Biochemical and immunochemical investigation of some South African strains of the human malaria parasite, *Plasmodium falciparum*

by

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LIST OF ABBREVIATIONS

ADP	-	Adenosine diphosphate
AMP	-	Adenosine monophosphate
ATP	-	Adenosine triphosphate
CS	-	Circum-sporozoite
CSP	-	Circum-sporozoite protein
DIC	-	Disseminated intravascular coagulation
DNA	-	Deoxyribonucleic acid
DDT	-	Chlorophenothane
FPIX	-	Ferriprotoporphyrin IX
IMP	-	Inosine monophosphate
MDR	-	Multi-drug resistance
MSA	-	Merozoite surface antigen
NIDTE	-	National Institute for Diseases in a Tropical Environment
PABA	-	Para-aminobenzoic acid
PBS	-	Phosphate buffered saline
PEG	-	Polyethylene glycol
PfHRP1	-	*Plasmodium falciparum* histidine rich protein
PfUP1	-	*P. falciparum* University of Pretoria isolate number one
PPM	-	Parasitophorous plasma membrane
PVM	-	Parasitophorous vacuolar membrane
RER	-	Rough endoplasmic reticulum
RNA	-	Ribonucleic acid
TEM	-	Transmission electron microscope

TSP	-	Transfer solid phase
WHO	-	World Health Organisation

CHAPTER 1

GENERAL LITERATURE REVIEW

Malaria (literally meaning "bad air", which refers to the old theory of the miasmatic origin of the disease) is one of the serious environmental diseases of man (1). Malaria is caused by a unicellular parasite of the genus *Plasmodium* and is biologically mediated through the bite of a female *Anopheles* mosquito (2). During the complex life cycle in man, the malaria parasite enters the the blood stream, infiltrate the liver and eventually infects the erythrocyte and undergoes rapid growth and development. The release of merozoites leads to a new wave of infection. The asexual blood stages are responsible for the clinical manifestations and complications such as anemia, disseminated intravascular coagulation syndrome (DIC), electrolyte disturbances and multi-organ failure (3).

1.1 Diagnosis of malaria

The first detection of the malarial parasite in an infected patient's blood was reported by Lavaren, a French surgeon in 1880 (4). Optical diagnosis was improved by Romanowsky in 1891 and the thick film technique was introduced by Ross in 1903 (5). Presently a microscopic examination of thick films, stained with giemsa can detect a 0,0004% parasitemia at 1000 times magnification in 30 to 60 minutes (5). A newly developed rapid diagnosis is based on staining the parasites with acridine-orange and detection of the parasites using fluorescence microscopy (6). This technique may replace the thick blood smears if a fast diagnosis is needed and no species identification of the parasite is required. Microscopical diagnoses are not always practical in a Third-world context due to lack

of microscopes and experienced technicians to use them. Alternative methods, like DNA probe-hybridiation may be useful, particularly if large scale screening is required (7). However, the expense problems with disposal, poor field applicability and the fact that final results are available only after a few days (if hybridizations are visualized on a photographic plate) renders this method impractical (7). Serology, e.g. using the ELISA method, can be used for the screening of a large number of samples. This technique can be used in a well-equipped laboratory as well as under field conditions. The antigenic diversity within the same parasite species and the fact that few antigenic determinants are shared in geographically distinct parasites, leads to standardization problems which have not yet been solved (8). A good test should be specific, sensitive, accurate, rapid, simple to learn and cheap. Presently, no tests comply to all of these criteria (8).

1.2 Epidemiology

Presently more than 2,5 billion people are living in malaria areas, mainly in Third-world countries with little or no measures to control the disease. *Plasmodium falciparum* is annually responsible for more than one million deaths of children under 5 years of age in Africa alone (9). In South Africa the geographic range of malaria embraces the tropical and sub-tropical regions. There is a small endemic area on the border next to Mozambique. The rest of the region stretches from Mtubatuba next to the Great Umfolosi on the east coast at the border of Mozambique. East from the Drakensberg and north from the Soutpansberg malaria is unstable with rapid seasonal epidemics. The area around the Limpopo in the north-west and around Upington are epidemic malaria areas. In Fig.1.1 the distribution of malaria in Southern Africa is illustrated.

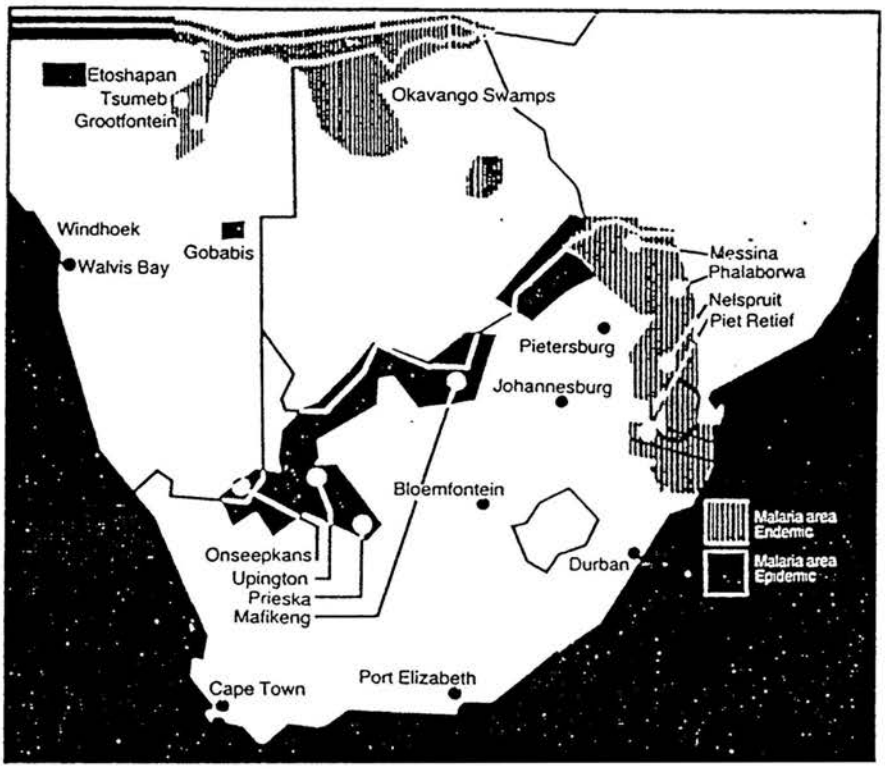

Figure 1.1 Malaria areas in Southern Africa (8).

Over the last decade (1980-1990) there has been an upsurge in malaria which can be attributed to a number of factors. Natural factors such as increased rainfall, increases the *Anopheles* mosquito's breeding sites, while the influx of refugees who harbor the malaria parasite, the increased incidence of drug-resistant malaria parasites and resistance of the vectors against insecticides are man-made problems (10). The number of districts that have incidence rates greater than 10 % have increased

yearly since 1986 (10). This increase in malaria notifications is shown in Fig 1.2.

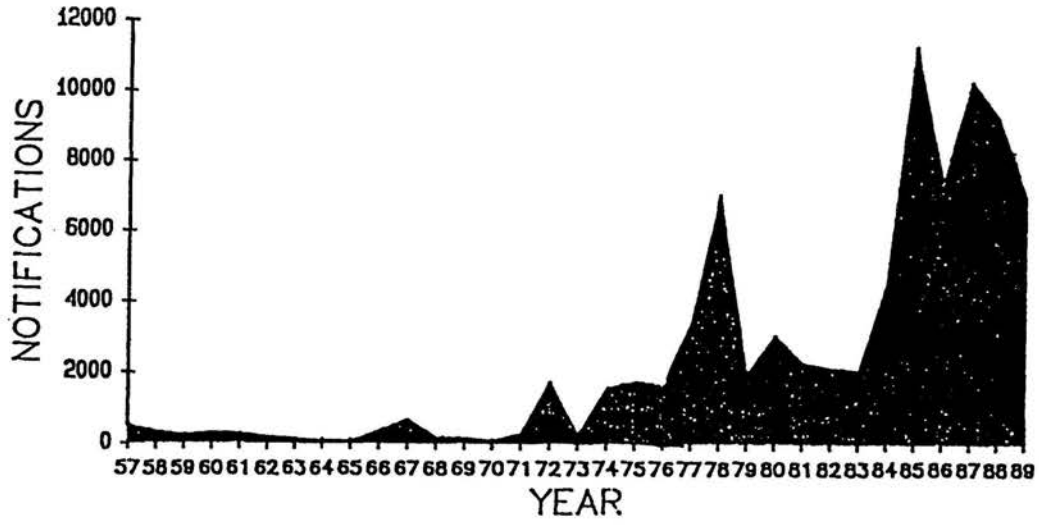

Figure 1.2 Malaria notifications in South Africa from 1957 to 1989 (10).

The re-organising of the active surveillance in 1975 led to a higher recorded incidence rate, but over the last decade in particular, a major increase was observed.

Malaria mostly occurs naturally through the exposure of a person to the feeding of an infected female *Anopheles* mosquito which transfers the malaria parasite among human victims (2).

1.3 Life cycle of the malaria parasites.

More than a hundred *Plasmodium* species exist of which only four are known to infect man (11). The malaria parasites are protozoa from the class sporozoa and genus *Plasmodium*. The life cycle of the four malaria

species are identical. Two processes, schizogeny and sporogony, are distinguished. Schizogony occurs in the human host and can be divided into the liver (tissue) and the blood stages. Sporogony starts in the host but ends in the vector (11).

Infection begins when a malaria-parasite-carrier mosquito injects sporozoites into the host during a bloodmeal. Sporozoites inoculated from the salivary glands of the mosquito, migrate to the liver where differentiation is initiated inside the hepatocytes and completed within 10-48 hours (11). Merozoites are released from the hepatic cells into the cardiovascular system. After infection of erythrocytes, the parasite will differentiate into the ring form, a name derived from the appearance of the Giemsa-stained parasite due to a ring of cytoplasm surrounding a large vacuole next to the deeply- stained nucleus. The parasite, feeding upon the hemoglobin of the host erythrocyte, deposits grains of pigment (haemozoin) in the cytoplasm and develops in an uninucleate trophozoite (12). The nucleus subsequently divides several times giving rise to a schizont. The erythrocyte containing mature schizonts ruptures and merozoites emerge. The merozoites have a short extra-cellular life of a few seconds during which they invade new erythrocytes by attachment to and invagination of the erythrocyte membrane. After a few cycles of schizogony the merozoites released from the erythrocytes changes into micro- or macrogametocytes (male or female). The rest of the cycle is completed inside the vector where macrogametocytes are fertilized by microgametocytes and form zygotes. The zygotes differentiate into ookinetes and eventually into sporozoites which migrate to the mosquito's salivary glands. Infection of a new host is now possible with the next bloodmeal (13). In Fig 1.3 the malaria life cycle is summarized.

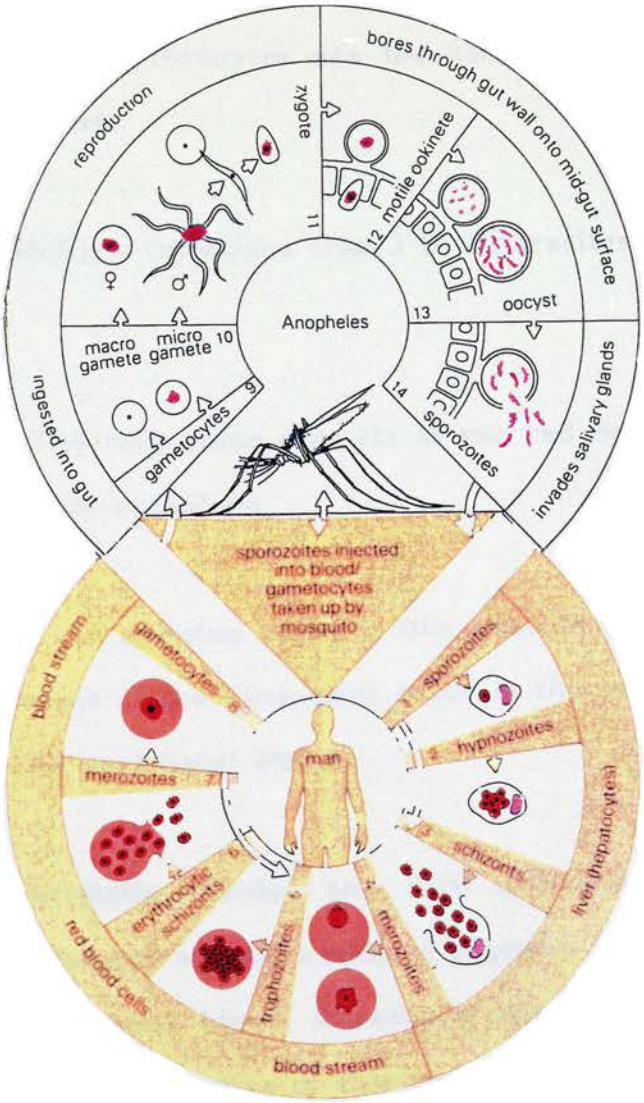

Figure 1.3 Life cycle of *Plasmodium* species (13).

1.4 The different malaria species.

Although the malaria parasites all belong to one genus, there are morphological differences in the life cycles of the different species. Even in the same species genetic variants are possible. The main differences between the 4 most important strains are as follows:

(A) Plasmodium falciparum.

Erythrocytes: Infected erythrocytes are the same size and colour as non-infected erythrocytes.

Multiple infections: Multiple infections from 2 to 6 parasites per cell have been recorded.

Young trophozoites: The ring stage consists of two red dots of chromatin and a thin circle of blue cytoplasm.

Growing trophozoite: The growing parasite fills the cell and Schuffner's dots (small, red pigments of the same size) occur in the cytoplasm. This stage is rarely found in peripheral blood.

Schizonts: The chromatin divides and 8-20 merozoites may form. The merozoites are arranged around a mass of pigment. These forms are also rarely seen in peripheral blood. Absence of late stage parasites in thick smears of patients is indicative of this type of parasite.

Gametocytes: The immature gametocyte is oval-shaped and lies to one side of the erythrocyte. The chromatin is pink and scattered among the pigment granules in the ectoplasm. As the mature gametocyte becomes concavely shaped the pigment granules gather in the middle half of the parasite. The immature macrogametocyte is spindle-shaped and only a small part of the erythrocyte can be seen at the edges of the parasite. The mature macrogametocyte is long and thin with chromatin granules concentrated in the middle of the parasite.

(B) Plasmodium vivax.

Erythrocytes: The infected erythrocyte is usually larger and irregular in shape compared to non-infected erythrocytes. The cell may be paler than non-infected erythrocytes, and Schuffner's dots can be seen in these cells.

Multiple infection: Common and easily seen in the ring stage.

Young trophozoites: The "signet" ring stage consists of a large red stained chromatin dot on a blue ring of cytoplasm. The ring is usually one third of the diameter of the erythrocyte. The cytoplasm is slightly broader opposite the chromatin dot. Small pseudopoda may develop in this stage and extend in various directions.

Growing trophozoites: The cytoplasm and the pseudopoda expand and small yellow-brown pigment granules form in the erythrocyte as the parasite digests the hemoglobin.

Schizont: The chromatin material divides and produces 12-24 separate particles. The pigment in the erythrocyte forms one or two conglomerates.

Gametocytes: Young gametocytes are round and the chromatin is centrally situated near a vacuole. The mature microgametocyte has a pale cytoplasm, a mass of chromatin that stains pink to dark red and pigment granules which are scattered inside the parasite. The macrogametocyte is larger and oval-shaped. The dark red chromatin lies near the edge of the parasite membrane and is free of vacuoles.

(C) Plasmodium malaria.

Erythrocytes: Infected erythrocytes are the same size or smaller than non-infected erythrocytes. Occasionally small red dots known as Ziemann's dots (irregular shaped red pigment) may be seen in the cytoplasm.

Multiple infection: This is rarely seen in this species.

Young trophozoites: In the ring stage the red chromatin dot is round or oval and the cytoplasm is small. The ring is about one fourth of the diameter of the erythrocyte. As the parasite ages, the cytoplasm elongates and forms a band across the erythrocyte with only a few pigment granules.

Growing trophozoite: The parasite grows larger and has a characteristic band form. The pigment may be scattered or arranged along the edge.

Schizont: The chromatin divides into 6-12 small chromatin masses in a rosette around a pigment conglomerate.

Gametocytes: The mature microgametocyte almost fills the erythrocyte and contains a round or an oval-shaped chromatin mass. The macrogametocyte is larger and has an oval-shaped chromatin mass which lies to one side of the erythrocyte with scattered pigment granules.

(D) Plasmodium ovale

Erythrocytes: The erythrocytes are larger than non-infected erythrocytes and are oval- or egg-shaped. The edges of the erythrocyte are commonly fimbriated and Schuffner's dots are abundant.

Multiple infections: This only occurs if the infection rate is high, but it is not a common phenomenon.

Young and growing trophozoites: The rings are small, about one third of the erythrocyte diameter. The cytoplasm is dark and pigment granules may form inside the erythrocyte.

Schizont: The roundly shaped parasites usually divide into 8 merozoites which rosette around the pigment mass in the center.

Gametocytes: They are similar to *Plasmodium malaria* although they appear smaller in the enlarged infected erythrocyte. A large amount of Schuffner's dots are present which assists with the classification of the parasites.

Fig.1.4 shows the bloodstages of the four stains of malaria that infects man.

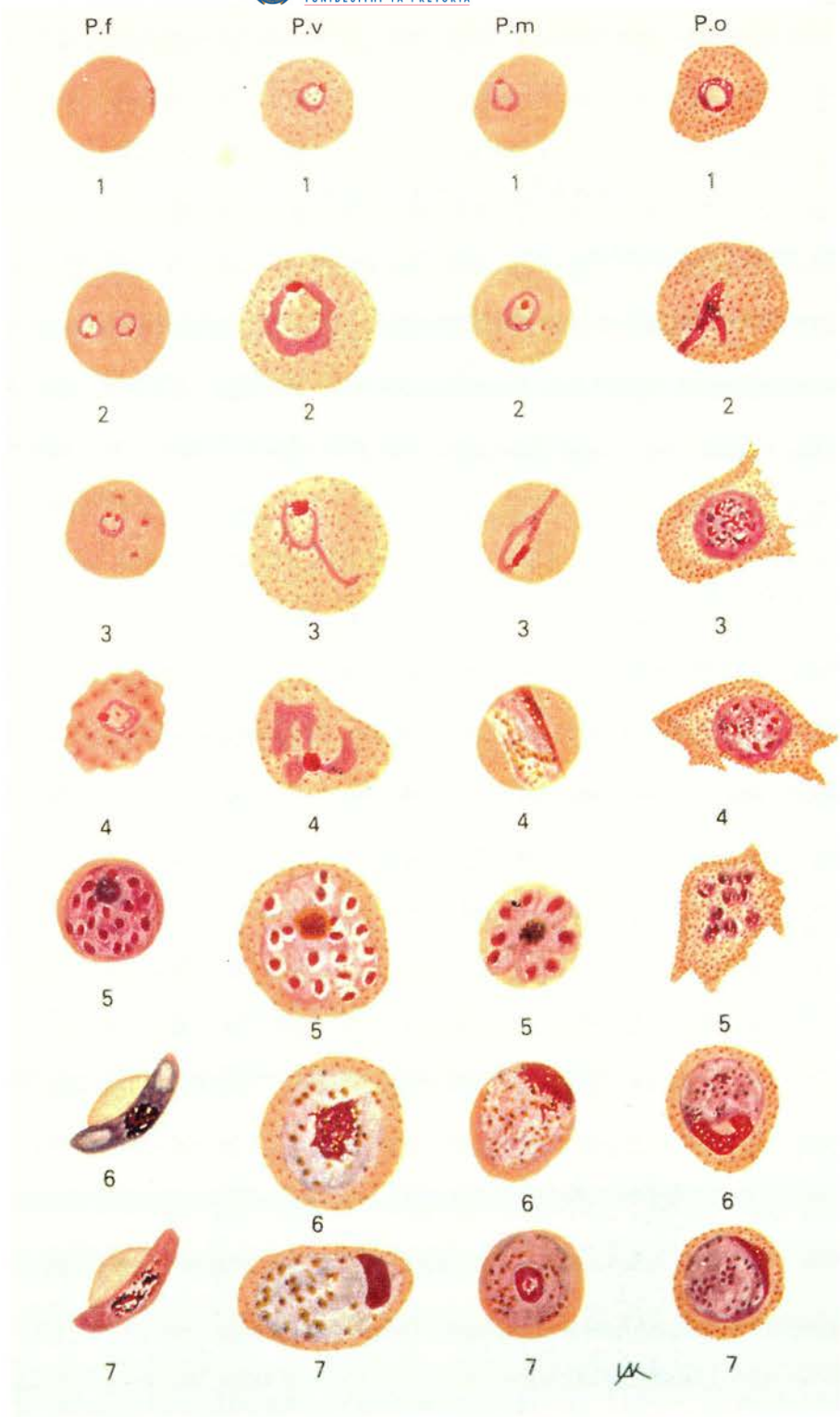

Figure 1.4 Comparison between the bloodstages of human malaria parasite strains after Giemsa staining.
(P.f.) Plasmodium falciparum. (P.v.) Plasmodium vivax (P.m.) Plasmodium malaria. (P.o.) Plasmodium ovale..
1. Ring stages
2. Young Trophozoite stage
3. Trophozoite stage
4. Young schizont stage
5. Schizont stage
6. Mature microgametocyte
7. Mature macrogametocyte

Taken from (39).

1.5 Pathology of malaria

1.5.1 Parasite induced membrane changes in the erythrocyte.

Electron microscopy of infected erythrocytes obtained from tissue culture reveal extensive changes of the membrane as well as lesions, depending on the infection stage (14). The lesions to the membrane can be attributed either to the merozoite penetration in the newly infected erythrocyte, or to the presence of mature trophozoites. The entry of the parasite into the erythrocyte is ordered and can be divided into 5 steps: (a) Merozoite recognition of the erythrocyte membrane. (b) Attachment of the parasite to a susceptible erythrocyte. (c) Junction formation. (d) Penetration of the parasite into the erythrocyte. (e) Sealing of the membrane after entry. These steps lead to a merozoite enclosed in a parasitophorous vacuolar membrane which differs from the erythrocyte membrane. The vacuolar membrane doesn't have spectrin and is initially almost devoid of intramembranous particles (15).

Export of malarial proteins from the parasitophorous vacuole membrane to the erythrocyte membrane involves diverse subcellular structures, or organelles (16). From electron microscopic evidence it seems that many of the membrane structures originate either from the parasite or the parasitophorous membranes via budding (16). According to Howard *et al.* (16) newly synthesized proteins from the intracellular *P. falciparum* parasite are translocated across the rough endoplasmic reticulum (RER) yielding asymmetrically-integrated proteins. The vesicle formed from the RER fuses with the parasitophorous plasma membrane (PPM) followed by subsequent blebbing from the PPM, fusion with the parasitophorous vacuolar membrane (PVM) and again blebbing from the PVM to reach the

erythrocyte cytoplasm on its way to fuse with the erythrocyte membrane (17). Parasite induced structures include single membrane clefts (Maurer's clefts) and electron-dense spheres. Maurer's clefts are diverse in shape and consist of erythrocyte membrane-bounded vesicles with a lumen of very low electron density (16). Spheres are electron-dense circular bodies which contain parasite proteins inside and on the surface of the organelle. These proteins are not expressed on the surface of the erythrocyte but play a structural and/or functional role in the protrusions found on the host erythrocyte membrane in certain *P. falciparum* species (16). PfHRP1 (*P. falciparum* histidine rich protein) are found inside the cytoplasm of the host erythrocyte which led to the hypothesis that it may be involved in the ferrying of other proteins from and to the erythrocyte surface. According to Sherman and Vinograd there is no biochemical evidence and little precedent for such trafficking, but it is not to say that it doesn't exist (18). If these structures are seen as part of the parasite organelles, then this is an extraordinary process in which an organism modifies its external environment, by releasing organelles into the host cytoplasm surrounding it (19).

P. falciparum and *P. malariae*-infected erythrocytes exhibit electron-dense excrescences or so called knobs on the surface of the erythrocyte membrane (20,21). Although there is much controversy surrounding this point, it is commonly believed that the knobs are proteins of parasite origin (22). The knob density increases during intracellular maturation of the parasite (21). It has also been observed that the distribution of knobs on the surface of the infected cell is not a random event. This implies that the knobs are produced in certain areas which may represent specific domains on the erythrocyte. The knobs are electron-dense,

conically-shaped, measuring 90-100nm in diameter and 30-40nm in height (21). It was also observed that not all the *P. falciparum* strains exhibit knobs on the erythrocyte surface (22). Loss of ability to produce knobs after lengthy culturing was noticed by Langreth *et al.* (22). By comparing the knob (K^+) and knobless (K-) strains of *P. falciparum*, It was observed that the knobless parasites lacked a unique histidine-rich protein which occurs in the electron dense cone which is bound to the skeleton of the erythrocyte (23). Considering the fact that the knobs appear only in the late stages of trophozoite and schizont stages and that only ring forms can be seen in bloodsmears from infected patients, these knobs are thought to mediate the binding of the cells to the capillary endothelium (21). The role of the knobs in sequestration of infected erythrocytes is not understood. It may play a role in endothelial adherence, although not all strains with knobs adhere, while some knobless strains do when evaluated *in vitro* (21).

Nearly a dozen proteins have been localized in regions near the knob, but only PfEMP 1, a protein with a molecular mass of >240 kDa has been claimed to be exposed on the surface of the infected erythrocyte. According to Sherman and Winograd alterations in Band 3, the principle protein of the erythrocyte, contributes to changes in the antigenicity of knobbly cells (18), as not a single monoclonal or monospecific polyclonal antiserum has been developed against the PfEMP1 protein and difficulty is experienced in cloning the gene for PfEMP1. Moreover Band 3 is clustered in the regions of the knobs and knobbly cells show an increase in binding to naturally occurring anti-Band 3 auto-antibodies.

Sherman and Winograd thus claim that the changes to the erythrocyte membrane proteins are parasite-induced changes to host proteins (18). This is contradictory to Newbolt and Marsh who concluded that modified host and neo-antigens (contributed by the parasite) co-exist on the erythrocyte surface (19). However, alteration to the erythrocyte membrane after invasion by *P. falciparum* parasites leads to changes in morphological and physiological properties (20).

The extent and the nature of the parasite-induced changes in the host erythrocyte membrane and cytoplasm suggests that a new entity is created with the "fusion" of the two interacting cells (malaria parasite and host erythrocyte). This new entity has special properties of both cells lacking in the original component parts. When one considers the intimate relationships in nature among symbiotic viruses, unicellular prokaryotes and eukaryotes and multicellular organisms this concept is not unusual.

1.6 Drug resistance

Using all the metabolic skills it has, the malaria parasite constantly adapts to its changing surroundings induced by the hosts immune system and the taking of antimalarial drugs as cure against the infection. While the seemingly limitless genetic ability of the parasite can be attributed for the multitude of drug-resistant mutants that have appeared, misuse of the drugs by man is much to blame (23).

In the 1960's chloroquine resistance in *P. falciparum* was reported for the first time (9). Twenty years later more than forty countries including South Africa face the same problem. In 1986 widespread resistance was

reported against sulphonamides and mefloquine, which is a second line drug treatment when chloroquine resistance is encountered (9). This led to the utilization of more toxic drugs (Fansidar) giving a 1 / 20 000 mortality rate (9).

Resistance is " the ability of the parasite to survive and/or multiply despite the administration and absorption of a drug given in equal or higher doses than usually recommended but within the limits of tolerance of the subject" (23). Evaluation of drug resistance can either be tested *in vivo* or *in vitro*. In the *in vivo* method the levels of blood stage parasites are monitored after the administration of a normal curative dose of the antimalarial drug (23). In this thesis a survey of drug resistance was made using the *in vitro* assay.

1.7 Immunology and vaccine development

Attempts to eradicate malaria using parasiticidal drugs and insecticides have only been partially successful and are now further hampered by the growing resistance against anti-malarial drugs (25). Inherent immunity against infection with *Plasmodium* does not exist, with one exception. Some rural Africans, lacking the Duffy antigen on the erythrocytes are highly resistant to *P vivax*. Certain enzyme deficiencies, eg. glucose-6-phosphate dehydrogenase and other genetic diseases like sickle cell anemia and thalassaemia, provide protection against infection with malaria (26). Experimental studies show that induced immunity against blood stages are short-lived, requires constant boosting for the maintenance of immunity and is much less effective against cloned than heterogeneous strains of parasites (25).

Protective immunity is usually parasite species specific, although cross reactions has been known to occur between rodent malaria species (27). The effectiveness of the response to an early infection with the malaria parasite may be limited to the intrastrain antigenic variants which are causing the infection (27).

Sporozoites entering the cardiovascular system of the host disappear rapidly, but their exact fate remains unknown in normal and immune hosts. However, the kuppfer cells appear to be the distal point of entry for

development in parenchyma cells (27). Circum-sporozoite protein (CS) the major protein on the sporozoite surface coat, can induce an immune response as is seen in endemically infected human populations. Vaccination with various attenuated sporozoites has been effective in avian malarias but not in human malaria. The reason for this is unknown (27). Research into the immune response to gametocytes has been largely neglected in comparison to the other parasite stages. Gametocytes may persist in the host for some time after clearance of the asexual stages, but their infectivity for mosquitoes decline (27).

The antigenicity of erythrocytes infected with the asexual blood stages of the parasite are demonstrated by the opsonization, agglutination and possible sensitization for destruction by antibody-dependent lymphocytes (27). At present it is not known how intra-erythrocytic parasites are killed *in vivo*, although phagocytosis of parasite infected erythrocytes by splenic and liver macrophages are evident in the immune host (28). $CD8^+$ T cells are activated by $CD4^+$ T cells in response to the malarial antigens and these $CD8^+$ T cells are either directly cytotoxic, and/or

secrete toxic cytokines. In doing so, CD4+ T cells control the parasite numbers, ensuring the survival of both the host and the parasite (27). However, the appearance of crisis forms of parasites in the blood could not be accounted for, until Pouvelle *et al.* observed that IgG molecules have access to the intra erythrocytic parasite via a parasitophorous duct (28). Until this discovery trophozoite and schizont surface antigens were never considered important as potential antigens for vaccine development. Using these parasitophorous ducts, toxins may be coupled to anti-parasitic antibodies, to home in at the parasite inside the erythrocyte (28).

The malaria parasite undergoes adaptive antigenic variation (29). Antigenic variation is divided into four categories: (a) The parasite is able to successively produce large quantities of different immunodominant surface antigens, possessing no shared determinants, to evade the immune response. Examples are the Circumsporozoite protein (CSP), merozoite surface antigen 1 (MSA1) and MSA2. An unusual finding in *Plasmodium* is that most antigens are immunodominant tandem repeats of oligopeptide sequence (30,31). (b) The parasite is able to switch the presenting variant specific antigen before the entire parasite population are wiped out by antibodies. This switch can be antibody- induced or spontaneous, resulting in a constant low rate of switching to create a small heterotype of parasites that will survive the specific host response better than homotypic parasites (30). This implies that the introduction of new forms of immune pressure (anti-malarial vaccines) may induce new variants, as has already been observed under laboratory conditions (31). (c) Expression of surface antigen genes in a sequential order to avoid gross population heterogeneity and rapid induction of antibody formation

against all the surface antigens. Parasites will continue to express the variants in the same sequence, even if they are transferred to another host. However transfer to an antibody-containing, variant specific host induces switch to the next variant in the sequence. This implies that the expression of antigens of a particular variant is predetermined, but is modulated by the immune milieu of the host. Chronically infected animals acquire an overall parasite immunity and their sera appear to recognize the transcended range of variant antigen expression. By sequentially expressing different gene products, a single infection produces a series of serologically distinct phenotypes of parasites. This strategy is used by the malaria parasite apparently to outpace the immune system of the host (31). (d) The parasite needs to feed (inside the erythrocyte) mate (in the vector) and absorb to specific targets without exposure of it's non-variant antigens (32). Furthermore by presenting immunodominant antigens on the surface that can be alternated, the parasite creates decoys to divert the immune response away from the constant inner-surface elements (31).

Various surface antigens like circumsporozoite protein (CS) from sporozoites, *Plasmodium* merozoite major surface antigen (MSA) and ring-infected erythrocyte antigen (RESA) are presently used to develop vaccines against the malaria parasite (33). None have yet proved to be effective for use (34). It is important to guard against the impression that an effective vaccine will eradicate malaria. Many good vaccines presently available are not widely used, especially where they are needed most, due to their cost and some Third world traditions (33).

1.8.1 Carbohydrates

Glucose is the main source of carbohydrate used by the malaria parasites (35). Constant supply of this metabolite is important for parasite survival because none is stored. Infected erythrocytes utilize 10 to 100 times the amount of glucose compared to non-infected erythrocytes (35). The plasma membrane of the asexual stages of *Plasmodium* seems to possess an active transport system for D-glucose consisting of a carrier mediated D-glucose co-transport mechanism which utilizes ATP (35). Other carbohydrate substrates utilized by *Plasmodium* include mannose and fructose by rodent parasite species.

The metabolites from glucose utilization vary between species and in many cases 10-20 % of the end products are not accounted for (35). The amount of glucose conversion to lactic acid seems to depend on the species and the culture conditions. Results indicate that no complete citric acid cycle exists in *Plasmodium* for only malic acid dehydrogenase could be identified in rodent and avian malaria parasites. No pentose phosphate cycle enzymes were identified either and it seems that ribose-1-phosphate and free base are obtained from ATP catabolites in the host erythrocyte (35).

1.8.2 Amino acids.

According to Sherman *et al*. intraerythrocytic parasites can either utilize the hemoglobin or free amino acids from the host amino acid pool (35). Infection of the erythrocyte by the malaria parasite destroys most of the energy coupled transport systems. It seems that free amino acids enter

the erythrocyte via a concentration gradient with the parasite acting as a metabolic sink. Carrier-mediated transport is therefore only required for the highly charged molecules (35). The metabolized hemoglobin is stored inside the parasite as a brown pigment (haemozoin) conglomerate consisting of ferriprotoporphyrin IX coupled to a parasitic polypeptide (35).

1.8.3 Nucleic acids

In the early 1940's it was shown that malaria parasites contained DNA by using Feulgen stain (36). According to Kemp *et al.* the parasite has 14 chromosomes consisting of about 60 genes. The RNA content is about 5 times greater than the DNA content and is mostly localized on abundant cytoplasmic ribosomes in the intraerythrocytic parasite (37) .

Purines, in contrast to pyrimidines, cannot be synthesized *de novo* in *Plasmodium* and are therefore salvaged for nucleic acid synthesis (37). Adenosine, inosine and hypoxanthine are taken up in relation to the stage of the parasite, whereas ATP, AMP and IMP are taken up in limited amounts (34). Apparantly thus, exogenous sources of purines are needed for nucleic acid synthesis. The erythrocyte purine pool, of which 80% exists in the form of ATP, is probably the main source. Indeed, according to Yamada and Sherman , up to 25 % of the purine requirements can be obtained from the erythrocyte pool with a concomitant sharp decline of the ATP level in infected erythrocytes (37).

In both the erythrocyte and the parasite, purine interconversion enzymes were identified and Yamada and Sherman proposed that adenylate nucleotide catabolism is towards the formation of hypoxanthine and that

the hypoxanthine present in the cytosol of the erythrocyte is taken up and consumed by the parasite (37).

The salvage pathway for purines are illustrated in Fig 1.5.

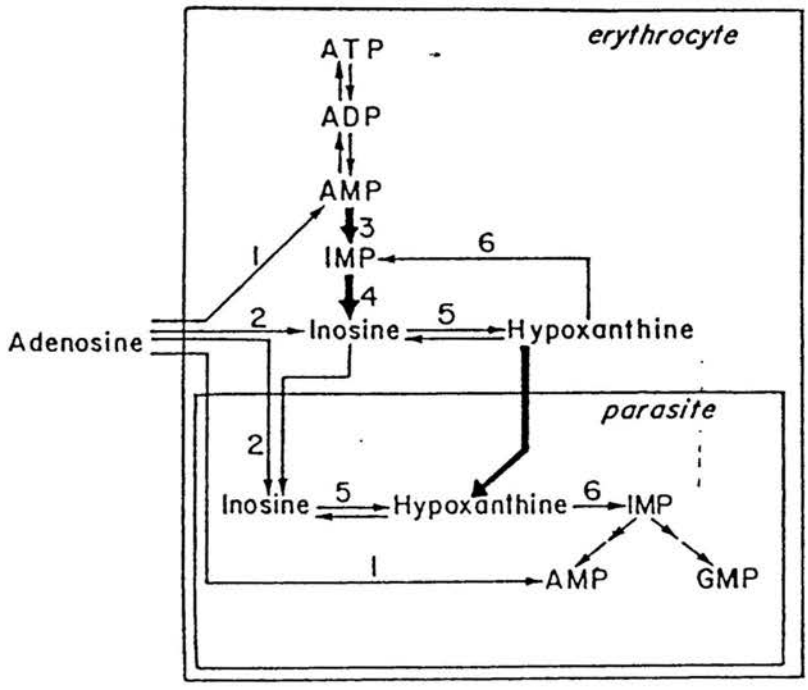

Figure 1.5 Purine salvage pathway in the erythrocyte and *Plasmodium* . 1. adenine kinase 2. adenosine deaminase 3. 5'AMP deaminase 4. 5'IMP deaminase 5. inosine nucleoside phosphorylase 6. hypoxanthine phosphorybosyl transferase (35).

1.9 Continuous culture of P. falciparum.

The first continuous culture of the blood stages of *P. falciparum* was achieved in 1976 by Trager and Jensen, who thereby revolutionized research on malaria (38). The relative slowness with which the techniques for the cultivation have been developed is mainly due to the inadequate knowledge of the biochemistry of the parasite, the host erythrocytes and their constituents (35). The parasites are dependent on the hosts erythrocyte which is demonstrated by their ability to invade and multiply only inside the erythrocyte. The integrity of the erythrocyte seen in relation to the supporting medium is of primary importance. The culture

system provided large amount for DNA and RNA isolation and metabolic experiments for research on the parasite for lethal malaria in man (7). The continuous *in vitro* culture of the sporogonic stages has not yet been achieved, although it seems that all the stages of sporogony are capable of being supported *in vitro* (39, 40).

The failure to eradicate malaria during the 1950 world campaign and the upsurge in malaria over the last few decades led to a revised strategy for malaria control. The inadequacy of the prevalent methods to contain the disease, highlights the need for a malaria vaccine (8). Vaccines, which are being tested at present, show some problems but the knowledge gained on the diversity and escape routes of the malaria parasite, should eventually lead to a method to control the disease.

1.10 Objectives

The first and foremost objective of the study was to establish long-term *in vitro* cultures of *Plasmodium falciparum* as a source of antigenic material for immunochemical studies. Wild isolates were obtained from malaria-infected patients during a national survey aimed at recording the distribution of resistance to chloroquine and its potential substitute, mefloquine (Chapter 2). Due to the initial difficulties experienced in establishing wild isolates in culture, investigations were undertaken to identify factors which may limit parasite growth. The influence of the gas composition of the medium and changes in the concentrations of metabolites and byproducts on parasite growth, received particular attention (Chapter 3). In order to determine antibodies specifically bound to membrane surface antigens or receptors, appropriate quantitative methods are needed. An existing micro-immuno-assay method was modified

and tested for its ability to detect the inverse relationship between thrombocyte count and thrombocyte-associated immunoglobulins of malaria-infected patients (Chapter 4). Lastly, different conditions for the fixation of malaria-infected erythrocytes were compared in order to select the most appropriate procedure. The aim was to investigate the ultrastructural detail of the parasite and provide a method to localize specific antigens *in situ* (Chapter 5).

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CHAPTER 2

DRUG SUSCEPTIBILITY AND SHORT-TERM CULTURE OF Plasmodium falciparum.

2.1 INTRODUCTION

Drug therapy provides a second defense front against malaria parasites after vector control (1). Eradication of the *Anopheles* mosquito is a sure way of controlling malaria infection. Campaigns undertaken in 1955 by the WHO using DDT had early success in Europe, North America and urban areas of tropical countries, but Africa, which is the central "hard core" of malaria, remained neglected (1). The eventual failure of the eradication campaigns were due to over-optimism of the potential of insecticides to eradicate the vector and inadequate understanding of the epidemiology (2). Safe and effective prevention and treatment of the victims of malaria are therefore contemporary priorities requiring constant research to assess and improve chemotherapy.

The continued long term use of a particular prophylactic such as chloroquine may eventually lead to complete resistance against this drug, by selection for resistant parasites. Chloroquine is the preferred drug for chemoprophylaxis even in areas where resistance towards the drug is becoming significant (3). The drug gave the best clinical results with the least complications. Quinine is also effective and safe but has a relatively short half-life in the body (10 h). In areas where resistance against chloroquine is pronounced proguanil is prescribed simultaneously

(4). The combination drug Fansidar (pyrimethamine and sulphadoxine) is also used in chloroquine resistant areas. This drug is taken once with the first signs of an infection because of its high toxicity. Mefloquine, a new drug which proved useful for prophylaxis is rather restricted to treatment of highly resistant malaria cases only (4). As drug resistance snowballs however, an effective vaccine against malaria is a growing need in the near future.

Continuous *in vitro* cultivation of *Plasmodium falciparum* opened up a new era in the development and evaluation of antimalarial chemotherapy (5). Large varieties of isolates can now be tested in a much shorter time. The amount of antimalarial drug used can be quantified and resistance to a variety of drugs can easily be determined. Using the principles of *in vitro* continuous culture for *P. falciparum*, a microtest was developed to test drug sensitivity (6). Standardized kits are now available for a large variety of drugs, e.g. chloroquine, amodiaquine, mefloquine and quinine. These are now widely used for the assessment of drug susceptibility of the parasites, and have replaced the use of the old macrotest. The major advantages of the microtest is that it is performed in microtiter plates which have been predosed, thus requiring less drug and allowing a large range of concentrations to be tested at the same time. Only 100 ul of blood is required which can be easily drawn from the finger (6).

As with most biological systems the *in vitro* technique also has some limitations or disadvantages. The most pronounced problem is the artificial time-course to drug exposure. The *in vitro* system does not allow for the fact that the chemotherapeutic drug is metabolised and/or changed and excreted. Another disadvantage of *in vitro* testing is the fact that some

of the effective antimalarial drugs are not selective and are toxic to the host. The *in vitro* system is also subject to artefacts such as drug adherence to the glass or polystyrene supporting medium (6). Although the test has been successfully used to test chloroquine and mefloquine resistance, it is not capable of determining susceptibility to combinations of sulpha-drugs and pyrimethamine, due to the high concentration of 4-aminobenzoic acid and folic acid in RPMI 1640 medium. Other tests have also been developed to test drug susceptibility. A 48 hour test, which is similar to the microtest, uses four times more blood and ten times more culture medium than the microtest and the medium must be supplemented with 10 % human serum. Although the test can evaluate chloroquine susceptibility it is mostly used for dihydrofolate reductase inhibitors like pyrimethamine using a special medium (6).

The first requirement for testing human antimalarial chemotherapeutic drugs is a growing malaria culture. Long term continuous culture will be discussed in Chapter 3, but the *in vitro* microtest requiring only short-term culture, will now be described.

2.1.1 Antimalarial drug classification and mechanism of action.

Antimalarial drugs can be categorized according to the stage of the parasite they affect. Specific antimalarial drugs can affect more than one stage.

i) Schizontocides.

(a) Tissue schizontocides used for causal prophylaxis.

These drugs eliminate the parasite in the pre-erythrocytic stage.

Proguanil, pyrimethamine and antifol combinations and certain antibiotics are included in this group. From these compounds only proguanil and pyrimethamine are prescribed primarily for prophylactic use (8).

As typical examples of "antifols", this group of compounds inhibits the activity of dihydrofolate reductase. They are not plasmodicidal but plasmodistatic and are only used as prophylactic antimalarial drugs. Blocking of the asexual cycle leads to the accumulation of late stage parasites in infected cells which eventually disintegrate. Resistance to proguanil and pyrimethamine have been reported between one and six years respectively since first used in therapy in 1952 (9). Pyrimethamine is absorbed slowly and has a prolonged biological activity. High doses lead to folic acid deficiency which inhibits hematopoiesis (8).

(b) Tissue schizontocides used to prevent relapses.

These drugs eliminate the hypnozoites or dormant liver forms of *P. vivax* and *P.ovale*. In this group only 8-aminoquinoline derivatives such as primaquine are presently in use. Little is known on the mode of action of 8-aminoquinolines, especially why they are more effective against tissue stages than blood forms of *Plasmodia* (10).

(c) Blood schizontocides used for clinical or suppressive cure.

Antimalarial drugs such as quinine, chloroquine and mefloquine belong to the blood schizontocide group. Intra-erythrocytic stages of *Plasmodium* are susceptible to these compounds. Schizontocides are also used in suppressive therapy to merely reduce the parasite load. A lowering of the parasitemia may result in a clinical cure as was demonstrated with

P.vivax infections. These drugs are also used to eliminate *P. falciparum* and *P. malaria* infections.

Although chloroquine causes a number of effects that singly or in combination may indicate to its primary mechanism, the detailed process is not yet understood. From early work it was hypothesized that chloroquine interacts with the DNA and inhibits DNA and RNA polymerase (10). Another theory holds that blood schizonticidal antimalarials compete with the parasite protein(s) which form complexes with the lytic ferriprotoporphyrin IX (FPIX), an oxidation product of hemoglobin digestion, to prevent its sequestration as inert haemozoin crystals. These FP IX drug complexes are membranolytic and cause parasite death by abolishing internal membrane permeability (11). The increasing amount of resistance against chloroquine as antimalarial has led to the use of tetracycline and quinine as the choice antimalarial drug combination.

Antibiotics have a weak antimalarial effect but act synergistically with quinine (12). Clindamycin, lincomycin, rifampicin and co-trimoxazole also show *in vitro* anti-schizontocidal effects against malaria and can be combined with quinine (12). All these antibiotics acts as inhibitors of prokaryotic protein synthesis. From experimental evidence it is suggested that the mechanism of protein synthesis of malaria parasites is of eukaryotic nature. Thus the inhibitory effect of antibiotics on malaria parasites probably result from the inhibition of mitochondrial protein synthesis which may also explain the relative slow clinical effect of antibiotics like tetracycline (8).

ii) **Sporotocides.** These drugs ablate transmission of the malaria parasite by inhibiting the formation of the oocysts and sporozoites in the infected vector. Primaquine is the major drug with this type of mechanism. The "antifols" also exhibit a sporontocidal action in the mosquito stages of all species.

iii) **Gametocytocides.** These antimalarial drugs will eliminate or sterilize the gametocytes at certain stages of development. Primaquine has this type of action against *P. falciparum*. Chloroquine and quinine shows gametocytocidal action against *P. vivax* and *P. malariae* but not against *P. falciparum*.

In Fig. 2.1 some of the structures of the commonly used antimalarial drugs can be seen.

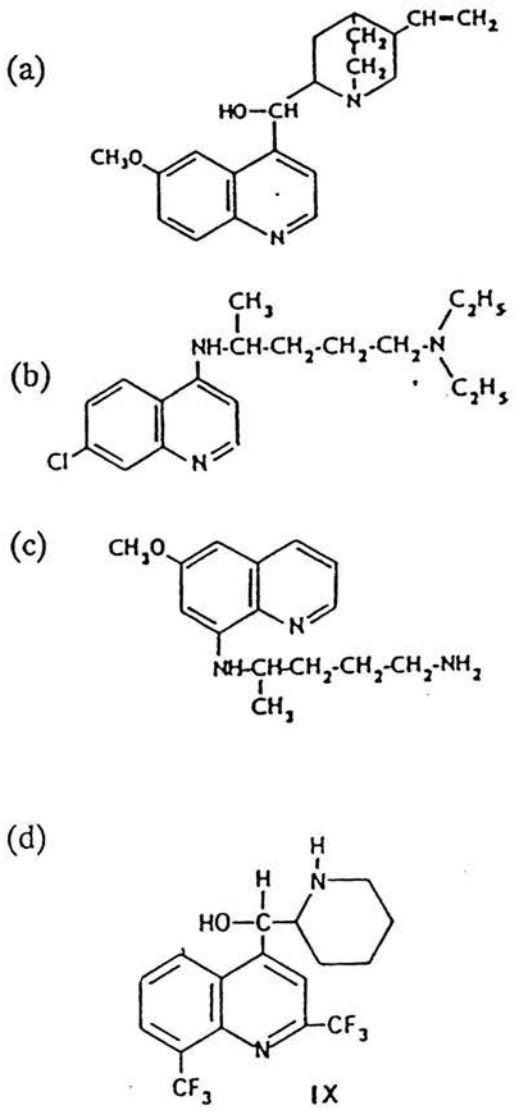

Figure 2.1 Structures of antimalarial compounds. (a) quinine (b) chloroquine (c) primaquine (d) mefloquine (WR 142490)

2.1.2 Chloroquine resistance.

Chloroquine accumulation in the acid vesicular compartment of the drug resistant parasites was found to be significantly less than in the susceptible parasites. Krogstad *et al*. (1987) showed that calcium channel blockers like Verapamil could reverse the resistant strain to be

chloroquine sensitive again (13). This led to the hypothesis that the mechanism of chloroquine resistance is based on a greatly increased drug efflux rate, a similar system to multi-drug resistance (mdr) in cancer cells. MDR protein, homologous to a family of ATP-driven transport proteins which act as transporters when overexpressed, expels drugs from the cell fast enough to protect against their toxic effects. Two genes (pfmdr1 and pfmdr2) were identified which coded for mdr proteins and it was found that some but not all resistant lines of parasites had amplified pfmdr1 genes and increased mdr expression. Foote *et al.*(14) found that sensitive strains can contain the mdr gene of "resistant" type sequence and concluded that other genes than pfmdr1 may be involved. A link between the mdr proteins coded for by the pfmdr1 gene and resistant parasites had been established but a more detailed understanding of chloroquine resistance requires that the other gene(s) are identified.

2.1.3 New drugs.

Research into new drugs such as the traditional Chinese remedy, qinghaosu, and mefloquine are important priorities, as new drugs are not common and malaria parasites have established resistance to most of the antimalarial drugs available (15). Mefloquine which is used in chloroquine resistant cases already proved ineffective to certain malarial species even before it was introduced. Arteeter a derivative from qinghaoso seems most effective against the blood stages of the malarial parasite, but is short lived and needs to be taken several times a day (15).

2.1.4 Objectives.

The objective of the study was to test drug susceptibility, (chloroquine and mefloquine) in *Plasmodium falciparum* infected patients in the North-Eastern Transvaal area. This project was part of a national survey in 1988 to record the chloroquine resistance status and to evaluate the use of mefloquine in chloroquine resistant parasites.

2.2 MATERIALS AND METHODS

2.2.1 Microtest.

The microtest kit for the assessment of chloroquine and mefloquine sensitivity in *Plasmodium falciparum* growth *in vitro* (supplied by the World Health Organization) was obtained from Professor Fripp, University of Medunsa, PO Medunsa, 2024.

2.2.2 Samples.

Venous blood samples were drawn in citrate/dextrose tubes from patients suffering from malaria. The blood was assayed as soon as possible, or maintained close to body temperature for a maximum of three hours, depending on the conditions of the field work. Persons that received chloroquine within the last 14 days were excluded from the test. The pre-selected patients were then subjected to a urine test for 4-aminoquinolines using Dill Glazko's solution, which indicates the use of prophylactic drugs and render the sample not useable.

2.2.3 Blood smears.

Thin and thick blood smears were made. A droplet of sample blood was placed on the microscope slide ± 1.5 cm from the frosted or marked section. The drop was smeared by using another microscope slide held at 45 ° angle. The smear slide was applied in front of the droplet blood allowing it to spread to the edges. The blood was then spread with a uniform forward stroke to cover 3/4 of the slide. After allowing time to dry, the cells were fixed with absolute methanol (E. Merck, SA Pty (Ltd), Midrand)

The thick smears were made by allowing a droplet of sample blood to dry on the microscope slide after spreading it with a matchstick.

2.2.4 Giemsa staining.

Giemsa stain was prepared fresh for staining of smears. Giemsa solution (500 µl) was diluted in 10 ml sodium phosphate buffer (0.01 M) pH 7,2. After staining the smear for 30 to 40 minutes the slides were rinsed in distilled water. Thick smears were not fixed but stained as described for thin smears. Stained thick and thin smears were then examined for *Plasmodium falciparum* parasites using a Nikon type 104 light microscope.

2.2.5 Dill Glazko's reagent (16).

Eosin (50 mg) was transferred to a small glass-stoppered separating funnel. Chloroform (100 ml) and 1N HC1 (1 ml) were added. The mixture was shaken for 3 minutes and the yellow chloroform layer was transferred to a brown glass-stoppered bottle.

Assay: To a small test tube containing 2 ml of urine, ten drops of the Dill-Glazko's reagent were added and mixed vigorously. A change from

yellow to violet red of the chloroform layer was taken as an indication of the presence of 4-aminoquinolines.

2.2.6 Preparation of growth medium.

The contents of 1 packet of RPMI 1640 powder (104 mg) were added to 10 ml sterile water and put in a Falcon tube (15 ml). To this was added 1 ml 7,2 % HEPES and 1 ml 2,4 % $NaHCO_3$ solution. The growth medium was sterilized by filtration through a 0,22 µm Millipore filter fitted onto a 20 ml syringe from which 0,9 ml aliquots were distributed in sterile 6 ml Falcon tubes for further use the same day (pH 7.4).

2.2.7 Performance of the microtest.

Blood taken from the *P. falciparum* infected patient in the field was processed as follows: Infected blood (100 µl) was added to one of the 6 ml Falcon tubes containing the growth medium. In each well of the appropriate columns of the chloroquine or mefloquine presealed microtiter plate, 50 µl of the blood/medium mixture was added. The plate was closed and gently shaken to ensure that the drug deposits in the wells were completely dissolved. The cultures were then placed in a desiccator with a paraffin candle and a flask containing copper sulphate solution. After the candle was lit, the desiccator lid was partially displaced, leaving only a small opening. The lid was closed just before the flame went out. The desiccator was subsequently placed in an oven (Labotec 382) at 37 °C and incubated for 24 hours. After incubation, as much as possible of the supernatant from each well was aspirated, thick blood smears were prepared from the residual contents of each well, stained for 30 minutes with Giemsa and subsequently viewed under a microscope. If the controls

revealed 20 or more schizonts of *P. falciparum* the culture was considered to be successful and the titration values interpreted as follows:

(a) Total inhibition (100%) of growth at 4.0 pmol chloroquine or mefloquine per well: Susceptibility to standard chloroquine, mefloquine treatment.

(b) Growth at 5.7 pmol or more chloroquine, mefloquine per well: Resistance of *P. falciparum* to chloroquine and mefloquine.

(c) Growth at 4.0 pmol chloroquine, mefloquine per well, but inhibition at 5.7 pmol: partial resistance, which may be reduced by treatment of the patient with higher dosages of the drug.

2.3 RESULTS

Of thirty two patients tested, 14 assays had to be abandoned due to too low parasite counts. Results from the eighteen patients are shown in Figure 2.2.

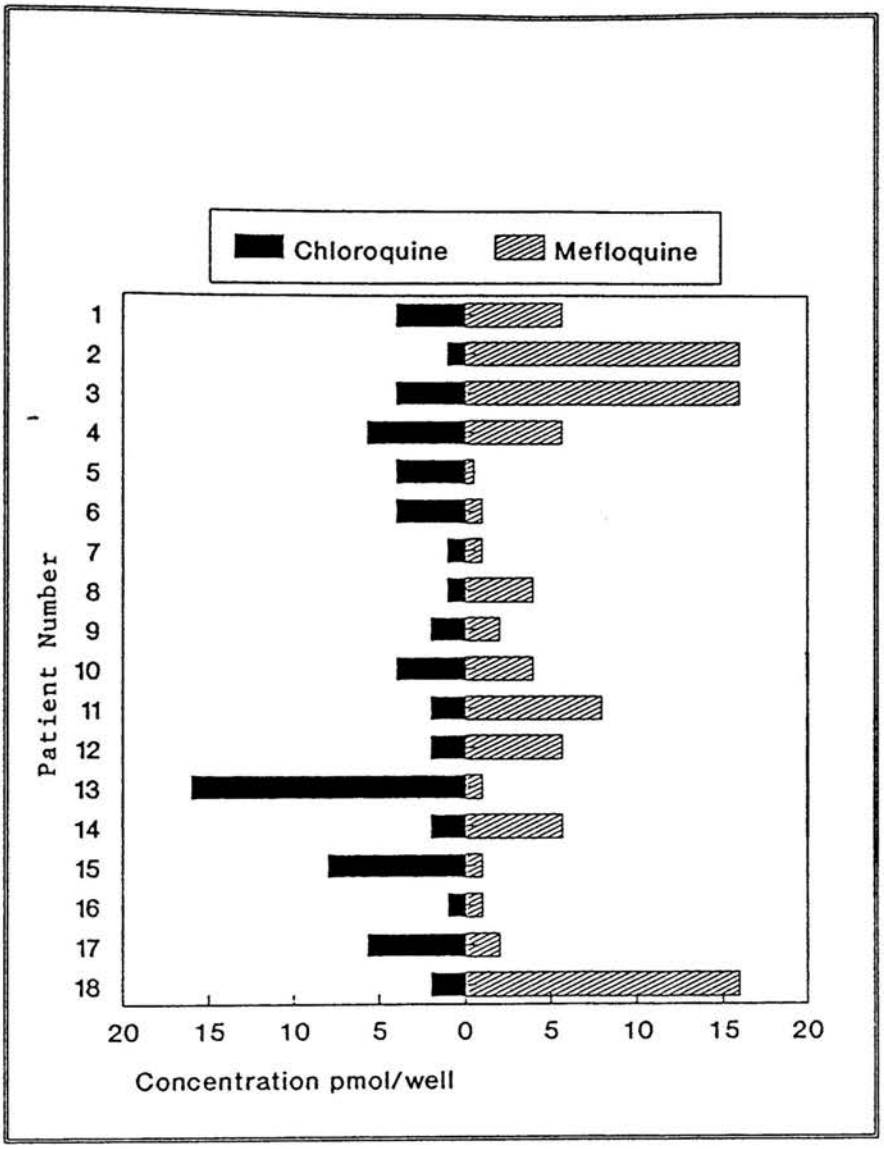

Figure 2.2 Growth inhibition from 18 patients tested for chloroquine and mefloquine resistance in North-Eastern Transvaal during 1988.

Patient 13 registered resistance to chloroquine at higher than 16 pmol/well, whereas number 15 was resistant up to 8 pmol/well. With the mefloquine test, patients 2, 3 and 18 were highly resistant and 11 only up to 8 pmol/well. It was also noticed that none of the isolates exhibited resistance to both chloroquine and mefloquine. Population pie charts of drug resistance towards chloroquine (Fig. 2.3 A) and mefloquine (Fig. 2.3 B) are shown.

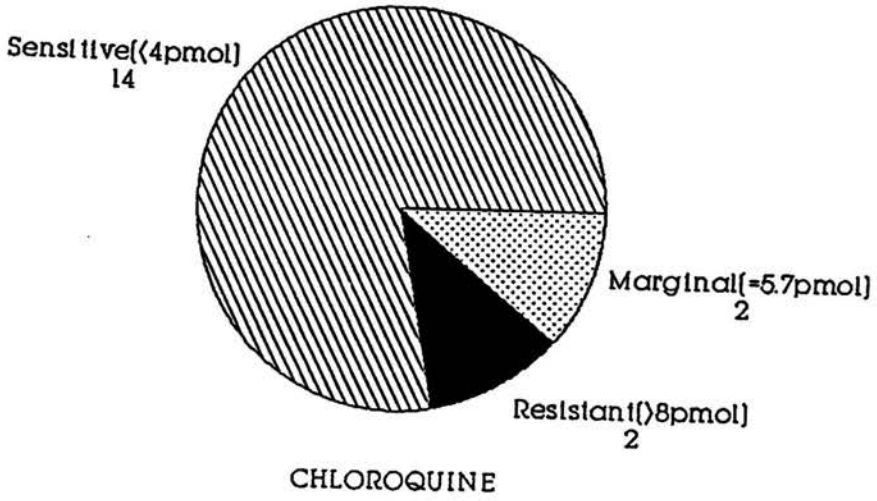

Figure 2.3(a) Population pie chart of chloroquine resistance in the North-Eastern Transvaal during 1988 (n=18).

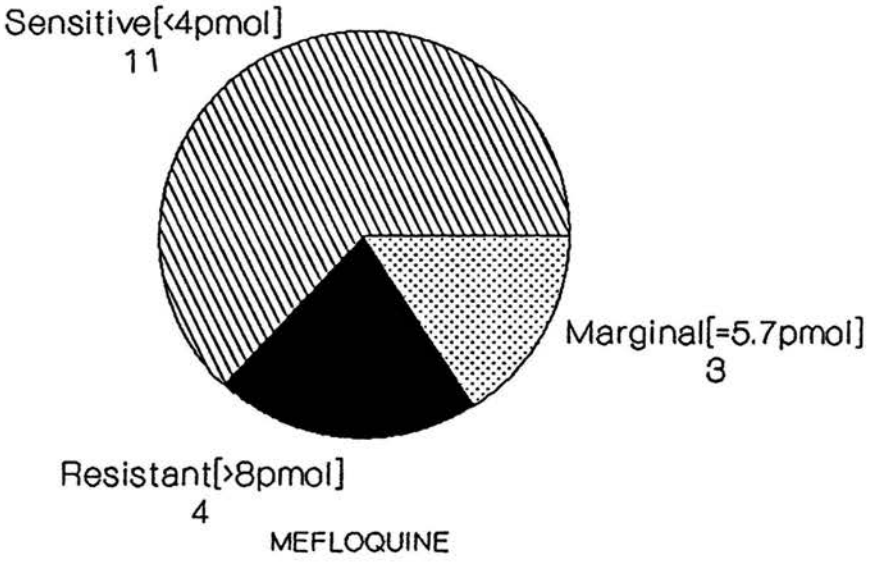

Figure 2.3(b) Population pie chart of mefloquine resistance in the North-Eastern Transvaal during 1988 (n=18).

Within the constraints of the small population tested, the results indicate a tendency towards higher resistance against mefloquine (22 %) than against chloroquine (11 %) among the patients tested.

2.4 DISCUSSION

The malarial parasite has the ability to adapt towards the constraints put on its propagation by chemotherapy and already shows resistance against a variety of antimalarial drugs. Chloroquine resistance has progressively increased over the last decade, especially in the Kwazulu area according to NIDTE surveys (17). In 1987, 25 % cases were found to be chloroquine resistant compared to 100% resistance measured in the Mamphene area in 1988 (17). Clinically the effectivity of chloroquine treatment (1500 mg per patient over three days) dropped from 100 % in 1983 to 85.2 % in 1987 (17). Chloroquine resistance measured for the whole Kwazulu area in 1988 was recorded at 15 % . In the North Eastern-Tranvaal, 15 to 30 clinical chloroquine resistant cases were recorded in 1988 by Hansford and Pammenter (18). This is a approximately 1 % of the total cases recorded compared to our 11 % determined in this investigation using the *in vitro* method. However, no direct extrapolation can be made between the clinical and *in vitro* determinations. Patients with malaria are not tested for the presence of prophylactic drugs before treatment is started, which may aid to cure the patient resulting in clinical resistance not being recorded. Patients also differ in their ability to metabolise drugs while in the *in vitro* system, no metabolism or excretion of the drugs takes place. Since 1988 no *in vitro* study has been undertaken to determine the chloroquine resistance in the North-Eastern Transvaal area. According to Hansford it would appear that the clinical recorded cases of resistance have declined over the last two years (Hansford, RIDTE, Box 33, Tzaneen, 0850, Personal communication, August 1991).

The use of mefloquine as a curative drug in areas where chloroquine resistance is a problem shows promise, as is shown in Fig. 2.2 where

no chloroquine resistant patients were found to be resistant against mefloquine. It was found that 4 of the 18 samples tested, showed parasite growth at mefloquine concentrations above 5.6 pmol, three of which at higher than 15 pmol per well. According to Graig Cranfield, Chairman of the WHO's committee on antimalarial drugs, some strains of parasites exhibited insensitivity against this drug even before it was introduced, which may explain our results (15). All of the samples that were chloroquine resistant in our tests were sensitive against mefloquine. This shows that chloroquine resistant malaria cases can be treated using mefloquine if the drug has the same effect *in vivo* as *in vitro*.

The possibility that antimalarials other than chloroquine could have been present in the samples using the *in vitro* assay led to the suggestion that the plasma from infected patients should be replaced with non-immune serum in the future (17). Freese *et al.* (17) found that the resistance micro-test gave better results with South African strains if the parasites were not grown in a burning candle oxygen depleted atmosphere, but in a specific gas mixture containing 3 % O_2, 4 % CO_2 and 93 % N_2. Finally Kouzretsov *et al.* (19) postulated that the parasite density was also a factor . He found that isolates with more than 100 000 parasites per ul blood needed more chloroquine for inhibition of the schizonts than the same isolates at a lower parasitemia.

It should be remembered that there is considerable variation in the infectious behaviour of malarial parasites from the same species, and field isolates generally contain genetically distinct clones of the parasite (6). The development of more sophisticated methods for the culturing and testing of parasite clones for resistance to drugs and other properties

for example by characterizing the genomic DNA by molecular biology techniques, should lead to a better understanding with beneficial implications for the chemotherapy of malaria.

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CHAPTER 3

CONTINUOUS CULTURING AND BIOCHEMISTRY OF THE ERYTHROCYTIC STAGES OF Plasmodium falciparum.

3.1 INTRODUCTION

Prior to 1976 *in vitro* erythrocytic cultures of *P. falciparum* were limited to short-term incubations (≈ 24 hours) (1). Trager and Jensen revolutionized research on human malaria with the introduction of a method for its continuous *in vitro* cultivation (2). The original method is still in use today but with a number of modifications which include the employment of static, continuous flow vessels (3) and modified T25 culture flasks instead of Petri dishes (4) and special gas mixtures instead of a desiccator and paraffin candle (5). Studies with various medium supplements, different sera or serum-free medium and other gas mixtures are under continuous investigation.

3.1.1 Factors affecting the continuous cultivation of P. falciparum.

(a) Erythrocytes

P. falciparum can be maintained in erythrocytes of the ABO bloodgroup. Invasion of the erythrocyte by merozoites and survival of intracellular forms are to some extent determined by the hemoglobin type (hemoglobin F, hemoglobin S, thalassaemia) and the presence of functional erythrocyte glycophorin and glucose-6-phosphate dehydrogenase (6). Freshly collected or stored blood are both suitable for culturing but it appears that

below 12 mM, since it is toxic to malaria parasites and compromise the capacity of the buffer system (12,13).

(c) Gas phase

The successful continuous culture of *P. falciparum* is dependent on the composition of the gas phase as well as the surface area of medium for adequate gas exchange (14). The solubility of the gasses in the medium, the dissolved gas gradient, the depth of the medium, and the hematocrit are critical to this exchange (15). According to Trigg (17) the above factors are probably the cause for failure of short term cultures at high altitudes. Concentrations of between 2-5 % CO_2 seem to be beneficial for parasite growth, but higher concentrations inhibit parasite development. This is probably due to the increased acidity of medium which contains a CO_2 / bicarbonate buffer system (15). Oxygen concentrations of between 5-10 % do not have a detrimental effect on continuous growth of *P. falciparum* in culture. Higher concentrations are inhibitory although the effect may take several days to manifest itself (15). The role of oxygen in the malaria parasite metabolism is unknown, but Scheibel *et al.* (15), showed that the parasite is microaerophilic. Continuous growth under total anaerobic conditions has not been obtained (17). Miyagami and Waki showed that *in vitro* cultivation of *P. falciparum* is possible under aerobic atmosphere in a CO_2 incubator (16).

(d) Serum

Continuous cultivation of the erythrocytic stages of *P. falciparum* was first achieved with complete RPMI 1640 medium supplemented with 15 % human type AB^+ serum (2). Any human serum type is suitable for the parasite culture as long as it is compatible with the erythrocytes being

the ATP content is of cardinal importance for invasion and development of intracellular parasites. ATP apparently maintains the erythrocyte cytoskeleton in the phosphorylated state, as well as the steady-state intracellular concentrations of Na^+ K^+ and Ca^{2+} via their respective pumps (8). Increased intracellular Ca^{2+} and sodium concentrations, often found in malaria parasite-infected erythrocytes, are associated with depletion of ATP (8). High intracellular calcium values lead to increased leakage of K^+ and proteolysis of ankyrin. When the intracellular concentration of Ca^{2+} exceeds 200 µM, intracellular proteins are adsorbed to the erythrocyte membrane and membrane proteins are crosslinked by transglutaminase which results in the rigidification of the erythrocyte membrane (7). The lack of invasion of ATP-depleted erythrocytes by malaria parasites was shown to be unrelated to cell shape which in turn is determined by the cytoskeleton (8). Trager has shown that ATP has a direct influence on the extracellular merozoite, since it is required to induce the formation of rings in a human erythrocyte extract (9). Yamada and Sherman studied the purine sources and requirements of avian malaria parasites and concluded that there was a dramatic decrease in erythrocyte ATP concentration during infection. They further estimated that the purine pool of the erythrocyte, mainly ATP, could supply up to 25 % of the purine requirement of the parasite (10).

(b) Culture medium

The studies of Trager and Jensen (2) have shown that RPMI 1640 medium with HEPES buffer was superior to HAM's F-12 Medium 199 for cultivation of *P. falciparum* (11). It is vital that the medium be replaced regularly to replenish spent nutrients and to keep the lactic acid concentration

used (15). Jensen (4) found that human serum varies remarkably in its ability to support parasite growth. However, this problem can be overcome if pooled serum from donors is used (15). The use of human serum in continuous cultures have many disadvantages. It is costly and it is difficult to obtain large quantities of naive serum in regions where donors may have been exposed to malaria. In addition, the danger that blood may be contaminated with hepatitis or AIDS is very real. These problems can be circumvented if human serum is replaced by animal serum. Reports have indicated that rabbit (18), horse (19) and bovine sera (20) are able to replace human serum in continuous *P. faciparum* cultures. Recently, Willet and Canfield (21) also succeeded to sustain parasite growth in serum-free medium supplemented with adenosine, fatty-acid free bovine serum albumin and unsaturated fatty acids (as oleic or cis-vaccenic), for more than four weeks.

(e) Parasite isolates

Since 1976, many different isolates have been established in culture (2). Despite these successes, however, it is evident that a number of isolates do not adapt easily to culture conditions (15). Generally, it is easier to initiate and maintain cultures from an established culture line than from a field isolate (15). Physical factors like the time between collection of blood and cultivation of the parasite isolate, the type and conditions of transport and the cryopreservation technique, may all affect the ability to establish a continuous culture (15).

3.1.2 Nutritional requirements of Plasmodium falciparum during in vitro culturing.

Within 1 hour after invasion, the parasite has a cup-shape and is devoid of intracellular organelles, usually associated with the merozoite

(microtubules, micronemes and rhoptries) (21). It not only ingests and digests erythrocyte cytoplasm, but also depends on nutrients from outside the erythrocyte to survive (19). According to Divo *et al.* (22), the parasite requires in addition to salts, glucose, and reduced glutathione also at least hypoxanthine, calcium pantothenate, cysteine, glutamate, glutamine, isoleucine, methionine, proline and tyrosine for continuous growth.

(a) Glucose

The asexual stages of malaria parasites as well as the erythrocyte have no carbohydrate reserves and consequently require a continuous supply of glucose from the medium to sustain growth (23). Although glucose is the main energy source for malaria parasites, other carbohydrates (mannose, fructose) and glycerol may also be utilized (23). In general, an infected erythrocyte uses 10-100 times more glucose than a non-infected erythrocyte (24).

(b) Amino acids

Malaria parasites and malaria-infected erythrocytes are able to fix CO_2, and synthesize a limited number of amino acids (alanine, aspartic acid, glutamic acid) (8). Although it is generally accepted that hemoglobin is the major source of amino acids, precise quantification of its contribution to the total amino acid requirement of the parasite has not been made (4). The utilization of exogenously supplied amino acids by malaria-infected erythrocytes is apparently dependent on the parasite species and stage of development (7).

(c) Nucleic acids

Malaria parasites are able to synthesize pyrimidines *de novo* but not purines (7). The most obvious purine source is the host erythrocyte. Approximately 80 % of the erythrocyte's purines are in the form of ATP and can potentially be utilized by the parasite (20). Yamada and Sherman (24), reported a dramatic decline in the ATP concentration of erythrocytes from hosts with malaria. They estimated that 25 % of the purine nucleotides required for the development of the uninucleate trophozoite to the schizont, could be obtained from the purine pool (mostly ATP) of the erythrocyte. Purine interconversion enzymes were identified in the erythrocyte and the parasite and it could be shown that hypoxanthine was taken up from the medium and utilized by the malaria parasite (24).

(d) Lipids

Erythrocytes and malaria parasites lack the necessary enzymes for *de novo* fatty acid synthesis (6). Consequently, both the malaria parasite and the host erythrocyte rely on the passive exchange of lipid precursors with the blood plasma (6). However, through the incorporation of acetate into existing fatty acids by means of limiting chain-lengthening reactions, the parasite is able to maintain a fatty acid composition distinct from that of the host erythrocyte (6). Additionally, the parasite membrane and host cell membrane are endocytosed whereby lipids are released for plasmodial lipid biosynthesis.

3.1.3. Objectives.

The technique of continuous cultivation of malarial parasites was mastered at NIDTE in Durban. Problems were, however, experienced in establish-

ing the same cultures in our laboratory. The objective of this study therefore was to investigate conditions which may be critical for the continuous culture of *P. falciparum*.

Experiments are described in this chapter in which the effects of the gas composition of the medium as well as changes in the metabolite concentration on parasite growth were investigated in *in vitro* cultures.

3.2 MATERIALS AND METHODS

3.2.1 Serum

(a) Human serum

Human type O Rh^+ blood was donated by healthy volunteers who neither had malaria before, nor came from a malaria endemic area. Blood was sampled in sterile blood donation bottles and left overnight at 4 °C to clot. Serum was collected after removal of erythrocytes and clots by centrifugation at 3000 ×g for 10 min (4 °C) in a Beckman J-6 centrifuge. Serum was frozen in sterile 50 ml tubes (Sterilin) at -20 °C until required.

(b) Bovine serum

Bovine serum (sterilized by gamma irradiation) was obtained from the Department of Veterinary Science at Onderstepoort, University of Pretoria. Complement proteins were inactivated by heat treatment of serum at 56 °C for 1 hour. After addition of human erythrocytes (3 ml/50 ml serum), the mixture was incubated in a rotating flask for 3 hours at 37 °C. At this stage another 2 ml of human erythrocytes were added to

the mixture and left for 12 hours at 4 °C. Erythrocytes were removed by centrifugation (3000 ×g for 10 min) and the serum was stored at -20 °C until required. (modified from 20)

3.2.2 Erythrocytes

(a) Collection and washing

Fresh erythrocytes were prepared on a weekly basis. Blood O Rh^+ was obtained from healthy volunteers by venipuncture and collected into tubes containing acid/citrate/dextrose as anticoagulant (Medsurge, Pretoria). After centrifugation, the erythrocytes were washed four times with RPMI 1640 medium without serum (washing medium). Care was taken to remove as much as possible of the buffy coat during each washing cycle. Erythrocytes were stored in washing medium at 4 °C.

(b) Removal of leukocytes.

Erythrocytes were obtained as described above. A glass syringe (10 ml) was plugged with cotton wool and sterilized by heat in an autoclave. A 1:1 mixture of α-cellulose and Sigmacell (α-SS) (Sigma) was suspended into water and autoclaved. A column of α-SS was packed into the sterile glass syringe using 2 cm^3 packing material for each 5ml washed erythrocytes (26). The column was first flushed with 10 ml washing medium before loading of the erythrocyte suspensions. Leucocyte- and platelet-free erythrocytes were collected after elution of the column with washing medium. A thick blood smear was made and stained with Giemsa to confirm that all the leucocytes were removed.

3.2.3 Media.

(a) Washing medium. (15)

RPMI 1640 powder (10.4g/L) with glutamine but without bicarbonate; Highveld Biologicals) was dissolved in 900ml double distilled water, and to this were added 5.94 g of HEPES (Merck) and 4g of glucose (Merck). The solution was diluted to 960 ml and sterilized by filtration through a 0.45 µm Millipore filter. The medium was completed by addition of 40ml sterile, 5 % (w/v) sodium bicarbonate solution and 0.4 ml 10 % (w/v) gentamycin solution (Highveld Biologicals).

(b) Culture medium.

Human or bovine serum was added at a concentration of 10 % (v/v) to the washing medium after equilibration with the special gas mixture (Afrox) as described below.

3.2.4 Gassing of cultures.

(a) Equilibration of medium with the special gas mixture.

A special gas mixture consisting of 5 % CO_2, 5 % O_2, and 90 % N_2 (Afrox) was bubbled through the washing medium with the aid of a sterile pipette with an in-line, sterile 0.22 µm Millipore GV filter. The medium was gassed for 15 min at a flow rate of approximately 5 L/min in a sterile hood at room temperature after which serum was added (10 % v/v) and the medium container sealed.

(b) Gassing of cultures in growing flasks (25)

The air above the cultures was displaced by ventilation of flasks with the sterilized gas mixture. The open end of the pipette was held at the back of the culture flask, above the medium, while gas was blown over the medium at a flow rate of approximately 40 L/min for 20 seconds.

3.2.5 Continuous cultivation of Plasmodium falciparum.

Freshly washed erythrocytes (O Rh^+) were diluted in gas-equilibrated medium to a hematocrit of 5 % and transferred to either a 100 ml or 250 ml culture flask (Sterillin) (5 ml or 20 ml erythrocyte suspension, respectively). Thawed or freshly infected blood, was mixed with non-infected erythrocytes (1:5) in the growth flask and gassed for 20 seconds as described above. After sealing, the growth flask was placed into an incubator at 37 °C and shaken at 200 rpm. Cultures were incubated for 24 hours before the medium was replaced and thin smears were made. Fresh erythrocytes were added every second day, depending on the parasitemia (4).

3.2.6 Gassing experiments

3.2.6.1 Optimization of the time required to displace the air with the special gas mixture.

The time required to displace the air above the medium was determined using non-infected erythrocyte cultures at a hematocrit of 5 %. The special gas mixture was blown at a rate of 40 L/min for 0, 5, 10, 20, 40, or 60 seconds into growth flasks, fitted with septum-containing stoppers. Aliquots of the medium were withdrawn through the septum and analyzed with a pH/blood gas analyzer (Radiometer, ABL30 Acid-Base Analyzer) to determine pH, Pco_2 and Po_2.

3.2.6.2 Determination of the gas profile under culture conditions.

Six cultures of non-infected erythrocytes (5 % hematocrit) were used. Three cultures were prepared in non-equilibrated medium whereas the other three contained gas-equilibrated medium. Flasks were gassed with the special gas mixture as described in 3.2.4 (b). The culture flasks were sealed and incubated at 37 °C in the shaking incubator (Gallenkamp, Plus series) , and analyzed after 1 and 24 hours with the gas analyzer as described above.

3.2.6.3 Effect of gassing method on parasitemia.

The experiment in 3.2.6.2 was repeated but non-infected erythrocytes were replaced by infected erythrocytes. Six *Plasmodium falciparum* (isolate PfUP-1) cultures, each containing 5 ml of a 5 % (v/v) erythrocyte suspension with a parasitemia of 1.2 % (21 % rings, 61 % trophozoites and 18 % schizonts) were used. Blood smears were made at 24 hour intervals for up to 96 hours to monitor parasite growth.

3.2.7 Cryopreservation of Plasmodium falciparum-infected

blood or cultures. Ring-stage infected blood was centrifuged (800g X 5 min) and washed 3 times with washing medium. The erythrocyte pellet was resuspended with an equal volume of washing medium containing 28 % glycerol and transferred to a 1 ml cryotube (Nunc). The sample was frozen by immersion into liquid nitrogen (25).

3.2.8 Thawing of cryopreserved infected erythrocytes.

Cryopreserved samples were removed from the liquid nitrogen tank and put into a waterbath at 37 °C to thaw. Two hundred microliters of 12 % NaCl was added for each 1ml of sample and incubated for 1 min at 37

°C. Nine ml 1.6 % NaCl was added next, and the suspension was centrifuged at 800 ×g for 5 min. The supernatant was aspirated and the pellet resuspended in 10 ml prewarmed (37 °C) washing medium. The erythrocytes were washed once more before it was used in culture experiments (25).

3.2.9 Medium supplements and parasite growth.

Medium was freshly prepared as described in section 3.2.3(a) with either 10 % human or bovine serum. Four cultures, containing medium supplements (Table 3.1) were prepared and incubated at 37 °C. Thin blood smears were made every 24 hours for up to 96 hours to determine parasitemia.

Table 3.1. Supplements added to freshly prepared medium.

Culture	Human Serum 10% v/v	Bovine Serum 10% v/v	Hypoxanthine
1	+	*	+
2	+	*	-
3	*	+	+
4	*	+	-

3.2.10 Metabolite concentrations in Plasmodium falciparum-infected cultures.

The concentrations of various metabolites in *Plasmodium falciparum* cultures were determined in two different experiments as shown in Fig 3.1.

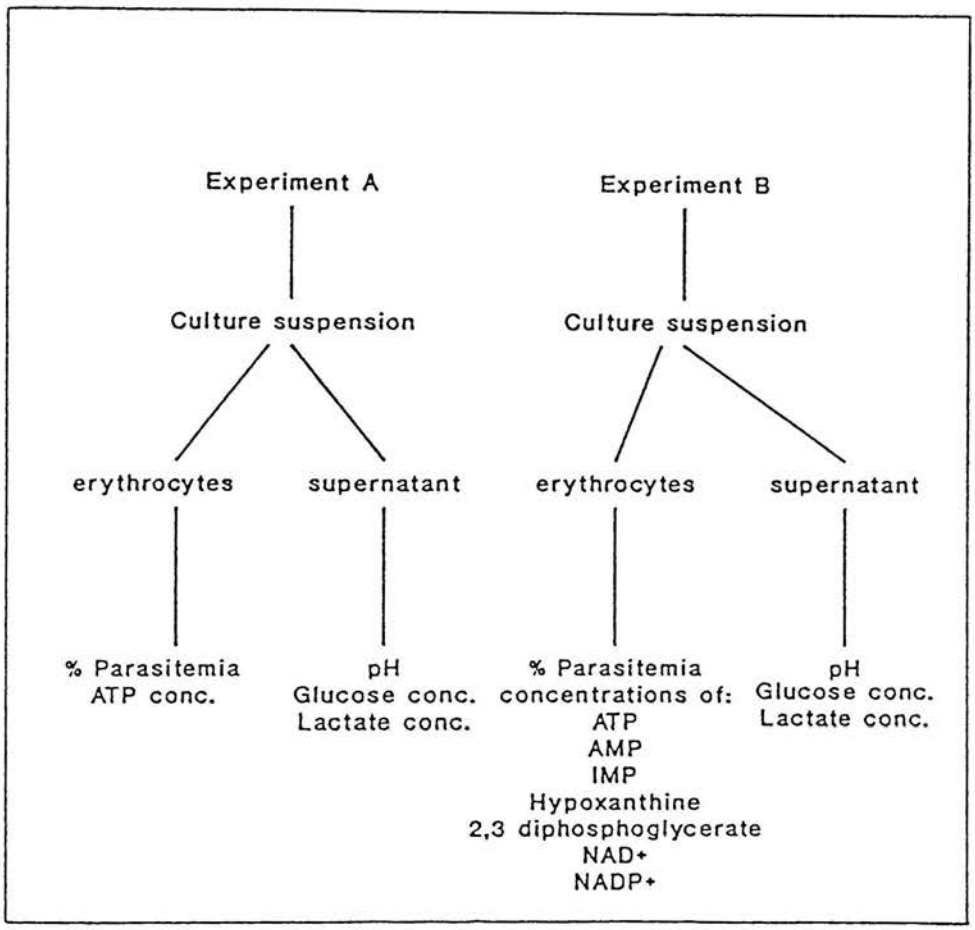

Fig 3.1 Diagrammatic representation of experiments to determine metabolite concentrations in Plasmodium falciparum cultures.

Experiment A

Two infected (0.2 % parasitemia) and two control cultures of 20 ml each, were initiated in 250 ml growth flasks using the method described in section 3.2.5.. The media of one each of the infected and control cultures were replaced every 24 hours. Cultures were lightly agitated to obtain a homogeneous culture suspension and 5 ml samples were removed every 24 hours for up to 96 hours. The erythrocytes and medium were

separated by centrifuging at 1000 ×g for 3 min at room temperature. The percentage parasetemia and pH and concentrations of ATP, lactate and glucose were then determined (section 3.2.10.1).

Experiment B

Four infected (0.8 % parasitemia) and four control cultures of 20 ml each, were initiated in 250 ml flasks using the method described in section 3.2.5. Medium was replaced at 24, 48, 60, 72, 78, 84, 90, and 96 hours. The pH of the spent medium was determined and then stored at -20 °C until assayed for metabolite concentrations (Fig 3.1. section 3.2.10.1). Five milliliter culture aliquots were removed at 24 hour intervals as described above. Erythrocytes and media were separated by centrifugation (1000 x g, 3 min). The erythrocytes were immediately processed (section 3.2.10.2) while the medium was stored at -20 °C until assayed (Fig 3.1; section 3.2.10.1). Starting values for glucose and lactate were determined after every medium change.

3.2.10.1 Assays on culture media.

(a) pH: The culture medium was collected in 10 ml tubes (Nunc), and the pH determined as quickly as possible with a pH meter (Zeiss model 300) at approximately 37 °C.

(b) Glucose concentration (28): The concentration of glucose in duplicate samples was determined with a Boehringer-Mannheim kit (Cat No 716 251). The assay is based on the formation of stoichiometric amounts of NADH from glucose in the presence of glucose-6-phosphate dehydrogenase/ATP and

hexokinase. The concentration of NADH in each sample was determined from its absorption at 340 nm using the following formula:

$C = V/\varepsilon d \times v \times \Delta A$

C = concentration (mmol/liter)

V = final volume of the sample (ml)

ε = molar absorption coefficient for NADH at 340 nm (6.3 L/mol.cm)

v = sample volume (ml)

d = light path (cm)

ΔA = absorption at 340 nm

(c) Lactate concentration. (29) The lactate concentration in duplicate samples was determined with a Boehringer-Mannheim kit (Cat. No 139 084). The assay is based on the formation of stoichiometric amounts of NADH from lactate in the presence of NAD^+, lactate dehydrogenase and glutamate-pyruvate transaminase. The concentration of NADH in each sample was determined as described above for glucose.

3.2.10.2 Assays on erythrocytes.

(a) Parasitemia. Thin blood smears were made as described in section 2.2.1. Approximately 1000 erythrocytes per slide were examined to determine the parasitemia.

(b) Purine nucleotide concentrations. The method was based on the separation of purine nucleotides in erythrocyte extracts, by high performance liquid chromatography. The concentrations were either determined from peak areas or enzymic assay of collected fractions. In some cases the con-

centrations of purine nucleotides were determined by direct enzymic assay of erythrocyte extracts. The methods employed are described below.

b (i) Extraction of purine nucleotides (31) Each erythrocyte fraction was divided into equal parts and transferred to Eppendorf tubes. After centrifugation at 8000 ×g for 15 min at 4 °C, extracts were made of the erythrocyte pellets, using perchloric acid (PCA), as described in Fig 3.2.

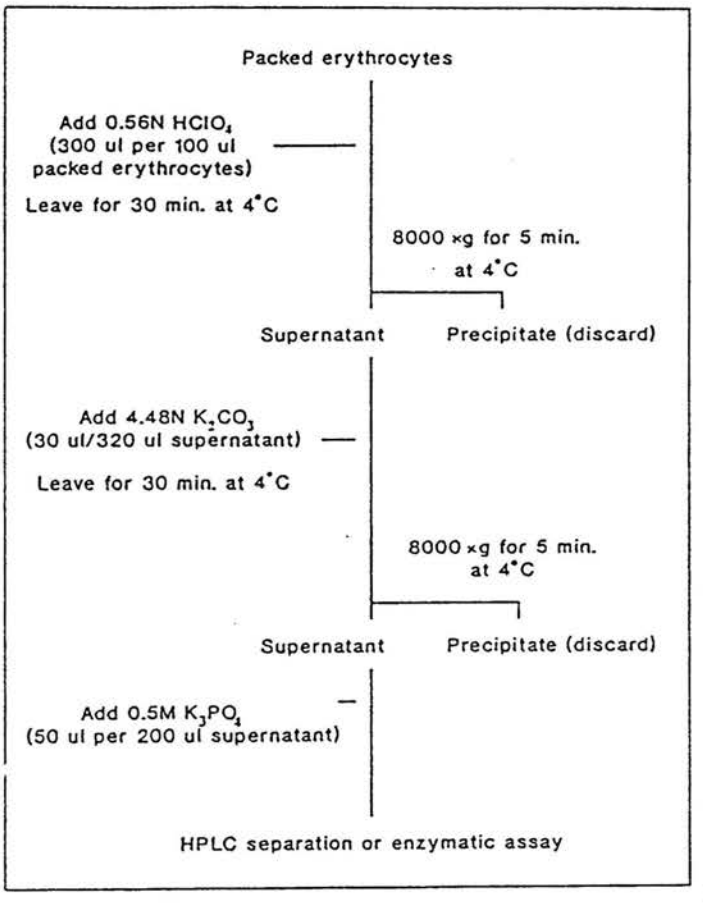

Figure 3.2 Extraction of purine nucleotides from erythrocytes (32).

b (ii) Chromatographic apparatus and conditions.

Chemicals: Nucleotide standards and coenzymes of the highest grade were purchased from Boehringer-Mannheim (Mannheim, West Germany). Analytical-grade potassium dihydrogen phosphate was obtained from Merck, HPLC-grade methanol was from Waters (Waters Assoc., Milford,

USA) while the double distilled water was prepared in the laboratory (Milli-Q, PB 430). Stock solutions of ATP, ADP, AMP, Hypoxanthine, NAD^+ and $NADP^+$ were prepared in 0.1 M KH_2PO_4 buffer solution (pH 6) and were stored at -20 °C. The concentrations of the stock solutions were determined spectrophotometrically.

Chromatographic apparatus: In experiment A, a Waters model ALC-204 HPLC instrument equipped with two 6000A model pumps and model 440 absorbance detector (254 nm) (Waters Assoc. Milford, USA) was used. Peak areas were integrated with a Spectra-Physics SP 4290 electronic integrator (Spectra-Physics, California). In experiment B, a Beckman system consisting of a model 110 pump, a model 166 fixed wavelength (254 nm) UV detector and model 210 sample injector was used. Integration of peak areas was performed on a Copam Personal Computer (model 286M-120) fitted with System Gold chromatography software from Beckman.

HPLC column: In experiment A, a self-packed 5 µm Nucleosil RP-18 (150 × 3.9 mm, Merck) analytical column was used. In experiment B, a 5 µm LiCrosorb RP-18 (150 × 4.6 mm) analytical column (Merck) and a Guard-Pak module from Waters (Waters Assoc. Milford, USA) fitted with RP-18 cartridges (No 88070), were used.

Buffers: In experiment A, the mobile phase consisted of a 0.1 M potassium phosphate buffer (pH 6.0). In experiment B the mobile phase was 0.1M potassium phosphate buffer (pH 6.5) in 1 % methanol. The buffers were filtered through 0.45 µm filters before use. The flow rate in both experiments was 1 ml/min and nucleotides in the eluants were detected at 254nm.

Peak identities: The identities of nucleotides were confirmed by spiking of extracts with standards and from their 250/260 absorbance ratios (33).

(c) Determination of 2,3-bisphosphoglycerate, hypoxanthine and inosine monophosphate concentrations. (3)

(i) 2,3-Bisphoshoglycerate. Triplicate samples of PCA extracts (section 3.2.10.2(b)) were spectrophotometrically assayed with a Boehringer-Mannheim kit (No 148 334). The assay is based on the formation of stoichiometric amounts of NADH from 2.3-bisphosphoglycerate in the presence of phosphoglycerate mutase/ phoshoglycerol kinase/glyceraldehyde-3-dehydrogenase/NAD^+/ATP. The concentration of 2,3 bisphosphoglycerate in each sample was determined from the absorption of NADH at 340nm with the following formula:

$$C = V \times F / \varepsilon \times d \times v \times 2 \times \Delta A$$

C = concentration (mmol/liter)

V = assay final volume (ml)

F = dilution factor

ε = molar absorption coefficient for NADH at 340 nm (6.3 L/mmol.cm)

d = light path (cm)

v = sample volume

ΔA = Absorption at 340 nm.

(ii) Hypoxanthine. (32) Triplicate PCA extracts (section 3.2.10.2(b)) were assayed for urate which is stoichiometrically produced from hypoxanthine in the presence of xanthine oxidase (E.C. 1.1.3.22; Boehringer Mannheim).

The amount of hypoxanthine was determined in each sample after addition of 5 µl xanthine oxidase (51 kU/l) to 25 µl erythrocyte PCA extract in 350 µl glycine buffer, pH 9.3. The concentration of hypoxanthine in each sample was determined from the absorption of urate at 280nm using the following formula:

$C = \Delta A \times F \times 10^3 / \varepsilon$ .....(I)

C = concentration (µmol/l)

F = dilution factor

ε = molar absorption coefficient for urate at 280nm = 7.0 L/umol.cm

Inosine 5'-monophosphate (32): The concentration of IMP in a sample can be determined by stoichiometric conversion to hypoxanthine which in turn is measured as urate as described above. The method is based on the hydrolysis of IMP in a 5 µl sample by 20 µl (1.5 kU/ml) alkaline phosphatase (E.C. 3.1.3.1; Boehringer Mannheim) in 1 ml (0.1 M) triethanolamine buffer (pH 7.6). The resulting inosine is converted to hypoxanthine by addition of 5 µl (125 U/ml) nucleoside phosphorylase (E.C. 2.4.2.1; Boehringer Mannheim) and 40 µl (0.1mol/L) EDTA. Hypoxanthine in turn is reacted with 4 µl (4 U/ml) xanthine oxidase (E.C. 1.1.3.22; Boehringer Mannheim) and converted to urate which is measured at 280nm. The absorbance reading was corrected for the hypoxanthine already present in the sample. The method was validated with samples containing known concentrations of IMP and hypoxanthine.

The concentration of IMP was determined with the same formula as for hypoxanthine (formula I).

3.3 RESULTS

3.3.1 Time required to displace the air above thin-layered cultures with the special gas mixture.

The composition of the air above the thin-layered erythrocyte culture in the growth flask is important for parasite growth (15). The air above the cultures was displaced with the special gas mixture and the time required to reach equilibrium was determined.

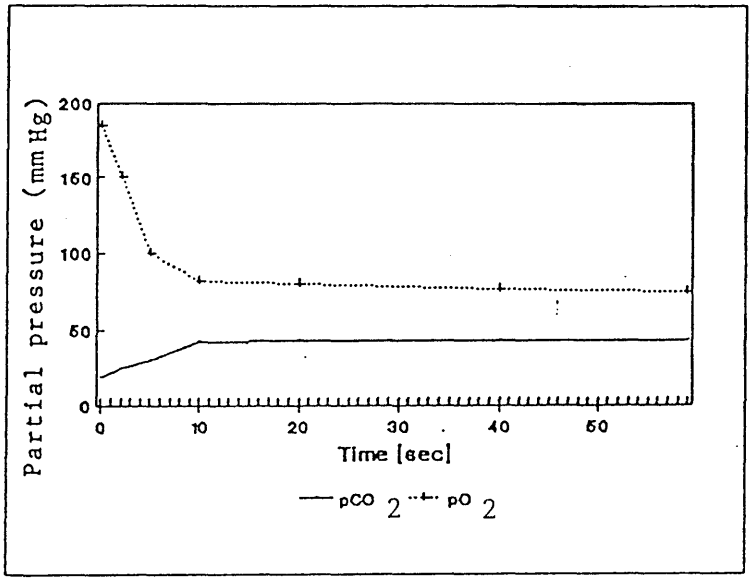

Figure 3.3 Time required to displace air in 250 ml growth flasks. Duplicate, non-infected erythrocyte cultures were gassed with the special gas mixture (5 % CO_2, 5 % O_2) at 40 L/min for 2 to 60 sec. Culture suspension samples were taken after 1 hour and the Pco_2 and Po_2 determined by a ALB30 Acid-Base analyser at 37 °C.

From Fig. 3.3 it is evident that the medium equilibrated with the gas mixture in a culture flask was reached within 10 sec. The starting values for Po_2 and Pco_2 in the culture suspension were 181.2 mmHg and 21.0 mmHg and reached at equilibrium, values of 83 mmHg and 46 mmHg, respectively.

3.3.2 Effect of gassing method on parasite growth.

Parasite cultures were prepared in air- and gas-equilibrated medium as described in section 3.2.6.2 and parasite growth was determined at 24 h intervals (Fig. 3.4 (a) and (b)).

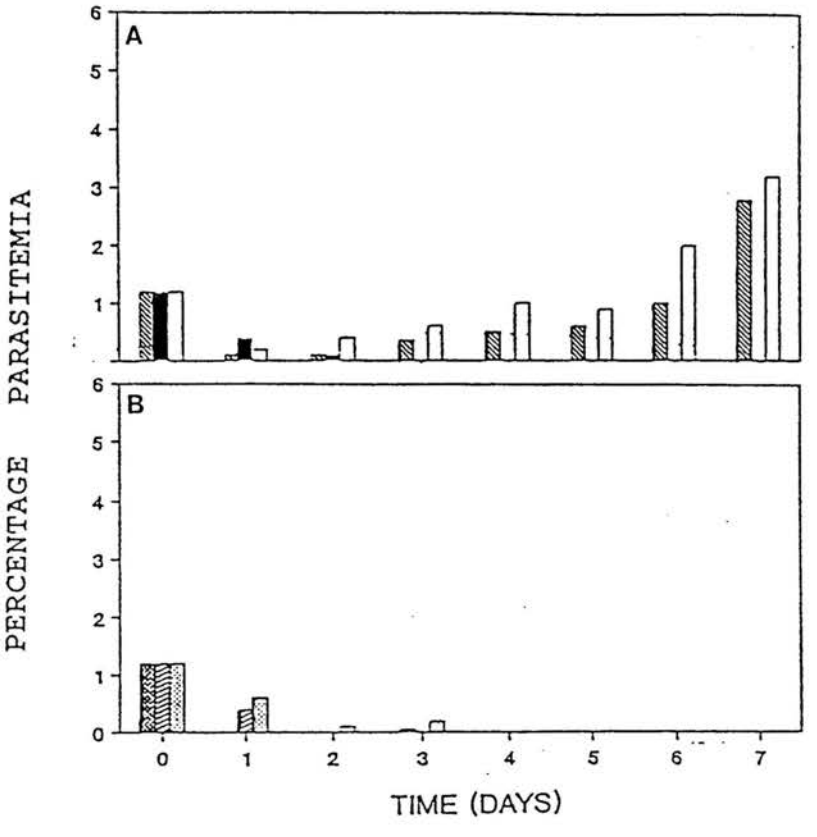

Figure 3.4 Parasite growth in air- and gas-equilibrated culture media. Two identical cryopreserved parasite cultures were started in (a) gas-equilibrated medium plus air displacement above the thin-layered cultures with the special gas mixture, (b) air-equilibrated medium and air displacement above the thin-layered culture using the special gas mixture. The percentage parasitemia in triplicate cultures was calculated by counting at least 2000 erythrocytes per blood smear.

Two of the three cultures developed successfully in the gas-equilibrated medium (a) whereas the cultures in the air-equilibrated medium (b) died within 3 to 4 days after initiation of the cultures.

3.3.3 Gas profile of medium under culture conditions.

Non-infected cultures were initiated as in section 3.3.2 and incubated at 37 °C. The partial pressures of O_2 and CO_2 were determined after 1 and 24 h of incubation. Results are given in Table 3.2.

Table 3.2 Gas analysis of culture media after 1 and 24 hours of incubation at 37 °C. Results are the average of of triplicate experiments.

Time	Gassing method	Pco_2 mmHg	Po_2 mmHg	pH
1 hour	(a)	21.0 ±1.1 (3.4 %)	173.0 ±0.1 (28.5 %)	7.30 ±0.02
	(b)	42.0 ±1.5 (6.9 %)	81.1 ±1.1 (13.3 %)	7.16 ±0.01
	(c)	50.0 ±0.6 (8.23 %)	70.8 ±2.4 (11.7 %)	7.12 ±0.01
24 hours	(a)	18.7 ±0.5 (3.08 %)	139.1 ±1.3 (22.9 %)	7.38 ±0.03
	(b)	36.5 ±1.4 (6.0 %)	82.6 ±6.4 (13.6 %)	7.2 ±0.01
	(c)	42.7 ±0.5 (7.0 %)	73.5 ±2.7 (12.1 %)	7.16 ±0.03

(a) No gassing
(b) Gassed by displacement of the air above the thin-layered culture in air-equilibrated medium with the special gas mixture.
(c) Gassed by displacement of the air above the thin layered culture in gas-equilibrated medium with the special gas mixture.

Values in brackets indicate the percentage of gas by volume at the mean atmospheric pressure of Pretoria (654mmHg; Weather Bureau) calculated with the following formula:

% Gas = Pco_2/ B - P_{H2O} × 100

B = barometric pressure at Pretoria

P_{H2O} = vapor pressure (47mmHg, at 37 °C)

After 1 hour of incubation the Pco_2 values of the gassed cultures were 50 mmHg and 42 mmHg in the gas- and air-equilibrated media, respectively, compared to a value of 21 mmHg for the non-gassed culture in air-equilibrated medium. The non-gassed culture in air-equilibrated medium had a Po_2 value of 173 mmHg. The corresponding values for the gassed cultures were 81.1 and 70.8 mmHg, respectively, in the air- and gas-equilibrated media. The pH of the gassed cultures were at 7.12 and 7.16 lower than that of the non-gassed culture (7.35). The Pco_2- and Po_2-values were generally lower and higher, respectively, after 24 h of incubation than the values after 1 hour of incubation. There was also corresponding increases in the pH of the media.

3.3.4 Parasite growth in media with different supplements.

Parasite growth was compared between media supplemented with human or bovine serum and with or without hypoxanthine (Table 3.3).

Table 3.3 Growth comparison between PfUP1-infected erythrocytes in medium supplemented with human or bovine serum and with or without hypoxanthine. Samples were assayed every 24 hours and at least 2000 erythrocytes in thin blood smears were counted to calculate parasitemia.

CULTURE NR	PARASITEMIA AFTER INCUBATION FOR:					RELATIVE PARA-SITEMIA
	0h	24 h	48 h	72 h	96 h	
1	0.4	1.20 ± 0.10	4.30 ± 0.50	22.30 ± 3.0	27.9 ± 0.90	46.5
2	0.4	1.23 ± 0.05	3.00 ± 0.70	9.10 ± 0.90	11.6 ± 0.60	19.3
3	0.4	0.80 ± 0.10	1.10 ± 0.10	3.00 ± 0.60	6.4 ± 0.60	10.7
4	0.4	0.46 ± 0.20	0.36 ± 0.20	0.70 ± 0.10	0.6 ± 0.10	1

Culture 1: Human serum with 44mg/L hypoxanthine
Culture 2: Human serum without hypoxanthine
Culture 3: Bovine serum with 44mg/L hypoxanthine
Culture 4: Bovine serum without hypoxanthine

From the results in Table 3.3 it is apparent that human serum sustains parasite growth better than bovine serum over an incubation period of 96 hours. This is true whether or not the media were supplemented with hypoxanthine. The relative parasitemia in medium supplemented with bovine serum and hypoxanthine, was 10.7-fold higher than in the absence of hypoxanthine. The corresponding increase in the relative parasitemia in human serum supplemented-medium was only 2.4-fold (from 19.3 to 46.5). However, the relative parasitemia in medium supplemented with human serum without hypoxanthine, was still 1.8-fold higher than in medium supplemented with bovine serum and hypoxanthine.

3.3.5 Metabolite concentrations of Plasmodium falciparum-infected cultures.

3.3.5.1 Experiment A

The home-made HPLC column was used to separate the purine nucleotides in perchloric acid (PCA) extracts of erythrocytes (Section 3.2.10). A typical elution profile is shown in Fig. 3.5.

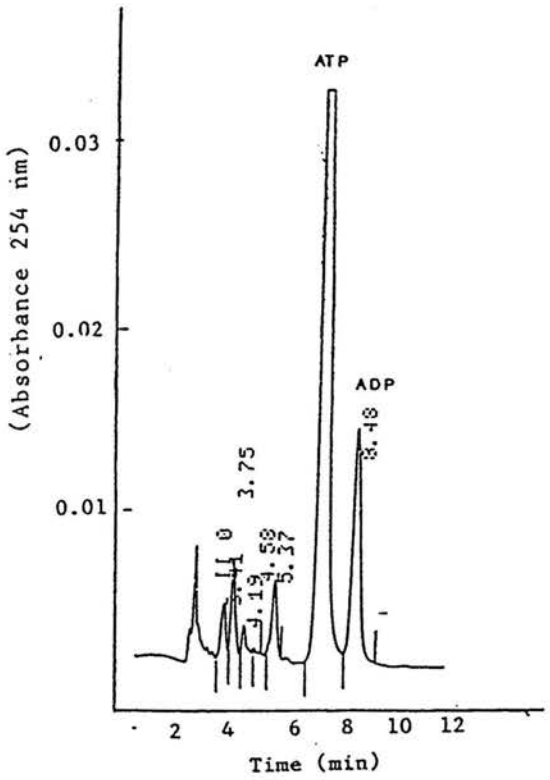

Figure 3.5 HPLC elution pattern of erythrocyte-PCA extracts conducted with a home-made 5µm Nucleosil column (250 × 3.9 mm). The mobile phase consisted of 0.1 M phosphate buffer (pH 6.0), and the flowrate was 1 ml/min. Five microliters samples were injected and the eluate was monitored at 254 nm.

From the elution pattern in Fig. 3.5 it is apparent that ATP is the main component in PCA extracts of erythrocytes. Other purine nucleotides and co-factors in the PCA extract were not determined for experiment A.

The concentrations of ATP and other metabolites detemined at 24 h intervals during culturing of *P. falciparum*- infected and non-infected (control cultures), are summarized in Fig. 3.6.

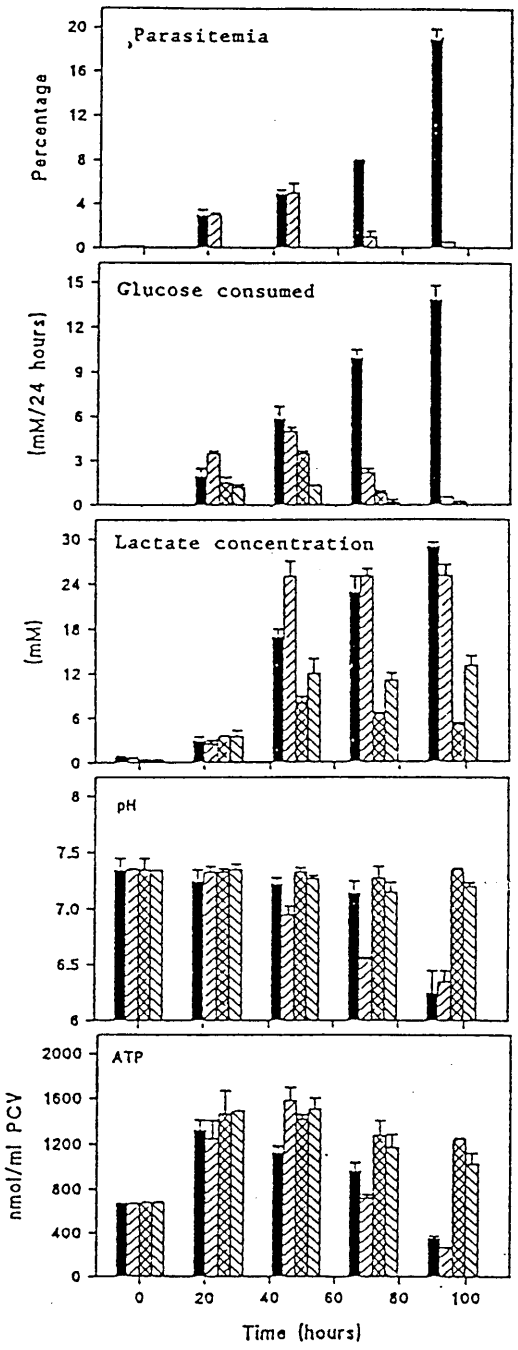

Figure 3.6 Comparison of various parameters during *in vitro* culture of *P. falciparum*-infected and non-infected erythrocytes under different conditions (Experiment A) ■ Parasite-infected erythrocyte cultures with daily medium replacement (In+R); ▨ Parasite-infected erythrocyte cultures without daily medium replacement (In-R); ▩ Non-infected erythrocyte cultures with daily medium replacements (No+R); ▧ Non-infected erythrocyte cultures without daily medium replacement (No-R).

0 hours:

At this time point the parasitemia of the unsynchronised PfUP1 culture was 0.3 %, the medium pH was 7.35 and the ATP concentration was approximately 670 nmol/ml PCV.

24 hours:

All the cultures consumed approximately the same amount of glucose, produced the same amount of lactate and had the same pH. The ATP concentrations of the infected cultures were slightly lower compared to the non-infected cultures. Parasitemia were the same for both types of infected cultures.

48 hours:

Both the infected and non-infected cultures with medium replacement showed higher glucose consumptions than the corresponding cultures without medium replacement. The infected cultures displayed higher glucose consumptions than the non-infected cultures. The accumulated lactate concentration was 25.4 mM for the infected cultures without medium replacement (In-R, lactate produced = 21.5 mM/24 h); whereas the infected cultures with daily medium replacement ($In^{+}R$), produced 16 mM/24 h (accumulated total = 22mM). The non-infected cultures with daily medium replacement ($No^{+}R$) produced 8 mM lactate/24 h (accumulated total = 11 mM) whereas the accumulated concentration for the non-infected

culture without daily medium replacement (No-R) came to 12 mM (lactate produced = 9 mM/24 h). At this point the pH of the In-R and In+R cultures were 7 and 7.25 respectively. The pH of the non-infected cultures were the same as for the 24 hour time point. The ATP concentration of the In+R culture decreased from 1300 at 24 hours to 1131 nmol/ml PCV. The ATP concentration of the non-infected cultures was similar to the values after 24 hours of incubation. The ATP concentration of the In-R culture was at 1580 nmol/ml PCV higher than at the 24 h time point. The parasitemia for both types of infected cultures was 5 %.

72 hours:

The parasetemia in the In+R cultures had increased to 8 % whereas the parasetemia in the In-R cultures decreased to 1.6 %. The glucose consumption in the In+R culture went up to 9.8 mM/24 h compared to the In-R culture whose consumption decreased to a value of 2.3 mM/24 h. The In+R cultures consumed ten times more glucose compared to the No+R culture. The No-R culture consumed 0.4 mM glucose/24 h compared to the 0.9 mM/24 h for the No+R culture. The accumulated lactate concentrations in the In-R and No-R culture were not changed from those at 48 h. The In+R and No+R cultures produced 22 mM (accumulated total = 41 mM) and 6 mM/24 h lactate (accumulated total = 17 mM) , respectively. The pH of the In-R and In+R cultures dropped to 6.35 and 7.2, respectively, whereas the pH in the other two cultures were approximately the same as before. The ATP concentrations of all the cultures decreased from their previous values: from 1131 to 968 and from 1581 to 719 nmol/ml PCV, respectively, for the In+R and In-R cultures. The

ATP concentrations changed from 1421 to 1282 and from 1508 to 1173 nmol/ml PCV for the No+R and No-R cultures, respectively.

96 hours:

The parasitemia in the In+R culture increased to 19 % compared to the In-R culture for which the parasetemia decreased to 0.5 %. The glucose consumed and the lactate produced in the In+R culture were 14 mM/24 h and 29 mM/24 h (accumulated total = 70 mM), respectively. Essentially no glucose was consumed by the remainder of the cultures over the assay period. The accumulated lactate concentrations of the In-R and No-R cultures were 25.9 and 13 mM, respectively. The No+R culture produced 5 mM lactate/24 h (accumulated total = 22 mM). The pH of the In+R and In-R cultures dropped to 6.25 and 6.3, respectively. The No+R and No-R cultures had a pH of 7.35 and 7.30, respectively. The ATP concentrations of both infected cultures decreased to under 400 nmol/ml PCV, while the concentrations of the non-infected cultures were above 1100 nmol/ml PCV.

3.3.5.2 Experiment B

A typical elution pattern for nucleotides and co-enzymes in erythrocyte-PCA extracts from a Licrosorb RP-18 column is shown in Fig. 3.7. IMP/GMP and AMP/hypoxanthine had the same retention times. NAD^+ was eluted approximately 24 min after injection of the sample.

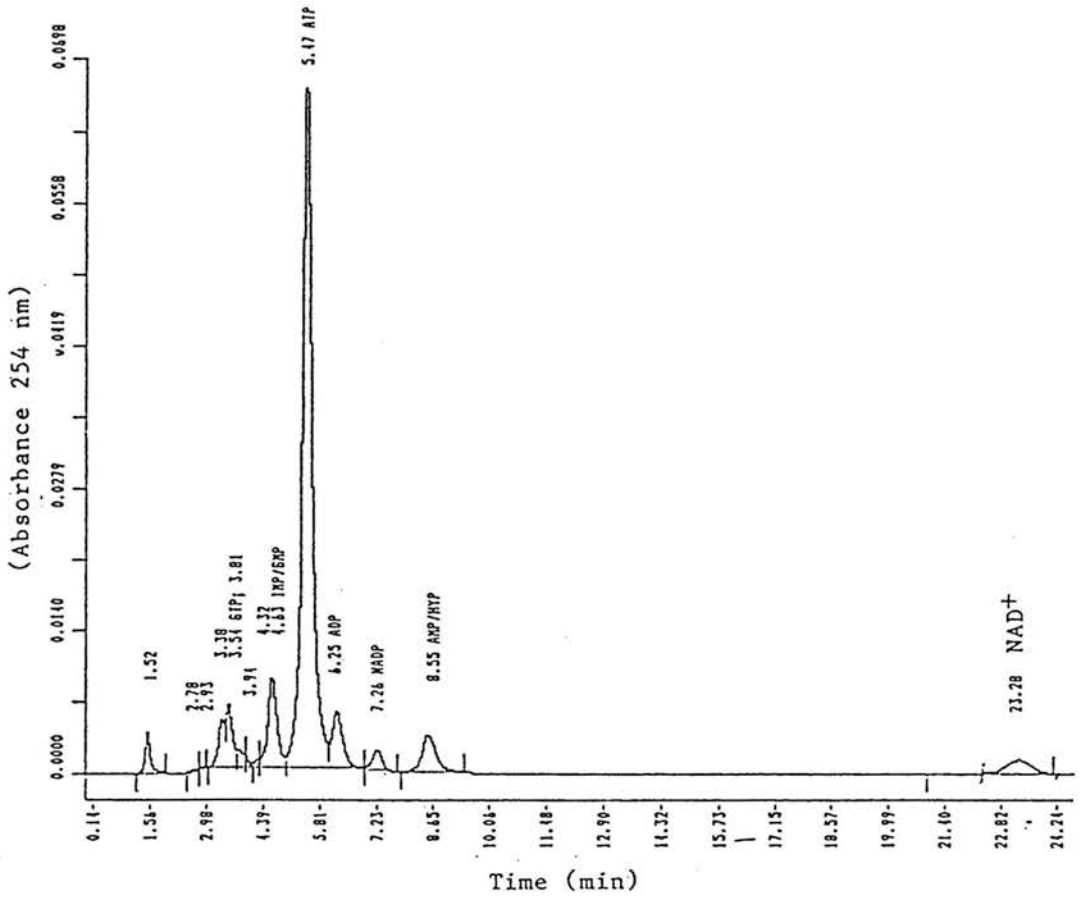

Figure 3.7 HPLC elution pattern of erythrocyte-PCA extracts from infected and non-infected cultures from experiment B (Section 3.2.10.2). The separation was on a commercial 5µm Licrosorb (Merck) column (150 × 4.6 mm). The mobile phase consisted of 0.1 M phosphate buffer (pH 6.5) containing 1 % (v/v) methanol at a flowrate of 1 ml/min. Five microlitre samples were injected and peaks were detected at 254 nm.

The concentrations of metabolites, determined by HPLC and enzymic assay in cultures of infected and non-infected erythrocytes, are shown in the Fig.3.8.

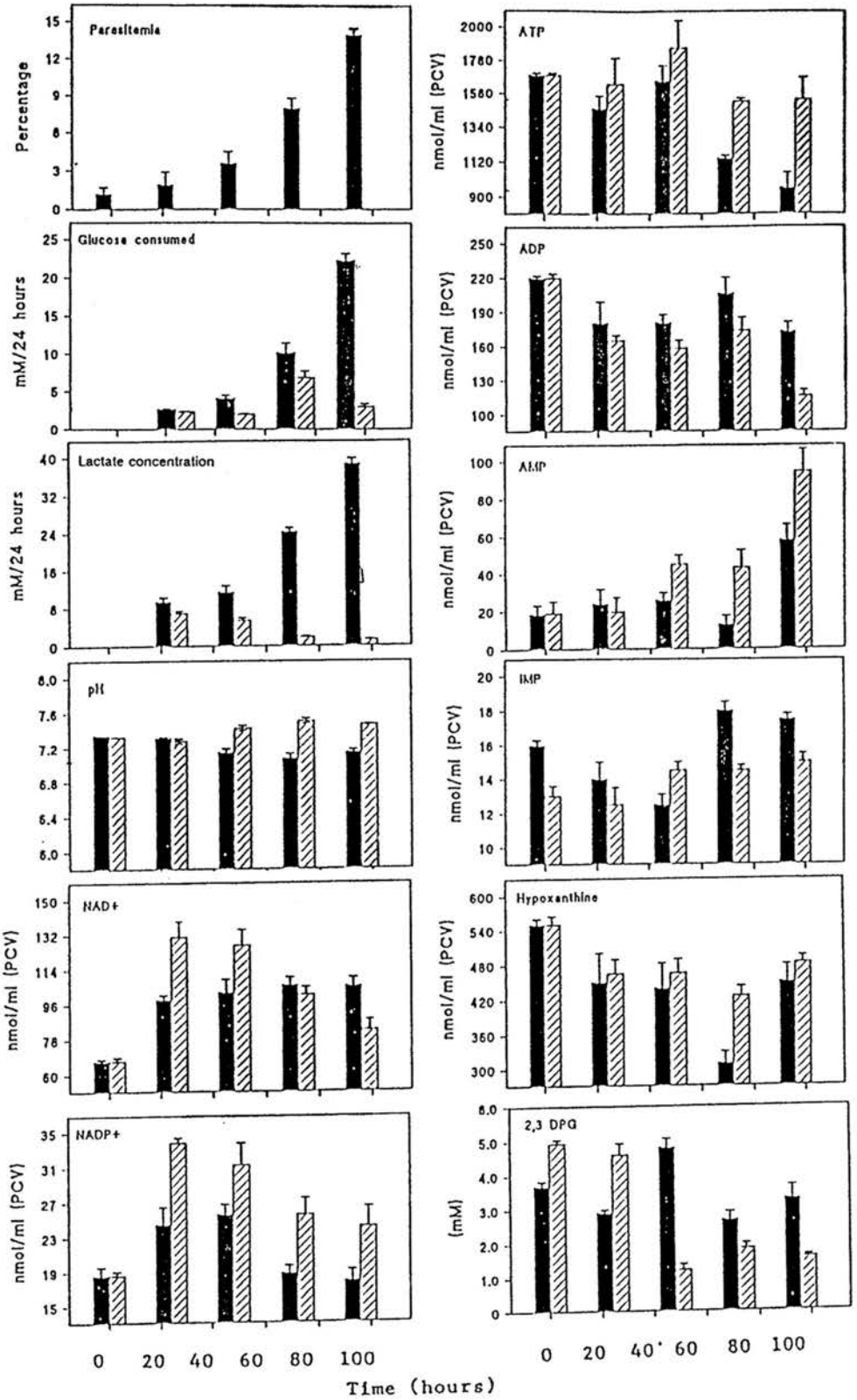

Figure 3.8 Comparison of various parameters during *in vitro* culture of *P. falciparum*-infected and non-infected erythrocytes. (Experiment B)
■ PfUP1-infected erythrocyte culture. ▨ Non-infected erythrocyte culture

Parasitemia: The parasite cultures were started at a parasitemia of 0.8 % and over four days increased to 14 %. Over the first and last 48 hours of culture the multiplication indexes were 5 and 3.5, respectively.

Glucose consumption: The glucose concentrations were determined every time the media were changed and values at 72 h (4.2 and 6.9 mM/12 h) and 96 h (4, 6, 5 and 7 mM/6 h) are the combined total for the preceding 24 hour culture period. The glucose consumption increased as the parasitemia increased. At 24 hours the parasitemia was 1.2 % and the glucose consumption of the infected and non-infected cultures were similar (4.2 mM/24 h). At a parasitemia of 14 % (96h) glucose consumption increased to 22 mM/24 h. The consumption of the control increased to 6 mM/24 h at 72 h but declined to 2.5 mM/24 h at 96 h.

Lactate: The lactate concentrations were detemined every time the media were changed and values at 72 h (9 and 15.2 mM/12 h) and 96 h (8, 12, 8.5 and 11.5 mM/6 h) are the combined total for the preceding 24 h culture period. The lactate production of the non-infected cultures decreased steadily (from 7 to 2 mM/24 h) while for the infected cultures it increased (from 9 to 40 mM/24 h) in conjunction with the parasitemia and glucose consumption. There does not seem to be a direct relationship between the glucose consumed and lactate produced for non-infected cultures. At 96 hours the infected erythrocytes produced 19 times more lactate than the non-infected erythrocytes.

pH: The pH was above 7.30 and 7.10 for the non-infected and infected cultures, respectively, throughout the whole experiment. At 96 hours the infected culture with a 14 % parasitemia had a pH of 7.1. It is ap-

parent that the regular and increased tempo of medium replacement assisted in the maintenance of the pH of the culture medium.

ATP: The ATP concentration for both cultures was 1690 nmol/ml PCV at initiation. The ATP content in the infected as well as the non-infected cultures started to decline after 48 h of culturing to reach values of 950 and 1505 nmol/ml PCV, respectively, after 96 h of incubation.

ADP: The initial value for both types of cultures was 220 nmol/ml PCV. The ADP content for both types of cultures was decreased at 24 hours after which it remained fairly constant (infected: 180 to 200 nmol/ml PCV; non-infected: 160 to 175 nmol/ml PCV) over the rest of the culturing period except for the non-infected culture whose content at 96 h was lower at 115 nmol/ml PCV.

AMP: The initial values for both types of cultures was 18 nmol/ml PCV which gradually increased to 93 nmol/ml PCV for the non-infected cultures over the 96 hours of culture. The concentration in the infected cultures increased sharply after 72 h of incubation to reach a value of 59 nmol/ml PCV after 96 h of culturing.

Hypoxanthine: The initial concentration was 550 nmol/ml PCV for both infected and non-infected cultures. From here it decreased progressively for the infected cultures to reach a low of 320 nmol/ml PCV at 72 hours before increasing again to 450 nmol/ml PCV at 96 hours. After declining to 420 nmol/ml PCV at 24 hours of culturing, the concentration for the non-infected controls remained fairly constant (420 to 480 nmol/ml PCV) for the remainder of the culture period.

IMP: The infected cultures had an initial value of 16 compared to 12.5 nmol/ml PCV for the non-infected cultures. After initially declining to 13 nmol/ml PCV (48 hours of culturing), the concentration in In+R cultures increased again to values of 18.5 and 17.8 nmol/ml PCV at 72 and 96 hours, respectively. The content of the non-infected control increased to 15 nmol/ml PCV after 48 h of incubation and remained at this value for the remainder of the culturing period.

2,3 DPG: The initial concentration in the non-infected cultures was 5 mM and was higher than the value for the infected cultures (3.5 mM). The value for non-infected controls declined sharply between 24 and 48 hours of culturing but remained fairly constant (1.5 - 2 mM) for the remainder of the culturing period. The concentration in the infected cultures varied between narrow limits (2.8 - 3.5 mM) except for the 48 hour time point at which a peak value of 4.5 mM was reached.

NAD+: The concentration in both the infected and non-infected cultures increased from the starting value of 68 nmol/ml PCV to reach values of 96 and 132 nmol/ml PCV, respectively, after 48 hours of culturing. Whereas the concentration in the infected culture remained fairly constant, the concentration in the non-infected controls declined steadily to a value of 87 nmol/ml PCV after 96 h of culturing.

NADP+: As with NAD^+ its concentration increased initially from 18.5 nmol/ml PCV and peaked after 24 to 48 hours of culturing for both infected and non-infected cultures. However, the concentration in both cultures progressively declined to 18 and 23 nmol/ml PCV for the infected and non-infected cultures, respectively, after 96 h of culturing. Table

3.4 shows the precursor-product relationship and metabolic status of the cultures from experiment B over a 96 hour cultivation period.

Table 3.4 Precurser-product relationship and metabolic status of *Plasmodium falciparum* cultures during 96 hours of culture (32).

TIME	LACTATE/GLUCOSE RATIO		ENERGY CHARGE RATIO Ø		ADENYLATE NUCLEOTIDES * (nmol/ml PCV)	
(hours)	Inf	Noninf	Inf	Noninf	Inf	Noninf
0	-	-	0.93	0.93	1921	1921
24	2.8	1.9	0.93	0.94	1677	1810
48	2.4	2.0	0.94	0.95	1856	2043
72	2.3	0.5	0.91	0.92	1361	1725
96	1.7	0.7	1.01	0.91	1033	1737
Normal values	2		0.9		range 1374-2031	

* Adenylate nucleotide concentration is the sum of the ATP, ADP and AMP concentrations

Energy charge = $\frac{ATP + 0.5\ ADP}{ATP + ADP + AMP}$

From Table 3.4 it can be seen that initially more lactate is produced in infected cultures than can be accounted for by glucose consumption alone. After 96 hours of cultivation the lactate:glucose ratio dropped below the normal 2:1 ratio expected for glycolysis. The ratio for the non-infected cultures , in contrast was initially 2:1 but was also lower at 72 hours and 96 hours. The adenylate nucleotide concentration of infected erythrocytes decreased almost 50 % compared to the non-infected erythrocytes which shows only a 10 % decrease over 96 hour of cultivation.

Table 3.5 Parasite stages in relation to parasitemia and multiplication index.

TIME (HOURS)	PARASITEMIA	STAGES			MULTIPLICATION INDEX	
		%R	%T	%S		
0	0.8	4	96	0		
						3,75
24	1.4	80	20	0	0-48h	
48	3.0	16	80	4		5,9
					24-72h	
72	8.2	45	35	20		
						4,7
96	14.0	71	18	12	48-96h	

Stages : R - Ring stages
T - Trophozoite stage
S - Schizont stage

Multiplication index = $\frac{\text{parasitemia over 48 h period}}{\text{initial parasitemia}}$

The increase in parasitemia over the 96 hour culturing period and the distribution of parasitic stages are presented in Table 3.5.

3.4 DISCUSSION

Trager believes that it is possible to establish and maintain any isolate of *P. falciparum* in culture. He attributes the inability of some cultures to grow, to poor culturing technique rather than to some inherent property of the parasite (3). However, persistent literature reports suggest that chloroquine-resistant strains of *P. falciparum* have a culturing advantage over sensitive strains and that strains from certain areas adapt easier to culture conditions than strains from other areas (17).

The *in vitro* culturing of malaria parasites in our laboratory was problematic from the beginning of this study. The technique was mastered at NIDTE (National Institute for Diseases in a Tropical Environment) in Durban and established cultures were brought back to our laboratory. However, no isolate could be successfully maintained for longer than two days, not even the well adapted and known Rockefeller isolate, FCR-3. Similar problems in maintaining malaria parasites in continuous culture for reasonable lengths of time were also experienced in other local laboratories (P.J. Fripp and M. Isaacson, personal communication).

Isolates of *P. falciparum* are usually maintained in vessels containing a thin layer of erythrocyte suspension (hematocrit: 5 - 10 %) with a large surface area to enable adequate exchange of gas between medium and the gas phase (17). Only 10 seconds of gassing at 40 L/min was required to equilibrate the media with the 5 % O_2 and 5 CO_2 gas mixture (Fig 3.3). This result demonstrated that the 20 seconds normally used to gas flasks was ample to displace the air above the thin-layered cultures and was not responsible for the inability to establish a malaria culture. However, as shown in Fig.3.4, parasite growth was only achieved once the medium was equilibrated with the gas mixture prior to cultivation. From this it is apparent that the gas composition of the cultures was not compatible with parasite growth when only the air above the cultures was displaced with the gas mixture. These results were in contrast to those at Durban, where flushing of growth flasks alone was sufficient to obtain satisfactory growth.

Consequently, the Po_2 and Pco_2 values of the medium, defined as the partial pressures of O_2 and CO_2 in the gas phase with which the media

are in equilibrium, were determined. The gas composition of cultures remained reasonably constant between 1 and 24 hours of cultivation except for the non-gassed cultures which showed a decline in both Po_2 and Pco_2 (Table 3.2). The Po_2 and Pco_2 were lower and higher, respectively, in flasks which contained gas-equilibrated medium and was flushed compared to flasks that were only flushed (Table 3.2). On the face of it, the Po_2 and Pco_2 values for the latter experiments do not differ markedly to account for the failure of cultures to grow in flasks that were only flushed with the gas mixture.

Scheibel *et al*. showed that the malaria parasite is a microaerophile, needing 3 % O_2 and 2 % CO_2 in the gas phase for optimum growth (15). In addition, their results demonstrated that high oxygen concentrations (17 - 21 %) are only tolerated by the parasite if the CO_2 concentration is also raised from 3 to 5 %. This was interpreted to be due to a rightward shift in the oxyhemoglobin dissociation curve by which the oxygen content of the erythrocytes is reduced (decreased hemoglobin affinity for oxygen). Since continuous parasite growth was only achieved in a gas-equilibrated and flushed medium (Fig.3.4), it appears that the higher oxygen and lower carbon-dioxide concentrations of the medium that was only flushed, (Table 3.2) are not compatible whith parasite survival. This result suggests that the local wild isolate of *P. falciparum* is more sensitive to the toxic effects of oxygen than the isolate of Scheibel *et al*. and that the composition of the gas mixture needs to be adjusted to take this into account. Furthermore, the higher partial pressures of oxygen and carbon dioxide in the gas mixture at Durban (35.7 mmHg, each) compared to Pretoria (30.4 mmHg, each), coupled to the 24-fold higher solubility of CO_2 in the liquid phase, may account for the suc-

cessful cultivation of parasites at Durban in medium that was only flushed with the same gas mixture.

Surprisingly, after a few weeks in culture, it was found that the parasites had adapted to such an extent to the culture conditions that it could be maintained in media that were only flushed with the gas mixture. In fact, cultures could be maintained in medium that was neither equilibrated nor flushed with the gas mixture, for up to three weeks (results not shown). It appears therefore, that the absolute concentrations of oxygen and carbon dioxide in the gas mixture are only critical during the initiation and adaptation stages of wild isolates of *P. falciparum*.

The development and survival of malaria parasites in culture is dependent on the integrity of the erythrocyte under long term *in vitro* conditions. This has to be viewed in relation to the medium by which it is surrounded. One of the most important medium supplements is the serum, whether it be human or bovine (20). From our results (Table 3.3) it appears that human serum is superior to bovine serum in the maintenance of *P. falciparum* in continuous cultures. For cultivation periods of less than 48 hours the differences in parasitemia are relatively small but at 72 and 96 hours it appears that bovine serum cannot cope with the nutritional demands of the growing parasite. Bovine serum contains $6x10^{-7}$ compared to the $1.5x10^{-4}$ mol/L hypoxanthine in human serum (20). This may explain why parasites grow better in human compared to bovine serum (Table 3.3), since malaria parasites need hypoxanthine as a purine precurser (24). Even after the addition of hypoxanthine, however, the parasitemia in cultures containing human serum was still 4-fold higher than in cultures containing bovine serum (Table 3.3). It is possible that

a further addition of hypoxanthine would have improved parasite growth in bovine serum-supplemented medium, but this was not investigated.

The superiority of the human serum compared to the bovine serum also might be due to the free fatty acid content of the serum. Willet and Canfield reported growth in medium without serum but with added free fatty acids (cis-vaccenic, oleic or linoleic) and adenosine (21). Stearic acid did not sustain parasite growth but was reported to be nearly as effective as plasma in supporting maturation during a 24 hour period (21). According to Divo, cultures may initially perform better on bovine than on human serum which may be explained by the high stearic acid content of the bovine serum (20).

Human serum also varies considerably in its ability to sustain parasite growth but still remains the serum of choice in many laboratories world-wide. However, in view of the difficulties experienced in obtaining naive human serum in endemic regions, it is apparent that studies with alternative sera and serum-free medium, should be actively and systematically pursued.

It is generally stated that continuous malaria cultures require regular replacement of medium and the addition of fresh erythorocytes (12). Zolg *et al*. devised a complicated medium exchange schedule based on predictions of the time taken by synchronised cultures to accumulate toxic levels of lactate (12). However, *in vitro* cultures are normally unsynchronised due to the presence of several different strains, each with its own characteristic development cycle time in the same isolate. The lactate concentrations and pH of media were therefore determined during two cycles (96 h) of a synchronous parasite growth. In addition, the ATP

concentration of erythrocyte populations (infected and non-infected) were also determined as indicator of the energy status and metabolic activity of the culture.

The parasite multiplication index for infected cultures whose media were replaced at 24 hourly intervals (In^+R) was 13 over the first 48 (0-48) hours of culture (Fig 3.6), but only 3.2 and 3.6 over the 24-72 and 48-96 hour periods, respectively. This indicated that less merozoites were produced per schizont and/or that fewer merozoites were able to invade erythrocytes over the latter half of the 96 hour culture period. Impaired growth has been attributed *inter alia* to exposure of cultures to lactate concentrations exceeding 12 mM by Zolg *et al* (12) and Jensen (27). The In^+R cultures and infected cultures whose media had not been replaced (In-R), contained 18 and 25 mM lactate, respectively, 48 h after initiation (Fig. 3.6). Growth in In-R cultures virtually ceased at this high lactate concentration as evidenced by the decreasing parasitemia and glucose consumption at 72 and 96 h of culture. The fact that In^+R cultures could tolerate lactate concentrations as high as 29 mM (Fig. 3.6) for periods of less than 24 h (Fig. 3.6), suggests that the time of exposure as well as concentration are important to parasite survival.

As expected, the pH's of the medium of both In^+R- and In-R cultures were lowered (pH 7.4 to 6.3) in the presence of high lactate concentrations ($\geq$ 25 mM, Fig. 3.6). The lower pH of the medium could have several effects on parasite metabolism and development. A higher H^+-concentration will shift the oxyhemoglobin dissociation curve to the right (decreased oxygen affinity of hemoglobin) and therefore reduce the oxygen content of the erythrocytes. If the lower threshold value of oxygen

is exceeded, the parasites will cease to develop due to their microaerophilic nature (15). In addition, lactate efflux from the erythrocyte is pH dependent and only stimulated if the external pH is higher than the intra-cellular pH (28). Thus, if the pH of the medium is less than that of the erythrocyte (pH 6.8), lactate will accummulate in the intra-erythrocytic compartment and lower its pH (28). As a consequence, activities of enzymes such as phosphofructokinase and hexokinase of the glycolytic pathway would be at least partially inhibited (27) and less ATP will be produced. In view of the importance of ATP for parasite development and maintenance of erythrocyte integrity (see Introduction) it is not surprising that the combination of a low pH and high lactate concentration will slow parasite development and growth. Although a higher concentration of buffer will counteract acidification of the medium, lactate *per se* has been shown to inhibit parasite growth (12). In addition, higher concentrations of HEPES were also shown to be inhibitory (6).

The ATP concentrations of both the infected and non-infected erythrocytes were very low initially but doubled during the first 24 hours of incubation at 37°C (Fig 3.6). This is explained by the fact that the erythrocytes were stored at 4°C for 2 weeks in medium without glucose prior to cultivation which results in a decline in the ATP concentration (29).. The ATP concentration in infected cultures declined over the last three days of culture, which may be due to the toxic effects of high lactate concentrations as discussed above. The low glucose consumption in non-infected cultures during the latter half of the incubation period, indicates a low level of ATP synthesis. The maintenance of a relatively high ATP concentration during this period therefore appears to be due

to a declining metabolic activity. In view of the importance of erythrocytic ATP for parasite development , a likely erythrocyte replenishment schedule is suggested, preferrably every 48 h regardless of the parasitemia.

From the results of experiment A, it is apparent that viable *in vitro* cultures of *P. falciparum* require regular replacement of medium. This is to ensure that lactate concentrations do not exeed 12 mM and a constant pH and high cellular ATP concentrations are maintained. At very high parasitemias this may become impractical and systems in which the medium is continuously replaced, may be more effective and convenient.

In experiment B, the medium of an unsynchronized *in vitro* culture of *P. falciparum*, was changed at increasing frequencies over a 96 h incubation period (two development cycles) in order to confirm that this procedure would yield uninhibited parasite growth. In addition, the efficiencies of glycolysis and the purine salvage pathway in providing ATP to the developing parasites, were probed by determining the concentrations of some of the key intermediates and the relationships between the precursors and products as well as the energy status of the cell.

The multiplication index, as a measure of parasite growth, changed from 3.7 to 5.9 to 4.7 over the 96 h cultivation period (Table 3.5). The amount of glucose consumed, increased with parasitemia which in turn produced higher amounts of lactate (40 mM/24 h at 96 h). Except in one case the lactate concentration per cultivation period never exceeded 12 mM, however, due to the frequency of medium replacements. In spite of the presence of these large amounts of lactate, the effect was minimal since medium pH did not drop below 7 (Fig 3.8). Although it is expected that

this pH may have little or no effect on glycolysis, it will favour the formation of deoxyhemoglobin which may affect parasite survival as discussed before (see above). It is uncertain what effects the small variations in 2,3 DPG concentration (3 to 5 mM) had on parasite survival (Fig 3.8).

A surprising finding is that more lactate was produced in infected cultures than can be accounted for by glycolysis alone (Table 3.4). This appears to be a parasite-specific phenomenon since the initial lactate-glucose ratio for non-infected erythrocytes in the same experiment, was normal. It would indicate that some lactate is derived from sources other than glucose. The lower than expected ratios during the later stages of cultivation in both infected and control cultures, on the other hand, suggest that glucose is also utilized for other purposes and not solely for glycolysis (Table 3.4). It is evident that further studies are required in order to confirm and explain these results.

The ATP concentrations in infected erythrocytes remained relatively constant over the first 48 h of cultivation (Fig. 3.8). The ATP concentration was approximately 30 % lower at 72 h of cultivation and even lower (900 nmol/ml PCV) after 96 h of cultivation. Considering the fact that only 14 % of the erythrocytes were parasitised at 96 h (Table 3.5), the implication is that non-infected erythrocytes also supply ATP to the parasite via hypoxanthine (6). The ability to accomplish this is dependent on their rate of glycolysis. The declining metabolic activity during the last 48 hour of cultivation (Fig 3.6, Fig 3.8, Table 3.4) thus may be at least partially responsible for the decrease in ATP concentration (see

below). Similar decreases in the ATP concentrations of whole erythrocyte populations under *in vivo* conditions, have been reported (6).

Apart from glycolysis, ATP is also produced by a parasite-specific purine salvage pathway: Hypoxanthine->IMP->Adenylosuccinate->AMP-> ADP ->ATP (6). It is apparent from Fig 3.8 that utilization of hypoxanthine increased until 72 h of cultivation, presumably due to conversion to IMP by hypoxanthine phosphoribosyl transferase. A similar increased utilization is noted for IMP but this lasted only until 48 h of cultivation. At 72 h of cultivation the IMP concentration was in fact 12,5 %higher than that at 0 h and coincided with a sudden 30 % drop in the ATP concentration. These observations imply that the reduced ATP concentration is partially due to inhibition of the purine salvage pathway (probably at the conversion of IMP->adenylosuccinate->AMP) which would explain the accumulation of IMP and decrease in adenylate nucleotide concentration. This conclusion is supported by the 27 % and near 50 % decrease in the adenylate nucleotide concentrations after 72 and 96 h of cultivation, respectively (Table 3.4). Further evidence of stress in the cultures is provided by the marked increase in the AMP concentration of infected cultures (probably due to the reaction: 2 ADP->ATP+AMP, catalyzed by adenylate kinase) after 96 h of cultivation.

It is predictable that a limited supply of ATP will act to constrain parasite development and multiplication (6).In addition, non-infected erythrocytes will be less susceptible to invasion by the parasite due to irreversible changes caused to the erythrocyte membrane by ATP depletion (see Introduction). This will reduce the multiplication rate even further (Table 3.5). In spite of the lower ATP concentration, the energy charge as a

measure of energy status of infected cultures, was within normal limits up to 96 h after initiation of cultures (Table 3.4).

Not much is known concerning the relationship between co-factors and malaria parasite growth, although it was found that levels of NAD^+, NADH and $NADP^+$ increased two-fold in erythrocytes infected with *P. lophurae* (6). The NAD^+ as well as the $NADP^+$ concentrations initially increased for both the infected and non-infected cultures (Fig 3.8). Thereafter the concentration of $NADP^+$ decreased over the last three days of culture of infected cultures. The NAD^+ concentration for infected erythrocyte cultures, however, remained constant despite the decline in the concentration of ATP which is needed in its biosynthesis. The $NADP^+$ declined for both the infected and non-infected erythrocytes over the last three days of culture. This could reflect another consequence of the limiting ATP supply since the biosynthesis of $NADP^+$ require an additional ATP molecule in comparison to NAD^+ - biosynthesis.

Malaria parasites have a low capacity to reduce $NADP^+$ due to the presence of a low activity glucose-6-phosphate dehydrogenase (6,22). However, a $NADP^+$-specific glutamic dehydrogenase could also serve in this capacity (6). NADPH is mainly derived from the hexose monoposphate shunt of the erythrocyte and used to reduce oxidised glutathione (GSSG -> GSH). The production of peroxide by the parasite tends to overload the reducing capacity of the erythrocyte. A depletion of reduced glutathione would lead to erythrocyte membrane damage and parasite death.

It is unknown at this stage whether the activity of adenylosuccinate synthetase of the parasite is also regulated by the AMP concentration as

in mammalian tissues (30). Even if this is the case, AMP does not seem to be responsible for the accumulation of the IMP since its accumulation occurs only 24 hours later (Fig 3.8). It is apparent from the results presented here that further studies are required to confirm that the decline in ATP concentration is caused by inhibition of the IMP->AMP step and to establish the nature of the inhibitor(s). Such studies would be greatly simplified by the use of synchronised and enriched cultures as well as isolated, stage-specific parasites to resolve *inter alia*, the respective contributions of hypoxanthine and glucose as well as the parasite-specific adenylate translocator (31) to the ATP pool of the parasite.

As demonstrated in this study, a high parasitemia (≥ 10 %) is accompanied by the production of toxic concentrations of lactate (≥ 12 mM) and a diminished capacity of the culture to supply the ATP necessary for parasite growth. The schedule of medium replacement and erythrocyte replenishment therefore should be continuously adjusted in accordance with the parasitemia for optimal growth in *in vitro* cultures.

The apparent simplicity of working with the erythrocytic stages of the malaria parasite, misled many investigators to believe that parasitized blood is equivalent to normal blood with parasites added (6). Using the *in vitro* technique developed by Trager and Jensen most of the accompanying pathology of the hematological system of the host is removed. However, in this artificial environment the metabolites which are consumed by the growing parasite are only those present in the medium or inside the host erythrocyte.

The metabolism of the parasite is very complex and in the enclosed environment (culture flask) the effect of one metabolite on all the others should be borne in mind. It is anticipated that a better understanding of the nutritional demands and metabolism of the parasite would lead to improvements in the culture conditions and the design of more effective anti-malarial drugs.

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CHAPTER 4

A MICRO-ENZYME-IMMUNOASSAY FOR THE DETERMINATION OF THROMBOCYTE-ASSOCIATED IMMUNOGLOBULINS IN MALARIA PATIENTS

4.1 INTRODUCTION

Platelets (thrombocytes) are involved in the pathology of malaria as a result of their role in the immune response, especially in the inflammation reaction (1). Platelets, if not activated, are round or oval discs of about 2,8 µm in diameter. They originate from megakaryocytes in the red bone marrow and have granules that contain hormones, nucleotides, calcium, synthesized proteins and enzymes (2). The normal concentration of platelets in blood is between 150 000 and 350 000 per cubic millimeter. They possess class1 MHC products and Fc receptors for IgG (Fc RIII) and IgE (Fc RII). Platelets can be sensitized via IgE binding of parasite antigens resulting in enhanced cytotoxicity against those parasites, as was illustrated with *T. gondii* and *T. cruzi* (3). Autoantibodies against platelets are seen in up to 70 % of cases of idiopathic thrombocytopenia. Removal of platelets is effected by splenic macrophages after infections or autoimmune diseases.

The surface of the cell membrane contains glycoproteins which adhere to damaged vessel walls. In addition, the membrane also has large amounts of phospholipids which can activate the "intrinsic" blood clotting system.

If the platelet count suddenly drops, recuperation will take about 5 days if the inhibiting agent is removed. Platelets have a half-life of 4 days and damaged or old platelets are sequestrated in the liver and spleen. Besides their role in hemostasis, platelets protect the body against foreign invaders by immune adherence, removal from circulation and enhancement of phagocytosis.

Severe malaria infection is usually associated with thrombocytopenia, although the mechanisms underlying the reduced thrombocyte survival remain poorly understood (4). Strong evidence exists for an immunoglobulin mediated peripheral destruction, as increased levels of thrombocyte-associated immunoglobulins (TAIg) have been measured in malaria patients (5). Whether immunoglobulin binds to thrombocytes non-specifically by means of adsorption of immunoglobulins and immune complexes, or specifically by anti-thrombocyte auto-antibodies induced by the *Plasmodium* infection, is presently not known. Quantification of the immunoglobulins bound to thrombocytes is a necessary first step towards providing an answer to this question.

Several protocols for the determination of thrombocyte-associated immunoglobulins have been established since 1973 and were classified in three categories by Kelton (6). The so-called "two-stage assay" is best suited for the purpose of determining the specificity of binding of immunoglobulins to thrombocytes as it allows antibody displacement studies by cross-reactive antigens. Dixon *et al*. (7) pioneered the "two-stage assay" in 1975, using complement fixation to quantify the immunoglobulin. Nel and Stevens (8) improved this method in 1980, using serum-coated polystyrene balls in a more sensitive and less complicated

test tube ELISA to determine immunoglobulin concentration. The aim of the present study was to further improve this ELISA method by employing a transferable solid phase system (Nunc-TSP, trade mark) compatible for use with conventional microtitre plates. This modification avoids tedious washing of thrombocytes during the assay. Moreover, the two stages of the assay, viz. labelled antibody incubation with thrombocytes and determination of the remaining immunoglobulin, are combined in one step (Fig. 4.1), with considerable saving in time. The potential application of this assay method was demonstrated in four malaria patients with severe thrombocytopenia. High values of TAIg were demonstrated for these patients while insignificant levels of immunoglobulin were detected on thrombocytes of healthy blood donors or recovered malaria patients.

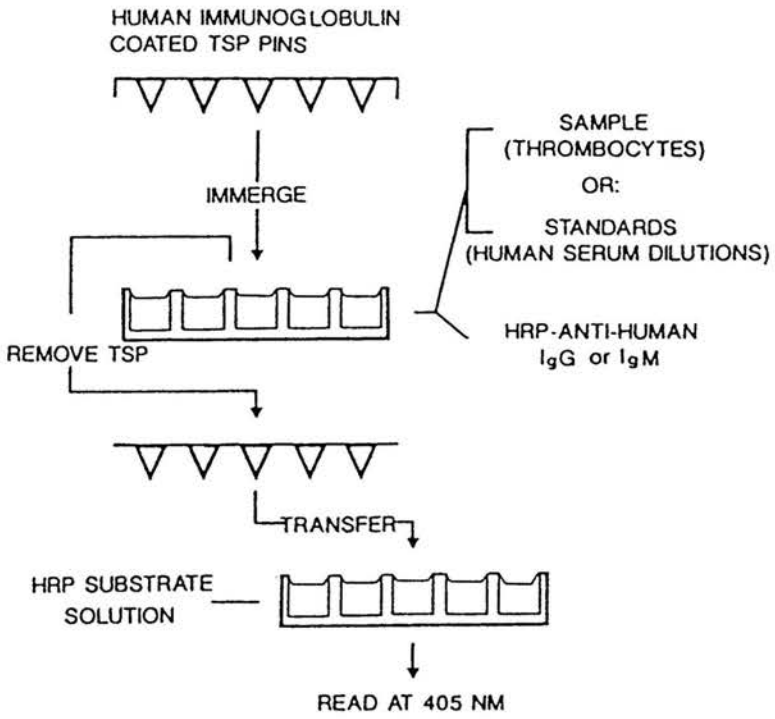

Figure 4.1 Diagrammatic presentation of the protocol for thrombocyte-associated immunoglobulin determination.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of partially purified immunoglobulin.

Venous blood (500 ml) was collected in a blood collection bag without anti-coagulant and left at 4 °C overnight. The serum was separated from the clot by centrifugation at 1000 g on a Beckmann J6 centrifuge. Solid Tris was used to increase the pH of human serum to 8. While stirring, polyethyleneglycol (Fluka) was slowly added up to a concentration of 30 g/100 ml (9). The solution was left at 10 °C for 2 hours before collection of the precipitate by centrifugation (1000 g x 15 min). The precipitate was washed twice, dissolved in 30 ml borate-buffered saline (BBS) and lyophilyzed. The sample was later dissolved (BBS) and purified on a Sephacryl S.300 column in batches of 5 ml. Fractions (2.5 ml) were collected and tested by ELISA for the presence of IgG and IgM as follows: From each fraction 100 µl was dispensed in wells of a microtitre plate. After 2 hours of incubation at room temperature the samples were flicked out and the wells washed and blocked using 0.5 % (w/v) casein in phosphate-buffered saline pH 7.4. Rabbit anti-IgM and -IgG conjugated peroxidase (Cappel, Worthington) at 1/5000 dilutions were used to detect the presence of IgG and IgM in the fractions. Orthophenylene-diamine (1mg/ml) (Sigma, St. Louis, M.O.) and urea-hydrogenperoxide (0.6 mg/ml) in 0.1 citrate buffer, pH 4.5, was used as substrate solution (100 µl/well) and the colour change measured at 405 nm with a Titertek multiscan MC (Flow Labs Inc., Helsinki, Finland). All washing and blocking steps were carried out using 0.5 % casein/PBS.

4.2.2 Comparison between coating buffers and pin coating samples.

Coating of the pins with partially purified immunoglobulin or serum diluted in either 0.05 M glycine buffer (pH 2.7/7.4) containing 0.15 M NaCl or 0.1 M bicarbonate buffer, pH 9.3, were compared. The serum was serially diluted to give IgG and IgM concentrations of between 4 and 90 ug/ml in the coupling buffers. The partially purified immunoglobulin fractions were reconstituted to the same range of concentrations. The coating of the pins and the ELISA were done as described in 4.2.4 using different coating buffers and concentrations of serum or partially purified immunoglobulins.

4.2.3 Thrombocyte preparation.

Blood samples, collected from malaria patients and healthy volunteers with citric acid/ citrate/ dextrose as anti-coagulant, were donated by Dr Louis Marcus (Niehaus & Botha Pathologists, Pretoria). Patients were selected irrespective of the severity of infection or type and stage of treatment. Individuals selected as controls never had malaria and exhibited a normal thrombocyte count. Thrombocytes were isolated by differential centrifugation, washed with filter-sterilized phosphate-buffered saline (PBS), pH 6.5, and finally made up to 10^7 thrombocytes/ml in 0,5 % casein-PBS (pH 7.4). Thrombocytes were counted using an automatic thrombocytometer (Beckman).

4.2.4 Immunoglobulin coated TSP.

Polystyrene TSP pins (Nunc, Roskilde, Denmark) were coated with the purified immunoglobulin at 100 µg/ml in glycine coating buffer (10) by incubation (5 hours, room temperature) of the TSP-pins in a pre-blocked microtitre plate filled with 200 µl/well coating solution. Blocking was done

by incubation of plates or TSP-pins in 0.5 % casein/PBS for at least 3 hours (room temperature).

4.2.5 Determination of thrombocyte-associated IgG and IgM.

All washing of plates or TSP-pins and dilution of biological reagents were performed with 0.5 % casein/PBS. Wells of a pre-blocked microtitre plate were filled with 100 µl of a dilution range of standard human serum (Hoechst-Behring; France) to obtain a standard curve of either IgG or IgM of 0-100 ng/well. The remaining wells received 100 µl of thrombocyte suspension samples from patients and healthy controls at 10^6 thrombocytes per well. Depending on whether IgG or IgM was determined, horse radish peroxidase conjugates of either sheep anti-human IgG (1:3000) or sheep anti-human IgM (1:2000) (Cappel, Worthington) were added at 100 µℓ per well prior to the immersion of the coated TSP-pins into the wells. After incubation for 3 hours at room temperature in a plate shaker, the TSP-pins were removed, washed and transferred to a microtitre plate filled with substrate solution (100 µl/well) consisting of orthophenylene-diamine (1 mg/ml) (Sigma, St. Louis, M.O.) and urea-hydrogenperoxide (0,6 mg/ml) in 0.1 M citrate buffer, pH 4.5. Colour development was measured at 405 nm with a Titertek Multiscan MC.

4.3 RESULTS

4.3.1 Preparation of partially purified immunoglobulin.

Polyethylene glycol (PEG) selectively precipitated the serum proteins depending on their molecular size and concentration. Using 30 % (w/v) PEG, most of the larger serum proteins were precipitated. To evaluate

the fraction collected, a sample was separated on Sephachryl S-300 and eluted fractions screened by ELISA for the presence of IgG and IgM. In Fig. 4.2 typical results are shown.

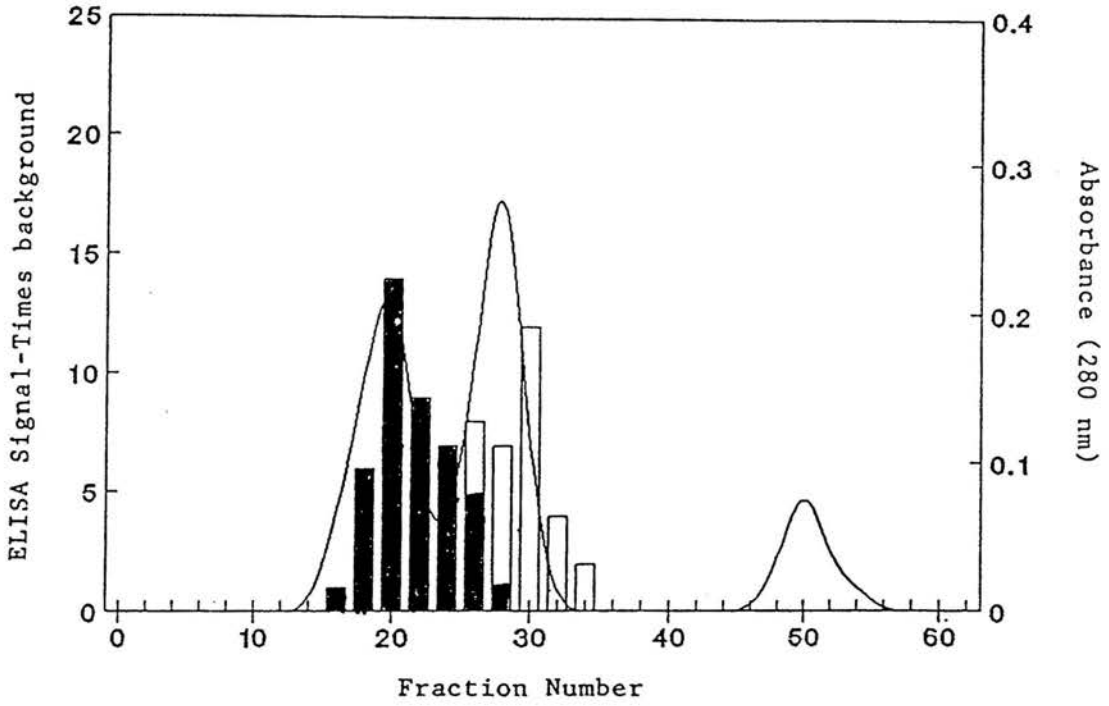

Figure 4.2 Elution pattern of the immunoglobulin fraction isolated from human serum on a 40x1.5 cm Sephacryl S-300 column. Flowrate was 0.5 ml/min and 2.5 ml fractions were collected. The ELISA signal is given as multiple of background measured at 405 nm. Fractions 15 to 30 showed IgM activity and fractions 23 to 35 exhibited IgG activity.

■ IgM

□ IgG

Fractions 15 to 36 were pooled, lyophilyzed and used as the partially purified immunoglobulin fraction.

4.3.2 Coating buffers and immunoglobulin source for coating pins.

Glycine and bicarbonate buffers were compared using serum and a partially purified immunoglobulin fraction as coating samples for TSP pins. The results for IgG and IgM are shown in Figs 4.3 and 4.4, respectively.

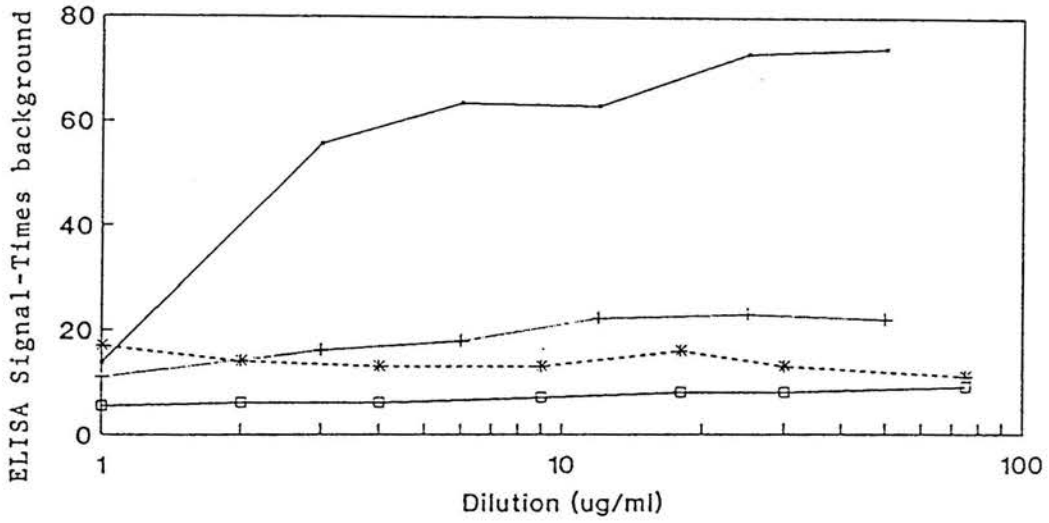

Figure 4.3 Comparison between coupling methods for IgG.
(a) Partially purified IgG/M coating with glycine coating buffer —
(b) Partially purified IgG/M coating with carbonate coating buffer —+
(c) Serum coating with glycine coupling buffer ···*··
(d) Serum coating with carbonate coupling buffer —□—

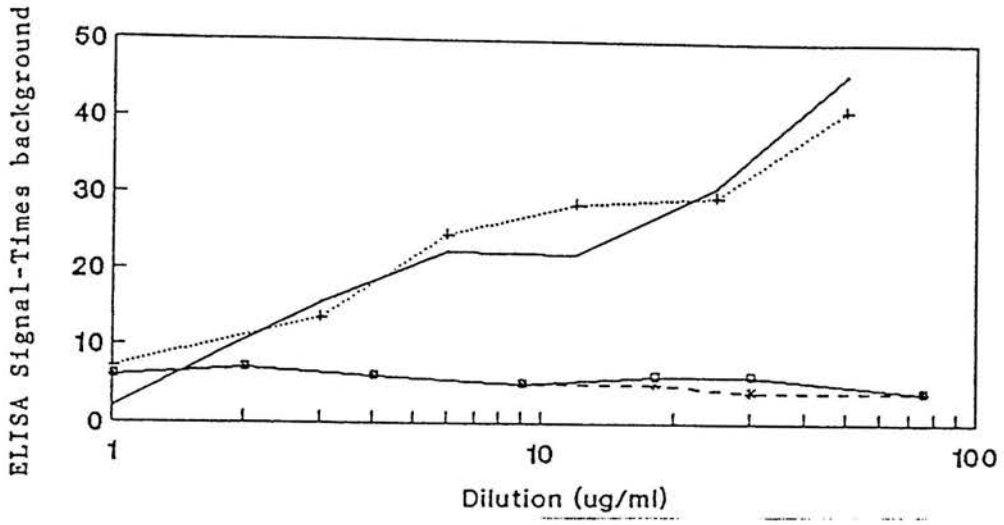

Figure 4.4 Comparison between coupling methods for IgM.
(a) Partially purified IgG/M coating with glycine coating buffer —+—
(b) Partially purified IgG/M coating with carbonate coating buffer —+—
(c) Serum coating with glycine coating buffer ···*···
(d) Serum coating with carbonate coating buffer —□—

From these results it is evident that partially purified IgG coated with glycine buffer generates higher signals than serum, or partially purified IgG coated with the carbonate buffer. Coating the partially purified fraction in either the glycine, or carbonate buffer generated equally good signals for IgM, while unpurified serum did not yield satisfactory results with either buffer system. The glycine buffer system therefore was used as coating buffer and partially purified immunoglobulin fraction as the immunoglobulin source for coating the pins in the assay.

4.3.3 Optimization of the coupling of immunoglobulins onto TSP-pins.

The ELISA signals obtained when using different concentrations of immunoglobulin for coating TSP-pins in an assay where thrombocytes or standard serum was omitted, are represented in Fig. 4.5. IgG produced

approximately a two-fold stronger signal than IgM, but both approached saturation at 200 µg/ml coating concentration.

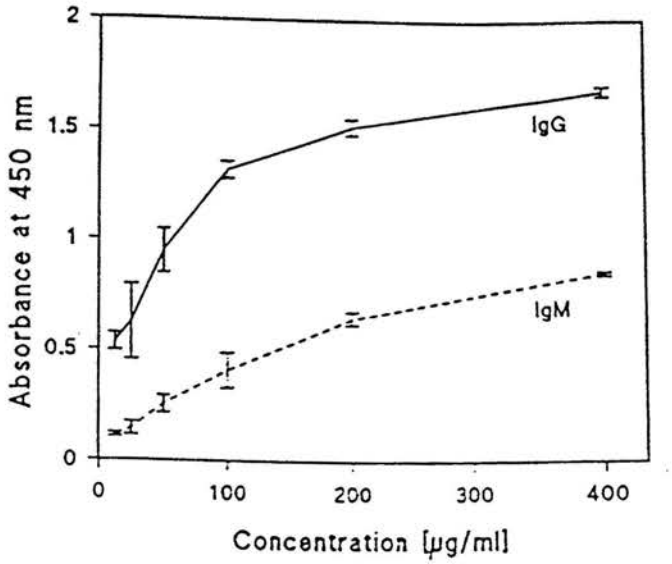

Figure 4.5 Optimization of the coupling of partially purified immunoglobulin onto the TSP-pins.

4.3.4 Standard curves.

A critical parameter determining the sensitivity of the assay, is the dilution of peroxidase-conjugate used as indicator. A dilution-response curve was obtained by titration of the indicator reagent, to determine the first order region. From the results in Fig. 4.6, values of 1:2000 for anti-IgM-and 1:3000 for anti-IgG-peroxidase were selected for the assay, as they occur in the first order region, generating a sufficient ELISA signal.

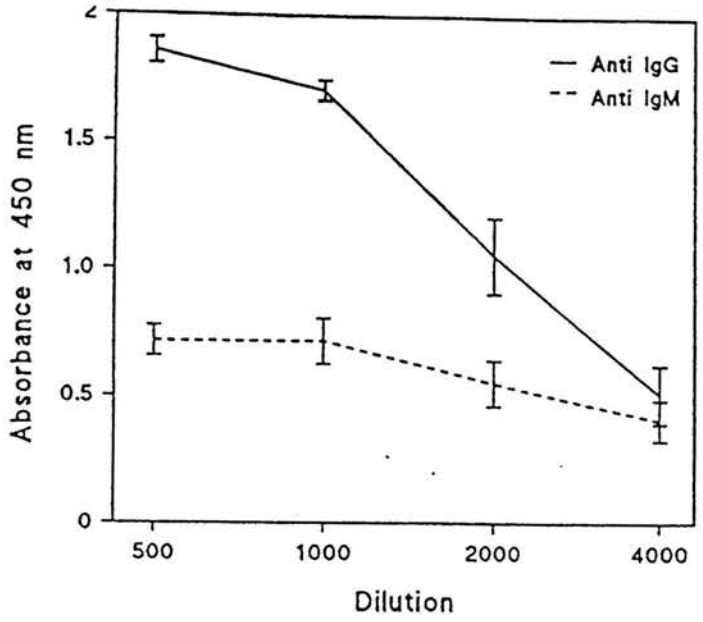

Figure 4.6 Optimization of the dilution of anti-IgG and -IgM-peroxidase.

Using these optimal dilutions of indicator antibody, standard curves for IgG and IgM were obtained by using standard human serum to inhibit binding of the former to the TSP-pins (Fig. 4.7 and 4.8).

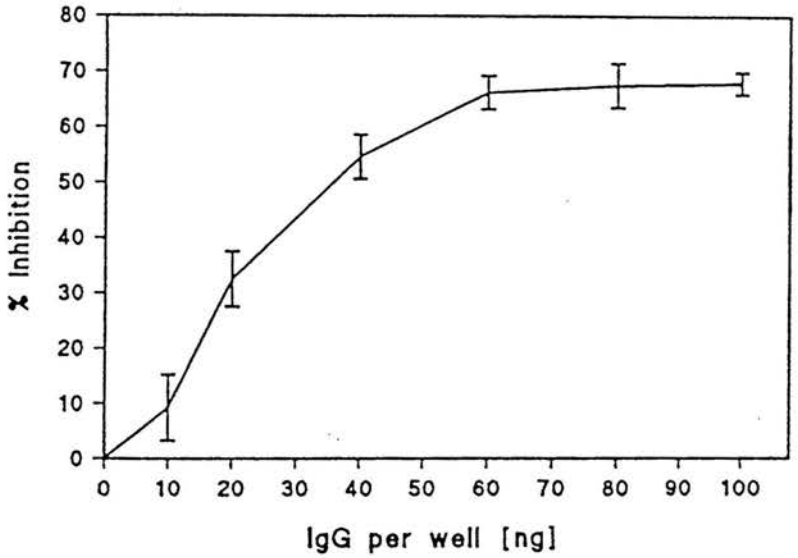

Figure 4.7 Standard curve for the quantification of TAIgG.

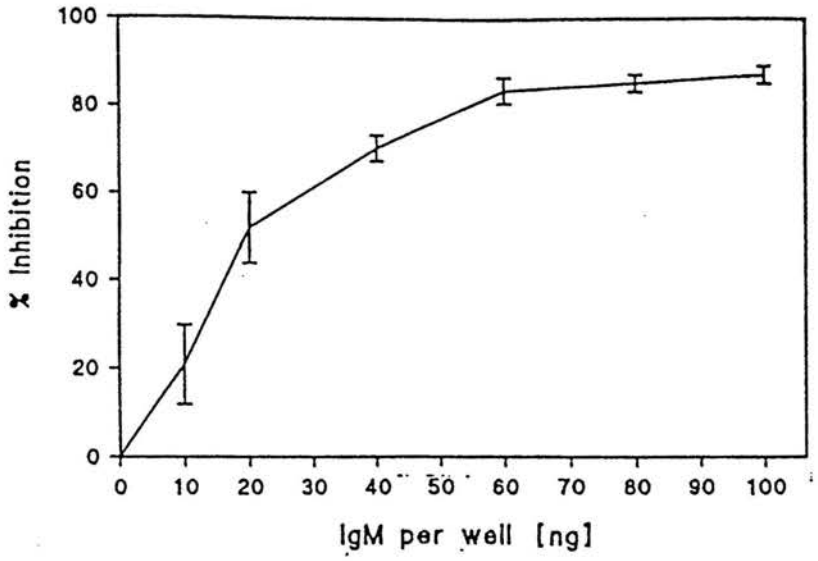

Figure 4.8 Standard curve for the quantification of TAIgM.

A linear response was obtained in the range of 0-20 ng per well for both IgM and IgG, rendering the assay useful for the determination of 5-50 ng of IgG or IgM.

4.3.5 Measurement of thrombocyte-associated IgG and IgM.

Results for 15 measurements of thrombocyte-associated immunoglobulins G and M, including four healthy controls, are summarized in Table 4.1.

Table 4.1. ELISA determination of thrombocyte-associated IgM (TAIgM) and IgG (TAIgG) in human blood.

Source of blood samples†	Thrombocyte count ($\times 10^9$/l)	IgM* ELISA signal inhibition (%)	IgM* TAIgM (ng/10^6 thrombocytes)	IgG* ELISA signal inhibition (%)	IgG* TAIgG (ng/10^6 thrombocytes)
Patient N	61	54	21 ± 6	16	12 ± 3
Patient G					
Day 0	54	57	26 ± 4	71	>60
Day 1	48	89	>60	75	>60
Day 2	53	88	>60	73	>60
Patient H					
Day 0	72	75	48 ± 5	26	17 ± 2
Day 1	89	10	5 ± 4	15	12 ± 4
Day 2	113	2	2 ± 4	15	12 ± 2
Patient F					
Day 0	91	80	55 ± 3	66	59 ± 2
Day 1	108	32	14 ± 4	61	52 ± 2
Day 2	142	1	1 ± 2	63	55 ± 4
Day 3	211	0	0 ± 1	20	15 ± 4
Healthy individuals ($n = 4$)	233 ± 30	(–)5 ± 6	0	(–)9 ± 1	0

*Values represent the mean of 3 measurements.
†Days since start of anti-malaria chemotherapy are indicated.

In three malaria patients, blood samples were obtained for up to three successive days after malaria treatment commenced, which enabled us to relate recovery of the patient with platelet counts and the level of thrombocyte-associated IgG and IgM. A severe decreased platelet count was registered among all four malaria patients. An inverse correlation between thrombocyte count and thrombocyte-associated immunoglobulins was observed in respect to both IgM and IgG.

4.4 DISCUSSION

In preliminary studies where human γ-globulin was bound to the bottom of ELISA plates instead of onto TSP-pins, high background values were obtained, which were not encountered by the TSP approach. While the approach of Nel and Stevens (8) required extensive washing of tubes and

γ-globulin-coated balls during the assay to prevent the background problem, the unique geometry of the TSP system apparently escaped the effect of sedimenting immune complexes which non-specifically adsorb to bed surfaces. Fragmentation of the IgM fraction was observed in the elution pattern from the isolated human serum (Fig.4.2). This may have been due to the freezing and lyophilyzation of the PEG precipitate. However this will have no effect on the assay as only the heavy chain is recognized by the anti-IgM peroxidase.

The inverse correlation between thrombocyte count and thrombocyte-associated immunoglobulin IgM was previously described in respect of thrombocyte-associated IgG in mice with experimental malaria (11). The remarkably rapid recovery of thrombocyte count with concomitant loss of platelet-associated immunoglobulin argues against an active auto-immune mechanism in the complication of thrombocytopenia in cases of malaria. It would seem unlikely that auto-antibody secretion by active plasma cells could so suddenly be switched off after removal of the parasite antigens. A more tangible explanation of the results would be that the clearance of parasite antigen by chemotherapy reduces the immune complex load in the circulation and accordingly lifts the burden on the platelets, which normally are able to carry immune complexes via so-called complement receptors on their outer surface (12). No immunoglobulins could, however, be detected on thrombocytes of healthy individuals or a recently recovered malaria patient, e.g. "F" in Table 4.1. The results indicate that the detectability of the method developed suffices for determining levels of TAIg typical of malarial thrombocytopenia. By using partially purified immunoglobulins a more sensitive method to that of Nel and Stevens is obtained (8). It requires relatively small volumes of blood

samples, comparable to the amounts required in the platelet-lysis method for TAIg determination developed by Hymes *et al*. in 1979 (13). The platelet-lysis approach, however, measures the total immunoglobulin found both inside and on the outer surface of the thrombocytes, which prevents its application to antibody displacement studies required for the specificity of TAIg.

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CHAPTER 5

ULTRASTRUCTURAL STUDIES OF ERYTHROCYTES INFECTED WITH Plasmodium falciparum.

5.1 INTRODUCTION

The erythrocyte membrane consists of a bilayer of phospholipids into which integral proteins are embedded. Peripheral proteins are bound by electrostatic interactions to the polar heads of phospholipids and/or to integral proteins. Most of the peripheral proteins are organized in a cytoskeletal network which underlies the cytoplasmic side of the membrane (1). Invasion of erythrocytes by malaria parasites results in modifications of the morphology and/or function of the host erythrocyte membrane including the lipid composition and fluidity (2). The concentration of fatty acids such as oleic and cis-vaccenic acids are increased whereas the concentration of arachidonic acid and cholesterol are decreased. The consequent increase in membrane fluidity affects the cytoskeleton-phospholipid interactions, leading to altered transport, enzymatic and osmotic properties of the host erythrocyte (2).

The invasion of the erythrocyte by a merozoite involves a series of complex interactions including recognition, attachment and membrane invagination (3). The invasive merozoite attaches to the erythrocyte by its apical end at which there is a pair of organelles called the rhoptries. Endocytosis is apparently induced by the secretion of a substance from the rhoptries that causes the erythrocyte to invaginate (4). After

internalisation, the parasite is surrounded by a parasitophorous vacuolar membrane (PVM) which is closely apposed to the parasite plasma membrane (3). Part of the PVM may originate from the erythrocyte membrane as evidenced by the reversal of the polarities of ATPase and NAD^+-oxidase in the PVM in comparison to the erythrocyte membrane (2).

5.1.1 Parasite-induced changes to the erythrocyte membrane.

P. falciparum and *P. ovale*-infected erythrocytes exhibit protrusions or knobs on the surface of the erythrocyte membrane (6). The knobs are electron-dense, conically shaped and measure 90-100nm in diameter and 30-40nm in height. Although there is much controversy surrounding this point, it is commonly believed that the knobs are proteins of parasitic origin which increase in number as the intracellular parasites matures (7). It has also been observed that the distribution of knobs on the surface of the infected cell is not random, implying that they are inserted into specific domains of the erythrocyte membrane. Not all strains of *P. falciparum* produce knobs on the erythrocyte surface and some strains have been shown to lose this ability after lengthy periods of *in vitro* culturing (8).

5.1.2 Intra-erythrocytic cytoplasmic organelles.

The presence of numerous membrane-bounded organelles in the cytoplasm of infected erythrocytes suggests a mechanism for the exchange of material between the parasite and erythrocyte membranes. Aikawa *et al.* (9) identified the following structures in the cytoplasm of infected erythrocytes by transmission electron microscopy (TEM).

(i) Maurer's cleft. These structures are exceedingly diverse in shape but all consist of a unit membrane-bounded vesicle containing lumen of a low density. The vesicle is often elongated but it can also appear as a horse shoe or a circle presumably, representing, different cross sections of the same organelle. Maurer's clefts occur in both knob and knobless clones of *P. falciparum* parasites (7).

(ii) Maurer's cleft with electron-dense material. Maurer's clefts with electron-dense material on the cytoplasmic face of the unit membrane are only seen in *P. falciparum*-infected erythrocytes (early to late-throphozoite stage) that exhibit knobs. The electron-dense material on these clefts is roughly 100 nm in diameter and is identical in shape, size and electron density as the material seen under knobs (7).

(iii) Golgi-like membrane stack. Membrane stacks of between 5 and 15 elongated vesicles, each demarcated by a single unit membrane, were observed inside the cytoplasm of knob-producing, *P. falciparum*-infected erythrocytes. Each vesicle is aligned roughly parallel to adjacent members on each side of the stack. The lumen of the vesicle within the membrane stack is of low density, i.e. identical to Maurer's clefts. It is unknown if these stacks perform similar functions as the Golgi apparatus found in other cells (7,9). The different structures of cytoplasmic organelles are illustrated in Fig. 5.1.

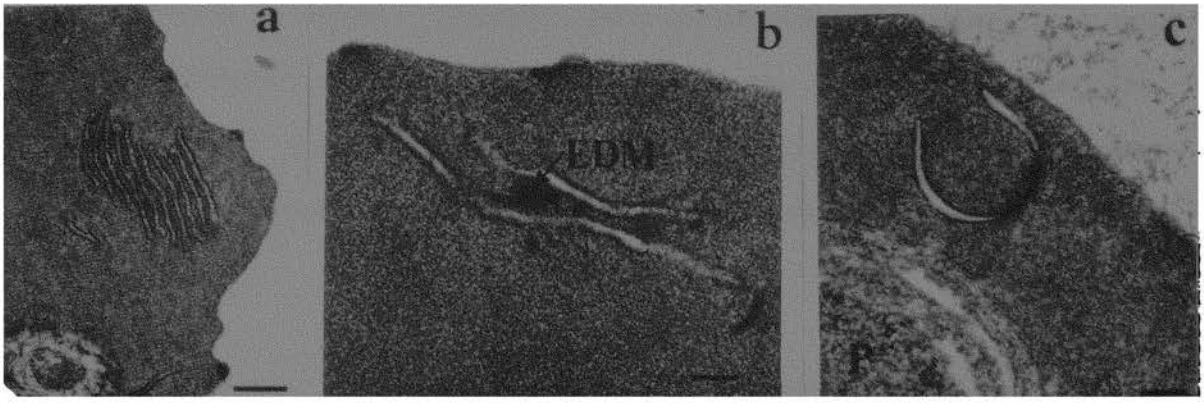

Figure 5.1 Transmission electron micrographs of organelles seen in the cytoplasm of infected erythrocytes. (a) Golgi membrane-like stack. Bar = 0.25 µm. (b) Maurer's clefts with electron dense matarial. Bar = 0.5 µm. (c) Maurer's cleft. Bar = 0.25 µm. Reproduced from (7).

5.1.3 Fixation of tissues for electron microscopy.

The high resolution obtainable with the electron microscope, necessitates that great care be taken with fixation procedures in order to preserve ultrastructural detail. Ideally, tissues used for microscopy should be preserved with chemicals that stabilize cellular structures as near as possible to the *in vivo* situation. However, this is only achievable within certain limits due to the high water content of biological materials (10). Fixation with buffered glutaraldehyde solution followed by buffered osmium tetraoxide (OsO_4), is a standard procedure used for fixation of biological materials for electron microscopical studies (11).

Neither osmium tetroxide nor glutaraldehyde alone is suitable as a general fixative (11). Fixation with glutaraldehyde which crosslinks the amino

groups of proteins, seems to be superior to OsO_4 for the preservation of membrane proteins (10). By using OsO_4 alone more than 40% of the membrane protein content can be extracted compared to 10% after glutaraldehyde fixation. Fixation with glutaraldehyde alone does not prevent extraction of lipids by organic solvents during pre-embedding procedures (10). Loss of cholesterol and phosholipid from the erythrocyte membrane is prevented by the inclusion of a secondary fixation with OsO_4,

Glutaraldehyde may react with amino groups in different ways depending on its concentration and that of the protein in the tissue as well as the temperature and pH during fixation (10). The temperature and pH of the fixation solution may influence the penetration and crosslinking rate of glutaraldehyde. The fixative should have the same tonicity as the cell to prevent water depletion or engorgement and ultrastructural changes (13). Coetzee *et al.* (11) found that the best results were obtained with 2-3 % (v/v) glutaraldehyde in phosphate buffer (pH 7.4). Penman *et al.* (12) have shown that the discoid shape of erythrocytes is better preserved when low concentrations of glutaraldehyde are stepwise added to suspensions of guinea pig erythrocytes with a hematocrit higher than 20 % (v/v).

5.1.4 Ultrastructural features of erythrocyte-stage parasites.

i) Merozoites. They are oval-shaped, 1,5 µm in length and 1 µm in diameter. Common organelles include a nucleus, rhoptries and micronemes which are bound by a pellicular complex consisting of an outer and two inner membranes. The outer membrane is about 7.5 nm thick in comparison to

the 15 nm of the fenestrated inner membranes. The outer membrane is covered with a proteinaceous surface coat with a thickness of approximately 20 nm. Although the function of the coat is not yet fully understood, it appears to play a role in the immune evasion strategy of the extracellular merozoite (13). The apical end of the merozoite is cone-shaped and displays two electron dense rhoptries, each forming a duct which extends to the tip of the apical end (15).

ii) Uninucleate trophozoites. Upon invasion of erythrocytes, the surface coat is shed and merozoites changes from oval to round and finally to irregular shaped parasites. The latter phenomenon is apparently due to the rapid degradation of the inner membranes and the microtubules of the pellicular complex (15). Once these structures are removed the parasite is only surrounded by a single plasma membrane and a PVM which at least partially originated from the host erythrocyte (15). Some trophozoites show prominent ameboid activity producing extensions and invaginations of the parasite cytoplasm. Ring forms seen in the early development stages of the trophozoite appear to result from extensions of the parasite cytoplasm.

Aikawa *et al.* (15) have shown that the host cytoplasm is invaginated through a circular structure in the pellicle which they named a cytosome. After invagination of cytoplasm through the cytosome and digestion in food vacuoles, hemozoin or pigment derives from digested hemoglobin. When sections of mammalian malaria parasites are stained with a high pH solution such as lead citrate, the pigment particles are dissolved leaving a clear area where they were previously located (16).

Although it was previously believed that *P. falciparum* did not contain mitochondria, Fry and Beesley (17) recently isolated these organelles from throphozoite stages of the parasite. Ribosomes are abundant in the throphozoites and are mostly in the free form. The nucleus contains a high percentage of euchromatin suggesting that the nucleus is undergoing preparation for nuclear division (13).

iii) Schizonts. They are defined as parasites containing more than one nucleus and are larger than merozoites and uninucleate trophozoites. During nuclear division considerable morphological changes are observed, starting with the formation of spindle fibers in the nucleus. Mitochondria increase in size and become irregular in shape, due to the formation of several buds before finally undergoing fission to yield many mitochondria. The cytoplasmic organelles surrounded by an outer and two inner membranes are formed next. The area covered by the inner membranes protrude outwards into the parasitophorous vacuole space to form new merozoites. As the merozoites grow and develop, the original schizont decreases in size until only a small residual body containing malaria pigment particles, finally remains (13,17)

5.1.5 Objectives.

The objectives of the study were firstly to identify by light microscopy the species of *Plasmodium* to which isolate PfUP1 belongs. The second objective was to compare the results obtained with two different fixation procedures with each other and to those in the literature in order to select the most suitable procedure for electron microscopy.

5.2 MATERIALS AND METHODS

5.2.1 *Parasite collection and in vitro culturing.*

Malaria-infected erythrocytes were collected from a black resident of Giyani, North-Eastern Transvaal by venipuncture into acid citrate dextrose collection tubes (Medical Home and Nursing Supplies, Pretoria). An *in vitro* continuous culture was initiated from cryopreserved blood as described in Chapter 3 (isolate PfUP1).

5.2.2 *Preparation of blood smears for light microscopy.*

Thin smears from *in vitro* cultures of PfUP1 on microscope slides were fixed and stained with Giemsa as described in Chapter 2. The blood smears were examined with a Nikon light microscope and photographed using Kodak Ectachrome 400 colour film.

5.2.3 *Slow fixation of infected erythrocytes.*

Erythrocytes were collected by centrifugation of an unsynchronized culture of PfUP1 at 1000 g for 5 minutes. The erythrocyte pellet was washed twice with RPMI 1640 without serum and diluted afterwards to 20 % hematocrit using the same medium. The erythrocyte suspension (2 ml) was cooled to 4°C and 10µℓ of a 2,7 % (v/v) glutaraldehyde solution (Poloron, Bio-rad) in 0,07M sodium phosphate buffer, pH 7,4 was slowly added to the erythrocyte suspension and mixed by gentle agitation. The addition of glutaraldehyde was repeated every hour for a total of ten hours. The final concentration of glutaraldehyde was 0,13 % (v/v). The cell suspension was incubated for a further 2 hours at 4 °C before the cells were processed for scanning and transmission electron microscopy.

5.2.4 Fast fixation of infected erythrocytes.

Erythrocytes were obtained from an unsynchronized culture and washed as above. The pellet was resuspended in RPMI 1640 without serum to give a 50 % hematocrit. The erythrocytes were fixed at room temperature by adding 1ml of a 2,5 % (v/v) glutaraldehyde solution (0,07M sodium phosphate buffer, pH 7.4) to 4 ml erythrocyte suspension. Fixation was carried out for 2 hours before the fixed erythrocytes were processed for scanning and transmission electron microscopy.

5.2.5 Processing of glutaraldehyde- fixed tissues for scanning electron microscopy.

Fixed erythrocyte pellets (400 µl) were suspended in 2 ml 0,07M sodium phosphate buffer, pH 7.4, and collected by filtration on a Whatman GF/A filter. After filtration the filters were placed in 1 % (v/v) OsO_4 for 1h. Dehydration was done by placing the filter with fixed erythrocytes for 10 minutes each in 30 %, 50 %, 70 %, 100 % (v/v) and absolute acetone. The samples were critically dried using liquid carbon dioxide and sputtered with gold. Non-infected cells were treated similarly to infected cells and used as controls. Fixed preparations were inspected with a Jeol JSM 840 scanning electron microscope operating at 12 kV and photographs were taken on ASA 125, Ilford FP4 film.

5.2.6 Processing of glutaraldehyde-fixed, infected tissues for transmission electron microscopy.

Fixed erythrocytes were suspended in 10 % (w/v) bovine serum albumin in 0,07M sodium phosphate buffer, pH 7.4 and placed in a 1,8 ml plastic Eppendorf tube. The erythrocytes were centrifuged at 8000 x g for 1 min and the excess solution, except for a 2 mm layer of albumin-buffer

on top of the erythrocytes, was aspirated. The tube was then slowly filled with 2.5 % (v/v) glutaraldehyde (1 ml) in 0.07M phosphate buffer, pH 7.4, without disturbing the erythrocytes. After incubation for 12 hours at 20 °C the albumin-imbedded erythrocyte pellet was removed from the Eppendorf tube and cut into approximately one mm blocks. After post-fixation in 1 % (v/v) OsO_4 for 1 hour at 20 ° C, the blocks were dehydrated by sequential treatment with 40 %, 60 %, 80 %, 90 % and 100 % acetone (5 min/step). Final embedding was in Quetol, which was prepared as follows:

Table 5.1 Quetol embedding resin. The first four components were mixed thoroughly before S1 was added.

Reagent	Mass
1. Quetol	3,90 g
2. Nadic Methyl Anhydrate (NMA)	4,46 g
3. Dodecyl Succinic Anhydrate (DDSA)	1,66 g
4. Araldite Cy212 resin (RD2)	0,20 g
5. Di-ethyl amino ethanol (S1)	0,10 g

Bovine serum albumin-embedded erythrocytes were incubated for 12 hours in Quetol embedding medium at 20 °C to allow infiltration of the tissue. The resin was polymerized in an oven at 60 °C for 48 hours before being cut into 70-100 nm sections on a Reicher Jung Ultracut E microtome, equipped with a diamond knife. The thin sections were mounted on G200 copper grids and contrasted with uranyl acetate (4%) and Reynolds lead citrate (18). Thin sections were inspected in a Philips EM301 transmission electron microscope operated at 60kV.

5.3.1 *Light microscopy of PfUP1-infected erythrocytes.*

The four species of malaria parasites which infects man differ with respect to their physiology and pathology but are best characterized on the basis of their morphology.

Three of the four blood stages of isolate PfUP1 are shown in Fig. 5.2. Typical *P. falciparum* parasites i.e. small rings, consisting of two red chromatin masses and a thin blue-coloured cytoplasm are present. The trophozoites are round and infected erythrocytes are the same colour and size as the non-infected erythrocytes. The schizonts rosett around masses of haemozoin pigment and consists of 18-20 merozoites each.

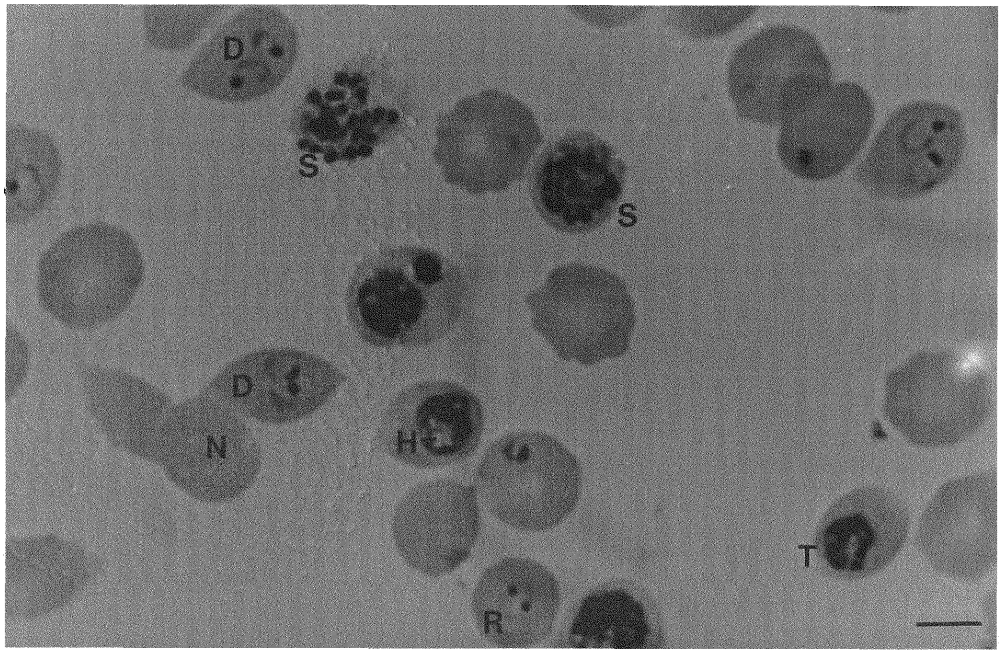

Figure 5.2 Light microscope photograph of the blood stages of isolate PfUP1. A thin blood smear was made from a continuous culture and stained with Giemsa. Blood smears were viewed under a Nikon light microscope using the 100 magnified oil immersion lens. D-double infection, H-haemazoin-pigment granules, N-normal erythrocyte, R-rings, S-schizont, T-trophozoite. (Bar = 7.5 µm.)

5.3.2 Scanning electron microscopy of PFUP1-infected erythrocytes.

Non-infected erythrocytes controls fixed by the slow method, are shown in Figure 5.3.. In the non-infected culture 95 % of the erythrocytes observed were discocytes.

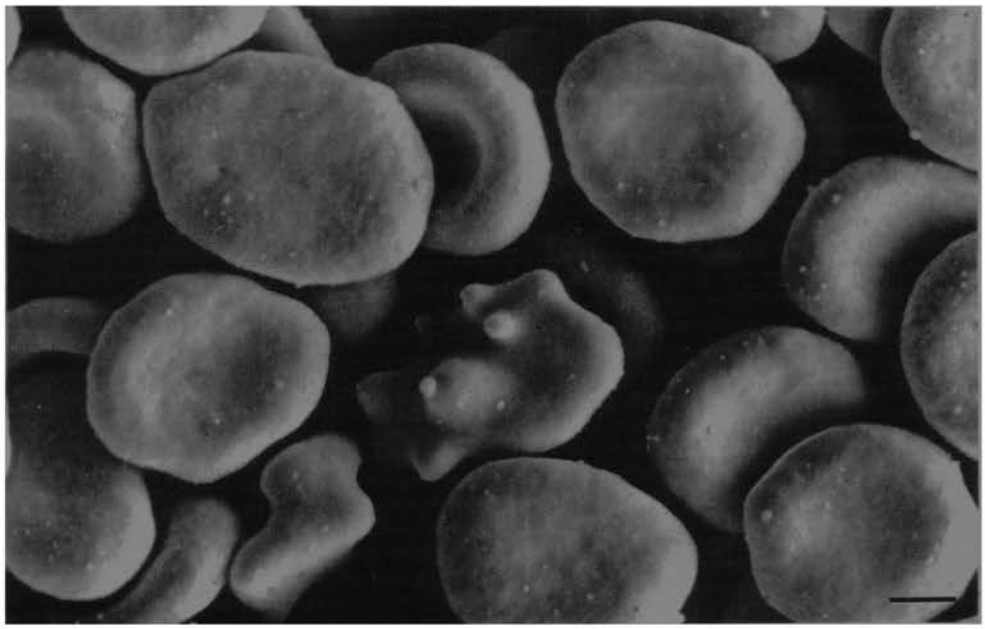

Figure 5.3 Scanning electron micrograph of non-infected erythrocytes after four days in continuous culture. The blood sample was fixed using the slow glutaraldehyde method (See text) (Bar = 2.8 µm.)

Scanning electron micrographs of PfUP1-infected erythrocytes fixed with the slow method, are shown in Fig. 5.4 (a) and (b).

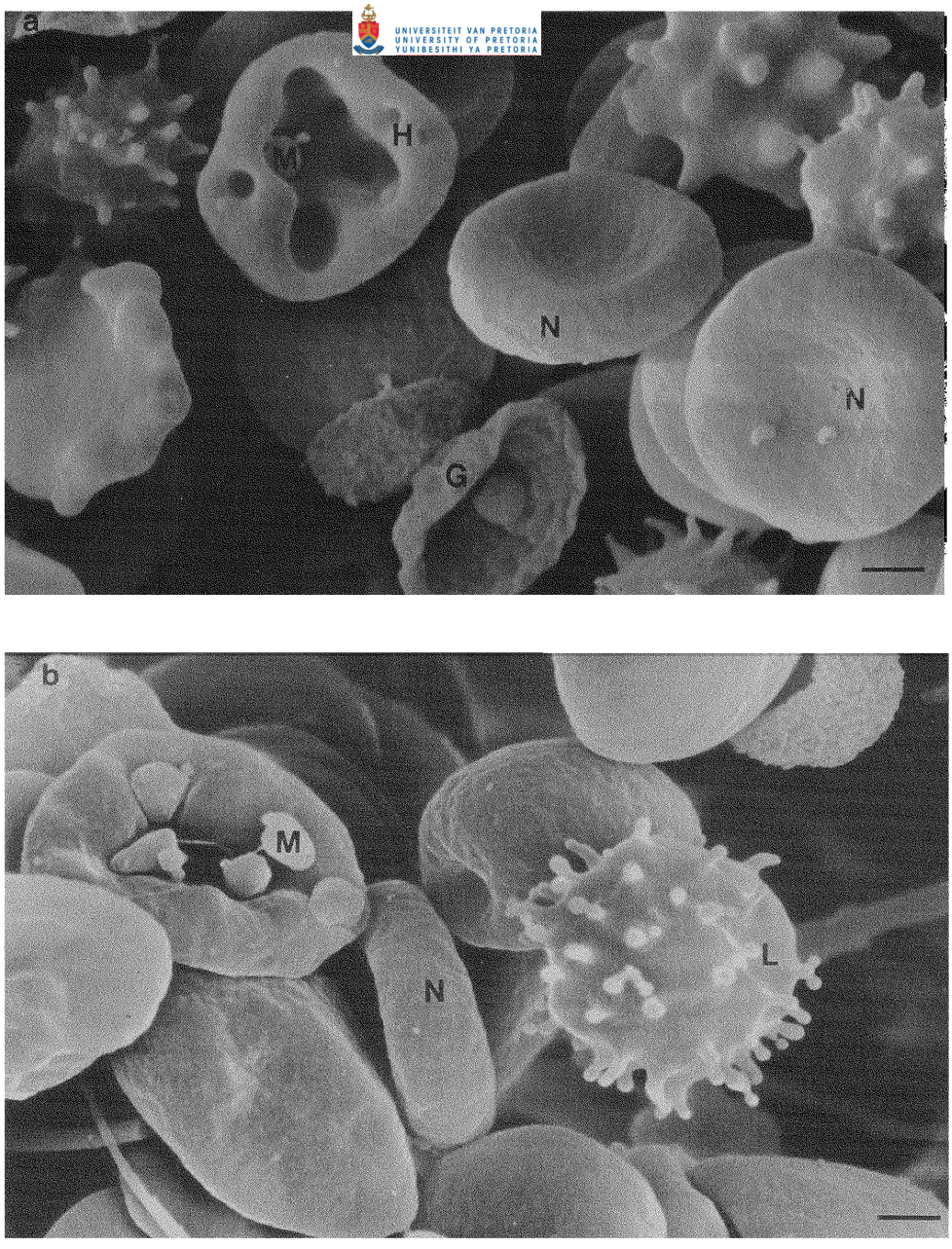

Figure 5.4 Scanning electron micrograph of PfUP1-infected erythrocytes fixed with the slow glutaraldehyde method. H-holes, M-merozoite, N-normal erythrocyte, L-lymphocyte, G-erythrocyte ghost. (a) Bar = 2.5 µm. (b) Bar = 2.0 µm.

The erythrocyte in Fig. 5.4 (a) is distorted and has invaginations as well as a single merozoite which is attached to the surface. A discocyte and echinocyte are also present in the same frame. In Fig. 5.4 five

merozoites are attached to a distorted erythrocyte. A lymphocyte is also present in the same micrograph.

Scanning electron micrographs of PfUP1-infected erythrocytes after fixation with the fast method are shown in Fig. 5.5.

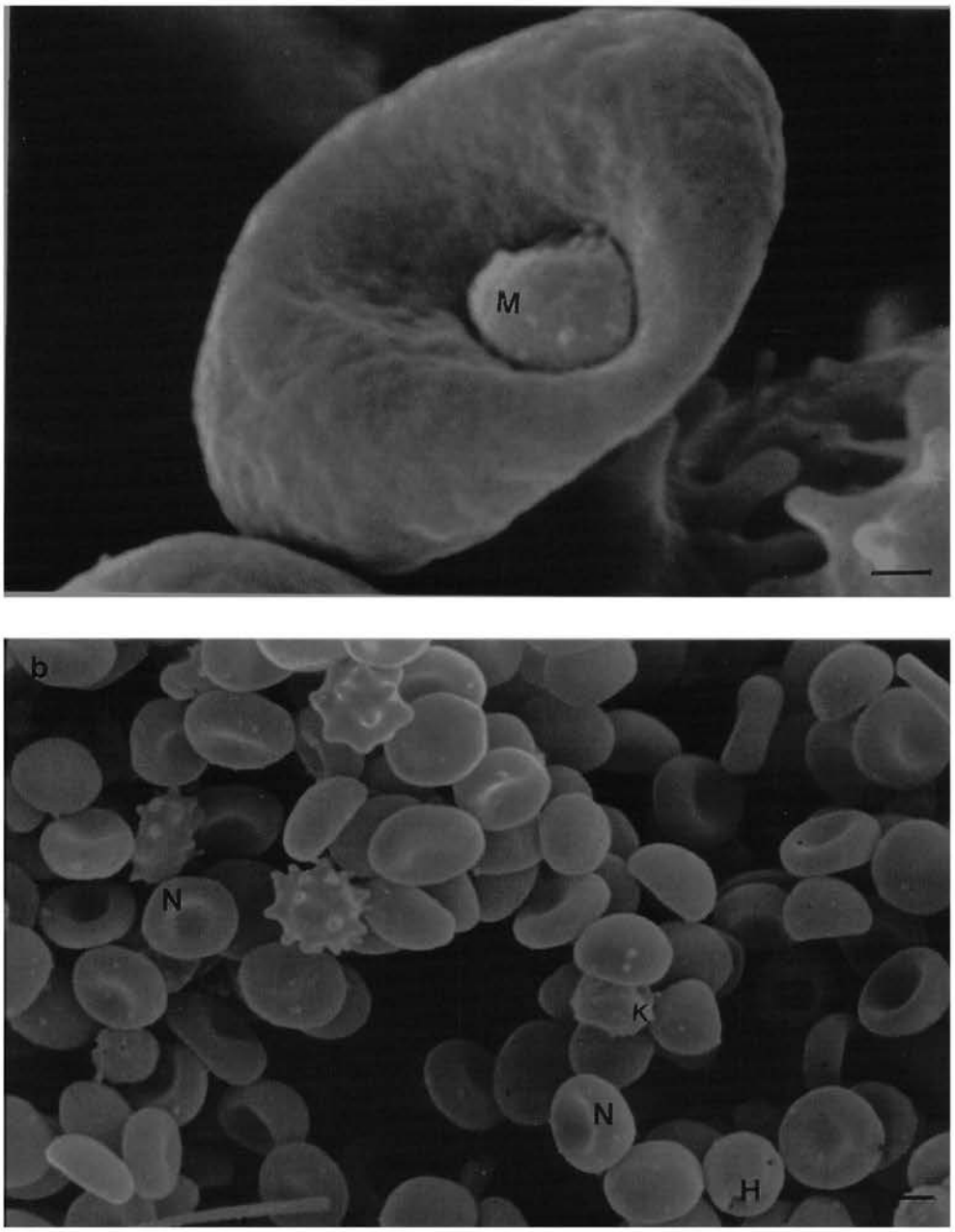

Figure 5.5 Scanning electron micrograph of PfUP1-infected erythrocytes fixed by the fast method (see text for details). (a) Shows a merozoite attached to an erythrocyte. (b) Infected erythrocytes shown at a lower magnification. N-normal erythrocyte, M-merozoite, H-holes, K-knobs. (a) Bar = 0.5 µm. (b) Bar = 10 µm.

In (a) a merozoite is shown attached to an erythrocyte. It is evident that most of the erythrocytes in (b) are discocytes when viewed at a low magnification. Erythrocytes with knobs (K) and another with holes (H) can also be discerned. Numerous holes with variable sizes from around 50 to 300 nm in diameter were observed during this study. The control fixed with the fast method (not shown) was identical to the control fixed with the slow method (Fig. 5.3), ie. no holes were observed.

Knobs were observed in PfUP1-infected erythrocytes which were fixed with either the slow or fast methods. Knobsizes were randomly determined from infected erythrocytes which were fixed with the slow method (Fig. 5.6). The mean value was 165.4 $\pm$ 16 nm (n = 15).

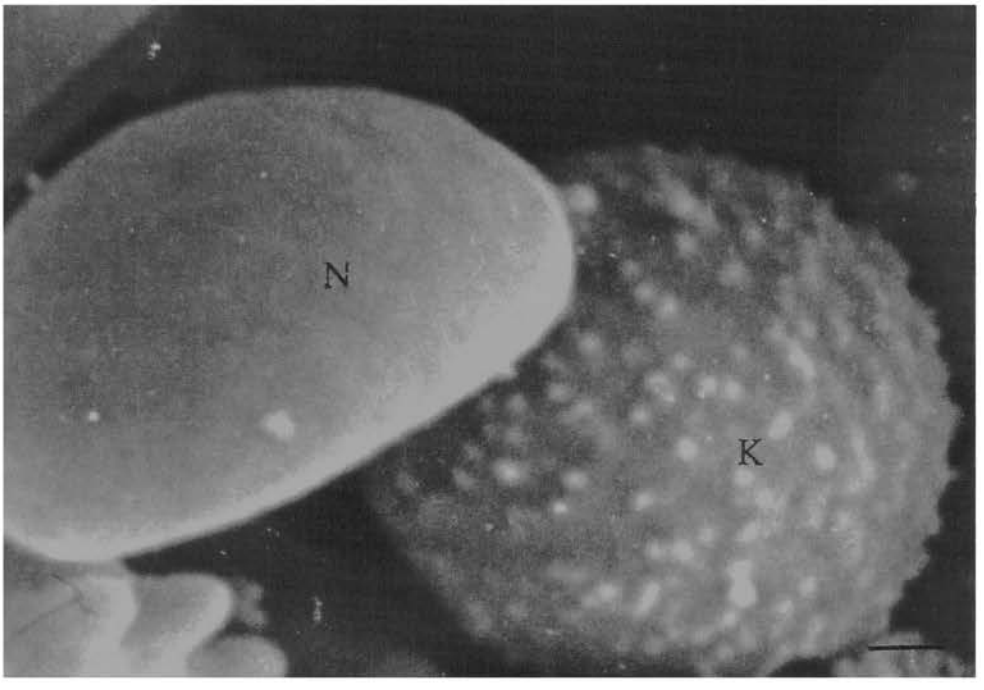

Figure 5.6 Scanning electron microscope photographs of PfUP1-infected erythrocyte with knobs. The blood sample was fixed by the slow method and processed as described in the text. N-normal erythrocyte, K-knob presenting erythrocyte. (Bar = 0.5 µm.)

5.3.3 *Transmission electron microscopy of PfUP1 infected erythrocytes.*

Transmission electron micrographs of erythrocytes infected with PfUP1 are shown in Fig. 5.7

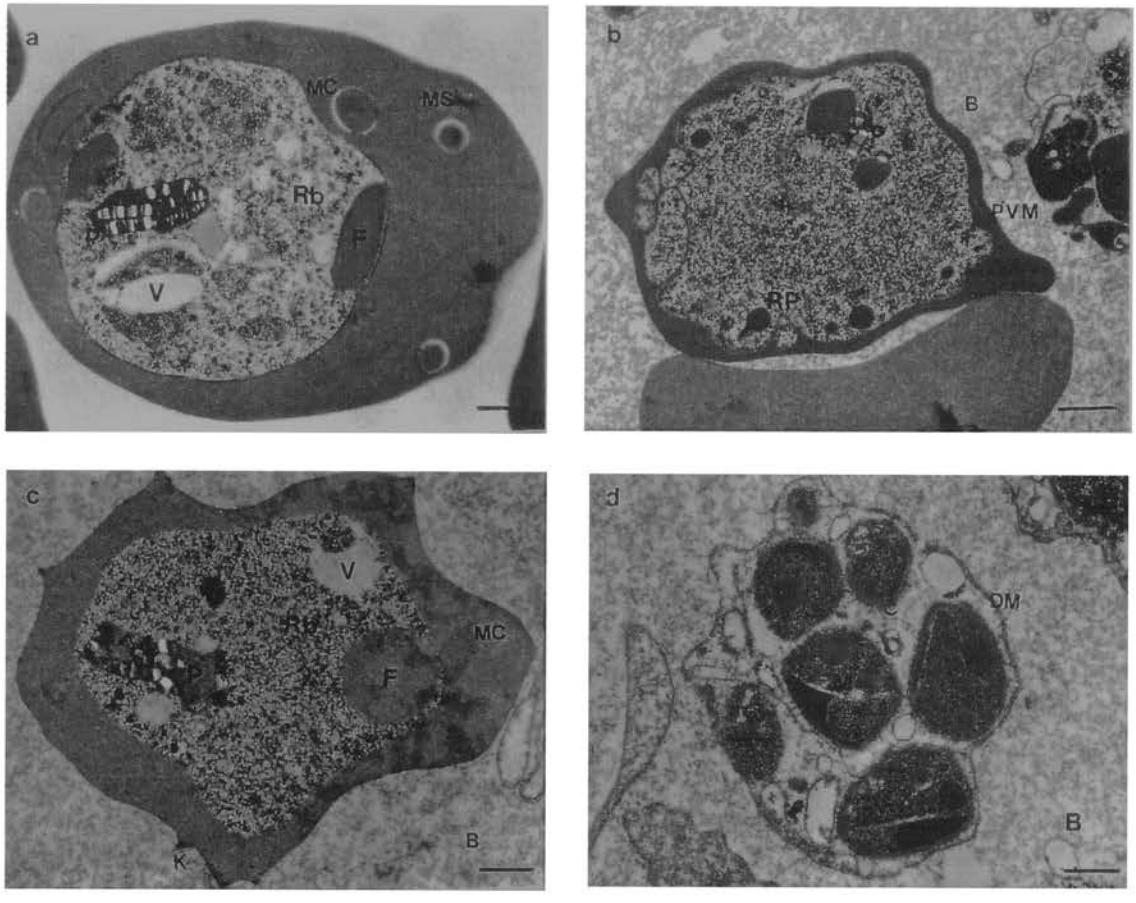

Figure 5.7 Transmission electron micrographs of PfUP1-infected erythrocytes. (a) fixed using the slow glutaraldehyde method; (b),(c) and (d) were fast fixed using 1 % glutaraldehyde solution. (b), (c) and (d) were imbedded using bovine serum albumin as supporting medium. B-background-glutaraldehyde fixed albumin, C-merozoite coat DM-double membrane, F-food vacuole, P-pigment granules, K-knobs, MC-Maurer's cleft, MS-membrane stacks, R-Rhoptry in merozoite, Rb-ribosomes, N-nucleus PVM-parasitophorous vacuolar membrane. V-Vacuole. RP-rhoptry precursors.

(a) Bar = 0.4 µm (b) Bar = 0.5 µm (c) Bar = 0.4 µm
(d) Bar = 0.5 µm

Trophozoites lack organelles normally seen in merozoites (rhoptries, micronemes, pellicular complex) but contain pigment granules and food vacuoles, and are uninucleate (a and c). The membranes in (a) are less well defined compared to (b), (c) and (d), eg. the PVM is not visible. The cytoplasm of the host erythrocyte in (a) contains organelles which are similar to Maurer's clefts and Maurer's clefts with electron dense material. Trophozoites are also irregular in shape and their ameboid activity produces invaginations and extensions of the parasite and the erythrocyte cytoplasm. In (b) an early schizont is shown having only a few rhoptries since the division of the nucleus is not yet complete. Oval-shaped merozoites measuring 1.5 nm in length, with rhoptries, micronemes and a pellicular complex are present at the late shizont stage (d).

5.4 DISCUSSION

The presence of malaria parasites in a patients blood is detected by light microscopy using either thick or thin blood smears stained with Giemsa. Only ring stages are observed in the peripheral blood of patient's infected with *P. falciparum* since erythrocytes infected with trophozoites or schizonts of this species (7) adhere to the epithelial cells of blood vessels.

Isolate PfUP1 could be identified as *P. falciparum* based on the following observations (Fig. 1.4, 5.2): Extracellular trophozoite and schizont stage parasites which only occur in *P. vivax* infections, were absent. Infected erythrocytes are the same size, colour and shape as non-infected

erythrocytes which excluded *P. vivax* and *P. ovale* whereas the absence of band-shaped trophozoites also excluded *P. malaria*. Ring-stage parasites contained two chromatin dots which are typical for *P. falciparum*.

Erythrocytes with holes were observed after fixation with both slow and fast methods (Fig. 5.4 and 5.5). Kreier *et al.* (4) demonstrated up to 4 holes in erythrocytes infected with different species of malaria parasites which he concluded: "appears to be associated with multiple merozoite infections". However, in our study up to 8 holes were sometimes observed after fixation with either the slow or fast methods. These many holes are unusual but could be caused by merozoites leaving partly fixed erythrocytes which are unable to reseal and/or merozoites attempting to enter partly fixed erythrocytes which is still flexible but not amenable to invasion.

Wild isolates of *P. falciparum* usually contain more than one strain of which some may be of the knobless variety (8). Although knobs were seen on some of the erythrocytes infected with isolate PfUP1, no correlation was attempted with the parasitemia to establish the proportion of knob-producing parasites, since an unsynchronized culture was used. The knob sizes (165.4 + 16 nm) in our study (Fig. 5.6) agree with that of the trophozoite-containing erythrocytes reported by Gruenberg *et al.* (diameter, 110-160 nm; 18) since the Knob sizes in schizont-bearing erythrocytes varied between 70-100 nm (18).

Parasites in Fig. 5.7 (a) and (c) can be identified as trophozoites due to the absence of typical merozoite intracellular organelles, their irregular shape and uninucleate character as well as the presence of food vacuoles with their associated hemozoin crystals. The parasite in Fig. 5.7(d) is

typical of merozoites which contain rhoptries, nucleus, mitochondria and is covered by a surface coat. The parasite in Fig. 5.7(b) displays many small rhoptries and is in the process of dividing, but fully developed merozoites are not present. The parasite is larger than a merozoite or an uninucleate trophozoite and is therefore at the schizont development stage.

Comparisons between the transmission electron micrographs in Fig 5.7 revealed the following: The trophozoite in (a) which was fixed using the slow method, displays Maurer's clefts and membrane stacks in the erythrocyte cytoplasm. Inside the trophozoite haemozoin pigment, food vacuoles and low electron dense-containing vacuoles can be discerned. However, the membranes appear to be undefined and rather fuzzy.

The trophozoite in (c) which were fixed using the fast method also displays Maurer's clefts in the erythrocyte cytoplasm and the same structures inside the parasite as seen with the slow fixation method. Knob-presenting and knobless erythrocytes were seen in both methods of fixation. The major difference between the slow and fast fixation method appears to be that the membranes are better preserved in the latter method. For instance, the PVM and pellicular complex can be clearly discerned in Fig. 5.7 (b and d) but not in (a).

Most of the fine structure of the parasite appears to have been lost using the slow fixation method, which makes the fast fixation method superior. The lack of membrane preservation with the former method may be due to a loss of unfixed soluble membrane components during the prolonged fixation or the acetone treatment (11).

Thus, the PfUP1 isolate is of the *P. falciparum* strain and could be a mixture of isolates of which some may have the ability to produce knobs and others not. The same cytoplasmic organelles as described in the literature were observed in our infected erythrocytes. Both fixation methods can be used for scanning electron microscopy, but when transmission electron microscopical examination is needed, the fast fixation method yielded better preservation of membranes.

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CHAPTER 6

6.1 CONCLUDING DISCUSSION

Parasitism involves the close relationship between two different kinds of organisms. One, namely the host, provides food and shelter for the other, the parasite (1). Although some parasites are beneficial to the host, the malaria parasite is not, causing much morbidity and mortality to the host (2). Malaria should be considered in any febrile patient who has travelled to an endemic area, as there is no specific clinical features for malaria infection (3). As we enter the last decade of this century, malaria is on the increase (See Fig. 1.2). The upsurge of the disease world-wide, especially Africa and Southern Africa can be attributed to the following: (a) The increasing population, especially in the high risk areas where malaria is endemic. (b) Increasing poverty and consequent lack of/or inefficient eradication programs. (c) Immigrants spreading the disease to areas where it was under control, or spreading of drug resistant strains to new areas (3). Furthermore, transmission is also expediated by the creation of agricultural communities in malaria areas, since it attracts more and more workers (4). (d) Insecticide resistance leading to an increasing mosquito population. (e) Drug resistance, which is one of the main problems in parasite control, as new drugs are not common and increasingly costly to develop.

By using our knowledge of the current status and occurrence of drug resistant isolates much of the morbidity and mortality can be avoided by correct treatment of the disease (5). With the development of techniques for the *in vitro* cultivation of malaria parasites a new era was entered

with respect to research on and diagnosis of malaria. Using the principles of the candle jar/petri dish method for the continuous cultivation of *P. falciparum*, a short term micro-assay was developed for the *in vitro* testing of the drug resistant status of malaria parasites (6). Although the system developed by Rieckman has some disadvantages as mentioned in section 2.1 the test can be used to determine the degree of susceptibility of malaria parasites to most of the antimalarial drugs, except to combinations of sulfa-drugs and pyrimethamine. A reduction in the concentration of 4-aminobenzoic acid (PABA) and folic acid in the RPMI 1640 medium to those found in human blood, made it possible to also employ this assay for the above drugs (7).

The field evaluation in 1988 revealed that 11 % of the cases in the North-Eastern Transvaal had parasites that were resistant to chloroquine (Fig 2.2 and Fig 2.3 a). Thus, one out of every ten patients infected with malaria, might respond only to drugs other than chloroquine. A bigger concern is the evidence that in 22 % of the cases, the malaria parasites were resistant to mefloquine. Mefloquine is a relatively new drug and not yet available in South Africa and already it appears to be ineffective. Perhaps in this case it is a matter of insensitivity to the drug rather than resistance (8). Padua crossed a cloned line of a highly drug-resistant isolate with a drug-sensitive isolate of malaria parasites and found that many clones of the progeny exhibited a level of resistance midway between that of the parents (9). During meiosis of hybrid zygotes, the various resistance genes apparently had undergone recombination, thus producing the intermediate forms of resistance (9). This may explain why certain isolates from North-Eastern Transvaal were more resistant to chloroquine and mefloquine than others. The *in vitro* method

also does not take cognisance of the immune system of the host or the possibility that metabolites formed *in vivo*, may be more effective than the drug itself (8). It is thus possible that *in vitro* assays could be biased and not a true reflection of the *in vivo* situation.

Interpretation of results of the micro-test is dependent on the ability to cultivate parasites in the control wells. It is thus important to ascertain that no prophylactics were taken (urine test) prior to the assay and to be aware that a high parasitemia needs more chloroquine than a lower parasitemia to inhibit schizont formation (6). The micro-test can be easily accomplished if the hematocrit is kept below 3 % and if fresh erythrocytes are added to reduce the parasitemia. The candle-jar can also be replaced by a dessicator filled with a special gas mixture (7). The short-term *in vitro* drug test is also more effective if the parasitised erythrocytes are washed prior to cultivation to remove any adsorbed inhibitory antibodies and if serum from a naive donor is used (7).

The *in vitro* testing of anti-malarial drugs thus broadens our knowledge of the isolates found in specific areas and will aid in the selection of the appropriate chemotheropeutic treatment as well as prophylactics. It is recommended that *in vitro* testing of the commonly used drugs such as chloroquine, quinine, sulpha-drugs and drug combinations should be continued on a regular basis to assist in the treatment of patients and to monitor changes in the antimalarial drug susceptibility profile, as well as to evaluate new antimalarial drugs (5).

The relatively late development of a long term *in vitro* culture method was mainly due to an inadequate knowledge of the biochemistry of the parasite (12). The extent of parasite growth depends on a number of

factors: strain, time of culture, suitability of the erythrocytes as well as the serum (7). Several modifications to the method developed by Trager and Jensen have now led to the development of semi-automatic apparatuses with various degrees of complexity, in order to maintain the physiological pH and remove toxic waste products (7).

The initial inability to establish a continuous *in vitro* culture in our laboratory appears to have been due to a difference in the composition of dissolved gasses between Pretoria and Durban. Parasite growth at Pretoria was only observed in flasks which contained gas-equilibrated medium and were flushed with the special gas mixture (Fig 3.4). The lack of growth in flasks that were only flushed, was most likely due to the toxic effect of a too high oxygen concentration which could not be prevented by the prevailing concentration of dissolved carbon dioxide. Equilibration of the medium with the gas mixture, prior to culturing of parasites, apparently compensated for the lower partial pressure of gases at Pretoria compared to Durban. The consequent higher concentration of dissolved carbon dioxide protected the parasites against oxygen toxicity. It appears that only wild isolates are extremely sensitive to the composition of dissolved gases since after a few weeks in culture, parasites could be maintained in medium that was only flushed but not equilibrated with the gas mixture. It is recommended that gas mixtures with lower concentrations of oxygen be used in future studies, especially if wild isolates need to be established.

Serum is one of the most important supplements of the culture medium (7). From our results in Table 3.3 it is evident that bovine serum can be used as a substitute for human serum. However, growth in human

serum-supplemented medium was superior to medium which contained bovine serum. Culture mediums also required the addition of hypoxanthine. It is possible that further additions of hypoxanthine to bovine serum-supplemented medium may further improve the growth rate due to the substantial lower content of hypoxanthine in bovine serum compared to human serum (Table 3.3).

One of the fundamental goals of studies on the biochemistry of *Plasmodium* is to search for metabolic differences between the host and parasite which can be exploited for the design of new drugs. Particularly striking is the molecular differences between the dehydrofolate reductases of host and malarial parasites, which contribute to the exquisite sensitivity of malaria parasites to pyrimethamine (12). There is no doubt that other as yet undiscovered differences exist between the host and *Plasmodium* which could be used in the development of specific antimalarial drugs.

The successful invasion of the host erythrocyte by a merozoite involves specific binding to a surface receptor followed by endocytosis (2). The intra-erythrocyte parasite derives its energy via conversion of glucose into lactate (both L and D isomers) (13). The avian parasite oxidizes a portion of the pyruvate into CO_2 and H_2O by means of a citric acid cycle, whereas rodent and primate parasites lack a complete citric acid cycle and have only lactate as end product (13). The only evidence for an energy-yielding electron transport chain is at best circumstantial. In all the malaria parasites studied, the only enzyme which could be associated with this system is cytochrome oxidase (13).

Malaria parasites can synthesise pyrimidines *de novo* but not purines (12). Purines are obtained via a parasite-specific salvage pathway in

which hypoxanthines, derived from the extracellular medium and the host, is converted mostly to ATP and small quantities of GTP (14).

One of the conclusions drawn from our results was that lactate concentrations above 12mM are inhibitory to parasite growth, in agreement with other researchers in the field (14). The rate of medium replacement is therefore largely determined by the parasitemia of the culture which should be kept below 10 %. The amount of lactate produced by the infected cultures in comparison to the non-infected cultures cannot be accounted for by glycolysis alone. For the first 48h of culturing, the lactate/glucose ratio exceeded 2 whereas during the last 48h it was below 2 (Fig 3.8 and Table 3.4). Confirmation of these results requires further investigation. As only a fraction or the available glucose is used this is clearly not a limiting factor in parasite cultures. The concentration of ATP inside the host erythrocyte is considered to be critical for parasite development (16). Its concentration is influenced by various factors which include the pH and lactate concentration of the medium as well as utilization by the erythrocyte and parasite (16). Over the first 48 hours of culture the ATP concentration in the infected cultures were relatively stable. However, over the last 48 hour period the ATP concentration decreased by 44 %, from 1690 to 950 nmol/ml PCV (Fig 3.8). Most importantly, this large decrease was obtained in a culture where only 14 % of the erythrocytes was infected. Similar results were also found for some *in vivo* infections (12). From our results (Fig 3.8), it seems that the purine salvage pathway is inhibited since, even though high concentrations of IMP were available, the ATP concentration was lower than in the non-infected controls. The total adenylate nucleotide concentration was as suspected lower, due to the excessive demand (Table 3.4). The

decrease in the multiplication index (Table 3.5), could thus be due to a limiting adenylate nucleotide pool as well as the lesser susceptibility of non-infected erythrocytes to invasion, caused by ATP depletion (see Introduction). At present the cause for the block in the purine salvage pathway is unknown and requires further investigation.

Haematological complications are the most common of all in malaria infections (16). According to Kelton, thrombocytopenia in malaria infection was first observed by Hill in 1964 and is prevalent in *P. falciparum* and *P. vivax* infections (16). Two major systems induce thrombocytopenia in malaria, namely the increased destruction of platelets either by hyperactivity of the reticulo-endothelium cells and/or immune destruction and disseminated intravascular coagulation (DIC) (16,17). Strong evidence exists for the immunoglobulin destruction of thrombocytes as increased concentrations of thrombocyte-associated immunoglobulins (TAIg) have been measured both by us (18) and Kelton *et al.* (16). The modified micro-enzyme-immunoassay for the determination of thrombocyte-associated immunoglobulins which we developed, simplified the assay considerably. The method is faster and more economical and accurate than any other method employed at this stage. It also uses less blood and can be employed in antibody displacement studies to determine the specificity of TAIg. This method also lends itself to quantification of autoimmune antibodies present on cells or antibodies specifically bound to membrane surface antigens or receptors. It could be shown with this method that the inverse correlation between thrombocytes and thrombocyte-associated immunoglobulins decreased while patients received treatment, which helped to monitor their recovery (Table 4.1).

The reason for the increased association of antibodies with thrombocytes is not yet understood but the following models need to be considered: (a) It is an autoimmune disease in which antibodies produced against the parasite cross-reacts with epitopes on the thrombocyte surface. (b) The antibodies are immune complexes consisting of parasite antigens and their antibodies which adhere to the thrombocyte surface. (c) Parasite antigens first adhere to the surface of thrombocytes before being bound by antibodies. Future studies should be aimed at the identification of the idiotypic specificity of the antibodies to discover whether or not cross-reacting epitopes are responsible for the platelet destruction.

The parasite morphology and the host species infected by the parasite are primary attributes considered in the taxonomy of *Plasmodia* (19). The light microscope is the principle tool used in assigning morphological descriptors to the parasites. However, over the last three decades the electron microscope with its capacity for detecting fine structures has become increasingly important to the researcher (25). Erythrocytes infected by *Plasmodium* exhibit various changes in its morphology. However, it is still not known why one strain of malarial parasites produce changes which is only specific for that strain (19).

Cytoplasmic organelles which are considered to be involved in parasite trafficking from and to the host cell membrane and the parasite, were described by Aikawa (19). Similar structures were seen in our studies. We found that the fast fixation method is the preferred method for electron microscopy. The slow fixation method, was only good enough for scanning electron microscopical studies. It is now possible to conduct

immunocytochemical studies and to investigate the fine structures of isolated parasites (Fig 5.7).

Thus, new techniques for studying the biology of the malaria parasite and their interactions with the host, opened new doors for research on malaria. Culturing techniques are currently used to screen parasites for drug susceptibility and effectivity of vaccine candidates (7). The drugs developed against the parasites are in a sense only an aid to nature (20). Without an active immune response from the host it is unlikely that any antimalarial drug used today will completely cure a patient of his malaria infection (20). It is furthermore misleading to compare the eradication of a disease such as smallpox, which is easily diagnosed and prevented by vaccination, to malaria (21). The inadequacy and high cost of the currently available control programmes coupled to the resistance of vectors and parasites to insecticides and drugs, respectively, makes this disease much more complex and highlights the need for a cheap, effective vaccine (22). However, we must guard against the impression that once an effective vaccine is developed the problems of malaria will disappear (23). As in the case with other vaccines, its distribution to all the areas where it is needed, is near impossible (23).

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SUMMARY

Malaria parasites are responsible for an increase in the morbidity and mortality in several tropical regions in Southern Africa. In this thesis, research was undertaken on *Plasmodium falciparum*, which is responsible for more than 95% of these cases.

Although pharmacological prophylaxis is available, a worldwide resistance against existing drugs have been encountered. A study on chloroquine-resistance in North-Eastern Transvaal in 1988, indicated that 11% of the strains in this area were resistant against chloroquine. Only 78 % of these parasites were sensitive to the new drug, Mefloquine, which could serve as a substitute in chloroquine-resistant infections. Furthermore, no strain was found to be resistant or insensitive to both anti-malarial drugs.

Malaria parasites can be obtained for research purposes from long term *in vitro* cultures. The initiation of cultures of some wild isolates of *P. falciparum* appears to be problematic at certain levels above sea-level. The gas composition of the medium was identified as a probable cause due to its dependence on the partial pressures of gasses at different heights above sealevel. The toxic effect of a too high oxygen concentration on the parasite, caused by the lower atmospheric pressure of the Highveld area, was prevented after the concentration of dissolved carbon dioxide in the medium was increased through equilibration with the special gas mixture.

Possible shortcomings in the long-term culture method for malaria parasites that could retard optimum growth, were also investigated. Local parasite isolates could be supported on medium enriched with bovine serum although the growth rate was lower than when human serum was

used. By increasing the frequency of medium replacement with progressing parasitemia, less stress were placed on the system since parasites was exposed for shorter periods and to lower concentrations of byproducts such as lactic acid. In addition, the ATP-concentration in infected cultures decreased by nearly 50 % over a growth period of 4 days. The stress in the host cell was reflected by the decrease in the total adenyl-nucleotide pool and the increase in AMP-concentration. An increase in the intracellular IMP-concentration indicated that the purine salvage pathway was inhibited which may explain the decrease in the ATP-concentration. It therefore appears that the regular replacement of medium and replenishment of erythrocytes only partially contribute to the successful establishment of malaria cultures. More research is necessary to identify the factors that are responsible for the inhibition of the purine salvage pathway.

The stress placed on the human body by the parasite is complicated by the thrombocytopenia observed in some infections. Increases in the concentrations of thrombocyte-associated immunoglobulin G and M which are a characteristic for this condition, can be determined by a modified micro-method developed in our laboratory. By monitoring the parasite-infected patient over a period of time with the micro-method, the increase in thrombocytes and decrease in thrombocyte-associated antibodies were correlated with the recuperation of the patient. This method does not destroy the thrombocytes, thereby allowing displacement studies to be undertaken with purified parasite antigens of synthetic peptides.

An investigation of parasite-infected erythrocytes by means of light microscopy, transmission and scanning electronmicroscopy, indicated that

the local isolate could be composed of a mixture of strains, of which some have the ability to induce knobs on the erythrocyte. Furthermore, the investigation illustrated that fast fixation with gluteraldehyde is superior to slow fixation when transmission electronmicroscopy is performed. However, no difference could be observed when scanning electron microscopy was performed on infected erythrocytes that had been fixed by either of these methods.

OPSOMMING

Malaria parasiete is verantwoordelik vir 'n toenemende morbiditeit sowel as mortaliteit in verskeie tropiese streke in Suider-Afrika waarvan meer as 95 % van die gevalle deur *Plasmodium falciparum* veroorsaak word. Navorsing in die tesis is gevolglik op hierdie parasiet toegespits.

Ten spyte daarvan dat farmakologiese profilakse toegepas word, kan malaria nog steeds opgedoen word as gevolg van 'n wêreldwye weerstandigheid teen bestaande geneesmiddels. 'n Ondersoek van die klorokien-weerstandigheidstatus in die Noord-Oostelike Transvaal het in 1988 getoon dat 11 % van die stamme in die area weerstandig is teenoor klorokien. Slegs 78 % van die parasiete was sensitief vir die nuwe middel, Meflokien, wat as moontlike plaasvervanger in klorokien-weerstandige parasietinfeksies gebruik kan word. Geen parasietstam was egter weerstandig of onsensitief teen beide middels nie.

Langtermyn kultuurkweking van malaria parasiete is essensieël vir die verkryging van uitgangsmateriaal vir navorsing op die parasiet. Dit blyk egter dat inisiasie van kulture van wilde stamme van *P. falciparum* problematies by sekere hoogtes bo seespieël is. Die gassamestelling van die medium is as 'n moontlike oorsaak geïdentifiseer aangesien dit afhanklik is van die parsiële drukke van gasse by verskillende hoogtes bo seespieël. Die toksiese effek van te hoë suurstofkonsentrasies op die parasiet a.g.v. die laer atmosferiese druk op die Hoëveldstreek, is oorkom deur die konsentrasie van opgeloste koolsuurgas in die medium te verhoog deur vooraf 'n spesiale gasmengsel daardeur te borrel.

Moontlike tekortkominge in die langtermyn kultuur metode vir malaria parasiete wat optimale groei kan belemmer, is ook ondersoek. Plaaslike parasietstamme kon op medium wat verryk is met beesserum onderhou word, alhoewel die groeitempo laer is as wanneer mensserum gebruik word. 'n Toenemende tempo van mediumvervanging soos die parasitemia in kulture toeneem, het getoon dat minder stres op die sisteem geplaas word deurdat die parasiete vir 'n korter periode en aan laer konsentrasies van byprodukte soos melksuur blootgestel word. Die ATP-konsentrasies in geïnfekteerde kulture daal egter met ongeveer die helfte oor 'n groeiperiode van 4 dae. Tesame hiermee, is daar ook 'n daling in die totale adeniel-nukleotied poel van die geïnfekteerde rooibloedsel en 'n styging in die AMP-konsentrasie wat die stres in die gasheersel weerspieël. 'n Verhoging in die intrasellulêre IMP-konsentrasie dui op 'n moontlike inhibisie van die purienherwinningspadweg wat die verlaagde ATP-konsentrasie mag verklaar. Uit die studie blyk dit dus of meer gereelde vervanging van medium en toevoeging van rooibloedselle slegs 'n gedeeltelike bydrae maak tot die suksesvolle vestiging van malariakulture. Die identifikasie van die faktore wat lei tot die inhibisie van die purienherwinningspadweg, verg nog verdere ondersoeke.

Die stres wat die parasiet in die menslike liggaam veroorsaak, word ook weerspieël deur onderandere die trombositopenie wat in sommige infeksies waargeneem word. Verhogings in die konsentrasie van trombosiet-geassosieërde immunoglobuliene G en M wat kenmerkend is van die toestand, kan gemeet word deur 'n gemodifiseerde mikrometode wat in ons laboratoriums ontwikkel is. Deur die parasiet-geïnfekteerde pasiënt oor 'n paar dae met die mikrometode te monitor, kon die verhoging in trombosiete en afname in trombosiet-geassosieërde antiliggame met herstel

van die pasiënt gekorreleer word. Die metode veroorsaak geen skade aan die trombosiete nie sodat antigeenverplasingstudies ook na die tyd gedoen kan word.

In 'n ondersoek van die parasiet met behulp van ligmikroskopie, deurstraal- sowel as skandeer-elektronmikroskopie, is gevind dat die plaaslike isolaat moontlik uit 'n mengsel van stamme bestaan, waarvan sommige die vermoë het om knoppe op die rooibloedsel te induseer. Verder het die ondersoek getoon dat indien deurstraal-elektronmikroskopie gebruik word, 'n vinnige fikseringsmetode met gluteraldehied beter is as 'n stadige fikseringsmetode. In vergelyking hiermee kon geen verskil waargeneem word met skandeer-elektronmikroskopie van geparasiteerde rooibloedselle wat met enige van hierdie metodes gefikseer is nie.