III. RESULTS

III.1 Preparation of Ovomucoid

Figure 5 shows the protein profile of crude OM eluted from a CM-cellulose (2.0x25.0 cm) column. The column was prewashed with 0.1 N acetic acid until the optical density at 280 nm of the eluted solution was less than 0.02. In each chromatographic run, the crude OM (500 mg in 3.0 ml distilled water) was then applied to the column. The eluted fractions, 3.0 ml/tube, was collected and their absorbance was measured at 280 nm. OM was found in the large peak (162). The OM-containing fractions were then pooled, dialyzed against distilled water and lyophilized. The total yield from four chromatographic runs was 800 mg. The impurities adsorbed in the column were washed out with 0.01 N NaOH before regeneration of the column.

III.2 Purification of Wheat germ Agglutinin

Figure 6 shows the elution profile of protein from crude aquous extract from homogenized wheat germ when passed through the ovomucoid-Sepharose 4B column. The unbound protein was washed out with PBS. The absorbed lectin was been eluted out from the column with 0.1 N acetic acid. As shown in the chromatogram, two peaks of proteins expressed as optical density at 280 nm were obtained the agglutinating activity as assayed in Section II.7 was found in the second small peak. These fractions were pooled, dialysed against

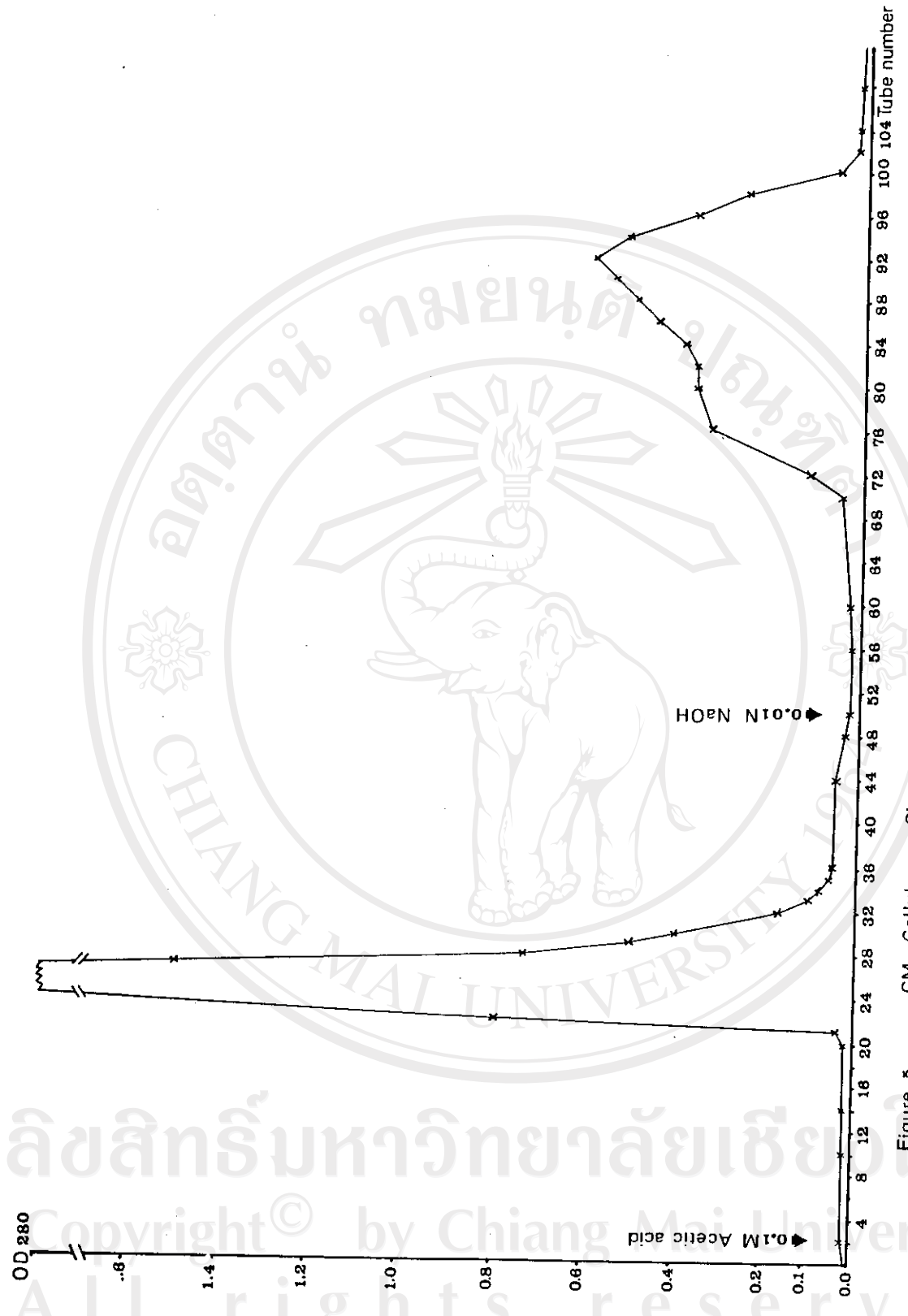

Figure 5 CM – Cellulose Chromatography of Crude Ovumucoid.

distilled water and then lyophilized. The white lyophilized powder of WGA (7 mg) was kept at 4°C for further study. Protein content in the preparation was determined by the method of Lowry et al.(179). The summary of WGA purification is presented in Table 3.

III.3 Preparation of Collocalia Mucoid

The protein concentration of the white lyophilized powder of collocalia mucoid was 3 mg/ml, determined by Lowry method (Section II.14). The sialic acid content was also determined by thiobarbituric acid method (section II.13.1) The sialic acid concentration was 10 %.

III.4 Extraction and Purification of PSA

After the extraction solution of pea seeds was precipitated with $(NH_4)_2SO_4$. The precipitate was separated by the centrifugation. The supernatant was checked for HA titer^{-1}. It's HA titer^{-1} was only 4, so it was discarded. The pellet was dialyzed and lyophilized. The lyophilized powder was dissolved in distilled water and applied to a Sephadex G-100 column. The column was eluted with PBS and 0.2 M glucose in PBS respectively. The optical density at 280 nm of the fractions was measured. Two protein peaks were obtained. The fractions were pooled and dialyzed against PBS before an assay of HA titer^{-1}. The large peak at void volume did not show any detectable hemagglutinating activity. Three fractions of the small peak were positive.

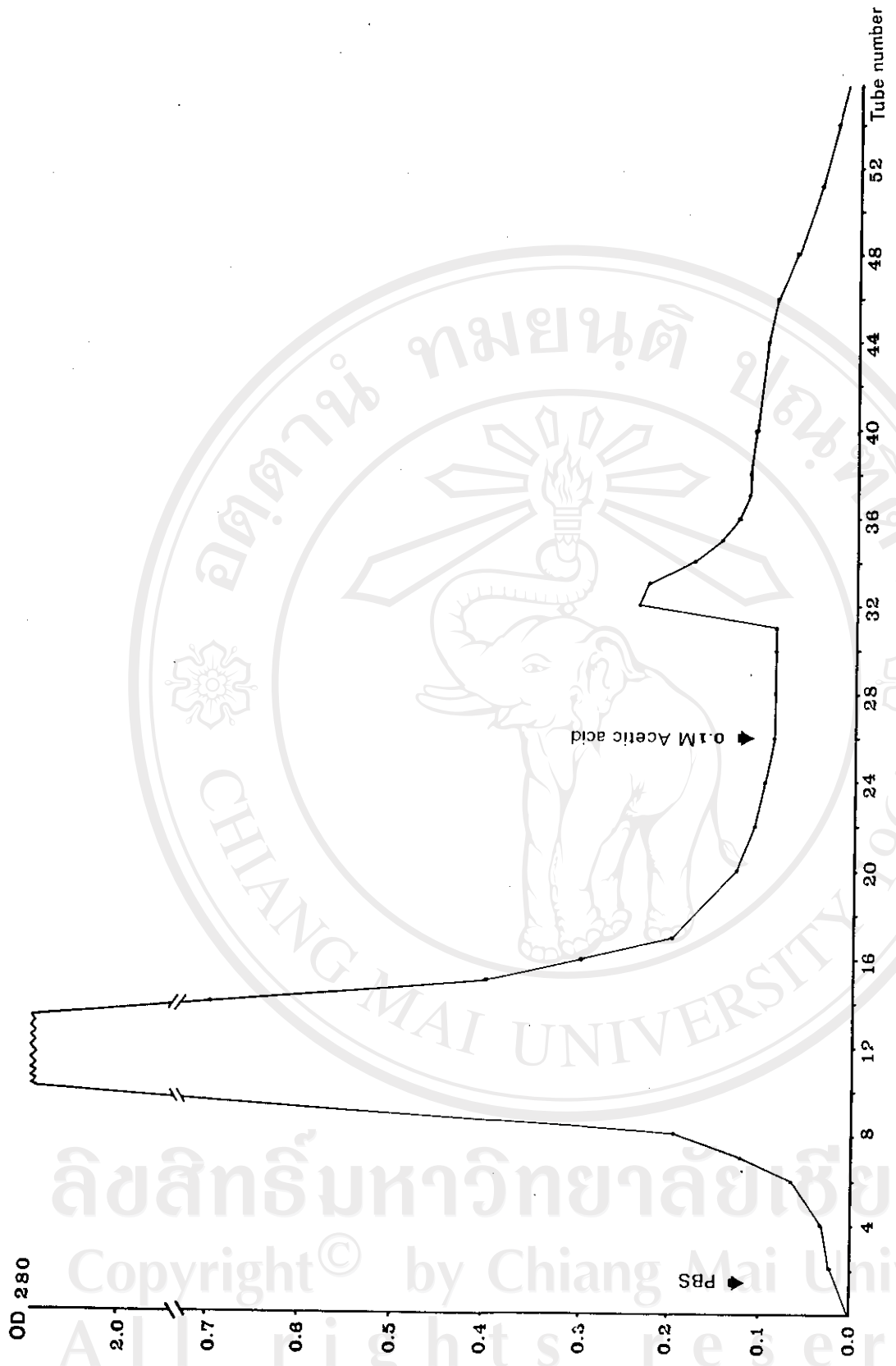

Figure 8 Affinity Chromatography of Crude WGA on OM-Sepharose 4B.

Table 3 Summary of Purification Steps of WGA.

Purification step	Total volume (ml)	Protein (mg/ml)	HA titer^{-1}	Specific activity*
Crude extract	1.5	6.5	16	2.4
OM-Sepharose 4B	6	0.2	128	640.0

* Specific activity = $\frac{\text{HA titer}^{-1}}{\text{mg/ml of protein}}$

Yield of WGA protein = 0.023 % of starting wheat germ

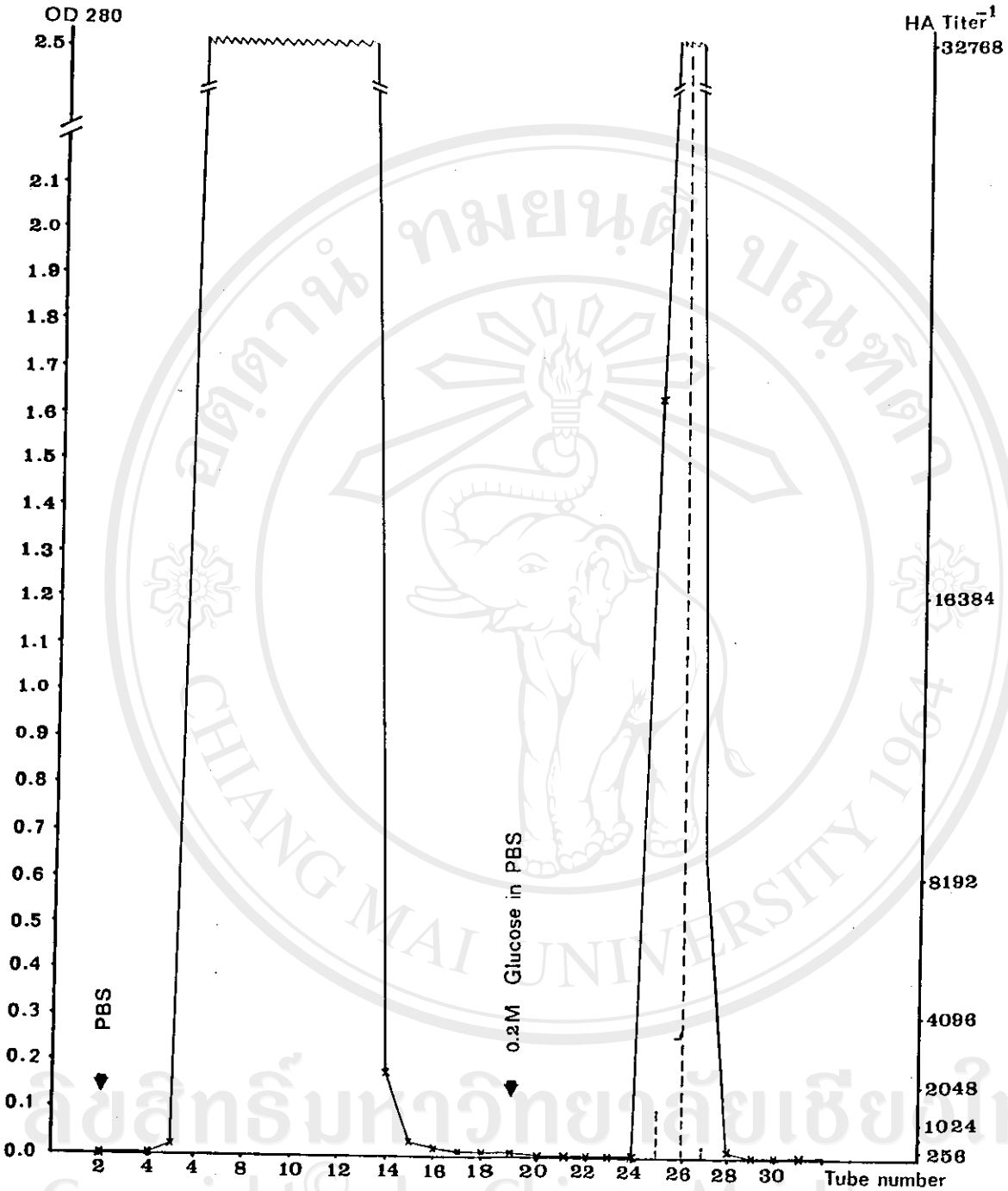

Figure 7 Affinity Chromatography of PSA on Sephadex G-100.
×——×O.D 280nm ; ----HA TITER^{-1}

The HA titer^{-1} values of the fraction were 1024, 32768, 256 (Figure.7). However, after standing in the room temperature for 1½ hr, the HA titer^{-1} values were reduced to 128, 512 and 128 respectively. This indicated the instability of PSA at room temperature. The summary of PSA purification is presented in Table 4.

III.5 Hemagglutinating Activity Assay

III.5.1 WGA

The protein concentration of the lection solution used in the HA assay was 7 mg/ml for crude WGA and 1 mg/ml for partially purified WGA. The HA titer^{-1} of crude and purified WGA were 8 and 128 respectively. The minimum protein concentration that caused the agglutination of human red blood cells was 45 µg from crude and 0.4µg for partially purified WGA. The agglutination potency of purified WGA was therefore about one hundred times higher than that of crude WGA.

III.5.2 PSA

The fractions which obtained from the second peak of the protein profile from Sephadex G-100 were dialyzed and then were brought for the HA assay. The HA titer^{-1} assayed was done at 4°C. The HA titer^{-1} obtained from the assay at RT was not stable, the positive agglutination could slowly altered to be a negative agglutination after the microtiter plate was left at room temperature longer than one hour.

Table 4 Summary of Purification Steps of PSA.

Purification step	Total volume (ml)	Protein (mg/ml)	HA $titer^{-1}$	Specific activity*
Crude extract	10	23.8	512	21.5
Sephadex G-100	15.6	1.26	32,768	26,006

Yield of PSA = 0.47 % of pea seeds

* Specific activity = $\frac{\text{HA titer}^{-1}}{\text{mg/ml of protein}}$

Table 5 The Comparison of Different Lectin Preparations.

Lectin preparations	sugar specificity	Minimum concentration of 1 HA (µg of protein/100 µl)
WGA (1^{st} experiment)	Sialic acid, GlcNAC and GalNAC	0.010
WGA (2^{nd} experiment)		0.037
LcH	glucose and mannose	1.56
PSA	glucose and mannose	0.40
TGA	Sialic acid	0.002

The HA assay, using fresh human red blood cells (group O, 0.4% suspension) was done by the same procedure (Section II.7)

III.5.3 LcH

The HA titer^{-1} of purified LcH (2 mg/ml protein) was 64. The effects of room temperature and 4°C on HA titer^{-1} were compared, It was shown that the suitable temperature for LcH assay was at 4°C.

III.5.4 IFV

The suspension of influenza virus used in this experiment was the mixture of 3 viral strains A/Phillippines /2/82 (H3N2), A/Bangkok/10/83 (H1N1) and B/Singapore/222/79 the HA titer^{-1} was 38,400. The viruses suspensions on dilution must be freshly prepared just before use. It was neccessary to do all the steps of the HA assay at 4°C to prevent the hydrolysis of sialic acid on red blood cells due to the action of active viral neuraminidase. Influenza virus particles caused hemagglutination by specific binding with sialic acid or sialoglycoproteins on surface red blood cells.

III.5.5 Horse Shoe Crab Lectin.

The clear supernatant of hemolymph after declotting and centrifugation was brought for the study. The HA titer^{-1} of the sample was 2048 (57.6 mg/ml protein). The lectin solution should be freshly prepared before use. Since the agglutination activity depends upon Ca^{2+} ions, all reagents were in Tris-HCl buffer containing 0.01 M $CaCl_2$. There was no effect of ambient temperature on HA or HAI assays, the experiment was therfore done at room temperature. The minimum protein of the crude lectin that caused the red cell agglutintion was 0.056 mg/well.

III.6 Hemagglutination Inhibition Assay

III.6.1 The WGA HAI titer^{-1} values of normal control samples varied between 32 to 128 ($\bar{X}$+SD = 72±29.79) and of the cancer serum samples from 32 to 256 ($\bar{X}$±SD = 139.29±82.31) (Table 6). The HAI titer^{-1} levels of cancer samples were statistically significant higher than those of the control as well as their serum NANA concentrations. In the experiment. the serum samples obtained from normal healthy subjects and breast cancer patients were warmed at 80 °C for 1 hr before HAI test. The HA inhibitory effect of serum from normal healthy subject increased about 8-10 folds where as appoximately 25 folds was observed in serum from cancer patients (Table 7). The frequencies of WGA HAI titer^{-1} of serum samples from healthy subjects and cancer patients are compared in Table 8. It was observed that the cancer serum samples both untreated and heated ones gave more frequencies of higher HAI titer^{-1} levels than the normal ones.

III.6.2 The obtained PSA-HAI titer^{-1} values normal control samples were between 80 to 320, and of cancer serum samples between 80 to 640 (Table 9 and 10). These results were read immediately after 4°C incubation with red cells. If the incubated plate was further left at room temperature, it appeared that the HAI titer^{-1} levels were slowly increased. The agglutinating red blood cells were separated

Table 6 The HAI Titer^{-1} of WGA and Concentration of Sialoglycoproteins as Sialic Acids in Serum Samples From Healthy Persons and Cancer Patients.

Sample source	Sample number	Serum NANA concentration (mg%)	HAI titer^{-1}
Normal healthy subjects	1	45.5	64
	2	54.5	64
	3	56.4	64
	4	56.4	128
	5	49.1	64
	6	51.8	64
	7	56.4	64
	8	58.2	128
	9	42.7	32
	10	45.4	32
	11	54.5	64
	12	52.7	128
	13	54.2	64
	14	49.1	64
	15	52.7	64
	16	56.1	64
		$\bar{X}\pm SD = 52.23\pm4.62$;	$\bar{X}\pm SD = 72.0\pm29.79$
Cancer patients	1	65.0	128
	2	71.0	64
	3	74.0	64
	4	73.0	64
	5	76.5	128
	6	74.0	128
	7	84.0	256
	8	69.0	64
	9	78.0	256
	10	81.0	256
	11	59.0	64
	12	57.0	64
	13	74.0	256
	14	76.0	256
	15	62.0	128
	16	54.0	64
	17	69.0	128
		$\bar{X}\pm SD = 70.38\pm8.48$;	$\bar{X}\pm SD = 139.29\pm82.31$

* The sialic acid content was determined by ion-exchange and resorcinol method.

Table 7 The HAI titer^{-1} of WGA and Concentration of Sialoglycoprotein expressed as Sialic Acids in 80 °C Heated Serum Sample From Normal Healthy Persons and Cancer Patients.

Sample source	Sample number	Serum NANA concentration (mg%)	HAI titer^{-1}
Normal healthy persons	1	45.4	1600
	2	54.5	800
	3	56.4	800
	4	56.4	800
	5	49.1	800
	6	51.8	1600
	7	56.4	400
	8	58.2	400
	9	42.7	800
	10	45.4	800
	11	54.5	400
	$\bar{X}$+SD = 51.89±5.39 ;		$\bar{X}$+SD = 836.36±417.78
Cancer patients	1	80.0	3200
	2	85.0	3200
	3	80.0	3200
	4	67.5	3200
	5	111.2	1600
	6	92.5	3200
	7	82.5	1600
	8	101.2	1600
	9	75.0	3200
	10	79.0	6400
	11	83.7	1600
	12	83.7	6400
	13	108.7	3200
	14	76.2	6400
	15	68.7	6400
	16	75.0	3200
	17	73.7	800
	18	108.7	3200
	19	96.2	3200
	20	82.5	3200
	21	75.0	3200
	22	75.0	800
	23	72.5	3200
	24	71.2	1600
	25	77.5	3200
	26	103.7	3200
	27	111.2	12800
	28	77.5	3200
	29	83.7	6400
	30	72.5	3200
	$\bar{X}$+SD = 83.36±13.36 ;		$\bar{X}$+SD = 3626.66±2365.11

Table 8 Frequencies of WGA-HAI Titer^{-1} Levels of Serum Samples From Normal Healthy Persons and Cancer Patients.

1) The data are derived from Table 6

HAI titer^{-1}	Number of control samples	Number of cancer samples
32	2(12.50%)	-
64	11(68.75%)	7(41.18%)
128	3(18.75%)	5(29.41%)
256	-	5(29.41%)

2) The data are derived from Table 7

HAI titer^{-1}	Number of control samples	Number of cancer samples
400	3(27.27%)	-
800	6(54.54%)	2(6.66%)
1600	2(18.18%)	5(16.66%)
3200		17(56.66%)
6400		5(16.66%)
12800		1(3.33%)

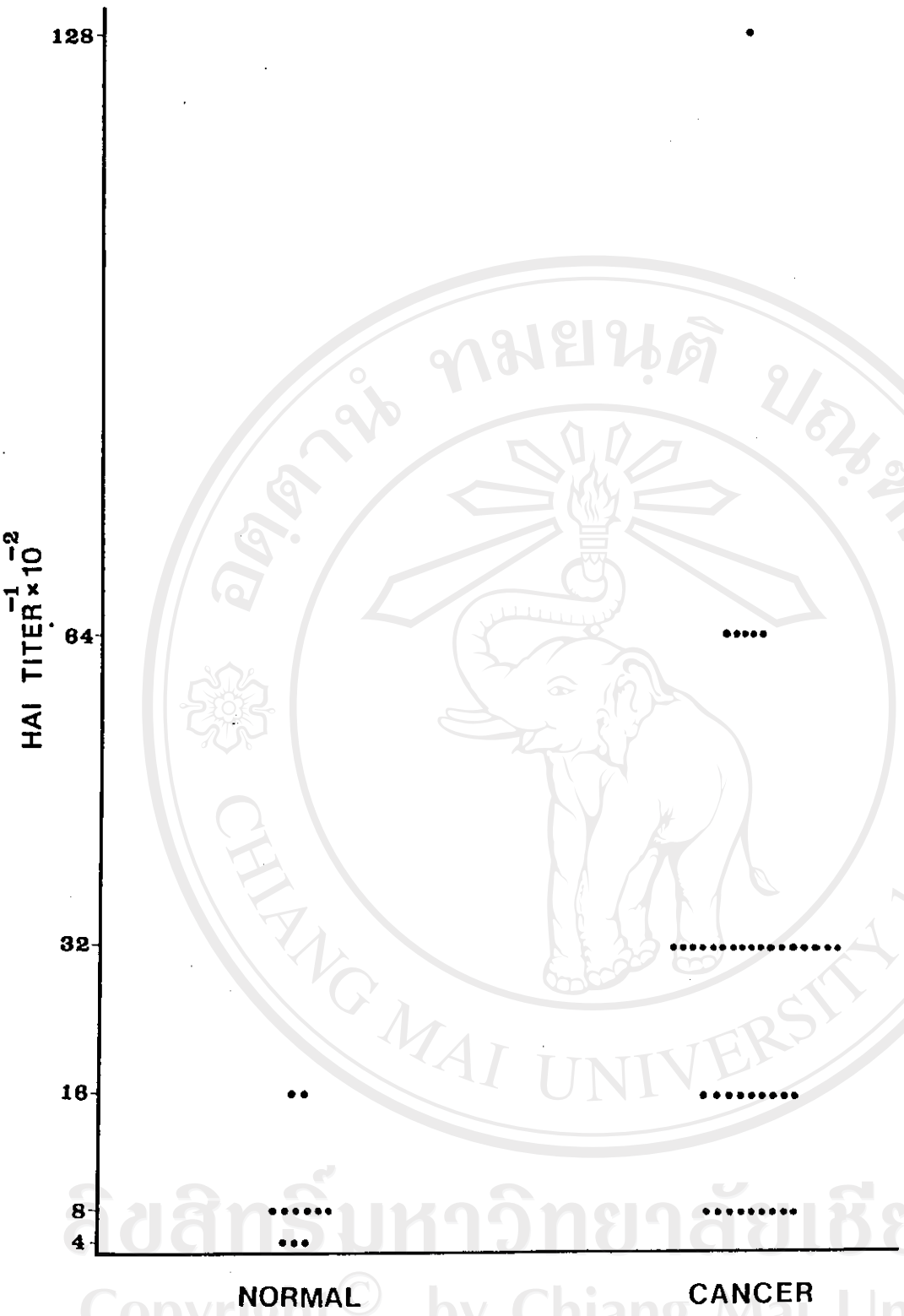

Figure 8 Distribution Diagram of WGA-HAI Titer^{-1} of Human Serum Samples From Normal Healthy Subjects and Cancer Patients. Each point (•) represents different individual sample.

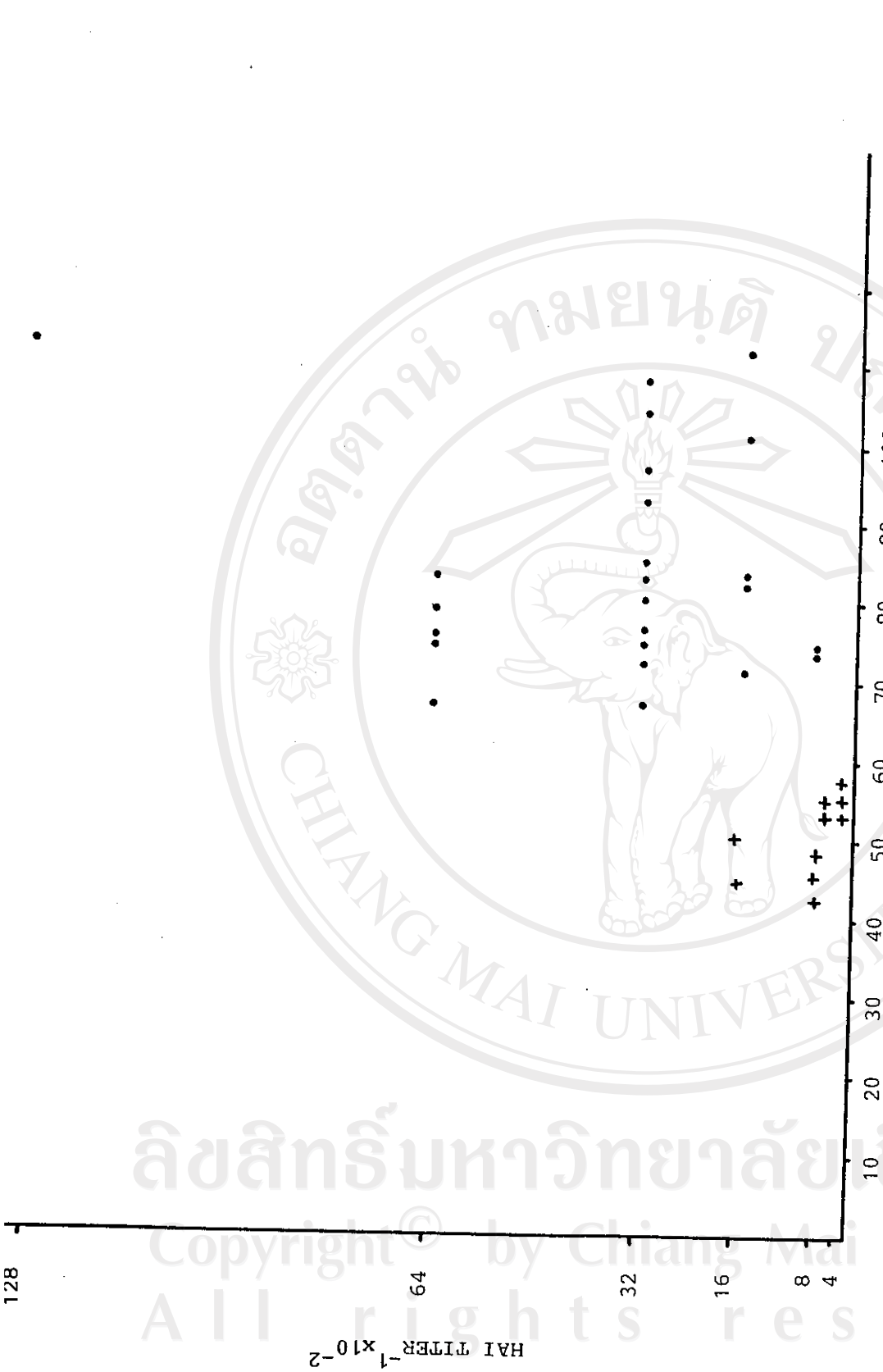

Figure 9 The Relationship Between WGA-HAI Titer^{-1} and Serum Sialoglycoprotein. Samples were obtained from normal healthy subjects (+) and cancer patients (•). Each point represents different individual sample.

from each other and become a red dot at the well bottom. The contamination of hydrolytic enzyme such as proteases, glycosidases may be the cause. There was no significant different in PSA-HAI titer^{-1} between the control samples and the cancer ones ($p < 0.01$).

III.6.3 The CCH-HAI titer^{-1} values of heated normal serum ranged from 160 to 320 at RT and from 640 to 1280 at 4°C. The HAI titer^{-1} values of a heated serum from cancer patients were between 80 and 320 at RT and 320 to 1280 at 4°C (Table 12). The HAI titer^{-1} of both normal and cancer serum samples assayed at RT were evidently lower than those obtained at 4°C. The end point agglutination of LcH could be clearly visualized after at least four and a half hour.

III.6.4 Table 14 shows the influenza viral HAI titer^{-1} of normal serum samples (N=17). The samples were preheated in two conditions: at 56°C 30 min and at 80°C, 1 hr, the untreated samples were used as the control. It showed that the heated treatments caused the increase of viral HA inhibitory activity of serum samples. The treatment at 80°C gave much stronger inhibitory effect than that treated at 56 °C. Table.15 shows the similar heating effect on the viral HI titer^{-1} using serum samples from cancer patients (N=35). The treatment at 80°C clearly gave the highest HI titer^{-1}, while treatment 56°C gave the slightly higher values than those obtained from the untreated corresponding samples. The distribution of HI titer^{-1} values of normal

Table 9 Comparison of Sialic Acid Concentration in Control Serum Samples and Their PSA-HAI Titer^{-1}

Sample number	Serum NANA concentration (mg%)	HAI titer^{-1}
1	51.4	160
2	48.8	320
3	41.2	320
4	45.9	320
5	42.2	160
6	49.0	160
7	47.9	320
8	34.5	160
9	36.7	320
10	43.1	320
11	45.0	160
12	34.5	80
13	53.6	160
14	39.3	160
15	42.1	320
	$\bar{X}\pm SD = 43.68\pm42.1$;	$\bar{X}\pm SD = 229.37\pm90.04$

Table 10 Comparison of Sialic Acid Concentration of Cancer Serum Samples and Their PSA HAI Titer^{-1}

Sample number	Serum NANA concentration (mg%)	HAI titer^{-1}
1	78.0	320
2	74.0	160
3	81.0	320
4	76.5	320
5	59.0	160
6	62.0	320
7	74.0	320
8	73.0	320
9	54.0	320
10	69.0	640
11	65.0	320
12	42.0	160
13	40.0	160
14	51.0	160
15	60.0	160
16	38.0	320
17	34.0	160
18	37.0	160
19	42.0	80
20	40.0	320
21	35.0	160
22	30.0	160
23	64.5	320
24	73.5	320
25	42.0	320
26	44.0	320
27	61.8	160
28	40.8	640
29	52.4	320
30	42.0	320
31	67.0	160
32	43.3	320
33	34.0	160
34	40.0	160
35	73.0	320
36	78.0	160
37	38.0	160
38	36.5	80
	$\bar{X}\pm SD = 53.82\pm16.03$;	$\bar{X}\pm SD = 256.84\pm124.99$

Table 11 Frequencies of PSA-HAI Titer^{-1} Levels of Serum Samples From Normal Healthy Persons and Cancer Patients. The data derives from Table 10

HAI titer^{-1}	Control samples	Cancer samples
80	1(6.66%)	2(5.26%)
160	7(46.66%)	16(42.10%)
320	7(46.66%)	18(46.37%)
640	-	2(5.26%)

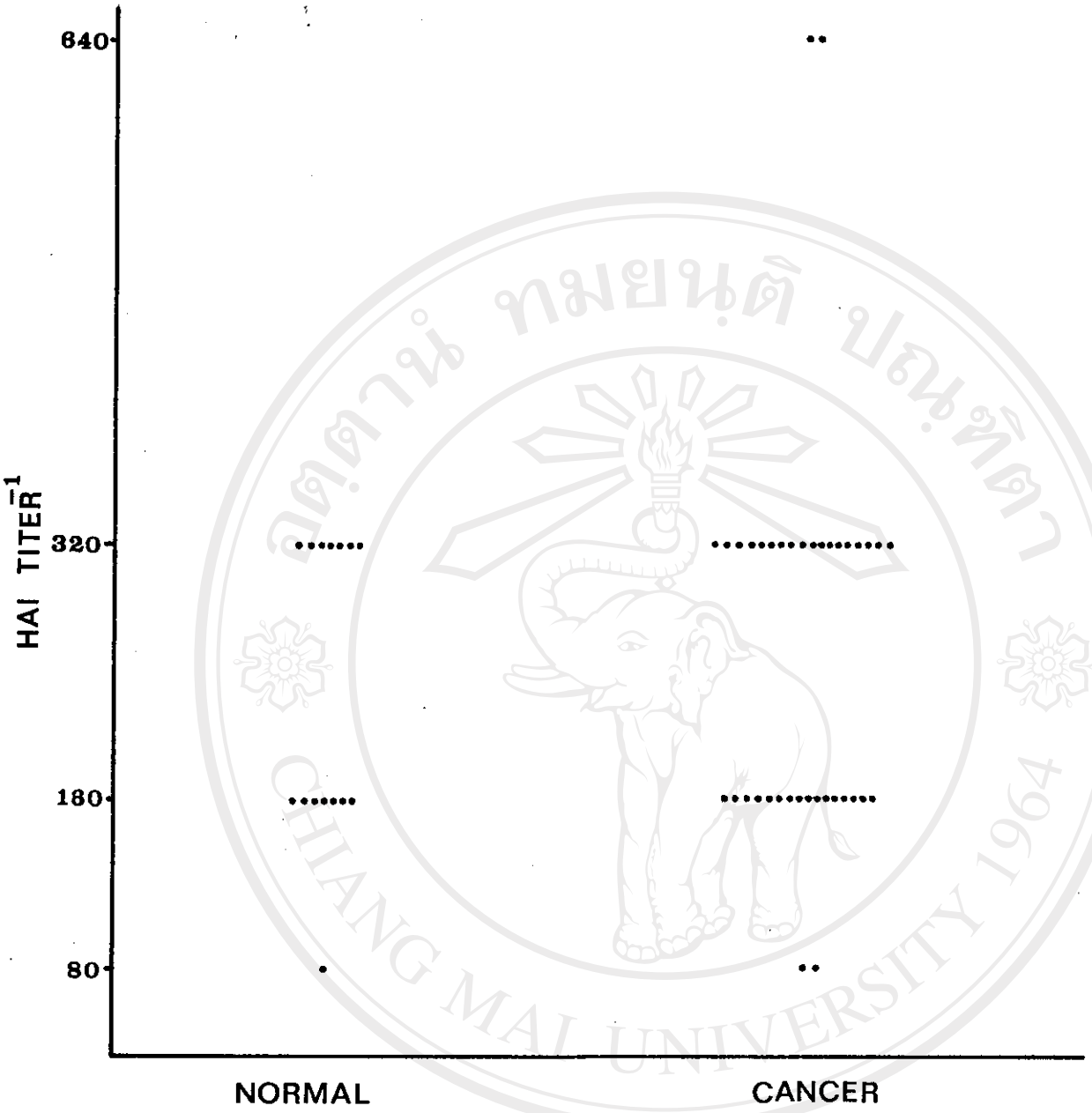

Figure 10 Distribution Diagram of PSA HAI Titer^{-1} of Human Serum Samples from Normal Subjects and Cancer Patients. Each point (•) represents different individual sample.

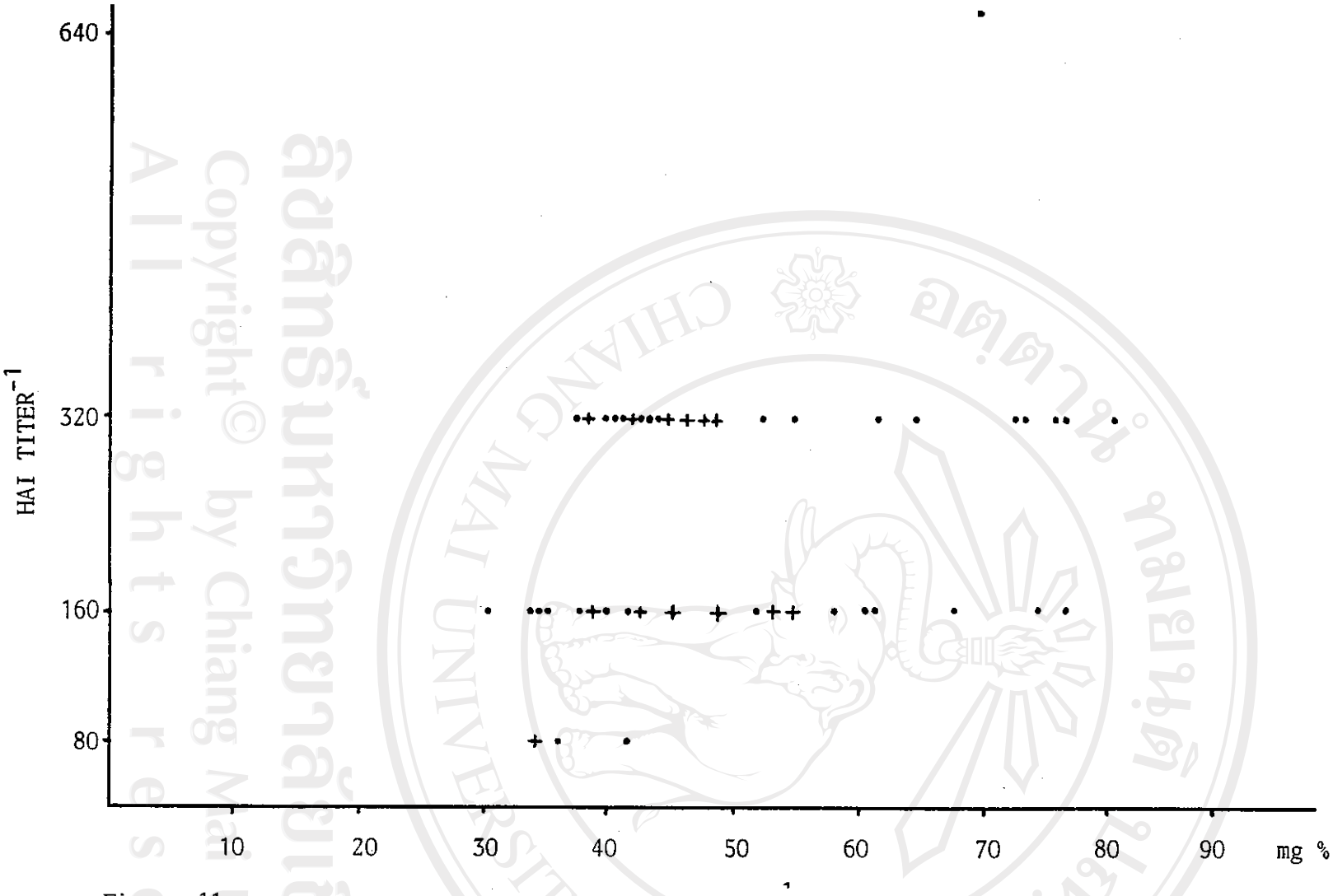

Figure 11 Figure 11 The Relationship Between PSA-HAI Titer^{-1} and were resents Serum Sialoglycoprotein. Samples were obtained from normal healthy subjects (+) and cancer patients (•). Each point represents individual samples .

Table 12 Effect of Agglutination Temperature on LcH-HAI Titer^{-1} of Heated Serum From Normal Subjects and Cancer Patients.

Sample number	Agglutination temperature		NANA
	Room temperature (RT) at 28 °C	4 °C	mg %
Normal serum samples			
1	320	640	45.4
2	160	1280	54.5
3	160	640	56.4
4	160	640	56.4
5	320	640	49.1
	$\bar{x}$±SD = 224±87.63	$\bar{x}$±SD = 768±286.22	52.38±9.87
Cancer serum samples			
1	320	320	69.0
2	320	1280	68.3
3	80	640	78.4
4	160	1280	74.6
5	160	1280	89.6
	$\bar{x}$±SD = 208±107.33	$\bar{x}$±SD = 960±452.55	75.98±8.67

and cancer serum samples after heating treatment is shown in Figure.12. Each point represents individual serum sample. Most of higher HAI titer^{-1} values of normal serum samples were in lower levels (1680-3200) while those of the cancer samples were evidently high (3200-12800). The relationship between serum sialoglycoprotein concentration and HAI titer^{-1} values is shown in Figure 13. The HAI titer^{-1} values are closely related to and clearly depended upon the concentration of serum sialoglycoprotein. Most of cancer serum samples that showed increased concentration of sialoglycoprotein also exhibited higher inhibitory effect to IFV-HA. Average serum sialoglycoprotein concentration in cancer patients was significantly higher than of normal subjects ($p < 0.01$).

III.6.5 Horse-shoe Crab Lectin

The data shown in Table 16 and 17 show the TGA HAI titer^{-1} of serum samples from cancer patients were markedly higher than that of the normal serum samples ($p < 0.01$). The highest HAI titer^{-1} of serum samples from cancer patients and from normal subjects were 40960 and 2560 respectively. The distribution diagram of individual HAI titer^{-1} values is shown in Figure 14. Most of HAI titer^{-1} level of normal serum were lower than 2560 while the HAI titer^{-1} of serum from cancer patients scattered in higher levels (2560). The serum sialoglycoproteins represented as total sialic scids (in mg% assayed by modified Ehrlich method, Sections II.13.2) of cancer samples were

Table 13 Effect of Heat Treatments on Influenza Virus HAI Capacity of Control Serum Samples.

Serum number	Serum NANA concentration (mg%)	HAI titer^{-1} Unheated serum	Heated serum at 56 °C	Heated serum at 80 °C
1	72.5	3200	3200	3200
2	61.5	3200	3200	3200
3	67.5	1600	1600	1600
4	81.2	3200	1600	6400
5	56.2	800	3200	3200
6	52.5	800	1600	1600
7	56.2	800	1600	1600
8	63.7	1600	3200	6400
9	80.0	1600	3200	6400
10	75.0	1600	1600	3200
11	61.8	800	1600	1600
12	65.0	800	3200	3200
13	65.0	800	3200	6400
14	72.5	800	3200	6400
15	70.0	1600	800	12800
16	50.0	1600	800	3200
17	54.0	1600	3200	3200
$\bar{X}\pm SD$ =	64.99±9.40;	1552.94±870.43;	2352.94±957.93 ,	4329.41±2871.36

Table 14 Effect of Heat Treatments on Influenza Virus HAI Capacity of Serum Samples From Cancer Patients.

Serum number	NANA (mg%)	Unheated serum	Heated serum at 56 °C	Heated serum at 80 °C
1	75.0	1600	3200	12800
2	73.7	800	1600	6400
3	96.2	1600	1600	6400
4	75.0	1600	3200	12800
5	77.5	3200	3200	12800
6	65.0	1600	3200	12800
7	68.7	1600	6400	12800
8	60.2	800	1600	12800
9	75.0	1600	1600	12800
10	82.5	800	1600	6400
11	76.2	1600	3200	6400
12	83.8	800	1600	6400
13	108.8	1600	3200	12800
14	101.2	400	1600	6400
15	82.5	800	1600	6400
16	82.5	800	1600	12800
17	92.5	3200	3200	6400
18	83.8	800	3200	6400
19	96.2	800	3200	12800
20	108.8	800	6400	12800
21	111.2	800	1600	12800
22	83.8	800	3200	6400
23	85.0	800	3200	12800
24	67.5.	400	3200	6400
25	75.0	800	3200	6400
26	70.0	800	3200	6400
27	77.5	800	3200	6400
28	103.8	800	3200	6400
29	71.2	800	800	12800
30	77.5	800	3200	12800
31	72.5	800	3200	6400
32	72.5	1600	1600	6400
33	80.0	1600	6400	12800
34	80.0	1600	6400	12800
35	111.2	1600	6400	6400
$\bar{X}\pm SD$ =	82.96±13.96	1120.00±547.08;	3085.71±1578.00;	9417.14±3377.91

Table 15 Frequencies of the Influenza Virus HAI Titer^{-1} Levels of Serum Samples Heated at Different Temperature. The data are derived from Tables 13,14.

FV-HAI Titer^{-1}	Number of serum samples corresponding to HAI titer^{-1}					
	Unheated samples		Heated at 56 °C		Heated at 80 °C	
	Control	Cancer	Control	Cancer	Control	Cancer
400	-	2(5.71%)	-	-	-	-
800	7(41.17%)	19(54.28 %)	2(11.76%)	1(2.85%)	-	-
1600	7(41.17%)	12(34.28%)	6(35.29%)	11(31.42%)	4(23.53%)	-
3200	3(17.64%)	2(5.71 %)	9(52.94%)	18(51.43 %)	7(41.17%)	
6400	-	-	-	5(14.28 %)	5(29.41%)	18(51.43 %)
12800	-	-	-	-	1(5.88%)	17(48.57%)

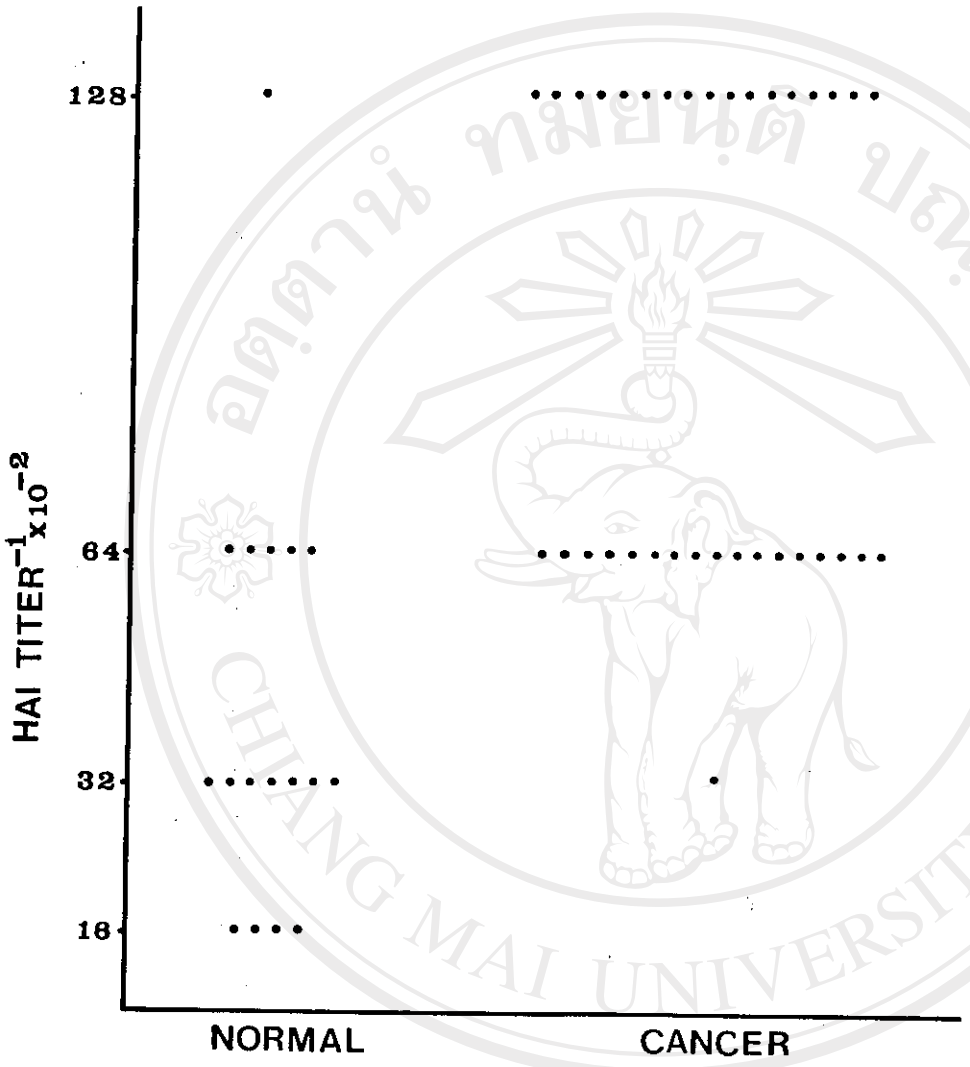

Figure 12 Distribution Diagram of lFV-HAI Titer^{-1} of Human Serum Samples. From Normal Healthy Subjects (+) and Cancer Patients (•). Each point represents different individual sample.

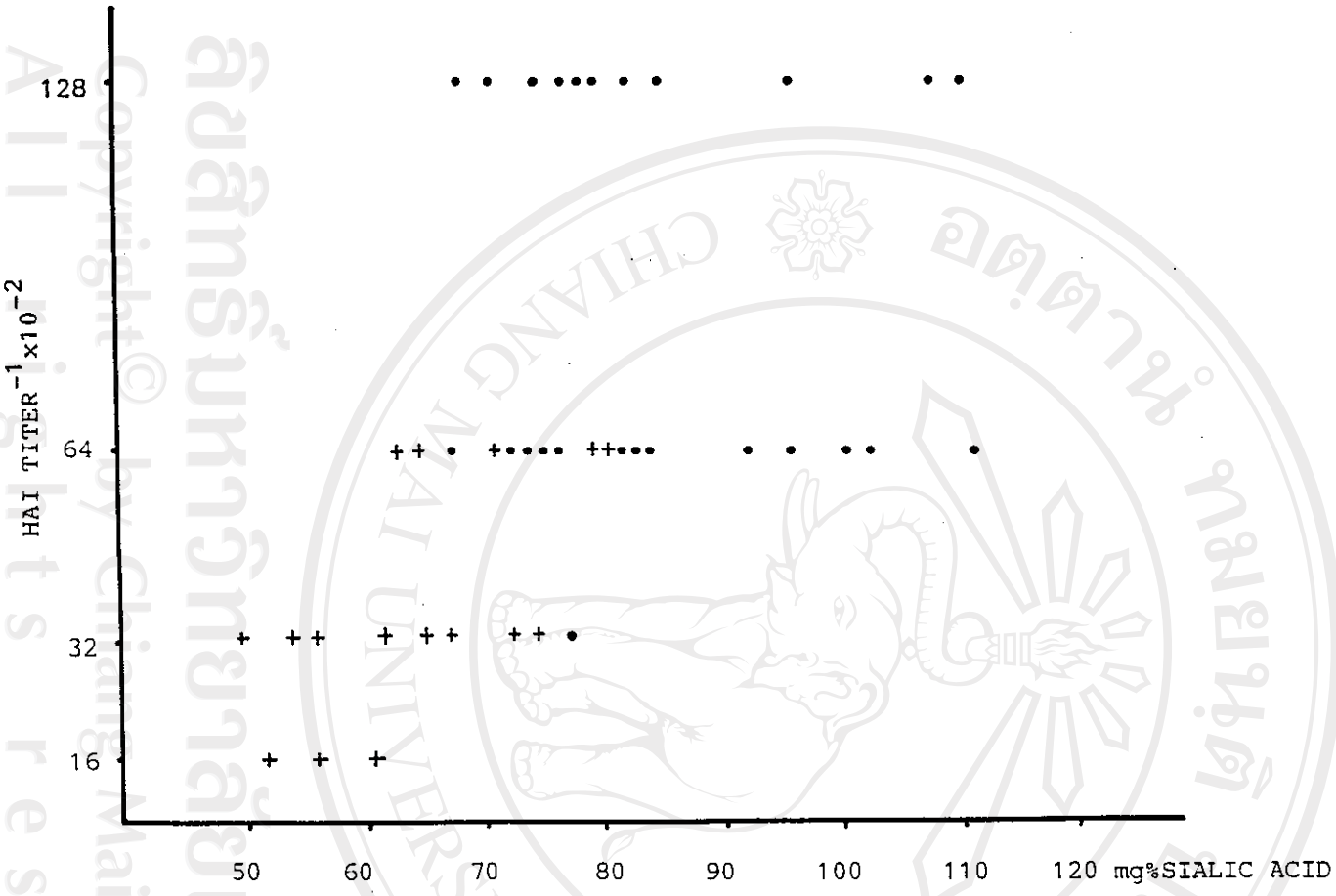

Figure 13 The Relationship Between lFV-HAI Titer^{-1} and Serum Sialoglycoprotein. Serum were obtained from normal healthy subjects (+) and cancer patients (•). Each point represents differents individual sample.

significantly higher than the normal serum sample. The relationship between sialic acids concentration and corresponding HAI titer^{-1} is shown in Figure 15. It indicated that HAI titer^{-1} of the samples varied proportionally with sialic concentration. The higher HAI titer^{-1} values were closely related to the concentration of serum sialoglycoproteins.

III.6.6 Effect of Neuraminidase on the Specificity of WGA and Influenza Agglutinin

As shown in Table 19 (a), red blood cells previously treated with 10 U N'ase caused no agglutination with WGA while the normal red cells could be evidently agglutinated with WGA at a high HA titer^{-1} of 128.

Table 19(b), shows the effect of N'ase on the WGA hemagglutination inhibitory activity of CM. Ten units of N'ase could reduce a 50% of total HAI activity of CM, while 20 U of the enzyme completely abolished the inhibitory action of CM.

Using IFV in hemagglutination test, N'ase treatment of CM completely could destroyed the inhibitory activity of CM.

III.7 Purification of Hores Shoe Crab Lectin (TGA)

III.7.1 After mixing the hemolymph (9.0 ml) with 1.5 ml of packed formalinized red blood cells at 4°C for 2

Table 16 Relationship of Sialic Acid Concentration in Control Serum Samples and Their Horse Shoe Crab HAI Titer^{-1}

Serum number	NANA (mg %)	HAI titer^{-1}
1	38.0	640
2	44.0	1280
3	39.0	1280
4	30.0	640
5	29.0	320
6	46.0	1280
7	33.0	640
8	37.0	640
9	30.0	1280
10	32.5	640
11	43.0	320
12	29.5	640
13	32.5	1280
14	31.0	1280
15	37.0	1280
16	34.0	640
17	37.0	640
18	39.0	1280
19	42.0	320
20	53.0	640
21	52.0	2560
22	44.0	2560
23	70.0	1280
	$\bar{X}\pm SD$ = 39.24±9.59	; $\bar{X}\pm SD$ = 1015.65±607.56

Table 17 Relationship of Sialic Acid Concentration and Their Horse Shoe Crab Lectin HAI Titer^{-1} of Cancer Serum Samples .

Serum number	NANA (mg %)	HAI titer^{-1}
1	67.0	5120
2	105.0	20480
3	67.5	5120
4	79.0	20480
5	69.5	10240
6	73.0	40960
7	72.0	40960
8	68.0	40960
9	73.5	5120
10	72.0	2560
11	57.0	2560
12	20.0	640
13	32.0	640
14	50.0	2560
15	58.0	2560
16	22.0	640
17	36.0	640
18	70.0	1280
19	42.0	2560
20	45.0	1280
21	40.0	2560
22	40.0	2560
23	38.0	1280
24	59.0	5120
25	68.0	20480
	$\bar{x}\pm SD = 56.94\pm19.96$;	$\bar{X}\pm SD = 9574.40\pm13325.23$

Table 18 Frequencies of Horse Shoe Crab Lectin HAI Titer^{-1} of Samples From Normal Healthy Subjects and Cancer Patients. The data are derived from Table 16,17.

HAI Titer^{-1}	Number of serum samples corresponding to HAI titer^{-1}	
	Control samples	Cancer samples
320	3(13.04 %)	-
640	9(39.13 %)	4(16%)
1280	9(39.13 %)	3(12%)
2560	2(6.06%)	7(28%)
5120	-	4(16%)
10240	-	1(4%)
20480	-	3(12%)
40960	-	3(12%)

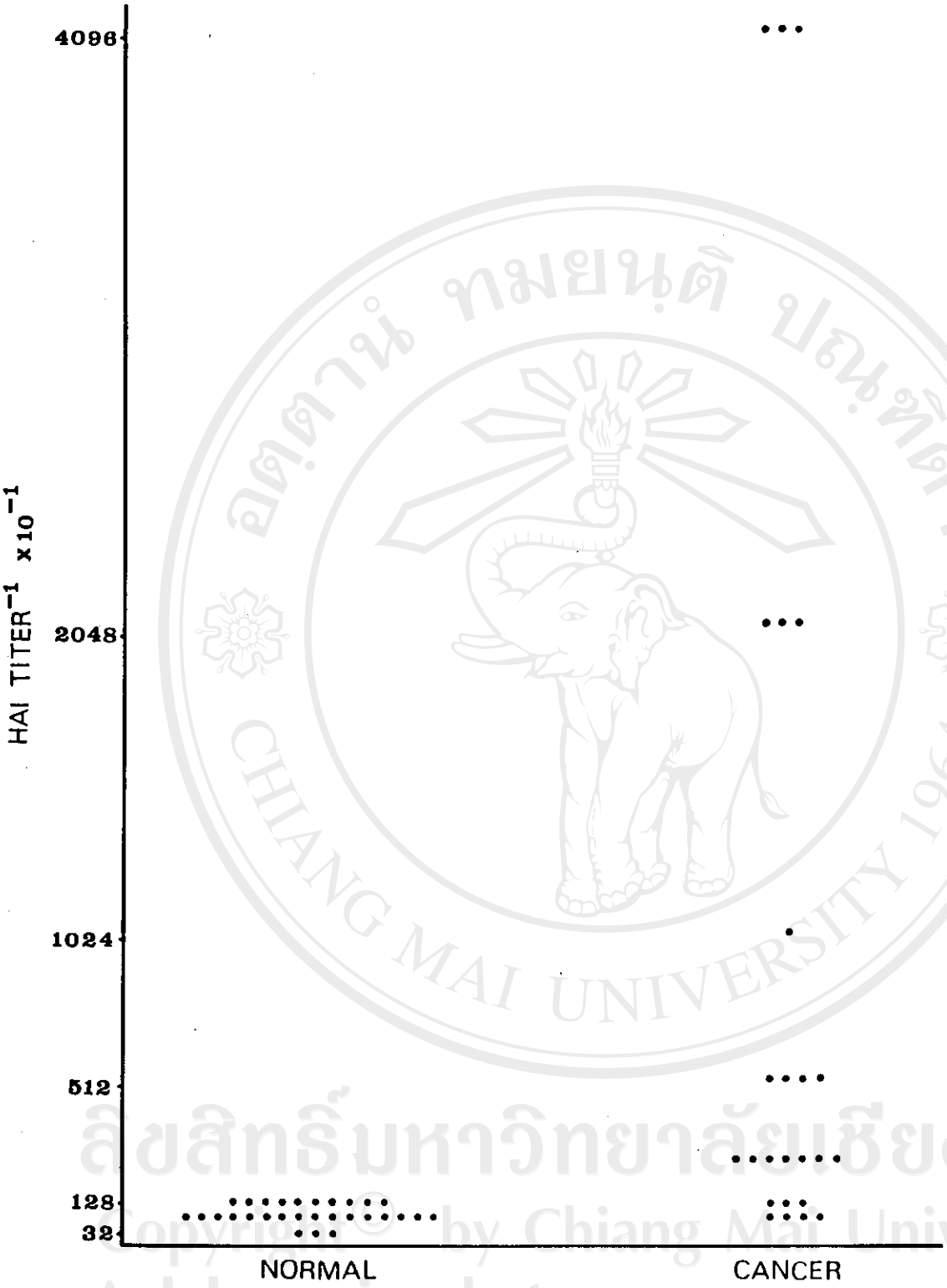

Figure 14 Distribution Diagram of the Hemolymph-HAI Titer^{-1} of Human Serum Samples From Normal Healthy Subjects and Cancer Patients. Each point (•) represents different individual samples.

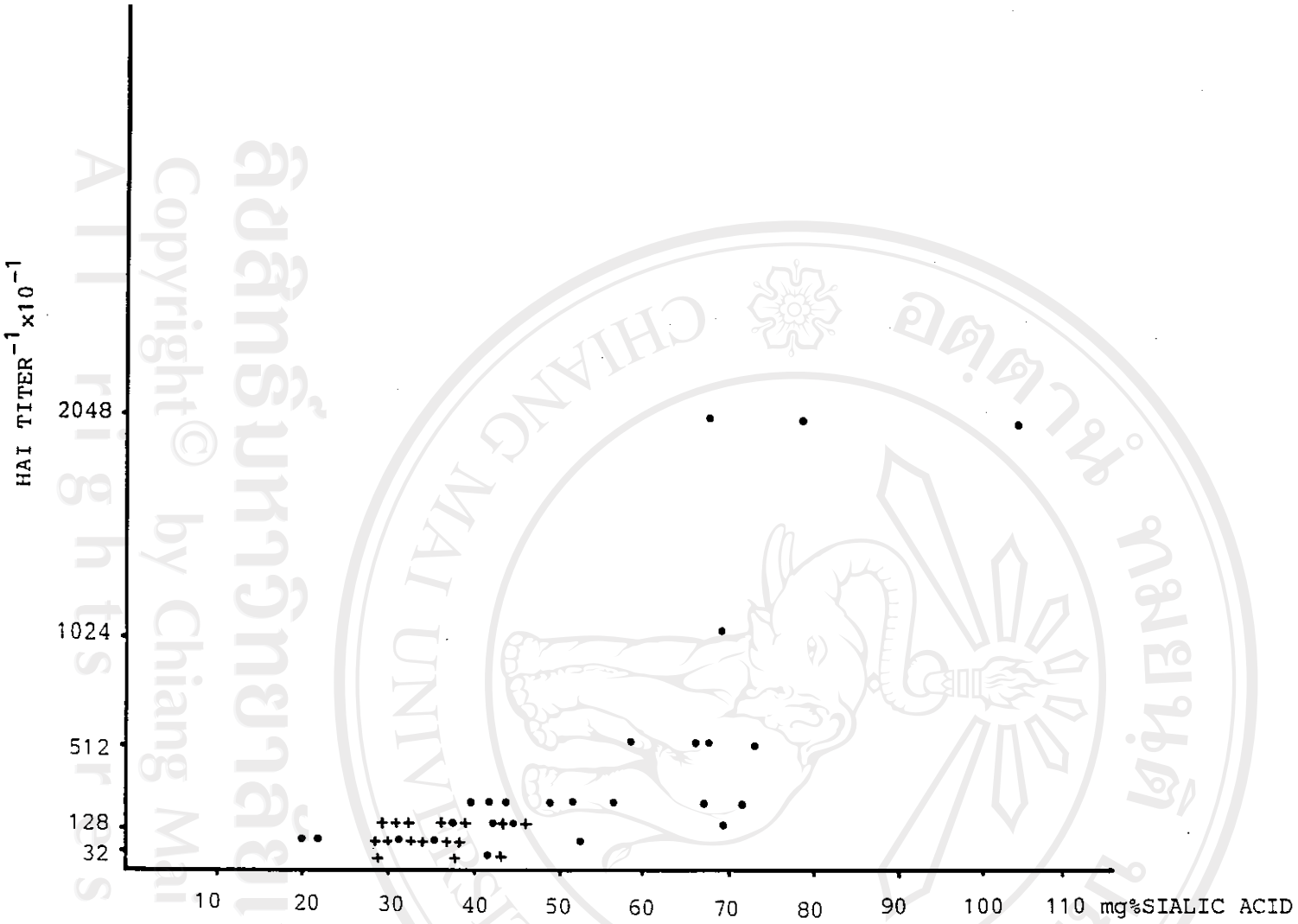

Figure 15 The Relationship Between the Hemolymph-HAI Titer^{-1} and Serum Sialoglycoprotein. Samples were obtained from normal healthy subjects (+) and cancer patients (•). Each point represents different individual sample.

Table 19(a) Effect of Neuraminidase Treatment of Human Red Blood Cells on Their Hemagglutination.

Tested cells	Treatment	WGA-HA titer^{-1}
Red blood cells	Without	128
Treated-Cells	with N'ase (10 U)	0

Table 19(b) Effect of Neuraminidase Treatment of CM on Its WGA-Hemagglutination Inhibition.

Tested inhibitor	Treatment	HAI titer^{-1}
Native CM	Without	64
Treated-CM	With N'ase 10 U	32
	With N'ase 20 U	0

Table 19(c) Effect of Neuraminidase Treatment of CM on Its Influenza Virus Hemagglutinin Inhibition.

Tested inhibitor	Treatment	HAI titer^{-1}
Native CM	Without	256
Treated-CM	With N'ase (20 U)	0

hr, the hemolymph was separated by centrifugation at 3,000 rpm 10 min. The protein concentration in the supernatant was determined by Coomassie brilliant blue method (micromethod; Section II.14.2). Table 20 shows that the lectin could be adsorbed on formalinized red cell. The protein concentration of hemolymph was reduced from 57.6 mg/ml to 44.8 mg/ml, and it's HA titer^{-1} also decreased from 512 to 64. The formalinized red cells was then twice washed with buffer A (Tris buffer with Ca^{2+} and low NaCl concentration) to eliminate the nonspecific proteins. The washing showed a little hemagglutinating activity. The red cells were again washed with buffer B, 5 times, the washings were collected by centrifugation. Their protein concentration and HA titer^{-1} were determined. It showed that small amount of protein was eluted into each washing and their HA activity could be detected. Their specific lectin activities were much higher. Fractions B_1-B_5 were pooled and then dialyzed against buffer A overnight at 4°C. However the HA titer^{-1} was then assayed again and found to be reduced.

III.7.2 After 17 hr incubation, the neuraminidase treatment of TGA-bound FRBC was stopped by centrifugation at 3,000 rpm, 10 min, and the supernatant was collected. The supernatant 5.0 ml, containing neuraminidase and free TGA was assayed for the lectin activity at room temperature. Its HA titer^{-1} was 256. After standing the sample at room temperature for about 7-8 hr, it was found that hemolysis of the agglutinated red cells occurred in the

Table 20 Summary of Purification of TGA .

Sample	Volume (ml)	Protein concentration (mg/ml)	HA Titer^{-1}	Specific activity***
Hemolymph before incubation	9.0	57.6	512	8.8
supernatant	9.0	44.8	64	1.4
Fractions eluted from FRBC by buffer A*				
A_1	4.0	2.6	2	0.8
A_2	4.0	1.4	0	0.0
Fractions eluted from FRBC by buffer B**				
B_1	4.0	0.7	256	355.5
B_2	4.0	0.3	32	106.6
B_3	4.0	0.4	32	80.0
B_4	4.0	0.6	32	53.3
B_5	4.0	0.1	32	320.0

* Buffer A = 0.05 M Tris-HCl buffer pH 7.8 containing 0.1 M NaCl and 0.1 M $CaCl_2$

** Buffer B = 0.05 M Tris-HCl buffer pH 7.8 containing 1.0 M NaCl and 0.1 M $CaCl_2$

*** Specific activity = $\frac{\text{HA titer}^{-1}}{\text{mg/ml of protein}}$

first three wells of HA the neuraminidase, which was hemolysis action on red cells. The enzyme had to be seperated from the lectin by further gel filtration step. The lectin-positive fractions were pooled and dialyzed against Tris-HCI buffer pH 7.8 contains 0.1 M $CaCl_2$ and 0.1 M NaCl and concentrated. The HA $titer^{-1}$ of the pooled fraction (0.75 ml) was 1024. Protein concentration, estimated by optical density at 280 nm, using bovine serum albumin as a standard, was 6.5 mg/ml. Its total volume was 0.75 ml. The yield was then 4.75 mg of protein.

III.8 Preparation of HRP-TGA Conjugates

The TGA preparation with high lectin activity was brought for the conjugation with HRP. HRP was to be first oxidized by periodate. The RZ of HRP used was initially 1.6 After periodate oxidation, the RZ of HRP-aldehyde product dropped to 0.6. However, the RZ of HRP-TGA conjugate rised up to 1.8 after coupling reaction. An excess HRP was seperated by Sephadex G-200 gel filtration. The elution pattern obtained showed two protein peaks measured as optical densities at 280 nm and at 403 nm (Figure 20). The enzyme and lectin activities aliquots from all the eluted fractions were assayed. Both the lectin activity and peroxidase activity could be found only under the first protein peak at the void volume, The highest HA $titer^{-1}$ of the eluted-fractions was 8. This fraction had also strong enzyme activity (RZ = 1.8). The large retarded protein peak

contained high amount of enzyme but no lectin activity and it was therefore assumed to be the excess peroxidase. The lectin and enzyme position fractions of the eluates were separately collected and further used for histological staining study.

III.9 Histochemical Staining

Breast and cervix cancer tissues were used for this conjugated-lectin staining study. The paraffin-embeded tissues specimens were prepared about 1-2 years ago by the routine laboratory procedure. After incubation with the HRP-TGA conjugates, the dark brown lining on cell membrance could be distinguishly seen (Figures 23a). Cancer cells exhibited a more density of the stained line than normal corresponding cells (Figure 23b). In some cancer tissue specimens, cytoplasm could be also stained and appeared as a brown area (Figure 23c). The negative control for staining was also done by using only the buffer and substrate mixture instead of the enzyme-conjugated lectin. No brown colour staining was observed in the control slide (Figure 21). Another negative control was incubated with neuraminidase to remove cell-surface sialic acid before it was simultaneously being stained with HRP-TGA conjugates. All the neuraminidase-treated specimens show negative staining neither on the cell membrance nor in the cytoplasm (Figure 22).

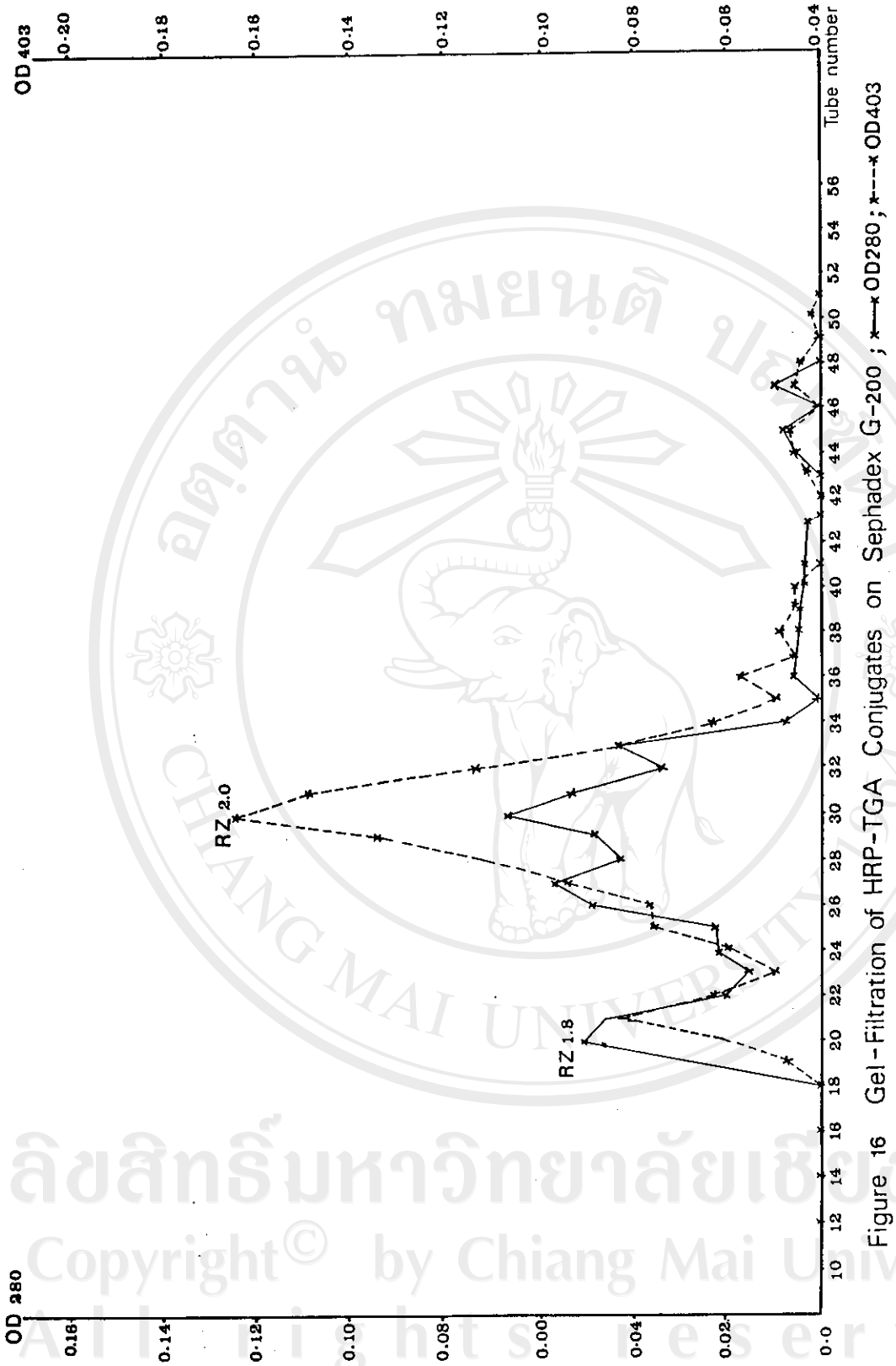

Figure 16 Gel-Filtration of HRP-TGA Conjugates on Sephadex G-200 ; ★—★ OD280 ; ★--★ OD403

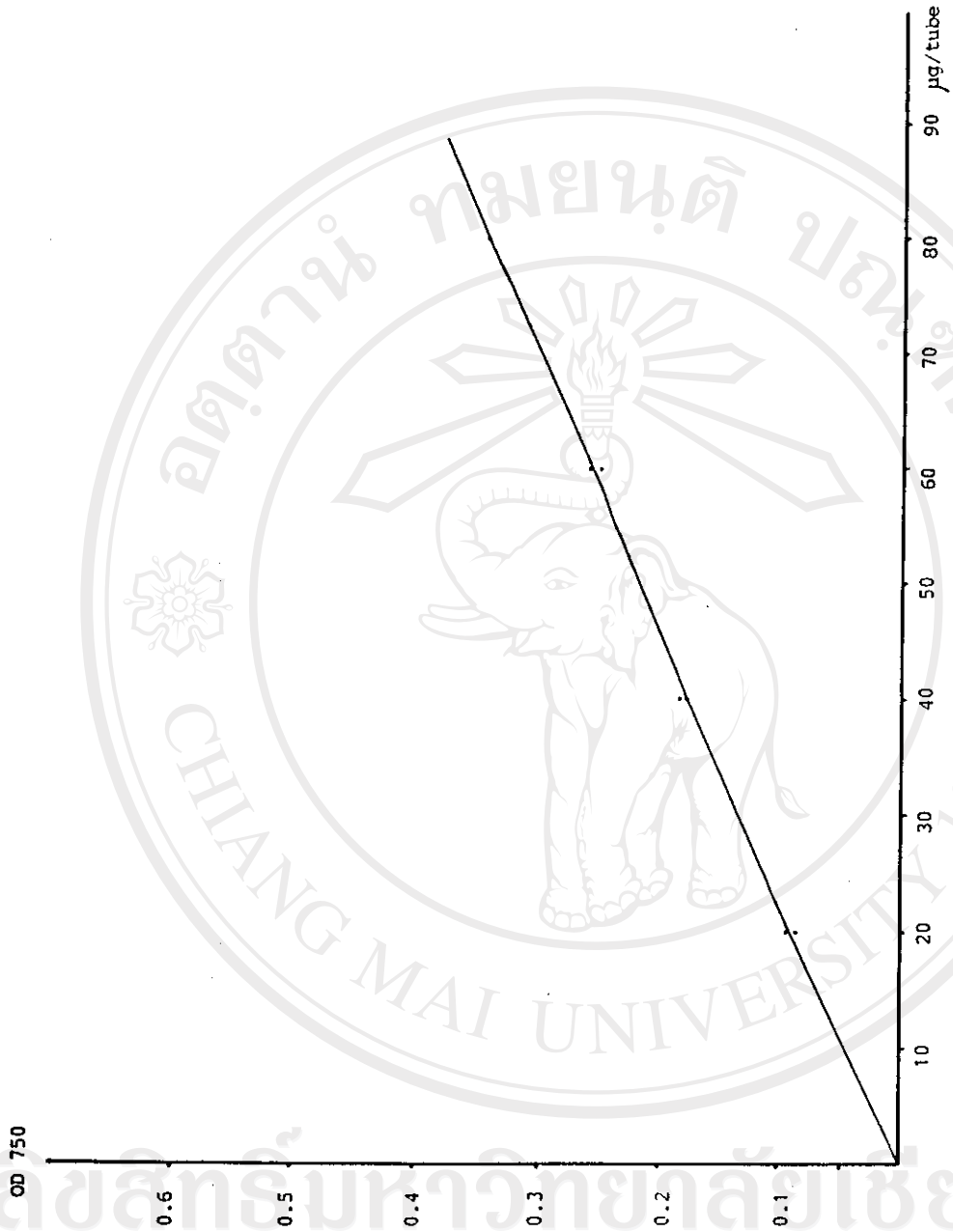

Figure 17 Standard Curve of BSA by the Lowry Method .

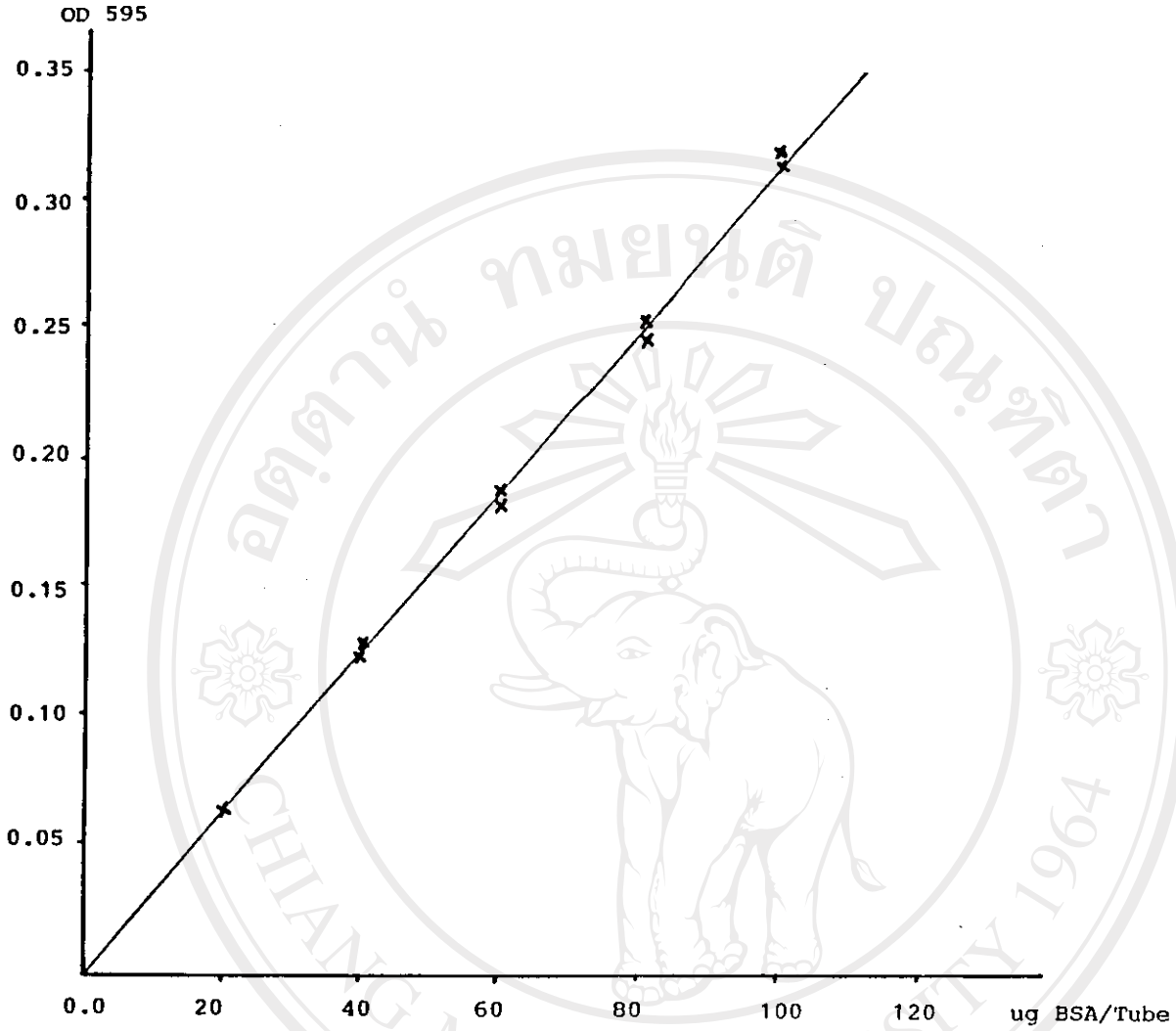

Figure 18 Standard Curve of BSA by Coomassie Brilliant-blue Method .

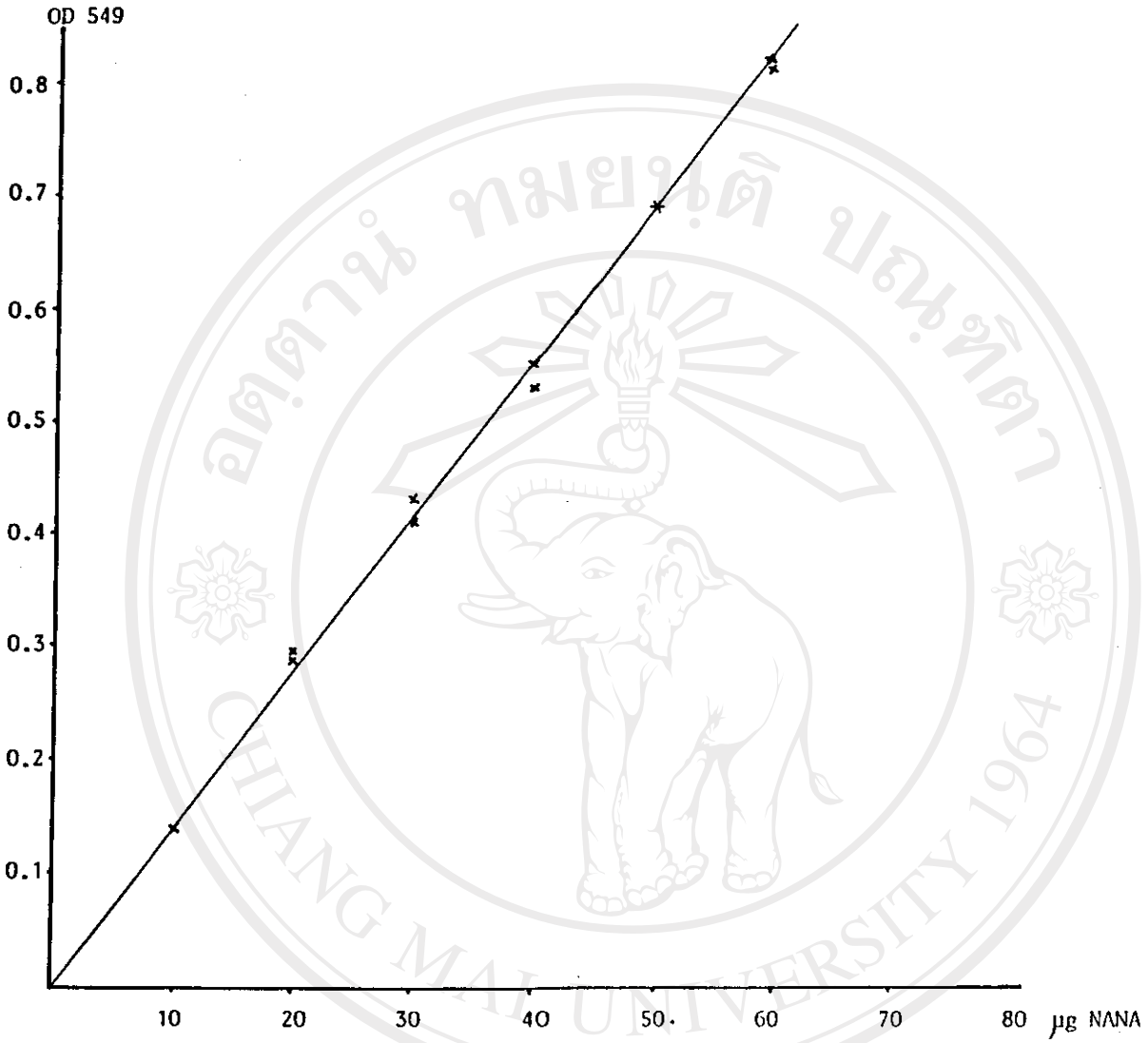

Figure 19 Standard Curve of Sialic Acid by TBA Method.

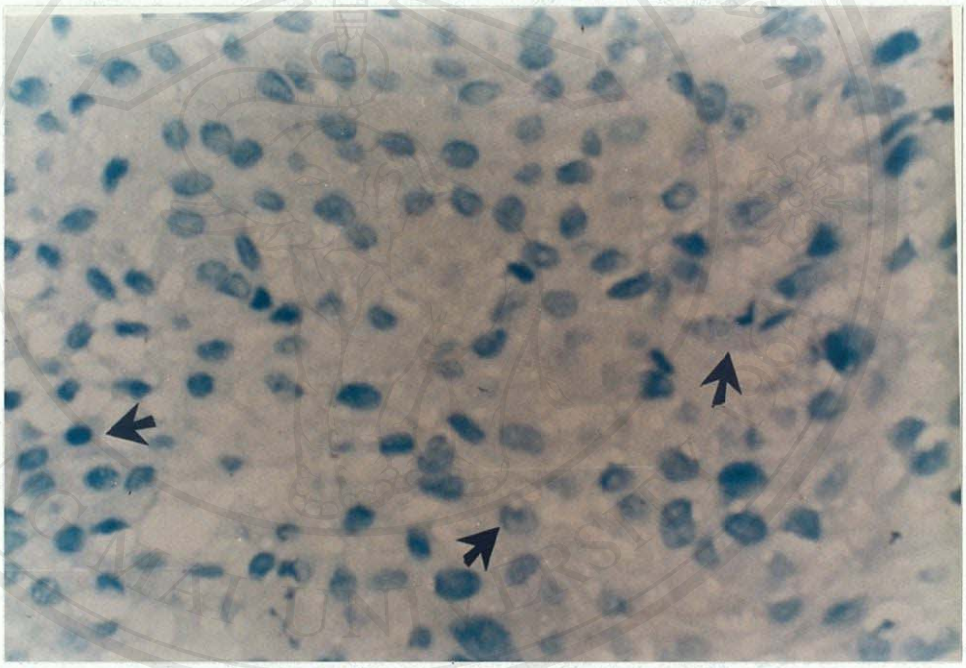

Figure 20 Light Micrograph of Paraffin Section of the Negative Control. The arrows show the cancer cells without positive stained lining on the cell membrane. Original Magnification x400.

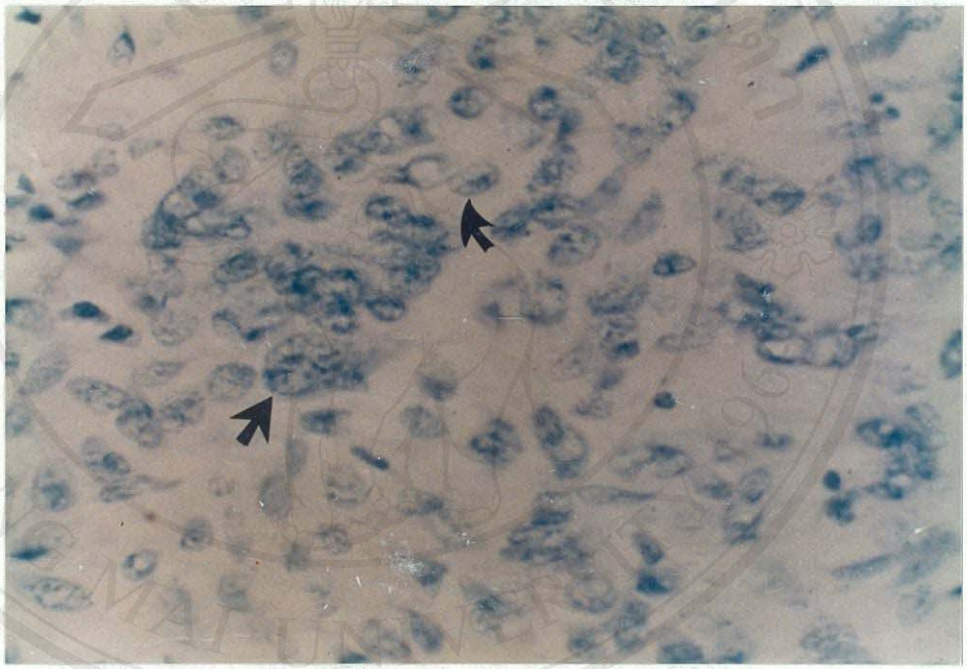

Figure 21 Paraffin Section of Neuraminidase-treated Cancer Tissues as Negative Control. The arrow shows the cancer cell membrane. Original Magnification x400.

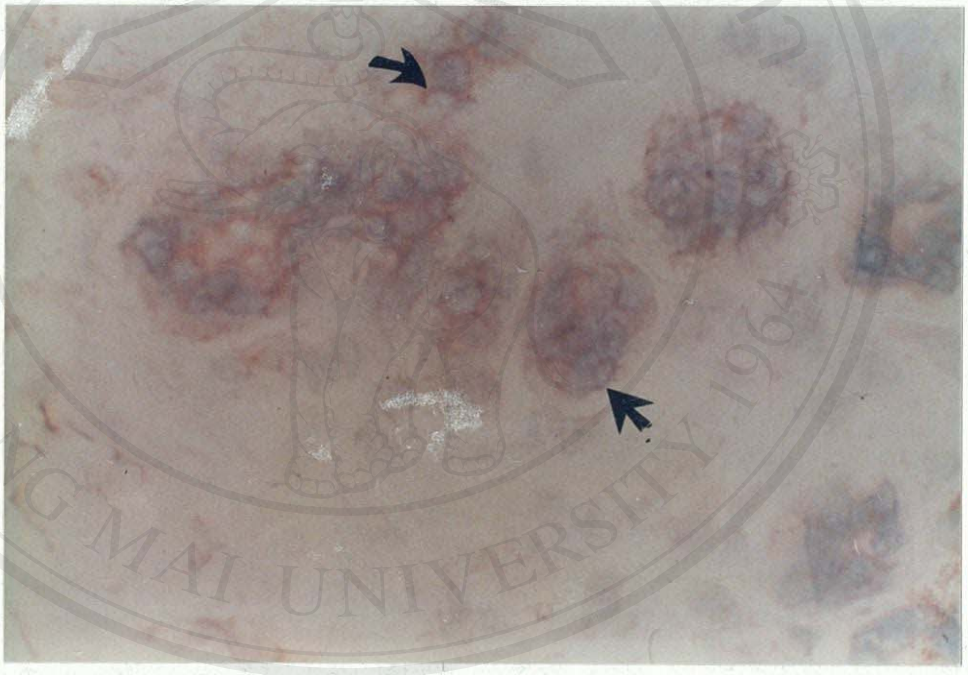

Figure 22 Breast Cancer Tissues Staining with HRP-TGA. The arrows show the intense positive stain on the cell surface as brown line. Original Magnification x400.

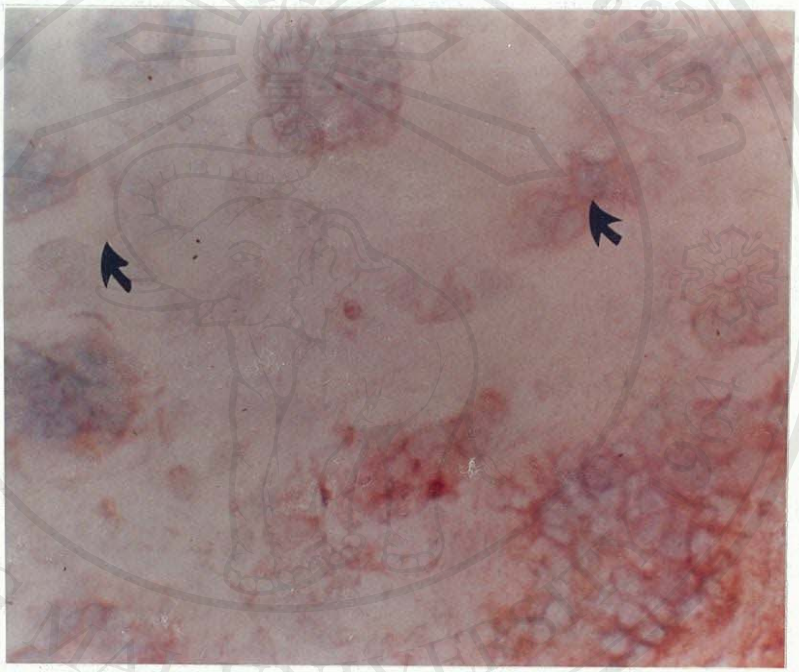

Figure 23 Cervix Cancer Tissues Stain with HRP-TGA. The right arrow shows the more intense positive stain of cancer cells than the normal cell (Left Arrow). Original Magnification x400.

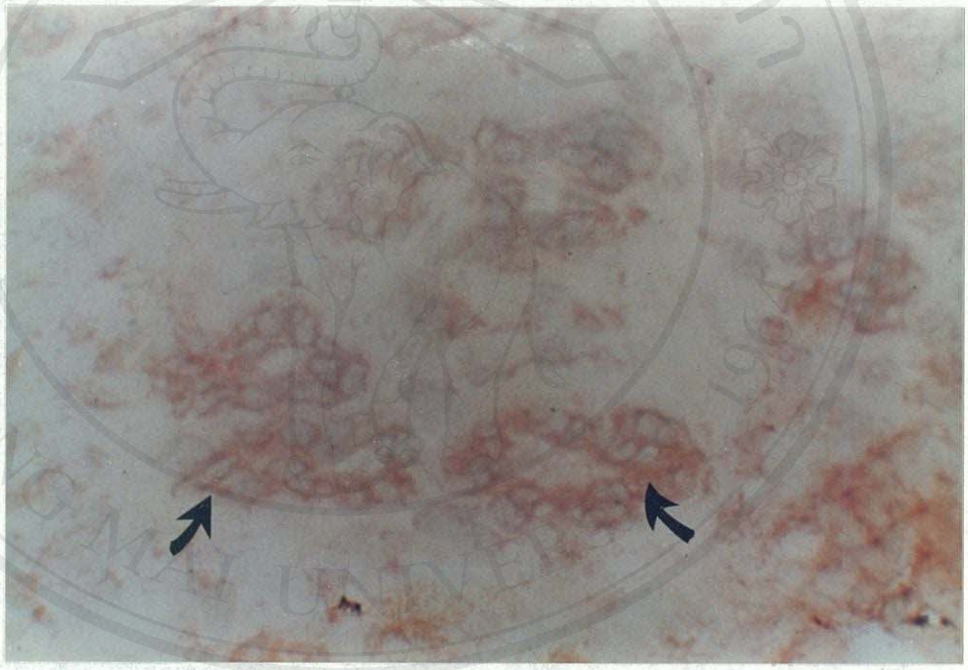

Figure 24 The Breast Cancer Tissues Stained with HRP-TGA. The right arrow shows the positive staining of cytoplasm. Original Magnification x400.