CHAPTER IV

RESULTS

1. Purification of dihydrofolate reductase from rat liver

The individual steps of the enzyme preparation were summarized in Table 1. The purification of DHFR from rat liver was performed as described in Methods Section 4. By these procedures, about 8-fold purification of rat liver DHFR was accomplished. The purified enzyme was free from contaminating substances such as haemoglobin. It was kept at $-70^{\circ}C$ under nitrogen gas and proved to be very stable. The activity was only slightly decreased after 9 months of storage. This purified enzyme was used in subsequent experiments including competitive binding assay.

2. Properties of DHFR from rat liver

2.1 Effect of pH on the activity of DHFR from rat liver

With sufficient amount of dihydrofolate and NADPH to saturate the enzyme, the dependency of the velocity of reaction to pH over a range from 4.5-9.0 was established. The data exhibited one broad optimum pH between 5.5-6.5 and flating shoulder range at pH 6.5-8.0. Under these conditions, the inhibition produced by 1×10^{-7}M pyrimethamine (expressed

Table 1 Purification of DHFR from rat liver.

Purification step	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)**	Yield (%)	Purification factor
Crude cell extract	1428	2441	1.1	100	1.0
Ammonium sulphate (45-85%)	636	900	1.4	37	1.3
Sephadex G-75	66	599	9.1	25	8.3

* One unit of enzyme is expressed as the amount that reducing 1 umole of NADPH per hr per ml enzyme under conditions of the standard asssy.

** Specific activity is expressed as units per mg of proteins.

as percentage of control) was markedly influenced between pH range of 6.0-6.5 (Figure 3a and 3b).

From this experimental result, it was determined that potassium phosphate buffer, pH 6.5 could be used as the assay buffer in subsequent experiments.

2.2 Effect of pH on the interaction of pyrimethamine to rat liver DHFR

In this experiment the concentration of pyrimethamine was varied and the enzymatic activity was measured at 3 different pH values. Results indicated the slightly higher efficiency of Tris-HCl buffer, pH 7.5 than phosphate buffer, pH 6.0 and citrate buffer, pH 5.5 compared to retained enzyme activity. However, stoichiometric amount of inhibitor which gave complete titration of enzyme activity was illustrated to be the same at all 3 pH values (using 2.81 units of rat liver DHFR) when the pyrimethamine concentration was increased to 11.3×10^{-7} M (Figure 4).

2.3 Effect of ethanol on the activity of DHFR from rat liver

In the experiment, adding ethanol to the reaction mixture caused reduction in DHFR activity. The enzyme activity decreased as the amount of ethanol increased and showed a linear relationship (Figure 5). When the amount of ethanol added was increased to 200 and 250 ul, the reaction mixture became turbidity due to colloidal proteins formation.

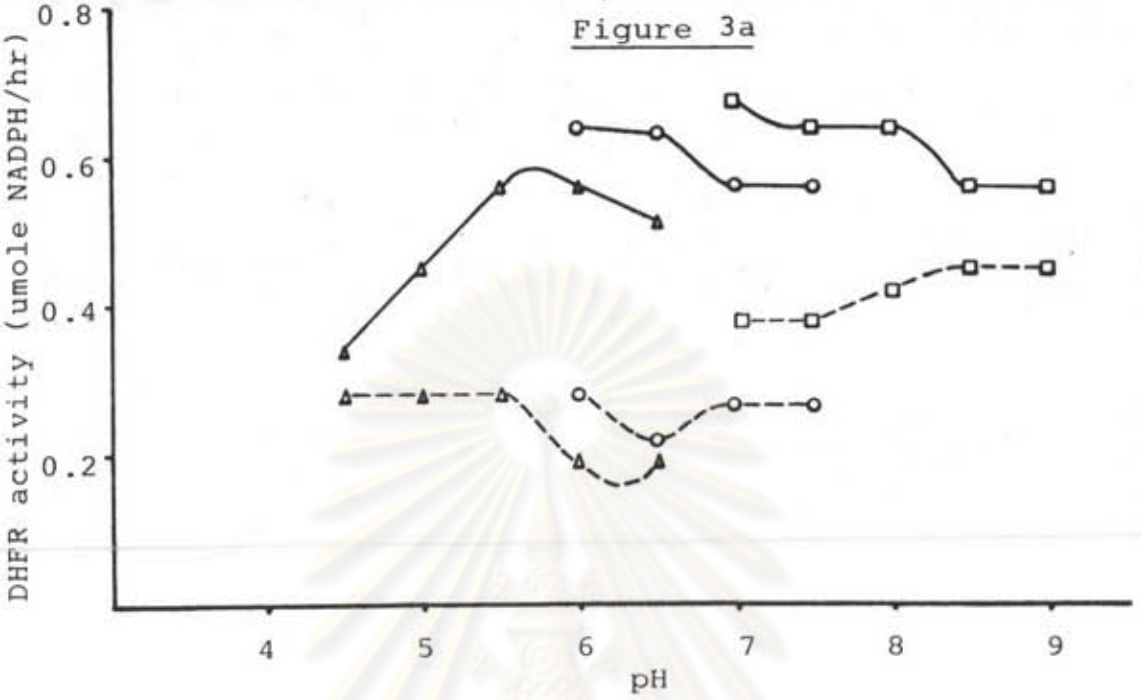


Figure 3a

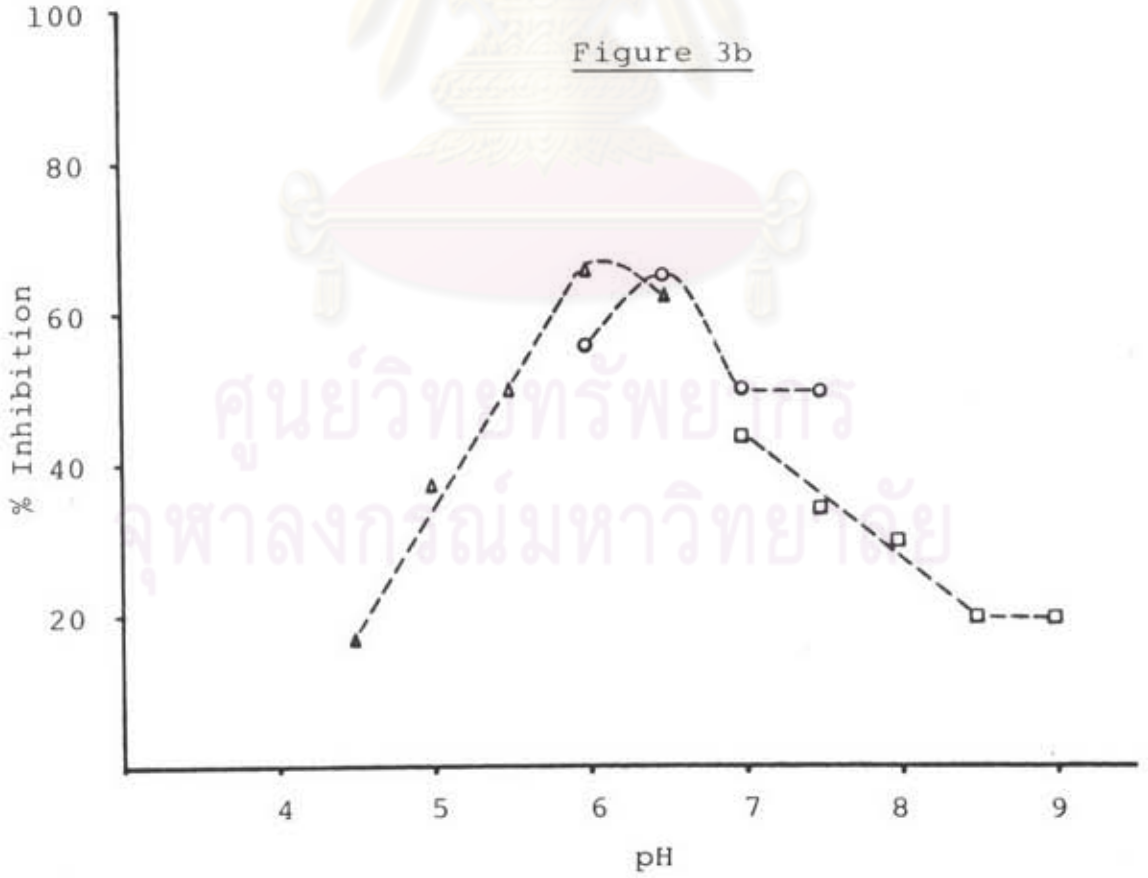


Figure 3b

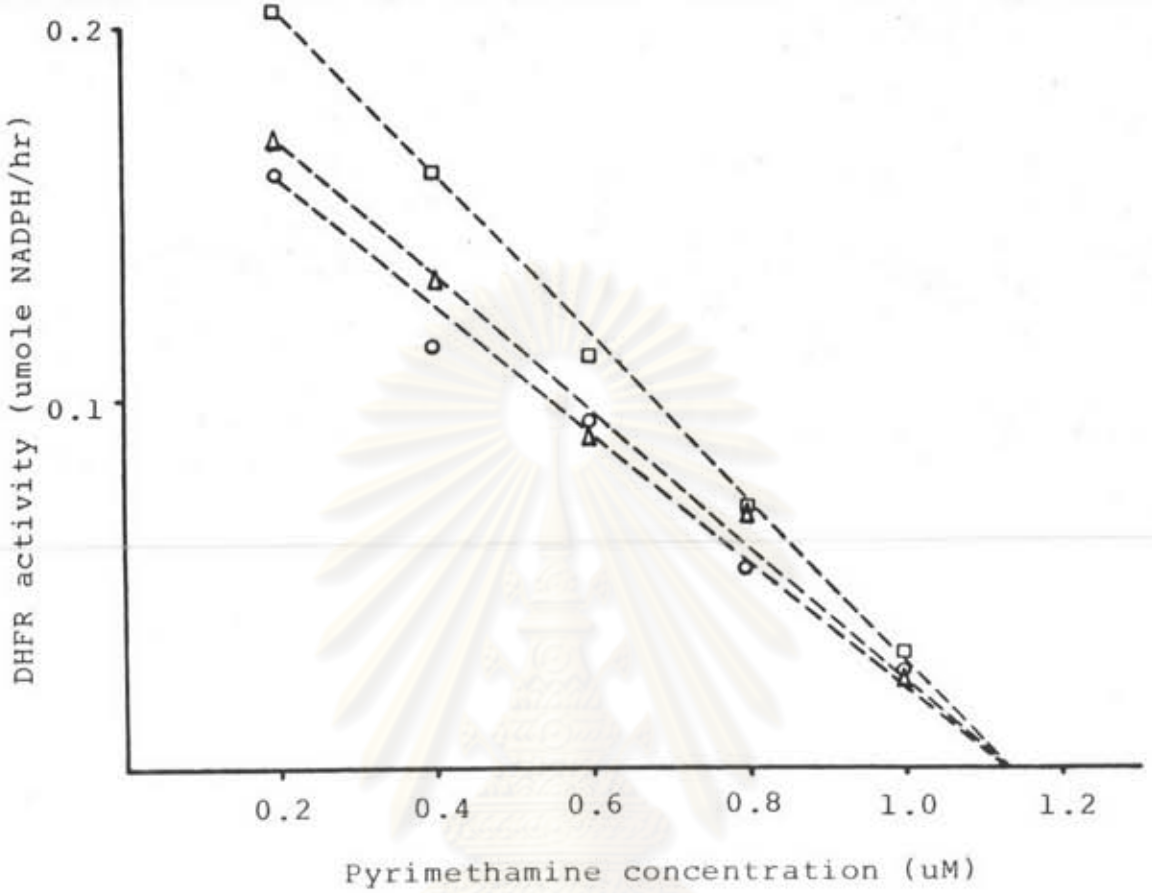


Figure 4 Effect of pH on the interaction of pyrimethamine to rat liver DHFR.

The assay mixture was identical with that described in Methods Section 2 (2.81 units of rat liver DHFR were used). Citrate buffer, pH 5.5 (△); potassium phosphate buffer, pH 6.0 (○) and Tris-HCl buffer, pH 7.5 (□) were used, with varying concentration of pyrimethamine.

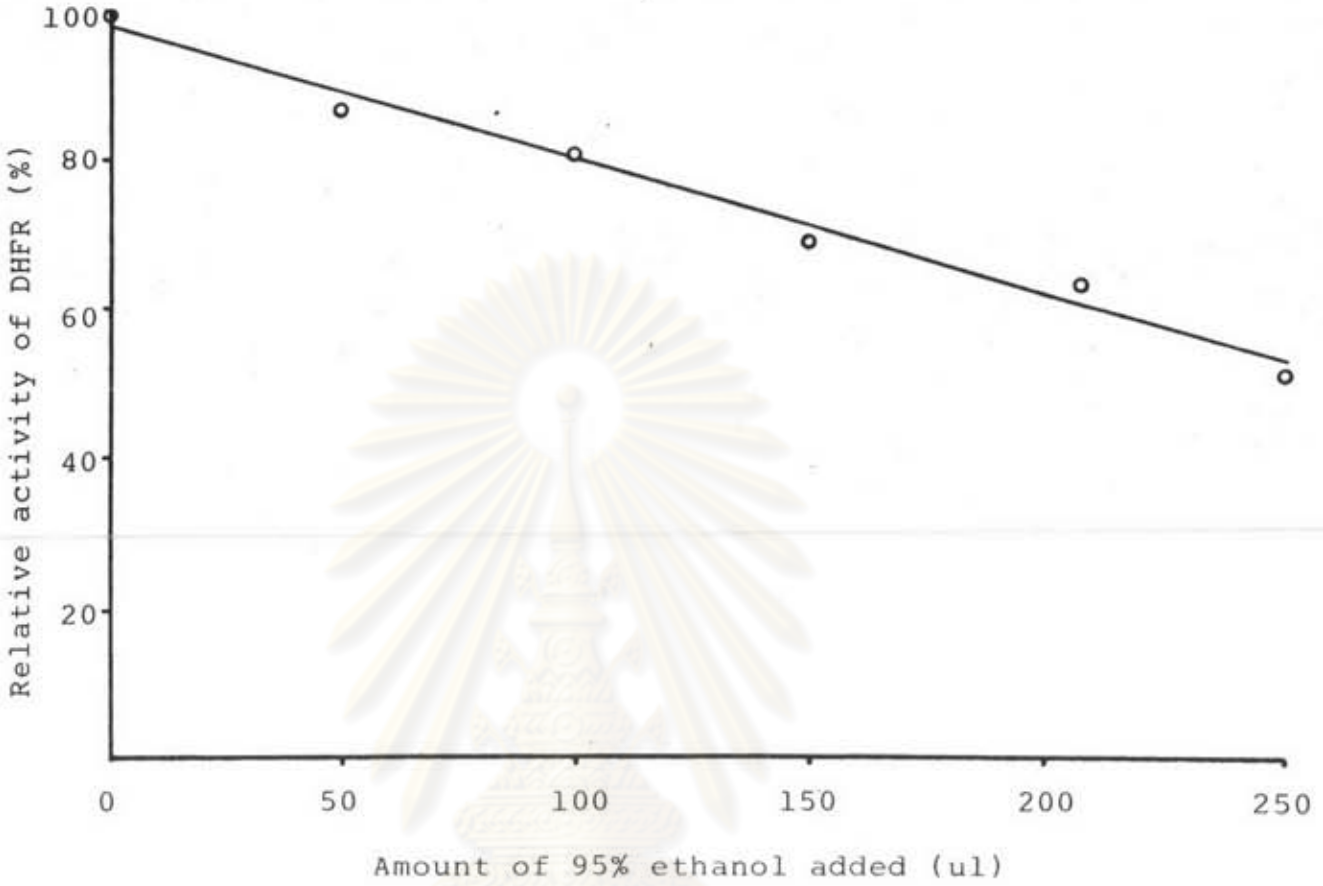


Figure 5 Effect of ethanol on the activity of DHFR from rat liver.

The assay mixture was identical with that described in Methods Section 2 (7.87 units of rat liver DHFR were used), except that adding and varying amounts of 95% ethanol were employed.

3. Optimization of the competitive binding assay for pyrimethamine

The assay was based on the competition binding between ^{14}C-pyrimethamine/pyrimethamine and DHFR. The conditions of competitive binding assay for pyrimethamine were studied as follows.

3.1 The amount of charcoal slurry for separation of free from bound pyrimethamine

A primary requirement for the assay of pyrimethamine by competitive binding is the separation of unbound and bound pyrimethamine. In the assay, the removal of unbound pyrimethamine at concentration as high as 2×10^{-5}M was accomplished by using 25 ul of 6 gram percent of charcoal slurry (1.5 mg charcoal) (Figure 6).

3.2 Effect of incubation time on binding of ^{14}C-pyrimethamine to rat liver DHFR

From figure 7, at 4°C, percent bound of pyrimethamine was only slightly decreased in a linear correlation when incubation time was increased to 120 min. While at 24°C, percent bound of pyrimethamine increased in linear relationship with incubation time within the range of 120 min. The capacity of binding between pyrimethamine and DHFR was gradually increased for a longer period of incubation.

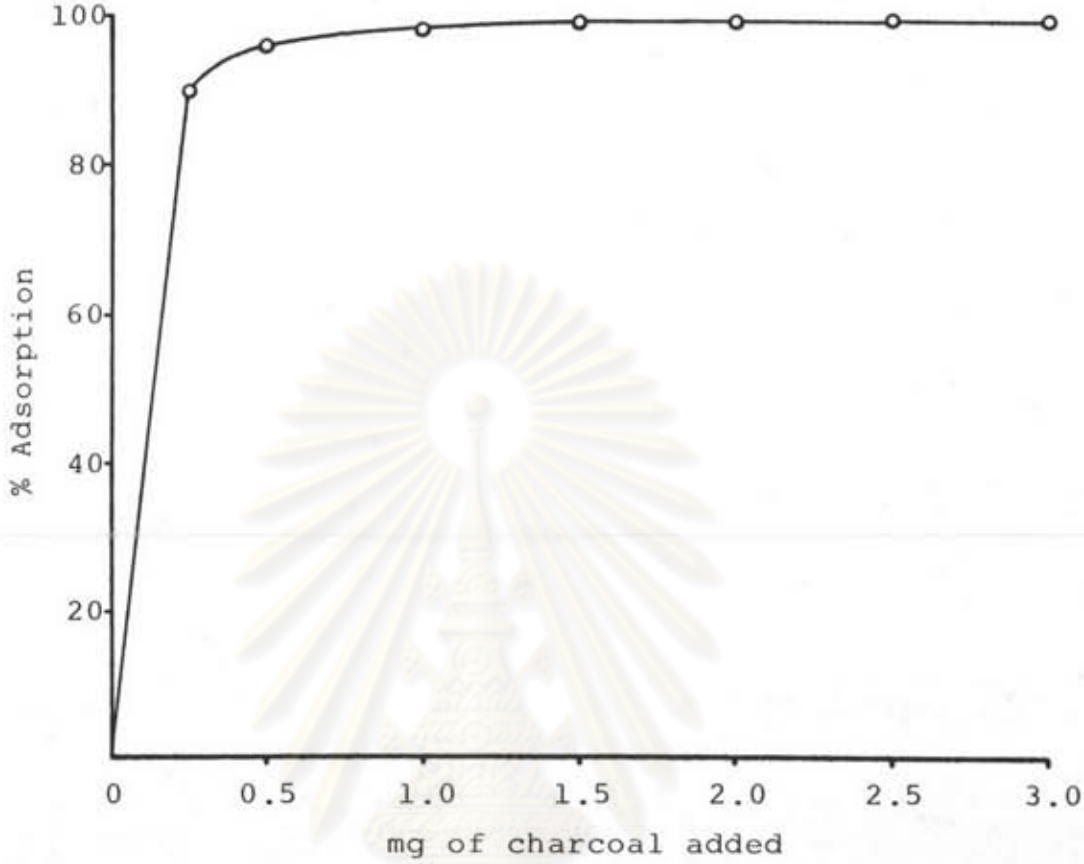


Figure 6 Adsorption of pyrimethamine by charcoal.

Assay protocol

100	mM	potassium phosphate buffer, pH 6.5
150	mM	KCl
1	mM	2-mercaptoethanol
0.1	mM	NADPH
0.02	mM	pyrimethamine
10	nM	^{14}C-pyrimethamine

Total assay mixture volume was 1 ml.
25 ul of various gram percents of charcoal slurry were added to assay mixture; after incubated for 2 min, 400 ul of incubation mixture were removed and counted in a liquid scintillation counter.

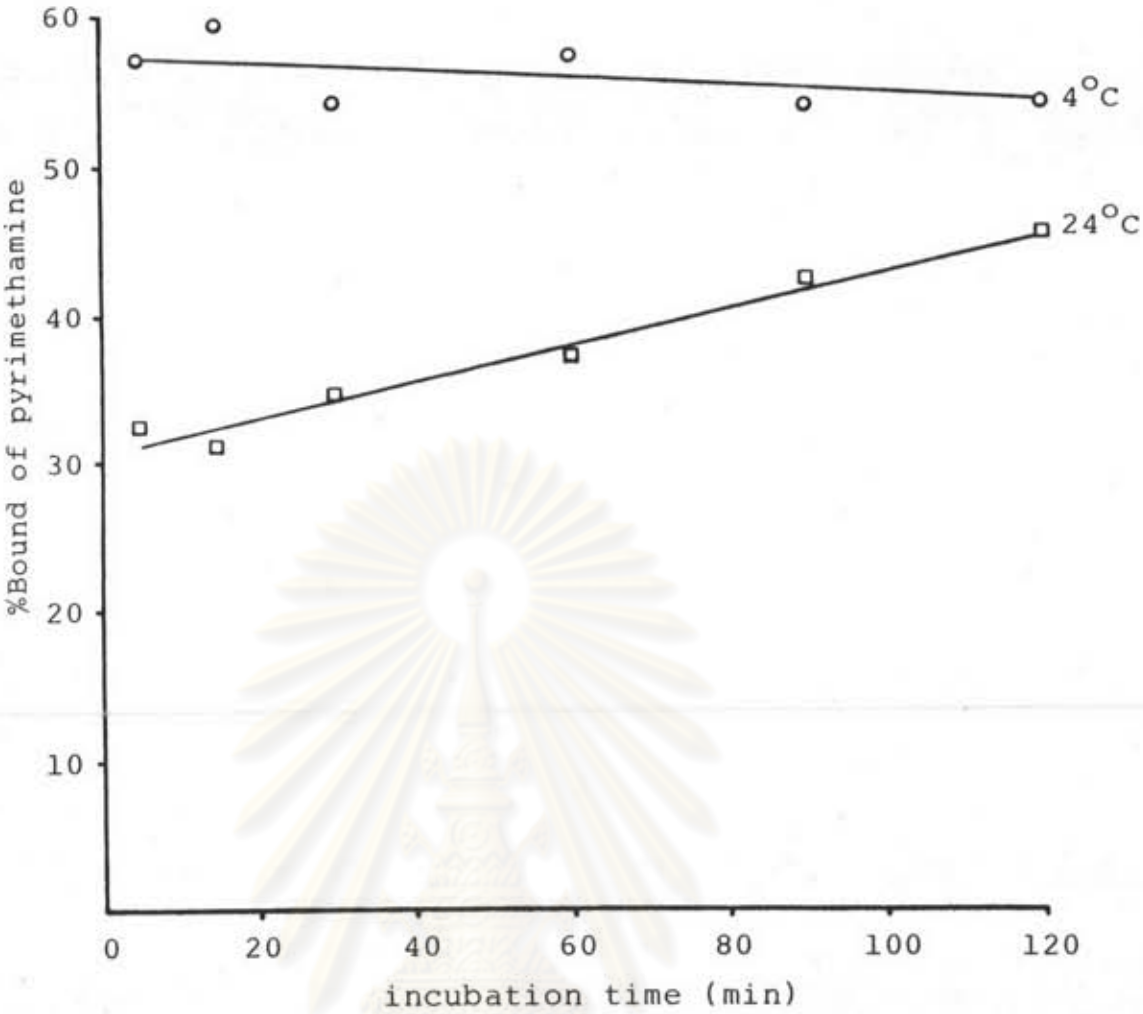


Figure 7 Effect of incubation time on binding of ^{14}C-pyrimethamine to rat liver DHFR.

Assay protocol

100 mM potassium phosphate buffer, pH 6.5
150 mM KCl
1 mM 2-mercaptoethanol
0.1 mM NADPH
10 nM ^{14}C-pyrimethamine
purified rat liver DHFR (23.7 units)
Total assay mixture volume was 1 ml.
Incubation were at 4°C (o) or 24°C (□)
25 ul of 10% charcoal slurry were added to assay mixture; after incubated for 2 min, 400 ul of incubation mixture were removed and counted for the radioactivity in liquid scintillation counter.

3.3 Effect of rat liver DHFR concentration on ^{14}C-pyrimethamine binding

The linear relationship of percent bound of pyrimethamine and the amount of enzyme in the assay were established for the whole range of enzyme concentration used (4.0-24.0 units) (Figure 8).

3.4 Effect of ^{14}C-pyrimethamine concentration on binding to rat liver DHFR

Graph plotted between ^{14}C-pyrimethamine concentration and ^{14}C-pyrimethamine bound to DHFR (dpm) showed a linear relationship for concentration up to 10 nM before reaching the plateau (Figure 9).

3.5 Effect of NADPH concentration on ^{14}C-pyrimethamine binding to rat liver DHFR

As shown in figure 10, percent bound of pyrimethamine to the binding protein was increased as concentration of NADPH increased until the level of percent bound of ^{14}C-pyrimethamine to the enzyme reached maximum at 50 percents of radioactivity when NADPH concentration reached 0.06 nM and remained constant when higher concentration of NADPH was added.

3.6 Effect of pH on binding of ^{14}C-pyrimethamine to rat liver DHFR

pH optimum of 6.5-7.0 was observed for binding of ^{14}C-pyrimethamine to rat liver DHFR (Figure 11).

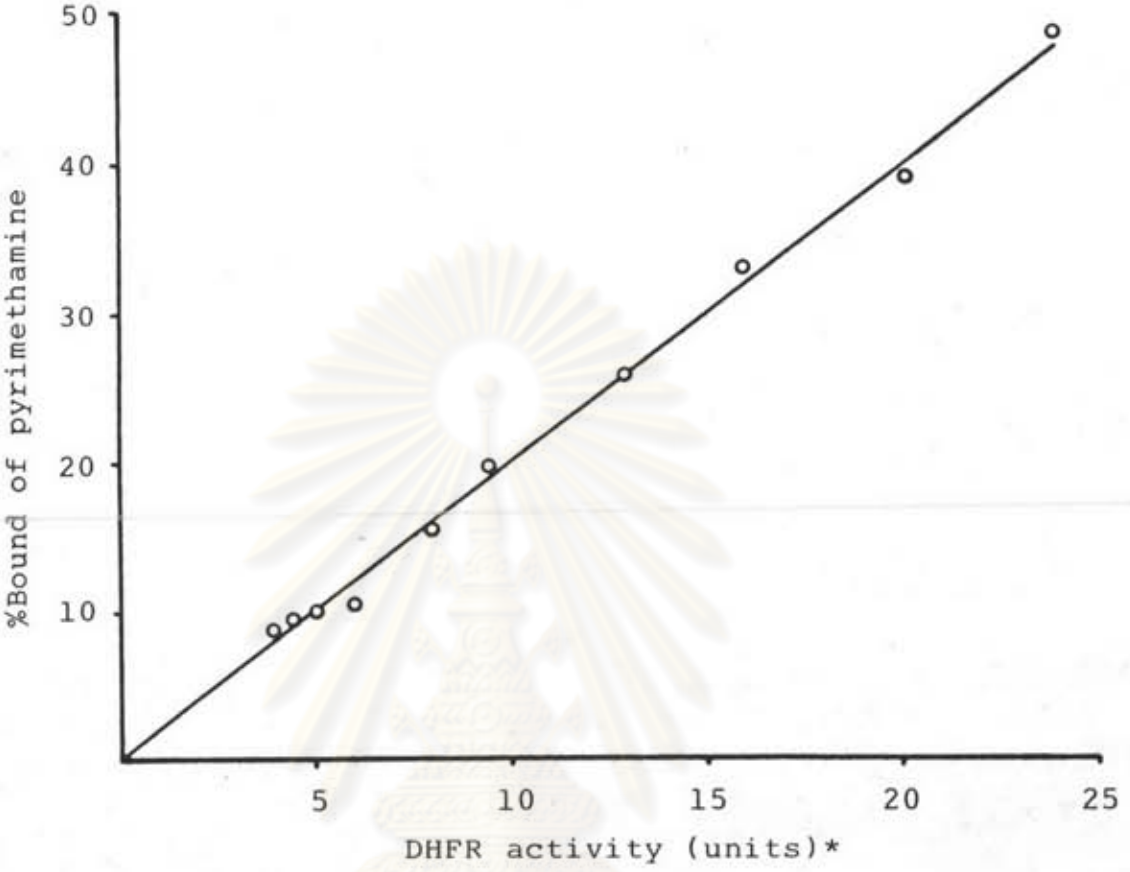


Figure 8 Effect of rat liver DHFR concentration on ^{14}C-pyrimethamine binding.

The assay protocol was identical with that described in Methods Section 5, except that various amounts of rat liver DHFR were employed as indicated in the figure. The concentration of ^{14}C-pyrimethamine was kept constant at 10 nM.

*units = umole NADPH reduced/hr/ml enzyme

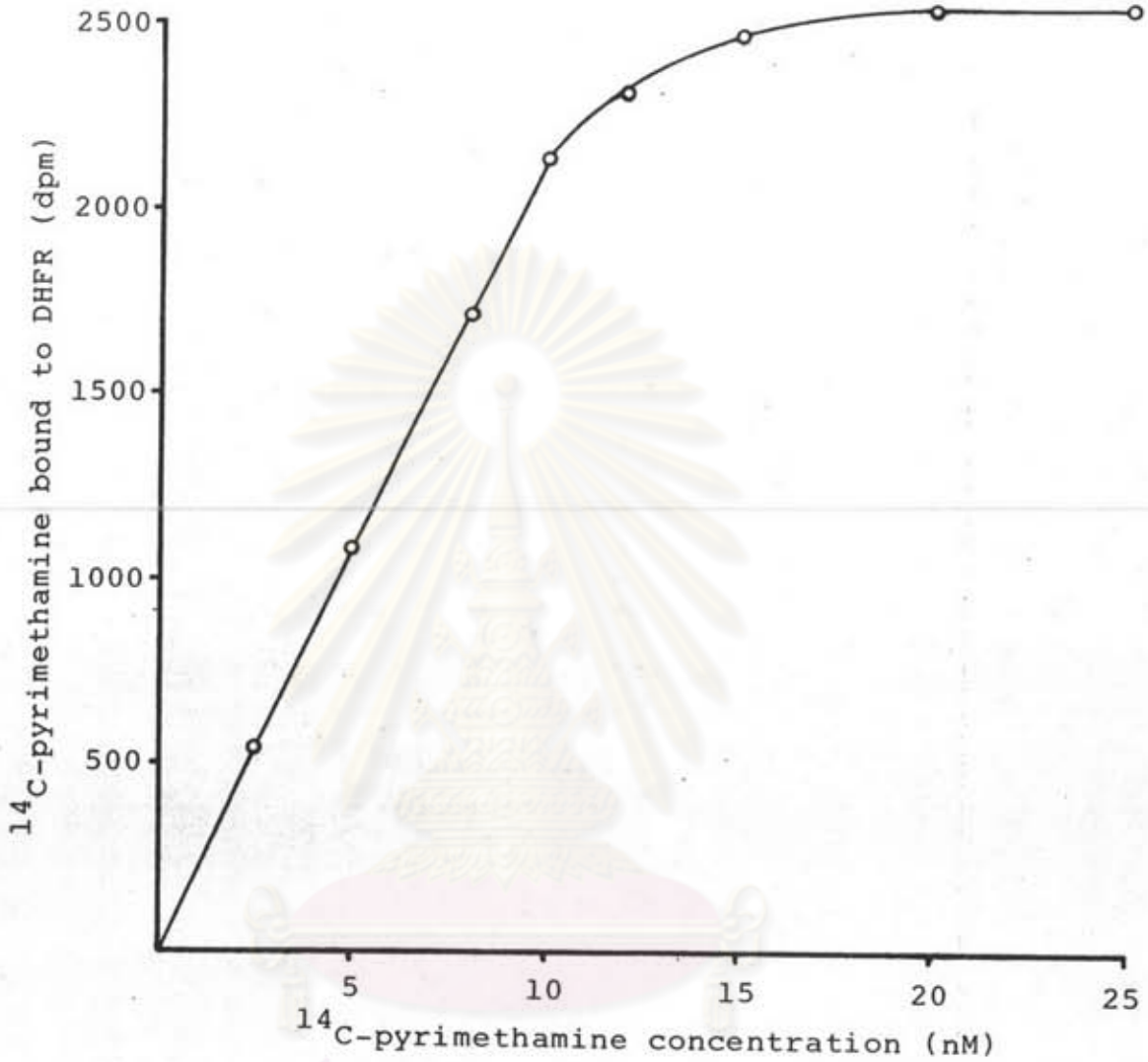


Figure 9 Effect of ^{14}C-pyrimethamine concentration on binding to rat liver DHFR.

The assay protocol was identical with that described in Methods Section 5, except that various concentration of ^{14}C-pyrimethamine were employed. The amount of rat liver DHFR was kept constant at 23.7 units.

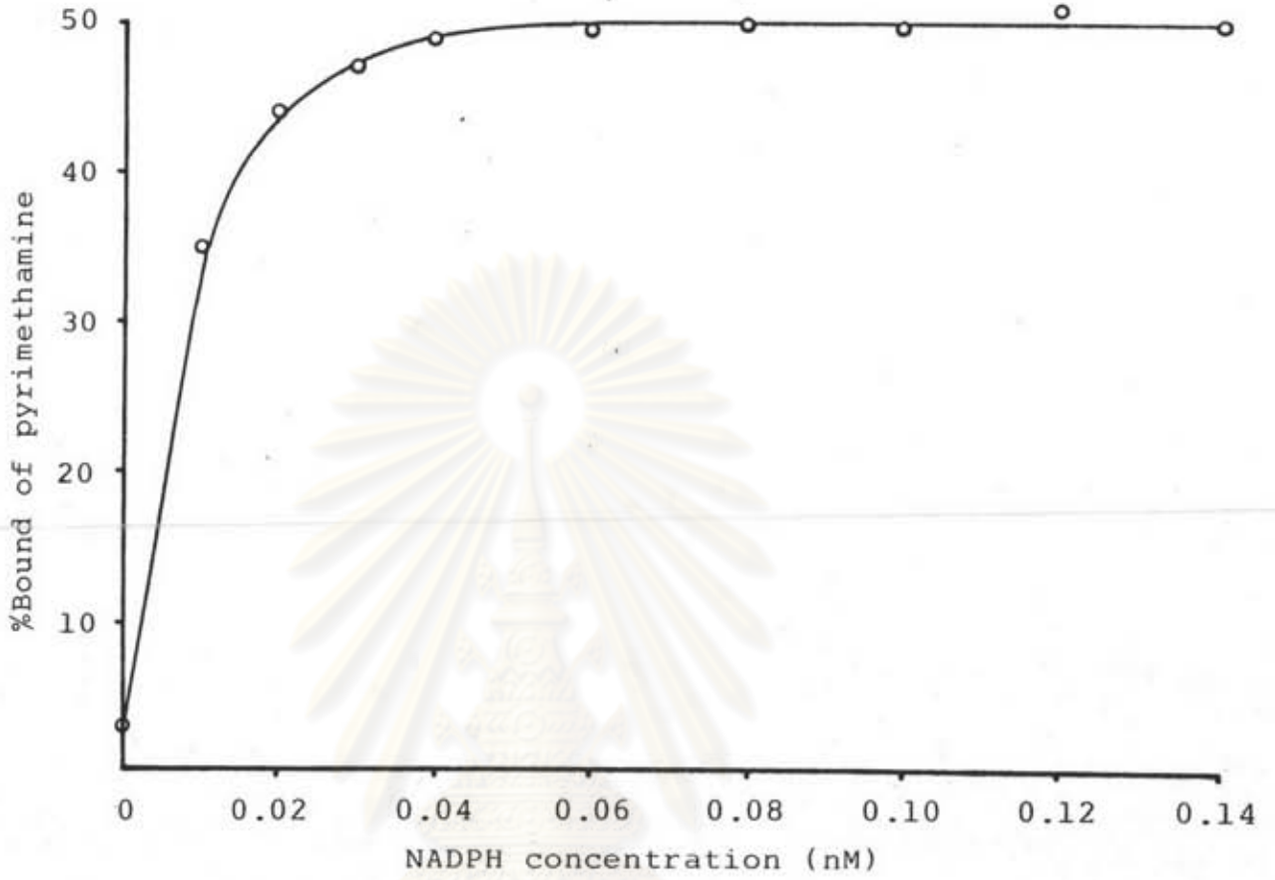


Figure 10 Effect of NADPH concentration on ^{14}C-pyrimethamine binding to rat liver DHFR.

The assay protocol was identical with that described in Methods Section 5, except that various concentrations of NADPH were employed as indicated in the figure. The amount of rat liver DHFR was kept constant at 23.7 units.

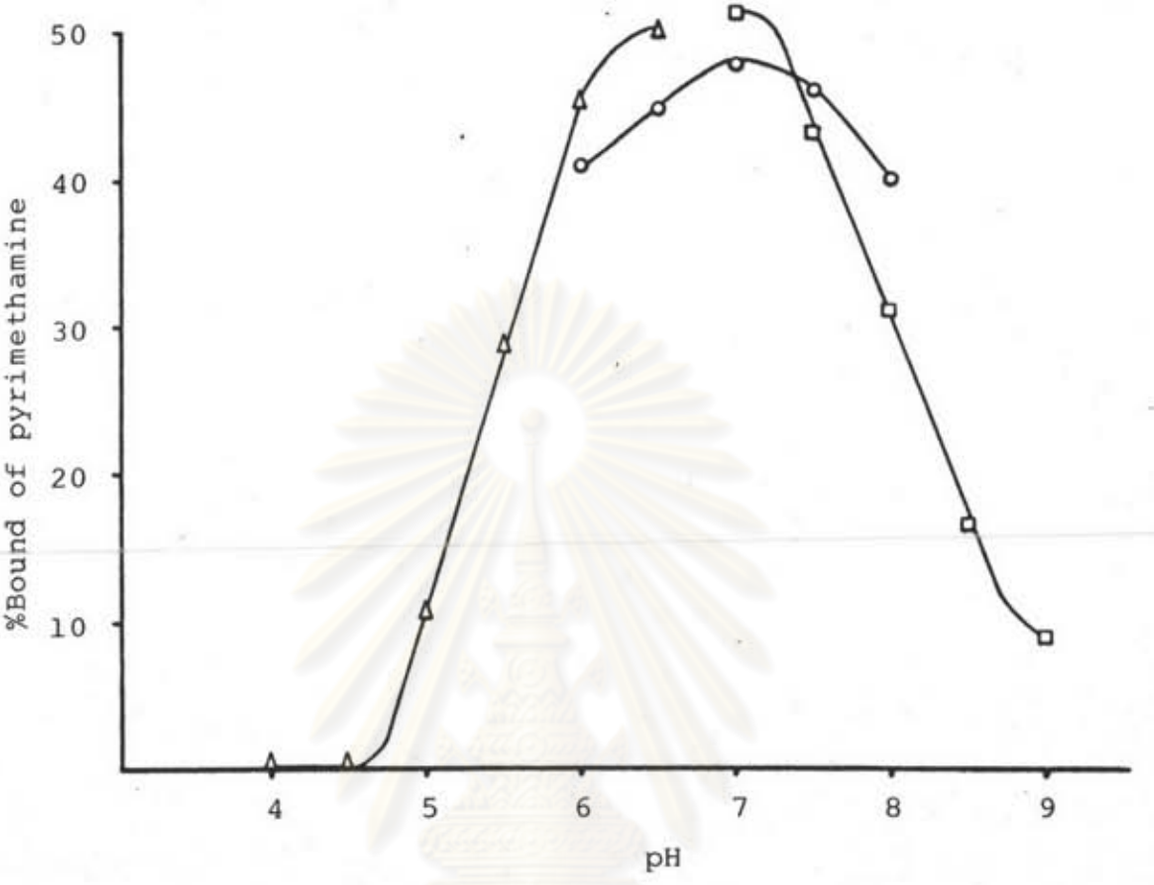


Figure 11 Effect of pH on binding of ^{14}C-pyrimethamine to rat liver DHFR.

The assay protocol was identical with that described in Methods Section 5, except that three kinds of buffer; citrate buffer (Δ), potassium phosphate buffer (○) and Tris-HCl buffer (□); at various pH were employed. The amount of rat liver DHFR was kept constant at 23.7 units.

4. Kinetic of binding between pyrimethamine and rat liver DHFR

A Scatchard analysis (Scatchard, 1949) of binding parameters between pyrimethamine and rat liver DHFR was performed at pH 6.5 and NADPH concentration of 0.1 nM, gave the binding constant (K_a) of 2.39 x 10^7 M^{-1}(Figure 12a). The presence of mouse plasma of 35 mg proteins or liver extract 100 ul (1:8 vol:vol ethanol extraction) in the assay mixture did not indicate any effect on the binding constant between pyrimethamine and rat liver DHFR. The values of K_a obtained as plotted in figure 12b and 12c still clearly showed a single homogeneous class of binding site with K_a of 2.45 x 10^7 M^{-1} and 2.23 x 10^7 M^{-1} in the presence of mouse plasma proteins and liver extract respectively.

5. Standardization of competitive binding assay for pyrimethamine

5.1 Standard curve for pyrimethamine determination

The competitive binding assay was performed according to the method described in Methods Section 6.1. The results from figure 13a and 13b showed that pyrimethamine could compete with ^{14}C-pyrimethamine which was bound to rat liver DHFR. The degree of the competition was dependent on pyrimethamine concentration. The linear relationship with C_o/C_x was in the range from 20 nM to 300 nM of pyrimethamine. No significant competition between ^{14}C-

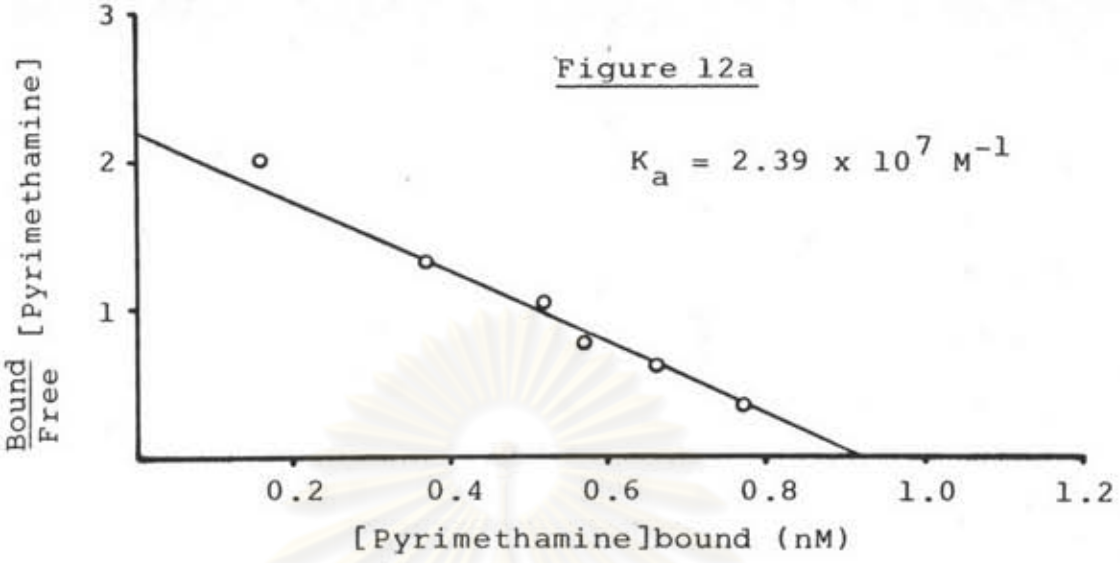


Figure 12a

$K_a = 2.39 \times 10^7\ M^{-1}$

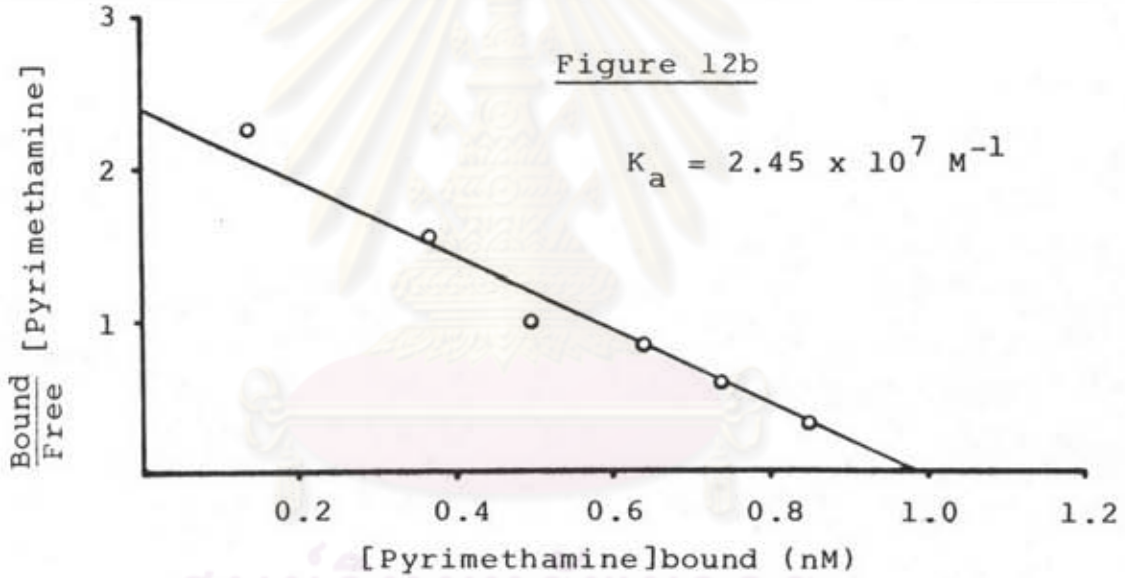


Figure 12b

$K_a = 2.45 \times 10^7\ M^{-1}$

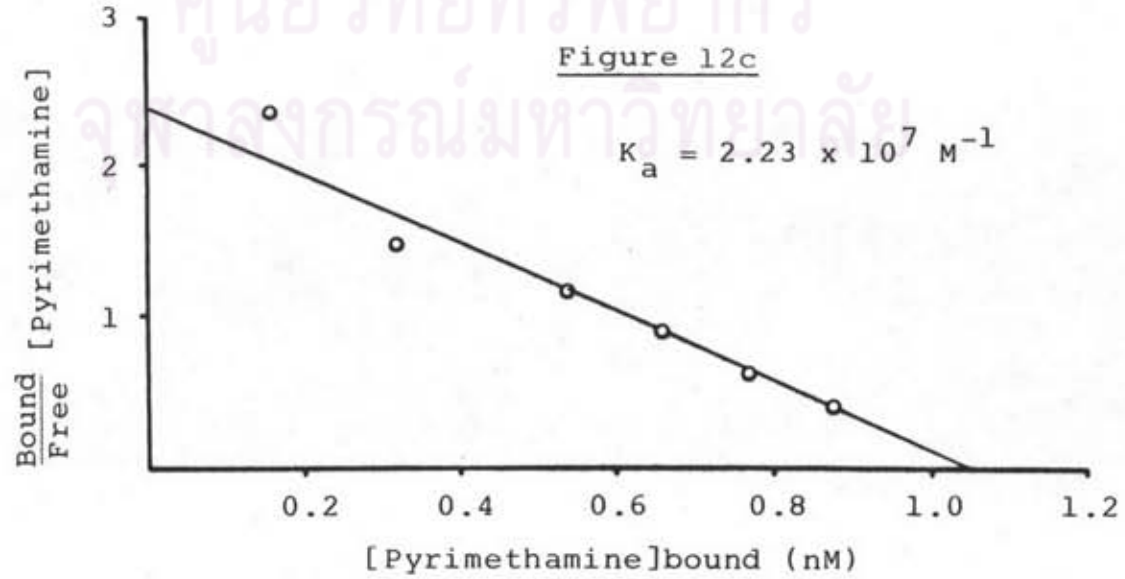


Figure 12c

$K_a = 2.23 \times 10^7\ M^{-1}$

pyrimethamine and standard pyrimethamine exhibited when the concentration of pyrimethamine was lower than 20 nM which represent the sensitivity of the assay. The sensitivity for the determination of pyrimethamine in the plasma and liver were 5 ng/ml plasma and 90 ng/g liver respectively.

From these experiments, the amount of pyrimethamine in samples could be detected by reading from standard curve. Also, the standard curve with the presence of mouse plasma (35 mg proteins) or mouse liver extract (100 ul of 1:8 vol:vol ethanol extraction) did not show significant differences from each other and from the controlled one as illustrated in figure 13a and 13b. The results in figure 13c and 13d also clearly showed that there were no significant effect in competitive binding for pyrimethamine of human plasma (39 mg proteins) and human serum (49 mg proteins).

5.2 Precision

The precision of competitive binding assay for pyrimethamine was determined in various background samples —— mouse plasma (35 mg proteins), mouse liver extract (100 ul of 1:8 vol:vol ethanol extraction), human plasma (39 mg proteins) and human serum (49 mg proteins). The assays were performed as described in Methods Section 6.2. The amount of pyrimethamine was detected within assay and between assay. Results in Table 2 showed that the percent coefficient of variations (%C.V.) within

Figure 13a

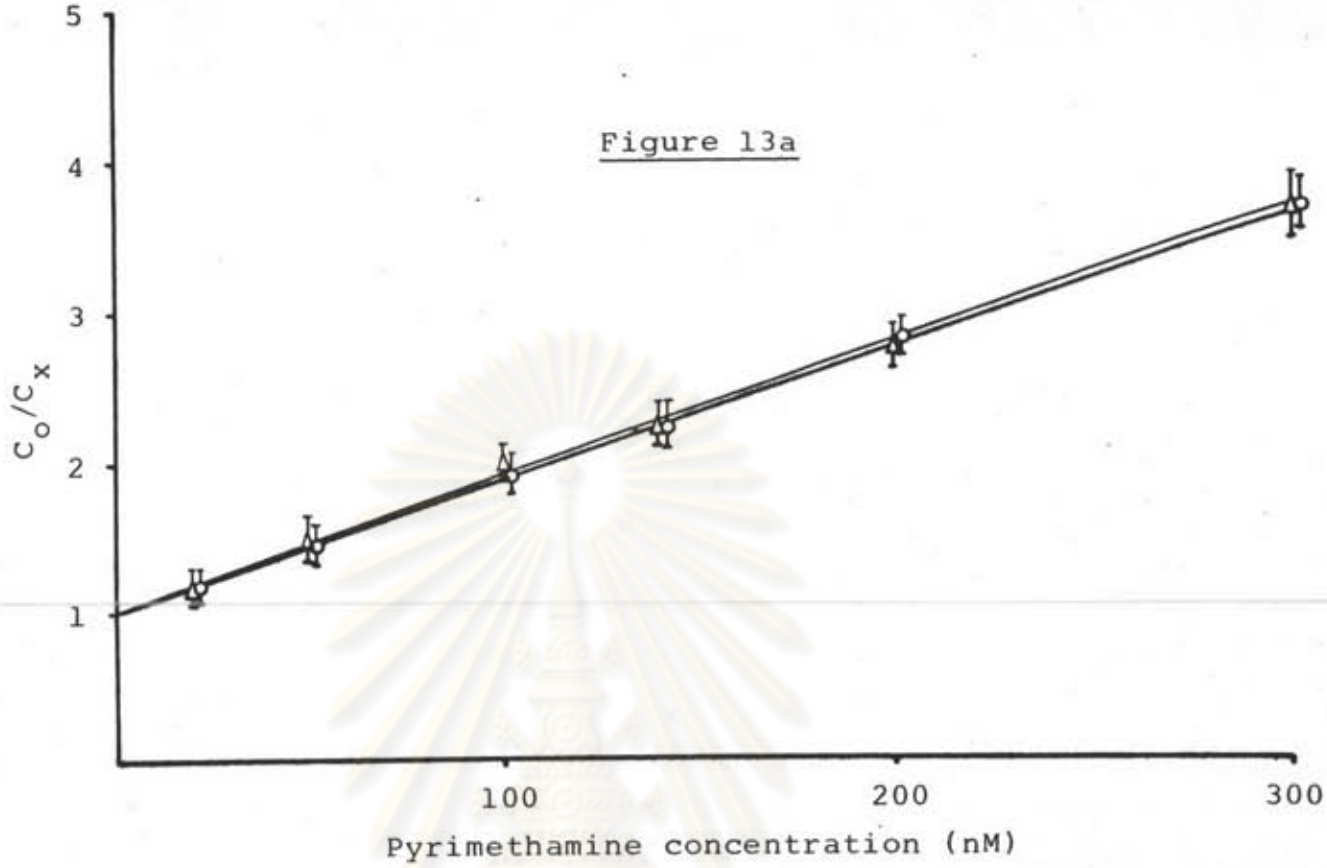
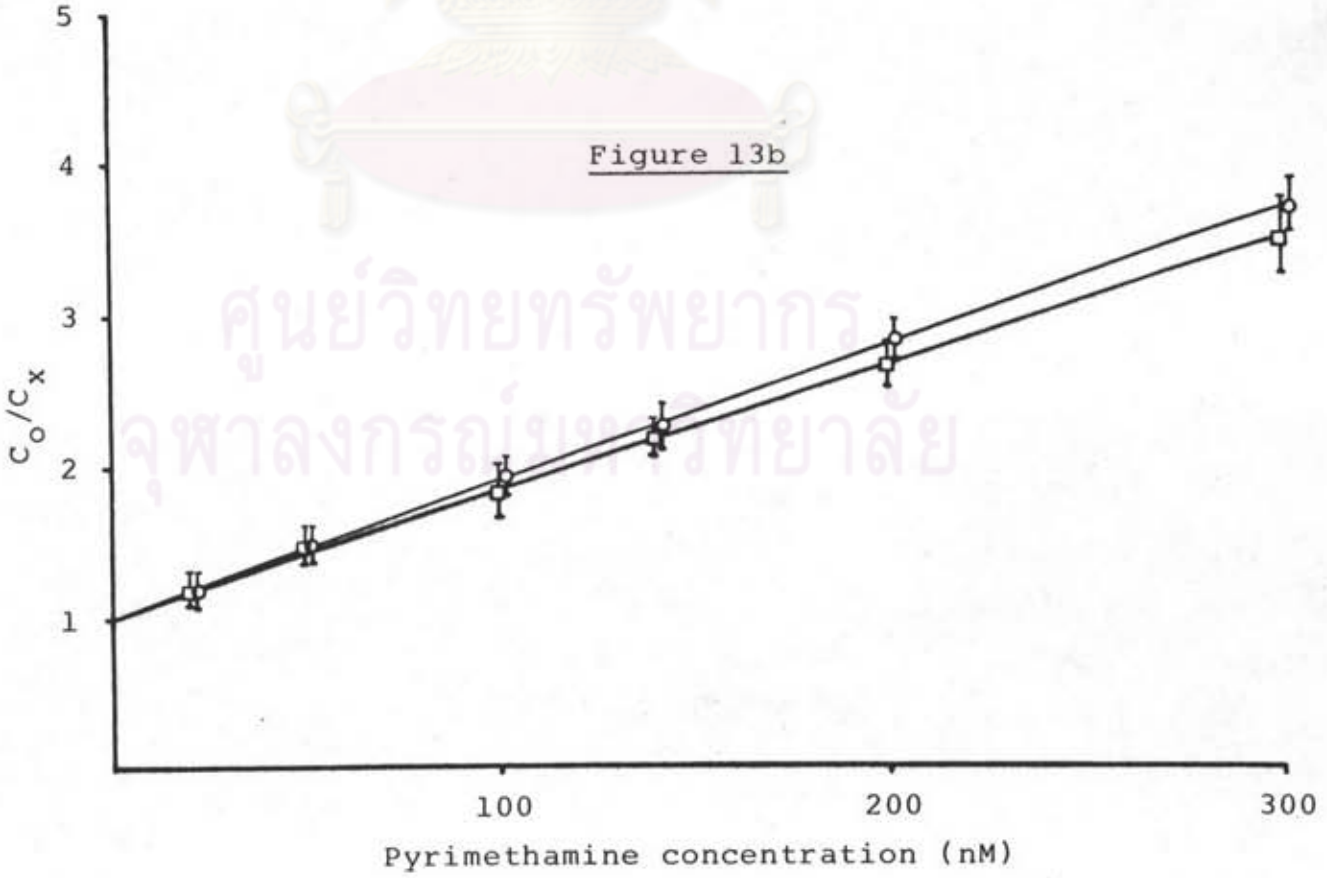


Figure 13b



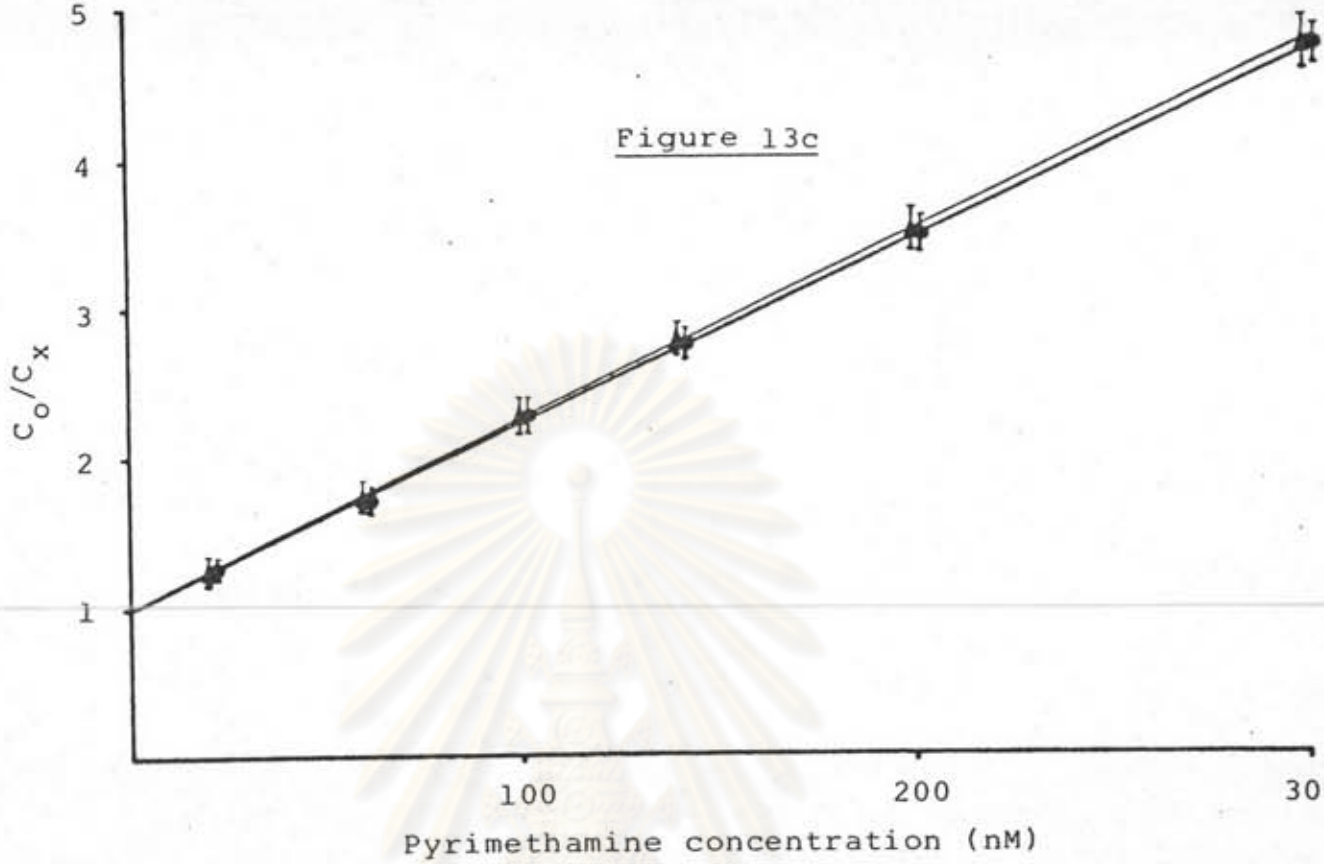


Figure 13c

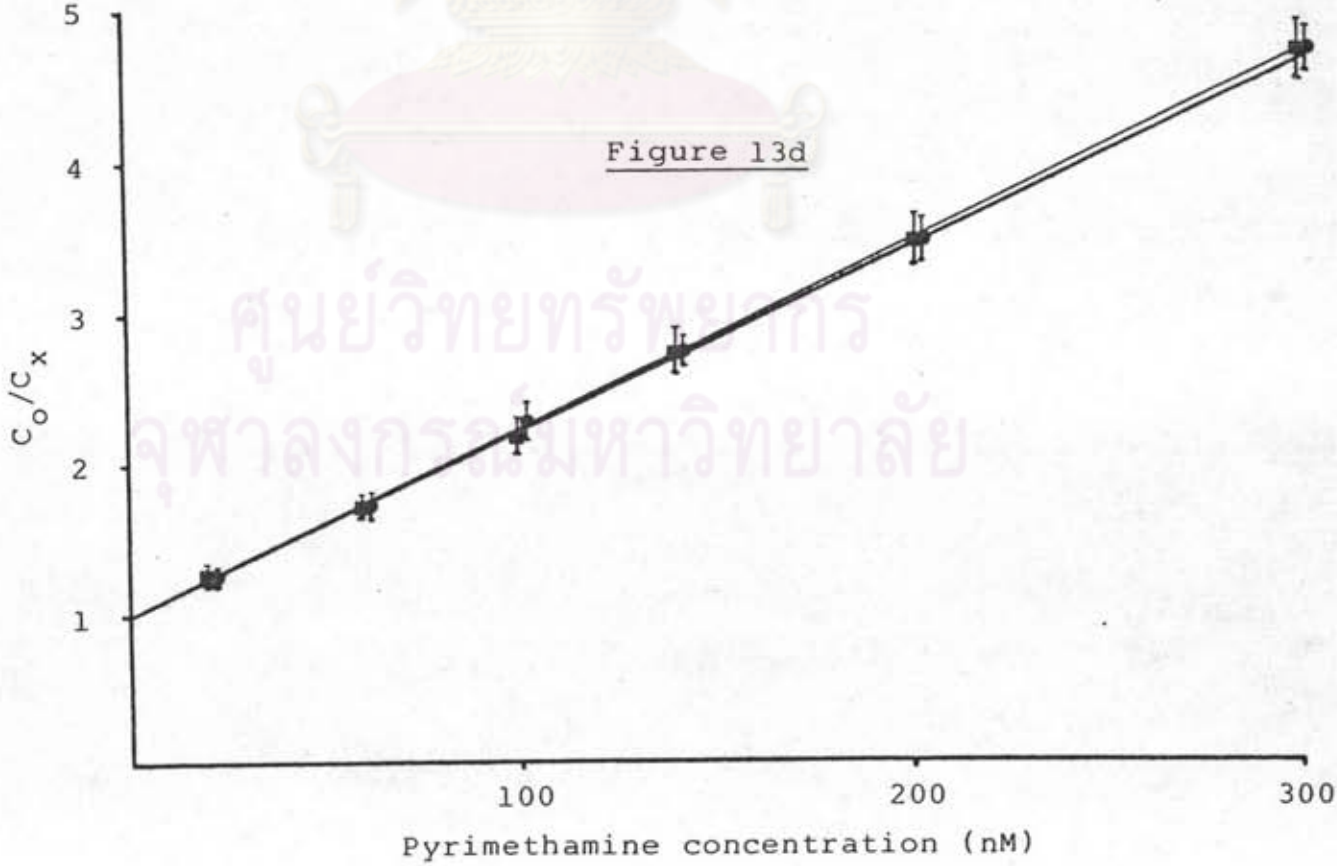
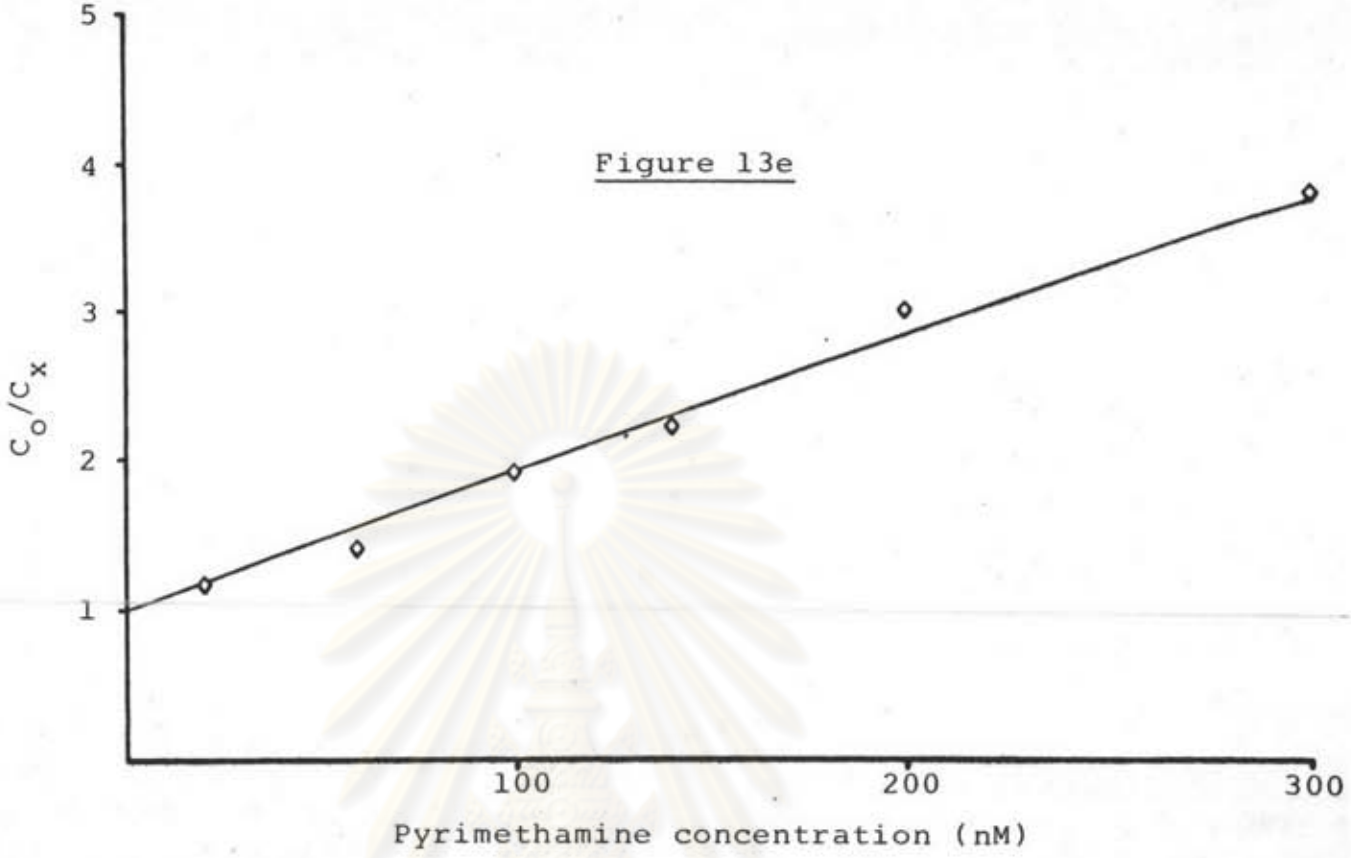


Figure 13d

Figure 13e



assay were 5.09, 5.58, 3.69 and 4.48; between assay were 5.81, 5.06, 6.07 and 6.27 when having mouse plasma, mouse liver extract, human plasma and human serum as background of samples respectively.

5.3 Accuracy

The accuracy of competitive binding assay for pyrimethamine was demonstrated with samples of 20, 60, 100, 140 nM of pyrimethamine added; then measured the amount of pyrimethamine and calculated the recovery. The results on %recovery are shown in Table 3. With the presence of mouse plasma (35 mg proteins), mouse liver extract (100 ul of 1:8 vol:vol ethanol extraction), human plasma (39 mg proteins) and human serum (49 mg proteins); %recovery was found to be in the range of 89.0-108.7, 84.5-99.4, 99.3-122.2 and 80.2-100.5 respectively.

5.4 Specificity

The specificity of the competitive binding assay for pyrimethamine was evaluated by comparing the concentration of pyrimethamine and other unlabeled folates required to decrease binding of the ^{14}C-pyrimethamine to rat live DHFR by 50% under standard assay conditions. Results are shown in Table 4. The concentration of pyrimethamine which decreased 50% binding capacity of ^{14}C-pyrimethamine to rat liver DHFR when having mouse plasma (35 mg proteins), mouse liver extract (100 ul of 1:8 vol:vol ethanol extraction), human plasma (39 mg proteins)

Table 2 Precision of competitive binding assay for pyrimethamine in various background of samples.

Background of samples	Amount of background samples	Amount of pyrimethamine					
		within assay			between assay		
		$\bar{X}$ (n=10)	S.D.	%C.V.	$\bar{X}$ (n=2)	S.D.	%C.V.
Mouse plasma	35 mg proteins	133.7	6.8	5.09	141.0	8.2	5.81
Mouse liver extract	100 ul of 1:8 vol:vol ethanol extraction	122.8	5.5	4.48	120.1	6.1	5.06
Human plasma	39 mg proteins	62.3	2.3	3.69	67.5	4.1	6.07
Human serum	49 mg proteins	52.7	2.4	4.48	57.5	3.6	6.27

Table 3 Accuracy of competitive binding assay for pyrimethamine in various background of samples.

Background of samples	Pyrimethamine added* (nM)	Pyrimethamine measured (nM)	%Recovery
Mouse plasma	-	141	-
(35 mg proteins)	20	175	108.7
	60	206	102.5
	100	225	93.4
	140	250	89.0
Mouse liver extract	-	119	-
(100 ul of 1:8 vol:vol	20	138	99.3
ethanol extraction)	60	178	99.4
	100	185	84.5
	140	222	85.7
Human plasma	-	76	-
(39 mg proteins)	20	96	100.0
	60	135	99.3
	100	209	118.8
	140	264	122.2
Human serum	-	61	-
(49 mg proteins)	20	65	80.2
	60	100	82.6
	100	157	97.5
	140	202	100.5

* n = 10

Table 4 Inhibition of ^{14}C-pyrimethamine binding by various kinds of folates and derivatives*.

Compound tested	50% Inhibition**			
	Mouse plasma (35 mg proteins)	Mouse liver extract (100uul of 1:8 vol:vol ethanol extraction)	Human plasma (39 mg proteins)	Human serum (49 mg proteins)
Pyrimethamine (M)	12.6×10^{-8}	12.2×10^{-8}	10.6×10^{-8}	19.2×10^{-8}
Folic acid (M)	3.3×10^{-5}	8.3×10^{-5}	2.9×10^{-5}	3.5×10^{-5}
Tetrahydrofolate (M)	10.4×10^{-5}	25.0×10^{-5}	5.6×10^{-5}	17.8×10^{-5}
5-Methyl-tetrahydrofolate (M)	8.6×10^{-5}	14.6×10^{-5}	10.6×10^{-5}	13.6×10^{-5}
Leucovorin (M)	1.1×10^{-4}	4.1×10^{-4}	2.0×10^{-4}	1.9×10^{-4}

* Sulphanilamides failed to inhibit ^{14}C-pyrimethamine binding at concentration up tp 1×10^{-3} M

** concentration of compound that inhibited 50% binding capacity of 10 nM ^{14}C-pyrimethamine to 23.7 units of rat liver DHFR under standard assay conditions

and human serum (49 mg proteins) were 12.6×10^{-8} M, 12.2×10^{-8} M, 10.6×10^{-8} M and 19.2×10^{-8} M, respectively, while that of folic acid were 3.3×10^{-5} M, 8.3×10^{-5} M, 2.9×10^{-5} M and 3.5×10^{-5} M; for tetrahydrofolate were 10.4×10^{-5} M, 25.0×10^{-5} M, 5.6×10^{-5} M and 17.8×10^{-5}M; for 5-methyl-tetrahydrofolate were 8.6×10^{-5} M, 14.6×10^{-5} M, 10.6×10^{-5} M and 13.6×10^{-5} M; and for leucovorin (5-formyl-tetrahydrofolate) were 1.1×10^{-4} M, 4.1×10^{-4} M, 2.0×10^{-4} M and 1.9×10^{-4} M. Sulphanilamide failed to inhibit ^{14}C-pyrimethamine binding to DHFR at concentration up to 1×10^{-3} M in all backgrounds.

6. Optimal amount of 95% ethanol used for the extraction of pyrimethamine from liver and red blood cells

Results from figure 14a and 14b illustrated that when 95% ethanol was used for the extraction of pyrimethamine from liver and red blood cells (containing 12.5 nM of ^{14}C-pyrimethamine); the ratio of liver homogenate or red blood cells to extracting ehtanol which yield maximum recovery of pyrimethamine (95% for liver homogenate and 85% for red blood cells) was found to be 1:5 (vol:vol) or more. However, when the concentration of pyrimethamine either in liver homogenate or red blood cells was increased to 12.5 nM of ^{14}C-pyrimethamine plus 0.1 mM of cold pyrimethamine; the ratio of liver homogenate or red blood cells to extracting ethanol used was increased to be 1:8 (vol:vol) for maximum 90% of pyrimethamine recovery from both sources.

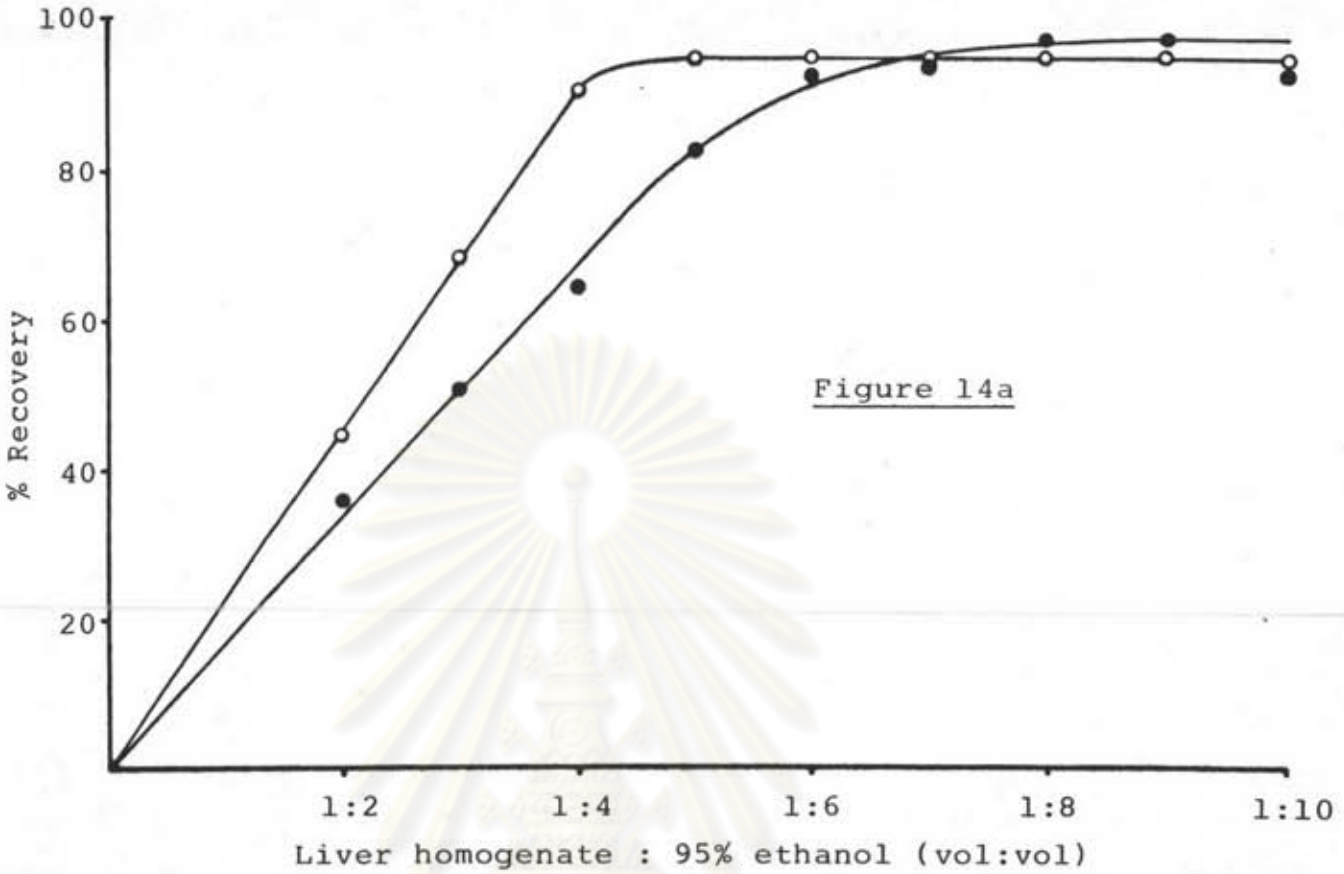


Figure 14a

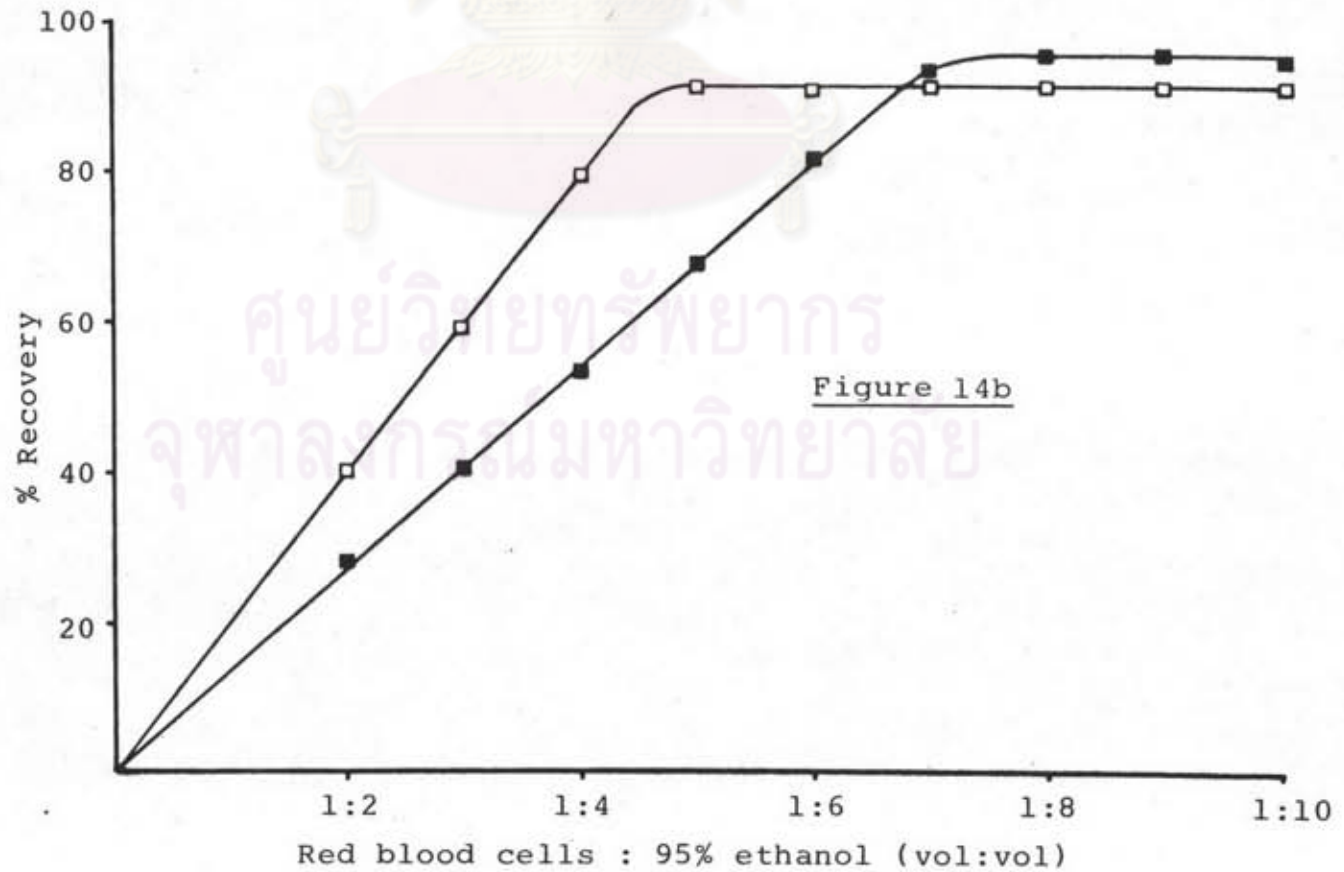


Figure 14b

7. Determination of pyrimethamine levels in plasma of *Plasmodium chabaudi* infected mice by competitive binding assay method

7.1 Pattern of *P. chabaudi* infection in non-pyrimethamine treated mice

Mice infected with *P. chabaudi* AS or *P. chabaudi* AS(Pr_1) which did not receive pyrimethamine showed similar changes in parasitemia pattern. Results are shown in figure 15a and 15b respectively. On the first day after mice were inoculated with approximately 10^8 parasitized erythrocytes, percent parasitemia was low (about 5 percents) and it slowly increased to about 10 percents on the second day. After that, percent parasitemia rapidly increased and when percent parasitemia reached about 80-90 percents, all of the infected mice died (approximately on day 4-5 after parasite infection).

7.2 Pattern of *P. chabaudi* infection and pyrimethamine turnover in mice treated with single dose of pyrimethamine orally (5 mg/kg body wt)

7.2.1 Pattern of *P. chabaudi* infection in mice treated with pyrimethamine orally (5 mg/kg body wt)

Figure 16a and 16b illustrated the changes in parasitemia of mice infected with *P. chabaudi* AS and *P. chabaudi* AS(Pr_1) which were treated with single dose of pyrimethamine orally (5 mg/kg body wt) on day 3 after

parasite inoculation. Percent parasitemia on day 3 at which pyrimethamine was administered were about 15-25% for mice infected with *P. chabaudi* AS and about 20-40% for mice infected with *P. chabaudi* AS(Pr_1). Within 6 hr after pyrimethamine administration, most of the parasitemia dropped slowly but some that were infected with *P. chabaudi* AS(Pr_1) showed a little increase in the parasitemia, as between 6 and 12 hr after pyrimethamine administration. Between 12 and 24 hr after treatment, most of the examination results had drastic decrease in the parasitemia. After 36 hr of drug treatment, most of the mice still showed decrease in the parasitemia but some showed no marked changes. The parasitemia of all mice started to rise again after 72 hr of pyrimethamine treatment for mice infected with *P. chabaudi* AS. A much shorter period of time for the increment of percent parasitemia was observed after 36 hr of drug treatment for mice infected with *P. chabaudi* AS(Pr_1). The recrudescent parasitemia peaks were observed between day 6 to 8 after parasite inoculation; then the parasitemia decreased and no significant amount of parasites were observed after day 12 for mice infected with *P. chabaudi* AS. For mice infected with *P. chabaudi* AS(Pr_1), the recrudescent parasitemia peaks were observed between day 6 to 7 after parasite inoculation and no parasites were observed after day 10.

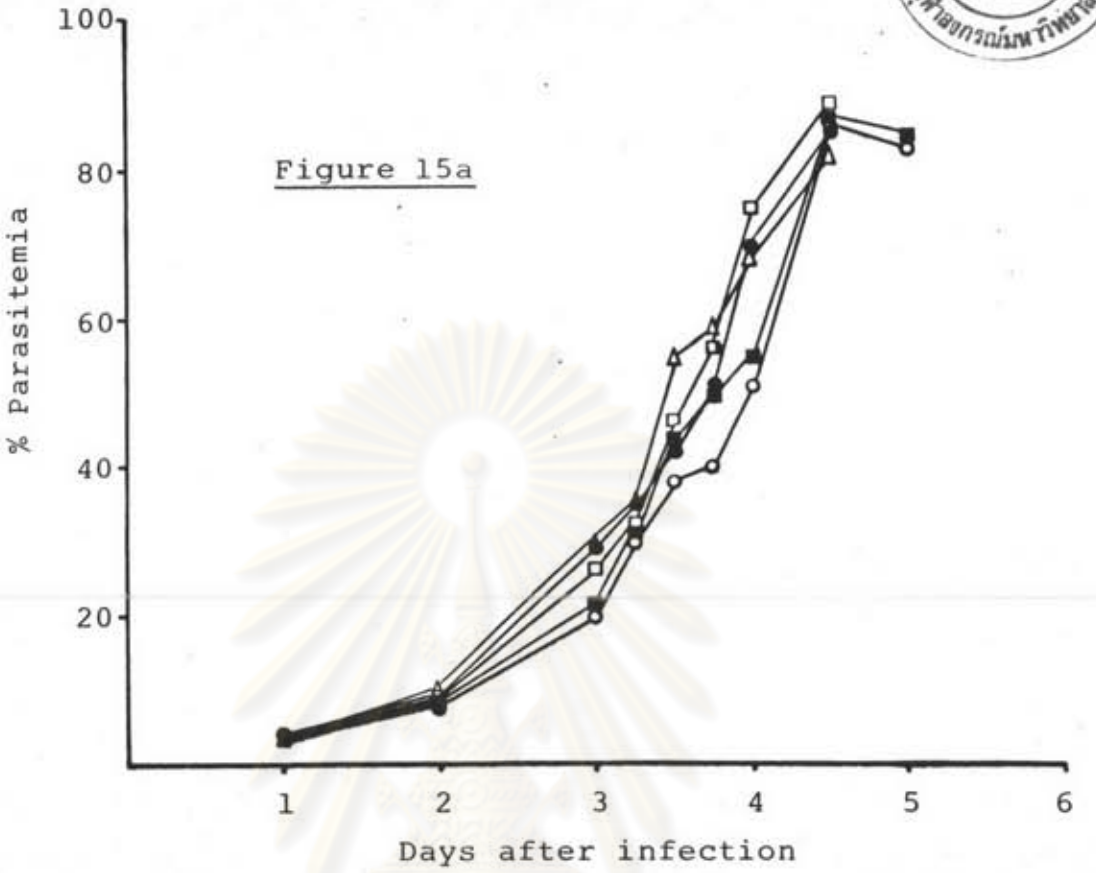


Figure 15a

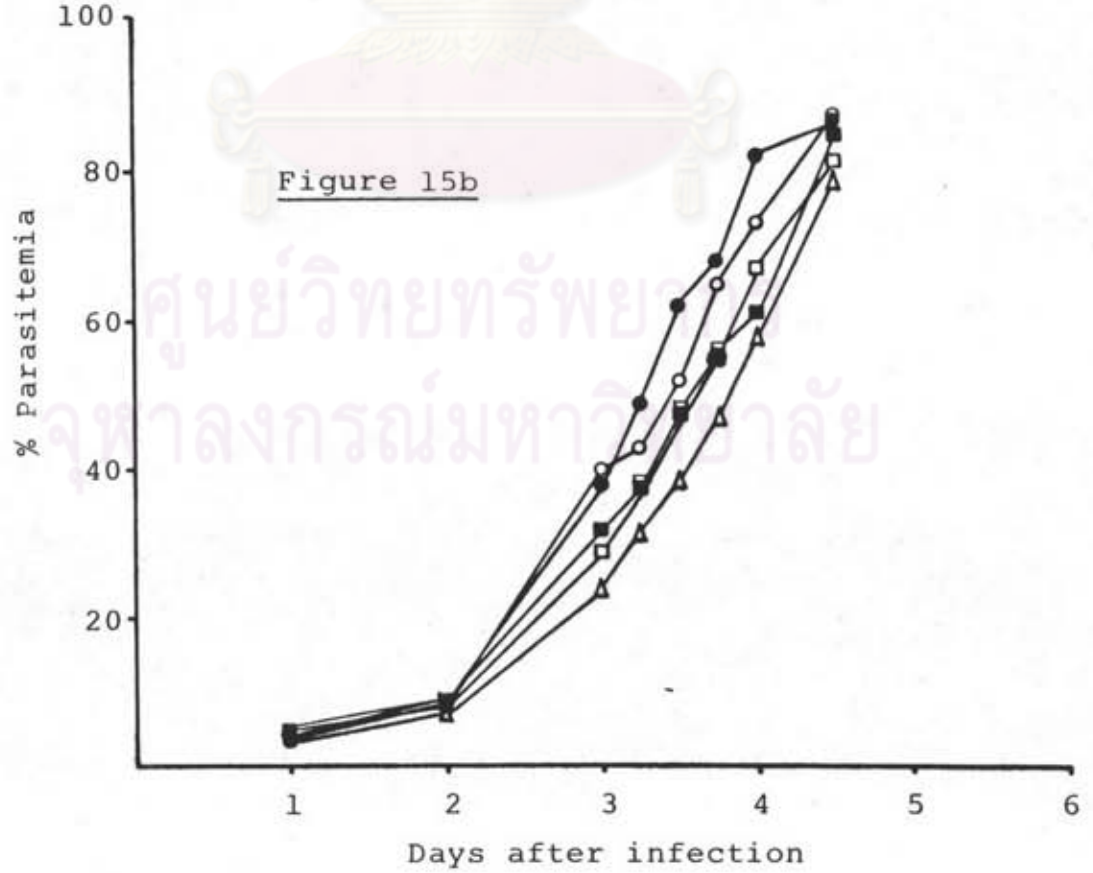


Figure 15b

Figure 16a

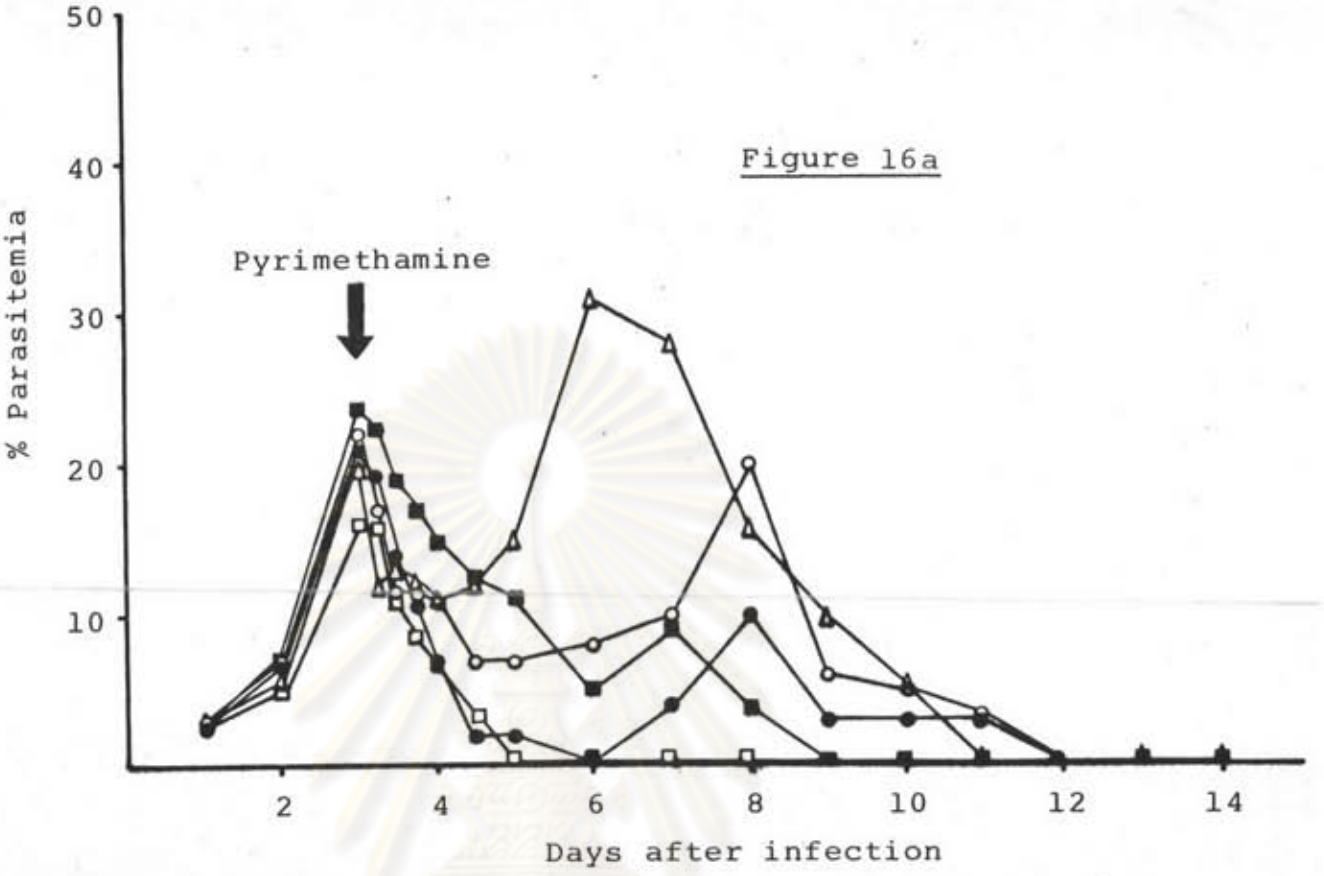
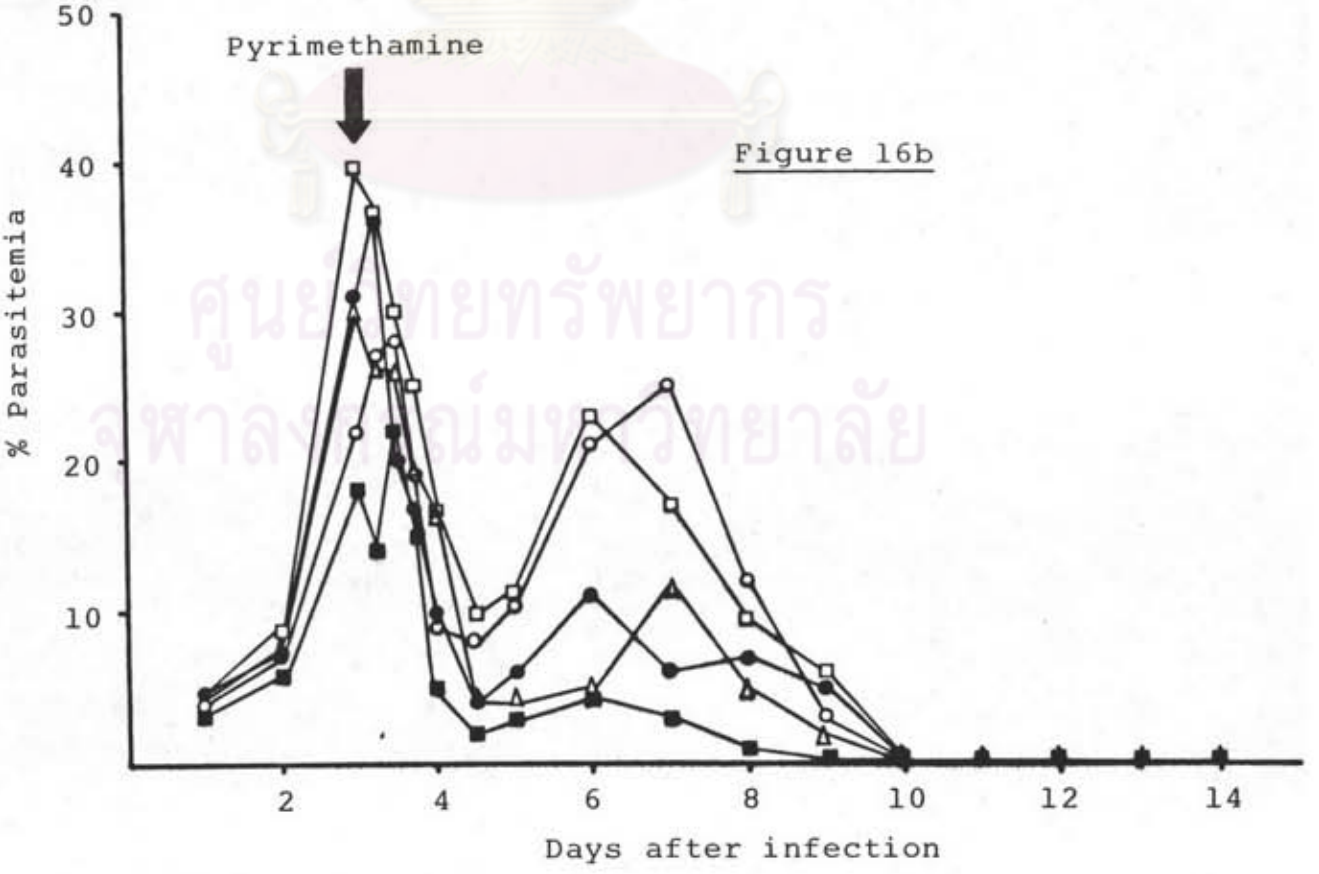


Figure 16b



7.2.2 Pattern of pyrimethamine turnover in mice treated with pyrimethamine orally (5 mg/kg body wt)

The levels of pyrimethamine in plasma of mice were determined after mice received single dose of pyrimethamine orally (5 mg/kg body wt). These determinations were done in uninfected mice, mice infected with *P. chabaudi* clone AS and AS(Pr_1). The infected mice were treated with pyrimethamine on day 3 after parasite inoculation of which the percent parasitemia was around 20-30. Results are shown in figure 17a, 17b and 17c. The levels of pyrimethamine in plasma of uninfected mice reached a peak at 1 hr after drug administration then the levels of pyrimethamine dropped and could not be observed at 18 hr after drug administration (Figure 17a). For mice infected with *P. chabaudi* AS and mice infected with *P. chabaudi* AS(Pr_1), the levels of pyrimethamine in plasma reached a peak approximately 3 hr after drug administration and it was also observed that the level of drug in plasma of mice infected with *P. chabaudi* AS remained in detectable levels at 48 hr after drug treatment while mice infected with *P. chabaudi* AS(Pr_1) the pyrimethamine levels in plasma was retained for a shorter period and could not be detected at 24 hr after drug administration (Figure 17b and 17c). Interestingly, the levels of pyrimethamine detected in plasma of mice infected with *P. chabaudi* AS were slightly higher than that in mice infected with *P. chabaudi* AS(Pr_1) and in uninfected mice respectively.

The levels of pyrimethamine were also measured in liver extract of mice after receiving 5 mg/kg body wt of pyrimethamine orally. As can be observed in figure 18a and 18b, the levels of pyrimethamine reached a peak at 1 and 3 hr after drug administration for uninfected mice and mice infected with *P. chabaudi* AS which were similar to the data observed in plasma. However, mice infected with *P. chabaudi* AS(Pr_1) gave a longer increment period of pyrimethamine in the liver (6 hr) (Figure 18c). The small amount of pyrimethamine could still be detected at 48 hr after drug administration. The noticeable higher levels of pyrimethamine were detected in liver extract of mice infected with *P. chabaudi* AS than in mice infected with *P. chabaudi* AS(Pr_1) and in uninfected mice which were similar to that observed in plasma of mice.

Since the detection values of pyrimethamine in red blood cells extract were low, so the significance of the data was obtained from the mean value (n=2) of the detection from concentrated pooled samples extract in each experimental sets as described in Method Section 8.3.2. However, the results illustrated in figure 19a, 19b and 19c still showed quite similar pattern as were found in plasma and liver extract of mice. The levels of pyrimethamine in red blood cells extract of mice infected with *P. chabaudi* AS were higher and retained for a longer period after drug administration than that of mice infected with *P. chabaudi* AS(Pr_1) and uninfected mice respectively. The levels of pyrimethamine in red blood cells extract of

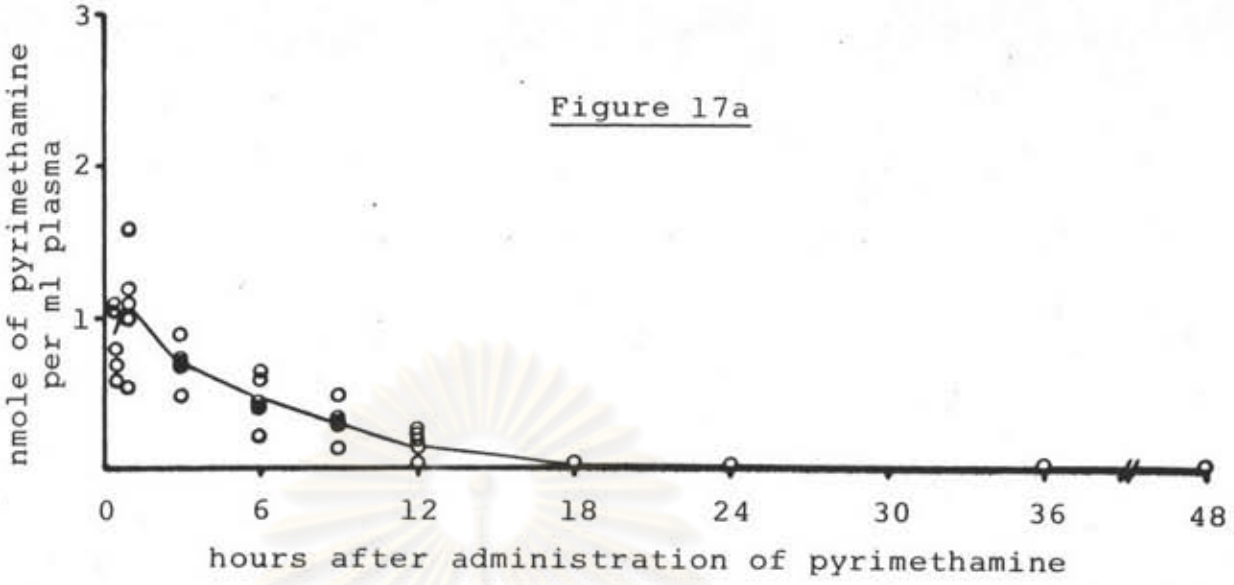


Figure 17a

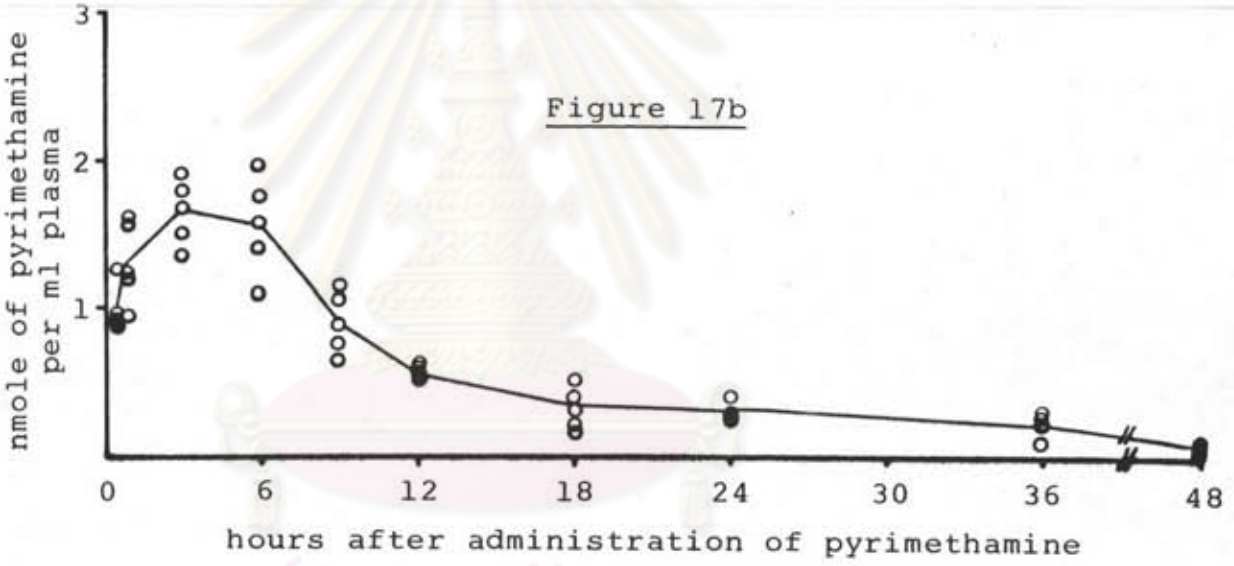


Figure 17b

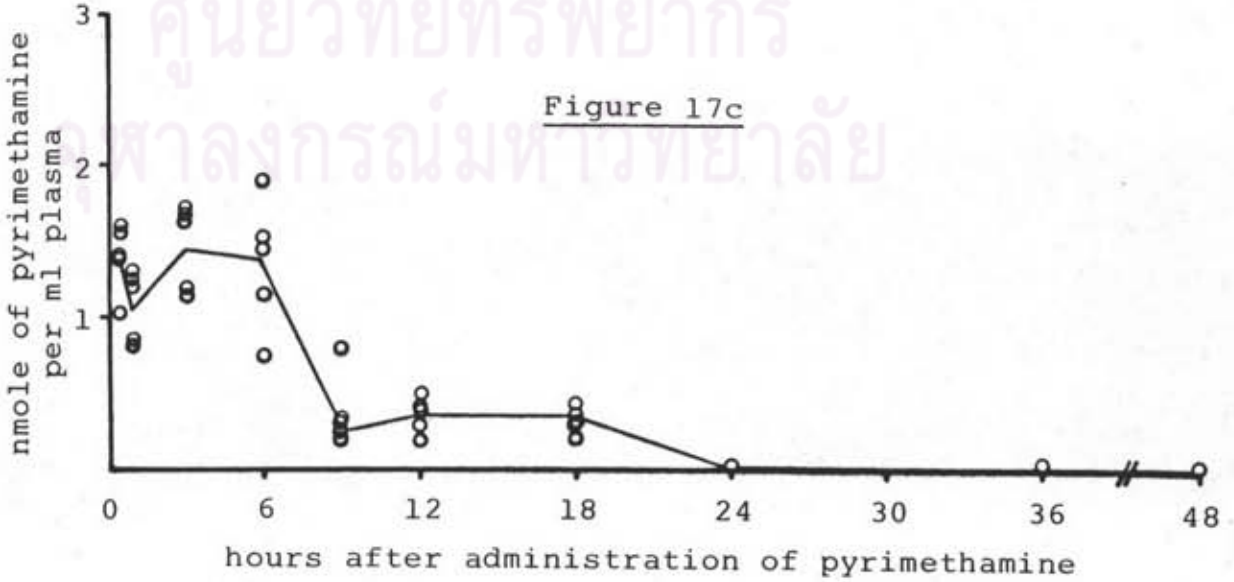


Figure 17c

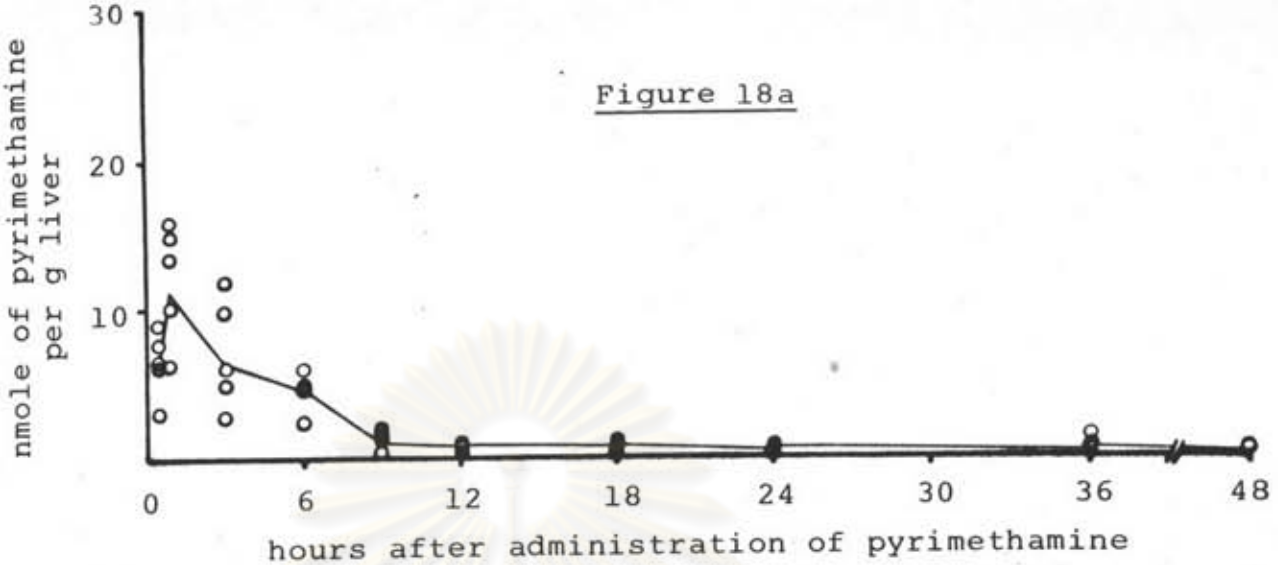


Figure 18a

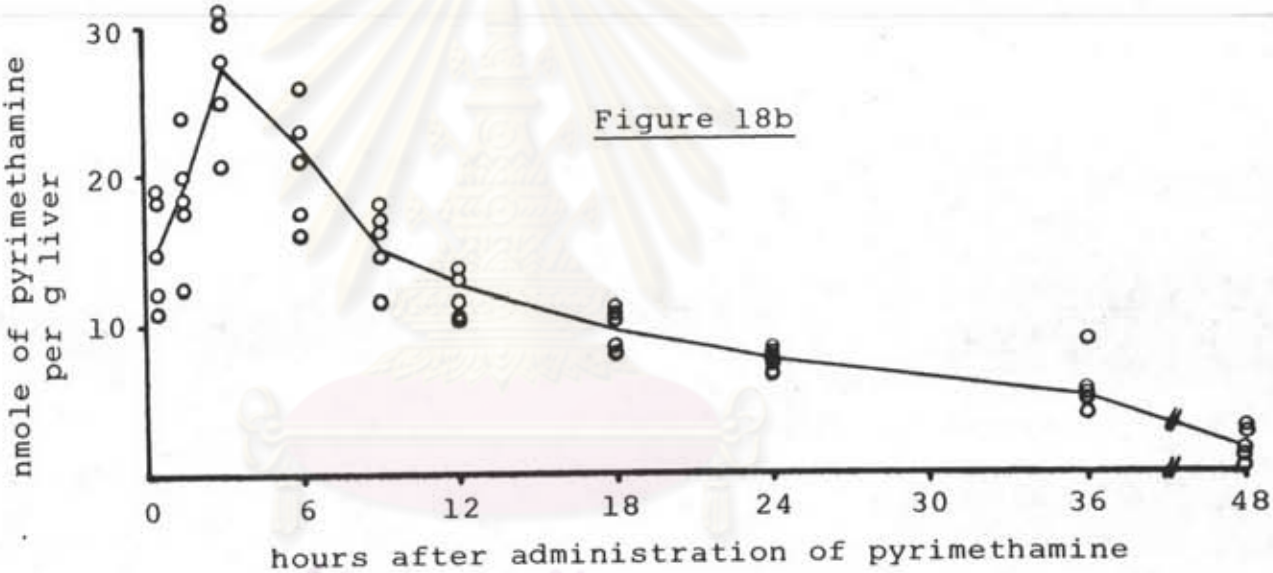


Figure 18b

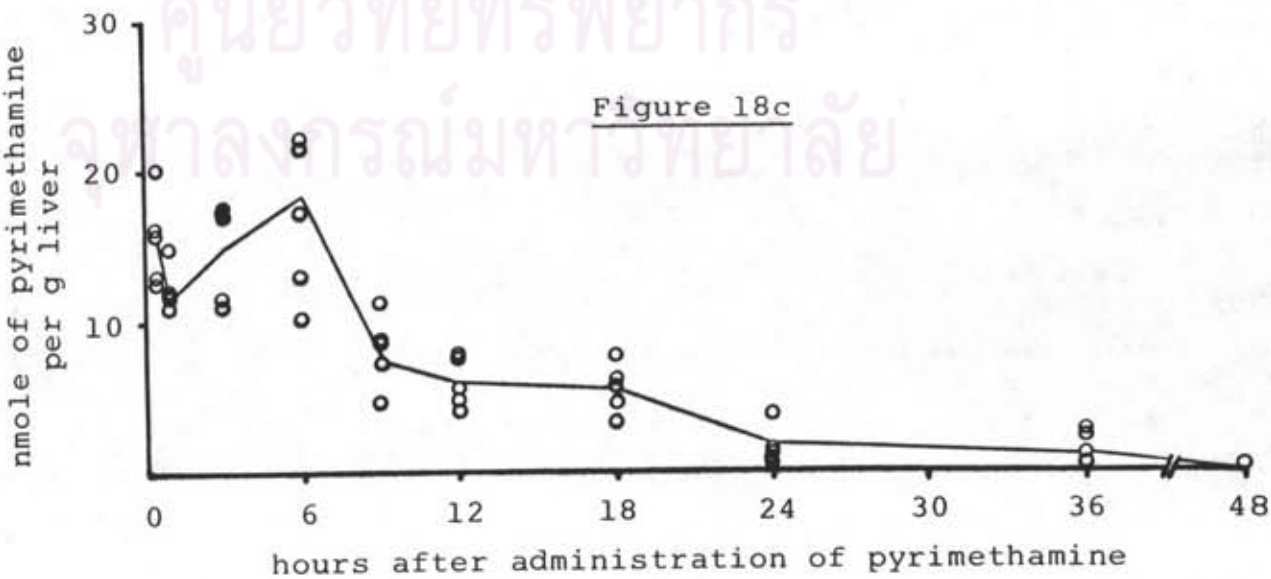


Figure 18c

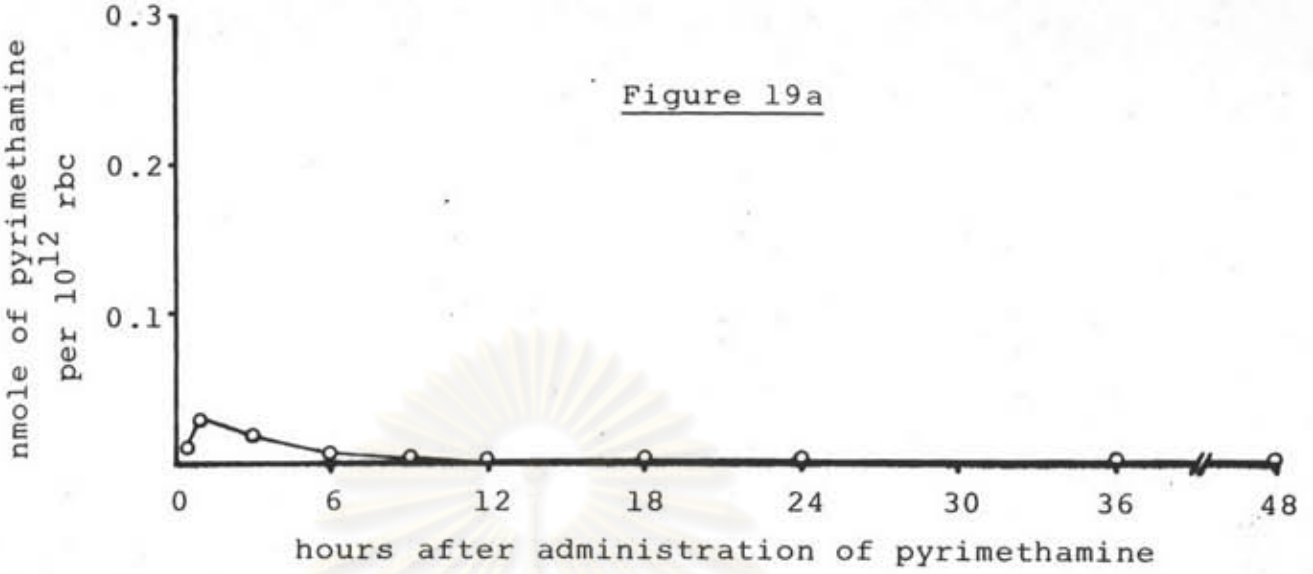


Figure 19a

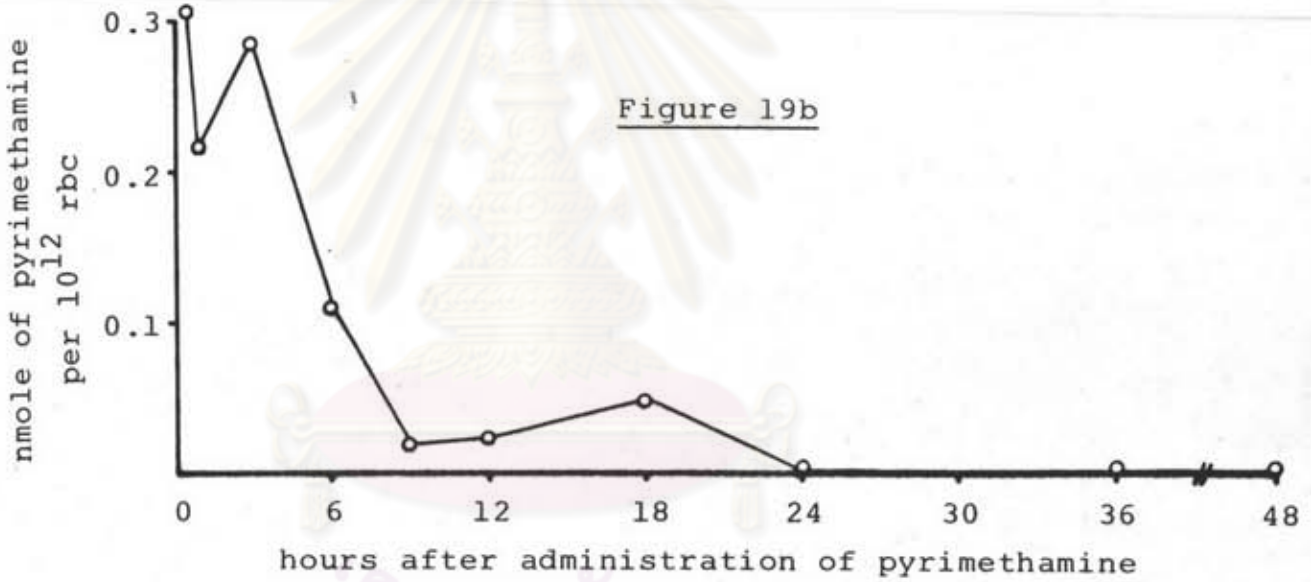


Figure 19b

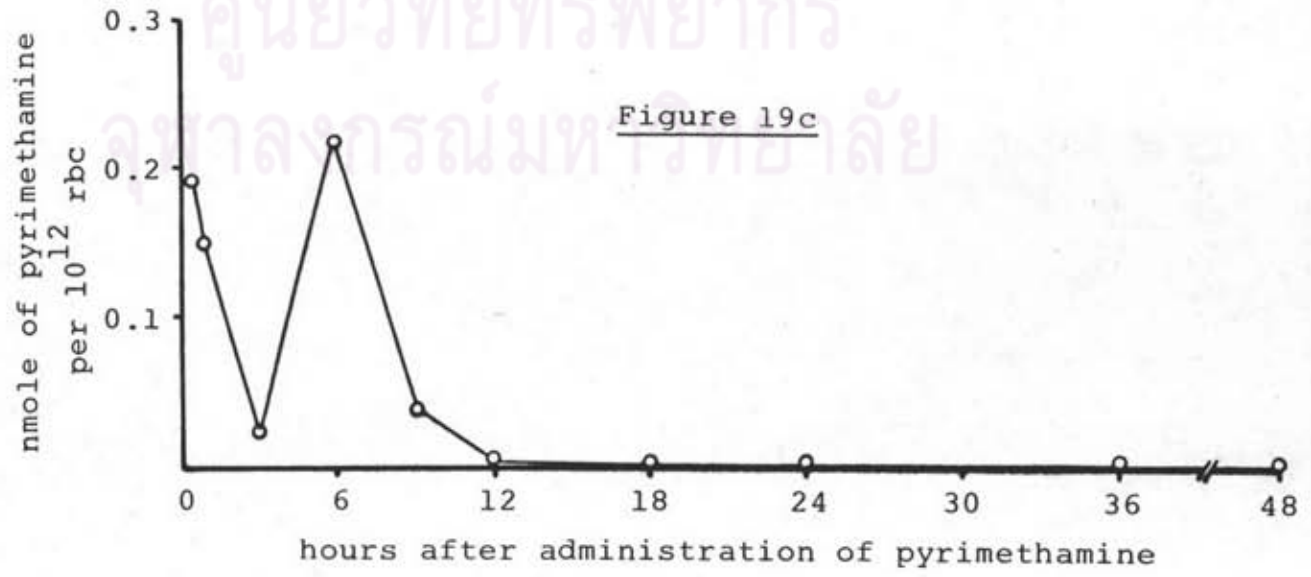


Figure 19c

uninfected mice were extremely low in comparison to the values which were found in both infected mice. They reached a peak at 1 hr and could not be detected at 12 hr after drug administration whereas for that of mice infected with *P. chabaudi* AS showed maximum peak at 3 hr and could not be detected at 24 hr. For that of mice infected with *P. chabaudi* AS(Pr_1), the maximum peak was at 6 hr and the clearance of pyrimethamine was at 18 hr.

7.3 Pattern of *P. chabaudi* infection and pyrimethamine turnover in mice treated with single dose of pyrimethamine intraperitoneally (5 mg/kg body wt)

7.3.1 Pattern of *P. chabaudi* infection in mice treated with pyrimethamine intraperitoneally (5 mg/kg body wt)

Figure 20a and 20b illustrated the changes in parasitemia of mice infected with *P. chabaudi* AS and *P. chabaudi* AS(Pr_1) which were treated with single dose of pyrimethamine (5 mg/kg body wt) intraperitoneally on day 3 after parasite inoculation. Percent parasitemia on day 3 at which mice were treated with pyrimethamine were about 15-25% and 20-40% for mice infected with *P. chabaudi* clone AS and AS(Pr_1) respectively. After mice received pyrimethamine, the changes in parasitemia pattern were mostly similar to that observed in mice treated with 5 mg/kg body wt of pyrimethamine orally. The decrease in para-

sitemia was observed. The parasitemia started to increase again between 12-24 hr after drug treatment and the rather high recrudescent parasitemia peaks were observed on day between 5-7 and no parasites were observed on day 12 after parasite inoculation; these were for mice infected with *P. chabaudi* AS (Figure 20a). For mice infected with *P. chabaudi* AS(Pr_1), the parasitemia started to rise again after 12-48 hr after pyrimethamine treatment and the recrudescent parasitemia peaks were observed on day 5-6, then the parasitemia dropped and could not be determined on day 11-12.

7.3.2 Pattern of pyrimethamine turnover in mice treated with pyrimethamine intraperitoneally (5 mg/kg body wt)

The levels of pyrimethamine in plasma of mice were determined after mice received single dose of pyrimethamine (5 mg/kg body wt) intraperitoneally. The levels of pyrimethamine in uninfected mice, mice infected with *P. chabaudi* AS and *P. chabaudi* AS(Pr_1) were illustrated in figure 21a, 21b and 21c respectively. The results indicated that pyrimethamine was rapidly uptaken into plasma when drug was administered by intraperitoneal injection. The values of pyrimethamine in plasma of uninfected mice at 0.5 hr after drug administration which was the first point of analysis were found to be highest; then gradually dropped and could not be detected any more at 18 hr. For those mice infected with *P. chabaudi* AS and *P. chabaudi*

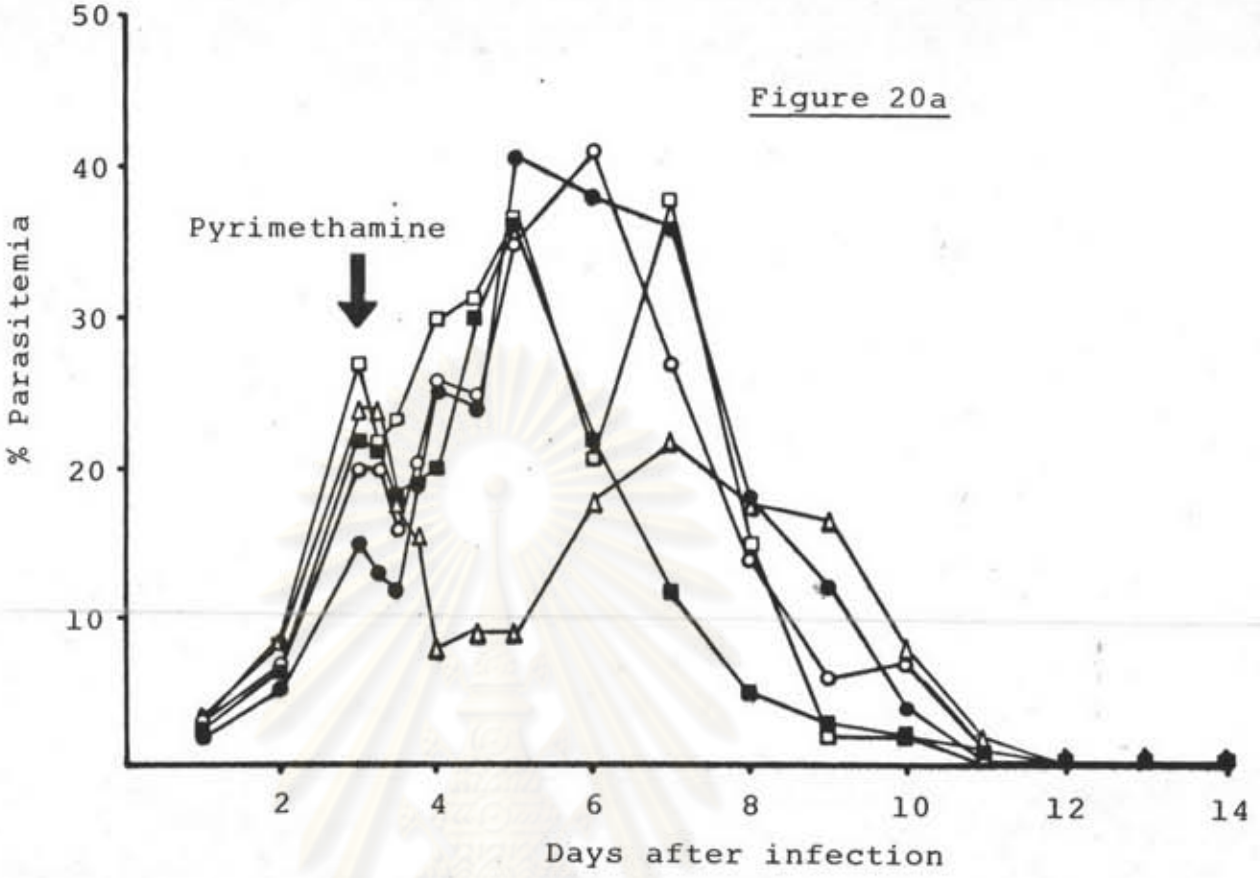


Figure 20a

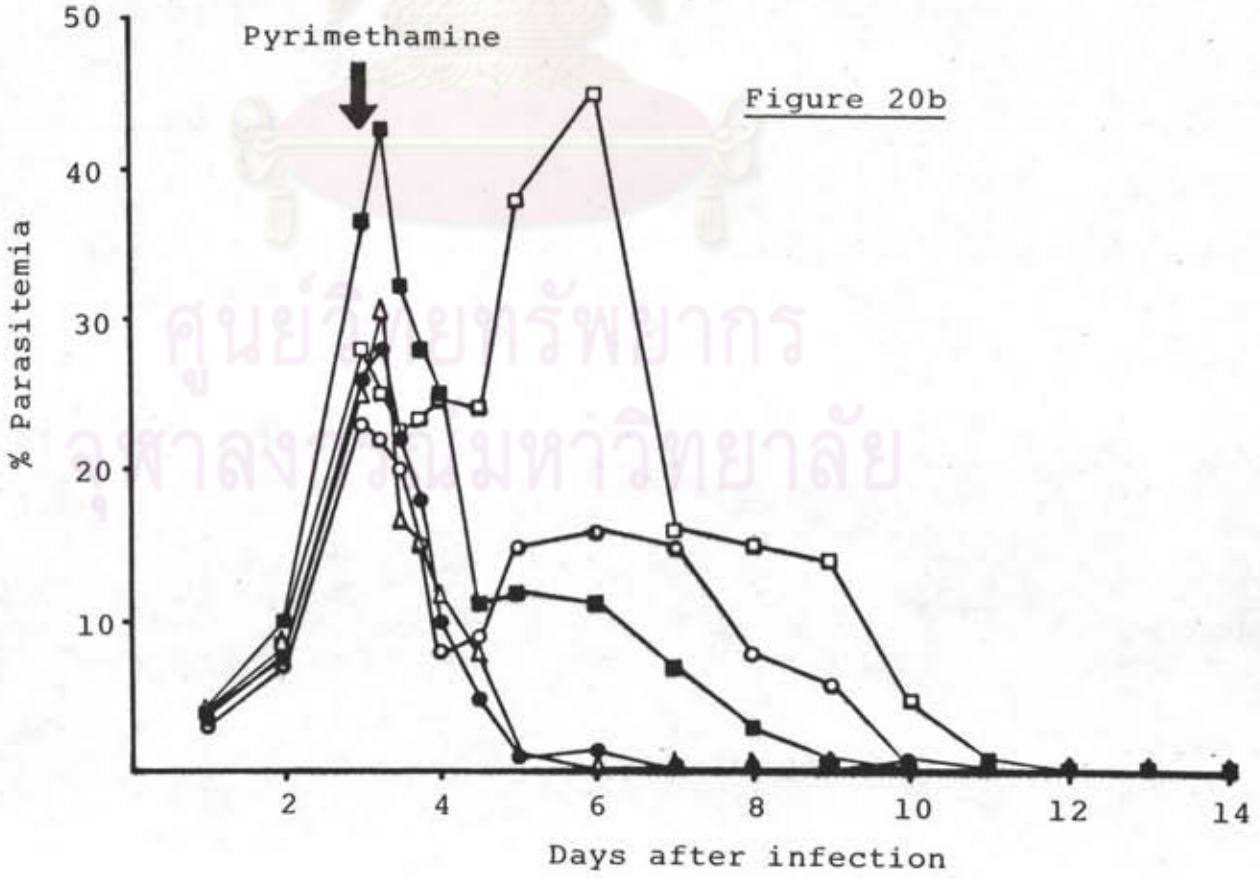


Figure 20b

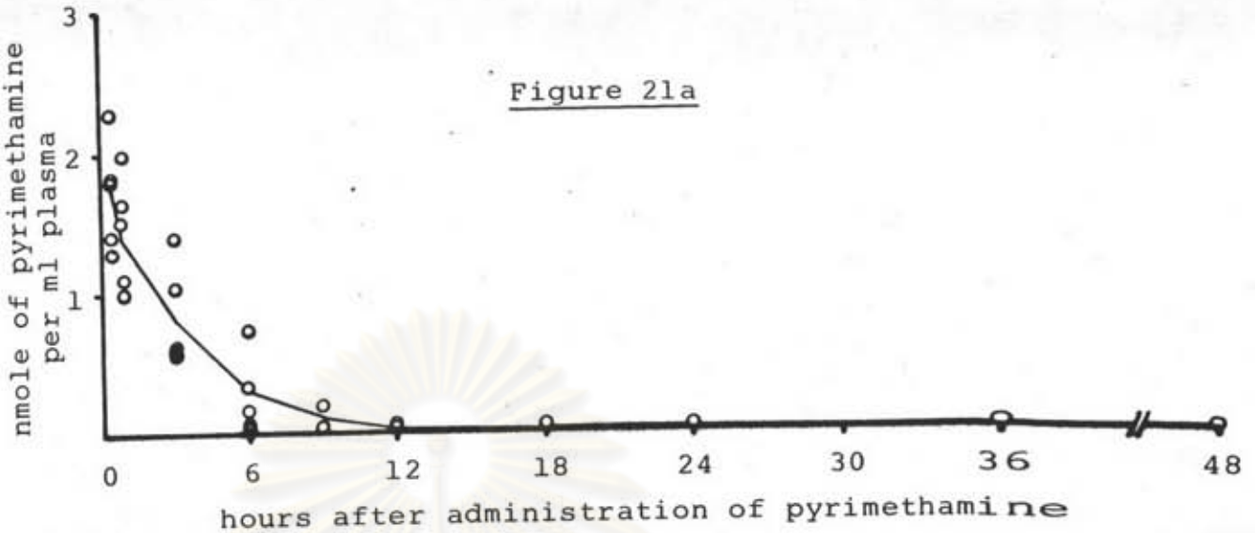


Figure 21a

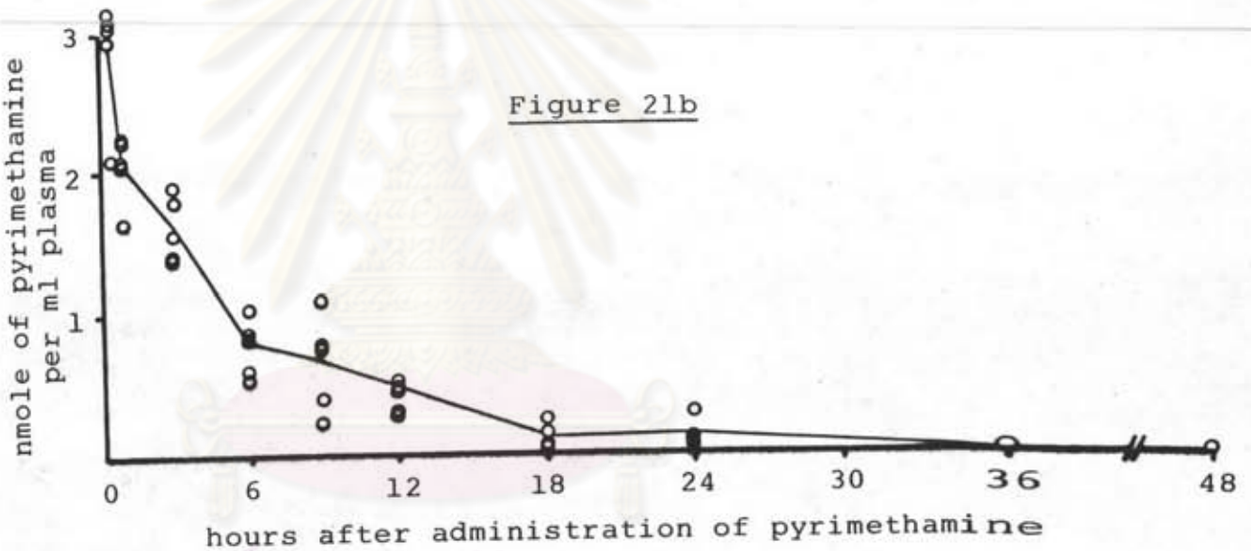


Figure 21b

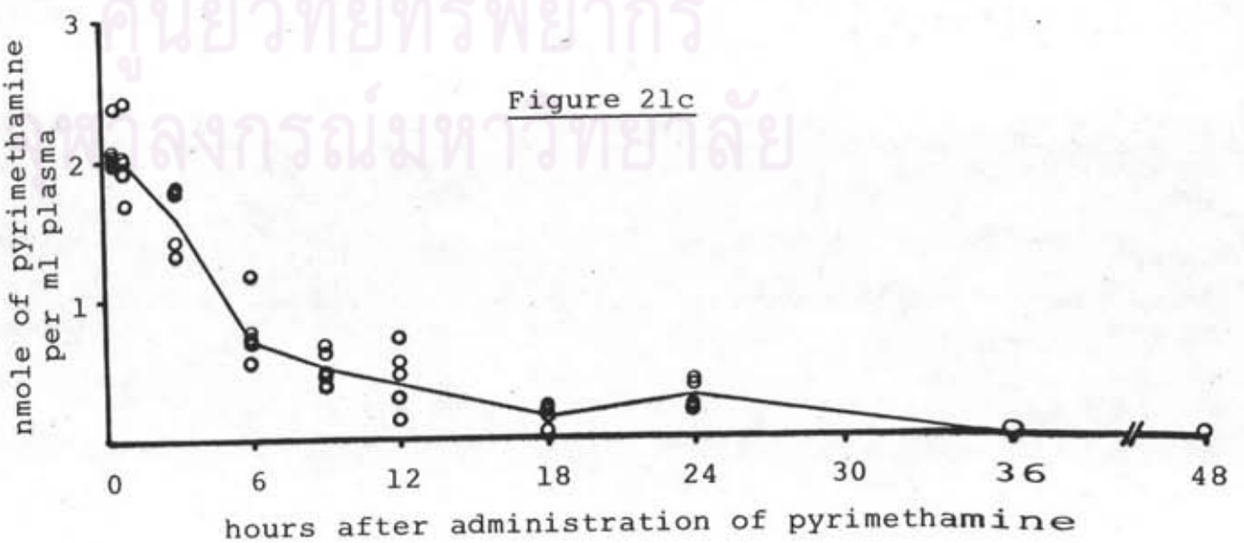


Figure 21c

AS(Pr_1), no drug could be detected at 36 hr after pyrimethamine administration. Hereagain, the levels of pyrimethamine detected in plasma of mice infected with *P. chabaudi* AS and *P. chabaudi* AS(Pr_1) were higher than that of uninfected mice.

7.4 Pattern of *P. chabaudi* infection and pyrimethamine turnover in mice treated with single dose of pyrimethamine intraperitoneally (30 mg/kg body wt)

7.4.1 Pattern of *P. chabaudi* infection in mice treated with pyrimethamine intraperitoneally (30 mg/kg body wt)

The changes in parasitemia for mice infected with *P. chabaudi* clone AS and AS(Pr_1) when treated with single dose of pyrimethamine (30 mg/kg body wt) intraperitoneally on day 3 after parasite inoculation were illustrated in figure 22a and 22b. Percent parasitemia on day 3 at which pyrimethamine was administered were in the range of 15-25% for mice infected with *P. chabaudi* AS (except mouse number 3 of which percent parasitemia was 6%) and 25-35% for mice infected with *P. chabaudi* AS(Pr_1) (except mouse number 4 of which percent parasitemia was 10%). After mice received pyrimethamine, they exhibited a rather similar pattern of parasitemia in comparison to that observed when mice were treated with 5 mg/kg body wt of drug either orally or intraperitoneally. However, the time for

the appearance of recrudescent peak of parasitemia were obviously longer in mice infected with both clones of *P. chabaudi* when treated with pyrimethamine 30 mg/kg body wt. The percent parasitemia of mice infected with *P. chabaudi* AS started to rise again after pyrimethamine treatment 2-5 days in comparison to the value observed after 5 days or longer which was found in mice infected with *P. chabaudi* AS(Pr_1). The parasitemia then decreased and could not be detected on day 11-14 for the infected clone *P. chabaudi* AS while slightly longer time of 12-15 days was found in mice infected with *P. chabaudi* AS(Pr_1).

7.4.2 Pattern of pyrimethamine turnover in mice treated with pyrimethamine intraperitoneally (30 mg/kg body wt)

Figure 23a, 23b and 23c illustrated the levels of pyrimethamine in plasma of uninfected mice, mice infected with *P. chabaudi* AS and mice infected with *P. chabaudi* AS(Pr_1) after receiving single dose of pyrimethamine (30 mg/kg body wt) intraperitoneally. No indication of maximum peak was found in uninfected mice. The first value of analytical levels of pyrimethamine detected at 0.5 hr after drug administration was found to be highest (Figure 23a). For mice infected with *P. chabaudi* clone AS and AS(Pr_1) at which pyrimethamine was administered on day 3 after parasite inoculation, the levels of pyrimethamine in plasma of mice reached maximum levels at 1 hr after drug administration (figure 23b and 23c). The levels of pyrime-

thamine in plasma of mice treated with pyrimethamine 30 mg/kg body wt were distinctly higher than that of mice treated with pyrimethamine 5 mg/kg body wt. The levels of pyrimethamine could still be detected significantly at 48 hr after drug administration for uninfected mice and mice infected with *P. chabaudi* AS and AS(Pr_1).

Figure 22a

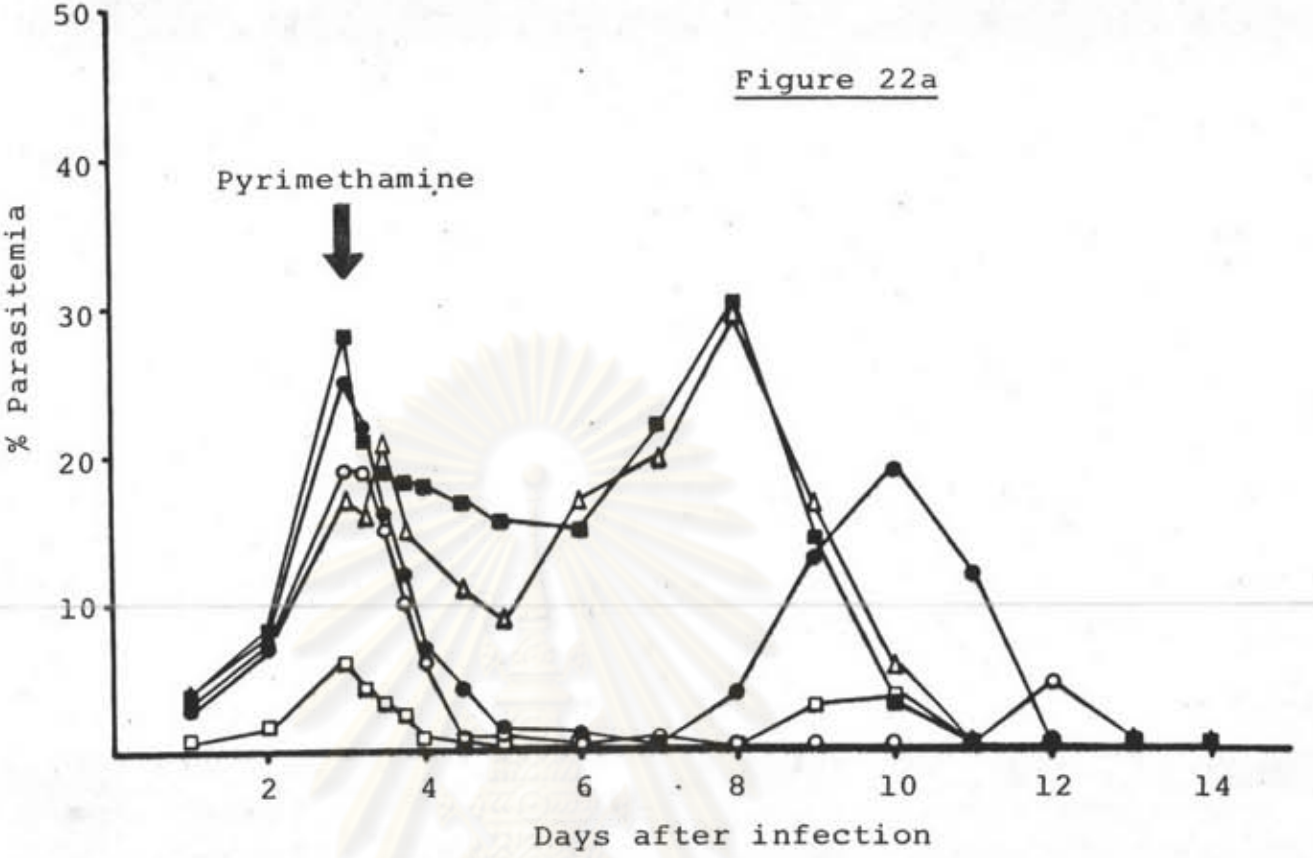
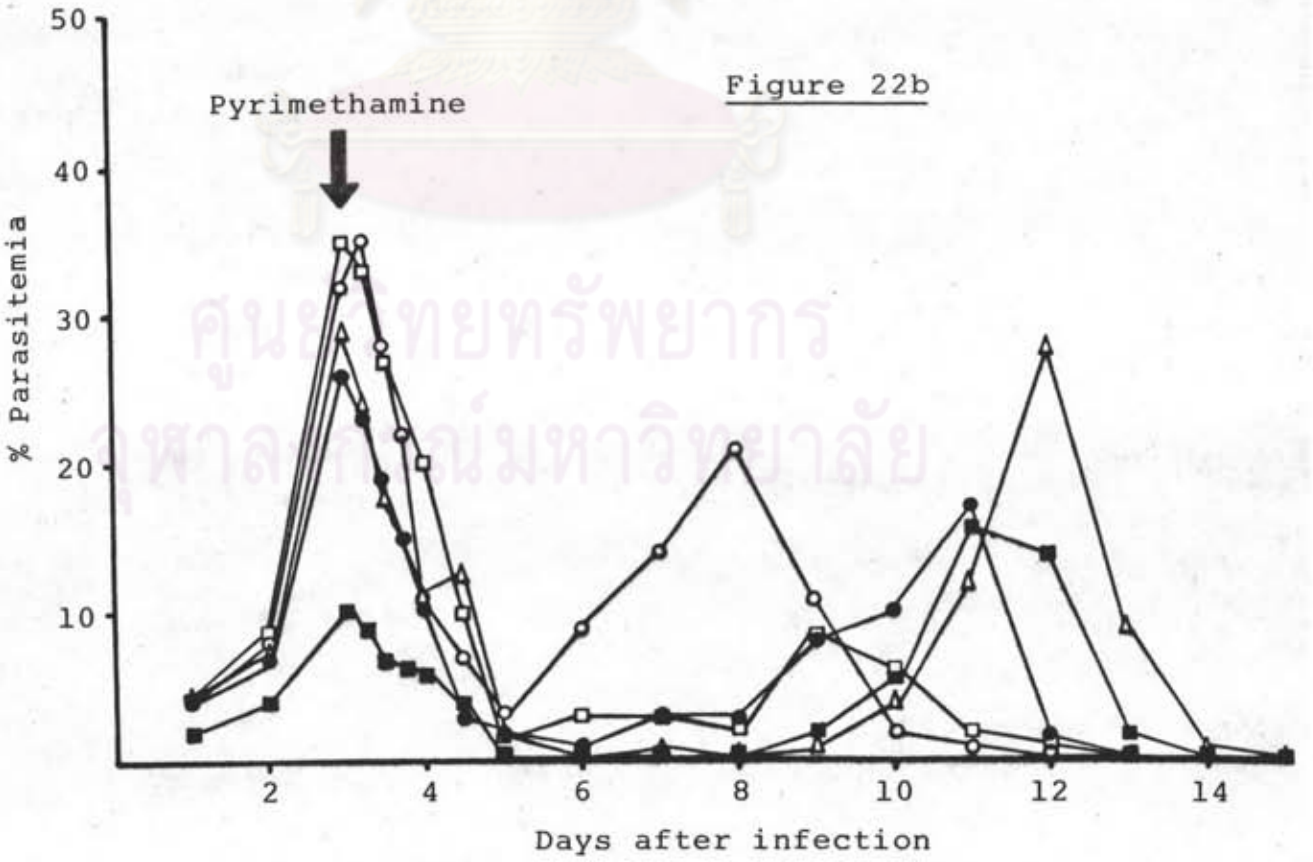


Figure 22b



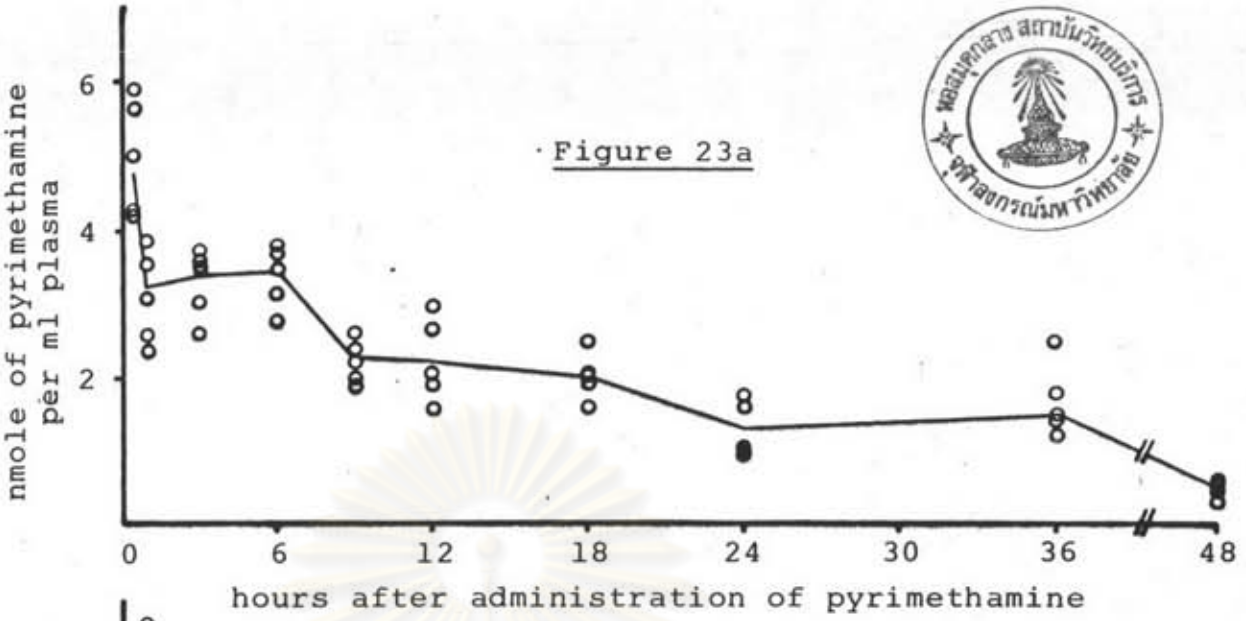


Figure 23a

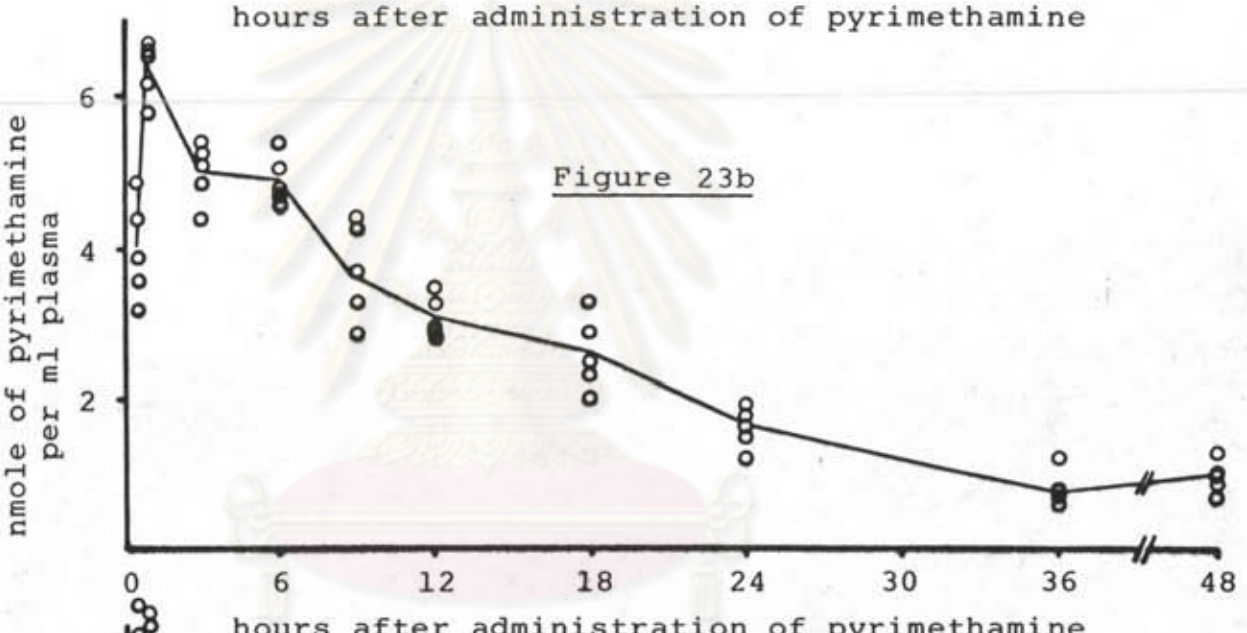


Figure 23b

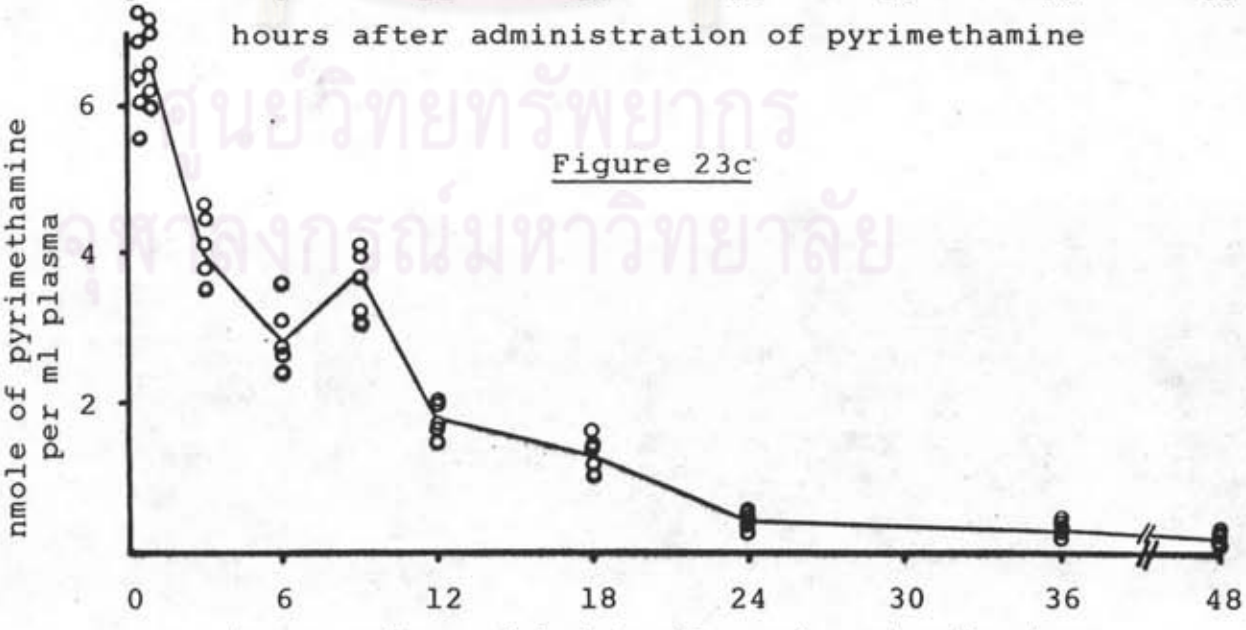


Figure 23c