EFFECT OF PYRIMETHAMINE ON GAMETOCYTOGENESIS, EXFLAGELLATION AND ASEXUAL GROWTH IN SOUTHERN AFRICAN ISOLATES OF *PLASMODIUM FALCIPARUM*

Joyce Mahlako Tsoka

Submitted in fulfilment of the
academic requirements for the degree of
Master of Science in Parasitology in the
Department of Zoology and Entomology,
University of Natal, Pietermaritzburg

January, 1995

ABSTRACT

Pyrimethamine efficacy was investigated *in vitro* on the blood asexual stages, the sexual stages and exflagellation in *Plasmodium falciparum*. Gametocytogenesis was stimulated following the standard methods on five isolates of *Plasmodium falciparum*. From these five isolates, RSA 2, 3 and 5 produced gametocytes which reached maturity within seven days and the gametocytes were able to exflagellate. Isolate MW2 produced young gametocytes which disappeared within ten days. NF54 produced mature gametocytes which lasted for 24 hours only.

There were no statistically significant differences between the static and the synchronization methods of gametocyte stimulation for any of the isolates. The effect of pyrimethamine was investigated by adding a known concentration of the drug (For RSA 2, MW2 and NF54, 100nmol/ℓ; RSA 3 and 5, 3000nmol/ℓ pyrimethamine) to the culture medium for seven days during gametocyte stimulation. The results of this investigation show that there was gametocytocidal activity on the isolates that were used and pyrimethamine also had a schizontocidal action on NF54 and the young gametocytes of this isolate were destroyed by the drug. At concentrations which were inhibitory to asexual parasites, the drug had a sporontocidal effect on isolate RSA 2 but not on isolate RSA 5. The pyrimethamine MIC values for asexual parasites ranged from 300nmol/ℓ to >3000nmol/ℓ (RSA 2 and 5 were not inhibited at 3000nmol/ℓ). These results are consistent with those found in previous studies when pyrimethamine resistance was first detected in South Africa.

The chloroquine MICs indicate a good correlation with the results obtained from previous drug sensitivity tests for all the isolates examined using both the 48-hour *in vitro* test and isotope incorporation for growth assessment. The isobolograms constructed to determine relationship between chloroquine and pyrimethamine indicated no synergism for isolates RSA 2 and 5, but the Σ relative IC_{50}s indicated a weak synergism. Both the isobolograms and the Σ relative IC_{50}s for the isolates RSA 6, 9 and 14 indicated an antagonistic action between chloroquine and pyrimethamine. The results obtained from this study have important implications for malaria control in South Africa.

PREFACE

This study represents original work done by the author and has not otherwise been submitted in any form for any degree or diploma to any University. Where the author used the work of others it is duly acknowledged in the text.

The experimental work described in this dissertation was carried out in the Department of Zoology and Entomology, University of Natal, Pietermaritzburg and in the laboratories of the National Malaria Research Programme of the South African Medical Research Council, Durban from January 1993 to December 1994 under the supervision of Professor Chris C Appleton (University of Natal) and Dr Janet A Freese (Medical Research Council).

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ACKNOWLEDGEMENTS

Without the input of the following people during the study period, this thesis would have not been completed:

Prof Chris C Appleton, my project supervisor, Department of Zoology and Entomology, Pietermaritzburg; for his critical assessment of my work and proper guidance during the study. Dr Janet A Freese, National Malaria Research Programme of the Medical Research Council, Durban; for her support and guidance, and her fruitful ideas.

The staff of the National Malaria Research Programme, Medical Research Council, Durban, especially Dr Brian Sharp, the head of the programme and Dr Dave le Sueur for the invaluable support they provided me during the initial stages of my project and for allowing me to use equipment and facilities in the laboratory. Tollie Rossouw, Sykes Saikoolal and David Mthembu for their assistance with various technical aspects of my project. Zandile Ntuli for her secretarial assistance. Eleanor Gouws of the CERSA Department for her assistance with analyzing my data. Finally, financial assistance for my research was provided by Ernst & Ethel Eriksen Trust Fund (first year), a University of Natal Graduate Bursary (first year), a FRD Research Studentship (first and second years), the Sales House Club (second year) and the National Malaria Research Programme of the Medical Research Council, Durban, (first and second years).

CHAPTER 1

INTRODUCTION

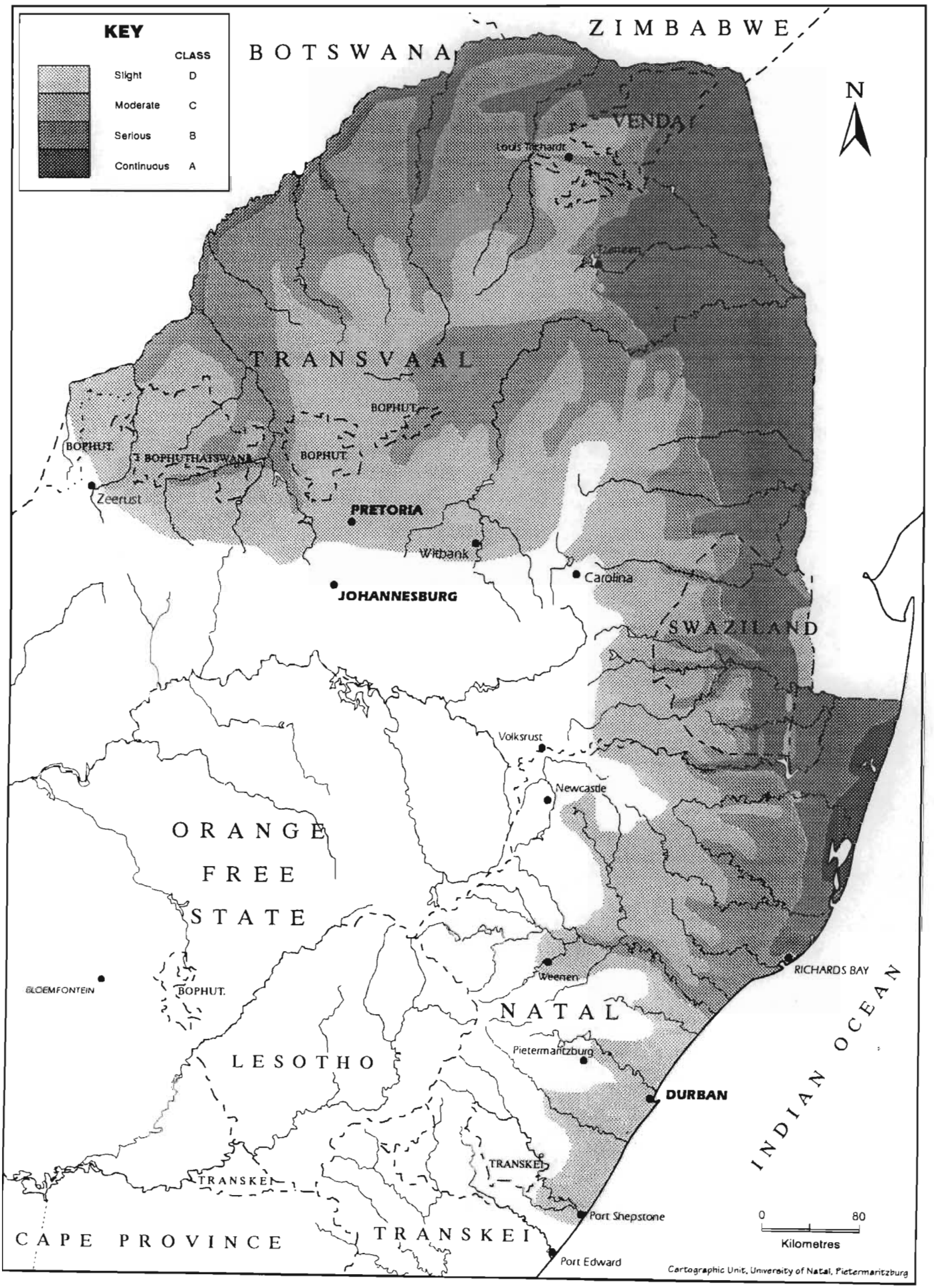
1.1 *PLASMODIUM FALCIPARUM* MALARIA IN SOUTH AFRICA

In South Africa at least 90% of malarial infections are caused by *Plasmodium falciparum* (Newlands, 1989). There is a limited distribution of malaria in this part of the African continent (Strebel *et al*, 1990) and the endemic malarial areas are the North and Eastern Transvaal and KwaZulu-Natal, especially near the borders of Mozambique and Zimbabwe (Frean & Blumberg, 1993; Hansford, 1993; le Sueur *et al.*, 1993) (Fig. 1- shows historical distribution of malaria, especially as modern borders of homelands are indicated).

In the Transvaal over 70% infections occur in the Barberton magisterial district. Most of these are found in the Malelane-Komatipoort sectors where intense irrigation is practised and to where a considerable proportion of farm workers migrate from Mozambique, Gazankulu, KaNgwane, Lebowa and Venda (Hansford, 1990). The main endemic area is KwaZulu-Natal. This is probably due to imported malaria via Mozambique migrants (about 90% of imported cases) (Ngxongo, 1993). High incidence rates during 1987 and 1988 occurred in the Ingwavuma and Ubombo districts where cases from these areas made up 88% of the total number of cases for KwaZulu-Natal (Ngxongo, 1993) due partly to irrigation spillage in the Mamfene sector of Ubombo district (Hansford & Muller, 1990). Epidemiological studies carried out in the KwaZulu part of the KwaZulu-Natal province have revealed that many malarious areas have been cleared of malaria and that the disease is now confined to the coastal region near the Mozambique border (Ngxongo, 1993).

FIG. 1. MALARIOUS AREAS OF THE UNION OF SOUTH AFRICA, 1938.

(Modified with permission from le Sueur *et al*, 1993).


KEY
CLASS
Slight D
Moderate C
Serious B
Continuous A
BOTSWANA
ZIMBABWE
N
VENDA
Louis Trichardt
TRANSVAAL
BOPHUT.
BOPHUTHATSWANA
Zeerust
PRETORIA
Witbank
JOHANNESBURG
Carolina
SWAZILAND
Volksrust
Newcastle
ORANGE
FREE
STATE
BLOEMFONTEIN
Weenen
RICHARDS BAY
NATAL
LESOTHO
Pietermaritzburg
DURBAN
INDIAN OCEAN
TRANSKEI
Port Shepstone
CAPE PROVINCE
Port Edward
0
80
Kilometres
Cartographic Unit, University of Natal, Pietermaritzburg

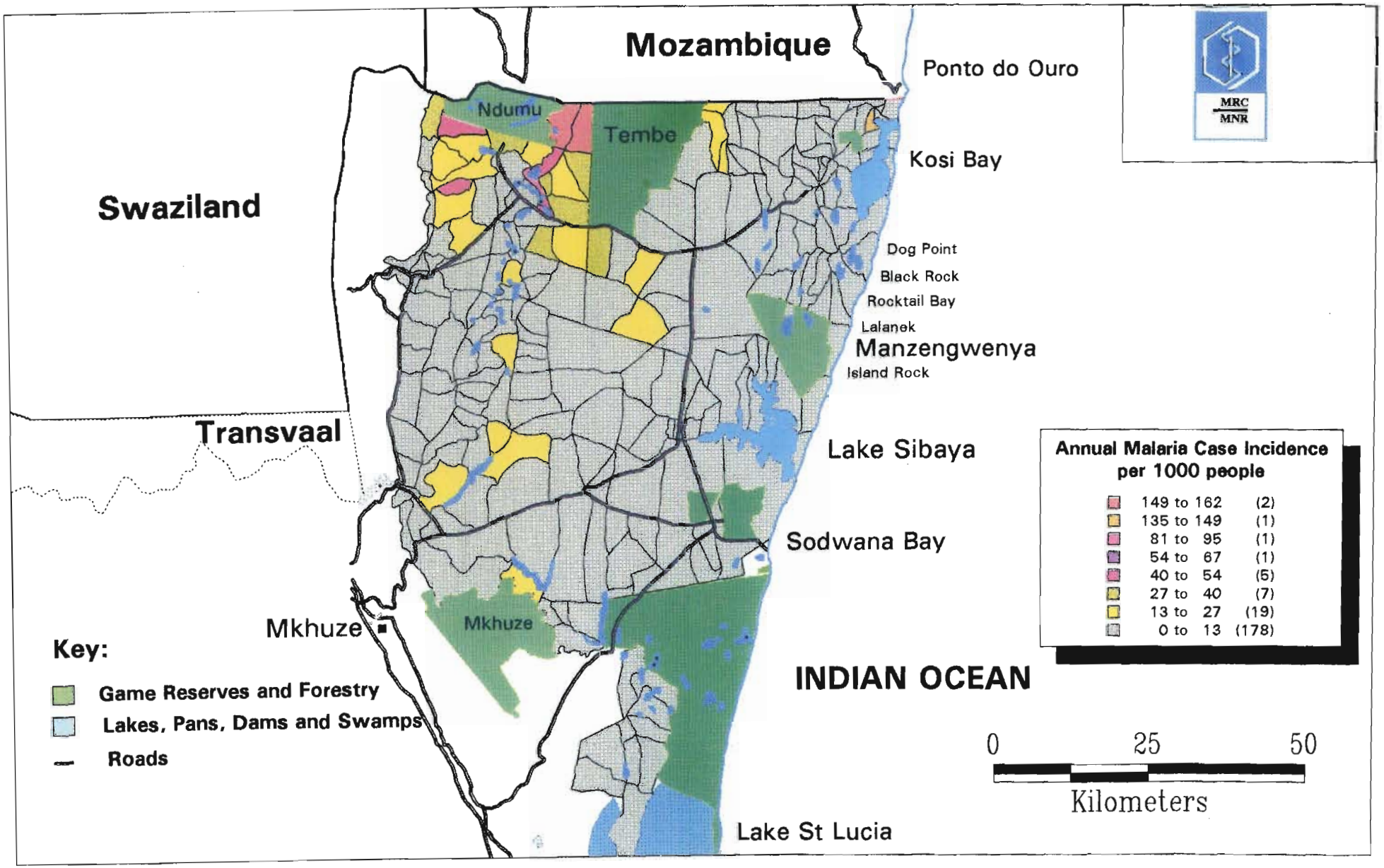
In South Africa malaria is transmitted mainly by *Anopheles arabiensis* and to some extent *Anopheles merus* (Sharp *et al*, 1984). Transmission is extremely variable from season to season (Frean & Blumberg, 1993; Hansford & Muller, 1990) and from locality to locality, being determined by various factors such as climate, human migration, environmental changes, agricultural development and changes in the malaria control policy (Frean & Blumberg, 1993). The majority of malaria cases (80%) are known to occur during the months January to June with peaks occurring in April to May (Ngxongo, 1993).

The Malaria Control Programme in KwaZulu-Natal has reduced the incidence of malaria to low levels in comparison to those experienced during the first three decades of this century (Fig. 1), due to the use of chemotherapeutic drugs and insecticides. Figure 2 shows the annual malaria case incidence from 1980 to 1991 in the Northern Natal Malaria Control Regions, the figures within parentheses indicate the number of places or polygons where cases were found (le Sueur & Ngxongo, 1994, unpublished). Hansford (1990) suggested that malaria control should be maintained essentially by a will to control, the availability of funds and the environmental planning.

FIG. 2. ANNUAL MALARIA CASE INCIDENCE FROM 1980 TO 1991 IN THE NORTHERN KWAZULU-NATAL MALARIA CONTROL REGIONS- the numbers in parentheses refer to the number of places or polygons captured in the map. (Source: le Sueur & Ngxongo, 1994, unpublished).

ANNUAL MALARIA CASE INCIDENCE 1980 TO 1991

Northern Natal Malaria Control Regions



D. le Sueur, National Malaria Research Programme & S.M. Ngxongo, KwaZulu Health, 1

1.2 LIFE CYCLE OF *PLASMODIUM FALCIPARUM*

Plasmodium falciparum malaria is a more serious and dangerous disease than those caused by the other three species of the genus *Plasmodium* infecting man; *P. ovale, P. vivax* and *P. malariae*. The incubation period lasts from 8 to 20 days and may run an acute course which may result in death if urgent treatment is not provided (Ash & Orihel, 1984; Garnham, 1988; Olsen, 1974).

1.2.1 In the human host

Plasmodium falciparum occurs in two phases; the pre-erythrocytic and the erythrocytic (blood) phases. The former takes place within parenchymal cells of the liver (Ash & Orihel, 1984; Garnham, 1988; and Olsen, 1974). The sporozoites introduced via the mosquito's bite are carried to the blood stream, then escape into the adjacent hepatocytes where they commence the schizogonic cycle. The pre-erythrocytic schizogony lasts for about five-and-a-half days. The spherical schizonts change shape in the course of their growth and burst to release merozoites (Beaudoin *et al*, 1988; Garnham, 1988; Newlands, 1989).

The blood phase starts when the merozoites enter the blood stream. Here each merozoite invades a red blood cell and develops into the asexual ring stage. The youngest rings are only 1.2μm in diameter. The ring stages tend to adhere to the surface of the erythrocyte and their nuclei are often divided. Amoeboid cytoplasmic strands, the trophozoites, grow out from the ring forms.

These trophozoites do not last very long, but can be readily observed in *in vitro* cultures of *P. falciparum* (Carter & Miller, 1979; Jensen & Trager,1977). The next stage is the erythrocytic schizont which develops from the trophozoite. Schizogony can be observed in cultures where immature schizonts appear within 12 hours or even less (Garnham, 1988). Schizonts contain a dark pigment and their nuclei divide several times forming merozoites which will either repeat the asexual stages or start the sexual cycle. After about eight to 10 days of asexual parasite growth, gametocytes, the sexual stages, appear. The early stages are almost triangular in shape with the longest side stretched into a bow and they appear as a spindle in later forms (Carter & Miller, 1979).

Gametocyte development can be observed clearly in cultures maintained under appropriate laboratory conditions. Gametocyte development in *in vitro* cultures takes seven to 10 days, and they pass through five developmental stages (Carter & Graves, 1988; Ponnudurai *et al*, 1986). These growth stages are classified according to their shape, pigment distribution and nuclear and cytoplasmic arrangement (Carter & Miller, 1979; Ponnudurai *et al*, 1986). The first stage (Stage I) takes about 24 hours to develop and is small and rounded. The parasite has a light staining nucleus and the cytoplasm is almost clear. The pigment is somewhat scattered within the parasite.

Stage II gametocytes can be distinguished within the erythrocyte by their tear-drop shape. These appear after two days. Stage III has rounded ends and the red blood cells become distorted. Stage IV gametocytes, like Stage III, take 24 hours to appear. The parasite at this stage has its two ends pointed like a spindle and the pigment is darker than in the young gametocytes.

The final developmental stage at which the parasite reaches maturity is Stage V. This stage takes 48 hours to develop. During this stage the sex of the parasite can be clearly distinguished. The female gametocyte (macrogametocyte) is more elongate than the male gametocyte, the nuclear material is centrally arranged and the pigment is very dark. The staining appears bluish and the parasite is crescentic. The male gametocyte or microgametocyte has a stumpy form and is brownish-pink in colour. Pigment is scattered along the length of the parasite (Carter & Miller, 1979; Ponnudurai *et al*, 1986).

1.2.2 In the mosquito

Further development of gametocytes takes place in the midgut of the mosquito. This happens only when mature gametocytes have been ingested by the mosquito. By a process called **exflagellation**, the male gametocytes produce microgametes. Macrogametocytes produce macrogametes by rounding up (Carter & Graves, 1988; Vanderberg, 1988). Exflagellation can be easily demonstrated by direct observation of wet mounted slide preparations under phase-contrast (Carter & Beach, 1977; Sinden, 1983; Sinden *et al*, 1984).

Following fertilization of a macrogamete by a microgamete a zygote develops which is called an **ookinete**. The ookinete divides several times and undergoes further development before transforming into young spherical **oocysts**. Sporozoite differentiation takes place at the surfaces of the cytoplasmic masses known as sporoblasts, formed by the segregation of the oocyst interior. The process may take four to 21 days *in vivo*. Further development of the sporozoites occurs within the salivary glands of the mosquito (Vanderberg, 1988) from where they are ready to be passed into the human host following a blood meal.

1.3 MALARIA CONTROL

Control of malaria in South Africa started as early as the 1930s when several experts were invited by the South African Government to assess and give advice on malaria (Swellengrebel, 1931). The complex life cycle of the malaria parasite makes control of the disease difficult. Malaria control involves the elimination of both the parasite and the mosquito vector.

1.3.1 Parasite Control

The are a number of factors to be considered in order to achieve successful parasite control and these include age of the patient, pregnancy and the tolerability of specific drugs (Frean & Blumberg, 1993). The species of *Plasmodium* to be treated and the developmental stage of the parasite are also important factors (Desjardins *et al*, 1988).

1.3.1.1 Asexual stages

It has been long established that the chemotherapeutic drugs developed for controlling asexual stages in the life cycle of the parasite, cannot be effectively used against other stages of development. Therefore more drugs need to be tested both *in vitro* (Wernsdorfer & Payne, 1988) and clinically so as to completely eradicate the parasites and their infections. There are several drugs that can be used for prophylaxis and treatment of asexual stages of the parasite. These have proved effective provided proper dosage and administration of the drugs are followed. One of the oldest drugs, quinine, is effective against all forms of malaria.

This drug kills the asexual blood stages as well as immature gametocytes of *P. falciparum*, and gametocytes of *P. malariae, P. ovale* and *P. vivax* (Black *et al*, 1986; Myint & Shwe, 1987; Newlands, 1989; White, 1988; WHO, 1982). However, quinine-resistant malaria has been reported in many parts of the world (Brasseur *et al*, 1987; Giboda, 1987; Hellgren *et al*, 1987; Ho *et al*, 1987; Howells, 1986) including some southern African countries such as Malawi and Zambia (Freese *et al*, 1994). A combined quinine/tetracycline treatment was recommended for malaria in Thailand in the early 1980s (Wernsdorfer, 1991). In South Africa it was shown recently that there is reduced sensitivity of *P. falciparum* to quinine *in vitro* (Freese *et al*, 1994).

Other antimalarials that can be used against the asexual blood stages of the parasite are; mefloquine, chloroquine, amodiaquine, proguanil, doxycycline, halofantrine, pyrimethamine and qinghaosu (QHS) (Hoffman *et al*, 1987; Wernsdorfer, 1986). According to Khaliq *et al* (1987) and Peto & Gilks (1986), amodiaquine is no longer effective against *P.falciparum* in Punjab. Bjorkman & Phillips-Howard (1990) and Wernsdorfer (1991) confirmed the reports on amodiaquine-resistant malaria. Freese *et al* (1994) suggested that amodiaquine would not be recommended for malaria chemotherapy in southern Africa due to growing evidence of resistance to it. Mefloquine was found to be highly effective against all the southern African isolates of *P. falciparum* tested *in vitro* (Freese *et al*, 1994) and this drug is used for both prophylaxis and treatment in South Africa. Mefloquine is fairly well tolerated, but severe side effects such as dizziness, epigastric pain and vomiting may result from the use of high doses during treatment (Ambroise-Thomas & Rossignol, 1986; Dept. Nat. Health & Pop. Dev., 1993; Edrissian *et al*, 1987; Ho *et al*, 1987).

Mefloquine should not be taken by pregnant women, persons with hypersensitivity to it, persons with neuropsychiatric or cardiac rhythm problems, babies or children weighing less than 15kg, pilots and others requiring fine or skilled co-ordination (Frean & Blumberg, 1993). Mefloquine resistance has been established in Thailand (Björkman & Phillips-Howard, 1990; Wernsdorfer, 1991), Mozambique, Central, East Africa (Frean & Blumberg, 1993), Zambia (Lemnge & Inambao, 1990)), Tanzania and most African countries (Bjorkman & Phillips-Howard, 1990).

Doxycycline is an effective prophylactic drug especially to people visiting chloroquine-resistant areas (Baker *et al*, 1993), but the drug cannot be taken by pregnant women or children under eight years (Frean & Blumberg, 1993). Proguanil is safe for pregnant women and children (Dept. Nat. Health & Pop. Dev., 1993) but this drug is no longer recommended on its own in South Africa but should be taken in association with chloroquine (Baker *et al*, 1993). Resistance to proguanil was established in the early 1950s and several reports have confirmed resistance to it in many parts of Africa (Bjorkman & Phillips-Howard, 1990).

Chloroquine is another effective antimalarial against all forms of malaria particularly in those areas where no resistance to this drug has been reported (Baker *et al*, 1993; Frean & Blumberg, 1993). Chloroquine is safe in pregnancy and for young children (Baker *et al*, 1993). The major problem with chloroquine is the development of resistance to it by the parasite. In southern Africa chloroquine-resistant falciparum malaria has been confirmed (Freese *et al*, 1988b; 1991; Herbst *et al*, 1985; 1987; Heymann *et al*, 1987; Sharp & Freese, 1990).

Present and previous reports (Deacon *et al*, 1994; Isaäcson *et al*, 1984) have revealed that Transvaal isolates of *P. falciparum* are also developing resistance to the drugs. Chloroquine-resistance has also become established in Cameroon in West Africa (Brasseur *et al*, 1987; Geary *et al*, 1987; Sinha & Gajanana, 1987) and in many parts of world especially, the southern hemisphere (Wernsdorfer, 1991). However, no evidence has been produced regarding the effectiveness of chloroquine on *P.ovale* and *P. malariae* (Wernsdorfer, 1991), but chloroquine-resistant *P. vivax* has been established in Papua New Guinea (Cooper, 1994).

Halofantrine has proved to be effective against southern African isolates of *P. falciparum* (Freese *et al*, 1993). This drug is known to have minimal side effects such as epigastric pain, vomiting and diarrhoea, and in certain predisposed individuals serious, including fatal in some cases have been reported. Halofantrine can be well tolerated (Ambroise-Thomas & Rossignol, 1986) and is the new drug under recommendation against the erythrocytic asexual stages of the parasite (Wernsdorfer, 1991).

Qinghaosu (QHS) is reported to be active against chloroquine-resistant falciparum malaria in China (Elford *et al*, 1987). QHS and its derivatives were first isolated in 1971 from *Artemisia annua*, a plant used in traditional Chinese medicine for 1 500 years (Howells, 1986). Although there are no reports involving the use of QHS in South Africa, this drug has been used in other African countries (TDR, 1994). Although drug-resistance is a problem for malaria chemotherapy, the use of drug combinations has been successful in many cases.

Fansidar (pyrimethamine/sulphadoxine) is still effective against chloroquine-resistant malaria (Watt *et al*, 1987; Wernsdorfer, 1986), though there has been reports about resistance to this drug compound in some parts of the world (Bjorkman & Phillips-Howard, 1990; Watt *et al*, 1987; Wernsdorfer, 1991). In South Africa recent studies have revealed low sensitivities to Fansidar by the isolates of *P. falciparum* (Freese *et al*, 1994) and this drug is no longer recommended for chemoprophylaxis by WHO because of severe side effects (Baker *et al*, 1993).

1.3.1.2 **Sexual stages**

Young gametocytes are killed by all schizontocidal drugs described above. Pyrimethamine is active against young gametocytes of *P. falciparum* but not against mature stages (Black *et al*, 1986). Pyrimethamine is effective against mature gametocytes of *P. vivax, P. ovale* and *P. malariae.* (Black *et al*, 1986; Desjardins *et al*, 1988). Pyrimethamine is no longer recommended on its own since there is widespread resistance to this drug (Baker *et al*, 1993; Peters, 1987). In South Africa recent studies involving drug tests showed that pyrimethamine failed to inhibit schizont growth (Freese *et al*, 1991).

Primaquine and pamaquine are effective against mature gametocytes of *P. falciparum*. These two drugs are restricted to gametocytes circulating in the blood stream and they do not affect gametogenesis, fertilization or sporogony (Meuwissen & Ponnudurai, 1988; Ponnudurai *et al*, 1989a).

1.3.1.3 Sporogonic stages

The major goal of using sporontocidal drugs is to prevent subsequent sporogonic stages in the mosquitoes after gametocytes have been ingested. One such drug is proguanil but this drug is no longer recommended on its own in South Africa because of its widespread resistance (Baker *et al*, 1993). Cycloguanil, the active metabolite of proguanil, showed a sporontocidal activity against some sensitive strains of *P. falciparum* (Teklehaimanot *et al*, 1985).

Pyrimethamine is active against the developing mosquito stages of the parasite (Black *et al*, 1986; Desjardins *et al*, 1988) but it does not prevent the development of oocysts in isolates of *P. falciparum* that are resistant to the drug (Teklehaimanot *et al*, 1985). Pyrimethamine and other parasite-specific inhibitors of dihydrofolate reductase and sulfonamides interfere with DNA synthesis by the parasite during sporogonic development in the mosquito (Carter & Graves, 1988).

1.3.2 Vector Control

Personal protection against and prevention of mosquito bites are important especially during the rainy season when mosquitoes are in abundance, due to the availability of more breeding places (Baker *et al*, 1993). Visits to endemic areas should be undertaken during the dry season or when rainfall is low. Avoiding malarious areas altogether is recommended for babies and children under five years, pregnant women, the elderly (above 65 years) and for immunocompromised individuals (Baker *et al*, 1993; Frean & Blumberg, 1993).

Mosquito attacks can also be avoided by not going out of doors between dusk and dawn as mosquitoes are most active at this time. Wearing long sleeves and long trousers at night can also help to prevent mosquito mouthparts from penetrating the skin (Dep. Nat. Health Pop. Dev., 1993). Protection against mosquitoes includes the use of bednets impregnated with an insecticide, the screening of windows and doors and the use of mosquito coils or the intradomiciliary spraying with knockdown insecticides of the resting places of mosquitoes (Frean & Blumberg, 1993; Wernsdorfer, 1986). One of the oldest insecticides is DDT which has been in use for more than 30 years, although there are some fears that the mosquitoes might be developing resistance to this chemical (Hansford, 1993; KwaZulu Dep. Health, 1991). Use of DDT is no longer being encouraged in South Africa since the components are environmentally unfriendly (Dr B.L. Sharp, pers. comm., 1994). Ficam and deltamethrin are other insecticides effective against the mosquitoes. Deltamethrin is cheap and recent studies (Dr B.L. Sharp, pers. comm., 1994) carried out in northern KwaZulu-Natal showed that this insecticide produced 100% mortality when applied for several weeks in mosquito-infested houses.

Lambda-cyhalothrin (ICON) is very effective not only against mosquitoes but it also controls bedbugs within the endemic malaria area. This pyrethroid is well-tolerated and non-staining and it can be applied on furniture as well. (le Sueur *et al*, 1993; Sharp *et al*, 1993). Repellents which contain di-ethyl toluamide are most effective but they have a short active life (Frean & Blumberg, 1993). Larvicides such as temephos can be applied to water to control the immature stages of mosquitoes. Malaria oil which is cheaper than other larvicides can be used effectively as well (KwaZulu Dept. Health, 1991).

1.4 SOUTHERN AFRICAN REFERENCE ISOLATES OF *P. FALCIPARUM*

The isolates of *P. falciparum* used in this study were obtained from malaria patients and maintained in culture before cryopreservation for future use (Freese *et al*, 1991). The isolates RSA 2, 3, 5, 6, 9 and 14 were collected from the former KwaZulu on 28/1/85, 11/3/85, 12/3/85, 10/7/84, 2/9/86 and 4/2/87 respectively and MW2 was isolated on 22/7/86 from a patient who imported malaria from Malawi. These reference isolates have been characterized previously according to their isoenzyme types (Freese & Markus, 1990), antigen variants (Freese *et al*, 1990) and drug sensitivity (Freese *et al*, 1991; Freese, 1994). Little variation among these isolates was revealed by the isoenzyme-typing technique (Freese & Markus, 1990), and the antigenic composition of most of them showed marked differences compared to those obtained in other areas (Freese *et al*, 1990). The responses of these reference isolates to chloroquine were as follows: RSA 2, 3, 5 were sensitive to the drug and RSA 6, 9, 14 and MW2 were found to be resistant (Freese *et al*, 1991).

1.5 OBJECTIVES

The objectives of this study are:

1. To produce gametocytes *in vitro* from reference isolates using different methods of stimulation.

2. To compare gametocyte production rates and exflagellation in these isolates.

3. To examine the effect of pyrimethamine on gametocytogenesis, exflagellation and asexual growth of *P. falciparum*.

4. To investigate the action of chloroquine alone, and in combination with pyrimethamine, on asexual stages of *P. falciparum*.

The study was conducted with the hope that the results will help in future malaria research in southern Africa. If mature gametocytes can be maintained under laboratory conditions for long enough, this would enable further studies involving the investigation of current and new drugs aimed at the sexual stages of the parasite and studies involving the infectivity of these gametocytes to mosquitoes.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Gametocytes are formed during the erythrocytic phase of the malaria parasite, *P. falciparum*, in the human host (Olsen, 1974). For the parasite to complete its life cycle, a second host, the mosquito is required. It is in this second host called the **vector**, that the gametocytes undergo further development by producing gametes which, upon fertilization of the macrogametes by microgametes, develop into a zygote. Following a series of divisions and developmental stages oocysts are formed which produce transmission stages, the sporozoites.

Earlier experiments had enabled production of gametocytes *in vitro*. From the observations of other workers it is possible to perform experiments on *P. falciparum* parasites and study their life cycle, especially the blood stages. Trager & Jensen (1976) developed techniques for culturing the blood stages of *P. falciparum in vitro*. Their methods were later modified by other investigators to optimise gametocyte production. According to Vanderberg (1988), gametocytogenesis *in vitro* involves two processes, the induction process which results in the formation of gametocytes from asexual parasites and the development of these gametocytes into mature and infective parasites.

2.2 CULTURE TECHNIQUES

Various techniques have been developed for culturing *P. falciparum* asexual blood stages. Jensen (1988) classified these techniques into two, that of short-term cultivation of human and numerous animal species of malaria, and that of the continuous cultivation of *P. falciparum*. **Rocker-dilution** procedures were used for short-term cultures, but these were found to be useless for *P. falciparum* which spends most of its 48-hour growth cycle of erythrocytic schizogony attached to the endothelium of the capillaries (Jensen, 1988).

RPMI 1640 culture medium replaced the standard **'Harvard medium'** which was used with a **custom built flow-vial** method. This method became outdated after the RPMI 1640 medium was developed (Jensen, 1988). The common method suitable for continuous culture is the **petri dish candle jar** method (Jensen & Trager, 1977). Here the complete medium (discussed later in this chapter) is used to dilute a suspension of infected and uninfected red blood cells to make a 12% erythrocyte suspension. Aliquots (1.5mℓ) of this suspension are then dispensed into 35mm plastic petri dishes. The dishes are placed in a glass desiccator with a candle. The candle is lit and the cover put on with the stopcock open. When the candle flame goes out the stopcock is closed and the jar is set at 37°C. However, observations involving maintaining *P. falciparum* in continuous culture showed that the petri dish candle jar method was not suitable for growing southern African isolates (Freese *et al*, 1988a). The method developed by Jensen (1981) also for maintaining continuous cultures of *P. falciparum* is said to be very simple and requires no peristaltic pumps or special filters to change the medium. This technique requires a daily maintenance of about 30 minutes.

Several variations of this system have been developed using tissue culture flasks of different volumes in place of glass vessels. A **semi-automated** cultivation apparatus for the *in vitro* culture of *P. falciparum* has been described by Ponnudurai *et al* (1982b). This method enables the change of the culture medium at preset intervals, but it is very expensive in design and construction. It can be used for synchronization of parasites (Ponnudurai *et al*, 1986). In all the culture techniques used, the important ingredient is the mixture of gases used in the range 2-5% carbon dioxide, 3-8% oxygen and the balance being nitrogen (Jensen, 1988).

2.3 PRODUCTION OF GAMETOCYTES

2.3.1 Isolates

The choice of a suitable isolate is of utmost importance because some isolates are known to have never produced gametocytes (Bhasin & Trager, 1984). According to Ponnudurai *et al* (1986) a good isolate should be sensitive to chloroquine, produce a reasonable number of gametocytes over a relatively long period and have asexual parasites which multiply relatively slowly allowing the sexual stages to mature over a two-week period. This is true of our southern African chloroquine-sensitive isolates of *P. falciparum* maintained in the Durban laboratory of the Medical Research Council, namely, RSA 2, RSA 3 and RSA 5 (Freese *et al*, 1988b). These isolates produce a large number of gametocytes when stimulated *in vitro*. Some studies have suggested NF54, NF7 and NF36, Tanzanian isolates I/CDC, Honduras I/CDC and Brazilian Ituxi 084 and IMTM22 to be good isolates (Vanderberg, 1988).

Graves *et al*, (1984) performed experiments comparing the rates of gametocyte production by five isolates of *P. falciparum*: two Brazilian and three African. They (Graves *et al*, 1984) showed that the isolates differed in their intrinsic rates of gametocyte production. Ituxi 084 was found to be significantly different from the other four isolates.

2.3.2 Culture medium

The widely used RPMI 1640 medium is supplied in two forms, liquid and powder (Jensen, 1988) and medium is prepared by using the method described by Jensen & Trager (1977). Briefly, the medium is dissolved in redistilled water and to it is added 5.95g N-2-hydroxy-ethylpiperazine-N-2-ethane-sulfonic acid commonly known as **HEPES** to give a final concentration of 25mM with a pH of 6.75. The solution is diluted to 960mℓ and sterilized through a 0.22μm millipore filter. It is dispensed aseptically in 96mℓ amounts and stored at 4°C. For use 96mℓ medium is supplemented with 4.2mℓ of 5% $NaHCO_3$ solution to give a medium without serum known as the **incomplete** medium.

2.3.3 Serum

To make the **complete** medium, 10% human serum is added to the incomplete medium (Jensen & Trager, 1977). The most widely used serum is type AB-positive or type A-positive serum (Carter & Miller, 1979; Jensen, 1988). Freese *et al* (1988a) used human type A-positive serum for routine cultures and AB-positive serum in newly initiated cultures to avoid damaging the infected cells of the patient. The serum is stored at -20°C.

2.3.4 Red blood cells

For *P. falciparum* cultures, the readily available O-positive and A-positive erythrocytes are used. Human O-positive cells are collected in lithium-heparin and stored as whole blood at 4°C for up to a week after collection (Freese *et al*, 1988a). Before use the blood is washed twice in incomplete medium.

2.3.5 Cultivation procedure

Mature gametocytes can be produced by using the static method described by Ponnudurai *et al* (1982b) or by the synchronization method described by Ponnudurai *et al* (1986). In the static method the parasites are cultured in tissue-culture flasks containing about 5% suspension of infected cells. Normal red blood cells and the culture medium are added. The flasks are gassed with 4% CO_2, 3% O_2 and 93% N_2. The lids of the flasks are tightly closed. The culture flasks are incubated at 38°C. When gametocytogenesis has been stimulated, the red blood cells are not replenished and during each daily medium change the flasks are handled with care to avoid disturbing the cells. Care is taken to ensure that the cultures are not exposed to low temperatures.

The stages of maturity of gametocytes are observed daily for three weeks. Gametocyte counts are made per 10 000 red blood cells. When using the synchronization method the cultures are harvested on day four after normal daily medium change. A 10% suspension of the cells is mixed with an equal amount of sterile 2% (w\v) gelatin (300 bloom). The mixture of gelatin-cells is allowed to stand for 30 minutes in a waterbath at 37°C. The supernatant fraction of the mixture containing schizont-trophozoite mixture is washed twice by centrifugation in culture medium.

Washed, fresh cells are added to the pellet to reduce the parasitaemia which is then suspended in complete medium to 5% and placed in a tissue culture flask which is gassed and incubated at 38°C. N-Acetyl glucosamine at a final concentration of 50mM is added 12 hours later. The medium containing the above concentration replaces the normal medium for the next 72 hours. Four to five hours after N-acetyl glucosamine is added a blood smear is made.

2.4 INFECTIVITY TO MOSQUITOES

Infectivity of cultured gametocytes of *P. falciparum* to mosquitoes depends on the choice of appropriate isolate with facilities for cryopreservation, good techniques for initiation of cultures, and the quality of serum used for culture and inclusion in the feed material for mosquitoes (Ponnudurai *et al*, 1989b). The species of the mosquito also plays an important role in infectivity (Klein *et al*, 1991a). When infecting mosquitoes, the culture medium containing mature gametocytes is fed to mosquitoes through membrane feeders usually under a water-jacket at 37°C for about 10 minutes (Burkot *et al*, 1984; Dearsly *et al*, 1991; Ponnudurai *et al*, 1982a). After the blood meal, the mosquitoes are maintained at 28°C on sugar water (Burkot *et al*, 1984) or a diet of raisins (Smalley & Sinden, 1977). After a few days the numbers of oocysts and sporozoites are counted in one of the following ways:

1. dissect the mosquitoes, fix in 10% formalin, dehydrate in 70% ethanol, stain in haematoxylin and examine their guts (Beier *et al*, 1991: Dearsly *et al*, 1991; Smalley & Sinden, 1977; Vermeulen *et al*, 1983).

2. use of monoclonal antibodies specific for the circumsporozoite (CS) protein by enzyme-linked immunosorbent assay (ELISA) (Klein *et al*, 1991a), immuno-radiometric assay (IRMA); these are known to detect as few as 100 sporozoites per mosquito, nitrocellulose (NC) membrane and immunofluorescence antibody assay (IFAA) which can be used to identify the species of mosquito involved in rodent and human malaria (Delves *et al*, 1989; Petros *et al*, 1989).

2.5 EFFECT OF SPORONTOCIDAL DRUGS ON INFECTIVITY

Young gametocytes are destroyed by all blood schizontocidal drugs. Mature gametocytes are sensitive to 8-aminoquinolines such as pamaquine and primaquine (Carter & Graves, 1988). The action of these drugs is restricted to gametocytes circulating in the blood and they do not affect gametogenesis, fertilization or sporogonic development. In some case studies, gametocytes from malaria patients have been shown to infect mosquitoes 20 days after treatment with chloroquine, quinine and quinine plus tetracycline (Klein *et al*, 1991b).

When patients were treated with primaquine, they did not demonstrate gametocytes or had abnormal gametocytes in their blood 24 hours after treatment and these gametocytes were not infective to mosquitoes (Klein *et al*, 1991b). However, not all antimalarials have sporontocidal properties. Chloroquine does not demonstrate sporontocidal or gametocytocidal activity on mature gametocytes (Herbst, *et al*, 1985) because chloroquine and other 4-aminoquinolines interfere with digestion of haemoglobin by the malaria parasites whether asexual or gametocyte. Once the parasites have passed the stage of digestion of haemoglobin at about six days of age, gametocytes of *P. falciparum* are no longer affected by chloroquine (Carter & Graves, 1988; Smalley & Sinden, 1977).

In South Africa pyrimethamine was used in combination with chloroquine for both prophylaxis and treatment of malaria until recently, presumably for its sporontocidal effect. Pyrimethamine and other parasite-specific inhibitors of dihydrofolate reductase and sulfonamides both interfere with the formation of precursors of DNA synthesis by the parasite when it recommences during sporogonic phase in the mosquito (Carter & Graves, 1988). When DNA synthesis has stopped and drug administration is discontinued, the gametocytes reach maturity and they regain their infectivity to mosquitoes.

The sporontocidal activity of pyrimethamine was confirmed by Teklehaimanot *et al* (1985). They found that pyrimethamine had a sporontocidal effect on the strains of *P. falciparum* that were sensitive to the drug but not on the resistant ones. Freese *et al* (1991) found that the asexual blood stages of the majority of southern African isolates tested had a reduced susceptibility to pyrimethamine *in vitro*. However, it is not known whether these culture-adapted isolates are resistant to the sporontocidal effect of pyrimethamine. Since the efficacy of pyrimethamine on its own has decreased dramatically, alternative drugs with tolerable side effects need to be investigated.

Chloroquine resistant *P. falciparum* has been reported in eastern Asia and South America (Wernsdorfer, 1991). Resistance to the sulfadoxine/pyrimethamine combination has been reported in some areas (Wernsdorfer, 1986; Watt *et al*, 1987). The question of drug resistance in *P. falciparum* is a problem not only to southern Africa (Freese *et al*, 1988b; 1991; Herbst *et al*, 1987) and other parts of Africa (Brasseur *et al*, 1987; Desjardins *et al* 1988; Freese, *et al*, 1988b; Hellgren *et al*, 1987), but to the whole world (Wernsdorfer, 1991).

The responses of *P. falciparum* isolates to sporontocidal drugs other than pyrimethamine need further investigation and the effects of these drugs on the gametocyte infectivity to mosquitoes also require future attention.

CHAPTER 3

GAMETOCYTE PRODUCTION

3.1 INTRODUCTION

Gametocytogenesis involves two important processes, the first is the development of the asexual stages and the second, maturity of the gametocytes. There are three main factors that influence the production of gametocytes of *P. falciparum* in culture; 1) the type of isolate used, 2) the duration of culture and 3) the conditions under which the culture is maintained (Vanderberg, 1988). The objectives of this study were:

-to stimulate gametocytogenesis by the static and the synchronisation methods,

-to compare the static and synchronization methods on the basis of gametocytogenesis and

-to characterize the southern African isolates of *P. falciparum* according to their production of gametocytes.

3.2 MATERIALS AND METHODS

3.2.1 Isolates of *Plasmodium falciparum*

Four southern African isolates; RSA 2, RSA 3, RSA 5, MW2 and one Amsterdam Airport strain (NF54) were used. The four southern African isolates were collected in lithium-heparin from malaria patients in the KwaZulu-Natal province (Freese *et al*, 1991) and NF54 (Ponnudurai *et al*, 1982b) was obtained from the Department of Immunology and Infectious Diseases, John Hopkins University, Baltimore, USA. These isolates were removed from cryopreservation and thawed at 37°C.

The infected cells were immediately washed successively in 12% sodium chloride (0.2 mℓ/1 mℓ cells), 1.6% sodium chloride (9 volumes) and 0.9% sodium chloride plus 0.2% glucose (9 volumes) before being cultured (Freese *et al*, 1988a).

3.2.2 Uninfected red blood cells

Human O-positive cells obtained from various donors working in the Malaria Laboratory of the South African Medical Research Council (Durban), were collected in lithium-heparin and stored as whole blood at 4°C. Before use, the cells were washed twice by centrifugation in incomplete culture medium (see Chapter 2). Care was taken when removing the buffy coat (Freese *et al*, 1988a).

3.2.3 Serum

Human A-positive serum obtained from "the Border Blood Transfusion Services", East London, was used in all the cultures. The serum was stored at -20°C.

3.2.4 Culture medium

The RPMI 1640 medium (Flow laboratories, Irvine, Scotland) buffered with HEPES was prepared following the method described by Jensen & Trager (1977) (see Chapter 2) and supplemented with glucose (4g/ℓ), hypoxanthine (44mg/ℓ), gentamicin (50mg/ℓ) and 10% human serum (Freese *et al*, 1988a; 1991).

3.2.5 Cultivation procedure

All the above ingredients were warmed to 37°C before culturing. The cultures were initiated in 50mℓ tissue-culture flasks containing 5mℓ of a 5% suspension of infected cells in the culture medium. The flasks were gassed with a mixture of 3% O_2, 4% CO_2, and 93% N_2 for 10 seconds (Fig. 3) and the lids were tightly closed. The flasks were then incubated at 38°C on a shaker platform. The shaker was switched off two hours before each daily medium change to allow the erythrocytes to settle. The flasks were gently tilted to allow spent medium to be aspirated off. Fresh, warm medium was added to the flasks which were then gassed before incubating them. The cultures were diluted with fresh erythrocytes every three to four days. Parasite growth was assessed by examining Giemsa stained smears prepared from settled cells. Parasite counts (Fig.4) were made daily per 10 000 red blood cells under X 1000 oil immersion microscopy. When the parasitaemia reached about 5%, the cultures were transferred to 250mℓ tissue-culture flasks (15mℓ medium, 5% haematocrit) and maintained as before.

3.2.5.1 Static cultures

Gametocytogenesis was stimulated in static culture flasks when the parasitaemia had reached 5% or more. The cultures were initiated at 5% haematocrit and the infected cells were diluted with O-positive red blood cells to 0.3-0.5% parasitaemia. No erythrocytes were added to these cultures once the experiment was set up. The medium was changed daily, care being taken not to disturb the settled layer of cells and exposure to lower temperatures kept to minimum. The flasks were maintained for up to 21 days. Gametocytaemia was determined daily per 10 000 red blood cells counted from Giemsa-stained smears.

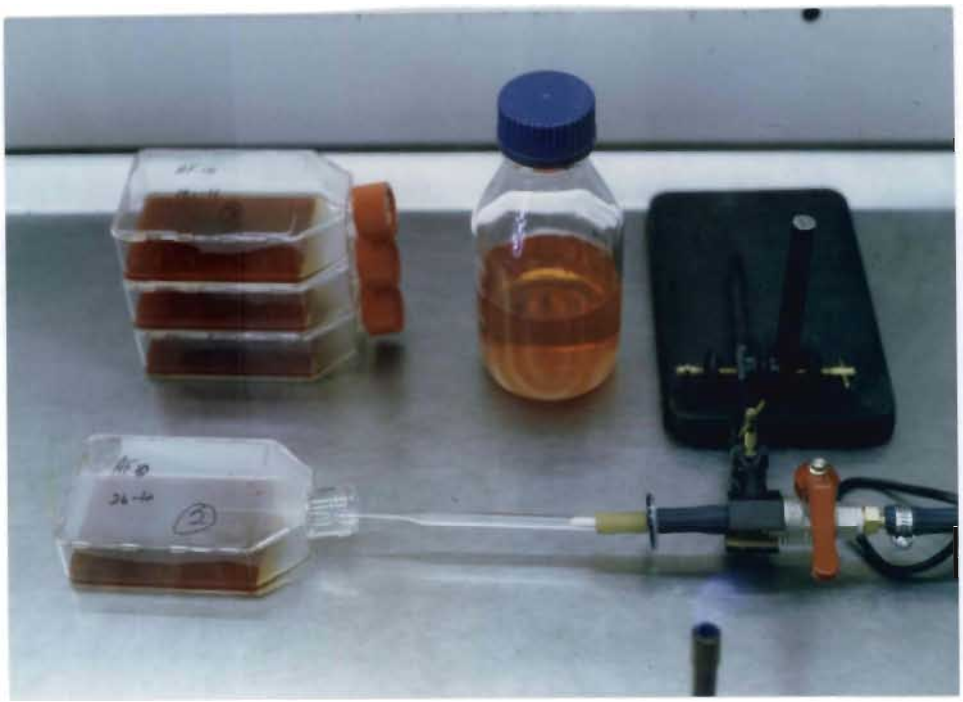
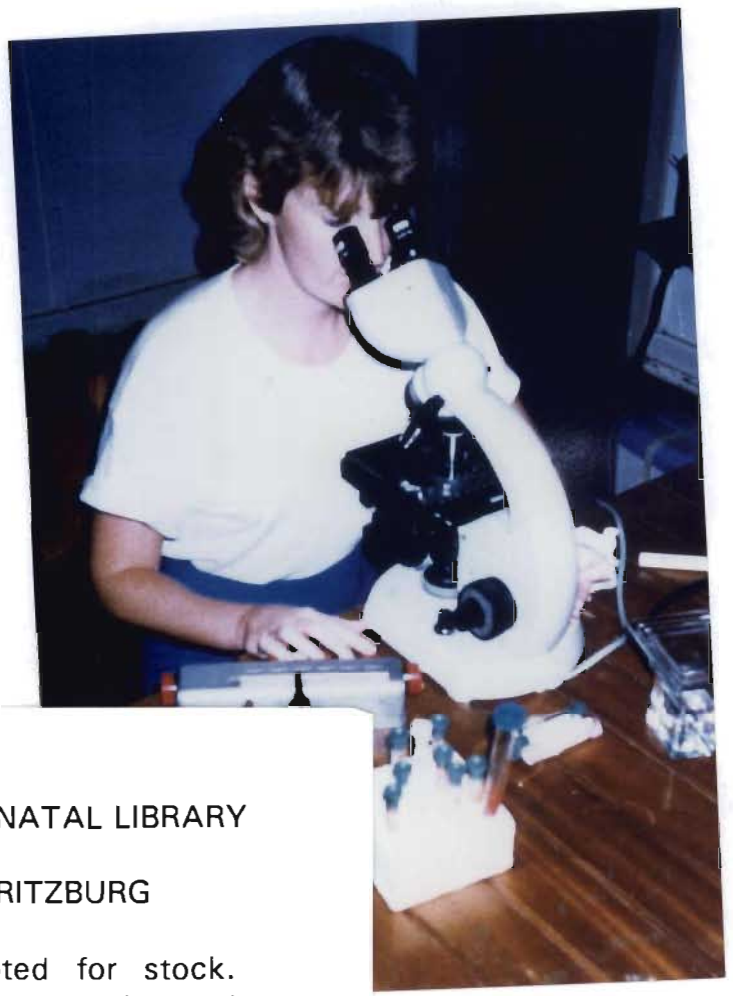


Fig. 3. Culture system, tissue-culture flasks being gassed with a mixture of 3% O_2, **4% CO_2 and 93% N_2.**



blood samples for presence of *Plasmodium*

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3.2.5.2 Synchronized cultures

In some cultures gametocytogenesis was stimulated by the synchronization technique as described by Ponnudurai *et al.* (1986). Briefly when the cultures had reached 5-10% they were harvested and a 10% suspension of cells was mixed in a 15mℓ test tube with an equal amount of sterile 2% gelatin (300 bloom) in incomplete medium.

This mixture was left for 30 minutes in a water bath at 37°C to allow the blood stages to separate. The supernatant fraction of the mixture which contained mainly schizonts, was washed twice in culture medium by centrifugation. Fresh, washed and warm red blood cells were added to the pellet to reduce the parasitaemia to 5% and increase the haematocrit to 5%. The cultures were put back into the culture flasks and incubated at 38°C after gassing with a mixture of 3% CO_2, 4% O_2 and 93% N_2.

Twelve hours later, N-acetyl glucosamine (NAG) at a final concentration of 50mmol/ℓ, was added to the cultures to eliminate the trophozoite and the schizont stages, leaving the ring stages intact. This NAG-containing medium at the above concentration replaced the normal medium for the next three days. A smear was prepared four to five hours after the initial addition of NAG. These cultures were maintained for 21 days and daily gametocyte growth was monitored by examining Giemsa-stained smears. For statistical analysis of the results refer to Chapter 5 section 5.2.1.

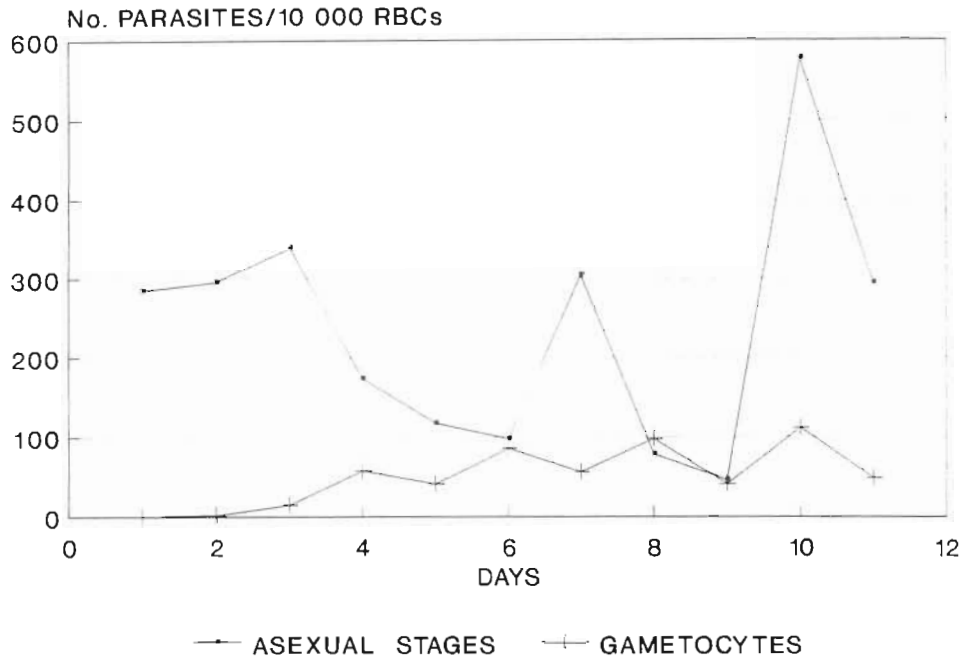
3.3 RESULTS

Figures 5-9 show the numbers of asexual parasites and gametocytes (all stages)/10 000 red blood cells produced in static cultures by RSA 2, 3, 5, MW2 and NF54 respectively. Isolate RSA 2 was maintained in culture for 11 days, isolates RSA 3, 5 and NF54 were cultured for 21 days and MW2 for 10 days. Isolate RSA 2 reached peak gametocytaemia of 1% on day ten (Fig. 5), while three isolates, RSA 3, 5 and NF54, attained their highest number of gametocytes (5.8%, 4.5% and 1.8% respectively) after eight days. Isolate MW2 was the only exception with peak gametocytaemia of 1.25% within two days. All the isolates showed a similar pattern: gametocytaemia increased with a decrease in the asexual stages and vice versa (Figs. 5-9).

Figures 10-14 compare the numbers of gametocytes produced/10 000 RBCs in static and synchronized cultures by NF54, RSA 2, 3, 5 and MW2 respectively. A peak gametocytaemia of 11% was obtained in the synchronized cultures of isolate NF54 (Fig. 10). Figure 11 shows the highest number of gametocytes on day one for synchronized cultures than in the static for isolate RSA 2. The remaining three isolates (RSA 3, 5 and MW2) produced high gametocytaemia (of 3.1%, 6.8% and 2.4%) on days 19, eight and two respectively (Figs. 12, 13 and 14). The two methods of gametocyte stimulation, the synchronization and the static, compared as follows :

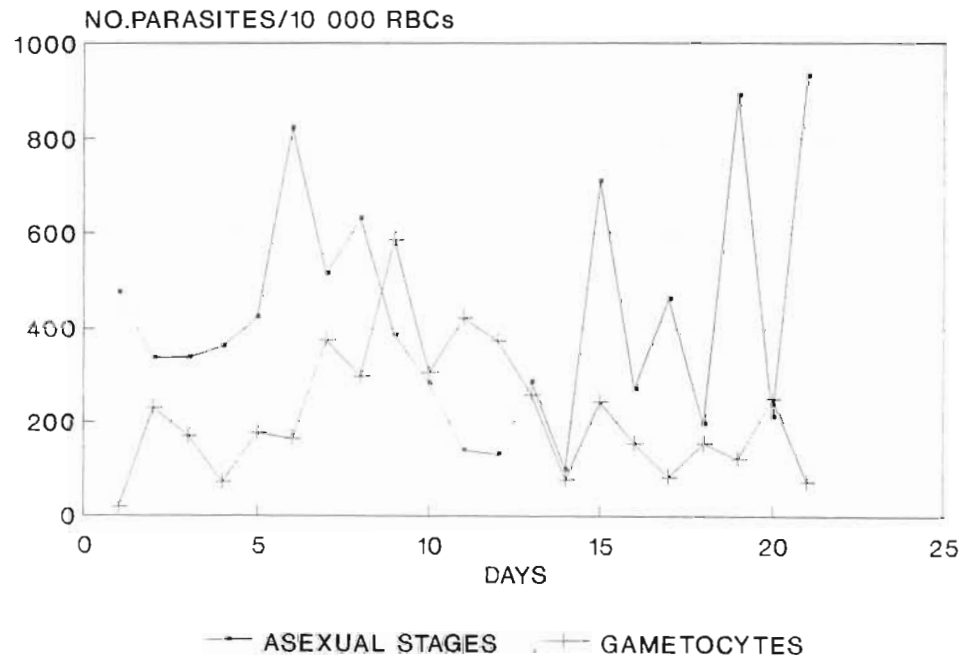
For RSA 2 and RSA 5 (Figs 11, 13), there were no statistically significant differences between the two methods (Appendix 1). For RSA 3, more gametocytes were produced in the static than in the synchronized cultures (Fig. 12) and there was a statistically significant difference between the two methods at p-value of <0.01. (Appendix 1).

FIG.5. Parasite counts of isolate RSA2



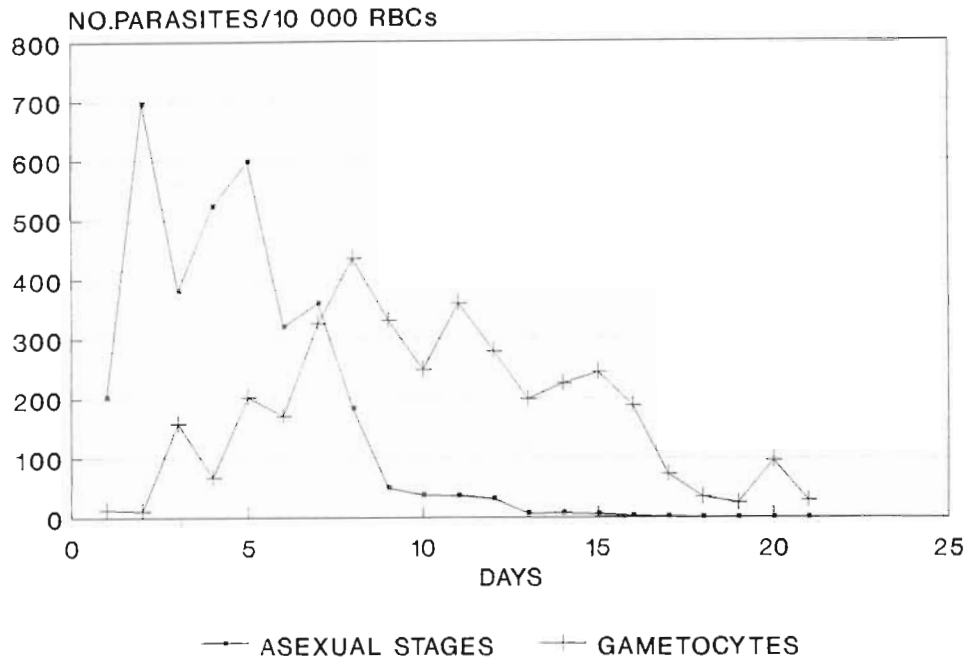
Static cultures

FIG.6 Parasite counts of isolate RSA3



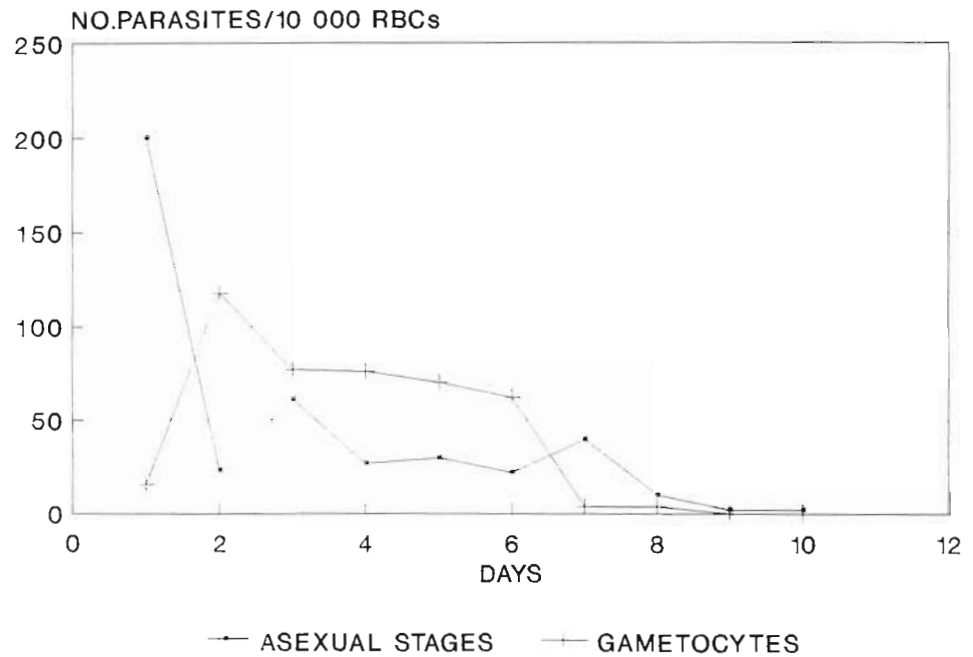
Static cultures

FIG.7. Parasite counts of isolate RSA5



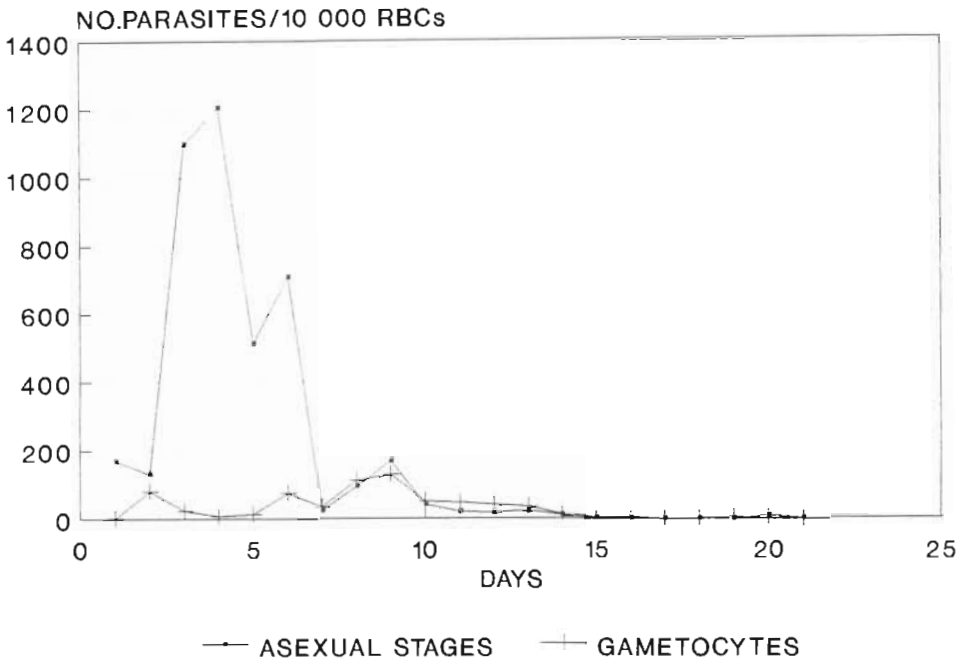
Static cultures

FIG.8. Parasite counts of isolate MW2



Static cultures

FIG.9. Parasite counts of isolate NF54



Static cultures

FIG.10.Gametocyte counts of isolate NF54

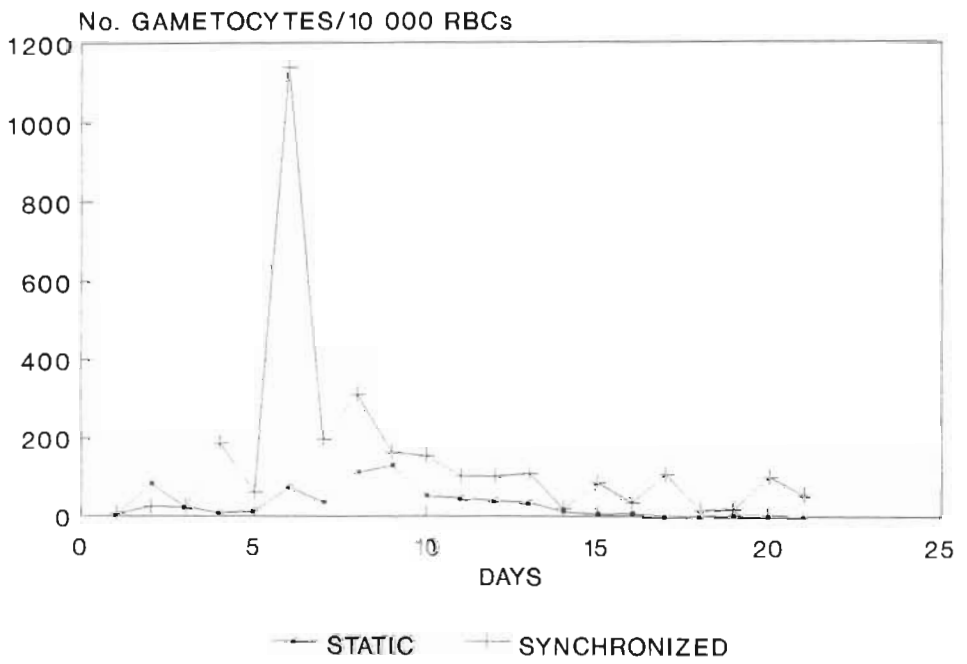


FIG.11.Gametocyte counts of isolate RSA2

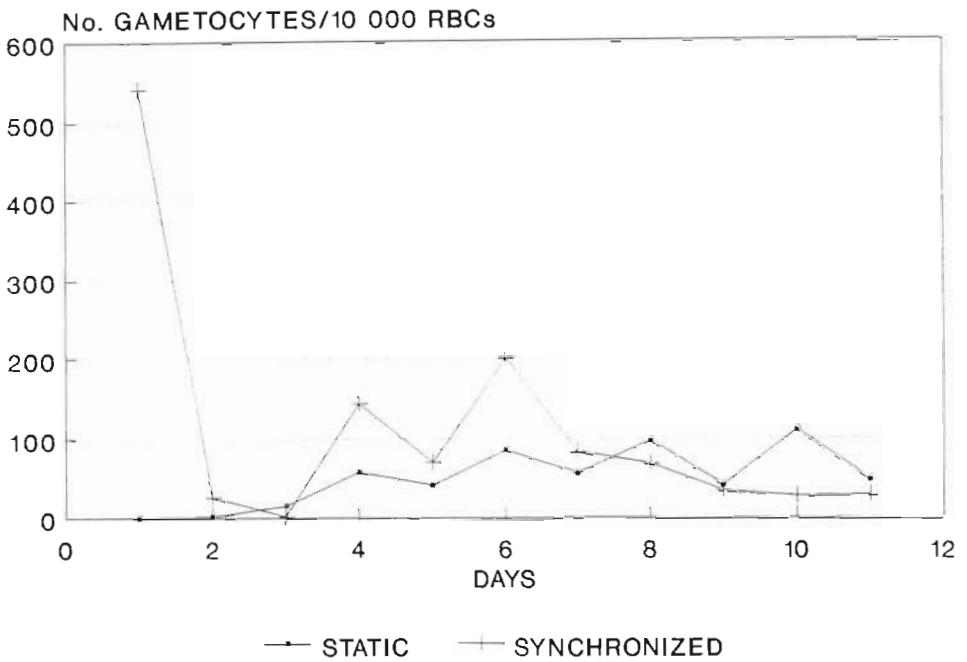


FIG.12.Gametocyte counts of isolate RSA3

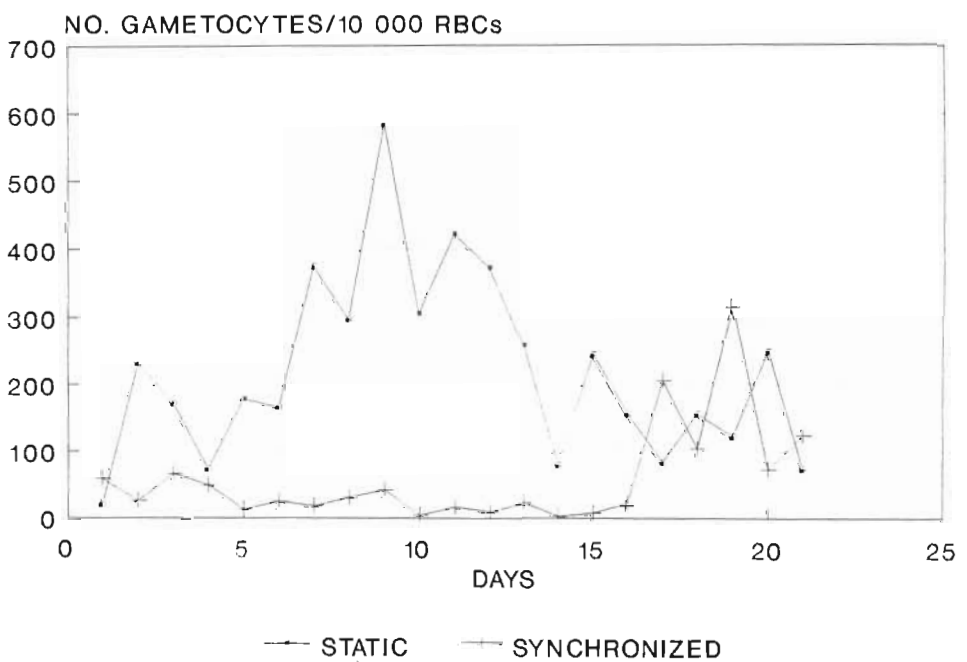


FIG.13.Gametocyte counts of isolate RSA5

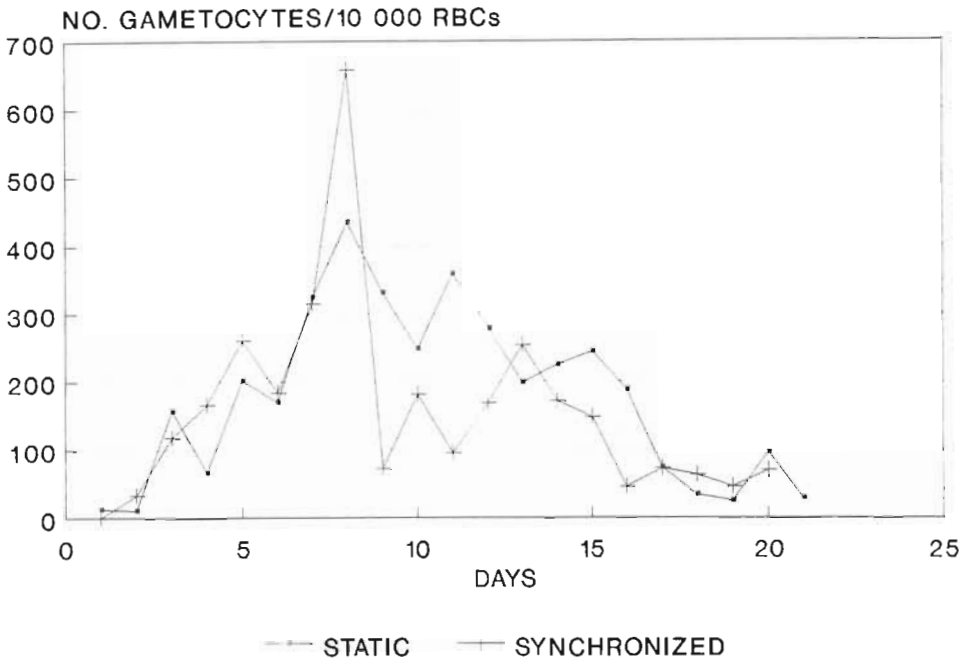
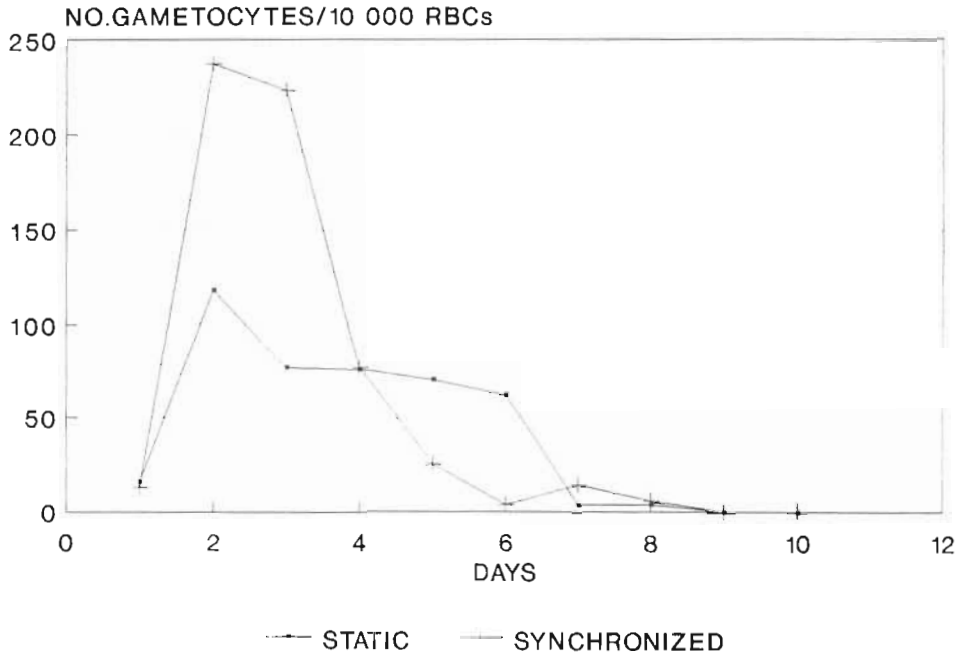


FIG.14. Gametocyte counts of isolate MW2



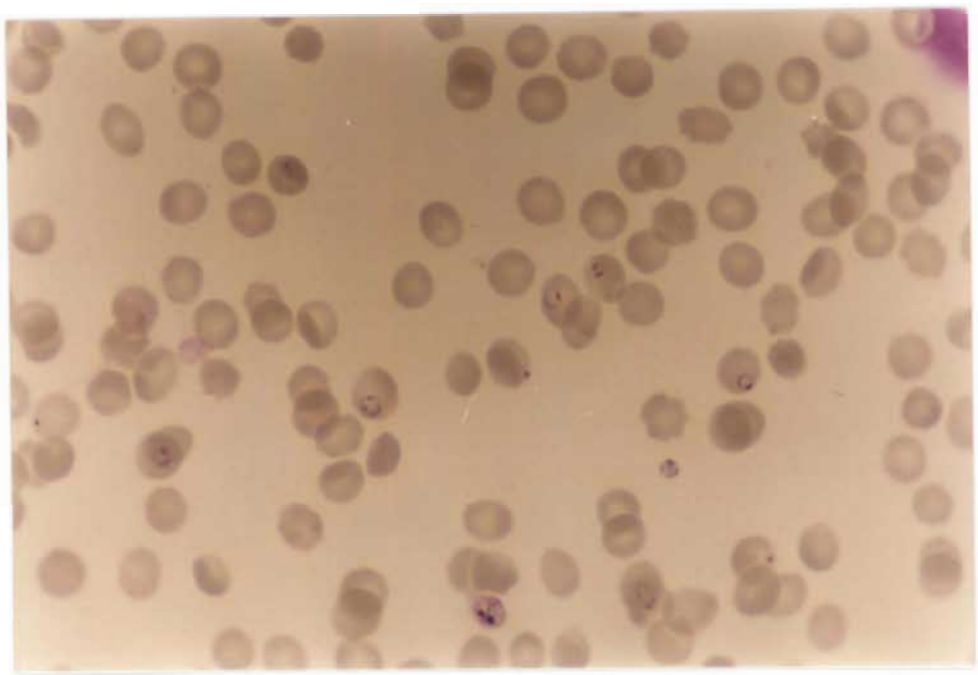


Fig. 15. The asexual blood ring stage. The micrograph was taken from *in vitro* static cultures of *P. falciparum* isolate RSA 2 (X1000 magnification).

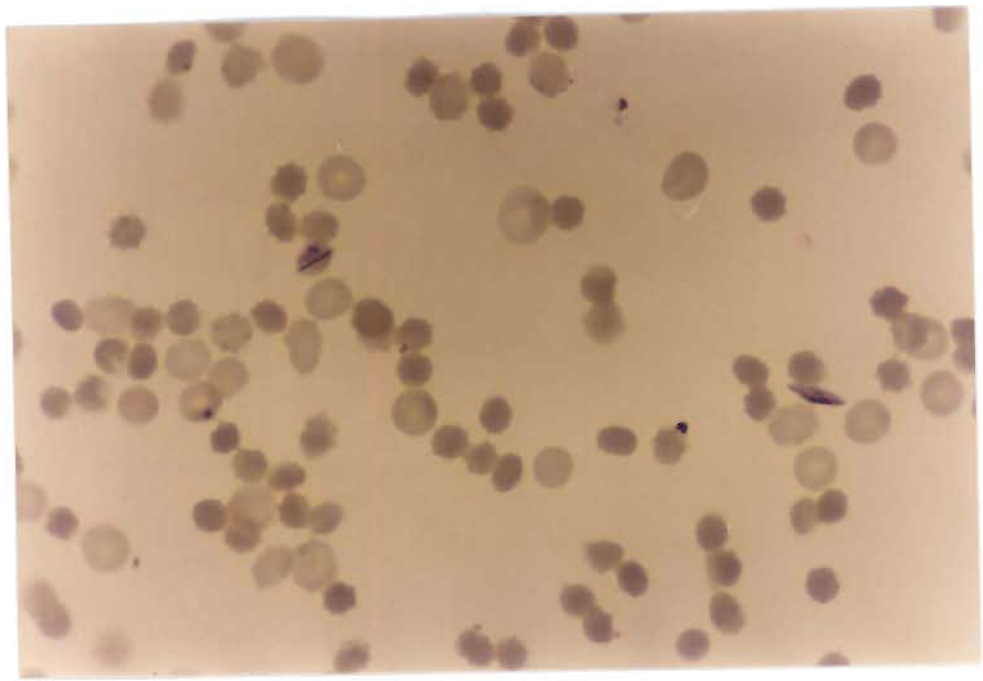


Fig.16. Gametocyte stage II (on the left) and stage III (on the right). The micrograph was taken from *in vitro* static cultures of *P. falciparum* isolate RSA 2 (X1000 magnification).

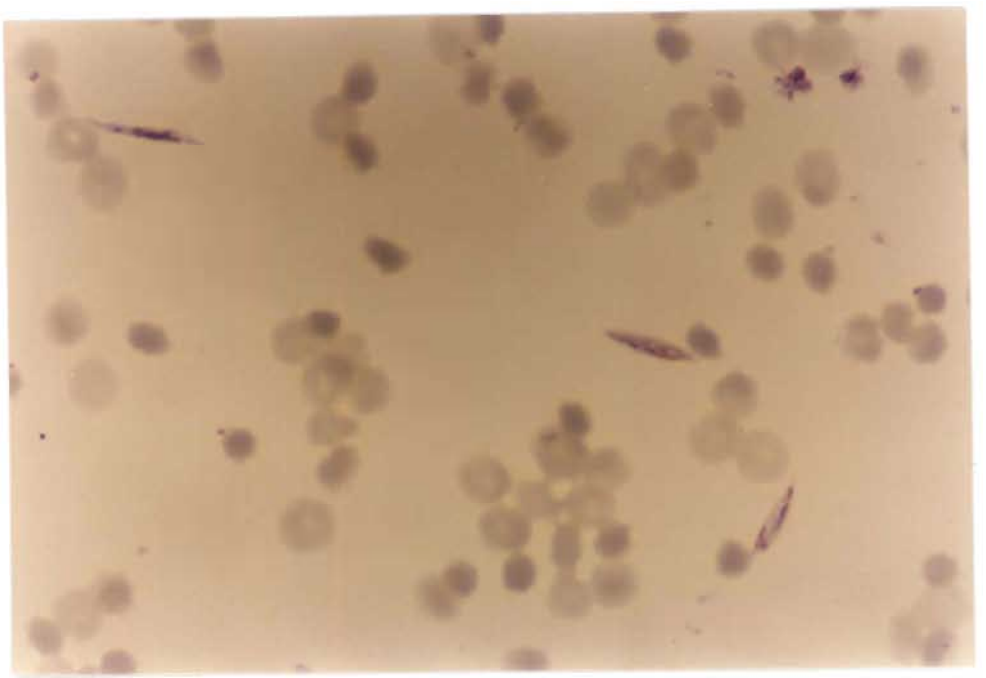


Fig. 17. Gametocyte stage IV (see Chapter 1 for description). The micrograph was taken from *in vitro* static cultures of *P. falciparum* isolate RSA 2 (X1000 magnification).

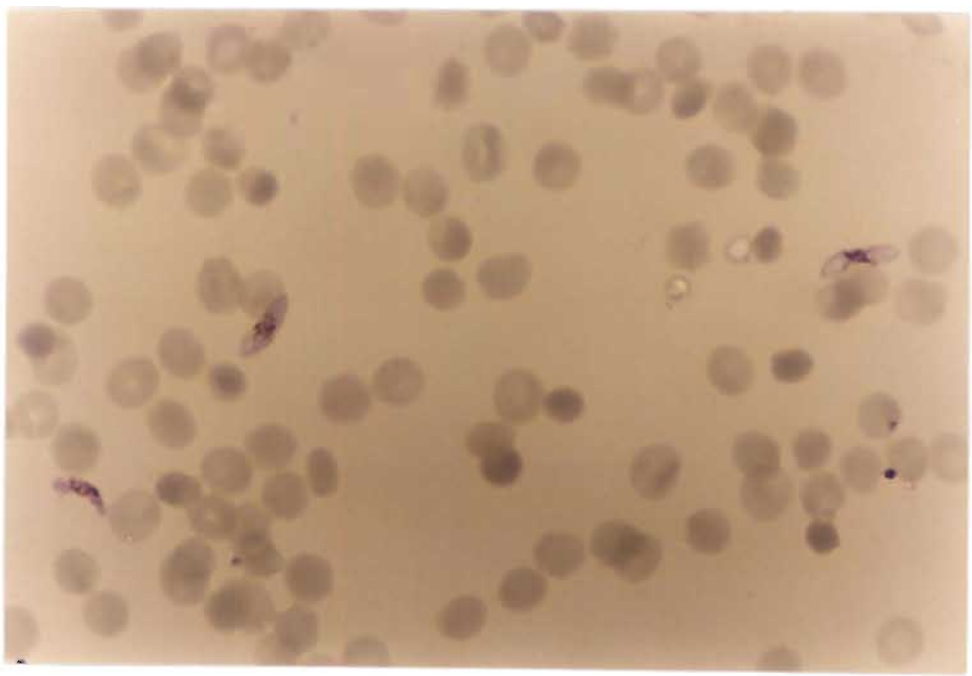


Fig. 18. Mature stage V gametocyte. The micrograph was taken from *in vitro* static cultures of *P. falciparum* isolate RSA 2 (X1000 magnification).

Higher numbers gametocytes were obtained in the synchronized than the static cultures of isolates NF54 and MW2 (Figs. 10 and 14 respectively). Appendix 1 shows that there were statistically significant differences between the two methods of gametocyte stimulation for both MW2 and NF54 ($p<0.05$ each). Figures 15-18 show the stages of the gametocyte development of *P. falciparum* (as described in Chapter 1, section 1.2.1) taken from the static cultures of the isolate RSA 2. The Stage I gametocyte is not shown because there is no clear distinction between the Stage I and the trophozoite morphologies.

3.4 DISCUSSION

Most isolates attain their peaks after eight days. The only exception was MW2 which reached a peak of 1.25% within two days. All the gametocyte stages (Figs. 15-18) were observed for both methods of stimulation. For the isolates RSA 2, RSA 3 and RSA 5 the gametocytes reached maturity within seven days of stimulation (Ponnudurai *et al*, 1986). This means that their ability to produce gametocytes is not reduced like MW2 whose gametocytes did not reach maturity within 21 days.

The fact that gametocytes were produced from isolate MW2 at all indicates that this isolate can still be stimulated to produce. However, it appears that something in the culture system is preventing these gametocytes from maturing. This appears to be the same for the Amsterdam airport strain, NF54, which formed mature Stage V gametocytes on day 12 in the synchronized cultures only and these were in very, very low numbers, i.e. 0.01%. The Stage V gametocytes in this isolate did not last long, disappearing after two days, and no mature gametocytes could be detected afterwards.

This isolate is maintained in several laboratories around the world and is known to be a good gametocyte producer (Ponnudurai *et al*, 1982a). However, it seems that local gametocyte culture conditions need to be optimised for this strain. The asexual stages of this isolate, however, became adapted to local culture conditions after being cultured for four weeks.

3.5 CONCLUSION

Of the 19 southern African reference isolates of *P. falciparum*, the three chloroquine-sensitive strains (RSA 2, RSA 3 and RSA 5) have been shown to be good producers of gametocytes when stimulated under appropriate laboratory conditions. Although MW2 and NF54 do produce gametocytes, they do so at a much slower rate. Emphasis should be based on the culture system ingredients (serum, RBCs and culture medium) to try and establish the requirements for these isolates.

For RSA 2, 3 and 5, it does not really matter what method of gametocyte stimulation is used since no significant differences were found between them (Appendix 1). Isolates MW2 and NF54 responded readily to the synchronized method. The extent to which individual isolates produce gametocytes may be subject to some degree of environmental changes. Cloned lines of the isolates (though not investigated in this case) may play a vital role in their variation in gametocyte production (Graves & Carter, 1988). Gametocytes produced from routine cultures can be used to carry out studies involving many aspects of malariology such as drug tests and infectivity to mosquitoes.

Large numbers of gametocytes are required to develop our field of study further especially the biochemical, the epidemiological and the physiological studies since not much research involving gametocytes has been done. Gametocytes are the transmission stages from man to mosquito. Knowing more about these infective stages of the parasite, will help in the prevention and treatment of malaria thus reducing transmission.

CHAPTER 4

EXFLAGELLATION

4.1 INTRODUCTION

The successful maturation of gametocytes leads to the production of gametes. For this to occur the gametocytes must first be ingested by mosquitoes or demonstrated under *in vitro* conditions. During the gamete formation process, known as **exflagellation**, the male gametocyte forms exflagellated microgametes or the male gametes. Under *in vitro* conditions, exflagellation needs a lower temperature and higher pH than those for gametocyte development. Exflagellation may be observed in the presence of **fetal bovine serum** (FBS) adjusted to pH 8.0 with 1.5% $NaHCO_3$ at 26°C (Carter & Beach, 1977). Unlike gametocytes which take hours to develop, gametes are reported to be produced within 10-20 minutes of stimulation (Carter & Beach, 1977). The purpose of this part of the study was to investigate whether exflagellation could be stimulated under *in vitro* conditions from laboratory-produced gametocytes.

4.2 MATERIALS AND METHODS

4.2.1 Experimental procedure

Exflagellation was stimulated by the method described by Carter & Beach (1977) in static cultures of those isolates which produced mature Stage V gametocytes (RSA 2, 3 and 5) and in synchronized cultures of RSA 2 and 5. Exflagellation was not stimulated in MW2 and NF54 cultures or in the synchronized cultures of RSA 3.

These isolates produced young gametocytes which lasted up to day seven only and thereafter these immature gametocytes either became morphologically abnormal or disappeared. When the gametocytes had reached maturity, the cells were evenly resuspended in their own culture medium and 0.3mℓ of the suspension was dispensed in a serum tube containing 3.0mℓ Foetal Bovine Serum (FBS) adjusted to pH 8.0 with $NaHCO_3$. The cells were then sedimented by centrifugation for 30 seconds. Most of the supernatant was removed leaving only a minimal amount to mix with the sediment. One drop of this cell-FBS mixture was placed on a slide covered with a coverslip and stored in a humid chamber at 26°C.

The preparation was observed under a phase-contrast microscope at X400 magnification (Carter & Beach, 1977; Dearsly *et al*, 1990; Sinden *et al*, 1984; Vermeulen *et al*, 1983) at 15 minutes intervals for 60 minutes. The numbers of exflagellating parasites were counted in 40 microscopic fields. In other experiments, the FBS was not used and gametocytes were suspended in the culture medium containing 10% human serum. This was initiated in two samples of isolate RSA 5 cultures. Exflagellation was assessed by counting the total number of exflagellations after 60 minutes in 30 microscopic fields.

4.2.2 Statistical analysis

A two-way Analysis of Variance was used to compare the isolates, RSA 2 and RSA 5. The two classification variables were ISOLATE and TIME. To avoid confusion the analysis is not shown here only the p-values are mentioned in the results section.

4.3 RESULTS

Exflagellation was observed in the static cultures of all the three isolates i.e. RSA 2, 3 and 5 (Table 1). In the synchronized cultures (Table 2), RSA 2 produced more exflagellations within 60 minutes than RSA 5. The difference was statistically significant at $p<0.0120$. However, the difference between the total number of exflagellations produced within 60 minutes in the static and synchronized cultures of each of RSA 2 and 5 respectively was not statistically significant.

The number of exflagellating parasites increased with an increase in observation time (Tables 1-2). Table 3 shows the total number of exflagellations counted after 60 minutes in both the static and the synchronized cultures of RSA 5. More exflagellations were obtained in sample 2 (67 for static and 204 for synchronized) than in sample 1 (55 for static and 99 for synchronized). Furthermore, more exflagellations were produced in the synchronized (99 and 204) than in the static (55 and 67) cultures of both samples (Table 3).

Because of the small sample size, the results obtained in the presence of **FBS** were not compared to those obtained when FBS was not added to the cultures. However, the total number of exflagellations obtained in the presence of FBS in static cultures was 60 compared to 55 and 67 obtained in the samples respectively (Tables 1 and 3). Higher total numbers of exflagellations were produced in the synchronized cultures without FBS than in the same cultures containing FBS (Tables 2 and 3).

Table 1. Number of exflagellations in 40 microscopic fields obtained in the presence of FBS in static cultures.

Observation Time (Minutes)	15	30	45	60	TOTAL
Isolate					
RSA 2	11	19	24	31	**85**
RSA 3	8	18	23	24	**73**
RSA 5	11	14	16	19	**60**

Table 2. Number of exflagellations in 40 microscopic fields obtained in the presence of FBS in synchronized cultures.

Observation Time (Minutes)	15	30	45	60	TOTAL
Isolate					
RSA 2	14	18	25	27	**84**
RSA 5	10	11	19	24	**64**

Table 3. The total number of exflagellations obtained in the absence of FBS after 60 minutes in both the static and the synchronized cultures of RSA 5 isolate.

	SAMPLE 1	SAMPLE 2
METHOD		
Static	55	67
Synchronized	99	204

4.4. DISCUSSION

Under laboratory conditions, exflagellation was demonstrated in RSA 2, 3 and 5 isolates. These three isolates produced mature gametocytes. These three isolates are also known to be chloroquine-sensitive (Freese *et al*, 1991). This indicates that all the three chloroquine-sensitive southern African isolates which originally came from the KwaZulu-Natal province can readily produce gametocytes which will under go exflagellation when stimulated. These results are consistent with those of Carter & Beach (1977) in that after 10 minutes exflagellation was observed. Furthermore, gametogenesis was observed both in the preparations containing FBS and human serum respectively.

The methods of gamete stimulation produced the same results for the same isolate (e.g. RSA 2 had 85 exflagellations per 40 microscopic fields after 60 minutes in the static system and 84 in the synchronized cultures), but there were marked differences in the total number of exflagellations within the synchronized culture system between RSA 2 and RSA 5 (84 total number of exflagellations produced after 60 minutes for RSA 2 and 64 for RSA 5) (Table 2). Although the two samples obtained from isolate RSA 5 were not statistically analyzed, more exflagellations were observed in sample 2 than in sample 1, and the synchronized cultures of both samples produced larger numbers of exflagellations than static cultures. Although the effect of FBS on gametogenesis was not statistically significant compared to that of human serum, Carter & Beach (1977) found no differences in the number of microgametes produced in either FBS or human serum. In contrast, Ponnudurai *et al* (1982a) discovered that more exflagellations were produced in the presence of FBS than in the cultures containing human serum.

4.5 COMMENTS

The next step following on from the experiments described in this Chapter, would be to try to infect mosquitoes with mature gametocytes in the laboratory. This would give an idea of how viable the gametocytes are, since it was observed (Vanderberg, 1988) that in some isolates of *P. falciparum* exflagellation never occurred but the gametocytes nevertheless produced heavy infections in mosquitoes. In other isolates where exflagellations were observed, the gametocytes did not become infective. Production of gametes *in vitro* can be regarded as an indication of successful gametocytogenesis, though not all gametocyte-producing isolates will readily undergo exflagellation. It would be interesting to investigate further the development of gametes *in vitro*. The remaining southern African isolates of *P. falciparum* should be stimulated to produce gametocytes and undergo exflagellation *in vitro*. Initiation of microgametogenesis using different sera requires further investigation since contradicting results were obtained previously on this subject.

CHAPTER 5

PYRIMETHAMINE TESTS

5.1 INTRODUCTION

Pyrimethamine was formulated with the primary aim of destroying all the asexual blood stages of the malaria parasite (Desjardins *et al*, 1988). This drug is not effective against sporozoites or mature gametocytes, but is effective against the young gametocytes of *P. falciparum*. It is also active against gametocytes of *P. ovale, p. vivax* and *P. malariae.* Pyrimethamine is also known to be effective against the primary exo-erythrocytic stages of all forms of malaria (Black *et al*, 1986; Desjardins *et al*, 1988). Pyrimethamine is effective as a drug because it interferes with the parasite's enzyme dihydrofolate reductase which is responsible for DNA synthesis, while it does not affect the same enzyme in the host (Carter & Graves, 1988; McCutchan, 1988).

It does however inhibit the enzyme histamine N-methyl transferase, which is responsible for the metabolism of histamine in mammalian tissues (Desjardins *et al,* 1988; Kemp *et al,* 1990). Like other antifolates, frequent administration of pyrimethamine over a long time may cause folate deficiency. It has been reported (Desjardins *et al*, 1988; Wernsdorfer, 1986; WHO, 1982) that a daily dose of 25mg taken weekly caused mild to moderate megaloblastic anaemia. Higher doses of this drug can cause side effects such as agranulocytosis. Pyrimethamine is an effective sporontocide but it has no effect on mature gametocytes or latent tissue stages (Black *et al*, 1986). The aim of this study was to investigate the effect of pyrimethamine on gametocytogenesis and exflagellation *in vitro*.

5.2 MATERIALS AND METHODS

The effect of pyrimethamine on gametocytes (all stages) was investigated in the established isolates of *P. falciparum* that had produced gametocytes i.e. RSA 2, 3, 5, MW2 and NF54 (Chapter 3) and undergone exflagellation viz. RSA 2 and RSA 5 (Chapter 4). For pyrimethamine-treated cultures, exflagellation was demonstrated on RSA 2 and 5 since MW2 and NF54 isolates produced only immature gametocytes in their cultures. The experiments were run in parallel to those set up in Chapters 3 and 4. For each reference isolate two tissue-culture flasks were prepared, one to serve as a control and the other for the drug test.

This can be termed **a seven-day drug test** because a known concentration of the drug was added to the flask each day for seven days, during stimulation of gametocytes. For RSA 2, MW2 and NF54, a culture medium containing 100nmol/ℓ pyrimethamine (Wellcome (PTY) LTD) solution prepared in 0.05% lactic acid was added during each daily medium change for seven days. For RSA 3 and 5, 3000nmol/ℓ of the drug was added. These concentrations were based on the isolates' reported **minimum inhibitory concentrations (MICs)** (Freese *et al*, 1991) which are defined as the lowest drug concentration at which no parasite growth occurred. The tissue culture flasks were gassed as normal and incubated in a static position at 38°C. The effect of pyrimethamine on the morphology of the different stages of gametocytes and the number of gametocytes was determined by examining Giemsa-stained smears. Parasite counts (all stages of gametocytes were counted) were made /10 000 red blood cells. When the gametocytes had reached maturity, in both the controls and the experiments, exflagellation was stimulated as described in Chapter 4.

5.2.1. Statistical analysis

5.2.1.1 Production of Gametocytes

All the different stages of gametocytes were counted. the data were first fitted to assess trends. It was clear from the trends in the data that polynomial regression had to be preformed. Different models were then fitted to the data, e.g. quadratic and cubic regression, using actual as well as log counts. The model with the best R^2 value, i.e. the best fitting model, was then used to describe the trend. In this case, cubic regression provided the best fit to the data. The model is described below:-

Production of gametocytes was assessed on different days using 4 different treatments, for 5 different isolates.

The treatments were:

a) Static treatment

b) Static control

c) Synchronized treatment

d) Synchronized control

The following analyses were done for each isolate separately:

1. Number of gametocytes/10 000 RBCs was plotted against time (in days) for the 4 treatments.

2. **A cubic regression model** on the log of the count, which proved to be the best fitting model, was fitted to each of the 4 curves. The **R^2 value** was calculated for each model to indicate how well it fitted the data.

3. From the cubic regression analysis, **predicted values** and **standard errors** were calculated.

The **least significant difference (LSD)** at the 5% level was calculated for the day that showed the greatest difference between treatments. The LSD was calculated for:

- Control v control
- Treatment v treatment
- Synchronized treatment v control
- Static treatment v control

LSD was calculated as follows:

$$LSD = t_{0.05} \sqrt{(SE_1^2 + SE_2^2)}$$

where SE = standard error for predicted value.

4. If the difference between the predicted means for the two treatments is greater than the LSD, i.e. $(P_1 - P_2) > LSD$ then the two treatments are significantly different at the 5% level of significance (where P_1 = predicted value for treatment 1 and P_2 = predicted value for treatment 2).

5.2.1.2 Exflagellation

The data that were analyzed did not follow a normal distribution, and **linear** models could not be fitted to the data. Therefore, a class of statistical models that generalizes classical linear models by the use of a transformation had to be applied. This is called **Generalised Linear models**, and it includes special cases of analysis of variance models, logit and probit models for quantal responses and multinomial response models for counts. In the case of these data, the model that provided the best fit to the data was the **Poisson regression**. The **Generalised Linear Models** procedure was used to analyze the data. A **Poisson regression** was fitted to the number of exflagellations counted in 40 microscopic fields every 15 minutes.

For each isolate a comparison was made over time, synchronized was compared to static, treated was compared to control, and interactions were investigated. This analysis was carried out on RSA 2 and RSA 5 isolates only because they had undergone exflagellation (Chapter 4).

5.3 RESULTS

Figures 19-23 show the plots for the log of the number of gametocytes against the period (in days) for which the parasites were maintained in culture. Each figure represents an isolate of *P. falciparum* and each includes the four comparisons mentioned above. At the previously determined minimum inhibitory concentration (MIC) of 100nmol/ℓ there was no significant difference between the gametocyte counts of the control and drug-treatments in the static cultures of isolate RSA 2, but for the synchronized cultures there was a significant difference ($p < 0.05$) (Table 4).

Gametocyte morphology was not affected by pyrimethamine in this isolate but the immature stages of gametocytes were affected and this resulted in lower mature gametocyte numbers. The cultures of isolate RSA 3 were highly affected by the drug at the MIC of 3.0μmol/ℓ (Fig. 20). The statistical analysis of the results for both the static and the synchronized cultures showed that there was a significant difference between the controls and the experiments ($p < 0.01$ and $p < 0.001$ respectively) (Table 5) with pyrimethamine resulting in few gametocytes. Figure 21 shows the plots for the cultures of isolate RSA 5. The morphology of the different stages of gametocytes was not affected by the drug but their numbers were affected.

The statistical analysis of the results for this isolate showed that there was a significant difference at $p<0.05$ between the static control and the drug-treated cultures and at $p<0.01$ for the synchronized control and the drug-treated cultures (Table 6) with treatment resulting in lower numbers of gametocytes. For isolate MW2 (Fig. 22), the synchronized cultures were not affected by the drug, but the static cultures were affected and there was a significant difference at 5% level of significance between the control and the treated experiments (Table 7). All the cultures (both static and synchronized) of NF54 (a foreign isolate, an Amsterdam Airport strain), were highly affected by the drug (Fig. 23).

The statistical analysis of the results showed that there was a significant difference between the controls and the experiments ($p<0.01$ each) (Table 8). There was no significant difference between the pyrimethamine-treated static and the pyrimethamine-treated synchronized cultures for all the five isolates (Tables 4-8). Appendix 7 shows the peak numbers of Stage II gametocytes counted per 10 000 RBCs for the five isolates, RSA 2, 3, 5 MW2 and NF54. the numbers within the brackets next to the control figures denote the days on which the peak values were attained.

Only Stage II gametocytes were counted to represent the immature stages of gametocytes because Stage I gametocytes look similar to the asexual trophozoite stage of the parasite. In the static cultures of isolate RSA 2 the number of Stage II gametocytes peaked to 16 (control) and 22 (pyrimethamine trial) on the eighth day after initiation of cultures. Pyrimethamine treatment of the synchronized cultures of isolate RSA 2 and all the cultures of other four isolates i.e. RSA 3, 5, MW2 and NF54, resulted in reduced numbers of young gametocytes (see Appendix 7).

Exflagellation was observed in all the control and pyrimethamine-treated cultures of both RSA 2 and RSA 5 respectively (Tables 9 and 10). The statistical analysis of the exflagellation experiments is as follows:

1. **Isolate RSA 2:**

There were significantly more exflagellations in the control group than those treated with pyrimethamine ($p < 0.01$), with no statistically significant differences between static and synchronized cultures (Fig. 24). The change over time was highly significant ($p < 0.001$).

2. Isolate RSA 5:

The synchronized treated cultures had significantly more exflagellations than any other combination ($p < 0.001$), among which there were no significant differences (Fig. 25).

Fig. 19. Log of the number of gametocytes/10 000 RBCs (LGAM) against time (days) for isolate RSA 2.

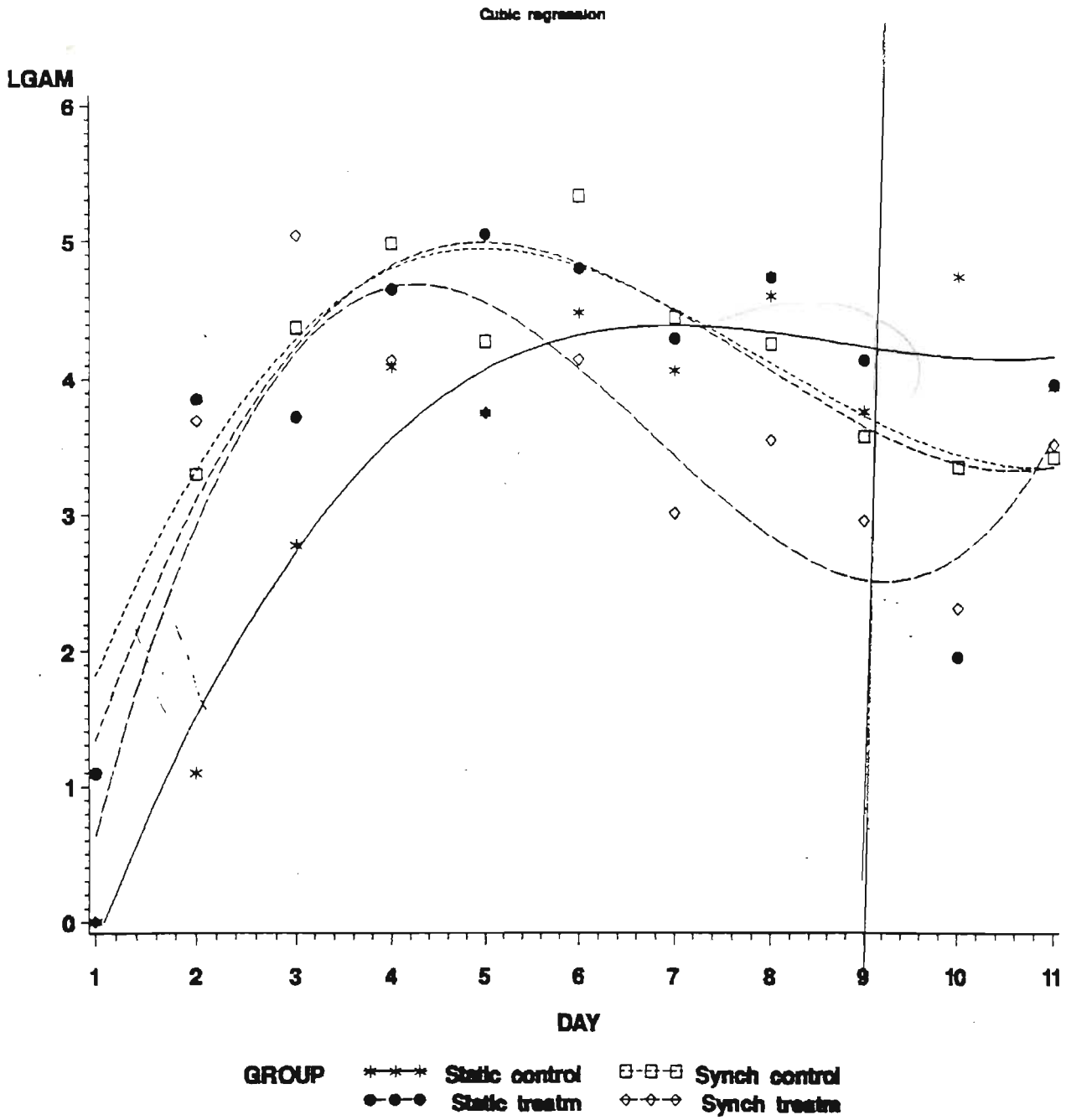


Fig. 20. Log of the number of gametocytes/10 000 RBCs (LGAM) against time (days) for isolate RSA 3.

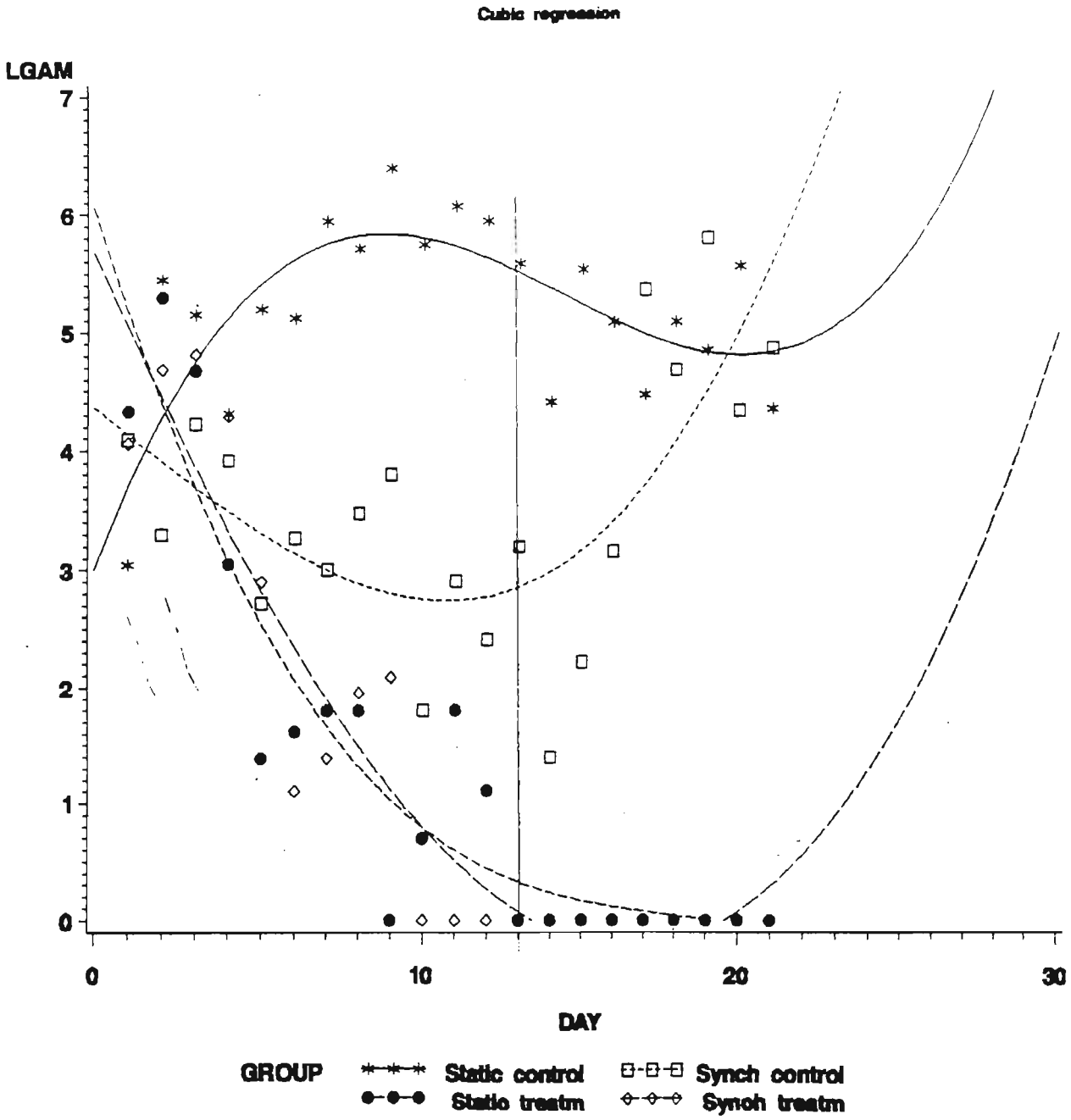


Fig. 21. Log of the number of gametocytes/10 000 RBCs (LGAM) against time (days) for isolate RSA 5.

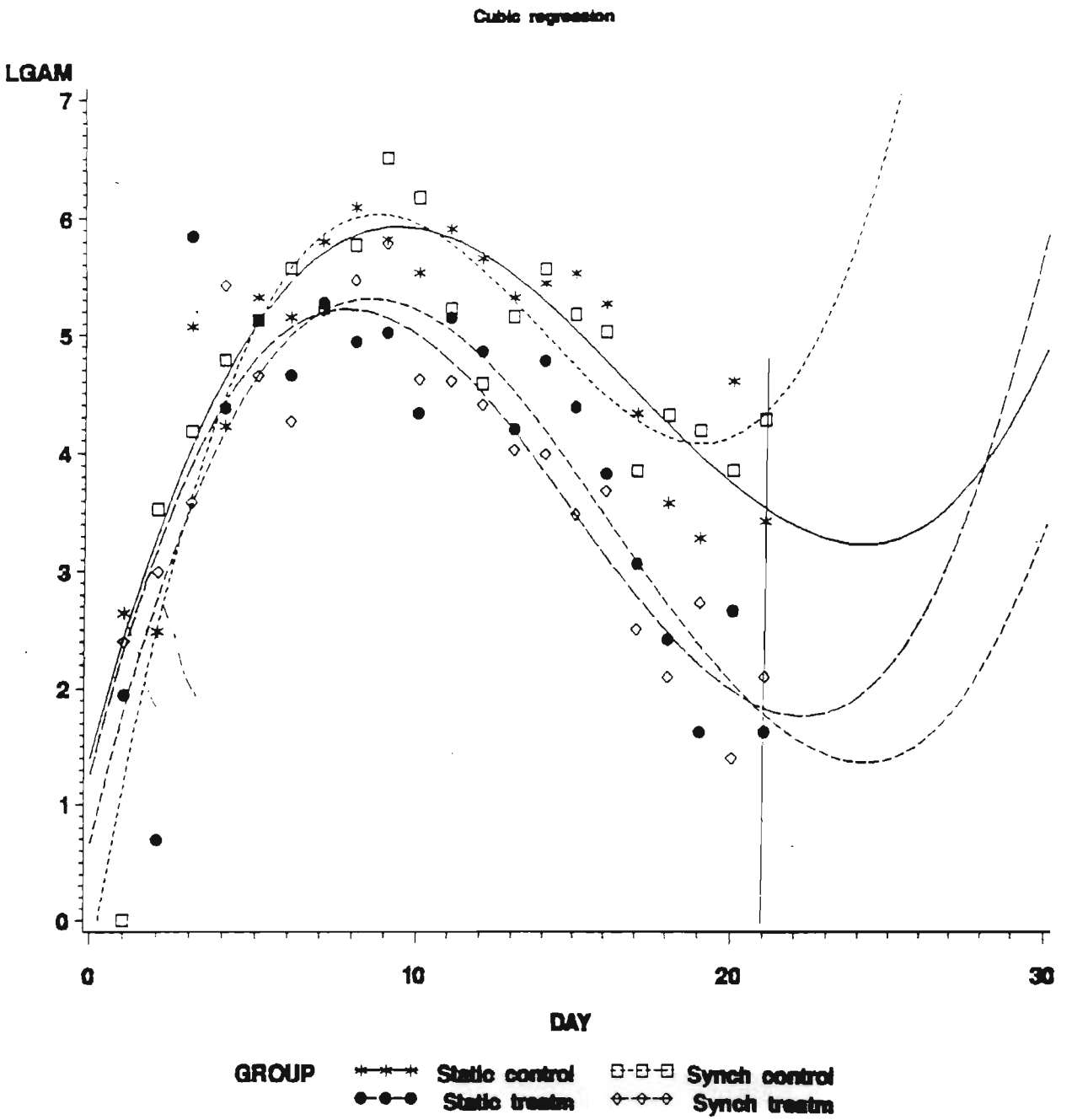


Fig. 22. Log of the number of gametocytes/10 000 RBCs (LGAM) against time (days) for isolate MW2.

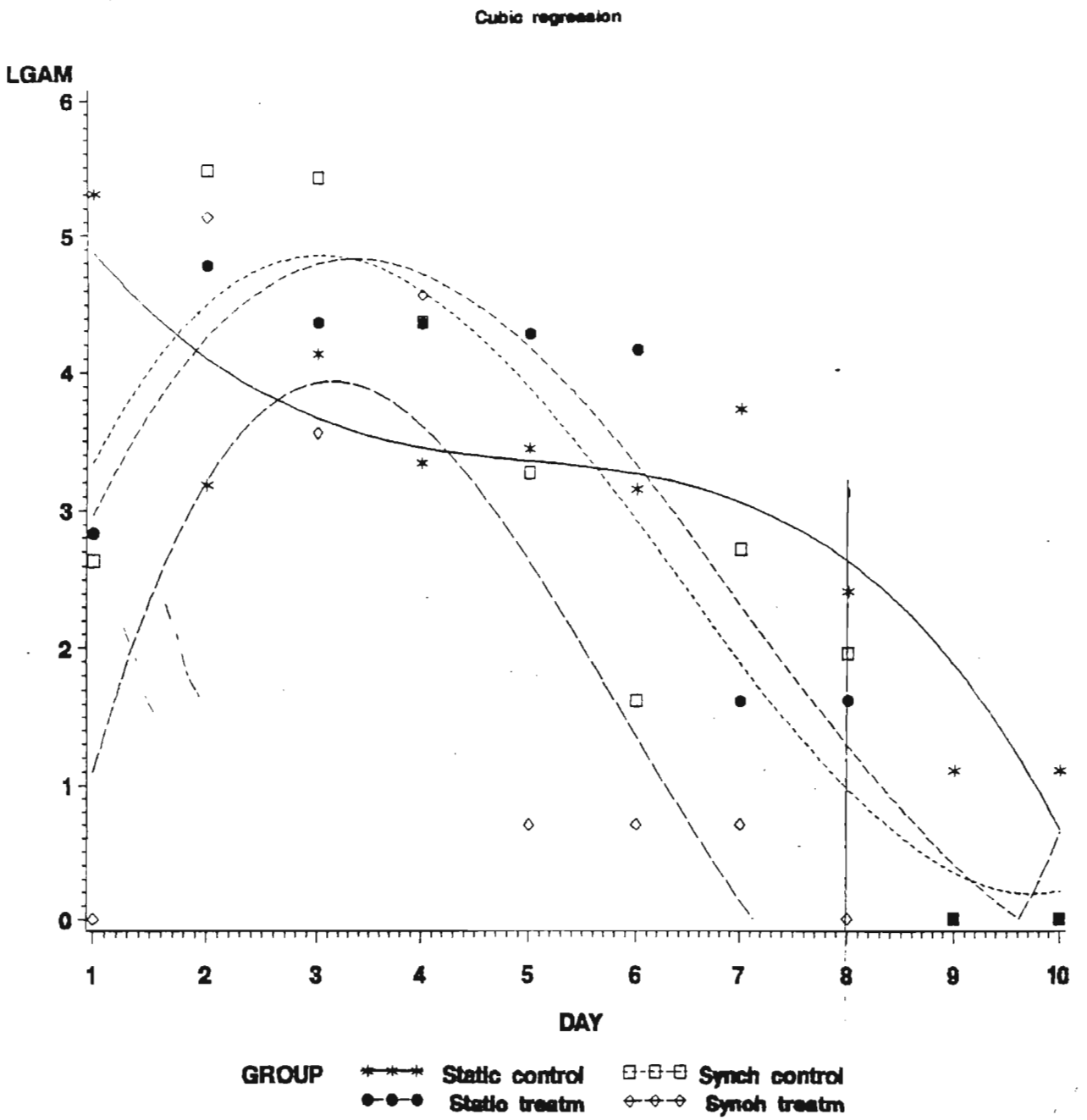


Fig. 23. Log of the number of gametocytes/10 000 RBCs (LGAM) against time (days) for isolate NF54.

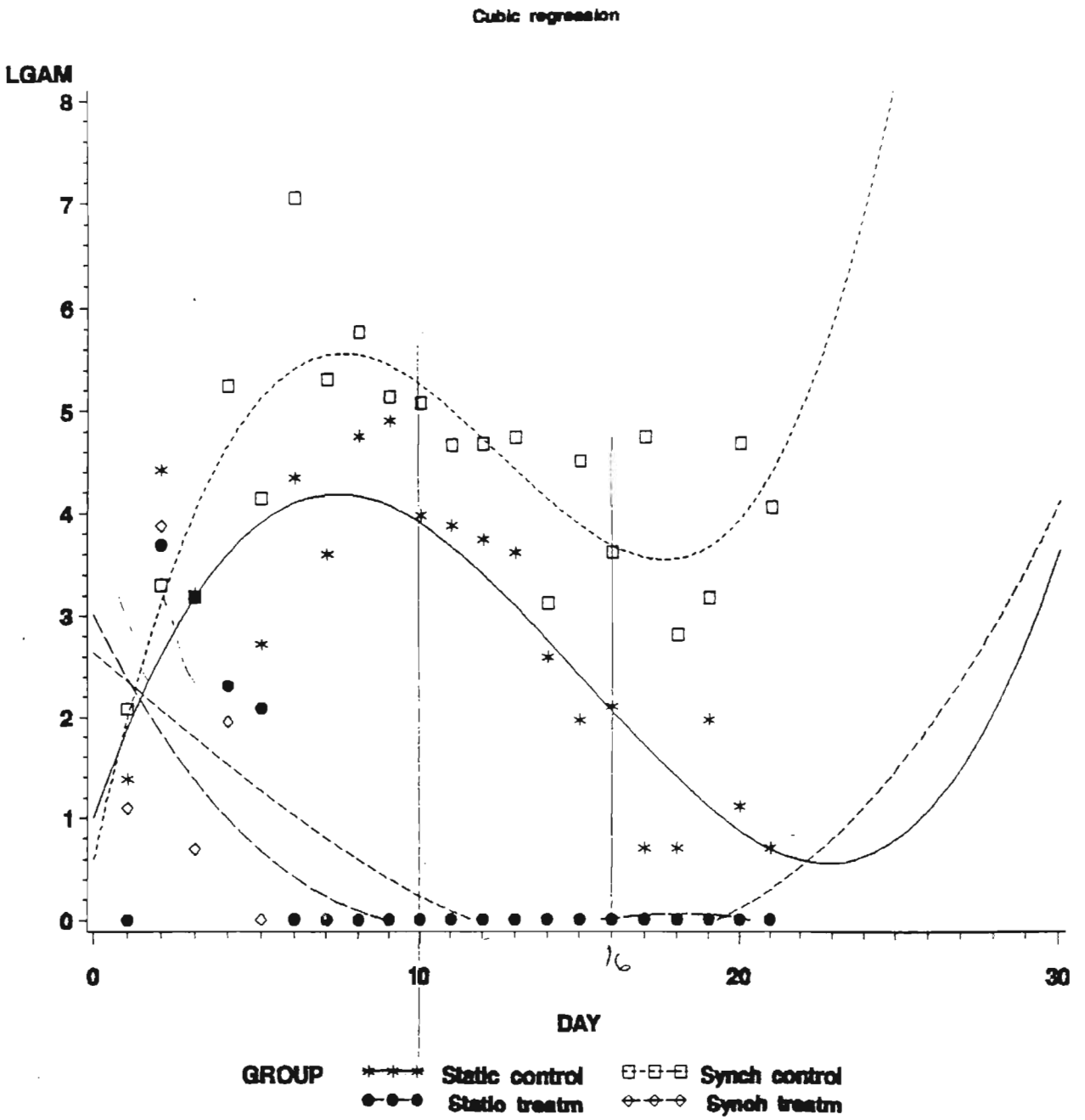


Table 4. Statistical analysis of the results shown for isolate RSA 2 in Fig. 19.

Comparison	Diff. in Pr. value	LSD	P-value
Ctrl v Ctrl	0.5	0.75	NS
Trt v Trt	1.1	1.32	NS
St. ctrl v Trt	0.6	1.1	NS
Syn. ctrl v Trt	1.2	1.05	<0.05

Table 5. Statistical analysis of the results shown for isolate RSA 3 in Fig. 20.

Comparison	Diff. in Pr. value	LSD	p-value
Ctrl v Ctrl	2.64	0.78	<0.01
Trt v Trt	0.25	0.69	NS
St. ctrl v Trt	5.16	0.64	<0.001
Syn. ctrl v Trt	2.77	0.81	<0.01

Key: St = Static cultures

Sy = Synchronized cultures

Diff. in Pr. = Difference in Predicted values

LSD = Least significant difference

NS = not significant

Table 6. Statistical analysis of the results shown for isolate RSA 5 in Fig. 21.

Comparison	Diff. in Pr. value	LSD	p-value
Ctrl v Ctrl	0.79	1.23	NS
Trt v Trt	0.06	1.54	NS
St. ctrl v Trt	1.75	1.57	<0.05
Syn. ctrl v Trt	2.48	1.19	<0.01

Table 7. Statistical analysis of the results shown for isolate MW2 in Fig. 22.

Comparison	Diff. in Pr. value	LSD	p-value
Ctrl v Ctrl	1.66	1.61	<0.05
Trt v Trt	1.27	2.06	NS
St. ctrl v Trt	1.34	1.21	<0.05
Syn. ctrl v Trt	0.95	2.32	NS

Key:

St = Static cultures

Sy = Synchronized cultures

Diff. in Pr. = Difference in Predicted values

LSD = Least significant difference

NS = not significant

Table 8. The statistical analysis of the results shown for isolate NF54 in Fig. 23.

Comparison	Diff. in Pr. value	LSD	p-value
Ctrl v Ctrl	1.61	0.89	<0.05
Trt v Trt	0.01	0.93	NS
St. ctrl v Trt	2.03	0.99	<0.01
Syn. ctrl v Trt	3.63	0.84	<0.01

Key: St = Static cultures

Sy = Synchronized cultures

Diff. in Pr. = Difference in Predicted values

LSD = Least significant difference

NS = not significant

Table 9. The number of exflagellations counted in 40 microscopic fields for isolate RSA 2.

Time (Min)	Static cultures		Synchronized cultures	
	Control	Treated	Control	Treated
15	11	10	14	10
30	19	15	18	11
45	24	17	25	16
60	31	19	27	16

Table 10. The number of exflagellations counted in 40 microscopic fields for isolate RSA 5.

Time (Min)	Static cultures		Synchronized cultures	
	Control	Treated	Control	Treated
15	11	9	10	19
30	14	13	11	24
45	16	15	19	31
60	19	20	24	35

Fig. 24. Poisson regressions of the number of exflagellations counted in 40 microscopic fields every 15 minutes for isolate RSA 2.

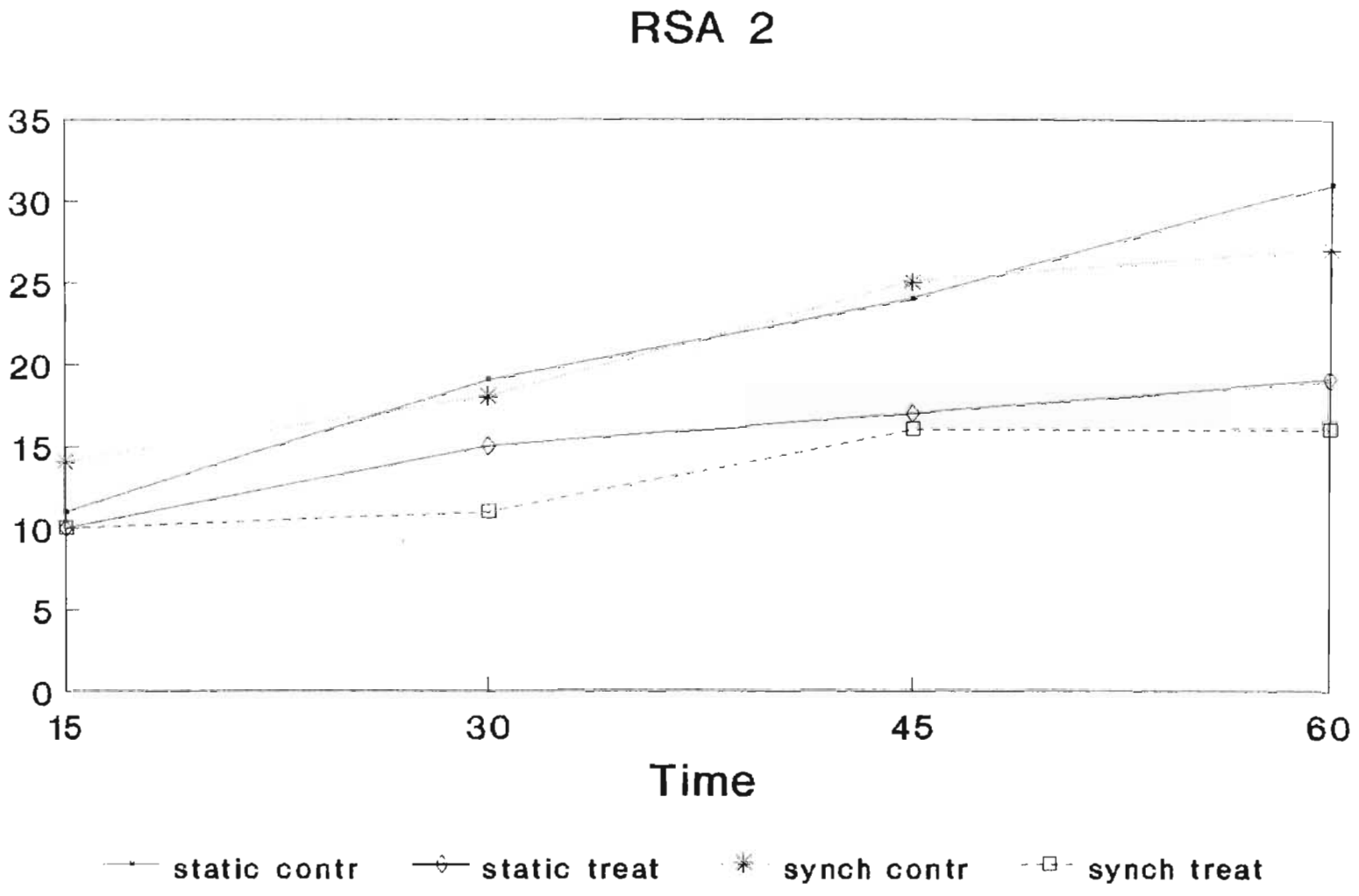

RSA 2
35
30
25
20
15
10
5
0
15
30
45
60
Time
static contr
static treat
synch contr
synch treat

Fig. 25. Poisson regressions of the number of exflagellations counted in 40 microscopic fields every 15 minutes for isolate RSA 5.

RSA 5
40
35
30
25
20
15
10
5
0
15
30
45
60
Time
static contr
static treat
synch contr
synch treat

5.4 DISCUSSION

The morphology of the different stages of gametocytes in all five isolates treated with pyrimethamine was not affected, but their numbers were (Figs. 19-23). The immature gametocyte stages were affected by the drug which resulted in lower numbers of mature gametocytes in all the isolates (also the synchronized cultures of isolate RSA 2) (Appendix 7). Tables 4-8 are confirming the statements above. There were no statistically significant differences within the static cultures of isolate RSA 2 between the control and treatment but there were significant differences within the synchronized cultures of the same isolate ($p < 0.05$). The differences were statistically significant between the control and the trial in the synchronized cultures of isolates RSA 3, 5 and NF54 at $p < 0.01$ each. Only in the synchronized cultures of isolate MW2 were the differences not significant.

The differences in gametocyte numbers between the control and treatment in the static cultures of the four isolates, RSA 3, 5, MW2 and NF54 were significant at p-values of < 0.001, < 0.05, < 0.05 and < 0.01 respectively. NF54 was highly affected by the drug (Fig. 23). Firm conclusions cannot be made at this stage about the resistance of this isolate to pyrimethamine since the MIC of this isolate was not determined. The pyrimethamine concentration used in this study for NF54 could be higher than the MIC of this isolate, thus resulting in visible reaction to the drug. The results confirm reports on cited literature that pyrimethamine acts on young gametocytes of *P. falciparum* (Black *et al*, 1986) and mature stages of gametocytes were not affected. This was expected as the drug does not act on mature gametocytes of this parasite (Desjardins *et al*, 1988). Exflagellation was demonstrated by all the cultures of both RSA 2 and RSA 5 (Tables 9 and 10).

There were no statistically significant differences between the static and the synchronized cultures of RSA 2, but there were between control and the pyrimethamine-treated cultures of this isolate. There were also statistically significant differences between the static and synchronized cultures of RSA 5, but none between the control and the treated cultures of both the static and the synchronized cultures of this isolate. For RSA 5, pyrimethamine had no effect on exflagellation at the concentration of 3000nmol/ℓ. Pyrimethamine is known to be an effective sporontocide (Wernsdorfer, 1986). However, previous findings by Teklehaimanot *et al* (1985), showed that it had a sporontocidal effect against the strains whose asexual erythrocytic stages are sensitive to the drug but not on the strains whose asexual blood stages are pyrimethamine-resistant. They concluded that there might be a cross-resistant relationship between asexual and sexual stages of the parasites in the strains examined. Peters (1987) also confirmed these observations on pyrimethamine resistance. He found that pyrimethamine failed to prevent sporogony in two malaria patients and he suggested that pyrimethamine was not suitable for a malaria control campaign since *P. falciparum* rapidly became resistant to it.

It is difficult at this stage to make firm conclusions about the relationship between the sporontocidal effect of pyrimethamine and pyrimethamine resistance in southern African *P. falciparum* since exflagellation was carried out on two isolates only. It would be interesting to investigate the sporontocidal effect of pyrimethamine on all 19 culture-adapted southern African isolates of *P. falciparum* available. Future investigations need to focus on the effect of pyrimethamine on oocyst development *in vivo*, the effect of other sporontocidal drugs such as proguanil, Fansidar and primaquine on gametocytes both *in vitro* and *in vivo* and their effects on infectivity in mosquitoes.

5.5 IMPLICATIONS FOR CONTROL OF MALARIA

Pyrimethamine resistance is a problem common to all malarial areas of the world (Coosemans *et al*, 1987; Petersen, 1987), including southern Africa (Freese *et al*, 1991). Pyrimethamine has been resistance by *P. falciparum* has in fact been confirmed at certain localities in all endemic regions including Africa (Black *et al*, 1986). This poses a huge problem for malaria control programmes since alternative drugs have to be developed. Pyrimethamine was used in combination with chloroquine in the hopes of interrupting malaria transmission. The results of this study indicate that pyrimethamine inhibits the development of gametocytes *in vitro* at concentrations that are inhibitory to the asexual parasites. However, previous studies have shown that southern African isolates have a wide range of MICs (Freese *et al*, 1991) and it is possible that the serum pyrimethamine levels achieved by the standard prophylactic and treatment doses of Darachlor would not be sufficient to inhibit gametocyte development *in vivo*.

CHAPTER 6

ACTION OF CHLOROQUINE AND PYRIMETHAMINE, ALONE AND IN COMBINATION, ON THE ASEXUAL GROWTH OF *PLASMODIUM FALCIPARUM*

6.1 INTRODUCTION

Chloroquine resistance is becoming common in southern Africa. Figures of 50% and 10% have been cited for Malawi (Newlands, 1989), and northern Namibia respectively (Isaäcson *et al*, 1984). In South Africa itself, a high number of cases in KwaZulu-Natal province are resistant (Freese *et al*, 1988b, 1991; Herbst *et al*, 1985, 1987). In South America, *P. falciparum* was found to be resistant to chloroquine in Brazil, Colombia, French Guiana, Guyana and Suriname (Black *et al*, 1986). Confirmation of resistance to pyrimethamine in South Africa (refer to Chapter 5) means that the drug cannot be relied upon when used alone (McCutchan, 1988; Newlands, 1989).

Drug combinations with pyrimethamine are thought to be more effective for chloroquine-resistant malaria. Examples are Fansidar (pyrimethamine/sulphadoxine), Darachlor (pyrimethamine/chloroquine) and Maloprim (pyrimethamine/dapsone). The latter combination was found to be highly synergistic (Scott *et al*, 1987). Although Fansidar resistance has been reported in some parts of the world (Hoffman *et al*, 1987; Watt *et al*, 1987), it is still effective against the parasite in most areas where chloroquine-resistance has been reported (Desjardins *et al*, 1988).

Multidrug resistance is posing another serious threat to malaria control schemes however and has been reported in various parts of the world, e.g. in Southeast Asia and South America (Desjardins *et al*, 1988; Watt *et al*, 1987; White, 1988). Fansidar was found to be effective for most patients with falciparum malaria in Indonesia (Hoffman *et al*, 1987). The use of the combination of chloroquine and pyrimethamine was popular in the 1950s and 1960s for the treatment of overt malaria in semi-immune patients (Peters, 1987) and it has recently been reported that in certain areas this chloroquine/pyrimethamine combination was still effective (Peters, 1987).

Previous studies have demonstrated the additive action of the chloroquine/pyrimethamine compound against the blood stages of *P. berghei* (Peters, 1987). The synergistic action between pyrimethamine and chloroquine has however not been investigated on the southern African isolates of *P. falciparum*. The aim of this study was to determine whether there was any synergism when these two drugs were used in combination. The study was relevant at the time it was carried out (1993) until recently (1994) when Darachlor was still the drug of choice in South Africa.

6.2 MATERIALS AND METHODS

The action of chloroquine and pyrimethamine alone and in combination on the asexual stages of *P. falciparum* was investigated following the methods described by Desjardins *et al* (1979) and Freese *et al* (1993) respectively.

6.2.1 *Plasmodium falciparum* Isolates

Five southern African isolates of *P. falciparum* were used: two chloroquine-sensitive (RSA 2 and 5) and three chloroquine-resistant; RSA 6, 9 and 14. The isolates were maintained in cultures as described previously in Chapter 3. For the drug tests, the cultures were diluted with fresh, washed, uninfected human type O-positive erythrocytes to a parasitaemia of 0.5-1% and suspended to 4% in complete culture medium (Freese *et al*, 1993).

6.2.2 Preparation of Drug Solutions

Chloroquine and pyrimethamine were mixed in the following fixed ratios of their approximate IC_{50}s (concentrations required to inhibit 50% of parasite growth (Freese *et al*, 1993)): 1:1, 1:3 and 3:1 as follows:

Chloroquine: Pyrimethamine (nM)

Isolate	1:1	1:3	3:1
RSA 2	30:30	30:90	30:30
RSA 3	10:2100	10:6300	30:2100
RSA 5	10:2100	10:6300	30:2100
RSA 6	150:60	150:180	450:60
RSA 14	60:30	60:90	180:30

The above combinations were prepared at **double strength** from stock solutions. Chloroquine solution was prepared in distilled water and pyrimethamine solution in 0.05% lactic acid. The drug combinations were then **double-diluted** six times in complete medium containing no hypoxanthine to yield seven dilutions of the drug combinations. For chloroquine alone, these concentrations (in nmoles) were prepared: 10, 20, 40, 80, 160, 320 and 640. For pyrimethamine, the following concentrations (in nmol/ℓ) were prepared: 6, 20, 60, 200, 600, 2000, 6000.

6.2.3 *In Vitro* Test

Each test was carried out in duplicate wells with the top row of a 96-well plate as control. On each plate there were two columns of chloroquine alone, two columns of pyrimethamine alone and two columns of each of the three fixed ratios of drug combination. To each well of a microtitre plate, 50$\mu\ell$ of a 4% suspension of infected red blood cells at a 0.5-1% parasitaemia and 50$\mu\ell$ of the drug or drug combination were added to yield a 2% suspension of infected red blood cells and the correct drug concentrations. The first row was the positive control and it contained no drug. The 11th column (wells C-H) were the negative controls and contained neither the drug nor the infected red blood cells only 2% normal red blood cell suspension in culture medium. The plates were incubated at 38°C in a desiccator gassed with for 24 hours (Fig. 26). After the incubation period, ^{3}H-hypoxanthine, 0.5μCi in 10$\mu\ell$ culture medium, was added to each well of the microtitre plate. The plates were again gassed and incubated for a further 18 hours.

6.2.4 Harvesting and Scintillation Counting

After the second incubation period, each plate was harvested on a **Titertek cell harvester** (Fig. 27). The contents of each well were aspirated and deposited onto small disks of glass fibre filter paper. The filter papers were then washed with distilled water and dried. The dried filter paper disks were placed in scintillation vials containing 4mℓ scintillation fluid and counted for 5 minutes in a **Beckman LS 1800 liquid scintillation counter** (Fig. 28).

6.2.5 Data Analysis

6.2.5.1 Calculation of IC_{50}s

The concentrations required to inhibit 50% parasite growth (IC_{50}s) for chloroquine alone, pyrimethamine alone and for their combination, for each of the five isolates were calculated by method one of Freese *et al* (1993). Briefly, the two percentage growth **(PCGROWTH)** values closest to 50%, one on either side, were selected, assuming linearity between these two points. The concentration corresponding to 50% growth was then calculated by **linear interpolation** between the two points and their corresponding concentration values i.e.

$$\frac{(y_1 - y_2)}{(x_2 - x_1)} = \frac{(y_1 - 50)}{(x_3 - x_1)}$$

or

$$x_3 = \frac{(y_1 - 50)}{(y_1 - y_2)} \times (x_2 - x_1) + x_1$$

where y_1 and y_2 are the two PCGROWTH values closest to 50%, one on either side. x_1 and x_2 are their corresponding concentrations, and x_3 is the concentration corresponding to 50% growth (IC_{50}).

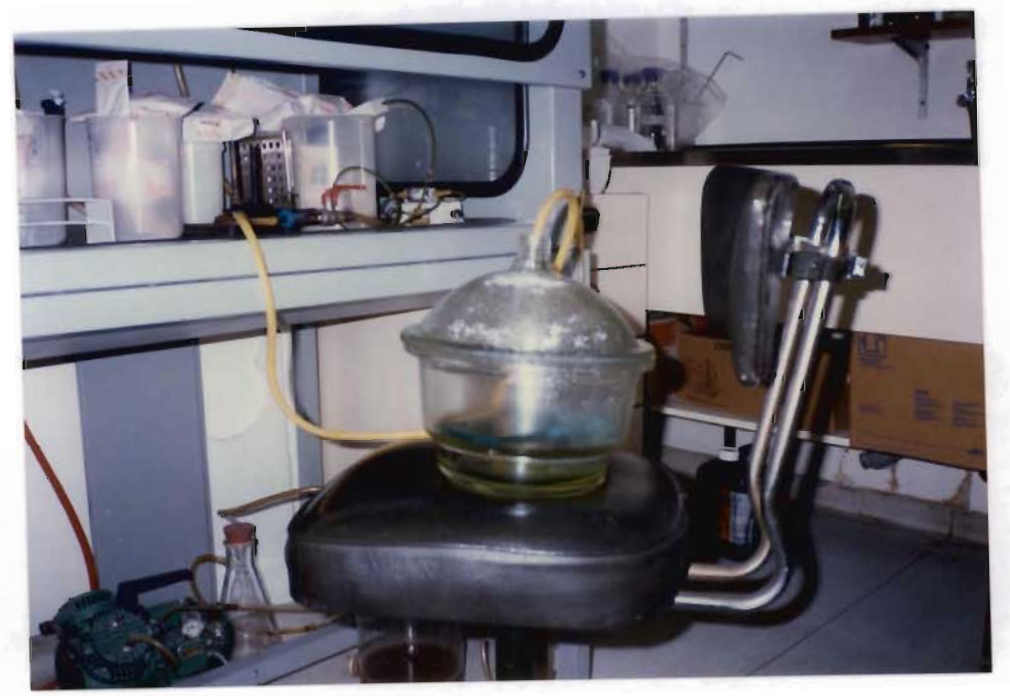


Fig. 26. Gassing the desiccator with 3% CO_2, 4% O_2 and 93% N_2.

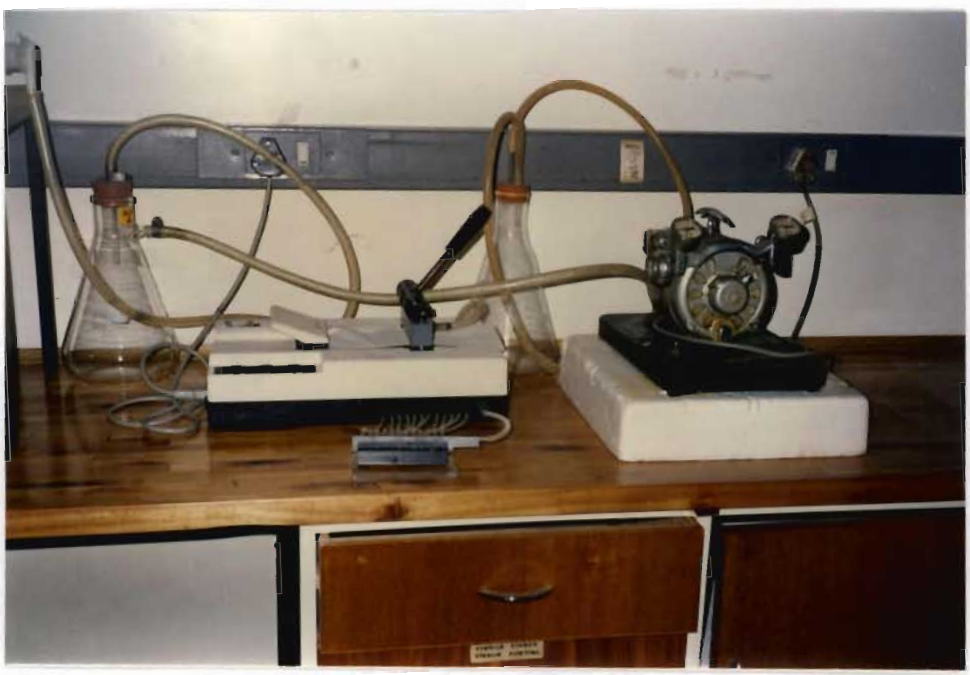


Fig. 27. Titertek Cell Harvester.

Fig. 28. Beckman LS 1800 Liquid Scintillation Counter.

The relative IC_{50}s were then determined from the calculated IC_{50}s by dividing the IC_{50}s of each drug in combination by the IC_{50} of the drug alone. The sum of the relative IC_{50}s was used to denote the degree of synergism. If Σ relative IC_{50} was smaller than one, synergistic action was indicated. When the sum equalled one the two drugs were additive, and when the sum was greater than one an antagonistic effect was indicated. Isobolograms were constructed by plotting the relative IC_{50}s for chloroquine against the relative IC_{50}s for pyrimethamine to show the synergistic effects of the two drugs (Martin *et al*, 1987). Concave curves depict synergism between the two drugs and convex curves depict antagonism.

6.2.5.2 Determination of the MICs

The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which growth of at least 95% of the parasites was inhibited. The mean of two **counts per minute values (CPM)** (of each isolate) corresponding to each drug concentration minus the mean of the six values corresponding to the negative control (non-parasitized erythrocytes) was expressed as a percentage of the control count. Taking the mean of the control count as 100 % growth (or 0% inhibition), the MIC for each isolate for chloroquine alone and pyrimethamine alone were determined.

6.3 RESULTS

6.3.1. Chloroquine sensitivity

The MICs and IC_{50}s for chloroquine are presented in Table 11. The MICs obtained for chloroquine reflect the results obtained previously by Freese *et al* (1991) i.e. the chloroquine-sensitive strain (RSA 5) had an MIC $<$ 160nmol/ℓ and the chloroquine-resistant strains (RSA 6, 9 and 14) had MICs $\geq$ 160nmol/ℓ (see Appendix 2). The comparison between the IC_{50} values determined in this study and those obtained in previous studies is shown in Appendix 3. The MIC and the IC_{50} values for RSA 2 isolate could not be determined since the range of concentrations used was too high to accommodate these values for this isolate. The IC_{50} for RSA 5 of 13nmol/ℓ falls within the same range of concentrations as that obtained previously for chloroquine-sensitive isolates (Freese, 1994) using the radio-isotope incorporation method. Again, the IC_{50} for isolate RSA 14 agrees well with that obtained by Freese (1994) using the radio-isotope incorporation method (see Appendix 3). Isolates RSA 6 and 9 have IC_{50}s that are lower than those determined previously (Freese, 1994: Freese *et al*, 1991).

6.3.2 Pyrimethamine sensitivity

The MIC and the IC_{50} values of the isolates RSA 2, 6, 9 and 14 for pyrimethamine are also represented in Table 11. It was not possible to determine these values for RSA 5 from the data obtained. Comparisons of the MICs and IC_{50}s obtained during this study and those obtained by Freese *et al* (1991) are shown in Appendices 4 and 5, respectively. The pyrimethamine MICs ranged from 300nmol/ℓ to $>$3000nmol/ℓ.

Table 11. The minimum inhibitory concentrations (MICs) and IC_{50}s for chloroquine and pyrimethamine (nM).

	CHLOROQUINE		PYRIMETHAMINE	
Isolate	**MIC**	**IC_{50}**	**MIC**	**IC_{50}**
RSA 2	**ND**	36*	>3000	194
RSA 5	40	13	**ND**	1426*
RSA 6	>320	44	300	9
RSA 9	>320	69	300	22
RSA 14	>320	70	300	5

Note: Chloroquine - MIC ≥160nmol/ℓ, indicates resistance to the drug.

- MIC ≤160, sensitivity to the drug is indicated.

Pyrimethamine - MIC ≥100nmol/ℓ, resistance to the drug is indicated.

- MIC ≤100nmol/ℓ, sensitivity to the drug is indicated.

ND - not determined

*** - from Freese (1994)**

They show a good agreement with the results obtained previously (Freese *et al*, 1991) except for RSA 5 which failed to be inhibited at 3000nmol/ℓ (see Appendix 4). This MIC value is high compared to the pre-determined MIC of 100nmol/ℓ (Freese *et al*, 1991). The IC_{50}s were all low compared to those obtained in previous drug sensitivity tests with the exception of RSA 2 which has an IC_{50} of 194nmol/ℓ when compared to 13nmol/ℓ of Freese *et al* (1991) (Appendix 5). The IC_{50} for RSA 5 could not be determined because the range of concentrations used for this study could not accommodate the IC_{50} for this isolate.

6.3.3 Synergistic action of pyrimethamine with chloroquine

It was possible to use the IC_{50} value to determine the effect of pyrimethamine on the sensitivity of a particular isolate to chloroquine. Tables 12-16 give the IC_{50}s and relative IC_{50}s of the pyrimethamine/chloroquine combinations for RSA 2, 5, 6, 9 and 14 respectively. The relative chloroquine IC_{50}s for RSA 2 (Table 12) and the relative pyrimethamine IC_{50}s for RSA 5 (Table 13) were calculated using previously determined chloroquine and pyrimethamine values indicated in Table 11 (Freese, 1994). The Σ relative IC_{50}s for the fixed chloroquine/pyrimethamine ratios (Table 12) for RSA 2 ranged from 0.609 to 1.794 and that for RSA 5 from 0.844 to 1.899 (Table 13). Higher Σs of relative IC_{50}s are shown by the chloroquine-resistant isolates (Tables 14-16).

Table 12. IC_{50}s and relative IC_{50}s for pyrimethamine/chloroquine combinations for isolate RSA 2.

			30:30	**30:90**	**90:30**	
IC_{50} (nM)	**PYR**	0	18,5	71,27	20,25	194,3
	CQ	36	18.5	23,76	60,77	0
Rel IC_{50}	**PYR**	0	0,095	0,367	0,104	1
	CQ	1	0,514	0,66	1,69	0
Σ		**1**	**0,609**	**1,027**	**1,794**	**1**

Table 13. IC_{50}s and relative IC_{50}s for pyrimethamine/chloroquine combinations for isolate RSA 5.

			10:2100	**10:6300**	**30:2100**	
IC_{50} (nM)	**PYR**	0	776	2295,48	807,27	1426
	CQ	12,45	3,7	3,65	11,53	0
Rel IC_{50}	**PYR**	0	0.544	1,609	0,566	1
	CQ	1	0.30	0,29	0,93	1
Σ		**1**	**0.844**	**1,899**	**1,496**	**1**

Table 14. IC_{50}s and relative IC_{50}s for pyrimethamine/chloroquine combinations for isolate RSA 6.

			150:60	**150:180**	**450:60**	
IC_{50} (nM)	**PYR**	0	13,9	33,42	11,79	9,11
	CQ	44,37	34,75	27,85	88,42	0
Rel IC_{50}	**PYR**	0	1,526	3,668	1,294	1
	CQ	1	0,783	0,628	1,99	0
Σ		**1**	**2,309**	**4,296**	**3284**	**1**

Table 15. IC_{50}s and relative IC_{50}s for pyrimethamine/chloroquine combinations for isolate RSA 9.

			150:60	**150:180**	**450:60**	
IC_{50} (nM)	**PYR**	0	22,43	24,27	8,6	21,52
	CQ	69,24	56,1	20,23	64,53	0
Rel IC_{50}	**PYR**	0	1,042	1,128	0,4	1
	CQ	1	0,81	0,292	0,932	0
Σ		**1**	**1,852**	**1,420**	**1,332**	**1**

Table 16. IC_{50}s and relative IC_{50}s for pyrimethamine/chloroquine combinations for isolate RSA 14.

			60:30	**60:90**	**180:30**	
IC_{50} (nM)	**PYR**	0	7,29	22,52	4,637	5,073
	CQ	70,06	14,59	15,02	27,82	0
Rel IC_{50}	**PYR**	0	1,437	4,439	0,914	1
	CQ	1	0,208	0,214	0,397	0
Σ		1	1,645	4,653	1,311	1

Figures 29-33 are the isobolograms depicting the interaction between chloroquine and pyrimethamine (see Appendix 6 for explanation of the graphs) for isolates RSA 2, 5, 6, 9 and 14 respectively. For RSA 2 and 5, the points on the graphs appear below and above the linear graph indicating no interaction. For RSA 6, 9 and 14, the points appear above the linear graph indicating an antagonistic interaction between the two drugs. The Σ relative IC_{50}s for the most effective combination for the five isolates used are represented in Table 17. RSA 2 and RSA 5 had the lowest Σ relative IC_{50} (0.61 and 0.84 respectively). RSA 6 had a lowest Σ relative IC_{50} of 2.3 and the other two isolates (RSA 9 and 14) each had the Σ relative IC_{50}s of the most effective drug combination of 1.3.

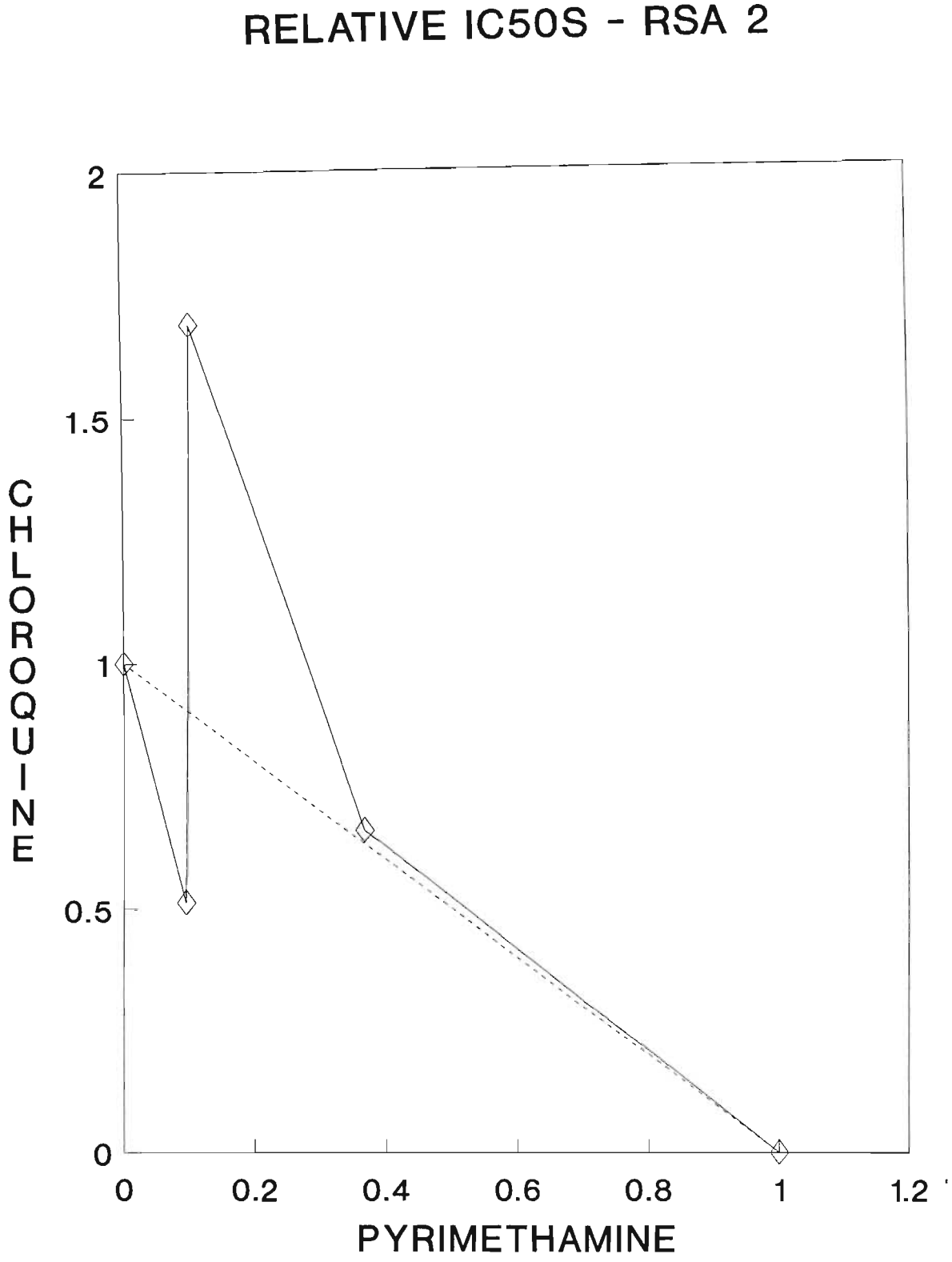


Fig. 29. Isobologram showing the relationship between chloroquine and pyrimethamine of chloroquine-sensitive isolate RSA 2.

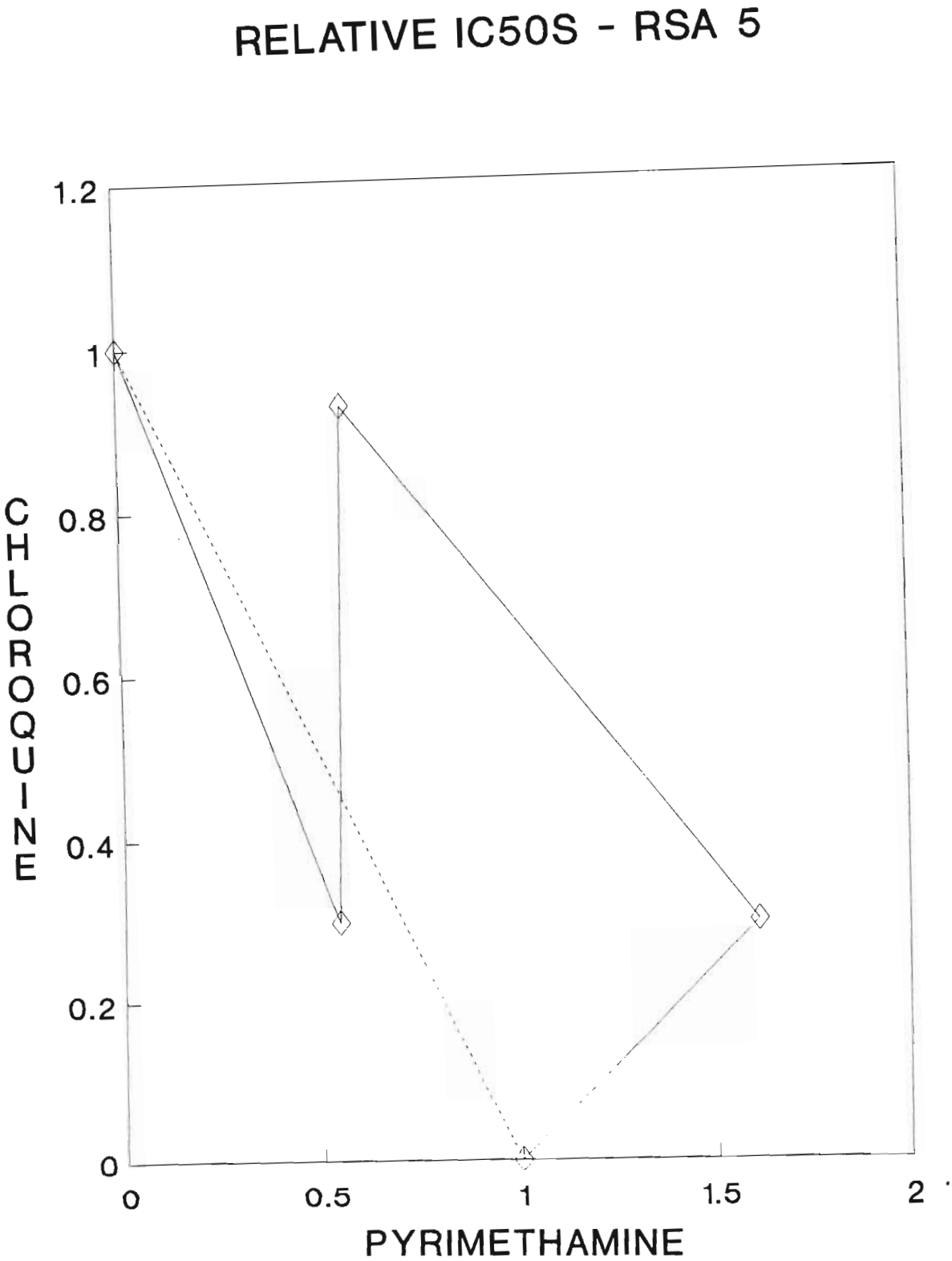


Fig. 30. Isobologram showing the relationship between chloroquine and pyrimethamine of chloroquine-sensitive isolate RSA 5.

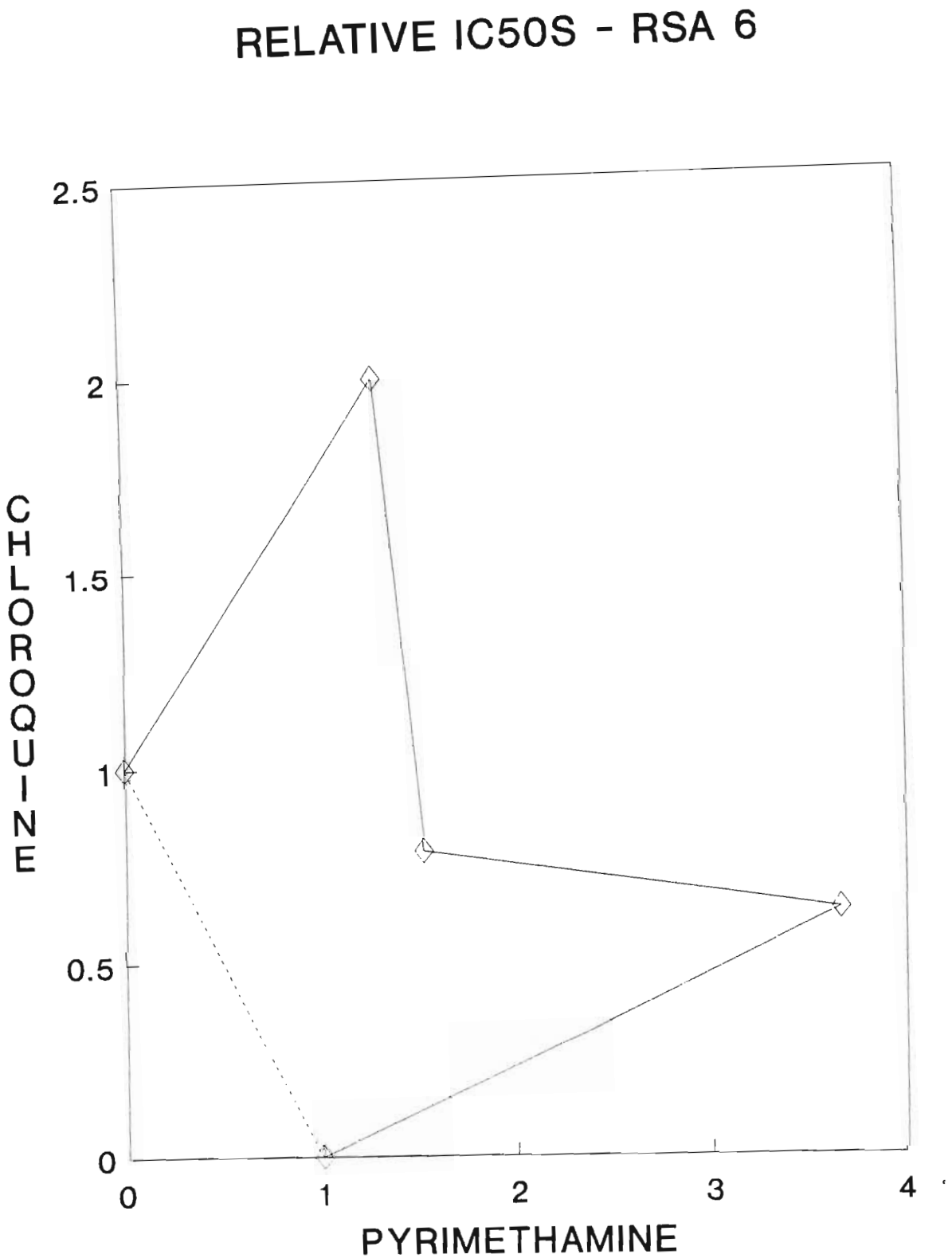


Fig. 31. Isobologram showing the relationship between chloroquine and pyrimethamine of chloroquine-resistant isolate RSA 6.

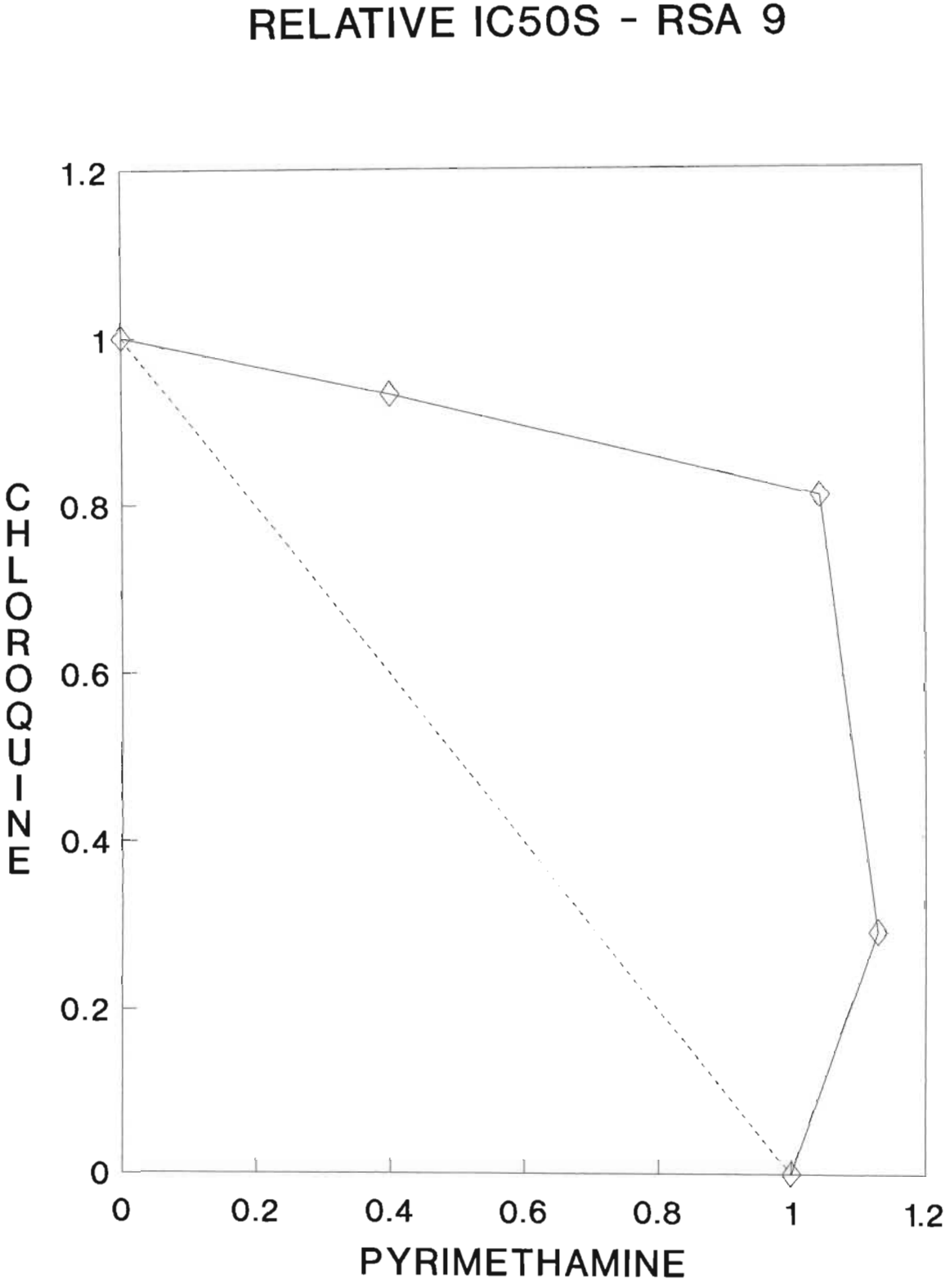


Fig. 32. Isobologram showing the relationship between chloroquine and pyrimethamine of chloroquine-resistant isolate RSA 9.

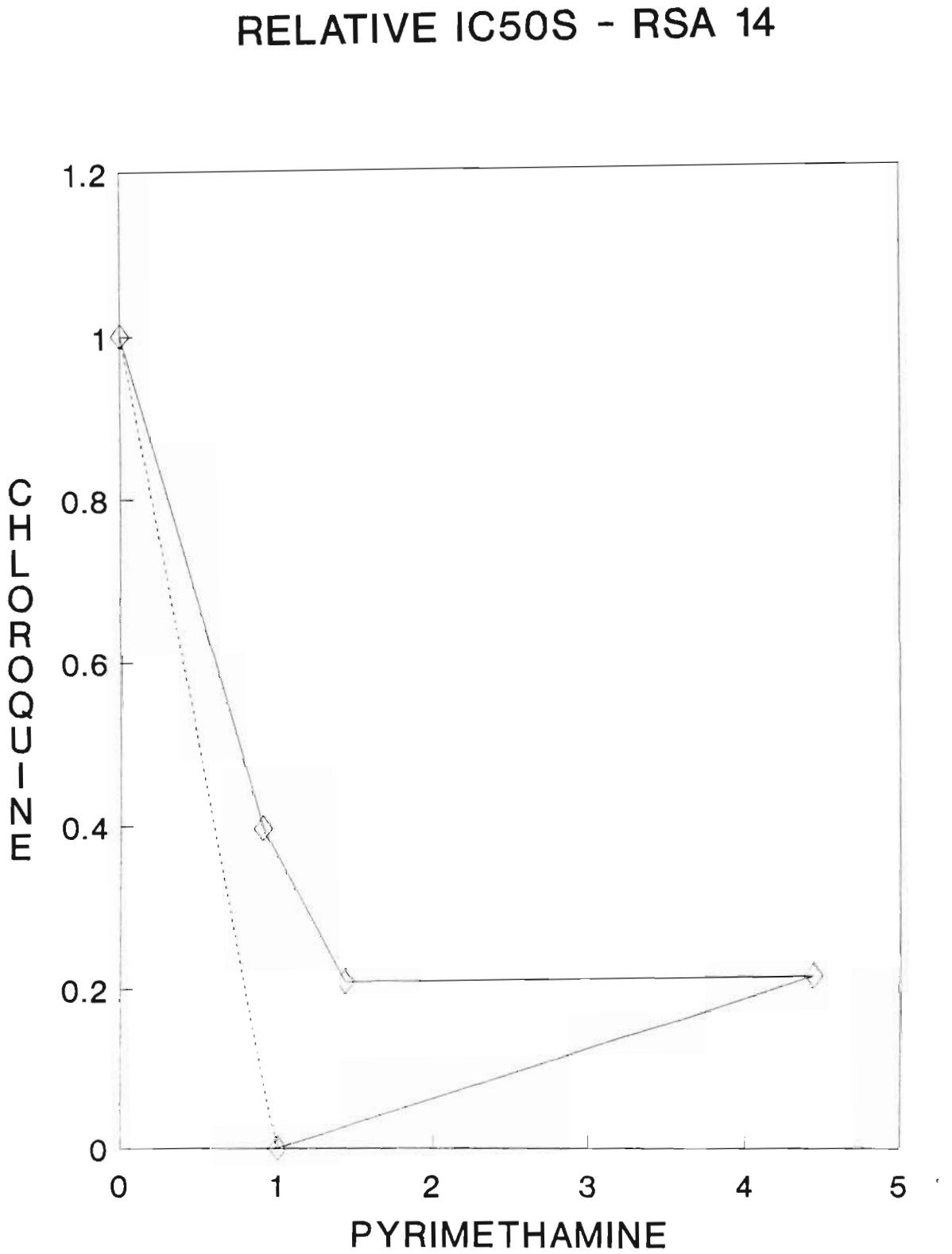


Fig. 33. Isobologram showing the relationship between chloroquine and pyrimethamine of chloroquine-resistant isolate RSA 14.

Table 17. The sum of relative IC_{50}s of the most effective drug combination for five isolates.

ISOLATE	CQ (nM)	PYR (nM)	SUM REL
RSA 2	30	30	0,6
RSA 5	10	2100	0,8
RSA 6	150	30	2,3
RSA 9	450	60	1,3
RSA 14	180	30	1,3

6.4 DISCUSSION

6.4.1 Antimalarial Activity

The chloroquine MICs show good agreement with results obtained from previous drug sensitivity tests for all isolates using both the 48-hour *in vitro* test (Freese *et al*, 1991) and growth assessment by isotope incorporation (Freese, 1994). The results indicate that RSA 2 and 5 are chloroquine-sensitive and that RSA 6, 9 and 14 are chloroquine-resistant as described in previous studies (Freese *et al*, 1988: 1991). The chloroquine IC_{50}s of RSA 5 and 14 are comparable with those determined by Freese *et al* (1991) using the same method of growth assessment but not the IC_{50}s of RSA 6 and 9 (Appendix 3).

The pyrimethamine MICs ranged from 300nmol/ℓ to >3000nmol/ℓ. The three isolates, all chloroquine-resistant (RSA 6, 9 and 14), were inhibited at 300nmol pyrimethamine/ℓ and two chloroquine-sensitive (RSA 2 and 5) were not inhibited at 3000nmol pyrimethamine/ℓ. These results are consistent with those obtained previously by Freese *et al* (1991). The pyrimethamine IC_{50}s (Table 11) of RSA 6, 9 and 14 are lower than those found by Freese *et al* (1991). The IC_{50} for RSA 2 is 194nmol/ℓ which is high compared to 31nmol/ℓ obtained in previous studies (Freese *et al*, 1991).

The pyrimethamine MICs and IC_{50}s (Table 11) show greater variation when compared to those obtained previously for the same isolates. This may be ascribed to the different methods used to determine the sensitivities, i.e. the isotope incorporation method used for this study and growth assessment by microscopy in a 48-hour *in vitro* test used in previous investigations by Freese *et al* (1991).

The isotope incorporation method detects sensitivities at lower concentrations than the 48-hour *in vitro* test (Freese *et al*, 1991). However, the difference in these values is insignificant because of the wide range of pyrimethamine sensitivities that have been shown to occur amongst the southern African isolates of *P. falciparum* (ranged from 10 to >3000nmol/ℓ) (Freese *et al*, 1991).

The IC_{50} value is a more sensitive measure of drug susceptibility than the MIC value and it is possible that these values and to a lesser extent, the MIC values, are showing differences in the clonal make-up of the isolates at different times. In other words, the proportions of clones with different sensitivities in these isolates vary at different times of testing, resulting in different IC_{50} and MIC values (Freese *et al*, 1991). Another explanation for these differences in MIC and IC_{50} values obtained at different times of testing is the possible cross-contamination of *P. falciparum* cultures maintained in the laboratory (Robson *et al*, 1992). To avoid cross-contamination between cultures, Robson *et al* (1992) suggested (1) that suitable markers should be identified for each laboratory line of parasites to allow monitoring at regular intervals, (2) that cloned isolates should be used for all laboratory studies and (3) that careful comparisons be made where the study involves drug resistance or antigen specificity. Results from characterization studies using southern African isolates of *P. falciparum* (Freese, 1994) suggested that cross-contamination may have occurred between these isolates. Recently Wooden *et al* (1993) developed a method for distinguishing *P. falciparum* strains in the laboratory based on the polymerase chain reaction (PCR) technique. This method could be used to determine whether or not contamination has taken place within the bank of southern African isolates of *P. falciparum*.

6.4.2 Synergistic action of pyrimethamine with chloroquine

The relationships between chloroquine and pyrimethamine are represented in Figs. 29-33 (see Appendix 6 for explanation of the graphs). Although the respective sums of relative IC_{50}s for RSA 2 and RSA 5 (0.6 and 0.8 respectively), (Table 17), indicate a weak synergism since it has been suggested (Hall *et al*, 1983) that a weak synergism occurs when the value of Σ relative $IC_{50} \geq 0.5$, The graphs for the two chloroquine-sensitive isolates (RSA 2 and 5) clearly show no synergism between pyrimethamine and chloroquine.

For the chloroquine-resistant isolates, RSA 6, 9 and 14, an antagonistic effect between the two drugs is indicated by Figs. 31-33 (refer to Appendix 6 for explanation of the graphs) and this is confirmed by their Σ relative IC_{50}s which are all above 1.0 (1.3 each for RSA 9 and 14 and 2.3 for RSA 6). It seems from these results, that the two drugs should not be used in combination, since an antagonistic action is exhibited. Although these results indicate an antagonistic action when chloroquine and pyrimethamine were used in combination, it has been reported that the two drugs had an additive effect on the blood stages of *P. berghei* (Peters, 1987).

6.5 CONCLUSION

Previous work based on malaria chemotherapy confirmed the existence of chloroquine-resistance parasites in South Africa. Herbst *et al* (1985, 1987) found that malaria patients from KwaZulu-Natal Province harboured chloroquine-resistant isolates of *P. falciparum* and that their levels of resistance to this drug were high.

These reports were later confirmed by Freese *et al* (1988) who reported high levels of chloroquine resistance at $\geq$32pmols per well in northern KwaZulu-Natal. From these findings there is absolutely no doubt that chloroquine resistant *P. falciparum* malaria is a serious problem in southern Africa. The continuous discovery of chloroquine resistant malaria in southern Africa and world-wide show that this drug can no longer be relied upon on its own.

The results of this and other studies indicate too that pyrimethamine, like chloroquine, is no longer useful for malaria prophylaxis on its own. Therefore, it is not surprising that the prescription of this drug is no longer recommended in this country. Although pyrimethamine resistance in southern Africa is not well documented, its resistance was discovered in many parts of the world as far back as the 1960s (Peters, 1987). These areas include many African countries like Gambia, Senegal, Ghana, Nigeria, Kenya and Tanzania as well as Malaya, India, Venezuela and many South American countries (Peters, 1969). Resistance to pyrimethamine was also established in Pakistan and Thailand (Doberstyn *et al*, 1979). These reports as well as the current one on the problem of drug resistance have serious implications for the control of malaria.

Initially, chloroquine was used in combination with pyrimethamine and marketed as Darachlor apparently because of their additive effect. It was thought that chloroquine with its blood schizontocidal effect would work with pyrimethamine, which is both a slow schizontocide and a sporontocide, against *P. falciparum* (Peters, 1987). A second reason for the formulation of this compound was to delay the emergence of resistance to either of the drugs (Peters, 1987).

Although this combination was very popular in the 1950s and 1960s, it had neither an additive effect nor any impact on the emergence of resistance which became established in many parts of the world (Desjardins *et al*, 1988; Peters, 1987). These present results confirm the lack of positive interaction between pyrimethamine and chloroquine. Future research on the chemotherapy of malaria should focus on combinations of both new and old drugs in an attempt to enhance their activity and to delay the development of resistance. Since this study was carried out on only five of the 19 available southern African isolates of *P. falciparum*, it would be valuable to investigate the action of pyrimethamine and chloroquine in combination on the remaining 13 isolates.

The isobolograms representing the results from the two chloroquine-sensitive isolates (RSA 2 and 5) show points on both sides of the middle line (Figs. 29-30) and those for the chloroquine-resistant isolates (RSA 6, 9 and 14), show points well above the midline (Figs. 31-33). Firm conclusions cannot therefore be drawn on whether or not the combination of the two drugs is additive or antagonistic. More studies need to be carried out to confirm the suggested antagonism but the interaction of the two drugs was definitely not synergistic.

CHAPTER 7

IMPLICATIONS FOR CONTROL OF MALARIA IN SOUTH AFRICA

7.1 GAMETOCYTOCIDAL EFFECT

The results from this investigation (see Chapter 5) indicate that pyrimethamine is still effective against the young stages of the gametocytes, thus preventing them from maturing. This confirms statements made earlier by Black *et al* (1986) that the immature stages of gametocytes of *P. falciparum* are sensitive to the drug. In conclusion to the observations made from this study, it can be stated that pyrimethamine may have a role to play in South Africa. The drug may not be useful against the asexual parasites as reduced susceptibility has been established *in vitro* (Freese *et al*, 1991), but it may be effective in blocking transmission by acting against the young gametocytes thereby decreasing the overall number of gametocytes.

7.2 SPORONTOCIDAL EFFECT

Pyrimethamine has been shown to inhibit the development of the sexual cycle of *P. falciparum* in the mosquitoes when administered to gametocyte carriers on whom the mosquitoes fed (Peters, 1987). Since this drug was effective against the developing mosquito stages it was known to play a role in interrupting transmission (Desjardins *et al*, 1988). The results observed from gametocyte exflagellation showed that one isolate, RSA 2, was affected by the drug at the previously determined MIC of 100nmol/ℓ and the other, RSA 5 (at the MIC of 3000nmol/ℓ) was not affected.

Since exflagellation was not investigated in the chloroquine-resistant southern African isolates and the sporontocidal effect of pyrimethamine was not investigated in this study either, firm conclusions cannot be made at this stage concerning the sporontocidal effect of pyrimethamine on these isolates. However, previous findings by Teklehaimanot *et al* (1985) showed that pyrimethamine had a sporontocidal action against the isolates whose asexual erythrocytic stages are sensitive to the drug but no sporontocidal activity on the isolates that have resistant asexual stages resistant. This investigation led to conclusions that a cross-resistant relationship exists between asexual and sexual stages of the parasites (Teklehaimanot *et al*, 1985). McCutchan (1988) also discovered that resistance to pyrimethamine was accompanied by resistance to their sporontocidal effect.

Previous case studies confirm a lack of sporontocidal action of pyrimethamine on *P. falciparum* resistant to the drug. Peters (1987) found that pyrimethamine failed to prevent sporogony in two malaria patients once resistance was established and that when repeated doses were administered to a Gambian baby, no effect was exerted on *P. falciparum*. Resistance to pyrimethamine was not only established in *P. falciparum*, but to other human species of *Plasmodium* as well. For example, a field study carried out in Venezuela showed that out of 15 cases that showed no response to pyrimethamine, 12 were associated with *P. vivax* and three with *P. falciparum* (Peters, 1987). Peters (1987) concluded that an increased dosage within tolerated limits will not help to improve the reduced parasite susceptibility if pyrimethamine is used on its own.

7.3 SYNERGISTIC EFFECT OF PYRIMETHAMINE AND CHLOROQUINE

The use of the combined chloroquine/pyrimethamine was introduced with the hope that the antimalarial action would be increased and that theoretically, resistance development to the components would be delayed. Unfortunately, studies showed that there was neither a synergistic effect nor an additive action between the two drugs (Peters, 1987) and resistance to both chloroquine and pyrimethamine is widespread (Björkman & Phillips-Howard, 1990; Wernsdorfer, 1991). The antagonistic effect of pyrimethamine on chloroquine requires further investigation as these two drugs are still available in the country and their effectiveness might be diminished if used in combination.

Until recently, the pyrimethamine/chloroquine combination was recommended for both prophylaxis and treatment in South Africa (Freese *et al*, 1991). These authors suggested too that the continued used of this drug combination would result in an increased prevalence of resistance malaria and that this would stabilize the level of resistance to both chloroquine and pyrimethamine. Fear was expressed that the effectiveness of other pyrimethamine combinations might also be placed in jeopardy. Although resistance to the chloroquine/pyrimethamine combination was established in Ghana, amongst army personnel and their families, it was found to be useful in certain areas, e.g. in Haiti on a three-weekly administration to malaria patients (Peters, 1987). Peters (1987) also reported that it was the massive deployment of pyrimethamine in association with chloroquine that was responsible for the rapid spread of pyrimethamine resistance in *P. falciparum* almost world-wide.

Prescription of this drug in South Africa alone or in combination with chloroquine may result in a regrettable experience because these results show that there was no synergistic action when pyrimethamine was used in combination with chloroquine. Pyrimethamine/chloroquine (Darachlor) is no longer recommended in South Africa since this drug combination has proved to have little effect on the parasites (Baker *et al*, 1993). Growing drug resistance has serious implications since developing and researching new drugs involve expensive measures.

The frequent monitoring for drug sensitivity should continue to be monitored both *in vitro* and *in vivo* where possible to prevent widespread resistance to other drug combinations with pyrimethamine such as Fansidar in South Africa. Freese *et al* (1994) suggested that continued monitoring of the susceptibility of *P. falciparum* is essential and that any current information involving drug sensitivity be made available to the general practitioners, health workers, drug policy makers and the malaria control personnel to ensure effective malaria control and treatment.

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APPENDIX 1

Statistical analysis of the results shown in Figs. 10-14 in Chapter 3.

Isolate	Comparison	Diff. in Pr. value	LSD	p-value
RSA 2	St vs Sy	0.5	0.75	**NS**
RSA 3	St vs Sy	2.65	0.78	**<0.01**
RSA 5	St vs Sy	0.79	1.23	**NS**
MW2	St vs Sy	1.66	1.61	**<0.05**
NF54	St vs Sy	1.61	0.89	**<0.05**

Key: St = Static cultures
Sy = Synchronized cultures
Diff. in Pr. = Difference in Predicted values
LSD = Least significant difference
NS = not significant

APPENDIX 2

Chloroquine sensitivity- MICs (nM)

Isolate	A	B	C
RSA 2	ND	60	40
RSA 5	40	100	20
RSA 6	320*	300	320
RSA 9	320*	600	320
RSA 14	320*	600	160

Key: A = results from this study obtained by Isotope incorporation method.
B = results from previous study obtained by growth assessment by microscopy in a 48-hr *in vitro* test.
C = results from previous study obtained by growth assessment by isotope incorporation method.
* = the isolate was not inhibited at this concentration.
ND = not determined.

APPENDIX 3

Chloroquine sensitivity- IC_{50}s (nM)

Isolate	A	B	C
RSA 2	ND	36	30
RSA 5	13	51	10
RSA 6	44	122	140
RSA 9	69	207	160
RSA 14	70	255	60

Key: A = results from this study obtained by Isotope incorporation method.
B = results from previous study obtained by growth assessment by microscopy in a 48-hr *in vitro* test.
C = results from previous study obtained by growth assessment by isotope incorporation method.
ND = not determined.

APPENDIX 4

Pyrimethamine sensitivity- MICs (nM)

Isolate	A	B
RSA 2	3000*	100
RSA 5	ND	3000
RSA 6	300	100
RSA 9	300	100
RSA 14	300	100

Key:

A = results from this study obtained by Isotope incorporation method.

B = results from previous study obtained by growth assessment by microscopy in a 48-hr *in vitro* test.

* = the isolate was not inhibited at this concentration.

ND = not determined.

APPENDIX 5

Pyrimethamine sensitivity- IC_{50}s (nM)

Isolate	A	B
RSA 2	194	31
RSA 5	ND	1426
RSA 6	9	49
RSA 9	22	46
RSA 14	5	26

Key:

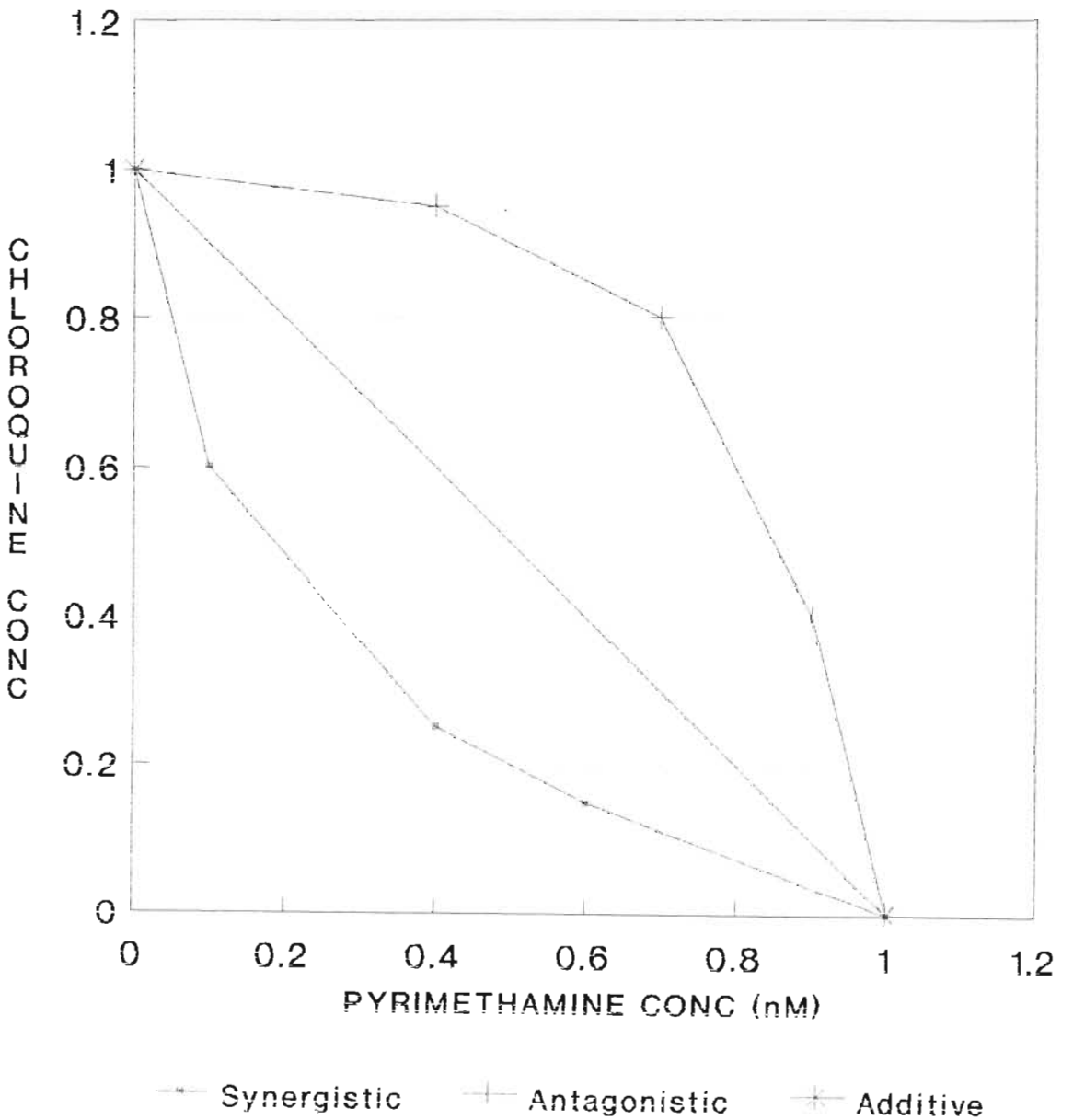
A = results from this study obtained by Isotope incorporation method.

B = results from previous study obtained by growth assessment by microscopy in a 48-hr in vitro test.

ND = not determined.

APPENDIX 6

An example of an isobologram.



APPENDIX 7

Peak numbers of Stage II gametocyte counts per 10 000 RBCs.

Isolate	Static cultures		Synchronized cultures	
	Control	Trial	Control	Trial
RSA 2	16 (8)	22	174 (1)	0
RSA 3	36 (6)	0	45 (19)	0
RSA 5	74 (7)	60	227 (9)	89
MW2	33 (2)	11	20 (2)	0
NF54	12 (9)	0	340 (6)	0

Key:

The numbers within brackets denote the days on which the peak was reached.