CHAPTER III
MATERIAL AND METHODS
ANIMALS

Adult rhesus monkeys, *Macaca mulatta* and assamese monkeys, *Macaca assamensis*, of either sex, weighing about 3-8 kg were maintained on normal diet (pellets, bananas and soaked grams) in the animal house of the Institute. They were free from tuberculosis as shown by tuberculin test and were kept under 12 hr photoperiodicity with fluorescent lights on from 7.00 hrs to 19.00 hrs, which ensured synchronicity of infection with malaria.

PARASITE

A strain of *Plasmodium knowlesi* (W₁, kindly donated by Professor P.C.C. Garnham) has been maintained by serial blood passages in normal monkeys or cryopreserved in buffered glycerol in liquid nitrogen (Jeffery, 1962) and used for initiating infection from time to time.

INOCULATION OF PARASITE

The monkeys were inoculated generally with a standard inoculum of $1 \times 10^6$ parasitized erythrocytes intravenously through cephalic vein.

PARASITAEMIA

Blood smears from infected monkeys were prepared from ear vein and stained with Giemsa stain. Percent infection (parasitaemia) was determined by counting parasitaemia/$10^4$ erythrocytes. Infection became patent on day 3-4. Mean patent period was 4-5 days and monkey died after attaining a maximum parasitaemia. The monkey generally remained in coma for 2-4 hrs before death.

COLLECTION OF BLOOD

Blood from infected monkeys was collected by cardiac or vein puncture in acid citrate dextrose (ACD) (Kessel, Lewis, Pasquel and Turner, 1965) or in 0.85% (w/v)
sodium chloride containing 2.0% (w/v) sodium citrate. For the biochemical study the blood was always stored at 4°C. Normal blood was collected in ACD or citrate-saline by cardiac or vein puncture.

**SEPARATION OF ERYTHROCYTES**

Blood was centrifuged at 800 g for 10 min in refrigerated centrifuge. Plasma and buffy coat were aspirated with pipette and the erythrocytes were suspended in 0.85% (w/v) sodium chloride or phosphate buffer saline, pH 7.2 and centrifuged at 800 g for 10 min. Red cells were washed thrice in this manner and the leukocytes were removed every time. Smears were prepared to check the contamination of leukocytes. If they were present the erythrocytes preparation was again washed till it was almost free from leukocytes. About 99% population of white cells was removed in this way.

**ISOLATION OF SCHIZONT-INFECTED CELLS**

Infected blood was collected in ACD under sterile conditions and centrifuged at 800 g for 10 min. Plasma and buffy coat were removed as above and the schizont-infected cells which form a brownish layer above the normal erythrocytes, were aspirated with pasteur-pipette and washed thrice in buffered culture medium.

**CULTURE MEDIUM**

For the isolation of merozoites of *P. knowlesi* in vitro the parasites were cultured in complete medium 199 and for in vitro studies of erythrocytes invasion complete medium RPMI-1640 was used. Commercially available media were designated complete when supplemented with the followings:
glucose ... ... ... 2 mg/ml
adenosine triphosphate (ATP) ... 0.3 mg/ml
adenosine 5'-monophosphate (AMP) 0.26 mg/ml
L-glutamine ... ... ... 0.2 mg/ml
reduced glutathione ... ... 0.1 mg/ml
calcium pantothenate ... ... 0.01 mg/ml
4-aminobenzoic acid (pABA) ... 0.01 mg/ml
biotin ... ... ... 0.002 mg/ml
penicillin ... ... ... 100 iu /ml
streptomycin ... ... ... 0.1 mg/ml
0.5% sodium bicarbonate ... 3.5% (v/v)

pH of the medium was adjusted to 7.4 with additional sodium bicarbonate. In the invasion experiments 25 mM of N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer was added. The medium was then filtered through a membrane filter. Always a freshly prepared medium was used.

ISOLATION OF MEROZOITES

0.05 ml of packed schizont infected cells were added to 3.0 ml medium 199 and 0.3 ml normal inactivated rhesus monkey serum in a 50-ml Erlenmeyer culture flask. The flasks were then kept at 37°C under an atmosphere of 7% CO₂, 1% O₂ and 92% N₂ and were occasionally shaken. The rupturing of schizonts was observed by preparing smears of culture suspension at different intervals. After 5-8 hrs incubation when most of the schizonts were ruptured, the cell suspension was centrifuged at 750 g for 15 min in a refrigerated centrifuge. The supernatant was then centrifuged at 2,000 g for 20 min and merozoites were collected in the sediment, spread on a slide and stained. For biochemical study, the sedimented merozoites were washed thrice with chilled normal saline and stored at -20°C until used.
INVASION OF ERYTHROCYTES

Normal monkey blood was collected in ACD and erythrocytes were separated from other blood components by repeated washings with phosphate buffer-saline, pH 7.2. Schizont-infected cells, collected as given above, were mixed with washed erythrocytes approximately in the ratio of 1 to 50. Then 0.1 ml of these packed cells was added to a 50 ml Erlenmeyer culture flask containing 3.0 ml of buffered medium RPMI-1640 and 3.0 ml serum. To study the effect of different enzyme blockers on invasion, 0.1 ml of desired concentration of inhibitor was added to experimental flasks while in controls 0.1 ml sterile water was supplemented. The flasks were then incubated at 37°C (or required temperature) under 7% CO₂, 1% O₂ and 92% N₂ (or candle gas). After 15 hrs (or different intervals) of incubation the culture suspensions were centrifuged at 1,000 g for 10 min. Smears were prepared and stained with Giemsa and the percentage of infected cells was determined.

SEPARATION OF CELL-FREE PARASITES, ERYTHROCYTE MEMBRANES AND HEMOLYSATE

Normal and P. knowlesi-infected blood from monkeys was collected in citrate-saline at 4°C either by vein-puncture or cardiac puncture and the erythrocytes were separated from other blood components by repeated washings with prechilled normal saline (as above). The washed erythrocytes were then lysed with 0.2% (w/v) saponin in 0.85% (w/v) sodium chloride with continuous stirring in an icebath for 30 min (volume of saponin solution added was double the volume of packed erythrocytes). The lysed suspension was then centrifuged at 7,000 g for 30 min. In case of normal cells, the cell membranes (ghosts) were sedimented and the supernatant, hemolysate, was pipetted out. The ghosts were washed with prechilled normal saline till they were free from hemolysate and
the supernatant was always added to hemolysate. Infected cells suspension formed three different layers: the uppermost layer or hemolysate was followed by ghosts and the parasites collected in the sediment. The parasites were separated from hemolysate and ghosts by repeated washings with cold normal saline by centrifugation at 7,000 g for 30 min and designated "cell-free parasites".

Erythrocytes, hemolysate, ghosts and cell-free parasites were assayed for the enzymes soon after preparation or stored at -20°C.

PREPARATION OF HOMOGENATE

Freshly harvested or stored erythrocytes, merozoites, parasites and ghosts were homogenized in Potter-Elvehjem homogenizer at 4°C in prechilled 0.25M sucrose solution or glass distilled water. The resulting homogenate was used as such or was centrifuged at 1,800 g for 15 min. The crude enzyme preparation present in the opalescent supernatant was used for enzyme assay.

SUBCELLULAR FRACTIONATION

The following procedure was employed for fractionation of *P. knowlesi* (Fig.1). The homogenate of cell-free parasites (prepared in 0.25M sucrose solution) was centrifuged twice at 600 g for 15 min (Remi, K23 centrifuge, using SOH rotor). The two pellets were combined, washed by suspending in 0.25M sucrose and again sedimented at 600g for 15 min to yield 'nuclear fraction'. The supernatants and washed solutions from 600 g centrifugations were combined and centrifuged twice at 10,000 g for 25 min (IEC centrifuge with No.873 fixed angle rotor). The two pellets were combined and washed with sucrose as before and sedimented at 10,000 g for 23 min. The sediment represented the 'mitochondrial fraction'. The combined supernatants were centrifuged at 24,000 g for
35 min (Sorvall centrifuge with SS-34 fixed angle rotor). The combined sediment after washings with sucrose was the 'lysosomal fraction'. The supernatants after 24,000 g centrifugations were then centrifuged at 105,000 g for 60 min (Beckman model L ultracentrifuge with No.40 fixed angle rotor). The pellet after washings represented the 'microsomal fraction' and the supernatant designated as the 'soluble fraction'. The different sediments were suspended in known volume of 0.25M sucrose and assayed for the enzymes.

Homogenate

600g for 15 min

Sediment (nuclear) Supernatant

10,000 g for 25 min

Sediment (mitochondrial) Supernatant

24,000 g for 35 min

Sediment (lysosomal) Supernatant

105,000g for 60 min

Sediment (microsomal) Supernatant

(soluble)

Fig.1. A flow-diagram for the subcellular fractionation of *P. knowlesi*. 


ENZYME ASSAYS

PROTEINASE

Proteinase activity was determined according to a slightly modified method of Anson (1938). The assay mixture contained 100 μ moles of buffer (for acid proteinase Walpole buffer, pH 5.0 and phosphate buffer, pH 7.2 for alkaline proteinase), 70 μ moles of cysteine hydrochloride (adjusted to required pH with NaOH), 0.05% (w/v) haemoglobin and 0.3 ml suitably diluted enzyme in a final volume of 2.0 ml. Tubes were incubated at 37°C for 60 min with occasional shaking. The reaction was stopped by the addition of 1.0 ml of 10% (w/v) prechilled trichloroacetic acid (TCA) and the precipitate was removed by centrifugation at 1,500 g for 15 min in the cold. The assays were run in duplicate and under optimal conditions of hydrolysis. In controls the enzyme was supplemented after the addition of cold TCA. A suitably diluted aliquot of the protein-free supernatant was taken for colour development with Folin and Ciocalteau's reagent (Lowry, Rosebrough, Farr and Randall, 1951).

Unit of enzyme was that amount which liberated one μ mole of tyrosine in 60 min.

ACID PHOSPHATASE

Acid phosphatase activity was assayed according to a slightly modified method of Nelson (1966). A typical reaction mixture in a final volume of 2.0 ml contained 0.5 ml suitably diluted enzyme, 6 μ moles of ethylenediaminetetraacetic acid (EDTA-sodium salt), 75 μ moles of sodium β-glycerophosphate (pH adjusted to 5.0) and 150 μ moles of Walpole buffer, pH 5.0. The tubes were incubated at 37°C for 60 min with occasional shaking. The reaction was stopped by the addition of 1.0 ml of 10% (w/v) cold TCA and the precipitate was removed by
centrifugation at 1,500 g for 15 min in the cold. The assays were run in duplicate and under optimal conditions of hydrolysis. The inorganic phosphate (Pi) liberated was measured in the clear supernatant by the method of Fiske and SubbaRow (1925).

One unit of enzyme activity was that amount of enzyme which liberated one μmole of Pi at 37°C in 60 min.

**ALKALINE PHOSPHATASE**

The enzyme activity was assayed according to a slightly modified method of Morton (1954). A reaction mixture of 2.0 ml contained 0.5 ml enzyme, 3 μ moles of magnesium acetate, 75 μ moles of sodium β-glycerophosphate (pH adjusted to 9.0) and 150 μmoles of Veronal buffer pH 9.0. The tubes were incubated at 37°C for 60 min with occasional shaking. The reaction was stopped by the addition of 1.0 ml of 10% (w/v) prechilled TCA and the precipitate was removed by centrifugation at 1,500 g for 15 min in the cold. The assays were run in duplicate and under optimal conditions of hydrolysis. The inorganic phosphate (Pi) liberated was measured in the protein free supernatant by the method of Fiske and SubbaRow (1925).

One unit of enzyme activity was that amount of enzyme which liberated one μmole of Pi at 37°C in 60 min.

**RIBONUCLEASE**

Ribonuclease (RNase) activity was determined according to a slightly modified method of Ishihara, Irie and Ukita (1967). A typical reaction mixture contained 0.5 ml suitably diluted enzyme, 200 μmoles of buffer (Walpole buffer, pH 5.0 for acid RNase and phosphate buffer, pH 7.5 for alkaline RNase), 0.2% (w/v) purified ribonucleic acid dissolved in buffer in a final volume of 2.0 ml. The incubation was carried out at 37°C for 30 min with occasional shaking. The tubes were then cooled in an icebath and the
reaction was stopped by the addition of 1.0 ml of MacFadyan reagent (0.75\% (w/v) uranyl acetate in 25\% (v/v) perchloric acid) followed by immediate shaking to mix the contents of tube. After a lapse of 30 min in cold, the contents were centrifuged at 1,500 g for 15 min and an aliquot of the clear supernatant was suitably diluted and the absorbancy was read at 260 nm in a Beckman model DU spectrophotometer. Controls were simultaneously run and their values were subtracted from the corresponding experimental values. The assays were always run in duplicate and under optimal conditions of hydrolysis.

One unit of ribonuclease enzyme activity was defined as that amount of enzyme capable of bringing about an increase in OD at 260 nm by 0.001 per min under experimental conditions.

DEOXYRIBONUCLEASE

The assay of deoxyribonuclease (DNase) was performed according to a slightly modified method of Irie, Yamada and Ukita (1966). The reaction mixture in a total volume of 2.0 ml contained 0.2\% (w/v) purified deoxyribonucleic acid dissolved in 200 \( \mu \)moles of buffer (Walpole buffer, pH 5.0, for acid DNase and phosphate buffer, pH 7.5 for alkaline DNase), 5\( \mu \)moles of magnesium chloride and 0.5 ml of suitably diluted enzyme. After incubation of the mixture at 37°C for 30 min the digest was chilled in icebath and the reaction was stopped by the addition of 1.0 ml of MacFadyan reagent. Contents of the tube were mixed immediately by thorough shaking of the tube. After 30 min in cold the tubes were centrifuged at 1,500 g for 15 min and an aliquot of the clear supernatant was suitably diluted and the absorbancy was measured at 260 nm in a Beckman model DU spectrophotometer. Controls were simultaneously run and their values were subtracted from the corresponding experimental values. The assays
were run in duplicate and under optimal conditions of hydrolysis.

One unit of deoxyribonuclease enzyme activity was the amount of enzyme capable of bringing about an increase in O.D. at 280 nm by 0.001 per min under experimental conditions.

**GLUCOSE - 6-PHOSPHATASE**

Glucose-6-phosphatase activity was determined according to a slightly modified method of Swansen (1950). A typical reaction mixture of 0.5 ml contained 0.1 ml diluted enzyme, 3.0 μmoles of tris-HCl buffer, pH 7.4, 10 μmoles of glucose-6-phosphate (sodium salt) and 10 μmoles of magnesium chloride. The tubes were incubated at 37°C for 30 min and then 1.0 ml of 10% (w/v) of prechilled TCA was added. The assay mixture was then diluted to 2.5 ml and centrifuged at 1,500 g for 15 min in the cold. The assays were run in duplicate and under optimal conditions of hydrolysis. The release of inorganic phosphate (Pi) was measured in the clear supernatant by the method of Fiske and SubbaRow (1925).

One unit of enzyme activity was that amount of enzyme which liberated one μmole of Pi at 37°C in 60 min.

**SUCCINIC DEHYDROGENASE**

The enzyme activity was assayed spectrophotometrically according to a modified method of Slater and Bonner (1952). The assay mixture in a final volume of 1.6 ml contained 50 μmoles of phosphate buffer, pH 7.2, 20 μmoles of neutralized potassium cyanide, 5 μmoles of potassium ferricyanide, 10 μmoles of sodium succinate and 0.5 ml of enzyme suspension. After 10 min of incubation at 37°C the reaction was stopped by the addition of 2.0 ml of 5% (w/v) prechilled TCA and the precipitated proteins were
centrifuged after one hour. The extinction of supernatant was measured at 400 nm in a Beckman model DU spectrophotometer using cuvettes of 1 centimeter light path. In controls the enzyme was added after the addition of TCA.

Enzyme activity was expressed as the number of μmoles of potassium ferricyanide reduced under the experimental conditions.

5'-NUCLEOTIDASE

5'-nucleotidase activity was determined according to a slightly modified method of Serrano, Das and Warren (1977). An assay mixture of 2.0 ml contained 0.5 ml of suitably diluted enzyme, 80 μmoles of tris-HCl buffer, pH 7.4, 10 μmoles of magnesium chloride, 5 μmoles of adenosine 5'-monophosphate (sodium salt) and 0.05% (w/v) sodium deoxycholate. After incubation for 60 min at 37°C the reaction was stopped by the addition of 1.0 ml of 1.5N perchloric acid and the precipitate was removed by centrifugation at 1,500 g for 15 min in the cold. The assays were run in duplicate and under optimal conditions of hydrolysis. The liberation of inorganic phosphate (Pi) in the supernatant was measured according to the method of Fiske and SubbaRow (1925).

One unit of enzyme activity was that amount of enzyme which liberated one μmole of Pi at 37°C in 60 min.

SPECIFIC ACTIVITY

Specific activity of the enzyme was expressed as units of enzyme per milligram protein.

ESTIMATION OF PROTEIN

The protein contents of the enzyme preparations were estimated colorimetrically using a slight modification of the Folin phenol method of Lowry, Rosebrough, Farr and Randall (1951).
8.0% (w/v) solution of anhydrous sodium carbonate was mixed with equal volume of a solution containing 0.06% (w/v) of cupric sulphate and 0.12% (w/v) sodium potassium tartarate. Solutions containing 10 to 150 micrograms of protein were mixed with 5.0 ml of the above reagent, incubated for 10 min at 37°C and then cooled to room temperature. 0.5 ml of the twice diluted Folin phenol reagent was added and the colour developed was read at 660 nm after 30 min against a proper blank in Klett Summerson photoelectric colorimeter using bovine plasma albumin as the standard. Under these conditions one klett reading was found to be equal to one microgram of the standard protein solution.

PREPARATION OF DIFFERENT HAEMOGLOBINS

Crystalline or denatured human haemoglobin was commercially available. The haemoglobin of other animals could not be procured, so it was prepared by the method given below:

10.0 ml blood from man, monkey, rabbit, guinea-pig, hamsters, rats and mice was collected in 1.0 ml of 2% (w/v) sodium citrate in 0.85% (w/v) sodium chloride and centrifuged at 800g for 10 min. After removing plasma and buffy coat, the erythrocytes were washed thrice in normal saline and lysed by repeatedly freezing and thawing and then centrifuged at 7,000 g for 30 min. The supernatant (hemolysate) was boiled for 15 min and the precipitate was homogenized; then 0.5 ml aliquot was added to the reaction mixture.

EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

Suitable aliquots of enzyme preparations were taken in a series of small test tubes and immersed in a water bath at the desired temperatures. The tubes were gently shaken for half a min and then corked so that no water
could evaporate. After heating for 20 min each tube was plunged into an icebath which quickly stopped any further destruction of enzyme. The corked tubes were tilted and rotated horizontally to collect condensed moisture on the wall and after removing the corks reaction mixture was added and incubated at 37°C for desired period.

**EFFECT OF ACTIVATORS, INHIBITORS, DRUGS AND ANTIBIOTICS**

Desired concentrations of activators, inhibitors, drugs and antibiotics were added to the tubes containing suitable enzyme preparations and incubated at 37°C for 10 min along with controls and then other constituents of assay mixture were added. The tubes were then incubated at 37°C for desired time (60 min /30 min ) and the enzyme activity was determined.

**PURIFICATION OF ENZYMES**

**AMMONIUM SULPHATE FRACTIONATION**

To the crude extract of the parasite ammonium sulphate was added slowly and with continuous stirring to make 20% saturation in cold. During the addition of ammonium sulphate the pH of the mixture was maintained at 5.0 in case of acid proteinase and acid phosphatase and 7.5 in alkaline proteinase. The mixture was allowed to stand at 5±1°C for 8 hrs and then centrifuged at 3,000 g for 20 min. The concentration of the supernatant was raised upto 40% saturation with additional ammonium sulphate and kept for 8 hours in cold. Precipitate was centrifuged and the supernatant was processed to 40-60% and then to 60-80% saturations with ammonium sulphate in the same way as in 20-40%. The precipitates obtained after different saturations were dialysed separately against buffer (acetate buffer for acid proteinase and acid phosphatase, and phosphate buffer for alkaline proteinase). The
unwanted proteins if precipitated during dialysis were centrifuged and the dialysates were made upto a known volume and the enzyme activity was determined. Further saturation of extract with ammonium sulphate to 100% yielded no precipitate; so a part of the mixture was dialysed and assayed. The dialysed fraction containing maximum enzyme activity was applied on DEAE- cellulose column for further purification.

**DEAE - CELLULOSE CHROMATOGRAPHY**

**PREPARATION OF THE DEAE-CELLULOSE COLUMN**

Five grams of DEAE-cellulose (exchange capacity 0.91 m equivalent per gram) were suspended in glass distilled water and allowed to swell for 3 to 4 hrs. The slurry was diluted to one litre, stirred and allowed to settle for about 20 min and decanted several times to remove non-sedimenting material. This process was repeated three times till the supernatant was free from turbidity. The resin was suspended in 500 ml 1N sodium hydroxide and allowed to remain in contact with alkali for 30 min. The adsorbent was then transferred to a Buchner funnel fitted with Whatman 3 mm filter paper and washed with glass distilled water after which the resin was suspended in 0.5N hydrochloric acid. A layer of glass wool was placed at the bottom of the column to hold the cellulose ion exchanger. The slurry was poured into the column and allowed to run under gravity. A circular piece of sponge was placed on the top of the column to prevent any disturbance of the resin during the addition of the eluting buffer. The chloride form of the resin thus obtained was washed free of hydrochloric acid with glass distilled water. The column was then equilibrated with 0.1M buffer (acetate buffer, pH 5.0 in case of acid proteinase and acid phosphatase and phosphate
buffer, pH 7.5 in case of alkaline proteinase and hereafter referred to as equilibrating buffer for respective enzyme) and finally transferred to the cold room (4-6°C). The flow rate of the eluate was adjusted to 1 ml/min. The regeneration of the resin after chromatography was carried out by passing sodium hydroxide, distilled water, hydrochloric acid, distilled water and buffer in the same order as given above. The same column could be used repeatedly by the recycling procedure.

**STEPWISE ELUTION**

For stepwise elution acetate or phosphate buffer containing concentrations of sodium chloride between 50-400mM were employed. The buffers with increasing concentration of chloride were passed through the column one after the other always maintaining 3-5 ml volume of buffered sodium chloride at the top of the exchanger. The dialysed supernate obtained after ammonium sulphate fractionation containing 9-12 mg protein, was applied to the top of the adsorbent using a pipette and allowed to run under gravity. When the enzyme had almost disappeared into the bed column 50 ml of equilibrating buffer was passed and the column was then fitted with a separating funnel for stepwise elution. The rate of elute was adjusted to 1 ml/min and 10 ml fractions were collected.