CHAPTER III

RESULTS

A. Immunological response of rabbits to $T_4.CH_3.HC1$-BSA and T_4-BSA conjugates.

The immunological response of rabbits to $T_4.CH_3HC1$-BSA were shown in Table 1 and Fig. 4. All of them responded well except rabbit No. 3 which was severely injured after the first booster injection so that no futher study could be followed.

Table I

Antibodies estimated in primary and secondary response of rabbits to $T_4.CH_3.HC1$-BSA conjugate.

Days after 1st immunization	% Bound		
	Rabbit 1	Rabbit 2	Rabbit 3
5	21.3	25.5	20.1
8	28.1	40.9	34.4
15	22.0	30.2	21.6
23	68.9	61.1	51.3
27	61.0	45.0	49.9
38	73.3	48.3	-
41	68.9	76.3	-
60	79.0	72.0	-
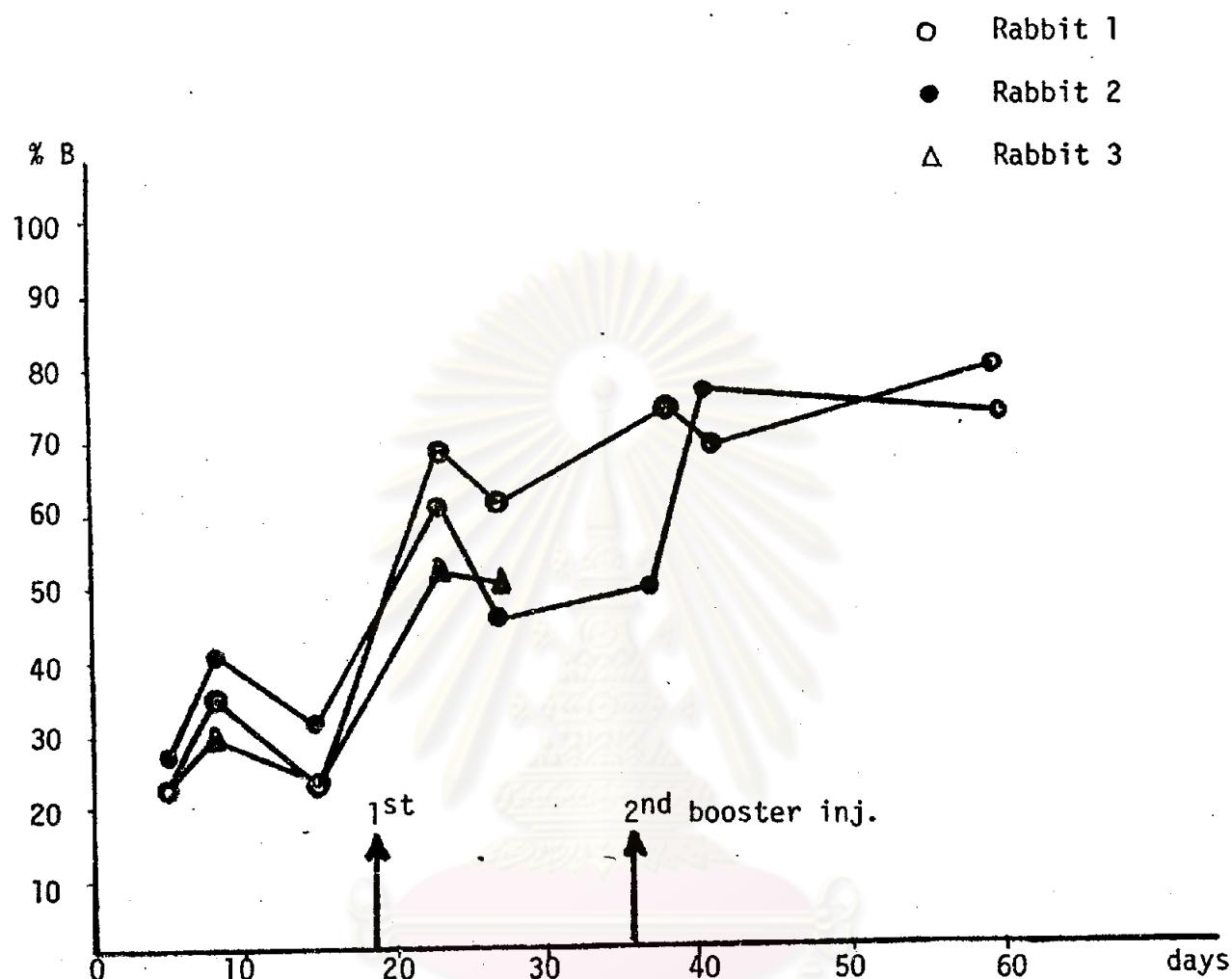
Fig. 4   Immunological response curve of rabbits to $T_4.CH_3.HCl-BSA$ conjugate.

Table II and Fig. 8 show the response of three rabbits to T_4-BSA conjugate.

Table II

Antibodies estimated in primary and secondary response of rabbits to T_4-BSA conjugate.

Day after 1st immunization	% Bound		
	Rabbit 4	Rabbit 5	Rabbit 6
7	28.5	19.1	23.5
10	33.3	26.3	38.0
18	21.6	39.7	36.1
39	78.2	83.2	87.5
53	76.2	82.7	84.9

B. Titre of antisera

The titre and specificity of the antisera from each animal has been separately studied. Titre was assessed by measuring the binding of T_4I^{125} by serial dilution of each antisera. Table III and Fig. 6 illustrates a comparision of the binding of T_4I^{125} to antisera obtained in 5 rabbits against $T_4.CH_3.HCl$-BSA and T_4-BSA conjugates at about 6 weeks of immunzation. $T_4.CH_3.HCl$-BSA and T_4-BSA antiserum bound 50% of the labelled T_4 at a final dilution of 1:1000 and 1:2000 per ml respectively.
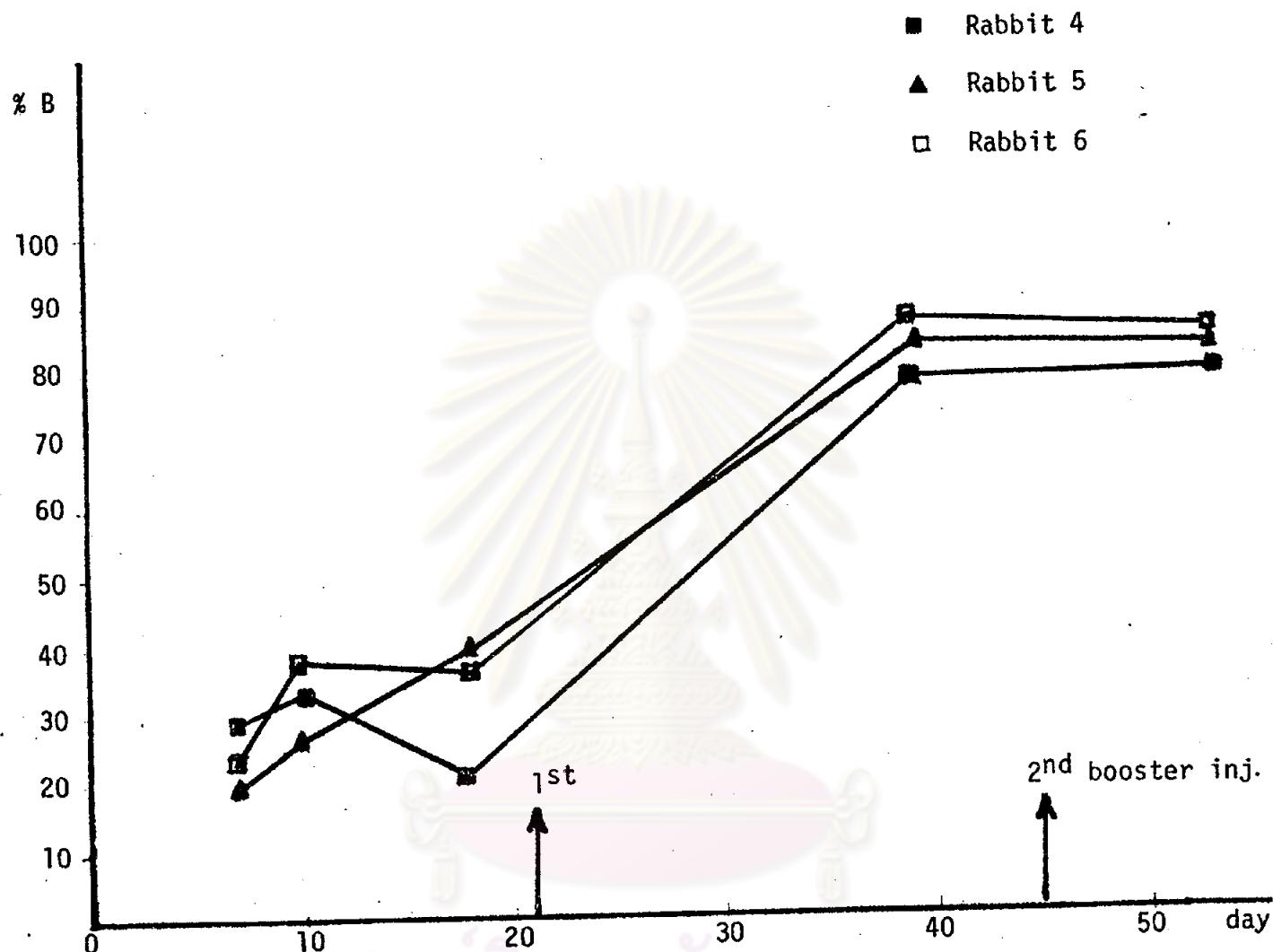
Fig. 5   Immunological response curve of rabbits to T_4-BSA conjugate.

Table 3

Comparision of % Bound $T_4 I^{125}$ to antisera obtained against $T_4 . CH_3 . HCl-BSA$ and T_4-BSA conjugates.

Antisera dilution	% Bound				
	Rabbit 1	Rabbit 2	Rabbit 4	Rabbit 5	Rabbit 6
conc.	79.71	75.21	78.9	83.7	85.9
1:10	72.42	68.74	77.9	81.9	84.1
1:100	50.66	46.90	62.9	67.0	78.2
1:300	38.54	35.19	47.3	47.9	57.4
1:600	21.69	19.44	23.3	28.9	31.1
1:800	13.50	12.13	-	-	-
1:900	-	-	17.8	20.0	22.5
1:1000	12.61	11.30	-	-	-
1:1200	-	-	14.6	16.0	18.0

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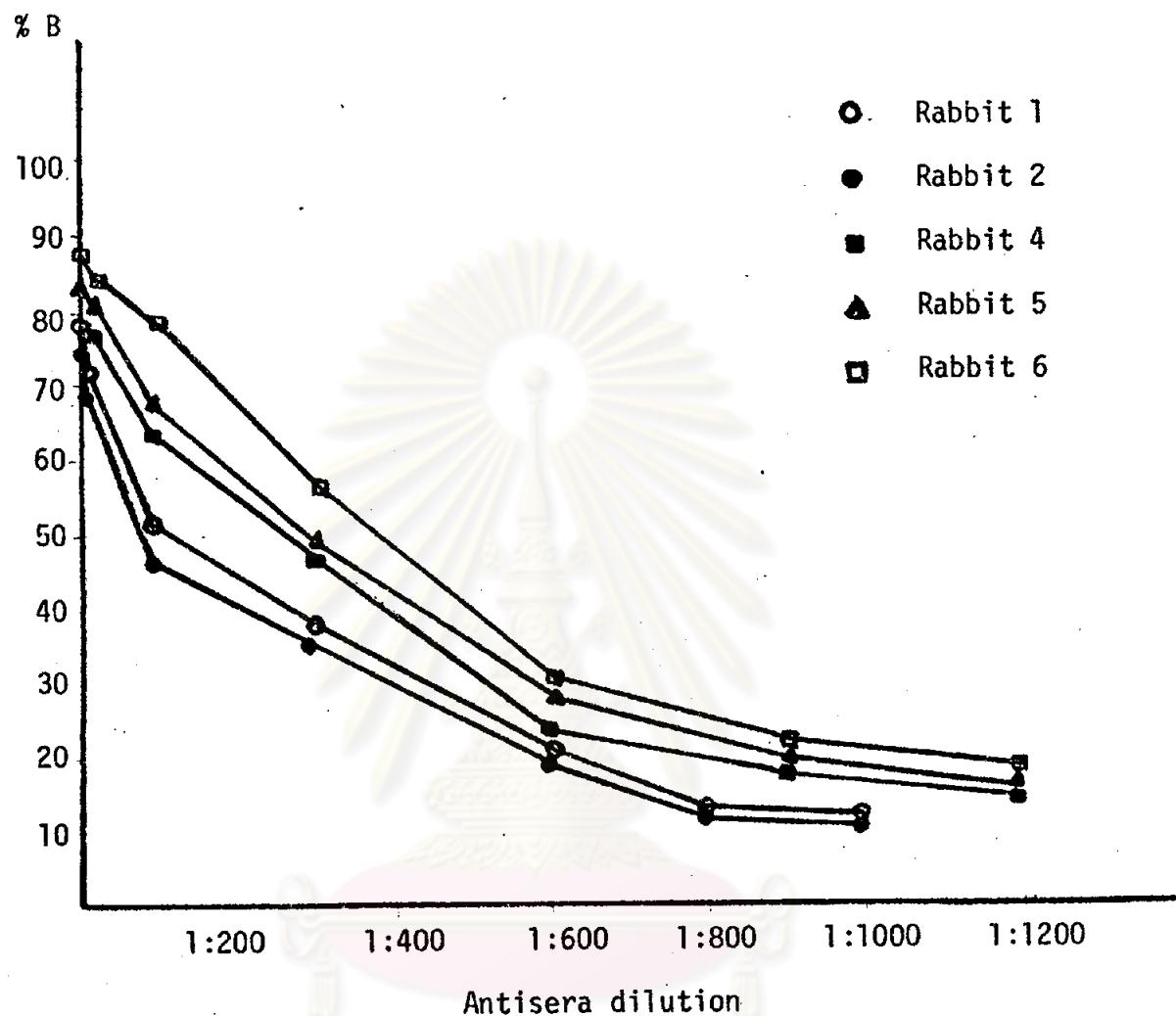
Fig. 6   Antibody titration curve. Serial dilutions of antisera
show progressively decreased binding of labelled T_4.

C. Scatchard Plot

Estimation of binding capacity, q, and eqrilibrium constant K, are obtained from a Scatchard Plot. Table 4, 5, 6, 7, 8 show the the data processing and Fig. 7, 8, 9, 10, 11 show the scatchard plot. From each curve one can estimate:

K = slope of curve

q = No of binding site by extrapolate the curve to the abcissa

Table 4
Data Processing

Std conc. (P)	B	B-NS	F	T	[B]	$(P+P^*)$	$[B](P+P^*)$	B/F
B_0	9384	4906	18659	28043	0.175	12.5	2.18	0.26
187.5	8049	3571	20232	28281	0.126	200.0	25.2	0.17
375	7055	2577	21335	28390	0.090	387.5	34.87	0.12
750	6412	1934	21635	28047	0.069	762.5	52.61	0.09
1500	5836	1358	22418	28254	0.048	1512.5	72.60	0.06
3000	5387	909	23023	28410	0.032	3012.5	97.50	0.03
6000	5016	538	23331	28347	0.019	6012.5	114.24	0.02

NS = 4478   T   = Total (count per

P^* 12.5 pg   [B]   = Fraction Bound B/

B   Bound (count per min) $[B](P+P^*)$ = Concentration of bound

F   Free ("   ")
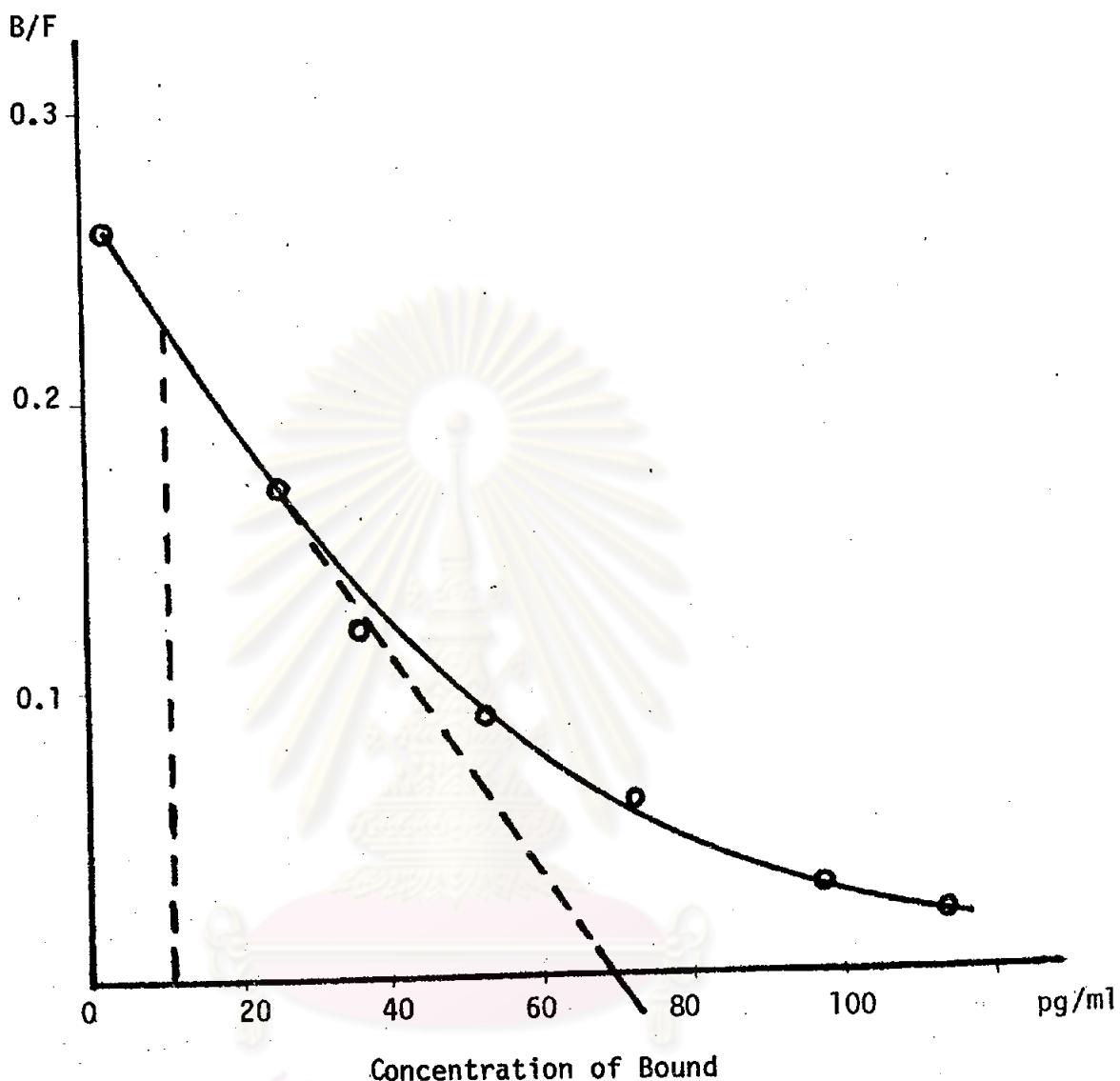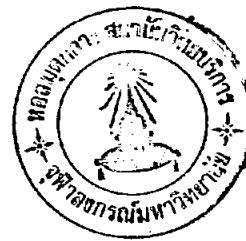
Fig. 7   Scatchard Plot of rabbit 1, $T_4.CH_3.HCl$-BSA antisera.

Rabbit 1

K = Equilibrium constant = slope of curve

$= \dfrac{0.225}{70-11} \quad = \quad \dfrac{0.225}{59}$

= 0.0038

$= 0.0038 \times 10^{-3} \times 10^{12} \quad$ ml/g

$= 776.93 \times 0.0038 \times 10^{9} \quad$ L/mole

$= 2.95 \times 10^{9} \quad\quad\quad$ L/mole

q = No of binding sites

= 70 pg/ml

$= 70 \times 10^{-12} \times 10^{3}$

$= 776.93 \times 70 \times 10^{-9} \quad$ mole/L

$= 5.4385 \times 10^{-5} \quad\quad$ mole/L

Table 5

Data Processing

Std. Conc.(p)	B	B-NS	F	T	[B]	$(p+p^*)$	$[B](p+p^*)$	B/F
B_0	8493	4516	19158	27651	0.163	12.5	2.03	0.24
187.5	7086	3079	21034	28120	0.109	200.0	21.8	0.15
375	6487	2510	21344	27831	0.090	387.5	34.87	0.12
750	5831	1854	21926	27757	0.067	762.5	51.09	0.07
1500	5144	1167	22890	28034	0.042	1512.5	63.53	0.05
3000	4553	742	23481	28034	0.026	3012.5	78.33	0.03
6000	4408	431	23313	27721	0.015	6012.5	90.19	0.02

NS	=	3977
P^*	=	12.5 pg
B	=	Bound (count per min)
F	=	Free (" ")
T	=	Total (" ")
[B]	=	Fraction bound, B/T
$[B](P+P^*)$	=	Concentration of bound
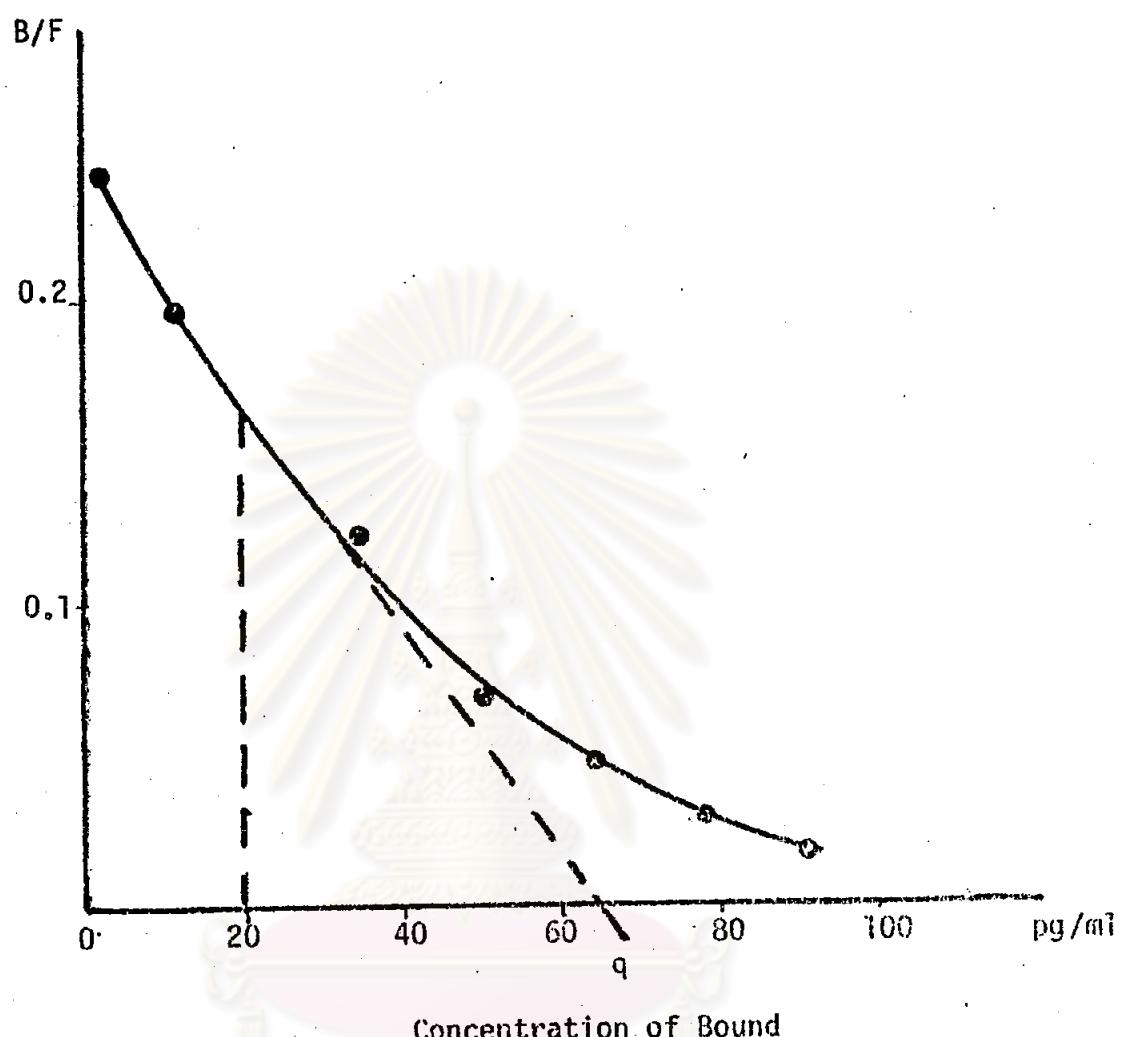
Fig. 8   The Scatchard Plot of rabbit 2, $T_4 \cdot CH_3 \cdot HCl-BSA$ antisera.

Rabbit 2

$$K = \frac{0.165}{65-20}$$

$$= \frac{0.165}{45}$$

$$= 0.0036 \qquad \text{ml/pg}$$

$$= 0.0036 \times 10^{-3} \times 10^{12} \quad \text{L/g}$$

$$= 776.93 \times 0.0036 \times 10^{9} \quad \text{L/mole}$$

$$= 2.79 \times 10^{9} \qquad \text{L/mole}$$

$$q = 65 \qquad \text{pg/ml}$$

$$= 65 \times 10^{3} \times 10^{-12} \qquad \text{g/L}$$

$$= 776.93 \times 65 \times 10^{-9} \qquad \text{mole/L}$$

$$= 5.05 \times 10^{-5} \qquad \text{mole/L}$$

Table 6

Data Processing

Std. Conc.(p)	B	B-NS	F	T	[B]	(p+p*)	[B](p+p*)	B/F
B_O	14434	9331	25911	40345	0.23	13.16	3.03	0.36
93.75	11440	6337	29270	40710	0.16	106.91	16.57	0.22
187.50	10305	5202	30486	40791	0.13	200.66	25.48	0.17
375	8754	3651	31864	40618	0.09	388.66	34.55	0.11
750	7808	2705	32905	40773	0.07	763.16	50.37	0.08
1500	7301	2198	33452	40753	0.05	1513.16	80.19	0.07
3000	7019	1916	33804	40823	0.04	3013.16	138.50	0.05
6000	6520	1417	34191	40711	0.03	6013.16	204.44	0.03

NS $=$ 5103

p^* $=$ 13.16 pg

B $=$ Bound (count per min)

F $=$ Free (" ")

T $=$ Total (" ")

[B] $=$ Fraction bound, B/T

[B](p+p*) $=$ concentration of bound
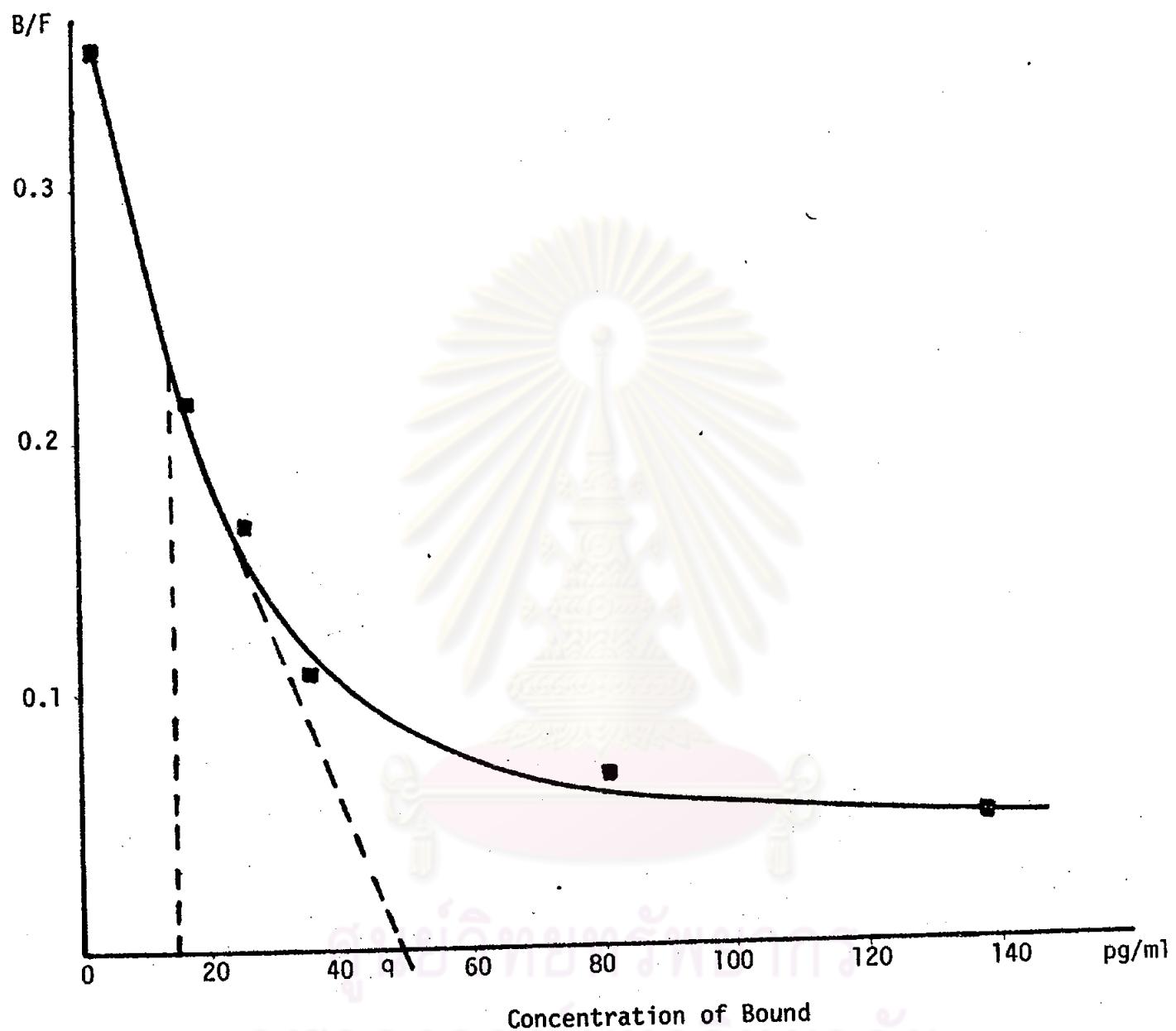
Fig. 9 The Scatchard Plot of rabbit 4, T$_4$-BSA antisera.

Rabbit 4

$$K = \frac{0.225}{50-15}$$

$$= \frac{0.225}{35}$$

$$= 0.00642 \qquad \qquad \text{ml/pg}$$

$$= 0.00642 \times 10^{-3} \times 10^{12} \qquad \text{L/mole}$$

$$= 776.93 \times 0.00642 \times 10^{9} \qquad \text{L/mole}$$

$$= 4.987 \times 10^{9} \qquad \qquad \text{L/mole}$$

$$q = \text{No of binding site}$$

$$= 50 \text{ pg/ml}$$

$$= 50 \times 10^{3} \times 10^{-12} \qquad \text{g/L}$$

$$= 776.93 \times 50 \times 10^{-9} \qquad \text{mole/L}$$

$$= 3.884 \times 10^{-5} \qquad \qquad \text{mole/L}$$

Table 7

Data Processing

Std. conc.(p)	Bound	B-NS	F	T	[B]	$(p+p^*)$	$[B](p+p^*)$	B/F
B_0	14289	9727	17357	31646	0.30	10.34	3.1	0.56
93.75	11939	7377	19731	31670	0.232	104.09	24.15	0.37
187.50	10300	5738	21696	31996	0.179	197.84	35.41	0.26
375.0	8154	3592	23417	31571	0.110	385.34	42.39	0.15
750.0	7347	2785	24358	31705	0.087	760.34	66.15	0.12
1500.0	6257	1695	25555	31812	0.053	1510.34	80.05	0.07
3000.0	5519	957	25921	31440	0.030	3010.34	90.31	0.04
6000.0	5276	714	26315	31691	0.022	6010.34	132.22	0.02

NS = 4562

p^* = 10.34 pg

B = Bound (count per min)

F = Free

T = Total

[B] = Fraction bound, B/T

$[B](p + p^*)$ = concentration of bound
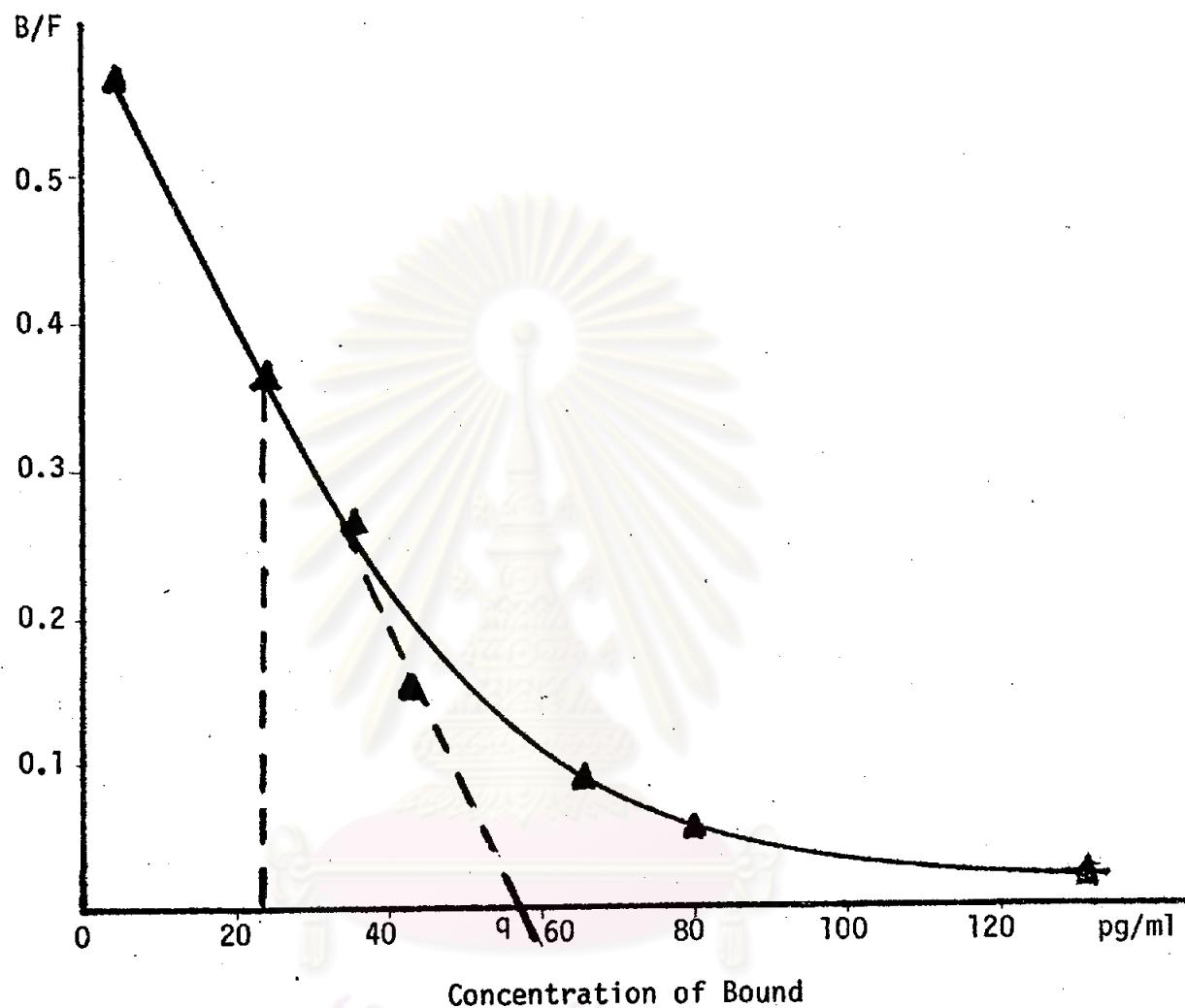
Fig. 10   The Scatchard Plot of rabbit 5, T_4-BSA antisera.

Rabbit 5

$$K = \frac{0.375}{57-24}$$

$$= \frac{0.375}{33}$$

$$= 0.01136 \qquad \text{ml/pg}$$

$$= 0.01136 \times 10^{-3} \times 10^{12} \qquad \text{ml/g}$$

$$= 776.93 \times 0.01136 \times 10^{9} \qquad \text{L/mole}$$

$$= 8.825 \times 10^{9} \qquad \text{L/mole}$$

$$q = 57 \qquad \text{pg/ml}$$

$$= 57 \times 10^{3} \times 10^{-12} \qquad \text{g/L}$$

$$= 776.93 \times 57 \times 10^{-9} \qquad \text{mole/L}$$

$$= 4.4285 \times 10^{-5} \qquad \text{mole/L}$$

Table 8

Data Processing

Std. conc.(p)	Bound	B-NS	F	T	[B]	$(p+p^*)$	$[B](p+p^*)$	B/F
B_0	18763	13659	21556	40319	0.34	13.16	4.47	0.63
93.75	15431	10328	24709	40140	0.257	106.91	27.47	0.42
187.50	13227	8124	27093	40320	0.201	200.66	40.33	0.30
375.0	11002	5899	29978	39980	0.147	388.16	57.05	0.19(
750.0	8887	3784	31111	39998	0.094	763.16	71.16	0.12
1500.0	7411	2308	31589	39000	0.059	1513.16	89.28	0.07
3000.0	6636	1533	33464	40100	0.038	3013.16	114.50	0.05
6000.0	6131	1028	33994	40125	0.025	6013.16	150.33	0.03

NS = 5013

p^* = 13.16 pg

B = Bound (count per min)

F = Free (")

T = Total (")

[B] = Fraction bound, B/T

$[B](p + p^*)$ = concentration of bound
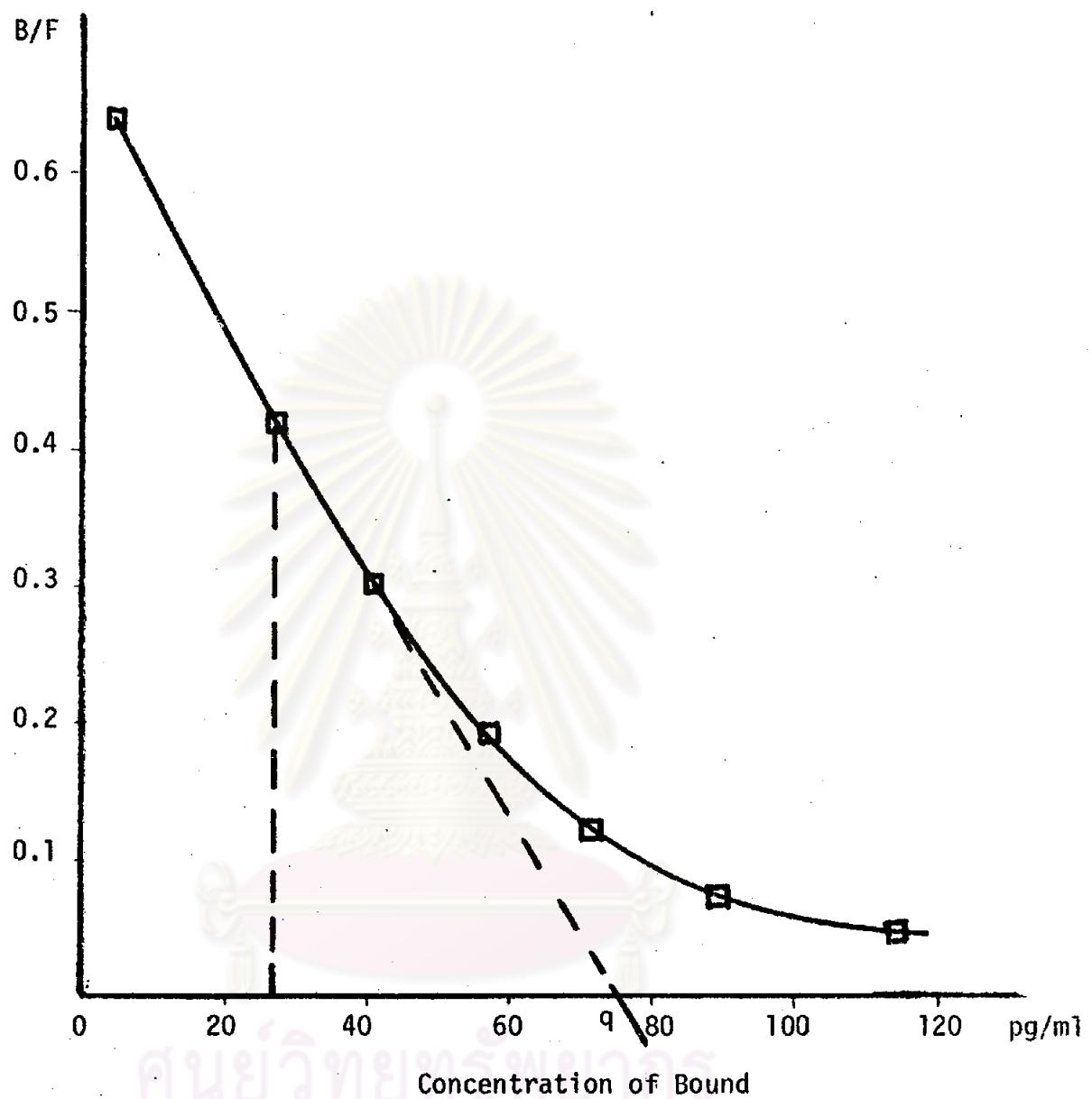
Fig. 11   The Scatchard Plot of rabbit 6, T_4-BSA antisera.

Rabbit 6

$$K = \frac{0.425}{76-27}$$

$$= \frac{0.425}{49}$$

$$= 0.00867 \qquad \text{ml/pg}$$

$$= 0.00807 \times 10^{-3} \times 10^{-2} \qquad \text{ml/g}$$

$$= 776.93 \times 0.00867 \times 10^{9} \qquad \text{L/mole}$$

$$= 6.735 \times 10^{9} \qquad \text{L/mole}$$

$$q = 76 \qquad \text{pg/ml}$$

$$= 76 \times 10^{-12} \times 10^{3} \qquad \text{g/L}$$

$$= 776.93 \times 76 \times 10^{-9} \qquad \text{mole/L}$$

$$= 5.904 \times 10^{-5} \qquad \text{mole/L}$$

D. Standard dose response curve

For six replicates analyzed in one assay the mean ($\pm$ SD) percent binding in the charcoal blank tube was 11.15 ± 0.44, cv = 3.9%.

The mean ($\pm$ SD) actual percent binding of the maximal binding tube (zero standard) were 21.82 ± 1.05, 20.16 ± 0.9 for $T_4.CH_3.HCl$-BSA antirera and 27.37 ± 0.94, 28.19 ± 0.9, 34.01 ± 0.78 for T_4-BSA antisera respectively as shown in Table 9, 10.

By paired t test analysis the percent binding in each of these standards were significantly different from that in the standard tube preceeding or following it.

Table 9

Standard dose response of $T_4.CH_3HCl$-BSA antisera

| Standard concentration	Rabbit 1			Rabbit 2		
ng/ml	% B	± S.D	%c.v	%B	± S.D	%c.v
B_0	21.82	1.05	4.8	20.16	0.90	4.5
0.156	21.01	1.09	5.1	20.25	0.85	4.2
0.625	18.28	0.77	4.2	18.48	0.94	5.1
2.5	15.50	0.87	5.6	14.92	0.79	5.3
10.0	10.04	0.56	5.5	10.30	0.58	5.7
30.0	5.51	0.37	6.7	5.44	0.37	6.9
60.0	3.54	0.62	17.5	3.47	0.70	20.3
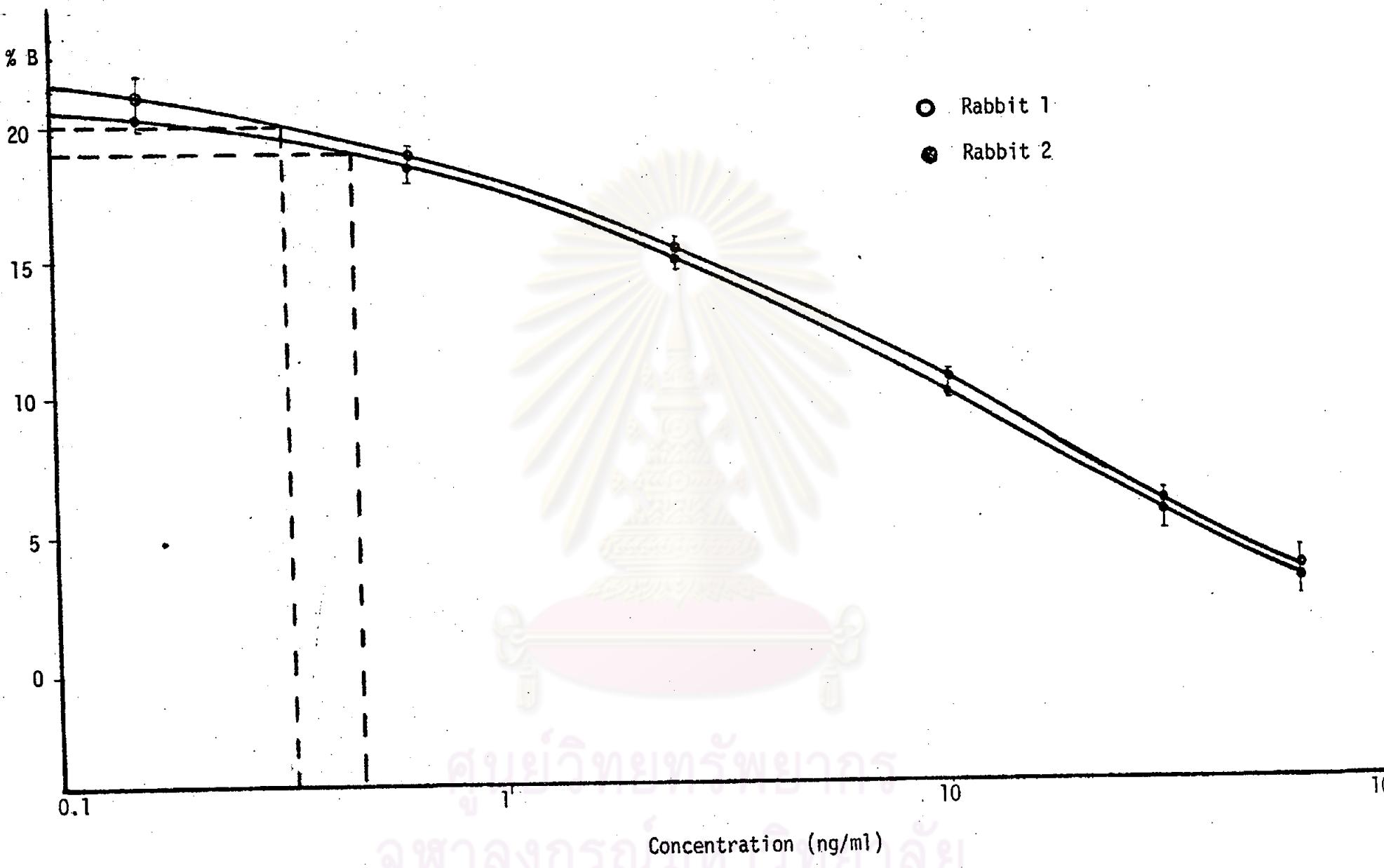
Fig. 12   Standard dose response of $T_4.CH_3.HCl$-BSA antisera.

Table 10

Standard Dose Response of T_4-BSA antisera

| Standard concentration(ng/ml)	Rabbit 4			Rabbit 5			Rabbit 6		
	% B	S.D	% C.V	% B	S.D	% C.V	% B	S.D	% C.V
B_0	27.37	0.94	3.4	28.19	0.90	3.2	34.01	0.78	2.3
0.156	27.49	0.85	3.0	27.61	1.10	4.0	33.96	1.42	4.2
0.625	24.79	1.0	4.0	25.14	0.87	3.5	31.24	0.78	2.5
2.5	19.17	0.52	2.7	19.88	0.63	3.2	25.48	1.09	4.3
10.0	11.71	0.72	6.1	12.33	0.78	6.4	18.02	1.13	6.3
30.0	6.49	0.38	6.4	6.63	0.47	7.1	11.51	1.07	9.3
60.0	3.59	0.41	11.4	3.78	0.49	13.2	7.75	2.06	26.6

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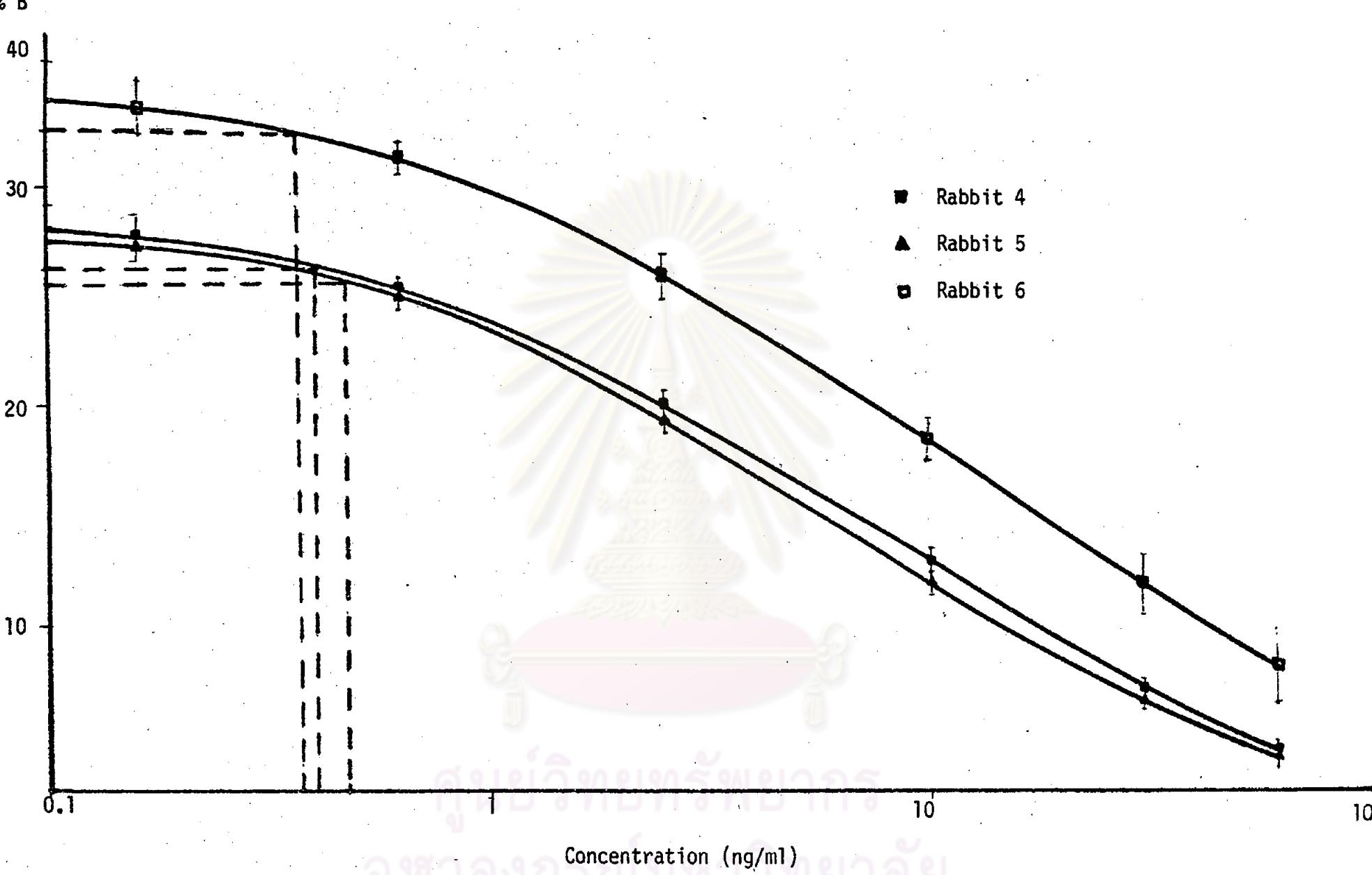
Fig. 13   Standard dose response of T_4-BSA antisera.

E. Intra-assay coefficients of variation

For six replicates analyzed in one assay the coefficients of variation were shown in Table 9, 10.

F. Sensitivity

When the maximal binding tube (zero Standard) of six replicates were analyzed in one assay the mean ($\pm$ S.D) actual percent binding were 21.82 $\pm$ 1.05%, 20.16 $\pm$ 0.9% for Anti $T_4.CH_3.HCl$-BSA and 27.37 $\pm$ 0.94%, 28.19 $\pm$ 0.9%, 34.01 $\pm$ 0.78% for Anti T_4-BSA. Therefore, the mean binding percentage minus 2 S.D were 19.72%, 18.36% and 25.49%, 26.39%, 32.45% respectively (Table 9, 10). Extrapolating from the mean standard curve the relative binding were approximately 0.33 ng/ml, 0.47 ng/ml and 0.40 ng/ml, 0.47 ng/ml, 0.37 ng/ml respectively (Fig. 12, 13).

From the paired t-test calculated the figure was 0.625 ng/ml. Therefore the sensitivity of our experiment performent was 0.625 ng/ml, which is the lower limit of our antisera detection.

G. Specificity (Fig. 14, 15 and Table 11, 12)

The ability of Monoidotyrosine (MIT) and L-Triiodothyronine (T_3) to compete with T_4I^{125} for binding to the $T_4.CH_3.HCl$-BSA antiserum and T_4-BSA antiserum were assessed by determining the amount of analogue required to cause a 50% decrease in the binding of T_4I^{125} and comparing it to the amount of L-T_4 required to produce the same decrease in binding of labelled T_4.

Table 11

Cross reactivity of MIT and T_3 to $T_4.CH_3.HCl-BSA$ antiserum

| Std. T_4 Conc^z ng/ml	B/B_0	Std.MIT ng/ml	B/B_0	Std T_3 ng/ml	B/B_0
B_0	1	B_0	1	B_0	
0.156	0.96	0.1	0.995	0.1	0.98
0.625	0.84	1	0.98	1	0.96
2.5	0.71	10	0.95	10	0.84
10	0.46	100	0.94	100	0.59
30	0.25	1000	0.92	1000	0.32
60	0.15	10000	0.86	10000	0.18
		100000	0.86	100000	0.14
		1000000	0.86	1000000	0.10

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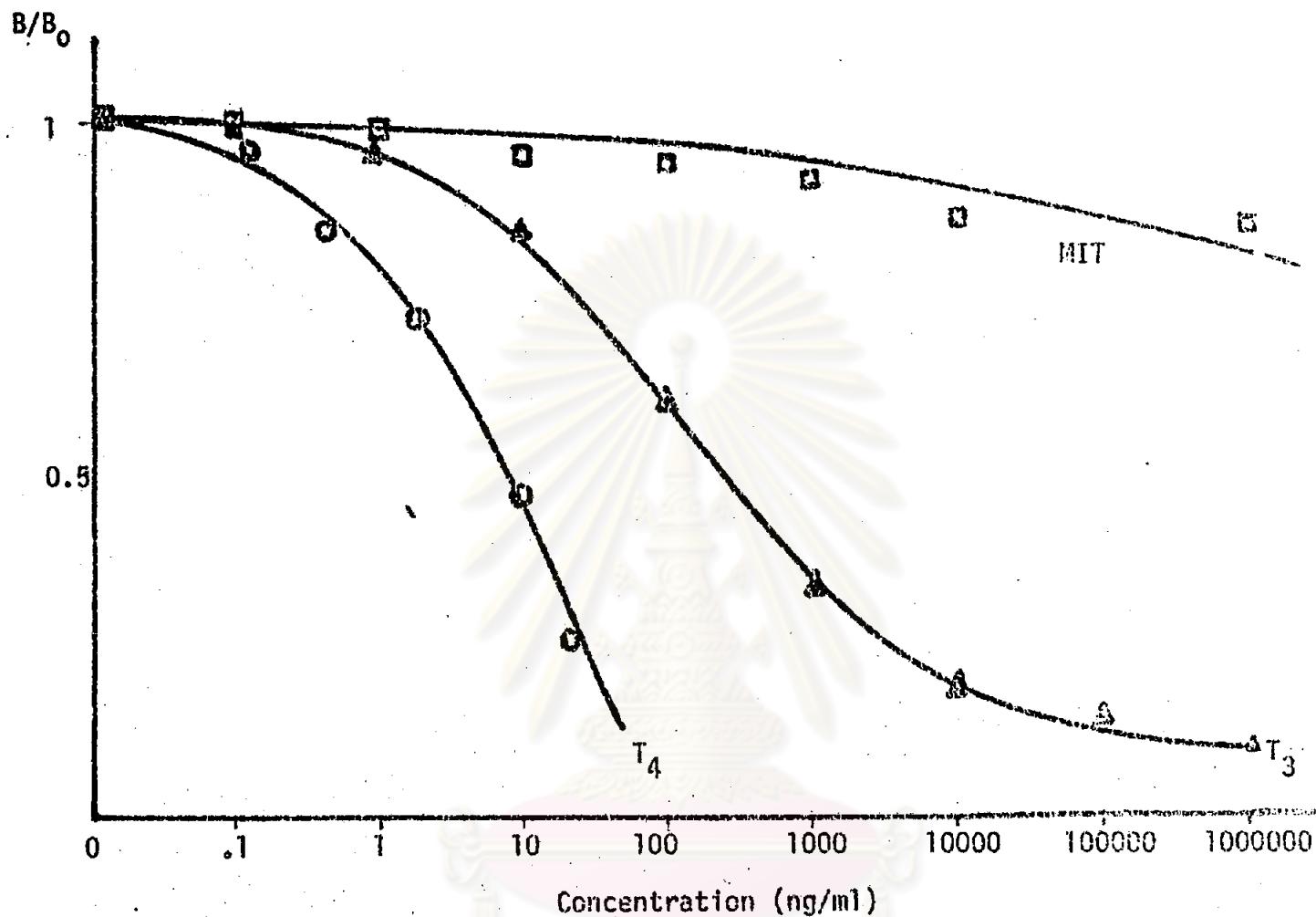
Fig. 14   Cross-reactivity of thyroid analogues with T_4 for $T_4.CH_3.HCl-BSA$
antisera binding site.

Data represented in Table 11, 12 and Fig. 14, 15 indicate that MIT & T_3 bind less than 0.0000075 and 0.0019, therefore they are not as effective as T_4 in competing for binding to the $T_4.CH_3.HCl$-BSA antiserum. For T_4-BSA antiserum MIT and T_3 are less than 0.000007 and 0.0018 respectively.

Table 12

Cross reactivity of MIT and T_3 to T_4-BSA antisera

| Standard T_4 ng/ml	B/B_o	Standard MIT ng/ml	B/B_o	Standard T_3 ng/ml	B/B_o
B_o	1	B_o	1	B_o	1
0.156	0.99	0.1	1.07	1	1.05
0.625	0.90	1	1.06	1	1.02
2.5	0.70	10	1.09	10	0.87
10.0	0.43	100	1.03	100	0.65
30.0	0.19	1000	1.03	1000	0.29
60.0	0.13	10000	0.96	10000	0.08
		100000	0.94	100000	0.04
		1000000	0.81	1000000	0.02
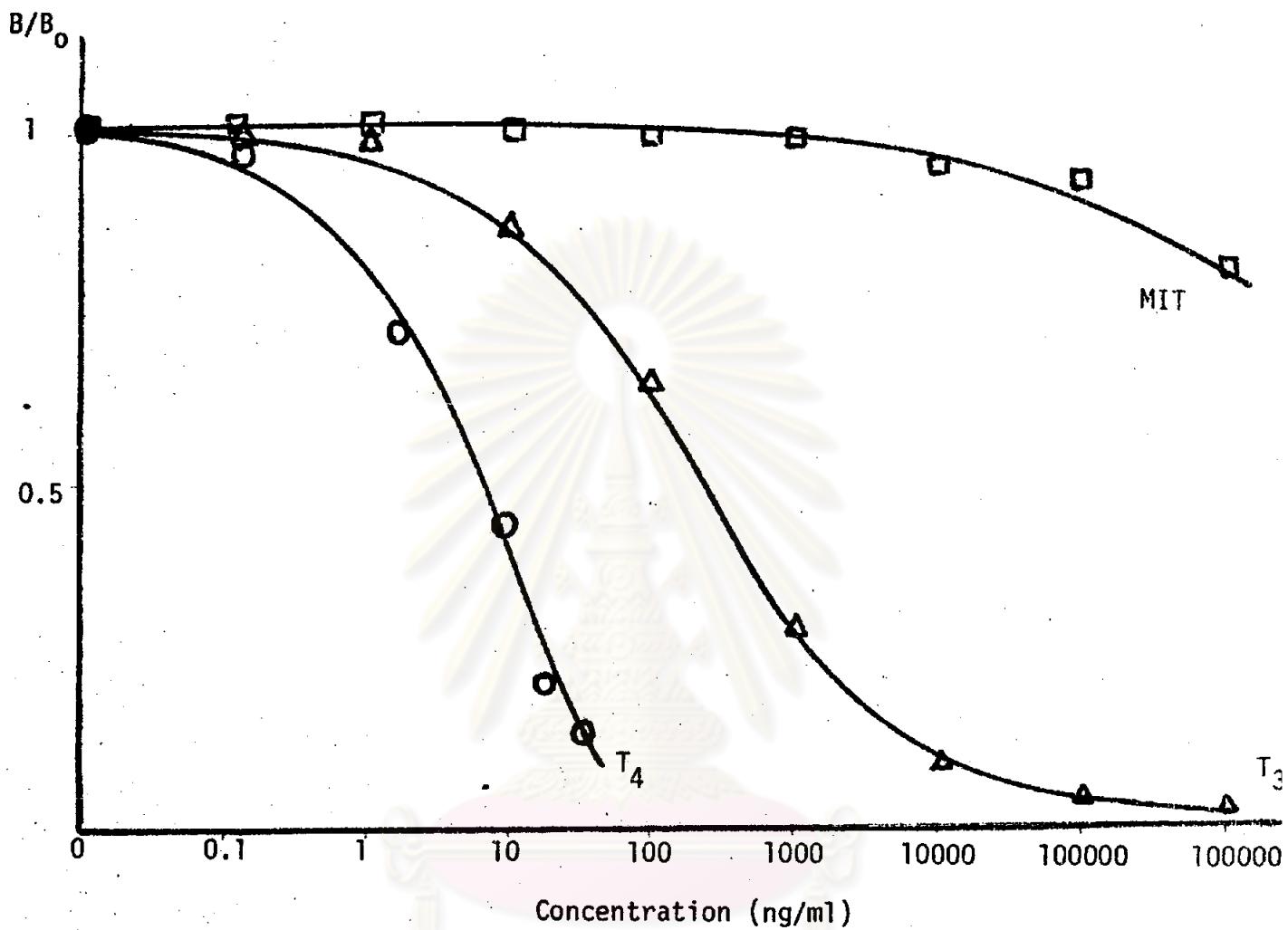
Fig. 15 Cross-reactivity of thyroid analogues with T_4 for T_4-BSA antisera binding site.

H. <u>Serum T$_4$</u>

Serum of hypothyroid, euthyroid, and hyperthyroid patients were assayed with both T$_4$.CH$_3$.HCl-BSA and T$_4$-BSA. The results were shown in Table 13.

Table 13

Serum T$_4$ assays with T$_4$.CH$_3$.HCl-BSA and T$_4$-BSA antiserum

Patients	T$_4$.CH$_3$.HCl-BSA antiserum Serum T$_4$ µg%	T$_4$-BSA antiserum Serum T$_4$ µg%
1	18.0	20.0
2	14.5	12.0
3	20.0	24.0
4	18.0	15.0
5	12.0	10.6
6	11.0	11.4
7	6.2	6.8
8	5.2	5.6
9	1.0	0.6

Analysis of sera from 45 euthyroid, 22 hypothyroid, and 51 hyperthyroid patients yielded mean values of 8.9 ± 2.6, 3.2 ± 1.3, and 23.5 ± 5.9 µg/100 ml, respectively.

I. Recovery and dilution experiments

When 2.5 ng/ml was add to normal and hypothyroid sera percent recovery was almost complete (Table 13).

50% Dilutions of normal and hyperthyroid sera resulted in the similar way.

Table 14

T_4 recovery experiment

Experiment	Serum T_4 concentration µg/100 ml			Recevery (%)
	Initial	Added	Final	
1	5.2	2.5	8.1	105
2	3.9	2.5	6.0	94
3	21	-10.5	11	104
4	5.6	- 2.8	2.3	82

J. Storage of antibodies obtained

Each antiserum was storaged 1 ml per ampoule, Lyophilized and store at -70°C.