MATERIALS AND METHODS
1. Maintenance of Laboratory Infections

(A) PARASITE

(i) Rodent Parasite:

A strain of *Plasmodium yoelii nigeriensis*, used in present study was received from Prof. P.C.G. Garnham in 1978. It has been maintained in the laboratory by weekly syringe passages in Swiss mice of either sex (20 ± 1 g). The animals (one or two) showing high parasitaemia were bled from the heart and the blood was diluted with appropriate quantity of the anti-coagulant (A.C.D.). The diluted inoculum was then injected intraperitoneally to the rodent host (Swiss mice).

(ii) Monkey Parasite:

*P. knowlesi* strain W1 was used for infection. 1 x $10^6$ - $1 \times 10^8$ parasitized red blood cells from infected donor monkey were injected intravenously to healthy monkey (*Macca mulatta*). When monkey showed high parasitaemia, it was bled from the vein and blood was diluted with the appropriate quantity of the anti-coagulant. The diluted inoculum was then injected intravenously in fresh healthy monkey.

(B) HOST

(i) Rodent Host:

Laboratory bred strain of Swiss mice was being employed in experiments with *P. yoelii nigeriensis*. Healthy
colony of this rodent was being maintained under standard temperature conditions in the 'Animal House' wing of this Institute (C.D.R.I.).

(ii) Monkey Host:

Rhesus monkey (*Macaca mulatta*) of both sexes (4-6 kg), negative by tuberculin test and chest x-ray, were used in the study. They were maintained under strict 12 hrs photoperiodicity and fluorescent light were switched on from 7.00 a.m. to 7.00 p.m. They were kept on standard animal feed supplemented with green vegetables, soaked grams and bananas.

(C) PREPARATION OF ANTICOAGULANT

The anticoagulant was prepared by dissolving following contents in 500 ml distilled water:

- **citric acid (anhydrous)** 3.65 gm
- **sodium citrate** 11.00 gm
- **dextrose** 12.25 gm

Above solution was autoclaved at 15 lbs pressure for 15 minutes.

(D) CRYOPRESERVATION OF THE PARASITE

Plastic vials, 2 ml in capacity, were used for freezing preservation of strain samples as described by Jeffery
The preservative was prepared as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Glycerol</td>
<td>20 ml</td>
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<tr>
<td>Phosphate buffer (pH 7.2)</td>
<td>80 ml</td>
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Buffered glycerol was dispensed 1 ml/vial and then the vials were autoclaved for 15 minutes at 15 lbs pressure. At the time of preservation, 1 ml of citrated blood is added to each vial containing 1 ml of buffered glycerol. The vials were then immersed in liquid nitrogen (-170°C). For initiation of infection from frozen samples, one of the vials was taken out from the liquid nitrogen container and gradually thawed to 37°C. The thawed blood was then injected in the animal.

(E) Examination of the Blood Smears

For recording the course of infection in Swiss mice thin blood smears were made from the tail vein of the animals and allowed to air dry. They were then fixed in absolute methyl alcohol (AR) for 1/2 minutes, stained with giemsa for 1/2 hr and then washed with phosphate buffer (pH 7.2). After drying, the blood smears were examined under oil immersion objective of the binocular microscope. The parasitaemia was then recorded by counting the number of infected cells in 50 fields of the oil immersion microscope and percent parasitaemia was then calculated. At least 50 fields were seen before labelling a smear to be negative.
(F) Preparation of Strain and Staining Buffer

Readymade giemsa stain (BDH) was used for staining the slides.

The stain was prepared by diluting the BDH liquid stain with buffer (pH 7.2) in the ratio 1:20 and then a little (5 ml) acetone was also added in the stain according to Walter Reed Army Institute of Research staining modification.

The buffer contents are as follows -

Solution 1 - Na$_2$HPO$_4$ 9.50 gm dissolved in 100 ml distilled water.
Solution 2 - KH$_2$PO$_4$ 9.07 gm dissolved in 1000 ml distilled water.

For preparing the working solution, 72 ml of $S_1$ and 28 ml of $S_2$ were added to 900 ml of distilled water and pH of the resulting solution was adjusted to 7.2.

2. Chemotherapeutic Drugs:

(A) Drugs

Commercial samples of the following antimalarial drugs were used in the chemotherapeutic studies.

Chloroquine (Resochin) Bayer (India) Limited, Bombay;
Amodiaquine (Camaquine) Parke-Davis (India) Limited, Bombay;
Mepacrine, British Pharmaceutical Laboratories, Bombay;
Pyrimethamine (Daraprim) Burrough Wellcome & Co. (India)
Private Limited, Bombay; Dapsone (Diamino-diphenyl sulfone) Bengal Immunity Co. Limited, Calcutta; Sulphadiazine, Medichem Pharmaceuticals, Ghaziabad and Sulphanilamide Cyper Pharma, New Delhi, DADDS (Acedapsone) received from Pharmaceuticals Division of the Institute. Pure samples of mefloquine hydrochloride (Ro-21-5998/lot A-461465) was received from MG.Roche-001, Products Limited, Basel. Metakelfin. Walter Bushnall Private Limited, Bombay.

(B) Antimalarial New Compounds

(i) Several compounds viz. 83/498, 80/693, 83/494, 83/496, 83/38, 82/142, 82/628, 82/143, 83/495, 42/183, 80/695, 83/495, 82/572, 81/35, 81/31 and 81/284 were received for testing from the Medicinal Chemistry Division of the Institute (C.D.R.I.).

(ii) Compound No. 1, 2, 4, 5, S12, S8, S4 were received from the Chemistry Department of the Lucknow University, for testing of antimalarial activity.

(C) Administration of Drugs

(i) Aqueous Solution

Various antimalarial drugs were dissolved in an appropriate quantity of distilled water to have the required amount in 0.5 ml liquid small quantity of methyl cellulose was added in water for making homogenate of those antimala-
rial compounds which were not soluble in water. The aqueous solution/suspension was administered orally.

(ii) Oil Solution/Suspension

Sulpha drugs were dissolved/suspended in ground nut oil in an appropriate amount to get required quantity of drug in 0.25 ml liquid. Drug was administered intramuscularly.

3. Experimental Studies

(A) Selection of Host

Pande and Dutta (1982) observed that Swiss mice was most suitable for infection of *P. yoelii nigeriensis* and the infection was invariably fatal. The course of infection of normal strain *P. yoelii nigeriensis* was observed in Swiss mice (20 gm). Animals were inoculated intraperitoneally with 1-10 million parasitised red cells per 20 gm wt. of the animal. The day on which the animals were infected was counted as Day 0 (D0) and subsequent days as D+1, D+2, D+3 and so on. The percent parasitaemia was recorded till death of the animal in case the host was susceptible.

Richardson (1946) reported that highly fatal infections could be produced with *P. knowlesi* infection in *Macaca mulata* (rhesus monkey).
The course of infection of W1 strain of *P. knowlesi* was observed in rhesus monkey (4-6 kg weight). Monkeys were inoculated intravenously with 1-10 million parasitized red cells. The parasitaemia was recorded daily till the death of animal.

**B. Chemotherapeutic Studies**

(a) **Against Rodent Parasite *P. yoelii nigeriensis***

(i) **Testing of long acting nature of antimalarial drugs:**

Antimalarial drugs were injected intramuscularly in oil vehicle to two groups of 8 Swiss mice each at 4 x M.E.D. and 8 x M.E.D. dose levels. The drugs were given on day -12, -9, -6, -3, and day 0. Mice in all batches were inoculated with *P. yoelii nigeriensis* parasitized R.B.C. (inoculum 10 million) on the same day. Course of infection in the treated groups were compared with untreated controls. Mice were observed till death or till day +21 post-inoculation.

(ii) **Blood Schizontocidal Activity:**

To determine the chemotherapeutic response to *P. yoelii nigeriensis* infection each antimalarial drug was administered in single dose or 4 dose levels. The activity was determined on the basis of (I) Mean survival time, (II) Minimum effective dose and (III) Percent suppression of parasitaemia in treated group when compared with untreated control.
(I) Determination of Mean Survival Times

Group of mice in four day test or in single dose treatment were inoculated on day zero with 1-10 million parasitized red cells and drugs were administered on day zero or from day 0 to +3 depending upon the type of experiment. The day of death of each animal was recorded daily till the period of day +21. The arithmetic average for each group was recorded as the mean survival time (M.S.T.).

(II) Determination of Minimum Effective Dose

The minimum effective dose of antimalarial drugs against sensitive and multiple resistant P. yoelii nigeriensis in Swiss mice host were determined. Animals were inoculated on day zero and drug was administered on day 0 in single dose treatment from day 0 to +3 in 4 day test. Blood smears of all animals were examined on day +4 and +7 to determine the erythrocyte infection rate (EIR). The lowest dose capable of suppressing the parasitaemia till day +7 was recorded as minimum effective dose (M.E.D.).

(iii) Efficacy of Single Dose Treatment

Antimalarial drugs were administered intramuscularly in oil at 20-640 mg/kg dose levels for each drug and for combination of sulphadoxine/pyrimethamine (fansidar) the doses ranging from 1.25 mg/kg sulphadoxine/0.625 mg/kg pyrimethamine to 40 mg/kg sulphadoxine/2 mg/kg pyrimethamine were
used. Doses of triple combination of sulphadoxine/pyrimetha-
mine/mefloquine ranging from 0.208 mg/kg sulphadoxine/0.083
mg/kg mefloquine/0.028 mg/kg pyrimethamine to 10 mg/kg sul-
phadoxine/1 mg/kg pyrimethamine/4 mg/kg mefloquine. For
metakelfin (sulphamethopyrazine + pyrimethamine) range
of doses from 35 mg/kg sulphamethopyrazine/1.5625 mg/kg
pyrimethamine to 500 mg/kg sulphamethopyrazine/25 mg/kg
pyrimethamine were given. Drugs were administered on day
0. 6-7 mice of each batch were infected intraperitoneally
with $1 \times 10^5$ inoculum.

The course of parasitaemia was recorded as the number
of parasitized red cell per 10,000 red cells. Smears were
made from the tail vein of animal. Blood smears were exa-
mined on day +4, +7, +10, +14 and +21. Survival of mice
were examined up to day +21. Mice which were survived beyond
+21 days after drug treatment challenged with standard ino-
culum ($1 \times 10^7$) of P. yoelii nigeriensis, and the parasita-
emias were checked on day +4. Mice which were protected after
1st challenge were rechallenged with antibiotic resistant
strain of P. yoelii nigeriensis. Parasitaemia was checked
on day +4 and mortality was recorded daily till day +21
post-inoculation.

(iv) Efficacy of New Compounds in Single Dose Treatment

Drug was administered orally on day zero to a group
of a 6-7 mice. All mice were infected with 10 million para-
site. The course of infection in the treated batches were compared with the untreated controls and all the mice were observed till day +21 post-inoculation.

(b) Against Monkey Parasite *P. knowlesi* (W1)

(i) Response of Antimalarial Drugs

Antimalarial drugs were administered when monkeys were patent. After patenty drugs were administered intramuscularly or orally for 3 days. Activity of antimalarial drugs were determined on the basis of parasiteamia clearance and recrudescence was observed till day +60 post inoculation.

C. Studies on Drug Resistance

(i) Selection of Sulphadoxine/Pyrimethamine/Mefloquine Resistant Strain of *P. yoelii nigeriensis*

The strain of *P. yoelii nigeriensis* was exposed to interrupted subcurative doses of sulphadoxine/pyrimethamine /mefloquine combination in sequential passages, in order to see whether resistance could be developed easily to the triple drug combination or not. The methodology for selection of resistant line was the same as used earlier for selection of mefloquine, pyrimethamine and pyrimethamine -mefloquine combined resistant lines of *P. berghei* in Swiss mice (Kazim et al., 1979; Agarwal et al., 1979; Puri and Dutta, 1982). After exposure of *P. yoelii nigeriensis* to
increasing doses of triple combination over 31 serial passages, the strain was tested for level of resistance by exposing the resistant line to various doses of the triple combination (sulphadoxine, pyrimethamine and mefloquine).

(ii) Cross Chemotherapeutic Studies against Multiple Resistant strain of *P. yoelii nigeriensis*

The cross sensitivity/resistance of the resistant strain to other antimalarial and drug combinations was determined on the basis of minimum effective dose (M.E.D.). The M.E.D. was determined by the 4 day treatment day (0-3) and compared with normal strain.

(iii) Selection of Chloroquine and Amodiaquine Resistant Strain of *P. knowlesi*

Using subcurative therapy from low dosages to high doses progressively or using relapse method for the selection of chloroquine and amodiaquine, attempt was made to select resistant strain of *P. knowlesi*.

During the course of subcurative and curative treatment in successive passages, several stabilates were collected from the major parasitaemia peaks during recrudescence episodes from different monkeys. Stabilates *viz* *W*₂ to *W*_₁⁹ were frozen in liquid nitrogen. These frozen stabilates of parasite were transferred to new monkeys for chemotherapy and determination of sensitivity of parasite to chloroquine and amodiaquine *in vivo*.
D. Immune Status of Rhesus Monkeys After Treatment

Two groups of monkeys were challenged with *P. knowlesi* (W1) after following treatment.

1. Treatment with suppressive chloroquine therapy.
2. Curative treatment or radical cure.

1. Challenge After Subcurative Treatment with Chloroquine

Successive subcurative doses of chloroquine were administered intramuscularly to 17 monkeys at varying degrees of parasitaemia. Subcurative therapy leads to chronicity of infection. Drug treated monkeys were challenged with *P. knowlesi* (1 x 10⁶ inoculum), and blood smears of the monkeys which survived, rechallenged with *P. knowlesi* were examined daily and parasitaemia was recorded for 30 days.

2. Challenge After Curative Treatment with Chloroquine

Monkeys which were cured with chloroquine were again challenged with W1 strain of *P. knowlesi* (1 x 10⁶ inoculum) and parasitaemia was recorded daily for 30 days. Monkeys which were protected from infection were rechallenged with *P. knowlesi*. 