# SERUM VITAMIN C AND SOME HAEMATOLOGICAL INDICES IN HEALTHY SUBJECTS AND IN PATIENTS WITH SICKLE CELL ANAEMIA

**BY**

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY.

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JULY, 1996

# DECLARATION

I hereby declare that this thesis has been produced by me and that the work has not been presented previously for any application for a higher degree. The thesis is an original work by me except for quotations and references which are duly acknowledged.

RAJI WASIU OLADIMEJI

# CERTIFICATION

This thesis entitled **SERUM VITAMIN C AND SOME HAEMATOLOGICAL INDICES IN HEALTHY SUBJECTS AND PATIENTS WITH SICKLE CELL ANAEMIA** by W.O. Raji meets the requirements governing the award of the degree of Master of Science in Biochemistry of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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# DEDICATION

To the sweet memory of my

late grand-mother,

Alhaja Raliat Omosanya Adepo.

# ACKNOWLEDGEMENTS

I wish to acknowledge with thanks the valuable suggestions and constructive criticisms by my Supervisors - Dr. D. A. Ameh, Head of Department of Biochemistry, Ahmadu Bello University and Prof. H. S. Isah, Head of Department of Chemical Pathology, Ahmadu Bello University Teaching Hospital, Zaria. I am also grateful to them for creating time to read through this report.

My sincere thanks go to the following staff of Haematology Department, Ahmadu Bello University Teaching Hospital, Zaria, Dr. D. M. Mukthar, Dr. A. I. Mamman and Dr. A. O. Ayansanwo for giving me access to their patients. I gratefully acknowledge the technical expertise of Mr. O. S. Adekunle in parts of the work. My profound gratitude go to the patients and the volunteers for their co-operation. I thank Dr. T. S. Akande who assisted me in bleeding the patients and the volunteers.

In carrying out this study, I have received immense encouragement, help and financial backing from my parents - Alhaji and Alhaja Raji, my uncles - Mr P. F. Adepo and Alhaji M. I. Raji; and my entire family.

Lastly, my heartfelt thanks go to my good friends and colleagues - Mr. J. Aladewolu, Mr. A. E. Adewuyi, Mr. J. A. Agboola, Mr. A. O. Owolabi, Miss F. J. Adeyemi, Miss Tawa Ajibola, Miss A.O. Alabi, Dr. T. Ige and Dr. R. A. Yusuf for their moral and emotional support. I also thank Mr. L. O. Omotosho for typing the manuscript, Mr. N. O. Omisanya who helped in drawing some of the figures and Mr O. S. Lamidi for the statistical analyses of the data. Above all, I thank the Almighty Allah for making everything possible.

# ABSTRACT

Scrum vitamin C, haemoglobin, packed cell volume and mean corpuscular haemoglobin concentration were measured in healthy subjects and in sickle cell anaemia (SCA) patients attending the Sickle Cell Clinic at Ahmadu Bello University Teaching Hospital, Zaria. The relationships between sex/age and these parameters were also examined. Sixty seven SCA patients homozygous for hacmoglobin-S and forty-four normal subjects homozygous for hacmoglobin-A were used in this study. There was no significant difference in the mean serum vitamin C levels between SCA patients and normal subjects ($0.603 \pm 0.07$ $V_s$ $0.602 \pm 0.05$). This suggests that SCA patients studied were vitamin C sufficient. On the basis of sex, no significant difference ($P>0.05$) was observed in the serum vitamin C levels between male and female SCA patients, however, the male normal subjects had a significantly lower ($P<0.05$) vitamin C levels than the female normal subjects. The mean serum vitamin C levels in normal male subjects was $0.479 \pm 0.06$ and the male SCA patients had $0.608 \pm 0.09$. There was no significant difference ($P>0.05$) between these observed serum vitamin C levels. Similarly, there was no significant difference ($P >0.05$) in serum vitamin C levels between female SCA patients and normal female subjects. Serum vitamin C decreased with increasing age among normal subjects and among SCA patients. However, no significant difference was found in serum vitamin C levels between SCA patients and normal subjects for the age groups (18-22 and 23-27) considered in this study. Mean haemoglobin (Hb) levels were significantly lower in SCA patients than the normal subjects and this difference persisted irrespective of age or sex. Packed cell volume (PCV) followed a similar trend as Hb in both SCA patients and normal subjects. The mean corpuscular haemoglobin concentration (MCHC) was independent of age and sex; and no significant difference was found between SCA patient and normal subjects. Correlation between serum vitamin C and Hb levels was negative in both sexes irrespective of state of health, (SCA patients and normal subjects having values of $r = -0.285$, $P<0.019$ and $r=-0.387$, $P<0.008$, respectively).

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# CHAPTER ONE

## 1.0 INTRODUCTION

Vitamin C also known as ascorbic acid (ascorbate) is a major water soluble dietary antioxidant found in the aqueous compartments of cells and extracellular fluids such as blood plasma, aqueous humour and the fluid lining the epithelium [1]. Among several other roles, ascorbic acid is effective at scavenging the majority of soluble radicals and is involved in the subsequent regeneration of $\alpha$-tocopherol by reducing the $\alpha$- tocopheryl radical at the membrane surface [1]. Consequently, ascorbic acid plays a prominent role in the protection of biological systems such as the inhibition of oxidative modification of low density lipoproteins in humans [2].

It is assumed in the general medical literature that many pathological conditions, including sickle cell anaemia, are associated with circumstances which promote peroxidation, inadequate antioxidant or antiperoxidant protection. Several genetic defects concerning the antioxidant enzymes are known. Mostly, these enzymatic deficiencies are associated with granulomatous diseases resulting mainly from altered red cell and phagocytic cell functions [3]. Oxidant stress may be important in deforming red blood cells (RBCs), and this could be worse in sickle cell anaemia (SCA). Circulating RBCs are particularly susceptible to lipid peroxidation because of their rich supply of polyunsaturated fatty acids, constant exposure to high oxygen tensions and the presence of haemoglobins which is a potent catalyst for the initiation of peroxidative reactions [4]. Normal RBCs are protected against peroxidative or oxidative damage by a number of antioxidants of

which vitamin C is one. The RBC membrane in patients with SCA is more susceptible to lipid peroxidation than is the normal [4, 5], leading to the formation of irreversibly sickled cells (ISC). However, vitamin C has been found to be the first plasma antioxidant to be depleted when lipid peroxidation is induced in vitro [6].

Based on these observations, which suggest a possible role for vitamin C in the protection of RBCs in patients with SCA; the present study which looks at the vitamin C status in this group of patients, seen at the Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, has been carried out to:

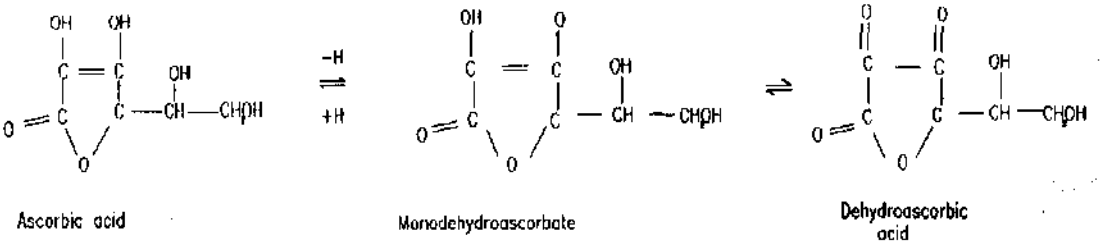
1. assess the serum level of vitamin C in patients with sickle cell anaemia who are permanently on drug, especially folic acid which makes excessive demands on ascorbic acid.
2. document the relationship between serum vitamin C (ascorbic acid) level and the manifestation of sickle cell disease. This could be of use to the physicians in their efforts to manage these patients.
3. find out if there is any age and sex differences in the serum level of vitamin C in people with sickle cell anaemia.
4. assess vitamin C antioxidant function in sickle cell disease.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

## 2.1 METABOLISM OF VITAMIN C

Vitamin C is essentially two compounds, L-ascorbic acid and its oxidised derivative, L-dehydroascorbic acid. Although upwards of 90 percent of vitamin C in animal tissues is in the form of ascorbic acid, both compounds have biological activity and are readily interconvertible by oxidation and reduction reactions through a short-lived intermediate, monodehydroascorbate (see equation 2.1).




Equation 2.1

### 2.1.1 Biosynthesis of vitamin C

Most plants and animals have the ability to synthezize vitamin C from either D-glucose or D-galactose through the glucuronic acid pathway (figure 2.1). There are, however, species like, humans, guinea pigs, apes, fruit-eating bats and red-vented bulbul (an Indian bird) which cannot synthesize the vitamin. In addition, there is evidence that insects and other invertebrates like fish and

microorganisms are also incapable of synthesizing vitamin C [7]. These organisms lack the enzymes involved in the biosynthetic pathway of the vitamin. Current evidence indicates that all vitamin C dependent species lack the enzyme L-gulonolactone oxidase (Fig.2.1) which catalyses the final step in the biosynthesis of vitamin C [8]. A study has provided evidence showing that scurvy, the condition which develops in the absence of adequate tissue concentrations of vitamin C, can be prevented by providing this enzyme, together with its substrate L-gulonolactone, to guinea pigs (9). In practice, however, scurvy is prevented by providing exogenous sources of vitamin C, and as a result this vitamin is considered to be an essential dietary requirement in the species lacking L-gulonolactone oxidase activity.

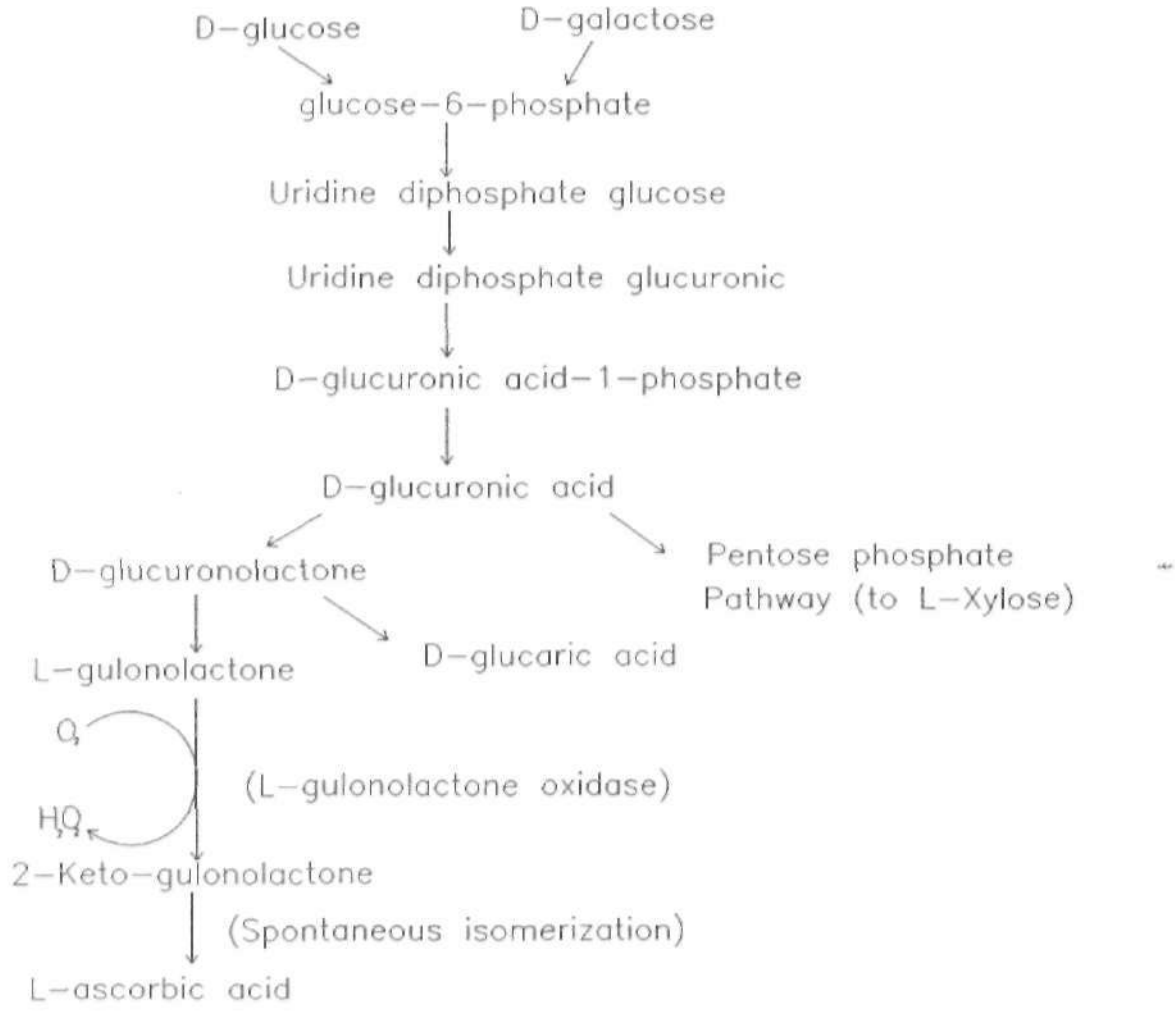



Fig. 2.1: Biosynthetic pathway of L-ascorbic acid (9).

At the chromosomal level the genes coding for the missing enzyme might be entirely absent as a consequence of deletion of genetic materials [9]. However, inability to demonstrate the biosynthesis of vitamin C in tissue homogenates does not necessarily signify absence of a particular enzyme. The defect could be due to the enzyme being inactivated by the products of the reaction.

Furthermore, the activity of L-gulonolactone oxidase may be subject to competitive inhibition with enzymes involved in the pentose phosphate (L-xylose) part of the biosynthetic fork [10]. In this context, it is noteworthy that vitamin C biosynthesis is known to occur in guinea pig embryos with rapidly differentiating tissues [9]. It is possible that the competitive activities of the enzymes involved in the formation of L-xylose are less in the embryos than in adults.

### 2.1.2 Absorption of Vitamin C

In humans and guinea pigs, the absorption of vitamin C occurs in the buccal mucosa, stomach and small intestine (Figure 2.2). The uptake of vitamin C from solutions introduced into to buccal cavity of human subjects has been shown to be pH-dependent [11]. Absorption also increases as the time of retention of the solution in the mouth is lengthened from one to five minutes. It is thought that the buccal absorption of vitamin C is mediated by passive diffusion through the membrane of the buccal mucosa cell. The rate and extent of diffusion are determined by the initial concentration of vitamin C in the buccal cells and by its rate of passage from the cells into the blood in the mucosal capillaries.

Gastrointestinal absorption of vitamin C in humans and guinea pigs has been found to be rapid and efficient, and an active carrier-mediated transport

system has been put forward by many workers [12, 13]. However, in species with no dietary requirement for vitamin C, such as rats and hamsters, the mode of intestinal absorption of the vitamin has been demonstrated to be by passive transport [9].

Using perfused intestinal segments, Stevenson and Brush [14] have revealed that the vitamin is absorbed from the distal region of the small intestine of the guinea pig by an active transport system which has the characteristics of a sodium-dependent coupled-carrier mechanism. These workers postulated that the mode of intestinal transport for a given species would reflect the presence or absence of a dietary requirement for vitamin C.

In man, the sodium-dependent active transport mechanism appears to occur at low concentrations of vitamin C [15]. However, this active absorption mechanism becomes saturated when the mucosa concentration of the vitamin is greater than 6 mM. It is thought that the absorption of vitamin C is facilitated by conversion to its oxidised form, dehydroascorbate. This may be due to the fact that unlike ascorbate, the dehydroascorbate, which is less ionised at physiological pH, has marked membrane penetrating power.

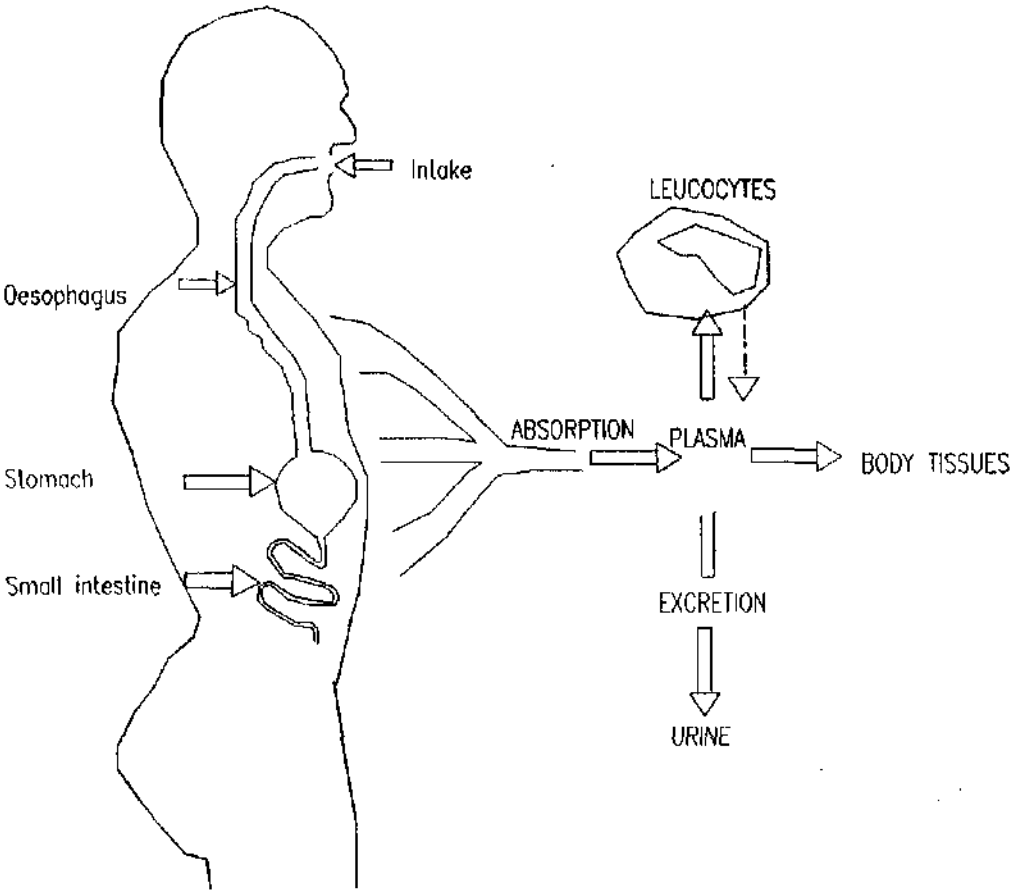



Fig. 2.2: The absorption and distribution of vitamin C (9)

### 2.1.3 Tissue Distribution of Vitamin C

Transfer of vitamin C occurs between plasma and leucocytes, and plasma and other tissues (Fig. 2.2). The lowest concentration of vitamin C appears to be in the plasma (9) (Table 2.1). This indicates that transfer of the vitamin from blood to tissue must occur against a concentration gradient and therefore to be mediated through active transport mechanisms. Although no particular organ acts as a storage reservoir for the vitamin, the tissues, and leucocytes in particular, must be regarded as a store house for it (9). This may account for the fact that scurvy only becomes clinically apparent after a subject has been on vitamin C free diet for several weeks.

Table 2.1: Vitamin C Content of Adult Human Tissues (9)

| Tissue | vitamin C (mg/100g wet tissue) |
|---|---|
| Pituitary gland | 40 - 50 |
| Adrenal glands | 30 - 40 |
| Eye lens | 25 - 31 |
| Brain | 13 - 15 |
| Liver | 10 - 16 |
| Spleen | 10 - 15 |
| Kidneys | 5 - 15 |
| Heart muscle | 5 - 15 |
| Lungs | 7 |
| Skeletal muscle | 3 |
| Testes | 3 |
| Thyroids | 2 |
| Leucocytes | 25 |
| Plasma | 0.4 - 1.0 |

### 2.1.4 Turnover and Body Pool Of Vitamin C

The average half-life of vitamin C in an adult human is about 16 days, with a turnover of 1 mg/kg/day and a body pool of 22 mg/kg (9). Baker, Saari and Tolbert (16) confirmed that in a healthy man with a dietary intake of about 100 mg vitamin C daily, the half-life of vitamin C is about 20 days and the body pool 2-3 g. The pool size may also be related to the fat-free body weight and was found to be 32-34 mg/kg fat-free mass in a study involving five men (16).

Baker and his associates (17) performed metabolic studies which revealed that there was a direct relationship between clinical signs of scurvy and the pool

size of vitamin C. Signs of scurvy were observed in all subjects when the body pool of the vitamin had been depleted to a level below 300 mg, and the clinical signs began to disappear when repletion took the vitamin C pool above the level of 300 mg.

Another study involving healthy male volunteers demonstrated that the half-life of vitamin C is inversely related to its dosage and that the pool could be increased by increasing the dosage (18). It also appears from this study that the saturated pool size could be reached by a daily intake of about 100 mg vitamin C in 95 percent of the healthy population.

## 2.2 THE ROLE OF VITAMIN C

The only role of vitamin C which has been categorically established is the ability of the vitamin to prevent and cure clinical scurvy, which is characterized by features such as swollen joints, muscular aches and bone pain, oedema, weakness, fatigue, anaemia and hyperkeratosis, especially around the hair follicles [9].

Elucidation of other possible roles of vitamin C can be approached in two ways, either by a study of the enzymes and the biochemical reactions in which the vitamin is believed to be involved, or by an investigation of the clinical signs and physiological disturbances which appear as the tissue become depleted of the vitamin.

### 2.2.1 Reduction Of Folic Acid

Anaemia is common in scurvy [19, 20]. Whilst there are many possible reasons for this, for example blood loss through haemorrhage, lack of iron uptake

and increased destruction of red cell [19], it is clear that a number of patients who are suffering from scurvy have a megaloblastic anaemia which is due to an effective deficiency of folic acid [21]. This leads to inability to synthesize sufficient nucleic acid and red cell production is decreased accompanied by the release of abnormal red cells known as megaloblasts, into the circulation [22]. Some of these patients respond to vitamin C, a few require folate [23]. Stokes and co-workers [21] presented evidence in one patient that the megaloblastic anaemia associated with scurvy is caused by an inability to maintain folic acid in the reduced tetrahydrofolate form, the active state of the vitamin. This metabolite is rapidly metabolised to the more oxidised folic acid which is then excreted in the urine. Vitamin C appeared to prevent this oxidation and leads to a rapid correction of the anaemia. It has also been noted that decreased vitamin C leads to an increase in urinary folic acid excretion which can be corrected by vitamin C therapy [24]. The possible role of ascorbic acid in maintaining the stability of tetrahydrofolate (Figure 2.3) was suggested by Vilter and co-workers [22]. About two decades ago, Barnerjee and Nandy [25] had found that guinea pigs deficient in vitamin C were still able to carry out the conversion of folic acid to tetrahydrofolate quite adequately. In the human, it has been noted that there is an association between plasma and leucocyte vitamin C levels and folic acid concentrations [26, 27]. This could mean that one vitamin affects the metabolic availability of the other or alternatively that a dietary source poor in one vitamin is also poor in the other and therefore deficiencies of folate might arise in association with, but metabolically independent of vitamin C depletions. Indeed,

Bates et. al (27) noted that vitamin C had no effect on folate levels in patients with low concentrations of both vitamins.

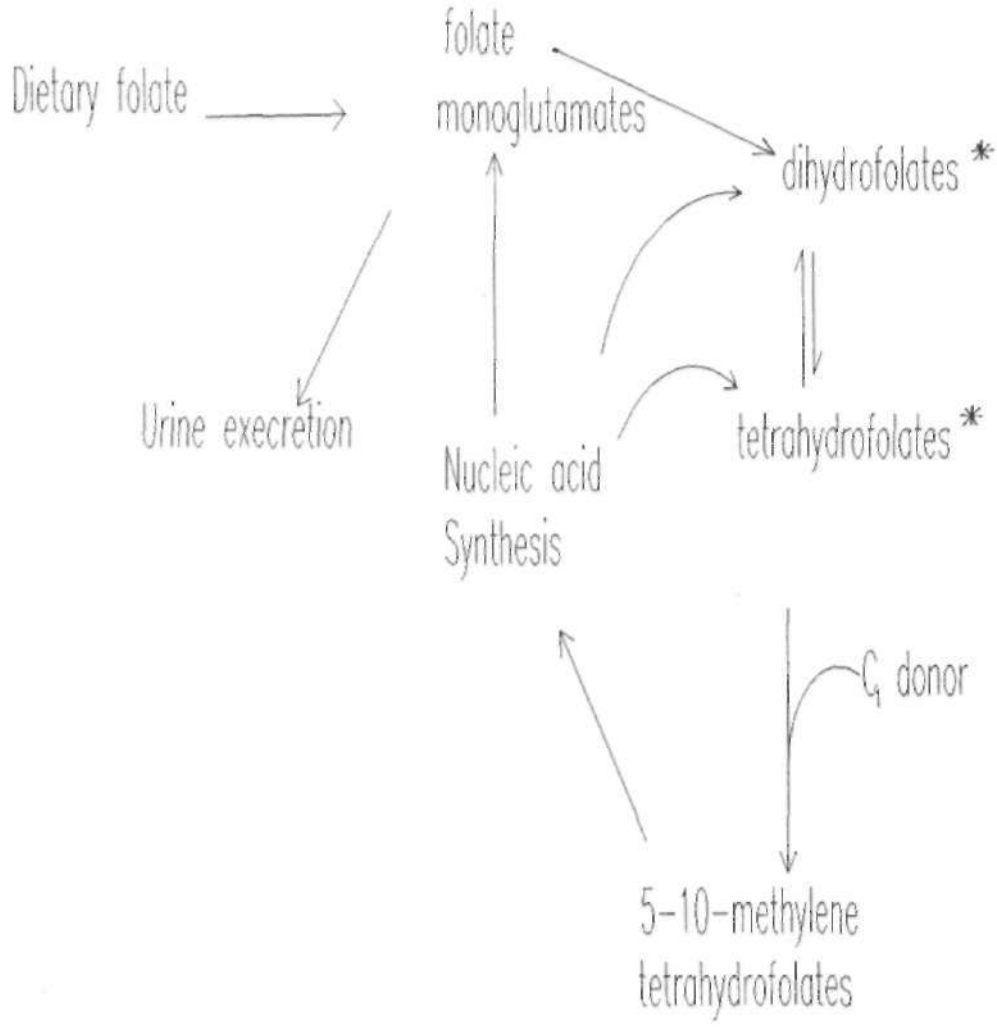



Fig. 2.3: Outline of folic acid metabolism (9)

The reduced active metabolites of folate (indicated by asterisks) are protected by vitamin C, the monoglutamate being rapidly excreted.

### 2.2.2 Vitamin C and Cytochrome P450 Hydroxylating Systems

Many drugs and other lipid - soluble potentially toxic agents such as bilirubin and steroid hormones produced by the body are modified prior to excretion by a mixed - function oxygenase (MFO) system. This is found predominantly in the liver microsomes but also in reticulo-endothelial tissues throughout the body [28]. This enzyme system requires a number of components which include enzyme for hydroxylating and demethylating substrates, flavoproteins, [a cytochrome protein (P450)], oxygen and provision of reducing agents, usually in the form of reduced NADP or NAD. A diagrammatic representation of the reactions involved is shown in figure 2.4.

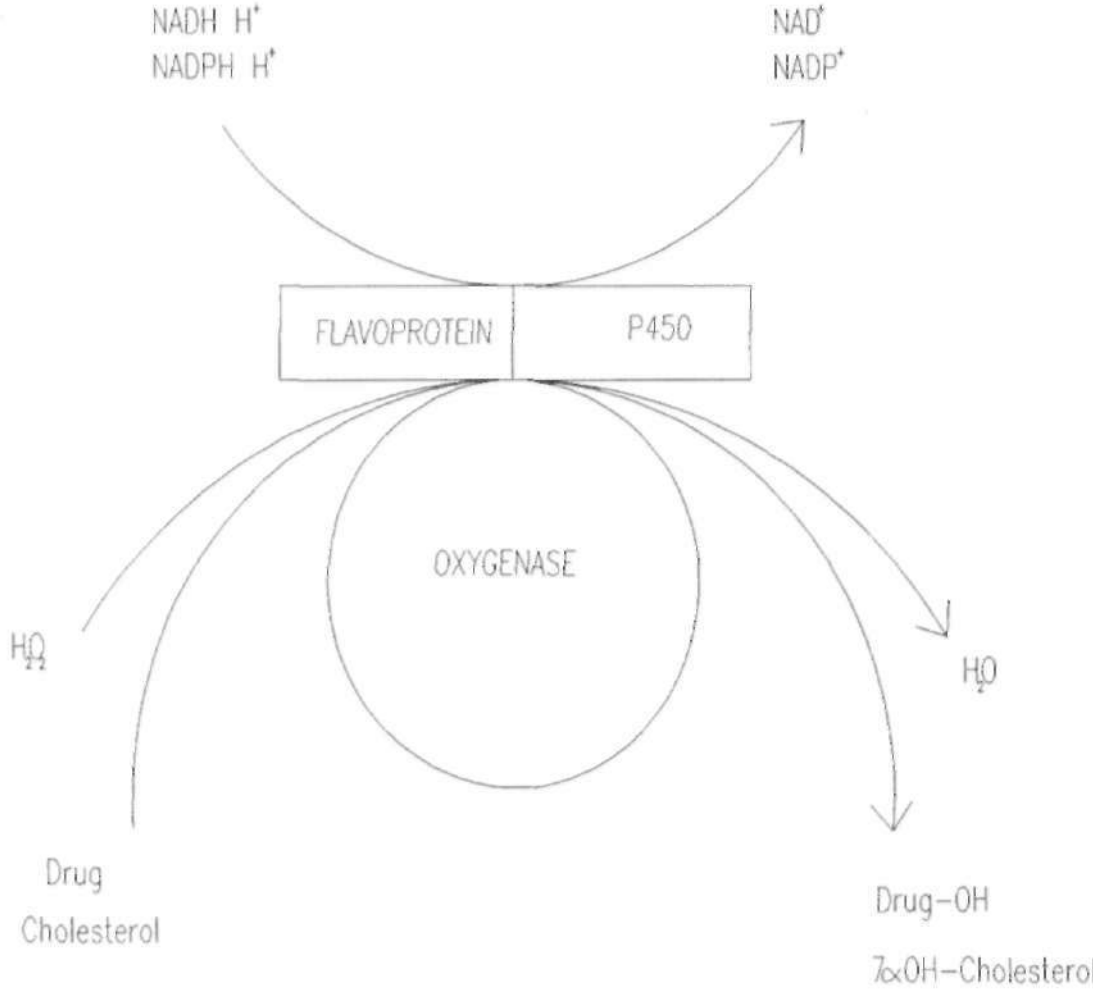



Fig. 2.4: Liver cytocbrome P450-dependent mixed - function oxygenase (MFO) (9). Level of P450 and oxygenase are increased by vitamin C.

There is considerable evidence linking changes in the activity of MFO with changes in vitamin C status. Animal studies have suggested that depletion of vitamin C is associated with a decrease in the activity of many of the enzymes involved in these reactions and also a decrease in the actual quantity and stability of the microsomal electron transport system and levels of cytochrome P450 [29, 30]. Not only does vitamin C deficiency lead to a decrease in these components, but there is also an association between the level of vitamin C reserves and the rate of metabolism of drugs both in animals [30] and in the humans [31, 32]. In patients with liver disease, the slowest rates of drug metabolism were found in those with the lowest vitamin C concentrations [32], although liver disease itself leads to a decrease in vitamin C reserves and can obviously also lead to a decrease in drug metabolism by that organ independent of vitamin C status. Such findings have led to the suggestion that vitamin C supplements may be required in diseases that affect the liver. In humans vitamin C status appears to affect the rate of drug metabolism and increased rates of drug breakdown can also affect vitamin C status as seen in those patients who are taking oral contraceptives [34, 35].

### 2.2.3 Relationship Between Vitamin C and Iron Absorption And Metabolisn

There is evidence in both animal and human that vitamin C can affect distribution of iron and its storage protein ferritin between the tissues and the plasma. In vitamin C deficiency, increasing quantities of iron are deposited in the tissues and serum levels of iron and ferritin bear little relationship to tissue iron reserves. The correlation can be restored by tissue vitamin C repletion [36]. vitamin C is also said to increase the intestinal uptake and peripheral metabolism

of iron [37]. Alternatively, pure iron deficiency anaemia in children leads to higher vitamin C reserves, whilst subsequent iron therapy, although not affecting plasma vitamin C, reduces cellular levels of vitamin C [38] and iron overload accelerates oxidation of vitamin C [39]. Because of this associations, vitamin C has been used to increase the absorption of iron from iron-fortified milk [40]. However, 500 mg a day of vitamin C in the diet did not improve the response of anaemic pregnant woman to iron, folic acid or vitamin $B_{12}$ in a study in India [41] and it seems unlikely that the anaemia seen in scurvy in many individuals is due mainly to iron deficiency [19]. Therefore, it seems that whilst vitamin C may be able to influence iron uptake from the gut, and affect its tissue distribution, the role of vitamin C in iron metabolism and particularly in promoting haemoglobin synthesis in iron-deficiency anaemia, remains unresolved.

### 2.2.4 Immunological/Antimicrobial Effects Of Vitamin C

It has been suggested [42] that vitamin C may be involved in resistance to certain human viral infections, although this may be somewhat controversial [43]. However, the role of the various parameters of both the innate and acquired immune systems has not been clearly elucidated.

Neutrophils contain ascorbic acid at levels that are 40 - 100 fold higher than the levels found in other tissues, and it has been suggested that vitamin C plays a role in the function of these phagocytes [44]. Ascorbic acid has been found to be capable of increasing migration and chemotactic activity of human leucocytes [45, 46]. Further more, the chemotactic activity of leucocytes from patients with Chediak-Higashi Syndrome (a disorder of the neutrophil function) is

improved by ascorbic acid [47]. Chretien and Garagusi [48] have reported that corticosteroid induced activity may be partially reversed by ascorbic acid administration. Miller [49] has suggested that ascorbate may act with $H_2O_2$ and lysozyme to kill those microbes which might normally be resistant to the effect of lysozyme. It has also been shown that ascorbic acid may decrease the activity of halide-$H_2O_2$-myelo-peroxidase system in leucocytes [50, 51].

Perhaps the most relevant assessment of phagocytes function is antimicrobial phagocytosis and killing. McCall et.al [50] have found that vitamin C has little or no effect on the ability of neutrophils to phagocytize Staphylococcus-aureus in vitro. On the other hand, Chatterjee et.al [7] have shown that neutrophils from scorbutic guinea pigs have a reduced capacity to ingest Bacillus-subtilis, and that vitamin C will partially reverse this effect.

## 2.3 THERAPEUTIC ASPECTS OF VITAMIN C

### 2.3.1 Infectious Diseases

One of the most investigated infections has been the common cold. There are indications of a change in the metabolism of vitamin C in common cold, with rapid loss of vitamin C from the leucocyte during the early stages of infection [52] and a decrease in plasma and urine vitamin C during experimentally induced infections [53, 54]. The need for very high intake to maintain the concentration of vitamin C in leucocytes during infection [52] provides scientific support for Pauling's [55] suggestions that vitamin C may be beneficial during the cold infection.

There are clear indications that the severity of symptoms are decreased by vitamin C treatment [56,57]. Not only are symptoms less severe, but there is the suggestion that complications such as pneumonia are less likely to develop in those on high - dose therapy [58]. In the lungs, ascorbic acid influences airway hyperactivity [59]. Increases in vitamin C concentrations have been associated with increased pulmonary function [60], which may suggests that ascorbate may attenuate deteriorations of lung function related to environmental stress or age.

Apart from common cold, other diseases associated with persistent infection, and which appear to be primarily a deficiency of the immune response mechanisms, have responded well to doses of vitamin C of the order of 1 g [61], although the response may not be the same in all individuals and may show some age dependence [62].

### 2.3.2 Cardiovascular Disease

The evidence that vitamin C supplementation is capable of having some effect in the hypercholesterolaemic subject is now gaining acceptance [63]. vitamin C may even be more effective than some drugs currently used to treat hypercholeslerolaemia [64]. It has also been suggested that vitamin C may be able to mobilise cholesterol from the arterial walls [65].

Not only is vitamin C implicated as a hypocholesterolaemic agent, but over the past few years evidence has accumulated suggesting that vitamin C has an effect on the metabolism of triglycerides. Thus, in hyperlipidaemic patients, vitamin C Supplements of 2-3 g daily for at least a year appear to be effective in reducing serum triglycerides (66). vitamin C administration (1 g/day) for six months to hyperlipidaemic subjects at the time of seasonal vitamin C deficiency brings about a reduction in the serum triglyceride of as much as 50 percent (67). Furthermore, experimental studies have shown that vitamin C deficiency results in hypertriglyceridemia, and this may be caused by a decrease in tissue lipolytic activity (68).

Hyperlipidaemia is not the only factor associated with arterial disease. Degenerative changes in the vessel wall and enhanced clotting tendencies have also been implicated. It is possible that an anti-atherosclerotic activity of vitamin C may also be mediated through these mechanisms as well as by a reduction in blood lipid levels. The vitamin may also be effective in reducing the incidence of thrombotic episodes. Spittle (65) found that the incidence of thrombi in vitamin C-supplemented group of patients with leg thrombosis, was only half that in the placebo group. Furthermore, the severity of thrombi in the supplemented group

was much less than in the control patients. Some of these effects may be mediated by increased fibrinolytic activity (69), although it has been suggested that these effects are not long lasting (70).

### 2.3.3 Malignant Disease

The antitumour activity of vitamin C and its oxidation products has been reported by a number of workers. Ascorbic acid, dehydroascorbic acid and 2,3-diketogulonic acid cause inhibition of growth of the transplanted solid sarcoma-180 mice, the highest activity being shown by dehydroascorbic acid (9). The inhibitory effect of vitamin C seems to be further enhanced by the addition of copper ions, as copper sulphate, suggesting the active agent might be an oxidation product of ascorbic acid. The synergistic effect of copper and vitamin C in inhibiting tumour growth is further evident in an in vitro study [71] involving malignant melanoma cells which are known to be rich in copper [72]. In this study, vitamin C was found to inhibit the growth of the melanoma cells 20-500 times more than normal cells, suggesting selective toxicity.

Furthermore, a synergistic killing of Ehrlich ascites carcinoma cells in vitro by vitamin C and 3-amino-1,2,4-triazole has been observed [72]. The experimental results indicated that vitamin C is lethal to Ehrlich ascites carcinoma cells and harmeless to normal cells.

## 2.4 Free Radicals and Vitamin C

Reactive oxygen species (ROS) have been implicated as important pathologic mediators in many clinical disorders. Oxygen free radicals damage

membranes, proteins and nucleic acids. The mechanism for radical production remains speculative because of the difficulty in measuring radical species in vivo. The formation of oxidants is balanced by a similar rate of their consumption by antioxidants that are enzymatic and/or non-enzymatic for maintaining equilibrium. An amount of oxygen free radicals production or impairment of defense mechanisms, leads to oxidative damage.

### 2.4.1 Vitamin C as a free radical Scavenger

The probable sequence of events leading from the pathological over-production of active oxygen products ($0_2^{\cdot}$ and $H_20_2$) to cell injury and death is summarized (figure 2.5). The first line of defense against these agents are the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase ($GSHP_x$) and catalase. If these enzymes are overwhelmed or otherwise compromised, iron is liberated from tissue stores of ferritin [74]. This iron can be redox-cycled by superoxide radical and can initiate lipid peroxidation.

However, vitamin C has been shown to be the first plasma antioxidant to be depleted in vitro [6]. It disappears faster than other antioxidants when plasma is exposed to ROS[75]. Lose of ascobate probably occurs by the following reactions:

$$2O_2^{\cdot} + ascorbic\ acid + 2H^{+} \rightarrow Semidehydroascorbate + 2H_2O_2$$

$$Semidehydroascorbate \xrightarrow{Disproportionation} \begin{matrix} Ascorbate \\ + \\ dehydroascorbate \end{matrix}$$

$$Dehydroascorbate \xrightarrow[break\text{-}down]{Non\ enzymatic} \left\{ \begin{matrix} oxalate \\ threonate \\ otherbreakdownproducts \end{matrix} \right\}$$

### 2.4.2 Relationship Between Vitamin C And Vitamin E

Once lipid peroxidation is in progress, its chain reaction spreads rapidly through cell membranes. The antioxidant vitamin E is a lipid soluble vitamin that inserts itself into the membrane to serve as a break. When a propagating lipid radical hits a vitamin E molecule, a very stable E radical is formed that does not participate in continuing the chain reaction. It goes up from the membrane to the aqueous interface, where it is reduced back to functional vitamin E by reacting with water - soluble vitamin C as indicated in Fig 2.5 [76]. The equation below gives the possible reaction between the two antioxidant vitamins.

$$Tocopherol\text{-}\dot{O} + ascorbate \rightarrow Semidehydroascorbate + tocopherol\text{-}Ol$$

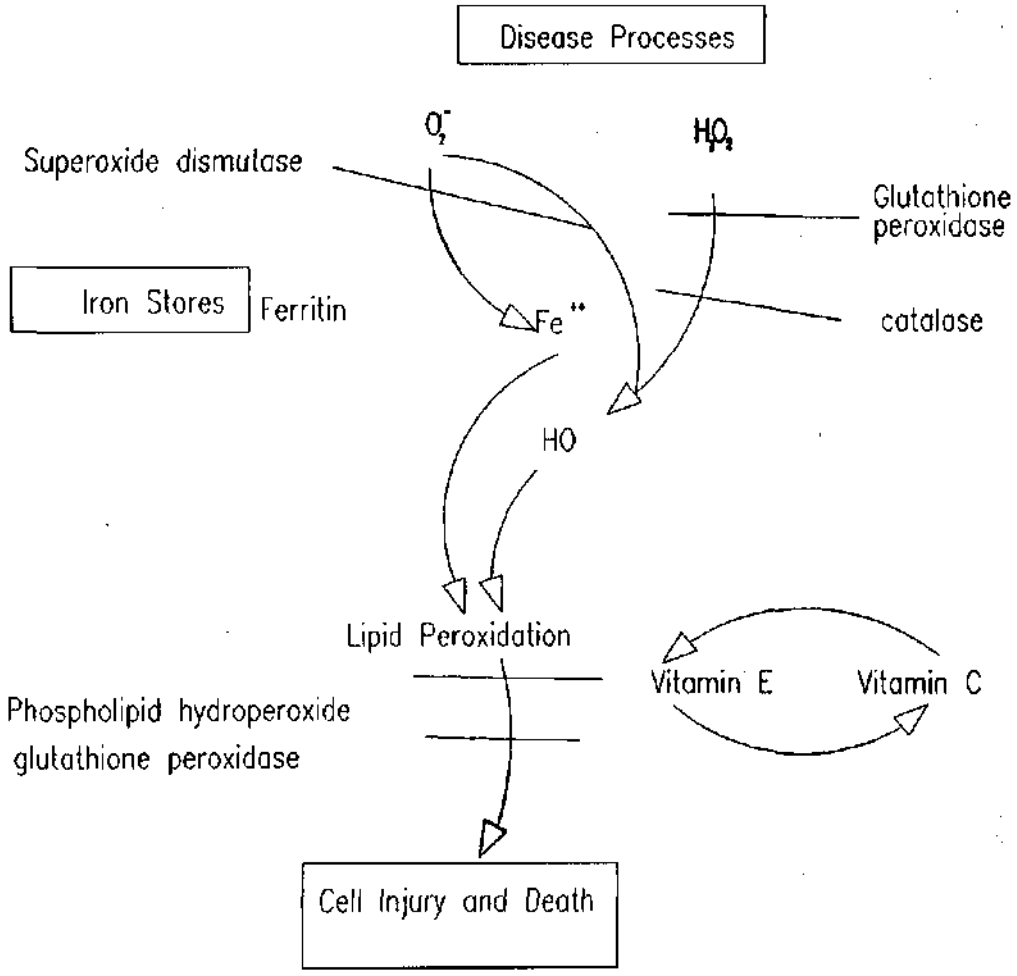



Fig. 2.5: How oxidant production leads to cell injury and death. (Adapted from Ref. 74)

## 2.5 What is Sickle Cell Disease/Anaemia?

Sickle cell disease is the condition resulting from the inheritance of two abnormal allelemorphic genes controlling the formation of the $\beta$-chains of

haemoglobin, at least one of which is the sickle gene; sickle cell disease, therefore, includes Hb-SS, Hb-SC, Hb-s/$\beta$ thalassaemia and other doubly heterozygous conditions. Thus, the condition resulting from the inheritance of two sickle genes (Hb-SS) is called sickle cell anaemia [83].

### 2.5.1 Biochemical genetics of Haemoglobin

The haemoglobin molecule is a tetramer composed of two dissimilar chains. Symbolically, haemoglobin A is designated as $\propto^{A}{}_{2}\beta^{A}{}_{2}$ while haemoglobin S is $\propto^{A}{}_{2}\beta^{S}{}_{2}$. Each chain is composed of approximately 140 residues of the twenty different amino acids found in animal proteins. These amino acid residues are arranged in a specific sequence. The abnormal electrophoretic mobility of Hb-S is due to the fact that the Hb-S molecule is more electropositive than is Hb-A, thus migrating more slowly toward the positive pole of the electric field. This charge abnormality connotes an abnormal amino acid composition of Hb-S. Hb-s differs from Hb-A by a single amino acid substitution in the beta polypeptide chains. At the sixth position of the amino terminal end, the glutamic acid residue normally present is substituted by the amino acid valine. This substitution not only alters the ionic charge of the entire molecule, but it also allows gelling of deoxygenated Hb-S (84).

### 2.5.2 Clinical Manifestation Of Sickle Cell Disease

Individuals homozygous for haemoglobin-S (sickle cell anaemia) have chronic, severe haemolytic anaemia. This disease begins in the first year of life after $\beta$-chains synthesis supersedes $\gamma$-chains synthesis. Patients tend to be thin and

gangling and appear chronically ill and younger than their true age. Splenomegally is frequent during childhood due to repeated infarctions but it is usually not present in the older patients. Severe jaundice betokens liver disease, usually the result of chronic anoxia, Salmonella osteomyelitis is an occasional complication. Impaired concentration of urine is probably the result of chronic anoxia (85).

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 Chemicals/Reagents

Grades and Sources of chemicals used.

1. Trichloroacetic acid, M & B Ltd, Daganham, England ( Laboratory reagent).
2. 2, 4 - Dinitrophenylhydrazine (Laboratory reagent) BDH chemicals Ltd, poole, England.
3. Acid - washed charcoal (Norit) (General purpose reagent) BDH chemical Ltd, poole, England.
4. Oxalic Acid (AnalaR) BDH chemicals Ltd, Poole, England.
5. L-Ascorbic Acid (AnalaR) BDH chemicals Ltd, Poole, England.
6. Thiourea (Laboratory reagent) Hopkin and Williams, Essex, England.
7. Sulphuric Acid(AnalaR) BDH Chemicals Ltd, Poole, England.

### 3.2 Equipment

1. Spectrophotometer, Model WPA 5101.
2. MSE bench centrifuge.
3. Microhaematocrit Centrifuge, type 346.
4. Hawksley microhaematocrit reader.
5. Gallenkamp water bath (B & T Model).

## 3.3 Subjects

Sixty-seven patients (aged 18 - 47 yrs, mean age = 21.9 yr; thirty males and thirty seven females) homozygous for haemoglobin S, who were attending Sickle Cell Clinic at the Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, were studied when they had no major clinical complications. Forty four healthy individuals (aged 18 - 43 yrs, mean age = 24.1 yr; twenty two males and twenty two females) homozygous for haemoglobin A served as control subjects. The healthy volunteers consisted of undergraduate medical students at the ABUTH and undergraduate students from both Samaru and Kongo Campuses of Ahmadu Bello University, Zaria.

All the subjects gave informed consent to participate in the study. None was taking any supplementation that contained vitamin C. This study was carried out in accordance with the guidelines on ethics of research involving human subjects.

## 3.4 Sample Collection

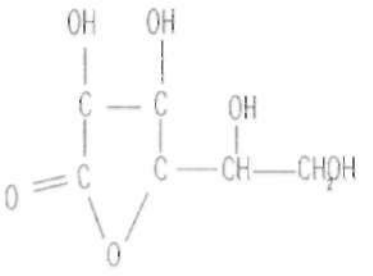
A venous blood sample (approximately 10 ml) was obtained from each volunteer at random. Three millilitres of the blood was collected into an evacuated EDTA tube for packed cell volume, haemoglobin and haemoglobin electrophoretic analyses; and the remaining 7 ml was collected into a clean plain tube to obtain the serum. The cells were separated from the serum by centrifugation within 1.5 hr after collection.

## 3.5 Determination of Ascorbic Acid

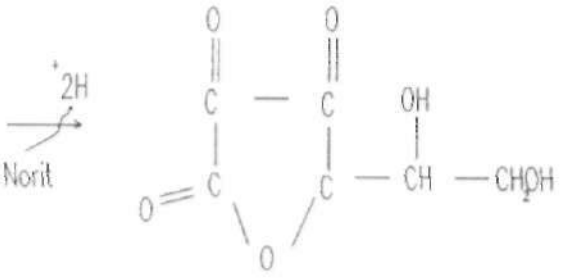
The method of Roe (77) was used to determine the serum vitamin C concentrations.

### 3.5.1 Principle

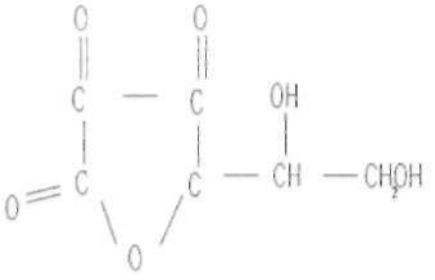
Charcoal was added to a protein-free serum filterate and the ascorbic acid in the filterate was oxidised to dehydroascorbic acid. Dehydroascorbic acid was coupled with 2,4 - Dinitrophenylhdrazine (DNPH) to form the 2,4 - dinitrophenylosazone. Treatment of the osazone with concentrated sulphuric acid yielded a reddish complex which was measured at 515 nm.



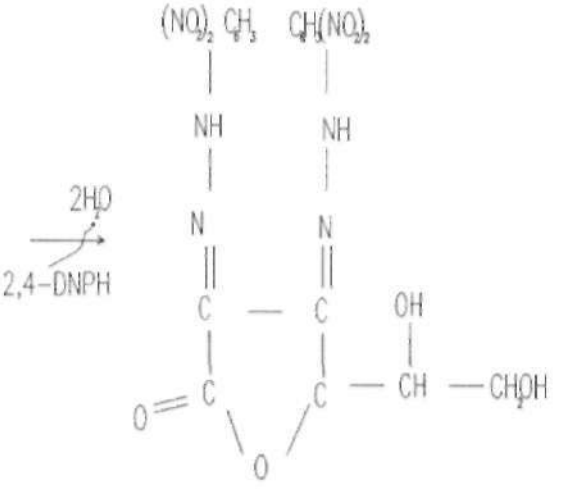
Ascorbic acid




Dehydroascorbic acid



Dehydroascorbic acid




Osazone

### 3.5.2 Preparation Of Reagents

1. Trichloroacetic Acid (7.5%): Seventy five grammes (75 g) of the acid was dissolved in water and diluted to 1 litre.

2. Trichloroacetic Acid (4%): Three hundred and fifty millilitres (350 ml) of water was added to 400 ml of the 7.5% solution and mixed properly.

3. Sulphuric Acid, 4.5 M: Twenty five millilitres (25 ml) of concentrated sulphuric acid was carefully added to 75 ml of water with mixing and cooling.

4. 2, 4 - Dinitrophenylhydrazine (DNPH): Two grammes (2 g) of 2, 4 - dinitrophenylhydrazine was dissolved in 100 ml of 4.5 M $H_2SO_4$. Four grammes (4 g) of thiourea was added and, when it dissolved completely, it was filtered through Whatman 42 filter paper.

5. Sulphuric Acid, 85%: To 100 ml of water 900 ml of concentrated sulphuric acid was added with mixing and cooling.

6. Oxalic Acid, 0.5%: 0.5 g of oxalic acid was dissolved in 100 ml of water.

7. Stock Ascorbic Acid Standard, 25 mg/100 ml: Twenty-five milligrammes (25 mg) of ascorbic acid was dissolved in 100 ml of 0.5% oxalic acid.

8. Working Ascorbic Acid Standard, 0.5 mg/100 ml:

   One millilitre (1 ml) of the stock standard was diluted to 50 ml with 4% trichloroacetic acid. This was prepared fresh for each run.

### 3.5.3 Procedure

Seven millilitres (7 ml) of 7.5% TCA was placed in a glass tube and 3 ml of serum was added dropwise. It was mixed vigorously, allowed to stand for 5 minutes and then centrifuged. The supernatant was transferred into a 60 ml plastic centrifuge tube containing 0.25 g of norit. Similarly, 10 ml of 4% TCA (blank) and 10 ml working standard were dispensed into plastic centrifuge tubes containing

0.25 g Norit. All tubes were shaken for 5 minutes, centrifuged, and then filtered through Whatman 42 filter paper. Two millilitres (2 ml) aliquots of each filterate were placed into two glass tubes. The tubes were marked as blank control ($A_{bb}$), blank Test ($A_b$), standard Control ($A_{sb}$), standard test ($A_s$), sample control ($A_{xb}$) and sample test ($A_x$).

One half of a ml (0.5 ml) of 2, 4 - DNPH reagent was added to Blank test, standard test, and sample test tubes, mixed thoroughly and were transferred into a water bath at 37°C and incubated for 4.5 hours. After incubation, all tubes were transferred to an ice bath and allowed to stand for 5 minutes. While still in ice bath, 2.5 ml of 85% $H_2SO_4$ was added to all tubes and mixed. To all tubes marked control, 0.5 ml of 2, 4 - DNPH reagent was added and mixed while still in ice bath. After 40 minutes all the tubes were removed from ice bath. The absorbance of the contents of each tube was read at 515 nm against a water blank.

The concentration of ascorbic acid in mg/100 ml serum was obtained from calculations using the formula (77):

$$Mg\ ascorbic\ acid/100\ ml\ Serum = \frac{(A_x - A_{xb}) - (A_b - A_{bb})}{A_s - A_{sb}) - (A_b - A_{bb})}\ 1.66$$

## 3.6 Determination Of Packed Cell Volume (PCV)

### 3.6.1 Principle

This is based on the volume occupied by blood cells which are thrown out of the plasma by centrifugal force. The volume of the packed cells, which is read on the microhaematocrit reader is usually expressed in percentage.

### 3.6.2 Procedure

A capillary tube was dipped slightly into a blood sample containing EDTA as anti-coagulant. The blood was drawn up the tube by capillary action. One end of the tube was then sealed using flame, after which it was placed in an hematocrit centrifuge and spun for about 5 minutes. The capillary tube was removed from the haematocrit centrifuge and transferred into the microhaematocrit reader, where the packed cell volume was read off.

## 3.7 Determination Of Haemoglobin Genotype

### 3.7.1 Principle

At alkaline pH (8.4 - 8.6) haemoglobin is a negatively charged protein and in an electric field will migrate toward the anode (+). Most structural variants of haemoglobin will separate due to surface charge differences, thus allowing identification of abnormal forms.

### 3.7.2 Procedure

Cellulose acetate strip was soaked in the tris buffer and gently blotted. The strip was placed in an electrophoretic tank and secured at each end by a double layer of Whatman Nº3 filter paper. Two microlitre of a 8-10 g/dl Hb solution was applied in a 2cm line midway between the centre of the strip and its cathodal end. Electrophoresis was carried out for 20mins at 5mA at a potential of 250-350v across the strip. At the end of the separation, the membrane was removed with forceps and stained with Ponceau S for 3-5min. The stained strip was dried in a hot air oven at 65°C for 4-6min (86).

## 3.8 Determination of Haemoglobin Concentration

### 3.8.1 Principle

Haemoglobin reacts with potassium ferricyanide to form a coloured complex containing cyanmet-haemoglobin which has maximum absorbance at 540 nm.

### 3.8.2 Procedure

Twenty microlitre of blood was added to 4ml of diluent (Potassium Ferricyanide 200mg, potassium cyanide 50mg, potassium dihydrogen phosphate 140mg in 1000ml of water; PH 7.0 -7.4). The tube containing the solution was stoppered with a rubber bung and inverted several times. The mixture was allowed to stand at room temperature for period of 8mins to ensure complete conversion of haemoglobin to cyanmethaemoglobin. The solution of cyanmethaemoglobin was compared with the standard cyanmethaemoglobin and a reagent blank in a photoelectric colorimeter at 540nm(86). The haemoglobin concentration in (g/dl) was obtained from the formula(86)

$$\text{Hb (g/dl)} = \frac{A_{540} \text{ of test sample} \times \text{Conc of std(mg/dl) x dilution factor}}{A_{540} \text{ of Std} \qquad 1000}$$

## 3.9 Estimation of Mean Corpuscular Haemoglobin Concentration (MCHC)

This is the measure of the actual concentration of haemoglobin in the erythrocytes. It was obtained from calculation, using the formula.

$$MCHC\ (g/dl) = \frac{haemoglobin\ in\ g/dl}{packed\ cell\ volume\ (\%)} \times 100$$

The samples were assayed for haemoglobin genotypes, haemoglobin concentration and MCHC at the Haematology Laboratory at ABUTH, Zaria, with the assistance of the technical staff in the laboratory.

### 3.10 Statistical Analysis

Statistical analyses of the data were performed by student's t-test, linear regression, and Pearson correlation. Significance level ($\propto$) equals 0.05 unless otherwise indicated.

# CHAPTER FOUR

## 4.0 RESULTS

The mean serum vitamin C level in normal subjects were not significantly different from those in the SCA patients (Table 1). The reference range for normal subjects was 0.462 - 0.742 mg/dl (95% confidence interval). Among the SCA patients, 34.3% had serum vitamin C values above their mean serum vitamin C levels while 38.6% of the normal subjects had values above their mean serum vitamin C levels (Fig.4.1). Exactly 49.3% of SCA patients and 52.3% of normal subjects had serum vitamin C $\geq$ 0.462 mg/100 ml (Fig. 4.1). All SCA patients had significantly lower Hb levels than all normal subjects. The pcv was also significantly lower ($p < 0.05$) in SCA patients compared with normal subjects (Table 1). However, there was no significant difference ($P > 0.05$) in MCHC values between SCA patients and the normal subjects.

The male normal subjects had a significantly lower mean serum vitamin C level ($P < 0.05$) than did the female normal subjects (Table 2). Among the males 45.5% had values above the mean serum vitamin C for male (FIG.4.2). In addition, 51.4% of normal females and 45.5% of normal males had serum vitamin C $\geq$ 0.462 mg/100 ml.

The normal males had a significantly higher Hb levels than the normal female subjects. Similarly, the pcv level was significantly higher ($P < 0.05$) in the normal males than in the normal females. However there was no significant difference in the levels of MCHC between both normal males and females (Table 2).

**TABLE 1:** **Serum Vitamin C and Some Haematological Parameters in Normal Subjects and Individuals with Sickle Cell Anaemia**

| | Vitamin C (mg/100ml) | Hb (g/dl) | PCV (%) | MCHC (g/dl) |
|---|---|---|---|---|
| Normal (n = 44) | $0.602 \pm 0.07^{a}$ | $13.55 \pm 0.22^{a}$ | $40.52 \pm 0.65^{a}$ | $33.47 \pm 0.04^{a}$ |
| Patients (n = 67) | $0.603 \pm 0.05^{a}$ | $8.57 \pm 0.18^{b}$ | $25.52 \pm 0.53^{b}$ | $33.59 \pm 0.03^{a}$ |

Values represent Mean ± SEM.

Means within the same column that bear different superscripts are significantly different ($P < 0.05$) while those within the same column bearing identical superscripts are not significantly different ($P > 0.05$).

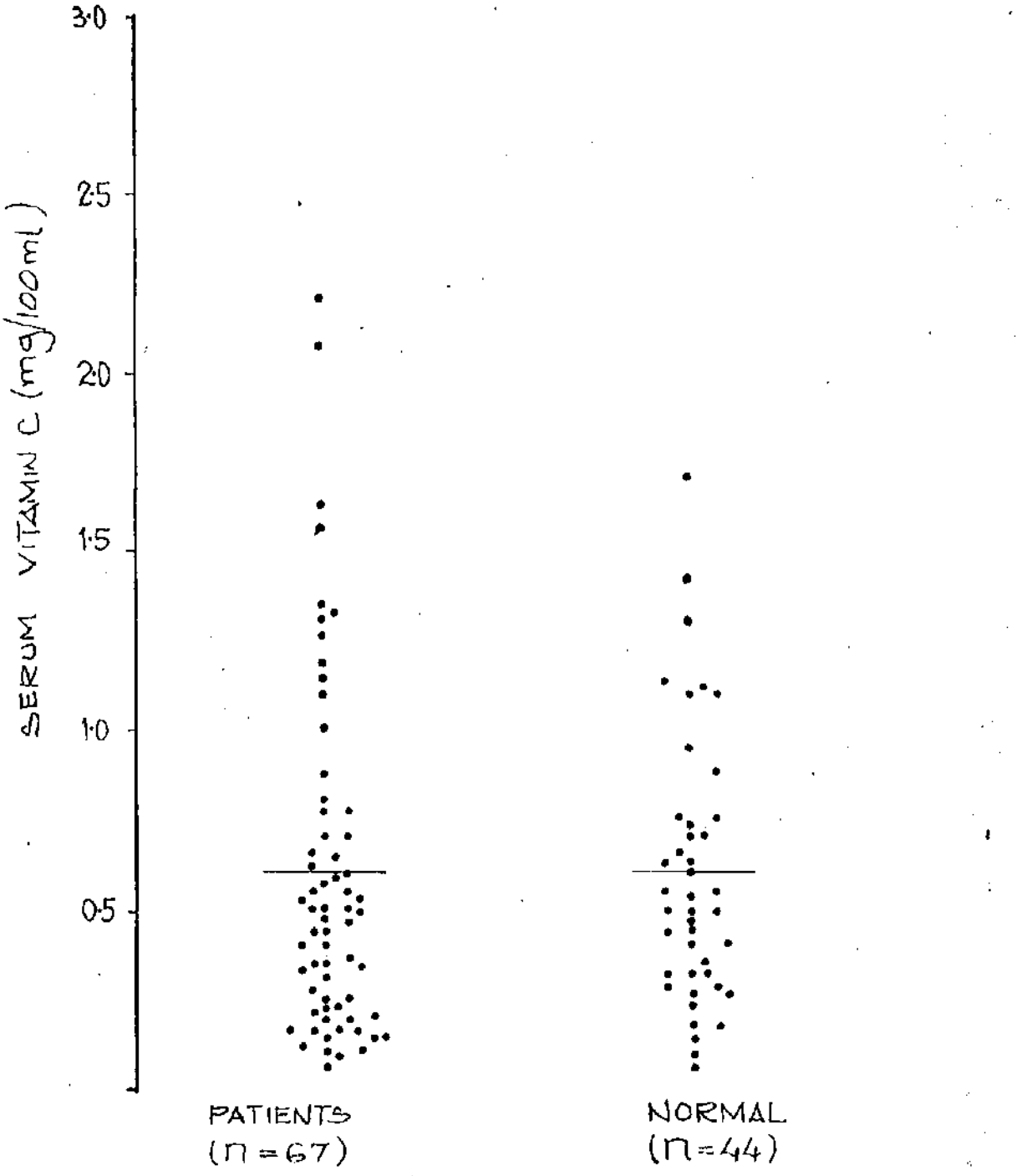



Fig 4·1 SERUM VITAMIN C (mg/100ml) IN NORMAL SUBJECTS AND SICKLE CELL ANAEMIA SUBJECTS.

**TABLE 2:** **Serum Vitamin C and Some Haematological Parameters in Male and Female Normal Subjects**

| | Vitamin C (mg/100ml) | Hb (g/dl) | PCV (%) | MCHC (g/dl) |
|---|---|---|---|---|
| Males (n = 22) | $0.479 \pm 0.06^{a}$ | $14.38 \pm 0.29^{a}$ | $43.09 \pm 0.86^{a}$ | $33.42 \pm 0.06^{a}$ |
| Females (n = 22) | $0.706 \pm 0.07^{b}$ | $12.72 \pm 0.26^{b}$ | $37.95 \pm 0.72^{b}$ | $33.50 \pm 0.05^{a}$ |

Values represent mean ± SEM.

Means within the same column that bear different superscripts are significantly different ($P < 0.05$) while those within the same column bearing identical superscripts are not significantly different ($P > 0.05$).

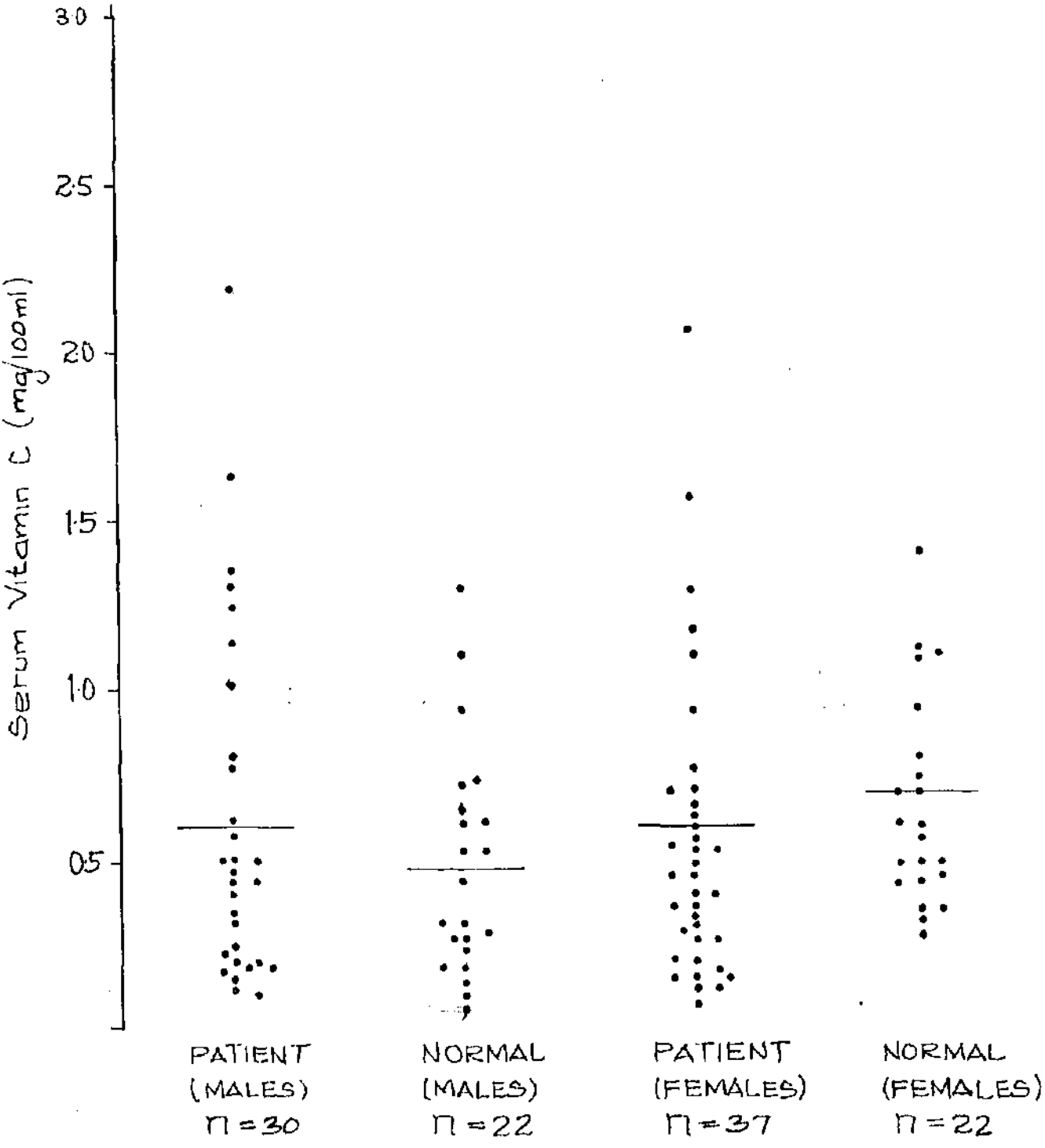



Fig. 4.2 SERUM VITAMIN C (mg/100ml) IN MALES AND FEMALES WITH AND WITHOUT SICKLE CELL ANAEMIA.

In the patients with SCA (Table 3), the mean serum vitamin C levels in males were not significantly different ($P>0.05$) from that in females. Thirty three percent of the male SCA patients had values above the mean serum vitamin C for male SCA patients while 37.8% of the female SCA patients had values above the mean serum vitamin C for the female SCA patients (Fig.4.2). Furthermore, 68.2% of female SCA patients and 46.7% of male SCA patients had serum vitamin C $\geq$ 0.462 mg/100 ml.

There was no significant difference in Hb and pcv levels between male SCA and female SCA patients. A similar observation was seen in the levels of MCHC between both male and female SCA patients.

Mean serum vitamin C levels were not significantly different ($P>0.05$) between normal males and males with SCA (Table 4). Reference range for normal men was 0.359 - 0.599 mg/dl. Thirty three percent of males with SCA had values above the mean serum vitamin C for male patients, while 45.5% of normal males had values above the mean for healthy males. In addition, 46.7% of the males with SCA had serum vitamin C $\geq$ 0.462 mg/100 ml and 45.5% of the normal males had serum vitamin C $\geq$ 0.462 mg/100 ml (Fig.4.2).

The mean Hb and pcv levels were significantly different ($P<0.05$) between men with and without SCA (Table 4). The mean MCHC levels however, was not significantly different ($P > 0.05$) between the two groups.

**TABLE 3:** **Serum Vitamin C and Some Haematological Parameters in Male and Female Patients with Sickle Cell Anaemia (SCA)**

| | Vitamin C (mg/100ml) | Hb (g/dl) | PCV (%) | MCHC (g/dl) |
|---|---|---|---|---|
| Males (n = 30) | $0.608 \pm 0.09^{a}$ | $8.89 \pm 0.26^{a}$ | $26.46 \pm 0.78a$ | $33.62 \pm 0.04^{a}$ |
| Females (n = 37) | $0.599 \pm 0.08^{a}$ | $8.31 \pm 0.22^{a}$ | $24.76 \pm 0.71^{a}$ | $33.58 \pm 0.05^{a}$ |

Values represent mean ± SEM.

Means within the same column bearing identical superscripts are not significantly different ($P > 0.05$).

**TABLE 4: Serum Vitamin C and Some Haematological Parameters in Normal Men and Men With Sickle Cell Anaemia (SCA)**

| | Vitamin C (mg/100ml) | Hb (g/dl) | PCV (%) | MCHC (g/dl) |
|---|---|---|---|---|
| Healthy men (n = 22) | $0.479 \pm 0.06^{a}$ | $14.38 \pm 0.29^{a}$ | $43.09 \pm 0.86^{a}$ | $33.42 \pm 0.06^{a}$ |
| Men with SCA (n = 30) | $0.608 \pm 0.09^{a}$ | $8.89 \pm 0.26^{b}$ | $26.46 \pm 0.78^{b}$ | $33.62 \pm 0.04^{a}$ |

Values represent mean ± SEM.

Means within the same column that bear different superscripts are significantly different ($P<0.05$) while those within the same column bearing identical superscripts are not significantly different ($P>0.05$).

As seen in the table 5, there was no significant difference ($P > 0.05$) in the mean serum vitamin C between females with SCA and normal females; although the latter had a slightly higher mean serum vitamin C level of $0.706 \pm 0.07$ mg/dl (0.566 - 0.846 mg/dl), than the former. Sixty eight percent and 51.4% of normal females and females with SCA respectively had serum vitamin C $\geq$ 0.462 mg/100 ml. Thirty Seven percent of females with SCA compared with forty five percent of normal females had values above their respective means of serum vitamin C (Fig.4.2).

The females with SCA had significantly lower mean Hb and pcv levels than did the normal females without SCA. The mean MCHC was not significantly different in ($P > 0.05$) females with and without SCA.

The mean serum vitamin C levels did not differ significantly with age between normal subjects and SCA patients for the two age groups considered (Table 6).
A significant difference in serum vitamin C levels occurred between the age group 18 - 22 and the age group 23 - 27 for SCA patients. However, no significant difference was observed between these two age groups for normal subjects.

Mean Hb and pcv levels differ significantly by age in both normal subjects and SCA patients. In the younger age group (18 - 22), the SCA patients had a significantly lower Hb level than did the normal subjects. Similarly, the pcv levels of the SCA patients was significantly lower ($P < 0.05$) than that of the normal subjects for this age group (18 - 22). In the older age group (23 - 27), a similar trend as seen in the younger age group was observed for both Hb and pcv levels. The mean MCHC levels also did not differ significantly ($P > 0.05$) by age between SCA patients and normal subjects for the two age groups considered (Table 6). Between the age groups there was no significant difference in Hb, pcv and MCHC levels for either normal subjects or SCA patients.

**TABLE 5: Serum Vitamin C and Some Haematological Parameters in Normal Women and Women with Sickle Cell Anaemia (SCA)**

| | Vitamin C (mg/100ml) | Hb (g/dl) | PCV (%) | MCHC (g/dl) |
|---|---|---|---|---|
| Healthy women (n = 22) | $0.706 \pm 0.07^{a}$ | $12.72 \pm 0.26^{a}$ | $37.95 \pm 0.72^{a}$ | $33.50 \pm 0.05^{a}$ |
| Women with Hb SS (n = 37) | $0.599 \pm 0.08^{a}$ | $8.31 \pm 0.22^{b}$ | $24.76 \pm 0.71^{b}$ | $33.58 \pm 0.05^{a}$ |

Values represent mean ± SEM.

Means within the same column that bear different superscripts are significantly different ($P < 0.05$) while those within the same column bearing identical superscripts are not significantly different ($P > 0.05$).

TABLE 6: Relationship Between Age, Some Haematological Parameters And Vitamin C Level Of Normal Subject And Sickle Cell Anaemia (SCA) Subjects.

| Age Group | Vitamin C (mg/100ml) | Hb (g/dl) | PCV (%) | MCHC (g/dl) |
|---|---|---|---|---|
| 18 - 22 | 0.672±0.07(AA)$^a$(20) | 12.99±0.31(AA)$^a$(20) | 38.95±0.93(AA)$^a$(20) | 33.46±0.06(AA)$^a$(20) |
| | 0.664±0.07(SS)$^a$(45) | 8.40±0.22(SS)$^b$(45) | 24.97±0.65(SS)$^b$(45) | 33.62±0.04(SS)$^a$(45) |
| 23 - 27 | 0.507±0.08(AA)$^a$(19) | 13.80±0.31(AA)$^a$(19) | 41.26±0.95(AA)$^a$(19) | 33.46±0.06(AA)$^a$(19) |
| | 0.342±0.14(SS)$^b$(13) | 8.90±0.41(SS)$^b$(13) | 26.53±1.22(SS)$^b$(13) | 33.65±0.07(SS)$^a$(13) |

AA - Homozygous haemoglobin - A (Normal)
SS - Homozygous haemoglobin - S (Sickle)

Values represents Mean ± SEM for the sample size indicated in the bracket. Mean values within the same column bearing different superscripts are significantly different ($P < 0.05$) while those in the same column bearing identical superscripts are not significantly different ($P > 0.05$).

Correlations between serum vitamin C and haemoglobin levels were examined in all SCA patients and normal subjects (Figs.4.3 and 4.4). In all SCA patients, serum vitamin C correlated significantly but negatively with haemoglobin ($r = -0.285$, $P < 0.019$, Fig. 4.3). In the normal subjects this relationship was significant and negative as well ($r = -0.387$, $P < 0.008$, Fig.4.4). A similar trend was observed in the correlation between serum vitamin C and pcv in both SCA patients and normal subjects. In the SCA patients, serum vitamin C correlated negatively and significantly with pcv ($P < 0.019$); and in the normal subjects ($P < 0.008$). Correlation between serum vitamin C and MCHC in the normal subjects was positive and not significant ($P = 0.89$); in the SCA patients the relationship between serum vitamin C and MCHC was positive and not significant as well ($P = 0.90$).

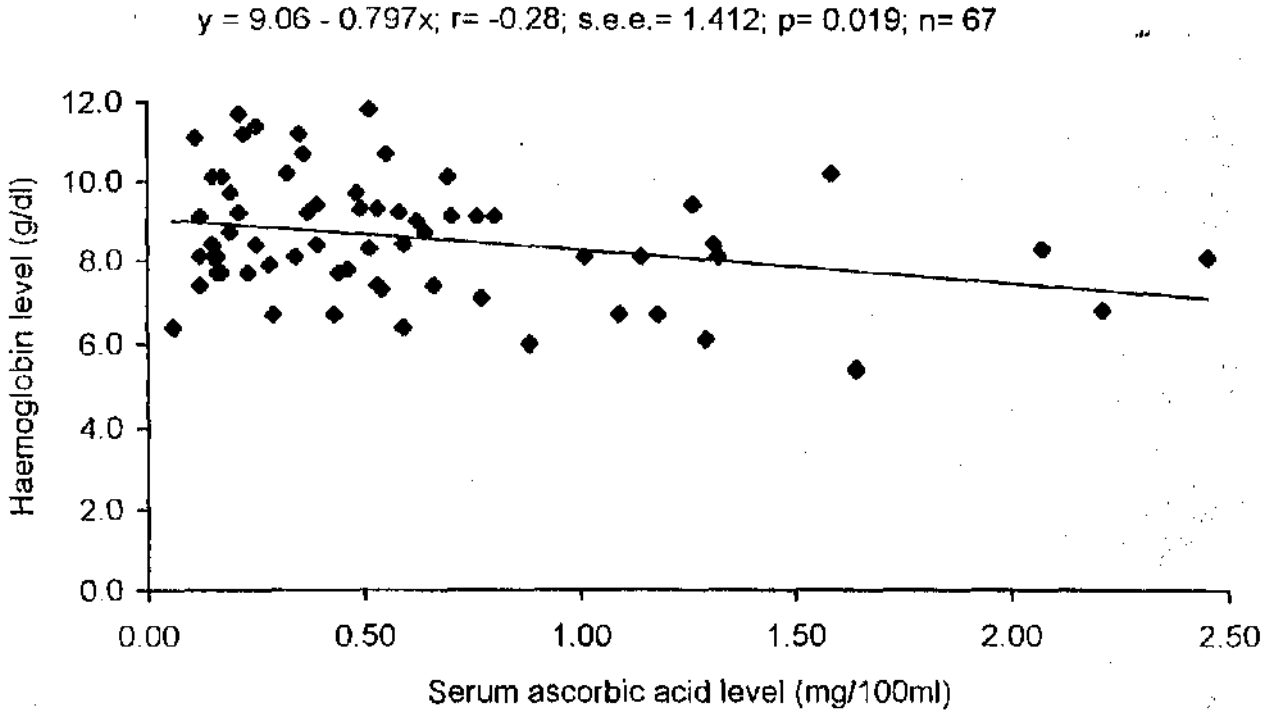



**Figure 4.3.** Relationship between haemoglobin concentration and serum ascorbic acid in patients with sickle cell anaemia

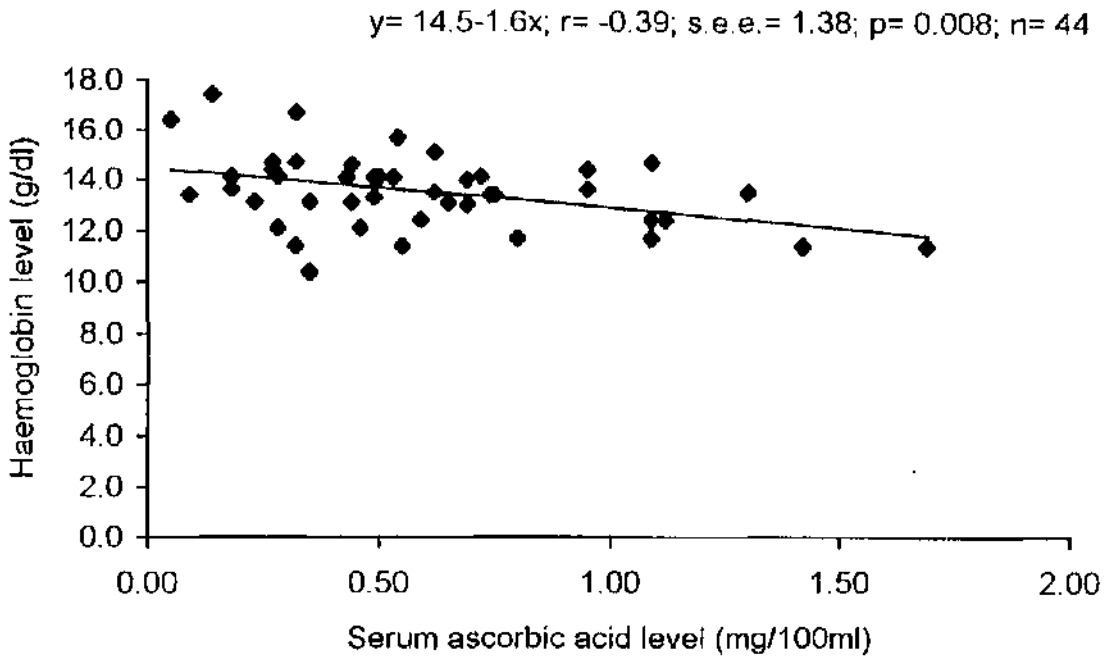



**Figure 4.4.** Relationship between haemoglobin concentration and serum ascorbic acid in normal subjects.

Table 7 shows the correlation between serum vitamin C and some haematological parameters for male normal subjects and male SCA patients. Correlation between the haematological parameters in turn can be seen in this table as well. A strong negative and significant correlation was observed between serum vitamin C and haemoglobin (Hb) in male SCA patients ($P<0.004$); this relationship was however, not significant in the male normal subjects ($P = 0.33$). In the male SCA patients, correlation between serum vitamin C and pcv was negative and significant ($P<0.004$) and in the normal males the association was not significant ($P = 0.38$). MCHC and vitamin C correlated negatively and significantly ($P < 0.037$) in male normal subjects, and this relationship was not significant in the male SCA patients (Table 7). No significant correlation was observed between MCHC and Hb in both normal male and male SCA patients. A similar trend was observed in the relationship between MCHC and pcv. However, there was a strong positive relationship between pcv and Hb in both male SCA patients and normal male subjects ($P<0.0001$; $P<0.0001$) respectively.

**TABLE 7: Correlation Beteen Vitamin C And Some Haematological Parameters In Normal Males And Males With Sickle Cell Anaemia(SCA)**

| | Vitamin C (mg/100ml) | Hb (g/dl) | PCV (%) | MCHC (g/dl) |
|---|---|---|---|---|
| Vitamin C (mg/100ml) | 1.000(AA)<br>1.000(SS) | | | |
| Hb (g/dl) | -0.217(AA)<br>-0.511**(SS) | 1.000(AA)<br>1.000(SS) | | |
| PCV (%) | -0.196(AA)<br>-0.512**(SS) | 0.996**(AA)<br>0.998**(SS) | 1.000(AA)<br>1.000(SS) | |
| MCHC (g/dl) | -0.445**(AA)<br>0.024(SS) | 0.049(AA)<br>0.100(SS) | 0.002(AA)<br>0.041(SS) | 1.000(AA)<br>1.000(SS) |

AA - homozygous haemoglobin - A (control)

SS - homozygous haemoglobin - S (patient)

** ($P < 0.05$)

Correlation coefficient of the various parameters studied in the female SCA patients and normal female subjects are shown in Table 8. Serum vitamin C and the Hb correlated negatively in both female SCA patients and normal female subjects, the association was not significant in either of the two categories of females. Correlation between serum vitamin C and pcv was not significant in female SCA patients and normal females ($P = 0.46$; $P = 0.12$) respectively. However, a positive correlation was seen between serum vitamin C and MCHC in female SCA patients ($P = 0.97$) and in normal females ($P = 0.58$). MCHC correlated positively with Hb and pcv in the two categories of females; the association was however not significant (Table 8). Like in the males, there was a strong positive and significant correlation between pcv and Hb in female SCA patients and normal female subjects ($P < 0.0001$; $P < 0.0001$) respectively.

**TABLE 8:** **Correlation Between vitamin C and Some Haematological Parameters in Females With and Without Sickle Cell Anaemia (SCA)**

| | Vitamin C (mg/100ml) | Hb (g/dl) | PCV (%) | MCHC (g/dl) |
|---|---|---|---|---|
| Vitamin C (mg/100ml) | 1.000(AA)<br>1.000(SS) | | | |
| Hb (g/dl) | -0.317(AA)<br>-0.122(SS) | 1.000(AA)<br>1.000(SS) | | |
| PCV (%) | -0.338(AA)<br>-0.123(SS) | 0.996**(AA)<br>0.998**(SS) | 1.000(AA)<br>1.000(SS) | |
| MCHC (g/dl) | 0.121(AA)<br>0.005(SS) | 0.415(AA)<br>0.037(SS) | 0.334(AA)<br>0.081(SS) | 1.000(AA)<br>1.000(SS) |

AA - homozygous haemoglobin - A (control)

SS - homozygous haemoglobin - S (patient)

** ($P < 0.05$)

## 4.1 Electrophoretic Patterns of Haemoglobin Variants

The electrophoretic patterns of haemoglobin for SCA patients and normal subjects are shown in Plate 1 and 2. The Hb-S of the SCA patients has a higher net electrical charge than Hb-A of the normal subjects thus Hb-S migrates at a slower rate than Hb-A giving the diagnostic SS pattern (Plate 1). The homozygous Hb-S like the homozygous Hb-A has a single band, on the countrary, the heterozygous Hb-AS has two bands. Another haemoglobin variant, Hb-F (Plate 1) has a single band like Hb-A and it is considered to be normal haemoglobin. The direction of migration is from left to right.

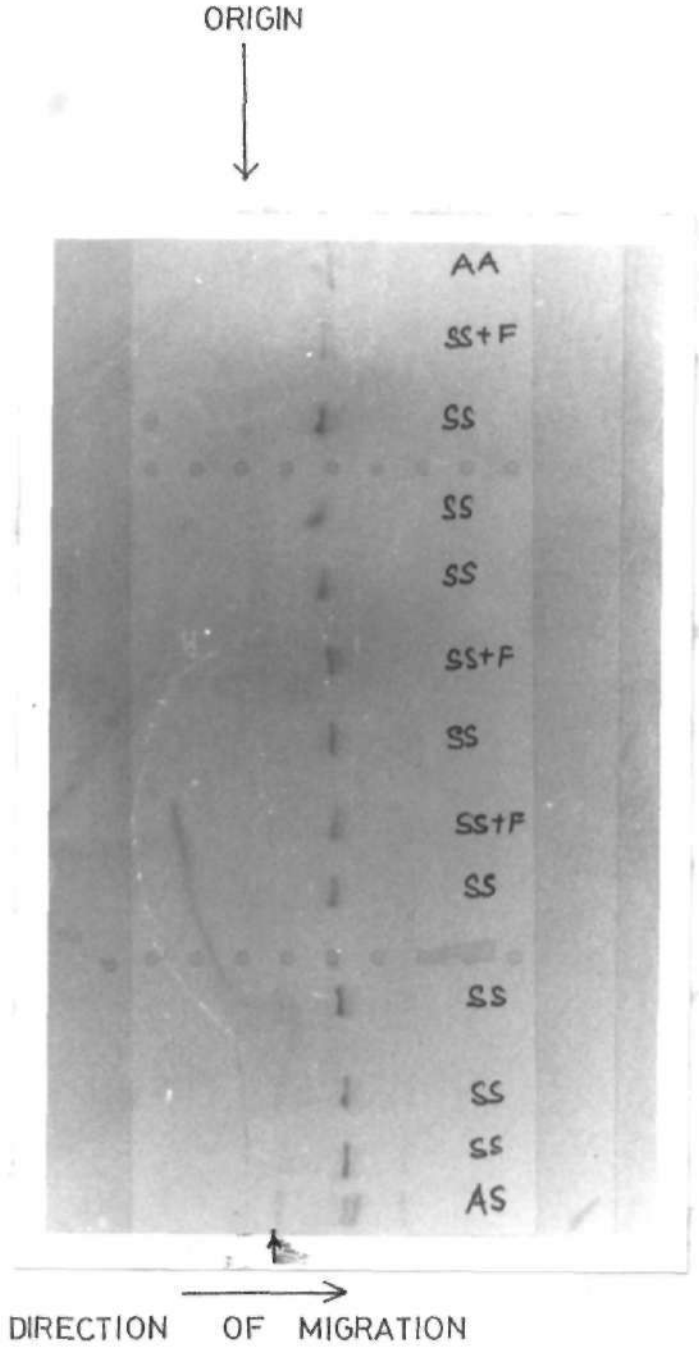



PLATE 1: A typical electrophoretogram of homozygous haemoglobin-S guided at the top by haemoglobin-A and at the bottom by heterozygous haemoglobin-AS as controls. The fetal haemoglobin is also shown. Hb-S is more electropositive than Hb-A thus migrating more slowly towards the positive pole of the electric field.

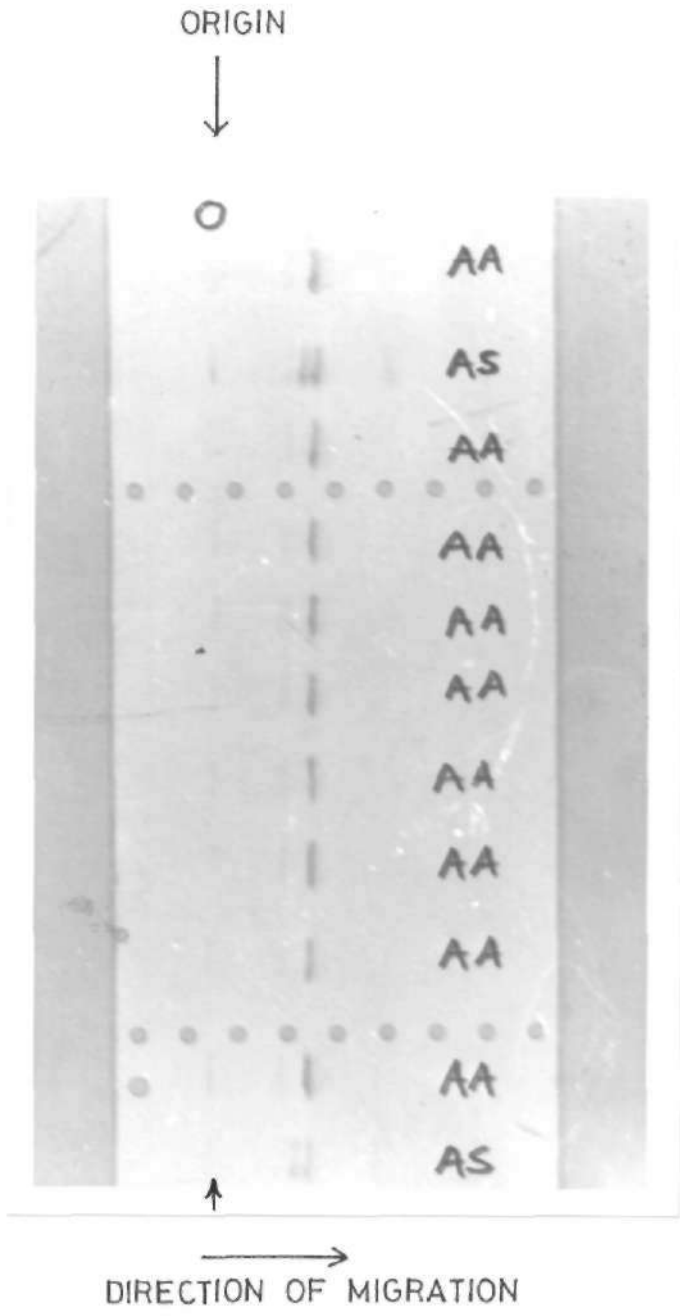



PLATE 2: A typical electrophoretogram of homozygous ofhaemoglobin-A guided at the bottom by heterozygous haemoglobin-As and at the top by haemoglobin-A. Hb-AS has double band while Hb-A has a single band.

# CHAPTER FIVE

## DISCUSSION

Results of this study show that patients with sickle cell anaemia were vitamin C sufficient when the reference range of serum vitamin C for normal subjects was used as index. The mean serum vitamin C levels of the SCA patients was indistinguishable from that of normal subjects. This finding may suggest that sickle cell disease does not have appreciable effect on serum vitamin C levels in these patients, and that oxidant damage to sickle cell may be minimal. Previous investigators (78) reported low serum vitamin C levels in SCA patients but the result obtained in this study disagrees with their observation; and this may be due to differences in dietary intake as well as differences in environment.

The female normal subjects had a significantly higher serum vitamin C than their male counterparts. This agrees with the report of Brook and Grimshaw (79) that both plasma and leucocytes vitamin C concentrations were lower in males than females and that the concentration decreased with increasing age in both healthy males and females. It has been shown that females have a higher renal threshold for ascorbic acid than the males which account for higher serum vitamin C levels in females at a given intake of the vitamin (80). Thus sex difference between male and female normal subject could reflect possible differences in the distribution volumes and in the metabolism of vitamin C between Sexes. However, there was no significant sex difference in the serum vitamin C levels between male and female SCA patients. The reason for absence of sex difference in serum vitamin C between male and female SCA patients is not known, though it may be due to the disease because it is clear that sex difference in serum vitamin C persist even into old age (79) in healthy individuals. Male SCA patients have slightly higher mean serum vitamin C levels

than the male normal subjects, although this difference was not significant. This slightly higher levels of serum vitamin C in male SCA patients is parallel to the pathophysiological stress to maintain tissue efficiency and integrity. Another possible suggestion for this slight difference could be that there is a slower utilization or metabolism of vitamin C in SCA patients. Similarly, in the females, there was no significant difference in the serum vitamin C levels in SCA patients and normal subjects; though a slightly higher serum vitamin C was observed in the female normal subjects. This finding suggests that the female SCA patients are vitamin C sufficient like their male counterparts. The little insignificant difference in serum vitamin C between female SCA patients and normal female subjects may suggest a slightly faster rate of turnover in female SCA patients.

It has been reported that the serum vitamin C levels decrease with increasing age (79). The results of this present study are in agreement with this obsevation. Among the normal subjects, the younger age group (18 - 22) had a higher serum vitamin C levels than those in the older age group (23 - 27). Similarly, among the SCA patients, the younger age group had a higher serum vitamin C levels than their counterparts in the older age group. However, there was no significant difference in serum vitamin C levels between SCA patients and normal subjects for the age groups considered in this study which may be due to the fact that the samples size was not large enough. The fall in serum vitamin C levels with increasing age observed among SCA patients and also among normal subjects may be as a result of some changes or differences in dietary habits between the younger and older age groups. It has been indicated that the serum vitamin C levels tended to decrease with age although a statistically significant correlation between age and ascorbic acid level has not been established (35).

Examining the relationship between haemoglobin (Hb) concentrations and serum vitamin C levels (Figs.4.3 and 4.4), it was found that Hb levels were more strongly associated, though negatively, with serum vitamin C levels in normal subjects than in the SCA patients ($r = -0.387$, $P < 0.008$ $V_s$ $r = -0.285$, $P < 0.019$). This result thus suggests that increasing serum vitamin C level through large oral intake, may be accompanied by a fall in Hb levels as a result of increased susceptibility of red cells to haemolysis. This agrees with the report that mega doses of vitamin C result in lysis of erythrocytes (9, 82) and this adverse effect of vitamin C may be due to its acidic nature. This observation is less pronounced in SCA patients than normal subjects. However, Latunde and Agboola (81) reported that ascorbic acid supplementation slightly increased the Hb levels of some healthy university students, though no supplementation was done in this study, moderate dose of vitamin C may raise Hb levels but mega dose will cause hemolysis as shown in the correlation graph in this study.

The mean Hb level of SCA patients was found to be significantly lower than that of the normal subject irrespective of age or sex. This finding was expected and indicates that the SCA patients are truly anaemic when compared with normal subjects. Furthermore, Hb-S electrophoretogram (Plate 1) indicates that the SCA patients are all homogygous for haemoglobin-S while the normal subject are all homozygous for haemoglobin-A (Plate 2). In the normal subjects, significant sex difference was observed in the haemoglobin concentrations. The normal males have higher haemoglobin concentration than the normal females. However, no sex difference was observed in the haemoglobin concentrations of male and female SCA patients. In addition, the normal male have significantly higher haemoglobin concentration than the male SCA patients. Similarly, the normal female have higher haemoglobin concentration than the female SCA patents. All these observations may

be attributable to the presence of sickle cell disease. There was no significant difference in the mean corpuscular haemoglobin concentration (MCHC) between SCA patients and normal subjects, and this was independent of age and sex. This suggests that though the RBCs of SCA patient have abnormal shape their haemoglobin concentration is comparable to those in the RBCs of normal subjects.

The packed cell volume (pcv) of SCA patients is lower than that of the normal subjects, age or sex notwithstanding. Normal males have significantly higher pcv than the male SCA patients and the normal females have significantly higher pcv than the female SCA patients as well. These observations further reflect the presence of sickle cell disease.

# CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

In conclusion, the data obtained in this study indicate that vitamin C deficiency defined by serum ascorbic acid level is rare in SCA patients in this enviroment, thus suggesting that there may be no association between serum ascorbic acid and the manifestation of sickle cell anaemia. The serum vitamin C level in SCA patients has been established and also found to be adequate when compared to that of normal subjects. The mean serum vitamin C levels in SCA patients and the normal subjects has been found to fall with increasing age. However, the sex differences in serum vitamin C levels was not pronounced between male and female SCA patients. On the contrary, the female normal subjects had a higher serum vitamin C levels than their male counterparts. Since there was no difference in the serum vitamin C levels between SCA patients and normal subjects; the implication of this may be that the RBCs of SCA patients are as well protected from oxidant damages (due to antioxidant function of vitamin C) as the RBCs of the normal subjects.

In view of the outcome of this study the following suggestions are put forward for the purpose of further work:

1. vitamin C supplementation studies, using moderate dose, should be carried out in *SCA patients to see if supplementation could lead to increase in Hb and pcv levels.* This will establish if there is beneficial effects of moderate vitamin C supplementation in SCA patients.
2. The concentration of vitamin C in the urine and leucocytes of SCA patient should be studied to see if the disease has any effect on urine and leucocyte ascorbic acid levels.
3. A similar study should be carried out in people with heterozygous haemoglobin (AS).

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