CHAPTER II

MATERIALS AND METHODS

INSTRUMENTS

1. Liquid scintillation counter (Nuclear Chicago Model 186 A)
2. Centrifuge (International Protable Refrigerated Centrifuge Model PR-2)
3. pH meter (Beckman)
4. Microhaematocrit centrifuge (Clay-Adams) with micro-capillary Reader (IEC)
5. Electrical balance (E. Mettler, Type H 16)
6. Super-mixer (Vortex-Genic)
7. Microscope (Leitz)

CHEMICALS

1. Adenosine-5′-triphosphate disodium salt (Sigma Chemical Company)
2. Buffered firefly lantern extract (Luciferase enzyme) (Sigma Chemical Company)
3. Sodium arsenate (May & Baker)
4. Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) (May & Baker)
5. Potassium monohydrogen phosphate G.R. (E. Merck)

6. Heparin sodium (Nordmark-Werke, Germany)
7. Potassium dihydrogen phosphate G.R. (E. Merck)
8. Powdered Wright's stain (E. Merck)
9. Absolute methyl alcohol, acetone-free (E. Merck)
10. Perchloric acid (May & Baker)
11. Sulphuric acid (May & Baker)
12. Sodium bicarbonate (BDH)
13. D-glucose (E. Merck)
14. Potassium chloride (E. Merck)
15. Calcium chloride (BDH)
16. Sodium chloride (E. Merck)
17. Sucrose (E. Merck)
18. Methylene blue (E. Merck)
19. Azure I (BDH)
20. Eosine (BDH)
21. Disodium hydrogen phosphate (E. Merck)
22. Glycerin (E. Merck)

All chemicals were of analytical grade.

PARASITES

1. Plasmodium berghei malaria (P. berghei) was obtained from the SEATO Medical Research Laboratory, Thailand.

2. Plasmodium knowlesi malaria (P. knowlesi) was obtained from the Liverpool School of Tropical Medicine, Liverpool, England.

METHODS

I. Experimental Subjects

1. Human blood samples

Blood samples were obtained from Thai blood donors at the Thai Red Cross Society.

Infected blood samples were taken from patients with P. falciparum malaria admitted into the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Bangkok.

2. Animals

2.1 The mice used for this work were albino mice of 20-25 g in weight. Mice were injected intraperitoneally with 0.05 ml of P. berghei-infected blood (diluted with 0.9% W/V aqueous sodium chloride solution) containing approximately 100 million parasites per ml from a donor mice.

Thin blood smear were stained for parasites every day. When parasitaemia were 10-70%, which was normally about 4-7 days after infection, the infected blood was inoculated into the recipient mice.

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

The healthy monkeys weighing between 2.8-4.0 kg were injected intravenously with 2.0-4.0 ml of whole blood containing 0.5-2.0 million P. knowlesi malaria from a blood donor monkey or 1-2 ml of frozen infected blood. The frozen infected blood was transfered from dry ice into a water bath at 37°C-40°C. Blood was then centrifuged at 25,000 rpm for 5 minutes. Red blood cell was washed with 0.9% normal saline solution for 3 times before it was infected into a recipient monkey.

II. Preparation of blood samples

1. Mice: Blood was withdrawn from mice infected with P. berghei malaria by the cardiac puncture. Twenty units of heparin sodium solution per ml of blood was used as an anticoagulant.

2. Monkeys: Blood was taken from the infected monkey with P. knowlesi by ear prick or venipuncture using the heparin as an anticoagulant.

3. Human blood was drawn from the vein and collected in the heparinized solution.

III. Study on ATP content of red blood cells

1. Method of extraction of ATP

One volume of heparinized blood samples 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 supernatant obtained by centrifugation were used for the estimation of ATP by the luciferase enzyme using the liquid scintillation counter as described by Stanley and Williams (1969).

2. Preparation of buffer solution

2.1 Sodium arsenate buffer (0.1 M)

A 0.1 M sodium arsenate buffer containing 40 mM magnesium sulfate was prepared from:

Sodium arsenate	31.201	g
Magnesium sulfate	9.857	g
Distilled water add to	1,000	ml

The pH of this solution was adjusted to 7.4 with N sulfuric acid. Any precipitation formed on standing was removed by filtering through a Whatman No. 1 filter paper.

2.2 Potassium phosphate buffer (0.01 M)

A 0.01 M potassium phosphate buffer containing 4 mM magnesium sulfate was prepared from:

Potassium phosphate	1.7418 g
Magnesium sulfate	0.9857 g

Distilled water add to 1,000 ml and the pH was also adjusted to 7.4 with N sulfuric acid.

3. Preparation of stock solution of ATP solution

A 10^{-4} M (10^{-7} mole/ml) solution of ATP was prepared using:

Adenosine triphosphate	5.512 mg
Potassium phosphate buffer add to	1,000 ml

This stock solution was kept in the freezer. The standard solution (10^{-9} - 10^{-10} mole/ml) was prepared shortly before experiment by the serial dilution of this stock solution with phosphate buffer, and stored in ice.

4. Preparation of the luciferase enzyme solution

Five ml of distilled water was added to a vial of luciferase enzyme, mixed, centrifuged at 1,500 rpm for 5 minutes. The supernatant was used for the determination of ATP. This solution was normally freshly prepared each day.

5. Preparation of a standard ATP curve

The standard solutions of ATP were prepared by diluting 0.05 ml of stock solution of ATP (10^{-7} mole/ml) with potassium phosphate buffer to 5 ml (10^{-9} mole/ml). The following standard solutions were prepared:

ATP concentration (mole/ml)	ml of Std. ATP (10^{-9} mole/ml)	ml of Phosphate buffer
$10^{-9.0}$	1.00	0.00
$10^{-9.3}$	0.50	0.50
$10^{-9.4}$	0.40	0.60
$10^{-9.6}$	0.25	0.75
$10^{-10.0}$	0.10	0.90

Each vial containing 1.0 ml arsenate buffer and 1.0 ml of distilled water was added with 1.0 ml of the different concentration of the standard solution of ATP. Fifty microlitre of luciferase enzyme solution was added to each of these vials, mixed well and counted for 6 seconds in the liquid scintillation counter (Nuclear Chicago Model 186 A).

Standard curve of ATP was obtained from plotting log count of activity per 6 seconds and -log ATP (mole/ml).

It is necessary to prepare the standard curve every day. Since the activity of enzymes decreased during incubation (Lyman and De Vincenzo, 1967). The enzyme solutions are stable for 24 hr at $0^{o}C$.

The calibration curve in this study is found to be linear over the wide range of 10^{-9} to 10^{-12} mole/ml.

6. Preparation of blood sample for determination of ATP content

One ml of the extracted blood was added to a vial contained 1.0 ml of arsenate buffer and 1.0 ml of distilled water. Fifty microlitre of luciferase enzyme solution was added and counted in the liquid scintillation counter. These volumes should be dispensed accurately since the arsenate affects the activity of the enzyme.

The ATP content was expressed as micromolar (μM) per 100 ml of red blood cell (RBC).

CALCULATION

$$\text{ATP content in RBC} = \frac{\text{dilution} \times 10^{8} \times 10^{-\log \text{ATP}}}{\frac{\text{Haematocrit}}{100}} \ \mu M/100 \text{ ml RBC}$$

IV. Determination of the haematocrit level

This test measures the proportion of red blood cells to plasma. The haematocrit reading gives the number

of millimeters of packed red cells per 100 millimeters blood indicating the volume (%) of packed red cells per 100 ml blood.

Microhaematocrit method

Two heparinized capillary tube (Jintan Terumo Co., Ltd) was filled with blood to a level of about two-thirds of the tubes. The dry end of the tube was sealed with the clay and centrifuged at 14,000 rpm in a micro-haematocrit centrifuge (Clay-Adams) for 5 minutes. The haematocrit concentration was measured using a special micro-capillary reader (IEC). The variation of the duplicate samples was within 1%.

V. Blood Examination

1. Blood smear and staining

Blood samples were smeared on the slides and stained by the Wright's stain for a thin blood film and Field's stain for a thick blood film (Hepler, 1973).

2. Parasite counts

Thin blood films from mice, monkey or man treated with Wright's stain were microscopically examined under the oil immersion lens. In every blood film the parasites were classified and enunnerated in three stages, (1) ring form (young trophozoites), (2) 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, not the number of parasites per 100 erythrocytes. This distinction is important owing to the frequency of multiple cell infections. The counts were made usually of 1,000 erythrocytes of 200 white blood cells in thin and thick films respectively.

3. Wright's stain method

Wright's stain solution. It was prepared from:

Powdered Wright's stain	0.3 g
Glycerine	3.0 ml
Methyl alcohol (acetone free) to	100 ml

The powder was mixed with 3 ml of glycerine and added a portion of methyl alcohol. The mixture was kept in a tightly stopped brown glass bottle for 1-2 weeks, and was filtered before use.

Buffer solution. Phosphate buffer pH 7.2 was prepared from:

Potassium dihydrogen phosphate	0.7 g
Disodium hydrogen phosphate	1.0 g
Distilled water add to	1,000 ml

Preparation method

The slide of the dry thin blood film was covered with Wright's stain for 3 minutes as fixative. Add buffer of about the same quantity as the stain and left for 3-6 minutes. Then wash stain off with tap water until smear is pink and translucent under microscope by oil immerssion lens.

VI. Separation of parasitized red blood cells from non-parasitized red blood cells

Infected red blood cells were separated from non-infected red cells using the sucrose in Krebs glucose-saline solution by a method which was modified from Williamson and Cover (1966).

Preparation of stock solutions

1. Krebs glucose-saline solution

Sodium chloride	5.54 g
Potassium chloride	0.35 g
Magnesium sulfate	0.29 g
Calcium chloride	0.28 g
Potassium dihydrogen phosphate	0.16 g
Sodium bicarbonate	2.10 g
Glucose	2.10 g
Distilled water add to	1,000 ml

2. M Sucrose solution

Sucrose	342.30	g
Distilled water add to	1,000	ml

Working solutions

Sucrose (0.7 M) in Krebs glucose-saline solution was prepared by adding 7 ml of M sucrose solution to 3 ml of Krebs glucose-saline solution.

Sucrose (0.8 M) in Krebs glucose-saline solution was prepared by adding 8 ml of M sucrose solution to 2 ml of Krebs glucose-saline solution.

Method of separation of parasitized RBC from non-parasitized RBC

Half ml infected blood was mixed with 1.5 ml of 0.7 M sucrose in Krebs glucose-saline solution. After mixing gently, the mixture was centrifuged at 1,000 rpm for 5 minutes. The upper layer of red blood cell was pipetted into another tube and both tubes were washed with 0.9% sodium chloride solution. The suspension was centrifuged at 2,300 rpm for 5 minutes and the supernatant was discarded. The red cells were then resuspended in 0.9% sodium chloride solution up to about 0.2 ml. Each suspension of red blood cell was determined for haematocrit, parasitaemia and ATP concentration.