Red Cell Membranes – A Methodological Approach

edited by

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Preface

The last ten years has seen a spectacular increase in biochemical and biophysical methodology, particularly applied to cellular systems. The study of red cell function has benefited from these new techniques, making it possible to investigate membrane properties to a new level of sophistication.

In our own laboratories we have had a number of visitors from clinical, zoological, pharmacological and biochemical backgrounds arrive to learn what seemed to us simple or even trivial techniques applied to red cell transport. Similarly we have visited others and puzzled over published papers to learn techniques which we wished to extend to our particular problem. From this the idea emerged of getting experts to write more extensively about particular methods to include the necessary, and often trivial, comments which are frequently lacking from conventional "methods" sections in published papers. Our aim was to give sufficient information on a particular technique or area to allow workers unfamiliar in that specific field to be able to use it with confidence. For this reason the text often contains explicit details, and should not be considered as a review. However, we hope that sufficient background information is given to put the matter into context.

The subjects covered differ tremendously in the range and depth, certain of them being very specific and limited, whilst others are so broad that the authors have inevitably been selective. We have tried to make contributions complementary, and hope that the inevitable overlap is small. The present work is obviously neither exclusive nor exhaustive, but we hope it may be useful, and that it will encourage the extension of the range of approaches available to the researcher interested in cell biology.

Clive Ellory
Jim Young

January 1982

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1

Cell Separation Techniques

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I. Introduction and Review of Red Cell Separation Methods

Interest in erythroid differentiation and red cell maturation over the past thirty years has led to the development of various methods of separating blood cells according to age. Most of the methods available can be used to separate reticulocyte-rich fractions from mature erythrocytes or the very young from the oldest red cell populations. Some of the methods can provide separation of cells at different stages of development or maturation in a quantitative manner. This article is written to briefly review the methods that have been introduced and are often used to separate red cells. A detailed description of one of the more recent methods available for separating red blood cells on a continuous density gradient is also presented.

Since Key's (1921) observation that reticulocytes are more buoyant than erythrocytes it is now generally accepted that the density of red cells increases as the cells age. Consequently, most age separation methods involve centrifugation techniques which separate cells according to their density. A review of the historical development of these has been provided by Harwood (1974). The most commonly used method is based on prolonged centrifugation of red blood cells (Chalfin, 1956; Rigas and Koler,

1961; Prentice and Bishop, 1965) and Murphy's (1973) modification of this technique has become the most popular in recent studies. The latter method simply involves prolonged high speed centrifugation (39 000 *g*, 1 h) of red blood cells (85% hematocrit) in plasma at 30°C. The use of a fixed angle rotor allowing internal circulation in the tube and separation at high temperature to reduce viscosity makes this the most effective packed cell separation method available. After centrifuging, the top and bottom 5 to 10% of cells are harvested to provide young and old red cell fractions respectively. The method is good for large volume separations of normal red cells with the main drawbacks being long centrifugation time, lower resolution and limited usefulness with some pathological blood samples.

Differential floatation of red cells by centrifuging with a non-aqueous mixture of phthalate esters has also been used to separate red cells into light and heavy fractions (Danon and Marikovsky, 1964; Joiner and Lauf, 1978). Since the specific gravity (SG) of most red blood cells is between 1·085 and 1·110 from youngest to oldest respectively, mixtures of *n*-dibutyl phthalate (SG 1·0416) and methyl phthalate (SG 1·189) can be prepared and used to separate a fraction of either the densest or lightest population of cells. These methods can successfully provide large quantity separations of red cells from each end of the age spectrum. Repeated centrifugation of separated fractions with a series of pthalate ester mixtures can provide increased resolution of density separation but this is more time consuming. In addition, exposure of red cells to the inorganic mixtures can damage them, lead to slight hemolysis and loss of cells.

Isopycnic continuous density gradient methods have been shown to provide a higher degree of separation than other centrifugation methods (Piomelli *et al*., 1967). Since the early sixties, bovine serum albumin was the most frequently used gradient material for this (Lief and Vinograd, 1964; Bishop and Prentice, 1966). However, factors such as batch-to-batch variation, osmolality, viscosity and preparation time led to further experimentation with other gradient materials. As a result, density gradient methods using Dextran (Schulman, 1967; Fitzgibbons *et al*., 1976), Ficoll (Boyd *et al*., 1967; Adams and Neelin, 1972; Rahman *et al*., 1973), Stractan II (Corash, *et al*., 1974; Pfeffer and Swislocki, 1976) and Percoll (Ellory and Wolowyk, 1979; Rennie *et al*., 1979) have been developed. Among these, Percoll is the most convenient to use and a detailed description of its use will be provided.

Other age-separation methods which do not directly depend on cell density have also been reported. Selective osmotic lysis (Marks and Johnson, 1958; Marks *et al*., 1958) is a method based on the fact that young red cells are more resistant to osmotic lysis in hypotonic saline than

old red cells. Thus separation by age is achieved by selective lysis of older cells. The main limitation of this method is that only intact young red cells can be collected, and these cells may be partially damaged by the osmotic insult and subsequent swelling. Gear (1977) describes a preparative electrophoretic method of separating red cells according to surface charge. This method could prove to be a useful means of separating reticulocytes from mature erythrocytes. However, effectiveness in age separation of mature erythrocytes is not clearly established and requires further evaluation in light of recent evidence that erythrocyte surface charge is not a function of cell age (Luner and Szklarek, 1976; Luner *et al.*, 1977). Finally, a counter-current distribution method of separating red blood cells of different ages by repeated partitioning of the cells between a dextran-polyethylene glycol, two phase system has also been described (Walter *et al.*, 1965; Walter and Selby, 1966; Walter, 1977). This process, however, involves a long time, an extensive apparatus and also is claimed to depend on cell charge. Another interesting method which may prove effective is described by Paul *et al.* (1978). The method depends on the magnetic properties of reduced haemoglobin and red cells are separated using a high gradient magnetic field.

II Percol Density Gradient Separation of Red Blood Cells

Various methods for the separation of red blood cells according to age have been described. The specific method used will depend on time, availability of equipment as well as the quantity and degree of separation required. In the following section, a detailed description of a rapid separation method using commonly available laboratory equipment is presented. The technique involves the use of a self-generated continuous density gradient of a new colloidal silica sol, Percoll (Pharmacia). The method is applied to normal and reticulocyte-rich blood samples.

A. Gradient Preparation

An iso-osmotic Percoll stock solution containing NaCl 150 mM and histidine 10 mM (pH 7·55) is prepared by adding 1 part (v/v) of 10 × concentrated histidine buffered saline solution to 9 parts of Percoll. The Percoll stock solution is then diluted to a starting density of 1·10 g/ml by the addition of isotonic histidine buffered saline solution (83 volumes of Percoll stock to 17 volumes of saline). These proportions can be varied to yield gradients that would best correspond to the density separation desired.

The self-generated continuous gradient is prepared by the method of Pertoft and Laurent (1977) by spinning 30 ml of the 1·10 g/ml Percoll solution at 14 000 rev/min (24 000 *g*) for 15 min at 20°C in a MSE centrifuge with a 8 × 50 angle head rotor. The break is kept off to minimize swirling and mixing of the gradient as the rotor decelerates. Since the gradient generated is dependent on the centrifugation conditions used, any modification in volume of gradient material, centrifugation speed, time, or type and angle of rotors used will result in a different gradient. However, modifications to the technique can easily be assessed with the aid of Sephadex density marker beads. The density marker beads are available in a kit (Pharmacia) of ten vials each containing different coloured beads with their buoyant densities calibrated for solutions of Percoll (the actual colour and density of beads may differ from those used in this article). A small quantity of beads in the desired density range is mixed and washed with distilled water by repeated centrifugation (a bench microcentrifuge being most convenient). Gradient formation is monitored by adding a pinch of coloured Sephadex density marker beads to the control tube. The

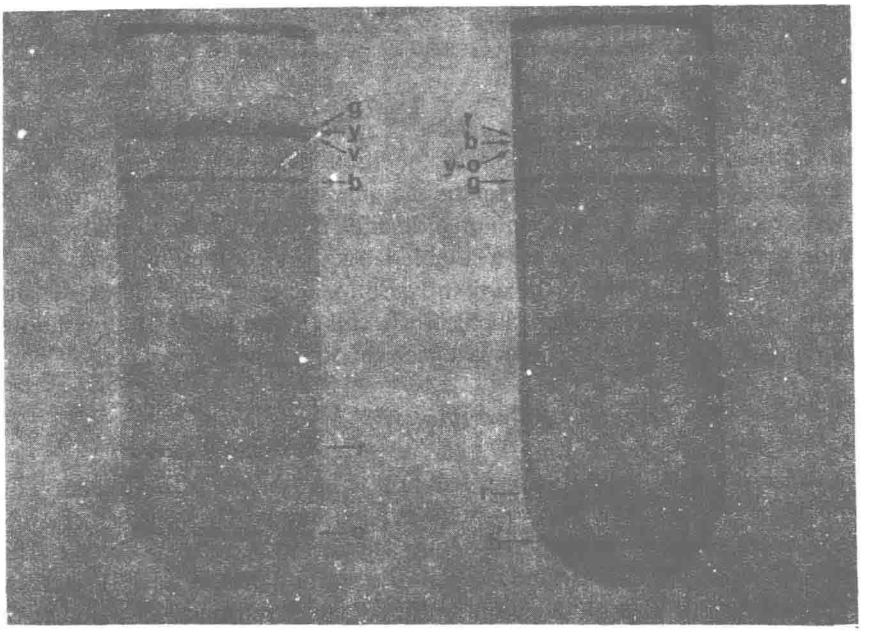

Fig. 1. Self generated Percoll gradients. SG is represented by the coloured density marker beads from top to bottom: Tube A—green (g) 1·036, yellow (y) 1·051, violet (v) 1·060, blue (b) 1·095, red (r) 1·100 and orange (o) 1·133; Tube B—red (r) 1·062, blue (b) 1·075, yellow–orange (y–o) 1·087, green (g) 1·098, red (r′) 1·122 and blue (b′) 1·141.

gradient thus formed provides maximum density separation between 1·087 and 1·10 g/ml (Fig. 1). The pre-formed gradient is stable for extended periods of time at room temperature. An alternative method of preparing a continuous density gradient of Percoll, using a gradient former attached to a peristaltic pump, is described by Rennie *et al.* (1979).

B. Preparation and Fractionation of Red Blood Cells

Heparinized whole blood is washed three times with saline by centrifugation (1350 *g*, 5 min) during which the plasma and buffy coat is removed by aspiration and a final suspension (15 to 20% haematocrit) is prepared with histidine buffered saline solution. Five ml of blood suspension is then carefully layered over the gradient (5 ml of saline on the control gradient) and centrifuged (800 *g*, 10 min) at room temperature, in a bench clinical centrifuge with swing-out rotor. Figure 2 shows the appearance of human red blood cells in the pre-formed density gradient before and after centrifugation.

The cells in the gradient are fractionated by upward displacement with

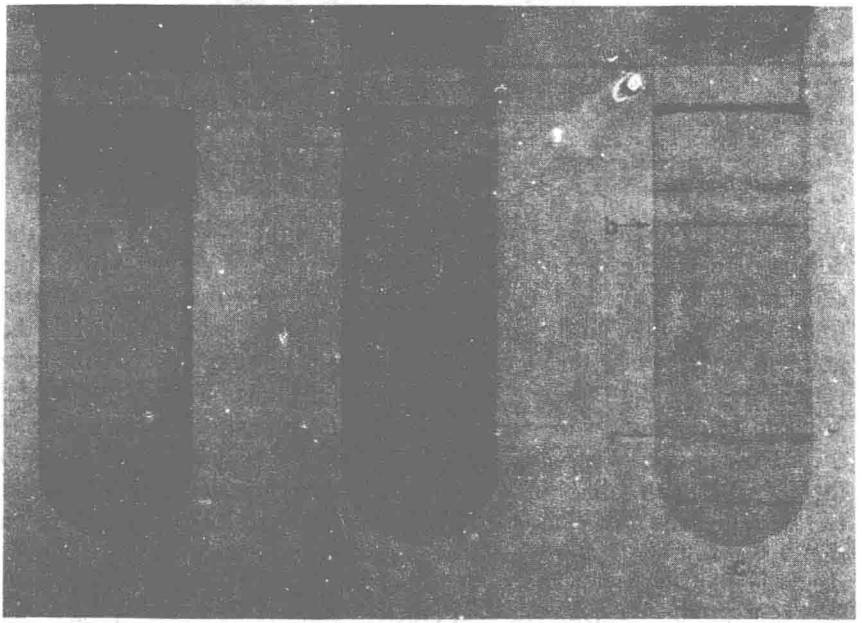

Fig. 2. Distribution of red blood cells before (A) and after (B) centrifugation (800 *g*, 10 min.). Control tube (C) contains density marker beads and is equivalent to tube A in Fig. 1.

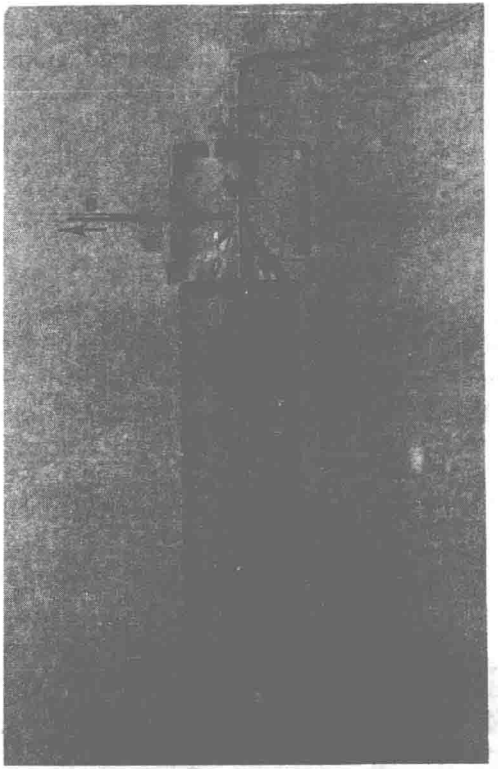

Fig. 3. Details of collecting apparatus for upward displacement of gradient material. Displacement fluid inflow tube (A); Gradient outflow tube (B).

Maxidense (Nyergaard) or a 60% sucrose solution. Figure 3 shows the details of a simple apparatus which is tightly fitted to the top of the centrifuge tube for collecting the fractions. Maxidense is slowly introduced to the bottom of the gradient, by gravity, through the central tube, and the gradient is funnelled through the collecting apparatus and out the side cannula to a standard fraction collector. With the aid of a drop counter and previous calibration of the centrifuge tubes, reproducible fractionation of the separated cells is possible. The collected blood fractions are then given three washes with saline to remove the gradient material. The Maxidense can be returned to its reservoir by simply applying a positive pressure on the outflow of the collecting apparatus with a syringe full of air. Other methods that could be used to collect fractions from the gradient tubes are: simple aspiration of fixed volumes (Bishop and Prentice, 1966), tube cutting (Rigas and Koler, 1961) or by piercing the bottom of the tube and allowing the contents to drip out (Winterburn and Batt, 1970).

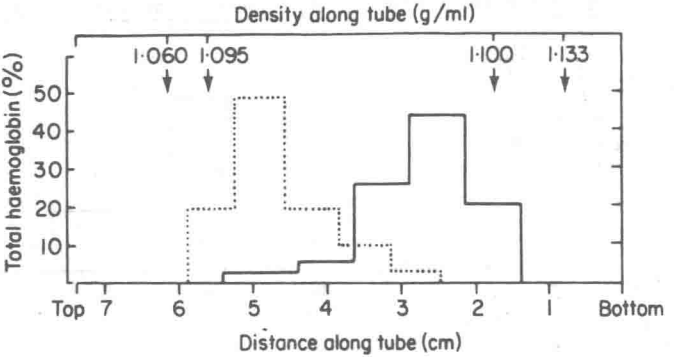

Fig. 4. Distribution of blood cells in Percoll density gradient, reticulocyte-rich blood (.....); normal blood (——). Haemoglobin determined by the method of Dacie and Lewis (1975). Reproduced with permission from Ellory and Wolowyk (1979), *Journal of Physiology*, **295**, p. 9–10.

When reticulocyte-rich human blood is fractionated in a Percoll gradient, good separation of the lighter reticulocytes from normal erythrocytes is obtained (Fig. 4). A shift in the gradient distribution of red cells is also obtained when goat blood is fractionated before and after rendering the goat anaemic by phlebotomy. Electrophoretic mobility measurements on the goat blood fractions (Fig. 5) indicates a significant difference in the charge density of cells in the top fraction from that in the other fractions. This along with the results of Rennie *et al.* (1979) showing that reticulocytes are concentrated at the top of their linear Percoll gradients suggests that the method can be used to separate reticulocytes from mature erythrocytes.

C. Red Blood Cell Age Markers

Age-dependent separation among red blood cell fractions has been confirmed for most of the above-mentioned separation methods by cohort labelling of red blood cells (usually with Fe^{59}) and then following the location of the labelled cells within fractions obtained at different points in time. As the labelled cells age, their position within the density gradient shifts from the top, shortly after labelling to the lower (more dense) fractions.

Along with cohort labelling studies, various physical and biochemical properties of red cells at different stages of maturation and/or senescence have been established and are now often used as markers in determining the degree of age separation achieved. Cohen *et al.* (1976) show a decrease in cell K^+, sialic acid, 2,3-diphosphoglycerate, ATP, cholesterol,

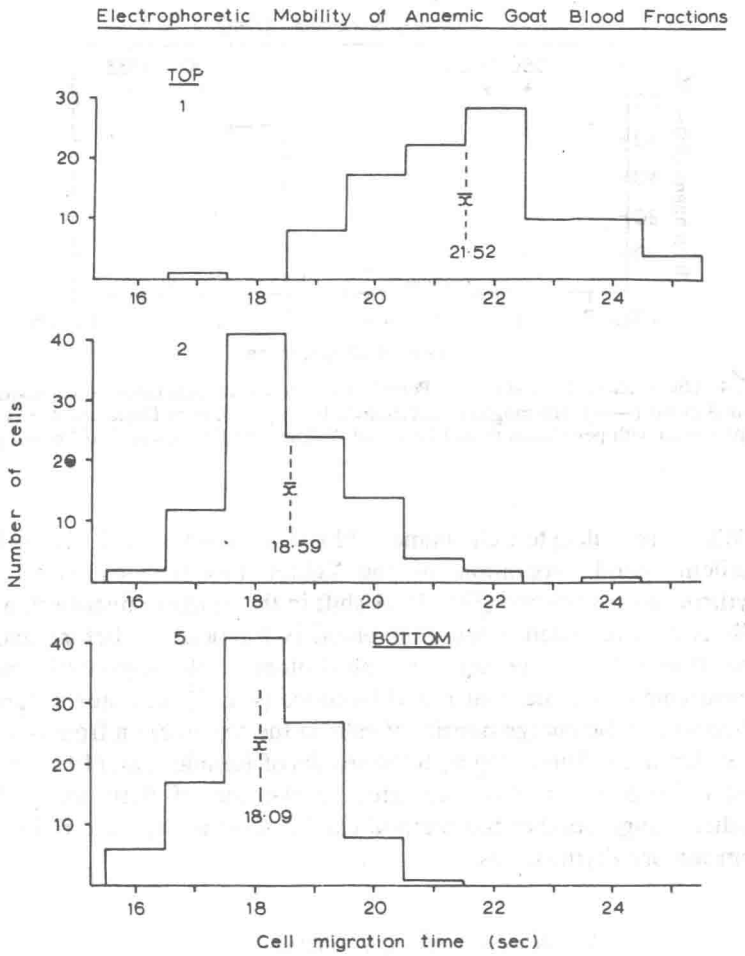

Fig. 5. Electrophoretic mobility of anaemic goat blood fractions measured according to the method of Bangham *et al.* (1958).

phospholipid and mean corpuscular volume as red cells age. Haemoglobin concentration increases, but because of a reduced cell volume, the mean corpuscular haemoglobin does not change with cell age. A number of enzyme activities, such as acetylcholinesterase (Herz *et al.*, 1968; Murphy, 1973; Cohen *et al.*, 1976), glucose-6-phosphate dehydrogenase (Marks *et al.*, 1958), isocitrate dehydrogenase (Turner *et al.*, 1974), glutamate oxaloacetate transaminase (Sass *et al.*, 1964), adenylate cyclase and protein kinase (Pfeffer and Swislocki, 1976) decrease with cell age.

Acetylcholinesterase is one of the most convenient markers to use