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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June 1980

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

## 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.

## CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

THE DEMOCRATIC REPUBLIC OF THE SUDAN

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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
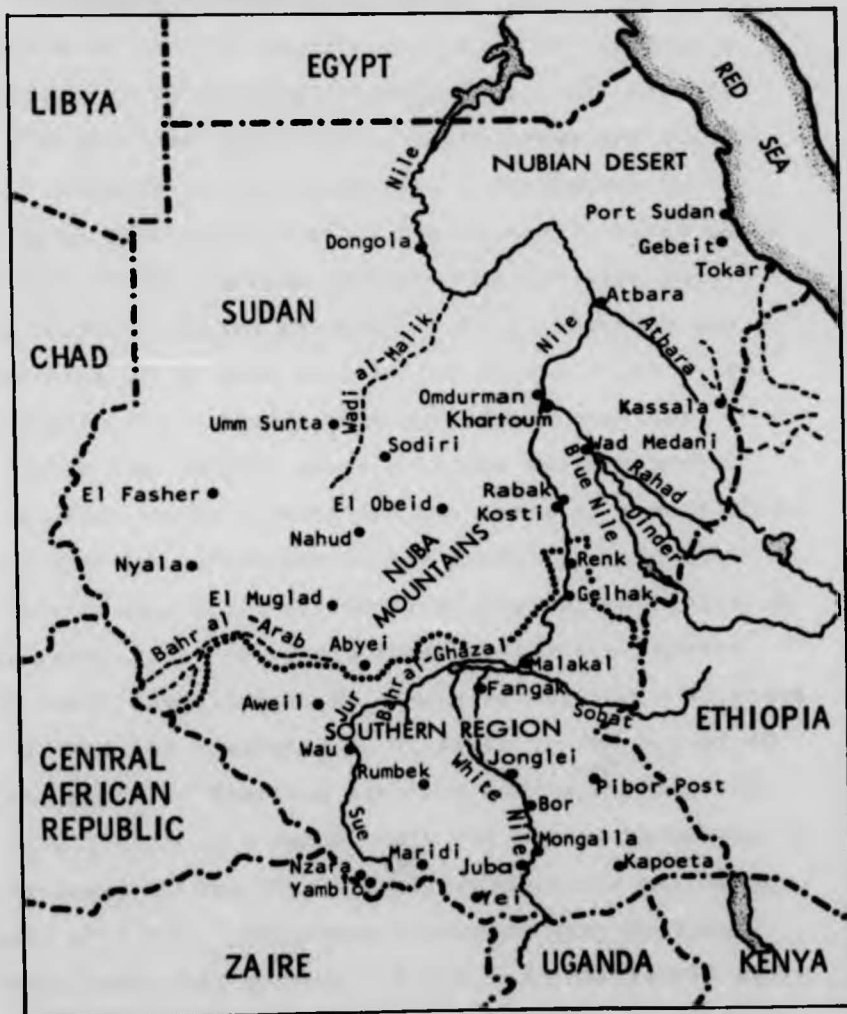
## 1.2 Population and Demography

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 most highly inhabited of all with 55.6 inhabitants per square kilometre of land, followed by the Blue Nile with 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

6

Table 1. Distribution of the Total Population by
Province in 1973

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

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° - 15° 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°C (126.5°F) in the summer to a
minimum of -2°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 con-
structing 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
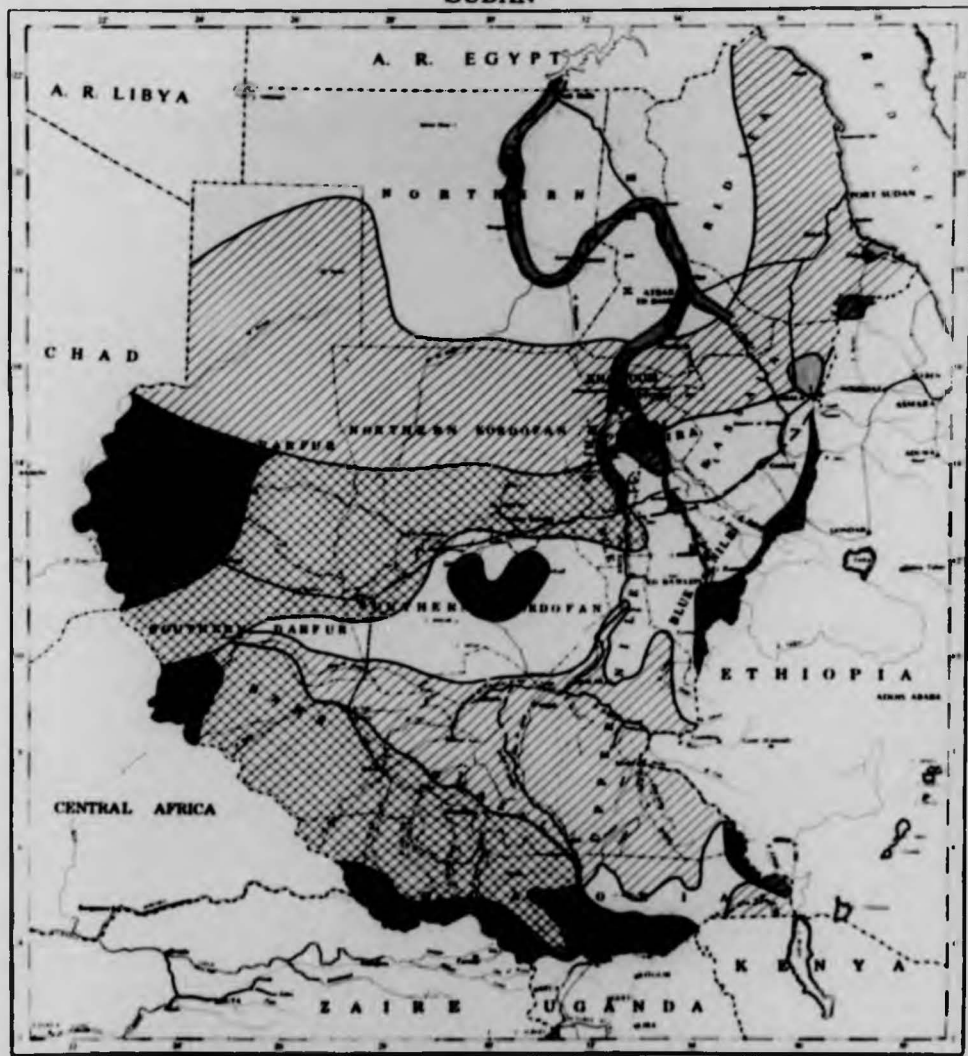
## 1.5 Communications

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 rail-
way.    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 While Nile between towns in the south.

## 1.6 Health Data

### 1.6.1 10 Common reported diseases

Disease resulting from poor environmental sani-
tation 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

| Diseases | TOTAL | | | | | | GRAND TOTAL | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Cases | | Deaths | | Rate | | | | |
| | M | F | M | F | M% | F% | Cases | Deaths | % |
| Malnutrition and Anaemia | 12,920 | 14,011 | 2664 | 2223 | 4.8 | 15.8 | 26,931 | 4887 | 18 |
| Respiratory and Measles | 9,310 | 9,042 | 1438 | 1377 | 15.4 | 15.2 | 18,352 | 2815 | 17 |
| Gastro-enteritis | 9,258 | 11,095 | 1811 | 1649 | 19.5 | 14.8 | 20,353 | 3460 | 17 |
| Heart and circulatory | 13,857 | 4,856 | 1328 | 1169 | 9.5 | 23.0 | 18,813 | 2497 | 13 |
| Infectious hepatitis | 1,457 | 723 | 151 | 126 | 10.3 | 17.4 | 2,180 | 277 | 13 |
| Cancer | 1,321 | 1,358 | 156 | 192 | 11.8 | 14.0 | 2,679 | 348 | 13 |
| T.B. | 8,447 | 3,553 | 747 | 629 | 8.8 | 17.7 | 12,000 | 1376 | 11 |
| Malaria | 10,906 | 9,150 | 762 | 642 | 6.9 | 7.0 | 20,056 | 1404 | 7 |
| C.S.M. | 3,899 | 1,716 | 193 | 91 | 4.9 | 5.3 | 5,615 | 284 | 5 |
| Accidents | - | - | - | - | - | - | 150,000 | 7500 | 5 |

Source:   Department of Statistics, Ministry of Health, Khartoum.

country as can be shown by the following figures regrouping the provinces:

Kassala and Northern                     89/100,000 population

Khartoum, Central and Blue Nile   131/100,000 population

Western Provinces                         56/100,000 population

Southern Provinces                       111/100,000 population

The total number of doctors in the country is 1287.   If we exclude those attached to the Medical Head-quarters (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
retired.   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 begining 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 prophy-
lactic 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 recommen-
ded 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 humi-
dity, 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 preci-
pitating intensive malaria transmission.

P. falciparum infections represent over 90 per
cent of all malaria infections, and hence the high mor-
tality 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 irri-
gation 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 distri-
bution 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 pro-
vinces of Blue Nile, Gezira, White Nile, Kassala, North
and South Kordofan, and North and South Darfur.    For the

**Fig. 4**

## PROVINCEWISE ANOPHELINE DISTRIBUTION IN THE SUDAN



○ A.arabiensis
✳ A.dathali
⊕ A.rufipes
■ A.pharoensis
△ A.funestus
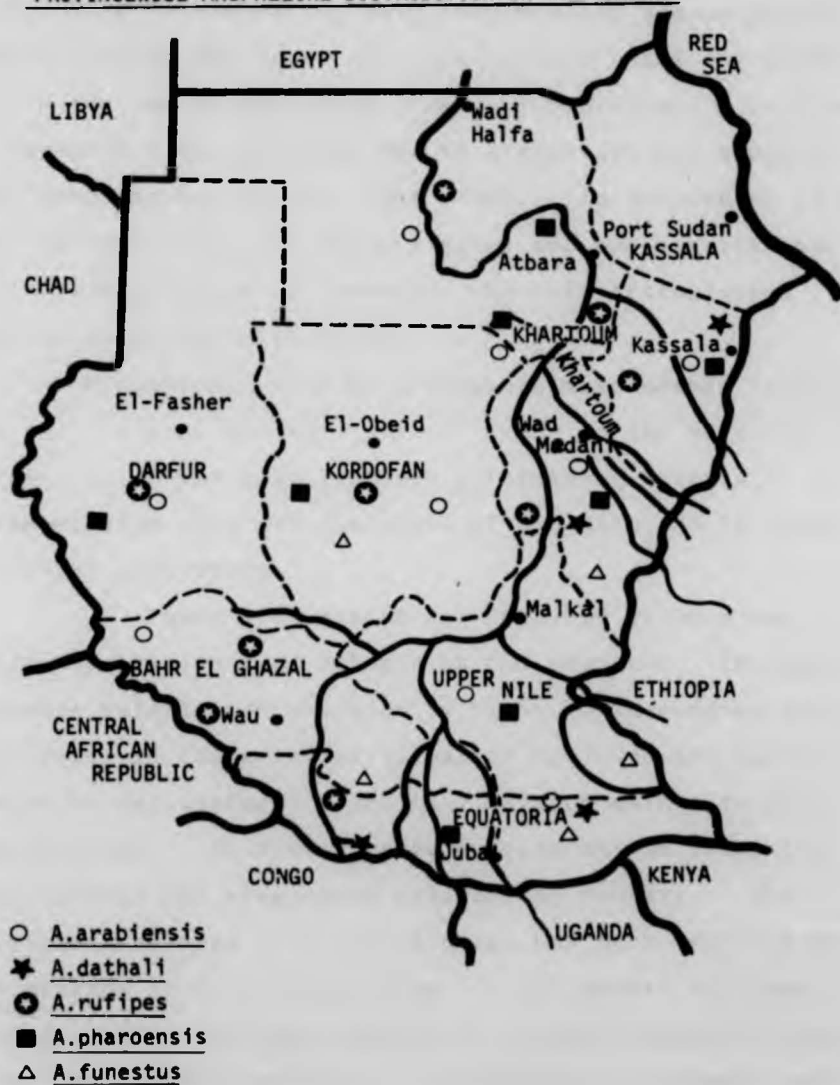
irrigated areas of the Blue Nile Province, Gezira, and White Nile Provinces the main transmission season starts shortly after the onset of rains in July until one month after the end of the rainy season in September. However, a favourable microclimate due to irrigation may support low transmission all 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 mesoendemicity 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
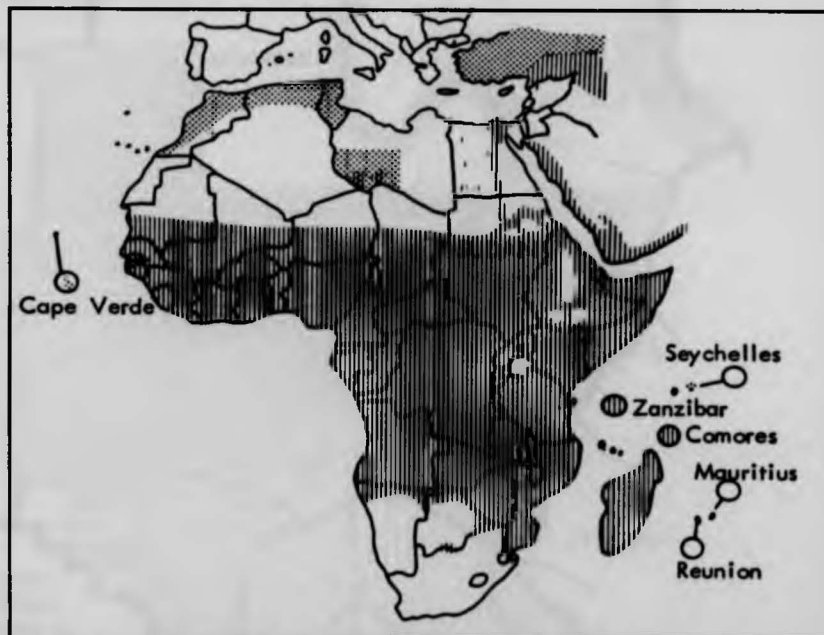


Areas in which Malaria has disappeared, been eradicated, or never existed

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
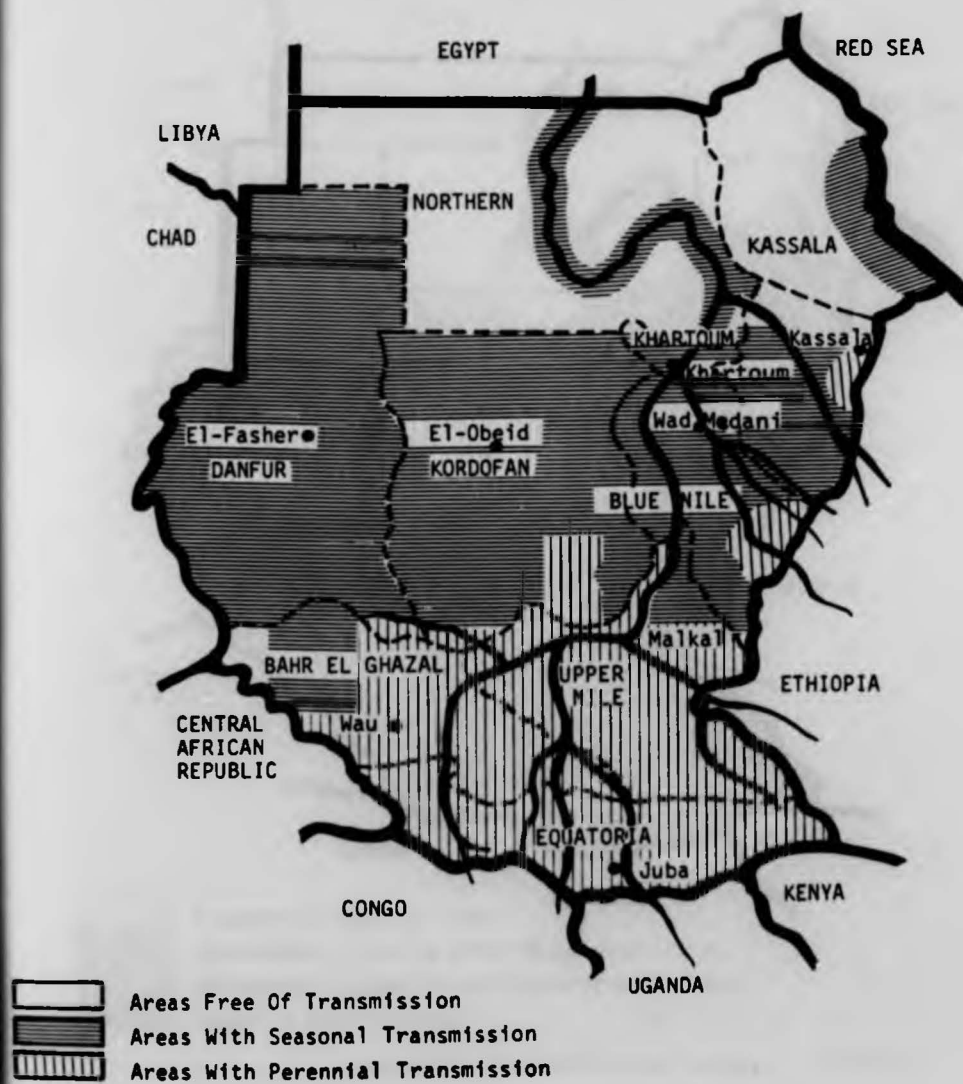
Fig. 7

## MALARIA ENDEMICITY IN SUDAN



Apparently Malaria Free
Hypoendemic Malaria With Mesoendemic Spots
Mesoendemic Malaria With Hyperendemic Spots
Hyper or Holo Malaria
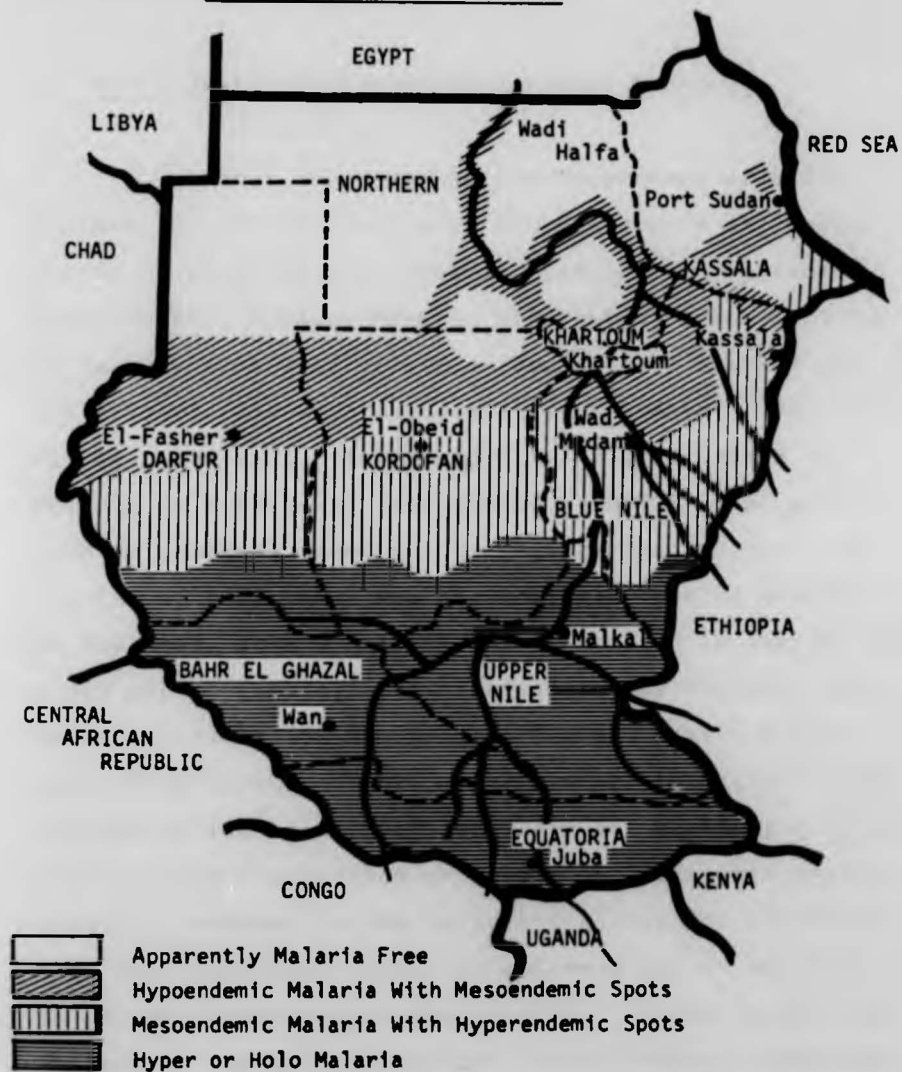
(Based on pre-eradication surveys - 1961/63)

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 possi-
bility 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 $gm/m^2$ 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 epide-
miological 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 com-
pletion 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 recom-
mendations 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 Eradi-
cation Service;

- Expansion of training facilities for all cate-
gories 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 suppor-
ted by the fact that the eight countries bordering the

Sudan with endemic malaria have no anti-malaria pro-
grammes.   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 assis-
tance 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 eradi-
cation, 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 entomo-
logical 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 Eradi-
cation Demonstration and Training Operation area. This
area covered 131 villages with a population of 83,000.
In this area a miniature scale Malaria Eradication Pro-
gramme was in operation. The activities included geo-
graphical 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 Eradi-
cation 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 inter-
rupt 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 authori-
ties of the council so as to form the nucleus for reorgani-
zation 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 aban-
doned 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 sur-
veillance 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

Sennar
Town

KEY

• •
• Localities where
malaria cases came from

Railway

—·—·— Sector limits
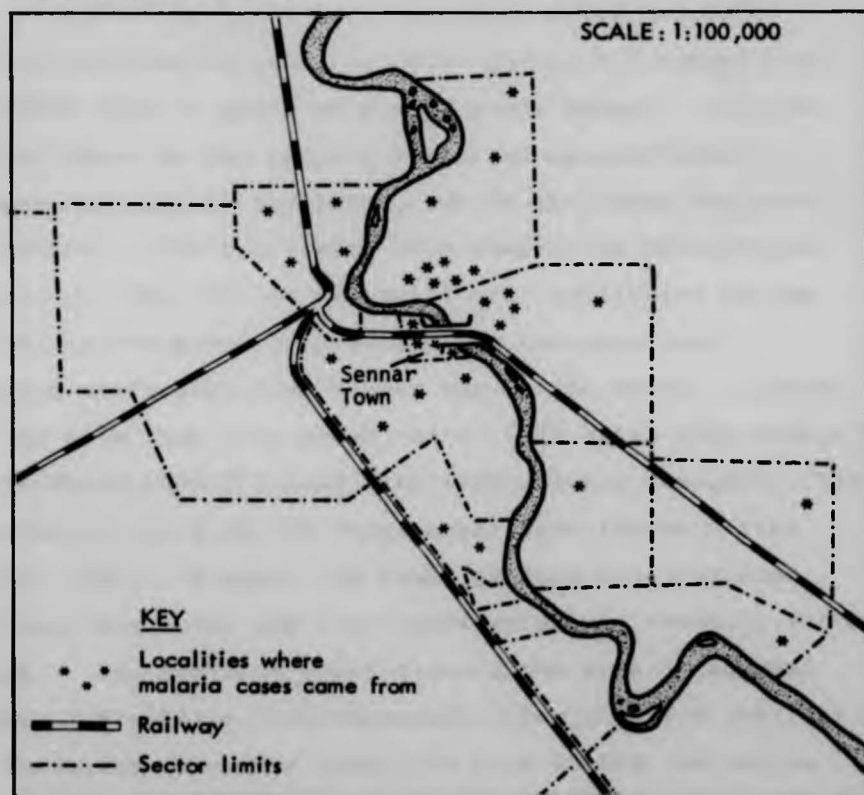
selected by the Malaria Control Division of the Ministry
of Health to be the first area to have a reorganized
malaria control programme.

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 intro-
duced.   The resident population of the area is approxi-
mately 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-

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* 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. falciparum 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 provin-
cial 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 alter-
native 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 Hasaheisa 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 Irri-
gated 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 para-
site 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 morbi-
dity 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 con-
trol activities.   The rapid reduction of malaria reser-
voirs was to be achieved by a mass single dose adminis-
tration of chloroquine and pyrimethamine to all of the
rural population and the population resident in the peri-
meter 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/$m^2$ 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/m$^2$, 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
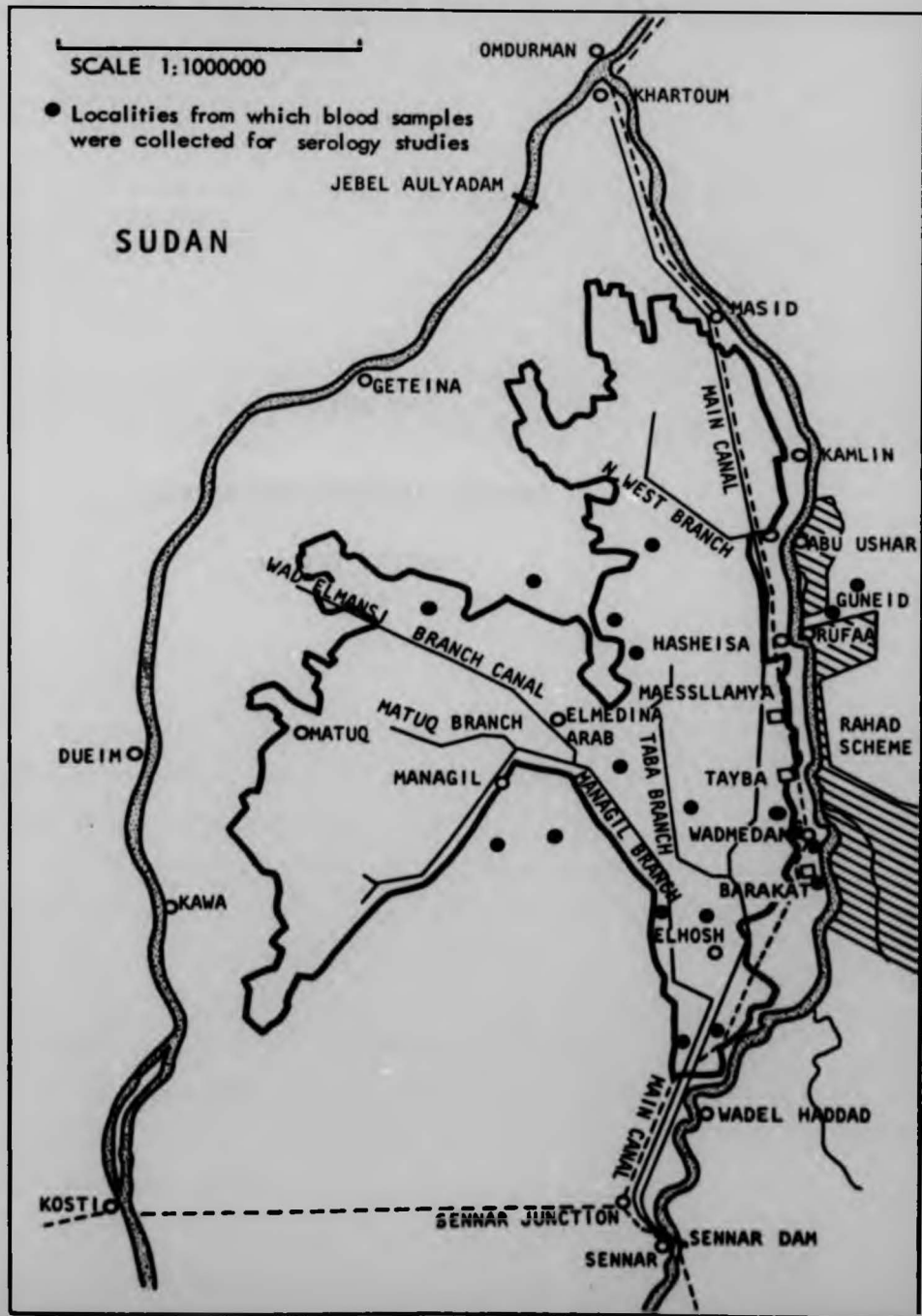
# CHAPTER TWO

# 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 sero-
logical techniques have been developed, none of them
is completely suitable to replace the microscopical exami-
nation 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 inclu-
ding 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. falci-
parum.    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 Heidlberger (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 stan-
dardization 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 dif-
ference 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 preci-
pitation in agar-gel of complexes formed between dif-
fusing 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 anti-
bodies against sporozoites (Voller, 1976). Sporozoites
develop a thread-like precipitate when incubated in serum
from rodents immunized with radiation attenuated sporo-
zoites. 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 immu-
nity, 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 sporo-
zoites. Nussenzweig et al. (1972a, b) in their experi-
ments 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 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 P. brasilianum or P. simium. Anti-sporozoites antibodies were also detected in the serum of squirrel monkeys which had acquired their P. brasilianum infection in nature, whereas the sera of laboratory-born control animals were consistently negative. Clyde et al. (1973) tested for the presence of antibodies against sporozoites in volunteers immunized with X-irradiated sporozoites of P. falciparum and then challenged by live sporozoites of 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 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 et al. (1973) succeeded in immunizing a human volunteer with sporozoites from X-irradiated mosquitoes infected with P. falciparum. Antisporozoite

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 antisporozoite 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. knowleri. 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 homo-
logous 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. cvnomolgi 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 unpro-
tected 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 har-
vested 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.    Wellde
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. falci-
parum 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 seroepi-
demiological studies. Using P. knowlesi as antigen
they tested sera collected from military recruits from
Argentina, Brazil, Colombia and the U.S.A.; they con-
cluded 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 anti-
bodies 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 un-
necessary to depend on the recorded malariometric sur-
veillance 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 intro-
duction 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 malariological conditions to permit better interpretation and understanding of the results in relation to other malariometric 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 P. falciparum antigen.
Mathews and Dilworth (1976) evaluated the use of P.
brasilianum antigen for the IHA test.   Their preliminary
study indicates the antigen can be used to detect anti-
bodies to P. malariae malaria with the IHA test, after
comparing it with antigens of P. falciparum and 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 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 P. falciparum and 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 pro-
bably 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 tech-
nique to detect circulating antigens and antibodies in
rodent malaria infections.   Their technique was to dis-
solve 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 anti-
gens 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 tech-
nique.    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. Fluorescin 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 parasi-
taemia.   Further successful development of the test
progressed by time and Collins and Skinner (1972) recom-
mended 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. cynomolgi
bastinanellii 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 yoeli, P.
malariae, two strains of P. cynomolgi and P. gallinaceum
to study the cross-reactions of malaria antisera.    Anti-
sera to four rodent malarias reacted only with P. berghei
antigen.  Antisera to three strains of P. cynomolgi reacted
to P. cynomolgi, but antisera to P. inui, P. shortti, and

P. malariae failed to react. Only P. malariae antisera reacted will

P. malariae antigen;   P. falciparum antisera did not
react.   The only reactor to P. gallinaceum antigen was
the P. gallinaceum anti-serum;   P. juxtanucleaye 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 plasmo-
dium and two simian species, P. cynomolgi bastianellii
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 anti-
body 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) evalu-
ated 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 anti-
sera, the use of mature schizonts as antigen raised the
antibody titres of most sera, which reflects a quanti-
tative 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 Rieck-
mann (1968) for the maturation of asexual parasites, he
cultured the blood obtained from a child with P. falci-
parum 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 parasito-
logically 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 cul-
ture of human P. falciparum, to be used in IFA test.
Kagan (1976) used the IFA test to detect prenatal infec-
tion. 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%

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Fig. 10

Principle of the indirect fluorescent antibody test (IFAT)
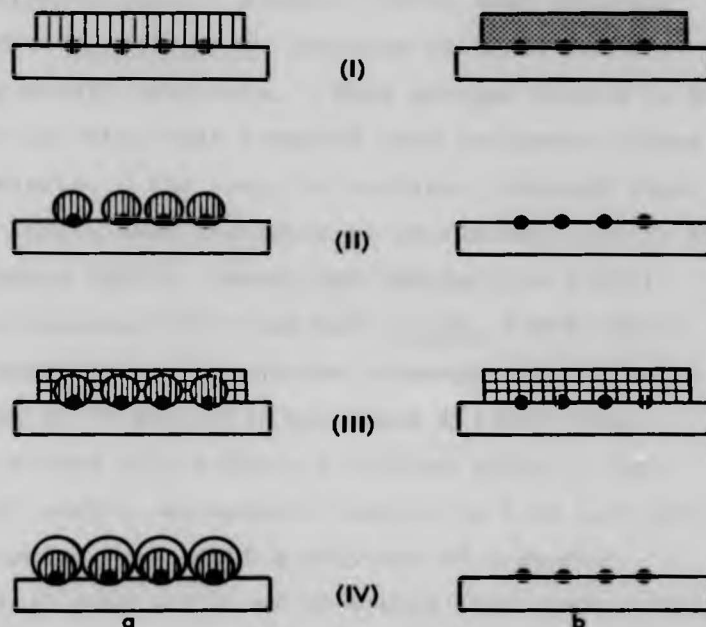


(I) Parallel preparations a and b which contain antigen (black discs) are treated respectivly with specific antiserum (hatched) and non immune serum (stippled).

(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 paper 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 demon-
strated 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. service-
men 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 intra-
muscularly 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 before-
hand, 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 anti-
body 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 bastianellii as anti-
gens, 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 neuro-
syphilis 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 presu-
mably 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 per-
sist 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 detec-
table even after 10 years from last exposure.    This
finding suggests that adult Africans returning home after
a period of absence in non-ma larious 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 resi-
dents 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. falcinarum 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 sus-
pects 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 demon-
strated 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 immuno-
globulin 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 anti-
bodies.   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 pro-
duction was greatly increased during experimental
infections of P. vivax and 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 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 immuno-
globulin 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 anti-
body 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 anti-
body. 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 sug-
gest 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 res-
ponse to hyperendemic malaria they found that the fluores-
cent 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 ntibodies 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 child-hood 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 areas below 750 feet, which have intense malaria trans-mission, 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 2 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 para-
sitaemia 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 sig-
nificant 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 fluores-
cent 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 popu-
lations.    In the unprotected population, this decrease
was followed by an increase, closely associated with the
parasitological findings, while in the protected popu-
lation 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 popu-
lation, 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 protec-
ted 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 unpro-
tected populations was the largest observed;   very signi-
ficant 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 pro-
grammes.    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 evi-
dence 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 fol-
lowed 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 trans-
mission 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 rela-
tively 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 posi-
tives 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-
firmed 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),
WHO (1974), Ambroise-Thomas (1974), Bruce-Chwatt (1974)
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 vir-
tually 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.

In Rumania by Lupascu et al. (1967, 1969), in Brazil by
Amato Neto et al. (1970), in U.S.A. by Fisher and Schultz
(1969), Sulzer and Wilson (1972) and Najem and Sulzer
(1976), in the U.K. by Dike and Draper (1970), Voller
(1971), Draper (1971) and Bruce-Chwatt (1972, 1974) and
in the U.S.S.R. by Duhanina and Zukova (1965), Tiburskaja
and Vrublevskaja (1965) and Lysenko et al. (1977).
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 poten-
tial 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 objec-
tive criteria of positive and negative reactions are
possible.   The antigen was prepared from P. falciparum
in chimpanzee.   A stable antigen fractionated by sequen-
tial 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 reproduci-
bility 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 seroepidemio-
logical 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 popu-
lations.    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 ≥ 1:80, the nine semi-immune patients had rapid ELISA antibody responses with titres ≥ 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 signifi-
cantly higher than those found by IFA.   They suggested
that further work must be done to determine the sensiti-
vity, specificity, reproducibility, and the predictive
value of negative and positive results of the ELISA test
for antibody to P. falciparum.   The test has great
potentiality depending on finding suitable soluble antigen.
This may be possible when large amounts of cultured para-
sites 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 et al. (1974), Voller et al. (1974), Wilson 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 corre-
      lated 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 <u>Materials and Methods</u>

- 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 miscroscope slide
- phosphate-buffered saline (PBS) at pH 7.6 (annex 1)
- electrical centrifuge
- chromatography papers (Whatman No. 3) cut in
  12½ 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 <u>Preparation of Antigen Slides</u>

Blood for preparing <u>P. fieldi</u> antigen was
obtained from a splenectomised rhesus monkey (<u>M. mulatta</u>).
This had been infected intravenously with <u>P. fieldi</u>
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 ear-
lobe 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 super-
natant 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 over-
night in a desiccator at $4^{o}C$.    After drying the boxes
were placed in polythene bags and stored in a deep freezer
at $-70^{o}C$ 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.
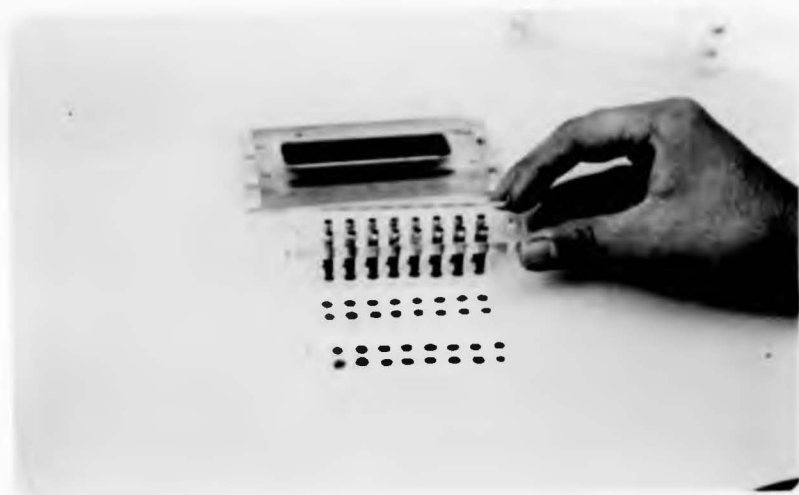
**Fig. 11**
**Preparation of malaria antigen slides for the IFA test.**

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 indivi-
dual.    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 immer-
sion 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$^o$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 appro-
ximate serum dilution of 1:16 (Figure 13). The blood
is left to elute overnight at 4$^o$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. 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 incu-
bation 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 micro-
scopic examination the slides were dried carefully from
excess glycerol with a tissue.

## 2.5 Microscopy

A Zeiss universal microscope with trans-
mitted fluorescent illumination was used. The exciting
light was produced by a 100W halogen (quartz iodine)
lamp. A BG38 and two Balzer FITC4 interference pri-
mary 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 <u>P.falciparum</u> at dilution 1:16 (x 250 magnification)



Fig. 15

IFA test : A positive <u>P.falciparum</u> at dilution 1:16 (x 400 magnification)

Fig. 14

IFA test : A positive <u>P.falciparum</u> at dilution 1:16 (x 250 magnification)
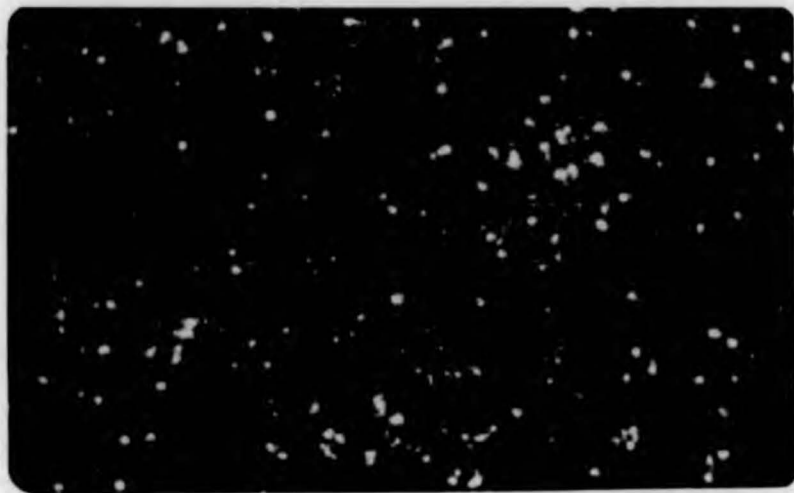


Fig. 15

IFA test : A positive <u>P. falciparum</u> at dilution 1:16 (x 400 magnification)
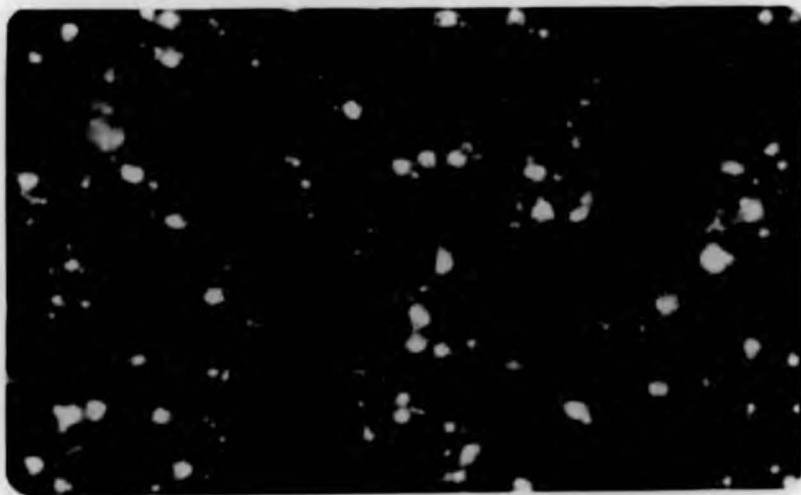
Fig. 14

IFA of A positive P. falciparum at dilution 1:16 (x 250 magnification)
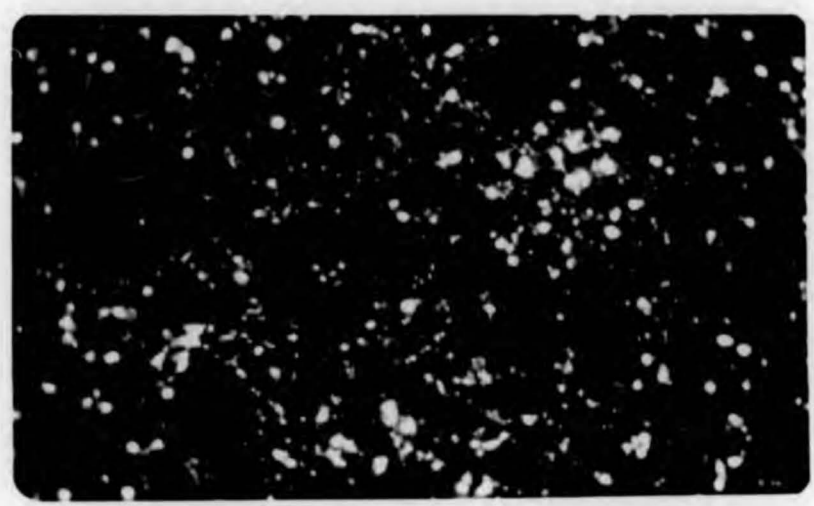


Fig. 15

IFA of a positive P. falciparum at dilution 1:16 (x 400 magnification)
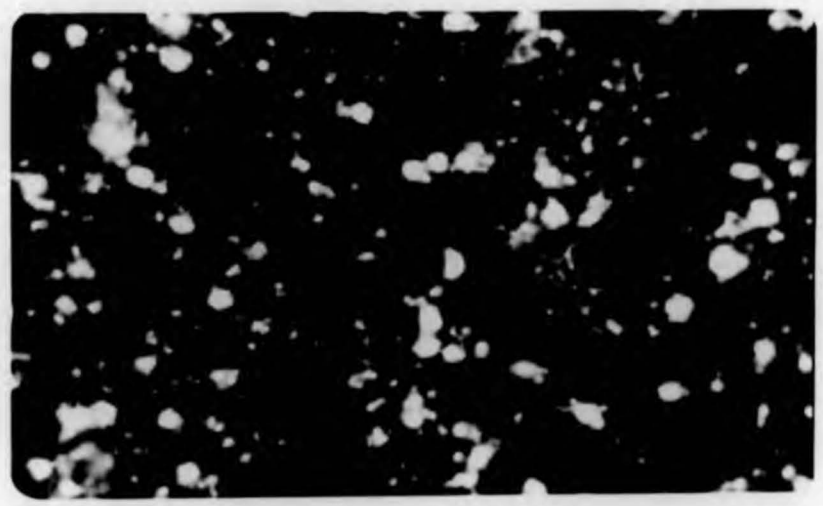
Table 3.    Total number of people tested in Gezira for
            the years 1977 and 1979 and percentage
            positive for antibodies

| Locality | Year | Total Population of localities surveyed | No. examined | % positive |
|---|---|---|---|---|
| South Gezira | 1977 | 4,564 | 282 | 62 |
|  | 1979 |  | 339 | 57 |
| Central Gezira | 1977 | 3,920 | 651 | 27 |
|  | 1979 |  | 570 | 75 |
| West Gezira | 1977 | 5,045 | 518 | 70 |
|  | 1979 |  | 213 | 40 |
| North Gezira | 1977 | 6,121 | 614 | 72 |
|  | 1979 |  | 674 | 63 |

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 col-
lected in 1979 showed 63% positives.   Again the dif-
ference 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 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 con-
forming 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 indi-
viduals 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

| Age-group | Year | No. examined | <16 | 16 | 64 | 256 | 1024 | 4096 | % +ve | GMT | % +ve excluding 16 | GMT excluding 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0-24 months | 1977 | 11 | 10 | 1 | - | - | - | - | 9 | 16 | 0 | - |
| | 1979 | 2 | 2 | 0 | - | - | - | - | 0 | 0 | 0 | - |
| 2-4 years | 1977 | 119 | 62 | 27 | 18 | 8 | 4 | - | 48 | 49 | 25 | 134 |
| | 1979 | 160 | 100 | 28 | 19 | 12 | - | 1 | 38 | 48 | 20 | 123 |
| 5-9 years | 1977 | 115 | 24 | 19 | 46 | 20 | 5 | 1 | 79 | 79 | 63 | 121 |
| | 1979 | 177 | 43 | 45 | 47 | 25 | 13 | 4 | 76 | 71 | 50 | 171 |
| 10-14 years | 1977 | 34 | 7 | 7 | 12 | 7 | 1 | - | 79 | 109 | 59 | 119 |
| | 1979 | - | - | - | - | - | - | - | - | - | - | - |
| 15-19 years | 1977 | 2 | 2 | - | - | - | - | - | - | - | - | - |
| | 1979 | - | - | - | - | - | - | - | - | - | - | - |
| 20-39 years | 1977 | 2 | 1 | - | - | - | - | 1 | 100 | 4096 | 100 | 4096 |
| | 1979 | - | - | - | - | - | - | - | - | - | - | - |
| > 40 years | 1977 | 1 | - | - | 1 | - | - | - | 100 | 64 | 100 | 64 |
| | 1979 | - | - | - | - | - | - | - | - | - | - | - |

Table 4.   Gezira Seroepidemiological Surveys 1977/79:   Prevalence of antibodies to P. falciparum by titre and age-group -   SOUTH GEZIRA

| Age-group | Year | No. examined | <16 | 16 | 64 | 256 | 1024 | 4096 | % +ve | GMT | % +ve excluding 16 | GMT excluding 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0-24 months | 1977 | 11 | 10 | 1 | - | - | - | - | 9 | 16 | 0 | - |
|  | 1979 | 2 | 2 | 0 | - | - | - | - | 0 | 0 | 0 | - |
| 2-4 years | 1977 | 119 | 62 | 27 | 18 | 8 | 4 | - | 48 | 49 | 25 | 134 |
|  | 1979 | 160 | 100 | 28 | 19 | 12 | - | 1 | 38 | 48 | 20 | 123 |
| 5-9 years | 1977 | 115 | 24 | 19 | 46 | 20 | 5 | 1 | 79 | 79 | 63 | 121 |
|  | 1979 | 177 | 43 | 45 | 47 | 25 | 13 | 4 | 76 | 71 | 50 | 171 |
| 10-14 years | 1977 | 34 | 7 | 7 | 12 | 7 | 1 | - | 79 | 109 | 59 | 119 |
|  | 1979 | - | - | - | - | - | - | - | - | - | - | - |
| 15-19 years | 1977 | 2 | 2 | - | - | - | - | - | - | - | - | - |
|  | 1979 | - | - | - | - | - | - | - | - | - | - | - |
| 20-39 years | 1977 | 2 | 1 | - | - | - | - | 1 | 100 | 4096 | 100 | 4096 |
|  | 1979 | - | - | - | - | - | - | - | - | - | - | - |
| > 40 years | 1977 | 1 | - | - | 1 | - | - | - | 100 | 64 | 100 | 64 |
|  | 1979 | - | - | - | - | - | - | - | - | - | - | - |

Table 5. Gezira Seroepidemiological Surveys 1977/79: Prevalence of antibodies to P. falciparum by titre and age-group - WEST GEZIRA

| Age-group | Year | No. examined | <16 | 16 | 64 | 256 | 1024 | 4096 | % +ve | GMT | % +ve excluding 16 | GMT excluding 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0-24 months | 1977 | 50 | 31 | 10 | 9 | - | - | | 38 | 31 | 18 | 64 |
| | 1979 | 5 | 4 | 1 | - | - | - | | 20 | 16 | 0 | 0 |
| 2-4 years | 1977 | 247 | 83 | 69 | 78 | 17 | - | | 66 | 41 | 39 | 82 |
| | 1979 | 40 | 32 | 2 | 4 | 2 | - | | 20 | 64 | 15 | 102 |
| 5-9 years | 1977 | 193 | 33 | 60 | 71 | 24 | 5 | | 83 | 51 | 52 | 102 |
| | 1979 | 138 | 76 | 17 | 28 | 12 | 5 | | 45 | 72 | 33 | 126 |
| 10-14 years | 1977 | 26 | 8 | 7 | 9 | 1 | 1 | | 69 | 47 | 42 | 94 |
| | 1979 | 30 | 16 | 4 | 7 | 3 | 0 | | 47 | 58 | 33 | 97 |
| 15-19 years | 1977 | 2 | - | - | 2 | 0 | 0 | | 100 | 64 | 100 | 64 |
| | 1979 | - | - | - | - | - | - | | - | - | - | - |
| 20-39 years | 1977 | - | - | - | - | - | - | | - | - | - | - |
| | 1979 | - | - | - | - | - | - | | - | - | - | - |
| > 40 years | 1977 | - | - | - | - | - | - | | - | - | - | - |
| | 1979 | - | - | - | - | - | - | | - | - | - | - |

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

| Age-group | Year | No. examined | <16 | 16 | 64 | 256 | 1024 | 4096 | % +ve | GMT | % +ve excluding 16 | GMT excluding 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0-24 months | 1977 | 5 | 4 | 1 | 0 | 0 | 0 | 0 | 20 | 16 | 0 | 0 |
| | 1979 | 6 | 3 | 3 | 0 | 0 | 0 | 0 | 50 | 16 | 0 | 0 |
| 2-4 years | 1977 | 60 | 35 | 12 | 9 | 4 | 0 | 0 | 42 | 42 | 22 | 98 |
| | 1979 | 82 | 40 | 22 | 17 | 3 | 0 | 0 | 51 | 32 | 24 | 79 |
| 5-9 years | 1977 | 346 | 105 | 91 | 104 | 38 | 7 | 1 | 69 | 52 | 43 | 106 |
| | 1979 | 586 | 210 | 188 | 114 | 48 | 24 | 2 | 64 | 47 | 32 | 136 |
| 10-14 years | 1977 | 164 | 28 | 36 | 66 | 30 | 4 | 0 | 83 | 65 | 61 | 108 |
| | 1979 | - | - | - | - | - | - | - | - | - | - | - |
| 15-19 years | 1977 | 4 | 0 | 0 | 1 | 3 | 0 | 0 | 100 | 181 | 100 | 181 |
| | 1979 | - | - | - | - | - | - | - | - | - | - | - |
| 20-39 years | 1977 | 19 | 1 | 3 | 5 | 5 | 3 | 2 | 95 | 189 | 79 | 308 |
| | 1979 | - | - | - | - | - | - | - | - | - | - | - |
| > 40 years | 1977 | 16 | 2 | 1 | 6 | 7 | 0 | 0 | 86 | 256 | 81 | 136 |
| | 1979 | - | - | - | - | - | - | - | - | - | - | - |

Table 7.    Gezira Seroepidemiological Surveys 1977/79:   Prevalence of antibodies to
P. falciparum by titre and age-group   -   CENTRAL GEZIRA

| Age-group | Year | No. examined | <16 | 16 | 64 | 256 | 1024 | 4096 | % +ve | GMT | % +ve ex-cluding 16 | GMT ex-cluding 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0-24 months | 1977 | 15 | 12 | 3 | 0 | 0 | 0 | 0 | 20 | 16 | 0 | 0 |
| | 1979 | 12 | 7 | 5 | 0 | 0 | 0 | 0 | 42 | 16 | 0 | 0 |
| 2-4 years | 1977 | 137 | 113 | 14 | 7 | 3 | 0 | 0 | 18 | 34 | 7 | 97 |
| | 1979 | 112 | 33 | 33 | 31 | 10 | 5 | 0 | 71 | 51 | 41 | 117 |
| 5-9 years | 1977 | 357 | 247 | 57 | 31 | 16 | 5 | 1 | 31 | 45 | 15 | 136 |
| | 1979 | 302 | 65 | 47 | 91 | 77 | 20 | 2 | 79 | 100 | 63 | 156 |
| 10-14 years | 1977 | 97 | 68 | 10 | 13 | 6 | 0 | 0 | 30 | 53 | 20 | 99 |
| | 1979 | 132 | 36 | 19 | 42 | 22 | 6 | 7 | 73 | 109 | 58 | 175 |
| 15-19 years | 1977 | 16 | 12 | 1 | 3 | 0 | 0 | 0 | 25 | 45 | 19 | 64 |
| | 1979 | 3 | 0 | 0 | 0 | 1 | 1 | 1 | 100 | 1024 | 100 | 1024 |
| 20-39 years | 1977 | 18 | 15 | 1 | 0 | 2 | 0 | 0 | 17 | 102 | 11 | 256 |
| | 1979 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 100 | 256 | 100 | 256 |
| > 40 years | 1977 | 11 | 7 | 0 | 3 | 1 | 0 | 0 | 36 | 91 | 36 | 91 |
| | 1979 | 8 | 0 | 0 | 3 | 2 | 1 | 2 | 100 | 362 | 100 | 362 |

Fig. 16

Total malaria antibodies by age - group

SOUTH GEZIRA   1977 ♣1979
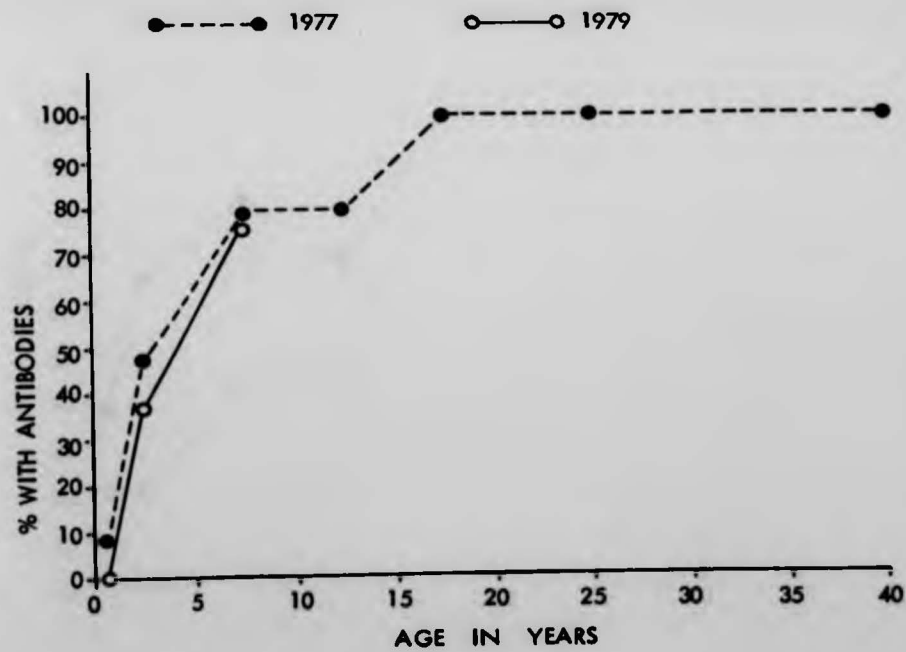
Fig. 17

Total malaria antibodies by age - group
WEST GEZIRA 1977 & 1979
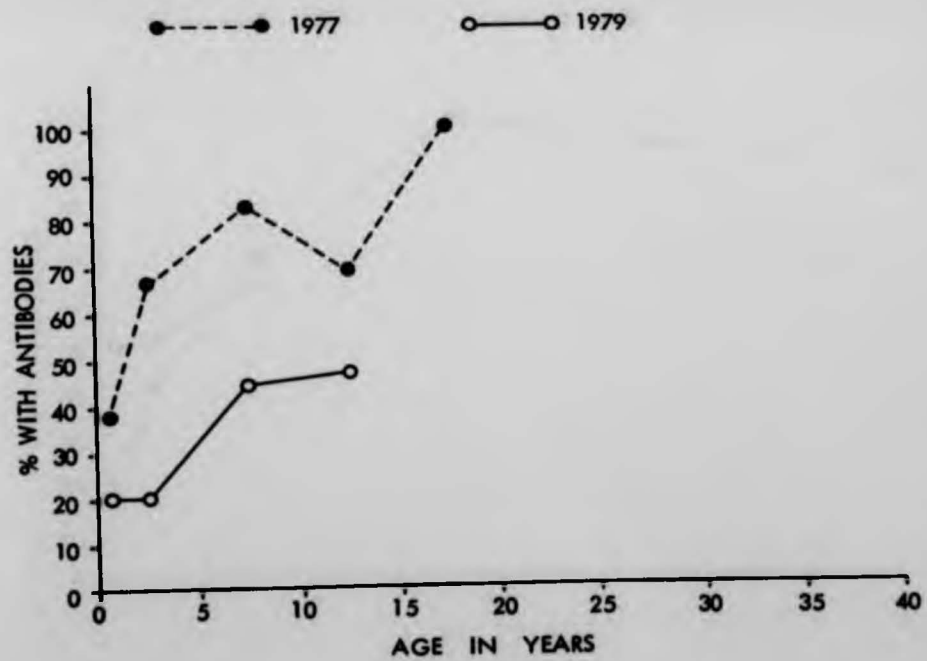
Fig. 18

Total malaria antibodies by age - group
NORTH GEZIRA 1977 & 1979
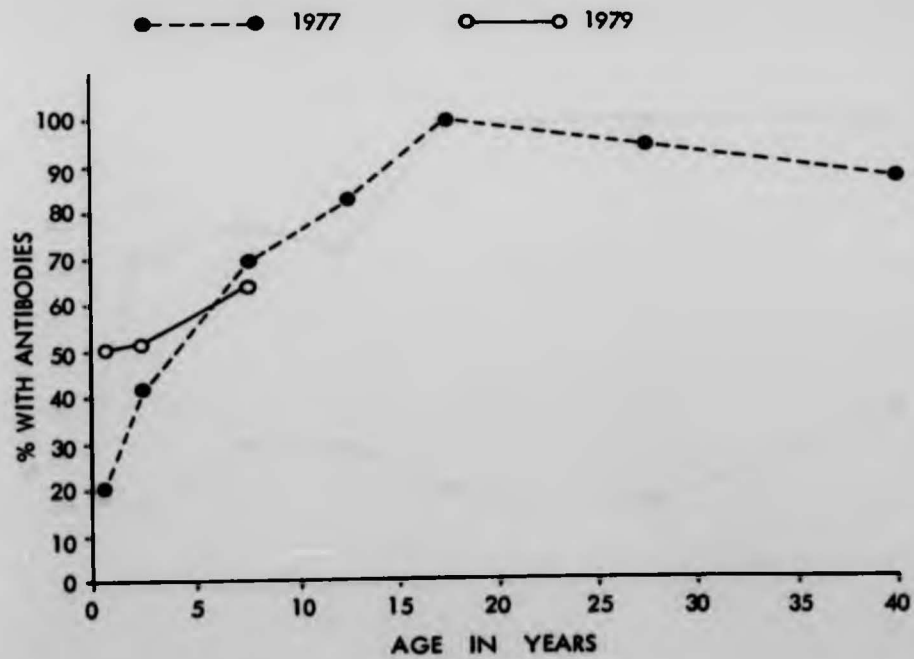
Fig. 19

Total malaria antibody by age - group
CENTRAL GEZIRA 1977 & 1979
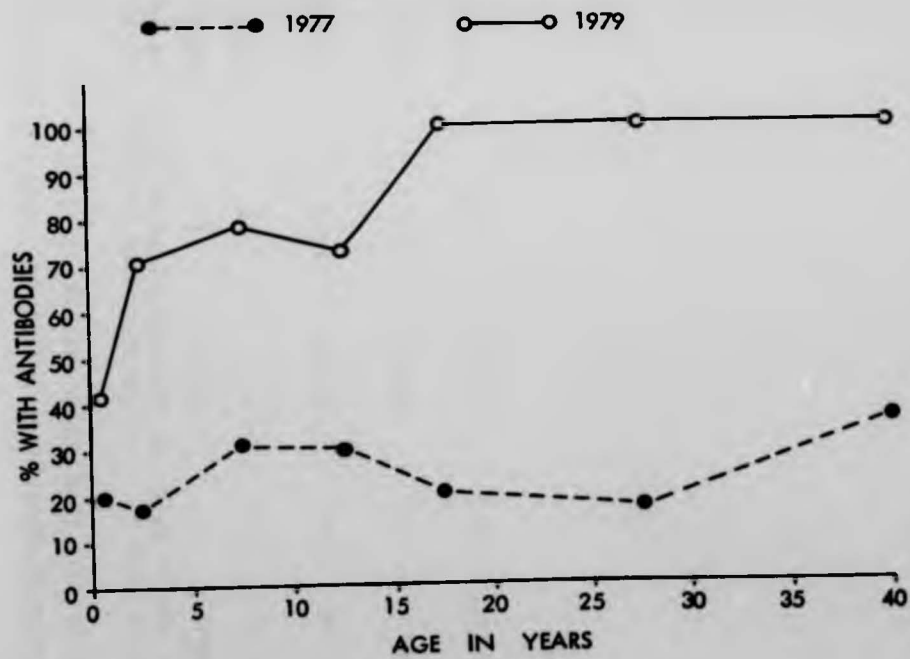
Table 8.    Gezira Seroepidemiological Surveys 1977/1979:  Change in antibody patterns in cases
repeated in 1979 from those surveyed in 1977

| Locality | -ve in 1977 and +ve in 1979 | | +ve in 1977 with increase in antibody titre in 1979 | | +ve in 1977 and -ve in 1979 | | +ve in 1977 with decrease in antibody titre in 1979 | |
|---|---|---|---|---|---|---|---|---|
| | Number | % | Number | % | Number | % | Number | % |
| South Gezira | 14/71 | 19.7 | 4/71 | 5.6 | 14/71 | 19.7 | 13/71 | 18.3 |
| Central Gezira | 92/168 | 54.8 | 25/168 | 14.9 | 18/168 | 10.7 | 2/168 | 1.2 |
| West Gezira | 6/99 | 6.1 | 13/99 | 13.1 | 45/99 | 45.5 | 18/99 | 18.2 |
| North Gezira | 16/122 | 13.1 | 11/122 | 9.0 | 34/122 | 27.9 | 20/122 | 16.4 |

Table 9.    Microscopical results of slide examination
            in Gezira 1977 and 1979

| Area | Year | Total No. examined | No. positive | Species | % positive |
|------|------|-----|------|------|------|
| South Gezira | 1977 | 282 | 1 | P. falciparum | 0.35 |
| | 1979 | 339 | 2 | P. falciparum | 0.59 |
| Central Gezira | 1977 | 651 | 0 | P. falciparum | 0.00 |
| | 1979 | 570 | 12 | P. falciparum | 2.10 |
| West Gezira | 1977 | 518 | 1 | P. falciparum | 0.19 |
| | 1979 | 213 | 5 | P. falciparum | 2.35 |
| North Gezira | 1977 | 614 | 1 | P. falciparum | 0.16 |
| | 1979 | 674 | 4 | P. falciparum | 0.59 |

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

| Year | Parasite rate during | | | | | | Slide positivity rate in P.C.D. centres in | | | | | |
| | January Survey | | | Sept./Oct. survey | | | Medina Arab Council | | | El Hosh Council | | |
| | Ex. | + | % | Ex. | + | % | Ex. | + | % | Ex. | + | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1975 | 2017* | 412 | 20.43 | 2301 | 82 | 3.56 | 11,809 | 1410 | 11.94 | 10,719 | 2396 | 22.35 |
| 1976 | 2197 | 115 | 5.23 | 2259 | 59 | 2.61 | 11,637 | 696 | 5.98 | 12,269 | 1353 | 11.03 |
| 1977 | 4040 | 80 | 1.98 | - | - | - | 12,890 | 164 | 1.27 | 8,518 | 345 | 4.05 |
| 1978 | 2855 | 5 | 0.18 | - | - | - | 9,557 | 132 | 1.38 | 8,800 | 119 | 1.35 |
| 1979 | 2528 | 17 | 0.67 | - | - | - | - | - | - | - | - | - |

* 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 inten-
sive control measures which started in mid 1975, and con-
trasts 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 res-
pectively 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 dis-
crepancy 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, par-
ticularly in 1979 showed that transmission had been con-
tinued 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 infec-
tions with malaria, so that new infections would be of
low density and could well be missed by standard tech-
niques 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 preva-
lence and from incidence data clearly shows that trans-
mission 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 trans-
mission 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 sen-
sitive 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 anti-malarial drugs as the capacity of a particular strain to survive when the drug has been administered to the verte-brate 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:

| Response | Recommended symbol | Evidence |
|----------|--------------------|----------|
| Sensitivity | S | Clearance of asexual parasitaemia within 7 days from the first day of treatment, without recrudescence. |
| Resistance | RI | Clearance of asexual parasitaemia as in sensitivity, followed by recrudescence |
| | RII | Marked reduction of asexual parasitaemia, but no clearance |

| Response | Recommended symbol | Evidence |
|---|---|---|
| Resistance | RIII | No marked reduction of asexual parasitaemia |

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 insecti-
cide, 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, C1-501) 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

Africa (Gambia, Senegal, Upper Volta, Ghana, Nigeria)
in Kenya and Tanzania (Peters, 1969).

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 inconclu-
sive (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 Kpainin, 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 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 pro-
duce a low level of parasitaemia and fever. The test
was run following precisely the technique that was des-
cribed by Rieckmann et al. (1968). The results obtained
indicated that the P. falciparum acquired by these
patients (one in Guinea and the other in Nigeria) respon-
ded 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 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 in vitro technique
(Rieckmann 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. falci
parum.

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 infec-
tions 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 chloro-
quine 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 success-
ful. 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 experi-
mental 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 res-
ponsiveness 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 poten-
tially dangerous possibility and will stimulate further
work in line with WHO guidelines.

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

Khan and Maguire (1978) reported a case of
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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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.

Morbidity and Mortality Weekly Report of U.S.A. (MMWR, 1978) reported three cases of chloroquine-resistant P. falciparum acquired in Africa; two cases from Kenya

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 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 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 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 sulfa-
methoxazole. 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 et al. (1979) and Campbell et al. (1979) respectively.
Fogh et al. (1979) referred to their case as the first
confirmed 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. falci-
parum is being transmitted in Africa.

Nuguyen-Dinh and Trager (1978) succeeded in
producing a chloroquine-resistant African strain of
P. falciparum using a continuous in vitro culture tech-
nique. The strain (FCR-3 CFMG) 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 expo-
sure 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 resis-
tance. 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).

144

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 treat-
ment he was found to be still harbouring asexual parasites
in his peripheral blood.·   Blood from this patient was

## 3.5 Chloroquine-resistant P. falciparum in South
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of chloroquine diphosphate, but after 5 days from treat-
ment he was found to be still harbouring asexual parasites
in his peripheral blood.·    Blood from this patient was

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 (Plaquenil)) and was
found to be resistant to both of them. The 7 cases res-
ponded 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 quadri-
maculatus infected with the resistant strain of P. falci-
parum 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 chlor-
proguanil 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 ino-
culation 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 propor-
tion.    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 O, 2, 3, 7, 14, 21 and 28,    Day O being the first
day of treatment.    Out of the 46 patients the parasit-
aemia 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. falci-
parum 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. falci-
parum there.   From one hundred and six persons with P.
falciparum infections, 50 patients were followed up to
give sufficient evidence.   The failure rate for chloro-
quine 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 pyri-
methamine 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 combi-
nation, nor was there evidence that the response to sul-
phonamide 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**



■ Sensitive

∗ Confirmed resistance

**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 hydroxy-
naphthalene 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 chloro-
quine 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

with chloroquine.

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. falci-parum 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 resis-tance 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), chlorguanil (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 hydroxy-
chloroquine (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 fre-
quency of unsatisfactory response to 1500 mg of chloro-
quine 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, chlorguanide, 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 sulphormethoxine.

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 dif-
ferent 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 adminis-
tration 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 cul-
ture.

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 adminis-
tration.

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 inhabi-
tants.    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
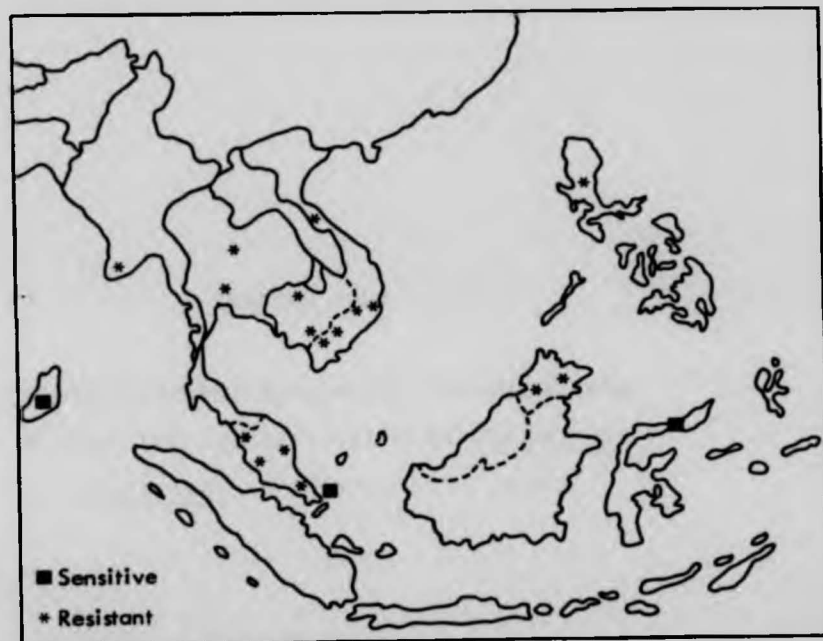


Updated from WHO 1973

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 prepar-
ations 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. falci-
parum 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 cit-
rated blood for over 2 weeks.     Bass and Johns (1912)
confirmed the previous results of Bass (1911) by culti-
vating 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.    Reinvasion
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
$37^{\circ}$ and $40^{\circ}$C were used, but the most rapid growth was
obtained at a temperature of $40^{\circ}$ to $41^{\circ}$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 $38^{\circ}$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 $39^{\circ}C$. 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 $39.5^{\circ}C$. 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 compli-
cated 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 $37^O$ to $41^O C$, 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 para-
sites without renewing the medium. They stated that
it is unnecessary to remove the leucocytes by centrifu-
gation 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 culti-
vation of malarial Plasmodia in vitro on February 1914
gave full details of the apparatus, materials and tech-
niques which should be used for cultivation of one gener-
ation of the parasites, and that for more than one gener-
ation 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 ½ inch wide, small cul-
ture tube, sucking pipette, pyrogallic acid, strong
caustic soda solution, human blood serum and 25% glucose
solution.    He used infected blood from the finger punc-
ture 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.

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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 suf-
fering 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 panto-
thenate 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 $39.5^o$ - $42^oC$,
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 mu l atta
and will also produce infection in man) gave growth and
good multiplication.   Rocker-dilution and rocker-perfu-
sion techniques, in which a slow flow of 5 per cent   $CO_2$
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 $7.4 \pm 0.1$ by equilibrating
the bicarbonate containing solution with 5 per cent $CO_2$ -
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 $38^{\circ}C$ and $39^{\circ}C$.

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 culti-
vation evaluation because of the distortion of parasites
from slow drying and the tendency of leucocytes to
agglutinate spontaneously or adhere to the sides of cul-
ture vessels. The results of counting should be calcu-
lated 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 morpho-
logical 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 culti- vation aiming to find out the effect of adenosinetri- phosphate, 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 para- sites and that heparinized blood is preferred for culti- vation 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 para-
sites from 3:1 to 6:1.    Geiman et al. (1966b) found
that addition of human plasma Fraction IV-4 to the syn-
thetic 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 splenec-
tomized 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 replace-
ment 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 tech-
nique 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 pro-
duced an increase in the multiplication rate over that
obtained when stearic acid was added alone. A new con-
tinuous perfusion system for the cultivation of the intra-
cellular 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 panto-
thenate. 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 $CO_2$ (5%) mixture was replaced by 90% N,
5% $O_2$ and 5% $CO_2$.

Diggs et al. (1971) obtained significant in
vitro reinvasion of foetal human red cells with P. falci-
parum 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 $37^{\circ}C$ in an atmosphere of 5% $CO_2$/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 parasi-
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% $CO_2$ 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% $CO_2$/95% air.     Using
this medium he managed to grow the parasites of P. falci-
parum for more than one cycle and was able to study the
invasion of fresh erythrocytes by the parasites in sub-
cultures.     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% $CO_2$ in air.
Mitchell et al. (1976) used both a modified Harvard medium,
and 199 medium freshly made up from the dry powder, supple-
mented with glucose, penicillin and buffered to pH 7.4
with $NaHCO_3$, 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% $NaHCO_3$
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 dif-
ferent 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 multi-
plication 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 supple-
mented 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 $CO_2$ incubator
(5% $CO_2$ in air) at 37$^O$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

RPMI 1640 powder medium dissolved in glass redistilled
water and supplementing it by HEPES (25 mM), and 5%
$NaHCO_3$ 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% $CO_2$ 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% $CO_2$/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% $CO_2$/air at $37^\circ C$ 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% $NaHCO_3$ with 10% human serum type AB. The culture medium was replaced daily and the flasks were gassed with a mixture of 6% $O_2$, 3% $CO_2$ 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% $NaHCO_3$.

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 incu-
bator 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 bicar-
bonate, 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 $CO_2$ in a low
oxygen tension atmosphere.    The desiccator was then
placed in an incubator at 38 - 39$^{o}$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% $O_2$/3 $CO_2$ was maintained by the
candle.    He concluded that in cultures exposed to 18%
$O_2$ 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 $O_2$ 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% $O_2$.    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.
falciparum in human red blood cells.    They stated that
best growth was obtained in all cases at 3% $O_2$ and 1%
to 2% $CO_2$, 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% $CO_2$ 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, $NaHCO_3$ and gentamycin was used. Humidified gas (3% $CO_2$, 10% $O_2$, 87% $N_2$) 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 $37^{o}C$ before use. A static water bath at $38^{o}C$ with a paraffin candle to produce the correct $CO_2$/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 obser-
vations 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 pro-
portion 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 dis-
advantages 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 recommen-
dation 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 intraperi-
toneally 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, Univer-
sity 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 pre-
paring 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 micro-
titre plates using different makes of plates and dif-
ferent media.    96 well, flat bottom, microtitre plates,
12.5 x 8.5 x 1.5 cm with a lid, were used.    Nunc (Inter-
Med, 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-gluocse | 4.0 g | 0.48% (w/v) |
| $NaH_2PO_4.2H_2O$ | 0.27 g ) | |
| $Na_2HPO_4.12H_2O$ | 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

3. N-Tris-(hydroxymethyl)-methyl-2-aminoethanesulphonic 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% $CO_2$,
5% $O_2$ and 90% $N_2$ was blown in and the bag was then
completely sealed and incubated at $37^{o}C$ in a dry incu-
bator.    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 asynchro-
nicity of the parasites made evaluation of growth diffi-
cult.   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 splenec-
tomised 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 Insti-
tute 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 modifi-
cation of the candle jar technique first described by
Jensen and Trager (1977). 96 well (flat bottom) micro-
titre plates were used. The plates were made by
either Nunc (Inter Med, Denmark) or Falcon (Microtest II,
Falcon Plastic Devision, 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
$NaHCO_3$ 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 pro-
duces a gaseous atmosphere of 3.0 to 3.5% $CO_2$ and 15 to
16% $O_2$. The amount of $CO_2$ and $O_2$ produced by the
burning candle were measured exactly, with the cooper-
ation of the Respiratory Physiology Department of the
Lung Unit, Brompton Hospital, London, using a centronic
type 806 mass spectrometer. The parasites were incu-
bated at 38 ± 0.5°C in a dry incubator or in a 'Milli-
pore' 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 $37^{\circ}C$ 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 $ED_{50}$, $ED_{90}$, slope of the curve
and the $\chi^2$ 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 stan-
dard 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 anti-
bodies 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.

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 anti-
bodies 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

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 tropho-
zoites 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 con-
trol and the ED50 was lower than that at shorter incu-
bation 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 ED50s.    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 concen-
tration 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)

| Culture No. | Control | Washed Blood | | | | $ED_{50}$ |
|---|---|---|---|---|---|---|
| | | $10^{-8.65}$ M | $10^{-8.35}$ M | $10^{-8.05}$ M | $10^{-7.75}$ M | |
| 21 | 27 | 17 | N.D. | 13 | 10 | $6.7 \times 10^{-9}$ |
| 23 | 80 | 61 | N.D. | 62 | 31 | $1.2 \times 10^{-8}$ |
| | | Unwashed Blood | | | | |
| 21 | 45 | 44 | N.D. | 21 | 12 | $9.6 \times 10^{-9}$ |
| 23 | 98 | 85 | N.D. | 81 | 90 | $6.2 \times 10^{-8}$ |

N.D. = not done

Fig. 23

P. coatneyi in the in vitro micro – test

Probit scale

Effect of Chloroquine CASE 21

o————o Unwashed blood

●――――――● Washed blood
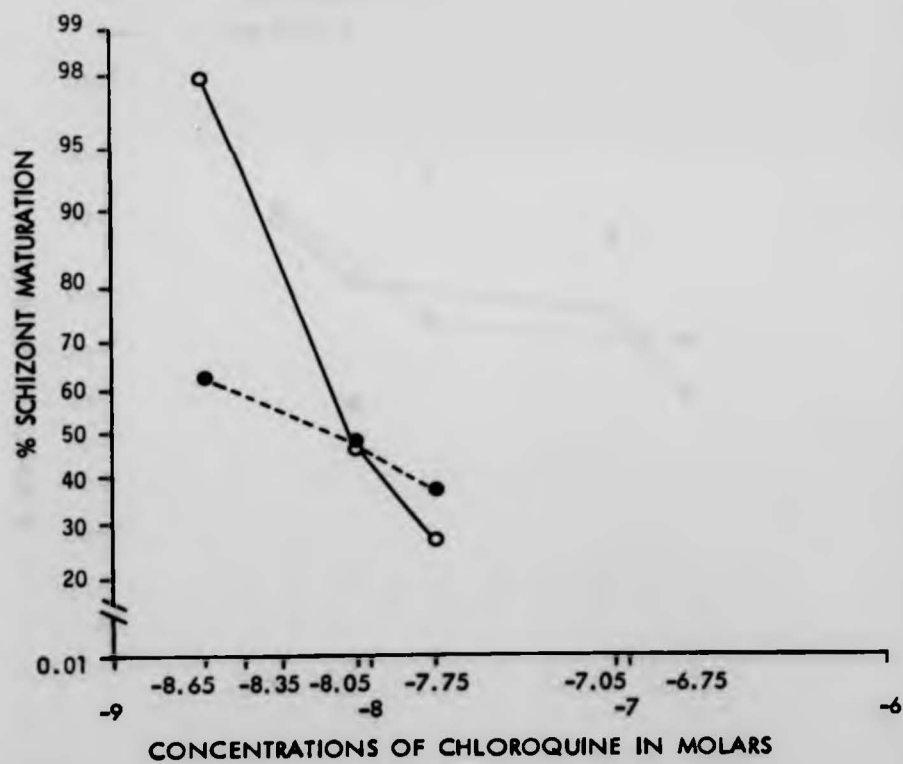
Fig. 24

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

Probit scale

Effect of Chloroquine CASE 23

o————————o Unwashed

●— — — — —● Washed



% SCHIZONT MATURATION

CONCENTRATIONS OF CHLOROQUINE IN MOLARS
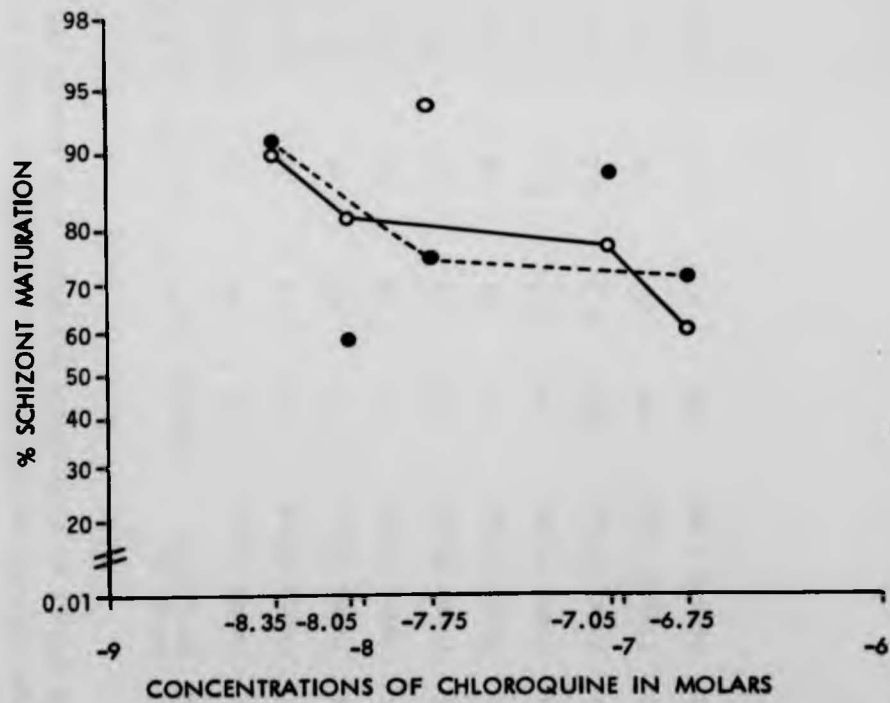
Table 12: Percentage schizont maturation of _P. coatneyi_ incubated at different periods of time under the effect of chloroquine phosphate (200 asexual parasites counted)

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

N.D. = not done

Fig. 25

P. coatneyi in the in vitro micro - test

Probit scale

Effects of Chloroquine CASE 27
Unwashed blood

o————o  28 hours incubation

o— — — —o  49 hours incubation
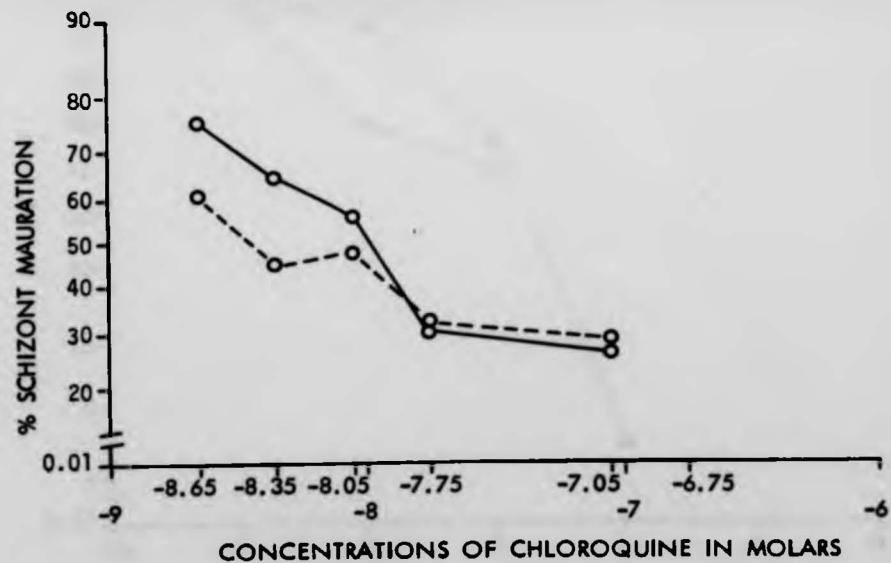
Fig. 26

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

Probit scale

| | Effect of | Pyrimethamine CASE 40 |
| △————————△ | 26 hours incubation |
| ▲————————▲ | 48 hours incubation |
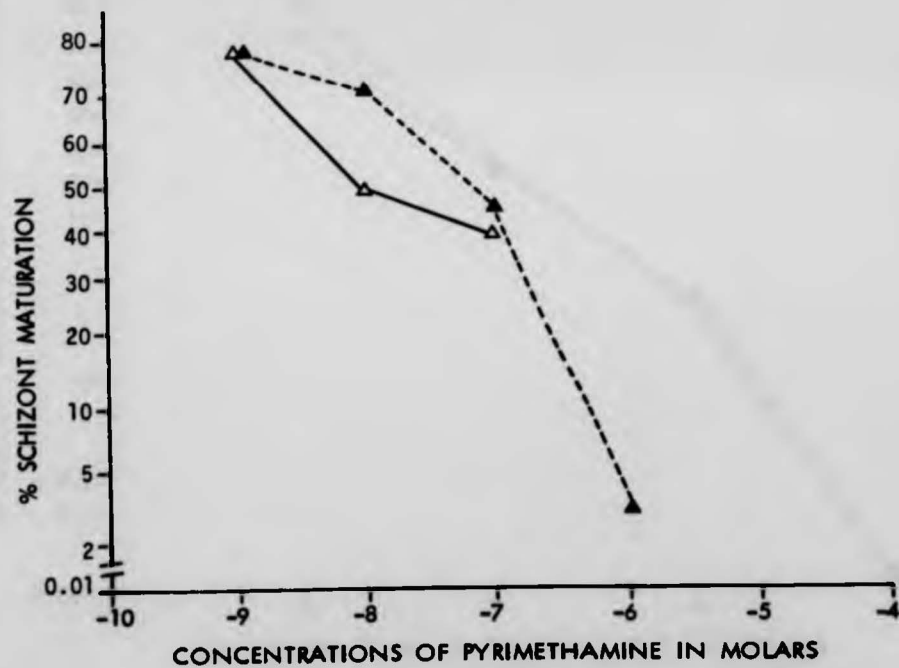
Fig. 27

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
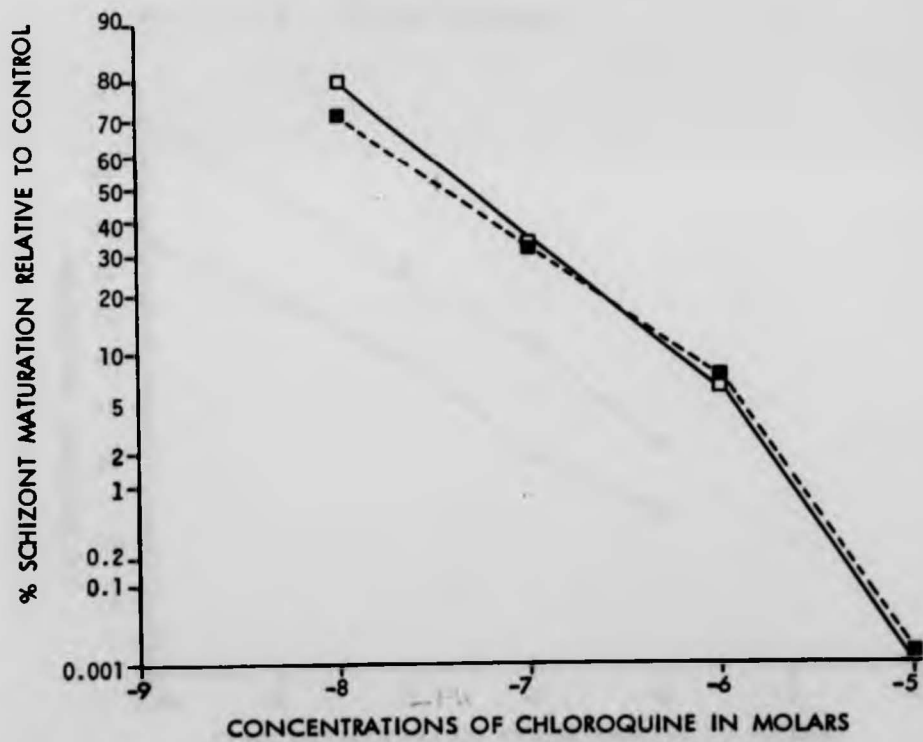
Fig. 28

P. coatneyi in the in vitro micro - test

Probit scale

Effect of Pyrimethamine CASE 43

△————△  28 hours incubation

▲------▲  48 hours incubation
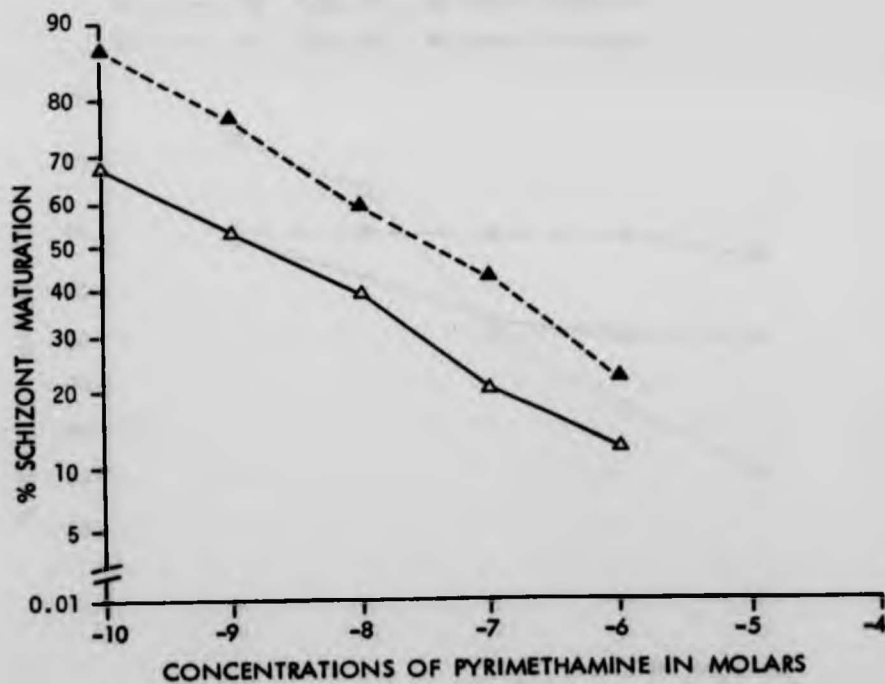
Fig. 29

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



CONCENTRATIONS OF PYRIMETHMINE IN MOLARS
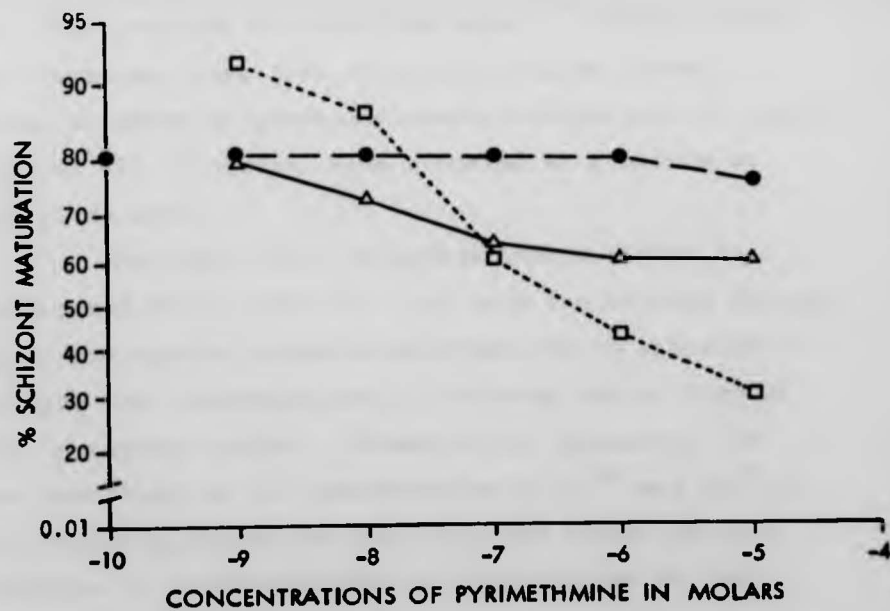
Table 13 and Figures 30, 31 and 32 show
the effect of chloroquine prepared in three different
ways on the maturation of the parasites to schizonts.
The three cultures with chloroquine show that a good
dose-response curve was obtained when the drug was
prepared in medium or when added in distilled water,
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 para-
sites 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 pyri-
methamine 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 cul-
tures 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)

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

Fig. 30

<u>P. coatneyi</u> in the <u>in vitro</u> micro - test
Three different ways of preparing Chloroquine
CASE 51

Probit scale

Drug in medium △————△   Drug inD.W. ●－－－－－●
                                    added to plate
Drug F.D.       □·········□



% SCHIZONT MATURATION RELATIVE TO CONTROL

CONCENTRATIONS OF CHLOROQUINE IN MOLARS
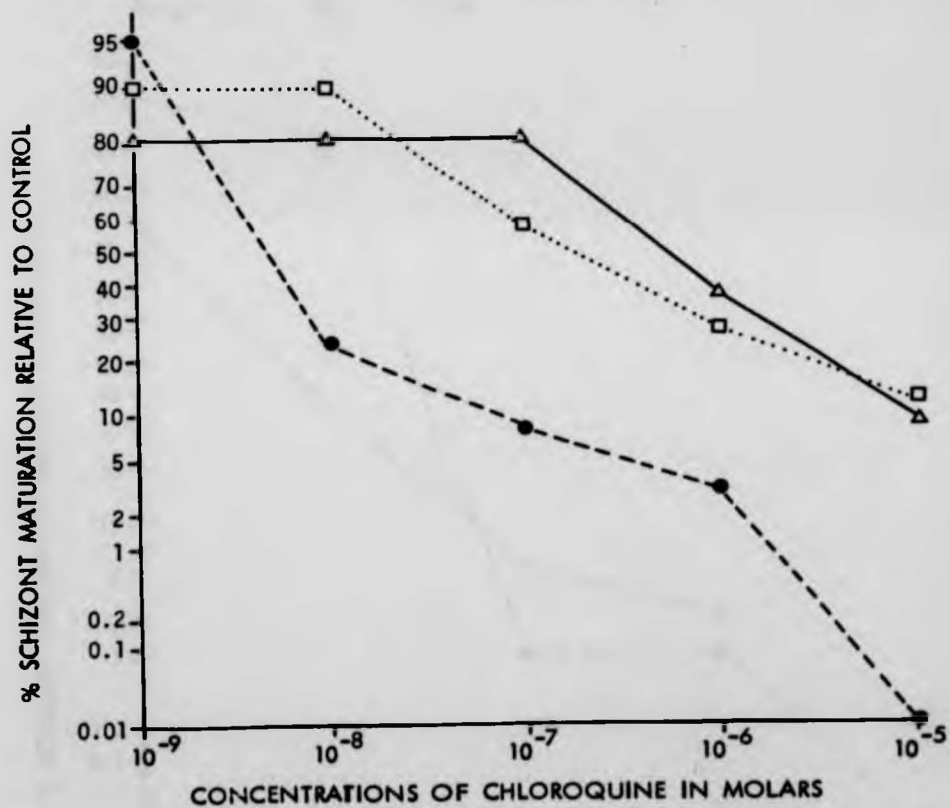
Fig. 31

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

Probit scale

Drug in medium △————△    Drug in D.W. ●------●
Drug F.D.          □··········□

Fig. 32

221
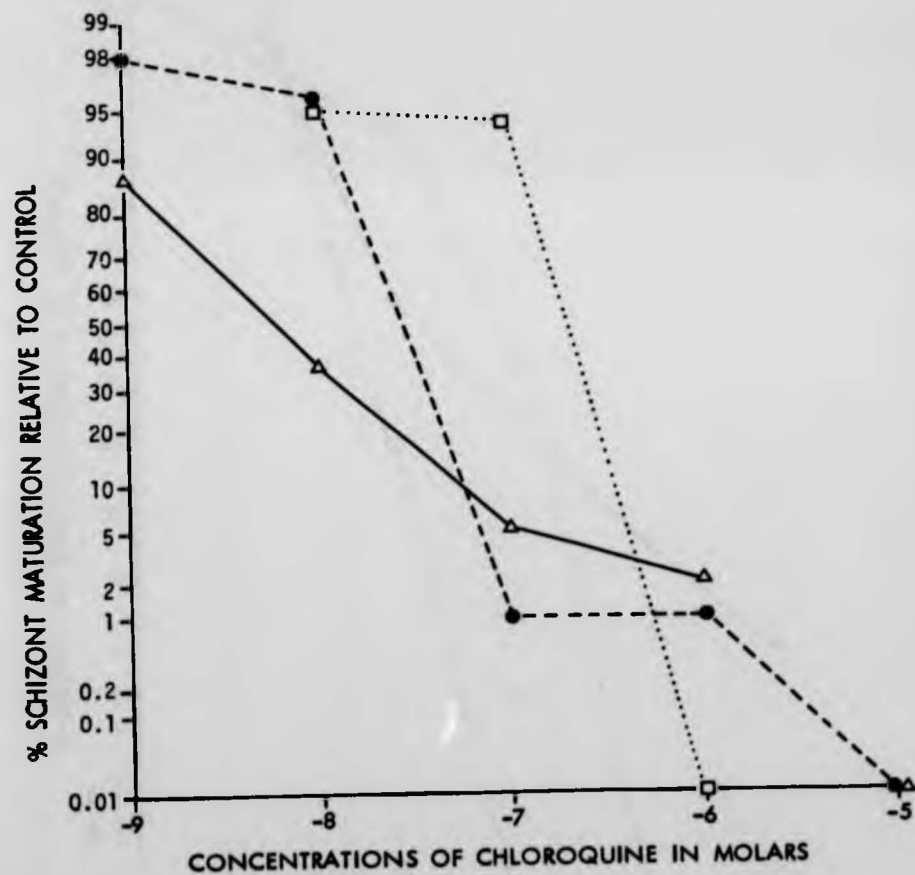
P. coatneyi in the in vitro micro - test
Three different ways of preparing chloroquine
CASE 53

Probit scale

●————————● Chloroquine disolved in medium
○———  ———○ Chloroquine added to plate in D.W.
□—————□ Chloroquine freez dried in plate
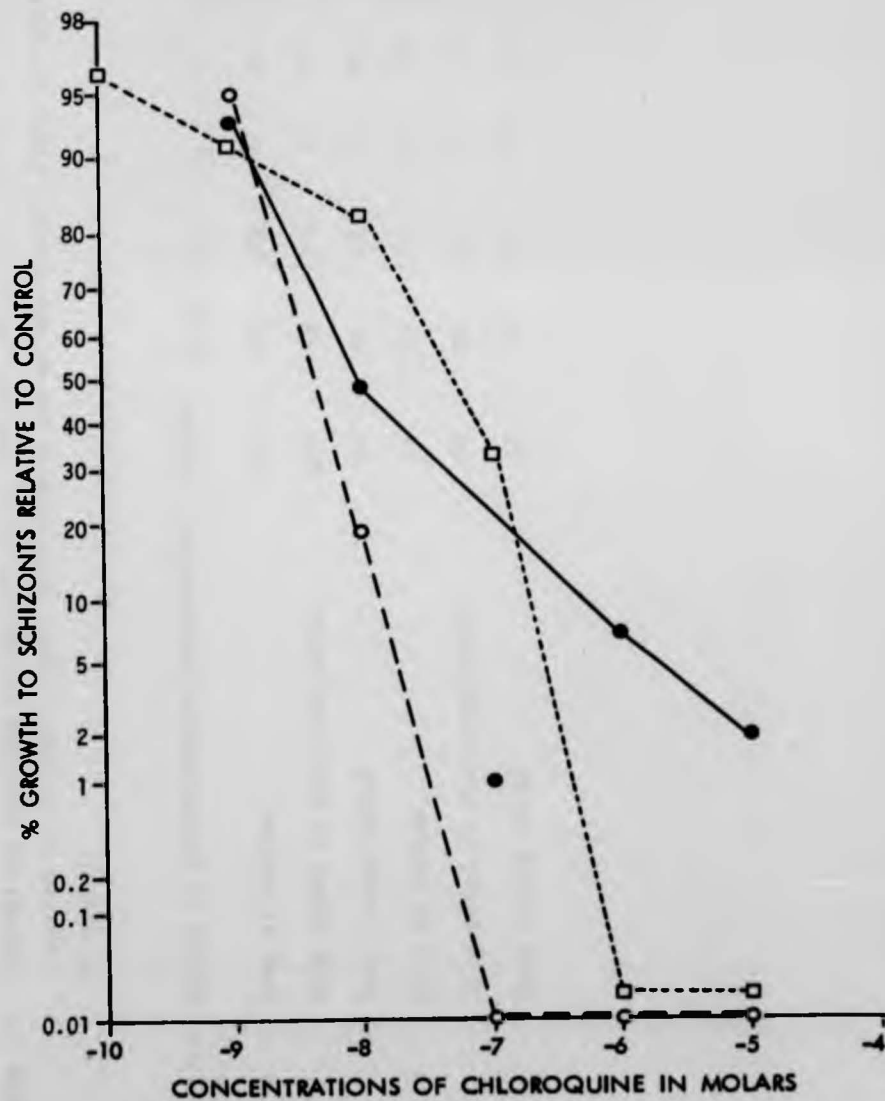
Table 14: Percentage schizont maturation of <u>P. coatneyi</u> under the effect of pyrimethamine prepared in different ways (200 asexual parasites counted after incubation for 40 hours)

| Culture No. | Method of pyrimethamine preparation | Control | $10^{-10}$ | $10^{-9}$ | $10^{-8}$ | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ | $ED_{50}$ |
|---|---|---|---|---|---|---|---|---|---|
| 52 | Drug in medium | 93 | 93 | 86 | 79 | 80 | 28 | 7 | $4.3 \times 10^{-7}$ |
| | Drug added in distilled water | 100 | 96 | 94 | 93 | 94 | 91 | 2 | $2.3 \times 10^{-6}$ |
| | Drug freeze dried | 94 | 91 | 95 | 86 | 91 | 22 | 3 | $5.2 \times 10^{-7}$ |
| 53 | Drug in medium | 98 | 96 | 75 | 86 | 80 | 11 | 2 | $1.5 \times 10^{-7}$ |
| | Drug added in distilled water | 99 | 99 | 99 | 92 | 58 | 66 | 0 | $3.2 \times 10^{-6}$ |
| | Drug freeze dried | 97 | 97 | 96 | 89 | 46 | 36 | 0 | $2.2 \times 10^{-7}$ |

Fig. 33

<u>P. coatneyi</u> in the <u>in vitro</u> micro - test.
Three different ways of preparing pyrimethamine
CASE 52
Probit scale

Drug in medium △————△    Drug in D.W. ●— — — —●
Drug F.D.      □----------□    added to plate



CONCENTRATIONS OF PYRIMETHIMINE IN MOLARS

Fig. 34

P. coatneyi in the in vitro micro - test 224
Three different ways of preparing pyrimethamine
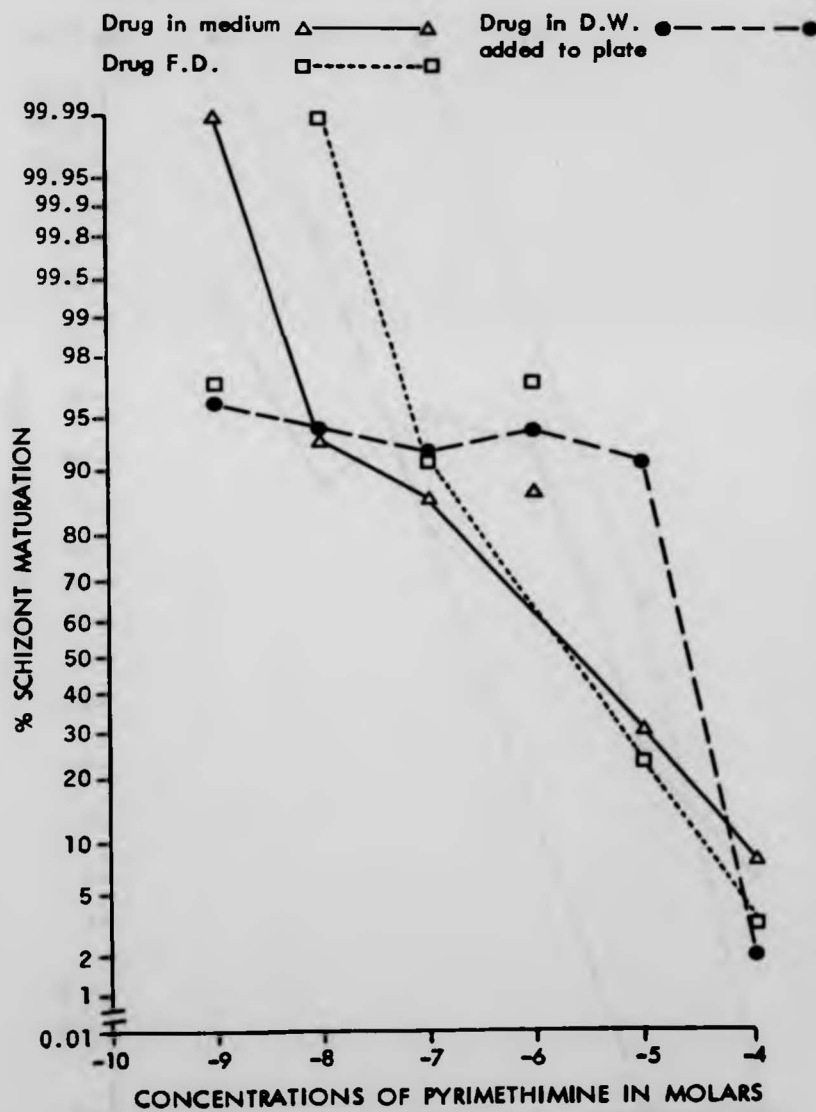
CASE 53

Probit scale

Drug in medium △————△    Drug in D.W. ●—— — ●
Drug F.D.        □--------□    added to plate
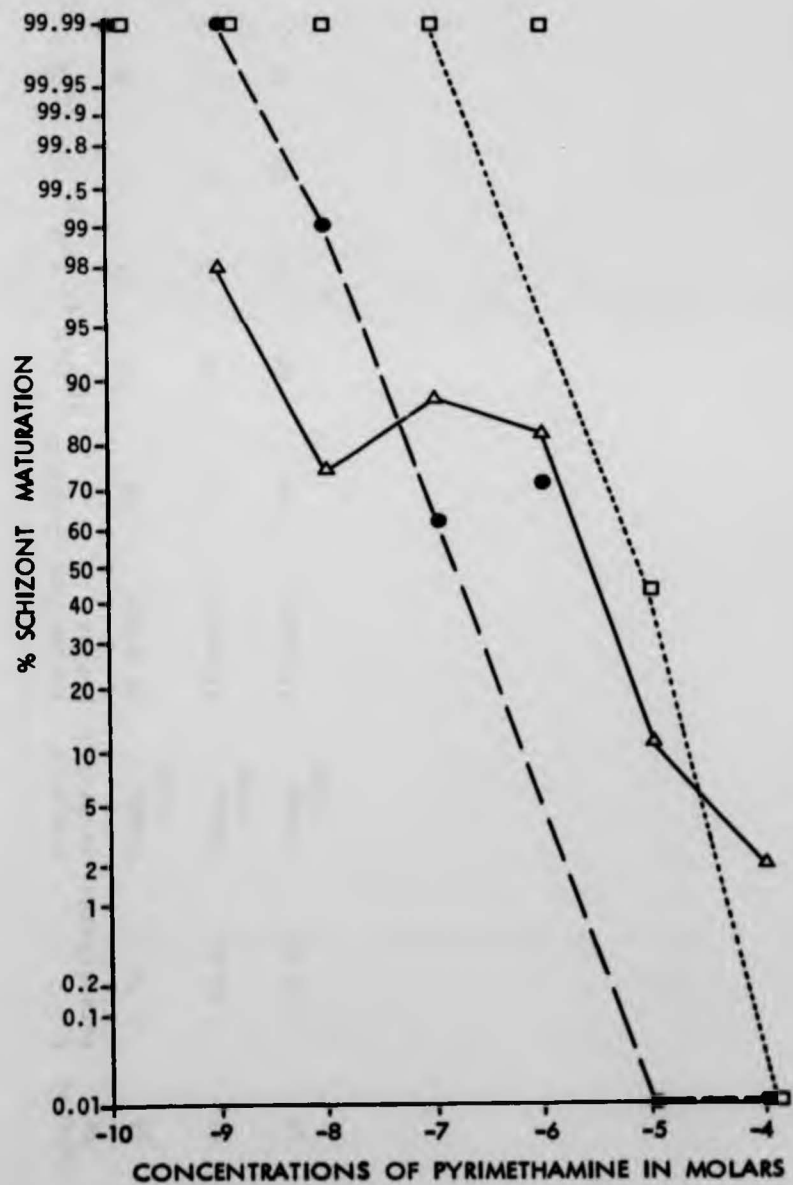
Table 15: Percentage schizont maturation of <u>P. coatneyi</u> under the effect of chloroquine phosphate comparing different levels of parasitaemia (200 asexual parasites counted)

| Culture No. | % Parasitaemia | Stage of parasites | Incubation period | Control | $10^{-7.6}$ | $10^{-7.0}$ | $10^{-6.4}$ | $10^{-5.8}$ | $10^{-5.2}$ | $ED_{50}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 54 | 1% | Young rings | 24 hours | 56 | 23 | 21 | 15 | 20 | 16 | $2.0 \times 10^{-8}$ |
| 57 | 10.4% | Young rings | 48 hours | 72 | 18 | 15 | 10 | 13 | 0 | $8.6 \times 10^{-10}$ |
| 58 | 13.4% | Young rings | 48 hours | 64 | 60 | 56 | 60 | 61 | 0 | $2.4 \times 10^{-6}$ |

Fig. 35

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

Probit scale

Pyrimethamine in D.W.

Case 54 O————————O    Case 57 ●— — — — — —●
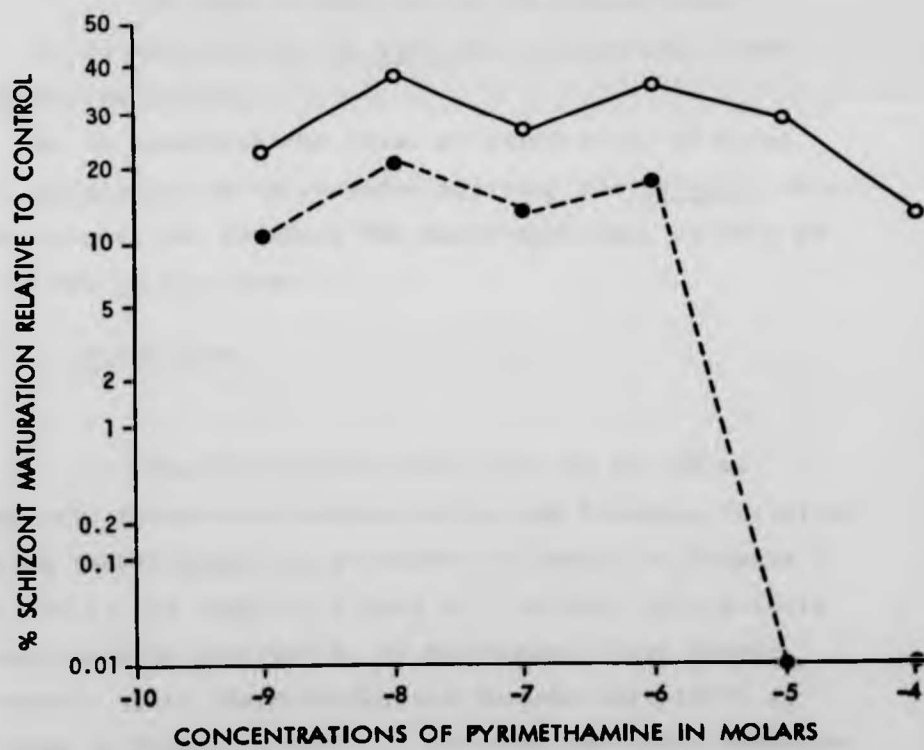
Parasites obtained from Aotus monkeys were always asynchronous and with high parasitaemia, and this was why none of them had grown beyond the trophozoite stages in the micro-test plates.

## 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 epi-
demics 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 Irri-
gated 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 insecti-
cide 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 out-patient 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 con-sidered 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 asso-ciated 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 sub-
jects 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 chloro-
form 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, 0.25ml 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:

| Molars | p-mol/ml | p-mol/well (50 µl |
|--------|----------|-------------------|
| $10^{-7.7}$ | 20 | 1 |
| $10^{-7.4}$ | 40 | 2 |
| $10^{-7.1}$ | 80 | 4 |
| $10^{-7}$ | 100 | 5 |
| $10^{-6.9}$ | 120 | 6 |
| $10^{-6.8}$ | 160 | 8 |
| $10^{-6.6}$ | 240 | 12 |
| $10^{-6.5}$ | 320 | 16 |
| $10^{-6.2}$ | 640 | 32 |

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 imme-
diately transferred into a sterile 25 ml Erlenmeyer
flask (stoppered) containing glass beads, and defibri-
nated by rotating the flask for 5 minutes.

Blood samples collected from a finger prick,
using a 100 µl Eppendorf pipette with a sterile tip con-
taining 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 desic-
cator 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 ($3\%$ $CO_2$ and $16\%$ $O_2$). 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 tem-
peratures between $37^{\circ}$ and $39^{\circ}C$ 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.

Presentation of the in vitro micro - test in culture of
P. falciparum - Sunnar 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
$38^{\circ}C \pm 0.5^{\circ}C$ 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 re-
suspend 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 shizonts 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 )
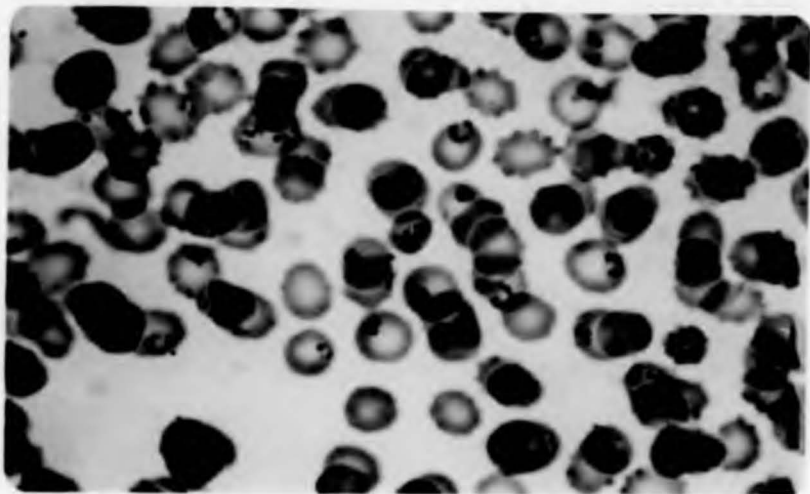


Fig. 38

A thick - blood film prepared from a culture plate after 24 hours of incubation showing maturation of P. falciparum to shizonts in an in vitro micro - test. ( x 1250 )
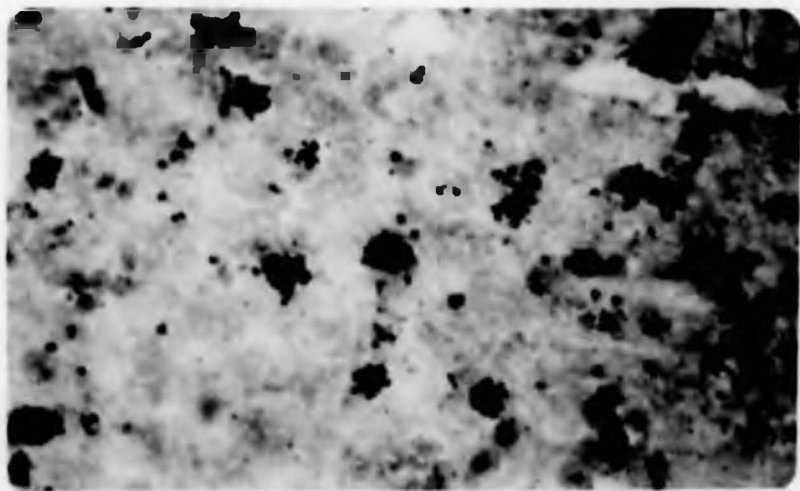
Fig. 39

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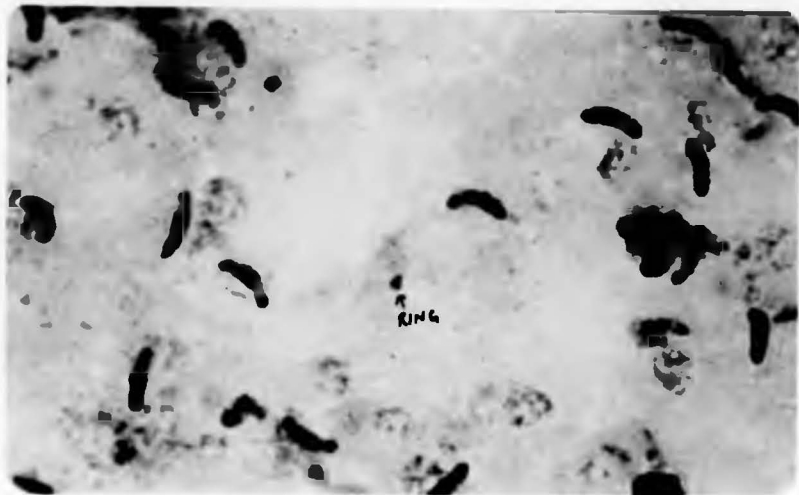


Fig. 40

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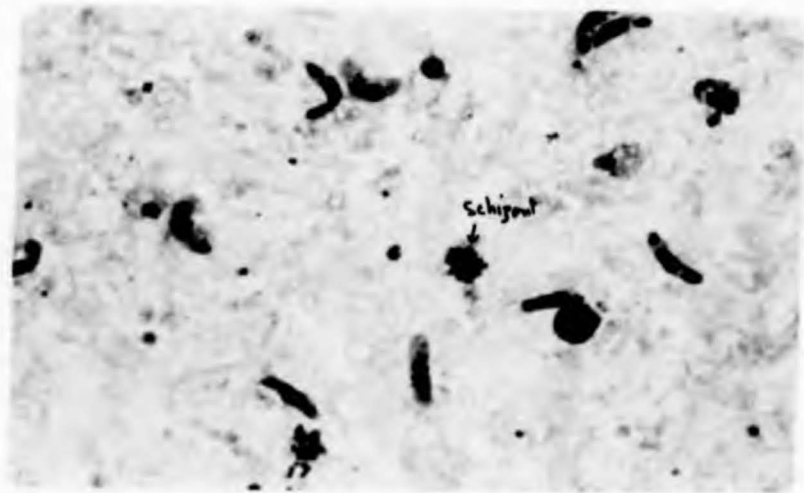
Fig. 39

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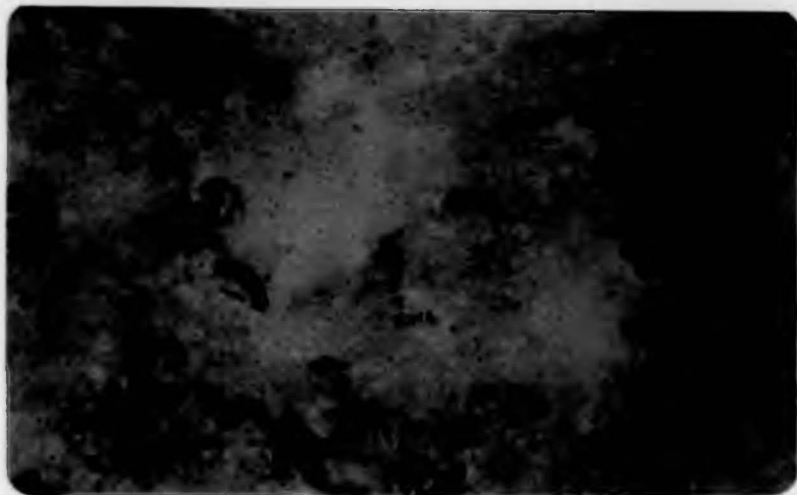


Fig. 40

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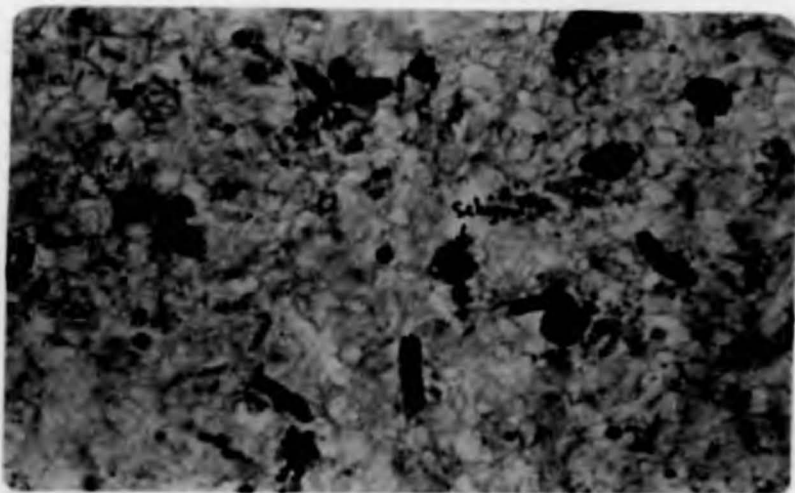
recorded.    The data for control growth were taken from the average of readings from the two control wells Nos. 2 and 3.    The percentage of maturation to schizonts in test wells relative to control samples was calculated by dividing the observed number of schizonts in each test well by the mean number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

till day 28. 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
$CO_2$, and it was found that after 4 to 6 hours after
start of culture the amount of $CO_2$ 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.

| Subject | Age in years | Sex | Parasites per µl | Result of urine test | Morphology of parasite |
|---------|--------------|-----|------------------|----------------------|------------------------|
| 1 | 11 | M | 72,500 | -ve | young rings |
| 2 | 9 | F | 38,250 | -ve | young rings |
| 3 | 14 | F | 21,400 | -ve | fleshy rings |
| 4 | 11 | F | 39,750 | -ve | young rings |
| 5 | 22 | F | 11,250 & 53,125G | -ve | fleshy rings and gametocytes |
| 6 | 15 | M | 77,700 | -ve | fleshy rings |
| 7 | 14 | F | 58,150 | -ve | young rings |
| 8 | 13 | M | 106,500 | -ve | young rings |
| 9 | 22 | F | 30,300 | +ve(trace) | fleshy rings |
| 10 | 12 | M | 33,950 | -ve | fleshy rings |
| 11 | 10 | M | 311,250 | -ve | fleshy rings; |
| 12 | 9 | M | 12,175 | -ve | young and fleshy rings |
| 13 | 14 | F | 22,650 | -ve | fleshy rings |
| 14 | 38 | F | 33,950 | -ve | fleshy rings; |
| 15 | 14 | F | 6,900 | -ve | fleshy rings |
| 16 | 42 | F | 14,675 | -ve | fleshy rings |
| 17 | 32 | F | 33,125 | -ve | young rings |
| 18 | 14 | M | 43,450 | -ve | young rings |
| 19 | 4 | M | 72,400 | -ve | young rings |
| 20 | 27 | M | 15,975 | -ve | young rings |
| 21 | 3 | F | 9,650 | -ve | young rings |
| 22 | 14 | M | 3,800 | +ve(trace) | young rings |
| 23 | 12 | F | 5,450 | -ve | young rings |
| 24 | 28 | F | 19,650 | -ve | young rings |
| 25 | 7 | F | 34,650 | -ve | fleshy rings |
| 26 | 17 | M | 6,450 | +ve(trace) | fleshy rings |
| 27 | 13 | M | 118,000 | -ve | young rings |
| 28 | 38 | M | 43,650 | -ve | young rings |
| 29 | 42 | M | 10,450 | -ve | young rings |
| 30 | 27 | M | 49,725 | -ve | fleshy rings |

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 con-
trols except in Subject 23 which showed only 46% matu-
ration 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 cul-
tured from a patient with parasitaemias of 311,250 per μl.

As can be seen from Table 17, 10 of the tests
had an $ED_{50}$ 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/μl
gave an $ED_{50}$ 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.

| Subject No. | Parasites per µl | Mean of Controls $K_1+K_2/2$ | % maturation of schizonts in 200 parasites p-mols of chloroquine/well | | | | | | | | | $ED_{50}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 4 | 5 | 6 | 8 | 12 | 16 | 32 | |
| 5 | 11,250 | 100 | 100 | 100 | 70 | 36 | 17 | 8 | 55 | 3 | 0 | 5.1 |
| 10 | 33,950 | 100 | 87 | 76 | 68 | 53 | 42 | 36 | 21 | 0 | 0 | 4.7 |
| 11 | 311,250 | 86 | 92 | 85 | 78 | 87 | 72 | 58 | 22 | 2 | 0 | 7.2 |
| 12 | 12,175 | 100 | 74 | 63 | 58 | 45 | 41 | 37 | 18 | 0 | 0 | 3.6 |
| 14 | 33,950 | 100 | 92 | 74 | 51 | 40 | 52 | 43 | 18 | 0 | 0 | 4.5 |
| 15 | 6,900 | 100 | 84 | 79 | 62 | 48 | 31 | 25 | 5 | 0 | 0 | 3.9 |
| 16 | 14,675 | 90 | 100 | 79 | 62 | 47 | 28 | 19 | 3 | 0 | 0 | 4.2 |
| 18 | 43,450 | 99 | 100 | 79 | 63 | 46 | 21 | 8 | 0 | 0 | 0 | 4.0 |
| 20 | 15,975 | 100 | 87 | 80 | 62 | 49 | 37 | 24 | 13 | 2 | 0 | 4.2 |
| 23 | 5,450 | 46 | 42 | 37 | 25 | 12 | 0 | 0 | 0 | 0 | 0 | 3.3 |
| 30 | 49,725 | 75 | 85 | 81 | 60 | 25 | 0 | 0 | 0 | 0 | 0 | 3.2 |

Table 18: Sensitivity of <u>P. falciparum</u> to chloroquine detected by a micro <u>in vitro</u> test. $ED_{50}$, $ED_{90}$ and $x^2$ calculated using a programme adapted to a Packard HP-33E calculator. Table showing method of calculations.

| Case No. | Doses | 1 p-mol /well | 2 p-mol | 4 p mol | 5 p mol | 6 p mol | 8 p mol | 12 p mol | $ED_{50}$ | $ED_{90}$ | Slope | $x^2$ | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | x log doses | 0.00 | 0.301 | 0.602 | 0.699 | 0.778 | 0.903 | 1.079 |  |  |  |  |  |
|  | y probit |  |  | +0.52 | -0.36 | -0.95 | -1.41 | +0.13 |  |  |  |  |  |
| 5 | $\hat{y}$ value of |  |  | -0.216 | -0.308 | -0.382 | -0.499 | -0.665 |  |  |  |  |  |
|  | h p² - 5 |  |  | +0.016 | +0.016 | +0.017 | +0.017 | +0.019 | 2.335 | 54.076 | -0.9405 | 135.086 | p<0.001 |
|  | y | 1.13 | 0.71 | 0.47 | 0.08 | -0.20 | -0.36 | -0.81 |  |  |  |  |  |
| 10 | $\hat{y}$ | 1.254 | 0.719 | 0.183 | 0.011 | 0.1297 | 0.352 | 0.665 |  |  |  |  |  |
|  | h | 0.028 | 0.019 | 0.016 | 0.016 | 0.016 | 0.017 | 0.019 | 5.071 | 26.595 | -1.7785 | 157.120 | p<0.001 |
|  | y | 1.41 | 1.04 | 0.77 | 1.13 | 0.58 | 0.20 | -0.77 |  |  |  |  |  |
| 11 | $\hat{y}$ | 0.847 | 0.379 | 0.090 | -0.241 | -0.364 | -0.558 | -0.832 |  |  |  |  |  |
|  | h | 0.024 | 0.019 | 0.019 | 0.019 | 0.019 | 0.021 | 0.024 | 9.915 | 57.95 | -1.6693 | 233.89 | p<0.001 |
|  | y | 0.64 | 0.33 | 0.20 | -0.13 | -0.23 | -0.33 | -1.34 |  |  |  |  |  |
| 12 | $\hat{y}$ | 0.847 | 0.379 | 0.090 | -0.241 | -0.364 | -0.558 | -0.0832 |  |  |  |  |  |
|  | h | 0.201 | 0.017 | 0.016 | 0.016 | 0.017 | 0.018 | 0.021 | 3.501 | 23.265 | -1.5562 | 81.041 | p<0.001 |
|  | y | 1.41 | 0.64 | 0.03 | -0.25 | 0.05 | -0.18 | -0.92 |  |  |  |  |  |
| 14 | $\hat{y}$ | 1.319 | 0.736 | 0.152 | -0.036 | -0.189 | -0.431 | -0.772 |  |  |  |  |  |
|  | h | 0.301 | 0.019 | 0.016 | 0.016 | 0.016 | 0.017 | 0.020 | 4.794 | 21.939 | -1.9377 | 12.676 | p<0.05 |
|  | y | 0.99 | 0.81 | 0.31 | -0.05 | -0.05 | -0.67 | -1.64 |  |  |  |  |  |
| 15 | $\hat{y}$ | 1.358 | 0.650 | -0.057 | -0.286 | -0.471 | -0.765 | -1.179 |  |  |  |  |  |
|  | h | 0.031 | 0.019 | 0.016 | 0.016 | 0.017 | 0.020 | 0.020 | 3.781 | 13.242 | -2.351 | 26.290 | p<0.001 |

**Table 18 continued**

| Case No. | Doses | 1 p-mol /well | 2 p-mol | 4 p mol | 5 p mol | 6 p mol | 8 p mol | 12 p mol | $ED_{50}$ | $ED_{90}$ | Slope | $\chi^2$ | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | y | | 0.81 | 0.31 | -0.08 | -0.58 | -0.88 | | | | | | |
| 16 | ŷ | | 0.941 | 0.073 | -0.206 | -0.434 | -0.794 | | | | | | |
| | h | | 0.024 | 0.018 | 0.018 | 0.019 | 0.022 | | 4.241 | 11.793 | -2.8817 | 6.176 | $p>0.25$ |
| | y | | 0.81 | 0.33 | -0.10 | -0.81 | -1.41 | | | | | | |
| 18 | ŷ | | 1.065 | 0.036 | -0.391 | -0.680 | -1.137 | | | | | | |
| | h | | 0.024 | 0.016 | 0.017 | -0.019 | 0.026 | | 3.909 | 8.750 | -3.6581 | 15.07 | $p<0.025$ |
| | y | 1.13 | 0.84 | 0.31 | -0.03 | -0.33 | -0.71 | -1.13 | | | | | |
| 20 | ŷ | 1.359 | 0.708 | 0.057 | -0.153 | -0.324 | -0.594 | -0.975 | | | | | |
| | h | 0.071 | 0.019 | 0.016 | 0.016 | 0.017 | 0.018 | 0.023 | 4.250 | 16.604 | -2.163 | 8.396 | $p>0.10$ |

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 chloro-
quine 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 x—·—·—·—x    Case No 10 ●————————●
Case No. 11 o············o    Case No. 12 △— — — —△
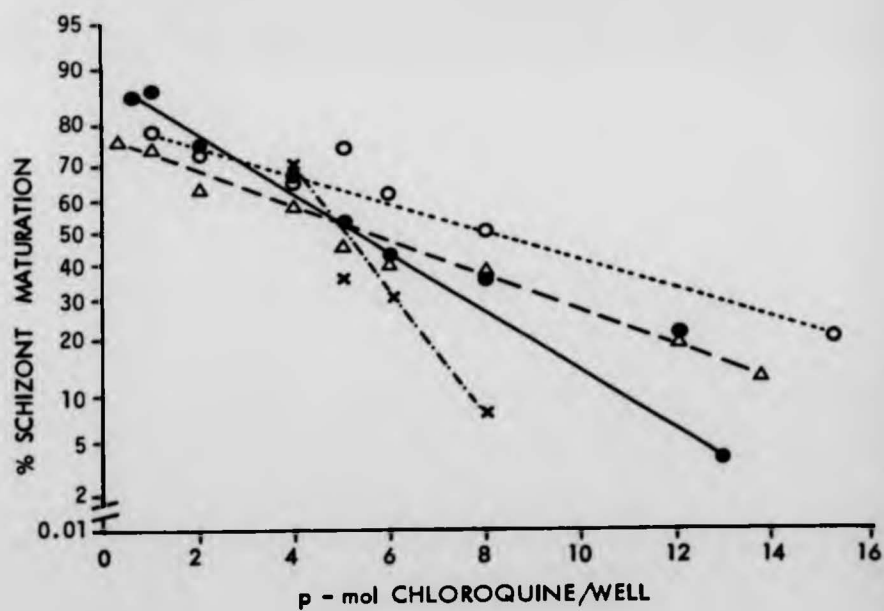
Fig. 42

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

Probit scale

Case No. 14 ●———————●     Case No. 15 ✗—·—·—·—·—✗
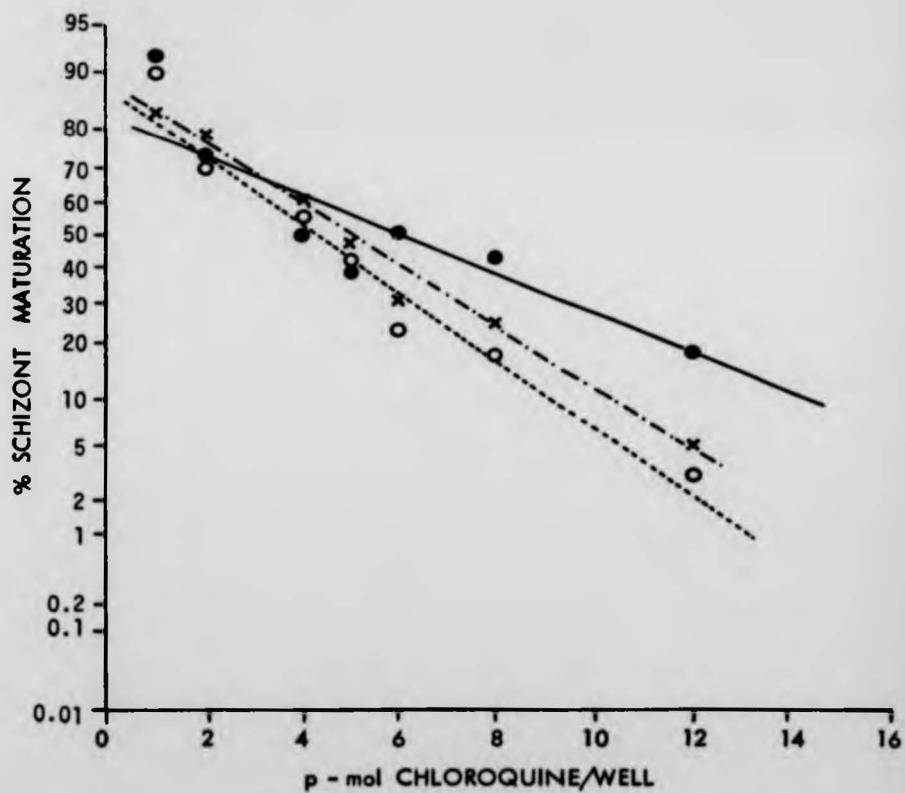
Case No. 16 ○----------○

**Fig. 43**

Dose—related inhibition of <u>P. falciparum</u> schizont maturation
in the <u>in vitro</u> micro - test. (Sennar area)

Probit scale

Case No. 18 ●————●     Case No. 20 ○— — — —○

Case No. 23 △—·— —·—·—△     Case No. 30 ✕----------✕
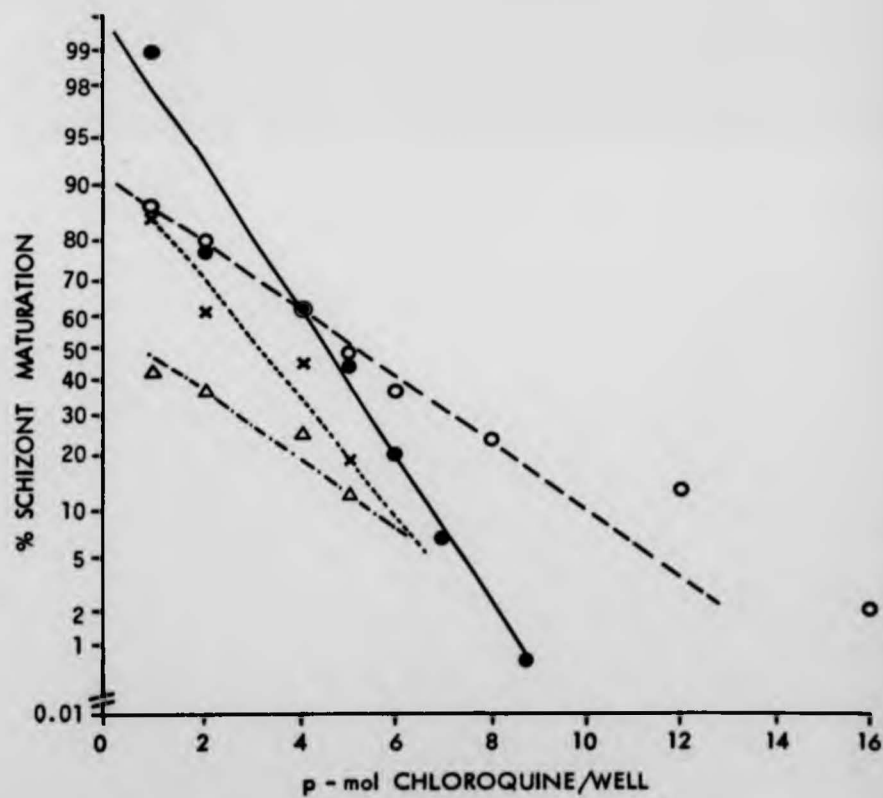
Table 19: Results of in vivo tests: 7-day test and those extended to 28 day test - Sennar Area, Sudan.

| Subject No. | Day 0 | Day 1 | Counts on 7-day test | | | | | | Counts for 28 days | | |
| | | | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | 2nd week | 3rd week | 4th week |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 38,250 | 40,575 | 350 | 25 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 7 | 58,150 | 3,750 | 150 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 9 | 30,300 | 2,725 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 11 | 311,250 | 38,200 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 12 | 12,175 | 725 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 13 | 22,650 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 14 | 33,950 | 7,225 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 15 | 6,900 | 250 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 16 | 14,675 | 675 | 50 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 18 | 43,450 | 1,150 | 75 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 2,175 |
| 20 | 15,975 | 1,725 | 75 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 465 |
| 22 | 3,800 | 100 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 23 | 5,450 | 875 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 24 | 19,650 | 3,950 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 27 | 118,000 | 325 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 780 |
| 29 | 10,450 | 575 | 75 | 25 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 30 | 49,725 | 5,325 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 375 | 00 |

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.

| Subject No. | Day 0 | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | 2nd week | 3rd week | 4th week |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Counts on 7-day test | | | | | | Counts for 28 days | | |
| 2 | 38,250 | 40,575 | 350 | 25 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 7 | 58,150 | 3,750 | 150 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 9 | 30,300 | 2,725 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 11 | 311,250 | 38,200 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 12 | 12,175 | 725 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 13 | 22,650 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 14 | 33,950 | 7,225 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 15 | 6,900 | 250 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 16 | 14,675 | 675 | 50 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 18 | 43,450 | 1,150 | 75 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 2,175 |
| 20 | 15,975 | 1,725 | 75 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 465 |
| 22 | 3,800 | 100 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 23 | 5,450 | 875 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 24 | 19,650 | 3,950 | 00 | 00 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 27 | 118,000 | 325 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 780 |
| 29 | 10,450 | 575 | 75 | 25 | 00 | 00 | 00 | 00 | N.D. | N.D. | N.D. |
| 30 | 49,725 | 5,325 | 00 | 00 | 00 | 00 | 00 | 00 | 00 | 375 | 00 |

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 cul-
turing 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 $NaHCO_3$ 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 $38^{\circ}C \pm 0.5^{\circ}C$ 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 sub-
jects who were followed for 28 days might indicate the
presence of resistance at the RI level. Since trans-
mission 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

RADIOCATIVE ASSAY FOR DETERMINATION OF CHLOROQUINE
ADSORPTION AND/OR DEGRADATION WHILE STORED AT $4^\circ$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 pre-
pared in RPMI 1640 culture medium at different concen-
trations and stored in glass and plastic tubes at $4^\circ$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 pre-
paration.

Radioactive chloroquine (CQ$^{14}$C) was used to
study what happens to chloroquine in medium RPMI 1640
stored in glass and plastic tubes at $4^\circ$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 specifi-
city 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 dif-
ferent antimalarial drugs.    Josephson et al. (1947)
used the ultra-violet spectrophotometry for the esti-
mation of basic organic compounds in biological material.

McChesney et al. (1961, 1962, 1965, 1967) carried
out laboratory studies on biological characteristics
and plasma levels of chloroquine and hydroxychloroquine and
their metabolism in man during and after repeated oral
dosage.    Fitch (1969) used radioactive chloroquine $^{14}$C
to study the processes which concentrate chloroquine in
mouse red blood cells infected with chloroquine-sensitive
or with chloroquine-resistant P. berghei.    Fitch (1970,
1972, 1973, 1977a, 1977b), Fitch et al. (1974a, 1974b,
1974c, 1975) studied different aspects of resistance,
chloroquine binding capacity, cross-resistance patterns
and accumulation of drug using radioactive chloroquine $^{14}$C.

## 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 Spectometer 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 µl of radioactive chloroquine phosphate $CQ-^{14}C$ (30 µCi/µmole;   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 $10^{-6.3}$   $10^{-6.6}$   $10^{-6.9}$ $10^{-72.}$ $10^{-7.5}$ $10^{-7.8}$
                                        12 ml   12 ml   12 ml   12 ml

                        12 ml RPMI   12 ml   12 ml   12 ml   12 ml

                        G2        P2   G3   P3   G4   P4   G5   P5   G6   P6
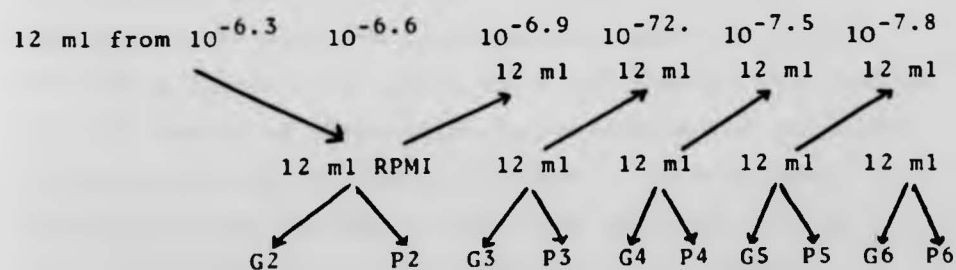
Prepare normal chloroquine phosphate solution by
weighing accurately 30 mg of the powder drug and dissolve
in 30 ml of D.W. to give cold chloroquine 1 mg/ml.

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 <u>Extraction of CQ$^{14}$C</u>

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 spectometer (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 spectometer.

## 5.4 RESULTS

Radioactivity of chloroquine was measured using a liquid scintillation spectometer and each vial was left for 20 minutes. The disassociation of $CQ^{14}C$ was recorded from the readings of the two spectometer 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 $^{14}$C 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 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 $4^{o}$C

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

Fig. 44

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

Experiment one

●- - -● Plastic tubes   ○———○ Glass tubes
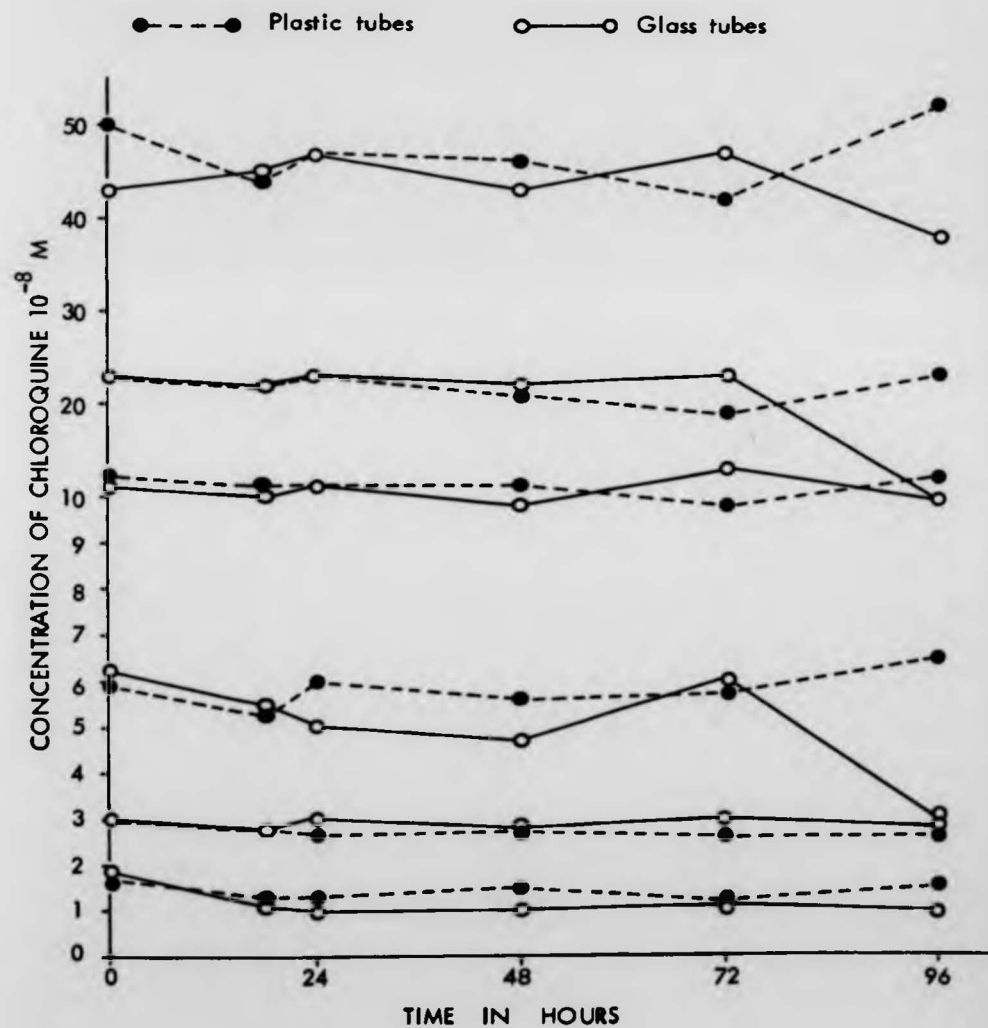
Table 21  SECOND EXPERIMENT:  Amount of chloroquine extracted from different concentrations of the drug prepared in plastic and glass tubes and stored at $4^{o}C$

| Time in hours between preparation & extraction | Plastic tubes Chloroquine concentrations x $10^{-7}$ M | | | | | | Glass tubes Chloroquine concentrations x $10^{-7}$ M | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 2.5 | 1.25 | 0.625 | 0.31 | 0.15 | 5 | 2.5 | 1.25 | 0.625 | 0.31 | 0.15 |
| 0 | 4.8 | 2.3 | 1.2 | 0.57 | 0.26 | 0.18 | 4.6 | 2.6 | 0.9 | 0.57 | 0.27 | 0.08 |
| 18 | 4.1 | 1.3 | 1.1 | 0.49 | 0.27 | 0.12 | 4.6 | 2.1 | 1.0 | 0.37 | 0.17 | 0.07 |
| 24 | 3.7 | 2.2 | 1.0 | 0.58 | 0.32 | 0.12 | 4.7 | 2.4 | 1.1 | 0.49 | 0.33 | 0.16 |
| 48 | 3.8 | 2.0 | 1.1 | 0.49 | 0.23 | 0.13 | 4.5 | 2.2 | 1.1 | 0.51 | 0.29 | 0.13 |
| 72 | 4.4 | 1.8 | 0.9 | 0.54 | 0.25 | 0.13 | 4.3 | 2.3 | 1.3 | 0.60 | 0.31 | 0.12 |
| 96 | 4.9 | 1.8 | 0.9 | 0.54 | 0.24 | 0.09 | 4.3 | 1.1 | 1.0 | 0.31 | 0.31 | 0.11 |

Table 22   THIRD EXPERIMENT:   Amount of chloroquine extracted from different concentrations of the drug prepared in plastic and glass tubes and stored at $4^{o}C$

| Time in hours between preparation & extraction | Plastic tubes Chloroquine concentrations x $10^{-7}$ M | | | | | | Glass tubes Chloroquine concentrations x $10^{-7}$ M | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 2.5 | 1.25 | 0.625 | 0.31 | 0.15 | 5 | 2.5 | 1.25 | 0.625 | 0.31 | 0.15 |
| 0 | 4.7 | 2.5 | 1.1 | 0.53 | 0.20 | 0.07 | 4.5 | 2.16 | 1.07 | 0.55 | 0.24 | 0.05 |
| 18 | 4.6 | 2.2 | 1.1 | 0.51 | 0.20 | 0.06 | 4.5 | 2.27 | 1.09 | 0.48 | 0.18 | 0.04 |
| 24 | 4.2 | 2.1 | 0.96 | 0.47 | 0.21 | 0.15 | 4.5 | 2.22 | 0.96 | 0.47 | 0.16 | 0.03 |
| 48 | 4.8 | 2.3 | 1.2 | 0.63 | 0.34 | 0.17 | 4.7 | 2.30 | 1.20 | 0.66 | 0.31 | 0.15 |
| 72 | 4.5 | 2.2 | 0.87 | 0.34 | 0.11 | 0.04 | 4.0 | 1.89 | 0.87 | 0.32 | 0.11 | 0.06 |
| 96 | 4.2 | 2.0 | 0.76 | 0.26 | 0.12 | 0.04 | 4.9 | 2.27 | 0.98 | 0.33 | 0.11 | 0.08 |

Fig. 45

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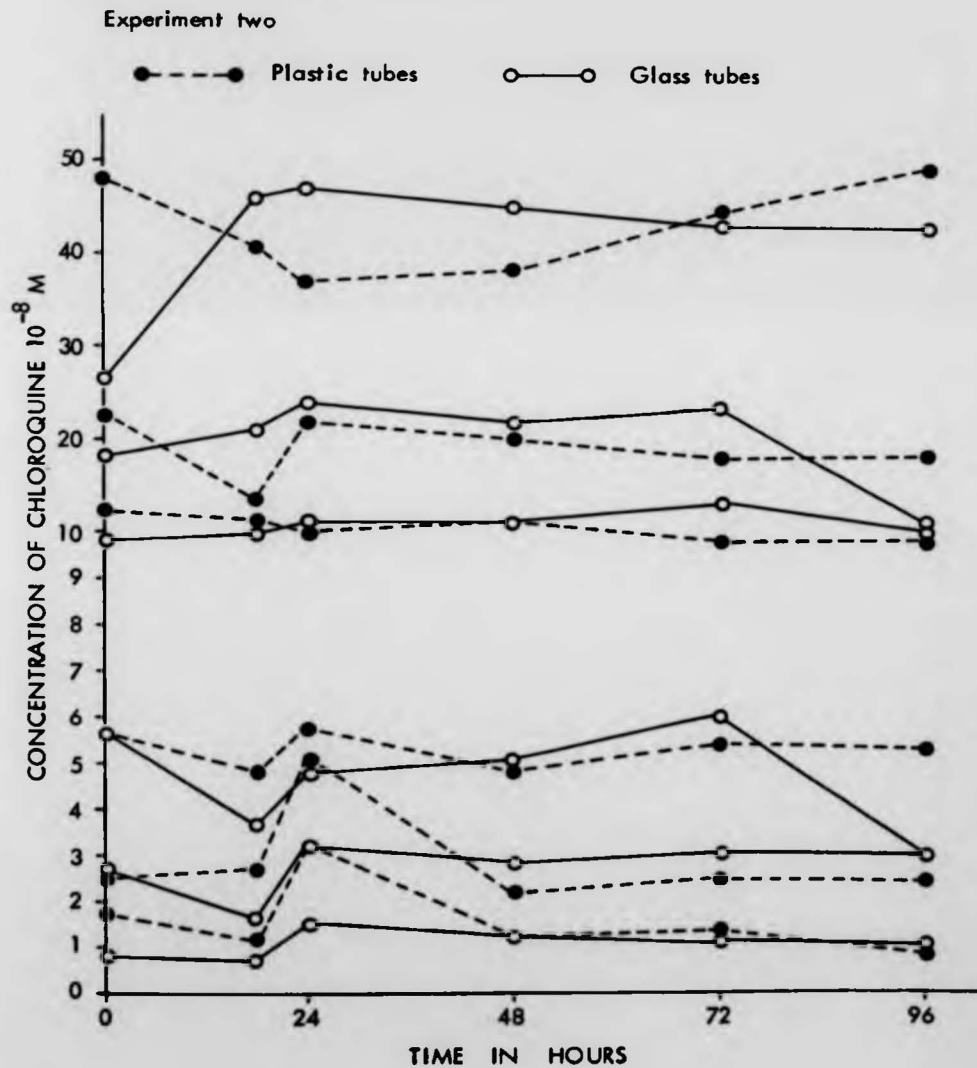
Experiment two

Fig. 46

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

Experiment three
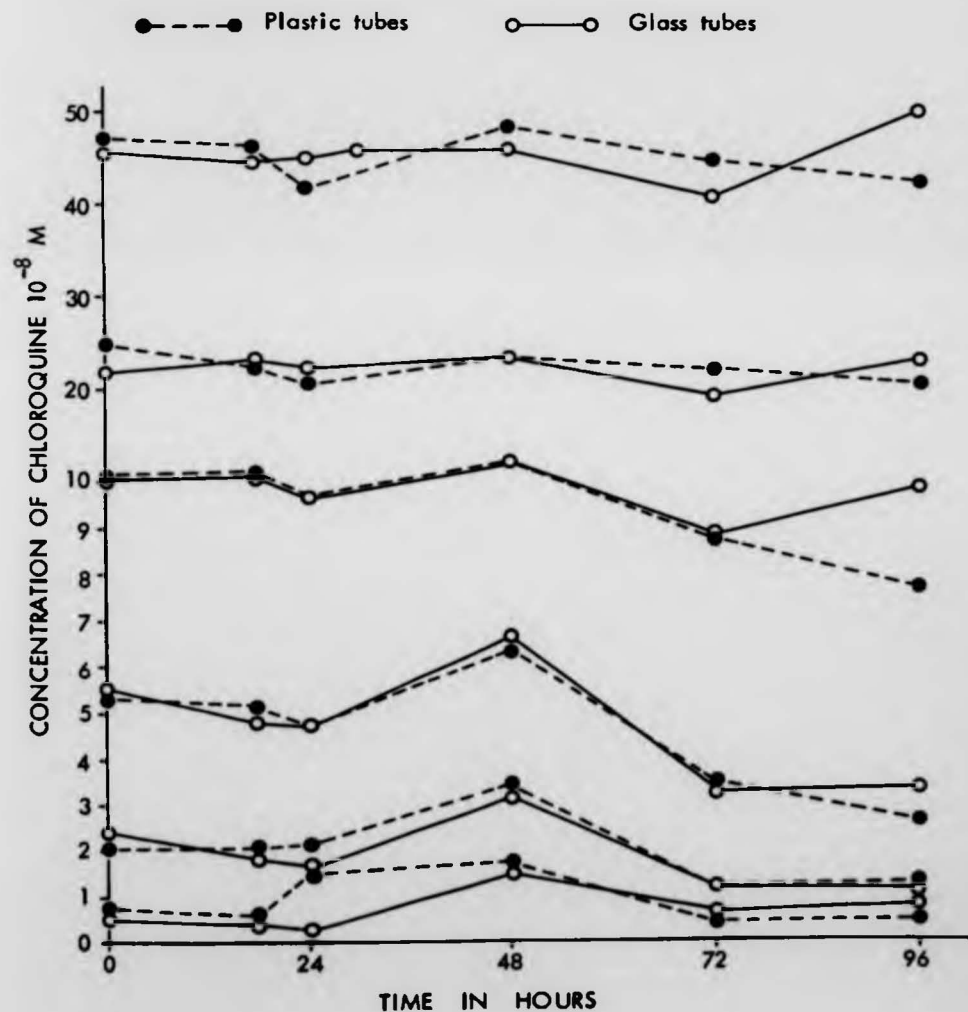
Table 23

Extraction of chloroquine using O.2M borate to remove
chloroquine metabolites from different concentrations
of chloroquine prepared in plastic and glass tubes and
stored at 4°C

| Time in hours between preparation & extraction | Plastic tubes Chloroquine concentrations x $10^{-7}$ M | | | | | | Glass tubes Chloroquine concentrations x $10^{-7}$ M | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 2.5 | 1.25 | 0.625 | 0.31 | 0.15 | 5 | 2.5 | 1.25 | 0.625 | 0.31 | 0.15 |
| 0 | 4.0 | 2.0 | 0.9 | 0.55 | 0.30 | 0.10 | 3.0 | 1.9 | 0.9 | 0.55 | 0.30 | 0.12 |
| 18 | 3.5 | 1.8 | 0.8 | 0.50 | 0.20 | 0.09 | 2.4 | 1.5 | 0.7 | 0.40 | 0.20 | 0.10 |
| 24 | 3.4 | 1.8 | 0.8 | 0.40 | 0.20 | 0.08 | 2.5 | 1.5 | 0.6 | 0.30 | 0.10 | 0.09 |
| 48 | 3.2 | 1.7 | 0.9 | 0.40 | 0.20 | 0.09 | 2.9 | 1.5 | 0.7 | 0.40 | 0.20 | 0.10 |
| 72 | 3.2 | 1.7 | 0.9 | 0.50 | 0.20 | 0.09 | 3.0 | 1.6 | 0.8 | 0.40 | 0.20 | 0.10 |
| 96 | 3.4 | 1.7 | 0.9 | 0.50 | 0.30 | 0.08 | 3.1 | 1.5 | 0.8 | 0.40 | 0.30 | 0.13 |
| 120 | 3.5 | 1.9 | 0.9 | 0.50 | 0.30 | 0.08 | 2.9 | 1.4 | 0.9 | 0.40 | 0.30 | 0.12 |
| 144 | 3.3 | 1.7 | 0.9 | 0.50 | 0.30 | 0.08 | 3.0 | 1.5 | 0.8 | 0.40 | 0.30 | 0.12 |
| 168 | 3.1 | 1.7 | 0.9 | 0.40 | 0.20 | 0.08 | 2.8 | 1.4 | 0.7 | 0.40 | 0.30 | 0.12 |

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.

| Time in hours between preparation & extraction | Plastic tubes Chloroquine concentrations x $10^{-7}$ M | | | | | | | Glass tubes Chloroquine concentrations x $10^{-7}$ M | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 2.5 | 1.25 | 0.625 | 0.312 | 0.15 | Mean | 5 | 2.5 | 1.25 | 0.625 | 0.31 | 0.15 | Mean |
| 0 | 3.2 | 5.5 | 6.9 | 9.3 | 18.3 | 6.7 | 8.3 | 12.3 | 16.5 | 18.1 | 7.2 | 12.9 | 31.1 | 16.4 |
| 18 | 12.8 | 23.7 | 12.0 | 18.4 | 19.4 | 31.1 | 19.6 | 9.6 | 12.4 | 17.6 | 25.3 | 32.3 | 51.1 | 24.7 |
| 24 | 16.3 | 12.5 | 18.4 | 12.0 | 7.5 | 42.2 | 18.2 | 7.5 | 7.7 | 15.7 | 22.1 | 15.1 | 35.6 | 17.3 |
| 48 | 12.1 | 14.5 | 9.3 | 10.0 | 10.7 | 10.0 | 9.5 | 10.3 | 10.7 | 14.7 | 12.5 | 15.4 | 11.1 | 10.8 |
| 72 | 12.8 | 21.7 | 28.8 | 22.7 | 33.3 | 35.6 | 25.8 | 13.1 | 13.5 | 7.5 | 18.9 | 22.6 | 35.6 | 18.5 |
| 96 | 4.4 | 18.4 | 23.7 | 22.7 | 33.3 | 37.8 | 23.4 | 12.8 | 42.0 | 21.1 | 50.4 | 24.7 | 36.0 | 31.2 |
| Mean | 10.3 | 16.1 | 16.5 | 15.9 | 20.4 | 25.6 | 17.5 | 10.9 | 17.1 | 15.8 | 22.7 | 18.8 | 33.4 | 19.8 |

Fig. 47

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

log probit scale
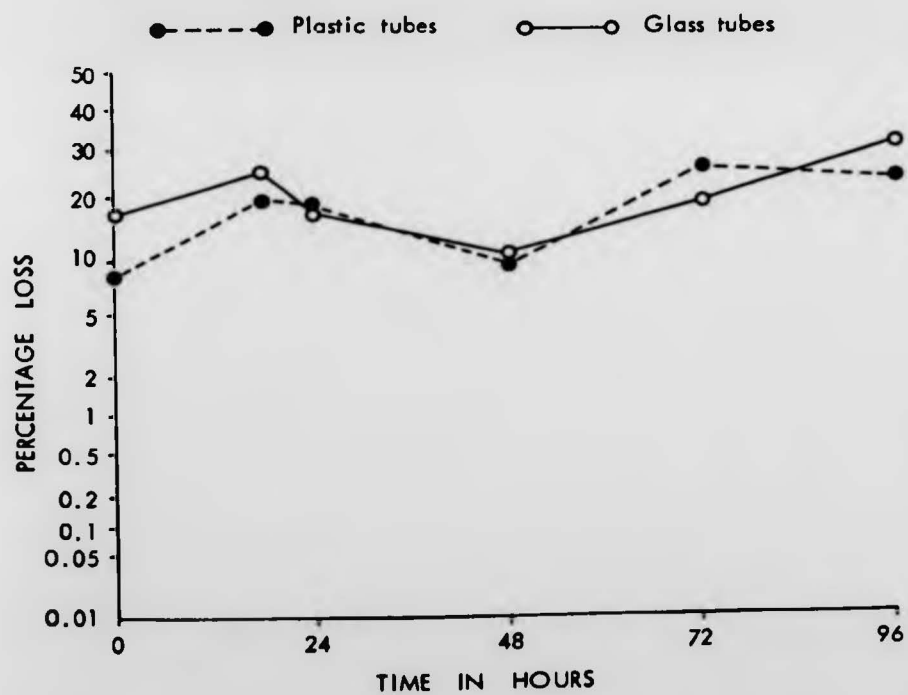
Fig. 48

Percentage loss of chloroquine $^{14}C$ by ~~degradation and/or~~ 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
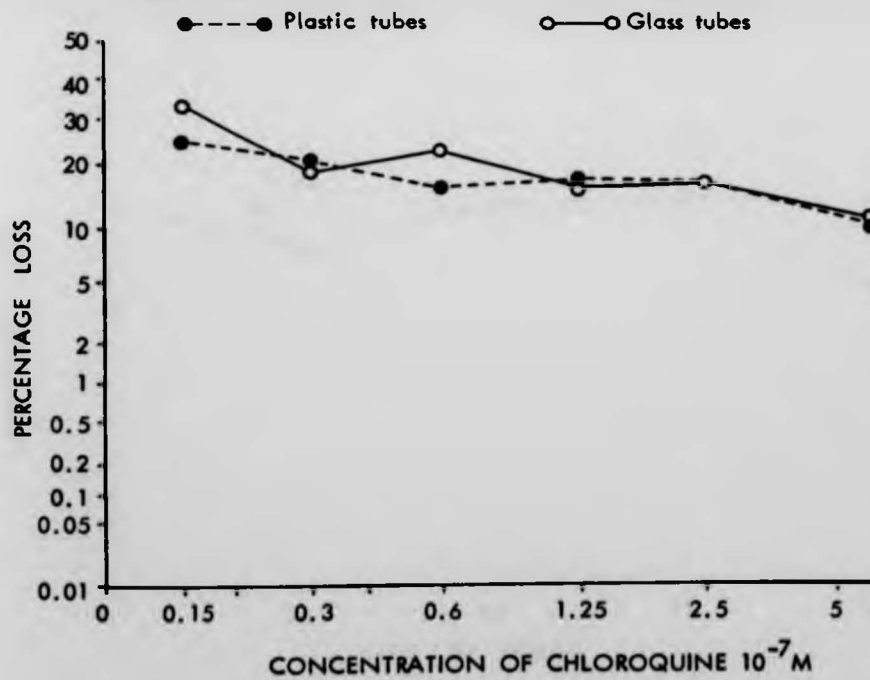
Fig. 49

Percentage loss of chloroquine $^{14}C$ at concentration $5 \times 10^{-7}M$ by adsorption to plastic and glass tubes at different periods.
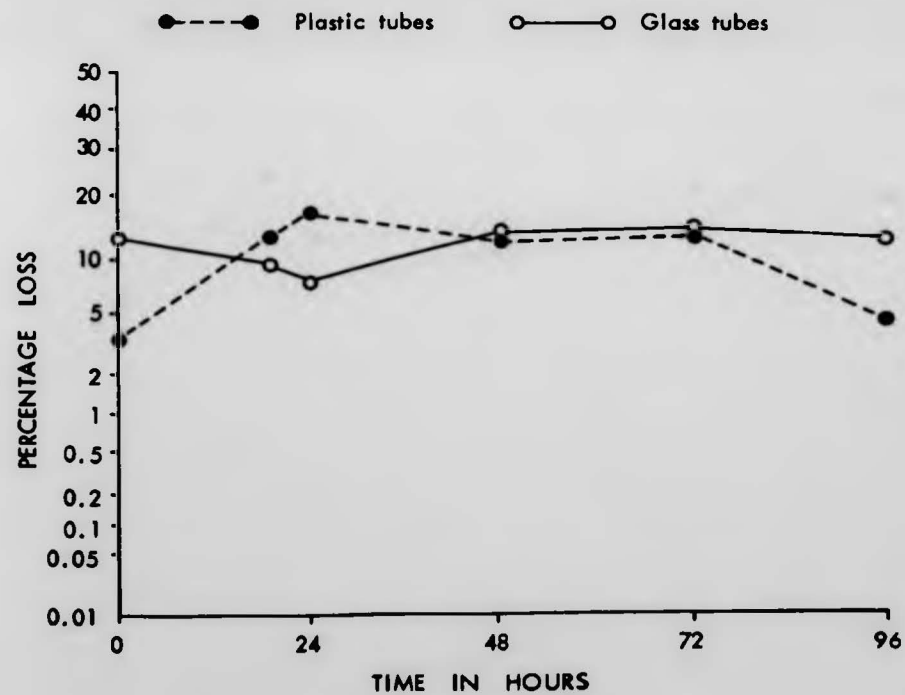
log probit scale

**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     ○——○ Glass tubes
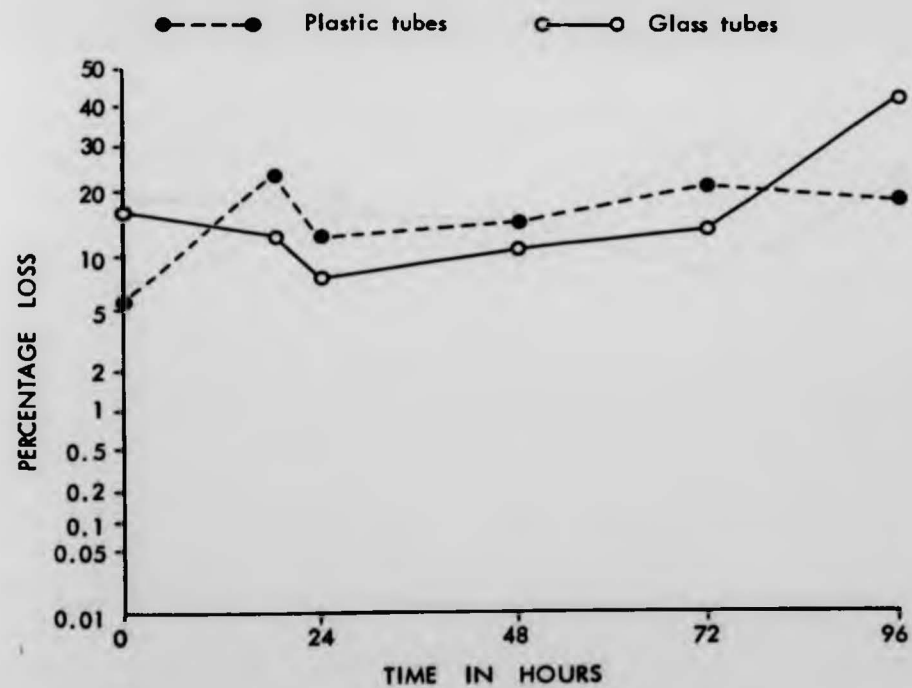
Fig. 51

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.
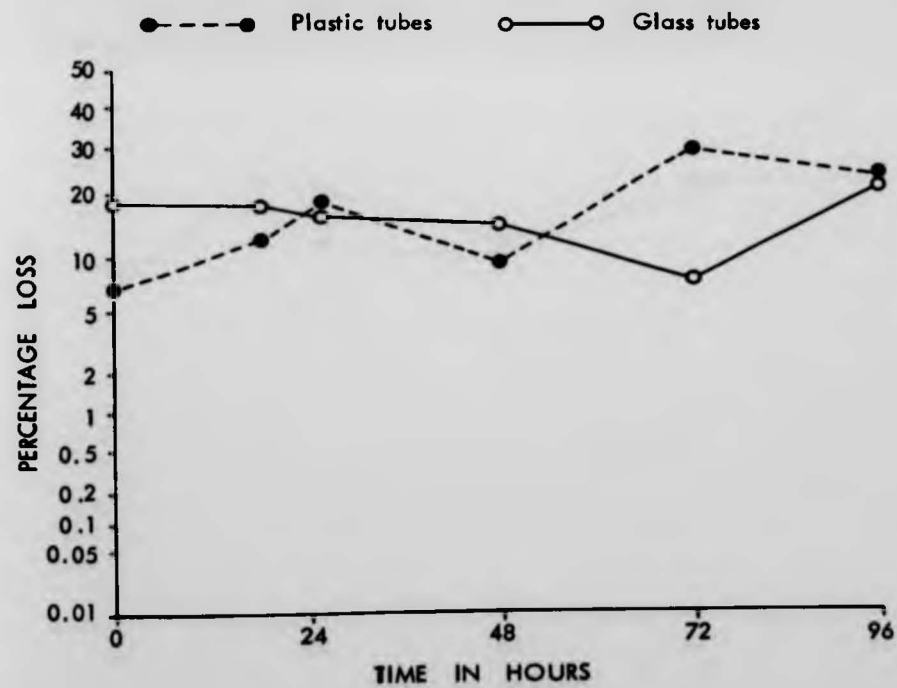
log probit scale

**Fig. 52**

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

log probit scale



● — — ●     **Plastic tubes**       ○——○     **Glass tubes**
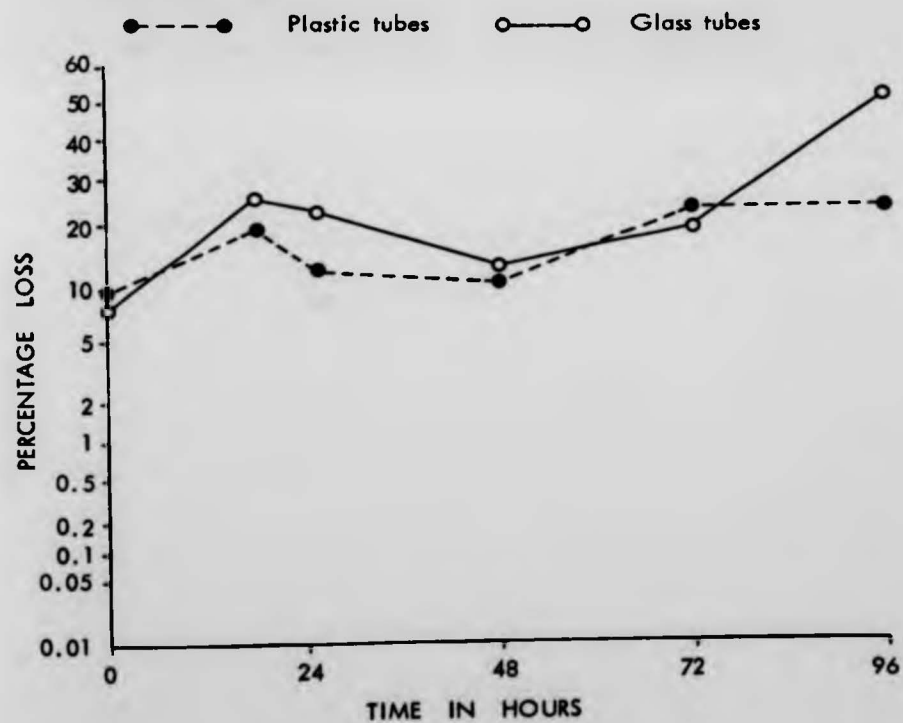
Fig. 53

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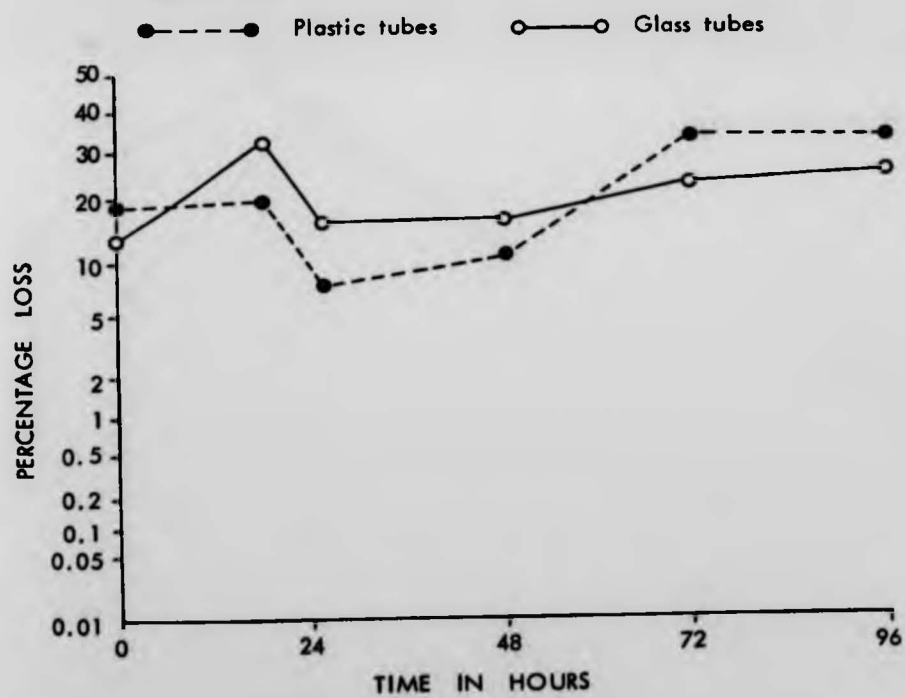
log probit scale

Fig. 54

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

log probit scale



●  — — ●  Plastic tubes        ○ —— ○  Glass tubes

Fig. 53

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

log probit scale

●  –  –  –  ●  Plastic tubes     ○ —— ○  Glass tubes

Fig. 54

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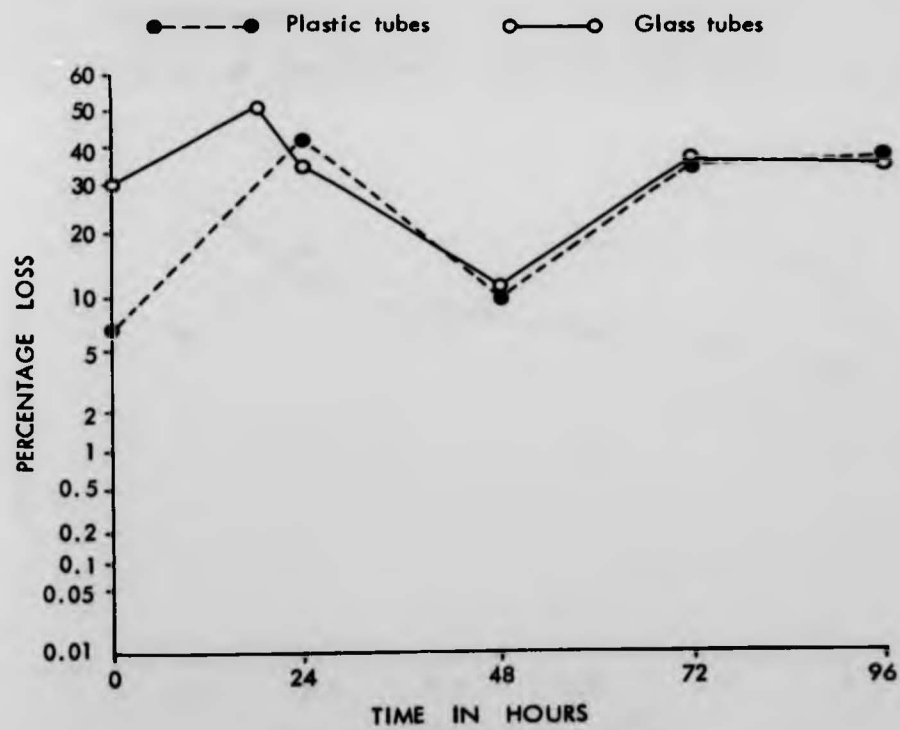
log probit scale

Fig. 55

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

log probit scale
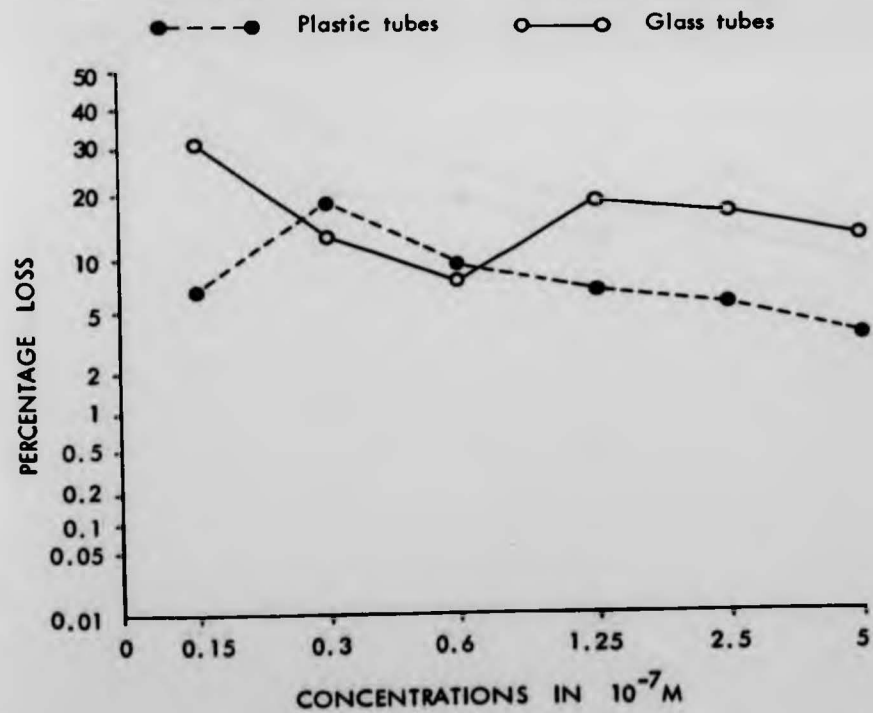
Fig. 56

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

log probit scale
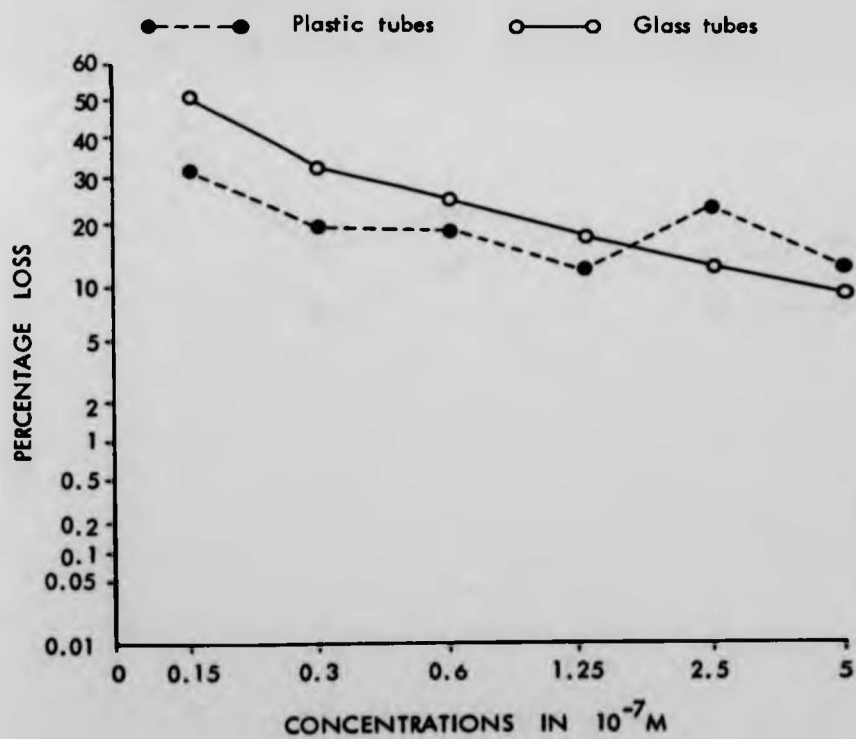
Fig. 57

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

log probit scale
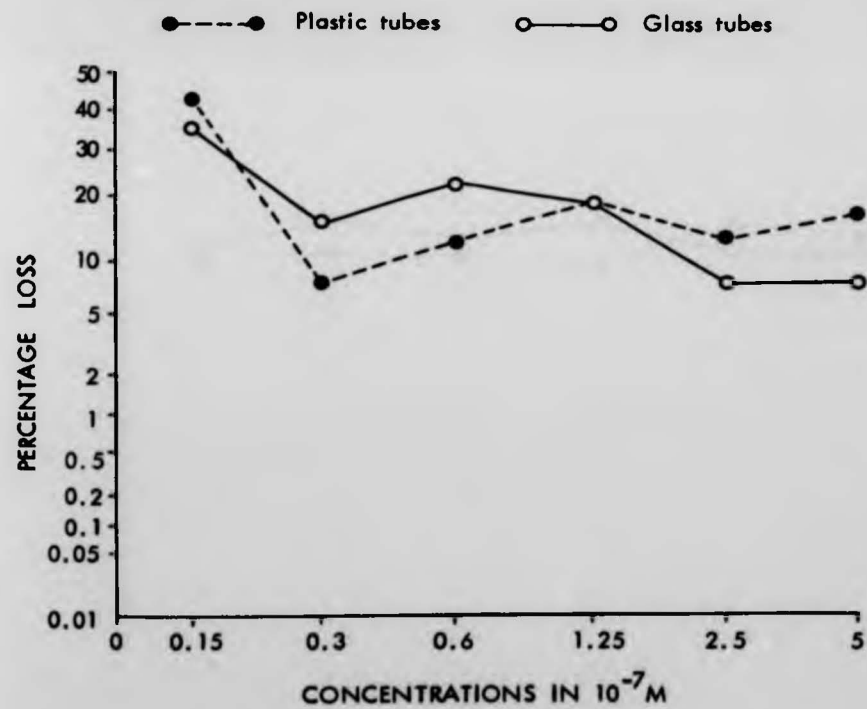
Fig. 58

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

log probit scale
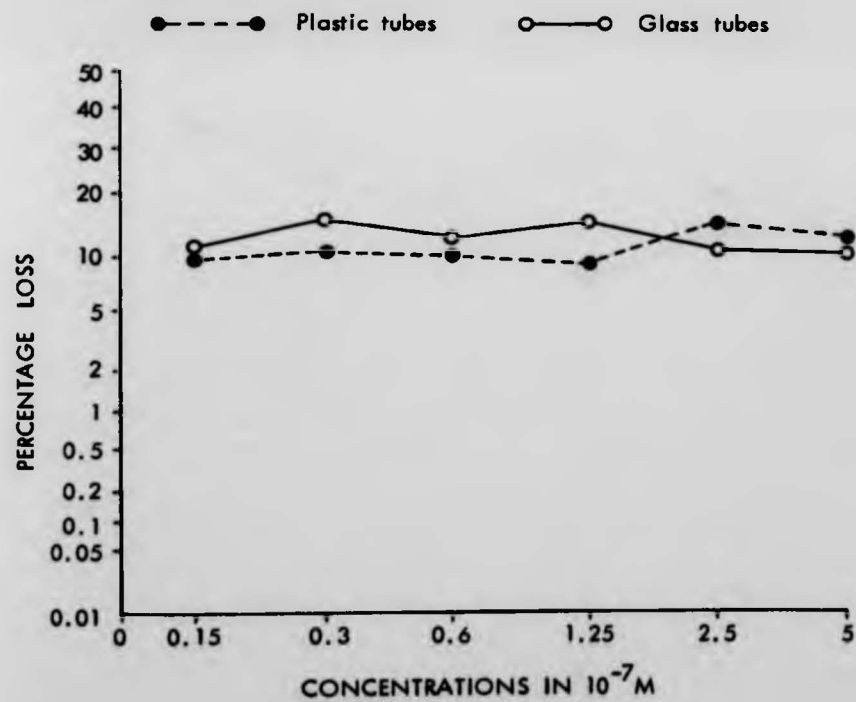
●─ ─ ─● Plastic tubes     ○───○ Glass tubes

Fig. 59

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

log probit scale

Fig. 59

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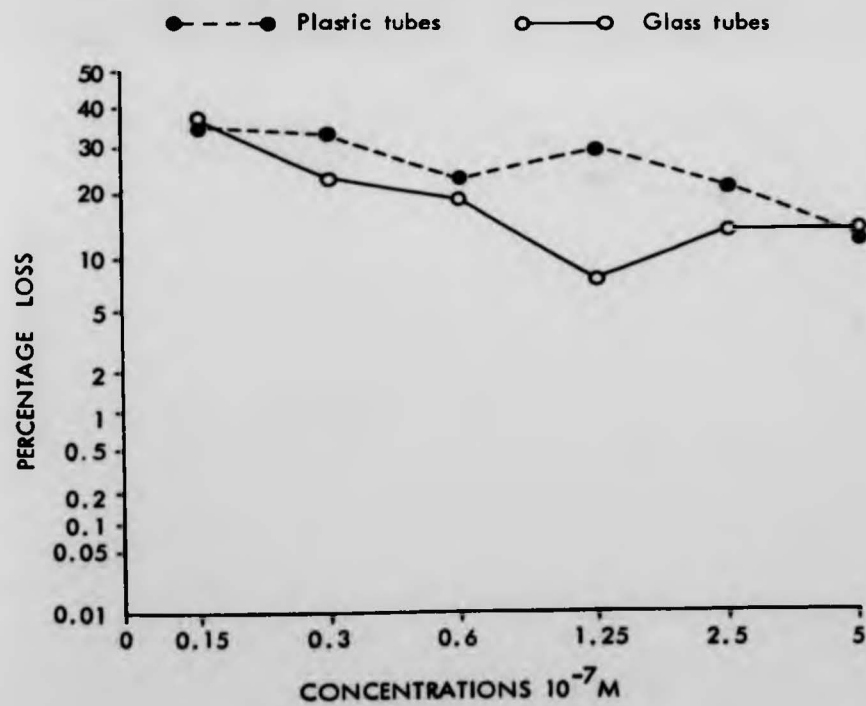
log probit scale

●  - - -  ● Plastic tubes          ○————○ Glass tubes
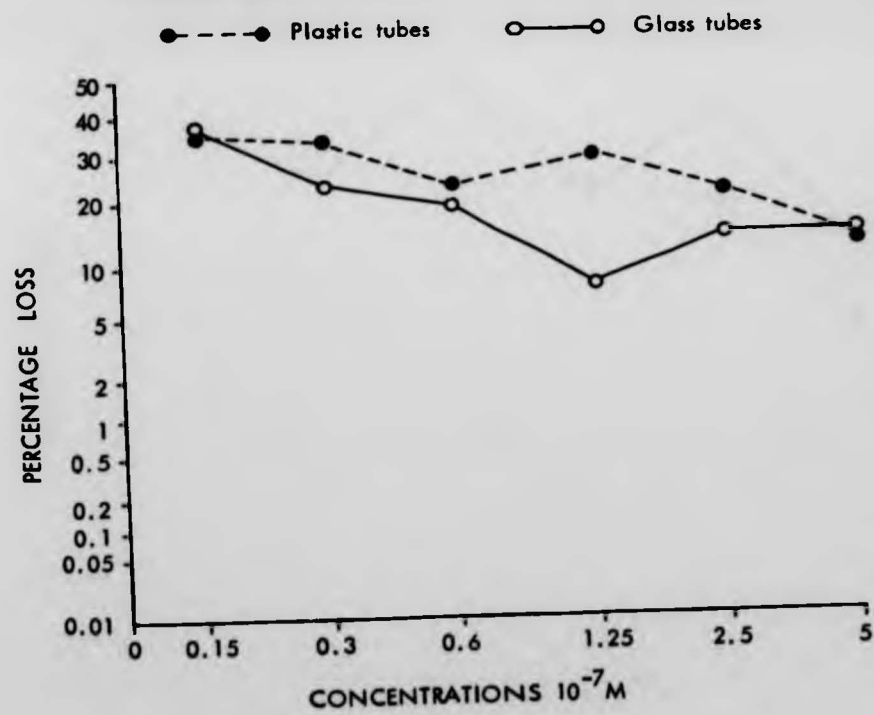
Fig. 60

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
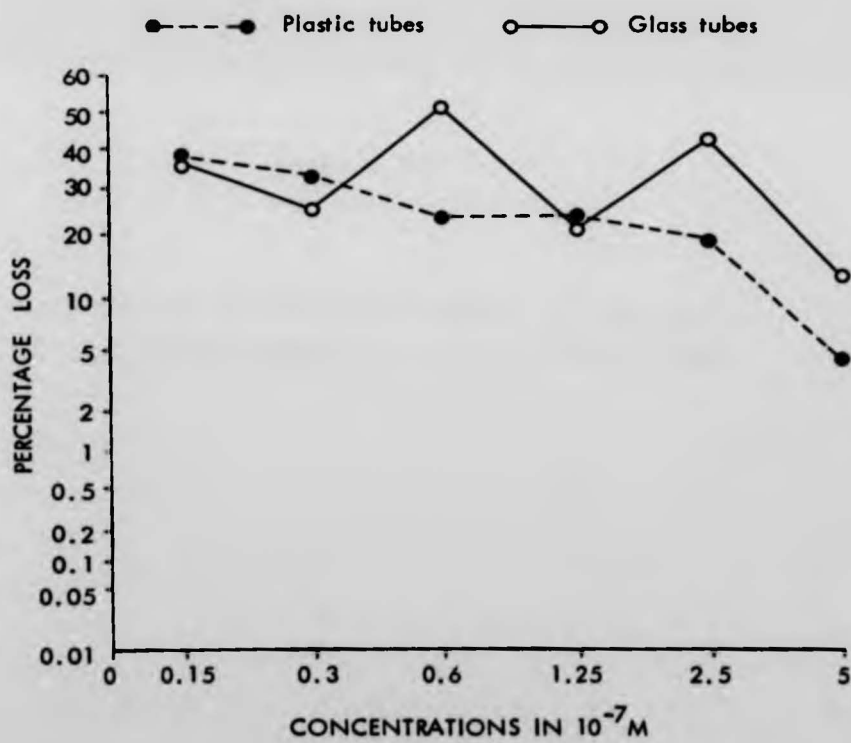
CHAPTER SIX

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 on-wards, 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 Zahar (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; Hadjini-
colaon, 1954, 1957) as well as BHC (Georgopoulos, 1954)
and dieldrin (Livadas, 1955). By the end of 1958, resis-
tance to DDT or dieldrin had developed in ten proved
malaria vectors in nineteen countries. A. sacharovi
in Greece and A. albimanus in El Salvador were 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 deve-
loped double resistance to both groups of the organo-
chlorine 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 resis-
tance in A. gambiae (species A) from Upper Volta.    Resis-
tance 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

| Species | DDT | Dieldrin/HCH | Organo-phosphorus | Carbamate |
|---|---|---|---|---|
| A.labranchiae | Algeria Tunisia | Morocco Algeria Tunisia | - | - |
| A.messae | Bulgaria | Romania Bulgaria | Romania | - |
| A.atroparvus | - | Romania Bulgaria | - | - |
| A.sacharovi | Greece,Iran Turkey,Iraq Syria, USSR Azerbaijan | Greece,Turkey Syria,Iraq | Turkey | Turkey |
| A.pulcher-rimus | Afghanistan Syria | Saudi Arabia | - | - |
| A.sergenti | - | Jordan | - | - |
| A.pharoensis | Egypt,Sudan Ethiopia | Egypt,Sudan Israel | - | - |
| A.culicifacies | Afghanistan Iran,Pakistan India,Nepal Sri Lanka | Afghanistan Pakistan India,Nepal Sri Lanka | India | - |
| A.stephensi | Afghanistan Iran,Iraq Pakistan Saudi Arabia India | Afghanistan Iran,Iraq Pakistan Saudi Arabia India | - | - |
| A.multicolor | Saudi Arabia | Saudi Arabia | - | - |
| A.d'thali | - | Iran | - | - |
| A.fluviatilis | India | Saudi Arabia | - | - |
| A.subpictus | Pakistan India,Nepal Indonesia | Pakistan India Sri Lanka Indonesia Bangladesh | - | - |
| A.hyrcanus | Pakistan Afghanistan Turkey | Turkey | Turkey | - |
| A.maculipennis | Iran,Turkey | Turkey | - | - |
| A.sinensis | Japan(Ryukyu) | Republic of Korea | Republic of Korea,Japan (Ryukyu) | - |
| A.annularis | Nepal,India Bangladesh | Indonesia Nepal | - | - |

Table 25  Insecticide resistance in anopheline mosquitoes:
Countries or areas

| Species | DDT | Dieldrin/HCH | Organo-phosphorus | Carbamate |
|---|---|---|---|---|
| A.labranchiae | Algeria Tunisia | Morocco Algeria Tunisia | - | - |
| A.messae | Bulgaria | Romania Bulgaria | Romania | - |
| A.atroparvus | - | Romania Bulgaria | - | - |
| A.sacharovi | Greece,Iran Turkey,Iraq Syria, USSR Azerbaijan | Greece,Turkey Syria,Iraq | Turkey | Turkey |
| A.pulcherrimus | Afghanistan Syria | Saudi Arabia | - | - |
| A.sergenti | - | Jordan | - | - |
| A.pharoensis | Egypt,Sudan Ethiopia | Egypt,Sudan Israel | - | - |
| A.culicifacies | Afghanistan Iran,Pakistan India,Nepal Sri Lanka | Afghanistan Pakistan India,Nepal Sri Lanka | India | - |
| A.stephensi | Afghanistan Iran,Iraq Pakistan Saudi Arabia India | Afghanistan Iran,Iraq Pakistan Saudi Arabia India | - | - |
| A.multicolor | Saudi Arabia | Saudi Arabia | - | - |
| A.d'thali | - | Iran | - | - |
| A.fluviatilis | India | Saudi Arabia | - | - |
| A.subpictus | Pakistan India,Nepal Indonesia | Pakistan India Sri Lanka Indonesia Bangladesh | - | - |
| A.hyrcanus | Pakistan Afghanistan Turkey | Turkey | Turkey | - |
| A.maculipennis | Iran,Turkey | Turkey | - | - |
| A.sinensis | Japan(Ryukyu) | Republic of Korea | Republic of Korea,Japan (Ryukyu) | - |
| A.annularis | Nepal,India Bangladesh | Indonesia Nepal | - | - |

Table 25 cont...

| Species | DDT | Dieldrin/HCH | Organo-phosphorus | Carbamate |
|---------|-----|--------------|-------------------|-----------|
| A.barbirostris | - | Indonesia | - | - |
| A.vagus | Bangladesh South Viet-Nam | Indonesia South Viet-Nam Philippines Malaysia | - | - |
| A.aconitus | Indonesia | Indonesia | - | - |
| A.splendidus | - | India | - | - |
| A.minimus | - | Indonesia | - | - |
| A.flavirostris | - | Philippines | - | - |
| A.sundaicus | Indonesia | Indonesia Malaysia (Sabah) | - | - |
| A.filipinae | - | Philippines | - | - |
| A.philip-pinensis | India | Malaysia (Sabah) | - | - |
| A.farauti Sp.No.1 | - | Solomon Islands | - | - |
| A.coustani | - | Saudi Arabia | - | - |
| A.gambiae A. | Togo South Africa Cameroon Central African Republic Upper Volta Nigeria Dahomey | Togo Cameroon Central African Republic Upper Volta Nigeria Senegal Guinea,Gambia Equatorial Guinea Sierra Leone Kenya,Liberia Ivory Coast Ghana,Congo, Dahomey,Mali Mauritania | - | - |
| A.gambiae B. | Sudan Senegal Swaziland | Upper Volta Nigeria Sudan,Senegal Swaziland,Chad Ethiopia Kenya Mozambique Southern Rhodesia Mauritania | - | - |
| A.funestus | - | Nigeria,Ghana Kenya Cameroon | - | - |

Table 25 cont..

| Species | DDT | Dieldrin/HCH | Organo-phosphorus | Carbamate |
|---|---|---|---|---|
| A.nili | - | Ghana | - | - |
| A.rufipes | - | Mali | - | - |
| A.albimanus | Haiti El Salvador Nicaragua Guatemala Honduras Mexico,Cuba Costa Rica Panama Dominican Republic Colombia | Haiti El Salvador Guatemala Belize Honduras Jamaica Ecuador Mexico Nicaragua Cuba Dominican Republic Colombia | El Salvador Guatemala Honduras Nicaragua | El Salvador Guatemala Honduras Nicaragua |
| A.quadrimacu-latus | USA Mexico | USA Mexico | - | - |
| A.pseudopuncti-pennis | Mexico Peru | Mexico Nicaragua Peru Venezuela Ecuador | | |
| A.aquasalis | - | Trinidad Venezuela Brazil | - | - |
| A.triannulatus | - | Venezuela Colombia | - | - |
| A.strodei | - | Venezuela | - | - |
| A.rangeli | - | Venezuela | - | - |
| A.albitarsis | Colombia Brazil | Venezuela | - | - |
| A.neomaculi-palpis | - | Trinidad Colombia | - | - |
| A.crucians | - | USA Dominican Republic | - | - |

Table reporduced from WHO 1976 Technical Report Series No. 585.

Table 26        Resistance of Anapheline Mosquitoes to Organophosphorous compounds, Carbamates and Pyrethroids

| | Malathion | Fenthion | Fenitrothion | Temephos | Chlorpyrifos | Propoxur | Pyrethroids |
|---|---|---|---|---|---|---|---|
| A.albimanns | R | S | R | S | R | R | S |
| A.culicifacies | R | S | R | | | | |
| A.stephensi | R | S | R | S | | R | S |
| A.gambiae | | | | | | | R |
| A.arabiensis | R | S | S | S | | S | R |
| A.sacharovi | S | R | R | | R | R | |
| A.maculipennis | S | S | R | | | R | |
| A.messeae | R | R | | | | | |
| A.sinensis | R | R | R | | | | |
| A.atroparvus | R | R | R | S | | R | S |
| A.hycranus | S | R | R | | | S | |

(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. gambiae "B") was first reported
to be resistant to DDT in Senegal in 1968 and in Swazi-
land in 1970.    Resistance of this species to dieldrin/HCH
was reported from Upper Volta, Nigeria, Senegal, Swazi-
land, Chad, Ethiopia, Kenya, Mozambique, Southern Rhodesia
and Mauritania.    Again no resistance to the organophos-
phorous 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 mala-
thion in susceptibility tests performed late in 1977
and during 1978 failed to give 100% mortality.    Resis-
tance 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 deter-
mining 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 pres-
sure.   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 popu-
lations, 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), Plapp
(1970) and Brown (1971) stated that the dominance of
resistant genes and their frequency were important fac-
tors 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 involve-
ment of more than one major genetic factor, moderate
pressure stimulate the nature of field selections where
with the residual insecticides, partial selection of sub-
sequent 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 insecti-
cides 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 resis-
tant 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 Oppenoorth 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 pene-
tration, 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 (Oppenoorth 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 expla-
nation 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 detoxi-
cation. There is some reason to believe that these two
mechanisms depend on an altered site of action, but defi-
nite proof is lacking. Milani and Travaglio (1957)
showed the effect of the kdr gene to be affected by modi-
fier 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
uticae by Smissaert (1964) and by others in ticks, green
rice leaf hopper, housefly. Recently it was shown in
A. albimanus by Ayad and Georghiou (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
Georghiou (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 theoreti-
cally 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 struc-
ture 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 specifi-
city 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 con-
ferred 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. albi-
manus 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 resis-
tance 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 confering 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 organo-
chlorine 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 resis-
tance 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 develop-
ment 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.

e. <u>A. arabiensis</u> SW9 + 12, a population resistant to DDT
and dieldrin but susceptible to malathion which
originates from Swaziland.

f. <u>A. gambiae</u> 16c, originated from Kano, Nigeria and
susceptible to DDT, dieldrin and malathion.

## 6.2.2 <u>Rearing Techniques</u>

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 monocups 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 temper-
ature.    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 main-
tenance of the colony, or for the provision of emerging
adults for testing with insecticides.

### 6.2.3 Chemicals Used

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

#### a. Organochlorines

DDT
1,1,1-trichloro,-2,2,-di(4-p-chlorophenyl)ethane.

dieldrin (DLD)
1,2,3,4,10,10-hexachloro-      6,7-epoxy-1,4,4a,5,6,7,
      -exo-
8,8a-octa-hydro-1,4-endo      -5,8-dimethanonaphthalene.

#### b. Organophosphates

Methoxy compounds
   Aromatic:   fenthion
O,O-dimethyl-O-(/4-methylthio/m-tolyl)phosphorothioate
            fenitrothion
   dimethyl-   3-methyl-4-nitrophenyl phosphorothioate
   Aliphatic:   malathion
                                        thiolo
O,O-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 world-
wide, 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 trans-
ferred 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 trans-
ferred 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.arabiensis 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 (morta-
lities 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, fenthion 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 offsprings 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 sus-
ceptible 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 con-
secutive 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 <u>Anopheles</u>
<u>arabiensis</u> from Fatasha, Shambat and Gezira
areas of the Sudan to the discriminating
dosages of DDT and DLD for 60 and 120 minutes

| Insecticide | | Locality | Exposure time in minutes | Total No. exposed | Percentage mortality |
|---|---|---|---|---|---|
| DDT | 4.0% | Fatasha | 60 | 238 | 68% |
| | | Fatasha | 120 | 125 | 94% |
| | | Shambat | 60 | 120 | 72% |
| | | Gezira | 60 | 249 | 61% |
| | | Gezira | 120 | 810 | 95% |
| DLD | 0.4% | Fatasha | 60 | 140 | 5% |
| | | Fatasha | 120 | 230 | 19% |
| | | Shambat | 60 | 100 | 38% |
| | | Gezira | 60 | 360 | 3% |
| | | Gezira | 120 | 480 | 4% |
| DLD | 4.0% | Fatasha | 60 | 214 | 25% |
| | | Fatasha | 120 | 143 | 38% |
| | | Shambat | 60 | 125 | 42% |
| | | Gezira | 60 | 139 | 4% |
| | | Gezira | 120 | 248 | 12% |

Table 28    Results of laboratory exposures of <u>Anopheles</u>
<u>arabiensis</u> from Fatasha Shambat and Gezira
areas of the Sudan to some of the organo-
phosphorous and carbamate insecticides for
varying times

| Insecticide | | Locality | Exposure time in minutes | Total No. exposed | Percentage mortality |
|---|---|---|---|---|---|
| Malathion | 5% | Fatasha | 60 | 380 | 100% |
| | | Shambat | 60 | 120 | 100% |
| | | Gezira | 60 | 678 | 43% |
| | | Gezira | 120 | 425 | 58% |
| | | Gezira | 300 | 348 | 72% |
| Fenitrothion | 1% | Fatasha | 60 | 125 | 100% |
| | | Shambat | 60 | 75 | 100% |
| | | Gezira | 30 | 265 | 100% |
| | | Gezira | 60 | 203 | 100% |
| Fenthion | 2.5% | Fatasha | 60 | 196 | 100% |
| | | Shambat | 60 | 96 | 100% |
| | | Gezira | 60 | 599 | 100% |
| Propoxur | 0.1% | Fatasha | 60 | 320 | 100% |
| | | Shambat | 60 | 80 | 100% |
| | | Gezira | 15 | 281 | 100% |
| | | Gezira | 30 | 259 | 100% |
| | | Gezira | 60 | 257 | 100% |

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

| Insecticide | | Strain | Total No. exposed | Percentage mortality |
|---|---|---|---|---|
| DDT | 4.0% | SB | 235 | 100% |
| | | SW9+12 | 189 | 60% |
| DLD | 0.4% | SB | 248 | 100% |
| | | SW9+12 | 320 | 0% |
| DLD | 4.0% | SB | 175 | 100% |
| | | SW9+12 | 80 | 0% |
| Malathion | 5.0% | SB | 100 | 100% |
| | | SW9+12 | 120 | 100% |

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

| Generation No. | Total No. exposed | Hours of exposure | Percentage mortality |
|---|---|---|---|
| 0 | 55 | 2 | 58.2% |
| 1 | 216 | 1 | 42.6% |
|   | 81 | 2 | 58.0% |
|   | 566 | 5 | 39.9% |
| 2 | 326 | 2 | 31.9% |
| 3 | 1399 | 2 | 15.4% |
| 4 | 1184 | 5 | 8.0% |
| 5 | 1269 | 5 | 2.7% |
|   | 1345 | 5 | 2.4% |
| 6 | 1058 | 5 | 1.2% |
| 7 | 1150 | 5 | 0.0% |

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

|  | $F_1$ | First backcross | Second backcross | Third backcross |
|---|---|---|---|---|
| Total no. tested | 1749 | 492 | 584 | 485 |
| Observed no. dead | 532 | 201 | 231 | 207 |
| % mortality | 30.4 | 40.9 | 39.6 | 42.7 |
| Expected no. dead | | 319 | 380 | 315 |
| % mortality | | 65 | 65 | 65 |
| $x^2$ | | 124 | 164 | 105 |
| P | | < 0.001 | < 0.001 | < 0.001 |
| Significance at the 1% level | | signi-ficant | signi-ficant | signi-ficant |

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 £% malathion for one hour

GEZ female x SW9+12 male

|  | $F_1$ | First backcross | Second backcross | Third backcross |
|---|---|---|---|---|
| Total no. tested | 1694 | 753 | 408 | 642 |
| Observed no. dead | 504 | 197 | 144 | 241 |
| % mortality | 29.8 | 26.2 | 35.3 | 37.5 |
| Expected no. dead |  | 489 | 265 | 417 |
| % mortality |  | 65 | 65 | 65 |
| $x^2$ |  | 497 | 157 | 211 |
| p |  | < 0.001 | < 0.001 | < 0.001 |
| Significance at the        level |  | significant | significant | significant |

The $x^2$ 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 <u>A. arabiensis</u> from the Sudan) to 5%
            malathion for one hour

GEZ female x SHAM male

|                            | $F_1$ | First backcross | Second backcross | Third backcross |
|----------------------------|-------|-----------------|------------------|-----------------|
| Total No. tested           | 891   | 465             | 654              | 589             |
| Observed no. dead          | 334   | 268             | 342              | 313             |
| % mortality                | 37.5  | 57.6            | 52.3             | 53.1            |
| Expected no. dead          |       | 316             | 445              | 401             |
| % mortality                |       | 68              | 68               | 68              |
| X                          |       | 22              | 74               | 60              |
| P                          |       | < 0.001         | < 0.001          | < 0.001         |
| Significance at the 1% level |     | significant     | significant      | significant     |

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 $X^2$ 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 back-
crossing 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)

| Temephos Concentration | GEZIRA | | SB | |
|---|---|---|---|---|
| | Total Tested | % Mortality | Total Tested | % Mortality |
| 0.000 | 46 | 0 | 50 | 0 |
| 0.002 | 50 | 0 | 52 | 0 |
| 0.003 | 44 | 9 | 47 | 2 |
| 0.004 | 40 | 53 | 48 | 21 |
| 0.005 | 158 | 44 | 113 | 38 |
| 0.0075 | 47 | 83 | 50 | 90 |
| 0.010 | 50 | 98 | 50 | 98 |
| 0.0125 | 96 | 93 | 49 | 96 |
| 0.025 | 334 | 98 | 143 | 100 |
| 0.05 | 172 | 99 | 75 | 100 |
| 0.125 | 48 | 100 | 46 | 100 |
| LC50 | 0.0053 | | 0.0054 | |
| LC90 | 0.0125 | | 0.0084 | |

Fig. 62

Susceptibility of larvae of two strains of <u>An.arabiensis</u> to Temephos
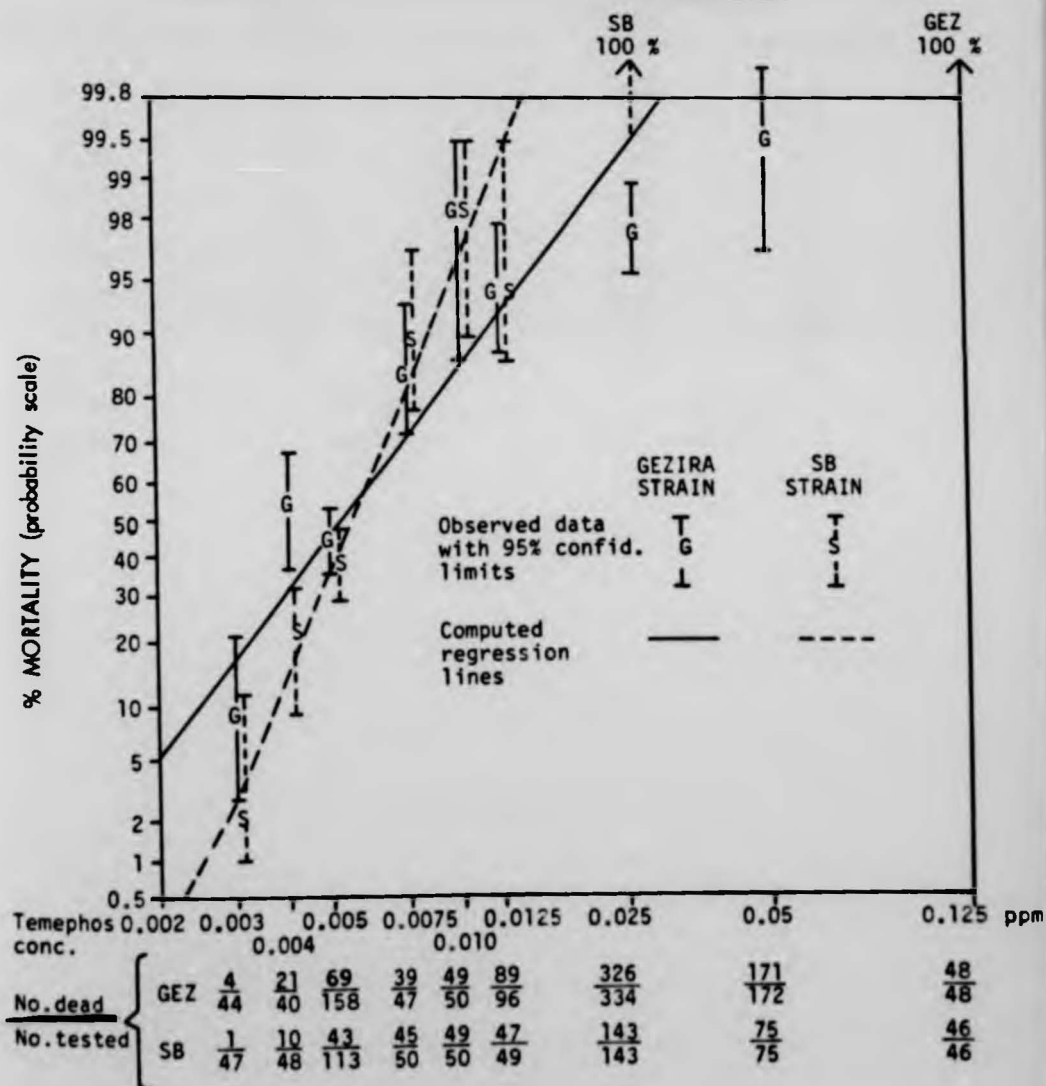


| Temephos conc. | | 0.002 | 0.003 0.004 | 0.005 | 0.0075 | 0.010 0.0125 | 0.025 | 0.05 | 0.125 ppm |
|---|---|---|---|---|---|---|---|---|---|
| No.dead No.tested | GEZ | $\frac{4}{44}$ | $\frac{21}{40}$ | $\frac{69}{158}$ | $\frac{39}{47}$ | $\frac{49}{50}$ $\frac{89}{96}$ | $\frac{326}{334}$ | $\frac{171}{172}$ | $\frac{48}{48}$ |
| | SB | $\frac{1}{47}$ | $\frac{10}{48}$ | $\frac{43}{113}$ | $\frac{45}{50}$ | $\frac{49}{50}$ $\frac{47}{49}$ | $\frac{143}{143}$ | $\frac{75}{75}$ | $\frac{46}{46}$ |

Table 35        Results of Testing two Populations of

A. arabiensis larvae (GEZIRA and SB) Against

Various Concentrations of Malathion

| Malathion Concentrations | GEZIRA | | SB | |
|---|---|---|---|---|
| | Total Tested | % Mortality | Total Tested | % Mortality |
| 0.00 | 48 | 0 | 54 | 0 |
| 0.05 | 74 | 1 | 42 | 0 |
| 0.10 | 98 | 10 | 48 | 10 |
| 0.15 | 146 | 15 | 148 | 20 |
| 0.20 | 92 | 42 | 120 | 51 |
| 0.25 | 75 | 75 | 50 | 80 |
| 0.30 | 225 | 95 | 123 | 98 |
| 0.35 | 186 | 98 | 98 | 100 |
| 0.40 | 100 | 100 | 73 | 100 |
| LC50 | 0.190 | | 0.187 | |
| LC90 | 0.293 | | 0.272 | |

Table 35    Results of Testing two Populations of
A. arabiensis larvae (GEZIRA and SB) Against
Various Concentrations of Malathion

| Malathion Concentrations | GEZIRA Total Tested | % Mortality | SB Total Tested | % Mortality |
|---|---|---|---|---|
| 0.00 | 48 | 0 | 54 | 0 |
| 0.05 | 74 | 1 | 42 | 0 |
| 0.10 | 98 | 10 | 48 | 10 |
| 0.15 | 146 | 15 | 148 | 20 |
| 0.20 | 92 | 42 | 120 | 51 |
| 0.25 | 75 | 75 | 50 | 80 |
| 0.30 | 225 | 95 | 123 | 98 |
| 0.35 | 186 | 98 | 98 | 100 |
| 0.40 | 100 | 100 | 73 | 100 |
| LC50 | 0.190 | | 0.187 | |
| LC90 | 0.293 | | 0.272 | |

Fig. 63

Susceptibility of larvae of two strains of <u>An. arabiensis</u> to Malathion



| No.dead | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| No.dead | GEZ | $\frac{1}{74}$ | $\frac{10}{98}$ | $\frac{22}{146}$ | $\frac{39}{92}$ | $\frac{56}{75}$ | $\frac{214}{225}$ | $\frac{182}{186}$ | $\frac{100}{100}$ |
| No.tested | SB | $\frac{0}{42}$ | $\frac{5}{48}$ | $\frac{30}{148}$ | $\frac{61}{120}$ | $\frac{40}{50}$ | $\frac{121}{123}$ | $\frac{98}{98}$ | $\frac{73}{73}$ |
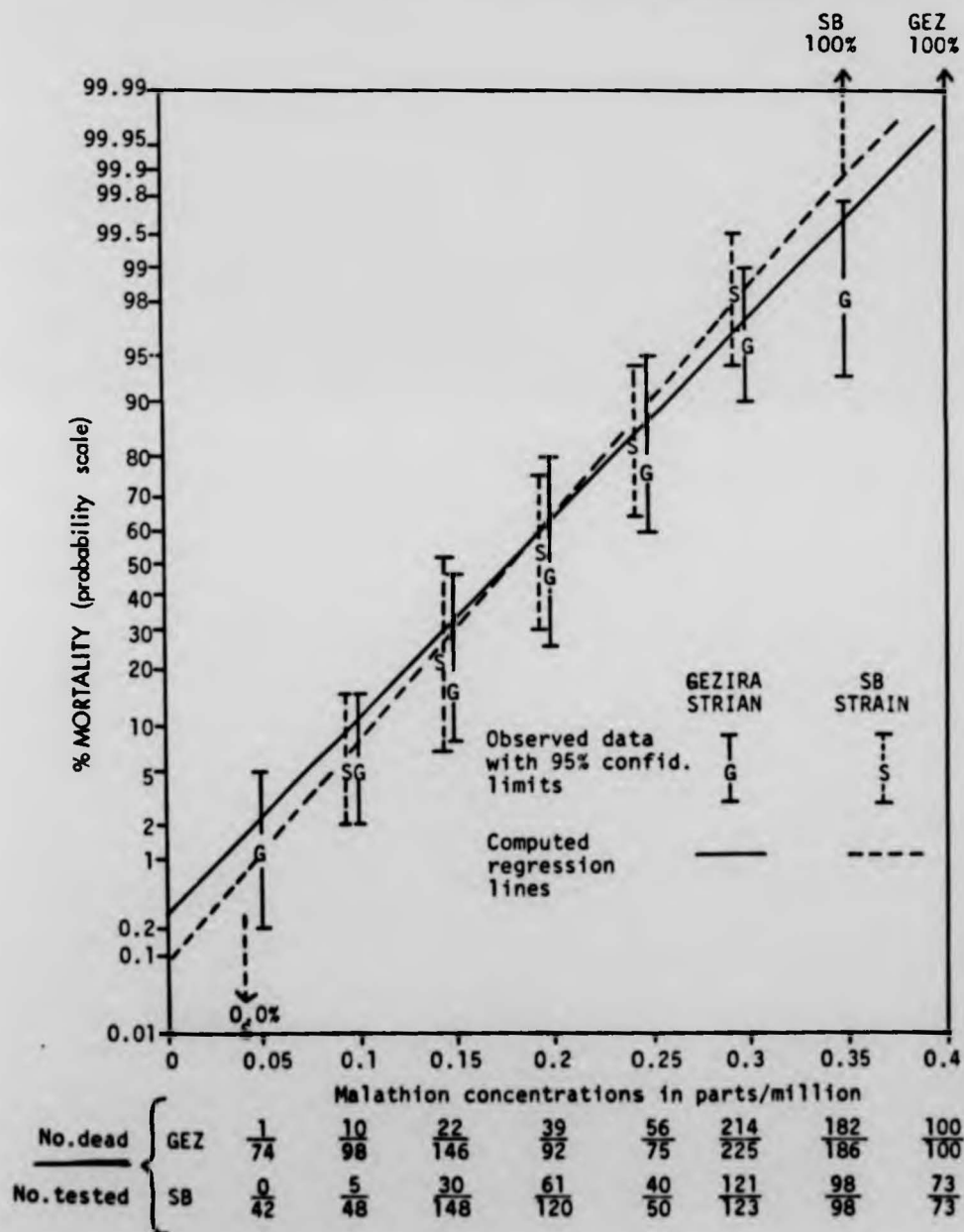
indicates that the resistance to malathion in the adult Gezira
population is not reflected in the larval stages.

## 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 back-
crossing 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 selec-
tion 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 larviciding 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 parasi-
tological data, obtained by microscopical examinations of
blood films alone, is not enough and could be misleading.  The
implementation of modern ser○logical techniques (the IFA test)
to study the incidence and prevalence of malaria, and to eva-
luate the control measures proved to be of great importance.

The level of sensitivity of P. falciparum to chloro-
quine which is the drug most widely used for malaria chemo-
therapy in the country must be investigated on a large scale
to establish baseline data and to enable application of effec-
tive 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 |
| $Na_2HPO_4$ | 25.6 gram |
| $NaH_2PO_4 \cdot 2H_2O$ | 3.12 gram |
| Distilled water | 20 litres |

Annex 2

Buffered Glycerol pH 8 - 9

| | |
|---|---|
| $NaHCO_3$ | 0.0175 gram |
| $Na_2CO_3$ | 0.016 gram |
| Glycerol | 90.0 ml |
| Distilled water | 10.0 ml |

Annex 3

Preparation of Acid Citrate Dextrose (ACD)

| | |
|---|---|
| Sodium citrate $(Na_3C_6H_5O_7 \cdot 2H_2O)$ | 2.25 grams |
| Citric acid $(H_3C_6H_5O_7 \cdot H_2O)$ | 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 $(Na_3C_6H_5O_7 \cdot 2H_2O)$ | 1.806 gram |
| Citric acid $(H_3C_6H_5O_7 \cdot H_2O)$ | 0.224 gram |
| Sodium phosphate $(Na_2HPO_4)$ | 0.153 gram |
| Dextrose (monohydrate) | 1.750 gram |
| Distilled water | 70 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:

$gx^2$

0.16

X

0.2

+

$g10^x$

R/S

÷

GTO00

R/S

-

$gx^2$

R/S

÷

STO + 0

GTO13

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 < 03% or > 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                    Repeat for each row of the

ENTER                       table

Key in X                    Use CHS for -ve signs

Σ +

CLX

f x̄     record result as log $ED_{50}$

g $10^X$ record result as $ED_{50}$

Key in 1.28

CHS

f x̄     record result as log $ED_{10}$

g $10^X$ record result as $ED_{10}$

f LR

x ⇄ y   record result as slope

f  CLR PRGM

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

Key in x                            Repeat for each row

f ŷ                                 of the table

Record results in Ŷ 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 O

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 calulater ON, switch to PRGM then key in:

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

Switch to RUN

g RTN

Store in memory the following:-

Chamber A background (9 x no. of minutes) STO 1

Chamber B background (22 x no. of minutes) STO 7

Count time in minutes STO 3

Total volume of heptane (2.5 ml) STO 4

Volume of Heptane/vial (1 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 (dpm)

Go to g RTN after each run.

Then to convert to μmols/l from dpm/ml

Store specific activity in μCi /μmol (30) in STO O

Then carry on the following steps:

Switch to PRGM

ENTER

EEX

3

X

ENTER

RCL O

÷

ENTER

Switch to RUN

g RTN

Store in memory the following:-

Chamber A background (9 x no. of minutes) STO 1

Chamber B background (22 x no. of minutes) STO 7

Count time in minutes STO 3

Total volume of heptane (2.5 ml) STO 4

Volume of Heptane/vial (1 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 (dpm)

Go to g RTN after each run.

Then to convert to μmols /l  from dpm/ml

Store specific activity in μCi /μmol   (30) in STO O

Then carry on the following steps:

Switch to PRGM

ENTER

EEX

3

X

ENTER

RCL O

÷

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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