Chapter II

Materials and Methods
Adenosine 5' triphosphate, casein, diphenyl oxazole (PPO), 1,4-bis (2,5-phenyloxazol) benzene (POPOP), 17β-estradiol, fluorescein iso thio cyanate (FITC) isomer I, phenyl methyl sulphonyl fluoride (PMSF), progesterone, testosterone, diethylstilbestrol, dexamethasone, Tamoxifen, 4-chloro-1-naphthol, benzamidine-HCl and poly L-aspartic acid were purchased from Sigma Chemical Co., St. Louis, USA. DEAE Cellulose (DE-52), GF/C glass micro fiber filters, Whatman filter papers (3mm), cellulose cc-31 and phosphocellulose were purchased from Whatman Biosys. Ltd., England. Sepharose 4B, and Sephadex series G-100, G-50, and G-25 were obtained from Pharmacia Fine Chemicals, Sweden. 2,4,6,7-[H] estradiol-17β (specific activity 101 Ci/m mol) and high specific activity tritiated amino acid mixture (Sp. act. 93-130 Ci/m mol) were purchased from Amersham. 35S Methionine (Sp. act. 1000Ci/m mol) was obtained from Board of Radiation and Isotope Technology (BRIT), Bhabha Atomic Research Centre (BARC), Bombay. Nitro cellulose membrane sheets were purchased from Kodak. Horse radish peroxidase-coupled anti rabbit IgG and tetra methyl benzidine (TMB) was purchased from Genei, Bangalore. Goat anti rabbit IgG and antibodies against estradiol coupled to BSA, raised in goat, were purchased from Lupin laboratories, Bombay. Dulbecco's modified Eagles medium was prepared in the laboratory. The medium was deficient in phe, leu, lys, tyr, pro and met. MALA-N (Indian Drugs and Pharmaceuticals Limited, Hyderabad), an oral contraceptive that contains Norgestrel and Ethinylestradiol, was obtained from the University of Hyderabad Health Centre. All other reagents used
were of analytical grade, purchased from local commercial establishments.

Water used in the preparation of buffer was processed as follows. Raw water was first subjected to pressure filtration in order to remove suspended elements. This was deionised using a Pure water DM-75 deionizer. The deionized water was subjected to single distillation using either a glass or a stainless steel unit. The single distilled water was redistilled using a glass/quartz unit. This is termed as double distilled water (DDW). Goat uteri were obtained from local slaughter, house transported on ice to the laboratory and stored at -75 C until further use.

BUFFERS USED IN THE EXPERIMENTS:

Buffers used in the purification of ER, E-RAF and nnaER:

**TEM:** 10mM Tris-HCl, pH 7.6, 1mM EDTA and 12mM monothioglycerol, containing 0.2mM PMSF.

**TEMN:** TEM with 50mM NaCl, pH 7.6.

**TCKM-Sucrose:** 50mM Tris-HCl, pH 7.5, 4mM MgCl$_2$, 20mM KCl, 1mM CaCl$_2$ and 250mM sucrose. This buffer was used in the isolation of nuclei from the uterus.

Buffers used in the purification of CANP:

**Homogenization buffer:** 20mM NaHCO$_3$, 1mM EDTA, pH 7.5.

pH 7.5 buffer: 20mM Tris-HCl pH 7.5, 100mM NaCl, 5mM EDTA and 10mM β-mercapto ethanol.

Substrate for CANP: This contained 0.21% casein, 5mM CaCl$_2$, 25mM β-mercapto ethanol and 0.1M sodium glycerophosphate, pH 7.5.
**SDS-PAGE buffers:**

**Acrylamide solution:** 30g of acrylamide and 0.8g of methylene bis acrylamide were dissolved in double distilled water (DDW) and the final volume was made upto 100ml.

Lower Tris (4X): 1.5M Tris-HCl pH 8.8 containing 0.4% SDS.
Upper Tris (4X): 0.5M Tris-HCl pH 6.8 containing 0.4% SDS.
APS solution: 2% APS solution (prepared freshly).
Reservoir Buffer: 0.1M Tris, pH 8.2 containing 0.8M Glycine.
Sample buffer: 1ml glycerol, 50μl β-mercapto ethanol, 3ml 10% SDS and 1.25ml upper tris solution (4X). Final volume was made upto 10ml with DDW.

**Buffers for Western transfer:**

Towbin buffer: 25mM Tris-HCl, pH 8.3, 190mM Glycine in 20% methanol.
Tris Buffered Saline (TBS): 10mM Tris and 0.9% NaCl pH 7.4.

Coating buffer: 10mM Sodium carbonate and 40mM Sodium bicarbonate pH 9.6, with 0.02% sodium azide.
PBS-Tween: 0.1M phoshate buffered saline pH 7.5 containing 0.05% Tween-20.

Bradford's reagent for protein estimation: 10mg of Coomassie Brilliant Blue G-250 was dissolved in 5ml of ethanol. To this 10ml of 85% (w/v) phosphoric acid was added. The resulting solution was made upto 100ml with DDW and was finally filtered through Whatman # 1 filter paper.

**Scintillation cocktails for radioactivity measurement:**

Toluene base: 5g PPO, 500mg POPOP per liter that contained Triton
X-100 and toluene in a ratio 33:67.
Brays Mixture: 60g naphthalene, 20ml ethylene glycol, 100ml methanol, 4g PPO, 200mg POPOP made upto 1 lit with dioxane.

Maintenance and surgery of animals:

3 months old female rats from an inbred colony of Wistar strain were used in the experimental studies. The animals were maintained under natural dark and light cycles (12 hours of light and 12 hours of darkness). Food (standard pellet food supplied by Hidustan Lever Ltd., India) and water were available ad libitum to the animals. Ovariectomy, adrenalectomy and the subcutaneous implantations of hormone-bees-wax pellets were done in rats under light ether anaesthesia. Adrenalectomized rats were given normal saline (0.9% sodium chloride) instead of water for drinking.

Preparation of hormone-wax pellets:

Estradiol-17ß, testosterone, Tamoxifen or Dexamethasone - bees wax pellets had a hormone to bees wax ratio of 1:1000. Progesterone: bees wax was in 1: 2 00 ratio. On an average the pellets to be implanted weighed between 50-70mg. Mala-N bees wax pellets were made in 1:1000 ratio with reference to its ethinylestradiol concentration. The steroid was extracted into alcohol before mixing with molten bees wax.

Detection of the stages of estrus cycle and pregnancy in rats:

The vaginal smears were observed under a light microscope to determine the stage of the estrus cycle of the rat (Zarrow et al., 1964). The vaginal smear taken during the proestrus contained leukocytes and numerous small, rounded nucleated epithelial cells. The vaginal smear of the estrus showed cornified
cells and those of **metestrus** had cornified cells along with large nucleated cells and a limited number of **leukocytes**. **Diestrus** vaginal smear contained large number of leukocytes, mucus and debris. The females in proestrus stage were caged with healthy males and the day one of pregnancy was determined following the detection of vaginal plugs.

**SDS-Polyacrylamide gel electrophoresis (SDS-PAGE):**

SDS-PAGE was performed as described by Laemmli (1970). The electrophoresis was performed by casting 10% gel to check the protein homogeneity and also for peptide mapping.

**Silver staining of SDS-gel:**

Silver staining of polyacrylamide gels was carried out according to the procedure described by Blum et al., (1987). The gels were fixed in a solution of 50% methanol and 12 % glacial acetic acid and 0.5ml of formaldehyde/ liter for 1 hour. After fixation of the proteins the gels were washed with 50% ethanol for 3 x 20 minutes. After **pre-treatment** with 0.2g/liter sodium thiosulphate (1min) the gels were washed 3 times with water and then impregnated for 20 minutes with 2g/ liter silver nitrate. Following this the gels were rinsed in water and developed with 60g/ liter sodium carbonate. The proteins appeared as silver stained bands.

**Radioimmuno assay of estradiol:**

The procedure described by Niswender et al., (1975) was followed.

Sample preparation: The hormone from ratr serum was extracted with diethyl ether solvent. 500µl of serum was mixed with equal
volume of ether and the tubes were left in a freezer at -75°C. The top organic layer that contained the hormone was collected by decanting it into an assay tube.

Estradiol Standards: Standards were prepared in the 20-200ng range by first making a stock of 200µg of estradiol/ml methanol and diluting it to make the corresponding working solution with 0.01M phosphate buffered saline containing 0.1% gelatin.

Labeled estradiol: H-estradiol of 40nm concentration was diluted 100 times and an aliquot of 100µl was added to each tube during the assay. The assay was carried out as follows.

All the standards were run in triplicates. To the standard tubes varying concentrations of unlabelled estradiol was added, in the 20-200pg range. This was followed by the addition of PBS to a final volume of 1ml. 100µl of labeled estradiol was added to the above mixture, mixed and to this 10µl of anti-estradiol-BSA raised in goats was added. The tubes were incubated at 4°C for 12 hours. At the end of the incubation, 500µl of dextran coated charcoal (0.1% dextran and 1% charcoal in PBS) was added to the tubes which were subsequently kept in ice for 30 minutes. The tubes were subjected to centrifugation at 5000xg for 5 minutes at 4°C. The supernatant was collected and the radioactivity associated with the samples measured using Bray's mixture (scintillation cocktail). The experimental samples were also treated in a similar fashion. Blanks were the tubes that contained labelled hormone alone. Total counts were calculated from the tubes that contained labelled hormone and estradiol antisera. Non specific counts were calculated from the tubes that
contained labeled hormone and control sera (goat IgG).

\[
\text{%binding} = \frac{(\text{Standard counts} - \text{Sample counts} - \text{Blank} + \text{NSB})}{\text{Total counts}} \times 100
\]

The graph was plotted with the % of binding on Y-axis and concentration of estradiol on X-axis.

**Preparation and ligand coupling to cyanogen bromide activated Sepharose:**

Cyanogen bromide (CNBr) activation of Sepharose 4B was performed as described by March et al.,(1974). One volume of Sepharose 4B along with equal volume of 2M sodium bicarbonate was taken in a beaker and was left to stir slowly. The rate of stirring was increased and 0.05 volumes of 2mg of CNBr/ ml of acetonitrile solution was added all at once. After stirring vigorously for 15 minutes, the slurry was transferred into a coarse sintered glass funnel and was washed with 10 volumes of 0.1M sodium bicarbonate pH 9.5.

The ligand was dissolved in one volume of 0.2M sodium bicarbonate, pH 9.5. Coupling of the activated Sepharose 4B was done for 20 hours in the absence and for an additional 4 hours in the presence of 1M glycine at 4°C. This gel was washed with 20 volumes each of 0.1M sodium acetate (pH 4.0), 2M urea and 0.1M sodium bicarbonate pH 10.0. All the three solutions contained 0.5M NaCl. The gel was stored suspended in TEM buffer containing 0.02% sodium azide at 4 C.

**Isolation of heat shock protein 90 (hsp-90):**

The hsp-90 was isolated from the the goat liver by the
procedure of Sullivan et al., (1985) with minor modifications. Goat liver was homogenized in +molybdate (+Mo) buffer (10mM potassium phosphate, pH 7.0 containing 10mM sodium molybdate and 10mM monothioglycerol). The homogenate was centrifuged at 12,000xg at 4°C for 20 minutes. The resultant supernatant was centrifuged at 100,000xg for 90 minutes in a Beckman L8-M Ultracentrifuge using a SW-28 rotor.

The liver cytosol thus obtained was stirred with 0.1 volume of phosphocellulose which was then filtered under vacuum. The filtrate was stirred with heparin agarose (40ml, also pre-equilibrated with +Mo buffer) for 20 minutes. The heparin agarose was washed with 20 volumes of +Mo buffer aided by vacuum filtration. The gel was resuspended in +Mo buffer. The heparin-eluate was mixed with DE-52 (30ml equilibrated with -Mo buffer) and stirred for 20 minutes.

The DE-52 was pelleted following centrifugation and the gel was washed with 20 volumes of -Mo buffer (phosphate buffer without molybdate) containing 0.3M potassium chloride; 2ml fractions were collected. The absorbance at 280nm was determined for each fraction. The peak fractions containing hsp-90 were dialyzed overnight against -Mo buffer and lyophilized.

**Immobilization of hsp-90 and E-RAF II on Sepharose 4B:**

Covalent coupling of E-RAF II and hsp-90 to CNBr activated Sepharose 4B was carried out following the method described by March et al., 1974.

**Isolation of uterine nuclei:**

The procedure described by Thampan (1985) was followed. The
goat uterus was homogenized in 20 volumes of TCKM-Sucrose buffer pH 7.5, using a Polytron homogenizer PT 45-80 and the homogenate was filtered through a cheese cloth. Following filtration the suspension was subjected to centrifugation at 1000xg for 10 minutes. The pellet was treated with Triton X-100, washed thrice with detergent free buffer and resuspended in 10ml of TCKM-Sucrose buffer. This was layered over 30ml of TCKM buffer containing 340mM sucrose and centrifuged at 1000xg for 10 minutes. The purified nuclear pellet was resuspended in TCKM-Sucrose buffer.

Isolation of DNA:

DNA was isolated following the method of Marmur (1961). The nuclei were isolated from goat liver following the method of Thampan (1985) and suspended in saline EDTA (0.15M NaCl, 0.1M EDTA, pH 8.0). To this a 25% SDS solution was added. The mixture was incubated for 10 minutes in a water bath at 60 C and was subsequently cooled to room temperature. Sodium perchlorate was added to the viscous suspension to a final concentration of 1M. This suspension was mixed thoroughly with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) in a ground glass stoppered flask for 30 minutes. The emulsion was centrifuged at 5,000xg for 5 minutes. The upper aqueous phase was carefully withdrawn. Two volumes of ethanol were gently layered on the aqueous phase to precipitate the nucleic acid. The precipitate was dispersed in saline citrate and was subjected to re-extraction with chloroform- isoamyl alcohol mixture until very little protein remained at the interphase. The DNA was reprecipitated with
ethanol.

Preparation of single stranded (ss) DNA-Sepharose:

DNA from the goat liver was extracted as described by Mannur (1961). The DNA was denatured by dissolving in 0.5M NaOH followed by heating in a boiling water bath for 15 minutes. Equal volume of 0.4M sodium bicarbonate solution of pH 9.5 was added. The denatured DNA was coupled to one volume of cyanogen bromide activated Sepharose 4B, prepared as described by March et al., 1974. The gel was suspended in TEMN buffer containing 0.04% sodium azide.

Preparation of DNA-Cellulose:

Rat liver DNA was extracted following the procedure of Marmur (1961). The DNA was adsorbed to cellulose (Whatman cc-31) as described by Alberts and Herrick (1971), with the modifications introduced by Thampan (1987). 60mg of goat liver DNA was denatured by dissolving it in 0.1N NaOH and then heating in a boiling water bath for 15 minutes. The solution was then cooled in an ice bath and the pH was adjusted to 7.0 with HCl. It was then mixed with a solution of native DNA (60mg DNA in 10mM Tris pH 7.5 and 1mM EDTA). 12g of cellulose was slowly added. The slurry was dried overnight at room temperature, lyophilized and suspended in 120ml of TEMN buffer. The suspension was stored in 3ml aliquots at -75 C.

Purification of E-RAF II:

The method developed by Thampan (1987) was followed. The experimental steps were all carried out at 0-4 C. Goat uterine cytosol was first chromatographed over a column of
p-aminobenzamidine-agarose equilibrated with TEMN (10mM Tris-HCl, pH 7.6, containing 1M disodium EDTA, 12mM monothioglycerol, 50mM NaCl and 0.2mM phenylmethylsulphonyl fluoride) buffer. The flow-through fraction from this column was collected. This step was chosen in order to reduce the protease contamination of the cytosol during the purification of the protein. The flow-through fraction was chromatographed over a 25ml column of Whatman DE-52, equilibrated with TEMN buffer. The flow-through fraction from the DE-52 column was chromatographed over a 10ml column of single stranded DNA Sepharose. The column was washed extensively with the buffer. Elution of E-RAF from the column was achieved using TEMN buffer containing 10mM ATP. Ammonium sulphate was added to this ATP-eluted fraction to give a final 70% saturation of the salt. The precipitated proteins were dissolved in a small volume of TEMN buffer and dialyzed extensively against the same buffer. The fraction was chromatographed over a 10ml column of hydroxylapatite equilibrated with 10mM sodium phosphate, pH 6.8. The column was washed with 200mM sodium phosphate and elution was carried out with 250mM sodium phosphate. The 250mM sodium phosphate-eluted fraction contained E-RAF II which appeared as a single band of 66kDa in silver stained poly acrylamide gels.

**Purification of regular estrogen receptor (ER):**

The procedure developed by Zafar and Thampan (1993) was followed. The goat uterine cytosol was chromatographed over pABA. The pABA flow-through fraction of the cytosol was mixed with a suspension of Whatman DE-52 in TEMN buffer, in a beaker kept in an ice bath. The protein fraction eluted from the matrix using
TEMN buffer containing 0.2M NaCl was collected. This was mixed with a suspension of phospho cellulose in TEMN buffer containing 0.2M NaCl. The gel was washed with this buffer and the receptor bound to the matrix was eluted with the buffer containing 0.3M NaCl. The NaCl concentration of this eluate was diluted to 50mM following which the material was mixed with a suspension of ss-DNA-Sepharose in TEMN buffer. The gel was washed with the buffer and the receptor bound to the DNA was eluted with the buffer containing 10mM ATP. The ATP-eluted fraction contained purified estrogen receptor, appearing as a single band of 66kDa in silver stained gels.

**Purification of non activated estrogen receptor (naER):**

The method developed by Anuradha et al., (1994) was followed. Minor modifications were introduced into this procedure in order to increase the yield of the protein. Goat uterus was homogenized (20%) in TEMN buffer using a Polytron PT 45-80 homogenizer. The cytosol, prepared following the method described by Van der Hoeven (1981) was chromatographed on a column of Whatman DE-52. After extensive washing, once each with TEM buffer and TEM buffer containing 0.3M NaCl, the proteins bound to the matrix were eluted using TEM buffer containing 0.5M NaCl. The NaCl concentration in the eluate was diluted to 0.05M and solid sodium molybdate was added to this preparation giving a final concentration of 10mM. This material was chromatographed over a column of hsp-90-Sepharose. The column was washed extensively and the proteins bound to hsp-90-Sepharose were eluted using zero molybdate TEM buffer. Fractions collected were analyzed for their
absorbance at 280nm. The peak fractions were chromatographed on a column of phosphocellulose equilibrated with TEM buffer to achieve final purification of the receptor. The naER was eluted from the phosphocellulose using a NaCl concentration between 0.6M and 0.7M. The purified protein appeared as 66kDa band in silver-stained SDS gels.

**FITC-Labeling of naER and E-RAF:**

naER and E-RAF were labeled with fluorescein isothio cyanate isomer I following the method of Nargessi and Landon (1981). The purified protein was dissolved in 0.1M NaHCO buffer pH 9.0 (1mg of protein was dissolved in 100μl of buffer). To this 100μl of 1mM solution of FITC in buffer was added and incubation was carried out for 12 hours at 30°C. The free FITC was removed by gel filtration on a Sephadex G-25 column. The labeled protein that was eluted in the void volume was collected and concentrated for further use.

**Antibodies:**

Equal volume of the antigen (1mg/ml) and complete Freund's adjuvant were mixed. The mixture was injected into the foot pad and four subcutaneous locations of a New Zealand white rabbit. Three booster injections with two weeks intervals between two boosters were given mixing the antigen with incomplete Freund's adjuvant. Bleeding of the rabbit from the ear vein was carried out a week after the third booster injection. and the serum was collected.

The serum collected was chromatographed over a DE-52 column, equilibrated with 10mM Tris-HCl buffer pH 7.6. The flow-through
fraction which contained Ig G was collected. Affinity columns were prepared by coupling the immunogen covalently to CNBr activated Sepharose 4B. The crude IgG fraction was chromatographed over the affinity column equilibrated with 10mM Tris-HCl pH 7.6. The column was washed with three column volumes of the buffer and the IgG bound to the column was eluted using 4M MgCl₂. The eluate was collected in an equal volume of 100mM Tris. The resulting mixture had a pH of 7.2. The eluted protein was concentrated by ultrafiltration and was chromatographed over a column of Sephadex G-25 equilibrated with salt free, 10mM Tris-HCl buffer, pH 7.6. The void volume was collected and used as a monospecific polyclonal antibody preparation.

**Western blotting:**

Western transfer of proteins from SDS gels to nitrocellulose membranes using a Bio-Rad transblot equipment was done as described by Towbin et al., (1979). The pure proteins were subjected to SDS-PAGE. Following electrophoresis the gel was equilibrated with Towbin transfer buffer for 30 minutes. Two pieces of Whatman No. 3 filter papers were cut to the size of the gel and the nitrocellulose membranes were saturated by soaking in transfer buffer for 30 minutes. A pre wetted fiber pad and the soaked filter paper sandwiched the gel and nitrocellulose sheet. Air bubbles were removed at each step of the assembly of the sandwich by ‘roll-pin’ exclusion using a glass pipette. The transfer was carried out for 5 hours at 60V (limit 0.21). The proteins transferred to the nitrocellulose membrane were exposed to the primary antibody (1:500 dilution) at room temperature for
12 hours, after pretreatment of the membrane with 3% BSA for one hour. The membrane was then coupled to HRPO-coupled anti rabbit IgG for 2 hours at room temperature. The membrane was washed with Tris buffered saline and stained with 4-chloro-1-naphthol.

Quantitation of proteins by Bradford's assay:

To 100μl of test sample 1ml of Bradford's reagent was added, mixed and the colour developed was read at 595nm after 5 minutes. BSA was used as the protein standard.

DNA estimation in rat uteri:

Rat uteri were homogenized (10%) in 10mM Tris-HCl ph 7.5. Equal volume of 10% trichloro acetic acid (TCA) was added to the homogenate following which the acidified homogenate was incubated in a boiling water bath for 15 minutes. Following centrifugation at 5,000xg, 0.1ml of the hot TCA extract was mixed with 5% TCA in order to make up the final volume to 1ml. 4ml of freshly prepared diphenylamine solution (3.0g of recrystallized diphenylamine in 300ml of glacial acetic acid and 8.25ml of concentrated sulphuric acid) was added to the 10 fold diluted TCA extract. The mixture was incubated in a boiling water bath for 10 minutes. The tubes were immersed in an ice water bath to facilitate rapid cooling and the absorbance was measured at 600nm. Calf thymus DNA was used as the DNA standard.

Purification of type I, III, and V collagens from goat uteri:

Different types of collagens were purified according to the procedure described by Miller et al., (1982) using neutral salt solvent.

Goat uteri were homogenized in TEMN buffer (0.05M Tris-HCl
pH 7.5, 1mM EDTA, 4.5M NaCl with 0.02M PMSF). Following centrifugation at 10,000xg for 10 minutes the pellet was collected. The pellet was subjected to washing with DDW to reduce the concentration of NaCl and was suspended in 20 volumes of solvent containing 0.05M Tris pH 7.5 and 1.0M NaCl. Extraction was carried out into the neutral salt solvent for 24 hours and the extraction mixture was filtered through a cheese cloth. The collagen solution was subjected to centrifugation at 100,000xg for 2 hours to get a clear supernatant that contained different types of collagens.

Collagens differential were separated by differential salt precipitation. Type III collagen was precipitated from the extract by the addition of solid NaCl to 1.8M. This was followed by the precipitation of collagen type I by increasing the salt concentration of the solution to 3.5M. Finally type V collagen was precipitated with 4M NaCl. Precipitates were recovered by centrifugation at 35, 000xg for 1 hour. All the 3 collagens were dissolved in 0.5M acetic acid and re-precipitated by the addition of 2M NaCl. The different collagens thus obtained were used in collagenase assay.

**Purification of collagenase:**

Crude collagenase was prepared from rat uteri as described by Weeks et al., (1976). The uteri were homogenized in 10 volumes of ice cold 0.01M calcium chloride solution containing 0.25% Triton X-100. The homogenate was centrifuged at 4 C for 20 minutes to obtain the 6,000xg pellet. The pellet was resuspended in 0.1M Tris-HCl buffer, pH 7.6 and incubated at 60 C for 4
minutes. After cooling in ice it was subjected to centrifugation at 10,000x g for 10 minutes and the supernatant collected was used for the assay of collagenase activity.

**Collaenase assay using type L, III and V as substrate:**

100μl of crude collagenase was incubated with 150μg of collagen at 37 C for one hour. Undigested collagen was removed by centrifugation at 5,000xg for 10 minutes. To the supernatant equal volume of 12N HCl was added at 100°C in order to hydrolyze the peptides released by the action of collagenses. The hydrolysate was evaporated at 130°C. The residue left behind was assayed for *hydroxyproline*.

**Measurement of hydroxyproline:**

*Hydroxyproline* (OHP) was assayed as described by Woessner et al., (1961). *Chloramine-T* solution was made by dissolving 1.41g of chloramine-T in 20ml of DDW to which 30ml of methyl cellosolve and 50ml of 0.26M citrate buffer pH 6.0 containing 0.21M acetic acid and 1.45M sodium acetate trihydrate were added. 1ml of chloramine-T solution was added to each tube containing the hydrolyzed peptide residues of collagen. Hydroxyproline was allowed to be oxidized for 20 minutes following which the reaction was terminated by the addition of 1ml of 3.15M perchloric acid. The contents of the tubes were mixed and allowed to stand for 5 minutes. Finally 1ml of 20% of *p*-dimethyl amino benzaldehyde dissolved in methyl cellosolve was added to the mixture and the tubes were placed in a water bath at 60°C for 20 minutes. The absorbance at 557nm was determined spectro photometrically.
**Purification** of calcium activated neutral protease (CANP) from goat uterus:

Goat uteri were obtained from a local slaughter house, transported in ice to the laboratory, and kept stored at -75°C until used. A 20% homogenate of the tissue in the homogenization buffer (20 mM NaHCO₃, 1 mM EDTA, pH 7.5) was made using a blender. The homogenate was filtered through glass wool and centrifuged at 12,000x g for 20 minutes. The supernatant was recentrifuged at 105,000x g for 2 hours using a sw-28 rotor in a Beckman ultracentrifuge, to obtain the uterine cytosol.

The cytosol thus obtained was chromatographed over a column of p-aminobenzamidine agarose (pABA) equilibrated with 20 mM Tris-HCl pH 7.5 containing 100 mM NaCl, 5 mM EDTA and 10 mM β-mercaptoethanol. The proteins bound to the pABA column were eluted with 2 mM benzmaidine-HCl in the same buffer. The eluate was chromatographed over a column of hydroxylapatite equilibrated with 10 mM sodium phosphate buffer following which the column was washed with the buffer. The enzyme bound to the column was eluted with 250 mM sodium phosphate buffer, pH 7.5. The sodium phosphate eluate was chromatographed over a column of DE-52, equilibrated with TN buffer (10 mM Tris, 100 mM NaCl pH 7.5). The protease bound to the column was eluted using a linear gradient of NaCl (100 mM to 1 M). Fractions (2 ml each) collected were subjected to CANP assay. All the isolation steps were carried out at 4°C, unless otherwise mentioned.

**CANP assay:**

Casein was used as the substrate and it was prepared as
described by Ishura et al., (1978). The preparation contained 0.24% casein, 5mM CaCl₂, 25mM β-mercaptoethanol and 0.1M sodium glycerophosphate, pH 7.5. To 500μl of the substrate, 250μl aliquots of the DE-52 fractions were added and the mixture was incubated at 30°C for 1 hour. The reaction was stopped by the addition of 750μl of 10% trichloroacetic acid (ice cold) following which the tubes were kept in ice for an additional 30 minutes. The supernatant was collected following centrifugation in a J2-21 Beckman refrigerated centrifuge using a JA 18.1 rotor. The absorbance at λ 280 nm of the TCA supernatants was measured in a Shimadzu spectrophotometer. The result of CANP action was examined using Bradford's assay (Bradford, 1976). One unit of the enzyme activity is expressed as equivalent to that activity which enhances digestion of casein by 0.1 absorption unit at 595nm in Bradford's assay.

E-RAF fragmentation by CANP and separation of the fragments on ss-DNA Sepharose:

To 100μg of E-RAF in the substrate buffer 200μl of enzyme fraction (10 units) was added. The mixture was incubated at 37°C for one hour. Reaction was stopped by the addition of 100μl of DE-52 slurry equilibrated with TEM buffer. Following centrifugation at 4,000x g for 5 minutes the supernatant was collected and chromatographed over a column of ss-DNA Sepharose. Flow-through fractions from this column were collected and the peptide bound to the DNA was eluted using TEM buffer containing 10mM ATP. Both the fractions were concentrated and subjected to SDS-PAGE analysis.
**nissociation of naER and E-RAF heterodimer:**

The purified naER (100μg in TEM) was incubated overnight with 20nm H-estradiol at 4°C. Following this an equal volume of dextran coated charcoal suspension (1% charcoal and 0.1% dextran in TEM buffer) was added to the incubation medium and the mixture was left in ice for 30 minutes. The supernatant was collected following centrifugation and was chromatographed over an E-RAF Sepharose column, E-RAF immobilized on CNBr activated Sepharose 4B. The column was washed with TEM and elution was carried out using varying concentrations of salt (sodium chloride 0.2M to 2M) and also with 10mM concentrations of various amino acids belonging to different chemical categories (leu, gly, cys, tyr, lys, asp, val, phe, met, and ile). The radioactivity associated with the eluates was measured using Bray’s mixture in a LS 1801 Beckman liquid scintillation counter.

**Experiments to identify the E-RAF fragment involved in the dimerization:**

To 0.5 ml of FITC labeled naER (100 μg) 100μg of E-RAF in 0.5ml of TEM was added. The mixture was incubated at 30°C for one hour. Both naER and E-RAF were dissociated by the addition of leucine to a final 10mM concentration. Separation of naER and E-RAF was carried out by the addition of 250μl of DE-52 slurry equilibrated with TEM (E-RAF appears in the flow-through fraction while the naER remains bound to DE-52). The mixture was subjected to centrifugation and the supernatant containing the E-RAF was collected. This E-RAF was treated with CANP as mentioned earlier and the fluorescence associated with the two fragments measured
in a fluorescence spectrophotometer (Shimadzu RF-5000) at excitation wavelength of 470nm and emission wavelength of 520nm.

Assay for dimerization between naER and one of the two E-RAF fragments:

To 100µg of FITC labeled naER in TEM an equal amount either of α or of β fragment of the E-RAF was added and the reaction mixture was incubated at 30°C for one hour. The complex was then subjected to gel filtration on a column of Sephadex G-100 equilibrated with the TEM containing 0.3M NaCl. The column was calibrated previously with blue dextran and the marker proteins and the fractions (4ml/fraction) were collected. An elution volume that was three times the void volume (bed size of 90ml and void volume of 28ml) was collected. Fluorescence associated with each fraction was measured as mentioned before.

Effect of estradiol on E-RAF-naER dimerization:

FITC-naER (100µg) was incubated with varying concentrations of unlabeled estradiol (0-20nM) overnight at 4°C which was followed by the addition of 100µg of E-RAF in TEM buffer to the incubation mixture. The incubation was continued for one hour at 30°C. Dissociation of both the proteins was carried out by the addition of leucine to a final 10mM concentration. Separation of E-RAF from naER was achieved following the addition of 100µl DE-52 slurry into the reaction mixture. The fluorescence associated with the DE-52 flow-through fraction (E-RAF) was measured in order to determine the extent of dimerization in the presence of varying concentrations of estradiol.
cross reactivity of naER, E-RAF, and the α and β fragments of E-RAF with anti polylaspartate antibodies:

Following SDS-PAGE, naER, E-RAF, and the α and β fragments of the E-RAF were transferred to nitrocellulose membranes as described by Towbin et al., (1971). The immuno blots were exposed to anti polylaspartate IgG for 12 hours at room temperature. Subsequently the membranes were exposed to HRPO coupled anti rabbit IgG for 2 hours. Blots were washed with Tris buffered saline and stained with 4-chloro-1-naphthol, a HRPO substrate. Assay for interdependence of E-RAF and naER for the nuclear migration of the heterodimer:

This experiment was designed in order to look into the conditions that influence the transport of naER and E-RAF to the nucleus. Goat uterine nuclei in sucrose buffer (50mM Tris-HCl pH 7.6 containing 2mM MgCl₂, 20mM KCl, 1mM CaCl₂, 250mM sucrose and 0.2mM PMSF) used in this procedure was prepared as mentioned earlier. To show the dependence of E-RAF on the transport of the heterodimer into the nucleus, 100μg of naER in 100μl of TEM was incubated with 20nM of ³H-estradiol 11(3, overnight at 4°C. To this equal volume of dextran coated charcoal was added. Following centrifugation, after an incubation in ice for 30 minutes, the supernatant was collected. This was mixed with varying concentrations of E-RAF (0-100μg in TEM) and incubated for another 30 minutes at 30°C. The nuclear suspension (200 μl/tube corresponding to 1mg of DNA in sucrose buffer) was added to the reaction mixture mentioned above and the incubation at 30°C was continued for one hour. The pellet was collected after
centrifugation at 1000g for 10 minutes and washed twice with the sucrose buffer. Radioactivity associated with the nuclei was measured following extraction with 1ml ethanol in a Beckman LS 1801 liquid scintillation counter using a toluene based scintillation cocktail containing 5g PPO, 500mg POPOP/ lit of toluene: Triton x 100 (67:33 v/v) mixture.

To show the dependence of naER on the nuclear transport of the heterodimer, 100μg of FITC labeled E-RAF was incubated with varying concentrations of naER-estradiol 17β complex (0-100μg) for 30 minutes at 30°C. To this 200μl of goat uterine nuclear suspension in sucrose buffer corresponding approximately to 1mg DNA was added. The mixture was incubated for one hour at 30°C, centrifuged and the 1000X g pellet was collected. The pellet was washed twice with the sucrose buffer. The FITC-labeled E-RAF, bound to the nuclei, was dissociated by the addition of 1ml of TEM containing 0.5M NaCl. The fluorescence in the supernatant was measured at excitation wavelength of 470nm and emission wavelength of 52 0nm.

Cross reactivity of a and β fragments with anti-ER and anti-E-RAF IgGs:

Both the fragments were transferred to nitrocellulose as mentioned above and the cross reactivity of the fragments with anti-ER or anti E-RAF IgG was studied following exposure of the blots first to the primary antibody and subsequently to HRPO-coupled anti rabbit IgG. The blots were stained with 4-chloro-1-naphthol.
In Vitro incorporation of labeled amino acids into E-RAF:

The uteri from rats belonging to different experimental groups were removed, cleared free of fat and connective tissue, slit longitudinally and immediately transferred to Dulbecco’s modified Eagles medium (1 uterus/ml) deficient in phe, leu, lys, tyr, pro and met. The uteri were incubated in the medium with a H-labeled amino acid mixture (leu, lys, phe, pro, tyr) and $^{35}$S met (5µCi each) at 37°C for 5 hours. Each incubated uterus was homogenized in 3.0 ml of TEMN buffer (10mM Tris-HCl, pH 7.6 containing 1mM EDTA, 12mM monothioglycerol, 50mM NaCl and 0.2mM phenyl methylsulphonyl fluoride). The homogenate was centrifuged at 10,000x g and the supernatant was collected. To 1ml of the supernatant, 50µl of a DE-52 slurry, equilibrated with TEMN, was added and mixed gently. The tubes were incubated in ice for 30 minutes, centrifuged and the supernatant was collected. This was mixed with anti goat uterine E-RAF IgG (final dilution of 1:200 with monospecific polyclonal antibody) overnight at 4°C. Afterwards goat anti rabbit IgG was added to the mixture (1:1000 final dilution) and the incubation was continued for another 2 hours. The tubes were centrifuged at 10,000 xg for 10 minutes and the precipitates collected. All the centrifugation protocols mentioned above were carried out using a JA 18.1 rotor in a Beckman refrigerated centrifuge. The precipitates were redissolved in 500µl of TEMN buffer and filtered through Whatman GF/C glass fiber filters using a Millipore (12 placed) filtration unit connected to a vacuum pump. The filters were washed twice with 10% TCA in order to remove the unincorporated, labeled amino
acids and twice with ethanol to dry them. The radioactivity associated with the dry filters was measured using a scintillation cocktail (5g PPO and 500mg POPOP/ liter of toluene: Triton X-100 67:33 (v/v) in a Beckman LS 1801 liquid scintillation counter.

**CNBr fragmentation of E-RAF II and analysis of the cross reactivity of the fragments with anti-ER IgG:**

E-RAF II was subjected to cyanogen bromide fragmentation following the method of Kasper (1970). The fragments were chromatographed over a column of anti ER IgG-Sepharose equilibrated with 10mM Tris-Hcl, 12mM mono thioglycerol, pH 7.2. The column was washed with this buffer. The protein fragments bound to the column were eluted with 4M MgCl₂. Both the flow through fraction and the 4M MgCl₂ eluate of the column were collected. The eluate was collected in a beaker containing equal volume of 100mM Tris. The resulting mixture displayed a pH of 7.2. Estrogen receptor was also treated with CNBr in a similar fashion and the resulting fragments were chromatographed on a column of anti ER IgG-Sepharose. The flow through and the 4M MgCl₂ eluate of the column were collected. The peptide fragments of ER and E-RAF were transferred to a nitrocellulose membrane which was in turn exposed first to anti E-RAF IgG for 12 hours and later to the secondary antibody, anti rabbit IgG coupled to HRPO for 2 hours. The proteins were stained using a HRPO substrate, 4-chloro-1-naphthol.

**ELISA FOR E-RAF:**

Indirect microplate ELISA procedure described by Voller et
al., (1987) was followed with modifications. Varying concentrations of E-RAF (0-200 ng) in 200μl coating buffer (10mM carbonate and 40mM bicarbonate buffer pH 9.6, having 2mM sodium azide) was coated on to a 96 well ELISA plate and the plate was incubated for 18 hours at 4°C. Following this the plate was blocked using 5% BSA in the coating buffer (200μl/well) for 4 hours. The plate was washed twice with phosphate buffered saline pH 7.6, containing 0.05% Tween-20. Following this 200 μl of rabbit anti goat E-RAF IgG was added (1: 500 dilution) to each well and the incubation was continued at 37°C for 6 hours. The plate was subjected to the washing procedure twice again with PBS-Tween, following which the secondary antibody (goat anti rabbit IgG coupled to HRPO) was added to the wells (200μl/ well). Following incubation for 2 hours at 37°C the plate was subjected to washing and the substrate for HRPO, 3, 3’,5, 5’-tetramethyl benzidine (TMB) was added to the wells (200μl/ well). The reaction was stopped after 30 minutes by the addition of 50μl of 1N sulphuric acid. The colour developed was read in a Thermomax microplate reader at A 450nm.

The tissue samples, for the measurement of E-RAF concentration, were homogenized (20% homogenate) in the coating buffer. The homogenates were centrifuged at 10,000 g and the supernatants collected. To 1ml of the supernatant 50μl of DE-52 slurry, equilibrated with the coating buffer, was added. The tubes were incubated in ice for 30 minutes and centrifuged following which the supernatant was collected. An aliquot of this supernatant was taken for the estimation of E-RAF through ELISA.