CHAPTER III

RESULTS

1. PgR in human breast tumor

The dextran - coated charcoal (DCC) method was used to assess the capacity of cytosolic progesterone receptor (PgR) in 120 specimens of human breast tumor. Ninety - five samples (79%) were classified as PgR positive, exhibiting an average binding capacity of ± SEM 335.2 ± 32.0 fmol/mg protein, with a range of 19.1 to 1,608.2 fmol/mg protein. The dissociation constant (Kd) of the binding was showed the high affinity type, ranging from .12 to 28.23 nmol/l, and the mean ± SEM at 8.58 ± 0.58 nmol/l.

2. The relationship between ER and PgR

2.1 Distribution of ER and PgR

The frequencies of finding ER and PgR derived from the same population of samples were shown in Table 1. Of 120 specimens investigated, 80 (66.7%) were ER positive (ER^+). ER and PgR were found together in 64 from 120 specimens (53.3%). Thus 80% of progesterone binding were found from ER^+ samples (64/80).

Table 1. Distribution of ER and PgR

Receptor Status	Number of Samples in Total (n = 120)	% of Total (n = 120)	% of ER^+ (n_1 = 80) (n_2 = 40)*
ER^+	80	66.7	100
ER^-	40	33.3	100*
PgR^+	95	79.2	-
PgR^-	25	20.8	-
ER^+ PgR^+	64	53.3	80.0
ER^+ PgR^-	16	13.3	20.0
ER^- PgR^+	31	25.8	77.5*
ER^- PgR^-	9	7.5	22.5*

* % of ER^- (n_2 = 40)

2.2 ER and PgR in malignant breast tumor

The majority of malignant breast tumor specimens investigated was infiltrating ductal carcinoma (IDC), 64 in 84 samples (76%). Table 2 showed that 55% of IDC samples showed ER^+ PgR^+ character (ER together with PgR). Three out of five medullary carcinoma were also ER^+ PgR^+. All together 46 cases of the total 84 malignant breast tumor samples (55%) showed ER together with PgR, and only 7 out of 84 case (8%) were both receptors negative.

2.3 ER and PgR in benign breast tumor

Table 3 showed the distribution of ER and PgR in 26 samples of benign breast tumor in the females. Six out of 8 cases of fibroadenoma contained both ER and PgR, whereas 4 from 11 of the fibrocystic disease showed both receptors. PgR^+ alone was found in 6/11 cases of fibrocystic disease. Thus about 53.8% (14/26) of these benign breast tumors were likly to be hormone dependent.

As for 3 samples of gynaecomastia, benign breast tumor found in Thai males, only one was ER^+ PgR^+, two were estrogen binding negative.

2.4 Content and affinity of ER and PgR in different types of breast tumor

These 120 human breast tumor specimens were classified into 3 types. Firstly there were 84 samples of the malignant breast

Table 2. ER and PgR in malignant breast tumor

Histopathology	Number of Samples	Receptor status			
		ER^+ PgR^+	ER^+ PgR^-	ER^- PgR^+	ER^- PgR^-
Infiltrating ductal carcinoma (IDC)	64	35	8	16	5
Medullary carcinoma	5	3	-	1	1
Papillary intraductal carcinoma	5	1	3	-	1
Adenocarcinoma	1	1	-	-	-
Mucineous carcinoma	2	1	-	1	-
Not identified	7	5	1	1	-
Total	84	46	12	19	7

Table 3. ER and PgR in benign breast tumor

Histopathology	Number of Samples	Receptor status			
		ER^+ PgR^+	ER^+ PgR^-	ER^- PgR^+	ER^- PgR^-
Fibroadenoma	8	6	1	1	-
Giant fibroadenoma	1	1	-	-	-
Fibrocystic disease	11	4	-	6	1
Cystosarcoma phylliodes	2	-	2	-	-
Not identified	4	3	-	1	-
Total	26	14	3	8	1
Gynaecomastia (Males)	3	1	-	1	1

tumors type, secondly there were 29 samples of benign breast lump including gynaecomastia in male, and thirdly there were 7 samples of not identified. Thable 4 demonstrated the significant difference in the Kd between ER and PgR in both malignant and benign types (P 0.005). PgR showed lower binding affinity than ER. When the content of ER and PgR were compared, the amount of ER in both benign and malignant were significantly lower than PgR (P 0.005). Different types of tumors: malignant or benign showed no effect on either Kd or content of both receptors.

2.5 Distribution of ER and PgR in patients of various age groups

Figure 2A showed that breast tumor incidence was highest in the age group of 41 - 50 years, about 35% of the total specimens observed. This investigation also showed that the distribution of both PgR and ER were maximum in this same age group (41 - 50 yr.) at the percentage of 30 and 22.5 of the total age group respectively (Fig 2B and C). The tendency to find both PgR and ER were slightly higher in the postmenopausal women (age group 51 - 60 and higher), comparing to the premenopausal group with less than 40 years of age.

3. Sedimentation analysis

In order to determine the sedimentation nature of estrogen and progesterone receptor protein in the cytosol fraction of breast

Table 4. Content and affinity of ER and PgR in human breast tumor.

Type of Tumor	Estrogen bindind		Progesterone binding	
	Kd (nmol/l)	fmol per mg protein	Kd (nmol/l	fmol per mg protein
Malignant (84)	n = 58 [a]3.83 ± 0.37 [b](0.13-13.10)	n = 58 152.6 ± 16.1 (11.8-662.5)	n = 65 8.14 ± 8.3* (0.13-27.27)	n = 65 318.9 ± 40.9* (19.1-1,609.2)
Benign (29)	n = 18 5.08 ± 0.82 (0.31-15.00)	n = 18 180.3 ± 34.3 (11.9-495.0)	n = 24 9.83 ± 1.11* (2.00-20.90)	n = 24 378.1 ± 59.4* (106.8-1,250.0)
Not identified (7)	n = 4 6.25 ± 1.19 (4.13 -9.69)	n = 4 213.5 ± 79.2 (63.4-450.7)	n = 6 9.55 ± 1.75 (3.42-12.68)	n = 6 272.0 ± 93.9 (89.1-615.1)

a = means ± SEM

b = range

* = $P < 0.005$ (the significance of difference between type of receptors was determined by t-test.

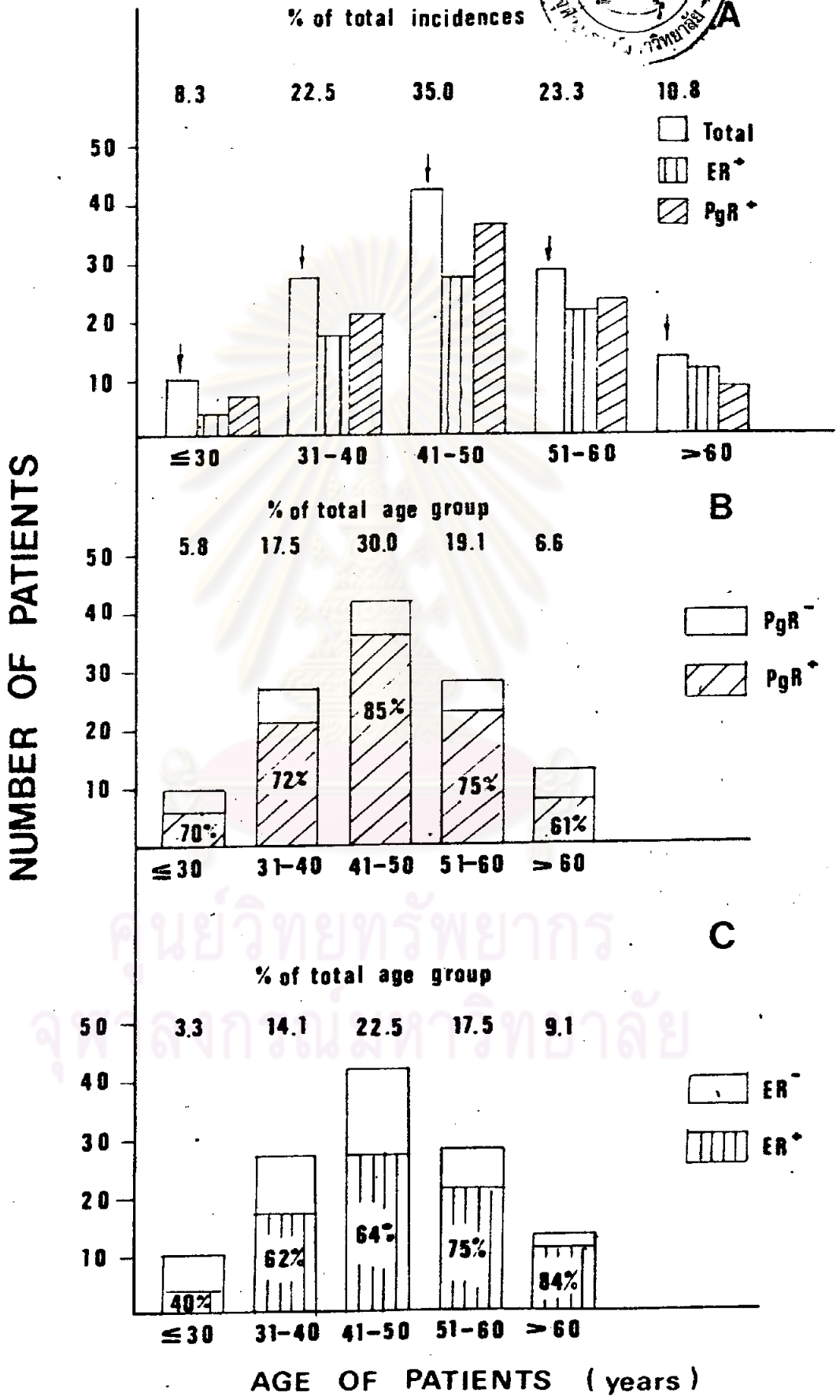

% of total incidences
8.3
22.5
35.0
23.3
10.8
Total
ER+
PgR+
50
40
30
20
10
≦30
31-40
41-50
51-60
>60
A
% of total age group
B
5.8
17.5
30.0
19.1
6.6
PgR-
PgR+
70%
72%
85%
75%
61%
≦30
31-40
41-50
51-60
>60
C
% of total age group
3.3
14.1
22.5
17.5
9.1
ER-
ER+
40%
62%
64%
75%
84%
≦30
31-40
41-50
51-60
>60
NUMBER OF PATIENTS
AGE OF PATIENTS (years)

Figure 2 Distribution of ER and PgR in patients of various age groups

ER and PgR were assayed in the same samples by DCC method (see in text, p. 10).

A. Number of ER^+ and PgR^+ samples according to the number of patients investigated in various age groups.

B. Distribution of PgR in various age groups presented in terms of per cent of total age group and per cent of each age group.

C. Distribution of ER in various age groups presented in terms of per cent of total age group, and per cent of each age group.

tumors tissues, $^{3}H - E_2$ and ^{3}H - Pg were used to label the binding proteins and seperated on the sucrose density gradient as described in Methods (p. 11 - 12).

3.1 Estrogen receptor

When the cytosolic estrogen receptor was analyzed on 5 - 20% sucrose gradient the peak of bound $^{3}H - E_2$ sediments approximately at 7.9 - 8.9S near the bottom of the gradient with a shoulder or another peak at 4 - 4.2S near the top of the gradient. (Fig 3 A, B and C). The 7.9 - 8.9S peak represented more specific binding ER than the 4 - 4.2S peak as indicated by a preincubation of an identical cytosol with a 100 fold excess of unlabelled estradiol. Unlabelled estradiol molecules can displace $^{3}H - E_2$ bound to 7.9 - 8.9S peak better than the 4 - 4.2S peak. Fig 3A showed 8.9S and 4S peaks together. In a sample of high level of the ER, the maximum dpm were 785 and 714 dpm per fraction respectively. Fig 3C, showed 7.9S and 4S peaks when a sample containing small amount of ER was used. The maximum dpm were 250 and 357 per fraction. The 7.9 - 8.9S peak showed more specificity than the 4S peak since unlabelled estradiol could eliminate 63.6% and 51.7% of the bound $^{3}H - E_2$ respectively. Thus, in these particular cytosols both 7.9 - 8.9S peak and 4S peaks represented specific $^{3}H - E_2$ binding molecules. In Fig 3B, this case showed only one peak of specific binding molecule at 4.2S, which unlabelled estradiol can

Figure 3. Sedimentation analysis of ER

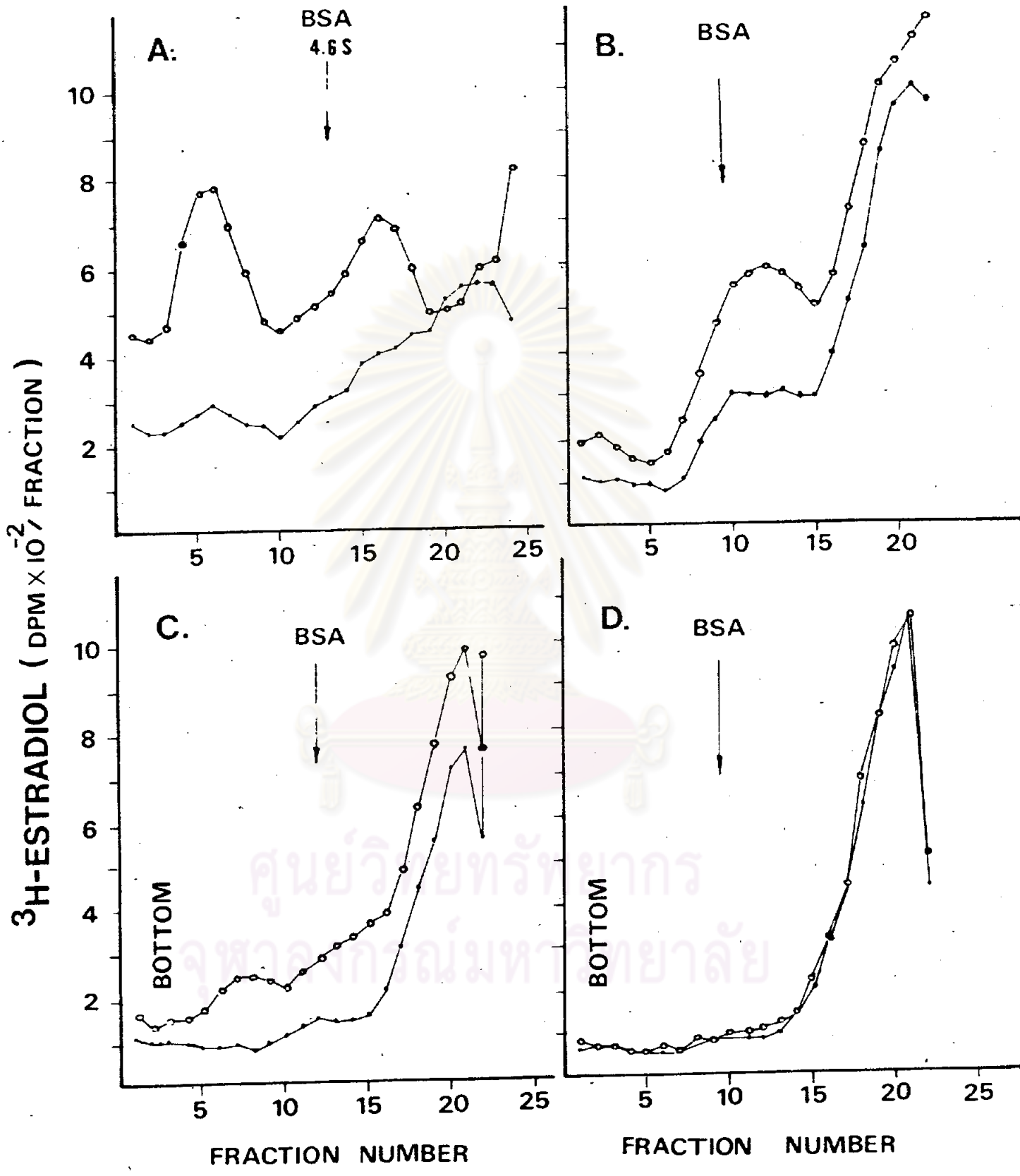
Cytosols from specimens were reacted with $^{3}H - E_2$ in the presence and absense of a 100 fold exceas of unlabelled estradiol for 4 h at 4 ^{o}C Unreacted ligand was removed by pellets of dextran-coated charcoal. 200 µl of the mixture was layered on the 5 - 20% sucrose (W/W). Estrogen receptor species were seperated in the SW 50.1 rotor at 48,000 rpm for 16 h at 4 ^{o}C ($W^2t = 1.45 \times 10^{12}$). The profiles shown with the open circles, (0), represent the association of $^{3}H - E_2$ alone; those shown with dot, (.), represent the association of $^{3}H - E_2$ in the presense of excess unlabelled estradiol. The efficiency of $^{3}H - E_2$ counting was 42%

A. ER^+ cytosol 1,216.2 fmol/mg protein

B. ER^+ cytosol 167.3 fmol/mg protein

C. ER^+ cytosol 105.5 fmol/mg protein

D. ER^- cytosol



replace 50% of $^{3}H - E_2$ in this peak. None of them were detected in the ER negative sample as shown in Fig 3D.

3.2 Progesterone receptor

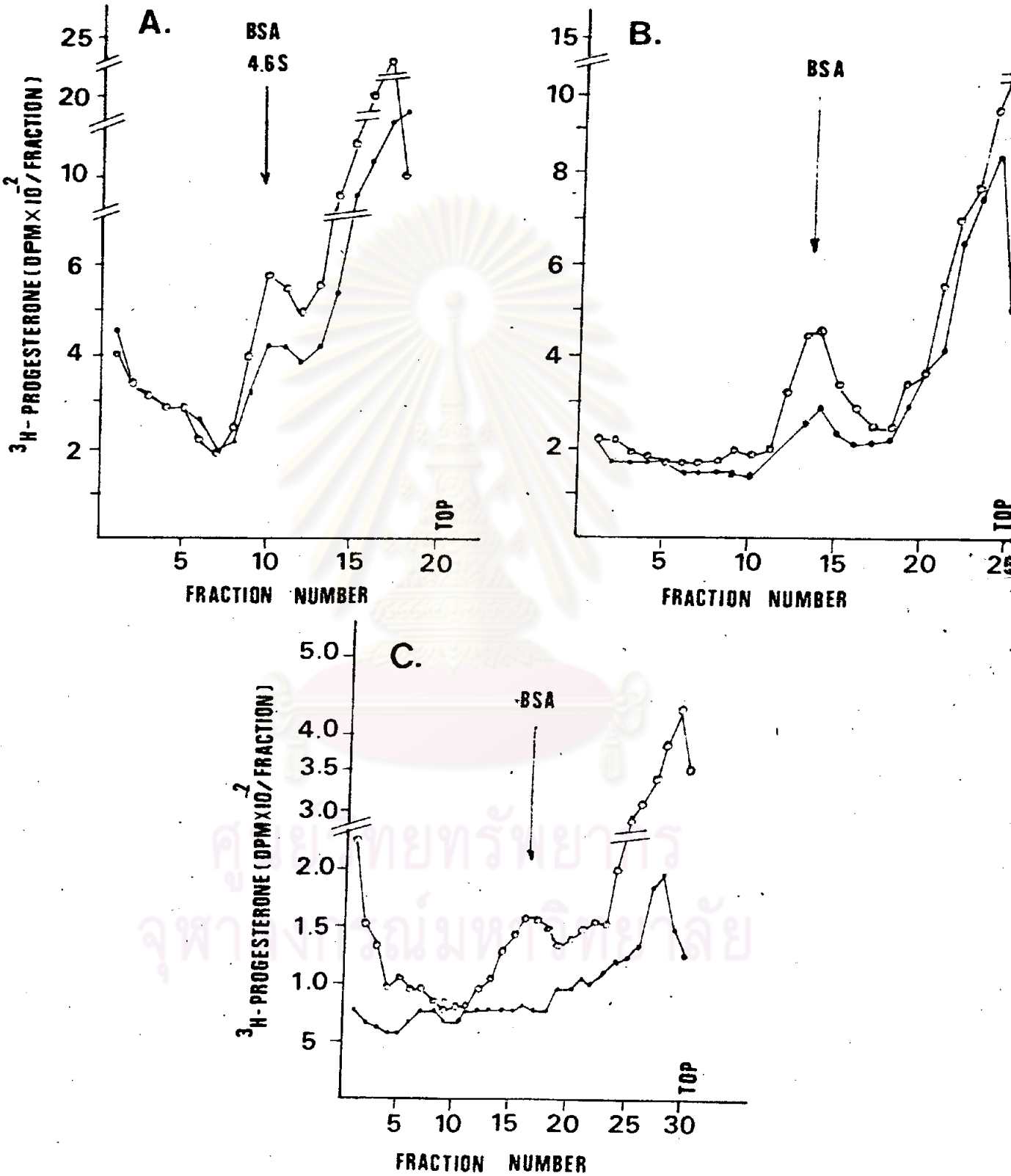
The sedimentation profiles (Fig 4A, B and C) obtained showed consistently a component which bound ^{3}H - Pg at the sedimentation coefficient about 4 - 4.6S. Each small figure represented for each tumor cytosol preparation, all of which only 4 - 4.6S peak but no 8S peak were detected. These 4 - 4.6S peaks of bound ^{3}H - Pg were displaced at about 37% by a 100 fold of non-radioactive progesterone preincubation. Thus the specificity of PgR binding to the 4 - 4.6S peak was found to be lower than that of 4 - 4.2 peak specific to $^{3}H - E_2$.

3.3 Effect of tamoxifen and progesterone on the $^{3}H - E_2$ binding profile

Figure 5 showed when tamoxifen, progesterone and unlabelled estradiol were used to determine their effect on the $^{3}H - E_2$ binding profile in one cytosol sample. This sample showed 2 peaks of bound $^{3}H - E_2$ at 8.9S and 4S. One hundred fold of unlabelled estradiol effectively displace $^{3}H - E_2$ binding to the 8.9S and 4S peak 63.6% and 51.7% respectively, whereas progesterone was not effective in competing with $^{3}H - E_2$. In this study, the presence of tamoxifen seemed to stabilize the $^{3}H - E_2$ binding with the 8S cytosol receptor.

Figure 4 Sedimentation analysis of progesterone receptor.

Cytosols from specimens were reacted with ^{3}H - Pg in the presence, (•), or absense, (0), of a 100 fold excess unlabelled progesterone for 4 h at 4 ^{o}C Unreacted ligand was removed by pellets of dextran-coated charcoal. Two hundred microliters of the mixture was layered on 5 - 20% linear sucrose gradient, centrifuged in the SW 50.1 rotor at 48,000 rpm ($W^2t = 1.45 \times 10^{12}$)for 16 h at 4 ^{o}C. The tubes were punctured for fractionation and the radioactivity was counted. The efficiency of ^{3}H - Pg counting was 38%.


A.
BSA
4.6S
25
20
10
6
4
2
TOP
5
10
15
20
FRACTION NUMBER
^{3}H-PROGESTERONE (DPM×10^{-2}/FRACTION)
B.
BSA
15
10
8
6
4
2
TOP
5
10
15
20
FRACTION NUMBER
C.
BSA
5.0
4.0
3.5
3.0
2.0
1.5
1.0
5
TOP
5
10
15
20
25
30
FRACTION NUMBER
^{3}H-PROGESTERONE (DPM×10^{-2}/FRACTION)

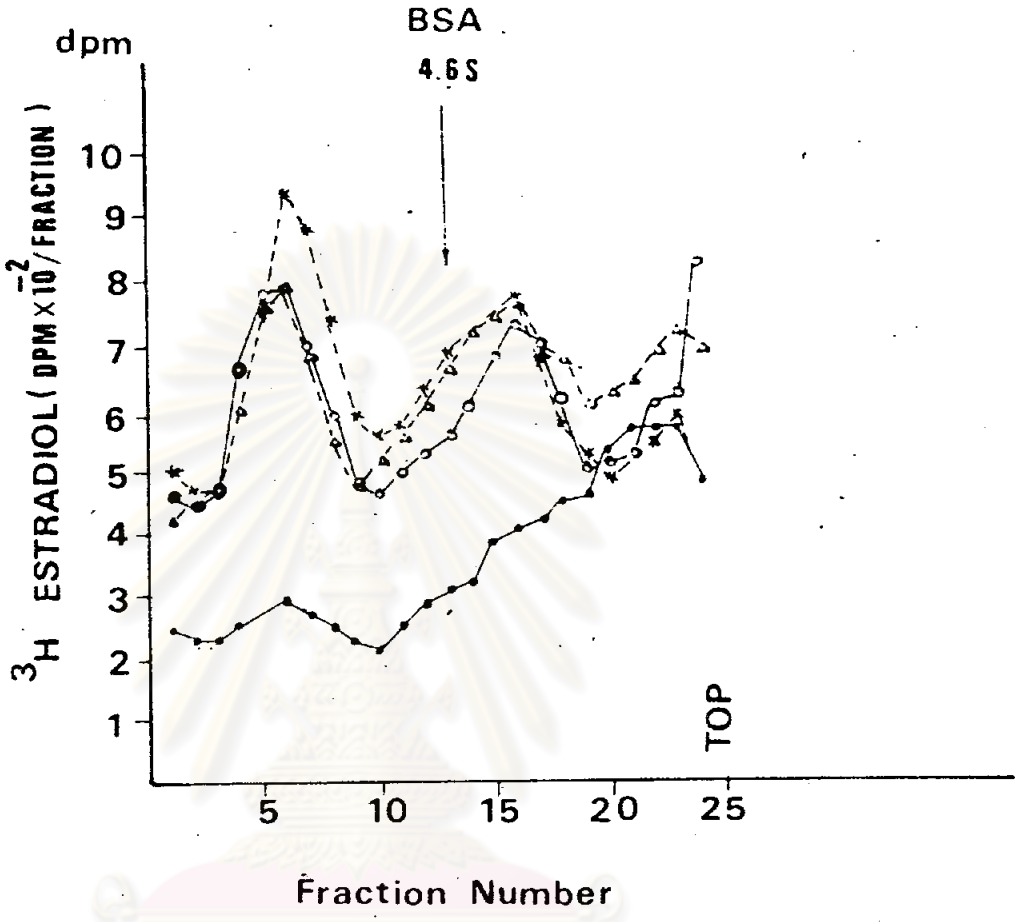


Figure 5. Effect of tamoxifen and progesterone on the ^3H-E_2 binding.

Cytosols of the same specimen reacted with ^3H-E_2 in the absence(o—o); or presence of 100 fold unlabelled 17β-estradiol,(——); tamoxifen,(• - •); and progesterone(▵-▵). Unbound ligand was removed by pellet of DCC. Sedimentation was carried out in the same condition previously described in Fig. 3. The efficiency of ^3H-E_2 counting was 42 %.

4. Competitive binding assay for ER and PgR by DCC method

Only a few cytosols demonstrating the binding activity for $^{3}H - E_2$ or ^{3}H - Pg were subjected to competition experiments. When the amount of cytosols were limited, only one concentration (100 fold of unlabelled meterial) of interesting substances were used to compete with a fixed amount of $^{3}H - E_2$ or ^{3}H - Pg. In the case that cytosols were available, orderly increasing amount of competing substance were used.

4.1 Competitive binding assay for estrogen receptor

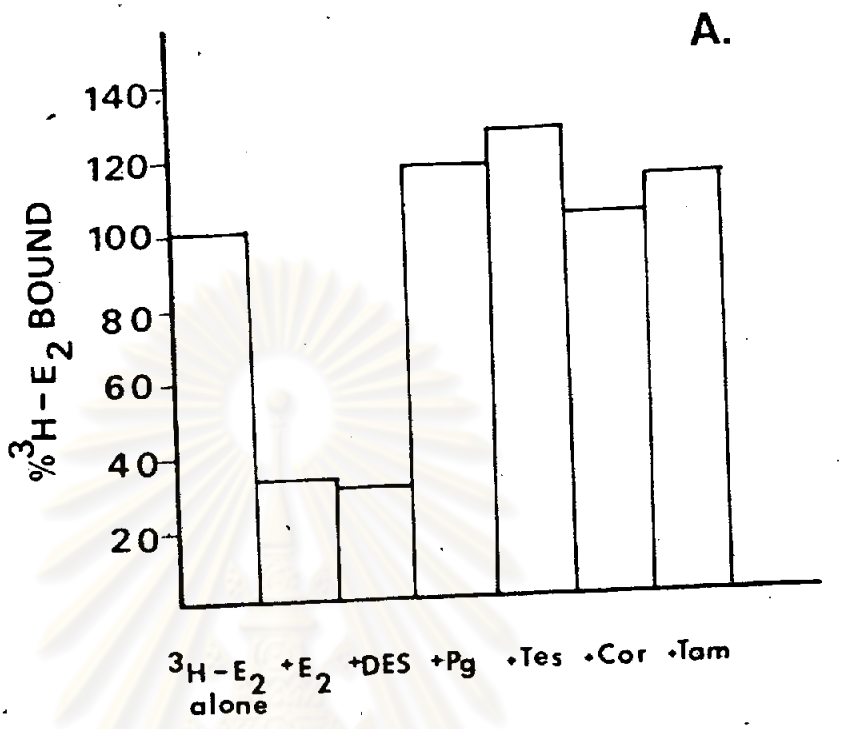
Figure 6A and B illustrated the results of experiments in which tumor cytosols were incubated with 100 pg of $^{3}H - E_2$ in the presence or absence of a 100 lold excess of non-radioactive estradiol, diethylstilboestrol, progesterone, testosterone, corticosterone and tamoxifen. Only estradiol and diethylstilboestrol (DES) were able to compete effectively at 66% and 70% respectively for the $^{3}H - E_2$ binding site. Fig 6A showed that the result of a cytosol, whose protein concentration is 10.2 mg/ml, containing high affinity estrogen receptor, Kd = 2.58×10^{-9} M, at a level of 1,216.2 fmol/mg cytosol protein. Only estradiol and diethylstilboestrol were able to compete effectively with $^{3}H - E_2$ (66% and 70%) Other steroids and tamoxifen were not reactive to compete for the specific $^{3}H - E_2$ binding in this cytosol. Fig 6B represented tumor cytosol which has 10.8 mg protein/ml cytosol with similar a affinity of estrogen receptor, Kd = 2.75×10^{-9} M, but lower amount of binding

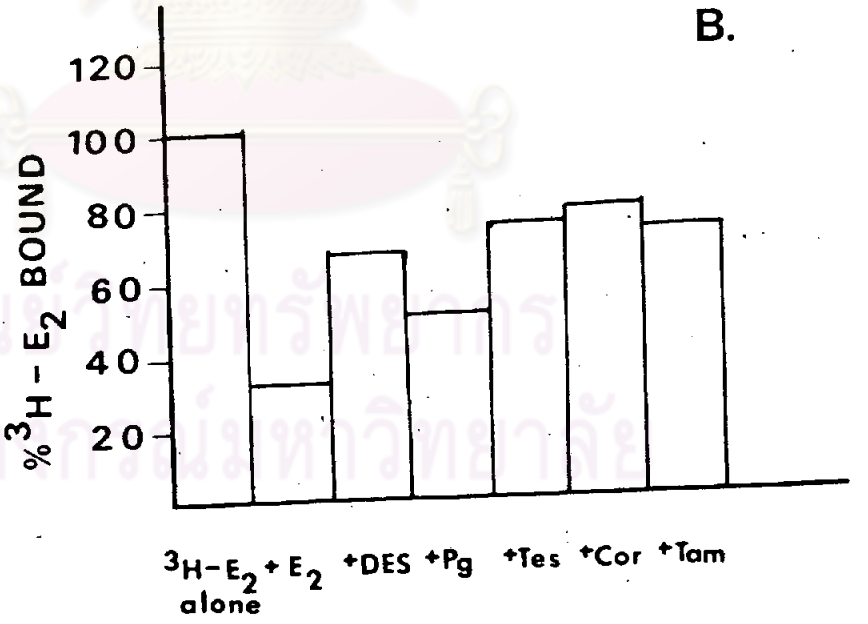
Figure 6 Competitive binding assay of estrogen receptor

Cytosols of specimens of IDC of human breast was reacted with $^{3}H - E_2$ in the presence or absence of 100 fold excess unlabelled estradiol (E_2), diethylstilboestrol (DES), progesterone (Pg) testosterone (Tes), corticosterone (Cor) and tamoxifen (Tam) at 20 ^{o}C 2 h. Unbound ligand was removed by DCC method and counted for radioactivity. Results were expressed as % bound of $^{3}H - E_2$ compared to the absence of various unlabelled competitors.

A. represented IDC cytosol, ER^{+}, Kd = 2.58 x $10^{-9}M$, binding site = 1,216 fmol/mg protein and protein concentration = 10.2 mg/ml.

B. represented IDC cytosol, ER^{+}, Kd = 2.75 x $10^{-9}M$, binding site = 110 fmol/mg protein and protein concentration = 10.8 mg/ml.





site 110 fmol/mg cytosol protein. In this case unlabelled estradiol (E_2) competed effectively with $^3H - E_2$ (68%), DES, tamoxifen and others were less effective, showing more or less 30% displacement. Figure 7 demonstrated the effect of increasing concentration of competitors on the ability of these compounds to compete for the $^3H - E_2$ binding. For this cytosol only estradiol and DES competed effectively for the specific $^3H - E_2$ binding. Less than 1 fold concentration of DES could effectively compete for 50% binding of $^3H - E_2$ where as the effective concentration of unlabelled E_2 must be at least 50 fold or more to displace 50% of $^3H - E_2$ binding.

4.2 Progesterone receptor

Figure 8A illustrated the results of experiments in which the cytosol (protein concentration = 6.8 mg/ml, Kd = 6.22×10^{-9} M, PgR = 61.2 fmol/mg protein) was incubated with 100 pg of ^3H - Pg in the presence or absence of 100 fold excess of unlabelled progesterone and other competitors. Progesterone and corticosterone partially competed with ^3H - Pg to 14% and 23% respectively. When this same cytosol was incubated with 100 pg of ^3H - ORG 2058 (which bind more effectively specific to PgR), Fig 8B showed that unlabelled ORG - 2058 was very effective and can displace 85% of the ^3H - ORG 2058 binding, whereas progesterone was much less effective (28%) and the others were not.

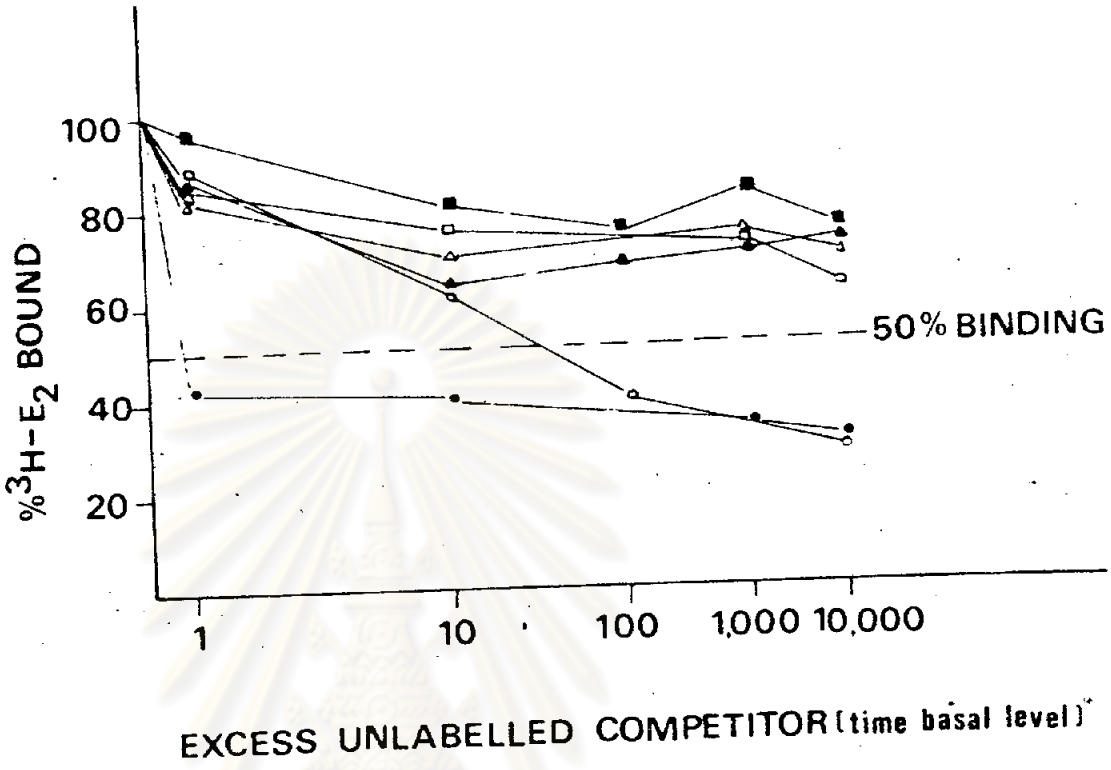


Figure 7. Effect of increasing concentration of competitor on the ability to compete for the $^{3}H-E_{2}$ binding.

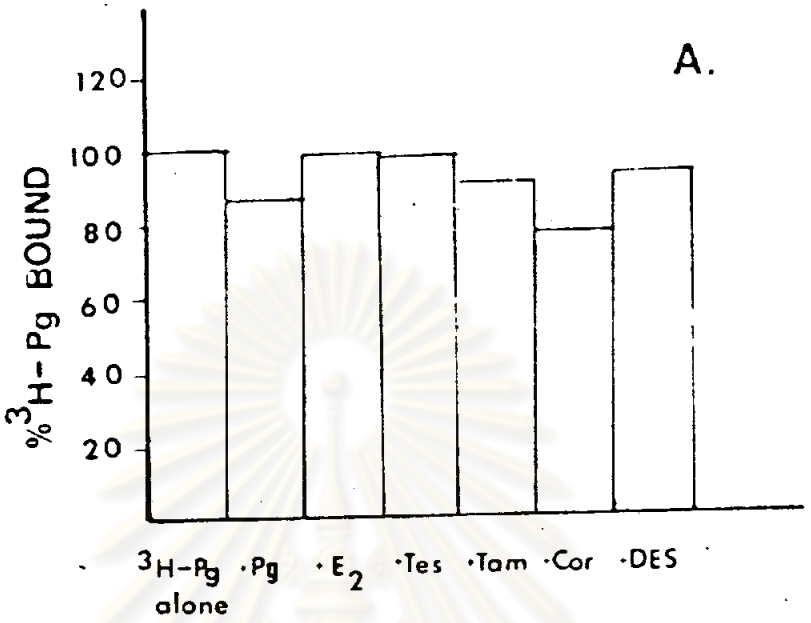
Binding of $^{3}H-E_{2}$ by cytosol from breast tumor was assessed in the presence of the increasing concentration of unlabelled estradiol (○), DES (●), tamoxifen (▲), testosterone (△), corticosterone (□), and progesterone (■). Incubation was performed at 20°C 2 h, see text for the method.

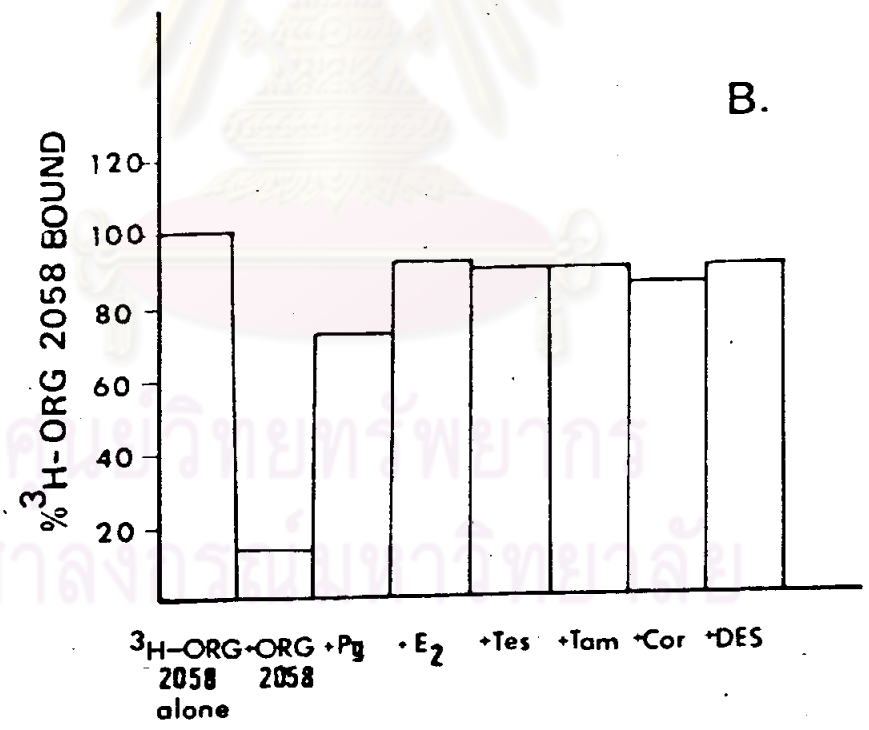
Figure 8. Competitive binding assay for progesterone receptor

Cytosols of specimens of IDC human breast, which PgR positive, Kd = 6.22×10^{-9} M, binding site = 61.2 fmol/mg protein and protein concentration 6.8 mg/ml, was reacted with ^{3}H - Pg or ^{3}H - ORG 2058 in the presence or absence of 100 fold excess unlabelled progesterone (Pg) or ORG 2058 respectively and with others; estradiol (E_2), diethylstilboestrol (DES), testosterone (Tes), corticosterone (Cor) and tamoxifen (Tam) at 20 ^{o}C 2 h Unbound ligand was removed by DCC method and counted for radioactivity. Results were expressed as % bound of ^{3}H - Pg or ^{3}H - ORG 2058 compared to the absence of various unlabelled competitors.

A. represented for competition of ^{3}H - Pg binding

B. represented for competition of ^{3}H - ORG 2058 binding.


A.
120
100
80
60
40
20
%3H-Pg BOUND
3H-Pg alone
+Pg
+E2
+Tes
+Tam
+Cor
+DES


B.
120
100
80
60
40
20
%3H-ORG 2058 BOUND
3H-ORG 2058 alone
+ORG 2058
+Pg
+E2
+Tes
+Tam
+Cor
+DES

5. Stability of PgR

Seperate aliquots of the same cytosols were assayed at various time intervals ($\frac{1}{2}$, 1, 2 and 3 month) for PgR content and Kd by DCC method. Table 5 showed that when 5 cytosols were stored at -70 °C within 2 months storage time after tissue collection, the Kd of PgR in 4 cytosols showed the per cent variation about ± 15% from the first determined value. When the storage time of the cytosols were increased to 3 months, the per cent of variation of Kd observed in the 3 samples were 17.6 - 32.9% increased based on the first determined value available.

Although the variation of Kd was abserbed and showed the lower affinity from the first determined values but the results suggested that PgR was moderately stable and the cytosol mights with ± 15% variation in assay values.

Table 5. Change in Kd of cytosolic PgR at various time of assay

The cytosols were prepared at 0 - 4 ^{o}C and stored seperately at -70^{o}C. PgR was assayed as described in Methods (p. 10) and the Kd of PgR was determined by the Scatchard analysis.

Sample No	Kd of PgR in 10^{-9} M Storage time (month)			
	$\frac{1}{2}$	1	2	3
1	1.36	1.16 (-14.7)*	-	1.60 (+17.6)
2	1.12	-	1.28 (+14.2)	-
3	2.29	2.01 (-12.2)	-	-
4	-	5.12	5.84 (+14.1)	6.70 (+30.9)
5	-	2.67	-	3.55 (+32.0)

( )* represented the per cent variation of Kd from the first determined value.