CHAPTER II

MATERIALS AND METHODS.

Instruments.

1. Microhaematocrit centrifuge (Clay-Adams)

2. Photometer (Bausch & Lomb)

3. Centrifuge (International Portable Refrigerated Centrifuge Model PR-2)

4. pH meter (Backman)

5. Automatic sample collecting apparatus (Ultrorac Fraction Collector Type 7000)

6. Well-type scintillation counter (Nuclear Chicago Model 181B)

7. Liquid scintillation counter (Nuclear Chicago Model 186A)

Radioisotopes.

1. Sodium chromate (^{51}Cr) solution B.P. (Amersham)

2. Ferric citrate (^{59}Fe) solution B.P. (Amersham)

Reagents.

1. Saponin
2. Normal saline solution
3. Nembutal sodium (Abbott)
4. Heparin (Nordmark)
5. Sodium arsenate (May & Baker)
6. Magnesium sulphate (May & Baker)
7. Potassium phosphate (E. Merck)
8. Adenosine triphosphate (Sigma Chemical)
9. Luciferase enzyme (Sigma Chemical)
10. Methylene blue (E. Merck)
11. Azure I (BDH)
12. Disodium hydrogen phosphate (E. Merck)
13. Potassium dihydrogen phosphate (E. Merck)
14. Eosine (BDH)
15. Stain powder (E. Merck)
16. Glycerine
17. Absolute methyl alcohol (E. Merck)
18. Potassium ferricyanide (BDH)
19. Potassium cyanide (May & Baker)

20. Sodium bicarbonate (BDH)

21. Sulphuric acid (May & Baker)

22. Perchloric acid (May & Baker)

Parasite.

Plasmodium knowlesi malaria was obtained from the Liverpool School of Tropical Medicine, Liverpool, England.

Animal.

Rhesus monkeys (Macaca mulatta) were purchased from the animal houses. They were the local monkeys obtained from the Southern Thailand.

Experimental Methods.

1. The parasite.

Plasmodium knowlesi was originally found as a naturally occurring infection in Macaca irus, the crab eating monkeys of South East Asian. In M. mulatta (Rhesus monkey) the infection, if untreated, was fatal in 5-8 days, and the erythrocytic cycle of schizogony was repeated every 24 hours. The most suitable model for laboratory studies is P. knowlesi

malaria in rhesus monkeys, since there are many features of the disease which resemble malignant tertian malaria. P. knowlesi stain was obtained from Liverpool School of Tropical Medicine, Liverpool, England.

2. Experimental animals.

Rhesus monkeys (Macaca mulatta) weighing between 2.8-4.0 kg were used. The animals were obtained from Southern Thailand.

2.1 Normal monkeys. The healthy monkeys were used as a control group for this study. They were free from the haematological and intestinal parasites during the course of the experiment. Animals were kept in the individual metabolic cages and received a conventional dict of vegetables, fruits and drinking water.

2.2 Infected monkeys. Healthy monkeys were infected with P. knowlesi by intravenously injection with 2.0-4.0 ml of whole blood containing 0.5-2.0 million trophozoites from a donor monkey or 1-2 ml of frozen infected blood. The frozen infected blood was transfered from dry ice into a waterbath at 37° - 40° C. Blood was then centrifuged at 25,000 r.p.m.

for 5 minutes. Red blood cell was washed with 0.9 % normal saline solution for 3 times before it was injected intravenously into a recipient monkey.

3. Study on ATP content of red blood cell.

Red blood cell ATP levels were determined by the luciferase enzyme using liquid scintillation counter (Stanley and Williams, 1969).

3.1 Preparation of buffer.

0.1 M. Sodium Arsenate Buffer.

Sodium arsenate	31.20	gm
Magnesium sulphate	9.86	gm
Distilled water q.s.	1000.00	ml

Adjust pH to 7.4 with 1 N. H_2SO_4.

Any precipitate which formed on standing was removed by filtering through a Whatman No. 1 filter paper.

0.01 M. Potassium phosphate Buffer.

Potassium phosphate	1.74	gm
Magnesium sulphate	0.986	gm
Distilled water q.s.	1000.00	ml

Adjust pH to 7.4 with 1 N. H_2SO_4.

3.2 Preparation of stock solution of ATP.

Adenosine triphosphate	5.512	mg
Potassium phosphate buffer q.s.	1000.00	ml

This stock solution has concentration of 10^{-7} mole/ml, was kept in freezer and standard solutions of ATP (10^{-9} - 10^{-10} mole/ml) were prepared shortly before required, by serial dilution of this stock solution with potassium phosphate buffer, and were kept on ice.

3.3 Preparation of the luciferase enzyme solution.

Five ml of distilled water was added to vial of luciferase enzyme, mixed well, centrifuged and kept on ice. Use only supernatant for determination of ATP.

3.4 Preparation of standard ATP curve.

A 10^{-9} mole/ml of ATP solution was prepared by diluting 1.0 ml of stock solution of ATP (10^{-7} mole/ml) with potassium phosphate buffer to 100.0 ml and used this ATP solution in preparing standard ATP curve.

Each vial containing 1.0 ml of sodium arsenate buffer and 1.0 ml of water, keep these vials cooled throughout assaying.

A series of various concentration was prepared by

adding standard ATP solution and potassium phosphate buffer to the vial containing sodium arsenate buffer and water. The amount of standard ATP solution and potassium phosphate buffer added as follow;-

ATP concentration (mole/ml)	ml of Std. ATP (10^{-9} mole/ml)	ml of Phosphate buffer
$10^{-9.0}$	1.0	0.0
$10^{-9.3}$	0.5	0.5
$10^{-9.4}$	0.4	0.6
$10^{-9.6}$	0.25	0.75
$10^{-10.0}$	0.1	0.9

Then 0.05 ml of luciferase enzyme solution was added to each vial, mixed well and count the number of photon produced within 6 seconds by using liquid scintillation counter (Nuclear Chicago Model 186A). Plot standard curve of ATP by using log count per 6 seconds and -log ATP (mole/ml) which will be a straight line. Using this standard curve for determination of ATP content in the sample.

3.5 Preparation of blood sample for determination of ATP content. One volume of whole blood was precipitated without delay with three volumes of ice-cold 6 % (w/v) perchloric acid. After thorough stirring which was necessary for good extraction of ATP, aliquots of the supernatant obtained by centrifugation were used for further diluting. One ml of the dilution was added to the vial containing sodium arsenate buffer and water and added 0.05 ml of luciferase enzyme solution and count by using liquid scintillation counter in the same manner of standard ATP solution. The amount of ATP per 100 ml of RBC was calculated from log count per 6 seconds and the standard ATP curve.

4. Study on trapped RBC in rhesus monkeys.

4.1 Labelling infected RBC with ^{51}Cr. (Silver, 1963)

(a) About 15 ml of infected blood are withdrawn from femeral vein and transfer to 4.5 ml of ACD solution.

(b) Centrifuge at 1,500 r.p.m. for 5 minutes. (International Portable Refrigerated Centrifuge Model PR-2).

(c) The plasma is removed to be used for the preparation of the subsequent washing solution (2-3 % in

normal saline solution). This solution is kept at low temperature, about 10° C.

(d) About 50 μCi of ^{51}Cr ($Na_2{}^{51}CrO_4$) is added to the packed cells, mixed well and the mixture is left at room temperature for about 20 minutes with gentle shaking at frequent intervals.

(e) After the incubation period the red blood cells are washed thrice with plasma-saline solution. A fraction of ^{51}Cr-RBC is kept to prepare the standard solution.

(f) The standard solution is prepared by making up 0.1 ml of ^{51}Cr-RBC to 50.0 ml in a volumetric flask with distilled water (a small amount of saponin has been added to lyse cells).

4.2 Labelling normal RBC with ^{59}Fe in vivo. The normal monkey was injected intravenously with a ^{59}Fe-ferric citrate solution containing 500 μCi of ^{59}Fe, about three weeks after the injection, blood was completely labelled with ^{59}Fe in vivo. The labelled blood was withdrawn and prepared as labelling infected RBC with ^{51}Cr from (a) to (f) except no $Na_2{}^{51}CrO_4$ is added to the red blood cell.

Mixed ^{51}Cr-labelled infected RBC with ^{59}Fe-labelled

normal RBC. A fraction of this mixed blood is kept to prepare the standard solution as in 4.1 (f). The residue is kept for injection into a common carotid artery of the experimental monkey.

4.3 Method of study in experimental monkeys.

4.3.1 Anaesthetic. The experimental monkey was anaesthetized with Sodium Pentobarbital (Nembutal Sodium; Abbott) at the dosage of 25 mg/kg body weight by injection intravenously.

4.3.2 Procedure for study on trapped RBC in experimental monkeys. The mixed solution of ^{51}Cr-infected RBC and ^{59}Fe-normal RBC was used as the tracer for detecting the trapped RBC in normal and a P. knowlesi-infected monkey.

After rapid injection of a known volume and radioactive concentration of a mixed labelled RBC solution into a common carotid artery of a monkey, a continuous series of samples was collected from both of the internal jugular veins which were connected with an automatic sample collecting apparatus (Ultrorac Fraction Collector Type 7000) by means of polyethylene tubes. Twenty samples were collected in the course of 1 minute starting at the time of injection. The sample was assayed in

well-type scintillation counter (Nuclear Chicago Model 181B) connected to pulse height analyzer, so that the radioactivity of ^{51}Cr and ^{59}Fe could be counted separately. (Operate instrument at H.V. = 680 volts, window width = 10. For ^{51}Cr, energy 0.32 Mev, count at gain 16 with window count, and for ^{59}Fe, energy 1.10 and 1.30 Mev, count both of gain 8 with base count and gain 16 with window count). Then the radioactivity in the sample (% of dose given) was plotted against time.

5. Method of parasite counts and haematological studies.

5.1 Parasite count. Parasite counts were made on blood sample obtained by ear prick or venipuncture. The first appearance of the parasite in the peripheral blood was detected by examination of thick blood film treated with Field's Stain. When the parasitaemia is higher, the parasite count was done on the thin blood film treated with Wright's Stain. In each film the parasites were classified and enumerated roughly under three different stages, namely, (1) early trophozoites (ring form), (2) late trophozoites (amoeboid form) and (3) schizonts. Gametocytes were not

included. The term " parasitaemia " is used throughout this work to denote the percentage of erythrocytes infected, and not the number of parasites per 100 erythrocytes. The distinction is important owing to the frequency of multiple cell infections. The counts were made usually of 1000 erythrocytes or 200 white blood cells in thin and thick films respectively.

5.1.1 Field's Stain.

Stain A.

Methylene blue	0.8	gm
Azure I	0.5	gm
Disodium hydrogen phosphate	5.0	gm
Potassium dihydrogen phosphate	6.25	gm
Distilled water q.s.	500.0	ml

Stain B.

Eosine	1.0	gm
Disodium hydrogen phosphate	5.0	gm
Potassium dihydrogen phosphate	6.25	gm
Distilled water q.s.	500.0	ml

After 24 hours, the solution A and B were filtered and were ready for use, they should be filtered if scums form at a later date.

Procedure for staining with Field's Stain.

The slide with dry thick blood film was dipped in a Stain A for 2-5 seconds and washed in tap water for 10 seconds, or until stain ceased to run from the film. Then it was dipped in Stain B for 1-3 seconds and washed in tap water for 5 seconds. Finally, the slide was placed on end to dry.

5.1.2 Wright's Stain.

Stain powder	0.3	gm
Glycerine	3.0	ml
Absolute methyl alcohol, acetone free	97.0	ml

The stain powder was grined with glycerine and added methyl alcohol into the solution. The mixture was kept in a tightly stopped brown glass bottle for 2-3 weeks, and was filtered before use.

Phosphate Buffer pH 7.2

Potassium dihydrogen phosphate	0.7	gm
Disodium hydrogen phosphate	1.0	gm
Distilled water q.s.	1000.0	ml

Procedure for staining with Wright's Stain.

The slide with dry thin blood film was covered with Wright's Stain for 3 minutes. An equal amount of phosphate

buffer was added and left for 3 minutes. Then it was washed by flooding with tap water and placed on end to dry.

The slides were examined under microscope by oil immersion lens.

5.2 Haemoglobin estimation. The haemoglobin level was determined by the cyanomethaemoglobin method (Oser, 1965).

Drabkin's Solution.

Potassium ferricyanide	200	mg
Potassium cyanide	50	mg
Sodium bicarbonate	1	gm
Distilled water q.s.	1000	ml

Procedure for determination of haemoglobin.

A 0.02 ml of well-mixed blood from an accurately calibrated pipet was added to 5.0 ml of Drabkin's Solution and mixed well. Let stand for 10 minutes and read in a photometer (Bausch & Lomb) at 540 mμ zeroing the photometer with the Drabkin's Solution. The percentage of absorption was read from the scale, and a calculation in grams of haemoglobin per 100 ml of blood was carried out if required or determined from standard table for haemoglobin.

5.3 Haematocrit level determination. Duplicate determinations of haematocrit levels were performed using a microhaematocrit centrifuge (Clay-Adams). Two thirds of the heparinized microhaematocrit tubes were filled with blood, one end was sealed with plasticene. The tubes were placed in opposite direction in a microhaematocrit centrifuge which were centrifuged at 11,800 r.p.m. for 5 minutes. Haematocrit levels were measured in percentage by a special reading provided with the centrifuge. The variation of duplicate samples was within 1 %.