A STUDY OF ENZYME VARIATION AND CHLOROQUINE SENSITIVITY OF PLASMODIUM FALCIPARUM IN VITRO

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DECLARATION

I hereby declare that this dissertation is my own work and that it has not been previously submitted for Degree purposes here or at any other University.

CONTENTS

Chapter 2, Experimental

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ABSTRACT

Blood samples from children with malaria were used to set up *in vitro* cultures of *Plasmodium falciparum* using fresh human serum to enrich the culture medium. A number of investigations aimed at improving the *in vitro* cultivation technique were carried out. Changing culture medium two times every 24 hours instead of once had a positive effect on the final parasitemia level. Any effect of extra glucose on parasite growth was not obvious within the two asexual cycles period considered. A number of human sera treated at 56° - 58°C for 30-45 minutes to destroy complement resulted in a parasitemia twice that obtained with ordinary sera. A further observation on change of serum was made on a continuous culture which had been maintained in two different human sera for the first 37 days *in vitro*. The cultures where serum was changed displayed a marked reduction in parasitemia though this was only temporary. However, when heat treated serum was used no decrease in parasitemia was apparent.

Starch-gel-electrophoreses of different isolates of *P. falciparum* from Zambia revealed enzyme variation in glucose phosphate isomerase (GPI), 6-phosphogluconate dehydrogenase (6-PGD), and adenosine deaminase (ADA).

Parasite lactate dehydrogenase (LDH) and peptidase-E (PEP-E) showed no variation. A rare enzyme form, 6-PGD-2, was observed at a frequency of 80% but a standard is required to confirm the enzyme type.

Five different isolates of P. falciparum were subjected to different low concentrations of chloroquine in vitro. Their growth was successfully inhibited by this drug and they were classified as being sensitive. Parasite growth was markedly reduced after 48 hours of drug administration and completely inhibited by 72 hours although for the last 24 hours culture was in drug-free medium.

ABBREVIATIONS

ADA	Adenosine deaminase
EDTA	Ethylendiaminetetraacetic acid
G-6-PD	Glucose-6-phosphate dehydrogenase
GPI	Glucose phosphate isomerase
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethane sulfonic Acid
LDH	Lactate dehydrogenase
MTT	{3- (4,5-Dimethyl Thiazolyl)-2,5-diphenyl Tetrazolium Bromide}
NAD^+	β-Nicotinamide adenine dinucleotide
$NADP^+$	β-Nicotinamide adenine dinucleotide phosphate
PEP-E	Peptidase-E
6-PGD	6-phosphogluconate dehydrogenase
PMS	Phenazine Methosulphate
RP-15-S	RPMI 1640 complete medium with bicarbonate and HEPES plus 15% human serum
TES	(N-tris {Hydroxymethyl} methyl-2-aminoethane sulfonic Acid)
Tris	Tris (Hydroxymethyl) Aminomethane

CHAPTER 1

I N T R O D U C T I O N

1.1 MALARIA AND ITS HISTORY

Malaria is a disease characterized by intermittent fevers, anaemia, and splenic enlargement (Russell et al., 1963). It is caused by parasites of the genus Plasmodium and class Sporozoa. Four species of plasmodia infect man:- P. falciparum, P. malariae, P. ovale and P. vivax. The word malaria comes from two Italian words mala and aria meaning bad air. The causative organisms of malaria were discovered in the nineteenth century. A French army surgeon, Charles Laveran, was the first man to see and describe malaria plasmodia as parasites in 1880. His observations were received with scepticism initially, however other scientists observed similar parasites later and the generic name Plasmodium was coined in 1886.

Mosquitoes were suspected to be the vectors of malaria long before the discovery of plasmodia. Although Patrick Manson was the first to put forward a hypothesis of mosquitoes acting as intermediate host to plasmodia in 1894, through his experiments with filaria, it was not until 1899 that Ronald Ross proved this to be the case. Ross was able to demonstrate elegantly the link between the two hosts, using bird malaria. He showed that gametocytes contained in a blood meal developed in the gut of anopheline Culex mosquito, and the resulting mature parasites (sporozoites) could then reinfect another bird. Ross then made similar observations on human malaria in Sierra Leone. At the same time Grassi, Bignami and

Bastianelli proved that mosquitoes were indeed vectors of human malaria by successfully following the cycle of falciparum plasmodia in Anopheles, and later that of P. vivax and P. malariae.

European physicians learned in the seventeenth century that the cinchona bark of a South American tree, Myroxylon peruiferum, was an effective remedy for intermittent fevers. The alkaloids of cinchona bark (quinine and cinchonine) were first isolated by two French pharmacists Pelletier and Caventou in 1820. Quinine was prepared commercially in the United States in 1823 but it was not until 1944 that Woodward and Doering first synthesized quinine. By 1944 quinine was used for malaria prophylaxis. The alternative means of reducing incidence of malaria was to destroy the second host, the mosquito, particularly by spraying breeding grounds. Insecticides like dichlorodiphenyltrichloroethane (DDT), benzene hexachloride (BHC) and dieldrin were discovered to have residual insecticidal properties during the second world war. Pyrethrum which had been discovered a few years earlier and was effective against adult mosquitoes was temporarily discarded for these better insecticides. Malaria eradication programmes utilized the three newly discovered insecticides with great success at first especially in South America, United States of America, Italy and Ceylon. However by 1951 some species of mosquito were reported to be resistant to DDT and the other two insecticides.

Chloroquine and other 4-amino-quinolines were also discovered during the second world war and have been in use ever since for malaria chemoprophylaxis.

1 Life Cycle

All malaria species require a vertebrate and an invertebrate host. Infection of the vertebrate host is initiated by a bite of a mosquito and the injection of sporozoites into the blood. The sporozoites so introduced are carried by the blood to various organs and tissues of the body. In mammals, the sporozoites invade the liver parenchymal cells where they divide asexually to form many daughter cells known as merozoites. Eventually, the merozoites are released from the tissues into the blood circulation where they invade the red blood cells. (see figure 1.1a).

The erythrocytic asexual life cycle of plasmodia involves three different parasite forms known as merozoites, trophozoites and schizonts. A very tiny ring form consisting of chromatin and cytoplasm inside an erythrocyte initiates the cycle of the malarial parasite in the blood. Each parasite grows and divides asexually within an erythrocyte, in a process called schizogony to produce a number of merozoites. Reinvasion of erythrocytes takes place when the erythrocyte ruptures, thereby releasing merozoites. Periodic fever-chill cycles accompany the synchronous asexual development and rupturing of erythrocytes by the parasites.

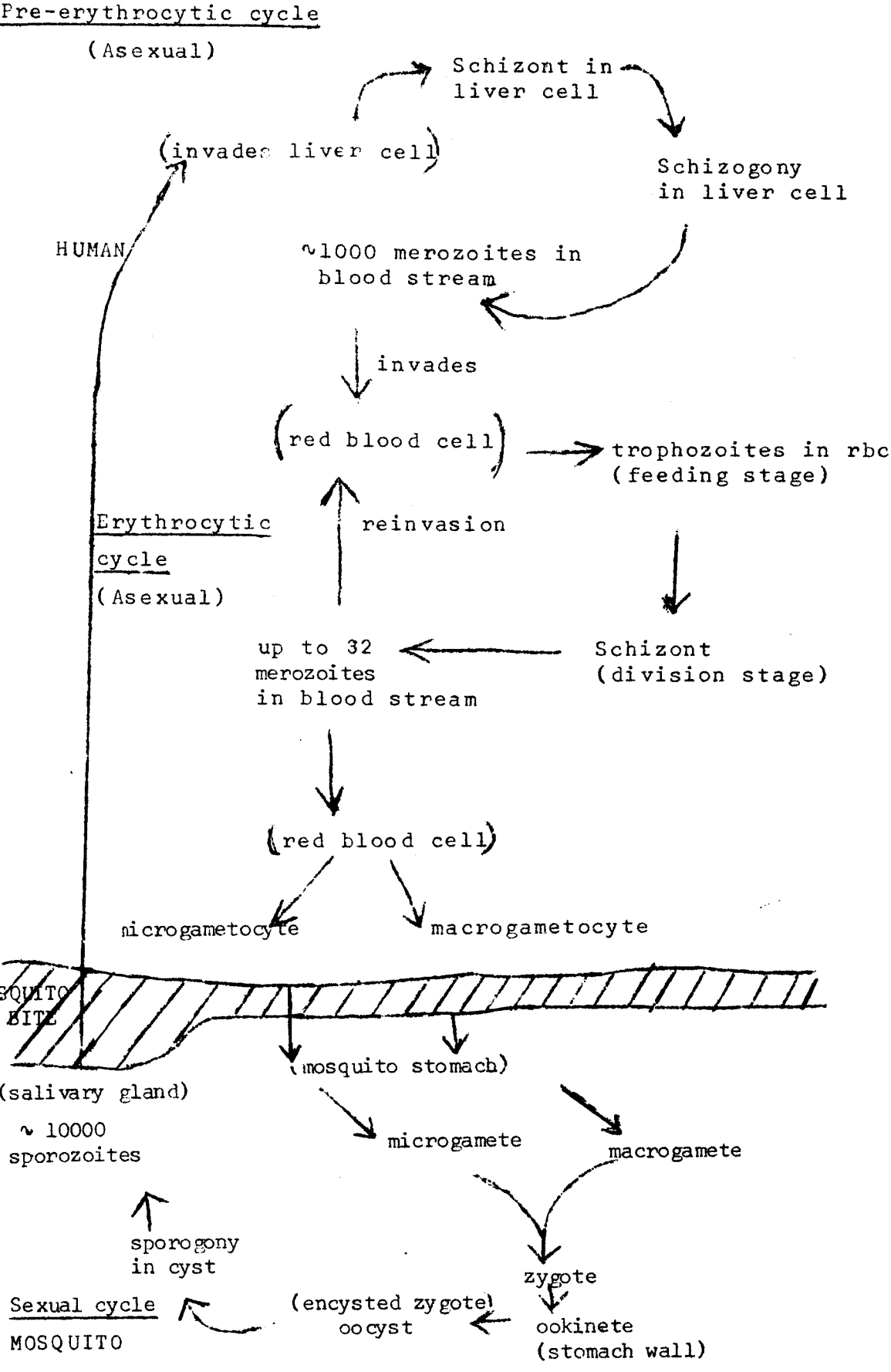


Figure 1.1a Life Cycle of the Malaria Parasite

It is not all merozoites that continue the synchronous cycle; some develop into sexual stages (gametocytes) within the invaded erythrocyte. A sexual cycle is initiated in the mosquito when the gametocytes are ingested during a blood meal. The gametocytes differentiate into gametes in the mosquito stomach; fertilization occurs and a zygote is formed. Sporozoites develop within the zygote by asexual multiplication.

.1.2 Morphology and growth of the blood stages

Histological staining methods reveal the general morphology of malarial parasites. Observing a stained blood film under the light microscope shows a clear cytoplasm and nucleus of the parasite. Electron micrographs make it possible to see the ultrastructure in detail (figure 1.1b).

Entry of malarial parasites into the host cells is aide by rhoptries and micronemes. The motile forms (merozoites) enter the host cell by invaginating the host cell membrane (Aikawa and Sterling, 1974). Kilejian (1976) isolated a histidine-rich protein from Plasmodium lophurae which was thought to be found in the rhoptries and micronemes and interaction of this protein with the erythrocyte membrane results in invagination. A similar protein was characterized by Kilejian and Jensen (1977) from P. falciparum. Miller (1977) suggested that the possible sequence of events in the invasion of erythrocytes by merozoites was:

(1) Attachment

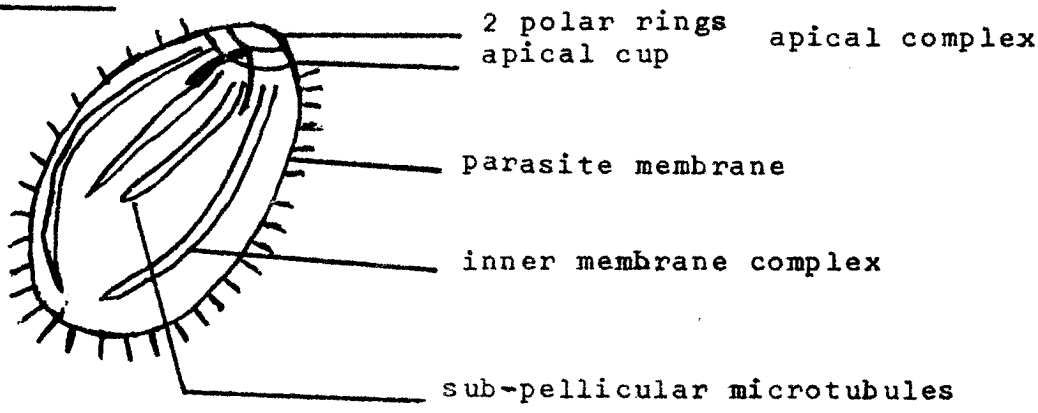
(2) Deformation of the erythrocyte membrane

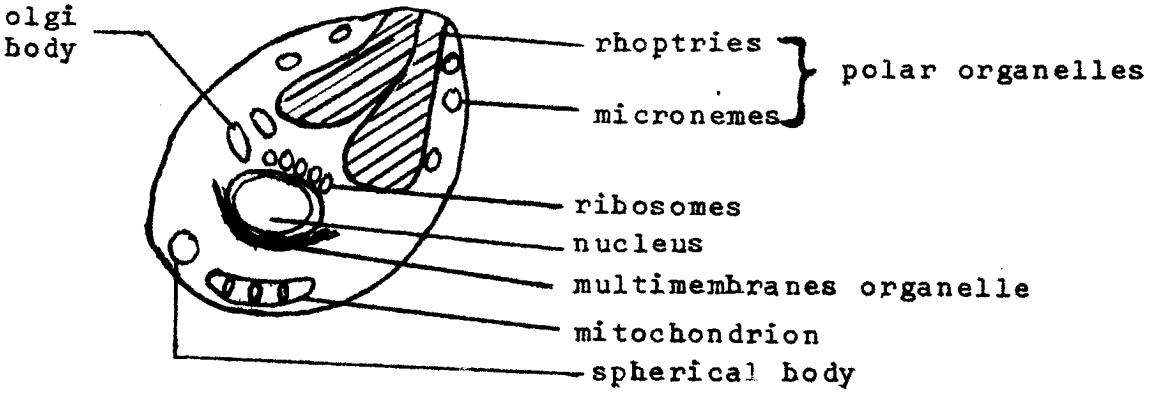
(3) Endocytosis

(4) Resealing of the membrane and vesicle.

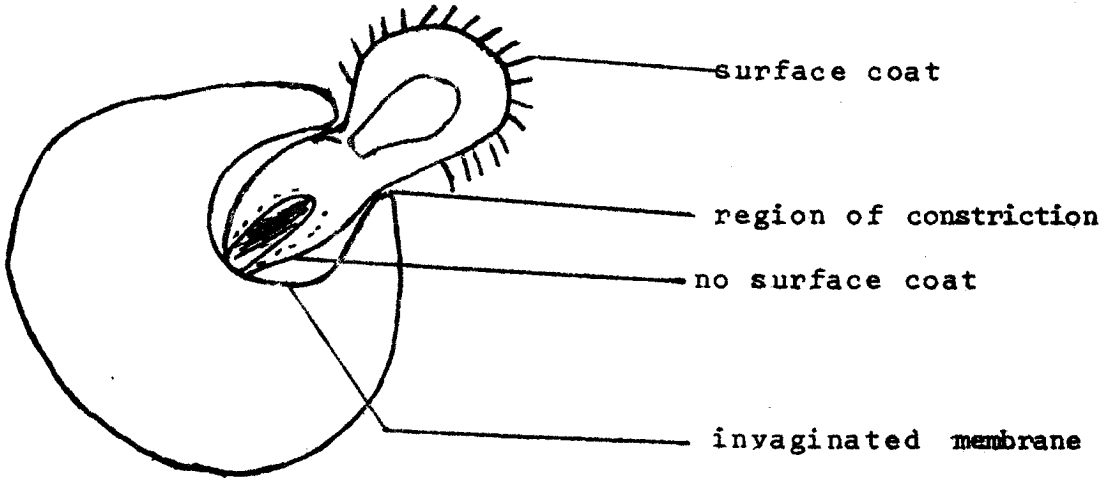
The host cell membrane invaginates and engulfs the merozoite as soon as it enters the host cell, thereby forming a parasitophorous vacuole (Bannister et al., 1975). A merozoite loses its rhoptries, micronemes and pellicular membranes when it invades an erythrocyte. An amoeboid trophozoite which is uninucleate results and lies within the parasitophorous vacuolar membrane (PVM). From here, it starts to ingest host cytoplasm using the cytostome (Aikawa, 1966; Langreth, 1976). Vacuoles are formed within which host haemoglobin digestion takes place and hemozoin (malaria pigment) is formed. The trophozoite feeds and increases in size and its nucleus enlarges. Ultimately, the trophozoite differentiates into a schizont which gives rise to merozoites by schizogony. In P. falciparum 32 merozoites can result, but normally only 8-24 are seen. The process of schizogony involves the formation of mitotic spindles, division of the nucleus, laying down of pellicular structure, development of rhoptrymicronemes at the periphery, and incorporation of nuclei and mitochondria into the divided cytoplasm.

Merozoite

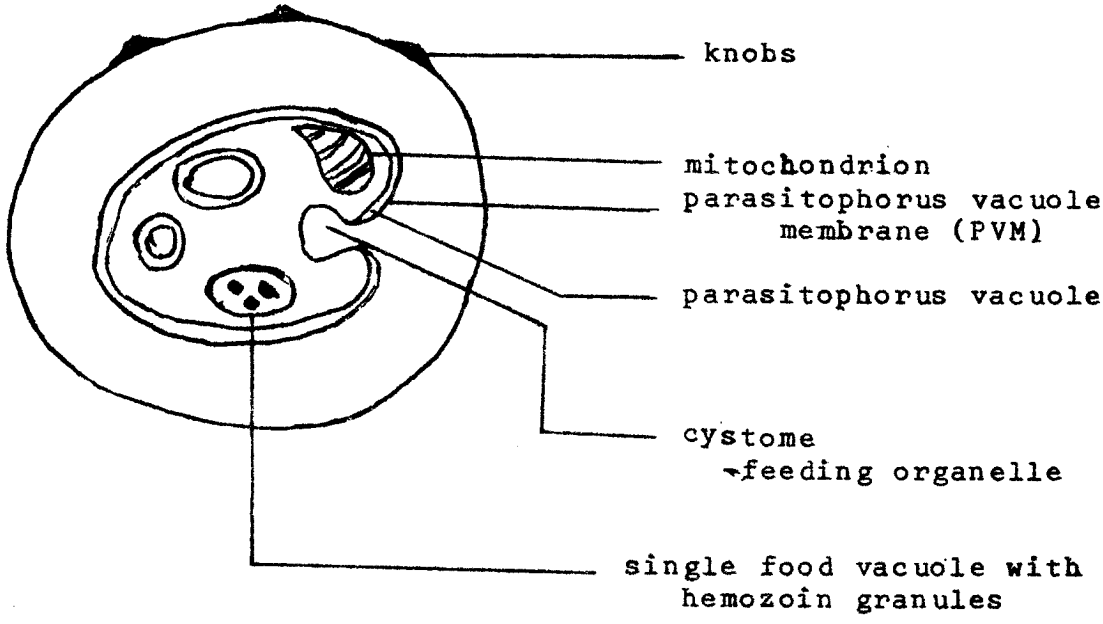




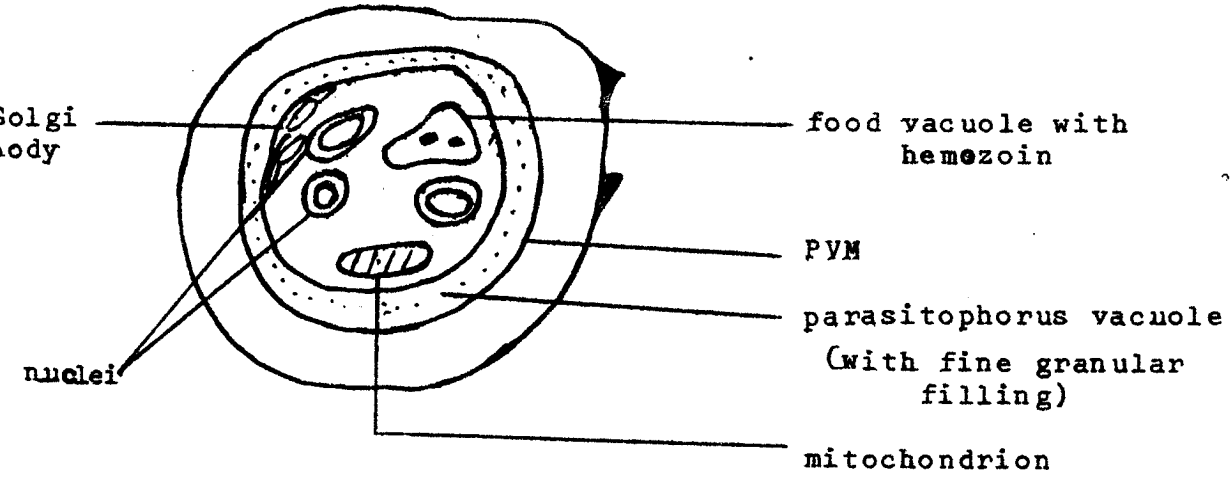
Penetration



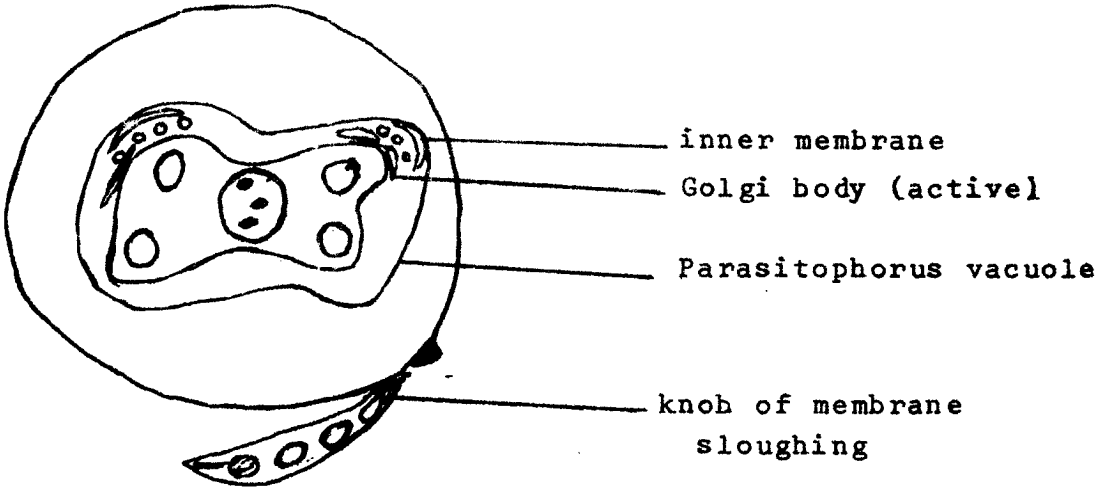
Trophozoite (Feeding stage)



Young Schizont



Schizont



1.3 Distribution of Malaria in Zambia

Plasmodium falciparum accounts for over 96% of the different species of human malaria in Zambia (Hira and Koularas, 1974). The remaining fraction constists mainly of *P. malariae* and a small proportion of *P. ovale*. These authors reported a very minute fraction of *P. vivax* though similar work done in two different areas of Zambia has shown no presence of this species (personal communication, Tropical Diseases Research Centre).

P. falciparum is found mainly in the tropical and subtropical regions where the temperatures are above 20°C most of the year. It is the most pathogenic of all human malaria species because it causes cerebral malaria, a condition which is sometimes fatal, especially in children. Elamin (1981) has shown that *P. falciparum* malaria can be fatal in adults, causing malarial encephalopathy. This author found cerebral malaria in adult Zambians to be common (7.4%). He also observed low values of immunoglobulins in the serum and cerebral spinal fluid of these patients.

The choice of *P. falciparum* for this study was due not only to the prevalence and pathogenicity but also to the success of the *in vitro* cultivation technique using this species.

The erythrocytic stages of other human malarial parasites have not been successfully cultivated *in vitro* except *P. malariae* in a mixed infection with *P. falciparum* (Chourdhuri et al., 1979). Nguyen-Dinh et al. (1981) have managed to grow *P. cynomolgi in vitro*; this is a vivax-type malarial parasite.

1.2.1 IN VITRO CULTIVATION OF THE BLOOD STAGE OF PLASMODIUM FALCIPARUM

The initial attempts to grow the erythrocytic stages of *Plasmodium falciparum in vitro* were made by Bass and Johns (1912). They observed the development of this malarial specie from ring form to schizont stage in a static layer of whole blood enriched with glucose. Unfortunately, the parasites developed through one asexual cycle only. Rieckmann et al. (1968) and Rieckmann (1971) modified this simple technique into a test system for studying antimalarial drug action *in vitro* in the field.

Later on, several other investigators managed to carry out a number of studies using short-term cultures (Cohen and Butcher, 1970; Trigg, 1976). Biological, biochemical, and physiological data obtained from parallel experiments on parasites maintained *in vivo* and *in vitro* were used to develop

methods for the in vitro cultivation of malaria parasites (Geiman et al., 1946; McKee et al., 1946; Trager, 1941). Insufficient knowledge of the biochemistry of the parasites and the blood and its components upon which the parasite depends, contributed to the slow rate of progress at which in vitro cultivation techniques were developed.

Most of the information on the biochemistry of malarial parasites was obtained using P. knowlesi. Harvard growth medium which was used for the original work on the in vitro cultivation of P. knowlesi (Ball et al., 1945) was based on chemical analysis of monkey plasma. Trigg (1968) and Cohen and Butcher (1971) obtained improved growth and multiplication of P. knowlesi using modified Harvard medium. Certain substances were identified as essential for growth of P. knowlesi, and a simplified medium was developed (Siddiqui et al., 1970; Trigg and Gutteridge, 1971).

Balanced salt solution to provide the correct ionic environment for the cultured cells, and a buffering system to maintain physiological pH are the basic requirements for a culture medium. All media that have given satisfactory results utilize a hydrogen carbonate and carbon dioxide system which keeps the medium pH at 7.4 and allows carbon dioxide fixation by the parasites (Sherman and Ting, 1968). Supplementing the growth medium with zwitterion buffers such as HEPES (N-2-Hydroxyethylpiperazine-N´-2- ethanesulfonic Acid)

or TES (N-tris [Hydroxymethyl] methyl-2-aminoethane sulfonic Acid) not only increases the buffering capacity of the medium but also aids in keeping physiological pH when cultures are removed from the carbon dioxide and air mixture.

The requirements of the parasite have been identified by detailed studies on parasite metabolism. A suitable in vitr medium must have similar physical and chemical properties to those of the host plasma. The most successful is a general tissue culture medium, RPMI 1640, however it lacks certain essentials which can only be provided by host serum.

Early in 1976 Jensen and Trager obtained a continuous culture of P. falciparum in vitro using a settled layer of human erythrocytes in RPMI 1640 culture medium supplemented with HEPES buffer (Trager, 1980). A gas phase of 2-3% carbon dioxide, 10% oxygen, 87% nitrogen was used and a provision was made for the slow flow of medium over the layer of erythrocytes. Improvement in growth rates was noted using small Petri dishes and manual change of medium. A vacuum desiccator, plus extinguished candle, was used to provide the correct carbon dioxide concentration. The Petri dish candle jar method though simple has been used in a number of studies on Plasmodium. These include the effect of antimalarials, investigation of parasite metabolism, effect of changes in culture medium, growth of the parasite in genetic variant erythrocytes (e.g. cells with sickle haemoglobin

or deficient in Glucose-6-phosphate dehydrogenase), and constituent isoenzymes and proteins.

Since the discovery of the Petri-dish candle jar method of Trager and Jensen (1976), tremendous improvements have been made to the technique to achieve high parasitemias in vitro (Chin, 1979; Osisanya et al., 1981; Butcher, 1976; Trager, 1979). These high parasitemias in vitro are necessary for biochemical investigations and large-scale production of antigens for a possible vaccine against malaria.

It is now possible to look into the aspects of the mode of action of drugs without the interfering effects of the host metabolism, and a higher concentration of drug can be used compared to that achieved in vivo. Parasite viability after drug pressure can also be checked.

The success of Trager and Jensen's technique has been confirmed in many other laboratories. Several isolates of P. falciparum have been maintained in continuous culture by this technique. The initial work used a South East Asian strain (FVO) which was originally passed through Aotus monkeys. Isolates from several parts of the world have since been propagated in vitro for periods ranging from a few weeks, months and some for several years. The ease with which some of the isolates can be maintained seems to vary (Jensen and Trager, 1978).

A number of investigations have been carried out on several factors related to the *in vitro* cultivation of *P. falciparum* (Jensen, 1979; Butcher, 1979; Siddiqui, 1980; Pasvol et al., 1980).

.2 Serum

Substitution of human serum by a commercially available serum has been pursued extensively because of non availability of suitable human serum in large amounts, some growth inhibitory effects encountered in the *in vitro* propagation of *P. falciparum* in malaria endemic areas, and the potential hazard of viral contamination.

After testing a number of different commercial sera, Jensen (1979) concluded that human serum was still the best for the continuous growth of *P. falciparum*. However, Siddiqui (1980) reported the successful substitution of human serum by commercial calf serum supplemented with human red cell extract fraction I for continuous cultivation of *P. falciparum in vitro*.

.3 Erythrocytes

Blood stored in acid-citrate-dextrose (ACD) for 14-35 days support growth of *P. falciparum in vitro*. Outdated blood from the Blood Bank is an ideal source of this. Jensen and

Trager (1977) found that red cells stored in ACD for 21-28 days supported growth of P. falciparum better than fresh cells. Butcher (1979) could not confirm the above finding. He found insignificant differences between the fresh cells and outdated ones in terms of supporting growth of P. falciparum in vitro.

Pasvol et al. (1980) noticed an increased susceptibility of reticulocytes and young red blood cells to invasion by P. falciparum. The author cautions investigators to be carefu when drawing conclusions on comparative studies of parasite counts of P. falciparum in vitro in as far as use of red blood cells of different ages is concerned. The possible reasons why young red cells are most susceptible to parasite invasion are:

(a) Ageing of red cells might be associated with loss of receptor sites on the red cell membranes,

(b) Young red cells are metabolically more active than old ones

(c) Red cells become progressively less deformable as they age.

Udeinya et al. (1981) showed that P. falciparum - infected erythrocytes bind specifically to cultured human endothelial cells by knoblike protrusions on the surface of the infected red cells. Loss of the knobs was seen on the surface of erythrocytes infected with trophozoites and later stages of

P. falciparum as reported by Trager (1980). The knobs on *P. falciparum* have been shown to be antigenic (Kilejian et al., 1977; Langreth and Reese, 1979).

4 Oxygen

21% oxygen is inhibitory to *P. falciparum* although this effect is noticeable only after several days (Butcher, 1979). The required range seems to be 5-10%. Trager and Jensen (1976) utilized 5% O_2, 7% CO_2 and 88% N_2 in their first successful continuous culture of *P. falciparum in vitro*.

1.3.1 METABOLISM OF PLASMODIUM

Basic nutritional requirements of malarial parasites are: glucose, malate, amino acids, purines and pyrimidines, p-aminobenzoic acid and vitamins. All malarial parasites have glycolytic enzymes of the Embden-Meyerhoff pathway. There is no complete sequence of glycolytic enzymes for any one malarial species though all the enzymes have been found in one species or another. In general, malarial parasites utilize mechanisms which are similar to those used by a number of other eucaryotes for the breakdown of glucose. Plasmodia growth and reproduction depends on simple sugars. *P. falciparum* can utilize glucose or maltose and produces lactate (Sherman, 1979).

Early biochemical studies on carbohydrate utilization in a number of malarial species, showed rapid disappearance of glucose and several other sugars including glycerol in suspensions of infected red blood cells, accompanied by an increase in oxygen uptake (Homewood, 1977). Glucose metabolism in different species of Plasmodia gives rise to different end products. Mammalian malarial parasites oxidise glucose to organic acids but in birds carbon dioxide as well as organic acids are produced.

The presence of citric acid cycle has only been demonstrated in rodent and avian malaria e.g. malic dehydrogena (Sherman, 1966) and succinic dehydrogenase (Seaman, 1953). Bicarbonate fixation has been demonstrated in several malaria species (Sherman, 1977). The pentose phosphate cycle is also active in Plasmodia. The presence of 6-phosphogluconate dehydrogenase has been widely documented and recently parasite glucose-6-phosphate dehydrogenase has been shown using polyacrylamide gel electrophoresis (Hempelmann and Wilson, 1981).

Amino acids for protein synthesis are mainly obtained from haemoglobin breakdown, using plasmodium-specific proteases However some incorporation of amino acids from the host plasma occurs, as shown in P. knowlesi (Sherman, 1977), and P. falciparum (Lovelace, 1976). Walsh and Sherman (1968) demonstrated that DNA synthesis by P. lophurae was not dependen

on the host cell nucleus. Konigk (1977) proved that plasmodia are unable to synthesize purines de novo. Nevertheless, plasmodia have the capacity for the de novo synthesis of pyrimidines.

.2 Genetics of Plasmodium

Investigation into the possibility of strain variation in P. falciparum began almost a decade ago (Carter and McGregor, 1973; Carter and Voller, 1975). However, this has not been shown in Southern Africa as yet, but in Gambia a report has been given on enzyme variation in P. falciparum (Carter and Walliker, 1977). A more recent study on enzyme patterns of P. falciparum using starch gel electrophoresis of six enzymes, has provided a comparative view of the genetic distribution of this species of malaria (Sanderson et al., 1981; Thaithong et al., 1981). The isolates used in this study were from Gambia, Tanzania, Congo, Thailand and Cambodia. Although enzyme polymorphism has been demonstrated, in particular using glucose phosphate isomerase, no evidence of genetic variation among the isolates tested was seen.

Enzyme polymorphism is common in some murine malarial parasites (Carter, 1978). This has been used as a tool in taxonomy since numerous subspecies of rodent malaria exist and identification based on parasite morphology is sometimes

difficult. Some enzyme forms have been shown to exist only in particular isolates.

A more sensitive technique of 2-dimensional polyacrylamide gel electrophoresis developed by O'Farrell in 1975, ha been used to detect variation in proteins of P. falciparum (Tait, 1981). Isoelectric focusing separates the proteins according to their isoelectric point in one dimension, and then sodium dodecyl sulphate-polyacryamide gel electrophoresi (SDS-PAGE) separates them according to molecular weight in th second dimension. The author took advantage of the inability of mature erythrocytes to synthesize proteins to preferential allow incorporation of ^{35}S-methionine into parasite protein. Autoradiography revealed variation in four different proteins between Gambian and South East Asian isolates; isolates from the same area were also seen to vary in a number of proteins.

.3.2 Isoenzymes

Isoenzymes are multiple molecular forms of an enzyme occuring in a single organism. The different proteins constituting a set of isoenzymes have similar but not necessa identical enzyme properties. For instance, they may catalyze the same reaction but differ in their kinetics. Although the may occur in the same cell, there may be differences in isoenzyme patterns between cells of different tissues or at

different developmental stages. Lactate dehydrogenase in man, is a good example of this phenomenon. Heart, kidney, liver and skeletal muscle shows five isoenzymes whereas red blood cells have only three isoenzymes. The amount of each isoenzyme differs also for example LDH-5 is the most intense band in liver and muscle but in heart and kidney it is LDH-1.

The term "isoenzymes" was first introduced by Markert and Moller in 1959. The use of zone electrophoresis in starch gel or acrylamide as a supporting media, and the development of sensitive and specific staining methods for a particular enzyme protein, have contributed considerably in the discovery of several isoenzyme systems. The first procedure for esterases was developed by Hunter and Markert (1957) and for dehydrogenases by Markert and Moller (1959).

The greatest advantage of enzyme electrophoresis technique is that crude homogenates of fresh tissues can be used directly without further purification and only small quantities are required. Early experiments on isoenzymes involved cutting of supporting matrix after electrophoresis, eluting the enzyme and assaying the resulting fractions for enzyme activity. Application of specific enzyme-staining methods for direct detection of individual isoenzymes in situ in the electrophoretic matrix, quickly supplanted the above approach.

Many enzyme proteins are made up of two or more polypeptide chains or subunits (multimeric). Homomeric isoenzymes have identical amino acid sequence whereas heteromeric isoenzymes differ. Generally, the number of subunits in the isoenzyme molecules and the number of structurally different polypeptide chains being synthesized will determine the pattern of primary isoenzymes seen after electrophoresis. Symmetrical isoenzyme patterns are observed if the polypeptides determined by the two alleles make equal contributions to the enzyme activity and there is a random combination of subunits. When the polypeptide chain coded by one of the alleles contributes less to the total enzyme activity, then asymmetrical isoenzyme patterns are seen. Identification of a variety of isoenzyme systems in numerous different organisms has been made possible by use of the general procedures for isoenzyme detection, in combination with other techniques at times (Wilkinson, 1968; Brewer, 1970). The phenomenon of multiple forms exists in non-enzymic proteins as well.

The generation of such multiple forms of a given enzyme or protein can be in a number of different ways. Harris (1969) classified the different possible causes of multiple molecular forms of functionally similar proteins into three categories:-

(a) Multiple gene loci coding structurally distinct *polypeptide chains of a protein*

Two or more gene loci may be involved in the determination of a set of isoenzymes or of functionally similar non-enzymic proteins. For example, three distinct loci ('A', 'B' and 'C') specify the various molecular forms of lactate dehydrogenase in man.

Each gene locus codes for the amino acid sequences of distinctive polypeptide chains. The several polypeptides may be associated together in the various members of the set of isoenzymes. These may be the individual isoenzymes, or several polypeptides could be associated together in various combinations. In the latter case, lactate dehydrogenase isoenzymes is an example (A_4, A_3B, A_2B_2, AB_3, B_4).

(b) Multiple alleles at a single locus

A number of different alleles may occur in a population of individuals at a given gene locus. Each allele codes for a structurally distinct form of the particular polypeptide chain, resulting in a different primary structure of the enzyme or

protein concerned in different individuals, depending on the allele present at the particular locus.

Generally, isoenzyme differences in a single individual due to presence of multiple gene loci, involve a greater amount of molecular difference than isoenzymes arising as a result of multiple allelism at a single locus. Multiple allelism results in differences between individual members of the population in the isoenzyme patterns they form.

(c) Secondary modifications of protein structures

The complexity of many isoenzyme systems cannot be explained in terms of multiple gene loci and multiple allelism at a single locus alone. There appears to be secondary modification of protein structures after primary synthesis of their polypeptide chains on the mRNA templates in the ribosomes. These secondary modifications may involve the removal of amide groups from glutamine or asparagine residues in the polypeptide sequences, the oxidation of sulphydryls, the addition of phosphate groups, the addition of

carbohydrate groupings of various structures, the cleavage of a polypeptide chain by proteolytic enzymes with loss of part of the amino acid sequence, and so on.

Appearances of multiple molecular forms of a particular enzyme or protein can also be due to differences in their three-dimensional conformation. These conformational isomers can exhibit different electrophoretic mobility because of the extent to which particular groups are exposed or concealed in their three-dimensional arrangements.

Electrophoresis of Enzymes

Several techniques can be employed in enzyme electrophoresis though gels are the most widely used supporting matrix. Horizontal or vertical starch gel electrophoresis, acrylamide gel, agarose gel and cellogel are commonly used. The fine gel matrix of starch or acrylamide has a molecular sieving effect which has an influence on the isoenzyme separations in terms of the net ionic charge and the molecular size differences. Molecular sieving effects in agarose gel or cellogel are minor because the gels are quite porous. Since enzymes are ampholytes they may possess a net negative or positive charge depending on the pH of the buffer in the supporting medium matrix.

The rate of migration depends on the net charge on the enzyme molecule and the electrical field strength applied through the electrodes. The migration speed is influenced by the pH of the buffer utilized during separation while the strength of the electrical field is dependent upon the voltage applied.

3.5 Detection of Isoenzymes

A variety of staining methods have been developed which fall into six groups:-

(1) Chromogenic staining methods

(2) Fluorogenic staining methods

(3) Radioactive staining methods

(4) Chemical detection staining methods

(5) Electron transfer dye staining methods

(6) Enzyme-linked staining methods.

In this work five enzymes are being investigated. These enzymes have been determined by other workers to be fairly easily detected in P. falciparum.

1.4 CHLOROQUINE

Chloroquine, also known as Resochin was first synthesised in 1934, in Germany, but was abandoned initially because it was thought to be too toxic. The second of the 4-aminoquinolines to be synthesised was sontochin (sontoquine). A series of clinical tests carried out during the second world war using these two drugs and other 4-aminoquinolines led to the selection of chloroquine and amodiaquine (Camoquin) as the best drugs for malaria prophylaxis and therapy. Both drugs are effective against asexual erythrocytic stage. They are effective against P. malariae and P. vivax gametocytes but not those of P. falciparum when mature. However, Sinden (1982) showed that immature gametocytes of P. falciparum are killed by chloroquine at a concentration of 0.1 nmole ml^{-1} (1×10^{-7} M).

Chloroquine and amodiaquine have superseded quinine and mepacrine in malaria treatment mainly because of:

(1) fast action on the asexual forms

(2) low toxicity

(3) lengthening the latency periods.

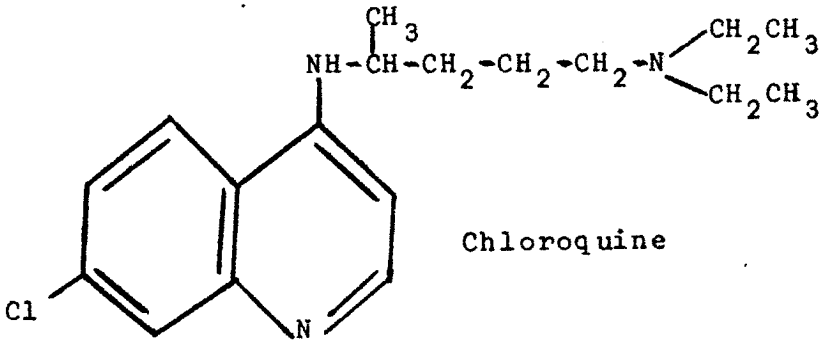
Both drugs are absorbed rapidly from the gastrointestinal tract and are concentrated in various tissues from which they are slowly metabolized and excreted.

Chloroquine has some minor side effects like nausea, slight headache and vomiting; some patients display an allergy reaction after chloroquine administration but this is only temporary.

1 Mode of Action of Chloroquine

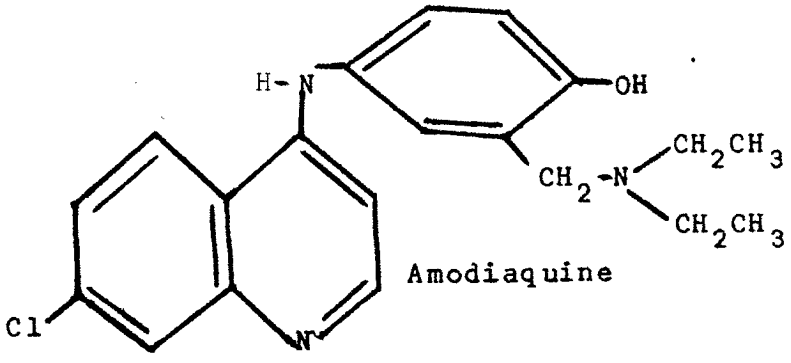
There are a number of postulates on the possible mechanism of action of chloroquine. Administration of 4-aminoquinolines and mepacrine is followed by hemozoin clumping, a phenomenon associated with the concentration of the drug in the parasite. Macomber et al. (1967) and Warhurst and Hockley (1967) suggested that the food vacuole where hemoglobin is broken down might be the first organelle to be affected by chloroquine, followed by changes in the nucleus and nucleolus. Interference with the digestive process leads to acute amino acid deprivation and cytolysosome formation Howells et al. (1970) suggested that this amino acid starvation is the primary effect of chloroquine on the malaria parasite.

Hahn et al. (1966) postulated that the quinoline ring of chloroquine is intercalated between the base pairs of double-stranded DNA. The binding affinity of chloroquine for DNA from P. knowlesi is of the same magnitude as that for DNA from the mammalian host (Cutteridge et al., 1972), this made Konigk et al. (1981) conclude that the binding of chloroquine to DNA is of no prime importance.



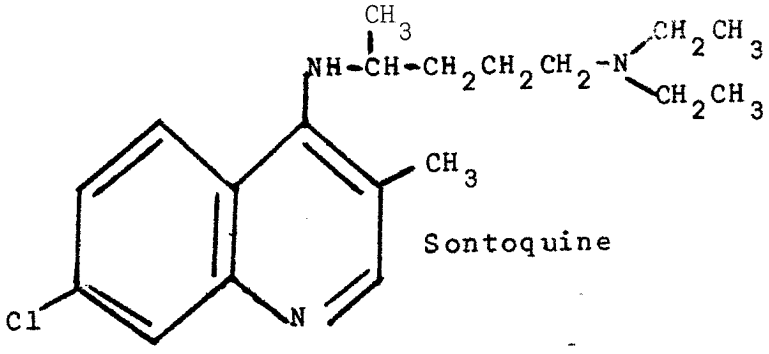
Chloroquine

7-Chloro-4-(4 -diethylamino-1 -methylbutylamino) quinoline



Amodiaquine

7-Chloro-4-(3 -diethylaminomethyl-4-hydroxyanilino) quinoline



Sontoquine

7-Chloro-4-(4-diethylamino-1 -methylbutylamino)-3-methyl-quinoline

Konigk et al. (1981) treated P. chabaudi - infected mice erythrocytes with chloroquine, mefloquine, primaquine, or floxacrine and showed that these drugs inhibit ornithine decarboxylase activity. This enzyme is inhibited by a number of amines. At pH 6-7 chloroquine is analogous to some polyamines since it occurs mainly as a divalent cation. The authors suggest that chloroquine induced changes of endomembrane structures of P. berghei (Ladda, 1966) and P. chabaudi (Wunderlich et al., 1981) may be due to lack of polyamines resulting from the inhibition of ornithine decarboxylase by chloroquine.

Chou et al. (1980) have identified the chloroquine receptor of malarial parasites as being ferriprotoporphyrin IX. The authors used mouse erythrocytes treated with a nonspecific protease from Streptomyces griseus or infected with chloroquine - sensitive Plasmodium berghei and demonstrated that these cells have a saturable process for the accumulation of chloroquine. Haemoglobin was found to be the substrate from which the receptor is created; the receptor is an aggregate of ferriprotoporphyrin IX. The saturable process for the accumulation of chloroquine was found to be present in drugs of the same class as chloroquine. The authors observed haemoglobin degradation and accumulation of malaria pigment in erythrocytes infected with chloroquine - susceptible P. berghei.

However, this process is not seen in erythrocytes infected with chloroquine - resistant P. berghei except when they revert to chloroquine susceptibility.

4.2 Drug Resistance

'Drug resistance is a state of insensitivity or of decreased sensitivity to drugs that ordinarily cause growth inhibition or cell death' (Goldstein et al., 1968 p. 508). Although chloroquine is widely used for chemoprophylaxis with great success, there is an alarming increase in the rate at which drug resistance is appearing. It is feared that this problem is spreading from South America and South East Asia to Africa.

An African strain of human malaria was used by Dinh and Trager (1978), to demonstrate the emergency of a chloroquine - resistant strain brought about artificially in a laboratory. The only report of chloroquine resistance in Zambia was that of a clinical case (Khan and Maguire, 1978) which lacked proper controls and is thought to be a false alarm. Recent work on drug resistance of some Thai isolates of P. falciparum using in vitro tests reveals that most Thai isolates are less sensitive to chloroquine and other anti-malarial drugs than African isolates (Thaithong and Beale, 1981).

The mechanisms involved in drug resistance by malarial parasites are not clearly known. The biochemical mechanism of resistance of malarial parasites to chloroquine is thought to involve decreased intracellular drug level. Another early theory was that chloroquine affected the use of food vacuole and haemoglobin; chloroquine resistant parasites were able to avoid this by transamination of citric acid cycle intermediates (Howells et al., 1970; Homewood et al., 1972). This was indicated by appearance of succinate dehydrogenase (Howells et al., 1970). Chloroquine resistant parasites do not digest adequate haemoglobin from the red blood cells and hence no accumulation of hemozoin occurs (Homewood et al., 1972). Spontaneous gene mutations in the nuclei of one or more cells is thought to be the most important mechanism of drug resistance (Beale, 1980). Different species of rodent malaria parasites show great variation in the response to chloroquine, whereas the sensitivity to pyrimethamine is uniform.

Investigation into the susceptibility of different isolates of P. falciparum to chloroquine was carried out here. This was studied using different low concentrations in the in vitro culture, and the parasite morphology and density were used to monitor the effect.

CHAPTER 2

E X P E R I M E N T A L

2.1 SAMPLING

Blood samples were normally taken from children with ages ranging from 3 months - 13 years attending the Paediatric Clinic of the University Teaching Hospital, Lusaka. A few samples were taken from adults attending the University of Zambia Clinic, Lusaka. 1-2 ml blood was withdrawn using a 21G sterile needle and syringed into a sterile bottle containing 0.5 ml. acid-citrate-dextrose (ACD) anticoagulant.

Initially blood was collected from any patient suspected to have malaria and admitted to the hospital. However, some samples were found to be negative. Later I tested all high fever suspected malaria patients in the Out-Patients Paediatric Department using Giemsa-stained thin smears, on two mornings a week. Patients positive for malaria had blood samples taken by the Doctor. Usually one or two samples were obtained in a morning (approximately 1 in 10 patients tested). There were more malaria cases during the months of March and April.

The blood collected into ACD was kept in a refrigerator at $4^{\circ}C$ for 2 up to 24 hours before separating the cells and putting into culture. A thin blood film fixed in methanol and stained with Giemsa stain was normally prepared before setting up culture to ascertain the degree of infection, and diluted as necessary.

2.2 CULTIVATION TECHNIQUES

1 Culture Medium

The preparation of the medium was as given by Jensen (1978). 10.4g powdered RPMI 1640 medium (Gibco, New York) with glutamine was dissolved in 900 ml double distilled water. 5.94g HEPES buffer was added to this and then the volume was adjusted to 960 ml with double distilled water. Once sterilized by millipore filtration through a 0.45 µm pore size filter, the medium was divided into 80 ml aliquots aseptically and kept at 4°C for up to four weeks. To avoid bacterial contamination, 720 µl of gentamycin sulphate (40 mg/ml) was added to 960 ml RPMI 1640 medium. 5% $NaHCO_3$ was sterilized through a syringe fitted with a 0.22 µm pore size filter unit, divided into 10 ml aliquots and stored for a period ranging from two to four weeks depending on the extent of exposure to air. $NaHCO_3$ was added to RPMI 1640 medium to a final concentration of 0.2% to give complete medium. At this stage the colour of the medium changes from pale yellow to orange. Human serum type AB was added to the complete medium to give a final concentration of 15% (RP-15-S). RP-15-S was kept at 4°C for up to one week.

2.2.2 Washing of blood cells

Outdated normal blood was obtained from the Blood Bank at the University Teaching Hospital, Lusaka. Blood group O was routinely used though other groups were occasionally used. The cells in either acid-citrate-dextrose (ACD) or citrate-phosphate-dextrose (CPD) were transferred aseptically into sterile 100 ml medium bottles and stored at 4°C. Erythrocytes stored in this fashion were suitable for culture when they were 14 days up to 40 days from the transfusion date. The blood was centrifuged in a sterile 15 ml screw-capped centrifuge tube at 1000xg for 10 minutes in the cold. A sterile Pasteur pipette was used to remove the plasma and buffy coat. The packed cells were then resuspended in RPMI 1640 complete medium without serum (volume added is equal to the initial volume of blood) and centrifuged at 1000xg for 10 minutes. The wash was repeated once again after removal of supernatant and buffy coat. The final pellet of packed cells was suspended in equal volume RP15S This red cell suspension was used to dilute parasitized blood.

2.2.3 Serum

Human serum, type AB normally, was obtained by collecting blood into a sterile bottle without anticoagulant. The clotted blood was left at 37°C for 1 hour and then at 4°C overnight.

The clear yellow upper layer was removed asceptically and centrifuged further to remove any contaminating cells. The serum was then divided into 10 ml aliquots and stored frozen at -18°C for several months. Once thawed, the serum was kept at 4°C for up to one week. Some of the serum was heat inactivated at 56° - 58°C for 30-45 minutes to destroy comple

.2.4 Preparation of Parasitized Cells

The parasitized blood was transferred aseptically into a sterile 15 ml screw-capped centrifuge tube and spun at 1000xg for 10 minutes in the cold. The plasma and buffy coat were removed with a sterile Pasteur pipette and the packed cells resuspended in RPMI 1640 complete medium without serum (RP); the volume added was equal to the initial volume of blood taken. This was centrifuged at 1000xg for 10 minutes, supernatant aspirated off using a sterile Pasteur pipette, an the washing repeated one more time. Once the supernatant had been removed, the packed cells were suspended in equal volume RPMI 1640 complete medium with 15% human serum (RP-15-S).

A thin blood film was then prepared, fixed in methanol and stained with Giemsa's stain (1:5 in phosphate buffer, pH 7.2) for 20 minutes. The level of parasitemia was determined by counting the number of viable parasites per 10^4 red blood cells. Parasites that appeared normal were taken to be viabl

Ring stage parasites were classified as viable if they had a clear nucleus and cytoplasm. For the schizonts, especially segmenters, clear healthy merozoites were considered. If the parasitemia was found to be too high, an appropriate amount of freshly washed erythrocytes was used to dilute the parasitized blood to between 0.3-1.5%.

Each millilitre of the processed infected blood was diluted with 6.0 ml RP-15-S medium to give a final cell suspension of 7%. The diluted blood was then dispensed into plastic culture dishes (35 x 10 mm), 1.5 ml into each dish. They were carefully placed in a desiccator and a white candle lit. The lid was replaced with the stop-cock tap open until the candle flame went off. The tap was then closed and the desiccator plus contents placed in an incubator at 37°C. The incubator was checked, and the temperature was maintained at $37 \pm 0.5^{\circ}C$.

2.5 Changing Culture Medium

The culture medium was changed at least once every 24 hours. A sterile Pasteur pipette was used to aspirate off the old medium. When most of the medium had been removed, a drop of cell suspension was removed aseptically and used for the preparation of a thin blood film which was fixed and later stained to monitor parasite growth.

1.5 ml fresh medium was added into each culture dish using a sterile pipette and the dish gently stirred. Fresh erythrocytes were routinely added every 3-4 days. On some occasions the medium was changed twice every 24 hours. Double the amount of medium recommended was sometimes added into each culture-dish on Saturday afternoon and left until Monday morning (D. Walliker, personal communication).

2.3 ISOENZYMES, ELECTROPHORESIS AND STAINING

Gels for enzyme electrophoresis were prepared according to Carter (1978). The buffer systems used and the conditions of electrophoresis for GPI and LDH were as given by Carter (1978). ADA, 6-PGD and PEP-E were studied using conditions similar to those given by Harris and Hopkinson (1976). Some modifications were included as recommended by Sanderson et al (1981). All chemicals used here were of analytical grade and were obtained from Sigma Chemical Company (London). Those used for making buffers were obtained from BDH (British Drug House, Poole).

2.3.1 Culturing

Samples of parasitized blood (2.1) were set up in *in vitro* culture (2.2). Isolates of *P. falciparum* were maintained *in vitro* for periods ranging from 24 hours to just over 2 weeks to get sufficient mature parasites (trophozoites and schizonts) required for enzyme electrophor Parasitemias between 1% and 5% with a high degree of schizon were used, depending on the enzyme under investigation. GPI and ADA could be identified using 1% parasitemia whereas the rest required 2.5% - 5% parasitemia.

2.3.2 Preparation of parasites for electrophoresis

Preparation of parasite material for electrophoresis was as given by Sanderson et al. (1981). Cultures showing the required parasitemia and mature stages were harvested from three or four culture dishes (35 x 10 mm) and centrifuge at 500xg for 10 minutes in the cold. A sterile Pasteur pipe was used to aspirate off the supernatant. The packed cells were then incubated with 0.15% saponin in complete RPMI 1640 medium (1 volume packed cells : 1.5 volumes saponin solution) at 37°C for 20 minutes. After resuspending the incubated material in ten volumes of complete RPMI medium and mixing well, it was centrifuged at 1000xg for 15 minutes. At this stage, four clear layers were seen.

The supernatant and red cell ghost layers were removed, leaving a grey-brown pellet of parasite material plus unlyzed red blood cells. The pellet of packed parasites was removed by a Pasteur pipette and distributed into a number of plastic vials. These were kept at -18°C for up to 48 hours or used immediately after freeze-thawing three times in a lysing solution (1 mM EDTA, 1 mM Tris and 1 mM Dithiothreotol). This step was performed quickly in a freeze at -15°C. A control sample of normal uninfected red blood cells was prepared in a similar manner to the experimental material. This normal blood was obtained from the Blood Bank as outdated blood.

.3.3 Enzyme Electrophoresis

The starch gels were prepared by dissolving 22.5g of hydrolyzed potato starch (Connaught, Ontario) in 250 ml of appropriate buffer, heating this with swirling on a bunsen flame until a clear viscous liquid was formed. Degassing was carried out using negative pressure from a vacuum pump for about a minute. Once all the small bubbles had disappeared, the gel was poured onto a gel mould made of a perspex rim (internal size 18 x 18 cm) supported on a glass plate (20 x 20 cm).* The thickness of the gel was 6 mm. The gel was left to settle at room temperature for 30 minutes.

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* These dimensions are as given by Carter (1978).

The gel was then transferred into a refrigerator at 4°C and left for another 2½ hours before use. If covered properl with a polythene sheet, the gel could be kept at 4°C for up to 2 - 3 days. Slots were made on the gel using a spatula with a small, even and sharp edge. A graduated perspex strip was used as a template when making slots in the gel. The saponin lyzed sample, after further lysis and repeated freeze thawing, was quickly absorbed onto filter paper strips (Whatman No. 3; 6 x 3 mm) and then applied into the slots on the gel. Up to five samples were applied on one gel at a time. The gel impregnated with the samples was then placed in a refrigerator at 4°C and electrophoresis allowed to run at the specified voltage and current for the time required.

.3.4 Buffer Systems and Staining Methods Used

(a) Adenosine Deaminase (ADA)

Electrophoresis

Electrode buffer: 0.1M phosphate pH 6.5

Gel buffer: 1 in 10 dilution of above

Current : 40mA

Voltage : 60V

Time of run: 18h.

or 240V/40mA for 4 hours

Reaction mixture

0.05M phosphate buffer pH 7.5	25 ml
Adenosine	15 mg
Nucleoside phosphorylase (40 units/ml)	16 µl
Xanthine oxidase (8.5 units/ml)	13 µl
MTT	5mg in 1 ml H_2O
PMS	5 µg in 1 ml H_2O
Agar 2%	25 ml.

(b) Glucose Phosphate Isomerase (GPI)

Electrophoresis

Electrode buffer: Tris/HCl, pH 8.0

Gel buffer: 1 in 5 dilution of above

Current: 60mA

Voltage: 240V

Time of run: 4.h

Reaction mixture

Tris/HCl buffer pH 8.0	25 ml
Disodium fructose-6-phosphate	50 mg
$NADP^+$	5 mg
$MgCl_2$	20 mg
MTT	5 mg
PMS	a microspatula tip (500 µg)
G-6-PD	10 µl
Agar 2%	25 ml

(c) Lactate Dehydrogenase (LDH)

Electrophoresis

Stock buffer	:	Tris citrate (55/33). 55g Tris + 33g citric acid monohydrate per litre.
Electrode buffer	:	1 in 2 dilution of above
Gel buffer	:	1 in 7 dilution of above
Current	:	70 mA
Voltage	:	60V
Time of run	:	14h

Reaction mixture

Tris/HCl pH 8.0	25 ml
Lithium lactate	200 mg
NAD^+	5 mg
MTT	5 mg
PMS	a microspatula tip
Agar 2%	25 ml

(d) Peptidase E (PEP-E)

Electrophoresis

Stock buffer	:	0.454M tris brought to pH 8.0 with 1M NaH_2PO_4.

Electrode buffer: 1 in 2 dilution of above

Gel buffer : 1 in 20 dilution of above

Current : 40 mA

Voltage : 90V

Time of run : 18h

Reaction mixture

0.02M citrate-phosphate buffer, pH 5.5	35 ml
Peptide (L-Leucyl-L-Alanine)	20 mg
Snake venom (amino acid oxidase)	6 mg
Peroxidase (Horse radish Type I)	6 mg
Manganese chloride	10 mg
O-dianisidine dihydrochloride	3 mg
Agar 2%	30 ml

(e) 6-Phosphogluconate Dehydrogenase (6-PGD)

Electrophoresis

Electrode buffer: 0.1M phosphate, pH 7.0
10 mg $NADP^+$ added to cathodal trough

Gel buffer : electrode buffer diluted 1 in 10. 5mg $NADP^+$ added to heated gel mixture just before final degassing.

Current : 40 mA

Voltage : 60V

Time of run: 17h

Reaction mixture

0.05M Tris/HCl buffer, pH 8.0	10 ml
Barium-6-phosphogluconate	50 mg
0.2M $MgCl_2$	5 ml
$NADP^+$	5mg in 1 ml H_2O
MTT	5 mg
PMS	a microspatula tip
Agar 2%	20 ml

2.3.5 Enzyme Staining Techniques

Two different staining methods were employed for the five enzymes studied:-

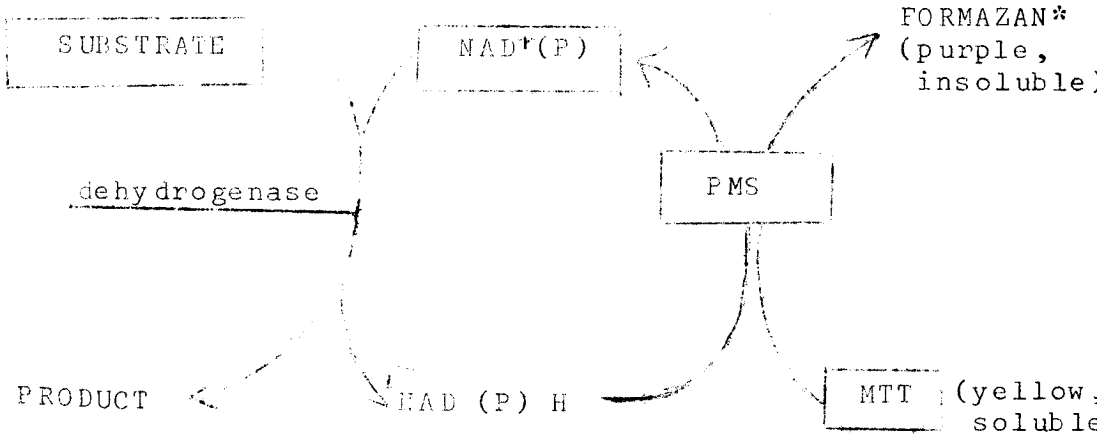
(a) Enzyme-linked staining method

(b) Electron transfer dye staining methods.

Two dyes are commonly used; methyl thiazoyl tetrazolium (MTT and 3-amino-9-ethyl carbazole. MTT is an acceptor for dehydrogenase reactions while the latter is for oxidases and peroxidases. O-dianisidine is greatly replacing 3-amino-9-ethyl carbazole and was used in the detection of PEP-E.

Tetrazolium salts

Electron donors reduce MTT giving a dark-blue-purple insoluble formazan. Phenazine methosulfate (PMS) catalyzes the redox reaction. The reaction of dehydrogenases utilizi the electron transfer dye MTT can be generalized thus:

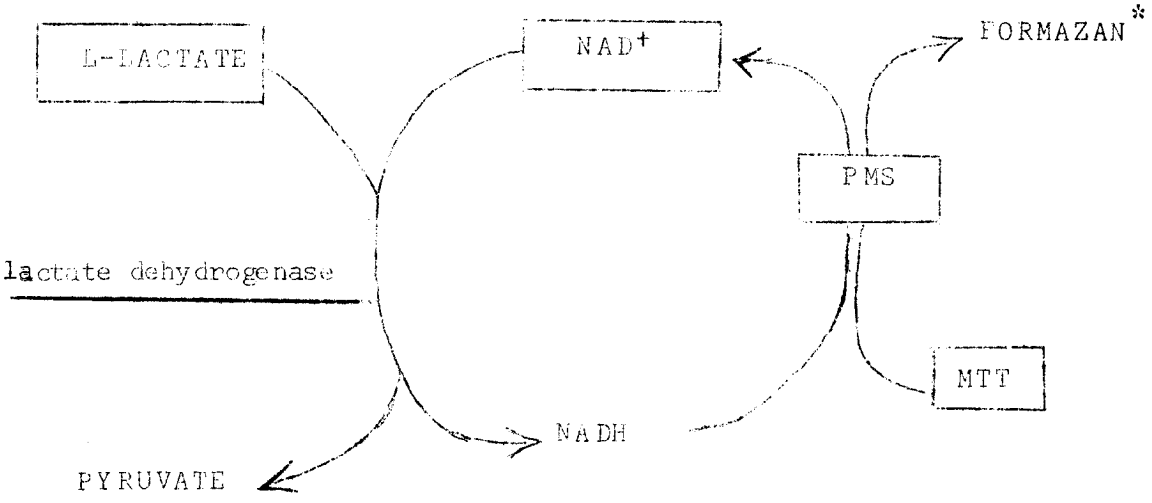


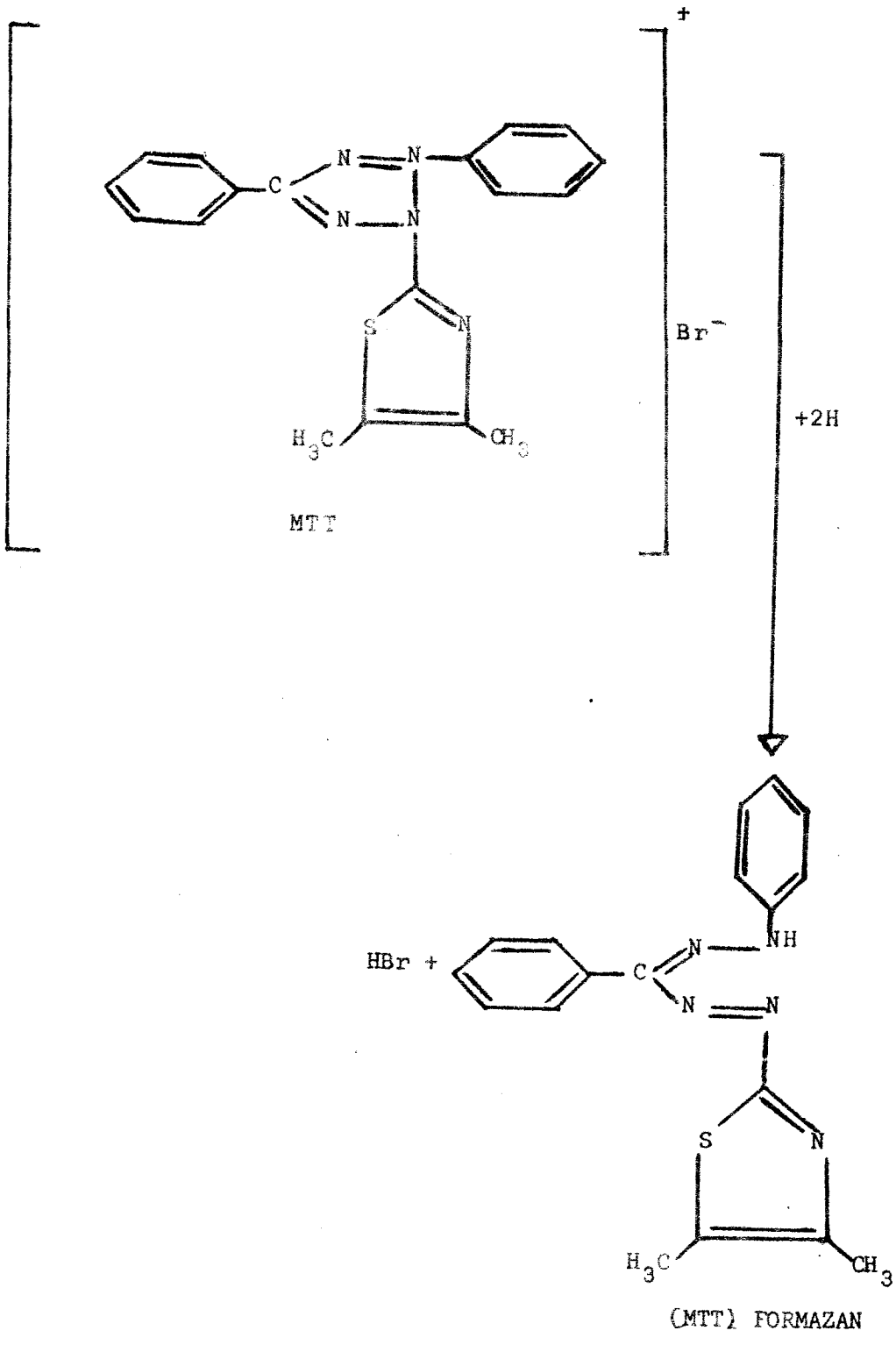
The specific reactions involved in the five enzymes ar

(1) Lactate dehydrogenase (LDH)

L-Lactate + $NAD^+ \rightleftharpoons$ pyruvate + NADH

Staining system



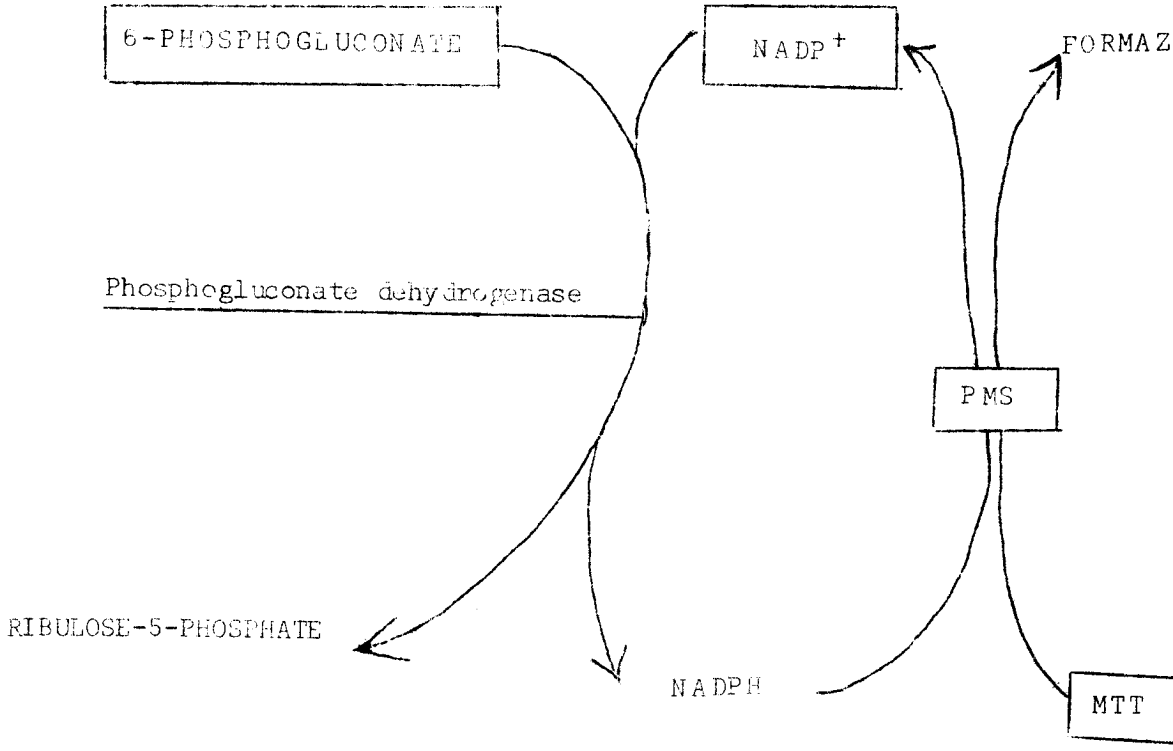


Formation of insoluble formazan by reduction of MTT.

(2) Phosphogluconate dehydrogenase (PGD)

6-phospho-D-gluconate + $NADP^+$ $\rightleftharpoons$ D-ribulose-5-phosphate + CO_2 + NADPH

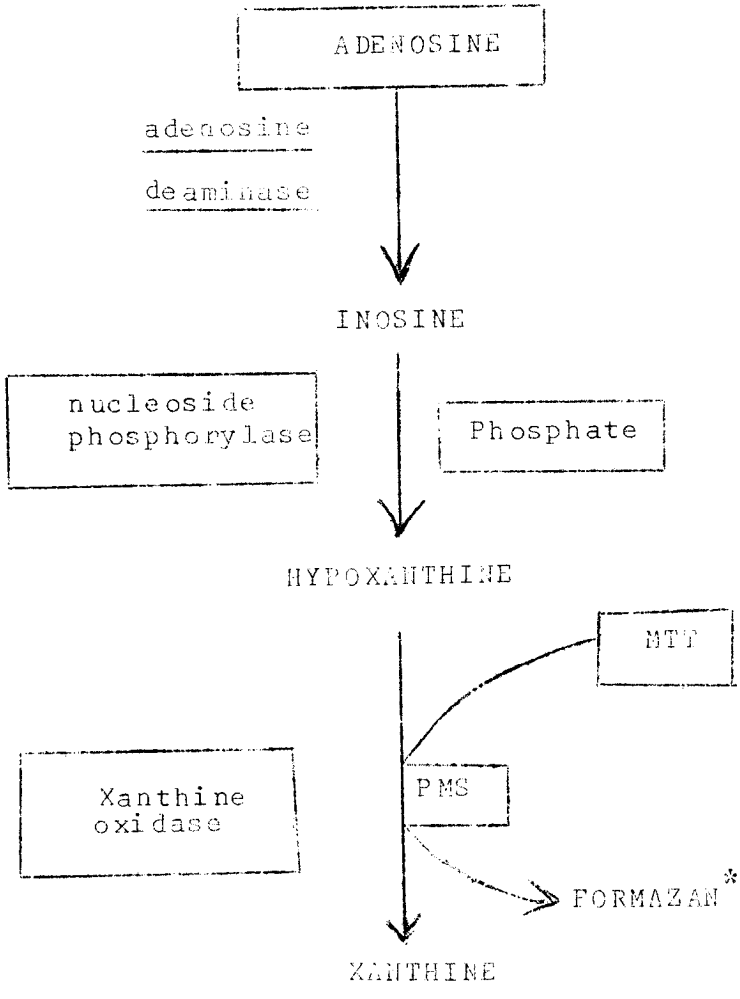
Staining system



(3) Adenosine deaminase (ADA)

Adenosine + H_2O $\rightleftharpoons$ inosine + NH_3

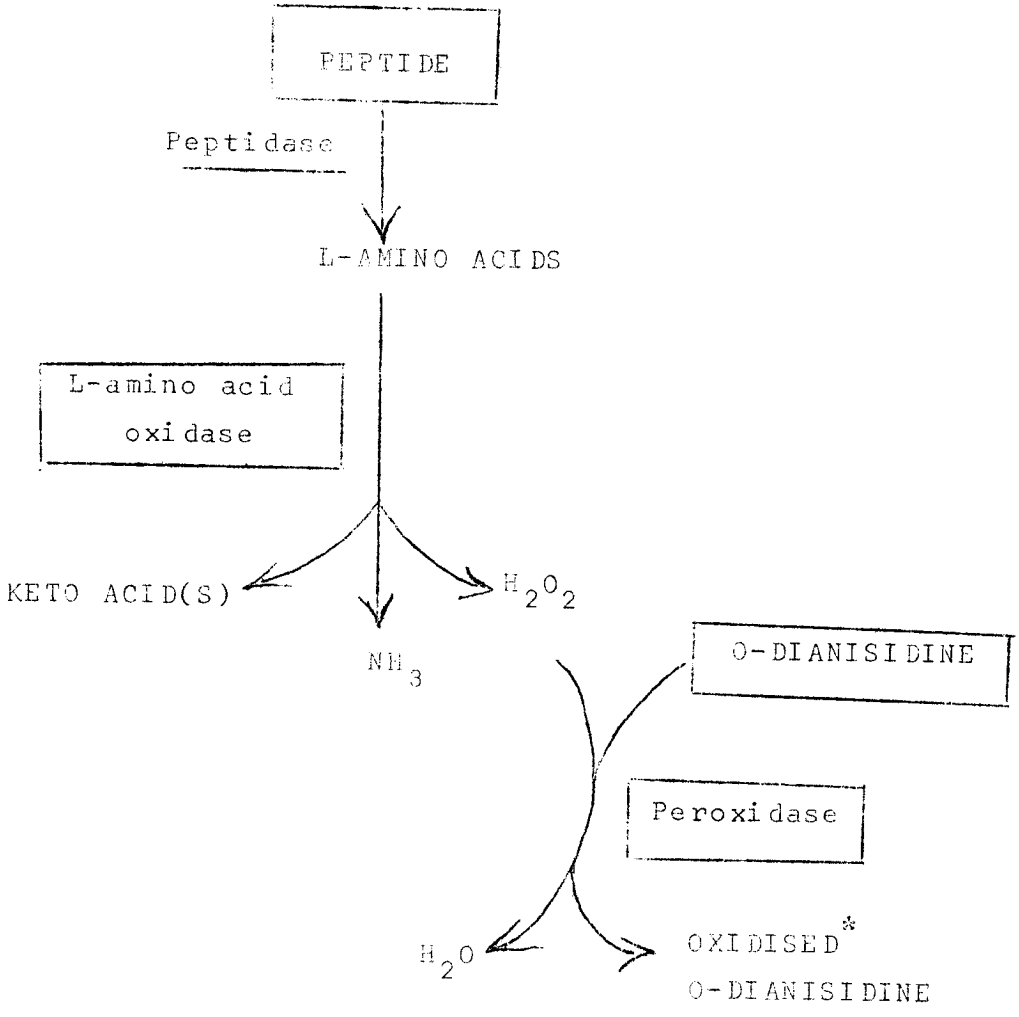
Staining system

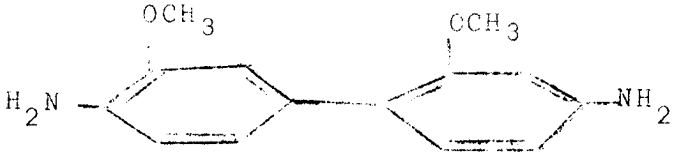


(4) Peptidases (A,B,C,E,F and S)

dipeptide + $H_2O \rightleftharpoons$ L-amino acids

Staining system





Structure of O-dianisidine

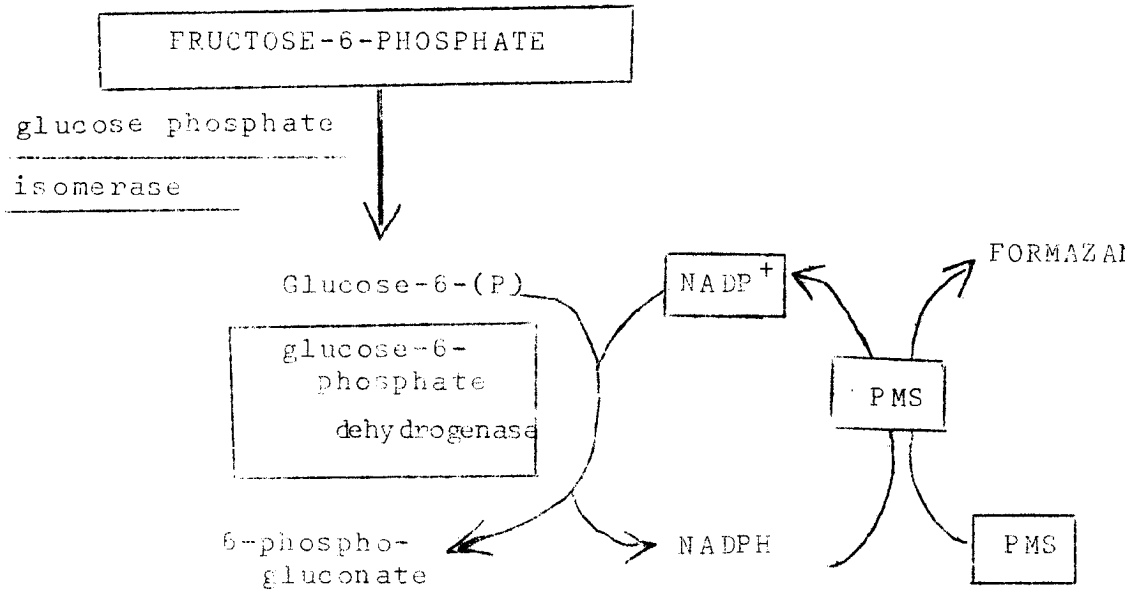
(5) Glucose phosphate isomerase (GPI)

Glucose phosphate isomerase (GPI) detection was by an enzyme-linked assay. The staining mixture utilized an exogenous enzyme in trapping and changing the product of the enzyme reaction under investigation into a coloured substance. Fructose-6-phosphate was used here as a substrate and glucose-6-phosphate dehydrogenase as the linking enzyme.

Glucose-6-phosphate

Staining system

Fructose-6-phosphate



2.4 CHLOROQUINE SENSITIVITY TESTS

Materials and Methods

.4.1 P. falciparum

Different isolates of P. falciparum were obtained on different occasions from the Paediatric Department of the University Teaching Hospital except for one isolate from a 20 year old male student collected at the University of Zambia Clinic. The technique used to grow the isolates is that described in 2.2.

.4.2 Antimalarial

Chloroquine diphosphate, 7-chloro-4-(4 -diethyl-amino-1 -methylbutylamino)-quinoline diphosphate (Resochin, Bayer) was dissolved in RPMI 1640 medium. A stock solution of 1×10^{-2}M concentration was prepared in RPMI 1640 without serum, sterilized through a syringe fitted with a millipore filter unit of pore size 0.22 µm and then kept at 4°C for up to four weeks. Serial dilutions were made from this stock solution in RPMI 1640 complete medium. The storage period once this last step was done was only one week. Four different concentrations of chloroquine were used:-

1×10^{-6}M, 5×10^{-7}M, 3×10^{-7}M and 1×10^{-7}M.

2.5 BLOOD SMEAR PREPARATION, MICROSCOPY, STAINING AND COUNTING

5.1 Thin blood film preparation

A sterile Pasteur pipette was used to place a drop of parasitized blood onto a clean glass microscope slide (about 5 mm from the end). Another microscope slide, held at an acute angle, was used to smear the blood quickly so as to obtain a thin film. This was allowed to dry rapidly by passing the slide over a flame for a few seconds. The blood smear was then fixed by irrigating it with methanol. After fixing for 5 - 10 minutes any excess methanol was poured off and the slide allowed to dry with the blood-side downwards to avoid dust. The smear was normally stained right away or kept in an air-tight container until staining was possible.

5.2 Staining

Since only a few slides were normally stained at a time only 8-12 ml of Giemsa stain was prepared. Giemsa stain concentrate was diluted 1:5 in phosphate buffer (pH 7.2). The diluted stain was poured over the fixed thin blood films and left for 15-20 minutes. The stained blood smears were then removed, excess stain poured off, and phosphate buffer used to wash them quickly (about 30 seconds).

2.5.3 Microscopy and Counting

The dried, stained, thin blood films were then examine directly under oil immersion.* The high power objective of a light microscope (Olympus, Model KHS) was used when observing films with oil immersion. The tail end of the blood smear was examined carefully and the number of parasi seen were counted per 10 000 red blood cells. If a slide w well prepared, the number of fields observed was normally 50 (approximately 200 Red blood cells/field). If a red blood cell contained more than one ring, only one of these was counted. The degree of infection was expressed in term of percent parasitemia. This index is taken to be number of parasites / 10 000 RBCS divided by 100.

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* Magnification 1000

CHAPTER 3

R E S U L T S

3.1 IN VITRO CULTIVATION

3.1.1 Improvements on the cultivation technique

A number of investigations aimed at improving the cultivation technique were considered. These included:- frequency of changing medium, increasing the glucose concentration, and using heat inactivated serum.

The frequency of changing medium did have an effect in the level of parasitemia attained in vitro. The medium was often changed twice every 24 hours in an attempt to obtain numerous parasites for enzyme electrophoresis. The medium was seen to be pink in colour if medium was changed twice in 24 hours whereas if changed only once it appeared pale yellow. Table 3.1a shows that changing medium more than once has a positive effect on the final parasitemia. A single isolate was used here to study effect of extra glucose (2 gl^{-1}) and changing medium two times every 24 hours. The effect of extra glucose does not seem to be obvious within 2 asexual cycles period; the parasitemia observed here is the same as that where no extra glucose was added.

Two different isolates of P. falciparum grown using the same medium, human blood cells and serum were seen to differ in their growth rates (table 3.1b).

Table 3.1a

Comparison of parasitemias observed when a single isolate of P. falciparum was grown under different conditions.*

Days in vitro	0	2	4	% Parasitemia
+ extra glucose ($2gL^{-1}$)	0.6	0.56	1.0	
Change once	0.6	0.56	0.9	
Change twice	0.6	0.9	1.5	

* Average value of 2 culture-dishes are given

Table 3.1b

Comparison of parasitemias observed when two different isolates of P. falciparum were grown under similar conditions.†

Days in vitro	0	1	2	3	4	Parasitemia
Isolate A	0.55	0.60	2.4	2.35	3.8	
Isolate B	0.45	0.5	1.35	1.5	1.7	

† Medium was changed twice every 24 hours. Values represent an average of 2 culture-dishes.

Table 3.1c

Multiplication rates observed in a continuous culture of *P. falciparum* within 48 hours and 96 hours

Days in vitro	% Parasitemia 0 hours	% Parasitemia 48 hours	Multiplication rate	% Parasitemia 96 hours	Multiplication rate
45	0.35	0.45	1,3	1.70	5
52	0,28	0.73	3	1,96	7
66	0,09	0.60	7	0,94	10
73	0,04	0.95	21	1,36	30

Note: Parasitemias are given as an average of 4 culture-dishes.

With a change of medium twice every 24 hours isolate A showed a 7-fold increase in parasitemia within 96 hours while isolate B displayed only a 4-fold increase within the same period.

Heat inactivated serum was used on some occasions with a hope of attaining high parasitemias in vitro. Figure 3.1a shows that P. falciparum grown in serum without complement resulted in a parasitemia twice that obtained with ordinary serum. This was a common observation in this laboratory even when different isolates and different human sera were used. Figure 3.1a and 3.1e show that P. falciparum grown in heat inactivated serum did better than those in ordinary serum, using two different isolates and two different human sera.

.1.2 Established Culture

Several attempts were made in this laboratory to obtain a continuous culture of Plasmodium falciparum. Initially, a number of isolates survived in vitro for a period ranging from one week to just over two weeks. Parasitemias never went above 6%, 2-3% being most common. A successful culture was started using a blood sample showing 10% parasitemia. This was diluted before putting into culture and propagated continuously for over 90 days.

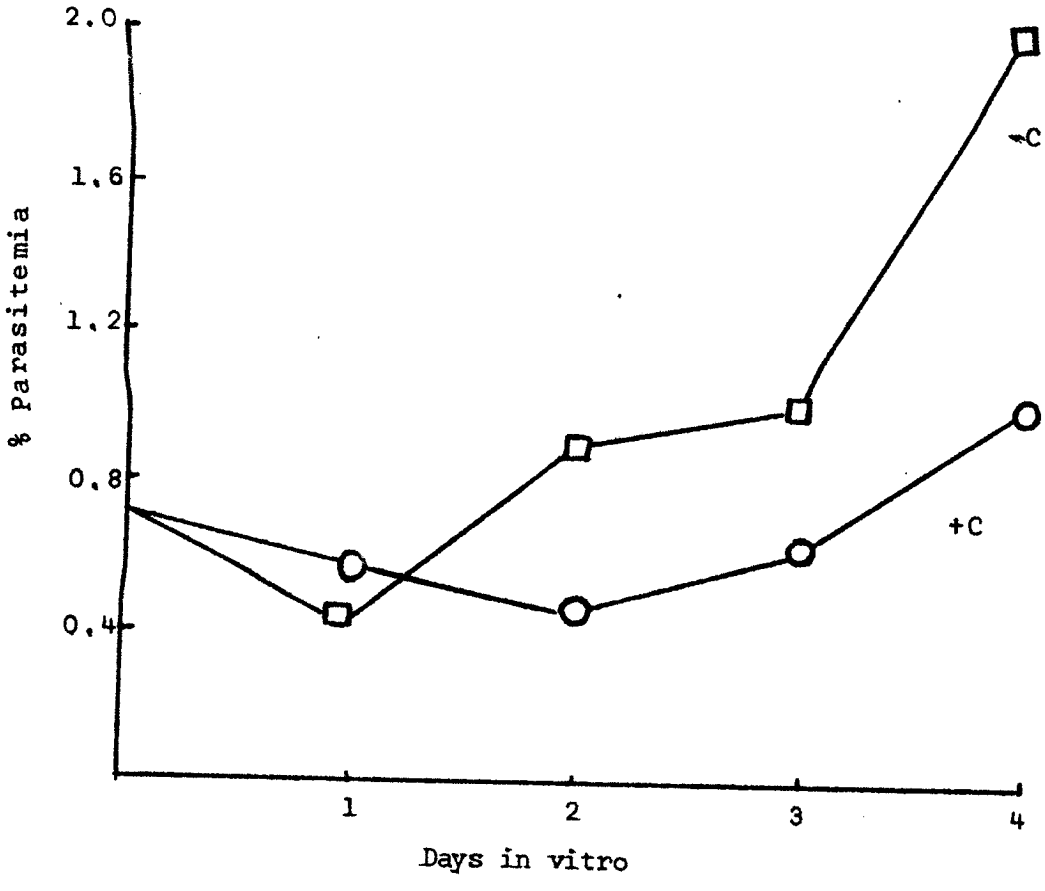

Figure 3.1a Comparison of parasitemia levels observed using human serum with or without complement

One of the human sera used for the growth of this successful culture had previously failed to support continuou growth of two other isolates. The medium employed for the cultivation of these two as well as the successful culture was from the same batch except that for the latter one a new batch of medium was used from day ten. The change of medium was found necessary because there had been obvious deterioration in the parasite morphology when the old batch of medium was used. When the new medium was utilized, there was a prompt improvement in the parasite morphology.

Serum used for growing this isolate was not heat treated. Two different human sera were used in the first 37 days *in vitro*, but since there seemed to be no difference in their ability to support growth, only one of them was used from here on. Data given in table 3.1c and figures 3.1b-3.1d are for this culture. Figure 3.1b represents typical growth rates in terms of percent parasitemia observed *in vitro*. The asexual erythrocytic cycle was synchronous in the initial cultivation period, new rings being observed every 48 hours; most erythrocytes contained 2 or more rings. This trend was not always true with all cultures, some of them were asynchronous right from the beginning, the asexual cycle taking less than 48 hours in some cases where the initi parasites were already mature ring size trophozoites, and sometimes more than 48 hours (see figures 3.1f and 3.1g).

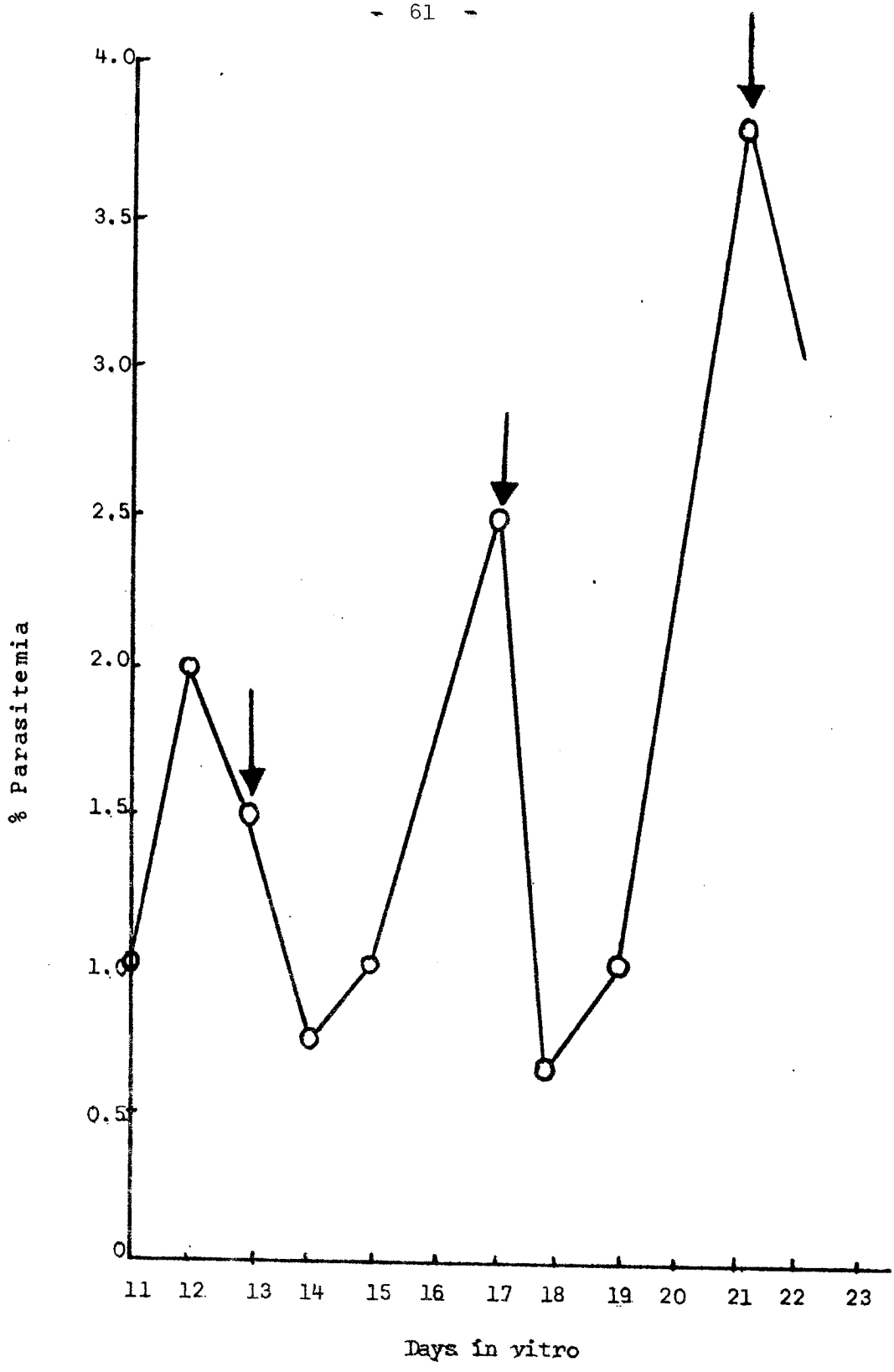

Figure 3.1b Plasmodium falciparum in continuous culture

A peak parasitemia was seen every 4 days. Fresh blood cells addition every 3-4 days reduced the parasitemias and provided the parasites with new erythrocytes to reinvade. 14-41 days old erythrocytes were used successfully in the dilution of cultures. A drop in parasitemia normally occured if no fresh red cells were added. Mature gametocytes were observed by day 7. Normal looking gametocytes were produced *in vitro* throughout the three months period. Table 3.1c shows that the multiplication rate of this isolate was very low initially. High multiplication rates within 48 hours and 96 hours were obtained after 10 weeks *in vitro*.

Serum Change

Figure 3.1c shows that changing serum can lead to a reduction in the parasitemia level *in vitro*. The percent parasitemia observed in the culture-dishes where serum was changed dropped to about half that seen in the ones where serum was not changed. This phenomenon was observed several times in this study whenever there was a change of serum. However, this decrease in parasitemia was only temporary as shown by the return to normal after a few days (figure 3.1d).

Some similar study done using a different isolate of *P. falciparum* and different serum showed that the observation made above is only seen when ordinary serum is used (figure 3.1e).

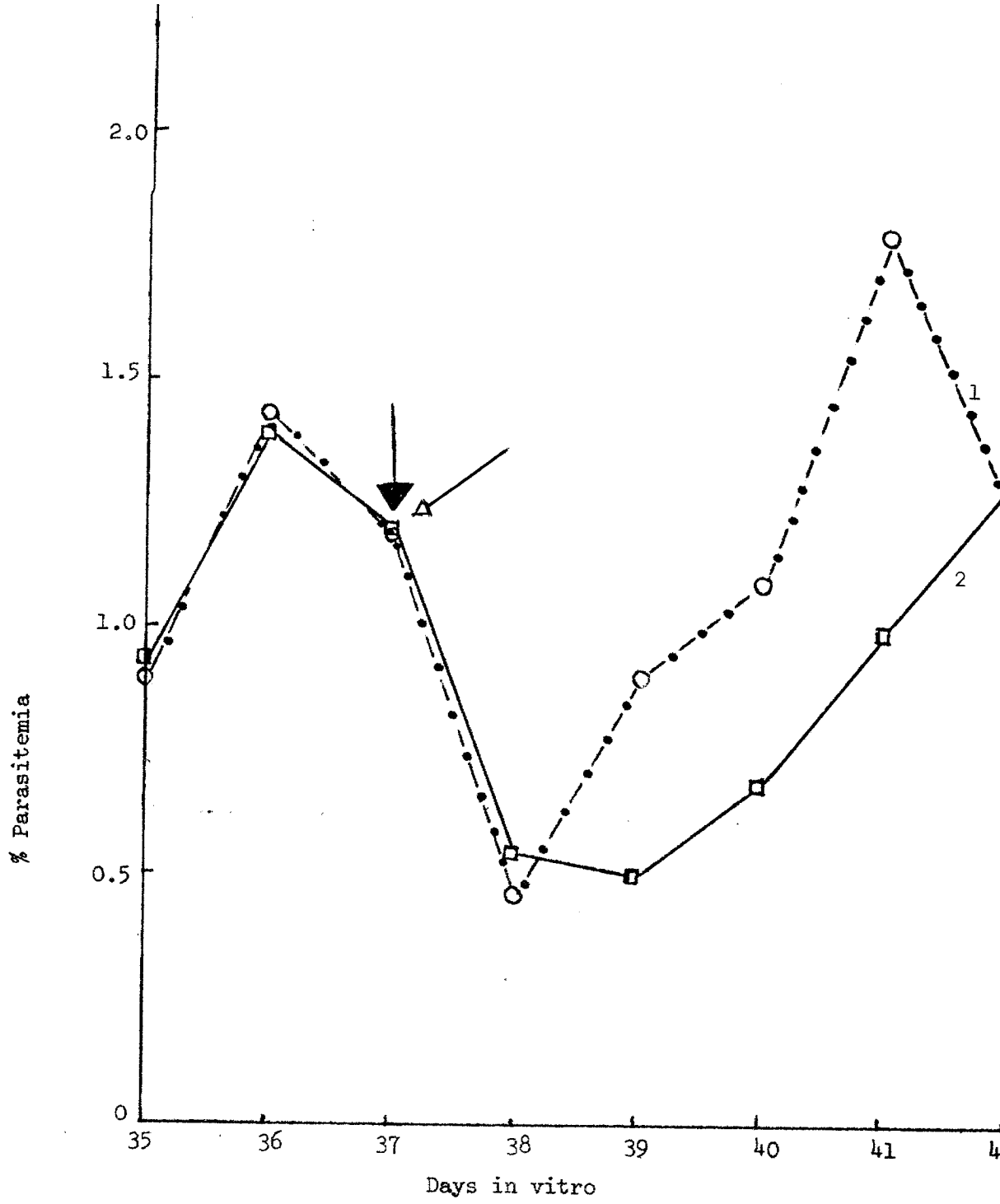

Figure 3.1c The effect of changing serum during cultivation of a single isolate of Plasmodium falciparum

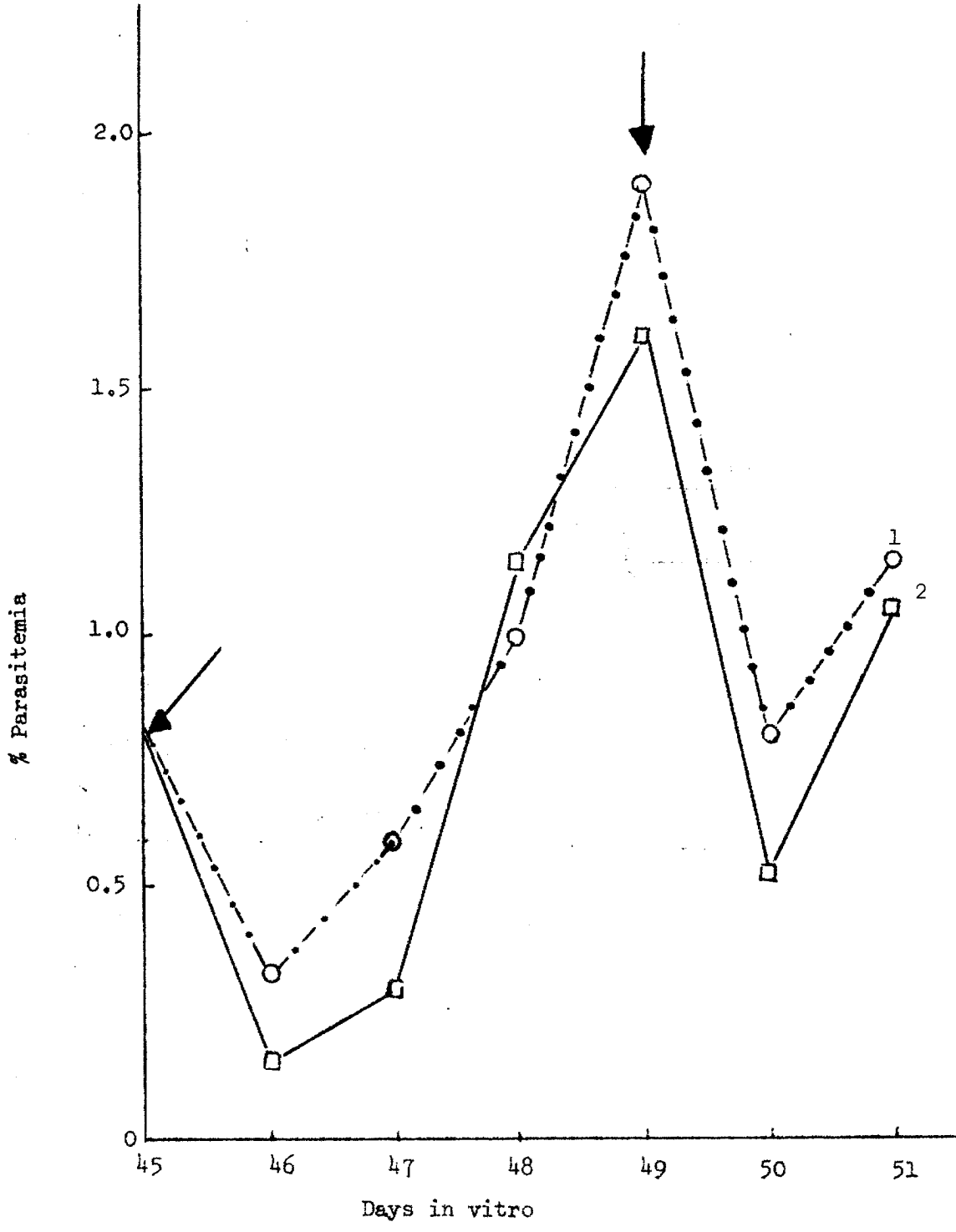

Figure 3.1d Multiplication observed in two cultures of P.falciparum showing stabilisation in serum from one donor

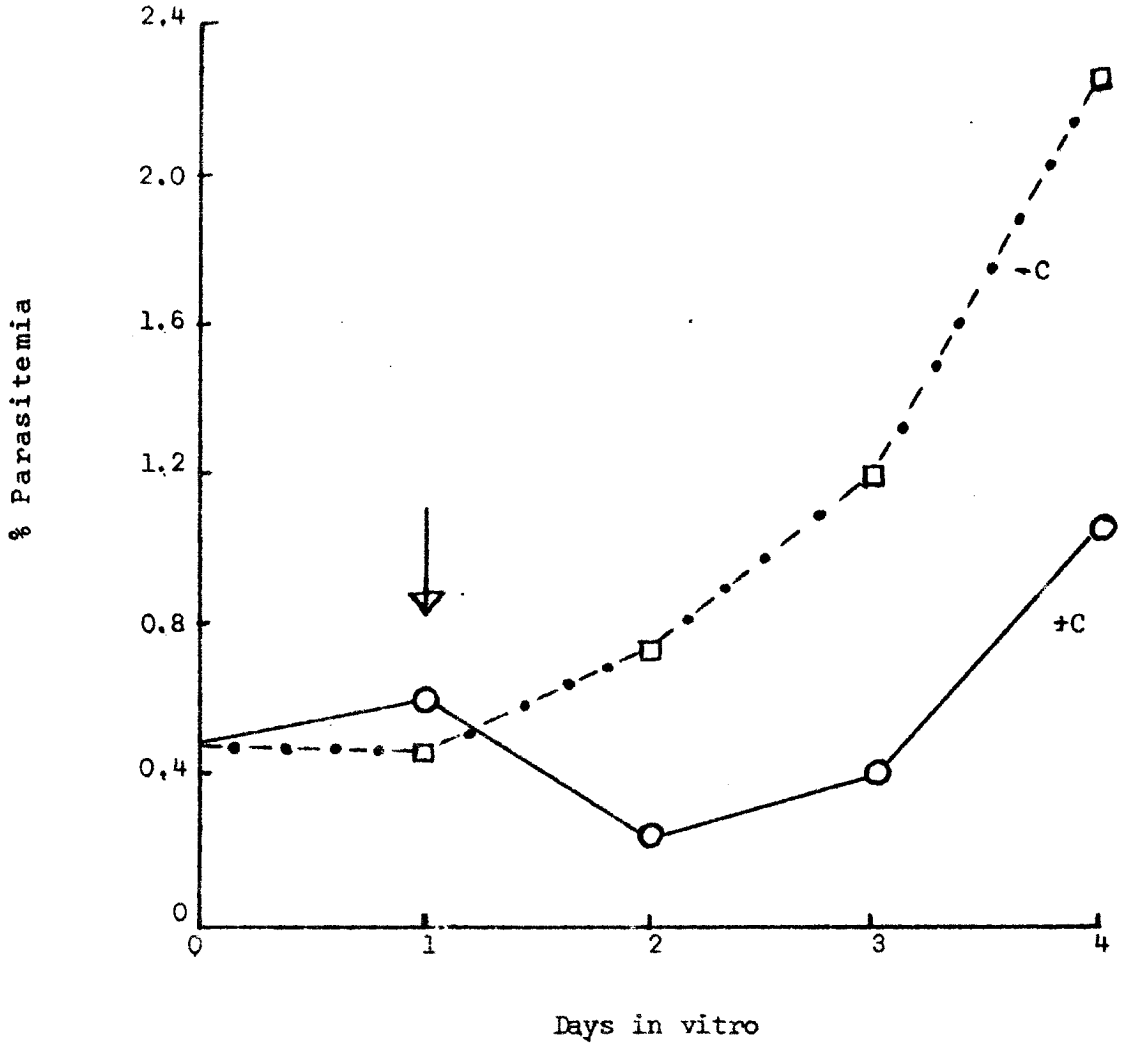

Figure 3.1e Reduction in parasitemia levels observed in P. falciparum in vitro upon change of serum

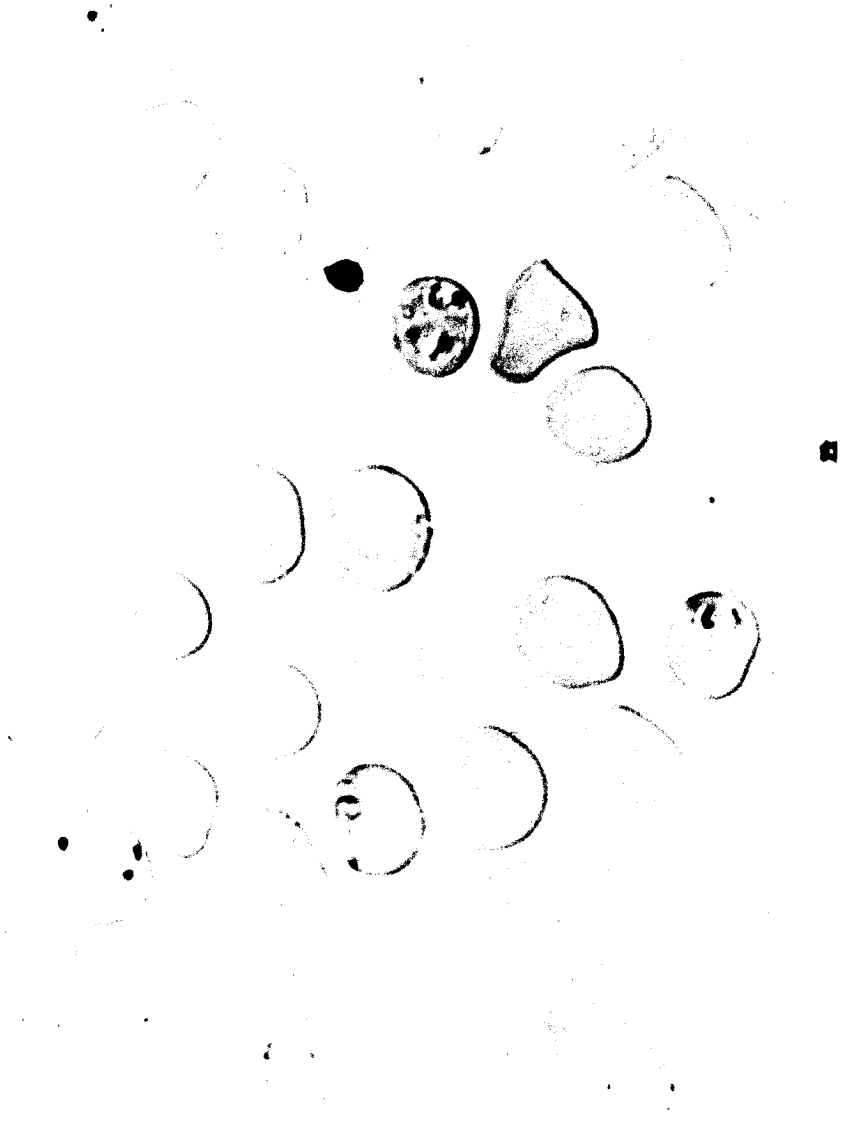

Figure 3.1f Photomicrograph of a 64 hours culture showing trophozoites of P. falciparum (Giemsa stained) Objective 100 x 1.25 (Oil immersion).

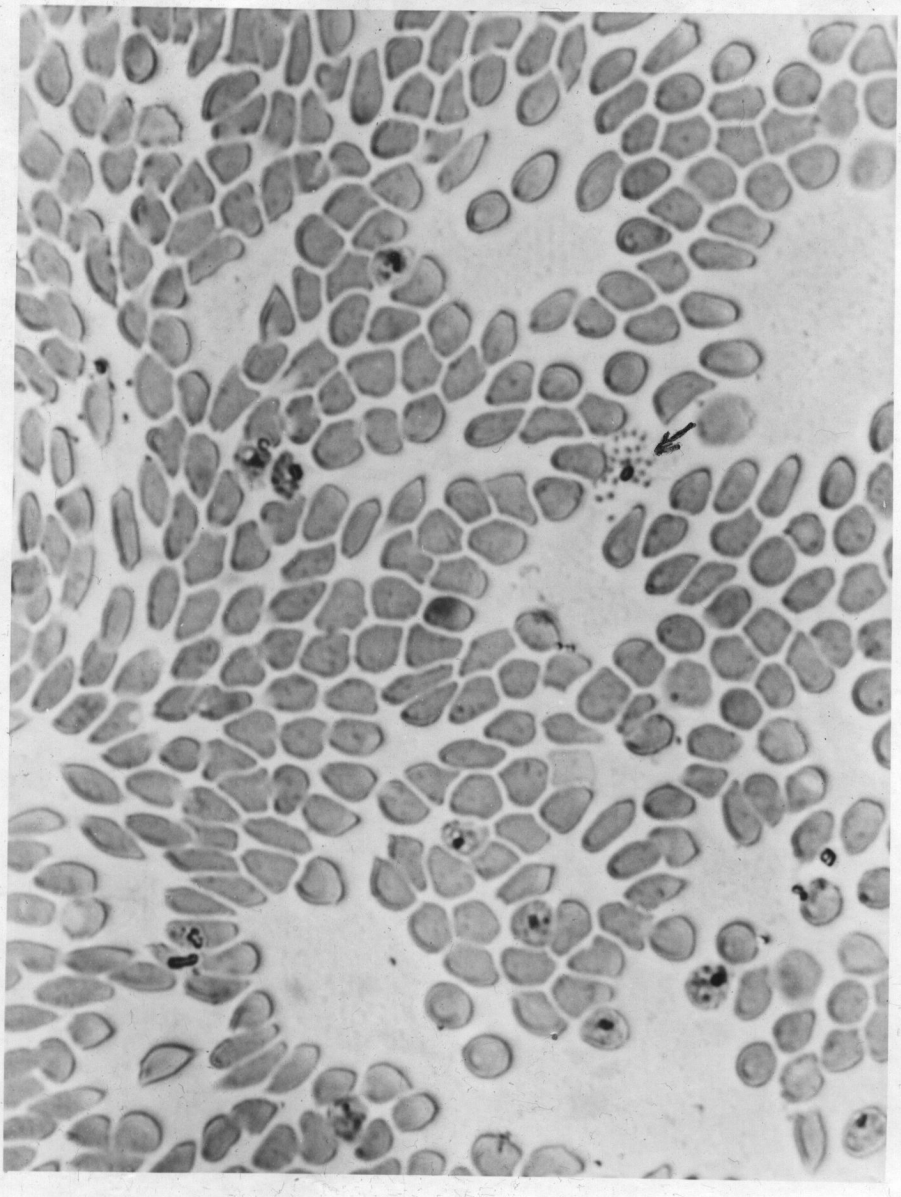

Figure 3.1g Photomicrograph of a 50 hours culture showing mature schizonts (segmenters) of P. falciparum (Giemsa stained).

Objective 40 x 1.25.

Arrow indicates inhibited schizont with 21 merozoites.

D I S C U S S I O N

Successful cultivation of P. falciparum in vitro requires a number of parameters other than reduced oxygen tension. Changing medium twice helps in the attainment of higher parasitemias in vitro (table 3.1a) because besides replenishing the nutrients, toxic end products of metabolism are removed. The pink colour of medium observed when there is more than one change of medium in 24 hours could be due to the production of less lactic acid in shorter time which is favourable for the parasites.

The fact that no apparent increase in parasitemia was obtained when extra glucose was employed (table 3.1a) does not imply there is no advantage in this. The observation here was based on a few days study and used isolates not yet established in culture. No adverse effect was seen within this short period either but the effect of prolonged use of extra glucose on isolates hoped to survive continuously in vitro was not essessed.

The difference in growth rate observed when two isolates of P. falciparum were grown under similar conditions (table 3.1b) can be explained solely by the difference in the 'strains' used.

Some isolates can adapt quickly to culture conditions and display a higher multiplication rate than others. These differences could be genetic and might depend on the degree of virulence of a given strain.

The observation that isolates of P. falciparum grown in heat inactivated serum did better than those in ordinary serum (figure 3.1a and 3.1e) implies that an immune reaction was responsible for the low parasitemias observed in vitro in the latter case. There is no difference in the first day in vitro but by the second and fourth days there is a marked difference in the parasitemia levels observed. This clearly implicates the participation of serum antibodies in the inhibition of merozoite dispersal upon rupture of erythrocyte containing segmenters right from the first asexual cycle (2 days). This supports the likelihood that some local serum is partially immune and might be responsible for the low survival rate of isolates in culture. Chulay et al. (1981) suggested that immune serum agglutinates merozoites thus inhibiting their entry into new erythrocytes. Some immune serum was found by Green et al. (1981) which inhibited merozoite dispersion from mature schizonts. This kind of inhibition was observed here as well (figure 3.1g).

The increase in multiplication rates observed (table 3.1 as the days in vitro increase, is due to the adaptation to

culture conditions by the isolate. The rate of multiplicat observed in week six in a 48 hours and 96 hours period was 1x and 5x respectively. However, by week ten *in vitro* the multiplication rate had increased to 21x and 30x in the abo periods of time. This clearly indicates that as the time *in vitro* increases there is an accompanying increase in the multiplication rate.

The failure of the other two isolates to grow continuo *in vitro* despite the use of the same serum that supported continuous growth of another isolate could be explained in terms of either medium or difference in the isolates used. The medium might have lost some nutrients due to prolonged storage, or the isolates differed intrinsically in their growt ability. Red cell lysis normally occured whenever a cultur was dying.

The drop in parasitemia observed when no fresh red blood cells are added once a peak has been reached is mainly due to the deterioration of erythrocytes with time at 37°C. Multiple infection of red blood cells with 2 or more rings, commonly encountered in this study, could be greatly reduced with agitation of the cultures. The static culture method employed here does not provide conditions similar to those encountered in the bloodstream. Consequently, the released merozoites invade the nearest red blood cell in static cultu

The interesting finding that changing serum is followed by a marked reduction in parasitemia (figure 3.1c) of P. falciparum in vitro, is difficult to explain because three parameters seem to be involved: erythrocytes, serum, and the parasite itself. The erythrocytes used to dilute the cultures on days 37 and 42 were from the same donor as those used in the two weeks preceding the change of serum. An initial reduction in parasitemia was seen in those culture dishes where serum was changed, however after 3-4 days recovery occured. The reduction in parasitemia seen again on days 46 and 47 with the level normalizing on day 48, and finally on day 50 only, seems to take place in stages and might involve a saturable process on the red blood cells.

A possible reason for the effect of serum change might be the involvement of serum antibodies which can interact with antigenic sites on the erythrocyte membrane. The decrease in parasitemia caused by serum change seems to require complement (figure 3.1e); when heat inactivated serum was used there was no reduction in parasitemia. This observation also supports the idea that an antigen-antibody reaction is involved, as this is likely to involve complement activity. Complement is a heat labile constituent of serum. It acts as a kind of catalyst for a range of antigen-antibody reactions (Cruickshank, 1970).

3.2 ISOENZYMES

At the commencement of this research project it was hoped that several isolates of Plasmodium falciparum would be analyzed for their enzyme patterns. However, this was not possible due to a number of problems encountered in the in vitro cultivation of the isolates put into culture here. The high parasitemias required for this kind of study were not easy to obtain in vitro. The suggested method of growing the parasites for at least two weeks (Thaithong et al., 1981) before enzyme typing could not be followed because most isolates did not survive this long. Immune serum was suspected to play a role in the low survival rates and parasitemias, so heat inactivated serum was employed in later work. This improved the levels of parasitemia attained (see figure 3.1a) and sufficient parasite material of a few isolates was obtained for enzyme studies.

Most of the isolates were analyzed for one or two enzymes only because of two reasons:-

(a) only one or two isolates were available from the hospital at one time,

(b) the processed crude red cell lysate could maintain enzyme activity when stored at $-15^{o}C$ for only 48 hours.

Table 3.2a shows the different enzyme variants observed in the isolates tested. Eight isolates were tested for GPI which was found to be easily detectable even when the parasitemia was low (≈ 1.5%). It was possible to study at least five isolates for 6-PGD because at one occasion three different isolates were run on the same gel.

The enzyme patterns observed on some of the stained gels for the five enzymes studied are shown in figures 3.2a - 3.2f.

Glucose phosphate isomerase (GPI) Figure 3.2a

Two forms of parasite enzyme, designated GPI-1 and GPI-2, and two forms of host GPI were seen, in one case host GPI-1 and 2 were seen together. Only one out of the eight isolates analyzed possessed both GPI-1 and GPI-2 Hemoglobin band appeared on the anodal end of gel just below parasite GPI-1 and on the same level in case of parasite GPI-2. The position of parasite GPI-1 was confirmed using a standard parasite preparation (isolate K1 from Thailand, donated by Dr D. Walliker) run under identical conditions.

Lactate dehydrogenase (LDH) Figure 3.2b

Parasite LDH appeared as one form only, LDH-1. Three forms of host LDH were normally seen, although LDH-4 was seen occassionally. Sometimes host LDH-2 appeared as two bands, one large and the other one small. Parasite LDH-1 position was confirmed using the above standard.

Table 3.2a

Enzyme Variation in P. falciparum in Zambia*

GPI			LDH			6-PGD			ADA			PEP-E		
1	2	1+2	1	2	1+2	1	2	3	1	2	1+2	1	2	3
4	3	1	5	0	0	1	4	0	2	1	0	2	0	0

* The figures in each column represent the number of isolates exhibiting the enzyme forms shown.

Table 3.2b

COMPARISON OF ENZYME VARIATION IN P. FALCIPARUM

	GPI			6-PGD			LDH			ADA			PEP-E		
ORIGIN	1	2	1+2	1	2	3	1	2	1+2	1	2	1+2	1	2	3
GAMBIA	23	6	25	46	2	1	38	3	13	49	2	2	52	0	0
TANZANIA	4	1	3	5	0	1	6	0	2	1	1	6	0	6	2
CONGO	3	3	2	4	0	0	6	2	0	6	1	1	6	0	1
ZAMBIA	4	3	1	1	4	0	5	0	0	2	1	0	2	0	0
THAILAND	110	38	30	64	0	0	143	0	0	4	0	3	64	0	0

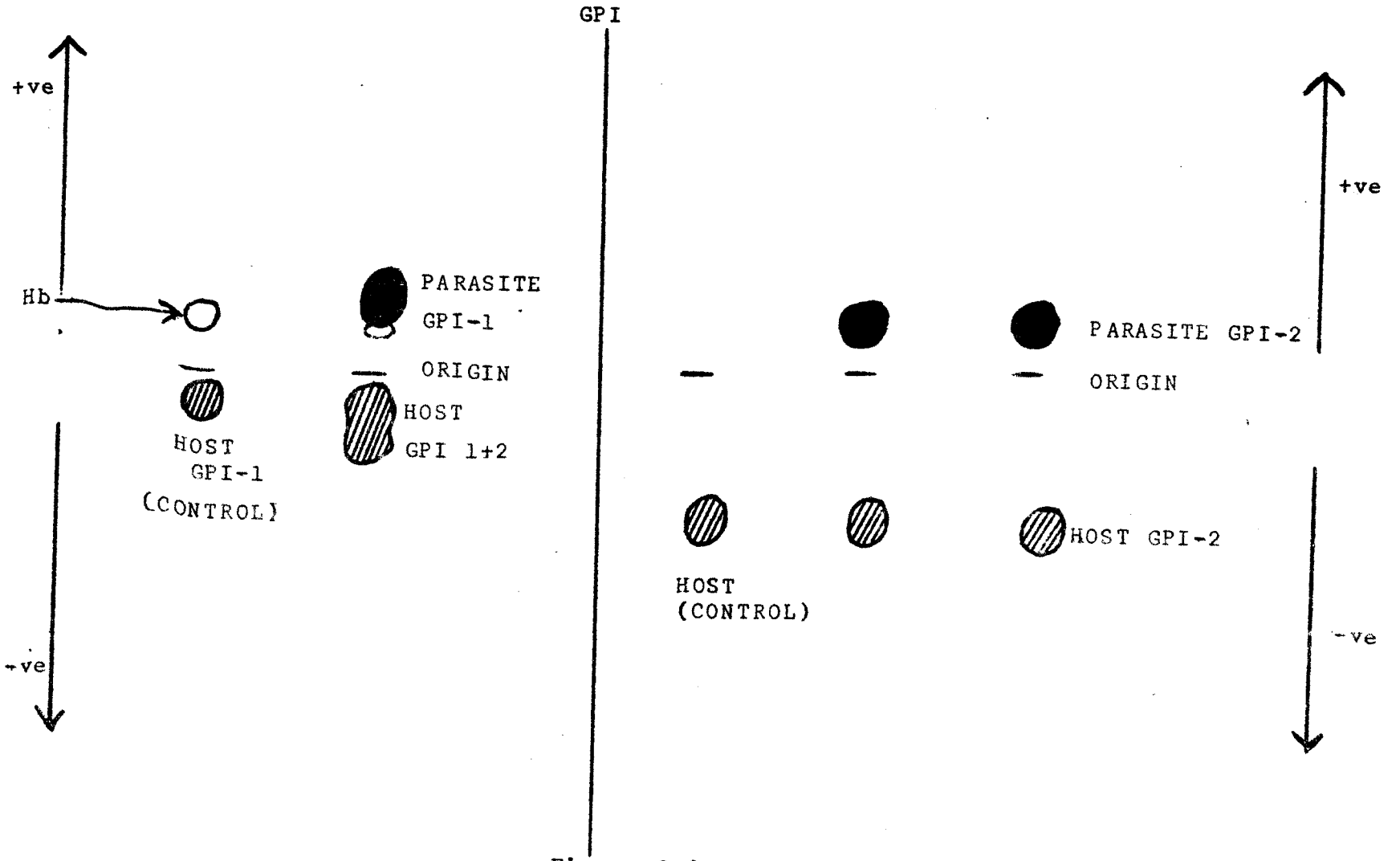
GPI
+ve
Hb
HOST
GPI-1
(CONTROL)
-ve
PARASITE
GPI-1
ORIGIN
HOST
GPI 1+2
HOST
(CONTROL)
PARASITE GPI-2
ORIGIN
HOST GPI-2
+ve
-ve

Figure 3.2a

6-phosphogluconate dehydrogenase (6-PGD) Figure 3.2c

Two variants of parasite PGD were present, PGD-1 and PGD-2. Host PGD was seen as a single band only and no variants were observed.

Adenosine deaminase (ADA) Figure 3.2d and 3.2e

Two forms of parasites ADA were observed. One of them, ADA-1 was seen in two different isolates as shown in figure 3.2d. Isolate 'a' failed to give a parasite enzyme band although it had been processed and stored in a similar manner with the other two. Host ADA-1 was always seen whereas ADA-2 was seen less frequently. The other form of parasite ADA, ADA-2 was seen in one isolate as shown in figure 3.2e.

Peptidase-E (PEP-E) Figure 3.2f

Parasite PEP-E-1 was the only form observed. The enzyme band appeared just below the hemoglobin spot. Host PEP-E was always present in two forms. Isolate 'a' again failed to reveal an enzyme activity band here.

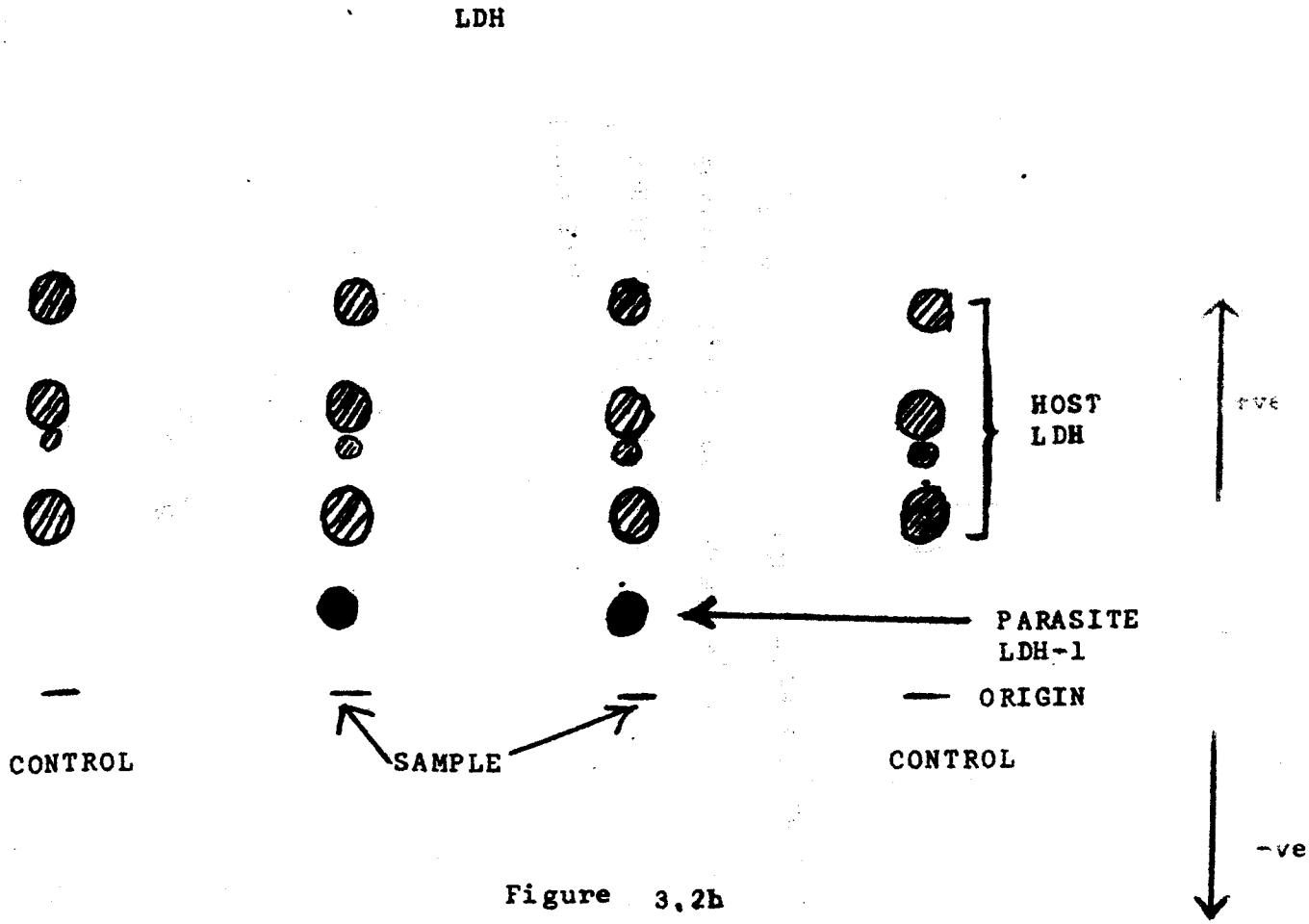
LDH
HOST
LDH
+ve
PARASITE
LDH-1
ORIGIN
CONTROL
SAMPLE
CONTROL
-ve

Figure 3.2b

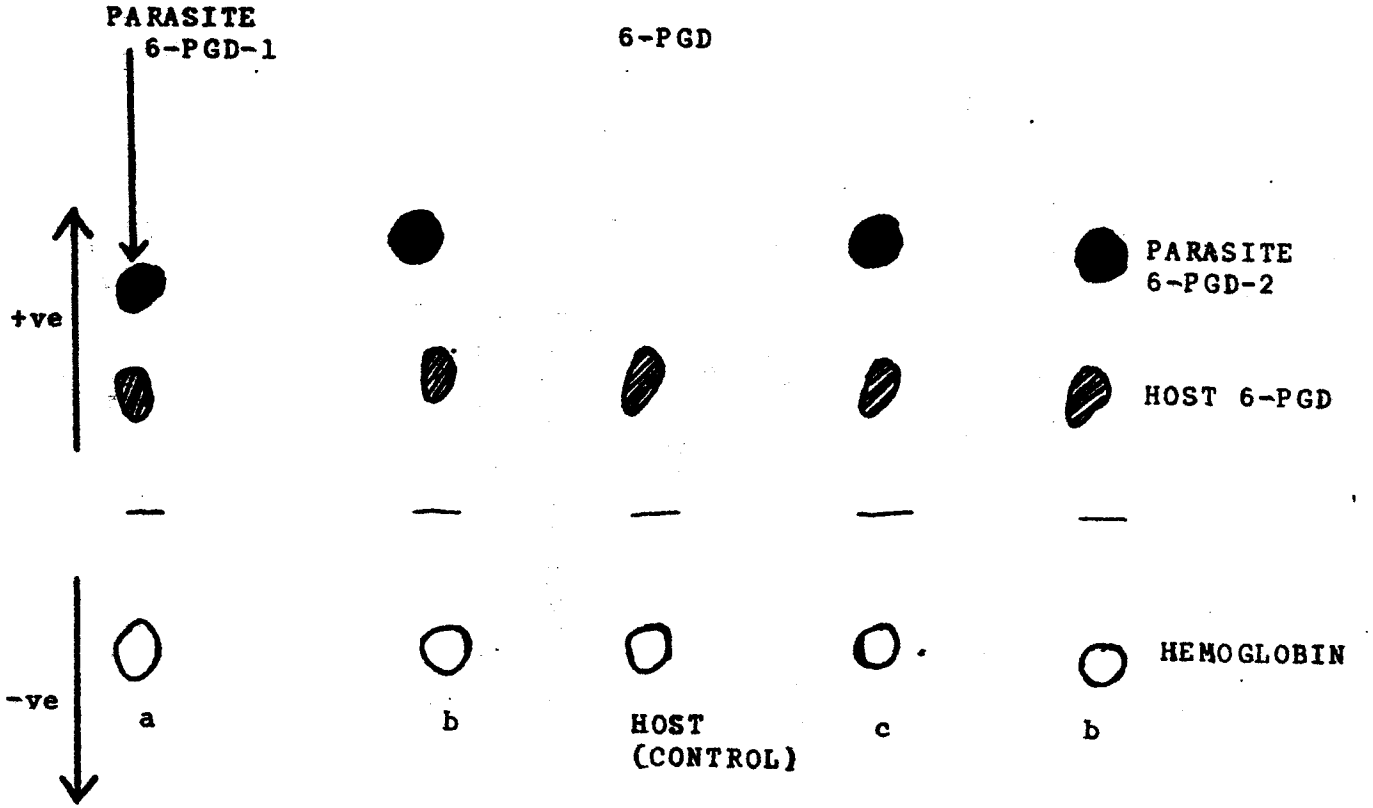
PARASITE
6-PGD-1
6-PGD
+ve
PARASITE
6-PGD-2
HOST 6-PGD
-ve
a
b
HOST
(CONTROL)
c
b
HEMOGLOBIN

Figure 3.2c

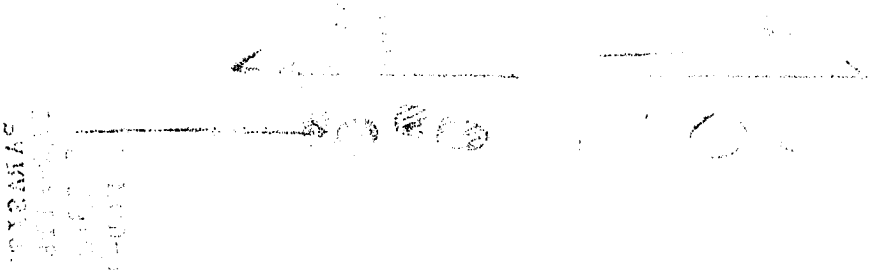

Figure 3.2d

ADA on 0.1M phosphate pH 6.5

Voltage 60V; Current 40mA; time of run 18h
a, b, c = different isolates of P. falciparum

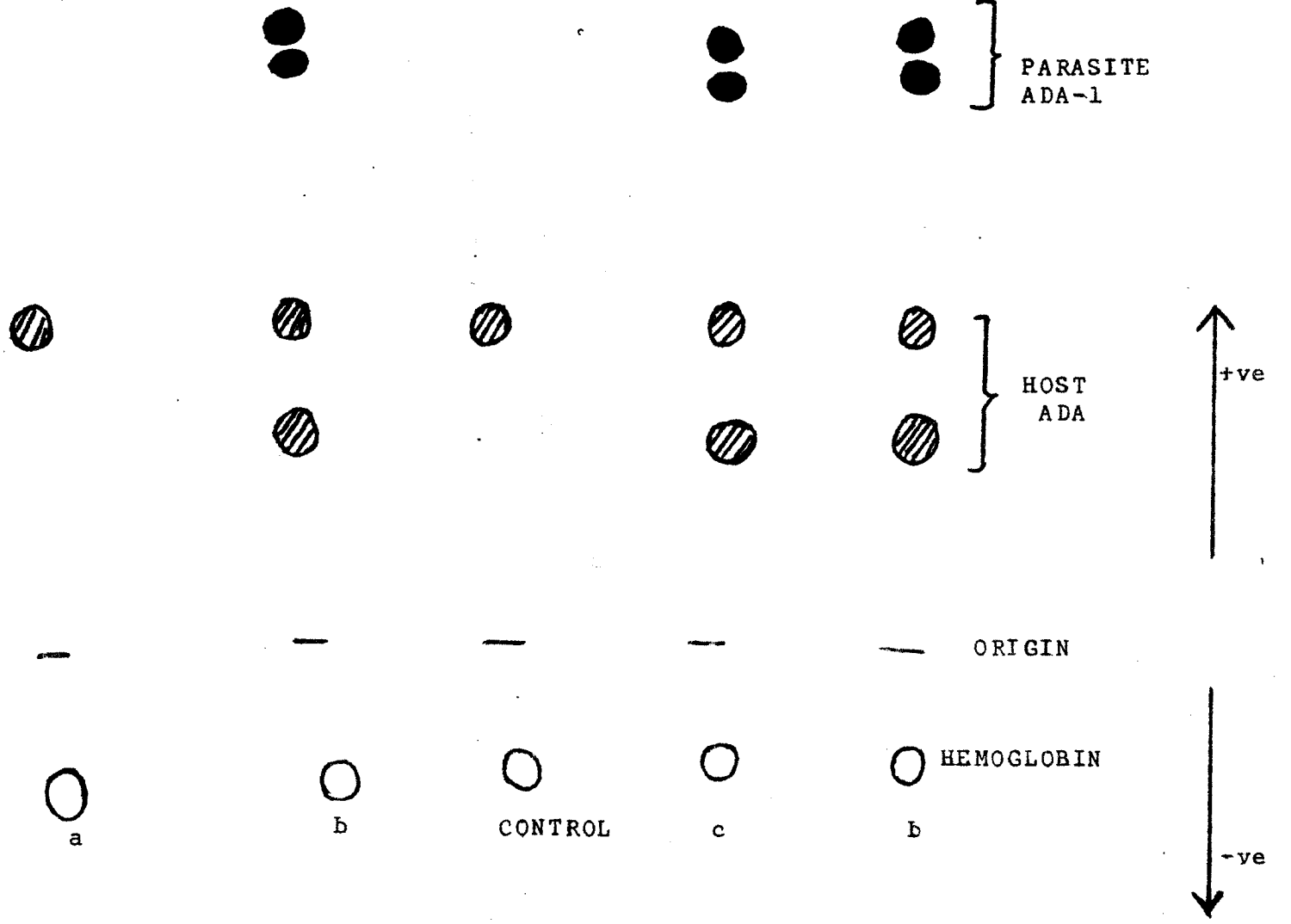
PARASITE
ADA-1
HOST
ADA
+ve
ORIGIN
HEMOGLOBIN
a
b
CONTROL
c
b
-ve

Figure 3.2d

Figure 3.2e

ADA on 0.1M phosphate pH 6.5

Voltage 240V; Current 40mA; time of run 4h

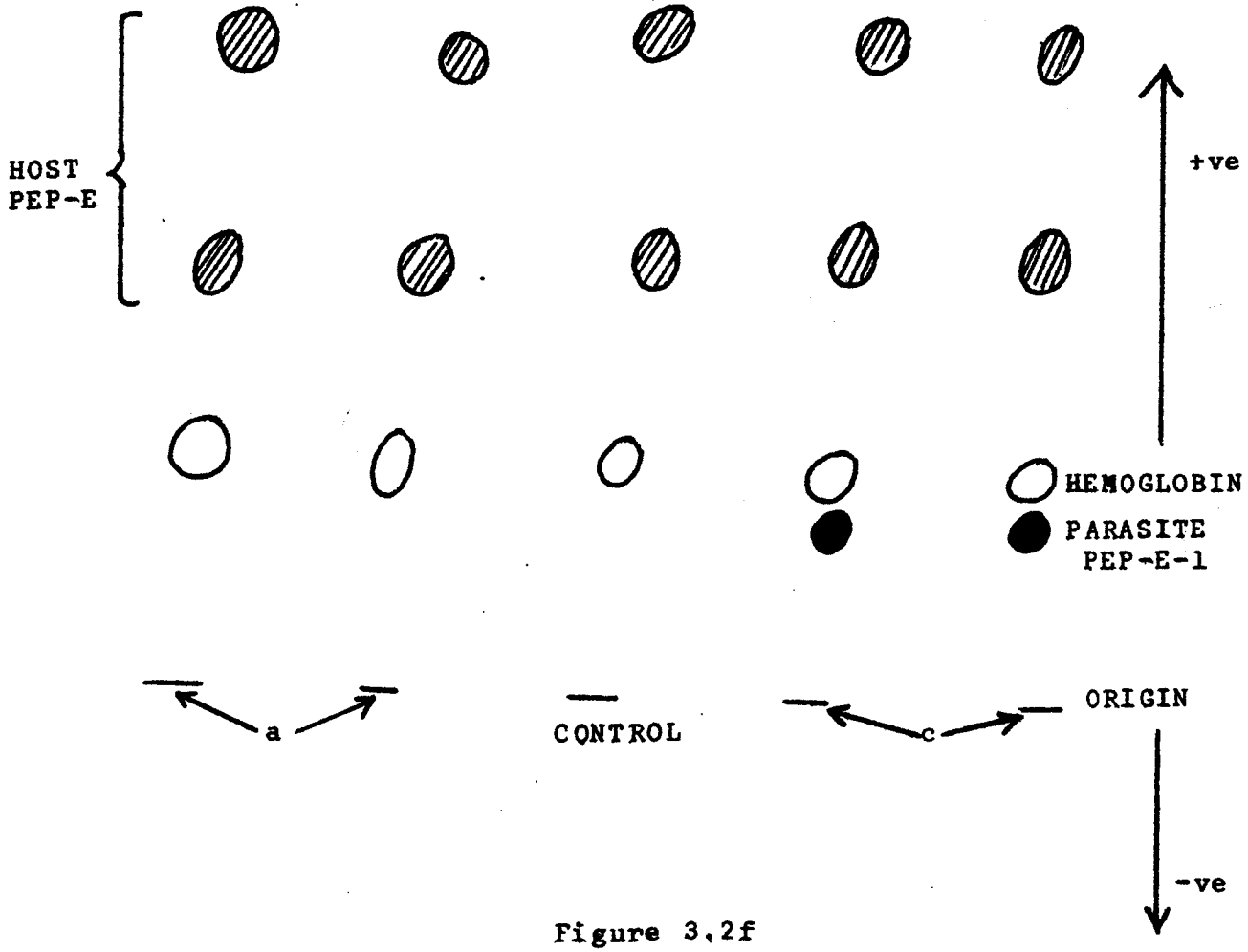
HOST
PEP-E
+ve
HEMOGLOBIN
PARASITE
PEP-E-1
a
CONTROL
c
ORIGIN
-ve

Figure 3.2f

D I S C U S S I O N

The time required for the enzyme bands to develop at 37°C varied with each enzyme. For 6-PGD, PEP-E, and GPI, enzyme activity bands develcped fast (30 minutes). ADA and LDH took a long time (1-2 hours).

The bands became more intense and clearer after being left in water at room temperature for some time. This was clearly so for GPI, LDH and PEP-E but for ADA the bands faded away. 6-PGD band was quite clear right from the start but when gel was washed with water the background became slightly dark. The band was still very conspicuous though because its colour was deep purple.

A number of control experiments were carried out to prove that the isoenzyme patterns seen were due to specific enzyme reactions and not artifacts. When a substrate or coenzyme was omitted from the enzyme assay mixture, then no enzyme activity bands were observed on incubation of the gel. Heat inactivating the enzyme either by heating the sample before application onto gel or by running electrophoresis without cooling, resulted in loss of enzyme activity.

The parasite enzymes have been assigned numbers according to the scheme presented in a paper on enzyme typing of Plasmodium falciparum by Sanderson et al. (1981).

Apart from including a control sample of uninfected human blood during enzyme electrophoresis, no standard parasite enzymes from the original enzyme studies was available for confirmation purposes for ADA, PEP-E and 6-PGD.

Table 3.2b gives a comparative view of the distribution of different enzyme forms of P. falciparum from different areas of the world. Data for Gambia, Tanzania and Congo was extracted from the paper mentioned above while those for Thailand are by Thaithong et al. (1981).

Results obtained in this laboratory (table 3.2a) indicate that 5 out of 8 isolates possessed GPI-1. This frequency is similar to that seen in Congo isolates (table 3.2b). Enzyme polymorphism involving GPI was seen at a low frequency in the Zambian isolates. The presence of only one form of parasite LDH in fresh culture material has also been reported by Thaithong et al. (1981). The isolates tested here revealed parasite PGD-1 and PGD-2 in the ratio 1:4. The occurence of PGD-2 at a frequency of 80% is in contrast with observations made elsewhere (table 3.2b). This variant of PGD has only been observed in Gambian isolates but at a very low frequency (table 3.2b).

3.3 CHLOROQUINE SENSITIVITY TESTS

P. falciparum isolates were put into culture for 96 hours to ensure optimal growth before chloroquine sensitivity tests were performed. In the initial stages of cultivation, the asexual erythrocytic cycle is found to be synchronous every 48 hours. It is therefore possible to obtain parasites predominantly in ring stage after 48 hours or 96 hours in culture. It is essential to use ring form parasites in this kind of study because the degree of schizont formation inhibition is a direct measure of the effectiveness of the drug.

Table 3.3a shows that all isolates responded well to chloroquine as shown by growth inhibition at the specified concentrations. One isolate was inhibited at a very low concentration of 1×10^{-7}M (0.03 $\mu g ml^{-1}$) chloroquine base. The rest of the isolates were not affected at this chloroqu concentration. During drug pressure and even after withdra of the drug, the parasites were still multiplying normally at 1×10^{-7}M. The drug was administered to a 96 hours cult displaying parasitemias ranging from 1.0% - 1.99% as shown in table 3.3b. Parasites in the control dishes grew well, producing normal looking schizonts and increasing in parasi

Table 3.3a

Chloroquine sensitivity of some Zambian isolates of *Plasmodium falciparum* in vitro

Case	Age (years)	Sex	Chloroquine concentration (moles l^{-1})			
			$1x10^{-7}$ (0.03 $\mu g ml^{-1}$)	$3x10^{-7}$	$5x10^{-7}$	$1x10^{-}$ (0.3 μ
1	20	M	✓	X	X	X
2	4	F	✓	X	X	X
3	3	M	X	X	X	X
4	10	M	✓	X	X	X
5	6	M	✓	X	X	X

Key: X = growth markedly reduced after 48 hours and complet inhibited by 72 hours.

✓ = growth not inhibited by the drug.

except on day 6 *in vitro* when there was a reduction in the parasitemia level. Since no fresh red cells had been added on day 4 when subjecting the culture to chloroquine, it was found necessary to do so on day 6.

At those concentrations where inhibition of parasite growth occured, parasites seen at 24 hours and 48 hours were vacuolated unhealthy rings, immature gametocytes at times and in some cases some abnormal schizonts with no clear merozoites. These unhealthy schizonts were normally very few compared to the rings. In these schizonts only one or two merozoites were seen with distinct nuclei. 72 hours later, the last 24 hours being in drug free medium, no more parasites were seen, not even immature gametocytes.

Table 3.3c shows parasitemia levels observed in one of the isolates tested. The isolate obtained from a four year old female was put into culture up to 48 hours before administration of medicated medium. The parasitemias at this point were all below 1.0%. Chloroquine at 1×10^{-7}M had no effect on the growth of the parasite *in vitro* (table 3.3c). Normal multiplication of parasites was seen in the control dishes as well. There was a steady reduction in parasitemias from the first 24 hours and by 72 hours the rest of the cultures were completely negative for parasites.

Case No. 3 Data

Table 3.3b

Chloroquine diphosphate effect on P. falciparum in vitro

Chloroquine concentration (moles l^{-1})	% Parasitemia			
	0 hours	24 hours	48 hours	72 hours
Control	1.37	1.6	1.36 ◁—	0.48
1×10^{-7}	1.15	0.63	0.16	0
3×10^{-7}	1.99	0.7	0.1	0
5×10^{-7}	1.0	0.46	0.08	0
1×10^{-6}	1.16	0.28	0.05	0

—▷ = Diluted 1:1 with freshly washed O^+ blood cells (18 days old)

Case No. 2 Data

Table 3.3c

Chloroquine diphosphate effect on P. falciparum in vitro

Chloroquine concentration (moles l^{-1})	% Parasitemia			
	0 hours	24 hours	48 hours	72 hours
Control	0.75	1.0	1.4	1.6
1×10^{-7}	0.45	0.85	1.15	1.5
3×10^{-7}	0.40	0.15	0.03	0
5×10^{-7}	0.45	0.2	0.01	0
1×10^{-6}	0.60	0.07	0	0

D I S C U S S I O N

Chloroquine is still being used with success in the treatment of malaria in Zambia, therefore it was decided to initially use 1 x 10^{-6} M (1.0nmole ml^{-1}) as highest concentration. All the isolates tested were sensitive to chloroquine. One of them (case number 3) can be considered to be very sensitive since it was inhibited at 1 x 10^{-7} M, a concentration at which chloroquine is normally found to be ineffective. Our results are encouraging in a situation where chloroquine resistance has become a common feature in some areas (Thaithong and Beale, 1981; Varnai et al., 1981; Ferraroni et al., 1981).

The only documented case of chloroquine resistance in Zambia is that reported by Khan and Maguire (1978). Using an *in vivo* test the authors implicated RII degree of chloroquine resistance without referring to the asexual parasitemia levels. The species of malaria involved and even the parasite forms observed are not mentioned. The authors show that blood films remained positive but they do not mention whether there was a clear reduction of asexual parasitemia. RII criteria is used in cases where there is a marked reduction of asexual parasitemia with no clearance (W.H.O. Technical Report Series (529), 1973).

Results obtained in this study suggest that chloroquine-resistant P. falciparum has not yet appeared in Zambia.† Another independent study carried out in a different area of Zambia using an in vitro macro-test where schizont maturation within 24 hours was followed, indicates that P. falciparum in this region is still sensitive to chloroquine (personal communications, Tropical Diseases Research Centre, Zambia).

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† Observations made by Doctors in Lusaka indicate infections not responding to chloroquine do occur at infrequent intervals but it has not been documented whether such people show lack of chloroquine absorption.

CHAPTER 4

GENERAL DISCUSSION OF RESULTS AND CONCLUSIONS

4.1 In Vitro Cultivation

The multiplication rates observed for the successful culture in vitro are not very high when compared with those obtained with line FCR-3 (Jensen and Trager, 1978). The rate of increase after four days (96 hours) observed in week six in vitro for the isolate grown here was only 5x while that of FCR-3 was 40x. The above authors encountered some strains of P. falciparum which did not multiply as well as FCR-3 also and one which did not get established in culture even after surviving for five weeks in vitro.

The increase in growth rate observed when medium is changed more frequently has been shown by other investigators as well. Butcher (1979) showed that changing medium two or three times every 24 hours leads to an increase in the level of parasitemia.

There is no documented literature which I am aware of that shows a decrease in parasitemia upon change of serum (figures 3.1c, 3.1d). This finding was purely coincidental and might have escaped attention if pooled serum was used instead. Pooled serum was not used here mainly because blood group AB donors are rare and no large - scale cultivation of parasites was carried out to warrant need for large amounts of serum.

It is hoped that investigators involved in studies that might require comparison of parasitemias do take note of this.

Ward et al. (1981) did not get better growth of P. falciparum in vitro when heat inactivated serum was used. This is in contrast to the findings of this study that serum inactivated by heat supported growth at two times the rate observed in ordinary serum. The above authors carried out a number of investigations into use of serum pretreated with immune complexes and C_2, C_3, C_4 and C_5 deficient serum as well. They concluded that none of the above including inactivation of complement by heating significantly influence the extent of penetration of red cells by P. falciparum. The suggestion by the authors that complement does not facilitate plasmodial infections seems to be in agreement with my finding. This is paradoxically so if the term facilitate is used to imply 'make easy' since the absence of complement seemed to encourage P. falciparum to invade erythrocytes more effectively.

The above finding coupled with the observation made upon change of ordinary serum (figure 3.1c) suggest strongly the involvement of the C system in an antibody-antigen reaction i the in vitro cultures of P. falciparum considered. The event lysis just prior to termination of a culture observed frequent

implicates the presence of specific antibodies (probably malarial) which would utilize complement when binding to an antigen.

Doubling the glucose concentration did not appear to increase the parasitemia of P. falciparum in short-term cultures here, but Osisanya et al. (1981) obtained results that indicated increase in glucose concentration was favorable in terms of economizing medium and time. The authors used strains already established in culture as well as newly isolated strains of P. falciparum. Unfortunately the period within which the data given by these authors was obtained is not mentioned, making it difficult for a reasonable comparison to be made.

Useful further investigations which could be carried out here include estimation of specific malaria antibodies (IgG) in Zambians, using a sensitive technique like radio-immunoassay, effect of glucose in long-term cultures, and use of techniques that would allow large increases in parasitemias in vitro.

.2 Isoenzymes

The results obtained here are similar to those given for other isolates in table 3.2b except for 6-PGD. The isolates tested here revealed PGD-2 mainly whereas those from other areas showed PGD-1 at a higher frequency (table 3.2b). PGD-3 was not present in the Zambian isolates. This was also the case with the Thai isolates (Thaithong et al., 1981). This variant was seen at a very low frequency in Gambian and Tanzanian isolates (Sanderson et al., 1981).

The technique of starch gel electrophoresis of enzymes has revealed genetically distinct and reproductively isolated populations in rodent malaria (Carter, 1978). However, although enzyme studies of P. falciparum have also shown genetic variation, according to work done by Sanderson et al. (1981) and Thaithong et al. (1981), isolates from Africa and South East Asia are thought to constitute a single inter-breeding species.

Enzyme polymorphism, involving especially GPI was commonly encountered in isolates from other areas of the world (table 3.2b) but this was present in only one isolate from Zambia. It is difficult to make meaningful comments on the ADA and PEP-E results at this stage because the number of isolates tested is small.

Some further investigations into isoenzymes of P. falciparum should aim at studying more isolates for ADA and PEP-B isoenzyme patterns as well as glutamate dehydrogen (GDH) and triose phosphate isomerase (TPI).

4.3 Chloroquine Sensitivity Tests

Our findings are similar to those of Richards and Maples (1979) except for a few observations. The results obtained by these authors show an increase in parasitemia level in the first 24 hours of drug pressure whereas the isolates tested here responded promptly as shown by the clea reduction in parasitemias during the same period. Even afte 72 hours, the above authors still observed some parasites at 10^{-6}M chloroquine though they were at a low parasitemia compared to the controls. The isolates tested here were completely inhibited after 72 hours at 10^{-6}M. Complete inhibition of parasite growth after 72 hours was also observe using 3×10^{-7}M and 5×10^{-7}M chloroquine base. We can concl that all the isolates tested here were far more sensitive tha that used by the above authors. The strain employed by these authors was originally isolated from a seaman who had been to Nigeria, by Dr. A. Reid of Liverpool School of Tropical Medic

It is interesting to note that Dinh and Trager (1978) also using the Petri-dish culture method found similar results i.e. 3×10^{-7}M ($0.1\ \mu g ml^{-1}$) was the lowest concentration of chloroquine which inhibited P. falciparum growth. They were using a stabilised African strain (FCR-3) obtained from Gambia. The degree of sensitivity is thus comparable to ours.

Reports of chloroquine resistant P. falciparum from Africa have been appearing frequently in scientific publications (Morbidity and Mortality Weekly Report, November 1978 ; Aronsson et al., 1981). Most of the cases reported involve European visitors to some areas of East Africa. The authors most often mention persistence of malaria symptoms despite administration and absorption of chloroquine as proved by the excretion of chloroquine in the urine. The only other comment frequently seen in these papers is that both the in vivo and in vitro tests proved that the P. falciparum considered was resistant to chloroquine. The source of chloroquine used is often not mentioned. This is important because the 2 optical isomers of chloroquine diphosphate for example, have been found to have different antimalarial activity (Haberkorn et al., 1980). The degree of resistance is sometimes mentioned without a good reason to substantiate the claim.

Not disputing the possibility of emergency of chloroquine resistant P. falciparum in Africa however, some articles on this subject will be considered here. Aronsson et al. (1981), Jensen et al. (1981) and Varnai et al. (1981) reported some clinical cases of chloroquine-resistant P. falciparum. Aronsson et al. (1981) found P. falciparum displaying R1 type of chloroquine resistance in three Swedish females. Two of them live in Madagascar while the third had gone to Kenya for a visit. The authors reported serum levels of chloroquine far above that considered to be effective against sensitive strains but incidents of recrudescence were present until quinine was administered. Varnai et al. (1981) reported a chloroquine resistant malaria case from Tanzania. The authors suggested RII and RIII degree of resistance. The authors mention that the patient's parasitemia still persisted after one week even after taking the recommended full dose of chloroquine. However, they do not mention whether there was a reduction in asexual parasitemia or not which would differentiate RII from RIII.

Jensen et al. (1981) reported a clinical case of chloroquine-resistant P. falciparum malaria acquired from FCR-3 strain in culture. Dinh and Trager (1978) showed this strain to be sensitive to chloroquine, 0.1 $\mu g ml^{-1}$ ($3 \times 10^{-7} M$) bringing about complete inhibition in vitro.

Jensen et al. (1981) showed that this strain required 0.3 $\mu g ml^{-1}$ (1×10^{-6} M) instead to bring about complete inhibition *in vitro*. The authors showed that this strain is now resistant to chloroquine both *in vivo* and *in vitro* as demonstrated by the failure of chloroquine to clear the infection accidentaly acquired by one of them in their laboratory. Dinh and Trager (1978) had shown that chloroquine resistance can be brought about artificially in the laboratory, using some parasites from FCR-3 strain and subjecting them to gradually higher concentrations of chloroquine for one month.

Further work on chloroquine should include both *in vivo* and *in vitro* tests. In cases where the 24 hours macrotest based on schizont inhibition is used, the patient should be followed up if results obtained incline towards possible chloroquine resistance. Routine field research should be carried out regularly to monitor the sensitivity of *P. falciparum* to chloroquine and other antimalarials.

APPENDIX

Appendix

Acid citrate dextrose (ACD)

1.34g dextrose (D(+) glucose)
1.32g sodium citrate
480mg citric acid

Diluted to 100 ml with distilled water

Giemsa Stain

Improved R66 solution was obtained from Hopkin and Williams (Essex, England). This stock concentrate was diluted 1:5 with phosphate buffer (pH 7.2) and the stain used within four hours.

Na_2HPO_4 - NaH_2PO_4 and Tris/HCl buffer solutions were prepared according to Sorensen (1955).

RPMI 1640 Culture Medium

RPMI, 1640 dry powdered synthetic medium (Gibco New York) contains the following:

Inorganic Salts

	mgl^{-1}
$Ca(NO_3)_2.4H_2O$	100.00
KCl	400.00
$MgSO_4$	48.84
NaCl	6000.00
Na_2HPO_4(anh.)	800.00

Other Components

Glucose	2000.00
Glutathione (reduced)	1.00
Phenol red	5.00

Amino Acids

L-Alanine	
L-Arginine (free base)	200.00
L-Asparagine	50.00
L-Aspartic acid	20.00
L-Cystine (2HCl)	65.15
L-Glutamic acid	20.00
L-Glutamine	300.00
Glycine	10.00

Amino Acids (Cont'd.)

L-Histidine (free base)	15.00
L-Hydroxyproline	20.00
L-Isoleucine (Allo free)	50.00
L-Leucine (methionine free)	50.00
L-Lysine HCl	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline (Hydroxy L-Proline free)	20.00
L-Serine	30.00
L-Threonine (Allo free)	20.00
L-Tryptophan	5.00
L-Tyrosine (Na salt)	28.94
L-Valine	20.00

Vitamins

Biotin	0.20
D-Ca pantothenate	0.25
Choline Chloride	3.00
Folic acid	1.00
i-Inositol	35.00
Nicotinamide	1.00

Vitamins (Cont'd)

P-aminobenzoic acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamine HCl	1.00
Vitamin B_{12}	0.005

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