COMPARATIVE STUDY OF SOME BIOCHEMICAL FACTORS IN HUMAN ERYTHROCYTES EXHIBITING "EARLY"AND "DELAYED" RE-INFECTION TO *placimodium falciparum*"

BY:

KENNETH ERICK OUMA WERIMO B.Sc.(Hons)-NAIROBI

A thesis submitted in partial fulfilment of the requirements of the Degree of Master of Science (BIOCHEMISTRY) of the [University of Nairobi]

1997

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DECLARATION

I, KENNETH ERICK OUMA WERIMO, hereby declare that this is my own work and it has not been presented to any other University.

Kenneth Erick Ouma Werimo

CANDIDATE

This thesis has been submitted for examination with our approval as University Supervisors.

Dr. J.B.L. Chek

SUPERVISOR

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CHAIRMAN, BIOCHEMISTRY DEPARTMENT.

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LIST OF ABBREVIATIONS

ACD -	Acid Citrate Dextrose
AS -	heterozygous sickle cell haemoglobin
G-6-PDH -	Glucose-6-phosphate dehydrogenase
G -	Gametocyte
GP -	Glycophorin
GR -	Growth Rate
HbF -	Fetal haemoglobin
HbS -	Sickle haemoglobin
Hem - B -	Haemoglobin buffer
K^+ -	Potassium
K_2HPO_4 -	Dipotassium hydrogen phosphate
$MgCl_2$-	Magnesium chloride
MgATP -	Magnesium - Adenosine Triphosphate
NADPH -	Reduced Nicotinamide adenine dinucleotide
PAL -	Pyridoxal hydrochloride
Pk -	Pyridoxal Kinase
PLP -	Pyridoxal phosphate
R -	Rings
RBC -	Red blood cell
S -	Schizont
SS -	Homozygous sickle cell haemoglobin
T -	Trophozoints
% P -	Percentage parasitaemia
6-PG	6-Phosphogluconate

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SUMMARY

Malaria remains the most important of all tropical diseases. Some 200 million individuals in 102 countries are at risk. Nearly 85% of the cases and 90% of the carriers are found in tropical Africa. In Kenya, out of a total population 30 million 22 million people are at risk of infection.

Previous studies at Saradidi, Western Kenya have indicated that malaria (*P. falciparum*), infection varies from individual to individual. In one such study a group of inhabitants within similar geographical environment and having similar incidence of malaria, were initially treated for malaria and later monitored for re-infection for a period of 96 days. Results from that study indicated that some individuals were re-infected by the malaria parasites much earlier than others. For comparative study of these inhabitants, those that were re-infected before 96 days were classified as "early re-infected group" whereas those that were re-infected after 96 days were classified as "delayed re-infected group"

Studies have shown that several factors in red blood cells determine the degree of infection by *P. falciparum*. Among the factors that have been shown to limit the invasion and growth of malaria parasites in red blood cells include; immunological factors, glucose-6-phosphate dehydrogenase, pyridoxal Kinase and haemoglobin variants among others.

The present study was conducted to establish the following:

(a) Whether there occurred significant difference in *in vitro* growth of *P. falciparum* in red blood cells obtained from person exhibiting "early" and "delayed" re-infection. Significant difference in *in vitro* growth would relate the difference in re-infection to factors within the red blood cell other than immunological factors.

(b) Whether there occurred a significant difference in

glucose-6-phosphate dehydrogenase and pyridoxal kinase activity in the red blood cells obtained from the two groups of persons.

(c) Determine haemoglobin variants in the red blood cells obtained from the two groups of persons.

The results in the present study showed no significant difference in percent parasitaemia, growth rate and (3H) Hypoxanthine
incorporation in the red blood cells from persons exhibiting "early" and "delayed" re-infection with *P. falciparum*.

Glucose-6-phosphate dehydrogenase and pyridoxal kinase activity showed a statistically significant higher means in the "Early" re-infected group compared to the "delayed" re-infected group. This suggests that the difference in malaria susceptibility by the red blood cells from the two groups of persons may be attributed to these enzymes.

Results from haemoglobin variant analysis showed that the "early" re-infected group had 69% with haemoglobin AA and 31% haemoglobin AS. In the "delayed" re-infected group 25% had haemoglobin AA and 75% haemoglobin AS. These results confirm earlier studies which support the view that haemoglobin AS has some protective advantage against malaria infection.

CHAPTER ONE

1:0 INTRODUCTION AND LITERATURE REVIEW

1:1 GENERAL INTRODUCTION

Malaria is caused by a single-celled blood parasite. The causative organisms are the protozoa of genus *plasmodium* and they include: *Plasmodium ovale, Plasmodium falciparum, Plasmodium vivax,* and *Plasmodium malariae,* these are are the most prevalent species in endemic areas usually accounting for more than 80-90% of malaria infections and is the main cause of mortality. *Plasmodium malariae* is found especially in Nyanza province, where it occurs in up to 10-15% in younger age groups. *P. ovale* occurs in some 5% of malaria infection and *P. vivax* is occasionally reported-more often from coastal areas (Brown *et.al,* 1991).

1:2 CURRENT WORLD MALARIA SITUATION

Malaria remains the most important of all tropical diseases. It is estimated that worldwide the number of clinical cases is over 100 million with one million deaths each year. Some 2,000 million individuals in 102 countries are considered at risk. Nearly 85% of the cases, and 90% of the carriers are found in tropical Africa, where 25-40% of the hospital admissions may be due to malaria and in some areas 20-30% of deaths in infancy and childhood are attributed to the disease.

In Kenya, out of a total population of 29 million, 22 million people are at risk of infection (Brown *et.al.,* 1991). Overall some 30% of the reported morbidity is from malaria whilst in some parts of the country, Malaria is the leading or second cause of both morbidity and mortality. This trend is increasingly getting worse in view of the development of resistance by the parasites to the available drugs especially chloroquine and development of resistance by mosquito vectors to insecticides.

The degree of endemicity or level of transmission of malaria in any region is determined by a variety of interrelated factors. These include the prevalence of infection in man-the reservoir; the species of endigenous anopheline mosquito-their relative abundance, feeding and resting behaviours, and individuals,

suitability as hosts for plasmodia-the vector; the presence of a susceptible human population-the new host; local climatic conditions, and local geographic conditions which determine breeding areas.

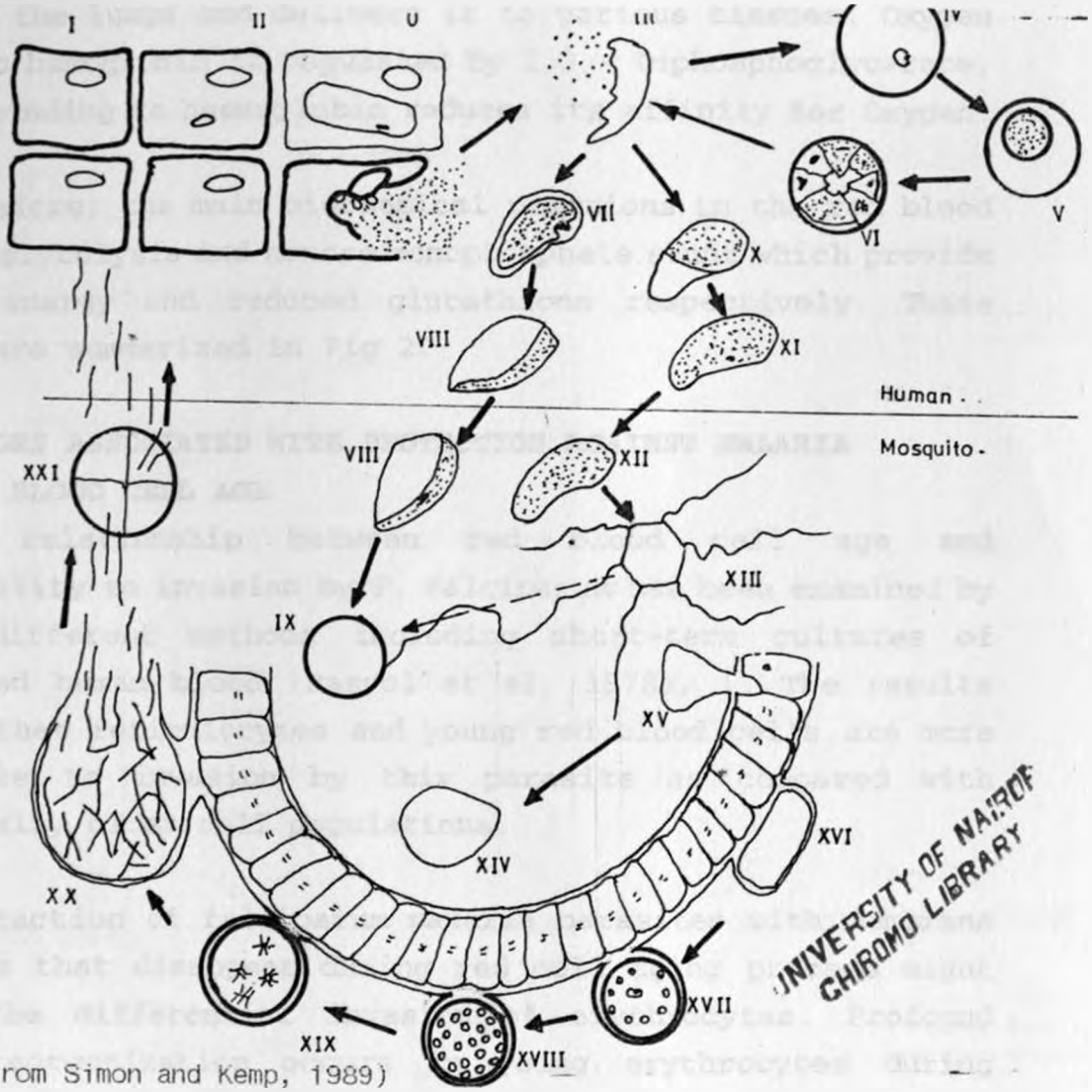
1:3 LIFE CYCLE OF MALARIA PARASITE

The life cycle of the malaria parasite involves three phases in the vector (mosquito) and two in the human host (Fig.1). Human infection begins when a female anopheline mosquito inoculates plasmodial sporozoites into the blood system while feeding. After a brief passage in the peripheral blood these organisms invade hepatocytes where they initiate the pre-clinical hepatic (exoerythrocytic) phase of the disease. Through the process of schizogony, sporozoites produce merozoites. In *P. falciparum* infections, the hepatic phase terminates at this stage. The clinical phase of malaria starts with the attachment of a released merozoite to a specific site which appears to differ for each species of malaria and the red blood cell concerned.

1:4 BIOCHEMISTRY OF HUMAN RED BLOOD CELL

The red blood cell, where the malaria parasite encounters the altered cellular functions is governed by variant genes and largely filled with haemoglobin. The surface membrane consists of glycolipids and glycoproteins. The membrane proteins control the transport of substances into and out of the cell, for instance potassium is pumped out and sodium in. This process requires energy which is provided by ATP generated through glycolysis, where glucose is broken down to form lactate. The hexose monophosphate shunt produces reduced NADP and reduced glutathione both of which prevent and repair oxidative damages to the cell membrane.

FIGURE 1: THE LIFE-CYCLE OF THE MALARIA PARASITE *P. falciparum*



(Adopted from Simon and Kemp, 1989)

Figure 1, shows the life-cycle of *P. falciparum.*

i) Parasite enters the blood stream of the host, invade hepatocytes (ii) where they undergo schizogony; merozoites are released which invade red blood cells and mature through the ring (iii) Trophozoite (iv) and schizont stages (v) merozoites are released (vi) which re-invade RBC (vii) the invaded merozoite can then develop as before or undergo sexual differentiation into immature macro- (viii) or micro-gametocyte (ix) These mature and are taken up by the feeding mosquito. The micro-gametocytes undergo rapid DNA replication and cell division to form flagellated cells (x) these are released and one invades the macro-gametocyte to form a diploid zygote (xi) which undergoes meiosis to develop into the Ookinete. (xv) These stages invade the wall of the mosquito gut to form the oocyst (xvi) which undergoes sporogony (xvii-xix) to produce haploid sporozoite (xx) which invade the salivary glands of the mosquito (xxi) from where they are injected into the human host and the cycle recommences.

The cytoplasm apart from being filled with metabolic enzymes is also filled with four-chain protein (haemoglobin) which binds oxygen in the lungs and delivers it to various tissues. Oxygen binding to hamoglobin is regulated by 2,3 - Diphosphoglycerate, which by binding to haemoglobin reduces its affinity for Oxygen.

Therefore, the main biochemical reactions in the red blood cells are glycolysis and hexose monophosphate shunt which provide cellular energy and reduced glutathione respectively. These pathways are summarized in Fig 2.

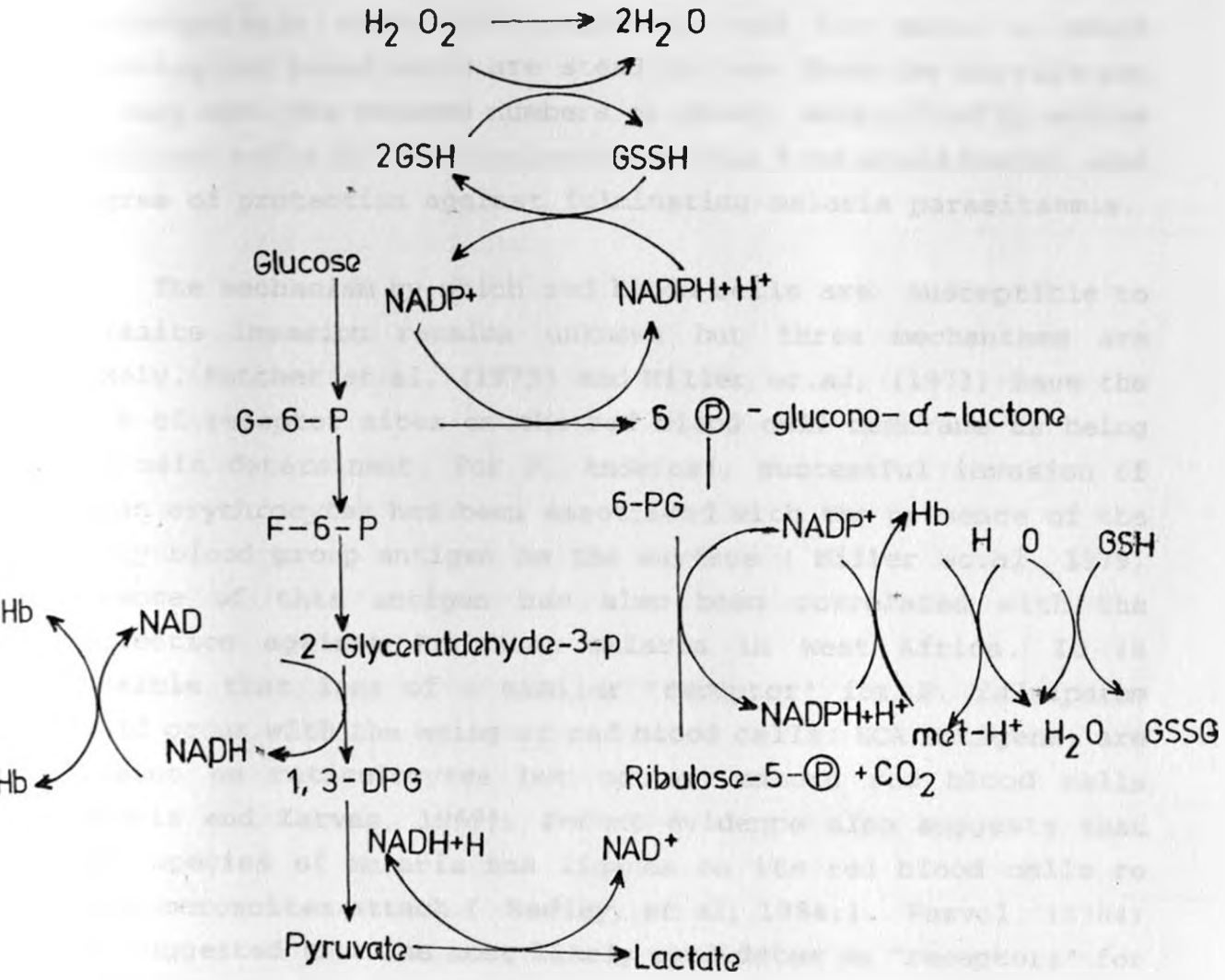
1:5 FACTORS ASSOCIATED WITH PROTECTION AGAINST MALARIA

1:5:1 RED BLOOD CELL AGE

The relationship between red blood cell age and susceptibility to invasion by *P. falciparum* has been examined by several different methods including short-term cultures of parasitized human blood (Pasvol *et al*, 1978a,). The results indicate that reticulocytes and young red blood cells are more susceptible to invasion by this parasite as compared with metabolically older cell populations.

Interaction of falciparum malaria parasites with membrane components that disappear during red cell aging process might explain the differential invasion of erythrocytes. Profound surface recrganization occurs in young erythrocytes during transition from the reticulocytes. Surface antigens such as HC-A disappear at this time.

FIGURE 2: SUMMARY OF GLYCOLYSIS AND HMP SHOWING HOW THE TWO PATHWAYS PROTECT THE ERYTHROCYTES



The importance of these pathways are the generation of ATP, NADH and reduced glutathione. These are important factors for the growth of the malaria parasite within the red blood cell.

Other studies have shown that older erythrocytes contain 30% less D-glucose residues on their surface than young cells. The relative delayed infection of "older" erythrocytes by *P. falciparum* might provide a temporary protective mechanism against malaria infection in new born infants. After birth, erythropoiesis shuts off completely and for about a month existing red blood cells are steadily lost from the circulation as they age. The reduced numbers of young, metabolically active red blood cells in the circulation at this time would confer some degree of protection against fulminating malaria parasitaemia.

The mechanism by which red blood cells are susceptible to parasite invasion remains unknown but three mechanisms are likely. Butcher *et.al*, (1973) and Miller *et.al*, (1973) have the role of receptor sites on the red blood cell membrane as being the main determinant. For *P. knowlesi*, successful invasion of human erythrocytes has been associated with the presence of the Duffy blood group antigen on the surface (Miller *et.al*, 1979. Absence of this antigen has also been correlated with the protection against *P. vivax* malaria in West Africa. It is possible that loss of a similar "receptor" for *P. falciparum* would occur with the aging of red blood cells. HCA antigens, are present on reticulocytes but not on mature red blood cells (Harris and Zervas, 1969). Recent evidence also suggests that each species of malaria has ligands on its red blood cells to which merozoites attach (Hadley, *et.al*, 1984;). Pasvol, (1984) has suggested that the most likely candidates as "receptors" for *P. falciparum* are glycophorins especially the glycophorin A(GP^a). Pasvol, (1984) also found that subaglutinating concentration of sera from patients whose red blood cells lacked GP^a (En1-cells) inhibited invasion.

The second mechanisms is based on the fact that young red blood cells are metabolically more active than old red blood cells (Sass *et.al*, 1964). A dependance of the invasion process on host cell mechanism might explain the prediletion by *P. falciparum* for young cells. The other possible mechanism for reduction of parasite invasion in older cells might be a simple mechanical effect. As red blood cells age, they become

progressively less deformable (La Celle and Orkin, 1970). This process is believed to be due to cross-linking of spectrin induced by ATP depletion or increase in calcium concentration within the red blood cell (Palek *et.al*, 1978). Entry of a merozoite into red blood cells involves an initial process of attachment, followed by internalization of the parasite. This causes marked deformation of young erythrocyte membrane of which an older less deformable erythrocyte membrane is not capable of parasite penetration.

1:5:2 RED CELL DISORDERS

Evolutionery results from natural selection, operating over a range of genetic diversity that arises from the mutation and recombination of genes. Variant genes that confer some selective advantage tend to increase in frequency whereas deleterious variants tend to be eliminated. Several inherited disorders of the red blood cells have been known to offer some protective advantage against lethal effects of malaria. These disorders are the sickle-cell anaemia and glucose-6-phosphate dehydrogenase .

1:5:2:1 HAEMOGLOBIN VARIANTS AND PROTECTION AGAINST MALARIA

Results from investigations of Raper (1959) on the interaction between *P. falciparum* malarial parasites and red cell genetic variants containing sickle-haemoglobin (HbS) suggested three possible mechanisms of intrinsic cellular resistance. First, there may occur "failure of infection" due to discrimination at the red cell membrane. Second, there may be an "abortive infection" due to poor intracellular growth of the parasite, and lastly there may be "suicidal infection" due to the destruction of the infected erythrocyte before completion of the parasites intracellular development. Friedman and Trager (1983) showed that the genetically controlled factor of sickle-haemoglobin (HbS) had protective effect against *P. falciparum*. Allison (1975) showed that individuals with sickle-cell trait were resistant to falciparum malaria. However, first attempts to grow *P. falciparum* in HbS containing red blood cells *in vitro* showed that haemoglobin type made no difference in parasite development (Raper, 1959). Luzzatto *et.al*, (1970) later found that parasitized deoxygenated sickle-cell trait red cells sickled

more rapidly than unparasitized cells. Trager and Friedman (1980) demonstrated the effect of sickling of red blood cells in confering resistance to malaria infection of SS and As cells in a 17% oxygen atmosphere. Some studies were also conducted in 3% oxygen tension with addition of cyanate, which increases the affinity of haemoglobin S for oxygen, making it less likely to aggregate at a given oxygen tension. At this culture condition (3% 0_2 and cyanate) the infected AS cells did not sickle allowing the parasites to survive. These studies confirmed that inhibition of parasite growth in sickle-cell was due to sickling of the red cell.

Sickling and damage to red cell membrane will cause K^+ to leak out of the cell. Since parasite metabolism requires high-potassium environment, leakage of K^+ inhibits parasites metabolism and thus its growth. This was also ascertained by the fact that maintaining a low oxygen tension with high cellular potassium level made the parasites to survive. These observations suggest that protection against malaria in sickle-cell heterozygotes occurs by the parasite in AS cell developing normally until the cell is sequestered in the tissues. Under low oxygen environment and low intracellular pH, the host cell sickles. These results in drop of potassium level with subsequent drop in parasite metabolism. The growth of the parasite is thus inhibited, Friedman and Trager (1983).

In addition to the sickle-cell disease and sickle-cell trait another red blood cell disorder that confer resistance to malaria is α-thalassaemia. Higgs *et al*,. (1981) found that children who carried both α-thalassaemia and sickle-cell trait experienced less clinical malaria than children with other haemoglobin genotype including children who were heterogenous for HbS. Amongst individuals with sickle cell trait, the percentage of HbS is greater in the red cells of those with normal X-globin genes than in those with α-thalassaemia. The mechanism of protection against malaria by α-thalassaemic cell arises from the abnormal sensitivity of the cell membrane to damage by oxidation of cell membrane molecules fragments and disrupts the integrity of the membrane. One of the factors in the cells that disrupts the

integrity of membrane is hydrogen peroxide. Malaria parasite generates hydrogen peroxide, therefore in sensitive thalassaemic cells, this will bring about damage of the cell membrane. This would therefore suggest that the parasites growth would greatly be inhibited. If there is high oxygen tension, and presence of chemicals that catalyse oxidation reactions, coupled with absence of reducing agents such as reduced glutathione and vitamin E, which protects cell membrane lipids against oxidation. Malaria parasites would be inhibited. Knyszynski *et.al*, (1979) reported on the increased phagocytosis of thalassaemic red cells by macrophages *in vitro*. These studies suggested that surface alteration, recognition and uptake by unstimulated phagocytes limit rate of invasion.

Faetal haemoglobin could also be linked to confering resistance to malaria infection since faetal protein has high affinity for oxygen, thus increasing the oxidative stress of the cells which affects the growth of the parasite. Malaria parasites in faetal red cells, like those of thalassaemic cells are highly sensitive to oxidative stress.

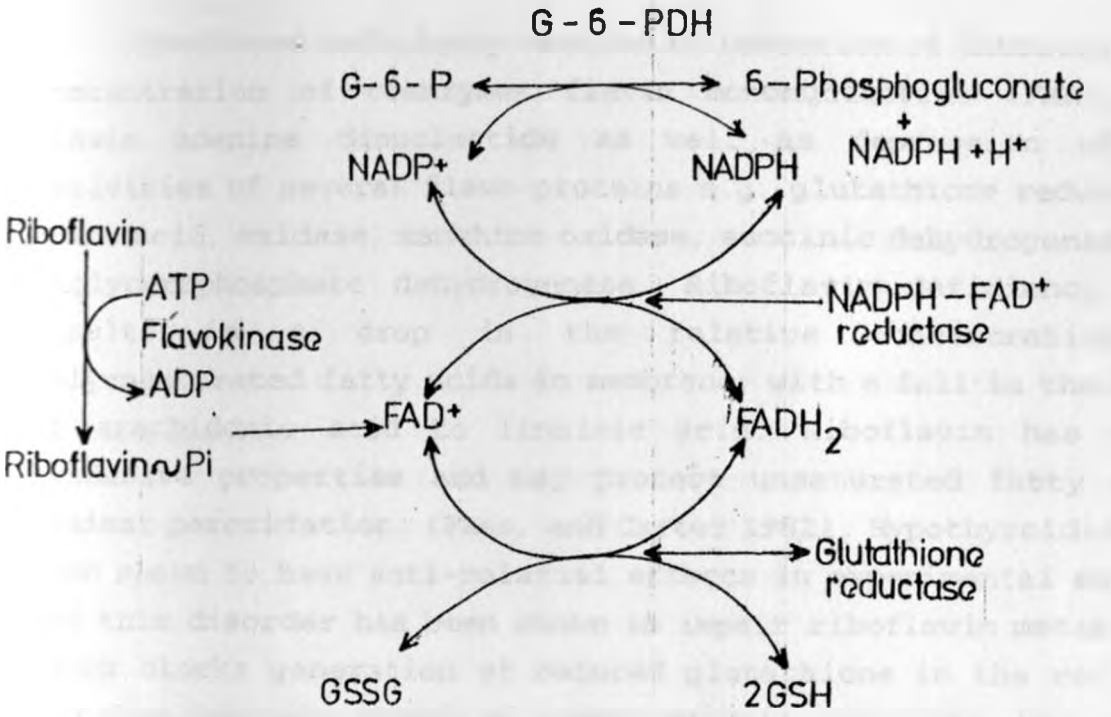
1:5:2:2 GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND PROTECTION AGAINST MALARIA

Glucose-6-phosphate dehydrogenase (G-6-PDH) deficiency of the erythrocyte is one of the commonest enzymatic abnormalities in humans, (Hempelman and Wilson, 1981). Population studies show a high correlation between the incidence of G-6-PDH deficiency and endemic *P. falciparum* malaria. G-6-PDH is the first enzyme in the hexose monophosphate shunt, which regenerates NADPH, a coenzyme that is essential for protection against and repair of oxidative damage. Motulsky, (1960); It has been suggested that possession of G-6-PDH deficiency conferred some degree of protection against *P. falciparum* on the affected individuals in a manner similar to the sickle-cell trait Motulsky, (1960),Allison and Clyde (1961). Rapidly growing parasites would have a requirement for products of the pentose phosphate pathway of which this enzyme catalyses the initial reactions. There is an increased amounts of G-6-PDH as a result of increased host response to parasitization. The parasite may also produce and

regenerate increased amounts of the reduced coenzyme NADPH. Other studies have however shown that the malaria parasite does not depend entirely on the red cell for its G-6-PDH requirement. Hempelman and Wilson, (1981) have shown that *P. Knowlesi* has its own G-6-PDH. The study also suggested that *P. falciparum* could be having its own G-6-PDH whose activity increases as the parasite grows, so as to replace reduced host cell activity due to digestion of host cell cytoplasm by the parasite.

The NADPH is required for bio-synthetic activities during rapid growth, especially the synthesis of lipids and proteins and for regeneration of reduced glutathione which provides the sulphur requirement of the parasites and helps to maintain the erythrocyte environment until maturity is reached. Additionally, the activity of this enzyme produces pentose phosphate sugars, a vital requirement for nucleotide synthesis, for the formation of coenzymes and nucleic acids which the parasite produces in large amounts. Friedman et al (1979) showed that parasites are sentitive to oxidative stress and their growth is inhibited in cells when reduced glutathione, a component of RPMI 1640 is left out of the culture medium. Other modifiers of inhibition is oxidant stress, and at higher oxygen levels (25-30%) parasite multiplication is inhibited. Rodent malaria parasites produce hydrogen peroxide. Accumulation of hydrogen peroxide would inhibit parasite growth. G-6-PDH generates reduced glutathione for maintainance of reduced cell environment. Other modifiers of inhibition in oxidant sensitive red cells are redox catalyst such as riboflavin, fava bean and manadione, (Friedman *et.al.*, 1979). Deficiency of G-6-PDH and other redox catalysts such as riboflavin will confer resistance to malaria infection.

Mechanism of G-6-PDH deficiency and riboflavin in confering resistance to malaria arises from their role in generation of reduced glutathione in the reaction shown in the diagram below.



The reduced glutathione reduces hyrogen peroxide to water as in the reaction below:

$$2GSH + H_2O_2 \xrightarrow[\text{Peroxidase}]{\text{Glutathione}} GSSG + H2O$$

This reaction is apparently important when hydrogen peroxide is too s
low to be dealt with efficiently by catalase (Cohen & Hochstein, 1963).

Glucose-6-phosphate is converted to 6-phosphogluconate with the action of the enzyme glucose-6-phosphate dehydrogenase. The reaction generates reduced NADPH-FAD+ reductase enzyme to generate $FADH_2$. By the reaction of Glutathione reductase, reduced glutathione if generated reduces the Oxygen stress. Glucose-6-phosphate dehydrogenase deficiency therefore inhibits this

reaction process resulting in high oxidant stress due to lack of reduced glutathione. The oxidant stress inhibits malaria parasite growth.

Riboflavin deficiency results in reduction of intracellular concentration of coenzymes flavin mononucleotide (FMN), and flavin adenine dinucleotide as well as depression of the activities of several flavo-proteins e.g. glutathione reductase, Amino acid, oxidase, xanthine oxidase, succinic dehydrogenase and α-glycerophosphate dehydrogenase. Riboflavin deficiency also results in a drop in the relative concentration of polyunsaturated fatty acids in membrane, with a fall in the ratio of arachidonic acid to linoleic acid. Riboflavin has anti-oxidative properties and may protect unsaturated fatty acids against peroxidation, (Fass, and Carter 1982). Hypothyroidism has been shown to have anti-malarial effects in experimental animals and this disorder has been shown to impair riboflavin metabolism which blocks generation of reduced glutathione in the red cell and thus inhibits growth of rodent malaria parasites. Due to the importance of riboflavin in the generation of reduced glutathione, its deficiency may render erythrocytes more susceptible to haemolysis and therefore not permit intracellular proliferation of plasmodia. Riboflavin deficiency also inhibits the production of reticulocytes, the prefered site of plasmodia invasion, in addition to making them more fragile. Glutathione reductase is elevated 2 to 3 times in infected erythrocytes, suggesting that this enzyme is essential in the metabolism of the parasite by maintaining a reduced environment which helps the parasite to cope with oxidative stress.

1:5:3 PYRIDOXAL KINASE IN PROTECTION AGAINST MALARIA

The vitamin B6 group-pyridoxine, pyridoxal, and pyridoxamine is essential for cellular metabolism, but there has been little investigation of the role of these compounds in malarial metabolism (Platzer *et.al*, 1978).

The first indication of a role of vitamin B6 in malarial cells was by Seeler (1944), who found that passive doses of pyridoxine markedly inhibited the activity of quinine and

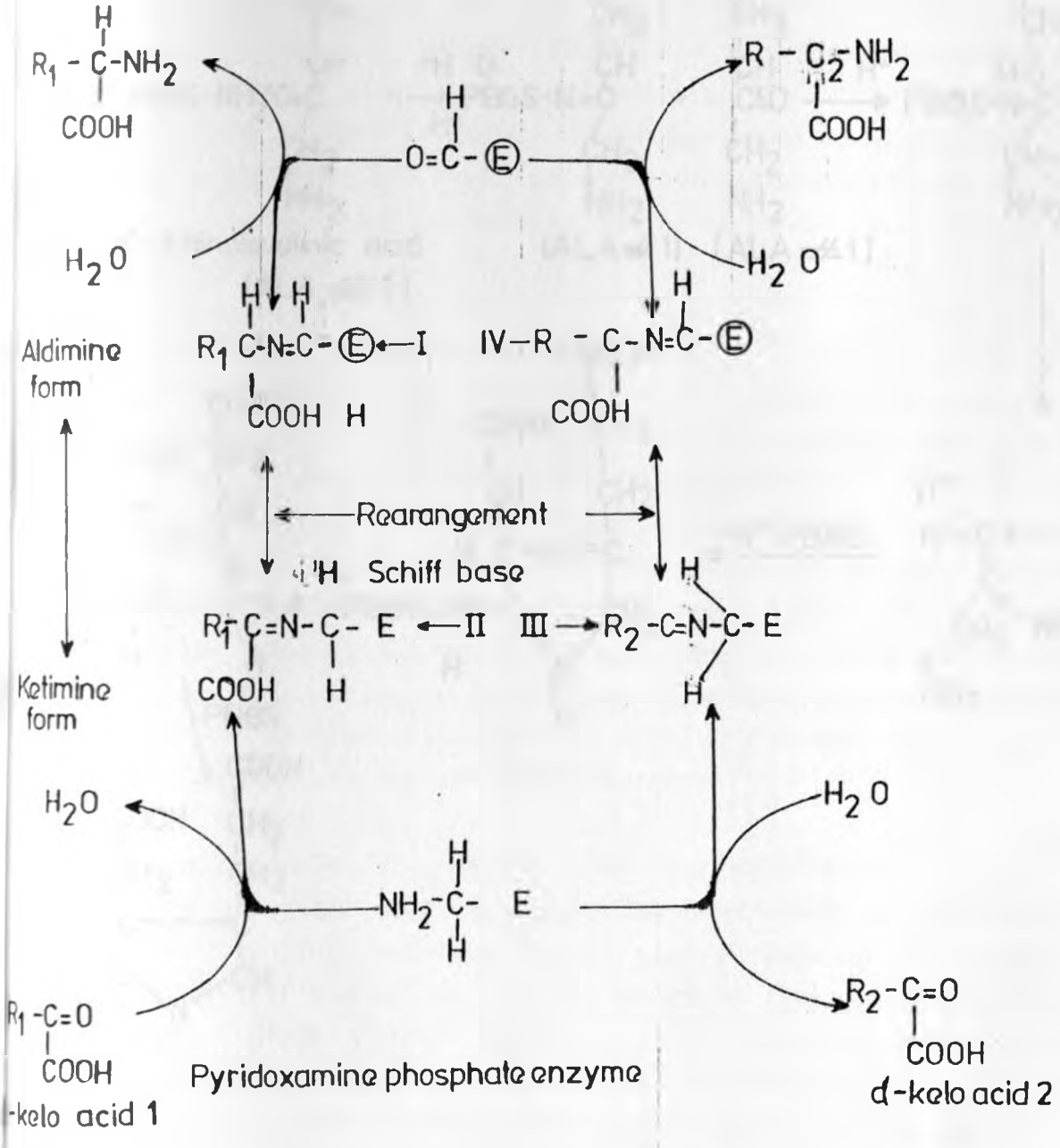
quinacrine against *Plasmodium lophurae*, in ducklings. Ramakrishnan (1954) reported that *P. berghei* infections developed poorly in rats maintained on diets deficient in pyridoxine. Although such evidence was equivocal, for instance the results may have reflected pharmacological effects unrelated to nutritional requirements or nutritional debilitation of the host animal, a requirement for vitamin B6 by malarial parasites was suggested. Many enzymatic reactions in amino-acid metabolism involve pyridoxal phosphate and the report of aspartate-alanine amino-transferase activity in *P. lophurae* by Sherman *et.al*, (1971) established a metabolic requirement for vitamin B6 and malaria parasite. The malaria parasite acquires the required vitamin B6 exogenously from the host cell. The first enzymatic step in B6 metabolism involves pyridoxal Kinase (E.C.2.7.1.35) which catalyses the reaction shown below:

$$\text{Pyridoxine} + \text{ATP} \xrightarrow[\text{Kinase Mg}^{++}]{\text{Pyridoxal}} \text{Pyridoxine-5-phosphate} + \text{ADP}$$

Pyridoxal Kinase (PK) phosphorylates vitamin B6 into its active coenzyme pyridoxal 5, phosphate which then participates as a schiff base (Fig. 3) in several key biochemical reactions within the cell (Inyama, 1991).It is reported that *P. falciparum* has been shown to lack pyridoxal Kinase and therefore depends on the host for its pyridoxal 5 phosphate requirement.

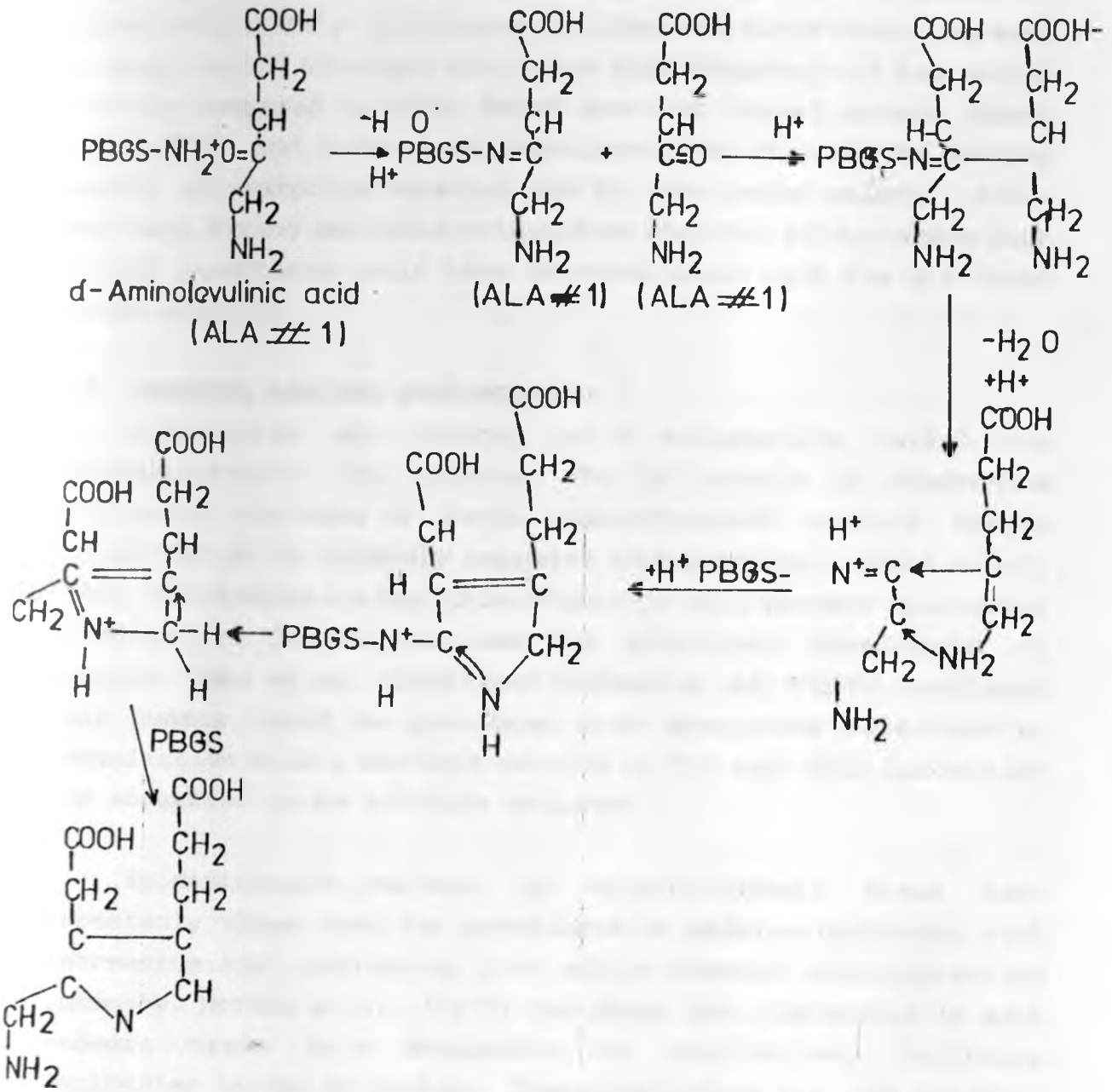
A part from acting as a schiff base, pyridoxal Kinase in erythroblast catalyses the synthesis of delta-amino levulinic acid which is the rate limiting step in heme biosynthesis (Fig. 4). The reactions are important for the parasites metabolism and therefore its dependence on the enzyme pyridoxal kinase. This would therefore imply that individuals whose red blood cells have low or lack this enzyme would be less susceptible to *P. falciparum* infection compared to those with high levels because the metabolic activity of the parasite would be inhibited in the former case.

FIGURE 3: PYRIDOXAL 5 PHOSPHATE ACTION AS A SCHIFF BASE IN TRANSAMINATION REACTION



Intermediate steps in the transformation of amino acid 1 via α-keto acid 2 to form amino acid 2. E represents aportion of the pyridoxamine phosphate-enzyme complex. (Adopted from Bichemistry, by Stryer 1988 Ed).

FIGURE 4: BIOSYNTHESIS OF PORPHOBILINOGEN FROM A-AMINOLEVULINIC ACID



d-Aminolevulinic acid (ALA ≠ 1) (ALA ≠ 1) (ALA ≠ 1)

Mechanism for the biosynthesis of porphobilinogen (PBG) two molecules of δ-aminolevulinic acid (ALA) as catalyzed by the enzyme porphobilinogen synthase (PBGS). The NH_2 group of the enzyme shown is probably the α-NH_2 of the lysyl residue in the enzyme molecule.

(Adopted from Biochemsitry by Stryer, 1988 Ed.)

There are two variants of pyridoxal Kinase in red blood cells, normal and low enzyme activity. The enzyme activity is stable and its activity decreases with red blood cell age (Chern and Beutler, 1975, 1979). This may explain the decrease in susceptability to *P. falciparum* by older red blood cells compared to young cells. Afro-Americans have high frequency of low enzyme activity compared to other North American racial groups (Churn *et.al*, 1974) and it has been postulated that this may be another genetic polymorphism selected for by falciparum malaria. Afro-Americans having had their origin from tropical Africa where high malaria prevalence could have favoured those with low pyridoxal Kinase activity.

1:6 IMMUNITY AGAINST SPOROZOITES.

Sporozoites are covered by a polypeptide called the circumsporozoite (CS) protein. The CS protein of *Plasmodium falciparum* contains a large immunodominant central domain consisting of 41 tandemly repeated tetrapeptides, (Dame *et.al*, 1984). Antibodies against this CS protein will prevent sporozoite infection of hepatocytes and the subsequent development of malaria. Dame *et.al*, (1984) and Hoffman *et.al*, (1987) confirmed that humans could be protected from sporozoite infection by immunization with a sub-unit vaccine of CSP and this protection was suggested to be antibody mediated.

Epidemiologic surveys in malaria-endemic areas have repeatedly shown that the prevalence of malaria decreases with increasing age, indicating that adults develop some protective immunity. Norden *et.al*, (1979) has shown that the adults in such endemic areas have antibodies to sporozoites, including antibodies to the CS protein. These antibodies are the possible mediators of protective immunity. Studies by Hoffmann,1987 have also shown that age-specific prevalence and titers of antibodies to the CS protein were inversely correlated with the age-specific prevalence *P. falciparum*. Hoffman *et.al*, (1987) demonstrated that levels of naturally acquired antibodies to the CS protein of *P. falciparum* do not protect against infection with *P. falciparum* during 96-day period of intensive malaria transmission. It is only under conditions of less exposure that such antibodies may

play a more significant role in preventing malaria infection.

The term semi-immune is often used by malariologists to describe adults from malaria- endemic areas who are less susceptible to malaria than are children. Cohen *et.al,* (1961) showed that there was naturally acquired protective immunity against blood stages of *P. falciparum*. Beaudoin *et.al,* (1977) suggested that repeated infection of humans with normal sporozoites, as occurs in malaria endemic areas, induces protective immunity. This could be the reason why adults in such endemic areas have decreased susceptibility to malaria infection.

Hoffman *et.al,* (1989) showed that non-repeat region. *T. epitopes* on the CS protein of *P. falciparum* have specific sites that are involved in providing help for production of antibodies these antibodies are involved in the protective cellular immune response. However, the problems that are encountered concerns the ability of the parasite to survive despite the development by the host of a degree of resistence to reinfection. The relative importance of individual component of the immune response in protection against malaria parasites are difficult to assess. The decreased susceptibility observed in some adults from malaria endemic areas may be either due to naturally acquired immunity or some other biochemical factors within the red blood cell.

1:7 CONCEPTUAL BASIS OF "EARLY" AND " DELAYED" RE-INFECTION WITH *P. falciparum*

When a female anopholine mosquito carrying sporozoites in her salivary glands feeds on a human, sporozoites pass into the bloodstream. Some of these sporozoites are transported to the liver, where they invade hepatocytes, initiating the cycle that culminates in malarial disease. When sporozoites have been injected in the human bloodstream, various factors determine the development of the disease. These factors include time. Hoffman *et.al,* (1987) conducted a study in Saradidi, Western Kenya to determine whether the level of naturally acquired antibodies to circumsporozoites protein would be predictive of protection against *P. falciparum* infection during a 98-day period when the rate of malaria transmission was high. In the study 93 adult male, life-long residents of Saradidi donated blood samples for

malaria smears. Each volunteer was then treated with a single dose of three tablets of pyrimethamine sulfadoxine (Fansidar, Roche), followed by 100 mg of doxycycline twice daily for 7 days. On the basis of local parasites susceptability to fansidar alone radical cures were expected for all volunteers. Each volunteer was visited for the next 96 days and blood smears were obtained on days 7, 14, 28, 42, 56, 70, 84, and 98 and on any day a volunteer complained of illness, Eight two volunteers completed the study. Thirty-two of the 82 (39%) had *P. falciparum* on day 0. None was positive on day 7, 14, or 28. Between day 42 and 96 there was a gradual increase in the cummulative proportion with parasitaemia. By day 96, 60 (75%) had become positive. The adults in this study had all life-long, intense exposure to malaria, yet 75% had *P. falciparum* re-infection within 96 days days after radical cure of infection. Twenty two (25%) did not develop re-infection after 96 days.

For purpose of the present study, the 75% of the volunteers who developed re-infections within 96 days are refered to as "early re-infected" group whereas the 25% who did not develop re-infection within the 98 days study period are refered to as "delayed re-infected" group.

2:0 CHAPTER TWO

2:1 GENERAL OBJECTIVE

To analyse and compare some biochemical parameters in red blood cells obtained from persons exhibiting "early" and "delayed" re-infection with *P. falciparum* . A systematic difference in a particular biochemical factor in the two groups of red blood cells might give a clue as to why these persons differ in susceptibility to malaria infection and may offer an important biochemical approach to designing more rational methods of control and treatment.

2:2 SPECIFIC OBJECTIVES

(a) To investigate *in vitro* growth rates of *P. falciparum* (strains K39 and M24) in red blood cells obtained from persons exhibiting "early" and "delayed" re-infection to *P. falciparum*.

(b) To determine the rate of incorporation of (3H) Hypoxanthine by the parasites cultured in different red blood cells and thus indirectly assess the activity of purine salvage enzymes.

(c) To determine the activity rates of the G-6-phosphate dehydrogenase and pyridoxal kinase enzymes in red blood cells from persons exhibiting "early" and "delayed" re-infection to *P. falciparum*.

1) Glucose-G-Phosphate dehydenase (G-6-PDH)
2) Pyridoxal Kinase (PK)

(d) To analyse by electrophoresis the haemoglobin variants in the red blood cells from persons exhibility "early" and "delayed" re-infection with *P. falcuparum*.

CHAPTER THREE

3:0 MATERIALS AND METHODS

3:1 CHEMICALS

All chemicals that were used in this study were of analytical grade obtained either from BDH chemicals Ltd (Poole, England) or Sigma chemical Company (U.S.A). Solutions were prepared with glass distilled water.

3:2 STUDY AREA

The study was conducted at saradidi (55km west of Kisumu) and 13km north of Lake Victoria.

The climate of the area has been described by Haddow (1942) and Joshi et (1975). Rainfall averages 1,200 -1,500 annually, with a "long rainy season" from march to May and a "short rainy season" from September to November. the amount and duration of the rainfall varies markedly among years.

The Luo tribe is the dominant ethnic group in saradidi. Most residents are subsistance farmers growing corn. Sorghum and vegetables.

3:3 SELECTION OF VOLUNTEERS

All blood samples were obtained according to the guidelines approved by the Ethical Review Committee on Clinical Research of the Kenya Medical Research Institute, Nairobi. Volunteers from Saradidi, Western Kenya who participated in similar studies previously participated again in this study. Human malaria infections in Saradidi consists of 87.1% *P. falciparum*, 1.6% *P. malarae*, 0.4% *P. ovale* and 11.0% mixed. (Spencer *et.al.*, 1987). The study group comprised 82 vounteers who had been previously studied to determine the incidence of *P. falciparum* infection. After radical cure, these individuals were monitored for 98 days to check on the re-infection by *P. falciparum*. Individuals who were reinfected during the 96 day study period were classified as "early" reinfected group whereas those who were re-infected after the 96 day study period were classified as "delayed" group (semi-immune). The semi-immune group were adults from malaria-endemic areas who were less susceptible to malaria than were children. Blood samples from "early" and "delayed"

volunteers were obtained to provide red blood cells for *in vitro* studies, enzyme activity (G-6-PDH and PK) and haemoglobin studies. These individuals had earlier been identified in a related study.

3:4:0 PLASMODIUM FALCIPARUM PARASITES

Two isolates of *P. falciparum* were used in this study. These were M24 (Malindi) and K39 (Kisumu). The parasites were initially obtained from infected people in Malindi and Kisumu and cryo-preserved in liquid nitrogen. Samples were reconstituted and cultivated *in vitro* using the method of Trager and Jensen, (1976) at the Biological Sciences Research Centre (BSRC) - Kenya Medical Research Institute (KEMRI) laboratories.

The cultures were maintained in 50 ml culture flask (lux 5350 USA).The growth rates were monitored through estimation of percent parasitemia (% P) on Giemsa stained thin films, subcultured in wells after attaining a parasitemia of (1.5-3%).

3:4:1 DETERMINATION OF PARASITAEMIA

The % parasitemia was determined by counting the number of parasitized red blood cells per 10,000 erythrocytes observed under oil immersion at a magnification of X100. The number of red blood cells in one field of view were counted and recorded. The following relationship was used to determine %P.

$$\% P = \frac{\text{No. of parasitized erythrocytes} \times 100}{(\text{RBCs per field}) \times \text{fields counted}}$$

Intraerythrocytic developmental stages were scored, viz rings,trophozoites,schizonts,and gametocytes.

3:4:2 GROWTH OF *P. falciparum* EARLY AND DELAYED REINFECTED PERSONS

To assess how the Early and Delayed re-infected red blood cells (RBC) affected *in vitro* growth of *P. falciparum*, blood samples from early and delayed re-infected persons were collected from volunteers and used for *in vitro* culture. Growth rates were assessed by two methods:

(a) Counting Giemsa stained thin blood films to determine parasitemia levels and assessment of parasite morphology.

(b) By the assessment of the rate of incorporation of radio-isotope [^{3}H] Hypoxanthine into parasite nucleic acid

3:4:2:1 *In vitro* GROWTH ASSESSMENT OF *P. falciparum* BY MICROSCOPY

Parasite cultures were set up in triplicate in 24 flat-bottomed well plates (diameter 16 mm - 25820 USA) starting with common parasitemia (0.2%) in all the blood samples from donors. The stock culture had percent parasitemia of 1.5-2.5% for both isolates K39 (Kisumu 39) and M24 (Malindi 24).

Complete culture medium containing AB$^+$ serum (CMS) was used to avoid possible agglutination of the blood cells.

Smears were made after 48 hours, and 96 hours. The smears were then stained with Giemsa and the number of parasitized cells per 10,000 erythrocytes counted and recorded.

The growth rates (GR) were calculated according to the formula adopted from Wilet and Canfield (1984).

$$GR = \frac{(P_f \times D)^{2/d}}{P_i}$$

where:

P_f = Final parasitemia

P_i = Initial parasitemia

D = Dilution factor

d = number of days of culture.

3:4:2:2 RADIOISOTOPE ASSESSMENT OF *in vitro* GROWTH OF *P. falciparum*

Assessment of in vitro growth rate by [^{3}H] Hypoxanthine uptake was performed according to the method described by Chulay *et.al.*, (1983) . Isolates (K39 and M24) were cultured as previously described by Trager and Jensen (1976).

[^{3}H] Hypoxanthine was used as a tool to measure growth *in vitro* because studies with other malaria parasites have indicated a critical role for this purine, Sharman,(1977). The radioactivity measured represented primarily [^{3}H] Hypoxanthine incorporated into parasite nucleic acid and thus a measure of the activity of purine salvage enzymes in the concerned erythrocytes,Chulay *et.al*,(1983)

Incorporation of [^{3}H] Hypoxanthine was measured by adding 0.5 µCi [G-^{3}H] Hypoxanthine (Specific activity, 1 Ci/mmol, Amersham Corp) in 60 µl complete medium to each parasite microculture. The [^{3}H] Hypoxanthine was then immediately added to the parasite microculture and the plate incubated for 12 hours to allow incorporation of [^{3}H] Hypoxanthine.

Radioisotopically labelled cultures were then harvested onto glass fiber filters (Mash 11 Glass filter No. 23-985-AH) using an automated multiple harvester (skatron).

The filter papers were dried at 37° C, and the paper discs transferred to tubes containing 1 ml scintillation fluid (containing PPO, methane, POPOP. toluene).

Radioactivity incorporation was counted in a liquid scintillation ß-counter (Beckman LS 1801 USA). Results were expressed as counts per minute (CPM) in parasitized erythrocytes minus counts per minute in counted non-parasitized erythrocytes. Control values were generally less than 30 CPM.

3:5 GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAY

Acid citrate dextrose (ACD) was used as anticoagulant in blood samples from volunteers.

Human blood 1 ml was obtained from branchial vein using a Terum disposable butterfly syringe. The blood was immediately transferred into small nunc tubes and containing the ACD at ratio of 0.15 of ACD per ml of whole blood (V/V) sealed tightly and cooled to 4° C. Enzyme assay was done within 2-3 days as this was the maximum time the blood took to arrive at KEMRI,Nairobi from the field.

The spectrophotometric readings used in this study were a modification of the methods of kornberg and Horecker, (1955) and Cohr and Waller, (1974).

The temperature of the reaction mixture was maintained at 30° C. The reaction mixture was prepared by adding 0.01 ml (10µl) blood directly to vial containing G-6-PDH assay solution (Catalog No 345-1) and mixed thoroughly to completely suspend erythrocyte. The mixture was then left to stand at room temperature (18-26° C) for 5 minutes. 20 ml G-6-PDH substrate solution was directly

added to reaction mixture and mixed gently by inverting several times. The reaction mixture was transferred to a water bath at 30° C and incubated for 5 minutes to attain thermal equilibrium and absorbances were read and recorded as [A] at 340 nm. These were initial A. Exactly 5 minutes later absorbances were again read and recorded. These were final A.

$$\Delta A \text{ per min} = \text{Final A} - \text{Initial A}/5$$

Glucose-6-phosphate dehydrogenase activity is expressed as U/g haemoglobin (U/gHb). Haemoglobin concentration was determined using a coulter counter (Model FN, coulter Instruments Inc. Hialeah, Florida, U.S.A.).The following relationship was used ass

$$\text{G-6-PDH [U/gHb]} = \frac{\Delta A \text{ per min} \times 100 \times 3.01}{0.01 \times 6.22 \times \text{Hb[g/dL]}} \times \text{TCF}$$

$$= \frac{\Delta A \text{ per min} \times 4839}{\text{Hb [g/dL]}} \times \text{TCF}$$

Where:

100	=	Factor to convert activity to 100 ml
3.01	=	Total reaction volume
0.01	=	Sample volume (ml)
6.22	=	Millimolar absorptivity of NADPH at 340 nm
Hb [g/dL]	=	Haemoglobin concentration determined for each specimen.
TCF	=	Temperature correction Factor [1 at 30° C]

3:6 PYRIDOXAL KINASE (PK)

The activity of PK was measured by flourometric method according to Kark *et.al.*, (1982). Briefly the flourometric assay

measured PK activity by determining the rate of conversion of pyridoxal to pyridoxal phosphate (PLP). A spectrophoto-fluorometer (Perkins-Elmer MPF-3 England) was used to measure flourescence.

3:6:1 BLOOD FOR PYRIDOXAL KINASE ASSAY

10 ml of blood was obtained from branchial vein using a butterfly disposable hypodermic syringe. The blood was immediately transferred into small blood bottles, containing heparin as anticoagulant, mixed well and cooled on ice. Hemolysates were prepared within 4 hours as recommended by Kark *et.al,* (1982.)

3:6:2 PREPARATION OF HEMOLYSATES FOR PYRIDOXAL KINASE ASSAY

10 ml blood was centrifuged at 8,000 RPM for 10 mins at 4°C. Serum and white blood cells were aspirated off. An equal volume of physiological saline was added and mixed. Centrifuging was again done at 8,000 RPM for 10 mins at 4° C. 1 ml of the packed cell volume (PCV) was mixed with 9 ml of 12 mm triethanolamine hydrochloride (TEA), pH 7.4 to give a 1:10 V/V dilution. The mixture was vortexed and allowed to stand at 4° C for 10 minutes, to allow complete hemolysis. The transparent, clear red solution was stored at -70° C for upto two weeks ready for enzyme assay.

3:6:3 PREPARATION OF REACTION MIXTURE

A bulk mixture was made of all components except the hemolysate sample.

16 ml of bulk mixture was then dispensed into 50 ml

centrifuge tube. The hemolysates were thawed and 4 ml added to the bulk mixture to make a total volume of 20 ml to start the reaction.

3:6:4 STOPPING THE REACTION AND EXTRACTING THE PYRIDOXAL PHOSPHATE

At time 0 Minutes, 3 ml aliquot from the hemolysate reaction mixture was taken and added into a test-tube containing 0.3 ml of 75% w/v trichloroacetic acid (TCA) to stop the reaction. The rest of the reaction mixture was incubated at 37° C in a waterbath and after 15, 30, 45, 60 minutes, a further 3 ml aliquot were taken and added into a test tube containing o.3 ml of 75% TCA to stop the reaction. Subsequently to extract the PLP, these samples (i.e. samples at 0, 15, 30, 45, and 60 minutes) were incubated at 50° C for 15 mins and span at 10,000 RPM at 4° C for 10 mins. Then 2 ml of clear supernant was taken from each sample and buffered to pH 7.4 with 1.6 ml of 3.3.M K_2HPO_4 and 0.1 ml of 20 mm KCN. The sample tubes were incubated at 50° C for 25 minutes, cooled and pH adjusted to 3.7 by adding 0.7 ml of 28 % H_3PO_4 and 2ml of 2M potassium acetate pH 3.7. Flourescence of these samples was measured at room temperature in a perkin-Elmer MPF-3 spectrophotometer at 320 nm excitation and 416 nm emission. Eight standard solutions of PLP ranging in concentration from 0-8.0μM were used. These standards were treated exactly as the reaction mixture aliquots from extraction of PLP to measurement of fluorescence. Relative units of fluorescence for the standards were plotted against corresponding concentration of PLP and a linear regression was performed. The slope of this line gave the ratio of fluorescence units to concentration units of PLP

For each reaction mixture the relative flourescence was plotted as a function of time, and a linear regression was performed. The slope of this line (units of flourescence/minute) gave the rate of formation of PLP (µM PLP/Ml/minute). Pyridoxal Kinase activity in µMoles of PLP/L/Min/10^9 red cells was obtained by dividing the rate of PLP formation by the number of red blood cells in the blood samples.

3:7:0 HAEMOGLOBIN ELECTROPHORESIS

Haemoglobin electrophoresis and identification was done as described by the Helena Titan IV citrate haemoglobin electrophoresis procedure.

3:7:1 PRINCIPAL OF THE METHOD

Very small samples of hemolysates prepared from whole blood are applied to the Titan IV citrate agar plate. The haemoglobins in the sample are separated by electrophoresis using citrate buffer, (pH 6.0 - 6.3) and stained with an O-Dianisidine staining solution. Separation of haemoglobins under those conditions depends both on the location of the substituted residue and on its electrophoretic charge. The method is based on the complex interactions of the haemoglobin with the electrophoretic buffer (acid pH and agar support).

3:7:2 BLOOD SAMPLE COLLECTION

Whole blood was collected in tubes containing anti-coagulant (EDTA).

3:7:2:1 Specimen preparation

Blood sample was centrifuged at 3500 RPM for 5 min. Plasma was removed and RBC washed in 0.85% saline (v/v) three times. After which the cells were centrifuged for 10 min. at 3500 RPM. One volume of purified water and 1/4 volume of toluene was added to washed RBC and vortexed at high speed for 1 minute. The sample was then centrifuged at 3500 RPM for 10 minutes. The top layer was removed with a capillary tube. The clear red solution was filtered through two layers of filter paper whatman # 1.

3:7:3 PREPARATION OF HEMO CONTROL

Standard AFSC hemo control was prepared by adding 1 part of the control to 1 part Hemo-lysate Reagent. This was mixed and allowed to stand for 5 minutes.

3:7:4 PREPARATION OF ZIP ZONE CHAMBER

100 ml of citrate Buffer pH 6.0 - 6.3 was poured into each outer section of the Zip Zone chamber. Two wet (in Buffer) spong wicks were placed in each outer compartments with the top surface protruding approximately 2mm above the inner chamber ridges. The sponge was gently pressed to assure complete saturation with buffer.

3:7:5 ELECTROPHORESIS

Haemolysate solutions were once more mixed to ensure complete lysis. Then 5 µl of each prepared hemolysate (field sample and control) was placed in separate wells of the Zip Zone sample well plate using the micro dispenser. The Zip Zone applicator was primed by quickly pressing the tips into the Zip Zone Applicator, it was then applied to the Titan IV citrate Agar

Plate by gently pressing the application tips down onto the gel surface. The samples were allowed to soak into the agar for about 1 minute.

The plate was quickly put in the Zip Zone chamber, Agar-side down, so that the agar later makes good contact with the top surface of the sponges. The first application point was nearest the anode . The chamber was closed and electrophoresis commenced at 100 volts/agar-plate for 30 minutes.

3:7:6 VISUALIZATION OF THE Haemoglobin BANDS

For visualization of the haemoglobin bands, a staining solution was prepared by mixing 5.0 ml, 0.2 % O-Deanisidine, 10ml 5 % acetic acid, 1ml 1 % sodium nitroferricyanide and 1ml 30 % hydrogen peroxide.

Upon completion of electrophoresis the agar plate was removed from the chamber and immersed in the stain, agar-side up. Staining was done for 5-10 minutes. The plates were rinsed in tap water until all the stain was washed off. The plate was laid on a fresh blotter, and dried at 50° C for 1 hour.

Haemoglobin bands were identified by reference to the control chart and control band.

3:7:8 DATA ANALYSIS

In vitro culture studies for % parasiteamia, Growth Rate, and [3H] Hypoxanthine incorporation in counts per minute (CPM) was compared in the two groups by of Students' t-test. P-values

of less than 0.05 were considered statistically Significant. the same tests were applied to enzymatic activity of G-6-PDH and pyridoxal phosphate. Comparison of haemoglobin types was done by looking at the percentage proportion of each haemoglobin type in the two groups of people.

CHAPTER FOUR

4:0 RESULTS

4:1 PERCENT PARASITAEMIA IN RED BLOOD CELLS OBTAINED FROM PERSONS EXHIBITING "EARLY" AND "DELAYED" RE-INFECTION BY *P. falciparum*

In vitro culture of malaria parasites in the red blood cells obtained from persons who had "early" and "delayed" re-infection by *P. falciparum* using both strains K39 and M24 developed through all the four stages of *Plasmodium falciparum* life-cycle i.e. the rings, trophozoites schizonts and gametocytes were observed in the culture (Plates 1-2).

Using strain K39 and M24, there was a significant increase in the *in vitro* percent parasitaemia of *P. falciparum* parasites in both the red blood cells obtained from "early" and "delayed" re-infected persons at 96 hours relative to 48 hours (Table 1) P less than 0.05. The *in vitro* growth of both strains of parasites (M24 and K39) obtained from "early" and "delayed" re-infected persons at 48 hrs and 96 hrs was similar (Table 2).

PLATE 1: Schizont stage of p.falciparum in culture

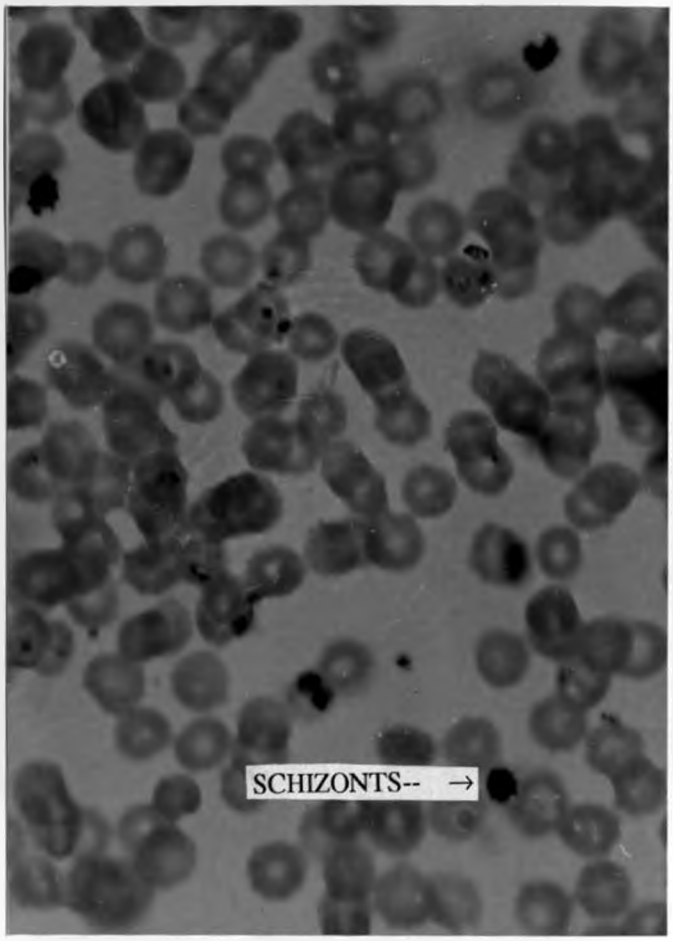


PLATE 2: Gametocyte stages of P. falciparum in culture

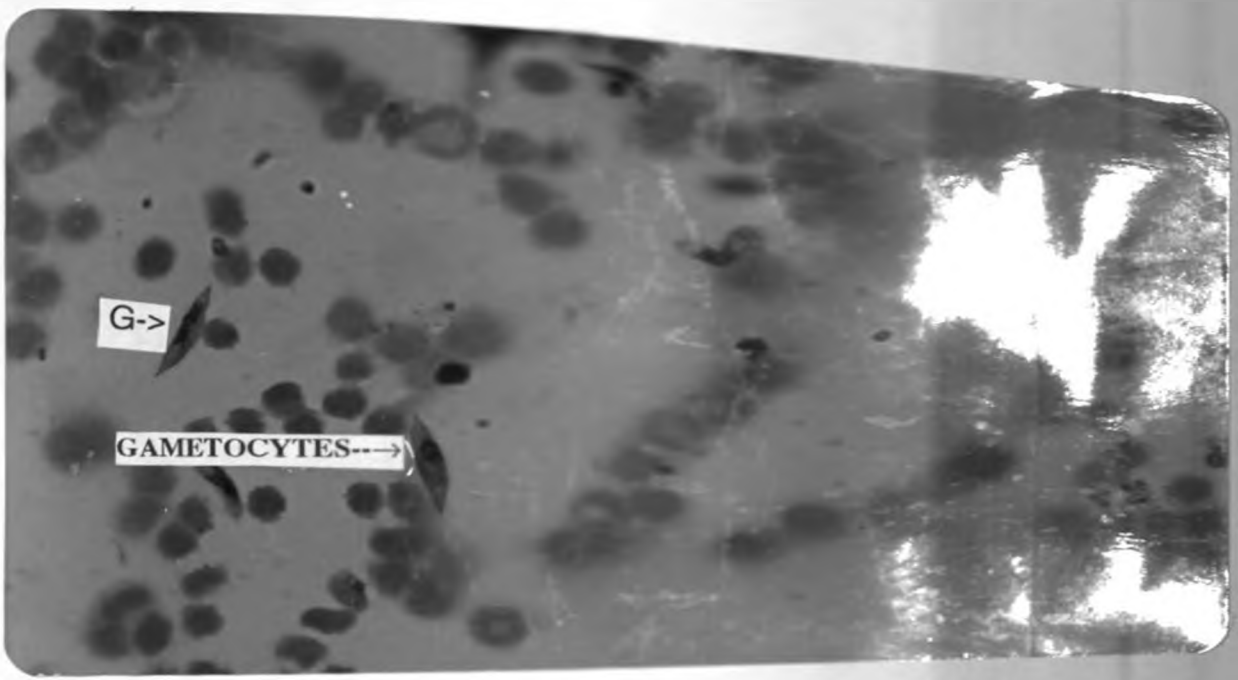


TABLE 1: Comparison of in vitro % Parasitaemia of *P. faciparum* using strain K.39 and M24 in red blood cells obtained from persons exhibiting "early" and "delayed" re-infection.

P. falciparum Strain	TIME IN CULTURE (HRS)	DELAYED RE-INFECTION	EARLY RE-INFECTION
		MEAN ± SEM	
K39	48 hrs	0.763 ± 0.15	0.939 ± 0.19
	96 hrs	2.26 ± 0.55 *	2.46 ± 0.67
M24	48 hrs	1.10 ± 0.27	0.920 ± 0.19
	96 hrs	3.07 ± 0.58 *	2.90 ± 0.54

* Figures are mean and SEM for nine determinations in each group. Significance of the means between the % parasitaemia in red blood cells obtained from "early" and "delayed" re-infected persons was tested by student's t-test.

A value of 0.6% parasitaemia under in vitro culture conditions is considered normal for *Plasmodium falciparum*. A % parasitaemia of less than 0.6 indicates inability of the parasites to multiply (Khan, 1992).

From total samples analysed in the "early" re-infected persons, 66% had percent parasitaemia abcve 0.6 and 33% below 0.6 using K39 at 48 hrs, whereas at 96 hrs 78% were above 0.6 and 22% below. In the "delayed" group at 48 hrs, 33% had percent parasitaemia above 0.6 and 66% were below 0.6. At 96 hrs 78% had percent parasitaemia of more than 0.6 and 22% below 0.6.

In delayed re-infected group strain K39 had % parasitaemia of 0.763±0.19 in the early re-infected group ($p>0.05$) thus showing no significant difference between the two groups.

The same observation occured using strain M24.

With strain M24, the "delayed" re-infected group had 86% above 0.6 and 14% below at 48 hrs. At 96 hrs, 87% were above 0.6 and 13% below. In the "early" re-infected group at 48 hrs, 62% were above 0.6 and 38% below. At 96 hrs 87% were above 0.6 and 13% were below.

4:2 GROWTH RATE IN RED BLOOD CELLS OBTAINED FROM PERSONS EXHIBITING "EARLY" AND "DELAYED" RE-INFECTION BY *P. falciparum*

The growth rate of *Plasmodium falciparum* in the red blood cells obtained from persons exhibiting "early" re-infection was similar at 48 hrs and 96 hrs using strain K39. Similarly strain M24 at 48 hrs mean growth rate was not different from that at 96 hrs in persons exhibiting "early" re-infection.

In the "delayed" persons the mean growth rate with strain K39 at 48 hrs was similar to the growth rate at 96 hrs. Using Strain M24 at 48 hrs, mean growth rate was significantly higher in relation to that at 96 hrs (Table 2).

TABLE 2: Comparison of *in vitro* growth rate in "early" and "delayed" persons using strain K39 and M24.

P. falciparum Strain	TIME IN CULTURE (HRS)	EARLY RE-INFECTION	DELAYED RE-INFECTION
		MEAN ± SEM	
K39	48 hrs	4.12 ± 0.72	3.58 ± 0.67
	96 hrs	3.08 ± 0.88 *	3.18 ± 0.57
M24	48 hrs	4.05 ± 0.79	5.37 ± 1.3
	96 hrs	5.18 ± 1.7 *	2.70 ± 0.61

* Values are mean growth rates and SEM for nine determinations in each group. Significance of the means between the growth rates in red blood cells obtained from "early" and "delayed" re-infected persons was tested by unpared student's t-test.

Taking growth rate of 3.0 as normal growth under *in vitro* culture conditions, the "delayed" re-infection group at 48 hrs had 55.6% of the total samples studied above 3.0, whereas 44.4% were below 3.0 using strain K39. The same result were observed at 96 hrs. Using same strain in the "early" re-infected group the same growth rate was observed at 48 hrs, whereas at 96 hrs, 33.3% had growth rate above 3.0 and 66.7% below. There was a general observation that using strain K39 in both the groups, growth rate was above 6.5 at 48 hrs and 96 hrs.

K 39 had a growth rate of 4.12 ± 0.67 in the delayed re-infected group ($p \geq 0.05$) showing no significant difference inthe two groups.

Using strain M24, in the red blood cells from "delayed" re-infected persons 25% were below and 75% above at 48% hrs. In the "early" re-infected persons 62.5% were above 3.0 and 37.5% below at 48 hrs. At 96 hrs in the "delayed" group, 37.5% were above 3.0 and 62% below with 50% above and 50% below in the "early" re-infected group.

4:3 (3H) HYPOXANTHINE INCORPORATION (IN COUNTS PER MINUTE (CPM) IN RED BLOOD CELLS OBTAINED FROM PERSONS EXHIBITING "EARLY" AND "DELAYED" RE-INFECTION BY *P. falciparum*

In the "delayed" persons using K39 at 48 hrs, the mean CPM was significantly lower than at 96 hrs. In the same group of red blood cells using strain M24 at 48 hrs the mean CPM was significantly lower than that at 96 hrs (Table 3).

Results observed in the red blood cells obtained from the "early" re-infected persons using k39 gave a similar mean Cpm at both the 48 hrs and 96 hrs. With strain m24 at 48 hrs the mean CPM was significantly lower than that at 96 hrs (Table 3).

There was no significant difference in mean CPM between the two groups of cells, except one shown by strain K39 at 48 hrs which showed a significant increase in hypoxanthine incorporation in the cells obtained from the "early" re-infected persons.

TABLE 3: Comparison of incorporation in "early" and "delayed" re-infected persons using strain K39 and M24.

P. falciparum Strain	TIME IN CULTURE (HRS)	EARLY RE-INFECTION	DELAYED RE-INFECTION
		MEAN ± SEM	
K39	48 hrs	9752 ± 1841	4697 ± 1264*
	96 hrs	10679 ± 1666	11118 ± 1092
M24	48 hrs	5258 ± 1666	3223 ± 800
	96 hrs	10483 ± 3090	8317 ± 1728

* Values are mean percent (3H)Hypoxanthine incorporation of RBC and SEM for nine determinations in each group. Significance of the means between the [3H] Hypoxenthine incorporation in red blood cells obtained from "early" and "delayed" re-infected persons was tested by unpaired student's t-test.

4:4 ENZYME ACTIVITY IN RED BLOOD CELLS OBTAINED FROM PERSONS EXHIBITING "EARLY" AND "DELAYED" RE-INFECTED BY *P. falciparum*

The activity of glucose-6-phosphate dehydrogenase and pyridoxal kinase were determined in the two groups of persons to find out if the activity of one of these two enzymes would contribute to the observed pattern of re-infection in these people.

The activity of glucose-6-phosphate in the "delayed" re-infection persons was significantly lower than that in the "early" re-infected persons. The enzyme activity in the "early" re-infection persons was about 51% higher than in the "delayed" group (Table 4).

Pyridoxal kinase activity in the red blood cells from persons exhibiting "early" re-infection by *P. falciparum* was also significantly lower than that from the red blood cells obtained from persons exhibiting "delayed" re-infection. The enzyme activity in "early" re-infected persons was 38% higher than in "delayed" persons.

TABLE 4: Comparison of Pyridocal kinase and G-6-PH activities in the red blood cells obtained frrom persons exhibiting "early" and "delayed" re-infection to *P. faciparum*.

ENZYME	EARLY RE-INFECTION	DELAYED RE-INFECTION
	MEAN ± SEM (U/gHb)	
G-6-PDH	10.764 ± 4.174	5.338 ± 3.994*
Pyridoxal kinase	2.17 ± 0.30	1.35 ± 0.80

* Values are means of enzyme activity and SEM of twelve determinations in each group. Significance of the means between the enzyme activity in red blood cells from the two groups of person was tested by unpaired student's t-test.

PLATE 3: Electrophoretic bands of different types of haemoglobin in in Delayed group

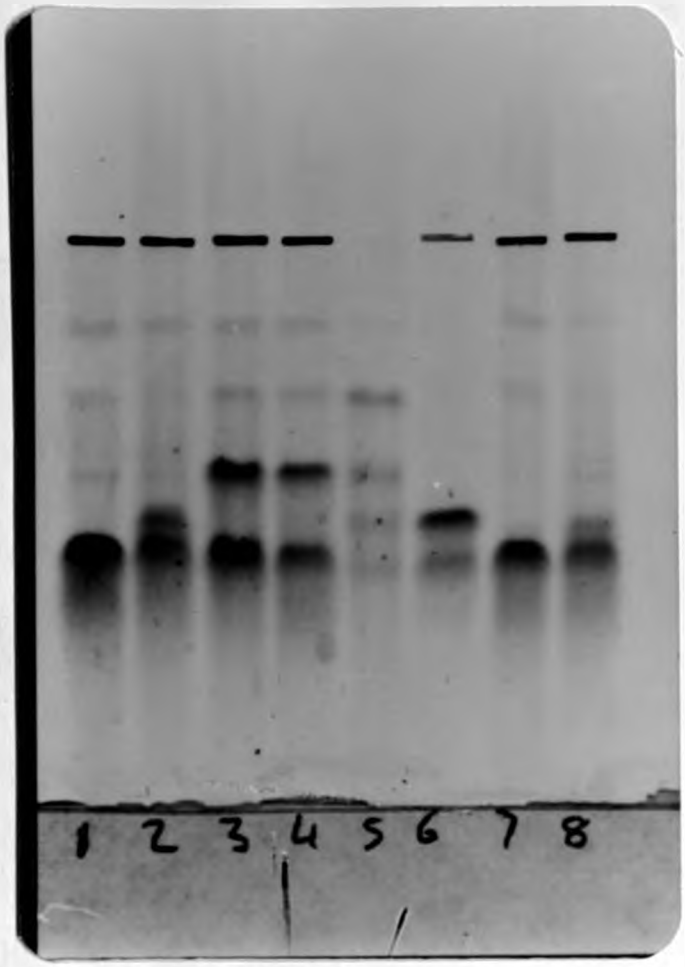
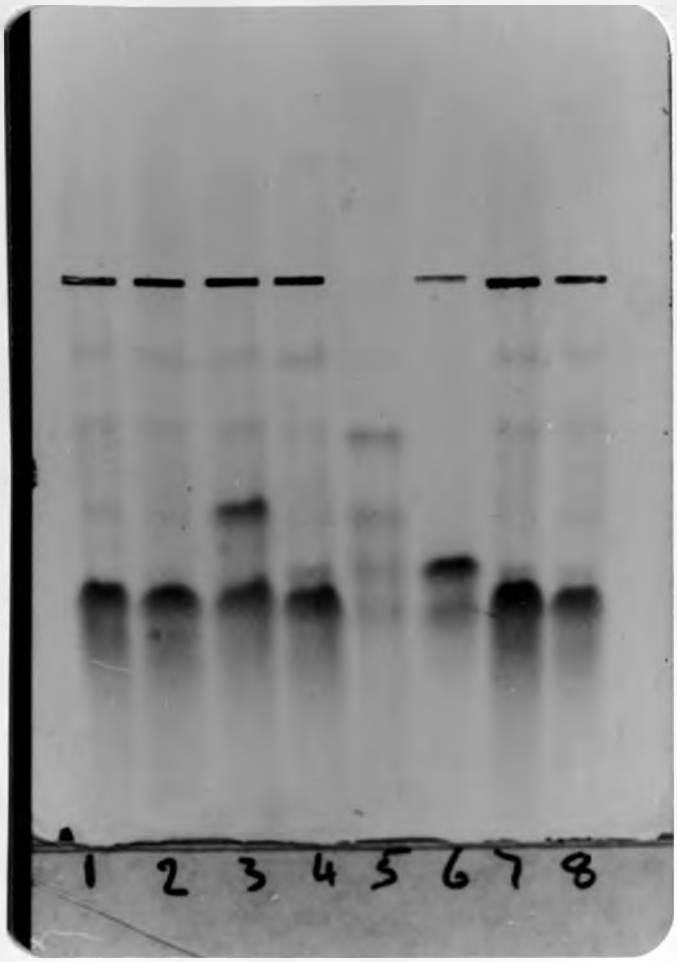


PLATE 4: Electrophoretic bands of different types of haemoglobin in Early group



CHAPTER FIVE

5:0 DISCUSSION

The results in the present study have shown that there is no significant difference in % paresitaemia, growth rate and (3H) Hypoxanthine incorporation using both strain K39 and M24 in red blood cells obtained from persons exhibiting "early" and "delayed" re-infection by *P. falciparum*.

The study has shown that the 'early" re-infected persons have a significantly higher enzyme activity than the "delayed" persons. The "early" re-infected persons have 51% higher G-6-PDH activity than the "delayed" ones,with a 38% higher pyridoxal Kinase activity than the 'delayed" persons.

The 'early" reinfected persons have 69% of its haemoglobin as AA and 31% as As. The "delayed" reinfected persons have their haemoglobin composed of 25%as AA and 75% as As.

5:1 PERCENT PARASITAEMIA AND GROWTH RATE IN RED BLOOD CELLS OBTAINED FROM PERSONS EXHIBITING "EARLY" AND "DELAYED" RE-INFECTION WITH *P. falciparum*

The general observation on the % parasitaemia indicates that both K39 and M24 increased with time in culture media in red blood cells obtained from persons exhibiting "early" and "delayed" re-infection . These results were expected as the parasites stayed longer in the culture media, they got adapted to the environment and hence increase in % parasitaemia from 48 hrs to 96 hrs. Strain M24 showed a higher % parasitaemia than K39.

Since M24 appeared to have had a higher % parasitaemia than K39, the possible explanation is that strain M24 being a coastal

strain is foreign to Western Kenyans. Having had no previous exposure to the strain the immunological players may be absent on the red blood cell membrane to offer any protection. Making the red blood cell more vulnerable to infection. Baird, (1995) indicated that the course of infection by *Plasmodium falciparum* among adults who lack a history of exposure to endemic malaria is fulminant, whereas the infection in adults living with higher- to holoendemic malaria is chronic and benign. In contrast K39 could have been recognized by some immunological receptors in the red blood cell membrane that limited the multiplication of the parasite.

The trend of percent parasitaemia was reversed in growth rate since growth dropped with time. Growth rate drop could possibly be due to either depletion of nutrients in the culture media or accumulation of waste products (toxins or growth inhibitors) released by the parasites themselves during their development in the culture media. Baird (1995) observed that *in vivo* some individuals may be infected with *P. falciparum* but show minimal or no signs of clinical malaria, even in the presence of high parasitaemic levels. The reason for this observation was speculated to be due to components of natural immunity to malaria (antitoxic and antiparasite) which inhibit the metabolic growth of parasites. As observed in the study, the growth rate in the red blood cells from "early" and "delayed" persons did not show any consistent pattern in the two groups.

It was expected that there would have been high growth rate in the "early" re-infected group than in the "delayed" group as previously observed *in vivo*. It may be speculated that growth rate is similar in the two groups of red blood cells due to

removal of all immunological components during washing of the red blood cells prior to culturing. Differences in the immunological components *in vivo* would cause a difference in growth rate.

It is probable that one of the possible factors causing "early" and "delayed" re-infection in red blood cells *in vivo* by *P. falciparum* is differences in immunological components between the two groups of persons.

5:2 HYPOXANTHINE INCORPORATION AS COUNTS PER MINUTE IN RED BLOOD CELLS FROM PERSONS EXHIBITING "EARLY" AND "DELAYED" RE-INFECTION TO *P. falciparum*

The "delayed" re-infection group had a lower hypoxanthine incorporation using both strains. There was a marked rise in CPM from 48 hrs to 96 hrs with both strains.

In the "early' infected group, strain K39 showed no marked increase in hypoxanthine incorporation from 48 hrs to 96 hrs. Strain M24 showed an increase in CPM from 48 hrs to 96 hrs. Various factors that affect (3H) Hypoxanthine incorporation by parasites in culture. Notable among these factors is the initial parasitaemia (Chulay *et.al*, (1983). The higher the initial parasitaemia, the lower the incorporation of hypoxanthine caused possibly by accumulation of acid metabolites. The culture in the present study was started with an initial parasitaemia of 0.2% which was suitable enough not to affect the growth of the parasites at 96 hrs. Another factor that affects hypoxanthine incorporation is availability of nutrients. The experiment was carried out using similar culture media so as not to affect the incorporation of the hypoxanthine.

The other factor that affect hypoxanthine incorporation is the extracellular concentration of purine salvage enzymes. Since both red blood cells and *P. falciparum* posses purine salvage enzymes, the red blood cells with high activity of these enzymes would be expected to have higher counts per minute than those with lower enzyme activity.

The probable conclusion is that the observed "early" and "delayed" re-infection of red blood cells by *P. faciparum* in these persons is not caused by different activities of purine salvage enzymes.

5:3 GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN RED BLOOD CELLS OBTAINED FROM PERSONS EXHIBITING "EARLY" AND "DELAYED" RE-INFECTION BY *P. faciparum*

The results observed in this study of glucose-6-phosphate dehydrogenase activity in red blood cells obtained from persons exhibiting "early" and "delayed" re-infection by *P. falciparum* shows that there is a relatively higher activity in the red blood cells from persons exhibiting "early" re-infection than those from "delayed" re-infection. The deficiency ratio in the two groups of persons are significantly different, with "delayed" group having a deficiency ratio of 44% compared to the "early" group which has a deficiency ratio of 0% (Table 4).

Aruwa (1977) in a study on Kenyan tribes inhabiting different geographycal areas, found that normal activity of G-6-PDH in human erythrocytes was 8.74 ± 0.48IV/gHb and deficient erythrocytes had enzyme activity of 3.36 ± 0.57IV/gHb.

Erythrocytes whose activity was greater than 9.265IV/gHb were classified as having high enzyme activity.

The observation in this study are in agreement with Aruwa's results conducted among the Western Kenya tribes who inhabit malarial region and were found to have a G-6-PDH deficiency ratio of 33% while the Kikuyu had defiency ratio of 5%. The Western Kenya tribes, due to constant exposure to infection by the malaria parasite are known to have a "delayed" infection rate compared to their central Kenya counterparts who due to non-exposure to the parasite exhibit early infection rate when exposed to the parasite. The possible mechanism causing these differences in susceptibility to infection may be explained by proposing that while the effect of G-6-PDH plays a vital role in parasite development, the effect of the enzyme is more influenced by production and availability of immunological components (cytokines etc) which are induced with constant exposure to parasite infection. This mechanism may be the crucial factor in the "delayed" group which has low G-6-PDH activity with high deficiency ratio compared to the "early" infected group which has high G-6-PDH activity and low deficiency ratio. It may be speculated that since both enzymatic and immunological components are protein in nature, the synthesis of high levels of enzymes will lower the overall synthesis of immunological components and since G-6-PDH is required by the parasite it will enhance its multiplication and growth. Likewise lower concenctration of enzyme will stimulate higher synthesis of the immunological components which will inhibit the multiplication and growth of the parasites.

The "early" re-infected group has 67% with high enzyme activity compared to 37.5% in the "delayed" group, this compares with the activity among the Western and Central Kenya tribes who could be classified as "delayed" and "early" re-infected groups respectively.

According to the conventional view of the development of acquired immunity, antigenic polymorphism governs susceptibility to infection for many years of heavy exposure. The parasite presumably manages evasion of an efficacious immune response through sheer diversity of epitopes. The chronically exposed host supposedly accumulates a repertoire of memory and affector cells capable of controlling infection by any given strain or variant of parasite. It may also be speculated that with constant exposure to the parasite, the body mechanism responds by lowered synthesis of the enzyme G-6-PDH thus a selective advantage for the red blood cell not to be severely infected by the parasite.

Tong *et.al,* (1992) in another study found that the chinese had a G-6-PDH activity of 8.10 ± 2.0410/gHb, while new born babies had activities of 6.46 ± 1.241v/gHb. In the same study adults were found to have enzyme activity of 5.24 ± 1.01v/gHb. The lower values detected in the new born babies compared to the average normal activity conforms with the known view that young babies below 6 months old are fairly protected from malaria infection, (Njoh and Bediako, 1991). This protection is explained by presence of low G-6-PDH and haemoglobin F (HbF) in the red blood cell, complemented with the natural immunity from the mother, all of which contribute to malaria protection to the infants.

Differences in the malaria susceptibility could be explained by a variation of the gene coding for enzyme G-6-PDH with a result that high activity will render the individual red blood cell more susceptible to *Plasmodium falciparum* while low activity will delay the multiplication and growth of the parasite. Prevalence of G-6-PDH or its deficiency has been carried out in various regions with values ranging from 2.5% to 6.5% in Punjab, India ,Chern and Beutler,(1976).

It is proposed that the effect of "early" and "delayed", re-infection by *P. faciparum* in the red blood cells obtained from the two groups of persons may be explained by differences in glucose-6-phosphate dehydrogenase and immunological components. High enzyme activity coupled with low immunological components favours "early" re-infection by *P. falciparum* whereas low-G-6-PDH activity with high immunological components results in "delayed" infection by the parasites.

It is suggested that further *in vivo* study of glucose-6-phosphate dehydrogenase be conducted to determine the critical activity that would limit the survival of the parasite *P. falciparum* in red blood cells from both groups of persons. A further study should be conducted to determine the concentrations of immunological components such as immunoglobulins, and proteins that participate in the immune response and relate them with enzyme concentration in the two groups of persons.

5:4 PYRIDOXAL KINASE ACTIVITY AND ITS CORRELATION WITH "EARLY" AND "DELAYED" RE-INFECTION BY *P. falciparum*

The results observed in the present study have shown that

the red blood cells obtained from persons exhibiting "delayed" re-infection by *P. faciparum* had a significantly lower mean pyridoxal kinase activity compared to those exhibiting "early" re-infection (Table 4).

Ching and Beutler, (1975) in their study on the activity of pyridoxal kinase in different races, found that American whites had activity of 1.37 ± 0.286, Afro-Americans had mean activity of 0.676 ± 0.282, Filipinos 1.074 ± 0.2.7, Asian Indians 1.119 ± 0.169 and Chinese 1.22 ± 0.339. These values indicate that pyridoxal kinase activity in red blood cells of American blacks was approximately 50% lower than that of American whites. It seems almost certain that the racial difference in pyridoxal kinase activity exists on a genetic basis with its correlation being influenced by the incidence of malaria prevalence. Afro-Americans have their origin in tropical Africa where there is high incidence of malaria infection. Their low pyridoxal kinase activity could have been a natural selection against malaria infection since lower activity of the enzyme is closely related to malaria protection. It may also be probable that lower activity of pyridoxal kinase which results in lower severity of malaria infection may be complemented by immunological components in the individuals concerned. The mechanism that may be operating could be affecting the synthesis of immunoglobin proteins which increase when synthesis of this enzyme decreases. The combined effect of lower pyridoxal kinase which the parasites requires and increased immunoglobins creates unfavourable condition for the multiplication and growth of the parasites.

The present results are in agreement with Ching and Beutller (1975) observations as red blood cells from persons who exhibited "early" re-infection had lower pyridoxal kinase activity than the red blood cells from persons exhibiting "early" re-infection. Though the two groups of persons inhabit the same geographical area, the "delayed" re-infection group could be likened to the Afro-Americans with lower enzyme activity thus "delayed" re-infection group could be compared to the white Americans with high enzyme activity thus high susceptibility to malaria infection.

Inyama, (1991) showed that *P. falciparum* lacks its own pyridoxal kinase enzyme. This enzyme participates in phosphorylating vitamin B6 into its active coenzyme pyridoxal 5-phosphate which then minimise the role of as a schiff base in several key biochemical reactions in the cell. An erythrocyte infected with a malaria parasite activity synthesizes several proteins needed by the parasite to complete its schizogonic cycle. An erythrocyte that lacks pyridoxal kinase or whose enzyme activity is low will inhibit the ability of the erythrocyte to synthesize vital proteins required by the parasite to complete its schizogonic cycle thus limiting parasite multiplication and growth. The effect of lack of this enzyme may be enhanced with immunological components as well.

As stated earlier, the difference in pyridoxal kinase activity in different races and individuals of the same race is basically genetic. It may be proposed therefore that the observation in this study between persons exhibiting "early" and "delayed" re-infection to *P. falciparum* could have occured due

to structural gene mutation coding for this enzyme with the "delayed" re-infection group having a lower enzyme activity and thus the protection against the malaria parasite, while the "early" re-infected group has high enzyme activity resulting in high susceptibility to malaria infection.

5:5 HAEMOGLOBIN VARIANT AND ITS RELATION TO *P. falciparum* INFECTION

The results from this study have shown that 31% of the persons whose red blood cells had "early" re-infection with *P. falciparum* had haemoglobin AS (sickle haemoglobin) compared to 75% of the persons whose red blood cells had delayed re-infection with the parasite. These results are in agreement with the work previously done by Gibbs *et.al.*, (1980) who found that in the general population 30% of the population had haemoglobin AS.

Friedman *et.al.*, (1979) defined the role of sickle haemoglobin (AS) in confering some protection against malaria infection. The high proportion (75%) of persons with haemoglobin AS in the "delayed" re-infection group agrees with previous studies which correlated delayance to malaria infection with presence of haemoglobin AS in red blood cells.

Allen *et.al*, (1992) observed that protection against malaria in children was due to sickle cell trait which they carried compared to those who did not carry the trait. Higgs *et.al*, (1981) also found that children who carried both α-thalassaemia and sickle-cell trait experienced less clinical malaria than children with other haemoglobin genotype including children who were heterogenous for HbS. Decreased invasion of red blood cells

containing sickle haemoglobin (HbS) under low oxygen tension, (Pasvol *et.al.*, 1978a) is probably due to a relatively rigid membrane which provide a unifying mechanism for decreased invasion of the cells compared to cells with normal haemoglobin.

The type of haemoglobin has significant influence on the re-infection of red blood cells by *P. falciparum*. High proportion of haemoglobin AS delayed the re-infection of the red blood cell by the parasite while high proportions of haemoglobin AA favoured the re-infection of the red blood cell by the parasite.

CHAPTER SIX

6:0 CONCLUSION AND RECOMMENDATION

The present study has shown that *in vitro* culture of *P. falciparum* being on artificial environment for the growth of the parasite is ideal for studying the develomental stages of the malaria parasites but it may not show the various differences that occur in different individual red blood cells *in vitro* as far as susceptibility to *P. falciparum* is concerned.The following conclusions were drawn from the study.

1) It was also shown that the levels of parasitaemia is not directly related to the growth rate of the parasites in the red blood cells from both "early" and "delayed" re-infected persons.

2) The study has shown a significant association between G-6-PDH activity in red blood cells with infection rate by *P. falciparum* with high activity favouring "early" infection whereas low enzyme activity inhibits parasite invasion resulting in "delayed" infection.

3) The results also show a significant association between pyridoxal kinase activity in red blood cells with infection time in culture by *P. falciparum*. High enzyme activity in red blood cells favours "early" infection by the parasite while low enzyme activity slows the multiplication and growth of the parasite hence "delayed" infection.

4) It has further been shown from the study that haemoglobin variant in the red blood cells will influence the invasion of the red blood cell by *P. falciparum*. Sickle haemoglobin (AS) confers

some protection against *P. falciparum* resulting in "delayed" infection by the parasite compared to the normal adult haemoglobin (AA) which favours the multiplication and growth of the parasite and hence "early" infection.

CHAPTER SEVEN

7:0 SUGGESTION FOR FURTHER STUDY

(1) Assessment of the critical levels of the enzymes G-6-PDH and *Pyridoxal kinase* in red blood cells from persons exhibitng "early" and "delayed" re-infection with *P. falciparum* that influence the "early" and "delayed" re-infection.

Knowledge of the critical levels of the enzymes needed by the parasites will contribute towards the treatment and management of the malaria parasites. This canbe achieved by developing multiple drugs that inhibit parasite development and also inhibit enzyme activity to a level that can met sustain the survival of the parasite.

(2) Determine the relationship between enzyme activity and immunological components such as immunoglobulin levels in the two groups of persons to establish if there is a complementary relationship between these factors in protectin against malaria.

APPENDIX:

PREPARATION OF STOCK SOLUTIONS

APPENDIX 1: PRAPARATION OF (3H) HYPOXANTHINE STOCK SOLUTION.

The [^{3}H] Hypoxanthine stock solution was prepared by mixing 0.5 µl Ci[G-^{3}H] Hypoxanthine, specific Activity 1 Ci/ mmol with CMS in a ratio of 1:48 (ie 1 µl [^{3}H] Hypoxanthine: 62.5 µl CMS) respectively.

APPENDIX 2 :PREPARATION OF ACID CITRATE DEXTROSE.

The anticoagulant was prepared as follows: sodium citrate (2.2g), citric acid (0.8g) and D-glucose (2.45g) were dissolved in distilled water and the volume made up to 100m

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