CHARACTERISATION OF KENYAN ISOLATES OF *PLASMODIUM FALCIPARUM* BY ISOENZYME ELECTROPHORESIS AND DRUG SENSITIVITY.

BY

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science of the University of Nairobi.

1989

DECLARATION

This Thesis is my original work and has not been presented for a degree in any other University.

Signed _____ Date 19/4/89

AYUB V.O. OFULLA

This Thesis has been submitted for examination with our approval as University supervisors:-

1. Signed _____ Date 4.5.89

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DR. BALDIP KHAN

DEDICATION

To those who still take science lightly.

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ABSTRACT.

Twenty one Kenyan isolates of Plasmodium falciparum were characterised by enzyme electrophoresis and antimalarial drug sensitivity.

For isoenzyme analysis, cellulose acetate and thin layer starch gel electrophoresis techniques were employed. P. falciparum isolates from malarious areas of Nyanza, Rift Valley and Coast provinces were cultivated in vitro and used in the isoenzyme study. The enzymes of uninfected red blood cells served as controls.

The enzymes studied were: Glucose phosphate isomerase (GPI)(EC 5.3.19), 6-phosphogluconate dehydrogenase (6PGD)(EC 1.1.1.44), Lactate dehydrogenase (LDH)(EC 1.1.1.27), NADP-dependent glutamate dehydrogenase (GDH)(EC 1.4.1.4), Adenosine deaminase (ADA)(EC 3.5.4.4) and Peptidase E (PEPE)(EC 3.4.11 or 13).

The sensitivities of cultured isolates to seven antimalarial drugs (chloroquine, amodiaquine, quinine, quinidine, mefloquine, pyrimethamine and sulphadoxine) were examined by the in vitro radioisotopic methods of Desjardins et al. (1979) with minor modifications.

Variant forms of the enzymes GPI and 6PGD were found in the Kenyan isolates of P. falciparum. GPI enzymes appeared as GPI-1 and GPI-2 variants and 6PGD as 6PGD-1 and 6PGD-2 variants. The level of enzyme polymorphism was greater in

GPI than in 6PGD. The isolates from Rift Valley province were found to possess a lower frequency of GPI-2 isoenzymes than isolates from Nyanza and Coast provinces.

The other four enzymes (GDH, LDH, ADA and PEPE) appeared to be invariant in all the isolates. ADA-2 forms were found in all the isolates while the other three enzymes appeared as GDH-1, LDH-1 and PEPE-1 types in all the isolates examined.

Considerable variation was found in the antimalarial drug response of the 21 parasite isolates. The Rift Valley province isolates showed significantly lower susceptibilities to most of the antimalarial drugs tested than the Coast and Nyanza province isolates. Coast Province isolates were found to be marginally more sensitive to the antimalarial drugs than the Nyanza province isolates.

CHAPTER ONE

INTRODUCTION

1.1 Literature Review

Plasmodium falciparum, the cause of malignant malaria, is classically recognised as a single species throughout the world. It has now been found to consist of several distinct strains by many workers. Strains differ in characters such as morphology, clinical manifestations of infection in man, drug-response and infectivity to different species of mosquito (Walliker, 1985). The parasite is antigenically an extremely complex organism which appears capable of changing its pattern of antigens to evade the immune defences of its host and which may exist as immunologically distinct strains in different geographical regions of the world (Carter and McGregor, 1973).

To gain more knowledge of the taxonomy and genetics of the parasite, it is imperative that strains of *P. falciparum* from different geographical regions of the world be characterised. Such knowledge will be important to the study of immunology, biochemistry, chemotherapy and epidemiology of malaria (Carter and McGregor, 1973; Walliker *et al.*, 1973; Walliker , 1976).

Malaria parasites show considerable genetic variation, not only between different species, but also within single species (Walliker, 1982). Genetic variation in *P. falciparum* is not uniform throughout the world; different

geographical regions of the world may have different strains of the species (Sanderson *et al.*, 1981). Furthermore considerable genetic variation in *P. falciparum* has been found to occur even within relatively small malarious areas (Thaithong *et al.*, 1984). This implies that malaria parasites have great potential for genetic variability which could be realised, for example, in the presence of drug pressure. Evidence exists that some *P. falciparum* strains differ in their drug-sensitivity and so they may require different control measures (Walliker, 1982; Thaithong, 1983). Thus a greater understanding of the genetic diversity of the parasite may help efforts to control the disease.

With such genetic diversity in *P. falciparum* it is important that reliable methods for characterising the different strains occurring in different geographical regions should be employed. Techniques are now available for studying variation in genetically determined characters such as enzymes and antigens. This has become possible because of the advent of techniques for *in vitro* culture and cloning of the blood forms of *P. falciparum* (Trager and Jensen, 1976; Wilson *et al.*, 1977; Rosario , 1982; Walliker, 1985; Rosario and Thaithong, 1986).

Various methods have been employed for characterising *P. falciparum*. These include morphology and growth rate studies, enzyme electrophoresis, antigen characterisation, two-dimensional protein electrophoresis, drug-sensitivity

studies and DNA studies (Wilson, 1980; Tait, 1981; Goman et al., 1982; Rosario, 1982; Graves et al., 1984; Fenton et al., 1985; Webster et al., 1985; Walliker , 1985; Rosario and Thaithong, 1986).

Enzyme electrophoresis has been used to identify individual gene differences between parasite strains and to examine gene frequencies in parasite populations (Carter and Walliker, 1977). Variant forms of enzymes as revealed by electrophoretic techniques often occur in parasite populations. Enzyme polymorphism has been found in all groups of parasitic protozoa including amoebae, trypanosomes, Leishmania, coccidia, Babesia and Plasmodia (Carter and Walliker, 1977; Al-Taqi and Evans, 1978; Godfrey, 1978; Gibson and Gashumba, 1983).

Enzyme electrophoresis has been applied to rodent species of Plasmodia (Carter and Walliker, 1975; Carter, 1978) and to P. falciparum (Carter and McGregor, 1973; Carter and Voller , 1975) and P. vivax (Myint-Oo, 1986). Isoenzyme forms are important for characterisation of parasites of the same or different species as they reveal the genetic diversity of such parasites (Thaithong, 1981). Therefore, their study can provide an insight into the genetic constitution of a parasite (Carter and Walliker, 1977). In the case of sexually reproducing P. falciparum, such knowledge is important for understanding how strains of the parasite freely interbreed in the field, a means through which certain undesirable parasite factors such as drug-

resistance, virulence and vector adaptability may spread in parasite populations.

Isoenzymes can act as reliable genetic markers of different strains or varieties and clones of parasite species. Such knowledge has been found invaluable in many of the genetic studies of malaria parasites in relation to their sensitivities to antimalarial drugs (Knowles et al., 1981; Knowles, 1982). Variant forms of enzymes are important diagnostic tools for identifying P. falciparum strains especially in mixed infections (Walliker , 1985). Isoenzymes have been used as markers in establishing and differentiating clones in the initial work on clones (Rosario, 1981; Graves et al., 1984).

Isoenzymes reflect the differences in the sequences of nucleotides that make up a gene and since enzymes are products of single genes, they are usually inherited in simple Mendelian fashion and have been found to be stable over many generations (Carter and Walliker, 1977; Walliker, 1982). Moreover, there is genetic proof of the haploidity of the blood forms of P. falciparum (Carter and McGregor, 1973; Walliker, 1982). This means that single clones of P. falciparum have single isoenzymes, thus making their characterisation by electrophoretic techniques possible (Carter and McGregor, 1973; Sanderson et al., 1981; Thaithong et al., 1981; Walliker, 1982; Rosario and Thaithong, 1986).

Isoenzyme analysis has been performed on P. falciparum from several countries such as Thailand, Gambia, Tanzania, Congo, Brazil and Cambodia (Carter and McGregor, 1973; Carter and Voller, 1975; Carter and Walliker, 1977 ; Sanderson et al., 1981; Thaithong et al., 1981, 1984; Myint-Oo et al., 1984; Walliker, 1985; Rosario and Thaithong , 1986). Six enzymes have been found to be most useful. These include: Glucose phosphate isomerase (GPI)(EC 5.3.19), 6-phosphogluconate dehydrogenase (6PGD)(EC 1.1.1.44), Lactate dehydrogenase (LDH)(EC 1.1.1.27), NADP-dependent glutamate dehydrogenase (GDH)(EC 1.4.1.4), Adenosine deaminase (ADA)(EC 3.5.4.4) and Peptidase E (PEPE)(EC 3.4.11 or 13) (Carter, 1978; Sanderson et al., 1981; Walliker, 1985).

Starch gel or cellulose acetate electrophoresis systems can be used to analyse the isoenzymes of the erythrocytic forms of P. falciparum (Thaithong et al., 1984; Walliker, 1985).

So far the results of the isoenzyme studies of P. falciparum from different countries reveal that GPI, LDH, ADA, and PEPE each occur as two variant forms. There is evidence of some regional variation in the frequencies of certain forms. Parasites characterised by GPI-1 are more common than those characterised by GPI-2 in Gambia, Tanzania and Thailand; and in Brazil, GPI-2 forms are more frequent (Walliker, 1985). ADA-2 occurs much more frequently in Brazil than in Gambia and Thailand, where the frequencies of

ADA-1 and ADA-2 are remarkably similar (Rosario and Thaithong, 1986). LDH-2 has been found almost exclusively among African isolates, the only exception being a report of its presence in five isolates from Burma (Walliker, 1985). Most cultured isolates show PEPE-1. PEPE-2 and PEPE-3 have been reported mainly among the freeze-dried samples from Tanzania, but also from among the isolates from Ghana, Indonesia and probably from Zaire. The frequency of PEPE among P. falciparum isolates is still uncertain in view of the few samples studied so far and also the difficulty in distinguishing its type-1 and type-2 forms (Sanderson et al., 1981).

No isoenzyme study of P. falciparum isolates from Kenya has been published and so the present study seeks to fill this gap.

Like isoenzyme electrophoresis, drug-sensitivity studies provide a way of indirectly examining the genetic variation of organisms. Resistance to both chloroquine and pyrimethamine has been shown to be due to stable gene mutations (Rosario, 1976; Padua, 1981) and more recent work by McCutchan (1988), and Ginsburg and Warhurst (1988) provide some clues on the biochemical pathways which could lead to pyrimethamine and chloroquine resistance in P. falciparum. Drug sensitivity characters, therefore, provide good strain markers (Walliker, 1985) and can be used for characterisation and identification of P. falciparum.

Drug-resistant forms of P. falciparum are now widespread in many parts of the world, including Africa. Such resistance may be due to nongenetic physiological adaptations of the parasite to the drug or to genetic changes such as mutations, followed by selection by the drug. Work with rodent malarial parasites, most of which have been found to be useful models for P. falciparum (Rosario, 1976; Thaithong et al., 1984), has shown that genetic changes are important causes of resistance to the two most commonly studied antimalarials, chloroquine and pyrimethamine (Walliker , 1980).

The molecular basis of all stable drug resistance (and of at least some of unstable resistance) probably consists in alterations of the parasite DNA (Molineaux, 1986; Beverley et al., 1986). Cloning of P. falciparum from naturally acquired infections has demonstrated clonal diversity within isolates, including diversity with respect to drug response (Rosario, 1981; Thaithong et al., 1984; Rosario and Thaithong, 1986). It is therefore likely that some lines of unstable resistance are mixtures of sensitive and resistant clones (Molineaux, 1986), thus pointing to the possibility of a genetic basis for all types of drug resistance.

The evidence available so far concerning Plasmodia suggests that resistance is commonly due to point mutations (Molineaux, 1986). It has been shown that resistance to the antifolate drug pyrimethamine arises by mutation and that

the genetic factors involved can undergo recombination with other markers in crosses between resistant and sensitive parasite lines (Walliker *et al.*, 1973).

Rosario (1976) found that the chloroquine resistance which developed in *P. chabaudi* was a stable character, inherited in simple Mendelian fashion, that could undergo genetic recombination with other markers, and that probably arose by mutation and selection in presence of the drug. Work by Knowles *et al.* (1981) and Knowles (1982) on strain hybridisation and progeny analyses of the rodent malaria, *Plasmodium yoelii*, provide more evidence that resistance to pyrimethamine has genetic basis. Knowles (1982) also investigated the probability of a genetic basis for cross-resistance between the antifolate drugs pyrimethamine and cycloguanil, independently selected for resistance to cycloguanil and pyrimethamine. He found that pyrimethamine and cycloguanil resistance was probably closely linked but suggested that more detailed genetic evidence was required to prove that they are allelic.

For strain characterisation, several *in vitro* test systems for drug-response are now available (Rieckman *et al.*, 1978; Desjardins *et al.*, 1979; Nguyen-Dinh and Trager, 1980; Nguyen-Dinh *et al.*, 1983; Spencer *et al.*, 1983b, 1984, 1986).

Routine testing for drug sensitivity of *P. falciparum* is an integral component of any rational malaria control programme. Such tests permit longitudinal comparison of

changes in drug susceptibility of parasites in one area as well as between areas (Spencer, 1985). Monitoring the responses to antimalarial drugs can enable us to resolve specific questions such as: whether resistant malaria is present in an area or is occurring in a particular group of people, whether resistance in an area or a population has reached a critical level requiring some specific action, how the parasite population in an area or a population of people responds to a drug, if there is a difference in drug response between parasite populations in different places or at different times, and which factors are associated with a variation in drug response (Spencer, 1985).

Longitudinal drug sensitivity studies were carried out in Kenya from 1980 through 1984 at a time when chloroquine-resistant falciparum malaria was emerging there (Spencer *et al.*, 1982; Spencer, 1985). The results of these investigations illustrated the progression of chloroquine resistance, the response of *P. falciparum* to other antimalarial drugs and the potential usefulness of the *in vivo* and *in vitro* tests. Pyrimethamine resistance was found to be widespread in Kenya (Spencer, 1985), and there was an excellent correlation between the *in vivo* response to pyrimethamine and *in vitro* results using a modified 48 hr test (Nguyen-Dinh *et al.*, 1982).

At present, it is not clear whether there is any direct biochemical correlation between isoenzyme variation and variation in drug sensitivity of *P. falciparum* (Thaithong,

1981). From genetic experiments with rodent Plasmodium species (Beale et al., 1978), it is known that variations in electrophoretic properties of enzymes are gene controlled. Thaithong (1981) reported that genes for drug resistance in rodent Plasmodium species segregated independently of those for the six enzyme variants studied. So, although the resistance to chloroquine and to pyrimethamine is probably gene controlled in P. falciparum also (Thaithong et al., 1984), there may not be genetic linkages between enzyme types and drug responses.

1.2. OBJECTIVES OF THE PRESENT STUDY.

The specific objectives of the study were:

1. To find out if distinct strains of P. falciparum occur within and among important endemic areas in Kenya.

2. To find out how much variation occurs within a single endemic area.

3. To compare isoenzyme profiles of drug-resistant isolates with those of drug-susceptible isolates.

4. To compare the potencies, and to find out the correlations (cross-resistance), between the different

antimalarial drugs used against the Kenyan isolates of P. falciparum.

1.3. DEFINITIONS.

The following are definitions of the terms as used in the present study (Carter, 1978):-

Isolate: a single sample of parasite material derived from a naturally infected host specimen on a unique occasion and preserved in the laboratory either by continuous in vitro cultivation or cryopreserved in liquid nitrogen. The parasite cells present in an isolate are not necessarily genetically homogenous and may indeed contain representatives of several distinct strains or clones.

Line: the progeny of parasites derived by in vitro cultivation on a unique occasion. Like an isolate, a line of parasites may not be genetically homogenous. The term is used in any circumstance in which attention is being drawn to a particular point of origin in the laboratory history of the parasites.

Strain: the progeny of parasites obtained from a particular isolate and having special properties distinguishing them from other members of the same species. In the context of

the present study, the term 'variant' has the same meaning as the term 'strain'.

Clone: the progeny of parasites descended from a single cell by asexual reproduction and therefore genetically identical to each other and to the parent cell.

CHAPTER 2

MATERIAL AND METHODS.

2.1. Source of Infected Blood Samples.

Venus blood samples previously collected from malaria patients as part of field studies and cryopreserved in liquid nitrogen were used. The infected blood samples were collected during the 1980 to 1986 period from malarious regions of Kenya namely: Nyanza province (Kisumu and Saradidi), Rift Valley province (Entasopia) and Coast province (Malindi, Jilore Primary School and Dindiri Primary School) (see Figure 1). Seven infected blood samples (isolates) from each of the three provinces were studied.

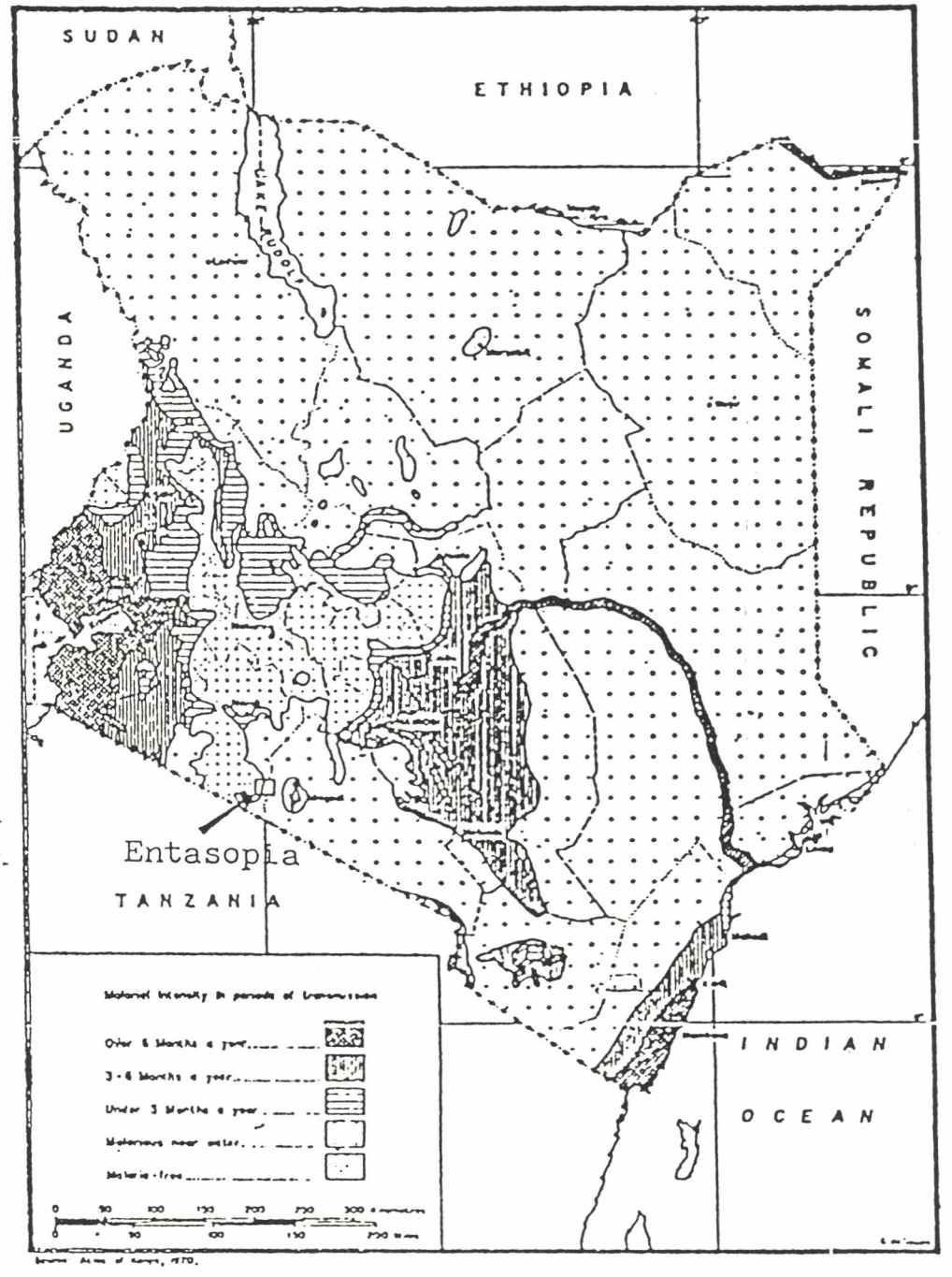
For cryopreservation in liquid nitrogen, Rowe's cryo solution (2.5% glycerol, 3% sorbital, 0.65% NaCl) or dimethyl sulfoxide (DMSO) were used (Diggs et al., 1977; Strome et al., 1977; Wilson et al., 1977b; WHO, 1981; Trager and Jensen, 1986).

2.2. *In Vitro* Cultivation of *Plasmodium falciparum* Isolates.

Among the human malaria parasites, only Plasmodium falciparum can be maintained in continuous in vitro culture (Trager and Jensen, 1976; Bull. WHO, 1977; Siddiqui and Palmer, 1981; Trager and Jensen, 1986).

The parasite isolates were thawed out from liquid nitrogen (WHO, 1981; Trager and Jensen, 1986) and then

Figure 1: Map of Kenya showing malarial intensity in periods of transmission.



Source: Vogel L.C. et al, 1974

cultivated in group O+ human erythrocytes by a modification of the methods of Trager and Jensen (1976) and Haynes et al. (1976) as described by Chulay et al. (1984), Watkins et al. (1984) and Spencer et al. (1985b) .

A 50% suspension of human type O+ erythrocytes was prepared by centrifugation in wash medium which consisted of powdered RPMI 1640 (GIBCO Laboratories, Grand Island, N. Y.) diluted in sterile water with 25 mM Hepes buffer (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; Calbiochem, La Jolla, California) and 25 mM $NaHCO_3$ (GIBCO).
Stock cultures were maintained either in 24-well microtiter plates or in tissue culture flasks as 6% erythrocyte suspension of RPMI 1640 medium diluted in sterile water with 25 mM HEPES buffer, 25 mM $NaHCO_3$ and 10% heat inactivated (40 minutes at 56°C) human serum. To guard against bacterial contamination, the medium used was fortified with gentamicin (10 ug/ml). The flasks were flushed with a gas mixture consisting of 3% CO_2, 5% O_2 and 92% N_2 (East African Oxygen, Nairobi, Kenya) at 37 °C.

During the continuous in vitro cultivation of the isolates, Giemsa-stained thin blood films of the parasite cultures were made to assess the parasite developmental stages, parasitaemias, growth rates (Santiyanont, 1985) and also to check if the cultures were free from bacterial, fungal or other contaminants.

Daily changes of the culture medium were carried out and fresh erythrocytes were added every three or four days

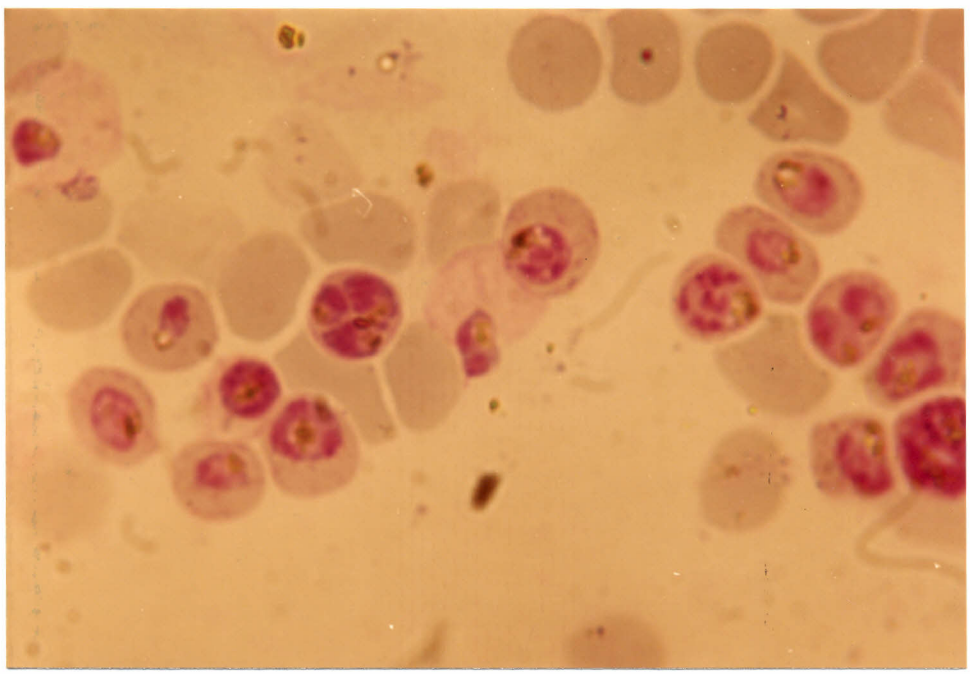
so that less than 3% of the cells were infected at any time. The dilutions were particularly necessary during the initial stages of culture adaptations of individual P. falciparum isolates and also for the preparation of the parasite isolates for in vitro drug sensitivity studies which required fast growing stock cultures. To obtain large numbers of parasites for electrophoretic analysis, culture volumes of fast growing parasite isolates were always increased, and dilutions sometimes delayed, but compensated for, by doing double medium changes (one in the early morning, the other in the evening) every day.

Aseptic techniques were used during the continuous cultivation of the malaria parasites. Glassware and plasticware used were sterile and all the preparations and filter sterilisation of solutions done inside laminar flow hood.

A number of experiences were encountered during the in vitro cultivation of P. falciparum isolates. For example, it was realised that using isolates with initial high parasitaemia (at least over 50 parasites per 10,000 red blood cells) and initially setting large volume cultures (at least 5ml) led to higher chances of in vitro parasites recovery and adaptation.

Faster growth was sometimes affected by simply putting cultures in the shaking incubator when the parasites were predominantly in schizont stages (Plate 1). Shaking cultures was sometimes found to lead to the precipitation of

PLATE 1. ERYTHROCYTIC SCHIZONT STAGES OF CULTIVATED *PLASMODIUM FALCIPARUM*.



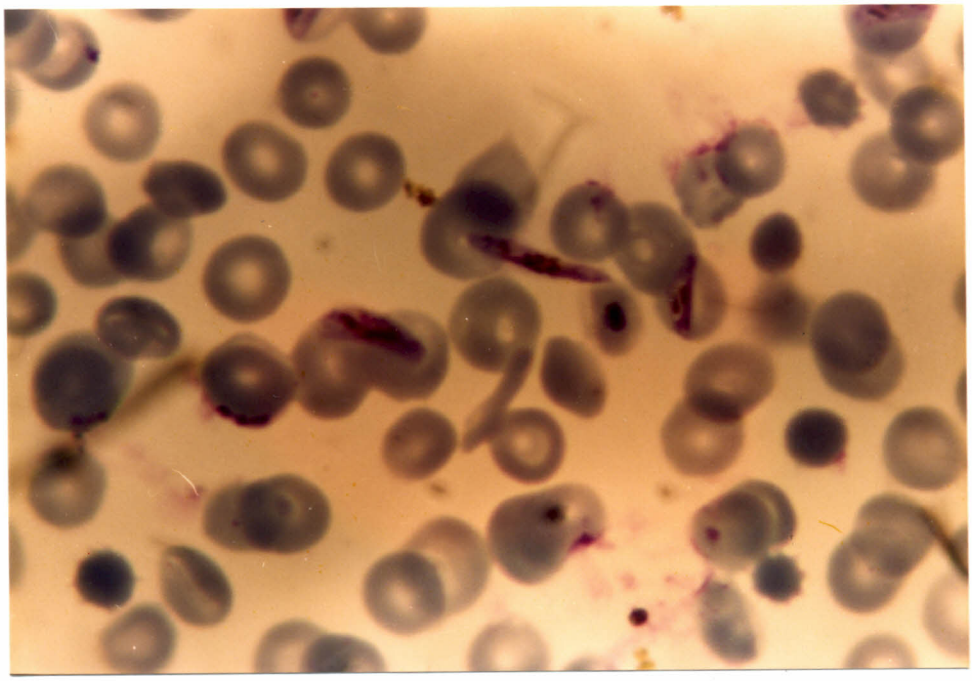
serum proteins in the cultures, although these deposits were never found to adversely affect the growth of parasite cultures.

Certain isolates readily produced gametocytes (Plate 2) in culture when compared with the other isolates examined. It was also observed that gametocytogenesis appeared to occur readily when cultures were poorly attended to, for example, when fast growing cultures were not diluted early enough or when their medium changes were delayed. It was clearly noted that gametocytes could withstand harsher culture conditions when compared to other asexual parasite forms in the cultures.

2.3. *In Vitro* Test for *Plasmodium falciparum* Sensitivity to Antimalarial Drugs.

The sensitivity to drugs of cultured isolates was examined using the radioisotope uptake method of Desjardins et al. (1979) with minor modifications. The drugs tested were: chloroquine phosphate (Dawa pharmaceuticals, Nairobi, Kenya), amodiaquine dihydrochloride (Parke Davis), quinine (Sigma Chemical Co. Ltd.), quinidine(Sigma Chemical Co. Ltd), mefloquine (Hoffmann-La Roche, Nutley, N. J.), tetracycline (Sigma Chemical Co. Ltd.), pyrimethamine (Burroughs Wellcome, Research Triangle Park, NC) and sulphadoxine (Hoffmann-La Roche, Nutley, N.J). The RPMI 1640 culture medium used contained no p-aminobenzoic acid

PLATE 2. ERYTHROCYTIC GAMETOCYTE STAGES OF CULTIVATED *PLASMODIUM FALCIPARUM*.



(PABA) and no folic acid (FA) which antagonises the antimalarial activity of sulphadoxine and pyrimethamine (Watkins et al., 1985).

The other modification involved the incubation of test cultures with drugs for 48 hours (Nguyen-Dinh and Payne, 1980; Nguyen-Dinh and Trager, 1980; Spencer et al., 1983b) before the addition of radiolabelled hypoxanthine. The 48 hour test allow for the determination of the susceptibility of P. falciparum to slow acting antifolates depending upon the reinvasion of the parasites (Spencer, 1985) and also making it possible to test the drug sensitivity of asynchronous parasites from in vitro cultures (Nguyen-Dinh et al., 1982; Spencer et al., 1983b).

The radioisotopic assay of Desjardins et al. (1979) can provide quantitative measurements of the antimalarial activity of large numbers of compounds, based on the inhibition of uptake of a radiolabelled nucleic acid precursor, hypoxanthine, by the parasite during short-term cultures. In the present study, serum rather than plasma was used to supplement the medium (Spencer et al., 1983).

Microculture techniques were used to measure the activity of the antimalarial drugs to the isolates examined. The antimalarial drugs chloroquine, amodiaquine, quinine, quinidine, mefloquine, pyrimethamine and tetracycline were dissolved at required stock concentrations in ethanol and water. However, sulphadoxine was first dissolved in

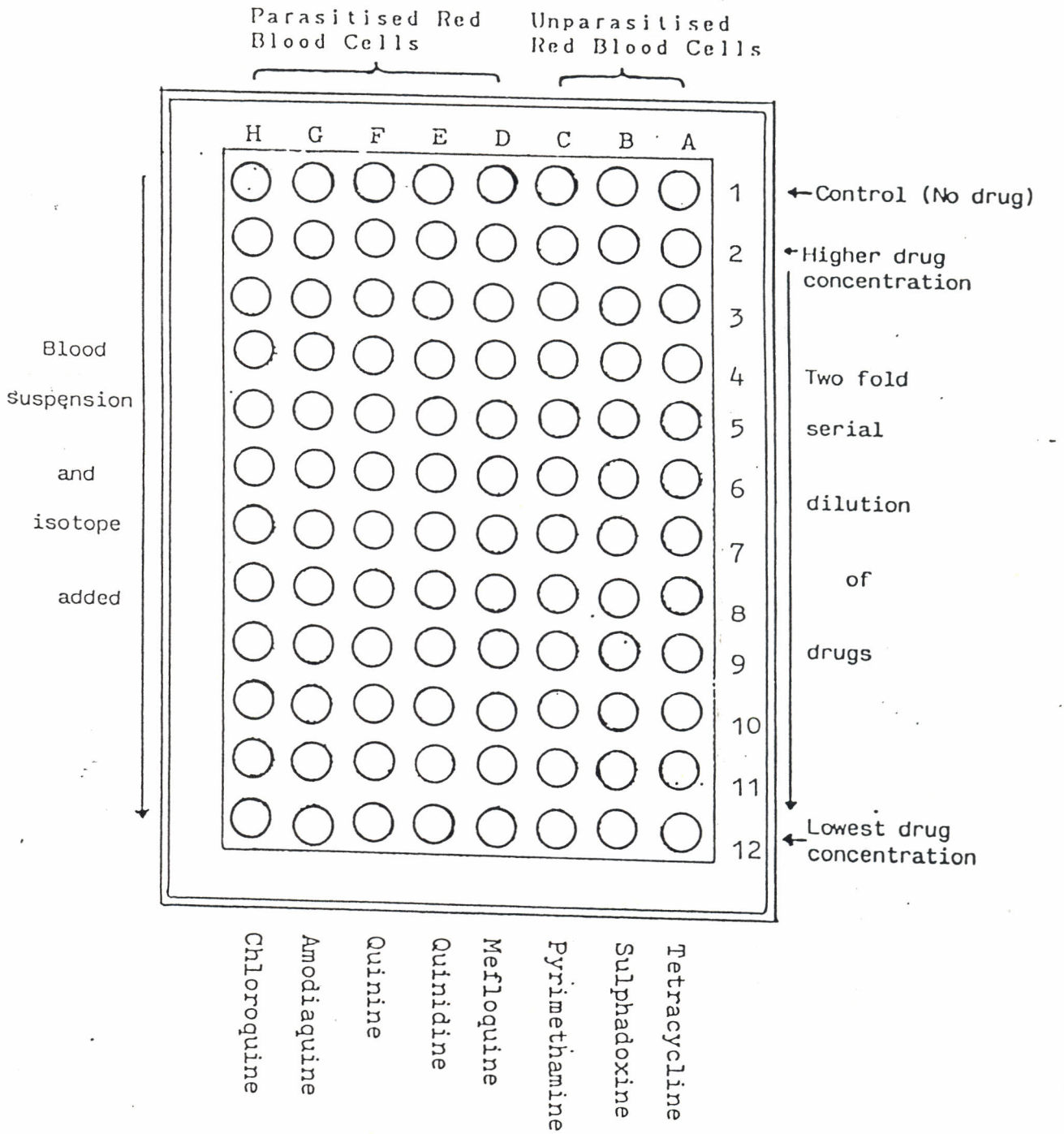
distilled water and then 1N NaOH was added dropwise to complete the solubilisation. It was then filter sterilised.

The concentration ranges of the drugs used were as follows: chloroquine, 1000-0.24 ng/ml; amodiaquine 1000-0.24 ng/ml; quinine, 2000-1.95 ng/ml; quinidine 1000-0.24 ng/ml; mefloquine, 250-0.12 ng/ml; pyrimethamine, 2048-0.0018 ng/ml; sulphadoxine, 5000-4.88 ng/ml; and tetracycline, 10000-9.77 ng/ml. These concentration ranges were obtained by further dilution of the stock concentrations with ethanol; the concentrations being selected so that the expected ID50 will fall in the middle of the ranges of dilutions (Webster et al., 1985b).

The microtiter plates contained 96 flat-bottom wells (see Figure 2), arranged in a matrix of eight rows (A through H) and 12 columns (1 through 12). When prepared, all wells of column 1 were used as controls (no drugs added). The first three wells: A, B, and C were used as UPRBC (Unparasitised Red Blood Cells) controls and the last five wells : D, E, F, G and H were used as PRBC (Parasitised Red Blood Cells) controls. All other wells of the rows A through H were charged with antimalarial drug dilutions. Wells of column 2 had the highest drugs concentrations. The drugs were serially diluted two-fold for the subsequent wells upto column 12 whose wells had the lowest drugs concentrations.

In preparation for addition to the microtiter plates, fast growing stock cultures (as indicated by doubling of the

Figure 2. The Drug Sensitivity Test Plate.



parasitaemia every 24 hours) were further diluted in culture medium containing sufficient noninfected type O^+ human erythrocytes to yield a final haematocrit of 1.5% and parasitaemia of 0.4%. 200 ul of the parasitised erythrocytes were then added into each of the wells of the 96-well plate except the 3 UPRBC control wells (in which 200 ul of 1.5% haematocrit suspension of UPRBC were added). The test was done in duplicate.

After the preparation, the plates were placed in a humified airtight box (Instrumentation Department, Washington D. C.), which was then flushed with a gas mixture of 3% CO_2, 5% O_2, and 92% N_2 and sealed. The box was then placed in an incubator at 37 °C for 48 hours.

Uptake of [G^3H] hypoxanthine (Amershen/Searle Corp., Arlington Heights, III) was used as an index of growth of the parasites. The isotope was supplied as a lyophylate (6.2 ci/mMol) in ampoules containing 5 mCi. The contents of a single ampoule were dissolved in 5 ml of sterile distilled water to provide a stock solution which was stored at 4 °C.

After the 48 hours incubation period, the plates were removed from the box and 25 ul of diluted radiolabelled hypoxanthine solution (1 volume of G^3H-hypoxanthine stock solution in 50 volumes of culture medium) added to each well. The plates were then returned to the box, which was again flushed with the gas mixture, sealed and incubated at 37 °C for an additional 18 hours.

At the end of the second incubation period, each plate was harvested on a MASH-II automated cell harvester (Microbiological Associates, Bethesda, Md.). This instrument aspirated and deposited the particulate contents of each of the wells onto small disks of filter paper (No. 934-AH, Whatman, Inc., Clifton, N. J.), which were then washed with copious volumes of deionised water. Each disk was dried and placed in a glass scintillation vial containing 1 ml of scintillation fluid. All 96 vials, corresponding to the 96 wells of the microtiter plate, were counted in a Beckman LS 1801 model liquid scintillation counter for two minutes.

The scintillation counter measures the incorporation of ^{3}H-hypoxanthine into parasite nucleic acids, with the results expressed as counts per minute (CPM).

Computation of the concentration of drug causing 50% inhibition of ^{3}H-hypoxanthine uptake (ID50) was modified slightly from the method of Desjardins et al. (1979).

The computation of the ID50 values for each isolate was done in duplicate. The parasitised control mean CPM values were used to estimate the midpoint (Y_{50}) as shown in the formula:-

$$\text{Midpoint } (Y_{50}) = (\text{PRBC} - \text{UPRBC})/2 + \text{UPRBC}$$

The ID50 value for each drug was then determined by interpolation between one data point above and below the Y_{50}. Interpolated ID_{50} values were obtained after

logarithmic transformation of both concentration and CPM values, using the formula of Sixsmith et al. (1982, 1984) as shown:

$$ID50 = \text{antilog}\left(\log X_1 + \left[\frac{(\log Y_{50} - \log Y_1)(\log X_2 - \log X_1)}{(\log Y_2 - \log Y_1)}\right]\right)$$

where X_1, Y_1, X_2, and Y_2 are the concentration and CPM values, respectively, for the data points.

2.4. Preparation of Host and Parasite Material for Electrophoresis.

Practically all of the procedures used for lysing red cells and obtaining plasmodial components from erythrocytic stages of the malarial parasites are variations of a few distinct techniques. These are hypotonic lysis, lysis by freezing and thawing, lysis with agents (such as saponin, NH_4Cl, or antiserum and complement), lysis by sudden decrease in pressure, lysis by ultrasound and, the most recent procedure, cultivation to permit the parasite to mature and be released spontaneously (Rutledge and Ward, 1967; Prior and Kreier, 1972; Kreier, 1977; Sirawaraporn, 1985).

Though several methods exist for 'freeing' parasites from infected red cells, saponin lysis has been found to be the most convenient and simple to use (Sanderson et al., 1981; Thaithong, 1985; Beaumelle et al., 1987). However, it has been recognised that parasites prepared by saponin lysis of parasitised erythrocytes are, with rare exceptions, contained within the collapsed erythrocyte membranes

(Kreier, 1977) and Sherman (1979) reported that saponin liberated parasites were leaky to macromolecules. But these situations do not seem to affect the isoenzyme electrophoresis of malaria parasites as was found in the present study.

The methods used for the preparation of host and parasite material for electrophoresis were based on those of Carter (1978), Sanderson et al. (1981), Thaithong (1981), Thaithong (1985), Walliker (personal communication, 1988) and Knowles (personal communication, 1988). Care was taken during the preparation of lysates to keep the samples cold by using ice so as to prevent deterioration of the enzymes.

Cultures exhibiting parasitaemias of at least 2.5% and with high proportions of schizonts were harvested from the culture flasks and centrifuged at about 1000 g for 10 minutes. The supernatant was discarded and the packed red blood cells incubated with 0.15% saponin in complete RPMI 1640 (1.5 volumes saponin solution: 1 volume packed cells) at room temperature for 10 minutes. The material was then resuspended in 8-10 volumes of cold complete RPMI 1640 (or phosphate buffered saline {PBS} pH 7.4) and after thorough mixing, it was again centrifuged at about 3000 g for 10 minutes at 4 °C. The supernatant and red cell ghosts were discarded, leaving grey-brown pellet of packed parasites.

The released packed parasites were washed three times with PBS and then lysed to release proteins for enzyme tests

or stored in nunc vials (with as little fluid as possible around the parasites) in liquid nitrogen.

To break up the parasites, three freeze-thaw cycles using -20 °C freezer were done. Detergents, such as Triton X 100, were found to be quite unnecessary. Having thawed the parasite material, a small volume of distilled water (1-2 X volume of the pellet) was added and mixed thoroughly. The mixture was centrifuged at 5000 g for 20 minutes at 4 °C. The supernatant was then removed and aliquoted as tiny beads into nunc vials and stored in liquid nitrogen for later enzyme tests.

Lysates of washed uninfected host red blood cells were also prepared to act as controls during electrophoresis. To lyse uninfected red blood cells, three cycles of freeze-thawing followed by centrifugation (2,500 g for 10 minutes at 4 °C) were found to be adequate. The control lysates were also stored as beads in liquid nitrogen for later enzyme tests.

2.5. Isoenzyme Analysis.

For isoenzyme analysis, both thin layer starch gel and cellulose acetate electrophoresis methods were employed. Initially it was proposed to use only cellulose acetate electrophoresis but after preliminary studies it was realised that four enzymes (lactate dehydrogenase, 6-phosphogluconate dehydrogenase, adenosine deaminase and peptidase E) were not being resolved well by this system.

So later, thin layer starch gel electrophoresis was adopted for the analysis of the four enzymes. Cellulose acetate electrophoresis was only used to analyse glucose phosphate isomerase, NADP dependent glutamate dehydrogenase and peptidase E.

The methods used for isoenzyme analysis were modifications of those of Carter and McGregor (1973), Carter and Walliker (1977), Carter (1978), Knowles *et al*. (1981), Sanderson *et al*. (1981), Thaithong (1981), Thaithong (1985), Walliker (personal communication, 1988) and Knowles (personal communication, 1988).

2.5.1. Cellulose Acetate Electrophoresis and Enzyme Assay.

Electrophoresis involves the migration of extracted proteins through a medium caused by application of an electric field across the medium. Cellulose acetate electrophoresis was used because it is more convenient, requires small amounts of material, and gives results in a shorter time than starch gel electrophoresis. Cellulose acetate plates offer much reduced adsorption of protein zones, so that clearer separation and lower background staining are obtained (Moss, 1979).

Electrophoresis tank (Zip-zone electrophoresis chamber; Helena Laboratories, Beamont, Texas, USA) and soaking containers were prepared with the same buffer appropriate to the enzymes (i.e. Helena Supre Heme Buffer). Backs of the cellulose acetate plates (Titan III iso vis plates; Helena

laboratories) were labelled and then lowered carefully into the soaking buffer. Soaking was done for at least 20 minutes , and not more than 3-4 hours.

While soaking was taking place, 1.2% agar underlays were prepared. This involved preparing the appropriate developers (staining solutions) for enzymes being investigated and then mixing them with about 8 ml of 38 °C warm 1.2% molten agar. These agar underlays were kept at 4° C in the dark if not used immediately.

Cooling sponges were then placed into central compartments of the electrophoresis tanks and wicks (to create connections between +ve and -ve electrode buffers and the cellulose acetate plate during electrophoresis run) were soaked in the buffer and then placed into position. Host and parasite lysate samples were prepared (using zip-zone sample well plate) ready for application in a listed order and kept cool with ice if not used immediately.

The soaked plates were removed from the buffers, blotted lightly and then placed (cellulose acetate sides up) on the alighning base(Helena laboratories model). Using the zip-zone applicator, samples were then loaded onto the soaked plates immediately. Loaded plates were then placed, cellulose acetate sides down and with the samples at the cathode end, on wicks in the electrophoresis chamber and weighed down with heavy slides.

The electrophoresis chamber was covered with a lid, the current switched on, voltage and time set. At the required

time, the current was switched off, electrophoresed cellulose acetate plates removed from the chamber and then placed, cellulose acetate side down, onto the appropriate agar underlays.

The development was allowed to take place in a dark incubator at 37 °C. The developing electrophoresed plates were checked for bands every 10 minutes. After optimum development, the reaction was stopped by fixing the bands with 4% acetic acid. The developed cellulose acetate plates were then photographed or preserved as records.

Staining Conditions for Isoenzymes.

The enzymes GPI, GDH and PEPE were run with Helena Supre Heme buffer made upto one litre. Titan III iso vis cellulose acetate plates were used in each case. All the other electrophoresis chemicals used were purchased from Sigma Chemical Company.

a. GPI

This enzyme was run for 10-15 minutes at 300 volts.

Enzyme assay solution:

Fructose 6-phosphate	50 mg
$MgCl_2$	20 mg
NADP	5 mg
Glucose 6-phosphate dehydrogenase	0.01 ml
MTT	5 mg
PMS	2 mg

made up in 4 ml of 0.05 M Tris HCl, pH 8.0. This enzyme stains quickly and overstaining was avoided.

b. GDH

This enzyme was run for 15-20 minutes at 300 volts.

Enzyme Assay Solution:

Monosodium glutamate	100 mg
NADP	5 mg
MTT	5 mg
PMS	2 mg

made up in 4 ml of 0.05 M Tris HCl, pH 8.0.

c. PEPE

This enzyme was run for 10-15 minutes at 300 volts.

Enzyme Assay Solution.

Peptide	20 mg
Snake Venom	5 mg
Peroxidase	20 mg
$MnCl_2$	10 mg
O-dianisidine	2 mg

made up in 4 ml of 0.02 M citrate phosphate, pH 5.5.

2.5.2. Thin Layer Starch Gel Electrophoresis and Enzyme Assay.

Starch gel electrophoresis has occupied a particularly important place in the study of multiple forms of enzymes. This is because the pore structures of the starch gels

(formed from partially hydrolysed starch) act as molecular sieves to separate components of protein samples on the basis of molecular size. The restricted diffusion of protein molecules then help in maintaining narrow zones during electrophoresis and the subsequent staining process (Moss, 1979).

Thin layer starch gels (1 mm thick) were used because they are easier and cheaper to prepare and stain, easier to keep cool during electrophoresis, and take less sample material than thick layer (5-10 mm) starch gels.

The hydrolysed starch used was commercially obtained from Connaught Medical Research Laboratories, Toronto, Canada.

The gel was prepared by heating a 9% suspension of the hydrolysed starch in buffer solution in a flat bottomed conical flask over a medium bunsen flame with constant swirling until a clear solution was obtained. De-gassing was then carried out using a vacuum pump while the mixture was still being swirled. Heating and suction were then removed and the resulting solution was poured rapidly into a 1 mm deep gel mould and immediately spread uniformly to attain 1 mm thickness using a special wide wedge-shaped perpex gel spreader. The gel was then covered with a glass plate and, after cooling to room temperature, stored at 4 °C until required. Gels were used not less than one hour after pouring and were sometimes stored for 24 hours.

The gel mould consisted of a perpex rectangular rim 1 mm thick, of internal dimensions 25 x 16 cm and external dimensions 32 x 18 cm. The rim was laid and fixed on the surface of a 0.5 cm thick glass plate. The wedge-shaped gel spreader was 18 cm wide at the sharp gel spreading edge.

The electrophoresis was done in a cold room at about 4 °C. Host and parasite lysate samples were prepared and transported to the cold room in ice and then absorbed into 0.8 mm long heat 'sterilised' cotton threads (no. 1,6 x stranded anchor embroidery cotton {or equivalent}) before their application into the gel along the chosen line of origin by use of fine tipped clean forceps.

The loaded gel was placed on a cooling plate between two electrode troughs each containing 500 ml of electrode buffer. Wicks (to create connections between +ve and -ve electrode buffers and the loaded gel) were laid over the cathode and anode edges of the gel and a glass plate pressed over the wicks. The lid of the electrophoresis chamber was then placed, the current switched on, the voltage and time set and the electrophoresis allowed to continue.

The electrophoresis apparatus consisted of the following items:

(a) A power supply capable of delivering currents of upto 150 mA and constant voltage of upto 300 volts.

(b) A pair of electrode troughs of 600 ml capacity, fitted with platinum electrode wires running the full length of the troughs and connected by leads to the power pack.

(c) Blotting paper wicks measuring 26 x 26 cm and 0.4 cm thick when wet.

(d) A central support to hold the lower cooling plate and gel plate just above the level of the electrode troughs.

Just before electrophoresis time was over, the specific enzyme developers (staining solution mixed with 0.9% agar gels) were prepared to be used as agar overlays for the development of the resolved bands. 0.9% agar was kept molten at 60 °C in the water bath. Meanwhile, the staining reagents for the enzyme being investigated were prepared and completely dissolved in 10 ml of specific incubation buffers and then kept in the dark at 4 °C if not used immediately.

When the electrophoresis running time was up, the electrophoresed starch gel plate was removed but still left at 4 °C. Very quickly, the 0.9% molten agar was cooled down to about 38 °C and then mixed thoroughly with 10 ml of the staining solution. Then immediately, this agar overlay developer was poured uniformly over the starch gel plate, taking care to avoid the formation of air bubbles in the setting agar. Once the overlay had set - this took about three minutes - the developing plate was transferred into a dark incubator set at 37 °C.

The progress of the developing bands was checked periodically. Care was taken not to jolt or displace the agar overlay when the development was continuing to avoid the creation of double band artefacts.

When the development was optimum (as could be seen by the colour development of the resolved bands), the reaction was stopped by immersing the developed plate in 5% acetic acid.

Development could take place either on the starch gels, on the agar overlays or on both of them depending on the enzyme being assayed. Therefore, it was always necessary to preserve the gel in which the bands developed best.

The stained gel was placed onto a filter paper and tightly overlayed with a cling film polythene sheet (Reynolds metal Co., Richmond, Virginia, U.S.A). It was dried for one and half hour using a slab gel dryer to preserve it permanently. The dried gel was then photographed or preserved as record.

Assay Conditions for Isoenzymes.

All the chemicals for enzyme assay were purchased from Sigma Chemical Company.

a. 6PGD

The assay conditions for this enzyme were adopted from those of Sanderson et al. (1981).

Gel buffer: 0.0175 M Tris -0.0063 M citrate, pH 6.4.

Electrode buffer: 0.22 M Tris -0.0785 M citrate, pH 6.2

Line of origin: 5 cm from cathodal end of the gel.

Voltage across gel: 180 volts.

Time of run: 4 hours.

Enzyme Assay Solution:-

50 mg barium 6-phosphogluconate

5 mg NADP

5 mg MTT

.5 mg PMS (added just before pouring the staining solution onto the gel)

All made up in 10 ml of 0.05 M Tris-HCl, pH 8.0 incubation buffer.

b. LDH

The assay conditions for this enzyme were adopted from those of Carter (1978).

Gel buffer: 0.065 M Tris -0.0222 M citrate, pH 6.5

Electrode buffer: 0.22 M Tris -0.0785 M citrate, pH 6.2

Line of origin: 3.5 cm from cathodal end of the gel

Voltage across gel: 60 volts

Time of run: 4 hours.

Enzyme Assay Solution:-

200 mg Lithium lactate

5 mg NAD

5 mg MTT

.5 mg PMS (added just before pouring the staining solution onto the gel).

All made up in 10 ml of 0.05 M Tris-HCl, pH 8.0 incubation buffer.

c. ADA

The assay conditions for this enzyme were adopted from those of Knowles et al. (1981).

Gel buffer: 1 in 10 dilution of electrode buffer

Electrode buffer: 0.1 M phosphate buffer, pH 6.5

Line of origin: 3.5 cm from cathodal end

Voltage across gel: 100 V.

Time of run: 3 hours.

Enzyme Assay Solution:-

15 mg	Adenosine
15 ul (0.6 unit)	Nucleoside phosphorylase
10 ul (0.1 unit)	Xanthine oxidase
5 mg	MTT
.5 mg	PMS (added just before pouring the staining solution onto the gel).

All made up in 10 ml of 0.1 M phosphate, pH 7.5 incubation buffer.

d. PEPE

The assay conditions for this enzyme were adopted from those of Thaithong (1981).

Gel buffer: 1 in 20 dilution of electrode buffer

Electrode buffer: 0.225 M Tris -0.22 M NaH_2PO_4, pH 8.0

Line of origin: 3.5 cm from cathodal end

Voltage across gel: 50 volts

Time of run: 14 hours.

Enzyme Assay Solution:-

20 mg Peptide substrate

6 mg Snake venom

4 mg Peroxidase

20 mg Manganese chloride

15 mg O-dianisidine

All made up in 10 ml of 0.02 M citrate phosphate incubation buffer, pH 5.5.

2.6. Identification of *Plasmodium falciparum* Enzymes.

The electrophoretic forms of each enzyme were identified by comparison with earlier works on P. falciparum enzyme typing such as those of Carter and McGregor (1973), Carter and Voller (1975), Sanderson et al. (1981), Thaithong (1981) and Myint-Oo et al. (1984). Each variant form of a given enzyme is numbered according to its position on the medium following standard conditions of electrophoresis (Thaithong, 1981; Walliker, 1985). The actual number signifies only the order of discovery (Thaithong, 1985). Standard parasite enzyme samples were not available and therefore unknown parasite forms were identified by comparing their mobility with the mobilities of the host enzymes (Thaithong, 1985).

CHAPTER 3

RESULTS

3.1. *In Vitro* Response of *P. falciparum* Isolates to Antimalarial Drugs.

The activities of the seven antimalarial drugs studied against 21 P. falciparum isolates from Kenya are shown in Table 1, Figure 3 and Figure 4. For the antimalarial drugs used in the present study, 100 nanoMoles was arbitrarily chosen to be the cut-off point between sensitive (<100 nM) and 'resistant' (>100 nM).

Considerable variation in antimalarial drug sensitivities was found among the 21 Kenyan isolates of P. falciparum examined. Of those investigated, the isolates from Rift Valley province were found to be the least susceptible to the antimalarial drugs followed by the isolates from Nyanza province and then the isolates from Coast province. These variations in drug sensitivities among the three provinces were confirmed by Kruskal-Wallis oneway nonparametric analysis of variance. Probability level was 0.01.

Of the seven drugs used in the study, the antimalarial activities of chloroquine, quinidine, pyrimethamine and sulphadoxine against the 21 Kenyan isolates of P. falciparum were found to vary between the three provinces considered. The Kruskal-Wallis oneway nonparametric analysis of variance confirmed this variation. The probability level was less than 0.05 for each of the four drugs.

Table 1: *In vitro* sensitivities of Kenyan *Plasmodium falciparum* to seven antimalarial drugs. ID50 values for 21 isolates.

Isolate	Antimalarial Drugs							Mean
	CH	A	Q	QD	M	P	SD	
Nyanza Province								
1. K39	14.2	3.5	81.1	40.0	11.2	341.0	1759.8	322
2. K67	6.5	6.5	102.6	21.4	14.9	0.04	199.2	50
3. K110	16.7	4.9	<1.5	36.3	11.8	269.3	931.3	182
4. K112	39.1	4.1	44.1	21.0	7.6	300.6	804.8	175
5. S104	397.5	12.0	334.6	104.8	1.3	73.6	9459.8	1483
6. S136	14.7	15.0	400.6	56.5	6.0	0.6	1506.8	286
7. S158	32.8	8.2	66.2	9.6	1.5	<32.2	<15.6	24
Mean	74.5	7.7	147.2	41.4	7.8	145.3	2096.8	360.1
Rift Valley Province								
8. ENT7	247.8	12.9	374.9	211.7	14.5	1074.6	>16111.4	2578
9. ENT11	200.9	4.9	78.5	17.1	3.5	348.8	4092.7	678
10. ENT24	176.2	6.2	217.4	75.7	14.9	582.9	>16111.4	2455
11. ENT30	127.1	6.6	31.4	72.5	8.5	328.3	874.3	207
12. ENT36	212.8	6.8	302.0	123.3	12.8	1084.8	2606.5	621
13. ENT37	181.3	13.5	572.4	172.5	43.7	784.9	>16111.4	2554
14. ENT41	285.9	8.0	376.1	162.1	11.6	3.7	1859.4	387
Mean	204.6	8.4	279.0	119.3	15.6	601.1	8252.4	1354.3
Coast Province								
15. M24	6.3	2.8	58.6	15.4	5.3	1.2	283.6	53
16. M423	22.9	3.5	147.7	14.6	3.3	0.01	293.2	69
17. JP1	<9.4	2.8	61.7	1.2	26.4	148.8	80.6	47
18. JP17/A	96.9	10.7	104.8	24.7	5.6	277.4	389.9	130
19. JP78	115.7	5.6	83.2	21.6	13.2	148.8	109.6	71
20. JP119	62.5	5.6	46.2	6.2	7.9	265.4	80.6	68
21. D7/22	<3.1	1.7	138.5	51.5	12.6	317.6	4506.3	719
Mean	45.3	4.7	91.5	19.3	10.6	165.6	820.5	165.4

CH = Chloroquine, A = Amodiaquine, Q = Quinine, QD = Quinidine, M = Mefloquine, P= Pyrimethamine and SD = Sulphadoxine. ID 50's in nano Moles.

FIG.3. SENSITIVITIES OF 21 P. FALCIPARUM ISOLATES FROM KENYA TO SEVEN ANTI-MALARIAL DRUGS IN VITRO

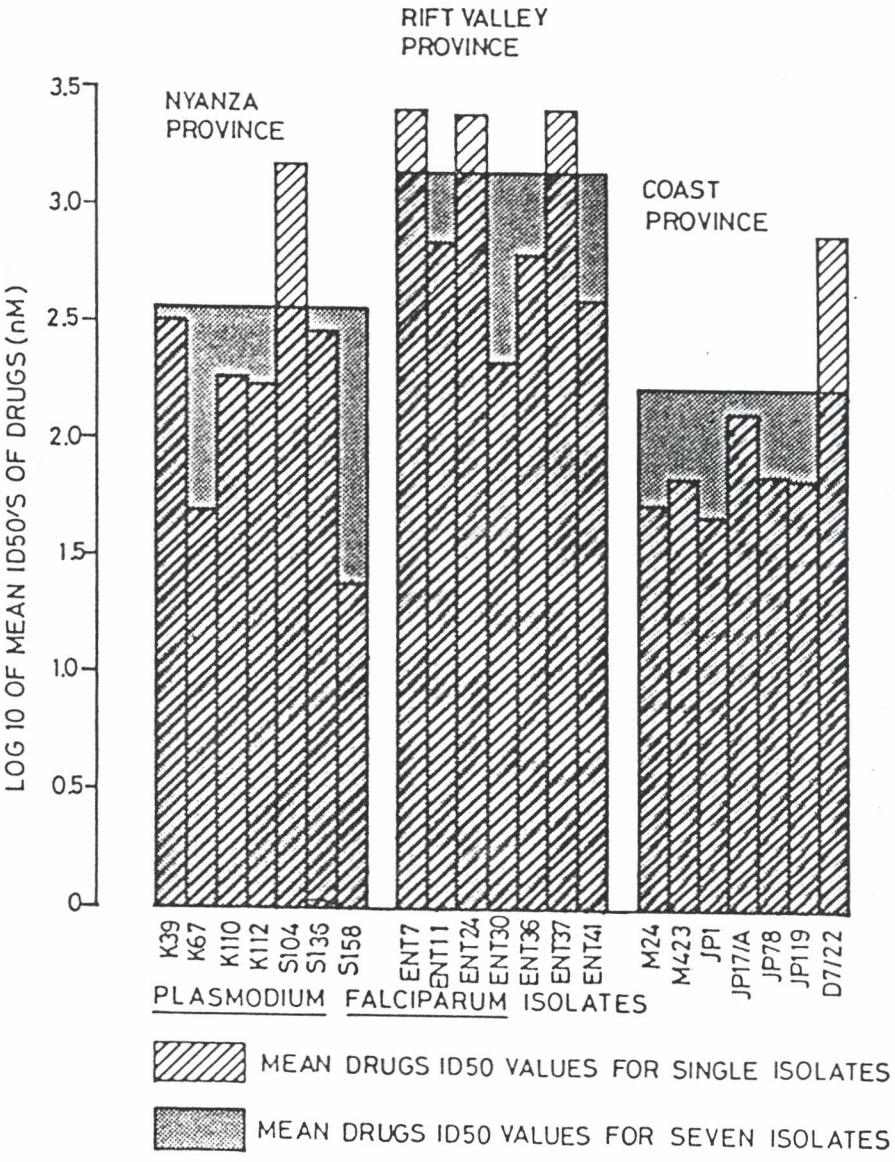
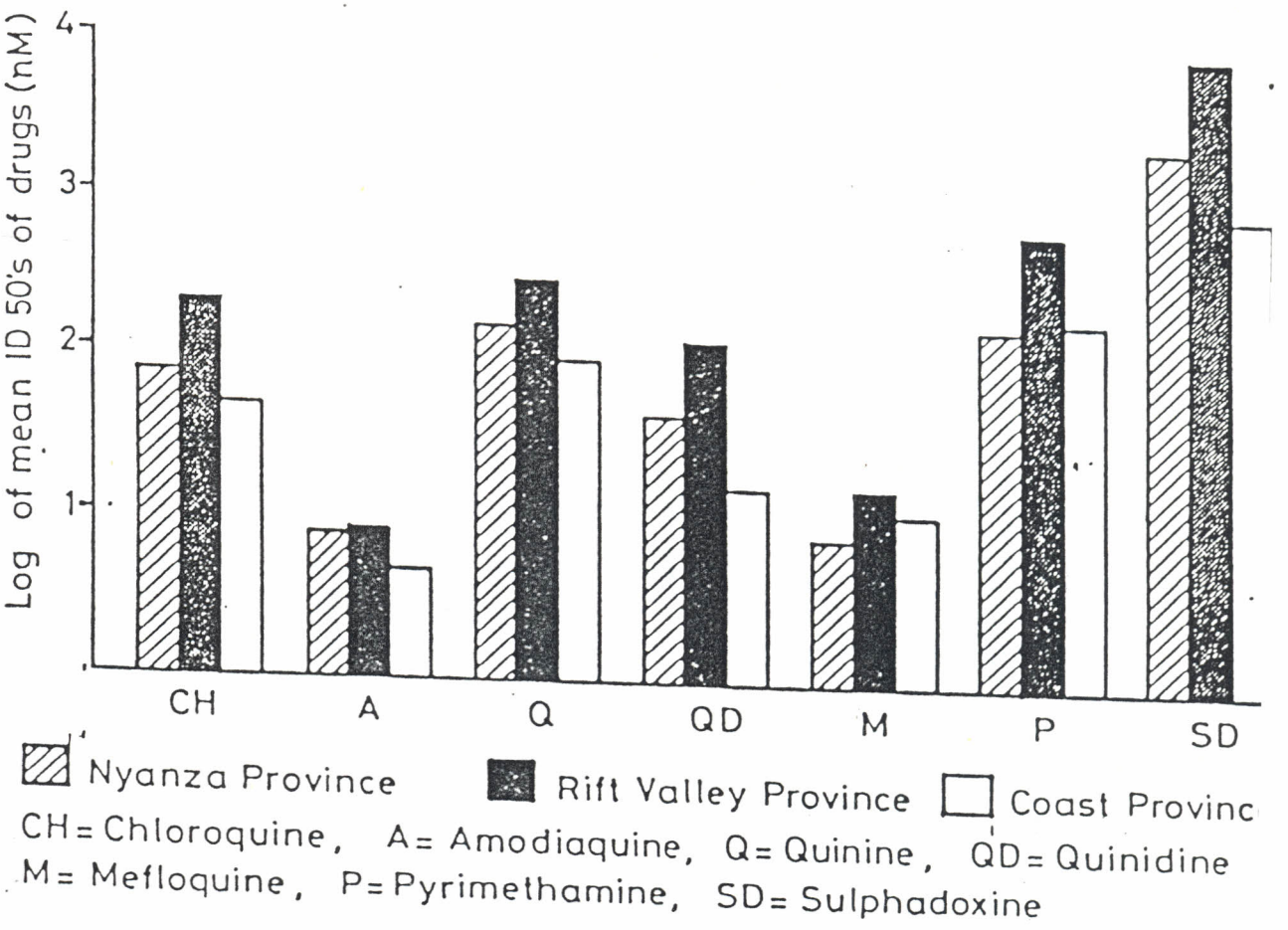


Figure 4. *In vitro* sensitivities of *Plasmodium falciparum* isolates from three provinces of Kenya to seven antimalarial drugs.



No variations were found in the antimalarial activities of amodiaquine, quinine and mefloquine against the 21 Kenyan isolates of P. falciparum between the three provinces considered. The Kruskal-Wallis oneway nonparametric analysis of variance confirmed this absence of variation. The probability levels for each of the drugs were all greater than 0.05.

It was observed that within the three provinces, the amount of variation in chloroquine sensitivity among isolates was inversely related to the level of chloroquine resistance. For example, the variation was lowest in Rift Valley province where the chloroquine resistance was highest (Figure 5).

To compare the possible correlations (or cross-resistance) and the potencies between the antimalarial drugs used against the 21 Kenyan isolates of P. falciparum examined, linear regression analysis and students t-tests were done on different drug pairs. The correlation coefficient (r) indicated the quality of fit achieved by the regression and values of r close to 1.00 indicated a better correlation (or cross-resistance) than values close to zero. Therefore, the drug pairs which generated linear regression curves with correlation coefficient (r) greater than 0.6 and slope probability levels less than 0.01 were considered to be correlated in their activities against the 21 isolates and are graphically presented in Figures 6 to 11.

FIG. 5. SENSITIVITIES OF 21 P. FALCIPARUM ISOLATES FROM KENYA TO CHLOROQUINE IN VITRO

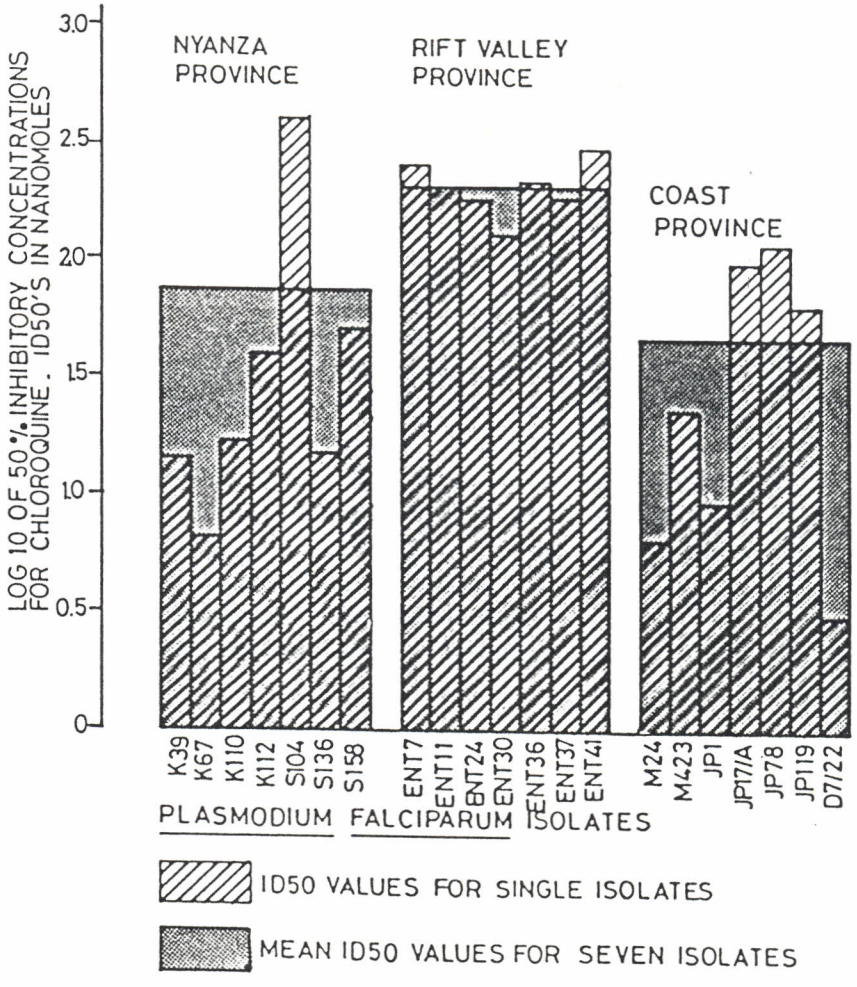


Figure 6: Correlation between in vitro sensitivities to Amodiaquine and Quinine among 21 Plasmodium falciparum isolates from Kenya.

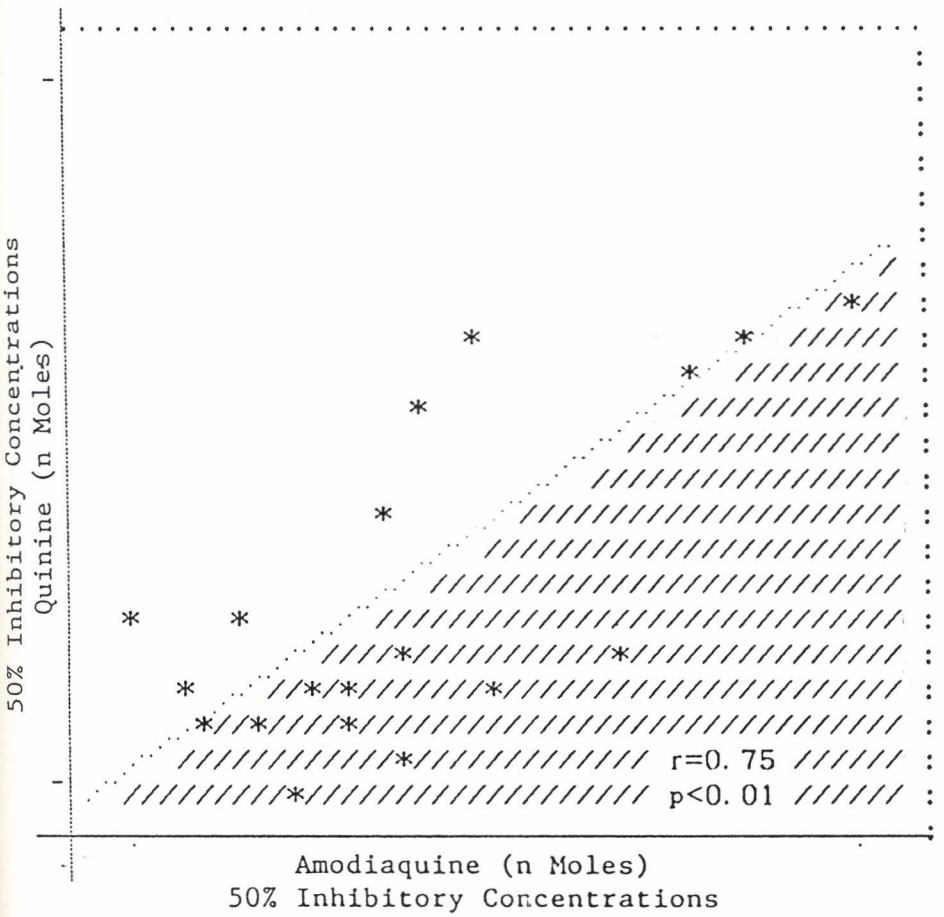


Figure 7: Correlation between in vitro sensitivities to Amodiaquine and Quinidine among 21 Plasmodium falciparum isolates from Kenya.

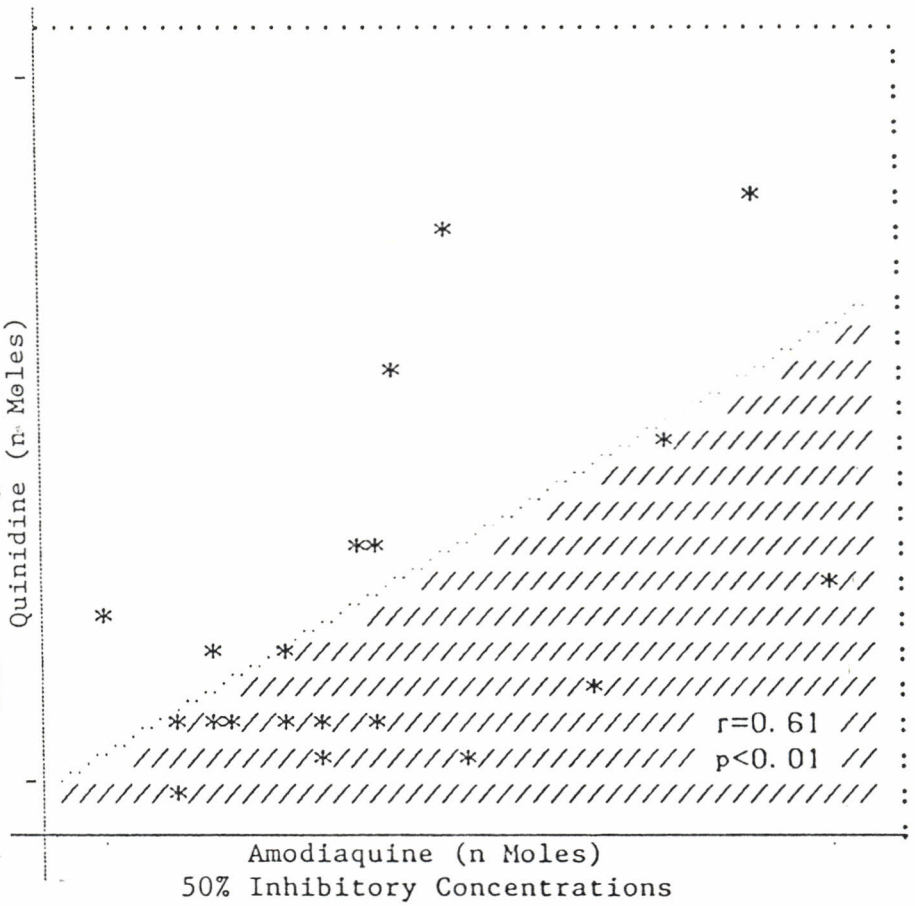


Figure 8: Correlation between in vitro sensitivities to Quinine and Quinidine among 21 Plasmodium falciparum isolates from Kenya.

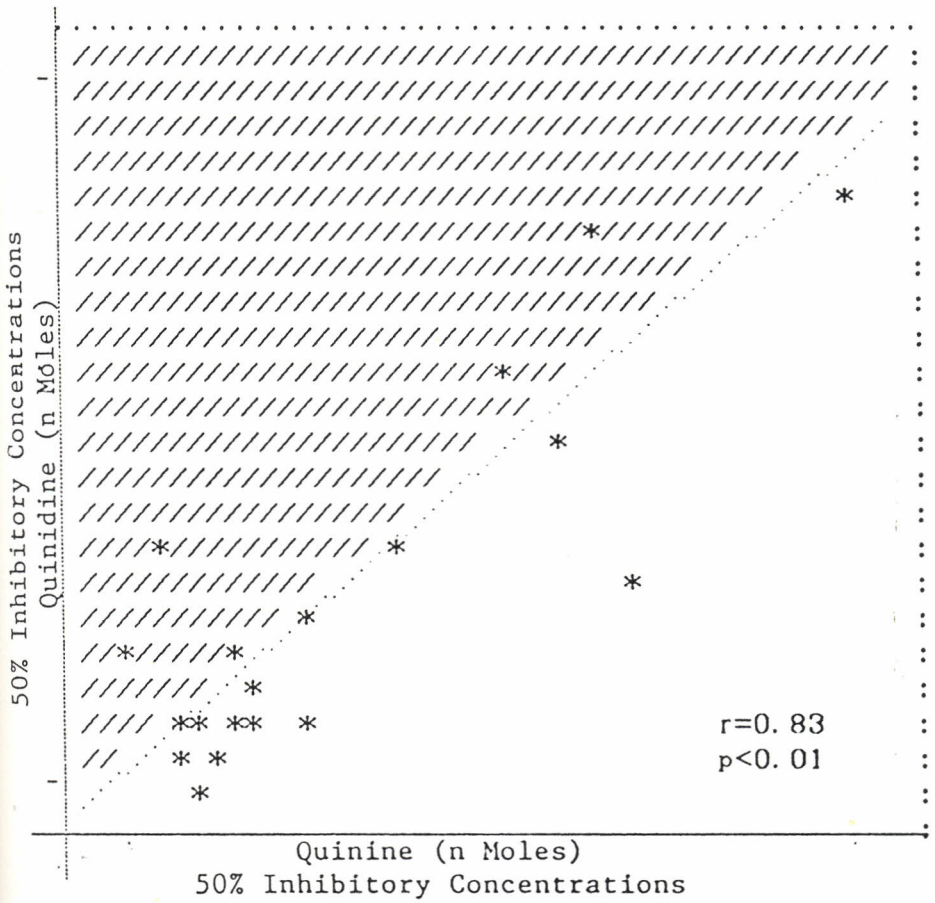


Figure 9: Correlation between in vitro sensitivities to Quinidine and Sulphadoxine among 21 Plasmodium falciparum isolates from Kenya.

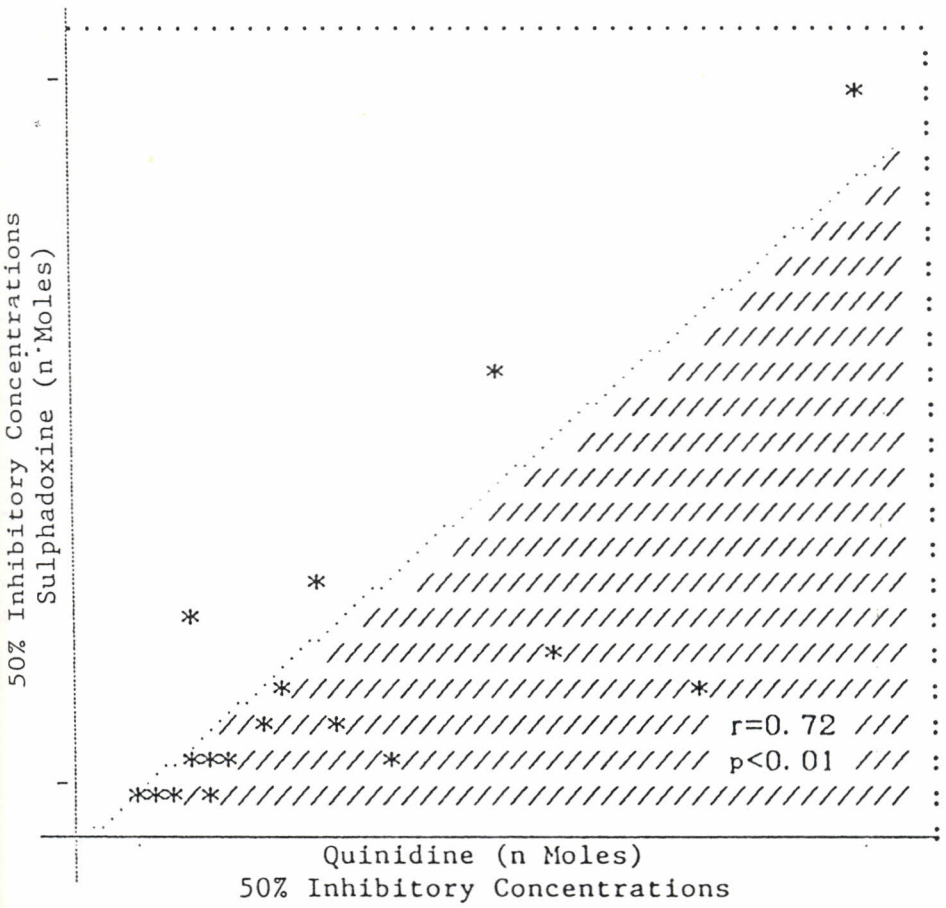


Figure 10: Correlation between in vitro sensitivities to Quinine and Sulphadoxine among 21 Plasmodium falciparum isolates from Kenya.

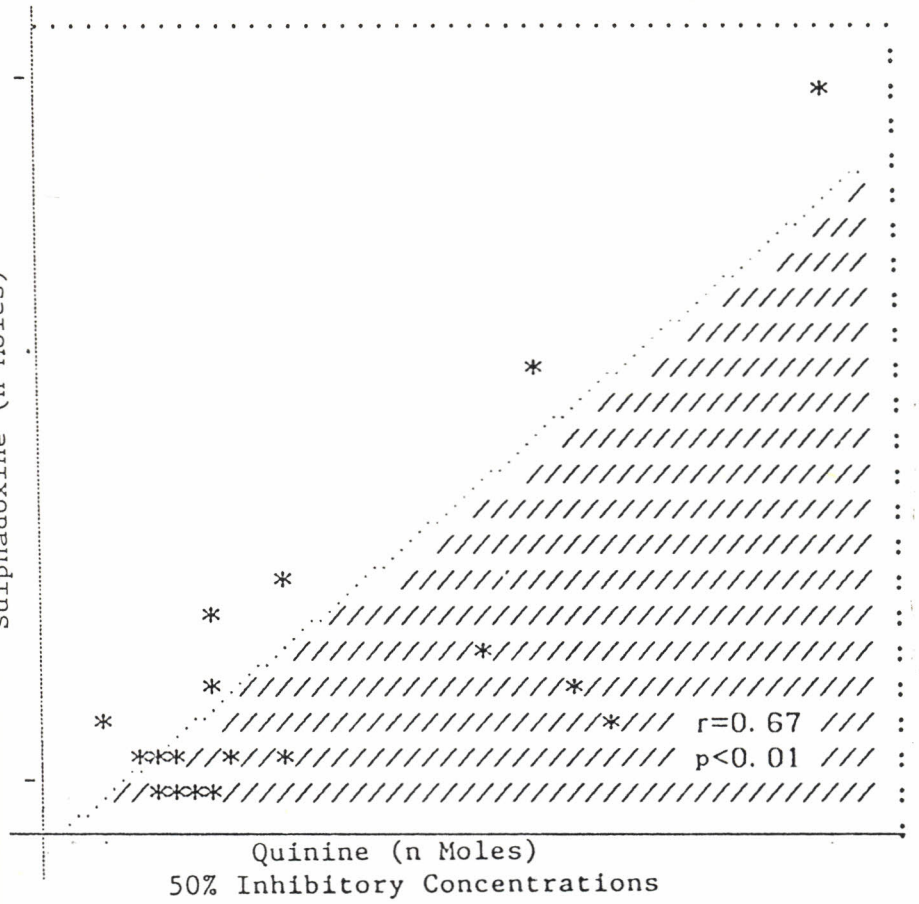
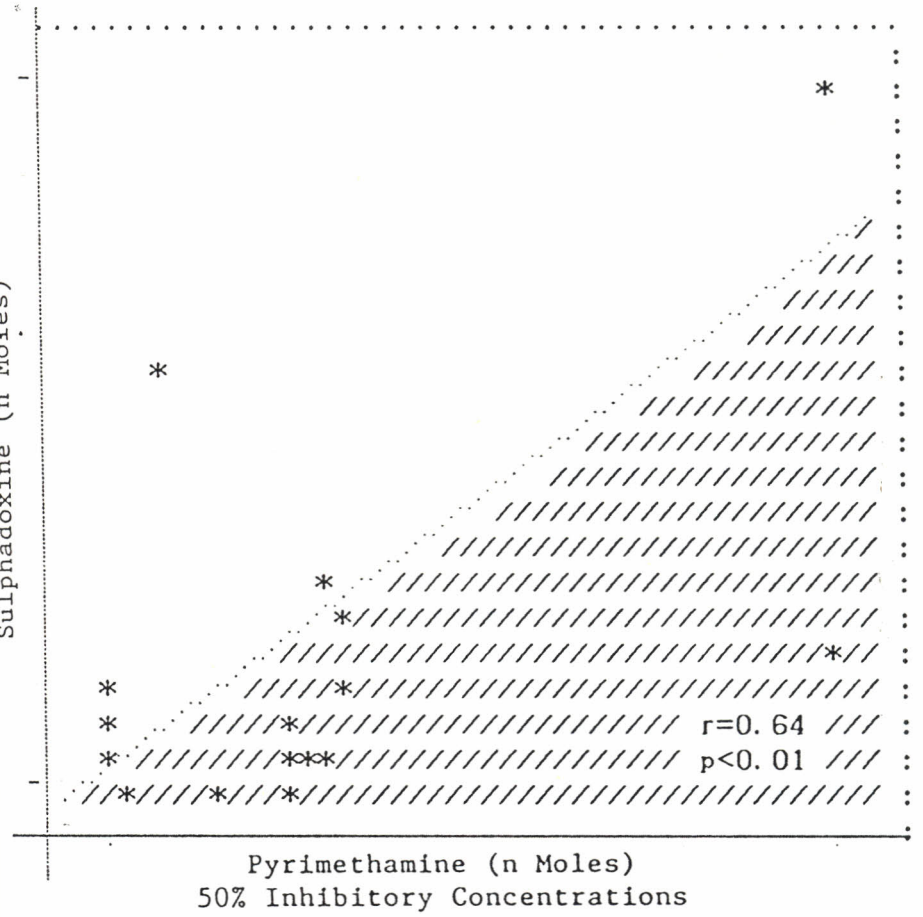


Figure 11: Correlation between in vitro sensitivities to Pyrimethamine and Sulphadoxine among 21 Plasmodium falciparum isolates from Kenya.



The shaded areas between the regression lines and the x or y axes corresponded to the more potent drugs among the different drug pairs as statistically confirmed by students t-test with probability levels of <0.05. For example, in Figure 8, the isolates ID50 values for quinine are plotted on the x-axis and ID50 values for quinidine are plotted on the y-axis and the shaded part is between the regression line and the y-axis meaning that quinidine is more potent than quinine.

Amodiaquine was found to be more potent than quinine and quinidine, and it had cross-resistance with the two antimalarials in their activities against the 21 Kenyan isolates considered (Figure 6 and 7).

Quinidine was found to be more potent than quinine, and sulphadoxine and it also had cross-resistance with the same two drugs in their activities against the 21 Kenyan isolates of P. falciparum (Figures 8 and 9).

Quinine was found to be more potent than sulphadoxine and it also had cross-resistance with the same antimalarial in their activities against the 21 Kenyan isolates of P. falciparum (Figure 10).

And lastly, pyrimethamine was found to be more potent than sulphadoxine and it also had cross-resistance with the same drug in their activities against the 21 isolates of Kenyan P. falciparum considered (Figure 11).

3.2. Enzyme Forms of *P. falciparum* Isolates

Results of the isoenzyme analysis of the 21 Kenyan isolates of P. falciparum examined are presented in Tables 2 and 3, in Figures 12 to 18 and in Plates 3 to 8.

Two forms of the enzyme GPI (GPI-1 and GPI-2) were found within the 21 Kenyan isolates from the three malarious regions considered. The frequencies of the two GPI isoenzymes differed between the provinces.

More GPI enzyme polymorphism was noted among Coast province isolates. Three Coast province isolates showed GPI-1, two isolates showed GPI- 2 and two isolates showed GPI-1 and GPI-2 combination. That is, there were five GPI-1 variants and four GPI-2 variants giving a total of nine GPI-1 and GPI-2 variants among the Coast province isolates (Table 3). The frequency of GPI-1 isoenzymes within the Coast province isolates was 56% while the frequency of GPI-2 for the same province was 44% (Figure 18).

GPI enzyme polymorphism was slightly lower among the Nyanza province isolates than among the Coast province isolates. Four Nyanza province isolates showed GPI-1 and three isolates showed GPI-2 isoenzymes (Table 3). The frequency of GPI-1 isoenzymes within the Nyanza province isolates was 57% while that of GPI-2 for the same province was 43%. These GPI isoenzyme frequencies were more or less similar to those for Coast province isolates (Figure 18).

Less GPI enzyme polymorphism was noted among Rift Valley province isolates (Table 3). Five isolates showed

Table 2. GPI, ADA, PEPE, GDH, 6PGD and LDH enzyme forms in 21 Kenyan isolates of *Plasmodium falciparum*.

GPI			ADA			PEPE		
1	2	1+2	1	2	1+2	1	2	3
57%	29%	14%	-	100%	-	100%	-	-

6PGD			GDH			LDH		
1	2	3	1	2	1+2	1	2	1+2
81%	19%	-	100%	-	-	100%	-	-

The percentages represent the proportions of the isolates examined showing the different isoenzyme types.

Table 3. *In vitro* Chloroquine sensitivity, GPI and 6PGD enzyme forms in 21 *Plasmodium falciparum* isolates from Kenya.

Isolate	Enzyme Type		Chloroquine Susceptibility	
	GPI	6PGD	ID50's(nM)	
Nyanza Province				
1.K39	1	1	14.2	Sensitive
2.K67	2	1	6.5	Sensitive
3.K110	1	1	16.7	Sensitive
4.K112	1	1	39.1	Sensitive
5.S104	1	2	397.5	Resistant
6.S136	2	2	14.7	Sensitive
7.S158	2	1	32.8	Sensitive
Rift Valley Province				
8.ENT7	1	1	247.8	Resistant
9.ENT11	1	1	200.9	Resistant
10.ENT24	1	1	176.2	Resistant
11.ENT30	1	1	127.1	Resistant
12.ENT36	2	1	212.8	Resistant
13.ENT37*	1,2	1	181.3	Resistant
14.ENT41	1	1	285.9	Resistant
Coast Province				
15.M24	1	2	6.3	Sensitive
16.M423	2	1	22.9	Sensitive
17.JP1	1	1	<9.4	Sensitive
18.JP17/A	2	1	96.9	Sensitive
19.JP78*	1,2	1	115.7	Resistant
20.JP119*	1,2	1	62.5	Sensitive
21.D7/22	1	1	<3.1	Sensitive

*Isolates with both GPI-1 and GPI-2 isoenzymes, meaning that they are not single strains.

Figure 12. Summary of GPI profiles in human red blood cells and in P. falciparum isolates from Kenya.

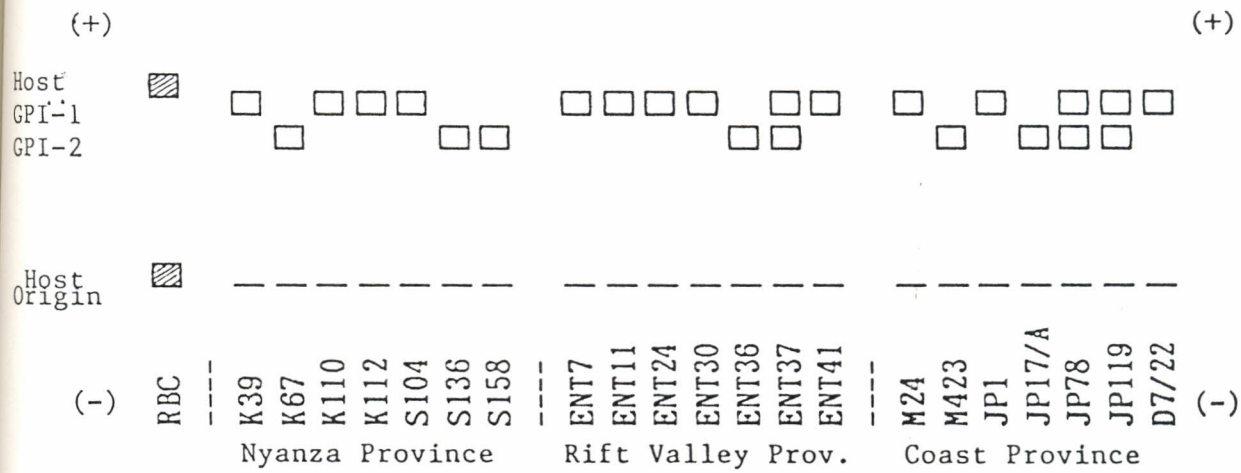


Figure 13. Summary of GDH profiles in human red blood cells and in P. falciparum isolates from Kenya.

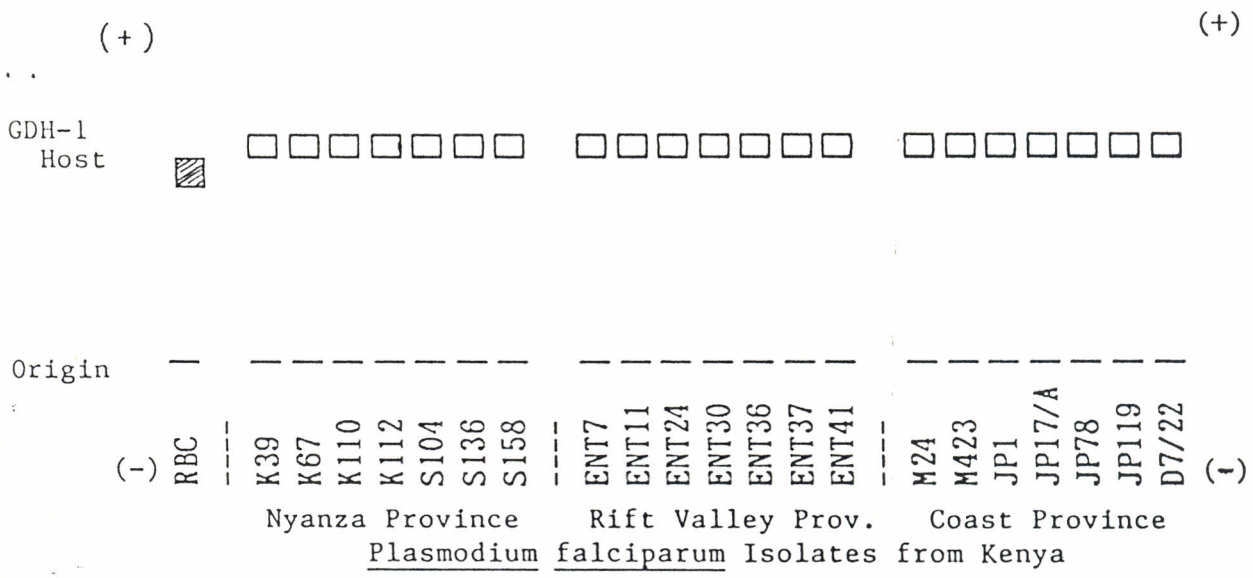


Figure 14. Summary of ADA profiles in human red blood cells and in P. falciparum isolates from Kenya.

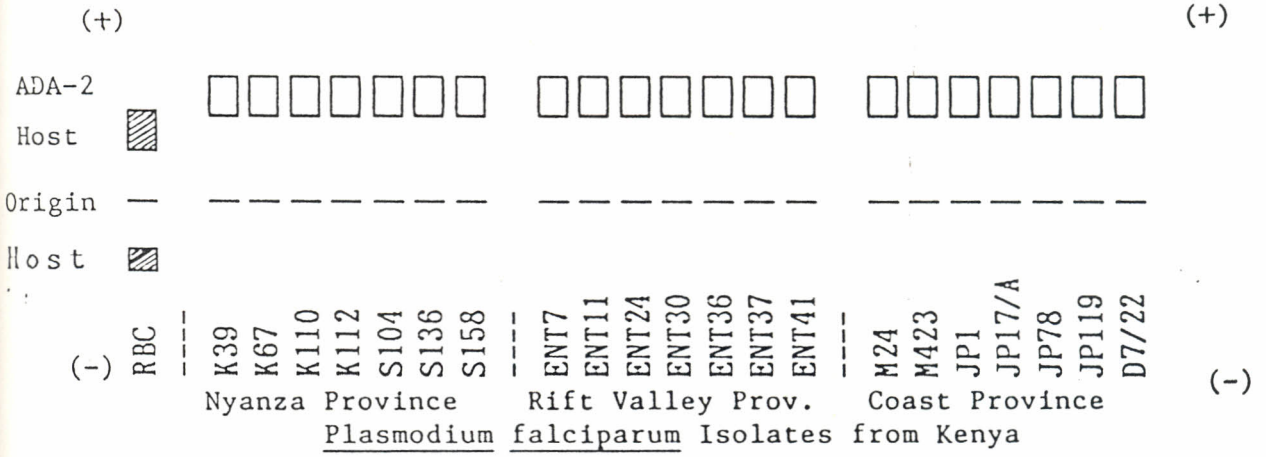


Figure 15. Summary of LDH profiles in human red blood cells and in P. falciparum isolates from Kenya.

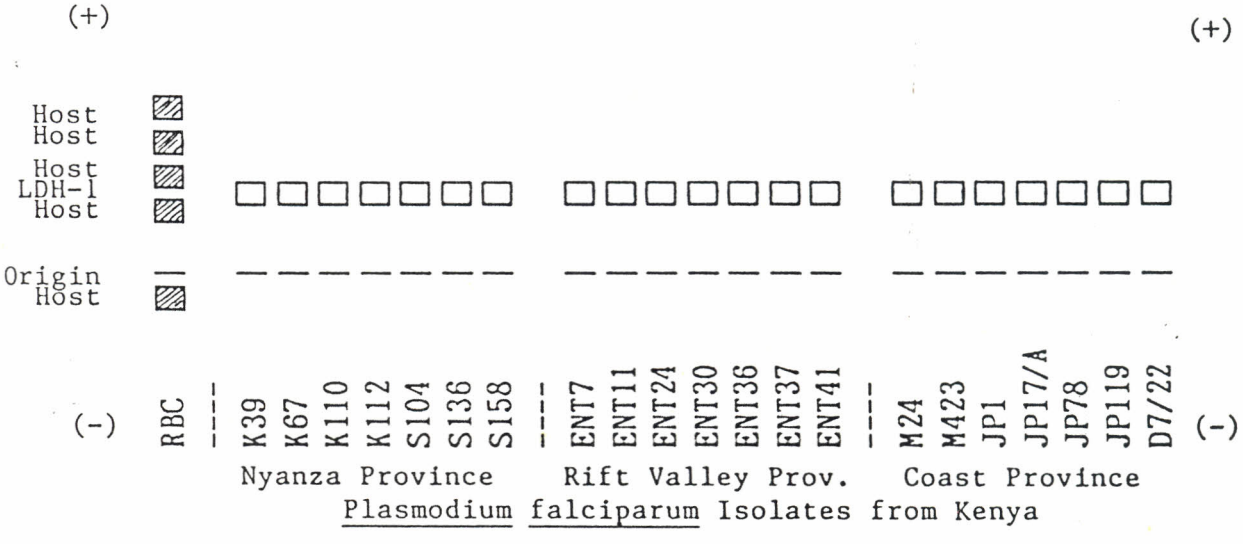
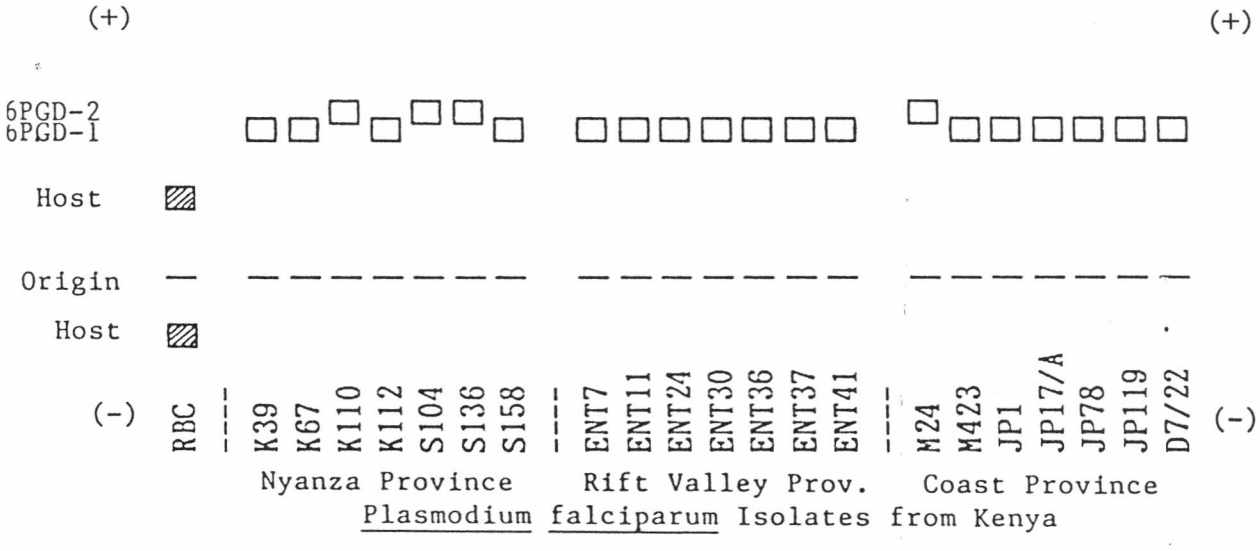


Figure 16. Summary of 6PGD profiles in human red blood cells and in P. falciparum isolates from Kenya.



re 17. Summary of PEPE profiles in human red blood cells and in
alciparum isolates from Kenya.

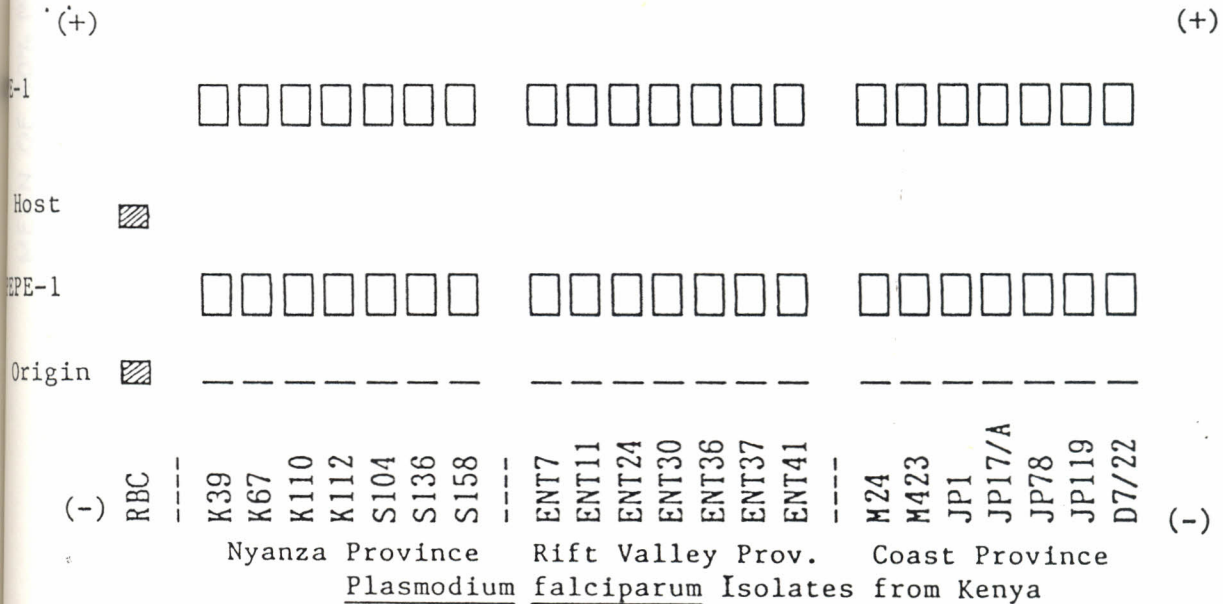


FIGURE 18. CORRELATION OF *IN VITRO* DRUG SENSITIVITY AND GPI ISOENZYMES OF *PLASMODIUM FALCIPARUM* ISOLATES FROM KENYA.

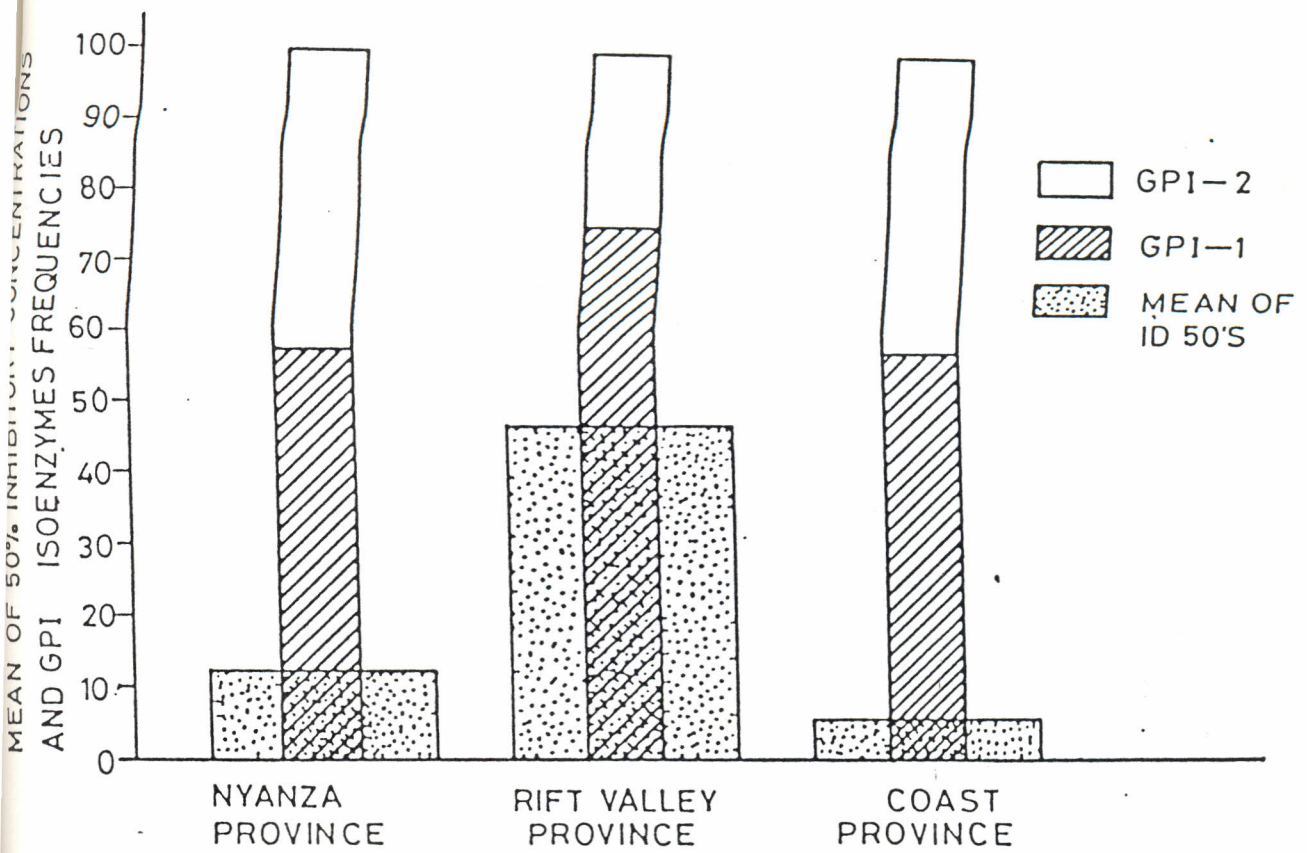


PLATE 3. ELECTROPHORETIC FORMS OF GPI IN HUMAN RED BLOOD CELLS AND IN *PLASMODIUM FALCIPARUM* ISOLATES FROM KENYA.

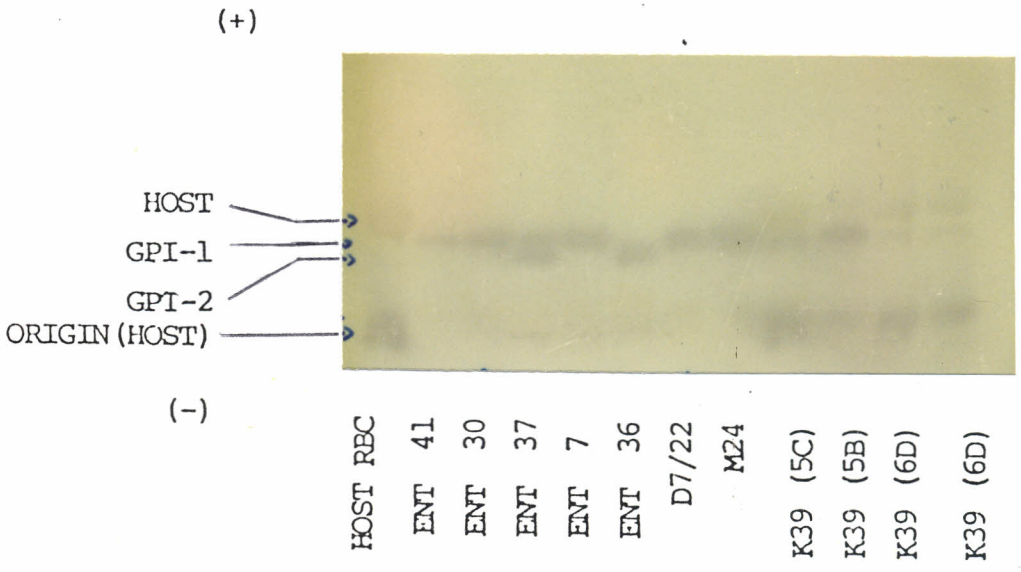


PLATE 4. ELECTROPHORETIC FORMS OF GDH IN HUMAN RED BLOOD CELLS AND IN *PLASMODIUM FALCIPARUM* ISOLATES FROM KENYA.

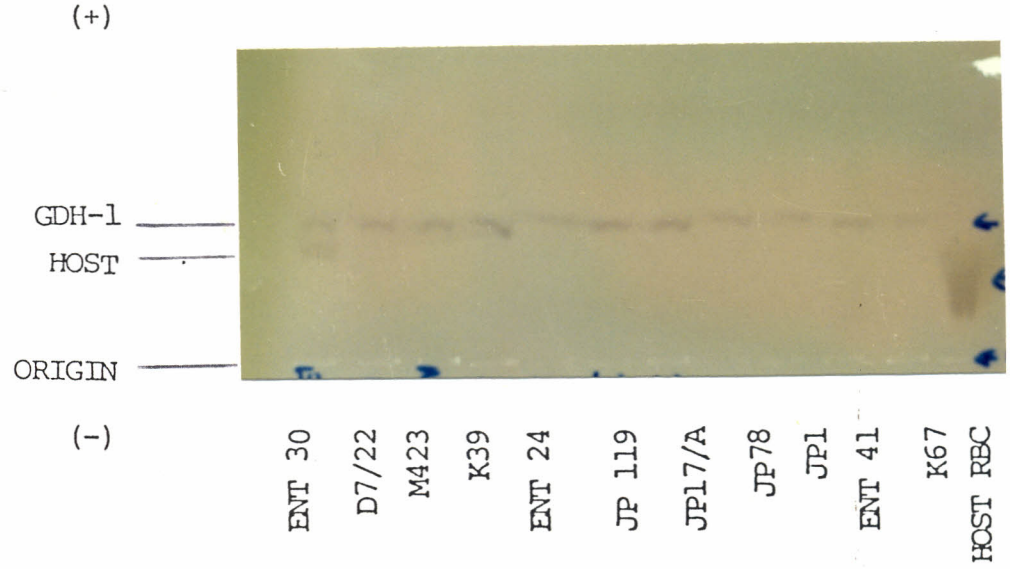


PLATE 5. ELECTROPHORETIC FORMS OF ADA IN HUMAN RED BLOOD CELLS AND IN *PLASMODIUM FALCIPARUM* ISOLATES FROM KENYA.

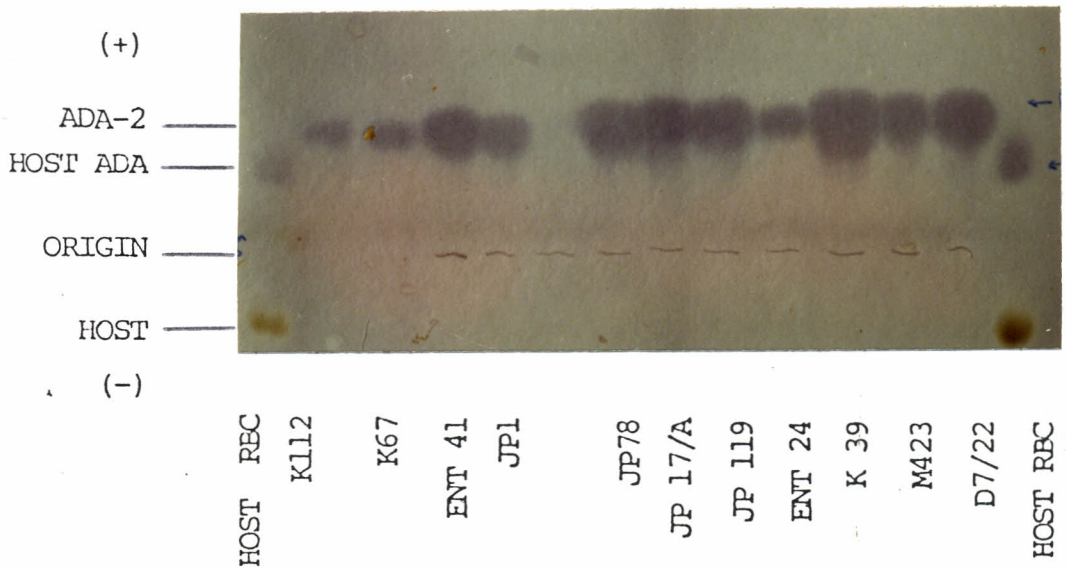


PLATE 6. ELECTROPHORETIC FORMS OF LDH IN HUMAN RED BLOOD CELLS AND IN *PLASMODIUM FALCIPARUM* ISOLATES FROM KENYA.

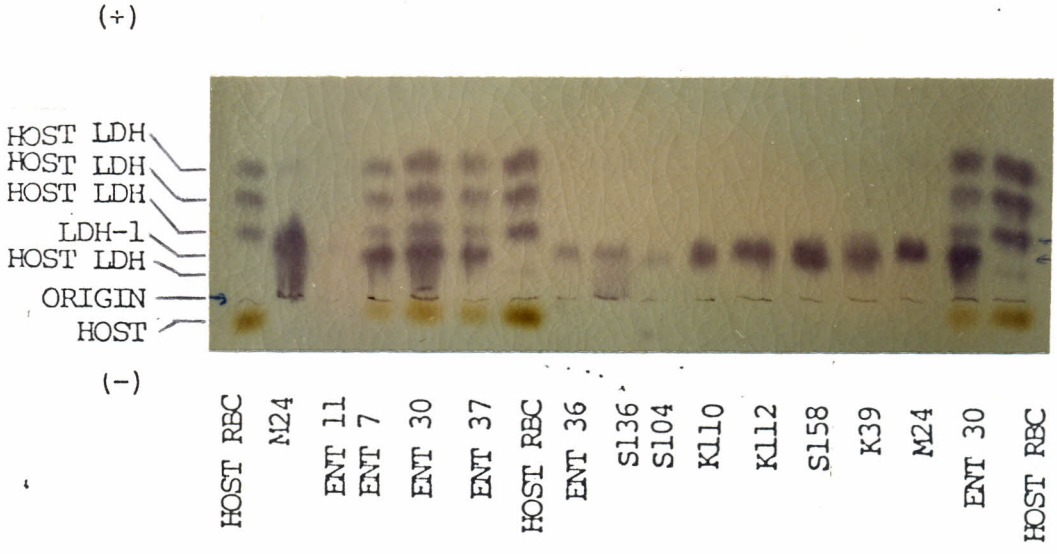


PLATE 7. ELECTROPHORETIC FORMS OF 6PGD IN HUMAN RED BLOOD CELLS AND IN *PLASMODIUM FALCIPARUM* ISOLATES FROM KENYA.

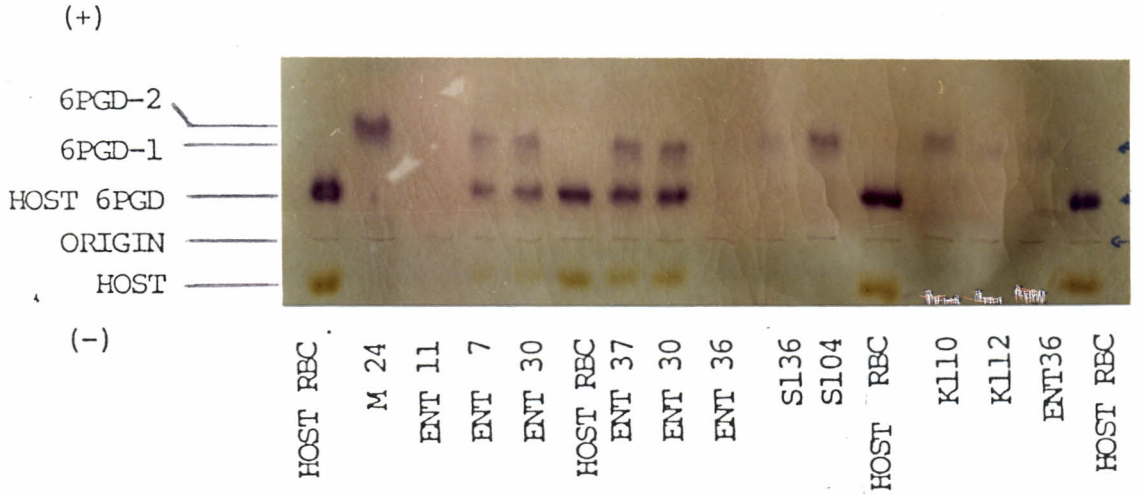
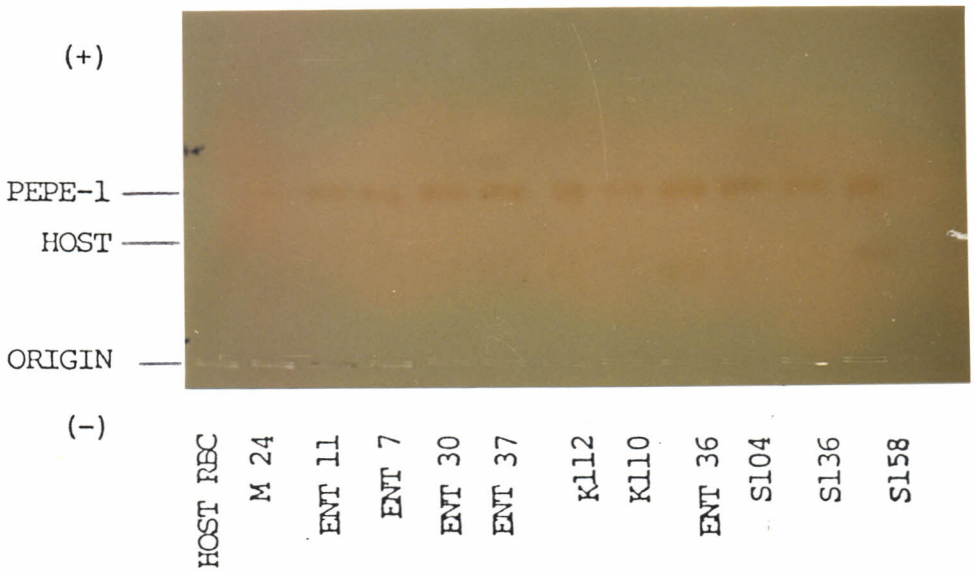


PLATE 8. ELECTROPHORETIC FORMS OF PEPE IN HUMAN RED BLOOD CELLS AND IN *PLASMODIUM FALCIPARUM* ISOLATES FROM KENYA.



GPI-1, one isolate showed GPI-2 and one isolate showed GPI-1 and GPI-2 combination (Table 3). That is, there were six GPI-1 variants and only two GPI-2 variants among the Rift Valley province isolates. The frequency of GPI-1 isoenzymes within the Rift Valley province isolates was 75%, greater than that for both Coast province and Nyanza province isolates (Figure 18).

Less variation was noted for the enzyme 6PGD among the isolates examined. All Nyanza province isolates had 6PGD-1 enzyme forms except three isolates which possesed 6PGD-2 enzyme forms. All Coast province isolates had 6PGD-1 forms of the enzyme except one isolate which had 6PGD-2 enzyme variant. All isolates from Rift Valley province had 6PGD-1 enzyme forms (Table 3).

GDH, LDH, ADA and PEPE appeared to be invariant in all of the 21 Kenyan isolates examined (Table 2 and Figures 13,14,15 and 17). GDH-1, LDH-1 and PEPE-1 were found in all the isolates except ADA which appeared as the isoenzyme ADA-2 in all the isolates examined.

3.3. Correlation of Drug Response and Isoenzymes of Kenyan Isolates of *P.falciparum.*

Isolates from the Rift Valley province were the least susceptible to most of the antimalarial drugs tested followed by the isolates from the Nyanza province and then the isolates from the Coast province (Table 1 and Figure 3). Correspondingly, there was least polymorphism in GPI enzyme

forms of the Rift Valley province isolates, some polymorphism in those from the Nyanza province while the Coast province isolates showed the greatest GPI enzyme polymorphism (Table 3 and Figure 18).

All isolates from Rift Valley province were found to be 'resistant' to chloroquine (Table 3 and Figure 5). The same isolates showed no variation in 6PGD and the highest frequency of GPI-1 isoenzyme forms compared to the isolates from the two other malarious regions considered (Table 3 and Figure 18).

CHAPTER 4

DISCUSSION AND CONCLUSIONS

4.1. *P. falciparum* Sensitivity to Antimalarial Drugs.

In vitro drug susceptibility tests have been found useful for epidemiological purposes and for evaluating new drug candidates (Desjardins et al. 1979; Spencer et al. 1984). In vitro and in vivo systems for evaluation of drug sensitivity against the same P. falciparum isolates have been found to produce similar results. Spencer et al. (1984) found that the in vivo response to pyrimethamine/ sulphadoxine was able to predict the in vitro response. The predictive value of the in vitro response to the in vivo response was also reported by Nguyen-Dinh et al. (1982), Spencer et al. (1983), Smrkovski et al. (1985) and Watkins et al. (1988). Therefore, results of the in vitro test presented in this study probably reflect the in vivo susceptibilities of the 21 Kenyan P. falciparum isolates studied.

Sixsmith et al. (1984) and Watkins et al. (1988) reported that the Malindi isolate M24 was sensitive to both chloroquine and pyrimethamine and that the Kisumu isolate K39 was sensitive to chloroquine but resistant to pyrimethamine. The two reference isolates (K39 and M24) were retested during the present study and the results confirmed the earlier reports, thus indicating the

reliability and the reproducibility of the antimalarial drug test systems used during the study.

The Kenyan isolates examined showed considerable variation in their sensitivities to the different antimalarial drugs studied (Table 1 and Figure 3). It was also observed that the amount of variation in chloroquine sensitivity among isolates was inversely related to the level of chloroquine resistance (Figure 5). A possible reason for this could be that in those provinces with low drug resistance (Coast and Nyanza) the isolates are a mixture of sensitive and resistant parasites whereas in the Rift Valley province most of the parasites have acquired resistance and hence the amount of variation among them is lower.

Even though there is a danger in regarding drug sensitivity data as good strain markers before their transmission through mosquitoes is done in order to confirm their genetic basis and stability (Rosario and Thaithong, 1986), it can still be suggested that antimalarial drug sensitivity test data are suitable for P. falciparum strain characterisation since there is evidence that most forms of drug resistance have a genetic basis (Walliker, 1980, 1982; Molineaux, 1986).

Naturally occurring P. falciparum infections always appear to be mixtures of genetically distinct populations differing in parameters such as enzyme forms and drug sensitivity (Thaithong, 1983). Therefore, the drug

sensitivity data for different isolates examined in this study does not necessarily depict the genotypes of homogenous parasite strains but could also be the products of mixed parasite strains occurring within single isolates. It could be difficult to consider such drug response data as genetic markers unless one can predict their long term stability by correlating them with other genetic markers such as single isoenzyme forms of single *P. falciparum* isolates. In this study, the drug response data was correlated with the single isoenzyme forms as explained in section 4.3.

Quinidine was found to be more effective against the isolates *in vitro* than quinine as had been observed by Schwartz (personal communication, 1988). The *in vitro* results of Spencer *et al.* (1983) indicated that amodiaquine was a more potent blood schizonticide than is chloroquine. Their results showed that most isolates were more sensitive to amodiaquine than to chloroquine, but a few isolates appeared to be equally sensitive to both drugs. The same situation was also observed during the present study. Amodiaquine was found to be an effective antimalarial drug against the 21 Kenyan isolates. The same finding was also reported by Watkins *et al.* (1984) and Spencer *et al.* (1983b, 1984). Unexpectedly, cross-resistance was not observed between the two 4-aminoquinolines (chloroquine and amodiaquine) used. Linear regression analysis r value was

low: 0.5, though the probability level of the slope was acceptable : 0.02.

In vitro drug sensitivity data of the Kenyan isolates of P. falciparum showed that mefloquine and quinidine are quite effective even against P. falciparum resistant to chloroquine.

There have been unconfirmed suggestions that there may be cross-resistance between mefloquine (a 4-quinolinemethanol) and quinine because the drugs are structurally similar (Spencer, 1985). In the present study, the cross-resistance between mefloquine and quinine was found to be rather poor. Linear regression analysis r value (0.45) was low, though the probability level of the slope (0.04) was acceptable. More evidence is required to ascertain the cross-resistance between these two drugs.

Sulphadoxine, an inhibitor of dihydropteroate synthetase, is a slow acting sulphonamide (Spencer, 1985) and this could be one of the reasons why its ID50 values were found to be higher in most of the isolates examined (Table 1).

It is also possible that the small amount of p-aminobenzoic acid (PABA) and folic acid (FA) in the blood products (red blood cells and serum) used in the culture systems could have contributed to the higher ID50 values of sulphadoxine, and probably to pyrimethamine, during the in vitro antimalarial tests (Chulay et al. 1984; Watkins et al. 1985). But above all, we cannot rule out the fact

that sulphadoxine, and even pyrimethamine, are weaker antimalarial drugs than the other drugs used in the study.

Another slow acting drug, tetracycline, was initially included in the study but abandoned later because its ID50 values to the isolates were found excessively higher than those for other test drugs. This means that it could not be successfully tested simultaneously with the other fast acting drugs in the same drug test plates since it needed longer times of incubation with the malaria isolate cultures.

Parasite resistance to the most commonly used dihydrofolate reductase inhibitor, pyrimethamine, has spread rapidly and its use is currently limited to combination therapy with other drugs (Sixsmith et al., 1984). Malaria resistance to pyrimethamine is widespread in Kenya (Nguyen-Dinh et al. 1982; Spencer, 1985). Therefore, it is not surprising that 70% of the isolates examined in the present study had low susceptibilities to pyrimethamine. ID50 values were higher than 100 n Moles (Table 1).

Despite widespread resistance to pyrimethamine alone, pyrimethamine/sulphadoxine (fansidar) is effective treatment for most P. falciparum malaria attacks in Kenya (Watkins et al., 1988). The present results show that there was some cross-resistance between pyrimethamine and sulphadoxine in their activities against P. falciparum isolates as may be expected in their therapeautic combination as fansidar. The

probability level of the linear regression analysis slope was 0.0019 and r value was 0.64.

There were no significant variations between the *in vitro* sensitivities of quinine against the isolates of *P. falciparum* from the three provinces of Kenya considered in this study. Kruskal-Wallis oneway nonparametric analysis of variance showed a probability level of 0.26. This is quite expected in view of the reports that quinine resistance, especially *in vivo*, is still rare (Spencer, 1985).

An unexpected cross-resistance between quinine and sulphadoxine (two structurally unrelated drugs) was observed in the present study (Figure 10). Linear regression r value was 0.67 and the probability level of the slope was 0.00092. The implications of this finding is still unclear in view of the fact that quinine and sulphadoxine are structurally unrelated drugs.

Chloroquine has always been the antimalarial drug of choice for semi-immune persons living in Kenya (Spencer *et al.*, 1982). It is the most widely used blood schizonticidal antimalarial drug and is the mainstay of antimalarial chemotherapy (Spencer, 1985). The observed poor response of the Kenyan Rift Valley province isolates to chloroquine in the present study may probably indicate that a more effective antimalarial drug may be required for controlling malaria in that region of Kenya during malaria epidemics.

Entasopia is situated on the South-Western side of the Rift Valley province (see Figure 1). When compared to

Nyanza and Coast provinces, regions of hyper-to holoendemic malaria, Entasopia is an area of seasonal malaria with higher transmission during the rainy seasons in May-June and November-December (Watkins *et al.*, 1988). It is therefore possible that most residents of Entasopia are non-immune to malaria. This could explain why most Entasopia *P. falciparum* isolates studied were not very sensitive to the antimalarial drugs examined. The earlier reports that drug-resistance tends to emerge in situations of relatively intense transmission but not as much in areas of the most intense transmission and immunity (Molineaux, 1986) could also explain the Entasopia situation.

4.2. Isoenzyme Analysis

It had been reported that cryopreservation and continuous *in vitro* cultivation procedures may favour certain parasite subpopulations (WHO, 1981; Thaithong *et al.*, 1984) and thus lead to genetic selection which can be reflected in the reduction of the genetic variants as compared to the freshly collected and immediately analysed isolate samples. This could have happened during the manipulation of the isolates examined prior to isoenzyme analysis in this study; but results from this study do not support this postulate as some isolates from Coast and Nyanza province had been cryopreserved and cultivated for over four years while isolates from Rift Valley province had

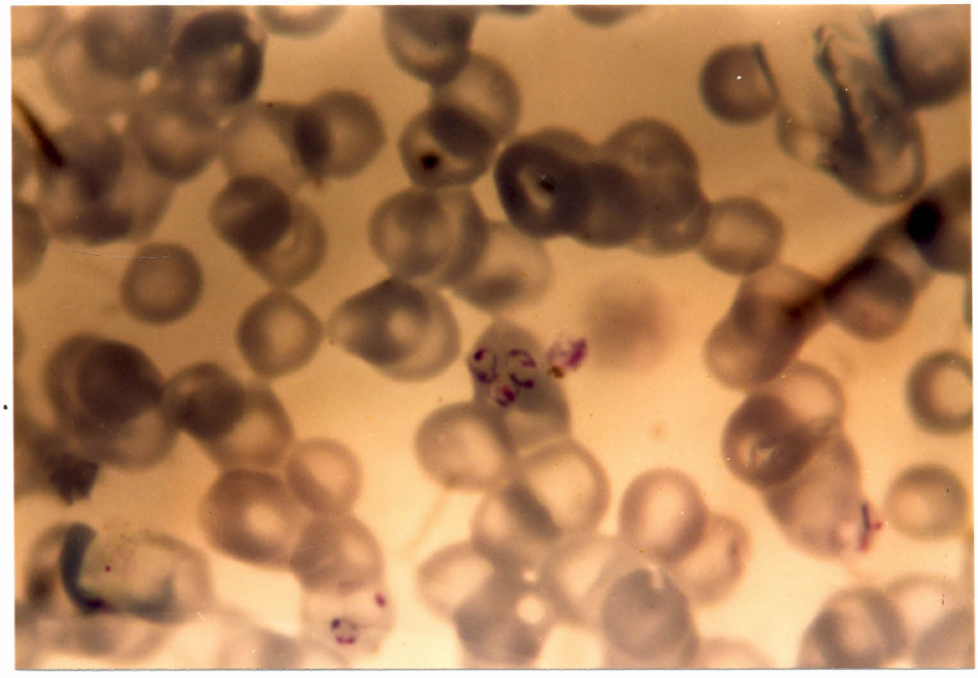
not been cryopreserved and cultivated for more than two years and yet it is the Rift Valley province isolates which showed least GPI enzyme polymorphism.

Several workers including Thaithong (1985) had reported that only cultures with a higher proportion of schizonts (Plate 1) were suitable for isoenzyme analysis. This was confirmed during the study as isolates harvested when the parasites were predominantly in the ring stages (Plate 9) never yielded isoenzyme results.

However, isolates harvested when the parasites were predominantly gametocytes (for example K67) were found to yield good electrophoretic forms of the enzymes investigated. This means that the gametocytes, just like the late trophozoites and schizonts, are also good sources of the enzymes examined in the study.

The occurrence of different isoenzyme forms in P. falciparum isolates from Kenya as has been found in this study indicates that different strains of the parasite occur in important endemic areas of Kenya. When compared to murine malaria, P. falciparum parasites do not exhibit many enzyme variants (Rosario and Thaithong, 1986) and P. falciparum from regions of the world studied so far could probably consist of a single interbreeding population (Carter and Walliker, 1977; Walliker , 1982). The same situation was found in the present study; among the isolates examined, only two of the six enzymes studied showed variants. The other four enzymes could have variants among

PLATE 9. ERYTHROCYTIC RING STAGES OF CULTIVATED *PLASMODIUM FALCIPARUM*.



Kenyan P. falciparum but probably present at a much lower frequency detectable only if a larger number of isolates are examined.

GPI and 6PGD showed variant forms of enzymes among the isolates examined. Enzyme polymorphism was greater in GPI than in 6PGD. Variations in GPI enzyme forms were found among the isolates from each of the examined provinces of Kenya. Coast province and Nyanza province isolates were found to possess considerably lower frequency of GPI-1 isoenzyme forms than the isolates from Rift Valley province- which had higher frequency of GPI-1 isoenzymes. These differences indicate that enzyme variations can occur among isolates within a single endemic area and also between different endemic areas.

GPI was examined using cellulose acetate electrophoresis procedures which were found adequate for its analysis. As had been reported earlier (Walliker, 1985; Rosario and Thaithong, 1986) two enzyme forms of GPI were found: GPI-1 and GPI-2. Isolates characterised by GPI-1 variants were more common than those characterised by GPI-2. Some few isolates such as ENT 37, JP78 and JP119 had GPI-1 and GPI-2 combination (Table 3). Maybe such single isolates which showed both GPI-1 and GPI-2 variants were few because of the selective effects of cryopreservation and continuous in vitro cultivation of the parasites.

6PGD enzyme forms never showed in the cellulose acetate electrophoresis system during this study. Maybe there was a

problem with electrode buffer used for the system. 6PGD enzyme forms were then examined by using the thin layer starch gel electrophoresis system with satisfactory results. All isolates examined had 6PGD-1 form of the enzyme except four isolates which showed 6PGD-2 enzyme variants. Carter and McGregor (1973) also found few 6PGD-2 enzyme variants among the African isolates of P. falciparum they examined.

ADA enzyme forms readily showed both in cellulose acetate and in thin-layer starch gel electrophoresis systems, but the resolved bands were stronger and clearer in the thin layer starch gel system. All isolates examined had ADA-2 enzyme variants. Earlier workers had reported the occurrence of both ADA-1 and ADA-2 in single isolates from Tanzania (Sanderson et al., 1981; Rosario and Thaithong, 1986) but this was not observed in the present study (Table 2).

Only host forms of the enzyme LDH could be resolved by the cellulose acetate electrophoresis during this study. So the final results of LDH enzyme forms were obtained from the thin layer starch gel electrophoresis procedures. Both host and parasite LDH enzyme forms showed readily in the thin layer starch gel system. Only LDH-1 enzyme variants were observed among the Kenyan isolates examined.

LDH-2 enzyme variants had been reported only from some freeze-dried isolates from Gambia, Tanzania and Congo and no cultured samples from Africa or any other country had shown this enzyme variant (Sanderson et al., 1981). This could be

because very few cultured isolates from Africa have been examined for isoenzymes compared to the greater number of freeze-dried isolates from the same region. Another reason could be that the isolates usually loose some of their enzyme variants during the *in vitro* cultivation processes, but this is unlikely because it would seem that the *in vitro* cultivation methods only selects for the LDH-1 enzyme variants.

Probably LDH-2 enzyme variants were not found during the present study because the number of isolates examined was small. Another possible reason could be that LDH-2 enzyme variants do not occur among Kenyan isolates of *P. falciparum*, but this could be more unlikely considering the fact that LDH-2 variants have been found in some *P. falciparum* from Africa.

GDH enzyme forms showed readily in cellulose acetate electrophoresis system. Only GDH-1 enzyme variants were found in the isolates examined. This finding is not unexpected from the reported very high frequencies of GDH-1 isoenzymes among the *P. falciparum* isolates from several African countries (Sanderson *et al.*, 1981).

PEPE enzyme forms were examined by using both cellulose acetate and thin layer starch gel systems. Both these methods worked differently for this enzyme for the isolates examined. Cellulose acetate electrophoresis showed only the upper bands of PEPE (Figure 17, plate 8) which are the same for PEPE-1 and PEPE-2 enzyme variants (Thaithong, 1985).

Thin layer starch gel electrophoresis system gave better resolution and showed both the upper and the lower PEPE bands but all of them were slow to develop, diffuse, large and difficult to analyse as to which enzyme forms they were, as has been reported by other workers (Sanderson et al., 1981; Thaithong, 1985).

The lower PEPE enzyme bands appeared as PEPE-1 in the gels when compared with the earlier reports of Sanderson et al. (1981) and Thaithong (1981) but no certainty can be attached to their actual forms in this study since they were found very difficult to analyse. But in all the isolates examined, the PEPE parasite bands were in the same positions, hence no variants were observed.

4.3. Correlation of Drug Response and Isoenzymes of Kenyan Isolates of *Plasmodium falciparum.*

Many workers have used isoenzymes as genetic markers when carrying out genetic and drug response studies of murine and human malaria parasites (Rosario, 1976; Knowles et al., 1981; Thaithong et al., 1984; Walliker, 1985). Essentially the same situation applied to this study; isoenzyme and antimalarial drug analyses of Kenyan P. falciparum isolates were done to investigate strain differences and also the possible correlation which might exist between certain isoenzymes and certain drug sensitivity patterns.

It is known that clones of asexual forms of P. falciparum contain only single isoenzyme types (Carter and McGregor, 1973; Walliker, 1982; Thaithong et al., 1984). But the converse may not be true, i.e, isolates with single and similar isoenzyme types may not be genetically homogenous clones. Thaithong (1983) found that some clones which had similar isoenzyme types showed different responses to similar antimalarial drugs. This means that even though the clones can be genetically similar with respect to their isoenzyme types, they can as well be genetically different with respect to their antimalarial drug responses. Thus differences in antimalarial drug responses between isolates can reflect strain differences if the isolates posses single isoenzymes (meaning that each of the isolates do not contain a mixture of strains but only single strains).

Drug sensitivity studies could therefore be used as an additional means of characterising isolates or clones which have been characterised by isoenzyme analysis, as was done in this study.

Of the 21 Kenyan isolates of P. falciparum examined in this study, isoenzyme analysis showed that only three isolates - ENT37, JP78 and JP119 - were "mixed infections" since each of them possessedtwo different GPI isoenzyme forms (Table 3). The other 18 isolates were further characterised by using their chloroquine sensitivity data (Table 3). The variations in their drug response data were taken to be reflections of the differences in their genetic

nature which could not be expressed by their isoenzyme types alone. The chloroquine ID50 value for each of the 18 isolates was found to be significantly different from the others. P value for X^2 test was < 0.01.

Although the number of the isolates studied was not large, it is clear from the data presented in Table 3 and Figure 18 that lower frequency in GPI-1 isoenzyme types were recorded from Coast and Nyanza province isolates than from Rift Valley province isolates. It was also observed that Coast and Nyanza province isolates were significantly more sensitive to the antimalarial drugs than isolates from Rift Valley province (Table 1 and Figure 3). Grand mean ID50 values for Coast and Nyanza province isolates were 165.4 n Moles and 360.1 n Moles respectively and these were found to be significantly different from the value for Rift Valley province isolates (1354.3 n Moles). P value for X^2 test was <0.001.

One can postulate from these data that the greater the GPI-1 isoenzyme frequency, the higher is the level of drug resistance in a population of parasites. But this hypothesis still awaits proof by further analysis of more isolates of P. falciparum from malarious regions of Kenya, especially from endemic areas with both drug resistant and drug sensitive isolates.

Earlier attempts had been made to correlate enzyme variation and variation in drug responses (Carter and Walliker, 1977). But these attempts seem to have been fruitless because they were aimed at finding genetic affinities between enzyme types and drug responses and yet

it is known that none of the six enzymes examined is concerned with drug resistance. It is also known that the genes for drug resistance in murine Plasmodium species (which serves as P. falciparum models) segregate independently of those for the six enzyme variants studied (Thaithong, 1981). From the results presented in Table 3 and Figure 18, it was realised that parasite drug resistance could also be a reflection of genetic selection which could be expressed as the lowering of the frequency of certain enzyme variants within a population of parasites as shown by GPI isoenzyme frequencies.

If drug-pressure is the cause of the genetic selection, then the selected parasite strains or clones should be less sensitive to the drugs (or to compounds closely related to the drugs) which 'selected' them and they should also possess fewer enzyme variants since the other variants were lost during the selection process. This could explain the drug response-isoenzyme patterns results as reported in this study.

It is now widely accepted that mutations in the parasites genome are probably the basis of all drug resistance. These mutations are probably spontaneous and drug pressure only acts by selecting pre-existing mutants which carry drug resistance genes (Molineaux , 1986). Spencer (1985) suggested that drug resistance might not confer a biological advantage in the absence of drug pressure since chloroquine-resistant P. falciparum did not

appear until the drug was used for treatment and chemoprophylaxis. This means that drug pressure may be necessary for the emergence and spread of drug resistant P. falciparum populations.

Molineaux (1986) reported that the response of P. falciparum to drugs shows significant geographic variation even before exposure to those drugs. This implies that there is a possibility that the malaria endemic regions considered in this study, i.e, Coast province and Nyanza province, originally had P. falciparum isolates differing in their drug response characteristics even before antimalarial drugs were used in the regions.

It is now difficult to ascertain whether the reported difference in the antimalarial drug responses between the endemic regions, i.e, Coast and Nyanza provinces, was caused by the differences in the antimalarials drug-use between the two provinces or not. This is because antimalarial drug-tests in Coast and Nyanza provinces in Kenya were only done long after antimalarial drugs had been extensively used in those regions (Vogel et al., 1974; Spencer, 1985).

4.4. CONCLUSIONS.

This study has shown that:

1. Characterisation by isoenzyme analysis and antimalarial drug sensitivity methods is suitable for identifying strains of *P. falciparum*.

2. Genetically diverse strains are present in Kenyan isolates of *P. falciparum* as shown by isoenzyme analysis and drug sensitivity results. This genetic diversity occur both within and between the three provinces of Kenya from which the isolates were collected.

3. The examined isolates of *P. falciparum* from Rift Valley province show a greater frequency of GPI-1 isoenzyme and also a lower susceptibility to most of the antimalarial drugs tested.

4. Correlations (cross-resistance) occur between some of the antimalarial drugs used against the examined isolates of *P. falciparum*.

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