CHAPTER II

MATERIALS AND METHODS

MATERIALS

1. Breast tumor specimens

Specimens of human breast tumors were obtained from

- Chulalongkorn Hospital
- Ramathibodi Hospital
- Vajira Hospital
- Rajvitee Hospital
- Bangkok Christian Hospital
- National Cancer Institute (Thailand)
- Surgical Pathology and Cytology Service

Excised tissues were put in a plastic bag placed on dry ice, and immediately brought to the laboratory at Department of Biochemistry, Chulalongkorn University, for preparation. Otherwise samples were stored frozen at -70 ^{o}C in a Forma-Bio-Freezer until used.

These tumor specimens were classified on the basis of the histopathology criteria from the hospital which was the source of that sample.

2. Chemicals

[1, 2, 6, 7 (n) $-^3H$] Progesterone (87 Ci/m mol) and [2, 4, 6, 7, (n) $-^3H$] estradiol (89 Ci/m mol) were obtained from the Radiochemical Center, Amersham, England. Unlabelled steroids namely progesterone, estradiol, testosterone, corticosterone were products of Sigma Chemical Co. DES (laboratory grade) was purchased from BDH. Tamoxifen [trans 1-(4-β-dimethylaminoethoxyphenyl) -1, 2, -diphenyl but - 1 - ene, Nolvadex; Imperial Chemical Industries] was the gift from Professor K. Griffith, Tenovus Institute for Cancer Rescarch, Wales. Other chemicals used in this study were of labolatory reagent grade.

3. Instruments

Centrifuge	:	Beckman Model J-21 C with JS 7.5 and JA 20 rotors.
Ultracentrifuge	:	Beckman L8 - 70 Ultracentrifuge with SW 50.1 rotor.
Counter	:	Packard Liguid Scintillation Counter (PRIAS)

4. Buffers

4.1 TED (Tris-EDTA-Dithiothreitol) buffer : 0.01M Tris: HCl [Tris (hydroxymethyl) - aminomethan from BDH], 1.5 mM EDTA

(ethylenediamine tetraacetic acid disodium salt from BDH), 0.5 mM-DTT (dithiothreitol from Sigma), pH 7.4

4.2 Buffer for tissue homogenization: 0.25 M Sucrose (from BDH) in TED buffer.

4.3 Dextran - coated - charcoal suspension (DCC suspension): 0.25 g of charcoal (Norit A) from Sigma, 0.025 g of dextran T 70 (Pharmacia Fine Chemical) in 100 ml. of TED buffer, pH 8.0.

METHODS

1. Protein and DNA analysis

Estimation of cytosol protein was done by the method of Lowry *et al*, (25) using crystalline bovine serum albumin (BSA, from Sigma) as standard. Nuclear pellet was determined for deoxyribonucleic acid (DNA) by the method of Giles and Mayers (26) using calf thymus DNA (Sigma) as standard.

2. Preparation of cytosol

Tissues were thawed at 4 $^{\circ}$C on ice bath. They were then debrised of necrotic and hemorrhagic segment, weighed and cut into small pieces. Homogenized buffer was added at a volume predetermined on the basis of tissue wet weight to 2 volume in ml/g (27). Homogenization was conducted in several 10 - 20 sec pulses, seperated by 20 - 30 sec cooling periods in a teflon - glass homo-genizer. A small ice bath was kept around the homogenizer at all

times to prevent the accumulation of heat (28). The tissue homogenate was then centrifuged at 25,000 g (18,000 rpm) in a Beckman Centrifuge J 21 C (JA 20 rotor) for 75 min. The supernatant fraction was removed without disturbing the lipid layer at the top of the tube. The cytosol obtained was stored in separate 0.5 ml aliquot at -70 °C until the receptor assay was performed. Nuclear pellet was kept at 4 °C for DNA determination.

3. <u>Progesterone receptor assay</u>

The assay conditions for PgR was the same as that used by Horwitz and McGuire (14) and Patanapanyasut (29). Either cytosol or assay buffer (100 µl) was incubate with increasing quantities of ^{3}H - Pg (20 - 150 pg in 100 µl of TED assay buffer) for 2 h at 20 °C in duplicate. The 500 µl of DCC suspension was then added, the mixture was incubated under vigorous shaking at 30 °C for 15 min, and then spun at 2,000 g 4 °C for 10 min in a Beckman Model J 21 C Centrifuge (JS 7.5 rotor). The supernatant fractions (500 µl) were counted for radioactivity of bound ^{3}H - Pg in 5 ml triton X - 100 scintillation cocktial [5.5 g PPO (2, 5 - diphenyloxazole) from Sigma, 0.1 g POPOP (1, 4 - bis [2 - (5-phenyl - 2 - oxazolyl)] - benzene) from Sigma, 333 ml Triton X - 100 (iso - octylphenoxypolyethoxyethanol from Packard) and 667 ml of toluene (from J.T. Baker)] with 40% counting efficiency in a Packard Liquid Scintillation Counter. The binding data were analyzed by the method of Scatchard (30, 31). The positive result (PgR^{+})

was $\geq$ 10 fmol/mg protein whereas values less than 10 fmol/mg protein were reported as negative (32).

4. Sedimentation analysis

Gradients of sucrose (5 - 20% w/w) were prepared in 4.6 ml linear gradient. The gradient were hand pipetted by five different sucrose concentration in TED buffer (0.5 ml 5.0%, 1.2 ml of 8.8%, 12.5% and 16.2%, and with 0.5 ml 20%) into each 5 ml pollyallomer tube. The gradients were allowed to diffuse for 24 h at 4 °C or 2 h at room temp before use. The method used in this study was according to McGuire (17). Samples for gradient analysis were prepared by incubating 250 µl of cytosol with 1 pmol of $^3H - E_2$ or ^3H - Pg for 4 h at 4 °C. Control cytosols were preincubated with 100 pmol of nonradioactive E_2 or Pg 15 min prior to addition of the corresponding $^3H - E_2$ or ^3H - Pg, respectively. After incubation the mixture were then incubated with the pellet of the 2,000 g of 0.5 ml of 0.25 g% DCC suspension at 4 °C for 30 min, and then centrifuged at 2,000 g 10 min at 4 °C to eliminate the free steroids. A 200 µl of the supernatant fraction removed was then layered onto the top of linear sucrose density gradient previously prepared. BSA ($S_{20,w}$ = 4.6) crystallized grade was used as the marker for sedimentation coefficient. The tubes were centrifuged in a SW 50.1 rotor at 48,000 rpm for 16 h (Setting $w^2t = 1.45 \times 10^{12}$) at 4 °C in a Beckman L8 - 70 ultracentrifuge.

<u>For the estrogen receptor analysis</u> : Gradients were fractionated by LKB peristaltic pump Model 2012 at 10 x 10 speed and a 7-drop fraction (approximately 200 µl) was collected into each scintillation vial. About 25 fractions were obtained from each tube and the radioactivity was determined in the presence of 5 ml triton X - 100 scintillation fluid.

<u>For the progesterone receptor analysis</u> : The bottom of the tube was punctured and 200 µl - fractions of the gradient were collected. All fractions were counted for radioactivity in 5 ml of triton X - 100 scintillation cocktail.

<u>For BSA standard marker</u> : The tube containing 200 µl of BSA (10 mg/ml) as the marker of sedimentation coefficient was parallelly run and collected in similar way and measured for the absorbance at 280 nm in a Beckman Model 25 spectrophotometer.

5. <u>Competitive binding studies</u>

The ability of various substances to compete with $^{3}H - E_{2}$ and $^{3}H - Pg$ for the binding site on the respective receptor protein was assessed according to Horwitz and McGuire (14) by incubation 100 µl of cytosol with $^{3}H - E_{2}$ or $^{3}H - Pg$ (100 pg/100 µl) for 2 h at 20 °C in the presence or absence of increasing concentration (100 pg - 1 µg/100 µl) of unlabelled estradiol or progesterone, DES, testosterone, corticosterone and TAM added 1 h prior to incubation. Free steroid was removed by treatment with DCC suspension.

The relative binding for each substance was determined as percent bound with respect to 100% bound in the absence of that compound assayed with $^{3}H - E_2$ or $^{3}H - Pg$ alone.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย