CHAPTER III

METHODS

1. Preparation of dihydrofolate (Futterman, 1957 and Blakely, 1960)

Ascorbic acid 1 g was dissolved with mechanical stirring in about 3 ml of water. While stirring, 1 M sodium hydroxide was added slowly until the pH rose to 6.0. The volume of the solution was brought to 10 ml by the addition of water and a solution of 38.2 mg of folic acid in 1.6 ml of 0.1 M sodium hydroxide. Mechanical stirring was recommenced and 400 mg of solid sodium dithionite added. When all the dithionite had dissolved stirring was continued slowly for 5 min at room temperature (22-25^{o}C). An ice-bath was then placed around the beaker and stirring continued until the temperature fell below 5^{o}C. Addition of 1 M hydrochloric acid at a rate of approximately 0.1 ml/min was then commenced while the solution was stirred rapidly at 0-5^{o}C. Addition of hydrochloric acid was continued until the pH fell to 2.8. After stirring the solution for a further 5 min to allow complete precipitation, the precipitate was recovered by centrifugation (5 min at 1,000 g, 4^{o}C) and the supernatant discarded. The precipitate, which was mainly amorphous but contained some crystalline material, was re-suspended in 10 ml of an ice-cold solution of 10

percent sodium ascorbate, pH 6.0, freshly prepared as previously described. The suspension was returned to a beaker cooled in an ice-bath with mechanical stirring and continuous pH measurement recommenced. The bulk of the precipitate was redissolved at this stage and the solution was between pH 5-6. If necessary, the solution would be adjusted to pH 6 by cautiously adding 1 M sodium hydroxide, and maintained at this pH while being stirred at $0^{o}C$ for a further 5 min. Under these conditions only crystalline material remained undissolved, causing marked birefringence. The dissolved dihydrofolate was crystallized from the solution by gradual addition of 1 M hydrochloric acid performed exactly as previously described. When crystallization was completed, (5 min after reaching pH 2.8) the heavy, white, birefringent suspension was centrifuged off as before and washed 3 times with ice-cold 0.001 M hydrochloric acid by suspending and centrifuging. The precipitate was added with ice-cold 0.001 M hydrochloric acid to make 50 percent suspension. This was kept at $-70^{o}C$ under nitrogen gas and could be kept for more than 3 months.

For dihydrofolate reductase activity assays, crystalline dihydrofolate was brought into the solution with 0.05 M Tris-HCl buffer, pH 7.5, 0.1 M with respect to 2-mercaptoethanol. The final dihydrofolate concentration was adjusted to 1 mM using the molar extinction coefficient of $2.84 \times 10^{4}\ M^{-1}cm^{-1}$ at 282 nm.

2. Dihydrofolate reductase activity assay

(McCullough, et al., 1971)

Dihydrofolate reductase activity was measured by a spectrophotometric method utilizing the decrease in absorbance that occurred at 340 nm when NADPH and dihydrofolate (DHF) were converted to NADP and tetrahydrofolate (THF), respectively (Perkins, et al., 1967). Assays were performed at 37°C. The standard spectrophotometric assay contained in a final volume of 1 ml : 100 mM potassium phosphate buffer, pH 6.5; 150 mM potassium chloride; 1 mM 2-mercaptoethanol; 0.10 mM NADPH; 0.05 mM dihydrofolate; and enzyme. Purified enzyme was added last to initiate the reaction.

Specific activity is expressed as umoles of substrate reduced per hr per mg of protein.

A unit of enzyme activity is expressed as that amount reducing 1 umole of substrate per hr under conditions of the standard assay.

3. Protein determination

Protein was determined by the biuret method (Zamenhof, 1957) in crude extract and amminium sulfate fraction. For sephadex fraction in purification of dihydrofolate reductase from rat liver, protein was determined by Lowry's method (Lowry, et al., 1951).

4. Purification of dihydrofolate reductase from rat liver (McCullough, et al., 1971)

Rat liver 6.0 g was used for the enzyme purification. All steps of purification were carried out at 4-10°C. Distilled water 25 ml was added to 6.0 g of liver and homogenized for 30 sec with a homogenizer. The pH of the homogenate was adjusted to pH 6.0 by cautious addition of 1.0 M hydrochloric acid. The cell homogenate was centrifuged in a Beckman refrigerated centrifuge, model 21-C at 27,000 x g for 20 min at 4°C. The supernatnat fluid was decanted through two layers of cheese cloth to remove lipid material. The supernatant was then fractionated with ammonium sulfate by slowly adding 25.8 g of the crystalline solid per 100 ml of homogenate. The mixture was stirred for 30 min at 4°C and then centrifuged for 20 min at 27,000 x g as before. The precipitate was discarded; to every 100 ml of the supernatant fluid were added 29.6 g of ammonium sulfate. The mixture was stirred at 4°C for 1 hr and centrifuged as before, except that in this case the supernatant fluid was discarded. The precipitate was dissolved in a minimum volume (3 ml) of 0.05 M Tris-HCl, pH 7.5, 0.1 M with respect to potassium chloride. This fraction is referred to as the 45 to 85 percent ammonium sulfate fraction. This fraction was then applied to a column of Sephadex G-75 (2.5 x 80 cm), which had been equilibrated with 0.05 M Tris-HCl, pH 7.5, 0.1 M with respect to potassium chloride. Tris-HCl of the same molarity was used to elute the enzyme from the column,

fractions of 5 ml each were collected. The elution was carried out at 4°C. Tubes containing the highest enzyme activity (about fraction 50-60) were pooled and used in the subsequent experiments. The enzyme was kept at -70°C until used.

5. Determination of pyrimethamine by competitive binding assay

The assay was based on competition between ^{14}C-pyrimethamine and unlabeled pyrimethamine for binding to dihydrofolate reductase, with subsequent removal of unbound drug by charcoal adsorption. The reaction mixture in a total volume of 1 ml containing 200 ul of 500 mM potassium phosphate buffer, pH 6.5; 100 ul of 1500 mM potassium chloride; 50 ul of 20 mM 2-mercaptoethanol; 100 ul of 1 M NADPH; 100 ul of 100 nM ^{14}C-pyrimethamine and initiated the reaction by adding 200 ul of purified rat liver dihydrofolate reductase (about 23.7 units); the tubes were mixed by vortex agitation. The assay was performed at 4°C.

After incubated for 15 min, 25 ul of 10% charcoal slurry was added (charcoal, 10 g/100 ml; bovine serum albumin, Fraction V, 2.5 g/100 ml; and high-molecular-weight dextran, 0.1 g/100 ml). Samples were again mixed by vortex agitation and further incubated for 2 min, then centrifuged at 2,500 x g, 4°C, for 30 min. A 400 ul aliquot of the supernatant was counted for radioactivity [bound DHFR-(^{14}C-pyrimethamine)] in 6 ml of toluene base scintillation fluid

Triton X-100 by using Packard PL-Tricarb liquid scintillation counter (efficiency 80%, error 0.5-4.5%).

The amount of pyrimethamine could be determined from standard curve between C_o/C_x and pyrimethamine concentration

$$\frac{C_o}{C_x} = \frac{\text{dpm without addition of cold pyrimethamine}}{\text{dpm with added cold pyrimethamine at various concentrations}}$$

6. Standardization of competitive binding assay for pyrimethamine

6.1 Determination of standard curve for pyrimethamine

A standard curve was constructed by performing each set of experiments as follows:

The reaction mixture in a total volume of 1 ml containing 200 ul of 500 mM potassium phosphate buffer, pH 6.5; 100 ul of 1500 mM potassium chloride; 50 ul of 20 mM 2-mercaptoethanol; 100 ul of 1 mM NADPH; 100 ul of 100 nM ^{14}C-pyrimethamine; 100 ul of known amounts of pyrimethamine in the range of (2-30) x 10^{-7}M; 100 ul of samples (mouse plasma, mouse liver extract (with 1:8 vol:vol 95% ethanol extraction), mouse red blood cells extract (with 1:8 vol: vol 95% ethanol extraction), human plasma and human serum) and initiated the reaction by adding 200 ul of purified rat liver dihydrofolate reductase (around 23-25 units). The assay was continued as described in Methods Section 5. The standard curve was plotted between C_o/C_x and pyrimethamine concentration.

6.2 Precision

The precision was done in 2 ways:

a) comparing the amount of pyrimethamine by repeated detection from the same batch of samples simultaneously —— within assay.

b) comparing the amount of pyrimethamine by repeated detection from the same batch of samples at different time intervals —— between assay.

The precision was calculated from coefficient of variation (C.V.) which is expressed as $\% \text{ C.V.} = \frac{\text{S.D.} \times 100}{\bar{X}}$

6.3 Accuracy

The assay was performed by adding 20, 60, 100, 140 nM of standard pyrimethamine in sample assay tubes, then detected the amount of pyrimethamine as described in Methods Section 5. Percent recovery was then calculated and the figures were used to indicate the accuracy of the method.

6.4 Specificity

The assay was performed by adding unlabeled pyrimethamine, folic acid, tetrahydrofolate, 5-methyl-tetrahydrofolate, leucovorin and sulphanilamide at various concentrations into the assay mixture and performed the assay as described in Methods Section 5. The specificity of this binding for pyrimethamine was evaluated by com-

paring the concentration of pyrimethamine and other unlabeled substances which was required to decrease binding of the labeled pyrimethamine by 50 percent under standard assay conditions.

The substances used for specificity determination were prepared as follows:

6.4.1 Pyrimethamine —— was dissolved initially in 0.5% lactic acid to a concentration of 10^{-3}M of drug.

6.4.2 Folic acid —— was dissolved in 0.1 M NaOH to give a concentration of 1 x 10^{-2}M.

6.4.3 Tetrahydrofolate and 5-Methyl-tetrahydrofolate —— were prepared by the same procedures. They were dissolved in 0.5 M phosphate buffer, pH 7.4 with respect to 0.16 M dithiothreitol (DTT) and kept under nitrogen gas at -20°C. The stock concentration was 2 x 10^{-2}M.

6.4.4 Leucovorin and Sulphanilamide —— were prepared for a concentration of 1 x 10^{-2}M and 2 x 10^{-2}M respectively by dissolving in distilled water.

All of the above stock solution were kept at -20°C.

7. Determination of the optimal amount of 95% ethanol used for the extraction of pyrimethamine from liver and red blood cells

The assay was performed by either adding 100 ul of 12.5 nM ^{14}C-pyrimethamine or 12.5 nM ^{14}C-pyrimethamine plus 0.1 mM cold pyrimethamine into 100 ul of liver homogenate (Methods Section 8.3.3) or packed red blood cells (Methods Section 8.3.2), then 95% ethanol was added at the ratio of liver homogenate or red blood cells to 95% ethanol of 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9 and 1:10 (vol:vol). The mixture was mixed vigorously and centrifuged at 3,000 g for 5 min. A 400 ul of the supernatant was counted for radioactivity in 6 ml toluene base scintillation fluid Triton X-100 by using Packard PL-Tricarb liquid scintillation counter. The optimal amount of 95% ethanol used for the extraction of pyrimethamine from liver and red blood cells was determined from percent recovery.

8. Determination of pyrimethamine level in mice

8.1 Animal preparation

Fisher albino mice; 3-4 month old, male and female weighing approximately 30-40 g were used. Mice were infected with *P. chabaudi* by intraperitoneal inoculation of infected erythrocytes having about 10^7-10^8 cells of plasmodium per mouse. Infected mice were fed by standard mouse diet and with 0.01% para-aminobenzoic acid as supplement (Peters, 1967). The environment of day and night was

created and controlled by automatic electrical switch in a specially designed chamber. The light was off between 8.00-17.30 hr simulating night time, and all the rest of the time simulating the day (Newbold, et al., 1982).

8.2 Drug administration

At day 3 after inoculation with (percent parasitemia around 20-30), mice were treated orally (5 mg/kg body wt) or intraperitoneally (5 mg/kg body wt or 30 mg/kg body wt) with a single dose of pyrimethamine. The pyrimethamine solution was prepared by suspending pyrimethamine powder in distilled water; 30 mg or 150 mg in 10 ml of water with the addition of one drop of Tween 80 and sonified for 5 min. The drug suspensions were kept at 4°C and were thoroughly shaken on a vortex mixer before administration.

8.3 Extractions of pyrimethamine from plasma, red blood cells and liver

At various intervals after treated with pyrimethamine, mice were sacrificed. Blood was collected separately for each mouse by cardiac puncture, using acid citrate dextrose (ACD) (sodium citrate 1.32 g, citric acid 0.48 g, dextrose anhydrous 1.40 g in 100 ml distilled water), 0.2 ml/ml blood, as an anticoagulant. Liver was also collected separately for each mouse. The samples were kept at 4°C until used.

8.3.1 Extraction of pyrimethamine from plasma

Plasma was removed from packed cells of blood sample by centrifugation at 3,000 g for 5 min at 4°C. This fraction of plasma was directly used in determination of the amount of pyrimethamine.

8.3.2 Extraction of pyrimethamine from red blood cells (Modified from Nuchadomrong, 1985)

Packed cells from Methods Section 8.3.1 were pooled together in each experimental set and were adjusted to 50% suspension by adding an equal volume of cold 5 mM phosphate-buffered saline (PBS), pH 7.4 and then passed through cellulose CF-11 column (Richards and Williams, 1973). The effluent from the column was centrifuged at 3,000 g for 5 min at 4°C to obtain packed red cells. The number of erythrocytes was counted as described in Methods Section 8.3.2.1 and parasitemia was determined from thin films stained with Giemsa stain. Packed red cells were extracted for pyrimethamine by adding 95% ethanol 1:8 (vol: vol) ratio, the solution was then shaken vigorously on a vortex mixture for 30 sec then centrifuged at 3,000 g for 5 min. The supernate was collected and kept at 4°C before being used to determine the amount of pyrimethamine.

8.3.2.1 Determination of erythrocytes

The amount of erythrocytes was determined by counting on a hematocytometer. The unit of

erythrocyte count was the number of cells/cubic millimeter of the suspension.

8.3.2.2 Erythrocyte staining

Percent parasitemia was determined by smearing the blood onto the slide as a thin film. The slide was air dried and thinly fixed in an absolute methanol for 1-2 min. The fixed specimen was stained in 3% giemsa solution for 30-60 min. After the slide was taken out, it was washed with water, air dried and examined under oil immersion lens of a light microscope. At least 500 erythrocytes were counted for parasitemia evaluation.

8.3.3 Extraction of pyrimethamine from liver

Liver from each individual mouse was chopped into small pieces and homogenized in ice-cold water (1 g of liver per 2 ml of water), using a homogenizer type NS1-12. Liver homogenate was used for extraction of pyrimethamine as previously described in Methods Section 8.3.2.

8.4 Determination of pyrimethamine

The amount of pyrimethamine was determined by competitive binding assay. The reaction mixture in a total volume of 1 ml containing 200 ul of potassium phosphate buffer, pH 6.5; 100 ul of 1500 mM potassium chloride; 50 ul of 20 mM 2-mercaptoethanol; 100 ul of 1 mM NADPH; 100 ul of 100 nM ^{14}C-pyrimethamine; 100 ul of assay sample and initiated

the reaction by adding 200 ul of purified rat liver dihydrofolate reductase (about 23.7 units). The assay was performed as the conditions described in Methods Section 5.

9. Determination of parasitemia in peripheral blood of mice infected with *P. chabaudi* AS and *P. chabaudi* AS(Pr_1) and treated with a single dose of pyrimethamine

At various intervals after *P. chabaudi* inoculation and treated with single dose of pyrimethamine, orally and intraperitoneally (Methods Section 8.1 and 8.2), parasitemia was determined for each animal (each experimental set consisted of 5 mice) by microscopic examination (oil-immersion lens) of red blood cells in peripheral (tail) blood smears stained with Giemsa (Methods Section 8.3.2.2). Peripheral blood smears were determined at 24 hr intervals for 14 days after *P. chabaudi* inoculation and treated with pyrimethamine. The determination was made more frequently at 6, 12, 18, 24 and 36 hr periods after pyrimethamine administration. After 14 days, mice were fed for at least 3 months with normal diet to see whether they would survive and be free from parasite. Each clone of *P. chabaudi* (AS and AS(Pr_1)) inoculation had a control group of 5 mice that received no drug treatment.