THE EFFECT OF IRON AND IRON CHELATORS ON THE GROWTH OF AN *IN VITRO PLASMODIUM FALCIPARUM* CULTURE

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ABSTRACT

The influence of iron on the outcome of various infections have been extensively reviewed. Clinical observations suggests that iron deficiency may be protective against malaria. Various researchers have shown that certain iron chelators blocked the proliferation of *Plasmodium falciparum in vitro* and *in vivo*. However the exact mode of malaria inhibition by iron chelators remains undefined. We have investigated the effect of iron chelators on the *in vitro* growth of *P. falciparum* .

Two strains of *P. falciparum* , the FCR-3 and the RSA-2 strains were cultured *in vitro* according to the method described by Freese *et al* (1988). The parasites were synchronized using the method of Lambros and Vanderberg (1979).

The sensitivity of both strains of parasites was evaluated against various concentrations of the following chelating agents, DFO, DFT, BIP, EDTA, ESTA, and DTPA. Desferrioxamine combined with unsaturated and iron saturated 2,2'-bipyridyl was tested on the FCR-3 strain. The chelator sensitivity tests were performed in 96-well microtiter plates. Incorporation of [^{3} H] hypoxanthine

was used as an index of growth. The parasites were harvested with an automated cell harvester. Results are expressed as a percentage of the controls with no chelator added. The dose response curves of the 6 chelators alone and those combined were determined.

IC_{50} values for the chelating agents when tested on both strains were determined.

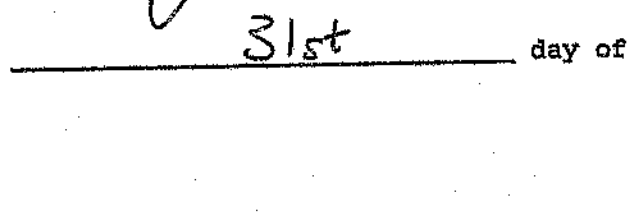
2,2'-Bipyridyl at various concentrations when combined with DFO, showed a relative increase in parasitaemia compared to the 2,2'-bipyridyl alone. Thus suggesting that these concentrations of 2,2'-bipyridyl antagonised the antimalarial effect of desferrioxamine. When 2,2'-bipyridyl was saturated with ferrous ammonium sulphate at equimolar concentrations and combined with desferrioxamine, the resultant dose response curve reflected the effect of desferroxamine alone.

The 6 chelators inhibited the *in vitro* growth of both strains of parasites. Iron deprivation was considered a possible mechanism of inhibition by the iron specific chelating agents. However if 2,2'-bipyridyl antagonised the antimalarial action of DFO instead of combining synergistically or additively with it, other possible

mechanisms of inhibition besides iron deprivation exists. The effective concentrations of DFO were compatible with the therapeutic concentrations used in patients with iron overload. These findings suggest prospects for future research.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



31st day of December, 1991.

LIST OF CONTENTS

PREFACE

I first became aware of the malaria problem when I visited the Eastern and Northern Transvaal and was advised to use prophylaxis against malaria. Subsequently I was made aware of the increasing spread of chloroquine-resistant malaria throughout the malaria endemic regions where *Plasmodium falciparum* is found.

Although chloroquine-resistant malaria was not reported in South Africa, its presence was confirmed in neighbouring states such as Angola, Namibia, Mozambique and in West Africa. I therefore realized that it was a matter of time before there was an emergence of chloroquine-resistant malaria within South Africa. My awareness of chloroquine-resistance was further increased after I performed a retrospective study based on the medical records of the malaria patients admitted to the Johannesburg hospital during 1988. To compound the chloroquine-resistant problem, a vaccine against malaria seems a long distance away. Therefore there is a need for alternate modalities for the therapy of malaria.

To study the parasites it was essential to establish an *in vitro* culture system of the parasites. With the guidance of Mrs. J Freese of the RIDTE in Durban, two

strains of parasites were successfully maintained in culture.

Portions of this dissertation have been presented at local and international conferences.

Jairam KT, Monteagudo FSE, Moch SL, Havlik I. Malaria at Johannesburg hospital : A retrospective study. South African Pharmacological Society Congress, Stellenbosch, 1989.

Jairam KT, Havlik I, Monteagudo FSE. *Plasmodium falciparum* : Inhibition of *in vitro* growth by iron chelators. South African Pharmacological Society Congress, Johannesburg, 1990.

Monteagudo FSE, Havlik I, Moch SL, Jairam KT. Aspects of malaria in Johannesburg. British Society of Parasitology, eleventh Malaria meeting, London, 1990.

Havlik I, Jairam KT, Van Zyl RL, Monteagudo FSE. Combined effect of iron chelators and antimalarials on an *in vitro* malaria parasite culture. Sixth Southeast Asian/Western Pacific Regional Meeting of Pharmacology, Hongkong, 1991.

Parts of this dissertation have been included in the following publications:

Jairam KT, Monteagudo FSE, Moch SL, Havlik I . Malaria at Johannesburg hospital : A retrospective study. S Afr Med J 1990; 78: 467-469.

Jairam KT, Havlik I, Monteagudo FSE. Possible mechanism of action of desferrioxamine and 2,2'-bipyridyl on inhibiting the *in vitro* growth of *Plasmodium falciparum* (FCR-3 strain) . Biochem Pharm 1991; 42(8):1633-1634.

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LIST OF ABBREVIATIONS

BIP	2,2'-bipyridyl
DFO	desferrioxamine
DFT	desferrithiocin
DTPA	diethylenetriamine pentaacetic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (B-aminoethyl ether) N,N,N',N',-tetraacetic acid.
g	weight in grams
g	centrifugation in gravity
HEPES	N-(2-Hydroxyethyl)piperazine-N'-ethane-sulphonic acid.
IC_{50}	drug concentration resulting in 50 % inhibition of parasite growth.
l	litre
ml	millilitre
mM	millimoles
MW	molecular weight
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
%	percent
PBS	phosphate buffered saline
ug	microgram
uM	micromole

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

SECTION 1.1 ASPECTS OF MALARIA

1. Distribution of malaria

Malaria is endemic in more than 100 countries in Asia, Africa, Oceania , certain Caribbean islands, Central and South America (WHO, 1988 a). It is the most important of the transmissible parasitic diseases in the tropics, causing significant morbidity and mortality especially among children . It is estimated that approximately 50 % of the world's population is at risk from malaria (Gilles, 1989).

2. Routes of infection

Malaria is usually acquired from a bite of an infected Anopheline mosquito (WHO, 1988 b). It is also transmitted, although rarely by transfusion of infected blood, use of contaminated syringes among drug addicts, and transplacental transmission (Harinasuta and Bunnag, 1988).

3. Types of malaria

The malaria parasite belongs to the genus *Plasmodium* of which 4 species, *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, regularly infect man (Warhurst, 1987; Garnham, 1988). *P. vivax* is the most extensively distributed and causes much debilitating disease. *P. ovale* is mainly confined to Africa and is less common. *P. malariae* also occurs widely and causes the most persistant infections, although the least severe (WHO, 1988 b). *P. falciparum* is the dominant malaria species and is responsible for the overwhelming majority of infections. It causes the most severe infections and is responsible for nearly all malaria related deaths (WHO, 1988 c). Mixed infections of *P. falciparum* and one of the other three species also occurs (Isaacson, 1987; Harinasuta and Bunnag, 1988).

4 . Life cycle of the parasite

The Plasmodium species affecting man live in the female Anopheline species during the sexual (sporogonic) phase of the cycle . Sporozoites which are produced from the sexual form migrate to the salivary gland of the mosquito. When the sporozoites are introduced into the human blood stream after the mosquito has had a blood

meal, they rapidly penetrate the parenchymal cells of the liver and undergo one cycle of asexual division which is described as pre-erythrocytic schizogony .

Many merozoites are released into the blood stream, about 5-20 days (depending on the species) after infection and invade circulating erythrocytes. The rapid intraerythrocytic multiplication of the merozoites (blood schizogony) is followed by the rupture of the host erythrocytes releasing another generation of the merozoites. These merozoites will also invade circulating erythrocytes , multiply, and release another generation of merozoites into the blood stream. The erythrocytic stages of schizogony are responsible for the pathological effects of malaria . The rupturing of the erythrocytes and the release of the parasite metabolic byproducts , produces the episodic chills and fever that characterizes several variants of this disease (WHO 1988 a, Garnham 1988) .

Some merozoites develop into male and female gametocytes (sexual stages) which fuse to form a zygote once inside the stomach of the mosquito, after it has had a blood meal from an infected person. The zygote or oocyst develops on the outside of the mosquito stomach and divides to produce sporozoites. These sporozoites will

travel to the salivary glands of the mosquito and are introduced into the blood stream of the next human host during the next blood meal of the mosquito (Warhurst, 1987 ; Garnham, 1988).

The life cycle of the parasite is represented in figure 1.1 (Moore *et al.*, 1990).

The tissue forms (hypnozoites) of *P. vivax* and *P. ovale* persist in the liver for months or even years and are responsible for the relapses of the malaria infection. *P. falciparum* and *P. malariae* do not generate these latent forms of malaria. In these species, recrudescence of the infection results from the persisting blood forms in untreated or inadequately treated patients (WHO, 1988 b).

5. Genetic and immunological factors

Susceptibility to malaria may be genetically determined . Persons originally from West-Africa are not susceptible to *P. vivax* malaria as they do not have Duffy blood group determinants. Bushmen and Caucasians, however, have these factors which seems to serve as the specific receptor on the erythrocytic surface for the *P. vivax* parasite. Certain abnormal haemoglobins do not support

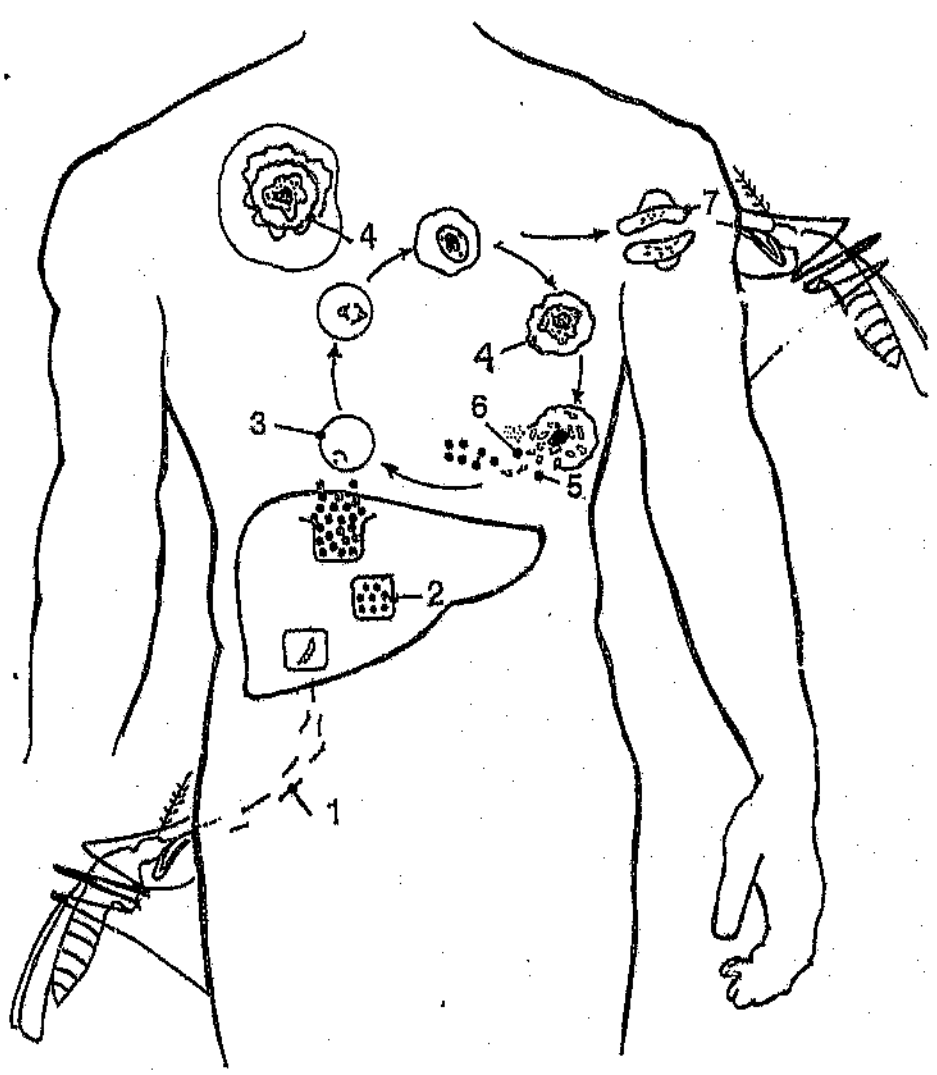

Figure 1.1: Life cycle of *Plasmodium falciparum*

Figure 1.1: Life-cycle of *PLASMODIUM FALCIPARUM*.

P. falciparum sporozoites (1) are injected into the human host by the bite of an infected Anopheline mosquito. Sporozoites circulate in the blood for not more than 60 minutes. An exocrythrocytic phase occurs in liver parenchymal cell (2). The hundreds of merozoites released from a liver cell are able to invade red blood cells (3) and to initiate the erythrocytic cycle. No further development of *P. falciparum* takes place in liver tissues. The asexual blood forms are responsible for the pathogenesis of the disease. The plasma membrane of the parasitized erythrocyte is altered as the parasite matures (4). *P. falciparum* differs from other human malarias as the erythrocytes infected with the mature stages are not present in the peripheral blood but are sequestered (4). Finally, the infected erythrocyte lyses and merozoites (5) and toxins (6) are released. Merozoites invade erythrocytes within seconds to repeat the erythrocytic cycle, with an average of 16-fold increase every second day, while others enter erythrocytes and differentiate into crescentic shaped gametocytes (7), the sexual forms which can complete the cycle within the mosquito.

the development of *P. falciparum* within the erythrocytes, and thus confer a degree of protection against falciparum malaria. In these situations the fatality rates tend to be lower e.g. sickle cell anaemia. Thalassaemia and glucose-6-phosphate dehydrogenase deficiency are also protective against malaria (Isaacson, 1987).

6. Clinical aspects of malaria

6.1 Diagnosis of malaria

Malaria cannot be definitively diagnosed clinically. A history of travel in a malaria endemic region , even a brief stopover should alert the doctor of the possibility of a malaria infection (Gilles, 1988; Gilles, 1989; Phillips, 1988). Malaria has also been contracted by persons who have never travelled to any malarious area, but who lived near or worked at airports in Western Europe. These cases of "airport malaria" are presumed to have been infected by mosquitoes trapped in parts of the aircraft, luggage , or in freight from a malaria endemic area and released on arrival (Isaacson,1987; Phillips, 1988). Cases of "imported malaria" are frequently reported from the United Kingdom in tourists who visited the tropics. Malaria can and does mimic many diseases therefore, the travel history is crucial (Gilles, 1988;

Phillips, 1988).

As soon as malaria is suspected blood smears are to be examined by an experienced microscopist. If the smear is negative, malaria should still be considered, as chemotherapy may suppress the parasitaemia. Thin and thick smears are considered a mainstay in the diagnosis of malaria (Shute, 1988 ; WHO, 1988 c). Diagnosis of the species of malaria is important as *P. falciparum* is resistant to chloroquine . The treatment selected should be based on known geographical resistance patterns (Gilles ,1988).

6.2 Pathophysiology of malaria

The clinical features of *P. falciparum* infections are associated with the asexual intraerythrocytic stages of the parasites (Robson, 1989). Sequestration is central in the pathophysiology of *P. falciparum* malaria. The mature stages of the parasitized erythrocytes withdraw from the circulation and adhere to post capillary venular endothelium in deep vascular beds.

These parasitized erythrocytes are tightly packed in the microcirculatory beds of the brain, heart, kidneys and gut. This results in organ dysfunction as the blood flow

to these organs is diminished. Sequestration is a feature unique to *P. falciparum* infections and is possibly responsible for the increased virulence of this species compared to other species of human malarias (Boonpucknavig and Boonpucknavig, 1988; Hall,1977).

The clinical aspects of malaria are reviewed in a paper which is a retrospective study based on the medical records of all the malaria patients admitted to the Johannesburg Hospital during 1988. This paper is reprinted with the permission of the Editor of the South African Medical Journal.

6.3 Reprint of article: (Jairam KT, Monteagudo FSE, Moch SL, Havlik I. Malaria at Johannesburg hospital : A retrospective study. S Afr Med J 1990; 78:467-469).

6.3.1 Summary

A total of 43 patients diagnosed as having malaria were admitted to Johannesburg Hospital during 1988;40 (94%) were infected with *P. falciparum*. Only 25 patients were recorded as having used prophylaxis of any kind; chloroquine alone and in combination was used as prophylaxis by 17. Patients were treated with quinine

(alone and in combination) in 67% of cases. In 4.5 of patients, chloroquine-resistant malaria was considered a possibility.

6.3.2 Introduction

Over the last 10-15 years the deterioration in the incidence of malaria in Africa has been partly due to the increasing spread of a *P. falciparum* population that is resistant to chloroquine and other drugs. There have been reports of chloroquine-resistant *P. falciparum* malaria from regions such as Ethiopia (Olatunde *et al.*, 1977), Kenya (Fogh *et al.*, 1979), East Africa (Jepsen *et al.*, 1983), northern Malawi (Wolfe *et al.*, 1985), and Mozambique (Schwalbach *et al.*, 1985) and chloroquine resistance has also been confirmed *in vitro* in southern Africa (Isaacson *et al.*, 1984 ; Freese *et al* , 1986).

The difficulty of malaria control in chloroquine resistant parasites is further compounded by the toxicity of alternative drugs that make it difficult to propose effective and safe medication for chemoprophylaxis and therapy.

A selected group of malaria patients and the drugs used in their therapy is reviewed . It is important to note that Johannesburg Hospital is a tertiary referral centre and the patients reported may not be representative of the general population.

6.3.3 Patients and methods

Johannesburg Hospital has 833 beds at present and during 1988 there were approximately 32051 admissions. The hospital serves as a tertiary referral centre and is a teaching hospital of the University of the Witwatersrand. The records of the Haematology Laboratory of the South African Institute for Medical Research in Johannesburg, which serves the hospital, were studied to identify all patients with positive malaria smears admitted during 1988. The medical records of these patients were then obtained from the Medical Records Department.

6.3.4 Results

There were 43 positive blood smears from 32 male and 11 female patients (median age 30 years; range:3-66years). The incidence of malaria in the different age groups is shown in Table 1.1.

6.3.4.1 TABLE 1.1

INCIDENCE OF MALARIA IN DIFFERENT AGE GROUPS

Age group (Yrs)	No of patients	%
0 -10	2	5
11-20	5	12
21-30	20	46
31-40	7	16
41-50	3	7
51-60	4	9
61-70	2	5

6.3.4.2 Types of malaria

Infection with *P. falciparum* was the most common type of malaria seen and occured in 40 patients (94%). patients were infected with *P. vivax* and *P. ovale* respectively, and in 1 patient the type of malaria was not identified.

The regions where the patients were thought to have contracted malaria are listed in Table 1.2. Six patients travelled through many malaria-endemic countries in Africa and so a definite area where they were infected

could not be determined (Trans-Africa). The three regions where patients most often contracted malaria were Botswana, Malawi and Zimbabwe. None of the patients in this study were thought to have contracted the disease in South Africa.

6.3.4.3 TABLE 1.2

SUSPECTED REGIONS WHERE MALARIA WAS CONTRACTED

Region	No of patients	%
Botswana	8	19
Malawi	7	16
Zimbabwe	7	16
Trans-Africa	6	14
Zambia	4	10
Swaziland	3	7
Unknown	3	7
Zaire	2	5
Ghana	1	2
Kenya	1	2
SWA	1	2
	13	

6.3.4.4 Drugs used as prophylaxis

Twenty-six patients (60%) had taken prophylaxis of some kind, while 10 patients had used no prophylactic agents (Table 1.3.). Data from 7 patients were not available. Only 13 of the 26 patients who had taken prophylactic measures had used the right dosage and, of these, only 11 continued taking medication after returning from an endemic malaria area. Chloroquine alone and in combination was used by 17 patients.

TABLE 1.3

DRUGS USED AS PROPHYLAXIS

Drugs	No of patients	%
Chloroquine	8	31
Chloroquine + Pyrimethamine	4	15
Chloroquine + Proguanil	4	15
Dapsone + Pyrimethamine	2	8
Chloroquine + Pyrimethamine + proguanil	1	4
Pyrimethamine & Sulphadoxine	1	4
Prophylaxis taken but details not available	6	23
Total	26	100

6.3.4.5 Presenting clinical features

Most patients presented with a 'flu-like illness - fever, headache and splenomegaly being the major presenting signs (- Table 1.4). The median period of duration of symptoms before diagnosis was confirmed was 4 days (range: 1-30 days).

TABLE 1.4

CLINICAL FEATURES ON ADMISSION TO HOSPITAL

Presenting features	No of patients	%
Fever	38	80
Headache	31	72
Splenomegaly	31	72
Rigors	22	51
GIT symptoms	21	49
Jaundice	20	47
Hepatomegaly	19	44
Photophobia	10	23
Myalgia	8	19
Tachycardia	8	19
Conjunctivitis	3	7
Hypotension	1	2

More than one sign was present in some patients.

6.3.4.6 Laboratory abnormalities and clinical complications

Laboratory abnormalities included thrombocytopenia, anaemia, bilirubinaemia and leucopenia (Table 1.5). Clinical complications included cerebral malaria, acute renal failure and adult respiratory distress syndrome (ARDS).

TABLE 1.5

LABORATORY ABNORMALITIES AND CLINICAL COMPLICATIONS

Complications	No of patients	%
Thrombocytopenia	24	56
Anaemia	20	47
Raised bilirubin levels	19	44
Leucopenia	16	37
Acute renal failure	4	9
Cerebral Malaria	4	9
Adult Respiratory Distress Syndrome (ARDS)	1	2
	16	

6.3.4.7 Drugs used f· ·rapy

In 42% of patients the possibility of chloroquine-resistant malaria was considered. This assessment was reached because the patient had become infected despite using adequate chloroquine prophylaxis, or because the patient did not respond to initial therapy using chloroquine, and was then treated with another drug. Quinine combined with tetracycline was most commonly used for therapy (Table 1.6).

TABLE 1.6

DRUGS USED FOR THERAPY

Drugs	No of patients	%
Quinine combined with tetracycline	23	53
Quinine alone	4	9
Chloroquine	4	9
Quinine combined with other drugs	2	5
Details of therapy not available	10	24

Twenty-nine (67%) patients were treated with quinine (alone or in combination) and side-effects occured in 12 (41%) (nausea occured in 5 (17%), cinchonism in 4 (14%), hypoglycaemia in 1 (3%), and cinchonism together with hypoglycaemia was seen in 2 (7%)).

While the data for the clinical course of 11 (26%) patients remains unknown, 31 (72%) patients recovered, with the median time spent in hospital being 7 days (range 2-17 days). One death was recorded in a woman diagnosed as having cerebral malaria, who presented late. She was in renal failure and coma on admission and subsequently died despite vigorous therapy.

6.3.5 Discussion

Three problem areas became apparent when reviewing the data : (i) timeous diagnosis and correct management of malaria; (ii) effective and safe prophylaxis; and (iii) degree of chloroquine resistance.

Major shortcomings in the management of malaria are a delay in diagnosis and inappropriate treatment (Hall, 1976). Rapid diagnosis can be made using thick and thin blood smears, but the clinical features with which the patient presents and , in particular, a history of travel from an endemic area are also important clues .

Morbidity due to malaria is serious, particularly if diagnosis is delayed. The median duration of symptoms before diagnosis was 4 days, with a range of 1 - 30 days in our study. Available statistics suggest that many deaths in short-stay travellers due to malaria occur after return to their country of origin as a result of inadequate diagnosis and medical care (Lobel, 1985).

In this study, 29 patients, including 4 with cerebral malaria, were treated with quinine alone or in combination. In 11 of these patients chloroquine therapy was started but, because of a lack of response, quinine was subsequently administered. In 8 patients quinine therapy was instituted immediately, possibly because the patients had contracted malaria despite apparently using adequate chloroquine prophylaxis. There appeared to be no additional reason why quinine was administered immediately to the remaining 10 members of this group.

In 42% of patients chloroquine-resistant malaria was considered possible. Although this was not proven, the assessment is in keeping with current opinion about the frequency of chloroquine resistance. The implications

regarding prophylaxis and therapy of malaria are significant. The incidence of side-effects due to quinine in this group was low and those reported resolved with dose reduction.

Our data indicate that only 26% of patients used adequate prophylaxis. Adequate prophylaxis is important, since surveys have indicated that patients with non-fatal infections have a much higher rate of use of chemoprophylaxis than those who die (Lobel, 1985) . Details of recommended prophylaxis, although contentious, are available (WHO, 1988 (a) ; Cook, 1988 ; WHO, 1989; Spracklen *et al.*, 1981 ; Spracklen and Monteagudo, 1986 ; Talmud *et al.*, 1989 ; Ross ,1987).

A common laboratory finding was thrombocytopenia, which was short-lived and was not associated with any bleeding disorder. Cerebral malaria was seen in 4 patients. This diagnosis was based on the clinical features with which the patients presented, such as impairment of consciousness, acute convulsions, focal cerebral signs , coma and positive smears for malaria (Warrell *et al.*, 1982 ; Schmutzhard and Gerstenbrand, 1984). ARDS was observed in 1 patient who needed to be ventilated and was treated in the respiratory intensive care unit. There was 1 death in a woman diagnosed as having cerebral malaria, who presented late.

Future research in the field of malaria could include a study of the characteristics of parasitized and non-parasitized red blood cells. This may assist in understanding the mechanisms of resistance to drugs. The above observations suggest that the risk of contracting malaria may be minimised by programmes directed at encouraging the use of correct chemoprophylaxis by travellers. Travellers should also be educated about the fatal risk of malaria, and knowledge of diagnosis and treatment of malaria by health care practitioners could be improved to better achieve this objective.

SECTION 1.2 THE ROLE OF IRON IN MALARIA

1. Importance of iron to man

1.1 The metabolism of iron in man (metabolic, enzymatic and storage iron compounds)

Iron is abundantly found on earth but because of its low solubility in nature, iron depletion is one of the most common forms of nutritional deficiency. Iron is important to man as it catalyses one-electron reactions and is required for oxygen transport (Hershko and Weatherall, 1988). Iron has the ability to accept and donate electrons easily, thus it has a key role in biologically important oxidative and reductive processes (Pearson and Robinson, 1976). Thus iron has efficient mechanisms to acquire, transport and store iron (Hershko and Weatherall, 1988).

Once iron is ingested it is kept soluble by substances such as haem or iron binding-proteins e.g. transferrin, lactoferrin and ferritin (Peto and Hershko, 1989). More than 80% of the body iron is involved in the support of erythrocyte production as haemoglobin is the major iron containing compound of the body (Hillman and Finch, 1985; Pearson and Robinson, 1976). Iron is also an

important component of myoglobin and various enzymes such as the heme containing enzymes e.g. catalase, peroxidases, the metall(flavoprotein , and mitochondrial enzymes (Hillman and Finch, 1985).

Body iron may be differentiated between essential iron containing compounds and those compounds in which excess iron is stored. Quantitatively haemoglobin dominates the essential fraction (Hillman and Finch, 1985). Approximately 25% of the total body iron is storage iron. This is found mainly within the liver, spleen, bone marrow and muscle (Pearson and Robinson, 1976).

1.2 The transport of iron in a mammalian cell
(The role of transferrin and the receptor in a mammalian cell):

Transferrin, an iron binding protein found in plasma, has two binding sites for ferric iron (Hillman and Finch, 1985). It transports iron to intracellular sites by means .of specific receptors found in the cellular plasma membrane. The iron-transferrin complex binds to one of these transferrin receptors and is taken up by receptor mediated endocytosis. The iron then dissociates from transferrin in an acidic intracellular compartment in a

pH dependent fashion (Brown *et al.*, 1983). The actual steps involved in the removal of iron from transferrin are not known. Konopka *et al.* (1980, 1981) have suggested that the removal of iron from transferrin to ferritin is possibly mediated by pyrophosphate together with other organic polyphophates, as well as ascorbate .

1.3 The effects of iron overload and iron deficiency

1.3.1 Iron overload

Iron overload may be due to excess iron accumulating from increased absorption via the gastro-intestinal tract, as in primary idiopathic haemochromatosis or as a result of transfusion therapy for B-thalassaemia (Longueville *et al.*, 1986). Some of the iron in the plasma will also bind non-specifically to serum proteins (especially albumin), forming low molecular weight iron complexes (Hershko and Peto, 1987). This iron promotes the formation of highly reactive hydroxyl radicals which stimulate the peroxidation of the membrane lipids causing tissue damage in haemochromatosis (Halliwell and Gutteridge, 1984). In addition these low molecular weight iron complexes are ready sources of iron to invading microbes.

1.3.2 Iron deficiency

The main observable effect of iron deficiency is a hypochromic microcytic anaemia. Many measurable non haematological effects may also occur, for instance in epithelial tissues, brain, and cell-mediated immunity (Oppenheimer and Hendrickse, 1983).

Iron deficiency affects cell-mediated immunity via altered lymphocyte morphology (Peto and Hershko 1989). Lymphocytes harvested from iron deficient patients show degenerative changes of their mitochondria on ultrastructural studies (Jarvis and Jacobs 1974). Iron deficiency also inhibits DNA synthesis in human haemopoetic tissues, possibly by a decrease in activity of the iron dependent enzyme, ribonucleotide reductase (E.C.1.17.4.1) (Hershko *et al.* ,1970; Hoffbrand *et al* ., 1976).

2. Iron and microorganisms

2.1 Mechanisms used by microorganisms to acquire iron from the host

Iron is an essential nutrient to all life forms except the one bacterium, *Lactobacillus plantarum* which utilises

cobalt instead of iron (Peto and Hershko, 1989).

Microorganisms can assimilate iron from the environment in many different ways, including incorporating the iron in solution (FeII, iron citrate, haem iron) or by chelating agents referred to as siderophores (Bullen, 1981).

Siderophores may be actively secreted by the bacterium itself or by other bacteria in the same environment. The production of these chelators is genetically determined and depends on the iron levels in the environment. By means of siderophores, microbes can overcome the host's iron binding factors (Peto and Hershko, 1989).

It is not necessary for certain pathogens to secrete siderophores to utilize transferrin-bound iron. *Neisseria meningitidis*, in response to iron deficiency develops a surface mechanism which recognizes transferrin and removes the iron bound to it (Peto and Hershko, 1989). It has also been observed that some virulent plasmids encode iron sequestering systems which enable bacteria to obtain iron in conditions of limited iron availability (Crosa JH, 1980). The genetic location of these systems whether chromosomal or extrachromosomal is unknown (Finkelstein et al., 1983).

2.2 Iron and the malaria parasite

2.2.1 A parasite derived transferrin receptor

The transferrin receptor is only present on the reticulocyte membrane and eventually disappears as the reticulocyte matures (Enns *et al.*, 1981).

There have been a number of reports on the possibility of a parasite derived transferrin receptor. Pollack and Flemming (1984) have shown that there is a subst ntially higher uptake of labelled iron from the extracellular fluid by *P. falciparum* parasitized erythrocytes compared to non-parasitized erythrocytes. Thus the possibility of a plasmodial derived transferrin-like receptor exists. These observations are confirmed by other studies which reported isolating a protein in parasite extracts which had the properties of a transferrin receptor (Rodriguez and Jungery, 1986; Haldar *et al.*,1986).

In contrast to the above findings Pollack and Schnelle (1988) failed to detect a transferrin receptor on the membrane of parasitized erythrocytes. They reported that transferrin binding to the "transferrin receptor" identified was non-specific as it was not saturable and was not limited to transferrin.

Subsequently, the presence of the transmembrane enzyme, transferrin reductase in *P. falciparum* infected erythrocytes, has suggested the presence of a parasite derived transferrin receptor (Fry, 1989).

Due to these conflicting observations there is still considerable confusion relating to the source of iron for the parasite. The formation of haemozoin, the malarial pigment, is some indication of haemoglobin catabolism (Rudzinska and Trager, 1959 ; Deegan and Maegraith, 1956). However, free haem is alleged to be toxic to the parasite (Orijh *et al.*, 1977). Moreover, as total haem is not measurably changed, it may be that the parasite does not utilise haemaglobin iron (Ball *et al.*, 1948) .

However controversy still surrounds the issue of the presence of a parasite derived transferrin receptor. Its presence is still not confirmed in parasitized erythrocytes.

2.2.2 The role of iron in the parasite

2.2.2.1 The role of iron as an essential nutrient (possible role of iron in DNA synthesis of the parasite)

In vitro studies suggest that the trophozoite stage is most vulnerable to the effects of iron chelators, implying that they may affect DNA synthesis (Fritsch and Jung ,1986; Raventos-Suarez *et al.*, 1982). Iron chelators probably affect the functioning of the iron dependent enzyme, ribonucleotide reductase, which is the rate controlling enzyme in DNA synthesis and requires iron as a cofactor (Reichard and Ehrenberg, 1983). This assumption has been made as iron deficiency also inhibits DNA synthesis in human hemopoeitic tissues, again probably by the same mechanism (Hoffbrand *et al.*, 1976).

In vitro observations have suggested that it is the erythrocytic instead of the extracellular iron which serves as the essential nutrient as iron deficient serum was equally effective as normal serum in supporting the *in vitro* growth of *P. falciparum* (Pollack and Flemming, 1984). This is further confirmed by the findings of Fritsch and Jung (1986), who found that parasitized erythrocytes in the trophozoite stage concentrated three times the amount of ^{14}C desferrioxamine compared to non-parasitized erythrocytes.

Clinical observations have reported similar findings. Microcytosis which is secondary to iron deficiency anaemia, inhibits the proliferation of the parasites

(Nurse, 1979). During microcytosis there is a decrease in the size of the normal erythrocytes, thus in the smaller intracellular environment there may be a depletion of the essential intracellular nutrients such as iron (Nurse, 1979). However a later study showed that the invasion by merozoites and development of *P.falciparum* in iron deficient erythrocytes was not limited by a decrease in erythrocyte size (Luzzi *et al.*, 1990). This suggests that the parasite is not dependent on haemaglobin iron.

2.2.2.2 Iron and free radicals

When the parasites are exposed to the synthetic iron chelator, L2-9, it forms a complex with the iron found within the parasite (Iheanacho *et al.* 1990). This complex is redox active and it generates oxygen derived free radicals by the iron catalysed Fenton reaction (Iheanacho *et al.*, 1990 ; Halliwell and Gutteridge, 1984). These radicals destroy the integrity of the membranes, thus destroying the parasite.

The destructive effect of the iron-chelator complex is further emphasized by the findings of Rice-Evans *et al.* (1988). The effects of the iron chelator desferrithiocin, and the ferrated complex ferrithiocin,

were observed on a model system of erythrocytes subjected to extracellular iron mediated oxidant stress . Membrane studies showed that ferrithiocin disrupted the structural integrity of the erythrocytic membrane and induced haemoglobin oxidation. It is therefore possible that ferrithiocin has similar effects on parasitized erythrocytes, especially if there is a progressive increase in redox-active iron during the development of the malaria parasite (Golenser *et al.*, personal communication). The presence of this iron increases the sensitivity of these stages to oxidant stress.

Klebanoff *et al.*, (1989) also reported that desferrioxamine is destructive via a mechanism involving free radicals. Desferrioxamine promotes the auto-oxidation of ferrous iron which results in the formation of destructive hydroxyl radicals. This effect is most pronounced at a pH of 5,5 , which is similar to the pH of the parasite food vacuole (Ginsburg, 1988). The possibility that desferrioxamine inhibits the growth of the parasite via this mechanism should be considered even if the study by Klebanoff *et al.* (1989) did not involve the malaria parasite.

3. Clinical observations on the outcome of iron supplementation

3.1 Iron supplementation and infections (clinical observations)

Barry and Reeve (1974, 1977) observed an increased incidence of Gram-negative sepsis in infants who received prophylactic iron dextran. This suggests that increased availability of iron to invading pathogens after iron administration may have caused the infection to progress. The converse is essentially what (Kochan, 1973) describes as "nutritional immunity" which emphasizes the importance of iron deprivation as a host defense mechanism in inhibiting the growth of invading pathogens.

A group of children received either iron fortified or non-fortified milk formulas and significantly fewer respiratory infections were observed in the iron fortified group up to 15 months later (Andelman and Sered, 1966). Burman (1972) observed no difference in the rate of infections between the control and iron supplemented groups in a similar study. The importance of the host iron status on the outcome of infections has been emphasized in many reviews (Brock, 1986; Bullen, 1981; Hershko *et al.*, 1988; Kochan, 1973; Peto and Hershko, 1989; Ward, 1986 ; Weinberg, 1974, 1975).

3.2 Iron supplementation and malaria (clinical observations)

Oppenheimer (1989) and Murray *et al.* (1978) stressed that iron supplementation programmes should be practised with caution in malaria endemic regions where iron deficiency anaemia was common. They noted that after the inhabitants of these regions received iron supplementation there seemed to be a recrudescence of the latent parasitaemia .

3.2.1 Pregnant women

Byles and D'Sa (1970), reported that there was a recrudescence of malaria in some women who received iron infusions when pregnant. A subsequent study observed that only women in their first pregnancy were at a risk of contracting malaria if they received iron infusions during pregnancy (Brabin *et al.*, 1983). In later reports (Oppenheimer 1986, 1989) again emphasized on the risk involved in pregnant women receiving iron supplements routinely in malaria endemic regions.

3.2.2 Children

Oppenheimer (1986a, 1986b) performed a study among infants who received either iron dextran or placebo when 2 months old. After 12 months, there were almost 50% more patients in the iron treated group who were positive for malaria. Although malaria did not prove to be fatal in either of the two groups, infants receiving iron supplements were at a higher risk of developing the disease. These observations give evidence for a possible protective role of iron deficiency against malaria.

A study involving children, between the ages of 6 months and 5 years concluded that there was a higher risk of fever associated with severe parasitaemia in iron-deficient children who received iron supplements during the rainy season (Smith *et al.*, 1989). Yet children below the age of 5 years are reported to be most vulnerable to iron deficiency anaemia and thus require iron supplements (Akenzua *et al.*, 1985; McGregor *et al.*, 1966).

Studies involving children older than 5 years , have suggested that mild to moderate iron deficiency would be treated, without an increased risk of contracting malaria as they are semi-immune (Bates *et al.*,1987; Harvey *et al.*, 1989).

3.2.3 Adults

Two studies performed by Murray *et al.*, (1975, 1978) also suggested the protective effect of iron deficiency in infections, including malaria. Their studies involved people in central African relief camps. In the first study, 72 adult patients were given diets supplemented with vitamins (Murray *et al.*, 1975). Clinical malaria was observed in 23 of the patients with their parasitaemia increasing from 2% to 15% within 5 days . It was suggested that the diet resulted in relative hyperferraemia which caused rapid multiplication of existing parasites.

The second study showed that after iron supplementation there were 15 cases of malaria in the iron-treated group (71 subjects) , while only 1 case was observed in the untreated control group (67 subjects) (Murray *et al.*, 1978).

In contradiction one study has reported the increased prevalence of malaria in iron deficiency (Masawe *et al.*, 1974). Malaria was observed in 24% of the iron deficient group compared to 5% in the group who had non-iron deficiency anaemias.

4 Iron chelators

4.1 Iron chelators used in man (iron overload)

Iron toxicity is a major life limiting complication of thalassaemia major and other iron loading anaemias managed by repeated blood transfusions (Hershko and Weatherall, 1988). The clinical manifestations of such iron overload can be overcome by treatment using iron chelating agents.

At present, desferrioxamine (DFO) is the only iron chelator commonly used for iron overload (Porter *et al.*, 1989). Desferrioxamine is a naturally occuring iron chelator which has an extremely high affinity for ferric iron (Hershko and Weatherall, 1988; Porter *et al.*, 1989). In hepatocytes, DFO is reported to chelate intracellular iron, prevent uptake of iron by cells and chelate transferrin-bound iron in the extracellular fluid (Baker *et al.*, 1985). It is administered parenterally (Cerami *et al.*, 1980).

Diethylenetriamine pentaacetic acid (DTPA) is used as an alternate to DFO in patients who are experiencing toxic side effects due to DFO therapy. It is a synthetic chelator which has a low affinity for ferric iron compared to DFO (Hershko and Weatherall, 1988). It is hydrophillic and acts in the extracellular fluid (Baker *et al.*, 1985).

Ethylenediaminetetraacetic acid (EDTA) has similar properties as DTPA (Baker *et al.*, 1985). Although both EDTA and DTPA causes significant iron excretion when administered parenterally, neither is specific for iron as DFO (Cerami *et al.*, 1980).

2,2'- Bipyridyl has a high affinity for ferrous iron and distributes in the membrane apolar phase. It blocks the transfer of iron from transferrin to the "iron acceptor" proteins by binding with the iron. It has not been used in humans (Nunez *et al.*, 1983)

4.2 The inhibitory effect of iron chelators on the growth of *P. falciparum* :*in vitro* observations

Raventos-Suarez *et al.*, (1982) showed that DFO inhibited the *in vitro* growth of *P. falciparum* . Similarly, desferrithiocin, desferricriocin and lactoferrin have been shown to suppress the growth of the parasites (Fritsch *et al.*, 1987).

The antimalarial properties of orally active iron chelators have been reviewed (Heppner *et al.*, 1988). Recently (Yinnon *et al.*, 1989) reported the antimalarial effects of the iron chelator, N,N'-bis(o-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid (HBED) *in vitro* .

It is possible that inhibition of the parasite growth may not be caused by iron chelation or deprivation alone. When L2-9, a synthetic iron chelator was combined with desferrioxamine, an antagonistic effect was observed instead of the expected additive or synergistic effect (Iheanacho *et al.*, 1990). There may therefore be another mechanism involved such as the generation of free radicals by an internal redox reaction of the preformed L2-9 and iron complex during a Fe^{+2} to Fe^{+3} transition. Desferrioxamine and desferrithiocin inhibited liver schizogony in both human and rodent malaria (Stahel *et al.*, 1988).

4.3 The inhibitory effect of iron chelators on the growth of *P. falciparum* : *in vivo* observations

Pollack *et al.*, (1987) reported that constant blood levels of desferrioxamine are needed to suppress *P.falciparum* in Aotus monkeys. Similarly, in mice infected with *Plasmodium vinckei*, constant blood levels of desferrioxamine are required to suppress the parasites (Fritsch *et al.*, 1985). Interestingly, suppression of parasite growth by DFO is independent of host iron status in rats infected with *Plasmodium berghei*. The inhibition is achieved without reducing transferrin saturation or by interfering with haemoglobin production (Hershko and Peto, 1988).

The role of iron in the malaria parasite is not well defined , although evidence for its effects exist. Iron chelators may be considered as prospective candidates for antimalarials.

CHAPTER 2 MATERIALS AND METHODS

SECTION 2.1 *IN VITRO* CULTIVATION OF THE ERYTHROCYTIC STAGES OF *P. FALCIPARUM*

1 Introduction

Prior to 1976 , the study of the immunology, physiology and biochemistry of the malaria parasite was restricted due to the difficulty of maintainig the parasite in isolation from the host. The relatively slow rate at which techniques for the successful continuous *in vitro* cultivation of the parasite were developed, was a result of inadequate knowledge of the biochemistry of the parasite and of blood and its constituents. During this period, the maintenance of several species of malaria parasites in laboratory animals greatly advanced many aspects of malaria studies, however knowledge of the human malaria parasite was limited .

During this period there was no completely successful method to cultivate the intracellular asexual and sexual stages of the parasite developing in circulating erythrocytes. These stages are vital as they are responsible for the pathogenesis of the disease, the immune response of the host and they are a major target

for antimalarial drugs in the treatment of the disease (WHO, 1972). Therefore techniques for the successful *in vitro* cultivation of the malaria parasites were urgently required.

William Trager began to tackle the problem of maintaining the intraerythrocytic stages in long term cultures in 1941. He began his investigations with *Plasmodium lophurae*, and was successful over 30 years later. Together with his assistant Jensen, he established the first long term culture of *P. falciparum* by technical modifications of previous methods which were used for short term cultures (Peters, 1986 a). The successful long term culture of *P. falciparum* has formed the basis of many recent advances in malaria epidemiology, parasitology, chemotherapy, immunology, and genetics.

2 Cultivation method

The parasites were cultured *in vitro* according to the method described by Freese *et al.* (1988), which is a modification of the method of (Trager and Jensen, 1976).

3 Parasite isolates

The parasite isolates that were cultivated were already established in culture. Two strains of *P. falciparum*, the FCR-3 and the RSA-2 were cultured. These strains were donations from Mrs. J Freese of the RIDTE in Durban.

4 Factors affecting the continuous *in vitro* cultivation of the erythrocytic stages of *P. falciparum*

4.1 Medium

The medium used in our studies was RPMI 1640, which was supplemented with HEPES buffer, glucose , hypoxanthine and gentamycin.

Routinely, 1 litre of medium was prepared by dissolving the following in 900ml of distilled autoclaved water:

10,4g RPMI 1640 powdered medium (Flow Lab)

5,94g HEPES buffer (Sigma)

4g glucose (Merck)

44mg hypoxanthine (Sigma)

The volume of this mixture was adjusted to 960ml by the addition of water. In our studies, this medium was referred to as incomplete or "washing" medium. This medium was prepared for cultivation purposes, by adding 4,2ml of a 5% sodium bicarbonate solution and 10ml of pooled human serum to 90 ml of incomplete medium. This was referred to as complete medium.

The pH of the incomplete medium was 6,75. The pH of the complete medium (supplemented with serum and sodium bicarbonate) was 7,4.

4.1.1 Filtration of medium

The incomplete medium used in our studies was filtered through a 0.22u pore membrane (Millipore), using a peristaltic pump (Millipore), and was dispensed into 100ml aliquots and stored at 4°C . The medium was filtered twice using a separate filter each time.

4.1.2 Preparation of a 5% sodium bicarbonate solution

Autoclaved distilled water was added to 5 grams of $NaHCO_3$ (Sarchem) up to 100ml. This solution was filtered through a 0.22 u pore membrane (Millipore) and dispensed into aliquots and stored at 4°C.

4.1.3 Preparation of gentamycin solution

Gentamycin sulphate (Sigma) was used prophylactically in our cultures at a concentration of 50mg per 1 litre of medium. The gentamycin sulphate stock solution was prepared at a concentration of 0,25 grams per 5ml of distilled water. This solution was filtered through a 0.22u pore membrane and stored at 4°C.

4.1.4 Storage of medium

The maximum storage period for powdered medium, incomplete medium and complete medium (at 4°C) is 12 months, 5 weeks and 1 week respectively.

4.2 Serum requirements

To maintain the parasites in culture, we used pooled human serum .

4.2.1 Preparation of pooled human serum

Human A" blood was collected from several donors in 10ml silicone coated tubes ("red capped" tubes, Radem Laboratories) which contained no preservatives. It was left overnight to clot at 4°C and the serum was

then separated and pooled. The pooled serum was inactivated by heating at 56°C for approximately 30 minutes. It was centrifuged at 1700 g for 15 minutes and the supernatant was collected.

4.2.2 Storage of serum

The serum was dispensed into sterile 10ml tubes and stored at -20°C.

4.3 Erythrocytes

4.3.1 Collection of erythrocytes

P. falciparum is maintained in continuous culture, using the erythrocytes from any of the ABO blood groups, provided it is compatible with the serum being used. Routinely in our studies O+ erythrocytes were used for culturing. O+ blood was collected in lithium-heparin tubes ("green capped" tubes, Radem Laboratories) from healthy donors. This blood was stored as whole blood, and was suitable for use 1 week after collection.

4.3.2 Preparation of erythrocytes for cultivation of parasites

The blood was prepared for culturing by washing with

incomplete medium. A certain volume (10 ml) of the whole blood was poured into a centrifuge tube (Costar), using a sterile disposable pipette (Sterilin) . The blood was centrifuged at 400 g for 5 minutes to separate the plasma from the cells. The plasma and buffy coat were removed by aspiration. The packed erythrocyte pellet remaining after aspiration was washed twice with 10 volumes of incomplete medium and centrifuged at 400 g for 5 minutes for each wash. After the second wash, the supernatant was removed and the erythrocytes were added to the culture.

The blood for serum and erythrocytes purposes was collected by the South African Blood Transfusion Services under the supervision of Mr. B. Nortman.

4.4 Gas requirements

P. falciparum parasites have very specific gas requirements. The gas mixture used to culture our parasites consisted of 3% oxygen, 4% carbon dioxide and 93% nitrogen (Afrox).

The gas initially passed through a sterile gas filter, (Millipore) and than flowed through a sterile pasteur

pipette which was inserted into the culture flask. Each culture flask was gassed for 20 seconds each time, and the cap was immediately replaced and tightened. Each time the culture flask was opened for any reason , it was regassed.

The gas filter and pasteur pipettes were sterilized by autoclaving at 120°C for 20 minutes. The gas filter was replaced once every month.

4.5 Parasites cultured in suspension

The parasite cultures were routinely maintained in suspension on an orbital shaker which agitated at 60 oscillations per minute.

5 Synchronization

In an established culture, all the developmental stages are generally seen in culture at any one time. Our cultures were routinely synchronized using the following method described by Lambros and Vanderberg (1979).

A 5% D-sorbitol solution was prepared at room temperature using autoclaved distilled water. This solution was filtered through a 0,22u pore membrane . A culture

was synchronized when it had a higher proportion of parasites in the ring stage. The culture was centrifuged at 400 g for 5 minutes which separated the parasitized erythrocytes from the medium. The supernatant was removed by aspiration. Ten volumes of the sorbitol solution was added to the erythrocyte pellet. This suspension was mixed thoroughly and left to stand at room temperature for 10 minutes. After 10 minutes the suspension was again centrifuged at 400 g for 5 minutes. The supernatant was removed and the packed erythrocyte pellet was washed twice with incomplete medium.

Freshly washed uninfected cells were added to the synchronized parasites to increase the haematocrit. Prewarmed complete medium was added to the parasitized erythrocytes. The parasites were then gassed as described and incubated at 37°C.

6 The routine maintenance of malaria cultures

6.1 The replacing of medium and preparation of thin smears

Routinely the culture medium was replaced and smears were prepared once every 24 hours.

The shaking platform was switched off one hour before changing the medium. The erythrocytes settled on the surface of the flask which facilitated preparing a smear. The medium was aspirated and discarded. A pasteur pipette was moved from the front to the back of the flask to lift cells from throughout the flask. Approximately 15ml of prewarmed complete medium was added to each flask using a separate pipette.

The parasites were cultured in a 5% suspension of erythrocytes in culture medium. Therefore in a tissue culture flask of a volume of 250ml , 15ml of complete medium was added to 750ul of packed parasitized erythrocytes.

6.2 Staining of thin blood smears

6.2.1 Preparation of stain

The blood smears were stained with Giemsa stain (Merck) which was diluted by a factor of 1 in 10 using phosphate buffer. The phosphate buffer was prepared by dissolving the following in 1 litre of distilled water: 5,79g KH_2PO_4 (Sarchem) and 7,25g Na_2HPO_4 $2H_2O$ (Merck). The pH of the buffer was between 7 and 7,4. The Giemsa stain was freshly diluted using the phosphate buffer immediately before using.

6.2.2 Procedure for staining smears

The thin smear was air dried and fixed with methanol for 1 minute. The excess methanol was removed, and the diluted Giemsa was applied for 15 minutes at room temperature. The Giemsa stain was then rinsed with water and the smear was air dried. The Giemsa stained thin smears were preserved by covering in xylene.

6.2.3 Determining the parasitaemia

The smears were observed under 1000 times magnification using oil immersion. The parasitaemia (the percentage of parasitized erythrocytes out of the total number of erythrocytes), was determined by counting a total of 2000 erythrocytes. The thin smears were routinely prepared and studied.

6.3 Subculturing of parasites

Routinely, if the parasitaemia was 5% or higher, the parasites were subcultured by a factor of 50% or lower. The parasitized suspension was centrifuged at 400 g for 5 minutes. The supernatant was removed by aspiration leaving behind the parasitized erythrocyte pellet. The volume of this pellet and parasitaemia was determined.

6.3.1 An example of subculturing of parasites

Assume that the parasitaemia determined is approximately 5 % and the volume of the parasitized pellet is 750ul. If a parasitaemia of 1 % in a volume of 750ul of packed erythrocytes is required, then in 750ul, of packed erythrocytes, a parasitaemia of 1 % is equivalent to 150 ul of packed erythrocytes. 150ul of the packed parasitized erythrocytes is removed and added to 600ul of newly washed uninfected erythrocytes. 15ml of prewarmed complete medium is added to the parasites which are then gassed and incubated at 37°C.

7 Cryopreservation of parasites

The parasites were cryopreseved using the method described by Freese *et al.* (1988). A solution of 28% glycerol in phosphate buffered saline (PBS) was used as a cryoprotectant.

7.1 Preparation of phosphate buffered saline

The PBS was prepared by dissolving 72g NaCl, (Sarchem), 18,55g $Na_2 HPO_4 .2H_2O$, (Merck) and 4,3g KH_2PO_4 (Sarchem), in 1 litre of distilled water. The buffer was diluted by

a factor of 1 in 10 using distilled water before it was added to the glycerol.

7.2 Preparation of 28% glycerol in phosphate buffered saline

The specific density of glycerol (Merck) is approximately 1.257g/ml. Thus 28g of glycerol was equivalent to a volume of 22,3ml which was diluted to 100ml using the prediluted PBS. This solution was filtered through a 0,22u pore membrane and stored at 4°C.

7.3 Parasite stage

The parasite cultures frozen in our laboratories were predominantly in the ring stage. The culture was centrifuged at 400 g for 5 minutes . The supernatant was removed and the volume of the packed cells was determined. The packed erythrocyte pellet was mixed with an equal volume of the 28% glycerol in phosphate buffered saline. Approximately 0,5ml of this suspension was aliquoted into NUNC cryotubes (Sterilab services). The parasites were placed into liquid nitrogen within 10 minutes of adding the glycerol mixture.

8 Thawing of the cryopreserved parasites

The cryopreserved parasites were thawed using the method described by Freese *et al.* (1988). Using this method, three solutions were required to thaw the cryopreserved parasites. These solutions were 12% NaCl; 1,6% NaCl; and 0,9% NaCl with 0,2% glucose. These solutions were prepared using sodium chloride (Sarchem), glucose (Merck) and autoclaved distilled water. The solutions were filtered through a 0,22u pore membrane .

8.1 Method of thawing (used in our laboratory)

1) After the frozen NUNC tubes were removed from the liquid nitrogen, the blood was rapidly thawed in a waterbath at 37°C with.. 30 seconds. The blood suspension was immediately transferred to a centrifuge tube (Costar).

2) To every 1ml of the blood suspension, 0,1ml of the 12% NaCl solution was added . This solution was added dropwise to the blood suspension, while mixing constantly. The suspension was left at room temperature for 3-5 minutes.

3) Nine volumes of the 1,6% NaCl solution was added to the suspension i.e. (9 volumes with respect to the erythrocyte pellet). This suspension was centrifuged at 400 g for 5 minutes.

4) The supernatant was removed, and 9 volumes of the final solution was added to the remaining erythrocyte pellet . The suspension was centrifuged at 400 g for 5 minutes. The supernatant was removed and newly washed fresh uninfected erythrocytes were added to the thawed parasites to increase the haematocrit.

5) 15ml of prewarmed complete medium supplemented with 20ml of serum per 1 litre of medium was added to the parasites which were then gassed for 20 seconds and incubated at 37°C.

SECTION 2.2 CHELATOR SENSITIVITY TESTS

1 Introduction

Iron is an essential growth nutrient to virtually all living organisms, with the possible exception of lactobacilli (Peto and Hershko, 1989). The influence of iron on infections has been well reviewed (Brock, 1986; Bullen,1981; Hershko *et al.*, 1988; Peto and Hershko, 1989). Although the immune system of the host clearly has a major role in the defence against infections, another vital factor is the availability of the host's iron to invading pathogens.

The malaria parasite is an intracellular obligate parasite and is therefore dependent on its host for essential nutrients. The host iron status probably has some effect on the proliferation of the malaria parasite. Clinical observations suggest that iron deficiency may be protective against malaria (Oppenheimer, 1986 a, 1986 b; Smith *et al.*, 1989; Murray *et al.*, 1975, 1978). Various researchers have also shown that certain iron chelators block the proliferation of *P. falciparum in vitro* (Raventos-Suarez *et al.*, 1982; Fritsch *et al.*, 1987; Heppner *et al.*,1988; Yinnon *et al.*,1989; Iheanacho *et al.*, 1990) and *in vivo* (Fritsch *et al.*, 1985; Pollack *et al.*, 1987; Hershko and Peto , 1988).

We investigated the *in vitro* sensitivity of *P. falciparum* to 6 chelating agents.

2 Basic requirements for the preliminary and final experiments

2.1 Chelators

The sensitivity of the malaria parasite was evaluated against 6 chelators:

1. Desferrioxamine (Ciba-Geigy).
2. Desferrithiocin (Ciba-Geigy).
3. 2,2'- Bipyridyl (Fluka Chemika).
4. DTPA. Diethylenetriamine pentaacetic acid (Fluka Chemika).
5. EDTA. Ethylenediaminetetraacetic acid (BDH).
6. EGTA. Ethylene glycol-bis (B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (Boehringer Mannheim).

2.2 Medium used to dissolve the chelators

The chelators were soluble in the culture medium.

Preparation of culture medium

The following were dissolved in 900ml of double distilled autoclaved water:

10,4g RPMI 1640	(Flow Lab)
5,94g Hepes buffer	(Sigma)
4g glucose	(Merck)

The volume was adjusted to 960ml with water. In our experiments this medium was referred to as hypoxanthine free incomplete medium. This hypoxanthine free incomplete medium was filtered through a 0,22 u filter (Millipore). When this medium is supplemented with human serum and $NaHCO_3$, it is hypoxanthine free complete medium. The preparation of the human serum and $NaHCO_3$ are described in Section 2.1 (cultivation method).

2.3 Chelator sensitivity tests (use of microtiter plates)

In our experiments, chelator sensitivity tests were performed in 96-well microtiter plates (Sterilin Ltd). The method used is described by Desjardins *et al.* (1979).

Microtitration techniques were used to measure the activity of the chelators. The microtiter plates consisted of 96 wells. The 96 wells were arranged in a matrix of eight rows (A to H) and 12 columns (1 to 12) as shown in the diagrams of the 96 well plates.

2.4 Parasites

2.4.1 Parasite inocula

The parasite inocula used in the experiments consisted of two isolates of *P. falciparum*, the FCR-3 and RSA-2. Both strains were grown continuously in stock cultures in our laboratories as described in Section 2.1.

2.4.2 Synchronization

We required the mature stages, i.e. the trophozoites and schizonts for the chelator sensitivity studies as shown in figure 2.1. The parasites used for the experiments were synchronised using the method of Lambros and Vanderberg (1979) as described in Section 2.1. which selectively destroys the mature forms but leaves the young ring form unaffected. The rings were left for 15 to 17 hours to mature to the mid and late trophozoite stages, used in the experiments.

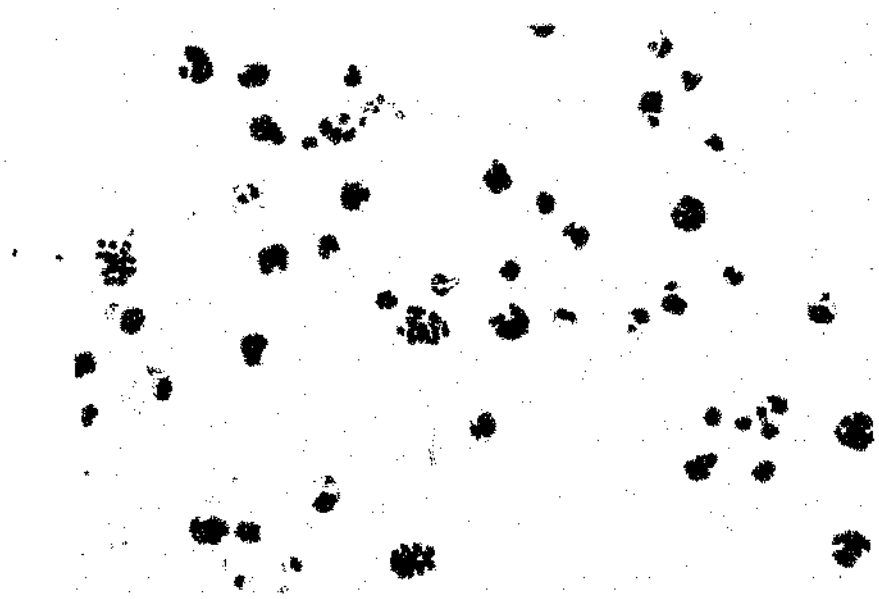

Figure 2.1 : Photograph of a thin blood smear depicting parasites synchronized to isolate the mature stages used in the chelator sensitivity tests.

2.4.3 Preparation of parasites for experiments (dilutions of stock cultures)

The stock cultures of the parasites were diluted to a final parasitaemia of 0,5% and haematocrit of 1% using newly washed O^- erythrocytes and hypoxanthine free complete medium.

2.4.3 (a) An example of a dilution of a stock culture:

In all the experiments, for each 96 well plate, a constant volume of 50ml of the parasite suspension with a parasitaemia of 0.5% and haematocrit of 1% was prepared. The stock culture (suspension) was centrifuged at 400 g for 5 minutes. The supernatant was removed and the volume and parasitaemia of the remaining pellet was determined.

Assume that the volume of the packed erythrocyte pellet is 500ul and the parasitaemia is 6%. If in 500ul of packed erythrocytes the parasitaemia is 6%, then 0.5% parasitaemia is equivalent to a volume of 42ul. 458ul of newly washed O^- erythrocytes and 49,5ml of hypoxanthine free complete medium are added to the 42ul of the pellet to make 50ml. Thus 500ul of packed erythrocytes in 50 ml of medium results in a 1 % haematocrit.

2.5 Preparation of an erythrocyte suspension

1% erythrocyte suspension in hypoxanthine free complete medium which served as an erythrocyte control was prepared for each experiment . For each 96 well plate, approximately 10ml of this suspension was prepared. 100ul of washed O" erythrocytes was added to 9,9 ml of hypoxanthine free complete medium.

3 Basic design

The following describes the basic design used to study the sensitivity of the parasites to the 6 chelators. This design was used in the preliminary studies and final experiments.

3.1 Preparation of the microtiter plate

1) The plates were prepared using strict aseptic techniques inside the laminar flow hood.

2) A Gilson pipette with sterile tips was used to add the chelator solutions and medium to the designated well.

3) 25ul of the appropriate chelator solution was placed into each well as indicated in the specific diagram of the 96 well plate. The 25ul of chelator solution was in a final volume of 250ul therefore the dilution factor is 1 in 10 .

4) In the wells denoted as control wells, 25ul of complete medium was added.

5) A constant volume of 200ul of the parasitized erythrocyte suspension described above in 2.4. was added to all the wells besides the erythrocyte control wells. In these wells, 200ul of the equivalent suspension of non parasitized erythrocytes described in 2.5. was added. These were added using a multichannel pipette with sterile tips. Thus the total volume in each well at this stage was 225ul.

6) Each concentration of a chelator was analysed in quadriplicate. The test wells contained 25ul of chelator solutions of different concentrations and 200ul of parasitized suspension. The parasite control wells contained 200ul of parasitized suspension and 25ul of hypoxanthine free complete medium. The erythrocyte

control wells contained 200ul of non parasitized erythrocytes and 25ul hypoxanthine free complete medium.
7) The plates were prepared as described above and were placed in a humidified airtight box. The box was flushed with the gas mixture for 5 minutes using the gas valves n the lid of the box. The valves were sealed and the box containing the 96 well plates was incubated at 37°C for 24 hours.

3.2 Preparation of isotope

The uptake of G-^{3}H hypoxanthine (Amersham) was used as an index of growth of the parasites.

1) The isotope was supplied as a lyophylate (1-5Ci/mmol) in ampoules containing 1 mCi. The contents of a single ampoule were dissolved in 2 ml of 50% ethanol to provide a stock solution which was stored at -20°C.

2) In our experiments for each 96 well plate, 200ul of the stock solution of the isotope was used. When preparing the isotope for addition to the microtiter plate, most of the ethanol was evaporated from the 200ul sample of the stock solution in a stream of nitrogen gas (Afrox).

3) Approximately 4,9ml of hypoxanthine free complete medium was added to the remaining isotope.

3.3 Labelling of parasites

1) After the 24 hour incubation period as described in 3.1.7 the plate was removed from the box.

2) 25ul of the isotope solution was added to each well. Thus the total volume in each well was; 25ul of chelator solution/complete medium + 25ul of isotope solution + 200ul of parasitized /non parasitized suspension = 250ul.

3) The plate was returned to the box which was then flushed with the gas mixture for 5 minutes. The gas valves were sealed and the box was incubated at 37°C for an additional 18 hours.

3.4 Harvesting of parasites and scintillation counting

1) At the end of the second incubation period, the plates were harvested using an automated cell harvester

(Titertek, Flow laboratories).

2) The cell harvester aspirated and deposited the particulate contents of each well onto small disks of filter paper. These paper disks were than washed automatically with distilled water.

3) The filter paper disks from the cell harvester were dried in a hot air oven. Each disk was placed in a glas. scintillation vial containing 5ml of Aquagel (Chemlab).

4) Each vial was assayed in a liquid scintillation counter for a period of 1 minute. Disintegrations per minute (DPM) values were obtained.

4. Preliminary studies

Preliminary studies were performed to assess if G ^{3}H hypoxanthine was a suitable index of growth of the parasites . In these investigations, the sensitivity of the RSA-2 strain was evaluated against the 6 chelators.

4.1 Preparation of chelators

The following formula was used to calculate the weight of the chelators required for the preparation of the stock solution of each chelator. The stock solution was then used to prepare further dilutions.

ug = Concentration x MW

where,

ug : Weight of the chelator required in micrograms

Concentration : required concentration of the stock solution.

MW : Molecular weight of the chelator in grams.

Chelator	Molecular weight (MW)
Desferrioxamine	656,8 g
Desferrithiocin	260,6 g
2,2'- Dipyridyl	156,18g
EDTA	372,24g
EGTA	380,35g
DTPA	393,0 g

The stock solutions were prepared in incomplete hypoxanthine free culture medium. The diluted solutions were prepared using the stock solutions and hypoxanthine free incomplete medium as a diluent. All solutions were filtered through a 0.22 u pore cellulose filter (Millipore). The concentrations of a specific chelator used in the preliminary studies are indicated in figure: 2.2. The plates were prepared and incubated as described in 3.1.1- 3.1.7. The subsequent steps in these experiments i.e. the labelling and harvesting of the parasites were those described in 3.2, 3.3, and 3.4.

The results of these studies are presented in the Results chapter.

5. Final experiments

5.1 Dose response curves

The sensitivities of both strains of parasites, the FCR-3 and RSA-2 were evaluated against the 6 chelators. The dose response curve of each chelator was drawn.

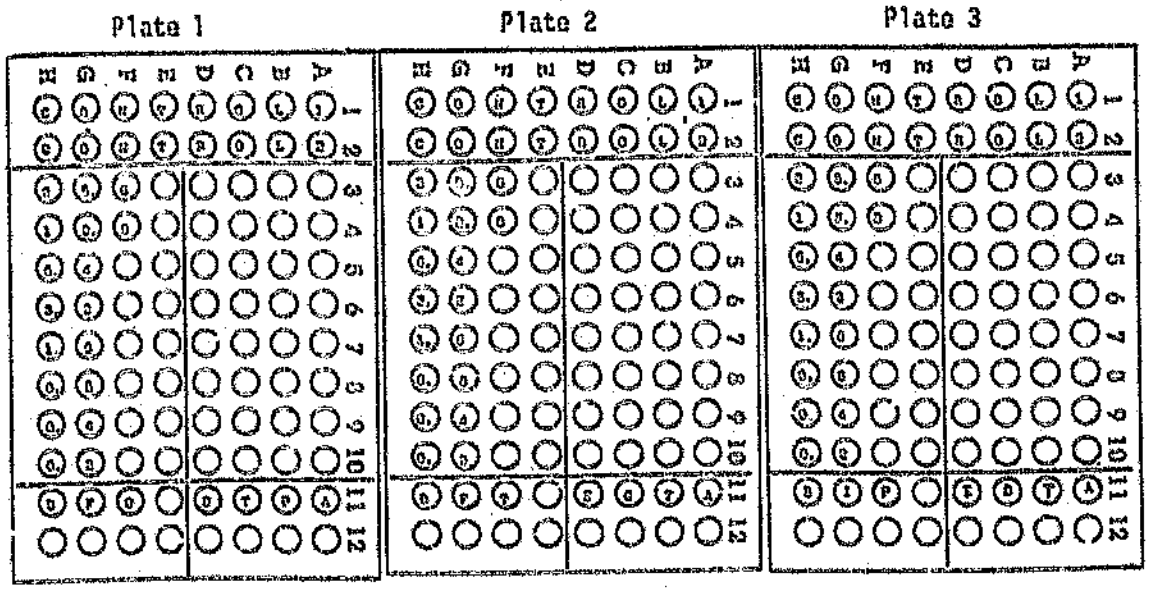

Figure 2.2: Detailed plan of the 96 well plates used to evaluate the sensitivity of the RSA-2 strain to the 6 chelators in the preliminary studies. In each plate, the effects of two chelators were studied:
Plate 1 - DFO and DTPA
Plate 2 - DFT and EGTA
Plate 3 - BIP and EDTA

Concentration range of the 6 chelators: 0,2-25,6 µM. Each concentration was analysed in quadriplicate.
Control 1: Erythrocyte control
Control 2: Parasite control

5.1.1 Preparation of chelators

The formula mentioned in 4. (preliminary studies) was used to calculate the weight of the chelators required for the preparation of the stock solution of each chelator. The stock and diluted solutions were prepared in incomplete hypoxanthine free culture medium. The various concentrations of each chelator resulting from the dilutions are stated on the respective diagrams of the 96 wells plates.

5.1.2 Preparation of microtiter plates

The concentrations of each chelator used is stated on the following diagrams

Chelator	Figure number
Desferrioxamine	2.3
Desferrithiocin	2.3
2,2'- Dipyridyl	2.4
EDTA	2.5
EGTA	2.5
DTPA	2.6

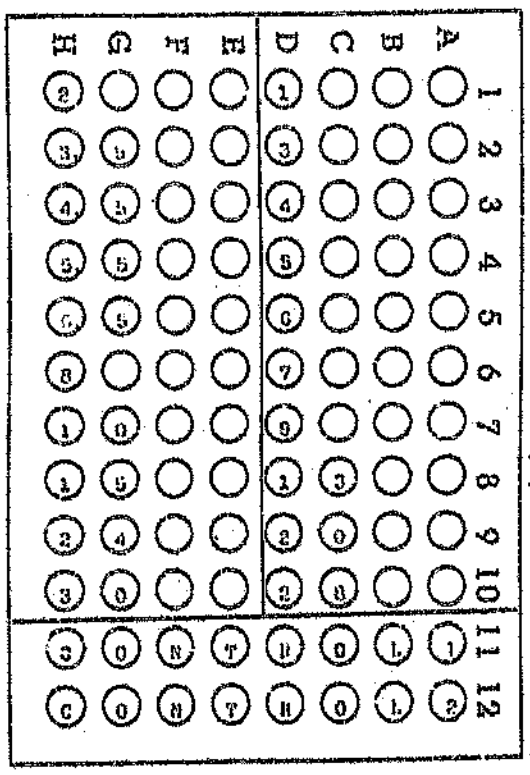

Figure 2.3: Detailed plan of the 96 well plate used to evaluate the sensitivity of the FCR-3 and RSA-2 strains to desferrioxamine and desferrithiocin. Concentration range: 1-30 µM. Each concentration was analysed in quadriplicate.
Control 1: Parasite control
Control 2: Erythrocyte control

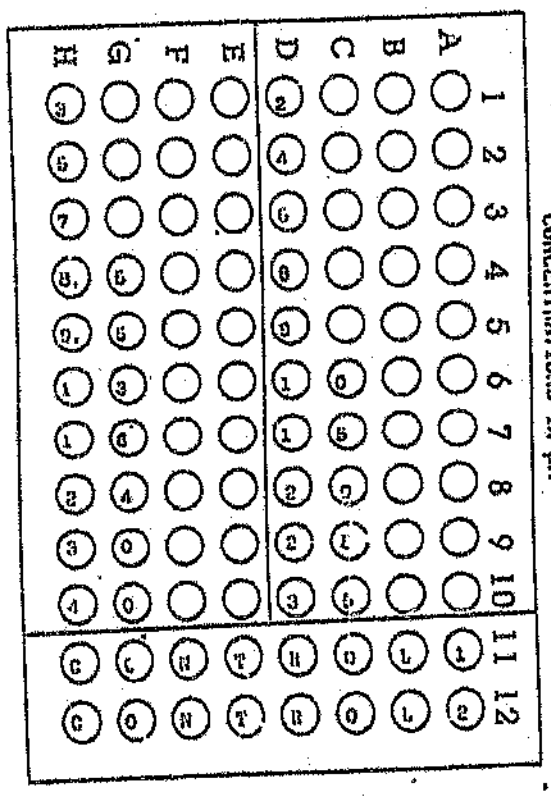

Figure 2.4: Detailed plan of the 96 well plate used to evaluate the sensitivity of the FCR-3 and RSA-2 strains to 2,2'-bipyridyl. Concentration range: 2-40 µM. Each concentration was analysed in quadriplicate.
Control 1: Parasite control
Control 2: Erythrocyte control

CONCENTRATIONS IN µM

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	○	○	○	○	○	○	○	○	(-)	(-)	(+)
B	○	○	○	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(-)	(+)
C	(0)	(0)	(0)	(2)	(8)	(2)	(8)	(5)	(0)	(0)	(0)	(0)
D	(2)	(6)	(9)	(1)	(1)	(2)	(2)	(3)	(5)	(0)	(R)	(R)
E	○	○	○	○	○	○	○	○	○	(0)	(T)	(T)
F	○	○	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(N)	(N)
G	(0)	(0)	(0)	(4)	(0)	(5)	(0)	(0)	(0)	(0)	(0)	(0)
H	(4)	(8)	(1)	(1)	(2)	(2)	(3)	(4)	(6)	(1)	(C)	(C)

Figure 2.5 : Detailed plan of the 96 well plate used to evaluate the sensitivity of the FCR-3 and RSA-2 strains to EDTA and EGTA. Concentration range: 70-1000 µM. Each concentration was analys . in quadriplicate.
Control 1: Parasite control
Control 2: Erythrocyte control

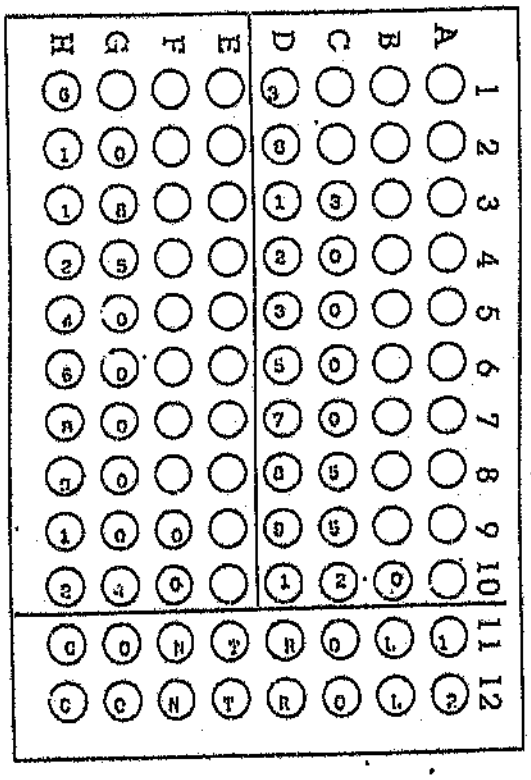

Figure 2.6: Detailed plan of the 96 well plate used to evaluate the sensitivity of the FCR-3 and RSA-2 strains to DTPA. Concentration range: 3-240 µM. Each concentration was analysed in quadriplicate.
Control 1: Parasite control
Control 2: Erythrocyte control

The plates were prepared and incubated as described in 3.1.1-3.1.7. The subsequent steps in these experiments i.e. the labelling and harvesting of the parasites were those described in 3.2, 3.3, and 3.4.

5.2 Combination of a chelator with an unsaturated chelator

The combined effect of desferrioxamine and 2,2'-bipyridyl was evaluated against the FCR-3 strain

5.2.1 Preparation of chelators

The stock and diluted solutions of desferrioxamine and 2,2' - bipyridyl were prepared in hypoxanthine free incomplete culture medium.

5.2.2 Preparation of microtiter plates

1) A defined combination of the two chelators was analysed in triplicate. The two chelators were combined as indicated in fig: 2.7 .

2) 25ul of each chelator solution was added to a well as indicated in fig: 2.7 . If the concentration of a chelator was stated as 0 uM, 25ul of complete culture

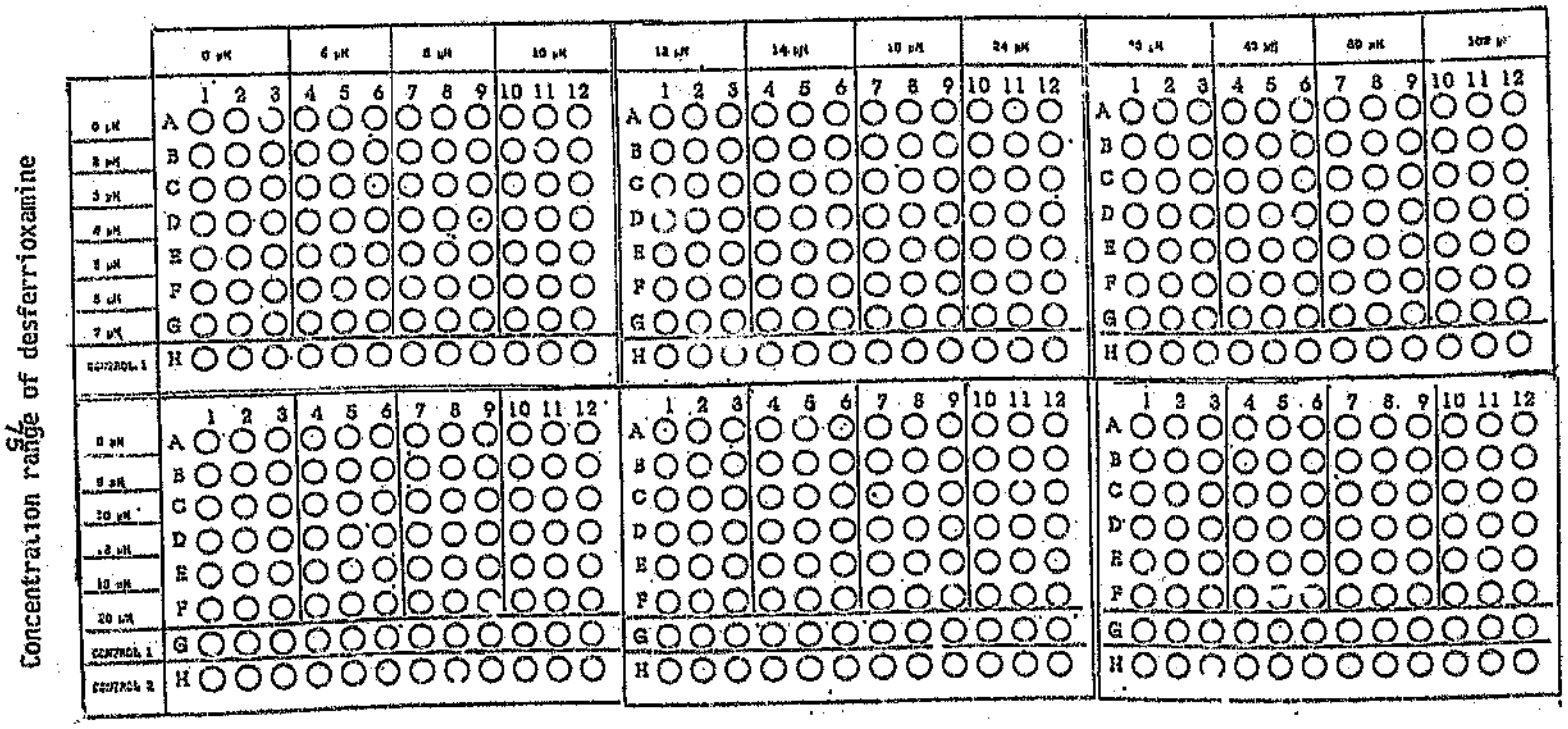

Figure 2.7: Detailed plan of the 96 well plates used to evaluate the sensitivity of the FCR-3 strain to the combined effect of desferrioxamine and 2,2'-bipyridyl. Each combination was analysed in triplicate.
Control 1: Parasite control
Control 2: Erythrocyte control

medium was added. The total volume of chelator solutions in each test well was 50ul, (25ul of each chelator). In the wells designated as parasite and erythrocyte control wells, 50ul of complete medium was added .

3) 175ul of the parasite suspension was added to each well, excluding the erythrocyte control wells in which 175ul of the equivalent non parasitized erythrocyte suspension was added. The parasite and erythrocyte suspensions are described in 2.4. and 2.5.

4) The chelator solutions and parasites were added to the the plates as described in the above steps . The plates were gassed and incubated as described in 3.1.7.

5) The subsequent steps in this experiment i.e. the labelling and harvesting of the parasites were those described in 3.2, 3.3, and 3.4.

5.3 Combination of a chelator with an iron saturated chelator

5.3.1 The spectroscopic titration of 2,2'- bipyridyl and ferrous ammonium sulphate

This titration d ined the molar concentration of ferrous iron required to completely saturate 2,2'-bipyridyl.

5.3.1.1 Preparation of solutions

The solutions of 2,2'- bipyridyl and ferrous ammonium sulphate (Sarchem) were prepared to a final concentration of 1 mM in distilled water.

5.3.1.2 Spectroscopic titration

A fixed concentration of 2,2'- bipyridyl (1 mM) was combined with various concentrations of ferrous ammonium sulphate. The absorbance of these solutions were measured at 520 nm, using the ultraviolet/visible spectrophotometer (Phillips, PU8700 series).

5.3.2 Combination of a chelator with an iron saturated chelator

The sensitivity of the FCR-3 strain was evaluated against the following:

a) Combination of 6 uM desferrioxamine and various concentrations of unsaturated 2,2'- bipyridyl.

b) Combination of 6 uM desferrioxamine and various concentrations of iron saturated 2,2'- bipyridyl.

5.3.2.1 Preparation of chelators and ferrous solutions

The stock and diluted solutions of desferrioxamine and 2,2'-bipyridyl were prepared in hypoxanthine free incomplete medium. The stock solution of ferrous ammonium sulphate was prepared in distilled water. The diluted solutions were prepared using the stock solution and hypoxanthine free incomplete medium as a diluent.

5.3.2.2 Preparation of microtiter plates

a) Combination of 6 uM DFO and various concentrations of 2,2'- bipyridyl.

1) Each combination of 6 uM DFO with a certain concentration of unsaturated 2,2'- bipyridyl was analysed in quadriplicate.

2) 25ul of the specific concentration of 2,2'-bipyridyl was added to the wells as indicated in fig: 2.8 . 25ul of complete medium was added to the 2,2'-bipyridyl.
25ul of the desferrioxamine solution (6 uM) was added to the 50ul in the wells as indicated in fig: 2.8 .

Concentrations of unsaturated and iron saturated 2,2'-bipyridyl in µM. Concentration range: 0-20 µM.

PLATE 1

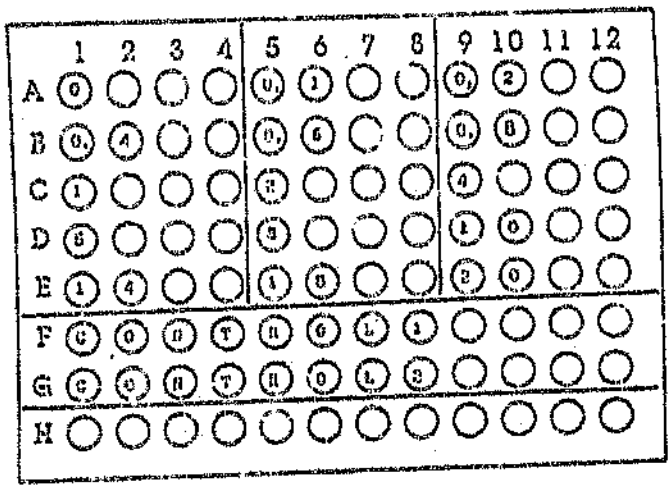

PLATE 2

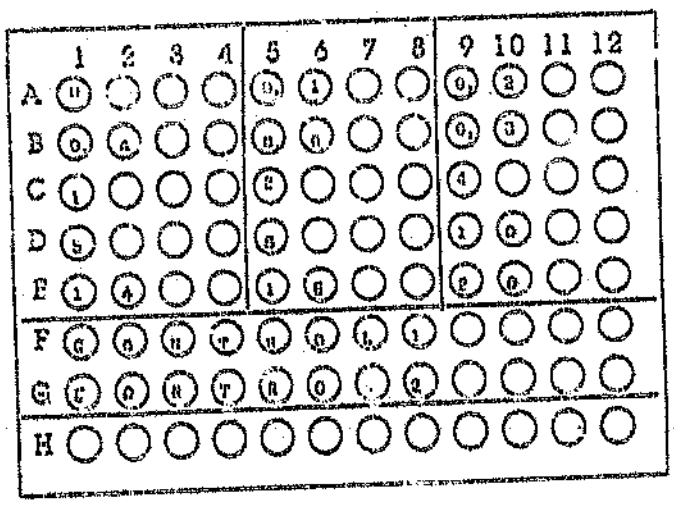

Figure 2.8 : Detailed plan of the 96 well plates used to evaluate the sensitivity of the FCR-3 strain to the combined effect of 6 µM desferrioxamine and various concentrations of unsaturated 2,2'-bipyridyl (plate 1) and iron saturated 2,2'-bipyridyl (plate 2). Each combination was analysed in quadriplicate.
Control 1: Parasite control
Control 2: Erythrocyte control

3) 75ul of complete medium was added to the wells designated as control wells (containing no chelators).

4) A constant volume (150ul), of parasite suspension was added to each well besides the erythrocyte control wells in which 150ul of the equivalent non parasitized erythrocyte suspension was added. These suspensions are described in 2.4. and 2.5.

b) Combination of 6 uM DFO and various concentrations of iron saturated 2,2'- bipyridyl.

1) Each combination of 6 uM DFO and a certain concentration of iron saturated 2,2'- bipyridyl was analysed in quadriplicate.

2) 25ul of a certain concentration of 2,2'- bipyridyl was added to the wells as specified in fig: 2.8 . 25ul of the equimolar concentration of the ferrous ammonium sulphate solution was added to the 2,2'- bipyridyl to form a complex. 250ul of the 6 uM DFO solution was added to the complex formed in each well.

3) 75ul of complete medium was added to the wells designated as control wells.

4) A constant volume of parasite suspension (150ul) was added to each well besides the erythrocyte control wells In these wells, 150ul of the equivalent non parasitized erythrocyte suspension was added. These suspensions are described in 2.4 and 2.5.

5) After both plates were prepared as described in a. and b. they were gassed and incubated as described in 3.1.7.

6) The subsequent steps of labelling and harvesting the parasites were described in 3.2, 3.3, and 3.4.

5.3.3 The spectroscopic titration of desferrioxamine and 2,2'- bipyridyl

This titration was performed to determine if a direct chemical interaction between 2,2'-bipyridyl and desferrioxamine existed.

5.3.3.1 Measurement of absorbance using distilled water to dissolve the chelators.

The absorbance spectrum of 2,2'-bipyridyl dissolved in water was measured. The changes in this spectrum was measured after an equal volume of desferrioxamine solution in water was added to the 2,2'-bipyridyl solution.

5.3.3.2 Measurement of absorbance using incomplete medium to dissolve the chelators.

The absorbance spectrum of 2,2'-bipyridyl dissolved in hypoxanthine free incomplete was measured. The changes in this spectrum was measured after an equal volume of desferrioxamine solution in hypoxanthine free incomplete medium was added to the 2,2'- bipyridyl solution.

6. Data analysis and expression.

1) Results of the scintillation counting were expressed as disintergrations per minute (DPM values).

2) The mean DPM value of the erythrocyte control wells was determined. This value was subtracted from the DPM value obtained for each chelator test well and parasite control well.

3) The mean DPM values were determined for each concentration of chelator tested, each combination of chelators, and the parasite control wells.

4) The mean DPM values of the chelator test wells were expressed as a percentage of the mean DPM of the parasite control (non-chelator control).

5) Dose response curves were generated using the Enzfitter Data Analysis program (Elsevier Biosoft) using the following sigmoid dose response equation:

Effect (%) = $100/ (1 + (C/IC_{50})^{P})$.

where:

100 represents the maximal effect achieved.

C represents the concentration of the chelator used.

IC_{50} represents the 50 % inhibitory concentration of the chelator.

P represents the slope.

This program is a non linear regression data analysis program which uses Marquart alogarithm. The reduced chi square is optimised to achieve the best estimates of the parameters of the sigmoid dose response equation.

The IC_{50} values are expressed as means with standard deviations.

6) The chelator combination curves were drawn using the percentage DPM values on the y axis and the various concentrations of the chelator added to the one fixed concentration of another chelator on the x axis.

CHAPTER 3

RESULTS

1. The dose response curves of the 6 chelators

The sensitivities of both strains , the FCR-3 and the RSA-2 were evaluated against the 6 chelators.

The results are tabulated and are also represented graphically. In the tables, the concentration of the chelator is stated in uM, and the parasite growth is estimated by [^{3}H] hypoxanthine incorporation as measured by DPM. The DPM values of the chelator test wells are compared to the mean DPM of the non chelator controls and expressed as a percentage thereof. These percentages for each concentration (or combination concentration) are expressed with standard deviation in tables .

In the graphs, the x axis represents the logged concentration of the chelator (log C). The y axis represents the DPM values (parasitaemia) obtained for the chelator test wells expressed as percentages of the DPM values (parasitaemia) obtained for the control parasites (Effect) .

The results of the preliminary studies in which, the sensitivity of the RSA-2 strain was evaluated against the 6 chelators will be presented first in the case of each chelator. The results of the final experiments will subsequently be presented. The results of the sensitivity of the FCR-3 strain will be followed by those of the RSA-2 strain.

1.1 Desferrioxamine

Preliminary study

In the preliminary studies, DFO inhibited the growth of the RSA-2 strain at concentrations of 2 uM and higher (refer to Figure 3.1).

FCR-3

Figure 3.2 shows the effect of increasing concentrations of DFO on the parasite counts. DFO inhibited the growth of this strain at concentrations of 1 uM and above.

RSA-2

Figure 3.3 represents the sensitivity of the RSA-2 strain to various concentrations of DFO. DFO inhibited the RSA-2 strain at concentrations of 4 uM and higher.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	16169 ± 498	100,0 ± 3,07
0,2	15810 ± 134	97,8 ± 0,83
0,4	15074 ± 619	93,2 ± 3,80
0,8	15659 ± 157	96,8 ± 0,97
1,6	15020 ± 601	92,9 ± 3,70
3,2	13756 ± 814	85,1 ± 5,03
6,4	7232 ± 1253	44,9 ± 7,75
12,8	719 ± 47	4,4 ± 0,29
25,6	272 ± 33	1,7 ± 0,20

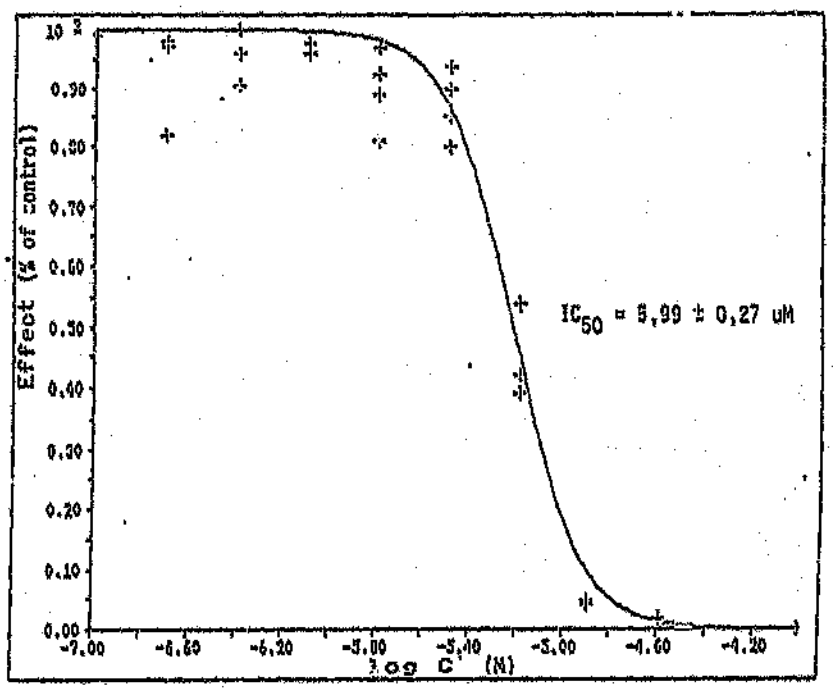

Figure 3.1 : The sensitivity of the RSA-2 strain to various concentrations of desferrioxamine (preliminary study).

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	45254 ± 1825	100,0 ± 4,03
1	35767 ± 484	79,0 ± 1,07
2	32000 ± 2329	70,7 ± 5,15
3	30713 ± 2985	67,9 ± 6,59
3,5	32588 ± 1242	72,0 ± 2,74
4	30277 ± 429	66,9 ± 0,95
5	19866 ± 3419	43,9 ± 7,6
5,5	19960 ± 1909	44,1 ± 4,22
6	16498 ± 2913	36,5 ± 6,44
6,5	8694 ± 1407	19,2 ± 3,11
7	6679 ± 1420	14,8 ± 3,14
8	5700 ± 256	12,6 ± 0,57
9	4635 ± 514	10,2 ± 1,10
10	4347 ± 268	9,6 ± 0,59
13	3549 ± 295	7,8 ± 0,65
15	2918 ± 445	6,4 ± 0,98
20	2 9 ± 212	4,5 ± 0,47
24	1.,7 ± 264	4,3 ± 0,58
28	1493 ± 210	3,3 ± 0,46
30	1981 ± 414	4,4 ± 0,91

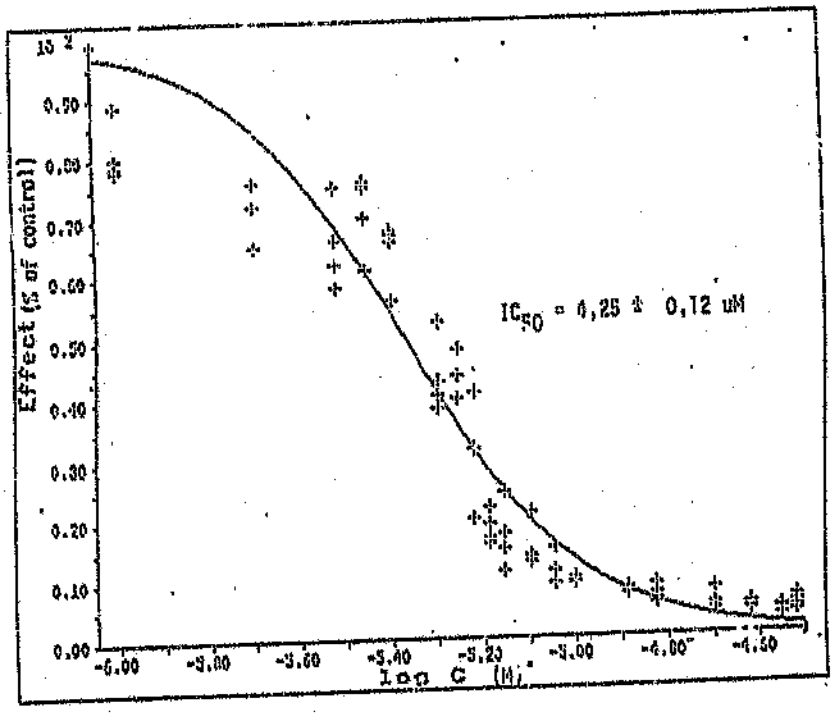

Figure 3.2: The sensitivity of the FCR-3 strain to various concentrations of desferrioxamine.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	29461 ± 1501	100,0 ± 5,09
1	29048 ± 1619	98,6 ± 5,49
2	27103 ± 245	91,9 ± 0,83
3	25393 ± 254	86,2 ± 0,86
3,5	27796 ± 722	94,3 ± 2,45
4	21983 ± 961	74,6 ± 3,26
5	19867 ± 1207	67,4 ± 4,09
5,5	16878 ± 1781	57,3 ± 6,05
6	13026 ± 844	44,2 ± 2,86
6,5	10355 ± 1894	35,1 ± 6,40
7	6535 ± 1183	22,2 ± 4,01
8	5552 ± 668	18,8 ± 2,27
9	2813 ± 240	9,5 ± 0,82
10	2977 ± 383	10,1 ± 1,29
13	1835 ± 62	6,2 ± 0,20
15	1622 ± 138	5,5 ± 0,47
20	1260 ± 36	4,3 ± 0,12
24	1030 ± 57	3,5 ± 0,19
28	775 ± 35	2,6 ± 0,12
30	810 ± 34	2,7 ± 0,12

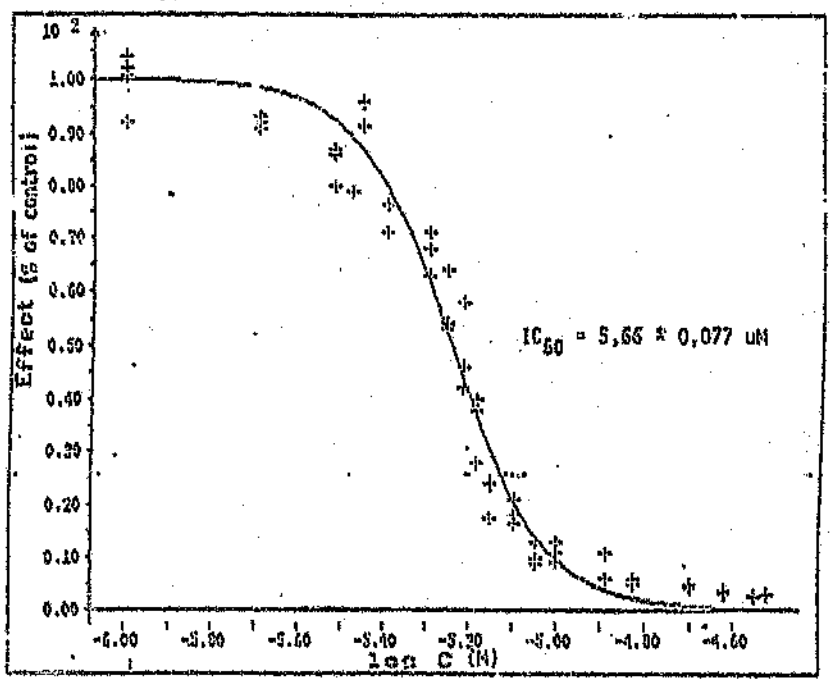

Figure 3.3: The sensitivity of the RSA-2 strain to various concentrations of desferrioxamine.

1.2 Desferrithiocin

Preliminary studies

The results of the preliminary studies show that DFT inhibited the RSA-2 strain at concentrations of 0.2 uM and higher (refer to Figure 3.4).

FCR-3

Figure 3.5 shows the inhibition of the FCR-3 strain by DFT. DFT inhibited this strain at concentrations of 1 uM and higher.

RSA -2

Figure 3.6 represents the inhibition of the RSA-2 strain by DFT. DFT inhibited this strain at concentrations higher than 1 uM.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	141538 ± 5991	100,0 ± 4,20
0,2	137252 ± 4894	96,9 ± 3,46
0,4	121927 ± 7768	86,1 ± 5,49
0,8	110116 ± 3120	77,8 ± 2,20
1,6	103489 ± 692	73,1 ± 0,49
3,2	88786 ± 9060	62,7 ± 6,40
6,4	80402 ± 5858	56,9 ± 4,14
12,8	5747 ± 787	4,1 ± 0,56
25,6	3362 ± 127	2,4 ± 0,09

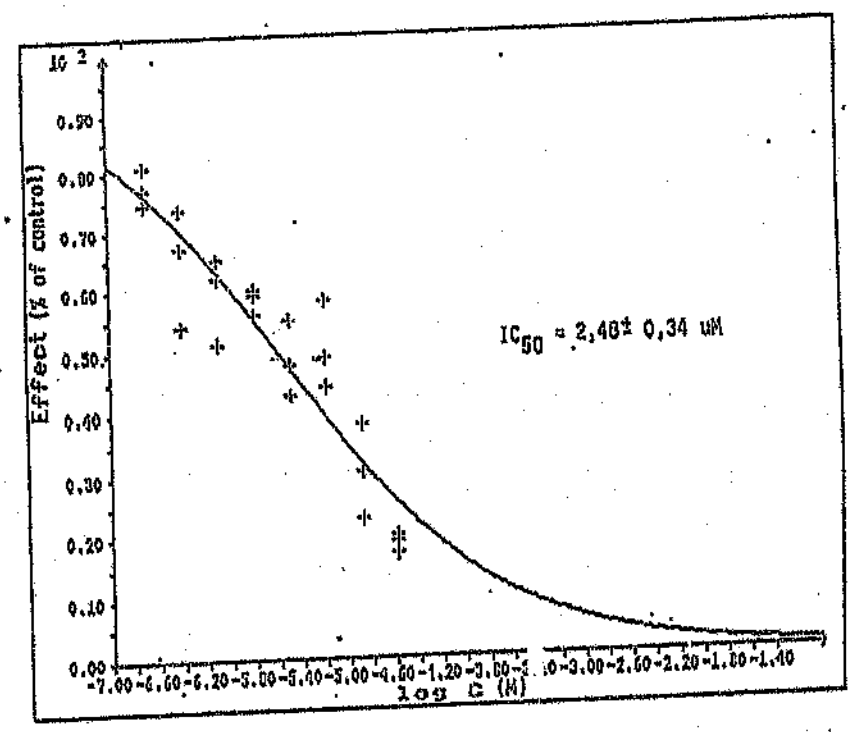

Figure 3.4 : The sensitivity of the RSA-2 strain to various concentrations of desferrithiocin (preliminary study).

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	47417 ± 747	100,0 ± 1,58
1	31471 ± 3269	66,4 ± 6,89
2	23442 ± 712	49,4 ± 1,50
3	26727 ± 1814	56,4 ± 3,80
3,5	18439 ± 1958	38,9 ± 4,13
4	16348 ± 2205	34,5 ± 4,65
4,5	15769 ± 4035	33,3 ± 8,51
5	14090 ± 2616	29,7 ± 5,50
5,5	13225 ± 1791	27,9 ± 3,78
6	14105 ± 734	29,7 ± 1,55
6,5	11395 ± 1616	24,0 ± 3,41
7	10828 ± 1492	22,8 ± 3,15
8	5821 ± 143	12,3 ± 0,30
9	4111 ± 635	8,7 ± 1,34
10	3049 ± 848	6,4 ± 1,79
13	1168 ± 151	2,5 ± 0,32
15	1264 ± 291	2,7 ± 2,67
20	693 ± 88	1,5 ± 0,19
24	658 ± 119	1,4 ± 0,25
28	476 ± 43	1,0 ± 0,09
30	574 ± 32	1,2 ± 0,07

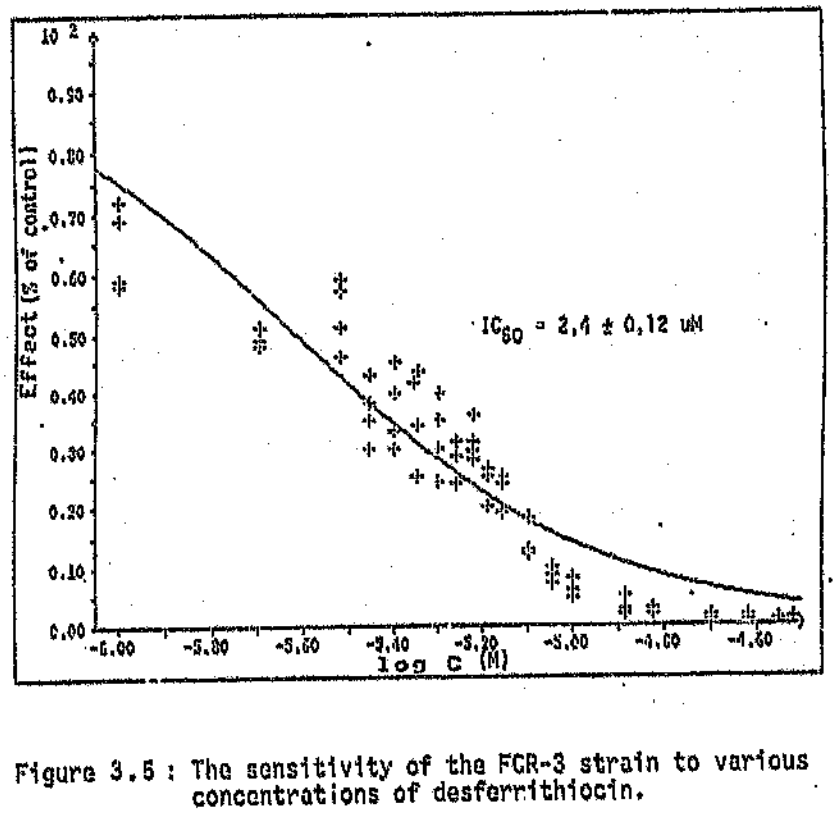

Figure 3.5 : The sensitivity of the FCR-3 strain to various concentrations of desferrithiocin.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	22111 ± 958	100,0 ± 4,33
1	19922 ± 1864	90,1 ± 8,40
2	18395 ± 737	83,2 ± 3 33
3	17094 ± 680	77,3 ± 3,10
3,5	14359 ± 355	64,9 ± 1,61
4	14076 ± 760	63,7 ± 3,44
4,5	13567 ± 843	61,4 ± 3,81
5	12275 ± 1202	55,5 ± 5,40
5,5	14346 ± 1005	64,9 ± 4,55
6	13002 ± 1390	58,8 ± 6,29
6,5	13715 ± 1056	62,0 ± 4,78
7	14208 ± 1300	64,2 ± 5,88
9	10013 ± 116	45,3 ± 0,52
10	8012 ± 1456	36,2 ± 6,58
13	1937 ± 271	8,8 ± 1,23
15	1359 ± 246	6,1 ± 1,11
20	618 ± 51	2,8 ± 0,23
24	623 ± 71	2,8 ± 0,32
28	518 ± 85	2,3 ± 0,38
30	418 ± 99	1,9 ± 0,45

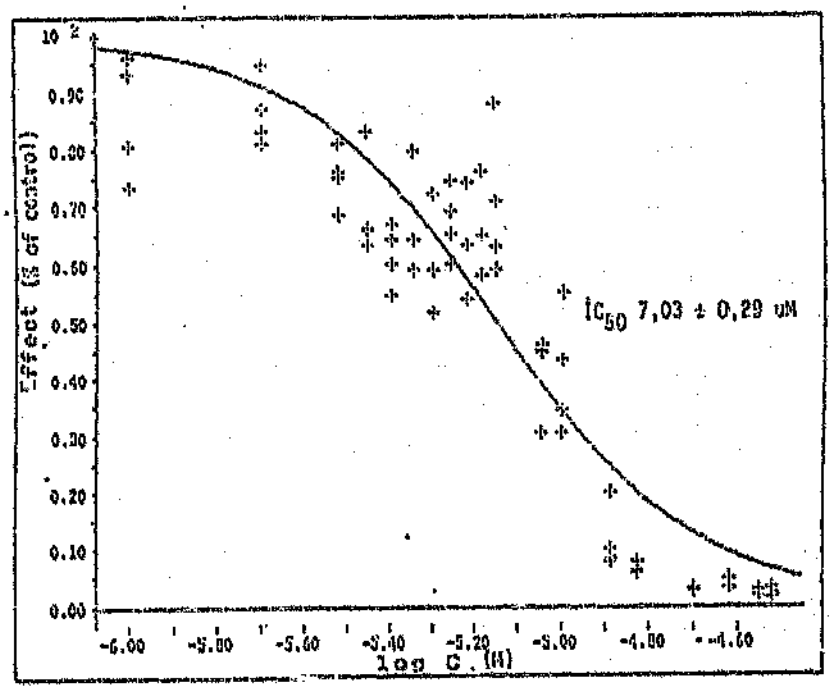

Figure 3.6 : The sensitivity of the RSA-2 strain to various concentrations of desferrithiocin.

1.3 2,2'- Bipyridyl

Preliminary study

In the preliminary studies, 2,2'- bipyridyl inhibited the RSA-2 strain at concentrations higher than 10 uM (refer to Figure 3.7).

FCR-3

Figure 3.8 represents the inhibition of the FCR-3 strain by 2,2'- bipyridyl. 2,2'- bipyridyl inhibited this strain at concentrations above 10 uM.

RSA-2

Figure 3.9 shows the response of the RSA-2 strain to various concentrations of 2,2'- bipyridyl. The RSA-2 strain was inhibited at concentrations higher than 10 uM.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	2000 ± 123	100,0 ± 6,15
0,2	1969 ± 97	98,5 ± 4,90
0,4	1915 ± 108	95,8 ± 5,40
0,8	1850 ± 34	92,5 ± 1,70
1,6	1860 ± 78	93,0 ± 3,90
3,2	1832 ± 87	91,6 ± 4,35
6,4	1761 ± 105	88,1 ± 5,25
12,8	1776 ± 70	88,8 ± 3,50
25,6	713 ± 75	35,7 ± 3,80

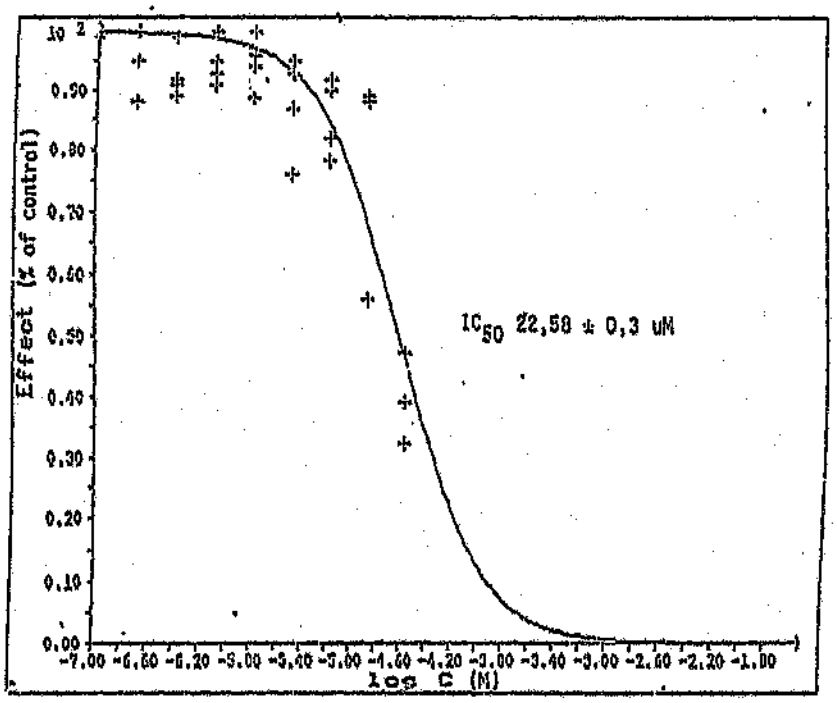

Figure 3.7 : The sensitivity of the RSA-2 strain to various concentrations of 2,2'-bipyridyl (preliminary study).

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	15564 ± 1023	100,0 ± 6,57
2	15340 ± 491	98,6 ± 3,16
3	13964 ± 881	89,7 ± 5,66
4	12518 ± 458	80,4 ± 2,94
5	13844 ± 807	88,9 ± 5,19
6	13684 ± 115	87,9 ± 0,74
7	13360 ± 146	85,8 ± 0,94
8	13530 ± 626	86,9 ± 4,02
8,5	13595 ± 599	87,3 ± 3,85
9	11628 ± 774	74,7 ± 4,97
9,5	12807 ± 722	82,3 ± 4,64
10	12734 ± 689	81,8 ± 4,43
13	12305 ± 710	79,1 ± 4,56
15	10293 ± 391	66, ± 2,51
18	10419 ± 222	66,9 ± 1,43
20	7472 ± 481	48,0 ± 3,09
28	6416 ± 646	41,2 ± 4,15
30	4597 ± 918	29,5 ± 5,89
35	2850 ± 77	18,3 ± 0,49
40	1446 ± 209	9,3 ± 1,3

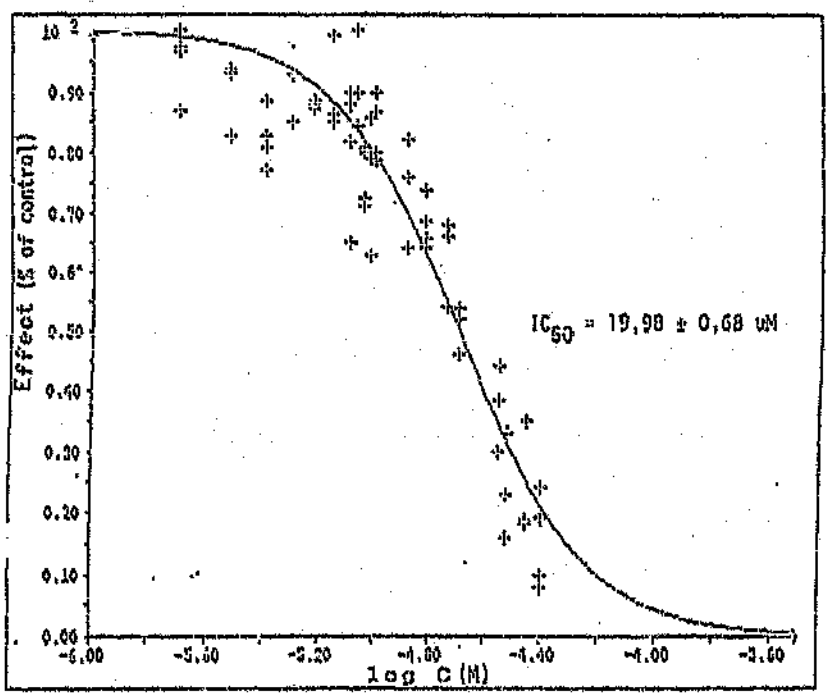

Figure 3.8 : The sensitivity of the FCR-3 strain to various concentrations of 2,2'-bipyridyl.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	50078 ± 3219	100,0 ± 6,43
2	46492 ± 1984	92,8 ± 3,96
3	47558 ± 1933	94,9 ± 3,86
4	44076 ± 1160	88,0 ± 2,32
5	47572 ± 2157	94,9 ± 4,31
6	46589 ± 2008	93,0 ± 4,01
7	43846 ± 1182	87,6 ± 2,36
8	40435 ± 1823	80,7 ± 3,64
9	42660 ± 3552	85,2 ± 7,09
9,5	43893 ± 981	87,6 ± 1,96
10	39713 ± 1574	79,3 ± 3,14
13	26497 ± 1653	52,9 ± 3,30
15	23819 ± 2099	47,6 ± 4,19
18	17850 ± 860	35,6 ± 1,72
20	16845 ± 560	33,6 ± 1,12
24	6343 ± 203	12,7 ± 0,41
28	3500 ± 104	6,9 ± 0,21
30	3291 ± 522	6,6 ± 1,04
35	2000 ± 473	3,9 ± 0,94
40	1626 ± 317	3,2 ± 0,63

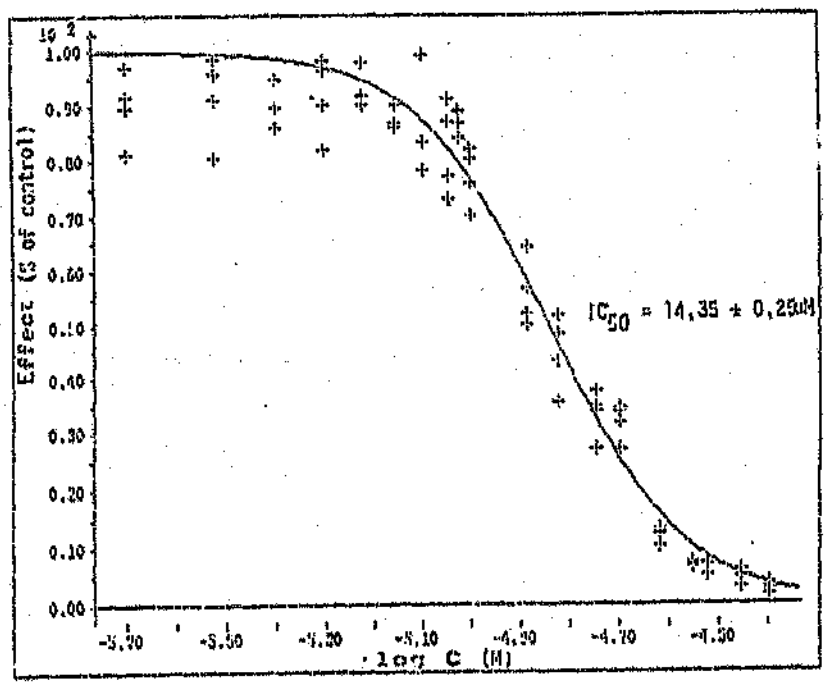

Figure 3.9: The sensitivity of the RSA-2 strain to various concentrations of 2,2'-bipyridyl.

1.4 EDTA

Preliminary study

In the preliminary studies, the highest concentration of 25 uM had no effect on the parasite growth. The dose response curve was a straight line (refer to Figure 3.10).

FCR-3

Figure 3.11 represents the response of this strain to increasing concentrations of EDTA. EDTA inhibited this strain at concentrations higher than 170 uM.

RSA-2

Figure 3.12 represents the inhibitory action of EDTA on the growth of the RSA-2 strain. EDTA inhibited the growth of this strain at concentrations higher than 100 uM.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	2000 ± 123	100,0 ± 6,15
0,2	1983 ± 70	99,2 ± 3,50
0,4	1870 ± 51	93,5 ± 2,55
0,8	1991 ± 24	99,6 ± 1,20
1,6	2024 ± 33	101,2 ± 1,65
3,2	1978 ± 50	98,9 ± 0,25
6,4	1880 ± 35	94,0 ± 1,75
12,8	1961 ± 7	98,1 ± 0,35
25,6	1964 ± 45	98,2 ± 2,25

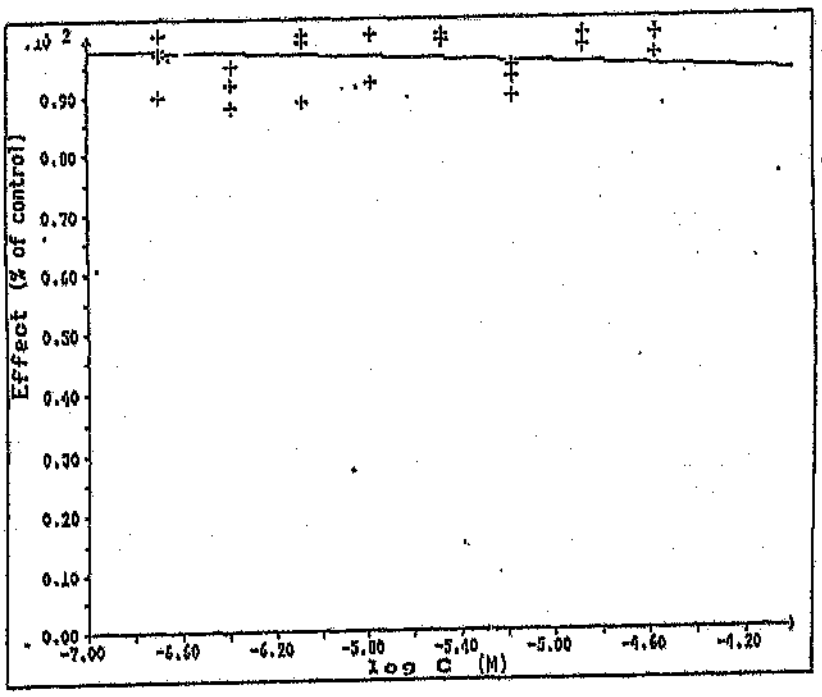

Figure 3.10: The sensitivity of the RSA-2 strain to various concentrations of EDTA (preliminary study).

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	5263 ± 567	100,0 ± 10,77
20	4652 ± 168	88,4 ± 3,19
40	4844 ± 366	92,0 ± 6,95
60	4669 ± 102	88,7 ± 1,94
80	4918 ± 144	93,4 ± 2,74
90	4884 ± 131	92,8 ± 2,49
100	4595 ± 77	87,3 ± 1,46
120	4609 ± 36	87,6 ± 0,69
140	4962 ± 25	94,3 ± 0,48
180	4138 ± 125	78,6 ± 2,40
200	4168 ± 199	79,2 ± 3,77
220	4473 ± 137	84,9 ± 2,60
300	17[illegible] ± 70	33,8 ± 1,34
350	591 ± 107	10,5 ± 2,04
400	434 ± 10	8,3 ± 0,20
500	322 ± 83	6,1 ± 1,57
600	319 ± 64	6,1 ± 1,22
800	454 ± 47	8,6 ± 0,89
1000	341 ± 41	6,5 ± 0,78

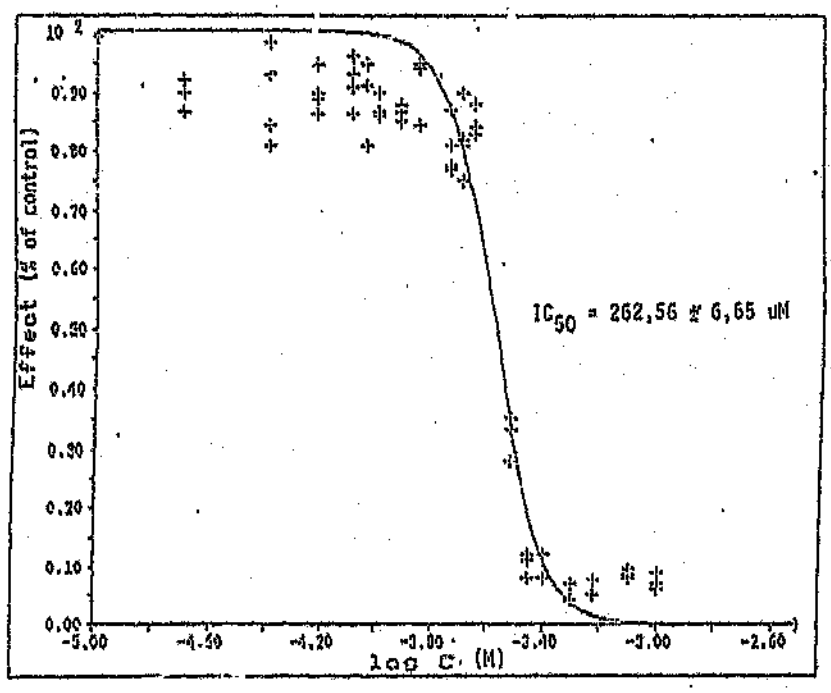

Figure 3.11: The sensitivity of the FCR-3 strain to various concentrations of EDTA.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	32925 ± 1759	100,0 ± 5,34
20	31382 ± 1089	95,3 ± 3,31
40	30393 ± 209	92,3 ± 0,64
80	30575 ± 3447	92,9 ± 10,47
100	27510 ± 1940	83,6 ± 5,89
120	23622 ± 4052	71,7 ± 12,31
140	25343 ± 827	76,9 ± 2,51
180	28916 ± 2045	87,8 ± 6,21
200	26357 ± 1394	80,1 ± 4,24
220	24438 ± 2374	74,2 ± 7,21
250	22598 ± 674	68,6 ± 2,05
280	21665 ± 484	65,8 ± 1,47
300	20628 ± 390	62,6 ± 1,18
350	5715 ± 910	17,4 ± 2,76
400	6622 ± 1020	20,1 ± 3,09
500	7159 ± 503	21,7 ± 1,53
600	860 ± 122	2,6 ± 0,26
800	356 ± 114	1,1 ± 0,35
1000	408 ± 65	1,2 ± 0,19

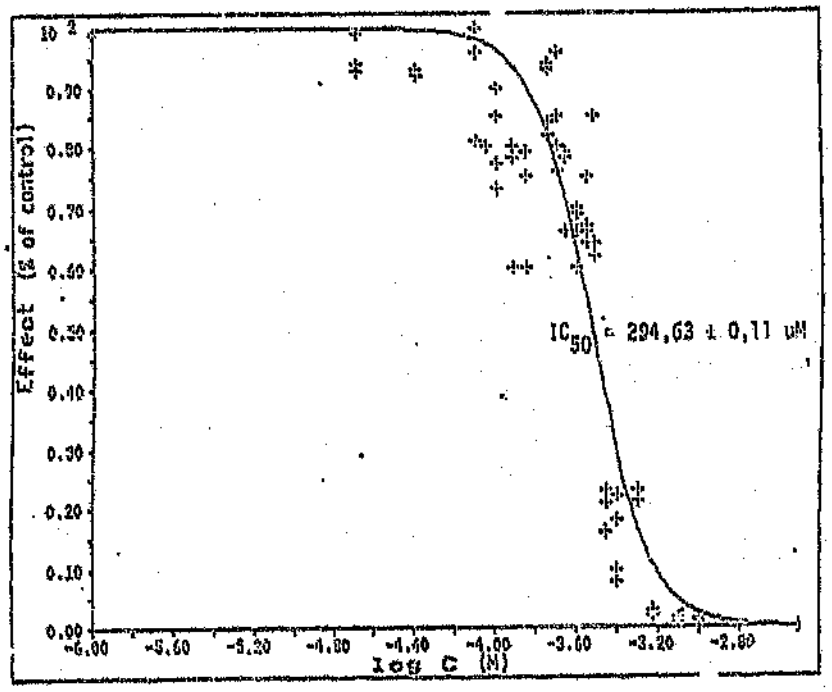

Figure 3.12: The sensitivity of the RSA-2 strain to various concentrations of EDTA.

1.5 EGTA

Preliminary study

The results of the preliminary studies showed that 25 uM EGTA had no effect on the growth of the RSA-2 strain . The dose resonse curve obtained was a straight line (refer to Figure 3.13).

FCR-3

Figure 3.14 represents the sensitivity of the FCR-3 strain to increasing concentrations of EGTA. EGTA inhibited the growth of this strain at concentrations higher than 200 uM.

RSA-2

Figure 3.15 represents the response of this strain to increasing concentrations of EGTA. EGTA inhibited the growth of this strain at concentrations higher than 100 uM.

CONCENTRATION (uM)	MEAN DPM + SD	% MEAN DPM + SD
0	141538 ± 5991	100,0 ± 4,20
0,2	137659 ± 1335	97,3 ± 0,94
0,4	126605 ± 2788	89,4 ± 1,97
0,8	126404 ± 1756	89,3 ± 1,24
1,6	132995 ± 240	93,9 ± 0,17
3,2	124421 ± 340	87,9 ± 0,24
6,4	138698 ± 3221	97,9 ± 2,28
12,8	127827 ± 675	90,3 ± 0,48
25,6	141045 ± 1218	99,7 ± 0,86

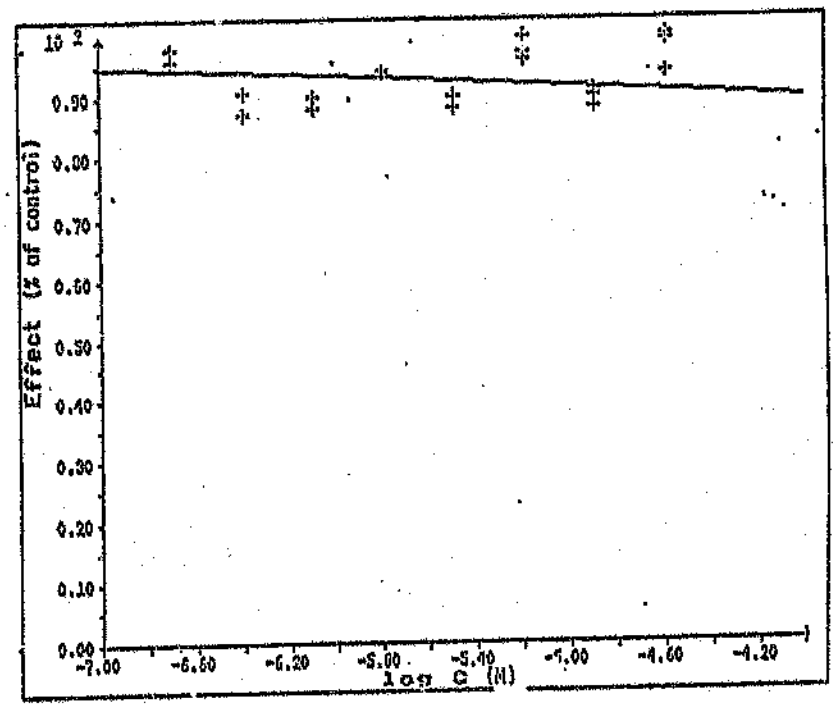

Figure 3.13: The sensitivity of the RSA-2 strain to various concentrations of EGTA (Preliminary study).

CONCENTRATION (μM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	4910 ± 337	100,0 ± 6,86
20	4765 ± 126	97,1 ± 2,57
40	4778 ± 364	97,3 ± 7,4
60	4675 ± 125	95,2 ± 2,54
80	4650 ± 120	94,7 ± 2,45
90	4761 ± 63	96,9 ± 1,29
100	4759 ± 123	96,9 ± 2,51
120	4489 ± 216	91,4 ± 4,39
140	4795 ± 69	97,7 ± 1,39
180	4714 ± 99	96,0 ± 2,01
200	4119 ± 22	83,9 ± 0,45
220	4514 ± 369	91,9 ± 7,52
250	3482 ± 120	70,9 ± 2,45
280	3346 ± 82	68,1 ± 1,67
300	2920 ± 188	59,5 ± 3,83
350	673 ± 182	13,7 ± 3,71
400	618 ± 147	12,6 ± 2,99
500	744 ± 40	15,2 ± 0,69
600	480 ± 143	9,8 ± 2,92
800	514 ± 11	10,5 ± 0,22
1000	551 ± 68	11,2 ± 1,38

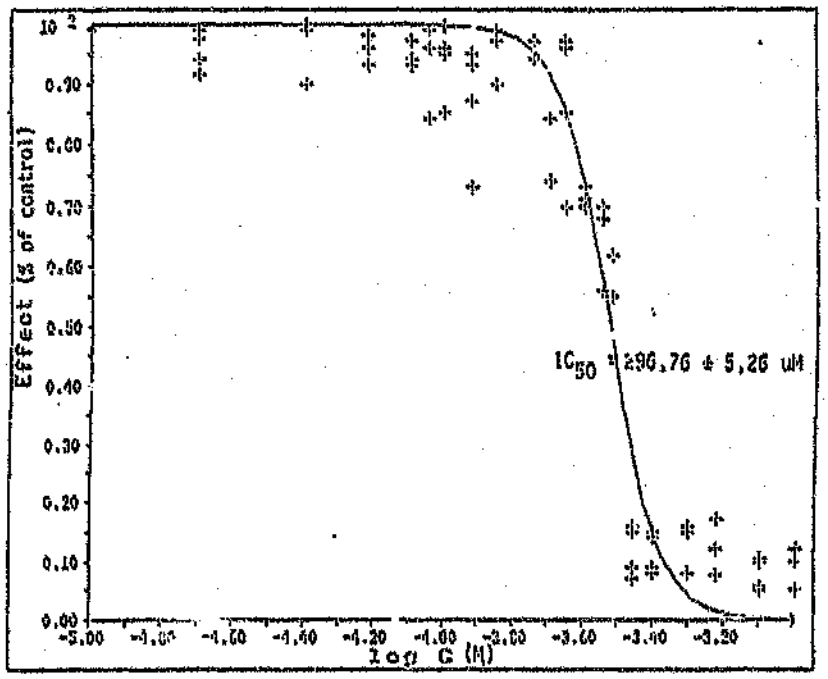

Figure 3.14: The sensitivity of the FCR-3 strain to various concentrations of EGTA.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SM
0	6544 ± 354	100,0 ± 5,41
20	5671 ± 18	86,7 ± 0,28
40	5458 ± 413	83,4 ± 6,31
80	5123 ± 122	78,3 ± 1,86
90	5557 ± 12	84,9 ± 0,19
100	5384 ± 61	82,3 ± 0,93
120	5481 ± 343	83,8 ± 5,24
140	5871 ± 177	89,7 ± 2,71
180	5834 ± 392	89,1 ± 5,99
200	3349 ± 251	51,2 ± 3,84
220	3286 ± 317	50,2 ± 4,85
250	2556 ± 100	39,1 ± 1,53
280	1909 ± 185	29,2 ± 2,82
300	1508 ± 103	23,0 ± 1,57
350	1339 ± 133	20,5 ± 2,04
400	1417 ± 116	21,7 ± 1,78
500	1124 ± 47	17,2 ± 0,71
600	972 ± 36	14,8 ± 0,55
800	1049 ± 44	16,0 ± 0,68
1000	875 ± 62	13,4 ± 0,95

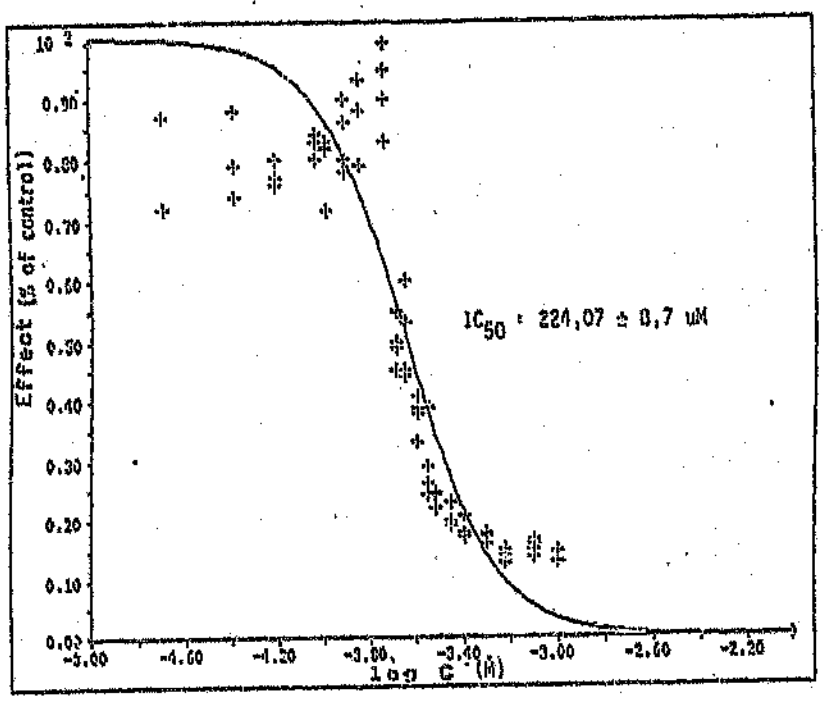

Figure 3.15: The sensitivity of the RSA-2 strain to various concentrations of EGTA.

1.6 DTPA

Preliminary study

The results of the preliminary studies indicated that 25 uM DTPA had no effect on the growth of the RSA-2 strain (refer to Figure 3.16).

FCR-3

Figure 3.17 represents the inhibitory action of DTPA on the growth of this strain. The dose response curve was a straight line indicating that the concentrations selected had no effect on the growth of this strain. The concentration range selected was between 0 and 240 uM.

RSA-2

Figure 3.18 shows the inhibitory effect of various concentrations of DTPA on the growth of the RSA-2 strain. The dose response curve obtained was a straight line.

CONCENTRATION (µM)	MEAN DPM + SD	% MEAN DPM + SD
0	16169 + 498	100,0 + 3,07
0,2	15654 + 757	96,8 + 4,68
0,4	15809 + 490	97,8 + 3,03
0,8	15730 + 337	97,3 + 2,08
1,6	15968 + 493	98,8 + 3,05
3,2	15691 + 466	97,0 + 2,88
6,4	15814 + 843	97,8 + 5,21
12,8	13631 + 752	84,3 + 4,65
25,6	15267 + 700	94,4 + 4,33

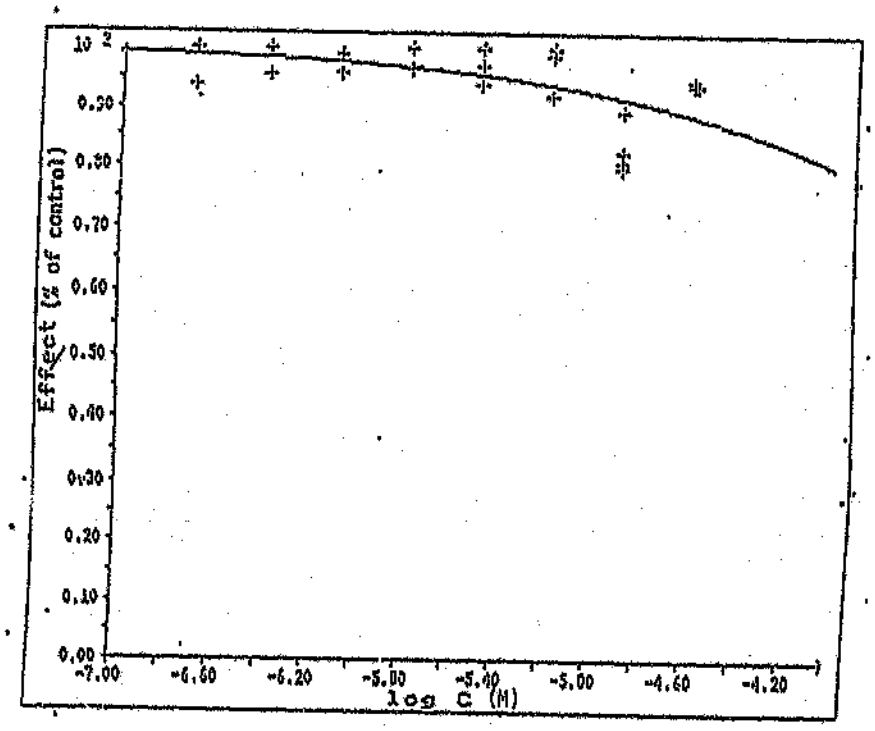

Figure 3.16: The sensitivity of the RSA-2 strain to various concentrations of DTPA (preliminary study).

CONCENTRATION (μM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	16413 ± 3200	100,0 ± 19,49
3	14590 ± 805	88,9 ± 4,91
6	13736 ± 616	83,7 ± 3,75
8	13087 ± 166	79,7 ± 1,01
10	13093 ± 757	79,8 ± 4,61
13	14500 ± 1155	88,3 ± 7,03
18	14928 ± 404	90,9 ± 2,46
20	14685 ± 381	89,5 ± 2,33
25	15293 ± 618	93,2 ± 3,76
30	15666 ± 838	95,4 ± 5,10
40	14253 ± 854	86,8 ± 5,21
70	15145 ± 702	92,3 ± 4,28
80	15827 ± 259	96,4 ± 1,58
85	14857 ± 327	90,5 ± 1,99
90	15501 ± 288	94,4 ± 1,75
95	14706 ± 230	89,6 ± 1,40
100	14767 ± 284	89,9 ± 1,73
120	14820 ± 433	90,3 ± 2,64
240	14429 ± 1393	87,9 ± 8,49

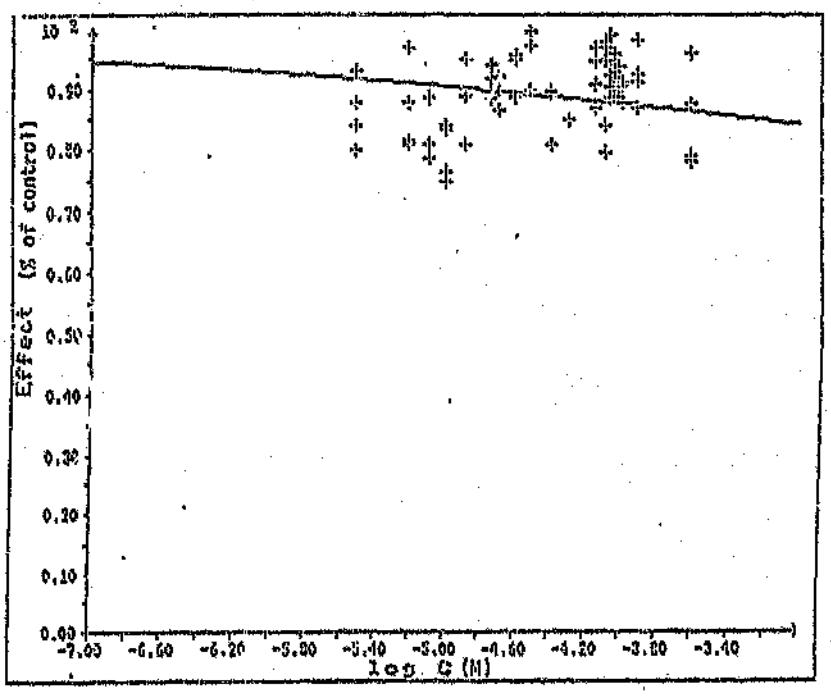

Figure 3.17: The sensitivity of the FCR-3 strain to various concentrations of DTPA.

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	31194 ± 2310	100,0 ± 7,41
3	27608 ± 1899	88,5 ± 6,09
6	28352 ± 1251	90,9 ± 4,01
8	25290 ± 173	81,1 ± 0,55
10	27288 ± 937	87,5 ± 3,01
13	27718 ± 2424	88,9 ± 7,78
18	29605 ± 1025	94,9 ± 3,28
20	27708 ± 2235	88,8 ± 7,16
25	28734 ± 2577	92,1 ± 8,26
30	27573 ± 2083	88,4 ± 6,68
40	30283 ± 526	97,1 ± 1,69
50	25918 ± 375	83,1 ± 1,20
60	29259 ± 1197	93,8 ± 3,84
70	28148 ± 670	90,2 ± 2,15
80	28094 ± 419	90,1 ± 1,34
85	27407 ± 2559	87,9 ± 8,20
90	28484 ± 181	91,3 ± 0,58
95	29114 ± 578	93,3 ± 1,8[illegible]
100	28214 ± 523	90,4 ± 1,[illegible]
120	29345 ± 794	94,1 ± 2,55
240	28809 ± 993	92,4 ± 3,18

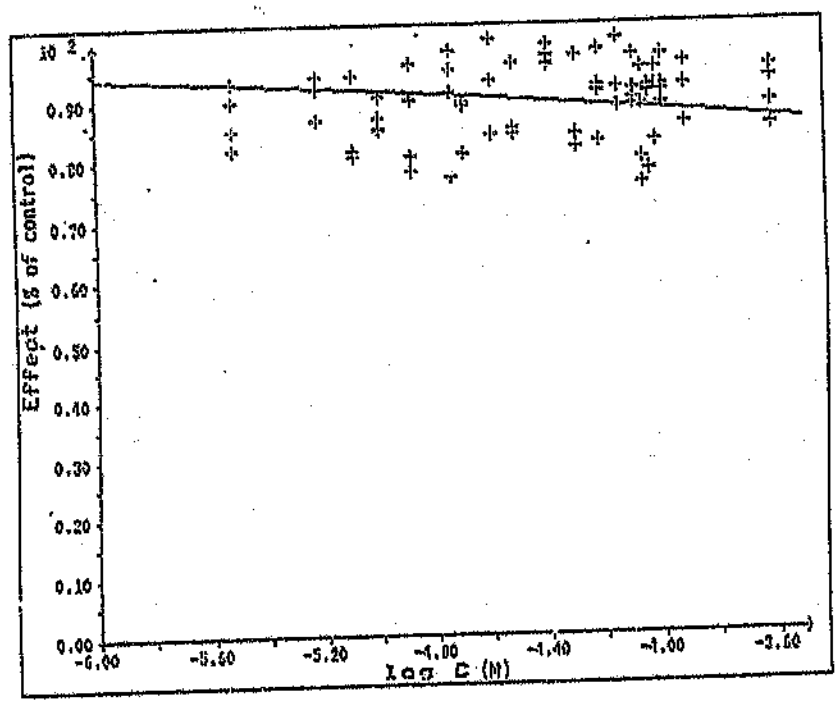

Figure 3.18: The sensitivity of the RSA-2 strain to various concentrations of DTPA.

2 Combination of a chelator with an unsaturated chelator

2.1 The dose response curves of DFO and 2,2'bipyridyl

Figures 3.19 and 3.20 represents the response of the FCR-3 strain to various concentrations of DFO and 2,2'-bipyridyl respectively.

The dose response curves of both DFO and 2,2'-bipyridyl using the FCR-3 strain was determined independently when performing this experiment. This eliminated any variations in the conditions of the experiment .

0 µM BIP +

CONCENTRATION (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
2	76778 ± 7798	87,2 ± 8,85
3	78112 ± 9364	88,7 ± 10,63
4	73919 ± 6446	83,9 ± 7,30
5	67251 ± 3364	76,3 ± 3,82
6	35011 ± 5873	39,7 ± 6,67
7	23494 ± 2204	26,7 ± 2,50
8	12607 ± 2595	20,6 ± 4,23
10	9403 ± 234	15,3 ± 0,38
12	9138 ± 1730	14,9 ± 2,82
18	6288 ± 917	10,3 ± 1,49
20	5048 ± 1047	8,2 ± 1,70
24	3842 ± 320	6,3 ± 0,52

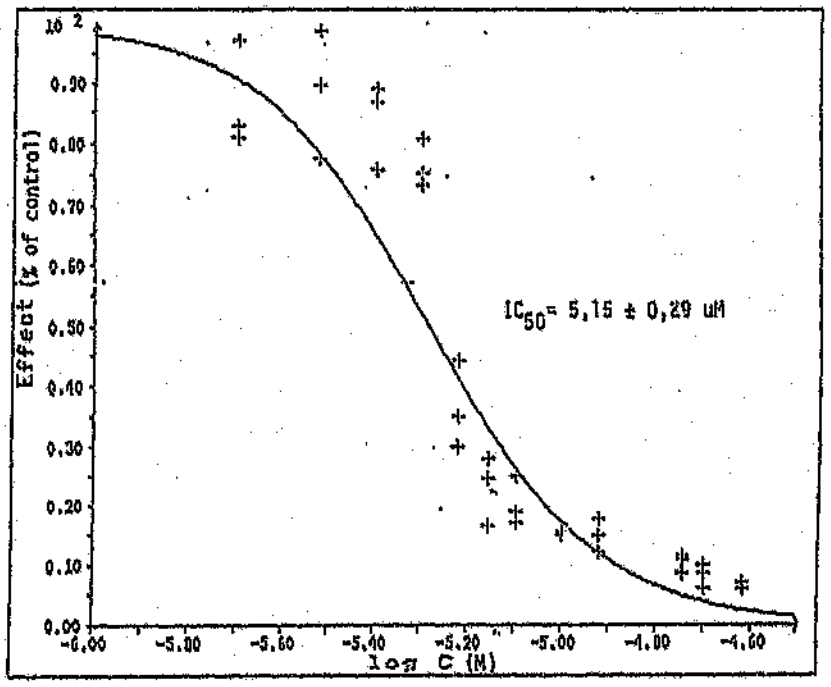

Figure 3.19: The sensitivity of the FCR-3 strain to various concentrations of desferrioxamine (combination of chelators).

0 µM DFO +

CONCENTRATION (µM)	MEAN DPM + SD	% MEAN DPM + SD
6	66108 + 7243	75,0 + 8,22
8	60938 + 1096	69,2 + 1,25
10	56094 + 8298	63,7 + 9,42
12	35666 + 1415	58,2 + 2,31
14	25981 + 4116	42,4 + 6,72
16	23002 + 4712	37,5 + 7,69
24	16217 + 3700	26,5 + 5,04
30	3116 + 349	4,0 + 0,45
40	1672 + 338	2,2 + 0,43
80	975 + 47	1,3 + 0,06
100	833 + 158	1,1 + 0,20

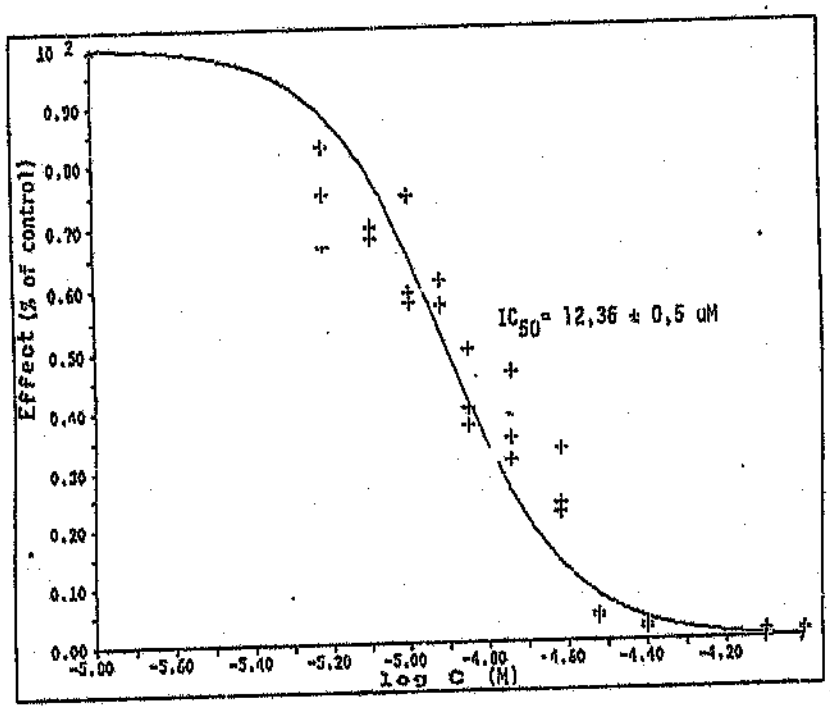

Figure 3.20 : The sensitivity of the FCR-3 strain to various concentrations of 2,2'-bipyridyl (combination of chelators).

2.2 The combination of desferrioxamine with 2,2'-bipyridyl

A fixed concentration of DFO was combined with various concentrations of 2,2'- bipyridyl within the range (0-100 uM). The effect of the combination of the two chelators are tabulated and are represented graphically.

The y axis of the graphs represents the effect. The effect is the resultant parasitaemia obtained (as a percentage of the control parasitaemia), after a fixed concentration of DFO was combined with various concentrations of 2,2'- bipyridyl.

The x axis represents the logged concentration of the 2,2'-bipyridyl with which the fixed concentration DFO was combined. If the log scale on the x axis is used, the concentration of 0 uM (2,2'-bipyridyl) is represented as 1 on the x axis and 1 on the y axis. Therefore when 2 uM DFO was combined with 0 uM 2,2'- bipyridyl, the resultant parasitaemia was represented as 1 on the x axis and y axis.

Figure 3.21 represents the combined effect of 2 uM DFO and various concentrations of 2,2'- bipyridyl on the proliferation of the FCR-3 strain.

The parasitaemia obtained after the FCR-3 strain was exposed to 2 uM DFO was equivalent to 80% of the control parasitaemia values. When 2 uM DFO was combined with 6, 8, and 10 uM 2,2'- bipyridyl , the resultant parasitaemias were approximately 95% of the control parasitaemia . Therefore instead of decreasing below 80%, the parasitaemias increased to 95% of the control parasitaemia.

2,2'- bipyridyl at various concentrations when combined with one fixed concentration of DFO e.g. 2 uM, showed a relative increase in parasitaemia compared to the 2,2'-bipyridyl or DFO alone, thus suggesting that these concentrations of 2,2'- bipyridyl antagonised the antimalarial action of DFO. This antagonistic effect of 2,2'-bipyridyl was less pronounced with higher concentrations of DFO.

2 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	76778 ± 7798	87,2 ± 8,85
6	84642 ± 2716	96,1 ± 3,08
8	76476 ± 5646	86,8 ± 6,41
10	83713 ± 3544	95,0 ± 4,02
12	48801 ± 2794	79,6 ± 4,56
14	48131 ± 2722	78,5 ± 4,44
18	34724 ± 2786	56,6 ± 4,55
24	13696 ± 3222	22,3 ± 5,26
30	6917 ± 593	8,8 ± 0,76
40	3591 ± 73	4,6 ± 0,09
80	1510 ± 68	1,9 ± 0,09
100	1200 ± 84	1,5 ± 0,11

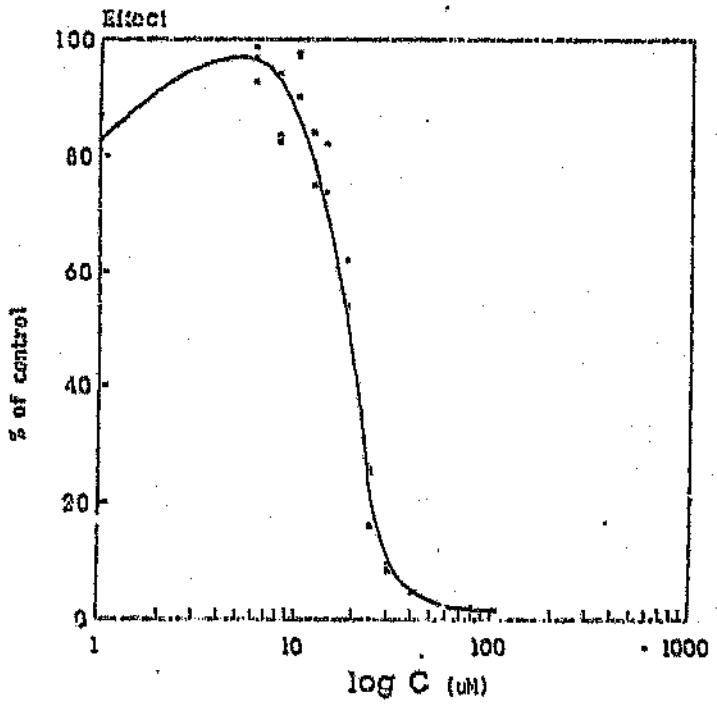

Figure 3.21: The sensitivity of the FCR-3 strain to the combined effect of 2 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

The parasitaemias resulting from the combined action of one fixed concentration of DFO and various concentrations of 2,2'- bipyridyl within the range (0-100 uM) is tabulated and is also represented graphically. The results of the following concentrations of DFO, each combined with the same concentrations of 2,2'- bipyridyl is represented in:

Desferrioxamine (uM)	Figure no
3	3.22
4	3.23
5	3.24
6	3.25
7	3.26
8	3.27
10	3.28
12	3.29
18	3.30
20	3.31
24	3.32

3 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	78112 ± 9364	88,7 ± 10,63
6	87014 ± 8112	98,8 ± 9,21
8	83243 ± 1073	94,5 ± 1,22
10	80472 ± 3024	91,3 ± 3,43
12	50171 ± 8936	81,9 ± 9,69
14	41436 ± 986	67,6 ± 11,56
18	16690 ± 2487	27,2 ± 4,06
24	12867 ± 5168	20,9 ± 8,43
30	7273 ± 1054	9,4 ± 1,36
40	3904 ± 417	5,0 ± 0,53
80	1634 ± 253	2,1 ± 0,33
100	1378 ± 69	1,8 ± 0,09

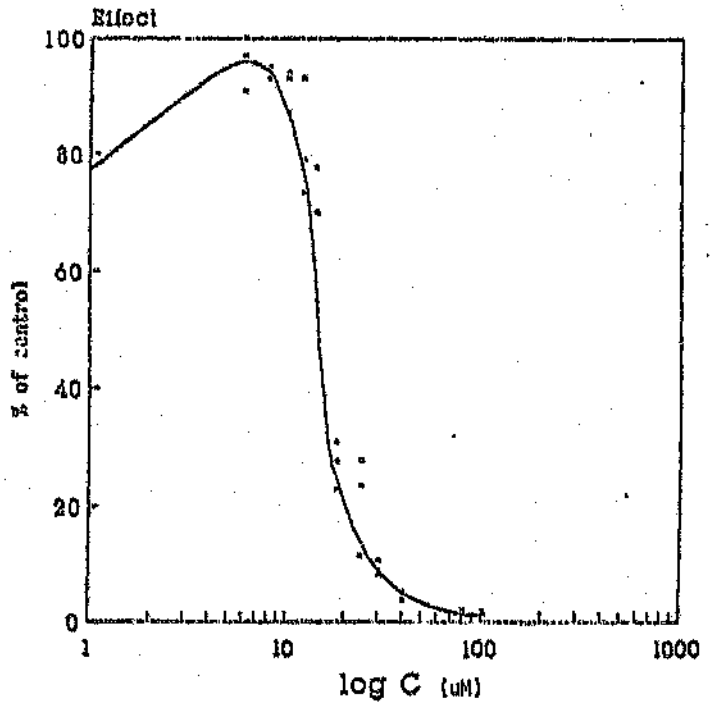

Figure 3.22: The sensitivity of the FCR-3 strain to the combined effect of 3 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

4 μM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (μM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	73919 ± 6446	83,9 ± 7,30
6	85412 ± 4188	96,9 ± 4,75
8	78372 ± 5619	88,9 ± 6,38
10	84519 ± 1502	95,9 ± 1,71
14	32634 ± 7394	53,2 ± 12,07
18	28587 ± 2882	46,6 ± 4,70
24	10992 ± 1202	17,9 ± 1,96
30	5360 ± 390	6,9 ± 0,50
40	3134 ± 594	4,0 ± 0,76
80	1414 ± 110	1,8 ± 0,14
100	1075 ± 102	1,4 ± 0,13

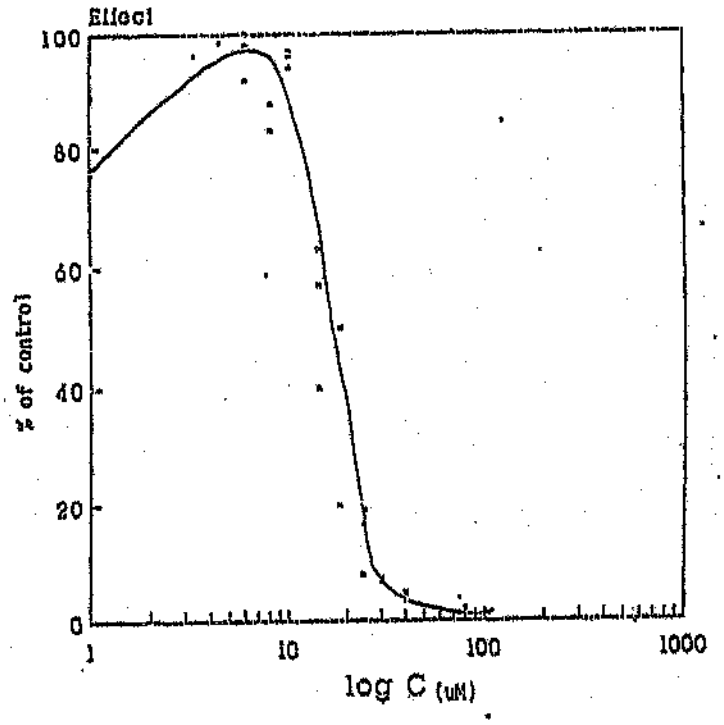

Figure 3.23: The sensitivity of the FCR-3 strain to the combined effect of 4 uM desferrioxamine and various concentrations of 2,2 '-bipyridyl.

5 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	67251 ± 3364	76,3 ± 3,82
6	77556 ± 3745	88,0 ± 4,25
8	65317 ± 9298	74,1 ± 10,55
10	62181 ± 5908	70,6 ± 6,71
12	51555 ± 5348	84,1 ± 8,72
14	50988 ± 1546	83,2 ± 2,52
18	14151 ± 4979	23,1 ± 8,12
24	13793 ± 3698	22,5 ± 6,03
30	5908 ± 1899	7,6 ± 2,44
40	3467 ± 197	4,5 ± 0,25
80	1448 ± 120	1,9 ± 0,16
100	1186 ± 142	1,5 ± 0,18

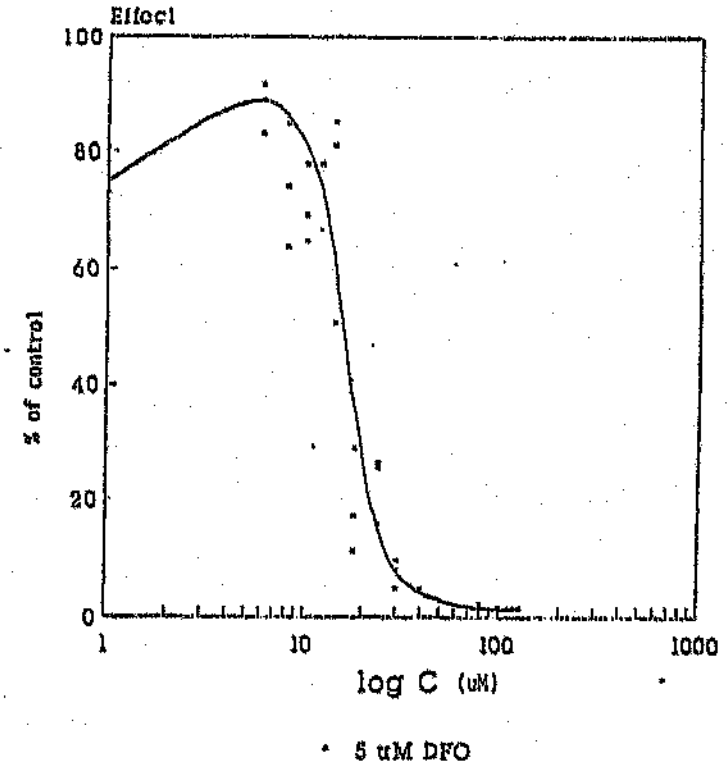

Figure 3.24 : The sensitivity of the FCR-3 strain to the combined effect of 5 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

6 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	35011 ± 5873	39,7 ± 6,67
6	70261 ± 3038	79,8 ± 3,45
8	61382 ± 3813	69,7 ± 4,33
10	62015 ± 15	70,4 ± 0,17
12	54774 ± 1030	89,4 ± 1,68
14	32554 ± 655	53,1 ± 1,07
18	9213 ± 1593	15,0 ± 2,59
24	7872 ± 3136	12,8 ± 5,12
30	4899 ± 363	6,3 ± 0,47
40	3238 ± 423	4,2 ± 0,54
80	1402 ± 23	1,8 ± 0,03
100	1399 ± 179	1,8 ± 0,23

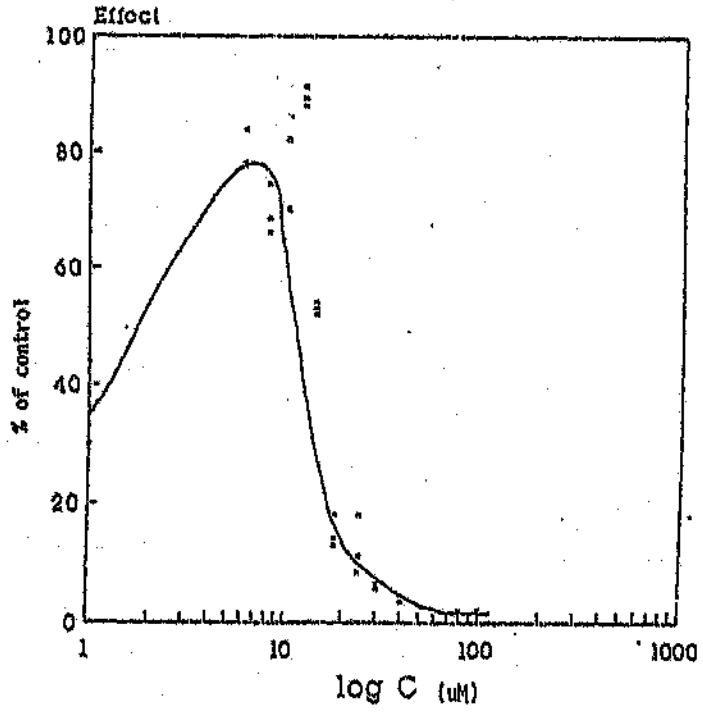

Figure 3.25 The sensitivity of the FCR-3 strain to the combined effect of 6 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

7 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	23494 ± 2204	26,7 ± 2,50
6	43502 ± 1801	49,4 ± 2,04
8	51064 ± 3286	57,9 ± 3,73
10	45184 ± 4378	51;3 ± 4,97
12	33104 ± 1922	54,1 ± 3,13
14	19181 ± 5263	31,3 ± 8,59
18	8091 ± 248	13,2 ± 0,41
24	6212 ± 622	10,1 ± 1,01
30	4227 ± 116	5,4 ± 0,15
40	2947 ± 206	3,8 ± 0,27
80	1629 ± 194	2,1 ± ,,25
100	1259 ± 58	1,6 ± 0,08

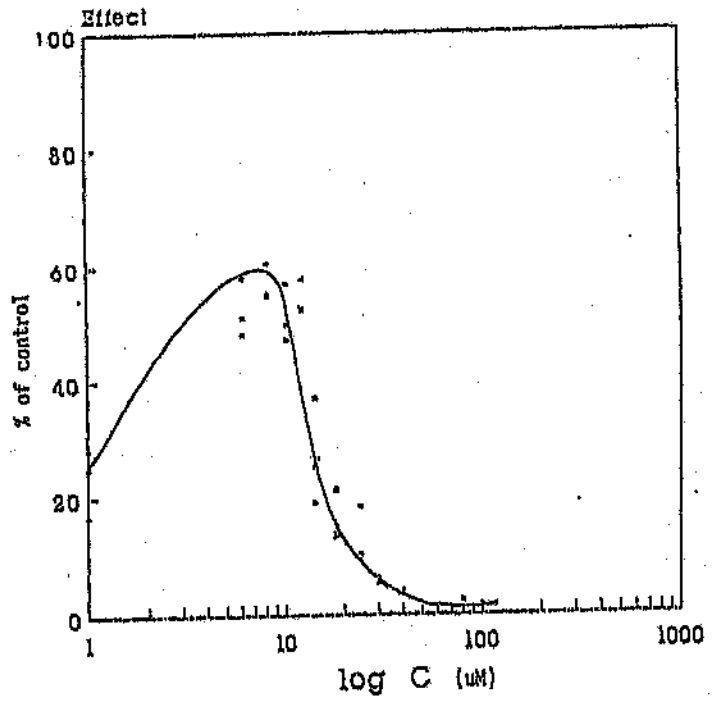

Figure 3.26: The sensitivity of the FCR-3 strain to the combined effect of 7 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

8 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	12607 ± 2595	20,6 ± 4,23
6	24639 ± 2148	40,2 ± 3,50
8	29575 ± 6020	48,2 ± 9,82
10	24297 ± 5690	39,6 ± 9,28
12	20339 ± 1047	26,9 ± 1,39
14	16843 ± 1617	22,3 ± 2,14
18	10119 ± 1573	13,4 ± 2,08
24	4135 ± 360	5,5 ± 0,48
30	3569 ± 1150	4,1 ± 0,09
40	2429 ± 316	2,8 ± 0,36
80	1256 ± 81	1,4 ± 0,09
100	1059 ± 139	1,2 ± 0,16

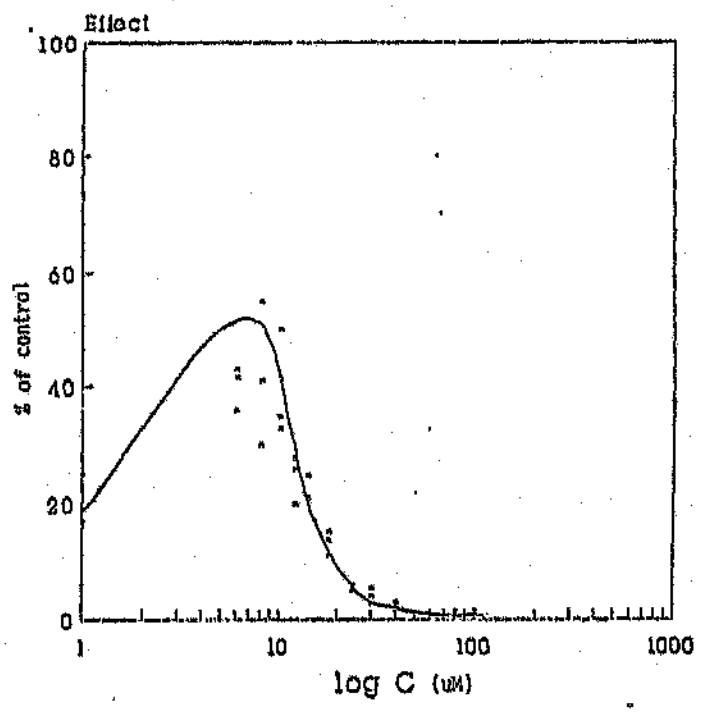

Figure 3.27: The sensitivity of the FCR-3 strain to the combined effect of 8 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

10 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	9403 ± 234	15,3 ± 0,38
6	17215 ± 2699	28,1 ± 4,40
8	26101 ± 2038	42,6 ± 3,37
10	23513 ± 5102	38,4 ± 8,30
12	17754 ± 1933	23,5 ± 2,60
14	12955 ± 1343	17,1 ± 1,78
18	7112 ± 2909	9,4 ± 3,85
24	6334 ± 208	8,4 ± 0,28
30	3728 ± 854	4,2 ± 0,97
40	2354 ± 211	2,7 ± 0,24
80	1327 ± 109	1,5 ± 0,12
100	1327 ± 317	1,5 ± 0,36

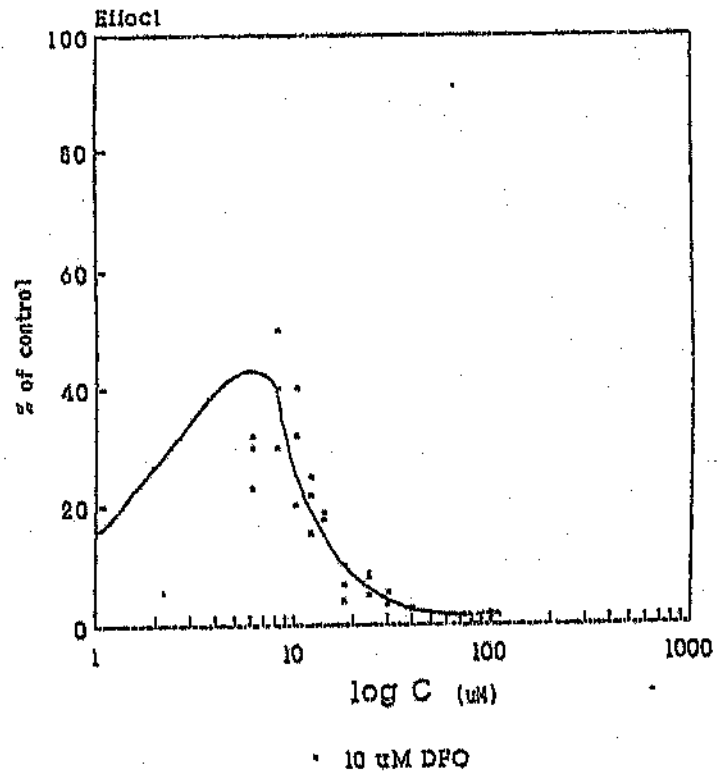

Figure 3.28: The sensitivity of the FCR-2 strain to the combined effect of 10 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

12 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	%MEAN DPM ± SD
0	9138 ± 1730	14,9 ± 2,82
6	13649 ± 483	22,3 ± 0,79
8	14129 ± 589	23,0 ± 0,96
10	12166 ± 1632	19,8 ± 2,66
12	12637 ± 435	16,7 ± 0,58
14	11019 ± 1500	14,6 ± 1,99
18	7173 ± 1935	9,5 ± 3,56
24	4938 ± 977	6,5 ± 1,29
30	3069 ± 593	3,5 ± 0,67
40	2041 ± 219	2,3 ± 0,25
60	1338 ± 129	1,5 ± 0,15
80	1309 ± 186	1,5 ± 0,21

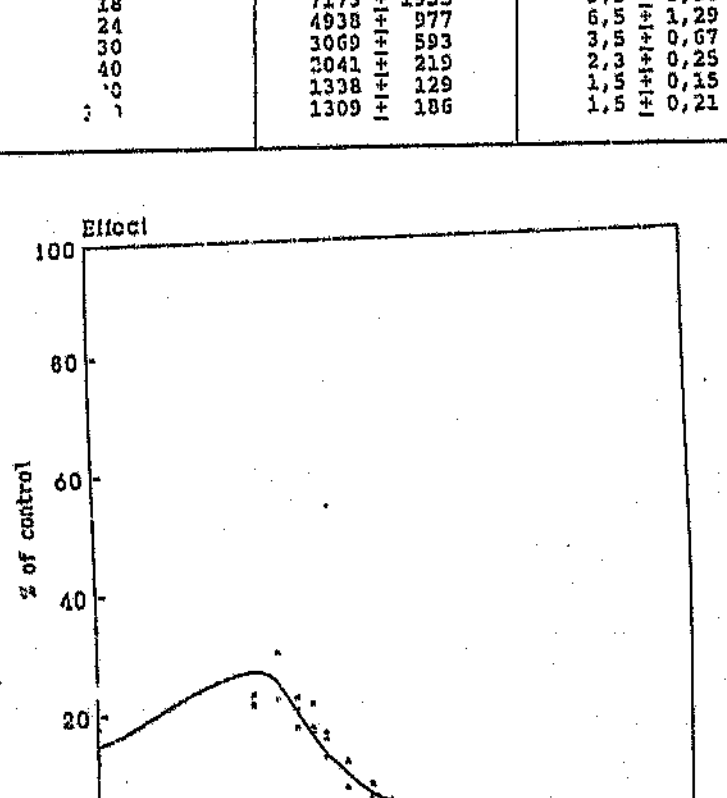

Figure 3.29: The sensitivity of the FCR-3 strain to the combined effect of 12 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

18 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	6288 ± 917	10,3 ± 1,49
6	12075 ± 370	19,7 ± 0,60
8	12701 ± 1095	20,7 ± 1,79
10	9567 ± 1545	15,6 ± 2,52
12	8962 ± 1243	11,9 ± 1,64
14	8741 ± 645	11,6 ± 0,85
16	7786 ± 766	10,3 ± 1,01
24	4267 ± 10	5,6 ± 0,01
30	2737 ± 314	3,1 ± 0,36
40	2277 ± 245	2,6 ± 0,28
80	1512 ± 54	1,7 ± 0,06
100	1279 ± 69	1,5 ± 0,08

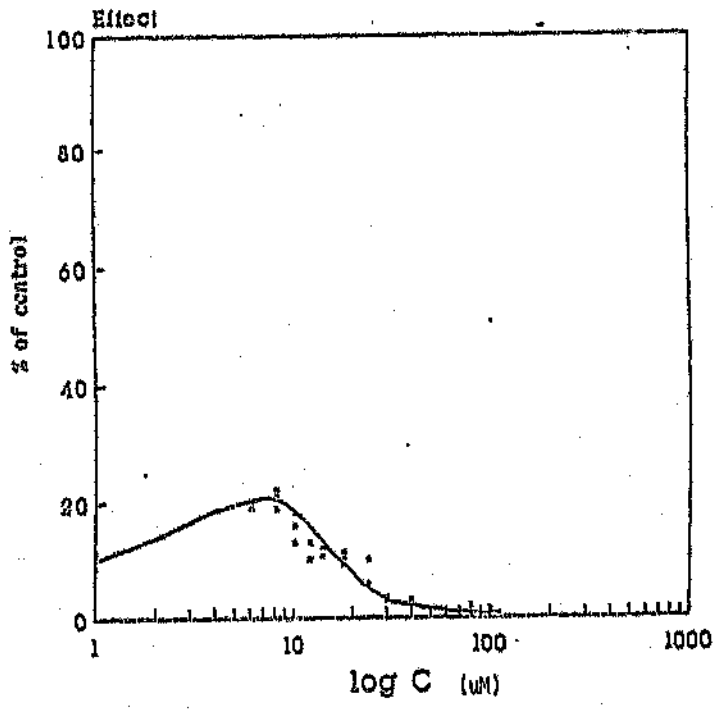

Figure 3.30: The sensitivity of the FCR-3 strain to the combined effect of 18 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

20 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	5048 ± 1047	8,2 ± 1,70
6	9606 ± 797	15,7 ± 1,30
8	11167 ± 754	18,2 ± 1,22
10	8773 ± 1769	14,3 ± 2,89
12	9449 ± 801	12,5 ± 1,06
14	7772 ± 522	10,3 ± 0,69
18	6469 ± 308	8,6 ± 0,41
24	4729 ± 107	6,3 ± 0,14
30	2939 ± 243	3,3 ± 0,28
40	1681 ± 155	1,9 ± 0,18
80	1234 ± 132	1,4 ± 0,15
100	1001 ± 84	1,1 ± 0,09

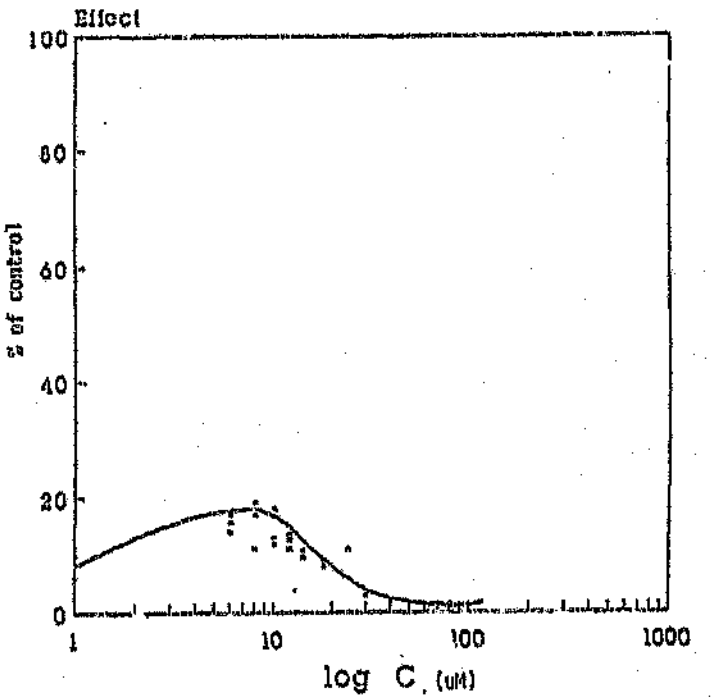

Figure 3.31: The sensitivity of the FCR-3 strain to the combined effect of 20 uM desferrioxamine and various concentrations of 2,2'-Bipyridyl.

24 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	3842 ± 320	6,3 ± 0,52
6	8301 ± 777	13,5 ± 1,27
8	8208 ± 544	13,4 ± 0,89
10	7066 ± 976	11,5 ± 1,59
12	7909 ± 742	10,5 ± 0,98
14	6973 ± 795	9,2 ± 1,05
18	6035 ± 564	7,9 ± 0,75
24	4094 ± 147	5,4 ± 0,19
30	3194 ± 123	3,6 ± 0,14
40	2204 ± 107	2,5 ± 0,12
80	1355 ± 55	1,5 ± 0,06
100	1379 ± 116	1,6 ± 0,13

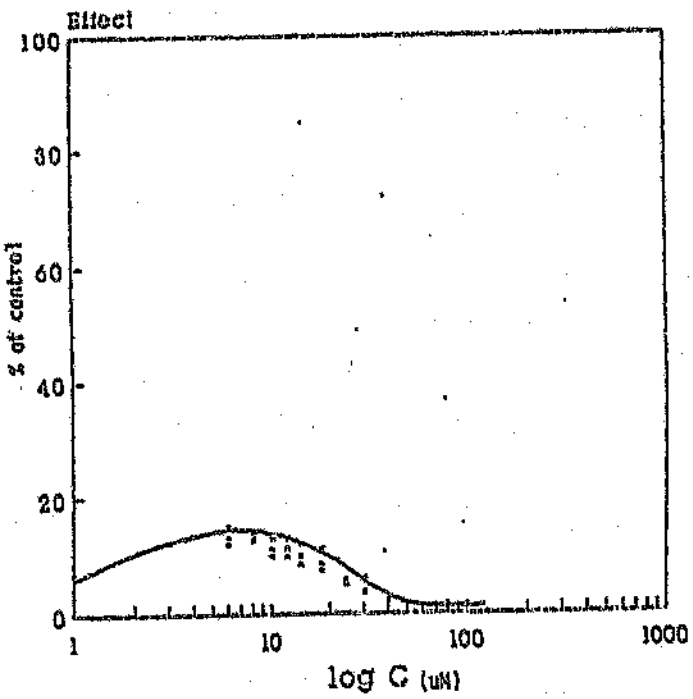

Figure 3.32: The sensitivity of the FCR-3 strain to the combined effect of 24 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

3 Combination of a chelator with an iron saturated chelator

3.1 The spectroscopic titration of 2,2'- bipyridyl and ferrous ammonium sulphate

Figure 3.33 represents the absorbance values measured of a fixed concentration of 2,2'- bipyridyl (1 mM) combined, with various concentrations of ferrous ammonium sulphate The y axis of the graph represents the absorbance measured. The x axis represents the various concentrations of ferrous iron added to the fixed concentration of 2,2'- bipyridyl.

When 1 mM ferrous ammonium sulphate was added to 2,2'-bipyridyl (1 mM), the absorbance reached a maximum value and remained constant at this value even after higher concentrations of ferrous ammonium sulphate were added. Therefore ferrous iron completely saturated 2,2'-bipyridyl in equimolar concentrations.

1 mM BIP added to:

CONCENTRATION OF FERROUS IRON ADDED (uM)	ABSORBANCE
50	0,042
100	0,036
200	0,062
250	0,063
380	0,064
500	0,064
750	0,067
880	0,065
1000	0,078
1250	0,074
1500	0,075
1750	0,070
2000	0,070
2250	0,070
2500	0,070
3000	0,070
3500	0,071

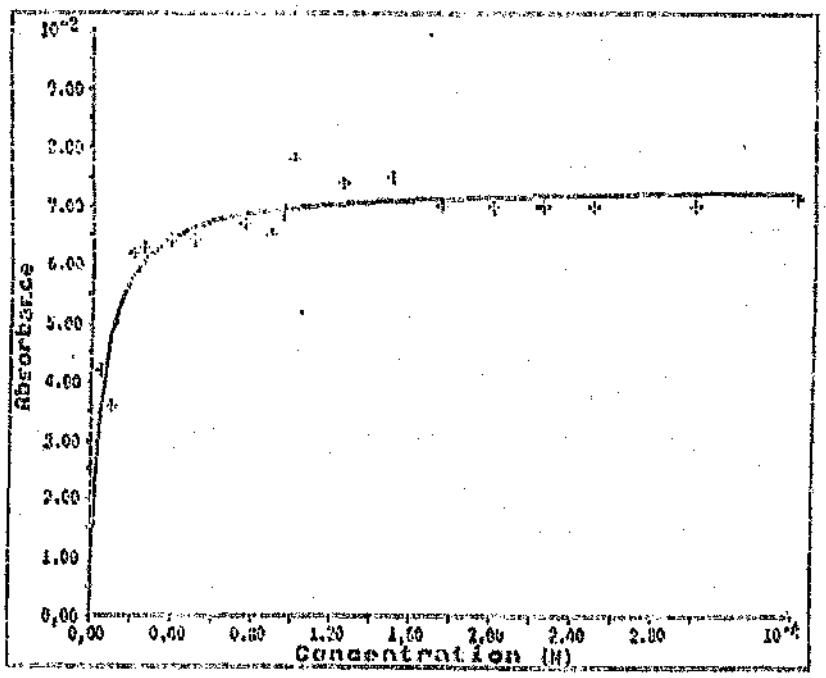

Figure 3.33: The absorbance values of 1 mM 2,2'-bipyridyl combined with various concentrations of ferrous iron (measured at 520 nm).

3.2 The combination of desferrioxamine with iron saturated 2,2'-bipyridyl

3.2.1 The combination of desferrioxamine with 2,2'bipyridyl

Figure 3.34 represents the combined effect 6 uM DFO and various concentrations of 2,2'- bipyridyl on the survival of the parasites. 2,2'-Bipyridyl antagonised the antimalarial action of 6 uM DFO.

6 µM DFO +

CONCENTRATION OF 2,2'- BIPYRIDYL ADDED (µM)	MEAN DPM ± SD	% MEAN DPM ± SD
0	31802 ± 5180	55,2 ± 8,98
0,1	31411 ± 4514	54,5 ± 7,83
0,2	29602 ± 5159	51,3 ± 8,95
0,4	27753 ± 4404	48,1 ± 7,64
0,6	39046 ± 8962	67,7 ± 15,55
0,8	35243 ± 4124	61,1 ± 7,15
2	36990 ± 3795	64,2 ± 6,58
4	47986 ± 8532	83,2 ± 14,80
8	43343 ± 4251	75,2 ± 7,38
10	50638 ± 1773	87,9 ± 3,08
14	30754 ± 3371	53,4 ± 5,85
18	31105 ± 6414	53,9 ± 11,13

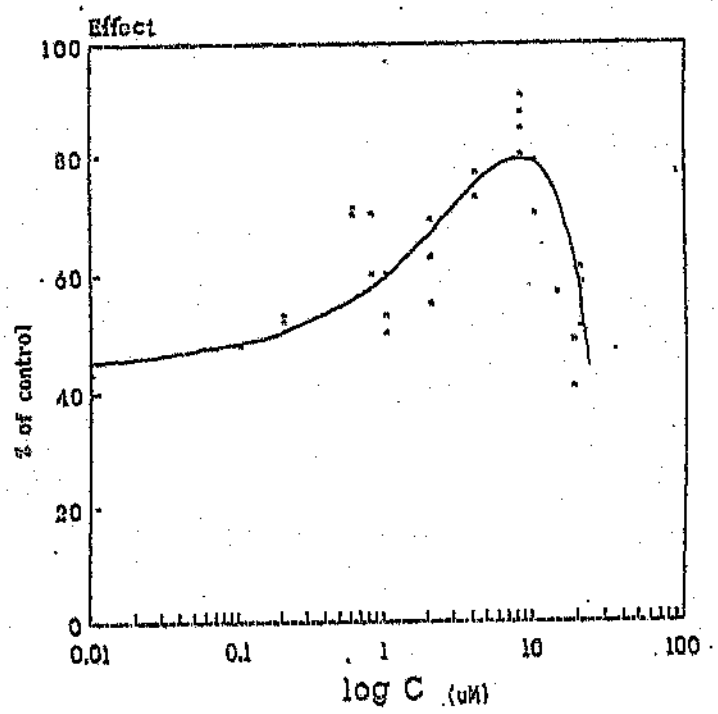

Figure 3.34: The sensitivity of the FCR-3 strain to the combined effect of 6 uM desferrioxamine and various concentrations of 2,2'-bipyridyl.

3.2.2. The combination of desferrioxamine with iron saturated 2,2'-bipyridyl

Figure 3.35 represents the sensitivity of the parasites to the combined effect of 6 uM DFO and various concentrations of iron saturated 2,2'- bipyridyl.
The dose response curve obtained was a straight line indicating that constant DPM values (parasitaemias) were obtained when various concentrations of iron saturated 2,2'-bipyridyl were combined with 6 uM DFO. The slope of the curve is not significant from zero. Essentially the curve represents the effect of 6 uM DFO alone on the growth of the parasites (The effect of 2,2'-bipyridyl was eliminated as it was saturated with iron).

6 µM DFO +

CONCEN. OF 2,2'-BIPYRIDYL ADDED (µM)	CONCEN. OF IRON ADDED	MEAN DPM ± SD	% MEAN DPM
0	0	35842 ± 1347	50,0 ± 1,88
0,1	0,1	34490 ± 1146	48,0 ± 1,59
0,2	0,2	34046 ± 2481	47,5 ± 3,46
0,4	0,4	29928 ± 1068	41,8 ± 1,49
0,6	0,6	40117 ± 620	55,9 ± 0,87
0,8	0,8	40923 ± 2660	57,1 ± 3,70
1	1	34195 ± 3100	47,7 ± 4,33
2	2	38574 ± 4456	53,8 ± 6,20
6	6	35144 ± 3680	49,0 ± 5,15
14	14	32183 ± 806	44,9 ± 1,12
18	18	32776 ± 3649	45,7 ± 5,09

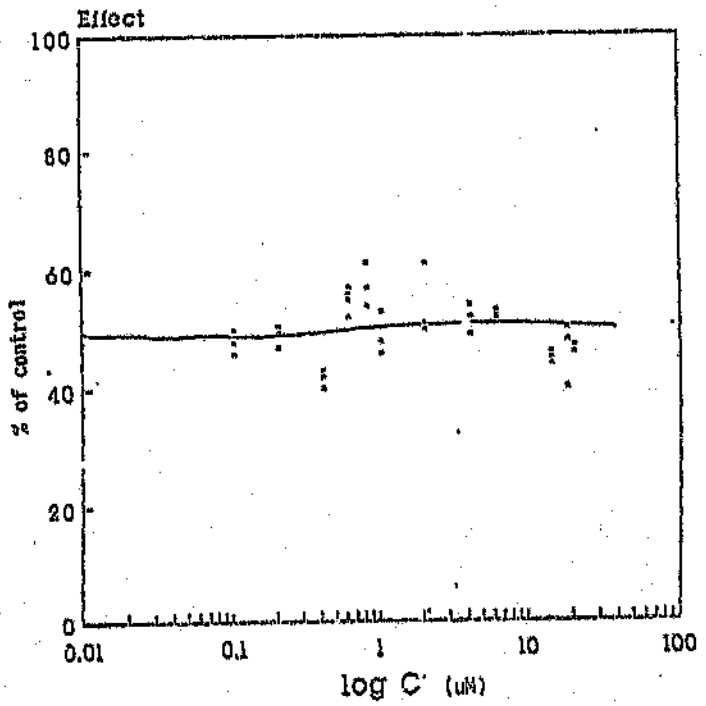

Figure 3.35: The sensitivity of the FCR-3 strain to the combined effect of 6 uM desferrioxamine and various concentrations of 2,2'-bipyridyl saturated with ferrous iron.

3.3. The spectroscopic titration of desferrioxamine and 2,2 '- bipyridyl

Figure 3.36 represents the absorbance spectrum of 2,2'- bipyridyl in water, using water as a base line. The solution absorbed maximally at 233 nm and 280 nm.

Figure 3.37 represents the absorbance spectrum of the 2,2'-bipyridyl solution diluted with an equal volume of a DFO solution in water. The DFO solution diluted with an equal volume of water was used as a base line. The maximum absorbance was observed at 233 nm and 280 nm.

Figure 3.38 represents the absorbance spectrum of 2,2'-bipyridyl in incomplete culture medium, using the incomplete medium as a base line. The solution absorbed maximally at 233 nm and 280 nm.

Figure 3.39 represents the absorbance spectrum of the 2,2'-bipyridyl solution diluted with an equal volume of the DFO solution in incomplete medium. The DFO solution diluted with an equal volume of incomplete medium was used as a base line. The maximum absorbance was observed at 233 nm and 280 nm.

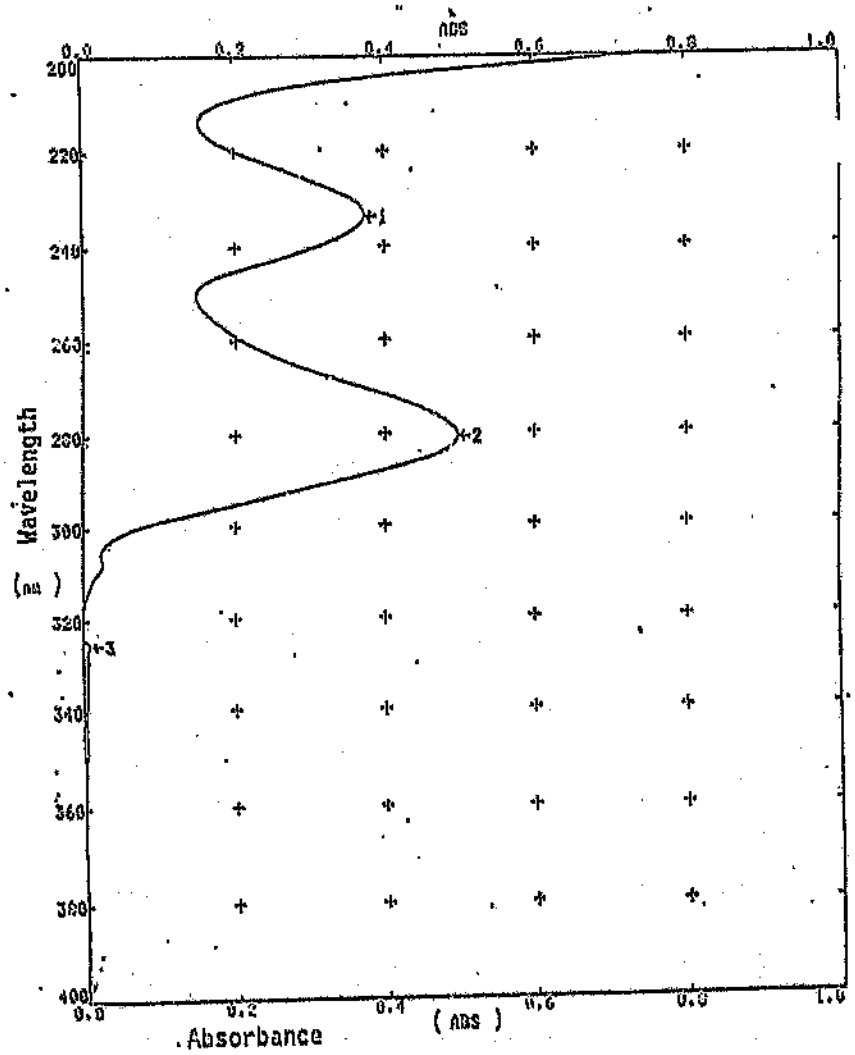

Figure 3.36 : The absorbance spectrum of 2,2'-bipyridyl. 2,2'-bipyridyl was dissolved in water. The absorbance of water served as a baseline.

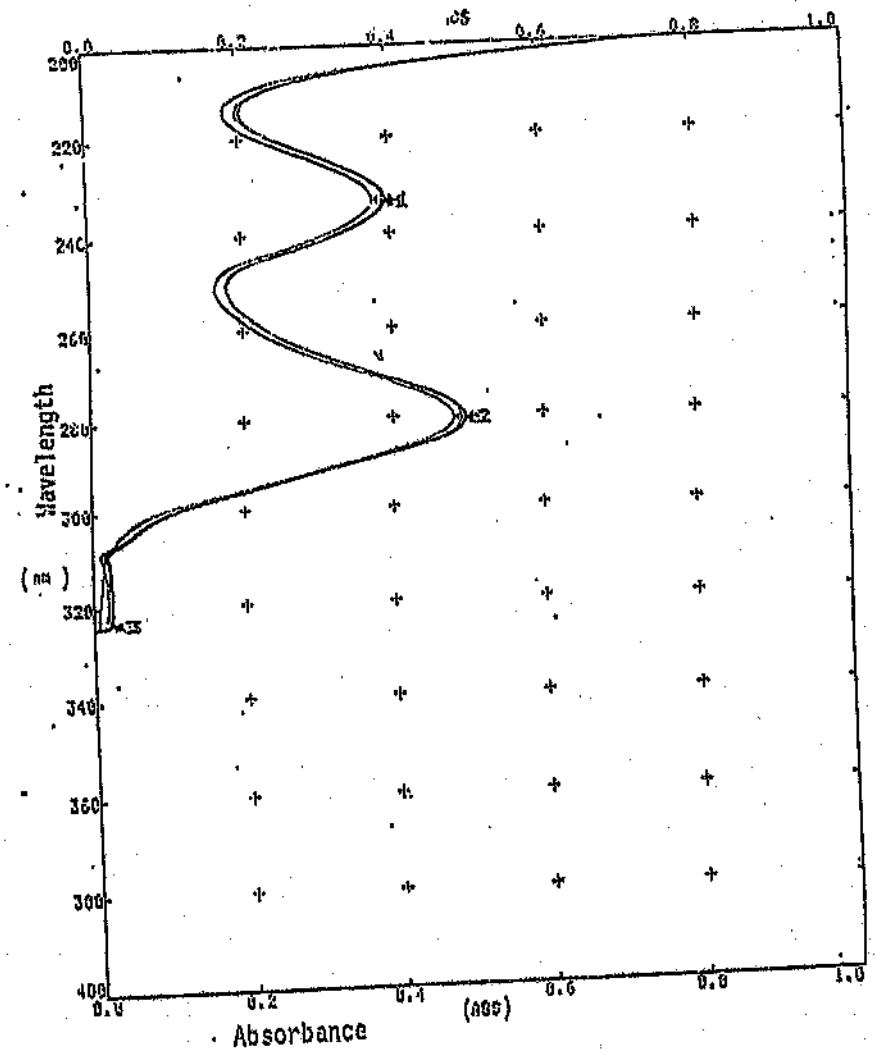

Figure 3.37: The absorbance spectrum of 2,2'-bipyridyl solution diluted with an equal volume of desferrioxamine solution. Both chelators were individually dissolved in water before combining. The absorbance of the desferrioxamine solution in water served as a baseline.

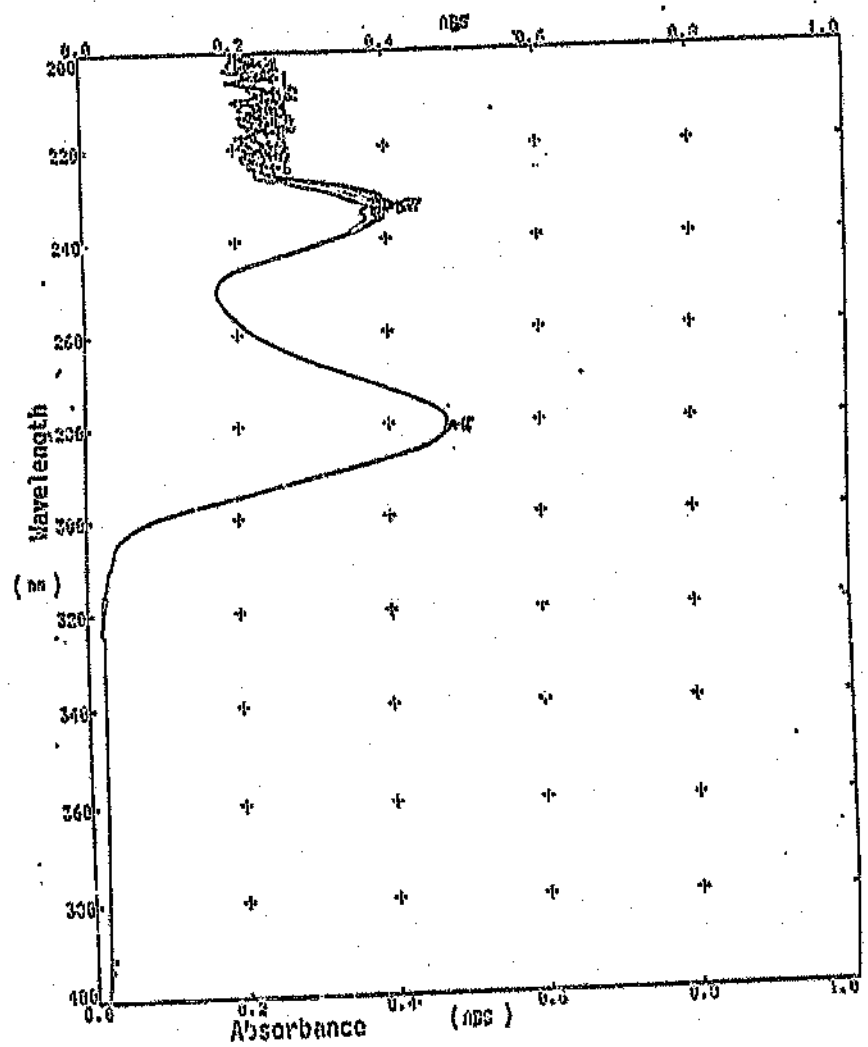

Figure 3.38 : The absorbance spectrum of 2,2'-bipyridyl. 2,2'-bipyridyl was dissolved in culture medium. The absorbance of the culture medium served as a baseline.

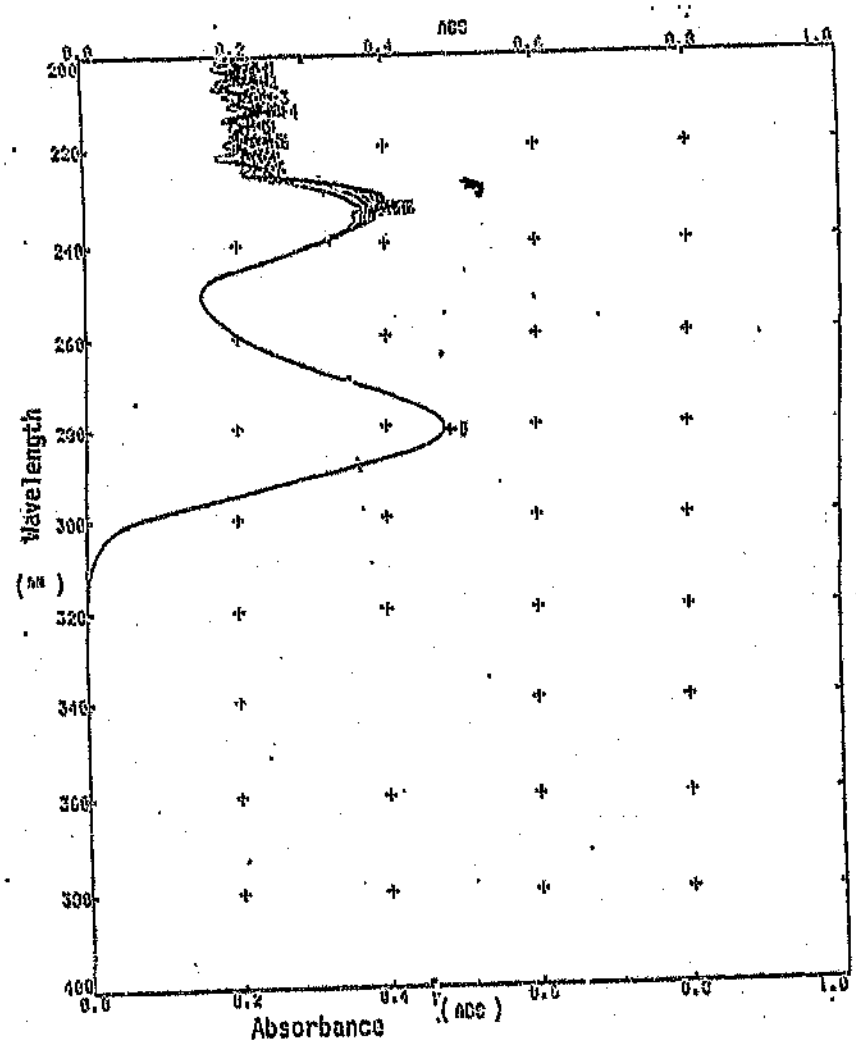

Figure 3.39 : The absorbance spectrum of 2,2'-bipyridyl solution diluted with an equal volume of desferrioxamine solution. Both chelators were individually dissolved in culture medium before combining. The absorbance of the desferrioxamine solution in culture medium served as a baseline.

CHAPTER 4 DISCUSSION

SECTION 4.1 DISCUSSION OF METHODS

A) DISCUSSION OF METHODS USED FOR THE CULTIVATION OF PARASITES

Prior to 1976 attempts to obtain continuous growth of the *P. falciparum* parasite were unsuccessful. Growth of the parasite was limited to the short term maintenance of human and certain animal plasmodia (WHO, 1972). In 1976, two groups working independently achieved the first successful continuous culture of *P. falciparum* parasites (Trager and Jensen 1976; Haynes *et al.*, 1976). These studies, (especially the findings of Trager and Jensen, 1976) revolutionised research on human malaria. Their methods have formed the basis of many of the recent advances in malaria biochemistry, immunology and pharmacology.

1 Characteristics of the parasite isolates used

Two strains of parasites, the FCR-3 and RSA-2 were routinely cultured in our laboratories. These were donations from J. Freese of the RIDTE in Durban.

Isolate characteristics as described by J. Freese:

FCR- 3

Origin: The Gambia

Jensen JB and Trager W. *Plasmodium falciparum* in culture: establishment of additional strains. Am J Trop Med Hyg 1978; 27:743-746.

Chloroquine sensitivity: MIC = 0.6 uM (resistant).

RSA- 2

Origin: Kwazulu

Date of collection: 28-1-1985.

Clinical data: Patient responded to treatment with 10 tablets of Darachlor (1 500 mg chloroquine base + 150 mg pyrimethamine base).

Chloroquine sensitivity: MIC = 0.06 uM.

2 Factors affecting the continuous cultivation of the erythrocytic stages of *P. falciparum*

2.1 Medium

The medium used to initiate the first continuous culture of *P. falciparum* was RPMI 1640 (Trager and Jensen, 1976). This medium was originally developed for the *in vitro* cultivation of leucocytes (Jensen, 1988). RPMI 1640 contains a hydrogen carbonate-carbon dioxide buffer system which maintains the physiological pH of the medium (WHO, 1972). This medium supplemented with 25 mM HEPES buffer remains the medium of choice.

2.1.1 Role of HEPES buffer

It is crucial that the pH of the medium be maintained between 7,3 and 7,5 for the successful *in vitro* growth of the parasites (Jensen, 1988; WHO, 1972). The parasites rapidly produces large concentrations of lactic acid which is a product of glucose metabolism (Trigg, 1985; Zolg *et al.*, 1984). This results in local and gross pH changes which is detrimental to the development of the parasites (Zolg *et al.*, 1984).

Therefore the long term maintenance of the parasites *in vitro* depends on overcoming the problems of drastic pH changes. The hydrogen carbonate - carbon dioxide buffering system found in RPMI 1640 is not sufficient to maintain the pH of the extracellular medium at 7,4 as the parasites consume glucose and produce lactate rapidly. To maintain the pH of the extracellular medium between 7,2 and 7,4, additional buffers are required (WHO, 1977).

In our studies, HEPES, a zwitterion buffer, was routinely used to supplement the hydrogen carbonate-carbon dioxide buffer system of RPMI 1640. The zwitterion buffer increases the overall buffering capacity of the medium and it does not interfer with normal cell physiology. It also ensures that the physiological pH within the culture flask is maintained once the flasks are opened for inoculation and removal of parasites (WHO, 1972).

HEPES is a suitable buffer as its pk at 37°C is approximately 7,3 which is very close to the pH required for optimum parasite growth (Trigg, 1985). Moreover Freese *et al.*, (1988) observed that RPMI 1640 supplemented with HEPES buffer resulted in a higher peak parasitaemia in local strains compared to supplementation with another buffer.

2.1.2 Importance of hypoxanthine supplementation

The method used for parasite cultivation in our studies, required that the medium should also be supplemented with hypoxanthine Freese *et al.* (1988). The parasite cannot synthesis purines de novo. The medium RPMI 1640 also lacks purines, therefore the parasite depends on its host for these. The only sources of purine precursors available to the parasite are the human erythrocyte and the serum which supplements the medium . Hypoxanthine is a precursor of adenine and guanine and it enters the parasite readily (Manadhar and Van Dyke, 1975). Supplementing the medium with a certain concentration of hypoxanthine increases the peak parasitaemia (Zolg *et al.*, 1982; Butcher 1979; Jensen 1979;). The hypoxanthine concentration of 44 mg per 1 litre of medium used in our studies was sufficient enough to enhance the parasitaemia (Zolg *et al.*, 1982).

2.1.3 Gentamycin used as prophylaxis

To avoid accidental contamination, gentamycin sulphate was used prophylactically in the culture medium.

2.2 Serum requirements

The continuous cultivation of *P. falciparum* requires that the basic culture medium, RPMI 1640 is supplemented with pooled human serum for parasite growth and the serum type selected is compatible with the erythrocytes being used (Divo and Jensen, 1982). In our studies, serum from healthy A $^+$ donors was used as it was compatible with the O $^+$ erythrocytes being used and the larger proportion of the Johannesburg population belong to this blood group.

In previous studies, 10ml of pooled serum was used to supplement 1 litre of medium (Divo and Jensen, 1982). At this concentration, most sera supports the growth of parasites that are already established in culture (WHO, 1977). Pooled human serum from a few donors is always more effective compared to serum obtained from a single donor (Divo and Jensen,1982; Jensen, 1979). Since the two strains of the parasites being cultured in our laboratories were already established in culture, 10ml of pooled heat inactivated human serum was routinely used to supplement 1 litre of medium.

In our studies the serum from freshly clotted blood was pooled, frozen and used. Reports from previous studies

indicate that serum collected under these conditions, produces optimal growth of the parasites (Zolg *et al.*, 1982; Divo and Jensen , 1982; Jensen , 1988). In our laboratory the serum was stored at -20°C in aliquots of volumes suitable for immediate use. Therefore once the serum was thawed, the entire aliquot was utilised. Repeated freezing and thawing is reported to result in a loss of nutrients (WHO, 1977). Temperatures higher or lower than -20°C is reported to be deleterious to the serum (WHO , 1972; Jensen ,1979).

2.3 Erythrocytes

P.falciparum is maintained in continuous culture, using the erythrocytes from any of the ABO blood groups provided these are compatible with the serum being used. There is no biological preference for any of the blood groups (Jensen 1988). With our experience, fresh or stored erythrocytes appeared to have the same ability to support the continous cultivation of the parasites, provided the erythrocytes were not stored in lithium-heparin for longer than 1 week.

2.4 Gas requirements

The *P. falciparum* parasite is an obligate microaerophillic and cannot proliferate under anaerobic conditions. The gas mixture used for the cultivation of the parasite consisted of oxygen, carbon dioxide and nitrogen (Scheibel *et al.*, 1979).

Although Trager and Jensen (1976) observed that the gas atmosphere in the candle jar i.e. (1.3 -3.3 % carbon dioxide and 14.5-17.5 % oxygen), is ideal for the establishment and maintenance of certain strains of parasites, Freese *et al*, (1988) reported that it was unsuitable for local strains. Therefore an alternate gas phase was selected.

Carbon dioxide concentrations between 2 and 5% are suitable for the growth of the parasites. Higher concentrations of carbon dioxide are inhibitory. The RPMI 1640 medium contains a hydrogen carbonate- carbon dioxide buffer system which is affected by high concentrations of carbon dioxide (Trigg, 1985). The carbon dioxide concentration should be sufficient to balance the bicarbonate content of the medium and maintain the correct pH (WHO, 1977).

The manner in which oxygen affects the metabolism of the malaria parasite is unknown. Oxygen concentrations equal to that in air (approximately 21%) is found to be inhibitory to the growth of the parasite, although this effect may take several days to become apparent (Scheibel *et al.*, 1979).

Therefore, low oxygen tensions are favourable to the metabolism of the parasites. The positive effect of low oxygen tensions is probably related to the maintenance of low redox potentials in the parasitic environment Trigg, 1985).

The maintenance of the correct pH and low redox potentials are essential. These are influenced by the concentrations of carbon dioxide and oxygen respectively in the gas ph. e. The concentration of the two gases are compatible and balance each other. Therefore, if the concentration of oxygen is low, the concentration of carbon dioxide is also low, so that the reduced oxygen supply is not compromised further.

Combining 3 % oxygen with 4 % carbon dioxide results in the pH of the medium being 7.42, which is the physiological pH (Scheibel *et al.*, 1979). In our studies the concentrations of 3 % oxygen and 4 % carbon dioxide

were selected. This gas mixture proved to be suitable for the establishment and maintenance of the local strains of parasites.

2.5 Parasites cultured in suspension

Cultivation of the parasites in suspension results in improved yields of parasites compared to static cultures (Freese *et al.*, 1988; Zolg *et al.*, 1982; Butcher, 1979; Butcher, 1981; Fairlamb, 1985;. In static cultures only the parasites at the top of the erythrocytic layer have good access to the nutrients within the medium which floats on top of the erythrocytic layer. Those parasites found deep in the erythrocyte layer have less access to the nutrients in the medium and their microenvironment soon becomes unsuitable for optimal proliferation. Toxic metabolites especially, lactic acid accumulates in the parasite microenvironment and this inhibits the optimum development of the parasite resulting in overall lower parasitaemia (Jensen, 1988). Furthermore the thin static layer has limited usefulness, as it cannot be easily scaled up to produce large quantities of parasites (Butcher, 1981).

Butcher (1981) reports that in cultures maintained in suspension (i.e.on an orbital shaker) there is increased

contact between the released merozoites and the uninfected erythrocytes. These contact points are more efficiently distributed in suspension cultures resulting in higher peak parasitaemia and multiplication rates. In static cultures there is reduced contact between the released merozoites and the available uninfected erythrocytes which yields higher percentages of multiple infected erythrocytes compared to suspension cultures.

The parasites in multiple infected erythrocytes are less likely to develop to the mature schizont stage and subsequently release merozoites. The presence of more than one parasite within an erythrocyte increases the stress on the nutritional capacity of the erythrocyte. Furthermore, the membrane of a multiple infected erythrocyte is too fragile to support the optimum development of two or more parasites (Butcher, 1981). Therefore these parasites rarely develop to maturity.

2.6 Synchronization

In an established culture , all the developmental stages are generally seen in culture at any one time. This situation however is not always suitable for studies in which a specific stage is required. In a synchronized culture, the nutritional and gas requirements of all the

parasites are constant as the parasites are in the same stage. In a non synchronized culture, the various stages of the parasite are present, thus the requirement of each parasite (stage) differs. Therefore the early stage of the parasite (i.e. the ring stage) may be deprived of nutrition and gas due to the increased requirements of the mature stages. Due to these factors it is essential to synchronize parasites regularly.

In our studies, the parasites were routinely synchronized, using the method described by Lambros and Vanderberg (1979). This method employs the use of an aqueous sorbitol solution which allows parasites in the early stages to survive. Normal erythrocytes are impermeable to sorbitol, but the developing parasite causes a change in the permeability of the erythrocytic membrane. Therefore washing the parasitized erythrocytes in the aqueous sorbitol solution lysed the membranes of the mature stages of the parasite as these membranes are most permeable to the sorbitol solution. The parasites in the early ring stage are isolated, as the parasitic membrane in this stage is not as porous and permeable as in the mature stages (Jensen, 1988).

2.7 The routine maintenance of the malaria cultures

2.7.1 The importance of replacing the medium

It is essential that the medium is replaced regularly e.g. once every 24 hours, as it is a limiting factor for the successful proliferation of the parasites. The frequency at which the medium is replaced depends on the parasitaemia. The nutritional capacity of the medium is exhausted by parasitized as well as non parasitized erythrocytes. Therefore if the medium is replaced once every 24 hours , a low parasitaemia is essential , since the metabolism of the parasites and erythrocytes limits the buffering capacity of the medium (Butcher, 1979). The accumulation of toxic metabolites especially lactic acid in the medium decreases the pH of the medium below 7.4 which prevents optimal multiplication of the parasites. In our studies the medium was replaced once every 24 hours at a low parasitaemias, and once every 12 hours at high parasitaemias.

2.7.2 The role of thin blood smears

In our studies, thin blood smears were prepared daily to determine the parasitaemia. These were carefully prepared, stained and studied. The thin smears are the

only indication of the parasitaemia and the condition of the parasites. Therefore the smears determines whether subculturing of the parasites is required.

The smears are especially crucial when preparing for an experiment. These smears indicate if the parasite stage is appropriate for a specific experiment and determines the parasitaemia of the stock cultures.

2.7.3 The importance of subculturing or diluting of parasites

In our studies if the parasitaemia of a culture was 5% and higher, it was advisable to subculture or dilute the parasites. At high parasitaemias the nutritional requirements of the parasites exceeds the capacity of the medium, especially if the medium is replaced only once every 24 hours . The accumulation of the lactic acid at high parasitaemias drastically reduces the pH of the medium which was unfavourable for the proliferation of the parasites (Zolg *et al.*, 1984). Moreover at high parasitaemias there is a reduced number of uninfected erythrocytes available to the released merozoites to penetrate. This has a direct influence on the new generation of parasites.

In our studies the parasites were subcultured by adding newly washed erythrocytes and fresh complete medium. It was crucial that large volumes of packed erythrocytes were added when subculturing for the new generation of merozoites to penetrate. The addition of fresh erythrocytes is important as erythrocytes deterioate *in vitro* . After 24 hours *in vitro* , erythrocytes show a decrease in glucose consumption and therefore a decrease in the rate of phosphorylation of (ADP), adenosine 5'-(trihydrogen pyrophosphate) to (ATP), adenosine 5'(tetrahydrogen triphosphate) (WHO, 1972). The fresh erythrocytes added when subculturing will have high ATP levels, which will promote the optimal developement of merozoites which will penetrate these erythrocytes. The added erythrocytes decreases the overall parasitaemia and at a low parasitaemia, less toxic metabolites are produced.

3 Cryopreservation and thawing of parasites

3.1 Cryopreservation of parasites

Continuous cultures of parasites are exposed to the risk of accidental bacterial or fungal contamination, therefore to avoid the total loss of a strain it is advisable to preserve some of the cultures.

At this stage there has been insuffic ent studies performed to elucidate optimum conditions for reliable and reproducible cryopreservation of the malaria parasites (WHO, 1981). In our studies, the method employed for the cropreservation of the parasites was a modification of the low glycerol method of (Rowe *et al.*,1968). The low glycerol method is also recommended for the cryopreservation of cultured parasites by Peters, (1988 b); Nguyen Dinh, (1988). The method described by Freese *et al*, (1988) was suitable for the cryopreservation of local strains.

In our studies, the volume of the cryoprotectant, 28 % glycerol in PBS added, was equal to the volume of the parasite suspension, resulting in a final concentration of 14 % glycerol. This concentration was suitable as only concentrations higher than 20 % glycerol were reported to be toxic to the parasites (WHO, 1972).

The glycerol serves as a cryoprotectant agent which prevents ice crystal formation and damage to the parasite membrane. In our studies only parasites in the ring stage were frozen . The parasites in the ring stage are frozen as the schizont and trophozoite stages are less resistant to the freeze-thaw process (Houba, 1988). This is also observed in the ultrastructural studies on *P.*

falciparum in which a higher percentage of the rings survived the freeze-thaw process (Diggs *et al.*, 1977). The ring stage of the parasite is also selected because it can be stored indefinately in liquid nitrogen without any loss of infectivity (Strome *et al.*, 1977; Houba, 1988).

In our studies, the parasites were placed in liquid nitrogen within 10 minutes of adding the glycerol mixture. It is advisable to attain the temperature at which the parasites are to be frozen rapidly, to prevent the parasites from metabolising the glycerol as it is cytotoxic to the parasites (WHO, 1981). Storage of the parasites in liquid nitrogen (-196°C) or in the gas phase of the liquid nitrogen (-185°C) results in optimal recovery of the parasites (Christofinis and Miller, 1983; Houba , 1988).

Parasites are not refrozen once thawed as this results in a significant loss of infectivity (Strom *et al.*, 1977). Therefore, during storage, the frozen samples are not exposed to ambient temperatures. Any elevation in temperature results in a substantial loss of viability (WHO, 1981; Houba ,1988).

3.2 Thawing of cryopreserved parasites

In our studies, cryopreserved parasites were thawed by adding sodium chloride solutions to the parasites. These solutions are added to prevent the cytotoxic damage by glycerol when the parasites are being thawed (Houba 1988).

B) DISCUSSION OF METHODS USED TO EVALUATE THE SENSITIVITY OF THE PARASITES TO THE CHELATORS.

1 The use of [^{3}H] hypoxanthine incorporation as an index of growth

In our studies, the growth of the parasites was assesed using [^{3}H] hypoxanthine (Amersham) as an index of growth. Using [^{3}H] hypoxanthine, it is possible to evaluate the chelators directly against the sensitivity of the parasite. This system offers the potential to detect a variety of antimalarials/iron chelators which may not be discovered in an *in vivo* system because of excess toxicity or unfavourable pharmacokinetics.

This method using radiolabelled hypoxanthine is quick, accurate and reliable for determining drug effects. It replaces the method of visual counting of parasites on thin smears which is tedious, time consuming and subjected to considerable error. Furthermore, the viability of the parasites cannot be assessed using light microscopy and the parasites counted on the thin blood smears may be metabolically dead. The uptake of labelled hypoxanthine is an indication of the viability of the parasites (Geary *et al.*, 1983).

Radiolabelled hypoxanthine is used as hypoxanthine is the

major purine base used by the parasite for the synthesis of adenosine and guanosine nucleotides and nucleic acids. Hypoxanthine is capable of crossing the malaria parasite membrane. It is incorporated into both ribonucleic and deoxyribonucleic acid and thus provides an index of the parasite metabolism. Thus this purine precursor has a crucial role to play in the metabolism of the malaria parasite (Manadhar and Van Dyke, 1975).

In our studies, the method using [^{3}H] hypoxanthine was based on the semiautomated microdilution technique described by Desjardins *et al.* (1979). The parasitaemia was determined by [^{3}H] hypoxanthine incorporation. The sensitivity of the parasites to a certain chelator was determined as a percentage of the hypoxanthine incorporation by the control parasites.

2 Basic requirements for the preliminary and final experiments

2.1 Medium used to dissolve the chelators

The 6 chelators were readily soluble in culture medium. The incomplete medium was used to prepare the stock and diluted solutions of the chelators to avoid any incompatibilities with the parasites. The culture medium

used for the experiments does not contain the "cold" hypoxanthine which was routinely added to it for culturing the parasites (refer to section 2.1 on Cultivation methods). The omission of the "cold" hypoxanthine from the medium eliminated the competition between the "cold" and labelled hypoxanthine as the parasites are unable to distinguish between the two and will thus use either one. Addition of purines e.g. hypoxanthine to the culture ium is shown to decrease the uptake of labelled hypoxanthine by the parasites, thus decreasing the parasite counts by the scintillation counter (Chulay *et al.*, 1983).

2.2 Chelator sensitivity tests (use of microtiter plate)

In our studies, chelator sensitivity tests were performed in 96 well microtiter plates. In each plate the sensitivity of one strain was evaluated against 20 different concentrations of a specific chelator. In this manner, extremely low and high concentrations of the chelator were tested. These concentrations would otherwise be totally unsuitable for *in vivo* studies. The low concentrations may not be sufficient enough to be effective in an animal model and the high concentrations would probably be to toxic to the animal.

In the 96 well plates, microlitres of the chelator solutions were used. Therefore smaller quantities of the chelators were required, which was economically advantageous.

2.3 Parasites

2.3.1 Synchrorization of parasites

Previous studies have reported that the mature stages of the parasite are susceptible to the effects of desferrioxamine (Raventos-Suarez *et al.*, 1982; Fritsch and Jung, 1986). To isolate the immature stages the parasites were synchronized using the method described by Lambros and Vanderberg (1979). Using this method, the aqueous sorbitol solution lysed the membranes of the mature stages i.e. the trophozoites and schizonts, thus isolating the young rings. These rings were left for 15 to 17 hours to mature to the mid and late trophozoite stages which are suitable for the chelator studies.

2.3.2 Preparation of the parasite suspension (dilution of stock cultures)

The parasite suspension which was added to the predosed 96 well microtiter plate was prepared by diluting the

stock cultures appropriately with erythrocytes and complete medium . In the chelator sensitivity studies the initial parasitaemia required was approximately 0.3%. The low parasitaemia is crucial as there is a reduced rate of [^{3}H] hypoxanthine incorporation by parasites that are cultured at a high initial parasitaemia. The accumulation of acidic metabolites i . the medium at high parasitaemias reduces the incorporation of the labelled hypoxanthine by the parasites (pH less than 7.3). At high parasitaemias the incorporation of [^{3}H] hypoxanthine is rate limiting, hence drug studies cannot be performed (Chulay *et al.*, 1903).

In our experiments, the haematocrit of the parasitized suspension was maintained at 1 %. It is important that the haematocrit is maintained at a low percentage. Non parasitized erythrocytes have a high concentration of many purines, thus there is reduced labelled hypoxanthine incorporation by the non parasitized erythrocytes. Therefore there is reduced hypoxanthine incorporation by the parasites in the presence of a large number of uninfected erythrocytes (Chulay *et al.*, 1983) .

2.4 The role of the erythrocyte control

In the experiments, each microtiter plate contained wells representing the erythrocyte control wells. The uptake of the labelled hypoxanthine by the non parasitized erythrocyte suspension represents the background ^{3}H hypoxanthine incorporation. When calculating the final parasiteamias, the DPM values (disintegration per minute) obtained for wells containing the parasitized suspension represents the DPM values of the parasitized and non parasitized erythrocytes found within the suspension. The DPM values of the non parasitized suspension is subtracted from the DPM values of the parasitized suspension. The final counts therefore only represents those of the parasitized erythrocytes.

2.5 Use of the humidified airtight box

In the experiments, the 96 well plates were placed in a humidified airtight box which was incubated at 37°C. The moisture was retained in the box thus preventing the contents of the plate evaporating. It was also essential that the box was kept airtight to maintain the specific gas atmosphere required by the parasites to proliferate successfully.

2.6 Period of exposure of the parasites to the iron chelators

In the experiments, the parasites were exposed for 42 hours to a specific chelator or combination of chelators. The parasites are exposed initially to the chelators alone for 24 hours and than an additional 18 hours to the chelators and isotope . Thus the parasites were harvested prior to schizogony and the invasion of erythrocytes by the new generation of merozoites . This prevented loss of radioactivity.

3 Concentrations of the chelators selected

3.1 Dose response curves of the 6 chelators

The concentrations of the chelators were selected depending on the results obtained in our preliminary studies and those reported by other similar studies.

In our experiments, the concentration range studied for both DFO and DFT was between 1 and 30 uM. Raventos-Suarez *et al.*, (1982) reported that 15 uM DFO completely inhibited the *in vitro* growth of the parasites. Fritsch *et al.*, (1987) concluded that 25-30 uM DFT completely inhibited the growth of the parasites

in vitro. The results of our preliminary studies suggested that 25uM DFO and DFT completely inhibited the *in vitro* growth of the parasites. Therefore lower concentrations were effective at inhibiting the growth of the parasites in a dose dependent manner and higher concentrations would completely inhibit the growth .

In our preliminary studies of 2.2'- bipyridyl, concentrations lower than 10 uM had no effect on the growth of the parasites. The highest concentration, 25 uM inhibited the growth but not completely. Therefore concentrations higher than 25 uM were required. The concentration range selected was between 2 and 40 uM.

The concentration range selected for those chelators which had a higher affinity for iron namely, DFO, DFT and BIP were similar.

In the preliminary studies 25 uM EDTA, EGTA and DTPA had no effect on the growth of the parasites. It is assumed that because these chelators have a very low affinity for iron, higher concentrations are required to inhibit the growth of the parasites. The concentration range selected for both EDTA and EGTA was between 20 and 1000 uM.

DTPA has a higher affinity for iron compared to EDTA and EGTA . DTPA binds to iron with a stronger dissociation constant (10^{28}) compared to EDTA (10^{25}) (Hershko and Weatherall, 1988). Therefore lower concentrations of DTPA are selected compared to EDTA and EGTA. The highest concentration selected for DTPA was 240 uM.

3.2 Combination of a chelator with an unsaturated chelator

From our results , we observed that chelators which have a high affinity and selectivity for iron, i.e. DFO, DFT and 2,2'- bipyridyl inhibited the growth of the parasites at relatively low concentrations compared to EDTA, EGTA and DTPA. It is assumed that because low concentrations of these iron specific chelators were required, their mode of inhibition was directly related to the parasite i.e. (the possible site of action was probably on the parasite) and to iron.

To elucidate possible mechanisms of inhibition of these chelators the combined effect of two iron specific chelators was studied. The two chelators selected to be combined were DFO and 2.2'- bipyridyl. From the results obtained both DFO and 2,2'- bipyridyl independently inhibited the growth of the parasites. However their

possible modes and sites of action differed. DFO has a high affinity for ferric ions (Hershko and Weatherall, 1988). Baker *et al.*, (1985) used hepatocytes as a model to describe the possible sites of action of DFO. DFO probably binds transferrin bound iron in the extracellular fluid, it chelates the iron which was being transferred between transferrin and the "iron acceptor" molecules in the plasma membrane and it chelates intracellular iron.

From our observations, both DFO and DFT had similar effects on the growth of the parasites. DFO was selected as DFT is an experimental chelator and information relating to its characteristics is not readily available. DFO on the other hand is clinically used as an iron chelator and is readily available.

2,2'- bipyridyl has a high affinity for ferrous ions. It prevents the transfer of iron from the transferrin molecule to the "iron acceptor" molecules in the plasma membrane by chelating the iron . Therefore it inhibits the intracellular uptake of iron (Nunez *et al.*, 1983).

If both chelators inhibited the growth of the parasites by iron chelation/deprivation, the combined effect of the two is expected to be synergistic or additive. However

our results proved that when combining the two chelators, 2,2'-bipyridyl antagonised the antimalarial action of DFO. To prove that 2,2'- bipyridyl, antagonised the antimalarial action DFO, the iron chelating effect of the former was eliminated by saturating it with ferrous ammonium sulphate.

3.3 Combination of a chelator with an iron saturated chelator

3.3.1 The spectroscopic titration of 2,2'- bipyridyl and ferrous ammonium sulphate

A titration was performed to determine the molar ratio at which ferrous iron saturated 2,2'-bipyridyl completely to form a complex. The absorbance was measured at 520 nm as the ferrous saturated 2,2'-bipyridyl complex absorbed maximally at this wavelength.

3.3.2 The combination of desferrioxamine and iron saturated 2,2'-bipyridyl

In the experiments, the concentrations of ferrous ammonium sulphate selected were low as large quantities of ferrous ammonium sulphate added to the incomplete

medium causes precipitation of the iron found in the medium. This renders the iron solution unsuitable for use as it is not clear. However lower concentrations dissolve readily in incomplete medium forming clear solutions.

In this investigation 6 uM DFO was combined with various concentrations of unsaturated and iron saturated 2,2'-bipyridyl. 6 uM DFO was selected as it is approximately the IC_{50} value obtained in this study for the strain being used (FCR-3).

3.3.3 The spectroscopic titration of desferrioxamine and 2,2'-bipyridyl

To exclude the posssibility of a direct chemical interaction of DFO with 2,2'-bipyridyl, which resulted in the antagonistic effect, changes in the absorbance spectrum of 2,2'-bipyridyl were measured after the addition of DFO.

SECTION 4.2 DISCUSSION OF RESULTS

1 Discussion of the dose-response curves of the 6 chelators

1.1 Desferrioxamine

Desferrioxamine has the desirable properties of a remarkably high affinity for ferric iron (Ka = 10^{31}) coupled with a low affinity for calcium (Ka = 10^{2}) (Klaassen CC, 1985). Baker *et al.* (1985) used hepatocytes as a model to suggest the possible sites of action of desferrioxamine as described in section 4.1.A Desferrioxamine does not remove iron from haemoglobin (Hershko and Weatherall, 1988; Porter et al., 1989; Klaassen CC, 1985).

From the results obtained, DFO inhibited the growth of the FCR-3 strain at concentrations of 1 uM and above. DFO inhibited the growth of the RSA-2 strain at concentrations of 4 uM and above. The difference in sensitivity between the two strains was also observed in the IC_{50} values obtained. The IC_{50} values determined for the FCR-3 and RSA-2 strains were 4,25 $\pm$ 0.12 uM and 5,66 $\pm$ 0.07 uM respectively.

The difference in the sensitivities of the two strains cannot be considered significant as it was only observed at concentrations below 5 uM. At concentrations higher than 5 uM, the response of the two strains appeared similar.

Other studies have also reported the inhibitory effect of DFO on the *in vitro* growth of *P.falciparum*. Raventos-Suarez *et al* . (1982) reported that 15 uM DFO completely inhibits the *in vitro* growth of *P. falciparum*. The inhibition is caused by iron chelation as iron saturated DFO has no effect on the growth of the parasites. Peto and Thompson (1986) concluded that DFO inhibits the growth of the parasite without interfering with the supply of iron from the incubation medium. This implies that the parasite is independent of the iron in the extracellular fluid. DFO is also reported to inhibit liver schizogony *in vitro* in human and rodent malaria (Stahel *et al.*, 1988).

In vivo studies have also reported the inhibitory effect of DFO. Pollack *et al.* (1987) reported that constant blood levels of DFO supressed *P. falciparum* in Aotus monkeys. Similarly, in mice infected with *Plasmodium vinckei*, constant blood levels of DFO suppress the

parasites (Fritsch *et al.*, 1985). DFO also suppresses parasite proliferation in rats infected with *Plasmodium berghei* (Hershko and Peto, 1988).

The results obtained from our studies and those reported by other studies indicated that DFO was effective at inhibiting the growth of the parasites *in vitro* and *in vivo*. The mechanism of inhibition remains undefined.

Peto and Thompson, (1986) concluded that it is the chelation of the intracellular iron that inhibits the growth of the parasites as medium supplementated with iron deficient serum results in the same growth rate as supplementation with normal serum. It is possible that the chelator crosses the parasite membrane and chelates with the intracellular iron. Fritsch and Jung (1986) reported the uptake of ^{14}C-Desferrioxamine B by *P. falciparum* infected erythrocytes. DFO B probably blocks the iron dependent enzyme, ribonucleotide reductase thus inhibiting DNA synthesis. The inhibition is most pronounced during the mid to late trophozoite stage when the parasites showed high metabolic activity especially in DNA synthesis (Fritsch and Jung, 1986; Raventos-Suarez *et al.*, 1982) .

The pool/nature of intracellular iron that is chelated by DFO remains undefined. DFO cannot remove iron from haemoglobin (Hershko and Weatherall, 1988). Furthermore the parasite cannot use haemoglobin iron as free haem is toxic to the parasites (Orijh *et al.*, 1977) and the erythrocytic haem is not measurably changed in the later stages-i.e. (trophozoites and schizonts) of the parasite when compared to the early stages i.e. rings (Ball *et al.*, 1948).

Perhaps the mode of inhibition is not related to iron deprivation alone. Klebanoff *et al.* (1989) reported the destructive action of DFO. DFO enhances the autooxidation of ferrous ions by chelation of ferric ions. In this manner DFO and ferrous ions acts as an oxygen radical generating system which contributes to the inhibitory action of DFO. This effect is most pronounced at the pH of 5,5 . It is possible that DFO inhibits the growth of the parasite using the same mechanism as the pH of the parasite food vacuole is reported to have a similar pH (Ginsburg, 1988).

Therefore DFO possibly inhibits the proliferation of the parasite by iron deprivation and by promoting the formation of destructive oxygen radicals.

1.2 Desferrithiocin

Desferrithiocin is an experimental drug . It inhibits the proliferation of an *in vitro P. falciparum* culture in a dose dependent manner, similar to DFO (Fritsch *et al* ., 1987). When DFT was tested in mice and dogs it was shown to be acutely toxic, thus this chelator is no longer under investigation (Porter J, 1989).

From our results obtained, DFT inhibited the growth of the FCR-3 strain at concentrations of 1 uM and higher. The IC_{50} value obtained for this strain was 2,4 ± 0.12 uM. DFT inhibited the growth of the RSA-2 strain at concentrations of 6 uM and higher. The IC_{50} value obtained for this strain was 7,03 ± 0.29 uM.

From these observations it appears that the sensitivity of the two strains to DFT differed considerably. FCR-3 appears to be significantly more sensitive to the effects of DFT. This is especially seen in the IC_{50} values obtained for each of the strains. The increased sensitivity could be due to the characteristics of the strain itself e.g. the mechanism of iron utilization by certain strains of parasites.

The inhibitory effects of DFT has also been reported by other studies. Fritsch *et al.* (1987) reported that 25-30 uM DFT prevented parasite multiplication *in vitro* . Stahel *et al.* (1989) reported that DFT inhibited liver schizogony of human and rodent malaria *in vitro*. What is the mechanism of inhibition of parasite proliferation caused by DFT ?

Being an experimental drug, information relating to the characteristics of DFT is not readily available. It binds to ferric iron with the same binding constant as DFO (Peter,1985). It can therefore be assumed, that like DFO, it probably inhibits parasite growth by iron chelation especially since the iron saturated DFT had no effect on the growth of the parasites (Fritsch *et al.*,1987).

Besides iron chelation, DFT probably inhibits the growth of the parasite by means of another mechanism. Rice-Evans *et al.* (1988) reported that DFT chelates the iron within normal erythrocytes forming a chelator-iron complex. This complex induces haemoglobin oxidation and disrupts the structural integrity of the erythrocytic membrane. It is therefore assumed that by means of the same mechanism, DFT destroys the parasite, since there is a progressive increase in redox-active iron during the

develoment of *P. falciparum* within the parasitized erythrocytes (Golenser *et al.*, personal communication). Therefore the mature stages of the parasites contains increased amounts of this redox-active iron, which probably increases the susceptibility of these stages to the effects of DFT.

1.3 2,2'- Bipyridyl

This chelator distributes in the membrane apolar phase. It prevents the transfer of iron from transferrin to the plasma membrane proteins by binding with the iron. Therefore it prevents the iron entering the intracellular compartment. It has a higher affinity for ferrous iron (Nunez *et al.*, 1983).

From our results, 2,2'- bipyridyl inhibited the growth of the FCR-3 strain at concentrations of 10 uM and higher. The IC_{50} value obtained for this strain was 19,98 ± 0.68 uM. 2,2'- Bipyridyl inhibited the growth of the RSA-2 strain at concentrations of 10 uM and higher. The IC_{50} value obtained for this strain was 14,35 ± 0.25 uM.

Although the RSA-2 strain appears to be more sensitive to the effects of 2,2'- bipyridyl, the difference cannot be considered significant. The slight difference in sensitivity could be related to the technical factors

such as the quality of the erythrocytes and serum used. The erythrocytes and serum are the only two components that may differ when the sensitivities of the two strains are being evaluated against the same chelator at the same time. The erythrocytes of certain donors are not effective at supporting the growth of the parasites. The serum of certain donors may contain anti-bodies or other inhibitory factors. The erythrocytes and serum are collected from various donors therefore these factors may influence the resultant sensitivity of a certain strain of parasites to a chelator.

Higher concentrations of this chelator were required to inhibit the growth of both strains of the parasites compared to DFO and DFT. It is possible that higher concentrations are required due to the site and mode of action of 2,2'- bipyridyl. 2,2'-Bipyridyl distributes in the lipid phase i.e. (the membrane apolar phase), but becomes water soluble when bound to iron . Once water soluble, it binds rapidly to available iron and prevents its uptake. This change in solubility once bound to iron explains the rapid onset of inhibition of iron uptake only after a period of time. This possibly explains the inhibition of the parasite growth only after a period of time i.e. concentrations between 0

and 10 uM did not affect the growth of the parasites. Concentrations higher than 10 uM, inhibits the growth of the parasite probably because the chelator becomes water soluble, inhibits the uptake of iron into the cell and is therefore effective at higher concentrations only (Nunez *et al.*,1983).

The above mechanism of action of 2,2'-bipyridyl was suggested using reticulocytes as a model, as these have transferrin receptors (Nunez *et al.*,1983). Whether this chelator inhibits the growth of the malaria parasite by preventing the intracellular uptake of iron using this mechanism remains unclear. This mode of inhibition of iron uptake is only possible if there is a transferrin receptor on the membrane of the malaria parasite. The presence of a transferrin receptor on the membrane of the malaria parasite has not been confirmed.

1.4 EDTA

EDTA is a synthetic chelator. It combines with ferric iron with a stability constant of 10^{25} which is low compared to DFO (10^{31}). EDTA has a low selectivity for ferric iron and a relatively high affinity for divalent cations such as Ca^{2+}, Mg^{2+}, and Zn^{2+} (Hershko and Weatherall, 1988). EDTA is

hydrophillic, acts in the extracellular fluid and presumably cannot penetrate cells (Baker *et al.*,1985). It has been used for many years as an industrial and analytical reagent because it chelates divalent metal ions (Klaassen , 1985).

We observed that concentrations lower than 170 uM had no effect on the growth of the FCR-3 strain. The IC_{50} value obtained for this strain was 262,56 ± 6,65 uM. Concentrations lower than 100 uM did not affect the growth of the RSA-2 strain. The IC_{50} value obtained for this strain was 294,63 ± 0.11 uM.

The sensitivities of both strains to EDTA appears to be similar. The differences observed in the IC_{50} values cannot be considered significant. The concentrations of EDTA required to inhibit the growth of both strains are extremely high compared to those of DFO, DFT and 2,2'-bipyridyl. This is especially evident in the IC_{50} values obtained for these chelators with respect to both strains.

Higher concentrations of EDTA are probably required due to the site and mode of action of the chelator. It is assumed that EDTA chelates all the available divalent

cations in the extracellular medium/fluid, thus depriving the parasites of essential nutrients. At low concentrations of EDTA, the growth of the parasite is not affected. At these concentrations, EDTA is not present in sufficient quantities to bind the abundant divalent cations present in the extracellular medium. However at higher concentrations there is sufficient EDTA available to bind most of the divalent cations in the medium thus causing a deficiency in essential nutrients which affects the normal proliferation of the parasites.

1.5 EGTA

EGTA, like EDTA is hydrophillic and acts in the extracellular fluid. It has a high affinity for calcium and a low affinity for iron (Baker *et al.*,1985).

From the results obtained, concentrations lower than 200 uM had no effect on the growth of the FCR-3 strain. The IC_{50} value obtained for this strain was 296,76 ± 5.26 uM. Concentrations lower than 100 uM did not affect the growth of the RSA-2 strain. The IC_{50} value obtained for this strain was 224,07 ± 8,7 uM. The RSA-2 strain appeared to be more sensitive to the effects of EGTA. This difference in sensitivity could be attributed to the previously mentioned technical factors namely, the

quality of the erythrocytes and serum used. The sensitivity of both strains to EDTA and EGTA is similar. This was evident in the IC_{50} values obtained for both chelators after evaluation against both strains which do not differ considerably from each other.

It is assumed that because EDTA and EGTA have similar site and mode of action, both chelators have the same effect on the proliferation of the parasites.

1.6 DTPA

Animal studies have shown that the spectrum of clinical effectiveness of DTPA is similar to that of EDTA (Klaassen , 1985).

The concentration range selected was between 0 and 240 uM. These concentrations did not have any effect on the growth of the parasites. The dose response curve obtained for both strains was a straight line which indicated constant parasitaemias were obtained at all the concentrations selected.

DTPA and EDTA have the same characteristics i.e. hydrophillic, acts in the extracellular fluid, cannot penetrate cells and a low affinity for ferric iron (Baker

et al., 1985). It is possible that due to their similar characteristics, DTPA has the same effect as EDTA on the growth of the parasites. This is observed in the following comparisons. At 100 uM of EDTA and DTPA the parasitaemias obtained for the FCR-3 strain was 86% and 91% respectively. The parasitaemias observed at 120 uM EDTA and DTPA were 88% and 90% respectively. These values are percentages of the control parasitaemia.

Therefore DTPA, EDTA and EGTA probably have similar effects on the growth of the parasites due to their common characteristics.

It appears that those chelators with a high affinity and selectivity for iron inhibited the growth of both strains of parasites at relatively low concentrations. These chelators were DFO, DFT and 2,2'- Bipyridyl. Chelators with a low affinity for iron and a strong affinity for metals such as calcium, magnesium and zinc required higher concentrations to inhibit the growth of the parasites. These chelators were EDTA, EGTA and DTPA.

2 Combination of a chelator with an unsaturated chelator

The chelators of interest would therefore be DFO, DFT and 2,2'- bipyridyl as these inhibited the growth of the

parasites at low concentrations. However the mechanism of inhibition caused by these chelators remain unclear.

To elucidate possible mechanisms of inhibition of these chelators, the combined effect of 2,2'-bipyridyl and desferrioxamine was studied. These chelators were selected as they differed in their sites and modes of action although independently they both inhibited the growth of the parasites. Baker *et al.* (1985) and Nunez *et al.* (1983) described the possible sites and modes of action of DFO and 2,2'- bipyridyl respectively.

If both these chelators inhibited the growth of the parasites by chelation of iron alone, the combined effect of the chelators is expected to be synergistic or additive.

2.1 Dose response curves of desferrioxamine and 2,2'-bipyridyl

The IC_{50} values obtained when evaluating the sensitivity of the FCR-3 strain to DFO and 2,2'- bipyridyl compared favourably to the values obtained in our earlier studies using this strain.

2.2 Combination of desferrioxamine and 2,2'-bipyridyl

The combined effect of the two chelators resulted in an antagonistic effect on the growth of the parasites. 2,2'- bipyridyl at various concentrations when combined with one fixed concentration of DFO , showed a relative increase in parasitaemia compared to DFO or 2,2'-bipyridyl alone, thus implying that these concentrations of 2,2'-bipyridyl antagonised the antimalarial action of DFO. This antagonistic effect was less pronounced when higher concentrations of DFO were combined with the same concentrations of 2,2'-bipyridyl.

This effect is explained in the following example.
The combined effect of 2 uM DFO and various concentrations of 2,2'- bipyridyl: The parasitaemia observed at 2 uM DFO is equivalent to 80% of the control parasitaemia. However when 6 , 8 , 10 uM 2,2'- bipyridyl are combined with 2 uM DFO , the resultant parasitaemias increased from 80 % to approximately 95% of the control parasitaemia. Therefore instead of decreasing below 80%, the parasitaemias increased .

3 Combination of a chelator with an iron saturated chelator

To prove that 2,2'-bipyridyl, antagonised the antimalarial effect of DFO, its iron chelating effect was eliminated by saturating it with equimolar concentrations of ferrous ammonium sulphate.

3.1 The spectroscopic titration of 2,2'-bipyridyl and ferrous ammonium sulphate

A spectroscopic titration proved that ferrous iron, saturated 2,2'- bipyridyl in equimolar concentrations.

3.2 Combination of desferrioxamine and iron saturated 2,2'-bipyridyl

2,2'- Bipyridyl when combined with 6 uM DFO antagonised the antimalarial effect of DFO as the DPM values (parasitaemias) increased relative to the effect of 6 uM DFO or similar concentrations of 2,2 '-bipyridyl alone. This is in accordance with our ealier findings.

However, when 6 uM DFO was combined with various concentrations of ferrous saturated 2,2'- bipyridyl the resultant dose response curve was a straight line meaning

that the slope was not significantly different from 0. The straight line implied that constant parasitaemias were obtained and reflected the effect of 6 uM DFO alone.

The following assumptions were made to describe the possible mechanisms by which 2,2'- bipyridyl antagonised the antimalarial effect of DFO.

Because the site of action of 2,2'-bipyridyl is within the cell membrane, it is assumed that it perhaps blocks the passage of DFO across the parasite membrane thus antagonising the effect of DFO . The DPM values (parasitaemias) increases as only the 2,2'- bipyridyl is now effective at preventing iron uptake and inhibiting parasite multiplication. At low concentrations 2,2'- bipyridyl is less effective than DFO at similar concentrations at inhibiting the growth of the parasites (refer to dose respon.e curves of both chelators) . Concentrations below 10 uM 2,2'- bipyridyl has no effect on the growth of the parasites and similar concentrations of DFO are effective at inhibiting parasite proliferation.

The next assumption, explaining the antagonistic effect caused by 2,2'- bipyridyl is based on the redox shunting of iron. Iron is in a continuous redox cycle, thus it

continuously undergoes oxidation and reduction forming ferric (Fe^{3+}) and ferrous (Fe^{2+}) ions (Klebanoff *et al.*,1985). Therefore, if the ferric ions are chelated after DFO is added, ferrous ions will not be generated and are thus not available for chelation by 2,2'-bipyridyl. Therefore the two chelators cannot combine additively or synergistically.

3.3 The spectroscopic titration of desferrioxamine and 2,2'-bipyridyl

To exclude the possibility of a direct chemical interaction of DFO with 2,2'-bipyridyl, changes in the absorbance spectrum of 2,2'-bipyridyl were measured after the addition of DFO. The ultraviolet spectrum of 2,2'-bipyridyl remained constant after DFO was added. Therefore desferrioxamine and 2,2'-bipyridyl do not interact chemically.

Both DFO and BIP were independently shown to inhibit the growth of the parasites . If the inhibition was due to iron deprivation alone, the combined effect of the two chelators would either be synergistic or additive. The fact that an antagonistic effect is observed, implies that other mechanisms of inhibition besides iron deprivation inhibits the growth of the parasites.

Previous studies using various iron chelators suggests other possible mechanisms of inhibition besides iron deprivation.

Iheanacho *et al.* (1990), reported that the combination of DFO and L2-9, a synthetic iron chelator resulted in an antagonistic effect. It is suggested that free radicals are generated by the internal redox reaction of the previously formed L2-9 and iron complex, during a Fe^{2+} to Fe^{3+} transition. These free radicals destroy the integrity of the parasite membrane.

Klebanoff *et al.* (1989) concludes that DFO promotes the autooxidation of ferrous ions at pH of 5 which generates oxygen derived free radicals. The possibility that DFO inhibits the growth of the parasite using this mechanism is considered a possibility because the pH of the malaria parasite food vacuole is approximately 5 (Ginsburg, 1988). Furthermore since radio-labelled DFO has been reported to accumulate in higher concentrations in parasitized erythrocytes compared to normal erythrocytes, this mechanism is a possibility (Fritsch and Jung, 1986).

At this stage the mechanism of inhibition by chelators is not clearly defined. However these findings suggest prospects for future research . In the future if an iron

chelator which is orally active, economical, free of toxic side effects and has a long plasma half life becomes available, it should be evaluated against the sensitivity of the malaria parasite. Iron chelators may form the basis of a prospective group of antimalarials.

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