CHAPTER II

MATERIALS AND METHODS
**MATERIALS**

2,4,6,7 $^{[3]H}$-estradiol-17β (sp.act. 101 Ci/mmol) was obtained from Amersham. γ-32p adenosine 5'-triphosphate (sp. act. 3000 Ci/mol) was purchased from Board of Radiation and Isotope Technology, Bhabha Atomic Research Centre, Bombay. Adenosine 5'-triphosphate (ATP) was purchased from Boehringer Mannheim. Phenylmethyl sulphonyl fluoride (PMSF) and silver nitrate were obtained from Merck (Germany). Diethylstilbestrol (DES), quercetin, 2,5-diphenyloxazole (PPO), 1,4-bis 2,5-phenyloxazoyl benzene (POPOP), dithiothreitol (DTT), estradiol-17p, testosterone, progesterone, dexamethasone, tamoxifen, Triton X-100, Tyrophostin 25, Nonidet P-40, 4-chloro-1-naphthol, phosphoaminoacids, Tween 20, bovine serum albumin (BSA), methyl αD-glucopyranoside, heparin disodium salt, fluorescein isothiocyanate (FITC) isomer I, adenosine 5'-monophosphate, cytidine 5'-monophosphate and glycopeptidase F (N-glycopeptidase F; EC. 3.2.2.18 and 3.5.1.52) were purchased from Sigma Chemical Co., St. Louis, U.S.A.. Sepharose 4B, Sephadex G-100, G-50 and G-25 were purchased from Pharmacia Fine Chemicals, Sweden. p-amino benzanidine agarose was purchased from Pierce Chemical Co. DEAE cellulose (DE-52), cellulose cc31 and phosphocellulose were purchased from Whatman Biosys. Ltd., England. Nitrocellulose membranes were purchased from Schleicher & Schuell Inc., U.S.A. X-ray film was obtained from Konica and Solidex X-ray developer from May and Baker, Photochem, India; fixer was the acid fixing salt with hardener purchased from Kodak, India. Hydroxylapatite (Bio Gel HTP) was purchased from Bio-Rad. A mouse monoclonal antibody (Mab 17) and a polyclonal antiserum (SB 208) that specifically recognizes the estrogen receptor were a kind gift of Dr. Richard J. Miksicek (Dept. Pharmacol. Sci., State Univ. of NY, Stony Brook, NY). Mab 17 has been shown to recognize an aminoterminal epitope (34-48 amino acids) of the human estrogen receptor. The polyclonal antiserum recognizes multiple determinants (Neff, et.al., (1994) Mol. Endocrinol. 8; 1215-1223). Goat anti rabbit IgG, horse radish peroxidase coupled anti rabbit IgG, horse radish peroxidase and concanavalin A were obtained from Banglore Genei, Bangalore. All other chemicals and reagents used were of analytical grade, obtained from local commercial establishments.
BUFFERS USED IN THE STUDY

Buffers used in the isolation of nuclei:

**Buffer A:** 50mM Tris-HCl, pH 7.6, containing 1mM CaCl2.2H2O, 2mM MgCl2.6H2O, 20mM KCl, 0.2mM phenyl methyl sulphonyl fluoride (PMSF) and 0.25M sucrose.

**Buffer B:** Same as buffer A except for sucrose which is 0.34M sucrose.

Buffers used in the purification of hsp 90:

**+MO buffer:** 10mM potassium phosphate buffer pH 7.0 containing 10mM sodium molybdate, 12mM monothioglycerol and 0.2mM PMSF.

**-MO buffer:** Same as +MO buffer except that, the buffer did not contain any molybdate salt.

Buffers used in the receptor purifications:

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

**TEMN buffer:** TEM buffer containing 50mM NaCl.

**TCMN buffer:** 10mM Tris-HCl, pH 7.6, containing 0.25M sucrose, 1mM CaCl2, 2mM MgCl2, 12mM monothioglycerol, 50mM NaCl and 0.2mM PMSF.

Buffers used in the purification of RNA polymerase II:

**Buffer 1:** 50mM Tris-HCl, pH 7.9, containing 20mM EDTA, 0.125% p mercaptoethanol, 25% glycerol, 0.2mg/ml soyabean trypsin inhibitor and 0.2mM PMSF.

**Buffer 2:** Same as buffer 1 except that the glycerol concentration was reduced to 10% and in addition it contained 0.2M ammonium sulphate.
Buffer 3: Same as buffer 1 except that 0.1 mg/ml soyabean trypsin inhibitor was used and 0.25 mM DTT replaced Pmercaptoethanol.

Buffers used in the western blotting:

Towbin buffer: 25 mM Tris buffer containing 192 mM glycine and 20% methanol; pH was adjusted to 8.3 with Tris/glycine.

TN buffer: 10 mM Tris-HCl, pH 7.6, containing 150 mM NaCl.

TNT buffer: TN buffer containing 0.1% Tween 20.

Bradford's reagent:

10 mg of coomassie Brilliant blue G-250 was dissolved in 5 ml of ethanol. To this 10 ml of 85% (W/V) phosphoric acid was added. The resulting solution was made up to 100 ml with DDW and was finally filtered through Whatman No. 1 filter paper.

TCMMN buffer: 10 mM Tris-HCl, pH 5.0 containing 1 mM each of MgCl₂, CaCl₂ and MnCl₂ 50 mM NaCl and 0.2 mM PMSF.

Buffers used in plasma membrane isolation:

1). 1 mM NaHCO₃ buffer pH 7.5.

2). Sucrose solutions of 0.25 M, 2.0 M, 63%, 49%, 41% and 37%, all made W/V in NaHCO₃ buffer pH 7.5.
PROCUREMENT OF TISSUE:

Goat uteri were obtained from a local slaughter house, brought in ice to the laboratory and stored at \(-75^\circ\text{C}\) until used in experiments.

PREPARATION OF GOAT UTERINE CYTOSOL:

Goat uterine cytosol was prepared as described by Van der Hoeven (1981). The uteri were minced finely. A 20\% homogenate of the tissue was made in TEMN buffer using a Waring blender. The homogenate was filtered through glass wool and the filtrate was centrifuged at 15,000 X g for 15 min. The post-mitochondrial supernatant was collected. Polyethylene glycol 6000 was added to the supernatant to a final concentration of 5\%. The suspension was allowed to stir for 30min at 4oC. Following centrifugation at 15,000 X g for 15 min, the supernatant was collected and used as the cytosol.

The conventional method was used in the initial experiments for obtaining uterine cytosol. In this method, the post-mitochondrial supernatant was centrifuged at 1,05,000 X g for 2 hrs using a SW 28 rotor in a Beckman ultracentrifuge. The ensuing supernatant was collected.

ISOLATION OF NUCLEI:

The goat uterine nuclei were purified following the method described by Thainpan (1985). Fresh uteri were used for the isolation of nuclei. The finely minced uteri were homogenized in 20 volumes of buffer A using a Polytron homogenizer (PT 45-80) at a setting of 4 (1 min X 5 with 5 min interval). The homogenate was filtered through
glass wool. The filtrate was centrifuged at 800 X g for 15 min. The pellet was treated with 0.1% Triton X 100 in buffer A. The pellet was washed twice with detergent free buffer A. The pellet was resuspended in 10 ml of buffer A, 5 ml of this suspension was carefully layered over 30ml of buffer B and centrifuged as before. The purified nuclear pellet suspended in buffer A, appeared to be free of any cytoplasmic contamination, when examined under a phase contrast microscope.

SOLUBILIZATION OF NUCLEAR MACROMOLECULES:

The nuclear pellet was suspended in TEM buffer containing 0.3M NaCl. This suspension was sonicated using a MSE sonicator fitted with a microtip (15 sec X 6 at a setting of 6 with an interval of 1 min). After sonication the material was centrifuged at 15,000 X g for 15 min. The concentration of NaCl in the supernatant was reduced to 0.05M following dilution with salt free buffer.

ISOLATION OF PLASMA MEMBRANE:

This was carried out following two different procedures.

PROCEDURE 1:

The method described by Yamashita and Field (1974) was followed. Buffers used in this procedure were: 1). 1mM NaHCO3 buffer pH 7.5. 2). Sucrose solutions of 63%, 45%, 41%, and 37%, all made w/v in 1mMNaHCO3 buffer pH 7.5.

Finely minced uteri were homogenized (20% homogenate) in the NaHCO3 buffer using a Polytron homogenizer. The homogenate was kept in ice for 30 min. to allow cell lysis. The homogenate was filtered through nylon cloth of 80-120μ mesh and the filtrate was subjected to centrifugation at 1900 X g for 25 min. The resulting pellet containing
most of the plasma membranes and nuclei were suspended in 63% sucrose solution. The
suspension was collected in centrifuge tubes (38ml capacity) of SW28 rotor in a
Beckman ultracentrifuge. Sucrose solutions of 63% (12ml), 45% (9ml), 41% (11ml)
and 37% (4ml) were layered successively over the sample. The tubes were centrifuged at
63,000 X g for 2hrs; plasma membranes with minimum contamination were obtained at the
interphase between 37% and 41% sucrose solutions. This band was collected and
recentrifuged at 30,000 X g. The pellet was used for further analysis.

PROCEDURE 2:

The yield of the plasma membranes was very low from the procedure 1. Therefore
another well cited procedure, described by Kidwai (1974), was followed. The buffers
used in this method were: 1mM NaHCO3, pH 7.5, containing either 0.25M sucrose or
2M sucrose.

The finely minced uteri were homogenized (20%) in 1mM NaHCO3 buffer pH 7.5,
containing 0.25M sucrose, using a Polytron homogenizer at a setting of 5 for 15 secs (x
5 with 60 secs intervals). The homogenate was filtered through a 80-120u. nylon mesh.
The filtrate was centrifuged at 7500 X g for 30 min. The resulting pellet was suspended
in 1mM NaHCO3 buffer containing 0.25M sucrose. This suspension was layered over a
0.25M to 2M sucrose density gradient. The tubes were centrifuged at 1,05,000 X g for
2.5hrs in a Beckman ultracentrifuge using a SW28 rotor. The plasma membrane formed
a band at the interphase of the loading and the gradient. This band was collected using a
Pasteur pipette, diluted to reduce the sucrose concentration and centrifuged again at
1,00,000 X g for 30min. The purified plasma membrane was collected as a pellet. This
preparation was used in the subsequent hormone binding studies.
CYANOGEN BROMIDE ACTIVATION OF SEPHAROSE 4B AND LIGAND COUPLING TO CNBr ACTIVATED SEPHAROSE 4B:

Cyanogen bromide activation of Sepharose 4B or agarose was carried out following the procedure described by March et al. (1974). One volume of Sepharose 4B was washed with three volumes of double distilled water. The matrix was further washed with 1M sodium bicarbonate. The matrix was mixed with 1M sodium bicarbonate and was allowed to stir slowly. The speed of stirring was increased followed by the addition of 0.05 volumes of 2 mg/ml of cyanogen bromide solution in acetonitrile, all at once. After stirring the slurry vigorously for 15 min, it was transferred to a coarse sintered funnel and was washed with 10 volumes of 0.1 M sodium bicarbonate, pH 9.5. Ligand was dissolved in 1 volume of 0.2M sodium bicarbonate pH9.5. Coupling of the ligand was done for 24 hrs in the absence and 4 hrs in the presence of 1M glycine at 4°C. This matrix 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 at 4°C as a suspension in the TEM buffer containing 0.04% sodium azide.

PURIFICATION OF HSP 90 AND PREPARATION OF HSP-90 SEPHAROSE:

The heat shock protein 90 was purified following the procedure described by Sullivan et al., (1985). Goat liver cytosol was made in the +Mo buffer. The liver cytosol was stirred with 0.1 volume of phosphocellulose equilibrated with +Mo buffer which was then filtered under vacuum. The filtrate was collected on heparin agarose equilibrated with +Mo buffer. The matrix was stirred for 20 min. After washing the matrix-bound the proteins were eluted with three volumes of -Mo buffer. The heparin-
agarose eluate was mixed with DEAE Sepharose equilibrated with -Mo buffer and stirred for 20 minutes. The matrix was pelleted following centrifugation and was washed with 20 volumes of -Mo buffer. After packing the matrix in a glass column the bound-proteins were eluted with -Mo buffer containing 0.3M KCl. The eluate was collected as 2ml fractions which were subjected to absorbance measurement at 280 nm. The peak absorbance fractions were pooled and concentrated.

Further purification was achieved by resolving the proteins on a precalibrated column of Sephadex G-100. The fractions which contained hsp-90 were pooled, dialyzed and lyophilized. The purified hsp-90 (judged by SDS-PAGE) was coupled to CNBr activated Sepharose 4B as described earlier.

**PREPARATION OF SINGLE STRANDED DNA SEPHAROSE COLUMN:**

**ISOLATION OF DNA:**

The DNA was isolated following the method of Marmur (1961). Nuclei were isolated from goat liver following the method of Thampan (1985). The purified nuclei were suspended in saline EDTA containing 25% sodium dodecyl sulphate. The mixture was incubated for 10 minutes in a water bath at 60OC following which it was cooled. Sodium perchlorate was added to the viscous suspension to a final concentration of 1M. The suspension was mixed thoroughly with equal volume of chloroform-isoamylalcohol (24:1 v/v) in a ground glass stoppered flask for 30 min. The emulsion was centrifuged at 5000 X g for 15 minutes. The upper aqueous phase was withdrawn carefully. Two volumes of ethanol was layered gently on the aqueous layer in order to precipitate the nucleic acids. The precipitate was dispersed in saline citrate and subjected to extraction with chloroform-isoamylalcohol (25:1 v/v) until very little protein remained at the interphase. The DNA was reprecipitated with alcohol.
COUPLING OF DNA SEPHAROSE 4B:

The DNA was covalently coupled to CNBr activated Sepharose 4B as described by March et al. The DNA was denatured before coupling was done. It was dissolved in 0.5 M NaOH, following which it was heated in a boiling water bath for 15 min. Equal volume of 0.4M sodium bicarbonate solution, pH 9.5, was added. The denatured DNA was coupled to one volume of CNBr activated Sepharose 4B. After coupling the gel was suspended in TEMN buffer containing 0.04% sodium azide and was stored at 4°C.

PREPARATION OF CONCANAVALIN A SEPHAROSE 4B:

Commercially available concanavalin-A was coupled to CNBr activated Sepharose 4B. 10mg of Con-A was coupled to 15ml of CNBr-activated Sepharose 4B as described by March et al., (1974) After coupling the gel was suspended in Tris-HCl pH 7.6 buffer containing 0.04% sodium azide.

PREPARATION OF ANTI PHOSPHOTYROSINE IgG SEPHAROSE:

Phospho tyrosine was coupled to BSA using the glutaraldehyde method described by Philanjanmeni et.al., (1985). The detailed method was described under the immunological method's section. The purified IgG was checked for its specificity for phosphotyrosine by western blotting. 30mg of IgG was coupled to 15ml CNBr activated Sepharose 4B as described by March et al., (1974). The gel was suspended in TEMN buffer containing 0.04% sodium azide.
SEPHADEX G-100 CHROMATOGRAPHY FOR STOKES RADIUS CALCULATION:

A Sephadex G-100 column (66 X 1.8cm) equilibrated with TEMN buffer containing 0.3 M NaCl was used for the determination of Stokes radius of the receptor protein. The column was precalibrated with proteins of known Stokes radii (BSA, ovalbumin and cytochrome C). The protein samples, concentrated to 100μl, were layered carefully over the matrix. The column was developed with TEMN buffer containing 0.3M NaCl. 1ml fractions were collected and assayed for the receptor activity. The Stokes radius was calculated as described by Siegel and Monty (1966).

PREPARATION OF HEPARIN-SEPHAROSE:

Commercially available disodium heparin (60 mg) was dissolved in 0.2M sodium bicarbonate buffer and was coupled to CNBr activated Sepharose 4B (30ml) as described earlier.
TRICHLOROACETIC ACID PRECIPITATION OF PROTEINS:

To one ml of protein (~100 µg) solution was added 500µl of 100% TCA to obtain a final 30% concentration of the acid. The tubes were left at -20°C for 15 min. The sample were thawed and the proteins were precipitated following centrifugation at 5000 X g for 15 min. The precipitate was washed twice with 100% ethanol to remove the acid. The precipitated protein was dissolved in the SDS sample buffer for electrophoresis.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS:

SDS-PAGE was performed as described by Laemmli (1970). The following solutions and buffers were made for gel polymerization and electrophoresis.

1. 30% Acrylamide solution: 30g of acrylamide and 0.8g of methylene-bis-acrylamide were dissolved in double distilled water (DDW) and the volume was made upto 100ml.

2. Lower Tris (4X1): 1 5M Tris-HCl, pH 8.8 buffer containing 0.4% SDS.

3. Upper Tris (4X): 0.5M Tris-HCl, pH 6.8 buffer containing 0.4% SDS.

4. Reservoir buffer (4X): 0.1M Tris buffer containing 0.8M glycine; pH was adjusted with Tris/glycine to 8.2.
5. **Gel running buffer (X):** 175ml of the reservoir buffer (4X) was diluted to 700ml along with 7ml of 10% SDS.

6. **APS solution:** 2% APS solution was prepared fresh immediately before use.

    10, 12 and **15% gels** were used in the studies.

**NON-DENATURING GEL ELECTROPHORESIS:**

Native gel electrophoresis was performed as described by Davis (1964). The following solutions were made in order to polymerize a native gel.

1. **Buffered TEMED:** 36.6g of Tris buffer was dissolved in 90ml of DDW and the pH was adjusted to 8.9 with HCl. Following the addition of 0.46ml of TEMED the volume was made up to 100ml.

2. **Acrylamide solution:** Prepared as described for the SDS-PAGE.

3. **Electrode buffer (10X):** 6g of Tris was dissolved along with **28.8g** of glycine in DDW. The volume was made up to 100ml.

4. **Gel running buffer (X):** 1:9 dilution was made with the electrode buffer using DDW.

5. **Sample buffer:** To 1ml of Tris-HCl, pH6.8, 1ml of glycine and 1ml of DDW containing 0.25mg bromophenol blue were added and mixed.
A 6% gel was polymerized by mixing the following solutions: 9.9ml of acrylamide, 4.5ml of buffered TEMED, 18.18ml of 0.2% APS solution and 17.4ml of DDW. The solutions were mixed and the gel was polymerized. The stacking gel was not made.

Samples for native gel electrophoresis were concentrated and made salt free by dialysis against 10mM Tris-HCl, pH 7.6. $^3$H estradiol binding to the native receptor was carried out by incubating the receptor overnight at 4°C with 20nM $^3$H estradiol before the mixture was loaded onto gels. After performing electrophoresis at 4°C, the gel was sliced into 3mm pieces which were then transferred to scintillation vials for radioactivity measurement using a solvent system of toluene and Triton X-100 (composition given elsewhere).

**ELECTROPHORETIC CONDITIONS:**

SDS-PAGE was performed at constant current (30mA) and native gel electrophoresis was carried out at constant voltage (120volts).

**SILVER STAINING OF GELS:**

The gels were stained with silver nitrate following the method of Blum *et al.*, (1987). The gels were transferred to the fixative solution which contained 50% methanol, 12% glacial acetic acid and 100μl of 37% formaldehyde/200ml. The gels were fixed for 1hr and were transferred carefully to a container having 50% ethyl alcohol solution, to remove SDS from the gel. After washing the gel for 30 minutes, the gels were incubated for one minute with sodium thiosulphate (200mg/lit). The gels were washed thrice, each for a duration of 2min, and were then incubated with a solution of silver nitrate (2g/lit containing 0.7ml of 37% formaldehyde) for a period of 30 minutes. The gel, washed thrice with DDW for durations of 2min each, was developed with a
solution of sodium bicarbonate (60g/lit containing 0.7ml of 37% formaldehyde).

The stained gels were dried using a BIO-RAD gel drier.

PROTEIN PURIFICATION METHODS

PURIFICATION OF NUCLEAR TYPE I AND TYPE II ESTROGEN RECEPTORS:

Purification of nuclear type I and type II estrogen receptors were done following the methods described by Karthikeyan and Thampan (1994a).

PURIFICATION OF NUCLEAR TYPE I ESTROGEN RECEPTOR:

The purified nuclei were sonicated in TEM buffer containing 0.3M NaCl, in order to solubilize the macromolecules. The sonicated nuclei were centrifuged at 10,000 X g for 15 minutes. The supernatant was collected and diluted to reduce the NaCl concentration. This diluted material was chromatographed over a ss-DNA Sepharose (25ml) column equilibrated with TEM buffer. The flow-through fraction from the ss-DNA column was collected and used in the purification of type II estrogen receptor. The ss-DNA Sepharose column was washed with 5 volumes of TEMN buffer. The DNA-bound receptor was eluted with TEMN buffer containing 10mM ATP. The ATP eluate was dialyzed extensively in order to remove the ATP.

The dialysate was chromatographed over a Whatman DE-52 column (20ml) equilibrated with TEM buffer. The column was washed with 5 volumes of TEM buffer. The DE-52 bound receptor was eluted with TEM buffer containing 0.2M NaCl. The eluate was collected in 2ml fractions, and subjected to DNA cellulose binding assay as
Final purification of the receptor was achieved upon performing a gel filtration on a Sephadex G-50 column (50 X 1cm) equilibrated with TEM buffer containing 0.3M NaCl. The peak fractions showing the receptor activity were pooled and layered over a column of Sephadex G-50. The column was developed with TEM buffer containing 0.3M NaCl. The void volume which contained the highly purified receptor was collected.

**PURIFICATION OF NUCLEAR TYPE II ESTROGEN RECEPTOR:**

Sodium molybdate was added to the ss-DNA Sepharose flow through fraction (from the previous experiment) to a final concentration of 10mM. This material was chromatographed over a hsp-90-Sepharose column (30ml) equilibrated with TEM buffer containing 10mM sodium molybdate. The column was washed with the same buffer and the hsp-90-bound receptor was eluted with TEM buffer containing zero molybdate. The eluate was collected in fractions, HAP binding assay was carried out as described elsewhere.

The fractions that displayed peak activity were pooled and rechromatographed on a column of phosphocellulose (20ml) equilibrated with TEM buffer. The column was washed with TEM buffer and the phosphocellulose-bound receptor was eluted with a 0-1M NaCl linear gradient in TEM buffer. The fractions collected were assayed for receptor activity using the HAP method.

**PURIFICATION OF NON-ACTIVATED ESTROGEN RECEPTOR:**

The naER from the goat uterine cytosol was purified following the method described by Anuradha et al., (1994), with slight modifications. Goat uterine cytosol was
prepared as described earlier following the method involving the use of PEG 6000. The cytosol was mixed with Whatman DE-52 (25ml) equilibrated with TEM buffer containing 0.3M NaCl. The matrix was washed twice with TEM buffer containing 0.3M NaCl. The DE-52 bound receptor was eluted with TEM buffer containing 0.5M NaCl. The eluate was diluted with salt free TEM buffer to reduce the NaCl concentration to 0.05M. Sodium molybdate was added to this to a final concentration of 10mM. The material was chromatographed over a column of hsp-90-Sepharose (30ml) equilibrated with TEM buffer containing 10mM sodium molybdate. The column was washed extensively and the hsp-90 bound receptor was eluted with TEM buffer free of molybdate salt. The eluate collected in fractions was subjected to HAP binding assay. The fractions containing peak activity were pooled and the pooled material was rechromatographed over a column of phosphocellulose (20ml) equilibrated with TEM buffer to achieve final purification of the receptor. The naER was eluted from the phosphocellulose column using a 0 to 1M NaCl gradient in TEM buffer. The eluate collected was subjected to HAP binding assay.

PURIFICATION OF PLASMA MEMBRANE ESTROGEN RECEPTOR:

The plasma membrane was isolated as described earlier. The purified plasma membranes were suspended in TEM buffer containing 0.3M NaCl and 0.2% NP-40 (v/v). This suspension was stirred for 20min at 4°C. The material was diluted with salt free buffer to reduce the NaCl concentration. This suspension was centrifuged at 10,000 X g for 15 min and the supernatant containing the solubilized proteins was collected.

The supernatant was chromatographed over a Whatman DE-52 column (25ml) equilibrated with TEM buffer. The column was washed with TEM buffer. The DE-52
bound receptor was eluted using a linear 0-1M NaCl gradient. The fractions collected were assayed for the receptor activity. The fractions which contained the peak activity were pooled, dialysed and chromatographed over a hsp-90 Sepharose column (30ml) equilibrated with TEM buffer containing 10mM sodium molybdate. The column was washed with TEM buffer containing 10mM sodium molybdate. The hsp-90 bound receptor was eluted with TEM buffer containing zero molybdate. The fractions collected were assayed for the receptor activity using the HAP method. The fractions that contained peak activity were pooled and chromatographed over a phosphocellulose column (25ml) equilibrated with TEM buffer. The column was washed with TEM buffer and the phosphocellulose-bound receptors were eluted with a 0-1M NaCl linear gradient in TEM buffer. The fractions collected were assayed for the receptor activity using the HAP method. The fractions which contained the receptor activity were analysed on a 10% SDS gel to judge the purity of the receptor preparation.

**PURIFICATION OF ESTROGEN RECEPTOR ACTIVATION FACTOR II (E-RAF II):**

The E-RAF II was purified following the procedure described by Thampan (1987; 1989). Goat uterine cytosol was prepared using PEG 6000 in TEMN buffer as described earlier. The cytosol was mixed with p-amino benzamidine Sepharose equilibrated with TEM buffer in a beaker kept in an ice bath for 20min. The unadsorbed fraction was collected carefully by decanting the solution. This fraction was mixed with 20ml of Whatman DE-52 matrix equilibrated with TEMN buffer. The DE-52 unbound fraction was collected and was chromatographed on a column of ss-DNA Sepharose (25ml) equilibrated with TEM buffer. The column was washed with TEM buffer extensively. The bound proteins were eluted with TEM buffer containing 10mM ATP. The proteins in the eluate were precipitated using ammonium sulphate at 70% saturation. The
protein pellet was redissolved in a small volume of TEM buffer and was dialysed against TEM buffer. The dialysate was mixed with 25ml of 60% hydroxylapatite (HAP) equilibrated with 10mM sodium phosphate buffer pH 6.8 containing 12mM monothioglycerol and 0.2mM PMSF. The HAP suspension was left in an ice bath for 20min. The HAP pellet was washed twice with 150mM sodium phosphate buffer pH 6.8 containing 12mM monothioglycerol and 0.2mM PMSF. E-RAF II was eluted using 250mM sodium phosphate buffer pH 6.8 containing 12mM monothioglycerol and 0.2mM PMSF. The eluate was dialysed against 10mM Tris-HCl pH 7.6 and the resulting protein appeared as a single band of 66 kDa when subjected to electrophoresis in 10% gels.

PURIFICATION OF DNA DEPENDENT RNA POLYMERASE II:

Goat uterine nuclear RNA Polymerase II was purified following the method described by Kim and Dahmus (1988).

Goat uterine nuclei were purified as described earlier. The purified nuclei were suspended in buffer 1. Triton X-100 was added to a final concentration of 0.25%. The suspension was allowed to stir for 10min at 4oC; while stirring polyethyeneimine (2μl/ml of 10% aqueous suspension) was added in drops. Stirring was continued for another 15min. at 4oC. The suspension was centrifuged at 8000 Xg for 10min. The pellet was recovered and resuspended in buffer 1. Following centrifugation at 14,600 X g for 15 min, the pellet was collected and suspended in buffer 2. Ammonium sulphate concentration in the suspension was made up to 0.2M. The suspension was centrifuged at 16,300 x g for 15 min and the supernatant was collected. The RNA polymerase II was precipitated following the addition of 0.25g/ml of ammonium sulphate. The mixture was stirred for 15 min and the pellet was collected following centrifugation of the
suspension at 25,000 X g for 30 min. The final pellet was suspended in buffer 3 having a final ammonium sulphate concentration of 0.12M.

The suspension was mixed in a beaker with a 50ml suspension of heparin Sepharose (prepared as described earlier) equilibrated with buffer 3 containing 0.12M ammonium sulphate. This was allowed to stir for 1hr at 4oC. The matrix was filtered under vacuum and was washed with three volumes of buffer 3 containing 0.12M ammonium sulphate. The heparin-bound enzyme was eluted with buffer 3 containing 0.7M ammonium sulphate. The eluate, collected through vacuum filtration, was diluted with buffer 3 to reduce the ammonium sulphate concentration to 0.1M. This diluted material was chromatographed over a 30 ml Whatman DE-52 column equilibrated with buffer 3 and the column was washed with two volumes of buffer 3. The DE-52 bound enzyme was eluted with buffer 3 containing 0.6M ammonium sulphate. Two ml fractions were collected and the absorbance at 280nm measured. The fractions showing the peak absorbance were pooled and dialysed. The enzyme was subjected to analysis on a 15% SDS gel and also on a non-denaturing gel.

**IMMUNOLOGICAL METHODS**

**DEVELOPMENT OF ANTIBODIES IN RABBITS AGAINST ER / PHOSPHO TYROSINE - BSA CONJUGATE / RNA POLYMERASE II.**

The purified proteins were mixed with equal volumes of Freund’s complete adjuvant. The mixture was injected into rabbits of an inbred strain, at 6 sub-cutaneous locations and also into the foot pad. Four booster injections were given using the protein solution mixed with an equal volume of Freund’s incomplete adjuvant at an interval of two weeks. Blood was drawn from the rabbit by cardiac puncture one
week after the fourth booster injection. The serum collected was stored at -20°C.

**PURIFICATION OF IMMUNOGLOBULIN:**

IgG was purified from the serum using the protein A agarose (Sigma) column adsorption method as described by Sambrook et al., (1989). One ml of the swollen gel packed in a Pasteur pipette plugged with glass wool was used. The column was equilibrated with 100mM Tris-HCl pH 8.0. The crude serum was mixed with 0.1 volume of 1M Tris-HCl pH 8.0 and chromatographed over a column of protein A agarose. The column was first washed with 10 volumes of 100mM Tris-HCl pH 8.0 followed by 10 volumes of 10mM Tris-HCl pH 8.0. The protein-A bound IgGs were eluted using 100mM glycine, pH 3.0. 500μl fractions were collected in microfuge tubes containing 50μl of 1M Tris-HCl, pH 8.0. The fractions were measured for their absorbance at 280nm. The peak fractions were pooled and the pH adjusted to 7.6. The material was dialysed against 10mM Tris-HCl, pH 7.6. The purified IgG fraction was stored in aliquots at -20°C.

**PREPARATION OF PHOSPHO TYROSINE-BSA CONJUGATE:**

Phospho tyrosine was coupled to BSA using glutaraldehyde, as described by Philajaniemi et al., (1987). 5mg of phospho tyrosine was dissolved in 3ml of 10mM sodium phosphate buffer, pH 7.4. This was mixed with 1mg of BSA in 1ml of 10mM sodium phosphate buffer pH 7.4. To the mixture was added, in drops, 300ml of 35% glutaraldehyde. The reaction was allowed to proceed for 24hrs in the dark following which it was stopped and the mixture was freeze dried. The lyophilized protein was dissolved in a small volume of TEMN buffer containing 0.3M NaCl and was chromatographed over a column of Sephadex G-25 in order to remove the uncoupled phosphotyrosine. The fractions containing the void volume from the column were
collected and dialyzed. Antibody in rabbits was raised against this protein conjugate as described. The IgG fraction was purified from the rabbit serum following chromatography on protein A agarose column as described earlier. The purified IgG was coupled to CNBr activated Sepharose 4B.

**WESTERN BLOTTING:**

Western transfer of proteins to nitrocellulose membrane, using a Bio-Rad Transblot equipment, was done as described by Towbin *et al.*, (1979) and Yonezawa *et al.* (1988). Following SDS-PAGE, the proteins in the gel were transferred to nitrocellulose membrane (Schleicher & Schuell, Inc.) using a Trans Blot Electrophoretic transfer cell (BIO-RAD 170-3910). The transfer was done for 3hrs at 70 volts (~0.25A), using Towbin buffer. After transfer the nitrocellulose membrane was incubated with the blocking buffer (3% BSA solution in TNT buffer) at room temperature for 60min. The nitrocellulose membrane was washed (6x5min each) using TNT buffer. The nitrocellulose membrane was then incubated overnight with primary antibody (in 3% BSA solution). After incubation the filter was washed (6x5min each) with TNT buffer. The nitrocellulose filter was incubated with secondary antibody (horse radish peroxidase conjugated anti rabbit IgG, in 3% BSA solution) for 2hrs. After incubation the membrane was washed (6x5min each) with TN buffer. The membrane was stained using 4-chloro-l-naphthol as substrate (10% methanol, 90% TN buffer, 0.25% H2O2 containing 0.3mg/ml of 4-chloro-l-naphthol).

**ENZYME ASSAY METHODS:**

Enzyme assays were carried out to check the purity of the plasma membrane in the preparation. The enzyme activities measured were succinic dehydrogenase, glucose-6-phosphatase, 5'-nucleotidase and cytidine tri-phosphatase.
SUCCINIC DEHYDROGENASE (SDH):

SDH activity was assayed according to Arrigoni and Singer (1962). The reaction mixture contained the following constituents:

**Reaction mixture:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM KH2PO4 buffer pH 7.6</td>
<td></td>
<td>0.2ml</td>
</tr>
<tr>
<td>100mM KCN</td>
<td></td>
<td>0.1ml</td>
</tr>
<tr>
<td>CaCl2 (4mM)</td>
<td></td>
<td>0.2ml</td>
</tr>
<tr>
<td>Dichloro phenol indophenol (DCPIP)</td>
<td></td>
<td>0.3ml</td>
</tr>
<tr>
<td>Succinate (0.5M)</td>
<td></td>
<td>0.1ml</td>
</tr>
<tr>
<td>DDW</td>
<td></td>
<td>2.0ml</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td>2.9ml</td>
</tr>
</tbody>
</table>

The reaction mixture was prepared immediately before use. For each assay the mixture was pipetted out into the cuvette in the following manner.

1. Reaction mixture - 2.9ml

2. Phenazine methosulphate (PMS; 10mM) - 0.05ml

3. **Sample (100μg protein)** - 0.05ml

The reaction was monitored at 600nm with 20 **secs** intervals and the rate of the reaction was calculated using a coefficient of extinction 16.9. The enzyme activity was expressed as **units/mg** protein/min, where one unit of the enzyme activity represented **1mmole** of the dye reduced per **min**. The mathematical formula used in the calculation of the enzyme activity is given below.
GLUCOSE 6-PHOSPHATASE:

Glucose 6-phosphatase activity (G-6-Pase) was measured according to Aronson and Touster (1974). The reagents used were:

1). Sodium glucose 6-phosphate 0.1M, pH 6.5.
2). Histidine, 35mM, pH 6.5. 3). Na-EDTA, 10mM, pH 7.0.

The enzyme activity was determined by measuring the rate of release of inorganic phosphate from glucose 6-phosphate. The assay mixture was made by mixing the reagents 1), 2), 3) and DDW in the ratio of 2:5:1:1.

50μl of the sample (25 μg protein) was mixed with 450μl of assay mixture and incubated for 30min at room temperature. After incubation 8% TCA (2.5ml) was added to stop the reaction. The inorganic phosphate released was estimated following Fiske-Subbarow's method (1925). The enzyme activity was expressed as mmoles of inorganic phosphate released /mg of protein/ min.

5' NUCLEOTIDASE:

5' nucleotidase enzyme activity was measured according to the procedure of Aronson and Touster (1974). The reagents used in the enzyme assay were:

1). 50mM Na-AMP, pH 7.0.
2). 0.5M glycine-NaOH buffer, pH 9.1.
3). 0.1M MgCl2 solution.

The assay mixture was made containing reagents 1), 2), 3) and DDW in the ratio of 1:2:1:5. 50μl of the sample containing 25μg of protein was mixed with 450μl assay
mixture. Incubation was carried out for 30 min at room temperature. The reaction was stopped by adding 2.5 ml of 8% TCA. The inorganic phosphate released was estimated as described earlier.

The enzyme activity was expressed as mmoles of inorganic phosphate released/mg of protein/min.

**CYTIDINE TRIPHOSPHATASE (CTPase):**

CTPase activity was estimated following the procedure of Perdue and Sneider (1970).

50 µl of the sample (25 µg of protein) was incubated for 30 min at 37°C with 1 ml of assay mixture [20 mM bicine (N,N,bis (2,hydroxyethyl) glycine), pH 7.8, containing 3 mM CTP as substrate, 3 mM MgSO4.H2O, and 250 mM sucrose]. 8% TCA was added to stop the reaction. The inorganic phosphate released was estimated as described elsewhere. The enzyme activity was expressed as mmoles of inorganic phosphate released /mg protein/min.

**INORGANIC PHOSPHATE ESTIMATION:**

The inorganic phosphate was estimated following the method described by Fiske and Subbarow (1925). The reagents used in the estimation of phosphate were:

ANSA reagent

- 7.312 g of sodium metabisulphite
- 0.25 g sodium sulphite
- 0.4 g of 1,2,4 ANSA (1,2,4 aminonaphthol sulfonic acid)
Sodium metabisulphite, ANSA and sodium sulphite were ground together to a fine mixture. 1g of this was dissolved in 25ml of DDW. 500µl of sample in TCA was vortexed with 1ml of acid molybdate. 200µl of ANSA reagent was added and incubated for 10min. After incubation the absorbance was measured at 600nm. Standard values were obtained using inorganic phosphorus solutions in the 1 to 10µg range.

**ESTROGEN RECEPTOR ASSAYS:**

**DNA CELLULOSE BINDING ASSAY:**

DNA cellulose was prepared following the method of Alberts and Herrick (1971), using goat liver DNA and Whatman cc-31. 60mg alkali-heat-denatured DNA was mixed with 60mg of native DNA and 12g of cellulose cc-31. The slurry was dried overnight, lyophylized and finally suspended in TEMN buffer. The suspension was stored, in 3ml aliquots, at -75oC.

The DNA cellulose binding assay was carried out as outlined by Thampan (1987). The estrogen receptor was incubated overnight at 4oC with 20nM 3H-estradiol, in a total volume of 200µl. The unbound hormone was separated from the bound using dextran coated charcoal (1% charcoal and 0.1% dextran suspended in TEMN buffer). The receptor-hormone complex was incubated with the DNA cellulose suspension for 30min at 30oC. The DNA cellulose pellet was washed twice with ice cold TEMN buffer. The labelled estradiol associated with the DNA cellulose pellet was extracted with 1ml of ethanol for the measurement of radioactivity.

**HYDROXYLAPATITE BINDING ASSAY:**

The HAP binding assay was carried out as described by Clark and Peck (1979).

Commercially available HAP (BIO-RAD) was used in the assay. 100µl of the
sample was incubated overnight at 4oC with 20nM final concentration of 3H-estradiol + 100 fold molar excess of diethylstilbestrol (DES). 250μl of a 60% HAP suspension in TEMN buffer was added and the mixture was incubated in ice for 15min. After washing twice with ice cold TEM buffer, the HAP pellet was extracted with 1ml ethanol. The radioactivity in the ethanol extract was measured.

**SATURATION KINETICS OF ESTRADIOL BINDING TO RECEPTORS:**

The saturation binding of the receptor was analyzed by incubating 100μl of receptor samples overnight with 2 to 40nM concentrations of 3H-estradiol + 100 fold molar excess of DES. HAP binding assay was carried out as described earlier. The saturation binding data was further analysed following the method of Scatchard (1949). The number of binding sites and equilibrium constant (Kd) were calculated from the Scatchard plot.

**NON-ACTIVATED ER ASSAY:**

100μl of receptor sample was incubated with 3H-estradiol giving a final concentration of 20nM, overnight at 4oC. The unbound hormone was removed following charcoal adsorption. The tubes were centrifuged at 5000 X g for 3min. The supernatant was added to the tubes containing E-RAF and was allowed to incubate at 4oC for 15min. DNA cellulose was added and the mixture was incubated at 30oC for 30min. The pellet was washed twice with ice cold TEMN buffer and the labelled estradiol associated with the DNA cellulose pellet was extracted with 1ml ethanol for the measurement of radioactivity.

**CYANOGEN BROMIDE FRAGMENTATION OF ESTROGEN RECEPTORS:**

Peptide mapping was carried out using CNBr as described by Kasper (1970). Chemical degradation and enzymatic digestion of proteins were employed in the study of
peptide map comparison CNBr has a specificity for methionyl residues. The cleavage of the protein was carried out in 70% formic acid. 100\(\mu\)g of pure protein was dissolved in 10\(\mu\)l of 70% formic acid. A 50 fold excess of CNBr (solid) was added and the mixture was incubated at room temperature for 1 hr. 1ml of DDW was added to stop the reaction. The peptides were lyophilized and analyzed on a 12% SDS gels.

**SUCROSE DENSITY GRADIENT ANALYSIS:**

The purified receptor (300\(\mu\)l) was layered over 4.5ml of a 5 to 20% linear sucrose density gradient prepared in TEMN buffer containing 0.3M NaCl. The tubes were subjected to centrifugation in a Beckman ultracentrifuge (L8-80M) using a vTi-80 rotor at 2,60,000 \(\times\) g for 2hrs. The fractions collected by gravity flow were subjected to estrogen receptor assays.

**CARBOHYDRATE DETECTION AND ESTIMATION:**

Carbohydrate moiety associated with the purified receptor preparation was detected using two procedures.

1). **Concanavalin A Sepharose chromatography:**

Concanavalin A (con A) was coupled to Sepharose 4B following the method described earlier. The column was equilibrated with TCMMN buffer. The purified proteins were chromatographed on a 3 ml column of con A Sepharose. The flow through volume from this column (including the washing volume) was collected in fractions. After washing the column the con A-bound proteins were eluted using the same buffer containing 0.3M methyl a-D-glucopyranoside. Fractions collected were analyzed for their absorbance at 280nm. This procedure was described originally by Lloyd (1970).
2). Glucoseprotein staining on blots:

This was carried out according to Olden and Yamada (1977). The purified protein, subjected to SDS-PAGE, was western-transferred to nitrocellulose membranes. The membranes were first blocked with 3% BSA in TN buffer for 1hr, following which, they were washed with TN buffer for 30min (3X10 min each). The membranes were further incubated with con A (2mg/ml) for 1hr. After washing for 25 min (5 X 5min each) with TN buffer, the membranes were incubated with HRPO (100 units/10ml) for 4hrs. The membranes were washed for 30 min (3 X 10min each), following which they were stained with 4-chloro-1-naphthol.

Carbohydrate content in the receptor proteins were estimated according to Dubois et al., (1956)

Standard glucose solutions of concentrations 1,2,5,10 and 20μg were made. 5% phenol reagent was prepared by dissolving 5g of phenol in 100ml of concentrated sulphuric acid.

To 2ml of the sample, containing 100μg of protein, was added 10ml of the phenol reagent. This was followed by the addition of 5ml of concentrated sulphuric acid. The vortexed samples were incubated at room temperature for 30mm. After incubation the absorbance in the samples at 490nm was measured. A standard graph was plotted with concentration versus absorbance. The carbohydrate content was calculated from the standard graph.

FLUORESCENT LABELLING STUDIES:

FITC LABELLING OF RECEPTORS:

Fluorescein isothiocyanate (FITC) labelling of proteins was carried out as described by Chard (1987). 1mg of protein was dissolved in 250μl of 0.15M
Na$_2$PO$_4$.2H$_2$O, pH 9.5. 100μl of FITC solution in the same buffer (1mg/ml) was added to the protein solution. The pH of the mixture was adjusted to 9.5 using 1M Tris. This was incubated for 2hrs at room temperature. After incubation the unbound FITC was removed following chromatography of the mixture over a column of Sephadex G-25. The covalent coupling of FITC with the protein was confirmed by viewing the SDS gel under an UV lamp.

**EXPERIMENTS TO STUDY THE INTERACTION OF THE RECEPTORS WITH RNA POLYMERASE II:**

To study the receptor interaction with RNA polymerase II the following experimental methodology was used. The FITC labelled receptors were incubated with RNA polymerase II in TEMG buffer, over a period of time, 0,5,10,15,20,30,40,50 and 60 min. at 37oC. After incubation, the tubes were cooled in ice; sodium chloride was added to the protein mixture to a final concentration of 0.5M in order to dissociate the receptor-RNA polymerase complex. Anti RNA polymerase IgG was added to this material and the dissociated RNA polymerase was immunoprecipitated as described elsewhere. The fluorescence associated with the immunoprecipitated RNA polymerase II was measured.

**PHOSPHORYLATION STUDIES:**

1). **Auto phosphorylation** of receptors:

10μl of the receptor protein (10μg) in the protein kinase assay buffer (10mM Tris-HCl, pH7.6 containing 6mM MgCl$_2$, 2mM CaCl$_2$, 12mM monothioglycerol, 0.2mM PMSF and 10μM ATP) was incubated with 5mCi/5ml [$\gamma$-32P] ATP for 1hr at 30oC. After incubation the reaction was terminated by the addition of 5ml of SDS
sample buffer (4X). The phosphorylated proteins were subjected to SDS-PAGE and the dried gels were exposed to X-ray films.

2). Detection of phospho amino acids:

The purified protein was allowed to phosphorylate in the presence of [γ-32P] ATP. After phosphorylation the proteins were precipitated with 5% TCA, washed twice with ethanol and suspended in 6N HCl (50μl). Protein hydrolysis was carried out at 110°C for 16hrs. The hydrolysed proteins were dissolved in 10μl of butanol:acetic acid:water (65:15:25) and spotted on a Whatman No. 1 chromatography paper, along with 20nM each of phosphotyrosine, phosphoserine and phosphothreonine as standards. The amino acids were allowed to separate by ascending chromatography using the butanol, glacial acetic acid, and water mixture (65:15:25). The chromatograms were dried, sprayed with ninhydrin (0.2% ninhydrin dissolved in acetone containing 0.1% pyridine) and finally exposed to the X-ray films.

3). Identification of the RNA polymerase II subunits phosphorylated:

10mg of the receptor was incubated with RNA polymerase II (20mg) in the presence or absence of estradiol. The phosphorylation was allowed to proceed as described earlier. The proteins, after phosphorylation, were subjected to SDS-PAGE followed by autoradiography.

MISCELLANEOUS:

Quantitation of proteins:

The protein estimation was carried out as described by Bradford et.al., (1976). To 100μl of the sample, 1ml of Bradford's reagent was added, mixed and the colour developed was measured at 595nm after an interval of 5min. BSA was used as the protein standard.
Radioactivity measurement:

Samples (ethanol extracts) were added to 3ml of scintillation cocktail [5g PPO/ 1 and POPOP/ 1 in tolueneTriton X-100 (67:33)]. The radioactivity was measured in a Beckman liquid scintillation counter (LS1701). Correction for quenching was carried out using the external standardization technique. Efficiency for tritium was 50-54%.

Measurement of fluorescence:

The fluorescence in the sample was measured at an excitation wave length of 492nm and emission wave length of 520nm, using a JASCO spectrofluorimeter.
Two dimensional gel electrophoresis:

Two dimensional gel electrophoresis was carried out as described by OTarrell® (1975). The buffers used were given below:

1. **IEF sample** solution: 9.5 M urea, 2% (w/v) NP-40, 2% ampholines (comprised of 1.6% of pH 4-6 and 0.4% of pH 3.5-10) and 5% β-mercaptoethanol.
2. Acrylamide solution: 28.38% (w/v) acrylamide and 1.62% bis-acrylamide were dissolved in distilled water.
3. **Stock NP-40** solution: 10% (w/v) NP-40 in distilled water.
4. Ampholines: Ampholines were used as 40% (v/v).
5. Ammonium persulphate solution: 10% (w/v) ammonium persulphate was dissolved in distilled water.
6. **Gel** overlay buffer: 8M urea in distilled water.
7. Anode electrode buffer: 0.01M phosphate buffer.
8. Cathode electrode buffer: 0.02M NaOH in distilled water (degassed and stored).
9. Sample overlay solution: 9M urea, 2% ampholines (comprised of 1.6% pH 4-6 and 0.4% pH 3.5-10)
10. Agarose solution: 1g of agarose in 100ml of SDS sample buffer (without bromophenol blue).

**Sample preparation:**

Lyophilized proteins (2µg) were dissolved in IEF sample buffer (50µl).

**First dimension:**

Isoelectric focusing gels were made in glass tubing (105 X 3 mm) sealed at the bottom with parafilm. The gel mixture (for 4 tubes) was made as given below:

- Urea: 2.25g
- Acrylamide: 0.67ml
- **NP-40** Stock solution (10%): 1ml
- Water: 0.99ml
- Ampholines pH 4-6: 200µl
- pH 3.5-10: 50µl

The gel mixture was degassed. 10ul of 10% APS solution was added followed by 7 ul of TEMED. The gel mixture was loaded into the glass tubes using a syringe with a long narrow gauge hypodermic needle. The gel was overlaid with gel overlay solution. After polymerization of the gel, the overlay solution was replaced by IEF sample buffer. The IEF sample buffer was replaced after 30 minutes with 0.02M NaOH. The parafilm at the end of the tubes were removed and placed in the electrophoretic apparatus. The lower reservoir was filled with 0.01M phosphate buffer and the upper reservoir was filled with 0.02M NaOH. The gels were pre-run for i) 15 minutes at 200 volts; ii) 30 minutes at 300 volts and iii) 30 minutes at 400 volts.

The samples were loaded, upon emptying the upper reservoir and the buffer on the top of the gel. The samples were overlaid with 10ul of sample overlay solution, then the tubes were filled with 0.02M NaOH. The upper tank was re-filled with 0.02M NaOH solution. The gels were subjected to electrophoresis at 400 volts for 12 hrs and then at 800 volts for 2 hrs.

The gels were extruded from the tubes using a syringe and a needle. The gel was equilibrated with 5ml of SDS sample buffer (without bromophenol blue) at room temperature for one hr.

Second dimension:

The second dimension gel electrophoresis was carried out as described by Laemmli (1970). 10% polyacrylamide gels of 3mm thickness were polymerized as described in this section. The upper gel was polymerized without any comb and to accommodate the IEF gel, little space was left, the IEF gel was carefully placed and was kept in position by pouring 1% agarose solution.

Electrophoresis and silver staining of the gel was carried out as described earlier.