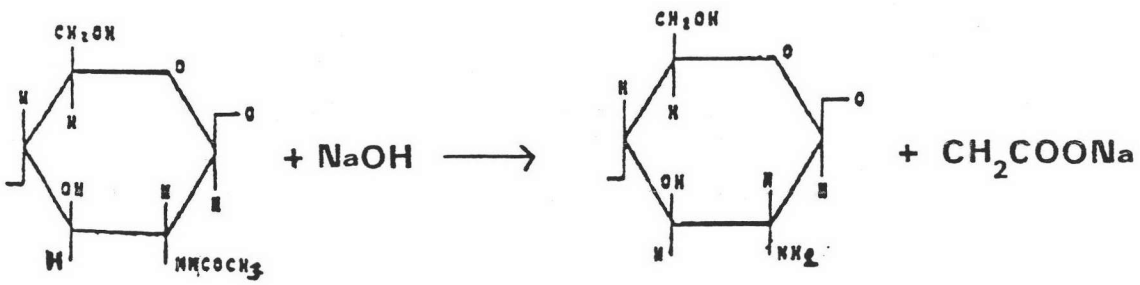
CHAPTER III

Results and Discussion

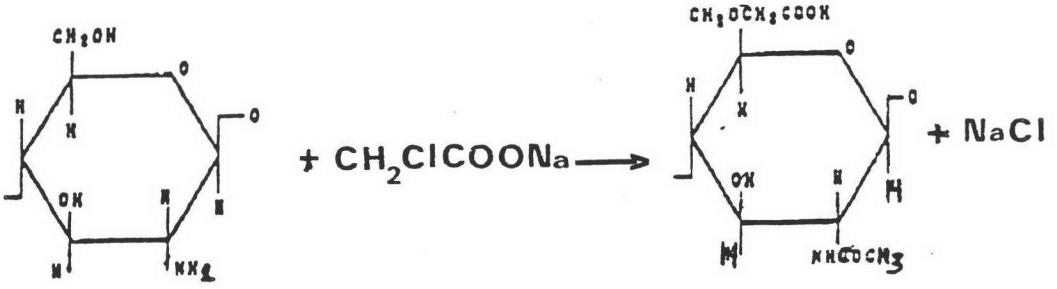
1) Preparation of Carboxymethylchitin

Carboxymethylchitin was prepared from chitin. In the step of mixing chitin with sodium hydroxide solution, chitin was changed to chitosan. This reaction was occurred by hydrolysis of the acetoamide group at carbon atom 2 in glucose residue of chitin and subsequent deacetylation to produce a free amino group. At the end of reaction there would be copolymer with N-acetyl glucosamine monomer in the structure. Binding of water and sodium hydroxide into the molecule of chitosan took times more than 30 minutes and stirring to let the binding of the molecule slowly occurred. The reaction was as follow:



Chitin Sodium hydroxide Chitosan Sodium acetate

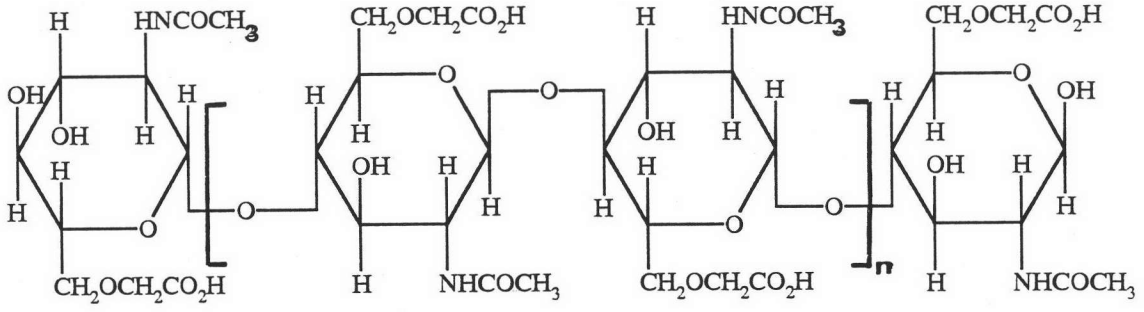
When the process above was done, solid of sodium monochloroacetate was added. Stirring was needed throughout the reaction which slowly took about 2-12 hours. The reaction was :



Chitosan Sodium monochloroacetate Carboxymethylchitin Sodium chloride

After the reaction was completely done, the excess of NaOH would be neutralized then the salt was wiped out. Neutralization of pH could be done by adding HCl. Carboxymethylchitin was separated by using acetone.

The Structure of carboxymethylchitin was :



Carboxymethylchitin was poly [N-acetyl-6-0 (carboxymethyl 1)-D-glucosamine]. From the structure above, carboxymethyl group took the place 6-0 (O-Substituted). Sometimes the place was took the place 2-N (N-Substituted) or both 6-0 and 2-N (N, O-Substituted) but rare because at 2-N or N-atom of every unit of copolymer was blocked by acetyl group. Therefore carboxymethyl group usually substituted at 6-0 position only.

This carboxymethylchitin was water soluble because there will be amino group in the form of primary amine ($-NH_2$) or secondary amine ($-NHCH_2$ COOH). The degree of water solubility of carboxymethylchitin was varied depending on molecular weight and degree of substitution.

The infraed spectra of chitin, carboxymethylchitin standard and carboxymethylchitin preparation was showed in Figure 7, 8 and 9 respectively.

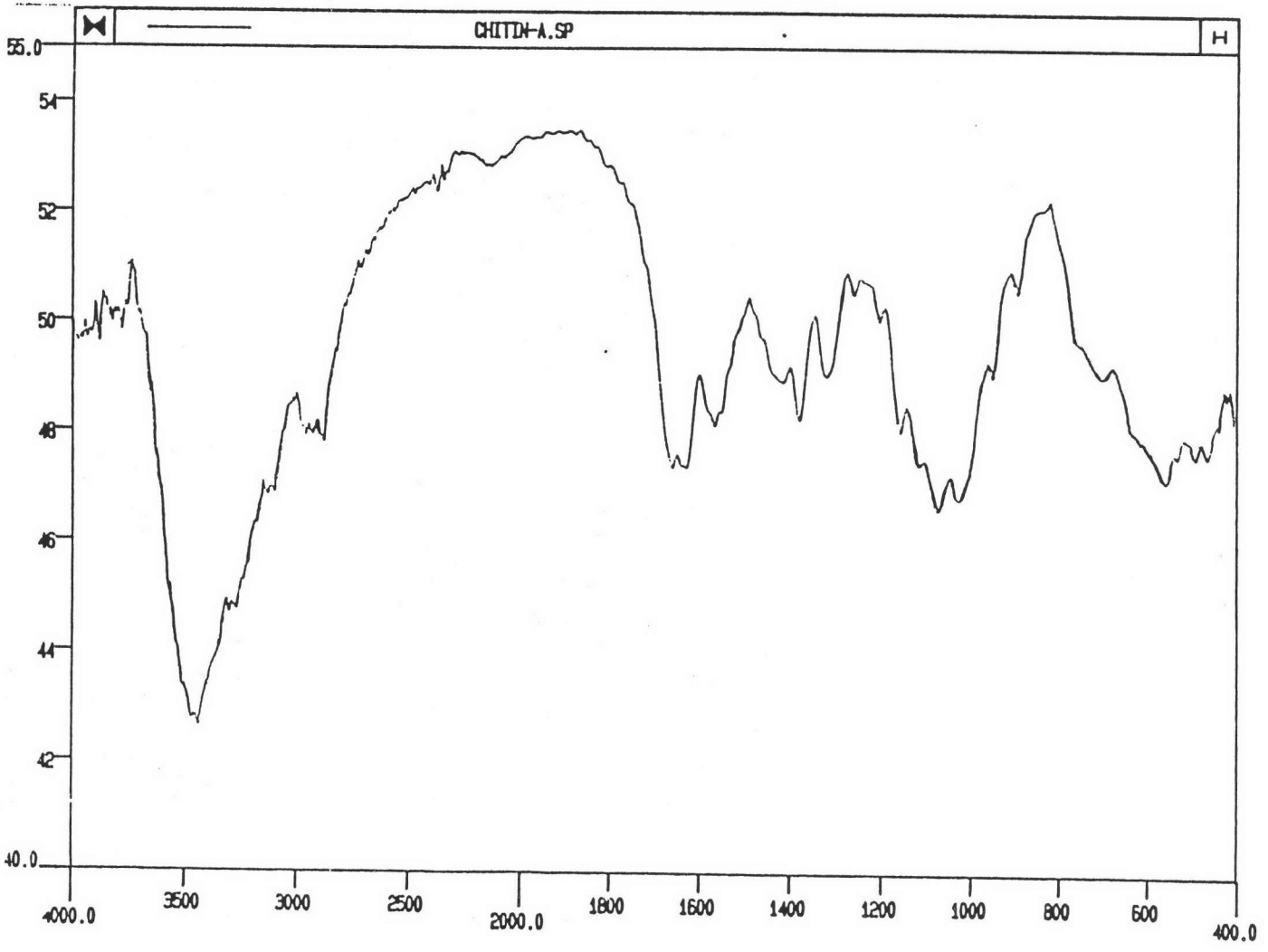


Figure 7 Infrared spectra of chitin

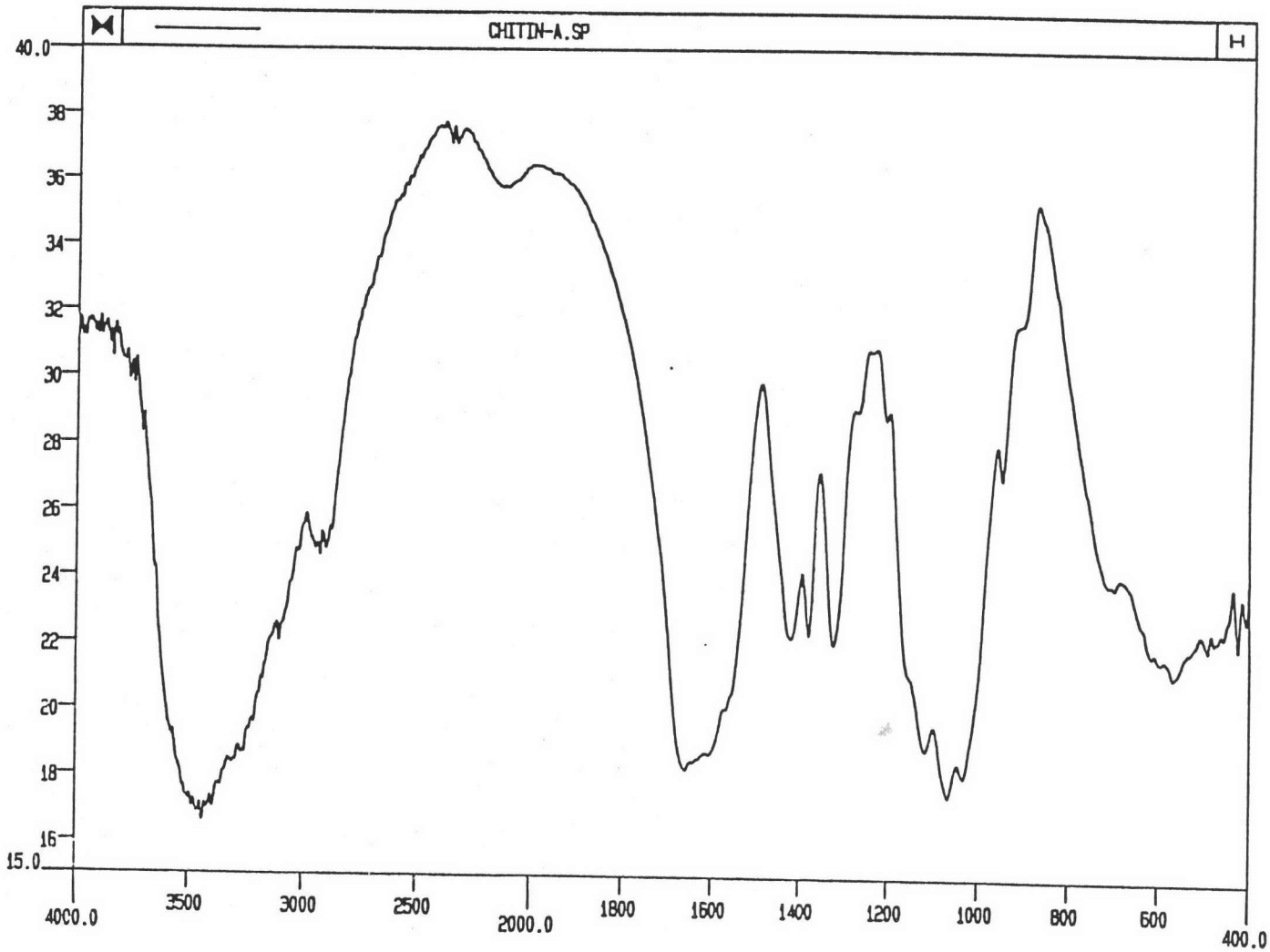


Figure 8 Infrared spectra of carboxymethylchitin standard

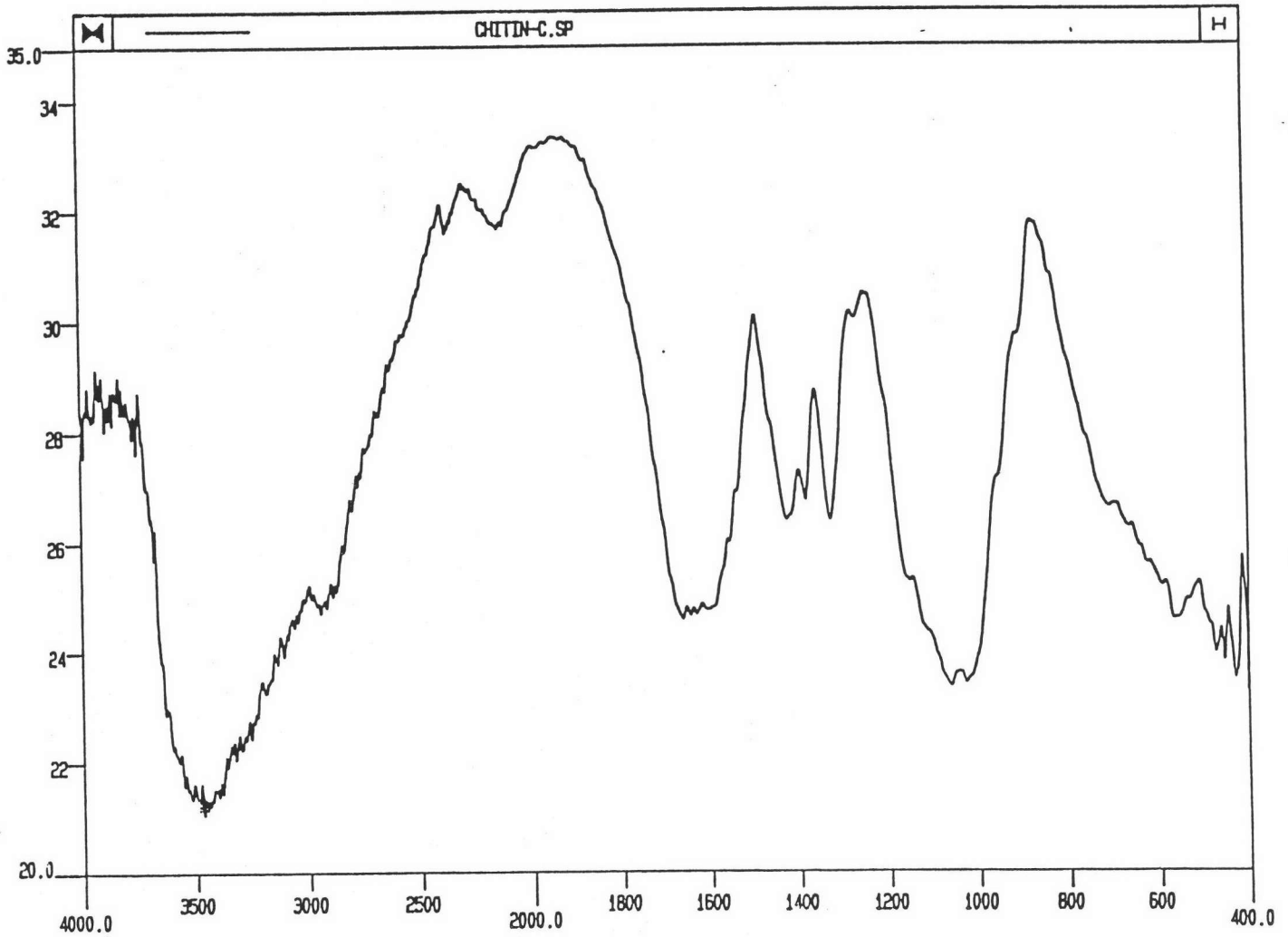


Figure 9 Infrared spectra of carboxymethylchitin preparation

2) Preparation of lecithin and carboxymethylchitin walled tetanus toxoid microcapsules.

Tetanus toxoid microcapsules were prepared by interfacial deposition technique. The microcapsules wall obtained by adding 0.2% lecithin in dichloromethane in equal volume to tetanus toxoid. During stirred mixture, w/o emulsion was obtained. As this, the lecithin would coat around tetanus toxoid droplets. To stabilize the lecithin wall, carboxymethylchitin in phosphate buffer solution pH 7.4 was added. At pH 7.4 lecithin molecule had a net positive charge while carboxymethylchitin molecule in the outer aqueous phase would interact electrostatically with the hydrophilic group of the lecithin molecules oriented at the oil-water interface to form a stable adsorbed layer on the surface of oil droplets. The spherical shape of vesicle were obtained by controlling the speed of stirring. In processing of preparation, adding another portion of carboxymethylchitin in phosphate buffer helped to strengthen the microcapsule wall. In according to the solubility of lecithin that was soluble in dichloromethane, an orangic solvent, but insoluble in water. Therefore, evaporation of dichloromethane from the system would necessary.

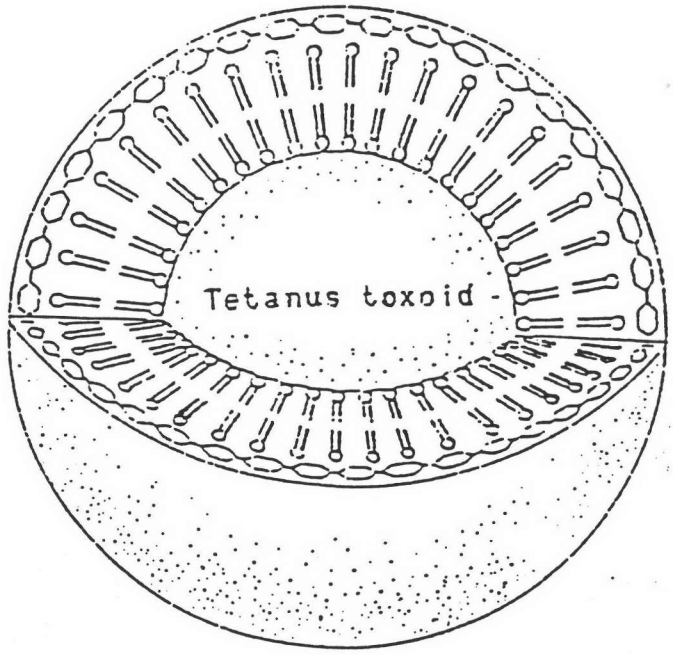


Figure 10 Structure of lecithin and carboxymethylchitin walled tetanus toxoid microcapsules by interfacial deposition technique.

3) Testing the quality of the Tetanus Toxoid Microcapsules Preparation.

3.1) Physical Testing

a) Particle Size Analysis of the Microcapsules

Tetanus toxoid microcapsules were separated from the supernatant by centrifugation technique at 15,000 rpm 15 minutes. The cumulative percentage undersize distribution was shown in table 1. Mean diameter was 0.37 micrometers ; mode was between 0.21-0.40 micrometers in 51.36% frequency ; and median was 0.33 micrometers.

Table 1 The cumulative percentage undersized distribution of tetanus toxoid microcapsules that obtained from 15,000 rpm centrifugation.

Size range (micrometer)	% Frequency	% Cumulative
0.01 - 0.20	44.80	44.80
0.21 - 0.40	51.36	96.16
0.41 - 0.60	2.56	98.72
0.61 - 0.80	1.12	99.84
0.81 - 1.00	0.16	100.00
Arithmetic mean	= 0.37	micrometers
Mode	= 0.21 - 0.40	micrometers
Median	= 0.33	micrometers

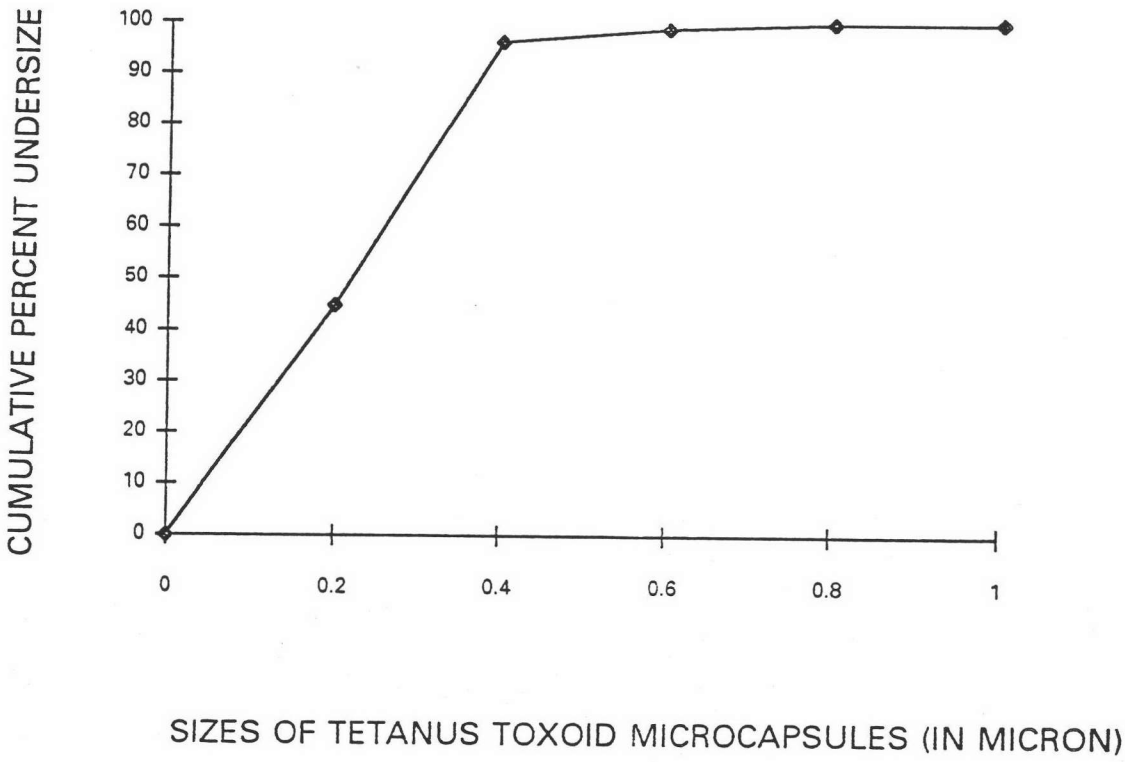


Figure 11 The cumulative percent undersize distribution curves of tetanus toxoid microcapsules.

b) Scanning Electron Microscopy

Scanning electron microscopy showed spherical and smooth walls particles of tetanus toxoid microcapsules. It was shown in Figure 12

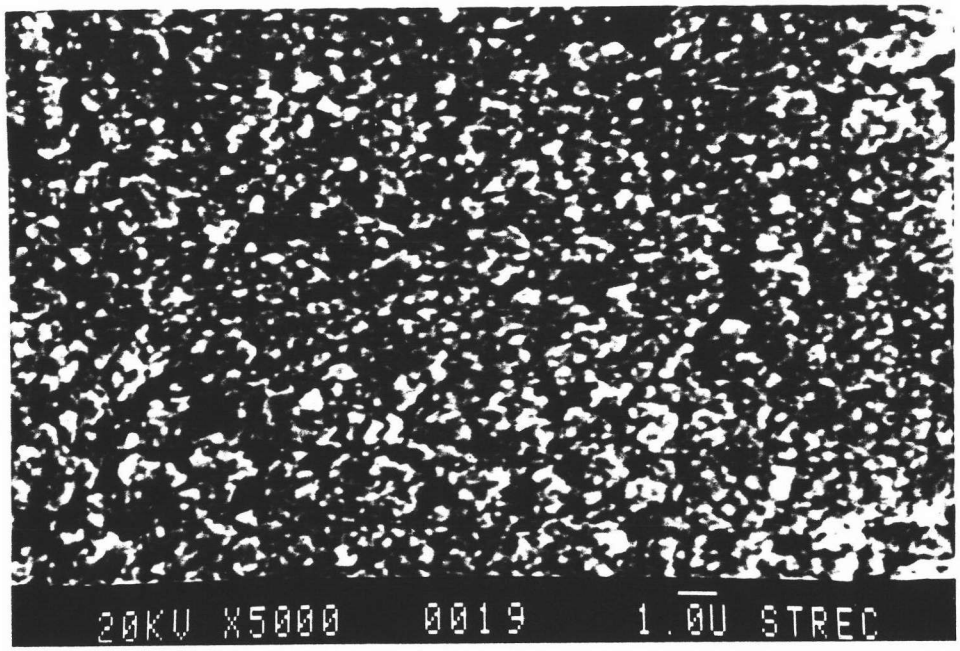


Figure 12 Scanning electron micrograph of tetanus toxoid microcapsules.

3.2) Animal Testing

a) $LD_{50/ml}$ of Tetanus Toxin

$LD_{50/ml}$ was the concentration of toxin that produced 50% mortality of mice injected with 0.5 ml toxin subcutaneously in five days .

Table 2 showed number of mortal mice after injection with 0.5 ml tetanus toxin in dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. And $LD_{50/ml}$ were calculated.

Table 2 Number of survived mice and determination of $LD_{50/ml}$ of tetanus toxin

Dilution	Mortality rate	Died	Survived	Accumulated Value			
				Died (D)	Survived (S)	Mortality ratio	Percent [D/(D+S)x100]
10^{-2}	10/10	10	0	40	0	40/40	100
10^{-3}	10/10	10	0	30	0	30/40	100
10^{-4}	10/10	10	0	20	0	20/40	100
10^{-5}	10/10	10	0	10	0	10/40	100
10^{-6}	0/10	0	10	0	10	0/10	0
10^{-7}	0/10	0	10	0	20	0/20	0
10^{-8}	0/10	0	10	0	30	0/30	0
10^{-9}	0/10	0	10	0	40	0/40	0

Calculation of $LD_{50/ml}$

Proportionate distance = $\frac{\text{mortality above 50\% - 50}}{\text{mortality above 50\% - mortality below 50\%}}$

= $\frac{100 - 50}{100 - 0}$

= 0.5

- log $LD_{50/ml}$ = - log dilution above 50% mortality + Proportionate distance

= - log 10^{-5} + 0.5

= 5.5

$LD_{50/ml}$ = $10^{-5.5}$

If desired concentration was,

1 $LD_{50/ml}$ tetanus toxin 1 ml should be diluted into $10^{5.5}$ ml

200 $LD_{50/ml}$ tetanus toxin 200 ml should be diluted into $10^{5.5}$ ml

200 $LD_{50/ml}$ tetanus toxin 1 ml should be diluted into $\frac{10^{5.5}}{200}$ ml

= 1581.14 ml

In table 2 the dilution of 10^{-5} tetanus toxin, gave 100 percent death and all of mice survived at dilutions of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}.

The dilution that produced 50% mortality in mice should be the dilution between 10^{-5} and 10^{-6}. Calculation of proportionate distance was 0.5 that indicated the desired concentration was more dilute $10^{0.5}$ times than 10^{-5} dilution so $LD_{50/ml}$ tetanus toxin was $10^{-5.5}$. If desired concentration was 200 $LD_{50/ml}$, the toxin could be diluted 1:1581.14 ml when the $LD_{50/ml}$ of toxin was $10^{-5.5}$ as the calculation.

b) Potency Testing

To determine and compare the potency of tetanus toxoid preparations in mice during 180 days after immunization, the mice were immunized with tetanus toxoid preparations and challenged with 200 $LD_{50/ml}$ of tetanus toxin at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180. The number of survived mice were recorded five days after each challenge.

Table 3 indicated that at day 0-3 all of the challenged mice in every vaccinated group (TT, TTM and TT+TTM) were dead. At day 7 five-tenth of mice in TT and TT+TTM groups were survived, while those immunized with TTM were dead.

At day 15, nine of TT immunized mice, five of TTM immunized mice, and eight of TT+TTM immunized mice were survived.

The result showed that there was 50% protection in TT and TT+TTM immunized mice were observed in 7 days, while those in TTM immunized mice were observed in 15 days.

At day 30, the mice protection in TT and TT+TTM group were maximum. All immunized mice that were challenged with 200 $LD_{50/ml}$ of tetanus toxin were survived, while nine of TTM immunized mice were survived.

At day 45 and 60, ten of TT immunized mice were survived. At day 75-90, the number of survived mice decreased, only eight at day 75 and five at day 90 were survived. At day 120-180 none were survived.

At day 45 and 180, preparations of TTM and TT+TTM gave the protection in all ten of immunized mice.

It should be considered that the longest duration of protection of TT in immunized mice was only 90 days. The highest protective effect of TT was

during day 30 until 60 and after that it decreased. But TT still showed partially protective effect until day 90.

TTM and TT+TTM could protect all of the immunized mice during day 30 until 180.

Therefore, TT and TT+TTM produced the shortest onset (7 days) and TTM produced the longest onset (15 days) of immune response. TT produced the shortest duration (90 day) while TTM and TT+TTM showed the longest duration (180 days) in immune response.

As the result of TT+TTM, at the early time it might be the immunizing effect of TT. In the middle period, not only the immunizing activity of TT but also the effect of TTM that produced longer immune response. Hence, to prepare an effective tetanus toxoid preparation, the shortest onset (TT) and longest duration of action (TTM), mixing in the ratio 1:1 should be done.

Figure 13 showed the comparison of the number of survived mice in potency testing of adsorbed tetanus toxoid (TT), tetanus toxoid microcapsule (TTM) and mixture of adsorbed tetanus toxoid and tetanus toxoid microcapsule (TT+TTM). There was significant difference (table 4) in number of survived mice in each time period during day 0 to 15 only pairs of day 7 and 0, day 7 and 3, day 15 and 0, day 15 and 3, and day 15 and 7.

But there was no significant difference in the number of survived mice both among tetanus toxoid preparation and each time period during day 30. to 75 (day 30, 45, 60 and 75).

On the other hand during day 90 to 180, there was significant difference in number of survived mice in tetanus toxoid preparation only pairs of TTM and TT, and TT+TTM and TT but TTM and TT+TTM were not significantly different.

In addition the result of the number of survived mice which were immunized with tetanus toxoid preparation stored for 3, 6 and 9 months (table 5-10) was similar to the result showed in table 3-4.

Table 3 Number of survived mice which were immunized with various tetanus toxoid preparations stored for 0 month

DAY* \ preparation	TT	TTM	TT+TTM	PBS pH 7.4
0	0	0	0	0
3	0	0	0	0
7	5	0	5	0
15	9	5	8	0
30	10	9	10	0
45	10	10	10	0
60	10	10	10	0
75	8	10	10	0
90	5	10	10	0
120	0	10	10	0
150	0	10	10	0
180	0	10	10	0

TT = Adsorbed tetanus toxoid

TTM = Tetanus toxoid microcapsules

TT+TTM = Adsorbed tetanus toxoid + Tetanus toxoid microcapsules ratio 1:1

PBS pH 7.4 = Phosphate buffer saline solution pH 7.4

* Each of ten immunized mice was challenged with 0.5 ml of tetanus toxin subcutaneously at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180.

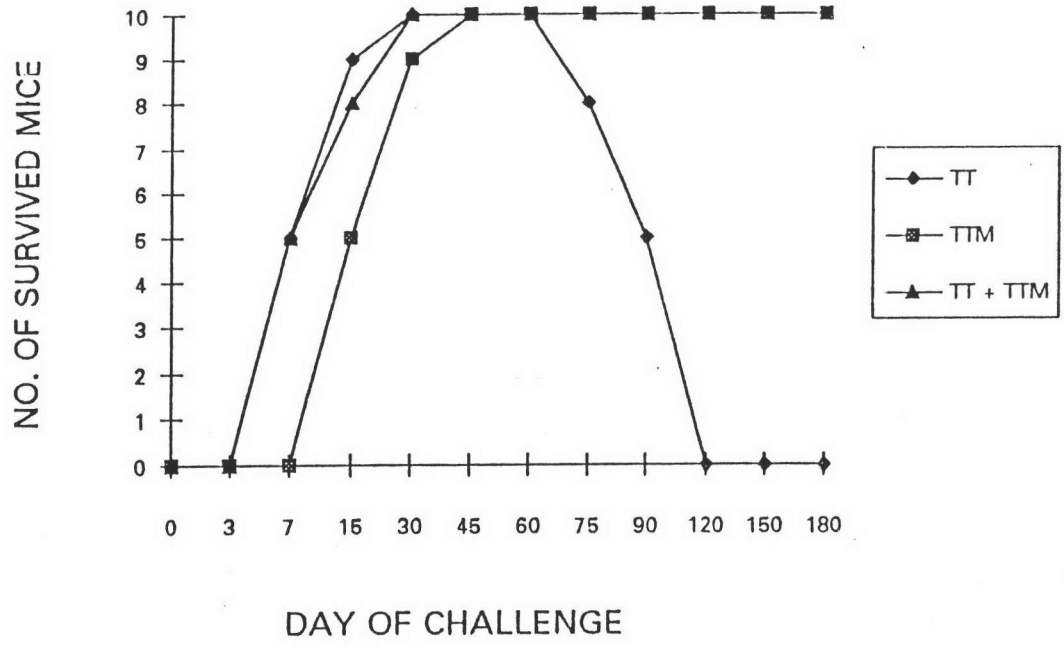


Figure 13 Comparison of the number of survived mice in potency testing of TT, TTM and TT+TTM that were stored for 0 month.

Table 4 Pairs of each time period (A) and tetanus toxoid preparations (B) that were significantly different in number of survived mice during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

B : Day 90 and 180

Preparation	TT	TTM	TT+TTM
TT	-	-	-
TTM	S	-	-
TT+TTM	S	-	-

S = Significant difference, the calculation was shown in appendix table 28-33

* Data of day 30 and 75 there was no significant difference in number of survived mice both among tetanus toxoid preparation and each time period.

Table 5 Number of survived mice which were immunized with various tetanus toxoid preparations stored for 3 months.

DAY* \ preparation	TT	TTM	TT+TTM	PBS pH 7.4
0	0	0	0	0
3	0	0	0	0
7	5	1	4	0
15	8	6	8	0
30	10	10	10	0
45	10	10	10	0
60	10	10	10	0
75	7	10	10	0
90	5	10	10	0
120	0	9	10	0
150	0	10	10	0
180	0	10	10	0

TT = Adsorbed tetanus toxoid

TTM = Tetanus toxoid microcapsules

TT+TTM = Adsorbed tetanus toxoid + Tetanus toxoid microcapsules ratio 1:1

PBS pH 7.4 = Phosphate buffer saline solution pH 7.4

* Each of ten immunized mice was challenged with 0.5 ml of tetanus toxin subcutaneously at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180.

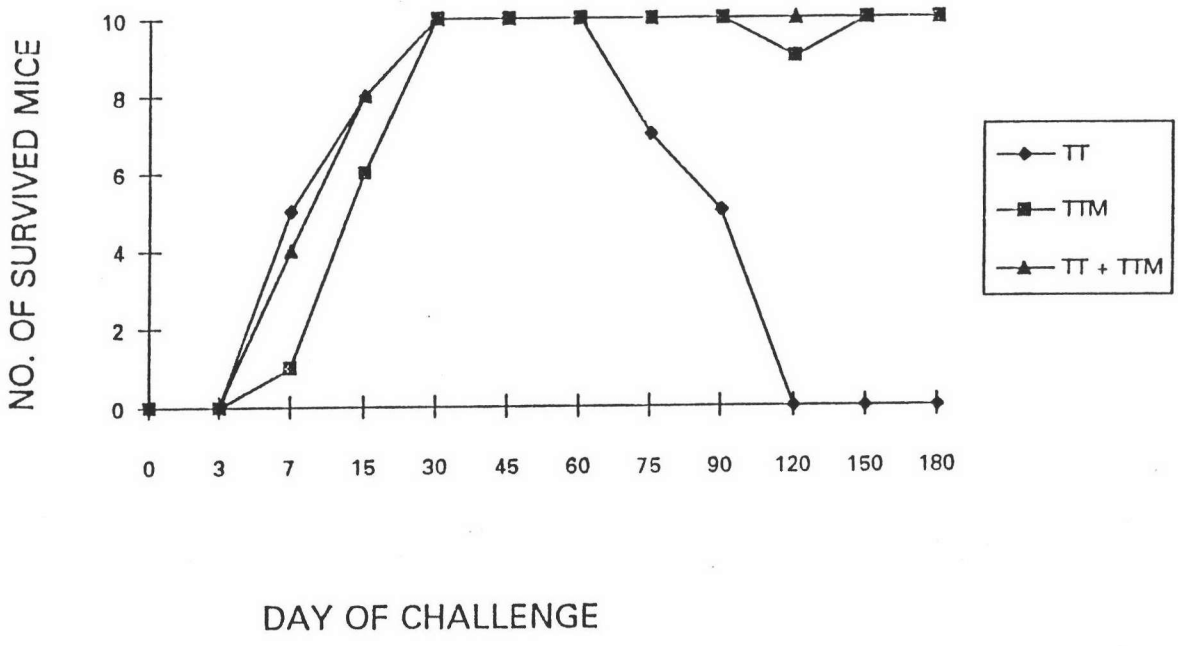


Figure 14 Comparison of the number of survived mice in potency testing of TT, TTM and TT+TTM that were stored for 3 months.

Table 6 Pairs of each time period (A) and tetanus toxoid preparations (B) that were significantly different in number of survived mice during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

B : Day 90 and 180

Preparation	TT	TTM	TT+TTM
TT	-	-	-
TTM	S	-	-
TT+TTM	S	-	-

S = Significant difference, the calculation was shown in appendix table 34-39

* Data of day 30 and 75 there was no significant difference in number of survived mice both among tetanus toxoid preparation and each time period.

Table 7 Number of survived mice which were immunized with various tetanus toxoid preparations stored for 6 months.

DAY* \ preparation	TT	TTM	TT+TTM	PBS pH 7.4
0	0	0	0	0
3	0	0	0	0
7	5	0	6	0
15	9	7	9	0
30	10	10	10	0
45	10	10	10	0
60	9	10	10	0
75	6	10	10	0
90	4	10	10	0
120	0	9	10	0
150	0	10	9	0
180	0	10	10	0

TT = Adsorbed tetanus toxoid

TTM = Tetanus toxoid microcapsules

TT+TTM = Adsorbed tetanus toxoid + Tetanus toxoid microcapsules ratio 1:1

PBS pH 7.4 = Phosphate buffer saline solution pH 7.4

* Each of ten immunized mice was challenged with 0.5 ml of tetanus toxin subcutaneously at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180.

Table 8 Pairs of each time period (A) and tetanus toxoid preparations (B) that were significantly different in number of survived mice during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

B : Day 90 and 180

Preparation	TT	TTM	TT+TTM
TT	-	-	-
TTM	S	-	-
TT+TTM	S	-	-

S = Significant difference, the calculation was shown in appendix table 40-45

* Data of day 30 and 75 there was no significant difference in number of survived mice both among tetanus toxoid preparation and each time period.

Table 9 Number of survived mice which were immunized with various tetanus toxoid preparations stored for 9 months.

DAY* \ preparation	TT	TTM	TT+TTM	PBS pH 7.4
0	0	0	0	0
3	0	0	0	0
7	4	2	5	0
15	8	6	8	0
30	10	9	10	0
45	10	10	10	0
60	9	10	10	0
75	5	10	10	0
90	3	9	10	0
120	0	10	10	0
150	0	10	10	0
180	0	10	10	0

TT = Adsorbed tetanus toxoid

TTM = Tetanus toxoid microcapsules

TT+TTM = Adsorbed tetanus toxoid + Tetanus toxoid microcapsules ratio 1:1

PBS pH 7.4 = Phosphate buffer saline solution pH 7.4

* Each of ten immunized mice was challenged with 0.5 ml of tetanus toxin subcutaneously at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180.

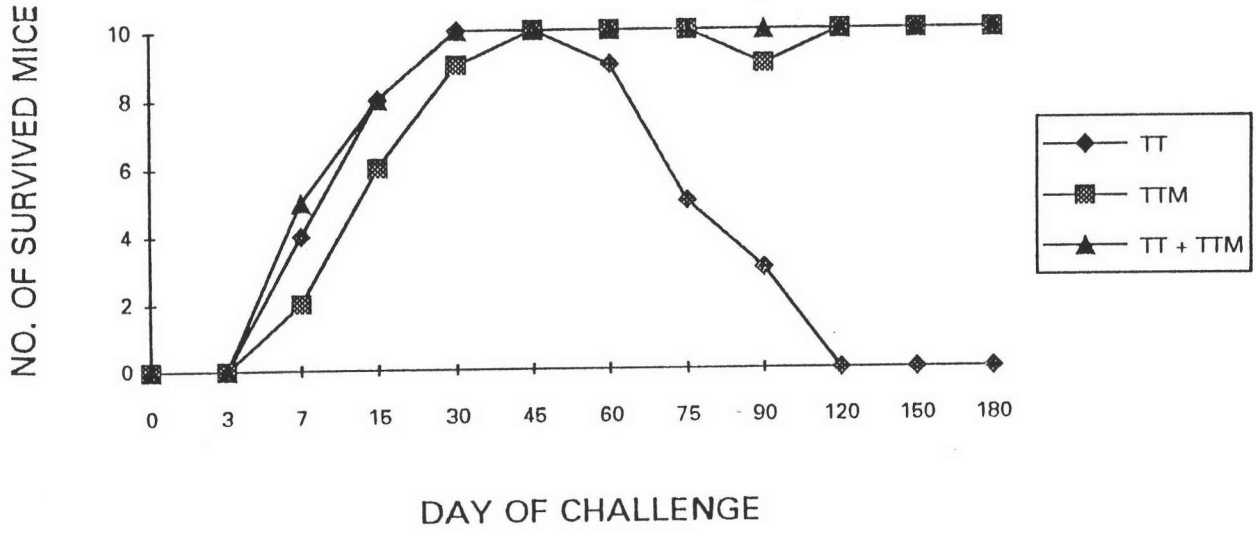


Figure 16 Comparison of the number of survived mice in potency testing of TT, TTM and TT+TTM that were stored for 9 months.

Table 10 Pairs of each time period (A) and tetanus toxoid preparations (B) that were significantly different in number of survived mice during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

B : Day 90 and 180

Preparation	TT	TTM	TT+TTM
TT	-	-	-
TTM	S	-	-
TT+TTM	S	-	-

S = Significant difference, the calculation was shown in appendix table 46-51

* Data of day 30 and 75 there was no significant difference in number of survived mice both among tetanus toxoid preparation and each time period.

Stability of potency testing

To determine and compare the stability of potency of tetanus toxoid preparations, the mice were immunized with tetanus toxoid preparation stored for 0, 3, 6 and 9 months and challenged with 200 $LD_{50/ml}$ tetanus toxin at day 0, 3, 7, 15 30, 45, 60, 75, 90, 120, 150 and 180. The number of survived mice were observed at five days after each challenge.

Table 11 indicated that at day 0-3 all of challenged mice with 200 $LD_{50/ml}$ tetanus toxin in every group of tetanus toxoid stored for 0, 3, 6 and 9 months were dead.

At day 7, five-tenth of mice immunized with TT stored for 0, 3, 6 months and four-tenth of mice immunized with TT stored for 9 months were surived.

At day 15, nine, eight, nine, and eight of TT immunized mice stored for 0, 3, 6 and 9 months were survived respectively.

At day 30-60, the protective effect of TT was maximum, all challenged mice were survived.

At day 75-180, the number of survived mice decreased at day 75. Eight, seven, six, five of mice immunized with TT stored for 0, 3, 6 and 9 months respectively were survived. At day 90, five, five, four, three of mice immunized with TT stored for 0, 3, 6 and months were survived respectively.

At day 120-180, none were survived.

As the result, TT showed the onset in 7 day and duration of immunizing activity was 90 days. The long duration of immunizing effect of TT in mouse protection was only 90 days. The highest protection of TT was during day 30-60 and after that it decreased. But it still showed partial protection until day 90.

Figure 17 showed the comparison of the number of survived mice in stability testing of TT that were stored for 0, 3, 6 and 9 months. There was significant difference (table 12) in number of survived mice in each time period. During day 0 to 15 only pairs of day 7 and 0, day 7 and 3, day 15 and 0, day 15 and 3, and day 15 and 7 there was significant difference.

During day 30 to 75 there was significant difference in number of survived mice between 30 and 75, 45 and 75 and 60 and 75.

During day 90 to 180 there was significant difference in number of survived mice between 90 and 120, 90 and 150 and 90 and 180.

During day 0 to 15, 30 to 75 and 90 to 180 there was no significant difference in number of survived mice in each month period.

Table 11 Number of survived mice which were immunized with tetanus toxoid stored for 0, 3, 6 and 9 months.

DAY* \ Month	0	3	6	9
0	0	0	0	0
3	0	0	0	0
7	5	5	5	4
15	9	8	9	8
30	10	10	10	10
45	10	10	10	10
60	10	10	9	9
75	8	7	6	5
90	5	5	4	3
120	0	0	0	0
150	0	0	0	0
180	0	0	0	0

* Each of ten immunized mice was challenged with 0.5 ml of tetanus toxin subcutaneously at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180.

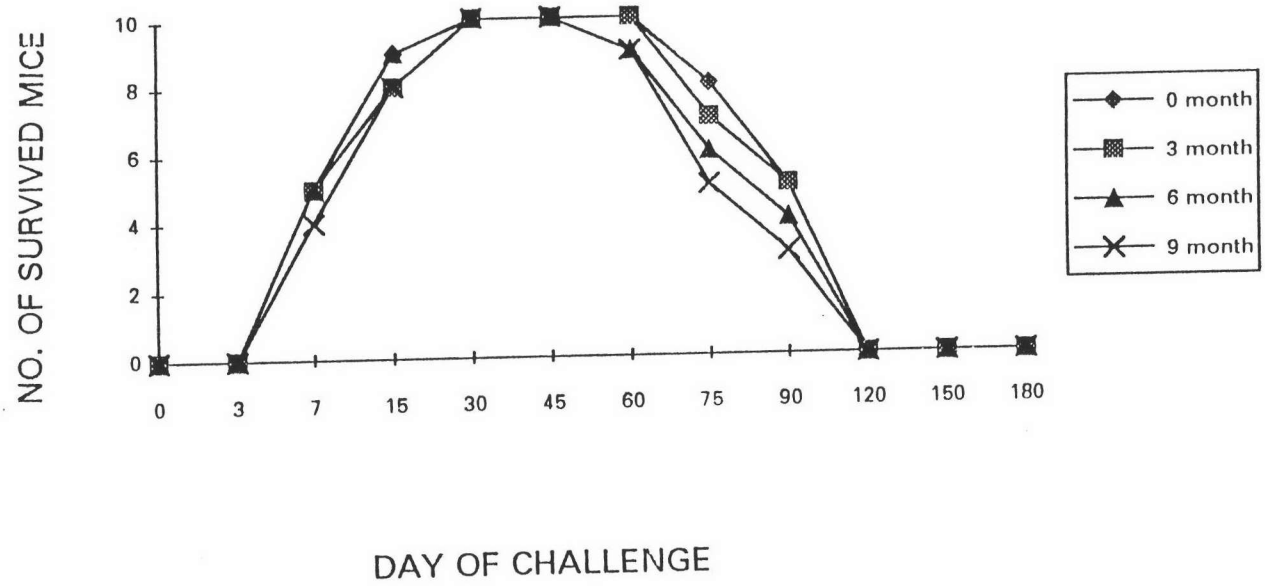


Figure 17 Comparison of the number of survived mice in stability testing of TT, that was stored for 0, 3, 6 and 9 months.

Table 12 Pairs of each time period (A, B, C) that was significantly different in number of survived mice during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

B : Day 30 and 75

Time period	30	45	60	75
30	-	-	-	S
45	-	-	-	S
60	-	-	-	S
75	-	-	-	-

C : Day 90 and 180

Time period	90	120	150	180
90	-	S	S	S
120	-	-	-	-
150	-	-	-	-
180	-	-	-	-

S = Significant difference, the calculation was shown in appendix table 52-57

* Data of day 0-15, 30-75, and 90-180 there was no significant difference in number of survived mice in each month period.

To determine and compare the stability of potency of tetanus toxoid microcapsules stored for 0, 3, 6 and 9 months. The mice were immunized with tetanus toxoid microcapsules stored for 0, 3, 6 and 9 months and challenged with 200 $LD_{50/ml}$ of tetanus toxin at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180. The number of survived mice were observed five days after each challenge.

Table 13 indicated that at day 0-7 nearly all of challenged mice in every group of tetanus toxoid microcapsules stored for 0, 3, 6 and 9 months were dead.

At day 15, five, six, seven and six mice immunized with TTM stored for 0, 3, 6 and 9 months were surived respectively.

At day 30-180, the preparation of TTM stored for 0, 3, 6 and 9 months gave protection in all of ten immunized mice.

As these result, TTM had the onset 15 day and duration in immunizing activity 180 days. TTM gave 100% protection in mice on day 30 through day 180 after immunization. The experiment was set for only 180 days but the result indicated that TTM should gave 100% protection in mice longer than 180 days.

Figure 18 showed the number of survived mice that were immunized with TTM and stored for 0, 3, 6 and 9 months. There was significant difference (table 14) in number of survived mice in each time period during day 0 to 15 between 15 and 0, 15 and 3, and 15 and 7. During day 30 to 75 and 90 to 180 there was no significant difference in number of survived mice that was tested against among each month period and each time period.

Table 13 Number of survived mice which were immunized with tetanus toxoid microcapsules stored for 0, 3, 6 and 9 months.

DAY* \ Month	0	3	6	9
0	0	0	0	0
3	0	0	0	0
7	0	1	0	2
15	5	6	7	6
30	9	10	10	9
45	10	10	10	10
60	10	10	10	10
75	10	10	10	10
90	10	10	10	9
120	10	9	9	10
150	10	10	10	10
180	10	10	10	10

* Each of ten immunized mice was challenged with 0.5 ml of tetanus toxin subcutaneously at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180.

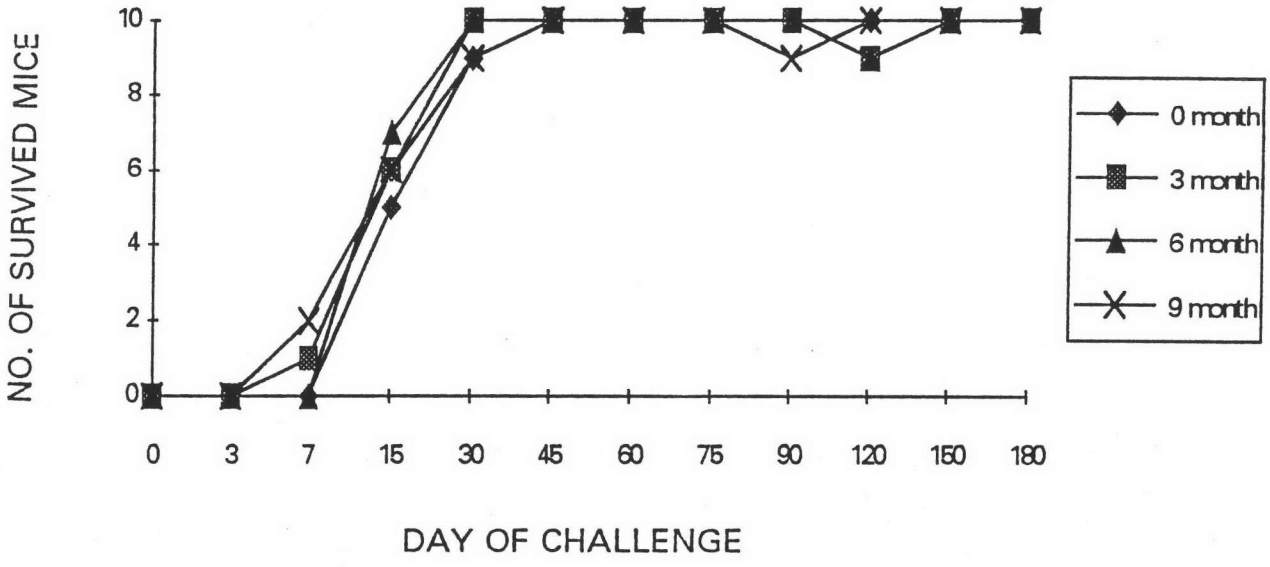


Figure 18 Comparison of the number of survived mice in stability testing of TTM, that was stored for 0, 3, 6 and 9 months.

Table 14 Pairs of each time period (A) that was significantly different in number of survived mice during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	-	-	-	-
15	S	S	S	-

S = Significant difference, the calculation was shown in appendix table 58-63

* Data of day 30-75, and 90-180 there was no siginificant difference in number of survived mice both among each month period and each time period.

To determine and compare the stability of potency of mixture of tetanus toxoid and tetanus toxoid microcapsules in the ratio of 1:1 (TT+TTM) stored for 0, 3, 6 and 9 months. The mice were immunized with TT+TTM stored for 0, 3, 6 and 9 months and challenged with 200 $LD_{50/ml}$ tetanus toxin at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180. The number of survived mice were observed five days after each challenge.

Table 15 indicated that at day 0-3 all of challenged mice in every group of TT+TTM stored for 0, 3, 6 and 9 months were dead.

At day 7, five, four, six and five mice immunized with TT+TTM stored for 0, 3, 6 and 9 months were survived respectively.

At day 15, the protective effect continuously increased, the number of survived mice were more than that of the earlier time, as eight, eight, nine and eight mice immunized with TT+TTM stored for 0, 3, 6 and 9 months respectively.

At day 30-180, the preparation of TT+TTM stored for 0, 3, 6 and 9 months gave 100% protection.

As these result, TT+TTM produce the onset of response in 7 days and duration of immunizing activity was 180 days. The long immunizing effect of TT+TTM in protecting the immunized mice was 180 days.

Figure 19 showed the comparison of the number of survived mice in stability testing of TT+TTM that was stored for 0, 3, 6 and 9 months. There was significant difference (table 16) in number of survived mice in each time period during day 0 to 15 between 7 and 0, 7 and 3, 15 and 0, and 15 and 7.

During day 30 to 75 and 90 to 180 there was no significant difference in number of survived mice that was tested against among each month period and each time period.

Table 15 Number of survived mice which were immunized with tetanus toxoid and tetanus toxoid microcapsules ratio 1:1 stored for 0, 3, 6 and 9 months.

DAY* \ Month	0	3	6	9
0	0	0	0	0
3	0	0	0	0
7	5	4	6	5
15	8	8	9	8
30	10	10	10	10
45	10	10	10	10
60	10	10	10	10
75	10	10	10	10
90	10	10	10	10
120	10	10	10	10
150	10	10	9	10
180	10	10	10	10

* Each of ten immunized mice was challenged with 0.5 ml of tetanus toxin subcutaneously at day 0, 3, 7, 15, 30, 45, 60, 75, 90, 120, 150 and 180.

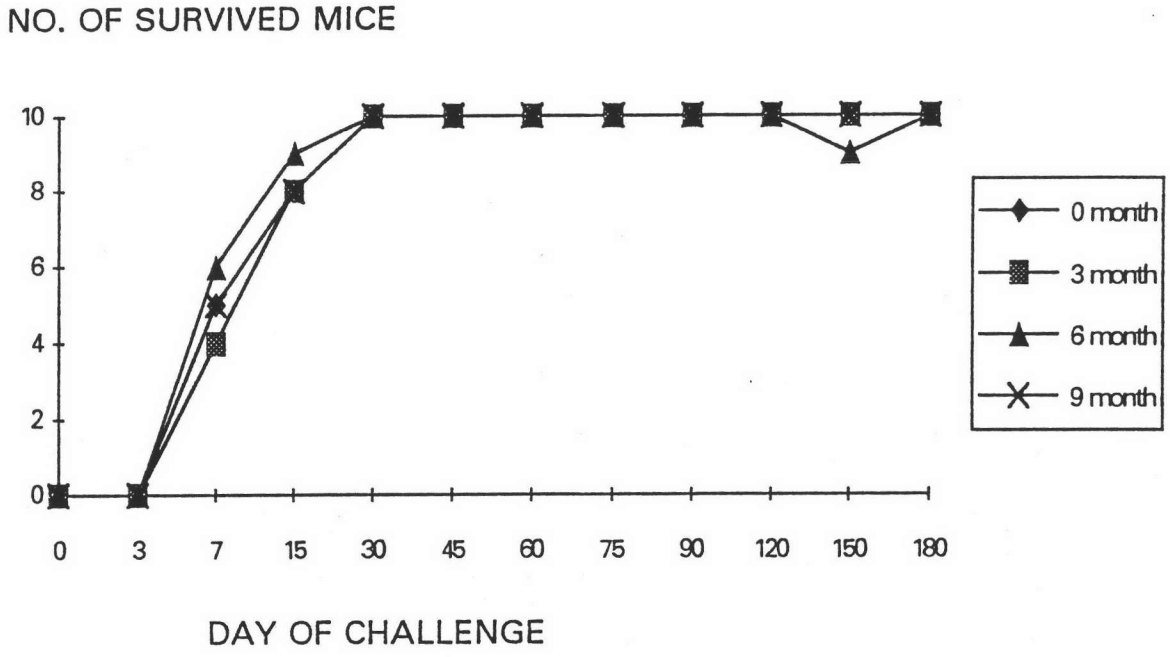


Figure 19 Comparison of the number of survived mice in stability testing of TT+TTM that was stored for 0, 3, 6 and 9 months.

Table 16 Pairs of each time period (A) that was significantly different in number of survived mice during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

S = Significant difference, the calculation was shown in appendix table 64-69

* Data of day 30-75, and 90-180 there were no siginificant difference in number of survived mice both among each month period and each time period.

C) Antibody titer determination

To determine the antibody titer of mouse anti-tetanus serum, serum was separated from blood collected from each group of immunized mice. Antibody titers of serum was determined by enzyme linked immunosorbent assay.

Table 17 showed the antiboty titers of mouse anti-tetanus sera obtained from mice immunized with adsorbed tetanus toxoid (TT), tetanus toxoid microcapsules (TTM), mixture of adsorbed tetanus toxoid and tetanus toxoid microcapsules in ratio 1:1 (TT+TTM).

At day 0-3, the antibody titers of sera from mice immunized with all tetanus toxoid preparation were 10.

At day 7, that immunized with TT and TT+TTM were 50 while that immunized with TTM was 10.

At day 15, the antibody titers of every sera was 250.

At day 30, the antibody titers of mouse sera immunized with TT was still 250, while that immunized with TTM and TT+TTM increased to 1250.

At day 45-180, the antibody titers of mouse sera immunized with TT was highest at day 45-60 and then rapidly decreased to 10 at day 120. For TTM group, the antibody titer was highest at day 75 and then decreased to 1250 at day 90-180. For TT+TTM group, the antibody titer was highest at day 60 and then decreased to 1250 at day 75-180.

Figure 20 showed the comparisons of the antibody titers of sera obtained from mice immunized with various preparations of tetanus toxoid. There was significant difference (table 18) in antibody titers of mouse anti-tetanus sera in each time period during day 0-15.

During day 30 to 75 there was no significant difference in antibody titers of mouse anti-tetanus sera both among tetanus toxoid preparation and each time period.

During day 90 to 180 there was significant difference in antibody titers of mouse anti-tetanus sera only pairs of TTM and TT, and TT+TTM and TT but TTM and TT+TTM was no significant difference.

Hence, the antibody titers of mouse anti-tetanus sera were detected in early period (day 0-15) in the group immunized with TT and TT+TTM, in middle period the antibody titers were higher that they should be the immunizing activity of TT and TTM. In final period (day 120-180) the result indicated the immunizing activity of long acting tetanus toxoid microcapsules.

In addition the result of the antibody titers of serum from mice immunized with tetanus toxoid preparation stored for 3, 6, and 9 month (table 19-24) was similar to the result showed in table 17-18.

Table 17 The antibody titers of sera from mice immunized with various tetanus toxoid preparations stored for 0 month.

Day \ preparation	TT	TTM	TT+TTM	PBS pH 7.4
0	10	10	10	10
3	10	10	10	10
7	50	10	50	10
15	250	250	250	10
30	250	1250	1250	10
45	1250	1250	1250	10
60	1250	1250	6250	10
75	250	6250	1250	10
90	250	1250	1250	10
120	10	1250	1250	10
150	10	1250	1250	10
180	10	1250	1250	10

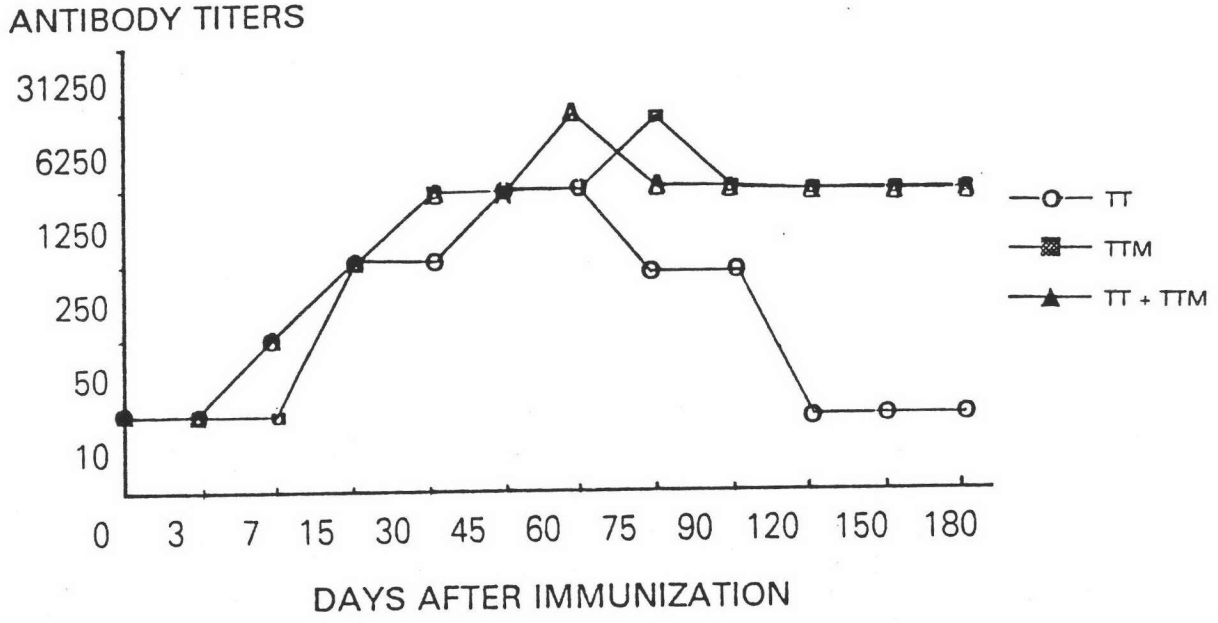


Figure 20 Comparison of the antibody titers of sera from mice iimmunized with various preparations of tetanus toxoid that were stored for 0 month.

Table 18 Pairs of each time period (A) and tetanus toxoid preparations (B) that were significantly different in antibody titers of mouse anti-tetanus sera during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

B : Day 90 and 180

Preparation	TT	TTM	TT+TTM
TT	-	-	-
TTM	S	-	-
TT+TTM	S	-	-

S = Significant difference, the calculation was shown in appendix table 70-75

* Data of day 30 and 75 there was no significant difference in antibody titers of mouse anti-tetanus sera both among tetanus toxoid preparation and each time period.

Table 19 The antibody titers of sera from mice immunized with various tetanus toxoid preparations stored for 3 months.

Day \ preparation	TT	TTM	TT+TTM	PBS pH 7.4
0	10	10	10	10
3	10	10	10	10
7	50	10	50	10
15	250	250	250	10
30	250	1250	1250	10
45	1250	1250	1250	10
60	1250	1250	6250	10
75	250	6250	1250	10
90	250	1250	1250	10
120	10	1250	1250	10
150	10	1250	1250	10
180	10	1250	1250	10

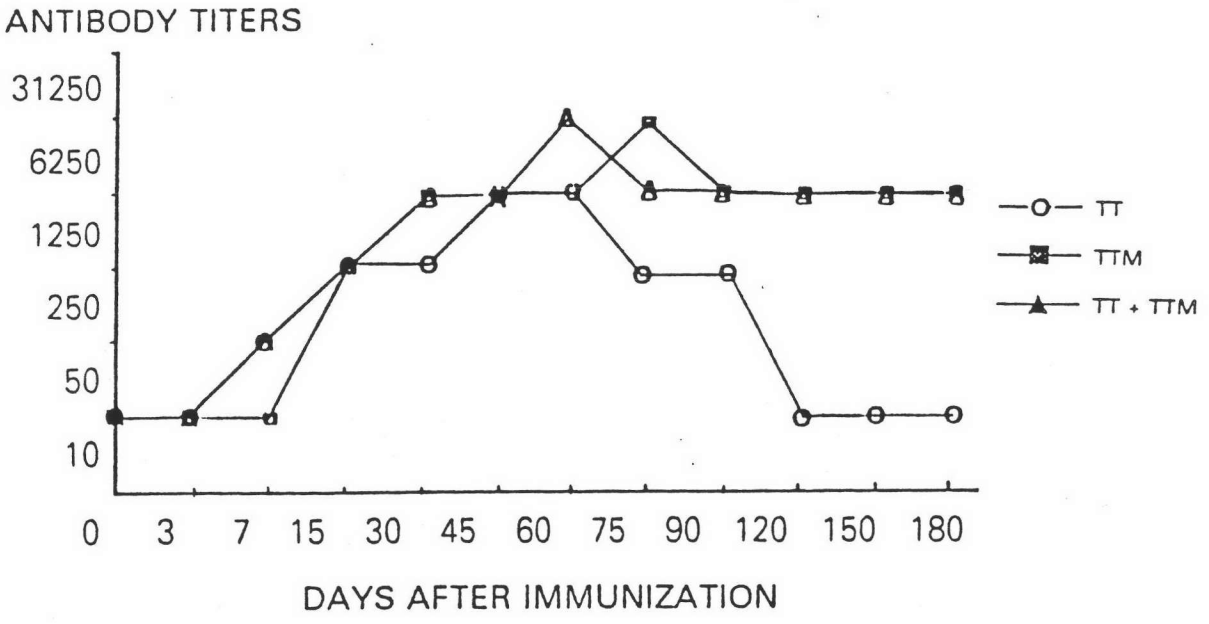


Figure 21 Comparison of the antibody titers of sera from mice immunized with various preparations of tetanus toxoid that were stored for 3 months.

Table 20 Pairs of each time period (A) and tetanus toxoid preparations (B) that were significantly different in antibody titers of mouse anti-tetanus sera during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

B : Day 90 and 180

Preparation	TT	TTM	TT+TTM
TT	-	-	-
TTM	S	-	-
TT+TTM	S	-	-

S = Significant difference, the calculation was shown in appendix table 76-81

* Data of day 30 and 75 there was no significant difference in antibody titers of mouse anti-tetanus sera both among tetanus toxoid preparation and each time period.

Table 21 The antibody titers of sera from mice immunized with various tetanus toxoid preparations stored for 6 months.

Day \ preparation	TT	TTM	TT+TTM	PBS pH 7.4
0	10	10	10	10
3	10	10	10	10
7	50	10	50	10
15	250	250	250	10
30	250	1250	1250	10
45	1250	1250	1250	10
60	1250	1250	6250	10
75	250	6250	1250	10
90	250	1250	1250	10
120	10	1250	1250	10
150	10	1250	1250	10
180	10	1250	1250	10

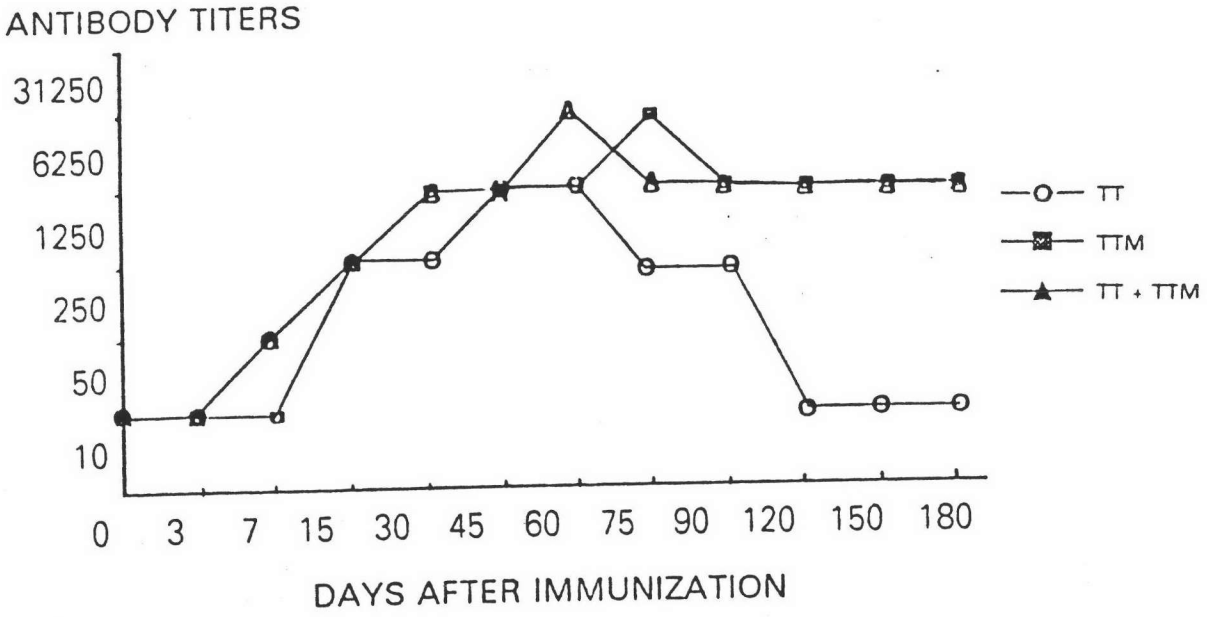


Figure 22 Comparison of the antibody titers of sera from mice immunized with various preparations of tetanus toxoid that were stored for 6 months.

Table 22 Pairs of each time period (A) and tetanus toxoid preparations (B) that were significantly different in antibody titers of mouse anti-tetanus sera during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

B : Day 90 and 180

Preparation	TT	TTM	TT+TTM
TT	-	-	-
TTM	S	-	-
TT+TTM	S	-	-

S = Significant difference, the calculation was shown in appendix table 82-87

* Data of day 30 and 75 there was no significant difference in antibody titers of mouse anti-tetanus sera both among tetanus toxoid preparation and each time period.

Table 23 The antibody titers of sera from mice immunized with various tetanus toxoid preparation stored for 9 months.

Day \ preparation	TT	TTM	TT+TTM	PBS pH 7.4
0	10	10	10	10
3	10	10	10	10
7	50	10	50	10
15	250	250	250	10
30	250	1250	1250	10
45	1250	1250	1250	10
60	1250	1250	6250	10
75	250	6250	1250	10
90	250	1250	1250	10
120	10	1250	1250	10
150	10	1250	1250	10
180	10	1250	1250	10

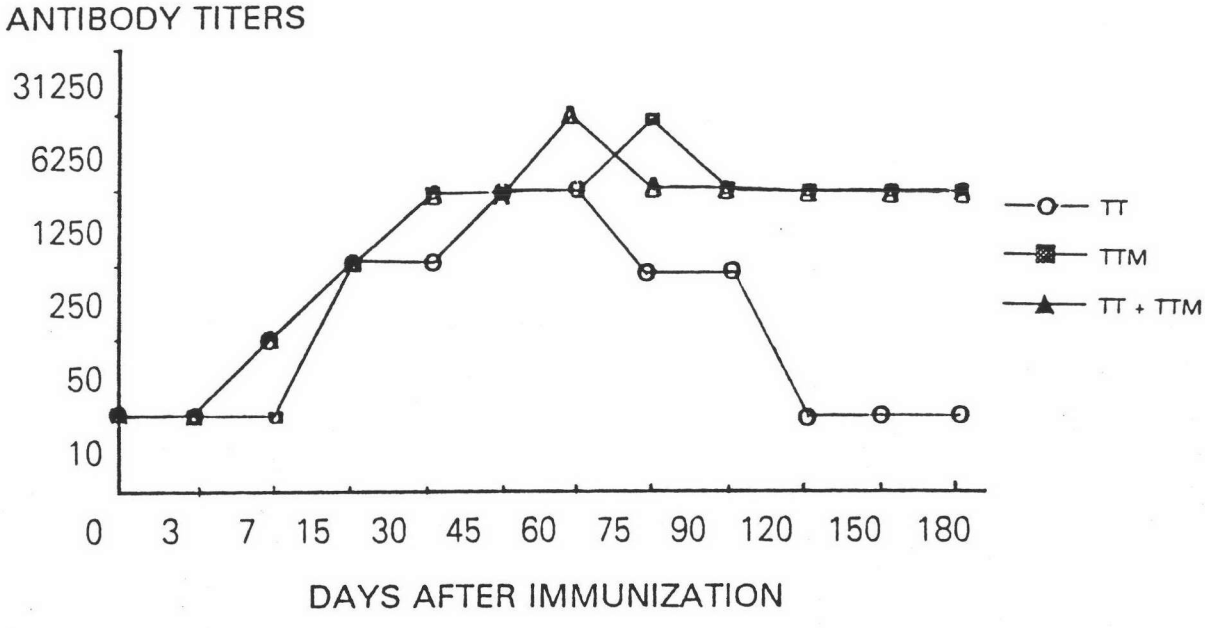


Figure 23 Comparison of the antibody titers of sera from mice immunized with various preparations of tetanus toxoid that were stored for 9 months.

Table 24 Pairs of each time period (A) and tetanus toxoid preparations (B) that were significantly different in antibody titers of mouse anti-tetanus sera during day 0 and 180*

A : Day 0 and 15

Time period	0	3	7	15
0	-	-	-	-
3	-	-	-	-
7	S	S	-	-
15	S	S	S	-

B : Day 90 and 180

Preparation	TT	TTM	TT+TTM
TT	-	-	-
TTM	S	-	-
TT+TTM	S	-	-

S = Significant difference, the calculation was shown in appendix table 88-93

* Data of day 30 and 75 there was significant difference in antibody titers of mouse anti-tetanus sera both among tetanus toxoid preparation and each time period.

Stability of tetanus toxoid preparation

Table 25 showed the antibody titers of sera from mice immunized with tetanus toxoid stored for 0, 3, 6 and 9 months.

At day 0-3, the antibody titers of sera from tetanus toxoid group were 10 when the vaccine was stored for 0, 3, 6 and 9 months.

At day 7, the antibody titer increased to 50.

At day 15-30, the antibody titer still increased to 250.

At day 45-60, the antibody titer increased to maximum level (1250)

At day 75-90, the antibody response was decreased and the anitbody titer was 250, at day 120-180 antibody titer was 10.

Figure 24 showed the comparison of the antibody titers of sera from mice immunized with TT stored for 0, 3, 6 and 9 months.

During day 0 to 180, there was no significant difference in antibody titers of mouse anti-tetanus sera both among each month period and each time period.

The calculation was shown in appendix table 94-99.

Table 25 The antibody titers of sera from mice immunized with tetanus toxoid stored for 0, 3, 6 and 9 months.

Days \ Months	0	3	6	9
0	10	10	10	10
3	10	10	10	10
7	50	50	50	10
15	250	250	250	250
30	250	250	250	250
45	1250	1250	1250	1250
60	1250	1250	1250	1250
75	250	250	250	250
90	250	250	250	250
120	10	10	10	10
150	10	10	10	10
180	10	10	10	10

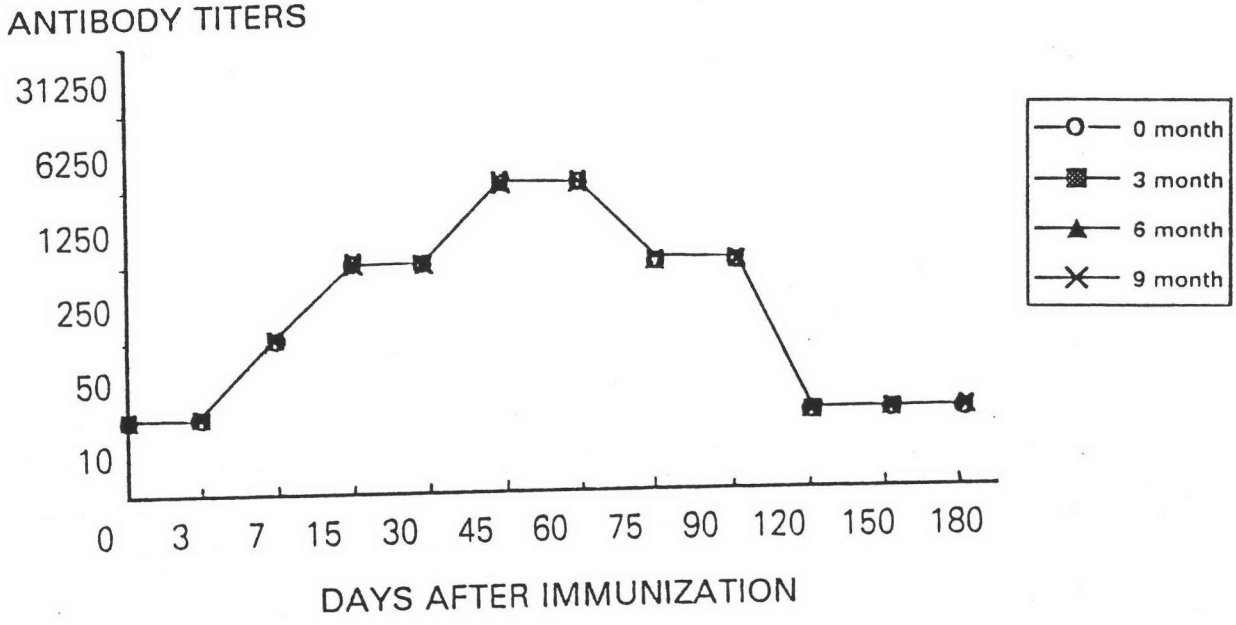


Figure 24 Comparison of the antibody titers of sera from mice immunized with TT stored for 0, 3, 6 and 9 months.

Table 26 showed the antibody titers of sera from mice immunized with tetanus toxoid microcapsules stored for 0, 3, 6 and 9 months.

At day 0-7, the antibody titers of sera from TTM group was 10 when the vaccine was stored for 0, 3, 6 and 9 months.

At day 15, the antibody titers of sera from TTM group increased to 250.

At day 30-180, the antibody titer was 1250, then increased to maximum level (6250) at day 75 and decreased to 1250 at day 90-180.

Figure 25 showed the comparison of the antibody titers of sera from mice immunized with TTM stored for 0, 3, 6 and 9 months.

During day 0 to 180, there was no significant difference in antibody titers of mouse anti-tetanus sera both among each month period and each time period.

The calculation was shown in appendix table 100-105.

Table 26 The antibody titers of sera from mice immunized with tetanus toxoid microcapsules stored for 0, 3, 6 and 9 months.

Days \ Months	0	3	6	9
0	10	10	10	10
3	10	10	10	10
7	10	10	10	10
15	250	250	250	250
30	1250	1250	1250	1250
45	1250	1250	1250	1250
60	1250	1250	1250	1250
75	6250	6250	6250	6250
90	1250	1250	1250	1250
120	1250	1250	1250	1250
150	1250	1250	1250	1250
180	1250	1250	1250	1250

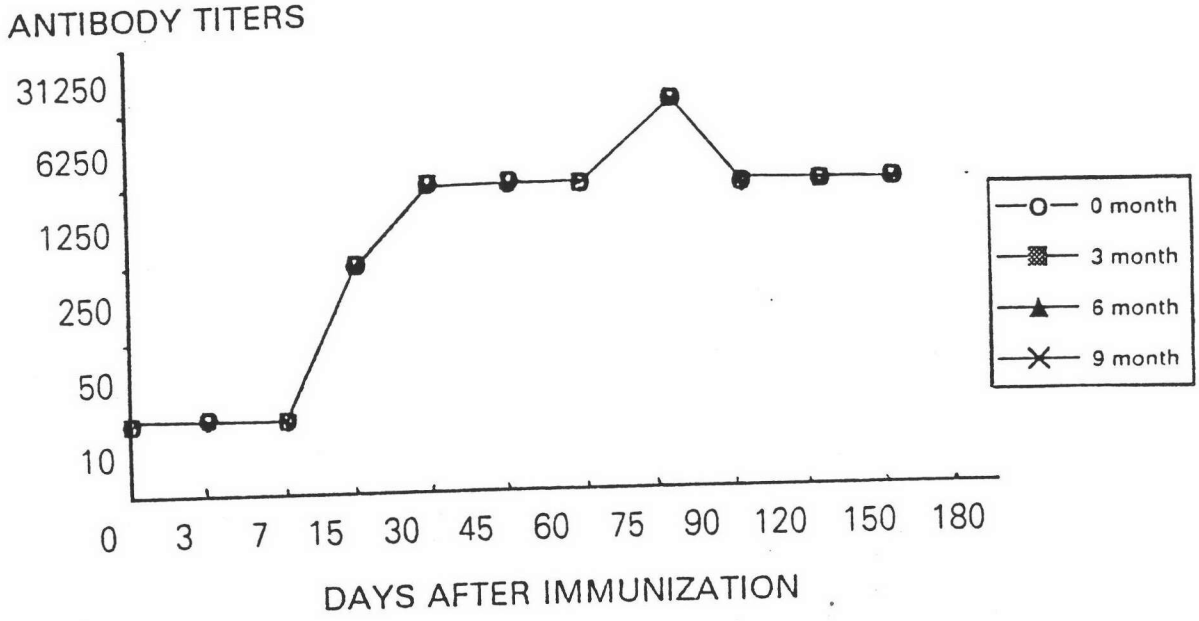


Figure 25 Comparison of the antibody titers of sera from mice immunized with TTM stored for 0, 3, 6 and 9 months.

Table 27 showed the antibody titers of sera from mice immunized with tetanus toxoid and tetanus toxoid microcapsules ratio 1:1 stored for 0, 3, 6 and 9 months.

At day 0-3, the antibody titer was 10.

At day 7-15, the antibody titers increased to 50 at day 7 and 250 at day 15.

At day 30, the antibody titer still increased to 1250, and reached the maximum level at day 60. After that antibody titer of TT+TTM decreased to 1250 at day 180.

Figure 26 showed the comparison of the antibody titers of sera from mice immunized with TT+TTM stored for 0, 3, 6 and 9 months.

During day 0 to 180, there was no significant difference in antibody titers of mouse anti-tetanus sera both among each month period and each time period.

The calculation was shown in appendix table 106-111.

Table 27 The antibody titers of sera from mice immunized with tetanus toxoid and tetanus toxoid microcapsules ratio 1:1 stored for 0, 3, 6 and 9 months.

Days \ Months	0	3	6	9
0	10	10	10	10
3	10	10	10	10
7	50	50	50	50
15	250	250	250	250
30	1250	1250	1250	1250
45	1250	1250	1250	1250
60	6250	6250	6250	6250
75	1250	1250	1250	1250
90	1250	1250	1250	1250
120	1250	1250	1250	1250
150	1250	1250	1250	1250
180	1250	1250	1250	1250

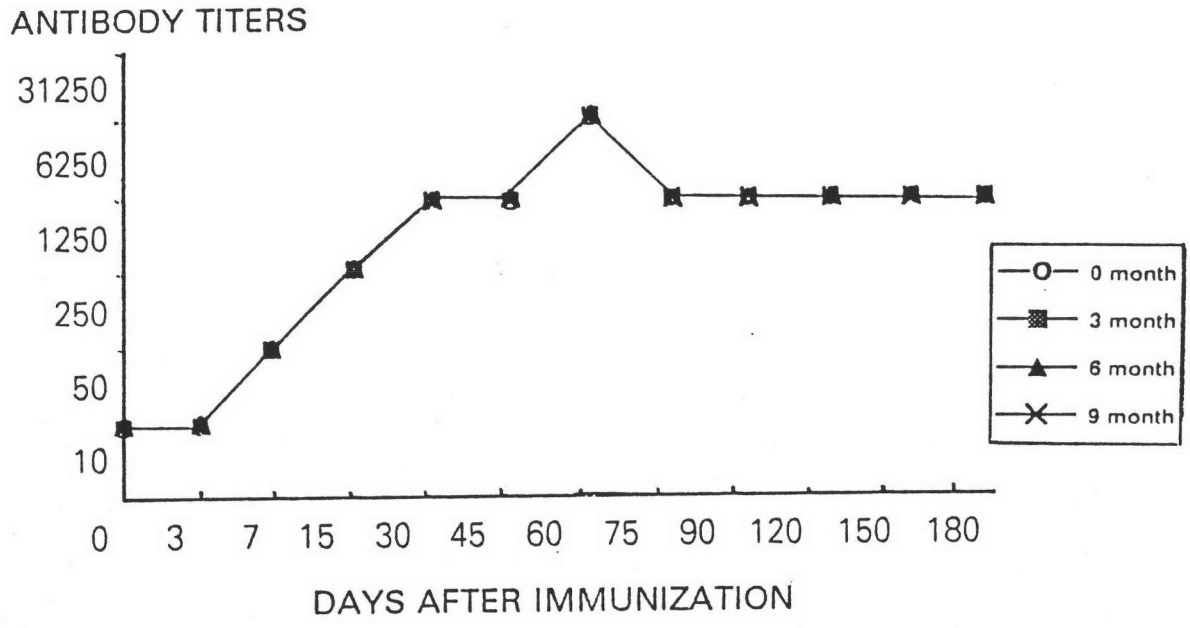


Figure 26 Comparison of the antibody titers of sera from mice immunized with TT+TTM stored for 0, 3, 6 and 9 months.

Correlation in Potency testing and Antibody titers

According to the result (table 3-10 and table 17-24), at the early time (day 0-3) all of the challenged mice in every vaccinated group (TT, TTM, and TT+TTM) were dead, the antibody titers of sera from immunized mice were 10. The antibody titers of sera from controlled mice were 10.

At day 7, five-tenth of mice in TT and TT+TTM groups were survived, while those immunized with TTM were dead. The antibody titers of sera of TT and TT+TTM immunized mice were 50 and that of TTM immunized mice was 10. It indicated that the antibody titer level at 50 was not high enough for protection.

At day 15, nine of TT immunized mice, five of TTM immunized mice and eight of TT+TTM immunized mice were survived. The antibody titers of every serum was 250. So the antibody titer level of 250 showed partial protection.

At day 30-60, the protection in TT immunized mice group were maximum. The antibody titers of TT was 250 at day 30 and 1250 at day 45-60. At day 75-180, the antibody titers were rapidly decreased to 250 at day 75-90 and 10 at day 120-180. The number of survived mice decreased to eight at day 75 and five at day 90. At day 120-180 none were survived. Therefore the duration of TT was short. It was only 90 days.

At day 30-180, the antibody titers of sera obtained from mice immunized with TTM and TT+TTM were 1250. For TT+TTM and TTM groups the antibody titer was highest at day 60 and 75 respectively.

Hence, when all of challenged mice were survived, the antibody titer was 1250 in TT group and 1250-6250 in TTM and TT+TTM groups.

In order to protect all mice from tetanus toxin, the titer must be higher than 250. For completely protection the antibody titer of serum should be 1250. After the

peak of antibody titers all mice were protected. While the antibody titer was decreased to the level of 250, most of the mice showed the risk of dead. When the antibody titer level of serum were lower than 250 all of the mice were dead.

The correlation between antibody titers of mouse sera and the antibody unit (antibody I.U.) were determined and the data were shown in appendix II. The human anti-tetanus serum was diluted in various concentration and reacted with tetanus toxoid in ELISA. The result showed that the antibody titer of mouse anti-tetanus sera of 1250 contained antibody approximately 2.5 I.U.

The Prolong Action of Tetanus Toxoid Microcapsules.

This investigation showed that tetanus toxoid microcapsules could produced immune response of longer duration than that produced by TT. So tetanus toxoid microcapsules was a sustained release dosage form and the antibody titers detected in sera from mice immunized with TTM showed high level for a long period. (180 days in the experiment performed.) In microcapsules preparation, tetanus toxoids were encapsulated in lecithin and carboxymethyl chitin as a polymeric membrane . It took time for the tetanus toxoid to diffuse through the polymeric membrane and slowly induced the immune response in mice and produced high response with long duration.