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THE USE OF SEROLOGY AND OF TESTS FOR DRUG 
AND INSECTICIDE RESISTANCE IN STUDYING PROBLEMS 
OF MALARIA CONTROL IN THE SUDAN

A thesis submitted for the 
Degree of Doctor of Philosophy 
in the Faculty of Medicine, 
University of London

by

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The Use of Serology and of Tests for Drug and Insecticide Resistance in Studying Problems of Malaria Control in the Sudan

By Mustafa Abdalla Sidahmed Akood

ABSTRACT

This thesis describes the application of new malariological techniques to study some of the problems facing, or which might face, the malaria control programme in The Gezira (Blue Nile Province) of The Sudan.

Serological tests, using the indirect fluorescent antibody test were carried out on a large sample of the population of the Gezira in 1977 and 1979 drawn from 18 localities in 4 different areas. The results showed a much higher level of prevalence and incidence of malaria than was detected by routine parasitological examinations. The percentage of those who had positive antibody reactions decreased in 1979 from that of 1977 in North, West, and South Gezira, but in Central Gezira there was a significant rise from 27% to 75%, indicating the presence of a high level of transmission. This coincided with malathion resistance of the vector in this area.

Eggs from females of Anopheles arabiensis, the main malaria vector there, which had survived field tests on malathion were brought to London and reared for further testing and genetic studies. A population which survived exposure to 5% malathion for 5 hours was selected. Crossing and backcrossing of this population with three different malathion-susceptible populations of A. arabiensis showed an involvement of one gene in the resistance, which appeared to be of dominant nature. Tests with Fenitrothion, Fenthion, and Propoxur gave total kills, showing no signs of cross-resistance. There was no indication of resistance to Malathion or to Temephos (Abate) at the larval stages.

The spread of drug resistant human malaria parasites is a possible danger for the future of malaria control in Africa. A micro in vitro test for drug resistance was developed using rodent and primate malaria parasites as models, as well as blood from human malaria cases in London. Radioactive chloroquine was used in a special study to estimate the adsorption of low concentrations of the drug to plastic and glass vials which might affect the results of the micro test. Approximately 20% loss was observed. A limited field trial of the micro-test in the Sennar Area (Blue Nile Province) showed no clear indication of drug resistance at the present time. These results confirmed those of in vivo tests done at the same time.
ACKNOWLEDGEMENTS

I would like to express my thanks and gratitude to my supervisors, Dr C.C. Draper and Professor G. Davidson for introducing me to some of the new and modern techniques used in malaria study which will be of great help to me in my future work for controlling malaria in my country. Their kind advice and constant encouragement gave me great faith in myself and the studies I was conducting.

I am greatly indebted to the British Council for granting me a fellowship, and to the Ministry of Health, Sudan Government, for giving me a leave of absence which enabled me to pursue this study.

Thanks are also due to all who provided me with the malaria parasites, without which most of this work could not have been done.

The statistical help and guidance rendered to me by Professor M. Healey and Mr T. Marshall of the Medical Statistics Department and Dr C.F. Curtis of the Ross Institute of the London School of Hygiene and Tropical Medicine are appreciated.

A Sincere thank you to Mrs R. Sawyer and the staff of the Ross Insectary, and to the staff of the animal house at Winches farm for their kind help with the keeping of the mosquitoes and animals which were used for the study.

Finally my special thanks to my wife Nalila whose encouragement, patience and assistance proved invaluable to the completion of this study.
OBJECTIVES OF THE STUDY

Malaria is endemic in the Sudan. It is considered number one health problem which causes the highest incidence of morbidity and mortality especially among infants and young children. All the country is under a very limited haphazard malaria control operation except in a few limited areas where proper control measures are taking place. The weapons which are used to control malaria are vector control by residual house spraying with insecticides for adult population and limited larviciding for larval control, besides the administration of antimalarial drugs to sick individuals and vulnerable groups of the population.

The only diagnostic facility available is the identification of the parasite by simple microscopy which requires a good stain and expert technicians to give reliable results.

Evaluation of the control measures in the country depends on parasitological findings obtained from the results of slide examinations.

Serological tests which are now widely used to detect malaria antibodies in populations and to evaluate the effect of eradication and control measures in malarious areas can be used in the Sudan for such purposes.

Part of this study was set out to review
existing immunodiagnostic tests, and to apply the indirect fluorescent antibody test (IFA) to study the incidence and prevalence of malaria in the Gezira Irrigated Area of the Sudan and to evaluate seroepidemiologically the effect of control measures on the malaria transmission in the area.

The alarming spread of chloroquine-resistant strains of *Plasmodium falciparum*, the most important tropical malaria parasite and the dominant one in the Sudan, in South East Asia and in the Americas, necessitates the investigation of the sensitivity of the local strains of *P. falciparum* to chloroquine in the African continent. To facilitate this on a large scale, part of this study was devoted to review the topic and to develop a simple micro-technique for *in vitro* drug sensitivity testing using rodent and primate malaria parasites as models, and to evaluate the use of such tests in the field.

The last part of the study was concerned with determining the susceptibility level of the local malaria vector (*Anopheles arabiensis*) to the insecticides used in the country and to other insecticides which might be used as alternatives after the appearance of resistant individuals to malathion after the fourth round of its application in the Gezira area.
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CHAPTER ONE

INTRODUCTION

THE DEMOCRATIC REPUBLIC OF THE SUDAN
CHAPTER ONE

INTRODUCTION

THE DEMOCRATIC REPUBLIC OF THE SUDAN
1. INTRODUCTION

THE COUNTRY

1.1 Location and Area

The Democratic Republic of the Sudan, lies in the eastern part of the African continent, separated from the Mediterranean at the north by the Arab Republic of Egypt. The country occupies the area between $3^\circ$ - $23^\circ$ north latitude and $22^\circ$ - $39^\circ$ east longitude. It shares frontiers with eight African nations: Egypt, Libya, Chad, The Central African Republic, Zaire, Uganda, Kenya and Ethiopia. The River Nile runs more than 4,000 miles from south to north. The Red Sea runs on most part of the north eastern side. Boundaries are natural or straight lines drawn by the colonial powers of the past, but to the nomadic population and their animals no such boundaries exist (Figures 1 and 2).

The country is of an area of approximately one million square miles (2,506,000 square kilometres). This area equals 1.7% of the world's land surface, 8.3% of the African continent, and ten times the size of the United Kingdom, so the Sudan is the largest country in Africa and the tenth largest in the world (Sudan Today, 1971).
Fig. 1.
Location of the Sudan in the African continent
Figure 2.
The Democratic Republic of the Sudan
1.2 Population and Demography

Estimated population of the country based on the results of the preliminary census of 1973 showed a figure close to 15 million (14,902,894). Out of this number 71% are rural population, 18.1% urban and 10.9% nomads. Looking to the population distribution by Provinces we find that 29.4% of the country's total population live in the western provinces of Kordofan and Darfur, 12.5% in Gezira province, 7.8% in Khartoum and the remaining 50.3% were distributed in the other 8 provinces (Table 1). Nomads were spread all over the country with the largest numbers in the western part.

The population ratio between the two sexes is almost 50:50. Distributing the population by age groups we find that 33.1% (almost one third of the total population) falls in the age group of 0 - 9, while those at the age between 10 - 19 years comprises 22.5%, those between 20 - 40 years are 27.3% and the remaining 17.1% falls in the age of 40 years and above. Khartoum province is the highly inhabited of all with 55.6 inhabitants per square kilometre of land, followed by the Blue Nile 28 inhabitants per square kilometre of land. All other provinces vary in their population densities between 3.2 and 6.5 inhabitants per square kilometre of land (Department of Statistics, 1975). If the total population of the country is divided equally over the land, the density on average will be 6.9 inhabitants per square kilometre of land. From the 1973 census
Table 1. Distribution of the Total Population by Province in 1973

<table>
<thead>
<tr>
<th>Province</th>
<th>Population</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khartoum</td>
<td>1,168,169</td>
<td>7.8</td>
</tr>
<tr>
<td>Blue Nile</td>
<td>969,474</td>
<td>6.5</td>
</tr>
<tr>
<td>White Nile</td>
<td>978,018</td>
<td>6.6</td>
</tr>
<tr>
<td>Gezira</td>
<td>1,865,499</td>
<td>12.5</td>
</tr>
<tr>
<td>Northern</td>
<td>998,883</td>
<td>6.7</td>
</tr>
<tr>
<td>Red Sea</td>
<td>465,043</td>
<td>3.1</td>
</tr>
<tr>
<td>Kassala</td>
<td>1,123,387</td>
<td>7.5</td>
</tr>
<tr>
<td>Kordofan</td>
<td>2,202,346</td>
<td>14.8</td>
</tr>
<tr>
<td>Darfur</td>
<td>2,181,161</td>
<td>14.6</td>
</tr>
<tr>
<td>Bahr El Ghazal</td>
<td>1,396,913</td>
<td>9.4</td>
</tr>
<tr>
<td>Upper Nile</td>
<td>798,251</td>
<td>5.4</td>
</tr>
<tr>
<td>Equatoria</td>
<td>755,750</td>
<td>5.1</td>
</tr>
<tr>
<td>Total</td>
<td>14,902,894</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Source: Department of Statistics, 1975.

Estimates based on Preliminary 1973 Census results.
it was revealed that the size of households in the urban areas is greater than that of the rural areas. Thus, in urban areas, the average household size ranges from 4.7 in the Red Sea province to 6.7 in Upper Nile province. In rural areas, it ranges from 2.6 in the Red Sea to 6.3 in Bahr El Ghazal. Among nomads it varies from 5.3 in Gezira (Blue and White Niles), to 7.5 in Kordofan. The reasons for the urban/rural/nomadic differences in average household sizes are probably due to social and economic factors. The fertility/mortality differentials between urban and rural areas can be one factor. The crude birth rate in the whole country is estimated to be 49 per 1000 population, while the crude death rate is 24 per 1000 population. From those two rates (birth and death) the crude rate of natural increase was calculated as 2.5%. The infant mortality rate is 140 per 1000 live births. The illiteracy in the country amounts to 82.9% of the total population, two thirds of this number are females.

1.3 Geography and Climate

The Sudan is a flat land consisting of vast clay plains, rolling stabilised dune land or desert. Hills lie at the extremities. Except for populated areas along the Nile, the remainder of northern and north-western parts are mainly desert. The central and north eastern parts are semi-desert. Between the two Niles (White and
Blue), and in a belt extending from east to west between north latitudes $10^\circ$ - $15^\circ$ you find the savannah belt. In the south and south-west parts is an ironstone plateau which merges gradually with the high lands of Ethiopia, Kenya and Uganda. This region is characterized by broad leaved thick forest intersected by rivers, streams and swamps (Barbour, 1961).

The climate ranges from humid tropical with long rainy season in the south, to semi-humid with seasonal rains during June to September in the centre, to desertic dry hot for the greater part of the year in northern parts of the country. Natural vegetation is governed by the amount of rainfall and soil. Temperatures range between a maximum record of $52.5\degree C (126.5\degree F)$ in the summer to a minimum of $-2\degree C$ in winter. The dry summer season extends from February to June, then gives way to the hot humid rainy season between June and September. The winter starts in October and extends to January, with the lowest temperatures during December. Rainfall varies between nil or traces in the north to 1,500 mm in the south (Meteorological Department, 1976).

1.4 Agriculture and Livestock

Out of the vast one million square miles area of the Sudan, 300 million acres are suitable for grazing, a further 100 million acres are arable and 100 million
more are of potential agricultural value. About 11 million acres of the arable land are already under cultivation. Different means and methods of irrigation are used to cultivate the land. These vary from water lifting using simple systems like shaduf and sagia for small pieces of land, to diesel or electrically operated pumps for large pump schemes along the banks of the Nile in the north, and both Blue and White Niles south of Khartoum. Flush irrigation is practised in the deltas of Gash and Baraka torrential Rivers. Gravity irrigation started by constructing the Sennar Dam on the Blue Nile in the year 1925. Water from the dam is used to irrigate Gezira/Managil scheme through canalization. Kashm El Girba Dam which was constructed in 1963 on the Atbara River irrigates an area of 480,000 acres. Natural floods and rains were also used for cultivation. The Roseires Dam on the Blue Nile is ready to irrigate 3 million acres either directly by gravity or by pumping from the reservoir. Cotton, wheat, dura (sorghum), ground nut, sesem, rice, sugar cane and kenaf are cultivated. Forests, fruit gardens are grown all over the country. Gum is found in the western part of the country (Figure 3).

No accurate animal census has ever been carried out in the Sudan, but it is generally believed on the basis of estimates that there are about 12 million cattle, 10 million sheep, 3 million camels and 8 million goats. The majority of these animals depend on shifting grazing for their food.
Fig. 3
Main agricultural regions of the Sudan.

MAIN AGRICULTURAL REGIONS
OF
SUDAN
1.5 Communications

Railways had begun in Sudan since 1873. The first line was only 54 kilometres long from Wadi Halfa in northern Sudan to Saras. This line started progressing southwards until it reached Khartoum in December 1899. Now there is a large network of railway connecting most large towns. The total length of this network is well over 5,000 kilometres.

Air transport started as early as the beginning of the twentieth century. Now Sudan Airways have 4 Boeings (2 of them 707 and two 737) and 12 F27s (Fokker Friendships). There is an international airport at Khartoum, a smaller one at Port Sudan and at Juba and about 21 domestic airports.

In a vast country like Sudan roads are very important. All seasons roads are only in the western part of the country, and in the north along with the railway. The only hard-top tarmac road is the one linking Khartoum to Wad Medani. By the end of this year, 1979, this will reach Kosti passing by Sennar, and also will reach Port Sudan passing through Rahad, Gedaref, Khashmel-Girba, Kassala and Haiya. Also the road to Shendi is nearly finished.

There are all seasons river routes on the Nile between Merowe and Dongola, and on the White Nile between Khartoum, Kosti, Malakal and Juba. Seasonal routes serve on tributaries of the White Nile between towns in the south.
1.6 Health Data

1.6.1 10 Common reported diseases

Disease resulting from poor environmental sanitation constitutes the bulk of the health problems of the Sudan.

The 10 most common reported diseases in order of their proportion to all reported cases are: malaria, dysentery, malnutrition and anaemia, respiratory tract infection, gastroenteritis of children, communicable eye disorders, T.B., heart diseases including hypertension, bilharzia, and cerebro-spinal meningitis. Measles, hepatitis and accidents which are known to be grossly under-reported, should be considered also as major health problems (National Health Programme, 1975) (Table 2).

1.6.2 Health facilities

- Hospitals (171) 1.1/100,000 population
- Health centres (144) 1/100,000 population
- Dispensaries (634) 4/100,000 population
- Dressing stations (1,505) 10/100,000 population
- Hospital beds (15,670) 104/100,000 population

Hospital beds ratio to population is very low compared to what exists in other countries. Furthermore, hospital beds are not evenly distributed throughout the
Table 2. Hospitalized Cases of 10 Common Diseases and Case Fatality Rates For the Whole Country 1976

<table>
<thead>
<tr>
<th>Diseases</th>
<th>TOTAL</th>
<th>GRAND TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Malnutrition and Anaemia</td>
<td>12,920</td>
<td>14,011</td>
</tr>
<tr>
<td>Respiratory and Measles</td>
<td>9,310</td>
<td>9,042</td>
</tr>
<tr>
<td>Gastro-enteritis</td>
<td>9,258</td>
<td>11,095</td>
</tr>
<tr>
<td>Heart and circulatory</td>
<td>13,857</td>
<td>4,856</td>
</tr>
<tr>
<td>Infectious hepatitis</td>
<td>1,457</td>
<td>723</td>
</tr>
<tr>
<td>Cancer</td>
<td>1,321</td>
<td>1,358</td>
</tr>
<tr>
<td>T.B.</td>
<td>8,447</td>
<td>3,553</td>
</tr>
<tr>
<td>Malaria</td>
<td>10,906</td>
<td>9,150</td>
</tr>
<tr>
<td>C.S.M.</td>
<td>3,899</td>
<td>1,716</td>
</tr>
<tr>
<td>Accidents</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Source: Department of Statistics, Ministry of Health, Khartoum.
country as can be shown by the following figures regrouping the provinces:

<table>
<thead>
<tr>
<th>Province</th>
<th>Population per 100,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kassala and Northern</td>
<td>89/100,000</td>
</tr>
<tr>
<td>Khartoum, Central and Blue Nile</td>
<td>131/100,000</td>
</tr>
<tr>
<td>Western Provinces</td>
<td>56/100,000</td>
</tr>
<tr>
<td>Southern Provinces</td>
<td>111/100,000</td>
</tr>
</tbody>
</table>

The total number of doctors in the country is 1287. If we exclude those attached to the Medical Headquarters (18), the Public Health (30) and Research (14), the ratio of doctors to the total population will be 8/100,000 population. The distribution of doctors in general is quantitatively and qualitatively very uneven, with 50% of all generalists and specialists concentrated in the two provinces of Khartoum and Gezira. The total number of medical assistants in the country is 1654 which gives a ratio of 11/100,000 population. There is a total of 514 technicians, 232 Public Health Officers, 271 Sanitary Overseers, 11,120 Nurses and 4,438 Village Midwives (National Health Programme, 1975).

1.7 History of Malaria and Malaria Control in The Sudan

1.7.1 History

Malaria was well-known in the country from very early days, even before naming it. Our ancestors used to give it many names. Some pertain to the season
of the year during which the disease appeared, "Wirdat El Karif" referring to the rainy season; to the symptoms manifested, "El Wirda Um Bard" meaning the fever with rigors; and to the periodicity of the fever, "El Wirda El Ghibbiya and El Wirda El Tiltia" referring to two days cycle (Quaterdian fever) and three days cycle (Quartan fever) (Abd El Halim, 1939).

Visitors since the 17th century reported the presence of malaria fevers in the country. James Bruce (1765 - 1775) who visited Abyssinia, the Sudan and Egypt recorded the presence of a disease in Sennar which was associated with intermittent fevers and particularly fatal during the rainy season; he emphasized that the disease is a form of malignant tertian-malaria (Recited by Bloss, 1941). Burckhardt (1819) mentioned a disease which he met with in Berber and Shendi in 1813. He wrote, "The people of Berber were on the whole a healthy race, probably due to the situation of the town on the edge of the desert. When the Nile was in flood, a fever called Wirda occurs among the people and occasionally became epidemic. It did not occur every year, but when it did there was a high mortality rate among those afflicted. This was almost certainly malaria."

Wadington (1820) wrote, "At Merrowe the army was by no means healthy, there were many sick of dysentries and intermittent fever." Of Dongla he wrote, "Some marshy ground exists which accounts for an intermittent fever
which generally prevails here after the inundation has
terminated. We found in consequence many of the inhabitants
and all the soldiers ill." Reculus (1885) wrote of
the area below Atbara River: "The inundations of the Nile
stretch far into the plains leaving stagnant pools here
and there, malignant fevers are very common and frequently
terminate fatally ..."

After the battle of Omdurman and the defeat
of the national army in 1898, the foreign troops did not
find an easy life in the Sudan. The intermittent fever
was the scourge of the troops. The following are extracts
from the reports of O/Cs of the army: "The closing months
of the year, which opened so auspiciously, saw, however,
the beginning of the struggle with an enemy even more dan­
gerous than the Dervish army and which found the medical
corps sadly unprepared." (Sudan Notes and Records, 1943).

Bimbasha Jennings reported that, "On November
22nd 1898 out of the garrison of Karkog with a strength
of 488 officers and men, 376 were on the sick list with
fever and 112 were fit for duty, while there were 213
cavalry horses, 363 camels and 8 mules to be attended to"
(Sudan Notes and Records, 1943).

In 1899 the report of the O/C reads as follows:
"The year was also marked by the expedition which resulted
in the death of the Khalifa and destruction or capture
of his entire army. Malaria fever was again a very
troublesome feature during the year. The mosquito is now
recognized as the causative agent and quinine in prophylactic doses has been used and yet, though the garrisons on the White and Blue Niles were reduced during the rains, there were 3490 admissions, 50 deaths and 42 invalids from malaria alone, not with-standing that the year was an exceptionally dry one."

The medical officer of health makes various recommendations relative to the measures of housing and drainage necessary to combat the disease. He recommended that flannel shirts should be used by the troops during the rainy season and strongly urges that in bad malarious stations each man should have his own mosquito curtain (Sudan Notes and Records, 1943).

1.7.2 The epidemiology of malaria in Sudan

Knowledge of the basic epidemiology of malaria in various localities of some provinces of the country is still lacking. From that available it can be concluded that malaria is endemic all over the country except in scattered limited areas in some parts of the Red Sea Province and the desert of the north western part.

1.7.3 Vectors, distribution and endemicity of malaria

a. Vectors

The chief malaria vector is Anopheles arabiensis (member of the A. gambiae group and used to be called
species B) throughout the country. The degree of malaria endemicity is mostly dependent on the amount of rainfall which offers the ideal places for the breeding of such a vector, and on the associated rise in the relative humidity, which offers a chance for its survival until it reaches the infective stage. The transition of the malaria endemicity from the hypoendemic situation in the north arid regions to the holoendemic situation in the tropical forests in the south, follows the characteristic transitional vegetation belts, if one takes a monthly average of relative humidity of 40 per cent to allow for certain appropriate microclimatic conditions (normally it should be 60 per cent) as the dominant factor in precipitating intensive malaria transmission.

*P. falciparum* infections represent over 90 per cent of all malaria infections, and hence the high mortality rate associated with it in the younger age groups of 0 - 5 years; the incidence among this group normally attains 90 - 95 per cent in the Sudan-savannah type of malarious areas with four or more months of intensive malaria transmission. Above this age, gradual immunity is developed, but the adult population will still be vulnerable to overt attacks of malaria particularly in the hyperendemic areas, though they are spared the pernicious forms of the disease. The pregnant mothers, however, suffer from severe anaemia. In the extreme south, where the transmission extends over eight months or more, the adult population achieves more solid immunity to the disease.
The high vectorial capacity of the main vector A. arabiensis, by reason of its preference to bite human beings, its prolific breeding in rain puddles and irrigation ditches around villages, its resting habits inside houses and its great susceptibility to malaria infections (as high as 30 per cent), is not matched by any other malaria vector in the world. A. pharoensis the main vector in Egypt (co-existing with A. arabiensis in Sudan, particularly in the irrigated and swampy areas) is a very poor malaria vector with a predilection to bite animals and rest outdoors. In the presence of A. arabiensis it can be ignored as a malaria vector in Sudan. The same can be said of A. nili in the southern regions. A. funestus, however, co-existing with A. arabiensis in the extreme south, must be playing a role in malaria transmission, particularly during the drier months of the year, and thus contributing to perennial intensive malaria transmission and holoendemicity (Figure 4).

b. Distribution and endemicity of malaria

The following is a brief summary of the distribution of the disease as known at present:

- Hyperendemic with holoendemic spots in the Southern Provinces. The transmission is perennial here.

- Mesoendemic with hyperendemic spots in the provinces of Blue Nile, Gezira, White Nile, Kassala, North and South Kordofan, and North and South Darfur.
Fig. 4

PROVINCEWISE ANOPHELINE DISTRIBUTION IN THE SUDAN

- A.arabiensis
- A.dathali
- A.rufipes
- A.pharoensis
- A.funestus
irrigated areas of the Blue Nile Province, Gezira, and White Nile Provinces the main transmission season starts shortly after the onset of rains in July until one month after the end of the rainy season in September. However, a favourable microclimate due to irrigation may support low transmission all the year round. In the centre of the savannah belt, in non-irrigated and non-riverian parts of northern fringe of savannah, the main transmission season coincides with rain.

- Hypoendemic with post-epidemic mesoendemic spots in the riverian and agricultural areas of the Northern Provinces. The main features determining malaria transmission here are the level of the Nile and the agricultural activities.

There are several agricultural schemes and major irrigation dams schemes in the country. In these schemes malaria transmission is high. Man-made malaria referring to the malarial situation produced and aggravated by agricultural practices, related mainly to irrigated areas. In recent years development of irrigated agriculture has progressed well in the country. The irrigated acreage under cultivation has been expanded on an average of 5 - 6% per annum. This growth has been accompanied by a rapid upsurge in the prevalence and incidence of man-made malaria. The problem is economic and social as well as medical in nature as it threatens the future economic growth of the country.
Due to ecological changes brought about by irrigated agricultural activity and the absence of traditional control measures, malaria changes from low meso-endemicity through hyper-endemicity to holo-endemicity. This is taking place in all irrigated areas of the Sudan.

Urban malaria is not well studied in the country. In several towns, A. arabiensis is breeding and the disease is bound to prevail (Figures 5, 6 and 7).

1.8 Malaria Control

Malaria control in the Sudan started as early as 1905 when by a Decree from the Governor General a Central Sanitary Board (C.S.B.) was formed. The C.S.B. was concerned with all matters concerning the general medical and sanitary policy as well as all important questions of public health such as the outbreak of an epidemic. Naturally, therefore, the C.S.B. was concerned with the situation of malaria in the country. Most old records refer to intensified anti-malaria work round Khartoum only. As to other parts of the country, the C.S.B. laid down rules and regulations for the prevention of malaria in irrigation schemes. Three important schemes which were the foci of malaria outbreaks, were the Gezira, the Basin irrigation in Dongola Province, and the Zeidab Agricultural Scheme. The C.S.B. issued in March 1914 its sanitary rules for canals and water courses. Malaria
Fig. 5.

Epidemiological assessment of status of Malaria in Africa
December 1977

Areas in which Malaria has disappeared, been eradicated, or never existed

Areas with limited risk

Areas where Malaria transmission occurs or might occur

from: WHO Weekly Epidemiological Record No. 22, 1979
Fig. 6

MALARIA TRANSMISSION IN THE SUDAN

Areas Free Of Transmission
Areas With Seasonal Transmission
Areas With Perennial Transmission
MALARIA ENDEMICITY IN SUDAN

Apparently Malaria Free
Hypoendemic Malaria With Mesoendemic Spots
Mesoendemic Malaria With Hyperendemic Spots
Hyper or Holo Malaria

(Based on pre-eradication surveys - 1961/63)
figures and reports were sent to the C.S.B. by M.O.H.s of the Provinces.

1.9 World Health Organization Assisted Projects

After independence the Government of Sudan decided in 1956 to study with WHO assistance the possibility of undertaking a malaria eradication programme. Consequently, a Pilot Malaria Eradication Demonstration Project in Blue Nile Province (Sennar) was started in the fall of 1956 with headquarters in Sennar. The aim was to demonstrate the feasibility of eradication of malaria by a residual insecticide. DDT 2 gm/m² was used as residual insecticide for spraying houses. By the end of 1960 there was not a single case of malaria in the area, so the project was terminated in January 1961. A new project, Malaria Eradication Survey Project, with headquarters at the Ministry of Health, Khartoum was started in February 1961. Its aim was to collect epidemiological and other data necessary for planning a country-wide malaria eradication programme. During the survey, valuable information was collected giving for the first time full knowledge about the epidemiology of malaria in all the provinces of the country. Since on the completion of the pre-eradication Survey Project financial, personnel and administrative conditions did not permit the launching of a malaria eradication programme, WHO
recommended the initiation of a Malaria Pre-eradication Programme. The Government of Sudan accepted the recommendations and a plan of operation was signed by the Government and WHO in June 1963. The plan envisaged to achieve the following main objectives:

- Development of a fully functioning technical and administrative machinery of a National Malaria Eradication Service;
- Expansion of training facilities for all categories of staff to be employed in the National Malaria Eradication Service;
- Development of rural health services (basic health services) to ensure adequate support for future malaria eradication in all its phases.

The review in late 1969 showed that though the malaria pre-eradication programme made some progress, yet technical, operational and administrative difficulties were yet to be overcome prior to the launching of a successful malaria eradication programme. Therefore it was not possible to fix a time limit for embarking on a malaria eradication programme.

In August 1970, following the recommendations of the World Health Organization Assembly on the change in the global strategy for malaria eradication, the Government of Sudan decided to discontinue the malaria pre-eradication programme in favour of a country-wide Malaria Control Programme. The decision was also supported by the fact that the eight countries bordering the
Sudan with endemic malaria have no anti-malaria programmes. The staff (national and international) of the malaria pre-eradication programme was absorbed in the newly created malaria division with headquarters in the Ministry of Health (Khartoum) with the responsibility of overall planning, technical supervision and assessment of malaria control activities in all provinces of the country. Provincial malaria divisions started in Gezira Province, Blue Nile Province, Khartoum and Kassala Province. Because of the high endemicity of malaria in Gezira, and because of its economic importance it was given first priority in applying intensive control measures. More effort was put on the control in Khartoum Province in Blue Nile and Kassala, the old staff belonging to the pre-eradication programme started the nucleus of the control units for those two provinces. Other provinces are still carrying on their haphazard irregular old style control which they were carrying on for almost the last 20 years under the supervision of the Public Health staff of the province and its councils.

The Malaria Training Centre in Sennar

This centre was established in 1963 with assistance from WHO, with the following objectives in its plan of operation:

- To train special categories of sub-professional
personnel of the public health and rural health services of the Sudan, such as public health inspectors and officers, sanitary overseers, medical assistants and laboratory technicians, in the methods of malaria eradication, in order to prepare them for their future role in the malaria eradication programme of the country.

Also to train special staff categories, such as entomological technicians and malaria microscopists, according to the requirements for the efficient running of the malaria eradication programme. To arrange in conjunction with the Ministry of Health authorities for seminars on malaria eradication, for the senior medical staff in charge of public health services in the provinces and districts.

- To co-ordinate the training with the expansion of malaria eradication activities in the country so as to be able to meet the requirements of the latter in trained personnel of different categories.

Since the first course in October 1963, emphasis was placed on training in malaria eradication techniques. Owing to the decision of the Government in August 1970 to reorganize the countrywide malaria control programme and postpone indefinitely the launching of the malaria eradication programme, the curricula of all succeeding courses were revised and more emphasis was given to malaria control techniques. The capacity of the training centre is just enough to conduct four regular courses per year with a maximum of twenty-four trainees in each course.
1.10 The Study Areas

a. Sennar

Sennar in Blue Nile Province was chosen as a study area for testing the sensitivity of *Plasmodium falciparum* to chloroquine. It is one of the small towns of the country, 176 miles south of Khartoum. It was the headquarters of the pilot project for studying the feasibility of malaria eradication in the country in 1956-1961 and the training centre for malaria control which started in 1963 is still functioning. The Blue Nile passes by and there is the giant Sennar Dam from which runs out the two major canals which irrigate the Gezira/Mangil scheme. It is connected to Khartoum by road and railways. There is a large hospital with more than two hundred beds. At the end of the pilot project, a small area some seventy kilometres long to the north and south of Sennar Town and bordering both banks of the Blue Nile River (a part of the former Pilot Malaria Eradication Demonstration Project area) was kept as a Malaria Eradication Demonstration and Training Operation area. This area covered 131 villages with a population of 83,000. In this area a miniature scale Malaria Eradication Programme was in operation. The activities included geographical reconnaissance, house spraying with residual insecticide, surveillance operations, routine entomological observations and epidemiological investigation. In
mid-1969 a review of the activities in this area revealed that the results achieved during the Pilot Malaria Eradication Demonstration Project could not be maintained and a low grade of transmission of acceptable level continued (2.4% to 4.3% positivity rate). The failure to interrupt transmission was attributed to the small size of the area and the influx of labourers and nomadic population. It was recommended that a buffer area of residual spraying at least fifteen miles deep should be created. In 1973 it was decided by the Headquarters of Malaria Control to hand over the MEDTO area to the health authorities of the council so as to form the nucleus for reorganization of the malaria control programme for the whole province. Since it became known to the field staff that the malaria pre-eradication programme has been abandoned and MEDTO area, Sennar, will be handed over to the health authorities, they became less enthusiastic and most of them applied to be transferred to general public health work. Since then no regular spraying, nor surveillance were conducted and malaria came back to the non-immune population of the area in an epidemic form (Figure 8).

b. Gezira irrigated area

Gezira irrigated area was chosen for the malaria serological studies and the entomological studies. This area, being of a major economic importance, was
Fig. 8.

Sennar Council - SUDAN

SCALE: 1:100,000

KEY

- Localities where malaria cases came from
- Railway
- Sector limits
selected by the Malaria Control Division of the Ministry of Health to be the first area to have a reorganized malaria control programme.

The area lying between the White Nile and the Blue Nile falls within 180 kilometres south of Khartoum and connected to it by a railway line which passes by Wad Medani south to Sennar. Also there is a tarmac road on which work is going on now to reach Sennar. In the Gezira there is the largest irrigated agricultural scheme not only in the Sudan, but in the whole continent of Africa. The cultivated area comprising two million acres* of land (12% of the total area cultivated in the Sudan) is irrigated by gravity from two main canals running northwards from Sennar Dam in the south. Cotton is the main cash crop grown there (75% extra long stable)** while wheat (50%);* ground nuts (15%), durra (sorghum) (12%)**. Vegetables are grown in large quantities (Sudan Gezira Board, 1971). Forests and fruit gardens are scattered all over the area, and rice cultivation was recently introduced. The resident population of the area is approximately 1.5 million, but thousands (nearly half a million) of immigrant labourers enter the area during the cotton sowing and harvesting seasons. The area is divided administratively into seven rural councils. Malaria control, mainly through mosquito control activities (house-

* 1.038 acres = 1 feddan = 0.42 hectares.
** Percentages out of the total quantity grown in the whole country.
spraying initially with BHC and later with DDT plus larviciding with oil) have been going on in the area over a number of years. Moreover, since 1957, agricultural aerial spraying with various chlorinated hydrocarbons and organophosphorus insecticides have been conducted. The whole area can be considered as one huge permanent breeding place. Breeding takes place all over the Gezira, in canals, irrigation ditches, drains, in seepages, in irrigation fields, in forests, in fruit gardens and in rainwater collections inside and around villages, producing not only the malaria vector *A. arabiensis*, but also other vigorously biting non-vector mosquitoes like *A. pharoensis* and *Aedes caspius*.

After the results of the susceptibility tests to insecticides which were conducted in the area in 1970, and which revealed that *A. arabiensis* had developed double resistance to the chlorinated hydrocarbons, dieldrin and DDT, house spraying was completely stopped. Due to the stoppage of spraying and the poor quality of larviciding malaria incidence nearly reached an epidemic level. The main transmission season spread from July to November, but due to favourable microclimatic conditions created by all year round irrigation, transmission can be considered as perennial. *P. falcinarum* is the predominant species of malaria parasite and accounts for over 90 per cent of the malaria cases. The activities of malaria control which were carried out by the Provincial
and Rural Councils public health staff were undertaken by the Malaria Control Division of the Ministry of Health in September 1970. The plan was to re-organize the control on scientific basis and to create a provincial malaria control unit to shoulder the responsibility after the method being streamlined by the expert staff of the Malaria Control Division of the Ministry of Health, Khartoum. The first task carried out by the staff of the Malaria Control Division after withdrawing house spraying and stopping oil larviciding, was to train the available larviciders and personnel in the area and to start a proper geographical reconnaissance to mark the breeding places and divide in reasonable working areas for mosquito men and larviciders. The use of an alternative residual insecticide had not been considered at this stage. Due to shortage of oil the anti-larval measures were suspended early in 1973. The only remaining measures for malaria control were larviciding as a trial using Abate in El Hosh rural council, light engineering methods, distribution of Gambusia fish in a few canals in Medina Arab rural council and Hasheisa rural council and individual case treatment provided by the health establishments of the Province. The deterioration of the situation has been further aggravated by an increasing number of seepage pools along irrigation canals, by the cessation of canal clearing (removal of vegetation), and by the introduction of rice cultivation which requires
continuous irrigation. The interaction of the above-mentioned factors has led to a significant rise of the malaria prevalence in the population of the Gezira Irrigated Area. A comparison of the parasite rates in 16 localities assessed in December 1961/January 1962 (during the pre-eradication survey) and in January 1975 reveals that the average parasite rate in children (2 - 9 years) has risen from 2.9% to 19.7%. It reveals that while 9 of the 16 localities surveyed in 1961/62 had zero parasite rates, none of the 16 localities was free from malaria in 1975 with the highest parasite rate of 59% in Sharafat Falata.

In 1971 and 1972 malaria was still largely a rural endemic disease in the Gezira Irrigated Area and in the Managil Extension. In 1973 and 1974 also the Provincial capital Wad Medani became subject to extensive malaria transmission. In 1974 the hospital of Gezira Province has reported 115,813 malaria cases as compared to 76,074 in 1973. Most of these cases were confirmed by blood examination. Apart from causing a high morbidity and a relatively high mortality, also among adults, malaria is responsible for much human suffering and for considerable losses in terms of manpower in an area which is the country's centre of socio-economic development. Therefore it was decided by the Government represented by the Malaria Control Division to implement an emergency programme for malaria control. A plan of action covering
the period 1st March 1975 to 30th June 1976 was drafted. The strategy of the plan of malaria control in the 1975/76 main transmission season was to aim at a rapid reduction of both malaria reservoirs and the transmitting potential vector in order to prevent malaria epidemics and to create a basis for future, continuous malaria control activities. The rapid reduction of malaria reservoirs was to be achieved by a mass single dose administration of chloroquine and pyrimethamine to all of the rural population and the population resident in the perimeter area of Wad Medani, exempting infants of 0 to 11 months of age. This mass drug administration was to coincide with the first round of residual spraying with malathion.

The first round of spraying using malathion 50% w.d.p. at a dose of 2 gram technical/m² was applied during the period June/July 1975; with the spraying mass drug administration was carried out as planned. A second round of spraying was applied in the same year during September/October 1975. The third round of spraying was conducted in the period June/July 1976, and from 1976 onwards one round of spraying was applied each year during the period June/July. Epidemiological evaluation after the second round of 1976 in the 16 villages from which base line data were collected show a dramatic drop in the parasite rate between children of 2 - 9 years of age from 19.7% before spraying to 3.5% after spraying and drug
distribution. Larviciding using Abate 500E in a dosage of 5 microlitres/m², applied by means of Hudson X-pert spray pumps fitted with cone-jet nozzle 5500 was carried out during the period November 1975/May 1976, and on the same period each year. Entomological surveys failed to reveal any adult mosquitoes in the area after the second round of malathion spraying.

Epidemiological evaluation using parasitological data from children 2 - 9 years of age survey results from 27 villages including the usual 16 indicator localities gave a parasite rate of 1.98% in January 1977 (6 months after the third round of spraying). In August of the same year after the fourth round of spraying the parasite rate of children 2 - 9 years of age dropped to 0.1%. (Table 9)

In June 1977, just before applying the fourth round of spraying, a high density of A. arabiensis was revealed in one locality called Barakat Falata south of Wad Medani town. This finding of resting A. arabiensis in sprayed huts inside the sprayed area, while all other localities gave zero results, alerted the entomologist on the project to look for signs of resistance to malathion. Tests conducted during the period September-October-November 1977 gave the first indication of A. arabiensis developing resistance to malathion. Adult mosquitoes collected from Barakat locality were tested on 3.2% and 5.0% malathion for one hour exposure. The average mortality on the two concentrations was 90%. These results were consistently obtained on several replicates. In December 1977 a test on 5% malathion for one hour exposure gave a mortality result of 78.6% (Figure 9).
Fig. 9. Gezira and Mangil irrigated areas

SCALE 1:1000000

- Localities from which blood samples were collected for serology studies

SUDAN
CHAPTER TWO

SEROEPIDEMIOLOGICAL STUDIES

IN THE GEZIRA
2. SEROEPIDEMIOLOGICAL STUDIES IN THE GEZIRA

2.1 Introduction

For the past 60 years many investigators have been concerned with the detection of malarial antibodies. Much interest has centred around the development of serological techniques which would be useful for individual or mass treatment, in establishing the efficacy of eradication measures and in detecting resumption in the transmission of malaria. In areas where malaria is or has been endemic, serology may be especially useful for the following purposes:

a. For the establishment of malarial endemicity rates including species prevalence and in particular, age-specific indices;

b. To assess changes in the degree of malaria transmission; usually during or after malaria eradication or control operations;

c. To identify areas or individuals requiring action with regard to malaria, especially during the later stages of malaria control programmes.

In areas where malaria is not endemic, serology can be of use for the following purposes:

a. For case-detection and identification, in some instances, of the species of malaria parasite responsible for the infection;
b. For screening of blood donors;
c. To exclude the diagnosis of malaria in patients with pyrexia of unknown origin.

Although in recent years, especially with the availability of good sources of antigens, several promising serological techniques have been developed, none of them is completely suitable to replace the microscopical examination of blood to detect the presence of plasmodia in fresh infections. During the past 16 years many writers have reviewed the serodiagnosis of parasitic infections including malaria serology (Tobie, 1964; Miller and Brown, 1969; Kagan and Norman, 1970; Bruce-Chwatt, 1970; Voller, 1971; Denham et al., 1971; Fife, 1971; Sulzer and Wilson, 1971; Kagan, 1972; Fife, 1972; Soltys and Woo, 1972; Capron et al., 1972; Sadun, 1972; Kagan, 1974; WHO, 1974; Meuwissen, 1975; Draper, 1976; Sadun, 1976; Voller, 1976; Lobel and Kagan, 1978).

2.2 Some Serological Tests

In this introduction I will review in brief some of the serological tests used in malaria.

2.2.1 Complement fixation test (CFT)

This is the earliest serological technique used to study malaria antibodies. Thompson (1919) used
antigens prepared from cultured malaria parasites to detect malaria antibodies in the serum of human patients using the CFT. More workers tried the technique but they failed to obtain specific reactions, so their work is not worth mentioning. It was not until the late 1930s that Coggeshall et al. (1938) used antigen from Macaca mulatta monkeys with an intense infection of Plasmodium knowlesi to test sera of monkeys during the acute and chronic stages of P. knowlesi infections. Their results indicated that specific antibodies appear in the early stages of the disease and persist during the course of the subsequent chronic infection. Eaton and Coggeshall (1939) applied the CFT to sera from human beings infected with P. knowlesi, P. vivax and P. falciparum. Using P. knowlesi antigen they obtained strong CF reactions with the three species showing that the test is group-specific rather than species specific. Eaton (1939) used the test to detect soluble malarial antigens in the serum of monkeys infected with P. knowlesi. He found that the amount of antigen in the serum is related to the parasite count during the acute phase of the infection. Results of reactions obtained with human sera and serum antigen from monkeys were generally weak or doubtful. Lippincott et al. (1945) used the CFT in human malaria with an antigen prepared from the chicken parasite P. gallinaceum which gave a weak reaction. Mayer and Heidinger (1946) used antigen prepared from
blood infected with *P. falciparum* and *P. vivax* to study the CF reactions of sera from cases of chronic relapsing vivax malaria, syphilitic sera, and sera from cases of naturally acquired *P. falciparum* infections. The test proved to be of only limited value. Davis (1948) used the CFT to study the antibodies in ducks against *P. lophurae* and in monkeys against *P. knowlesi* after immunization by means of formalin-killed parasites emulsified with certain adjuvants. The *P. lophurae* antigen fixed complement with homologous sera and also with heterologous sera of monkeys immunized or infected with *P. knowlesi*. The *P. knowlesi* antigen, on the other hand, reacted similarly with the homologous monkey antisera, but did not cross-react with the heterologous duck sera. Rein et al. (1949) studied for 18 months the course of the CF reaction with plasmodial antigen in each of 87 prisoner volunteers experimentally infected with sporozoites of the St. Elizabeth strain of *P. vivax*. Positive serologic reactions were associated with all but 4 of their 199 malarial attacks. They concluded that the CFT for malaria might prove useful in identifying well developed or recently subsided vivax infections but would be of no value in establishing a diagnosis either early in an attack or during the long latent period between early and late activity. Kent and Fife (1963) described the technique for a precise CFT to be applied generally in the serodiagnosis of infectious diseases. They gave
an account of detailed quantitative methods for standardization and control of the haemolytic system. Schindler and Voller (1967) compared the results obtained by CFT and the indirect fluorescent antibody (IFA) methods in a longitudinal study of simian malaria infection. They concluded that there is a clear difference in peak titres recorded by CF and IFA and that IFA is a more sensitive test than the CF. Voller and Schindler (1967) carried out a study to determine the value of the monkey malaria parasite *P. cynomolgi bastianellii*, as antigen for comparative measurement of human malarial antibody by the IFA and CF tests. They concluded that there is no cross-reaction between human antibody provoked by *P. falciparum* infection and *P. cynomolgi bastianellii* antigen in the complement-fixation reaction. Voller (1976) in a review of serological tests, wrote that the CFT is quite selective in that it allows serological discrimination between *P. vivax* and *P. falciparum*; however, its relative insensitivity and tendency to yield false positives has meant that it has not been widely applied.

2.2.2 *Agar gel diffusion test*

Methods of immunodiffusion rely on the precipitation in agar-gel of complexes formed between diffusing soluble antigen and antibody. The method readily
permits the analysis of multiple antigen/antibody systems but is less suitable for quantitative work as reported by Mancini et al. (1965). Zuckerman and Spira (1965) were the first to report the use of agar-gel diffusion in malaria. By this test they compared different plasmodial antigens and they concluded that there were both species specific and shared antigens between different plasmodia. Zuckerman et al. (1969) using gel-diffusion to study rodent malaria reported that precipitins appear within 1 - 2 weeks following inoculation of rats with *P. berghei*. McGregor et al. (1966, 1968, 1970), McGregor and Wilson (1971) and Wilson et al. (1969) demonstrated the presence of circulating precipitating antibodies to *P. falciparum* in the sera of Gambians, West Africa, believed to be immune to malaria by gel-diffusion techniques. Children with severe *P. falciparum* infection show the presence of soluble antigens in their blood. The antigens were classified into four groups on the basis of their thermostability. Using this technique which is a modification of Ouchterlony (1948) method of double diffusion in agar-gel, it was shown that many sera of the hyper-immune population of Gambia react with antigen extracts to form multiple lines of precipitation, each of which represents the interactions of an antibody with its specific antigen. Wilson et al. (1973) demonstrated the stability and fractionation of malarial antigens from the placenta of Africans heavily infected with *P. falciparum*. 
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using the gel-diffusion technique.

This technique is mainly applied to unravel the complex antigenic structure of *P. falciparum* and the reciprocal antibody responses, but its low sensitivity limits its value in individual case detection.

2.2.3 Circumsporozoite precipitation test (CSP)

This is a test which is specific for antibodies against sporozoites (Voller, 1976). Sporozoites develop a thread-like precipitate when incubated in serum from rodents immunized with radiation attenuated sporozoites. Vanderberg *et al.* (1969) demonstrated *in vitro* CSP reaction, which they said may also occur *in vivo* in animals immunized with X-irradiated sporozoites, since there appears to be a correlation between CSP reactivity and protective immunity. They also concluded that experimental procedures that tended to produce high CSP titres also produced a higher degree of protective immunity, whereas procedures that decrease one also decreased the other. Spitalny and Nussenzweig (1972) showed that the CSP antibody titre increases after sequential inoculations by the intravenous route with mature sporozoites. Nussenzweig *et al.* (1972a, b) in their experiments with non-irradiated viable *P. berghei* sporozoites in rats, and with *P. brasilianum* and *P. simium* X-irradiated sporozoites in monkeys proved that a single intravenous
infection of 10,000 non-irradiated, viable sporozoites of \textit{P. berghei} consistently induces detectable CSP antibodies in rats. Also circumsporozoites antibodies were present in the serum of monkeys immunized with X-irradiated sporozoites of either \textit{P. brasilianum} or \textit{P. simium}. Anti-sporozoites antibodies were also detected in the serum of squirrel monkeys which had acquired their \textit{P. brasilianum} infection in nature, whereas the sera of laboratory-born control animals were consistently negative. Clyde \textit{et al.} (1973) tested for the presence of antibodies against sporozoites in volunteers immunized with X-irradiated sporozoites of \textit{P. falciparum} and then challenged by live sporozoites of \textit{P. falciparum} through mosquito bites using the CSP technique. They concluded that antibodies appear in the serum after the challenge. Spitalny and Nussenzweig (1973) studied the relationship between protective immunity and anti-sporozoite (CSP) antibody in mice. Nussenzweig \textit{et al.} (1973) gave evidence that sporozoites of human and simian malaria are immunogenic since the injection of relatively small doses of parasites into rats rapidly induced the formation of appreciable amounts of CSP antibodies. They stated that an intravenous booster given to the rats resulted in a significant increase in titre of CSP antibodies. Clyde \textit{et al.} (1973) succeeded in immunizing a human volunteer with sporozoites from X-irradiated mosquitoes infected with \textit{P. falciparum}. Anti-sporozoite
antibodies, demonstrated through the CSP reaction, developed slowly with succeeding immunization, after 11 of which a serum dilution end-point of 1:40 was attained. The volunteer was exposed at various times to non-irradiated mosquitoes carrying infective sporozoites of the same strain and of strains from different geographical areas, and did not develop malaria. The CSP gave equal reaction with all strains. Nussenzweig and Chen (1974) showed that non-irradiated sporozoites of primate and human plasmodia elicit CSP antibodies in insusceptible hosts. In further experiments Clyde et al. (1975) succeeded in immunizing man against P. falciparum and P. vivax by use of attenuated X-irradiated sporozoites. The duration of protection was reflected by a positive species-specific CSP reaction. Cochrane et al. (1976) used electron microscopy to study the antibody induced ultrastructural changes of malaria sporozoites. They found that a thick surface coat was formed as a result of binding of antibodies to the sporozoites during the CSP reaction. Nardin and Nussenzweig (1978) applied the CSP method to determine the stage and species specificity of the antibodies. The stage and species specific antigens on the surface membrane of sporozoites of malaria parasites were tested against infected red blood cells of P. berghei, P. knowlesi and P. falciparum using the CSP and IFA tests. Stage and species anti-sporeozoite antibodies were detected in the three malaria species tested. Nardin et al. (1979a) tested serum samples from 158 West Africans for antibodies specific
for *P. falciparum* using the CSP assay. Their results showed that serum samples obtained from the older groups had higher levels of CSP reactivity, while none of the samples from the children in the 5 - 9 and 10 - 15 year age groups gave positive reactions. Nardin et al. (1979b) demonstrated that young sporozoites of *P. knowlesi* from 11-day-old oocysts failed to produce a clearly positive CSP reaction, even with undiluted antiserum from immunized monkeys while 20-day-old sporozoites obtained from the salivary glands of the same group of mosquitoes gave a CSP titre of 1:64 with the same antiserum.

From the above review it can be concluded that the CSP test which is stage and species specific is unsuitable for use in seroepidemiological studies because it only detects antibodies formed against sporozoites and misses most of these antibodies in younger children even in hyperimmune populations.

2.2.4 Schizont-infected-cell agglutination test (SICA)

Eaton (1938) first demonstrated that antibodies in sera taken from monkeys infected with *P. knowlesi* show aspecific agglutinating properties which are detectable both by macroscopic and microscopic methods when reacted with *P. knowlesi* antigen. He noted that agglutinins persist in the sera of chronically infected animals for a year or longer, and that immune serum agglutinates mature
intracellular or extracellular parasites but does not agglutinate unparasitized cells or cells containing young rings or immature parasites. Also he showed that the agglutinins are species specific and that they were present in high levels in monkeys given repeated infections. Brown and Brown (1965) tested schizont-infected erythrocytes of monkeys heavily infected with *P. knowlesi* against antisera of homologous and heterologous parasites. Using this technique, which they called SICA, frequent antigenic variation has been demonstrated in blood induced *P. knowlesi* infections, which implies that it probably occurs in human malaria also. Brown *et al.* (1968) used the SICA test to distinguish among variants of one strain and to demonstrate that a chronic blood-induced *P. knowlesi* infection consists of a succession of antigenically distinct populations, each stimulating a specific agglutinin response. They concluded that the SICA test is highly specific and that each relapse is a new variant. They showed that by repeated antigenic changes of this sort the parasites may survive the host's immune response. Brown *et al.* (1970a and b) described the activity in the SICA test of sera taken at various times from monkeys, sensitized by drug-suppressed infections of *P. knowlesi*, by inoculation of dead parasitized cells in Freund's "incomplete" adjuvant (FIA), or by dead parasitized cells in Freund's "complete" adjuvant (FCA). The test showed that homologous challenge of monkeys sensitized with
dead parasites in Freund's adjuvant resulted in the appearance of a new antigenic variant, as in monkeys sensitized by drug-cured infections, and that this change took place even when relatively few parasites were inoculated. Butcher and Cohen (1971) in their development of a technique for a short term culture of *P. knowlesi* showed that SICA titres are independent of the inhibitory effect of immune sera on *in vitro* growth of *P. knowlesi*. Agglutinating antibodies have not been detected with any of the human malaria infections.

2.2.5 *Indirect haemagglutination test (IHA)*

The principle of this test is that red cells coated with antigen will agglutinate in the presence of antibody specific to that antigen. Desowitz and Stein (1962) were the first to introduce the passive haemagglutination test (PHA) in the field of malaria serology. They stated that the tanned red cell haemagglutination test is a sensitive technique for measuring antibodies developed against a wide variety of antigens, and that it will detect low concentrations of antibody. Only minute quantities of antigen are required. The malaria parasite they used was *P. berghei*. Stein and Desowitz (1964) modified the test by using formalized tanned sheen cells to determine the reactions of various plasmodium antigens and sera of human patients with malaria. As antigens, aqueous extracts of *P. berghei, P. cynomologi, P. coatneyi* and *P. vivax* were used. Serum from patients infected
with *P. vivax* and *P. falciparum* were tested. Positive results were obtained with heterologous antigen, and titres as high as 1:25600 were being detected with homologous antigen. Desowitz and Saave (1965) applied the IHA test in the field to study the immunity to malaria in protected and unprotected populations of Australian New Guinea. Using *P. cvnomo* and *P. coatneyi* as antigens the test revealed that 71% to 92% of the unprotected population gave positive HA reactions. 70% of the children in the protected population, between 1 and 4 years old, gave negative reactions compared to 17% to 29% negatives of the same age-group among the unprotected population. Bray and El-Nahal (1966a, b) using sensitized tanned sheep erythrocytes with plasmodial antigens of *P. berghei* showed that the test could be used with rodent malaria infections and was species specific. Desowitz *et al*. (1966) applied the IHA test in further studies on the immuno-epidemiology of human malaria and the immune response in experimental malaria. They claimed that the IHA test is quite reliable and gives interpretable results. Rogers *et al*. (1968) modified and evaluated the IHA test for malaria. They prepared their antigen from mature schizonts of *P. knowlesi* harvested from the blood of *Macaca mulatta* monkey. The plasmodia were freed from red cells with distilled water and then disrupted in a pressure cell. A 2% NaCl extract of the disrupted plasmodia was used to sensitize human, group O, tanned erythrocytes. They reported that their test detected antibody titres of 1:16 or greater
in 98% of slide-proved cases of malaria and in less than 1% of sera from persons without a history of malaria, showing that the test is reliable and sensitive. Welde et al. (1969) used a lysate of erythrocytes parasitized with *P. berghei* and *P. falciparum* as antigens in their test which appeared to give sensitive, specific and reproducible results. They utilized only small amounts of both antigen and serum. 93 of 94 sera from *P. falciparum* infections gave positive reactions, most of them reacted at a relatively high titre, when tested against *P. falciparum* antigen.

Meuwissen and his team (1972a, b; 1973a, b; and 1974) tried to perfect the technique and adapt it to field conditions with reasonable reproducibility. They developed a method for the preparation of lyophilized sensitized fixed erythrocytes, that remain stable in vacuum-sealed ampoules, as antigen. *P. cynomolgi* and *P. falciparum* were used as antigens for sensitizing cells. Testing sera from *Aotus trivirgatus* infected with *P. falciparum*, they found that the homologous antigen gave a higher reaction than the heterologous *P. cynomolgi* antigen. Comparing six different sensitizing antigens, they found that the best reactivity is obtained with *P. falciparum* cells obtained from infected *Aotus trivirgatus* and recommended its use for field application. Results of duplicate tests with lyophilized sensitized cells in the IHA test indicate the good reproducibility of the test.
Comparative studies of IHA test and IFA test on field material from Tanzania showed that the IHA test seemed to be less reactive than the IFA test with anti IgG conjugate, particularly in parasite carriers. The American team, Kagan et al. (1969a, b) carried on, developing the IHA test for the application in seroepidemiological studies. Using *P. knowlesi* as antigen they tested sera collected from military recruits from Argentina, Brazil, Colombia and the U.S.A.; they concluded that their serological data compared well with data obtained by active surveillance for malaria and that the test is both sensitive and specific. Antibody titrated from plasma eluted from filter paper gave good results and they recommended the technique to be used to determine positive serological rates in a population, which could indicate the extent of malaria transmission. Matthews, Fisher and Kagan (1970a, b) applied the test to measure the persistence of malaria antibody in Tobago, West Indies, following a successful eradication programme, and in 4 provinces of Philippines comprising 20 localities. In Tobago, using *P. knowlesi* as antigen, they demonstrated the fall of malaria antibodies to negligible levels within 15 years after the last clinical case was detected, while in Philippines the study revealed that malaria antibody was present to some degree in all the 20 locations sampled. This strengthened their previous recommendation that the IHA test can be applied in epidemiology to delineate malarious areas, to measure the level of malaria endemicity
and to detect small foci of malaria transmission. Goldsmith et al. (1971) used the test in Oaxaca State, Mexico, to get information on the prevalence of antibodies to seven human parasitic infections including malaria. Kagan (1972) discussed the use of the IHA test as a seroepidemiological technique for malaria. In addition to the previous usages mentioned before, he recommended its use for an independent determination of the intensity and distribution of malaria making it unnecessary to depend on the recorded malariometric surveillance information; identification of population groups with specially high rates of malaria infection; to assess the coverage of the standard surveillance methods; to determine the role of migrants in the introduction of malaria from malaria endemic areas to receptive areas with little or no malaria; and for surveillance in areas of low endemicity. Since large numbers of specimens can be titrated readily at small expense, blood can be collected by finger prick on filter papers, and the antigen prepared from P. knowlesi is genus specific and will react equally well with all the four plasmodium species causing human malaria, the test can reliably be used for epidemiological purposes. Frashy and Kagan (1972) introduced the use of stable sensitized cells as antigen. Human 'O' erythrocytes were stabilized with pyruvic aldehyde, tanned, fixed with glutaraldehyde, and sensitized with antigenic preparations of P. knowlesi.
Lobel et al. (1973) in their attempt to interpret IHA titres for the study of malaria epidemiology suggested that the technique may become an important procedure in the armamentarium of malaria epidemiologists, but further laboratory investigations would be necessary to improve the standardization of test results. Also, field studies needed to be carried out in areas with different malarious conditions to permit better interpretation and understanding of the results in relation to other malarious measurements and to determine the optimum ways in which the method can be applied to the study of malaria epidemiology. Mathews et al. (1973a, b) developed an antigen from *P. falciparum* and one from *P. vivax* to use in the IHA test for human malaria. This proved to be more sensitive than *P. knowlesi*. Farshy and Kagan (1973) improved the reactivity of the indirect microhaemagglutination test by the use of a combination of the cells sensitized with both *P. knowlesi* and *P. falciparum* antigen for detecting antibodies to *P. falciparum*. The use of this antigen of stable sensitized cells will increase the reactivity of the test, reduce the routine daily work and may improve the reproducibility of the test. Mathews et al. (1975) evaluated the use of antigens prepared from *P. falciparum* and *P. vivax* cell in the IHA test for malaria. Their results showed that the sensitivity of antigens used is above 90% in homologous infections and between 72% and 76% in heterologous infections, and that the test is
reproducible at different times.

Mathews et al. (1976) demonstrated the presence of high levels of malaria antibodies in West African children using the IHA test with \textit{P. falciparum} antigen. Mathews and Dilworth (1976) evaluated the use of \textit{P. brasilianum} antigen for the IHA test. Their preliminary study indicates the antigen can be used to detect antibodies to \textit{P. malariae} malaria with the IHA test, after comparing it with antigens of \textit{P. falciparum} and \textit{P. vivax}. Lobel et al. (1977) used the IHA test in a longitudinal seroepidemiological malaria survey in Guyana. Their results did not suggest recent malaria transmission in the studied sectors where indigenous malaria cases had not been detected since 1968. Ceneviva and Camargo (1979) used parasitized chicken erythrocytes as a source of \textit{P. gallinaceum} antigen in IHA test to detect IgM antibodies in human malaria. The test yield positive results only when infection was patent, or for a short period thereafter. This is because the test detects only IgM anti-plasmodial antibodies and not IgG-antibodies, both in \textit{P. falciparum} and \textit{P. vivax} infections. They reported that the test was very sensitive, with positive results in every case with patent parasitaemia.

Although most of the previous workers thought that the IHA test is the most sensitive seroepidemiological tool at their time, the work of Wilson et al. (1971), Voller et al. (1974) Meuwissen et al. (1974), Wilson et al. (1975) and Corville-Brogger (1978) showed clearly
that the IHA test misses antibodies in young children because they have less avid antibodies and also it misses positive cases at the early stage of the infection, while the IFA test readily demonstrated the presence of antibodies in both cases. This is why the test was not suitable for application in malaria epidemiology studies.

2.2.6 Counter immunoelectrophoresis (CIE)

The use of CIE in detecting malaria antibodies is very restricted and not widely used because it is probably inferior to immunofluorescence as mentioned by Ambroise-Thomas (1976), IHA or the ELISA method as reported by Voller (1976). It was first applied by Targett (1970) to compare immunoglobulin concentrations, antibody titres and the antigenicity of different asexual forms of P. falciparum parasite. He used LKB 'immunophor' apparatus with 1.5% Difco Nobel agar in pH 8.6 veronal buffer (1 = 0.045). Zaman et al. (1972) used the technique to detect circulating antigens and antibodies in rodent malaria infections. Their technique was to dissolve Ionagar (Oxide No. 2) in a 1:2 dilution of barbitone buffer (pH 8.6, ionic strength 0.083) in distilled water to give a final concentration of 1.5%. The agar was then poured into plastic plates to a depth of 1 mm and wells were cut in pairs, about 6 mm apart from centre to centre and 3 mm in diameter. 8 microlitres volume of
antigen or antiserum was introduced into the appropriate well and electrophoresis was carried out in a Hyland electrophoresis unit with constant output current of 50 milliamperes at 120 volts for 90 minutes. Bidwell et al. (1973) and Bidwell and Voller (1975) used the CIE technique to detect antibodies in monkeys infected with human malaria. They carried out their electrophoresis at 100 volts for 45 minutes. They concluded that results are obtained quickly and only small amounts of serum and antigen are needed since no peripheral diffusion occurs, and that the method is particularly useful when weak antigens are encountered. Seitz (1975) demonstrated malarial antigens and antibodies in the sera of rats and mice with the CIE technique. They concluded that the CIE method is more sensitive when compared with double diffusion technique. Draper (1975) reviewed the use of the technique in immunodiagnosis in tropical and other infections including malaria and concluded that it was insensitive for general use in malaria.

2.2.7 The indirect fluorescent antibody test (IFA)

Historical

Ingram et al. (1961), Tobie and Coatney (1961), Tobie et al. (1962), Voller (1962) and Voller and Bray (1962) were the pioneer workers who introduced fluorescent labelling for detecting malaria antibodies. They applied
both the direct and indirect method, using a thin malaria film as an antigen. Fluorescein isothiocyanate (FITC) was their staining conjugate. The test was applied by Kuvin et al. (1962a, b) to study antibody production in human malaria and the course of antibody production and serum globulin levels in normal volunteers infected with human and simian malaria. Voller (1964a) tried to develop the test to differentiate between strains of *P. falciparum* with limited success. Collins et al. (1964a, b, c) used it to demonstrate the production and persistence of specific antibody in patients infected with *P. malariae* and *P. falciparum*. Voller (1964b) gave a detailed description of the techniques of both direct and indirect methods and their use in malaria research. Coudert et al. (1965) applied the test to study the presence of malarial antibody in serum of 39 patients. Using *P. cynomolgi* from the blood of *Macaca mulatta* as antigen they found that all the 39 patients had positive reactions. McGregor et al. (1965) measured the immune response to hyperendemic malaria in Gambia, West Africa by the IFA test. Lunn et al. (1966) used the IFA test to study the changes in antibody titres during the course of prolonged infections with *P. vivax* and *P. falciparum* in human volunteers. Their results showed that antibodies first appeared 3 to 9 days after the onset of patent parasitaemia in both types of infections, reached maximum titres within 8 to 21 days of patency, and that antibodies
persist up to 252 days after the end of patent parasitaemia. Further successful development of the test progressed by time and Collins and Skinner (1972) recommended the test to be used in malaria serological surveys since it proved to be one of the most suitable techniques for the detection and measurement of malarial antibody and it has been shown to give a reliable indication of the malarial experience in populations in areas endemic for malaria. Ambroise-Thomas (1973, 1974) reviewed the use of IFA test in seroimmunological studies of malaria indicating the main results achieved by using the test in human malaria. He summarized the usage of the test in the following points:

- as a diagnostic tool in countries where malaria is not endemic;
- for detecting antibodies in persons who have been infected while abroad and have been treated before parasitological diagnosis was confirmed;
- for detection and diagnosis of relapses and prevention of their occurrence;
- for detection of asymptomatic parasitaemia in blood donors and prevention of transfusion-induced malaria;
- as a means of evaluation of the efficacy of control and eradication programmes.
Choice of species for antigen and cross-reactions

Tobie and Coatney (1961) studied the cross-reactions between human, simian and rodent malaria. Using antigens of thin blood films containing either *P. vivax*, *P. cynomolgi bastianellii* or *P. berghei* they tested serum from a volunteer who had a long-standing infection with *P. vivax*. Their preliminary observation showed that *P. vivax* and *P. cynomolgi bastianellii* parasites fluoresce essentially the same after the application of labelled *P. vivax* antibody, pointing to the possibility that each species may possess an antigen or antigens in common. The rodent parasite *P. berghei* fluoresced less than the human parasite, *P. vivax*, after testing both species against *P. vivax* labelled antibody.

Tobie et al. (1962) utilized the technique to carry out studies on cross-reactions between human and simian malaria in human volunteers. Using *P. cynomolgi* and *P. vivax* antigens they concluded that the cross-reactions which they observed by the use of IFA test appeared to be dissimilar in certain respects but similar in others to those obtained by earlier investigators using the CFT. Higher antibody titres were obtained when sera from volunteers were allowed to react with the homologous species of parasite than when the same sera were allowed to react with heterologous antigen species. Coudert et al. (1965) used *P. cynomolgi* from the blood
of infected *M. mulatta* to study the presence of malarial antibody in the serum of 39 malaria patients. They got positive reactions in all of the sera from the 39 patients. Collins et al. (1966) studied the cross-reactions between human and simian malaria using the IFA test. Using antigens of *P. falciparum, P. malariae, P. vivax, P. fieldi, P. gonderi, P. inui, P. coatneyi, P. knowlesi, P. cynomolgibastianelli* and *P. brasilianum*, they tested serum samples from 11 patients having past experience with malaria. The test reactions showed that experience with *P. falciparum* and *P. malariae* produced a response to all the plasmodium antigens tested. In patients having experienced only *P. falciparum*, cross-reactions were highest with *P. fieldi*, whereas in those having experienced only *P. malariae* cross-reactions were high not only with *P. brasilianum* but also with *P. fieldi*. Meuwissen (1966) used thin smears of antigens prepared from blood infected with *P. fieldi, P. ovale* and *P. vivax* to test the presence of antibodies in human malaria patients. He concluded that *P. fieldi* appeared to be good cross-reacting antigen for serological investigations of human malaria.

El-Nahal (1967) used antigens of *P. berghei yoelli, P. malariae*, two strains of *P. cynomolgib* and *P. gallinaceum* to study the cross-reactions of malaria antisera. Antisera to four rodent malarias reacted only with *P. berghei* antigen. Antisera to three strains of *P. cynomolgib* reacted to *P. cynomolgib*, but antisera to *P. inui, P. shortti*, and *P. malariae* failed to react. Only *P. malariae* antisera reacted with
P. malariae antigen; P. falciparum antisera did not react. The only reactor to P. gallinaceum antigen was the P. gallinaceum anti-serum; P. juxtanucleæ failed to react. This suggests that exoerythrocyte schizonts may prove to be species-specific antigens in IFA methods.

Sulzer and Wilson (1967) introduced the use of a thick smear antigen in the test instead of the thin smear antigen which is in use before. Meuwissen (1968) used antigens prepared from the four species of human plasmodium and two simian species, P. cynomolgi bastianelli and P. fieldi, in thin smears to measure the antibody response of patients with natural malaria infections. He observed from his results that all patients developed fluorescent antibodies against each of the six plasmodium species used as antigens, and that most homologous antibody titres were higher than heterologous antibody titres, and that of the two simian plasmodium species used, P. fieldi appeared to be the more sensitive for detecting human malarial antibodies. Sulzer et al. (1969) evaluated the use of their thick-smear antigen. They found that it is very sensitive, with a false positive rate of less than 1%, and a detection rate of known malaria infections of 95%. This technique uses less blood for antigen and made possible the use of multispot slides. P. vivax and P. brasilianum antigens were found to be more specific in reaction than P. falciparum antigen.

Kielman et al. (1970a, b) introduced and evaluated the
use of *P. gallinaceum* as antigen in human malaria IFA test. It was found to give weak reactions. Targett (1970) used antigens prepared from blood containing mainly schizonts stages of *P. falciparum* from cultured blood in Harvard medium for 22 hours, and peripheral forms of parasites of *P. falciparum* before culture in a comparison of immunoglobulin concentrations, antibody titres and antigenicity of different asexual forms of the malaria parasite. With each of the conjugated antisera, the use of mature schizonts as antigen raised the antibody titres of most sera, which reflects a quantitative difference between the antigenicity of schizonts and of trophozoites. Sulzer et al. (1971a) prepared and tried a multi-species antigen for use in IFA test for human malaria. Infected blood with *P. falciparum*, *P. malariae* and *P. vivax* was pooled and thick smears of antigen were prepared from the pooled blood. The antigen gave good reaction with all species of human malaria.

Lopez-Antunano (1974) boosted the IFA test by preparing a *P. falciparum* antigen from parasites in *in vitro* cultures. Using the method described by Rieckmann (1968) for the maturation of asexual parasites, he cultured the blood obtained from a child with *P. falciparum* infection of $10^5$ parasites per cm, in a water bath at $37^\circ$C for 24 hours. After incubation the blood was centrifuged to remove the plasma, and then washed five
times in phosphate buffer saline (PBS) to remove the patient's serum. Thick blood films were prepared, dried and stored at -70°C ready for use. Testing the antigen against *falciparum* control sera and sera from parasitologically confirmed malaria cases he obtained consistently reproducible results which were comparable with those obtained when an *Aotus-falciparum* antigen was used. Sulzer (1975) proposed the establishment of a central facility to provide malaria antigen to all institutions interested in malaria serology. Such facility would make possible more widespread use of serological methods and would be a step towards the standardization of the application of the IFA test.

Thomas and Pannampalam (1975) produced a thick smear antigen from schizonts obtained by *in vitro* culture of human *P. falciparum*, to be used in IFA test. Kagan (1976) used the IFA test to detect prenatal infection. He concluded that the detection of specific IgM antibody in the newborn indicates an infection acquired during foetal life. Sulzer and Latorre (1977) described a simplified method for *in vitro* production of schizonts of primate malarias to be used as antigen in serological tests. For 9 out of the 10 patients examined the system was perfectly homologous. Titres were found to range between 1:40 to > 1:640. Hall *et al.* (1978) used *P. falciparum* parasites from a continuous culture to prepare antigen slides. Two strains were used, the Southeast Asian Camp strain and the African Z strain. Testing
serum from a Nigerian patient against the two antigens, higher titres were obtained with the Camp than with the Z antigen, suggesting that *P. falciparum* strain differences may be detectable by the IFA test.

**The technique and its development**

The advantage of the IFA test to the direct FA which requires the labelling with fluorescein of each serum individually, is that a quick sandwich method is utilized testing very small quantities of sera on one antigen slide labelled with small amount of conjugate. Illumination with UV-blue light permits visual observation of the fluorescent dyes attached to the complex. The amount of fluorescence is proportional to the quantity of antibody absorbed onto the slide-antigen from the test sera. Certain technical improvements have been made and these facilitate large-scale application of the technique (Figure 10). Kuvin *et al.* (1962), Tobie *et al.* (1962) and Voller (1962) used thin blood film as antigen. Sulzer and Wilson (1967) and Sulzer, Wilson and Hall (1969) introduced the use of washed blood to make thick smear antigens which allowed carrying out multiple tests per one antigen slide having a number of thick smear spots. Using the thick smear antigen it was found that the false positive rate is less than 1% at the 1:16 dilution level, and the detection rate of known malaria infections was 95%
serum from a Nigerian patient against the two antigens, higher titres were obtained with the Camp than with the Z antigen, suggesting that *Plasmodium falciparum* strain differences may be detectable by the IFA test.

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Fig. 10
Principle of the indirect fluorescent antibody test (IFAT)

(I) Parallel preparations a and b which contain antigen (black discs) are treated respectively with specific antiserum (hatched) and non immune serum (stippled).

(II) The immune serum combines with the antigen in a while the non immune serum is removed from b by washing.

(III) The fluorescent labelled specific antiserum (chequered) is applied.

(IV) The fluorescent antiglobulin has combined with the antibody attached to the antigen in a and is washed away from b.

(Reproduced from Naim R.C., 1962 ch 6 in Fluorescent Protein Tracing)
with serum sample of low titre. *P. vivax* and *P. brasilianum*
antigens were found to be more specific in reaction than
*P. falciparum* antigen. Targett (1970) used antigens
prepared from *P. falciparum* infected blood in culture
containing mainly schizonts. This antigen turned to be
more sensitive than that prepared from peripheral forms
of the parasite. The usage of antigens prepared from
schizonts stages made available by continuous culture by
Lopez-Antunano (1974), Thomas and Pannompalam (1975),
Sulzer and Latoree (1977) and Hall *et al.* (1978) was a
bonus to developments in antigen preparation. O'Neill
and Johnson (1970 and 1971) developed a simple semi­
automatic method with multispot antigen slide. This
method will enable one operator easily to test 120 sera
a day consuming very small quantities of reagents.
Microscopical examination of 12 slides (144 spots) takes
about two hours (ten minutes per slide). Turner (1971)
used an 8-place antigen slide for multitesting. Voller
and O'Neil (1971) introduced the usage of a 12 spot
antigen slide which is suitable for application of the
IFA test in a large scale. The collection of blood spots
from a finger prick in filter paner in the field was a
major step in making the test useful as seroepidemiological
tool for field application. Using the micro-technique
by eluting the blood from the filter paper and preparing
the dilutions in a microtitre plate helped in fastening
the procedure of the test and enabled one technician to
test large numbers of sera in one day. Reading the test is time consuming and depends on the judgement of the examiner at different times in different laboratories making comparisons of results not practicable.

Manawadu and Voll er (1971, 1973) introduced the use of fibre-optic probe for reading the results of the test. They fitted the fibre-optic probe into a 10x focusing microscopic eyepiece connected with a photomultiplier which in turn coupled to a digital photometer. This increased the efficiency of reading the test quantitatively.

Effect of drug treatment and persistence of malarial antibody

Voller and Wilson (1964) were the first to apply the IFA test in a field study to find the effect of drug treatment on malarial antibody production in infants and their mothers in Gambia, West Africa. Their tests revealed that malarial antibody was not found in any of the infants who had been on antimalarial prophylaxis since birth, in contrast malarial antibody was demonstrated in all but one of the unprotected children. The average titre of the protected mothers was much lower than that of the unprotected group. Wilson et al. (1970) determined the malaria antibody patterns in U.S. servicemen after chemotherapy by the IFA test. They concluded that the malaria antibody declined within 6 months from
relatively high levels to low levels after radical treatment and presumed cure. Ambroise-Thomas et al. (1971) carried out systemic serological studies using the IFA test on 17 patients who were successfully infected with *P. vivax* and treated either with quinine intramuscularly or chloroquine orally after eight to 10 paroxysms. All the subjects studied were serologically negative before inoculation, the appearance of fluorescent antibodies coincides with parasitaemia or takes place slightly beforehand, a relapse is immediately followed by an increase of antibody titres, and after treatment there was no patent parasitaemia and there was a rapid and marked decrease of serological titres. Collins et al. (1975) used the IFA test in an attempt to determine the evolution of the antibody response in volunteers who had no prior experience with malaria and whose primary attack was of limited duration, as would be the case with many people to whom antimalarials are readily available. Volunteers were infected via the bites of infected mosquitoes with six different strains of *P. vivax*. It was shown that although there was always a detectable IFA response, it is often of short duration.

Kuvin and Voller (1963) studied 26 West Africans who had been living in a nonendemic area (Britain). Using *P. falciparum* and *P. cynomolgi bastianelli* as antigens, they demonstrated that malarial antibody in these persons persisted for as long as 7 years after they had
left Africa. Titres to *P. falciparum* were recorded as 1:50, 1:100 and 1:50 in three West Africans who had been in Britain for at least 5 years. Collins *et al.* (1964) studied the development and persistence of antibodies to *P. falciparum* in 7 patients being treated for neurosyphilis at varying periods of time with a Colombian strain of *P. falciparum*. Using the homologous species as antigen, they demonstrated malarial antibody titres ranging from positivity in an undiluted serum sample to 1:160, 20 months after the original infection. Kuvin *et al.* (1962) studied malarial-antibody levels in presumably non-immune volunteers after a single exposure to infection. By the IFA test malarial-antibody titres of 1:20 and 1:40 were demonstrated in two volunteers with *P. vivax* as antigen 150 days after inoculation with the sporozoites of the Venezuelan strain of *P. vivax*. Lunn *et al.* (1966) studied the changes in antibody titres during the course of prolonged infections with *P. vivax* and *P. falciparum* in human volunteers. Their results showed that antibodies first appeared three to nine days after the onset of patent parasitaemia in both types of infection, reached maximum titres of 1:640 to 1:2560 within 8 to 21 days of patency, and that antibodies persist up to 252 days after the end of patent parasitaemia.

Luby *et al.* (1967) found a low level of malarial antibody detectable by IFA test 13 years after the original transmission of an infection of *P. vivax* in persons in the Lake Vera, California, who were exposed to *P. vivax* malaria within a discrete period of time,
were successfully treated and had not been re-exposed to infection. This low level of malarial antibody was detectable with P. vivax, but not with P. fieldi antigen. Fasan et al. (1972) studied the malarial antibody level in 52 healthy Western Nigerian Yoruba students resident in Washington D.C., U.S.A. using the IFA test. Their studies revealed that the malarial antibodies fall slowly and steadily over many years though still detectable even after 10 years from last exposure. This finding suggests that adult Africans returning home after a period of absence in non-malarious areas may become susceptible to and develop clinical malaria on re-exposure. Bruce-Chwatt et al. (1972) carried out seroepidemiological studies in Indian and Pakistani immigrants who are residents in Bradford, U.K. About 50% of them showed a positive IFA response at a low titre against P. falciparum and P. vivax antigens. Although there is little evidence of persistence of malaria infection in them, the test indicates the long duration of antibody acquired ten to fifteen years ago. Collins et al. (1968) studied the persistence of malarial antibody response in sera from 95 patients having had induced infections with P. falciparum, P. malariae, P. vivax or P. ovale. The intervals between termination of the infections and acquisition of the sera ranged from approximately 6 months to 26 years. Specific serum dilution end points high enough to be considered positive (1:20 or greater) were found as late as 8 years after termination of P. falciparum infection and 13 years
after termination of *P. malariae*. For *P. vivax*, positive responses could not be found three years after termination of the infection. With patients who had experienced *P. malariae* infections which had not been terminated by curative antimalarial treatment, sera collected 15 to 24 years after the last known patent parasitaemia frequently yield a positive FA response. Draper and Sirr (1980) tested sera from neurosyphilitics who had been treated with prolonged attacks of malaria, sometimes with several species, before having radical treatment, 78 per cent were found to have detectable antibodies 11 to 20 years after their last malaria episode, and 70% after 20 years or more, including one after 30 years.

Detection of malaria antibodies in soldiers and travellers returning to non-malarious areas

Most of these studies were carried out in U.S.A. servicemen returning home from Vietnam. Fisher *et al.* (1969), Wilson *et al.* (1969), Gleason *et al.* (1969, 1970), and Wilson *et al.* (1970) reported cases of *P. vivax* and *P. ovale* in combat troops who have been in Vietnam for periods of a maximum of 12 months, and who have no history or complaint of malaria. Luby *et al.* (1967) reported two cases of introduced *P. vivax* malaria at Fort Benning, Georgia. Investigations eliminated travel to areas endemic for malaria, transfusions, and recent inoculations,
as modes of acquisition of the disease. Screening suspects and contacts parasitologically and serologically using the IFA test, the index case was found to be a serviceman who had been stationed in Korea for one year. This man was not suffering from malaria and blood films from him were negative, but his IFA test titre was high. Leibovitz et al. (1969) studied the prevalence of malarial FA in Vietnam returnees with no history of overt malaria. Out of 183 returnees tested one week after their return to the U.S.A., 26 per cent were found positive when tested against P. falciparum antigen and P. vivax antigen. All of the 17 positive controls with overt malaria demonstrated antibodies, whereas none of 50 individuals with no geographical connection with an endemic area gave any fluorescent reactivity.

Immunoglobulin response to malaria and its relationship to antibodies

The immunoglobulin response to malaria infection and its relation to the antibody detected by IFA are of much interest. Anti-IgM, IgG and IgA specific immunoglobulin conjugates are now available commercially and this facilitates the study of these immunoglobulins with the IFA test. Kuvin et al. (1962) notes in experimental infections that the increase in total gammaglobulin was correlated with the rise in malaria antibody titre, but
that the excess gamma globulin did not consist entirely of malarial antibody. Abele et al. (1965) extended the work to show that increase in beta_2-M macroglobulins closely coincide with the appearance of malaria antibody detected by IFA. Lunn et al. (1966) studied the changes in antibody titres and serum protein fractions during the course of prolonged infections with vivax or falciparum malaria. They found that the initial increase in gamma globulins closely coincided with the appearance of antibodies. They reported a transient rise in the gamma globulins and alpha_2 globulins, a transient decrease in the albumin and alpha_2 globulins, and no consistent change in the beta globulins during each episode of parasitaemia. Tobie et al. (1966a, b) reported that IgM globulin production was greatly increased during experimental infections of \textit{P. vivax} and \textit{P. cynomolgi}. Increases in IgG were also large but not as striking as the IgM levels. Increases in antibody levels were correlated with increases in immunoglobulin. In the same year they published findings that suggested dead plasmodia elicited slight antibody responses in \textit{P. vivax} infections. They concluded that the antibody response was probably due to asexual forms and not to sporozoites or exoerythrocytic parasites. Turner and Voller (1966) compared IgD, IgA, IgG and IgM immunoglobulin levels of Nigerian adults with those of British adults. IgD and IgA levels were similar in both groups, but IgG and IgM levels were significantly higher.
among Nigerians than British. No correlation of immunoglobulin levels with malarial antibody titres was found. They concluded that the elevated immunoglobulin levels could be due to diseases other than malaria. McFarlane and Voller (1966) in continuation of their work in the studies on immunoglobulins of Nigerians found that IgA, IgG and IgM levels were not significantly different in persons with or without peripheral parasitaemia, although the IgA and IgM levels were higher and the IgG levels lower in the group with detectable parasitaemia. The IFA titres were greater in those persons with positive blood films. Cox, Crandall and Turner (1969) used anti-IgM, IgG and IgA immunoglobulin conjugates to study the IgM, IgG and IgA response to rodent malarial infections as detected by IFA. They used as models mice infected with P. vinckei and P. chabaudi. During the early stages of infection, IgM antibody levels were higher than those of IgG, but the IgG levels soon rose to higher levels than the IgM. There was no apparent decline in IgM 39 days after infection nor increase in antibody levels after challenge. Harvey et al. (1969) reported a case of congenital malaria in the United States. P. malariae was diagnosed microscopically in the mother who had no clinical evidence of malaria for over 20 years. Using anti-IgM conjugate they demonstrated malarial IgM antibody in both the mother and child. Gilles et al. (1969) studied the relationship between malaria, anaemia and
pregnancy in Nigeria. Malarial antibody titres were determined before and during pregnancy. Although many of the women developed peripheral parasitaemias during pregnancy, the IFA titres did not show consistent changes. There was a progressive fall in both IgG and IgM levels during pregnancy. Their studies demonstrated clearly that the known reduction of effective immunity during pregnancy was not reflected in IFA titre levels, so the IFA titres may not be a clear indication of the immune state of the individual to malaria. Williams and McFarlane (1969) reported on malarial antibody in maternal and cord sera of Nigerian mothers and their infants. Titres in the IFA test ranged from 1:1280 to 1:5120 in the mothers and from 1:640 to 1:2560 in the cord bloods. All of the malarial antibody was found in the IgG fraction, although the presence of IgM was demonstrated by immuno-electrophoresis in both maternal and cord serum. The authors concluded that malarial immunity of the newborn Nigerian is due to passively acquired maternal IgG antibody. McFarlane et al. (1970) measured IFA responses and immunoglobulin levels of a Nigerian population. They found that the development of IFA titres paralleled the development of IgG. Serum IgG was lowest when the children were between four weeks and four months of age and reached adult levels at about five years. Adult levels of serum IgM were reached at one year. They suggest that the IgM detected at birth may be indicative of
congenital infection. Targett (1970) measured antibody titres of children and adults living in the malarious endemic region of Gambia, West Africa. Malarial antibody was detected with antisera to IgG, IgM and, at low levels, to IgA but not with antisera to IgD or IgE. Adults in whom IgM antibodies were detected had a mean IgM concentration significantly higher than that for individuals who were IgM antibody negative, and there was a positive correlation between IgG fluorescent antibody titres and total IgG in the children examined. This correlation was not found with the adult sample, and results from the serial examinations showed that, although IgG fluorescent antibody titres generally rose as a result of acute infection, there was no corresponding increase during the same period in total IgG levels. Collins et al. (1971) used specific immunoglobulin conjugates to study antibody patterns and serum proteins in experimentally induced human malaria. In those infected with *P. vivax*, the IgG response rose and peaked slightly earlier than the IgM response, while in *P. falciparum* infections, both peaked at approximately the same time. Regardless of whether or not the patients were treated, the IgM and IgA responses returned to low levels, while the IgG levels remained elevated. They suggested that these responses could be of practical use in distinguishing between recent infection and past experience with malaria.

Voller, Lelijveld and Matoba (1971) surveyed the IgG, IgM and malarial IFA levels of several groups
at different altitudes in northeastern part of Tanzania. IgG antibodies were found to rise steeply from the early childhood in the low area, and the plateau reached by about 5 years of age was maintained into adult life, while the IgM levels increased with age. In the middle area the pattern of IgG is similar to that in the low area but the plateau was not reached until 10 - 16 years, again IgM levels increase with age. However in the highest area the IgG levels were low, but the mean levels of total IgM in the children were somewhat higher than in the other two areas.

Kagan (1976) summarized the value of detecting IgM in the IFA test for diagnosis of parasitic infections. He said that the detection of specific IgM antibody in the newborn indicates an infection acquired during foetal life. Cornille-Brøgger (1977) titrated IgM antibodies to *P. falciparum* by the IFA test. 10 sera with high levels of serum IgM from adult Nigerians were tested. The comparison of the titres observed in eight whole sera and their separated IgM fractions showed slightly higher IgM antibody titres in the purified IgM fraction. In another series of experiments, IgG fractions were added to homologous and heterologous whole sera to detect possible inhibitory effects of a higher IgG concentration in the IFA-IgM test. The results showed there is no evident inhibitory effect of IgG or IgM antibodies.
Seroepidemiological field applications of the IFA test

a. Studies in endemic areas

Serological surveys on relatively large numbers of people have been conducted using the IFA test in endemic malarious areas specially in Tropical Africa, Central and South America. Voller and Bray (1962) were among the pioneers in applying the test in the field of seroepidemiology of malaria in Liberia, West Africa. They demonstrated the presence of antibodies in the semi-immune population and in cord blood from immune mothers. McGregor et al. (1965) followed them in an identical study in Gambia, West Africa. Measuring the immune response to hyperendemic malaria they found that the fluorescent antibody levels were high in newborns, fall in the weeks immediately following birth, remain low for the first year of life and thereafter rise progressively throughout childhood into adult life. Haverson et al. (1968) carried out an assessment of current malarial endemicity in Bathurst, Gambia, West Africa. Collins et al. (1967) and Voller and Bruce-Chwatt (1968) did studies in Nigeria. Collins et al. (1970) and Armstrong (1972) conducted surveys in Ethiopia. In Tanzania the effect of malaria in populations living in different altitudes was studied by Lelijveld (1971), Otieno et al. (1971), Voller et al. (1971, 1972) and Draper et al.
(1972), Edrission and Afshar (1973, 1974) applied the test to detect low malaria parasitaemia and to study the malaria prevalence in Iran.

In all the previously-mentioned areas of study it is shown that new-born babies had a high IFA titre due to the presence of antibodies transported through the placenta from their mothers. These titres decrease with time after birth and remain low for about a year after which they increase rapidly throughout childhood due to exposure to infections, then more slowly until the adult plateau levels were achieved.

Voller, Lelijveld and Matoba (1971) surveyed the IgG, IgM and malarial IFA levels of several groups at different altitudes in northeastern Tanzania. Malaria antibody was detectable in almost everyone tested in the area below 750 feet, which have intense malaria transmission, and mean titres increased with age. In the area at 1800 - 3000 feet with sporadic transmission, only half of the children under 2 years old had detectable antibody; however, the older age groups were virtually all positive at levels lower than those in the lower area. In the area above 4500 feet with little or no transmission very few of those under 2 years old had malaria antibody and only one-third of the older persons were positive at very low levels. In the low area a steep rise in the mean IgG occurred from early childhood and the plateau reached by about five years was maintained into adult life.
while the IgM levels increased with age. In the middle area the pattern of IgG is similar to that in the low area, but the plateau was not reached until 10 - 16 years and there was a slight increase again to a peak held by the adults over 30 years of age, the same holds for IgM levels. However in the highest area the IgG levels were low, but the mean levels of total IgM in the children were somewhat higher than in the other two areas.

Collins et al. (1977) studied the passage of malaria antibodies from mothers to infants in Middle America. Their results indicate that, even though the levels of antibody response in mothers living in a malarious area of El Salvador were relatively low, approximately 26% of the infants had detectable antibody responses to 6 weeks following birth. The study also suggests that the transplacental movement of antibody which provides a significant degree of protection for the newborn population in hyperendemic areas, would not be the case in an area of low endemicity where infrequent and relatively brief antepartal exposure of the mother to vivax malaria does not necessarily assure the presence of antibody in the infant. Sulzer et al. (1975, 1978) studied a focus of hyperendemic malaria in the Peruvian Amazon area using both stained blood smears and IFA test. Their results illustrated that the two methods complement each other, and that 97% of the cases were P. malariae, 3% P. vivax and no P. falciparum. Jeffery et al. (1975) studied the
malaria endemicity in Mato Grosso, Brazil. Collins et al. (1968) applied the IFA test to study the relationship between fluorescent antibody response and ecology of malaria in Malaysia. Three areas were selected for the study. The hyperendemic area showed high rates of parasitaemia coupled with high mean IFA responses, the hypoendemic area had low rates of parasitaemia coupled with very low mean IFA responses, while area three, where control measures had been in effect, the rate of parasitaemia was low whereas the IFA responses were high in regard to the percentage positive; the mean titres were lower, however, than those of the hyperendemic group. Thomas and Dissanaike (1977) studied malaria endemicity among Orang Asli (Malaysian Aborigines) by IFA tests. Out of 288 Orang Asli studied, fluorescent antibodies were detected in 89% with P. falciparum antigen and in 62% with P. brasilianum antigen. 18 blood films were positive for P. falciparum; 2 of them had mixed infection with P. vivax. Of 17 sera from cord blood, 16 had significant levels of P. falciparum antibody and 14 of P. malariae antibody, the levels being the same as those of the mothers, but none of these babies had congenital malaria. Brown et al. (1976) evaluated malaria fluorescent antibody patterns in 22 remote islands populations of the western Pacific (New Hebrides, Solomons, Western Carolines and New Guinea). Antibody prevalence data confirmed the presence of significant endemic malaria.
among most of the visited islands in the New Hebrides, and a variable but smaller amount of malaria in the Solomons. Malarial infection occurred in from 40% to 100% of the New Guinea villages that were visited, and was entirely absent from the Western Caroline Islands. In evaluating a control programme Molineaux et al. (1978) carried out a longitudinal serological study of malaria in infants in the West African savannah. Two infants populations were studied, one exposed to intense malaria transmission and the other protected. The IFA test revealed that the IgG and IgM levels increased with age and were systematically, though only slightly, lower in the protected children, and that the titres were high at birth and decreased rapidly afterwards in both populations. In the unprotected population, this decrease was followed by an increase, closely associated with the parasitological findings, while in the protected population the decrease continued to very low levels. Cornille-Brøgger et al. (1978) in a longitudinal study in the West African savannah to see the changing patterns in the humoral immune response to malaria before, during and after the application of control measures showed that the IFA test is the most sensitive serological tool to be used. They found that in the unprotected population, all were positive by the age of one year; there was a rapid increase in titre with age to a plateau by the age of 5 - 8 years. After 20 weeks of control, the
titre decreased in the 0 - 28 year age groups, after 50 weeks of control there was a further decrease in antibody titres in the 0 - 28 year age groups and a smaller decrease in those aged 29 years and over. After 70 weeks of control there was again a decrease in antibody titres affecting all age groups. At the end of the intervention phase, the IFA titres in the protected population had decreased to the lowest mean value observed whereas in the unprotected population, after a slight decrease at the end of the dry season, the titres had reached their highest value at the fifth serological survey. The difference between the protected and unprotected populations was the largest observed; very significant in all age groups, it was especially large in the younger age groups. The IFA test using P. falciparum as antigen showed a positive association between the test result and concurrent P. falciparum parasitaemia up to the age of 5 years and no association for older age groups.

b. Evaluation of control and eradication programmes

The IFA test is used by many workers to follow the effect of control on the population, and the track of disappearing malaria after successful eradication programmes. Ambroise-Thomas et al. (1972) carried out a seroepidemiological study on disappearing malaria in Corsica. Their studies showed that there is a spread of
malaria foci in Southern area of Bastia and in the middle part of the Eastern plain, but the Southern part of the island remained free from malaria. Bruce-Chwatt and Draper (1973) using the IFA test demonstrated a clear evidence of eradication of malaria from Mauritius. They stated that on the serological findings it is unlikely that there has been any malaria transmission in the last five years, as no specific antibodies were detected in children born during this period. They found a high prevalence of antibodies in subjects over 20 years old, born before effective malaria control was started, and a lower prevalence in those aged five to 19 years, born in the period when malaria control was becoming increasingly effective and approaching the point of complete cessation of transmission. Bruce-Chwatt et al. (1975) applied the IFA test to study the disappearing malaria in Greece. Nearly three thousand serum samples from the population of Hemathia below the age of 20 years, born since the introduction of intensive malaria control measures followed by surveillance activities, were tested, and none of them showed the presence of malarial antibodies. They concluded that there can have been very little or no transmission of malaria in the area of the survey during recent years. Warren et al. (1975a, b, 1976) carried out sero-epidemiological studies using the IFA test in Middle America where the malaria transmission has been reduced to a very low level in many localities due to the use of
residual insecticides and the treatment of fever cases. Their studies revealed that malaria transmission varied from one locality to another even in a geographically small area, and that it may be quite localized in some situations. The study revealed the presence of a relatively large number of positive responses to *P. falciparum* although most of the species diagnosed were *P. vivax*, but there is the possibility that antibodies to *P. falciparum* reach higher titres and persist for longer periods than *P. vivax*, especially in adults who have lived most of their lives in areas endemic for *P. falciparum*. The serology test showed some false negatives in people who are microscopically positive, and a number of false positives in people with no known history of malaria. The false negatives may be due to sampling people early in their infections since the study area was one of low endemicity, while the false positives, which are mainly in adult sera, may be due to a prior malaria experience which even a brief current exposure to the non-specific antigen might well produce the positive IFA titre. The results were helpful in defining the intensity of the outbreaks, the parasite species involved, and the localized character of the epidemics.

Ambroise-Thomas *et al.* (1976) studied the malarial antibodies in 18 localities in Tunisia to confirm the presence or absence of malaria transmission and to measure a regression of sero-positivity after full or
partial interruption of malaria transmission following
the implementation of a malaria eradication programme
since 1967. They concluded that the serological pic-
ture corresponds to that of disappearing malaria in the
absence of new infections. Lysenko et al. (1977) con-
irmed the eradication of P. vivax and P. falciparum in
the Moldavian S.S.R. and showed that sporadic trans-
mission of P. malariae by the mosquito vector is possible.

Screening of blood transfusion donors

The use of the IFA test to screen blood trans-
fusion donors has been reviewed by WHO (1969), Voller
(1971), Bruce-Chwatt (1972), Sulzer and Wilson (1972),
and Lobel and Kagan (1978). They all agreed that the IFA
test proved to be a very efficient and reliable tool in
detecting malarial antibodies in blood transfusion donors
especially those who had previous experience of infections
with P. malariae, and those whose parasitaemia is too low
to be detectable in a stained thick blood smear. They
mentioned that, although a positive IFA test does not
necessarily indicate a current malaria infection, exclusion
of all donors with positive malarial antibody will virtu-
ally eliminate the risk of transfusion malaria. The
practical application of the test to screen blood donors
in non-malarious areas was carried out by Coudert et al.
(1966), Bertaud (1967) and Ambroise-Thomas (1969) in France.

Sulzer and Wilson (1972) in their review of the IFA test, confirmed the value of this method in investigating the source of infection in 10 cases of transfusion malaria in U.S.A. They detected antibodies in one donor in each of 9 cases. They stated that an antibody titre of 1:256 or higher is suggestive of a current malaria infection if there has been no specific treatment during the previous 6 months. In France Ambroise-Thomas et al. (1971) investigated over 1000 blood donors who had lived in malarious areas overseas. Using P. cynomolgi as an antigen for IFA test, they found that in 58.2% of potential blood donors, who were not exposed to the infection for less than 5 years, the test was negative and that those subjects could be used as blood donors. On the other hand in 28.2% of subjects previously exposed to malaria and living in France for over 5 years, the IFA test was still positive; consequently the use of their blood would be unsafe because of the possibility of transfusion malaria. They concluded that the IFA test could be used for screening prospective blood donors, not
only to eliminate those who might be carriers of malaria parasites, but also not to reject, because of the history of previous travel in malarious areas, a sizeable proportion of volunteers.

2.2.8 **Soluble antigen fluorescent antibody test (SAFA)**

Sadun and Gore (1968) and Sadun *et al.* (1969) introduced the SAFA technique for malaria serology. In this test a soluble antigen is coated on to cellulose discs to which test sera are added directly. Subsequent processing of the test is similar to that of slides but the fluorescence is measured by a fluorometer and objective criteria of positive and negative reactions are possible. The antigen was prepared from *P. falciparum* in chimpanzee. A stable antigen fractionated by sequential elution with chromatography from a DEAE Sephadex A25 column gave fractions that were very active in this test. They estimated that 50,000 tests can be performed with the amount of antigen normally collected from one infected chimpanzee. In the first studies, comparing the test with IHA test, it was claimed that the SAFA test possessed a high degree of sensitivity, specificity and reproducibility of results. However the test was not followed because of difficulties of producing large amounts of antigen. Today after the success in culturing human malaria parasites in continuous *in vitro* cultures the test might be of great value if further development was done.
2.2.9 The enzyme-linked immunosorbent assay (ELISA)

The ELISA is the most modern technique applied for serological testing of malarial antibodies. The principles of the test are similar to the IFA test except that an enzyme is used to label the antiglobulin instead of the FITC. A macro method using tubes or the micro method in plates coated with soluble antigens are in use. The test serum reacts with the sensitized solid phase, and after washing, an enzyme labelled antiglobulin is incubated. After another washing, the enzyme substrate is added. The colour reaction can be estimated visually or read quantitatively in a photometer.

Engvall and Perlmann (1971, 1972) were the first to develop the technique for measuring antigens and antibodies to Trichinella spiralis. Voller et al. (1974) were the first to apply ELISA as a seroepidemiological tool. They used a micro-plate method (MICRO-ELISA) with a crude P. falciparum antigen prepared from Aotus monkeys to compare sera from two Colombian populations. One group resident in an area where malaria transmission had been interrupted had much lower MICRO-ELISA values than the other group who lived in a similar area but which still had high malarial endemicity. Voller et al. (1975) using the tube or macro ELISA method with heterologous antigen prepared from the simian malaria parasite P. knowlesi tested sera from human populations.
in Iran and Tanzania. Sera from European people who were never exposed to malaria were used as controls. Results showed that sera from the people of malarious areas of Tanzania had higher ELISA values than sera from European controls, while the sera of the Iranian group had a wider range of reactivity reflecting the lower malarial endemicity there. Spencer et al. (1979a, b) used parasites from an in vitro culture of *P. falciparum* as an antigen for the micro-ELISA test. Serum from 50 persons from the southeastern United States and serum specimens collected weekly from four non-immune and nine semi-immune patients infected with *P. falciparum* were tested. None of the 50 sera from the United States had ELISA antibody titres \( \geq 1:80 \), the nine semi-immune patients had rapid ELISA antibody responses with titres \( \geq 1:2560 \), following patent parasitaemia. ELISA titres remained elevated despite disappearance of patent parasitaemia, and declined gradually following curative antimalarial therapy. The ELISA responses observed in the four non-immune patients were more variable, though positive titres appeared rapidly with patent parasitaemia. To compare the micro-ELISA with cultured *P. falciparum* as antigen with the IFA test, 261 sera collected in Vietnam and 351 sera samples eluted from filter papers collected in Honduras were examined by both methods of measuring anti-body to *P. falciparum*. The results of the two test methods did not correlate well. Discordance between the
two tests was observed in 23.0% and 29.4% of the two groups of the sera examined respectively. The major differences occurred in younger persons (less than 30 years in the Vietnam specimens and less than 16 years of age in those from Honduras); in these age groups seropositivity rates by ELISA examinations were significantly higher than those found by IFA. They suggested that further work must be done to determine the sensitivity, specificity, reproducibility, and the predictive value of negative and positive results of the ELISA test for antibody to \textit{P. falciparum}. The test has great potentiality depending on finding suitable soluble antigen. This may be possible when large amounts of cultured parasites become available.

2.3 Choice of Test for the Study

Studying carefully all the serological tests applied in the field of malaria as reviewed by Kagan and Norman (1970), Bruce-Chwatt (1970), Voller (1971), Kagan (1972), Sadun (1972), Kagan (1974), WHO (1974), Sadun (1976), Voller (1976) and Lobel and Kagan (1978), it was found that the two most widely used techniques were the IHA and IFA tests. Wilson et al. (1971), Meuwissen \textit{et al.} (1974), Voller \textit{et al.} (1974), Wilson \textit{et al.} (1975) and many other workers compared the results obtained when testing sera by both methods. It was
concluded that:

- in primary infection, it may very well take longer for the IHA test to become positive than for the IFA test;

- free circulating, soluble antigens in the sera of patients during parasitaemia might inhibit the IHA test more than the IFA test. Soluble antigens could be demonstrated in *P. falciparum* infections and the presence of S antigens was directly correlated with the degree of parasitaemia at the time of examination;

- after a malaria infection, the IHA test may revert to negative sooner than the IFA test;

- antimalarial drug administrations may have a greater influence on the IHA antibody titre level than on the IFA titre level.

From the above it can be concluded that the IFA test is the most specific and sensitive test for use as a seroepidemiological tool in malaria. The developments introduced to the test like multi-spot antigen slides, micro methods for titration of sera, availability of conjugates of high standard commercially, and possibility of testing sera collected in filter paper from the field made the IFA test, the test of choice to carry on this study. The disadvantages of the IFA test are that it needs much equipment, depends on quality of antigen used and microscope and its interpretation is subjective.
2.4 Materials and Methods

- 76 x 25 mm pre-cleaned washed, glass microscope slides
- small perspex trough (2.5 x 8 cm x 1.0 cm deep) on wheels (Haemobile)
- an iron applicator with 16 protruding pegs from a perspex block (8 on each side) for preparing 16 thick blood films on normal glass microscope slide
- phosphate-buffered saline (PBS) at pH 7.6 (annex 1)
- electrical centrifuge
- chromatography papers (Whatman No. 3) cut in 121 x 10 cm
- disposable lancets (Hawkesley and Sons Ltd)
- metal punch to cut a disc of 1.5 cm diameter
- humidifier chamber.

The haemobile and the iron applicator were constructed at the workshop of the School.

2.4.1 Preparation of Antigen Slides

Blood for preparing *P. fieldi* antigen was obtained from a splenectomised rhesus monkey (*M. mulatta*). This had been infected intravenously with *P. fieldi* infected blood stored in liquid nitrogen in 15% glycerol saline in the Ross Institute of Hygiene of the London School of Hygiene and Tropical Medicine. The course of
infection was monitored by daily examination of Giemsa-stained thick and thin blood films prepared from the earlobe of the monkey. Nine days after inoculation the parasitaemia had reached 12%. At a time at which most of the parasites were in the schizont stage the monkey was tranquilized with phencyclidine hydrochloride (sernylan) 1 mg/kg body weight intramuscularly. 50 ml of blood was withdrawn from the femoral vein in 15% acid citrate dextrose (ACD) (Annex 3). The blood is divided into 25 ml tubes, centrifuged at 2000 rpm for 10 minutes to separate the red cells from the serum. The supernatant fluid is discarded using a pipette and the red blood cells are resuspended in phosphate-buffered saline (PBS) (see Annex 1) pH 7.6 and centrifuged again at 2000 rpm for 10 minutes for washing. The centrifuging and washing procedure is repeated 4 times. After the final wash a thick film was prepared from the sediment-red cells which were now pooled in one tube, stained with Giemsa and examined using x 100 oil immersion lens of a microscope. The number of schizonts per oil immersion field were counted, and the blood was diluted with PBS till the thick Giemsa stained film showed about 10 schizonts per oil immersion field. The pooled diluted red blood suspension is kept in a refrigerator at 4°C ready for preparation of the antigen slides. Clean, washed and polished microscope slides were spread on the laboratory bench in rows which will enable the haemobile to move in
between. 8 - 10 ml amounts of blood were transferred from the refrigerator to the haemobile. The antigen spots were prepared by dispensing drops of blood by means of the applicator to the clean slides (Figure 11). The slides are then allowed to dry at room temperature, wrapped individually in absorbent toilet paper and packed in groups of 10. The wrapped slides are then kept in 50s in their original boxes and left to dry overnight in a desiccator at 4°C. After drying the boxes were placed in polythene bags and stored in a deep freezer at -70°C for future use.

Blood for preparing *P. falciparum* was obtained from Dr A. Voller of the Nuffield Institute. The infection was maintained in an *Aotus trivirgatus* (owl monkey). The method for preparing the antigen slides was exactly the same as that followed for preparing the *P. fieldi* antigen.

2.4.2 Collection of Blood Samples

Eighteen localities were chosen from four areas in the Gezira Irrigated Area (see study area Chapter One, Figure 9) which represents most of the rural councils in the area. In these localities blood surveys have been carried out each year on children aged 4 to 9 years since 1974 in order to have a record of parasite rate among this age-group. Blood from a finger prick using a disposable
Fig. 11
Preparation of malaria antigen slides for the IFA test.
Fig. 11
Preparation of malaria antigen slides for the IFA test.
lancet was collected on a chromatography filter paper 12½ x 10 cm in size. Two samples each forming a circle of about 1.5 cm in diameter were taken from each individual. Thick and thin blood smears were also prepared. Samples in both filter paper and microscope slide were numbered, and the name of the person, his number, sex and age were recorded on a form in which there is the name of the locality, the council and the date. Sample spots from 5 persons can be collected in one paper with the numbering in pencil at the left hand side (Figure 12). The blood in the filter papers and slides was left to dry at room temperature away from dust and house flies. After they dry the slides were wrapped individually in toilet paper, and the filter papers were placed in self-sealing polythene bags. In the laboratory at Wad Medani town the blood on the filter papers, well labelled, is stored at 4°C in a refrigerator, and the blood slides were processed for microscopical examination. The thin films were fixed with methanol and stained with 5% Giemsa at pH 7.2, while the thick films which are on the same slide were stained without fixing for 30 minutes. After washing and drying the blood films were examined with a Nikon compound microscope using x 100 oil immersion lens. Thick films were examined first, and when they were found positive, the thin film would be examined to identify the parasite species. A hundred oil immersion fields were examined using the thick film before saying
that the film was negative. These surveys were carried out twice, June - August 1977 and December 1978 - January 1979, in the same localities (Figure 9) trying as much as possible in the second survey to examine the same persons who were sampled in the first survey. The results of the blood film examinations and the filter papers with the blood spots were brought to the School of Hygiene and Tropical Medicine, London, for carrying out indirect fluorescent antibody tests on them and stored at -20°C.

2.4.3 Performance of the Indirect Fluorescent Antibody Test

Blood spots from each locality were tested separately. Sealed plastic bags containing the filter papers were taken out of the freezer and the papers were spread in a desiccator containing silica gel at room temperature for about 30 minutes to avoid condensation of vapour on the papers. With a calibrated puncher, circles of 1.5 cm diameter were cut from the blood spot in the filter paper. The disc of blood in the filter paper which contained the equivalent of 50 µl of blood is placed inside an LP3 plastic tube (6 x 1 cm) carefully numbered according to the sample number. The 50 µl sample is eluted in 0.4 ml of diluent PBS to give an approximate serum dilution of 1:16 (Figure 13). The blood is left to elute overnight at 4°C in refrigerator. After elution the filter paper was taken out of the tube and the
Fig. 12
Collection of blood spots in filter paper per serological test.

Fig. 13
Dilution of blood spots with PBS in plastic tubes.
**Fig. 12**

Collection of blood spots in filter paper per serological test.

**Fig. 13**

Dilution of blood spots with PBS in plastic tubes.
Fig. 12
Collection of blood spots in filter paper per serological test.

Fig. 13
Dilution of blood spots with PBS in plastic tubes.
blood is ready for testing. The antigen  *P. f. fieldi* slides were taken out of the freezer, unwrapped and quickly placed inside a desiccator containing silica gel to avoid condensation of water on the antigen slide. After drying the antigen slides were taken out of the desiccator, marked and numbered using a 'Texpen' or any other water insoluble ink marker. The slides are then placed in a humid chamber and the sample dilutions were added to the antigen spots using a 25 µl Eppendorf pipette and a separate tip for each sample. Care was taken to add the sample to its coinciding antigen spot number. The serum was then left to incubate with the antigen at room temperature for 30 minutes. After incubation the slides were transferred from the humid chamber to a Coplin jar full of PBS, rinsed quickly and refilled with fresh PBS. The Coplin jar was then placed on a mechanical shaker for 30 minutes during which time the PBS was changed twice. Sheep anti-human immunoglobulin conjugate (Wellcome Laboratories) was prepared by adding to 0.1 ml of conjugate 2.6 ml of PBS and 0.3 ml of 1% counterstain Evans blue to give a final dilution of 1:120. The slides were then removed from the wash and replaced in the moist chamber. Using a pipette the conjugate was distributed evenly over all the antigen slides making sure it covered all antigen spots. The slides were covered and incubated again for 30 minutes at room temperature. Excess conjugate was removed by gentle shaking in PBS in a Coplin jar, rinsed rapidly and the jar was refilled with
fresh PBS and placed on the shaker for 30 minutes with two PBS changes. The slides were dipped in acetone for 3 seconds to wash excess stain off, then in PBS. Excess fluid was removed from the slides by careful blotting, and the slides were put in a dry perspex chamber. A drop of buffered glycerol (pH 8 - 9) was placed on each slide and a coverslip was superimposed. Positive control serum sample, negative control serum and a saline-solution control were included with each test. Prior to microscopic examination the slides were dried carefully from excess glycerol with a tissue.
2.5 Microscopy

A Zeiss universal microscope with transmitted fluorescent illumination was used. The exciting light was produced by a 100W halogen (quartz iodine) lamp. A BG38 and two Balzer FITC4 interference primary excitation filters plus a 500 barrier filter were used. Specimens were examined with a x 25 NA 0.60 and/or X40 NA 0.75 Neofluor objectives coupled with a x 10 widefield eyepiece. All specimens showing the presence of fluorescence (apple green) were marked as positives (Figures 14 and 15).

All samples were first screened using the P. fieldi antigen to exclude the negatives and to have an idea of the level of antibody titre of each positive case. The intensity of fluorescence at dilution 1:16 was graded by eye from + to ++++, comparing it with the dilutions of a known positive control. All positives were then titrated in 4-fold serum dilutions 1:16 to 1:16384 and examined using P. falciparum antigen (95% to 97% of malaria cases in Gezira were P. falciparum).

The range of dilutions used depended on the initial grading. Experience showed that a + was equivalent to a titre 1:16, ++ = 1:64, +++ = 1:256; ++++ = 1:1024; and +++++ = 1:4096.

2.6 Results

Table 3 summarises the total numbers of people
Fig. 14
IFA test: A positive *P.falciparum* at dilution 1:16 (x 250 magnification)

Fig. 15
IFA test: A positive *P.falciparum* at dilution 1:16 (x 400 magnification)
Fig. 14
IFA test: A positive *P. falciparum* at dilution 1:16 (x 250 magnification)

Fig. 15
IFA test: A positive *P. falciparum* at dilution 1:16 (x 400 magnification)
Fig. 14
1a. A positive *P. falciparum* at dilution 1:16 (x 250 magnification).

Fig. 15
1b. A positive *P. falciparum* at dilution 1:6 (x 400 magnification).
Table 3. Total number of people tested in Gezira for the years 1977 and 1979 and percentage positive for antibodies

<table>
<thead>
<tr>
<th>Locality</th>
<th>Year</th>
<th>Total Population of localities surveyed</th>
<th>No. examined</th>
<th>% positive</th>
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<td>282</td>
<td>62</td>
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<td></td>
<td>1979</td>
<td>3,390</td>
<td>339</td>
<td>57</td>
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<tr>
<td>Central Gezira</td>
<td>1977</td>
<td>3,920</td>
<td>651</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>3,920</td>
<td>570</td>
<td>75</td>
</tr>
<tr>
<td>West Gezira</td>
<td>1977</td>
<td>5,045</td>
<td>518</td>
<td>70</td>
</tr>
<tr>
<td></td>
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<tr>
<td>North Gezira</td>
<td>1977</td>
<td>6,121</td>
<td>614</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>6,121</td>
<td>674</td>
<td>63</td>
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</tbody>
</table>
tested, in which all ages are combined, with positive antibody reactions in each of the four localities in 1977 and 1979. Out of 282 sera tested from South Gezira in 1977, 62% showed positive antibody reactions, while the 339 sera tested from the same area in 1979 showed a percentage positive of 57%. Although there was a 5% drop in the percentage of people with positive antibodies, the difference is not significant at 95% level ($p > 0.1 < 0.5$). The 614 samples of 1977 from North Gezira showed 72% positive, and the 674 sera collected in 1979 showed 63% positives. Again the difference here is not significant ($p > 0.1 < 0.5$).

In West Gezira, 518 sera tested in 1977 with 70% positivity, but the 213 samples of 1979 resulted in 40% positives showing a significant decrease ($p > 0.05 < 0.1$ at 95%).

In Central Gezira, the case is different from the previous three areas; while the other areas show a decrease in the percentage of people with positive antibodies reactions in 1979 from those examined in 1977, Central Gezira showed a significant rise from 27% positives out of 651 samples in 1977 to 75% positives out of 570 sera in 1979 (at the 95% level $p > 0.02 < 0.05$).

The distribution of antibodies to \textit{P. falciparum} by titre and age-group is shown in Tables 4, 5, 6 and 7 for the 4 areas, and illustrated in Figures 16, 17, 18 and 19. The Geometrical Mean Titre (GMT) was generally low in all areas at both times in all age groups. It
never exceeded 80 in the age-group 0 - 9 years. When the GMT was calculated excluding those positive at 16, in some of which the fluorescence might be non-specific, it did not go beyond 180 in the 0 - 9 age-group. A small rise in GMT in 1979 was observed in Central Gezira conforming with the increase in the percentage positive with antibody reactions.

In 1979 the sampling was concentrated on younger age-groups and an attempt was made to repeat as many individuals as possible from those who were surveyed in 1977. Table 8 shows the change in antibody patterns in subjects who were surveyed on both occasions. In South, West and North Gezira, the number of individuals who lost antibodies since the 1977 survey was higher than those who gained antibodies. The loss was significant in West Gezira ($p > 0.05 < 0.1$), but not in South and North Gezira. In Central Gezira a greater proportion of the population who were retested in 1979 gained antibodies. 54.8% of those who were negative in 1977 became positive while only 10.7% of those positive in 1977 lost their antibodies. The difference was significant when tested at the 95% confidence limits ($p > 0.05 < 0.1$).

Parasitological Results

Table 9 summarises the microscopical results of slide examinations. The numbers positive were very low in the 4 areas in both surveys in 1977 and 1979.
The positivity rate ranges between 0% to 2.35%, which was far below the antibody positivity rates detected by the IFA test.

Table 10 shows the annual parasite rates in school children 2 - 9 years in 27 indicator localities surveyed annually during the month of January by the malaria control team in Gezira. These 27 localities include the 18 localities surveyed in this study in 1977 and 1979. The pre-control result of January 1975 showed a parasite rate of 20.4%, then a clear decrease from 5.2% during the first year of control to 0.67% in January 1979. The table also summarises the slide positivity rates in passive case detection posts (PCD) in two councils of Gezira. 9 of the 18 localities surveyed in our study fall inside those two councils. Again it can be seen from the table that the slide positivity rate which was 22.4% before the application of control measures in 1975 dropped to 1.4% in 1978.
Table 4. Gezira Seroepidemiological Surveys 1977/79: Prevalence of antibodies to *P. falciparum* by titre and age-group - SOUTH GEZIRA

<table>
<thead>
<tr>
<th>Age-group</th>
<th>Year</th>
<th>No. examined</th>
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<th>16</th>
<th>64</th>
<th>256</th>
<th>1024</th>
<th>4096</th>
<th>% +ve</th>
<th>GMT</th>
<th>% +ve excluding 16</th>
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<td>2-4 years</td>
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<td>123</td>
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<td>5-9 years</td>
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<td>24</td>
<td>19</td>
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<td>5</td>
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Table 4. Gezira Seroepidemiological Surveys 1977/79: Prevalence of antibodies to *P. falciparum* by titre and age-group - SOUTH GEZIRA

<table>
<thead>
<tr>
<th>Age-group</th>
<th>Year</th>
<th>No. examined</th>
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<th>16</th>
<th>64</th>
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<th>GMT</th>
<th>% +ve excluding 16</th>
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### Table 5. Gezira Seroepidemiological Surveys 1977/79: Prevalence of antibodies to *P. falciparum* by titre and age-group - WEST GEZIRA

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Table 6. Gezira Seroepidemiological Surveys 1977/79: Prevalence of antibodies to P. falciparum by titre and age-group - NORTH GEZIRA

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<th>GMT</th>
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<td>0</td>
<td>50</td>
<td>16</td>
<td>0</td>
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</tr>
<tr>
<td>2-4 years</td>
<td>1977</td>
<td>60</td>
<td>35</td>
<td>12</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>42</td>
<td>42</td>
<td>22</td>
<td>98</td>
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<tr>
<td></td>
<td>1979</td>
<td>82</td>
<td>40</td>
<td>22</td>
<td>17</td>
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<td>0</td>
<td>51</td>
<td>32</td>
<td>24</td>
<td>79</td>
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<td>5-9 years</td>
<td>1977</td>
<td>346</td>
<td>105</td>
<td>91</td>
<td>104</td>
<td>38</td>
<td>7</td>
<td>1</td>
<td>69</td>
<td>52</td>
<td>43</td>
<td>106</td>
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<td></td>
<td>1979</td>
<td>586</td>
<td>210</td>
<td>188</td>
<td>114</td>
<td>48</td>
<td>24</td>
<td>2</td>
<td>64</td>
<td>47</td>
<td>32</td>
<td>136</td>
</tr>
<tr>
<td>10-14 years</td>
<td>1977</td>
<td>164</td>
<td>28</td>
<td>36</td>
<td>66</td>
<td>30</td>
<td>4</td>
<td>0</td>
<td>83</td>
<td>65</td>
<td>61</td>
<td>108</td>
</tr>
<tr>
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<td>1979</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>15-19 years</td>
<td>1977</td>
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<td>0</td>
<td>1</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>20-39 years</td>
<td>1977</td>
<td>19</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>2</td>
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</tr>
<tr>
<td>&gt; 40 years</td>
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<td>16</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>86</td>
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<td>81</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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Table 7. Gezira Seroepidemiological Surveys 1977/79: Prevalence of antibodies to *P. falciparum* by titre and age-group - CENTRAL GEZIRA

<table>
<thead>
<tr>
<th>Age-group</th>
<th>Year</th>
<th>No. examined</th>
<th>&lt;16</th>
<th>16</th>
<th>64</th>
<th>256</th>
<th>1024</th>
<th>4096</th>
<th>% +ve</th>
<th>GMT</th>
<th>% +ve excluding 16</th>
<th>GMT excluding 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24 months</td>
<td>1977</td>
<td>15</td>
<td>12</td>
<td>3</td>
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<td>0</td>
<td>0</td>
<td>20</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>12</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>42</td>
<td>16</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>2-4 years</td>
<td>1977</td>
<td>137</td>
<td>113</td>
<td>14</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>18</td>
<td>34</td>
<td>7</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>112</td>
<td>33</td>
<td>33</td>
<td>31</td>
<td>10</td>
<td>5</td>
<td>71</td>
<td>51</td>
<td>41</td>
<td>117</td>
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<tr>
<td>5-9 years</td>
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<td>247</td>
<td>57</td>
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<td>16</td>
<td>5</td>
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<td>45</td>
<td>15</td>
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</tr>
<tr>
<td></td>
<td>1979</td>
<td>302</td>
<td>65</td>
<td>47</td>
<td>91</td>
<td>77</td>
<td>20</td>
<td>79</td>
<td>100</td>
<td>63</td>
<td>156</td>
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</tr>
<tr>
<td>10-14 years</td>
<td>1977</td>
<td>97</td>
<td>68</td>
<td>10</td>
<td>13</td>
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<td>53</td>
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<td>99</td>
<td></td>
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<td></td>
<td>1979</td>
<td>132</td>
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<td>19</td>
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<td>6</td>
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<tr>
<td>15-19 years</td>
<td>1977</td>
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<td>1</td>
<td>3</td>
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<td>0</td>
<td>25</td>
<td>45</td>
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<td>64</td>
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<td></td>
<td>1979</td>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>100</td>
<td>1024</td>
<td>1024</td>
</tr>
<tr>
<td>20-39 years</td>
<td>1977</td>
<td>18</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>17</td>
<td>102</td>
<td>11</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>100</td>
<td>256</td>
<td>100</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>&gt; 40 years</td>
<td>1977</td>
<td>11</td>
<td>7</td>
<td>0</td>
<td>3</td>
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<td>0</td>
<td>36</td>
<td>91</td>
<td>36</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>100</td>
<td>362</td>
<td>100</td>
<td>362</td>
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</tr>
</tbody>
</table>
Fig. 16

Total malaria antibodies by age-group
SOUTH GEZIRA 1977-1979

% with antibodies

AGE IN YEARS
Fig. 17

Total malaria antibodies by age-group
WEST GEZIRA 1977 & 1979

% WITH ANTIBODIES

AGE IN YEARS
Fig. 18

Total malaria antibodies by age-group
NORTH GEZIRA 1977 & 1979

% WITH ANTIBODIES

0 10 20 30 40 50 60 70 80 90 100

0 5 10 15 20 25 30 35 40

AGE IN YEARS
Fig. 19

Total malaria antibody by age-group
CENTRAL GEZIRA 1977 & 1979

% WITH ANTIBODIES

AGE IN YEARS
Table 8. Gezira Seroepidemiological Surveys 1977/1979: Change in antibody patterns in cases repeated in 1979 from those surveyed in 1977

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number %</td>
<td>Number %</td>
<td>Number %</td>
<td>Number %</td>
</tr>
<tr>
<td>South Gezira</td>
<td>14/71 19.7</td>
<td>4/71 5.6</td>
<td>14/71 19.7</td>
<td>13/71 18.3</td>
</tr>
<tr>
<td>Central Gezira</td>
<td>92/168 54.8</td>
<td>25/168 14.9</td>
<td>18/168 10.7</td>
<td>2/168 1.2</td>
</tr>
<tr>
<td>West Gezira</td>
<td>6/99 6.1</td>
<td>13/99 13.1</td>
<td>45/99 45.5</td>
<td>18/99 18.2</td>
</tr>
<tr>
<td>North Gezira</td>
<td>16/122 13.1</td>
<td>11/122 9.0</td>
<td>34/122 27.9</td>
<td>20/122 16.4</td>
</tr>
</tbody>
</table>
Table 9. Microscopical results of slide examination in Gezira 1977 and 1979

<table>
<thead>
<tr>
<th>Area</th>
<th>Year</th>
<th>Total No. examined</th>
<th>No. positive</th>
<th>Species</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Gezira</td>
<td>1977</td>
<td>282</td>
<td>1</td>
<td>P. falciparum</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>339</td>
<td>2</td>
<td>P. falciparum</td>
<td>0.59</td>
</tr>
<tr>
<td>Central Gezira</td>
<td>1977</td>
<td>651</td>
<td>0</td>
<td>P. falciparum</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>570</td>
<td>12</td>
<td>P. falciparum</td>
<td>2.10</td>
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<tr>
<td>West Gezira</td>
<td>1977</td>
<td>518</td>
<td>1</td>
<td>P. falciparum</td>
<td>0.19</td>
</tr>
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<td>1979</td>
<td>213</td>
<td>5</td>
<td>P. falciparum</td>
<td>2.35</td>
</tr>
<tr>
<td>North Gezira</td>
<td>1977</td>
<td>614</td>
<td>1</td>
<td>P. falciparum</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>674</td>
<td>4</td>
<td>P. falciparum</td>
<td>0.59</td>
</tr>
</tbody>
</table>
Table 10**  Parasite Rates in school children (2-9 years) of 27 indicator villages* and slide positivity rates in P.C.D. centres of two rural councils in Gezira Province - Sudan

<table>
<thead>
<tr>
<th>Year</th>
<th>January Survey</th>
<th>Sept./Oct. survey</th>
<th>Medina Arab Council</th>
<th>El Hosh Council</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ex.</td>
<td>+</td>
<td>%</td>
<td>Ex.</td>
</tr>
<tr>
<td>1975</td>
<td>2017*</td>
<td>412</td>
<td>20.43</td>
<td>11,809</td>
</tr>
<tr>
<td>1976</td>
<td>2197</td>
<td>115</td>
<td>5.23</td>
<td>11,637</td>
</tr>
<tr>
<td>1977</td>
<td>4040</td>
<td>80</td>
<td>1.98</td>
<td>-</td>
</tr>
<tr>
<td>1978</td>
<td>2855</td>
<td>5</td>
<td>0.18</td>
<td>12,890</td>
</tr>
<tr>
<td>1979</td>
<td>2528</td>
<td>17</td>
<td>0.67</td>
<td>-</td>
</tr>
</tbody>
</table>

* Only 16 indicator villages were included in the January survey of 1975.

** From the Annual Report of Malaria Control Programme, 1979.
2.7 Conclusion

The results of serological surveys carried out in mid 1977 and early 1979 from 18 localities in 4 areas representing most of the Gezira rural councils suggested strongly that the prevalence of malaria as shown by the IFA test is quite high despite the intensive control measures which started in mid 1975, and contrasts with the parasitological results shown in Tables 9 and 10. The parasite rates among children of 2 - 9 years of age dropped from nearly 2% in 1977 to about 0.2% in 1978, and then slightly rose to about 0.7% in 1979 (Table 10). The slide positivity rates in Passive Case Detection (PCD) centres in Medina Arab and El Hosh Rural Councils was 1.27% and 1.38% in 1977 and 1978 respectively for Medina Arab, and 4.05% and 1.35% in 1977 and 1978 for El Hosh (Table 10), while antibody positivity rate ranges between 27% to 75% in the 4 areas surveyed.

There are a number of reasons for this discrepancy between antibody rates and parasite rates. As has already been discussed, it is known that antibodies may persist for ages, especially if malaria infections were not adequately treated and radically cured, and that antibodies in older children and adults might be due to infections experienced before control started. However, a significant number of antibody reactions was found in the two youngest age groups (0 - 24 months and
2 - 4 years) in all areas, who have been born after the control started. These ranged from 20% to 70%.
The rise in the percentage positive with antibodies, particularly in 1979 showed that transmission had been continued recently.

Although the antibody rates clearly indicated that transmission is being recently going on, actual parasite rates were very low. Several factors might have contributed to this. Older children examined would be semi-immune from their experience with previous infections with malaria, so that new infections would be of low density and could well be missed by standard techniques used for parasite examination. Another factor is that most of those semi-immune individuals get a mild infection with no severe symptoms and thus they do not report to malaria PCD posts, especially if they are far from where they live. However this might not be applied in case of the youngest age group (0 - 24 months). A more important factor depressing the parasite rates might be the widespread availability and use of antimalarial drugs which can easily be obtained in most areas and which is the drug of choice for treatment of any fever, bone-ache or even headache. Even small doses of drugs can depress the parasitaemia to sub-microscopical level, but would not prevent the development of antibodies.

The continuation of transmission in all areas was confirmed by figures for incidence in Table 8. In
all areas there were conversions from negative to positive reactions for antibodies and also some individual increases of titre which could be due to fresh infections.

The above evidence from cross-sectional prevalence and from incidence data clearly shows that transmission was continuing in all areas. This was least in West Gezira and the figures might suggest a drop in the transmission rate. This might be attributed to the fact that the intensity of irrigation in this area is less than in other areas, and that rice plantation which was introduced to other parts of Gezira did not reach the western part yet. In North and South Gezira it seems that the level of transmission remains nearly the same; however in Central Gezira the figures suggest that there has been an increase in transmission from 1977 to 1979. This is an area where resistance of the vector A. arabiensis to malathion has been detected during 1977 (Chapter 6).

These studies have confirmed the values of using antibodies studies for monitoring malaria transmission particularly at lower levels. Parasite rates measure only the point prevalence of malaria and for reasons given above may underestimate this. On the other hand antibody rates give a measure of the period prevalence in that they show not only those who have active infections, but also those who had infection or infections in previous year or years. For incidence studies measurement of antibodies is obviously more sensitive than looking for parasites.
CHAPTER THREE

RESISTANCE OF HUMAN MALARIAL PARASITES TO ANTI-MALARIAL DRUGS WITH SPECIAL REFERENCE TO AFRICA
3. INTRODUCTION

The problem of drug resistance of human malaria parasites became significant only since 1948 - 1950 with the discovery of proguanil resistance in *Plasmodium falciparum* and *P. vivax* in Malaya (WHO, 1961). Further observations on proguanil resistance were reported in local strains of *P. falciparum* in Assam, New Guinea, Vietnam and several parts of Malaya. Local strains of *P. falciparum* and *P. malariae* from Indonesia were also reported to be resistant to this drug. Reports of cross-resistance to pyrimethamine of proguanil-fast strains of *P. falciparum* and *P. malariae* came from different parts of Africa, Venezuela and New Guinea. During this period observations of lessened sensitivity of some strains of malaria parasites to quinine, pamaquine and mepacrine were reported from different parts of the world (WHO, 1961). The real danger showed its head when reports about chloroquine-resistant strains of *P. falciparum* started to come from different parts of the world specially South East Asia, South America and Tropical Africa.

3.1 Definition of Drug Resistance

Pampana (1963) defined resistance to antimalarial drugs as the capacity of a particular strain to survive when the drug has been administered to the vertebrate host at a dose that would normally destroy the
parasites in the same stage of life cycle.

The WHO Scientific Group on Resistance of Malaria Parasites to Drugs (WHO, 1965) defined resistance as the "ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject".

3.2 Grading of the Response of P. falciparum malaria to the 4-aminoquinolines administered in vivo

a. Standard technique with 28 days' follow up

In clinical practice, as far as resistance is concerned, one meets with an entire spectrum of response ranging from the anticipated norm to a complete failure. The response of P. falciparum infections to the standard 1.5 gramme of chloroquine base given during 3 days was graded by a WHO scientific group on Chemotherapy of Malaria (WHO, 1967) as follows:

<table>
<thead>
<tr>
<th>Response</th>
<th>Recommended symbol</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>S</td>
<td>Clearance of asexual parasitaemia within 7 days from the first day of treatment, without recrudescence.</td>
</tr>
<tr>
<td>Resistance</td>
<td>RI</td>
<td>Clearance of asexual parasitaemia as in sensitivity, followed by recrudescence</td>
</tr>
<tr>
<td></td>
<td>RII</td>
<td>Marked reduction of asexual parasitaemia, but no clearance</td>
</tr>
</tbody>
</table>
b. Alternative test with single-dose treatment

WHO (1973) adopted an alternative test using a single dose of 10 mg of chloroquine base per kilogram of body weight instead of the 3-day regimen. The observation period is limited to 7 days and the test is interpreted as the Standard Field Test.

3.3 In vitro testing for drug resistance

Rieckmann et al. (1968) employed a simple in vitro test which is a modification of the technique described by Bass and Johns (1912), to study the effects of chloroquine, quinine, and cycloguanil upon the maturation of asexual erythrocytic forms of two strains of *P. falciparum*. The two strains used were Uganda I strain which was first obtained from a child in Kampala by Martin and Arnold (1967) and which was known to be sensitive to chloroquine. The other strain is a drug-resistant strain from Malaysia, termed the Malayan (Camp) strain first found by DeGowin and Powell (1965). The blood used in these studies was obtained from volunteers; each group infected with one of the two strains. The experimental system which excluded the influence of host immunity on
drug response consists of the following steps:

1. Small samples of venous blood (10 to 15 ml) were obtained from persons having patent parasitaemia.

2. Blood was defibrinated (swirled gently for 5 minutes in 25 ml Erlenmeyer flasks containing glass beads).

3. One ml aliquots of each sample of defibrinated blood were placed in screw-cap, flat-bottomed glass vials (6 cm tall, 1.5 cm in internal diameter) that contained added glucose (5 mg) and either no drug (control samples) or drug added in various amounts (experimental samples).

4. Blood in the vials was swirled gently to mix the contents well, and the vials were then placed in an incubator at 38.5° to 40°C for 24 hours (without agitation during incubation).

5. The vials were shaken gently after incubation to remix the contents well.

6. Giemsa-stained thick films of blood were prepared.

7. One hundred asexual parasites, observed consecutively, were examined and classified according to morphological characteristics evident on examination with conventional light microscopy.

For convenience, solutions of glucose and of drugs were added to vials days or weeks in advance of the experiments and water was then allowed to evaporate at a temperature not exceeding 40°C.

They reached, at the end of the experiment, the conclusion that the two strains of P. falciparum which they used for their experiment and which had been studied
in vivo, a chloroquine-sensitive strain from Uganda (Uganda I; Martin and Arnold, 1967) and a chloroquine-resistant strain from Malaya (Malayan (Camp), DeGowin and Powell, 1965) behaved differently in vitro with the addition of the different antimalarial drugs they used. Chloroquine, quinine and cycloguanil inhibited the maturation of trophozoites of the Uganda strain to a greater extent than the maturation of trophozoites of the Malayan strain. This proves that the Uganda strain is a chloroquine-sensitive one and can be used as a base to compare the sensitivity of other African P. falciparum strains to chloroquine.

3.4 Chloroquine-resistant P. falciparum in Africa

For the control and eradication of malaria from hyperendemic regions of tropical Africa, antimalarial drugs were used in addition to measures directed against the mosquito. Individuals having different degrees of immunity need different dosages of antimalarial compounds to suppress or cure their infections.

The appearance of chloroquine-resistant strains of P. falciparum in areas where there is persistence of malaria transmission in so-called "problem areas" in spite of eradication procedures with drugs and residual insecticide, implies a great danger to the countries concerned like those of South and Central America, South East Asia, and Africa. The World Health Organization foreseeing
the danger of chloroquine-resistant strains of *P. falciparum* spreading in areas of Tropical Africa where chloroquine is widely used for prophylaxis, suppression and treatment of malaria cases with a very high percentage of the cases caused by *P. falciparum* stimulated interest in fundamental research to assess the response of *P. falciparum* to chloroquine and other 4-aminoquinoline drugs used. A quick need to establish a baseline for the response of the parasite to chloroquine was felt by all countries concerned, and both *in vivo* and *in vitro* tests to draw that line and to discover any strain of *P. falciparum* resistant to chloroquine as soon as it appeared were on the run.

The first *P. falciparum* strain which showed a distinctly lower sensitivity to proguanil was isolated by Covell et al. (1949) in Lagos, Nigeria. Other foci of proguanil resistance were later exposed during field trials of proguanil derivative, cycloguanil pamoate (Camolar, Cl-501) by Payet et al. (1966) in Senegal and Lunn et al. (1964) in Southern Rhodesia. Clyde (1965) reported the appearance of proguanil resistance in Tanzania during the course of a trial in the field of cycloguanil pamoate. Avery Jones (1954) was the first to report pyrimethamine resistant *P. falciparum* in Kenya. The second report came in the same year from Tanzania by Clyde (1954). Primary pyrimethamine resistance is now widespread in *P. falciparum* infections in much of West
The first report of possible chloroquine-resistance in Africa reached the WHO in a communication made as early as 1964 from Ouagadougou Hospital in Upper Volta. Late in the same year, a WHO consultant visited the region to investigate the evidence for the alleged chloroquine-resistance. Since the consultant had no opportunity at that time to carry out field tests, the results of the investigation were considered inconclusive (WHO, 1965).

In 1965 Loasch and N'Guyen published a detailed report about the resistant cases they reported before to WHO in 1964. In the study they investigated the response of *P. falciparum* to chloroquine in 15 children whom they kept in Ouagadougou Hospital in the central part of Upper Volta. Nine of the cases demonstrated breakthrough while under massive suppressive doses of chloroquine. In the other six cases the *P. falciparum* infection could not be controlled by large dosages of chloroquine. In one of these cases there was no reaction whatsoever to treatment, and the other five relapsed soon after the infection had apparently been controlled. They concluded that their results gave good reason to suspect that one or several chloroquine-resistant strains of *P. falciparum* exist in this part of West Africa.

Schwendler (1965) reported chloroquine-resistant
strains of *P. falciparum* among the indigenous (semi-immune) population of Ghana. Jeffery and Gibson (1966) following the procedures recommended by the WHO Scientific Group on Resistance of Malaria Parasites to Drugs (1965), studied and followed 65 cases out of 78 cases of *P. falciparum* in Ouagadougou region of Upper Volta, West Africa and 72 cases from Kpawin, Liberia. All cases were treated with a regimen of 10 mg/kg body weight dose of chloroquine base. They concluded that all cases were cleared of parasitaemia within the three-day limit and thus no resistance to chloroquine can be claimed among these subjects studied. In 1967 a WHO Scientific Group on the Chemotherapy of Malaria (WHO, 1967) concluded that all the scattered reports about the presence of chloroquine-resistance in various parts of Africa, e.g. Upper Volta, Liberia, Ghana, Eastern Nigeria, Conakry, Guinea, Malawi, Tanzania, Uganda and Madagascar, had shown no substantial evidence of resistance after critical field assessments. Also the report recommended that detailed studies of the response of *P. falciparum* to 4-aminoquinolines have yet to be carried out in many parts of tropical Africa (WHO, 1967).

Himpoo and MacCallum (1967) reported three cases of *P. falciparum* with possible chloroquine-resistance from Zambia. The parasites in the three patients did not respond to normal regimen of chloroquine, but they were radically cured when quinine was used. Wolfe and Hudleston (1969) carried out a study on 61 malaria patients who all
had *P. falciparum* infection, to see the response of the parasite to chloroquine treatment. Of the 47 patients who completed the treatment and the necessary follow-up period, 46 were free of parasites on the 7th day after commencement of treatment. The remaining case, who was free of parasites on the fourth to the sixth day, had one parasite in the blood smear on the seventh day. This study which was carried in the Kabwe (Broken Hill) hospital in Zambia failed to substantiate the report of Himpoo and MacCallum (1967) of a suspected chloroquine-resistant strain of *P. falciparum* in the area of Kabwe, Zambia.

Peters (1969) in his paper 'Drug Resistance in Malaria - A perspective', which he read in a meeting of the Royal Society of Tropical Medicine and Hygiene in December 1968, stressed that up to the present time none of the reports of chloroquine-resistant *P. falciparum* infections which came from East and West Africa had been substantiated by critical evaluation and no evidence of resistance has been found in these localities. Bruce-Chwatt (1970) discussed all reports on resistance of *P. falciparum* to chloroquine in Africa and he also concluded that *P. falciparum* strains in Africa are still susceptible to treatment by a single dose of 10 mg/kg body weight of chloroquine base, and so there is no evidence from studies carried out *in vivo* (i.e. in man) of chloroquine-resistance in Africa.

Peters and Seaton (1971) employed the Rieckmann
test using chloroquine-containing tubes and control tubes containing glucose only supplied to them by Riechmann himself to test the in vitro sensitivity to chloroquine of \textit{P. falciparum} obtained from two non-immune white patients who became infected in West Africa and developed clinical malaria after their return to Liverpool. Both men had been taking suppressive proguanil but in each case the infection appears to have broken through to produce a low level of parasitaemia and fever. The test was run following precisely the technique that was described by Rieckmann et al. (1968). The results obtained indicated that the \textit{P. falciparum} acquired by these patients (one in Guinea and the other in Nigeria) responded in a manner almost identical to the Uganda I strain (Martin and Arnold, 1967). The infections responded promptly to "standard" oral treatment with chloroquine (1.5 g base over three days) which produced a radical cure.

Bruce-Chwatt and Roberts (1972) criticized severely a report published by Voshinaga et al. (1970). Their claim that they found a chloroquine-resistant strain of \textit{P. falciparum} in Kenya was proved to be false because of the unconventional method which they used for examining the patients' blood for their experiment's evaluation, and their failure to state the amount of chloroquine base they used for treatment.

Rieckmann (1972) used his \textit{in vitro} technique (Rieckmann \textit{et al.}, 1968) to assess the sensitivity of
P. falciparum to chloroquine at Kisumu, Kenya and Lagos, Nigeria. Blood from 26 children from Kenya and 8 children from Nigeria was cultured in vitro using concentrations of chloroquine of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 nmol/ml of blood. The results showed that all cases from both areas are sensitive to chloroquine and there was no sign of a chloroquine-resistant strain of P. falciparum.

Ansdell et al. (1974) reviewed malaria cases in Birmingham, United Kingdom in the period 1968 - 1973. Of the 70 malaria patients who all acquired their infections abroad, 44 were in the Indian subcontinent, 23 in Africa and three in New Guinea. Out of those 70 patients 48 had P. vivax, 20 P. falciparum and one each P. ovale and P. malariae. Four of the patients with malignant tertian malaria due to P. falciparum did not respond to chloroquine in a dose of 25 mg/kg body weight over three days. They only improved when they were given quinine. All the four patients had contracted the infection in Nigeria.

Denis et al. (1974) treated and followed 41 subjects with falciparum malaria in Ethiopia for 28 or more days after giving them a single 10 mg/kg body weight dose of chloroquine. Recrudescences were detected in 11 cases out of the 41 subjects studied and, in every case, it occurred during the fourth week after treatment. Regarding all their patients as non-immune they concluded
that the parasites had shown low-grade of refractoriness to chloroquine in vivo. Also they attempted in vitro testing with parasites isolated from 37 of the 41 patients and blood specimens from 10 of the 11 patients who had relapses were cultured during both the initial and recrudescent parasitaemias. They had successful growth in only 25 cases, and 9 isolates showed development in chloroquine concentrations of 0.5 to 1.0 millimicromoles per cc. of blood. 3 isolates with development at the 1.0 millimicromole level were from patients who experienced a recrudescence of parasitaemia. These results of the in vitro tests agreed with those of the in vivo suggesting the presence of a chloroquine responsive isolate of *P. falciparum* in Ethiopia which is between the sensitive Uganda I strain and the resistant Malayan (Camp) strain.

Armstrong *et al.* (1976) studied in vivo 150 subjects in four localities of Ethiopia. All the 150 subjects who had *P. falciparum* malaria were treated with the single test dose of chloroquine (10 mg base/kg body weight) (WHO, 1965) and followed for periods of 6 or 11 days. The results showed that all test subjects were cleared from trophozoites by day 3 after treatment. Palmer *et al.* (1976) carried out in vitro tests for chloroquine sensitivity of *P. falciparum* in three regions in Ethiopia out of the four regions in which previous in vivo tests were carried out by Armstrong *et al.* (1967). Rieckmann technique (Rieckmann, 1968) was followed in the
study. Out of 114 blood cultures, 82 only were successful. Preliminary results obtained from one area showed that 0.5 nmol/ml of chloroquine failed to prevent the formation of schizonts in 6 of 24 cultures. In the other two areas, out of the 58 successful cultures, schizogony was completely inhibited in 43 of the experimental samples at 0.5 nmol/ml, in 8 additional samples at 0.75 nmol/ml and in 5 samples inhibition occurred only at 1.0 nmol/ml, while in the last 2 samples schizonts were seen even at 1.0 nmol/ml of chloroquine. They concluded that their results were initially suggestive of decreased drug sensitivity when compared to the Uganda I strain of *P. falciparum*, although growth of schizonts in vials containing up to 1.0 nmol/ml of chloroquine may be the cut-off point between sensitive and resistant falciparum infections under the RI classification used by WHO (1968).

Olatunde (1977) reviewed the subject of *P. falciparum* resistance to chloroquine in the world referring to all studies and reports from 1961. In Africa he wrote, "Within the past three to four years, however, there have been a number of indications which suggest the presence in Africa of strains of *P. falciparum* more resistant to chloroquine than had been earlier reported or confirmed". He mentioned that the Ethiopian report of the presence of strains of *P. falciparum* malaria more resistant to chloroquine than was earlier confirmed is the most convincing. He summarized the experience
of five medical practitioners concerning malaria responsiveness to chloroquine in four different hospitals in the South-western States of Nigeria between 1972 and 1974. Out of their 7 malaria patients who were given 25 mg chloroquine base/kg body weight divided over three days, 3 showed delayed response and 4 showed no response at all. All were treated with other antimalarial drugs. He concluded that although clinical observation is of doubtful value in the absence of blood examination both before and after treatment, it is hoped that the previously mentioned reports will alert malariologists, public health practitioners and governments in Africa to this potentially dangerous possibility and will stimulate further work in line with WHO guidelines.

Smalley (1977) studying the effect of chloroquine on the development of \( \text{P. falciparum} \) gametocytes in The Gambia, West Africa, first established a base line data for the chloroquine sensitivity of asexual parasites of \( \text{P. falciparum} \). Using the \textit{in vitro} test of Rieckmann \textit{et al.} (1968) he found that in almost all cases 0.5 nmols of drug per ml of blood completely inhibited the formation of schizonts.

Khan and Maguire (1978) reported a case of \( \text{P. falciparum} \) resistant to chloroquine in Zambia at the degree RII category according to the classification of resistance by WHO (1965). They reported that they suspected more resistant cases, but because the patients
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Khan and Maguire (1978) reported a case of P. falciparum resistant to chloroquine in Zambia at the degree R11 category according to the classification of resistance by WHO (1965). They reported that they suspected more resistant cases, but because the patients
were very ill they were unable to prove the resistance according to the WHO criteria (WHO, 1965).

Omer (1978a, b) studied the response of *P. falciparum* to oral chloroquine in two regions of the Sudan, one region in Southern Sudan (Bor) and the other in Northern Sudan (Gezira). 40 patients in Bor district with *P. falciparum* were treated with 25 mg chloroquine phosphate base per kg body weight orally divided over a period of three days. None of the 40 patients who were followed for 7 days after taking the drug showed asexual parasites in his blood. 506 patients in Gezira district were studied and a 28-day follow-up was carried out. All patients except three responded completely to oral chloroquine showing high sensitivity of the parasite to chloroquine. In one patient the parasitaemia did not clear but there was marked reduction, indicating the parasite may present an RII resistance (WHO, 1973). In the other two patients the parasites disappeared between days 3 and 14 of follow-up, but in both trophozoites were present on day 28. They were considered to have stage RI resistance (WHO, 1973), but the possibility of reinfection could not be ruled out. On the whole the study showed that *P. falciparum* in those two regions is highly sensitive to chloroquine.

and one from Tanzania. One of the Kenyan two cases developed the disease after his return to New York. He was treated with chloroquine on two occasions, and each time his malaria recrudesced. He was finally treated successfully with a drug effective against chloroquine resistant \textit{P. falciparum}. The other patient who had been touring in Kenya, and had been taking the recommended chloroquine prophylaxis while in Kenya and for 2 weeks after returning to Denmark developed \textit{P. falciparum} malaria 19 days after his return to Denmark. He was treated with chloroquine, but he had four recrudescences in about four months. After the fourth recrudescence he was treated with pyrimethamine and sulfadoxine, and no parasites were seen in his blood 4 days later. After 4 months no recrudescence has been detected, and the patient is presumed cured. The Tanzania case appeared in Atlanta, Georgia. He reported with \textit{P. falciparum} malaria to an Atlanta hospital after 6 days from coming back to the States from his safari. After one month from taking the chloroquine treatment the malaria recrudesced. The patient was then treated with trimethoprin and sulfamethoxazole. By the second day of therapy, the smears became negative. The patient remains asymptomatic. These three cases were reported in detail by Kean (1979), Fogh \textit{et al.} (1979) and Campbell \textit{et al.} (1979) respectively. Fogh \textit{et al.} (1979) referred to their case as the first confirmed \textit{P. falciparum} malaria in Kenya, resistant to
chloroquine at the level of RI delayed recrudescence according to the WHO criteria (WHO, 1973). The results of the in vivo and in vitro observations on the Tanzanian (later named I/CDC) strain provide the most conclusive evidence that chloroquine-resistant *P. falciparum* is being transmitted in Africa.

Nguyen-Dinh and Trager (1978) succeeded in producing a chloroquine-resistant African strain of *P. falciparum* using a continuous in vitro culture technique. The strain (FCR-3 CFMC) was originally a chloroquine-sensitive one isolated in 1976 from a patient in the Gambia, West Africa, and since then was kept in continuous culture in their laboratory. The parasite growth used to be completely inhibited by a 48-hour exposure to a concentration of 0.1 µg of chloroquine base per millilitre of medium in repeated experiments over 8 months. By progressively increasing the concentration of chloroquine in the culture medium they managed to grow the parasite in the presence of 0.13 µg/ml and they called the resistant strain R-FCR-3. Their study in vitro indicates that an African strain of *P. falciparum* has the genetic capability to develop chloroquine resistance. They concluded that epidemiological factors unfavourable to the emergence of resistant strains might account for their absence from Africa so far, but this situation is not necessarily stable, and chloroquine resistance represents a real threat to Africa (Figure 20).
Fig. 20.

Localities in Africa where the response of P. falciparum to Chloroquine has been critically studied - 1979

- Strain proved sensitive
- Strain suspected resistant at RI level
- Strain proved resistant

Up dated from WHO 1973
3.5 Chloroquine-resistant *P. falciparum* in South and Central America

Maberti (1960) brought to notice instances of alleged failure of chloroquine to clear pyrimethamine-resistant trophozoites of *P. falciparum* in a limited area of western Venezuela. Moore and Lanier (1961) reported the first substantial observation of possible chloroquine-resistance in *P. falciparum* from Colombia. They studied two cases of non-immune patients who had recurrent attacks of *P. falciparum* malaria in spite of oral treatment by chloroquine in a dose of 1.5 gramme base over three days. Both cases were cured by a 10-day course of quinine. The WHO Regional Office for the Americas sent a team to investigate the focus from which the above two reported cases acquired their infection in Colombia, but the team came up with no solid evidence about a chloroquine-resistant strain of *P. falciparum* in that area (WHO, 1961).

Young and Moore (1961) studied a case of a patient who acquired *P. falciparum* infection in Colombia in May 1960 and his parasites did not react to the normal chloroquine treatment. After coming to the U.S.A. he had a recrudescence of malaria and received 2.1 grammes of chloroquine diphosphate, but after 5 days from treatment he was found to be still harbouring asexual parasites in his peripheral blood. Blood from this patient was
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injected into 7 neurosyphilitic patients. In all seven patients the normal curative dose of 1.5 grammes and abovenormal dose of 2.1 grammes of chloroquine phosphate base did not cure the malaria infection. They concluded from their findings that this strain of *P. falciparum* is resistant to chloroquine. This strain was tested against other members of the 4-aminoquinoline group (amodiaquine (Camoquin) and hydroxychloroquine (Plaquinil)) and was found to be resistant to both of them. The 7 cases responded to quinine and mepacrine but relapses occurred (Young, 1961). Out of these cases a single case treated with pyrimethamine appeared to respond normally. Young (1962) studied the suppressive action of chloroquine and amodiaquine on *P. falciparum* strain of Colombia which proved resistant to radical cure with those two drugs. The study was carried out on 8 neurosyphilitic patients. 3 were given 300 mg of chloroquine base weekly, 3 given 400 mg of a modiaquine base weekly, and 2 were given no drug and kept as controls. Three days after starting the drug the patients were bitten by *Anopheles quadrimaculatus* infected with the resistant strain of *P. falciparum* of Colombia. The average plasma daily levels of the drug were much greater than the 10 - 30 μg/litre, considered to be the amount which cures infections with susceptible strains of *P. falciparum*. All the patients showed parasitaemias and fevers at about the same time. The infections were challenged with other drugs. Two
patients each were given either mepacrine (Catabrine), pyrimethamine (Daraprim), proguanil (Paludrine) and chlorproguanil (Lapudrine). All of the infections were eliminated, but those receiving mepacrine and chlorproguanil relapsed indicating that the parasite has cross-resistance, while the infections treated with pyrimethamine or proguanil cleared with no relapses, showing that there is no cross-resistance to those two drugs by the Colombia chloroquine-resistant strain of *P. falciparum*.

After the previous three mentioned cases of chloroquine-resistant *P. falciparum* from Colombia, so many other resistant cases were reported. Box et al. (1963) reported two resistant cases from Porto-Velho in Brazil. Powell et al. (1963) carried out some studies on the chloroquine-resistant strain of *P. falciparum* from Colombia by injecting infected blood of that strain in three non-immune volunteers. Their studies confirmed the findings of the previous workers who reported the presence of the resistant strain in Colombia.

Walker and Lopez-Antunano (1968) studied the response of 18 different strains of *P. falciparum* to chloroquine, pyrimethamine and combined pyrimethamine-sulphonamide. The 18 strains were taken from persons presumably infected in Brazil, Colombia and Venezuela. Infections with these strains were induced by blood inoculation in 131 adult male patients in a hospital in Brazil. The response to chloroquine was studied in 90
attacks. The initial impact of parasitaemia was normal in most patients, but was delayed in a significant proportion. The rate of recurrence was high. The response to pyrimethamine alone was studied in 46 attacks. 2 strains were found to be sensitive and 6 moderately resistant. The response to the combined drug was studied in 44 attacks. The parasitaemia usually cleared on the third day and the cure rate was high, 41 out of 44.

Comer et al. (1968) reported chloroquine-resistant P. falciparum strain on the Pacific Coast of Colombia. The strain appeared in infections in three North American servicemen with no previous history of malaria. A survey was carried out in the area where the infections were acquired. 186 residents were investigated, 57 of them showed positive thick blood films with P. falciparum infections. Those patients were treated with 1.5 grammes of chloroquine base for adults, adjusted for weight, over a three-day period. Thick and thin blood films were examined from 46 patients on days 0, 2, 3, 7, 14, 21 and 28, Day 0 being the first day of treatment. Out of the 46 patients the parasitaemia in 3 did not clear at all, in 4 it relapsed in 7 days and in 17 it relapsed during the 28 days observation period. This showed a 52% failure of treatment with chloroquine. All of the failure cases were among children and adults of less than 18 years of age. This constitutes the first report of chloroquine-resistant malaria from
the Pacific Coast of the Western Hemisphere.

Rieckmann and Lopez Antunano (1971) used the in vitro technique to study the response of *P. falciparum* to chloroquine in 30 patients in Brazil. The results of the study indicated that each of the 30 patients was infected with a chloroquine-resistant strain of *P. falciparum*. The failure to cure 28 out of the 30 cases by the oral administration of standard regimen of chloroquine supported, in general, the in vitro findings. The 2 patients whose infection was cured by chloroquine had the disease for about 2 - 3 months and consequently had acquired a substantial degree of partial immunity before participating in the study. This partial immunity did not affect the in vitro study results.

Young and Johnson (1972) carried out a survey in Panama to find out the response of *P. falciparum* in that area to chloroquine. A patient whom they treated with quinine after he relapsed twice after 1.5 grammes of chloroquine in 1969 gave them an indication of the presence of a chloroquine-resistant strain of *P. falciparum* there. From one hundred and six persons with *P. falciparum* infections, 50 patients were followed up to give sufficient evidence. The failure rate for chloroquine was 84% and the failure rate for amodiaquine was 76% and was higher in adults than in children. Quinine sulfate was used to cure the relapsing cases with 100% success. Johnson et al. (1972) tried a combined therapy
to cure the Panama chloroquine-resistant *P. falciparum*: Sulphadimethoxine (Madribon) or Sulphormethoxine (Fanazil) plus pyrimethamine (Daraprim) and Primaquine. The parasitaemias responded rapidly to a combination of either of the two sulphonamide drugs combined with pyrimethamine and primaquine. However, more than one-third of the infections relapsed. In some cases there were multiple failures with these drugs. They concluded that there was no evidence of cross-resistance between the 4-aminoquinoline drugs and the sulphonamide combination, nor was there evidence that the response to sulphonamide combinations was lessened by repeated treatment with the same drugs (Figure 21).

3.6 Chloroquine-resistant *P. falciparum* in South East Asia

Young et al. (1963) reported their studies of a chloroquine-resistant strain of *P. falciparum* which originated in Thailand. The non-immune patient had no previous history of malaria. After staying 10 days in Thailand he left the country and started a course of 15 mg primaquine daily for 10 days. While he was in Thailand he took 0.3 grammes of chloroquine base, once weekly for 2 weeks only. He had his first attack on the 14th day from the date he entered Thailand. Ten days after the onset of clinical symptoms, he was admitted to hospital and his blood film was found positive for *P. falciparum*. He was treated with 1.5 grammes chloroquine
Fig. 21.
Distribution of Chloroquine-resistant Falciparum infections observed in Central and South America—1979

Updated from WHO 1973
base over a period of 3 days. He had 5 recrudescences, after each of which he was given a treatment of chloroquine. After the fifth recrudescence he was given 1.3 grams of quinine over 2 days, parasites cleared, but reappeared again in his peripheral blood. Then he was given a second dose of quinine 0.65 g in 2 days, and again the parasites appeared after a clearance on the first day. His parasitaemia only cleared without recrudescences after taking 14.0 g of quinine over 7 days. Infected blood was drawn from this patient, deep frozen and shipped to Atlanta. Six volunteers were injected with the infected blood and several antimalarial drugs were used for their treatment. The parasites showed poor or no response to the usual therapeutic doses of chloroquine, amopyroquine, pyrimethamine, proguanil, and a new hydroxynaphthalene derivative. The parasites responded rapidly to mepacrine but relapsed. The infections were cured by regimens of 8 g or more of quinine.

Jeffery et al. (1963) confirmed that the strain of *P. falciparum* reported to be resistant to chloroquine by Young et al. (1963) from Thailand, is truly resistant to chloroquine and extended to be multiresistant to amodiaquine, pyrimethamine and proguanil. Eyles et al. (1963) reported the presence of chloroquine-resistant *P. falciparum* in the State of Battembang in Cambodia. They followed three patients who contracted the disease there while taking chloroquine prophylactically, and they did not respond to normal therapeutic regimen of treatment
Montgomery and Eyles (1963) reported ten cases of chloroquine-resistant *P. falciparum* among British Army men who were operating in North Perlis (Thai-Malaysian border area) of Malaya. All the non-immune members of the unit were taking a daily dose of 200 mg of proguanil (Paludrine) as prophylactic, 53 proved cases of malaria were admitted to medical units, and 35 of these infections were found to be due to *P. falciparum*, 10 of which showed evidence of resistance to chloroquine. All resistant cases were treated by a daily dose of 2000 mg of quinine for 7 days. Powell *et al.* (1963) isolated the first highly resistant strain of *P. falciparum* from the non-immune American combat troops who were in operation in the Central Highlands of Vietnam.

Contacos *et al.* (1963) studied the response of five Asian strains to chloroquine. Their results indicate that four of these strains (Cambodian II, Malayan I, II and III) are definitely resistant to normally curative doses of chloroquine, i.e. 0.6 or 1.5 g of the base. Moreover, they concluded that early minimal resistance to chloroquine was obtained from the Cambodian I strain.

Both papers of Montgomery and Eyles (1963) and Contacos *et al.* (1963) about chloroquine-resistance in Malaya were questioned by Wilson and Edeson (1964). They mentioned that these drug-resistant strains could
have been present for a considerable time; but they were noticed only because non-immune troops entered the area and some of those infected were not cured by the standard course of chloroquine.

DeGowin and Powell (1964) studied the response of the Malaya (Camp) strain to different anti-malarial drugs in 29 healthy, non-immune volunteers. Oral administration of 1.5 g base of chloroquine over 3 days failed to cure infection with this strain in each of 6 volunteers. Chloroquine given orally in twice the recommended dose also proved ineffective. Usually effective doses of amodiaquine, hydroxychloroquine, quinacrine (Mepacrine), chloroguanil (Proguanil), and pyrimethamine did not eliminate infections with this strain. Quinine sulphate, 10 grains thrice daily for 7 or 10 days effected radical cure in 12 of 13 volunteers.

Powell et al. (1964) reported a chloroquine-resistant P. falciparum case in a non-immune, 34 years old, Caucasian of the United States Marine Corps, who had been stationed for almost one year near Nha Trang in the Republic of Vietnam. He had been taking 300 mg of chloroquine base weekly during his stay there. While stationed in Vietnam he developed the first acute clinical attack of malaria. Standard regimens of 1.5 g of chloroquine base administered orally over three days on three occasions were always followed by recrudescences. A course of 2.7 g of chloroquine base over 7 days was administered after the third recrudescence. The patient
was transferred to the U.S.A. There he was cured by six dosages, 5 grains each, of quinine. Infected blood from this patient was injected intravenously in seven non-immune volunteers. After the onset of the disease in them they were given the usually recommended regimens of hydroxychloroquine, chloroquine, amodiaquine and mepacrine, and all failed to effect a radical cure.

Powell et al. (1964) using infected blood from a patient whose *P. falciparum* strain from Thailand proved to be chloroquine-resistant, induced the infection into 24 non-immune volunteers (22 Caucasians and 2 Negroes) by intravenous inoculation of infected blood containing approximately 500,000 asexual parasites. The strain was named The Thailand (JHK) strain. Nine volunteers were treated, during acute attacks with a three-day course of chloroquine; eight received 1500 mg and one received 3000 mg. Radical cure was achieved in none of these individuals. Two volunteers were treated with hydroxychloroquine (1500 mg given over three days). The results were similar to those obtained with chloroquine. Three volunteers received 1400 mg base of Amodiquine over three days and none reached a state of radical cure. Two were given Mepacrine (2198 mg given over seven days) and both showed recrudescences of the disease. No radical cure resulted after treating 2 volunteers with proguanil (2610 mg base over 10 days) and four volunteers who received a three-day course of pyrimethamine (150 mg base). Quinine consistently effected radical cure of infections with the Thailand (JHK) strain.
DeGowin et al. (1965) infected volunteers with the Malayan strain (now called Malayan Camp), and treated them with normal regimen of chloroquine base normally used for radical cure. The parasites showed clear resistance to the dosages used. Hydroxychloroquine, Amodiaquine, Mepacrine, proguanil, pyrimethamine and quinine were all tried in dosages which usually give radical cure in sensitive strains of P. falciparum, but the Malayan parasite showed no response to any of them.

Legters et al. (1965) confirmed the high resistant level of the Vietnam P. falciparum strain to chloroquine and to all other used 4-aminoquinolines.

Bourke et al. (1966) reported the results of their survey in three areas of Southern Thailand near the Thai-Malaysian border. In 79% of the 307 cases with P. falciparum infections the parasites showed reduced sensitivity of 4 mg per lb of chloroquine base, and in approximately 53% of the 111 schoolchildren with P. falciparum the parasites exhibited reduced sensitivity to 10 mg chloroquine base per lb.

Cadigan et al. (1968) carried out surveys in selected villages of 5 provinces in Thailand. Out of 2,863 persons examined, 732 cases of P. falciparum were found. All patients were treated with chloroquine but only those who could be followed up for the requisite period were included in the treatment study. The frequency of unsatisfactory response to 1500 mg of chloroquine base ranged from a high of 85% in the north to a
low of 6% in the south. Mahoney (1968) reported a resistant case of *P. falciparum* to chloroquine in a European teacher in Malaysia. Samples of the patient's blood were forwarded to Bethesda U.S.A. where it was injected in to volunteers and proved to be resistant to chloroquine, clorguanide, pyrimethamine and mepacrine, but not resistant to quinine.

Kellett, Cowan and Parry (1968) reported a case of chloroquine-resistant *P. falciparum* malaria in the United Kingdom. The patient, a 26-year-old white male, had been in the jungle of the Grike area of Malaysia for 3 weeks. Parasitaemia rapidly cleared after treatment with pyrimethamine and sulphhemethoxine.

Rieckmann et al. (1968) developed an in vitro technique to study the effects of antimalarial drugs upon the maturation of asexual erythrocytic forms of *P. falciparum*. The experimental system which is simple and easy to use in the field, is a modification of the technique described by Bass and John (1912). The technique involved the following steps:

Small samples of venous blood (10 to 15 ml) were obtained from persons having patent parasitaemia.

Blood was defibrinated (swirled gently for 5 minutes in 25-ml Erlenmeyer flasks containing glass beads).

One-ml aliquots of each sample of defibrinated blood were placed in screw-cap, flat-bottomed glass vials (6 cm tall, 1.5 cm in internal diameter) that contained
added glucose (5 mg) and either no drug (control samples) or drug added in various amounts (experimental samples).

Blood in the vials was swirled gently to mix the contents well, and the vials were then placed in an incubator at 38.5°C to 40°C for 24 hours (without agitation during incubation).

The vials were shaken gently after incubation to remix the contents well.

Giemsa-stained thick films of blood were prepared.

One hundred asexual parasites, observed consecutively, were examined and classified according to morphological characteristics evident on examination with conventional light microscopy.

For convenience, solutions of glucose and of drugs were added to vials days or weeks in advance of the experiments and water was then allowed to evaporate at a temperature not exceeding 40°C. The use of vials prepared in this way yielded results similar to those noted during studies in which glucose and drug were added just before incubation. Stock solutions of glucose (reagent grade), containing 20 g of glucose per 100 ml, were prepared; 25 µl of this solution was placed in each vial. This amount of glucose was selected on the basis of extensive preliminary studies. Stock solution containing appropriate concentrations of the drug was prepared; 20 µl of each solution was added to each
experimental sample within 6 hours after preparation of the stock solution. It was found that maturation of parasites was retarded at temperatures below 37°C and markedly inhibited at temperatures exceeding 42°C.

This technique was first used to study the effect of chloroquine, of quinine, and of DHT upon maturation of asexual parasites in vitro of two strains of P. falciparum that had been studied in vivo: a chloroquine-sensitive strain from Uganda and a chloroquine-resistant strain from Malaya. Chloroquine, quinine and DHT inhibited the maturation of trophozoites of the Uganda strain to a greater extent than the maturation of trophozoites of the Malayan strain. This supports the in vivo findings and shows that this system can be used for differentiation of drug-sensitive from drug-resistant strains of P. falciparum.

Rieckmann et al. (1969) applied their technique (Rieckmann et al., 1968) to study the effect of quinine and chloroquine in vitro upon a strain of chloroquine-resistant P. falciparum that displays a relative resistance to quinine in vivo. The strain is a variant of the Malayan (Camp) strain. The studies displayed that the variant strain also displays a relative resistance to quinine in vitro and that the effect of chloroquine against the variant strain in vivo or in vitro are not markedly different from those exerted by this agent against asexual erythrocytic forms of the parent Malayan (Camp) strain.
Colwell et al. (1972a) carried out studies to evaluate the reliability and reproducibility of the in vitro technique in detecting chloroquine-resistant strains of *P. falciparum* in Central Thailand. They tested in vitro and in vivo 57 subjects. 55 out of those subjects showed clear resistance to chloroquine both in vitro and in vivo. The remaining two subjects who were cured with conventional chloroquine administration had parasites which were resistant in vitro. Schizogony was observed in vials containing concentrations of chloroquine approximately 7-fold that of the inhibitory concentration for the Ugandan strain. Limitations to the in vitro studies were the high parasite densities and the requirement for an adequate stage of in vivo trophozoite maturity before obtaining specimens for culture.

In the same year more work was carried out by Colwell et al. (1972b) in four localities of Thailand to get more information about the distribution of chloroquine-resistant strains in the area. Both in vitro and in vivo methods were applied. Successful in vitro cultures were observed in 66 per cent of 253 attempts. The proportions of in vitro chloroquine-resistance among the cultures which were successful ranged from 85% in the southeastern region to 100% in the central region. None of the cases studied exhibited a radical cure after chloroquine administration.
Siddiqui et al. (1972) used a model in vitro system to test the susceptibility of five strains of *P. falciparum* to seven antimalarial drugs. The first *P. falciparum* strain was from a blood of a *Hylobates larlar* which was infected from a volunteer; the second is Uganda-Palo Alto strain; the third is Malayan-Camp strain; the fourth is a Vietnam-Oak Knoll strain; and the fifth is a Vietnam-Monterey strain. All the strains were kept by passaging in owl monkeys. Chloroquine, amodiaquine, pyrimethamine, quinine, cycloguanil, trimethoprim, and dapsone were the seven antimalarial drugs used. The in vitro technique used was different from that of Rieckmann et al. (1968). In this technique the blood was diluted with culture medium, and gentle agitation of the culture tubes in the presence of a gaseous phase to provide a physiological environment was carried out. It was found that the Uganda-Palo Alto strain of *P. falciparum* is the most sensitive and the Vietnam-Monterey strain is the most resistant to chloroquine among the five strains studied. The two strains showed the same response to quinine, cycloguanil and dapsone; both were susceptible to quinine and resistant to cycloguanil and dapsone. The Uganda strain was quite resistant to pyrimethamine and trimethoprim, while the Vietnam strain was quite susceptible to both drugs. Both strains were found to be susceptible to amodiaquine.

Andre et al. (1972) conducted a survey in West
Malaysia to determine the magnitude of the prevalence of chloroquine-resistant *P. falciparum* among the inhabitants. Both *in vivo* (following the procedure set down by WHO, 1965) and *in vitro* (Rieckmann *et al.*, 1968) techniques were carried out. Four states of Malaysia were chosen for the study. A total of 6,880 individuals were examined for malaria, with an overall prevalence of 20%. Of 864 *P. falciparum* cases, 395 met all the criteria of the 7-day study. The equivalent of a single adult dose of chloroquine (600 mg base) was given to 217 of the participants; of these, 36% were positive on the seventh day following treatment. 178 people were treated with 25 mg/kg chloroquine base over a 3-day period, with 3% being found positive on day 7. It can be seen from these results that a low amount of resistance exists. Blood from 30 cases was cultured *in vitro*. Only 13 cultures were successful. Out of these 9 were considered to be sensitive, 1 to be intermediate in its response to chloroquine, and 3 were classified as resistant.

Shute *et al.* (1972) revealed the presence of 4-aminoquinoline (Amodiaquine) resistant *P. falciparum* in the Philippines. Six cases were studied and their level of resistance was at both RI and RII levels. One of the six cases had contracted his infection in Laos. The other cases were from two islands in the Western Philippines. All cases were treated and followed in Manila where there is no malaria transmission and so re-infection can be
excluded. In all cases, the parasites appeared to be highly susceptible to quinine therapy. Lewis et al. (1973) reported a strain of *P. falciparum* in West Malaysia which is resistant to chloroquine suppression (weekly dose of 5 mg base/kg, Avlochor, I.C.I.) but susceptible to chloroquine treatment (25 mg base/kg chloroquine over a 3-day period).

Clyde et al. (1973) used a strain of *P. falciparum* from Burma to study its susceptibility to chloroquine. The strain transmitted by the vector *Anopheles stephensi* to volunteers proved to be resistant to chloroquine, amodiaquine and pyrimethamine. They concluded that the implications of spread of chloroquine-resistant *P. falciparum* from South East Asia to the Indo-Pakistan subcontinent, should malaria eradication programmes falter, are alarming. Sehgal et al. (1973) reported the first appearance of chloroquine-resistant strains of *P. falciparum* in the eastern states of Assam and Meghalaya of India. They suspected that the origin of this strain might be Burma. Rooney (1978) reconfirmed the presence of chloroquine-resistant *P. falciparum* in Assam and Nagaland states of India applying both in vivo and in vitro methods.

Sucharit et al. (1974) carried out a study on 20 patients from Thailand. Studies were carried out both in vitro and in vivo using chloroquine as the test drug. The results demonstrated RI in vivo study corresponding to 2.5 - 3 millimicromoles of chloroquine per ml
of blood in vitro which could inhibit the maturation of parasites.

Vaiera and Shute (1976) used Rieckmann's in vitro technique in studying the response of *P. falciparum* to chloroquine in the Philippines. 51 cases were studied, but cultures from 34 only grown successfully. Growth was inhibited in 18 cases by a dose of 0.75 nmol of chloroquine per ml blood, 16 developed in 1.0 nmol/ml, 12 in 1.5 nmol/ml, 10 in 2.0 nmol/ml, 3 in 2.5 nmol/ml and one only continued growing up to the concentrations of 3.0 nmol/ml. 18 cases were followed for in vivo evaluation for 28 days. 8 cases showed no recrudescence of infection, but in the other 10 recrudescences were detected indicating parasite resistance to the drug. In each of the 18 cases, the in vivo response followed the in vitro prediction (Figure 22).
Fig. 22.
Distribution of Chloroquine-resistant Falciparum infections observed in South East Asia - 1979

■ Sensitive
• Resistant

Updated from WHO 1973
CHAPTER FOUR

THE DEVELOPMENT OF A MICRO TECHNIQUE FOR TESTING THE SUSCEPTIBILITY OF *PLASMODIUM FALCIPARUM* TO ANTIMALARIAL DRUGS
IN VITRO METHODS FOR STUDY AND CULTIVATION OF PLASMODIA

4.1 Introduction

Since 1880 when Laveran discovered the human malaria plasmodia, many attempts to cultivate malaria parasites were reported in the history of malariology. All trials, although not so successful, gave leading threads to other workers, and the first reliable procedure for propagation of parasites in vitro was reported in 1943.

The history of the cultivation of malaria parasites from the circulating blood might arbitrarily be divided into four periods:

4.1.1 The period between 1880 and 1911
4.1.2 The period between 1911 and 1941
4.1.3 The period between 1941 and 1960
4.1.4 The period between 1960 and the present.

4.1.1 The period between 1880 and 1911

Thayer and Hewetson (1895) reviewed the uniformly unsuccessful attempts of many observers to cultivate the malaria parasite in vitro using varied methods. They cited that Coronado in 1891 claimed to have cultivated malarial Plasmodium from an infected source in water. Sacharow repeated the Coronado experiments without success.
Sacharow and Rosenbach reported their success in keeping malarial Plasmodia alive for several days in leeches that had been allowed to draw blood from malarial patients. Blumer, Hamburger and Michel carried on experiments with leeches during the period 1892 - 1893. Miller (1891) reported also that he had cultivated the parasites, but his experiments which have been repeated, have not been confirmed. Laveran (1893) reported malaria parasites to be visible in hanging drop preparations for as long as ten days, but he confessed that he failed to satisfy himself that any multiplication took place, nor, indeed that the parasites remained alive.

4.1.2 The period between 1911 and 1941

Bass (1911) reported what was considered as the first successful cultivation of malarial parasites infecting man in vitro, i.e. Plasmodium vivax, P. falciparum and P. malariae. In this preliminary report Bass did not reveal his culture technique in detail, but he mentioned that he used citrated or defibrinated blood as his culture medium. Anaerobic conditions were maintained throughout, and he claimed that he kept cultures in citrated blood for over 2 weeks. Bass and Johns (1912) confirmed the previous results of Bass (1911) by cultivating P. falciparum and P. vivax in vitro in human blood. They grew the parasites in red blood cells in the presence
of Locke's solution, free of calcium chloride and in the presence of ascitic fluid. Defibrinated blood was used. The defibrinating tube was charged with one tenth of a cubic centimetre of 50% solution of dextrose for each ten cubic centimetres of blood before the blood was drawn in. The column of blood must be one to two inches deep to give a column of serum one half to one inch deep above the cells and parasites when the latter have settled. They observed that young parasites increase in size slowly during the first twenty-four hours, but segmentation begins in about thirty-six hours from the smallest ring form stage and parasites containing up to about twenty segments were found in cultures. Reinversion takes place, and a four generations culture of aestivo-autumnal parasites in a blood from which leucocytes were removed was carried out successfully. Temperatures of 37°C and 40°C were used, but the most rapid growth was obtained at a temperature of 40°C to 41°C.

Thomson and McLellan (1912) followed Bass's technique and they successfully cultivated one generation of P. falciparum in vitro. They incubated their culture at 38°C and they used Giemsa stained smears to read their results. They found that sporulation occurred after 26 hours of incubation. Lavinder (1913) in a trial to confirm Bass and Johns' findings carried out three cultures of malarial plasmodium from human cases. Two cases were P. falciparum and the third was P. malariae.
He followed exactly the technique described earlier by Bass and Johns without any deviation. In the first case of *P. falciparum* culture which he started from a rather heavy infection with small ring forms, only larger pigmented forms were observed after incubating the blood at 40°C for a period he called "late afternoon and next morning". On later examination, after the culture was returned to the incubator, he found that growth had ceased at the previous stage. The second *P. falciparum* case was also a heavy infection with non-pigmented small rings. In this case the blood after defibrination, was centrifuged to get rid of the serum and to eliminate leucocytes so as to grow the first and second generations. The parasites went to sporulation stage, but the merozoites for some unknown reason did not enter fresh blood cells, and growth ceased at this stage. He reported, with no reasons, that his culture of the third case which was a light infection of *P. malariae* was not successful.

Carter and Thompson (1913) tried to cultivate *P. vivax* using Bass's technique, but they only succeeded in growing it to three-fourths the parasite's full size.

Thomson and Thomson (1913) tried to cultivate *P. vivax* *in vitro* from a malaria patient who had resided for some time in the island of Java. They again adapted Bass and Johns' technique with a little modification of adding more dextrose to the blood than what was recommended by them. They started their culture with blood containing
numerous young ring parasites. They incubated their culture at a temperature of 39°C. They succeeded in reaching one cycle of sporulation and reinvasion of uninfected red cells with young parasites. The conclusion they reached is that maximum sporulation occurs between twenty and twenty-nine hours of incubation. Also they reached the conclusion that growth of the parasites is more rapid in culture tubes than in the circulating patient blood. After 120 hours of incubation they noticed that the parasites had changed much in appearance and all became heavily pigmented and compact.

Zieman (1913) tried to culture in vitro tertian malaria parasites from an East African who came to his out-patients' clinic. He followed Bass and Johns' method but instead of adding 0.1 c.cm of 50% dextrose to 10 c.cms of blood, he tried 8, 10 and 12ccms of blood with 0.1 c.cms of dextrose. He followed the culture for 4 days, and he claimed that he observed clear growth, sporulation, reinvasion and sexual forms (male and female). He kept his incubating temperature constant at 39.5°C. From his observations he suggested that if 0.1 c.cm of 50% dextrose is added to 8 c.cms of parasitized blood, better growth was obtained than if this amount is added to 10 c.cm of blood. Joukoff (1913) tried cultivation of the mosquito cycle of malarial parasites, but his results have not been confirmed.
Thomson and Thomson (1913) used a less complicated method of Bass and Johns to observe and compare the growth and sporulation of the Benign "vivax" and Malignant "falciparum" Tertian malarial parasite in the culture tube and in the human host. Using defibrinated blood in small sterile tubes (one inch column of blood) incubated at 37° to 41°C, corpuscles settle in a short time leaving about half-inch of clear serum at the top, they claimed that they have grown four complete generations of parasites without renewing the medium. They stated that it is unnecessary to remove the leucocytes by centrifugation and that they do not see why the growth should not continue indefinitely, provided fresh serum and corpuscles be added. They found that parasites develop even in the very deepest layer of the column of corpuscles. In general they concluded that the morphology of Benign tertian vivax is identical in culture tubes to that in human host, while in the case of P. falciparum you see all stages in culture tubes, but you only find rings in the peripheral blood of human host, except in cases of severe infections, and the remaining stages develop in the internal organs.

Bass et al. (1913 - 1914) in their paper on cultivation of malarial Plasmodia in vitro on February 1914 gave full details of the apparatus, materials and techniques which should be used for cultivation of one generation of the parasites, and that for more than one generation of parasites. He laid stress on complete sterility,
importance of dextrose, temperatures of incubation and their influences on rate of growth of the parasite. He stated that at 41°C the segmentation cycle is reduced to about thirty hours, and usually less than forty-eight hours when the temperature is 40°C. It averages forty-eight hours when the incubation temperature is 39°C and at 37°C most cultures require more than forty-eight hours for complete segmentation from youngest ring forms.

Row (1917) gave a detailed description of a simpler method than that of Bass by means of which he succeeded in culturing malarial parasites with ease and studying their morphology. His apparatus consisted of an ordinary potato culture tube 1 inch wide, small culture tube, sucking pipette, pyrogallic acid, strong caustic soda solution, human blood serum and 25% glucose solution. He used infected blood from the finger puncture made for the blood films, drawing it in a small capillary pipette. The blood is poured into a sterile dry test tube and shaken for defibrination. A drop of glucose solution is used for a nippleful of the serum introduced in the culture tube. Pyrogallic acid and caustic soda were used to secure a supply of sufficiently good anaerobic conditions. Four species of plasmodia were cultured using this technique and incubated at 37°C. He produced coloured plates of his results showing that the four species reached sporulation in the period 24-72 hours of incubation.
importance of dextrose, temperatures of incubation and their influences on rate of growth of the parasite. He stated that at 41°C the segmentation cycle is reduced to about thirty hours, and usually less than forty-eight hours when the temperature is 40°C. It averages forty-eight hours when the incubation temperature is 39°C and at 37°C most cultures require more than forty-eight hours for complete segmentation from youngest ring forms.

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Sinton (1922a, b) developed a simplified method for cultivating *P. falciparum* which could be carried out in any clinical laboratory. In his method he reduced the amount of apparatus needed, and lessened the amount of manipulation of the cultivation so as to reduce the risk of contamination. Also he managed to grow the parasites from a few drops of blood obtained from a finger prick. The optimum temperature used was 37°C. He applied his culture method for the diagnosis of malarial infection in cases in which the parasites were difficult to find or could not be found even in thick blood films although the cases were obviously suffering from malaria fever. He reported that quinine treated cases are not successful in culture. Bass (1922) used his cultivation technique for studying the effect of quinine upon the growth of malarial plasmodia *in vitro*. Serum from people who had quinine was used in medium. He concluded that there is a direct protoplasmic poisoning effect upon the parasites and that sufficient amount of quinine which will prevent the growth and finally kill the plasmodia was taken up within a period of twenty minutes.

4.1.3 The Period between 1941 - 1960

Trager (1941 and 1943) studied the conditions and factors affecting the survival and development *in vitro* of an avian malaria parasite (*Plasmodium lophurae*) and he found out that, red cell extract, suitable
concentration of glucose, glutathione, calcium pantothenate and serum or plasma plus a balanced salt solution are essential components for the culture medium. Gentle agitation of the suspension, aeration with low oxygen tension, renewal of the medium, provision of fresh red blood cells and an incubation temperature of 39.5° - 42°C, all favoured survival of the parasites. Ball and co-workers (1945) made a major advance for the culture of malaria parasites in vitro by developing the Harvard medium which consisted of a balanced salt solution of high sodium content, dextrose and supplies of vitamins, purins, pyrimidines and amino acids. The success of this medium was measured when in vitro cultivation of the erythrocytic forms of the malaria parasite P. knowlesi (which has a 24-hour cycle, produces heavy infection of red cells in the monkey, Macaca mulatta and will also produce infection in man) gave growth and good multiplication. Rocker-dilution and rocker-perfusion techniques, in which a slow flow of 5 per cent CO₂ in 95 per cent air is passed into the culture vessel, were also developed by the same team at the same time as the medium.

Black (1945, 1946) cultivated P. falciparum (New Guinea strains) in vitro and tested the effect of antimalarial drugs in their development. The blood for culturing was collected from patients who harbour the parasite in their circulation and who already had
antimalarial treatment. 50 per cent glucose solution was used as the culture medium.

McKee et al. (1946), Geiman et al. (1946) and Anfinsen et al. (1946) used in vitro cultures of P. knowlesi for studying the chemistry of the parasite, perfecting the rocker-dilution and rocker-perfusion techniques and to find a method by which the buffering capacity of the Harvard medium would be increased without detectable toxicity to the parasite. Their results revealed that monkey red blood cells parasitized with P. knowlesi consume oxygen in the presence of glucose, lactate, glycerol and amino acids as substrates. Glucose, which is the most essential ingredient for the cultivation of malaria parasites was metabolised in large quantities with production of lactic acid which accumulated in dilution cultures and caused a drop in the pH to a level below that required for cellular integrity. The pH of the medium was maintained at 7.4 ± 0.1 by equilibrating the bicarbonate containing solution with 5 per cent CO₂ - 95 per cent air. In their cultures they used sodium citrate, balanced oxalates and heparin as anticoagulants. The incubation temperatures of their cultures ranged between 38°C and 39°C.

Geiman (1949) in his review of all the work done and development of the in vitro methods used for the cultivation and study of plasmodia described a parasite control method which will enable research workers to
evaluate quantitatively and qualitatively their *in vitro* and *in vivo* experiments. The method will help to follow the course of infection in experimental hosts, and to follow the survival, growth and multiplication of the parasites during short and long term *in vitro* experiments. He mentioned that in the early stages of their work that parasite counts in thin films made on microscopic slides gave variable and inaccurate results. The parasitized cells, being of a different density than normal red cells, accumulate in greater numbers around the edges and the tail of the thin film. Also he stated that thick films are not satisfactory for *in vitro* cultivation evaluation because of the distortion of parasites from slow drying and the tendency of leucocytes to agglutinate spontaneously or adhere to the sides of culture vessels. The results of counting should be calculated as parasites per cubic millimeter and also as a total percentage of parasites. An important part of this parasite control method of Geiman is the morphological description of the parasites at the beginning, during and at the end of the experiment. He divided his table of counts into rings, trophozoites, schizonts, segmenters, gametocytes and degenerate parasites. In his review he cited that successful growth, reinvasion and multiplication of *P. vivax* have been obtained in three experiments. He stressed on the point that minimum of time and handling of the parasitized blood between the
host and incubating culture vessel will ensure the best results.

Trager (1950, 1952, 1954) continued his in vitro studies using the avian malaria P. lophurae as his model managed to keep the parasites alive for 16 days in cultures incubated at 40°C. He tried to grow the intracellular parasite on an extracellular cultivation aiming to find out the effect of adenosinetriphosphate, malate and coenzyme A concentrates on the growth of the parasites. He reached the conclusion that the addition of these substances to the culture medium which contain extract of duck erythrocytes, was in favour of the survival and development of the parasites and that heparinized blood is preferred for cultivation experiments.

4.1.4 The period between 1960 - 1980

Geiman et al. (1966a) improved the culture medium which they developed before and studied the effect of improvement in seven different species of malarial parasites. They came to the conclusion that glucose which is an essential element for the cultivation of malaria parasites was metabolized in large quantities with production of lactic acid which accumulated in dilution cultures and caused a drop in the pH to a level below that required for cellular integrity. To overcome
the problem of deleterious effects of lowered pH during culture, they found that glycylglycine (0.005M) when added to the improved Harvard medium would increase the buffering capacity without detectable toxicity to the parasites. They increased the ratio of medium to parasites from 3:1 to 6:1. Geiman et al. (1966b) found that addition of human plasma Fraction IV-4 to the synthetic medium would increase the growth and multiplication rates of the parasites in culture tubes.

Geiman and Meagher (1967) gave a push to the in vitro culture for the study of human plasmodia by introducing the new world monkey Aotus trivirgatus as a laboratory animal which is susceptible to P. falciparum from man. They succeeded in infecting, and maintaining the infection by passage of P. falciparum to splenectomized monkeys. This suggests that the erythrocytes and plasma of the Aotus monkey are similar in constituents to human blood.

Trigg (1967) in order to obtain large numbers of schizonts which can be used to extend investigations on metabolic and antigenic problems in human malaria, carried out in vitro cultures of P. knowlesi and P. falciparum. He modified the Harvard medium and reduced the number of stock solutions to four (I - balanced salt solution, II - glucose-acetate solution, III - vitamin concentrate, and IV - purines and pyrimidines). To avoid the clumping of parasitized cells, which may be due
to either the transfer of agglutinins or histones from the host's serum into culture or to a fibrinogen effect, he replaced the host serum with normal AB serum after thorough washing of the erythrocytes in the medium. Siddiqui et al. (1967) reported the successful replacement of monkey plasma by stearic acid for intracellular in vitro cultures of P. knowlesi.

Trigg (1968a, b, and 1969a, b) in his study of the factors affecting the cultivation of P. knowlesi in vitro using a modified Harvard medium and rocker-dilution technique found that the optimal growth and multiplication were obtained when fresh normal plasma was used. He reported that addition of 0.16 mM cholesterol and 0.026 mM stearic acid bound to lipid-deficient plasma protein produced an increase in the multiplication rate over that obtained when stearic acid was added alone. A new continuous perfusion system for the cultivation of the intracellular erythrocytic stages of malaria parasites was described in detail. He also reported that enhancement of growth and re-invasion of new host cells was obtained when P. knowlesi was incubated in low oxygen tension. He stated that lipids present in the plasma are important for the growth of P. knowlesi in vitro. Stearic acid and cholesterol are required by the parasite but do not account for all the growth promoting properties of plasma. It was also demonstrated that vitamin B_{12} is required. Growth and multiplication were obtained using a proprietary
tissue-culture media, e.g. '199' and 'NCTC 135' in an improved perfusion apparatus which consists of a sandwich of three chambers, of which the outer two contained medium and the inner contained the infected blood sample. Siddiqui et al. (1969) used a simplified medium for the in vitro culture of *P. knowlesi*. The medium contains inorganic salt solution, glucose, glycerol, sodium acetate, isoleucin, methionine, p-aminobenzoic acid, pyrimidines, biotin and glycylglycine. The medium is thus designated as basal simplified medium. Siddiqui et al. (1970a, b) reported for the first time the in vitro culture of *P. falcinarum* from experimental animals and compared it to a few cases in man. They used the modified Harvard medium and in two experiments in which they used the perfusion jar technique they reported a definite twofold increase. In the same year they used a commercially available culture medium to test the susceptibility of human malarial parasites to antimalarial drugs. They used CMRL-1066 produced by the Grand Island Biological Company, and compared it to the Harvard medium. They concluded that both were similar in their effect upon the growth of the asexual stages of the parasite. Trigg and Gutteridge (1971) developed a dilution medium for the cultivation in vitro of the erythrocytic stages of *P. knowlesi*, which in the presence of plasma gave better results than the Harvard medium during one asexual cycle and also allowed the subculture of the parasites through
three asexual cycles. The simplified medium contained a balanced salt solution, glucose, isoleucine, methionine, adenosine, p-aminobenzoic acid, biotin and calcium pantothenate. Butcher and Cohen (1971) reported an average multiplication rate for *P. knowlesi* of at least six-fold in 24 hour culture in a modified medium developed from the original medium of Geiman and co-workers (Ball et al., 1945; Anfinsen et al., 1946; Geiman et al., 1946; McKee et al., 1946; Leiman, Siddique and Schnell, 1966). In this medium they replaced the lactalbumin hydrolysate by a synthetic amino acid mixture, they selected the normal rhesus or horse sera by screening for their ability to support parasite growth in vitro, added Glutamine, ATP, AMP and coenzyme A, increased the biotin concentration 10 times (from 0.4 to 4.0 µg/l final medium), folic acid (100 µg/l final medium) was added to the vitamin solution, and the air and CO₂ (5%) mixture was replaced by 90% N, 5% O₂ and 5% CO₂.

Diggs et al. (1971) obtained significant in vitro reinvasion of foetal human red cells with *P. falciparum* in human blood. By suspending washed erythrocytes from heparinized human cord blood and from patients with acute falciparum malaria in tissue culture medium 199 for 50 hours they reached a five-to-eightfold increase in parasitized cells. Phillips et al. (1972) have been able to subculture the parasite through almost 3 cycles in vitro with a marked increase in numbers during the
first subculture but with the numbers being only main-
tained during the second subculture. The growth medium
was tissue culture medium '199' supplemented with 5%
inactivated foetal calf serum, glucose to give a final
concentration of 3 g/l, and sodium bicarbonate to give a
pH of 7.4 at 37°C in an atmosphere of 5% CO₂/95% air.

Siddiqui and Schnell (1972) conducted few
experiments on the amino acid requirements for the develop-
ment of *P. falciparum* in their plasma-free culture system.
They concluded that the parasites grow better in the
presence of amino acids, isoleucine and methionine.

Siddiqui and Schnell (1973) found that the buffering
capacity of glycylglycine has not been entirely satisfac-
tory for in vitro cultivation of *P. falciparum*, where 48
hours of incubation is required for the completion of one
developmental cycle. They tried three different buffers
(BES, TES and HEPES) to replace glycylglycine, and they
concluded that out of the three buffers TES gave better
growth and multiplication of the parasites. Siddique
et al. (1974) cultured *P. falciparum* in vitro at parasit-
taemias ranging from 20% to 70%. They used the modified
Harvard medium supplemented with TES as a buffering agent,
using 500 ml side arm flasks fitted with stoppers with
entry ports for a 95% air/5% CO₂ mixture. A 1:18 dilution
of blood in medium was adopted for routine cultivation.

Trigg (1975) used a modified medium which he
called Mill Hill medium, constituted from balanced salt
solution, organic carbon, amino acids, purines and pyrimidines and vitamins. The medium is equilibrated at 37.5°C with a moist stream of 5% CO₂/95% air. Using this medium he managed to grow the parasites of *P. falciparum* for more than one cycle and was able to study the invasion of fresh erythrocytes by the parasites in subcultures. Dennis *et al.* (1975) isolated merozoites of *P. knowlesi* which they grew *in vitro* using 199 tissue culture medium at 37°C and an atmosphere of 5% CO₂ in air. Mitchell *et al.* (1976) used both a modified Harvard medium, and 199 medium freshly made up from the dry powder, supplemented with glucose, penicillin and buffered to pH 7.4 with NaHCO₃, to study the effect of human IgG on the *in vitro* development of *P. falciparum*.

Trager and Jensen (1976) succeeded in maintaining *P. falciparum* in continuous culture for more than three months in human erythrocytes incubated at 38°C in RPMI 1640 medium supplemented with Hepes buffer (25 mM), 0.2% NaHCO₃ and 10% type AB human serum. An atmosphere of 7% carbon dioxide and low oxygen (1 to 5 per cent) was maintained during the culture. Human erythrocytes were added at 3- or 4-day intervals, and the parasites continued to reproduce in their normal asexual cycle of approximately 48 hours but were no longer highly synchronous.

Trager (1976) using the rocker dilution flasks and his flow vials compared 4 of the commercially available culture media (RPMI 1640 + 25 mM HEPES,
Dulbecco's-High glucose, modified Ball-Geiman, and modified Ball-Geiman + 25 mM HEPES). For the continuous growth and multiplication of *P. coatneyi* and *P. falciparum* it was found that RPMI 1640 always had superiority over the other media tried.

Wilson and Phillips (1976) used a micro in vitro culture technique to test inhibitory antibodies in human sera to wild populations of *P. falciparum*. Microtissue culture plates and medium TC-199 were used. 10 μl of parasitized blood were added to 150 μl of medium. 100 μl of serum from different sources was added to different wells. They concluded that parasites grow from rings to schizonts, but merozoites failed to invade unparasitized red cells. Haynes et al. (1976) reported that they succeeded in maintaining the growth and multiplication of *P. falciparum* in vitro for more than 3 weeks using modified tissue culture medium 199 and a low oxygen tension. Smalley (1976) obtained gametocytes in an in vitro culture of *P. falciparum* using 199 medium supplemented with 200 mg% glucose, 30 mg% glutamine, 25% (v/v) foetal calf serum and 2.5% (v/v) of washed packed blood cells. The culture was maintained in a CO₂ incubator (5% CO₂ in air) at 37°C and the medium was replaced every second day. Some of their cultures produced up to 32% morphologically mature gametocytes. Jensen and Trager (1977) described their candle-jar method in which they cultured *P. falciparum* in plastic petri dishes using
RPML 1640 powder medium dissolved in glass redistilled water and supplementing it by HEPES (25 mM), and 5% NaHCO₃ solution to give a pH of 7.4. 11 ml human serum type AB was added to 100 ml of medium. Outdated erythrocytes were added for reinvasion of parasites and they concluded that they proved to be better than freshly prepared erythrocytes. Mitchell et al. (1977) to obtain merozoites for vaccination of monkeys against P. falciparum, cultured P. falciparum parasites, obtained from Gambian children with parasitaemias of 2 - 32%, in RPMI 1640 augmented with 5% human AB serum and 5% foetal calf serum, incubated at 37°C in conical flasks under 5% CO₂ in air. The medium was replaced 1 - 3 times during the 30 - 50 hours of culture, after which more than 40% of the parasites were mature schizonts with 8 or more nuclei.

Pasvol et al. (1977) in their study of the effects of foetal haemoglobin on susceptibility of red cells to P. falciparum used 5 ml of leucocyte-depleted red cells infected with late ring stages of P. falciparum washed and suspended in Ehrlenmeyer flasks with 100 ml supplemented medium 199. The flasks were gassed with 5% CO₂/95% air and incubated at 37°C with gentle shaking. After 24 hours when the parasites had grown to schizont stage, the red cells were recovered by centrifugation and reconstituted to the original 5 ml volume with medium 199. A mixture of parasitized cells and uninfected red
cells which were to be examined was placed into the wells of a microtissue culture plate (250 μl per well) and incubated in 5% CO₂/air at 37°C without shaking. At various times blood films were made and either stained with Giemsa or treated by the acid-elution method.

Carter and Beach (1977) reported that gametocytes of *P. falciparum* grown from parasites maintained by continuous culture *in vitro* are able to develop to the point at which they can be stimulated to undergo gametogenesis (exflagellation). The medium they used for their culture was RPMI 1640 powdered medium supplemented with HEPES buffer (25 mM) and 0.2% NaHCO₃ with 10% human serum type AB. The culture medium was replaced daily and the flasks were gassed with a mixture of 6% O₂, 3% CO₂ and 91% N. For maintenance the parasite cultures were diluted with fresh red cells every 4 - 8 days. Gametocytes from the culture were stimulated to undergo gametogenesis by dispensing 0.3 ml of the suspension in a serum tube, into 3.0 ml of foetal bovine serum (FBS) adjusted to pH 8.0 with 1.5% NaHCO₃.

Rieckmann et al. (1978) published a detailed description of an *in vitro* microtechnique to measure the sensitivity of *P. falciparum* to antimalarial drugs. In this technique the chloroquine sensitivity was determined by measuring the extent to which normal maturation of trophozoites to schizonts was inhibited by various concentrations of chloroquine. Various amounts of chloroquine diphosphate (0.1 to 25.0 ng), dissolved in
25 μl of distilled water, were added to flat-bottomed wells of microtitre plates which were dried in an incubator and then stored at room temperature for further use. At the start of the experiment, 50 μl of culture medium RPMI 1640 supplemented with 2 mg/ml sodium bicarbonate, 6 mg/ml HEPES buffer, and 4 μg/ml gentamicine sulphate were added to each well and stirred briefly to dissolve the chloroquine. Using Eppendorf pipette 5 μl of parasitized blood was added to each well of the plate. The plate, covered with a lid, was then shaken and placed in a vacuum glass desiccator containing a paraffin candle which will provide the CO₂ in a low oxygen tension atmosphere. The desiccator was then placed in an incubator at 38 - 39°C for 24 - 30 hours. After incubation thick blood films prepared from each well were dried and stained with Giemsa, and examined for parasite maturation. The number of schizonts per 500 white blood cells was determined in control and drug samples.

Friedman (1978) used the petri dish and candle method to study the erythrocytic mechanism of sickle cell resistance to malaria. RPMI 1640 plus 25 mM HEPES with 10% human serum type AB was used as medium. Gentamicin sulphate (40 μg/ml) was added to the culture and an atmosphere of 18% O₂/3 CO₂ was maintained by the candle. He concluded that in cultures exposed to 18% O₂ P. falciparum develops equally well in cells from
homozygous S/S individuals, cells from trait carriers (S/A) and in normal A/A cells. When the O₂ tension is reduced to 5%, a level which occurs physiologically in many tissues of the body, some remarkable differences are seen. Development in A/A cells is just as good as with 18% O₂. In S/S cells sickling occurs and the parasites are quickly destroyed. In the heterozygous S/A cells, containing about 40% sickle haemoglobin, the parasites are able to invade and become young rings, but they fail to develop further and die. Jensen and Trager (1978) established additional strains of P. falciparum in continuous culture using human type AB* erythrocytes in RPMI 1640 medium supplemented with HEPES buffer (25 mM) and 15% or 10% human type AB serum. They used both the flow vial and petri dish-candle jar methods for culture. Four lines, named FCR-2, FMG, 6252 and FSG, were established successfully in culture. Normal-looking gametocytes were seen in all four strains. Scheibel et al. (1979) employed the system of Jensen and Trager (1977) of the petri dish-candle jar and RPMI 1640 to study the microaerophilic requirements of P. falcinarum in human red blood cells. They stated that best growth was obtained in all cases at 3% O₂ and 1% to 2% CO₂, but the parasite can tolerate oxygen levels as low as approximately 0.5% or less without ill effects and growth rates at these microaerophilic levels remain high. They mentioned that at these low levels of oxygen, an optimum level of 1% to 3% CO₂ is needed,
and that oxygen levels of 17% to 21% decrease parasite survival.

Trager (1979) improved the continuous flow method using a supplemented RPMI 1640 medium to get an increase in the parasites numbers to eightfold per cycle with parasitaemias reaching 10% to 15%. Jensen et al. (1979) described their semiautomated apparatus which changes the culture medium and redistributes the infected erythrocytes at preselected intervals. Again RPMI 1640 medium supplemented with HEPES buffer, NaHCO₃ and gentamycin was used. Humidified gas (3% CO₂, 10% O₂, 87% N₂) flows continuously through the culture vessel. Using this technique they managed to produce approximately 10 ml of packed erythrocytes per week with parasitaemias between 12% and 16%. Lopez Antunano and Wernsdorfer (1979) used the in vitro microtechnique to test the response of chloroquine-resistant P. falciparum to mefloquine and to evaluate the application of the test using isolates of P. falciparum from Brazil and Colombia. They used disodium salt of ethylenediamine tetra acetic acid (EDTA) as an anticoagulant and 10.4 g RPMI 1640 dried medium supplemented with 2 g of sodium bicarbonate, 6 g of HEPES buffer powder and 4 mg of gentamycin in 1 litre of double-distilled water as medium. The microtitre plates they used were already dosed with different concentrations of the drugs to be tested and dried at 37°C before use. A static water bath at 38°C with a paraffin candle to produce the correct CO₂/air mixture was used for incubation. Their incubation period varied between 24 - 30 hours.
4.2 Development of an in vitro microtechnique

Malaria in the African continent is still the major disease which causes the highest incidence of morbidity and mortality. WHO (1974) estimated that 96 million cases of malaria occur annually in Africa alone, with one million deaths involving mostly young children. In most of the African countries antimalarial activities are limited to reducing malaria morbidity and mortality through the administration of antimalarial drugs. The 4-aminoquinolines are the most widely used drugs.

The appearance of resistant strains of *P. falciparum* to chloroquine and amodiaquine in South East Asia, the Indian continent and the Americas, and the evidence of their spread as reviewed in Chapter 3 of this study, constitute a serious problem at all levels of malaria control. The timely detection and investigation of both individuals and foci with chloroquine-resistant *P. falciparum* is essential for the application of effective counter measures to stop its spread. For this reason the World Health Organization is actively promoting an international programme for the assessment and monitoring of the response of *P. falciparum* to chloroquine.

The WHO recommended two standard types of tests to be employed in all the world for the assessment of the response of *P. falciparum* to chloroquine and other
antimalarial drugs:

a) the WHO in vivo standard (seven days) and extended (28 days) test (WHO, 1973); and

b) the in vitro standard test (WHO, 1977) developed on the basis of the work of Rieckmann et al. (1968).

Both of these tests have their disadvantages when applied in the field.

The in vivo test requires repeated observations or hospitalization of the patients which are costly and receive less acceptance from the community. The human element affects the results of the test (e.g. absorption of the drug and the immune status of the subject). The funds and personnel needed for the in vivo test limit the application of it to a small proportion of participants. Recurrence of parasitaemia within 28 days after administration of chloroquine in vivo provides only presumptive evidence of a drug-resistant infection in areas where re-infection cannot be ruled out.

The in vitro macro-method of Rieckmann et al. (1968), which was adopted by WHO (1977), overcomes most of the disadvantages of the in vivo test, but it has disadvantages of its own. These can be summarized in the following points:

- at least 10 - 12 ml of venous blood were needed for the test; this virtually excludes infants
and young children as well as persons with a high degree of anaemia;
- blood samples must contain at least medium or large rings;
- tests were limited to blood samples with parasite densities between 1000 and 80,000/μl of blood.

To overcome some of the above mentioned disadvantages the WHO Scientific Group on the Chemotherapy of Malaria (WHO, 1973), strongly recommended the development of a micro technique for in vitro drug sensitivity testing. On the basis of the WHO recommendation the following study was carried out.
4.3 Laboratory Cultures

4.3.1 Rodent malaria - Materials and Methods

In-bred white mice - Swiss albino TO strain of both sexes were used. The mice were 3 - 5 weeks old and weighing between 18 - 22 grams before inoculation with the parasites. The mice were infected intraperitoneally with red cells parasitized by either P. berghei berghei (strain obtained frozen in liquid nitrogen from the Ross Institute of Hygiene of The London School of Hygiene and Tropical Medicine), or with one of three strains of P. chabaudi (Lump 1370 derived from Edinburgh stabilate 314 obtained from Dr Targett at Winches Farm; a chloroquine-sensitive line called Stabilate 665 from Edinburgh; and a chloroquine-resistant line 668 derived from the previous sensitive line). Both the last two strains were obtained frozen in liquid nitrogen from Dr Walliker of the Institute of Animal Genetics, University of Edinburgh. The cages in which the mice were kept were labelled with the date, time of inoculation and the strain the mice were inoculated with. The malaria parasites were routinely passaged every 3 or 4 days into fresh mice. Infection was monitored by preparing thick and thin blood films from a drop of blood obtained by a slight snip of the tip of the tail of the mice with a pair of scissors. The blood films were
stained with 5% Giemsa and examined under the microscope. Blood for culture was obtained under aseptic conditions by cardiac puncture using a disposable 2 ml syringe damped with heparin. The mice were anaesthetized by chloroform in a glass chamber. Usually about 1 to 1.5 ml of blood was obtained from an infected mouse. After determining the percentage of red cells parasitized, which was usually high, up to 30%, and the morphological stages of the parasites, the blood was cultured in microtitre plates using different makes of plates and different media. 96 well, flat bottom, microtitre plates, 12.5 x 8.5 x 1.5 cm with a lid, were used. Nunc (InterMed, Denmark) and Falcon (Microtest II, Falcon Plastics Division, BioQuest, California, USA) plates were used.

The different media used were:

- 0.5% glucose (5 mg/ml), prepared from 5% glucose stock solution
- 199 medium (Burroughs Wellcome, UK), complete x 1 liquid medium buffered with 4.4% sodium bicarbonate
- 199 medium + glucose 5 mg/ml
- 199 medium + glucose 5 mg/ml + 10% foetal calf serum
- Eagle’s minimum essential medium (MEM) (Wellcome Reagents Ltd, Beckenham, Kent, UK) complete x 1 liquid medium with 4.4% sodium bicarbonate
- Coombs and Gutteridge medium which was prepared as follows:
Component | Amount used | Concentration after addition of blood
---|---|---
Eagle's MEM\(^1\) | 750 ml | x 1.33 diluted
D-glucose | 4.0 g | 0.48% (w/v)
Na\(_2\)H\(_2\)PO\(_4\)·2H\(_2\)O | 0.27 g | 9.7 mM
Na\(_2\)HPO\(_4\)·12H\(_2\)O | 2.6 g | 9.7 mM
Glycylglycine | 1.3 g | 10 mM
HEPES\(^2\) | 2.4 g | 10 mM
TES\(^3\) | 9.2 g | 40 mM
Adenosine | 4.0 mg | 4.0 µg/ml
p-Aminobenzoic acid | 0.1 mg | 0.1 µg/ml
Biotin | 0.1 mg | 0.1 µg/ml
Glass distilled water | to 875 ml | -

1. Minimal Essential Medium
2. N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

The following combinations of blood in medium were tried:

- 5 µl of blood in 50 µl of medium
- 10 µl of blood in 100 µl of medium
- 20 µl of blood in 100 µl of medium
- 25 µl of blood in 175 µl of medium
- 50 µl of blood in 200 µl of medium
- 100 µl of blood in 100 µl of medium

Eppendorf pipettes (Brinkmann Instruments, Wesbury, NY, USA) were used to add the medium and the blood to the
plates. After preparing the culture plates, they were put inside a strong autoclavable plastic bag, sealed with an electric soldering iron, except for a small opening through which a gas mixture of 5% CO₂, 5% O₂ and 90% N₂ was blown in and the bag was then completely sealed and incubated at 37°C in a dry incubator. After 24 hours of incubation, the bags were removed from the incubator, opened with a pair of scissors, and the plates were sampled to see if there was any growth. To prepare the thick blood films from the plates, the supernatant from each well was withdrawn using an Eppendorf pipette, and 3 to 4 thick films were prepared on one slide. A clean tip was used for each well to avoid transporting parasites from one well to another. Each slide was labelled with the date, culture number and each smear was numbered with its well number. The slides were left to dry for 3 to 5 hours, or overnight before staining. 5% Giemsa at pH 7.2 for 30 minutes, was used for staining the culture slides. The growth of parasites which were asynchronous in mice was measured by counting 200 asexual parasites and enumerating them morphologically into rings, trophozoites, early schizont and late schizonts. This was compared with the preculture slide to see if there were any changes in the relative distribution of the different stages, which could indicate if there was any growth or reinvasion.
Results

5 cultures of *P. berghei* and 17 of *P. chabaudi* were attempted using the different media mentioned before. No success was obtained in any of the cultures. Staining with Giemsa was affected by heparin, especially in thin films, which made it difficult to read most of the culture slides. High parasitaemia and the asynchronicity of the parasites made evaluation of growth difficult. It was observed that thick blood films from cultures washed away during staining if they were not dried overnight or at least for about 5 to 6 hours before staining. 5 cultures of *P. chabaudi* were attempted using the modified medium of Coombs and Gutteridge (1975) but none of the 5 cultures showed any growth.

4.3.2 Primate Malaria

*P. fieldi* and *P. coatnevi* - Materials and Methods

Six rhesus monkeys (*Macaca mulatta*), 3 males and 3 females weighing between 4.6 and 9.0 kilograms, were used in the experiments. Two monkeys were splenectomised and infected with a strain of *P. fieldi* obtained originally from Dr Meuwissen, Nijmegen, Holland, which was stored frozen in liquid nitrogen in the Ross Institute of Hygiene. The parasitized blood was thawed
quickly and injected intravenously through the saphenous vein. The other four monkeys were left intact, and infected with *P. coatneyi*. The parasites were obtained frozen in liquid nitrogen from Dr L.H. Miller of the National Institutes of Health, Bethesda, Maryland, USA. This parasite, originally described by Eyles et al. (1962) from Malaya, closely resembles *P. falciparum* in its morphology and periodicity. It is quite synchronous with only ring and trophozoite stages seen in the peripheral blood, except in cases of severe chronic infections with high parasitaemia when schizonts and gametocytes appear in the circulating blood. The first monkey, M331, weighing 6 kg, was infected with the parasites intravenously through the saphenous vein. The monkeys were anaesthetized by phencyclidine hydrochloride (Sernylan) 1 mg/kg body weight, intramuscularly. The parasites were passaged into the other monkeys, which were clean from any other infection, by the same route at approximately monthly intervals. The parasitaemia was monitored by examining Giemsa-stained thick and thin blood films prepared from a blood drop from the ear-lobe of the monkeys. High parasitaemias were controlled by suppressive doses of 3 to 5 mg/kg body weight of chloroquine phosphate given intramuscularly. Monkeys with high parasitaemia (> 20%) suffer from severe anaemia, and two of them died within a period of 2 to 4 weeks after inoculation. When the parasitaemia
reached 0.5% to 5% and most of the parasites were rings or trophozoites, blood was drawn for culture aseptically, using a 20 ml disposable syringe, from the femoral vein after anaesthetising the monkey. Different anticoagulants were used for collecting the blood. The blood was then transported from the Winches Farm at St Albans to the Ross Institute in London in a 20 ml screw cap glass tube at ambient temperature. Different variables were studied using the two primate malarias *P. fieldi* and *P. coatneyi* as models.

**Culture Technique**

The culture procedure followed was a modification of the candle jar technique first described by Jensen and Trager (1977). 96 well (flat bottom) microtitre plates were used. The plates were made by either Nunc (Inter Med, Denmark) or Falcon (Microtest II, Falcon Plastic Devisioin, BioQuest, USA). The medium used was that first developed at the Roswell Park Memorial Institute (RPMI 1640 growth medium) obtained from Gibco BioCult, Glasgow, Scotland, UK. The medium which contains glutamine was buffered with a 7.5% solution of NaHCO₃ to a final concentration of 2 mg/ml and a 1 molar solution of HEPES to give a final concentration of 25 mM. For detailed method see Annex 5. 50 µl of the complete
medium, which had been sterilized by filtering through a 0.22 µm pore millipore Swinnex filter (Millipore Corporation, Bedford, UK), were added to the wells of the plate with an Eppendorf pipette. The blood was added in quantities of 5 µl to each well. Different concentrations of either chloroquine phosphate or pyrimethamine were used in the tests. The methods of preparing and adding these are described below. After preparing the culture plates they were placed inside a vacuum desiccator of about 12 inches diameter in which a white paraffin wax candle was placed. The top cover of the desiccator was adapted with a stop-cock opening. The candle was lit, and the top was placed firmly on top with the stop-cock open. Just when the candle went out the stop-cock was closed. The burnt candle produces a gaseous atmosphere of 3.0 to 3.5% CO₂ and 15 to 16% O₂. The amount of CO₂ and O₂ produced by the burning candle were measured exactly, with the cooperation of the Respiratory Physiology Department of the Lung Unit, Brompton Hospital, London, using a centronic type 806 mass spectrometer. The parasites were incubated at 38 ± 0.5°C in a dry incubator or in a 'Millipore' portable incubator, which can be run from a voltage of 240 V to 12 V, fitted with a purpose built small vacuum box to act as a candle jar. At the end of the incubation period the plates were taken out, the supernatant in each well was withdrawn using an
Eppendorf pipette and a thick blood film was prepared from each well. A clean tip was used for each well to avoid transporting parasites from one well to the other. Three to four films were prepared in each slide. The films were left to dry at room temperature or inside the incubator at 37°C for 4 to 5 hours before staining. 3% Giemsa stain at a pH of 7.2 to 6.6 was used to stain the culture slides for 30 minutes. Gentle washing under tap water to remove excess stain was carried out with the utmost care so as not to wash off the blood films from the slides. Morphological stages of 200 asexual parasites were enumerated to evaluate the development of the parasites during the culture period. The results for each well were expressed as the percentages of schizonts present in 200 asexual parasites counted. A schizont being considered as a parasite in which the chromatin divided into more than two pieces.

Using the estimated final concentration of drug in each well, the ED₅₀, ED₉₀, slope of the curve and the $x^2$ for the goodness of fit were calculated with a computer programme written by Professor M. Healey (Medical Statistics Department, London School of Hygiene and Tropical Medicine). The programme used the standard maximum likelihood method for fitting a probit curve.
The variables studied and their results:

7 cultures of *P. fieldi* were attempted with no success, while 40 cultures of *P. coatneyi* were attempted with some positive results to study the following variables:

a. Effect of anticoagulants:

Blood for culture was collected from monkeys in the different anticoagulants mentioned below:

- Heparin (Evans Medical Ltd, Liverpool, UK)
  1000 IU per ml used as 10 IU/ml of blood
- Disodium salt of ethylene-diamine tetraacetic acid (EDTA or Sequestrene) used at a concentration of 25% in blood
- Acid-citrate-dextrose (ACD) used to a final concentration of 15% in blood (Annex 3)
- Citrate-phosphate-dextrose (CPD) used to give a final concentration of 14% in blood (Annex 4).

It was observed that the growth of parasites collected in heparin or sequestrene was inhibited, and parasite counts after the incubation period were less than those of the pre-culture, indicating the toxication and death of the parasites during incubation. Also, heparin was found to affect staining with Giemsa of the cultured parasites giving the whole parasitized cell a faint pink colour. Blood collected in CPD always clotted in the plates while preparing the cultures or
during the incubation period. Best growth was obtained with blood collected in ACD.

b. Effect of pH on staining:

Different pHs were tried to find the best pH for staining cultured parasites. 3% Giemsa stain at pH 7.2, 7.1, 7.0, 6.8 or 6.6 for 30 minutes was tried for thick blood films prepared from culture plates after incubation. Observations showed that best appearance of the parasites with well-stained chromatin, cytoplasm and pigment was obtained at a pH of 6.8.

c. Washing blood:

To avoid possible interference of host antibodies with the growth of the parasites in vitro, the parasitized blood was washed prior to the set up of cultures with sterile PBS (Annex 1). The PBS was sterilized by autoclaving at 15 lb/square inch for 30 minutes. The blood was first spun in a 20 ml sterile screw-cap glass tube in a centrifuge at 2000 rpm for 10 minutes. The plasma was removed with a sterile pipette without disturbing the packed red cells at the bottom of the tube. 10 ml of sterile PBS were added, and it was then centrifuged for another ten minutes at 2000 rpm, after which the supernatant was discarded. This was repeated three times before culturing the blood.

Only 2 cultures out of 5 attempts using washed and unwashed blood and different concentrations
during the incubation period. Best growth was obtained with blood collected in ACD.

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Only 2 cultures out of 5 attempts using washed and unwashed blood and different concentrations
of chloroquine phosphate in medium gave comparable results. Table 11 and Figures 23&24 show that washing the blood from its plasma reduces the parasite growth rate in vitro. Comparing the ED50s of washed and unwashed blood it was found that the ED50 was higher for the unwashed blood than that for the washed blood. Also it was observed that some bacterial contamination always takes place in wells of washed blood during the incubation period. This was attributed to hazards of contaminating the blood during the process of washing even when utmost care was taken to keep a high level of sterility.

d. Period of incubation and stage of parasites:

Parasites at different stages and different ages were incubated for periods ranging between 24 to 48 hours. Young as well as fleshy rings and trophozoites were cultured. Better growth was observed after 48 hours' incubation if the parasites were young rings at the start of the culture. This can be seen from Table 12 and Figures 25 to 29. Higher percentages of schizont maturation were observed specially in the control and the ED50 was lower than that at shorter incubation periods. But, as can be seen from culture 43 in the same table, when culture was started with fleshy rings, full maturation to 100% was obtained in controls after 28 hours but at 48 hours some increase in maturation was seen in the drug wells, with slightly
higher EDSOs. When trophozoite stages were cultured, maturation to schizonts was observed in all test wells, indicating that drug had little or no effect at all on the parasites which started dividing before the drug reached them.

e. Different preparations of chloroquine and pyrimethamine:

Different ways of preparing the drug were tried out to find one which can easily be applied in the field. Three different methods were tried in this study:

1. Drug prepared straight in the culture medium. This was done by preparing a strong stock solution in distilled water, e.g. $10^{-4}$ Molar solution, and then this was further diluted in complete medium to give the range of concentrations required. From each concentration 50 μl were placed in each well of the microtitre plate prior to adding the blood.

2. Appropriate dilutions of the drug were made in distilled water and 5 μl amounts of each concentration were added to the plate wells already containing 50 μl of complete medium.

3. Freeze dried. This was done by preparing different concentrations of the drug in distilled water, then 20 μl of each concentration was transferred to plate wells. This was freeze dried in an Edwards shelf freeze-drier (Edwards High Vacuum Ltd, UK). The freezing was by evaporative cooling and the plates were left overnight at a vacuum of $10^{-3}$ Torr.
Table 11: Percentage schizont maturation of *P. coatneyi* comparing growth in washed and unwashed blood under the effect of different concentrations of chloroquine phosphate (200 asexual parasites counted)

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Control</th>
<th>Washed Blood</th>
<th>Unwashed Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$10^{-8.65}$</td>
<td>$10^{-8.35}$</td>
</tr>
<tr>
<td>21</td>
<td>27</td>
<td>17</td>
<td>N.D.</td>
</tr>
<tr>
<td>23</td>
<td>80</td>
<td>61</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. = not done
Fig. 23

*P. coatneyi* in the *in vitro* micro-test

Probit scale

Effect of Chloroquine CASE 21

- - - - - Unwashed blood
- - - - - Washed blood
Fig. 24

P. coatneyi in the in vitro micro-test

Probit scale

Effect of Chloroquine CASE 23

- - - - - - Unwashed

- - - - - - Washed
Table 12: Percentage schizont maturation of *P. coatneyi* incubated at different periods of time under the effect of chloroquine phosphate (200 asexual parasites counted)

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Period of incubation</th>
<th>Stage of parasites</th>
<th>Control</th>
<th>$10^{-8.65}$</th>
<th>$10^{-8.35}$</th>
<th>$10^{-8.05}$</th>
<th>$10^{-7.75}$</th>
<th>$10^{-7.05}$</th>
<th>ED$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>28 hours</td>
<td>Young rings</td>
<td>51</td>
<td>41</td>
<td>35</td>
<td>30</td>
<td>16</td>
<td>14</td>
<td>$1.1 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>Young rings</td>
<td>71</td>
<td>43</td>
<td>32</td>
<td>34</td>
<td>22</td>
<td>20</td>
<td>$4.5 \times 10^{-9}$</td>
</tr>
<tr>
<td>29</td>
<td>24 hours</td>
<td>Young rings</td>
<td>67</td>
<td>60</td>
<td>61</td>
<td>48</td>
<td>40</td>
<td>42</td>
<td>$1.4 \times 10^{-7}$</td>
</tr>
<tr>
<td></td>
<td>42 hours</td>
<td>Young rings</td>
<td>82</td>
<td>68</td>
<td>60</td>
<td>56</td>
<td>40</td>
<td>44</td>
<td>$9.1 \times 10^{-9}$</td>
</tr>
<tr>
<td>30</td>
<td>24 hours</td>
<td>Young rings</td>
<td>41</td>
<td>36</td>
<td>39</td>
<td>35</td>
<td>29</td>
<td>22</td>
<td>$1.1 \times 10^{-7}$</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>Young rings</td>
<td>67</td>
<td>41</td>
<td>38</td>
<td>39</td>
<td>27</td>
<td>22</td>
<td>$1.0 \times 10^{-8}$</td>
</tr>
<tr>
<td>42</td>
<td>24 hours</td>
<td>Young rings</td>
<td>86</td>
<td>N.D.</td>
<td>N.D.</td>
<td>69</td>
<td>28</td>
<td>5</td>
<td>$4.7 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>45 hours</td>
<td>Young rings</td>
<td>100</td>
<td>N.D.</td>
<td>N.D.</td>
<td>72</td>
<td>32</td>
<td>7</td>
<td>$3.6 \times 10^{-8}$</td>
</tr>
<tr>
<td>43</td>
<td>28 hours</td>
<td>Fleshy rings</td>
<td>100</td>
<td>67</td>
<td>53</td>
<td>39</td>
<td>20</td>
<td>12</td>
<td>$1.4 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>Fleshy rings</td>
<td>100</td>
<td>87</td>
<td>77</td>
<td>59</td>
<td>43</td>
<td>22</td>
<td>$3.1 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

N.D. = not done
Fig. 25

P. coatneyi in the in vitro micro-test

Probit scale

Effects of Chloroquine CASE 27
Unwashed blood

- - - - - - - - 28 hours incubation
- - - - - - - - 49 hours incubation

% SCHIZONT MAURATION

CONCENTRATIONS OF CHLOROQUINE IN MOLARS
Fig. 26

*P. coatneyi* in the *in vitro* micro-test

Probit scale

Effect of Pyrimethamine CASE 40

- \(\triangle\) 26 hours incubation
- \(\triangle\) 48 hours incubation

% SCHIZONT MATURATION

CONCENTRATIONS OF PYRIMETHAMINE IN MOLARS
Fig. 27

P. coatneyi in the in vitro micro-test

Probit scale

Effects of Chloroquine in medium CASE 42

- 24 hours incubation
- 45 hours incubation

% SCHIZONT MATURATION RELATIVE TO CONTROL

CONCENTRATIONS OF CHLOROQUINE IN MOLARS
Fig. 28

P. coatneyi in the in vitro micro-test

Probit scale

Effect of Pyrimethamine CASE 43

\[ \begin{align*}
\Delta \quad \Delta & \quad 28 \text{ hours incubation} \\
\Delta \cdots \cdots \Delta & \quad 48 \text{ hours incubation}
\end{align*} \]

% SCHIZONT MATURATION

CONCENTRATIONS OF PYRIMETHAMINE IN MOLARS
Fig. 29

*P. coatneyi* in the *in vitro* micro-test

Probit scale

Effect of Pyrimethamine

- △-△ Case 46 - 46 hours incubation
- •-• Case 47 - 28 hours incubation
- □-□ Case 48 - 48 hours incubation

% SCHIZONT MATURATION

CONCENTRATIONS OF PYRIMETHAMINE IN MOLARS
Table 13 and Figures 30, 31 and 32 show the effect of chloroquine prepared in three different ways on the maturation of the parasites to schizonts. The three cultures with chloroquine show that a good dose-response curve was obtained when the drug was prepared in medium or when added in distilled water, but not with freeze-dried preparation. However ED$_{50}$s for distilled water were always slightly lower than for medium. This suggests that there might be more loss of drug by adsorption when prepared in medium than from that prepared in distilled water. It also seems that there was some loss of active drug on freeze drying as shown by generally greater maturation of parasites at all dilutions, when compared with medium or distilled water.

For the effect of pyrimethamine (Table 14 and Figures 33, 34 and 35) not much can be said because the percentage of parasites that matured to schizont in the five low concentrations of the drug was as high as that in control wells. Growth of the parasites was only inhibited at the concentration of $10^{-6}$ and $10^{-5}$ M. This could be due to the fact that the effect of pyrimethamine is mostly exerted on the parasites at the reinvasion state, although some inhibition of maturation appears to occur with the two higher concentrations.

f. Level of parasitaemia:

The effect of different levels of parasitaemia on the maturation of the parasites and the effect of
different concentrations of drugs on them was not studied simultaneously. However comparisons of different cultures suggested that at very high parasitaemias (over 10%) a smaller proportion of parasites mature to schizonts as compared to lower parasitaemias. Also it could be observed that the drug was less effective. Some results of cultures of different parasitaemias which suggest this are given in Table 15 and Figure 35.

4.3.3 Human Malaria

Nine cases of *P. falciparum* malaria, and three of *P. vivax* obtained from blood of patients who reported to outpatient clinics and were admitted as malaria patients in different hospitals in London, were tried in culture. None of them grew to more than trophozoites. This was due to several reasons, such as blood being kept overnight in Sequestrene before culture, or high parasitaemia with very young rings. Also none of the *P. vivax* cultures showed any growth *in vitro*.

*P. falciparum* from continuous cultures and monkeys

Attempts to culture parasites of *P. falciparum* which was maintained in continuous cultures, had given no success. This might be due to the fact that the parasites were already adapted to a serum supplemented medium.
Table 13: Percentage schizont maturation of *P. coatneyi* under the effect of chloroquine phosphate prepared in different ways (200 asexual parasites counted)

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Method of chloroquine preparation</th>
<th>Control</th>
<th>$10^{-9}$</th>
<th>$10^{-8}$</th>
<th>$10^{-7}$</th>
<th>$10^{-6}$</th>
<th>$10^{-5}$</th>
<th>ED$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>Drug in medium</td>
<td>99</td>
<td>90</td>
<td>81</td>
<td>81</td>
<td>36</td>
<td>9</td>
<td>$6.8 \times 10^{-7}$</td>
</tr>
<tr>
<td></td>
<td>Drug added in distilled water</td>
<td>90</td>
<td>77</td>
<td>24</td>
<td>8</td>
<td>3</td>
<td>0</td>
<td>$6.5 \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>Drug freeze dried</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>38</td>
<td>18</td>
<td>8</td>
<td>$5.7 \times 10^{-8}$</td>
</tr>
<tr>
<td>52</td>
<td>Drug in medium</td>
<td>92</td>
<td>80</td>
<td>33</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>$6.4 \times 10^{-7}$</td>
</tr>
<tr>
<td></td>
<td>Drug added in distilled water</td>
<td>100</td>
<td>98</td>
<td>96</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>$2.8 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>Drug freeze dried</td>
<td>95</td>
<td>95</td>
<td>89</td>
<td>88</td>
<td>0</td>
<td>0</td>
<td>$2.0 \times 10^{-7}$</td>
</tr>
<tr>
<td>53</td>
<td>Drug in medium</td>
<td>98</td>
<td>91</td>
<td>48</td>
<td>11</td>
<td>7</td>
<td>2</td>
<td>$1.6 \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>Drug added in distilled water</td>
<td>95</td>
<td>95</td>
<td>88</td>
<td>18</td>
<td>1</td>
<td>0</td>
<td>$4.4 \times 10^{-7}$</td>
</tr>
<tr>
<td></td>
<td>Drug freeze dried</td>
<td>99</td>
<td>98</td>
<td>90</td>
<td>80</td>
<td>23</td>
<td>1</td>
<td>$2.6 \times 10^{-7}$</td>
</tr>
</tbody>
</table>
Fig. 30

*P. coatneyi* in the *in vitro* micro-test

Three different ways of preparing Chloroquine

CASE 51

Probit scale

Drug in medium \(\triangle\)  \(\triangle\)  Drug in D.W.  \(-\)  \(-\)

Drug F.D.  \(\square\)  \(\square\)  added to plate

% SCHIZONT MATURE relative to control

CONCENTRATIONS OF CHLOROQUINE IN MOLARS
Fig. 31

P. coatneyl in the in vitro micro-test
Three different ways of preparing Chloroquine
CASE 52
Probit scale

Drug in medium Δ——Δ Drug in D.W. •—•
Drug F.D. □——□

% SCHIZONT MATURATION RELATIVE TO CONTROL

CONCENTRATIONS OF CHLOROQUINE IN MOLARS
P. coatneyi in the in vitro micro-test
Three different ways of preparing chloroquine

CASE 53

Probit scale

- Chloroquine dissolved in medium
- Chloroquine added to plate in D.W.
- Chloroquine freeze-dried in plate

% GROWTH TO SCHIZONTS RELATIVE TO CONTROL

CONCENTRATIONS OF CHLOROQUINE IN MOLARS
Table 14: Percentage schizont maturation of *P. coatneyi* under the effect of pyrimethamine prepared in different ways (200 asexual parasites counted after incubation for 40 hours)

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>Method of pyrimethamine preparation</th>
<th>Control</th>
<th>10^-10</th>
<th>10^-9</th>
<th>10^-8</th>
<th>10^-7</th>
<th>10^-6</th>
<th>10^-5</th>
<th>ED50</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>Drug in medium</td>
<td></td>
<td>93</td>
<td>93</td>
<td>86</td>
<td>79</td>
<td>80</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Drug added in distilled water</td>
<td></td>
<td>100</td>
<td>96</td>
<td>94</td>
<td>93</td>
<td>94</td>
<td>91</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Drug freeze dried</td>
<td></td>
<td>94</td>
<td>91</td>
<td>95</td>
<td>86</td>
<td>91</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>53</td>
<td>Drug in medium</td>
<td></td>
<td>98</td>
<td>96</td>
<td>75</td>
<td>86</td>
<td>80</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Drug added in distilled water</td>
<td></td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>92</td>
<td>58</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Drug freeze dried</td>
<td></td>
<td>97</td>
<td>97</td>
<td>96</td>
<td>89</td>
<td>46</td>
<td>36</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 33

P. coatneyi in the in vitro micro-test.

Three different ways of preparing pyrimethamine

CASE 52

Probit scale

Drug in medium \( \triangle \) \( \text{Drug in D.W.} \) \( \bullet \) \( \text{Drug F.D.} \) \( \square \)

added to plate

CONCENTRATIONS OF PYRIMETHIMINE IN MOLARS

% SCHIZONT MATURATION
Fig. 34

P. coatneyi in the in vitro micro-test
Three different ways of preparing pyrimethamine

CASE 53

Probit scale

Drug in medium \(\triangle\) --- \(\triangle\) Drug in D.W. \(\bullet\) --- \(\bullet\) added to plate

% SCHIZONT MATURATION

CONCENTRATIONS OF PYRIMETHAMINE IN MOLARS
Table 15: Percentage schizont maturation of *P. coatneyi* under the effect of chloroquine phosphate comparing different levels of parasitaemia (200 asexual parasites counted)

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>% Parasitaemia</th>
<th>Stage of parasites</th>
<th>Incubation period</th>
<th>Control</th>
<th>10^-7.6</th>
<th>10^-7.0</th>
<th>10^-6.4</th>
<th>10^-5.8</th>
<th>10^-5.2</th>
<th>ED_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>1%</td>
<td>Young rings</td>
<td>24 hours</td>
<td>56</td>
<td>23</td>
<td>21</td>
<td>15</td>
<td>20</td>
<td>16</td>
<td>2.0 x 10^-8</td>
</tr>
<tr>
<td>57</td>
<td>10.4%</td>
<td>Young rings</td>
<td>48 hours</td>
<td>72</td>
<td>18</td>
<td>15</td>
<td>10</td>
<td>13</td>
<td>0</td>
<td>8.6 x 10^-10</td>
</tr>
<tr>
<td>58</td>
<td>13.4%</td>
<td>Young rings</td>
<td>48 hours</td>
<td>64</td>
<td>60</td>
<td>56</td>
<td>60</td>
<td>61</td>
<td>0</td>
<td>2.4 x 10^-6</td>
</tr>
</tbody>
</table>
Fig. 35

*P. coatneyi* in the *in vitro* micro-test

Probit scale

Pyrimethamine in D.W.

Case 54 O—— O Case 57 ————

% SCHIZONT MATURATION RELATIVE TO CONTROL

CONCENTRATIONS OF PYRIMETHAMINE IN MOLARS
Parasites obtained from Aotus monkeys were always asynchronous and with high parasitaemia, and this was why none of them had grown beyond the trophozoite stages in the micro-test plates.

4.4 Trial of In Vitro Micro-Test in the Field - Sennar Area

The main objectives of this study were:

a. to evaluate the in vitro micro-technique under field conditions;

b. to establish the level of sensitivity of local P. falciparum to chloroquine applying the in vitro micro-technique, the standard WHO macro-technique as well as the WHO in vivo test.

4.4.1 Study Area

The study area, which used to be called Malaria Eradication Demonstration and Training Operation Area (MEDTO Area) was described in detail in Chapter 1 (1.10.a) and shown in Figure 8. In this area malaria was formerly reported to be mesoendemic with hyperendemic spots (Wernsdorfer and Wernsdorfer, 1967) as shown in Figures 6 and 7. The area was under extensive spraying operation since 1958 and up to 1971. This had led to the virtual elimination of indigenous malaria and in most villages of the area both the active and passive
case detection failed to reveal a single case of malaria among the resident population. By the end of 1971 the vector A. arabiensis was reported as highly resistant to both BHC and DDT (Malaria Pre-eradication Programme Annual Report, 1971). This was thought to be related to aerial spraying with various insecticide compounds applied in large agricultural schemes, which mainly grow cotton in this area. When the area was handed over to the provincial health authorities, all antimalaria activities came to a standstill. In 1975, focal epidemics of malaria occurred in a number of villages which forced the health authorities to resume spraying operations with an alternative insecticide. Malathion, which was applied in the same year in the Gezira Irrigated Area (see Chapter 6) was used at a dosage of 2 grams of active ingredient per square meter. This continued annually, but in 1978 the late delivery of the insecticide stopped the operations.

Materials and Methods

Parasitological findings

Prior to the studies, parasitological surveys were carried out in Sennar town and in five villages which were reputed to be the most malarious localities of the district by a WHO team helped by the staff of the METC Sennar. The results of slide examinations from Sennar
town and two villages were absolutely negative. In the other three villages the parasite rates were below 5%, indicating a rather low degree of endemicity. From a total 1515 blood slides collected from fever cases during the survey, some 131 were found positive, representing a slide positivity rate of 8.65% (Kouznetsov et al., 1979).

The patients studied

Prior to the drug sensitivity studies, well-trained malaria microscopists were stationed in the outpatient clinics of Sennar Hospital, Sennar Health Centre, Genina village dispensary and METC laboratory. Their task was to take thick blood films from all people with fever reporting to those outpatient clinics, stain them rapidly for 10 - 20 minutes with 10% Giemsa, and examine 20 - 25 microscopical fields using an oil immersion lens of a binocular compound microscope. Subjects having asexual forms of *P. falciparum* in their blood were considered to be suitable candidates for the study. (Figure 8)

Most of the positive cases came from peripheral areas of Sennar district or had contracted the infection outside the district. Most of the patients, mainly all of whom were adults, were suffering from high fever associated with other typical clinical symptoms of acute malaria. Some of the subjects presented severe clinical manifestations of malaria as is seen in non- or semi-immune people. This can be attributed to the high degree
of protection of the population for about 13 years.

Urine test for presence or absence of chloroquine

The Dill and Glazko's test (Lelijveld and Kortmann, 1970) was carried out on urine specimens of subjects who were positive for *P. falciparum* prior to bleeding them for culture or to include them in the in vivo studies. Also the test was carried out on subjects who were studied in vivo on days one through seven after the start of treatment.

The method of preparation of the reagent used in this colour test was as follows:

"50 mg of eosin (yellowish) were weighed and transferred to a small glass-stoppered separatory funnel. 100 ml of chloroform (reagent grade) and 1 ml of 1N hydrochloric acid were added, and the mixture was shaken by hand for a few minutes until the chloroform assumes a light-yellow colour due to solution of the eosin. The chloroform layer was allowed to separate, and was transferred to a brown glass-stoppered bottle for storage."

To a small test-tube containing about 2 ml of urine, 10 drops of the Dill-Glazko reagent were added and the contents were mixed by vigorous shaking for a few minutes. A colour change from yellowish to violet-red in the precipitated chloroform layer was taken as an indication of the presence of chloroquine in the urine.
The test was reported to give a high proportion of false positives and false negatives when tried in Malaysia (Dondero and Marappam, 1974). The results obtained during this study were quite good and reproducible with a prominent colour change as that reported by Ghosh, Roy and Sitaraman (1976) in India.

Preparation of medium and chloroquine phosphate

The medium was prepared as described in Annex 5. After preparation of the medium, chloroquine concentrations in the medium were prepared as follows:

51.6 mg of chloroquine phosphate were weighed accurately and dissolved in 1 litre of distilled water in London. This will give $10^{-4}$ Molar solution. In the field, a tenfold dilution was done by taking 1 ml of the $10^{-4}$ Molar solution into 9 ml of medium to give $10^{-5}$ Molar stock solution which is equivalent to $10^4$ p-mol of chloroquine/ml. 1 ml of stock solution was put into 14.6 ml of medium to give approximately 640 p-mol/ml. This was then diluted in a serial of twofolds in medium to give 320, 160, 80, 40 and 20 p-mols/ml. To prepare the 240 and 120 p-mols/ml, 0.25 ml of the stock solution was taken in 10.4 ml of medium, this will give 240 p-mols/ml, then a twofold dilution from this will give 120 p-mols/ml. A concentration of 100 p-mols/ml was also prepared by taking
0.1 ml of the stock solution in 9.9 ml of medium. These concentrations 20, 40, 80, 100, 120, 160, 240, 320 and 640 p-mols/ml were prepared in plastic or glass vials/tubes with tight plastic screw tops, well marked with the chloroquine concentration inside and stored at 4°C ready for use. Sterility was obtained by filtering all the liquids through millipore 0.22 µm pore filters into sterilized plastic or glass containers. For the test 50 µl of each concentration was added by means of an Eppendorf pipette to each well of the plate. The dosage of chloroquine per well will be expressed in an ascending order of concentrations as 1, 2, 4, 5, 6, 8, 12, 16 and 32 p-mols/well. This was calculated instead of Molars to follow Rieckmann (1978) and WHO procedure so as to standardize interpretation of the results. An equivalent table will be as follows:

<table>
<thead>
<tr>
<th>Molars</th>
<th>p-mol/ml</th>
<th>p-mol/well (50 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-7.7}$</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>$10^{-7.4}$</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>$10^{-7.1}$</td>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>$10^{-6.9}$</td>
<td>120</td>
<td>6</td>
</tr>
<tr>
<td>$10^{-6.8}$</td>
<td>160</td>
<td>8</td>
</tr>
<tr>
<td>$10^{-6.6}$</td>
<td>240</td>
<td>12</td>
</tr>
<tr>
<td>$10^{-6.5}$</td>
<td>320</td>
<td>16</td>
</tr>
<tr>
<td>$10^{-6.2}$</td>
<td>640</td>
<td>32</td>
</tr>
</tbody>
</table>
Blood was collected from the subjects selected for the study, in 1 ml syringes containing 0.15 ml ACD (Annex 3) from one of the arm veins. 12 to 15 ml of blood was collected from the vein in a 20 ml syringe from subjects who were chosen for the comparative study of the macro and micro-techniques. The blood was immediately transferred into a sterile 25 ml Erlenmeyer flask (stoppered) containing glass beads, and defibrinated by rotating the flask for 5 minutes.

Blood samples collected from a finger prick, using a 100 µl Eppendorf pipette with a sterile tip containing a drop of ACD were tried from people who reported to METC laboratory.

Culture Techniques

Prior to culturing the blood, a micro-titre culture plate was dosed with 50 µl of medium alone in wells Nos. 2 and 3 and 50 µl of medium containing drug at different concentrations, starting with the lowest concentration, in wells Nos. 4 to 12. Well No. 1 was left as a reservoir for the blood. The number of rows of the plate dosed depend on the number of cases available, each row for one case. Then with a 5 µl Eppendorf pipette, 5 µl of parasitized blood were transferred from well No. 1 to each of the wells Nos. 2 - 12. The plate was then covered with its lid, gently shaken and placed in a
vacuum desiccator of 12 inches diameter. Both plastic and glass desiccators were used. The top of the desiccator was adapted with a stopcock. Inside the desiccator two white paraffin candles were used to provide an atmosphere of low oxygen tension and high carbon dioxide tension (3% CO₂ and 16% O₂). The candles were lit before the introduction of the culture plate, then the culture plate was gently centred and balanced at the bottom of the desiccator, the top of the desiccator was placed firmly in its place with the stopcock open. The stopcock was closed just before the flame went out. The desiccator was then placed in a dry incubator at temperatures between 37° and 39°C for a period depending on the pre-culture size and age of the parasites.

Figure 36 shows the technique of the microtest.

A portable Millipore incubator, which can be run from mains or batteries, was used after construction of a small box to replace the candle jar. Cultures were run simultaneously on a duplicate from each subject in parallel with those incubated in the dry standard incubator.

For comparison blood from subjects found suitable for the macro in vitro test, was processed following the instructions of the WHO (1977) produced with the kit.

1 ml aliquots of the defibrinated blood were placed using a sterile 1 ml pipette into the screw cap,
Fig. 36
Preparation of the in vitro micro-test in culture of *P. falciparum* - Sennar area.
Fig. 36
Preparation of the in vitro micro-test in culture of
P. falciparum - Sennar area.
flat-bottomed test vials which are already dosed with 5 mg glucose alone for controls, and 5 mg glucose with different concentrations of chloroquine for testing. The blood in the vials was swirled gently to mix the contents well. The vials were then placed in the provided rack and placed in the dry-air incubator at 38°C ± 0.5°C for 24 hours.

After the end of the incubation period, the plate was taken out of the desiccator and with the aid of a 50 µl Eppendorf pipette the supernatant was removed. Thick blood films were prepared from wells Nos. 2 - 12. A clean tip was used for each well so as to avoid transferring parasites from one well to another. Three to four blood films were placed on each glass slide which was then duly marked with the date, the patient and well numbers. Slides were left to dry at room temperature for at least six hours before staining. 3% Giemsa at pH 6.8 was used for staining the films for a period of 30 minutes. The slides were gently washed under a tap to remove excess stain. The same procedure was followed in preparing thick blood films from the vials of the macro-test, but instead of removing the supernatant, the vials were shaken to resuspend cells in plasma before preparing the films on slides. Counts were done using light microscope with an oil immersion lens (Figures 37 to 40).

The number of schizonts seen in 200 asexual parasites were
Fig. 37
A thin-blood film stained with Giemsa showing fleshy and young rings of *P. falciparum* before culturing *in-vitro*. (x 1250)

Fig. 38
A thick-blood film prepared from a culture plate after 24 hours of incubation showing maturation of *P. falciparum* to schizonts in an *in-vitro* micro-test. (x 1250)
Fig. 37
A thin blood film stained with Giemsa showing fleshy and young rings of *P. falciparum* before culturing in vitro. (x 1250)

Fig. 38
A thick blood film prepared from a culture plate after 24 hours of incubation showing maturation of *P. falciparum* to schizonts in an in vitro micro-test. (x 1250)
Fig. 39
A thick-blood film showing sexual and asexual forms of P. falciparum before in vitro culture. (x 1250)

Fig. 40
A thick-blood film showing sexual and asexual forms of P. falciparum in an in vitro micro-test after 24 hours incubation. (x 1250)
Fig. 39

A thick-blood film showing sexual and asexual forms of *P. falciparum* before *in vitro* culture. (x 1250)

Fig. 40

A thick-blood film showing sexual and asexual forms of *P. falciparum* in an *in vitro* micro-test after 24 hours incubation. (x 1250)
recorded. The data for control growth were taken from the average of readings from the two control wells Nos. 2 and 3. The percentage of maturation to schizonts in test wells relative to control samples was calculated by dividing the observed number of schizonts in each test well by the mean number of schizonts from the two control wells and the result was multiplied by hundred and expressed as a percentage.

**In vivo studies**

The response of *P. falciparum* to chloroquine in vivo was determined in 17 selected subjects by means of the WHO standard field test (seven-day test) (WHO, 1973) and observations in 10 of them were extended for 28 days (WHO, 1973). Selected subjects who showed asexual forms of *P. falciparum* in their peripheral blood, and their urine gave a negative reaction in the Dill-Glazko test were given chloroquine on three consecutive days in doses of 10 mg base/kg body weight on days 1 and 2 and 5 mg base/kg body weight on day 3. All the patients were hospitalized for the first seven day periods. The Dill-Glazko test was carried out on urine specimens obtained on day 1 through 7 after the start of treatment. The parasitaemia was monitored by counting the number of asexual parasites against 300 leucocytes in Giemsa stained thick blood films prepared from the patients on day 1 to 7 and then weekly
The resulting figure of counting parasites against 300 leucocytes was multiplied by 25 to give the count of parasites per μl of blood assuming that the normal local leucocyte count is to be in the region of 7500 per μl. For selecting subjects for the in vivo study, the clinical condition of the patients was at all times considered to be of first priority.

4.5 Results

In vitro cultures - micro technique

Out of the 30 cultures attempted (Table 16) only 11 of them gave readable results with good maturation in the controls as shown in Table 17 and Figures 41, 42 and 43. None of those cultured and incubated in the Millipore incubator showed growth beyond the trophozoites stage. This was due to the fact that the candle box was leaking CO₂, and it was found that after 4 to 6 hours after start of culture the amount of CO₂ dropped from 3.5% to only 0.5%.

Out of the 19 unsuccessful cultures, 3 have shown traces of chloroquine when their urine was tested with the Dill-Glazko, 2 experienced a power cut of 3 hours during incubation period, 4 were discarded because of bacterial contamination and 6 gave 100% maturation in all wells even those with high concentrations of
Table 16: Subjects from whom blood was tried for *in vitro* micro-test - Sennar Area, Sudan.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age in years</th>
<th>Sex</th>
<th>Parasites per ul</th>
<th>Result of urine test</th>
<th>Morphology of parasite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>M</td>
<td>72,500</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>F</td>
<td>38,250</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>F</td>
<td>21,400</td>
<td>-ve</td>
<td>fleshy rings</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>F</td>
<td>39,750</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>F</td>
<td>11,250 &amp; 53,125G</td>
<td>-ve</td>
<td>fleshy rings and gametocytes</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>M</td>
<td>77,700</td>
<td>-ve</td>
<td>fleshy rings</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>F</td>
<td>58,150</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>M</td>
<td>106,500</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>F</td>
<td>30,300</td>
<td>+ve(trace)</td>
<td>fleshy rings</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>M</td>
<td>33,950</td>
<td>-ve</td>
<td>fleshy rings</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>M</td>
<td>311,250</td>
<td>-ve</td>
<td>fleshy rings;</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>M</td>
<td>12,175</td>
<td>-ve</td>
<td>young and fleshy rings</td>
</tr>
<tr>
<td>13</td>
<td>14</td>
<td>F</td>
<td>22,650</td>
<td>-ve</td>
<td>fleshy rings</td>
</tr>
<tr>
<td>14</td>
<td>38</td>
<td>F</td>
<td>33,950</td>
<td>-ve</td>
<td>fleshy rings;</td>
</tr>
<tr>
<td>15</td>
<td>14</td>
<td>F</td>
<td>6,900</td>
<td>-ve</td>
<td>fleshy rings</td>
</tr>
<tr>
<td>16</td>
<td>42</td>
<td>F</td>
<td>14,675</td>
<td>-ve</td>
<td>fleshy rings</td>
</tr>
<tr>
<td>17</td>
<td>32</td>
<td>F</td>
<td>33,125</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>18</td>
<td>14</td>
<td>M</td>
<td>43,450</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>19</td>
<td>4</td>
<td>M</td>
<td>72,400</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>20</td>
<td>27</td>
<td>M</td>
<td>15,975</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>21</td>
<td>3</td>
<td>F</td>
<td>9,650</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>22</td>
<td>14</td>
<td>M</td>
<td>3,800</td>
<td>+ve(trace)</td>
<td>young rings</td>
</tr>
<tr>
<td>23</td>
<td>12</td>
<td>F</td>
<td>5,450</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>24</td>
<td>28</td>
<td>F</td>
<td>19,650</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>25</td>
<td>7</td>
<td>F</td>
<td>34,650</td>
<td>-ve</td>
<td>fleshy rings</td>
</tr>
<tr>
<td>26</td>
<td>17</td>
<td>M</td>
<td>6,450</td>
<td>+ve(trace)</td>
<td>fleshy rings</td>
</tr>
<tr>
<td>27</td>
<td>13</td>
<td>M</td>
<td>118,000</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>28</td>
<td>38</td>
<td>M</td>
<td>43,650</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>29</td>
<td>42</td>
<td>M</td>
<td>10,450</td>
<td>-ve</td>
<td>young rings</td>
</tr>
<tr>
<td>30</td>
<td>27</td>
<td>M</td>
<td>49,725</td>
<td>-ve</td>
<td>fleshy rings</td>
</tr>
</tbody>
</table>
chloroquine. All these 6 last cultures were cultured using medium stored for 3 to 4 days in a refrigerator at 4°C. The remaining 4 cultures did not grow beyond the late trophozoite stage showing distinct clumping of the pigment. These might have needed a longer incubation period than the 24 hours given.

The results of the 11 good tests were analysed using Professor Healey's programme mentioned in the section on rodent malaria. The programme was also adapted to a Hewlet-Packard HP-33E portable calculator so as to be used in the field (Annex 6). The analysed results were shown in Tables 17 and 18. All cases show a high maturation percentage in schizonts in controls except in Subject 23 which showed only 46% maturation to schizonts. All tests showed schizonts maturation up to concentrations of 5 p-mol per well. In two cases (23 and 30) growth was inhibited at 6 p-mols chloroquine per well, in one case (18) inhibition took place at 12 p-mols per well, 3 showed slight growth in 16 p-mols per well. One of the 3 samples which show slight schizont maturation at 16 p-mol/well was cultured from a patient with parasitaemias of 311,250 per ul.

As can be seen from Table 17, 10 of the tests had an ED50 in the range of 3.2 to 5.1 p-mols chloroquine per well with an arithmetic mean of 4.07 p-mols/well. Subject number 11 which had a parasitaemia of 311,250/ul gave an ED50 of 7.2 p-mols/well suggesting a relationship between dose and parasite number which had already been
Table 17: Results of *in vitro* micro-test for the susceptibility of *P. falciparum* to chloroquine, Sennar Area, Sudan.

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Parasites per µl</th>
<th>Mean of Controls</th>
<th>% maturation of schizonts in 200 parasites</th>
<th>( \text{ED}_{50} ) p-mols of chloroquine/well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( K_1 + K_2/2 )</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>11,250</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>33,950</td>
<td>100</td>
<td>87</td>
<td>76</td>
</tr>
<tr>
<td>11</td>
<td>311,250</td>
<td>86</td>
<td>92</td>
<td>85</td>
</tr>
<tr>
<td>12</td>
<td>12,175</td>
<td>100</td>
<td>74</td>
<td>63</td>
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<td>14</td>
<td>33,950</td>
<td>100</td>
<td>92</td>
<td>74</td>
</tr>
<tr>
<td>15</td>
<td>6,900</td>
<td>100</td>
<td>84</td>
<td>79</td>
</tr>
<tr>
<td>16</td>
<td>14,675</td>
<td>90</td>
<td>100</td>
<td>79</td>
</tr>
<tr>
<td>18</td>
<td>43,450</td>
<td>99</td>
<td>100</td>
<td>79</td>
</tr>
<tr>
<td>20</td>
<td>15,975</td>
<td>100</td>
<td>87</td>
<td>80</td>
</tr>
<tr>
<td>23</td>
<td>5,450</td>
<td>46</td>
<td>42</td>
<td>37</td>
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<tr>
<td>30</td>
<td>49,725</td>
<td>75</td>
<td>85</td>
<td>81</td>
</tr>
</tbody>
</table>
Table 18: Sensitivity of *P. falciparum* to chloroquine detected by a micro in vitro test. $ED_{50}$, $ED_{90}$ and $x^2$ calculated using a programme adapted to a Packard HP-33E calculator. Table showing method of calculations.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Doses</th>
<th>1 p-mol/well</th>
<th>2 p-mol</th>
<th>4 p-mol</th>
<th>5 p-mol</th>
<th>6 p-mol</th>
<th>8 p-mol</th>
<th>12 p-mol</th>
<th>$ED_{50}$</th>
<th>$ED_{90}$</th>
<th>Slope</th>
<th>$x^2$</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>x log doses</td>
<td></td>
<td>0.00</td>
<td>0.301</td>
<td>0.602</td>
<td>0.699</td>
<td>0.778</td>
<td>0.903</td>
<td>1.079</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y probit</td>
<td></td>
<td></td>
<td></td>
<td>+0.52</td>
<td>-0.36</td>
<td>-0.95</td>
<td>-1.41</td>
<td>+0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\hat{y}$ value of p² - 5</td>
<td></td>
<td></td>
<td></td>
<td>-0.216</td>
<td>-0.308</td>
<td>-0.382</td>
<td>-0.499</td>
<td>-0.665</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h</td>
<td></td>
<td></td>
<td></td>
<td>+0.016</td>
<td>+0.016</td>
<td>+0.017</td>
<td>+0.017</td>
<td>+0.019</td>
<td>2.335</td>
<td>54.076</td>
<td>-0.9405</td>
<td>135.086</td>
<td>$p&lt;0.001$</td>
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<tr>
<td>y</td>
<td>10</td>
<td>1.254</td>
<td>0.719</td>
<td>0.183</td>
<td>0.011</td>
<td>0.1297</td>
<td>0.352</td>
<td>0.665</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h</td>
<td></td>
<td>0.028</td>
<td>0.019</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016</td>
<td>0.017</td>
<td>0.019</td>
<td>5.071</td>
<td>26.595</td>
<td>-1.7785</td>
<td>157.120</td>
<td>$p&lt;0.001$</td>
</tr>
<tr>
<td>y</td>
<td>11</td>
<td>0.847</td>
<td>0.379</td>
<td>0.090</td>
<td>-0.241</td>
<td>-0.364</td>
<td>-0.558</td>
<td>-0.832</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h</td>
<td></td>
<td>0.024</td>
<td>0.019</td>
<td>0.019</td>
<td>0.019</td>
<td>0.019</td>
<td>0.021</td>
<td>0.024</td>
<td>9.915</td>
<td>57.95</td>
<td>-1.6693</td>
<td>233.89</td>
<td>$p&lt;0.001$</td>
</tr>
<tr>
<td>y</td>
<td>12</td>
<td>0.64</td>
<td>0.33</td>
<td>0.20</td>
<td>-0.13</td>
<td>-0.23</td>
<td>-0.33</td>
<td>-1.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h</td>
<td></td>
<td>0.021</td>
<td>0.017</td>
<td>0.016</td>
<td>0.016</td>
<td>0.017</td>
<td>0.018</td>
<td>0.021</td>
<td>3.501</td>
<td>23.265</td>
<td>-1.5562</td>
<td>81.041</td>
<td>$p&lt;0.001$</td>
</tr>
<tr>
<td>y</td>
<td>14</td>
<td>0.847</td>
<td>0.379</td>
<td>0.090</td>
<td>-0.241</td>
<td>-0.364</td>
<td>-0.558</td>
<td>-0.832</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h</td>
<td></td>
<td>0.201</td>
<td>0.017</td>
<td>0.016</td>
<td>0.016</td>
<td>0.017</td>
<td>0.018</td>
<td>0.021</td>
<td>3.501</td>
<td>23.265</td>
<td>-1.5562</td>
<td>81.041</td>
<td>$p&lt;0.001$</td>
</tr>
<tr>
<td>y</td>
<td>15</td>
<td>1.358</td>
<td>0.650</td>
<td>-0.057</td>
<td>-0.286</td>
<td>-0.471</td>
<td>-0.765</td>
<td>-1.179</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>h</td>
<td></td>
<td>0.031</td>
<td>0.019</td>
<td>0.016</td>
<td>0.016</td>
<td>0.017</td>
<td>0.020</td>
<td>0.020</td>
<td>3.781</td>
<td>15.242</td>
<td>-2.351</td>
<td>26.290</td>
<td>$p&lt;0.001$</td>
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</table>
Table 18 continued

<table>
<thead>
<tr>
<th>Case Doses No.</th>
<th>1 p-mol /well</th>
<th>2 p-mol</th>
<th>4 p mol</th>
<th>5 p mol</th>
<th>6 p mol</th>
<th>8 p mol</th>
<th>12 p mol</th>
<th>$ED_{50}$</th>
<th>$ED_{90}$</th>
<th>Slope</th>
<th>$\chi^2$</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma$</td>
<td>0.81</td>
<td>0.31</td>
<td>-0.08</td>
<td>-0.58</td>
<td>-0.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>16</td>
<td>$\gamma$</td>
<td>0.941</td>
<td>0.073</td>
<td>-0.206</td>
<td>-0.434</td>
<td>-0.794</td>
<td>4.241</td>
<td>11.793</td>
<td>-2.8817</td>
<td>6.176</td>
<td>p&gt;0.25</td>
<td></td>
</tr>
<tr>
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<td>0.024</td>
<td>0.018</td>
<td>0.018</td>
<td>0.019</td>
<td>0.022</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\gamma$</td>
<td>0.81</td>
<td>0.33</td>
<td>-0.10</td>
<td>-0.81</td>
<td>-1.41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>$\gamma$</td>
<td>1.065</td>
<td>0.036</td>
<td>-0.391</td>
<td>-0.680</td>
<td>-1.137</td>
<td>3.909</td>
<td>8.750</td>
<td>-3.6581</td>
<td>15.07</td>
<td>p&lt;0.025</td>
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</tr>
<tr>
<td>$h$</td>
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<td>0.016</td>
<td>0.017</td>
<td>-0.019</td>
<td>0.026</td>
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</tr>
<tr>
<td>$\gamma$</td>
<td>1.13</td>
<td>0.84</td>
<td>0.31</td>
<td>-0.33</td>
<td>-0.33</td>
<td>-0.71</td>
<td>-1.13</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>20</td>
<td>$\gamma$</td>
<td>1.359</td>
<td>0.708</td>
<td>-0.153</td>
<td>-0.324</td>
<td>-0.594</td>
<td>-0.975</td>
<td></td>
<td></td>
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<tr>
<td>$h$</td>
<td>0.071</td>
<td>0.019</td>
<td>0.016</td>
<td>0.016</td>
<td>0.017</td>
<td>0.018</td>
<td>0.023</td>
<td>4.250</td>
<td>16.604</td>
<td>-2.163</td>
<td>8.396</td>
<td>p&gt;0.10</td>
</tr>
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</table>
summarized by Wernsdorfer (1978). Figures 41, 42 & 43 show the fit of the data in a dose-relation probit scale. ED$_{50}$s obtained by the programme in the portable calculator were near to those of the computer (Table 18).

**Macro-technique**

All the 10 isolates, attempted *in vitro* with the WHO macro-method gave no growth. Few reached the trophozoite stage.

**In vivo tests**

The response of *P. falciparum* to chloroquine was assessed in 17 subjects using the 7-days test, and in 10 subjects of those 17 the extended 28-days test was carried out. The parasite counts in the tested subjects ranged from 2800 to 311,250 per µl of blood (Table 19). Asexual parasitaemia disappeared from the peripheral blood of 15 subjects by day 3, while 2 subjects showed a low parasite count of 25 per µl of blood by day 3 and they were found negative by day 4. After the disappearance of the asexual parasites by 96 hours from all 17 subjects, they were not seen again in the blood smears during the remainder part of the 7-days observation period. All subjects responded to chloroquine treatment, with no difference between persons
Fig. 41

Dose-related inhibition of *P. falciparum* schizont maturation in the *in vitro* micro-test. (Sennar area)

Probit scale

Case No. 5 •——• Case No. 10 •——•
Case No. 11 •——• Case No. 12 •——•
Fig. 42
Dose-related inhibition of *P. falciparum* schizont maturation in the *in vitro* micro-test. (Sennar area)

Probit scale

Case No. 14 •——•
Case No. 15 x———x
Case No. 16 o———o
Fig. 43

Dose-related inhibition of *P. falciparum* schizont maturation in the *in vitro* micro-test. (Sennar area)

Probit scale

Case No. 18 • — — — — Case No. 20 ○ — — — ○
Case No. 23 △ — — — △ — — — Case No. 30 □ — — — —□

% SCHIZONT MATURATION

p - mol CHLOROQUINE/WELL
Table 19: Results of in vivo tests: 7-day test and those extended to 28 day test - Sennar Area, Sudan.

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Counts on 7-day test</th>
<th>Counts for 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 3</td>
<td>Day 4</td>
<td>Day 5</td>
</tr>
<tr>
<td>2</td>
<td>40,575</td>
<td>350</td>
<td>25</td>
<td>00</td>
</tr>
<tr>
<td>7</td>
<td>3,750</td>
<td>150</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>9</td>
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</tr>
<tr>
<td>20</td>
<td>1,725</td>
<td>75</td>
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</tr>
<tr>
<td>22</td>
<td>100</td>
<td>00</td>
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</tr>
<tr>
<td>23</td>
<td>875</td>
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</tr>
<tr>
<td>30</td>
<td>5,325</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
</tbody>
</table>

N.B. Parasite count against 300 leucocytes x 25

- 00 out of 100 microscopical field
- ND = not done
Table 19: Results of *in vivo* tests: 7-day test and those extended to 28 day test - Sennar Area, Sudan.

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Counts on 7-day test</th>
<th>Counts for 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>2</td>
<td>38,250</td>
<td>40,575</td>
</tr>
<tr>
<td>7</td>
<td>58,150</td>
<td>3,750</td>
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<td>9</td>
<td>30,300</td>
<td>2,725</td>
</tr>
<tr>
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<td>311,250</td>
<td>38,200</td>
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<td>33,950</td>
<td>7,225</td>
</tr>
<tr>
<td>15</td>
<td>6,900</td>
<td>250</td>
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<tr>
<td>16</td>
<td>14,675</td>
<td>675</td>
</tr>
<tr>
<td>18</td>
<td>43,450</td>
<td>1,150</td>
</tr>
<tr>
<td>20</td>
<td>15,975</td>
<td>1,725</td>
</tr>
<tr>
<td>22</td>
<td>3,800</td>
<td>100</td>
</tr>
<tr>
<td>23</td>
<td>5,450</td>
<td>875</td>
</tr>
<tr>
<td>24</td>
<td>19,650</td>
<td>3,950</td>
</tr>
<tr>
<td>27</td>
<td>118,000</td>
<td>325</td>
</tr>
<tr>
<td>29</td>
<td>10,450</td>
<td>575</td>
</tr>
<tr>
<td>30</td>
<td>49,725</td>
<td>5,325</td>
</tr>
</tbody>
</table>

N.B. Parasite count against 300 leucocytes x 25

- 00 out of 100 microscopical field
- ND = not done
having heavy or light parasitaemia. This confirmed the observation of Dr Dawi (Director of Sennar Hospital), 1979 (personal communication) that all *P. falciparum* cases in Sennar Area respond quickly to chloroquine which was given orally or intramuscularly at a dosage of 25 mg/kg body weight over three days. The Dill and Glazko test was positive in all 17 subjects within 96 hours and continued positive in 14 subjects up to 144 hours.

Out of the ten subjects for whom the follow up was extended for 28 days, 4 were found positive with asexual forms of *P. falciparum* in their peripheral blood (40%) (Table 19). One of them was positive on the third week, while the other three were positive during the last follow up by the end of the fourth week. This might indicate presence of resistance at RI level as Omer (1978) reported from the Gezira about 160 kilometres north of this area, but since malaria transmission was going on in the area, reinfection could not be excluded, and those subjects might have contracted new infection after being radically cured from their previous one.

Comparison of in vivo and micro-in vitro results

Results obtained from 9 subjects who were studied *in vivo* (6 of them followed for 28 days) and whose blood was cultured *in vitro* using the micro technique were compared. 6 of the 9 subjects showed a complete disappearance of asexual parasitaemia by day 2
after the start of treatment, while the other 3 who showed a very low level of parasitaemia on day 2 were cleared by day 3 after commencement of treatment. Two of the subjects became positive in the last week of the 28-days follow up (Table 19). Complete arrest of schizont formation in vitro was observed at 6 p-mol per well in 2, at 12 p-mol per well in one, and at 16 p-mol per well in the other remaining six cases. The cases who were found positive after being cleared of parasitaemia for 3 weeks developed schizonts in vitro at concentrations of 8 p-mols of chloroquine and 12 p-mols of chloroquine per well (Table 17).

4.6 Conclusion

Cultures using the rodent malaria as a model to develop a micro in vitro technique gave no success but helped in mastering the technique of in vitro culturing and of handling parasitized blood from small animals like mice and keeping high levels of sterility of glassware and reagents used in culturing parasites.

Although there had been no success in culturing the primate malaria P. fieldi from M. mulatta, some important variables and their effect on the maturation of parasites to schizonts were learnt from the cultures of P. coatneyi. Those variables can be summarized in the following points:
- ACD is the best anticoagulant to be used for collecting blood for culture.
- Giemsa stain at a pH of 6.8 produces the best staining results of cultured parasites.
- Parasites in the fleshy ring or trophozoite stage give a better growth than those in the young ring stage.
- Young parasites "small rings" need a longer incubation period (more than 24 hours).
- Unwashed blood gives better maturation to schizonts than washed blood.
- Small amounts of blood, as little as 5 µl, can easily grow in 50 µl of RPMI 1640 growth medium.
- Drugs can be prepared either in medium, distilled water which is added to medium, or is freeze-dried in the wells of the plate. Allowance should be made for loss of drug by adsorption to containers and to plates.
- High parasitaemia (> 10%) affects the rate of maturation of parasites and reduces the effect of drug on the parasites.

From the field studies in Sennar Area the following conclusions can be drawn:

- The indigenous population of the Sennar Area, having been under protection from malaria by extensive control measures for 13 years, experienced great reduction in their collective immunity and appeared to be non- or semi-immune. The pronounced symptoms of malaria in
patients with severe clinical manifestations, and the high level of parasitaemia among older age groups, were clear evidence of loss of acquired immunity.

The fact that all attempts to assess the response of P. falciparum to chloroquine in vitro with the macro-test method gave unsuccessful results, made it impossible to compare the results of the micro-method with the macro-method. The reason or reasons for this failure were not properly investigated because of the time limit and the difficulty of getting a new test kit from WHO in such a short period. More work could be done in the future to establish the possible reasons for this failure.

The limited success of the micro in vitro test has confirmed its potentiality for field screening of human subjects in order to establish base line data on the sensitivity of local strains of P. falciparum to chloroquine, the quick detection of the appearance of any resistant strains, and for screening new antimalarial drugs.

The micro-test proved to be very simple and could make possible the screening of many individuals in a short time.

Liquid or dry RPMI 1640 medium, buffered with HEPES and NaHCO₃ with the addition of Gentamycin to reduce the hazards of contamination, produced good results and could be used in future studies.
The candle in a desiccator, if tightly closed, produced the required gaseous atmosphere for the maturation of parasites.

Incubation in a dry incubator at 38°C ± 0.5°C was quite successful, but if a portable small incubator which can be run from different sources of power is developed, it will reduce the chances of losing cases because of power cuts which are quite normal in developing countries, and also will spread the use of the test in areas where there is no electricity.

The results obtained from the micro-test which show that growth of parasites was not inhibited in 8 out of the 11 good cultures at the concentration of 12 p-moles of chloroquine per well might be interpreted as an indication of the presence of a resistant strain in the area because it is double the minimum inhibition dose for susceptible strain as suggested by Rieckman et al. (1978). However the fact that chloroquine loss by adsorption to containers and plates, which was demonstrated in Chapter five of this work, renders such interpretation as invalid. Allowance for such loss, which can be as high as 40% in low concentrations of the drug, must be catered for. This could be done by adding an excess of the drug to the medium, the distilled water, or before freeze drying but more work needs to be done on this.

Results of the in vivo studies with WHO
standard 7-day field test, and the extended 28-day test indicate clearly the absence of chloroquine resistance at RII and RIII levels; however appearance of asexual parasites in the peripheral blood of 4 out of 10 subjects who were followed for 28 days might indicate the presence of resistance at the RI level. Since transmission was continued in the area reinfection cannot be completely excluded. The results of Omer (1978) in the Gezira irrigated area, north of Sennar, using the WHO extended field test, showed 2 cases with an RI response and one case with an RII response out of 506 subjects. However, since these studies were performed during the peak of the transmission season, the possibility of reinfection could not be ruled out. Nevertheless the results in the Sennar patients are in agreement with those from the Gezira patients, which both indicate that the local strains are sensitive to chloroquine, and that if there is any chloroquine-resistant strains present in the area, their frequency should be very low.

Comparing the results of the micro-tests conducted in Sennar Area with the laboratory tests of Rieckmann et al. (1978), it was observed that Sennar strain falls in sensitivity between the chloroquine-sensitive African Palo Alto, Uganda Strain (FUP) and the chloroquine-resistant Oak Knoll, Vietnam Strain (FVO). Rieckmann reported that the FUP strain is at least 10 times more sensitive to chloroquine than the
resistant FVO strain, while Sennar results are nearly 2.5 times less sensitive than the FUP strain. This again indicates that there might be differences between sensitive strains from one geographical area to another, and the discriminating dose which will completely inhibit the maturation of parasites in vitro using the micro-technique must be established in the field.

- The Sennar results obtained from this study were in agreement with the results of the studies of Kouzentsor et al. (1979) which were carried out in the same area. They also concluded that isolates from Sennar, are approximately two times less sensitive than the highly sensitive FUP strain and over five times more sensitive than the FVO strain.

- The results of this study correlate with those of Dennis et al. (1974) and Palmer et al. (1976) in Ethiopia. They observed that Ethiopian strains of *P. falciparum*, studied with the macro-technique, appeared to be less sensitive than other African strains and somewhere on the borderline between sensitivity and resistance to chloroquine.

- More studies using both the micro in vitro tests beside the in vivo test must be carried out in different areas of the Sudan to establish base line data on the sensitivity of *P. falciparum* to chloroquine and to see if there are variations in the strains from one area to another.
CHAPTER FIVE

RADIOACTIVE ASSAY FOR DETERMINATION OF CHLOROQUINE ADSORPTION AND/OR DEGRADATION WHILE STORED AT 4°C IN GLASS AND PLASTIC TUBES
5.1 INTRODUCTION

It was observed in the field while testing the sensitivity of Plasmodium falciparum to chloroquine using the *in vitro* microtechnique in Sennar area (Blue Nile Province, Sudan), that chloroquine phosphate prepared in RPMI 1640 culture medium at different concentrations and stored in glass and plastic tubes at 4°C loses its effectiveness and that malaria parasites grow from ring stage to schizonts in 24 hours even at higher concentrations of the drug after three days from preparation.

Radioactive chloroquine (CQ$^{14}$) was used to study what happens to chloroquine in medium RPMI 1640 stored in glass and plastic tubes at 4°C for different lengths of time.

Simple and reliable methods for the measurement of the concentration of therapeutic agents in biological materials were in use since early 1943 when Masen (1943) determined quantitatively the amount of Atabrin in blood and urine.

Brodie *et al.* (1943) followed in the same year estimating Atabrin in biological fluids and tissues, and
quinine in human plasma.

Auerbach et al. (1944) used a Photofluorometric method for the determination of Atabrin. Brodie et al. (1945) applied a technique for the appraisal of specificity for the estimation of Cinchona alkaloids. In 1947 Brode et al. published a set of 5 papers dealing with the estimation of basic organic compounds in biological material using different methods and techniques for different antimalarial drugs. Josephson et al. (1947) used the ultra-violet spectrophotometry for the estimation of basic organic compounds in biological material.


5.2 MATERIALS AND REAGENTS

1. Radioactive chloroquine $^{14}$C (30 μCi/μmole; New England Nuclear Corp.).
2. Normal chloroquine phosphate solution (1 mg/ml; Winthrop Labs).
3. RPMI 1640 tissue culture medium supplemented with sodium bicarbonate, hepes buffer and gentamycin.
4. 2N NaOH.
5. n-Heptane with 1.5 ml isoamyl alcohol/100 ml (analytical grade).
6. Isoamyl alcohol
7. Toluene
8. 0.2M borate (0.2M boric acid + 1M NaOH) pH 9.3
9. 10 ml glass tubes (10 cm x 1.5 cm) with plastic screw tops
10. 6 ml plastic tubes (4.5 cm x 1.6 cm) with plug in plastic tops
11. Whirlimixer (electrical mixer)
12. Scintillation vials (standard).

The results were read in Liquid Scintillation Spectrometer Packard Tri-Carb Model 574 and a Packard 33E portable calculator was used for programming the calculations (Annex 7).

5.3 METHOD

5.3.1 Preparation of CQ-\(^{14}\)C dilutions in RPMI 1640 medium

To 26.72 ml of RPMI 1640 add 20 ul of radioactive chloroquine phosphate CQ-\(^{14}\)C (30 \(\mu\)Ci/\(\mu\)mole; New England
Nuclear Corp.) to give a concentration of drug of $5 \times 10^{-7}$ M = $10^{-6.3}$. From the above solution take 6 ml in glass tube and label it G1 and 6 ml in plastic tube and label it P1. Then take 12 ml of the same solution $10^{-6.3}$ and add it to 12 ml of medium to give $10^{-6.6}$ dilution (two-fold dilution) and carry on this process to give 12 ml of $10^{-6.9}$, $10^{-7.2}$, $10^{-7.5}$, and $10^{-7.8}$.

Divide each quantity into two halves 6 ml each in glass tubes and plastic tubes and label them G2, P2, G3, P3, G4, P4, G5, P5, G6 and P6.

12 ml from $10^{-6.3}$ $10^{-6.6}$ $10^{-6.9}$ $10^{-7.2}$ $10^{-7.5}$ $10^{-7.8}$

12 ml RPMI 12 ml 12 ml 12 ml 12 ml

G2 P2 G3 P3 G4 P4 G5 P5 G6 P6

Prepare normal chloroquine phosphate solution by weighing accurately 30 mg of the powder drug and dissolve in 30 ml of D.W. to give cold chloroquine 1 mg/ml.

To 12 glass tubes (10 ml capacity) labelled G1 - G6 and P1 - P6 add 0.1 ml of cold chloroquine using 1 ml pipette and to each add a sample of 1 ml from the corresponding glass or plastic testing tubes. Repeat this after 18 hours, 24, 48, 72, 96, 120, and 144 hours.
5.3.2 Extraction of CQ$^{14}$C

The radioactive chloroquine $^{14}$C was extracted from the medium applying the procedure used by Brodie et al. (1947). To 10 ml clean glass tubes with screw tops 0.1 ml of cold normal chloroquine phosphate 1 mg/ml was added. The nonradioactive chloroquine was added to minimize adsorption of radioactive chloroquine $^{14}$C to glassware. To each glass tube containing 0.1 ml non-radioactive chloroquine add 1 ml of medium containing $^{14}$C from the test tubes both glass and plastic and label each glass tube with its corresponding number of test tube using letter G for glass and P for plastic and stating the code number of chloroquine concentration and the number of hours at which the sample is taken. To each sample tube add 1 ml of 2N NaOH to raise the pH value to 10 or 11, then add 2.5 ml of n-Heptane with 1.5 ml isoamyl alcohol/100 ml as an extraction solvent. n-Heptane being a low polar solvent necessitates the addition of isoamyl alcohol to reduce the adsorption of $^{14}$C to glass surface. Each sample tube is then shaken mechanically for 20 minutes using an electrical whirlimixer. After the solvent was separated by settling, 1 ml was taken in 10 ml of toluene (scintillation fluid) in scintillation vials. Each vial is marked with the marking on the test tube from which the sample was extracted.

A liquid scintillation spectrometer (Packard
Tri-Carb model 574) was used for measuring the amount of $^{14}$C by reading the radioactivity in each vial for 20 minutes. This procedure was done at 0 hours, 18 hours, 24 hours and each 24 hours for seven days for each test tube glass and plastic with the six different concentrations of chloroquine.

An experiment with triple extraction from each test tube, adding 2.5 ml of n-heptane with isoamyl alcohol and taking out 2 ml sample of solvent layer after each extraction, and pooling them in one tube was carried out. To the 6 ml pooled solvent, 10 ml of 0.2M borate (sodium borate/boric acid) buffer at pH 9.3 were added to remove chloroquine metabolites leaving unchanged chloroquine in the heptane. After shaking each tube for 20 minutes, 4 ml of heptane solvent layer were taken into a scintillation vial containing 16 ml toluene (scintillation fluid). Each vial was labelled according to the label in the test tube from which the chloroquine was extracted. The scintillation vials were read for 20 minutes each in a liquid scintillation spectrometer.

5.4 RESULTS

Radioactivity of chloroquine was measured using a liquid scintillation spectrometer and each vial was left for 20 minutes. The disassociation of CO$^{14}$C was recorded from the readings of the two spectrometer channels in a printer. The total disassociation per
minute and the amount of chloroquine recovered in heptane were calculated using a simple programme adapted to a Packard 33 E portable calculator (Annex 7).

The first experiment (Table 20 and Figure 44) showed that almost the whole amount of chloroquine dissolved in the medium was extracted in heptane at 0 hours at all 6 concentrations in both plastic and glass tubes. At 18 hours there was a slight loss from all concentrations. A marked loss which was more clear in glass than in plastic tubes appeared at 48 and 96 hours in most of the concentrations.

The second experiment (Table 21) and the third experiment (Table 22) which were exact replicates of experiment one, showed more or less the same pattern as that of experiment one (see figures 45 and 46).

Table 23 showed extraction of chloroquine using 0.2M borate to remove chloroquine metabolites. The results showed a loss of more than 20% in all concentrations at all times. The loss was slightly greater from glass tubes than from plastic ones.

Table 24 summarizes the percentage loss of chloroquine 14C from plastic and glass tubes by time and by initial dilution added. The results were the mean of three replicate experiments. Figures 47 and 48 summarizes the mean % loss from all concentrations at different intervals of time, and the mean percentage of loss from each concentration, summing up the loss at all intervals of time for 96 hours, respectively. In both cases the total loss from glass at 24 and 96 hours was a little more than from plastic, and the same can be seen at the lower concentrations of chloroquine e.g. 0.15 x 10^-7 M. Also it could be seen from Figure 48 that the
total amount of loss at lower concentrations was greater than that from higher concentrations from both plastic and glass tubes.

Figures 49 to 60 show the percentage loss by degradation and/or adsorption of each concentration of chloroquine at 6 different periods of time and the percentage loss at each period of time from the 6 different concentrations of the drug prepared in RPMI 1640 medium in plastic and glass tubes.

5.5. CONCLUSION

From the previous results it can be concluded that loss of chloroquine by adsorption to plastic and glass surfaces was not clearly marked and the differences between the two containers is not so significant although a slight hint can be drawn to the fact that glass loses more drug than plastic. The summary in Table 24 and Figures 47 and 48 showed a mean loss of 17.5% from plastic and 19.8% from glass tubes due to degradation of the drug and/or adsorption to surfaces of the containers. The human error in the extraction process can affect the results as can be seen from most of the figures presented in the results, but still the signs of loss of the chloroquine up to 20% from the original concentrations will affect the interpretation of the results of drug resistance tests carried out in vitro miro-tests. More work should be carried out to clarify this fact.
Table 20  FIRST EXPERIMENT: Amount of chloroquine extracted from different concentrations of the drug prepared in plastic and glass tubes and stored at 4°C

<table>
<thead>
<tr>
<th>Time in hours between preparation &amp; extraction</th>
<th>Plastic tubes Chloroquine concentrations x $10^{-7}$</th>
<th>Glass tubes Chloroquine concentrations x $10^{-7}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5  2.5  1.25  0.625  0.31  0.15</td>
<td>5  2.5  1.25  0.625  0.31  0.15</td>
</tr>
<tr>
<td>0</td>
<td>5.0  2.3  1.2  0.60  0.30  0.17</td>
<td>4.3  2.3  1.1  0.62  0.30  0.18</td>
</tr>
<tr>
<td>18</td>
<td>4.4  2.2  1.1  0.53  0.28  0.13</td>
<td>4.5  2.2  1.0  0.55  0.28  0.11</td>
</tr>
<tr>
<td>24</td>
<td>4.7  2.3  1.1  0.60  0.27  0.13</td>
<td>4.7  2.3  1.1  0.50  0.30  0.10</td>
</tr>
<tr>
<td>48</td>
<td>4.6  2.1  1.1  0.56  0.27  0.15</td>
<td>4.3  2.2  0.9  0.47  0.28  0.10</td>
</tr>
<tr>
<td>72</td>
<td>4.2  1.9  0.9  0.57  0.26  0.12</td>
<td>4.7  2.3  1.3  0.60  0.30  0.11</td>
</tr>
<tr>
<td>96</td>
<td>5.2  2.3  1.2  0.65  0.26  0.15</td>
<td>3.8  0.98  0.98  0.29  0.28  0.09</td>
</tr>
</tbody>
</table>
Fig. 44

Adsorption of chloroquine $^{14}\text{C}$ to glass and plastic tubes as determined by radio-active-assay.

Experiment one

- Plastic tubes
- Glass tubes

![Graph showing the adsorption of chloroquine $^{14}\text{C}$ to glass and plastic tubes over time. The graph displays the concentration of chloroquine $10^{-8} \text{M}$ against time in hours. The data is represented by two lines: one for plastic tubes and one for glass tubes.]
Table 21  SECOND EXPERIMENT: Amount of chloroquine extracted from different concentrations of the drug prepared in plastic and glass tubes and stored at 4°C

<table>
<thead>
<tr>
<th>Time in hours between preparation &amp; extraction</th>
<th>Plastic tubes Chloroquine concentrations x 10⁻⁷ M</th>
<th>Glass tubes Chloroquine concentrations x 10⁻⁷ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.57 0.26 0.18</td>
<td>0.57 0.27 0.08</td>
</tr>
<tr>
<td>18</td>
<td>0.49 0.27 0.12</td>
<td>0.49 0.37 0.17</td>
</tr>
<tr>
<td>24</td>
<td>0.58 0.32 0.12</td>
<td>0.49 0.33 0.16</td>
</tr>
<tr>
<td>48</td>
<td>0.49 0.23 0.13</td>
<td>0.51 0.29 0.13</td>
</tr>
<tr>
<td>72</td>
<td>0.54 0.25 0.13</td>
<td>0.60 0.31 0.12</td>
</tr>
<tr>
<td>96</td>
<td>0.54 0.24 0.09</td>
<td>0.31 0.31 0.11</td>
</tr>
</tbody>
</table>
Table 22  THIRD EXPERIMENT: Amount of chloroquine extracted from different concentrations of the drug prepared in plastic and glass tubes and stored at 4°C

<table>
<thead>
<tr>
<th>Time in hours between preparation &amp; extraction</th>
<th>Plastic tubes Chloroquine concentrations x 10^-7 M</th>
<th>Glass tubes Chloroquine concentrations x 10^-7 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.7 2.5 1.1 0.53 0.20 0.07</td>
<td>4.5 2.16 1.07 0.55 0.24 0.05</td>
</tr>
<tr>
<td>18</td>
<td>4.6 2.2 1.1 0.51 0.20 0.06</td>
<td>4.5 2.27 1.09 0.48 0.18 0.04</td>
</tr>
<tr>
<td>24</td>
<td>4.2 2.1 0.96 0.47 0.21 0.15</td>
<td>4.5 2.22 0.96 0.47 0.16 0.03</td>
</tr>
<tr>
<td>48</td>
<td>4.8 2.3 1.2 0.63 0.34 0.17</td>
<td>4.7 2.30 1.20 0.66 0.31 0.15</td>
</tr>
<tr>
<td>72</td>
<td>4.5 2.2 0.87 0.34 0.11 0.04</td>
<td>4.0 1.89 0.87 0.32 0.11 0.06</td>
</tr>
<tr>
<td>96</td>
<td>4.2 2.0 0.76 0.26 0.12 0.04</td>
<td>4.9 2.27 0.98 0.33 0.11 0.08</td>
</tr>
</tbody>
</table>
Fig. 45

Graph showing adsorption of chloroquine $^{14}\text{C}$ to glass & plastic tubes as determined by radio-active-assay.

Experiment two

- Plastic tubes
- Glass tubes

TIME IN HOURS

CONCENTRATION OF CHLOROQUINE $10^{-8} \text{M}$
Fig. 46

Adsorption of chloroquine $^{14}$C to glass and plastic tubes as determined by radio-active assay.

Experiment three

- Plastic tubes
- Glass tubes
Table 23

Extraction of chloroquine using 0.2M borate to remove chloroquine metabolites from different concentrations of chloroquine prepared in plastic and glass tubes and stored at 4°C

<table>
<thead>
<tr>
<th>Time in hours between preparation &amp; extraction</th>
<th>Plastic tubes Chloroquine concentrations $x 10^{-7}$M</th>
<th>Glass tubes Chloroquine concentrations $x 10^{-7}$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5 2.5 1.25 0.625 0.31 0.15</td>
<td>5 2.5 1.25 0.625 0.31 0.15</td>
</tr>
<tr>
<td>18</td>
<td>4.0 2.0 0.9 0.55 0.30 0.10</td>
<td>3.0 1.9 0.9 0.55 0.30 0.12</td>
</tr>
<tr>
<td>24</td>
<td>3.5 1.8 0.8 0.50 0.20 0.09</td>
<td>2.4 1.5 0.7 0.40 0.20 0.10</td>
</tr>
<tr>
<td>48</td>
<td>3.4 1.8 0.8 0.40 0.20 0.08</td>
<td>2.5 1.5 0.6 0.30 0.10 0.09</td>
</tr>
<tr>
<td>72</td>
<td>3.2 1.7 0.9 0.40 0.20 0.09</td>
<td>2.9 1.5 0.7 0.40 0.20 0.10</td>
</tr>
<tr>
<td>96</td>
<td>3.2 1.7 0.9 0.50 0.20 0.09</td>
<td>3.0 1.6 0.8 0.40 0.20 0.10</td>
</tr>
<tr>
<td>120</td>
<td>3.4 1.7 0.9 0.50 0.30 0.08</td>
<td>3.1 1.5 0.8 0.40 0.30 0.13</td>
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<tr>
<td>144</td>
<td>3.5 1.9 0.9 0.50 0.30 0.08</td>
<td>2.9 1.4 0.9 0.40 0.30 0.12</td>
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<tr>
<td>168</td>
<td>3.3 1.7 0.9 0.50 0.30 0.08</td>
<td>3.0 1.5 0.8 0.40 0.30 0.12</td>
</tr>
<tr>
<td></td>
<td>3.1 1.7 0.9 0.40 0.20 0.08</td>
<td>2.8 1.4 0.7 0.40 0.30 0.12</td>
</tr>
</tbody>
</table>
Table 24  Percentage loss of chloroquine $^{14}$C from plastic and glass tubes by time and by initial dilution added. Means of three replicate experiments.

<table>
<thead>
<tr>
<th>Time in hours between preparation &amp; extraction</th>
<th>Plastic tubes</th>
<th>Glass tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine concentrations x $10^{-7}$M</td>
<td>5  2.5  1.25  0.625  0.312  0.15</td>
<td>Mean</td>
</tr>
<tr>
<td>0</td>
<td>3.2  5.5  6.9  9.3  18.3  6.7  8.3</td>
<td>12.3 16.5 18.1 7.2 12.9 31.1</td>
</tr>
<tr>
<td>18</td>
<td>12.8 23.7 12.0 18.4 19.4 31.1 19.6</td>
<td>9.6 12.4 17.6 25.3 32.3 51.1</td>
</tr>
<tr>
<td>24</td>
<td>16.3 12.5 18.4 12.0 7.5 42.2 18.2</td>
<td>7.5 7.7 15.7 22.1 15.1 35.6</td>
</tr>
<tr>
<td>48</td>
<td>12.1 14.5 9.3 10.0 10.7 10.0 9.5</td>
<td>10.3 10.7 14.7 12.5 15.4 11.1</td>
</tr>
<tr>
<td>72</td>
<td>12.8 21.7 28.8 22.7 33.3 35.6 25.8</td>
<td>13.1 13.5 7.5 18.9 22.6 35.6</td>
</tr>
<tr>
<td>96</td>
<td>4.4 18.4 23.7 22.7 33.3 37.8 23.4</td>
<td>12.8 42.0 21.1 50.4 24.7 36.0</td>
</tr>
<tr>
<td>Mean</td>
<td>10.3 16.1 16.5 15.9 20.4 25.6 17.5</td>
<td>10.9 17.1 15.8 22.7 18.8 33.4</td>
</tr>
</tbody>
</table>
Fig. 47

Percentage loss of chloroquine $^{14}C$ by adsorption in plastic and glass with time. (Mean of the percentage loss from different (6) concentrations)

Log probit scale

```
\begin{align*}
\text{PERCENTAGE LOSS} \\
0 & 0.01 & 0.05 & 0.1 & 0.2 & 0.5 & 1 & 2 & 5 & 10 & 20 & 30 & 40 & 50 \\
\text{TIME IN HOURS} \\
0 & 24 & 48 & 72 & 96
\end{align*}
```

- Plastic tubes
- Glass tubes
Fig. 48
Percentage loss of chloroquine $^{14}C$ by adsorption to plastic and glass tubes by initial concentrations.
(Mean of the percentage lost from each concentration at 6 different intervals of time)
log probit scale

- Plastic tubes
- Glass tubes

CONCENTRATION OF CHLOROQUINE $10^{-7}$ M
Fig. 49
Percentage loss of chloroquine $^{14}$C at concentration $5 \times 10^{-7}$M by adsorption to plastic and glass tubes at different periods.

Log probit scale

- Plastic tubes
- Glass tubes
Fig. 50

Percentage loss of chloroquine $^{14}$C at concentration of $2.5 \times 10^{-7}$M by adsorption to plastic and glass tubes at different periods.

Log probit scale

- - - Plastic tubes  O---O Glass tubes
Fig. 51

Percentage loss of chloroquine $^{14}$C at concentration of $1.25 \times 10^{-7}$M by adsorption to plastic and glass tubes at different periods.

Log probit scale

- - - Plastic tubes  o--o Glass tubes

![Graph showing percentage loss over time](image-url)
Fig. 52

Percentage loss of chloroquine $^{14}$C at concentration of $0.625 \times 10^{-7} \text{M}$ by adsorption to plastic and glass tubes at different periods.

log probit scale

- Plastic tubes
- Glass tubes
Fig. 53

Percentage loss of chloroquine $^{14}\text{C}$ at concentration of $0.31 \times 10^{-7} \text{M}$ by adsorption to plastic and glass tubes at different periods.

Log probit scale

- Plastic tubes
- Glass tubes

![Graph showing percentage loss over time for plastic and glass tubes]
Fig. 54

Percentage loss of chloroquine $^{14}C$ at concentration of $0.15 \times 10^{-7}M$ by adsorption to plastic and glass tubes at different times.

Log probit scale

- Plastic tubes
- Glass tubes

TIME IN HOURS

PERCENTAGE LOSS
Fig. 53

Percentage loss of chloroquine $^{14}\text{C}$ at concentration of $0.31 \times 10^{-7}$ M by adsorption to plastic and glass tubes at different periods. Log probit scale

- Plastic tubes
- Glass tubes

TIME IN HOURS

PERCENTAGE LOSS

0.01

0.05

0.1

0.2

0.5

1

2

4

8

16

32

64

128

256

512
Fig. 54

Percentage loss of chloroquine $^{14}$C at concentration of $0.15 \times 10^{-7}$ M by adsorption to plastic and glass tubes at different times.

Log probit scale

- Plastic tubes
- Glass tubes

TIME IN HOURS

PERCENTAGE LOSS
Fig. 55
Percentage loss from different concentrations of chloroquine $^{14}$C
by adsorption to plastic and glass tubes at zero hours.

Log probit scale

- Plastic tubes
- Glass tubes

PERCENTAGE LOSS

CONCENTRATIONS IN $10^{-7}$ M
Fig. 56

Percentage loss from different concentrations of chloroquine \(^{14}\text{C}\) by adsorption to plastic and glass tubes at 18 hours.

Log probit scale

- Plastic tubes
- Glass tubes
Fig. 57

Percentage loss from different concentrations of chloroquine $^{14}$C by adsorption to plastic and glass tubes at 24 hours.

Log probit scale

- Plastic tubes
- Glass tubes

CONCENTRATIONS IN $10^{-7}$M

PERCENTAGE LOSS
Fig. 58

Percentage loss from different concentrations of chloroquine $^{14}$C by adsorption to plastic and glass tubes at 48 hours.

Log probit scale

- - - - Plastic tubes
- - - - Glass tubes
Fig. 59

Percentage loss from different concentrations of chloroquine $^{14}\text{C}$ by adsorption to plastic and glass tubes at 72 hours.

log probit scale

- Plastic tubes
- Glass tubes
Fig. 59

Percentage loss from different concentrations of chloroquine $^{14}$C by adsorption to plastic and glass tubes at 72 hours.

log probit scale

- Plastic tubes
- Glass tubes

![Graph showing percentage loss from different concentrations of chloroquine C by adsorption to plastic and glass tubes at 72 hours. Log probit scale.](image-url)
Fig. 60
Percentage loss from different concentrations of chloroquine $^{14}C$ by adsorption to plastic and glass tubes at 96 hours.

Log probit scale

- Plastic tubes
- Glass tubes
CHAPTER SIX

STUDIES ON THE SUSCEPTIBILITY OF ANOPHELES ARABIENSIS FROM THE GEZIRA TO INSECTICIDES
6.1 INTRODUCTION

By the end of the 1930s and the beginning of the 1940s, DDT (dichloro-diphenyl-trichloro-ethane) and other synthetic insecticides like HCH (hexachlorocyclo-hexane), dieldrin, chlordane, etc., with residual action were discovered. The magnificent results obtained after field application of these insecticides, gave the human population of the globe great hopes in ridding themselves from their fierce insect enemies which affect both their health and food. These hopes did not last long, because just a few years after the field application of DDT, Wiesmann (1947) reported the first incidence of resistance to the insecticide in house flies. From that date onwards, the history of insecticide development has also been the history of development of insecticide resistance. Reports of the vastly increased problem of resistance developing in different species of pests, to different types and numbers of insecticides started coming from all over the world. The World Health Organization shouldered the responsibility of announcing and updating records of resistance cases pouring from all over the world. The latest reviews of the WHO (1976) and Georghiou and Taylor (1976) showed the present situation regarding the status of resistance in the anopheline mosquitoes with special reference to the malaria vectors all over the globe.
6.1.1 Detection of Resistance

Up to the time of the development of the WHO standard technique for the detection of resistance (1963) which was based on the principle described by Busvine and Nash (1953), resistance was reported on observations based on facts of control failures. Assessment of the changes in the susceptibility levels of insects detected by the WHO technique were expressed as the lethal concentration which kills 50% of the tested population (LC$_{50}$) (WHO, 1963) or the lethal time which kills 50% of the tested population (LT$_{50}$) (Pennel et al., 1964). By applying these indices for practical testing, it was pointed out that these values had limited significance in measurement of resistance levels in heterogeneous populations. Davidson (1958), Dyte and Blackman (1967), Dyte (1970) and others pointed to the danger of complete reliance on assessments based solely on LD$_{50}$ and LT$_{50}$ which could be misleading depending on the level of heterogeneity of the populations tested in which the presence of resistant individuals may be missed altogether. To avoid this confusion, Davidson (1960), Dyte (1970), and Davidson and Zuhar (1973) adopted the use of discriminating dosages, known to kill the susceptibles of a particular species. One of the advantages of the use of discriminating dosages is the early detection of resistance even in a population with a very small proportion of resistant individuals.
They suggested continuous exposure of the offspring of the survivors to the same discriminating dosage used for their parents, to confirm suspected cases of resistance.

6.1.2 Resistance of Anopheline Mosquitoes to Insecticides

6.1.2.a History

The history of insecticide-resistance in anopheline mosquitoes, and its distribution in the world was reviewed by Busvine and Pal (1969). Georgopoulos (1951) was the first to report resistance to DDT in *A. sacharovi* from Greece. The resistance of this species soon extended to chlordane (Georgopoulos, 1954; Hadjinicolaon, 1954, 1957) as well as BHC (Georgopoulos, 1954) and dieldrin (Livadas, 1955). By the end of 1958, resistance to DDT or dieldrin had developed in ten proved malaria vectors in nineteen countries. *A. sacharovi* in Greece and *A. albimanus* in El Salvador are the only two cases in which resistance to both DDT and dieldrin has developed within the same species and in the same country (Zulueta, 1959). By 1968, 38 species of anopheline were reported to be resistant to DDT and/or dieldrin all over the world (Busvine and Pal, 1969).

6.1.2.b. Present status

WHO (1976) and Georghiou and Taylor (1976) reported that among the 42 cases of resistance in anopheline
mosquitoes recorded in 1975, 41 species were resistant to dieldrin and 24 to DDT, 21 of the latter having developed double resistance to both groups of the organochlorine insecticides. The first anopheline to develop multiple resistance to organophosphorus and carbamate insecticides was A. albimanus in Central America (WHO, 1976). Resistance to one or more organophosphorus compounds was reported in A. hyrcanus and A. sacharovi from Turkey, in A. culicifacies from India, in A. sinensis from Ryuku Islands, and in A. messeae from Romania. Ramsale (1975) reported survivors to the discriminating dosages of fenitrothion, fenthion and propoxur in A. sacharovi from Turkey. The first indication of malathion resistance in A. culicifacies from the Gujarat State of India was reported by Rajagopal (1977). Manouchehri et al. (1976a, b) reported resistance to malathion in A. stephensi from Bandar Abbas, Southern Iran (Tables 25 & 26).

6.1.2.c. Resistance in anopheline mosquitoes in Africa with special reference to the Sudan

The major vectors of malaria in the Anopheles gambiae complex were reported to be resistant to dieldrin since 1955, but remained susceptible to DDT until 1968. Hamon et al. (1968) reported a high level of DDT resistance in A. gambiae (species A) from Upper Volta. Resistance of the same species to DDT was reported from Togo in 1969 and 1970, and from Senegal in 1968, and from South
Table 25  Insecticide resistance in anopheline mosquitoes:
Countries or areas

<table>
<thead>
<tr>
<th>Species</th>
<th>DDT</th>
<th>Dieldrin/HCH</th>
<th>Organophosphorus</th>
<th>Carbamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. labranchiae</td>
<td>Algeria</td>
<td>Morocco</td>
<td>Algeria</td>
<td>Tunisia</td>
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<tr>
<td></td>
<td>Tunisia</td>
<td></td>
<td></td>
<td></td>
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<td>A. messae</td>
<td>Bulgaria</td>
<td>Romania</td>
<td>Bulgaria</td>
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<td></td>
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<td>A. sergenti</td>
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<td>A. multicolor</td>
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<td>A. fluviatilis</td>
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<td>A. subnictus</td>
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<td>Pakistan</td>
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<td>A. hyrcanus</td>
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<td>Nepal, India</td>
<td>Indonesia</td>
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<td>Nepal</td>
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</table>
Table 25  Insecticide resistance in anopheline mosquitoes: Countries or areas

<table>
<thead>
<tr>
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Table reproduced from WHO 1976 Technical Report Series No. 585.
Table 26: Resistance of Anopheline Mosquitoes to Organophosphorous compounds, Carbamates and Pyrethroids

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(Updated from WHO, 1976 technical report series 585)
Africa, Cameroon, Central African Republic, Upper Volta, Nigeria and Dahomey by 1975 (WHO, 1976). This species was also reported to be resistant to dieldrin/HCH in Togo, Cameroon, Central African Republic, Upper Volta, Nigeria, Senegal, Guinea, Gambia, Equatorial Guinea, Sierra Leone, Kenya, Liberia, Ivory Coast, Ghana, Dahomey, Congo, Mali and Mauritania. No resistance to the organophosphorous or carbamate was reported. *A. arabiensis* (previously *A. gambiaca* "B") was first reported to be resistant to DDT in Senegal in 1968 and in Swaziland in 1970. Resistance of this species to dieldrin/HCH was reported from Upper Volta, Nigeria, Senegal, Swaziland, Chad, Ethiopia, Kenya, Mozambique, Southern Rhodesia and Mauritania. Again no resistance to the organophosphorous or carbamate was encountered by this species. *A. arabiensis*, the main malaria vector in the Sudan, showed resistance to HCH in 1968, and to DDT in 1970. Malathion was introduced in 1975 in the Gezira irrigated area to combat the epidemic that broke out in 1974-75. After the fourth round of spraying in July 1977, adult mosquitoes were caught resting in sprayed surfaces in September 1977. Discriminating concentrations of malathion in susceptibility tests performed late in 1977 and during 1978 failed to give 100% mortality. Resistance was further confirmed by subsequent laboratory tests on the progeny of surviving females. (Figure 61)
Fig. 61
Status of susceptibility of *A. arabiensis* to insecticides in the Sudan - 1979

- DLN Resistant
- DLN Susceptible
- DDT Resistant
- DDT Susceptible
- Malathion Resistant
- Malathion Susceptible
6.1.3 Nature and Causes of Resistance

Georghiou (1972) reviewed the factors determining the development of resistance to pesticides in insect populations. The most essential requisites for resistance development in populations were the presence of the genetic potential and sufficient selection pressure. Wilson Jones and Davidson (1958) put the assumption that resistant genes were rare in nature, but the rapid development of DDT resistance in houseflies and mosquitoes, and also the high frequency of dieldrin resistant genes observed in A. gambiae (Service and Davidson, 1964) and A. funestus (Service, 1964) from unsprayed areas invalidated the earlier assumptions. Porter (1964) considered the DDT and dieldrin resistant genes to exist in a low frequency in untreated populations, being maintained in a balanced polymorphism. Keiding (1975) during his study of the selection process, suggested the possibility of variations in the genetic potential to develop different levels of resistance among species. Macdonald (1959), Georghiou (1969), Planp (1970) and Brown (1971) stated that the dominance of resistant genes and their frequency were important factors in determining the speed of resistance development. In addition, the importance of population size, rate of its growth, relative isolation of populations, inbreeding, stage of life at which selection is exerted (e.g. eggs,
larvae, adults before or after mating, or egg-laying), proportion of the population and the number of generations subjected to selection pressure, were all cited as factors influencing the development of resistance. Georghiou (1972) considered that the fact where there is involvement of more than one major genetic factor, moderate pressure stimulate the nature of field selections where with the residual insecticides, partial selection of subsequent generations and the migrants are affected. Such selections could preserve both recessive and dominant factors, which may interact to increase the resistance levels. He also stated that previous exposure to insecticides both related and unrelated to the selecting agent, enhance the speed of resistance development to the latter. He concluded that the speed of resistance development is initially slow in previously unselected populations when the major resistant factors are increasing and after which the process is accelerated. Keiding (1975) discussed the importance of types of insecticides used, nature of resistant mechanisms involved, and the sequence of use and combinations of insecticides.

6.1.4 Resistance Mechanisms

Recent years have seen important advances in the understanding of the mechanisms causing resistance. The progress made has mainly been due to the improved
toxicological, biochemical, physiological and genetic techniques. Plapp (1976) and Oppenoorh and Welling (1976) made the most recent reviews on the aspect of resistance mechanisms. Chadwick (1955) and Winteringham (1969) broadly categorised the factors influencing the fate of insecticide within an animal. They claimed that, on theoretical basis, any changes affecting penetration, distribution and target site interaction of an insecticide contribute to resistance. Of those changes so far established, reduced penetration, increased detoxication, and altered site of action have been cited as of prime importance (Oppenoorh and Welling, 1976).

6.1.5 Biochemical Basis of Resistance

Many causes of resistance have been defined, although there are still several types that defy an explanation in biochemical terms. Among the latter are such important resistance mechanisms as that for dieldrin, and the mechanism of DDT resistance due to the gene for knockdown resistance (kdr) that does not involve detoxication. There is some reason to believe that these two mechanisms depend on an altered site of action, but definite proof is lacking. Milani and Travaglio (1957) showed the effect of the kdr gene to be affected by modifier genes. Keiding (1963) has suggested a greater abundance of the gene kdr in Europe based on studies on
houseflies. Grigolo and Oppenoorth (1966) showed a DDT-ase of low activity to enhance the effect of this gene. On the other hand, an altered site of action as a cause of resistance has been definitely established with cholinesterase inhibitors. Originally this was found in the OP resistant spider mite Tetranychus urticae by Smissaert (1964) and by others in ticks, green rice leaf hopper, housefly. Recently it was shown in A. albimanus by Ayad and Georgiou (1975). In these cases, a mutant acetyl cholinesterase (AChE) is produced that is inhibited more slowly by the insecticides than the normal enzyme in susceptible strains.

6.1.5.a Reduced penetration

Reduced penetration is another mechanism of resistance, though of less importance. Generally it is important because it increases resistance brought about by other factors. Several authors have related reduced insecticidal penetration in a number of insects with different genes. This mechanism was shown to be non-specific and selected by a variety of insecticides in houseflies by Hoyer and Plapp (1968), Plapp and Hoyer (1968), Sawicki and Farnham (1968), Sawicki (1970) and Georgiou (1971).

6.1.5.b Increased detoxication

The enzymes or classes of enzymes which play an
important role in increased detoxication are:
- DDT-ase (DDT-dehydrochlorinase), affecting DDT and several analogues;
- hydrolases, affecting phosphate esters or carboxylic ester groups in organophosphorous compounds and in some pyrethroids;
- glutathion-S-transferase, affecting organophosphorous compounds;
- oxidases, affecting carbamates, organophosphorous compounds, DDT and its analogues, as well as pyrethroids.

Lipke and Kearns (1960), Perry (1964), O'Brien (1967) and Oppenoorth and Welling (1976) reviewed studies on DDT-ase which had been primarily confined to the houseflies. Considering the differences encountered in the enzymes within various strains of houseflies, similar variations in other species have been suggested by Oppenoorth and Welling (1976).

The group of oxidases is of special importance since various oxidases can attack a wide variety of insecticides. Oppenoorth and Welling (1976) have updated and reviewed information correlating mixed function oxidases (MFOs) activity with resistance to varying groups of insecticides, i.e. organochlorines, organophosphates, carbamates and pyrethroids.

Both carboxylesterases and phosphatases fall within the category of hydrolases. The commonest
organophosphate resistance factor is considered to be a gene controlling the modified carboxylesterases. Oppenoorth and Welling (1976) has shown the theoretically possible primary sites of attack on malaoxon, the toxic analogue of malathion. On the other hand they doubted, in view of the reduced vulnerability of the phosphorothioates to phosphatase attack, due to the electrophilic atom, the possibilities of these compounds being metabolised by these enzymes to any considerable extent. They indicated the possible misinterpretations that could occur in attempting deductions as to the nature of metabolic pathways, purely based on the structure of the metabolites formed. Having analysed the studies made on the effect of a number of malathion analogues on the different species by many workers, Oppenoorth and Welling (1976) suggested that the specificity of carboxylesterases in resistant strains is more related to the size of the alkoxy groups than to the carboxylester groups of the insecticides concerned.

The significance of the glutathion-S-transferase (GSH) enzyme in OP resistance has not yet been fully established. Lewis (1969), Lewis and Sawicki (1971) and Oppenoorth et al. (1972) identified a gene controlling this enzyme. In addition, two additional genes, one of which was associated with carboxylesterases, was also reported. The role of the enzyme and its activity was demonstrated in a multiresistant strain of houseflies by Lewis and Sawicki (1971), and its effect on parathion in
a malathion resistant strain, was shown by Oppenoorth et al. (1972).

6.1.6 Types of Resistance

6.1.6.a Cross-resistance

Busvine (1968), Sawicki (1975) and Oppenoorth and Welling (1976) defined cross-resistance as that conferred by a single defence mechanism towards a group of compounds, which are usually the selecting agent as well as those related to it, in a strain of insects. This type of resistance was studied by Davidson in a DDT resistant population of A. sundaicus (1957), A. albimanus (1963a), and A. quadrimaculatus (1963b), and also by Davidson and Jackson on A. stephensi (1961). Busvine (1968) considered the studies above to be insufficient for identification of the resistance mechanism involved. Davidson (1956, 1963a) demonstrated a typical type of dieldrin resistance spectrum in A. gambiae and A. albimanus respectively.

6.1.6.b Multiple resistance

This was defined as the resistance where two or more independent defence mechanisms existing together each impart resistance (cross-resistance) to a different group of insecticides (Busvine, 1968; Sawicki, 1975; Oppenoorth and Welling, 1976). It was pointed out by
Sawicki (1975) that the nature of this type of resistance can be variable and could develop in several stages as a consequence of either simultaneous or the consecutive use of several insecticides. Oppenoorth and Welling (1976) discussed the question of distinguishing between cross and multiple resistance in instances where linked but distinct genetic factors may be mistaken for a single factor conferring cross-resistance. Ariaratnam and Georghiou (1971) selected with propoxur in the laboratory a resistant A. albimanus population from El Salvador which had shown resistance to organochlorine and tolerance to propoxur, carbaryl, malathion, fenitrothion and others in the field. Georghiou (1975) correlated the multiresistance in Culex p. quinquifasciatus in California with the extensive usage of agricultural insecticides in addition to direct larvicidal pressure.

6.1.6.c Multiplicate resistance

Busvine (1971) has described multiplicate resistance in a strain of insects as that resulting when two or more mechanisms co-exist in the same organism and impart resistance towards the same insecticide. Almost all studies made so far have been limited to houseflies. One of the most important reasons for this is the ease of maintenance of houseflies in the laboratory. In these studies, multiplicate resistance has been resolved into its components, and the individual mechanisms studied. Then, by the sequential recombination of the components,
the effect of their interactions has been established. A review of some of these interactions between various mechanisms of resistance to insecticides in insects was given by Sawicki (1975). Keiding (1967) in a detailed discussion on the sequential resistance development in houseflies has drawn attention to the fact that resistance mechanisms quite unrelated to selecting agents can also be selected.

6.2 MATERIALS AND METHODS

6.2.1 Anopheles arabiensis Populations Used for the Study

a. A. arabiensis from the Gezira Irrigated Area (Sudan) brought to London in January 1978 as eggs laid by females which survived exposure to 5% malathion for one hour in the field.

b. A. arabiensis from Fatasha area (Sudan) eggs laid from wild caught females and brought to London in September 1977.

c. A. arabiensis from Sennar and Shambat areas (Sudan) brought as eggs laid by wild caught females in January 1980.

d. A. arabiensis SB, a population susceptible to DDT, dieldrin and malathion, selected from a cross between susceptible males from Mauritius with dieldrin resistant females from Kano, Nigeria.
c. *A. arabiensis* SW9 + 12, a population resistant to DDT and dieldrin but susceptible to malathion which originates from Swaziland.

f. *A. gambiae* 16c, originated from Kano, Nigeria and susceptible to DDT, dieldrin and malathion.

6.2.2 Rearing Techniques

The adult mosquitoes used for the study were all maintained in the Ross Institute insectary, where the temperature ranges between 25 - 27°C, and the relative humidity was kept at the range 70 - 80%. A 12-hour day and 12-hour night were maintained with the aid of an automatic time-switch lights. Cages in which adults were retained vary in size from 8 inch cubes to 39 inch cubes, depending on the density of the mosquitoes available. Males were supplied with a 20% glucose solution, while females were fed twice a week on anaesthetized guinea pigs placed on the top of the cage. Most of the times, when the adult females were slow or reluctant to feed on guinea pigs, feeding on human blood was largely resorted to. The females were collected by an aspirator in monoculars with mosquito netting on both sides and held against the arm until they were fully fed. Also when mating in cages was not established, induced copulation technique, a modification of that described by Baker et al. (1962) was carried out. Human feeding and
induced mating were resorted to for all adult survivors from insecticide selections. It was established that newly colonized females required two blood meals before the first oviposition so all survivors from insecticide selections were fed twice before the induced copulation. The oviposition interval was three days under laboratory conditions. A five inch enamel bowl, lined with filter paper and half filled with water, was provided in each cage 48 hours after feeding for egg-laying. After oviposition the eggs were transferred to the bowls in the larval room to hatch. Larvae were reared in plastic bowls 12 inches in diameter and 5 inches deep, covered with pieces of beaded-weighted netting. The bowls were filled with tap-water to a depth of 1.5” to 2” a day prior to its use so as to bring the water to room temperature. A piece of turf was placed in the bowls as a possible source of nourishment for the first instar larvae. Usually an average of 200 - 300 larvae were reared in a bowl which was labelled, dated and properly covered with the netting. Second and subsequent stages of larvae were fed on ground Farex (a proprietary cereal babyfood with added minerals and vitamins) twice daily. Sufficient quantities were supplied, depending on the numbers and age of the larvae concerned, to ensure a yield of uniform sized larvae and pupae of more or less the same age at the same time. Excess amounts of Farex were avoided because they cause scum formation. Once pupation occurred,
the pupae were strained off into enamel bowls and placed in appropriate labelled cages for either continued maintenance of the colony, or for the provision of emerging adults for testing with insecticides.

6.2.3 Chemicals Used

The impregnated papers of insecticides used for testing were provided by the World Health Organization, Geneva.

a. Organochlorines

**DDT**

1,1,1-trichloro,-2,2,2-trichloroethylene.

**dieldrin** (DLD)

1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octa-hydro-1,4-endo-5,8-dimethanonaphthalene.

b. Organophosphates

**Methoxy compounds**

**Aromatic:** fenthion

0,0-dimethyl-O-(4-methylthio/m-tolyl)phosphorothioate

**fenitrothion**

dimethyl-3-methyl-4-nitrophenyl phosphorothioate

**Aliphatic:** malathion

0,0-dimethyl-S-[1,2-di(ethoxycarbonyl)ethyl]phosphorothioate.
c. **Carbamates**

**propoxur**

2-isopropoxyphenyl N-methyl carbamate.

d. **Malathion and temephos (Abate)** as solutions in ethanol supplied by WHO were used for larval testing.

6.2.4 **Insecticide Susceptibility Tests**

6.2.4.a **Adult stage**

The standard World Health Organization Adult Mosquito Susceptibility Test (WHO, 1963) employed worldwide, particularly under field in almost all vector control programmes, was adopted in this study. 25 to 30 one-day-old adults (males and females) were introduced into holding tubes, 12.5 cm long by 4.5 cm in diameter, lined with clean white paper. These were then transferred to exposure tubes of the same dimensions as the holding ones lined with the appropriate insecticide impregnated paper. Discriminating dosages of DDT, dieldrin, malathion, fenitrothion, fenthion and propoxur were used. After the mosquitoes were exposed to the insecticides for the required test period, they were transferred again to the clean holding tubes. A piece of cotton wool, soaked in 10% glucose solution was placed on the netting of the holding tube and the mosquitoes were
kept for a holding period of 24 hours after which the mortality in each one was recorded.

6.2.4.b Larval stages

A modified technique of that of WHO (1963), and French and Kitzmiller (1965) was adopted. Early fourth stage larvae were collected from the larval bowls by means of a pipette into small plastic bowls. 25 larvae were counted into each bowl. Care was taken to transfer the minimum amount of water with the larvae from the larval bowl. In a 300 ml capacity plastic bowl, 249 ml of distilled water (D.W.) were placed. To each bowl 1 ml of the test insecticide with the appropriate concentration was added. The insecticides were supplied by the WHO, and dilutions from them were done in ethanol. One bowl, to which 1 ml of ethanol alone (without insecticide) was added, was used as control. The larvae were strained into a small nylon net and rinsed with D.W. before they were introduced into the test solution. The larvae were left exposed in the solutions for a period of 24 hours, after which mortality counts were made.

6.2.5 Selection for insecticide resistance

For the genetic studies of the Gezira population to malathion, a homozygote resistant population was needed. This was done by mass selection over a
number of generations. One day old, unmated males and females were exposed to 5% malathion for varying times of 1, 2 up to 5 hours. The survivors males and females were artificially mated with each other and inbred. The progeny were again tested with the same dose for 5 hours and the survivors mated and inbred until zero percent mortality was reached.

For genetic studies, females from the Gezira resistant strain which survived exposure to 5% malathion for 5 hours were mated with males of the susceptible populations SB, SW 9 + 12, SHAM and 16c. The offsprings of these crossings were exposed to 5% malathion for one hour, and again females which survived this exposure were backcrossed with males from the susceptible populations. The backcross progeny were exposed to the discriminating dosage and the survivors females were kept for further backcrossing to the susceptible parents males. This was repeated for three successive generations.
6.3 Results

6.3.1 Adults Testing

Summary of the results of testing the susceptibility of populations of *A. arabicnsis* from Fatasha, Shambat and the Gezira areas of the Sudan against the discriminating dosages of DDT and DLD are shown in Table 27. Populations from the three areas showed a high level of resistance to DLD (mortalities 4% to 38%) and a moderate level of resistance to DDT (mortalities 61% to 72%) when exposed for one hour.

Tests with the discriminating dosages of malathion, fenitrothion, fenithion and propoxur showed that populations of the three areas are highly susceptible to the four insecticides tested, except that the Gezira population showed resistance to 5% malathion when exposed to it for one hour (mortality 43%). For results see Table 28. Mass selection from the Gezira population using 5% malathion with exposure periods ranging from 1 to 5 hours resulted in a homozygous resistant population. The offspring of the sixth generation gave a 0% mortality when exposed to 5% malathion for 5 hours (Table 30).

Table 29 shows that SB population is highly susceptible to DDT, DLD and malathion, while SW 9 + 12 which is susceptible to malathion showed a high level of resistance to both DDT and DLD.

Results of the crossing of the Gezira resistant females with susceptible males from SB, SW 9 + 12 and Shambat populations and the backcrosses of the progeny for three consecutive generations to determine the number of genetic factors concerned are shown by Tables 31, 32 and 33. The
Table 27 Results of laboratory exposures of *Anopheles arabiensis* from Fatasha, Shambat and Gezira areas of the Sudan to the discriminating dosages of DDT and DLD for 60 and 120 minutes

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Locality</th>
<th>Exposure time in minutes</th>
<th>Total No. exposed</th>
<th>Percentage mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDT 4.0%</td>
<td>Fatasha</td>
<td>60</td>
<td>238</td>
<td>68%</td>
</tr>
<tr>
<td></td>
<td>Fatasha</td>
<td>120</td>
<td>125</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td>Shambat</td>
<td>60</td>
<td>120</td>
<td>72%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>60</td>
<td>249</td>
<td>61%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>120</td>
<td>810</td>
<td>95%</td>
</tr>
<tr>
<td>DLD 0.4%</td>
<td>Fatasha</td>
<td>60</td>
<td>140</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>Fatasha</td>
<td>120</td>
<td>230</td>
<td>19%</td>
</tr>
<tr>
<td></td>
<td>Shambat</td>
<td>60</td>
<td>100</td>
<td>38%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>60</td>
<td>360</td>
<td>3%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>120</td>
<td>480</td>
<td>4%</td>
</tr>
<tr>
<td>DLD 4.0%</td>
<td>Fatasha</td>
<td>60</td>
<td>214</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Fatasha</td>
<td>120</td>
<td>143</td>
<td>38%</td>
</tr>
<tr>
<td></td>
<td>Shambat</td>
<td>60</td>
<td>125</td>
<td>42%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>60</td>
<td>139</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>120</td>
<td>248</td>
<td>12%</td>
</tr>
</tbody>
</table>
Table 28 Results of laboratory exposures of *Anopheles arabiensis* from Fatasha Shambat and Gezira areas of the Sudan to some of the organophosphorous and carbamate insecticides for varying times

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Locality</th>
<th>Exposure time in minutes</th>
<th>Total No. exposed</th>
<th>Percentage mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malathion 5%</td>
<td>Fatasha</td>
<td>60</td>
<td>380</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Shambat</td>
<td>60</td>
<td>120</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>60</td>
<td>678</td>
<td>43%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>120</td>
<td>425</td>
<td>58%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>300</td>
<td>348</td>
<td>72%</td>
</tr>
<tr>
<td>Fenitrothion 1%</td>
<td>Fatasha</td>
<td>60</td>
<td>125</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Shambat</td>
<td>60</td>
<td>75</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>30</td>
<td>265</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>60</td>
<td>203</td>
<td>100%</td>
</tr>
<tr>
<td>Fenthion 2.5%</td>
<td>Fatasha</td>
<td>60</td>
<td>196</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Shambat</td>
<td>60</td>
<td>96</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>60</td>
<td>599</td>
<td>100%</td>
</tr>
<tr>
<td>Propoxur 0.1%</td>
<td>Fatasha</td>
<td>60</td>
<td>320</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Shambat</td>
<td>60</td>
<td>80</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>15</td>
<td>281</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>30</td>
<td>259</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Gezira</td>
<td>60</td>
<td>257</td>
<td>100%</td>
</tr>
</tbody>
</table>
Table 29  Results of laboratory exposures of *Anopheles arabiensis* SB and SW9+12 strains to the discriminating dosages of DDT, DLD and Malathion for 60 minutes

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Strain</th>
<th>Total No. exposed</th>
<th>Percentage mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDT 4.0%</td>
<td>SB</td>
<td>235</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>SW9+12</td>
<td>189</td>
<td>60%</td>
</tr>
<tr>
<td>DLD 0.4%</td>
<td>SB</td>
<td>248</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>SW9+12</td>
<td>320</td>
<td>0%</td>
</tr>
<tr>
<td>DLD 4.0%</td>
<td>SB</td>
<td>175</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>SW9+12</td>
<td>80</td>
<td>0%</td>
</tr>
<tr>
<td>Malathion 5.0%</td>
<td>SB</td>
<td>100</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>SW9+12</td>
<td>120</td>
<td>100%</td>
</tr>
</tbody>
</table>
Table 30  Results of selection pressure of 5% malathion for varying times on *Anopheles arabiensis* from the Gezira-Sudan to select a homozygous resistant population

<table>
<thead>
<tr>
<th>Generation No.</th>
<th>Total No. exposed</th>
<th>Hours of exposure</th>
<th>Percentage mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55</td>
<td>2</td>
<td>58.2%</td>
</tr>
<tr>
<td>1</td>
<td>216</td>
<td>1</td>
<td>42.6%</td>
</tr>
<tr>
<td></td>
<td>81</td>
<td>2</td>
<td>58.0%</td>
</tr>
<tr>
<td></td>
<td>566</td>
<td>5</td>
<td>39.9%</td>
</tr>
<tr>
<td>2</td>
<td>326</td>
<td>2</td>
<td>31.9%</td>
</tr>
<tr>
<td>3</td>
<td>1399</td>
<td>2</td>
<td>15.4%</td>
</tr>
<tr>
<td>4</td>
<td>1184</td>
<td>5</td>
<td>8.0%</td>
</tr>
<tr>
<td>5</td>
<td>1269</td>
<td>5</td>
<td>2.7%</td>
</tr>
<tr>
<td></td>
<td>1345</td>
<td>5</td>
<td>2.4%</td>
</tr>
<tr>
<td>6</td>
<td>1058</td>
<td>5</td>
<td>1.2%</td>
</tr>
<tr>
<td>7</td>
<td>1150</td>
<td>5</td>
<td>0.0%</td>
</tr>
</tbody>
</table>
Table 31 Summary of the results of exposures of the progenies of three consecutive backcrosses with selection (involving the resistant GEZ strain of A. arabiensis from the Sudan and the susceptible SB strain of A. arabiensis from Mauritius/Nigeria to 5% malathion for 1 hour

**GEZ female x SB male**

<table>
<thead>
<tr>
<th></th>
<th>F1</th>
<th>First backcross</th>
<th>Second backcross</th>
<th>Third backcross</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. tested</td>
<td>1749</td>
<td>492</td>
<td>584</td>
<td>485</td>
</tr>
<tr>
<td>Observed no. dead</td>
<td>532</td>
<td>201</td>
<td>231</td>
<td>207</td>
</tr>
<tr>
<td>% mortality</td>
<td>30.4</td>
<td>40.9</td>
<td>39.6</td>
<td>42.7</td>
</tr>
<tr>
<td>Expected no. dead</td>
<td>319</td>
<td>380</td>
<td>315</td>
<td></td>
</tr>
<tr>
<td>% mortality</td>
<td>66</td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>$X^2$</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>significant</td>
<td>significant</td>
<td>significant</td>
<td></td>
</tr>
</tbody>
</table>

The $X^2$ for heterogeneity is equal to 1.08 with $p > 0.5$ (not significant).
Table 32: Summary of the results of exposures of the progenies of three consecutive backcrosses with selection (involving the resistant GEZ strain of *A. arabiensis* from the Sudan and the susceptible SW9+12 strain of *A. arabiensis* from Swaziland to 1% malathion for one hour.

GEZ female x SW9+12 male

<table>
<thead>
<tr>
<th></th>
<th>F1</th>
<th>First backcross</th>
<th>Second backcross</th>
<th>Third backcross</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. tested</td>
<td>1694</td>
<td>753</td>
<td>408</td>
<td>642</td>
</tr>
<tr>
<td>Observed no. dead</td>
<td>504</td>
<td>197</td>
<td>144</td>
<td>241</td>
</tr>
<tr>
<td>% mortality</td>
<td>29.8</td>
<td>26.2</td>
<td>35.3</td>
<td>37.5</td>
</tr>
<tr>
<td>Expected no. dead</td>
<td>489</td>
<td>265</td>
<td>417</td>
<td></td>
</tr>
<tr>
<td>% mortality</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>$\chi^2$</td>
<td></td>
<td>'&lt; 0.001'</td>
<td>'&lt; 0.001'</td>
<td>'&lt; 0.001'</td>
</tr>
</tbody>
</table>

The $\chi^2$ for heterogeneity is equal to 22.75 with $p < 0.001$ showing a significant difference.
### Table 33
Summary of the results of exposure of the progenies of three consecutive backcrosses with selection (involving the resistant GEZ strain and the susceptible SHAM strain (both are *A. arabiensis* from the Sudan) to 5% malathion for one hour

#### GEZ female x SHAM male

<table>
<thead>
<tr>
<th></th>
<th>F1</th>
<th>First backcross</th>
<th>Second backcross</th>
<th>Third backcross</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. tested</td>
<td>891</td>
<td>465</td>
<td>654</td>
<td>589</td>
</tr>
<tr>
<td>Observed no. dead</td>
<td>334</td>
<td>268</td>
<td>342</td>
<td>313</td>
</tr>
<tr>
<td>% mortality</td>
<td>37.5</td>
<td>57.6</td>
<td>52.3</td>
<td>53.1</td>
</tr>
<tr>
<td>Expected no. dead</td>
<td>316</td>
<td>445</td>
<td>401</td>
<td></td>
</tr>
<tr>
<td>% mortality</td>
<td>68</td>
<td>68</td>
<td>68</td>
<td>60</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significance at the 1% level</td>
<td>significant</td>
<td>significant</td>
<td>significant</td>
<td></td>
</tr>
</tbody>
</table>

The $\chi^2$ for heterogeneity = 3.4 and $p > 0.10$ (not significant)
observed percentage mortalities of the backcrosses with the three susceptible populations is significantly different from that expected in a 1:1 ratio such as should be the case when crossing resistant homozygous with susceptible homozygous in the presence of a single resistant gene. However, the observed mortalities still suggests the involvement of a single semi- or dominant resistant gene. The X² test for the heterogenity of the tested populations showed that there is no significant difference in the progeny of backcrosses of surviving females of (GEZ ♀ x SB ♂) with SB ♀ and the (GEZ ♀ x SHAM ♂) with SHAM ♂, while there is a significant difference when backcrossing the surviving females of (GEZ ♀ x SW 9 + 12 ♂) with SW 9 + 12 ♂. This shows the variability of results when crossing and backcrossing a resistant strain population from one area to susceptible strain populations from different geographical areas.

Results of crossing the Gezira malathion resistant population with a malathion susceptible strain of A. gambiae (16c) from Kano-Nigeria gave 100% mortality when the offspring was exposed to 5% malathion for 1 hour.

6.3.2. Larval Testing

Comparing the results of testing Gezira population and SB population against Temephos (Abate) and Malathion, it was shown that there is little difference in susceptibility between the two populations to temephos (Table 34 and Figure 62) while their level of susceptibility to malathion is virtually the same (Table 35 and Figure 63). This
Table 34  Results of Testing two Populations of  
*A. arabiensis* Larvae (GEZIRA and SB) Against  
Concentration of Temephos (Abate)

<table>
<thead>
<tr>
<th>Temephos Concentration</th>
<th>GEZIRA</th>
<th>SB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Tested</td>
<td>% Mortality</td>
</tr>
<tr>
<td>0.000</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>0.002</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>0.003</td>
<td>44</td>
<td>9</td>
</tr>
<tr>
<td>0.004</td>
<td>40</td>
<td>53</td>
</tr>
<tr>
<td>0.005</td>
<td>158</td>
<td>44</td>
</tr>
<tr>
<td>0.0075</td>
<td>47</td>
<td>83</td>
</tr>
<tr>
<td>0.010</td>
<td>50</td>
<td>98</td>
</tr>
<tr>
<td>0.0125</td>
<td>96</td>
<td>93</td>
</tr>
<tr>
<td>0.025</td>
<td>334</td>
<td>98</td>
</tr>
<tr>
<td>0.05</td>
<td>172</td>
<td>99</td>
</tr>
<tr>
<td>0.125</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>LC50</td>
<td>0.0053</td>
<td></td>
</tr>
<tr>
<td>LC90</td>
<td>0.0125</td>
<td></td>
</tr>
</tbody>
</table>
Susceptibility of larvae of two strains of *An. arabiensis* to Temephos

![Graph showing susceptibility of larvae to Temephos]

- **Temephos concentrations** (ppm): 0.002, 0.003, 0.005, 0.0075, 0.0125, 0.025, 0.05, 0.125
- **No. dead**
  - **GEZ**:
    - 4
    - 21
    - 69
    - 39
    - 49
    - 326
    - 171
    - 48
  - **SB**:
    - 17
    - 40
    - 13
    - 45
    - 49
    - 47
    - 143
    - 46
- **No. tested**
  - **GEZ**:
    - 44
    - 40
    - 158
    - 47
    - 50
    - 96
    - 334
    - 172
  - **SB**:
    - 48
    - 113
    - 50
    - 50
    - 49
  - 143
  - 75
  - 46

Observed data with 95% confidence limits. Computed regression lines.
Table 35

Results of Testing two Populations of *A. arabiensis* larvae (GEZIRA and SB) Against Various Concentrations of Malathion

<table>
<thead>
<tr>
<th>Malathion Concentrations</th>
<th>GEZIRA</th>
<th></th>
<th>SB</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>% Mortality</td>
<td>Total</td>
<td>% Mortality</td>
</tr>
<tr>
<td>0.00</td>
<td>48</td>
<td>0</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>74</td>
<td>1</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>0.10</td>
<td>98</td>
<td>10</td>
<td>48</td>
<td>10</td>
</tr>
<tr>
<td>0.15</td>
<td>146</td>
<td>15</td>
<td>148</td>
<td>20</td>
</tr>
<tr>
<td>0.20</td>
<td>92</td>
<td>42</td>
<td>120</td>
<td>51</td>
</tr>
<tr>
<td>0.25</td>
<td>75</td>
<td>75</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>0.30</td>
<td>225</td>
<td>95</td>
<td>123</td>
<td>98</td>
</tr>
<tr>
<td>0.35</td>
<td>186</td>
<td>98</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>0.40</td>
<td>100</td>
<td>100</td>
<td>73</td>
<td>100</td>
</tr>
<tr>
<td>LC50</td>
<td>0.190</td>
<td></td>
<td>0.187</td>
<td></td>
</tr>
<tr>
<td>LC90</td>
<td>0.293</td>
<td></td>
<td>0.272</td>
<td></td>
</tr>
</tbody>
</table>
Results of Testing two Populations of *A. arabiensis* larvae (GEZIRA and SB) Against Various Concentrations of Malathion

<table>
<thead>
<tr>
<th>Malathion Concentrations</th>
<th>GEZIRA</th>
<th>SB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Tested</td>
<td>% Mortality</td>
</tr>
<tr>
<td>0.00</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>74</td>
<td>1</td>
</tr>
<tr>
<td>0.10</td>
<td>98</td>
<td>10</td>
</tr>
<tr>
<td>0.15</td>
<td>146</td>
<td>15</td>
</tr>
<tr>
<td>0.20</td>
<td>92</td>
<td>42</td>
</tr>
<tr>
<td>0.25</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>0.30</td>
<td>225</td>
<td>95</td>
</tr>
<tr>
<td>0.35</td>
<td>186</td>
<td>98</td>
</tr>
<tr>
<td>0.40</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>LC50</td>
<td>0.190</td>
<td></td>
</tr>
<tr>
<td>LC90</td>
<td>0.293</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 63

Susceptibility of larvae of two strains of An. arabiensis to Malathion

![Graph showing susceptibility of larvae to Malathion concentrations.](image)

- **Observed data with 95% confidence limits.**
- **Computed regression lines.**

<table>
<thead>
<tr>
<th>Malathion concentrations in parts/million</th>
<th>GEZ</th>
<th>SB</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. dead</td>
<td>1/74</td>
<td>0/42</td>
</tr>
<tr>
<td>No. tested</td>
<td>10/98</td>
<td>5/48</td>
</tr>
<tr>
<td></td>
<td>22/146</td>
<td>30/148</td>
</tr>
<tr>
<td></td>
<td>39/92</td>
<td>61/120</td>
</tr>
<tr>
<td></td>
<td>56/75</td>
<td>40/50</td>
</tr>
<tr>
<td></td>
<td>214/225</td>
<td>121/123</td>
</tr>
<tr>
<td></td>
<td>182/185</td>
<td>98/98</td>
</tr>
<tr>
<td></td>
<td>100/100</td>
<td>73/73</td>
</tr>
</tbody>
</table>
indicates that the resistance to malathion in the adult Gezira population is not reflected in the larval stages.

6.4. Conclusion

The previous results indicate clearly that the A. arabiensis population from the Gezira area which showed signs of resistance to malathion in the field could end in a homozygous resistant population if exposed to a high selection pressure using malathion. The laboratory selection yielded a population which gave 0% mortality when exposed to the discriminating dose of malathion after the sixth generation.

Backcrossing with malathion susceptible populations of A. arabiensis revealed the possible involvement of a semi- or dominant single gene. The low mortality observed when backcrossing the surviving females with their parent susceptible males compared to the expected mortalities might be due to a high mortality amongst the susceptible individuals in the larval stages. Rearing single colonies and testing the ones from the bowls which give a good yield of adults (>70%) will definitely give better results. The limited time and the difficulty encountered in rearing colonies from single females made it difficult to pursue further work. This could be done in the future in the Sudan so as to obtain a clearer conclusion. Also the difficulty in obtaining a susceptible population from the same area of resistance made the interpretation of the results not so conclusive. Future work, involving the selection of a susceptible homozygous population as well as a resistant one from the same area for studying the genetic pattern of resistance, should be carried out.
This study shows that there is no cross resistance to the other organophosphorous compounds and carbamates.

Larval tests with malathion and temephos show that the Gezira malathion resistant population is as susceptible as the SB malathion susceptible population to both insecticides. This will indicate that residues of agricultural residual insecticides in mosquito breeding places are unlikely to be the source of the selection which produced the adult resistance. However the effect of aerial agricultural spraying on the adult mosquito resting or flying in the fields should not be ignored.

The fact that larvae from adults which gave 0% mortality when exposed to 5% malathion for 5 hours were highly susceptible to temephos and malathion is a hopeful indication that control with malathion spraying would still have an effect on the transmission of malaria.
7. **FINAL CONCLUSIONS**

The results of this study gave insight into and revealed some of the problems facing or which might face the malaria control programmes in the Sudan.

The study revealed that the evaluation of the effect of control measures on the level of transmission using parasitological data, obtained by microscopical examinations of blood films alone, is not enough and could be misleading. The implementation of modern serological techniques (the IFA test) to study the incidence and prevalence of malaria, and to evaluate the control measures proved to be of great importance.

The level of sensitivity of *P. falciparum* to chloroquine which is the drug most widely used for malaria chemotherapy in the country must be investigated on a large scale to establish baseline data and to enable application of effective countermeasures as soon as individuals and/or foci with a resistant strain are detected. The micro *invitro* technique developed in this study, and proved to be feasible for field application, will be of great help in carrying out such studies.

Malathion resistance which appeared in a population of *A. arabiensis* (the main malaria vector) in the Gezira irrigated area after the fourth round with this insecticide endangers the future control of malaria in the country. The Sudan with its limited resources and funds cannot afford to shift to alternative insecticides which are more expensive than malathion and more toxic to humans and animals. Fast detection of such resistance and the study of its genetic implications will help in quickly reducing such danger by
applying an effective alternative insecticide in the foci of resistance before its spread. Knowledge has been gained in this study about the level of susceptibility of *A. arabiensis* to other organophosphorous compounds and carbamate insecticides which could be used as an alternative to malathion.
Annex 1

Preparation of Phosphate Buffered Saline (PBS) pH 7.6

NaCl 170 gram
Na₂HPO₄ 25.6 gram
NaH₂PO₄·2H₂O 3.12 gram
Distilled water 20 litres

Annex 2

Buffered Glycerol pH 8 - 9

NaHCO₃ 0.0175 gram
Na₂CO₃ 0.016 gram
Glycerol 90.0 ml
Distilled water 10.0 ml

Annex 3

Preparation of Acid Citrate Dextrose (ACD)

Sodium citrate (Na₃C₆H₅O₇·2H₂O) 2.25 grams
Citric acid (H₃C₆H₅O₇·H₂O) 0.80 grams
Dextrose (monohydrate) 2.20 grams
Distilled water 100 ml

To be used at 15% in blood.
Annex 4

Preparation of Citrate Phosphate Dextrose (CPD)

Sodium citrate \( \text{(Na}_3\text{C}_6\text{H}_5\text{O}_7\cdot2\text{H}_2\text{O)} \) \( 1.806 \text{ gram} \)
Citric acid \( \text{(H}_3\text{C}_6\text{H}_5\text{O}_7\cdot\text{H}_2\text{O)} \) \( 0.224 \text{ gram} \)
Sodium phosphate \( \text{(Na}_2\text{HPO}_4 \) \( 0.153 \text{ gram} \)
Dextrose (monohydrate) \( 1.750 \text{ gram} \)
Distilled water \( 70 \text{ ml} \)
Use as 10% in blood.

Annex 5

Preparation of Dried RPMI 1640 medium

Dissolve the packet of 10.4 grams in 1 litre of glass double distilled water
From above solution take 90 ml in small bottle.
Add 2.66 ml of NaHCO\(_3\) (7.5% solution) to give 2 mg/ml.
Add 2.5 ml of Hepes buffer (1 molar solution) to give 25 mM solution (6 mg/ml).
Add 0.5 ml of Gentamycin (10 mg/ml) to give 50 µg/ml.
Then add more medium to complete to 100 ml.
Filter with a millipore filter in sterilized bottle.
Annex 6

Programme for probit regression calculations using a Hewlet-Packard HP-33E calculator used to calculate the dose related curves of the effect of chloroquine on *P. falciparum*.  
1. Turn calculator ON, switch to PRGM, key in the following steps:

   \[
   \begin{align*}
   &g x^2 \\
   &0.16 \\
   &x \\
   &0.2 \\
   &+ \\
   &g 10^x \\
   &R/S \\
   &+ \\
   &GTO00 \\
   &R/S \\
   &- \\
   &g x^2 \\
   &R/S \\
   &+ \\
   &STO + 0 \\
   &GTO13
   \end{align*}
   \]

   Switch to RUN.

2. For each set of data:

   Use f log button on calculator to convert the doses to log doses.

   Tabulate x (log dose), n (total number of cells
counted), $S$ (number surviving) for each dose level.

If the survival rate at zero dose is less than 100\%, multiply all the n's by it before proceeding.

Tabulate $p = S/n$ for each row and cross out any row for which $p < 0.03$ or $> 0.97$. If there are fewer than 3 rows left, abandon this set of data. Otherwise look up in the probit table the values corresponding to $p$'s and tabulate probit values $-5 = (Y's)$.

Then carry the following steps on the calculator:

3. 

- $f$ CLR REG
- Key in $Y$ ENTER
- Key in $X$ SUM
- $CLX$
- $f \times$ record result as $\log ED_{50}$
- $g 10^X$ record result as $ED_{50}$
- Key in 1.28
- $CHS$
- $f \times$ record result as $\log ED_{10}$
- $g 10^X$ record result as $ED_{10}$
- $f LR$
- $x \div y$ record result as slope
- $f$ CLR PRGM

4. For $x^2$ carry on the following steps:

- Key in $x$
- $f \hat{y}$
- Record results in $\hat{Y}$ column

Repeat for each row of the table

Use CHS for -ve signs

Repeat for each row of the table

Record results in $\hat{Y}$ column
R/S
Key in n ) for each number
R/S )
Record result in h column
f CLR REG
GTO 14
Key in Y
R/S
Key in \( \hat{Y} \)  
Repeat for each row of the table
R/S
Key in h
R/S
RCL 0
Record result as \( x^2 \)
Record degrees of freedom as 2 less than number of rows in the table.
Annex 7

Packard 33E Programme to Calculate Chloroquine Quantities
Extracted from Solutions

Switch calculator ON, switch to PRGM then key in:

1. ENTER
2. RCL 1
3. -
4. STO O
5. R/S
6. ENTER
7. RCL 7
8. -
9. STO 6
10. R/S
11. RECL 0
12. ENTER
13. RCL 6
14. ÷
15. ENTER
16. 58.4
17. X
18. CHS
19. ENTER
20. 101.67
21. +
22. ENTER
23. 100
24. ÷
25. STO 2
26. R/S
27. RCL 6
28. ENTER
29. RCL 3
30. ÷
31. ENTER
32. RCL 4
33. X
34. ENTER
35. RCL 5
36. ÷
37. ENTER
38. RCL 2
39. ÷
40. R/S
Switch to RUN

Store in memory the following:

Chamber A background \((9 \times \text{no. of minutes})\) STO 1
Chamber B background \((22 \times \text{no. of minutes})\) STO 7
Count time in minutes STO 3
Total volume of heptane \((2.5 \text{ ml})\) STO 4
Volume of Heptane/vial \((1 \text{ ml})\) STO 5

To operate the programme keep switch in RUN

Key in count A
R/S gives background correction for A channel
Key in count B
R/S gives background correction for B channel
R/S gives relative efficiency count
R/S gives total disassociation per minute in extract \((dpm)\)

Go to g RTN after each run.

Then to convert to \(\mu\text{mols/l from dpm/ml}\)
Store specific activity in \(\mu\text{Ci/\mu mol}\) \((30)\) in STO 0

Then carry on the following steps:

Switch to PRGM

ENTER
EEX
3
x
ENTER
RCL 0
+
ENTER
Switch to RUN
g RTN
Store in memory the following:
Chamber A background \((9 \times \text{no. of minutes})\) STO 1
Chamber B background \((22 \times \text{no. of minutes})\) STO 7
Count time in minutes STO 3
Total volume of heptane \((2.5 \text{ ml})\) STO 4
Volume of Heptane/vial \((1 \text{ ml})\) STO 5
To operate the programme keep switch in RUN

g RTN
Key in count A
R/S gives background correction for A channel
key in count B
R/S gives background correction for B channel
R/S gives relative efficiency count
R/S gives total disassociation per minute in extract \((\text{dpm})\)
Go to g RTN after each run.
Then to convert to \(\mu\text{mol s/1}\) from \(\text{dpm/ml}\)
Store specific activity in \(\mu\text{Ci/\mu mol} \ (30)\) in STO 0
Then carry on the following steps:
Switch to PRGM
ENTER
EEX
3
X
ENTER
RCL 0
+
ENTER
22.2
*
ENTER
EEX
5
*
R/S
Switch to RUN
g RTN
Enter the reading of total dpm
R/S gives amount of chloroquine extracted.
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Trigg, P.I., 1969b. The use of proprietary tissue-culture media for the cultivation *in vitro* of the


V. In vitro effects of immune serum on sporozoite. Military Medicine, 134: 1183-1190.


