increasing density. The entire elutriator system is filled with Seligmanns buffer. 20 – 25 ml of buffer containing between $10^7 - 10^8$ human lymphocytes (isolated by Ficoll-Hypaque density gradient centrifugation) in 25 ml are added to the chamber via the mixing reservoir with the rotor spinning at 3000 rpm and a flow rate of 11 min/ml. 50 ml of buffer is flowed through the chamber to wash the cells. 15% albumin solution (in Seligmanns buffer) is allowed to flow into the chamber in place of the buffer with the same flow rate. 50 ml of albumin eluate is collected and buffer

allowed to reflow through the chamber at the same rate 50 ml of 17% albumin solution is pumped into the chamber and the eluate collected. This process is repeated with two further albumin solutions of 19 and 21%. Four 50 ml fractions are thus obtained which are diluted to 100 ml with buffer and pelleted by centrifugation for identification. The rotor, buffer and albumin solutions are all maintained at 4°C throughout the elutriation procedure.

T cells are identified by E-rosetting and B cells by EAC-rosetting. Fraction 1 (15% albumin) contains mostly T cells with 2% B cells. Fraction 2 (17% albumin) contains no cells. Fraction 3 (19% albumin) contains B cells with 4% T cells and some contamination with monocytes and other non-rosette forming cells. Fraction 4 (21% albumin) contains cell clumps and granulocytes. Viability of the elutriated cells is the same as before elutriation.

CHAPTER 6

Aqueous two-phase partition

6.1. Introduction

One of the classic separation techniques in chemistry is the differential partition of solutes between two immiscible liquid phases. Such techniques have been adopted by biochemists particularly for the separation of lipids which dissolve much more readily in organic solvents than in water. Clearly this approach is of little use for cell separation since cells will not survive in organic solvents. It is however possible to produce two immiscible phases with certain combinations of aqueous solutions, which, being around 90% aqueous are compatible with cells. The aqueous phases are formed by mixing solutions of certain high molecular weight polymers which, as shown in Fig. 6.1, can be arranged in a hydrophobic ladder in a similar fashion to organic solvents. There is thus a difference in 'hydrophobicity' between the upper and lower phases and it is this difference that provides the basis for the partition of cells.

Aqueous two-phase partition was developed in Sweden in the late 1950s by Per Åke Albertsson (Albertsson 1971) for the separation of proteins and other soluble material and was adapted for the separation of cells by Harry Walter (Walter 1977). The two polymers that have been almost exclusively used for cell separation are poly-(ethyleneglycol) (PEG) and dextran. When aqueous solutions of these are mixed together above certain critical concentrations, two

phases are formed; the upper phase is PEG-rich and the lower phase is dextran-rich. When cells are added to the phase system they distribute themselves (partition) between the interface and one of the bulk phases, which is usually the upper, PEG-rich phase. The extent to which the cells partition into the upper phase for any given phase system and set of conditions (temperature, etc.) is dependent on the surface properties of the cells. Cells with different surface characteristics have different affinities for the upper phase, and therefore partition to a lesser or greater extent. This differential partition is used to physically separate cells with different surface properties simply by separating the upper phase from the interface. The degree of purification of a particular cell type depends on the difference in partition of that cell type and the rest of the cells present. In a single partition experiment the degree of purification of a particular cell type is usually low. It is therefore often necessary to conduct several partition steps in sequence to increase purification. This involves transferring the upper phase with partitioned cells to fresh lower phase, and adding fresh upper phase to the interface and lower phase and repeating the partition process. This can be carried manually or automatically on a thin-layer countercurrent distribution apparatus.

Aqueous two-phase partition has several significant advantages

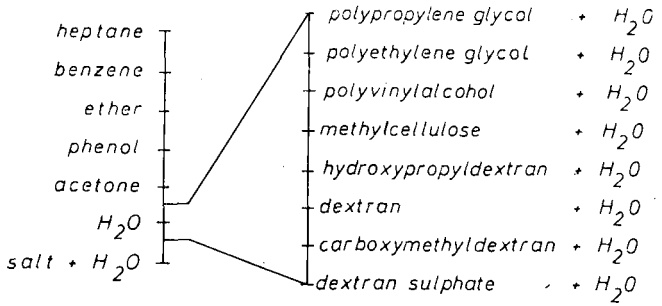


Fig. 6.1. Hydrophobic ladder of organic solvents (left) and aqueous solutions of polymers (right).

over all other cell separation techniques which will become evident throughout this chapter. Without doubt the biggest of these is the sensitivity of the technique. Equation 1 shows a simplified relationship between partition (K) and the factors that influence partition.

$$K = \exp. \frac{a + \gamma A}{kT}$$

where a is the difference in electrostatic free energy between the phases, γ is the difference in interfacial free energy between the phases, A is the surface area of the cell, k is the Boltzmann constant and T is the absolute temperature.

The main point to note is the exponential nature of the relationship which means the technique is extremely sensitive. Aqueous two-phase partition is for example more sensitive than electrophoresis (which is the only comparable technique for separating cells on the basis of general surface differences), largely because electrophoretic mobility is a linear relationship. (See also Brooks et al. (1985).)

6.2. *Factors that influence partition*

6.2.1. Polymer composition and concentration

Both the molecular weight and concentration of the polymers used have major influences on the partition of cells. Increasing the molecular weight and/or the concentration of either polymer decreases the partition of cells. This phenomenon is best explained by referring to a phase diagram, like the one shown in Fig. 6.2.

The phase diagram is characteristic for polymers of a given molecular weight at a particular temperature. Changing the polymer molecular weights or the temperature, changes the phase diagram, i.e. the properties of the phase system. The curved line, called the binodal, represents the lowest concentrations of the two polymers which when mixed together form a two-phase system. Thus all points

above the binodal represent polymer concentrations that give rise to two-phases. Points below the binodal represent polymer concentrations that do not form two phases but form a single homogeneous solution. The point which corresponds to the lowest concentration of both polymers that will form a two-phase system is called the critical point.

The further the distance a particular combination of polymer concentrations is from the binodal, the higher is the interfacial tension between the two phases. Since cells partition between the interface and the top phase, increasing the interfacial tension, increases the number of cells remaining at the interface and thus decreases partition.

Increasing the molecular weight of either polymer also increases the interfacial tension and hence reduces partition. The separation of the two polymers into the two phases is not totally exclusive. The up-

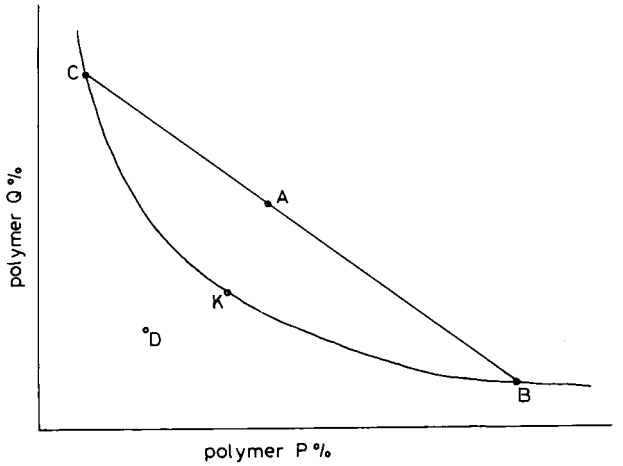


Fig. 6.2. Phase diagram. The curved line (binodal) represents lowest concentrations of polymers P and Q (e.g. dextran and PGG) that will produce a two-phase system at a particular temperature. D = single homogenous solution. A = two phase system. B and C = concentrations of P and Q in upper and lower phase of phase system A. K = lowest concentrations of P and Q that will produce a two-phase system.

per phase consists largely of PEG but there is always a small percentage of dextran present. The same is true for the lower phase which is dextran-rich with a small percentage of PEG. The concentration of PEG and dextran in both phases at equlibrium can be easily measured (see 6.3.3) and a line connecting the point on the phase diagram can be drawn to produce a 'tie line' (Fig. 6.2). The ratio of the lengths of each half of a tie line, on either side of the point corresponding to the total concentration of polymers, is the ratio between the volume (or weight) of the two phases.

The construction of a phase diagram although not essential for conducting partition cell separations can sometimes be useful, particularly when trying to find optimum separation conditions. The phase diagram shows at a glance the distance a particular phase system is from the critical point and the phase volume ratios.

It is important to remember that a phase diagram produced for a particular polymer does not apply to other polymers, either of different molecular weight, or even of the same molecular weight from different manufacturers' batches. Because partition is so sensitive, very small changes in the average molecular weight of the polymers such as can occur between different batches is sufficient to affect partition.

6.2.2. Temperature

Temperature is an important factor in the partition of cells. Lowering the temperature has the effect of shifting the binodal of the phase diagram toward lower polymer concentrations. Polymer concentrations that are monophasic at room temperature will often form two phases at a lower temperature. This means that by working at lower temperatures such as 4°C, low polymer concentrations can be used.

It is important to control the temperature during partition experiments since temperature fluctuations will affect the partition of cells. It is usually sufficient to conduct experiments in constant temperature rooms, ice buckets, water baths, etc.

6.2.3. Ionic composition

The osmotic strengths of polymer solutions around the concentrations used for cell partition separations (5 – 6%) are around 50 mOsm and are therefore too low for most cells. The osmotic strength of phase systems can be increased by the inclusion of various salts such as NaCl or phosphate, in the form of phosphate buffer, (which also serves to buffer the phases at the required pH). The incorporation of salts into phase systems has major effects on the nature of the phase system and cell partition. The reason for this is that the ions of the salts themselves partition between the two phases. Some ions such as chloride have an equal affinity for both phases while others partition more into one phase than the other, for example phosphate partitions in favour of the lower phase. Both anions and cations can show asymmetric partition. When different salts are added to phase systems the partition of cells in the phase systems is invariably changed, even when the ionic and osmotic strengths are kept constant. These changes in partition occur as a result of the asymmetric partition of certain ions, which create an electrostatic potential difference between the phases, that is, the phases are charged with respect to each other. Since cells carry a net surface charge (usually negative), they will be attracted to the phase of opposite potential. The degree of attraction depends on the amount of cell surface charge and the value of the electrostatic potential difference. Every salt produces a characteristic potential difference between the phases which is the sum of the partition of the constituents ions. However, the partition of ions is affected, albeit to a small extent, by the polymer concentration and composition. For constant salt composition and concentration the electrostatic potential difference increases in phase system further from the point (Table 6.1). Thus two phase systems with identical salt compositions but different polymer composition will have slightly different potential differences between the phases. Although these effects are small they can be important if the partition of cells is being compared in phase systems of different polymer composition. The potential difference also in-

TABLE 6.1
Electrostatic Potential Difference between Top and Bottom Phases (Top Phase Positive)

Phase system	Potential difference (mv)
5:3.5 1	+0.68 ± 0.09
5:4 1	+1.13 ± 0.26
7:4.4 1	+2.50 ± 0.14
5:3.3 5	+0.04 ± 0.01
5:4 5	+0.04 ± 0.06

Phase compositions were as follows: (5:3.5 1) 5% dextran, 3.5% polyethylene glycol, and 0.11 M Na phosphate buffer, pH 6.8 (5:4 1) 5% dextran, 4% polyethylene glycol, and the same salt concentration and composition as above. (7:4.4 1) 7% dextran, 4.4% polyethylene glycol, and the same salt concentration and composition as above. (5:3.5 5) and (5:4 5) 5% dextran, 3.5 or 4% polyethylene glycol, respectively, and 0.15 M CaCl and 0.01 M Na-Phosphate buffer, pH 6.8. From Walter (1977), Plenum Press – with permission.

The results presented in this Table are quantitatively correct but are probably not qualitatively correct due to the type of electrodes used. For a detailed discussion of this see Bamberger et al., (1985).

creases with increasing concentration of asymmetrically partitioning salts.

The two principle salts used in cell partition are NaCl and sodium or potassium phosphates. NaCl produces no effective potential difference between the phases. Phosphate ions however have a higher affinity for the lower phase than the upper phase and thus produce a significant, and measurable potential difference between the phases, the upper phase being positive with respect to the lower phase (Table 6.1). By mixing NaCl and Na/K phosphate in different amounts a range of potential differences can be obtained from zero to about 2.8 mV (Fig. 6.3).

The partition of cells in phase systems with high potential differences ('charged systems') is largely influenced by surface charge whereas partition in phase systems with little or no potential difference ('uncharged or zero potential systems') is largely unaffected by surface charge.

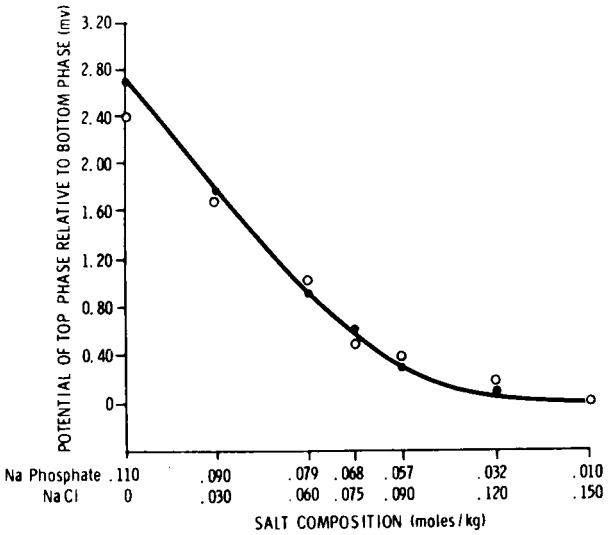


Fig. 6.3. Potential difference between top and bottom phase of a two-phase system as a function of salt composition. Polymer concentration is 5% dextran, 4% PEG. The electrodes contained either 1.5 M KCl (○——○) or 3 M KCl (●——●) (Reiherman et al., 1973).

If salts other than NaCl and phosphate are being used, the potential difference between the phases may need to be measured. (This is relatively simple to do and is described in Section 6.3.5).

6.2.4. Polymer ligands

Ligands can either be coupled to dextran or PEG, but more usually PEG is used because it is cheaper and the chemistry is easier. When ligands having an affinity for cell surfaces, such as fatty acids, are used, the partition of cells is affected by increasing their affinity for the phase with the ligand attached. Other ligands that do not have a particular affinity for cell surfaces can also affect partition by changing the properties of the phases, for example DEAE-dextran or sulphonated-PEG will alter the electrostatic potential difference be-

tween the phases. The most commonly used ligands for cell separation are fatty acids coupled to PEG. The fatty acid-linked PEGs greatly increase the affinity of cells for the upper phase by the fatty acids inserting into the plasma membrane and effectively coating the cells with PEG. The use of cell surface-specific ligands (affinity partition) is discussed in Chapter 10.

6.3. *Cell partition*

Cells partition between the interface and the upper phase in most PEG-dextran phase systems (Fig. 6.4). The partition of a particular

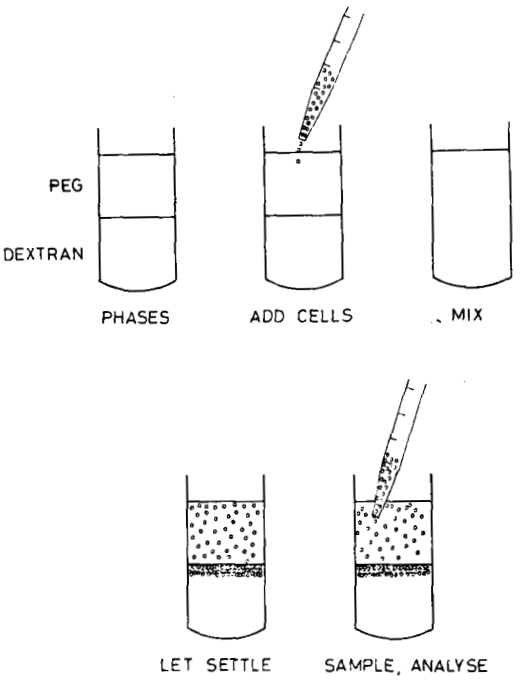


Fig. 6.4. Principle of single-tube partition of cells.

cell population in a given phase system is described by the partition coefficient P, which is the number of cells in the upper phase per cent of the total number of cells added to the phase system. A partition coefficient above 50% indicates that the cells have a higher affinity for the upper phase than the interface. A partition coefficient below 50% indicates that the cells have a higher affinity for the interface than the upper phase.

The partition coefficient of cells is independent of the phase volume ratio. The concentration of cells in the top phase varies inversely with the upper phase volume, i.e. when the upper phase volume is halved, the concentration of cells in the upper phase is doubled.

The mechanism of cell partition involves the attachment of cells to droplets of PEG and dextran that are formed as an emulsion when the phases are shaken (Raymond and Fisher, 1980) (Fig. 6.5).

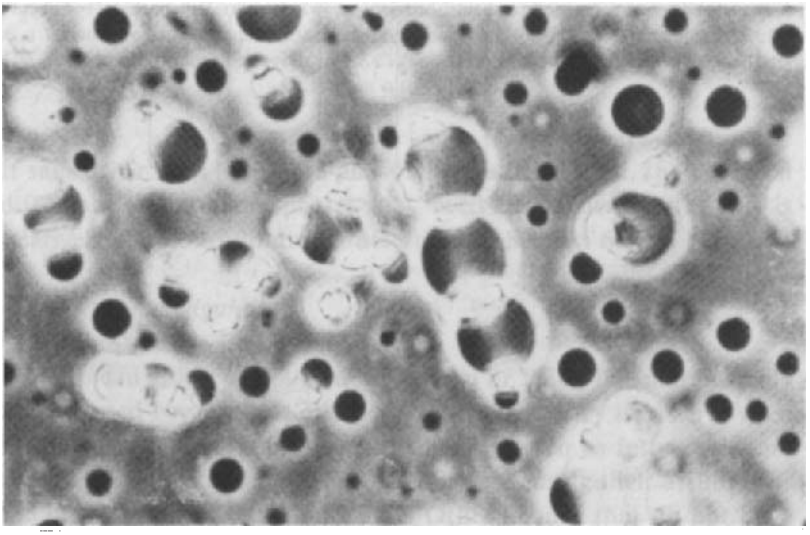


Fig. 6.5. Light micrograph (phase contrast × 800) of rat RBC's attached to outer surfaces of dextran globules present in top phase of a 5% dextran: 4% PEG phase system. Raymond and Fisher (1980).

6.3.1. Measurement of partition coefficient-single tube partition

The partition coefficient of cells is measured as shown in Fig. 6.4. A phase system is made by weight, usually 5 or 10 g in a plastic screw-top tube and equlibrated to the required temperature. Cells are added as a packed pellet or alternatively can be added by resuspension in a constituent of the phase system (e.g. phosphate buffer) that has been omitted when the phases were made. The tubes are shaken both to mix the cells with the phases and to produce the phase emulsion. The shaking action used must be repeatable, i.e. inversion of the tube, 20 – 30 times or vortexing. The method of shaking can affect partition and the same method should always be used. The mixed cells and phase system are allowed to settle in order for the two phases to separate and the cells to partition. The time allowed for settling is usually taken as the time for phase separation to reach equilibrium. This is when the phases are visibly clear and only tiny microdroplets remain. At phase equilibrium the cells remaining in the upper phase, i.e. those partitioned, are not attached to droplets and therefore no cells are rapidly falling to the interface. The number of cells in the upper phase at this time is relatively stable to allow accurate reproducible sampling of the upper phase. Cells will gradually fall, under the influence of gravity, to the interface and after a long period (several hours or even days) all cells will be at the interface. Cell partition is thus a non-equilibrium process. The time of phase equilibrium depends on the polymer concentration, the volume of the phases and the interfacial area to phase volume ratio.

The volumes of the phases are noted and a measured aliquot of cells is removed from the centre of the top phase and the cell number counted. Knowing the number of cells added the partition coefficient is calculated as total number of cells in top phase over total number of cells added $\times$ 100.

6.3.2. Manipulation of phase composition to change cell partition coefficients

Any given cell population has a characteristic partition coefficient in

a particular phase system. The partition coefficient of cells can be changed by altering the composition of the phase system. As described above, changing the polymer concentrations, polymer molecular weights and the salt composition will affect the partition coefficient. Increasing polymer molecular weights or increasing the concentrations of either polymer will decrease the partition coefficient. Conversely, decreasing the polymer molecular weights or concentrations will increase the partition coefficient. Increasing the ratio phosphate/NaCl concentration will generally increase the partition whereas decreasing this ratio will decrease partition. The extent of the changes in partition will however be different for cells with different surface characteristics. This phenomenon is used to produce separation of different cell types. Thus, if in a population consisting of two cell types, the partition coefficients of both are the same in one phase system, manipulation of the phase components will often result in divergence of the partition coefficients of the two cell types which will allow separation.

Greatest subfractionation of cell populations is achieved with phase systems in which one cell type has a partition coefficient above 50% and the other below 50%.

If the aim of a particular experiment is to separate different cell populations, then the phase composition can be varied at will in order to achieve the desired separation. However in some cases one may wish to fractionate cells on the basis of particular, albeit general, surface characteristics, e.g. surface charge or hydrophobicity. In these cases the phase composition cannot be varied at will, but components must be changed such that the overall characteristics of the phases remains the same, i.e. charged, uncharged, etc.

6.3.2.1. Uncharged (low potential) phase systems. Uncharged phase systems typically contain 0.1 – 0.15 M NaCl and 0.01 M Na/K phosphate buffer. The osmotic strength of these phase systems is around 175 mOsM and can be increased by the addition of sucrose.

The partition coefficient of the cells should be adjusted by changing the polymer concentrations. With these conditions, partition, and separation of cells is predominantly a function of non-charge

related surface properties. An uncharged phase system in which human monocytes have a partition coefficient of 50% is shown in 6.3.4.3.

6.3.2.2. Charged phase systems. A phase system can be produced that partitions cells largely on the basis of surface charge by incorporating phosphate at around 0.1 M with a low concentration of NaCl of less than 0.03 M. Cells are initially partitioned in an uncharged phase system and the concentration of one or both of the polymers gradually increased until a point is reached where all cells just fail to partition, i.e. a coefficient of zero. These polymer concentrations are used to produce the charged phase system by inclusion of an appropriate concentration of phosphate to produce the required partition coefficient. A charged phase system in which human monocytes have a partition coefficient of 50% is shown in 6.3.4.3.

6.3.2.3. Non-specific affinity ligand systems. This type of phase system usually refers to partition mediated by the inclusion of PEG-fatty acid conjugates, palmitate being the most common fatty acid used. The salt composition used is the same as an uncharged phase system with the polymer concentrations the same as a charged phase system, i.e. a system where all cells just remain at the interface. A small amount of PEG-palmitate is then included until the desired partition coefficient is obtained. Typical concentrations of PEG-palmitate used are between 0.0001 – 0.001%.

A PEG-palmitate phase system in which human monocytes have a partition coefficient of 50% is shown in 6.3.4.

6.3.3. Stock solutions

1. Dextran

Dextran is a high molecular weight glucose polymer available in very pure form from Pharmacia. The molecular weight most commonly used is 500 000, which is available under the name of T500. Details of the molecular weight average and molecular weight distribution of dextran in each batch is supplied by Pharmacia. These values will

vary from batch to batch and the variation is usually sufficient to affect cell partition. It is advisable therefore to use the same batch of dextran for a given set of experiments and when the batch is changed, to check whether the same partition coefficient is obtained. Dextran is hygroscopic and therefore the powdered form supplied will have an unknown water content. In order to accurately produce a stock solution, the precise concentration of the solution made up must be determined by using polarimetry. A stock solution is prepared at 20% (w/w) in water by weighing about 215 g of dextran and dissolving this in 785 g water. Dextran dissolves slowly at room temperature and it is often convenient to leave the solution stirring slowly overnight. The solution can be heated to 90°C for 1 h in a water bath which allows storage at 4°C for about 2 weeks. Alternatively the stock solution can be sterilised by autoclaving.

The actual concentration of the dextran solution is determined by weighing accurately, approximately 5 g of the solution in a 25 ml volumetric flask. After making up the volume to 25 ml, the rotation is measured on a polarimeter with a Na lamp. The concentration is calculated from; $[P]_v = ø25/l[\alpha]w$ where $[P]_v$ is the concentration % (w(v), ø is the measured optical rotation, l is the sample tube length in decimeters, $[\alpha]$ is the specific optical rotation (degrees/percent w/v decimeters) at 25°C with a Na lamp. For dextran this value is 1.99, w is the weight of solution (5 g) made up to 25 ml, (Albertsson, 1971; Bamberger et al., 1985). The concentration as % (w/w), $[P]_w$ can be calculated from; $[P]_w = [P]_v/\varrho$ where ϱ is the density of the dextran solution (g/ml). ϱ can be calculated from $[P]_v$ by $(0.997 + 0.391\ [P]_v)/100$.

2. PEG

PEG is available from a variety of sources in a range of molecular weights. Molecular weights used for cell partition are 4000 – 8000. The purest source obtainable should be used. BDH supply a biochemical grade PEG which is suitable, as is the PEG from Sigma which is produced by Union Carbide. Recently the Union Carbide PEG 6000 which has been extensively used for cell partition was

found to have an average molecular weight close to 8000 and has therefore been renamed PEG 8000.

The average molecular weights of PEG can vary between batches and the same batch should therefore be used for a series of experiments. Since PEG is very cheap it is advantageous to buy a large quantity from the same batch. PEG is not hygroscopic and a 40% (w/w) stock solution can be prepared by dissolving 400 g in 600 g water. Stock solutions can be stored at 4°C for about 4 weeks.

The concentrations of PEG solutions can, if required, be determined by measuring the refractive index. Solutions can be autoclaved or stored frozen.

3. Buffers and salts

NaCl is conveniently stored as a 1 M solution. Phosphate buffers can be conveniently stored at 0.2 M stock solutions. These solutions will keep almost indefinitely at room temperature in the dark.

6.3.4. Phase systems

Phase systems are made up by weight. The easiest way is to zero an empty conical flask on a top-pan balance and add the required weights of solutions. The 20% stock solution of dextran is very viscous and can only be pipetted with large bore pipettes. Large disposible plastic Pasteur pipettes are the most suitable. Once weighed out, the phase system should be shaken and allowed to equilibrate at the required temperature.

6.3.4.1. Tube phase systems. Phase systems that are to be used for tube separations can be produced in several ways. Total weights of around 5 – 10 g are used which can be weighed out directly, although this is not recommended since weighing errors can be large with small amounts. The preferred way is to remove 5 – 10 g aliquots from an equilibrated phase system while the system is thoroughly stirred. An alternative method is to separate the upper and lower phases after they have become translucent at the required temperature, and produce the phase system in the tube by remixing

the two phases. The separated phases can be stored at 4°C for several weeks or can be frozen in plastic containers.

6.3.4.2. Multiple partition phases. For multiple partition, principally using countercurrent distribution (see 6.6.), phase systems are produced in bulk, usually 200 – 600 g. The freshly prepared phase system is left at the required temperature (usually 4°C) overnight to equilibrate. The system is then vigourously shaken, poured into a separating funnel and the phases left to separate until they become translucent. For a 600 g phase system at 4°C about 5 days is required for complete separation to occur. Once separated, the lower phase is drained into a flask making sure that the interface and contaminants that collect at the interface are not included. These are drained into a separate container and discarded. The upper phase is collected by pouring through the top of the funnel.

The separated phases can be stored in the countercurrent apparatus (4°C) or can be stored frozen in plastic containers. The separated phases can be autoclaved if required although some caramelisation of dextran in the lower phase may occur, particularly in systems with high phosphate concentrations. There is evidence that such caramelisation may in some instances affect partition (Sharpe et al., 1985). If sterile phases are required they can be filtered through a 0.45 μm Millipore filter.

For separation of mammalian cells, serum is usually included in the phases at around 5% (w/v). This can be added as the phase systems are weighed out although this can lead to contamination problems since the phases are left for 4 – 5 days to separate. A better method is to make the phase system slightly concentrated and add the serum immediately before use to produce the correct phase concentration.

6.3.4.3. Phase recipes. Below are three examples of recipes for phase systems that I have used to partition human monocytes. Each phase system gives a partition coefficient of around 50%. Each phase system has an osmolarity of 280 – 300 mOsM. Note that these systems will only produce a partition coefficient for human

monocytes of around 50% with the batches of Dextran and PEG I used. With different batches, the polymer concentrations will probably need changing to obtain the desired partition.

1. Uncharged phase system
4.8% PEG 4000 (BDH), 5.5% dextran T500, 0.05 m/kg NaCl, 0.01 m/kg potassium phosphate buffer pH 7.0, 4.8 sucrose and 5% FCS.

21.59% dextran		152.82 g
40.00% PEG		72.00 g
1.0 M NaCl		30.00 g
0.2 M phosphate buffer		30.00 g
sucrose (solid)		29.00 g
distilled water		256.18 g
	Total	570.00 g
FCS (added as 3 g to 57 g)		30.00 g
	Total	600.00 g

2. Charged phase system.
5.8% PEG 4000 (BDH), 5.8% dextran T500, 0.0015 m/kg NaCl, 0.09 m/kg potassium phosphate buffer pH 7.0, 5% FCS.

21.59 % dextran		161.18 g
40.00% PEG		87.00 g
1.0 M NaCl		1.80 g
0.2 M phosphate buffer		52.00 g
distilled water		268.02 g
	Total	570.00 g
FCS (added as 3 g to 57 g)		30.00 g
	Total	600.00 g

3. PEG-palmitate phase system
5.8% PEG 4000 (BDH), 5.8% dextran T500, 0.05 m/kg NaCl, 0.01

m/kg potassium phosphate buffer pH 7.0, 5.3% sucrose, 5% FCS and 0.0041% PEG-palmitate.

21.59% dextran		161.18 g
40.00% PEG		87.00 g
1.0 M NaCl		30.00 g
0.2 M phosphate buffer		30.00 g
sucrose (solid)		32.00 g
PEG-pal. (30% esterified)		00.075 g
distilled water		229.745 g
	Total	570.000 g
FCS (added as 3 g to 57 g)		30.00 g
	Total	600.00 g

The concentration of PEG-palmitate used here is higher than is normally required because fatty acids bind to serum and thus a large proportion of the ester is neutralised.

6.3.5. Where to start

Because of the large number of variables in cell partition it can be very difficult for someone without partition experience to begin to produce phase systems to separate particular cell populations. The easiest way to begin is to use single 10 g tube systems and conduct a series of experiments with systems of different phase composition. Initially, aim to obtain a partition coefficient of 50% for the cell population. Once this is achieved manipulation of the polymer concentrations or salt composition will produce the required changes in partition to produce separation. For an initial series of phase compositions I would suggest conducting separations in low potential and charges phase systems in parallel with the following five phase systems based on dextran T500 and PEG8000.

4% dextran/3.5% PEG
4.5% dextran/4% PEG

5% dextran/4% PEG
5% dextran/4.5% PEG
5.5% dextran/4.5% PEG

For 10 g systems in test-tubes use a vertical settling time of around 30 – 60 min or a horizontal time of 10 – 15 min. The differences in partition between each low potential and charged system is an indication of the cell surface charge.

6.3.6. Measurement of electrostatic potential difference between phases

Details of several methods that can be used to measure the electrostatic potential difference between phases have been described by Bamberger et al. (1985). Detailed below is a simple method I have used.

Two standard calomel half-cells are used consisting of two glass U-tubes containing a reservoir of 3 M KCl overlying the calomel which is connected to a copper electrode via mercury. The calomel paste is prepared from 3 M KCl and solid mercurous chloride. One half cell is connected to the positive, and the other to the negative terminal of an electrometer. Before use, the half-cells are allowed to equilibrate for several days whilst connected together with a salt agar bridge consisting of 1% (w/v) agar and 3 M KCl solidified inside 1 mm diameter plastic tubing.

Each sample containing 4 ml of top phase and 4 ml of bottom phase in a 10 ml vial is kept at the appropriate temperature. The samples are connected together using salt-agar bridges. An initial zero reading is taken with all the connecting bridges in the top phases. Alternate bridges are then inserted through the interface into the bottom phase to connect all six samples in series. The reading is noted and divided by six to give a value for the interfacial potential difference for one sample. (Fig. 6.6)

6.4. *Single-step cell separation*

Single-step cell partition experiments conducted basically as shown in Fig 6.4 can be used to enrich cell populations for a particular cell type. Separation of one cell type relatively uncontaminated with other cell types is however somewhat difficult to achieve with a single partition step. To obtain 100% purification of one cell type from a mixture, the respective partition coefficients of the cell type required and all other cells would need to be 100% and 0%. This means that all cells of one cell type are either at the interface or the upper phase and totally free of all other cell types. Such a situation very rarely occurs, the partition coefficients of cells cannot usually be made disparate enough to allow anymore than an enrichment of one cell type. If enrichment is all that is required then single-step partition can prove adequate.

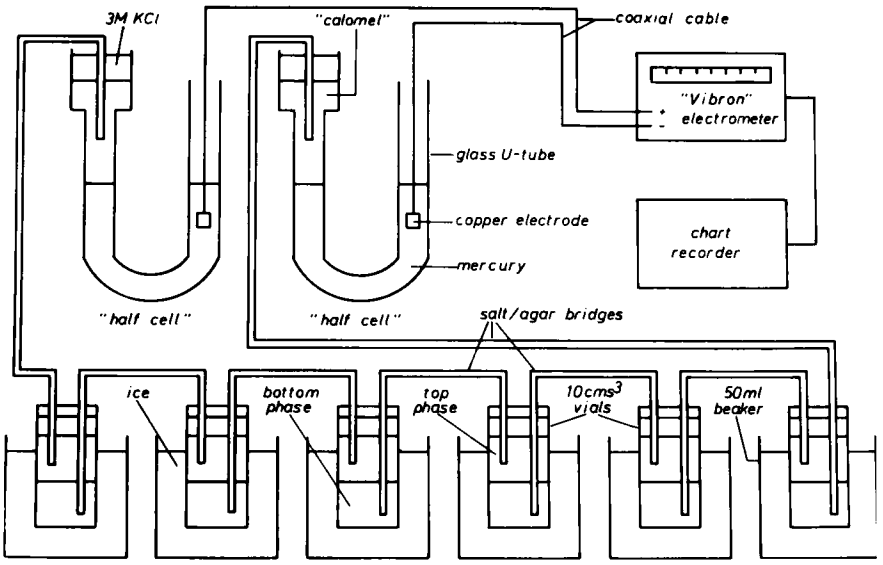


Fig. 6.6. Apparatus for measuring the electrostatic potential difference between the upper and lower phase of an aqueous two-phase system.

6.5. Two-step partition

If adequate separation cannot be obtained with single-step partition two-step partition can be tried. Here the upper phase after the first partition is removed and added to fresh lower phase. To the interface (with cells) and lower phase remaining after the first partition is added fresh upper phase. The tubes are shaken and the cells repartitioned. The effect of two-step partition is to enrich for cells with disparate partition coefficients, thus cells with a high partition coefficient will be concentrated in the upper phase after the second partition and will be relatively free of cells with appreciably lower partition coefficients which will be concentrated at the second interface. Very little use has been made of this approach either to produce highly purified cell populations or to produce populations enriched for a particular type that can be used for further purification. One example of the use of two-step partition to produce highly purified populations of two different cell types is described below. In this particular example only two cell types are present in the population but these have a wide range of partition coefficients. The two cell types are precursors of terminally differentiated cells types (spores and stalk cells) which form during development of the cellular slime mould *Dictyostelium discoideum*. At the stage of development investigated, the two cell types have different partition coefficients with the prespore type having a lower partition coefficient than the prestalk type in uncharged phase systems (Sharpe et al., 1982). In order to obtain purified populations of both cell types, each free from contaminations by the other, the cells most disparate in partition coefficient are separated and cells with intermediate coefficients discarded. Two different phase systems are used for both partition steps, each having the same salt composition (low potential) but different polymer concentrations. The system with the higher polymer composition (5.5%/5.5%) is used to purify the prestalk cells. The system with the lower polymer composition (5.1%/5.1%) is used to purify the prespore cells. Cells at 10 h development are resuspended in water at a known concentration and equal amounts added to each

of the two 5 g phase systems which have previously been prepared suitably concentrated and left at 4°C overnight. The tubes are shaken by inverting 30 times and allowed to separate for 6 min at 4°C in a horizontal position. The tubes are gently tilted to the vertical position and the volumes of each phase noted. 10 μl is removed from the centre of both upper phases and the cell number counted. The upper phase of the 5.5%/5.5% system is carefully removed and added to an equal volume of separated lower phase from a system with the same polymer composition. The upper phase of the 5.1%/5.1% system is removed and discarded. To the remaining interface (+ cells) and lower phase is added an equal volume of upper phase previously separated from a system of the same total composition. Both tubes are inverted 30 times and allowed to separate horizontally at 4°C for 6 min.

About 8% of the cells partition into the upper phase at the first step in the 5.5%/5.5% system. When these cells are re-partitioned, all (100%) re-partition into the upper phase. These are a pure population of prestalk cells. In this example the second partition step is not needed since the 8% cells that partition in the first step are pure prestalk cells as demonstrated by their 100% partition in the second step. About 40% of cells partition in the 5.1%/5.1% system leaving 60% at the interface. When the interface cells are re-partitioned about 40% re-partition. This figure is however difficult to quantify accurately because a portion of cells at the interface are always inadvertantly removed with the first upper phase. The cells remaining at the interface after the second partition do appear to be a pure population of prespore cells when assayed for DNA composition (Watts et al. 1988).

The main problem with two-step cell partition is that it is difficult to remove all the upper phase without either disturbing the interface, especially when the interface + cells is diffuse as happens when short settling times are used, i.e. less than 10 min. Removal of the upper phase can be improved by using positive displacement pipettes.

6.6. *Multiple partition – thin-layer countercurrent distribution*

It is not difficult to imagine that by increasing the number of partition steps the separation of cells with different partition coefficients is increased. Thus if enough partition steps can be carried out, cells differing only slightly in partition coefficient can be separated. There is a limit to the number of partition steps that can be manually conducted in tubes as described in 6.5, three or four being about the maximum. Various designs of automated apparatus have therefore been produced to carry out multiple partition steps. The most successful of these is the thin-layer countercurrent distribution apparatus or 'TLCCD'. In order to reduce the time of each partition step, the interfacial area to volume ratio of the phase is kept at a maximum by having the phases as a 'thin-layer'.

The heart of the TLCCD apparatus is a plastic rotor which is split into two halves. Accurately radially machined into each rotor half are shallow cavities which have the same cross-sectional area. When

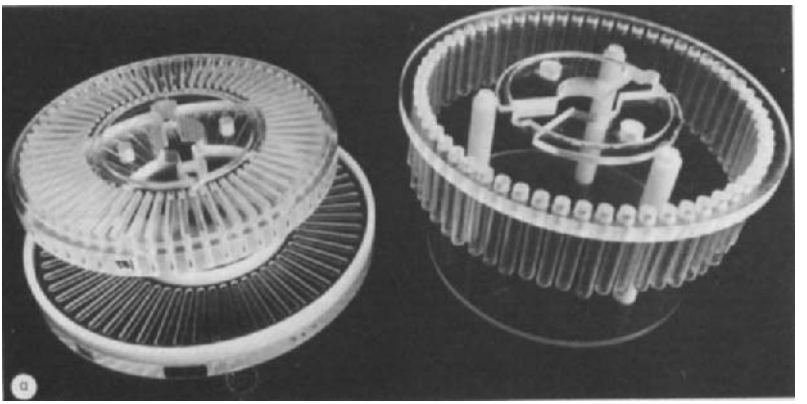


Fig. 6.7a. Bioshef TLCCD rotor and unloader. Left; two halves of the rotor, the upper half is inverted to show detail of the cavities. Right; rotor unloader, 60 plastic tubes held in a plastic ring which is bolted onto the rotor. Inversion of the rotor/ring assembly allows the contents of each chamber to empty into a tube.

the two rotor halves are together the cavities form chambers which hold the phases. The bottom half of the rotor holds the lower phase and interface and the top half holds the upper phase (Fig. 6.7 and 6.8). The principle of TLCCD is shown in Fig. 6.9. Cells type 'A' represent a theoretically homogeneous cell population with a partition coefficient of 50%. After the first partition step when 50% of cells are in the upper phase and 50% at the interface, the upper phase and cells is transferred to fresh lower phase in an adjacent chamber. At the same time, fresh upper phase is introduced to the lower phase and interface in the first chamber. The phases are shaken and the cells partition in the two chambers with the same partition coefficient to produce the distribution shown in Fig. 6.9. The process is automatically controlled and can be repeated any number of times between 1 and 59 with a 60 chamber rotor. At the end of the required

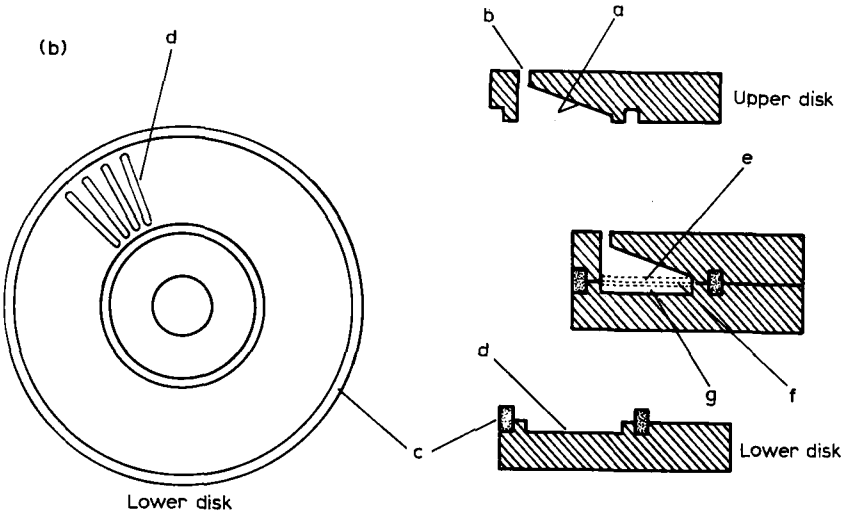


Fig. 6.7b. Detail of arrangement of cavities in a TLCCD rotor. (a) Sloped upper surface of upper cavity to allow emptying of chamber contents. (b) Emptying and filling hole. (c) Removable Teflon rings. (d) Lower cavity which holds the bottom phase and interface. (e, f, g) Relative positions of phases and interface.

number of steps the contents of each chamber is removed by injecting a buffer solution into the chambers and inverting the rotor so that the contents of each chamber fills a separate plastic tube (Fig. 6.7). Fig. 6.9 shows the distribution of cells type 'A' after 59 partition steps (transfers), a symmetrical bell-shaped curve with a peak in the centre fraction. This distribution is characteristic for a homogeneous population with a partition coefficient of 50%. Cells with different partition coefficients produce distributions in different fractions. For example, Fig. 6.10 shows the countercurrent distribution of cells type 'B' which represent a homogeneous population with

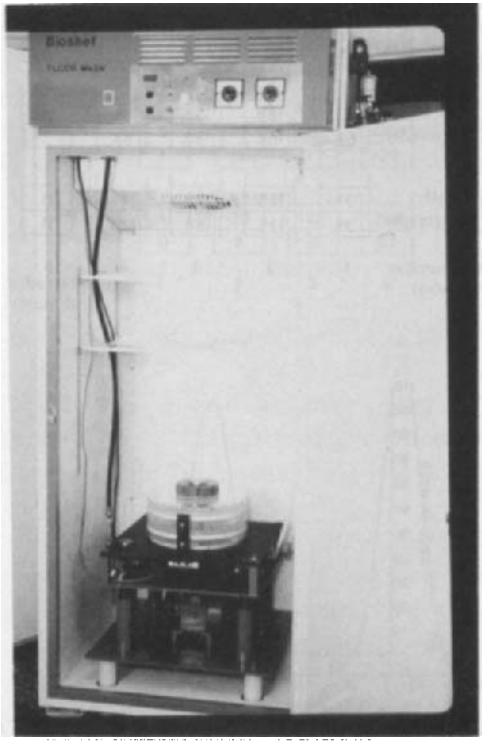


Fig. 6.8. Bioshef TLCCD apparatus.

a partition coefficient of 80% (i.e. a higher affinity for the upper phase than type 'A'). The resulting distribution after 59 transfers is a peak in fraction 48 which is skewed slightly toward the centre fractions and is in fact based on a Poisson distribution. Cells with partition coefficients greater than 50% produce distributions with peaks

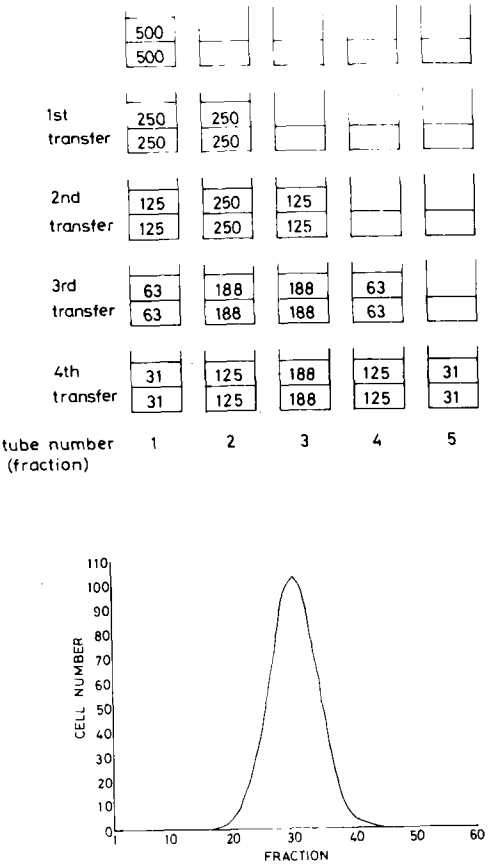


Fig. 6.9. Principle of countercurrent distribution. Cells types 'A' are a theoretical homogeneous cell population with a partition coefficient of 50%. Four transfer (partition) steps are shown and the resulting distribution after 59 transfers is shown.

in fractions higher than the centre fraction. Cells with partition coefficients lower than 50% produce distributions with peaks in fractions lower than the centre fraction. The ability of TLCCD to separate cells that have different partition coefficients (i.e. different surface properties) is clearly shown in Fig. 6.11 which is the distribution obtained after 59 transfers from a 50/50 mixture of cells of type 'A' and 'B'.

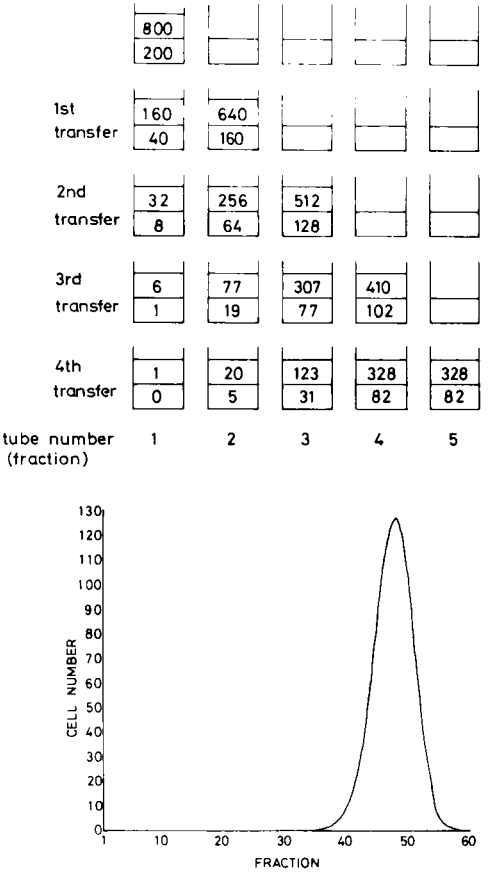


Fig. 6.10. Countercurrent distribution of a theoretical homogeneous cell population 'B' with a partition coefficient of 80%.

EQUAL MIXTURE OF CELLS TYPE A and B	4th transfer	6	25	38	25	6	% of A
		0	3	15	41	41	% of B
	tube number	1	2	3	4	5	

Fig. 6.11. Number of cells of each type, 'A' and 'B', in each fraction after four countercurrent partition steps of a 50:50 mixture of both cell types.

Fig. 6.12 shows the arrangement of the partition and transfer processes in a section of a rotor. This corresponds to the first transfer step of cells 'A' shown in Fig. 6.9.

Automated TLCCD apparatus is available from two sources, Bioshef (Dept. of Biochemistry, University of Sheffield, U.K.) and The Workshop, Chemical Centre, University of Lund, Sweden. Both current models operate with similar mechanisms although there are important differences in rotor construction and design. Fig. 6.8 shows the Bioshef apparatus. Further details can be obtained from the above addresses. The rotors from both these machines can also be used manually, which although extremely time consuming, can be useful in accessing the potential of the technique.

6.6.1. Operation of a TLCCD apparatus

1. Preparation of phase systems.
Each TLCCD chamber requires around 1.5 ml of phase system and the phases must therefore be prepared in bulk as described in 6.3.4.2.

2. Loading the rotors.
Lower phase is loaded into every chamber. The volume of phase being determined by the lower chamber volume which should be filled to about 80 – 90% with lower phase. The phases are loaded via a repeating syringe such as a Gilson Repetman. An equal volume of upper phase is added to all chambers except chamber number one to which cells in upper phase are added.

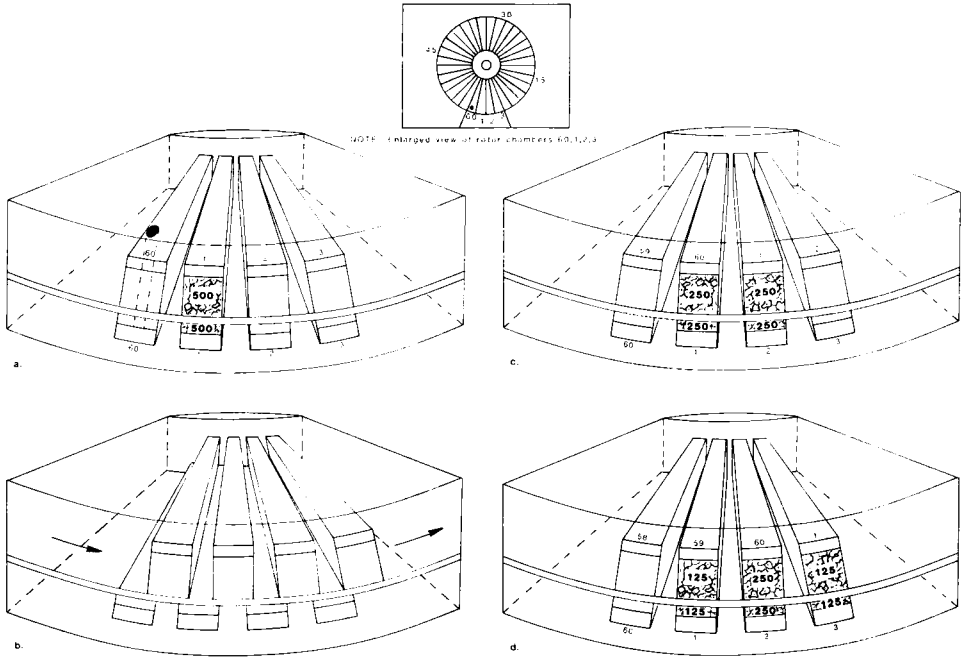


Fig. 6.12. Principle of thin-layer countercurrent distribution (TLCCD) shown diagramatically in a portion of a rotor. (a) Cells type 'A' with a partition coefficient of 50% after one partition step (no transfers). (b) Transfer in progress. (c) Distribution of cells after second partition step. (d) Distribution of cells after third partition step (second transfer). Goodman et al. (1986).

3. Preparation of cells.
Prior to loading, cells should be washed in upper phase and resuspended in upper phase at the required density. The minimum number of cells that can be loaded is solely determined by the sensitivity of the assay used to estimate the number of cells in each fraction. The maximum number that can be loaded into a single chamber for cells of around 10 μm is about 5×10^8. The limit is determined by the volume of the interface with adsorbed cells which must be retained in the lower half of the rotor. If too many cells are loaded, cells adsorbed at the interface may be transferred with the upper phase. The number of cells can be increased by loading in several chambers without undue loss of resolution.

4. Unloading.
After completion the contents of each chamber are removed by injecting about 1.5 ml of buffer into each chamber which 'breaks' the phases into a single phase. The fraction unloader is bolted onto the rotor and the assembly inverted so that the contents of each chamber drains into a disposable plastic tube (Fig. 6.7). The number of cells in each tube is determined and the distribution of cells plotted. The volume of each fraction obtained is around 3 ml.

6.7. Fractionation of human peripheral blood lymphocytes with TLCCD

Several independent studies have demonstrated the fractionation of lymphocytes with TLCCD (Walter et al., 1979, 1980; Malstrom et al., 1980). Fig. 6.13 shows the results obtained by Malstrom et al., (1980). The phase system used was a charged type consisting of 5% (w/w) dextran T500, 4% (w/w) PEG 8000, 0.85 M sodium phosphate buffer, 0.015 M sodium chloride and 5% (w/w) FCS. 119 transfer TLCCD was carried out at 4°C. There is clear separation of B and T cells, B cells identified as surface immunoglobulin positive ($SmIg^+$) are concentrated in the early fractions. T cells identified by

E-rosetting and a-naphthyl acetate esterase (ANAE$^+$) are concentrated in the later fractions. Other lymphocyte cell populations can also be detected in localised areas of the distribution, for example natural killer cells in fractions 90 – 120 and supressor T cells in fractions 80 – 85.

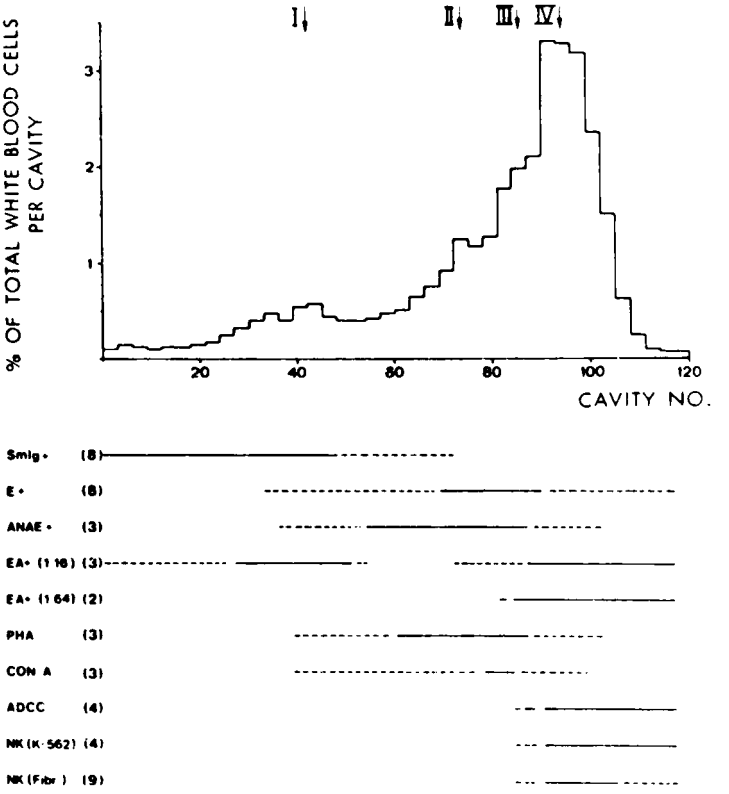


Fig. 6.13. Summary of CCD separations of human peripheral blood lymphocytes. The average distribution curve of separated cells after 119 transfers is shown. The areas, in which lymphocytes with distinctive markers and mediating various functional activities were recovered, are indicated below the curve. (– – –) indicates 50 – 100% of the proportions of activity of unseparated cells. (——) indicates above 100% of the proportion of activity of unseparated lymphocytes. Academic Press – with permission.

6.8. *Separation of differentiating* D. discoideum *cells*

The two-step partition separation of prespore and prestalk cells of the cellular slime mould *D. discoideum* described in 6.5 was based on

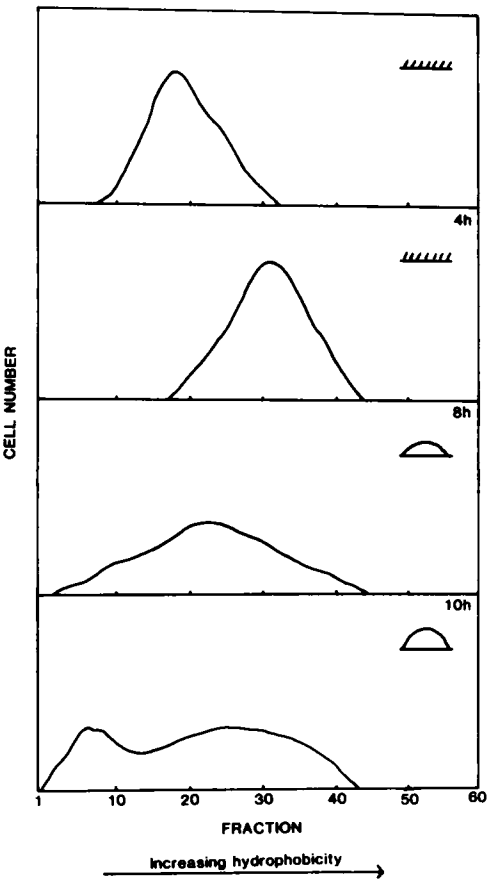


Fig. 6.14. Separation of *D. discoideum* cells at different times (stages) of development by TLCCD. The morphology of the cell aggregates is shown in the top right of each profile. 0–4 h = smooth lawn in cells, 8 h = stream aggregates, 10 h = tight aggregates. Total developmental time with the conditions used is approximately 24 h Sharpe et al., (1982).

earlier results obtained with TLCCD (Sharpe et al., 1982). TLCCD was used in this instance to detect differential changes in surface properties during early stages of *D. discoideum* development. Such changes were first detected at 8 h development and the changes were great enough by 9 h development to produce two clearly separable cell populations (Fig. 6.14). 59 transfer TLCCD with a 5.5% dextran T500, 5.5% BDH PEG 4000, zero potential phase system was used. The cell populations separated at 9 h development were shown to be precursors of the two differentiating cell types, prespore and prestalk cells. (Sharpe et al., 1982, 1985). Several other separation techniques have been used to try and separate these cells during development, the most successful being isopycnic centrifugation on Percoll gradients. The earliest developmental stage that the two types can be separated on Percoll is around 12 h. Clearly, separation on the basis of cell differences as detected by TLCCD can identify and separate the cell types earlier in development.

6.9. Effect of protease treatment on cell partition

The disruption of many tissues into single cell suspensions and detachment of cultured cells from plastic surfaces necessitates the use of proteolytic enzymes such as trypsin. Exposure of cells to such enzymes will clearly affect the surface properties and hence will also affect partition. The extent of the enzyme induced surface changes and altered partition depends on several factors, including cell type, source of protease and concentration, incubation time, temperature, and presence of protease inhibitors.

There are unfortunately few published accounts of the effect of protease treatment on cell partition, however experiments on the partition of cultured human bone cells have clearly demonstrated the effect of trypsin treatment on cell partition (Sharpe et al., 1985).

Human bone cells (osteoblasts) grown in culture are detached from tissue culture plastic by incubation with 0.5% trypsin for 30 min at 37°C. After thorough washing in PBS around 10^6 cells are

resuspended in top phase (4.9% dextran T500, 4.9% PEG 4000) and loaded on a 60 cavity TLCCD rotor. After 59 transfer (8 min settle, 10 min shake cycle) at 4°C the cells are unloaded and the number in each fraction counted on a Coulter counter. Typically two populations of cells are seen as shown in Fig. 6.15. The proportions of cells in each population however, is dependent of the time of incubation with trypsin (Fig. 6.15). Increasing the incubation time results in proportionally more cells in the second population (higher fraction number). Since the phase system used is of a low potential type, cells in the second population probably have a relatively more hydrophobic surface than cells in the first population. It thus appears that incubation with trypsin progressively removes surface protein, producing a more hydrophobic cell surface. Fractionation of the cells into two populations is therefore based on selection of surface differences which are created by removal of surface protein.

Analysis of various functional properties of the two cell popula-

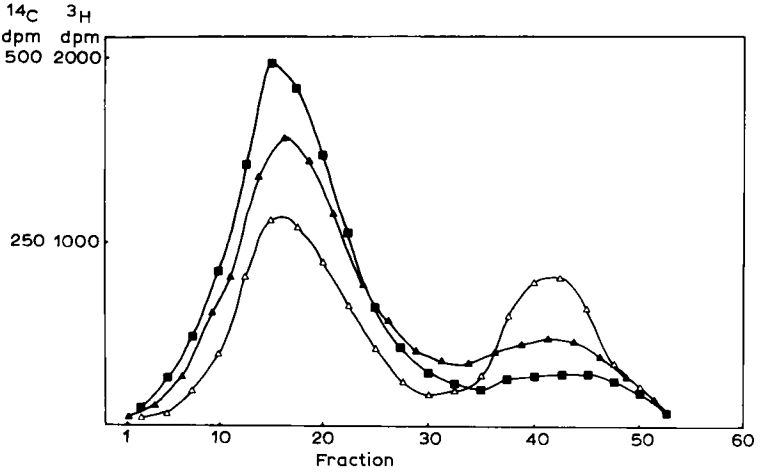


Fig. 6.15. Effect of protease treatment on the separation of human osteoblast cells in culture. Cell number determined by incorporation of radiolabelled leucine (either carbon-14 or tritium). ■——■, 15 min, [^{3}H]leucine; ▲——▲, 1 h [^{14}C-]leucine; △——△, 2 h [^{3}H]leucine. Conducted as a separate experiment. Sharpe et al., (1986).

tions has revealed that cells in the first population are rapidly growing 'young' cells and those in the second population are slow growing 'older' cells (Sharpe et al., 1986). It appears that 'older' cells are more sensitive to trypsin and therefore more protein is removed from

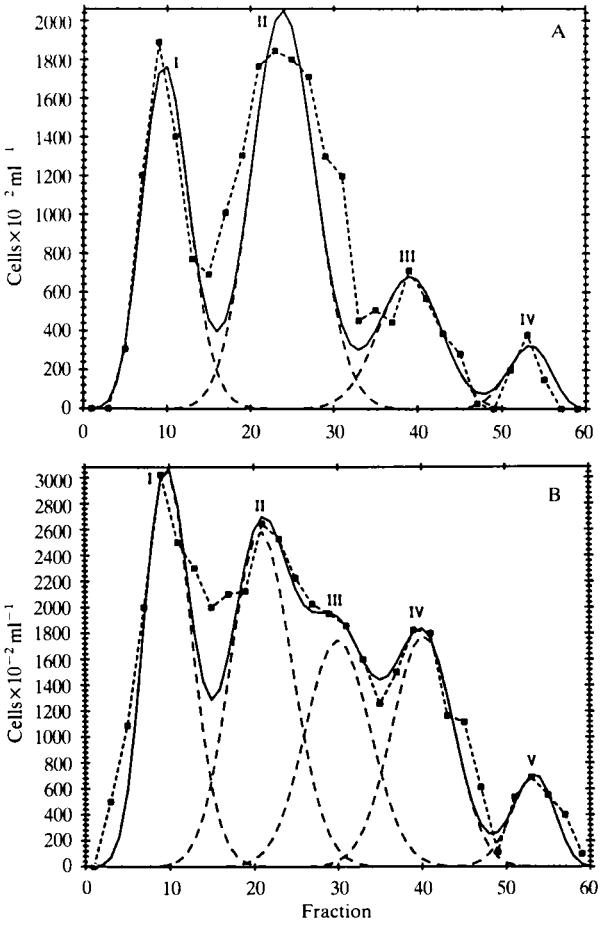


Fig. 6.16. TLCCD profiles of cells dissociated from different limb-bud regions after 59 partition steps. Experimental points; - - - - -, individual cell populations; – – –, sum of individual cell populations.
(A) Stage-21 progress zone; (B) stage-21 subprogress zone. Cottrill et al., (1986).

their surface, producing a more hydrophobic surface and giving them a higher partition coefficient. 'Young' cells, being less affected by the trypsin retain more of their surface protein and have a lower partition coefficient. This separation of these two cell populations, which appear to be functionally different, is based on differential sensitivity to trypsin.

There is some evidence that mild protease treatment does not unduly affect surface properties. Disruption of chick limb mesenchymal cells from chick embryos by incubation with 2% trypsin for 5 min at 40°C in FCS does not appear to affect the surface properties, as detected by TLCCD, (Cottrill et al., 1986).

6.10. Computer analysis of TLCCD profiles

The principle of TLCCD detailed in 6.6 was described in terms of partition of theoretical cell populations where all cells had effectively identical surface properties, and produced profiles based on Poisson distributions (e.g. Figs 6.9 and 6.10). Such profiles are however rarely obtained since surface differences between cells in a population are more often than not detected by a partition process. TLCCD cell profiles are most often broad peaks such as those shown in Fig. 6.13 and 6.14. A broad TLCCD cell profile can be considered to be formed from the sum of each individual peak based on a Poisson distribution formed from cells detected as having identical surface properties. The extent to which a profile deviates from the theoretical is an indication of the heterogeneity (with respect to surface properties) of the cell population. A computer programme originally devised by Blomquist and Wold (1974) and modified by Ford, Treffry and Sharpe (unpublished) has been produced, the sum of which gives the best fit (5%) to the experimental data. An example of the use of this programme is shown in Fig. 6.16 where possible cell sub-populations are identified from a broad TLCCD profile produced by chick limb bud cells (Cottrill et al., 1986).

Electrophoresis

7.1. Introduction

The majority of cells carry a net surface negative charge at physiological pH. The intensity of this surface charge density, varies for different cell types and thus allows separation of the different types by rate of migration in an electric field. The density of surface charge does not directly reflect the chemical composition of the cell surface since a layer of counter ions (ions of opposite charge) surrounds the cell forming an electric double layer. The electrokinetic or 'zeta' potential is the potential at the outer region of this double layer and therefore represents the apparent cell surface charge. Electrophoretic mobility depends upon this zeta potential.

The velocity of a cell in an electric field in a homogeneous medium in the absence of any effects of ions is described by:

$$v = eE/6\pi nr$$

where v is the velocity of migration, e is the charge on the cell, E is the field strength, n is the medium viscosity and r is the radius of the cell.

The electrophoretic mobility of the cell is:

$$v = V/E$$

When counter ions are present, the electrophoretic mobility is dependent on the zeta potential, ζ. Thus:

$$v = \zeta \epsilon E / 6\pi r$$

where ϵ = the dielectric constant.

The relationship between electrophoretic mobility, ionic composition of the medium, surface charge, surface shape and cell size is complex and there is no completely satisfactory formulation relating these factors. The ionic strength of the medium I, which in practice is measured as conductivity Λ has an effect on the zeta potential. The effect of changes in ionic strength can be calculated from the relationship:

$$V_2/V_1 = [I_1/I_2]^{1/2} \text{ or } V_2/V_1 = [\Lambda_1/\Lambda_2]^{1/2}$$

Because of these effects the ionic strength of the medium is kept as low as possible, usually by inclusion of non-ionic sugars (e.g. sucrose) which maintain the required osmotic strength while keeping the ionic strength low to ensure maximum migration.

There are four basic electrophoretic methods that have been used for cell separation; free-flow electrophoresis, density gradient electrophoresis, endless belt electrophoresis and stable-flow-free boundary electrophoresis (STAFLO). Of these, free-flow electrophoresis is the most successful and the most extensively used method.

There are three major practical problems to be overcome in electrophoretic separation of cells. These are cell adherence, effects of gravity and effects of temperature. Because cells readily adhere to a wide variety of surfaces, electrophoresis on solid surfaces as used for soluble materials cannot be used for cell separation. The problems of adherence are overcome by electrophoresing the cells in a liquid medium.

Cells will naturally sediment under the influence of gravity and in order to reduce any major effects of gravity that may affect separation cells are electrophoresed either in a density gradient (density gradient electrophoresis) or in a constant flow of medium.

When an electric current is applied across a liquid medium, the temperature of the medium will gradually increase. In order for efficient separation and maintainance of viability, this increase in temperature must be prevented. The cell electrophoresis apparatus all have cooling systems to dissipate the heat generated in the medium.

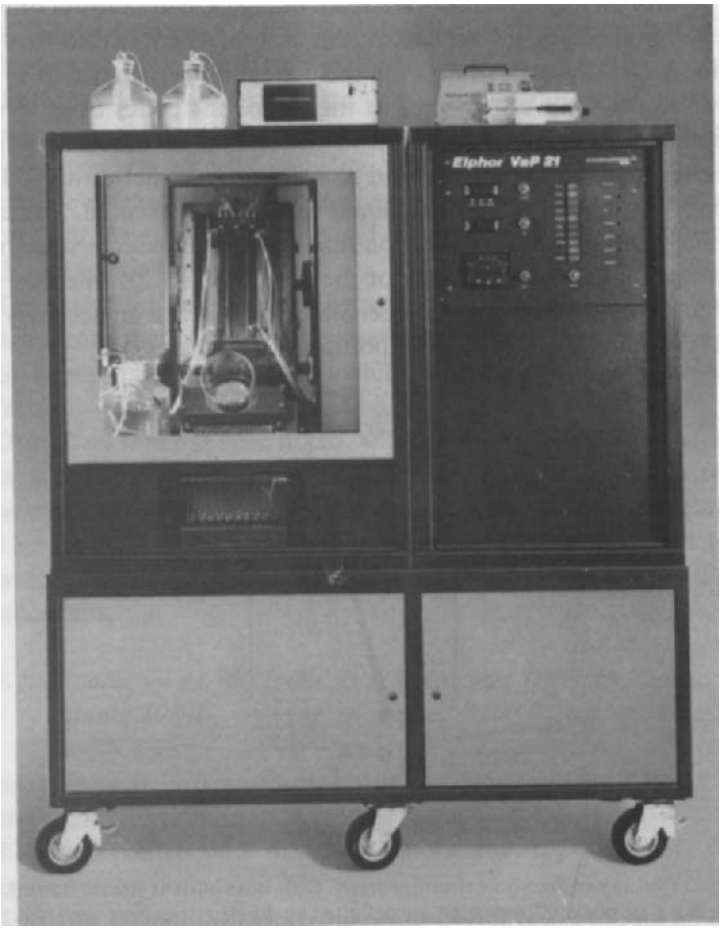


Fig. 7.1. Elphor Vap 21 free-flow electrophoresis apparatus. Courtesy of Bender and Hobein.

7.2. *Free-flow electrophoresis*

Free-flow electrophoresis was originally developed by Kurt Hannig in the early 1960s, resulting in the commercial production of the Elphor Vap apparatus by Bender and Hobein, which is the most extensively used cell electrophoresis apparatus.

The Elphor Vap apparatus is shown in Fig. 7.1. It consists of two vertical glass plates, 0.5 mm apart. An accurately controlled flow of buffer passes downwards between the plates and perpendicular to an electric field. Cells are injected at the top of the plates close to the cathode. As the cells flow down in the stream of buffer they are deflected toward the anode and the amount of deflection (electrophoretic mobility) is proportional to the zeta potential. Beneath the plates are a series of collection tubes (up to 90) which collect the deflected cells across the width of the plates (Fig. 7.2). The flow of buffer is contolled by a 90-fold peristaltic pump with an accuracy of ± 1%, which is located at the bottom of the plates.

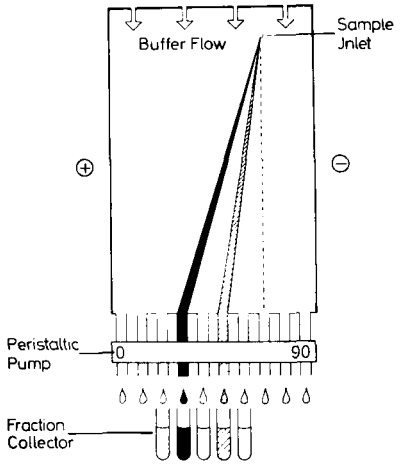


Fig. 7.2. Principle of free-flow electrophoresis. Cells in a constant stream flowing between two glass plates 0.5 mm apart are deflected by the electric current applied across the flow. The degree of deflection depends on the cell surface charge. Deflected cells are collected in fractions as they leave the plates.

Free-flow electrophoresis is a continuous process which can accomodate $3-50 \times 10^9$ cells injected per hour with an operation time of between 6 – 12 h. Cells are continuously applied via automatically controlled pulse-free injection. Cells can be fractionated into up to 90 fractions with 1 mm distance between each fraction.

An efficient cooling system is employed which enables a constant temperature (usually 4°C) to be maintained between the plates. The efficiency of the cooling system enables a wide range of flow rates to be used. High flow-rates are used for separation of large cell numbers and lower flow-rates are used for maximum separation of smaller numbers of cells.

High ionic strength buffers can be used in this particular apparatus because of the efficient cooling and the ability to operate with either high voltage or high current.

The retention time of cells between the glass plates is 1 – 5 min which means that high cell viabilities are maintained, although if low ionic strength buffers are used viability and recovery can be severely affected.

One effect that has been noted, which results in a loss of fractionation, is the broadening of the cell bands or streams. This can be compensated by ensuring that the glass surfaces have a similar zeta potential to the cells. This can be achieved by coating the plates with an appropriate protein solution such as 3% BSA in the case of mammalian cells.

7.2.1. Separation of rat T and B lymphocytes (Hansen and Hannig 1984)

Electrophoresis buffer: 15 mM triethanolamine, 4 mM potassium acetate, 240 mM glycine, 11 mM glucose.

Electrode buffer: 75 mM triethanolamine, 20 mM potassium acetate.

The osmolarity of the electrophoresis buffer is 290 mOsm and has a low ionic strength. This low ionic strength has a destabilising effect

on the cells and they must therefore be transferred very gradually to this buffer by first resuspending in a 1:1 mixture of this and a balanced salt solution. Cells should not be exposed to the electrophoresis buffer for longer than 1 h and they should be fractionated in tubes containing 1 ml of 1% BSA or 5% FCS in balanced salt solution.

Electrophoresis is carried out at 5°C with a buffer flow rate of 550 ml/h at a voltage of 1000 V (effective field strength of 70 – 100 V/cm) yielding a current of 200 mA. Before beginning the separation the apparatus is standardised by electrophoresing fresh or glutaraldehyde-fixed red blood cells. The distribution of the red cells is examined for position, band resolution and skewedness which should be consistent for each separation.

A lymphocyte preparation free of red cells is prepared, the cells

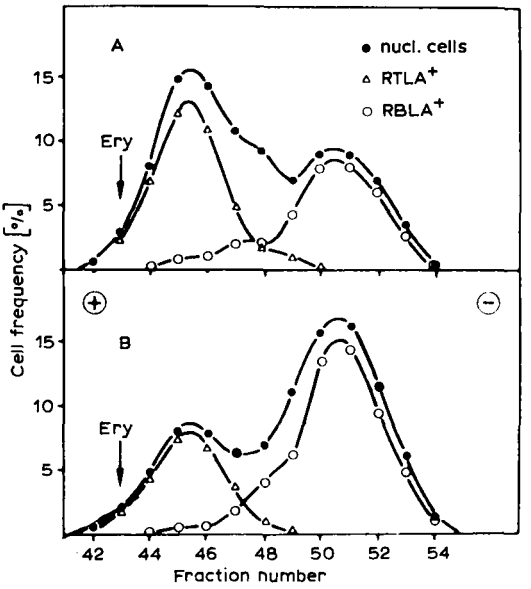


Fig. 7.3. Free-flow electrophoresis of rat lymphocytes from (A) lymph nodes; (B) spleen. RBLA = rat B lymphocyte specific antigens. RTLA = rat T lymphocyte-specific antigens. Antigens detected by direct immunofluorescence with FTC or TRITC conjugated antisera raised in rabbits. Hansen and Hannig, (1984), Academic Press – with permission.

washed, resuspended in the electrophoresis buffer at around $2-6 \times 10^7$ cells/ml and injected into the apparatus. 20 fractions are collected in 10 ml tubes and the number of cells in each are counted. Two peaks of cells are obtained as shown in Fig. 7.3. The distribution of cells with surface antigens specific for T and B cells are tested and cells with low electrophoretic mobility (the peak in the high numbered fractions) are 95% pure B lymphocytes. Cells with high electrophoretic mobility (the peak in the low fraction numbers) are 95% pure T cells. The viability of cells in all fractions is around 90% when tested by trypan blue exclusion. The recovery of cells is between 80 – 85% per fraction which appears to be a reflection of the low ionic strength buffer. Dead cells (i.e. intact but non-viable) aquire a high negative charge.

7.2.2. Separation of human T and B lymphocytes (Hansen and Hannig 1984)

Although free-flow electrophoresis produces a very effective separation of rat T and B cells, human T and B cells cannot be separated in this way because their differences in zeta potential are too small. In an attempt to resolve this problem Hansen and Hannig have developed 'antigen-specific electrophoretic cell separation' (ASECS). This method is based on the fact that immunoglobulins have a lower charge than that on cell surfaces. Cells with antibodies bound to their surfaces thus have a lower overall charge and hence a lower electrophoretic mobility than cells without antibodies. In this example, human lymphocytes are incubated with rabbit anti-human IgM for which B cells have receptors whereas T cells do not. 5×10^7 cells are incubated in a 1:4 dilution of antibody for 30 min at 4°C. Free antibody is removed by washing, and in order to determine the percentage of cells with antibody bound, the cells are further incubated with a 1:2 dilution of TRITC-conjugated goat anti-rabbit IgG for 30 min at 4°C and viewed under a fluorescence microscope. After washing the cells are resuspended in 2 ml electrophoresis buffer and filtered through a wet plug of cotton wool in a Pasteur pipette. Elec-

trophoresis is carried out at 4°C with 900 – 1000 V, a flow rate of 500 ml/h and sample injection of 6 ml/h. 20 fractions are collected in 10 ml tubes, the number and fluorescence intensity of cells determined. Fig. 7.4 shows the results obtained. B cells (+ antibody) are collected in the low electrophoretic mobility fractions and T cells in the high mobility fractions.

Smolka et al. (1979) have described a method for altering the surface charge of cells by specific labelling with poly(vinylpyridine) and poly(glutaraldehyde) microspheres. Binding of the microspheres

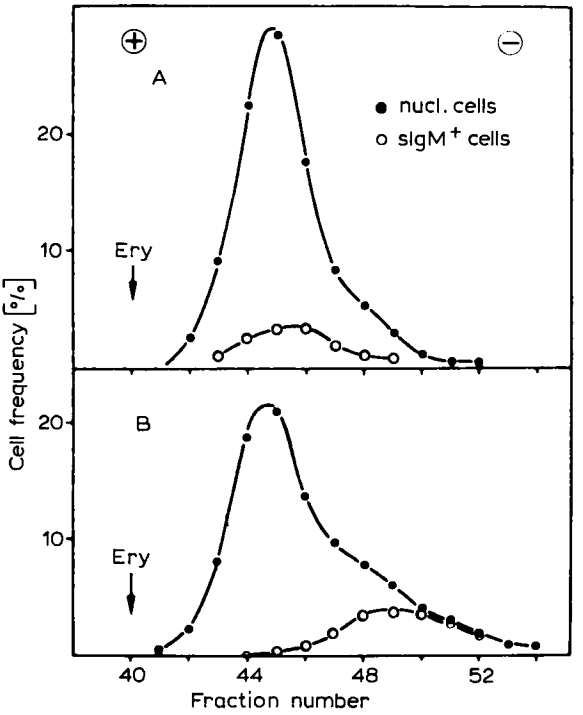


Fig. 7.4. Free-flow electrophoresis of human peripheral blood lymphocytes (A) separation of untreated cells. B cells identified by indirect immunofluorescence with antiserum antiserum against IgM. (B) separation of antibody-labelled cells (ASECS) using rabbit anti-human IgM antiserum and TRITC-conjugated goat anti-rabbit Igb. Hansen and Hannig, (1984), Academic Press – with permission.

reduces the surface charge of cells (in this case red cells) which can enable electrophoretic separations to be achieved where the differences in surface charge between cells are too small to produce effective separation.

Fig. 7.5. Biostream electrophoresis apparatus. Courtesy of CJB Developments.

7.3. Biostream

The Biostream free flow apparatus has recently been developed in the U.K. by John Brown PLC and marketed by CJB Devolpments (a subsidiary). Biostream was produced principally for the large scale electrophoretic separation of proteins but can also be used for cell separation. There is as yet however little published information on the separation of cells.

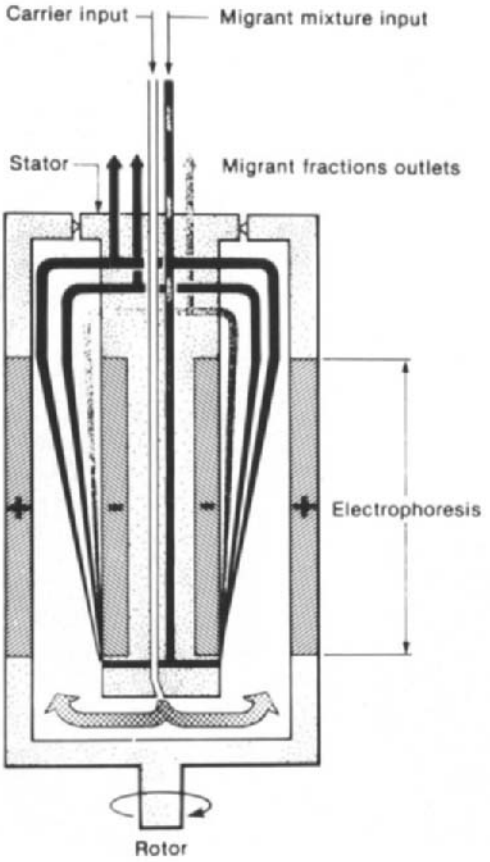


Fig. 7.6. Principle of the Biostream. Courtesy of CJB Developments.

The Biostream apparatus is shown in Fig. 7.5 and its cut-away shown in Fig. 7.6. It consists of two concentric steel cylinders, an outer cylinder which rotates and an inner, stationary cylinder. The carrier buffer and cells flow upward in an annulus between the two cylinders with the flow (up to 3 l/h) being stabilised by the rotation of the outer cylinder (130 – 160 rpm). Electrolyte solutions flow in two compartments in the outer and inner cylinders such that the carrier buffer and cells are sandwiched between the two electrolyte solutions. The electrolyte solutions are isolated from the carrier buffer and cells by dialysis membranes. The cells in the carrier buffer are more or less attracted to the anode, the outer electrode, moving at different rates towards the outer wall of the annulus. Concentric bands of cells are thus formed within the annulus. At the top of the stationary cylinder there is an assembly of 30 discs called maze plates. The concentric streams of cells flow upward and into these plates. Cells in different divergent, concentric streams flow into different maze plates. Each maze plate has a single outlet allowing col-

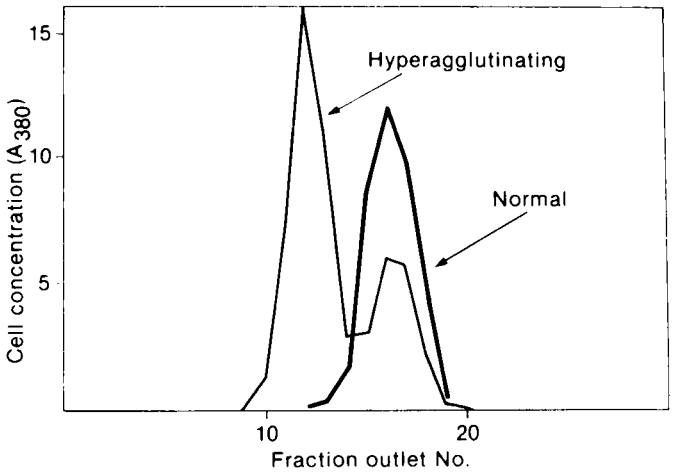


Fig. 7.7. Separation of abnormal hyperagglutinating RBC's from normal RBC's by electrophoresis on a Biostream apparatus. Courtesey of Harwell Laboratory and Blood Products Laboratory (U.K.).

lection of the cells flowing into each plate. 30 cell fractions are thus obtained, from the 30 maze plates.

The Biostream can handle very large numbers of cells, for example in the example shown in Fig. 7.7, 1.0×10^8 cells/min were electrophoresed. Fig. 7.7 shows the separation of normal and abnormal red blood cells obtained on the Biostream. Viability of the cells was maintained and recovery was close to 100%.

The use of the Biostream for cell separation has yet to be fully evaluated as only a few different cell types have been tested. There should be few problems of maintaining cell viability since the average total time a single cell is in the apparatus is 2 – 3 min. Although small cell numbers can be separated, the apparatus is designed for large scale industrial-type separations and significant cell wastage can occur when operated with small cell numbers.

7.4. Density gradient electrophoresis

In density gradient electrophoresis, cells are electrophoresed downward through a density gradient of liquid medium. The gradient is such that sedimentation velocity of the cells (at unit gravity) is minimised and thus rate of migration is related to cell charge (zeta potential). Electrodes are located with the cathode at the top of the gradient and the anode (toward which the cells are attracted) at the bottom. There are two important practical difficulties to be overcome in density gradient electrophoresis. The cells must be layered onto the gradient, as in centrifugation as a narrow band and the gradient must be fractionated, undisturbed. Gradient materials used for centrifugation have been tried for electrophoresis and it appears that Ficoll, in spite of its high viscosity is most suitable.

A wide variety of different types of apparatus have been used for density gradient electrophoresis, such as the Boltz-Todd device, the STAFLO apparatus, the LKB column and the Tulp apparatus. Details and further references on each of these can be found in a review by Tulp (1975). The following description of density gradient

electrophoresis will be limited to one particular device, the Buchler-Polyprep 200 (Buchler Instruments).

The Polyprep apparatus is shown in Fig. 4.6 in Chapter 4. It consists of a glass column surrounded by a cooling jacket with an inner cooling cylinder running down its centre. The gradient is formed in the hollow cylinder (cross-sectional area 17.6 cm), which is siliconised. A porous glass membrane separates the lower electrolyte from the dense bottom solution of 10% Ficoll, 5.1% sucrose (80 ml). A 100 ml continuous, linear density gradient of 2.5 – 6.25% Ficoll made 300 mOsm with sucrose producing an inverse sucrose gradient (6.3 – 5.72%) covering a density range of 1.0397 – 1.0480 g/ml, is layered above the bottom solution in the hollow chamber and over layered with 125 ml of upper electrolyte solution. About 5 – 10 ml of cell sample is layered at the interface between the top of the gradient and the upper electrolyte via Teflon tubing. The upper and lower electrolyte solutions are circulated at 1.2 ml/min with electrophoresis being carried out for 4.5 h at 4°C with a constant 20 mA current. The gradient is unloaded by pumping a dense solution through 2 at a rate of 1.0 ml/min which forces the gradient through 4 at a rate of 2.9 ml/min, where it is collected as a series of fractions (Catsimpoolas and Griffith, 1977).

Upper electrolyte: 6.8% sucrose in electrophoresis buffer
Lower electrolyte: 5.1% sucrose in electrophoresis buffer
Electrophoresis buffer: (20 litres per experiment), 0.2 g KCl, 1.5 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.12 g sodium acetate and 10 g glucose per litre. Gradient prepared from, 50 ml of 2.5% Ficoll/6.35% sucrose and 50 ml of 6.25% Ficoll/5.72% sucrose, both in electrophoresis buffer.

7.4.1. Separation of mouse T and B cells. (Platsoucas et al., 1976; Catsimpoolas and Griffith, 1977)

The separation of mouse (CBA/H/T6j) spleen lymphocytes obtained with the Polyprep apparatus using the general method described

above is shown in Fig. 7.8. T cells have a higher electrophoretic mobility (θ-positive, FITC-conjugated anti-theta alloantisera) than B cells (Ig-positive, FITC-conjugated rabbit anti-mouse immunoglobin antibody). Overlapping of the two cell types occurs in the centre fractions although a high percentage of these cells are both

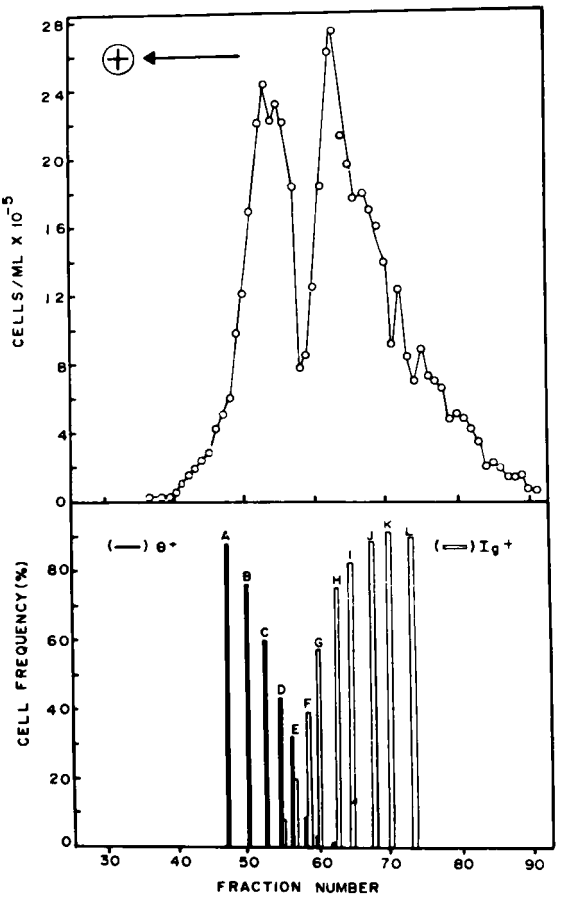


Fig. 7.8. Electrophoretic distribution profile of mouse (CBA/H/T6j) spleen lymphocytes and the presence of θ and Ig-positive cells in pooled fractions. (Platsoucas et al. (1976), Catsimpoolas and Griffith (1977)).

θ- and Ig-negative. The recovery of cells after electrophoresis, counting and washing was 70 – 90% and viability was treater than 90%.

Specific surface methods – an introduction to Chapters 8 – 13

The methods discussed in Chapters 6 and 7 were methods of separating cells that differed in their surface characteristics where the nature of these differences were not known i.e. cells could be separated without prior knowledge of the existence of any differences. Clearly these methods are powerful tools in cell biology research particularly for identifying and separating subpopulations of cells having the same phenotype e.g. cells at different stages of development. For the separation of cells where known differences in surface character exist there are more powerful methods than those discussed in Part 2. These methods separate cells having known specific differences in surface character. The basis of all these separation methods is that a molecule is available that has an affinity for the surface molecule(s) that are different. The binding of these molecules is then used to 'mark' the cells and by employing a variety of methods the marked cells can be distinguished and separated. The three most widely used molecules to mark cells are antibodies specific for a particular cell surface determinent (antigen), hormones and other molecules that have cell surface receptors and lectins that bind to particular carbohydrate sequences on cell surfaces. All of the specific surface separation methods described below can utilise these interactions. The difference between each method is how the cells marked with these molecules are separated from the unmarked cells. In principle these are extremely powerful separation methods because they are based on very specific biological interactions. The

main hurdle that has had to be overcome is the method of separating the marked cells from unmarked cells, which, to be successful in separating a wide range of different cell types, has in some instances required the development of very complex and expensive equipment.

CHAPTER 8

Affinity chromatography

8.1. Introduction

Affinity chromatography of cells is basically the same as affinity chromatography of soluble molecules such as proteins. The general procedure is to use a column of beads to which an affinity ligand has been attached. The cell population is passed through the column and cells with an affinity for the ligand bind to the beads and remain in the column. Cells lacking this affinity pass directly through the column. The cells attached to the beads are then removed and eluted from the column. Although this method is very successful for the separation of a variety of soluble molecules, when applied to cell separation many problems can arise. The extent of these problems is greatly dependent on the particular cells being used. Although there are many excellent examples of affinity chromatography separations published in the literature particularly for separating lymphocytes, many workers have been unable to obtain adequate separation, recovery or viabilities with this method.

The most common problem appears to be non-specific binding of cells to the column material. Where this occurs the purity of the cells separated is greatly reduced. The extent of non-specific binding is greatly dependent upon the particular cells and is probably the major explanation of why there are very good examples of separations with certain cells whereas some cell populations cannot be separated. In

terms of equipment required, affinity chromatography is one of the cheapest surface separation methods since the only equipment is a small column. This is the major advantage of this method since it allows any laboratory to conduct trial experiments to determine if the particular cells of interest can be separated with very little expenditure. Added to this is the fact that the method itself is very simple and conditions that should in theory achieve binding and elution of a specific cell population can quickly be found. Therefore although there are reservations concerning the general applicability of this method, because of its simplicity and cheapness it is well worth considering as a first option in trying to separate cells with a specific surface 'marker' molecule.

8.2. Types of ligand interaction

The three major types of ligand interaction used are antibody/antigen, ligand (e.g. hormone)/receptor and lectin/carbohydrate. Either molecule of these pairs can be coupled to adsorbant and separate cells by binding to its partner on the cell surface. Most commonly the molecules that are linked to the adsorbant cells are antibodies, hormones and lectins.

8.2.1. Antibody/antigen interactions

Antibodies can easily be attached to many different types of beads either by chemical coupling or adsortion. The simplest chemical coupling method involves the use of cyanogen bromide to activate polysaccharide (e.g. Sepharose, Sephadex, etc.) beads which are then able to react with free amino groups. Activation of Sepharose 6MB beads produces reactive imidocarbonate and cyanate ester groups which in mild alkali conditions will react with free amino groups to produce isourea linkages (Axen et al., 1967). This reaction unfortunately involves the use of highly toxic cyanogen bromide. Sepharose 6MB beads are however available already activated with

cyanogen bromide which are ready to be coupled to proteins.

Antibody coupled to macrobeads will bind cells with a surface antigen recognised by the antibody (Fig. 8.1). This method gives effective depletion of one particular cell type from the whole population. Removal of bound cells can however be difficult since the antibody – antigen interactions are often very strong (association constant $> 10^9\ M^{-1}$) and a suitable competing agent may not be available. Very often the only way of eluting antibody-bound cells is to elute with high concentrations of unbound antibody but these may not always be available. An alternative to direct antibody coupling to the adsorbant, is to use Protein A. Protein A is an IgG binding protein from *Staphylococcus aureus* which binds to the Fc region of IgG-type antibodies. Cells can be incubated with antibody and then passed through a column of protein A-linked adsorbant. The protein A will bind the cells with antibody on their surface and these will be retained on the column. These cells can now be competitively eluted with IgG (Fig. 8.2).

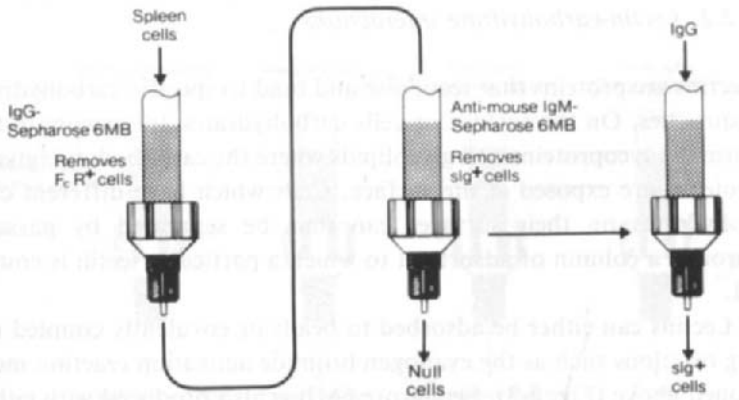


Fig. 8.1. Principle of affinity separation of cells on IgG-coupled Sepharose 6MB. Courtesy of Pharmacia.

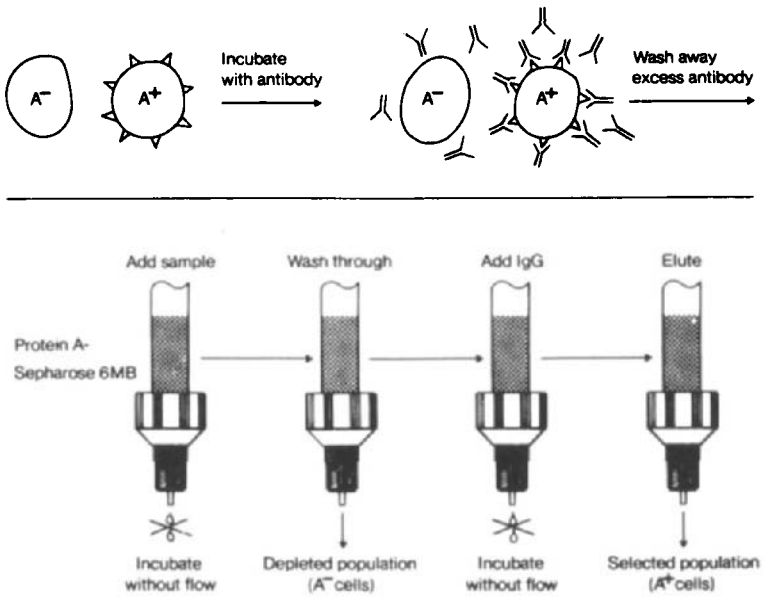


Fig. 8.2. Affinity of T cells on Protein A-Sepharose 6MB. Courtesy of Pharmacia.

8.2.2. Lectin-carbohydrate interactions

Lectins are proteins that recognise and bind to specific carbohydrate sequencies. On the surface of cells carbohydrates are usually in the form of glycoproteins and glycolipids where the carbohydrate (glyco) moieties are exposed at the surface. Cells which have different carbohydrates on their surfaces can thus be separated by passage through a column of adsorbant to which a particular lectin is coupled.

Lectins can either be adsorbed to beads or covalently coupled using reactions such as the cyanogen bromide activation reaction mentioned above (Fig. 8.3). Sepharose 6MB is also produced with either Helix pomatia (specific for *N*-acetyl-a-D-galactosamine) or wheat germ (specific for *N*-acetyl-glucosamine) lectins. Affinity separation

using lectins can only be used where a particular cell type of interest shows a specific interaction with a particular lectin. Hence this method is not as generally applicable as antibody/antigen interactions. The advantage of the lectin system is that elution of bound cells is relatively straightforward since simple mono- and disaccharides will compete with cells for lectin binding.

Table 8.1 shows a list of lectins and their carbohydrate specificities that are currently available (Sigma and others). The ones that have been most extensively used for cell separation are, Concanavalin A; Helix pomatia; lentil lectin; peanut lectin; soybean lectin; Vicia villosa lectin and wheat germ lectin. Although many of these lectins show the same carbohydrate specificity they often have different binding affinities (association constants) for the same carbohydrate. For example, the association constant for the binding of methyl-a-D-glucoside the lentil lectin is $1.0 \times 10^2\ M^{-1}$ and to concanavalin A is $5.4 \times 10^3\ M^{-1}$ (Lis and Sharon 1977).

8.2.3. Ligand-receptor interactions

Where a particular cell type has a surface receptor for a ligand such as a hormone this interaction can be used for affinity cell separation.

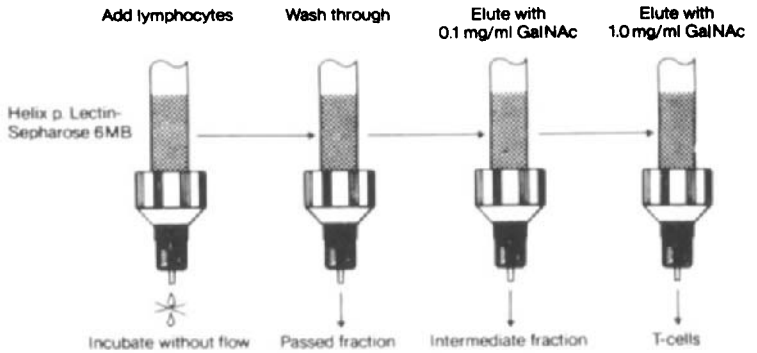


Fig. 8.3. Separation of T cells by affinity chromatography on *Helix pomatia* lectin Sepharose 6MB. Courtesy of Pharmacia.

TABLE 8.1
Lectins and their binding specificities (Sigma)

Lectin	Sugar specificity
Abrus precatorius	D-gal
Agglutinin	
Abrin A (toxin)	D-gal
Abrin C (toxin)	D-gal
Agaricus	β-D-gal(1-3)-D-galNAc
Anguilla anguilla	α-L-Fuc
Arachis hypogaea	β-D-gal(1-3)-D-galNAc
Bandeiraea simplicifolia	
BS-1	α-D-gal; α-D-galNAc
BS1-B_4	α-D-gal
BS1-AB_3	α-D-gal; α-D-galNAc
BS1-A_2B_2	α-D-gal; α-D-galNAc
BS1-A_3B_1	α-D-gal; α-D-galNAc
BS1-A_4	α-D-galNAc
BS11	D-glcNAc
Bauhinia purpurea	0-β-D-gal(1-3)-D-galNAc
Caragana arborescens	D-galNAc
Cicer arietinum	Fetuin
Codium fragile	D-galNAc
Concanavalin A	α-D-man, α-D-glc
Succinyl-Con A	α-D-man, α-D-glc
Datura stramonium	$(D\text{-}glcNAc)_2$
Dolichos biflorus	α-D-galNAc
Erythrina corallodendron	β-D-gal(1-4)-D-glcNAc
Erythrina cristaglli	a-D-gal(1-4)-glcNAc
Euonymus europaeus	α-D-gal(1-3)-D-gal
Glycine max	D-galNAc
Helix aspersa	D-galNAc
Helix pomatia	D-galNAc
Lathyrus odoratus	α-D-man
Lens culinaris	α-D-man
Limulus polyphemus	NeuNac
Bacterial agglutinin	D-galNAc; D-glcNAc
Lycopersicon esculentum	$(D\text{-}glcNAc)_3$
Maclura pomifera	α-D-gal; α-D-galNAc
Mycoplasma gallisepticum	Glycophorin
Naja mocambique mocambique	–
Naja naja kaouthia	–
Perseau americana	–
Phaseolus coccineus	–

TABLE 8.1
(continued)

Lectin	Sugar specificity
Phaseolus limensis	D-galNAc
Phaseolus vulgaris	
PHA-E	Oligosaccharide
PHA-L	Oligosaccharide
Phytolacca americana	$(D\text{-glcNAc})_3$
Pisum sativum	α-D-man
Psophocarpus tetragonolobus	D-galNAc-D-gal
Ptilota plumosa	α-D-gal
Ricinus communis	
Toxin RCA_{60}	D-galNAc; β-D-gal
Aglutinin RCA_{120}	β-D-gal
Robinia pseudoaccacia	–
Sambucus nigra	β-D-gal(1-4)-D-glc
Solanum tuberosum	$(D\text{-glcNAc})_3$
Sophora japonica	β-D-galNAc
Tetragonolobus purpureas	α-L-fuc
Triticum vulgaris	$(D\text{-glcNAc})_2$NeuNAc
Ulex europaeus	
UEA I	α-L-fuc
UEA II	$(D\text{-glcNAc})_2$
Vicia faba	D-man, D-glc
Vicia villosa	D-galNAc
A_4	D-galNAc
B_4	D-galNAc
Vigna radiata	α-D-gal
Viscum album	β-D-gal
Wisteria floribunda	D-galNAc

The purified ligand is immobilised by coupling to an adsorbant i.e. to cyanogen bromide activated Sepharose, and used to retain cells on a column which have the surface receptor. The major problem with this interaction is the availability of ligand (e.g. a hormone) both for coupling and elution of bound cells.

8.3. *Types of adsorbant*

In order for cells to be able to pass rapidly through the column there must be large, uniform pores between beads. Large beads must therefore be used of around 250 – 300 μm diameter. A variety of different beads have been used in affinity cell separations including, glass and plastic beads, cross-linked dextrans such as Sephadex G-200, acrylamide, agarose in the form of Sepharose 6MB macrobeads and Affi-gel (Biorad). Examples of the use of each of these different types of beads can be found in a review by Platsoucas and Catsimpoolas (1980). The use of three types of beads is detailed below.

8.4. *Sepharose 6MB macrobeads*

As mentioned above, both Sepharose and Sephadex beads have previously been used for affinity chromatography of cells. Indeed Sephadex G200 beads have also proved successful for separating cells on the basis of size. For affinity chromatography it is essential that cells do not become trapped in the column hence large beads producing large pores are more ideal than smaller sized beads such as Sephadex G200 (40 – 120 μm). It is also important that the beads should pack evenly in the column so cells can pass through freely without getting trapped. Sepharose 6MB macrobeads have been produced specifically for affinity chromatography of cells by Pharmacia. They are large, uniform size, beads between 250 – 350 μm diameter that appear to exhibit a low level of non-specific adsorbance. Furthermore the beads can be obtained in different forms i.e. with lectins or antibodies already attached or with chemically reactive groups such as cyanogen bromide to allow the coupling of specific ligands using simple reaction procedures.

8.4.1. Generalised methodology for Sepharose 6MB macrobeads

Described below is the generalised methodology for the use of

Sepharose 6MB macrobeads as described by Pharmacia. Following this are described several examples (including details of reaction conditions etc.) of the use of these beads for various cell separations.

8.4.1.1. Coupling of proteins to cyanogen actived Sepharose 6MB. The commonest affinity ligands that are used in affinity cell separation are proteins i.e. antibodies, lectins and hormones, and since the easiest way of coupling these to suitable adsorbants is to couple to cyanogen bromide activated Sepharose, this method will be described in detail. For coupling of proteins to other polysaccharide based adsorbants using this reaction the reader is referred to Axen et al., (1967).

The size of the column of adsorbant used for affinity cell separation need only be small between 1 – 5 ml bed volume, depending on the number of cells to be separated. The column itself must be fitted with a suitable large pore bed support net of around 80 μm to allow free passage of cells, and if made of glass it must be siliconised.

Any buffer can be used to carry out the coupling reaction as long as there are no free amino groups as in Tris buffers, although coupling is most effective at pH 8 – 10 in high ionic strength solutions (0.5 M). The degree of substitution obtained for proteins is usually of the order to 80 – 95%. Highly substituted ligands are useful when depleting a population of a particular cell type that binds to the affinity ligand. However when the bound cells are required and must therefore be eluted, it is often an advantage to use adsorbants with lower substition of ligand to facilitate elution of bound cells. The simplest way of ensuring a lower level of substitution is to prehydrolyse the beads by incubation in buffer at high pH. 50% of original activity can be lost in 4 h incubation at pH 8.3.

8.4.1.2. Separation conditions. After thorough washing, the adsorbant should be packed into a small column under gravity flow and equibrated with at least 10 volumes of buffer. The volume of adsorbant required depends on its binding capacity and the number of cells to be separated. As a general guide about 1×10^8 cells can be

separated using 2 – 5 ml of adsorbant with 1 mg/ml of bound affinity ligand.

For conducting separations under sterile conditions the adsorbant may be sterilised with 1% formaldehyde for 30 min at room temperature provided the attached ligand remains stable. When not in use, the addition of bacteriostatic agents such as sodium azide at low concentrations (0.05%) will help maintain sterility of adsorbant solutions.

The separation conditions can be varied to a large extent without critically affecting the separation. In general therefore, conditions can be selected that maintain viability of the particular cells being separated. Factors that will influence separation are processes that involve surface antigens such as capping, shedding or internalisation. These processes can be inhibited during separation by inclusion of metabolic inhibitors such as sodium azide (0.02%) or conducting the separation at temperatures below 37°C where possible. Exposure of cells for long periods in sodium azide can cause damage and a recovery period after separation will be necessary.

Although the medium used as a column buffer can be of virtually any constitution, it should not contain carbohydrates or serum when utilising lectin interactions, or contain serum when Protein A is used as these will interact with the affinity ligand. Protective colloids such as Ficoll 70 (0.3%), serum albumin (0.3%) and gelatin (0.25%) are often included in the medium. The buffer medium used should be at the same temperature as the adsorbant in the column to avoid bubbles forming.

8.4.1.3. Sample application. The buffer above the equilibrated adsorbant bed is removed and the cell suspension added to the top of the bed. The volume of suspension added should ideally not be greater than 30% of the bed volume. Once all the cell suspension has drained into the adsorbent bed the flow should be stopped and the cells allowed to incubate in contact with the affinity adsorbant. An incubation period of 10 – 20 min is usually adequate but longer incubation times may be more effective where a population is being

depleted of the cells that bind to the ligand. More efficient cell adsorption may sometimes be obtained by gently mixing or stirring of the adsorbant bed although care should be taken since excessively violent mixing may cause cell damage. The period of incubation without buffer flow can have pronounced effects on the separation obtained. Table 8.2 shows the effect of incubation time on the separation of B lymphocytes (sIg^+) from human peripheral blood lymphocytes. Although the percentage of B cells binding to the adsorbant increased with time of incubation from 2 – 20 min the maximium recovery of B cells eluted from the column was obtained with an incubation period of 10 min. The total cell recovery low decreased with increased incubation time.

8.4.1.4. Elution of cells. After allowing sufficient time for incubation, unbound cells can be washed off the column with about 20 volumes of buffer with a flow rate of 2 – 10 ml/min. The gentlest method of recovering bound cells is via competitive elution. This is most effective where lectin interactions are used since simple carbohydrates will compete for binding. A solution of the appropriate carbohydrate between 0.1 – 10 mg/ml is usually sufficient to elute bound cells by gently flowing 20 – 30 volumes of solution through the column. Different lectins do however have very different binding constants (see 8.2.2) which means that the optimal concentration of competing carbohydrate solution will vary depending on the lectin used since cells will bind to different lectins with greater or lesser affinity. T lymphocytes bound to wheat germ-Sepharose 6MB can be eluted with a solution containing *N*-acetyl-glucosamine at 25 mg/ml (Hellstrom et al., 1976a; Kimura et al., 1979). Human blood lymphocytes have also been fractionated on Helix pomatia lectin-Sepharose 6MB by elution with DGalNAc at 0.1 mg/ml in PBS/HSA (Hellstrom et al., 1976, 1984).

One way to optimise the elution conditions is to continuously monitor the number of cells, bound and unbound, recovered from the column as a percentage of the total added. Conditions are then chosen which give the highest possible recovery of bound cells in as

TABLE 8.2
Effect of on-column incubation time on cell separation on Protein A-Sepharose 6MB

	Non-bound cells				Desorbed cells				
On-column incubation time	No. cells in fraction ($\times 10^{-6}$)	Percent of no. added	sIg^+ (%)	E^+ (%)	No. cells in fraction ($\times 10^{-6}$)	Percent of no. added	sIg^+ (%)	E^+ (%)	Total cell recovery (%)
2 min	30	88	14	47	3.8	11	66	1	99
10 min	28	82	9	61	4.3	13	70	4	95
20 min	22	65	4	64	4.9	14	46	2	79

1.5 ml column of Protein A-Sepharose 6MB (equilibrated with Tris-Hanks buffer containing 0.3% Ficoll 70 and 0.02% NaC3) was loaded with 3.4 × 10 human peripheral lymphocytes, pretreated with rabbit anti-human Ig, and incubated for the stated time at room temperature before washing out non-bound cells with starting buffer. Bound cells were desorbed with bovine IgG (20 mg/ml) at 37°C, aided by gentle resuspension of the beads. The cells applied consisted of 18% sIg^+ and 45% E^+. (Pharmacia).

short a time as possible. Alternatively a rapid batch technique can be used that has been described by Dillner et al., (1980). A small volume of beads (about one tenth) are mixed with one tenth the number of cells and incubated for different times. The beads are then viewed under a light microscope, the number of cells bound per bead scored and the optimum incubation time to achieve maximum binding obtained. Incubation of the beads with cells bound, with various concentrations of sugar solutions for different times, followed by scoring of the number of cells per bead, can be used to determine appropriate elution conditions.

Stepwise elution with increasing concentrations of carbohydrate can be useful for identifying subpopulations of cells bound to the adsorbant i.e. cells with different affinities for the lectin (Hellstrom et al., 1976a, b). Different putative subpopulations obtained should however always be shown to be functionally different by other criteria in order to rule out the possibility of elution of the subpopulations being an artifact. Competitive elution may be used to remove cells bound via antibody or ligand/receptor interactions. This method is not always convenient particularly where solutions containing milligram quantities of specific antibodies or hormone receptors are required. Removal of cells bound to protein A Sepharose can be achieved by competitive elution with IgG at a concentration of 20 mg/ml.

Recovery of bound cells may be improved by incubating the column with eluting solution without flow prior to elution. A high buffer flow rate may also increase recovery as may increasing the temperature of elution buffer (Nicola et al., 1978). Mechanical treatment of the adsorbant with cells bound may also enhance recovery. Gently stirring or vortexing are often used although this is not always advisable where the cells are likely to be fragile.

8.4.1.5. Column regeneration. One of the advantages of affinity chromatography is that the adsorbants with specific ligands attached are often reusable. Protein A Sepharose 6MB should be regenerated by removing the bound IgG by washing with at least 10 volumes of

0.1 M glycine – HCl buffer pH3 containing 0.5 M NaCl followed by equilibration with the storage buffer at neutral pH. Most other adsorbants can be regenerated by washing with at least 10 volumes of 0.1 M Tris – HCl buffer pH 8.5 containing 0.5 M NaCl followed by at least 10 volumes of 0.1 M sodium acetate buffer pH 4.5 containing 0.5 M NaCl. Where the adsorbant is a lectin with high association constant, regeneratioin may require incubation with 0.1% Nonidet P40.

8.4.2. Immunoadsorbant separation

This section describes in detail the coupling of antibody to CNBr activated Sepharose and use of this adsorbant for cell separation (from Hubbard et al., 1984).

8.4.2.1. Coupling. 1. 1 g CNBr activated Sepharose 6MB (3 ml swollen gel) is swollen in 1 mM HCl for 15 min.
2. The swollen gel is washed with 200 ml 1 mM HCl on a sintered glass filter.
3. The gel is washed with 10 ml of coupling buffer (usually 0.1 M $NaHCO_3$ – 0.5 M NaCl, pH 8.0) and transferred to 3 ml of the same buffer containing the antibody at a concentration of between 1 – 10 mg/g dry Sepharose after dialysis against coupling buffer.
4. The suspension is rotated for 2 h at room temperature or overnight at 4°C.
5. Before adding the antibody solution to the gel the absorbance at 280 nm is read.
6. Unbound ligand is removed from the reacted gel by washing with 20 ml of 0.1 M acetate (or citrate), 0.5 M NaCl pH 4.0 followed by 20 ml coupling buffer on a sintered glass funnel. This cycle is repeated 3 times.
7. The absorbance at 280 nm of the washings is read and the coupling efficiency determined by comparing with the initial adsorbance at 280 nm. The coupling efficiency should be around 80 – 95%.
8. Unreacted groups remaining on the gel are blocked by incubation

with 0.2 M glycine (pH 8) or 1 M ethanolamine for 2 h at room temperature.
9. The gel is washed with 50 ml of coupling buffer and either used immediately or stored at 4°C in buffer containing 0.05% sodium azide.

8.4.2.2. Cell separation. 1. The adsorbant is poured into the column and allowed to pack under gravity flow before washing with 10 volumes of media.
2. The media is allowed to drain out of the column until the level just touches the top of the gel and $1-5 \times 10^7$ cells/ml (10^8 total maximum) are added and allowed to pass into the gel. At this point the flow can be stopped if an incubation period is to be used.
3. After incubation or directly after the last of the cell suspension has entered the column. 2 volumes of media are flowed through the column to remove unbound cells.
4. A further 10–20 volumes of media are flowed through to wash the gel and remove any further unbound cells.
5. Bound cells are eluted with 1 mg/ml antibody in medium via competition with bound antibody. Alternatively, bound cells can be mechanically removed by pouring the gel into a dish containing a few ml of cold medium and agitated by swirling. The beads are allowed to settle and the free cells recovered in the supernatant.
6. The gel is regenerated by washing with 2–3 volumes of 0.1 M glycine, 0.5 M NaCl, pH 2.5 followed by at least 10 volumes of medium and is stored as before.

8.4.2.3. Examples of immunoaffinity cell separations. There are now many excellent examples of cell separation on immunoadsorbant affinity columes using the methodology described above. The vast majority of these examples involve the separation of various cells of the immune system. Described below are two such examples, one involving immune system cells, T and B cells and the second from separation of non-immune related cells namely anterior pituitary cells.

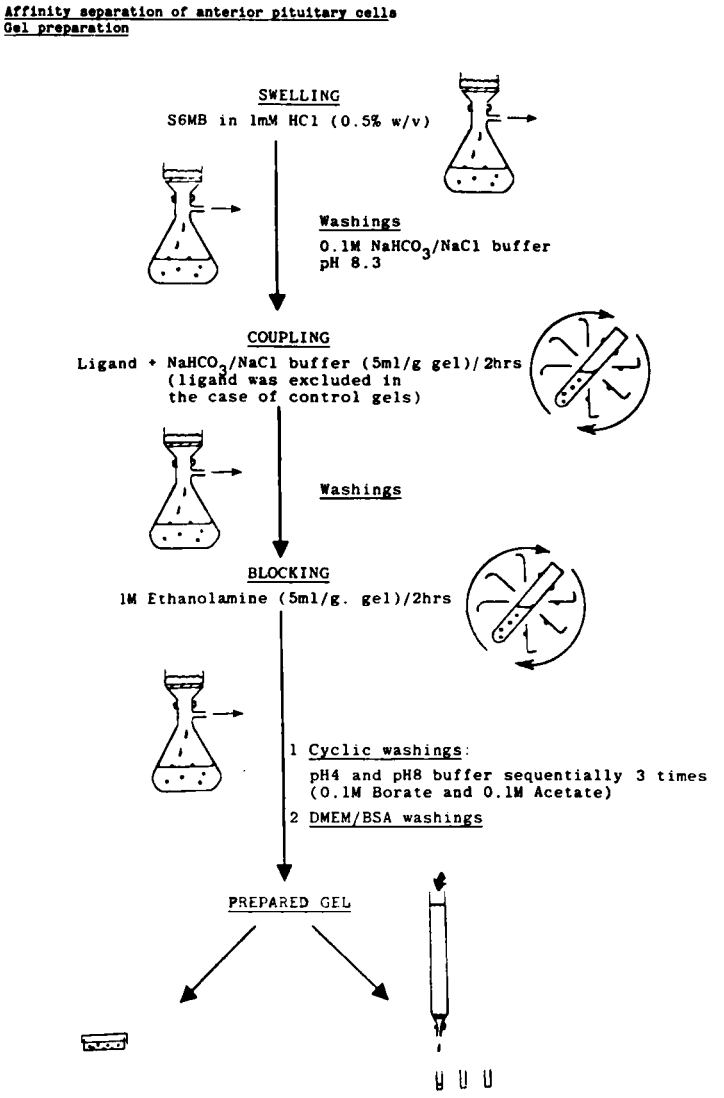


Fig. 8.4. Protocol for affinity separation of anterior pituitary cells. Courtesy of Pauline Dobson.

1. Separation of T cells. (Marshak-Rothstein et al., 1979). 10^7 mouse lymph node cells were applied to a 2.5 ml column of monoclonal antibody against T cell-specific Thy-1 antigens, coupled to Sepharose 6MB (1.5 mg antibody protein/ml gel). Non-bound cells comprised 90% non-T cells contaminated with only 2% T cells as assayed by lysis by anti-Thy-1 antibody and complement. Adsorbed cells were recovered by transferring the gel to a tube and vortexing for 10 s. The recovered cells represented 70% of the T cells in the original population and were 90% viable.

2. Separation of anterior pituitary cells. (Dobson and Brown, 1981, and in preparation). Fig. 8.4 shows the reaction protocol adopted for the preparation of Sepharose 6MB coupled to various ligands used in the affinity separation of different cell types of the anterior pituitary. Several different ligands have been used, including anti-growth hormone antibodies raised against the growth hormone secreting rat tumour GH, thyrotrophin-releasing hormone (TRH) and somatostatin. The results obtained with anti-GH cell antiserum are described below and with thyrotropin-releasing hormone and somatostatin in section 8.4.5.2. The cell separation procedure is the same for each ligand coupled.

A portion of gel is packed into a column (K9/30, Pharmacia) fitted with a 80 μm nylon net and 10^7 trypsin-digested rat anterior pituitary cells which have been applied in 2 ml. The cell suspension is allowed to enter the gel and the eluted with DMEM containing 0.5% BSA. 100 fractions are collected before the addition of 0.25% trypsin. Once the column is filled with trypsin solution (30 fractions), the flow is stopped for 5 min before elution with DMEM containing 10% FCS. 3 drop fractions are collected throughout at a rate of 6 drops/min. 1 ml of DMEM/BSA is added to each fraction and each divided into two portions. One portion is sonicated and stored at $-20°C$ for assay of cell function (principally by RIA) and the other is cultured for investigation of cellular responsiveness.

Fig. 8.5 shows the results obtained with anti-GH cell antibody as the ligand. The cellular concentrations of three hormones, prolactin, thyroid stimulating hormone and growth hormone were assayed in

each fraction by RIA. The majority of prolactin and thyroid stimulating hormone were found in cells in the early fractions that were eluted before addition of trypsin. Fractions obtained after incubation of the column in trypsin contained high concentrations of growth hormone and little of the other two hormones. Thus it appears that the anti-GH cell antibody bound to the beads has retained cells containing (and presumably secreting) growth hormone.

Preparation of the gel takes about 5 h and although the gel can be stored at 4°C, ligand is progressively lost.

8.4.3. Protein A separation (Ghetie and Sjoquist, 1984)

8.4.3.1. Coupling. Protein A Sepharose 6MB can be obtained from Pharmacia or can be prepared as follows.

1. 5 g of CNBr-activated Sepharose is swelled and incubated overnight with 10 mg protein A (from *Staphylococcus aureus*) in 10 ml 0.1 M carbonate-bicarbonate buffer, pH 8.8 containing 0.5 M NaCl (coupling buffer). The gel is washed and resuspended for 2 h in 1 M glycine buffer pH 9.0. The gel is washed sequentially with coupling

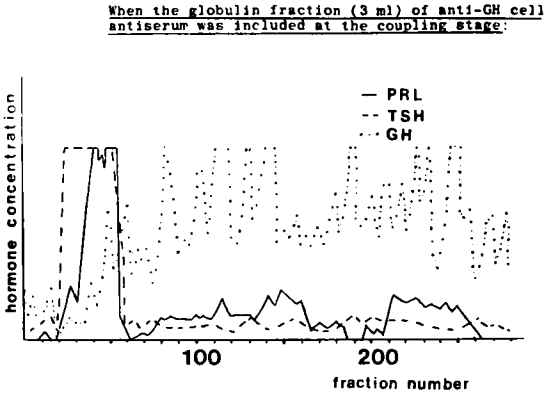


Fig. 8.5. Fractionation of rat anterior pituitary cells on anti-GH, cell antibody coupled to Sepharose 6MB. PRL = prolactin, TSH = thyroid stimulating hormone, GH = growth hormone. Courtesy of Pauline Dobson.

buffer, 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl and again coupling buffer. The gel is finally resuspended in an appropriate volume of culture medium (or other suitable medium in which the cells are to be suspended). Using this method approximately 2 mg of protein A binds to 1 ml packed gel. Lower or higher substitutions can be obtained by altering the Protein A/Sepharose ratio.

8.4.3.2. Checking binding ability of adsorbant. This is a method described for checking the ability of the adsorbant to bind cells with surface IgG. The cells used in this example are red blood cells which are easily obtained. Throughout the procedure the cells are washed and resuspended in culture medium (serum free) which in this case happens to be the medium in which the cells of interest were suspended for application to the column. The red blood cells could equally be washed in isotonic buffer or alternatively cells other than red blood cells could be used providing a suitable IgG is available.

The Protein A-Sepharose 6MB in culture medium is poured into a small column with a nylon screen. The ability of the adsorbant to interact with cell bound IgG is checked before introducing the cell suspension. A suspension of 2% chicken (CRBC) and sheep (SRBC) red blood cells in a 2:1 ratio is prepared. The suspension is incubated with a subagglutinating dose of anti-SRBC rabbit IgG (5 μg/ml suspension) for 30 min at room temperature after which the cells are washed with medium. 0.75 ml of the suspension is applied to the column (in this particular case a 1 $\times$ 7 cm column) and the flow halted for 15 min at room temperature. Non-adsorbed cells are washed off in medium and the percentage of CRBC and SRBC eluted is determined (CRBC are nucleated). The column is washed with 20 ml medium and the gel gently suspended with a Pasteur pipette to remove adsorbed cells. The eluted cells are collected and the percentages of CRBC and SRBC are determined. If the adsorbant is binding correctly results similar to those shown in Table 8.3 should be obtained.

The column is finally washed in 30 ml medium, 10 ml of 0.2 M acetic acid to remove antibody and finally in 50 ml medium.

8.4.3.3. Cell separation. Cells at a maximium density of 2×10^7/ml packed gel in a total volume smaller than the volume of gel in the column are applied to the column. The flow is stopped for 15 min at room temperature and non-adsorbed cells are washed off with about 50 ml of medium. Bound cells can be eluted by competition with free IgG or mechanically by resuspension of the gel. Table 8.4 shows the recoveries and viabilities of mouse spleen lymphocytes coated with rabbit anti-mouse IgG and eluted from a Protein A-Sepharose 6MB column by three different methods.

TABLE 8.3
Separation of chicken (CRBC) and sheep red blood (SRBC) cells on Protein A Sepharose after incubation with anti-SRBC. Ghetie and Sjoquist, (1984), Academic Press – with permission.

Sample	CRBC (%)	SRBC (%)
Initial mixture	67	33
Nonadherent cells	95	5
Adherent cells	5	95

TABLE 8.4
Recoveries of mouse spleen lymphocytes coated with rabbit anti-mouse IgG and eluted from a Protein A Sepharose 6MB column by three different methods. Ghetie and Sjoquist, (1984), Academic Press – with permission.

Method of elution	Cells eluted (%)	Viability (%)[a]		
		Before elution	After elution	After culture
Dog IgG[b]	61	95	89	65
Stirring	74	95	86	ND[c]
Resuspending	100	95	83	45

[a] Determined by trypan blue method.
[b] Only 10% of the mouse lymphocytes were eluted with fetal calf serum or rat IgG.
[c] Not determined.

1. 2 ml dog IgG at 20 mg/ml in medium is flowed into the column and the flow halted for 1 h at 37°C. The flow is restarted and cells eluted with a total of about 15 – 50 ml depending on the size of the column.
2. The same method as (1) is used except that the closed column is stirred on a Vortex mixer for 3 min at low speed prior to elution.
3. Medium is added to the column and the gel resuspended with the aid of a small coil of wire while medium is simultaneously flowed through at room temperature.

As Table 8.4 shows, a higher recovery of cells is obtained the more mechanical dispersion of the gel is employed. This is however at the expense of cell viability.

8.4.4. Lectin separation

Sepharose 6MB is available with either wheat germ or Helix pomatia lectin already coupled. These preparations may require washing with high and low pH buffers before use to remove any free lectin present. It has also been reported that these preparations are in some instances not reusable (Hellstrom et al., 1984). These authors have described in detail the methodology for coupling lectins to Sepharose 6MB which is reproduced below.

8.4.4.1. Coupling. 1. 1 g of CNBr activated Sepharose is swollen in 1 mM HCl and washed on a glass filter with 1 mM HCl followed by 0.1 M $NaHCO_3$ – 0.5 M NaCl, pH 8.6.
2. The gel (3 ml) is incubated with 6 ml of lectin solution containing 20 mg lectin in 0.1 M $NaHCO_3$ – 0.5 M NaCl, pH 8.6 overnight at 4°C with gentle stirring.
3. The gel is extensively washed with coupling buffer and remaining active groups blocked by incubation for 2 h at 20°C in 0.5 M glycine – 0.1 M $NaHCO_3$, pH 8.3.
4. The gel is washed 3 times with alternatively low and high pH buffer (0.1 M acetate buffer pH 4.0 and 0.1 M borate buffer pH 8.0 both containing 0.5 M NaCl).

5. Before use the gel is washed and equilibrated overnight at 4°C in the solution in which the cells will be applied to the column.
6. The ability of the gel to bind cells can be checked by incubating a small proportion of cells with beads prior to running the column and observing the number of cells bound per bead circumference under a microscope. The concentration of carbohydrate needed to remove bound cells can be estimated by adding solutions of different concentrations and monitoring the release of cells from the beads (Dillner-Centerlind et al., 1980).

A more exact determination of the elution conditions can be obtained by eluting cells bound on a column by progressively increasing concentrations of carbohydrate from 0.001 – 1.0 mg/ml. The concentration at which no further cells are eluted is the concentration to use. The presence of any cells remaining on the column that cannot be eluted can be checked by dispersion of the gel and viewing of the beads under a microscope (Hellstrom et al., 1984).

The degree of substitution obtained should be in the region of 5 mg/ml gel with the method above. The gel can be reused several times by following steps 4 and 5.

8.4.4.2. Separation of lymphocytes on Helix pomatia *– Sepharose 6MB. (Hellstrom et al., 1984).* 1. 3 ml of HP – Sepharose prepared as above, at 1 mg/ml are transferred to a small column and washed with 10 ml TH-HSA-NaN_3 (Tris-buffered Hanks salt solution pH 7.4 containing 0.2% human serum albumin and 0.02% NaN_3).
2. 2.5×10^7 neuraminidase treated human peripheral blood lymphocytes in 0.2 ml TH-HSA-NaN_3 are run into the column and allowed to bind for 10 – 15 min.
3. Unbound cells are washed off with 50 ml TH-HSA-NaN_3.
4. The column is eluted with 50 ml 0.1 mg/ml D-GalNAc in TH-HSA-NaN_3.
5. 10 ml of the same medium containing 1.0 mg/ml D-GalNAc is added to the column and allowed to incubate for 5 – 10 min without flow. 40 ml of the same solution is added and the gel suspended by stirring and the solution drained off.

The majority of cells that pass directly through the column without binding are B cells with surface bound immunoglobulins. 10 – 15% of the total T cells present are eluted by 0.1 mg/ml D-GalNAc which also contain a small percentage of B cells which are presumed to be a subset with receptors for the lectin. 45 – 60% of the T cells are eluted by 1.0 mg(ml D-GalNAc after resuspension of the gel.

T cells prepared by E-rosetting (Dillner-Centerlind et al., 1980) can also be fractionated into different subpopulations on the same adsorbant, however in this case the two elution steps required higher concentrations of D-GalNAC, 1.0 and 5.0 mg/ml. Similarly T cells can also be fractionated into 2 subpopulations on wheat germ Sepharose 6MB (5 mg/ml) after elution with D-GlcNAc at 25 mg/ml. The majority of T cells pass directly through the column and have little affinity for the lectin. 15 – 25% however bind to the adsorbant and are eluted as above. These two populations also show functional differences (Hellstrom et al., 1984).

8.4.5. Ligand-receptor separations

Fewer applications of cell affinity separations based on specific ligand/receptor interactions have been made. The same basic coupling reactions to those described for antibodies and lectins can be used for linking protein ligands to the beads.

8.4.5.1. Separation of neurone cells. (Dvorak et al., 1978). Neurones have an affinity for αBT toxin, which binds to a surface located receptor and can be used as an affinity ligand bound to Sepharose 6MB to separate the cells.

1. Coupling. In this example the particular coupling reaction used was not the extensively used cyanogen bromide reaction. Instead the toxin was coupled via azo-linkage to one of its two histidine residues. This reaction described in detail by Cuatrecasas (1970), involves the incubation of the toxin (protein) in borax buffer pH 8.0 with diazotised *p*-aminobenzoylhexylamino Sepharose 6MB. The

basic reason for using this reaction is that the histidines of the toxin are located well away from the active site of the molecule and hence coupling via these should have little affect on the activity of the toxin.

2. Cell separation. 1. Ganglion cells are prepared from 19 day chick embryos where the neurones contain about 2×10^5 αBT receptors per cell. After trypsinisation (0.025%) the cells are dissociated in Pucks saline G containing 2 mg/ml BSA, 20 μg/ml DNase and 10 units nerve growth factor (Medium A), filtered through nylon gauze (50 μm), centrifuged and resuspended at 1×10^6 cells/ml in medium A.
2. The derivatised beads in a small column are washed with PBS then with medium A and the flow rate adjusted to 1 – 2 ml/h.
3. The cell suspension is loaded at 4°C and the column washed with medium A, until few non-neuronal cells can be detected in the eluate.
4. PBS containing DNase (4 μg/ml) and finally PBS/DNase containing 0.1% trypsin.
5. The flow is stopped, the temperature raised to 37°C and incubation allowed for 2 – 3 min.
6. FCS is added and the cells eluted with 10 ml F15 medium (Gibco) containing 5% FCS.

The original cell suspension contained about 10^5 neurones and the final suspension eluted from the column contained 95% neurones.

8.4.5.2. Separation of anterior pituitary cells. (Dobson and Brown, 1981, and in preparation). Conditions similar to those described in 8.4.2.3.2. have been used to couple thyrotropin-releasing hormone and somatostatin to Sepharose 6MB in order to separate populations of anterior pituitary cells that interact with these hormones. These are principally mammotrophs in the case of TRH and somatotrophs in the case of somatostatin.

As Fig. 8.6 shows, two peaks of mammotrophs are obtained with TRH-ligand, as identified by the presence of prolactin. One population elutes with non-mammotroph cells in the early fractions while

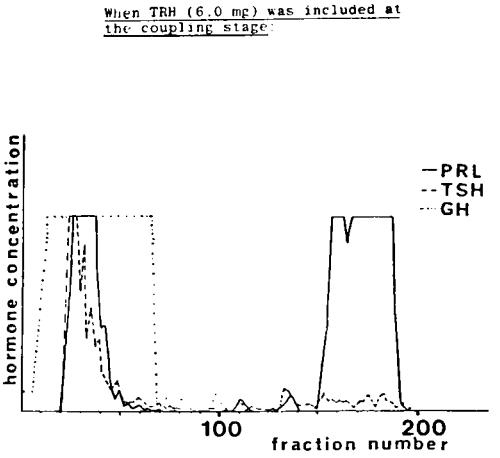


Fig. 8.6. Fractionation of rat anterior pituitary cells on thyrotrophin-releasing hormone coupled to Sepharose 6MB. PRL = prolactin, TSH = thyroid stimulating hormone, GH = growth hormone. Courtesy of Pauline Dobson.

the majority of the mammotrophs elute as a highly enriched population in the later fractions after trypsin incubation.

The responsiveness of the cells eluted in the higher fraction numbers was tested by culturing the separated cells, stimulating them with TRH and measuring the release of prolactin against a suitable control. The separated mammotrophs responded to TRH stimulation by increased prolactin release. Full details of this experiment are given in the legend to Fig. 8.7.

8.5. Affi-gel

Affi-gel beads, produced by Biorad are 250 – 300 nm diameter and consist of cross-linked acrylic derivative with antibody immobilised to the surface. Three different preparations of Affi-gel are available, with either anti-mouse, anti-rabbit or anti-FITC. The beads can be sterilised with 1% aqueous formaldehyde and after washing with

buffer containing 0.1% sodium azide, can be poured directly in the column that is supplied. Affi-gel beads can be used to separate cells for which specific antibodies are available. The antibodies must have been produced in either a rabbit or a mouse, or alternatively may be

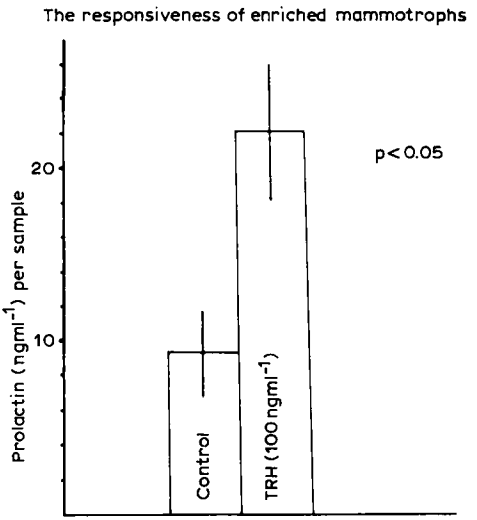


Fig. 8.7. The responsiveness of mammotrophs separated from rat anterior pituitary by affinity chromatography on Sepharose 6MB-coupled-thyrophin-releasing hormone.

The elutes from the TRH-S6MB column were collected into plastic tubes, and to these were added 1 ml DMEM. After mixing (to resuspend the cells), aliquots of 0.5 ml suspension were dispensed into the wells of Linbro plates. To these were added 0.5 ml DMEM containing 30% foetal calf serum (FB), and 200 μgml^{-1} gentamicin (i.e. final concentrations were half these values). After 3 days in culture, those monolayers identified by RIA as having a delayed peak of mammotrophs were removed from the plates with trypsin and pooled. The pooled fractions were washed and then incubated for 5 h in DMEM containing 20% FB. The cells were washed 3 times with DMEM containing 0.5% BSA, and incubated in this medium for 30 min at 37°C. Samples of 30 000 cells were incubated with 1 ml DMEM/BSA $\pm$ 100 ngml^{-1} TRH (contained in LP3 tubes) and shaken slowly in a 37°C waterbath for 30 min. After this incubation, the cells were pelleted by centrifugation (200 g/3 min) and portions of the supernatants were taken and assayed for PRL content by RIA. Results are expressed as mean $\pm$ SD; n = 4. Dobson and Brown, in preparation.

from any source with FITC attached. The antibodies coupled to the beads will act as second antibodies and bind to the appropriate first antibodies on the cell surface i.e. anti-mouse Affi-gel beads will bind cells with mouse antibodies on their surfaces. The basic methodology for the use of Affi-gel is similar to Sepharose 6MB. Cells are added to a small column of gel, unbound cells washed off with buffer and incubated without flow for 20 – 30 min. Bound cells are eluted by competition with a solution of first antibody or alternatively by mechanical agitation of the gel. An example of results obtained with the FITC Affi-gel system is shown in Table 8.5. Mouse spleen lymphocytes are preincubated with commercially available FITC-conjugated monoclonal antibody to mouse Lyt 1 antigen which is a marker for differentiating T cells. The T cells possessing the Lyt 1 antigen on their surfaces bind to the anti-FITC Affi-gel beads and can thus be separated from other T cells that pass directly through the column.

8.6. Immobilisation of ligands through cleavable bonds

In an attempt to reduce the problems associated with the recovery of cells bound to ligand-coupled beads, several groups have investigated the use of coupling reactions that link ligands to beads and produce

TABLE 8.5

Separation of Lyt 1^+ T cells from mouse spleen lymphocytes by affinity chromatography on anti-FITC Affi-gel beads after preincubation with FITC-conjugated monoclonal antibody to mouse Lyt 1 antigen. Courtesy of Biorad

	Total	FITC Labeled	Unlabeled
Start	7.6×10^7	37.9%	62.1%
First eluant	4×10^7	–	100%
Second eluant	2×10^7	100%	–
Total recovery	78.9%		

easily cleavable bonds. Cells attached to ligands can therefore be recovered from the beads by cleavage of the bonds.

Early experiments utilised cleavable disulphide bridges which can be cleaved with thiol (Kiefer, 1975; Cambier and Neale, 1982). More recently mercury-sulphur bonds have been used which are cleaved more easily than S-S bridges with thiol (Bonnafous et al., 1985). The coupling of ligands via mercury-sulphur bridges to trisacryl beads and their subsequent applications for cell separation have recently been described in detail by Bonnafous et al., (1985) and will not therefore be described here.

8.7. Glass and plastic beads

Glass and plastic beads between 200 – 300 nm diameter can be used as adsorbents for affinity cell separation. Proteins, particularly antibodies will adsorb onto the beads and can effect separations of antibody positive cells. This method is generally considered to be inferior to the other types of beads discussed above since the ligands cannot be chemically coupled to the beads. Non-specific adsorption of cells also appears to be more of a problem with glass and plastic beads.

Glass beads (available from Sigma) should be siliconised before use and may also require acid washing. Plastic beads can be obtained sterile (e.g. Nunc Biosilon Beads) and should be thoroughly washed in buffer before use. For most proteins a concentration of 1 mg/ml should be sufficient to saturate the bead surfaces after incubation overnight at 4°C or 30 min at 45°C followed by 30 min at 4°C (Wigzell, 1976; Hubbard et al., 1984). Elution of bound cells can either be by competition or mechanical agitation.

Affinity separation on fibres and surfaces

9.1. Introduction

The fractionation of cells on fibres and surfaces is basically very similar in principle to the affinity chromatography methods described in the previous chapter. Instead of using a column of beads with affinity ligands attached, solid surfaces such as plastic Petri dishes and fibres are used. The fibres and surfaces can be used with specific ligands coupled or in some cases can be used in their natural state, for which particular cells such as monocytes and macrophages have an affinity.

9.2. Separation on unmodified fibres

Where cells have a natural affinity for fibres they can be separated by using unmodified fibres. The use of this method has largely been restricted to the separation of lymphoid cells which have a natural affinity for nylon. The same basic method is however applicable to any cells that have an affinity for nylon or indeed any other fibres. The metodology described below is taken from work by Litvin and Rosenstreich (1984) on the separation of T lympocytes. The method is based on the fact that T cells do not adhere to nylon whereas other lymphoid cells such as B cells, monocytes etc. do adhere.

1. Nylon fibre (Scrubbed nylon fibre, 3 denier, type 200) is boiled in distilled water for 10 min.
2. After cooling, fresh water is added and the fibre boiled again. This is repeated 3 – 5 times.
3. The washed fibre is wrapped in a clean cloth, the excess water is squeezed out and the fibre dried for 2 – 3 days at 37°C.
4. If required sterile, the fibre can be wrapped in aluminium foil and dry heat sterilised at 120°C for 18 h.
5. 0.4 g of fibre is teased apart in medium containing 1% glutamine, penicillin, streptomycin and 20 mM HEPES.
6. The fibre is loosely packed into a small 5 ml column or syringe taking care to ensure that the fibre is continuously saturated with medium.
7. Wash the fibre with 50 ml of medium containing 5% FCS, cover, and incubate in a 5% CO_2 incubator for 1 h at 37°C.
8. Immeditely before use, wash the column with 50 ml of medium with 5% FCS at 37°C.
9. Add 1 ml of cell suspension (lymphoid) containing $1 - 6 \times 10^7$ cells in medium plus FCS to the top of the column (outflow sealed) and allow to perculate through the fibre.
10. A further 2 ml of medium plus FCS is added and the column incubated without flow for 1 h at 37°C.
11. Nonadherent cells are removed by slow addition of 10 ml medium plus FCS at 37°C collecting 10 ml effluent as a single fraction.
12. Adherent cells are removed by gently tapping the column and compressing the fibre with a small plunger. The fibre is then teased apart in the column with forceps and the fibre washed with 30 ml medium plus FCS at 4°C with the effluent 30 ml being collected.

The nonadherent cell population consists mainly of T cells. Certain T cell subsets may be specifically retained on the column, particularly Fc receptor positive T cells. The recovery of T cells is low, between 60 – 75% of the total T cell population and the viability depends on the source of the cells. The T cells can be further purified by a second passage through the column. The adherent cells consists of monocytes/macrophages, bound T cells, B cells and neutrophils.

The purification of T cells is highly dependent on the concentration of FCS in the medium. Between 5 – 10% should be used to obtain optimal purification since at lower concentrations there is increased non-specific adherence of cells and at higher concentrations fewer adherents are able to bind to the fibres. Other fibre materials such as glass wool, rayon and cotton balls can also be used to separate T cells as described.

9.3. Separation on modified fibres

The same method as that described above for unmodified fibres can be used with modified fibres that have cell specific ligands attached. This method is therefore essentially the same as the affinity chromatography methods described in Chapter 8. The specificity of this method is however rather low, around 40 – 60% and hence is usually inadequate for isolation of purified cells (Rutishauser and Edelman, 1977; Edelman and Rutishauser, 1974). A novel method of using modified fibres to obtain more specific cell separations has been developed by these same authors.

The basic feature of this method is that nylon fibres with ligands attached are strung in a geometric pattern that prevents non-specific trapping of cells and allows microscopic observation. Cells are allowed to collide with the fibres and those with the appropriate surface receptors bind to the fibre surfaces leaving unbound cells to be washed away. Bound cells can be characterised directly or mechanically removed from the fibres.

The major drawback of this method is that only a very small percentage of the cells capable of binding will actually become attached to the fibres. Even under optimal conditions the binding can be as low as 1 – 10% although the purity of cells obtained is very high. Cell recovery can be increased by using meshes of nylon fibres which can retain as many as 10^7 cells although the purity obtained is reduced to 60 – 75%. The capacity can also be increased by using multiple meshes. Edelman and Rutishauser (1974) have described a

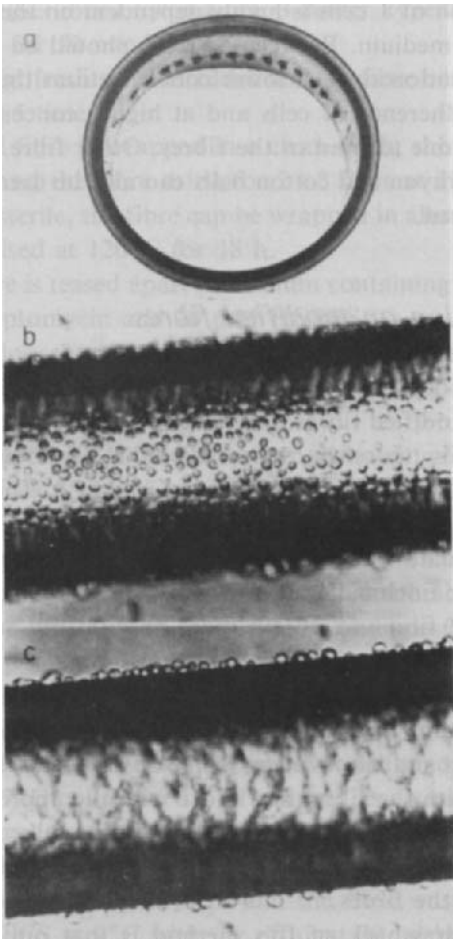


Fig. 9.1. (a) Petri dish containing polyethylene ring strung with nylon filament. (b) Mouse thymocytes bound to a Con A-derivatised nylon fibre. The field is focussed on the face of the fibre at × 480 magnification.
(c) Mouse thymocytes bound to a Con A-derivatised nylon fibre. The field is focussed on the edge of the fibre at × 360 magnification. Edelman, (1973), Academic Press – with permission.

simple apparatus which houses several fibre meshes on top of each other which can retain up to 2×10^7 bound cells.

One of the major advantages of this separation method is that the cells attached to fibres can be visualised with a light microscope enabling studies of cell-fibre interactions (Fig. 9.1). There are several criteria that must be satisfied in order for fibres of a particular material to be used for cell separation. They must not adsorb cells non-specifically, they must have easily reactable groups on the surface and must be relatively strong. Nylon is one of the few materials that exhibit these properties in fibre form. A range of different diameters of nylon fibres are available but the size generally used is $125-250$ μm diameter which is available as size 50 transparent sewing nylon. The fibres are strung under tension in a small frame that will fit into a 35×10 mm Petri dish. The circular frame should be of soft plastic (polyethylene, polypropylene or Teflon). Holes are pierced in appropriate positions in the frame with a hot needle and the fibre sewn into the frame. The tension of the fibres should be such that they are taut but do not cause buckling of the frame. Fig. 9.1 shows an example of fibres strung on a frame. For a frame of diameter that fits into a 35×10 mm Petri dish, 24 fibres are adequate for cell separations. The fibres should always be parallel and within the focal plane of the viewing microscope. The chemical modification of the fibres is conducted on the fibres strung in the frame (9.3.2 below).

9.3.1. Binding and recovery of cells

The binding of cells to the fibres is dependent on the rate and angle of collision between fibre and cell, the dwell time of the cell on the fibre and settling due to gravity. If cells are allowed to settle onto the fibres under the influence of gravity the amount of non-specific binding is greatly increased. The fibre array immersed in the cell suspension must therefore be shaken. Too violent shaking however results in no cell binding since the dwell time is too short. Shaking the fibre array immersed in cell suspension on a reciprocal shaker with a $3-4$

cm horizontal stroke at 70 – 90 oscillations/min for 15 – 30 min produced the best results. The volume of cell suspension should be just enough to ensure that the fibres are completely immersed at all times.

The composition of the medium in which the cells are suspended follows the same constraints as affinity chromatography and depends upon the particular ligand attached to the fibres e.g. should not contain high serum or carbohydrate concentrations when lectins are used. Most cells will bind at 25°C. At lower temperatures binding is decreased and at higher temperatures the amount of non-specific binding may increase.

After incubation for 15 – 30 min, the unbound cells can be removed by transferring the fibre array to a dish containing fresh medium (use of a larger volume of medium will facilitate removal). Gentle swirling of the array in several fresh aliquots of medium will remove all the unbound cells. The number of cells bound to fibres can be determined after removal by using any of the previous described counting methods. Alternatively the number bound can be determined by directly counting those bound to a measured segment of fibre. This is best achieved by using an inverted microscope and counting the number of cells bound to one edge of a known length of fibre (Fig. 9.1). The total number of cells bound is proportional to this edge count but the factor used to convert this count to total number must be previously obtained from a count of the total number removed.

Bound cells are removed from the fibres by plucking each fibre three times with a needle. This shearing action produces a lesion in the surface membrane which can result in the viabilities of recovered cells being as low as 5%. Much higher viabilities can be obtained by removing the cells into medium containing 10% serum followed by incubation in this medium for 30 min, at 37°C. Clearly this is only suitable for cells which are generally prepared with serum based medium. Although not documented, viabilities of cells not prepared in serum based medium e.g. plant cells, yeasts, bacteria etc. may therefore be very low unless they are stabilised by removal in a compatible medium.

9.3.2. Modification of fibres

Several different reactions for the coupling of ligands to nylon fibres have been described (Rutishauser and Edelman, 1977) which are summarised below. In common with affinity chromatography the principle reactions used are those which enable proteins to be attached to the fibres.

Before modification, contaminants on the surface of the fibres must be removed. The fibres are thus soaked for 10 min in carbon tetrachloride and then in petroleum ether. If desired, the number of amino and carboxyl groups on the fibres can be increased by partial hydrolysis with 3 M HCl for 30 min at room temperature. The fibres should be thoroughly washed in distilled water before use.

9.3.2.1. Non-covalent attachment of proteins. Incubation of the fibres in a solution of protein at 0.025 – 1.0 mg/ml in 0.15 M NaCl for 30 min at room temperature will produce adsorption of around 10^{11} protein molecules per cm of fibre. The protein-adsorbed fibres should be thoroughly rinsed and stored in 0.15 M NaCl. The adsorption of protein is stable for about 4 h in physiological medium.

The amount of protein adsorbed can be controlled by inclusion of a spacer protein such as BSA. A cylindrical fibre with a diameter of 0.125 mm can adsorb 17 ng BSA or 20 ng ligand protein per cm. The amount of ligand protein adsorbed can thus be varied by inclusion of the appropriate concentration of BSA.

9.3.2.2. Covalent coupling of protein. Proteins can be cross-linked to the fibres using carbodiimide. Water soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene-sulphonate (Aldrich) at 0.25 – 2.5 mg/ml in 0.15 M NaCl pH 6 – 7 at a ratio of 5:1 with protein is added to the array of fibres and incubated at room temperature for 30 min. The derivatised fibres are washed with 0.15 M NaCl and used immediately. This method produces fibres with high densities of bound protein although around 20% of the protein bound will be adsorbed (not covalently linked).

9.3.2.3. Activation of carboxyl groups. The fibre carboxyl groups can be activated so that they can react with free amino groups by incubating the fibre array for 10 min at 25°C in a 1:500 dilution of thionyl chloride in dioxane.

9.3.2.4. Attachment of DNP. The antigenic hapten, 2,4-dinitrophenyl (DNP) can be attached to fibres in several ways. By linking DNP to BSA and allowing the BSA to adsorb to the fibres DNP can be non-covalently attached. DNP is coupled to BSA by allowing 1 g of 2,4-dinitrobenzenesulphonic acid, 2 g BSA and 1 g K_2CO_3 to react in 100 ml water at 37°C for 1 h. The reaction is stopped by addition of 1 g glycine and extensively dialysed against water. The DNP-BSA can also by covalently coupled to the fibres using the carbodiimide reaction. DNP can be directly coupled to the fibres via the fibre amino groups. 40 mg DNP-SO_3H and 80 mg K_2CO_3 are dissolved in water and incubated with the fibres for 1 h at 25°C.

DNP can also be coupled to fibres via a spacer arm using the reaction described by Kiefer (1973). The use of a spacer arm can be advantageous in the removal of cells since for example lymphocytes binding to spacer-DNP fibres is stable at 4°C but not above 25°C and this can be used to effect cell removal.

9.3.2.5. Coating with gelatin. Fibres coated with gelatin bind cells to a much lesser degree than native nylon. Thus by using derivatised gelatin, for example DNP-gelatin, to coat the fibres, the amount of non-specific cell binding can be greatly reduced. The fibres are coated by simply dipping the fibre array in 5% gelatin at 40 – 50°C followed by rapid centrifugation to remove excess gelatin. Since gelatin melts at 37°C, warming gelatin fibres with cells bound, to this temperature produces gentle release of cells. Cell removal by plucking is also less harmful to cells because of the soft gelatin.

9.4. Separation on unmodified surfaces

This method has been used principally to separate monocytes and macrophages which adhere to glass and plastic surfaces. Although generally described as attachment to plastic, the attachment is most often to plastic coated with serum proteins since the plastic dishes are always incubated with serum (usually autologous). The method outlined below has been described by Mosier (1984) for the separation of macrophages from spleen.

Sterile plastic culture dishes or tissue culture washed glass Petri dishes are preincubated with complete culture medium (including FCS and any antibiotics) for 30 min at 37°C in a 5% CO_2 incubator. The actual culture medium used will depend on the source of the cells. A maximum of around 3×10^7 cells per 60 mm dish are added in serum and the dish contents swirled and incubated for 1 h at 37°C in a CO_2 incubator. Non-adherent cells can be removed by swirling the dish and aspirating the free cells. The dishes are washed several times with cold PBS to remove loosely adherent cells. The adherent cell population consists of about 5% spleen cells and 50–70% peritoneal exudate cells.

The adhered cells are recovered by addition of 0.01 M EDTA in PBS at 37°C for 15 min with repeated vigourous pipetting across the surface of the dish. The resuspended cells are removed from the dish by aspiration. A quick look using an inverted microscope will reveal how many cells are left adhered to the dish. These can be removed by gentle scraping with a piece of rubber, a 'rubber policeman'.

The viability of separated cells is usually very high around 90% but the purity of the macrophages is never 100% and depends on the proportion of other adherent cell types present e.g. monocytes.

A similar protocol can be used to separate monocytes from erythrocyte depleted blood. Douglas et al. (1981) also described the separation of monocytes by attachment to plastic dishes upon which BHK-21 fibroblasts have previously been grown. The BHK cells produce a microexudate which remains on the surface of the dishes after the cells have been removed and after extensive washing with EDTA

in PBS. Monocytes bind to this exudate whereas other potentially adherent cells such as platelets do not. Then bound monocytes can be recovered with EDTA-PBS. The recovery of monocytes obtained is around 50% of the total in the blood.

Fischer and Koren, (1981) have separated monocytes with recoveries around 70% by adherence of erythrocyte depleted blood to dishes coated with autologous serum.

9.5. Separation on ligand coated surfaces

Ligands, principally proteins, when adsorbed on the surface of plastic or glass dishes can be used to separate a cell population with surface receptors specific for the ligand because these cells will preferentially adhere to the dish surface and can be washed free of unbound cells. This method is extensively used to obtain enriched cell populations prior to analysis or separation via flow cytometry. The method is often termed 'plating', or where antibodies are used as ligands, 'panning'. The main type of ligand used in these separations are antibodies, either general anti-immunoglobulins or cell-type specific antibodies e.g. monoclonals.

Antibody protein will readily adsorb to tissue dishes when incubated at room temperature for 1 h or overnight at 4°C. Antibody is not wasted since any that does not bind is retrieved and can be reused. Antibody-treated dishes should be thoroughly washed with PBS and can be stored in PBS at 4°C for about 1 month. Cells will not generally bind to ligand-coated plates at low temperatures. Room temperature or 37°C can be used, although the optimal temperature for binding of any particular cell type to ligand will need to be determined by trial and error. Incubation of cells for too long on the plates will increase the amount of non-specific binding. If necessary the cell binding can be conducted in a CO_2 incubator and operating under sterile conditions is clearly no problem.

Described below is an example of the use of this method to separate mouse Ig^+ and Ig^- lymphocytes using anti-Ig-adsorbed dishes, from the work of Mage (1984).

9.5.1. Non-adherent cells

100 mm diameter dishes are coated with goat anti-mouse Ig as described above. A mouse spleen cell suspension in 5 ml medium (RPMI 1640) containing 1×10^8 cells is pipetted onto the dish and allowed to incubate for 30 min at room temperature. The non-adherent (Ig^-) cells are resuspended by swirling the dish for 30 s and poured off into a second antibody-coated dish and incubated for a further 30 min. This is repeated for a third time. The nonadherent cells obtained from each dish are 90% Ig^- and over 90% viable. These can be further fractionated into Lyt-2^+ and Lyt-2^- sub-populations by incubation with anti-Lyt-2 antibody prior to plating on Ig coated dishes. The cells with anti-Lyt-2 antibodies bound will now adhere to the Ig coated dish and can be separated from Lyt^- cells which do not adhere.

9.5.2. Adherent cells

To obtain a pure population of adherent (Ig^+) cells it is necessary to dilute the Ig antibody coated onto the dishes which reduces the chances of non-specific adherence and also facilitates release of bound cells. Dishes are prepared by allowing the Ig antibody solution, diluted with normal goat Ig (1:7) to adsorb. Cells are incubated on three sets of dishes as described for non-adherent cells. The dishes containing bound cells are thorougly washed in PBS and the adherent cells are removed from each dish by forcefully pipetting medium against the bottom of the dish until all the adhered cells are free from the dish. The resulting cells obtained are 90% Ig^+ and over 90% viable.

9.6. Separation on Sephadex G-10

Monocytes and macrophages can be separated from lymphocytes by passage through a column of Sephadex G-10. The basis of this

separation involves both adherence and entrapment of monocytes and macrophages in the G-10 matrix, while the lymphocytes pass directly through the column. A similar method is used for both cell types, details of macrophage separation can be found in Mishell and Mishell, (1984), details of monocyte separation are given below, from Chien and Ashman (1984).

Sephadex G-10 is autoclaved for 30 min, washed with warm sterile Hanks BSS containing 15% FCS and packed into a small column to

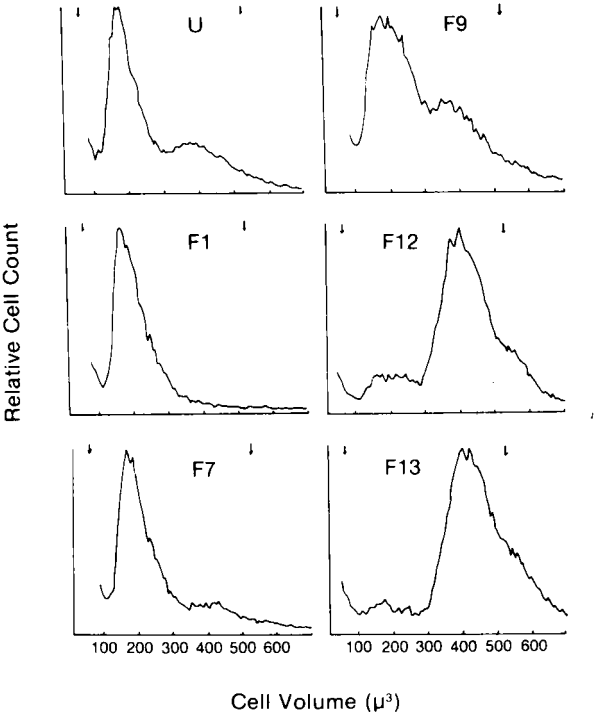


Fig. 9.2. Separation of monocytes from human peripheral blood on a 5 ml column of Sephadex G-10. Cell counts and volumes measured on a Coulter Counter with Channalyser. Left arrow = calibration with 4.9 μm diameter beads. Right arrow = calibration with 10.18 μm beads. Monocytes identified in the second peak by esterase staining. U = unfractionated; F = fraction. Chien and Ashman, (1984).

give a bed volume of 5 – 10 ml. Mononuclear cells at $5.0 - 10 \times 10^7$ cells/ml in medium at 37°C are layered onto the column, the excess medium drained off and the cells allowed to incubate for 5 min. Non-adherent cells are eluted with a flow rate of 2 – 8 min/ml in 4 bed volumes of medium at 37°C and a further 6 bed volumes at room temperature. These washings are collected and the size distribution of cells analysed. When equal numbers of monocytes and lymphocytes (detected by differences in size) appear in the same fraction, elution is stopped and the Sephadex emptied into ice-cold medium and gently agitated for 30 min. The Sephadex is returned to the column and the cells eluted with ice-cold medium. The whole separation process takes around 45 min. The results obtained by Chien and Ashman are shown in Fig. 9.2. The Sephadex was removed for the ice-cold incubation after 11 fractions had been collected (each fraction being approximately one bed volume). Fractions 1 and 4 contained 63% of the total cells applied only 0.13% of which were monocytes (esterase-positive cells), the bulk being esterase negative (71% of total esterase-negative cells). Fractions 13 and 15 contained 90% monocytes representing 45% of total monocytes applied (5% of total cells). The total cell recovery was 72% with the monocytes being 90 – 96% viable. Chien and Ashman found that larger bed volumes of Sephadex could accomodate proportionally larger cell numbers without affecting the separation.

CHAPTER 10

Affinity partition

10.1. Introduction

Affinity partition is the most recent technique described in this book, the first demonstrations of affinity partition separation of cells being presented at the 4th International Conference on Partition in Aqueous Two-Phase System held in Lund, Sweden in 1985. The principle of affinity partition is the same as all affinity separation methods in that surface specific molecules are used to label particular cells. The presence of the molecules on cell surfaces is identified by increased partition into the upper phase of an aqueous two-phase system.

The principle of affinity partition is shown in Fig. 10.1. Cells are partitioned in an aqueous two-phase system (Chapter 6) where all the cells *just* fail to partition into the upper phase. If an affinity ligand such as an antibody which has been covalently coupled to poly-(ethyleneglycol), PEG, is now included in the phase system cells which are recognised by the ligand will effectively become coated with PEG molecules and their affinity for the PEG-rich upper phase of the phase system will be increased. Thus in such a system only cells which bind the affinity ligand will partition into the upper phase and can be separated by physical removal of this phase. This is the theory of affinity partition in its simplest, most useful form which to date has not been thoroughly tested for cell separation, although is well

established for separation of soluble molecules (Flanagan, 1984). To date three affinity separations have been tested. Two of these studies are essentially the same, involving the covalent coupling of IgG antibodies against human red cells to PEG (Sharp et al., 1985; Karr et al., 1985) and were conducted as demonstrations of the feasibility of cell affinity partition. The third study by Walter and Pangburn, (1985), Pangburn and Walter (1987) used a novel method which does not involve chemical coupling of ligand to phase system components. In Walter's system, normal human red cells were separated from abnormal red cells, sensitive to complement, by allowing the deposition of complement C3b onto the surface of the abnormal cells. The attachment of the complement proteins is sufficient to change the cell surface properties allowing the abnormal cells to be distinguished

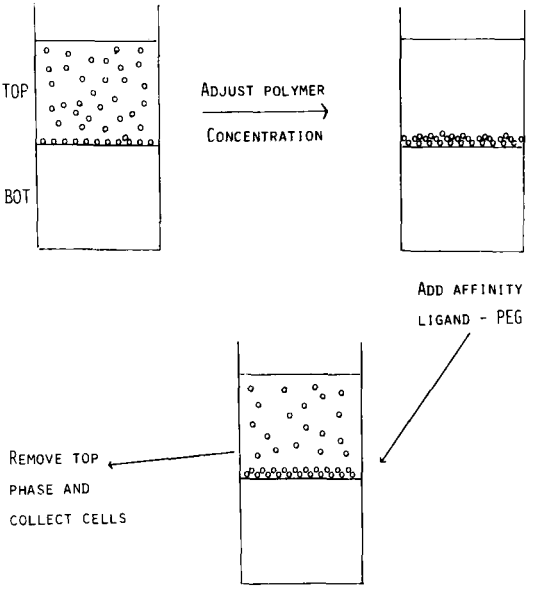


Fig. 10.1. Principle of affinity partition.

from normal red cells in a charged phase system. This affinity separation system may be more generally applicable by allowing C3b to bind to cell surface specific antibodies attached to cells. In some cases the mere binding of antibody onto the cell surface may also be sufficient to affect partition enough for separation.

10.2. Antibody affinity cell partition. (Sharp et al., 1985; Karr et al., 1985).

The major part of any antibody-affinity cell separation is the synthesis of the affinity ligand i.e. antibody coupled to PEG. A variety of reactions can be used, the basis of each being the production of reactive PEG species. Each PEG molecule has two terminal hydroxyl groups, one at each end of the polymer chain, which are ideally suited for chemical activation. For cell partition it is essential that only one antibody molecule is coupled to each PEG molecule via one activated terminal hydroxyl group. Coupling of antibody to both needs will result in cross-linking between cells. The easiest way to couple antibodies (or any other ligand), to one end of PEG molecules is to use monomethyl PEG which has one terminal hydroxyl group replaced by a methyl group. Reactions can be used which couple antibody to the hydroxyl group and not to the methyl group. A number of reactions have been documented by Milton-Harris's group in Alabama specifically for the production of PEG affinity ligands for affinity partition (Milton-Harris et al., 1984, 1985; Shafer and Milton-Harris, 1986). The simplest reaction utilises activation of PEG with cyanuric chloride which allows antibodies to be coupled via their lysine groups (Fig. 10.2). A typical reaction procedure is detailed below. It is important to note that the molecular weight of the PEG used has an important effect on partition. Larger molecular weight PEGs show increasing inhibition of attached antibody activity which may be caused in part by steric effects (Table 10.1).

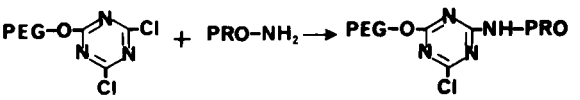


Fig. 10.2. Coupling of cyanuric chloride activated-PEG to protein via lysine groups. Reaction of Abuchowski et al., (1977).

TABLE 10.1
Effects of PEG substitution on the ability of antibody to agglutinate RBCs

PEG M.W.	*Lysines modified (%)*	*Minimal hemagglutination concentration (μg/ml)**
Control	0	< 0.2
(A) 5000	27	23 ± 8
(B) 5000	40	19 ± 8
(C) 5000	51	5 ± 2
(D) 1900	45	1 ± 0
(E) 1900	70	3 ± 1

* lowest antibody concentration exhibiting appreciable haemagglutination in microtitre assay ($\bar{x}$ ± SD, n = 3). Karr et al., (1986).

10.2.1. Activation of PEG with cyanuric chloride (Abuchowski et al., 1977)

9.5 g monomethyl PEG (M.W. 1900 – 5000) is dissolved in 100 ml of benzene and 15 ml of benzene is distilled off to remove water. This solution is slowly added to 100 ml of benzene containing 4.6 g cyanuric chloride and 5 g anhydrous sodium carbonate. The solution is stirred at room temperature for 48 h and then filtered. The product is precipitated by adding the reaction mixture to 300 ml dry hexane and reprecipitated twice in toluene by addition of two volumes of hexane.

10.2.2. Coupling of antibody to actived PEG (Sharp et al., 1986, Karr et al., 1986)

Around 10 mg of antibody is dissolved in 0.5 ml 0.05 M $Na_2B_4O_7$ pH 9.2 and added to 1.5 ml to 0.1 M borate buffer. 1 ml of cyanuric chloride-activated PEG in borate buffer equimolar relative to antibody lysine groups (approx. 90 per molecule), is added at 4°C and the mixture stirred for 1 h. Unattached PEG can be removed by diafiltration with an Amicon PM-30 membrane (30 000 M.W. cut-off) with 10 volumes of 0.05 M borate buffer and 0.025 M sodium azide, giving a final volume of 2 ml. With these conditions, up to 50% of the lysine amino groups are modified. The degree of modification can be estimated by assaying the percentage of unmodified amino groups using the Biuret or Habeeb methods (Habeeb, 1969; Abuchowski et al., 1977, Shafer and Milton-Harris, 1986). The effect of antibody-PEG coupling can be monitored by comparing the partition coefficients of the PEG-antibody and free antibody. Table 10.2 shows the partition of three IgG coupled PEGs with different substitutions. Increasing the degree of substitution increases the partition but decreases the IgG activity.

10.2.3. Affinity partition of red blood cells (Sharp et al., 1986; Karr et al., 1986)

The ability of PEG coupled-rabbit IgG against human red cells to increase the partition coefficient of human red cells was first tested in tube experiments with a phase system consisting of 4.6% dextran T500, 3.9% PEG 8000, 150 mM NaCl, 7.3 mM Na_2HPO_4, 2.3 mM NaH_2PO_4 pH 7.2. 0.5 ml of upper phase containing 2×10^7 human red cells was incubated with 0.2 ml PEG-antibody for 15 min at 37°C. The cells were pelleted at $1000 \times g$ for 10 min., washed in fresh upper phase, resuspended in 1 ml upper phase. 1 ml of lower was added, the phases mixed by 20 inversions and allowed to settle for 15 min. The mixing and settling was repeated before 0.7 ml of top phase was removed and the number of cells counted. Fig. 10.3

TABLE 10.2

The effect of PEG molecular weight and concentration on the partition coefficient of IgG and haemagglutination ability after coupling 5% T500, 3.4% PEG 8000, 130 mM sodium chloride and 10 mM sodium phosphate buffer pH 7.2. Sharp et al. (1986). Academic Press – with permission.

Expt.	PEG mol. wt. (g/mol)	PEG: lysine molar ratio	IgG activity[a] (mg/ml)	Partition coefficient	Attached PEG mol/mol	
					Measured	Calculated[b]
1	0	0	0.03	1.1 ± 0.05	0	0
2	200[c]	5	0.03	1.2 ± 0.05	nd[d]	nd
3	200	1	0.09	1.4 ± 0.05	nd	nd
4	200	3	0.09	1.85 ± 0.1	nd	nd
5	200	5	0.06	2.3 ± 0.1	nd	nd
6	1900[c]	5	0.04	1.28 ± 0.5	nd	nd
7	1900	1	0.3	5.9 ± 0.5	27	2 – 3
8	1900	3	0.9	18.5 ± 2	31	4 – 5
9	1900	5	None	40.3 ± 5	43	5 – 6
10	5000[c]	1	0.03	1.2 ± 0.05	nd	nd
11	5000	0.2	n.d.	3.7 ± 0.2	nd	nd
13	5000	0.6	0.3	7.8 ± 0.7	nd	nd
12	5000	1	None	11.0 ± 2	nd	nd

[a] Activity is expressed as the minimum concentration sufficient to cause haemagglutination.
[b] The effective number of moles of PEG attached per mole of IgG
[c] PEG-cyanuric chloride was inactivated by hydrolysis.
[d] Not determined.

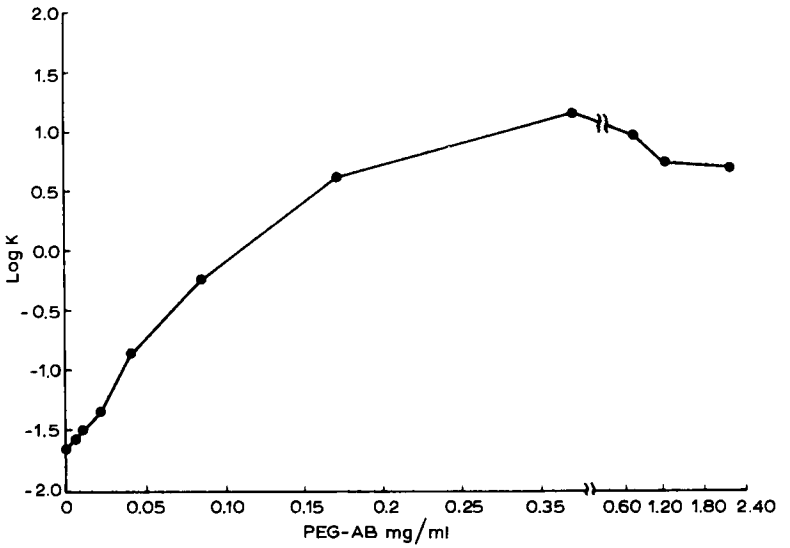


Fig. 10.3. Effect of incubation concentration of PEG-AB (derived from rabbit) on the upper-phase affinity of human RBC. Log k = log partition coefficient. Karr et al. (1986).

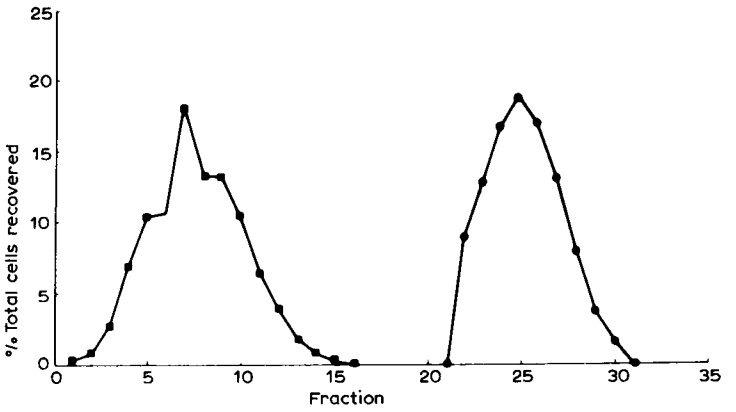


Fig. 10.4. Separation of human (●) and sheep (■) RBCs by 30-transfer TLCCD in a two-phase system containing PEG-rabbit and anti-human antibody. (a) control, no affinity ligand; (b) 1.3 mg/ml PEG-antibody. Karr et al., (1986).

shows the observed increases in partition coefficient of human red cells with different preparations of rabbit anti-human IgG-PEGs. In each case increasing modification of PEG with antibody increases the partition of the red cells. PEG 5000 is more effective than PEG 1900. In control experiments, sheep red cells were partitioned with the same antibody-PEG conjugates and no changes in partition were observed.

In order to demonstrate the separation of human red cells from a mixture of human and sheep red cells, thin-layer countercurrent distribution (TLCCD) was used. The same phase composition was used as in the tube experiments, containing 1.3 mg/ml PEG-antibody. 7×10^7 human and sheep red cells were partitioned with 30 transfers on a Bioshef MkII TLCCD apparatus. The results obtained are shown in Fig. 10.4. With no PEG-antibody present there is little or no separation of the two cell types. When the mixture of cells is incubated with PEG-antibody for 15 min at 37°C prior to TLCCD, clear separation of the two cell types is observed.

Note added in proof: Further examples of affinity partition cell separations were described at the 5th International Conference on Partition in Aqueous Two-Phase Systems held at Oxford, U.K. in August 1987. Proceedings of this Meeting are to be published by Plenum Press entitled 'Advances in Separations Using Aqueous Phase Systems in Cell Biology and Biotechnology'. Editors D. Fisher and I.A. Sutherland.

CHAPTER 11

Flow sorting

11.1. Introduction

Flow sorting is the general name used to describe cell separation via automated cell sorters, which are often referred to as fluorescence activated cell sorters or FACS. By far the most extensive use of flow sorting in cell separation is the separation of cells specifically 'marked' with a fluorescent molecule, hence the common name of fluorescence activated cell sorting. The same apparatus can however also be used to separate cells of different sizes based on their light scattering properties.

Flow sorting is without question one of the most powerful methods of cell separation currently available. Much use of this technique has been made in recent years for separating cells which have surface antigens for a specific antibody. By either coupling a fluorescent molecule to the antibody or by using a fluorescent second antibody, the cells can be separated on the basis of the fluorescence emitted. In principle, this kind of cell separation could just as easily be conducted using methods already described such as affinity chromatography. The overwhelming advantages of flow sorting however are that it is extremely sensitive and since the cells are continuously in a liquid environment there are no problems of non-specific adsorption etc. In many laboratories flow sorting is now the method of choice for cell separations involving antibodies against

surface antigens. The major problem for a laboratory that does not have access to a flow sorter is that they are very expensive, being by far the most expensive cell separation method currently available.

Because the operation of flow sorters is usually carried out by skilled operators, I have omitted the majority of detail regarding the operation. The discussion below is limited to the principle of flow sorting and examples of the kind of separations that can be obtained.

11.2. Principle

Flow cytometry is a technique of automated cell analysis. Where the analysed cells are also separated or 'sorted' the term flow sorting is used. Cells in a liquid medium flow one by one at high speed through an intense beam of light usually from a laser. As each individual cell passes through the beam, optical and electrical signals are produced, which are accurately measured. The signals may represent fluorescence, light scatter or light absorbance. Individual cells exhibiting particular properties e.g. a particular level of fluorescence emission, can be selected. After passing through the beam, the cell stream is broken into droplets, each droplet containing a single cell. Droplets containing selected cells are then electrically charged. The stream of droplets passes between two electrically charged plates causing the charged droplets, containing the selected cells, to be deflected away from the main stream into a separate collecting vessel (Fig. 11.1). Flow sorters can be considered to consist of two parts, one where the analysis and sorting is carried, the other, an electronic part containing an inbuilt computer, where the signals are received, processed, displayed and cells selected for sorting. A small accessory computer is also usually connected to the electronic part so that data can be stored and plotted (Fig. 11.2). Flow sorters are currently produced by three manufacturers, Becton Dickinson, Coulter and Ortho Diagnostic Systems.

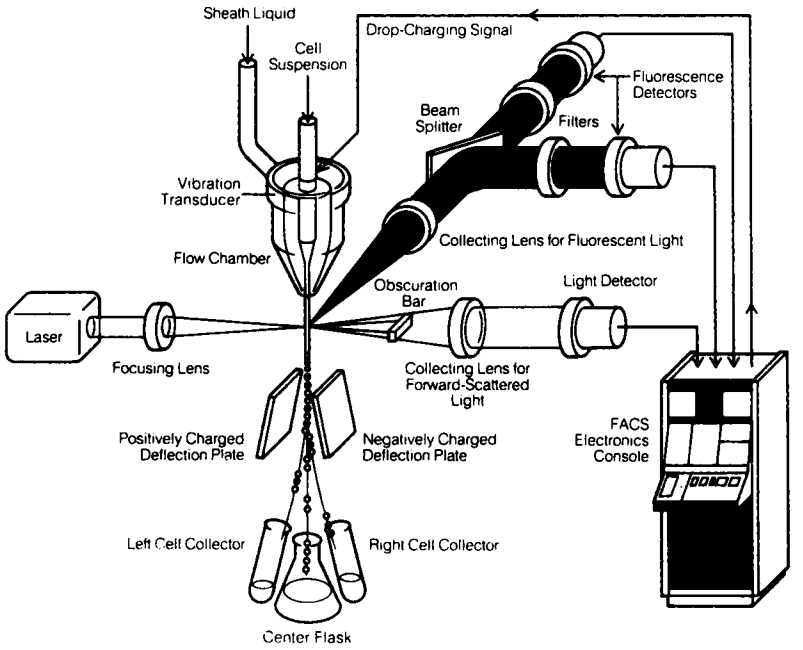


Fig. 11.1. Principle of flow sorting. Courtesy of Becton Dickinson.

11.3. Signals produced

The two types of signals that are used as parameters for cell sorting, produced as cells pass through the laser beam, are optical signals and electrical signals. Electrical signals are measured using the same principles as Coulter cell counters giving values of cell number and size (volume). The optical signals produced are of two types, light scatter and fluorescence.

The signal outputs from the flow sorter can be presented in four ways which are shown in Fig. 11.3. Each of the plots in Fig. 11.3 is from the same data with two output signals, 90° angle scatter (FL2) and forward (low angle) light scatter (FSC). Each dot on the dot plot

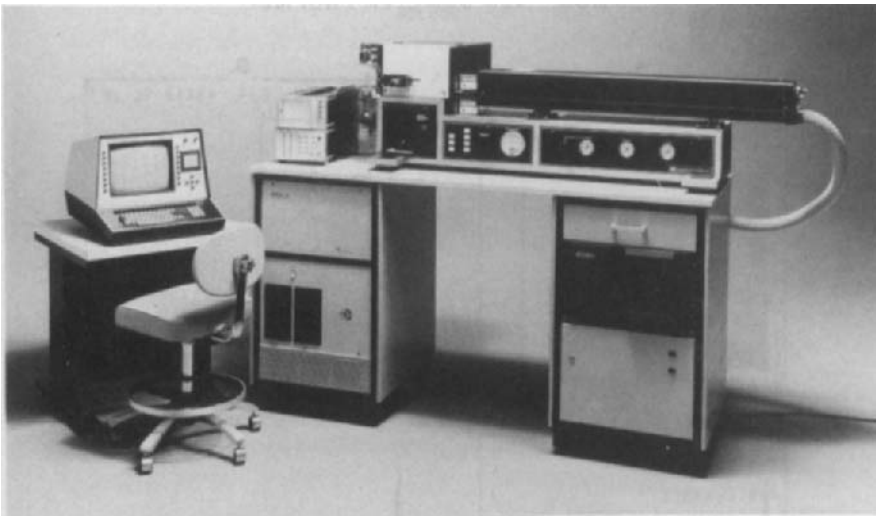


Fig. 11.2. Coulter Epics 541 flow sorter.

A, represents a single cell. The dots can be grouped in areas of similar intensity to produce the contour plot B. A histogram C, produced from the same data clearly shows that two major cell populations have been detected which have different sizes. A histogram can only show one parameter in this case forward scatter which is proportional to cell size. A clearer picture of the cell distribution is obtained from the net, or three dimensional plot, D, which shows both the size and fluorescence signals together with cell number (z-axis) as cell peaks. The signals monitored to produce these plots can be combinations of any of those described below, which most of the modern flow sorters will produce.

11.3.1. Light scatter

As the cell passes through the laser beam light is scattered and the light scattered at angles of less than 2° (small angle scatter) is proportional to cell size for spherical cells. Light is also scattered at 90°

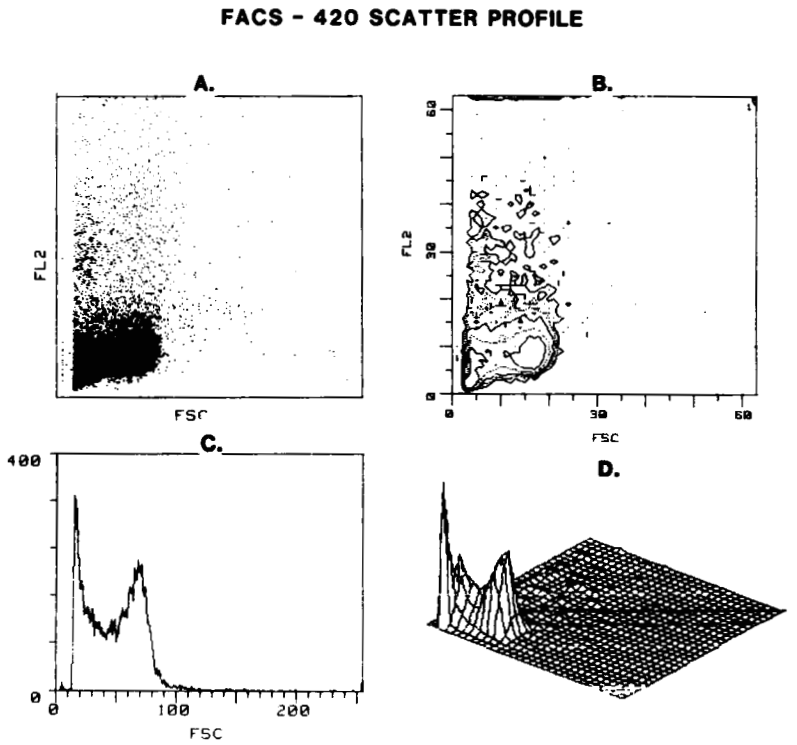


Fig. 11.3. Signal output displays from a flow cytometer (Becton Dickinson FACS 400). FL2, 90° angle scatter; FSC, forward scatter. A = dot plot, B = contour plot, C = histogram, D = net or 3D plot

which is related to the cellular contents, for example lymphocytes produce lower 90° scatter than monocytes (Fig. 11.4). The combination of these independently measurable values provides a great deal of information concerning cell size and morphology. The scatter signals received and processed can be used as a basis for cell sorting i.e. different sized cells or cells with different morphologies. The scatter signals received are analysed and displayed as a dot plot or a histogram as shown in Fig. 11.3. Each dot or point represent a value of the light scatter by a single cell. Fig. 11.4 shows a typical

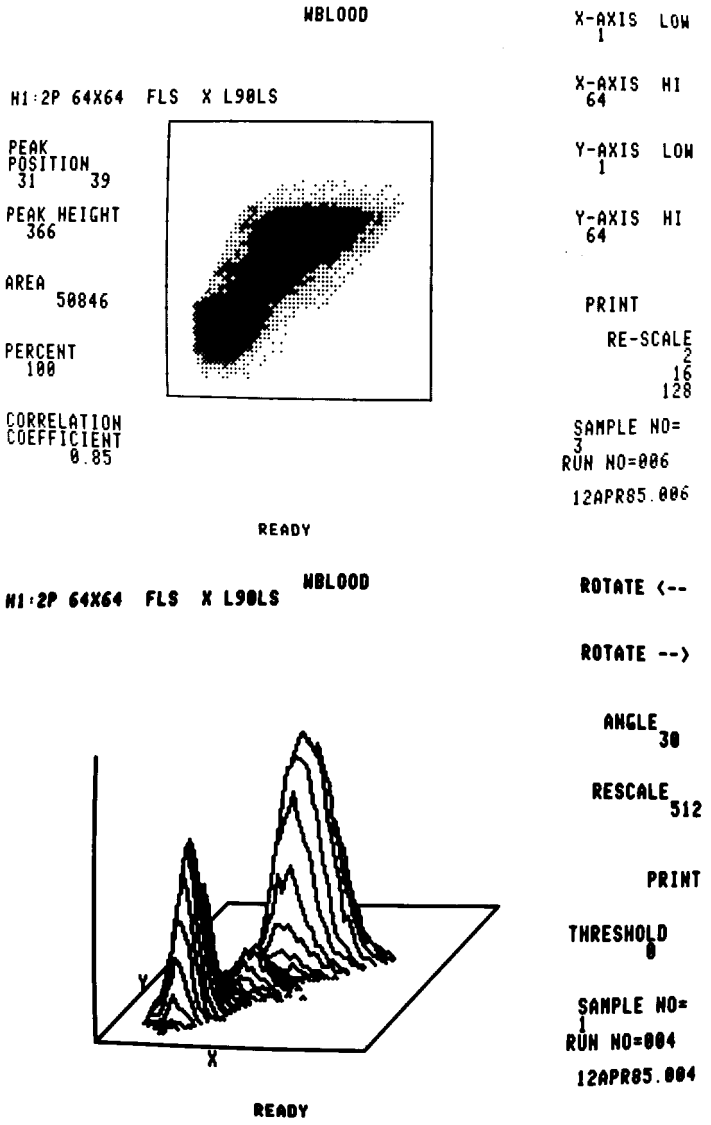


Fig. 11.4. Dual parameter scanning (small and 90° scatter) of human lymphocytes on a Coulter EPICS V flow sorter. (a) Contour/dot plot, (b) net plot, rear peak = granulocytes, middle peak = monocytes, front peak = lymphocytes.

output from both small angle and 90° scatter signals presented together (dual parameter). The cells in this example are human lymphocytes run on a Coulter Epics V flow sorter. Three distinct cell populations can be visualised representing lymphocytes, monocytes and granulocytes that differ in size and cellular contents. This data can be used to sort the cells by selecting for droplets containing one cell population to be charged positive, another population to be charged negative leaving the third uncharged.

11.3.2. Fluorescence

When a cell containing a fluorescent dye (fluorochrome) passes through the beam it absorbs light at the incident wavelength and emits the light at longer wavelength. The light is emitted throughout 360° but is collected through a detector system at 90° to the laser beam. If several different fluorochromes are present, each emits a different wavelength which can be detected separately. The photons of light received by the detectors are converted by photomultiplier tubes into electrical signals which are processed by the electronic part of the sorter into a digital signal. The digital signals are displayed in any of the forms shown in Fig. 11.3. Each dot on a plot display is the fluorescence emitted by a single cell.

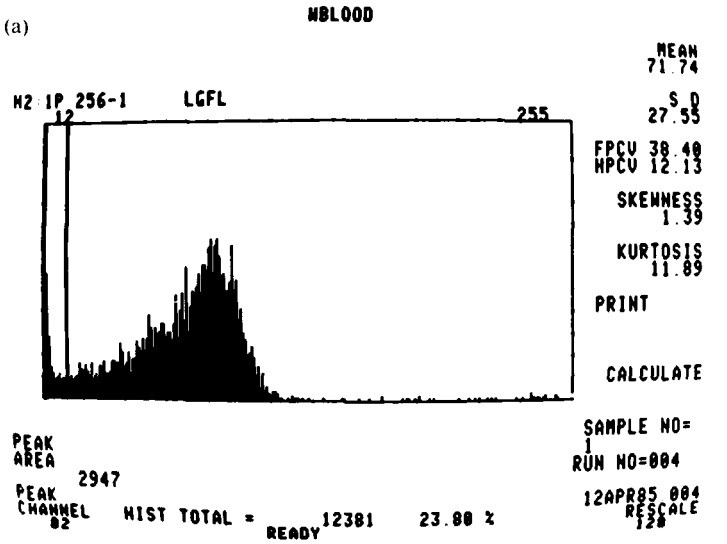
11.3.3. Setting parameters for cell sorting 'gating'

As many as six different measurements can be made simultaneously on each individual cell in the stream e.g. size, cell contents, fluorescent emission at different wavelengths on some of the latest models of flow sorters. Where several parameters are analysed simultaneously, this is known as multiparameter analysis. A particular population of cells identified, with certain characteristics, can be selected to be sorted by selecting upper and lower limits of the particular parameter(s). This is loosely termed 'gating'. The limits are selected by moving cursers on the oscilloscope screen displaying the particular parameter(s). This information is relayed via the inbuilt computer to

the droplet charger which conveys an electrical charge to each of the droplets containing the selected cells which are subsequently deflected from the main stream by the charged plates in a collecting vessel (usually a test tube).

If required, the sort gates selected can be very close together so that a very small population of cells can be separated. This, together with the overall sensitivity of detection of signals means that very pure cell populations can be separated. The speed of sorting is between 3000 – 5000 total cells per second with lower rates of around 3000 cells/s. being most commonly used to ensure that each droplet only contains one cell. However if the population required represents only a small percentage of the total population, say 10% the time required to separate 10^7 of these cells would be around 9 h at 3000/s. This time period can present problems where particularly labile cells are being used. Separation of large numbers of pure cells which requires long periods of sorting is often unavoidable. Ideally it would be convenient to sort overnight in such cases, however since

(a)



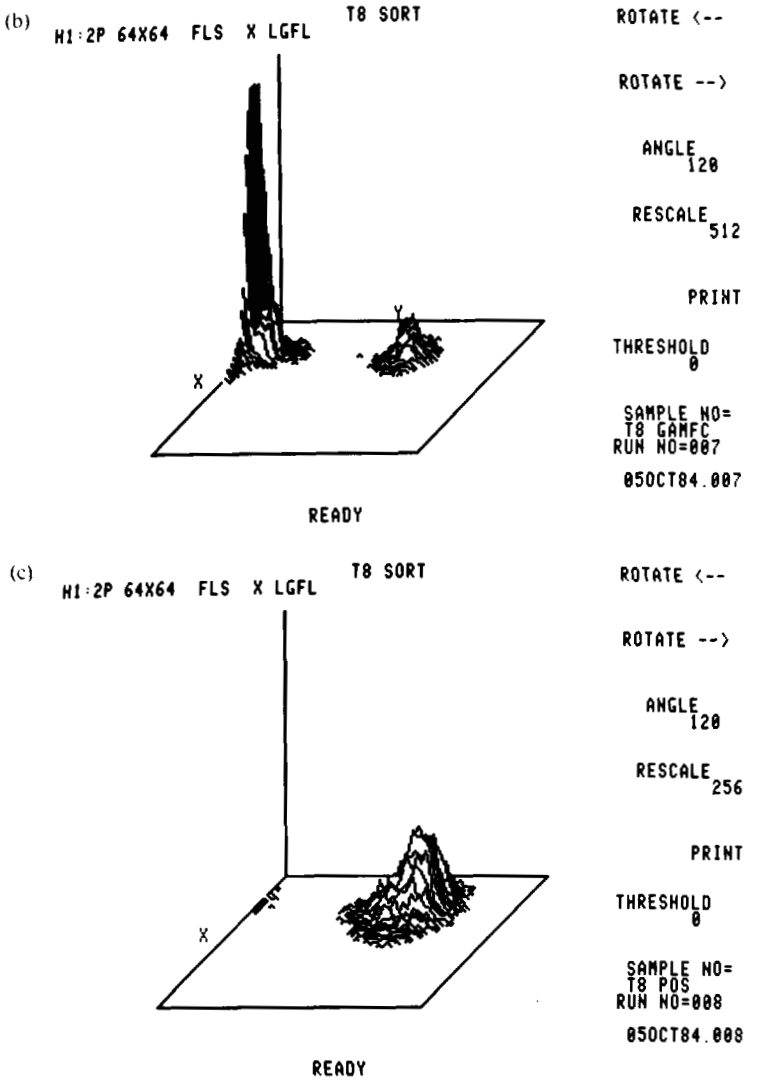


Fig. 11.5. Selection of cells for separation by flow sorting 'cell sort'. (a and b) Cells before sorting, solid line at position '12' in (a) is position of gate to exclude all cells to left of line. (c) Cells (T8-antibody positive) after sort, reapplied to sorter.

blockages in the nozzle orifice can frequently occur the operator must give fairly constant attention. Fig. 11.5 shows an example of a cell sort (Coulter Epics V).

11.4. Cell separation

11.4.1. The cells

The commonest uses of flow sorting for cell separation are either based on surface antibody/antigen interactions or on DNA content. This latter application is discussed in Chapter 13. The following description is therefore limited to the cell separation using fluorescent antibodies. Ideally single cell suspensions are used in flow sorting although the presence of cell clumps is less critical than in other separation methods since cell clumps can be identified by their light scattering and eliminated from the sort. Larger clumps should be avoided if possible since these will cause clogging of the nozzle orifice.

To ensure that each droplet contains only one cell the droplets are produced at a rate of 40 000 per second whereas the flow of cells in the stream is 3500 – 5000 per second. When the instrument receives a signal to say that a cell in a droplet is required three consecutive droplets are electrically charged to ensure that the droplet containing the cell is not missed. As a safeguard to prevent the situation of more than one of the three droplets containing a cell, the instrument is programmed to default the charging if a second cell is detected soon after the first.

Three sorted cell populations can be collected at the same time. A population charged positive will be deflected one way into a collection vessel and a different population charged negative can be deflected the other way and collected in a different vessel. The undeflected cells can also be collected in the central stream.

As a control, a sample of cells from each sorted population should be reanalysed to determine the effectiveness of the separation. The medium in which the cells flow can contain a wide variety of consti-

tuents without affecting the separation, thus isotonic buffers, culture media etc. can be used. The number of cells in the suspension can be highly variable, between $5 \times 10^5 - 10^7$ cells/ml.

11.4.2. The stain

The stain is commonly in the form of a fluorescent first antibody that binds to specific antigens on the cell surface or a fluorescent second antibody that binds to a first antibody on the cell surface. Because of the sensitivity of the technique, the antibodies should be of the purest possible source. Polyclonal antibodies are likely to produce brighter and more stable staining than monoclonals since they will probably recognise more than one determinant on an antigen. The source of the antibody can also be important since antibodies from different sources show different levels of non-specific binding to cells with surface IgG or Fc receptors. For example rabbit Fc reacts well with mouse Fc receptors but reacts less with goat and not at all with chicken Fc (Scher and Mage, 1984).

Antibodies bound to cell surfaces are detected by the emission of fluorescence. As already mentioned, the fluorescent dye used can either be coupled directly to the first antibody or to a second antibody which binds to the first antibody on the cell surface. Direct conjugation of dye to the first antibody is probably the better method although it is not the most convenient. A relatively large amount of antibody is needed for the coupling reaction although the reactions are relatively simple (Nakames and Pierce, 1966; Coons et al., 1941). The advantage of direct conjugation is that there is a better chance of avoiding non-specific staining which can occur with second antibodies. Indirect staining using a fluorescent second antibody is the most convenient method since a wide variety of fluorescent labelled second antibodies are available. Hence, with this method no coupling reactions need to be carried out. The cells are incubated with the first antibody e.g. a mouse monoclonal antibody. After thorough washing, the cells are incubated with fluorescent conjugated rabbit anti-mouse antibody which binds to the Fc portion of

TABLE 11.1
Fluorescence data for dye-substrate complexes used in flow sorting

Dye	Substrate	Fluorescence maxima		Laser line	Range
		Excitation emission			
		nm	nm	nm	nm
P I	DNA, RNA	540	625	514, 488, 531*	550 – 590
E Br	DNA, RNA	518	610	514, 488	550 – 590
MMC	DNA	421	575	457	510 – 530
Hoechst	DNA	352	460	351 – 364	380 – 420
Dapi	DNA	365	450	351 – 364	380 – 420
7-a A	DNA	550	655	514, 488, 531*	550 – 590
A-F	DNA	480	550 – 600	488	510 – 530
P-F	DNA	570	625	514, 488, 568*	590 – 610
A O	DNA	500	530	488	510 – 530
	RNA	500	640	488	590 – 610
FITC	protein	494	517	488	510 – 520
TMRITC	protein	545	575	514, 531*	550 – 570
XRITC	protein	572	595	568*	580 – 590
RITC	protein	545	600	514	600 – 620
PE	protein	488 – 545	580	488	550 – 600
Texas red	protein	595	615	568*	600 – 620
Lucifer yellow	protein	280 – 430	540	351 – 364	500 – 550
Adriomycin	protein	472	570	488	520 – 550

Codes used: P I, = propidium iodide; E Br, = ethidium bromide; MMC, = mithramycin; Hoechst, = Hoechst 33342 and 33258; Dapi, = 4′,6-Diamidino-2-phenylindole; 7-a A, = 7-amino actinomycin; A-F, = acriflavine-feulgen; P-F, = pararosoniline-feulgen; A O, = acridine orange; FITC, fluorescein isothiocyanate; TMRITC, = tetra methyl rhodamine isothiocyanate; XRITC, = (substituted) rhodamine isothiocyanate; RITC, = rhodamine isothiocyanate; PE, *phycoerythrin*.
Other fluorescent reagents: F D A, = flourescein diacetate; DTAF, = dichloro triazinyl amino flourescein; SITS, = disodium 4 acetamideo-4′-isothiocyano stilbene-2,2′-disulfonic acid; DANS, = 1-dimethylamino naphthalene-5-sulphonic acid; RB-200, = lissamine rhodamine B; mepacrine; proflavin; isoflavin; plant chloroplasts.

the mouse monoclonal and thus labels the cell surface with dye. The use of this indirect staining method increases the possibility of non-specific staining because two antibodies are being used. The method as well as being very convenient does have the advantage that more dye can be associated with the cell since more than one labelled second antibody can bind to a first antibody, particularly if the second antibody is polyclonal. This results in an amplification step which is extremely useful for staining cells that have few antigenic sites for a particular antibody.

If the number of sites on a particular cell are so few that even with indirect staining the fluorescence emitted is lower than the detection sensitivity further indirect staining steps can be used or alternatively an avidin (streptavidin)-biotin amplification system can be used (Wilchek and Bayer, 1984, Shamsuddin and Harris, 1983).

A list of dye – substrate complexes used in flow sorting is shown in Table 11.1.

Magnetic cell sorting

12.1. Introduction

In magnetic cell sorting cells are labelled with specific surface binding molecules which have magnetic properties (via iron groups) and can thus be sorted by their attraction to magnets. This is a very simple and efficient method of cell separation. The only criterion for separation is that a magnetic surface labelling molecule is available.

Unlike FACS, magnetic methods cannot produce detailed analysis of cell populations. The magnetic methods are simple 'sort' methods to separate a particular population. Although not as sophisticated as FACS, magnetic based separations do have the advantage of producing very pure populations of separated cells very rapidly with very simple, cheap equipment.

A variety of magnetic particles (microspheres) have been produced for cell separation. Ideally they should be small, between 0.2 – 2 μm diameter, must not form clumps, must not bind non-specifically to cells, must adsorb proteins or contain reactive groups and must be easy to prepare. The microspheres should have magnetic moments small enough to prevent clumping but large enough to provide magnetophoresis (movement toward and retention on a magnet) when attached to cells. Two basic types of particles have been used, those which have permanent magnetic moments (usually containing magnetite) and paramagnetic particles which have no permanent

magnetic properties but in which magnetism can be induced by an applied magnetic field. Two opposing forces interact in a suspension of microspheres, thermal energy which drives dissociation and attraction energy. If the attraction energy is lower than the thermal energy the microspheres will repel each other and thus will not form clumps. The attraction energy is lower with thicker polymer coatings around the particle and with increasing distance between the enclosed dipoles. Thus the diameter of the microspheres D, must be;

$$D > (4\pi M^2/18\mu_0 K_T)\, d^2$$

where k_T = thermal energy; M = magnetism of particle; μ_0 = permeability of free space and d = diameter of magnetic core (Kronick, 1980).

From this equation it can be seen that the required size for non-clumping microspheres increases rapidly with the diameter of the magnetic particle core. For a maximum diameter of 2 μm, the magnetic core diameter must be less than 50 nm. Such a microsphere has an iron content of 0.01%. There are several variables determining the minimum number of microspheres attached to cells to produce magnetophoresis. Kronick (1980) calculated a value of a minimum number of 10 microspheres (2 μm diameter as described above) per 10 μm diameter cell for the apparatus shown in Fig. 12.2.

12.2. Types of apparatus

The apparatus used is very simple and there are no major design constraints. Described below are two types of apparatus that have been used.

12.2.1. High gradient apparatus

This apparatus described by Melville et al., (1975); Owen, (1978,

1983), Molday and Molday, (1984), and Owen and Sykes, (1984), utilises a small glass column (around 10 cm × 0.5 – 1 cm i.d.) loosely packed with fine stainless steel wire (approx. 25 μm diameter), Fig. 12.1. An electromagnet is used to apply the magnetic field to the column and cells with microspheres attached are attracted to the large surface area of the magnetised wire matrix. The magnetic fields set up by magnetisation of the wire die off extremely rapidly with increasing distance from the surface of the wire. This produces a large field gradient in the spaces between the wires which is of the order of 10^6 Gauss/cm., considerably more than can be obtained without the wire, even with a powerful magnet. Care in packing the wire should however be taken to avoid trapping of cells in the wire matrix. Since the high

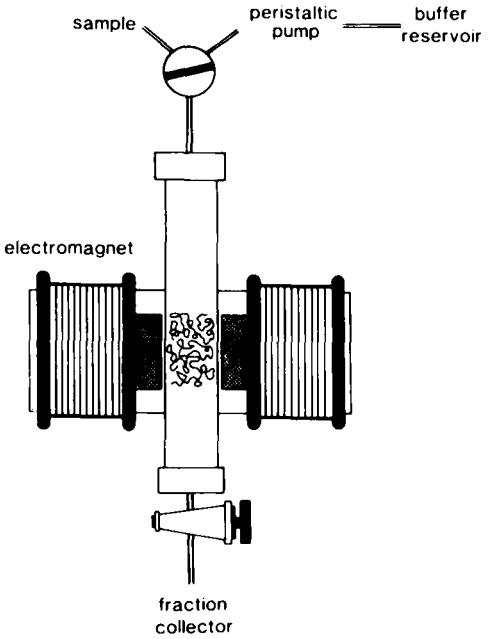


Fig. 12.1. Apparatus for magnetic cell sorting. Molday and Molday, (1984).

field gradient extends only a very short distance, the column of wire should be long enough to ensure that cells come in contact with the field.

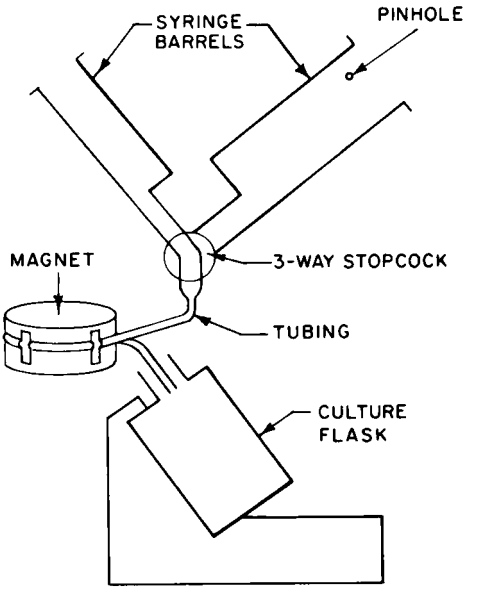
Cells previously incubated with microspheres are pumped through the column with the electromagnet on. Cells passing directly through the column are collected and the column washed by several passages of buffer/medium to remove entrapped cells. Cells bound to the wire are removed by switching off the electromagnet and eluting with buffer/medium. Mechanical agitation of the wire may sometimes be necessary in order to remove all bound cells.

For any particular cell population the optimal separation conditions have to be obtained by passing the same cell preparation through the column with various flow rates and strengths of magnetic field.

12.2.2. Kronick apparatus (Kronick, 1980)

This apparatus is shown in Fig. 12.2. The cells are allowed to flow via gravity through 1 mm i.d. × 1. 7 mm o.d. plastic tubing which is wrapped around an electromagnet. The outlet of the tubing is inserted into a collecting vessel such as a sterile culture flask. Two syringe barrels are connected to the tubing via a three-way tap. The smaller of the barrels (1 ml) is used to wash the system and to determine the total volume of liquid in the tubing. This is done by filling the system with water and allowing an air bubble to enter from the other empty syringe. The amount of liquid collected while the air bubble flows through the tubing is the volume of liquid in the tubing. The larger of the syringe barrels is used for the cell sample. This has a small hole wich allows any entrapped air bubbles to be removed, by pushing the liquid with the syringe plunger, which because of the hole, can be retracted without pulling back the liquid.

A suspension of cells previously incubated with microspheres is allowed to flow through the tubing with the electromagnet on. Fresh buffer/medium is flowed through to elute any non-bound cells. The cells retained in the tubing by the magnetic field are eluted by



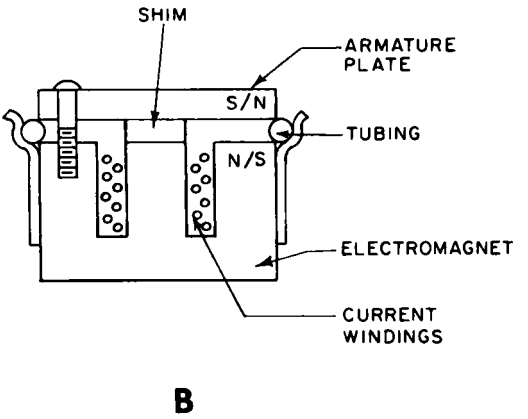


Fig. 12.2. Magnetic cell sorting apparatus of Kronick, (1980). A; layout of apparatus, B; detail of electromagnet. Plenum Press – with permission.

repeatedly flowing buffer/medium through the tubing with the electromagnet off. The elution fluid can if necessary be pumped through the tubing with the syringe plungers. The syringe barrels should ideally be similar to those shown in Fig. 12.2, which have offset outlet holes to prevent cells and microspheres from becoming trapped. The tubing itself should be arranged in level stretches to prevent cell settling.

12.3. Preparation of magnetic microspheres

A variety of different methods have been developed for the production of magnetic microspheres for cell separation. The majority of these reactions involve the production of magnetic 'hydrogel' beads. Hydrogel is the name given to beads produced from insoluble water-swollen polymers that have a minimum interaction with cells (Wichterle and Lim, 1960). Magnetic hydrogels are prepared from a variety of different polymers by co-polymerisation with an iron based core, most often magnetite.

12.3.1. Redox polymerisation (Kronick et al., 1978; Kronick 1980)

This reaction involves the coating of magnetite particles with a polymer matrix consisting of hydroxyethyl methacrylate, *N,N*-methylene bisacrylamide and methacrylic acid.

1. A monomer mixture is prepared comprising, by weight, 70% hydroxyethyl methacrylate, 10% *N,N*-methylene bisacrylamide and 20% methacrylic acid.
2. 50 mg of 50 nm magnetite particles (Wright Industries, Brooklyn, NY) and 50 mg $Na_3P_2O_7$ are homogenised in 40 ml water and sonicated in a 50 ml round bottom flask under nitrogen.
3. 30 mg of $Na_2S_4O_8$ are added and a 100 μl aliquot is removed for analysis of the dissolved monomer content by reading the O.D. at 210 nm after centrifuging out the magnetite (specific adsorbance = 8×10^{-4} cm^{-1} M^{-1}).

4. The reactants are slowly stirred under nitrogen with a paddle stirrer for 2 h.
5. The beads produced are 99% magnetic and are magnetically precipitated by placing the side of the bottle against the edge of a large magnet.
6. The precipitate is washed in 10 mM phosphate buffer at pH 7.0 and dialysed against the same buffer.

The microspheres produced are now derivatised for coupling of proteins and other molecules via free amino groups.

7. 250 mg dry weight beads, 0.1 g diaminoheptane and 100 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 15 ml of 10 mM phosphate buffer pH 5.0 is shaken with 5 mm glass beads for 5 h at 4°C. This reaction amidates carboxyl groups of the methacrylic acid with the diamine, and introduces a spacer arm with a free amino group.
8. If the attachment of the beads to cell surfaces is to be monitored by light microscopy, a fluorescent label can be coupled to a proportion of the free amino groups at this point. 250 mg dry weight beads from 7 are throughly washed (magnetically) in 10 mM sodium borate buffer pH 8.5 and resuspended in 170 ml of the buffer. A few glass beads are added and the mixture shaken at room temperature for 3 h. The microspheres are precipitated magnetically and thoroughly washed in neutral buffer until no free dye can be detected.
9. Proteins can either be coupled to the beads from step 7 or the fluorescent labelled beads from step 8.
10. 250 mg dry weight of beads are resuspended in 15 ml of 10 mM phosphate buffer at pH 7.0 containing 1% distilled glutaraldehyde. The suspension is shaken with glass beads for 2 h at room temperature.
11. The microspheres are washed with phosphate buffer and can be stored at 4°C for several months.
12. 1 mg protein is added to 50 mg activated microspheres in 2 ml of 10 mM phosphate buffer at pH 6.9 – 7.0 and the suspension shaken overnight at 4°C.
13. The microspheres are washed with phosphate buffer by repeated

magnetic precipitation and are ready to use.
14. The amount of protein bound to any particular preparation of activated microspheres can be determined by using labelled protein or other protein assays.

12.3.1.1. Separations with redox prepared microspheres. 1. Separation of neuroblastoma cells having surface gangliosides. (Kronick et al., 1978; Kronick, 1980).

This separation is based on the affinity of the cholera enterotoxin, choleragen, for gangliosides. Choleragen is coupled to microspheres using the method described above. $2-5 \times 10^6$ C-1300 neuroblastoma cells (10 – 20% of which bind the toxin) are incubated with 0.1 – 1.0 mg of the choleragen conjugated microspheres for 30 min at 4°C. Unbound microspheres are removed by centrifuging the suspension twice on FCS at 800 g for 10 min. The cells are resuspended in 2 ml Dulbeccos BME and applied to the Kronick type apparatus described above. The unbound cell fraction obtained contains between 12.5 – 15% of the choleragen positive cells. The retained fraction contained 99 – 99.5% choleragen positive cells. Both fractions of cells are viable and can be recultured. Fig. 12.3 shows the specificity of labelling of the neuroblastoma cell surface ganglioside with choleragen labelled microspheres.

2. Separation of brain oligodendrocytes. (Kronick 1980).

This separation is based on binding of antibody against oligodendrocytes which are then incubated with microspheres to which a 2nd antibody has been coupled.

An enriched glial fraction is prepared from brain cells of 10-day-old rats. The cells are incubated with rabbit serum against bovine oligodendrocytes and washed. The cells are further incubated with microspheres bearing goat anti-rabbit immunoglobulin (prepared as above) for 30 min at 4°C. Unbound microspheres are removed by centrifugation through FCS and passed through the Kronick apparatus. 90% of the oligodendrocytes are retained by the magnet.

12.3.2. Dextran microphores (Molday and MacKensie, 1982; Molday and Molday, 1984)

These microspheres are prepared by reacting ferrous chloride with ferric chloride and dextran in alkaline conditions.

The dextran is first derivatised with diaminoethane by oxidising 20 g dextran T-40 in 100 ml of 0.05 M acetate solution (pH 6.5) with 2.14 g sodium periodate for 1.5 h at 23°C. The solution is dialysed overnight against 2 l water at 4°C. The oxidised dextran T40 is

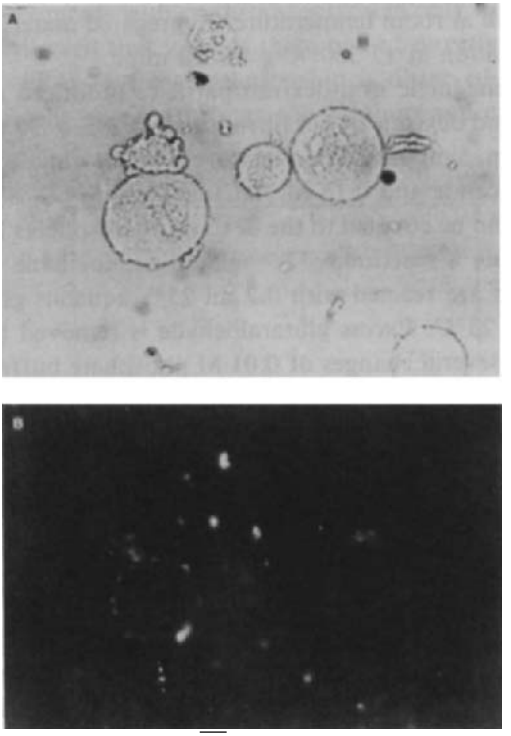


Fig. 12.3. Specific labelling of cells with magnetic microspheres. A. C-1300 neuroblastoma cells incubated with fluorescent choleragen-coated microspheres viewed in a light field; B, dark field illumination of A. Kronick, (1980). Plenum Press – with permission.

diluted with water to 180 ml and 20 ml of 6 M diaminoethane, previously titrated to pH 8.7 with acetic acid added. The mixture is stirred for 1 h at 23°C and the reaction stopped by the addition of 0.75 g $NaBH_4$. The solution is dialysed and lyophilized.

A stock solution of dextran is prepared from 1 vol 50% (w/w) diaminoethane dextran and 4 vols of 50% (w/w) underivatised dextran. A freshly prepared 5 ml aqueous solution of iron chloride containing 0.75 g $FeCl_3 \cdot 6H_2O$ and 0.32 g $FeCl_2 \cdot 4H_2O$ is mixed with 5 ml of stock dextran solution and stirred vigourously at 23°C. 10 ml of 7.5% (v/v) NH_4OH is rapidly added and the black suspension stirred for 1 h at room temperature. Aggregated material is removed by centrifugation at 17 300 × *g* for 10 min.

The ferromagnetic iron-dextran particles produced are separated from unbound dextran by gel filtration on a 2.5 × 33 cm Sephacryl S-300 column. 5 ml of reaction mixture is applied and eluted with 0.1 M sodium acetate and 0.15 M NaCl at pH 6.5.

Proteins can be coupled to the dextran microspheres by a two-step glutaraldehyde reaction. 2 ml diaminoethane derivatised microspheres are reacted with 0.2 ml 25% aqueous glutaraldehyde for 1.5 h at 23°C. Excess glutaraldehyde is removed by dialysis at 4°C against several changes of 0.01 M phosphate buffer pH 7.0 for 15 h. 2 mg of protein is added to 2 ml of microspheres and stirred for 12 – 16 h at 23°C. The reaction is stopped by the addition of 0.05 M glycine and the protein bound microspheres separated from unbound protein by gel filtration on a 1.5 × 25 cm Sephacryl S-300 column in PBS. The protein conjugated microspheres elute in the void volume with a iron dextran concentration of 3 – 4 mg/ml (dry weight).

12.3.2.1. Separation with dextran microspheres. (Molday, 1984; Molday and Molday, 1984). This separation was originally published as an example to demonstrate the retaining capacity of dextran microspheres in conjuction with the high gradient apparatus. Glutaraldehyde fixed human erythrocytes (1.4×10^7 in 150 μl) are incubated with 100 μl of rabbit anti-human RBC antiserum for 30

min at 25°C. After thorough washing in PBS aliquots of 2×10^7 cells in 200 μl are incubated with 100 μl of Protein A or goat anti-rabbit Ig coupled dextran microspheres at 3 – 4 mg/ml for 0.5 – 1 h at 23°C. The cells are thoroughly washed in PBS, resuspended in 0.5 ml PBS and passed through the high gradient column apparatus containing 30 mg (25 μg diameter) stainless steel wire in a 10 × 0.6 cm glass column with a magnetic field of 10 kilogauss midway between 2 cm diameter pole faces set at a gap of 10 mm. Between 96 – 98% of the erythrocytes are retained on the column and almost all of these can be recovered.

Control experiments with erythrocytes treated only with Protein A microspheres showed that 97% of these passed directly through the column (Fig. 12.4). A linear relationship is observed between the percentage of cells passing through the columns and either 10 g of

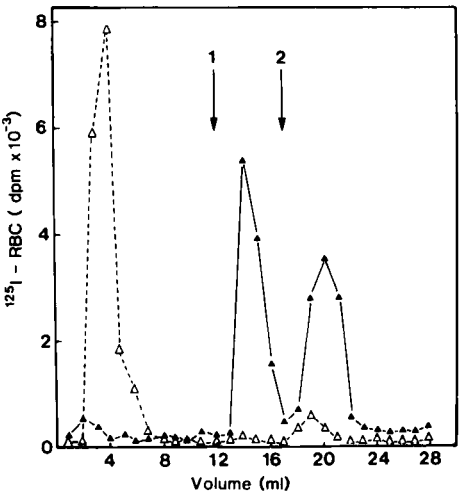


Fig. 12.4. High gradient magnetic capture (HGMC) of human RBCs treated with immunospecific ferromagnetic iron dextran microspheres. (▲——▲) [^{125}I]RBC sequentially treated with rabbit antihuman RBC antiserum and Protein A-ferromagnetic microspheres. (△——△) RBC treated only with Protein A-ferromagnetic microspheres. Molday and Molday, (1984).

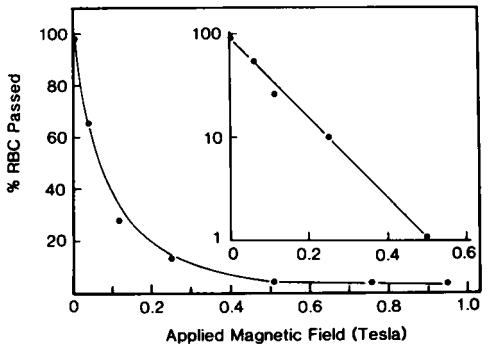


Fig. 12.5. The effect of applied magnetic field on the percentage of labeled RBC passing through a high gradient magnetic capture apparatus (Fig. 12.1). Molday and Molday, (1984).

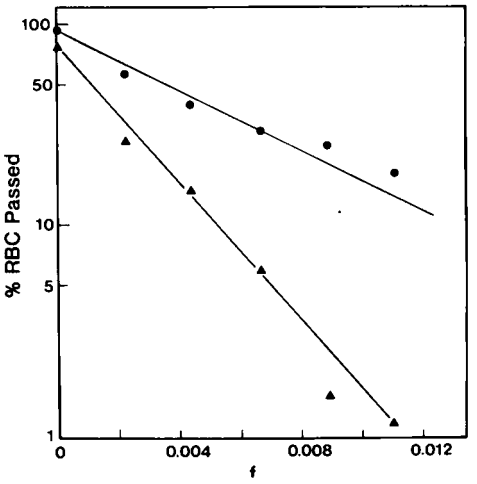


Fig. 12.6. The effect of the fraction of volume occupied by stainless steel wire on the percentage of RBC passing through the HGMC column, (Fig. 12.1), in the presence of magnetic fields of 0.25 tesla (▲——▲) and 0.65 tesla (●——●). Molday and Molday, (1984).

the magnetic field strength or the fraction of the volume occupied by the wire (Figs. 12.5 and 12.6). Kronick and Gilpin, (1986) have also described a method for producing dextran based superparamagnetic magnetite microspheres which they have used to separate *Legionella* from other water bacteria. These particles do not attract each other in the absence of a magnetic field and are therefore less prone to clumping.

12.3.3. Polyglutaraldehyde spheres (Margel et al., 1979)

Magnetic polyglutaraldehyde spheres can be prepared by the alkaline polymerisation of aqueous glutaraldehyde with ferrofluid particles and detergent. These microspheres have the advantage that proteins can easily be coupled by linking the protein amino groups to the aldehyde groups on the microspheres. The microspheres do however aggregate in the presence of high salt concentrations.

100 ml of a solution containing 5% aqueous glutaraldehyde, 1% Aerosol 604 (American Cyanamid), and 5% (w/v) Ferrofluid ('magnetite particles' Ferrofluidics) at pH 11 is shaken for 24 h at room temperature under nitrogen. The pH is periodically checked and readjusted to 11 by addition of NaOH. After shaking, the mixture is extensively dialysed against water and the spheres are separated from the reactants by means of a magnet and are thoroughly washed in water before being stored in phosphate buffer containing 0.25 M sucrose. About 200 mg of spheres are produced with an average diameter of 200 nm $\pm$ 200. Different size spheres can be produced by altering the concentrations and pH of the reaction mixture. The ability of these microspheres to specifically separate cells has been tested as follows. 0.2 mg of goat anti-rabbit antibody in 0.2 ml PBS was added to 0.2 ml of microspheres (2 mg) and the mixture gently shaken for 2 h at 4°C. 10 mg glycine and 20 mg BSA were added and stirred for another hour at room temperature. Unbound antibody was removed by passing the suspension through a Sepharose 4B column. 1.0×10^7 human glutaraldehyde-fixed red blood cells in 0.5 ml PBS were incubated

with 0.2 mg rabbit anti-human antiserum for 30 min at room temperature. The cells were washed 3 times in PBS, added to the microsphere suspension and shaken for 1 h at 4°C. The cells were separated from unbound microspheres by differential pelleting 3 × at 500 × *g*. Mixtures of normal and labelled cells were made with varying ratios and gently stirred for 2 h in a glass vial fitted with a horseshoe magnet (300 Gauss). The cells attracted to the magnet were diluted in PBS and the separation repeated. On average 95% of the unlabelled cells were separated from the mixtures by attraction of the labelled cells to the magnet.

12.3.4. Albumin microspheres (Widder et al., 1981)

Magnetic microspheres can be prepared from albumin by the emulsion polymerisation of ferrofluid particles with a mixture of albumin and cotton seed oil. In the reaction outlined below, Protein A is included in the reaction mixture to produce Protein A-microspheres. 0.5 ml of an aqueous suspension (190 mg dry weight) is prepared containing 60% human serum albumin, 19% ferrosoferric oxide and 15% Protein A. 60 ml of cottonseed oil is added and the emulsion sonicated for 1 min with 60 W. The homogenate is added to 200 ml of continuously stirred cottonseed oil at 120 – 125°C for 10 min. The resulting microspheres are washed 4 times with excess ether and separated by centrifugation at 2000 × *g* for 15 min. The microspheres are dried and stored at 4°C.

In a similar experiment to the one described above using polyglutaraldehyde microspheres, albumin/Protein A microspheres were used to separate chicken red blood cells from sheep red blood cells using rabbit anti-chicken antibodies, which bind to Protein A. Using a 400 Gauss magnetic field, 97% of the chicken cells were attracted to the magnet along with 9.5% of the sheep cells.

The main advantage of these microspheres are that they are simple to produce and do not involve chemical coupling reactions for attaching proteins, the protein is actually incorporated into the microsphere.

12.3.5. Colloidal protein microspheres (Owen and Liberti, 1986)

A recent article by the above authors edited by Pretlow and Pretlow describes the use of colloid protein-magnetite particles for cell separation. The particles are produced by the precipitation of magnetite in the presence of protein. The precipitation is effected by the addition of a base to a solution of ferric and ferrous iron chlorides and protein. Protein cross-linking reactions can be used to attach cell surface specific molecules to the particles. For example antibodies and Protein A were attached with SPDP (succinimidyl-propiono-dithiopyridine) in a reaction involving cleavage of protein disulphide bonds with SPDP and DTT and the activation of antibody and/or Protein A with SPDP. Biotin can also be attached to the particles by using commerically available activated biotin (biotin *N*-hydroxysuccinimide). Owen and Liberti, (1986) tested the ability of antibody, Protein A and biotin microspheres to deplete a population of mouse spleen cells of T cells using a high gradient apparatus. With the biotin coupled microspheres, avidin and biotinated anti-Thy-1.2, monoclonal antibody against T cells, 75% of the T cells in the cell population were recovered, 90% of which (68% of total) were retained by the magnetic wire column. 34% of non-T cells were also retained by the magnetic column and 64% passed through the column. The results obtained with Protein A microspheres, linked to T cells via anti-Thy1.2, avidin and biotinated rabbit gammaglobulin were less impressive with a similar retention of T cells but with only 27% of non-T cells passing through the column. The highest contamination of the non-T cell population passing through the column with T cells was obtained with antibody coupled particles (goat-anti-rabbit particles), which were linked to the cells via Thy-1.2 avidin and biotinated rabbit gammaglobulin. 11% of the T cells passed through the column with 37% of the non-T cells.

12.3.6. Immunoferritin

The major disadvantage of all the methods described above is that

each necessitates the synthesis of magnetic microspheres with ligands that are specific to a particular cell population. The use of ferritin as a paramagnetic source to label cells for magnetic separation is particularly attractive therefore because of the availability of a large variety of ferritin conjugates. The feasibility of magnetically separating cells with ferritin conjugates attached to their surfaces has been studied by Owen and Lindsey, (1983). They used a high gradient apparatus consisting of 10 cm of 25 μm diameter stainless steel wire packed in 1 cm diameter column. Erythrocyte ghosts were prepared from human blood and ghost suspension containing 0.1 – 0.3 mg protein, were incubated for 1 h at 4°C in 0.5 ml PBS containing 0.25 mg ferritin-Con A conjugate. The ghosts were then washed in PBS by centrifugation at 11 000 × g for 30 min and resuspended in 1.1 ml PBS. The ghost suspension was pumped into the column with PBS containing 0.5% BSA as the running buffer at a flow rate of 0.3 ml/min. Around 5 – 10 times the bed volume was collected until the eluate was free of ghosts. The column was removed from the magnetic field and bound ghosts eluted with 3 – 8 bed volumes of PBS-BSA. The retention of ferritin labelled ghosts was compared with a control which comprised the same labelled ghosts with the inclusion of alpha-methyl-D-glucoside as a competing sugar for Con A binding. 12% of the control ghosts and 95% of the labelled ghosts were retained. A 50:50 mixture of the two would be expected to result in a 90-fold enrichment of labelled cells. The total recovery of cells was between 60 – 90%.

The main drawback of this method is that relatively little iron is bound to the surface. This means that very high magnetic fields (70 kG in the example above), and low flow rates must be used in order for cells to be retained. Low flow rates increase the amount of non-magnetic retention.

12.3.7. Other reactions

Rembaum et al., 1982 have described a method for coating magnetic polystyrene microspheres with polacrolein. The reaction involves exposure of a mixture of magnetic polystyrene microspheres, acrolein

and polyethylene oxide to cobalt radiation (1 Mrad for 5 h). The microspheres produced can easily be reacted with proteins.

Graham and Selvin, (1982) have used paramagnetic rare earth ions, erbium and dysprosium to label cells for magnetic separation.

12.3.8. Dynabeads M-450

A most recent development in the field of cell separation using magnetic microspheres is the availability of commercially-produced immunomagnetic particles, 'Dynabeads M-450' together with custom-built apparatus for their separation. The beads and apparatus are produced by Dynal. Dynabeads are uniform, magnetic polystyrene beads with a diameter of 4.5 μm that are supplied in an uncoated form or precoated with various anti-mouse second antibodies, which are ideal for cell separations utilising monoclonal antibodies. Dynabeads are also produced that are coated with monoclonal antibody against CD8 antigen present on T cells which can be used for rapid isolation of these cells or coated with anti-HLA Class II B-chain monoclonal antibody for isolation of HLA Class II lymphocytes. Details of the conditions for the coupling of proteins to Dynabeads and the cell separations protocols are supplied with the beads.

The apparatus produced by Dynal, the 'Magnetic Particle Concentrator' (MPC), consists of a simple permanent magnet (8500 – 9300 Gauss) supported on a clamp which holds either one (MPC 1) or six (MPC 6) test tubes containing the cell/Dynabead mixture.

Separation of cells in different phases of the cell cycle

13.1. Introduction

The classic division cycle or 'cell cycle' is shown in Fig. 13.1. The detailed study of cell division processes of most cells necessitates the physical separation of cells at different stages in the cell cycle. Once isolated such cells can either be studied directly or can be used to initiate synchronous cultures. Separation of cells in the different phases is particularly attractive because it does not involve alteration of the cycle by inhibitors which is the other method normally used to obtain cells in a particular phase.

The isolation of cells in a particular phase of the cell cycle, free of cells in all other phases represents a particular testing application for most cell separation methods. The differences in size and surface character between cells in different phases are small in comparison with differences between different cell types. The ability of a particular method to separate cells in different cell cycle phases to a high degree of purity is therefore a good demonstration of the sensitivity of a separation method. By far the most succesful methods in current use are *centrifugal elutriation* which separates the cells on the basis of size differences, and *flow cytometry* which separates the cells on the basis of DNA content. Of these, flow cytometry is a more analytical technique and its greatest use is in monitoring the proportion of cells in each phase. Elutriation has the capability of

separating large numbers of cells in a particular phase which can then be used to start synchronous cultures.

Of the separation methods discussed in this volume, all those except the specific surface affinity methods (excluding flow cyometry) can separate cells on the basis of phase of the cell cycle. The use of each of these methods to carry out such separations is discussed below both for the intrinsic value of obtaining cells in different cell cycle phases and also as a comparison of the sensitivity of each method.

Although it is not within the scope of this volume to discuss the identification of cells in different cell cycle phases the reader should be aware of these. Cells can be identified by DNA content where G2 cells have twice the DNA content of G1 cells, with S phase cells distributed between these. By continuously labelling with a precursor of DNA such as ^{3}H-thymidine all cells that undergo DNA synthesis (S phase) during the labelling period can be detected by quantifing the radiolabel or by visualising via autoradiography. By using pulse labelling techniques cells labelled in S phase can be detected as they progress through the other phases of the cycle. The third method is to identify the cells in mitosis by scoring the mitotic index i.e. number of cells undergoing mitosis.

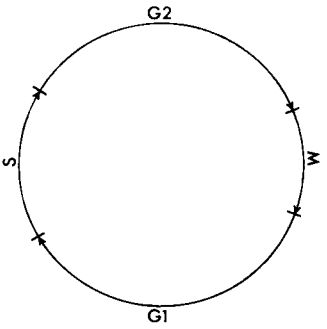


Fig. 13.1. The cell cycle.

13.2. Centrifugation

Cells in G1 phase are generally smaller than cells in S phase which are smaller than cells in G2 phase, since G1 cells have most recently divided. This size difference provides the basis for separation using all the methods ultilising differences in rate of sedimentation, e.g. centrifugation, elutriation and unit gravity sedimentation.

Centrifugation, being the longest established, was the first of these methods to be used for cell cycle separations. Although, as described below, centrifugation can separate cells in the different phases, the

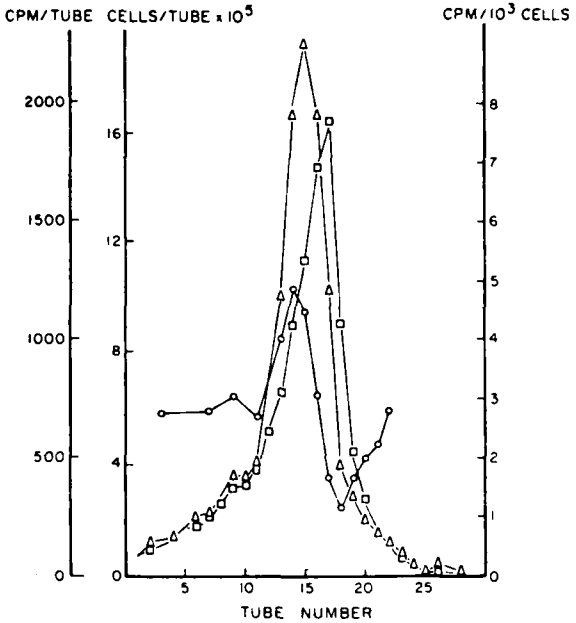


Fig. 13.2. Distribution of neoplastic murine mast cells (P81SY) on a 5 – 20% sucrose gradient after centrifugation at 1000 × *g* for 8 min. (□——□) Number of cells per tube, (△——△), radioactivity per tube, (○——○), specific radioactivity per tube in counts/min/cell. Cells labelled with a 60 min pulse of ^{3}H-thymidine. Morris et al., (1967). Academic Press – with permission.

purity of cells obtained in any one phase is generally low compared with the more recent methods.

In an early study, Morris et al., (1967) centrifuged cells from a growing culture of neoplastic murine mast cells (P815Y) on a 5 – 20% sucrose or 5 – 20% Ficoll gradient for 8 min at around 1000 × *g*. The cell distributions obtained were the same in both gradients and are shown in Fig. 13.2. It is clear that there is considerable overlap between cells in the different phases. A value of 80% of the cells in fraction 15 is given as being those in S phase. No details were presented concerning cell recovery and viability.

A more recent example is taken from the work of Woffendin and Griffiths (1982) who describe the separation of a fraction of cells in G1 phase which are used to produce synchronous cultures of *Dictyostelium discoideum* cells. An exponentially growing culture is centrifuged at a very low *g* force (35 × *g*) for 2 min. The supernatant produced contains a high proportion of non-viable cells which are discarded. The pellet is resuspended in culture medium and centrifuged for 2 min at a slightly lower *g* force of around 30 × *g*. The cells obtained in the supernatant (i.e. the smallest cells) are viable and constitute around 10% of the total cell population. When the cells from the supernatant are cultured, a high degree of synchrony is obtained with the cells undergoing DNA synthesis before dividing, indicating that they are in G1 phase (Fig. 13.3).

When using such low *g* forces it is essential that the speed of the centrifuge is monitored very accurately by using a stroboscope. The type of centrifuge tube used (glass or plastic) may also affect the separation obtained and both should be tried. The use of this method to separate G1 phase cells of another organism, *Acanthamoeba castellanii* is described by Chagla and Griffiths, (1978).

13.3. Centrifugal elutriation

Centrifugal elutriation is rapidly becoming the method of choice for isolating large numbers of cells in a particular phase of the cell cycle

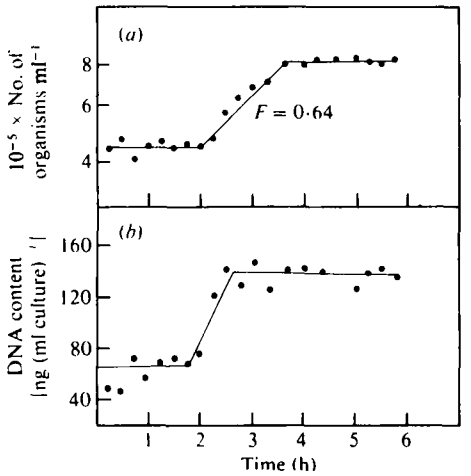


Fig. 13.3. Degree of synchrony obtained with *D. discoideum* cells in S1 phase separated by centrifugation. (a) Cell number, (b) DNA content. Woffendin and Griffiths, (1982). Society of General Microbiology – with permission.

for direct study or initiating synchronous cultures. The reason for this is the sensitivity of the method and the ability to control very accurately the elution of cells of different sizes i.e. a large number of fractions can be obtained each with a small increment in size. This means that very pure fractions of cells in one phase can be obtained.

In common with separation of any cells using elutriation, one of the biggest advantages is that the separation can be carried out in culture medium. Thus growing cells can be fractionated directly in their growing medium and are not perturbed in any way, other than perhaps a lowering of the temperature to stop cells cycling.

An illustration of the separation that can be obtained is produced by Beckman in their Applications Data Sheet DS-594, authors Griffith and Adams. Here Chinese hamster ovary (CHO) cells growing exponentially in Hams F-10 medium plus 15% FCS are harvested and resuspended in 10 ml medium at 1×10^7 cells/ml. The 10 ml suspension is introduced into a standard elutriation chamber with a

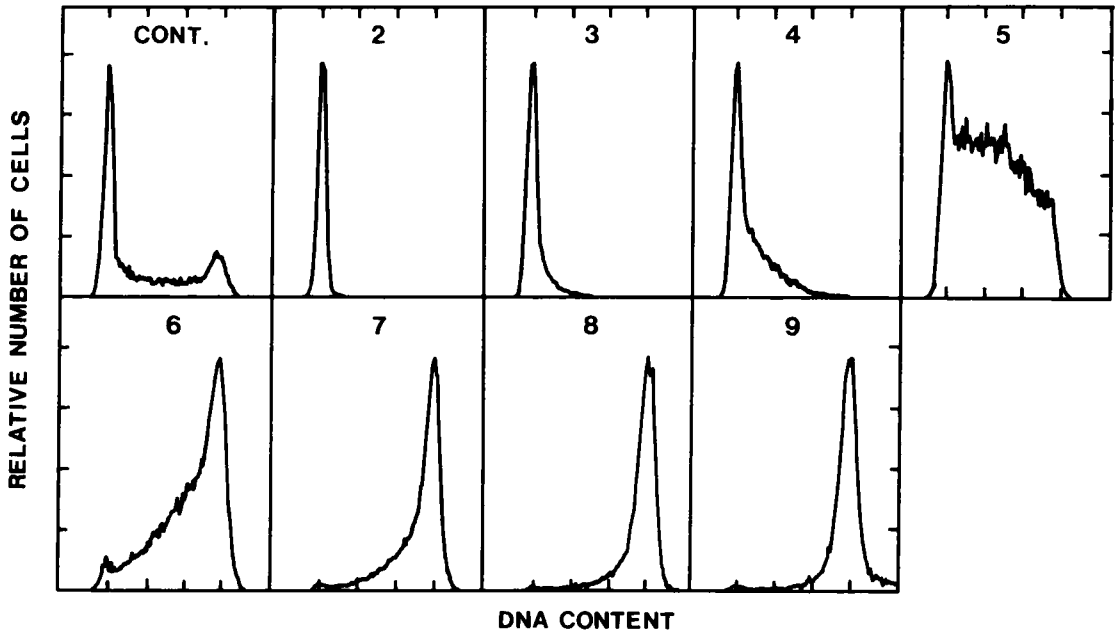


Fig. 13.4. DNA histograms of exponentially growing CHO cells fractionated by centrifugal elutriation. Cont = original population, 2–9 elutriated fractions. Griffith and Adams, Beckman Applications Data Sheet DS-594.

rotor speed of 2000 rpm and an initial flow rate of 10 ml/min. The rotor is maintained at 4°C and growth medium is pumped continuously from a reservoir at 4°C through the chamber. Five minutes after the cells have filled the chamber the flow rate is increased in 2 ml/min increments up to 28 ml/min. 100 ml of eluate is collected per increment in flow rate and kept at 4°C prior to analysis. The number and volume of cells in each fraction is measured together with their DNA content. A similar analysis is also carried out on the unseparated cell population. Figs. 13.4 and 13.5 show the results obtained by Griffith and Adams. Unelutriated cells show a broad size distribution compared with all the elutriated fractions. The average volume of elutriated cells increases as expected with higher fraction number. The volume of elutriated cells exactly doubles from fraction 2 to fraction 9. The DNA analysis shows that fraction 1 contains mostly debris and unelutriated cells. Fraction 2 contains 99% small

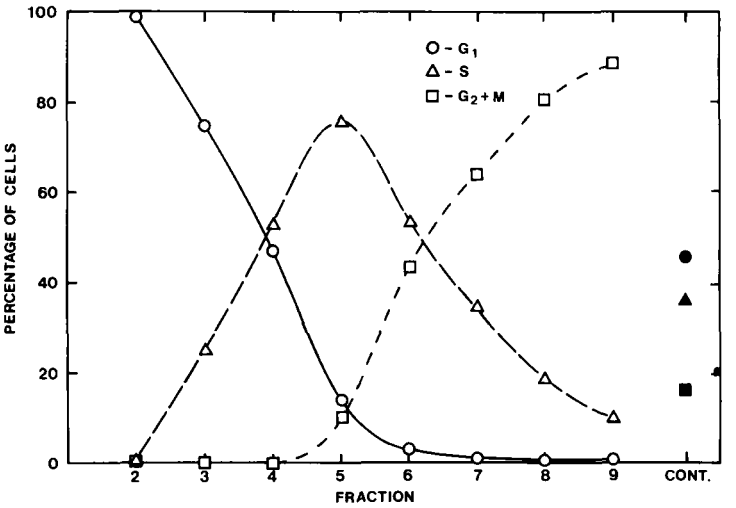


Fig. 13.5. Cell cycle distributions of elutriated fractions from Fig. 13.4, (○——○) percentage of cells in G1 phase; (△——△) S phase; and (□——□) G2 + M phase. ●▲■ Cell cycle phase distribution of original unelutriated population. Griffith and Adams, Beckman Applications Data Sheet DS-594.

cells having a DNA content consistent with them being in G1 phase (i.e. diploid 2n). Fraction 3 contains 25% early S phase cells, fraction 4, 53% S phase and fraction 5, 76% S phase. Fractions 7 – 9 contain 64% G2 and 80% M phase.

Results such as these are typical of those that can be obtained from most cultures of mammalian cells and indeed many other cells. Gandour and Walker, (1983) obtained an almost identical separation from a culture of murine macrophage cells (P388D1). Creanor and Mitchison, (1978) described the use of the elutriator rotor to separate a population of G1 phase cells to establish synchronous cultures of yeast. 7.0×10^9 yeast cells are loaded in culture medium into the chamber with the rotor spinning at 3700 rpm with a flow rate of 22 ml/min. Increasing the pump speed to 33 ml/min can elute a fraction of around 2×10^8 cells in G1 phase which are used to establish synchronous cultures. The degree of synchrony obtained is shown in Fig. 13.6. In this particular example, the medium and rotor were maintained at a constant 32°C and the cells continue to cycle.

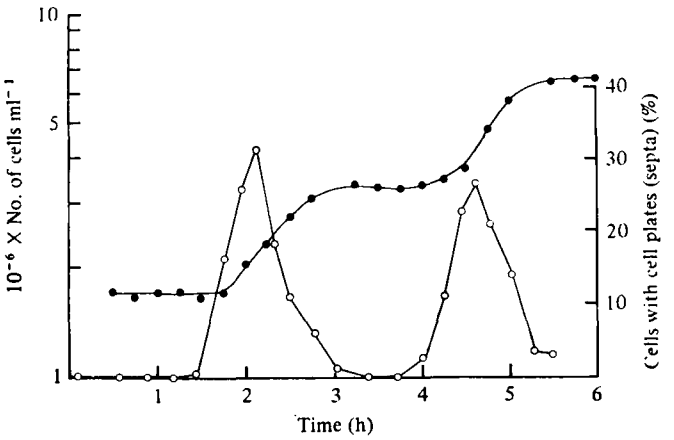


Fig. 13.6. Synchronous cultures of *S. pombe* (972h-) initiated by S1 phase cells separated by centrifugal elutriation. (●——●), cell number; (○——○) cell plate index. Creanor and Mitchison, (1978). Society of General Microbiology – with permission.

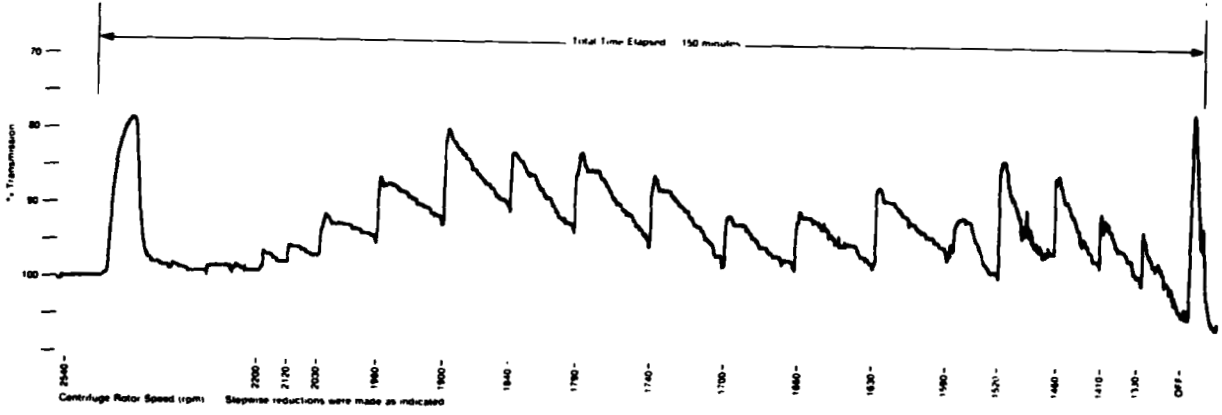


Fig. 13.7. Complete fractionation of CHO cells by centrifugal elutriation. Initial peak is small particulate debris which flows though the rotor during the load phase. First cells recovered when rotor speed reduced to 2200 rpm. % transmission cell number. Cells recovered per centrifuge setting is proportional to area of each corresponding cell peak. Grabske, Beckman Applications Data DS-503A.

Creanor and Mitchison further showed that if left in the rotor with medium circulating at 33 ml/min, the yeast cells continue to grow with a normal doubling time of 150 min.

The majority of cell cycle separations have been conducted by increasing the flow rate as this avoids the problems that can occur when cells are elutriated by decreasing the rotor speed. Grabske, (Beckman Applications Data DS-503A) has described the elutriation of CHO cells from a growing culture into 16 fractions by decreasing the rotor speed. As described in Chapter 5, the centrifuge needs to be modified in order to elutriate cells by decreasing rotor speed. This principally involves fitting the speed control with a 2000 ohm ten turn potentiometer in series with a 3000 ohm resistor. $2-4 \times 10^8$ cells are loaded into the chamber at 2540 rpm with the flow rate of medium set at 15 ml/min. The rotor speed is decreased in a series of steps equivalent to 0.5 mm/h of sedimentation velocity. The distribution of cells elutriated is shown in Fig. 13.7. To establish synchronous cultures, the first fraction can be used or if more cells are required, two or three of the first can be pooled.

13.4. Unit gravity sedimentation

The separation of cells in different phases of the cell cycle by unit gravity sedimentation has been described by Macdonald and Miller, (1970). A 'muffin' type apparatus similar to the Celsep apparatus (see Fig. 4.11), was used in these experiments. The method described by Macdonald and Miller using mammalian cells grown in culture is outlined below. Earles L-cells (L60T) grown in suspension culture and pulse labelled with tritiated thymidine for 15 min are harvested in exponential phase, washed, filtered to remove clumps and resuspended at 2.5×10^5 cells/ml. This cell density is well below the streaming limit for this apparatus. 20 ml of the cell suspension is layered onto a linear gradient of 15 – 30% FCS in PBS at 4°C in a closed chamber, diameter 13.8 cm, heigh 7.9 cm. Sedimentation is allowed to proceed for 3.5 – 4 h before the gradient is unloaded in

the tilted position into 15 ml fractions. An aliquot of cells is removed from each fraction for cell number and volume determination. The remaining cells are fixed, stained and prepared for autoradiography to determine to percentage of labelled cells and the mitotic index. The results obtained by Macdonald and Miller are shown in Fig. 13.8). The distribution of S phase and mitotic cells is considerably

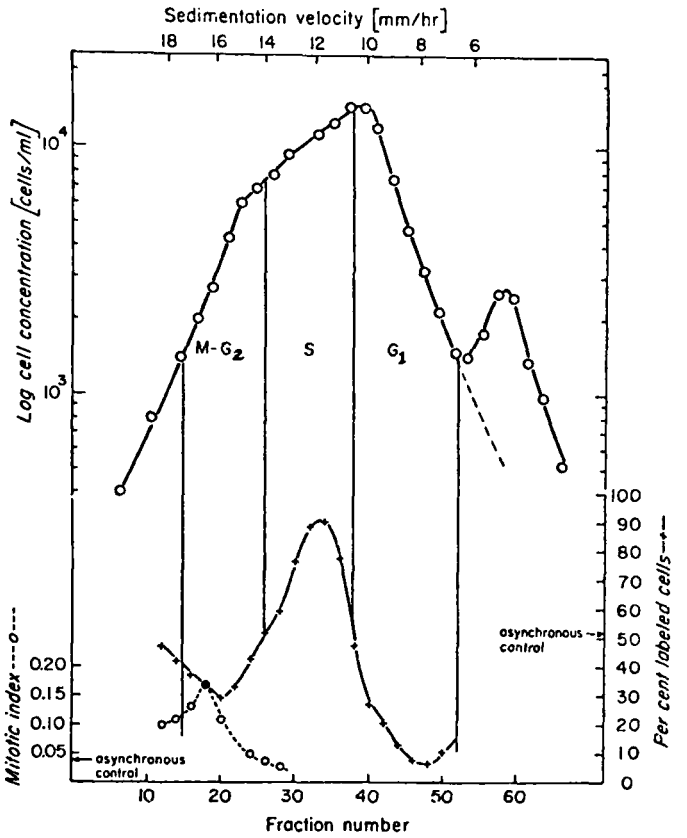


Fig. 13.8. Distribution of L-cells separated by unit-gravity sedimentation on a linear, 15 – 30% gradient of FCS in PBS (O——O) Log cell number (cells/ml); (x——x) percent labelled cells – 15 min pulse of ^{3}H-thymidine; (– – 0 – –) mitotic index. MacDonald and Miller, (1970). The Rockefeller University Press – with permission.

more homogeneous than the population as a whole. G1 and S phase cells can be obtained with a purity of around 90% whereas G2 and M phase cells can only be obtained with a lower purity of around 70%. The reduced purity of the G2-M phase cells is partly caused by the presence of cell clumps and polyploid cells in that region of the gradient.

The percentage of cells in each phase can be calculated from the distribution, and values of 18.4, 48.4 and 33.2% are obtained for

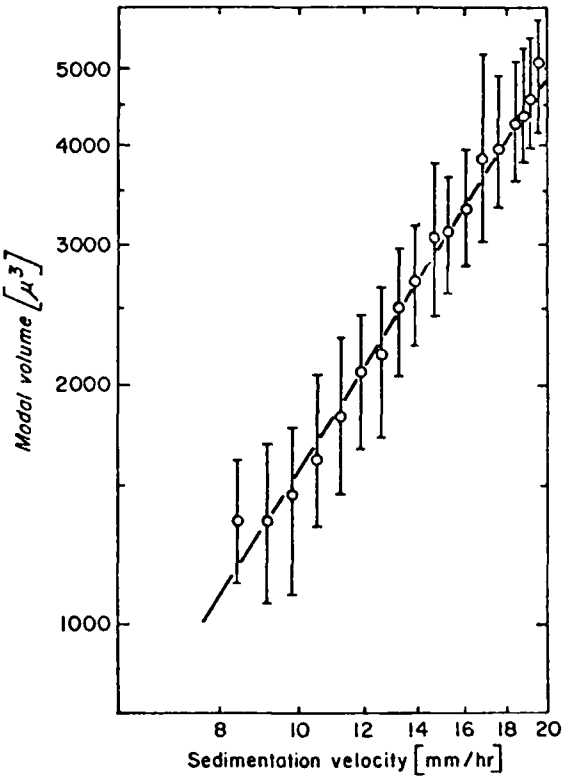


Fig. 13.9. Correlation between log modal cell volume and log sedimentation velocity of cells fractionated as in Fig. 13.8. Dashed line = least squares fit to the data; vertical bars = volume dispersion for each fraction. MacDonald and Miller, (1970). The Rockefeller University Press – with permission.

G2-M, S and G1 phases respectively. These agree well with the predicted percentages from the length of each phase which are, 19, 49 and 32% respectively.

Little use, other than this work, has been made of the potential of unit *g* sedimentation for separating cells in different phases of the cell cycle. Considering the very low cost of the apparatus which is at least a quarter of the cost of an elutriation system, together with the separations obtained it is surprising that more use of this method has not been made. Macdonald and Miller have reported that the distributions obtained are highly reproducible.

One possible problem that may arise with this method is the influence of density differences which may reduce resolution. This clearly may vary for different cell populations, however for the L-cells used above there appears to be no affect of cell density. Fig. 13.9 shows the relationship between cell volume and sedimentation velocity for cells in each fraction. The resultant straight line has a slope and standard deviation of 1.55 ± 0.07, which is not significantly different from the predicted value of 1.50. Thus the cell volume is proportional to sedimentation velocity for cells in each fraction, indicating that cell density differences have not influenced the separation.

A more serious limitation which can also equally apply to elutriation, is possible heterogeneity of cell volumes at any single point of the cell cycle. This would result in broadening of the distribution and thus loss of resolution.

13.5. Phase partition

The technique of aqueous two-phase partition has not been used to separate cells in different phases of the cell cycle although analysis of several distributions obtained has revealed that cells have partitioned according to cell cycle phase. In principal aqueous partition should be able to separate cells in different phases since specific changes in surface properties occur as cells cycle and although such

changes are often subtle, they should be reflected in the partition coefficient. Walter et al., (1971, 1973) partitioned *Chlorella pyrenoidosa* from sychronous culture at different times after cell division. The phase system used consisted of 5% (w/w) dextran T500, 4% (w/w) PEG 6000 (Carbowax 8000) and 0.01 M potassium phosphate buffer pH 7.8. 120 transfer TLCCD was carried out at 4°C in the dark. *Chlorella* were subjected to TLCCD at 6, 8, 14 and 16 h after cell division (0 h). The resulting profiles are shown in Fig. 13.10. The distribution of unsynchronised cells clearly shows two distinct peaks (sub-populations). The peak to the left in the lower fraction numbers (with the lowest partition) corresponds to the 'youngest cells' i.e. those just divided, which can be clearly seen from the distribution of synchronous cells at 6 h. The peak to the right in the higher fraction numbers (with the highest partition) corresponds to cells just prior to cell division and these cells are significantly larger than cells in the left peak. However the high partition peak in the unsynchronised population is further to the right of any peaks produced by the synchronous cells. Walter et al., suggest that the explanation for this is that in non-synchronously growing *Chlorella* there is a time lag in the changes in surface properties just prior to and just after division. The cell cycle of *Chlorella* differs from the classic cycle in that it does not have a G2 phase.

The major changes in the distributions of synchronised cells occur between 9 – 14 h. Although no explanation is offered for this, one possibility is that this may represent cells entering S phase, which occurs around 10 h after division and lasts for about 6 h.

None of the distributions obtained are homogeneous with respect to surface properties suggesting that even though the cultures are growing synchronously, there is heterogeneity at the cell surface.

The phase system used in these experiments was of a charged type which suggests that the young cells with a low partition have a lower surface charge than the older cells with a high partition.

Another example of the partition of cells being cell cycle related is work conducted by myself and D.J. Watts (Sharpe and Watts, 1984). Here, an exponentially growing culture of *Dictyostelium*

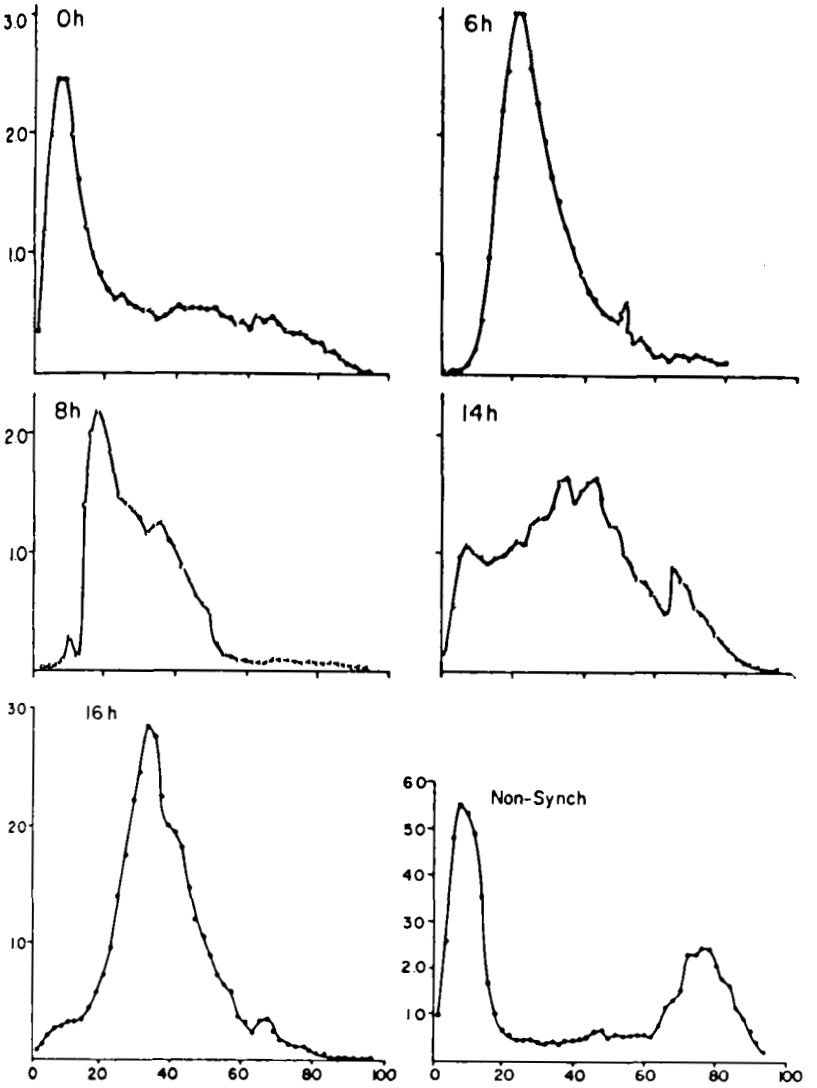


Fig. 13.10. TLCCD profiles of synchronised *Chlorella pyrenoidosa* at different times after cell division in a 5% dextran, 4% PEG, 0.01 M potassium phosphate buffer pH 7.8 phase system at 4°C in the dark. Non-synch = normally grown (non-synchronised) cells. Abscissa – fraction number; ordinate = A590 (cell number). Walter et al., (1973). Academic Press – with permission.

discoideum cells were partitioned in a low charge phase system composed of 5.5% (w/w) dextran T500, 5.5% PEG 4000, 0.05 M NaCl, 0.01 M Na_2SO_4 and 0.001 M phosphate buffer pH 7.8. The distribution obtained is somewhat broader than the distribution that would be obtained for a homogeneous population. This indicates that the cells are heterogeneous with respect to surface properties. In order to determine whether this heterogeneity was related to cells in different phases of the cycle, the DNA content of cells in different regions of the distribution was measured and the distribution of cells in S phase detected by incorporation of radiolabelled thymidine given as a short pulse during growth. The results obtained are shown in Fig. 13.11. The DNA content of cells in the centre fractions was higher than cells at either side. The incorporation of radiolabelled thymidine was highest in cells at the extreme left of the distribution. Labelled thymidine was also incorporated to some extent in all cells which is

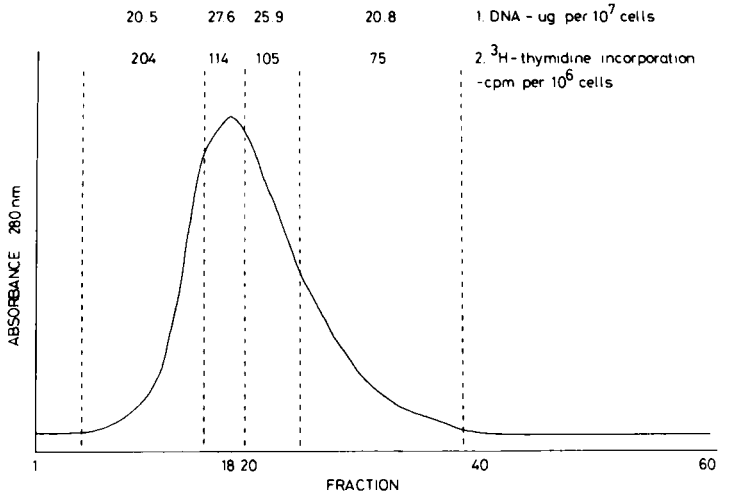


Fig. 13.11. TLCCD profile of exponentially growing *D. discoideum* amoebae. Fractions were pooled as shown and the DNA content (pg/cell) and radioactivity-counted following a 30 min pulse of ^{3}H-thymidine (CPM/10^7 amoebae) during growth. Phase system = 5.5% dextran, 5.5% PEG, 0.05 M NaCl, 0.01 M Na_2SO_4, 0.001 M phosphate buffer pH 7.8 at 4°C. Sharpe and Watts, (1984).

indicative of the high proportion of mitochrondrial DNA in this organism which replicates independently of the cell cycle. From these results it can be deduced that as shown in Fig. 13.12, cells are distributed according to phase of the cell cycle with S phase to the extreme left, G2 cells in the centre and G1 cells to the extreme right. The location of mitotic cells was not determined. Clearly aqueous partition is able to detect surface differences between cells in different phases and partition them accordingly. It seems reasonable to assume, although yet to be tested that where growing cells produce a heterogeneous distribution, the basis of the heterogeneity will be cells in different phases of the cell cycle. The practical use of this to obtain pure populations of cells in a particular phase has again not been fully evaluated. In this example there is considerable overlap between cells in each phase. If the computer program described in Chapter 6 is used to analyse the distribution it can be seen that only a small number of pure S phase and G1 phase cells can be obtained in this particular example (Fig. 13.13).

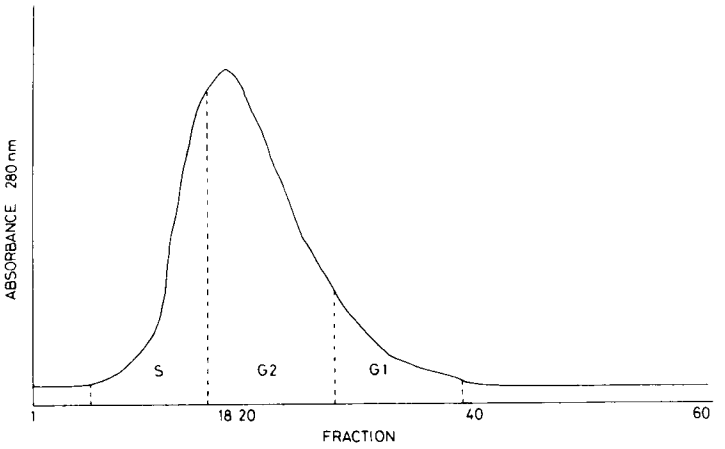


Fig. 13.12. Cell cycle distribution of *D. discoideum* amoebae from Fig. 13.11.

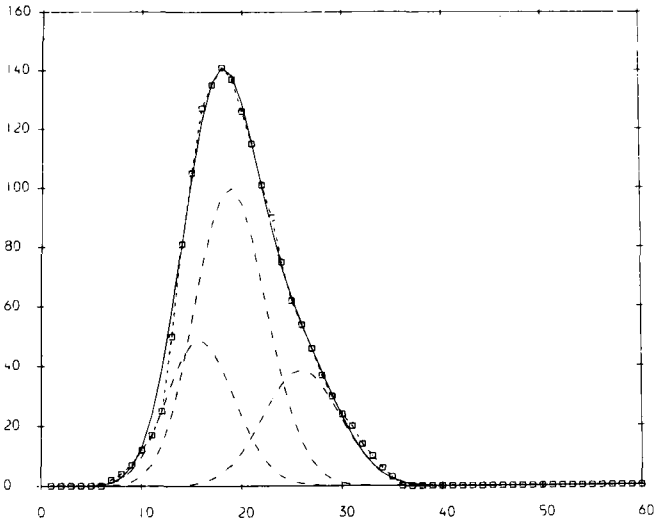


Fig. 13.13. Computer analysis of the TLCCD distribution from Fig. 13.11. Abscissa = fraction number; ordinate = cell number (normalised). (□——□) actual cell numbers from experiment; (– – –) cell sub-populations predicted by computer; (———) sum of computer predicted sub-populations.

13.6. Flow sorting

Flow sorting (flow cytometry) is the most sensitive method of determining the proportion of cells in a particular phase of the cell cycle and can also be used in certain instances to sort cells in a particular phase for initiating synchronous cultures. The method involves staining the DNA in intact cells by incorporating a specific DNA dye and detecting the emission of light from the dye as the cells pass through the lazer. A profile of cells having different DNA contents is obtained, a typical profile from mammalian cells is shown in Fig. 13.14. Since cells in G1 phase have half the DNA content of cells in G2, two cell populations are detected with the second peak (G2) in a fraction number exactly double that of the first peak (G1). An intermediate region of cells between the two peaks is detectable which is the S

phase cells. The percentage of cells in each of these regions is easily calculated by the apparatus. Gates can be set to select a particular population for sorting. Before discussing the DNA dyes used and the conditions where viable cells can be sorted it is worth noting one factor that can affect the interpretation of the distributions. This main factor is polyploidy. This is where cells have more than one nucleus. Many cell populations have a small percentage of polyploid cells. Cells containing 3 or more nuclei are easily detected because they produce peaks in progressively higher fraction numbers. Problems arise when a large proportion of the cells are polyploid (e.g. certain tumour derived cells) because the apparatus cannot distinguish between mononucleate cells in G2 and binucleate cells in G1, both of which have the same DNA content. A dual scan of the dye and small angle scatter can resolve this problem when there is a difference in size between the mono- and binucleate cells. The percentage of each of these cells in the × 2 DNA region can be measured by sorting these cells and scoring the percentage of binucleate cells under a

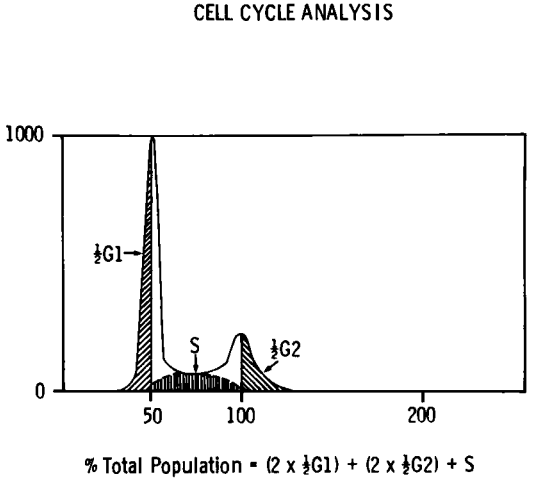


Fig. 13.14. Typical profile of cell number and DNA content of mammalian cells after flow cytometry (stained for DNA). Percentages of cells in each phase calculated as shown. Total cell population = (2 × G1) + (2 × G2) + S. Courtesy of John Lawry.

microscope, illuminating the cells at the correct wavelength for the dye used.

Dual parameter analysis can be carried out with cells stained with acridine orange, which binds to both DNA and RNA. Green fluorescence is emitted by the double-stranded DNA and red fluorescence is emitted by the single-stranded RNA when excited at 640 nm. Dual parameter analysis of green (IGFL) and red (IRFL) fluorescence, as shown in Fig. 13.15, shows mitotic cells with increased red fluorescence and decreased green fluorescence relative to cells in G2 phase. The dyes most commonly used to specifically stain DNA are mithramycin, ethidium bromide, Hoechst 33258, 33342 and DAPI (4,6-diamidino-2-phenylindole). The wavelengths used

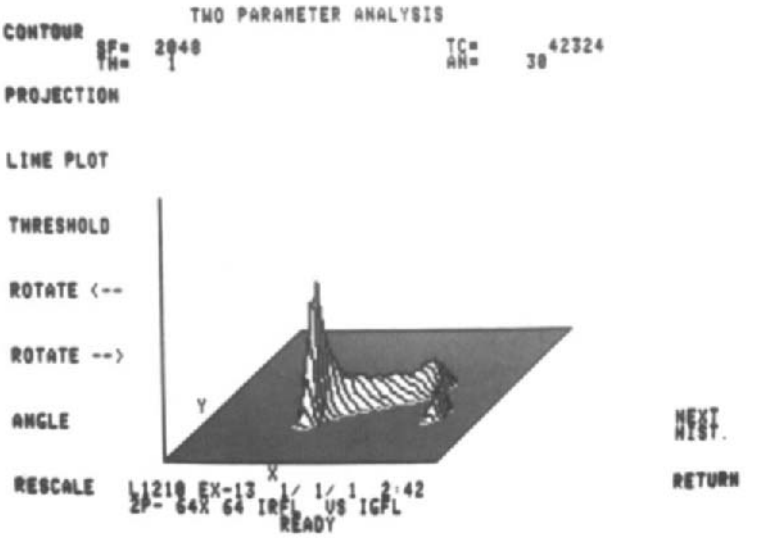


Fig. 13.15. Dual parameter flow cytometric analysis of acridine orange stained cells. IGFL = green fluorescence, IRFL = red fluorescence. (Coulter EPICS V Flow Sorter).

with these dyes are shown in Table 11.1. The uptake of these dyes by cells is low and in many cases needs to be enhanced by either fixing the cells in ethanol or by treatment with a mild detergent. Obviously ethanol-fixed cells can only be used for analysis purposes and not for initiating synchronous cultures. The same is partly true of detergent treated cells. Both the Hoechst dyes can however be incorporated into cells in sufficient quantities for fluorescence detection without prior fixing or detergent treatment. Arndt-Jovin and Jovin, (1977) have described the use of both the Hoechst dyes to label the DNA of several mammalian cell lines and sort cells in different phases of the cell cycle. The sorted cells could be recultured and cells in each phase had comparable viabilities, of around 90% and comparable growth rates. The labelling was carried out at 37°C in medium containing 10 μM dye for 1.5 h and sorting was carried out at 4°C to prevent transport of the dye out of the cells. Mithramycin is the most commonly used dye for cell cycle analysis. A concentration of 100 μg/ml in buffer (0.1 M Tris/HCl pH 7.4, 0.1 M NaCl and 15 mM MgCl) can be used, and often ethidium bromide at around 35 μg/ml is included in the mithramycin solution. The ethidium bromide increases the fluorescence emitted but also reduces the specificity because ethidium bromide also binds to RNA. Other dyes that can be used are acridine orange and propidium iodide. An evaluation of all the DNA fluorochromes can be found in Taylor and Milthorpe, (1980). When using these dyes the cells must either be fixed in ethanol (25 – 70%) or alternatively incubated with 0.2% Triton in 0.1% NaCl prior to staining. Ethanol fixation has the advantage that once fixed, cells can be stored for prolonged periods. Some cell clumping may however occur with ethanol fixation (Taylor and Milthorpe, 1980).

Accurate estimates of the percentages of cells in G1 and G2 phases can be obtained from the flow cytometric distribution. The estimation of the numbers of S phase cells is however less accurate since these represent a diffuse region between the G1 and G2 peaks (Fig. 13.14). A method has recently been developed for obtaining accurate determination of cells in S phase. Cells are incubated with a short

pulse of bromodeoxyuridine (BrdU) which is an analogue of thymidine and is specifically incorporated into DNA during synthesis (S phase). A monoclonal antibody against BrdU (anti-BrdU) conjugated to fluorescence (Becton Dickinson) is used to detect BrdU in-

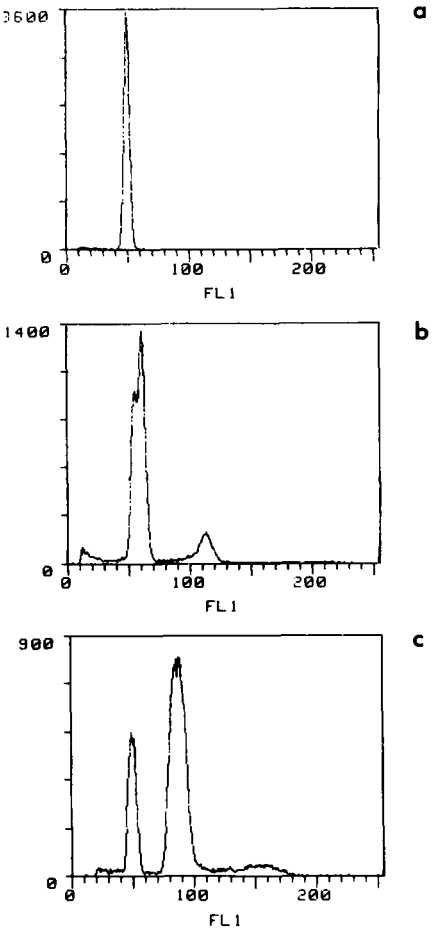


Fig. 13.16. Flow cytometry of cells stained for DNA with mithramycin/ethidium bromide. (a) Peripheral blood lymphocytes (single peak of diploid cells); (b) (c), cells from two different human breast tumours showing different percentages of aneuploid cells. (FACS 400 Flow Sorter). Courtesy of John Lawry.

corporated into DNA. Dual parameter analysis can be used to detect G1 and G2 phase cells stained with a fluorochrome and S phase cells stained with fluorescent antibody.

Flow sorters have proved particularly useful for the study of tumour cells. In many tumours, a percentage of the cells become aneuploid and such cells can easily be detected by flow cytometry after staining of their DNA. An example of this kind of analysis is shown in Fig. 13.16. Peripheral blood lymphocytes show a single peak of diploid (FIg. 13.16a), G1 phase cells when stained with mithramycin/ethidium bromide. Cells isolated from two breast tumors show different percentages of aneuploid cells (Fig. 13.16b, c). The occurrence of aneuploid cells can in some instances be useful as an early marker of tumour formation. The number of aneuploid cells is expressed as the ploidy index (PI) where:

$$PI = \frac{\text{(peak channel of aneuploid population)} \times 2}{\text{peak channel of diploid population}}$$

13.7. Mitotic wash-off

When cells attached to a solid surface divide, they round-up and as a consequence become less firmly attached. The mitotic cells can be washed free of the surface and the attached (non-mitotic) cells. The strength of attachment of mitotic and non-mitotic cells varies depending on the type of cells and therefore the conditions required to wash the mitotic cells free can vary according to the cell type. In some cases simple swirling of culture over the cell layer is sufficient whereas in other cases incubation with proteases such as trypsin is required (Adams, 1980). Adams also describes the methods than can be used to obtain synchronous cell cultures that do not involve separation of cells.

Appendix

Suppliers' addresses

Albright and Wilson Ltd.,
Marchon Division,
Whitehaven,
U.K.

American Cyanamid Co.,
Stamford,
CO.,
U.S.A.

Beckman Instruments
International SA.,
17 rue des Pierres-du-Niton,
PO Box 76,
1211 Geneva 6,
Switzerland.

Beckman Instruments Inc.,
Spinco division,
1050 Page Mill Road,
PO Box 10200,
Palo Alto,
CA. 94304,
USA.

Becton Dickinson Laboratory
Systems (BDLS) Europe,
Antwerpsesteenweg 259,
B-2800 Mechelen,
Belgium.

Bender and Hobein GmbH,
Postf. 150229,
Lindwurmstrasse 71,
8000 Munchen 15,
W. Germany.

Biorad Laboratories,
Caxton Way,
Holywell Industrial Estate,
Watford,
Herts.
WD1 8RP.
U.K.

Bio-Sep,
872 Autumn Drive,
Walnut Creek,
CA 94598,
U.S.A.

Bioshef,
Department of Biochemistry,
University of Sheffield,
Sheffield,
S10 2TN.
U.K.

Biotech Instruments Ltd,
183 Camford Way,
Luton,
Bed,
LU3 3AN,
U.K.

CJB Developments Ltd,
Airport Service Road,
Portsmouth,
Hampshire,
PO3 5PG
U.K.

Coulter Electronics Ltd,
Northwell Drive,
Luton,
Beds,
LU3 3RH,
U.K.

Coulter Electronica Inc,
590 West Twentieth Street,
Hialeah,
FL 33010,
U.S.A.

De Koningh BV,
Postbus 347,
6800 AH Arnhem,
The Netherlands.

Du Pont Company,
Diagnostic and Bioresearch
Systems Division,
SORVALL Centrifuges,
Wilmington,
Delaware 19898,
U.S.A.

Dynal A.S.,
P.O. Box 158,
Skøyen,
N-0212 Oslo 2,
Norway.

Dynal Inc.,
1 Executive Drive,
P.O. Box 1568,
Fort Lee,
NJ 07024,
U.S.A.

Dynal,
c/o Bio-services,
Suite 2,
Station House,
24 – 26 Grove St.,
New Ferry,
Wirral L62 5AZ,
U.K.

Ferofluidics,
Burlington,
MA,
U.S.A.

Medilog bv,
Marijkelaan 11,
Postbus 95,
2420 AB Niewkoop,
The Netherlands.

Pharmacia Fine Chemicals AB,
Box 175,
S-751, 04 Uppsala 1,
Sweden.

Shermond Scientific Supply Ltd.,
Shermond House,
80 Stoneham Road,
Hove BN3 5HH,
U.K.

Wescor,
459 South Main Street,
Logan,
Utah 84321,
U.S.A.

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