The Effects of Ovarian Steroids on Erythrocytes and Coagulation Markers in Women with Sickle Cell Disease

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Abstract
Sickle cell disease (SCD) is an inherited haemoglobin disorder characterised by chronic haemolysis, anaemia and recurrent episodes of occlusion in the microvasculature by poorly deformable erythrocytes. There is experimental and clinical evidence suggesting that ovarian hormones, principally oestradiol and progesterone, modify the process of sickling. As red cells do not appear to express steroid receptors, it has been postulated that ovarian steroids may influence the clinical nature of SCD by modulating the cell membrane of sickle erythrocytes or by adversely affecting haemostatic balance. This is of clinical and therapeutic relevance as the use of hormonal contraception, in particular the combined oral contraceptive pill (COCP), in women with SCD remains the subject of considerable uncertainty and controversy. The use of depot medroxyprogesterone acetate has been shown in two studies to safely reduce the incidence of painful crises, but there is still anxiety that women with SCD who use the COCP may have an additional risk of thromboembolic disease.

This thesis examines the in vitro and ex vivo effects of oestradiol and progesterone on the cell membrane of erythrocytes and on molecular markers of coagulation from women with SCD. The menstrual pattern in women with SCD and the influence of the menstrual cycle on painful crises were investigated. Data were also collected on the effects of the menopause in women with SCD.

Oestradiol and progesterone associated loosely with the red cells and no specific receptors sites were identified in the sickle erythrocytes. The association was greater with oestradiol than progesterone and for both hormones, this association was significantly diminished in erythrocytes from women with Hb SS compared to Hb SC or Hb AA. Incubation with oestradiol and progesterone did not inhibit sickling or alter the plasma cell membrane thickness or morphology of the erythrocyte as examined by transmission electron microscopy. The deformability and osmotic fragility of sickle erythrocytes was not affected by in vitro incubation with therapeutic concentrations of oestradiol and progesterone. The more acute effect of incubation in vitro may, of course, differ from the effect of longer term exposure to synthetic steroids in vivo.
This was therefore further explored in the *ex vivo* situation, when the use of different types of exogenous contraceptive steroids in women with SCD was also found not to affect red cell deformability.

Women with SCD exhibit a degree of hypercoagulability and increased platelet activation even in the quiescent steady state. However, it is reassuring that the haemostatic markers, which measure thrombotic tendency, were not significantly higher in women with SCD who used the COCP compared to those who used non-hormonal forms of contraception. Menstrual clustering of crises was reported by 37% of women with natural menstrual cycles and these were usually of moderate severity, controllable with oral analgesia. The frequency of painful crises decreased following menopause and the commonest post-menopausal complaints were of joint pains and vaginal dryness. Post-menopausal women with SCD also appear to have an increased risk of developing osteoporotic fractures.

In conclusion, this sequence of studies establishes an ethical basis for a future patient-level interventional study using therapeutic preparations of oestradiol and progestogen relevant to effective contraception for women with SCD.
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The research work pertaining to the submission of this thesis was conducted at the Royal Free Hospital (RFH), London between 1996 and 1998. All the radio-labelled receptor binding studies and the laboratory measurements of red cell deformability and osmotic fragility were performed by the author. Measurement of haemoglobin S by electrophoresis was performed by technical staff in the Haematology Laboratory at the RFH and assessment of markers of thrombosis, fibrinolysis and platelet activation was mostly done by laboratory staff of the Haemophilia Centre and Haemostasis Unit of RFH. Electron microscopy pictograms were processed by staff of the Electron Microscopy Laboratory of the RFH. Statistical guidance was provided by Dr Richard Morris of the Department of Primary Care and Population Studies at the RFH. The author was responsible for the design of the studies, data collection, analysis and interpretation and for the production of all the manuscripts arising from this thesis.

This work is dedicated to my wife Maud, who has been my steadfast companion through difficult times and to my little daughter Helienke Su-lin.
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1.1 The historical milestones in Sickle Cell Disease (SCD)

The first recorded description of Sickle Cell Disease (SCD) in Africa is attributed to Africanus Horton, who in 1874, described fevers of crises, shifting joint pains and abnormality of the blood. Lebby (1846) and Hodenpyl (1898) of North America, both reported autopsy findings, suggestive of SCD, in a runaway slave executed for murder and in a 32 year old man who died after complaining of joint pains, pleuritic symptoms and jaundice. Herrick of Chicago (1910) published the first generally accepted scientific case report of this condition which was entitled “Peculiar elongated and sickle shaped red blood corpuscles in a case of severe anaemia”. In this paper, he described how he examined a blood film from a young Grenadian student who had complained of fever and cough and noted the unusual elongated shape that has become pathognomonic of SCD. Haemoglobin S (Hb S) was first observed as a molecular abnormality on electrophoresis by Linus Pauling and colleagues (1949) and Ingram later (1956) identified this abnormality as the replacement of glutamic acid by valine in the β globin chain of Hb S. In 1964, Makio Murayama expounded the modern hypothesis that deoxygenated Hb S polymerises to form rigid microfilaments which produce distorted sickle shaped red blood cells.

1.2 Sickle cell syndromes and their pathophysiology

The sickle cell syndromes refer to a group of inherited disorders of haemoglobin synthesis which present clinically with vaso-occlusive and haemolytic symptoms. Within this group, there are approximately 40 different variants, all of which share the abnormal haemoglobins caused by the substitution of valine for the glutamic acid residue at the sixth amino acid position of the β globin chain of the haemoglobin molecule. Of the these 40 variants, three are more commonly found and are usually grouped together as sickle cell disease (SCD) in the literature. These three are:

a) sickle cell anaemia (SCA), the homozygous state for the sickle cell gene (Hb SS genotype)

b) sickle cell haemoglobin C disease (Hb SC), which indicates simultaneous heterozygosity for both haemoglobin S and C genes (haemoglobin C results from the
substitution of lysine for glutamic acid at the sixth amino acid position of the \(\beta\) globin chain)
c) sickle cell \(\beta\) thalassaemia (Hb S\(\beta\) thal) which results from being doubly heterozygous for the genes for haemoglobin S and for \(\beta\) thalassaemia. \(\beta\) thalassaemia is characterised by globin chain imbalance resulting from inadequate \(\beta\) chain synthesis and the syndrome of sickle cell \(\beta\) thalassaemia itself, is highly variable depending on the nature of the \(\beta\) thalassaemia gene.
The less common types of sickle cell syndrome include the heterozygous combinations Sickle \(O_{\text{ARAB}}\) (SO\(\text{ARAB}\)) and Sickle D (Hb SD) disease.

The sickle cell gene is widely distributed throughout the world with particularly high prevalence in sub-Saharan Africa, Saudi Arabia, Central and South India, Sri Lanka, the Mediterranean, Turkey and Greece. Subsequently, the gene has also been distributed to the United Kingdom, the USA, the Caribbean and South America as a result of forced and voluntary migration of peoples of African and Asian origins.

Genetic studies using restriction enzyme cleavage of the \(\beta\) globin deoxyribonucleic acid (DNA) have identified more than a dozen \(\beta\) gene clusters or haplotypes, with most S genes occurring on four such haplotypes known as the Senegal, Benin, Central African region (or Bantu) and the Cameroon types (Noguchi et al 1993). A different haplotype has been described in sickle cell patients of Saudi Arabian and Indian origins (Padmos et al 1991). There is some evidence that the severity of SCD may be affected by the haplotype in which the S gene appears (Powars 1991). Discussion of SCD pathophysiology has generally been dominated by an emphasis on the phenomenon of Hb S polymerisation producing a series of events that both curtail red cell survival and impede blood flow in the microvasculature (Schecter et al 1987). Deoxygenated haemoglobin S forms fibrous precipitates consisting of a helical array of six filaments of haemoglobin molecules. These aggregates distort red cells into relatively rigid characteristic shapes producing microvascular stasis and eventually lead to repeated vicious cycles of deoxygenation, further sickling and infarction. Deoxygenated Hb S molecules co-polymerise most effectively with other Hb S molecules and, in decreasing order, with Hb C, D, \(O_{\text{ARAB}}\), A, J and F (Charache and
These in vitro observations would seem to predict to some extent the clinical severity of the disorders involving these variants. However, despite this relatively simple situation at the molecular level, it is clear that there is a wide clinical diversity of SCD, both between individuals having the same genotype and for the same individual through the course of their lives.

Of particular relevance to this thesis are the complex and only partly understood impressions of the variations in disease severity in women associated with physiological and pharmacological variations in ovarian steroid hormone levels, with a particular focus on what might be associated with the use of hormonal contraceptive preparations.

Recent evidence has indicated that a more complex interaction of cellular, environmental and genetic modulators may be responsible for the variations in the pathology seen in practice (Steinberg and Hebbel 1983). Sickle red cells have been shown to have a number of membrane abnormalities (Hebbel 1991) including abnormal cation homeostasis, which results in a subpopulation of abnormally dense cells. An important constituent of this subpopulation are the irreversible sickled cells (ISC’s), which are dehydrated with elevated mean cell haemoglobin concentrations (MCHC) and altered monovalent cation contents (low total sodium and potassium ions due to slightly increased sodium but very low potassium).

Sickle red blood cells also have a dysfunctional lipid bilayer which, when exacerbated by sickling, membrane protein defects and abnormal cell-to-cell interactions, may stimulate both endothelial and macrophage adherence (Hebbel 1991). It is postulated that these membrane defects modulate the disease severity conferred by the genetic determinants of the haemoglobin polymerisation and therefore produce the wide clinical diversity of the disease.
1.3 SCD in the United Kingdom

In the United Kingdom, the majority of people with SCD are of West African and Afro-Caribbean origin and within these ethnic groups, the condition occurs in approximately 1 in 60 births to parents of African descent and 1 in 300 births to parents of Afro-Caribbean descent respectively (Serjeant 1992).

In 1986, it was estimated that there were at least 5000 such individuals in the United Kingdom (Brozovic and Davies 1987) and by the year 2000, this estimate doubled to 10,000 (Streetly et al 1997). Despite this, its public profile in terms of scientific research, support services and general public awareness is comparatively low, possibly due to the fact that SCD predominantly affects people of minority ethnic groups. The situation, however, is changing and the World Health Organisation (WHO) has recognised SCD as of a high priority and a major health problem. The first Sickle Cell Centre in the United Kingdom was opened in Brent, North-West London in October 1979 and in the same year, the Sickle Cell Society was formed in London as a registered charity to work directly for the relief of SCD sufferers and to campaign for improvements in the services available to them.

1.4 Clinical manifestations of SCD

Patients with SCD have characteristic haemolytic and vaso-occlusive symptoms. Infants appear to be in normal health at birth, as fetal haemoglobin (Hb F), which does not “sickle”, is present in abundance. The earliest clinical signs of the condition, anaemia and haemolysis, occur at two to three months of age, when the proportion of Hb F begins to fall. Often the first clinical manifestation is the infarction of metacarpal, metatarsal or phalangeal bones, causing the so-called “hand foot syndrome”. Due to repeated infarctions, the spleen regresses and may not be palpable after the age of 10 years. During the first few years of life, children are prone to acute pneumococcal and other septicaemias (Bromberg 1974) and the mortality rate in untreated SCD may be as high as 15-20% in the first decade of life. However, with early diagnosis and treatment, including the use of prophylactic penicillin, this has decreased to less than 2% in industrialised countries (Vichinsky et al 1988). After the age of five years, the main complications are the vaso-occlusive painful crises and the consequences of organ and tissue damage due to these repeated infarctions.
Episodes of crisis are characterised by the onset of pain, which results from tissue ischaemia and can occur in any part of the body, most commonly affecting the joints and muscles of the back, arms and legs. In any crisis, especially with underlying infection, the rate of haemolysis may increase, necessitating blood transfusion. Bone infarcts are common (Diggs et al 1937) leading characteristically to aseptic necrosis of the femoral and humeral heads. Cerebral infarcts can also occur and are especially important in children (Russell et al 1976), when regular transfusion therapy is then indicated.

Increased cardiac output is necessary to compensate for the reduced oxygen content of the arterial blood. The cardiovascular system is strained by the sickle erythrocytes’ propensity to occlude small vessels and myocardial infarctions are more common in these individuals (Lindsay et al 1974). Due to its hypoxic, acidic and hypertonic microenvironment (Allon 1990), the kidney is also a major target organ prone to the effects of sickling, and renal failure is another important cause of death in the middle-aged individual with SCD. Priapism (Emond et al 1980), leg ulcers (Gueri and Serjeant 1970), sickle retinopathy (Goldberg 1971) and acute pulmonary lesions (Oppenheimer and Esterley 1971) are all characteristic problems in SCD.

An important feature of the clinical course for patients with SCD is its variable nature. Some affected individuals suffer an unremitting chronic course with frequently recurring crises and organ damage from infarction, while others have few problems with their illness. In general, patients with compound heterozygous conditions (eg Hb SC and Hb Sβ thal) have milder manifestations of the disease than those with the homozygous Hb SS genotype, although this does not apply with regard to pregnancy. Women with Hb Sp⁰ thalassaemia possess no Hb A and often have as severe clinical symptoms in pregnancy as women with Hb SS, unlike those with Hb Sβ⁺ thalassaemia who have Hb A levels of 20-30% and thus generally have a milder form of the condition.
1.5 The hormonal influences on SCD

Circumstantial evidence and anecdotal case reports have suggested a possible hormonal effect explaining to some extent the fluctuating nature of this condition. It is recognised that pregnant women with SCD are more prone to sickling crises and suffer major maternal and fetal complications (Tuck et al 1983, Charache and Niebyl 1985, El Shafei et al 1992, Dare et al 1992, Leborgne-Samuel et al 2000, Sun et al 2001). The use of exogenous steroids such as oestrogens and progestogens, contained in the combined oral contraceptive pills (COCP’s), seems to modify the clinical nature of SCD, as will be discussed later. The menstrual cycle, puberty and the menopause also appear to have an effect on the frequency of painful crises.

1.5.1 Pregnancy

Pregnancy is a considerable undertaking for women with SCD with high rates of maternal and perinatal mortality and morbidity. Yater and Mollari (1931) published the first report of pregnancy in a woman with SCD, prior to which very few women are thought to have survived to reproductive age. Before the introduction of filter paper electrophoresis in the 1950’s, the diagnosis of SCD was made on the basis of in vitro sickling and clinical manifestations. The first reported series on pregnant women with SCD (diagnosed by their clinical histories) was by Eisenstein et al (1956) from Harlem Hospital in New York, who documented a maternal mortality rate of 10.5% and perinatal mortality rate of 22.5%.

It has been generally observed that pregnant women with SCD have increased incidences of sickling crises, infection (urinary tract, pneumonia, pyelonephritis and puerperal uterine sepsis), acute chest syndrome and anaemia (Tuck et al 1983, Charache and Niebyl 1985, El Shafei et al 1992, Dare et al 1992, Leborgne-Samuel et al 2000) based on experiences in the United Kingdom, Bahrain, USA, Guadeloupe and Nigeria, although these series have shown widely varying maternal and fetal outcomes. More recent reviews have suggested that while the infants of mothers with SCD have a tendency to be premature and small for gestational age (SGA), the perinatal morbidity and mortality may not be significantly different to ethnically matched controls (Smith et al 1996, Sun et al 2001). With improvements in obstetric and neonatal care in specialised centres accustomed to the management of SCD, as
well as with advancements in transfusion medicine, the maternal and perinatal mortality have respectively decreased to 1.7% and 11% in more recent reviews from 4.1% and 52.7% in the 1970's (Powars et al 1986, Sun et al 2001). However, it should be remarked that such rates are still respectively one thousand-fold and ten-fold higher than current total maternal and perinatal mortality rates in the United Kingdom (Report on CEMD 1997-1999, McFarlane and Mugford 2000). In fact, there were three cases of haemoglobinopathy-associated maternal mortality reported in the Confidential Enquiry into Maternal Deaths in the United Kingdom 1991-1993 (HMSO 1996), of which, two were due to sickling crises (Hb SS and Hb SC) and one, to hepatic sickle cell sequestration syndrome (Hb Sβ thalassaemia). The reduction in the number of such cases in the more recent Reports on Confidential Enquiry into Maternal Deaths in the United Kingdom (none in 1994-1996 and only one case in 1997-1999) may be due to a more aggressive transfusion regimen in pregnant women with SCD. Interestingly, retrospective data suggested that prophylactic transfusions decreased the incidence of maternal sickling, but did not affect the eventual fetal outcome (Howard et al 1995). Selective transfusions in pregnant SCD women with low haemoglobin may reduce painful crises, but as yet, there is insufficient prospective data in randomised controlled trials to recommend a regimen of prophylactic blood transfusions in these women (Mohamed 2000). However, the fluctuating nature of the disease make such trials difficult to conduct in practice.

There is a wide geographical variation in morbidity and mortality, with the highest documented maternal mortality (9.2%) occurring in Africa (Dare et al 1992). Comparison of outcomes of pregnancy between Hb SS and Hb SC genotypes is confusing. Some authors report a worse outcome in women with Hb SS (Leborgne-Samuel et al 2000) while others claim that maternal mortality and morbidity is higher in the latter group (Pritchard et al 1973). Many investigators, however, note no difference in outcome of the pregnancy between the two groups of women (Horger 1972, Dare et al 1992, Sun et al 2001), in contrast to their behaviour outside pregnancy.
Currently, there is no specific evidence directly linking the elevation in physiological levels of oestrogen and progesterone associated with pregnancy to the complications reported above. The increase in the incidence of sickling that occurs in pregnancy is multifactorial and is likely to be influenced by pregnancy associated vessel wall changes, alterations in coagulation factors and blood flow. The higher incidence of sickling in pregnancy may also be associated with the increased plasma and whole blood viscosity and impaired erythrocyte deformability noted in pregnant women with sickle cell disease. Cross sectional observational data show that the red cell transit time (RCTT), venous haematocrit and the corrected high shear blood viscosity are significantly higher in women with SCD compared to non sickle controls at similar gestational ages (Howard 1995). However, comparative studies have not been performed with non pregnant women with SCD and it is not known if the parameters in pregnant women with SCD documented in Howard’s thesis (1995) were due to the disease process or to pregnancy or if indeed, these effects altered progressively with advancing gestational age.

1.5.2 Puberty

Some children with SCD exhibit reduced gonadal function, possibly due to primary gonadal failure (Abbasi et al 1976, Osegbe and Akinyanju 1987). Levels of luteinising hormone (LH) are described as being increased in both boys and girls with homozygous Hb SS younger than 10 years of age (when compared with matched controls of the same age and at the same stage of development of secondary sexual characteristics), with these values returning to the normal range in older affected children (Olambiwonmu et al 1975), suggesting a transient impairment of gonadal function during the first decade of life. Changes in the levels of follicle stimulating hormone (FSH), however, are inconsistent between the two sexes.

Contrary to this, a concept of hypothalamic hypogonadism was proposed by El Hazmi and colleagues (1992), who noted lower LH, FSH, testosterone and cortisol levels compared to age and sex matched controls in both male and female Hb SS patients of Saudi Arabian origin. In this case-control study, patients with more severe clinical histories show more frequent abnormalities in these measurements when compared to
those with milder disease manifestations, implying that the severity of the course of the disease exert an adverse effect on endocrine function.

Along with the above findings, there seem to be a corresponding delay in the development of secondary sexual characteristics (pubic hair and breast growth) in girls with SCD (Platt et al 1984, Soliman et al 1999). In the Cooperative Study for Sickle Cell Disease, the attainment of Tanner Stage III for pubic hair in boys and girls was reported to be retarded in all genotypes, the delay being greater in Hb SS and Sβ° thalassaemia than in SC or Sβ+ thalassaemia (Platt et al 1984). More recently, this observation was confirmed by Soliman and colleagues (1999) who noted that despite modern management with regular transfusions and desferrioxamine, girls with SCD have markedly delayed breast development and menarche. Children with SCD of short stature have lower levels of serum insulin-like growth factor 1 (IGF 1) compared with children with constitutional delay of growth and it is thought that this delayed growth pattern may be attributable to delayed puberty and reduced synthesis of IGF 1.

Singhal et al (1994), in a study examining the growth pattern in 44 Jamaican children with Hb SS and Hb SC (compared to 44 Hb AA controls), remarked that the onset of the adolescent growth spurt was delayed in these children with Hb SS compared to Hb AA, but the actual growth pattern was normal and that there was no difference in the final height by the age of 17.9 years. The growth spurt did not appear to be delayed in children with Hb SC.

Menarche in girls with SCD is also delayed when compared to non SCD controls, who have a mean age of menarche of 13.1 years with a standard deviation of 1.7 years (Alleyne et al 1981). In fact, the mean age of menarche in SCD girls has been reported in various studies to range from 13.9 years in the African-American population (Jiminez et al 1966) to 16.1 years in the Jamaican population (Graham et al 1986). The age of menarche in girls with Hb SS is also significantly later (15.4 vs 13.7 years) compared with that in the Hb SC group (Singhal et al 1994). However, there is no specific published evidence that the hormonal changes associated with puberty alter the process of sickling.
1.5.3 The menstrual cycle

There is little reported data on the actual pattern of menstrual loss in women with SCD or if there exists any association between the menstrual cycle, the presence or absence of dysmenorrhoea and sickling crises.

Anecdotal reports claim that painful crises sometimes cluster around menstruation and that the onset of menstruation may be a "trigger" to painful crises, but published studies have so far been unable to demonstrate a clear relationship. Samuels-Reid and Scott (1985a), in a retrospective review, based on a single questionnaire, of a group of 52 women with Hb SS, Hb SC disease and Hb Sβ thalassaemia, attempted to correlate the frequency of sickle crises with menstruation. Their data suggest that crises may be precipitated cyclically by the process of menstruation in about a third of cases and these occur especially during and after menstruation, when oestradiol and progesterone levels are relatively low. Unpublished observations of admissions to the Sickle Cell Day Care Centre of the Medical Research Council Laboratories in Jamaica report no significant relationship between menstruation and sickling crises (Serjeant 1992), although that does not exclude a causative relationship in certain individuals. More recently, Serjeant and colleagues (1994) examined the timing of 50 episodes of painful crises in relation to natural menstrual cycles in women with SCD not using hormonal contraception. They reported a trend of painful crises occurring more frequently (18/50 episodes) between days 1-6 of the cycle, although the difference was not statistically significant ($p=0.3$, $\chi^2$ test).

Based on retrospective data which relied on women's recollection, menstrual cycles are thought to be more irregular, prolonged, painful and heavy in women with SCD compared with controls (Samuels-Reid and Scott 1985b).

It is possible that any menstrual clustering of sickling crises may be associated with exacerbations of anaemia although this has not been established objectively. Red cell deformability is impaired in certain conditions associated with elevated oestrogen and progesterone levels such as pregnancy, but this cannot be easily extrapolated to the lesser hormonal fluctuations that occur with the menstrual cycle. Moreover, painful crises, when they are associated with the menstrual cycle, seem to cluster during or after menses (Samuels-Reid and Scott 1985a, Serjeant et al 1994), when oestrogen
and progesterone levels are comparatively low, and hence apparently the opposite trend to that observed in pregnancy.

1.5.4 The menopause
Apart from anecdotal reports, there is little specific data on the effect of the menopause on the quality of life, symptomatology, or bone mineral density in women with SCD. There is observational data suggesting that sickling crises may become less frequent and less severe after the age of 40 years and Serjeant (1992) considered this to be due to a true amelioration rather than the preferential survival of more mildly affected individuals.

1.5.5 The combined oral contraceptive pills (COCP's)
The available literature on the interactions between the COCP and SCD is limited and these interactions could be categorised as follows:

a) whether the oestrogen and progestogen components of the COCP influence the pathophysiology of the sickling process, and

b) whether SCD itself increases the risk of thromboembolic disease (TED) associated with the use of the COCP.

The use of the low dose COCP, Microgynon (ethinyl oestradiol 30 μg and levonorgestrel 150 μg) and of depot progestogen contraception may reduce the incidence of sickle cell crises, as reported in a study from Panama (de Abood et al 1997). In this study, the authors randomly allocated Hb SS women with painful crises to receive either depo-medroxyprogesterone acetate (DMPA) (n=13) or Microgynon (ethinyl oestradiol 30 μg and levonorgestrel 150 μg) (n=14) for a year, with Hb SS patients who had been surgically sterilised (n=16) as controls. No statistically significant changes in haematological (Hb, haematocrit and reticulocyte count) or coagulation parameters (prothrombin time and thromboplastin time) were noted between the three groups. At the end of one year, 70% of the DMPA group were pain free (p=0.014, Students’ t test) and only 16% of those still reporting painful crises rated them as intense. Similarly, about 55% of the patients receiving Microgynon also reported a reduction in crisis frequency after a year. However, 50.5% of the control
group also had an amelioration of crises, which the authors attributed to closer medical care and psychosocial support.

In his study, Lutcher (1976) reported that 11 out of 12 women with SCD had no increase in severity or frequency of crises while using the COCP and that there were no significant differences in tests of coagulation between SCD women who were COCP users and those who were not. Freie (1983), in a detailed review of the literature, maintains that the use of the COCP in women with SCD is not associated with significant adverse effects and advocates that low dose COCP should be the contraceptive of choice in these women.

It has been long recognised that the use of the COCP is associated with a risk of thromboembolic disease in the general population (Spitzer et al, 1996, Farmer et al, 1997). The anxiety that women with SCD who use the COCP may augment their risk of venous thromboembolism has led to the majority of manufacturers listing SCD as a relative contraindication to its use (APBI, 2001). Anecdotal case reports have described a possible association in contraceptive pill users between venous and arterial thromboses and the sickle cell gene (Haynes and Dunn, 1967, Greenwald, 1971, Hargus et al, 1977). However, these case reports describe four cases of pulmonary embolism and one cerebrovascular accident in individuals who were simply heterozygous for the S gene, raising some doubts as to its relevance to the events. Furthermore, these cases involved the older COCP’s, which contained a higher dosage of oestrogen no longer germane to current clinical use.

67 (45%) of 149 women with sickle haemoglobinopathies (Hb SS, SC and Sβ thalassaemia) studied in a North London population (Howard et al, 1993) used the COCP for a total of 148 woman-years. Two of the 67 women described having developed deep venous thrombosis (DVT) requiring anticoagulation during COCP use, although the authors were unable to confirm this with hospital records due to the confidential nature of the interviews. While the numbers are small (n=67 in the COCP group) and the groups not strictly comparable, it is interesting to note that 5.9% of the COCP users had an increase in the frequency of painful crises, while 3.6% of the intrauterine contraceptive device (IUCD) users (n=28) reported the same. The authors
concluded that while an increased risk of crises and venous thrombosis with COCP usage could not be excluded, the use of COCP in women with this condition should not be considered absolutely contraindicated and that any complications should be balanced against the substantial risk associated with (unplanned) pregnancy for these women.

Thus, there is currently insufficient scientific data to support the assumption that women with SCD who use the COCP's have an augmented risk of developing TED (Guillebaud 1993, Midence and Elander 1994). Moreover, the reliability (with a failure rate of 0.1-1.0 per 100 woman-years), widespread availability and reversibility of the COCP make this mode of contraception particularly important to women with SCD.

1.5.6 Progesterone and its' derivatives

Isaacs and Hayhoe (1967) discovered that incubation in vitro with progesterone at the concentration of approximately 1 µg/ml, prevented and, indeed, possibly reversed sickling, while comparable red cell preparations containing prednisolone and oestrone remained sickled. In the same study, similar findings were noted ex vivo when a female patient with Hb SS was treated intramuscularly with the more polar 17 OH progesterone and the authors postulated that these steroids produced a stabilising effect on the erythrocyte membrane.

In a subsequent study, Isaacs and colleagues (1972) randomised 28 women with Hb SS to receive either progesterone in oil (10 mgs in 1 ml) or saline (1 ml) as control, while 16 male patients with Hb SS also had either testosterone in oil (10 mgs in 1 ml) or saline as control (1 ml). These intramuscular injections were given once a week for six months. About 80% of those who received steroids reported a reduction in severity of pain and a similar percentage in the control group receiving saline recorded poor response to treatment. In fact, 22 out of the 31 patients defaulted from receiving weekly injections of saline after the first two weeks, complaining that they had had no improvement in symptomatology. Although the data were not statistically analysed, Isaacs and co-authors (1972) postulated again that this clinical improvement might be due to the stabilisation of the sickle cell membrane by these steroids.
In vitro sickling was also thought to be inhibited by medroxyprogesterone acetate (Perkins 1971) and the progesterone derivative, megestrol (Adadevoh and Isaacs 1973).

Studies by Lundh and Gardner (1970) demonstrated no alteration in the morphology or clinical behaviour of Hb SS erythrocytes when treated with progesterone but the experiments were conducted at higher concentrations than the in vitro studies by Isaacs and Hayhoe (1967), suggesting that the anti-sickling effects may be dose dependant. Raper and colleagues (1970) were also unable to reproduce the anti-sickling effect of ovarian steroid hormones in vitro and suggested that earlier reported findings by Isaacs and Hayhoe (1967) may be artefactual due to the “sphering” action of the oily base in which the steroid preparations were dispensed.

De Ceulaer and colleagues (1982) reported a small but well conducted placebo controlled crossover study involving 23 women with homozygous sickle cell disease (Hb SS) treated with DMPA over two years. Certain haematological parameters, such as fetal haemoglobin, total haemoglobin, red cell count and red cell survival time, increased, while reticulocytes, irreversibly sickled cells (ISC’s) and bilirubin levels fell. The incidence of painful crisis was significantly less in the DMPA phase of the study (29 vs 58 episodes), suggesting a possible inhibition of in vivo sickling. The beneficial haematological changes observed were attributed either to an increased production of fetal haemoglobin or a membrane stabilising effect and were thought to be independent of the effect of DMPA on reducing menstrual loss. More recently, in a study from Panama involving 43 Hb SS women with frequent painful crises, de Abood et al (1997) noted that 70% of those given DMPA (n=9/13) were symptomatically improved, although no alterations in haematological parameters were noted.

Nascimento and colleagues (1998) looked prospectively at the effect of the progestogen implant, nomegestrol acetate, in women with Hb SS with low disease activity (n=30) over 9 months. They found that women in the treatment group (n=20) had a reduction in the frequency of crises compared to the control group (n=10). Improvements in haematological parameters, such as increased levels of fetal
haemoglobin as well as a reduction in ISC’s, paralleled the observed clinical improvement in the treated Hb SS women. The serum glucose in the treated group was higher compared to baseline (although the $p$ value was not significant) and the authors postulated that the increased glucose levels may result in the formation of glycosylated haemoglobin, which is a more stable molecule with a higher affinity for oxygen. They concluded that nomogestrol acetate was safe and effective in women with SCD, with no adverse haematological or biochemical changes.

In Howard and colleagues’ (1993) observational study of a North London population of 149 women with sickle haemoglobinopathies, 26 women (17%) were found to be using injectable DMPA with no reported clinically significant side effects.

Progestogen only contraception has a Pearl Index (number of pregnancies per 100 woman-years of use) of between 0.1-3.0, with the main side effects being erratic vaginal bleeding (20%) and the formation of ovarian cysts (20%) (Glasier 1996). Depot medroxyprogesterone is particularly effective (Pearl Index of 0.1-1.2) and has been licensed as a contraceptive in the United Kingdom since 1984.

1.5.7 Testosterone

In in vitro experiments, testosterone and norandrostenolone inhibit and even reverse sickling in suspensions of sickle cells while comparable red cell preparations containing prednisolone and oestrone remain sickled (Isaacs and Hayhoe 1967). The authors repeated these findings in vivo by injecting two male patients with testosterone propionate and postulated that these steroids produce a stabilising effect on the erythrocyte membrane. In a later clinical study of 44 patients, of whom 16 were male, Isaacs and co-authors (1972) also noted that weekly injections of 10 mg testosterone in these men for four to six months reduced the incidence of painful crises.

Dromostanalone, a synthetic androgen was thought to stimulate erythropoiesis and thus increase the level of haemoglobin in 11 out of 12 patients so treated (Zanger et al 1974). While this may have potential beneficial effects in individuals with SCD, the increase in red cell mass observed in response to androgen use, may also in itself, precipitate painful crises (Mentzer et al 1969, Zanger et al 1974). Moreover,
predictable virilising side effects such as hirsutism has limited the use of androgens in women with SCD.

1.5.8 The biosynthesis of ovarian steroid hormones

Progestérones are C$_{21}$ steroids derived from cholesterol, secreted by the corpus luteum, placenta and the ovarian follicle. The synthesis of the androgens, which contain 19 carbon atoms, commences with the hydroxylation of progesterone at the C-17 position, followed by cleavage to yield androstenedione and further reduction to form testosterone. Natural oestrogens, such as 17 $\beta$ oestradiol, are C$_{18}$ steroids produced in the theca and granulosa cells of the ovary by the aromatisation of androgens in a complex process that involves three hydroxylation steps (Granner 1996) (figure 1.5.8).
Transformations in the biosynthesis of ovarian steroids.

Figure 1.5.8: The biosynthesis of ovarian steroid hormones from cholesterol
1.6 Red cell membrane abnormalities in SCD

1.6.1 Current views on the plasma membrane

The plasma membrane represents the outer limit of the living cell and controls the ease with which substances enter the cell, providing it with selective permeability. The plasma membrane contains many diverse molecules on its surface, which confer upon it the capacity to interact with other cells and with the extracellular environment. The fluidity and permeability of the plasma membrane is determined by the ratio of cholesterol to phospholipid in its structure. The membrane possesses enzymatic pumps which control the levels of Na, K, Ca and other ions both in the intracellular and extracellular environments. It also contains the enzyme adenosine triphosphatase (ATPase), which breaks down adenosine triphosphate (ATP), thereby providing energy for active transport (pumping), endocytosis, and other energy consuming membrane functions. Some of the molecules that extend from the surface of the plasma membrane are receptors capable of selectively linking with substances outside the cell including receptors on other cells. Many essential cell functions such as conduction, phagocytosis, antibody production, antigen recognition and hormone induced activities, are receptor mediated. Some receptors are shared by many cell types such as insulin receptors needed in carbohydrate metabolism, while others are specific, for example, erythropoietin receptors on erythroblasts.

The plasma membrane is predominantly protein (50-60% of the dry weight) and this composition reflects its high metabolic activity and structural stability. Most proteins associated with the cell membrane are regarded as intrinsic (integral) to the membrane as they can only be removed by drastic procedures of extraction, digestion or denaturation. Other proteins are easily removed and are considered extrinsic (peripheral). Many of the intrinsic proteins are amphipathetic i.e asymmetrical or polarised, with hydrophilic groups at one pole and hydrophobic groups at the other. Proteins embedded in the lipid bilayer matrix are involved in diverse biological processes including those associated with signal transduction and ion transport (figure 1.6.1).

Lipids constitute 20-30% of the dry weight of red cell membranes. The lipids that make up the biomembrane lipid matrix of erythrocytes and platelets consist of
glycerol and sphingosine. Lipids are asymmetrically arranged in the biomembrane matrix so that phosphotidyl choline (lecithin) and sphingomyelin make up 90% of the outer membrane surface while the inner surface consists of phosphatidyl serine, phosphatidyl ethanolamine and smaller amounts of phosphatidyl choline, sphingomyelin and phosphatidyl inositol. Phospholipids also tend to be amphipathetic: the glycerol end is water soluble, carrying phosphate and other ionised groups, while the fatty acid end is lipid soluble and hydrophobic. Other lipids which constitute the cell membrane include cholesterol and minor components linked to protein or carbohydrate as lipoprotein or lipopolysaccharide. Carbohydrate accounts for less than 10% of the weight of plasma membranes in most cells (Cormack 1987).

The plasma membrane is approximately 7.5 nm (75 Å) in thickness with a range between 6-9 nm (60-90 Å) when examined using Transmission Electron Microscopy (TEM). It is seen as a trilaminar structure which is termed the unit membrane, with outer darker lines approximately 2 nm (20 Å) wide and an inner lighter line approximately 3.5 nm (35 Å) wide. The plasma membrane can be isolated for study by using cell disruption techniques followed by differential centrifugation. Its composition and function have been studied by a number of techniques such as Infra-Red Spectroscopy and the use of fluorescent probes.
Figure 1.6.1: The plasma cell membrane (from Junqueira LC, Carneiro J and Kelly RO, Basic Histology, 7th edition, Appleton and Lange 1992)
1.6.2 Electron microscopy (EM)

Two types of electron microscopy (EM) techniques can provide morphological and analytical information on cells and tissues. These are:

a) Transmission electron microscopy (TEM)
b) Scanning electron microscopy (SEM).

The primary advantage of EM over light microscopy is that the wavelength of the EM beam is approximately 1/2000th that of light microscopy, thereby increasing the resolution by a factor of 1000. With EM, a resolution of about 0.2 nm is possible and a magnification of 500,000 can be achieved.

TEM, with a wavelength in the electron beam of approximately 0.1 nm, has a theoretical resolution of 0.05 nm. Electron beams are invisible to the naked eye and the images they form must be revealed by causing them to strike a fluorescent screen, after which they are recorded on a photographic plate. Stability of the specimen is crucial and efforts must be made to protect the specimen against sublimation, distortion and other damage by the electron beam and vacuum. The specimen must be sufficiently thin for the electron beam, which is easily absorbed, to pass through and create an image. Electron microscopic sections are approximately 0.025 μm thick. The thin sections have little intrinsic contrast and must therefore be stained with electron absorbing heavy metals to provide the contrast necessary to reveal the details of the cell structure.

Because of the great resolution of the TEM, the quality of fixation (ie the degree of preservation of the subcellular structures) must be the best achievable. Preparation of specimens usually begins with glutaraldehyde fixation followed by a buffer rinse and fixation with osmium tetroxide. Glutaraldehyde preserves protein constituents by cross linking them and the osmium reacts with lipids, as well as imparting electron density to cell and tissue structures, thus enhancing image formation. The dehydration process is identical to that used in light microscopy and the tissue is infiltrated with a monomeric resin, usually an epoxy resin, that subsequently polymerises.
SEM provides three dimensional, high resolution images of the surface of cells and tissues, from which cytochemical features can be localised. The sample is fixed, dehydrated by critical point drying, coated with an evaporated gold carbon film, mounted on an aluminium stub and placed in the SEM chamber. Mounts of tissue cultures are placed on the stage of the SEM and a slender electron beam is directed upon the surface, scanning the preparation. As the electron probe strikes the surface of the specimen, it generates different kinds of signals. Electrons reflected from the surface (backscattered electrons) and electrons forced out of the surface (secondary electrons) are collected to form a three dimensional image on a high resolution cathode ray tube (television tube). X rays are generated when the electron beams strike atoms having a mass greater than that of sodium (molecular weight 23). As each element is a source of X rays of distinctive wavelength, analysis of the X ray pattern of a tissue provides data on the concentration and distribution of elements (Ross et al 1995a).

1.6.3 Assessment of the sickle cell erythrocyte membrane using light and electron microscopy

In the earlier in vitro and ex vivo studies assessing the effect of ovarian steroids on sickling, many authors used light microscopy to examine the morphology of sickle red cells (Isaacs and Hayhoe 1967, Lundh and Gardner 1970, Raper et al 1970, Isaacs et al 1972).

Bertles and Dobbler (1969) investigated the structure of cytoplasm and cell membrane of intact sickle cells under oxygenated and deoxygenated conditions using TEM. They noted the following:

1) Oxygenated reversibly sickled cells (RSC’s) resembled normal erythrocytes in that they have homogenous, coarse-grained cytoplasm and a well defined cell membrane of around 8 nm (80°A). Oxygenated irreversibly sickled cells (ISC’s) display wavy, blurred margins and generally obscured membrane detail. Polymerised filaments are never found in the cell cytoplasm of either oxygenated RSC’s or ISC’s.

2) In deoxygenated RSC’s, the cytoplasm contained filaments which were arranged in a loose and random pattern. These filaments were more tightly and regularly packed in deoxygenated ISC’s.
The authors suggest that the well organised pattern of filament formation as well as the differing phospholipid ratios peculiar to deoxygenated ISC’s make them more rigid, less deformable and hence more prone to intravascular sequestration and sickling.

Allan and co workers (1982), using both light and electron microscopy techniques reported that when sickle cells undergo repeated oxygenation-deoxygenation cycles, they lose 2-3% of their membrane lipid bilayer as spectrin-free, haemoglobin containing spicules in the form of rods and microspheres. They also noted that the most striking feature of the spicules is the absence of normal membrane cytoskeletal peptides (such as spectrin, ankyrin, band 4.1 and actin) and concluded that the spicules are composed of Hb S polymers, which project through gaps in the actin-spectrin framework and cause a herniation of the lipid bilayer. This may undermine the integrity of the bilayer-cytoskeletal interface and alter irreversibly sickle cell morphology.

Liu and colleagues (1991) observed that deoxygenation lead to the formation of spicules projecting from the sickle cells and when the cells were further subjected to repeated oxygenation-deoxygenation cycles, the spicules became rounded vesicles and detached from the erythrocytes. The authors agreed with Allan’s earlier suggestion that deoxygenated Hb S polymerise through the gaps in the spectrin-actin meshwork and cause a herniation through the membrane lipid bilayer. Repeat cycles lead to the disruption and the uncoupling of the lipid bilayer which eventually detaches from the cytoskeleton.

The rate and duration of deoxygenation also appear to influence sickle cell morphology (Kaul and Xue 1991). With fast deoxygenation, most sickle erythrocytes are characterised by granular, irregular surface contours and small projections on SEM. TEM shows the presence of heterogenous polymer domains which are randomly and haphazardly distributed. When deoxygenation continues for 30 minutes, the original granular cells become elongated with long spicules visualised on SEM, while TEM demonstrates that these spicules contain tightly packed and aligned polymers similar to early descriptions by Bertles and Dobbler (1969).
These morphological changes can be explained on the basis of the rate of polymerisation. Rapid polymerisation produces homogenous nucleation and the formation of independently arranged polymers resulting in the granular morphology, whereas slower rates of deoxygenation lead to the creation of regularly arranged and tightly packed polymers. Gradual deoxygenation over 30 minutes, which simulates the \textit{in vivo} conditions, produces the typical sickle cells and “holly leaf” cells (Eaton and Hofrichter 1987). The presence of characteristic morphological cell types is also associated with certain changes in cellular viscosity: the granular sickle cells produced by rapid deoxygenation appear to be most viscous while the elongated, spiculated sickle cells show a reduction in viscosity.

Most red cells do not sickle \textit{in vivo} in the steady state because of the extremely short transit time involved (< 1 second) (Mozzarelli et al 1987), although disruption to this steady state by various conditions such as low oxygen tension and acidosis may precipitate the vicious cycle of hypoxia, Hb S polymerisation and the occurrence of vaso-occlusive episodes (Lipowsky et al 1987, Kaul et al 1989). As yet, however, there has been little published data on the effect of ovarian steroid hormones on the morphology of sickle cell as assessed by SEM and TEM.
Figure 1.6.3: Scanning electron microscopy (SEM) picture of sickled red cells
1.6.4 Fluorescent probes and membrane fluidity

The fluorescent probe method has assumed an important position in biomembrane research as one of the few techniques permitting the study of structure and function of biological membranes ex vivo. Probes such as ANS (1-anilinophthalene-8-sulfonate) bind covalently to membrane proteins as well as to lipids and are sensitive to changes in the probe environment (Slavik 1982). The interpretation of these results, however, is usually the weakest point of the method.

Information provided by fluorescent probes relates to the molecular environment around the probe and reflects its various interactions with the surrounding molecules. The most fruitful approach has been based on understanding the spectroscopic properties of the probe as revealed under a great variety of simple conditions (e.g., different solutions), followed by the use of simple but well-defined heterogeneous systems such as liposomes (artificial simple membranes) and finally by extending the complexity of the model to a level where the observed properties in the membrane are interpretable with some precision. Different fluorescent probes have been used to "view" different areas of the cell membrane. Pyrene, for example, provides a measure of the lateral diffusion through the membrane, while 1,6-diphenyl 1,3,5-hexatriene (DPH) examines the interface between the two layers of lipids in the cell membrane (McCLean and McGrath 1993).

Using the fluorescent probe DPH, certain steroids such as progesterone and testosterone have been shown to alter the dynamics and fluidity of artificial unilamellar membranes created by dispersing egg yolk phosphotidyl choline in 0.2 M Tris Cl buffer and later adding cholesterol (Whiting et al 1995). Progesterone decreases membrane fluidity, while both 17α and 17β oestradiol appear to increase membrane fluidity. This effect, however, was not reproducible with natural biological membranes, nor has it been examined using normal erythrocyte or indeed sickle cell membrane.

Jacobsohn and colleagues (1994) noted that cholesterol type compounds limit the intercalation between the lipid bilayer and hence reduce membrane fluidity.
In premenopausal women, the fluctuating ovarian steroid levels during the follicular and luteal phases of the menstrual cycle have been found to have little effect on red cell ghost membrane fluidity, as assessed by DPH (Berlin et al 1989). Instead, the membrane fluidity is influenced more by the phospholipid contents and the cholesterol to phospholipid ratio of the red cell membrane.

There is evidence that the synthetic oestrogen, ethinyl oestradiol, could alter ileal cell membrane fluidity, accompanied by a marked decrease in bilayer phospholipids and an increase in the cholesterol/ phospholipid ratio (Schwarz et al 1988). Ileal cells, however, are nucleated, and the effects demonstrated may be mediated through a different pathway to that applicable to the non-nucleated cells such as erythrocytes.

1.6.5 Infra-Red Spectroscopy (IRS) (and its application in the examination of the sickle cell membrane)

The earlier static view of the structure of bio-membranes based upon electron microscopy has now been complemented by a more dynamic technique. In many cases, the lipids and proteins are able to undergo rotational and lateral diffusion within the plane of the lipid matrix of the plasma membrane. Chemical bonds undergo various forms of vibration, such as stretching, twisting and rotation. The energy of most molecular vibrations corresponds to that of the infra-red region of the electromagnetic spectrum.

The two main types of spectroscopic methods based on the vibration of the atoms of a molecule are the Infra-Red (IR) and Raman Spectroscopy. Raman Spectroscopy is sensitive to vibrations that modulate bond polarisability. Vibrations that lead to changes in the dipole moment of a molecule can be detected and measured using Infra-Red Spectroscopy. Thus for a molecule such as a protein, there are many vibrations that can result in a complex spectrum, but fortunately these vibrations can be localised to specific bonds or groupings such as C=O and O-H groups.

Fourier Transform Infra-Red Spectroscopy (FTIRS) is a technique for the study of proteins where high quality spectra can be obtained with very small amounts of material (1 nM) in a variety of environments such as aqueous solutions, lipids,
crystals and organic solvents. When FTIRS is applied to the study of biomembranes, bands are observed arising from the lipids, proteins and chromophore (light producing) probe. The structures of a large number of membrane proteins have now been investigated (Braiman and Rothschild 1988, Chapman et al 1989) and these show the orientation of the different secondary structures within the lipid bilayer of the biomembranes. Many biological reactions are the result of interaction between two or more proteins. Techniques for studying such interactions, to determine how the structure of one protein modulates the structure of another, are limited. $^{13}$C labelled protein can be used to aid the study of such interactions (Haris et al 1992). When unlabelled proteins interact, the overlap of absorbance of the amide I band, for example, is so great that it is impossible to distinguish the individual bands from each protein. However, if one of the proteins is $^{13}$C labelled, then its absorbance can be clearly separated from the amide I absorption of the other.

Kucuk et al (1992), using FTIRS, showed that oxysterols, the breakdown products of cholesterol, are present in normal and sickle erythrocytes and that there is an increase in the amount of oxysterols in sickle cell “ghost” membrane, suggesting greater oxidative damage or change in the cell membrane. The authors postulated that the membrane abnormalities observed in sickle red cells, such as reduced deformability, vesiculation (Allan et al 1982), changes in cation homeostasis (Hebbel et al 1985) and abnormal adhesions to endothelium (Hebbel et al 1980), may be partially due to the formation of oxysterols. Using a similar technique, Rooney et al (1985) showed that the insertion of oxysterols into the cell membrane alters the fluidity in normal red cells and reduces the mobility of the acyl chain in the lipid membrane. Szostek et al (1991) examined the effect of inserting oxysterols into ghosts cell membranes prepared from normal and sickle erythrocytes. They reported that red cells with enhanced cholesterol have lower C-H stretch band frequency, while those with lower cholesterol content have higher C-H stretch band frequency, suggesting reduced membrane fluidity in the former.
1.7 Red cell deformability

1.7.1 The concept of red cell deformability

The flow behaviour of blood in vitro can be of relevance to two clinical issues in SCD:

a) the diagnosis and monitoring of disease processes and

b) the extrapolation to and prediction of blood flow in vivo.

Erythrocyte deformability and plasma viscosity constitute the two major physical properties of blood that influence its flow in capillaries. The ability of normal erythrocytes, of average diameter 8 μm (Ross et al 1995b), to traverse through the microcirculation, where capillary diameters may be as low as 3 μm, depends on their ability to deform. The normal shape of the red blood cell with an excess surface area to volume ratio of approximately 1.5 : 1, creates a “fluid drop” biconcave appearance, which allows for deformation. The main determinants of red cell deformability include cell geometry, the viscosity of the intracellular contents and the visco-elastic property of the membrane (which is influenced by its cholesterol to phospholipid ratio). The cell membrane deformability is affected by the levels of cellular ATP, pH and pCO₂ (Weed and Lacelle 1969, Weed 1970) and alterations in these variables by chemical agents or in haematological disorders could impair this.

1.7.2 Red cell filterability as a function of deformability

The ability of erythrocytes to deform in order to pass through microcapillaries can be replicated to a certain extent in vitro, by assessing the ease with which a standardised suspension of red cells is able to filter through pores of a diameter of 5 μm or less. This has the advantage of being able to measure a relatively large number of red cells in order to obtain a result representative of the mean cell deformability of the blood sample. Most commercially available filtration systems employ polycarbonate or metal filters with pores of diameter 3-8 μm and length 10 μm, which model flow conditions in the capillaries. For clinical studies, membranes with pore diameter of 5 μm are most attractive as this approximates to the mean diameter of nutritive capillaries in vivo. Compared with filters of 3 μm, filtration through 5 μm pores is relatively independent of mean cell volume (MCV) and temperature (Stuart et al
1985a) and less likely to cause haemolysis (Reinhart and Chien 1985). For the study of erythrocyte deformability per se, the filtration of suspensions of relatively "pure" red cells in buffer through filters of 5 μm at high driving pressure (20 cm of water) has been recommended (ICSH 1986). Certain confounding variables such as the patient's age, race, smoking habit, MCV, temperature and pH of the laboratory specimens are known to influence this measurement and need to be standardised (Hofrichter et al 1974, Bookchin et al 1976, Stuart 1985c).

Although filtration of whole blood may be regarded as a global test of potential flow resistance in the microcirculation, it is a composite of red and white cell deformability and therefore of less specific relevance to sickle cell disease than the use of "pure" red cell suspensions.

1.7.3 The St George's Filtrometer

Filtration of red cell suspensions may be performed in assembled systems using commercially available instruments such as the Hemorheometre (IMH, St Witz, France) (Hanss 1983), the Erythrometre (SEFAM, Nancy, France) (Stoltz et al 1984) and the St George's Filtrometer (Carri-Med Ltd, Dorking, UK) (Dormandy et al 1985). Most instruments require the removal of white cells from the red cell suspensions to less than 0.025 x 10^9/l by pre-filtration with cotton wool and washing in buffer (Stuart et al 1985b, ICSH 1986).

The St George's Filtrometer (figure 1.7.3) has been shown, in comparative studies with the Hemorheometre, the Contraves LS 30 Rotational Viscometer and the Radial Spreading Rheometer, to be the most sensitive instrument in the measurement of red cell deformability (Staubli et al 1986). It measures the rate of pore clogging by red cells and then extrapolates back to the initial flow rate, which is relatively independent of residual white cells. If the transit time of red cells through a pore is to be measured, then ideally only one cell should pass through each pore. However, as increasing numbers of cells filter through, the rate of flow may be affected by clogging of the pores by a minority of rigid red cells (Skalak et al 1983).

The vertically situated 5 μm polycarbonate filter system of the St George's Filtrometer has the advantage over horizontally placed membrane filter systems such
as the Hanss Hemorheometre, which suffer from this sedimentation artefact (Dormandy et al 1985).

With the St George’s Filtrometer, the filtration parameters Red Cell Transit Time (RCTT) and Clogging Rate (CR) have been shown to be sensitive and reproducible in the measurement of red cell deformability (Dormandy et al 1985). CR refers to the relative decrease in the initial filtration rate with the cumulative volume of suspension filtered, while RCTT is calculated as a fraction of the initial filtration rate and haematocrit of the red cell suspension (Dormandy et al 1985). While viscosity measurements are useful as global tests reflecting the rheology of sickle cell erythrocytes, filtration techniques, especially through 5 μm pores, appear more sensitive to changes in intracellular polymerisation of small amounts of Hb S (Green et al 1986) and are considered the most appropriate for clinical studies (Stuart and Kenny 1981). The filtration rate may be influenced by contaminating leucocytes (Stuart et al 1985b) and hence the acute response to vaso-occlusive crises, in the form of leucocytes and plasma proteins, may produce a spurious impression of impaired red cell filterability.

Using a pore diameter of not less than 5 μm maximises the sensitivity to changes in MCHC and cytoplasmic viscosity and hence, reduces dependence of filtration parameters on cell size (Reinhardt and Chien 1985, Stuart et al 1985b). Filtration then becomes a function of polymerisation of Hb S, which is influenced by oxygen tension (Messer and Harris 1970), temperature (Hofrichter et al 1974) and pH (Bookchin et al 1976), all of which must be standardised.
Figure 1.7.3: The St George’s Filtrometer (Carri-Med, Dorking, UK)
1.7.4 Others methods of measuring red cell deformability

The deformation of red cells can also be examined using more sophisticated techniques either in bulk suspensions or using micropipette filters. The former include laser diffractometry techniques using the Ektacytometer (Technicon International, St Denis, France) in which red cells are suspended in a high viscosity medium and sheared in a rotational system where they diffract a helium-neon laser beam. Alterations in cell dimension with increasing shear stress, as determined by changes in the ellipticity of the diffraction patterns, provide a measure of the cellular deformability (Mohandas 1988). The Ektacytometer, however, requires a fairly substantial amount of Hb S polymer to produce a detectable decrease in deformability (Sorette et al 1987). Micropipette studies also provide useful information on the visco-elastic properties of single red cells (when aspirated into pipette of diameter 3-6 μm) and the visco-elastic behaviour of the cell membrane (when parts of the cells are aspirated into pipettes of about 1 μm) (Mohandas and Hebbel 1994). However, micropipette techniques may be unable to detect localised membrane lesions if the major part of the sickle red cell membrane has normal cellular deformability (Mohandas and Bessis 1979).

As such the practical applicability of these methods is limited. The procedures are tedious and more importantly, only measure a small number of cells, which is a significant drawback, as it is well recognised that the red cell population in an individual with SCD is heterogeneous and changing (Stuart et al 1984).

1.7.5 Red cell deformability in SCD

Intracellular polymerisation of sickle haemoglobin with deoxygenation induces the morphological transformation from the flexible biconcave shape to the rigid sickled shape. Repeated cycles of sickling produce a complex array of secondary effects on the cell, altering its shape, size, hydration and deformability. The higher cytoplasmic viscosity of sickle cells caused by the polymerisation of deoxygenated Hb S and cytoplasmic dehydration, as well as oxidative damage to membrane cellular structure, contribute towards the impairment of cellular deformability. This has been demonstrated using ektacytometry (Clark et al 1980), micropipette aspiration (Nash et
al 1984, Evans et al 1984) and by filtration methods (Stuart 1985, Staubli et al 1986) as described earlier.

1.7.6 The effects of modulating agents on sickle red cell filterability
Numerous agents with presumed effects on the filterability of sickle erythrocytes have been documented. In in vitro studies, the calcium antagonist, bepridil, [beta-(2-methylpropoxy) methyl-N-phenyl-N-(phenylmethyl)-1-pyrrolidineethanamine monochloride monohydrate] is thought to improve red cell deformability by increasing the surface area to volume ratio and creating a stomatocytic effect. Bepridil does not appear to inhibit intracellular haemoglobin S polymerisation (Johnson et al 1994) and no clinical studies in patients have so far been reported.

Incubation with zinc (Brewer and Oelshlegel 1974, Brewer et al 1977, Taylor et al 1991) and benzaldehyde (Kenny and Stuart 1983) also improve the filterability of red cells from women with Hb SS, when measured using the St George’s Filtrometer. The mechanism of action is unclear: the effect may be due to membrane stabilisation or mediated through an increase in the oxygen affinity of sickle erythrocytes, thereby reducing intravascular sickling. Clinical claims of reduction in painful crises have not been confirmed in larger controlled studies.

Cetiedil, an ethyl ester (Stuart et al 1988), pentoxyfylline, a xanthine derivative (Bilto et al 1988, Stuart et al 1988) and piracetam, an acetamide (Stone et al 1988) have all been shown to have beneficial effects on red cell filterability in in vitro studies, but clinical benefits again have not been proven in individuals with SCD.

1.7.7 The effects of steroid hormones on red cell filterability
In an ex vivo study involving red cells from 34 non sickle cell women using the COCP’s, Oski and colleagues (1972) noted that erythrocytes from women exposed to mestranol (a synthetic oestrogen) and norethindrone (a synthetic progestogen) had prolonged filtration times compared to red cells from 36 comparable controls. This impairment in erythrocyte deformability was observed one to three days following the initiation of treatment and the changes were reversed within two to three days of discontinuing therapy. Conversely, Durocher et al (1975) found no significant
difference in filterability between non-sickle erythrocytes from COCP (n=20) and non-COCP (n=18) users, but did report that filtration time was significantly longer in the third trimester of pregnancy (n=28). However, the increase in filtration time in women in the third trimester of pregnancy may be due to the confounding influence of the higher MCV with advancing gestation rather than to the increasing levels of pregnancy-associated steroid hormones.

1.8 Osmotic fragility in SCD and the agents which modulate osmotic fragility

The osmotic fragility refers to the resistance of erythrocytes to haemolysis when exposed to a series of saline solutions of decreasing osmolality. Normal red cells, because of their biconcave geometry and flexible shape, can withstand a certain degree of oncotic stress before lysis. Sickle erythrocytes are osmotically resistant (Figueirdo and Zago 1985) due to a combination of inherent membrane damage limiting the passive diffusion of water and their increased surface area relative to cellular volume.

Isaacs and Hayhoe (1967) postulated that progesterone, testosterone and norandrostenolone inhibit sickling by stabilising the red cell membrane although the effect of these steroids on the osmotic fragility of sickle cells is unclear.

In a placebo controlled cross-over trial involving 23 Jamaican women with sickle cell anaemia, De Ceulaer and colleagues (1982) implied that the reduction in the frequency of crises and the improved red cell survival observed in those treated with intramuscular DMPA, may be due to a lower rate of haemolysis. However, the authors did not specify if that was mediated through an effect on the osmotic fragility of sickle red cells.

More recently, there have been studies on agents, predominantly ovarian steroids and their derivatives, which appear to modulate the osmotic fragility of normal erythrocytes. It is however unwise to extrapolate these findings to sickle erythrocytes due to the inherent differences in their geometry and membrane deformability.
Kaya and Saito (1985), for example, noted that progesterone reduces the osmotic fragility of fresh unincubated red cells at the concentration of $1.25 \times 10^{-5}$ to $2 \times 10^{-4}$ M, while 17 $\alpha$ hydroxyprogesterone has virtually no effect.

Androgens such as danazol are thought to stabilise the red cell membrane and increase the resistance of erythrocytes to osmotic lysis (Ahn et al. 1989). The appearance on electron microscopy of extra folds in the red cell membrane of patients taking danazol led Ahn and colleagues to conclude that this attenuated androgen increases the red cell membrane surface area relative to its volume, thus rendering the cell less susceptible to osmotic lysis. Interestingly, in a preliminary study exploring a different mechanism, Olawoye (1989) noted a consistent enhancement of membrane $Ca^{2+}$ ATPase activity by progesterone and testosterone. He suggested that this has the effect of increasing calcium efflux out of the erythrocyte, thus reducing intracellular pressure and making the erythrocytes more osmotically resistant.

Reticulocytes and red cells from patients following splenectomy have a greater surface area of membrane relative to the cell volume when compared with normal erythrocytes and therefore are osmotically resistant (Dacie and Lewis 1995). “Target” cells, formed by the accumulation of lipids in liver disease, as well as cells of abnormal geometry such as leptocytes which are unusually flattened, also tend to be osmotically resistant and may introduce bias into osmotic fragility tests (Cooper 1970). As both splenectomy and deranged liver functions are relatively common in patients with SCD, it is important to exclude these confounding factors when examining effects on osmotic fragility of sickle cells in isolation.
1.9 The binding of ovarian steroids to red cells

1.9.1 Ovarian steroid receptors

The traditional concept of steroid action involves binding to intracellular protein receptors of the target organ, following which steroid-receptor complex travels to the nucleus, promotes the formation of mRNA and modulates gene transcription (figure 1.9.1). The biological response of such a receptor to steroid hormones is a saturable phenomenon with a finite binding capacity.

By definition, a “true” receptor should have:

a) high ligand affinity
b) high ligand specificity
c) high degree of tissue specificity and
d) limited binding capacity (i.e. saturable).

The activity of a hormone at the target cell is determined by its concentration, its binding affinity to the receptor, the number of receptor sites occupied and the duration of binding. Steroid specific receptors display high affinities and specificities for a particular hormone or class of hormones, which allow a given target cell to respond to a hormonal signal without interference from other signals. The oestrogen receptor, for example, has a strict ligand requirement, recognising oestradiol ten times more than oestrone and a thousand times more than androgens, while cortisol and progesterone are not at all recognised. In biological terms, oestradiol is therefore, more potent than oestrone while testosterone can exert oestrogenic effects only at high pharmacological concentrations (King 1988).

The first successful demonstration of the specific attachment of a hormone to its target tissue was achieved when Jensen and Jacobson (1962) injected tritium labelled oestradiol into a rat and recorded initial high concentrations of radioactivity in muscle, kidney and liver, followed by rapid decrease which paralleled plasma levels. In contrast, the oestrogen responsive tissues such as the uterus and vagina attained and maintained higher levels of radioactivity for longer periods, due to selective “trapping” of the hormone by a protein unique to the target cell. The authors applied the term “receptor” to the protein since they were able to show that the binding was
specific to oestradiol, that there was an associated genomic action in response to oestradiol binding and no chemical alteration occurred to the steroid molecule as a result of binding to the receptor.

Two oestrogen receptors, $\alpha$ (ER $\alpha$) and $\beta$ (ER $\beta$) have been identified and these are expressed in different concentrations in various tissues such as the uterus and ovary (Ganong 2001). Similarly, there also appears to be two forms of progesterone receptors, A ($\text{PR}_A$) and B ($\text{PR}_B$). The presence of a receptor in a tissue, however, does not necessarily mean that the receptor serves a purpose in that site or that it exerts a biological effect. A large number of oestrogen receptors is expressed in the liver of the cockerel, for example, but administration of oestradiol appears to lead only to the synthesis of the egg protein vitellogenin by this organ (Blankenstein and Mulder 1988).
Figure 1.9.1: The traditional concept of steroid hormone action, where $E$ represents oestrogen, $R_c$, the receptor protein and $R_n$, the active nuclear form of the receptor protein
1.9.2 The characterisation of steroid hormone receptors

In biological systems, steroid receptors exist in the presence of other binding components which complicate the analysis of receptor binding parameters. In this thesis, a single receptor system is assumed and in such a system, the total number of receptors (Rₜ) is determined under equilibrium conditions by adding steroid until saturation or near saturation is obtained (Figure 1.9.2a). The point of saturation is equal to the number of receptor sites (n) or Rₛ, while the dissociation constant (Kₐ) is the concentration of steroid at which 50% of the receptor sites are occupied.

The amount of bound ligand (RS) can be related mathematically to free ligand, total receptor (Rₜₒₜ) and the dissociation constant (Kₐ) of the receptor-ligand complex by using a variation of the Michaelis-Menten equation:

\[ [RS] = \frac{[Rₜₒₜ][S]}{Kₐ} + [S] \]

A Scatchard plot of bound ligand/free ligand on the ordinate vs bound ligand on the abscissa yields a straight line with a slope of \(-1/Kₐ\), the intercept on the abscissa being equal to the value of Rₜₒₜ.

Steroid receptor specificity can be examined using competition studies, where receptor preparations are incubated with a single saturating concentration of radioligand, followed by the addition of different concentrations of unlabelled ligands. Total binding (TB), in this case, is the sum of non-specific (NS) and receptor specific (RS) binding. RS and NS binding can be quantified by means of competitive inhibition of radio-labelled steroid binding by the non-labelled steroid. NS refers to the ligand binding to non-receptor sites which is usually of low affinity and high capacity relative to the RS sites. This may be composed of a) true non-specific binding to the tissue in question, b) free radioligand not effectively removed during "washing" and c) non specific binding to separation material eg filters and test tubes.

In figure 1.9.2b, the curve RS + NS represents TB ie the amount of \[^3\text{H}\] radio-labelled ligand that is bound to both specific receptor sites and non specific sites and thus contains both saturable and non-saturable components. The addition of an excess (100-1000 times) of non radio-labelled hormone will displace the high affinity
hormone RS sites but not the low affinity NS sites. The competing non radio-labelled ligand essentially occupies all high affinity specific receptor sites but does not interfere appreciably with the binding of $[^3\text{H}]$ ligand to non-specific sites. NS binding sites are then measured as the radioactive steroid still bound in the presence of excess (approximately 100 x $K_d$) non radio-labelled competitive ligand. RS binding is calculated as the difference between TB and binding that occurs in the presence of an excess amount of unlabeled ligand (which is NS binding). RS binding sites are estimated by subtracting NS from RS + NS.

The radioisotope most commonly used is tritium $[^3\text{H}]$, which is incorporated into molecules either by direct synthesis or by catalytically induced tritium exchange with hydrogen atoms. Advantages of tritium labelling are that the labelled product is biologically indistinguishable from the native compound, that high specific activity (upwards of 30 Ci/mmol) can be obtained and stable storage for months is possible. The deterioration of the radioligand can be reduced by storage at $-70^\circ\text{C}$. In this thesis, centrifugation and repeated buffer washes are used to separate bound from free radioligand. As radio-labelled ligands can also bind non specifically to non biological substances such as test tubes, the critical aspect was to ensure rapid careful rinsing of the centrifuge tube and pellet to remove as much unbound radioligand as possible without allowing the rinsing buffer to remain in contact for more than a few seconds, which might result in the disruption of pellet integrity.
Non-Competitive Inhibition

A Saturation Plot

B Scatchard Plot

Noncompetitive inhibition of receptor binding. Symbols: • = no inhibitor added; o = inhibitor added at 1 nM; □ = inhibitor added at 10 nM.

Figure 1.9.2a: The saturation of steroid receptor binding in a single receptor system

Saturation and Scatchard analyses of specific and nonspecific binding.

Figure 1.9.2b: Specific (RS) and non specific binding (NS) of hormone receptor sites
1.9.3 The binding of ovarian steroids to haemoglobin and to the red cell membrane

Certain actions of ovarian steroid hormones, such as the oestrogen-induced alterations on neurotransmitter discharge (Nabekura et al 1986) and increases in the Ca$^{2+}$ influx in human spermatozoa associated with progesterone (Blackmore et al 1990) are so rapid that it is difficult to believe that these are mediated solely through the classical concept of gene transcription. Evidence has been accumulating that steroid hormones may not require entry into the cell but can exert non-classical non-genomic action at the membrane level through a variety of mechanisms. These mechanisms, which include modulation of membrane fluidity and activation of gamma amino butyric acid GABA$_\alpha$ plasma membrane receptors (Majewska 1992), have been shown to occur in a variety of tissues such as brain and smooth muscle and even in neural tissue preparations lacking cell nuclei (Brann et al 1995).

The actual mechanism by which ovarian steroids influence erythrocyte function is unclear. The traditional concept of steroid action involving intracellular receptor binding and gene transcription may not apply in the case of erythrocytes, as mature red cells are naturally anucleate and do not appear to express steroid receptors. In non-nucleated cells, it is possible that steroid hormones exert their effect by modulating membrane fluidity as postulated by some investigators (Wilmer 1961), although whether this effect would influence the biological action of erythrocytes is not known.

Early experimental evidence initially suggested that the haemoglobin of intact red cells may bind oestrogens (Bischoff and Bryson 1960). Later, Ige and Adadevoh (1975) demonstrated that oestradiol, progesterone and testosterone bind equally to the intact erythrocyte and to the actual haemoglobin molecule in solution. This binding is constant over a wide range of steroid concentrations and there is a suggestion that these hormones bind more strongly to Hb S and C molecules than to Hb A. The temperature of incubation also appears to influence binding and increasing the temperature from 24°C to 36°C seems to reduce the binding of progesterone to Hb S and A (Ige and Adadevoh 1975).

Most authors are of the opinion that steroid hormones become superficially bound onto the red cell membrane or cytoskeleton and that this binding is of a high capacity.
but non-saturable nature (Devenuto et al 1969, Brinkmann et al 1972, Puca and Sica 1982, Jacobsohn et al 1994). However, some of the studies had methodological flaws: many of the experiments were not conducted at physiological temperature and the authors often did not take into account the gender of the individuals from whom the samples were obtained or if the donors were using exogenous steroid hormones.

1.10 Contraceptive steroids, coagulopathy and SCD

1.10.1 Coagulation markers and COCPs' in non SCD women

It is recognised that the use of COCP's is associated with an increased risk of thromboembolic disease (TED) in the general population, with a rate of around four per 10000 woman-years of use (Spitzer et al 1996, Farmer et al 1997). The use of the COCP's has also been associated with alterations in both procoagulant and fibrinolytic activities (Kluft and Lansik 1997). Surrogate markers for the procoagulant, anticoagulant and fibrinolytic pathways, such as Fibrinogen, Prothrombin Fragment 1+2 ($F_{1+2}$), Plasmin α1 AntiPlasmin Complex (PAP), Protein S (PS), Platelet Factor 4 (PF 4) and β Thromboglobulin ($β$ TG), are all informative about the haemostatic systems in vivo (Kluft and Lansik 1997). $F_{1+2}$ measures procoagulant activity, while PAP assesses fibrinolysis; PS is an inhibitor of coagulation and both PF 4 and $β$ TG measure platelet activation.

Several studies have assessed changes in the levels of these protein markers in a population of women without SCD using modern COCP's (containing less than 50 μg oestrogen) in an attempt to evaluate the relative prothrombotic risk of “pill” users (Task Force on Oral Contraceptives 1991, Campbell et al 1992). It is currently believed that the procoagulant, anticoagulant and fibrinolytic changes induced by the use of COCP's result in an overall equivocal effect on haemostasis (Task Force on Oral Contraceptives 1991, Campbell et al 1992, Notelovitz et al 1992, Quehenberger et al 1996). Second and third generation progestogen components of modern COCP's appear to have no measurable effect on platelet aggregation and on molecular markers of coagulation and fibrinolytic activity (Winkler 1993, Kuhl 1996).

Many women are still understandably wary about the use of the COCP despite recent reassuring studies (Kaper et al 2000, Farmer et al 2000), which suggest that third
generation combined oral contraceptives are associated with similar risk estimates of thromboembolic disease as the second generation, and that the interpretation of epidemiological data which originally contributed to the pill scare of 1995-1996 (Jick et al 1995, Spitzer et al 1996) was probably due to bias rather than causation.

1.10.2 Markers of thrombosis and fibrinolysis in SCD

There is evidence that individuals with SCD exhibit abnormalities of coagulation and fibrinolysis even in steady state, but particularly during acute sickling crises (Nagel and Fabry 1988, Kurantsin-Mills et al 1992, Nsiri et al 1996, Wright et al 1997). In these individuals, the free Protein S and C levels are lower than in matched controls (Francis 1988, El Hazmi et al 1993, Wright et al 1997, Liesner et al 1998, Westerman et al 1999), while the levels of Thrombin Anti Thrombin complex (TAT) and F_{1+2} are increased compared with individuals without the condition (Liesner et al 1998, Westerman et al 1999). In children with SCD, blood transfusions appear only partially to reverse these abnormalities (Liesner et al 1998). Conversely, some investigators claim that while chronic intravascular coagulation is indeed elevated in patients with SCD, levels of these markers are not significantly different in the steady state and during painful crises (Green and Scott 1986, Westerman et al 1999), suggesting that sickling crises per se are not “thrombotic” events.

When stratified by genotype (Hb SS, SC and AS), there is also evidence that procoagulant activity (as indicated by F_{1+2} and TAT complexes) is increased in individuals with Hb SS compared with those with Hb SC and Hb AS (Helley et al 1997, Setty et al 2001). These authors claim that the procoagulant properties which characterise Hb SS also affect Hb SC and AS to a lesser degree and that the more hypercoagulable state in the former may be attributable to a greater exposure of phosphotidyl serine at the surface of sickle red cells from individuals with Hb SS. A similar view was also proposed by Westerman et al (1999), who noted increased levels of antiphospholipid antibodies, in particular Ig G to phosphotidyl serine, in individuals with homozygous Hb SS.

It is interesting to note that the Factor V Leiden mutation, which plays a role in thromboembolic disease in the general population, is rare in African-Americans with
SCD with a prevalence of 0.6% compared to white Caucasians in whom it is found in 3-7% of individuals (Kahn et al 1997).

1.10.3 Platelet activity in SCD
In individuals with SCD, the levels of PTG, PF 3 and PF 4, all molecular markers of platelet activation, are increased when compared with age, race and sex matched controls, although sickling crises do not necessarily precipitate further rise (Watson et al 1983, Green and Scott 1986, Famodu and Oduwa 1995). Experimental evidence suggests that extracellular proteins such as thrombospondin (Brittain et al 1993) and fibrinonectin (Kasschau et al 1996) released from activated platelets in patients with SCD may be the initiating event in sickle vasculopathy. Interestingly, these factors seem to promote increased adherence of sickle erythrocytes to human microvascular endothelial cells, compared with non-sickle erythrocytes, (Brittain et al 1993).

1.11 The endothelium and SCD
Considerable evidence now suggests that interactions between sickle erythrocytes and vascular endothelium play an important role in the clinical manifestations of SCD.
Single-cell micropipette measurements of erythrocyte adhesion (in human plasma) to bovine endothelial cells reveal that the force necessary to detach adherent red cells is greater for sickle than for normal red cells, $3.08 \pm 1.14$ vs $2.19 \pm 1.36 \times 10^{-6}$ dyne (Mohandas and Evans 1984). Hebbel and colleagues (1980) reported a strong correlation between the tendency of erythrocytes from patients with SCD to adhere to cultured endothelial cells and the clinical severity of their condition. Activated endothelial cells are procoagulant and favour the adhesion of phagocytes and sickle cells. The adherence of sickle cells to the endothelium may augment circulatory stasis, thereby promoting the polymerisation of Hb S and the occlusion of the microvasculature. Up-regulation of the adhesive and haemostatic properties of the endothelial cells occurs in viral infections, which explains why infections such as with the herpes virus, can precipitate vaso-occlusive complications.

Kaul and Hebbel (2000), using a transgenic sickle mouse model and directly visualising the microcirculation in a living open cremasteric muscle preparation, postulated that the cycle of hypoxia followed by reperfusion may be an important
factor in the pathophysiology of endothelial adhesion in SCD. A distinct inflammatory response in the endothelium, with increased leucocyte migration and adhesion was observed after three hours of mild hypoxia followed by reoxygenation. The authors also noted that abnormal migration and adhesion of leucocytes can be prevented by infusion of a monoclonal murine P-selectin antibody before reperfusion. They proposed that erythrocytes periodically attach themselves to the endothelial cells and that a transient log jam of relatively rigid deoxygenated red cells occurs in hypoxic conditions. Following reperfusion, the red cells are likely to adhere to inflamed endothelium which becomes increasingly loaded with large leucocytes. Repetitive episodes of localised ischaemia and reperfusion would eventually set up a low grade chronic inflammatory tissue injury state.

It is possible that the presence of circulating activated endothelial cells in SCD reflects the procoagulant status of the peripheral circulation. Solovey et al (1997) reported finding activated endothelial cells, probably of vascular origin, in the circulation of patients with SCD. The authors suggested that there may be a mechanistic relation between activated endothelial cells of microvascular origin and painful crises in patients with SCD. Factors that enhance the activation of endothelial cells also increase the adherence of sickle cells to endothelium. Individual variation in the expression of the adhesion molecules on the endothelium may explain the clinical variability of the disease. Using circulating endothelial cells (CEC’s) as a surrogate marker for vessel wall endothelium in individuals with Hb SS, Solovey (1998) noted that these markers were present in 83 ± 19% of the cases studied during sickling crises compared to 66 ± 13% during steady state (p=0.023).

The complex interplay between red cell adhesiveness, plasma factors and endothelium appears to be important (Mohandas and Evans 1984, Hebble 1997) and further studies of circulating endothelial cells may yield new insights into the clinical variability of the condition, methods of predicting painful crises and even techniques for reducing the responsiveness of endothelial cells in vivo. The activation of endothelial cells have additional pathophysiological effects, such as the release of von Willebrand’s factor (vWF) and the modulation of the expression of tissue factor and thrombospondin (TSP), all of which favour platelet-endothelial interactions. The cumulative effects of
these processes may be to bring about a procoagulant state, which would explain some of the severe thrombotic events that are common in patients with SCD.

It may soon be possible to develop laboratory procedures to measure the effects of agents which can modulate the adhesions between sickle cells and endothelium, such as lubricants, corticosteroids, antibodies against adhesive molecules and anti-inflammatory agents (Platt 1994). Conditions such as the acute chest syndrome and stroke, in which early markers of disease have also been identified, could also be investigated with regard to the state of endothelial cell activation in vivo (Adams et al 1992, Styles et al 1996).

1.12 Fluctuations of ovarian steroids during the menstrual cycle, pregnancy and the menopause; therapeutic levels of contraceptive steroids

Fluctuations occur in the plasma levels of ovarian steroid hormones during the female menstrual cycle. The variation in the plasma concentrations of 17β oestradiol and progesterone during the menstrual cycle is shown in figure 1.12 (Chabbert Buffet et al 1998). The reference range for plasma testosterone in premenopausal females is less than 3 nmols/ml (1 x 10⁻⁹ g/ml) (Milgrom 1990, Grudzinkas 1997) and the author has assumed 30 pmols/ml (1 x 10⁻¹¹ g/ml) as the mean baseline plasma testosterone in normal menstruating women. The levels of ovarian steroids in maternal blood during normal pregnancy (Grudzinskas 1997) are depicted in table 1.12. The plasma concentrations of oestradiol and progesterone in post-menopausal women are thought to be respectively 100 times (approximately 18-90 fmols/ml or 5-25 pg/ml) and 20 times (approximately 1.5 fmols/ml or 0.5 pg/ml) lower than peak levels in premenopausal women (Sherman 1987).

The plasma concentrations of synthetic oestrogens and progestogens in therapeutic use (for example in COCP’s) are notoriously difficult to replicate, with large inter- and intra-individual variations (Fotherby 1995, Carol et al 1992). A review by Orme and colleagues (1983) suggests that peak plasma levels achieved after long term administration of COCP’s containing 50 μg of ethinyl oestradiol rarely exceed 200 pg/ml (approximately 600 fmols/ml), while steady state plasma concentrations of
norethisterone and levonorgestrel range from 5-20 pmols/ml (1.6-15.2 ng/ml) and 2.5-15 pmols/ml (0.8-4.5 ng/ml) respectively.

Damber (1979) attempted to compare the relative biological potency of various oestrogens by quantifying the rise in pregnancy zone protein (PZP) induced by oestrogen stimulation and concluded that the synthetic oestrogen, ethinyl oestradiol was approximately 500 times more potent than the naturally occurring 17 β oestradiol.

Interestingly, there does not appear to be significant variation in plasma levels of gonadotrophins and ovarian steroid hormones in women of different ethnic origins (Salmon et al 1976, Romani et al 1977, Dada et al 1981).

Communication between the author and contraceptive pill manufacturers suggested that the approximate plasma levels of 17 β oestradiol and progesterone suitable for in vitro incubation experiments so as to simulate therapeutic concentrations were around approximately 30-35 µM (1 x 10^{-5} g/ml) (Schering Health Care Ltd., UK, personal communication 1996). For the in vitro experiments, the incubation with testosterone was performed at 0.3 µM (1 x 10^{-7} g/ml), arbitrarily chosen to represent a therapeutic concentration at least 100 fold the physiological levels for women.

1.13 Summary of literature review

Thus there is existing evidence suggesting links between ovarian steroid hormones, red cell function and the clinical nature of the sickle cell disease. However, a coherent understanding of the potential interaction(s) between ovarian (contraceptive) hormones and SCD has not yet been established and is therefore explored in this thesis.
Figure 1.12: Fluctuations in plasma concentrations of ovarian steroids during the normal menstrual cycle (Chabbert Buffet et al 1998)
Table 1.12: Steroid hormone levels in maternal blood during normal pregnancy

<table>
<thead>
<tr>
<th>Trimester</th>
<th>Oestradiol</th>
<th>Progesterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>2-10 nmol/l</td>
<td>30-45 nmol/l</td>
</tr>
<tr>
<td>Second</td>
<td>13-30 nmol/l</td>
<td>70-150 nmol/l</td>
</tr>
<tr>
<td>Third</td>
<td>20-80 nmol/l</td>
<td>150-600 nmol/l</td>
</tr>
</tbody>
</table>
CHAPTER 2: THE HYPOTHESIS

2.1 Statement of the hypothesis

2.2 Aims of the thesis
2.1 Statement of the hypothesis

It is hypothesised that:

a) oestradiol and progesterone have direct effects on erythrocytes from women with SCD, which may, in turn, influence the clinical manifestations of the condition. These effects can be demonstrated by variations in haematological parameters corresponding to the hormonal variations found during the menstrual cycle, in women using exogenous contraceptive steroid hormones and in the post-menopausal state

b) there is a specific interaction between the effects of oestradiol and progesterone on sickle red cells and their concurrent effects on blood coagulation factors

c) by characterising these interactions, there is a potential for using them therapeutically to ameliorate the clinical course in women with the condition.
2.2 Aims of the thesis

The aims of this thesis are to:

a) explore a sequence of *in vitro* and *ex vivo* phenomena intended to demonstrate the potential for such interactions to be present:

   i) to characterise the nature of binding of oestradiol and progesterone to red cells from SCD women

   ii) to investigate the ability of oestradiol and progesterone to inhibit red cell sickling

   iii) to investigate the effect of oestradiol and progesterone on membrane morphology of erythrocytes from women with SCD, as assessed by EM

   iv) to investigate the effects of oestradiol and progesterone on red cell deformability and osmotic fragility in erythrocytes from women with SCD

   v) to evaluate red cell deformability of erythrocytes from women with SCD using various exogenous steroid hormones

   vi) to investigate the effects of various exogenous ovarian steroids on markers of coagulation and platelet activation

   vii) to investigate the effects of the menstrual cycle and the menopause on sickling crises

b) to draw conclusions concerning the effects of ovarian steroids on erythrocytes and coagulation factors in women with SCD and hence, to establish an ethical basis for the construction of a patient level interventional study using therapeutic preparations of oestradiol and progesterone relevant to effective contraception and pregnancy planning for women with SCD.
CHAPTER 3: GENERAL METHODOLOGY

3.1 Ethical approval and consent

3.2 Patient groups

3.3 ICSH Guidelines for measurement of blood viscosity and erythrocyte deformability

3.4 Statistical methods
3.1 Ethical approval and consent

All the women with SCD included in this thesis participated voluntarily following interview and recruitment by the author. Verbal and/or written consent was given prior to entry into the study. Approval was obtained from the local ethical committees of the patients' hospitals prior to recruitment and the consultant staff managing these patients were fully informed of their participation. Confidentiality was maintained by identifying each subject by initials only and this code was kept in a locked filing cabinet in the author's room at the Royal Free Hospital.

3.2 Patient groups

Women with SCD (genotypes Hb SS, Hb SC and Hb Sβ thalassaemia confirmed by electrophoresis) were recruited from the haematology clinics of the Royal Free, the North Middlesex and St Thomas' Hospitals, London.

The inclusion criteria for all the studies (with exception of Chapter 11) were as follows:

a) not pregnant and had not been pregnant in the three months preceding the study
b) in steady haematological state *ie* no major sickling crisis in the three months preceding the study
c) had not received any blood transfusion in the three months preceding the study
d) normal liver function tests (LFT's) and not previously having undergone splenectomy
e) non-smoker
f) women using exogenous steroid hormones, such as the combined oral contraceptive pills (COCP's), had done so for at least three months prior to inclusion into the study.

At the first visit, a full history, including frequency of sickling crises, as well as the obstetric and gynaecological histories, was taken by the author. The proforma is shown as Appendix 8.
Four groups of women with SCD were included in this thesis:

Group 1: women of reproductive age using the COCP (n=10)

Group 2: women of reproductive age using progestogen only (PO) contraception (n=18)

Group 3: women of reproductive age not using exogenous steroid hormones (n=42)

Group 4: post-menopausal women (n=16), of whom two were using hormone replacement therapy (HRT).

A fifth group (group 5) consisted of women of Afro-Caribbean origin without haemoglobinopathy (Hb AA genotype) not using exogenous steroid hormone preparations (n=11) who were recruited from gynaecological and family planning clinics to form a comparative group.

3.3 ICSH Guidelines for measurement of blood viscosity and erythrocyte deformability

An Expert Panel on Blood Rheology, established by the International Committee for Standardisation in Haematology (ICSH), convened in 1986 to make recommendations on the laboratory assessment of erythrocyte deformability and blood rheology. A document was subsequently produced to define standardisation of laboratory conditions and instrument criteria (ICSH 1986) and the methods described herein (sections 3.3.1-4) adhere as closely as possible to these recommendations.

3.3.1 Venesection

Prolonged use of a tourniquet increases haematocrit and it is recommended by the ICSH (1986) that a tourniquet should only be used to allow localisation of the vein prior to insertion of the needle and that it should be removed at least five seconds prior to the actual withdrawal of blood. Blood should be taken from the antecubital vein using a large bore sterile needle to prevent shear damage to erythrocytes.

However, venous access in patients with SCD may be difficult as peripheral veins are often thrombosed due to their frequent use for venous sampling and transfusion. Therefore, standardisation regarding venesection using the antecubital vein may not always be feasible.
When possible, blood samples used for research in this thesis were collected from an antecubital vein using a size 21 G needle. Otherwise, blood samples were drawn from an indwelling subclavian venous catheter or from a peripheral vein other than the antecubital vein. If a tourniquet was used to locate the vein, then this was removed prior to actual venesection.

3.3.2 The timing of venesection

Circadian rhythms have been shown to exist in humans for blood volume, serum protein concentration and blood haematocrit. These factors were standardised for by removing the plasma proteins and by manipulating the haematocrit of the final red cell suspension used in the experiments. There does not appear to be a circadian rhythm in red cell deformability (Howard RJ, MD thesis 1995).

For the studies described in this thesis, it was not always possible to standardise for the time of collection as venesection was dependent on the time the patient arrived for her appointment. Most of the samples were taken between 1400 and 1700 hours, when patients attended the haematology clinics. Some samples were taken from patients at home and the author took these between 1400 and 1700 to correspond to the times that the other samples were drawn. Blood samples from the control patients were taken at the afternoon gynaecological and family planning clinics.

3.3.3 The collecting device and anticoagulant

It is recommended that blood should be collected with a plastic or glass syringe using minimal suction on the plunger to avoid trauma to the blood cells. None of the commonly used anticoagulants other than citrate or oxalate, which may cause shrinkage of the red cell, have a significant effect on red cell deformability (Dormandy 1981).

Venous blood samples in this thesis were collected using a 10 ml plastic syringe (Sherwood Medical, Gosport, UK) with minimal suction and then transferred into a 2.7 ml plastic collection tube (Sarstedt, Monovette, Leicester, UK). Unless otherwise mentioned, the anticoagulant used throughout the *in vitro* and *ex vivo* experiments was potassium ethylene diamino tetra acetic acid (EDTA) as a liquid at a
concentration of 3.4-4.8 mmols/l of blood. EDTA was used as this has been previously shown to be superior to lithium heparin (Dormandy et al, 1985) as the anticoagulant in studies on red cell deformability.

### 3.3.4 Subsequent processing of blood samples

Storage has an effect on the physical properties of whole blood (Dormandy, 1981). Passive storage of blood impair red cell filterability and viscosity (Poraicu et al, 1983), although this effect appears to be less marked in sickle erythrocytes (Adewuyi and Awarun, 1990). Depletion of red cell ATP by in vitro incubation without substrate for up to 24 hours can alter erythrocyte geometry, although parameters of red cell deformability such as red cell transit time (RCTT), seem to be unaffected (Staubli et al, 1986). Nevertheless, it has been recommended that all measurements should be carried out within four hours of venesection if the samples are maintained at room temperature (ICSH, 1986).

All samples in the studies in this thesis were processed within four hours to minimise the depletion of red cell ATP as recommended (ICSH, 1986). After collection, blood samples were kept at room temperature. Blood samples were mixed evenly prior to experiments to ensure that any rheological abnormality was uniformly distributed within the red cell population.
3.4 Statistical methods

All data were subjected to the Kolmogorov-Smirnov test to ensure that they conformed to a normal distribution. Where data were normally distributed, and a "subject" and a "control" group were involved, *t* tests were an appropriate method for detecting differences between the two samples. Paired *t* tests were used when the same group was tested before and after a procedure (eg before and after incubation) and unpaired *t* tests were used when two different groups were involved (eg comparing levels of haemostatic markers in two groups of women). When three groups of data were involved, the data were subjected to analysis using the ANOVA, followed by the Tukey-Kramer Multiple Comparison test if the difference was statistically significant. However, in cases where the numbers were small and/or the data were not normally distributed, then non-parametric statistics were utilised for analysis. In these cases, the Wilcoxon rank sum test was used for paired or dependent samples, while the unpaired or independent samples were analysed using the Mann-Whitney U test. The Kruskal-Wallis test was used for non-parametric analysis of three groups of data.

The data reported in the studies described in chapters 4-9 and 11 were normally distributed and were therefore analysed using parametric statistics. Data for the measurement of markers of coagulation (chapter 10) followed a non-parametric distribution and were analysed using the Kruskal-Wallis test. In accordance with generally accepted convention, a *p* value of <0.05 was taken as indicating a statistically significant difference (Bradford Hill 1978, Faragher and Marguerie 1998).

For this thesis, data are presented as mean ± standard error of mean (SEM) when data are normally distributed, and as median and interquartile ranges (IQR's) when the data are non-parametric. Histograms are used to depict graphically normally distributed data, while scatterplots are used to present non-parametric data.

Data analysis was performed using GraphPad Prism 2 software (San Diego, CA, USA).
CHAPTER 4: THE *IN VITRO* RECEPTOR BINDING CHARACTERISTICS OF OESTRADIOL AND PROGESTERONE TO RED CELLS FROM WOMEN WITH SCD

4.1 Introduction

4.2 Method

4.3 Results
4.1 Introduction
An understanding of the interactions between red cells and ovarian hormones can be reached by characterising and comparing the binding of oestradiol and progesterone to intact erythrocytes from ethnically matched women with Hb SS, Hb SC and normal control volunteers with Hb AA genotypes.

Most steroid receptors exist in the presence of other binding components which could complicate the analysis of receptor binding parameters, but for the purpose of this thesis, the author has assumed a single component system kinetics ie that the system contains only one type of receptor site.

In a single component system, the total amount of receptor (Rtot) is determined under conditions of equilibrium by adding steroids until saturation or near saturation is achieved. Non-specific binding (NS) is the result of the ligand binding to non-receptor sites which are usually of low affinity and high capacity relative to the receptor specific sites (RS) (see section 1.9.2).

RS and NS bindings can be quantified by means of competitive inhibition of radio-labelled steroid binding by the non-labelled steroid. Centrifugation and repeated buffer washes are used to separate bound from free radioligand, bearing in mind that radioligands can also bind non-specifically to biological (tissue) and non-biological (test tube) material. In this series of experiments, the concentration of the radio-labelled steroids was held constant while increasing the concentration of the unlabelled ligand, thus in effect, increasing the ligand concentration by diluting the specific activity of the radioligand.

Although mature red cells are naturally anucleate and do not express hormone receptor sites, it has been known that steroids can associate with circulating erythrocytes from non Hb SS volunteers (Willmer 1961, Vermeulen 1961). Most authors are of the opinion that steroids become superficially bound or incorporated into the red cell membrane and that this "binding" is of a high capacity but non-saturable nature (Denevuto et al 1969, Brinkman et al 1972, Jacobsohn et al 1994). However, many of the earlier studies did not standardise the gender of the individuals
from whom the blood was obtained nor whether these donors had been previously treated with exogenous steroid hormones. Moreover, many of these experiments were not performed at the physiological temperature of 37°C.

Given that steroids can bind to normal erythrocytes and possibly influence their biochemical and biophysical properties, the objective of the studies reported in this chapter is to characterise and compare the binding of oestradiol and progesterone to intact erythrocytes from ethnically matched women with Hb SS, Hb SC and “normal” volunteers of Hb AA genotype.

4.2 Method

Preparation of ovarian steroids

Radio-labelled [2,4,6,7-^H]-oestradiol (81 Ci/mmol) and [1,2,6,7-^H]-progesterone (92 Ci/mmol) were purchased from Amersham International plc (Aylesbury, Bucks., UK) and were of at least 98% radiochemical purity. Non-radioactive oestradiol and progesterone were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Radio-labelled and non radio-labelled steroids were prepared as described in Appendix 1.

Preparation of erythrocyte suspensions

Blood was taken from premenopausal Afro-Caribbean female volunteers with haemoglobin genotypes Hb SS (n=11), Hb SC (n=4) and Hb AA (n=7), who were not using contraceptive steroid hormones and had not had sickling crises, been transfused or pregnant in the preceding three months. In order to standardise the exposure of red cells to endogenous ovarian hormones, venesection was performed in the early follicular phase of the menstrual cycle (days 1-7), when the female plasma levels of oestrogen and progesterone were at their nadir ie oestradiol 1 x 10^-10 g/ml (approximately 30 nM) and progesterone 1 x 10^-9 g/ml (approximately 3 μM) (Chabbert Buffet et al 1998) (see section 1.12). This was confirmed by the concurrent measurement of serum oestradiol and progesterone. All the experiments were performed within four hours of venesection.

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Suspensions of "pure" red cells were prepared in order to exclude the confounding influences of leucocytes and plasma proteins. To filter off leucocytes, cotton wool (Gossypium barbadense) was taken from an IMUGARD IG500 leucocyte removal filter (Terumo Corporation, Tokyo, Japan) and one g of this was loosely packed to the five ml mark of a vertically clamped five ml plastic syringe, the plunger having been discarded. The cotton wool was initially saturated with 5 to 6 ml of buffer and allowed to drain for two to three minutes until the buffer no longer dripped from the syringe nozzle. 2 ml of whole blood was then added to the top of the damp cotton wool. When the blood had soaked into the cotton wool, a further 5 ml of buffer was added over one to two minutes (figure 4.2a). The effluent was collected, washed twice in buffer and then centrifuged at 700 g for one minute to remove residual platelets and plasma protein. The erythrocytes were then resuspended in Hepes buffer and the packed cell volume (PCV/haematocrit) of this suspension measured by the microhaematocrit method as recommended by ICSH (1986). All measurements of PCV/haematocrit in this thesis were performed by the author using a manual microhaematocrit reader (Hawksley, England) (appendix 4). The author's coefficient of variation (CV) of this method was 1.63%.

Following this, the buffy layer was removed by a micropipette and the remaining erythrocyte column washed three times with Hepes buffered saline (20 mmol/l HEPES, pH 7.4 ± 0.05 and osmolality 295 ± 5 mmol/kg) and resuspended to make up the appropriate haematocrit. The method of preparation of the Hepes buffer is described in appendix 2 and its pH and osmolality were rechecked prior to each experiment. The buffer was prefilted through 0.2 μm membranes (Acrodisc PF, Gelman Sciences, Godalming, UK) prior to use, to remove impurities which could influence measurements. The accuracy of leucocyte removal was checked by flow cytometry in the special haematology laboratory of the Royal Free Hospital. After filtering and washing the red cells twice in buffer, the white cell count in the solution was 0.1 x 10⁹/l.

The red cell suspensions were then incubated with 1μCi [³H]-oestradiol (1.25 nM) or [³H]-progesterone (1nM) in glucose-supplemented Hepes in a final volume of one ml
per tube for one hour at 37°C. The samples were centrifuged at 1000 g at 4°C for ten minutes, after which the supernatant was separated from the cell pellet. An aliquot of the supernatant (800 µl) was assayed directly for radioactivity, while the cell pellet was resuspended to 1 ml with glucose-supplemented Hapes prior to assay of radioactivity in a 500 µl aliquot. The radioactivity of all samples was quantified using a liquid scintillation counter (Beckman LS 5000CE) (figure 4.2b) and 5 ml Ultima-Gold scintillant (Canberra Packard, Pangbourne, Berks., UK) per sample, with a count time of five minutes per vial. Percentage [3H]-steroid binding was calculated as the proportion of radioactivity associated with the cell pellet divided by the sum of the radioactivity in the pellet and supernatant fractions, corrected for "non-specific" binding of the steroids to the borosilicate assay tubes. (The mean non-specific binding to the assay tubes was 2.0% for oestradiol and 6.9% for progesterone). In all cases, steroid binding was expressed in fmol steroid bound per million cells. This value was derived by multiplying the percentage binding of [3H]-oestradiol/progesterone by the total amount of oestradiol/progesterone in each assay tube and correcting for the cell density at a constant haematocrit of 20%. Cell densities were estimated as 0.73 x 10^6 cells/ml for Hb AA erythrocytes and 2.01 x 10^6 cells/ml for Hb SS erythrocytes at the 20% haematocrit.

The duration of incubation in the experiments conducted in this thesis varied from 30 minutes to 24 hours. The red cell suspensions involved in these studies were supplemented with 10 mmol/l glucose as substrate, to allow aerobic metabolism and to circumvent the potential confounding effect of ATP depletion which may induce functional and geometrical changes.
Figure 4.2a: The removal of leucocytes by filtration through cotton wool
Figure 4.2b: The Beckman LS 5000CE Liquid Scintillation Counter (Beckman Instruments Inc., Fullerton, CA 92634-3100 USA)
The effect of temperature
To assess the effect of temperature on steroid binding to erythrocytes, the binding of \[^{3}\text{H}\]-oestradiol and of \[^{3}\text{H}\]-progesterone were compared at 4°C and 37°C. The erythrocytes were incubated at 4°C and at 37°C in the presence of tritiated 1.25 nM oestradiol and 1 nM progesterone for one hour, after which the samples were centrifuged and both supernatants and cell pellets were assayed for radioactivity. For the former, red cells were incubated in a thermostatically controlled refrigerator, while a thermostatically controlled water bath set at 37°C (± 0.5°C) was used for the latter.

The effect of sequential buffer washes
To investigate the dissociation of steroid binding with sequential “washes”, erythrocytes were incubated with 1.25 nM or 1 nM \[^{3}\text{H}\] oestradiol and progesterone at 37°C for one hour and then “washed” by successive resuspension in and precipitation (by centrifugation) from glucose-supplemented Hepes buffered saline. The proportion of the total added radioactivity associated with pellets washed zero, one, two and three times was calculated, as described above, to assess the dissociation of \[^{3}\text{H}\]-steroids from the erythrocytes associated with successive washes.

The effect of differing steroid concentration
In order to evaluate the binding affinity for steroids, erythrocytes were incubated at 37°C for one hour with increasing concentrations of non-radioactive oestradiol and progesterone (10 nM, 100 nM, 1 μM and 10 μM) while keeping the concentration of the appropriate \[^{3}\text{H}\]-steroids constant at 10nM. Absolute amounts of steroid bound by the red cells were calculated by multiplying the steroid concentration (in pmoles per assay tube) by the percentage binding, calculated as described above.

As it was difficult to prepare large quantities of red cells from women with SCD, the initial binding studies described above were performed using erythrocytes from the four women of Hb AA genotype recruited from gynaecological and family planning clinics. Each of the studies was repeated three times using red cells from a different woman of Hb AA genotype on each occasion.
The effect of haemoglobin genotype

To compare the steroid binding to erythrocytes from women with different haemoglobin genotypes, red cells from women with Hb SS (n=11), Hb SC (n=4) and Hb AA (n=7) genotypes were incubated with 1.25 nM $[^3]H$-oestradiol or 1 nM $[^3]H$-progesterone at 37°C for one hour. Binding of each steroid was then calculated as described above.
4.3 Results

All data were normally distributed and were therefore analysed using parametric statistical tests. There was no significant difference between the binding of either oestradiol or progesterone to erythrocytes (n=3) incubated at 4°C vs 37°C (oestradiol, \( p=0.83; \) progesterone, \( p=0.98; \) paired t-tests; figure 4.3a). All subsequent experiments were therefore performed at the physiological temperature of 37°C. Sequential post-incubation washes with glucose-supplemented Hepes progressively decreased the binding of both oestradiol and progesterone to erythrocytes by up to 90.7% and 81.8% respectively, as shown in figure 4.3b \( (p<0.001 \) for both steroids, ANOVA). The apparent “non-specific” binding of oestradiol and progesterone to the borosilicate assay tubes was also decreased by up to 97.8% with successive washes. Hence, these experiments demonstrate that the binding of these steroids can be displaced by successive suspension and resuspension in buffer.

There was no significant difference in the percentage binding of oestradiol and progesterone to erythrocytes when the concentration of the appropriate non-radioactive steroid was increased from 10 nM to 10 \( \mu M \) (oestradiol, \( p=1.00; \) progesterone, \( p=0.88; \) ANOVA). The absolute amount of steroid bound increased in direct proportion to the total amount of steroid added for both oestradiol and progesterone and the binding of both hormones appear to be of a non saturable nature (figure 4.3c).

For erythrocytes from each of the three genotypes studied, the binding of oestradiol was significantly greater than that of progesterone \( (p<0.005 \) for all three genotypes, unpaired t-tests). The binding of oestradiol to erythrocytes from women with Hb SC was not significantly different from that observed in Hb AA controls. However, the oestradiol binding was 23% lower in women with Hb SS compared to controls \( (p=0.012) \). Similarly progesterone binding to red blood cells did not differ between women with Hb SC and Hb AA genotypes, but was decreased by 28% in cells from Hb SS patients \( (p=0.025; \) tables 4.3a and b, figure 4.3d).
Furthermore, the percentage binding of steroids in the Hb SS samples is likely to have been overestimated as the binding of sex steroids to red cells were compared at a constant haematocrit. In fact, the preliminary data suggest that the abnormal geometry of sickle erythrocytes allowed nearly three times as many cells to be packed into a similar haematocrit (2.01 x 10^6 vs 0.73 x 10^6 cells per ml at 20% haematocrit for Hb SS and Hb AA erythrocytes respectively). This calculation was based on using the counting chamber of a haemocytometer to count the number of cells in 1 μl of 0.02% haematocrit suspension and then extrapolating to estimate the number of erythrocytes in the Hb SS and Hb AA suspensions.

The author has not excluded the possibility that the abnormal geometry and “packing” of sickle erythrocytes as well as the higher number of cells in the Hb SS samples could introduce confounding variables which may interfere with the binding of ovarian steroid hormones.
Figure 4.3a: The effect of incubation temperature on the binding (mean ± SEM) of oestradiol and progesterone
Figure 4.3b: The effect of buffer washes on the binding (mean ± SEM) of oestradiol (solid line) and progesterone (broken line) at 37°C (* \( p < 0.05 \), ** \( p < 0.01 \); ANOVA)
Figure 4.3c: The effect of final steroid concentration on the total binding (mean ± SEM) of oestradiol (solid line) and progesterone (broken line) at 37°C.
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<thead>
<tr>
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<tbody>
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Table 4.3a: The percentage binding of oestradiol to red cells from women of different Hb genotypes

<table>
<thead>
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<th>Hb AA</th>
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<td></td>
<td>41.11</td>
</tr>
<tr>
<td>25.50</td>
<td></td>
<td>23.60</td>
</tr>
<tr>
<td>22.50</td>
<td></td>
<td>24.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.00</td>
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<td>41.60</td>
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<td>Mean</td>
<td>21.42</td>
<td>28.63</td>
</tr>
<tr>
<td>SEM</td>
<td>1.11</td>
<td>1.65</td>
</tr>
</tbody>
</table>

Table 4.3b: The percentage binding of progesterone to red cells from women of different Hb genotypes
Figure 4.3d: The percentage binding (mean ± SEM) of oestradiol and progesterone to red cells from women of different Hb genotypes at 37°C (** p<0.01, ANOVA)
CHAPTER 5: THE EFFECT OF INCUBATION WITH OESTRADIOL, PROGESTERONE AND TESTOSTERONE ON CELL MEMBRANE THICKNESS IN DEOXYGENATED SICKLE CELLS ASSESSED USING TRANSMISSION ELECTRON MICROSCOPY (TEM)

5.1 Introduction

5.2 Method

5.3 Results
5.1 Introduction

Mature erythrocytes are anucleate and do not appear to express steroid receptors. Thus the classical concept of receptor mediated genomic actions, in which the steroid hormone-receptor complex travels to the nucleus where it modifies gene transcription, does not apply in red cells. The data from chapter 4 suggest that oestradiol and progesterone become superficially bound onto the red cell membrane in a high capacity but non-saturable manner. The series of experiments in the current chapter are designed to examine if such binding of ovarian steroid hormones (following incubation with therapeutic concentrations of oestradiol, progesterone and testosterone) modulates the physical properties of the red cell membrane in women with SCD.

5.2 Method

Suspensions of “pure” erythrocytes were prepared from women with Hb SS genotype (n=5) by filtration through cotton wool and resuspension in buffer as described previously (see section 4.2). Aliquots were incubated at 37°C for one hour with Hepes buffer (control), 35 μM oestradiol, 30 μM progesterone and 0.3 μM testosterone in glucose-supplemented Hepes buffer. These concentrations were chosen to simulate therapeutic concentrations which are approximately 100 to 10,000 fold the peak concentrations reported for the normal systemic female plasma (Stewart et al 1993). Sickling was induced by passing humidified 95% (v/v) nitrogen slowly through the erythrocyte suspensions in sealed beakers for 30 minutes. The presence of sickled cells in each aliquot was verified by light microscopy. The sickled red cells were then fixed in 1.5% (v/v) gluteraldehyde and 1% (v/v) paraformaldehyde in phosphate buffer for two hours and prepared for transmission electron microscopy (TEM) using the Philips 201 TEM Electron Microscope (Eindhoven, Netherlands).

For each aliquot preparation ie control, oestradiol, progesterone and testosterone, ten individual sickle red cells were initially selected from the TEM pictograms (figure 5.2). Five measurements from different sites of the cell membrane of each of the ten sickle red cells were then taken by technical staff of the Electron Microscopy Laboratory of the Royal Free Hospital using the analySIS 2.1 (Soft Imaging Software...
GmbH, Munster, Germany) programme. The mean membrane thickness of sickle red cells from the different aliquot preparations were then calculated and statistically compared. The changes in membrane thickness induced by incubation with ovarian steroid hormones were calculated by subtracting the membrane thickness of the red cells of the control sample from those of the oestradiol, progesterone and testosterone samples, respectively, and these were also statistically compared.
Figure 5.2: Transmission electron microscopy (TEM) image of the cell membrane (arrow) and cytoplasm (c) of a sickle erythrocyte (with measurement grid)
5.3 Results

The mean cell membrane thickness (in nm ± SEM) of sickle red cells from five women with Hb SS after incubation with oestradiol, progesterone, testosterone and Hepes buffer only are presented in Table 5.3a. The data are normally distributed and when analysed using Repeated Measures ANOVA, showed no statistically significant differences between the four groups (p=0.4).

The difference in cell membrane thickness induced by ovarian steroid hormones was calculated by subtracting the measurements of sickle red cells incubated with oestradiol, progesterone and testosterone from that of the control sample incubated with Hepes buffer only (Table 5.3b). Positive values indicate a greater thickness and negative values represent a lesser thickness of the cell membrane compared to control sample. No statistically significant change in cell membrane thickness was noted following incubation with oestradiol, progesterone and testosterone (p=0.41, Repeated Measures ANOVA), at concentrations which approximate pharmacological plasma levels.
Table 5.3a: Mean cell membrane thickness ± SEM (nm) of sickle red cells from five Hb SS women incubated with Hepes buffer only (control), oestradiol, progesterone and testosterone. There was no statistically significant difference ($p=0.4, \text{Repeated Measures ANOVA}$) in cell membrane thickness between the four groups.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Control</th>
<th>Oestradiol</th>
<th>Progesterone</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.44 ± 0.07</td>
<td>5.25 ± 0.11</td>
<td>5.37 ± 0.06</td>
<td>5.52 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>5.57 ± 0.09</td>
<td>5.44 ± 0.09</td>
<td>5.33 ± 0.13</td>
<td>4.87 ± 0.08</td>
</tr>
<tr>
<td>3</td>
<td>5.43 ± 0.10</td>
<td>5.65 ± 0.15</td>
<td>5.68 ± 0.13</td>
<td>5.67 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>5.86 ± 0.09</td>
<td>5.72 ± 0.09</td>
<td>5.73 ± 0.09</td>
<td>5.65 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>5.53 ± 0.07</td>
<td>5.56 ± 0.09</td>
<td>5.28 ± 0.04</td>
<td>5.15 ± 0.11</td>
</tr>
</tbody>
</table>

Table 5.3b: Mean change in cell membrane thickness (nm) of sickle red cells following incubation with oestradiol, progesterone and testosterone. There was no statistically significant difference ($p=0.41, \text{Repeated Measures ANOVA}$) in the mean change in cell membrane thickness following incubation with these different ovarian steroid hormones.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Oestradiol</th>
<th>Progesterone</th>
<th>Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.19</td>
<td>-0.07</td>
<td>+0.08</td>
</tr>
<tr>
<td>2</td>
<td>-0.13</td>
<td>-0.24</td>
<td>-0.70</td>
</tr>
<tr>
<td>3</td>
<td>-0.14</td>
<td>-0.13</td>
<td>-0.21</td>
</tr>
<tr>
<td>4</td>
<td>+0.22</td>
<td>+0.25</td>
<td>+0.24</td>
</tr>
<tr>
<td>5</td>
<td>+0.03</td>
<td>-0.25</td>
<td>-0.38</td>
</tr>
</tbody>
</table>
CHAPTER 6: THE EFFECT OF INCUBATION WITH OESTRADIOL AND PROGESTERONE ON THE INHIBITION OF SICKLING IN ERYTHROCYTES FROM WOMEN WITH SCD

6.1 Introduction

6.2 Method

6.3 Results
6.1 Introduction

It has been documented that in vitro incubation with progesterone (Isaacs and Hayhoe 1967), medroxyprogesterone acetate (Perkins 1971) and the progestogen, megestrol (Adadevoh and Isaacs 1973) prevent and even possibly reverse sickling. Conversely, comparable red cells suspensions are reported to remain sickled when incubated with oestrone (Isaacs and Hayhoe 1967). Other authors (Lundh and Gardner 1970, Raper et al 1970) have been unable to reproduce the antisickling effects of progesterone and its derivatives and have suggested that the earlier reported findings were artefactual, due the “sphering” effect of the oil base of the progesterone preparations.

The aim of this series of experiments is to assess if incubation with therapeutic concentrations of contraceptive steroids reduced sickling in red cells from women with SCD.

6.2 Method

Aliquots of erythrocyte suspensions from women with Hb SS (n=4) and Hb AA controls (n=3) were incubated at 37°C for one hour with 35 μM oestradiol or 30 μM progesterone in glucose-supplemented Hepes buffer as previously described. Sickling was induced by passing humidified 95% (v/v) nitrogen slowly through the erythrocyte suspensions in sealed beakers for 30 minutes and the presence of sickled erythrocytes was confirmed by light microscopy. The erythrocytes were then fixed anaerobically in 1.5% (v/v) gluteraldehyde and 1% (v/v) paraformaldehyde in phosphate buffer and the percentage of sickled cells, in three fields at 640 times magnification, was compared between the four Hb SS and three Hb AA women and, for each genotype, between the three treatment groups (no steroid vs oestradiol vs progesterone). For this study, the term “sickled cells” included typical sickle cells, holly leaf shaped and elongated cells with one or more spicules, as defined by Kaul and Xue (1991).
6.3 Results

The ability of oestradiol and progesterone to inhibit sickling in vitro was assessed in women with SCD (n=4) and compared to a control group of women with Hb AA (n=3) genotype. For each subject, the number of cells counted in each field of view of the light microscope ranged from 71 to 326 with a median of 200. Erythrocytes obtained from the three Hb AA volunteers did not sickle after slow deoxygenation irrespective of whether or not the red cells had been pre-incubated with steroids. For Hb SS erythrocytes pre-incubated without exogenous steroids, 61.10 ± 7.70% of cells were sickled following passage of nitrogen. However, there was no statistically significant difference in the percentage of sickling for cells pre-incubated for one hour with 35 μM oestradiol or with 30 μM progesterone ($p=0.98$; ANOVA; figure 6.3a).
Figure 6.3a: The percentage sickling (mean ± SEM) of red cells after incubation with oestradiol and progesterone at 37°C in four women with Hb SS
CHAPTER 7: THE EFFECT OF INCUBATION WITH OESTRADIOL AND PROGESTERONE ON THE OSMOTIC FRACTILITY OF ERYTHROCYTES FROM WOMEN WITH SCD

7.1 Introduction

7.2 Method

7.3 Results
7.1 Introduction
The osmotic fragility test is a measure of the resistance of erythrocytes to haemolysis when exposed to a series of saline solutions of decreasing osmolarity. Normal red cells, because of their biconcave and flexible shape, can withstand a limited degree of osmotic stress before lysis. Sickled shaped erythrocytes are osmotically resistant (Figueirido and Zago 1985) due to a combination of inherent membrane damage limiting the passive diffusion of water and to the intracellular polymerisation of Hb S which increases the surface area relative to the cell volume. Osmotic fragility is most accurately described in terms of Mean Corpuscular Fragility (MCF), the saline concentration at which 50% of the cells haemolyse at standard pH and temperature (Dacie and Lewis 1995).

There is experimental evidence that sex steroids such as progesterone (Kaya and Saito 1985) and androgens (Ahn et al 1989) may alter the osmotic fragility of red cells from individuals with non Hb SS genotype. The author wished to investigate if incubation with therapeutic concentrations of oestradiol and progesterone affected the MCF of erythrocytes from women with SCD.

7.2 Method
Erythrocytes from women with Hb SS (n=7) and Hb AA (n=4) genotypes were pre-incubated at 37°C for 24 hours with 35 μM oestradiol or 30 μM progesterone and glucose-supplemented Hepses buffered saline under sterile conditions. These steroid concentrations were chosen, in order to approximate therapeutic concentrations, which are 1,000 to 10,000 times the peak concentrations reported for women not taking exogenous hormone preparations (Stewart et al 1993). As previously indicated, plasma concentrations of oestradiol and progestogens in therapeutic use (as found, for example with the COCP’s), are difficult to reproduce, with large inter- and intra-individual variations (Orme et al 1983, Fotherby 1990, Carol et al 1992, Fotherby 1995) but encompass the concentrations used in the studies described in this thesis. Incubation for 24 hours metabolically stresses the red blood cells and accentuates any abnormality in the function of the plasma membrane.
A graded series of saline solutions of decreasing tonicity (10 to 1 g/l) was prepared as described by Papert et al (1947) and later modified by Dacie and Lewis (1995). The hormone treated erythrocytes were then transferred to tubes containing decreasing concentrations of saline solutions. After careful mixing, the cell suspensions were left to equilibrate for 30 minutes and then centrifuged at 1000 x g for five minutes. The supernatants were decanted from the pellets and the absorbances of the supernatant evaluated at 540 nm, standardised against the 10 g/l saline supernatant as an assay blank (assumed to correspond to 0% haemolysis).

Optical density (OD) of the supernatant was read using a Philips PU 8720 UV/VIS Scanning Spectrophotometer (Mercer Row, Cambridge, UK) and this reflected the degree of haemolysis of the red cells. The percentage lysis was calculated by dividing the OD of the supernatant obtained from a particular saline osmolality by the OD of the 1 g/l standard, which was assumed to represent 100% haemolysis. Osmotic fragility curves were constructed by plotting the percentage lysis on the abscissa against the concentration of saline solutions on the ordinate. The MCF value, which is the saline concentration at which 50% of the cells haemolyse at standard pH and temperature, was then obtained from these curves using the radioimmunoassay template of GraphPad Prism 2 software (San Diego, CA, USA) for the analysis of semi-logarithmic sigmoidal data.

7.3 Results
The mean corpuscular fragility (MCF) at 50% haemolysis (g/l) of red cells from seven pre-menopausal women with Hb SS pre-incubated for 24 hours with Hepes buffer (control), oestradiol and progesterone are presented below in table 7.3a.

The MCF of oestradiol and progesterone loaded sickle cells were compared to sickle red cells incubated with Hepes buffer only (controls) using the two-way analysis of variance (ANOVA). In women with SCD, there appeared to be no significant difference in the MCF values between erythrocytes pre-incubated for 24 hours with Hepes buffer only, 35μM oestradiol and 30μM progesterone within and between subjects (p=0.552, two-way ANOVA).
The two-way ANOVA was also used to analyse similar data of red cells from four Hb AA women incubated with Hepes buffer only, oestradiol and progesterone shown in table 7.3b ($p=0.830$).

The osmotic fragility curves of the sickle red cells and red cells from Hb AA women, constructed by plotting the percentage lysis against the corresponding concentration of Na Cl solution, are represented in figures 7.3a and b. Osmotic fragility curves for erythrocytes from women with Hb SS show a left shift compared to the curves for erythrocytes from women with Hb AA, indicating that sickle cells, in general, show a decrease in osmotic fragility. However, there was no significant difference in the MCF values between red cells incubated with no hormones, 35 µM oestradiol and 30 µM progesterone in either of the two groups of women.
Table 7.3a: Mean corpuscular fragilities (MCF, mean concentration of saline in g/l required to induce 50% haemolysis) of Hb SS red cells (n=7) incubated with Hepes buffer (control), 35 μM oestradiol and 30 μM progesterone

<table>
<thead>
<tr>
<th>Patient</th>
<th>Control</th>
<th>Progesterone</th>
<th>Oestradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.285</td>
<td>0.295</td>
<td>0.289</td>
</tr>
<tr>
<td>2</td>
<td>0.332</td>
<td>0.340</td>
<td>0.336</td>
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<td>3</td>
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<td>0.270</td>
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<td>0.314</td>
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<td>SEM</td>
<td>0.020</td>
<td>0.020</td>
<td>0.019</td>
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</table>

Table 7.3b: Mean corpuscular fragilities (MCF, mean concentration of saline in g/l required to induce 50% haemolysis) Hb AA red cells (n=4) incubated with Hepes buffer (control), 35 μM oestradiol and 30 μM progesterone

<table>
<thead>
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<th>Subject</th>
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</tr>
</thead>
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<td>0.332</td>
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<tr>
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<td>0.42</td>
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<td>0.385</td>
</tr>
<tr>
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<td>0.485</td>
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<td>0.482</td>
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<td>0.453</td>
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<td>0.015</td>
<td>0.017</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Hb SS</td>
<td>Hb AA</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>---------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.8 ± 0.1</td>
<td>4.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>2.9 ± 0.1</td>
<td>4.5 ± 0.2</td>
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</tr>
<tr>
<td>Progesterone</td>
<td>2.9 ± 0.2</td>
<td>4.6 ± 0.2</td>
<td></td>
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</tbody>
</table>

Table 7.3c: Mean values ± SEM of MCF (g/l) in red cells from women with Hb SS (n=7) and Hb AA (n=4) genotypes following 24 hour incubation with Hepes buffer only, 35 μM oestradiol and 30 μM progesterone
Figure 7.3a: The osmotic fragility curves of sickle red cells from seven Hb SS women incubated with Hepes buffer (dotted line), oestradiol (solid line) and progesterone (dashed line) at 37°C for 24 hours. Values represent mean ± SEM, with triplicate determinations for each sample.
Figure 7.3b: The osmotic fragility curves of red cells from four Hb AA women incubated with Hepes buffer (dotted line), oestradiol (solid line) and progesterone (dashed line) at 37°C for 24 hours. Values represent mean ± SEM, with triplicate determinations for each sample.
CHAPTER 8: THE EFFECT OF INCUBATION IN VITRO WITH OESTRADIOL, PROGESTERONE AND TESTOSTERONE ON RED CELL DEFORMABILITY IN WOMEN WITH SCD

8.1 Introduction

8.2 Method

8.3 Results
8.1 Introduction

The ability of erythrocytes to deform in order to pass through small capillaries can be replicated by measuring the ease with which a set volume of red cell suspension is able to pass through a filter with pores of diameter 5 μm or less. In this study, the ability of erythrocytes to deform was assessed by the filtration of red cells through the St George's Filtrometer (see section 1.7.3), one of the most sensitive instruments available for the measurement of erythrocyte deformability (Staubli et al 1986). The author has used the filtration parameters Red Cell Transit Time (RCTT) and Clogging Rate (CR), which have been shown to be sensitive and reproducible (Dormandy et al 1985). 

In vitro incubation with various agents such as zinc (Brewer et al 1977, Taylor et al 1991), cetiedil (Stuart et al 1988), pentoxyfylline (Bilto et al 1988, Stuart et al 1988) and piracetam (Stone et al 1988) appear to modulate sickle erythrocyte deformability but until now, similar in vitro experiments have not been performed using steroid hormones.

8.2 Method

Erythrocyte suspensions were prepared from ten Hb SS women and ten Hb AA controls as described in chapter 4. The aliquots at 5% haematocrit were incubated at 37°C for 30 minutes with glucose-supplemented Hepes buffer (which served as the control) and two concentrations respectively of 17 β oestradiol, progesterone and testosterone. Prolonged incubation without substrate for greater than 24 hours could result in physiological and geometrical changes secondary to ATP depletion and the loss of membrane lipid. A decrease of approximately 30% of cellular ATP is accompanied by a significant deterioration in membrane deformability as measured with a micropipette (Weed and Lacelle 1969) and for this reason, incubated erythrocyte suspensions were supplemented with 10 mmol/l glucose as substrate to allow aerobic metabolism and a limited incubation time was used.

Incubation with 17 β oestradiol was performed at 3.5 nM (1 x 10⁻⁹ g/ml) (peak plasma levels in the luteal phase of the menstrual cycle) and at 35 μM (1 x 10⁻⁵ g/ml) (equivalent to therapeutic concentrations). Progesterone loading was done at 0.3 μM (1 x 10⁻⁷ g/ml) (the peak luteal plasma levels) and at 30 μM (1 x 10⁻⁵ g/ml) (equivalent
to therapeutic concentrations). Testosterone incubation was done at 30 pM (1 x 10^-11 g/ml) (the physiological level in menstruating women) and at 0.3 µM (1 x 10^-7 g/ml) (chosen to represent at least a hundred fold the normal premenopausal female levels).

After incubation, the red cells were washed three times in buffer to remove excess hormones and diluted to a final haematocrit of 2.5% for sickle cells and 10% for normal erythrocytes. These values have been demonstrated to result in the lowest coefficient of variation for the measurement of RCTT and CR for SCD erythrocytes (Stone PCW, personal communication, 1996) and normal erythrocytes (Keidan et al 1987) respectively.

The final aliquots were well mixed in air and warmed to 37°C prior to passage through the Filtrometer, which was operated at a negative pressure of 4 cms of water. The perspex housing of the instrument was unclamped and the outflow channel filled with deionised water, ensuring that no air bubbles were present. A few drops of pre-filtered buffer were used to top up the outflow channel. Each measurement was done using a new 5 µm diameter polycarbonate membrane filter (Co Star Scientific Corp., High Wycombe, UK) from a single batch (Lot 3965) to avoid variation between batches (ICSH 1986). The filter was then put in place using a small brush and the two halves of the perspex housing was reassembled. Pre-filtered buffer was flushed through the system twice and the system filled to the indicator mark on the capillary tube (placed 5 mm from the edge of the detector assembly) ensuring that the whole system was free of air bubbles (see section 1.7.3 and figure 1.7.3).

Using the computer programme, a standardisation procedure was employed to standardise the filter using the buffer. This was repeated until two measurements fell within the pre-determined range of 1% which was installed on the computer software. Following this, the buffer was withdrawn from the system using the syringe and the air-mixed red cell suspension inserted, ensuring that no air bubbles were present in the filter chamber. The haematocrit value in the software programme always defaulted to 10% and when the actual PCV (determined by microhaematocrit) of the suspension differed from this, this value was substituted into the programme, thereby correcting
the measurement of the red cell deformability to the haematocrit. Following each measurement, all remaining suspension was removed from the filter chamber using a syringe and the perspex housing was unclamped and rinsed in distilled water. Confounding variables known to influence measurements of erythrocyte deformability such as age, race, temperature and MCV were all standardised. The full details of the technique of using the St George’s Filtrometer and calculation of RCTT and CR are described in appendix 10.

8.3 Results

The comparable demographics of ten Hb SS and ten Hb AA women were shown in table 8.3a. The data followed a normal distribution and are presented as mean ± SEM. The mean haemoglobin level was lower in the sickle cell group but was corrected for by adjusting the haematocrit of the final red cell samples prior to the filtration studies. The serum oestradiol and progesterone values confirmed that the women in both groups were in the early follicular phase of their menstrual cycles at the time that the study was undertaken.

Women with Hb SS genotype

The RCTT and CR values of red cell suspensions incubated in Hepes buffer (control) and in different concentrations of exogenous steroid hormones are presented in tables 8.3b and c. Repeated Measures ANOVA was used to compare the values in each of the columns of tables 8.3b and c and no statistically significant differences in RCTT and CR values of red cell suspensions incubated in different concentrations of oestradiol, progesterone and testosterone were found (RCTT, \( p = 0.628 \); CR, \( p = 0.787 \); ANOVA).

The RCTT and CR for red cell samples with added hormones were then each compared with the values of the “control” sample containing sickle erythrocytes and Hepes buffer only. The changes in RCTT and CR for the Hb SS women for each hormone concentration were obtained by subtracting the RCTT and CR of the “control” sample from those of the Hb SS samples. Negative values indicate a lower reading and positive values indicate a higher reading of RCTT and CR after the
incubation with the steroids (tables 8.3d and e). When analysed using Repeated Measures ANOVA, the changes in RCTT and CR following incubation with different concentrations of hormones did not reach statistical significance (RCTT, $p = 0.073$; CR, $p = 0.470$).

**Women with Hb AA genotype**

The RCTT and CR values of red cell suspensions incubated in Hepes buffer (control) and in different concentrations of exogenous steroid hormones are presented in tables 8.3f and g. When analysed using Repeated Measures ANOVA, the RCTT and CR values with and without incubation in steroid hormones were not statistically different from each other (RCTT, $p = 0.703$; CR, $p = 0.502$). The change in RCTT and CR values with incubation in steroid hormones compared with Hepes buffer are presented in tables 8.3h and i. Addition of pharmacological concentrations of 17 ß oestradiol, progesterone and testosterone to normal erythrocyte suspensions does not result in statistically significant changes in RCTT and CR (RCTT, $p = 0.74$; CR, $p = 0.112$; ANOVA).

There are wide confidence intervals in all groups of data, particularly with the Hb SS samples, which inevitably comprises an unknown combination of true biological variation between individuals and technical error of the measurement.

**Figures 8.3a and b** show the mean change ($\pm$ SEM) in RCTT and CR values with the incubation of oestradiol, progesterone and testosterone, compared with the control samples.

The unpaired $t$ test was then used to compare the data from women with Hb SS and Hb AA to see if the addition of these hormones produced more change in red cell deformability parameters in one group compared to the other. There was no statistically significant difference between the changes in RCTT and CR in women with Hb SS compared to women with Hb AA ($p > 0.1$, unpaired $t$ test). The incubation with oestradiol, progesterone and testosterone therefore does not produce more
change in red cell deformability measurements in women with Hb SS compared to Hb AA women.
Table 8.3a: The demographics of ten Hb SS women and ten Hb AA controls from whom red cell suspensions were prepared for deformability studies.

<p>| Table 8.3a: The demographics of ten Hb SS women and ten Hb AA controls from whom red cell suspensions were prepared for deformability studies |
|-------------------------------|----------------------|-------------------|-------------------|
|                                | Hb SS (n = 10)      | Controls (n = 10) | t                 | p     value |
| Age (years); mean ± 2 SD       | 25.50 ± 6.50        | 30.60 ± 6.60      | 0.50              | p &gt; 0.2   |
| Hb (g/ml); mean ± 2 SD         | 9.30 ± 2.50         | 11.97 ± 1.90      | 2.65              | p &lt; 0.02  |
| MCV (fl); mean ± 2 SD          | 87.60 ± 7.10        | 85.00 ± 10.4      | 0.64              | p &gt; 0.2   |
| MCHC (g/dl); mean ± 2 SD       | 31.30 ± 4.50        | 33.50 ± 5.0       | 0.48              | p &gt; 0.2   |</p>
<table>
<thead>
<tr>
<th>Patient</th>
<th>Control</th>
<th>E 35 μM</th>
<th>E 3.5 nM</th>
<th>P 30 μM</th>
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<td>19.068</td>
</tr>
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<td>4</td>
<td>73.401</td>
<td>79.930</td>
<td>73.671</td>
<td>69.367</td>
<td>74.441</td>
<td>62.181</td>
<td>73.216</td>
</tr>
<tr>
<td>9</td>
<td>36.625</td>
<td>23.946</td>
<td>34.463</td>
<td>26.759</td>
<td>29.362</td>
<td>30.362</td>
<td>32.465</td>
</tr>
</tbody>
</table>

**Table 8.3b:** Red Cell Transit Time (RCTT) (secs) of erythrocyte suspensions from ten Hb SS women incubated with steroid hormones.

E=oestradiol, P=progesterone, T=testosterone

<table>
<thead>
<tr>
<th>Patient</th>
<th>Control</th>
<th>E 35 μM</th>
<th>E 3.5 nM</th>
<th>P 30 μM</th>
<th>P 0.3 μM</th>
<th>T 0.3 μM</th>
<th>T 30 pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4.3031</td>
<td>3.570</td>
<td>3.369</td>
<td>3.873</td>
<td>2.043</td>
<td>3.059</td>
<td>3.527</td>
</tr>
<tr>
<td>3</td>
<td>1.816</td>
<td>1.974</td>
<td>2.066</td>
<td>2.389</td>
<td>3.258</td>
<td>2.379</td>
<td>2.400</td>
</tr>
<tr>
<td>4</td>
<td>2.232</td>
<td>2.279</td>
<td>2.297</td>
<td>2.529</td>
<td>1.891</td>
<td>1.995</td>
<td>2.192</td>
</tr>
<tr>
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<td>2.541</td>
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<td>2.261</td>
<td>1.994</td>
<td>2.008</td>
<td>2.428</td>
</tr>
<tr>
<td>6</td>
<td>2.862</td>
<td>2.654</td>
<td>2.551</td>
<td>2.436</td>
<td>2.375</td>
<td>2.437</td>
<td>2.631</td>
</tr>
<tr>
<td>7</td>
<td>2.415</td>
<td>3.115</td>
<td>2.479</td>
<td>2.325</td>
<td>2.502</td>
<td>2.496</td>
<td>1.812</td>
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<tr>
<td>8</td>
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<td>2.228</td>
<td>2.934</td>
<td>3.483</td>
<td>3.168</td>
<td>3.003</td>
<td>2.923</td>
</tr>
<tr>
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<td>3.027</td>
<td>3.120</td>
<td>3.864</td>
<td>3.331</td>
<td>3.402</td>
<td>3.402</td>
<td>3.023</td>
</tr>
<tr>
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<td>2.462</td>
<td>2.963</td>
<td>2.643</td>
<td>3.062</td>
<td>2.722</td>
<td>2.204</td>
<td>2.625</td>
</tr>
</tbody>
</table>

**Table 8.3c:** Clogging Rate (CR) (/ml) of erythrocyte suspensions from ten Hb SS women incubated with steroid hormones.

E=oestradiol, P=progesterone, T=testosterone
### Table 8.3d: The change in RCTT (secs) of red cells from ten Hb SS women incubated with various ovarian steroids, compared with Hepes buffer only.

E=estradiol, P=progesterone, T=testosterone

<table>
<thead>
<tr>
<th>Patient</th>
<th>E 35 μM</th>
<th>E 3.5 nM</th>
<th>P 30 μM</th>
<th>P 0.3 μM</th>
<th>T 0.3 μM</th>
<th>T 30 pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-3.687</td>
<td>-3.778</td>
<td>-1.920</td>
<td>-2.717</td>
<td>-2.932</td>
<td>-1.761</td>
</tr>
<tr>
<td>2</td>
<td>+0.553</td>
<td>+2.525</td>
<td>+7.085</td>
<td>+5.614</td>
<td>+5.910</td>
<td>+3.993</td>
</tr>
<tr>
<td>3</td>
<td>-2.771</td>
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<td>-5.752</td>
<td>-4.069</td>
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</tr>
<tr>
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<td>+1.529</td>
<td>+0.270</td>
<td>-4.034</td>
<td>+1.009</td>
<td>-11.220</td>
<td>-0.185</td>
</tr>
<tr>
<td>5</td>
<td>+1.785</td>
<td>+1.832</td>
<td>+3.038</td>
<td>+0.696</td>
<td>+0.025</td>
<td>+1.683</td>
</tr>
<tr>
<td>6</td>
<td>+2.350</td>
<td>-0.230</td>
<td>-1.512</td>
<td>+0.647</td>
<td>+0.036</td>
<td>-0.964</td>
</tr>
<tr>
<td>7</td>
<td>+3.641</td>
<td>-2.641</td>
<td>-0.879</td>
<td>+4.232</td>
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<td>+1.430</td>
</tr>
<tr>
<td>9</td>
<td>-8.356</td>
<td>-12.938</td>
<td>-13.584</td>
<td>+0.989</td>
<td>-17.876</td>
<td>-1.409</td>
</tr>
<tr>
<td>10</td>
<td>-0.836</td>
<td>-1.293</td>
<td>-1.338</td>
<td>+0.099</td>
<td>-1.790</td>
<td>-0.141</td>
</tr>
<tr>
<td>Mean</td>
<td>-0.723</td>
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<td>-2.083</td>
<td>+0.400</td>
<td>-3.264</td>
<td>-0.346</td>
</tr>
<tr>
<td>SEM</td>
<td>1.122</td>
<td>1.398</td>
<td>1.707</td>
<td>0.948</td>
<td>2.097</td>
<td>0.738</td>
</tr>
</tbody>
</table>

### Table 8.3e: The change in CR (/ml) of red cells from ten Hb SS women incubated with various ovarian steroids, compared with Hepes buffer only.

E=estriadiol, P=progesterone, T=testosterone

<table>
<thead>
<tr>
<th>Patient</th>
<th>E 35 μM</th>
<th>E 3.5 nM</th>
<th>P 30 μM</th>
<th>P 0.3 μM</th>
<th>T 0.3 μM</th>
<th>T 30 pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+0.731</td>
<td>+0.955</td>
<td>+0.103</td>
<td>-0.043</td>
<td>+0.669</td>
<td>+1.219</td>
</tr>
<tr>
<td>2</td>
<td>-0.734</td>
<td>-0.934</td>
<td>-0.430</td>
<td>-4.303</td>
<td>-1.244</td>
<td>-0.776</td>
</tr>
<tr>
<td>3</td>
<td>+0.158</td>
<td>+0.249</td>
<td>+0.573</td>
<td>+1.442</td>
<td>+0.563</td>
<td>+0.584</td>
</tr>
<tr>
<td>4</td>
<td>+0.047</td>
<td>+0.0648</td>
<td>+0.297</td>
<td>-0.341</td>
<td>-0.237</td>
<td>-0.039</td>
</tr>
<tr>
<td>5</td>
<td>+0.100</td>
<td>-0.098</td>
<td>-0.180</td>
<td>-0.447</td>
<td>-0.434</td>
<td>-0.013</td>
</tr>
<tr>
<td>6</td>
<td>-0.208</td>
<td>-0.311</td>
<td>-0.426</td>
<td>-0.587</td>
<td>-0.425</td>
<td>-0.231</td>
</tr>
<tr>
<td>7</td>
<td>+0.700</td>
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<td>+0.088</td>
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<td>-0.603</td>
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<td>+0.569</td>
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<td>+0.089</td>
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<td>+0.305</td>
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<td>+0.375</td>
<td>-0.004</td>
</tr>
<tr>
<td>10</td>
<td>+0.500</td>
<td>+0.181</td>
<td>+0.600</td>
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<td>+0.163</td>
</tr>
<tr>
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<td>+0.508</td>
<td>+0.123</td>
<td>+0.031</td>
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<tr>
<td>SEM</td>
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<td>0.170</td>
<td>0.127</td>
<td>0.554</td>
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</tbody>
</table>
### Table 8.3f: Red Cell Transit Time (RCTT) (secs) of erythrocyte suspensions from ten Hb AA women incubated with steroid hormones.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Control</th>
<th>E 35 µM</th>
<th>E 3.5 nM</th>
<th>P 30 µM</th>
<th>P 0.3 µM</th>
<th>T 0.3 µM</th>
<th>T 30 pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>10.263</td>
<td>11.708</td>
<td>10.561</td>
<td>10.204</td>
<td>11.932</td>
<td>10.817</td>
<td>10.057</td>
</tr>
<tr>
<td>5</td>
<td>8.496</td>
<td>8.269</td>
<td>8.633</td>
<td>8.167</td>
<td>8.896</td>
<td>8.237</td>
<td>8.177</td>
</tr>
</tbody>
</table>

### Table 8.3g: Clogging Rate (CR) (/ml) of erythrocyte suspensions from ten Hb AA women incubated with steroid hormones.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Control</th>
<th>E 35 µM</th>
<th>E 3.5 nM</th>
<th>P 30 µM</th>
<th>P 0.3 µM</th>
<th>T 0.3 µM</th>
<th>T 30 pM</th>
</tr>
</thead>
<tbody>
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<td>0.968</td>
<td>1.518</td>
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<td>1.026</td>
<td>0.934</td>
</tr>
<tr>
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<td>3.158</td>
<td>3.437</td>
<td>3.855</td>
<td>3.873</td>
<td>3.675</td>
<td>3.497</td>
</tr>
<tr>
<td>3</td>
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<td>3.681</td>
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<td>1.939</td>
<td>1.078</td>
<td>0.587</td>
</tr>
<tr>
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<td>2.394</td>
<td>3.026</td>
<td>2.378</td>
<td>2.529</td>
</tr>
<tr>
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<td>3.226</td>
<td>2.823</td>
<td>3.426</td>
<td>2.791</td>
<td>2.723</td>
<td>2.927</td>
<td>3.026</td>
</tr>
<tr>
<td>6</td>
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<td>2.183</td>
<td>0.830</td>
<td>1.615</td>
<td>2.100</td>
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<tr>
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<td>3.025</td>
<td>2.825</td>
<td>2.125</td>
<td>2.527</td>
</tr>
<tr>
<td>8</td>
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<td>4.060</td>
<td>4.213</td>
<td>4.477</td>
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<td>2.688</td>
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<tr>
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<td>2.323</td>
<td>2.685</td>
<td>3.418</td>
<td>2.480</td>
<td>3.773</td>
<td>3.631</td>
<td>3.530</td>
</tr>
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</table>
Table 8.3h: The change in RCTT (secs) of red cells from ten Hb AA women incubated with various ovarian steroids, compared with Hepes buffer only.
E=oestradiol, P=progesterone, T=testosterone

<table>
<thead>
<tr>
<th>Patient</th>
<th>E 35 μM</th>
<th>E 3.5 nM</th>
<th>P 30 μM</th>
<th>P 0.3 μM</th>
<th>T 0.3 μM</th>
<th>T 30 pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>+0.044</td>
<td>+0.595</td>
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<td>+1.445</td>
</tr>
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<td>-3.270</td>
</tr>
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</tr>
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<td>-0.503</td>
<td>-0.299</td>
<td>-0.199</td>
</tr>
<tr>
<td>6</td>
<td>+1.448</td>
<td>+0.096</td>
<td>+0.880</td>
<td>+1.367</td>
<td>+0.468</td>
<td>+0.358</td>
</tr>
<tr>
<td>7</td>
<td>+0.258</td>
<td>+0.311</td>
<td>+0.911</td>
<td>+0.711</td>
<td>+0.011</td>
<td>+0.413</td>
</tr>
<tr>
<td>8</td>
<td>-1.103</td>
<td>-0.950</td>
<td>-0.686</td>
<td>-0.750</td>
<td>-0.836</td>
<td>-1.234</td>
</tr>
<tr>
<td>9</td>
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<td>-0.137</td>
<td>-0.374</td>
<td>+0.630</td>
<td>-0.167</td>
<td>-2.333</td>
</tr>
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<td>10</td>
<td>+0.362</td>
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<td>+0.158</td>
<td>+1.450</td>
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<td>+1.208</td>
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<tr>
<td>Mean</td>
<td>+0.270</td>
<td>+0.109</td>
<td>+0.285</td>
<td>+0.358</td>
<td>-0.058</td>
<td>-0.113</td>
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<tr>
<td>SEM</td>
<td>0.251</td>
<td>0.288</td>
<td>0.247</td>
<td>0.392</td>
<td>0.411</td>
<td>0.456</td>
</tr>
</tbody>
</table>

Table 8.3i: The change in CR (/ml) of red cells from ten Hb AA women incubated with various ovarian steroids, compared with Hepes buffer only.
E=oestradiol, P=progesterone, T=testosterone

Mean -0.386 -0.029 -0.183 -0.328 -0.114 -0.396
SEM 0.431 0.271 0.280 0.368 0.346 0.253

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Mean changes in red cell transit time (RCTT) following incubation with exogenous steroids compared with Hepes buffer only

Figure 8.3a: The mean change in Red Cell Transit Time (RCTT) ± SEM following incubation with various concentrations of exogenous steroid hormones, compared with Hepes buffer only. Dotted line indicates no change. Hb AA red cell suspensions are represented by solid squares and Hb SS by open circles. E=oestradiol, P=progesterone, T=testosterone
Mean changes in clogging rate (CR) following incubation with exogenous steroids compared to Hepes buffer only.

**Figure 8.3b:** The mean change in Clogging Rate (CR) ± SEM following incubation with various concentrations of exogenous steroid hormones, compared to Hepes buffer only. Dotted line indicate no change. Hb AA red cell suspensions are represented by solid squares and Hb SS by open circles.

E=oestradiol, P=progesterone, T=testosterone
CHAPTER 9: RED CELL DEFORMABILITY IN WOMEN WITH SCD USING THE COMBINED ORAL CONTRACEPTIVE PILLS (COCP'S) AND PROGESTERONE ONLY (PO) CONTRACEPTIVES

9.1 Introduction

9.2 Method

9.3 Results
9.1 Introduction

In an *ex vivo* observational study, Oski and colleagues (1972) demonstrated that erythrocytes of non sickle women exposed to COCP’s containing Mestranol and Norethindrone were less deformable compared to red cells from control women who were non “pill” users. Durocher (1975), on the other hand, noted no significant difference in filterability between red cells from COCP users and non-users. The particular oestrogens and progestogens examined in the aforementioned studies, however, are no longer germane to current contraceptive practice.

The *ex vivo* study described in this chapter represents a methodological progression from the experiments described in chapter 8. The aim of the author was to evaluate the effect of oestrogens and progestogens used in modern contraceptive preparations on sickle cell deformability, when the exposure was through the contraceptive medication taken by the women herself.

9.2 Method

The erythrocyte deformability of SCD women using exogenous contraceptive hormones was measured using the St George’s Filtrometer. The study was observational and involved 30 women with SCD recruited from the haematology clinics of two London teaching hospitals. Inclusion criteria were established as before in chapter 3.

The SCD women in the study comprised three different groups:

Group 1 Women using a monophasic COCP containing 30 μg ethinyl oestradiol (specifically chosen so that erythrocytes from these women had been exposed to a constant dosage of synthetic oestrogen and progestogen)

Group 2 Women using PO contraception (*i.e.* the progestogen only pill, intramuscular depot medroxyprogesterone acetate or the progestogen releasing IUCD releasing 20 μg of levonorgestrel daily)

Group 3 Women not using exogenous steroid hormones, who served as controls.
All women were non-smokers in steady haematological state and those on contraceptive steroid hormones had used them for at least three months.

Blood samples were drawn from the women at standardised times as follows:

Group 1 (Women using COCP’s) Day 10 of the 21 day pill packet cycle
Group 2 (Women using PO contraception) Day 10 from the onset of bleeding, unless amenorrhoeic, in which case venesection was performed at the patient’s convenience
Group 3 (Women not using hormones) Within days 1-7 of the natural menstrual cycle.

Women in the control group (Group 3) had venesection performed in the early follicular phase as the erythrocytes would then be subjected to the lowest plasma levels of endogenous oestradiol and progesterone (Carr 1998, Chabbert Buffet 1998). Women in Groups 1 and 2 had blood drawn on Day 10 to ensure adequate and standardised exposure to contraceptive steroids. All samples were processed within four hours of venesection. Suspensions of sickle erythrocytes were prepared by filtration through cotton wool, washing three times with Hepes buffered saline and diluted to a final haematocrit of 2.5% as described previously.

Filtration measurements were performed using the St George’s Filtrometer with a 5 μm polycarbonate membrane from a single batch (Lot 3965) as described previously. The RCTT and CR were measured in triplicate for each woman.
9.3 Results

The mean RCTT and CR values for women with SCD using the COCP’s, PO contraception and non-hormonal contraception (n=10 for each group) are presented in table 9.3. Three readings done for each woman and the mean of these three readings was taken, in order to minimise observer error. The mean ± SEM for each group (ie COCP’s, PO contraception and non-hormonal contraception) were then calculated. The data, which were normally distributed, were subjected to analysis by ANOVA, followed where appropriate, by the Tukey-Kramer Multiple Comparison test.

Demographic characteristics such as age, smoking habit and MCV were similar in all three groups.

High values for RCTT and CR or increases in these values would be indicative of poorly deformable red cells. There was a large SEM for the RCTT in the control group of SCD women using non-hormonal contraception and this was due to the inclusion in the results of one particular individual with an unexplained exceptionally high value. Were she to be excluded from this control group, the mean RCTT would have been 21.565 seconds with a SEM of 2.28. The mean RCTT and CR values, without the exclusion of this individual reading, are lower in groups 1 and 2, compared to group 3. This suggests an improvement in red cell deformability in women on contraceptive steroids, although the differences do not reach statistical significance using the one-way ANOVA.

The histograms of RCTT and CR in women with SCD using COCP, PO and non-hormonal contraception are depicted in figures 9.3a and b.
Table 9.3a: The mean ± SEM values for Red Cell Transit Time (RCTT) and Clogging Rate (CR) of three groups of women with SCD (n=10 in each group) using differing exogenous contraceptive steroids. (Higher values of RCTT and CR indicate poor erythrocytes deformability)
Red Cell Transit Time (RCTT) in women with SCD using contraceptive steroids

Figure 9.3a: The histograms of Red Cell Transit Time (RCTT) values of women with SCD using exogenous contraceptive steroids (*p=0.98, ANOVA). Values represent mean ± SEM. Higher values represent poor erythrocyte deformability.
Clogging Rate (CR) in women with SCD using contraceptive steroids

Figure 9.3b: The histograms of Clogging Rate (CR) values of women with SCD using exogenous contraceptive steroids (*p=0.75, ANOVA).
Values represent mean ± SEM. Higher values represent poor erythrocyte deformability
CHAPTER 10: MEASUREMENTS OF MARKERS OF THROMBIN GENERATION, FIBRINOLYSIS AND PLATELET ACTIVATION IN WOMEN WITH SCD USING THE COCP’S AND PO CONTRACEPTIVES

10.1 Introduction

10.2 Method

10.3 Results
10.1 Introduction

Coagulation markers such as activation peptides, enzyme-inhibitor complexes and markers of platelet activation are informative about the level of activity of the haemostatic systems in vivo. Prothrombin Fragment 1+2 (F1+2) is generated when prothrombin is cleaved to thrombin and therefore is a marker of thrombin generation. Plasmin-antiplasmin complexes are generated during activation of the fibrinolytic activity. Platelet factor 4 (PF 4) and β Thromboglobulin (β TG) are derived from the α-granules of platelets and increased levels indicate platelet degranulation and therefore act as surrogate markers of platelet activation. Protein S, together with activated Protein C, regulates the activity of factors Va and VIIIa.

Women with SCD, even in steady state, exhibit accelerated coagulation and fibrinolysis in vivo. In these individuals, protein S levels are lower than in controls (Francis 1988, El Hazmi et al 1993), whilst the levels of PF 4 and β TG, markers of platelet activation, are increased compared to age, race and sex matched controls (Green et al 1986, Kurantsin-Mills et al 1992).

However, there is little published data on how the levels of activation markers relate to various hormonal contraceptive preparations in women with SCD. This study aims to establish whether differing hormonal contraception preparations in women with SCD have effects on markers of platelet activation, thrombin generation and fibrinolysis.

10.2 Method

Women with SCD of reproductive age and in steady haematological state were recruited from haematological clinics of three London teaching hospitals. Inclusion criteria were as described previously in chapter 3. All the women were non-smokers who had no family history of TED and those using hormonal contraception had done so for at least three months prior to the study.

Certain markers of coagulation such as Factor VIII (FVIII) and von Willebrand’s Factor (vWF) seem to vary with the menstrual cycle and it is recommended that blood sampling
for assessment of clotting factors be conducted no later than day seven of the natural menstrual cycle (Edlund et al 1996).

Three groups of women with SCD were recruited and blood samples drawn at specific times in each of these groups for the analysis of markers of haemostasis and platelet activation.

- **Group 1 (Women using monophasic COCP's)** Day 10 of the 28 day pill packet cycle
- **Group 2 (Women using PO contraception)** Day 10 from the onset of bleeding, unless amenorrhoeic, in which case venesection was performed at the patient’s convenience
- **Group 3 (Women not using hormones)** Within days 1-7 of the natural menstrual cycle

Women in the control group (Group 3) had venous samples drawn in the early follicular phase of the menstrual cycle when serum levels of oestradiol and progesterone were at their lowest. Women in groups 1 and 2 had venesection performed on day 10 to ensure adequate and standardised exposure to contraceptive steroids. For these coagulation studies involving the measurement of markers of thrombosis, fibrinolysis and platelet activation, blood was transferred, following venesection, into a 3 ml plastic collection tube (Sarstedt, Monovette, Leicester, UK) pre-filled with the anticoagulant trisodium citrate 0.109 M.

Six laboratory markers of platelet activation, thrombin generation and fibrinolysis were analysed: Prothrombin Fragment $1+2$ ($F_{1+2}$), Plasmin $\alpha 2$ Antiplasmin Complex (PAP), Platelet Factor 4 (PF 4) and $\beta$ Thromboglobulin ($\beta$ TG), together with fibrinogen and Protein S (PS). $F_{1+2}$ and fibrinogen are measures of procoagulant activity, while increased fibrinolysis is indicated by elevated PAP levels. PF 4 and $\beta$ TG are molecular markers of platelet activation (Kluft and Lansik 1997). PS is an inhibitor of coagulation and PS assays were performed to establish if women with SCD using hormonal
contraception have a disproportionate fall in the levels of this natural anticoagulant, which might, therefore exacerbate their hypercoagulable state.

Preparations for the analysis of haemostatic markers
Venous samples were collected from free flowing venepuncture into tubes containing pre-chilled anticoagulant. For F$_{1+2}$, PAP complexes, PS:Ag and PF 4, the anticoagulant used was trisodium citrate 0.109 M, while specially prepared β TG anticoagulant (100 μl per tube) was used for β TG assays. The samples were then transported in ice at 4°C and processed within one hour of venepuncture. For the measurement of F$_{1+2}$, PAP complexes and PS:Ag, samples were centrifuged at 2000g for 20 minutes at 4°C, following which the supernatant plasma was stored at −80°C until assayed. For PF 4 and β TG, double centrifugation was performed at 2000g for 5 minutes at 4°C and the supernatant was then stored at −80°C as above.

F$_{1+2}$ and PAP complexes were measured by ELISA (Behring Diagnostics UK, Walton Manor, Milton Keynes, UK). PF 4 determinations were performed using an ELISA kit manufactured by Diagnostica Stago, Asnieres, France. Free PS Ag were measured using the ELISA method described by Murdock (Murdock et al, 1997) and the results were expressed as percentage of total Protein S. β TG assays were performed using an “in house” ELISA method developed at the Royal Free Hospital (appendix 6).

The author performed one of each assay and the remainder of the measurements were undertaken by the staff of the Haemophilia and Haemostasis Unit at the Royal Free Hospital.
10.3 Results

A total of 44 women with SCD were studied. 42 of the 44 women studied were of the Hb SS genotype and there were two women with Hb SC disease (these were in groups 1 and 2 respectively).

10 women were using COCP's which contain 30 µg oestradiol (group 1), 18 were using PO contraception (group 2) and 16 were using barrier methods or no contraception (group 3).

Contraceptive usage in groups 1 and 2 can be further summarised as follows:

COCP (n=10)
Seven of the group were using Microgynon 30 (ethinyl oestradiol 30 µg and levonorgestrel 150 µg) (Schering Health Care Ltd., Burgess Hill, UK) and three were on Cilest (ethinyl oestradiol 30 µg and norgestimate 250 µg) (Janssen-Cilag, High Wycombe, UK).

PO contraception (n=18)
11 were using Depo-Provera (depot medroxyprogesterone acetate 150 mg/ml) (Pharmacia & Upjohn Ltd., Milton Keynes, UK), three the Mirena intrauterine contraceptive device (releasing 20 µg levonorgestrel per day) (Schering Health Care Ltd., Burgess Hill, UK) and four women, the progestogen only pill Micronor (Janssen-Cilag, High Wycombe, UK) which contains 350 µg norethisterone.

The mean age was similar in the three groups (27.5, 25.7, 26.2 years; p>0.05).

The data on markers of coagulation were non-parametric in distribution and the median values and interquartile ranges of the haemostatic variables measured in the three groups of women are shown in table 10.3. The data show that free PS levels were decreased and PF 4 levels increased in all three groups compared with previously established reference ranges for individuals not on the COCP or with SCD. The levels of F₁+₂ were increased in the COCP and control groups while the PO groups had levels which were on the upper limit of normal. PAP and β TG levels in all three groups were within the laboratory reference range. Overall, there was no statistically significant difference
between the three groups of SCD women in all the five haemostatic variables measured $(p>0.05$, Kruskal-Wallis test). The scatter plots of the two parameters, Free PS and PF 4 are depicted in figures 10.3a and b below.
<table>
<thead>
<tr>
<th>Normal reference range</th>
<th>Fibrinogen</th>
<th>Free PS</th>
<th>F1/2</th>
<th>PAP</th>
<th>PF 4</th>
<th>β TG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COCP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>2.50 (2.23-2.60)</td>
<td>18.00 (17.25-19.00)</td>
<td>1.70 (1.04-2.44)</td>
<td>553.50 (480.25-724.50)</td>
<td>111.00 (77.50-112.00)</td>
<td>21.00 (18.05-54.75)</td>
</tr>
<tr>
<td>PO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>2.45 (2.20-2.78)</td>
<td>20.00 (18.25-22.00)</td>
<td>1.09 (0.65-1.47)</td>
<td>530.00 (384.00-617.50)</td>
<td>103.00 (61.50-112.75)</td>
<td>18.00 (11.63-25.88)</td>
</tr>
<tr>
<td>No Hormones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>2.65 (2.23-2.60)</td>
<td>18.00 (16.00-21.25)</td>
<td>1.60 (1.05-2.48)</td>
<td>534.00 (474.50-704.50)</td>
<td>103.50 (82.50-116.50)</td>
<td>16.80 (10.80-25.53)</td>
</tr>
</tbody>
</table>

Table 10.3: The median values and interquartile ranges (IQR) of the haemostatic variables in 44 women with SCD using COCP’s (n=10), PO (n=18) and non-hormonal contraception (n=16). There was no statistically significant difference between the groups (p>0.05, Kruskal-Wallis) NB: free Protein S data are presented as percentage of total Protein S. The laboratory reference range for PS at the Haemophilia Centre and Haemostasis Unit of the RFH is correct at the time of submission of this thesis but has since been changed to 69-137 iu/dl as results are now expressed as absolute levels based on a free PS standard curve.
Figure 10.3a: The scattergrams of protein S levels in women with SCD using COCP (n=10), PO (n=18) and non-hormonal contraception (n=16) with the medians being depicted as short bold lines
Figure 10.3b: The scattergrams of PF 4 levels in women with SCD using COCP (n=10), PO (n=18) and non-hormonal contraception (n=16) with the medians being depicted as the short bold lines.
CHAPTER 11: AN INVESTIGATION OF THE NATURAL MENSTRUAL PATTERN IN WOMEN WITH SCD: THE ASSOCIATION BETWEEN THE MENSTRUAL CYCLE AND PAINFUL CRISES

11.1 Introduction

11.2 Method

11.3 Results
11.1 Introduction

There have been anecdotal reports that painful crises sometimes cluster around menstruation and that the onset of menstruation may precipitate sickling crises, but published studies have so far been unable to demonstrate a clear relationship (Samuels-Reid and Scott 1985a, Serjeant et al 1994). The author has therefore conducted a prospective observational study to investigate the following:

1) The natural menstrual pattern in women with SCD
2) Is there an association between the menstrual cycle and painful crises?
3) Subjective estimations of menstrual blood loss in women with SCD.

11.2 Method

Women with SCD who had natural menstrual cycles and were not on exogenous steroid hormones, attending haematology clinics of four London hospitals, participated in the study. The subjects comprised a cross sectional sample of women in steady haematological state, women with occasional crises managed as outpatients and those with frequent crises requiring inpatient stay. They were asked to complete a prospective six month menstrual diary (appendix 7) which recorded the occurrence of painful crises, the presence or absence of dysmenorrhoea and the estimation of menstrual blood loss.

The severity of painful crises was defined as follows:

0 = none; 1 = mild, requiring simple oral analgesia eg paracetamol; 2 = moderate, requiring stronger oral analgesia eg dihydrocodeine phosphate; 3 = severe, requiring opiates and inpatient stay.

Menstrual loss was subjectively graded as light, moderate, heavy and flooding as judged by the woman herself and by the amount of sanitary protection used. Throughout the duration of the study, direct contact and telephone communication were maintained with the women to encourage compliance. At the end of six months, the completed menstrual diaries were returned to the author for analysis.
11.3 Results

82 women with SCD agreed to participate and 42 women returned completed menstrual charts which had sufficient information for data analysis. Parametric statistics were used for analysis of the data, which were normally distributed.

Menstrual cycles
A total of 213 continuous prospectively recorded natural cycles were evaluated in the 42 women with SCD. The mean age of the participants was 26.2 ± 3.5 years and their mean age of menarche was 13.9 ± 2.7 years. 30% of the women studied had regular menstrual cycles (defined as ± 2 days) over the six month observation period. The mean cycle length was 28.9 ± 5.9 days, whilst the mean duration of menstrual loss was 4.7 ± 1.2 days. 87% of the women reported either light or moderate menstrual loss, 11% had heavy menses and only 2% complained of flooding.

Menstrual clustering of crises
37% of the women had definite painful crises with every menstrual cycle; however, these were usually moderate (grade 2) and treated with oral analgesia. Only one woman required inpatient treatment with every menses - this was a patient who normally had frequent severe crises and was about to embark on a prophylactic blood transfusion regimen. 21% of the study group had at least three crises documented in the six month of the study period although none of these were sufficiently severe to warrant in patient treatment. 42% of the women reported no association between menses and painful crises.

Of the 16 documented cases where painful crises were cyclical and coincided with menses, three patterns were identified (figure 11.3): 61.5% occurred only during menstrual flow, whilst 34.5% started premenstrually (between day 21 and 28) and continued during menses. In 4% of cases, painful crises commenced premenstrually and appeared to abate with menstrual flow.
Figure 11.3: The timing of painful crises (bold lines) in relation to menstrual flow (shaded bar) in 16 women with definite cyclical crises. 61.5% occurred only during menstrual flow, 34.5% started premenstrually and continued throughout menses and 4% started premenstrually and abated with menses.
CHAPTER 12: THE MENOPAUSE IN WOMEN WITH SCD:
SYMPTOMATOLOGY, BONE MINERAL DENSITY STUDIES AND THE
USE OF HORMONE REPLACEMENT THERAPY (HRT)

12.1 Introduction

12.2 Method

12.3 Results
12.1 Introduction

There is observational data suggesting that sickling crises may become less frequent and less severe in both men and women after the age of 40 years and Serjeant (1992) considered this to be due to a true amelioration rather than the survival to older age of more mildly affected individuals. Approximately 20% of women with SCD in the London area are currently over 45 years of age (Streetly et al 1997) and as the mean life expectancy increases, more women with this condition will spend a greater portion of their lives after the menopause. There is not only limited data on the effects of the menopause on SCD, but also a general reluctance to prescribe hormone replacement therapy (HRT) to these women.

The aim of the study described in this chapter was to evaluate the symptoms of oestrogen deficiency in a group of post-menopausal women with SCD. There is consistent data indicating a prevalence of reduced bone mineral density (BMD) in young girls with SCD (Brinker et al 1998), as well as in adults with β thalassaemia major, another severe haemoglobinopathy (Jensen et al 1998). Many SCD women complain of chronic bone pain unrelated to sickling crises and the author also wished to investigate the association between chronic bone pain and bone mineral density measurements in post-menopausal women with SCD.

The World Health Organisation (WHO) defines “osteoporosis” as having a T score of equal to or further than 2.5 standard deviations (SD) below the mean peak bone mass for normal young women (WHO 1994). Individuals with a T score of −1 SD below the mean are considered as having low risk of osteoporotic fractures, while intermediate risk (osteopenia) is suggested by having a T score of between −1 and −2.5 SD. However, many clinicians now feel that the Z score (defined as the number of standard deviations above or below the age and sex matched reference range) provides a more predictive and practical assessment of the individual’s fracture risk, particularly in women over the age of 50 years (Fogelman and Blake 1998). Strictly speaking, however, the term osteoporosis describes a clinical condition in which pathological fractures have occurred in the presence of reduced BMD. There is thus
some dispute about the WHO use of terminology, which describes a numerical expression of BMD only.

The BMD measurements in this thesis were expressed both as T scores, which compares the individuals to their expected peak bone mass and hence indicates their absolute fracture risk, as well as the Z scores (representing the number of standard deviations above or below the age and sex matched reference range), which is informative about their relative fracture risk.

12.2 Method
Post-menopausal women with SCD recruited from haematology clinics of three London teaching hospitals were questioned regarding symptoms associated with oestrogen deficiency such as vasomotor disturbances (hot flushes and nocturnal sweats), urogenital problems (atrophic vaginitis and urinary urgency), musculoskeletal symptoms (joint and bone pains) and psychological complaints (anxiety, depression, forgetfulness, insomnia, poor concentration). This is a modified version of the Kupperman Index, which is a weighted 11 point questionnaire that quantifies the incidence or severity of hot flushes, nocturnal sweats, sleep disturbances, anxiety, depression, fatigue, arthralgia and vaginal dryness (Wiklund et al 1993).

Age at the menopause and the frequency of painful crises following the menopause were also ascertained. Knowledge of and interest in HRT were documented.

BMD, the most important predictor of fracture risk (Cummings et al 1996), was measured using dual energy x-ray absorptiometry (DEXA) at standard reference regions ie lumbar vertebrae L1-4 and hip (femoral neck, greater trochanter and intertrochanteric regions and Ward’s triangle). These measurements were undertaken in the Medical Physics Department of the Royal Free Hospital using a HOLOGIC QDR - 4500W fan beam X ray densitometer (S/N 47096) (Waltham, MA, USA) which utilises a multi element array and a fan beam geometry for performing the scan. The scanner uses X rays of energies 70 and 140 kVp. In each case, bone densities were
expressed as standard deviation (SD) scores (the T and Z scores) matched for ethnicity (African-American), sex, age, weight and height drawn from the normative reference data for this equipment.

The post-menopausal symptomatology and patients' personal data were recorded by the author on to proformas, an example of which can be seen in appendix 9.

12.3 Results
The questionnaires were completed by the author for 16 post-menopausal women with Hb SS. The mean weight and height were 68.00 ± 3.22 kg and 163.91 ± 1.82 cm, respectively.

The mean age for the post-menopausal group was 52.50 ± 2.80 years, while the mean age of menopause was 48.20 ± 4.17 years. 14 women had naturally occurring menopause, while two had undergone bilateral oophrectomies at the time of hysterectomies for benign gynaecological conditions. All 16 women felt that the frequency of sickle cell crises had decreased noticeably following the menopause. The commonest complaints were backache and joint pains (16/16) and vaginal dryness (12/16). Only one patient had hot flushes and no one admitted to having psychological symptoms.

Knowledge of HRT was generally poor, with 14 out of 16 women knowing little about treatment principles and modalities. Only two women (12.5%) understood that HRT relieves vasomotor symptoms, reduces the risk of osteoporotic fractures and is likely to be cardioprotective. The same two women were using Prempak C (cyclical conjugated equine oestrogen 625 µg and norgestrel 150 µg) (Wyeth Laboratories, Maidenhead, Berks., UK) and Evorel transdermal patches (releasing 25 µg oestradiol per day) (Janssen-Cilag, High Wycombe, UK) respectively. The use of HRT by these two women was not associated with an exacerbation of their previous frequency of sickling. One other woman had previously used HRT for several months, but discontinued because of unacceptable side effects of weight gain and headaches. Of the 14 who were not on HRT, 12 were not interested, while two had had negative
responses to their enquiries about HRT from their general practitioners and were therefore reluctant to consider it.

11 women, including the two on HRT, had DEXA bone density scans performed and all had been in steady haematological state for at least one year at the time of their measurements. The T and Z scores of lumbar spine and hip regions of the group are shown in table 12.3.

The post-menopausal women had a mean lumbar spine T score of -1.37 SD (osteopenia) and a mean hip T score of -0.18 SD (within normal limits). Five of the eleven postmenopausal women had lumbar spine BMD in the osteopenic range, while two had measurements in the osteoporosis range (T scores lower than -2.5 SD).

Although the mean hip T score of the postmenopausal group was within the normal reference range, four women had BMD values indicating osteopenia in this region.

The mean group Z score of the lumbar spine was -0.35 SD and that of the hip region was 0.50 SD, suggesting low fracture risk (Z greater than 0). Within the group, however, three of the eleven had Z scores at the lumbar spine of lower than -1 SD, indicating an increased relative risk of osteoporotic fracture, while one had a significantly reduced Z score of -2.71 SD.

All individuals in the post-menopausal group showed statistically lower T scores in the lumbar spine compared to the hip region (p=0.014, unpaired t test). All 11 postmenopausal women also had lower Z scores in the lumbar compared to the hip regions but the difference did not reach statistical significance (p=0.057, unpaired t test). The scatter plots of T and Z scores of lumbar and hip bone mineral densities of the 11 post-menopausal women are shown in figures 12.3a and b.
### Table 12.3

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Lumbar spine T score</th>
<th>Hip T score</th>
<th>Lumbar spine Z score</th>
<th>Hip Z score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1.45</td>
<td>-1.56</td>
<td>-0.41</td>
<td>-0.8</td>
</tr>
<tr>
<td>Patient 2</td>
<td>-0.51</td>
<td>0.72</td>
<td>0.57</td>
<td>1.50</td>
</tr>
<tr>
<td>*Patient 3</td>
<td>-0.52</td>
<td>0.73</td>
<td>0.88</td>
<td>1.78</td>
</tr>
<tr>
<td>Patient 4</td>
<td>-1.02</td>
<td>0.41</td>
<td>0.04</td>
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</tr>
<tr>
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<td>-3.78</td>
<td>-2.34</td>
<td>-2.71</td>
<td>-1.74</td>
</tr>
<tr>
<td>Patient 6</td>
<td>-1.84</td>
<td>-1.29</td>
<td>-1.81</td>
<td>-1.29</td>
</tr>
<tr>
<td>Patient 7</td>
<td>1.09</td>
<td>1.64</td>
<td>1.65</td>
<td>2.06</td>
</tr>
<tr>
<td>Patient 8</td>
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<td>-0.07</td>
<td>1.17</td>
</tr>
<tr>
<td>Patient 9</td>
<td>-2.14</td>
<td>-0.05</td>
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<td>0.74</td>
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<tr>
<td>*Patient 10</td>
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<td>0.95</td>
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<tr>
<td>Patient 11</td>
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<tr>
<td>Mean</td>
<td>-1.37</td>
<td>-0.18</td>
<td>-0.35</td>
<td>0.50</td>
</tr>
<tr>
<td>SEM</td>
<td>0.41</td>
<td>0.37</td>
<td>0.41</td>
<td>0.41</td>
</tr>
</tbody>
</table>

*T (indicating absolute fracture risk) and Z (indicating relative fracture risk) scores of the lumbar and hip regions of 11 post-menopausal women with SCD

* = on HRT
Figure 12.3a: The scattergram of T (absolute fracture risk) scores in post-menopausal women with SCD (n=11), arranged in ascending order of values. The T scores in the lumbar region are significantly lower than the hip (p=0.014, unpaired t test). The bold lines indicate −1 and −2.5 SD, respectively. Solid squares represent the hip while open circles represent the lumbar region.
Figure 12.3b: The scattergram of $Z$ (relative fracture risk) scores in post-menopausal women with SCD ($n=11$), arranged in ascending order of values. The $Z$ scores in the lumbar region are lower than the hip in all cases but the difference was not statistically significant ($p=0.057$, unpaired $t$ test).

Solid squares represent the hip while open circles represent the lumbar region.
CHAPTER 13: DISCUSSION

13.1 Introduction

13.2 The binding of ovarian steroid hormones to erythrocytes from women with SCD

13.3 Light and electron microscopy changes in the cell membrane of sickle red cells incubated with steroid hormones

13.4 The effect of in vitro incubation with oestradiol and progesterone on the inhibition of sickling

13.5 The effect of in vitro incubation with oestradiol and progesterone on the osmotic fragility of red cells from women with SCD

13.6 The effect of in vitro incubation with oestradiol and progesterone on red cell deformability in women with SCD

13.7 The deformability of erythrocytes from women with SCD taking various contraceptive steroid hormones

13.8 The measurement of markers of thrombin generation, fibrinolysis and platelet activation in women with SCD using various contraceptive steroid hormones

13.9 An investigation of the natural menstrual pattern in women with SCD and the effect of menstruation on sickling crises

13.10 The menopause in women with SCD: symptomatology, bone mineral density studies and the use of hormone replacement therapy (HRT)
13.1 Introduction
A sequence of studies was undertaken to explore physical and functional evidence of the influence of oestrogen and progesterone on erythrocytes from women with SCD. The initial studies assessed evidence of functional changes in SCD erythrocytes incubated in vitro with ovarian steroid hormones. Subsequent studies assessed red cell function and coagulation markers at the ex vivo level in SCD women who were taking exogenous hormonal preparations. The final studies involved clinical observations of the correlations between various physiological hormonal variations and the clinical manifestations of SCD.

The hypothesis proposes that oestradiol and progesterone have direct effects on the cell membrane of erythrocytes and on the molecular markers of coagulation in women with SCD, which in turn, influence the clinical nature of the condition. The aims of this thesis have been to explore a sequence of in vitro and ex vivo features intended to demonstrate the potential for such an interaction to be present. Should these interactions prove favourable or at least not adverse, the aim of this work is then to provide an ethical basis for an interventional study using therapeutic contraceptive preparations of oestradiol and progesterone in women with SCD.

One of the difficulties encountered has been to establish the correct concentration of steroid hormones appropriate to use in the in vitro studies, since therapeutic plasma levels vary significantly between and within individuals (but encompass the concentrations used in the studies performed by the author).

While the present results suggest that steroid hormones have no adverse effect on red cell sickling in vitro, two caveats must be added. Firstly, the lack of effect in the in vitro experiments may reflect the relatively acute nature of the incubation with steroids applied in these experiments, which in most instances, was about one and a half hours. However, the binding studies described in chapter 4 suggest that this duration was sufficient to attain substantial degrees of steroid binding. Secondly, the in vitro studies undertaken used natural steroids rather than the synthetic analogues employed in some of the previous studies (Isaacs and Hayhoe 1967, De Ceulaer et al.
Clearly, any effects of chronic treatment with synthetic steroids in vivo on the susceptibility to sickling may differ from the acute effects of incubation in vitro with natural steroids. Notwithstanding these caveats, the data suggest that the beneficial clinical effects of steroid administration in vivo noted by previous authors (De Ceulaer et al 1982, De Abood et al 1997, Nascimento et al 1998) on the susceptibility to sickling may not be mediated through direct effects of the steroids at the level of the erythrocyte.

The assessment of molecular markers of thrombosis, fibrinolysis and platelet activation do not support earlier anecdotal reports (Haynes and Dunn 1967, Greenwald 1971, Hargus et al 1977) that the use of the COCP poses an additional risk of thromboembolic disease in women with SCD.

The studies correlating the occurrence of clinical sickling episodes with phases of the menstrual cycle and with the post-menopausal state should, of course, be interpreted in purely endocrine terms. It is interesting to note that 58% of the premenopausal SCD women experienced sickling crises with at least half of their menses, while the group of post-menopausal SCD women reported a reduction in sickling episodes following the menopause. Both observations would suggest a reduced occurrence of sickling episodes in circumstances where physiological oestrogen and progesterone levels are at their lowest.

The lower than expected BMD observations in post-menopausal women with SCD is interesting and brings up the possibility that the vaso-occlusive phenomenon may predispose to an increased risk of osteoporotic fractures in this group of individuals.
13.2 The binding of ovarian steroid hormones to erythrocytes from women with SCD

These studies represent the first report of the binding characteristics of the sex steroids, oestradiol and progesterone, to erythrocytes from women with Hb SS, Hb SC and Hb AA genotypes at physiological temperature. In all cases, venesection was performed by the author during the early follicular phase of the menstrual cycle to ensure low levels of endogenous ovarian steroids, so that any binding of endogenous ligands, which may complicate the binding studies, was minimal.

There appears to be no significant difference in the binding of oestradiol and progesterone to intact red cells at 4°C and 37°C. The data demonstrate, for the first time, decreased binding of both oestradiol and progesterone to erythrocytes from women with Hb SS compared to normal Hb AA controls at the physiological temperature of 37°C. These findings disagree with Ige and Adadevoh’s study (1975), which noted increased binding of testosterone, oestradiol and progesterone to haemoglobin S compared to haemoglobins C or A. However, their experiments involved lysed red cells and did not take account of the gender of the individuals from whom the blood samples were drawn or if they were using exogenous steroid hormone preparations.

The rapid loss of oestradiol and progesterone binding with sequential "washes" concurs with the earlier findings of a loose association of testosterone and androstenedione with normal erythrocytes as described by Brinkman and colleagues (1972) and between cortisol with normal erythrocytes as observed by Vermeulen (1961). In the present experiments performed at 37°C, the binding of oestradiol and progesterone appears to occur in a low affinity, non-saturable manner, and the steroids rapidly dissociate from the erythrocytes when the cells are "washed". No saturable specific oestradiol or progesterone binding sites were identified in these cells, which agrees with Brinkman (1972) and Ige and Adadevoh (1975). It seems likely, therefore, that sex steroid hormones interact with erythrocytes in a non-specific biophysical manner, either by adhering to the outer lipid leaflet of the plasma membrane or by intercalating into the membrane lipid bilayer (Vermeulen 1961). It is possible that red
cells may merely function as a passive transport medium for steroid hormones (Koefoed and Brahm 1994).

In each of the three Hb genotypes studied, the binding of oestradiol to erythrocytes was 44 ± 4% greater than that of progesterone. This finding was unexpected since progesterone, being a more hydrophobic molecule than oestradiol, might be expected to intercalate more efficiently into the hydrophobic moiety of the plasma membrane. This increased binding of oestradiol could reflect a hydrophilic interaction between the two oestradiol dipoles and the charged phospholipid head groups of the erythrocyte plasma membrane. It was interesting to note that the binding of both oestradiol and progesterone, at a physiological temperature, was significantly diminished in erythrocytes from women with homozygous Hb SS compared with Hb SC and Hb AA genotypes. It is recognised that the plasma membrane of sickle cells has a higher sterol content compared with the membrane of normal erythrocytes (Kucuk et al 1992, Connor et al 1997). In a separate study, Jacobsohn et al (1994) noted that increasing the cholesterol content of artificial lipid membranes and erythrocyte membranes of oxen restricted the “uptake” of oestradiol. One could attribute this phenomenon to the possibility that cholesterol and oestradiol compete for the same position in the membrane lipid bilayer. In the context of the present data, the lower binding of oestradiol to erythrocytes from women with Hb SS compared to Hb AA genotypes may be due to the higher sterol content of the sickle cell plasma membrane inhibiting the intercalation of oestradiol and progesterone. An alternative explanation is that the loss of erythrocyte membrane lipid asymmetry in the homozygous Hb SS state, with abnormal exposure of phosphatidyl ethanolamine and phosphatidyl serine on the outer membrane lipid leaflet (Lubin et al 1987, Test and Mitsuyoshi 1997), results in a change in phospholipid charge distribution which could decrease the adsorption of oestradiol and progesterone.

The statistical power (the ability of the statistical test to lead to rejection of the null hypothesis when the hypothesis is false) of the studies in this chapter was calculated to be 95% i.e. only a 5% chance of making a type II error.
In summary, the data reported suggest a low affinity, non-saturable association between ovarian steroids and erythrocytes at a physiological temperature, with decreased binding of both oestradiol and progesterone to cells from Hb SS patients, possibly due to differences in the lipid composition of the plasma membrane. However, the nature of this interaction does not result in altered erythrocyte function and certainly has no demonstrable effect on the susceptibility to sickling, osmotic fragility or red cell deformability, as will be discussed in later sections (13.3-8) of this chapter. While this has reassuring clinical implications, particularly in the context of HRT (which predominantly involves natural sex steroids), extrapolations of these findings to synthetic steroids, contained in the formulations of the COCP’s (eg ethinyloestadiol and levonorgestrel), should still be made with some caution.

As mature erythrocytes are anucleate and there appears to be no interaction between mature sickle red cells and ovarian steroids, further in vitro research could be considered with precursor red cells, such as erythroblasts, which do possess nuclei. These, however, could only be harvested from bone marrow and supply of precursor red cells could pose a logistical problem.

13.3 Transmission electron microscopy (TEM) changes in the cell membrane of sickle red cells incubated with steroid hormones
The mean cell membrane thickness (in nm) of deoxygenated sickle red cells does not alter significantly when incubated with approximate therapeutic concentrations of oestradiol, progesterone and testosterone. In each case, the author took five measurements of cell membrane thickness from ten individual sickle red cells and these measurements were felt to represent the average membrane thickness of the sickle red cell. These findings would be in keeping with the general trend suggesting that ovarian steroid hormones do not appear to produce an effect on the physical characteristics of sickle red cells.
13.4 The effect of *in vitro* incubation with oestradiol and progesterone on the inhibition of sickling

Incubation with approximate therapeutic concentrations of oestradiol and progesterone had no significant effect on the inhibition of sickling. The lack of effect with *in vitro* incubation with steroid hormones contrasts with previous reports that progestogens could inhibit erythrocyte sickling (Isaacs and Hayhoe 1967, De Ceulaer et al 1982). The present data could simply reflect the relatively acute nature of the incubation with steroids, which was only 1.5 hours in total, although the binding studies in chapter 4 suggest that this duration was sufficient to attain substantial degrees of steroid binding. Furthermore, the present study used natural steroids rather than the synthetic analogues employed in endocrine therapies and any effects of chronic treatment with synthetic steroids *in vivo* on the susceptibility to sickling may differ from the acute effects of incubation *in vitro* with natural steroids.

While ovarian steroid hormones can associate with erythrocyte membranes as noted in chapter 4, their biological effect(s) on these cells is less certain. Sickling appears unaffected by *in vitro* incubation with therapeutic concentrations of ovarian steroids.

13.5 The effect of *in vitro* incubation with oestradiol and progesterone on the osmotic fragility of erythrocytes from women with SCD

The osmotic fragility test is a measure of the resistance of erythrocytes to haemolysis when exposed to a series of saline solutions of decreasing osmolality. Sickle-shaped cells are more osmotically resistant than normal erythrocytes because of their greater surface area relative to cell volume and the increased susceptibility of the plasma membrane to oxidative damage. While it is clear that ovarian steroid hormones can associate with erythrocyte membranes, their physical and biological effect(s) on red cells is less certain. The data presented suggest that the osmotic fragility of erythrocytes from women with the Hb SS and Hb AA genotypes appears unaffected by *in vitro* incubation with supraphysiological concentrations of ovarian steroids. In this study, the red cells from women with Hb SS and Hb AA genotypes had been incubated for 24 hours to exaggerate any potential effects of ovarian steroids, nevertheless, the osmotic fragility remains quite a gross test of membrane stability. A
longer incubation duration may simulate the *in vivo* chronic exposure to ovarian steroids but any difference detected then may also reflect ATP depletion secondary to such prolonged incubation.

**13.6 The effect of *in vitro* incubation with oestradiol and progesterone on red cell deformability in women with SCD**

As erythrocytes are anucleate, it had been suggested that the effect of steroids on erythrocytes, if any, is to alter the dynamic properties of the lipid bilayer of the cell membrane. Rooney (1985) claimed that increasing the cholesterol content of the normal erythrocyte membrane decreases membrane fluidity, while Szostek (1991) maintained that the alteration in membrane fluidity is marginal when cholesterol derivatives are inserted into the red cell membrane. There are structural similarities between cholesterol and ovarian steroid hormones (section 1.5.8). Moreover, the author has demonstrated a non-specific interaction between ovarian steroid hormones and the sickle red cell (chapter 4), possibly by intercalation into the membrane lipid bilayer as suggested by Vermeulen (1969). Thus, incubation with ovarian steroid hormones may affect membrane fluidity and hence the deformability of red cells. However, when confounding variables such as age, race, smoking habit, temperature, pH and MCV, which may influence red cell deformability (Stuart 1985) were controlled, the author’s data demonstrate an absence of impairment of erythrocyte deformability by therapeutically relevant concentrations of oestrogen and progesterone. In the analysis of the data, the large standard error of mean (SEM), particularly in the Hb SS group, is noted. This suggests that red cell deformability may not be a reliable or reproducible test in sickle cells, with large intra-individual variations being present. In fact, the coefficient of variation (CV) of this method as documented in validation studies in appendix 10 is greater than 10%.

It was reassuring that, despite theoretical concern, these contraceptive steroids do not appear directly to impair red cell deformability in sickle or normal erythrocytes. Stone (personal communication 1996) suggested that deformability studies with deoxygenated sickle cells would not be technically feasible due to the extreme rigid nature of the cell membrane. The author did attempt to repeat the deformability
studies with deoxygenated sickle red cells but discontinued after three such experiments, as the rigid sickle erythrocytes severely clogged up the polycarbonate Nucleopore membrane of the St George’s Filtrometer and both RCTT and CR measurements were found to be extremely inconsistent.

Incubation with certain antisickling agents such as cetiedil and bepridil seems to improve red cell deformability in vitro, but these effects could be mediated through a different mechanism, such as the inhibition of calcium induced potassium loss, increasing cation flux (Bilto et al 1988) or the prevention of cellular dehydration (Stuart et al 1988). The data herein seem to support the fact that oestradiol and progesterone only associate loosely with sickle red cells and hence do not alter their biological function.

13.7 The deformability of erythrocytes from women with SCD taking various contraceptive steroid hormones

Both the oestrogen and progestogen components of contraceptive preparations are thought to have detrimental effects on red cell deformability (Devenuto et al 1969, Oski et al 1972) in non-sickle women. The in vitro studies on erythrocytes (chapter 8) from women with the Hb SS genotype suggest that the incubation with contraceptive concentrations of oestradiol and progesterone do not alter red cell deformability in these women (Yoong et al 1998). As a progression of this sequence of experiments, the influence of contraceptive steroids on red cell deformability in the ex vivo environment was evaluated. The deformability of Hb SS erythrocytes is dependent on $pO_2$ and temperature (Stuart and Johnson 1987) and these variables have been standardised by performing all ex vivo experiments at 37°C and atmospheric $O_2$ tension, which is more representative of the physiological state. The large SEM (which was greater than 10%) for RCTT in the control group (using no hormonal contraception) is explained by the inclusion in the results of one individual with a particularly high measurement. The mean RCTT and CR values are indeed improved in the two groups using contraceptive steroids but the difference between the three groups does not reach statistical significance when analysed using ANOVA ($p>0.05$).
It is possible that contraceptive steroids do affect red cell deformability as previously noted (Denevuto et al 1969, Oski et al 1972) but the inherent decreased deformability due to the Hb S gene may itself exert a greater influence than that of the steroid hormones. The limitations of the relatively small numbers in this study is acknowledged and the methodology was not ideal, this being a cross-sectional observational study. However, given that the safety of COCP use in women with SCD had yet been established at that stage, a randomised crossover study would not have been ethical.

The author does not recommend further research on the in vitro or ex vivo measurement of cell deformability as the techniques for the assessment of these parameters are complex and do not appear to have robust reproducibility, with CV’s the technique of greater than 10%. The statistical power of the deformability studies would be in the region of 50% and hence, the possibility of making a type II error, high.

13.8 Measurement of markers of thrombin generation, fibrinolysis and platelet activation in women with SCD using various contraceptive steroids

The use of the COCP in women with SCD is controversial: there has long been concern regarding the potential synergistic effects of the coagulation abnormalities associated with COCP use and the hypercoagulable state reported in individuals with SCD. Some clinicians have justified the use of the COCP’s in women with SCD on the basis that the significant maternal and fetal morbidity and mortality associated with pregnancy outweigh the increased risk of thromboembolism associated with this effective form of contraception. Many of the concerns about COCP use, however, have not been based on scientific data but on theoretical caution.

The single study that examined the influence of contraceptive steroidson haemostasis in women with SCD (Lutcher 1976), involved only 12 women using an older high dose combined oral contraceptive pill, no longer relevant to modern clinical practice. Interestingly, Lutcher’s study shows no significant difference in coagulation profiles
or blood viscosity measurements between women with SCD who used the COCP’s and those who did not.

In their observational study on contraceptive use in women with SCD, Howard and colleagues (1993) document two suspected but unconfirmed episodes of deep venous thrombosis in 148 woman-years of modern COCP usage (<50 μg ethinyl oestradiol). This was obviously higher than that reported in non SCD users (Farmer et al 1997) but Howard’s data are based on anonymous confidential interviews with patients and the two thromboembolic episodes could not, therefore, be objectively verified by reference to their hospital records.

The reduced levels of PS and increased levels of PF 4 in this study (indicating reduced natural anticoagulation and increased platelet activation) appear to agree with findings in subjects with SCD reported by previous authors (Green et al 1986, Francis 1988, Kurantsin-Mills et al 1992, El Hazmi et al 1993). The levels of fibrinogen, PAP and β TG (markers of procoagulation activity, increased fibrinolysis and platelet activation, respectively) in the three groups of women with SCD using the COCP’s, PO contraception and non-hormonal contraception studied are within the normal reference range.

The measurements of molecular markers reported by the author in this thesis are stratified by gender and also standardised for the time of venesection and the type of exogenous contraceptive steroids used. As such, it is difficult to relate this data to previously reported studies, which did not take these factors into account.

Only women using the most common hormonal contraceptive preparations in current use, which provide a “constant” dose of oestradiol and progestogen are included. However, due to marked individual variation in absorption and metabolism, wide ranges in the plasma levels of steroids of up to ten fold in hormonal contraceptive users may occur (Fotherby 1995).

The author recognises that the data presented here are cross-sectional and reflect the findings in a relatively small group of 44 women. Moreover, surrogate laboratory
markers cannot reliably predict clinical thrombotic risk. Furthermore, the possibility of a complex interaction between the use of hormonal contraceptives in women with SCD and other features relevant to thromboembolism, such as vessel wall endothelial changes and impairment to vascular flow, was not explored in these studies. Within these limitations, the data do not support the suggestion that there is a higher risk of thromboembolism in women with SCD who use the COCP's compared to those who do not, as the levels of the measured haemostatic variables are not significantly different between the groups. The decreased free protein S levels and elevated β TG levels noted in all three groups (using COCP’s, PO and non-hormonal contraception) suggest a degree of hypercoagulability that exists in individuals with SCD even in the steady haematological state. However, a randomised interventional trial would be necessary to evaluate further the safety aspect of COCP use in this group of women.

13.9 An investigation of the natural menstrual pattern in women with SCD and the effect of menstruation on sickling crises

This prospective study using menstrual dairies provided a more accurate and reliable evaluation of the natural menstrual pattern in women with SCD than those previously published (Samuels-Reid and Scott 1985a, Samuels-Reid and Scott 1985b). Although conventional teaching has been that the variation in natural cycle length in women is considerable, (with a mean of 29.6 days and the 5th and 95th centiles being 23 and 39.4 days, respectively) (Davey 1995), there is, in fact, little published data on the length of menstrual cycles in different ethnic groups.

30% of the women studied had natural cycles which could be strictly classified as being regular (ie ± 2 days) but the variation within the total group was not large, with the mean cycle length being 28.9 days and the group range being from 23-35 days. The data collected disagree with that of Samuels-Reid and Scott (1985b) who, based on a single retrospective questionnaire, documented that 77.1% of SCD women had regular menstrual cycles.

In this study, the menstrual loss was assessed by the women themselves as being light, moderate, heavy or flooding. It has been documented that there is poor correlation
between subjective and objective assessments of menstrual loss, particularly when the perceived loss was thought to be “excessive” (Haynes et al 1977, Fraser et al 1984). Only 50% of women generally who complain of “menorrhagia” have measured menstrual blood loss of greater than 80 ml (Fraser et al 1984). Conversely, 96% of women who rate their menstrual loss as “light” or “moderate” have objective menstrual blood loss measurements which correspond to their perceived loss (Fraser et al 1984). The subjective assessment of menstrual loss therefore appears to be more reliable in women who consider their menstrual loss to be within normal limits. In this study, 87% of women rated their loss as either “light” or “moderate” and the subjective assessment by these women was likely to correspond to objectively measured loss. The collection of soiled sanitary protection required for the objective laboratory measurement of menstrual blood by the alkaline haematin method (Hallberg and Nilsson 1964) would have been tedious and, in the author’s opinion, unlikely to have been accepted by the study group, especially when the majority of the women did not complain of heavy periods. The author did not use non-laboratory pictorial assessments of menstrual loss such as the Pictorial Blood Loss Assessment Chart (PBAC) (Higham et al 1990) as the accuracy of this method may be limited by the variety and size of tampons and pads currently available on the retail market. Moreover, the aims of the study were to investigate the menstrual pattern and cyclical association of menses with painful crises and not specifically to assess objectively the menstrual blood loss in this group of women.

The data suggest that over the six month duration studied, 37% of the women had sickling crises, albeit not severe, with every period. A further 21% reported episodes of mild to moderate sickling pain associated with menstrual loss in at least 50% of the menstrual cycles. These figures are higher than those previously reported by Samuels-Reid and Scott (1985a) and by Serjeant et al (1994). Most sickling crises in the study group occur either premenstrually or during menstrual flow, when plasma levels of endogenous ovarian steroid hormones are low. However, this evidence does not in itself allow the extrapolation that sickling would be inhibited by the higher levels of oestradiol and progesterone that occur in the luteal phase of the natural menstrual cycle.
Why some women should have sickling pain in association with menses is uncertain. It could be speculated that the extravascular fluid retention associated with menses reduces the circulating plasma volume, which may lead to "sludging" and erythrostasis, hence increasing the susceptibility to intravascular sickling. In any case, women with SCD in whom severe sickling pain is precipitated by menses could benefit from the use of continuous COCP therapy or DMPA injections to suppress ovulation and to decrease the frequency of, or to abolish menstruation. Both modalities of treatment have been shown to reduce the severity and frequency of painful crises (De Ceulaer 1982, Nascimento et al 1997).

13.10 The menopause in women with SCD: symptomatology, bone mineral density studies and the use of hormone replacement (HRT)

Approximately 10-15% of women of Northern European ethnic origin currently use HRT for the alleviation of distressing oestrogen deficiency symptoms around the menopause (Hope and Rees 1995). Women of ethnic minorities often exhibit a reluctance to use hormone replacement: only about 0.1% of post-menopausal African-American women, for example, use HRT (Nicholson et al 1999). In fact, women of African-American origin often do not find menopausal symptoms bothersome (Holmes-Rovner et al 1996) and have a significantly more positive attitude towards the menopause (Sommer et al 2000), which may explain the low prevalence of HRT use in this group of women.

There is little published literature providing comparable statistics on the menopause specifically in women of African and Afro-Caribbean origin living in the United Kingdom, who constitute the group studied in this thesis. The author has therefore used as a basis for comparison, previously published studies using observational data on post-menopausal African-American women.

The data presented in this thesis indicate symptom prevalence and HRT usage (12.5%) among SCD women which is broadly similar to the data for women of Northern European ethnic origin reported from the United Kingdom by Hope and Rees (1995). Of course, the small numbers involved in this study may not be extrapolated to post-menopausal women with SCD in general.
The mean age of menopause in the SCD women studied is 48.2 years, which is lower than that previously observed in an unselected African-American population (49.3 years) (Bromberger et al 1997).

All the women questioned reported a welcome reduction in the frequency of sickling crises after the menopause. It is possible that this cohort of SCD post-menopausal women represent an atypical subset of survivors from their generation and may exhibit a number of subtle haematological differences compared with the majority of SCD sufferers.

Only two out of the 16 (12.5%) post-menopausal SCD women surveyed understood that HRT relieved oestrogen deficiency symptoms and protected the cardiovascular and skeletal system, compared with 30% of the general British public having this knowledge (Hope and Rees 1995). This is in keeping with published reports that post-menopausal African-American women appear less knowledgeable about HRT compared to their Caucasian counterparts (Holmes-Rovner et al 1996). Interestingly, the use of HRT in these two women was not associated with an exacerbation of their previous sickling crises.

It is known that black women, in general, achieve greater peak bone mass as adolescents compared to women of other ethnic groups (Aloia et al 1996) and this may explain the reason why the incidence of hip fractures reported in black women is half that of Caucasians (Farmer et al 1984). More specifically, the NHANES III study (which used the same Hologic Densitometer as in this thesis) estimated that the prevalence of hip T score of <-2.5 SD below the mean in women over 50 years of age of African-American ethnic group was half that of white women (10% vs 21%) (Looker et al 1997). A similar trend has been described when comparing the BMD in different ethnic groups in the UK: lumbar spine and femoral neck bone mineral density is higher in Afro-Caribbean compared to Caucasian or Asian women (Tobias et al 1994). Up to now, however, there has been no published data on the prevalence of osteoporosis in post-menopausal women with SCD.
In this thesis, the T score (the number of standard deviations above or below the mean peak bone mass for normal young women) and Z score (the number of standard deviations above or below the age and sex matched reference range) are used to define the risk of osteoporotic fractures. With regard to bone density and fracture risk, some of the best data come from the Study for Osteoporotic Fractures (Cummings et al 1993), which found that the risk of hip fracture increased 2.6fold for every reduction of one SD in the femoral neck or hip region.

The author of the thesis has ensured that the BMD measurements in this study were matched particularly for ethnicity using HOLOGIC's standard normative data. Over a third (four out of eleven) of the post-menopausal women with SCD studied have an increased predicted risk of osteoporotic fractures in the lumbar spine compared to age and ethnically matched peers (Z<-1). Furthermore, about one fifth (two out of eleven) had lumbar spine T scores of less than -2.5, denoting osteoporosis. While the numbers of postmenopausal SCD women studied are admittedly small, the proportion of them having an increased risk of pathological hip fractures appears higher than expected when compared to the 10% reported in African-American women in the NHANES III study.

All the individuals in the post-menopausal group showed significantly worse T scores in the lumbar spine compared to the hip region (unpaired t test, \( p=0.014 \)). Z scores were also generally lower in the lumbar spine compared to the hip with the difference almost reaching statistical significance (unpaired t test, \( p=0.057 \)). This was interesting considering the fact that the sites of sickling pain most commonly recorded are the lumbar spine (49%) and hip (30%) (Serjeant et al 1994). These observations are also unusual when compared with post-menopausal Caucasian women appear to have higher BMD in the lumbar spine than the hip region (Felson et al 1993).

Why these findings should occur is uncertain. Weinstein and Bell (1988) noted that the actual rate of bone formation in healthy black (non SCD) adults is 35% lower than that in white ethnic groups \( (p<0.001) \) and deduced that the greater bone mass in the former group was due to a reduced rate of bone resorption. Osteoblastic activity may be further suppressed in systemically debilitating diseases such as SCD. In fact, young
girls with SCD already exhibit significantly lower lumbar spine BMD (up to 16%) compared with age, sex and ethnically matched controls without SCD (Brinker et al 1998), which would predict a lower peak bone mass being achieved at the end of adolescence. It would be reasonable to postulate that this lower peak bone mass achieved in young women will contribute towards the large number of post-menopausal women with SCD having low bone densities. The increased erythropoiesis and marrow hyperplasia associated with SCD could itself also predispose to a decrease in trabecular network, resulting in osteopenia (Johanson 1990).

While the pathophysiology of bone disease associated with SCD and that of osteoporosis is different in a number of respects, a presumption can be made that prophylactic attempts to minimise development of osteoporosis could be particularly relevant in women with SCD.

It can be assumed that women with SCD are still vulnerable to the same long-term sequelae of oestrogen deficiency as apply to all post-menopausal women and should therefore be informed about the general benefits and risks of HRT. While it remains a contentious area, there is no evidence so far to indicate that hormone replacement should be avoided in post-menopausal women with SCD. In fact, there may be a role for HRT in these women as both observational and randomised controlled trial data suggest that hip fractures are reduced in HRT users, although therapy has to be continued for between five and ten years to be effective (Cauley et al 1994, Writing Group for the Women’s Health Initiative Investigators 2002). On the other hand, the increase in the incidence of thromboembolic events (by two to three fold) especially in the first year of HRT use (Writing Group for the Women’s Health Initiative Investigators 2002, Nelson et al 2002) could pose additional worry in potential users. Transdermal oestrogen replacement therapy (particularly when used in conjunction with oral progestogens) avoids the hepatic first pass effects, thus producing minimal alterations in haemostatic variables (Fox et al 1993, Kroon et al 1997) and may be the safer option in post-menopausal women with SCD desiring hormone replacement. The development of selective oestrogen receptor modulators (SERMS) (Purdie 1997,
Fontana and Delmas 2003) may also prove beneficial in postmenopausal SCD women who require long term bone protection.
CONCLUSIONS
Conclusions

*In vitro* data suggest that oestradiol and progesterone associate loosely with erythrocytes from women with SCD and can be removed by sequential “washes”. The association is non-specific and there are no saturable steroid receptors demonstrated on sickle erythrocytes. This association is significantly diminished in erythrocytes from women with homozygous Hb SS compared with Hb SC and Hb AA genotypes, possible due to differences in the lipid composition of the plasma membrane. No genomic effects are likely to occur as a result of this association and the biological characteristics of SCD red cells (as represented by osmotic fragility and red cell deformability tests), do not appear to be affected by ovarian steroid hormones. It is acknowledged, however, that these are gross tests of cell function and may not be able to detect the more subtle changes which may occur at the intracellular level.

Although SCD is itself a condition involving a “hypercoagulable” state, markers of coagulation are not further increased in women with SCD exposed to contraceptive steroid hormones. About 37% of SCD women with natural menstrual cycles have crises, of moderate severity, associated with menstruation. Painful crises appear to be improved by the onset of the menopause. A larger than expected proportion of post-menopausal women with SCD have reduced BMD (Z scores) compared to sex and ethnically matched controls, although this finding cannot necessarily be attributable directly to the haematological condition itself. The Z scores of the lumbosacral spine are significantly lower than those of the hip and this may reflect the fact that the sites of sickling pain most frequently reported are the lumbar spine (49%) and femoral (30%) regions (Serjeant et al 1994).
With respect to the hypothesis which this work set out to test:

a) oestradiol and progesterone have direct effects on erythrocytes from women with SCD, which may, in turn, influence the clinical manifestations of the condition

Oestradiol and progesterone associate loosely with sickle red cells and evidence of specific receptors is not found with sickle red cells. The association is significantly lower in women with Hb SS compared to Hb SC or AA. These hormones do not appear to affect the biological characteristics of sickle red cells.

Clinical manifestations of sickle cell disease associated with the physiological hormonal variation of natural menstrual cycles and of the menopause, seem on first analysis, to be conflicting. A third of women appear to have increased crises with menstruation when oestrogen and progesterone levels are at their nadir, whilst the postmenopausal women with even lower prevailing hormone levels, report fewer crises.

b) there is a specific interaction between the effects of oestradiol and progesterone on sickle red cells and their concurrent effects on blood coagulation factors

Individuals with SCD have higher baseline levels of coagulation markers but ovarian steroid hormones do not further elevate the baseline levels.

c) by characterising these interactions, there is a potential for using them therapeutically to ameliorate the clinical course in women with the condition

As contraceptive steroid hormones do not appear to adversely affect the biological characteristics of red cell nor the coagulation markers in women with SCD, the use of COCP and HRT, both of which be of considerable practical relevance in this group of women, should be investigated further. Whether or not they confer specific additional
haematological benefits to women with SCD remains unproven by the features explored by the author. At the very least, the laboratory observations do not suggest any overwhelming evidence of actual or potential harm from pharmacological concentrations of oestradiol and progesterone in women with SCD.

Thus the sequence of observations and conclusions described above achieve the aims of this thesis which were to explore the potential for interactions between ovarian steroid hormones and erythrocytes in women with SCD and thereby, to establish an ethical basis for devising a patient level interventional study using therapeutic preparations of oestradiol and progesterone relevant to effective contraception and hormone replacement for these women.
Further Research Proposals

Having demonstrated that the use of COCP does not significantly increase the levels of haemostatic markers in women with SCD, interventional studies with therapeutic preparations of contraceptive steroids can be commenced. In order to recruit sufficient numbers and to include SCD women of different ethnic origins, with a full spectrum of disease manifestations, the author is of the opinion that these studies need to be collaborative and multi-centred.

BMD is reduced in post-menopausal SCD women. Case-control trials on the use of HRT and SERMS in the prevention of bone mineral density changes in these women would be of considerable interest. This is an emerging area of study which is only recently of practical relevance given the increasing life expectancy of adults with SCD in developed countries. The author has also completed a pilot study in premenopausal SCD women with back pain not associated with sickling crises, which shows that the BMD in this group of women is also lower than in ethnically matched controls. This brings up the possibility that there is a potential problem of osteoporosis in women with SCD which may only be partially explained by hormonal factors. Furthermore, it suggests that the very prevalent symptoms of disabling bone pain in people with SCD may be contributed to by pathological mechanisms other than the characteristic one of ischaemic infarction during acute sickling crises.
REFERENCES


Allon M. Renal abnormalites in sickle cell disease. Arch Int Med 1990; 150: 501-504


Baum KF, Dunn DT, Maude GH and Serjeant GR. The painful crises of homozygous sickle cell disease. A study of risk factors. Arch Int Med 1987; 147: 1231-1234

Berlin E, Bhatheja SJ, Judd JT, Nair PP, Jones DY and Taylor PR. Dietary fat and hormonal effects on erythrocyte membrane fluidity and lipid composition in adult women. Metabolism 1989; 38(8): 790-796


Bromberg PA. Pulmonary aspects of sickle cell disease. Arch Intern Med 1974; 133: 652-656


Campbell SJ, Mackie IJ, Robinson GE and Machin SJ. Contact factor mediated fibrinolysis is increased by the combined oral contraceptive pill. Br J Obstet Gynaecol 1993; 100: 79-84

Carol W, Klinger G, Jager R, Kase R and Brandstadt A. Pharmacokinetics of ethinylestradiol and levonorgestrel after administration of two oral contraceptive preparations. Experimental & Clinical Endocrinology 1992; 99: 7-12


Charache S and Conley CL. Rate of sickling of red cells during deoxygenation of blood from persons with varying sickling disorders. Blood 1964; 24: 25-31


Dada OA, Lapido OA, Osinusi BO, Osotimehin BO and Nduka EU. Circulating blood levels of gonadotrophins and prolactin in the normal menstrual cycle. Int J Gynecol Obstet 1981; 19: 291-294

Dare FO, Makinde OO and Faasuba OB. The obstetric performance of sickle cell disease patients and homozygous hemoglobin C patients in Ile-Ife, Nigeria. Int J Gynecol Obstet 1992; 37: 163-168


de Abood M, de Castillo Z, Guerrero F, Espino M and Austin KL. Effect of Depo-Provera or Microgynon on the painful crises of sickle cell anaemia patients. Contraception 1997; 56(5): 313-316

De Ceulaer K, Gruber C, Hayes RJ and Sergeant GR. Medroxyprogesterone Acetate and homozygous sickle cell disease. Lancet 1982; 2 (8292): 229-231


Eaton WA and Hofrichter J. Hemoglobin S gelation and sickle cell disease. Blood 1987; 70:1245


Francis RB. Protein S deficiency in sickle cell anaemia. J Lab Clin Med 1998; May: 571-576


Green MA, Noguchi CT, Marwah SS, Keidan AJ and Stuart J. Polymerisation of Hb S and loss of sickle erythrocyte deformability at arterial oxygen tension. Blood 1986; 68 (suppl 1): 62a


Hanss M. Erythrocyte filterability measurement by the initial flow rate method. Biorheology 1983; 20: 199-211


Haynes RL and Dunn JM. Oral contraceptives, thrombosis and sickle cell hemoglobinopathies. JAMA 1967; 200: 994


Herrick JB. Peculiar elongated and sickled shaped red blood corpuscles in a case of severe anemia. Arch Int Med 1910; 6: 517-521


Hodenpyl E. A case of apparent absence of spleen with general compensatory lymphatic hyperplasia. Medical Record 1898; 54: 695-698

Hofrichter J Ross PD and Eaton WA. Kinetics and mechanism of deoxyhemoglobin S gelation; a new approach to understanding sickle cell disease. Proceedings of the national Academy of Science (USA) 1974; 71: 4864-4868


Horton JAB. The diseases of tropical climates and their treatments. Churchill Livingstone, London 1874


188


Ingram VM. A specific chemical difference between the globins of normal and sickle-cell anemia haemoglobin. Nature 1956; 178: 792-794


Isaacs WA, Effiong CE and Ayeni O. Steroid treatment in the prevention of painful episodes in sickle cell disease. Lancet 1972; ii: 570-571

Jensen EV and Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Prog Horm Res 1962; 18: 387-414


Kaul DK, Fabry ME and Nagel RL. Erythrocytic and vascular factors influencing the microcirculatory behaviour of blood in sickle cell anaemia. Ann NY Acad Sc 1989; 565: 316

Kaul DK and Xue H. Rate of deoxygenation and rheologic behaviour of blood in sickle cell anemia. Blood 1991; 77: 1353-1361

Kaul DK and Hebbel RP. Hypoxia/ reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice. J Clin Invest 2000; 106: 411-420


Koefoed P and Brahm J. The permeability of the human red cell membrane to steroid sex hormones. Biochim Biophys Acta 1994; 1195: 55-62


Lindsay J, Meshel JC and Patterson RH. The cardiovascular manifestations of sickle cell disease. Arch Int Med 1974; 133: 643-651


Liu SC, Derick LH, Zhai S and Palek J. Uncoupling of the spectrin based skeleton from the lipid bilayer in sickled red cells. Science 1991; 252: 574-576


Lutcher CL. Blood coagulation studies and the effect of oral contraceptives in patients with SCA. Clin Res 1976; 24: 47A


193

Menzter CA, August CS and Nathan DG. Androgens in sickle cell anaemia. Blood 1969; 34: 733-734

Messer MJ and Harris JW. Filtration characteristics of sickle cell: rates of alteration of filterability after deoxygenation and reoxygenation, and correlations with sickling and unsickling. J Lab Clin Med 1970; 76: 537-547


Mohandas N and Evans E. Adherence of sickle erythrocytes to vascular endothelial cells: requirements for both cell membrane changes and plasma factors. Blood 1984; 64: 282-287


Morrison JC and Wiser WL. The use of prophylactic partial exchange transfusion in pregnancies associated with sickle cell hemoglobinopathies. Obstet Gynecol 1976(a); 48: 516-520


Nash GB, Johnson CS and Meiselman HJ. Mechanical properties of oxygenated red blood cells in sickle cell disease (SS). Blood 1984; 63: 73-82


Oppenheimer EH and Esterley JR. Pulmonary changes in sickle cell disease. American Review of Respiratory Diseases 1971; 103: 858-859

Orme MLE, Back DJ and Breckenbridge AM. Clinical pharmacokinetics of oral contraceptive steroids. Clinical Pharmacokinetics 1983; 8: 95-136


Oski FA, Lubin N and Buchert ED. Reduced red cell filterability with oral contraceptive agents. Arch Int Med 1972; 77: 417-419


Petitti N and Etgen AM. Progesterone promotes rapid desensitisation of $\alpha_1$ adrenergic receptor augmentation of cAMP formation in rat hypothalamic slices. Neuroendocrinology 1992; 55: 1-8


Poraicu D, Sandor S and Menessy I. Decrease of red blood cell filterability seen in intensive care. II. Red blood cell crenelation *in vivo* as morphological evidence of increased red blood cell viscosity in low flow state. Resuscitation 1983; 10(4): 305-316


Puca GA and Sica V. Identification of specific high affinity sites for the estradiol receptor in the erythrocyte cytoskeleton. Biochem Biophys Res Com 1981; 2: 682-689


Rambo CO and Szego CM. Estrogen action at endometrial membranes: alteration in luminal surface detectable within seconds. Cell Biology 1983; 97: 679-685


Romani P, Robertson DM and Diczfalusy E. Biologically active luteinising hormone (LH) in plasma: II. Comparison with immunologically active LH levels throughout the human menstrual cycle. Acta Endocrinol 1977; 84: 697


Samuels-Reid J and Scott RB. Characteristics of menstruation in sickle cell disease. Fert Ster 1985b; 43: 139-141


200


Solovey A, Giu L, Key NS and Hebbel RP. Tissue factor expression by endothelial cells in sickle cell anaemia. J Clin Invest 1998; 101(9): 1899-1904


Sorette MP, Lavenant MG and Clark MR. Ektacytometric measurement of sickle cell deformability as a continuous function of oxygen tension. Blood 1987; 69: 316-323


Stone PCW. Personal communication. 1996


202


Stuart J. Design principles for the clinical and laboratory studies of erythrocyte deformability. Clin Hemorheol 1985; 5: 159-169


Stuart J, Stone PCW and Bilto YY. Effects of oxypentifylline and cetiedil citrate on the filterability of valinomycin dehydrated sickle cells. Clin Hemorheol 1988; 8: 105-112


Sun PM, Willburn W, Raynor BD and Jameson D. Sickle cell disease in pregnancy: twenty years of experience at Grady Memorial Hospital, Atlanta, Georgia. Am J Obstet Gynecol 2001; 184(6): 1127-1130


Test ST and Mitsuyoshi J. Activation of the alternative pathway of complement by calcium loaded erythrocytes resulting from loss of membrane phospholipid asymmetry. J Lab Clin Med 1997; 130: 169-182


Whiting KP, Brain PF and Restai CJ. Steroid hormone induced effects on membrane fluidity. Biochem Soc Trans 1995; 23: 438S


Willmer EN. Steroids and cell surfaces. Biological Reviews of the Cambridge Philosophical Society 1961; 36: 368-398


Zanger B, Alfrey CP, McIntire LV and Leverett LB. The effects of dromostanalone in sickle cell anaemia. J Lab Clin Med 1974; 84: 889
APPENDICES

1 Preparation of steroids (non radio-labelled and radio-labelled)

2 Preparation of HEPES buffered saline

3 Osmotic fragility test protocol

4 Measurement of packed cell volume (PCV/haematocrit)

5 Protocol for fixation of samples for SEM and TEM

6 RFH “in house” ELISA assay for measurement of β TG

7 Menstrual chart

8 Proforma for premenopausal women

9 Proforma for post-menopausal women

10 Instruments and validation of experiments
Appendix 1

Preparation of oestradiol, progesterone and testosterone
All steroid solutions were prepared as advised by the manufacturer, Sigma Chemicals Co (St Louis, Mo, USA).

Physiological levels of oestradiol, progesterone and testosterone in women are $1 \times 10^{-9}\ g/ml$ (3 nM), $1 \times 10^{-7}\ g/ml$ (0.3 μM) and $1 \times 10^{-11}\ g/ml$ (30 pM) respectively (Chabbert Buffet 1998). Oestradiol and progesterone incubation were both performed at $1 \times 10^{-5}\ g/ml$ (30-35 μM), which was equivalent to the approximate therapeutic levels as suggested by Schering Chemicals (Schering Health Care Ltd., West Sussex, UK). Testosterone incubation was done at $1 \times 10^{-7}\ g/ml$ (0.3 μM), so chosen to approximate supraphysiological levels in women.

Validation experiment to determine the optimal % (v/v) of ethanol to be used as solvent for the steroid solutions
The % (v/v) of ethanol (Hayman Ltd., UK) which could be safely used as a diluent for preparation of steroid solutions, so as not to produce artefactual effects on red cells, was investigated.

A red cell suspension was prepared as described earlier. Five aliquots of red cells were prepared with differing % (v/v) of ethanol alcohol and the preparations viewed under light microscopy.

1. Red cells and distilled water only: red cell lysis.
2. Red cells, 1% (v/v) [or 1 : 100] ethanol alcohol in Hepes and distilled water: red cell lysis.
3. Red cells and Hepes buffered saline only (control): normal red cells seen.
4. Red cells and 1% (v/v) [1 : 100] ethanol in Hepes: normal red cells seen.
5. Red cells and 10% (v/v) [or 1 : 10] ethanol in Hepes: crenated red cells, due to hypertocity of solution.

Hence, 1% (v/v) [or 1 : 100] ethanol in Hepes does not appear to produce gross artefactual changes to red cells.
Preparation of stock hormone solutions

The solvent used in all cases was ethanol alcohol $C_2H_5OH$. The author has established that to preserve the integrity and geometry of red cells and to prevent haemolysis and crenation, the final dilution of ethanol alcohol in the solution must be $>1:100$.

1. Primary stock solutions were prepared by dissolving 100 mg of oestradiol, progesterone and testosterone in 10 ml of ethanol alcohol. The steroid concentration is therefore $1 \times 10^{-2}$ g/ml in 10 ml of alcohol.

2. The first dilution is that of $1:10$ with Hepes buffered saline *ie* 1 ml of primary stock in step 1 with 9 ml Hepes buffer saline to make up a 10 ml solution. Steroid concentration would be $1 \times 10^{-3}$ g/ml with ethanol concentration of $1:10$.

3. The second dilution is a further $1:10$ dilution with Hepes buffered saline *ie* 1 ml of step 2 with 9 ml of Hepes buffered saline to make up a 10 ml solution. The steroid concentration would be $1 \times 10^{-4}$ g/ml with ethanol concentration of $1:100$.

4. This is the final stock solution which is stored at $4^\circ C$ and re-prepared every week. The ethanol concentration of $>1:100$ does not adversely affect red cell membrane.

5. Further dilutions are made of the stock solution as required.
Appendix 2

HEPES BUFFERED SALINE

HEPES buffered saline (20 mmol/l Hpes), pH 7.4 ± 0.05 and osmolality 295 ± 5 mmol/kg was used for the washing and subsequent suspension of erythrocytes as per ICSH guidelines (1986).

PREPARATION OF HEPES BUFFER

The molecular weight of Hepes free acid is 238 (ie 238 g of acid dissolved in 1 l solvent produces a 1 Molar solution). Hence, 4.76g free acid in a final total of 1 l will give a 20 mM solution.

1 4.76 g of Hepes solid was dissolved in 900 ml of normal saline (0.9%).

2 Sodium hydroxide (NaOH) pellets was dissolved in normal saline to produce a strong NaOH solution.

3 This was titrated into the Hepes solution made in step 1 until a pH of 7.4 is achieved.

4 The final solution was topped up to a volume of one litre by adding 50 ml of normal saline and 50 ml of distilled water.

5 The osmolality was checked using an osmometer and a few mls of distilled water or normal saline was titrated into to solution to achieve an osmolality of 295 ± 5 mmol/kg.

6 The Hpes buffer was prefiltered through 2 μm pores to remove any impurities.

7 The solution was then decanted into 10 ml tubes.

NB To ensure that the buffer is free from bacterial contamination, it must used within 1 month if stored at 4°C. For longer term storage, the buffer must be kept at -20°C, or autoclave the solution in a glass bottle. The pH and osmolality of one sample was checked prior to every set of experiments.
SAMPLE

Lithium Heparin form patient and a normal control. The test should be carried out within 2 hours of collection with blood stored at RT or within 6 hours. If the blood has been kept at 4°C.

PRINCIPLE

Fresh blood or blood incubated for 24 hours at 37°C is suspended in a graded series of buffered saline solutions and the lysis is measured spectrophotometrically. Two main abnormalities may be noted:

a) Red cells having increased resistance to lysis i.e. Reticulocytes hypochromic cells as in iron deficiency and thalassaemia, sickle cells. Decreased fragility is also found after splenectomy and in liver disease.

b) Red cells having increased fragility i.e. spherocytes as found in hereditary spherocytosis, certain acquired haemolytic anaemias and some congenital non-spherocytic anaemias.

REAGENTS

1. STOCK SOLUTION Phosphate Buffered Sodium Chloride pH 7.4 100g/l.
   
   Dissolve: NaCl 90g
   Na₂HPO₄ 13.65g
   NaH₂PO₄ 2H₂O 2.43g
   in distilled water and adjust the final volume to 1 litre.

2. WORKING SOLUTION Phosphate Buffered Sodium Chloride pH 7.4 10g/l (PBS)
   Dilute stock solution 1/10

NB Crystals which form on storage at 4°C must be dissolved by warming stock solution at 37°C and mixed before use.
2. Using a positive displacement pipette add 321 of fresh heparined blood (test to one set and control to the other) to each tube and gently invert to mix thoroughly.

3. Incubate at RT for 30 minutes, remix and centrifuge at 2500 rpm for 5 minutes.

4. Read the absorbances of the supernatants at 540nm against the 10g/l saline supernatant as blank.

**CALCULATION**

The optical density (OD) of the 1g/l saline solution represents 100% lysis Express the OD’s of all the other supernatants as a percentage of this value.

Plot the result on special graph paper provided with % lysis on Y axis and NaCl concentration on X axis. Determine the mean cell fragility ie. Saline concentration at which 50% lysis occurs.

**INCUBATED OSMOTIC FRAGILITY**

1. Incubate about 1ml of heparinised test and control blood in sterile bijou bottles at 37 C for 24 hours.

2. After thorough mixing add 0.032ml of test and control blood to 4ml aliquots of saline solutions as for the fresh osmotic fragility and proceed as before.

**NORMAL RANGE**

Mean red cell fragility 4.0 – 4.45g/l NaCl

Incubated mean red cell fragility 4.65 – 5.9g/l NaCl

**REFERENCES**

3. Prepare the following concentrations from the 10g/l working solution

<table>
<thead>
<tr>
<th>Conc NaClg/l</th>
<th>H2O (ml)</th>
<th>g/l PBS(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>2.0</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>3.0</td>
<td>14</td>
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<td>7.5</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>8.5</td>
<td>3</td>
<td>17</td>
</tr>
</tbody>
</table>

Can be stored for several months at 4°C. Discard if mould develops

**CONSUMABLES**

- Plastic universals: 20ml
- Plastic tubes: 10ml
- Pipette tips: 200ul, 5000ul

**METHOD**

1. Prepare dilutions of PBS as described, aliquot 4ml of each dilution including 10g/p into 2 sets of 10ml tubes.
Appendix 4

Measurement of packed cell volume (PCV/haematocrit)

It is recommended that for rheological studies, the PCV of specimens should be determined manually by microhaematocrit centrifugation at 10000 to 15000 x g for 5 minutes (ICSH 1986). Although PCV measurements by microhaematocrit for samples of sickle cell blood has been reported to be associated with inaccuracies due to high levels of plasma trapping (England et al 1972), this has not been confirmed by other authors (Pearson and Guthrie 1982). Mean trapped plasma values for individuals with homozygous sickle cell disease in the latter study were found to be 1.7%, only marginally more than for normal blood samples.

Method

All haematocrit measurements in this thesis were performed by the author. Blood samples were mixed well in air and when containing sickle haemoglobin, were oxygenated. Blood was then transferred into a capillary tube (Bilbate Ltd., Daventry, England) and the end sealed using a Bunsen burner flame. The sample was then spun using a microhaematocrit centrifuge (Hawksley, England) at 15000g for 5 minutes. The height of the column of blood cells was then read using a microhaematocrit reader (Hawksley, England) and a magnifier.

Reproducibility of the technique was assessed by drawing one sample of blood from a female volunteer and preparing 10 replicate capillary tubes from this. The microhaematocrit was calculated for each capillary tube as described above. The coefficient of variation (CV), which is expressed as a percentage of the mean of the distribution $\text{SD/mean} \times 100$, is a measure of the reproducibility of the method and was found to be 1.63%.
PROCCESSING CELL SUSPENSIONS FOR SCANNING ELECTRON MICROSCOPY

Procedure
The samples are fixed in 1.5% glutaraldehyde for at least 2 hours. They are then deposited on to 13mm 0.6µm Nucleopore polycarbonate filters which have been mounted in Millipore filter holders. This is done by applying a drop of the cell suspension to the filter holder using a fine tip pipette. The cells are then flushed with 20mls of freshly distilled water to attach them to the filter membrane.

The filters are removed from the filter holders then postfixed with osmium tetroxide for 1 hour. The filter is then gently washed with distilled water and dehydrated through graded acetone (30%, 50%, 70%, 90% and 100% HPLC grade) being careful at all times not to remove the cells with vigorous treatment. The filters were then treated with tetramethylsilane (Sigma) and then allowed to air dry on blotting paper.

The filters were then attached to aluminium stubs with double sided tabs (TAAB) and then coated with gold using an SC500 (EMScope) sputter coater. The stubs were examined and photographed using a Philips 501 scanning electron microscope.

Reagents
Glutaraldehyde
(20mls 20% paraformaldehyde [Analar BDH] + 16mls 25% glutaraldehyde [TAAB] + 59mls phosphate buffered saline [Oxoid])

Osmium tetroxide
1% osmium tetroxide [Analar BDH] + 1.5% potassium ferricyanide [BDH] in PBS [Oxoid]

Health and Safety
COSH Quick Check
Glutaraldehyde Harmful by inhalation. Harmful if in contact with skin. Prolonged skin contact may cause dermatitis.
Paraformaldehyde High toxicity, severe irritant, animal carcinogen - potentially exposed employees require health surveillance.
Osmium tetroxide Harmful by inhalation and if swallowed. Corrosive to eyes and skin causing burns. Stains skin black. Low concentrations of vapour affects the eyes. Use only in a fume cupboard.
Potassium ferricyanide Low toxicity. May be harmful if ingested in large quantities. Irritating to eyes, respiratory system and skin.
Acetone Highly flammable. Irritant. Irritating to respiratory system and skin. Risk of serious damage to eyes.
Tetramethylsilane Extremely flammable. Harmful by inhalation, in contact with skin and if swallowed. May develop pressure in the bottle. Store at 2 to 8°C.

Reagent and equipment suppliers
TAAB - TAAB Laboratories Equipment Ltd, Unit 3 Minerva house, Calleva Ind. Park, Aldermaston, Reading, Berks. RG7 4QW
Oxoid - Oxoid Ltd, London, SE1 9HF
BDH - Merck Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leics, LE17 4XN
Sigma Chemical - Company Ltd.
Appendix 6

**An ELISA technique for estimating β TG at the Royal Free Hospital**

β TG “in house” assay is performed as described: purified serum Ig G was prepared by the n-Octanoic (Caprylic) Acid method 14 (rabbit antiserum to human β TG was obtained from Stago Diagnostics, through Shield Diagnostics Ltd., The Technology Park, Dundee, UK). The biotinylation process of the peroxidase-conjugated antibody had been extensively modified from that of Hnatowich et al. The 1st International Standard (National Institute of Biological Standards and Controls, Potters Bar, UK) for β TG (83/501) was used as the standard (where 1 IU/ml = 1 ng/ml). A standard curve was constructed to cover a range of 6 IU/ml to 0.3 IU/ml. Samples were diluted 1/50 and 1/1000. Control samples were made from the standard (2.4 iu/ml and 4.8 iu/ml).

Antiserum to β TG was used to prepare both the coat and conjugated antibodies. 100 μl of diluted coat antibody was added to each well of a microtitre plate. The plate was sealed and left at 4°C overnight. The coated plate was washed five times before addition of 100 μl of patient, control or standard curve dilutions were added in 100 μl volumes to the plate. The plate was sealed and incubated on a plate shaker for one hour at room temperature, after which the plate was washed a further five times. 100 μl of diluted biotinylated antibody/HRP-SA was added to each well. The plate was again incubated for one hour on a plate shaker at room temperature. Just before the end of incubation, the substrate solution was prepared by dissolving one 10 mg OPD tablet (Sigma Chemical Company Ltd., Fancy Rd., Poole, Dorset, UK) in 15 ml substrate buffer. The plate was then washed for a final five times. 100 μl of substrate solution was added to each well at timed intervals and after ten minutes, the reaction was stopped by the addition of 100 μl of 1.5 M sulphuric acid to each well at the same interval. The plate was read within 30 minutes at 492 nm. The plate reader softwear calibrated the β TG standard curve (optical density against IU/ml on a semi-log scale) and then calculated the mean test and control results from the curve.
Appendix 7

PATIENT'S MENSTRUAL DIARY

EXPLANATORY NOTES:

We are doing a study to investigate the pattern of menstruation in women with Sickle Cell Disease and if crises may be triggered off by certain phases of the menstrual cycle - some patients find that their Sickle Cell Disease is made worse by menstruation. We would be grateful if you could fill in this menstrual chart as accurately as possible for the duration of this study (6 months).

Below are some guidelines on how to fill in the chart:

**Bone pain:**
0 = None
1 = Mild, requiring simple painkiller tablets eg Paracetamol
2 = Moderate, requiring stronger painkillers eg DF 118
3 = Severe, requiring inpatient stay / painkiller injections (Pethidine)

**Tiredness:**
A = Mild
B = Moderate
C = Severe

**Day of month:**
This refers to the calendar month eg January 1 - 31

**Menstruation:**
A = Flooding
B = Heavy
C = Moderate
D = Light

**No. of pads and tampons:**
Just fill in the number used each day
| Month:       | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 |
|-------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---|
| Menstruation|   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| Bone Pain   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| Tiredness   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| No. Of Pads |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| No. Of Tampons |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

| Month:       | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 |
|-------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---|
| Menstruation|   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| Bone Pain   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| Tiredness   |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| No. Of Pads |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| No. Of Tampons |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
Appendix 8

Premenopausal women with SCD
(Initial Visit)
Name:

Hospital: Hospital no:

Address:

Tel no:

DOB:

Smoking:

Type of Sickle Cell Disorder:

Medication incl. Hormones:

No of admissions due to sickle crises:

No of crises in the past 12 months: major=

Minor=

No of episodes of jaundice:

Chronic complications:

Steady state Hb:
Obstetric history
No of pregnancies:
No of miscarriages:
No of TOP's:
No of livebirths:
No of SB's:
No of NND's:

<table>
<thead>
<tr>
<th>Year</th>
<th>Planned/unplanned</th>
<th>Outcome</th>
<th>Crises in pregnancy</th>
</tr>
</thead>
</table>

Gynae history
Menarche:
Cycle:
No of periods in the past 12 months:
Period pain (1/2/3):
Menstrual loss (1/2/3):

Contraceptive history
Age of onset of contraception:
Current contraception:
Duration of use:

Previous contraception used:

Reason for stopping:
Appendix 9

PROFORMA FOR POSTMENOPAUSAL SCD PATIENTS

Name: DOB/Age:

Hospital No:

Age at time of Menopause:

Frequency of crises since menopause:

Frequency of crises prior to menopause:

Menopausal Symptoms

Vasomotor :
Vaginal Dryness:
Urinary:
Musculoskeletal:
Psychological:
Libido:

Weight:
Smoking Habit:
Alcohol:

PMH

Medication:

HRT and knowledge
Appendix 10

INSTRUMENTS AND VALIDATION OF EXPERIMENTS

The St George’s Filtrometer and the measurement of erythrocyte deformability

The St George’s Filtrometer (Carri-Med, Dorking, UK) is an instrument that assesses erythrocyte deformability using a filtration technique which passes red cells through 5 μm membranes. It measures three time intervals for the passage small quantities of filtrate, allowing the measurement of small volumes (3 x 20 μm) at the beginning of the filtration procedure. It uses a vertically mounted filter to minimise changes in the cell concentration of the sample due to sedimentation. As there is batch to batch variation in the geometry and distribution of the filter pores, all filters should be taken from the same batch number (ICSH 1986). The filter used is positioned in the filter chamber which is supplied with outlets leading to the capillary tube filling channel and outflow channel.

The filtered volume is measured using four pairs of fibre-optic light sources and detectors aligned along a 1.6 mm diameter horizontal glass capillary tube. By detecting the change in the intensity of light reflected by the internal surface of the capillary wall (ie at the glass and air, or glass and fluid interface when the sample meniscus passes the detectors) the flow rate along the capillary tube is measured in milliseconds. The results are calculated using a BBC microcomputer with the appropriate software.

The effective filtration pressure can be set by varying the height of the receiving reservoir. Prior to the first use of the instrument, a pressure head of 3 cm water was calculated and the water level was marked on the reservoir bowl. Before operating the instrument each day, the water level was topped up to this level with de-ionised water.

The St George’s Filtrometer suffers from difficulty of maintaining the oxygen tension and temperature of the sample. Filtration is a function of polymerisation of Hb S and since the latter is dependent on oxygen tension (Messer and Harris 1970) and temperature (Hofrichter et al 1974), these variables should be controlled. For the experiments in this thesis, the pH was controlled by the Hepes buffer and all samples were well mixed in room air to ensure uniform exposure to atmospheric oxygen, prior to measurement.

Although a water jacket is sometimes provided with the Filtrometer to help maintain filtration temperature, the author did not have access to this. Moreover, the water jacket does not control the temperature at the membrane where the erythrocytes pass through the pores and its value may be limited.
Using the computer programme, a standardisation procedure was employed to standardise the filter using the buffer. This was repeated until two measurements fell within the pre-determined range of 1% which was installed on the computer software. Following this, the buffer was withdrawn from the system using the syringe and the air-mixed red cell suspension inserted, ensuring that no air bubbles were present in the filter chamber. The haematocrit value in the software programme always defaulted to 10% and when the actual PCV (determined by microhaematocrit) of the suspension differed from this, this value was substituted into the programme, thereby correcting the measurement of the red cell deformability to the haematocrit. For the experiments in this thesis, all haematocrit values were between 2.5-3% for sickle cell suspensions and 10% for normal red cell suspensions (Stone 1996, personal communication). Following each measurement, all remaining suspension was removed from the filter chamber using a syringe and the perspex housing was unclamped and rinsed in distilled water.

**Analysis of the software results**

The filtration rates of the sample are expressed in relation to the buffer filtration rates at each of the three filtered volumes. Assuming a constant clogging rate (ie a linear decrease in relative filtration rate with cumulative volumes filtered) and knowing the volumes involved, the initial relative filtration rate of the cell suspension (rFRo) is calculated.

The mean red cell transit time (RCTT) is then expressed using the formula:

\[ RCTT = \frac{1}{rFRo} - 1 + \frac{1}{\text{haematocrit}/100(\%)} \]

The clogging rate (CR) is calculated from the rFRo and the relative filtration rate (RFr=suspension flow rate/standardisation flow rate) when a known volume of sample (v) has been filtered using the formula:

\[ CR = 1 - \frac{rFR/rFR}{v} \]
Assessment of reproducibility: this was assessed by calculating the coefficient of variations (CV) of the method and of the instrument.

The CV of the method was assessed by taking 20 ml venous blood from a female volunteer and dividing this into 10 x 2 ml aliquots. Erythrocyte suspensions were then processed separately from each 2 ml sample (including the leucocyte filtration procedure) and these 10 different aliquots passed through the St George’s Filtrometer. The RCTT and CR measurements were performed for each sample and the CV calculated by the equation CV = Standard deviation/mean x 100. The CV’s for RCTT and CR were 12.41% and 13.76% respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CR (/ml)</th>
<th>RCTT (secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.323</td>
<td>9.509</td>
</tr>
<tr>
<td>2</td>
<td>2.685</td>
<td>10.476</td>
</tr>
<tr>
<td>3</td>
<td>3.418</td>
<td>12.114</td>
</tr>
<tr>
<td>4</td>
<td>2.480</td>
<td>10.040</td>
</tr>
<tr>
<td>5</td>
<td>3.773</td>
<td>10.320</td>
</tr>
<tr>
<td>6</td>
<td>3.631</td>
<td>10.993</td>
</tr>
<tr>
<td>7</td>
<td>4.530</td>
<td>10.223</td>
</tr>
<tr>
<td>8</td>
<td>4.665</td>
<td>10.970</td>
</tr>
<tr>
<td>9</td>
<td>5.413</td>
<td>10.008</td>
</tr>
<tr>
<td>10</td>
<td>5.985</td>
<td>9.910</td>
</tr>
</tbody>
</table>
The CV for the instrument was calculated by performing replicate measurements on eight aliquots prepared from a single red cell suspension from the same female volunteer. The CV’s for RCTT and CR were 5.8% and 9.8% respectively.

<table>
<thead>
<tr>
<th>Aliquot</th>
<th>CR (ml)</th>
<th>RCTT (secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.757</td>
<td>8.469</td>
</tr>
<tr>
<td>2</td>
<td>1.167</td>
<td>8.002</td>
</tr>
<tr>
<td>3</td>
<td>0.890</td>
<td>8.166</td>
</tr>
<tr>
<td>4</td>
<td>1.670</td>
<td>13.902</td>
</tr>
<tr>
<td>5</td>
<td>1.370</td>
<td>8.193</td>
</tr>
<tr>
<td>6</td>
<td>1.279</td>
<td>7.910</td>
</tr>
<tr>
<td>7</td>
<td>1.432</td>
<td>7.977</td>
</tr>
<tr>
<td>8</td>
<td>1.133</td>
<td>7.002</td>
</tr>
</tbody>
</table>
The liquid scintillation counter Beckman LS 5000CE

The Beckman Model 5000 CE Liquid Scintillation System (Beckman Instruments Inc., Fullerton, CA 92634-3100 USA) is a microprocessor-controlled spectrometer designed to provide highly accurate automated counting of the level of radioactivity in radio-labelled samples. The instrument usually calculates counts per minute (cpm) but the results can also be reported in disintegrations per minute (dpm), which is a more reliable result since it incorporates correction for variables that can distort the result. To use the liquid scintillation instrument, the samples in capped vials, are placed together with a scintillant into a rack which is then transferred into the sample changer compartment. The automated counting mode was used and the instrument contained a 30 microcurie (1.11 MBq) $^{137}$Cs source enclosed in a lead container. Gamma ray emission at any exterior panel is less than 0.5 rem per hour.

The theory of liquid scintillation counting

Liquid scintillation involves the detection and counting of radioactive decay. The radioactive sample to be counted is combined with a "scintillation cocktail" which usually contains a solvent, an emulsifier and a fluor. The scintillation cocktail used in the receptor binding experiments was Ultima Gold (Canberra Packard, Pangbourne, Berks., UK). The cocktail serves to convert the energy emitted during the radioactive decay process into light which is then detected by the LS counter. An LS counter is capable of measuring alpha particles (positively charged helium nuclei), beta particles (electrons), positrons (positively charged electrons), Auger electrons, conversion electrons and Compton electrons. Part of the energy emitted by the radioactive decay is absorbed by the solvent molecules, causing them to become excited and the number which become excited is proportional to the energy of the radioactive decay being measured. The energy is transferred between the solvent molecules as they are the dominant species in the cocktail. Eventually, excited solvent molecules will transfer their energy into the fluor molecules. The whole process occurs in less than $10^{-12}$ secs. The fluor molecule is now in the excited state and the solvent molecule returns to normal. The excited fluor molecules return to their ground state by emitting light. Each fluor molecule emits one photon at a wavelength between 380 to 420 nm, depending on the fluor (not the radioactive sample). The amount of light emitted from the vial is proportional to the energy of the particle, so that the higher the energy of the particle the more solvent it is able to excite and
therefore, the more light it will produce. The light is then directed to two photomultiplier tubes which convert it into a measurable electrical pulse. The pulses from the photomultiplier tubes are analysed, converted into digital form and stored in the appropriate channel of a multichannel analyser corresponding to the particle energy.

During sample measurement, the multi channel analyser accumulates the number of pulses (or counts) in each channel. A plot of the counts vs channel number yields a spectrum of the radioactive decay particle in the sample. In the experiments, tritium $[^3\text{H}]$, which decays with emission of $\beta$ energy at 18.3 keV, was used. The data in the multi channel analyser could be used to determine the energy of the particle in the sample and the count rate (cpm) of the radioactive decay in the sample. Cpm is the total number of pulses in the channels of the multichannel analyser divided by the total time in minutes of obtaining the count.

Quench

A liquid scintillation process is a direct relationship between the amount of radioactivity of the sample and the amount of light being emitted from the vial. In practice, a number of factors act to reduce the amount of light being emitted and this phenomenon is called quenching. Quenching causes two effects: a reduction in the measured cpm of the sample (lower efficiency) and a shift in the end point of the pulse height spectrum to lower the channel numbers. The two types of quench are chemical and colour quench. Chemical quench is caused by compounds such as oxygen, water, emulsifiers used in the aqueous cocktail solutions. Some chemicals such as water and alcohol are weak quenching agents and several ml have little effect of the cpm recorded. Other such as phenol and acetone are strong quenchers. Chemical quench can interfere with transfer of energy form the radioactive decay to the solvent, transfer from solvent to solvent etc. Colour quench is caused by the introduction of colour from the sample (haemoglobin or chlorophyll etc) and the light emitted from the flour is absorbed by the coloured solutions and does not reach the photomultiplier tubes. Blue solutions quench very little whereas yellow solutions quench heavily.

The end result of quench is that less light arrives at the photomultiplier tubes and therefore fewer cpm are recorded by the instrument. All samples are quenched to some
degree from the sample or even oxygen. Hence in order to express the data in units that allow comparison (ie independent of quench levels), the data must be converted from cpm to a value that correctly reflects disintegrations that actually occur. This value is called disintegrations per minute or dpm. The ratio of cpm to dpm is called counting efficiency:

\[
\text{Counting Efficiency (CE)} = \frac{\text{cpm}}{\text{dpm}} = \frac{\text{events observed by the instrument}}{\text{actual disintegrations that occurred}}.
\]

Once cpm is known, then dpm can be calculated if the CE is known. CE is normally determined from a quench curve set up in the LS instrument prior to beginning to count the experimental sample. A quench curve establishes the counting efficiency for a given value of quench as measured by the H#. \(^3\)H is a weak \(\beta\) emitter (18 keV) and the \(\beta\) decay can be quenched easily so that little or no light is produced.

The efficiency was calculated as part of counting using the H coefficient method.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>haemoglobin A</td>
</tr>
<tr>
<td>(^6)A</td>
<td>angstrom</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>(\beta) TG</td>
<td>(\beta) thromboglobulin</td>
</tr>
<tr>
<td>C</td>
<td>haemoglobin C</td>
</tr>
<tr>
<td>COCP</td>
<td>combined oral contraceptive pill</td>
</tr>
<tr>
<td>CR</td>
<td>clogging rate</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DPG</td>
<td>diphosphoglycerate</td>
</tr>
<tr>
<td>D</td>
<td>haemoglobin D</td>
</tr>
<tr>
<td>DMPA</td>
<td>depot medroxyprogesterone acetate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamino tetra acetic acid</td>
</tr>
<tr>
<td>(F_{1+2})</td>
<td>Prothrombin fragment 1+2</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>FTIRS</td>
<td>Fourier Transform Infra-Red Spectroscopy</td>
</tr>
<tr>
<td>Hb</td>
<td>haemoglobin</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-2- hydroxyethylenepiperazine-N-2 ethanesulphuric acid</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>ISC's</td>
<td>irreversibly sickled cells</td>
</tr>
<tr>
<td>IUCD</td>
<td>intrauterine contraceptive device</td>
</tr>
<tr>
<td>LH</td>
<td>luteinising hormone</td>
</tr>
<tr>
<td>MCF</td>
<td>mean corpuscular fragility</td>
</tr>
<tr>
<td>MCHC</td>
<td>mean cell haemoglobin concentration</td>
</tr>
<tr>
<td>MCV</td>
<td>mean cell volume</td>
</tr>
<tr>
<td>NS</td>
<td>non-specific (binding)</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PAP</td>
<td>plasmin (\alpha)2 antiplasmin complex</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCV</td>
<td>packed cell volume (haematocrit)</td>
</tr>
<tr>
<td>PF 4</td>
<td>platelet factor 4</td>
</tr>
<tr>
<td>PO</td>
<td>progesterone only</td>
</tr>
<tr>
<td>pO2</td>
<td>partial pressure of oxygen</td>
</tr>
<tr>
<td>PC</td>
<td>protein C</td>
</tr>
<tr>
<td>PS</td>
<td>protein S</td>
</tr>
<tr>
<td>RCTT</td>
<td>red cell transit time</td>
</tr>
<tr>
<td>RS</td>
<td>receptor specific (binding)</td>
</tr>
<tr>
<td>S</td>
<td>haemoglobin S</td>
</tr>
<tr>
<td>SC</td>
<td>haemoglobin SC</td>
</tr>
<tr>
<td>SCA</td>
<td>sickle cell anaemia (Hb SS)</td>
</tr>
<tr>
<td>SCD</td>
<td>sickle cell disease</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean, scanning electron microscopy</td>
</tr>
<tr>
<td>TAT</td>
<td>thrombin antithrombin complex</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>thal</td>
<td>thalassaemia</td>
</tr>
</tbody>
</table>
LIST OF PUBLICATIONS ARISING FROM THIS THESIS


Yoong WC, Buscombe JR and Tuck SM. Bone Mineral Density in women with Sickle Cell Disease. (Manuscript submitted to Maturitas).