# CHAPTER III

## MATERIALS AND METHODS

(1) Experimental animals

The studied were performed in rhesus monkey (Macaca mulatta) with body weight ranging between 2.5 to 5.0 Kg. They were bought from Southern Thailand. The monkeys 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 diet of vegetables, fruits and drinking water.

(2) Preparation of ethyl palmitate solution

10-20% Ethyl palmitate solution was prepared by the following method :

| | | |
|---|---|---|
| Ethyl Palmitate | 10-20 | gm |
| Tween 20 | 0.1 | gm |
| Dextrose (5%) q.s. ad | 100 | ml |

The emulsion then was homogenized for 15 minutes and sterilized in the autoclave. Prior to use, the emulsion was incubated at $37^{0}$ C.

(3) Preparation of microaggregate human serum albumin

(Ilo and Wagner, 1963)

Ten ml of 30% Human Serum Albumin (HSA) was diluted with 0.9% NaCl to the final solution of 100 ml of 3% HSA, the concentration of HSA was then 30 mg/ml. It has been reported

that the particle size of aggregated 3% HSA solution was the most suitable one for studying the R.E.S. phagocytic activity when compared with 1, 2, 3, 4, 6, and 8% aggregated HSA. The pH of the diluted HSA solution was then adjusted to 10.0 with 0.2 N NaOH and the optical density ranged from 0.0367 to 0.047 at a wavelength of 525 mμ with Beckman spectrophotometer. After measuring the optical density, the HSA solution was placed in a water-bath at $70^{0}$ C and shaken continuously at a rate of 100 times per minutes for 20 minutes and followed immediately by shaking for an additional 100 times per minutes for 15 minutes at $79^{0}$ C. If shaking properly, the optical density would increase 0.026 ± 0.005 units (mean ± 1 SD) above the initial value after cooling with tap water. The HSA solution was centrifuged at 3,000 rpm for 20 minutes, and then the supernatant fluid was decanted and replaced by an equal volume of 0.1 N $Na_2CO_3$ and was left overnight at $4^{0}$ C until the precipitate was almost completely redissolved with a slightly opalescent solution.



The diluted HSA solution could be stored at $4^{0}$ C in a refrigerator without changing its physiological behavior for 8 months.

(4) Radioiodination of microaggregated albumin

The process was a modification of Mc Farlane's iodine monochloride system (1958) as described by Briner (1968).

Materials:

Reagents:

(a) Iodine monochloride (Bale et al, 1962)

Stock solution

| | | |
|---|---|---|
| KI | 0.5550 | gm |
| $KIO_3$ | 0.3567 | gm |
| NaCl | 29.53 | gm |
| HCl conc. (sp. gr. 1.18) | 21.00 | ml |
| Water for Injection | 250.00 | ml |
| $CCl_4$ | 3-4 | ml |

Dissolving the KI, $KIO_3$ and NaCl in approximately 150 ml of Water for Injection, the solution was then added with concentrated HCl and Water for Injection to the final volume of 250 ml. Free iodine which was present could be removed by repeating shaking the solution with $CCl_4$. Dissolved or suspended $CCl_4$ after vigorously shaking was removed out by bubbling a current of air saturated with water vapor to volatilize the organic solvent.

If carefully prepared this stock solution contains 2.5 mg of iodine per ml and remins stable for at least 6 months at $4^0$ C in a tightly closed container.

The solution contained 0.02 M ICl, 2.0 M NaCl, 0.02 M KCl and 1.0 M HCl.

Working solution

Two ml of stock solution was diluted with 3 ml of 2.0 M NaCl.

(b) Borate Buffer

| | |
|---|---|
| NaCl | 4.676 gm |
| $H_3BO_3$ | 6.184 gm |
| Distilled Water | 250.00 ml |

The pH was adjusted with 1.60 M NaOH to 8.0. The solution contained 0.32 M NaCl, 0.40 M $H_3BO_3$ and approximately 0.04 M NaOH.

(c) $^{131}I$ in diluted NaOH

The required amount was 5-6 mCi.

(d) Amberlite IRA-400 anion exchange resin

Method, the anion exchange resin was prepared by hydrating it first in distilled water and then transferred to normal saline overnight. A volume of approximately 6 ml (wet) of this resin was packed into the 10 ml disposable syringe fitted with a two-way stopcock at the tip of the syringe. An additional volume of 50 ml of NaCl injection was passed through the column.

The process of labelling :-

Three test tubes were set and filled with

(a) first test tube : 30 mg of microaggregated HSA, 1.5 ml of borate buffer.

(b) second test tube : 5 or 6 mCi $^{131}I$ in NaOH and 1.5 ml of borate buffer.

(c) third test tube : 0.3 ml of working ICl

All tubes were chilled at $4^{0}$ C for 15 minutes and then with rapid sequence, the contents of tube three was transferred into tube two, and the combined contents of tube two was transferred to tube one. The final mixture in tube one was allowed to stand for 3 minutes at room temperature (approximately 28-$30^{0}$ C). The contents in tube one were passed through the ion exchange resin Amberlite IRA-400, chloride form, at a rate of 15-20 drops per minute while excess radioactive iodide was removed by ion exchange resin. The final $^{131}$I-AA solution was made up to 15 ml by adding 0.9% NaCl for injection. The concentration of $^{131}$I-AA was 2 mg/ml.

(5) Preparation of $^{51}$Cr-labelled red blood cells

Approximately 10 ml of blood with ACD solution as an anticoagulant (2 gm disodium citrate, 3 gm dextrose, water to 120 ml) is delivered into a centifuge tube and the red cells are spun down at slow speed. The supernatant plasma ACD is removed and use to prepare the washing solution, which consists of about 3 ml of plasma and 100 ml of sterile isotonic saline. A sterile isotonic solution of $Na_2{}^{51}CrO_4$ of high specific activity containing about 50 $\mu$Ci. $^{51}$Cr is added to the red cells and left at room temperature for about 30 minutes. The red cells are washed thrice with the plasma saline mixture, the centifuging being done at not more than 1,000 rpm.

(6) Procedure of experiments in monkeys

(6.1) Anesthesia

Each experimental monkey was anesthetized with Sodium Pentobarbital (Nembutal Sodium : Abbott) at the dosage of 25 mg/Kg body weight given intravenously.

(6.2) Monkeys treated with ethyl palmitate (Ethpalm monkeys)

The anesthetized monkeys were injected intravenously with 0.75, 1.5 and 2.0 mg per Kg body weight of 10, 15 and 20% ethyl palmitate for 2 consecutive days and were left 48 hours before the study on the liver blood flow, phagocytic activity and erythrophagocytosis.

(a) Studies on the effect of ethyl palmitate on the liver blood flow and phagocytic activity of reticuloendothelial system in monkeys

$^{131}$I-AA was given intravenously to experimental monkeys 0.03 mg/Kg. One ml of heparinized blood sample were taken at 1½, 3, 5, 7 and 9 minutes after injection. The blood sample was centrifuged and plasma was counted with a well type scintillation counter. Thirty minutes later, a large dose of $^{131}$I-AA (5 mg/Kg) was administered and 1 ml of heparinized blood sample were taken at 0, 3, 5, 8, 12 and 15 minutes. All blood samples were centrifuged and 0.5 ml of plasma was passed through anion exchange resin (Amberlite IRA-400, Chloride form) in order to remove free iodine which has been splitted out by the metabolic activity of R.E.S. The plasma was washed out with distilled water to yield a final content volume of 10 ml or more by testing the excess solution with 20% trichloroacetic acid. After centrifugation the spunned sample was counted in a well type scintillation couter.

The logarithm (base 10) of plasma radioactivity was plotted as a function of time on a graph paper, and for the dose

of 0.03 mg of $^{131}$I-AA/Kg and the phagocytic index (K) was estimated from equation (2)

$$K = \frac{\log C_1 - \log C_2}{t_2 - t_1}$$

The corrected phagocytic index ($K_e$) was calculated by the equation

$$K_c = 2.28\ K$$

The weight of the liver and spleen were calculated from the body weight using the relationship between the body weight and the weight of the liver and spleen as described by Areekul (1973).

$$\text{Weight of liver} = 67.61 \times (\text{body weight})^{0.33}$$

The blood volume is calculated by

$$\text{Blood volume (ml)} = \frac{\text{Plasma volume (ml/Kg)}}{1 - \text{Haematocrit}}$$

The liver blood flow is calculated by

$$\text{Liver blood flow (ml/min)} = K_e \times \text{blood volume}$$

$$\text{Liver blood flow (ml/min/gm liver)} = \frac{K_e \times \text{blood volume}}{\text{weight of liver}}$$

For the dose of 5 mg of $^{131}$I-AA/Kg, the phagocytic index (K) was estimated from equation

$$K = \frac{\log C_1 - \log C_2}{t_2 - t_1}$$

and the half-disappearance time (T½) was calculated by

$$T\frac{1}{2}\ (\text{min}) = \frac{0.693}{K}$$

(b) Studies on the effect of ethyl palmitate on the inhibition of erythrophagocytosis

Two ml of $^{51}$Cr-labelled red cells were injected intravascularly into the anesthetized monkeys. One ml of heparinized blood sample were taken at 30, 60, 90, 120, 180 and 240 minutes after injection. All blood sample were centrifuged and 0.5 ml of packed red cells were counted in a well type scintillator counter.

The logarithm (base 10) of packed red cell radioactivity was plotted as a function of time on a graph paper, the half-disappearance time (T½) of the radioactivity from the circulation was determined.

(7) Haematological Studies

The following studies were performed in the monkeys treated with ethyl palmitate.

(a) Haemoglobin estimation The haemoglobin level was determined by the Cyanmethaemoglobin method.

(b) Packed cell volume estimation Two thirds of the heparinized micro-haematocrit tubes were filled with blood, one end was sealed with plasticene. The tubes were placed in opposite direction in a microhaematocrit centrifuge (Clay Adams). Then they were centrifuged at 11,800 rounds per minute (12,000 gm) for 5 minutes. Pack cell volume were estimated in percentage by a special reading scale provided with the centrifuge.