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AN INVESTIGATION OF P-GLYCOPROTEIN IN

PLASMODIUM FALCIPARUM AND THE

ISOLATION OF HAEMOZOIN

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A DISSERTATION SUBMITTED TO THE FACULTY OF MEDICINE
UNIVERSITY OF THE WITWATERSRAND,
JOHANNESBURG,
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ABSTRACT

Chloroquine-resistant *Plasmodium falciparum* accumulate significantly less chloroquine than susceptible parasites, and this is thought to be the basis of their resistance (Fitch, 1970). Martin *et al.* (1987), recently demonstrated that in the presence of verapamil, a calcium channel blocker, chloroquine-resistant *P. falciparum* becomes chloroquine-sensitive, with an increase in the chloroquine accumulation The mechanism of such reversal has yet to be elucidated. The aim of the research proposal, is firstly to investigate the antimalarial properties of B8509-035 (Byk-Gulden), a novel calcium channel blocker, and to investigate its efficacy as a chloroquine-reversing agent. The investigation was based on the uptake of [G-^{3}H]-hypoxanthine by the parasite during short-term culture in the presence of the drug. The results indicate a strong antimalarial effect of B8509-035 with an IC_{50} range of 0.77μM to 5.78μM. The studies involving combination of chloroquine and B8509-035 indicate a mild synergistic effect.

The chloroquine resistance phenotype in *P. falciparum* has been likened to a form of multidrug resistance (MDR) found in some mammalian tumour cell lines. The concept of MDR in cancer cells involves an efflux pump which

mediates the extrusion of a variety of stucturally and functionally unrelated drugs. This efflux pump is termed P-glycoprotein which has been clearly implicated in MDR. It has been proposed that the expression of *pfmdr*1 gene of *P. falciparum* is linked to chloroquine resistance (Foote *et al.*, 1989, 1990). In this study murine monoclonal antibodies (C219 and JSB-1) specific for P-glycoprotein were utilized to analyze the protein product of the *pfmdr* 1 gene and whether this gene expresses P-glycoprotein as known in cancer lines.

The results indicate the presence of P-glycoprotein on both chloroquine sensitive and -resistant strains, which was irrespective of the level of resistance. We therefore concluded that the presence of P-glycoprotein does not confer reversal of resistance. This glycoprotein may have another function, like that of removal of toxins from the parasite.

The third part of the proposal describes a new method of haemozoin isolation. While isolating parasitic membrane proteins for the P-glycoprotein investigation, we realized, that haemozoin could also be isolated. Haemozoin (malaria pigment) is the brown-black product of haemoglobin degradation that appears in increasing amounts in the intra-erythrocytic growth stages of members of the genus Plasmodium. A continuing question has been: How is haemozoin formed and how does chloroquine interfere with its formation?
Due to the conflicting reports on the nature of malarial pigment it seemed worthwhile to reinvestigate the characteristics and composition of haemozoin

derived from the human malarial parasite *P. falciparum*. I sucessfully isolated haemozoin, and subjected it to SDS-PAGE, from which we concluded that protein does not form an integral part of the structure of haemozoin, as previously believed, but that it perhaps binds to haemozoin nonspecifically due to the charge on the haemozoin molecule.

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.



8th day of September, 1992

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PAPERS PRESENTED AT CONGRESSES

Parts of this dissertation have been presented at local and international conferences:

Hempelmann E., Bischof D., De Almeida M., Monteagudo F.S.E.

Haemoglobin degradation *in vitro* by *Plasmodium falciparum*

17th International Congress of Chemotherapy, June 23-28, 1991, Berlin

De Almeida M., Hempelmann E., Havlik I., Monteagudo F.S.E.

Membrane protein profile of *Plasmodium falciparum* using SDS Gel-Electrophoresis

Annual Congress of the South African Pharmacological Society, October 7-10, 1991, San Lameer

Field S., Papadellis K., Hempelmann E., De Almeida M.

Blood group polymorphism in Southern Africa and innate resistance to *Plasmodium falciparum*

30 Jahre Biochemie Studium in Tuebingen, July 1-3, 1992, Tuebingen

Havlik I., Hempelmann E., De Almeida M., Botha G., Butkow N.

The effect of a novel calcium channel blocker, B8509-035, in inhibiting the *in vitro* growth of *Plasmodium falciparum*

Vth World Conference on Clinical Pharmacology and Therapeutics, July 26-31, 1992, Yokohoma

De Almeida M., Hempelmann E., Havlik I.

Immunostaining of *Plasmodium falciparum* for P-glycoprotein

Annual Congress of the South African Pharmacological Society, September 14-16, 1992, Bloemfontein

PUBLICATION

Hempelmann E., De Almeida M., Havlik I.

A simple method for the isolation of pigment (submitted for publication)

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LIST OF ABBREVIATIONS

DTT . . . . . Dithiothreitol (Cleland's Reagent)

EDTA . . . . . ethylenediaminetetraacetic acid disodium salt

g . . . . . weight in grams

g . . . . . centrifugation in gravity

H_2O_2 . . . . . Hydrogen peroxide

HEPES . . . . . 4-(2-hydroxyethyl)-1-piperazinethane-

. . . . . sulphoric acid

IC_{50} . . . . . drug concentration resulting in 50%

. . . . . inhibition of parasite growth

ℓ . . . . . litre

μg . . . . . microgram

ml . . . . . millilitre

mM . . . . . millimoles

μM . . . . . micromole

MTT . . . . . 3-(4,5 dimethylthiazol-2-yl)-2,5-

. . . . . diphenyltetrazolium bromide

NADP . . . . . nicotamide adenine dinucleotide

NADPH . . . . . nicotamide adenine dinucleotide, reduced

% . . . . . percent

PBS . . . . . phosphate buffer saline

PMS . . . . . phenazine methosulphate

RPMI . . . . . Roswell Park Memorial Institute

SDS . . . . . Sodium dodecyl sulphate

TEMED . . . . . NNN'N'-tetramethylenediamine

TRIS . . . . . hydrozymethylaminomethane

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INSTRUMENTS AND EQUIPMENT

Balance	Oertling
Centrifuge	Sorval Ecospin
Immunoblot apparatus	Bio-Rad Trans-blot
Incubator	Gallencamp
Microbalance	Mettler AE 240
Microcentrifuge	Beckman microfuge B
Microscope	Nikon
pH meter	Beckman 40 pH meter
Power pack	LKB 2197 power supply
Sterile filter pump	Millipore
UV/Visible Spectrophotometer	Philips PU8700
Microtititre plates	Sterilin

CHEMICALS

Acetic acid . . . . . Merck

Acetone . . . . . .Saarchem

Acrylamide . . . . . BDH

Ammonium acetate . . . . . Merck

Ammonium persulphate . . . . . Bio-rad

Aquagel . . . . . Chemlab

Bisacrylamide . . . . . BDH

C219 . . . . . Centocor

4-Chloro-1-Naphtol . . . . . Merck

Citric acid . . . . . Merck

Dextrose . . . . . Merck

Diaminobenzidine . . . . . .Sigma

DTT . . . . . Bio-Rad

EDTA . . . . . .Boehringer Mannheim

Formaldehyde . . . . . .Labchem

Gelatine . . . . . .Sigma

Gentamicin sulphate . . . . . .Sigma

Giemsa . . . . . BDH

Glucose . . . . . Merck

Glucose-6-phosphate . . . . . .Sigma

Glycerol . . . . . .Saarchem

Glycine . . . . . Bio-rad

HCl . . . . . Merck

Haematoxylin . . . . . Sigma

HEPES . . . . . Boehringer Mannheim

Horseradish peroxidase . . . . . Sigma

[^{3}H] hypoxanthine . . . . . Amersham, UK

Hypoxanthine . . . . . Sigma

JSB-I . . . . . Boerhinger Ingelheim

KCL . . . . . Saarchem

KH_2PO_4 . . . . . Saarchem

Methanol . . . . . Saarchem

$MgCl_2$ . . . . . Merck

MTT . . . . . Sigma

NaOH . . . . . Merck

$Na_2HPO_4.2H_2O$ . . . . . Merck

NADP . . . . . Sigma

NADPH . . . . . Boehringer Mannheim

$NaHCO_3$ . . . . . Saarchem

PAGE blue . . . . . BDH

Phenol red . . . . . Sigma

PMS . . . . . Sigma

RPMI 1640 . . . . . Highveld Biological

Saponin . . . . . Merck

SDS . . . . . Bio-Rad

D-sorbitol . . . . . Saarchem

TEMED . . . . . Bio-Rad

Trichloroacetic acid . . . . . Saarchem

Tris . . . . . Saarchem

Tween 20 . . . . . Merck

Urea . . . . . Isolab

CHAPTER ONE

1 INTRODUCTION

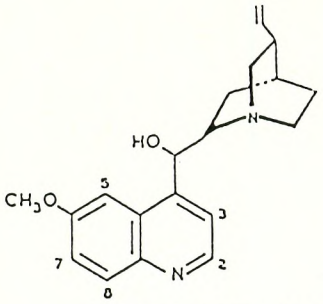
Today, of all the parasitic diseases that beset man in the warmer parts of the world, malaria is still the major cause of morbidity and mortality. The disease is spreading as current drugs are becoming less effective due to the wide-spread drug resistance.

Malaria treatment has one of its roots in ancient China. By 200BC the herbal remedy, qinghaosu was being used as a febrifuge (Peters, 1987), but remained unknown outside China until 1975. Another traditional plant product was found in South America in 1600. The Indians near Loxa, Peru, cured intermittent fever with powder from the bark of the *Quinquina* tree. It is told that an earthquake provoked great destruction at Loxa, where many cinchona trees fell into a small lake. The water polluted with the cinchona bark became very bitter and almost undrinkable. Nevertheless, an Indian with a violent fever quenched his thirst by drinking this water, and within a day or two he was completely cured (Wernsdorfer, 1988). The use of the "fever tree" bark was introduced into European medicine by Jesuit missionaries. The cinchona bark contains various alkaloids, including quinine, which was first isolated by Pelletier and Caventou in 1820 (Bruce-Chwatt, 1988). The compound was analysed in 1929 by P.Rabe in Germany and synthesized in 1944 by Woodward in the USA (Bruce-Chwatt, 1988).

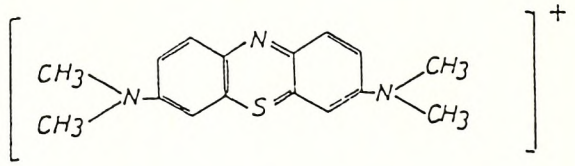
1.1 MODERN CHEMOTHERAPY

The first period of modern chemotherapy was influenced by the growing dye industry and their products. Chemotherapy as it is known today, started in 1891 with the first synthetic drug ever produced; methylene blue. From his studies P.Ehrlich had predicted that the antimalarial potency of methylene blue would be enhanced by the addition of a dicationic aliphatic chain (Guttmann and Ehrlich, 1891). Consequently, methylene blue was modified with a dicationic chain to produce Paludenblue which was found to increase its antimalarial activity, but the therapeutic usefulness was still poor compared to quinine. Nevertheless the importance of a quinoline ring in Quinine and of the dicationic chain in Paludenblue for their antimalarial action was recognised.

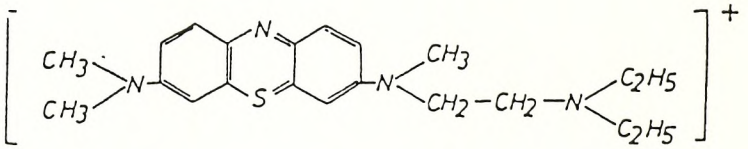
The first synthetic antimalarial, an 8-aminoquinoline, Plasmochin (Pamaquine) was synthesized at the end of 1924, by adding the side chain of Paludenblue to a quinoline ring (fig. 1). Andersag and his co-workers synthesized a 4-aminoquinoline drug by replacing the hydrogen of carbon-4 of quinoline with a diethylamino-1-methyl-butylamino group.. The above compound was named Resochin and later renamed chloroquine (Schulemann, 1932). The significance of chloroquine was at first not recognised and was only appreciated following screening of 16,000 compounds by the US Army in 1943, resulting in clinical application of chloroquine by 1947 (Loeb, 1946; Hahn, 1975).



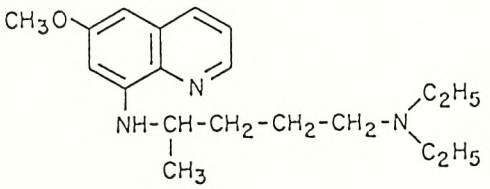
QUININE (1630)



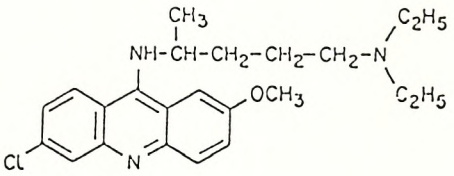
METHYLENBLUE (1891)



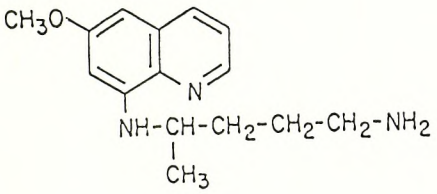
PALUDENBLUE



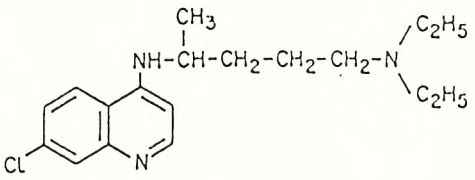
PAMAQUINE (1926)



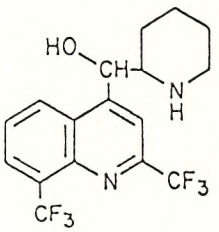
MEPACRINE (1935)



PRIMAQUINE (1951)



CHLOROQUINE (1945)



MEFLOQUINE (1972)

FIGURE 1: The development of Quinoline containing antimalarials

1.1.1 QUINOLINE CONTAINING DRUGS

Chloroquine is an amino derivative of quinine, both containing the quinoline group as the backbone. Quinoline containing drugs acting on *P. falciparum* can be divided into two groups based on their chemical structure (fig. 1) and their effects on the morphology of the parasite. The first group (4-aminoquinoline) is exemplified by chloroquine. This group consists of two highly electronegative (easily protonatable) nitrogen atoms. Below pH 5 chloroquine is doubly protonated, whereas quinine is less protonated and mefloquine singly protonated (Yuthavong *et al.*, 1985). The morphological effect caused by these antimalarials is a swelling and fusion of the adjacent digestive vacuoles, resulting in pigment clumping.

The second group (8-aminoquinoline) is exemplified by primaquine. These compounds have only one highly electronegative nitrogen atom. In this group, pigment clumping does not occur but the digestive vesicles may swell and the pigment within them becomes less electron dense. Pigment clumping produced by compounds in the first group will be competitively inhibited by these compounds, indicating that the same receptor site may be involved (Wernsdorfer and Trigg, 1988).

1.2 POSSIBLE MODE OF ACTION OF CHLOROQUINE

The mode of the antimalarial action of chloroquine is not yet understood, although it has been detailed in many textbooks and reviews since 1950 (Bowman and Band, 1980; Mahony and Eaton, 1981; Goodman and Gilman, 1991). The intraerythrocytic development of malaria parasites is characterized morphologically by three stages, and the susceptibility of the parasites to chloroquine at the different stages is still debated. Some modes of the antimalarial action of chloroquine seem to confirm the concept that chloroquine is a drug which inhibits the transition of trophozoites to schizonts. It was demonstrated that the trophozoite and schizont stages were considerably more sensitive to chloroquine than the ring stage (Yayon and Ginsburg, 1983). In contrast, experiments carried out by Zhang *et al.* (1986) showed that in *P. falciparum* cultures containing a therapeutic concentration of chloroquine (250nM), the development of trophozoites to schizonts, the reinvasion and the formation of new rings from schizonts was not inhibited. This report is in agreement with the results of others (Langreth *et al.*, 1978).

The effect of chloroquine on the parasite appears to be dependent on the concentrations of the drug within the parasite (Macomber *et al.*, 1966). It is possible that several mechanisms are operating at various times (Yuthavong *et al.*, 1985). The intercalation into DNA and inhibition of

nucleic acid synthesis occurs at a relative high concentration of 10^{-4} M (O'Brien *et al.*, 1966). The increase of the pH in the parasite's acid vesicle and inhibition of lysosomal functions, occurs at a chloroquine concentration of 10^{-6}M (Ginsburg and Geary, 1987). Binding to ferriprotoporphyrin IX to form a membrane toxic complex occurs at 10^{-7} M, and membrane destabilisation by interactions with phospholipids occurs at 10^{-7}M (Lullmann-Rauch, 1979).

1.2.1 FERRIPROTOPORPHYRIN IX HYPOTHESIS

Fitch and colleagues showed that chloroquine uptake is attributable to two processes: one is saturable with a high affinity and specificity for chloroquine whereas the other is not saturable and of low affinity to the drug (Fitch,1969). Normal uninfected erythrocytes are devoid of the high affinity sites, and such sites are significantly reduced in erythrocytes containing resistant parasites. Fitch and his colleagues have extended their hypothesis to suggest that the high affinity binding site for chloroquine is ferriprotoporphyrin IX (FP) and that the selective antimalarial action of chloroquine is due to the complexing of the drug with this product of haemoglobin degradation (Fitch, 1986). Erythrocytes infected with chloroquine-susceptible *P. falciparum* produce FP, and some of the FP is available to bind chloroquine with high affinity (Chou *et al.*, 1980).

1.2.1.1 CHLOROQUINE - FERRIPROTOPORPHYRIN COMPLEX

Fitch *et al.* (1982) and Chou and Fitch (1981) provided evidence that FP and a chloroquine-ferriprotoporphyrin (CQ-FP) complex both lyse isolated *P. falciparum* parasites. The amount of lysis was found to be dose dependent. Formation of a CQ-FP complex reduced, but did not eliminate the toxicity of FP. Since there is evidence indicating that a CQ-FP complex forms when CQ-sensitive parasites are exposed to CQ, Fitch *et al.* (1982) suggest that accumulation of this complex may account for the chemotherapeutic effect of CQ against *P. falciparum*.

Susceptibility to FP toxicity is a common characteristic of biological membranes. To avoid this toxicity, parasites which digest haemoglobin and produce FP must destroy or sequester it in a non-toxic form. Fitch and his colleagues propose that sequestration of FP in malarial pigment serves this function. If the selective antimalarial action of CQ is due to the formation of a CQ-FP complex, then failure to form the complex would cause chloroquine resistance. However, there are certain inconsistencies with this hypothesis. For example, it is not clear how FP is capable of binding large amounts of drug which are concentrated in the digestive vacuoles, as the existence of free FP is still unproven (Banyal and Fitch, 1982). It is most unlikely that there is any significant amount of free FP (chloroquine binds only to free FP but not to FP complexed in pigment (Zarchin *et al.*,1986)), for the following reasons:

FP is produced at a rate which would increase its vacuolar concentration by 280μM in 1 min. Yet much lower concentrations of FP are sufficient to lyse cellular membranes (Dutta and Fitch 1984; Fitch *et al.*, 1982). Thus FP must be rapidly and efficiently sequestered into the non-toxic pigment to avoid damage to the vacuolar membrane and the concentration of free FP should therefore be much lower than that needed to account for chloroquine accumulation by ligation to FP.

The concentration of chloroquine in the food vacuole can be 100μM, however the concentration of FP is likely to be much below the required level (Vander Jagt, *et al.*,1986), as one molecule of chloroquine can bind 2 or 3 molecules of ferriprotoporphyrin IX (Chou *et al.*, 1980). Furthermore, related drugs don't bind to ferriprotophorphyrin (Warhurst,1987; Geary *et al.*, 1987), and chloroquine analogues eg: aminodiaquine or quinacrine show competitive inhibitory effects on chloroquine binding to FP (Fitch, 1972). Ultrastructural studies of chloroquine - treated infected red blood cells did not reveal any damage to the food vacuole membrane (Yayon *et al.*, 1984) as would be expected from the known toxicity of CQ-FP complexes for membranes.

1.2.1.2 DRUG RESISTANCE

The ferriprotoprophyrin IX hypothesis attempts to explain drug resistance as follows:

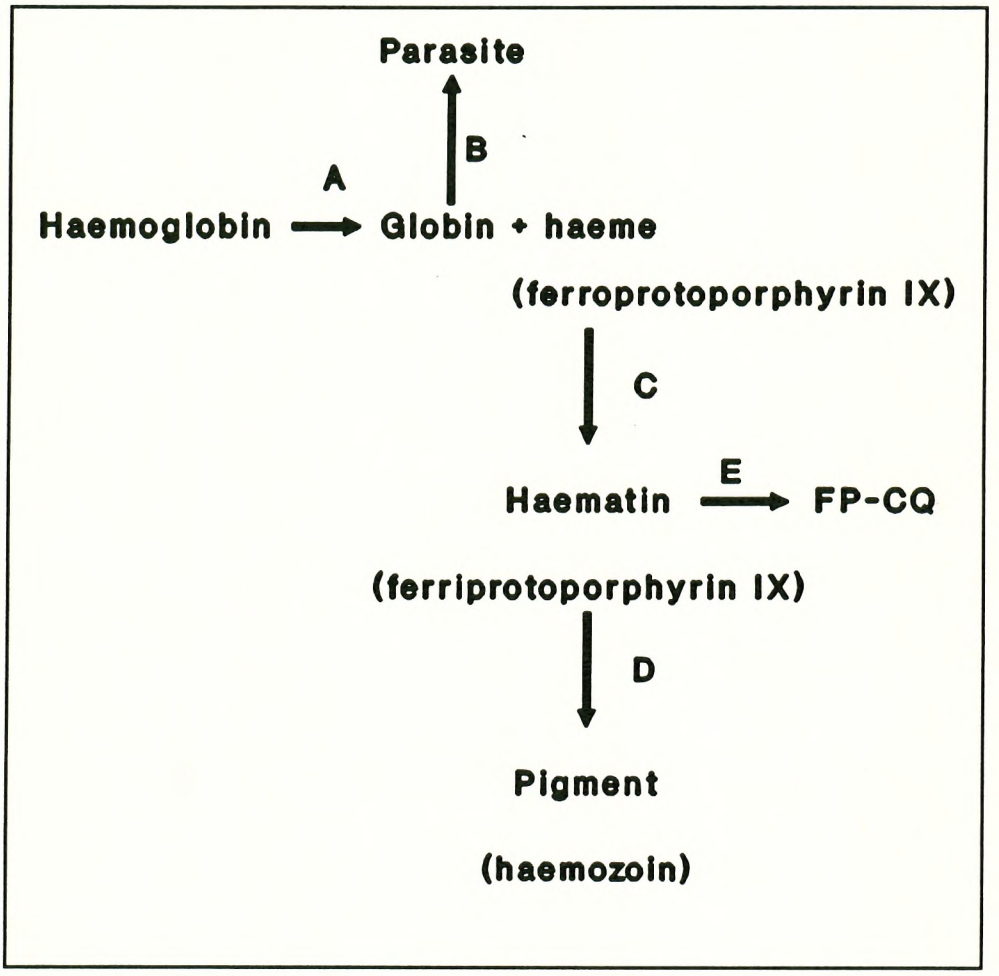


FIGURE 2: Proposed mechanism for haemoglobin breakdown

Haem consists of porphyrin to which a ferrous ion has attached forming ferroprotoporphyrin IX. Haematin is formed by the addition of an OH-group to ferroprotoporphyrin IX and the oxidation of the ferrous iron to the ferric form. Parasites which (via mutation and/or selection) delay the conversion of haematin to haemozoin (D) relative to the rate of formation of haematin from haem (C) would have in their food vacuole a substance that complexes readily with chloroquine (via E) and thereby, be protected to a certain degree from chloroquine's toxic action (fig.2) (Schueler and Cantrell, 1964). The authors believe that the CQ-FP complex is less toxic than free FP. However, there is no direct evidence that haematin exists in parasitized red cells of chloroquine resistant strains to a greater degree than in normal strains.

McNamara *et al.* (1967) showed that sensitive and resistant strains of *P. falciparum* do not differ in the extent of haemoglobin degradation and the accumulation of chloroquine is reversible eg: NH_4Cl can displace chloroquine quantitatively. On the other hand, Zarchin *et al.* (1986) demonstrated that the quinoline - containing antimalarial drugs, such as chloroquine, inhibit amino acid production at the same concentrations at which they inhibit parasite growth, but have no effect on the endogenous parasite protein degradation. They postulate that the parasite feeding on the host cell cytosol is the primary target for the antimalarial action of these drugs. A more acid pH will result in a greater susceptibility of amino acid production to chloroquine. Zarchin *et al.*

(1986) postulates that the decreased susceptibility of ring stage parasites to chloroquine can be caused by its lesser ability to concentrate the drug or by its lesser dependence on amino acid production.

1.2.2 LYSOSOMOTROPIC HYPOTHESIS

Substances that are selectively taken up into lysosomes are called lysosomotropic. Lysosomes are digestion organelles of cells, which have a pH of about 4-5, and contain 40 or more acidic digestive enzymes (De Duve *et al.*, 1974).

There is little doubt that chloroquine accumulates in the food vacuoles, and as a result this hypothesis suggests that chloroquine functions as a lysosomotropic agent, and might interfere with lysosomal functions by raising its pH (Homewood *et al.*, 1972a), and inhibiting various lysosomal hydrolases (Matsuzawa and Hostetler, 1980).

It has been postulated that the acid content of the food vacuole could be responsible for the concentration of chloroquine, since this drug and other 4-aminoquinolines are weakly basic and capable of gaining or losing protons from their amino groups depending on the environmental pH (Homewood *et al.* 1972a; de Duve, 1974; Warhurst and Thomas, 1978). Chloroquine passes into the acidic digestive vacuole of the parasite in the monoprotonated

form, and is di-protonated in the acidic environment of the food vacuole, thereby rendering itself incapable of passing back through the membrane, and at the same time depleting the vesicles of hydrogen ions. The pH of the vesicles increase, resulting in an inhibition of lysosomal enzymes (Howells, 1987).

1.2.2.1 DRUG RESISTANCE

This hypothesis has been further elaborated by Ginsburg and co-workers, in an attempt to explain the chloroquine resistance present in certain strains of *P. falciparum* . Ginsburg (Ginsburg, 1988; Yayon *et al.*, 1985), has proposed that the food vacuole membrane consists of two transport systems - a Ca^{2+}/H^{+} antiport and a Ca^{2+} channel (fig. 3). The vacuolar H^{+} pump driven by ATP maintains the proton gradient across the membrane. Due to the high concentration of H^{+} in the food vacuole, this would drive Ca^{2+} into the vacuole in exchange for H^{+} by means of the so called "Ca^{2+}/H^{+} antiport". The Ca^{2+} would then be driven out of the vacuole through the calcium channel along its gradient, assisted by the membrane potential which is maintained by the H^{+} pump. This cycling results in the leaking of H^{+} out of the vacuole and an increase in Ca^{2+} within the vacuole. The final consequence would be an increase in pH, and therefore a decrease in the accumulation of chloroquine, as chloroquine requires an acidic medium in

order to be concentrated. Inhibitors of either the Ca^{2+} channel and/or the Ca^{2+}/H^{+} exchange antiport system, such as verapamil or diltiazem through their effect on Ca^{2+}/H^{+}, should reduce the proton leak and result in a more acidic vacuolar pH and therefore promote chloroquine accumulation. From this hypothesis one concludes that the chloroquine sensitive parasites may be lacking in this Ca^{2+}/H^{+} antiport. This has not yet been experimentally demonstrated and the suggested Ca^{2+} transport systems have not yet been identified in lysosomal membranes.

The above hypothesis suggests that drug resistance may stem from an extensive translation and transcription of the transporter-proteins involved in increasing the H^{+} -leak in resistant parasites. Gene amplification studies are needed to test this hypothesis. Yayon *et al.* (1985) and Geary *et al.* (1990) stated that within the therapeutic range of chloroquine (up to $5x10^{-7}M$), the vacuolar pH remained virtually constant. It was noted that marked alkalinization of the food vacuole of the intraerythrocytic parasite only took place at chloroquine concentrations of $10^{-5}M$ or higher. Another controversy is apparent from the work done by Mikkelson's group (Mikkelson *et al.*, 1986), where it was found that *P. chabaudi* parasites freed from the erythrocyte maintained their intracellular pH at a alkalinity 0,2 to 0,7 units greater than the external pH. This is consistent with the fact that the pH of the

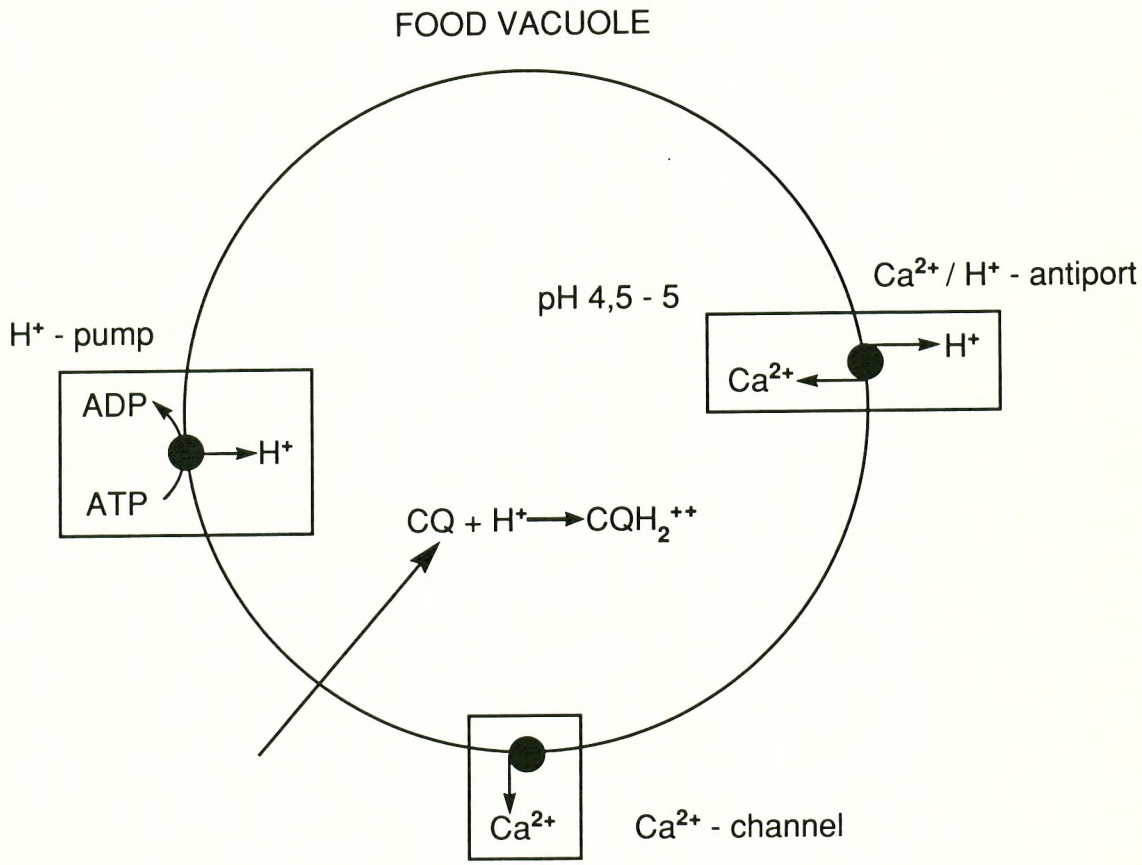


FIGURE 3: Lysosomotropic Hypothesis

erythrocyte is 7,1 and the cytosol of the parasite has a pH of 7,4. If this is the case, one may ask what is the driving force that causes chloroquine to move from the cytosol of the erythrocyte across the parasitic membrane and into the cytosol of the parasite, if it is passing through a more alkaline medium? In this explanation one is assuming that both the mode of action of chloroquine and the reversal of chloroquine resistance due to verapamil follow the same mechanism or pathway. However, it may be that both these factors function independently of one another.

1.2.3 DNA INTERCALATION HYPOTHESIS

Chloroquine and related drugs can bind to DNA isolated from plasmodia. A hypothesis has been proposed to explain the mode of the antimalarial action of chloroquine (Hahn, 1975) suggesting that:

1) The 7-chloroquinoline ring of chloroquine is intercalated between base pairs and the cationic aliphatic side chain protrudes beyond the contour of the double helix to form a DNA-chloroquine complex.

2) This interferes with the template function of DNA during DNA replication and, therefore, chloroquine inhibits the growth of the malaria parasite.

However this has now been shown to be unlikely since chloroquine binds equally well to both mammalian and plasmodial DNA, and the related drug mefloquine does not interact with DNA *in vitro* (Peters *et al.*, 1977). The concentrations of chloroquine sufficient to produce intercalation into DNA (10^{-3}M-10^{-4}M) are several orders of magnitude greater than those that inhibit the growth of chloroquine susceptible strains (10^{-6}M-10^{-7}M) (Krogstad and Schlesinger, 1987). Also there are no known differences in the DNA of resistant versus susceptible parasites (Krogstad and Schlesinger, 1987).

There is no doubt that inhibition of DNA transcription is one of the modes of action of chloroquine, but only at extraordinary high concentrations of the drug since the drug is accumulated in the DNA-free food vacuole (Geary *et al.*, 1986).

1.2.4 PERMEASE HYPOTHESIS

Warhurst (1988) has postulated that chloroquine may enter the parasite via a permease (carrier protein) which transports the diprotonated drug. The structural requirement of this permease is such that it would have to accommodate the two protonated nitrogen atoms of chloroquine, and it would indicate that it might normally function in the transport of basic

amino acids. This permease in chloroquine-resistant parasites extends to the vacuolar membrane.

Monoprotonated chloroquine diffuses into the vacuole, where it becomes diprotonated and concentrated, and is then pumped out of the parasite by the permease. Again, there is little experimental evidence to support this hypothesis. Moreover, Warhurst (1988) fails to explain how the permease hypothesis may account for the reversal of chloroquine resistance with the use of calcium channel blockers. There is the additional possibility that the permease is similar to the multiple-drug-resistance (MDR) glycoprotein of cancer cells, which would explain the reversal of chloroquine resistance with the use of calcium channel blockers. Such a protein may be present in the normal cell in very small quantities, functioning in cellular detoxification and might be over-expressed in the resistant cell - probably as a result of gene amplification.

1.2.5 MICROSOMAL MONOOXYGENASE INHIBITORS

Salganik *et al.*, (1987) have suggested that microsomal monooxygenases may be responsible for the chloroquine resistance of *P. berghei* malarial parasites. Microsomal monooxygenases (e.g., cytochrome P-450), metabolize xenobiotics. Through oxidation the hydrophobic xenobiotics are converted into

more polar metabolites, resulting in their elimination. Arylhydrocarbon hydroxylase (AHH) and aminopyrine N-demethylase (AND), are two monooxygenases that inactivate chloroquine, and this activity was found to be present in *P. berghei* (Salganik *et al.*, 1987). Spectroscopic studies provided evidence for the presence of monooxygenase and of cytochrome P-450 in *P. berghei* species. This experiment also indicated that the activity of AHH and AND was much greater in the chloroquine-resistant strains and that the degree of resistance was related to the monooxygenase activity.

Salganik also examined the effect of a number of mouse liver microsomal monooxygenase inhibitors, where copper(lysine)$_2$ and phenylhydrazine were the most effective, and completely suppressed the AHH activity of *P. berghei*. In the presence of a monooxygenase inhibitor chloroquine activity would be prolonged by suppressing its metabolism and therefore chloroquine resistance would be reserved. Rabinovich *et al.* (1987) carried out an experiment on a chloroquine resistant strain of *P.berghei* which demonstrated that copper(lysine)$_2$ or chloroquine on their own exhibited no antimalarial activity. The administration of a combination of chloroquine and the copper-lysine complex, considerably decreased the parasitaemia level of the infected mice. It would be interesting to examine the activity of AHH and AND enzymes in *P. falciparum*. However,this hypothesis fails to explain the restoration of the parasites sensitivity to chloroquine with the use of calcium channel blockers.

1.2.5.1 DRUG RESISTANCE

It is however interesting to note that verapamil is metabolized in the liver by N-demethylation to produce norverapamil (Gillman *et al.*, 1991). The resistance to chloroquine, which is reversed by verapamil, could therefore be explained on the grounds that both the chloroquine and verapamil would compete for the enzyme, resulting in a decreased enzymatic inactivation of chloroquine, leading to increased levels of chloroquine and therefore reversing the chloroquine resistance. By means of induction, the concentration of these enzymes would be higher in the chloroquine resistant parasites. This hypothesis states that the decreased levels of chloroquine in resistant parasites, are due to increased chloroquine metabolism, and it may explain the role played by verapamil in reversing the resistance.

1.2.6 INHIBITION OF A HAEM POLYMERASE ENZYME

Haemoglobin is rapidly and completely digested by the parasite (Hempelmann *et al.*,1986), whereby haem is incorporated into waste product. The parasite needs to inactivate the haem, which can damage biological membranes and inhibit a variety of enzymes (Vander Jagt *et al.*, 1986). The parasite has evolved a unique pathway for the detoxification of haem by incorporating it into

an insoluble crystalline material called haemozoin or malaria pigment. These crystals remain in the food vacuole until the infected red cell bursts.

A haem polymerase enzyme has been identified and characterized from extracts of *P. falciparum* trophozoites, and it was shown that this enzyme is inhibited by quinoline-containing drugs such as chloroquine and quinine (Slater and Cerami, 1992). Chloroquine at a concentration of 120μM caused a 50% inhibition of haem polymerase reaction. This inhibition occurs at a pH and drug concentration similar to that estimated in the malaria food vacuole of a susceptible malaria trophozoite (Ginsburg and Geary, 1987). It is therefore proposed that at these concentrations haem polymerase will be inhibited, thereby disrupting the enzymatic conversion of haemoglobin-derived haem into non toxic pigment.

The different hypotheses discussed are set out in fig. 4.

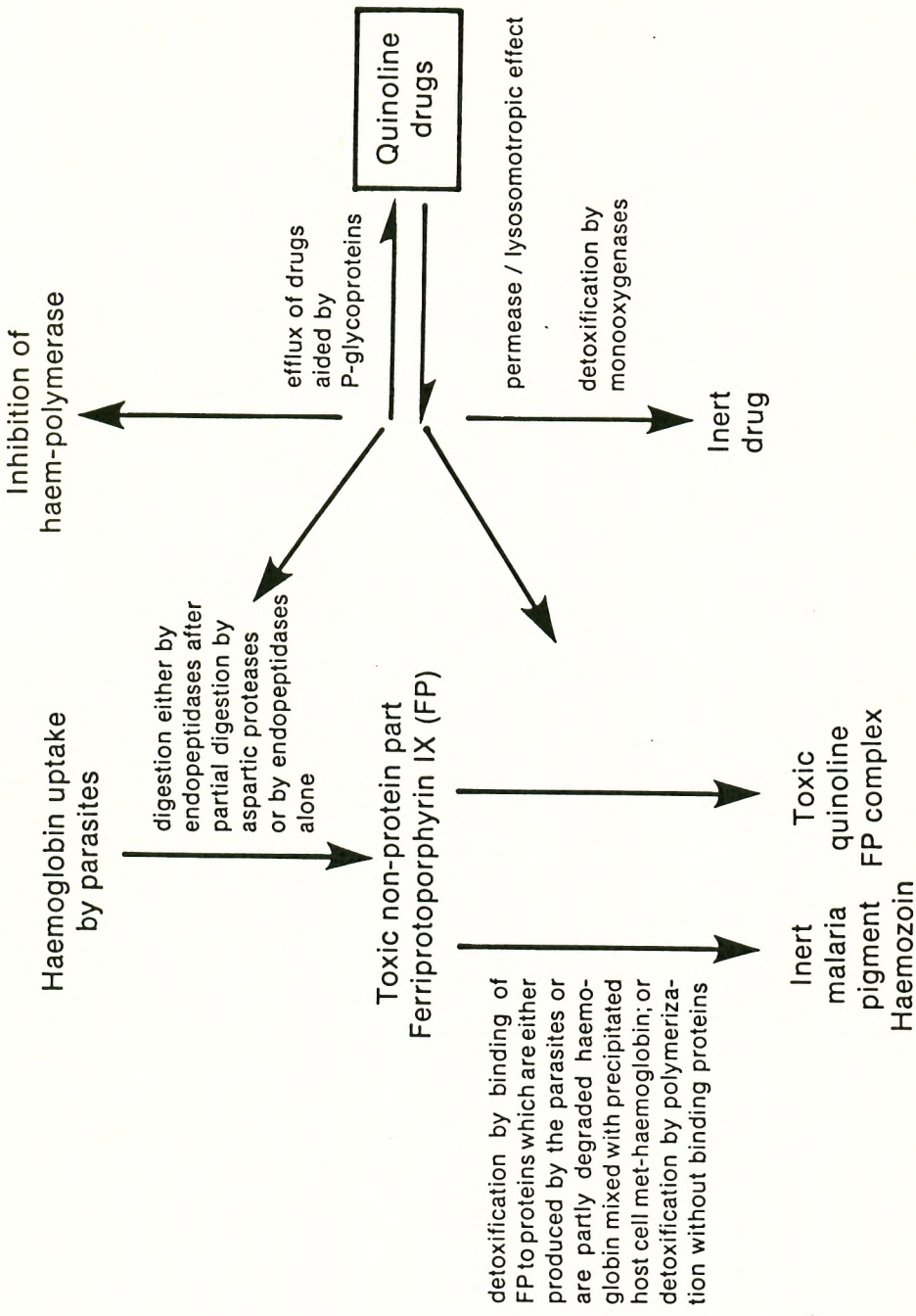


FIGURE 4: Summary of Hypotheses

1.3 CHLOROQUINE RESISTANCE IN *P. FALCIPARUM*

Drug resistance in *P. falciparum* has become one of the major obstacles to malaria control in wide parts of eastern Asia and South America, and is now also affecting many countries in Africa.

Chloroquine resistant malaria was first reported in Colombia and Thailand in 1959 (Moore and Lanier, 1961; Young *et al.*, 1963), and subsequently chloroquine resistance has been found over a wide area of South America and South-east Asia. Today, the problem of chloroquine resistant *P. falciparum* extends eastwards across the Indochinese peninsula, Southern China, Malaysia, Indonesia and across Papua New Guinea; that is, as far eastwards in the Southwest Pacific as malaria itself (Wernsdorfer and Trigg, 1988).

While not necessarily running exactly in parallel with resistance to chloroquine, it is interesting to observe that resistance to quinine has frequently appeared in an area where a high level of chloroquine resistance to either of these compounds has been limited to this parasite, such a resistance to quinine has often been found to follow resistance to chloroquine. Until 1991 this has not been proven in man.

1.3.1 NEW ANTIMALARIALS UNDER DEVELOPMENT

In response to the threat caused by the impending loss of chloroquine as the drug of choice for the treatment of acute falciparum malaria, and the toxicity of quinine as an alternative drug for treatment, an approach was being made that involved exploiting the highly potentiating action of a dihydrofolate reductase inhibitor, pyrimethamine, with another compound that blocked the incorporation of *p*-aminobenzoic acid by the malaria parasites, i.e. a sulphonamide or sulphone. A combination of pyrimethamine with dapsone was launched under the name Maloprim® by the Wellcome Foundation in 1968 as a prophylactic, but originally with the aim of avoiding the problem of pyrimethamine resistance itself.

Meanwhile the US Army Research and Development Command had established a massive programme based in the Walter Reed Army Institute of Research for the screening and development of antimalarial compounds. Of the new drugs to emerge from this programme, one compound closely related to quinine has come to the forefront. Mefloquine has now been studied in several hundred individuals with acute falciparum malaria in three continents and has been proved to be both well tolerated and highly effective against nearly all infections with chloroquine-resistant (as well as chloroquine-sensitive) strains of *P. falciparum* (WHO, 1983). Already there are indications that a number of

strains of *P. falciparum* highly resistant to chloroquine will respond to mefloquine *in vitro* that is significantly below that previously anticipated from all available data (Smrkovski *et al.*, 1982).

Research teams in the People's Republic of China discovered that a plant traditionally employed for the treatment of "fever" for over 2000 years, Qinghaosu contained a sesquiterpene lactone, now named Artemisinine, that is an extremely potent and rapidly acting blood schizontocide (Gu *et al.*, 1984). While the rapidity of action of these sesquiterpenes makes them invaluable for therapy in acute falciparum malaria (Li *et al.*, 1982), they too suffer from the likelihood that their widespread use alone could lead to the emergence of parasites resistant to them.

As has been pointed out, *P. falciparum* can develop resistance to any antimalarial drug if it is used alone. One of the worst problems, is the misuse of drugs. This applies to almost all drugs and is not in any way limited to antimalarials. One of the prime means of preventing or combatting the problem of drug resistance in malaria is to control their distribution and ensure that they are correctly deployed.

1.3.2 BIOCHEMICAL ASPECTS OF DRUG RESISTANCE IN MALARIA PARASITES

Beale (1980) has considered a number of different mechanisms which can account for changes in drug sensitivity of various organisms. Spontaneous gene mutation was considered as likely to be the most important mechanism for Plasmodium species (Beale, 1980). Present evidence suggests that resistance to pyrimethamine or chloroquine is inherited, as a Mendelian character, and the presence of resistance-transfer factors has so far not been demonstrated. These mechanisms can lead to various means of biochemical expression of drug resistance. These include increase in target enzyme in the parasite so as to overcome inhibitory activity of the drug, alteration of the target enzyme leading to decreased affinity for the drug, decrease in drug uptake by the parasite, bypass of metabolic lesion by alternative metabolic pathway, and inactivation of the drug through parasite metabolism. The biochemical basis for chloroquine resistance is still unclear, reflecting incomplete understanding of its mechanism of action.

1.3.3 CHLOROQUINE RESISTANCE AND THE CALCIUM-CHANNEL BLOCKERS

Martin *et al.* (1987) postulated that the mechanism by which *P. falciparum* acquires multidrug resistance may be similar to that by which neoplastic mammalian cells develop simultaneous resistance to multiple structurally unrelated drugs. The multidrug resistance in neoplastic cells has been correlated with an increase in the number of P-glycoproteins in the cell membrane which serve to pump many different hydrophobic molecules out of the cell (Stark, 1987). The consequence of this is an enhanced efflux of drugs from the cell such that they fail to attain intracellular toxic concentrations. Blockade of the efflux channels in cultured neoplastic cells reverses resistance, and Martin *et al.* (1987) similarly demonstrated that verapamil, a calcium channel blocker, reversed chloroquine resistance in *P. falciparum*. Commenting on the findings of Martin *et al.* (1987), Ryall (1987) observed that an increase in drug efflux is not known to occur in chloroquine-resistant plasmodia. Verapamil, a weak base, is also capable of inhibiting lysosomal function in mammalian cells, and Ryall (1987) suggested that the reversal of chloroquine resistance in *P. falciparum* might relate to an effect of verapamil on the acid vesicles of the parasite.

An alternative to the mechanism of chloroquine resistance proposed by Martin *et al.* (1987) is a reduction rather than increase in chloroquine "channels" in the

infected erythrocyte, proposed by Warhurst (1988). Warhurst postulated that resistance could result from a reduction or loss of the permease, or that the affinity of the permease for chloroquine might be reduced. The relationship, if any, of chloroquine "permeases" or of proteins analogous to the P-glycoprotein of mammalian cells in chloroquine-resistant *P. falciparum* remains speculative.

1.4 P-GLYCOPROTEIN IN CANCER

The multiple drug resistance which occurs in certain neoplastic cells has been shown to be due to enhanced efflux of cytotoxic drug from the cells preventing intracellular concentrations of the drug achieving therapeutic levels. Multiple drug resistance (MDR) is expressed as the ability of cells exposed to a single drug to develop resistance to a broad range of structurally and functionally unrelated drugs due to enhanced outward transport of drugs mediated by a membrane glycoprotein "drug transport pump".

In neoplastic cells calcium channel blockers (e.g., verapamil), reverse drug resistance by inhibiting the efflux mechanism. In these neoplastic multidrug resistant cells there is an overexpression of a 150-170-kilodalton membrane glycoprotein termed permeability (P)- glycoprotein. This

glycoprotein is either not present, or not detectable because of its very low concentrations in sensitive cells (Safa *et al.*, 1987; Gottesman and Pastan, 1988). The degree of drug-resistance was found to correlate with expression of P-glycoprotein. Low levels of P-glycoprotein have been found in the drug sensitive lines, and an overexpression has been detected in drug-resistant cells. It is this overexpression that causes multidrug resistance. Evidence suggests that the P-glycoprotein functions as a transporter of antitumour agents on the plasma membrane of multidrug-resistant tumour cells (Ames, 1986). Photoaffinity labelling of multidrug-resistant cancer cells has shown that the calcium channel blockers bind to the exporting P-glycoprotein and compete with the chemotherapeutic drug being exported (Cornwell *et al.*, 1986; 1987a).

1.4.1 STRUCTURE OF P-GLYCOPROTEIN

P-glycoprotein is an integral plasma membrane glycoprotein that spans the lipid bilayer (fig. 5). A gene coding for the P-glycoprotein has been isolated from a drug-sensitive and a drug-resistant line (Gros *et al.*, 1986; Chen *et al.*, 1986). Sequence analysis done on the P-glycoprotein gene has shown that there are 1276 amino acids in the mouse sequence, and 1280 amino acids in the human sequence, and the molecular masses are 140 and 141 kDa respectively. The P-glycoprotein is a duplicated molecule, where each

half consists of a large hydrophobic domain, and a conserved hydrophilic cytoplasmic domain containing an ATP-binding site (fig. 6) (Gerlach *et al.*, 1986; Cornwell *et al.*, 1987b). With the use of monoclonal antibodies, it has been shown that the ATP-binding domain on the P-glycoprotein is localized on the cytoplasmic side of the cell membrane. Two groups have shown that mutation of one or both nucleotide-binding consensus sequences results in failure to confer drug resistance. These results suggest that both ATP-binding sites are required, and these may functionally interact to effect active drug efflux (Rothenburg and Ling, 1989).

The duplicated moiety may be considered to have a N-terminal portion which is hydrophobic, and a C-terminal portion which is hydrophilic. The C-terminal portion of the two halves of the molecule are highly homologous and each contains the consensus sequence for an ATP-binding site, while the corresponding N-terminal portions show less striking amino-acid sequence homology (Gerlach et al., 1986). It appears that the P-glycoprotein has homologous gene structure to the HlyB gene product, which is a bacterial membrane protein required for secretion of α-hemolysin from *E. coli*. It is also highly homologous to a number of ATP-binding bacterial transport proteins. It is believed that the P-glycoprotein forms a channel in the plasma membrane, and it utilizes energy derived from ATP-hydrolysis to transport drugs out of cells. However, it still remains to be determined

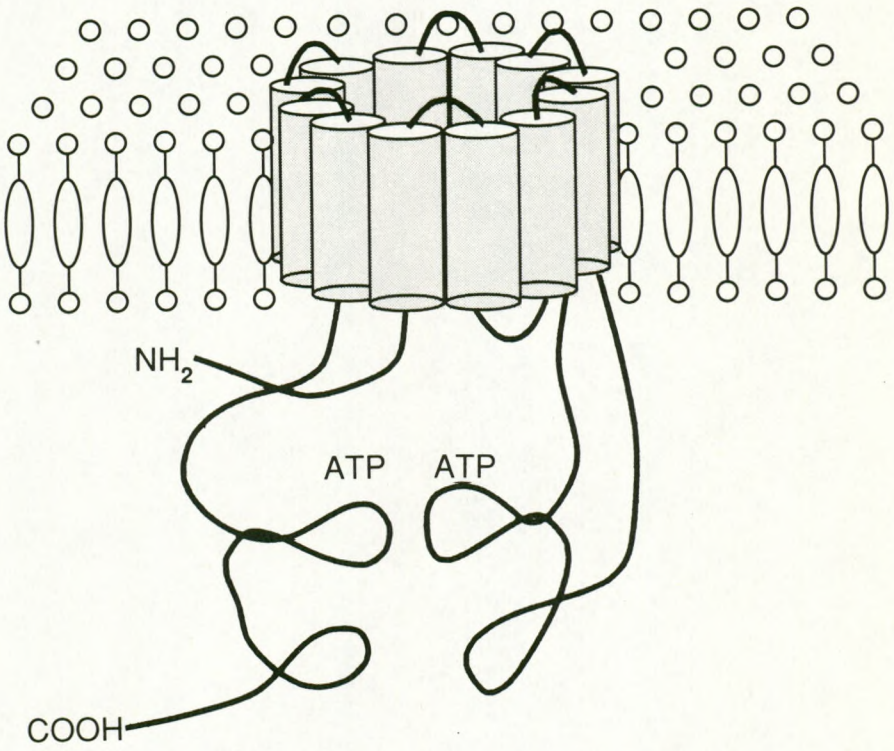


FIGURE 5: Proposed structure of P-glycoprotein in the lipid-bilayer (from Bradley *et al.*, 1988)

whether ATP-hydrolysis by P-glycoprotein is coupled to drug efflux, as predicted by the current model (Hamada and Tsuruo, 1988). The numbers of P-glycoprotein molecules required to form one channel is not known.

1.4.2 BINDING OF DRUG TO P-GLYCOPROTEIN

One can speculate that P-glycoprotein contains a multiplicity of drug-binding sites and this would explain the fundamental question of how a single protein can mediate transport of a diverse group of cytotoxic compounds. How the drugs bind to the P-glycoprotein is uncertain. Since the drug molecules have to be released at the cell surface, the drug-binding to P-glycoprotein must be reversible. However, one must account for the fact that both neoplastic cells and *P. falciparum* become resistant to a number of unrelated drugs. This would then mean that the P-glycoprotein molecule must have binding-sites for a diverse group of drugs, probably within its hydrophobic domain.

1.4.2.1 COMMON BINDING SITE

Photoaffinity-labelling with radioactive vinblastine analogues has shown specific binding-sites for vinblastine (Cornwell *et al.*, 1986; 1987b). This

photoaffinity labeling can be inhibited by drugs such as daunomycin or vincristine as well as several chemosensitizing agents such as verapamil, quinidine, reserpine, and azidopine (Gottesman *et al.*, 1988). The ability of drugs and reversing agents to inhibit each others' binding to P-glycoprotein suggest that they may be competing for a common binding site. Thus, one mechanism of MDR reversal by chemosensitizers and non-toxic drug analogs may be explained on the basis of competition for drug binding, which results in a decrease in efflux rate and a higher intracellular level of toxic drugs in MDR cells.

1.4.2.2 DIFFERENT BINDING SITES

However, it has been shown that colchicine and actinomycin - D do not compete for the vinblastine binding site (Cornwell *et al.*, 1986). One may postulate that P-glycoprotein might consist of a number of binding sites. The second version of the model for P-glycoprotein, suggests that the drug may bind irreversibly to this glycoprotein and the entire drug-glycoprotein complex is removed from the cell. This drug-binding protein is present in the drug-sensitive cells, but in the drug-resistant cells it is overexpressed, and it must be produced in sufficient quantities, as it is being continuously exported (Gerlach *et al.*, 1986). This hypothesis, however needs further investigation.

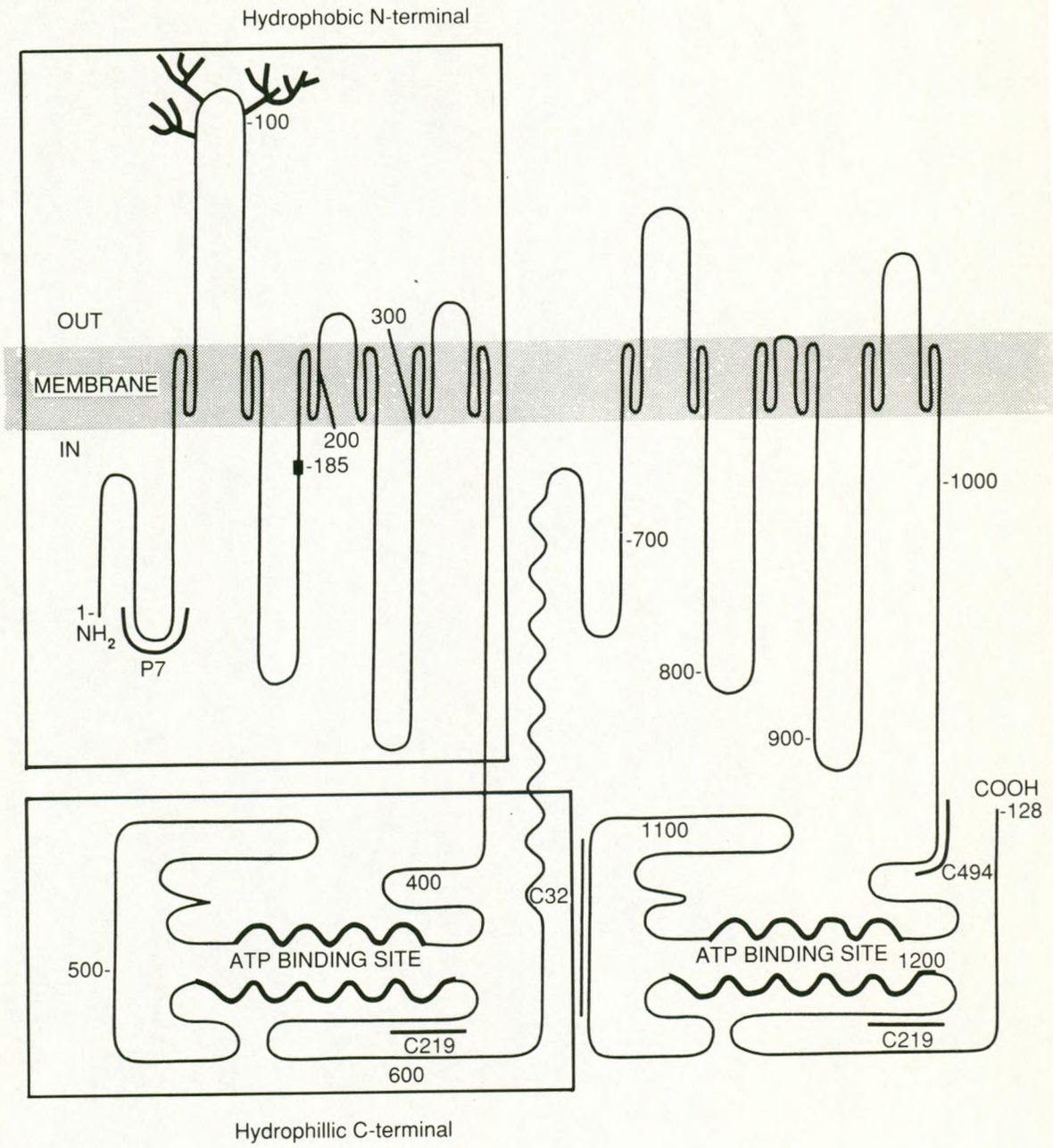


FIGURE 6: Structure of P-glycoprotein (from Juranka *et al.*, 1989)

Besides having detected P-glycoprotein in neoplastic and *P. falciparum* cells, the occurence of P-glycoprotein in normal tissues is under investigation. Out of 17 different mammalian tissues that were examined, 6 showed high levels of P-glycoprotein. These tissues were liver, pancreas, kidney, colon, jejenum, and adrenal. These findings suggest that P-glycoprotein may have a physiological role in secretory processes, other than multidrug resistance (Thiebaut *et al.*, 1987).

In summary: there is experimental evidence to support a model of P-glycoprotein as an ATP-driven drug efflux pump. Still unresolved are the mechanistic details of this drug efflux and the enigma of the broad specificity and differences in the drug resistance profiles of MDR cell lines.

1.5 SIMILARITIES BETWEEN DRUG RESISTANCE IN CANCER AND MALARIA

Extensive research on cancer lines, has increased our knowledge of P-glycoprotein. However the research done on *P. falciparum* with respect to the P-glycoprotein is still in its infancy. Findings of P-glycoprotein in cancer, may open new avenues for research in malaria, since the chloroquine resistance in *P. falciparum* shares features with resistance tumour cells. Martin *et al.* (1987) demonstrated that in the presence of verapamil,

chloroquine-resistant *P. falciparum* becomes chloroquine-sensitive with an increase in the chloroquine accumulation. As a result, Martin *et al.* (1987) proposed that multiple drug resistance which has been observed in different isolates of *P. falciparum* may share the same biochemical basis. In this experiment, it was demonstrated that verapamil was capable of reversing chloroquine resistance in clones of *P. falciparum* obtained from Asia and Brazil, but had no effect on the susceptibility of chloroquine-sensitive clones. This is analogous to findings in cancer research. Examples of a drug effective in reversing resistance in both cancer and malaria would be cyclosporin A which has antimalarial activity *in vitro* and *in vivo* (Scheibel *et al.*, 1987). Cyclosporin A also reverses vincristine resistance of tumour cells, probably by a Ca^{2+}/calmodulin dependent process (Slater *et al.*,1986).

1.5.1 THYMIDYLATE SYNTHASE - DIHYDROFOLATE REDUCTASE INHIBITORS

In most organisms, thymidylate synthase (TS) and dihydrofolate reductase (DHFR) are separate, monofunctional enzymes. DHFR is a monomer of about 20 kDa (Sirawaraporn *et al.*, 1990), and TS is a dimer of identical subunits of about 35 kDa each (Sirawaraporn *et al.*, 1990). However, in protozoa the enzymes exist on the same 55-70 kDa polypeptide chain, with the DHFR domain at the amino terminus and TS at the carboxy terminus, separated by a

junction peptide. The TS-DHFR of *P. falciparum* is one of the few well-defined drug targets for malaria. The DHFR inhibitors pyrimethamine and cycloguanil have been used as antimalarial agents for several decades, but the emergence of resistant parasites has reduced the utility of these drugs.

Inhibition of dihydrofolate reductase results in a reduction in the pool of tetrahydrofolate co-factors which are needed in most cells for the *de novo* synthesis of purines, methionine and thymidylate and for the interconversion of glycine with serine. Malaria parasites do not synthesize purine *de novo* (Sherman, 1979). Thus, the presence of enzymes of the thymidylate synthase cycle and the lack of utilization of pyrimidines by the malaria parasite (Sherman, 1979) indicate that the high affinity of parasite dihydrofolate reductase to pyrimethamine should result in inhibition of DNA synthesis by this drug.

1.5.1.1 RESISTANCE TO PYRIMETHAMINE

Most strains of *P. falciparum* have now become resistant to pyrimethamine (Peterson *et al.*, 1990). In the same manner, cancer cells have become resistant to methotrexate, another DHFR-inhibitor (Kartner *et al.*, 1983). Peterson *et al.* (1990) has shown that parasites that are resistant to the DHFR-inhibitor, cycloguanil (the active metabolite of proguanil), have a pair

of point mutations from Ala-16 to Val-16 and from Ser-108 to Thr-108, but these parasites are not resistant to pyrimethamine. However, resistance to pyrimethamine is confered by a single Asn-108 mutation, with only a moderate decrease in susceptibility to cycloguanil. Parasites with mutations from Ser-108 to Asn-108 and Ile-164 to Leu-164 have significant cross resistance to both drugs (Wellems *et al.*, 1991). Studies have failed to demonstrate an increase in the copy number of the DHFR gene in pyrimethamine-resistant isolates. It is also not known whether cycloguanil and pyrimethamine share the same binding site on the DHFR-enzyme (Peterson *et al.*, 1990). Available evidence suggests that DHFR point mutations act by inhibiting pyrimethamine binding in the active site of the enzyme. It may be , that the point mutations observed in the resistant parasites result in a structural change to the DHFR-enzyme, inhibiting the binding of pyrimethamine and cycloguanil.

1.5.2 CALCIUM CHANNEL BLOCKERS

Calcium channel blockers and calmodulin affect intracellular calcium levels, and as a result it has been speculated that efflux of cytotoxic drugs might be controlled by a calcium-calmodulin complex or other calcium-dependent processes (Tsuruo *et al.*, 1982). Kessel and Wilberding (1985) measured Ca^{2+} uptake, but failed to demonstrate differences in calcium flux between

drug-sensitive and multidrug-resistant cells or any effect of calcium antagonists on calcium flux in these cells. The ability of verapamil to reverse resistance did not correlate well with effect on calcium channels or calmodulin (Tsuruo *et al.*, 1982). However, one must not exclude the possibility of Ca^{2+} involvement in drug transport processes.

Calcium channel blockers and calmodulin inhibitors may alter drug uptake and retention through membrane effects, as both have lipophillic portions that are compatible with membrane binding. Cornwell *et al.* (1987a), showed that verapamil binds specifically to isolated membrane vesicles of multidrug-resistant cells and inhibits vinblastine labelling of P-glycoprotein in membrane vesicles. Similarly the malarial parasite actively accumulates Ca^{2+}, which is an essential requisite for the growth of the malarial parasite (Tanabe *et al.*, 1982). Malaria infected erythrocytes change their membrane transport to accumulate Ca^{2+} from the extracellular medium (Tanabe *et al.*, 1982). Calcium plays a crucial role in the maturation of *P. falciparum*, and calcium mediated changes are responsible for the loss of deformability observed with maturation to the trophozoite stage (Krogstad *et al.*,1991). Classical antimalarial drugs such as chloroquine and quinine have been shown to inhibit calmodulin activity (Tanabe *et al.*, 1989). Calcium channel blockers when used at high enough concentrations will inhibit the growth of the parasite by themselves. The inhibitory effect of verapamil on its own may be due to its binding and antagonization of calmodulin

(Scheibel *et al.*, 1987). Due to this, it was speculated that the efflux of drugs might be controlled by calcium-dependent processes. However till now, there has been a failure to demonstrate any difference in calcium flux between drug-sensitive and multidrug-resistant cells. Ye and Van Dyk (1989) reported that chloroquine resistance in falciparum malaria was reversed by using the R(+) isomers of verapamil congeners, gallopamil and devapamil. These R(+) isomers essentially do not inhibit cardiac calcium channels as do the S(-) isomers of verapamil congeners (Bayer *et al.*, 1975). One may therefore conclude that the R(+) isomer which essentially does not bind to the calcium channel produces a complete reversal of chloroquine resistance. Perhaps, verapamil is able to convert chloroquine resistant strains into chloroquine sensitive, by its added inhibitory effect on the malarial parasite (Ryall, 1987).

1.6 MULTIDRUG RESISTANCE: DIFFERENCE BETWEEN CANCER AND MALARIA

Although multidrug resistance in cancer and malaria appear to have some features in common, there are major differences. In cancer, multidrug resistance has been shown to be associated with reduced drug accumulation in different cell lines of hamster, mouse and human origin, where the rate of

accumulation is consistently reduced in multidrug-resistant cells, when compared to the respective drug-sensitive parent (Kartner *et al.*, 1983; Fojo *et al.*, 1985). It was suggested that the multidrug-resistance was due to the decreased drug influx. However, the measured influx of these drugs appeared to be relatively unaltered in multidrug-resistant cells. Different studies have attributed influx to either passive diffusion or mediated transport (Skovsgaard, 1987).

Sirotnak *et al.*, (1986) noted that reduced accumulation of colchicine in cancer cells is thought to be directly related to decreased influx. However, this is very difficult to prove experimentally, because influx measurements are complicated by rapid and substantial adsorption of hydrophobic drugs to the cell surface. In malaria, the initial rate of chloroquine uptake is the same in both chloroquine-resistant and -sensitive strains of *P. falciparum* (Krogstad and Schlesinger, 1987; Geary *et al.*, 1987). Chloroquine resistance is also not associated with resistance against 8-aminoquinolines (Geary *et al.*, 1987).

1.7 P-GLYCOPROTEIN IN MALARIA

The quest for a P-glycoprotein in malarial parasites has been initiated only lately, after it was found that verapamil which reverses drug resistance in cancer cells also enhances the action of chloroquine in resistant malaria parasites (Martin *et al.*, 1987). The chloroquine resistance phenotype in *P.*

falciparum has been likened to a form of MDR found in some mammalian tumour cell lines. There are two key features from which this similarity stems. First, resistance to the cytotoxic drug is characterized by enhanced efflux of the drug in drug-resistant cells compared to their drug-sensitive counterparts (Fojo *et al.*, 1985; Krogstad *et al.*, 1987; Wellems *et al.*, 1990). Secondly, reversal or chemosensitizing drugs reinstalls the sensitivity to a cytotoxic drug-resistant cell (Fojo *et al.*, 1985; Martin *et al.*, 1987; Kyle *et al.*, 1990). It is possible that the underlying mechanisms giving origin to the two common phenotypic features of chloroquine-resistance and MDR may be similar.

Some antimalarial compounds such as chloroquine, quinine and quinidine are capable of sensitizing MDR tumour cells (Fojo *et al.*, 1985), while some classical anticancer drugs such as daunomycin and vinblastine can render chloroquine-resistant *P. falciparum* susceptible to chloroquine (Krogstad *et al.*, 1987). However, not all drugs capable of chemosensitizing MDR tumour cells are similarly efficacious for chloroquine-resistant *P. falciparum*. Reversal of resistance is a result of enhanced retention of the cytotoxic compound.

The effector molecule(s) involved in expulsion of chloroquine from resistant *P. falciparum* has not been identified. Chloroquine-resistant *P. falciparum* isolates are able to expel 40 to 50-fold more chloroquine than chloroquine-sensitive isolates and this process has a half-life of 1 to 2 min (Krogstad *et al.*, 1987). Ginsburg and Stein (1991) devised a kinetic model which predicts that the half-

time of drug efflux from parasitized cells should be directly correlated with the vacuolar drug concentration, i.e., exactly as found by Krogstad *et al.* (1987). The transport process of drugs in MDR tumour cells is a much more rapid process than occurs in chloroquine-resistant *P. falciparum* and this is perhaps not unexpected as the tumour cell lines are usually much more resistant to the cytotoxic drugs than is the case in chloroquine-resistance (Karcz and Cowman, 1991).

1.7.1 AMPLIFICATION OF THE PARASITES MULTIDRUG RESISTANT GENE

Foote and colleagues (1989) first demonstrated amplification of segments of DNA on chromosome 5 containing the *pfmdr1* gene in some but not all chloroquine-resistant isolates of *P. falciparum*. The *pfmdr1* gene is the *P. falciparum* homologue of the MDR-gene, where *pfmdr* stands for *Plasmodium falciparum* multidrug resistance. In the present context homology refers to the corresponding similarity in position and origin of the respective amino acids within a protein product. Sequence analysis of the *pfmdr* gene indicates that, like cancer, it is a duplicated molecule, where each half consists of a large hydrophobic domain (N-terminal), and a conserved hydrophillic cytoplasmic domain (C-terminal). Chen *et al.* (1986) compared the two halves of *pfmdr* and human MDR 1, and found that these sequences are 59% similar, the greatest

similarity being present in the hydrophillic regions, where it is believed to code for the nucleotide-binding (ATP-binding) sites.

If the mechanism of these *pfmdr* genes in *P. falciparum* is similar to that of MDR genes in human multidrug- resistant tumour cells, the model predicts that the gene would be expressed at a higher level in drug-resistant cells than in drug-sensitive cells. Wilson *et al.* (1989) indicates that the *pfmdr1* gene is expressed in both W2-mef (mefloquine resistant) and W2 (mefloquine sensitive) lines. The W2-mef clone showed a two to fourfold increase in the copy number of the *pfmdr1* gene when compared to its parent clone W2. It is interesting to note that the mefloquine-resistant line derived by the selection was more sensitive to chloroquine than was the original parent-line (Oduola *et al.*, 1988). This suggests that the amplification of *pfmdr1* in W2-mef was not associated with an increase in chloroquine resistance (Wellems *et al.*, 1990).

Foote *et al.* (1989) found that in certain chloroquine-resistant clones, the *pfmdr* gene is amplified. In sensitive isolates no gene amplification was found, and two resistant isolates (CSL2 and V1) apparently have the same copy number as the sensitive isolates. Whereas another resistant line, K1, is believed to have deleted *pfmdr* and that the gene is not essential for growth *in vitro*.

This was also found in cancer where multidrug-resistance did not necessarily correlate with P-glycoprotein gene amplification (Riordan *et al.*, 1985; Scotto *et al.*, 1986).

The questions of particular interest are, therefore, why does amplification of *pfmdr1* occur and is it in any way related to chloroquine-resistance. While the significance of *pfmdr1* gene amplification is not understood, it is clear that amplification of *pfmdr1* is insufficient for chloroquine-resistance.

1.7.2 MUTATIONS OF PFMDR 1 LINKED TO CHLOROQUINE-RESISTANCE

The presence of the *pfmdr1* gene in some chloroquine-resistant isolates at a copy number equivalent to that of chloroquine-sensitive parasites led Foote *et al.* (1989) to suggest that perhaps the *pfmdr1* gene of chloroquine-resistant parasites was mutated at one or more positions within the primary sequence.

The amino acid sequence for the *pfmdr* gene of the K1 isolate is Tyr-86 Tyr-184 Ser-1034 Asn-1024 Asp-1246 and the amino acid sequence for the 7G8 is Asn-86 Phe-184, Cys-1034 Asp-1042, Tyr-1246 The amino acid sequence for the sensitive isolates, D10 and 3D7 are, Asn-86, Tyr-184 Ser-1034, Asn-1042 Asp-1246. The only difference between the K1 (resistant)

isolate and the sensitive strains is at amino acid number 86, where the change is from Asn (sensitive) to Tyr (resistant).

Table 1. Amino acid changes in different *P. falciparum* isolates

Amino Acid	86	184	1034	1042	1246
D10	Asn	Tyr	Ser	Asn	Asp
3D7	Asn	Tyr	Ser	Asn	Asp
K1	Tyr	Tyr	Ser	Asn	Asp
7G8	Asn	Phe	Cys	Asp	Tyr

However, the 7G8 resistant isolate consists of 4 amino acid changes at number 184, 1034, 1042, and 1246. The amino acid changes included Tyr to Phe, Ser to Cys, Asn to Asp, Asp to Tyr, from the sensitive to the 7G8 resistant strain respectively. One may therefore conclude that the *pfmdr* of the resistant isolates differ from the *pfmdr* sequence of the sensitive strains by 5 key positions.

It is speculated that these changes are found in domains of the molecule that may be involved in substrate specificity.

The presence of sequence differences in the *pfmdr1* gene enabled the accurate prediction of the chloroquine resistance or sensitivity status of a further 34 of 36 isolates in a study based on the sequence of distinct regions of *pfmdr1* alone (Foote *et al.*, 1990).

The presence of mutations in other members of the P-glycoprotein-like family may provide some insight into the functional significance of mutations in *pfmdr1*. In human P-glycoprotein, for example, a single mutation resulting in a glycine to valine substitution at position 185 dramatically alters its substrate specificity (Choi *et al.*, 1988). A valine at this position predisposes for preferential colchicine resistance while cells expressing wild-type P-glycoprotein exhibit preferential resistance to vinblastine. In the case of the mouse *mdr1* gene product, a mutation at position 939 results in a P-glycoprotein capable of conferring resistance to vinblastine but which has lost the ability to confer cross resistance to colchicine and adriamycin. It seems reasonable to suggest that mutations in *pfmdr1* may also lead to functional alterations. Whether these changes are in any way linked to the intracellular distribution of chloroquine has not been demonstrated.

1.7.3 THE GENETIC BASIS OF CHLOROQUINE-RESISTANCE

Chloroquine-resistance is known to have arisen from two foci: one in South America and one in Southeast Asia in the late 1950's and early1960's (Foote *et al.*, 1989). It has also not been possible to select *in vitro*, a chloroquine resistant *P. falciparum* line from a chloroquine-sensitive parent, suggesting that multiple genetic events are required. It, therefore seems likely that the genetic basis of chloroquine-resistance involves alterations in more than one gene.

However, a single genetic cross performed by Wellems and colleagues (Wellems *et al.*, 1990) suggests that the inheritance of chloroquine-resistance in *P. falciparum* behaves as a single genetic locus. They could not find a linkage between the rapid efflux of drug in chloroquine-resistant isolates and the MDR-like *P. falciparum* genes or amplification of these genes. Subsequent analysis of the progeny of this cross has localized a gene to a 400-kb region of chromosome 7 that is linked to the rapid efflux phenotype that characterizes chloroquine resistance (Wellems *et al.*, 1991).

The suggestion from the genetic cross that a single locus independent of *pfmdr* genes is involved in the rapid efflux phenotype is in contrast with the report that mutations in *pfmdr1* are strongly linked to the chloroquine-resistant phenotype.

1.7.4 EXPRESSION AND LOCALIZATION OF THE PFMDR 1 GENE PRODUCT

Cowman *et al.* (1991) has recently showed that the gene product of *pfmdr1* is a polypeptide of approximately 160 kDa and has been called Pgh1. Analysis of this protein in a number of *P. falciparum* isolates has revealed that all parasite lines regardless of their chloroquine-resistant or sensitivity status express Pgh1. Pgh1 is defined as the P-glycoprotein homologue or the P-glycoprotein-like-protein present in the malaria parasite. This Pgh1 was identified with antibodies raised to a fragment of the *pfmdr1* gene product expressed in *E. coli*. Many chloroquine-resistant isolates express about the same level of Pgh1 as do their chloroquine-sensitive counterparts.

The chloroquine-resistant cloned line, FAC8, which has three copies of the *pfmdr1* gene expresses approximately threefold more Pgh1 than other chloroquine-resistant isolates (Cowman *et al.*, 1991). Since FAC 8 was not more resistant to chloroquine than other chloroquine-resistant lines expressing lower levels of Pgh1, there is no correlation between the level of chloroquine-resistance and the amount of Pgh1 expressed. This may suggest that Pgh1 is not directly involved in the chloroquine-resistance phenotype and overexpression of this protein is not necessary for chloroquine-resistance.

Using antibodies raised against the *pfmdr1* gene product, it was possible to

localize Pgh1 to the membrane of the digestive vacuole of trophozoites (Cowman *et al.*, 1991).

A transport molecule, present on the surface of the digestive vacuole, would be able to remove chloroquine from its site of action. This mechanism by itself however, would not remove chloroquine from the parasitized cell. Another point of consideration is the direction of the Pgh1. If the direction of transport of Pgh1 is into the digestive vacuole, it may be required for the transport of proteins usually required for the function of this organelle. The protein product of the *pfmdr1* gene of *P. falciparum*, has been circumstantially linked with chloroquine resistance, but there has been no direct functional demonstration of its role in resistance to this drug. Sequence data of the *pfmdr1* gene indicates the existence of a P-glycoprotein homologue (Foote *et al.*, 1990). However, the genetic cross between chloroquine-resistant and -sensitive cloned parasite isolates has suggested that the P-glycoprotein homologues are not linked to the chloroquine resistance phenotype (Wellems *et al.*, 1990). One may conclude that the Pgh1 protein of *P.falciparum* is a member of the MDR ATP-binding superfamily and most of these molecules are involved in the transport of molecules across membranes, including amino acids and proteins.

1.7.5 MONOCLONAL ANTIBODIES

A 40-42 kDa component was identified by the C219 (Centocor P-glycoCHEK) monoclonal antibody in both *P. berghei* sensitive and resistant strains and *P. falciparum* clones W2 and D6 (Grogl *et al.*, 1991). On visual inspection, the chloroquine resistant *P. berghei*-line showed consistently greater expression of the 40-42 kDa component than the chloroquine-susceptible *P. berghei*-line. However, in *P. falciparum* there was no consistent difference between the chloroquine-resistant W2 and -susceptible D6 clones after C219 labelling of the 40-42 kDa component.

Speculatively, parasites express distinct P-glycoprotein precursors with different molecular sizes that can be products of distinct P-glycoprotein genes. The presence of P-gp like components in Plasmodium supports the hypothesis that this glycoprotein is an evolutionarily conserved determinant, responsible for the transport of toxic substances, as has been demonstrated in higher eukaryotes.

In mammalian MDR tumour cells, it has been shown that expression of P-glycoprotein is sufficient to confer the drug resistance phenotype (Guild *et al.*, 1988) and that the level of plasma-membrane-associated P-glycoprotein approximates the level of drug resistance observed. The recent studies on the genetic basis of chloroquine-resistance (Wellems *et al.*, 1990, 1991) and the demonstration of mutations in *pfmdr1* linked to chloroquine-resistance (Foote

et al., 1990) suggest that expression of mutant forms of Pgh1 alone is insufficient for chloroquine-resistance. Similarly, analysis of the expression and subcellular localization of Pgh1 in *P. falciparum* also supports the notion that overexpression of Pgh1 on the digestive vacuole membrane is not correlated with the level of chloroquine-resistance (Cowman *et al.*, 1991). Therefore, it is possible that Pgh1 may not function in a manner analogous to the mammalian P-glycoprotein which is involved in drug resistance.

1.8 MALARIAL PIGMENT

The human malaria parasite *P. falciparum* has a 48-h life cycle within host erythrocytes. The erythrocyte cycle is responsible for all of the clinical manifestations of falciparum malaria. The intraerythrocytic malaria parasite develops within a cell that contains a single major cytosolic protein, haemoglobin. The organism avidly ingests host haemoglobin and degrades it in a specialized proteolytic organelle called the digestive food vacuole (Rudzinska, 1965). As the parasite has a limited capability for *de novo* synthesis, or exogenous uptake of amino acids, the haemoglobin catabolism is the main source of amino acids for growth and maturation (Sherman, 1977).

Malarial pigment is an end product of the digestion of haemoglobin by the parasite. More than 30 years before the discovery of the malaria parasite, by Alphonse Laveran in 1880, malaria pigment was noticed in the organs of autopsied patients by Meckel (1847). During the course of investigations in the 20th century, it became clear that malarial pigment, also known as haemozoin, is a haem-containing protein-like material derived from the catabolism of haemoglobin, the chief protein constituent of the host red cell (Yamada and Sherman 1979).

1.8.1 DEGRADATION OF HAEMOGLOBIN

Haemoglobin is a haemoprotein (MW = 64 500), composed of four polypeptide chains, each in association with a haem group. Normal erythrocytes contain 18mM of haemoglobin monomers, each of which is composed of 143 amino acid residues, and the parasite degrades 25-75% of it (Ball *et al.*, 1948; Groman, 1951; Roth *et al.*, 1986). This estimation may be too low, as it is impossible to isolate the very late stages without other intermediate stages.

Evidence of the consumption of haemoglobin by malaria parasites during their growth in red cells has previously been derived from four sources of evidence. Firstly, the pallor of infected red cells, containing, for example, the larger forms of *P. vivax*, are supposed to be due to loss of haemoglobin from the red cell. The formation of malarial pigment and its identification with haematin indicate that the parasite utilizes haemoglobin, and accumulates the non-protein part.

Secondly, as found in avian and rodent erythrocytes infected with their respective species of malaria parasites (Sherman *et al.*, 1968), human erythrocytes infected with *P. falciparum* can also produce amino acids at a rate some hundredfold higher than do uninfected erythrocytes.

Thirdly, the composition of the amino acid pool of infected erythrocytes is similar to the amino acid composition of haemoglobin (Cenedella *et al.*, 1968; Zarchin *et al.*,1986).

Fourthly, the infection of erythrocytes containing radiolabelled haemoglobin is followed by the appearance of labelled amino acids in parasite proteins (Theakston *et al.*, 1970).

Haemoglobin degradation occurs predominantly during the trophozoite stage of the erythrocytic life cycle of *P. falciparum*. Trophozoites ingest erythrocyte cytoplasm which is incorporated into a large central food vacuole, where the haemoglobin rich cytoplasm is degraded (Aikawa, 1971). In the food vacuole, the process of haemoglobin breakdown leads to the release *in situ* of globin and ferroprotoporphyrin IX. The globin is hydrolysed into its constituent free amino acids, and the ferroprotoporphyrin IX (haem) moiety is autoxidized to ferriprotoporphyrin IX (ferrihaemic acid) and later to haematin (Fitch *et al.*, 1982). Malaria parasites lack haem oxygenase (Eckman *et al.*, 1977) which would detoxify the iron porphyrin of ferriprotoporphyrin. Haematin is a toxic substance which may be rendered both safe and insoluble by conjugation with a nitrogenous moiety to form the typical malarial pigment, haemozoin (Peters, 1987).

1.8.2 BINDING OF CHLOROQUINE BY HAEMATIN

Blood schizontocidal antimalarials may compete with the complexing protein (s) to form complexes with ferriprotoporphyrin IX, and prevent its sequestration as haemozoin (reviewed in chapter 1.2.1). The complexes are membranolytic, like free ferriprotoporphyrin IX, and lead to death of the parasite through breakdown of internal membrane permeability barriers (Fitch, 1983). The hypothesis advanced by Schueler and Cantrell (1964) and Cohen *et al.* (1964) that the binding of chloroquine by haematin might account for chloroquine resistance directed attention to haemoglobin digestion and its waste product "malarial pigment" as being possibly of significance in both the mode of action of chloroquine and in resistance to it. A possible mechanism for resistance is alteration in the protein (s) responsible for sequestering ferriprotoporphyrin IX as malaria pigment. They may be produced in greater quantity or with higher affinity for the iron porphyrin (Fitch, 1983).

1.8.3 CONTROVERSY OVER PIGMENT COMPOSITION

Carbone was the first to suggest the presence of the metalloporphyrin haematin (also known as ferrihaemic acid, ferriheme hydroxide and oxyhemin, all terms for ferriprotoporphyrin IX (FP) with a hydroxyl ion coordinating the fifth position of ferric ion) within the malaria pigment granule (Carbone, 1891).

Whereas the identity of haematin within haemozoin appears clear, there remains much controversy over other constituents of the malarial pigment granules.

It has previously been believed that malarial pigment is formed from a mixture of partially degraded molecules of haemoglobin, that is, it consists of haemin plus denatured protein derived from globin (Sherman *et al.*, 1968). However Homewood *et al.*, (1972b) queried this view and suggested that all preparations of pigment so far examined were heavily contaminated with membranes and that analysis of such preparations could lead to erroneous results. Homewood *et al.*, (1975) purified malarial pigment from red blood cells infected with *P. berghei* by treatment with sodium dodecyl sulphate (SDS) solution, to obtain pure membrane - free pigment. The purified pigment retained the apparently crystalline structure of pigment within the parasite. It contained approximately 1% iron, all of which could be accounted for in terms of haemin.

1.8.4 PROTEIN MEASUREMENT

The iron of malarial pigment is indeed in the ferric state. Thus at some point in the process of haemoglobin digestion and pigment formation the ferrous iron of haem is oxidised to ferric iron. The stage of digestion at which this occurs is not known. While several researchers maintain pigment is haematin alone (Fitch and Kanjananggulon 1987, Homewood *et al.*, 1972b), some evidence has

been reported that pigment is not chemically pure haematin (Morselt *et al.*, 1973), and consists of FP self-aggregates in a non-covalent complex of FP and protein (Balasubramanian *et al.*, 1984). Deegan and Maegraith (1956b) showed that pigment is not solely the iron protoporphyrin portion of haemoglobin, as had been assumed, but that it contains also a proteinaceous moiety. This conclusion was fully confirmed and extended by Sherman and Hull (1960). The presence of a protein component is well documented (Deegan and Maegraith 1956b, Sherman *et al.*, 1965), and evidence that the protein component is related to the host haemoglobin has been presented by Sherman and others (Sherman *et al.*, 1965, Sherman *et al.*, 1968, Yamada and Sherman 1979). Yamada and Sherman (1979) produced purified pigment from *P. lophurae* and subjected it and its associated protein components to SDS-polyacrylamide gel electrophoresis. The major proteinaceous components were of 21 kDa and 15 kDa molecular weight; it was concluded that, whilst the 21 kDa protein could be of parasite origin, the 15 kDa protein was possibly denatured globin monomers from the host cell haemoglobin.

Devine and Fulton (1941) found crude haemozoin from *P. knowlesi* to be one-sixth pigment (haematin), with 5 times its weight in insoluble "parasitic" protein. In recent studies, Ashong *et al.* (1989) demonstrated that the purest preparations of pigment contained approximately 43% FP and 57% protein, assuming that haemozoin consisted only of these two components. Their results confirmed that other components must make up less than 10% of the purified

material. A glycine-rich polypeptide of around 14 kDa molecular weight, which is synthesized by the parasite was also described (Ashong *et al.*, 1989). The iron porphyrin was not covalently bound to the protein. This suggests that the 14 kDa protein must have a very high affinity for FP. Ashong and his colleagues support the idea that the intraerythrocytic malarial parasite, incapable of cleaving the haem ring, detoxifies the iron porphyrin residium from haemoglobin catabolism in a crystalline complex with a specific parasite-derived protein.

Haemozoin has been implicated in the mode of action of chloroquine and resistance to this antimalarial drug has been associated with altered pigment formation. There have been suggestions that pigment may contain a protein component synthesized by the parasite (Moore and Boothroyd 1974, Fitch 1983). However, after fully reviewing the literature, the question has been raised whether any of the protein components found in isolated pigment may actually be contaminated artifacts that bind to haematin (FP) after disrupting the parasite.

1.9 OBJECTIVES OF THE PROJECT

A. To maintain an *in vitro* culture of both chloroquine sensitive and resistant *P. falciparum* parasites.

B. To determine the IC_{50} of B8509-035 on a chloroquine resistant strain, and its ability to reverse chloroquine resistance.

C. To investigate the existence of P-glycoprotein in malaria parasites with the use of monoclonal antibodies.

D. To isolate haemozoin and analyze its protein composition.

CHAPTER TWO

2 *IN VITRO* CULTURE OF *P. FALCIPARUM*

2.1 METHODOLOGY

The first report of the cultivation of intraerythrocytic parasites was given by Bass and John (1912), who obtained limited reproduction of *P. falciparum* and *P. vivax* in a simple suspension of defibrinated blood with supplememtary glucose. While their system proved of value for some elementary studies, the parasites never survived beyond one or two cycles at the best. A modification of their system did, however, later form the basis of a widely employed *in vitro* field test for evaluating the response of *P. falciparum* to chloroquine (Rieckmann *et al.*, 1968).

The next investigator to tackle the problem of maintaining the intraerythrocytic stages in the long term was William Trager. Starting in 1941 with the avian parasite *P. lophurae*, his efforts were rewarded over 30 years later when, with his assistant Jensen, he was able to establish long-term cultures of *P. falciparum* by adopting several simple but vital technical modifications (Trager and Jensen 1976). Almost simultaneously, a second group, working at the Walter Reed Army Institute of Research in Washington, also reported that they could keep *P. falciparum* alive *in vitro* (Haynes *et al.*, 1976).

"As with so many other things, it's all very simple once you know what to do. I doubt whether I can convey to you my feelings during February and March of 1976 when I had just two flow cultures going....(Jensen and Trager 1978).

2.2 STRAINS

Three *P. falciparum* strains were maintained *in vitro*, with slight modification (Freese *et al.*, 1988) to the original candle jar method (Jensen and Trager, 1977). FCR-3 is a chloroquine-resistant strain originally isolated from The Gambia (Jensen and Trager, 1978) with an IC_{50} of 96.32nM. This strain is known as the International Reference strain. Unlike RSA-2, this strain has lost the ability to produce gametocytes, due to its long adaptation to culture conditions. RSA-2 is a local chloroquine-sensitive strain with an IC_{50} for chloroquine of 40nM. The RSA-2 strain was isolated in Kwazulu on the 28th January 1985, from a patient who responded to treatment with Daraclor (1500 mg chloroquine base + 150 mg pyrimethamine base). The 3D7 strain is a chloroquine sensitive strain with an IC_{50} of 2nM, isolated from a patient in the Netherlands (Walliker *et al.*, 1987), and kindly donated to us by Dr Walliker from Edinburgh University.

2.3 CRYOPRESERVATION OF PARASITES

The mature stages of *P. falciparum* do not survive freezing and thawing, resulting in the cryopreservation of only the ring stage parasites. The parasitized cells were pelleted and diluted 1:1 with 28% (v/v) glycerol in phosphate buffered saline (PBS), placed in nunc-tubes and stored in liquid nitrogen.

2.3.1 PHOSPHATE BUFFERED SALINE (PBS)

Made up to PBS using autoclaved distilled water, and sterile filtered

NaCl.....0.123M

$Na_2 HPO_4$ X $2H_2O$.....8.315mM

$KH_2 PO_4$.....3.159mM

2.4 THAWING OF CRYOPRESERVED PARASITES

Three solutions were utilized to thaw cryopreserved parasites (Freese *et al.*, 1988)

a) 12% (w/v) NaCl

b) 1,6% (w/v) NaCl

c) 0.2% (w/v) dextrose in 0,9% (w/v) NaCl

The above solutions were made up in distilled water and sterilized through a 0.22μm millipore filter. The frozen nunc tubes were removed from the liquid nitrogen and placed in a water-bath (37°C) to defrost. Once defrosted, the blood suspension was transfered to a centrifuge tube. To every 1ml of blood suspension 0,1ml of 12% (w/v) NaCl was added dropwise and the suspension mixed. It was left to stand at room temperature for 3-5 min. 9 volumes of the 1,6% NaCl solution was added to the suspension and centrifuged at 400g for 5 min. 9 volumes of solution C was added to the pellet and centrifuged at 400g for 5 min. Freshly washed uninfected erythrocytes were added to the pellet to increase the haematocrit. Culture medium was added as explained under 2.1.5. In order to increase initial growth, RPMI 1640 medium containing 20% serum was utilized instead of the usual 10%.

2.5 CULTIVATION PROCEDURE

The parasites were cultured in 800ml (175cm^2) tissue-culture flasks containing 25ml of a 5% suspension of infected O^+ erythocytes in culture medium. Flasks were gassed after every medium change with a mixture of 3% oxygen, 4% carbon dioxide and 93% nitrogen (Afrox) and lids were tightly secured. The cells were incubated at 37°C, either in a static position or agitated on a shaker (Butcher, 1981). The shaker was switched off 1 hour prior to each medium change to allow the erythrocytes to settle. Flasks were slightly tilted, and spent medium was aspirated off. Twenty-five ml of fresh complete medium warmed to 37°C was added. Fresh red blood cells were added every 2 to 4 days to maintain a haematocrit of 5-10%, and

a parasitaemia of 1-10%. If parasites were harvested, the parasitaemia was allowed to go up to 30%.

2.5.1 PREPARATION OF CULTURE MEDIUM

Incomplete medium was made up in 1ℓ autoclaved, distilled water as follows:

RPM1 1640.....10,4g

Hepes buffer.....5,94g

Glucose.....4,0g

Hypoxanthine.....0,044g

Gentamicin.....0,050g

Culture medium was sterilized by filtering through a Sterivex-GS 0,22μm filter. It was dispensed aseptically in 90ml amounts and stored at -20°C until used. Complete medium was prepared by adding 10ml of human AB^+ plasma and 4,2ml of a 5% $NaHCO_3$ solution to 90ml of incomplete medium. Complete medium was stored at 4°C for no longer than one week.

2.5.2 RED BLOOD CELLS

Human O^+ erythrocytes was collected in CPD-A (citrate phosphate-dextrose-adenine) and stored at 4°C for a maximum of three weeks. Before addition to culture, erythrocytes were washed twice by centrifugation at 400g for 5 min in incomplete RPMl 1640 medium. Care was taken to remove the buffy coat. The unused washed erythrocytes were stored at 4°C for no longer than 24 hours.

2.5.3 PLASMA PREPARATION

Human AB^+ plasma was pooled from 3 individuals and inactivated at 56°C in a water bath for 2 hours. The inactivated plasma was centrifuged at 800g for 10 min. The supernatant was aliquoted into 10ml centrifuge tubes and stored at -70°C (Hui *et al*, 1984).

2.6 ASSESSMENT OF PARASITE GROWTH

Parasite growth and morphology was assessed by examination of Giemsa-stained smears prepared from the settled cells. Slides were fixed with 100% methanol for a few seconds and stained for 15 min with a 1:10 dilution of Giemsa in phosphate buffer. Slides were rinsed off with water, and examined microscopically with an oil immersion lens (1000X magnification). Ten fields were studied. For each field the

number of infected and non-infected cells in half of the field was counted and multiplied by two. The parasitaemia was then calculated using the following formula:

$$\% \text{ parasitaemia} = \frac{\text{infected cells X 100}}{\text{total cells}}$$

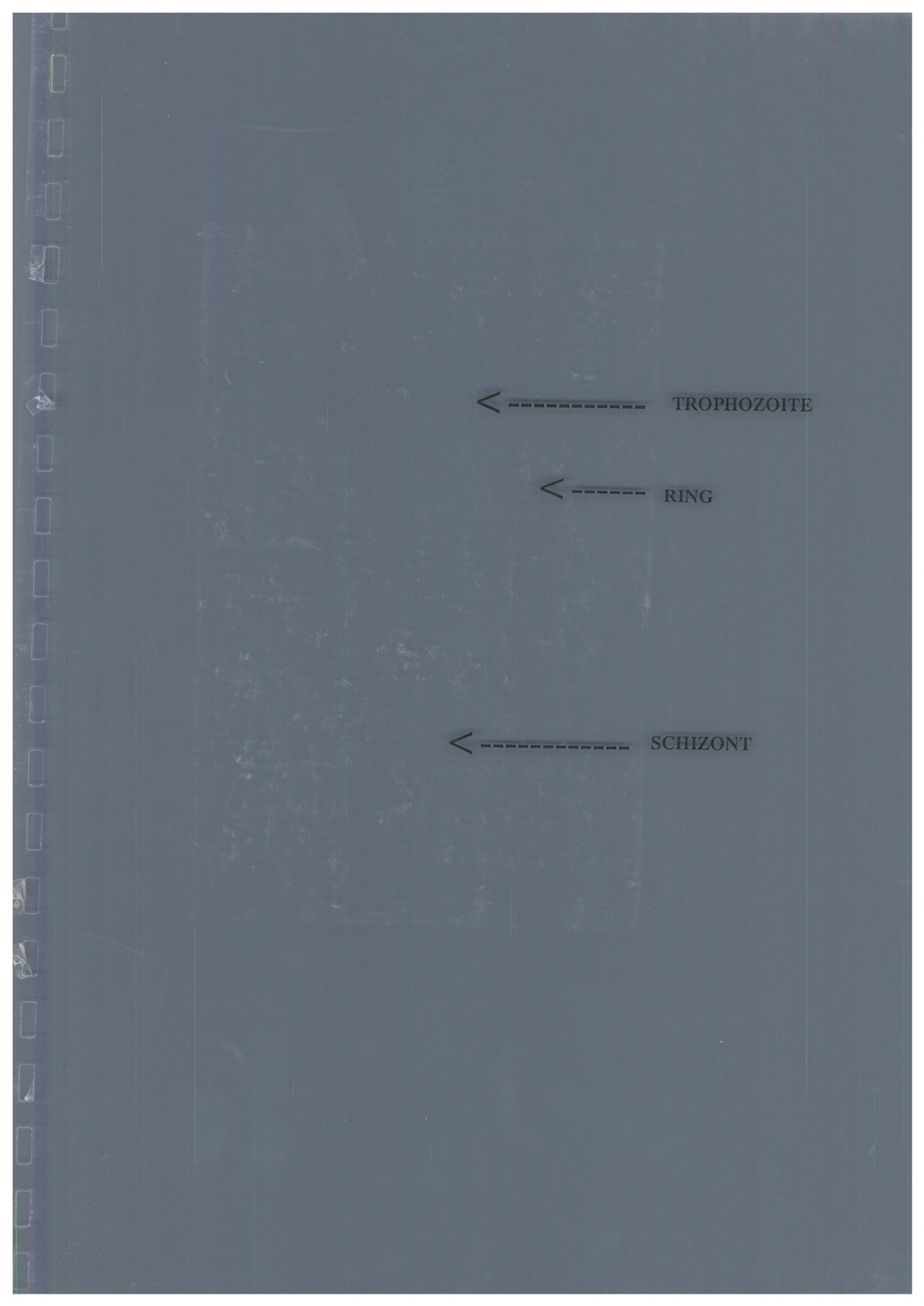
2.7 SYNCHRONIZATION

The culture was synchronized with 5% D-sorbitol (w/v) according to the method described by Lambros and Vanderberg (1979). Synchronization was carried out when 50% or more of the parasites in culture were in the early ring stage, as the mature stages are selectively lysed by the sorbitol. The culture was centrifuged at 400g for 5 min and the medium removed. The pellet, consisting of uninfected and parasitized erythrocytes, was resuspended in sorbitol, in 10 times the pellet volume and kept for 10 min at room temperature. The suspension was then centrifuged at 400g for 5 min and the pellet re-established into culture. This treatment maximises the parasite growth and permits efficient harvesting of specific stages.

2.8 RESULTS

Figure 7 represents a Giemsa stained smear from a mixed *P. falciparum* culture *in vitro* as seen under a microscope at 1000X magnification. The photo clearly indicates the three stages of the parasite namely; the ring, trophozoite, and schizont stage. Such a mixed culture would be treated with D-sorbitol to obtain a synchronized culture as indicated in figure 8. It was noted that higher parasitaemias could be obtained with a synchronized culture.

Figure 9 represents a culture producing gametocytes *in vitro*. The FCR-3 strain no longer produces these gametocytes, however the two chloroquine-sensitive strains namely; RSA-2 and 3D7, produced this sexual form when culturing conditions were not optimum. To optimize culturing conditions and therefore eliminate gametocyte production, the cultures were kept on an orbital shaker for 24hr and only removed for feeding purposes. It was also noted that their was a reduction to complete disappearance in the formation of gametocytes if the incomplete medium was saturated with CO_2. The gassed medium had a lower pH and required further addition of $NaHCO_3$ to maintain a pH of 7.4.


TROPHOZOITE
RING
SCHIZONT

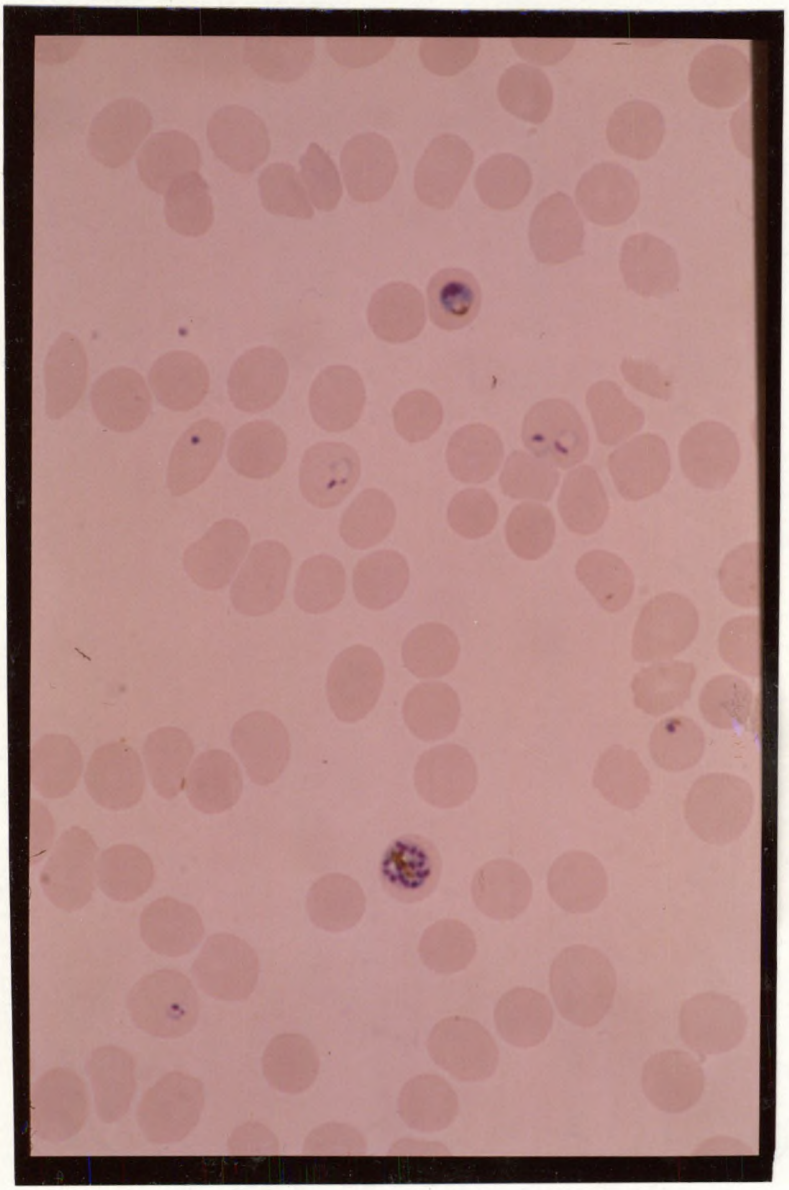


FIGURE 7: Mixed *in vitro* culture of *P. falciparum*

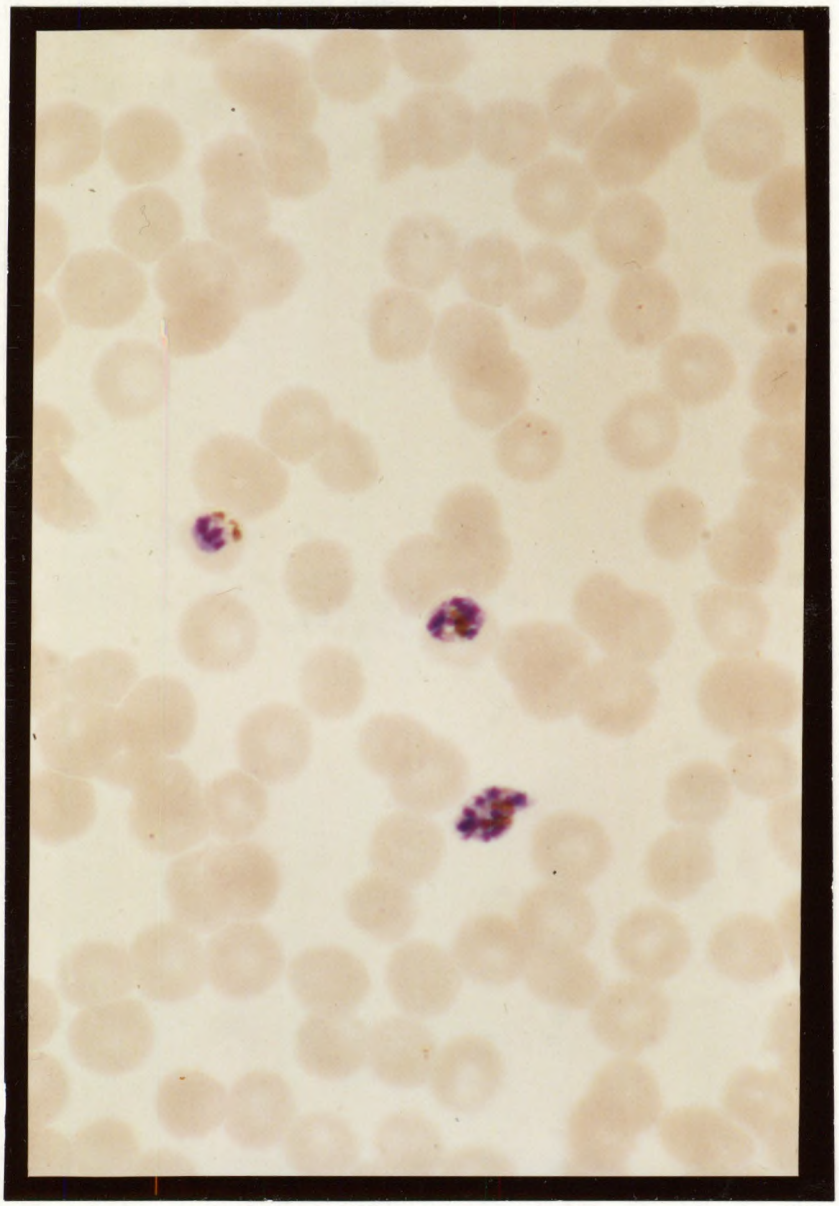
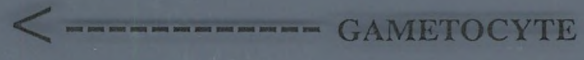


FIGURE 8: Synchronized *in vitro* culture with *P. falciparum* in late trophozoite stages


GAMETOCYTE

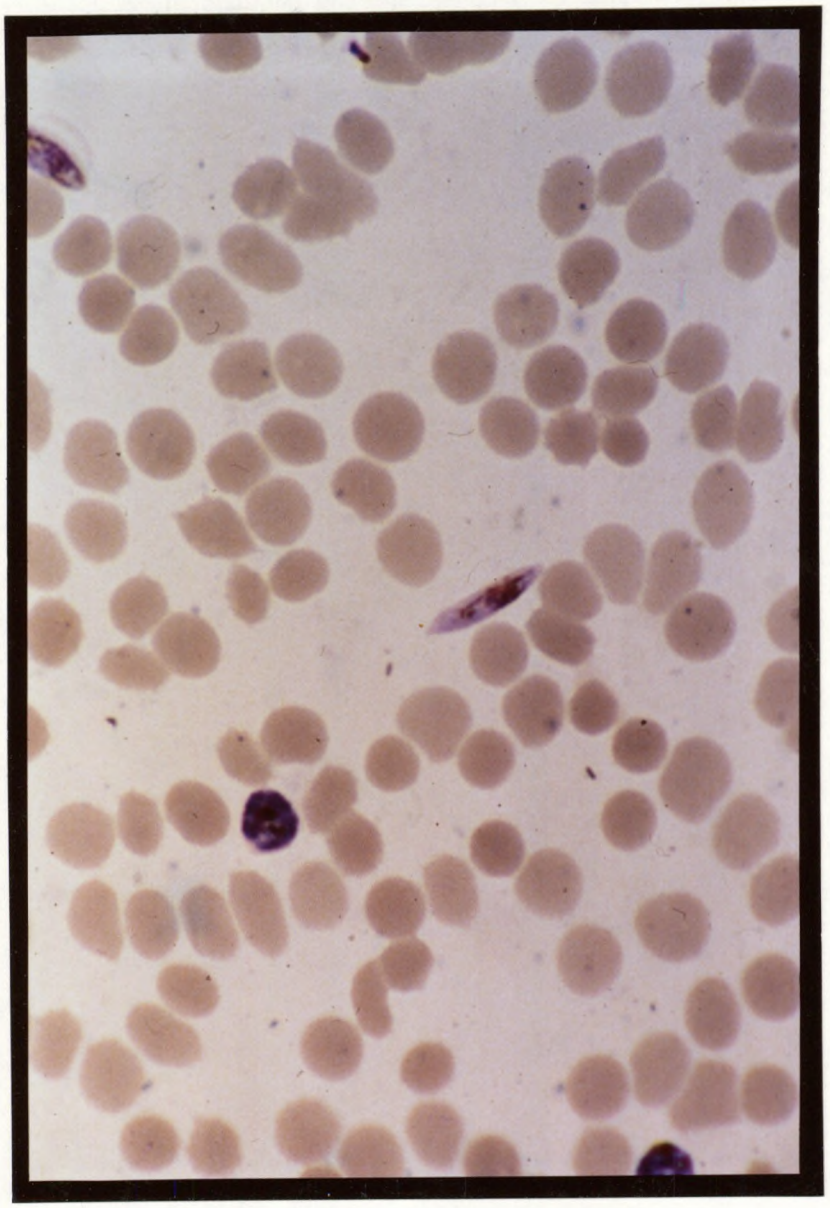


FIGURE 9: *In vitro* culture of *P. falciparum* showing gametocyte

CHAPTER THREE

3 CALCIUM CHANNEL BLOCKER - B8509-035

The study involves an investigation of the antimalarial properties of a novel calcium channel blocker - B8509-035; its efficacy alone and as a chloroquine reversing agent. The [G-^{3}H]-hypoxanthine method described by Desjardins *et al.* (1979) provided a quantitative measurement of the antimalarial activity of B8509-035. The assay is based on the inhibition of uptake of a radiolabelled nucleic acid precursor by the parasite during short-term cultures in 96-well microtitration plates.

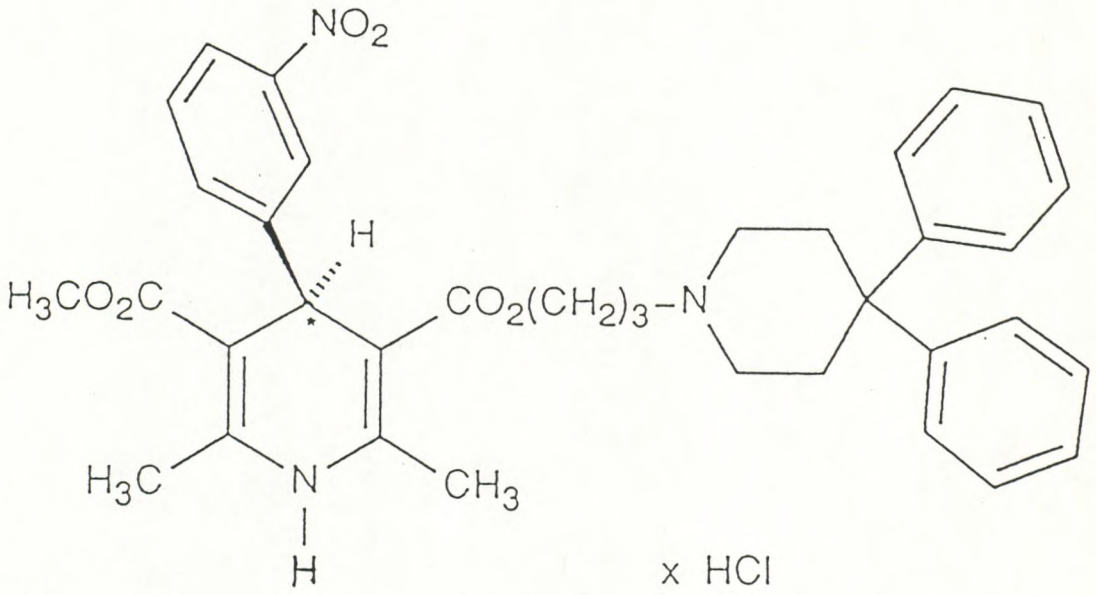
B8509-035 is the (-)-enantiomer of a new Ca^{2+}/calmodulin antagonist with dihydropyridine structure, which has cardiovascular activity at least an order of magnitude less than that of the more potent (+)-enantiomer. The cytotoxic effect of B8509-035 was studied in vincristine and daunomycin resistant cell lines. Addition of B8509-035 caused a reduction in vital cells. Addition of vincristine in concentrations of $5X10^{-9}$ M or 10^{-8} M daunomycin in the presence of B8509-035 caused almost complete cell killing (Haussermann *et al.*, 1990).

PRODUCT INFORMATION.

Chemical name: (-)-3-Methyl-5-[3-(4,4-diphenyl-1-piperidinyl)-propyl]-1,4-dihydro-2,6-dimethyl-4-(3-nitro-phenyl)-pyridine-3,5-dicarboxylate-hydrochloride,

Molecular formula: $C_{36}H_{39}N_3O_6$ X HCL

Structural formula:



Molecular weight: 646,19 (free base: 609,73)

3.1 METHODOLOGY

3.1.1 SECTION A - COMBINATION STUDY

Section A comprises a combination study with fixed ratios of chloroquine given concurrently with B8509-035, the calcium channel blocker. The study investigates the chloroquine-reversal properties of a new calcium-channel blocker on a chloroquine-resistant strain.

Section B deals with the varying IC_{50}'s of B8509-035 and its dependance on inoculum size.

3.1.2 PREPARATION OF PARASITES

The parasite inocula used in these experiments consisted of the FCR-3 (chloroquine-resistant) isolate of *P. falciparum*. The strain was grown continuously in stock cultures by a modification of the methods of Trager and Jensen (1976) (2.1.5). In preparation for addition to the microtitration plates, 50$\mu\ell$ of packed parasite from a stock culture with a 7% parasitaemia and 5% haematocrit was further diluted in 89ml culture medium containing 850$\mu\ell$ noninfected type 0-positive human erythrocytes to yield a final haematocrit of 1% and a parasitaemia of 0.5%.

The stock cultures were synchronized with sorbitol to ensure most of the parasites were in the ring stage at beginning of experiment. The culture medium utilized was prepared as explained under 2.6, with the omission of hypoxanthine. This ensured that the viable parasites took up the radiolabelled hypoxanthine.

3.1.3 PREPARATION OF DRUGS

The individual concentrations of chloroquine (CQ) and B8509-035 for the fixed ratio combination study were as follows;

chloroquine (nM)	0	50	100	150	200	250	300	350
B8509-035 (μM)	4	3.5	3	2.5	2	1.5	1	0

The chloroquine and B8509-035 concentrations were multiplied by 20 (dilution factor) and therefore became;

chloroquine (μM)	0	1	2	3	4	5	6	7
B8509-035 (μM)	80	70	60	50	40	30	20	0

The dilution factor became 20, as the final volume in each well was 250$\mu\ell$, and the volume of each drug added to the wells was 12.5$\mu\ell$.

The above individual concentrations for chloroquine and B8509-035 were prepared in incomplete hypoxanthine-free medium. The stock solutions were filtered through a 0.22μm millipore filter before diluted.

The serial dilutions for the chloroquine and B8509-035 combinations were prepared as indicated in tables (2-9).

TABLE 2: Dilution for B8509-035 at a concentration of 4μM

Tube no.	**medium ($\mu\ell$)**	**solution $\mu\ell$**	**conc. B8509-035 μM**
1	500	500 of 80μM	4.00
2	250	750 tube 1	3,00
3	250	750 tube 2	2.25
4	250	750 tube 3	1.69
5	250	750 tube 4	1.27
6	250	750 tube 5	0.95
7	250	750 tube 6	0.71
8	250	750 tube 7	0.53
9	250	750 tube 8	0.40
10	250	750 tube 9	0.30

TABLE 3: Dilutions for combination study of chloroquine at 50nM, and B8509-035 3.5μM

Tube no.	**medium ($\mu\ell$)**	**solution ($\mu\ell$)**	**conc. chloroquine (nM)**	**conc. B8509-035 (μM)**
1	500 of 1μM	500 of 70μM	50.00	3.50
2	250	750 tube 1	37.50	2.63
3	250	750 tube 2	28.13	1.97
4	250	750 tube 3	21.09	1.48
5	250	750 tube 4	15.82	1.11
6	250	750 tube 5	11.87	0.83
7	250	750 tube 6	8.89	0.62
8	250	750 tube 7	6.67	0.47
9	250	750 tube 8	5.01	0.35
10	250	750 tube 9	3.75	0.26

TABLE 4: Dilutions for combination study of chloroquine at 100nM, and B8509-035 3μM

Tube no.	**medium ($\mu\ell$)**	**solution ($\mu\ell$)**	**conc. chloroquine (nM)**	**conc. B8509-035 (μM)**
1	500 of 2μM	500 of 60μM	100.00	3.00
2	250	750 tube 1	75.00	2.25
3	250	750 tube 2	56.25	1.69
4	250	750 tube 3	42.19	1.27
5	250	750 tube 4	31.64	0.95
6	250	750 tube 5	23.73	0.71
7	250	750 tube 6	17.80	0.53
8	250	750 tube 7	13.35	0.40
9	250	750 tube 8	10.01	0.30
10	250	750 tube 9	7.51	0.23

TABLE 5: Dilutions for combination study of chloroquine at 150nM, and B8509-035 2.5μM

Tube no.	**medium ($\mu\ell$)**	**solution ($\mu\ell$)**	**conc. chloroquine (nM)**	**conc. B8509-035 (μM)**
1	500 of 3μM	500 of 50μM	150.00	2.50
2	250	750 tube 1	112.50	1.88
3	250	750 tube 2	84.38	1.41
4	250	750 tube 3	63.28	1.06
5	250	750 tube 4	47.46	0.79
6	250	750 tube 5	35.60	0.59
7	250	750 tube 6	26.70	0.45
8	250	750 tube 7	20.02	0.33
9	250	750 tube 8	15.02	0.25
10	250	750 tube 9	11.26	0.19

TABLE 6: Dilutions for combination study of chloroquine at 200nM, and B8509-035 2μM

Tube no.	**medium ($\mu\ell$)**	**solution ($\mu\ell$)**	**conc. chloroquine (nM)**	**conc. B8509-035 (μM)**
1	500 of 4μM	500 of 40μM	200.00	2.00
2	250	750 tube 1	150.00	1.50
3	250	750 tube 2	112.50	1.13
4	250	750 tube 3	84.38	0.84
5	250	750 tube 4	63.28	0.63
6	250	750 tube 5	47.46	0.48
7	250	750 tube 6	35.60	0.36
8	250	750 tube 7	26.70	0.26
9	250	750 tube 8	20.02	0.20
10	250	750 tube 9	15.02	0.15

TABLE 7: Dilutions for combination study of chloroquine at 250nM, and B8509-035 1.5μM

Tube no.	**medium ($\mu\ell$)**	**solution ($\mu\ell$)**	**conc. chloroquine (nM)**	**conc. B8509-035 (μM)**
1	500 of 5μM	500 of 30μM	250.00	1.50
2	250	750 tube 1	187.50	1.13
3	250	750 tube 2	140.63	0.84
4	250	750 tube 3	105.47	0.63
5	250	750 tube 4	79.10	0.48
6	250	750 tube 5	59.33	0.36
7	250	750 tube 6	44.50	0.27
8	250	750 tube 7	33.37	0.20
9	250	750 tube 8	25.03	0.15
10	250	750 tube 9	18.77	0.11

TABLE 8: Dilutions for combination study of chloroquine at 300nM, and B8509-035 1μM

Tube no.	**medium ($\mu\ell$)**	**solution ($\mu\ell$)**	**conc. chloroquine (nM)**	**conc. B8509-035 (μM)**
1	500 of 6μM	500 of 20μM	300.00	1.00
2	250	750 tube 1	225.00	0.75
3	250	750 tube 2	168.75	0.56
4	250	750 tube 3	126.56	0.42
5	250	750 tube 4	94.92	0.32
6	250	750 tube 5	71.19	0.24
7	250	750 tube 6	53.39	0.18
8	250	750 tube 7	40.05	0.13
9	250	750 tube 8	30.03	0.10
10	250	750 tube 9	22.53	0.08

TABLE 9: Dilution for chloroquine at a concentration of 350nM

Tube no.	**medium ($\mu\ell$)**	**solution $\mu\ell$**	**conc. chloroquine nM**
1	500	500 of 7μM	350.00
2	250	750 tube 1	262.50
3	250	750 tube 2	196.88
4	250	750 tube 3	147.66
5	250	750 tube 4	110.74
6	250	750 tube 5	83.06
7	250	750 tube 6	62.29
8	250	750 tube 7	46.72
9	250	750 tube 8	35.04
10	250	750 tube 9	26.28

3.1.4 PREPARATION OF MICROTITRATION PLATES

The microtiter plate consisted of 96 flat-bottom wells, arranged in a matrix of eight rows (A through H), and 12 columns (1 through 12) (Fig.10). The plates were prepared and harvested using strict aseptic techniques inside a laminar flow hood. Four 96-well microtitration plates were used to run the experiment.

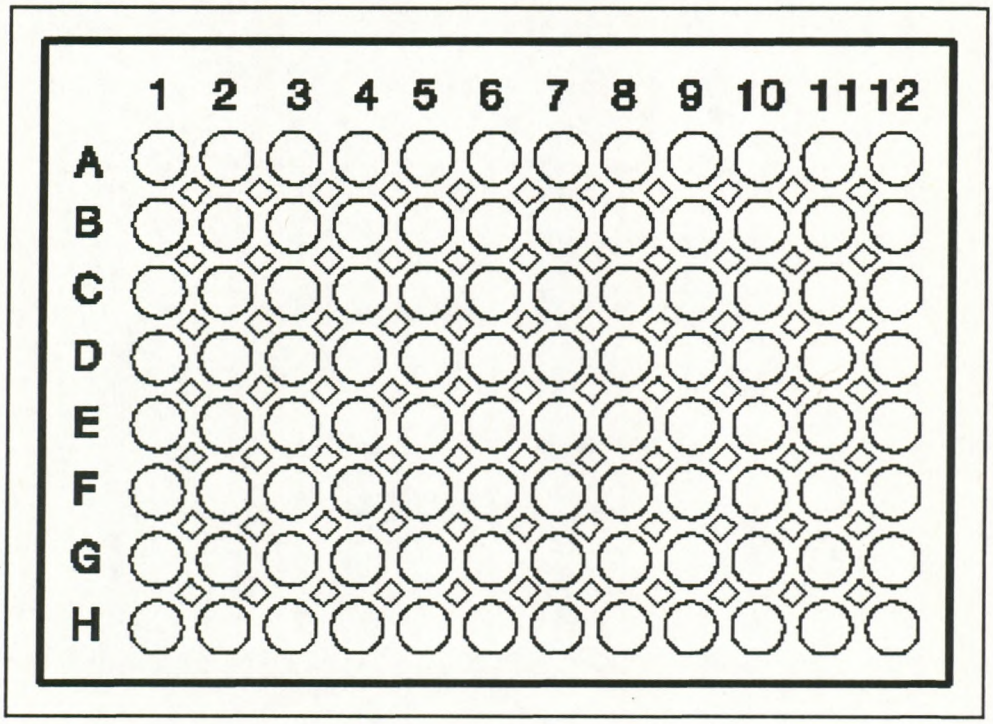


FIGURE 10: 96-well microtitration plate

The drug combinations in each plate were as follows:

Plate 1:	chloroquine 0	B8509-035 4μM
	chloroquine 50nM	B8509-035 3.5μM
Plate 2:	chloroquine 100nM	B8509-035 3μM
	chloroquine 150nM	B8509-035 2.5μM
Plate 3	chloroquine 200nM	B8509-035 2μM
	chloroquine 250nM	B8509-035 1.5μM
Plate 4:	chloroquine 300nM	B8509-035 1μM
	chloroquine 350nM	B8509-035 0

There were ten dilutions to each drug combination (table 2-9), and each dilution was done in quadruple. 200$\mu\ell$ of a synchronous parasitized erythrocyte suspension with a 1% haematocrit and 0.5% parasitaemia was aseptically added to each well. To the red blood cell control wells (row G, column 9-12), 200$\mu\ell$ of a noninfected erythrocyte suspension was added. The noninfected erythrocyte suspension included 100$\mu\ell$ packed red blood cells added to 9.9ml of complete hypoxanthine-free-medium. Twenty five $\mu\ell$ of the serial drug dilution was added to the corresponding well, except the parasitized red blood cell control (row H) and the red blood cell control wells (row G, column 9-12).

To these wells 25$\mu\ell$ of incomplete medium was added to make up the volume. After preparation as above, the plates were placed in a candle jar (Jensen and Trager, 1977), and incubated at 37°C for 24hr.

3.1.5 PREPARATION OF ISOTOPE AND LABELLING OF PARASITES

Uptake of [G-^{3}H]-hypoxanthine was used as an index of growth of the parasites. The isotope is supplied as a lyophylate (1-5 Ci/mmol) in ampoules containing 3.7mCi. The contents of a single ampoule were dissolved in 2.0ml of 50% ethanol to provide a stock solution which was stored at -20°C. In preparation for addition to the microtiter plates, the ethanol was evaporated from 0.2ml sample of stock solution, and 4,9ml of culture medium was added to the remaining 0.1ml of the isotope in water. The final isotope solution consisted of 74μCi [G-^{3}H]-hypoxanthine per ml of culture medium.

After the 24-hr incubation period described above, the plates were removed from the candle jar and 25$\mu\ell$ of the isotope in culture medium (1.85μCi) was added to each well. The plates were then returned to the candle jar and incubated at 37°C for an additional 18hr.

3.1.6 HARVESTING PARASITES AND SCINTILLATION COUNTING

At the end of the second incubation period, each plate was automatically harvested (TITERTEK Cell Harvester, Flow Laboratories) onto glass-fibre filters (Whatman). This instrument aspirated and deposited the particular contents of each of the wells onto small disks of filter paper. Each filter disk was dried and placed in a glass scintillation vial containing 5ml Aquagel®. All 96 vials corresponding to the 96 wells of the microtiter plate were counted on a liquid scintillation spectrometer for 1 min.

3.2 RESULTS

3.2.1 DATA ANALYSIS

A print-out was collected from the scintillation spectrometer with the counts in disintegrations per minute (dpm) for each vial corresponding to each well on the plate. For each plate the mean of the red blood cell control (RBC) and the parasitized red blood cell control (PRBC) was used.

The % effect for each dilution was calculated using the following formula:

$$\text{Effect (\%)} = \frac{\text{DPM - RBC}}{\text{PRBC - RBC}} \times 100$$

where;

DPM = Disintegrations per minute

RBC = Red blood cell control

PRBC = Parasitized red blood cell

The concentration-response curves for the active compounds over the specified range were characteristically sigmoidal after logarithmic transformation of the concentration and were interpreted by non-linear regression analysis, using the following formula:

Effect (%) = $Q/(1+((10\text{^}(C)/IC_{50})\text{^}-P))$ where;

IC_{50} = 50% inhibitory concentration of drug

Q = maximium effect

P = gradient

C = concentration

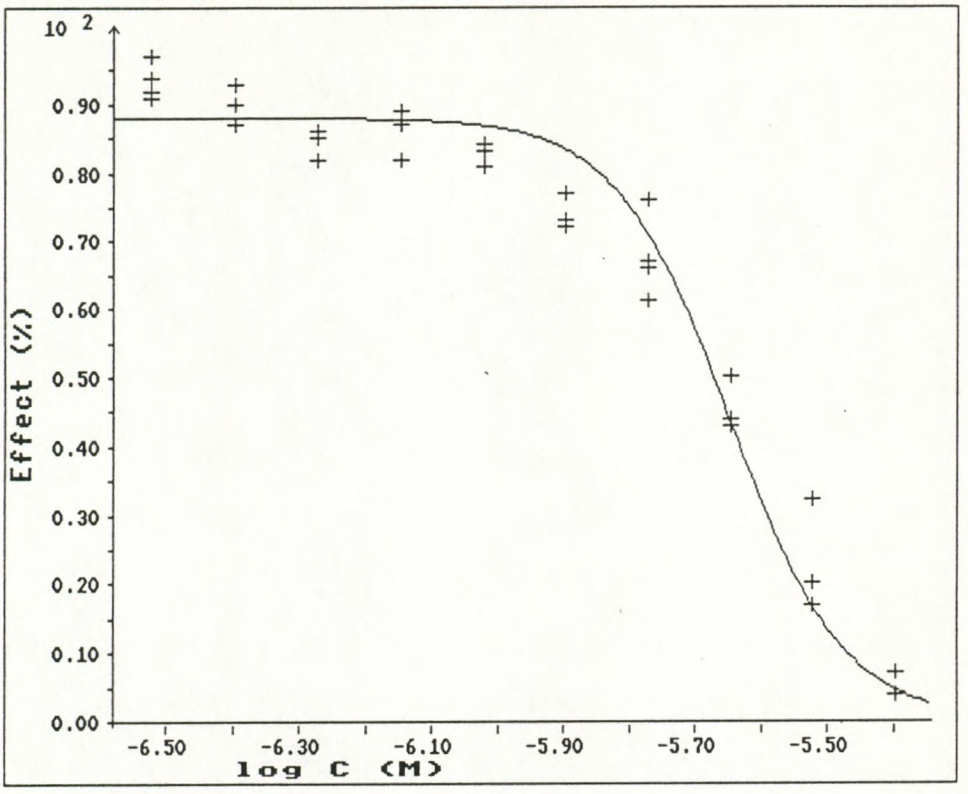
The computer program, Enzfitter® (Elsevier Biosoft) was used to generate the graphs. The output included a graph of the data with the fitted curve and accompanying estimate of the IC_{50}.

The drug concentrations, % effect and standard deviations are indicated in tables 10-17.

The dose response curves are presented in figures 11-18.

TABLE 10: Effect (%) for B8509-035 4μM

B8509-035 μM	Effect (%)				Mean	SD.
4.00	5.50	3.16	2.57	2.75	3.50	1.36
3.00	18.98	19.39	15.53	-	17.97	2.12
2.25	49.76	43.01	41.97	43.22	44.49	3.55
1.69	67.04	60.07	65.17	-	64.09	3.60
1.27	72.29	71.95	76.78	-	73.67	2.69
0.95	82.99	80.77	83.62	83.33	82.68	1.23
0.72	82.23	86.43	89.00	86.88	86.14	2.83
0.54	87.54	82.94	84.52	-	85.00	2.34
0.40	86.70	89.89	93.19	86.51	89.07	3.16
0.30	96.88	91.32	94.09	91.85	93.54	2.52



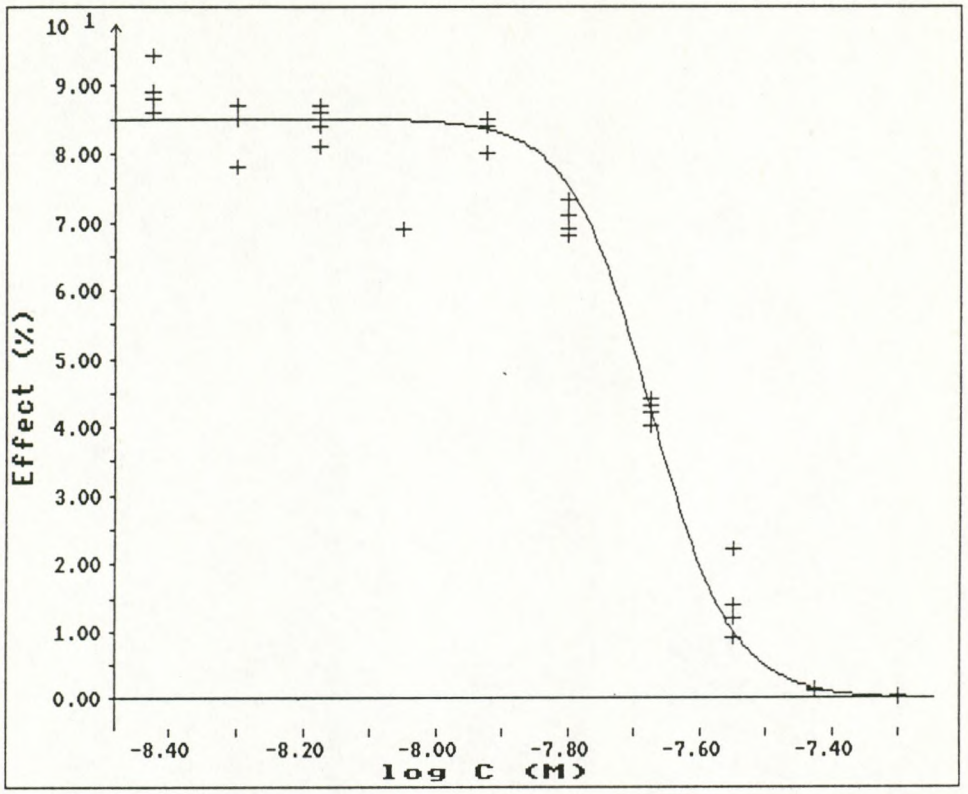
IC_{50} B8509-035 = 2.24 $\pm$ 0.046μM

FIGURE 11: Dose response curve for B8509-035 at 4μM

TABLE 11: Effect (%) for chloroquine 50nM and B8509-035 3.5μM

B8509-035 μM	chloroquine nM	Effect (%)				Mean	SD.
3.50	50.00	0.0	0.0	0.0	0.0	-	-
2.63	37.50	0.0	0.0	0.0	0.0	-	-
1.97	28.13	10.79	12.82	8.00	-	10.54	2.42
1.48	21.09	42.94	39.11	41.69	42.29	41.51	1.68
1.11	15.82	70.19	67.14	68.99	72.77	69.77	2.36
0.83	11.87	85.21	79.48	84.77	84.01	83.37	2.64
0.62	8.90	54.63	51.72	47.93	-	51.43	3.36
0.47	6.67	83.79	80.74	87.00	85.62	84.29	2.71
0.35	5.00	84.54	78.17	84.86	86.43	83.50	3.65
0.26	3.75	94.73	87.97	88.84	86.16	89.43	3.71

For serial dilution of the drugs, please refer to tables 3-8



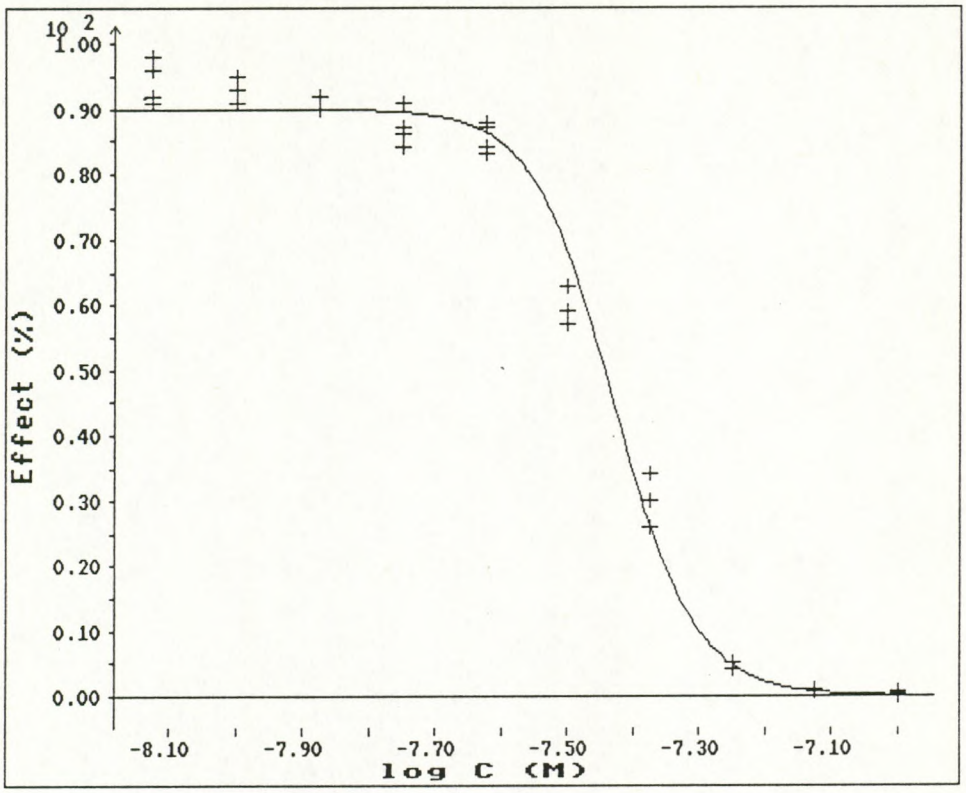
IC_{50} CQ = 21,89 $\pm$ 0,389nM

IC_{50} B8509-035 = 1,53 $\pm$ 0,027μM

FIGURE 12: Dose response curve for chloroquine 50nM and B8509-035 3,5μM

TABLE 12: Effect (%) for chloroquine 100nM and B8509-035 3μM

B8509-035 μM	chloroquine nM	Effect (%)				Mean	SD.
3.00	100.00	0.0	0.0	0.0	0.0	-	-
2.25	75.00	0.0	0.0	0.0	0.0	-	-
1.69	56.25	3.75	2.51	3.26	2.79	3.08	0.55
1.27	42.19	24.76	29.05	25.37	33.07	28.06	3.84
0.95	31.64	56.91	63.07	58.54	-	59.51	3.18
0.71	23.73	82.57	83.93	86.44	79.89	83.21	2.73
0.53	17.80	87.12	85.76	90.51	83.92	86.83	2.78
0.40	13.35	90.17	92.21	89.75	-	90.71	1.32
0.30	10.01	95.32	93.44	90.82	94.87	93.61	2.02
0.23	7.51	98.39	92.38	96.49	91.00	94.57	3.45



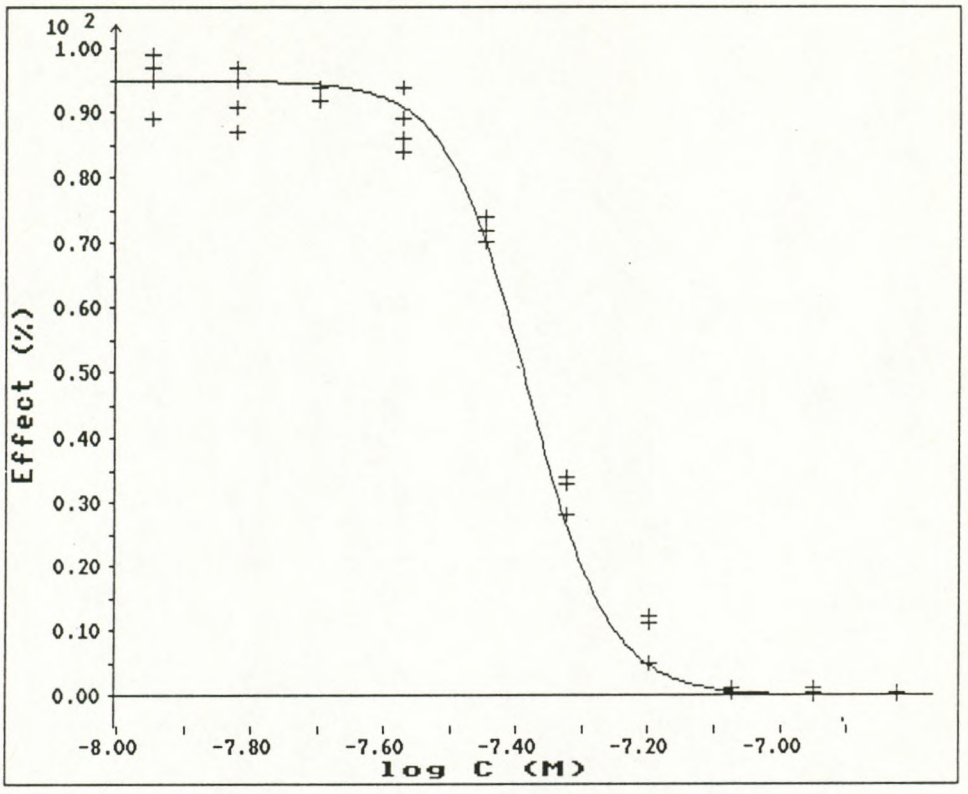
IC_{50} CQ = 37.17 $\pm$ 1.102nM

IC_{50} B8509-035 = 1.12 $\pm$ 0.033μM

FIGURE 13: Dose response curve for chloroquine 100nM and B8509-035 3μM

TABLE 13: Effect (%) for chloroquine 150nM and B8509-035 2.5μM

B8509-035 μM	chloroquine nM	Effect (%)				Mean	SD.
2.50	150.00	0.0	0.0	0.0	0.0	-	-
1.88	112.50	0.0	0.0	0.0	0.0	-	-
1.41	84.30	0.0	0.0	0.0	0.0	-	-
1.06	63.28	10.21	10.71	3.67	3.83	7.11	3.87
0.79	47.26	33.28	31.86	27.22	-	30.79	3.17
0.59	35.60	71.83	73.78	69.99	73.45	72.26	1.74
0.45	26.70	87.30	85.18	89.16	83.50	86.29	2.47
0.33	20.02	91.85	92.46	92.02	93.55	92.47	0.77
0.25	15.02	95.43	91.25	97.36	-	94.68	2.57
0.19	11.26	98.83	97.16	94.97	-	96.99	1.94



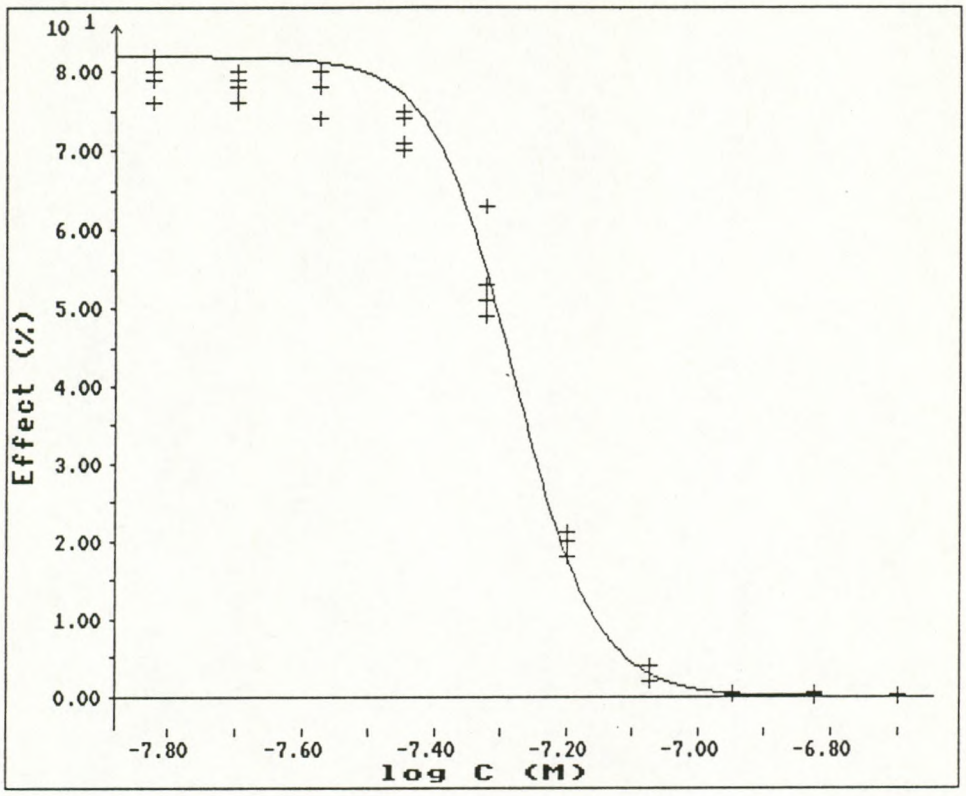
IC_{50} CQ = 41.36 $\pm$ 2.862nM

IC_{50} B8509-035 = 0.69 $\pm$ 0.048μM

FIGURE 14: Dose response curve for chloroquine 150nM and B8509-035 2.5μM

TABLE 14: Effect (%) for chloroquine 200nM and B8509-035 2μM

B8509-035 μM	chloroquine nM	Effect (%)				Mean	SD.
2.00	200.00	0.0	0.0	0.0	0.0	-	-
1.50	150.00	0.0	0.0	0.0	0.0	-	-
1.13	112.50	0.0	0.0	0.0	0.0	-	-
0.84	84.38	2.44	1.05	0.56	0.55	1.15	0.89
0.63	63.28	19.14	19.78	17.38	-	18.77	1.25
0.48	47.46	44.60	50.49	48.04	52.88	49.00	3.58
0.36	35.60	74.13	70.22	74.32	69.43	72.03	2.56
0.26	26.70	73.71	78.04	79.61	-	77.12	3.05
0.20	20.02	79.07	75.35	77.32	79.47	77.8	1.88
0.15	15.02	80.04	81.91	78.96	75.52	79.11	2.69



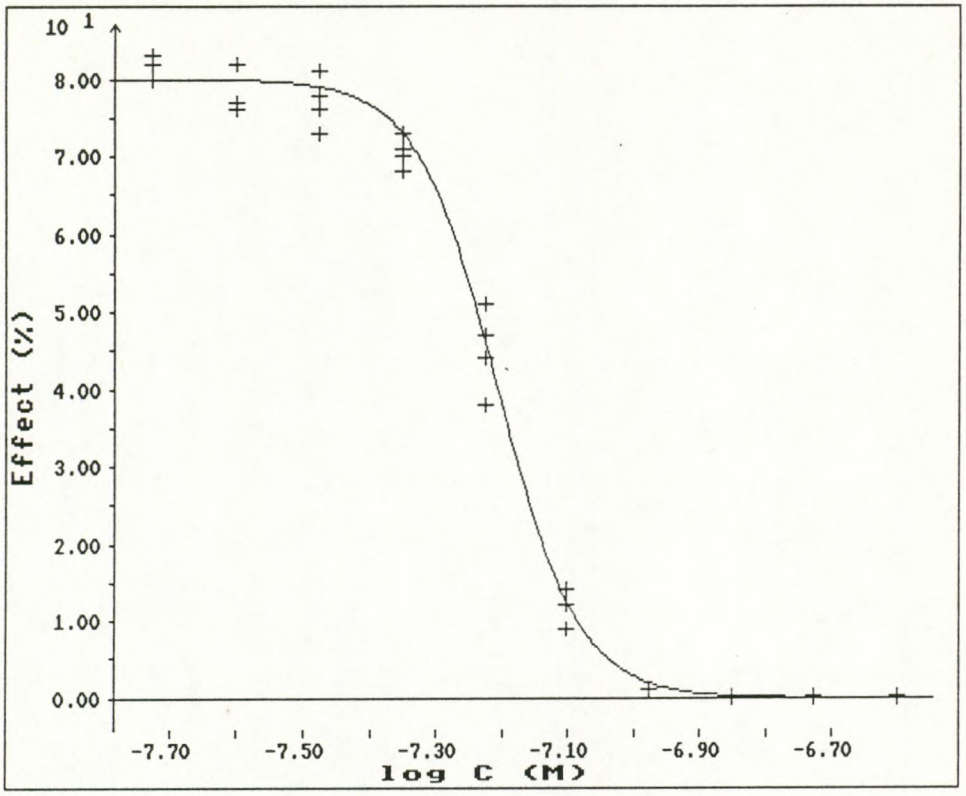
IC_{50} CQ = 52.51 $\pm$ 2.313nM

IC_{50} B8509-035 = 0.53 $\pm$ 0.023μM

FIGURE 15: Dose response curve for chloroquine 200nM and B8509-035 2μM

TABLE 15: Effect (%) for chloroquine 250nM and B8509-035 1.5μM

B8509-035 μM	chloroquine nM	Effect (%)				Mean	SD.
1.50	250.00	0.0	0.0	0.0	0.0	-	-
1.13	187.50	0.0	0.0	0.0	0.0	-	-
0.84	140.63	0.0	0.0	0.0	0.0	-	-
0.63	105.47	0.0	0.0	0.0	0.0	-	-
0.48	79.10	13.30	10.93	8.23	-	10.82	2.53
0.36	59.33	50.19	46.18	43.17	-	46.51	3.51
0.27	44.49	70.48	69.25	72.27	68.10	70.03	1.78
0.20	33.37	73.12	76.09	80.41	77.45	76.77	3.03
0.15	25.03	82.14	81.42	75.48	76.86	79.00	3.29
0.11	18.77	79.66	82.85	81.60	82.82	81.73	1.50



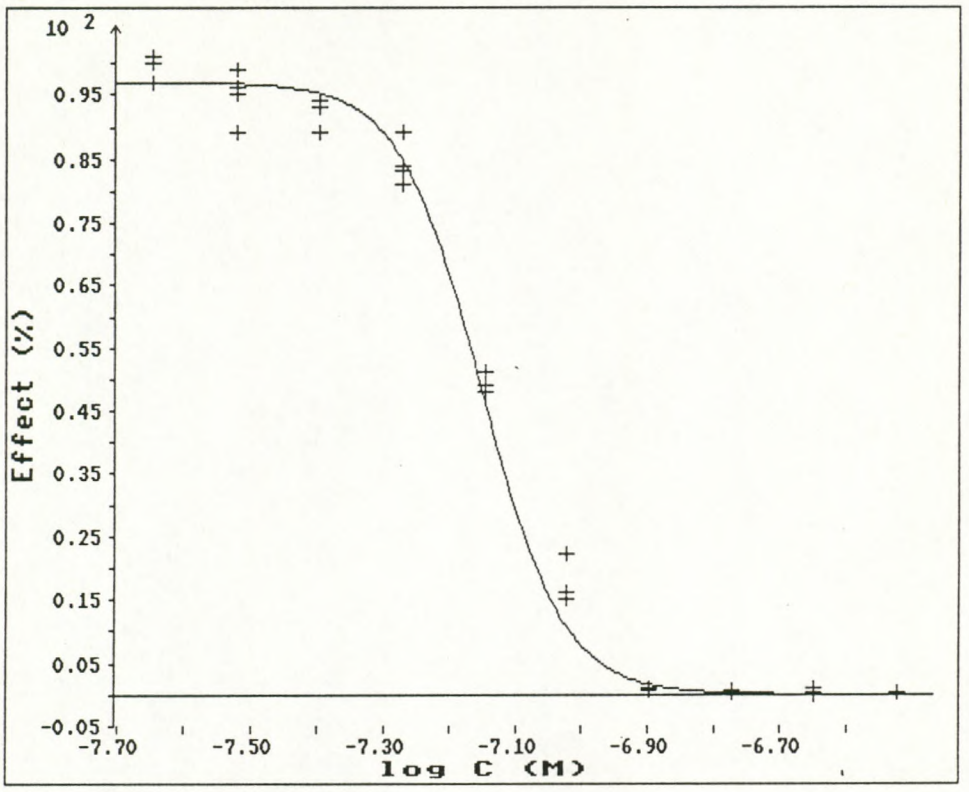
IC_{50} CQ = 62.08 $\pm$ 2.060nM

IC_{50} B8509-035 = 0.37 $\pm$ 0.012μM

FIGURE 16: Dose response curve for chloroquine 250nM and B8509-035 1.5μM

TABLE 16: Effect (%) for chloroquine 300nM and B8509-035 1μM

B8509-035 μM	chloroquine nM	Effect (%)				Mean	SD.
1.00	300.00	0.0	0.0	0.0	0.0	-	-
0.75	225.00	0.0	0.0	0.0	0.0	-	-
0.56	168.75	0.0	0.0	0.0	0.0	-	-
0.42	126.56	0.0	0.0	0.0	0.0	-	-
0.32	94.92	13.87	14.73	14.10	-	14.23	0.45
0.24	71.19	49.89	46.95	48.71	-	48.52	1.48
0.18	53.39	81.31	89.06	83.62	82.77	84.19	3.38
0.13	40.05	92.54	89.33	94.06	-	91.98	2.41
0.10	30.03	96.05	95.37	90.33	98.99	95.19	3.59
0.08	22.23	100.17	97.17	100.63	97.31	98.82	1.84



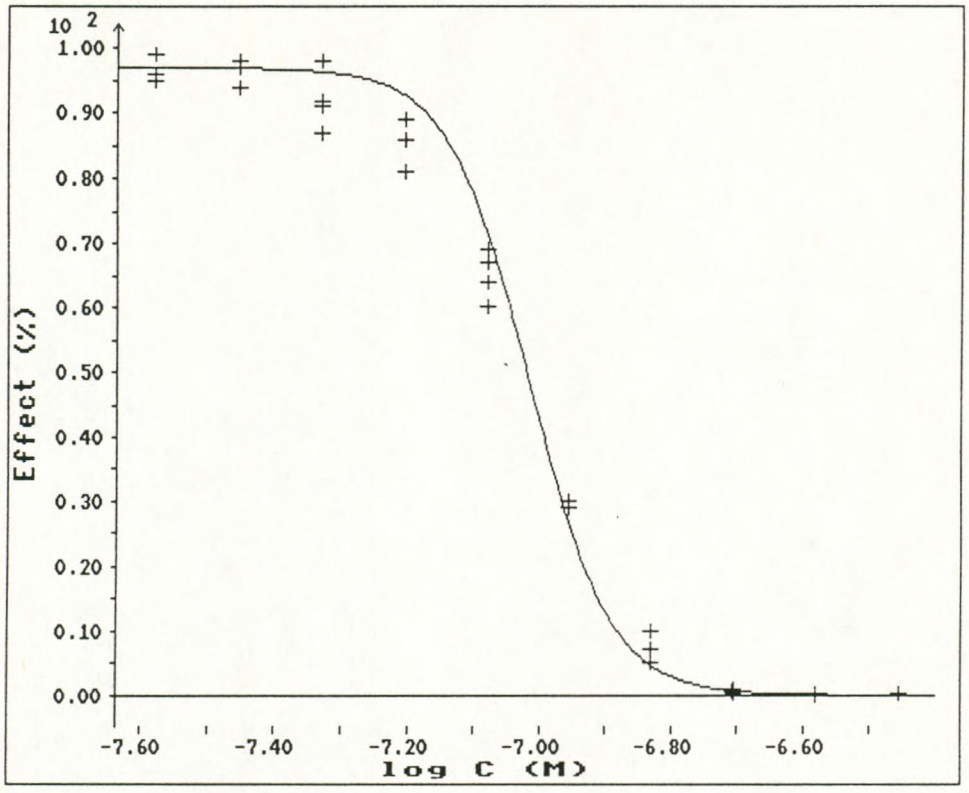
IC_{50} CQ = 70.32 $\pm$ 5.165nM

IC_{50} B8509-035 = 0.23 $\pm$ 0.017μM

FIGURE 17: Dose response curve for chloroquine 300nM and B8509-035 1μM

TABLE 17: Effect (%) for chloroquine at 350nM

chloroquine nM	Effect (%)				Mean	SD.
350.00	0.0	0.0	0.0	0.0	-	-
262.50	0.0	0.0	0.0	0.0	-	-
196.88	0.0	0.0	0.0	0.0	-	-
147.66	5.61	3.31	8.53	8.90	6.59	2.63
110.74	29.04	28.50	28.43	28.49	28.62	0.28
83.06	66.00	63.40	69.14	-	66.18	2.89
62.29	86.23	85.25	88.53	-	86.67	1.68
46.72	91.70	97.72	90.45	-	93.29	3.88
35.04	97.14	95.92	92.74	95.95	95.44	1.88
26.28	95.68	98.69	93.88	98.49	96.69	2.34



IC_{50} CQ = 96.32 $\pm$ 5.934nM

FIGURE 18: Dose response curve for chloroquine at 350nM

The dose response curves may be reviewed as follows;

CQ nM	350	300	250	200	150	100	50	0
B8509-035 μM	0	1	1,5	2	2,5	3	3,5	4
IC_{50}								
CQ nM	96.32	70,32	62,08	52,51	41,36	37,17	21,89	-
B8509-035 μM	-	0,23	0,37	0,53	0,69	1,12	1,53	2,24

The relative IC_{50}'s for the isobologram (Fig. 19) are as follows:

CQ	1	0,73	0,64	0,55	0,43	0,39	0,23	0
B8509-035	0	0,10	0,17	0,24	0,31	0,50	0,68	1
Σ	1	0,83	0,81	0,79	0,74	0,89	0,91	1

On the isobologram the relative IC_{50} for chloroquine was plotted against the relative IC_{50} for B8509-035 (as shown above). When the points fall below the straight line then a synergistic effect is prevalent, if above the line then the combination is antagonistic and if on the line then an additive effect is present when both drugs are given concurrently.

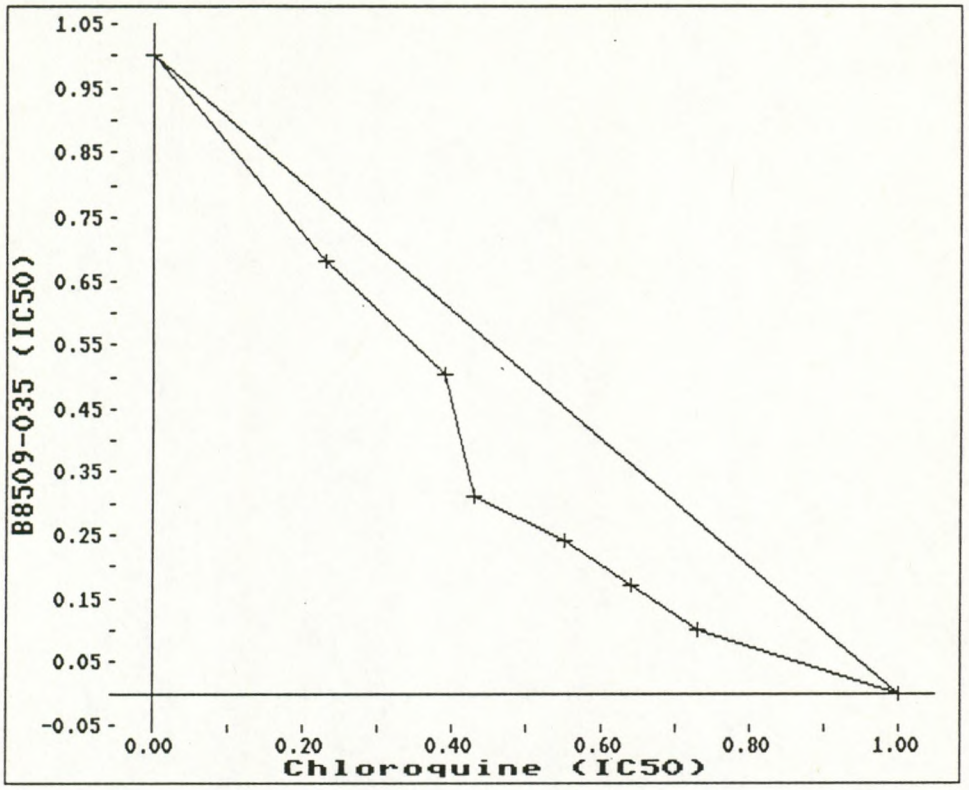


FIGURE 19: Isobologram for chloroquine versus B8509-035

3.3 SECTION B - IC_{50} FOR B8509-035

A standard IC_{50} for B8509-035 alone on the FCR-3 strain was determined with another three experiments. The methodology was as explained under section A, and will therefore not be covered here.

The only difference was that the parasitaemias and haematocrit of the diluted culture added to the plates was changed as follows;

a) for experiment one the culture had a 5% parasitaemia and 1% haematocrit, instead of the standard 0.5% parasitaemia and 1% haematocrit.

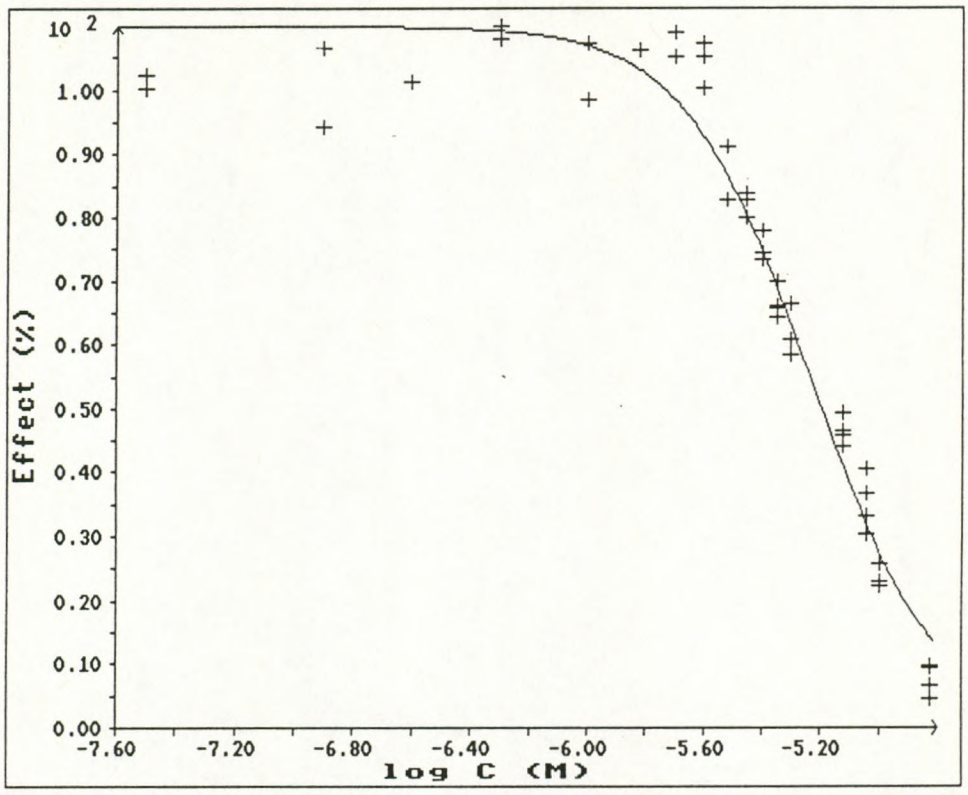
b) experiment two had the standard values i.e., 0.5% parasitaemia and 1% haematocrit.

c) experiment three had a 0.5% parasitaemia and a 1.5% haematocrit.

3.4 RESULTS

TABLE 18: Effect (%) for B8509-035 at 5% parasitaemia and 1% haematocrit

B8509-035 μM	Effect (%)				Mean	SD.
20.00	1.89	1.84	2.99	2.62	2.33	0.56
18.00	3.51	3.44	2.65	2.25	2.96	0.62
15.00	9.75	9.46	6.67	4.46	7.59	2.51
10.00	25.95	22.95	22.35	-	23.75	1.93
9.00	40.41	33.13	36.83	30.29	35.17	4.41
7.50	46.50	43.90	49.07	45.80	46.32	2.14
5.00	66.19	60.87	58.16	-	61.74	4.09
4.50	66.10	65.63	69.74	64.22	66.42	2.35
4.00	73.27	74.44	78.02	-	75.24	2.48
3.50	79.82	82.94	83.75	-	82.17	2.07
3.00	91.26	91.16	82.89	-	88.44	4.80
2.50	100.06	107.14	105.16	-	104.12	3.65
2.00	113.45	109.12	105.98	105.16	108.43	3.75
1.50	106.31	114.93	117.11	116.28	113.66	4.98
1.00	98.64	119.19	107.24	-	108.36	10.32
0.50	109.95	115.09	110.21	107.74	110.75	3.10
0.25	110.76	101.16	110.88	-	107.60	5.58
0.125	106.66	94.35	114.90	-	105.30	10.34
0.0625	119.62	111.69	113.13	-	114.81	4.22
0.03125	100.23	102.17	110.49	-	104.30	5.45

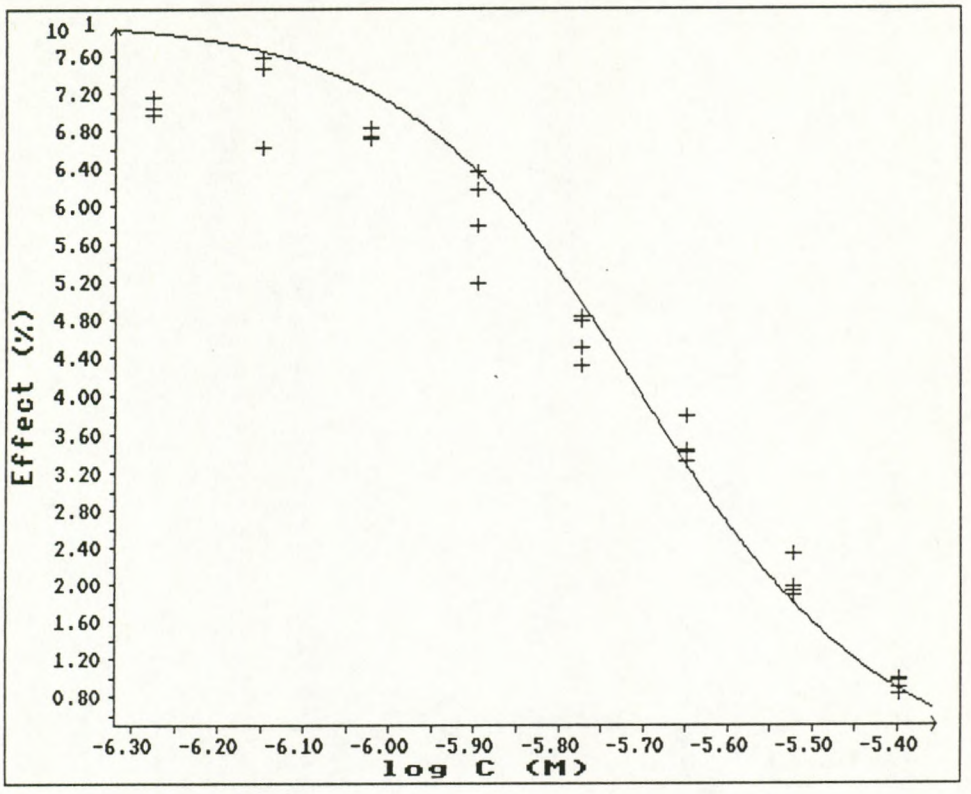


IC_{50} B8509-035 = 5.78 $\pm$ 0.201μM

FIGURE 20: Dose response curve for B8509-035 at 5%parasitaemia and 1% haematocrit

TABLE 19: Effect (%) for B8509-035 at 0.5% parasitaemia and 1% haematocrit

B8509-035 (μM)	**Effect (%)**				**Mean**	**SD.**
4.00	9.12	10.06	9.64	8.38	9.3	0.72
3.00	23.40	19.34	18.90	19.89	27.18	2.05
2.25	38.01	33.18	34.28	34.38	34.96	2.10
1.69	43.13	48.25	45.03	47.79	46.05	2.41
1.27	51.74	61.68	58.05	63.62	58.77	5.22
0.95	67.19	67.46	68.21	67.45	67.58	0.44
0.71	66.07	75.75	74.71	-	72.18	5.31
0.53	70.53	71.68	69.62	-	70.61	1.03

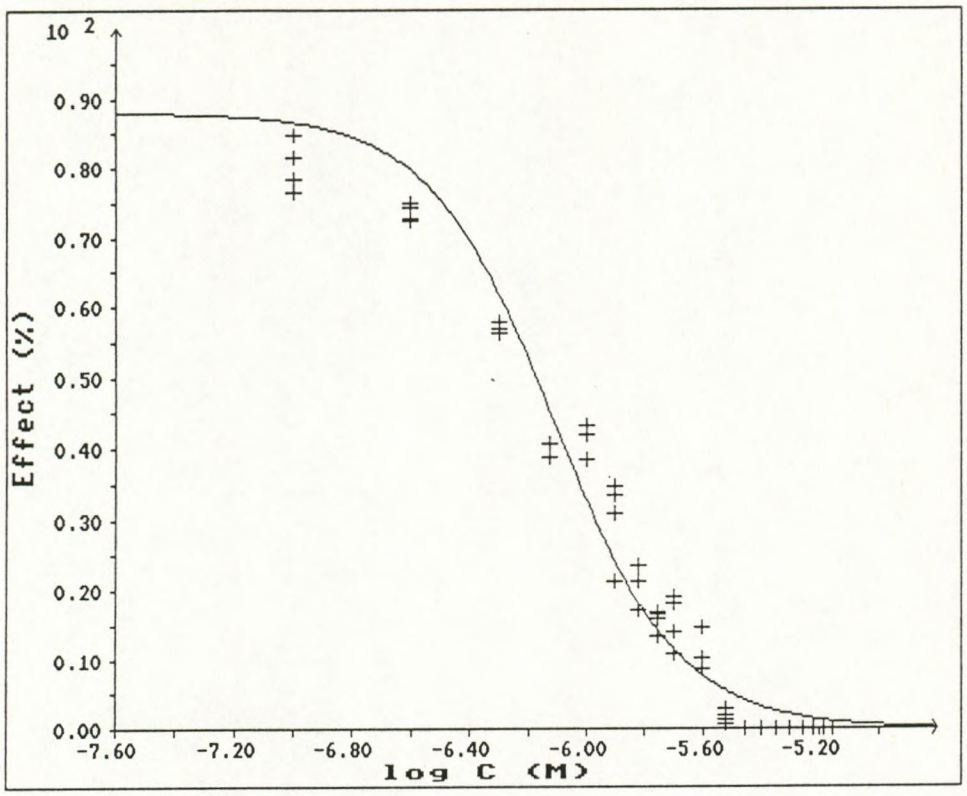


IC_{50} B8509-035 = 1.99 $\pm$ 0.102μM

FIGURE 21: Dose response curve for B8509-035 at 0.5% parasitaemia and 1% haematocrit

TABLE 20: Effect (%) for B8509-035 at 0.5% parasitaemia and 1.5% haematocrit

B8509-035 (μM)	Effect (%)				Mean	SD.
7.0	0.0	0.0	0.0	0.0	-	-
6.5	0.0	0.0	0.0	0.0	-	-
6.0	0.0	0.0	0.0	0.0	-	-
5.5	0.0	0.0	0.0	0.0	-	-
5.0	0.0	0.0	0.0	0.0	-	-
4.5	0.0	0.0	0.0	0.0	-	-
4.0	0.0	0.0	0.0	0.0	-	-
3.5	0.0	0.0	0.0	0.0	-	-
3.0	1.35	1.95	0.75	2.73	1.70	0.85
2.5	8.72	14.60	10.29	-	11.20	3.05
2.0	19.05	10.89	18.17	14.11	15.56	3.78
1.75	13.18	16.83	15.95	16.41	15.59	1.65
1.50	23.55	21.30	17.09	17.16	19.78	3.19
1.25	34.75	33.35	30.77	21.37	30.06	5.93
1.00	41.75	43.11	38.52	-	41.13	2.36
0.75	38.81	38.84	40.62	38.87	39.29	0.89
0.50	56.14	56.89	57.74	-	56.92	0.80
0.25	74.33	72.73	74.99	72.52	73.64	1.21
0.10	76.61	78.55	81.62	84.91	80.42	3.63



IC_{50} B8509-035 = 0.87 $\pm$ 0.051μM

FIGURE 22: Dose response curve for B8509-035 at 0.5 % parasitaemia and 1.5 % haematocrit

In summary; the varying IC_{50}'s for B8509-035 on the FCR-3 strain were as follows:

a) 5% parasitaemia, 1% haematocrit = $5.78 \pm 0.201\mu M$

b) 0.5% parasitaemia, 1% haematocrit = $1.99 \pm 0.102\mu M$

c) 0.5% parasitaemia, 1.5% haematocrit = $0.87 \pm 0.051\mu M$

3.5 DISCUSSION

The literature is well documented with examples of calcium-channel blockers, and their reversal of chloroquine resistance in *P. falciparum* (Martin *et al.*, 1987). The reversal of sensitivity to chloroquine has been attributed to an efflux pump, that would export the drug (e.g. chloroquine) out of the cell therefore not obtaining therapeutic levels. Reversal of sensitivity by calcium channel blockers has been explained on the basis that the calcium channel blocker competes with the therapeutic drug for the efflux pump, resulting in an overall decreased efflux of the therapeutic drug.

In the above experiments, the IC_{50} for chloroquine alone was 96,32nM and for B8509-035 alone ranged from $0.87\mu M$ to $5.78\mu M$. This variation in the IC_{50} for B8509-035 can be explained mainly by variations in inoculum size (inoculum size is a product of haematocrit and parasitaemia). This inoculum size effect has been described for chloroquine (Geary *et al.*, 1990). The

respective IC_{50}'s dropped considerably when the 2 drugs were given concurrently (Table 10-17). The IC_{50} for chloroquine dropped from 96,32nM to 21,89nM when given concurrently with B8509-035. The IC_{50} for B8509-035 also dropped from 2,24μM when given alone to 0,23μM when given with chloroquine. It can be seen from the isobologram (Fig.19) that there is a synergistic effect i.e. the sum of the relative inhibitor concentrations is smaller than one. This translates, that to obtain the same therapeutic effect, the dose for each compound when given in combination is lower than the necessary dose if each compound was given alone. The results conform to the predicted reversal of chloroquine resistance with calcium channel blockers. This reversal has been attributed to the presence of an efflux pump. Hofmann and colleagues (personal communication from drug company) concluded that B8509-035 is an inhibitor of P-glycoprotein in multidrug resistant cancer cell lines. However, the chloroquine reversal caused by B8509-035 in malaria parasites is not that pronounced, and this could perhaps be accounted for, by the strong intrinsic antimalarial property of B8509-035. Calcium channel blockers on their own have an intrinsic antimalarial property, (Scheibel *et al.*, 1987) which is not related to reversal of resistance. Using the same experimental system, other workers found that the IC_{50} for diltiazem and nifedipine (calcium channel blockers) were 14.1μM and 20.8μM respectively (Krogstad *et al.*,1991). If we compare this to the IC_{50} of B8509-035 (IC_{50} = 2,24μM) one would have to conclude that B8509-035 has a stronger intrinsic antimalarial effect.

I would conclude by stating that B8509-035 causes a weak reversal of chloroquine resistance which may be due to, either its potent intrinsic antimalarial effect, and/or the efflux pump on the parasite membrane has a lower specificity for the molecular structure of B8509-035 and therefore requires certain concentrations to achieve this effect. At the concentrations that are needed to reverse chloroquine resistance, the parasites are already dead.

CHAPTER FOUR

4 P-GLYCOPROTEIN

The stated hypothesis, is that the *P. falciparum* strains which are resistant to the actions of chloroquine, have an overexpression of a membrane glycoprotein which pumps the drug out. This glycoprotein (receptor) would not be expressed in the chloroquine sensitive strains. The resistance to chloroquine is reversed by calcium channel blockers e.g. B8509-035, due to the specific competition for the receptor resulting in increased levels of therapeutic drug within the parasite.

The presence of P-glycoprotein in malaria parasites was investigated with the aid of monoclonal antibodies (C-219 and JSB-1). Two methods were employed;

Section A = Western Blotting

Section B = Immunohistochemistry Staining

4.1 METHODOLOGY

4.1.1 SECTION A - WESTERN BLOTTING

4.1.2 PARASITE ISOLATION

The isolation of malaria parasites free from the host erythrocytes is a difficult process because the intraerythrocytic parasite has a food vacuole containing host derived material and the parasitophorus membrane originates partly from the erythrocyte membrane. Ideally, we would like to obtain parasites for biochemical studies, as free from host proteins as possible. *P. falciparum* parasites were isolated by one of two methods:

a) Gelatine sedimentation followed by saponin lysis of erythrocytes

b) Differential Freezing Method

Both methods were carried out on the mature stages of the parasite. The latter method was experimentally established in our laboratory.

4.1.2.1 GELATINE SEDIMENTATION

Gelatine sedimentation was carried out in order to enrich infected cells from 10% parasitaemia to greater than 75% (Jensen, 1978). 1% (w/v) gelatine was dissolved in incomplete RPMI 164O medium by warming to 56°C and sterile filtered through a 0.22μm millipore filter. The culture was centrifuged at 400g for 5 min. To 2ml pellet, 6ml of 1% gelatine and 12 ml incomplete medium was added, mixed, vortexed and centrifuged for 5 min at 400g. To 2ml of pellet, 5 ml 1% gelatine and 5ml incomplete medium were added. The suspension was aliquoted into centrifuge tubes, each containing 5-7ml. The tubes were then placed in a 37°C water bath until sedimentation occured. A brownish supernatant remained after approximately 30 min. The uninfected erythrocytes and those parasitized with young rings rapidly settled out to form the sediment, while the cells infected with the mature trophozoites and schizonts remained in suspension, due to decrease cell deformability and their distorted shape. This supernatant was collected and centrifuged for 10 min at 400g. The pellet was concentrated with trophozoite and schizont enriched parasites, which could either be reestablished into culture, or further used for parasite isolation.

4.1.2.2 SAPONIN LYSIS

In order to lyse the erythrocytes and release the parasites, the pellet obtained from the gelatine sedimentation or a pellet from a culture with high parasitaemia was saponin treated (Zuckerman *et al.*, 1967). Two times the pellet volume of a 0,05% (w/v) saponin solution was added, mixed and left at 37°C for 5 min. Effective lysis of the erythrocytes obtained after centrifugation (400g for 5min) of the pellet was determined by light microscopy.

The "free parasites" were washed three times in PBS to remove haemoglobin and were stored at -70°C until used.

4.1.2.3 DIFFERENTIAL FREEZING METHOD

A culture with a 10% late stage parasitaemia was centrifuged and a 3 times sucrose-tris-EDTA (0,25M sucrose, 5mM tris-HCL, 1mM EDTA, pH=7,4) solution was added to the pellet volume and placed at -70°C for at least 1 hour. After thawing, haemolysis was evident. The suspension was centrifuged at 400g for 10 minutes and the red supernatant discarded. The pellet which contained the "free parasites" was washed a number of times until it became free of evident haemoglobin contamination. The parasite pellet was stored in one times the pellet volume of 0,1% (w/v) sodium dodecyl sulphate at -70°C until required.

4.1.3 PARASITE LYSIS

The "free parasites" were lysed by freeze-thawing. The suspension was placed in liquid nitrogen until completely frozen and then immediately placed in a water-bath at 37°C until thawed. This procedure was repeated three times. The resulting homogenate was centrifuged at 900g for 10min, and the supernatant aliquoted into ependorf tubes and stored at -70°C until utilized. The sample contained parasitic membrane and cytoplasmic proteins, which were analysed by polyacrylamide gel electrophoresis.

4.1.4 HIGH RESOLUTION SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

To analyse the protein composition of the sample, we utilized a polyacrylamide gel which has a gradient of increasing acrylamide concentration and hence decreasing pore size. Sodium dodecyl sulphate (SDS) was used as the dissociating agent.

4.1.4.1 CASTING GELS

A 3 - 20% linear gradient gel was prepared as follows (Hempelmannn, 1984):

3% Gel

- 2ml of 0,8M Tris-HCl pH 8,6
- 0,75ml of an acrylamide and bisacrylamide solution (38,9g acrylamide, 1,1g bisacrylamide made to 100ml)
- 7,25ml H_20
- 8mg ammonium persulphate (PER)

20% Gel

- 2ml of 0,8M Tris-HCl pH 8,6
- 5ml of an acrylamide and bisacrylamide solution
- 3ml H_20
- 8mg PER

It cannot be emphasized enough that both acrylamide and bisacrylamide monomers are highly toxic either by skin absorption or by inhalation of monomer powder. The effects range from skin irritation to central nervous system damage. Therefore

disposable plastic gloves were worn at all times when handling monomers in either solid or liquid form.

Polymerization of acrylamide is initiated by the addition of ammonium persulphate. In addition 5μl N,N,N',N'- tetramethylethylenediamine (TEMED) was added as an accelerator of the polymerization process. Increases in either the TEMED or ammonium persulphate concentration increase the rate of polymerization. The concentration chosen was to enable the handling for nearly 10 min. If after 10 min the polymerization was not complete, the amount or quality of PER or TEMED was insufficient.

4.1.4.2 BUFFERS

Electrode Buffer

The electrode buffer was prepared as follows:

4,8g Tris was added to 3,0g glycine and the volume made up to 1ℓ with distilled water. 0,5g SDS was added to 500ml of the above solution which constituted the cathode buffer. The remaining 500ml was used as anode buffer.

Sample Buffer

To 20$\mu\ell$ of sample (constituting parasitic proteins) 20$\mu\ell$ of sample buffer was added, which was prepared as follows:

1,2g..... urea

1.8ml... H_20

0.05g....SDS

4 mg ....DTT

1 drop of phenol red

4.1.4.3 APPARATUS

The apparatus utilized to carry out the electrophoresis was that of Studier (1973) as indicated in figure 23. Essentially the apparatus consisted of two buffer reservoirs, the upper of which was notched, supported on an integral plastic stand. The gel was formed between two glass plates each about 0,3cm thick. One plate was rectangular in shape and the second was the same size but with a notch cut in one edge. The two plates were placed together to form the gel holder with a plastic spacer running down each vertical side of the sandwich. Sample wells were formed in the gel during polymerization using a plastic sample comb; sample well number and dimensions could then easily be altered by changing the number and dimensions of the comb teeth. The polymerized gel was held between its glass plates, and was attached to

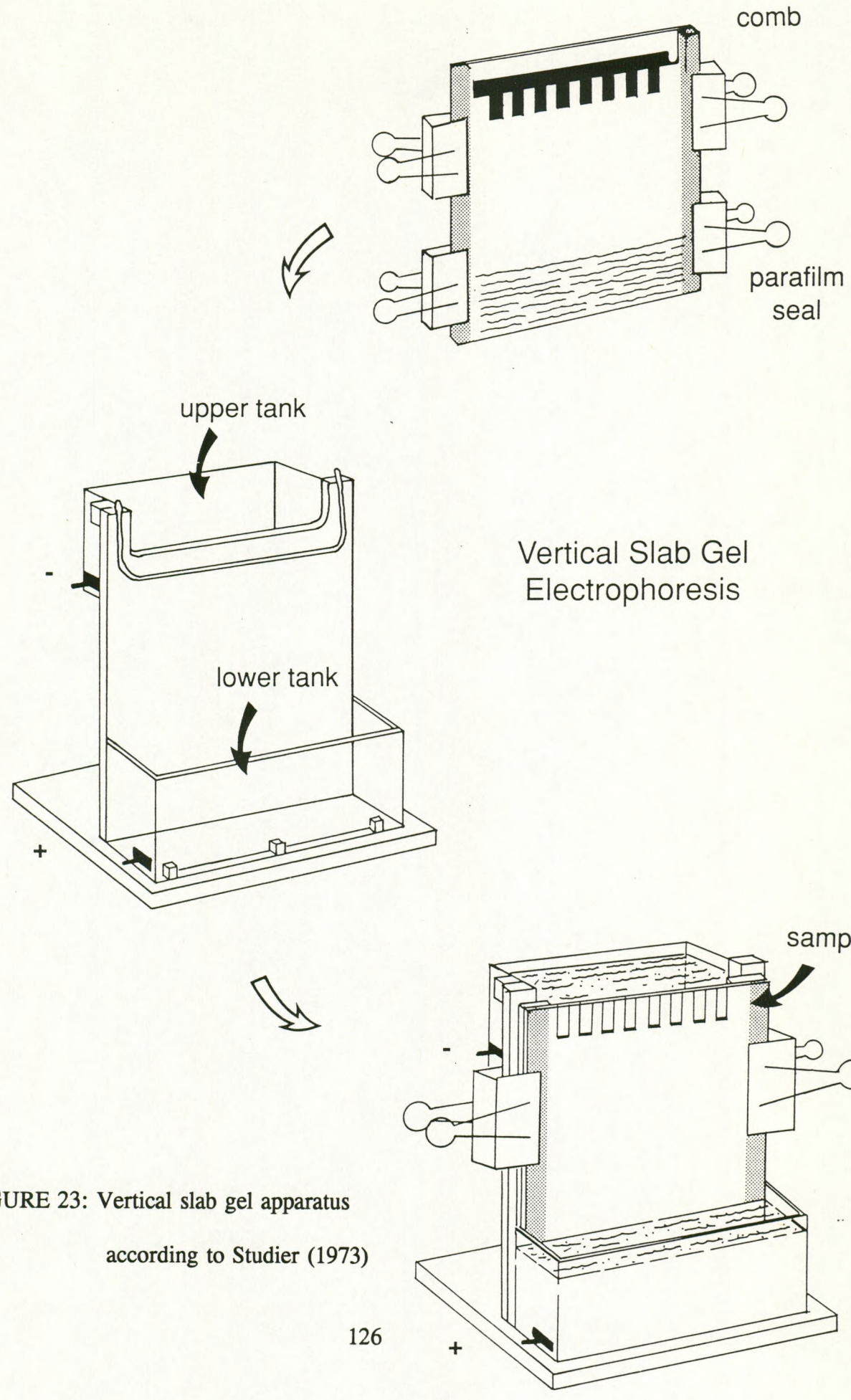


FIGURE 23: Vertical slab gel apparatus according to Studier (1973)

the electrophoresis apparatus by means of strong metal clips in such a way that the notched glass plate was aligned with and adjacent to the notch in the upper buffer reservoir to allow contact with the top of the gel and the upper reservoir buffer. The bottom of the gel was immersed in the buffer in the lower reservoir. Since many samples could be analysed on one slab gel, the platinum electrodes were placed so as to be equidistant from each sample which means that they are positioned along the length of both upper and lower buffer reservoirs.

Furthermore, if the heating effect was significant, there would be a temperature gradient from the centre of the gel to the surface which would cause proteins to migrate faster at the gel centre than at the surface. The increased temperature may also inactivate labile proteins. To alleviate these problems the run was carried out at 4°C.

4.1.4.4 ELECTROPHORETIC PROCEDURE

It was most important to ensure that the glass plates were perfectly clean to obtain good gel adhesion to the glass. The plates were cleaned by soaking them overnight in sodium hydroxide, followed by rinsing them with water and then with methanol. The plates were then placed down onto clean tissue paper with the side which was to be in contact with the gel uppermost. It was swabbed with methanol and allowed to air-dry. The glass plate sandwich which would form the gel mould was assembled.

The glass plates were usually held the correct distance apart by thin plastic spacers which must be of uniform thickness, both with respect to each other and along their length to ensure good contact with the plates and a gel of uniform thickness. The clamped plate assembly was held vertically during pouring of the gel. Having selected an appropriate resolving gel concentration, the gel mixture was prepared by adding the correct volumes of all components, adding the TEMED, mixing it in and pouring the gel solution without delay between the glass plates to within 0,5cm of the top. The two chambers of the linear gradient maker were of exactly the same cross-section and were joined by an inter-connecting tunnel controlled by a two-way tap. The outlet from the gradient maker was connected via fine-bone Tygon tubing to a peristaltic pump and through this to the glass-plate sandwich. The most concentrated acrylamide solution entered the glass-plate sandwich first and ran down the inside of the unnotched glass plate to reach the gel bottom. The tip of the Tygon tubing was moved along the unnotched plate back and forth so as to ensure the formation of a good gradient. As the level of acrylamide mixture rose in the sandwich, the acrylamide concentration steadily decreased. Immediately the gradient gel had been poured, the gradient maker was flushed out with water to prevent acrylamide polymerizing in the apparatus. Immediately a sample comb was inserted between the glass plates and into the gel mixture. The teeth of the comb should fit snugly against the glass plates. Special care was taken to ensure that air bubbles were not trapped beneath the comb otherwise irregularly shaped sample wells would be formed. The assembly was left undisturbed for the gel to polymerize (10-30min) as was evidenced by the appearance of a sharp boundary below areas of gel/air interfaces. If the gel

was to be used immediately the sample wells were rinsed out with reservoir buffer. The gel could also be stored at 4°C if it was to be used at a later stage. The slab gel was clamped to the electrophoretic apparatus as described under "apparatus", and the run was continued for 3 hours at 150V. During electrophoresis, heat was generated by the passage of electric current through the gel. Since the mobility of migrating ions is increased and the risk of denaturation of proteins increases as the temperature rises, it is apparent that the temperature must be low (4°C) and constant if electrophoretic separations are to be reproducible. The position of the dye front was intermittently assesed and was taken as an indication of the termination of the run.

Slab gels were recovered by removing the side spacers and gently levering the glass plates apart at the end, away from the notch to avoid damage to the fragile notched end. Once the slab gel was recovered it was essential to mark which end was which. In addition it was sometimes necessary to mark the position of the tracking dye for later R_f determinations.

4.1.4.5 STAINING PROCEDURE

Coomassie Blue was utilized as the protein stain to evaluate the protein bands. It can detect as little as 0.5μg/cm of protein. The gel was normally left overnight in the stain and then transferred to the destaining solution.

Preparation of Stain

A) 0,750g of PAGE BLUE 83 was dissolved in 1ℓ methanol

B) 150g of trichloroacetic acid (Saarchem) was dissolved in 1,5l H_2O

Solution A and B were dissolved independently and then mixed together.

Preparation of Destain

* 30% methanol
* 65% H_2O
* 5% acetic acid

4.1.4.6 GLUCOSE 6 PHOSPHATE DEHYDROGENASE (G6PD) AS MARKER ENZYME

Hempelmann and Wilson (1981) found evidence for the existence of a parasitic G6PD in *P. falciparum*. G6PD was used as a marker enzyme to control the purity of the isolated parasites.

4.1.4.6.1 Sample Preparation

A culture with a 10% parasitaemia was centrifuged and a sucrose-tris-EDTA (0,25M sucrose, 5mM Tris-HCL, 1mM EDTA, pH=7,4) solution was added to the pellet and placed at -70°C for at least 1 hour. Haemolysis was evident after thawing. The suspension was centrifuged at 400g for 10 min and the red supernatant collected and used as a sample. The pellet contained isolated host cell "free" parasites. The purified parasites were lysed by the freeze-thaw method. The samples were placed in liquid nitrogen, then quickly defrosted; this procedure was repeated twice. The lysed parasites were centrifuged for 5 minutes. The supernatant was used for electrophoretic separation.

Ten per cent glycerol was added to the sample to increase its density. Phenol red was added to both samples serving as a front marker during electrophoretic separation.

4.1.4.6.2 Separation of enzyme

A polyacrylamide vertical gel was used to separate the enzymes of erythrocytes and *P. falciparum*.

Preparation of 7,5% Polyacrylamide Gel

- 5ml of 30% (w/v) acrylamide solution
- 5ml distilled water
- 10ml of 300mM Tris-HCL buffer, pH 8,9 (gel buffer)
- 20mg ammonium persulphate
- 20μl TEMED

30% (w/v) Acrylamide Solution

- 29,2g acrylamide
- 0,8g bisacrylamide
- added distilled water to make volume to 100ml

Tris-HCL Buffer (Separation Buffer)

- 4,8g Tris
- 3,0g glycine
- volume made up to 1ℓ with distilled water

4.1.4.6.3 Procedure

i) Two prewashed glass plates with 0,75mm separators were clamped together.

ii) The bottom of the plates was sealed using grease on the corners and parafilm along the bottom.

iii) All the chemicals for the polyacrylamide gel were combined, except for the TEMED.

iv) To 2ml of the polyacrylamide gel solution, 5ℓ TEMED was added. The solution was poured along the sides of the glass plates. Polymerization was allowed to take place (10 min) and the bottom of the plates was sealed.

v) To the remaining solution 20$\mu\ell$ TEMED was added. The solution was poured between the plates, air bubbles were avoided and the 8 laned comb was inserted into the gel and clamped down.

vi) Polymerization took place within 10 min and the gel could either be stored or used after 1 hour.

vii) The comb and parafilm were removed and the wells washed with buffer.

The separation was carried out with a Studier Apparatus (fig.23)

- 500ml of the Tris-glycine buffer was poured into each of the apparatus tanks
- 10-50$\mu\ell$ of sample was loaded into the gel slots
- The separation was run at 150V for 3 hours at 4°C.

4.1.4.6.4 G-6-PD Visualization

Glucose 6 Phosphate Dehydrogenase Staining Solution

- 10-40 ml of 50mM Tris-HCL, pH 8,0
- 15mM glucose 6 phosphate
- 2,69mM NADP
- 0,24mM MTT
- 0,0326mM PMS
- 42mM $MgCl_2$

The gel was placed in the above solution and incubated at 37°C. The parasitic enzyme has a low activity therefore it was stained for approximately half an hour or longer. Gel was removed from staining solution when a clear band was visible.

4.1.4.7 PROTEIN MEASUREMENT

It was necessary to control the amount of protein added to each well, i.e. the concentration of protein in each sample. The standard Bradford (1976) method was utilized.

4.1.4.7.1 Procedure

i) 100$\mu\ell$ of standards and appropriately diluted samples were placed in dry, clean test tubes. 100$\mu\ell$ of sample buffer was used as "blank".

ii) 5ml of diluted dye reagent was added to each test tube. One volume of dye was diluted with 4 volumes of double distilled water and then filtered through a 0.22μm millipore filter.

iii) The contents were mixed gently by inversion of the test tube.

iv) After a period of from 5 minutes to one hour, the OD_{595} was measured versus the reagent blank.

v) The wavelength versus the concentration of standards was plotted and the unknowns were read from the standard curve.

4.1.4.8 ELECTROBLOTTING

This technique was employed to transfer the separated proteins from the gel to a thin support matrix (nitrocellulose membrane), to which they bind and are immobilized (Towbin *et al.*, 1979). This technique is needed if the reagent used does not penetrate into the gel (e.g. antibodies).

i) The transfer buffer for the immunoblot was prepared as follows (modified from Towbin *et al.*, 1979);

48mM.....Tris

39mM.....glycine

1,3mM....SDS

200ml....methanol (20%v/v)

The above ingredients were made up to 1ℓ with double distilled water. Before adding the 200ml of methanol the tris, glycine, and SDS were dissolved in approximately 200ml of double distilled water. The transfer buffer pH (pH=9,2) cannot be adjusted with the addition of either acid or base. Improperly prepared buffer would cause excess heat generation. Only high quality reagent grade methanol was utilized. Contaminated methanol would result in increased transfer buffer conductivity, as well as poor transfer of macromolecules. Methanol (20% by volume) was added to the

transfer buffer since it minimizes swelling or shrinking of the gel during blotting and increases the binding capacity of nitrocellulose for protein.

ii) Following the gel electrophoresis, the gels were equilibrated in transfer buffer. Equilibration facilitated the removal of electrophoresis buffer salts and detergents. If the salts were not removed, they would increase the conductivity of the transfer buffer and the amount of heat generated during the transfer. Equilibration allowed the gel to adjust to its final size prior to electrophoretic transfer. The length of time required for equilibration was dependent on the gel thickness. The gel was normally left in the transfer buffer for 5 min.

iii) The nitrocellulose membranes were cut to the dimensions of the gel. The membrane was soaked by slowly sliding it into transfer buffer and allowing it to soak for 15-30 min. Complete wetting of the membrane was important to ensure proper binding. To avoid membrane contamination, forceps were used when handling membranes.

iv) The filter paper was cut to the dimensions of the gel. Four pieces of filter paper per gel were used for each gel/membrane sandwich. The filter paper was completely saturated in transfer buffer.

4.1.4.8.1 Assembly of the unit

i) Pre-soaked sheets of filter paper were placed onto the platinum anode. A glass tube was rolled over the surface of the filter paper to exclude all air bubbles. The air bubbles block the transfer of molecules.

ii) The pre-wetted nitrocellulose paper was placed on top of the filter paper. Again all the air-bubbles were rolled out.

iii) The equilibrated gel was carefully placed on top of the transfer membrane, and the gel was aligned on the center of the membrane. Transfer would be incomplete if any portion of the gel was outside the blotting media.

iv) Another sheet of pre-soaked filter paper was placed on top of the gel, and air bubbles from between the gel and filter paper were carefully removed.

v) Large gels were transfered for 30 minutes to one hour at 15-25V.

vi) Following transfer the unit was disconnected and the filter paper discarded. The transfer efficiency was monitored by staining the gel with Coomassie blue R-250 protein stain.

4.1.4.8.2 India ink

The nitrocelllulose paper was placed in india ink to stain for the proteins. The india ink was prepared by adding 300$\mu\ell$ of tween 20 and 200$\mu\ell$ of india ink to 100ml of PBS.

4.1.4.9 IMMUNOENZYME METHOD

Immunoenzyme detection involves the detection of a specific antigen with a colour reaction. The antigen in question is the P-glycoprotein (P-gp). Electroblotting transfers the antigen to the nitrocellulose paper (fig. 24). Use is made of a specific antibody or primary antibody to detect the antigen. The secondary antibody detects the primary antibody. The peroxidase-labelled secondary antibody is made visible by a colour reaction after the addition of a substrate. This colour change is indicative of the presence of the antigen.

We made use of two primary antibodies, namely P-glycoCHEK C219 and JSB-1. The Centocor P-glycoCHEK C219 is a murine monoclonal antibody (IgG2a) specific for P-glycoprotein and can be used in immunoblotting procedures. The C219 antibody was originally developed by Kartner *et al.* (1985).

The JSB-1 monoclonal antibody was recently characterized by Scheper *et al.* (1988), and then demonstrated its ability to detect P-glycoprotein using immunoprecipitation and immunohistochemistry. The antibody specifically recognizes a conserved epitope

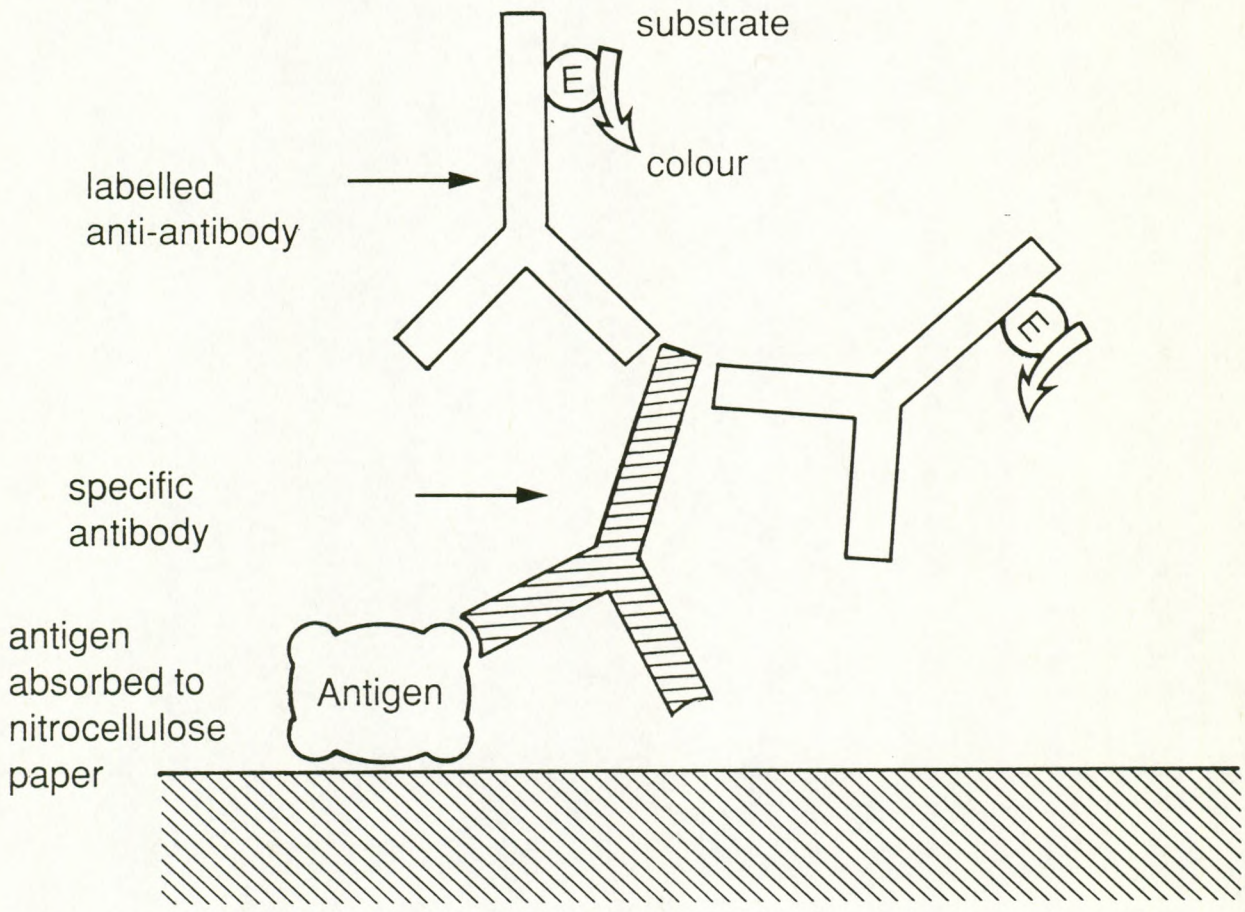


FIGURE 24: Immunoenzyme staining (modified from Peters and Baumgarten, 1984)

of the membrane associated internal domain of the P-gp 170-180 kD of different species including hamster and human.

4.1.4.9.1 Procedure (modified from Peters and Baumgarten, 1984)

i) The nitrocellulose filter was incubated in blocking buffer overnight at 37°C. Since immunostaining is based upon specific antibody binding to particular antigens, it was important to minimize non-specific binding of the antibody to the filter matrix.

Blocking Buffer (PBS - Tween 20)

8,277g.....NaCl

2,900g.....$Na_2HPO_4.12H_20$

0,200g.....KH_2PO_4

The above ingredients were dissolved and made up to 1ℓ with distilled water. To 100ml of PBS 50$\mu\ell$ Tween 20 (0,05%v/v Tween 20) was added and this constituted the blocking buffer.

ii) After having blocked the non-specific binding, the nitrocellulose filter was incubated in the primary antibody solution, which was made up in blocking

buffer. The concentration of the respective antibody was 4$\mu\ell$/ml. This was left at 4°C for 20 hours.

iii) The nitrocellulose filter was washed for 3 h at room temperature in six changes of PBS. The washing removed any traces of unbound primary antibody.

iv) The nitrocellulose filter was then incubated for 2 hr at room temperature with a 1:3000 dilution of goat anti-mouse IgG-peroxidase diluted in PBS. This constituted the secondary antibody which attached to the primary antibody.

v) After several washes, the nitrocellulose filter was incubated with enzyme substrate for 30 min.

4.1.4.9.2 Enzyme Substrate Preparation

The enzyme substrate buffer was prepared by dissolving 0,130g ammonium acetate and 0,060g citric acid in 100ml distilled water. The pH of the buffer was 5.0.

To 20ml of the buffer, the following was added;

- 50$\mu\ell$ of 3% H_2O_2

- 200$\mu\ell$ of 1% 4-Chlor-1-Naphthol (10mg in 1mℓ methanol).

As soon as a colour band appeared on the nitrocellulose paper, the reaction was stopped by rinsing with water.

4.1.3 SECTION B - IMMUNOHISTOCHEMISTRY

This technique was carried out to evaluate and confirm the results obtained from western blotting. Parasites were concentrated by the gelatine method (4.1.2.1). A smear of the concentrated parasites was made on a slide, which was then covered with formaldehyde and placed at -70°C. This made the cells permeable, so that the antibody could enter into the cytoplasm. The P-gp is situated in the cytoplasmic part of the cell membrane, and perforation of the cell membrane is therefore necessary. The sample material was fixed with acetone on the slide for 20 min at -20°C. Excess acetone was allowed to evaporate. The method employed was a modification of that described by Nakame amd Pierce (1966).

i) The preparation was covered according to the size of the section with antibody solution and incubated in a humid chamber for 1 hr at room temperature.

ii) Slides were washed three times with PBS and were wiped clean except area of section.

iii) The section was then covered with a suitable volume of peroxidase-labeled anti-mouse-Ig antibody and allowed to incubate in a humid chamber for 1 hr at room temperature.

iv) Slides were washed again as described above.

v) The preparation was overlayed with a substrate solution and incubated at room temperature until a clearly visible redbrown colour developed. A negative control did not show any colour change during this incubation period. The substrate solution was subsequently removed with PBS and the preparation was counterstained with haematoxylin for about 10 min. The haematoxylin solution was removed with PBS and the preparation was examined under the microscope.

4.1.3.1 SUBSTRATE SOLUTION

25mg 3,3'-diaminobenzidine-tetrahydrochloride was dissolved with 50ml Tris-HCL, (0,05mol/ℓ; pH 7.3) and 50$\mu\ell$ H_2O_2 (30% (v/v)) was added. Solution was prepared freshly every time.

4.2 RESULTS

SECTION A

The malaria culture was concentrated by gelatine sedimentation (4.1.2.1) as demonstrated in fig. 25. The object was to isolate parasites free from host contamination. Gelatine sedimentation was a means of reducing the concentration of uninfected red blood cells, which would interfere with the isolation procedure, and increase that of infected host cells. With time it was noticed that a solution of sucrose-tris-EDTA (4.1.2.3) added to the pellet of a culture, reduced aggregation of contaminants especially haemoglobin.

Figure 26 represents a photomicrograph of a smear made after lysing the erythrocytes and washing the pellet a couple of times to remove haemoglobin and other host contamination (4.1.2.2). The Glucose-6-Phosphate-Dehydrogenase (G6PD) assay was carried out on the isolated parasites to ensure that the isolation had been complete, and that no remaining host contamination was present. The parasite has its own G6PD which has a higher molecular weight to the erythrocyte G6PD. As a result G6PD was used as a cytoplasmic marker enzyme. Figure 27 represents the separation of both erythrocyte and parasitic G6PD on a polyacrylamide gel (4.1.2.2). Lane B is the erythrocyte (host) G6PD and lane A the parasitic G6PD. Parasitic isolated material as indicated in fig. 26 was subjected to freeze-thawing (4.1.3), i.e., lysing the parasites. This parasitic lysate was examined for the presence of host

G6PD. If the parasitic lysate had been contaminated with host G6PD, then a band would show up in lane A next to the host G6PD band, which was run as control. One may therefore conclude, that the isolated parasites were free of contaminating host cytoplasm.

Approximately 20$\mu\ell$ of the parasitic lysate was separated by SDS-PAGE (4.1.4). This represented 10μg of parasitic protein as determined by the Bradford method (4.1.4.7). Red blood cell membranes were always run concurrently as controls. Lane A of fig. 25 illustrates parasitic proteins separated on a polyacrylamide gel, and lane B, red blood cell membrane proteins. As may be seen from the photograph, the spectrin band is missing in lane A, indicating no or only minimal contamination by host membrane proteins. The spectrin is characteristic of the presence of red blood cell membrane protein. Also, another point to consider is the minimal haemoglobin presence in lane A. High and low molecular weight markers (Bio-Rad) were employed (Fig. 26) to ascertain the molecular weights of the parasitic proteins, as indicated in figure 27. The parasitic proteins on the SDS-gel were transfered onto nitrocellulose paper (4.1.4.8). The efficacy of the transfer was analyzed by staining the proteins on the nitrocellulose paper with india ink (4.1.4.8.2) (Fig.28). Lane A in figure 27 are the parasitic proteins stained with amido black after immunoblotting, and lane B, the erythrocyte membrane proteins. The parasitic proteins were then probed with both monoclonal antibodies (C219 and JSB-1) for the detection of P-glycoprotein (4.1.4.9). The range of molecular sizes that have been reported for P-glycoprotein in tumour lines have been from between 130-200 kDa (Greenberger *et*

However, our results indicate a molecular weight of 40-42 kDa for the parasitic P-glycoprotein (Fig. 32). Lane B in figure 32 represents the FCR-3 strain, lane C the RSA-2, lane D the 3D7 strain and lane A the negative control. The negative control constituted parasitic proteins, treated in exactly the same manner as the other three samples, just that the monoclonal antibody was omitted. This ensured that the 40 kDa obtained in lane B, C, and D, was not due to nonspecific binding of the secondary antibody. It must be noted that approximately 10μg of protein was utilized for all three strains. It was alarming to note that the IC_{50}'s for the three strains ranged from 96.32nM (FCR-3), 40nM (RSA-2) to 2nM (3D7), and yet, their was no noticeable difference in the labelling of the 40 kDa band. These results confirm previous results by Grogl *et al.* (1991) on the D6 and W2 strains. One must therefore conclude that the P-glycoprotein-like-compound is present on both chloroquine resistant and -sensitive strains of *P. falciparum*.

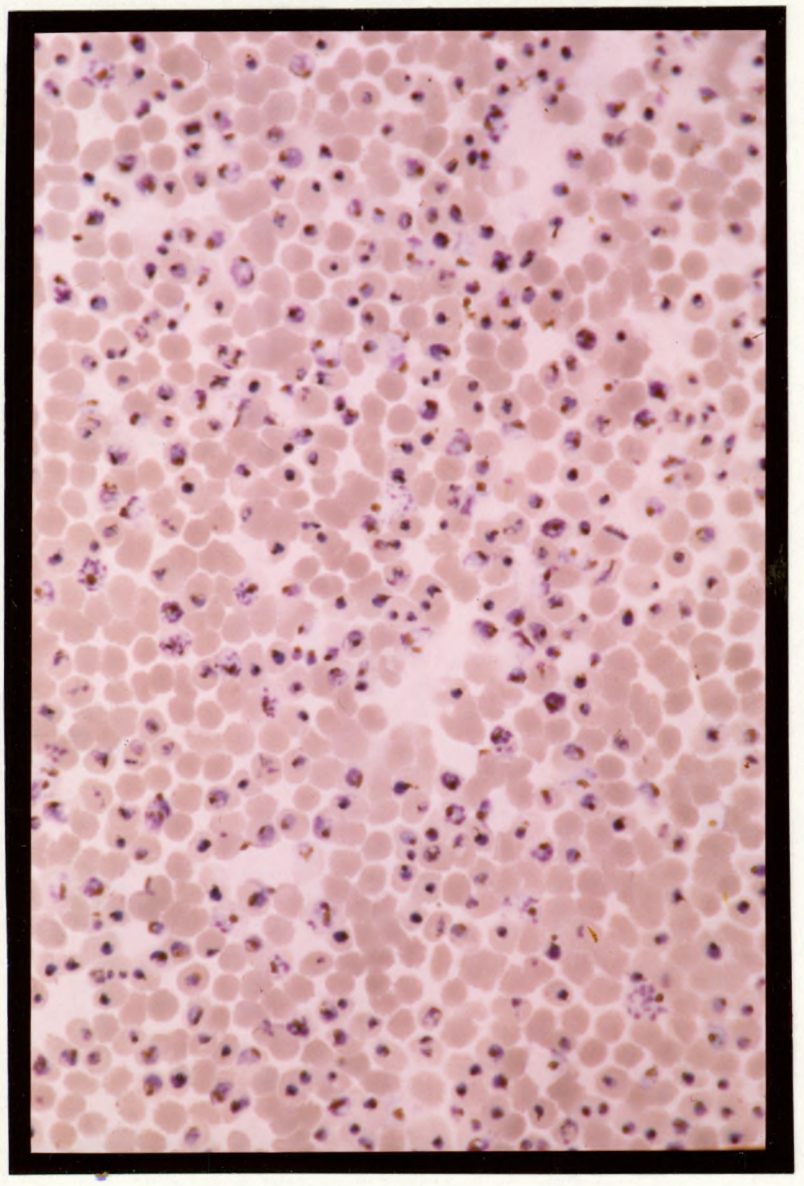


FIGURE 25: Gelatine Sedimentation

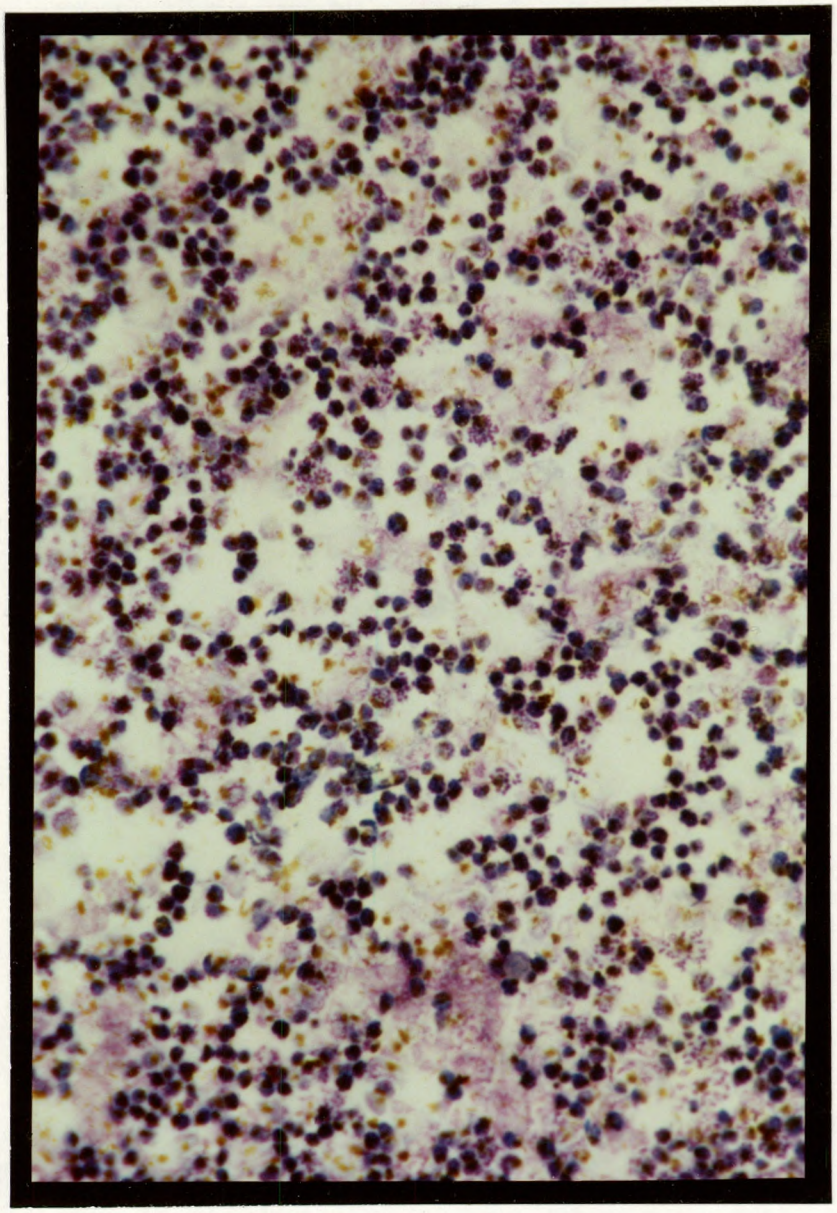
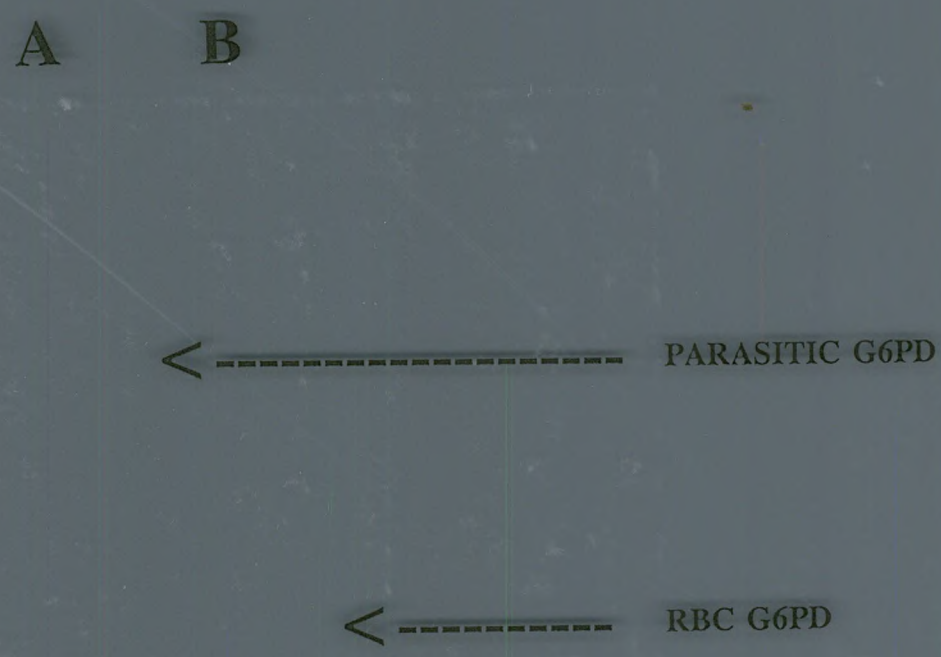


FIGURE 26: Isolation of parasites


A
B
PARASITIC G6PD
RBC G6PD

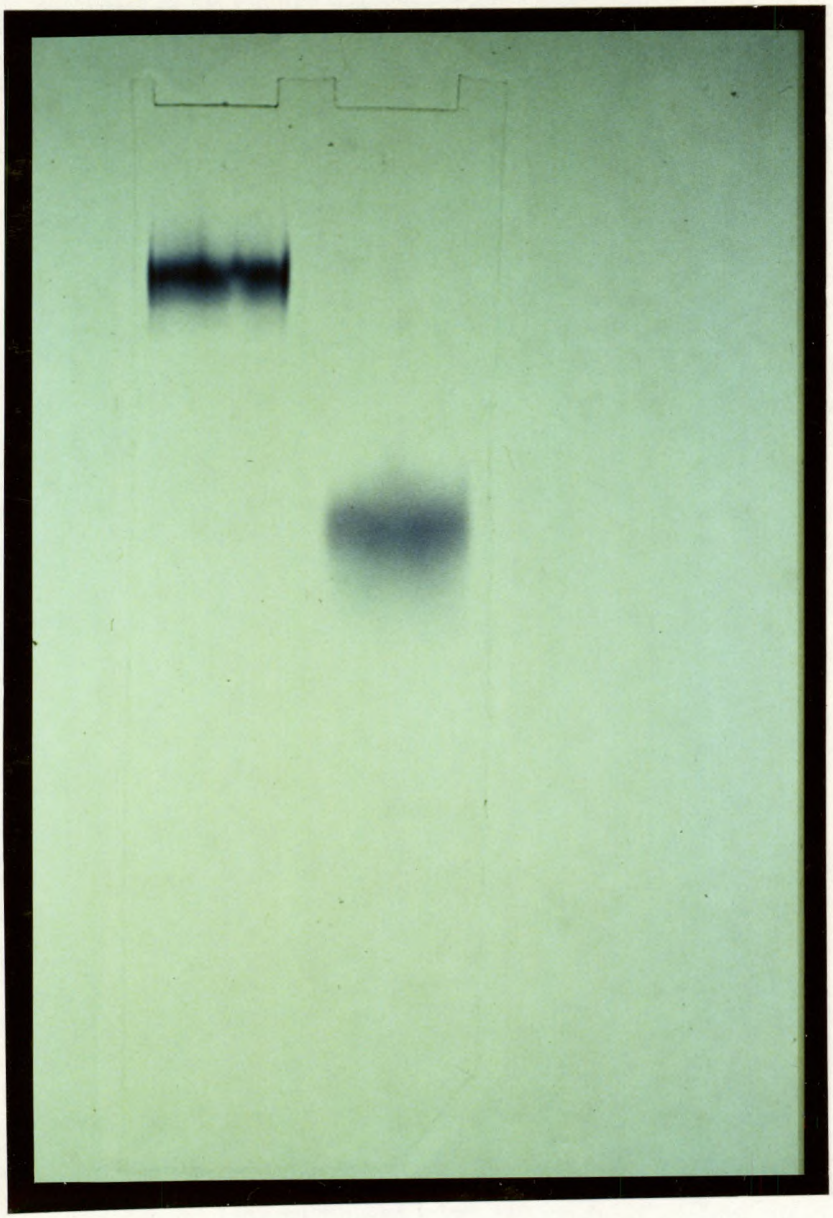
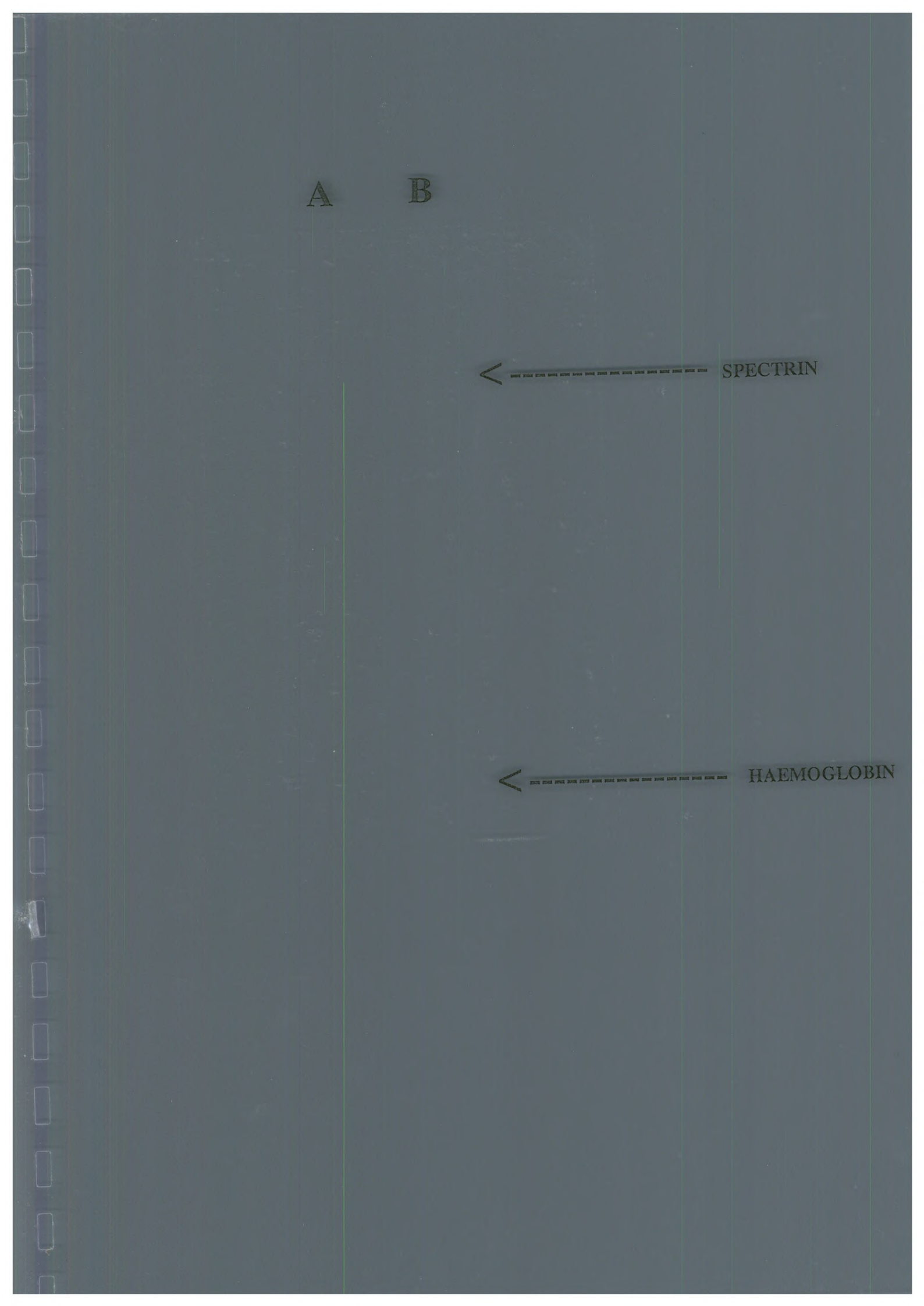


FIGURE 27: Glucose-6-Phosphate-Dehydrogenase


A
B
SPECTRIN
HAEMOGLOBIN

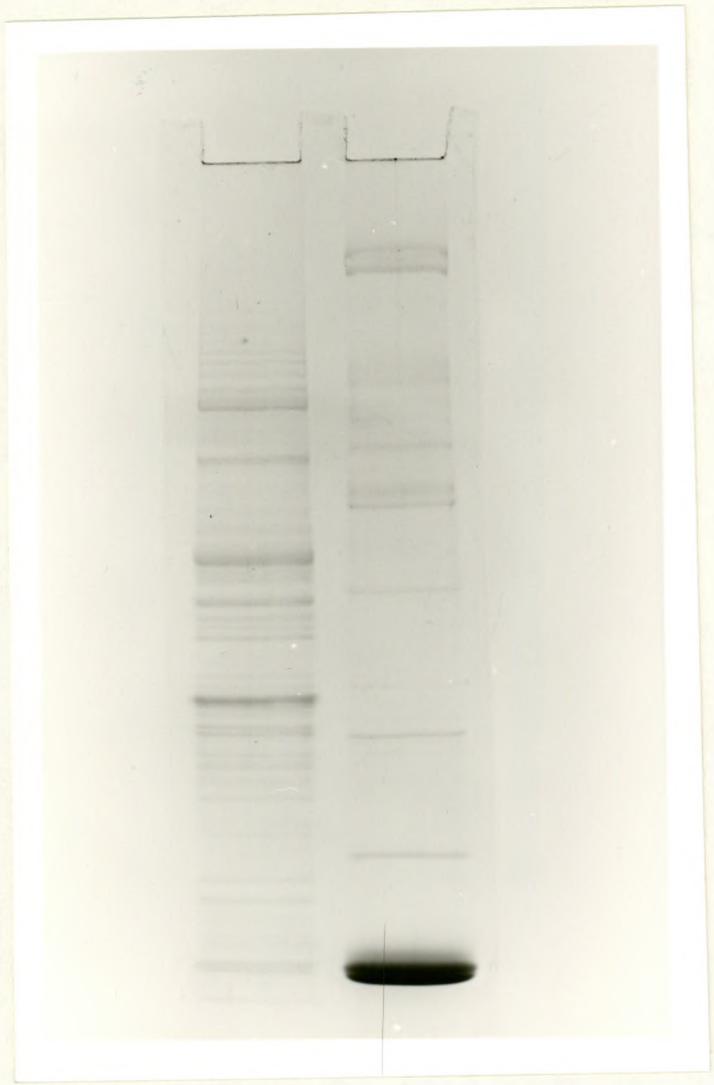
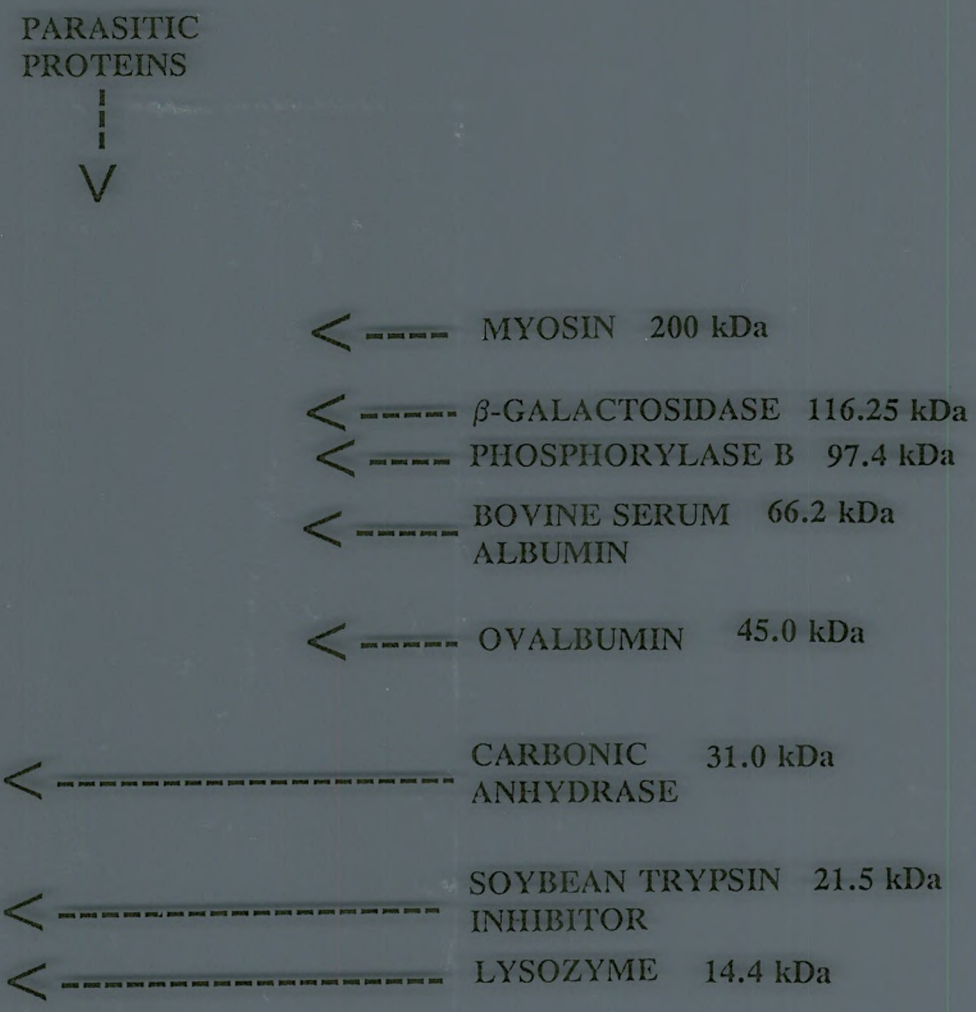


FIGURE 28: Parasitic proteins separated by SDS-PAGE


PARASITIC
PROTEINS
MYOSIN 200 kDa
β-GALACTOSIDASE 116.25 kDa
PHOSPHORYLASE B 97.4 kDa
BOVINE SERUM ALBUMIN 66.2 kDa
OVALBUMIN 45.0 kDa
CARBONIC ANHYDRASE 31.0 kDa
SOYBEAN TRYPSIN INHIBITOR 21.5 kDa
LYSOZYME 14.4 kDa

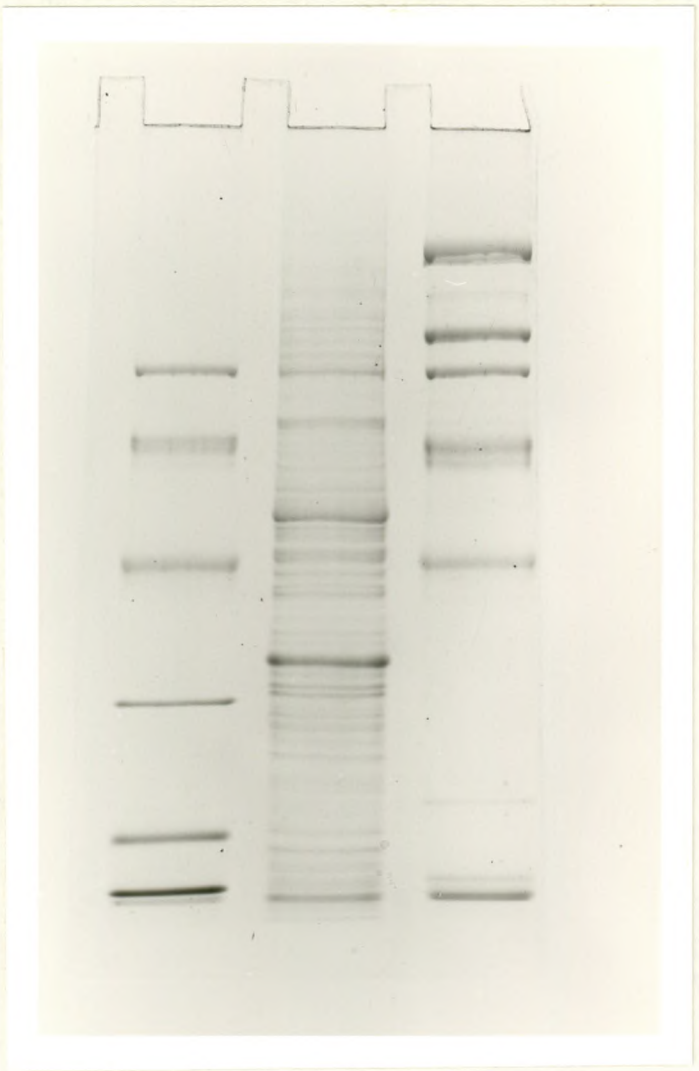


FIGURE 29: Parasitic proteins and molecular weight markers on SDS-PAGE

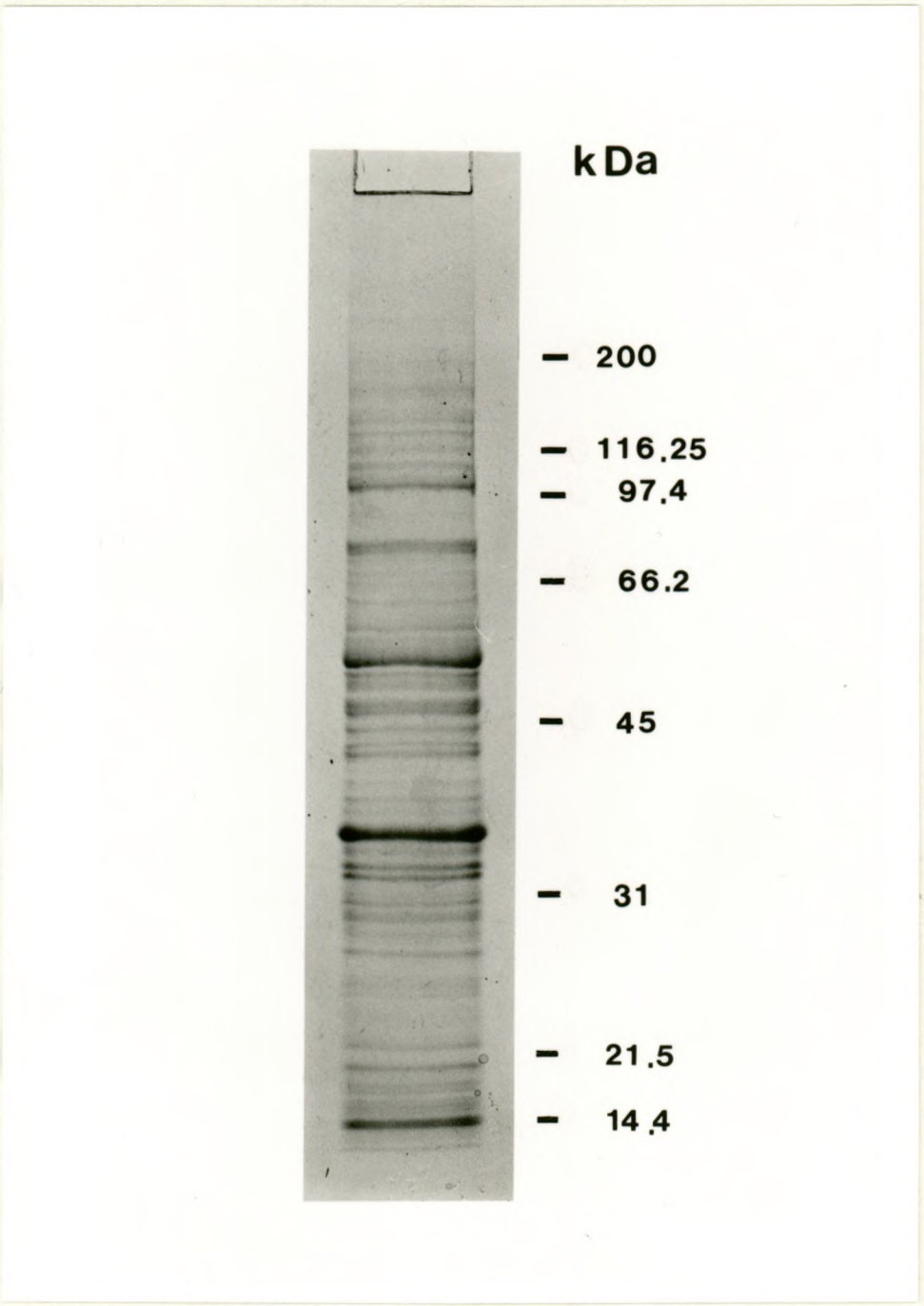


FIGURE 30: Molecular weights of parasitic protein

A B A

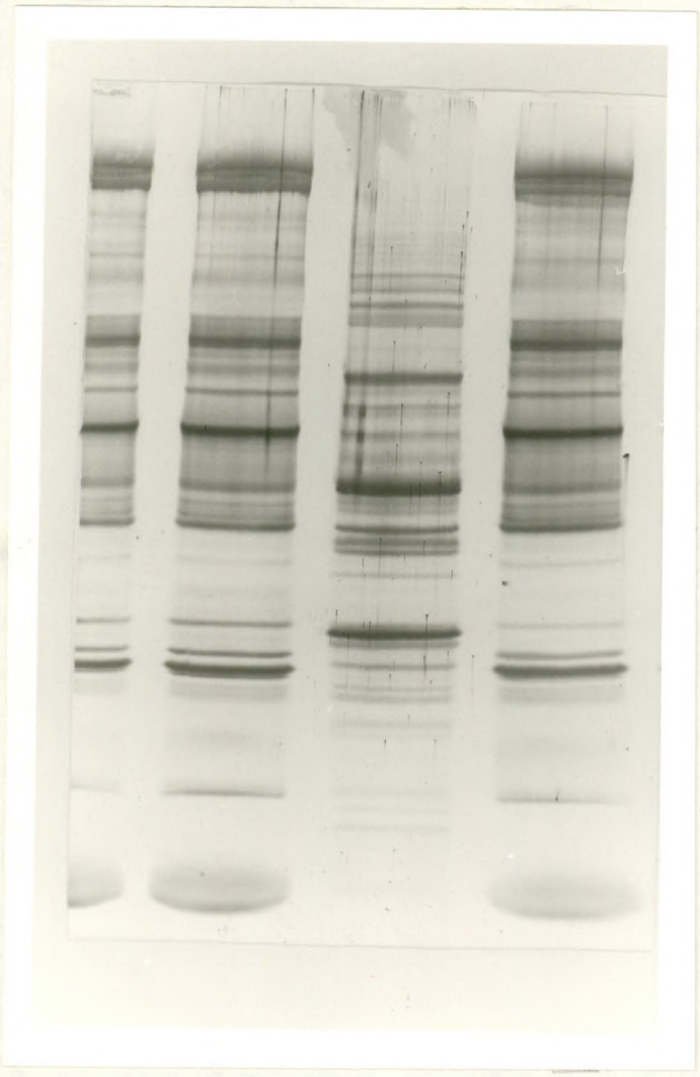


FIGURE 28: Immunoblot of parasitic protein onto nitrocellulose paper

Lane A = parasitic proteins; Lane B = erythrocyte membrane proteins

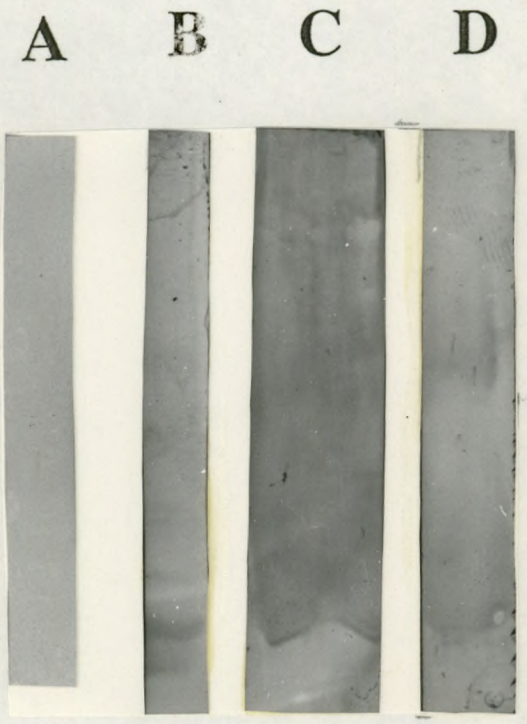


FIGURE 29: Labelling of the 40-42 kDa-protein by a monoclonal antibody

Lane A = negative control; B = FCR-3 strain; C = RSA-2 strain;

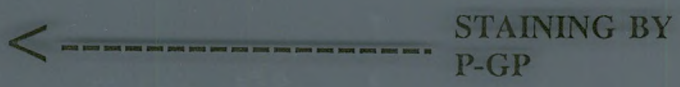
D = 3D7 srain

4.2.2 IMMUNOHISTOCHEMISTRY-SECTION B

In this section we investigated the presence of P-glycoprotein using the method, of immunohistochemistry staining as explained under 4.1.3. The advantage of this technique as opposed to western blotting, is that, the investigation is done directly on the whole cell as it exists in culture. It overrides technique (eg: isolation of cell components) errors that may be prevalent in the western blottting method. However, the one problem encountered with this technique was that the cells needed to be permeabilized for the antibody to react. This is because the monoclonal antibodies (C219 and JSB-1) specifically bind to epitopes of P-glycoprotein exposed on the cytoplasmic side of the plasma membrane (Kartner *et al.*, 1985). Another reason for verifying our previous results (section A) was that we were able to obtain positive and negative control slides for P-glycoprotein. Prof. Volm from Heidelberg University was kind enough to donate slides of colchicine-resistant and -sensitive chinese hamster ovary (CHO) tumour cells. These slides were immunohistochemically stained as explained under 4.1.3. Figures 33 to 36 (3000X) are photomicrographs of P-glycoprotein staining on CHO tumour cells. Figure 33 presents labelling of P-glycoprotein on resistant CHO cells by monoclonal antibodies (C219 and JSB-1). In the same token figure 34 presents the absence of labelling of P-glycoprotein on a sensitive CHO cell line. In figure 33 and 34, methylene blue was used as counterstain, whereas haemotoxylin was used as the counterstain for the other micrographs (Fig. 35, 36).

Figure 37 demonstrates the labelling of P-glycoprotein on the FCR-3 strain (chloroquine-resistant) by monoclonal antibodies. Figure 38 was performed as a control. Here the FCR-3 strain was utilized and the slide was subjected to exactly the same treatment as for figure 37, except that the monoclonal antibody was omitted. One can see a lack in staining of the parasite. The parasite is only recognised by its predominant haemozoin (malaria pigment), which is identified by the black spot within the parasite.

Figures 39 and 40 demonstrate the 2 chloroquine sensitive strains (RSA-2 and 3D7), and they too are stained with the monoclonal antibodies. As in the results presented in section A there is no noticeable difference in the staining of the three strains of *P. falciparum*. These results confirm our previous results, and one may conclude that the P-glycoprotein-like-compound is present on both chloroquine resistant and - sensitive strains of *P. falciparum*. It must be emphasized that these results were obtained with both the monoclonal antibodies i.e., C219 and JSB-1.


STAINING BY
P-GP

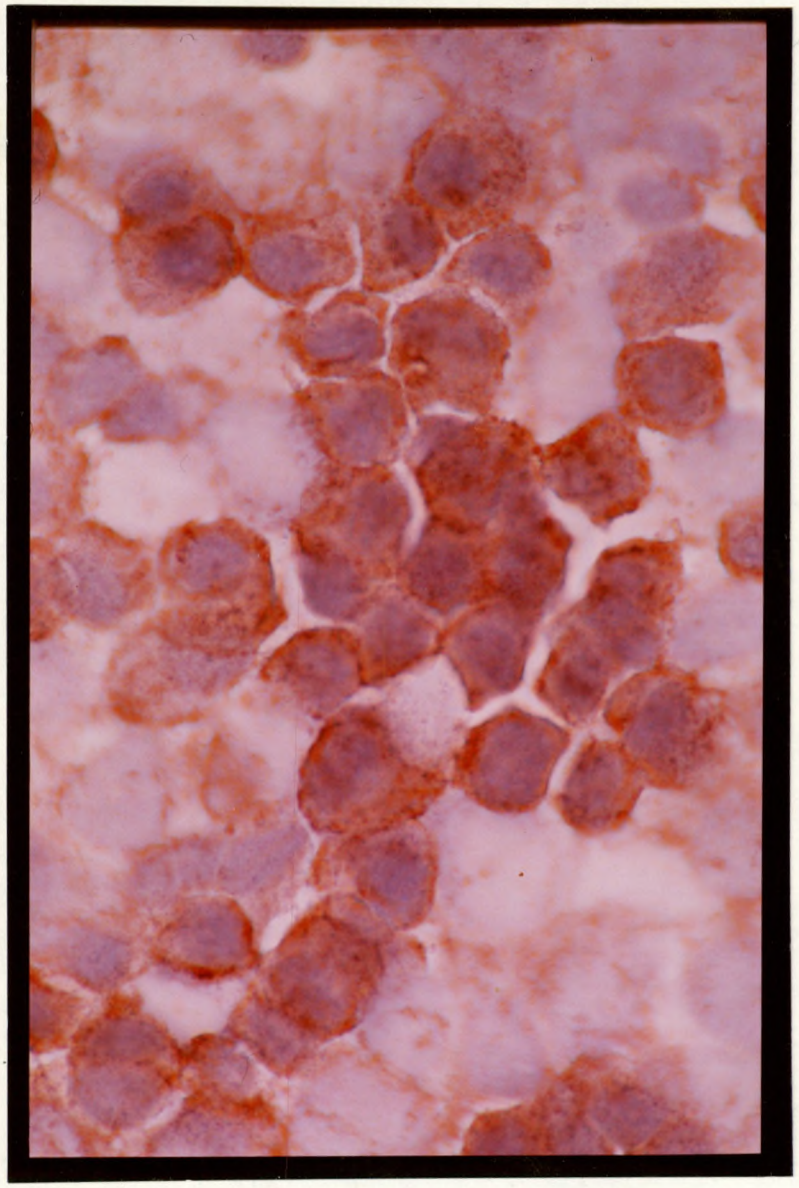


FIGURE 33: Resistant CHO cancer cells

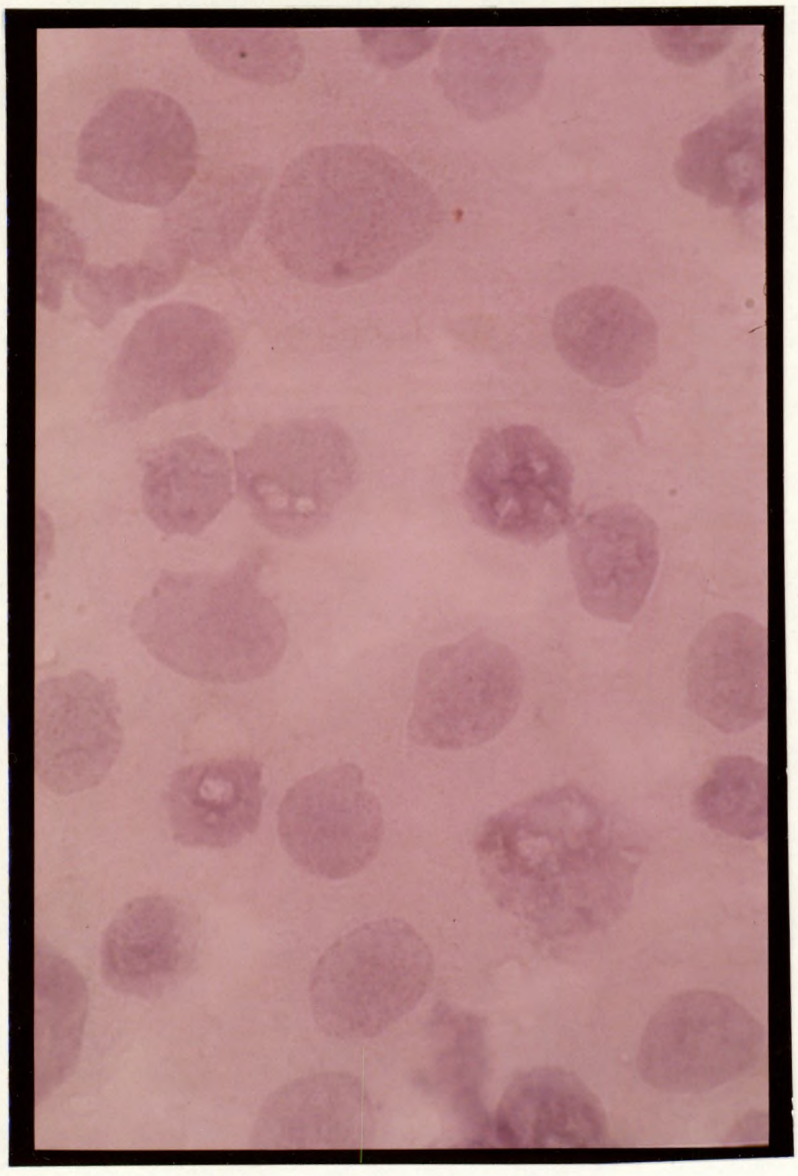


FIGURE 34: Sensitive CHO cancer cells


STAINING BY
P-GP

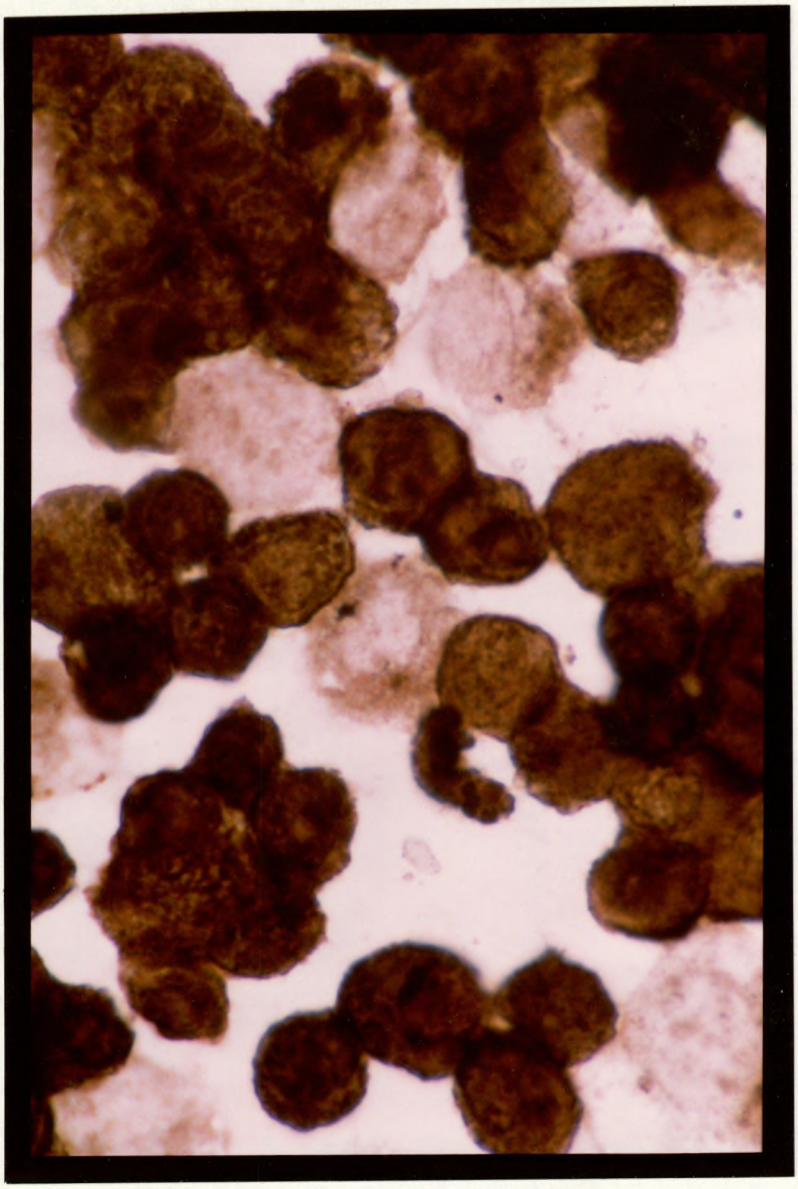


FIGURE 35: Labelling of P-glycoprotein on resistant CHO cancer cells

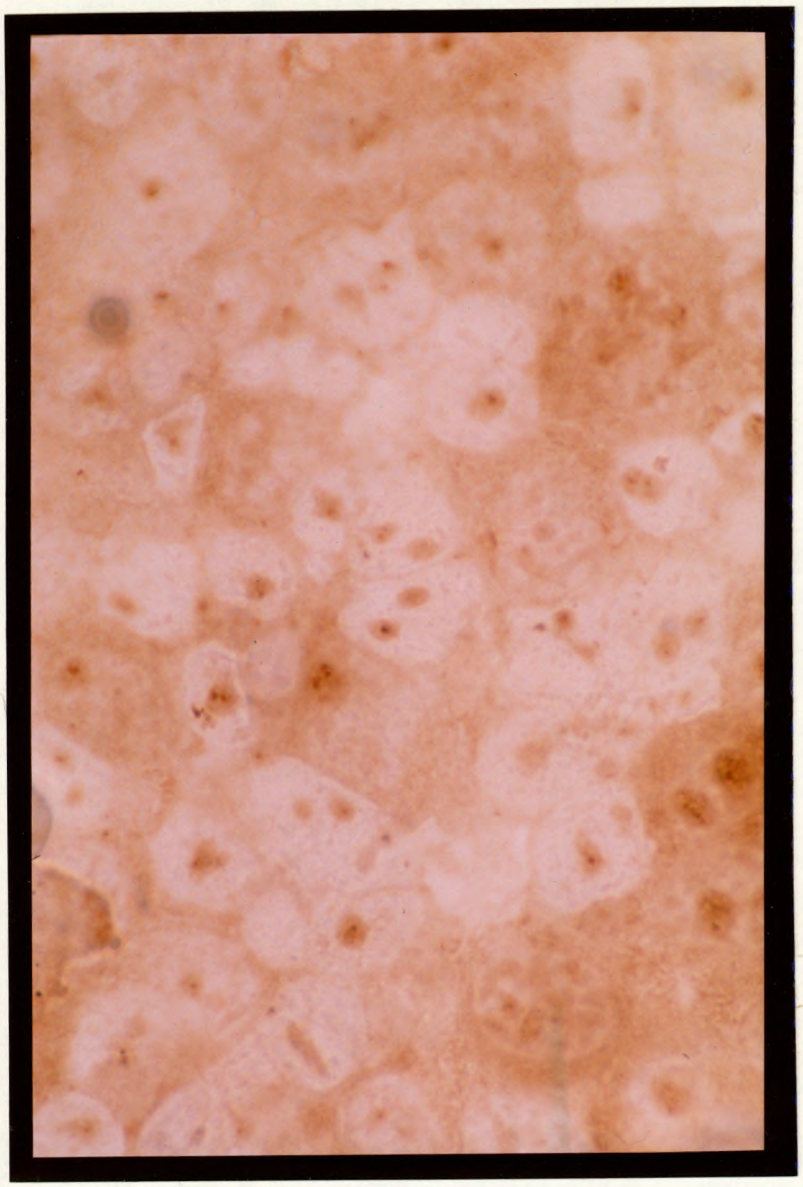
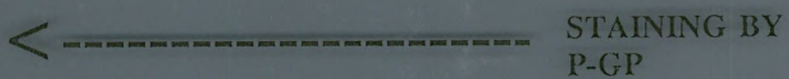


FIGURE 36: Absence of labelling on sensitive CHO cancer cells


STAINING BY
P-GP

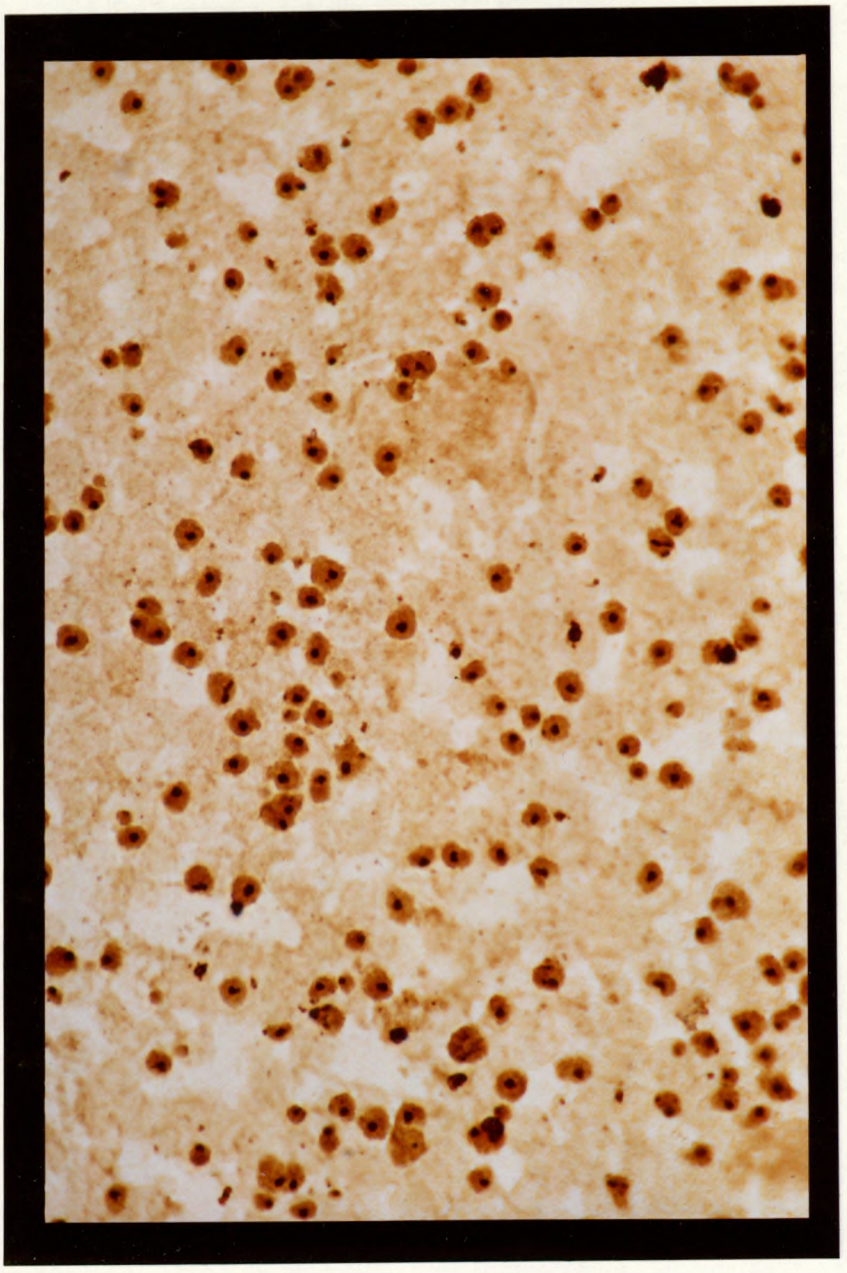
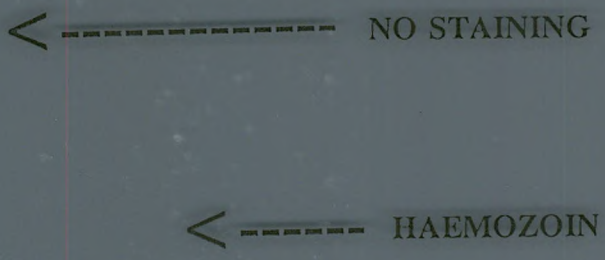


FIGURE 37: Labelling of FCR-3 strain by monoclonal antibody


NO STAINING
HAEMOZOIN

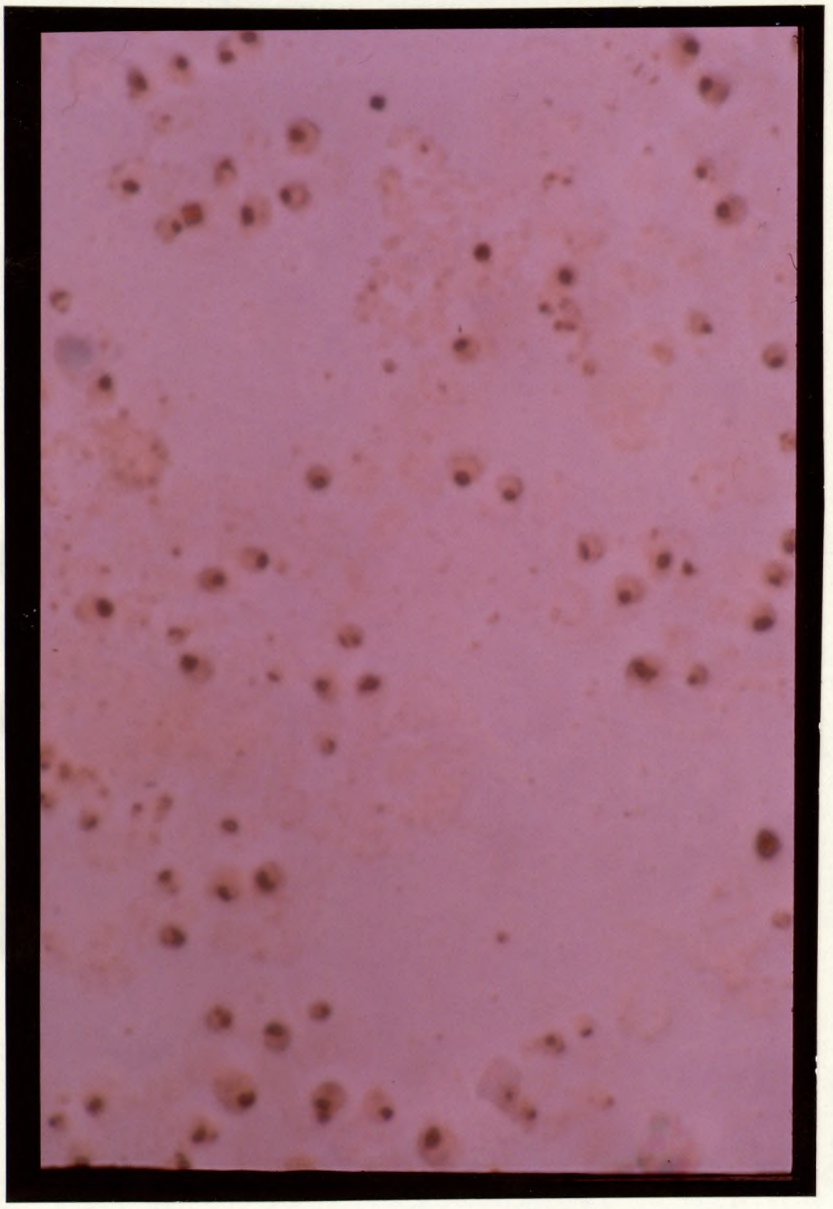


FIGURE 38: Control slide for P-glycoprotein labelling on FCR-3 strain


STAINING BY
P-GP

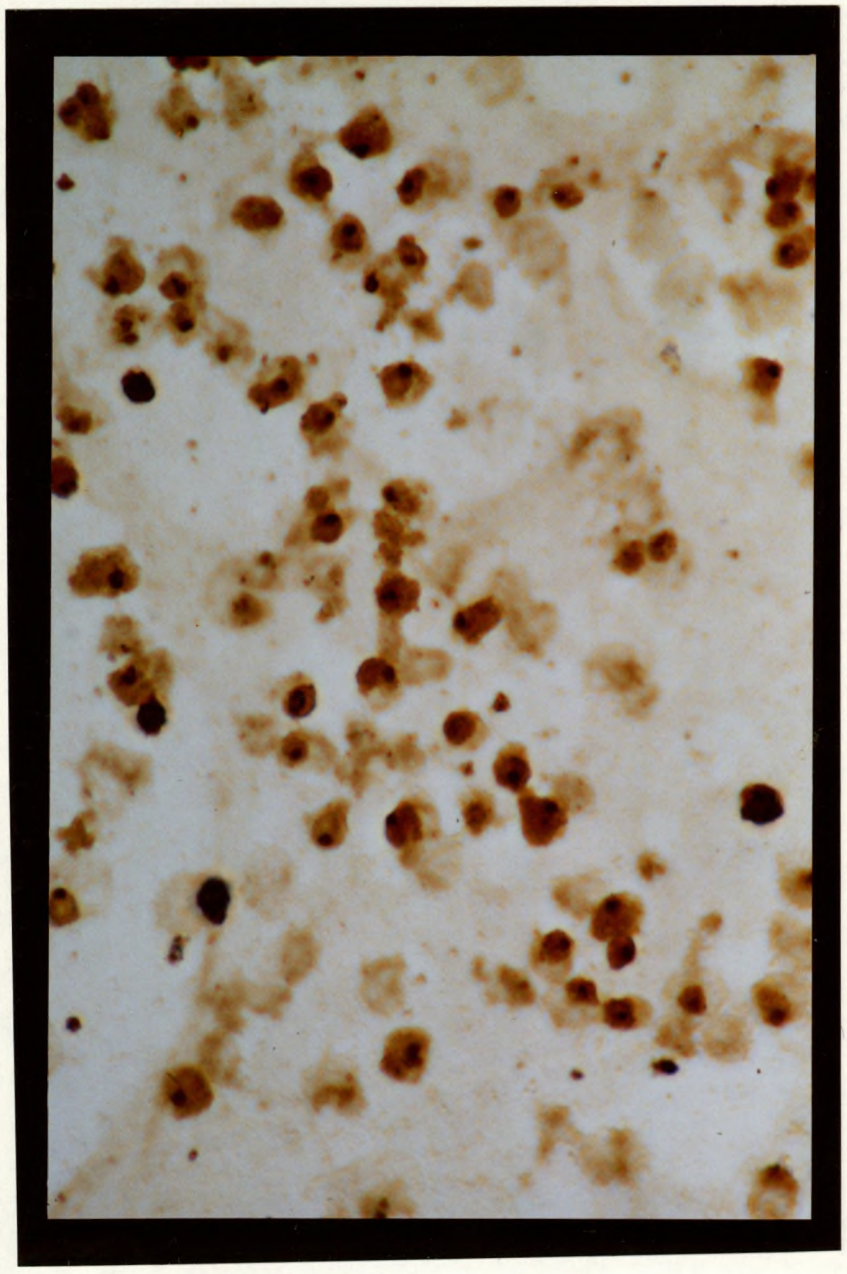
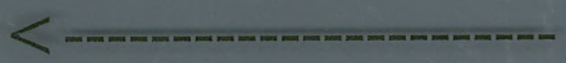


FIGURE 39: Labelling of RSA-2 strain by monoclonal antibody


STAINING BY
P-GP

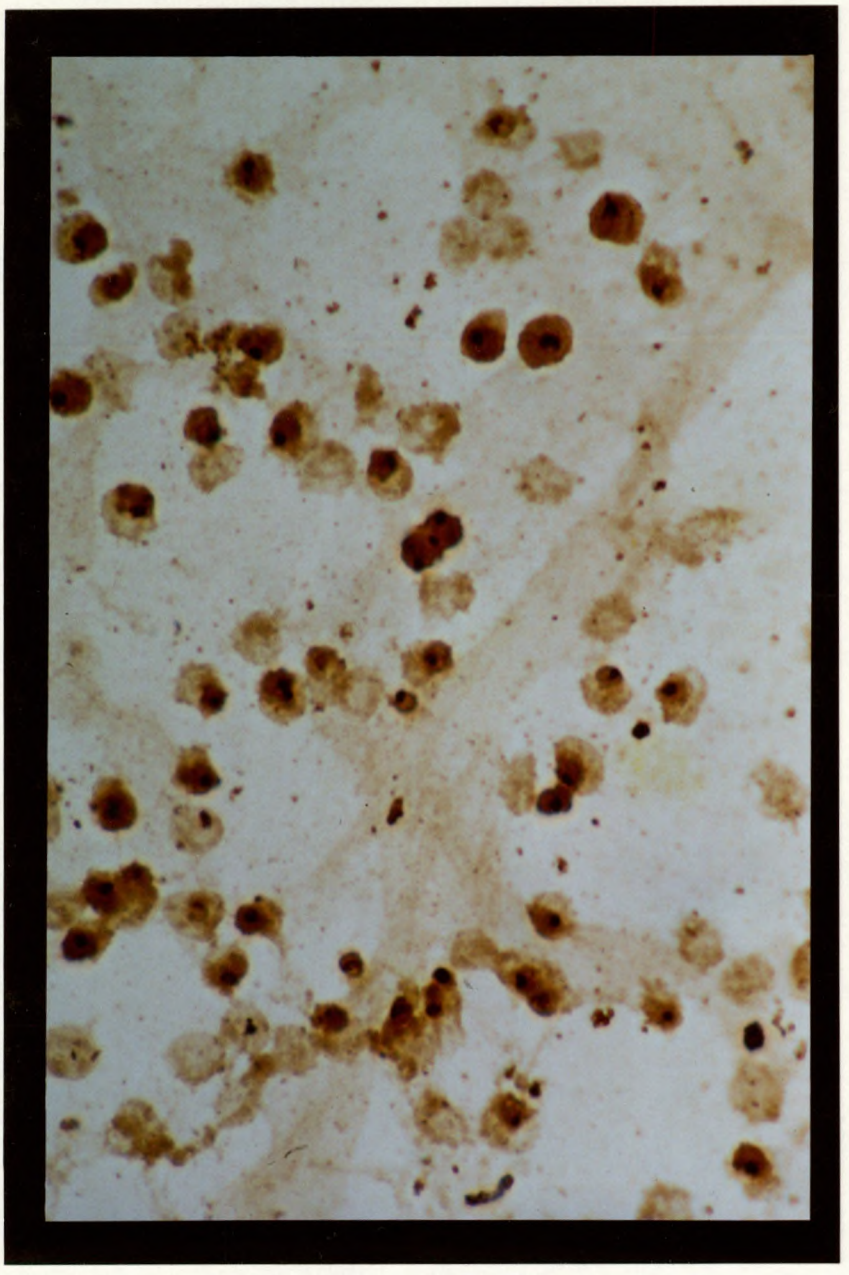


FIGURE 40: Labelling of 3D7-strain by monoclonal antibody

4.3 CONCLUSION AND DISCUSSION

Resistance to antimalarial drugs of diverse chemical classes is highly reminiscent of the human multidrug resistance (MDR) phenomenon observed in cancer cells. Studies with resistant *P. falciparum* suggest at least one analogous mechanism of resistance, the reversal of resistance to chloroquine *in vitro* by a variety of calcium antagonists (e.g. verapamil and B8509-035). The mechanism of MDR in neoplastic cells is thought to be mediated in part by enhanced transport of drug that reduces the intracellular accumulation to sublethal levels. This efflux mechanism has been associated with the overexpression of P-glycoprotein, a 170 kDa membrane transport protein that contains two nucleotide-binding domains, consistent with its role as an energy dependant "drug efflux pump". One would expect the same mechanism to be present as classical anticancer drugs such as daunomycin and vinblastine can render chloroquine-resistant *P. falciparum* susceptible to chloroquine (Krogstad *et al.*, 1987).

The results presented here indicate a P-glycoprotein-like-component in *P. falciparum*, which is present irrespective of drug-resistance and has a molecular weight of 40-42 kDa. One may also draw the conclusion that this expression fails to explain reversal of resistance, as there was no consistent difference in the level of expression on different strains that cover a broad range of chloroquine sensitivity (from 96.32nM to 2nM). These results are however, in accordance with previous results of Grogl *et al.* (1991). There are two aspects to consider, firstly the molecular weight

obtained was 40-42 kDa and not 170 kDa as expected, and secondly the presence of this band on both chloroquine resistant and -sensitive strains.

It has been accepted that C219 represents a ubiquitous probe for the detection of P-glycoprotein, since cDNA sequence analysis has confirmed that the epitope recognized by the monoclonal antibody is present in all P-glycoprotein molecules whose sequence is known. It must be remembered, however, that C219, as any other monoclonal antibody, has an inherent specificity for an epitope-type, and not necessarily a single protein entity. Thus, a potential problem for the interpretation of results from these is the diversification in the molecular weight of the polypeptides recognised by C219 and JSB-1 in *P. falciparum*. By applying the appropriate controls, however, we excluded the possibility that C219 and JSB-1 is binding nonspecifically to parasite proteins (4.1.4.9.1), as well as to prosthetic groups such as common carbohydrate epitopes and phosphates. For this reason the immunohistochemistry method was also employed. It is posssible that parasites selectively express or control a multigenic family of transport molecules with different molecular weights, to include a common epitope recognized by C219 and JSB-1.

There are identical amino acids between the mouse MDR 1 protein and *pfmdr* 1 (59% identity). The *pfmdr* 1 fragment contains an additional 45 amino acids not in the mouse MDR 1 gene (Wilson *et al.*, 1989).

The above results (Wilson *et al.*, 1989) indicated that the *pfmdr* 1 gene was expressed in both drug-sensitive and drug-resistant parasites, just that the expression was higher in the resistant strain. From this, one may speculate

that the C219 and JSB-1 (both mouse antibodies) are recognizing this 59% similarity in the amino acids. The methods used are not sensitive enough to differentiate between levels of expression.

Based on its mobility on gel electrophoresis, a range of molecular sizes between 130-200 kDa has been reported for P-glycoprotein. Glycosylation and phosphorylation are at least two of the post-translational modifications resulting in modified P-glycoprotein with higher molecular weights. Speculatively, parasites express distinct P-glycoprotein precursors with different molecular sizes that can be products of distinct P-glycoprotein genes. In addition, differential levels of modification may suggest different functional levels in the sensitive and resistant cells. This molecular diversity could possibly be driven by differences in the environment (host) and toxins encountered. The presence of P-glycoprotein-like-components in Plasmodium supports the hypothesis that this glycoprotein is an evolutionarily conserved determinant responsible for the transport of toxic substances, as has been demonstrated in higher eukaryotes. The modification of such a glycoprotein to transport cytotoxic drugs may result in the expression of one of probably many different mechanisms of drug resistance in parasites that could be similar to MDR phenotype in neoplastic cells.

Kartner *et al.* (1983) found that there was a reproducible difference in molecular-weight of the P-glycoprotein detected in colchicine- and daunorubicin-resistant cells, the daunorubicin resistance-associated protein being lower i.e., by 5 kDa. This diffuse nature of the P-glycoprotein band in SDS-PAGE indicates a molecule that may

be heterogeneous in molecular weight. This is consistent with the notion that variation in the structure of the P-glycoprotein might account for variation in the specificity of the barrier to accumulation of various drugs. Such variability, could be accounted for if the P-glycoprotein were to present a class of similar proteins with different specifications, the appropriate member (s) of the putative gene family being expressed or amplified under a given selective pressure. The possibility of minor differences in the peptide sequence of P-glycoprotein in cancer-resistant lines and *P. falciparum* resistant-strains is of interest.

A comparative study has shown that much of this heterogeneity can result from different experimental protocols (Greenberger *et al.*, 1988a). The mobility of P-glycoprotein in different gel systems is unusually sensitive to the conditions employed. The full-length P-glycoprotein cDNA sequences isolated from mouse and human cells predict a P-glycoprotein molecular size of circa 140 kDa (Chen *et al.*, 1986; Gros *et al.*, 1986). The higher molecular weights observed for the mature glycoprotein result from extensive P-glycoprotein post-translational modification. The carbohydrate moiety can account for 30-40 kDa of the mature P-glycoprotein.

It has been shown that the multiple forms of P-glycoprotein observed in a number of MDR mouse cell lines are likely the products of different P-glycoprotein genes that can undergo differential N-linked glycosylation (Greenberger et al., 1988). A vinblastine-selected cell line and a colchicine-selected cell line both express a P-glycoprotein precursor of 125 kDa that can undergo rapid differential N-linked

glycosylation to a mature P-glycoprotein of molecular size 135 kDa respectively (Greenberger *et al.*, 1987).

P-glycoprotein glycosylation has also been studied in MDR KB cells (Richert *et al.*, 1988). Only a single P-glycoprotein precursor of 140 kDa is observed in these cell lines that undergoes maturation to a final molecular size of 170 kDa through N-linked glycosylation. In the same mode, the P-glycoprotein in the malaria parasite could be "disintegrated" to form the lower molecular weight component. Future research could possibly involve the investigation of glycosylation in the parasitic P-glycoprotein, which may lead to the understanding of the function of the 40-42 kDa molecular weight protein.

The amount of protein used in western blotting for the detection of P-glycoprotein on cancer lines was approximately 50μg (Kartner *et al.*, 1985). However, we have only made use of approximately 10μg of protein. This did not affect the detection of the 40 kDa band. We do however, believe that higher protein concentrations would have given a stronger band.

The problem of chloroquine-resistance is neither simple nor straightforward. If one endeavours to explain the reason for the labelling by the monoclonal antibodies of both chloroquine resistant and -sentitive strains, one would start by comparing it to MDR in cancer. Chloroquine-resistant isolates of *P. falciparum* do not display such striking cross-resistance (vincristine and colchicine) as occurs in mammalian MDR.

It is clear that acquisition of chloroquine resistance does not guarantee resistance to either quinine, amodiaquine, or mefloquine, but it does appear to predispose the parasite to resistance to these other drugs (Fitch C.D., 1972).

In mammmalian MDR tumour cells, it has been shown that expression of P-glycoprotein is sufficient to confer the drug resistance phenotype and that the level of plasma-membrane-associated P-glycoprotein approximates the level of drug resistance observed (Guild *et al.*, 1988). This is not the case in the malaria parasite. The recent studies on the genetic basis of chloroquine-resistance (Wellems *et al.*, 1990,1991) and the demonstration of mutations in *pfmdr* 1 linked to chloroquine-resistance (Foote *et al.*, 1990) suggest that expression of mutant forms of Pgh 1 (chapter 1) alone is insufficient for chloroquine-resistance.

Analysis of this protein in a number of *P. falciparum* isolates revealed that all parasite lines regardless of their chloroquine-resistant or sensitivity status express Pgh 1. One must keep in mind that the Pgh 1 was identified with antibodies raised to a fragment of the *pfmdr* 1 gene product expressed in *E. coli*, and the result is therefore not unexpected, if one considers all the strains contained the *pfmdr* 1 gene product. It was also reported by Foote *et al.* (1989) that the presence of the *pfmdr* 1 gene in some chloroquine-resistant isolates had a copy number equivalent to that of chloroquine-sensitive parasites. Amino acid analysis encoding the *pfmdr* gene in FCR-3, RSA-2 and 3D7 strain would probably indicate the same copy number, explaining the equivalent expression of P-glycoprotein.

It is possible that P-glycoprotein may not function in a manner analogous to the mammalian P-glycoprotein's which are involved in drug resistance. What then is responsible for the common phenotypic features of chloroquine-resistance and MDR?

It is generally accepted that the pH gradient between the medium and the parasite digestive vacuole is the driving force for accumulation of chloroquine. It has been shown that the intravacuolar pH of several chloroquine-resistant isolates is somewhat higher than that of chloroquine-sensitive isolates, and that this might account for the observation that chloroquine does not accumulate significantly within the digestive vacuoles of chloroquine-resistant parasites (Geary *et al.*, 1990). One prediction that may follow from this model is that the so-called reversal agents might function by decreasing the pH of the relatively more basic food vacuole compartment of chloroquine-resistant parasites. The idea of altered intravacuolar pH is interesting in light of recent observations that P-glycoprotein activity results in alkalinization of the drug-accumulating compartment in mammalian MDR tumour cells (Thiebaut *et al.*, 1990).

Warren *et al.* (1991) found that multiple drug-resistant human lymphoblastic leukemic cells in which P-glycoprotein was expressed had a lower content of lysosomal enzymes, such as N-acetylglucosaminidase and -galactosidase, and the relative rates of secretion of these enzymes were significantly greater than those of its drug-sensitive counterpart. The presence of P-glycoprotein in the plasma membrane may,

in some indirect manner, lead to increased exocytosis of lysosomal enzyme, ultimately resulting in a significant depletion of enzyme.

Genetic analysis has also shown that neither of two known *P. falciparum* MDR-like genes (*pfmdr* 1 and *pfmdr* 2) can be linked to resistance (Wilson *et al.*, 1989; Foote *et al.*, 1989,1990). A mechanism other than one involving amplification or mutation of *pfmdr* 1 must therefore account for rapid drug efflux from chloroquine-resistant parasites.

One may conclude that *P. falciparum* contains a P-glycoprotein-like-component. The exact function of the component is yet unknown. It is possible that it functions as a transporter of substances eg., toxins out of the parasite, or amino acids into the parasite.

CHAPTER FIVE

5 PIGMENT ANALYSIS

Haemozoin (malaria pigment) is the brown-black product of haemoglobin degradation that appears in increasing amounts in the intra-erythrocytic growth stages of members of the genus Plasmodium. A continuing question has been; How is haemozoin formed and of what is it composed?

Due to the conflicting reports on the nature of malarial pigment and the possible interaction of pigment with chloroquine, it seemed worthwhile to reinvestigate the characteristics and composition of haemozoin derived from the human malarial parasite *P. falciparum*.

5.1 METHODOLOGY

5.1.1 PIGMENT ISOLATION

The two important factors which must operate in any extraction of haemozoin, regardless of the source are; (a) the material prior to extraction must be made as free from haemoglobin as possible; and (b) the extracting solution must be one in which any residual haemoglobin, of however minute a concentration will

be stable. The more difficult achievement is the production of haemoglobin-free material prior to extraction.

The method to isolate malarial pigment was developed in the Department of Experimental and Clinical Pharmacology (paper in preparation).

PHOSPHATE BUFFERED SALINE

8,0g NaCL

0,3g KCL

0,73g Na_2HPO_4 x $2H_20$

0,20gKH_2PO_4

The above ingredients were dissolved in distilled water and the volume made up to 1ℓ.

5.1.2 PIGMENT ISOLATION MEDIUM

P. falciparum parasites were isolated and lysed (4.1.2 and 4.1.3). The pellet was washed in two to three times its volume with pigment isolation medium. Extraction medium constituted 0.1% SDS in 10% PBS. The suspension was centrifuged at 900g for 10 min and the supernatant discarded. A methanol-fixed Giemsa-stained slide of the pellet was prepared to determine the purity of

the pigment. The washes were repeated several times, until only pure pigment remained. The pure isolated pigment was stored at -70°C.

5.1.3 SDS-GRADIENT GEL ELECTROPHORESIS, pH 11.2

5.1.3.1 STOCK SOLUTIONS

PHOSPHATE BUFFER

300mM Na_2HPO_4 adjusted to pH 11,2 with NaOH

ACRYLAMIDE STOCK SOLUTION

38,9g acrylamide and

1,1g bisacrylamide was

dissolved in distilled water and the volume made up to 100ml

5.1.3.2 CASTING GELS

A 3-20% linear gradient gel was utilized to separate the components of the isolated pigment (modified from 4.1.4).

3% Gel

5ml phosphate buffer (300mM, pH 11,2)

0,75ml acrylamide stock solution

4,25ml water

10mg ammonium persulphate

The above ingredients were mixed well

20% Gel

5ml phosphate bufer

5ml acrylamide stock solution

10mg ammonium persulphate

5.1.3.3 BUFFERS

Anode Buffer

15ml phosphate buffer (300mM, pH11,2) was made up to 500ml with distilled water.

Cathode Buffer

15ml phosphate buffer (300mM, pH 11,2) was made up to 500ml with distilled water. To this, 500mg of SDS was added.

Sample buffer

1,2g urea

100mg SDS

900$\mu\ell$ phosphate buffer (300mM, pH 11,2)

900$\mu\ell$ water

a trace of phenol red

5.1.3.4 SAMPLE PREPARATION AND APPLICATION

Blood

20$\mu\ell$ of freshly washed erythrocytes was diluted in 480$\mu\ell$ of 10% PBS (1:24 dilution) and insoluble part removed by centrifugation. 20μl of the diluted blood was added to 20$\mu\ell$ of sample buffer and vortexed. 20$\mu\ell$ was applied to the gel.

Pigment

20$\mu\ell$ of the sample buffer was added to 10$\mu\ell$ of isolated pigment pellet and vortexed. 20$\mu\ell$ was applied to the gel

5.1.3.5 GEL ELECTROPHORESIS

A detailed description with apparatus of the electrophoretic procedure has been discussed (4.1.4.3).

The separation was done at a constant voltage of 95V for approximately 4 hours at 4°C. To prevent overheating the voltage was kept below 100V.

5.1.4 DETECTION OF PROTEIN BANDS

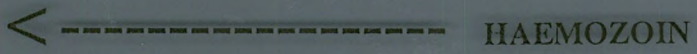
The protein bands were detected with Coomassie blue stain. Each gradient gel was loaded in such a way that one half was the mirror image of the other half. One half of the gel was placed in Coomassie blue stain and the other half in destain. The gels in Coomassie blue stain were transferred to destain after approximately 12 hr and remained there until the background became clear.

5.2 RESULTS

In order to be able to evaluate that the isolation procedure had not in any way detracted from the crystalline structure of haemozoin, a smear of a normal culture was prepared, stained with giemsa and then examined under polarized light (Fig 41). Figure 43 is a photomicrograph of isolated pigment. As can be seen its stucture resembles that of intact haemozoin within the parasite (Fig. 41). Figures 44a, and b, are micrographs, representing the isolated pigment as in figure 43, but subjected to different intensities of polarized light. Figure 45 represents polarized pigment in isolated parasites. Again one cannot differentiate between the haemozoin in figure 41 (intact parasite) and the isolated haemozoin as in figure 44a, b, and figure 45.

As the extraction medium contained sodium dodecyl sulphate which is known for dissolving protein, a protein assay (4.1.4.7) was carried out on each extraction supernatant to ensure that integral protein within the haemozoin was not being dissolved. Figure 42 is the standard curve used to determine the protein content. Supernatant 1 (i.e., the first wash) contained 14,59$\mu g/100\mu\ell$. Supernatant 2 and 3 contained 25,47$\mu g/100\mu\ell$ and 2,65$\mu g/100\mu\ell$ respectively.

After separating the components of the haemozoin by SDS-PAGE, the gel was stained for protein (Fig. 46). Lane A is the haemoglobin (run as control), Lane B is the isolated haemozoin. Both lane A and B were placed in destain. Lane C and D was placed in Coomassie blue (protein stain). Lane C represents the isolated pigment as for lane B, and lane D represents the haemoglobin as for lane A. Lane C, which is the haemozoin stained for protein, did not stain unlike the control (lane D). From these results we concluded that protein does not form an integral part in the structure of haemozoin.


HAEMOZOIN

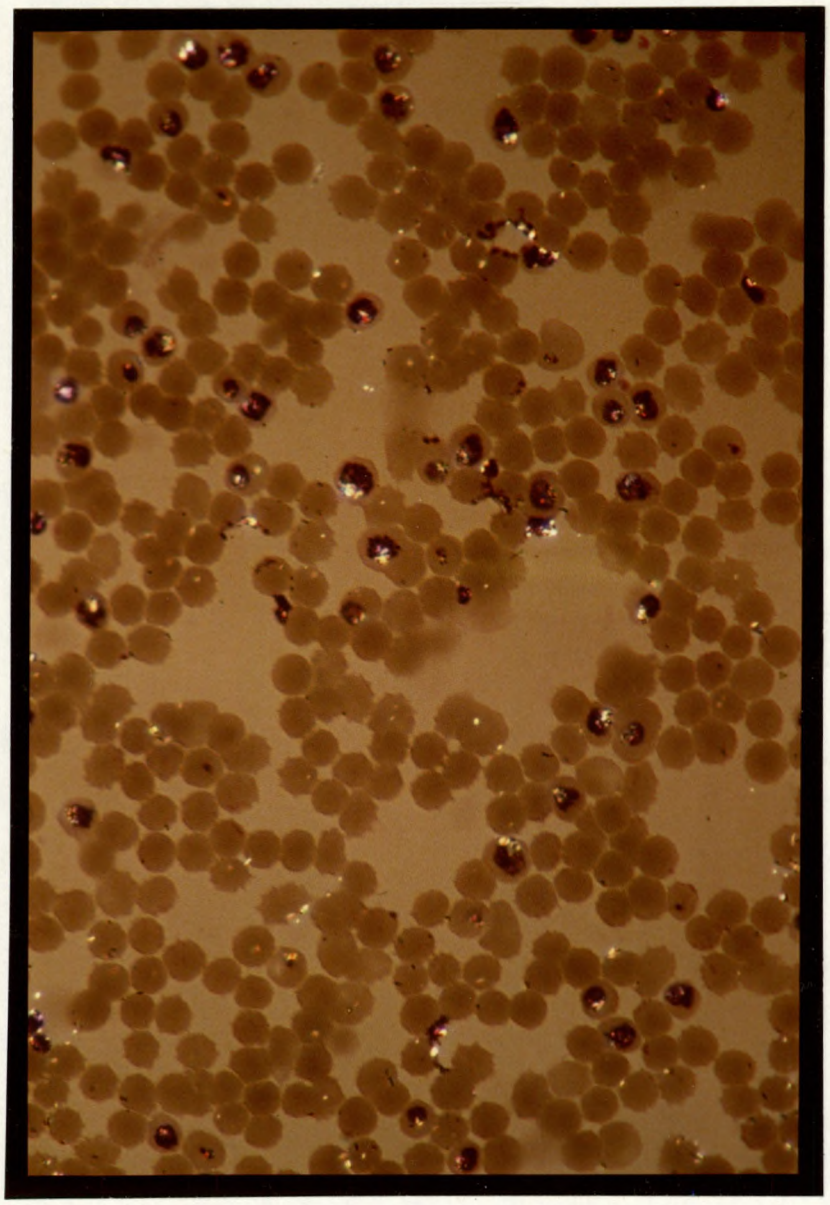


FIGURE 41: Polarized light on culture showing the crystalline form of haemozoin as present *in vitro*

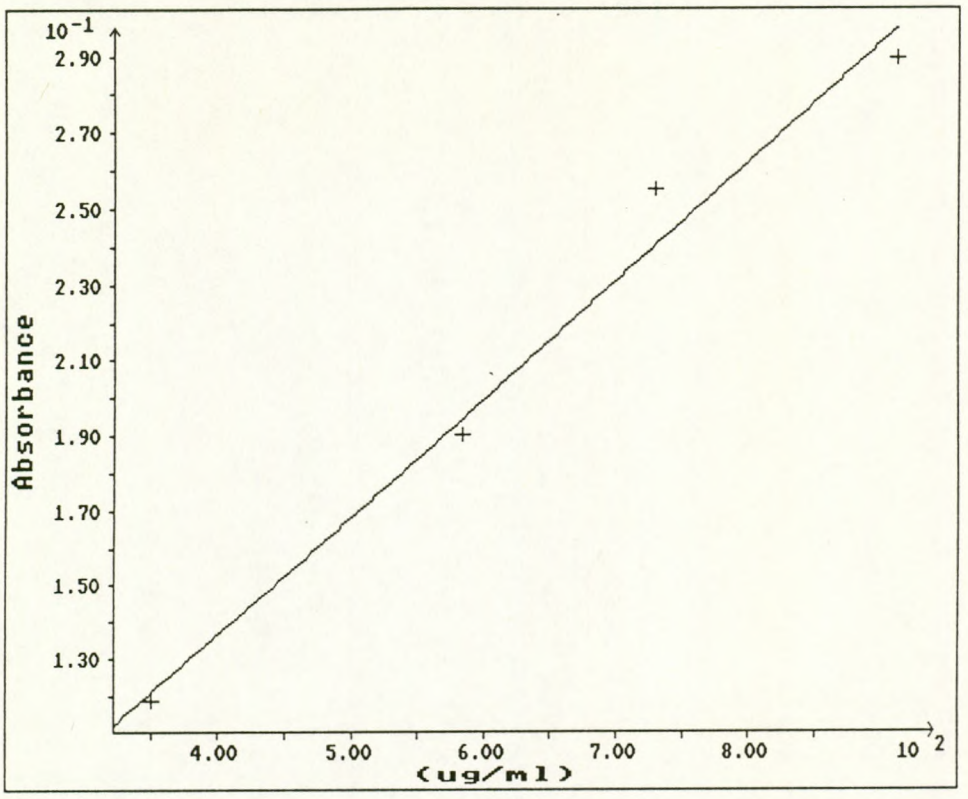


FIGURE 42: Standard curve for protein measurement

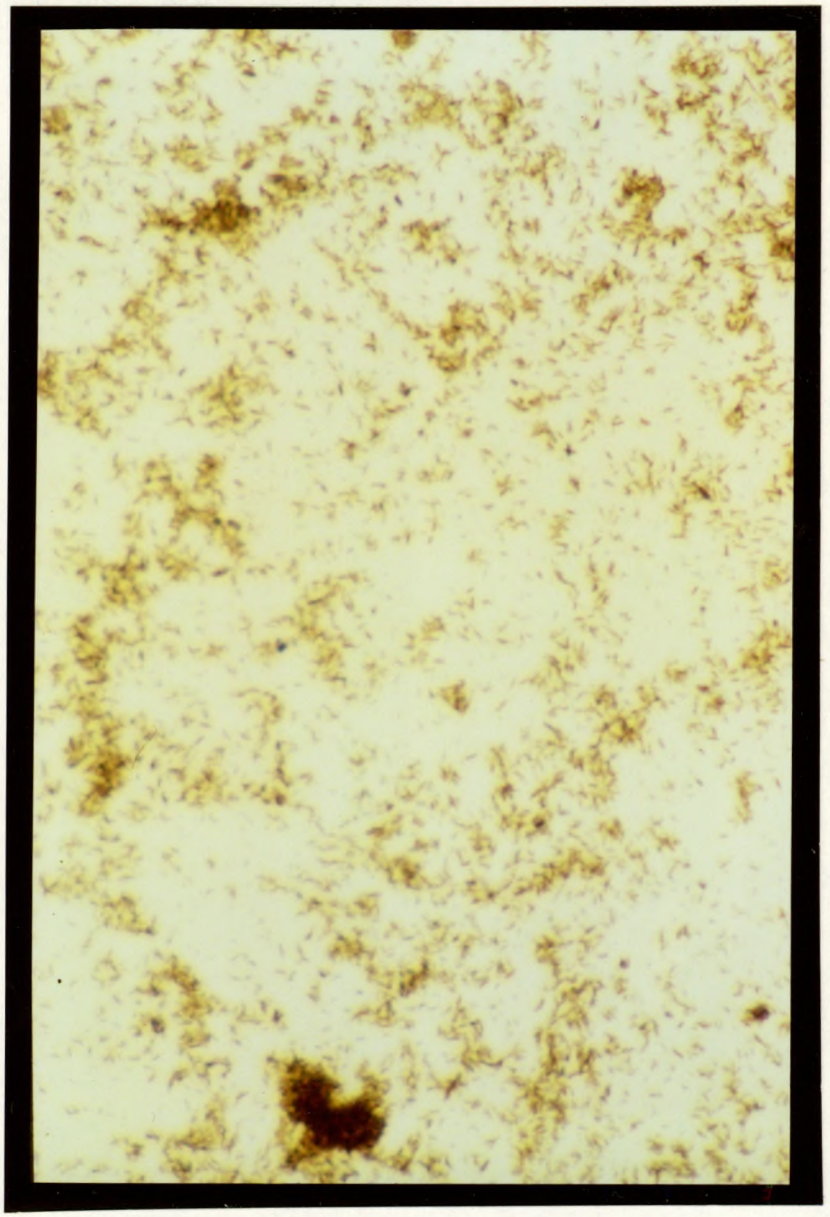


FIGURE 43: Isolated haemozoin

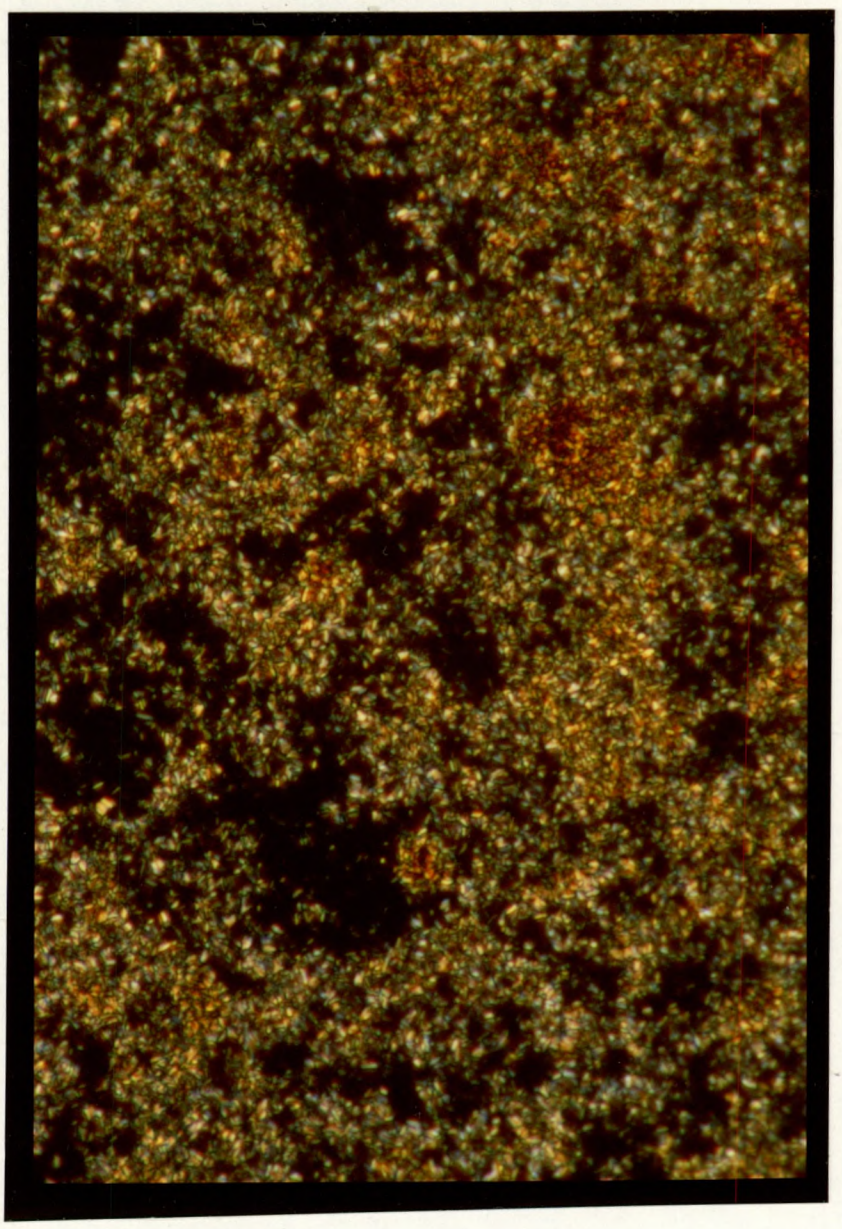


FIGURE 44A: Different concentrations of isolated haemozoin, showing its intact crystalline shape

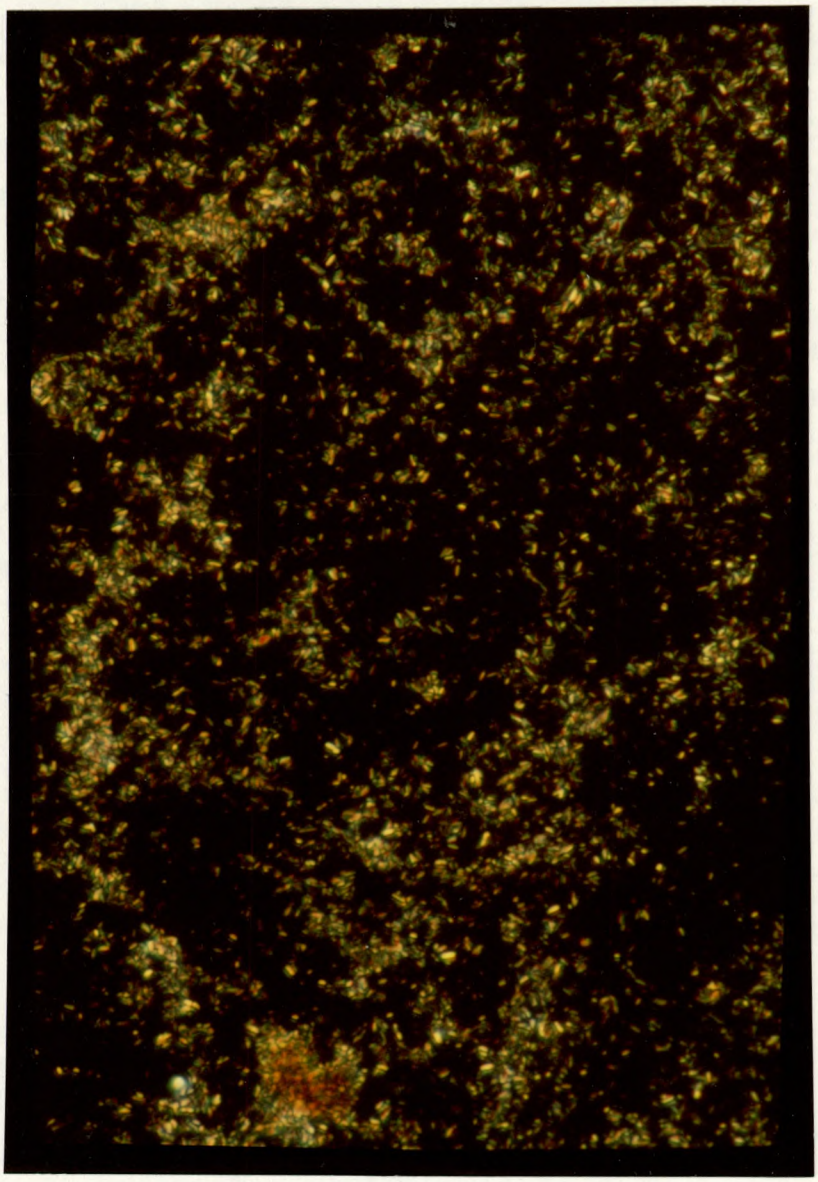


FIGURE 44B

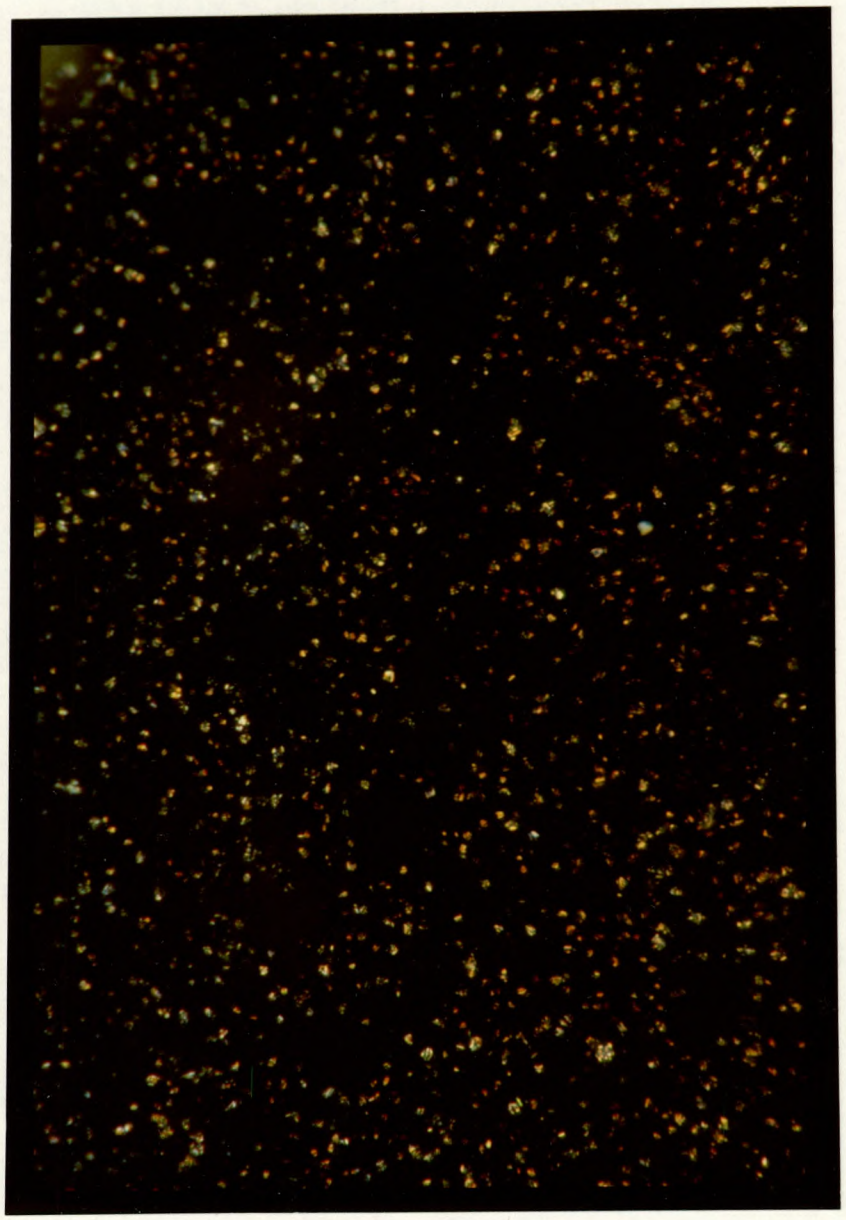


FIGURE 45: Polarized pigment in isolated parasites

A B C D

DESTAIN

COOMASSIE BLUE STAIN

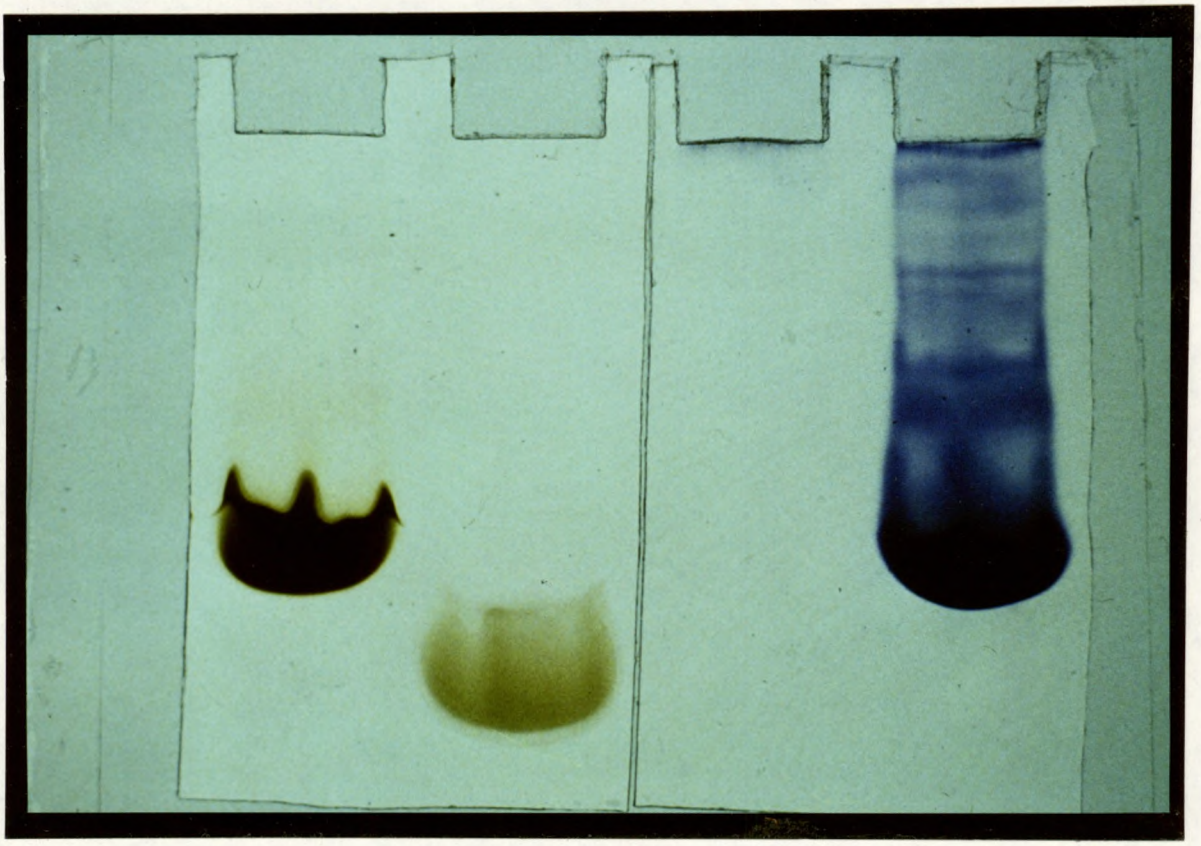


FIGURE 46: Protein stain of haemozoin (malaria pigment)

Lane A = haemoglobin (control); B = isolated haemozoin;

C = isolated pigment; D = haemoglobin

5.3 DISCUSSION

Since the free amino acids present within the erythrocyte are not sufficient to serve as a source for plasmodial protein synthesis, and since *de novo* biosynthesis of amino acids is severely restricted, the principal protein of the erythrocyte, haemoglobin, remains the most abundant reservoir of amino acids available to the growing plasmodia. A conspicuous feature of the intraerythrocytic development of the malarial parasite is the deposition of golden brown- black pigment granules (haemozoin). It was already assumed by the early malariologists that haemozoin is a breakdown product of haemoglobin (Ross, 1923); however, there was no experimental evidence for this until the turn of the century. Brown (1911) extracted malaria pigment from livers and spleens obtained from *P. falciparum* infected patients using alcoholic KOH or dilute alkali. The spectral properties of these extracts were identical to pure haematin. Based on this similarity, he proposed that the malarial parasite contained proteolytic enzymes capable of splitting haemoglobin into protein and haematin; the protein was used by the parasite, whereas the haematin simply accumulated as a waste product. Brown never assumed that haemozoin was pure haematin, and considered it probable that haematin was associated with other materials. Other investigators (Devine and Fulton, 1941; Devine and Fulton, 1942; Rimington and Fulton, 1947) solubilized malaria pigment from *P. kowlesi, P. berghei* and *P. gallinaceum* in 0.25 mol/litre Na_2CO_3 or 0.01 mol/litre NaOH and came to a similar conclusion: haemozoin was haematin.

However, Deegan and Maegraith (1956b) questioned such equivalence. They concluded that the extraction procedures employed by previous workers were too harsh and that during solubilization the haem was cleaved from an associated polypeptide constituent. Using milder extraction methods (0.1 mol/litre borate buffer, pH 9.2) they found, after spectral analysis, that *P. malariae*, *P. falciparum*, and *P. cynomolgi* haemozoin's contained haematin coupled to a denatured polypeptide (Deegan, 1956a; Deegan and Maegraith, 1956b). According to Deegan and Maegraith (1956b), the combined form of haematin would contribute to the insolubility of malaria pigment. However, under *in vitro* conditions Yamada and Sherman (1979) have shown that dimeric haematin, a constituent of haemozoin, undergoes spontaneous aggregation at pH 12. Titration of such alkaline solutions of solubilized haemozoin to physiological pH values caused the pigment to precipitate. Haematin treated in an identical manner behaves similarly. This indicates that protein is not needed to form insoluble haemozoin. Thus, the particulate quality of haemozoin *in vivo* largely reflects the phenomenon of iron-porphyrin self-aggregation.

Following these lines of reasoning and using similar extraction techniques plus amino acid analysis as well as peptide fingerprints, it was claimed that *P. lophurae* haemozoin was haem-containing protein (Sherman and Hull, 1960; Sherman *et al.*, 1965; Sherman *et al.*, 1968). The insolubility of haemozoin (except in highly alkaline solutions) made determination of its molecular size by conventional ultracentrifugation difficult; however, using an indirect method (comparison of

haem-iron; dry weight of haemoglobin and haemozoin) Sherman *et al.* (1968) claimed that haemozoin had, on the average, a significantly smaller molecular size than haemoglobin. Thus the conclusion reached was that haemozoin was partially degraded haemoglobin. Homewood *et al.*, (1972) challenged this view and suggested that the amino acids and peptides found in the haemozoin preparations of Sherman *et al.* (1968) were not an integral part of the pigment, but instead were derived from membrane contaminants. Indeed, subsequent electron microscopic investigations with *P. knowlesi* haemozoin prepared by the Sherman-Hull method showed membrane contaminants (Yamada and Sherman, 1979).

Homewood *et al.* (1972, 1975) attempted to purify *P. berghei* haemozoin by repeated washings with 0.1% sodium dodecyl sulphate (SDS), and in some instances followed this detergent treatment with a 2-day incubation in a solution of pancreatin. The resultant product retained the *in vivo* crystalline appearance of haemozoin (Moore and Boothroyd, 1974; Moore *et al.*, 1975), contained ferric-iron, had an absorption maximum at 650nm, and rotated the plane of polarized light. Yamada and Sherman (1979) believe that malaria parasites complex free ferriprotoporphyrin IX (which is lytic to membranes) with protein (s) to give an inert crystal; haemozoin. As a result a possible mechanism for resistance would be alteration in the protein (s) responsible for sequestering ferriprotoporphyrin IX as haemozoin. However, it is known that haemin (ferriprotoporphyrin IX) is insoluble at low pH (such as that found in the food vacuole), and therefore, does

not need to be complexed with protein to render it insoluble and therefore inactive.

In our study the haemozoin was isolated by a modification of the method applied by Homewood *et al.* (1972). To investigate that the crystal remained intact during the isolation procedure, polarized photographs of the isolated pigment were taken (fig. 44a,b and 45). From figures (43 and 45) one can see that the crystals retained their morphological shape as found in intact parasites, during the isolation procedure. The extraction medium utilized, contained 0.1 % SDS. This would have removed proteins that were present in the lysate after lysing the parasite. One may argue that SDS being notorious for solubilizing protein would also solubilize protein associated with the haemozoin. However, if protein formed an integral part of the haemozoin, and if this protein was solubilized by the SDS the haemozoin crystal would have disintegrated. The physical properties of haemozoin do not change following the complete removal of protein. The figures clearly demonstrate a native intact crystal which has not been tampered with. We believe that non-specific protein which attaches to the haemozoin due to its inherant charge would be removed by the SDS. The carboxy side-chains in ferroprotoporphyrin IX (figure 47) will become hydrolysed ($O=C-O^-$) in an acidic environment (food vacuole has a pH 5). Nitrogenous compounds will be attracted by the negative charge. These are most likely the proteins that were detected by earlier researches (Sherman and Hull, 1969).

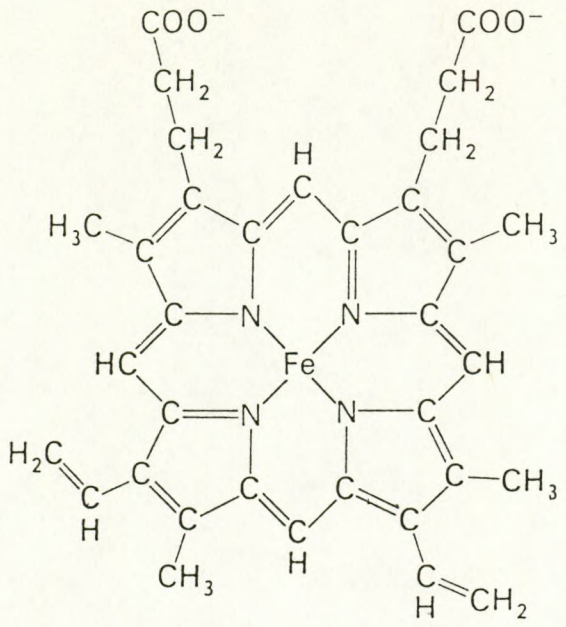


FIGURE 47: Ferroprotoporphyrin structure

Goldie *et al.* (1990) used a sucrose gradient to remove membranes, but did not remove proteins from the lysate. These researchers stipulate that haemozoin is 65.1% protein. It appears illogical that the parasite would give up so much protein for the haemozoin (waste product), when it cannot synthesize amino acids *de novo*, and therefore ingests the host's haemoglobin. Ferriprotoporphyrin IX is also known to interfere with the Bradford colorimetric protein assay (Bio-Rad Bulletin 1069, 1979) by giving anomalous high readings. Assuming protein makes up a large portion of haemozoin, conflicting results exist as to the origin of this protein. Goldie *et al.* (1990) conclude that almost all of the protein component of haemozoin comes from haemoglobin. However, Ashong *et al.* (1989) concluded that the protein component of *P. falciparum* haemozoin was a parasite synthesized

polypeptide of 14 kDa. This contradiction must arise from incorrect isolation procedures.

The protein concentrations in our consequent washes decreased. Figure (46) indicates that the isolated haemozoin was void of protein. The isolated haemozoin is insoluble and the only way one could get it to run on a gel was to prepare a sample and running buffer with a pH=11.2. At this pH the haemozoin dissolved and its components could then be separated on a SDS -polyacrylamide gel. Haemoglobin was run as the control. When the gel was stained for protein, the haemoglobin stained, but the dissolved haemozoin did not stain for protein. Sherman isolated *P. lophurae* haemozoin by Kilejian's technique (Kilejian, 1974) for obtaining dense granules. Both crude and purified haemozoin (contaminating dense granules were removed by acetic acid treatment, a technique that leaves insoluble haemozoin) were analysed by SDS polyacrylamide gel electrophoresis and isoelectric focusing. No evidence for haemoglobin degradation products was found; however, even with "purified" haemozoin, contaminating proteins (mainly histones) were present. The evidence suggests that haemozoin is almost entirely haematin. It seems probable that the method of isolation, or the choice of solvent of the malaria pigment has a great influence on the results, leading to the confusion that still exists over the composition of haemozoin.

Sherman (1977) believes the relationship of haemoglobin to haemozoin to be the following: when the intraerythrocytic plasmodium takes up host cell haemoglobin, the latter is deprived of those physiological mechanisms that serve to protect it from oxidation; consequently, haemoglobin auto-oxidizes to methaemoglobin and non-enzymatically dissociates into ferrihaem (haematin) and globin. As the haematin concentration increases, there is self-aggregation, especially below pH 8. Spontaneous granule deposition occurs and the accumulated haematin is haemozoin.

The evidence presented by our study permits the following conclusion: haematin (ferriprotoporphyrin IX) associates non-specifically (non-covalent complex) with proteins and this may occur *in vivo* but, may be a consequence of breaking the cell to obtain haemozoin. *In vivo*, the topographic isolation of such proteins prevents their attachment to haematin; however, lysis of the cell releases these proteins and thereby permits new haem-protein associations. At physiological pH, haematin aggregates are insoluble and therefore non-toxic; therefore to account for its physiological activity, a polypeptide association with haematin is unnecessary. Thus, the physicochemical properties of haemozoin strongly suggest its equivalence to haematin. *In vivo*, some of the haematin may be associated with minor amounts of enzymatic or other proteins present in the digestive vacuoles. Proteins almost certainly associate with haemozoin, *in vivo*, but they are not integral to the haemozoin structure and therefore have no function (Goldberg and Slater, 1992). Indeed, elemental analysis of purified haemozoin from both *P. berghei* and *P.*

falciparum is consistent with haem being the only component present (Slater *et al.*,1991; Fitch and Kanjananggulon, 1987).

One other conclusion that may be drawn from the uncertainty of the composition of haemozoin, is that results are dependant upon the methodology utilized. For example, if haemozoin was isolated in the presence of 10mM KCN, either in acid or alkaline solutions, it showed higher iron:dry weight values, than did material prepared without KCN (Yamada and Sherman, 1979). Extraction of the pigment in solvents which degrade haemoglobin, eg. phenol,0.1N NaOH, cannot be used to ascertain the properties of haemozoin, for the pigment in such solvents shows a spectrum identical to haemoglobin (Sherman and Hull, 1960).

The present investigation of the pigment granules leaves the question of the exact nature of the malaria pigment unanswered, except to say that a small contribution has been made with regard to the protein component of haemozoin. It is also interesting to note that haemozoin remains for years in the spleen and other organs in which it is deposited and this clearly indicates that the human organism possesses no adequate means for its disposal (Brown, 1911), and therefore the intricate composition of haemozoin.

CHAPTER SIX

6 REFERENCES

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