DEFORMABILITY AND MICROCIRCULATORY FLOW OF RED BLOOD CELLS IN NEWBORN INFANTS

Submitted by

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DEDICATED TO

MY MOTHER *AIJAZ A. AGHA*

&

MY FATHER *AGHA K.HAIDER*

DECLARATION

I declare that the work described in this Thesis was carried out by me in the Department of Child Health, Royal Hospital for Sick Children, Glasgow and Paediatric Department of the Queen Mother's Hospital, Yorkhill, Glasgow. The laboratory and clinical work was carried out without technical assistance, except where acknowledged in the text.

This work has not been submitted in candidature for any other degree, and is not concurrently being submitted for any degree to other Universities. Parts of the reported work were presented at the Spring Meeting of the Royal Society of Medicine, Forum on Clinical Haemorheology,Scarborough,1989 and at the Annual Meeting of the British Society for Haematology, Cardiff, 1988 and published as abstract in the following scientific journal.

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SUMMARY

Deformability of red blood cells is defined as their ability to deform (ie; to assume new shapes) in response to forces applied on the cell. This is essential for effective blood flow, particularly in the microcirculation, where red blood cells of about 8 *um* diameter flow through 3 to 4 *um* diameter vessels. Thus deformability of red blood cells is an important determinant of flow through microcirculation, of oxygen transport and release to the tissues and also of whole blood viscosity and red cell life span.

Filtration techniques are widely used as models of the microcirculation and for measurement of red cell deformability. These are based on measurements of the flow of suspensions of cells through the filter membranes. The filtration method developed by Jones *et al* (1984 and 1985) was used in this thesis to measure the red cell deformability. The factors other than red cell deformability, which influence the rate of filtration were eliminated so that a sensitive filtration technique for measuring red blood cell deformability was used.

This study describes the fetal red cell filterability measured in varying *in vitro* pH and temperature conditions which are important in newborn infants *in vivo*. The series of experiments performed on blood samples from newborn infants to see the effect of changing pH and temperature showed that the RBCs are sensitive to pH and temperature changes. Filterability of red blood cells is decreased at low pH and temperature when compared with normal blood temperature and pH. The effect of low pH is reversible and possibly correctable.Thus acidosis and hypothermia seen in newborn infants could affect the deformability of red cells and this might be a contributary factor in clinical complications seen in these babies.

Filterability of red cells from fetuses, newborn infants and adults was measured. It was found to be directly related to the gestational age of the infant with no significant difference between red blood cells of gestational age matched fetuses and newborn infants. However, RBCs from healthy newborn term infants showed significantly decreased filterability than those from adult RBCs. Thus the fetuses and infants who were transfused with adult blood showed considerably improved flow properties (four fold increase in filterability with adult

blood transfusion) when compared to gestational age matched untransfused fetuses and infants. The filterability of red cells was negatively related to the mean cell volume (MCV) of RBC. However, multiple regression analysis showed that gestational age of the infant and MCV were independent determinants of filterability. Therefore, apart from their larger size, neonatal RBCs are inherently less filterable than those of adults.

The influence of HbF on the filterability of fetal and neonatal erythrocytes was also assessed. During filtration through 3 *um* pores, cells with a higher proportion of adult haemoglobin emerge from the filter sooner than the less mature cells with a higher proportion of fetal haemoglobin.

Various clinical parameters were correlated with the red cell deformability in newborn infants. Preterm babies with major complications of preterm delivery ie; respiratory distress syndrome and intraventricular haemorrhage when compared with similar babies without these complications showed decreased red cell filterability but the recorded differences were not statistically significant. However red cell filterability was decreased in small for date (SFD) infants compared to gestational age matched appropriate for gestational (AGA) infants.

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

The science of deformation and flow of fluid through channels under the influence of a driving pressure is known as Rheology(Greek,rhein,to flow). Since one of the most important properties of blood is that it flows in the circulatory system of arteries, veins and capillaries to deliver oxygen to the vital organs, it is apparent that the rheological properties of blood play an important role in governing flow dynamics and circulatory transport in health and disease.The study of these properties is called haemorheology.

In recent years, there has developed an increasing interest in haemorheology and this has contributed to a new understanding of the role of blood fluidity as it affects the microcirculation. A variety of haemorheological factors has been uncovered as being of significance in many physiological and pathological flow conditions.

Definition of viscosity

All fluids resist attempts to alter their shape to a greater or lesser extent and this resistance to flow is the measure of fluid viscosity. During flow, as the layers of fluid move parallel to each other at different rates, a velocity gradient is formed between these layers and is known as shear rate (s^{-1}). The force required to produce this velocity gradient is called shear stress (Nm^{-2}). The unit for measuring shear rate is reciprocal seconds (S^{-1}) and the Systeme Internationale (SI) units of shear stress are pascals (Pa); one pascal equals one newton per square metre ($N.m^{-2}$). Due to the small shear stresses involved in haemorheology, millipascals (mPa) are used rather than pascals. The SI units of viscosity are millipascal-seconds (mPa.s); one millipascal-second is equal to one centipoise (cP) in centimetre-gram-second (c.g.s.) units.

Viscosity of the fluids (η) may thus be defined as the ratio of shear stress to rate. The unit of viscosity being pascal seconds (Pa.s).

$$\text{Viscosity (mPa.s)} = \frac{\text{Shear Stress (mPa)}}{\text{Shear rate } (S^{-1})}$$

According to Hagen-Poiseuille equation,the flow rate of fluids through narrow tube depends on the pressure gradient (driving pressure, P) along the tube;the length and radius of the tube (l and r); and the viscosity of the fluid (η):

$$\text{Flow rate} = \frac{\Delta P \times \pi \times r^4}{8 \times l \times \eta}$$

In his *Principia* of 1686, Sir Isaac Newton hypothesized that the shear rate in a fluid was directly proportional to the shear stress ie; the viscosity was constant regardless of the flow conditions. Newtonian fluids are thus defined as fluids with constant viscosity despite different shear conditions. Water and plasma are Newtonian fluids. In non-Newtonian fluids, viscosity varies with shear conditions. Shear rate increases with flow rate and with decrease in vessel diameter.

1.1 . Blood Viscosity

Whole blood, which is a mixture of plasma and blood cells, is a non-Newtonian fluid. Hence as the blood flows from wide bore/high pressure arteries to narrow bore capillaries and to wide bore/low pressure veins there are large changes in shear rate and concomitant changes in

whole blood viscosity. Vessel diameter not only influences the shear forces within the blood,but also requires deformation of individual red and white blood cells when it decreases below the cell diameter(6 to 8 um), as in nutritive capillaries:this also greatly alters the flow resistance of the blood.

The major determinants of blood viscosity are plasma viscosity, red cell concentration, red blood cell aggregation, red blood cell deformation, the number of leucocytes and their flow properties.

1.1A. Plasma Viscosity:

This is dependent mainly on the concentration of plasma proteins, especially high molecular weight proteins, ie., fibrinogen and some globulins. In normal plasma ,albumin contributes only 36% of the difference between plasma and water viscosity, although it comprises 60% of total plasma protein by weight. This is due to its low molecular weight and relative symmetry. Serum globulins have a greater effect, especially the larger macroimmunoglobulins. Fibrinogen contributes 22% of the plasma water viscosity difference, although only 4% of the total protein by weight (Lowe, 1987a). Plasma viscosity increases linearly with increasing total plasma proteins

and plasma fibrinogen concentration. Total plasma protein, concentration of individual plasma proteins and plasma viscosity are very low in preterm infants, increase with increasing gestational age and reach the highest values in adults (Linderkamp *et al*, 1984b).

Table 1.1

Plasma Protein Data from Linderkamp *et al* (1984b)

	Neonates			
	a) Preterm	b) Preterm	c) Term	d) Adults
Gestation age (wk)	24-30	31-36	38-41	
Number (n)	10	10	10	10
TotalPlasma Protein (g/dl)	4.5±0.4	5.1±0.5	5.8±0.6	7.4±0.8
Plasma Fibrinogen (mg/dl)	171±52	233±54	252±60	346±55
Plasma Viscosity (cp)	1.04±.06	1.16±.08	1.25±.09	1.46±.11

1.1B. Red cell concentration or Haematocrit:

In the normal infant red blood cells (RBC) constitute the largest percentage of blood cells and the haematocrit is the major determinant of blood viscosity. As the haematocrit rises, the increase in blood viscosity accentuates (Chien, 1981). Oski and Naiman(1982) have shown that neonatal polycythaemia (defined as a haematocrit over 0.65 g/l) is the main cause of hyperviscosity syndrome in newborn babies. Developmentally, the haematocrit increases from 0.33/l at the 12th week of gestation to 0.45/l at the 28th week (Walker & Turnbull, 1955) and 0.52/l at full term (Guest & Brown, 1957). A postnatal haematocrit of 0.45-0.65/l is considered normal in infants (Linderkamp, 1987). In adults the normal value is 0.40/l - 0.60/l. Table 1.2 shows the haematological data observed in preterm and term infants and adults.

Table 1.2: Haematological Data for blood from Preterm and Term Infants and Adults. (Linderkamp *et al*, 1986b)

	Neonates (wks of gestation)			Adults
	24-30w *n*=10	31-36w *n*=10	38-41w *n*=10	*n*=10
Hematocrit(%)	43.5± 2.2	48.0±2.8	49.4±4.7	44.3±3.1
MCV (fl)	123.2±10.1	117.2±7.8	107.8±5.7	90.1±4.9
MCH (pg)	40.5± 2.6	38.5 2.2	35.7±1.9	29.9±1.9
MCHC (g/dl)	32.6± 1.7	32.9±1.5	33.0±1.5	33.2±1.3
HaemoglobinF(%)	92.4± 6.7	85.7±6.1	70.3±8.2	0.8±0.4
Reticulocytes (% of RBC)	11.1+ 4.4	6.8±2.9	3.2±1.4	1.1±0.3
Nucleated RBCs (% of WBC)	70.3±56.7	18.7±13.8	6.3±2.8	0.0±0.0

1.1C. Red Cell Aggregation

Fibrinogen and some globulins can be adsorbed onto red cell surfaces and bridge adjacent RBCs to cause their aggregation (Chien, 1975;Schmid-Schonbien, 1976). The normal aggregation process requires contact of more than a second between adjacent red cells and therefore occurs

only during blood stasis or at low shear rate (Linderkamp, 1987). Red cell aggregation increases with increasing gestational age (Linderkamp *et al*, 1984a) and is less in neonates than in adults (Schmid-Schonbien *et al*, 1973; Linderkamp *et al*, 1984a). Decreased red cell aggregation, in general correlates with the fibrinogen concentration in newborn infants. However, preterm infants tend to have weaker red cell aggregation than full term neonates at similar fibrinogen concentrations. A special type of fetal fibrinogen is found to be contributing to the decreased aggregation of these neonatal red cells (Rampling, 1985). It is characterized by an increased sialic acid concentration relative to the normal adult fibrinogen. The increased change due to sialic acid is thought to reduce the interaction between fibrinogen in pretern infants and their red cell membranes (Rampling *et al*, 1984).

Linderkamp *et al* (1984a) have shown in cross-suspension studies (neonatal red cells in adult plasma and adult red cells in neonatal plasma) that neonatal and adult red cells have the same aggregation patterns when they are suspended in the same plasma. Moreover, neonatal and adult red cells demonstrate the same strong aggregation when they are suspended in high molecular weight dextran. This shows that specific plasma properties are responsible for the decreased red cell

aggregation in neonates, while specific neonatal red cell properties do not affect aggregation.

1.1D. Red Blood Cell Deformability:

The red cell, which consists of a haemoglobin rich fluid surrounded by a flexible membrane, is highly deformable (Chien, 1981). It is this property of the erythrocyte which enables it to pass through vessels smaller than its own diameter. Arterial blood flow in the macrocirculation is also facilitated by the deformability of erythrocytes since they can adopt an ellipsoid shape and rotate (tank-tread) their membranes in response to the high extrinsic shear stress (Schmid-Schonbein & Gaehtgens , 1981).

Deformability of red cells is an important determinant of blood flow in the microcirculation, of oxygen transport and release to the tissues and of the red cell life span (Delobel *et al*, 1981, Kon *et al*, 1983; Linderkamp *et al*, 1986c). No significant difference in deformability between red cells from fetuses, preterm and full term neonates and adults was found when cells were studied under defined shear conditions in rheoscope (Linderkamp *et al*, 1986a), and ectacytometer (Coulombel *et al*, 1982) or viscometer (Linderkamp *et al*, 1981 and 1984b). On the other hand, red cell deformability when assessed by

filtration techniques, showed that the filtration rates of red cells are markedly lower in full term neonates than in adults (Gross & Hathaway, 1972; Tillman *et al* 1981; Reinhart *et al*, 1985). The red cells of preterm infants show even lower filtration rates than those of full term infants (Holland *et al*, 1985; Linderkamp *et al*, 1986b).

1.1E. White Blood Cells and their flow properties:

White blood cells have negligible effects on bulk blood viscosity but have important effects on blood flow in narrow vessels, due to their higher internal viscosity and their adhesiveness when activated (Lowe, 1987a). Neonatal blood contains more leucocytes and more immature granulocytes than adult blood. In small preterm infants, this count is even higher (Linderkamp *et al*, 1986b). White cells have similar diameters to red cells (6.2-7.5 *u*m) but being spheres have twice their volume and are therefore much more rigid, due to their rigid nuclei and granular cytoplasm. The adhesive and poorly deformable white cells can have important effects on microcirculatory blood flow (Chien, 1988). An abnormally high leucocyte count could lead to hyperviscosity states eg; leukaemias.

This thesis will be concerned largely with the contribution of red cell deformability to the flow properties of blood in circulation. There follows a

summary of the clinical conditions , in particular important perinatal and neonatal problems which are associated with the altered rheological factor.

1.2. Abnormal Rheology in relation to Perinatal and Neonatal Disorders:

It has been demonstrated that fetal development is dependent on the uteroplacental blood flow. When this flow is reduced, fetal development is impaired. Kaibara *et al* (1985) found a positive correlation between erythrocyte filterablility of maternal blood and birth weights of infants. Reduced maternal erythrocyte filterability has been observed in pregnancies with intrauterine growth retardation (Thorburn *et al*, 1982; Buchan 1984a).

1.2A. Intrauterine Growth Retardation:

The two most readily identifiable groups of infants born in a growth retarded state are those born to mothers with pre-eclampsia and to those who are heavy smokers during pregnancy.

a) Pre-eclampsia: Pre-eclampsia has a complex aetiology but from the fetal point of view, is seen as a condition of reduced blood flow in the placental

microcirculation. There is an increase in the blood viscosity and a reduction in erythrocyte deformability in pre-eclamptic women (Thorburn *et al*, 1982; Buchan, 1982) as well as in the babies from such mothers (Buchan, 1984a). Furthermore, these infants have a lower birth weight than gestation matched healthy controls (Buchan, 1984a).

b) Smoking in Pregnancy: The association between cigarette smoking in pregnancy and fetal intrauterine growth retardation is well established (Pirani, 1978). The haemor heological changes seen in adult smokers include an increased haematocrit, plasma fibrinogen, plasma viscosity, whole blood viscosity and red cell aggregation (Dintenfass, 1975). Buchan (1983), demonstrated lower erythrocyte deformability values in smoking mothers. Infants born to smoking mothers have a higher haematocrit and whole blood viscosity, and a lower erythrocyte filterability and mean birth weight (Buchan, 1983).

1.2B. Infants of Diabetic Mothers (IDM):

Pramanik & Mohandas (1984) suggested that RBCs of infants of diabetic mothers are less deformable than that of normal infants. The biochemical basis for this is an alteration in the lipid bilayer, associated with increased cholesterol and cross-linking of lipids in the red blood

cells of infants of diabetic mothers. Such red cells have membranes with decreased elasticity (Linderkamp and Meiselman, 1982). The haematocrit and plasma viscosity of these infants may be increased (Violaris *et al*, 1986). Hyperviscosity resulting from increased haematocrit, increased plasma viscosity and decreased red cell deformability may contribute to the high risk of thrombo-embolic complications to these infants (Linderkamp, 1987).

1.2C. Neonatal Jaundice following the use of Oxytocin in labour:

A relation between oxytocin administration in labour and the subsequent development of neonatal jaundice is well documented (Beazley and Alderman, 1975; Chalmers *et al*, 1975 and Freidman *et al*, 1978). Various causes are suggested such as enhanced placento fetal transfusion (Oski, 1975) and increased osmotic fragility (Singhi and Singh, 1977). In 1979, Buchan reported that infants born after oxytocin-induced labour, showed clear evidence of increased haemolysis associated with significantly decreased erythrocyte deformability. In *vitro* studies described in this publication, showed a time and dose related reduction in erythrocyte deformability in response to oxytocin. Buchan (1979) suggested that the vasopressin-like action of oxytocin causes osmotic swelling

of erythrocytes leading to decreased deformability and hence more rapid destruction with resultant hyperbilirubinaemia in the neonate.

1.2D. Asphyxiated and acidotic babies:

A decrease in pH causes a significant decrease in the filterability of fetal and adult erythrocytes (Gross and Hathaway, 1972). Buchan (1984b) recorded a greater increase in viscosity and a greater decrease in red cell filterability in fetal blood with falling pH than in adult blood. A linear association between pH and red cell filtration rates has also been shown in cord blood of infants with fetal distress (Buchan, 1984b). Even the stress of a normal vaginal delivery decreases red cell filterability below that of infants born by elective caesarian section (Kaibara *et al*, 1981) . Asphyxiated babies are prone to develop polycythaemia. Polycythaemia and decreased red cell deformability with acidaemia may further increase the risk to the hypoxic fetus of circulatory failure, decreased cerebral perfusion and brain damage.

1.2E. Hyperviscosity Syndrome:

This is a serious condition affecting 1-5% of all infants (Hathaway, 1983; Wiswell *et al*, 1986). Abnormal rheology of neonatal blood may result from polycythaemia,

decreased cell filterability, increased red cell aggregation, changed distribution of leukocytes and raised plasma viscosity. The effects could lead to life-threatening complications such as convulsions, congestive heart failure, respiratory distress, necrotising enterocolitis, acute renal failure and peripheral gangrene, (Hathaway, 1983).

1.2F. Neonatal Septicaemia:

Rivers *et al* (1985) have shown that in serious neonatal septicaemia, red cell deformability may be markedly decreased as a result of membrane alterations and loss of normal membrane elasticity. These alterations are probably caused by endotoxins (Linderkamp *et al*, 1981). Red cell aggregation also increases due to increased plasma macromolecules eg; fibrinogen (Rivers *et al*, 1985). These altered rheological blood properties may contribute to circulatory compromise in septicaemia.

1.2G. Vitamin E Deficiency:

An anaemia secondary to vitamin E deficiency has long been recognised in premature infants (Oski and Barness, 1967). The haemolytic process may be accentuated by medicinal iron and by incubation of red cells with hydrogen peroxide. Lubin and Chui (1982) found decreased filtration rates of red cells from vitamin E deficient infants after

incubation of these cells with hydrogen peroxide. This was associated with the formation of aggregates of high molecular weight proteins and decreased ATPase activity in red cell membranes. These changes which follow peroxidant injury, probably contribute to the shortened red cell survival in vitamin E deficiency state (Lubin and Chui, 1982).

1.2H. Other Microcirculatory Complications in Neonates:

Red cell filterability is found to be impaired in newborn babies, in particular infants born after preterm delivery (Gross and Hathaway, 1972; Reinhart *et al*, 1985; Holland *et al*, 1985; Linderkamp, 1986b). This correlates with short gestation as does intracranial ischaemia/haemorrhage, jaundice, anaemia and necrotising enterocolitis.

The high risk of intracranial haemorrhage in preterm infants may result in part from poor red cell aggregation, impaired passsage of their large red cells through narrow cerebral capillaries and increased blood viscosity (Linderkamp and Betke, 1985a). Irrespective of the cellular pathological causes for the prematurity-related impairment of red cell filterability, this finding may reflect important *in vivo* phenomena: the pathogenesis of

haemolysis, jaundice and circulatory stagnation, thrombosis and haemorrhage in neonatal life may depend in part on this malfunction of fetal red cells after birth (Holland *et al*, 1985).

1.3. Decreased Red Cell Deformability and some important Paediatric Disorders:

1.3A. Iron Deficiency Anaemia:

Iron deficient red blood cells are less deformable than normal cells (Yip *et al*, 1983, Tillman and Schroter, 1980). The diminished deformability of the erythrocytes of these patients is due to an unfavourable ratio of cell surface area to microcytic cell volume (Tillman and Schroter, 1980). Linderkamp *et al* (1985b) found that the surface area of iron deficient red cells was up to 20% larger than that of normal RBCs, the membrane elastic shear modulus was increased by 20% (ie, elasticity was impaired) whereas the time constant for recovery from extensional deformation was shortened by about 20% when compared to normal RBC.

1.3B. Sickle Cell Anaemia:

There is an altered red blood cell shape and decreased deformability both at normal and at reduced oxygen tension in sickle cell anaemia (Stuart & Johnson, 1987).

Irreversibly sickledcells cannot deform at all and therefore plug narrow capillaries. Subsequent red cells are blocked, release their oxygen, become sickled and plug adjoining vessels, thereby initiating a sickle crisis (Linderkamp, 1985c).

1.3C. Thalassaemia:

Tillmann and Schroter (1979) have shown that red cell deformality is diminished in β Thalassaemia. Microcytosis and diminished fluidity of the haemoglobin were suggested to be responsible for the reduced filterability of red blood cells from the β Thalassaemia patients.

1.3D. Some Other Paediatric Problems:

Decreased red cell deformability is recorded in many other conditions, such as advanced liver disease, nephrosis, renal failure, immune haemolysis, intrinsic RBC defects and cystic fibrosis (Gross and Hathaway, 1974).

1.4. Altered Blood Viscosity and some important medical Disorders

1.4A. Diabetes Mellitus:

A number of published studies have reported an elevated blood viscosity associated with diabetes while many other reported studies have often produced conflicting

results. There is general agreement that diabetics have increased levels of plasma and serum viscosity, red cell aggregation, low-shear blood viscosity and high shear blood viscosity at standard haematocrit compared with non-diabetics (Lowe, 1987b). These changes reflect increased levels of fibrinogen and serum acute phase globulins (Barnes *et al*, 1977; Schmid-Schonbein and Volger, 1976). Blood filterability is decreased in diabetics (Schmid-Schonbein and Volger, 1976) and correlates with poor control and with complications (Barnes *et al*, 1977).

1.4B. Cardiovascular Diseases:

The incidence of coronary heart disease is correlated strongly with an increase in the factors that increase whole blood viscosity and there is evidence that this also affects the subsequent prognosis (Dormandy, 1987). Haemor heological abnormalities have also been observed in adult patients suffering from peripheral vascular diseases (Alderman *et al*, 1981) and blood filterability is an important indicator of prognosis in this disease (Reid *et al*, 1976a). The filterability of red cells decreased in patients with cerebro-vascular accidents as compared to controls (Lorient-Roudant *et al*, 1981).

1.4C. Venous Thrombosis:

Lowe (1987b) suggested that the increased viscosity of whole blood is a major factor in the onset of venous thrombosis.

1.4D. Renal Disease:

Decreased red cell filterability is found in chronic renal failure, and this may contribute to the reduced erythrocyte survival and anaemia in these patients (Lowe, 1987b).

1.5. What is Red Cell Deformability?

In order to transport oxygen to the individual cells of the various tissues of the body - the red cell must deform both in major and minor transport channels. The young erythrocyte enters and the old worn-out cell leaves the circulation via sinusoids on the basis of deformation characteristics (Branemark, 1981). Transport via the major vessels and the distribution within the microvasculature compartments of different tissues is strongly influenced by cytorheological phenomena.Figures 1.1 & 1.2 are the artist's impression of the different flow conditions encountered in microcirculation.

Figure 1.1 Different geometries adopted by red blood cells in microcirculation.

1) Optimally deformed bullet shaped RBCs charactistically seen in high speed flow conditions.This allows rapid passage of individual cells down a narrow tube.

2) A reduced flow rate referred to as "granular flow" due to occlusion of capillary by a slowly passing granulocyte. Note the cell-free bolus of plasma seen in front of red cells which gradually gets larger as plasma flows faster than the red cells around the occlusion.

3) Very slow blood flow leaving the red cells collected together with very little plasma.

4) Pre-stasis with rouleaux formation. The pressure due to blood flow behind these red cells could pack them tightly up against the vessel wall leading to complete stasis.

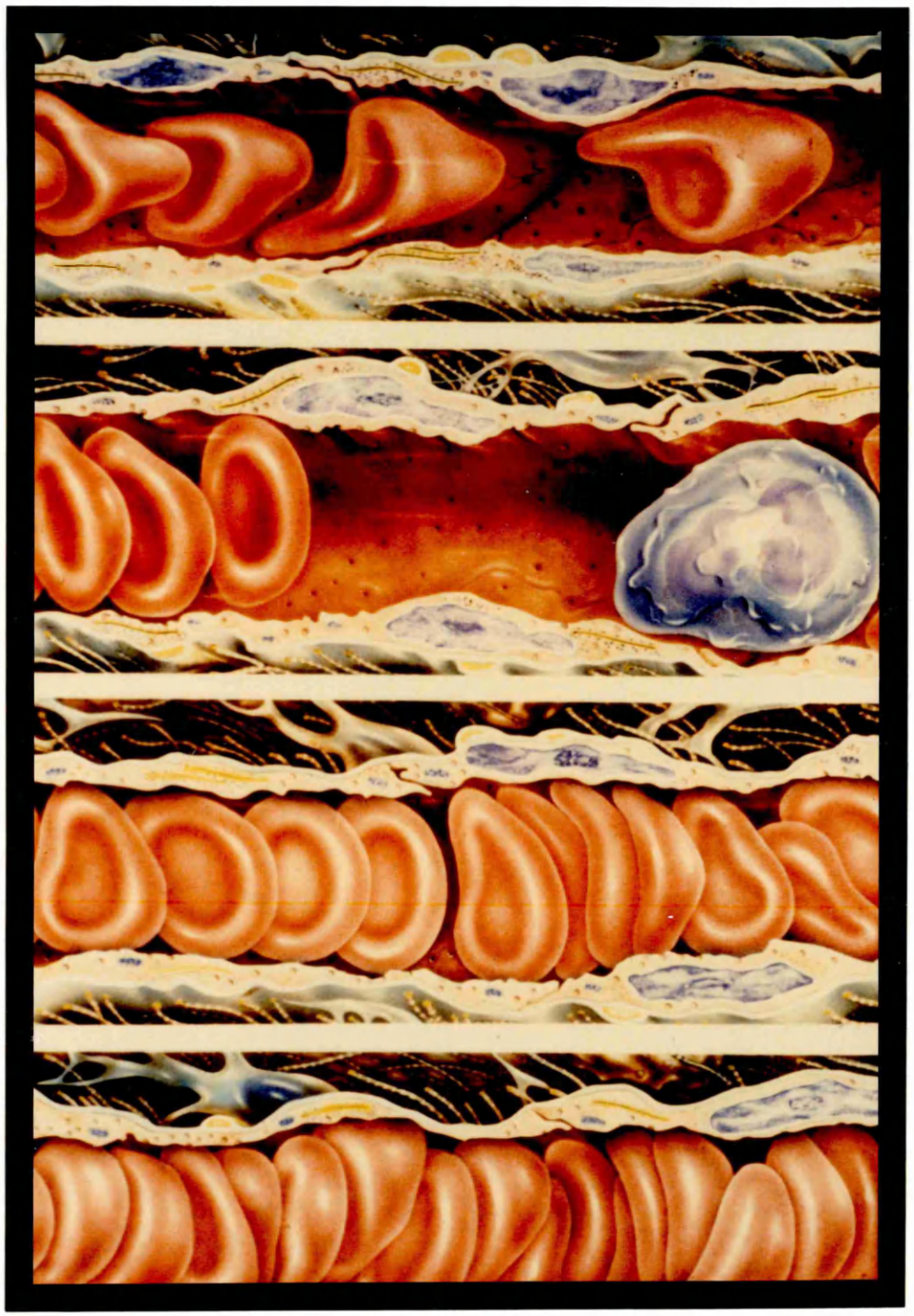
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Figure 1.2 demonstrates different flow conditions within small blood vessels representing:

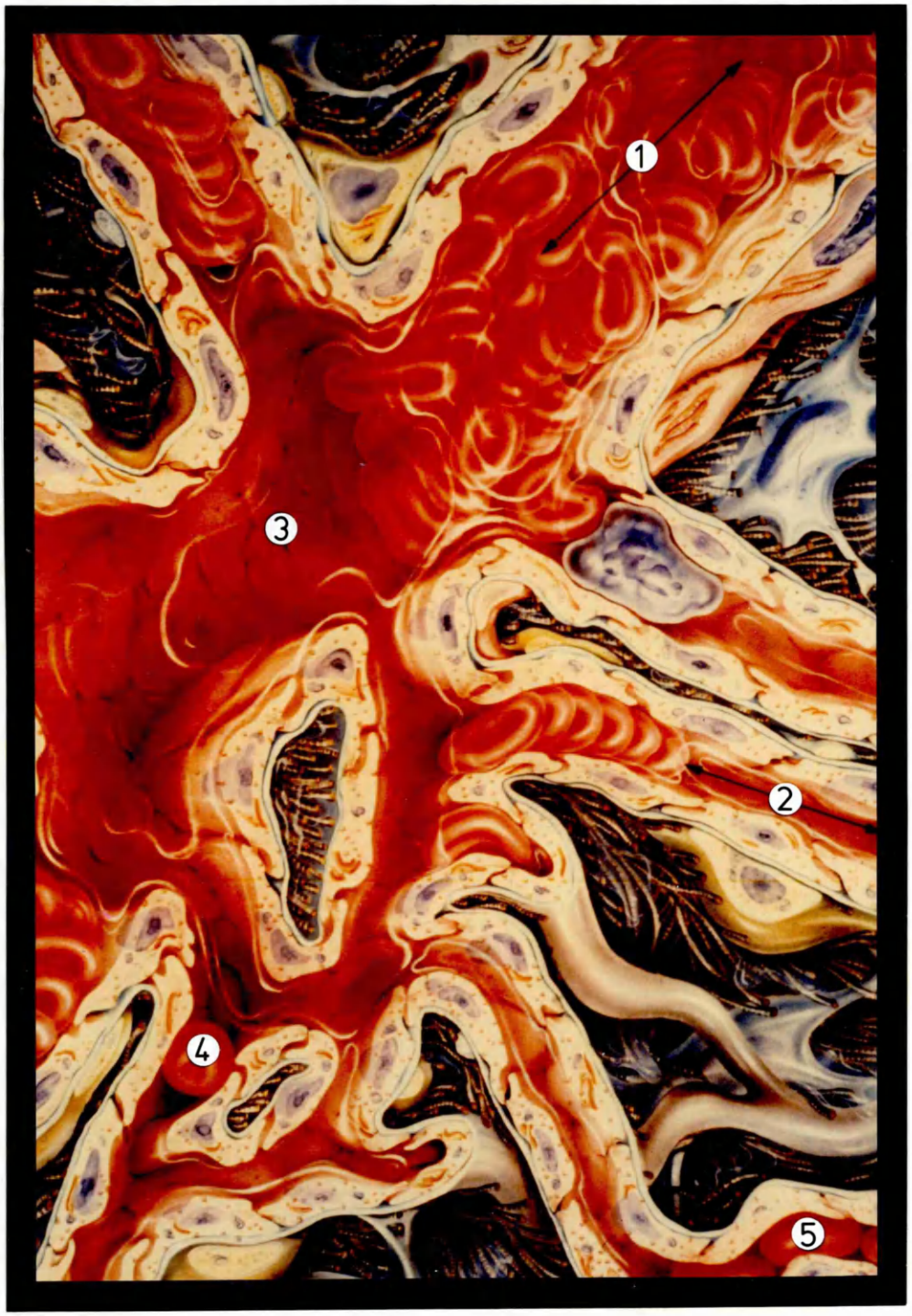
1 - Reticulated rouleaux formation in prestasis.

2 - Granular blood flow.

3 - Cell Free Plasma.

4 - Occlusion of a narrow vessel by a poorly deformable red cell.

5 - Normal cells obstructed by the reduced lumen of the vessel due to swollen endothelial cells.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1.6. Factors Determining Red Cell Deformability:

The deformation of red cells in bulk suspension is influenced by several extrinsic factors eg; shear conditions, haematocrit and viscosity of the medium and by intrinsic factors ie; the internal viscosity, cell geometry and the membrane flexibility (Stuart, 1985a).

1.6A. Intrinsic Deformability of Red Cells:

The intrinsic properties are of primary importance for the red cell deformation in small-geometry vessels.

(a) Intracellular Fluid Viscosity: This is dependent on the concentration of haemoglobin and its physiochemical properties. For normal red cells, the mean corpuscular haemoglobin is approximately 32 g/dl, corresponding to an internal viscosity of about 7 cP. Internal viscosity of the RBC increases non-linearly with haemoglobin concentration, the rise being very steep as haemoglobin is elevated beyond the normal value (Chien, 1981). Filterability of the red cell falls with increasing MCHC (Stuart, 1985a). The relationship between the intracellular fluid viscosity and haemoglobin may change in haemoglobinopathies. For example; deoxygenated haemoglobin in sickle cell disease has a high internal viscosity value at a given haemoglobin, leading to a

reduced red cell deformability. Changes in cell cation and water flux may result in cell dehydration, increased internal viscosity and decreased deformability (Stuart & Ellory, 1987). These changes may be relevant to decreased deformability in sickling disorders (Stuart & Johnson, 1987) and vascular diseases (Lowe, 1987b). Increased internal viscosity and decrease deformability can be due to inclusion bodies, including malarial parasites (Miller *et al*, 1971); nuclear fragments in reticulocytes (Leblond and Coulombe, 1979); Heinz bodies (Lucas *et al*, 1983); globin chain precipitates (Tillman & Schroter, 1983) and postsplenectomy inclusions (Robertson *et al*, 1981).

(b) Red Cell Geometry: The normal red cell has a surface area of approximately 140 um^2 and a cell volume of approximately 90 um^3. Since a sphere with a volume of 90 um has a surface area of only 97 um^2, the normal human RBC has an excess surface area of about 40%, which allows its deformation into a variety of shapes without area expansion. Loss of the biconcave,discocyte shape of the erythrocyte can compromise the advantageous surface area-to-volume geometry of the normal cell (Stuart, 1988). Alterations in shape when secondary to a congenital abnormality of the erythrocyte result in reduced red cell deformability eg; hereditary spherocytosis. Acquired changes in erythrocytes shape that may also adversly

influence deformability include the spur cells of liver disease (Garnier *et al*,1983), the loss of surface membrane during splenic conditioning in hypersplenism (Cooper *et al*,1974), the stomatocytes and macrocytes of alcoholism (Wisloff *et al*,1979) and the burr cells of severe renal failure (Kikuchi *et al*,1982).

(c) Red Cell Membrane: The red cell membrane consists of a lipid bilayer and proteins located on the membrane surfaces as well as extending into the hydrophobic cell. The viscoelastic properties of the red cell membrane renders it easy to bend which makes the cell readily deformable at constant surface area. There is increasing evidence that the lipid composition of the membrane, especially the cholesterol:phospholipid ratio, is a major determinant of membrane deformability and hence whole-cell deformability (Garnier et al, 1985). Alterations of the molecular organisation of the red cell membrane in disease states would lead to abnormalities of its rheological properties (Chien, 1981).

1.6B. Extrinsic Factors:

The degree of deformation is dependent on extrinsic influences on RBC's as well. For example, shear stress which is a product of the shear rate and external fluid viscosity, acting on the cell surface ie; plasma viscosity.

As the diameter of the vessel is reduced to sizes smaller than the red cell diameter, the layer of fluid surrounding the cell becomes progressively thinner, therefore the shear rate in this thin layer and the shear stress acting on the cell surface becomes elevated. A similar increase in the shear stress results in bulk flow. When there is an increase in haematocrit this leads to a greater degree of RBC deformation.

The ability of the red cell to deform in response to an elevation of shear stress leads to an interesting autoregulatory behaviour which prevents an excessive elevation of whole blood viscosity when there is an increase in external fluid viscosity or a decrease in diameter of vasculature or a rise in haematocrit. If the red cells were not deformable, such mechanisms requiring an enhanced RBC deformation due to increased shear stress would not be operative and each of these external disturbances would have caused a much greater increase in whole blood viscosity (Chien, 1981).

When a red cell enters a narrow capillary, pore or micropipette, it is deformed by extension and folding. Four processes are involved in this kind of deformation (Evans *et al*(1984):

1. Cylindrical membrane extension without change in the surface area (resisted by extensional or shear rigidity).
2. Membrane bending and folding of the whole cell (resisted by bending rigidity).
3. Rotation ("tank-treading") of the membrane around the cell content (resisted by membrane viscosity).
4. Shear and displacement of the cell contents (resisted by haemoglobin viscosity).

At low pressure and in narrow channels, membrane extension prevails, whereas at higher pressure and in wider channels, the cell folds. The pressure at which folding begins is important for the entrance of red cell, since the resistance to membrane folding is markedly less than the resistance to membrane extension.

1.7. Measurement of Red Cell Deformability:

Human red blood cells, when subjected to extrinsic shear stress in circulation, are able to deform because of their low cytoplasmic viscosity, high ratio of surface area to cell volume and viscoelastic membrane. Loss of deformability may arise from impairment of any one or a combination of these properties. Similarly, the methods and instruments available for measuring erythrocyte

deformability are sensitive to these properties to different degrees, so that no one type of method is equally sensitive to all aspects of erythrocyte deformability.

Basically, the methods for measuring red cell deformability fall into two main categories. One is the method in which erythrocyte suspensions are sheared in large geometry systems in bulk suspensions and include techniques such as viscometry and laser diffractometry. Other methods are those in which erythrocytes are made to traverse narrow channels eg; micropipettes or filter pores. These techniques measure various ranges of determinants of red blood cell deformability and employ different methodologies for monitoring the resulting deformation. Each method is one way of expressing different aspects of the deformability of the cells under investigation. A standardisation of the conditions of measurement by all techniques has been attempted by the International Committee for Standardisation in Haematology (ICSH, 1986).

1.7A. Viscometry:

Red cell deformability is an important determinant of the whole blood viscosity and measurement of whole blood viscosity can be used as a measure of the red cell deformability if corrected for haematocrit or plasma viscosity. Alternatively, the viscosity of washed cell

suspensions in buffer and standard haematocrit (ICSH, 1986) can be used. Measurements at high shear rate will reflect the internal viscosity of the red cells whereas, measurements at a low shear rate provides information on cell geometry and membrane properties.

1.7B. Laser Diffraction Ellipsometry (Ectacytometry):

The Ectacytometer (Technicon Internation Division, Saint Denis, France) is an instrument for measuring cell elongation under different conditions of flow. It combines viscometry with laser diffractometry. The erythrocytes are suspended in a high viscosity medium (polyvinyl pyrolidone or dextran in buffer) and are subjected to shear forces in a translucent cylindrical viscometer. A helium-neon laser beam passes at right angles through the cell suspension during rotational shear (Stuart, 1985). The pattern of diffracted light is measured by an image analyser to give a value for cell elongation ie; Elongation Index (EI).

In one mode the red cells are subjected to a progressive increase in shear stress to obtain a deformation stress pattern. On a second mode, the cells are subjected to constant high shear stress while the osmolality of the suspending medium is progressively increased. Cell elongation can thus be studied under hypo, iso and hyper-tonic conditions and an osmotic deformability

spectrum generated. This osmoscan plot is sensitive to cell shape, the elasticity of the membrane and the internal viscosity of the cell.

This is a very sophisticated and versatile technique with advantages of a small blood sample and particularly low co-efficient of variation (=1%) for replicate estimations but its high initial cost makes it very expensive for routine use.

1.7C. Rheoscopy:

The deformability of a single cell is observed and measured using a counter-rotating cone-plate Rheoscope. Red cells are subjected to constant shear forces and are photographed with a suitably mounted microscope. The cell deformation (D) is defined as the ratio (L-W/L+W) where L is the projected length of the cell in the direction of flow and W is the width perpendicular to the direction of flow. The D value increases with increasing cellular deformation and is zero in the absence of deformation and the cell appears circular (Linderkamp *et al*, 1982).

1.7D. Centrifugation:

Flexible cells pack easier than hardened cells. This packing of cells at high gravitational forces can be used as a measure of their deformability.

1.7E. Cell Transit Time Analyser:

This instrument measures the flow of 1000 cells through 10-20 pores (5 *um* diameter) in a nuclepore polycarbonate membrane. The Cell Transit Time Analyser measures changes in the electrical conductance when a pore is occupied by a single erythrocyte, the time of passage being longer for less deformable erythrocytes. Measured transit time is then displayed as a histogram.

1.7F. Single Erythrocyte Rigidometer:

The single Erythrocyte Rigidometer (Hoechest Ag, Frankfurt, West Germany) measures the time required for 200 individual erythrocytes to pass through a single pore of 3-6 *um* diameter (Stuart, 1985). The passage through the pore, located in a plastic (Macroford N) membrane is measured by change in the electrical resistance.

1.7G. Micropipette Aspiration:

Glass micropipettes of 1 *um* or 3-4 *um* in diameter can be used to aspirate part of the red cell membrane or all of the red cell, and the aspiration pressure can then be measured or the deformability is estimated by the time taken for entry at a given pressure. Micropipette studies have given useful information on the visco-elastic behaviour of the cell membrane and single cells. The

number of cells that can be examined by this technically demanding procedure is likely to remain small and not suitable for attaining average values for a population of cells.

1.7H. Filtration Methods:

These are the most widely used techniques for estimating red blood cell deformability. The method involves measuring the rate of flow of suspension of red cells through a filtration membrane. The relative importance of cell geometry, internal viscosity and membrane properties depend on the diameter of the pores in the filtration membrane.

The most commonly used filtration systems employ polycarbonate or metal filters with pore diameter 3 to 8 *um* and length about 10 *um*. These systems model flow conditions in nutritive capillaries,whose minimum diameter (approximately 3 *um*) is less than the resting diameter of red or white blood cells (6 to 8 *um*). The filtration methods are easy to perform, are cheap and have the clinical advantage of measuring a relatively large number of cells to obtain a result representative of the mean red cell deformability in the blood sample.

The work described in the present thesis analyses the

deformability of red blood cells using a filtration method. There now follows a brief description of theoretical models used in filtration studies.

1.8 . Theoretical Models for measuring Red Cell Deformability using Filtration Method

Since the development of a simplified filtration apparatus (Reid *et al*, 1976b), the measurement of erythrocyte deformability by flowing blood through 3-5 um diameter pore of polycarbonate membranes has gained considerable clinical importance. In the method described by Reid *et al*, (1976b), whole blood was filtered under a negative pressure and the passage time for a single volume of blood was taken as a measure of flow rate.

A mathematical model was described by Blackshear *et al* (1979), for the flow of cells suspension through membranes, that allowed the calculation of the relative resistance of flow of a suspension to that of buffer at a constant pressure.

In 1981, Skalak described the resistance of cells to flow through Nucleopore Membranes in terms of pressure required to maintain a constant flow rate of cell

suspension and suspending medium. Cokelet (1981), described a similar mathematical model but with different terminology. In this model, the proportion of pores, occupied by a given population of cells or suspending medium, that are evacuated per second is described by a proportionality constant (k) which describes the conductance of the cell or medium throught the pore. The theoretical models described so far, assumed a steady rate of flow of homogeneous suspensions of red cells. But Buchan (1980), and Alderman *et al*, (1981) highlighted the influence of leucocytes on the filterability of whole blood through 5 *um* Nuclepore membranes, using the technique of Reid *et al*, (1976b). They found that the measurement of red cell filterability is very sensitive to the changes in leucocyte concentration and concluded that the pore blocking by the white blood cells should be kept in consideration while measuring red cell filterability.

In 1984 and 1985, Jones *et al* described mathematical models which extended the steady state treatment described by Blackshear (1979), for analysing flow profiles at constant pressure with either 3 *um* or 5 *um* Nuclepore membranes. In this approach, the rate of occupation of pores by particles, (assuming there are two types of particles), from the flowing suspension is given by:-

$$dP_1/dt = k_1 (T-P_1-P_2) - k_2 P_1$$

$$dP_2/dt = k_3 (T-P_1-P_2) - k_4 P_2$$

Where P_1 and P_2 represent the number of pores occupied by type 1 and type 2 particles respectively. T = total number of pores in the membrane. K_1 and k_3 are the proportion of unoccupied pores being occupied every second by type 1 and type 2 particles respectively. K_2 to K_4 are the proportions of pores occupied by type 1 and type 2 particles respectively being evacuated per second.

The rate of flow of medium (Buffer) is given by

$$dv/dt = Q (T-P_1 - P_2)$$

where v = volume of the suspending medium collected at time t second and Q = flow rate of suspending medium through a single pore.

The flow profile of suspensions with one type of particle would be obtained by setting $K_3 = 0$ in the relevant equations. Values for the proportionality constants k_1 to k_4 are obtained by comparing the measured values of v at different times with those predicted by integration of these equations. Reference to computer programmes for achieving these comparisons were given by

Jones *et al*, (1984). These authors concluded that the flow profile of dilute suspensions of washed but unfractionated blood cells could not be fitted to a mathematical model based on the existance of one homogenous population of cells. All flow profiles appeared to be adequately described by a model based on the existance of two populations of particles each with differing flow properties.

All of these mathematical models are basically similar although the approach and terminology differs. A fundamental difference between the various mathematical models is the difference in the theoretical approach. The model described by Blackshear, (1979), Cokelet, (1981) and Shalak, (1981 and 1983) all assume that the number of pores being occupied by the cells and subsequently evacuated is equal to the number of cells that are filtered through the pores. Conversely, Jones *et al*, (1984, 1985) described the calculation of the number of occupation and evacuation events in a given time interval from the observed change in the rate of flow of the suspending medium, presumed to occur largely through the pores that are occupied by the cells. This approach predicts that a number of cells flow through a single pore without significant interruption by the suspending medium. This kinetic phenomenon is visualised as a clustering of cells

around a pore and the calculated size of these kinetic clusters increases with the increasing number of cells in the suspension. The second slower particles probably contain white cells. The results can be expressed as the time taken by a single cell to cross a pore in the filter membrane.

This Transit Time for red cells is calculated by $1/(K_2 \times N)$ where N is the number of cells in a Type 1 cluster. A combination of kinetic analysis and electron microscopic examination of used membranes confirmed that with 5 *um* membranes the slower particles were white cells whereas, with 3 *um* membranes, the slower particles were largely clusters containing less deformable red cells.

All mathematical models can be extended, in theory to include any number of particle types in the suspension. In this way, a good fit between the experiment and theory is expected. However, increasing the complexity of a model can be counter productive when an over complex model fails to provide a realistic physical interpretation.

Kooshesh (1989) described the use of statistical calculations (the theory of runs) for selecting the simplest mathematical model, based on either of the two mechanisms described above, that gives an acceptable fit

between the experiment and theory. This author found a consistantly better fit with the models based on the assumption of clustering and described an automated apparatus for collecting flow profiles of suspensions and an analytical procedure that allowed the calculation of the pore transit time of the red cells which is independent of the number of white cells in the suspension and of the viscosity of the suspending medium. In this thesis the method described by Jones *et al* (1984,1985) and Kooshesh (1989) is applied for recording and analysing the flow profiles of the red cell using the filtration technique for measuring red cell deformability.

CHAPTER 2

MATERIALS AND METHODS

Materials and Method

The work described in this thesis is based on the measurement of filterability of red blood cells using the method developed by Jones *et al* (1984,1985).

2.1 Filters

Nuclepore polycarbonate membrane filters (Nuclepore Corporation, Pleaston, California, USA;Lot numbers 62A2B4, 62A5B30 and 62EOB3) were purchased from Sterlin Ltd., Feltham, England. They have the nominal pore diameter of 3 *um* and pore density of 1 x 10^6 cm. Each membrane has the diameter of 25 mm and thickness of 10 *um*. The filters of 3 *um* size were used throughout the study. The upper surface of the membrane is shiny and the pores are mostly uniform, though some inhomogeneity and pore coincidence is well documentaed (Schmid-Schonbein et al, 1973; Stuart, 1985a). The lower surface of the filter membrane is dull and pores are irregular (see figure 2.1).

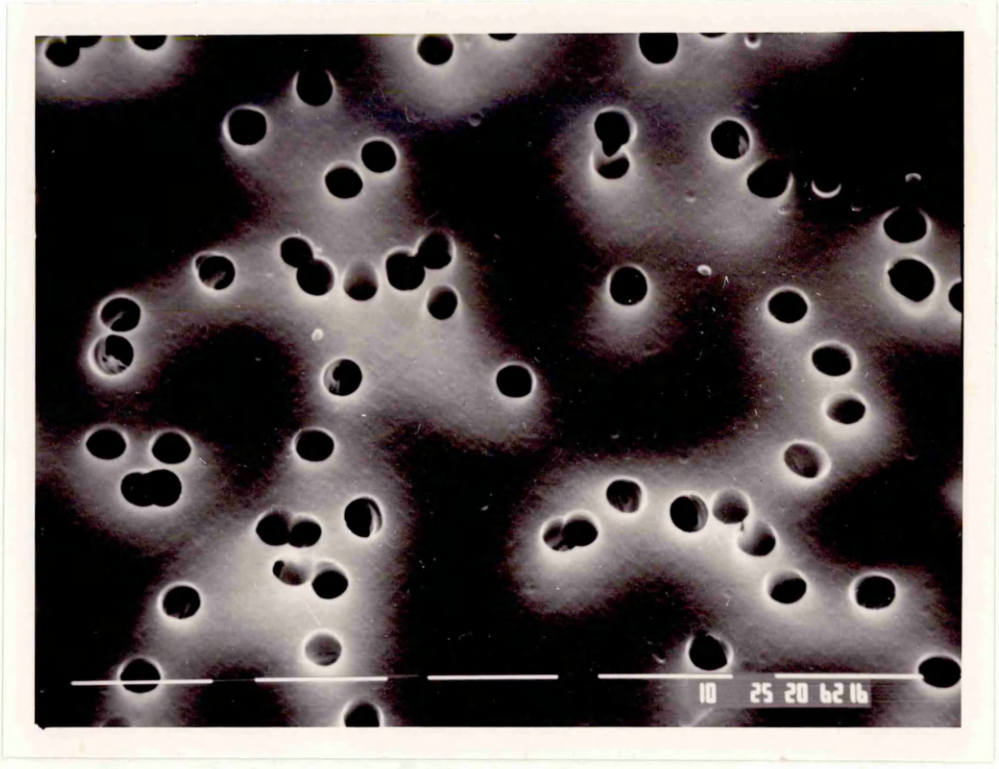


Figure 2.1a *Scanning Electron Micrograph of the upper surface (shiny side) of a 3um Nuclepore membrane filter.*

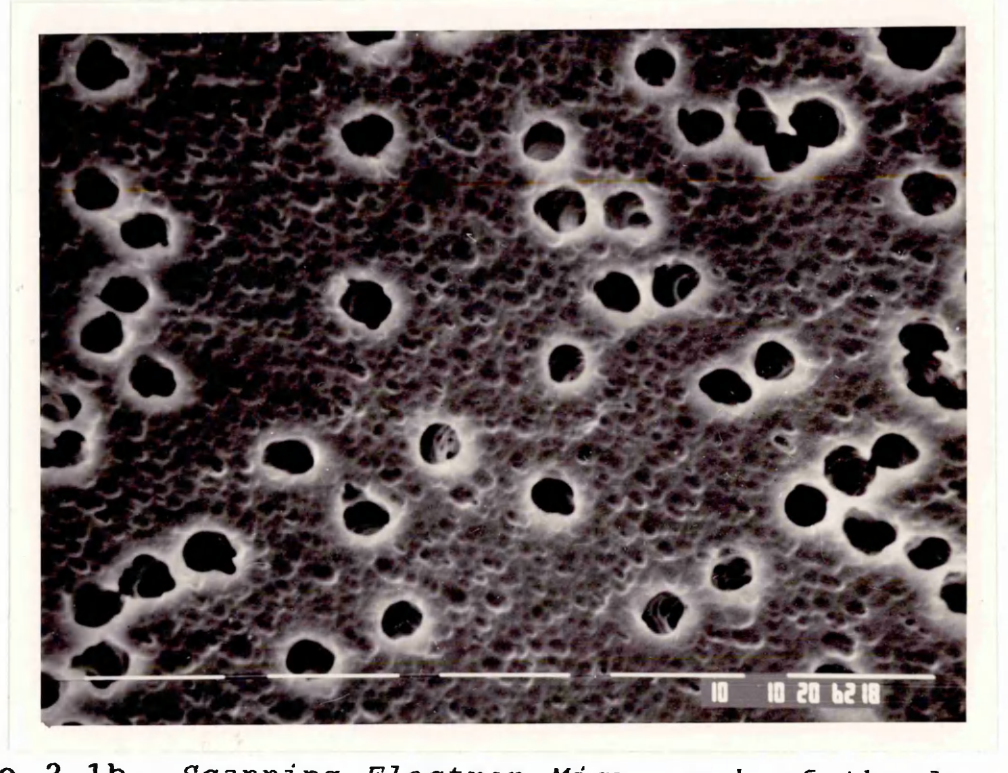


Figure 2.1b *Scanning Electron Micrograph of the lower surface (dull side) of a 3um Nuclepore membrane filter.*

2.2 Chemicals

Phosphate buffered saline (PBS) was used to prepare cells suspensions. It contained 0.12M sodium chloride, 0.02M di-sodium hydrogen orthophosphate and 0.005M Potassium dihydrogen orthophosphate (Besis and Mohandas, 1975). The pH of the buffer was measured using Horiba pH meter (Model M.8L). The pH of this buffer is 7.4 and osmolality 295 mmols/kg. Phosphate of buffered saline solutions made up of 0.02M sodium di-hydrogen orthophosphate and 0.02M di-sodium hydrogen orthophosphate mixed with 0.12M sodium chloride were used to alter the pH of the suspensions. These chemicals were obtained from British Drug Houses (BDH) Chemicals Ltd., Poole, England and were of AnalaR specification.

Filterability of erythrocytes through 3 *um* pores is sensitive to the volume/surface ratio of the cells. Hence factors, such as pH, temperature and buffer composition, that affect the hydration of the cell have to be carefully selected and controlled. The temperature of system was maintained at 37°C throughout the experiments. The buffer used for the work described in this thesis contained 20-30 mM phosphate with an osmolality of 295 mmol/kg adjusted with sodium chloride.

Buffers of different pH were prepared from suitable mixtures of 0.02M NaH_2PO_4/Na_2HPO_4 in 0.12 M NaCl. The volumes required to achieve the required pH values are shown in Table 2.1 .

Table 2.1

Mixtures of sodium phosphate salts required to give buffers of different pH values.

pH	Volume (ml) of NaH_2PO_4 added to 100ml Na_2PO_4
7.0	61.0
7.2	40.0
7.4	24.0
7.6	15.5
7.8	10.0
8.0	6.5
8.6	0.0

2.3 Ultrafiltration Cell

An ultrafiltration cell was manufactured by the Biochemistry Department of the University College, Cardiff. It is an airtight apparatus for filtration of blood, made up of perspex and consisting of two parts. The base, for holding the membrane filter and the upper double-walled portion which holds the cell suspension filled in for filtration. The upper section has an outer closed covering with two connections for a water pump in which water is run to maintain and change the temperature. The inner upper section is fixed over the membrane filter during the experiment. It has a connection on top for the pressure line. The base of the apparatus can be dismantled after each experiment for cleaning and replacing the filter membrane.(see Figure 2.2).

2.4 Study Population and Blood Samples

Fetal and neonatal blood samples were collected with approval of the Ethics Committee,Royal Hospital for Sick Children,Glasgow . Parental consent was also obtained before sampling. Placental blood was collected from the umbilical cord by drip method soon after cord clamping, at the time of delivery .Fetal blood samples were obtained when *in utero* fetal blood sampling was performed

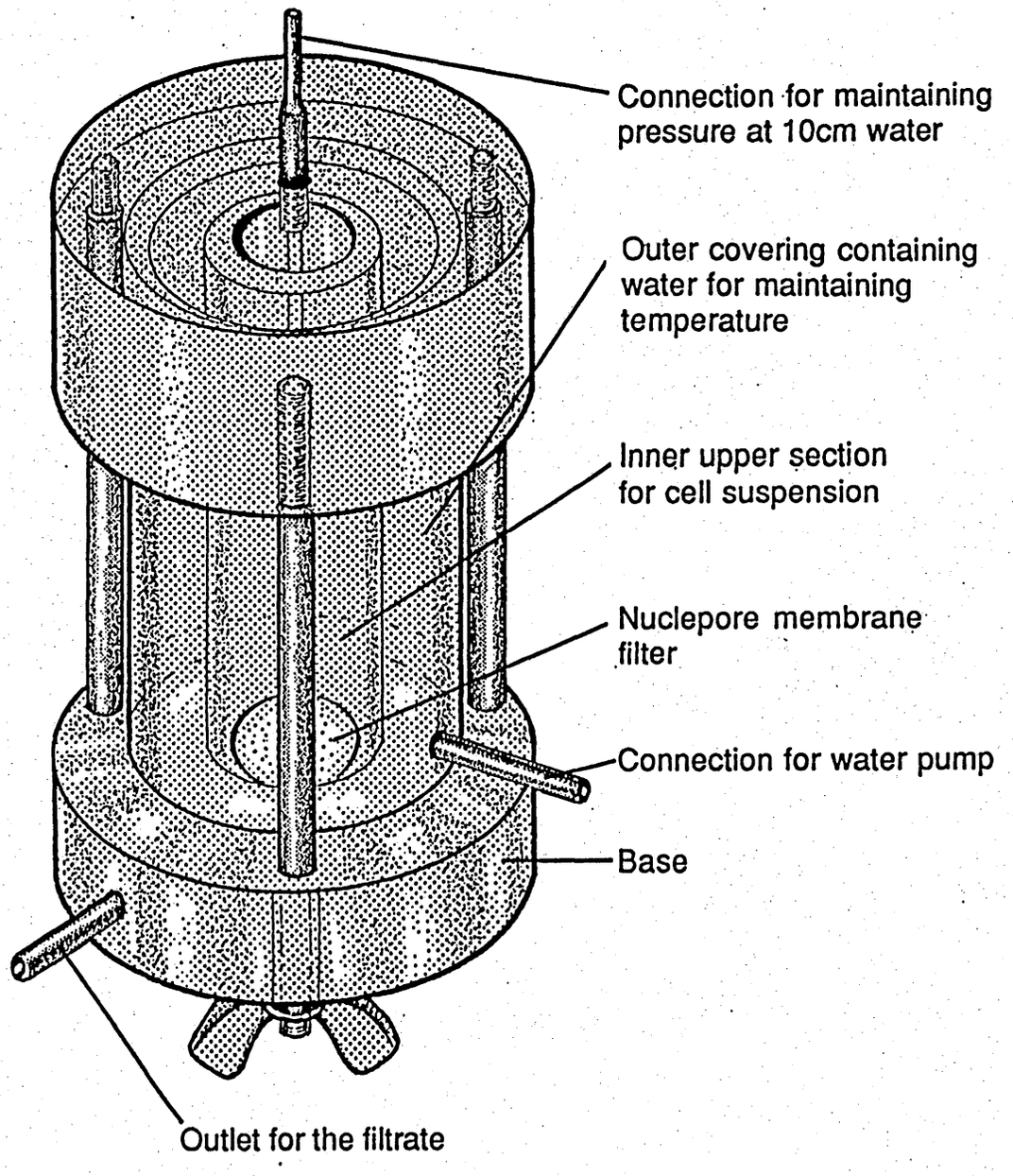


Figure 2.2 *Ultrafiltration cell used to filter red blood cells suspension*

for diagnostic and therapeutic purposes [see Nicolaides & Rodeck (1987) for the details of the procedure]. In other cases, blood was taken from babies either by umbilical arterial catheter, placed for routine intensive care management or by venepuncture when blood was taken for routine diagnostic blood testing. pH of whole blood was measured by means of Corning 178, blood gas analyser. Each sample was anticoagulated with EDTA (Ethylenediaminotetra-acetic acid, 1 mg/ml). Full blood count was performed in Haematology Department by Model S Coulter Counter. Red cell count and haemoglobin values obtained from such count were used for preparing cell suspensions.

2.5 Blood Cell Suspensions

Cell suspensions were prepared by diluting whole blood to a known number of red cells. By using the following formula,

$$N \times V = N' \times V'$$

where N = Number of red cells in blood sample,
V = Volume of blood required for making dilution,
N' = Number of red blood cells required for suspension and
V' = Volume of buffer used for suspension.

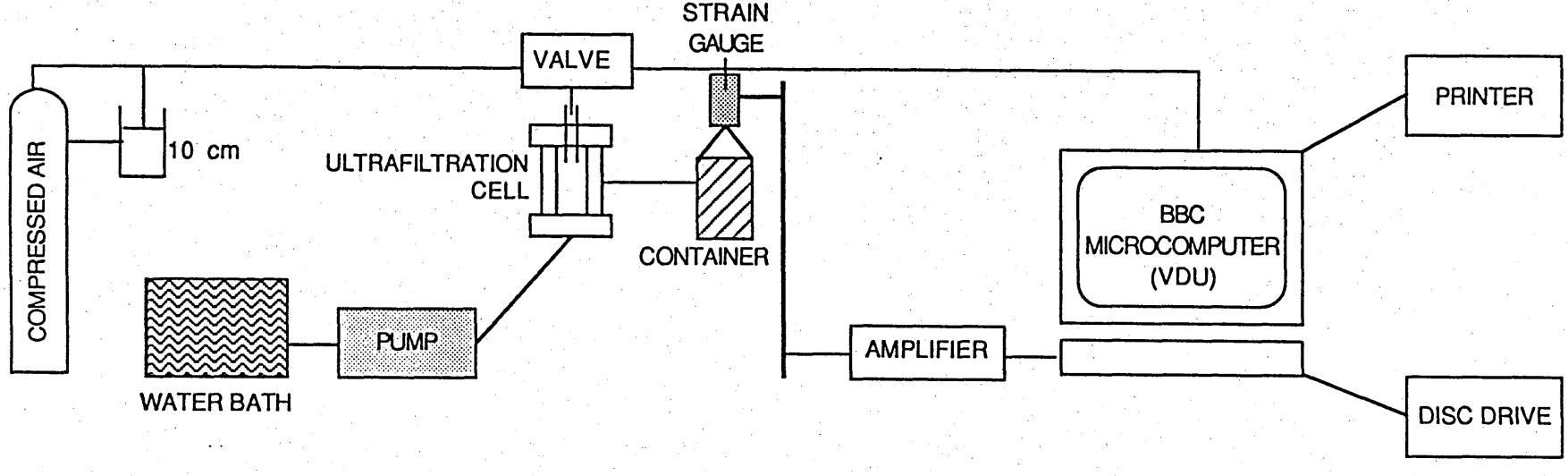
All dilutions were prepared with phosphate buffered saline that had previously been filtered through 0.2 *um* filters (Acrodisc-DLL;Gelman Sciences Inc., Michigan 48106, USA). 10 or 20 mls of suspension prepared in sterile container (Sterilin Ltd., England) contained 30 x 10^6 red blood cells/ml.

2.6 Filtration Assembly

Nuclepore polycarbonate membranes were assembled in the ultrafiltration cell. In this assembly there are 6.3 x 10^6 pores available at the beginning of each experiment. The pressure is kept constant ie., 10 cm of water for the duration of the experiment. Each membrane was calibrated first by measuring the rate of flow of prefiltered buffer through the system. A suspension of the blood cells (volume = 10 ml) was then filtered at the same temperature and pressure through the 3 *um* membrane filter and flow profiles recorded. During the study, babies who had received adult blood transfusion were found to have faster flow rates and ten mls of suspension flowed too quickly to record flow profiles for one minute. In such cases 20ml of suspension was used.

The complete flow profiles were recorded by collecting the eluate into a single container which was weighed continuously by a strain gauge transducer (UFI,

Figure 2.3 *Schematic representation of the apparatus*



Poiden Controls Ltd., Graham Bell House, Roper Close, Roper Road, Canterbury CT2 7EP). The transducer collects, amplifies and feeds the readings into a BBC Master Series Microcomputer. The hardware was manufactured at the University College, Cardiff. The start of the experiment is synchronised with application of positive pressure by the computer through a magnetic valve (H. Kuhnke Ltd., Unit 11A, Lane End Industrial Park, High Wycombe, Bucks. HP14 3JG) installed in the pressure line. A printed copy of the flow profiles is recorded and used for manual entry to the main frame computer for further analysis. The information is also stored on the floppy disc. The information from the disc can be transferred to the main frame computer directly if a kermit link is provided between BBC microcomputer and the mainframe computer. The software for the automated apparatus was developed at the University College, Cardiff. The whole system is represented diagramatically in Figure 2.3 and shown in Figure 2.4 & 2.5 .

In each experiment, a complete flow profile is constructed from recording the mean weight of suspension which is passed through the filter over a 0.1 second interval and repeated every second. The weight of suspension passed through the filter is summated and visually displayed at five second intervals. The time span

Figure 2.4 *Automated apparatus used to measure flow profile of red blood cells.*

A. Water Bath

B. Water Line

C. Filtration Cell

D. Valve

E. Strain Guage

G. Air Line

H. Pressure (10cm of water)

I. Amplifier

J. Disk Drive

K. BBC Microcomputer

L. Air Cylinder

M. Printer


A
B
C
D
E
G
H
I
J
K
L
M

Figure 2.5

Automated apparatus for measuring the flow properties of red blood cells.

A. Water Bath for maintaining temperature at 37° C

B. Water Line

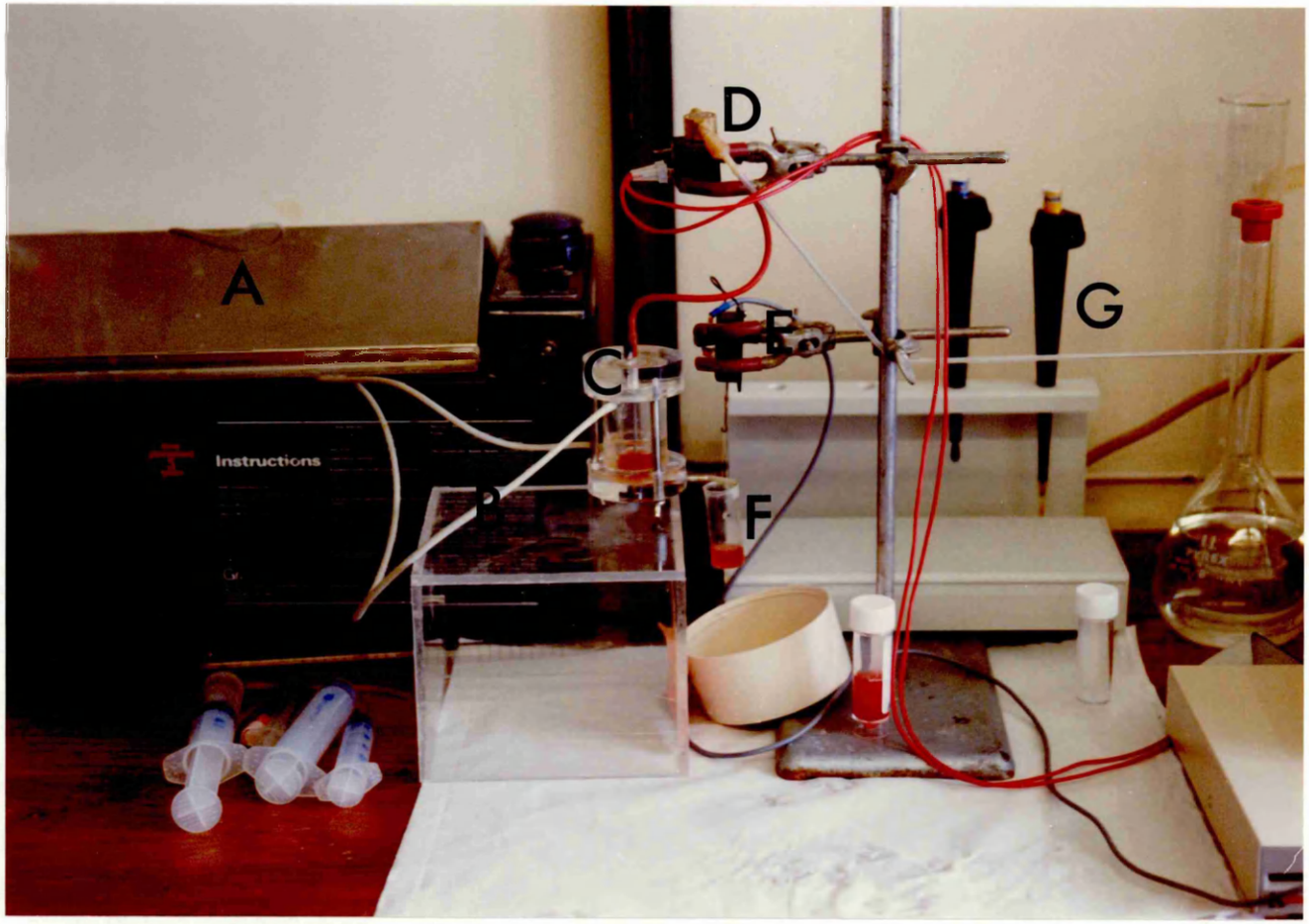
C. Ultrafiltration Cell

D. Magnetic Valve

E. Stean Gauge

F. Container with filtrate

G. Air Line for keeping positive pressure of 10cm water

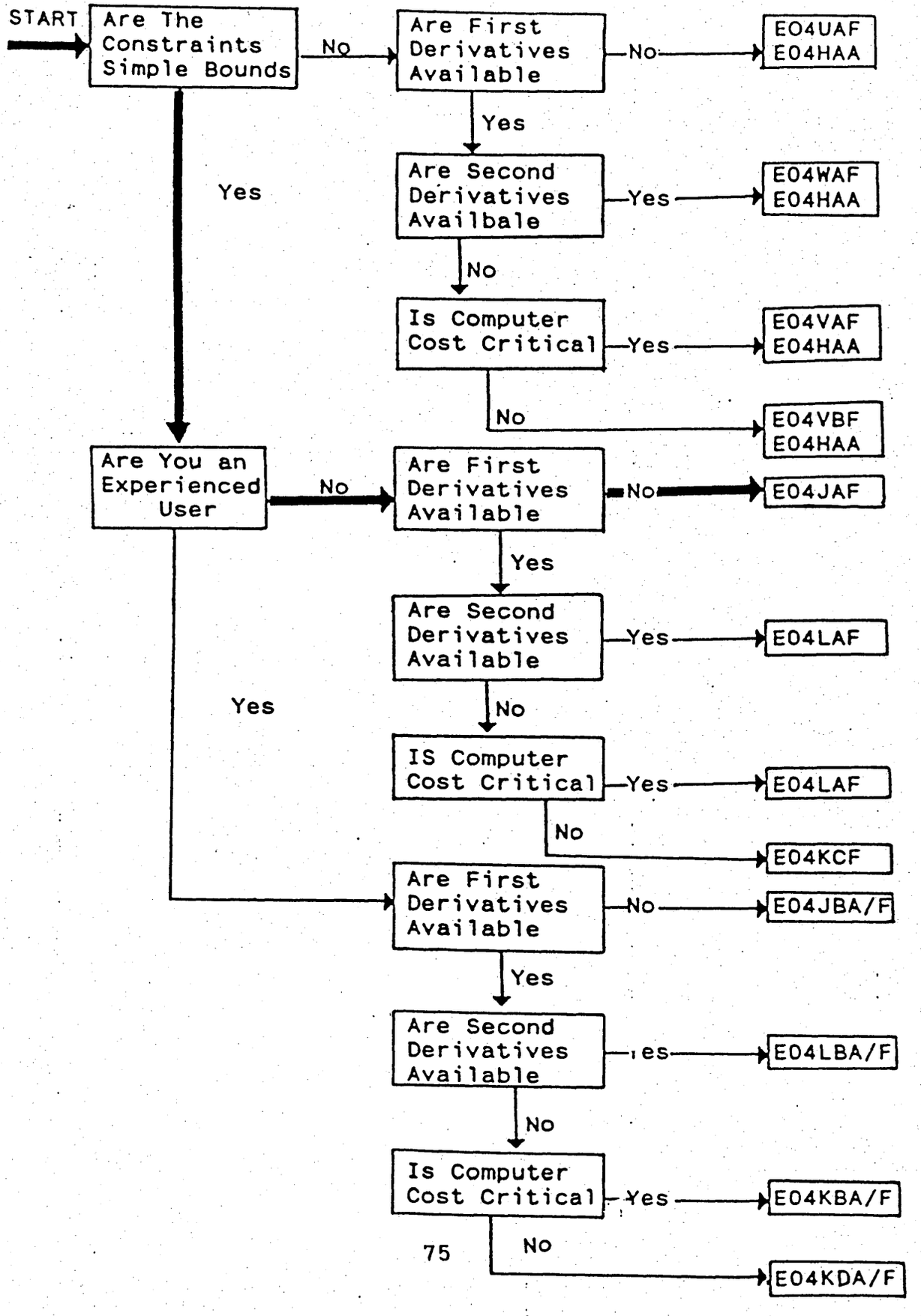

A
Instructions
C
D
E
F
G

for the complete experiment is chosen by the user. The minimum time for recording a flow of suspension is about 0.1 second as the resolution of time sampling is 10 ms.

2.7 Analysis of Flow Profiles

The flow properties of different particles predicted by any mathematical model can be derived by least-squares fitting of the experimental flow profiles to the appropriate set of equations described theoretically for that model. The minimization procedure was obtained form the NAG library (Numerical Algorithms Group Inc., Mayfield House, 256 Banbury Road, Oxford OX2 7DE) and available on the main frame computer. The NAG library is an immense library of algorithms for performing a wide variety of mathematical manipulations. In order to fit a mathematical expression to a series of experimentally determined parameters one requires a routine for minimising the sums of squares of the deviations of theoretical values from experimental measurements. In the method used in this thesis, the equations are based on rate constants that cannot have negative values. Accordingly a minimisation procedure is required that allows the user to place simple bounds on these constants. The choice of algorithm was then directed by a flow chart provided in the NAG-Library literature (figure 2.6).

Figure 2.6 *SELECTION CHART FOR CONSTRAINED PROBLEMS*



The bold line in this figure indicates the route taken that leads to the decision to use routine E04JAF. In order to use the algorithim the user must write a short programme in FORTRAN to read the experimental data, call the NAG subroutine E04JAF and print the results of minimisation procedure and any consequential calculations. The user must also provide a FORTRAN subroutine, to calculate theoretical flow profiles from rate constants provided by E04JAF and calculate the deviations of each value from the experimental one provided from the main programme. The programmes used are the same as described by Jones,(1984) and Kooshesh (1989). The complete algorithm used in the analysis of flow profiles is provided in the appendix to this thesis. These programmes are suitable for translation by any Fortran compiler. For local use, they have been modified for the VME system to allow interactive running.

2.8 Scanning Electron Microscopy

A few nuclepore membranes were examined by electron microscopy before and after filtration of red blood cells. Samples were fixed in 1.5% gluteraldehyde, washed in 0.1M sodium cacodylate buffer and then dehydrated in graded ethanol. They were mounted on aluminium stubbs using silver paints, then dried in a Emscope CPD 750 dryer and sputter

coated with gold in Polaron E, 5000 sputter coater. Microscopic examination was then performed by Jeol T200 scanning electron microscope. Figure 2.7 shows the electron microscopic photograph of the 3 um Nuclepore membrane after the filtration experiment .

2.9 Reproducibility of the System

Eight separate experiments were performed from one blood sample under same conditions. Suspensions were prepared from diluted whole blood, containing 30 x 10^6 red cells/ml. All experiments were done at 37°C temperature, pH of 7.4, pressure of 10 cm H_2O and flow profiles recorded for 60 seconds and analysed by main frame programme.The results are collected in Table 2.2

TABLE 2.2

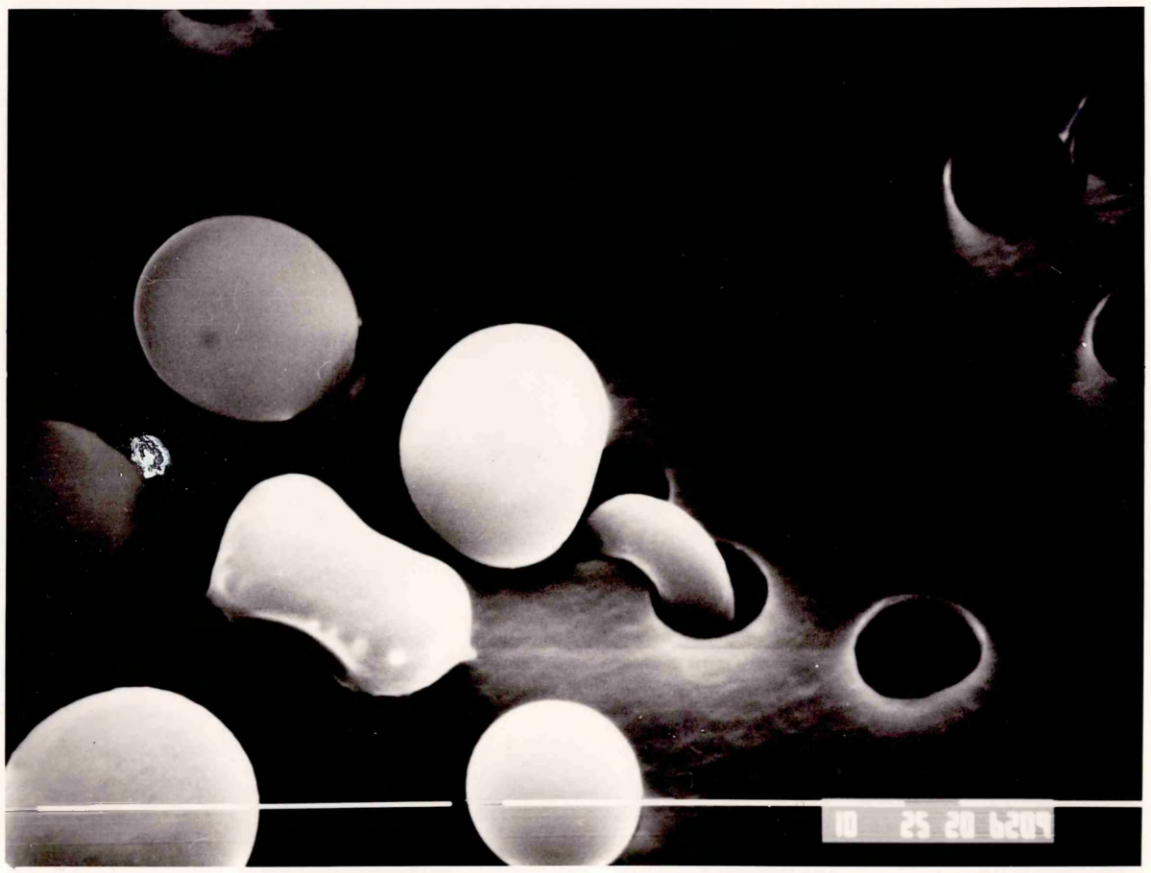
Filtration Parameters for red cell suspensions

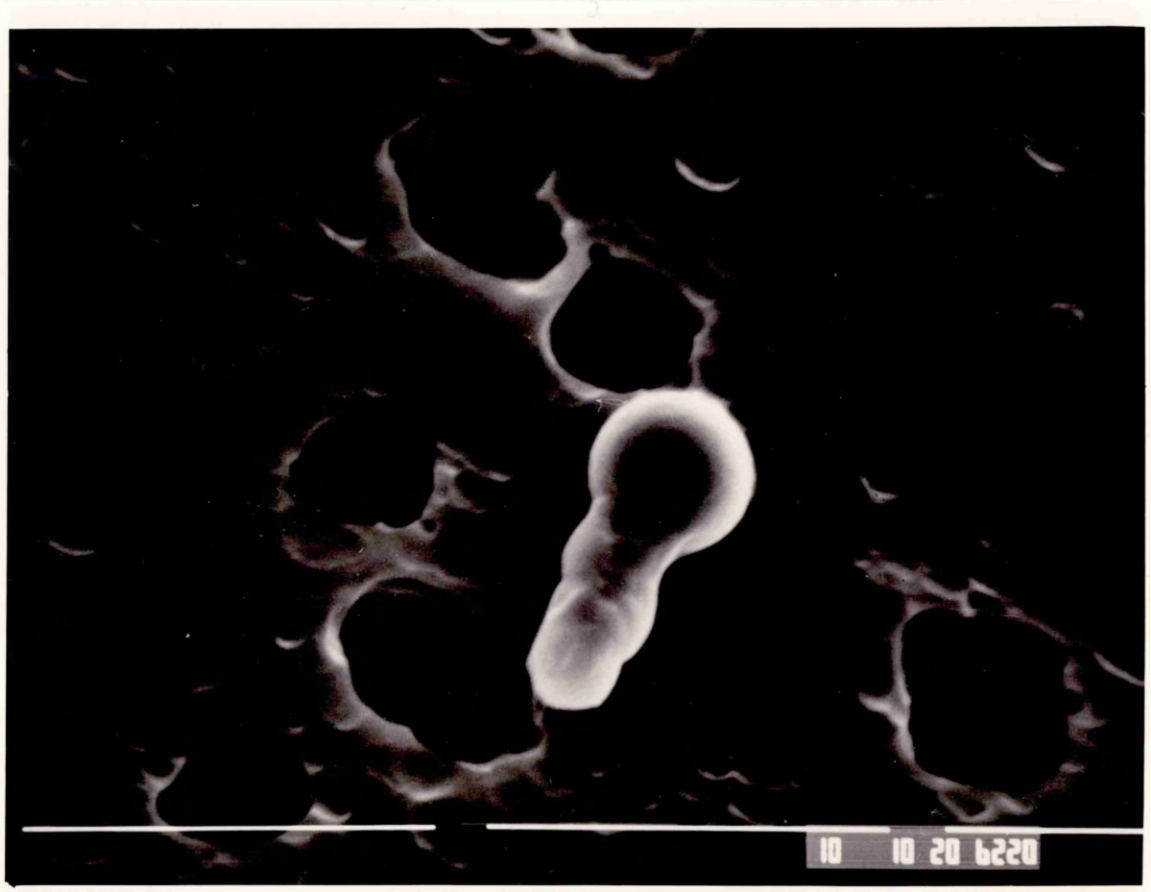
Number = 8

	Mean	STD	SEM	CV(%)
Pore Transit Time(s-)	2.77	0.0817	0.0289	2.9

Figure 2.7a *Scanning Electron Microsopic picture of the Nuclepore membrane from the upper surface showing the deformation and entry of red blood cells in 3um pores.*

Figure 2.7b *Scanning Electron Microscopic picture of the lower surface of Nuclepore membrane showing the exit of the deformed red cell from the 3um size pore.*





2.10 Fetal Haemoglobin (HbF) Estimation:

Estimation of the fetal haemoglobin was performed in the whole blood and the filtered red blood cells using the method des cribed by Phillips *et al* (1986). HbF was estimated by an alkali denaturation procedure. 10 *u*l of whole blood was diluted (and red cells lysed) in 3 ml ammoniated water (25mmol/l) and the optical density measured at 542 nm with a Pye-Unicam SP8-100 spectrophotometer. Denaturation was initiated by adding 50 *u*l 4 mol/l sodium hydroxide, and the decline in optical density was monitored over 12 minutes at 25°C. Computer analysis allows estimation of the percentage of HbF in the original sample by least square fitting of the data to a double experimental equation describing the simultaneous denaturation of HbA and HbF. The curve-fitting procedure uses subroutine E04JAF (Figure 2.6) from the library of Numerical Algorithms Group (Mayfield House, 265 Banbury Road, Oxford).

Since the number of red cells was quite low in the filtrate and HbF estimation was not possible using this method, in later experiments, the modified method described by Phillips *et al* (1988) was employed which allows quantitation of % HbF in the very small amounts of Hb present. Using this technique, the cells were lysed in 250

*u*l ammoniated water (25 mmol/l) in a microcuvette and 5 *u*l 4 mol/l NaOH added to initiate denaturation. The denaturation reaction was followed at 415 nm with a Pye Unicam SP8-100 spectrophotometer over a 12 minute period. Calculation of the HbF percentage was done using the same computer analysis as described in previous method.

CHAPTER 3

RESULTS

SECTION A. LABORATORY CONDITIONS FOR MEASUREMENT OF FILTERABILITY OF RED BLOOD CELLS.

ATP depletion is associated with decreased deformability of the red cell (Weed *et al*,1969). Therefore a decline in the metabolic activity during storage *ex vivo* can lead to complex changes in the flow properties of red blood cells and it is important that samples are collected and stored in a way that will not cause deterioration of the cells before routine testing of the samples can be performed.

The pore transit time calculated for erythrocytes from blood collected from healthy adult volunteers increases with decreasing pH and temperature (Stuart *et al*, 1985; Kooshesh, 1989). Similar studies on newborn infants' whole blood cells were performed by Buchan (1980) using 5*um* Nuclepore membrane and observations were made that the fall in pH leads to decrease in erythrocytic filterability but the temperature change does not have significant effect on red cell filtration.

The Arhenius equation, which describes the influence of temperature on simple rate constants, is often applicable to more complex systems such as enzyme reactions

(Gutfreund, 1972), and even intact biological systems such as the rate of walking of ants (Levy *et al*, 1959). The effect of changing temperature on the filterability of erythrocytes appears complex and dependent on the diameter of the pores in the filter membrane. Changes in the Index of filtration (Hanss, 1983) with temperature are predicted by the Arhenius equation:-

$$Ln\ (1/IF) = A + Ea/RT$$

Where A is a temperature-independent constant, R is the gas constant (8.314 kJ/mol/°K), T the absolute temperature and Ea is termed the energy of activation for the particular reaction (in this case - the filterability of erythrocytes). The effect of temperature on the filterability of erythrocytes through 3 *um* membranes is however more complex and has been described by Kooshesh (1989) and will be discussed in more detail later in this thesis.

The detailed investigations, described above, of the effect of pH and temperature on the filterability of erythrocytes were performed with human adult cells. No such information is available for neonatal cells and it is of more than academic interest. Attempts to relate red cell filterability to the onset or prognosis of any disease

state should perhaps consider the flow of the cells under conditions likely to prevail *in vivo* rather than those under standard laboratory conditions. The present work was undertaken in order to derive some factor(s) for predicting the filterability of erythrocytes *in vivo* from measurements performed *in vitro* at controlled temperature and pH. This approach will allow calculation of a "correction factor" for adjusting the pore transit time measured at pH 7.4 and temp of 37° C to that expected at the temperature and pH recorded *in vivo*. To this end, experiments to investigate the influence of pH on erythrocyte filterability were conducted with varying mixtures of 0.02M sodium phosphate salts and 0.12M NaCl. This concentration of phosphate is still within the recommended range quoted by Keidan *et al* (1987) for maintaining the mean corpuscular volume of RBC equal to that in whole blood. Sodium salts were used throughout to avoid differences in Na^+/K^+ at the different pH values. Temperature was adjusted through a thermostatically controlled water chamber around the filtration cell .

Note: The flow of red cells from the filter membrane is defined mathematically by the expression:-

dCells/dt = K_2 * N * Number of pores occupied by a cluster of cells.

The curve fitting procedure calculates values for k_2 and N (the number of cells in each cluster) and the Pore Transit Time is then defined as $1/(k_2 * N)$ [see Jones *et al*, 1985]. The Pore Transit Time is the time taken by a single cell to traverse a pore. Pore transit time is equivalent to the concept of half-life of reactions used to describe the rate of loss of drugs from the body or the rate of decay of radioactive isotopes. For investigating factors that influence the rate of flow of cells, the rate constant $k_2 N$ is the appropriate descriptor of these properties. For the sake of clarity, this rate constant will be referred to as 1/Pore Transit Time (1/PTT).

3.1. Effect of Storage on filterability of neonatal erythrocytes.

Blood was collected into plastic tubes containing EDTA (1mg/1ml blood) and kept at room temperature for varying times to suit the plan of each experiment. Suspensions were prepared and a filtration profile recorded as described in chapter 2. The results are presented in Table 3.1 and show that the calculated pore transit time is found to be constant for up to 4 hours after venepuncture. Further storage of the blood leads to a variable and unpredictable decline in the filterability of the red cells.

Table 3.1

Effect of storage at room temperature on filterability of fetal and neonatal erythrocytes.

Gestational Age (weeks)	Pore Transit Time (s)			
	Hours after venepuncture ...<2	4	6	24
22	2.41	2.39	5.21	6.10
25	1.22	1.35	1.48	2.05
28	1.54	1.60	2.05	2.88
30	2.84	2.84	3.05	8.13
29	1.41	1.40	1.48	ND

3.2 Effect of pH and temperature on the filterability of red blood cells

3.2A. Effect of changing pH on the filterability of neonatal erythrocytes:

Suspensions containing 30 x 10^6 erythrocytes/ml were then prepared in each buffer and filtration profiles

recorded at 20°C, 30°C and 37°C. The results are summarised in figures 3.1 to 3.3 . Values for individual blood samples are shown at 20°C and 30°C but mean values have been used at 37°C for the sake of clarity. All data have been analysed by linear regression and the calculated values for each slope are given in Table 3.2.

Table 3.2

Effect of pH on filterability of neonatal erythrocytes.

Temperature [°C]	N	Slope of Regression Line of 1/Pore Transit Time against pH
20	42	0.80 ± 0.17
30	42	0.93 ± 0.15
37	76	0.91 ± 0.11

Note: Values are quoted with the standard deviation estimated from the regression line.There is no significant difference between the lowest and highest values for the slope (P = 0.21)

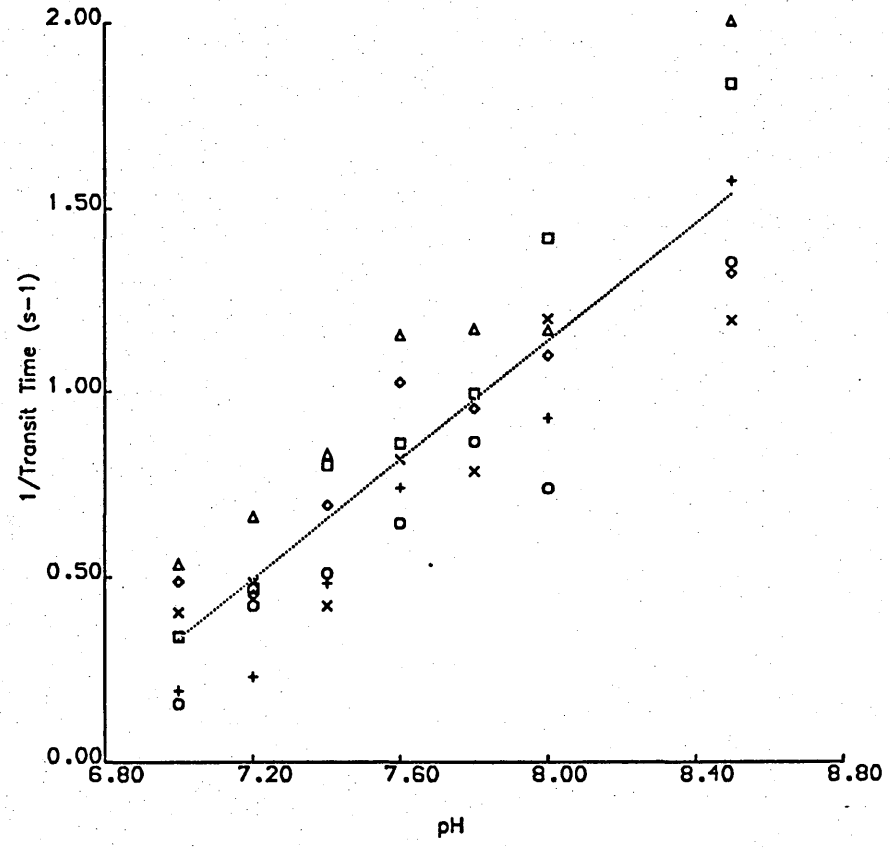


Figure 3.1: *The effect of pH on the rate of flow of neonatal erythrocytes through 3 um Nuclepore filter membrane at 20° C. Different symbols represent different blood samples. The flow rate of erythrocytes is expressed as [1/Pore Transit Time(1/PTT)].*

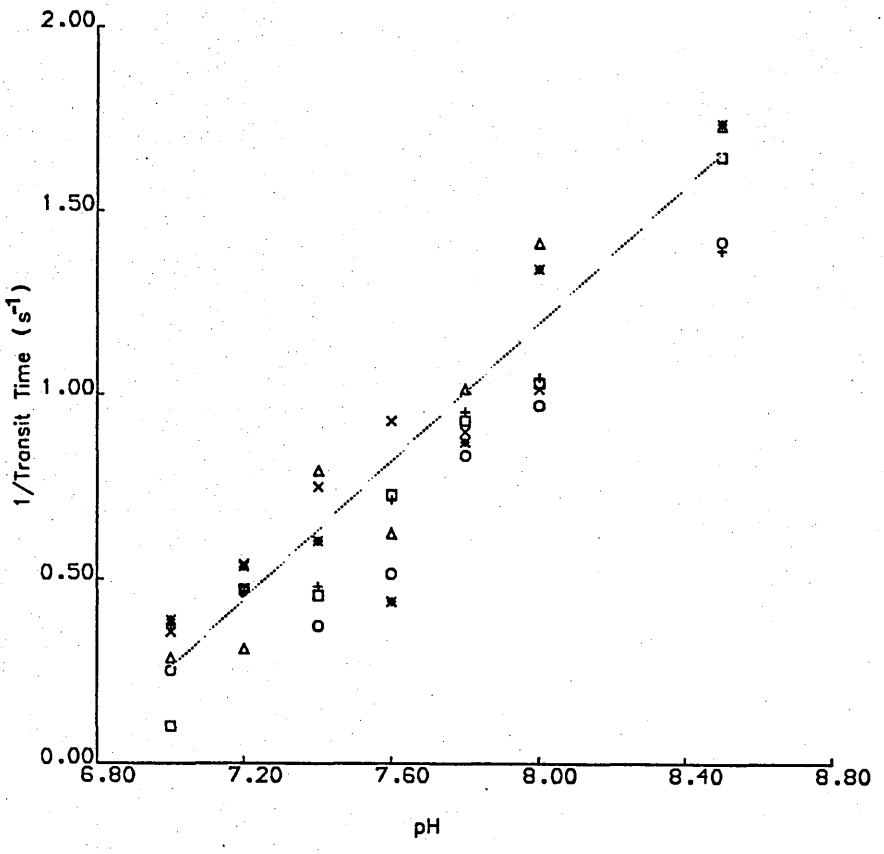


Figure 3.2: *The effect of pH on the rate of flow of neonatal erythrocytes through 3 um Nuclepore filter membranes at 30° C. Different symbols represent different blood samples. The flow rate of erythrocytes is expressed as [1/Pore Transit Time(1/PTT)].*

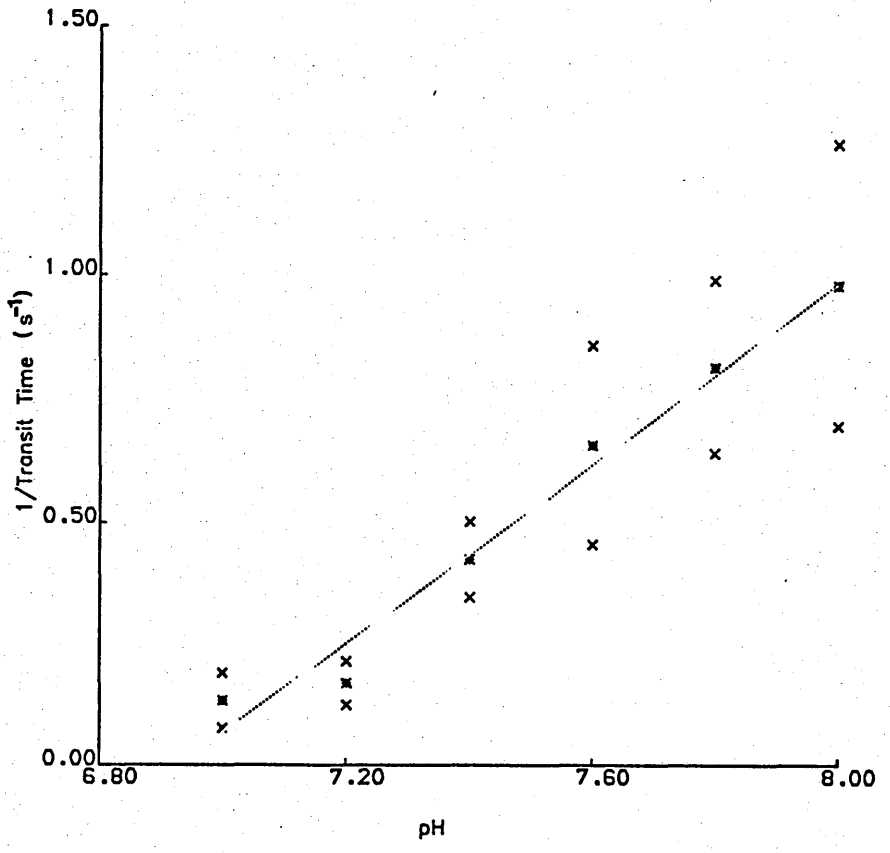


Figure 3.3: *The effect of pH on the rate of flow of neonatal erythrocytes through 3 um Nuclepore filter membranes at 37° C. Values are means ±SD .*

3.2B. Reversibility of the effect of pH on the filterability of neonatal erythrocytes.

Blood samples from six individual babies were diluted with 30 mM phosphate buffer in saline at pH 7.0, 7.2 and 7.4 to give 30 x 10^6 red cells/ml. Flow profiles were recorded and the Pore Transit Time calculated in the usual way. Samples of the same blood were also stored at pH 7.0 and 7.2 in suspensions containing 150 x 10^6 red cells/ml for 1 hour at room temperature. These were then diluted to 30 x 10^6 red cells/ml in buffer at pH 7.4 and the Pore Transit Time immediately measured at that higher pH value and 37°C. The results [Table 3.3] show that the decrease in filterability at low pH is rapidly reversed when the pH is restored to 7.4 at 37°C.

Table 3.3

Reversibility of the effect of pH on the filterability of neonatal erythrocytes.

pH	N	Pore Transit Time (s) [Mean ± SEM]
7.0	6	5.8 ± 1.44
7.2	6	2.5 ± 0.46
7.4	6	1.4 ± 0.28
7.0/7.4	6	1.5 ± 0.27
7.2/7.4	6	1.6 ± 0.21

3.2C. Effect of Temperature on the filterability of Neonatal Erythrocytes

Blood samples from nine babies were diluted in phosphate buffer in saline to contain 30 x 10^6 erythrocytes/ml. Filtration profiles were recorded at five different temperatures and the Pore Transit Time for the red cells calculated. The results are presented graphically in Figure 3.4 and as an Arhenius plot in Figure 3.5 . The equivalent Arhenius plot for the flow rate of buffer is presented in Figure 3.6 . The energy of activation [Ea], calculated from the slopes of these graphs, for red cell and buffer flow rate are 38.4 kJ/mol and 5.6 kJ/mol respectively.

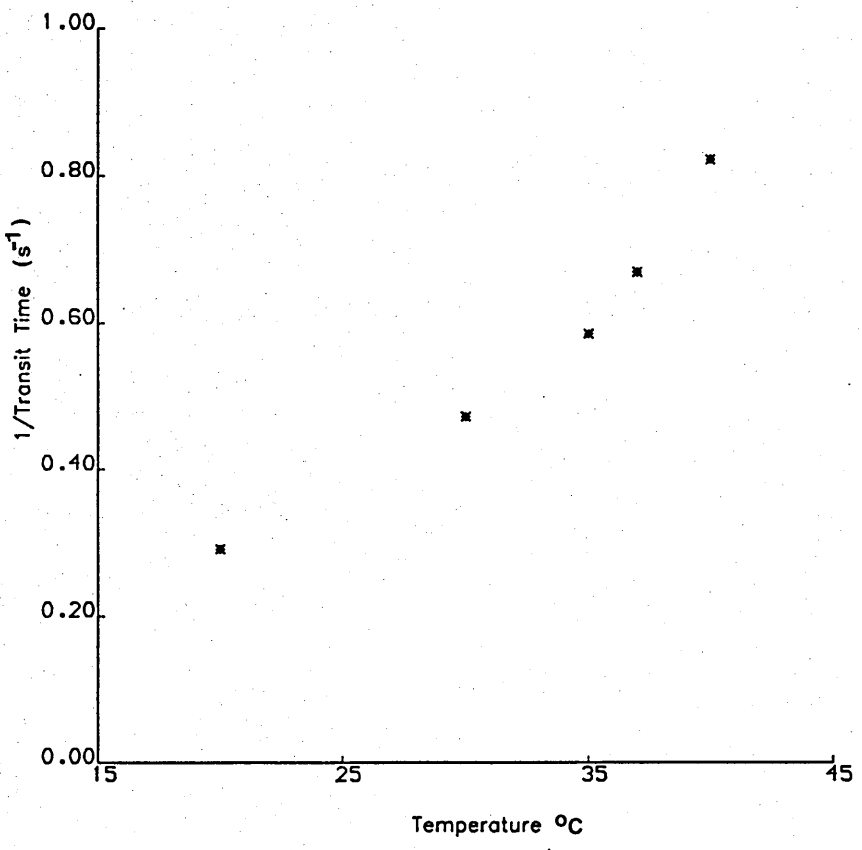


Figure 3.4: *The effect of temperature on the rate of flow of neonatal erythrocytes through 3 um Nuclepore filter membranes at pH 7.4 . The rates of flow of erythrocytes are expressed as 1/Pore Transit Time (1/PTT).*

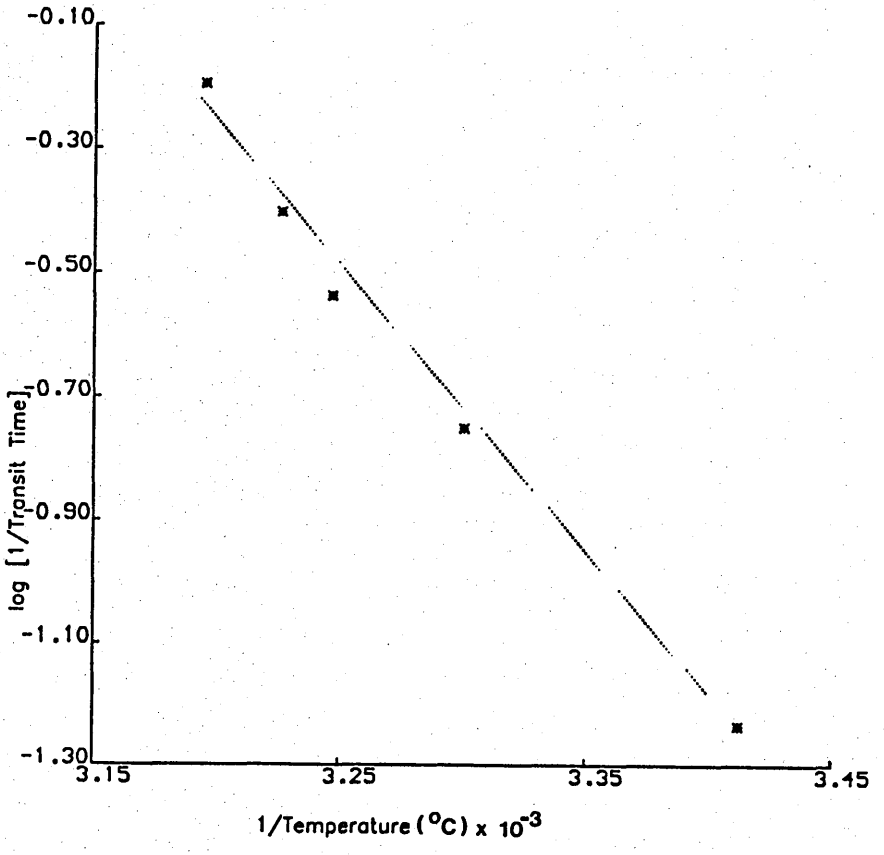


Figure 3.5: *Arhenius Plot for filterability of neonatal erythrocytes through 3 um Nuclepore filter membranes at pH 7.4.*

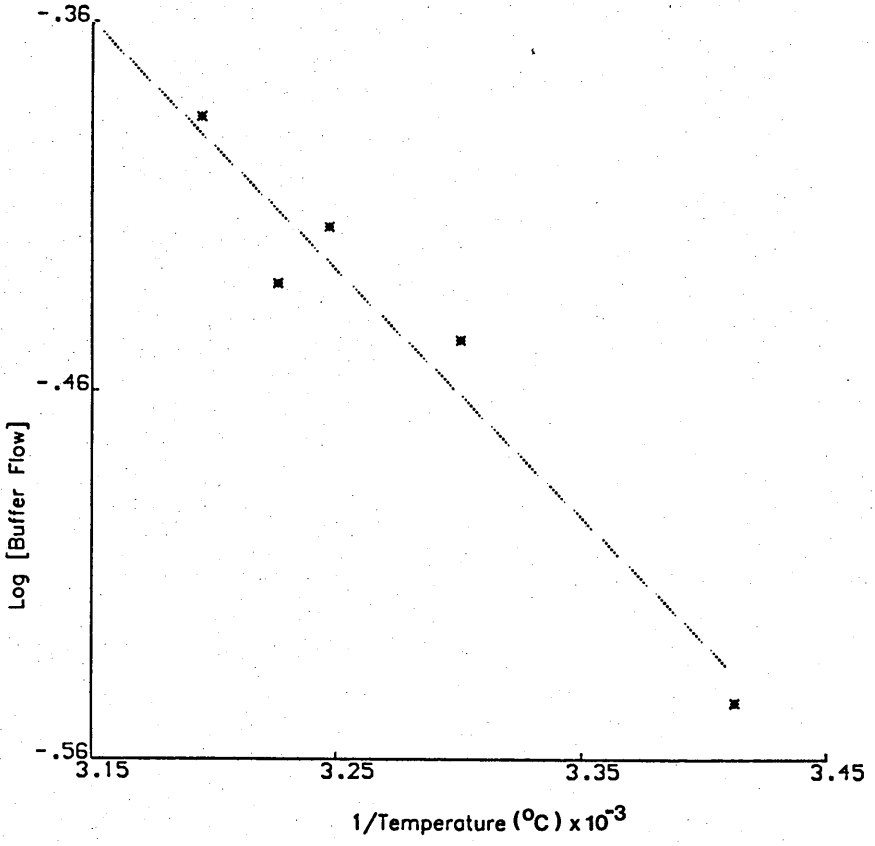


Figure 3.6: *Arhenius Plot for the flow rate of buffer through 3 um Nuclepore filter membranes.*

3.5. Correction factor for pH and temperature changes:

The present work was undertaken to calculate a "correction factor" for adjusting a Pore Transit Time *in vitro* to a Pore Transit Time *in vivo*. The method used here is NOT affected by white cells or plasma viscosity (see Kooshesh, 1989) and employs 3 um membranes to mimic the minimum sized capillaries likely to be encountered by the blood cells *in vivo*. Over the relatively narrow pH range studied the rate of flow of neonatal erythrocytes decreases linearly with decreasing pH. Hence the effect of decreasing the surface area/volume ratio outweighs the decrease in internal viscosity as the cells swell at low pH (see Orringer, 1985). Linear regression analysis yields an equation that relates the Pore Transit Time and pH at any temperature:-

$$1/\text{Pore Transit Time} = 0.94 * \text{pH} - 5.95$$

Defining Standard Pore Transit Time (PTT) as that measured at pH 7.4 and Pore Transit Time *in vivo* (PTT_{iv}) as that predicted at a given pH *in vivo* (pH_{iv}) :-

$$1/PTT_{iv} = 1/PTT - [0.94 * (7.4-pH_{iv})]$$

The energy of activation for the filterability of neonatal erythrocytes is much higher than that expected for a simple change in viscosity. This undoubtedly reflects the importance of cell size in determining the overall transit time of these cells through such small pores. The Pore Transit Time at any temperature (T) is given by the equation:-

$$1/PTT = K * e^{[-Ea/RT]}$$

$$= K * e^{[-4618/T]}$$

and the "correction" equation for temperature is:-

$$1/PTT_{iv} = 1/PTT * e^{[-4618*(1/T-1/310)]}$$

The combined "correction" equation for both pH and temperature is given by:-

****

$$1/PTT_{iv} = [1/PTT - (0/94*(7.4-pH_{iv})] * e^{[-4618*(1/T-1/310)]}$$

****

This equation has been programmed into a BBC-Master Series PC and is routinely used for predicting PTT_{iv} from a measured PTT.

SECTION B. Filterability of Red Blood Cells in Preterm & Term Infants, Fetuses and Adults.

A total of 108 fetuses and babies, varying in gestational age from 18 weeks to 42 weeks were studied with the approval of RHSC Ethics Committee. The mean gestational age distribution is presented in Figure 3.7. Blood samples from six healthy adult volunteers were also obtained. Fifteen samples were taken from fetuses *in utero* at gestational ages between 18 and 33 weeks. These fetal blood samples were taken for diagnostic and therapeutic purposes - the residue of blood was used, for filtration studies. The remaining 93 blood samples were taken from newborn infants within a few days of delivery. All experiments were performed within 4 hours of collecting the sample.

Number(N) = 108

Gestational Age (*weeks*)	Number	Histogram	
18	1	+	+ Fetus
20	2	++	* Newborn infant
24	5	++++*	
26	10	++*********	
28	8	********	
30	23	++**********************	
32	14	+**************	
34	20	+********************	
36	5	*****	
38	9	*********	
40	6	******	
42	5	*****	

Figure 3.7 *Histogram of the newborn infants and fetuses in the study showing the frequency distribution.*

3.4. Clinical Data of Fetuses in the study:

The fetuses in the study are those who underwent fetal blood sampling *in utero*. The details are shown in table 3.4.

TABLE 3.4 Clinical details of fetuses in the study.

Case No.	GA (*weeks*)	Indication for FBS*	FBS* results / Final outcome
102	18	High AFP+	Triploidy Pregnancy/ Termination of pregnancy
103	21	Maternal Age Low AFP	Normal female karyotype/ Normal pregnancy
108	22	High AFP	Normal male karyotyping/ Spontaneous abortion at 25 weeks of gestation
106	24	High AFP	Normal male karyotype/ Still birth at 28w
117	24	Raised Rh antibody	No Rhesus Incompability/ Normal pregnancy
114	33	Abnormal ultrasound scan	Diaphragmatic Hernia/ Normal progress,baby well after hernia repair

104	23	Raised Rh antibody	Rhesus incompatibility/ Normal progress
107	24	Rhesus in-compatibility	IUT* / Spontaneous abortion at 25w
109	25	Rhesus incomp.	IUT/Normal progress
110	25	Rhesus incomp.	IUT/Normal progress
111	26	Rhesus incomp.	IUT/Normal progress
112	29	Rhesus incomp.	IUT/Normal progress
113	29	Rhesus incomp.	IUT/Normal progress
115	30	Rhesus incomp.	IUT/Normal progress
116	30	Rhesus incomp.	IUT/Normal progress

*FBS:Fetal blood sampling

+AFP :Alpha fetoprotein

xIUT :Intrauterine transfusion

3.5. Clinical Data of Preterm and Term Infants in the study:

93 newborn infants were studied with gestational ages varying from 24 to 42 weeks. Table 3.5 shows the details of these infants.

TABLE 3.5

Clinical data of the newborn infants in study.

	24-29weeks	30-36weeks	37-41weeks
Number	28	45	20
Sex			
Male	20	27	9
Female	8	18	11
Mean Birth weight(*kg*)	1.10	1.79	2.96
Mode of delivery			
SVD	10	22	11
Forceps/ventouse	2	2	5
Breech	6	2	0
Elective Caesarian	4	4	2
Emergency caesarian section	6	15	2
Apgar at 1 minute			
1-3	5	7	1
4-6	13	19	2
7-10	10	20	17
Apgar at 5 minutes			
1-3	2	0	0
4-6	2	7	2
7-10	24	38	18

Rescusitation needed			
Mucus extraction	1	4	6
Facial oxygen	0	11	3
Bag & mask ventil-lation	0	5	0
Elective Intubation	15	5	1
Intubation required	12	14	2
No treatment needed	0	6	8

3.6 General Haematological Data:

General haematologial data from the preterm and term newborn infants and healthy adult volunteers is presented in Table 3.6 . The red cell count increased throughout the gestational age and reached the highest values in the adults. Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and white cell count (WCC) decreased as the gestational age increased and reached the lowest values in adults. Mean corpuscular haemoglobin concentration (MCHC) was similar in preterm and term infants and adults. Haemoglobin and haematocrit increased with gestational age in babies but values in adults were lower than in babies.

The filterability of red cells measured as Pore Transit Time through the filters was found to be much

higher in preterm babies with gestational age less than 30 weeks than term babies and in adults the values were very low.

Table 3.6. Haematological data and filtration time of red blood cells(*mean±SD*).

	Neonates (wk of gestation)			Adults
	24-29 w	30-35 w	37-42 w	
	n=20	n=40	n=20	n=6
Red cells count ($x10^{12}/l$)	4.2±0.6	4.4±0.6	4.8±0.7	5.1±4.6
Haematocrit(%)	51.0±7.1	51.7±7.8	56.1±8.3	45.9±3.1
Haemoglobin (g/dl)	16.8±2.4	17.1±2.8	18.6±3.6	14.7±1.6
MCV (fl)	120.7±8.9	115.9±7.6	114.5±7.0	90.9±2.9
MCH (pg)	39.7±2.9	38.3±2.8	38.1±2.8	29.2±1.5
MCHC (g/dl)	32.9±0.7	33.1±1.1	33.2±0.7	32.1±0.9
White Blood Cells($x10^{9}/l$)	15.0±9.9	13.9±9.2	13.4±5.9	6.1±1.6
Transit Time (Seconds)	2.03±1.0	0.74±0.4	0.59±0.4	0.17±0.1

values are mean±SD.

3.7. Statistical analysis of results

All statistical analyses of the results were performed with Minitab (Ryan *et al*,1985). The student's t-test was used to assess the significance of differences between mean values in most cases. In the presence of sufficient evidence of non-Gausian distribution, the Mann-Whitney non-parametric test was employed. Correlation and regression analysis were used to find the association between different variables. Correlation tells us how much association there is between two variables; but regression goes further. It gives us an equation that uses one variable to help explain the variation in another variable. The statistical significance (*P value*) of the correlation was derived from the correlation coefficient (r) using the standard statistical values (see appendix 3).

3.8. Definition of pore transit time *in vivo* (PTTiv)

Previous work has established that the pore transit time of neonatal red blood cells depends on the pH and temperature of the test system. Pre-term infants are often acidotic and/or hypothermic and a pore transit time measured under standard conditions *in vitro* may not be a realistic expression of the deformability of red cells *in vivo*. For this reason, all statistical tests were performed with the measured pore transit time (PTT) and that adjusted to conditions *in vivo* (PTT_{iv}) using correction factors described earlier.

3.9. Effect of transfusion of adult red cells on the filterability of fetal and neonatal blood

As shown in Table 3.6 there was a very significant difference in the mean pore transit time between adult red cells and the red blood cells of infants. In order to evaluate the effect that blood transfusion might have on the pore transit time of the neonatal red cells, statistical analysis was performed on twenty fetuses and infants (Mean GA:29.6$\pm$3.3) who had been transfused before the measurement of filterability. In all of these cases there was a marked difference in filterability from those not transfused (Mean GA:31.6$\pm$3.3). The mean pore transit time of red blood cells in untransfused and transfused fetuses and newborn babies were 1.31 $\pm$ 1.45 and 0.28 $\pm$ 0.30 s^{-1} respectively. The difference in these mean values are highly significant (P value > 0.0001 by t-test OR non-parametric Mann-Whitney test). Figures 3.8 and 3.9 show plots of pore transit time *in vivo* (PTT) and *in vitro* (PTT_{iv}) against the gestational age of the infants. Figures 3.10 and 3.11 are the respective plots of log transformation of pore transit times versus the gestational age. Subsequent to this observation, analysis was limited to the untransfused infants.

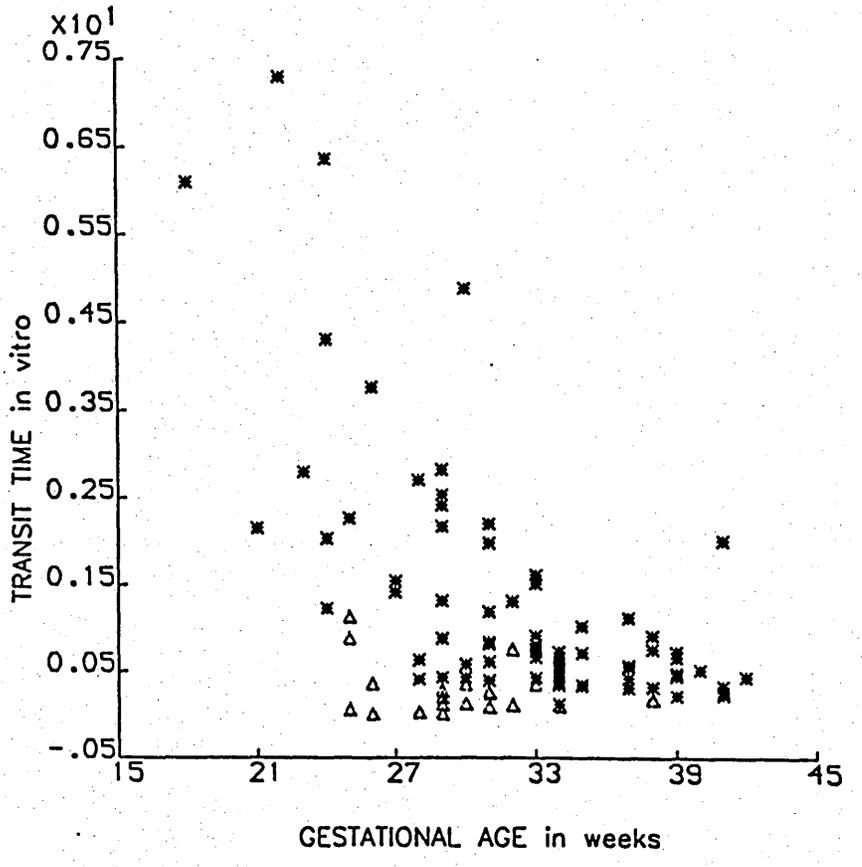


Figure 3.8 *Pore Transit Time in vitro (PTT) in Transfused (Δ) and Non-Transfused (✳) cases plotted against the Gestational Age.*

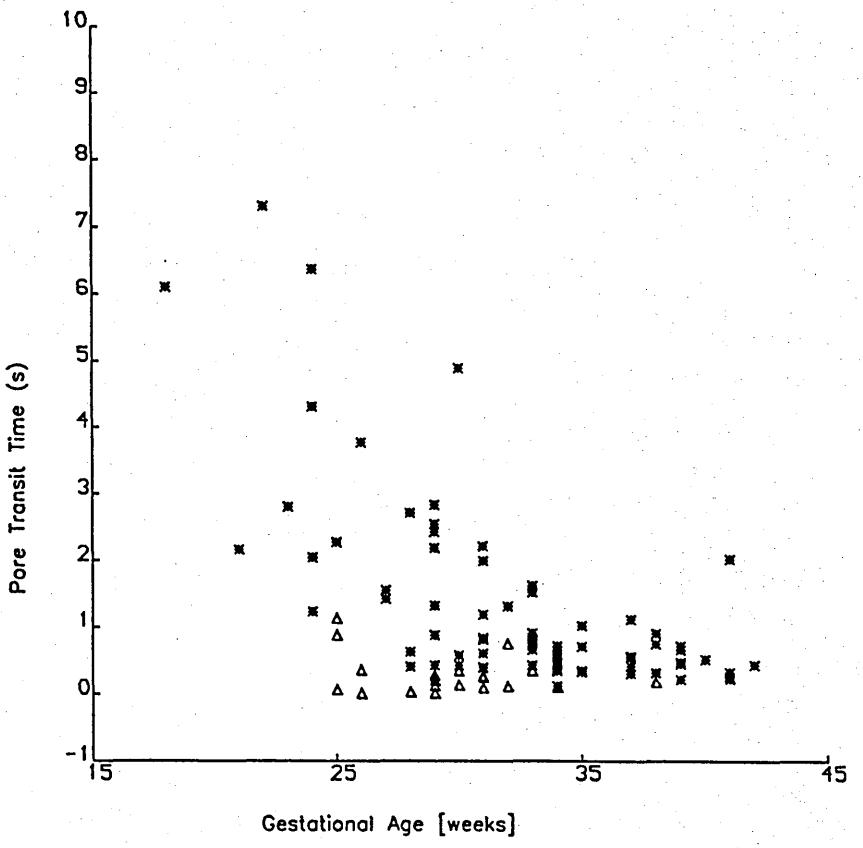


Figure 3.9 *Corrected Pore Transit Time in vivo ($PTT_{i\,v}$) in Transfused (Δ) and untransfused (*) fetuses and infants plotted against their gestational age.*

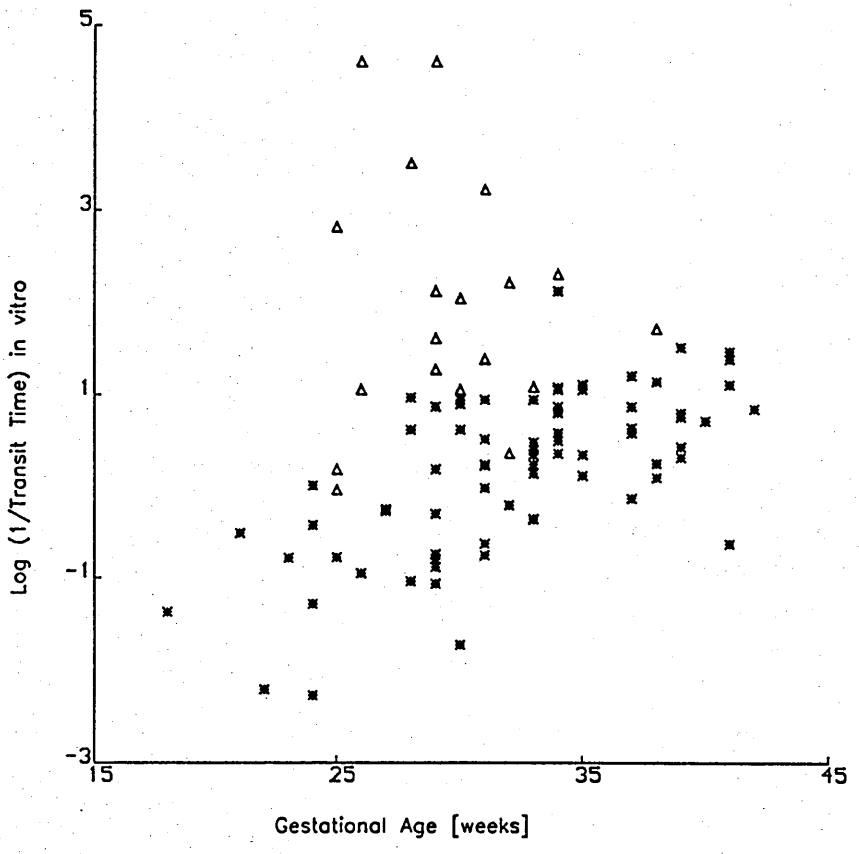


Figure 3.10 *Log of filterability rate of red blood cells (Log 1/PTT) plotted against the gestational age of transfused (△) and untransfused (*) fetus and infants.*

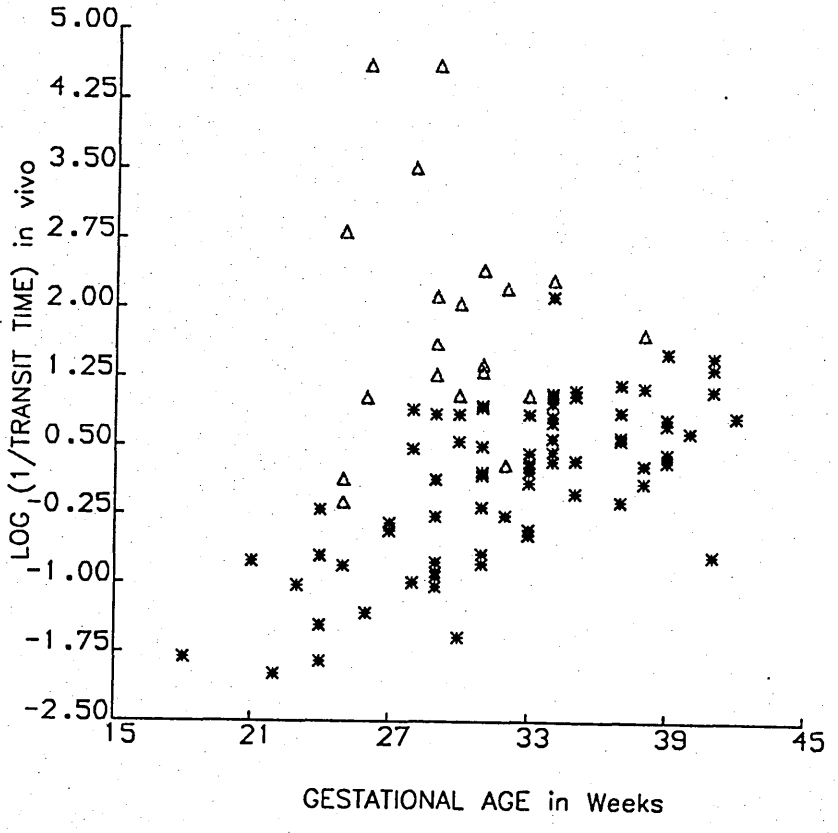


Figure 3.11 *Log of corrected filterability rate of red cells (Log 1/PTTiv) plotted against the gestational age of transfused (△) and untransfused (*) fetuses and infants.*

3.10 Statistical distribution of calculated transit times in all of the un-transfused study infants

The distribution of the values for pore transit time around the mean of 1.31 s^{-1} was investigated using the Normal Score test (nscore). In this test a sample of random numbers with a mean of 0 and standard deviation 1.0 is selected with the number of values equal to the population being investigated. Both populations are then arranged in ascending order and plotted against each other in a two-dimensional graph. A progressive deviation from linearity in this plot is evidence that the distribution of the measured parameter values is not "normal" (Gaussian) and statistical tests based on an assumption of normality are not valid. Under these conditions, it is permitted to select mathematical transformations of the original data that yields a population of normally distributed values and perform the tests on this transformed data. Figures 3.12 & 3.13 show a normal score (Nscore) Plot for PTT and PTT_{1v} respectively. Clearly both experimental parameters are not distributed normally and are not suitable for many conventional statistical test procedures.

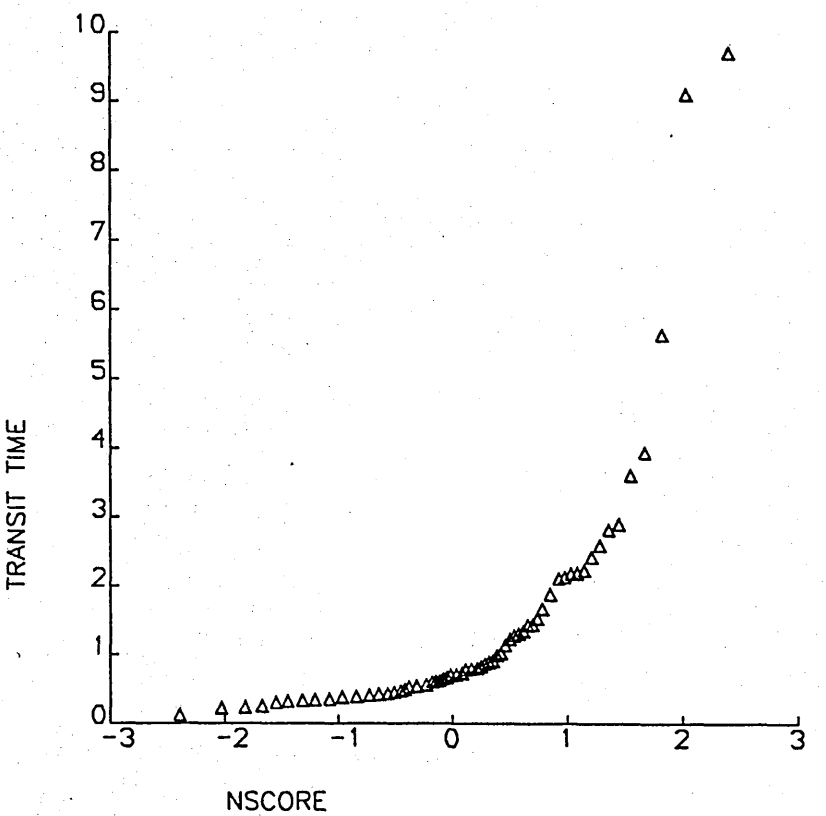


Figure 3.12 *Normal score plot of in vitro Pore Transit Time (PTT)*

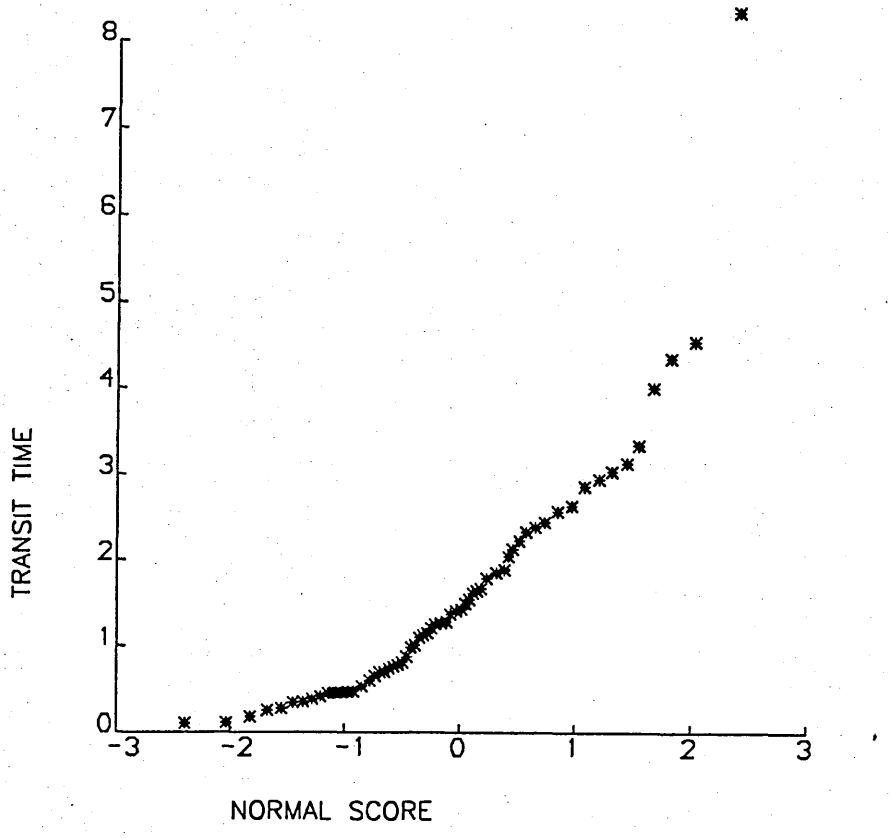


Figure 3.13 *Nscore plotted against the corrected Pore Transit Time (PTTiv).*

Before seeking mathematical transforms of the transit times to yield a Gaussian distribution, it is worth considering the meaning of this experimental parameter.

It represents the time taken by a red cell to cross a single pore in the filter membrane. The computer algorithms actually calculate the rate at which a cell crosses a pore and this is a better representation of the flow properties of the red cell. This rate is in fact the reciprocal of the transit time and has units of s^{-1}. Using this parameter to express the property of the red cell is analogous to quoting the speed of a car on the highway rather than the time it takes to travel from A to B. However, as both expressions of transit time are not distributed normally, their reciprocals also show a skewed distribution.

Figures 3.14 & 3.15 show the Nscore Plots for log (1/PTT) and log ($1/PTT_{iv}$) respectively and establish that the distribution of transit times can be described as log-normal.

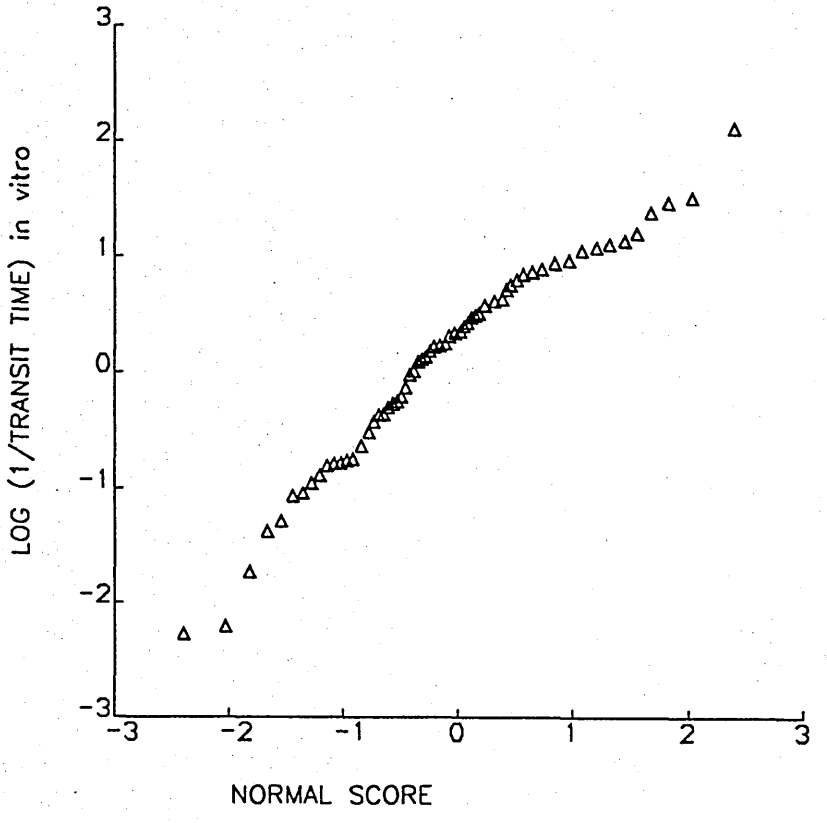


Figure 3.14 *Normal score plot of Log transformation of 1/Pore Transit Time (Log 1/PTT).*

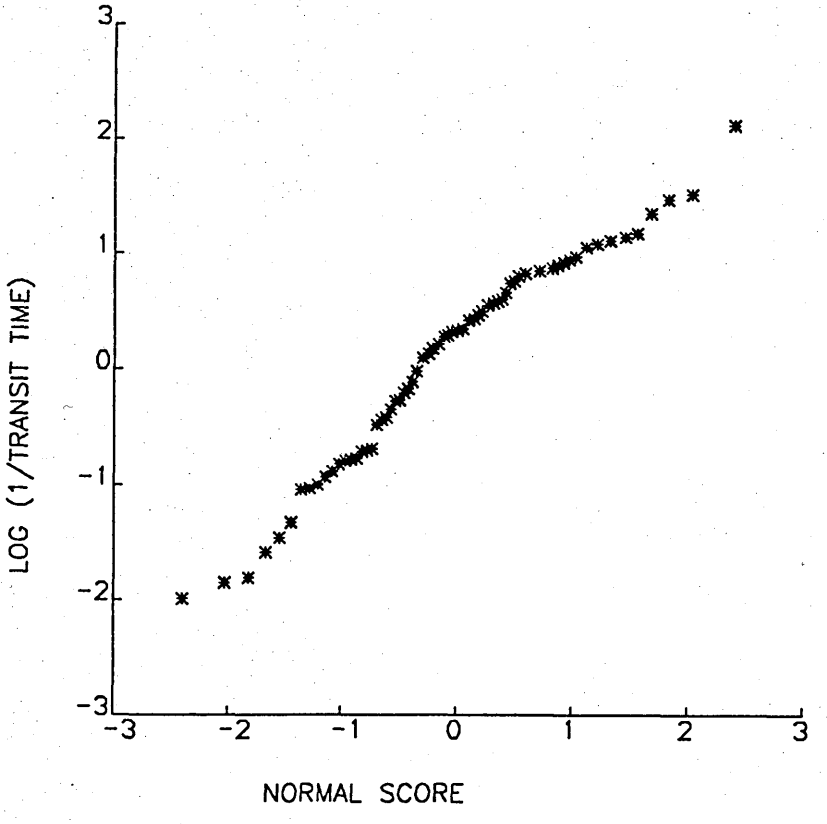


Figure 3.15 *Normal score plot of Log transformation of 1/Pore Transit Time in vivo (Log 1/PTTiv).*

3.11. Gestational Age of the Fetus and Newborn infant and Red Cell Filterability

Filterability of RBCs was correlated against the gestational age of fetuses and newborn infants in study. The relationship between the Pore Transit Time and the gestational age of the infant is shown in Figure 3.16. A linear regression of log (1/PTT) against gestational age is presented in table 3.7 and shown in Figure 3.17.

TABLE 3.7

Regression analysis of log 1/Pore Transit Time and Gestational Age (GA).

The regression equation is

Log (1/PTT) = -3.45 + 0.113 x GA

Predictor	Coefficient	Stdev	t-ratio
Constant	-3.455	0.464	-7.44
Gestational Age	0.113	0.014	7.87

Correlation Coefficient (r) = 0.64

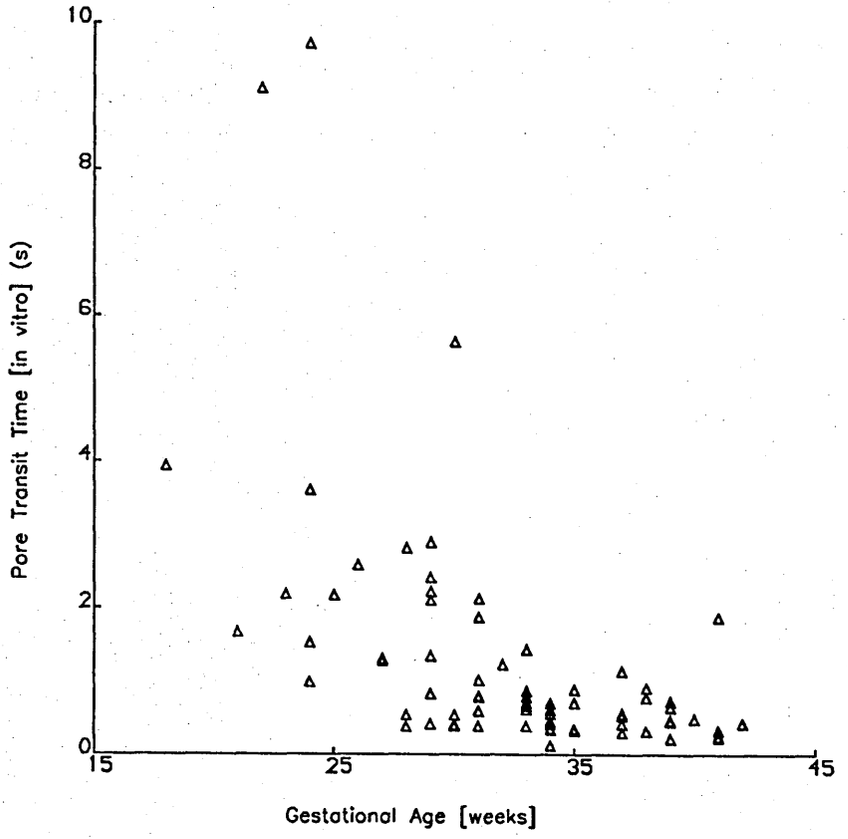


Figure 3.16 *Pore Transit Time (PTT) plotted against the gestational age of the fetuses and newborn infants.*

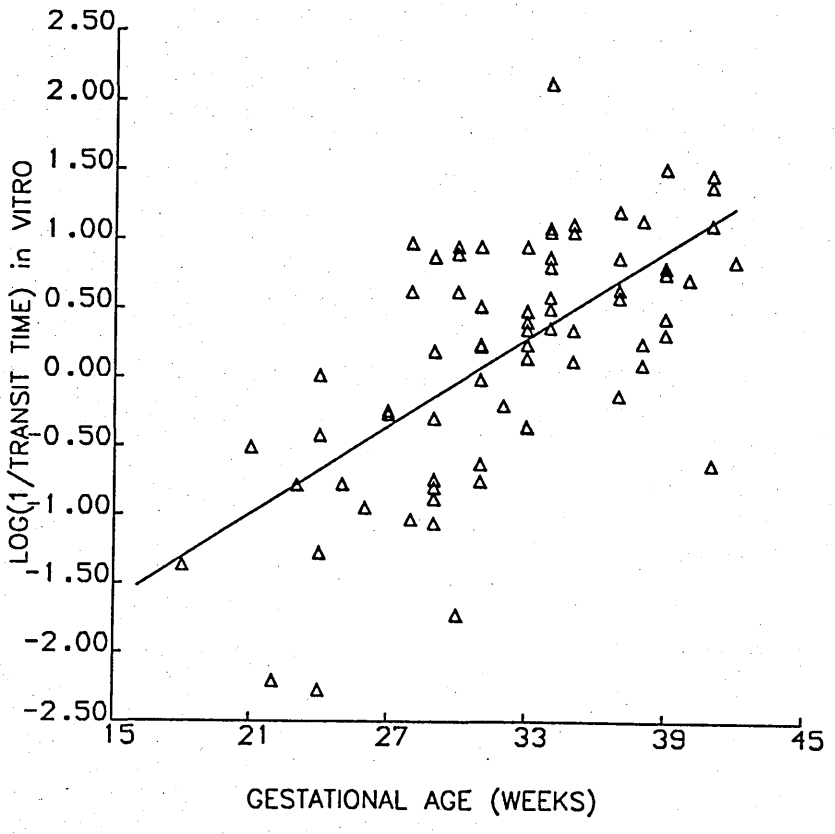


Figure 3.17 *Log transformation of filterability rate of red cells (Log 1/PTT) plotted versus the gestational age.*

The positive correlation found between the gestational age and the rate of filtration of red cells(1/PTT) is statistically significant (P value < 0.05).

Similar relationship between PTT_{iv} (corrected pore transit time) and the gestational age is seen in Figure 3.18 .The regression analysis between the gestational age and Log of corrected filtration rate of the red cells ($1/PTT_{iv}$) is presented in table 3.8 and plotted in Figure 3.19.

TABLE 3.8

Regression analysis of Log 1/Pore Transit Time (Log $1/PTT_{iv}$) and Gestational Age(GA).

The regression equation is

Log $(1/PTT_{iv})$ = -3.72 + 0.120 x GA

Predictor	Coefficient	SD	t-ratio
Constant	-3.721	0.452	-8.23
Gestational Age	0.120	0.014	8.56

Correlation Coefficient *(r)* = 0.68

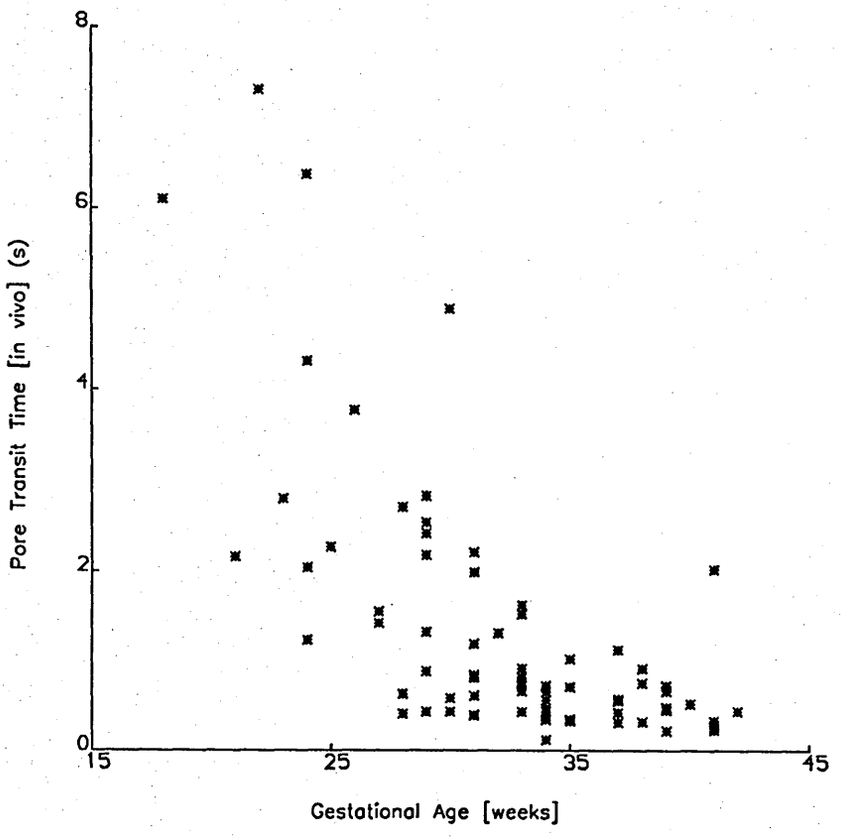


Figure 3.18 *Red cell Pore Transit Time in vivo (PTTiv) plotted versus the gestational age of the fetuses and newborn infants.*

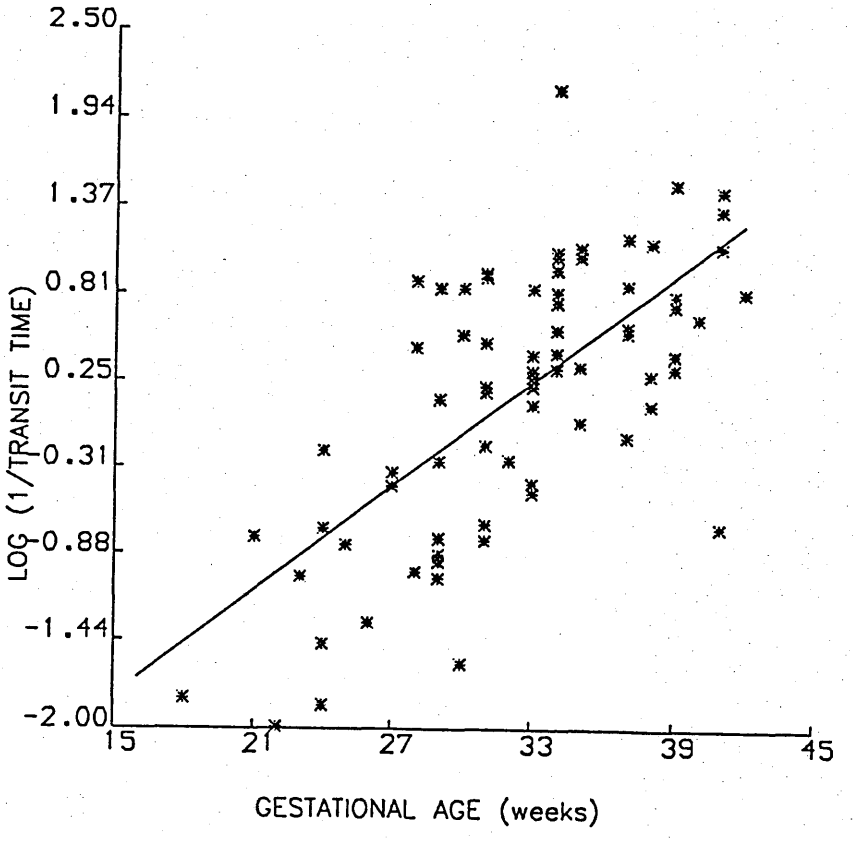


Figure 3.19 *Correlation between Log transformation of corrected Pore Transit Time (Log1/PTTiv) and the gestational age.*

Again there is strong positive correlation between 1/red cell pore transit time (rate of filtation) and the gestational age of the baby showing decreased filterability of RBC in premature newborn infants. The probability that these correlations arose by chance is < 0.05 in both cases.

3.12 Sex of the newborn infant and RBC deformability

Thirty two female and forty male gestational age matched newborn infants who were not transfused with the adult blood were studied and filterability of red cell compared using the student's t-test . The statistical analysis showed no significant difference between both groups. The results are presented in Table 3.9 .

Table 3.9 Filterability of RBCs in male and female babies

Sex	No	GA *weeks* mean±SD	Filterability of RBCs (Log $1/PTT_{1v}$) mean±SD
Male	40	32.28±4.40	0.258±0.871
Female	32	32.40±4.32	0.237±0.694

P = 0.90

3.12 Mean Cell Volume (MCV) of RBC and the Filterability

Since the mean cell volume of red cell decreases with the increasing gestational age in infants and reaches lowest values in adults (Table 3.6), the effect of MCV on filterability of RBC was analysed to see any correlation between the two parameters.

The relationship between RBC pore transit time and the corresponding 1/log transformation with the mean corpuscular volume is shown in Figure 3.20 a & b respectively. The regression analysis is described in table 3.10.

Table 3.10 Regression analysis of Log (1/PTT) and MCV

The regression equation is

Log (1/PTT) = 4.90 - 0.0399 x MCV

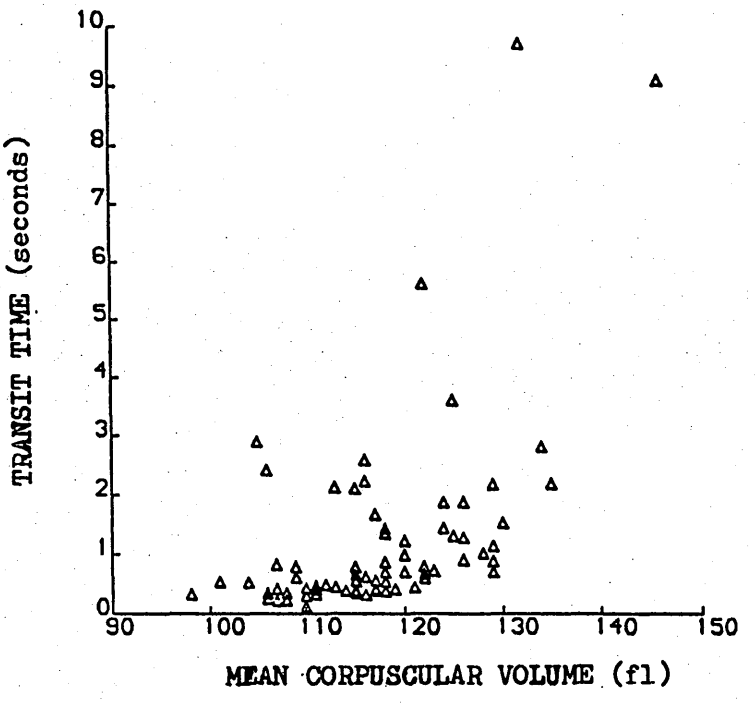
Predictor	Coefficient	SD	t-ratio
Constant	4.899	0.761	6.44
MCV	-0.039	0.006	-6.27

Correlation Coeffient (r) = 0.56 $P < 0.05$

Figure 3.20(a) *Pore Transit Time in vitro (PTT) plotted versus the Mean Corpuscular Volume (MCV).*

Figure 3.20(b) *Correlation plot of Log 1/Pore Transit Time in vitro (1/PTT) and Mean Corpuscular Volume(MCV) of the fetal and neonatal red blood cells.*

Transit Time in Vitro vs MCV



LOG (1/TRANSIT TIME) in VITRO v MCV

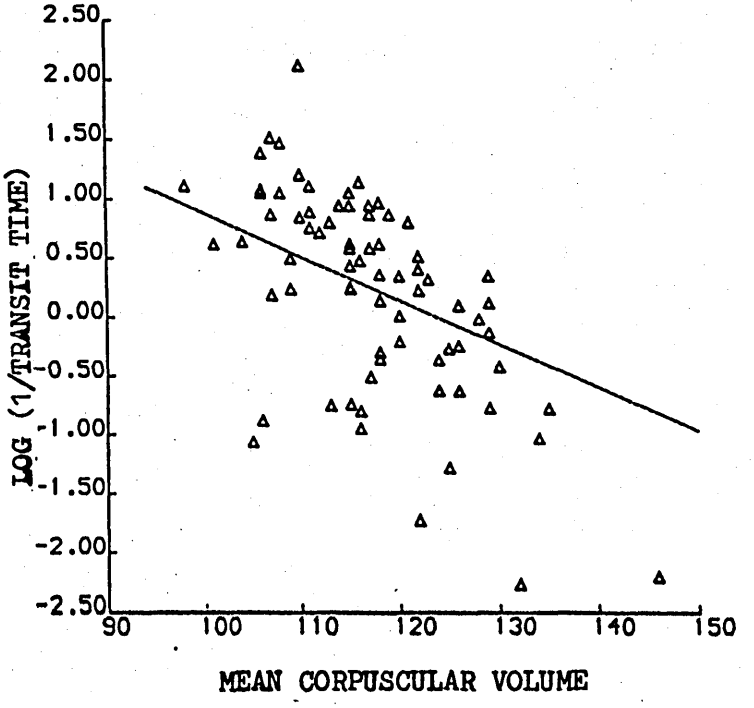


Figure 3.21 (a & b) plots the corrected Pore Transit Time (PTT_{iv}) and corresponding 1/log transformation against the mean corpuscular volume and table 3.11 presents the regression analysis. The regression gives a negative correlation coefficient of 0.59 with the probability that this correlation arose by chance is < 0.05.

TABLE 3.11

Regression analysis of Log ($1/PTT_{iv}$) and MCV

The regression equation :

Log ($1/PTT_{iv}$) = 5.17 - 0.0426 x MCV

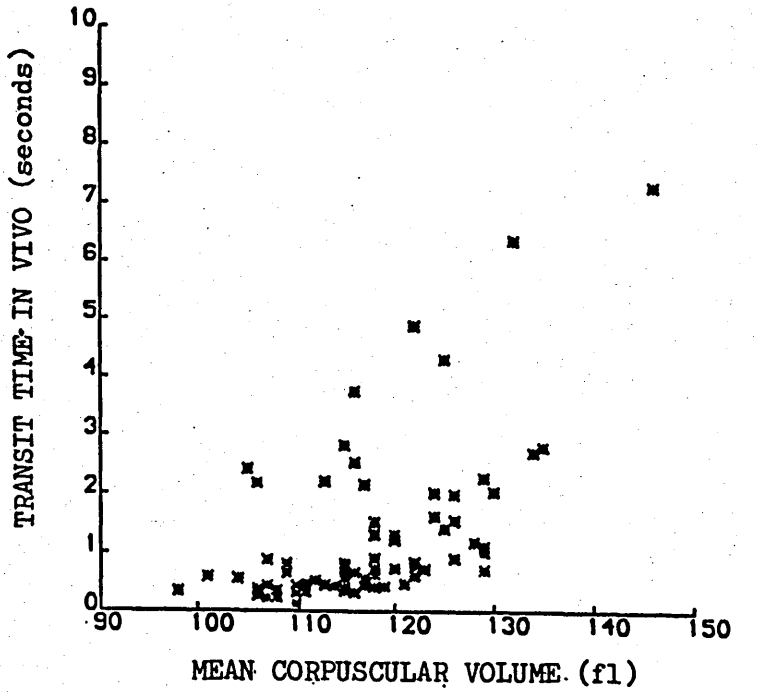
Predictor	Coefficient	SD	t-ratio
Constant	5.169	0.749	6.90
MCV	-0.0426	0.006	-6.80

Correlation coefficient = 0.59

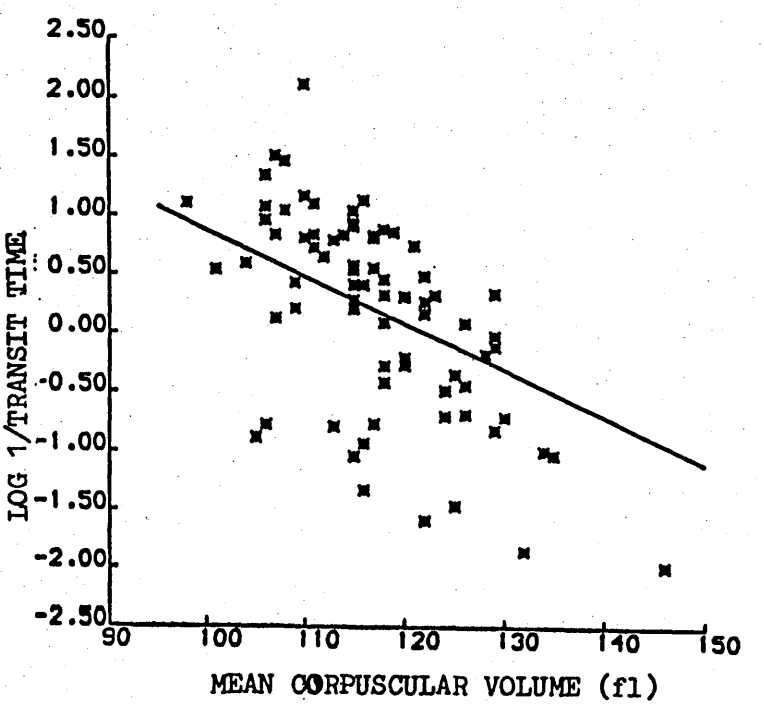
Figure 3.21(a) *Pore Transit Time in vivo(PTTiv) plot versus the Mean Corpuscular Volume(MCV).*

Figure 3.21(b) *Correlation plot of Log 1/Pore Transit Time in vivo(Log 1/PTTiv) and the Mean Corpuscular Volume(MCV). Note the negative relationship between the filtration rate (1/PTTiv) and MCV.*

Transit Time in Vivo v MCV



Log (1/Transit Time) in VIVO v MCV



3.14 Mean Cell Volume as the determinant of RBC filterability in relation to the gestational age.

There is a strong negative correlation between MCV and the gestational age. To investigate the possibility that the negative correlation between MCV and red cell filterability is coincidental with the negative correlation of MCV with gestational age (Correlation coefficient for this regression is -0.49), both parameters were examined in a multiple regression analysis. The results of this regression are collected in Table 3.12.

TABLE 3.12

Correlation of log (1/PTT) with gestational age and MCV

The regression equation :

$\log (1/tt_{s\,t}) = 0.07 + 0.0806 \times ga - 0.0209 \times MCV$

Predictor	Coefficient	SD	t-Ratio
Constant	0.075	1.127	0.05
Gestational Age	0.0806	0.0162	5.42
MCV	-0.0209	0.0065	-3.40

Correlation Coefficient $r = 0.69$

The results of a multiple regression of log ($1/PTT_{iv}$) versus gestational age and MCV are collected in Table 3.13.

TABLE 3.13

Correlation of log ($1/PTT_{iv}$) with gestational age and MCV

The regression equation :-

log ($1/PTT_{iv}$) = 0.07 + 0.0855 x ga - 0.0226 x MCV

Predictor	Coefficient	SD	t-ratio
Constant	0.07	1.069	0.06
Gestational	0.0855	0.0153	5.96
MCV	0.0226	-0.0062	-3.85

Correlation Coefficient r = 0.73

Both tables 3.12 & 3.13 show that the t-ratio for gestational age and MCV remain high for both transit times during a multiple regression analysis . Hence the influence of gestational age and MCV on the filterability of neonatal red blood cells are independent and statistically significant. This was further confirmed

by using correction factors. Correlation coefficient (r= 0.54) remained statistically significant (p <0.05) when the filteration was corrected for MCV and then correlated with gestational age . Similarly, as shown in Figure 3.22 , the correlation persisted when Log(1/PTT) was corrected first for gestational age and then correlated with mean cell volume (r = 0.59, p <0.05).

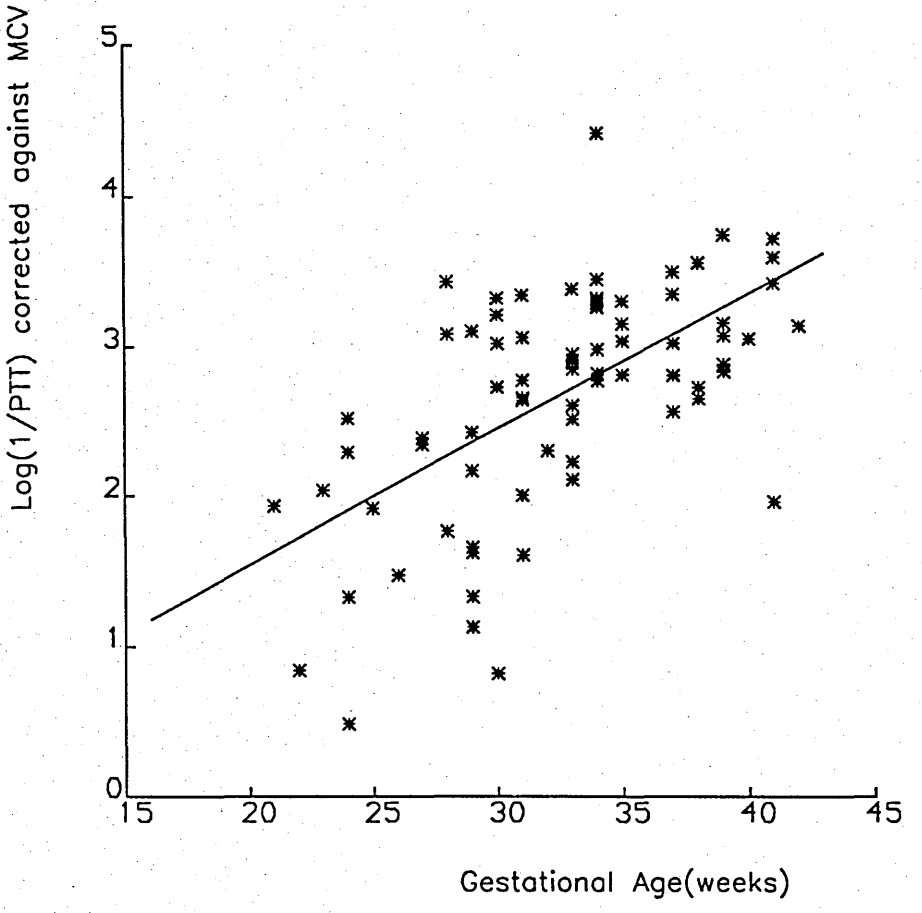


Figure 3.22 *Log 1/Pore Transit Time corrected against the MCV and then correlated with the gestational age. Note the positive correlation between the filtration rate and the gestational age even without the MCV factor.*

Filterability of fetal and neonatal red cells when compared with adult red blood cells, demonstrated that adult red cells filter much faster (Table 3.6). The main determinant factor for this difference is the mean corpuscular volume (MCV). When the filtration rate(1/PTT) is plotted against the MCV of fetal, neonatal and adult red blood cells on the same plot (Figure 3.23), the negative correlation between MCV and red cell filterability (Log1/PTT) remained and the same line of best fit applies to the adult RBCs which have lower MCV and higher filtration rates. The regression analysis is presented in table 3.14 .

TABLE 3.14 Regression analysis of Log (1/PTT) and MCV of Infants & Adults RBC.

Regression equation is

Log (1/PTT) = 7.51 - 0.0622 MCV(Infants+Adults)

Predictor	Coefficient	SD	t-ratio
Constant	7.5087	0.801	9.37
MCV(Infant+Adult)	-0.0622	0.006	-9.08

Correlation Coefficient $r = 0.69$ ($P < 0.05$)

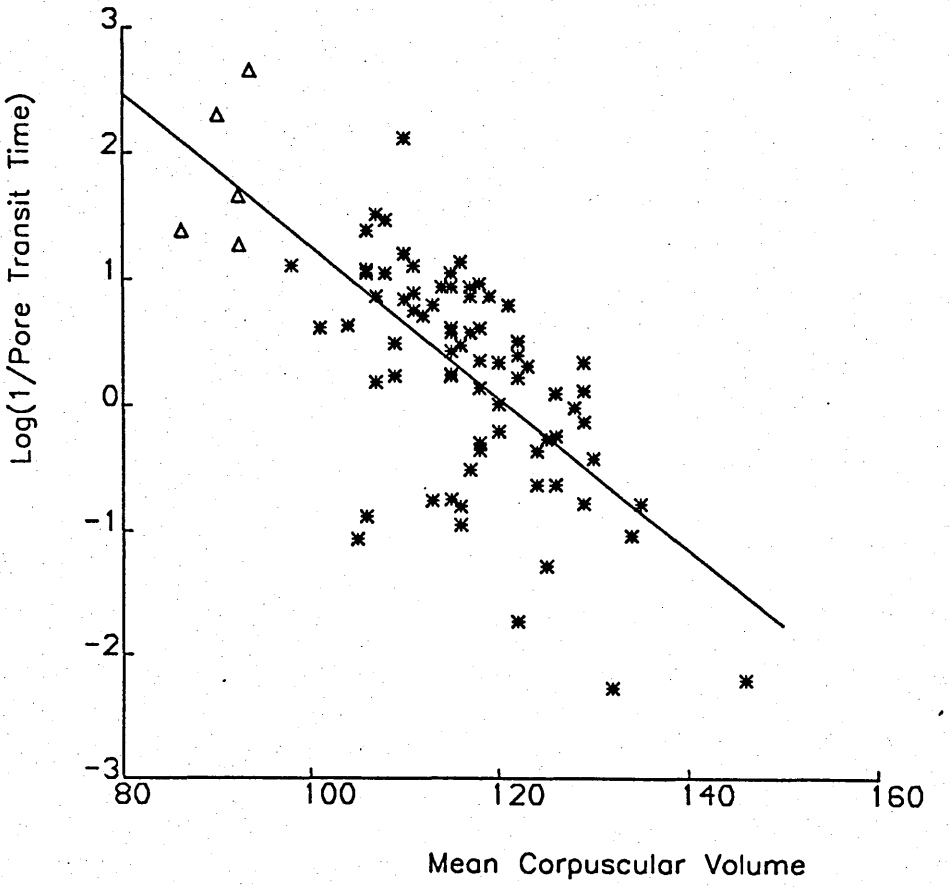


Figure 3.23 *Correlation plot of Log(1/PTT) versus the mean corpuscular volume(MCV) of fetal, neonatal and adult red blood cells. Note the adult(△) and fetal & neonatal (*) points are plotted on the same line of best fit.*

3.15 Red Cell Count and the Filterability of RBC

The correlation between log (1/PTT) and the red cell count (RCC) is shown in table 3.15 and plotted in Figure 3.24. The probability that this correlation arose by chance is < 0.05. However, there is also a correlation between RCC and gestational age and the question arises whether the correlation between transit time and RCC is coincidental with this correlation.

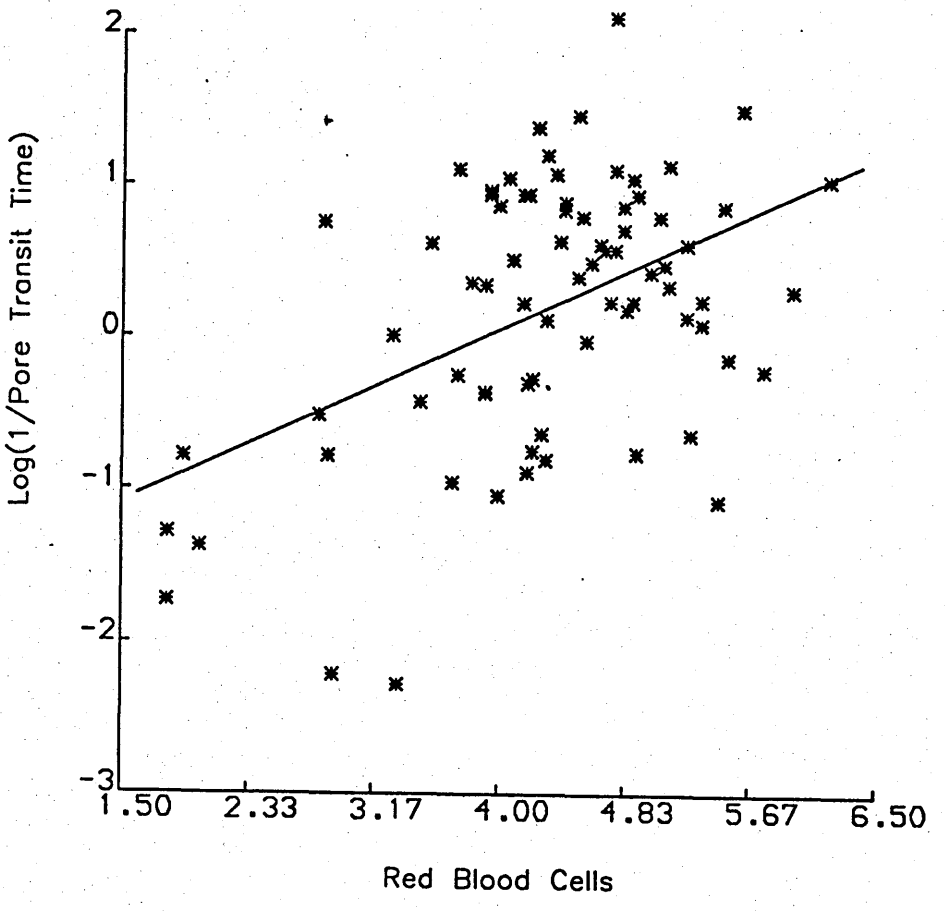


Figure 3.24 *Correlation plot of Log(1/PTT) versus the red cell count. Note the pos itive correlation between the filtration rate and the red cell count.*

A multiple regression of log(1/PTT) versus gestational age and red cell count is also summarised in table 3.15 . This analysis shows that the statistical significance of the correlation with RCC disappears with the inclusion of gestational age. Figure 3.25 also confirms this. Hence unlike MCV, the RCC count is not an independent determinant of pore transit time in these infants.

TABLE 3.15

Correlation of log (1/PTT) with RCC and gestational age

The regression equations are:-

log (1/PTT) = -1.77 + 0.456 x RCC (r = 0.48)

log (1/PTT) = -3.33 + 0.134 x RCC + 0.0916 x ga (r = 0.65)

Predictor	Coefficient	SD	t-ratio (Multiple)	t-ratio (Single)
Gestational Age	0.0916	0.0189	4.84	7.85
RBC	0.134	0.108	1.24	4.72

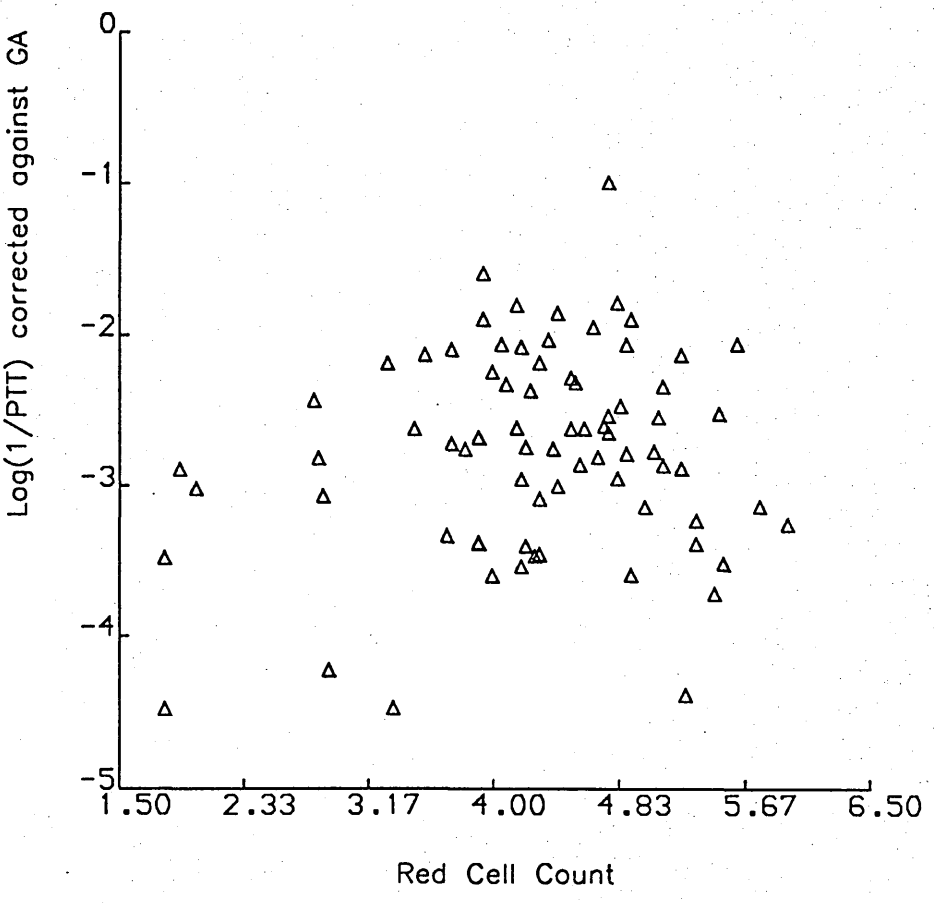


Figure 3.25 *Correlation plot of filtration rate (Log 1/PTT) of RBC and the red cell count corrected against the gestational age. Note the disappearance of any relationship seen in figure 3.24.*

3.16 Mean corpuscular haemoglobin concentration (MCHC) and the filterability of RBC .

There was no correlation between the filterability of red blood cells (Log 1/PTT) and the mean copuscular haemoglobin concentration . The results of this analysis are presented in Table 3.16.

TABLE 3.16

Regression analysis between the filterability of RBC and MCHC .

The regression equation is

Log (1/PTT) = 0.47 - 0.0076 x MCHC (r = 0.00)

Predictor	Coefficient	SD	t-ratio
Constant	0.469	3.255	0.14
MCHC	-0.007	0.098	-0.08

3.17 White Cell Count and the filterability of RBC's

The filtration procedure was designed to allow measurement of the flow properties of red blood cells which is unaffected by the presence of white cells. Figures 3.26 & 3.27 confirm that there is no correlation between either version of log (1/PTT) and the white cell count (WBC). The correlation coefficient for these analyses are .07 and .08 respectively. The probability that these correlations arose by chance is > 0.05. Figure 3.28 shows the electron microscopic photographs of Nuclepore membrane filters after the filtration of blood suspension, confirming that the cells filtering through the pores are red blood cells .

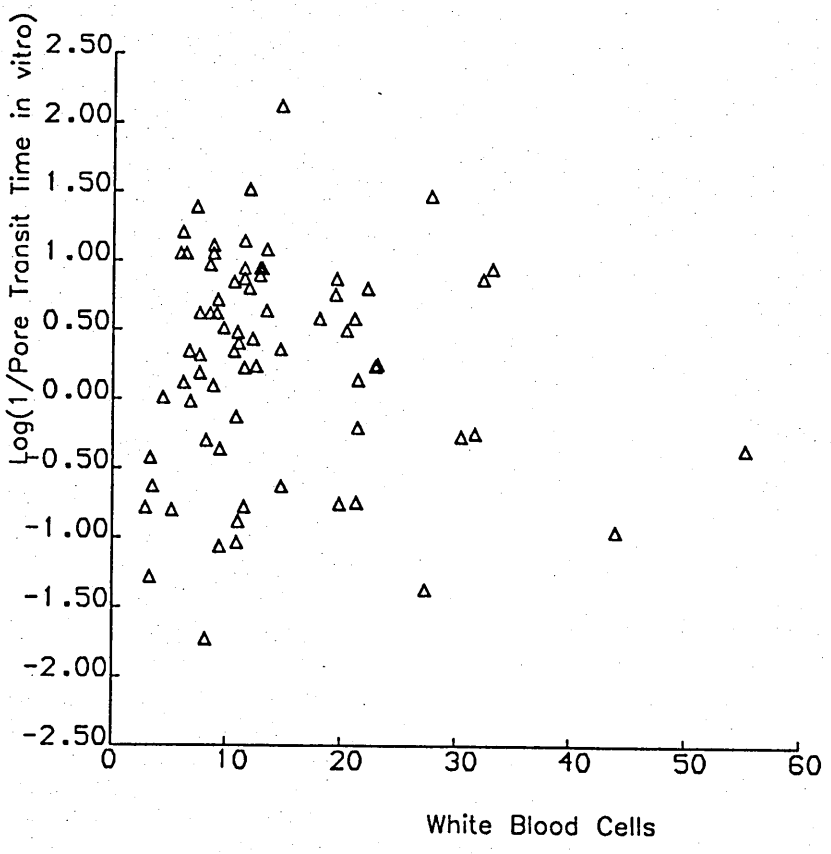


Figure 3.26 *Regression plot of Log 1/PTT versus the white cell count. Note there is no definite correlation between these two factors.*

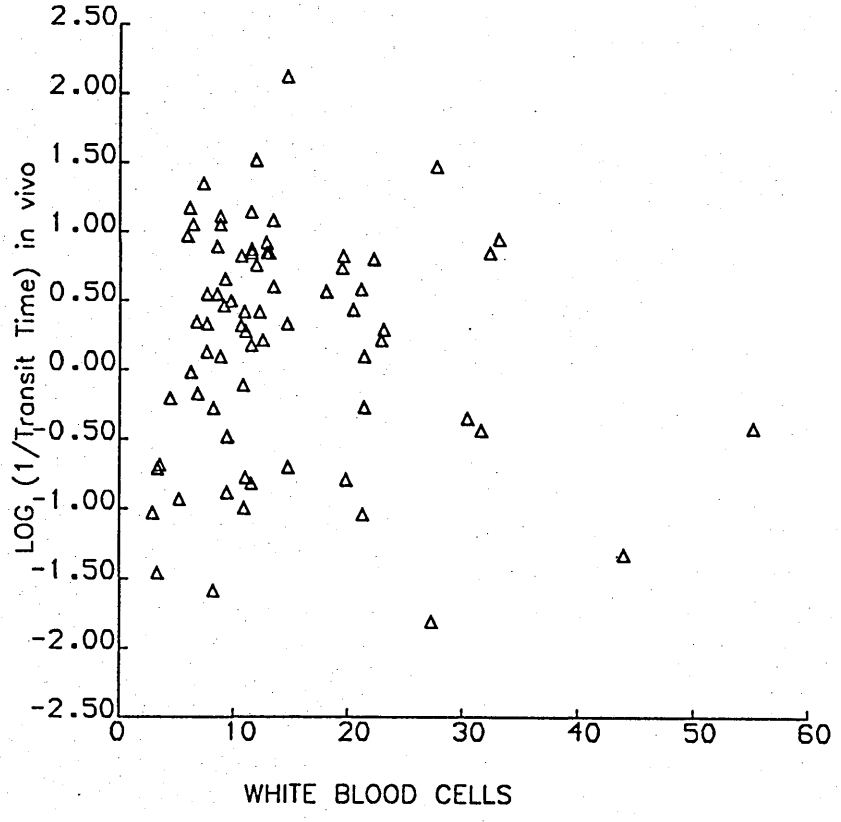
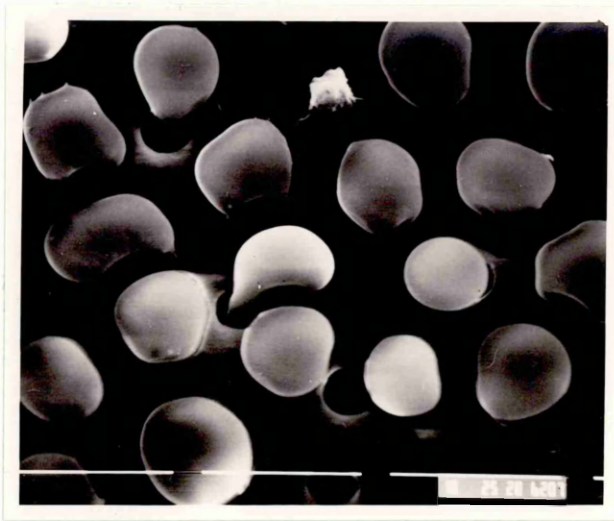
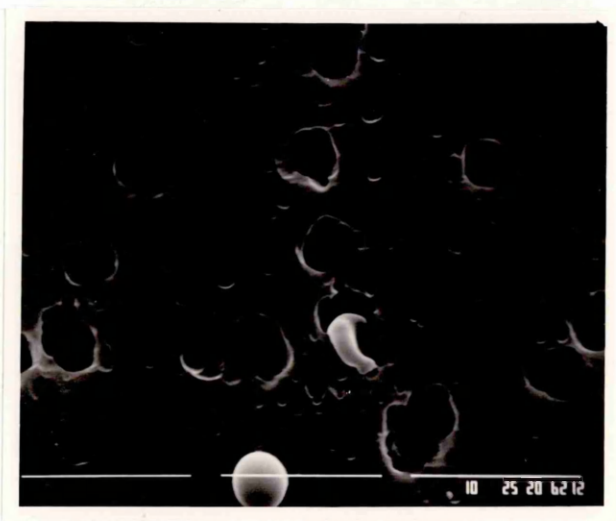


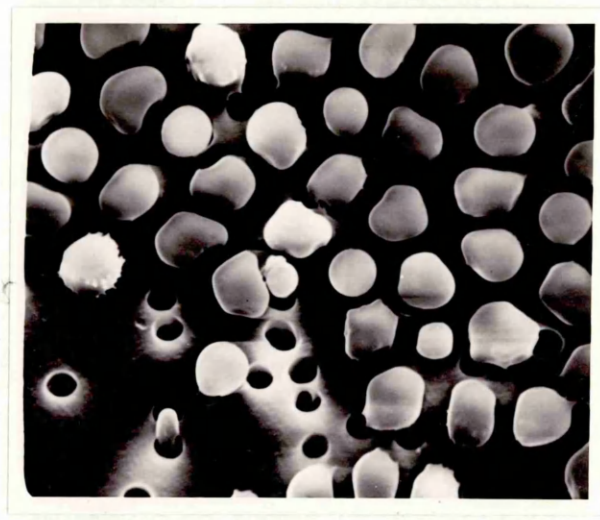
Figure 3.27 *Regression plot of corrected Log (1/PTTiv) versus the white blood cell count.*



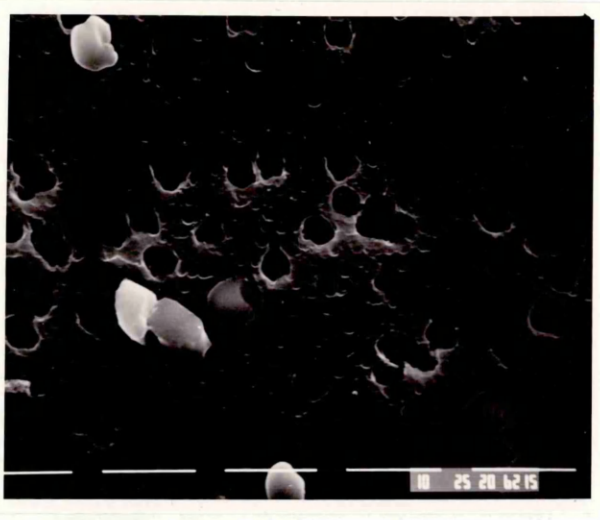
a. *Upper surface of the Nuclepore filter membrane. Magnification x 3500*



b. *Lower surface of the Nuclepore filter membrane Magnification x 3500*



c. *Upper surface x 2000*



d. *Lower surface x 2000*

Figure 3.28 *Scanning electron microscopic photographs of 3um Nuclepore filter membrane after filtration of blood suspension .*

3.18 RBC filterability from Fetus compared to Newborn Infant

There was no statistically significant difference between red cell filterability in blood samples collected from fetuses collected *in utero* and those from gestational age matched newborn infants .The results are collected in Table 3.17 .

TABLE 3.17 Two sample t-test between fetal and infants' red blood cell filterability .

	N	GA *weeks*	Log1/Pore Transit Time (PTT)
Fetal red cell filterability	8	25.12±4.16	-1.055±0.813
Infant red cell filterability	12	26.50±2.24	-0.399±0.713
		P = 0.15	

Values are ± SD .

3.19 Effect of Fetal Haemoglobin (HbF) on the filterability of erythrocytes

Tillman *et al* (1980) suggested that the presence of HbF in the red blood cells of newborn infants might contribute to their rigidity. It was postulated that if the high fetal haemoglobin in the infant's red cells affected their deformability, the cells with lower fetal haemoglobin might filter faster ie; the HbF percentage in the suspensions collected in the early filtrate whould be lower than the later samples. Experiments were done on fetal and neonatal blood samples to estimate percentage of fetal haemoglobin (HbF). Fetal haemoglobin was estimated by alkaline denaturation procedure using the method described by Phillips *et al* (1986) . The experiments were designed to establish the effect of HbF on filterability of red blood cells. The HbF content of a whole blood sample was measured and a cell suspension was prepared for filtration. Aliquots of the filtrate were collected at 15 seconds interval in separate containers. HbF was estimated on each 15 second filtered suspension sample. Since the red cell count in each 15 second filtered sample was very low (sometimes 5 x 10^6/ml), the estimates of HbF were difficult by this method. Other methods were therefore tried including electrophoresis and Kleihauer acid elution technique which involves staining

for cells containing fetal haemoglobin (Kleihauer & Betke,1958) . Finally the modified alkali denaturation procedure described by Phillips *et al* (1988) was selected for HbF estimation in whole blood and each 15 second filtered suspension. The results are presented in Table 3.18 and shown graphically in Figure 3.28 . The results of this initial study show that the HbF content of the RBCs filtered increases gradually with the highest fetal haemoglobin concentration observed in suspension collected after sixty seconds .

TABLE 3.18

Fetal haemoglobin percentage in whole blood and filtered suspensions collected after 15 seconds interval.

Sample no.	GA(*weeks*)	Type of sample	Hb F%
1	40	whole blood	74.8%
1a	40	15 sec filtered suspension	69.0%
1b	40	30 sec filtered suspension	89.1%
1c	40	45 sec filtered suspension	91.3%
1d	40	60 sec filtered suspension	91.0%
2	37	whole blood	75.2%
2a	37	15 sec filtered susp.	50.0%
2b	37	30 sec filtered susp.	47.0%
2c	37	45 sec filtered susp.	60.7%
2d	37	60 sec filtered susp.	63.2%
3	33	Whole blood	88.3%
3a	33	15 sec filtered suspension	65.8%
3b	33	30 sec filtered suspension	82.7%
3c	33	45 sec filtered suspension	70.0%
3d	33	60 sec filtered suspension	95.5%

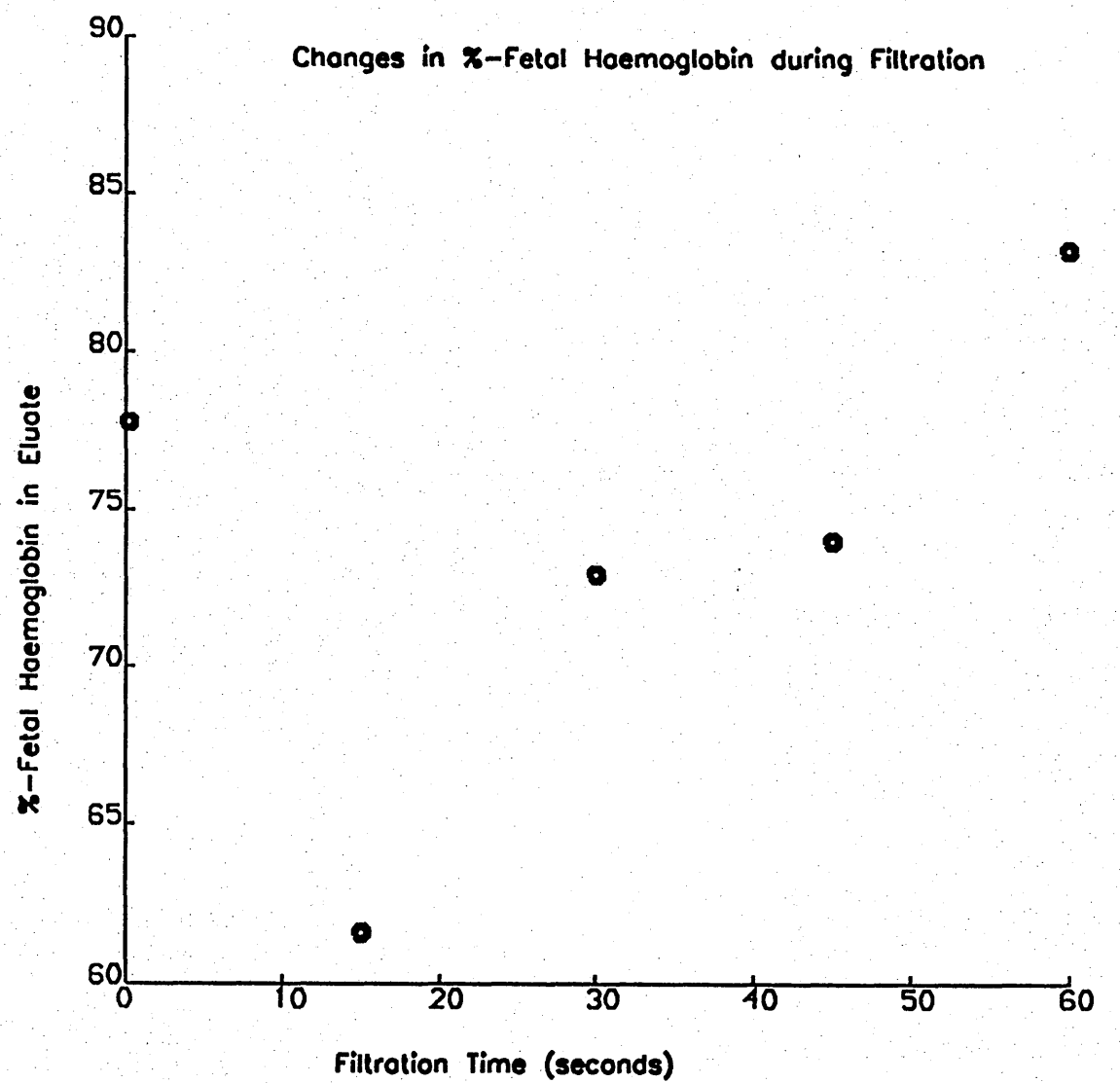


Figure 3.29 *Fetal haemoglobin percentage (HbF%) plotted against the filtration time. Each experimental point represents the mean of HbF% values measured in the blood before the filtration and then in the elute after 15 seconds interval .*

3.20 Filterability of red blood cells and clinical outcome in newborn infants

Infants, both preterm or at term who had not received blood transfusion were selected to compare their clinical outcome with red cell pore transit time. The pore transit time of red cells from infants with each major clinical complication was compared with the relevant control group and for this analysis, each clinical condition was considered in isolation and coincidence with any other complication was ignored.

3.20A. Intrauterine growth retardation

Erythrocyte filterability was measured in thirteen newborn infants who suffered from intrauterine growth retardation.The birth weight of these infants was below the 10th centile when plotted on the growth centile charts prepared by Gairdner and Pearson (1971).Of these 13 IUGR infants,2 were smaller of twins, 2 suffered from pre-eclampsia and mothers of 6 IUGR infants smoked more than 20 cigerettes per day during the pregnancy.In the remaining 3,no probable cause of IUGR could be found.The filterability of red cells in infants with intrauterine growth retardation was compared with sixty one gestational age matched appropriate for gestational age babies .There was a statistically significant difference in red cell

filtration in both groups .The results are presented in table 3.19 .

TABLE 3.19

	No	Gestational Age *weeks*	Pore tansit Time	Pore Transit Time *in vivo*
Small for date infants	13	32.62±4.40	1.25±0.73	1.43±0.97
Appropriate for gestational age infants	61	32.62±5.20	0.96±0.84	1.01±0.73
		P = 0.023		

Values are ± SD.

3.20B Respiratory Distress Syndrome

Fifty five of all the 73 preterm babies in this study suffered from respiratory distress syndrome (RDS) which is one of the major complications of preterm delivery. Most of these babies were less than 33 weeks of gestational age. The severity of respiratory distress syndrome varied from mild to severe , depending upon the the clinical condition, radiological appearance and the ventilatory requirements (Roberton,1987). Of the 55 RDS babies 19 suffered from mild, 17 from moderate and 19 from severe respiratory distress syndrome. Most of the

babies suffering from moderate to severe respiratory distress required ventilatory management and 8 of these developed pneumothorax while on ventilators. None of the term infants and eighteen preterm babies showed any feature of respiratory distress syndrome. Thirty eight preterm babies suffering from RDS who had not received any blood transfusion before the filtration study were compared with the "non RDS" babies. Table 3.20 shows the comparison between pore transit time of red cells in both groups.

Table 3.20.

Relationship between red cell pore transit time and respiratory distress syndrome.

	N	GA *mean* (weeks)	Transit Time *in vitro* (s)	Transit Time *in vivo* (s)
Respiratory Distress Syndrome	38	30	1.173±0.91	1.287±0.96
Non RDS (Control group)	22	33	0.779±0.62	0.804±0.61
Moderate to severe RDS	21	29	1.381±0.91	1.553±1.0
Control group	22	33	0.779±0.62	0.804±0.61

Using the non-parametric Mann-Whitney test or Student's t-test on log transformations, there was no significant difference ($P = 0.07$) in filterability of red cells of babies without RDS and babies with respiratory distress syndrome of all degrees of severity (mild , moderate and severe). However, when babies having moderate to severe RDS were compared with "non RDS" babies, the difference in filterability appeared to be statistically significant ($P = 0.01$). Although both RDS and non RDS babies were group matched for gestational age, and the range of gestational age was similar in both groups, there was a difference in mean gestational age. Therefore the transit time was corrected for gestational age in both groups and the difference in pore transit time of red cells which was statistically significant disappeared.

3.20C. Cerebral Ischaemia and Intraventricular Haemorrhage

Intraventricular haemorrhage (IVH) and ischaemia which is a serious complication of preterm delivery. was correlated with filterability of red blood cells.Of 73 preterm babies in this study 14 suffered from Intracerebral ischaemia and /or Intaventricular haemorrhage . This condition was diagnosed by ultrasound examination by the unit personnel and confirmed on independent ultrasound film examination by the radiologist .

The haemorrhage was graded according to the classification of Papile *et al*(1978) [See Appendix II]. Of these 14 infants in study, 4 had grade I ;3 grade II;4 grade III;2 grade IV intraventricular haemorrhage and one developed leucomalacia. Eight preterm babies between 24 and 34 weeks gestation who had not been transfused before the filtration study and had not suffered from IVH were selected to compare filterability of red cell with age matched untransfused "non IVH" babies. The red cells in these babies filtered poorly as compared to the control babies but the difference was not statistically significant (*P = 0.79*). Results are represented in table 3.21 .

Table 3.21 Correlation between red cell transit time and the incidence of intraventricular haemorrhage.

	N	GA mean (weeks)	Transit Time *in vitro*	Transit Time *in vivo*
Intraventricular Haemorrhage and Cerebral Ischaemia	8	29.4	1.26±0.76	1.45±0.94
Control Group	40	30.5	0.96±0.75	1.02±0.81

3.20D. Jaundice

Of the total 93 infants in this study, 76 suffered from jaundice and 51 of these jaundiced babies required phototherapy. The incidence in preterm infants was 89% (65/73) and in term infants 55% (11/20). The jaundiced infants who were not transfused with adult blood before the study were compared with the gestational matched non-jaundiced infants in relation to pore transit time of red cells. There was no statistically significant difference between both groups as shown by Table 3.22.

Table 3.22

	No	GA	Filtration rate of RBC (1/PTT)	Corrected filtration rate ($1/PTT_{iv}$)
Jaundice infants	68	33	0.313±.796	0.264±.820
Control group	10	35	0.195±.623	0.145±.659

P = 0.61

Other clinical parameters including bronchopulmonary dysplasia, patent ductus arteriosus, septicaemia, fetal distress and smoking in mothers were also considered to

establish any correlation with the filterability of red cells but no such relationship was found. However conclusions can not be drawn on these results as the number of samples in both study and control groups were not properly matched (see appendix IV for detailed results).

CHAPTER 4

DISCUSSION

GENERAL DISCUSSION

The term red cell deformability is not mathematically defined and it is not surprising that studies on the deformability of neonatal red cells may seem to have given conflicting results. There is no significant difference in the deformability between red cells from fetuses, preterm and term infants and adults when the cells are studied under defined shear conditions in a rheoscope (Linderkamp *et al*, 1982, 1986a), an ektacytometer (Coulombel *et al* 1982; Matovcik *et al*, 1986) or a viscometer (Linderkamp *et al*, 1984b). However, many factors which are important determinants of deformability of the red cells, exert minor or no influence on rheoscope, ektacytometer and viscometery results. These factors include RBC diameter, surface area and presence of poorly deformable cells.

The filtration method is the most commonly employed method for measuring the deformability of red cells. Filterability of red cells measures their ability to pass through narrow pore capillaries and reflects the ease with which they flow through the microcirculation *in vivo*. Filtration rates of term infants' red cells through 3 & 5 um filters are markedly lower than for adult red cells

(Gross and Hathaway, 1972; Tillman *et al*, 1977;Buchan,1980 and Reinhart *et al*, 1985). Holland *et al* (1985) and Linderkamp *et al* (1986b) reported even lower red cell filtration rates in preterm infants than in term neonates and adults. However, the correlation between filtration rates and other parameters were found to be different by these working groups. Although Gross & Hathaway, (1972) and Holland *et al* (1985) did not find any significant correlation between the red cell filtration and mean corpuscular volume, Linderkamp *et al* (1986b) and Reinhart *et al* (1985) showed that the filtration rates are inversely related to the mean corpuscular volume (MCV) of red cells from preterm infants, term neonates and adults. This impaired filterability of neonatal red cells was considered to be due to the large cell volume of neonatal red cells. Linderkamp *et al* (1986c) also showed that the negative pressure required to aspirate red cells completely into pipettes of 3.3 *u*m diameter is markedly greater for neonatal red cells compared with adult and this aspiration pressure for neonatal and adult cells increased with increasing cell volume. These observations suggest that the greater mean aspiration pressure for neonatal red cells resulted from their larger volume.

Speculations have been raised that decreased filterability of the neonatal red blood cells could lead to

impaired microcirculatory dynamics in narrow vessels and predispose to the complications seen in infants after preterm delivery (Gross and Hathaway, 1972; Holland *et al*, 1985). Linderkamp and Betke (1985a) suggested that the high risk of intracranial haemorrhage in small preterm infants may in part result from poor red cell aggregation, impeded passage of their large cells through narrow cerebral capillaries and raised blood viscosity due to rapid transfusion of adult blood. However, there is no evidence that neonatal red cells are indeed disadvantageous for adequate circulation and none of the published studies have demonstrated a correlation between decreased deformability of neonatal red cells and the major clinical complications seen in babies.

The present study was designed to measure the filterability of red blood cells in preterm and term babies, fetuses and adults; - to determine the physico-chemical factors affecting it and also to explore a correlation, if there is any, between filterability of red cells and the clinical problems in newborn infants.

Methodology used to study red cell deformability

Since the rheological behaviour of red cell suspension depends on the external fluid viscosity, haematocrit and red cell aggregation in addition to the

degree of red cell deformation, filterability tests with the aim of assessing red cell deformability should be designed to eliminate variations in other parameters. Because of the variabilities of plasma proteins which cause red cell aggregation, tests should be performed on suspensions of red cells in isotonic buffered solutions rather than whole blood (Chien, 1981).

The significant effect of white blood cells on filtration of whole blood while studying RBC deformability had been pointed out by Buchan (1980) and Alderman *et al* (1981). Any technique used for the preparation of a pure erythrocyte suspension carries the inherent risk that the subpopulation of less deformable erythrocytes may be selectively removed. A second risk is that the preparation method may alter the rheology of the erythrocytes (ICSH, 1986). Another problem with removing leukocytes is the size of the samples that one has to work with. Removal of WBCs using methods like filtration through cotton wool or cellulose are not suitable for a small blood sample.The method used in this thesis is the one described by Jones *et al* (1984, 1985) and Khooshesh (1989). The calculation of the pore transit time of red cells (filterability of red cells) is independent of the number of white cells in the suspension (Chapter 3,section B) and of the viscosity of the suspending medium (Koosesh, 1989). This method can

therefore be used with diluted whole blood, thus needing only a few microlitres of blood and removal of white blood cells is not required before filtration studies on the red blood cells.

The Filters: Various types of filters have been used for studying RBC deformability by the filtration method eg; Silver filters, Millipore filters and Nuclepore filters. The Nuclepore polycarbonate sieves have the advantage that the channels are relatively straight and are of 10 *um* length which is in accordance with the recommendations of the International Committee on Standardization in Haematology (1986). Filters with a 3 *um* pore diameter were used in this study since this is approximately the critical size for passage of normal human red blood cells, thus allowing for a more sensitive detection of reduced deformability.

Population in Study: Fetal, preterm and term infant and adult blood samples were taken for this Red Cell Deformability study. Until recently, blood from the fetus *in utero* has been inaccessible for direct observation and measurements. Most of the previous rheological studies were done on umbilical cord blood samples obtained at birth. What changes come about during labour is not known and, just as maternal haemorheology is altered by

intravenous and other therapies of labour (Buchan, 1980), so fetal haemorheology may also be altered. The fetuses studied here were those who underwent fetal blood sampling *in utero* and the red cell deformability measurements performed on such samples have not previously been reported. The correlations performed between the clinical complications in the newborn infants and red cell deformability were based on deformability measurements of venous blood samples from these preterm and term infants.

Effect of storage,pH and temperature on red cell deformability: The results presented in this thesis confirm the earlier reports that the filterability of neonatal erythrocytes is reduced on prolonged storage. With the methodology used in this study, blood can be collected into EDTA and kept for upto 4 hours at room temperature without loss of filterability. If samples are kept for longer periods of time then the cells begin to show signs of metabolic decline and their flow properties deteriorate.

Erythrocyte filterability is determined by the red blood cell membrane visco-elasticity, cytoplasmic viscosity and cell geometry. All of these determinants are influenced by the metabolic competence of the cell in maintaining the level of ATP. This nucleotide determines the level of phosphorylation of spectrin (Palek *et al*,

1978) which in turn may influence the mechanical properties of the membrane cytoskeleton (*Fairbanks et al,* 1981). The biconcave shape of the human red cell is maintained by ATP requiring reactions that are assumed to occur at the membrane-cytoplasm interface (Weed *et al,* 1969). Again ATP is involved in ion pumps that control the intracellular concentration of calcium, potassium and sodium (see Smith *et al,* 1983). The failure of the calcium pump leads to an accumulation of the cation and an increase in covalent cross-linking of spectrin with resulting loss of deformability of the membrane (Lorand, 1979). An increase in intracellular calcium also leads to the stimulation of potassium loss through the so-called Gardos channel with resultant dehydration of the cell and increase in internal viscosity (see Stuart & Elroy, 1987). A decline in the ATP-dependent sodium/potassium pump also leads to a rise in intracellular sodium with resulting swelling and reduced filterability through 3 *um* but not 5 *um* pores (Stuart *et al,* 1985b).

The results presented here also show that the calculated RBC pore transit time for blood from newborn infants increases with decreasing pH and temperature. These results are to be expected according to classical physiology. Hence the haemoglobin exists in equilibrium with hydrogen ions and the position of that equilibrium is

described by the conventional equilibrium constant:-

Hb^{n+} ------ $Hb^{(n-1)+} + H^+$

$K = [Hb^{(n-1)+}] \times [H^+] / [Hb^{n+}]$

$pK = \log (1/K)$

This equilibrium is displaced to the left by:-

(i) Decrease in pH (increase in $[H^+]$

(ii) Increase in pK by decreasing temperature

These effectors will therefore increase the concentration of positive charges on haemoglobin in solution (or decrease the concentration of negative charges). Since the red cell controls the concentration of metal cations by active pumps, this increase in the positive character of the protein is offset by an increase in the intracellular concentration of Cl^- and/or HCO_3^-. The increase in osmolality of the cell contents is then corrected by the entry of water and a resulting increase in Mean Corpuscular Volume (MCV). This is the classical Gibbs Donnan equilibrium effect and will influence the pore transit time of erythrocytes due to changes in cell size and internal viscosity. Results, reported by Kooshesh (1989), are consistent with the hypothesis that the pH effect is explained by the ionisation of a single group . This effect is produced by changes in MCV as pH has no influence on filterability through 5 um pores (Stuart *et al*, 1985b).

The effect of pH and temperature on neonatal blood cell rheology has been reported previously (Buchan, 1980). This work was performed using a whole-blood filtration technique with 5 *um* filter membranes and this method is known to be sensitive to haematocrit, white cell count and plasma viscosity (Buchan, 1980; Alderman *et al*, 1980). Despite these drawbacks some durable conclusions were drawn from this early work. Hence a decline in pH is deleterious to erythrocyte rheology but the effect of temperature is confined mainly to changes in plasma viscosity (see Buchan, 1980). The flow of adult erythrocytes through 5 *um* membranes is relatively insensitive to changes in temperature (Schmid-Schonbein, 1973; Stuart *et* al, 1985). Although the results of Hanss & Koutsouris (1984) show an effect with an energy of activation [Ea] of 11.3 kJ/mol (see Kooshesh, 1989) i.e; this degree of sensitivity to temperature probably reflects a combination of decreasing viscosity and MCHC with increasing temperature. This is partly offset by an increase in MCV. The energy of activation is therefore a little higher than that found for the viscosity of simple salt solutions (see above) or solutions of plasma proteins (see Rampling & Whitingstall, 1987).

The results presented in chapter 3, section A of this thesis confirm the earlier reports of decreased

deformability of fetal red blood cells in acidotic conditions (Gross and Hathaway, 1972; Buchan, 1980) and demonstrates that low temperature also adversely effects the erythrocytic filterability. The influence of pH and temperature was found to be reversible. Any experimental procedure cannot duplicate exactly the hypothermia/acidosis which might occur *in vivo* but can point to the direction and approximate magnitude of changes in erythrocyte filterability that are likely to occur in such clinical disorders. The implications of these findings are interesting. It is well documented that infants with acidosis and hypothermia are prone to develop clinical complications (Roberton, 1986), a hypothesis can be made that the clinical complications seen in preterm babies eg; intracerebral ischaemia, haemorrhage and necrotising enterocolitis could be related to decreased deformability of red cells in these acidotic and hypothermic babies. Hypoxic acidosis and hypothermia by causing pronounced decrease in erythrocyte deformability, combined with the already disturbed microcirculatory blood flow, lack of autoregulation and hypotension could initiate thrombosis in the microcirculation of these sick babies. Capillary thrombosis might rapidly lead to haemorrhage in a situation complicated by secondary coagulation failure (Turner, 1976; Foley & McNicol, 1977;Turner *et al*,1981).

Differences in red cell filterability in fetuses, preterm and term infants and adults

The filterability of red cells is directly related to the gestational age of the infant, with no significant difference between cells sampled *in utero* or *ex utero* for age matched infants. Hence there is no evidence that the "trauma" of birth has a long-term deleterious effect on the rheology of erythrocytes. However even cells from healthy term infants are considerably more resistant to flow than those from adult blood and the transfusion of infants with adult blood therefore produces a marked improvement in the flow properties of red cells. In this study, the filterability of erythrocytes increased four-fold after transfusion with adult blood cells.

The accepted explanation for the decreased filterability of erythrocytes seen in preterm infants is the age related decrease in MCV. Hence the plot of filterability against MCV shows a linear decline from the high values of MCV seen in the most immature infants to the lowest MCV seen in adults. These results accord well with those already reported by Linderkamp (1987). However, multiple regression analysis of the data reported here shows clearly that the gestational age of the infant and

the MCV of the erythrocytes are independent determinants of red cell filterability. Therefore, apart from their larger size, neonatal red cells are inherently less filterable than those of adults.

The other factors which are known to influence red cell deformability were considered as possible causes. Cytoplasmic viscosity of the cell is one such main determinant of cell filterability. In human red cells, the internal viscosity is mainly determined by the haemoglobin concentration (Chien 1981) and also by the type of haemoglobin ie; haemoglobin F (Tillman, 1980). MCHC was not found to be statistically different in different groups studied, thus it is not a factor responsible for the difference in RBC deformability. The influence of HbF on the filterability of fetal and neonatal erythrocytes was assessed in a preliminary study. The slower rate of filtration of cells containing a high proportion of fetal haemoglobin can be seen within a single blood sample. Hence, during filtration through 3 um pores, cells with a higher proportion of adult haemoglobin emerge from the filter sooner than the less mature cells with a higher proportion of fetal haemoglobin. It is not yet clear whether a solution containing fetal haemoglobin is more viscous than one containing adult haemoglobin or whether both filterability and fetal haemoglobin content are

markers of MCV and/or the state of maturity of the erythrocyte. Data presented by Linderkamp *et al* (1986c) suggest that the viscosity of fetal haemoglobin may be marginally higher than that of adult haemoglobin but this difference was not statistically significant.

Another possible explanation for the difference in RBC filterability between fetal, neonatal and adult cells might be the difference in surface area/volume relationship in the fetal, infants and adult red cells (Linderkamp O, personal communication). A recent study by Stadler and Linderkamp, (1989) has demonstrated that the neonatal RBC when compared to adult RBCs show 18.6% larger volume, 12% greater surface area and 11.4% wider diameter. Because of their larger size, neonatal red cells have a greater minimal cylindrical diameter, defined as the smallest cylinder a red cell can enter without a loss of volume (3.04 ± 0.25 *um* in neonatal red cells; 2.81 ± 0.23 *um* in adult RBCs). Filtration studies through 3 *um* size pores might be influenced by the greater minimal cylindrical diameter of neonatal RBCs.

Red cell membrane properties could also influence the deformability of fetal and adult erythrocytes. A marked increase in the quantity of both cholesterol and phospholipid is characteristic of neonatal red cells

(Matovcik & Mentzer,1985). This increase is greater in preterm than in term infants and is in part, secondary to the increased surface area of the larger neonatal red cell. However, based on theoretical calculations, the increase in lipid content exceeds that required for the surface area by at least 20% (Neerhout, 1968 & 1971). Moreover, it is known that fetal erythrocytes have a diminished ability to handle oxidant stress and exhibit other membrane lipid alterations (Neerhout, 1968).

The filtration technique is very sensitive to the presence of a small percentage of poorly deformable RBCs which may gradually block more and more pores, thereby hindering the passage of subsequent RBCs and decreasing overall filtration rates. Fetuses and newborn infants of lower gestational ages have a high nucleated red cell count and reticulocytes which are more resistant to passage through narrow channels than mature RBCs. These might act as poorly deformable cells thus showing differences in overall red cell filterability among different gestational age subjects.

The life span of a preterm RBC is about 40-60 days, compared to 60-80 days in a term infant and 120 days in adults (Pearson,1967;Roberton,1986). The prolonged pore transit time of neonatal RBCs through filters suggests that

the passage time through the small splenic slits is also prolonged (Cokelet *et al*, 1981), thus explaining the shortened life span of neonatal RBC as compared to the adult. In preterm infants, RBC survival time is even shorter than in term infants which agrees with the RBC deformability findings of this study (deformability of RBC in preterm infants < term infants < adults). A normal RBC passes through the spleen about 3,600 times during its life and each passage requires marked deformation. The minimum size of human splenic capillaries is about 3 *um* (Weiss and Tavassoli, 1970) and the decreased deformability through these capillaries probably leads to destruction of the RBCs in the spleen.

Clinical Outcome:

Intrauterine growth retardation (IUGR) in newborn infants is associated with neonatal hyperviscosity and its complications. Placental insufficiency and fetal hypoxia is the usual final common pathway leading to growth retardation in infants. Placental insufficiency may be caused by infection,pre-eclempsia or maternal smoking in pregnancy.Reduced maternal red cell filterability has been observed by Thorburn *et al*(1982) and Buchan(1984a),in pregnancies with intrauterine growth retardation.Buchan (1984a) also observed decreased red cell filterability and

increased blood viscosity in cord blood of infants of mothers who were smokers during pregnancy and those who developed pre-eclempsia during pregnency.The results presented in this thesis (Chapter 3,section B) confirm that the deformability of red cells is decreased in intrauterine growth retardation independent of the causal factors. Because of the delayed brain growth,growth retarded fetuses are at greater risk of delayed development and neurological problems.Whether decreased brain perfusion resulting from increased haematocrit and decreased red cell deformability is involved in cerebral dysfunction of these infants is unknown.

Infants born after preterm delivery suffer from various clinical complications - more so than the term newborn infants. Since the deformability of preterm infants RBCs is decreased as compared to the term infant, the hypothesis was made that this decreased RBC deformability in the preterm infant might be a factor contributing to the poorer clinical outcome.

In this thesis, the red cell deformability, which could possibly be a factor in theory, to the development of major clinical problems such as intracerebral ischaemia/ haemorrhage, respiratory distress syndrome and jaundice was studied. Though the deformability of red cells was found

to be decreased in intraventricular haemorrhage /intracerebral ischaemia and respiratory distress syndrome babies as compared to gestational age matched babies without these problems, the difference was not statistically significant. However, to confirm these findings a larger study population is needed.

Speculations about decreased RBC deformability and impaired microcirculatory flow in narrow channels leading to clinical complications in preterm babies may not be true since RBC filterability is also found to be decreased in normal fetuses of lower gestational ages. There is no evidence that fetal and neonatal RBCs are indeed disadvantageous for adequate circulation since the circulation in the neonate is characterised by low vascular resistance and high flow conditions (WU *et al*, 1980). Moreover, the minimal capillary size in fetus and preterm newborn infants may also be larger than in term infants and in adults.

The capillary network in the fetal and newborn infant's brain has been examined extensively in relation to intraventricular haemorrhage. Pape & Wigglesworth (1979) has characterized the elaborate capillary bed of germinal matrix as a "persisting imature vascular rete". Transmission electron microscopic studies of the germinal

matrix revealed many small larger diameter vessels with features of immature vessels (Pinar *et al*, 1985). Studies have also shown that matrix microcirculation is composed of simple endothelial-lined vessels, often of a larger size than capillaries but not recognizable as arterioles or venules because of absence of muscle and collagen. Trommer *et al*, (1987) demonstrated that the capillaries in the brain of a newborn beagle puppy are also relatively larger in size. No published studies were found in the literature about capillary size in general microcirculation of human fetal and preterm newborn infants. However, animal studies performed on fetal and newborn mammals present interesting findings. A recent study by Porter and Bankston (1987) on myocardial capillary diameter and RBC size in fetal and neonatal rat has shown that there is a gradual decrease in capillary diameter during late fetal and early neonatal life. The average capillary diameter showed a significant decrease from 4.74 *um* $\pm$ 0.28 *um* in fetal rat to 3.61 *um* $\pm$0.20 *um* in the + 7 day neonatal rat ie; a decrease in diameter of 24%. This decrease in diameter was found to occur concomitantly with the decrease in the mean diameter of circulating red blood cells. The mature erythrocytes of rats have been measured to have a mean diameter of 6.8 *um* and are capable of deformability to pass through a capillary as small as 2.45 *um* in diameter (Henquell *et al*, 1976). The mean diameter of the RBC was found to decrease

from 10.31 um in fetal rat to 6.68 um in adult ie; a 35% decrease in diameter.

The pattern of decrease in RBC diameter in fetal to neonatal and adult rats is similar to that observed in human fetal, preterm and term newborn infants and adult red cells in the present study (Chapter 3, Section B) and this leads to the speculation that it would be a functional necessity in the human fetus and preterm infant to have a capillary diameter larger than that found in term infants and adults.

Future Developments

The work presented in this thesis describes the flow characteristics of fetal, neonatal and adult red cells through 3 um pore size filters which is the minimum size of capillaries in the human adult. Since the exact size of capillaries in general is not known for the human fetus and newborn infant and since animal studies point to a larger capillary size in the fetus and newborn than in the adult, it will be very important to study the microcirculation in the fetus and newborn infant before any conclusion can be made about the role of decreased deformability of red cells in these subjects.

The white cell count in normal preterm infants is higher than in term infants and adults (Chapter 3, table 3.3). The present study measures the filterability of RBCs only, but if we consider the microcirculatory flow of whole blood the WBCs deformability will also be important. White cells exhibit 700-1000 times more resistance to passage through 5 um filters pores than red cells (Chien *et al*, 1983). Considering the fact that the WBC count is much higher in preterm infants than in normal infants and adults, white cells may be a major determinant of microcirculatory flow *in vivo*. The methodology used in this thesis could also be used for measuring the filterability of white cells using 5 *um* nuclepore membranes. A study of fetal and preterm infant's white blood cell deformability would lead to a more detailed understanding of microcirculatory blood flow in fetuses and immature infants.

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APPENDICES

APPENDIX I

Algorithm for Analysing Flow Profiles for Cell Suspensions

```
C     CALCULATION OF 6 VARIABLES USING  INTEGRATED FORMS
C     OF EQUATIONS FOR THREE PARTICLES  AND OMEGA
C     OMEGA IS USED AS THE INTIAL FLOW RATE IF THIS IS LOWER
C     THAN THAT OF BUFFER. IT IS PARTICULARLY USEFUL FOR 5 MICRON
C     FILTRATION. THE USER CHOOSES WHETHER TO :-
C       (1) FIX OMEGA AT 1.0
C       (2) FIX OMEGA AT <1.0
C       (3) ALLOW OMEGA TO FLOAT BETWEEN 0 AND 1.0
C     FOR ALL WORK DESCRIBED IN THIS THESIS OMEGA WAS FIXED AT 1.0
C     AND N FIXEWD AT 4

      IMPLICIT DOUBLE PRECISION(A-H,O-Z)
      CHARACTER*20 NAME,DATE
      COMMON ITM,IBLANK,E,TIME,THEORY,R,XT,CELLS,T
      COMMON/AREA6/ OMEGA
      COMMON/AREA3/ NOMEGA,JCALL
      COMMON/AREA4/ TYPE1,TYPE2,TYPE3
      DIMENSION IW(12),BL(10),BU(10),W(170),X(10),A(10),
     &TIME(500),THEORY(500),E(500),R(500)

      LW=170
      LIW=12
      IBOUND=0
      DO 13 I = 1 , 500
      TIME(I) = 0.0
   13 E(I) = 0.0
      WRITE(4,100)
  100 FORMAT(1X,20X,'CURVE FITTING FOR FILTRATION')
      WRITE(4,110)
  110 FORMAT(1X,20X,'*****************************')
C
C
  120 CONTINUE
C     ENTER NAME AND DATE OF SAMPLE
C
      READ(5,20,END=21000) NAME
   20 FORMAT(A20)
      READ(5,20) DATE
C
C     ENTER FLOW RATE OF BUFFER, NUMBER OF POINTS, NUMBER OF PORES
C     AND NUMBER OF CONSTANTS
C
      READ(5,*) XT,ITM,CELLS,T,N
C     READ CODES AS FOLLOWS:-
C     0     1.0   ..... NO OMEGA FACTOR REQUIRED
C     1     0     .....  OMEGA IS AN UNKOWN VARIABLE
C     2     0.43 ..... OMEGA = 0.43 [OR ANY NUMBER < 1.0]
      READ(5,*) NOMEGA,OMEGA
      IF(NOMEGA.EQ.1) N = N + 1
C     ENTER ESTIMATES FOR N VARIABLES
      READ(5,*)(A(I),I=1,N)
C     ENTER TIME AND VOLUME COLLECTED
      READ(5,*)(TIME(I),E(I),I=1,ITM)
      WRITE(4,65) NAME,DATE
      WRITE(4,70) XT
   65 FORMAT(1X,//,' Name = ',A20,5X,'Date = ',A20)
   70 FORMAT(1X,/,' XT =',F7.3,//)
      DO 50 I = 1,N
   50 X(I) = A(I)
C     SET LIMITS ON VARIABLES
      DO 3 I = 1 , N
      BL(I) = 1D-06
   3  BU(I) = 1D+06
```

```
      BU(1) = CELLS * XT/T
      BU(3) = BU(1)
      BU(5) = BU(1)
      IF(NOMEGA.EQ.1) BU(N) = 1.0
      IFAIL=1
      JCALL = 0
      CALL E04JAF(N,IBOUND,BL,BU,X,F,IW,LIW,W,LW,IFAIL)
      IF(NOMEGA.EQ.1) OMEGA = X(N)
      N1 = 0
      IF(NOMEGA.EQ.1) N1 = 1
      WRITE(4,71)  IFAIL, F
   71 FORMAT(1X,//,'   IFAIL =',I2,1X,
     &'SUMS OF SQUARES = ',D10.4)
      WRITE(4,600)
  600 FORMAT(1X,//,32X,'VARIABLE',18X,'BEST ESTIMATE')
      DO 611 I = 1 , N - N1
      WRITE(4,610) I , X(I)
  610 FORMAT(1X,33X,'K',I1,D35.3)
  611 CONTINUE
      WRITE(4,612) OMEGA
  612 FORMAT(1X,30X,'OMEGA',D35.3)
      WRITE(4,620)
  620 FORMAT(1X,///)
      IF(IFAIL.EQ.1.OR.IFAIL.EQ.9) GO TO 20000
C     CALCULATE BEST THEORETICAL CURVE
      JCALL = 1
      CALL FUNCT1(N,X,F)
      WRITE(4,699)
  699 FORMAT(11X,'TIME',9X,'VOLUME',4X,'THEORETICAL',7X,'RESIDUAL')
      WRITE(4,698)
  698 FORMAT(11X,'================================================',/)
      WRITE(4,701)(TIME(I),E(I),THEORY(I),R(I),I=1,ITM)
  701 FORMAT(3F15.2,F15.4)
      IF(NOMEGA.GT.0) THEN
        TORR = T * (1-OMEGA)/(OMEGA*XT*CELLS)
        IF(N.GT.1) TORR1 = 1/X(2)
        IF(N.GT.3) TORR2 = 1/X(4)
      ELSE
        CLUST=CELLS/(TYPE1+TYPE2+TYPE3)
        TORR = 1/(X(2)*CLUST)
        IF(N.GT.4) TORR1 = 1/(X(4)*CLUST)
       ENDIF
      WRITE(4,740)TYPE1,TYPE2,TYPE3
  740 FORMAT(1X,//,' Type One Particles   = ',D10.3,/,' Type Two',
     &' Particles   = ',D10.3,/,' Type Three Particles = ',D10.3)
      WRITE(1,762) TORR,TYPE2,TORR1,X(3)
  762 FORMAT(F10.3,2D10.3,F10.4)
      WRITE(4,760)TORR,TORR1,TORR2
  760 FORMAT(1X,/,' Pore Time(1) = ',F7.4,'  Pore Time'
     &,' (2) = ',F8.3, ' Pore time(3)',F8.3)
      IF(NOMEGA.EQ.0) WRITE(4,761) CLUST
  761 FORMAT(1X, '  Cluster Size = ',F10.1)
20000 CONTINUE
      GO TO 120
21000 CONTINUE
      STOP
      END
      SUBROUTINE FUNCT1(N,XC,FC)
      IMPLICIT DOUBLE PRECISION(A-H,O-Z)
      COMMON ITM,IBLANK,E,TIME,THEORY,R,XT,CELLS,T
      COMMON/AREA3/ NOMEGA,JCALL
      COMMON/AREA6/ OMEGA
      COMMON/AREA4/TYPE1,TYPE2,TYPE3
      DIMENSION E(500),TIME(500),THEORY(500),R(500),XC(N),YA(500)
      AK1 = 1.0D-10
      AK2 = 0.0
      AK3 = 1.0D-10
```

```
      AK4 = 0.0
      AK5 = 1.0D-10
      AK6 = 0.0
      N1 = N - NOMEGA
      IF(N1.GT.0) AK1 = XC(1)
      IF(N1.GT.1) AK2 = XC(2)
      IF(N1.GT.2) AK3 = XC(3)
      IF(N1.GT.3) AK4 = XC(4)
      IF(N1.GT.4) AK5 = XC(5)
      IF(N1.GT.5) AK6 = XC(6)
      IF(NOMEGA.EQ.1) OMEGA = XC(N)
      PT = XT * OMEGA
      ALPHA=AK1+AK2+AK3+AK5
      BETA=AK3+AK4+AK5
      GAMMA=AK5+AK6
      ALPHA1=ALPHA-GAMMA
      BETA1=BETA-GAMMA
      FC=0.0
      DO 5000 J=1,ITM
      EXA=ALPHA*TIME(J)
      EXB=BETA*TIME(J)
      EX5=GAMMA*TIME(J)
      IF(EXA.GT.88) EXA=88
      IF(EXB.GT.88) EXB=88
      IF(EX5.GT.88) EX5=88
      FA = 1 - DEXP(-EXA)
      FB = 1 - DEXP(-EXB)
      F5 = 1 - DEXP(-EX5)
      A = 1-AK3/BETA1 -AK1/ALPHA1 + AK1*AK3/(ALPHA1*BETA1)
      AA = F5/GAMMA
      B = AK3/BETA1 - AK1*AK3/(BETA1*(ALPHA-BETA))
      BB = FB/BETA
      C = AK1/ALPHA1 - AK1*AK3/(BETA1*ALPHA1)
      C = C + AK1*AK3/(BETA1*(ALPHA-BETA))
      CC = FA/ALPHA
      A = A * AK5/GAMMA
      B = B * AK5/GAMMA
      C = C * AK5/GAMMA
      ADD1 = (1-AK5/GAMMA) * (AK3/BETA - AK1*AK3/(BETA*(
     &ALPHA-BETA)))
     & * FB/BETA
      ADD2 = (1-AK5/GAMMA) * (AK1/ALPHA - AK1*AK3/(ALPHA
     &*BETA) + AK1*AK3/BETA*(ALPHA-BETA))
     & * FA/ALPHA
      ADD3 = TIME(J) * (1-AK5/GAMMA) * (1+AK1*AK3/(ALPHA*BETA)
     & - AK1/ALPHA - AK3/BETA)
      THEORY(J) = PT*(A*AA + B*BB + C*CC + ADD1 + ADD2 + ADD3)
      R(J)=THEORY(J)-E(J)
      FC=FC+R(J)*R(J)
5000  CONTINUE
      IF(JCALL.EQ.0) GO TO 4000
      TN = T * AK5/GAMMA
      XEND = TIME(ITM)
      EXA = ALPHA*XEND
      EXB = BETA*XEND
      EX5 = GAMMA*XEND
      IF(EXA.GT.88) EXA = 88
      IF(EXB.GT.88) EXB = 88
      IF (EX5.GT.88) EX5 = 88
      FA = 1-DEXP(-EXA)
      FB = 1-DEXP(-EXB)
      F5 = 1-DEXP(-EX5)
      TYPE3 = TN*F5
      T1 = AK6*TN
      T2 = XEND - F5/GAMMA
      TYPE3 = TYPE3 + T1*T2
      P2 = F5*TN*AK3*AK4/(GAMMA*BETA1) - FB*TN*AK3*AK4/(BETA*BETA1)
```

```
     *+AK3*TN*DEXP(-EX5)/BETA1 -AK3*TN*DEXP(-EXB)/BETA1
      T1 = FB*AK3*T*(1-AK5/GAMMA)/BETA
      T2 = AK3*AK4*XEND*T*(1-AK5/GAMMA)/B
      T3 = FB*AK3*AK4*T*(1-AK5/GAMMA)/(BETA*BETA)
      W = P2 + T1 + T2 + T3
      T1 = T*AK1*FA*(1-AK5/GAMMA-AK3/BETA+AK3*AK5/(BETA*GAMMA))/ALPHA
      T2 = T*AK1*AK5*(DEXP(-EX5)-DEXP(-EXA))*(1-AK3/(BETA-GAMMA))
     & /(GAMMA*ALPHA1)
      T3 = T*AK1*AK3*(DEXP(-EXB)-DEXP(-EXA))*(1-AK5/GAMMA +
     &BETA*AK5/(GAMMA*BETA1))/(BETA*(ALPHA-BETA))
      T4 = T*AK1*AK2*XEND*(1-AK5/GAMMA-AK3/BETA+AK3*AK5/(
     &BETA*GAMMA))/ALPHA
      T6 = FA*T*AK1*AK2/ALPHA
      T6 = T6*(AK3/(ALPHA*BETA) - 1/ALPHA + AK5/(ALPHA*GAMMA)
     & - AK3*AK5/(ALPHA*BETA*GAMMA) -AK5/(ALPHA1*GAMMA)
     & + AK3*AK5/(GAMMA*BETA1*ALPHA1) - AK3/(BETA*(ALPHA-BETA))
     & + AK3*AK5/(GAMMA*BETA*(ALPHA-BETA))
     & - AK3*AK5/(GAMMA*BETA1*(ALPHA-BETA)))
      T7 = T*FB*AK1*AK2*AK3/(BETA*(ALPHA-BETA))
      T7 = T7 * (1/BETA - AK5/(GAMMA*BETA) + AK5/
     & (GAMMA*BETA1))
      T8 = T*F5*AK1*AK2*AK5/(GAMMA*GAMMA*ALPHA1)
      T8 = T8 * (1-AK3/BETA1)
      R1 = T1 + T2 + T3 + T4 + T6 + T7 + T8
      W = P2
      TYPE2 = W/THEORY(ITM)
      TYPE1 = R1/THEORY(ITM)
      TYPE3 = TYPE3/THEORY(ITM)
 4000 RETURN
      END
```

Example Data File For a Profile with 30 Experimental Points

```
A.N.OTHER
TODAY
0.33 30 150E06 6.28E06 4
0 1
0.57 .105 .033 .0064
     1.000     0.210     2.000     0.320     3.000     0.410
     4.000     0.480     5.000     0.550     6.000     0.620
     7.000     0.660     8.000     0.710     9.000     0.760
    10.000     0.800    11.000     0.830    12.000     0.860
    13.000     0.900    14.000     0.930    15.000     0.960
    16.000     1.000    17.000     0.990    18.000     1.030
    19.000     1.070    20.000     1.090    21.000     1.120
    22.000     1.150    23.000     1.180    24.000     1.190
    25.000     1.230    26.000     1.240    27.000     1.260
    28.000     1.290    29.000     1.310    30.000     1.330
```

Result file04 from this data file

CURVE FITTING FOR FILTRATION

Name = A.N.OTHER Date = TODAY

XT = 0.330

IFAIL = 0 SUMS OF SQUARES = 0.1594D-02

VARIABLE	BEST ESTIMATE
K1	0.109D+01
K2	0.685D+00
K3	0.163D+00
K4	0.314D-01
OMEGA	0.100D+01

TIME	VOLUME	THEORETICAL	RESIDUAL
1.00	0.21	0.21	-0.0030
2.00	0.32	0.32	0.0006
3.00	0.41	0.41	-0.0004
4.00	0.48	0.49	0.0057
5.00	0.55	0.55	0.0023
6.00	0.62	0.61	-0.0087
7.00	0.66	0.66	0.0039
8.00	0.71	0.71	0.0014
9.00	0.76	0.75	-0.0053
10.00	0.80	0.79	-0.0056
11.00	0.83	0.83	0.0012
12.00	0.86	0.87	0.0057
13.00	0.90	0.90	-0.0019
14.00	0.93	0.93	-0.0010
15.00	0.96	0.96	-0.0014
16.00	1.00	0.99	-0.0130
17.00	0.99	1.01	0.0245
18.00	1.03	1.04	0.0114
19.00	1.07	1.07	-0.0025
20.00	1.09	1.09	0.0032
21.00	1.12	1.12	-0.0015
22.00	1.15	1.14	-0.0066
23.00	1.18	1.17	-0.0119
24.00	1.19	1.19	0.0025
25.00	1.23	1.22	-0.0133
26.00	1.24	1.24	0.0008
27.00	1.26	1.26	0.0047
28.00	1.29	1.29	-0.0014
29.00	1.31	1.31	0.0023
30.00	1.33	1.34	0.0060

Type One Particles = 0.169D+08
Type Two Particles = 0.701D+07
Type Three Particles = 0.141D-01

Pore Time(1) = 0.2328 Pore Time (2) = 0.000 Pore time(3) 0.000
Cluster Size = 6.3

APPENDIX II

Classification of cerebroventricular haemorrhage (Papile *et al.*, 1978)

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1. GLH

2. IVH without ventricular dilatation

3. IVH with ventricular dilatation

4. IVH with parenchymal haemorrhage

Note: Bilateral haemorrhages graded according to size of the larger haemorrhage.

APPENDIX III

The values to be exceeded for the coefficient of simple correlation to be significant.

Number of Points on Scatter Graph	10% Significance Level	5% Significance Level	1% Significance Level
3	0.988	0.997	1.00
4	0.900	0.950	0.99
5	0.805	0.878	0.95
6	0.729	0.811	0.91
7	0.669	0.755	0.87
8	0.621	0.707	0.83
9	0.582	0.666	0.79
10	0.549	0.632	0.76
11	0.521	0.602	0.73
12	0.497	0.576	0.70
13	0.476	0.553	0.68
14	0.457	0.532	0.66
15	0.441	0.514	0.64
16	0.426	0.497	0.62
17	0.412	0.482	0.60
18	0.400	0.468	0.59
19	0.389	0.456	0.57
20	0.378	0.444	0.56
21	0.369	0.433	0.54
22	0.360	0.423	0.53
23	0.352	0.413	0.52
24	0.344	0.406	0.51
25	0.337	0·396	0·50
26	0.330	0.388	0.49
27	0.323	0.381	0.48
28	0.317	0.374	0.47
29	0.311	0.367	0.47
30	0.306	0.361	0.46
35	0.283	0.335	0.43
40	0.264	0.312	0.40
45	0.249	0.294	0.38
50	0.235	0.279	0.36
60	0.215	0.255	0.33
70	0.198	0.236	0.30
80	0.185	0.220	0.28
90	0.175	0.207	0.27

APPENDIX IV

TABLE 1

Red Cell Filterability & Bronchopulmonary Dysplasia (BPD)

	No.	GA *weeks* mean±SD	Pore Transit Time *in vivo* (PTT_{iv})	Log1/Pore Transit Time *in vivo* (Log $1/PTT_{iv}$)
BPD Infants	6	28.5±1.8	1.39±0.86	-0.23±0.64
Control Groups (Non BPD Infants)	22	28.0±1.6	1.66±1.02	-0.34±0.78
		P = 0.74		

TABLE 2

Red Blood Cell Filterability and Patent Ductus Arteriosus (PDA)

	No.	GA *weeks* mean±SD	Pore Transit Time *in vivo* $PTT_{i\,v}$	Log 1/Pore Transit Time *in vivo* ($Log1/PTT_{i\,v}$)
Infants with PDA	5	29.6±0.5	1.12±0.65	-0.08±0.29
Infants without PDA	12	29.3±0.4	1.86±0.93	-0.31±0.87
		p = 0.56		

TABLE 3

Comparison of filterability of RBC in Septicaemia and non Septicaemic Infants

	No.	GA *weeks* mean±SD	Pore Transit Time *in vivo* (PTT_{iv})	Log of $1/PTT_{iv}$
Infants who developed septicaemia	4	30.7±7.2	1.77±1.32	-0.47±0.83
Infants who didn't suffer septicaemia	31	33.2±3.8	0.93±0.81	0.38±0.83
		p = 0.15		

TABLE 4

Red Cell Filterability in Infants of Smoking and Non Smoking Mothers

	No.	GA *weeks* mean±S	Pore Transit Time *in vivo* (PTT_{iv})	Log of 1/Pore Transit Time *in vivo* (Log $1/PTT_{iv}$)
Infants of smoker mothers	16	31.5±4.2	1.06±0.78	0.11±0.62
Infants of non smoker mothers	62	33.0±4.4	0.98±0.83	0.28±0.83
		p = 0.37		

TABLE 5

Filterability of red cells in infants who had fetal distress as compared to normal deliveries

	No.	GA	$PTT_{i\,v}$	$Log(1/PTT_{i\,v})$
FD	15	32.5±4.1	0.89±0.89	0.37±0.75
Non FD	63	32.7±4.5	1.02±0.80	0.21±0.81
		p = 0.47		