Biochemical Estimation of Prolactin

Binding Sites
OBJECTIVES AND PLAN OF WORK

The plasma membrane is the reception site for the peptide hormones, but the hormonal activity is elicited in the cells upon internalization of the hormone-receptor complex (Kahn et al., 1981). For manifestation of the hormone effect, amount of hormone in circulation and the responsiveness of the target tissue to the specific hormone are the two important factors. This in turn depends upon the receptor level of that hormone. Any deviation of the receptor level on either side will influence the occurrence of the disease. As discussed earlier the reproductive endocrine system which changes during the life time of women may contribute considerably to the aetiology and pathogenesis of breast disease. The role of PRL in the pathogenesis of human breast cancer is still not fully elucidated. There are some studies which have reported the presence of PRL-R in human breast tumours. They all had used crude membrane preparation for determining the specific binding of PRL-R. No study has used the purified plasma membrane for this purpose.

The aim of this study was therefore to biochemically determine the PRL binding sites in the purified plasma membrane and crude membrane of the breast tissue.

We have followed the method of sucrose density gradient centrifugation (Neville DM Jr., 1960) for the preparation of purified plasma membrane from normal, benign and malignant breast tissue. The separation of cellular organelles depended upon the isopyinic densities. Crude membranes were prepared according to the method of Peyrat et al. (1982). Binding studies were done in both the types of membranes for PRL receptors.
INTRODUCTION

Breast, as mentioned earlier in Chapter II, may be the target organ for various peptide hormones and may undergo changes in response to secretion of gonadotropic hormones. In human mammary tumours the existence of PRL-R has been reported (L-Hermite Baleriaux et al., 1987). There are evidences to suggest that looking for the binding site for PRL in normal and pathological state could probably establish some therapeutic importance.

The binding activity of PRL to a specific cellular receptor has been identified in several organs of various species though the effect of PRL in all these organs is not completely known.

PRL receptor, the binding unit has been identified and purified by different biochemical techniques mainly from the rabbit mammary gland (Necessary et al., 1984; Djiane et al., 1985; Sakai et al., 1986) and some of their physico-chemical properties have been determined (Haeuptle et al., 1983; Katoh et al., 1984; Djiane et al., 1985). PRL receptors are hydrophobic glycoproteins which possess very high affinity for PRL and related hormones possessing similar biological activity, for example GH and placental lactogenic hormones. Digestion with proteases and phospholipase C destroys the activity of the receptors (Shiu and Friesen, 1974). Like the other membrane proteins, it has its hydrophobic portion embedded in the lipid layer of the plasma membrane while its hydrophilic portion, the carbohydrate moiety, protrudes outwards.
The association and dissociation constants of $^{125}$I labelled PRL are time and temperature dependent and the binding is influenced strongly by pH (optimum at 7.5-8.5). The ionic condition that is required is of calcium and magnesium ions and it remains unaffected by the presence of the wide range of low molecular weight compounds tested like steroids and nucleotides. Binding of the hormone to the receptor does not result in the destruction of biological activity of the hormone and a large portion of the hormone remains bound to the receptor inspite of mechanical stress due to homonization during membrane preparation. This is due to the slow rate of dissociation of PRL from its receptor (Birkinshaw and Falconer, 1972) or due to the irreversible nature of PRL binding to its receptor (Van der Gugten et al., 1980).

PRL-R are very short-lived molecules in the mammary gland. When the mammary gland explants are cultured in the presence of cycloheximide, 50% of PRL-R disappears from the membranes in less than 3 hrs, but the time increases to 24-48 hrs in the absence of the inhibitor. When the transcriptional activity is blocked by actinomycin D, the level of PRL is only slightly modified though the milk protein synthesis is completely prevented (Shiu and Friesen, 1980). It has been suggested that the short life of PRL-R is affected probably at translational or degradative steps. Degradation of the receptor occurs in the lysosomes as inactivation of these organelles by amines, such as chloroquines, ammonium chloride, results in a rapid increase in the PRL-R level.

The target tissues that are regulated by the hormones have the ability to respond to changes in ligand concentration by regulating their number or affinity of their surface receptors. The regulation of PRL-R concentration by
various tissues appears to be quite complex (Posner et al., 1975, 1976). Other hormones also modify the number of PRL-R depending on the target organ studies (Yen et al., 1974; Kelly et al., 1977, 1978). In vivo, both 'up regulation' (Posner et al., 1975; Djiane et al., 1977) and 'down regulation' (Djiane et al., 1979) occur of these PRL-R. In vitro, PRL-R shows 'down regulation' in explant cultures having a relatively rapid turnover rate (Djiane et al., 1979, 1982). The down regulation has been shown to be a multistep process starting by an internalization of the hormone receptor complex and resulting in its breakdown within the lysosomes.

The mechanism involved in hormonal transduction remains unknown, however, the possible step that follows after the hormone binding to its receptor has been stated as their internalization and association partly with golgi elements and partly with lysosomes (Kelly et al., 1980). The biological activity could be induced by the hormone-receptor complex either acting directly at the level of the nucleus or the lysosomal degradation of PRL and/or the alteration of PRL owing to its association with golgi could also result in products that might act on nucleus.

The concept that peptide-hormone receptors are preferentially concentrated on plasma lemma seems less plausible now by a number of studies that have demonstrated the binding of labelled hormones to the intracellular membranes (Djiane et al., 1981; Posner et al., 1982). Djiane et al. (1981) by performing discontinuous sucrose gradient ultracentrifugation and purifying the plasma membrane and golgi component showed that despite the initial step of binding of PRL to the plasma membranes, most of the PRL-R appeared
Biochemical estimation

to be located within the cell rather than at the level of plasma membrane. Purified golgi fraction of rat liver cells were found to be richer in PRL-R and have higher affinity for PRL than plasma membrane fractions (Kelly et al., 1983). These studies also lend support to the view that the down regulation of PRL receptors occurs initially at the cell periphery reinforcing the theory of the internalization of the hormone-receptor complexes and their subsequent degradation. In the lactating mammary cell about 70% of the PRL-R are intracellular receptor and thus these receptors are not recognised by the hormone in their first step of binding (Djiane et al., 1985). However, in another study smooth membrane preparations of pregnant and lactating ewes were found to be richer in PRL-R than the microsomal preparations (Emane et al., 1986).
MATERIALS AND METHODS

Materials

Patients admitted to the 'indoor wards' of the King George's Medical College, who underwent breast biopsies or mastectomy were included. Biopsy piece thus obtained from operation theatre was divided into two pieces. One was sent for histopathological study while the other adjacent one was immediately frozen at -20°C till further biochemical processing.

Methods

Isolation of plasma membrane

Plasma membrane fractions were obtained from human mammary tissue by a method of Neville DM Jr. (1960) as modified by Emmelot et al. (1964). All the operations during membrane preparation were carried out in a cold room at 4°C. Tissues from -20°C were thawed and then washed with ice-cold 0.25M STM buffer (pH 7.4) consisting of sucrose (0.25 M), Tris HCl (4 mM), and MgCl2 (1mM). It was cut into small pieces removing all the visible fat. These were again washed and weighed. It was then minced finally with scissors on glass plate kept on ice. Minced tissue was suspended in homogenization medium (1 gm/4 ml of 0.25 STM buffer) and homogenized in an all glass homogenizer (50 ml capacity) of the Potter-Elvehjoein type with Teflon pestle normally using five up and down strokes at medium speed. The homogenate was filtered through prewetted cheese cloth (four layers). While homogenising, protease inhibitor in sufficient amount was added drop wise. The filtered homogenate was centrifuged at 280 g for 5 min. at 2°C in an RC5C centrifuge. The intact cells, debris and nuclei settle down. The super-

93
natant was carefully decanted off and then centrifuged at 1500 g for 10 mins at 2°C in the same centrifuge. The resultant supernatant was discarded and the pellets were pooled, suspended in homogenizing medium and washed twice or thrice. Plasma membranes were isolated from the fraction by sucrose density gradient. Briefly, the pellet was suspended in a mixture of 0.25 M STM (density 1.02, volume 1.5 ml) and 2M STM (density 1.24, volume 5.5 ml). This mixture overlaid by 0.25 M STM was ultracentrifuged at 25000 rpm at 2°C in an SW28 rotor of Sorvall ultracentrifuge. Pelicle of plasma membrane was formed at the interface (density 1.18 g/cm³). This was carefully aspirated by a pasteur pippette and resuspended in 0.25 M STM buffer. This preparation was washed with normal saline and then stored in ice for further biochemical estimations.

**Crude Membrane Preparation**

Crude membranes were prepared following the method of Peyrat et al. (1982). Briefly, tissues were treated in the same fashion as above except the homogenization step. In this case, the buffer (pH 7.8) contained Tris (0.025 M), EDTA (3 mM), dithiothreitol (1 mM) and azide (0.01%).

Homogenate was centrifuged at 800g in RCSC for 10 min. at 2°C. The supernatant thus obtained was ultracentrifuged at 1,05,000 g for 60 min. at 2°C in a swing-out rotor of Beckmen Ultracentrifuge. The pellet was suspended in 25 mM Tris containing 10 mM MgCl₂ (pH 7.6).

Protein was measured according to Lowry et al. (1951) with Bovine serum albumin as the standard.
Enzyme Assay

The marker enzymes were assayed qualitatively so as to ascertain the purity of the plasma membrane thus prepared. 5'-Nucleotidase (EC 3.1, 3.5) assayed according to the method of Serrano et al. (1977). The assay mixture consisted of Tris- HCl (100 mM) buffer (7.4), 10 mM MgCl2, 5 μM 5'AMP sodium salt and suitably diluted enzyme in a final volume of 0.5 ml (100-200 μg of protein). The reaction mixture was incubated for 30 min. at 37°C. It was terminated by adding 1 ml of prechilled 10% TCA and the liberated phosphate in the supernatant was estimated and OD was read at 620 nm.

Alkaline phosphatase (EC 3.1, 3.1) assayed according to the method described by Bessey et al. (1946) with slight modification. The reaction mixture in a total volume of 2 ml consisted of 200 mmoles bicarbonate buffer (pH 9.5) + 3 umoles p-nitrophenyl phosphate as substrate and suitably diluted enzyme. After incubation at 37°C for 30 min., the reaction was stopped by adding 2 ml of 0.1N NaOH. The appearance of p-nitrophenol was read at 405 nm.

To assay Glucose 6-phosphate, the reaction consisted of the same reagents as was taken for 5' nucleotidase, except the substrate which was 0.1 M glucose-6-phosphate here. All the other steps were the same. Phosphate liberated was estimated and OD was read at 620 nm.
Electron Microscopy

Membranes were pelleted by centrifugation and were fixed in buffered 3% glutaraldehyde (pH 7.2-7.4) for 1 to 2 hrs. The fixed pellets were washed several times in cacodylate buffer, post fixed in buffered (pH 7.2) 1% osmium tetraoxide solution for 1 hr at 4°C. It was again washed to remove excess of OsO₄ with buffer and dehydrated by several changes of graded concentrations of acetone, treated with various combinations of acetone and Durcapan ACM mixture and then embedded in Durcapan ACM mixture. The ultrathin sections were stained with uranyl acetate and lead citrate and examined under transmission electron microscope (Philips 301).

Alternatively, the plasma membrane preparations were diluted 10 times and a drop of suspension was placed on formvar coated grid. The grids were dried and then floated on a drop of 1% aq. phosphotungstic acid, again dried, and observed under the electron microscope.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemali (1970). The concentration of resolving acrylamide gel was 10% and that of stacking gel was 5%. Sample was solubilized in 5x sample buffer consisting of 0.08 M Tris (pH 6.8), SDS (2% w/v) glycerol (10% v/v) and beta-mercaptoethanol (5%), and heated for 2-3 min. in a boiling water bath prior to loading on gels. Running buffer containing 0.025 M Tris, 0.112 M glycine and 0.1% SDS (pH 8.5) was used. The gels were stained with Coomassie Brilliant Blue R-250.
**Immunoblotting**

Malignant plasma membranes preparations (purified, 2 samples) were electrophoretically transferred from polyacrylamide SDS gels to nitrocellulose paper as described by Towbin et al. (1979). Briefly, membranes proteins were electrophoresed in the first dimension by the method of Laemali (1970). The proteins were then transferred to nitrocellulose paper. The electrode buffer used contain 25mM Tris, 192 mM glycine, 20% (v/v) methanol (ph 8.3). Staining of blotted proteins was done by amido black (0.1% in 45% methanol, 10% acetic acid) and then destained with 90% methanol and 2% acetic acid. The adjacent strip of nitrocellulose sheet having transferred proteins was treated by DNP labelled immunocytochemical technique previously described in chapter III except the inhibition of endogenous peroxidase step. The concentration of primary antibody (aPRL) was 100 times higher than that used for paraffin sections. After staining the paper with DAB it was dried and approximate Mol wt. of the bands were measured with reference to monkey erythrocyte membrane.

**Binding Studies**

Membranes prepared as described previously were frozen immediately at -20°C till the binding assay was carried out. No membranes were kept frozen for more than 2-3 days. Besides the membranes of different lesions of human breast, crude membranes of mammary glands of rat 5 days after partution and livers of rats were also prepared so as to check the proper working of the reagents. Hormone used for the binding assay, i.e. h-PRL was purchased from Bioproducts-Iodination grade lot No. i017 Batch 016 + 017.
**Iodination of PRL**

PRL hormone was labelled with $^{125}$I following the method of Greenwood et al. (1963), using chloramine T as the oxidising reagent. Four $\mu$g of PRL hormone dissolved in 40 $\mu$l of 0.05 M phosphate buffer (pH 7.4) was taken in a vial and 1.5 mCi of Na$^{125}$I was added. 5 $\mu$g/10 $\mu$l of chloramine T solution was then added and the mixture was carefully vortexed. After 3 minutes, the reaction was stopped by adding excess of bovine serum albumin in 0.05M phosphate buffer pH 7.4. Before transferring the mixture to the column for purification of labelled hormone, 10 $\mu$l of mixture was taken out in duplicate to determine the specific activity.

Purification of the labelled hormone was done by column chromatography. For this purpose, a column (0.5 x 50 cm) of sephadex G-75 was used. Prior to the use, 2% BSA in 0.5 ml volume of phosphate buffer (0.05 M, pH 7.4) was allowed to pass out of the column so as to check the uniformity of the column. It was then thoroughly washed with the same buffer containing 0.1% BSA. After loading the iodination mixture on the column the column was eluted and 1 ml fractions collected. The radioactivity was measured by $^{125}$I counts.

**Freeing of Receptors from the bound endogenous PRL**

As PRL does not dissociate from its receptors during the process of membrane preparation, a certain portion of the PRL receptors may be occupied by the endogenous circulating PRL. Thus these receptors could not be determined by the assay till the bound PRL is removed. For this, high
concentration of MgCl₂ was used (Kelly et al., 1979). Briefly, to one volume of membrane preparation six volumes of 3M MgCl₂ was added. The mixture was vortexed and left for 3 min. at 4°C. The suspension was diluted with 4 times the volume of ice cold 25 mM Tris buffer containing 10mM MgCl₂. The whole suspension was centrifuged and at 25,000 g for 30 min. at 2°C. The resulting pellet was resuspended in the above buffer and proceeded for the binding assay. About 50-60% of the total protein present in the preparation was lost during the above procedure.

**Binding Assay**

Method of L'Hermite-Baleriaux et al. (1987) was followed for the binding studies. Briefly, the membrane preparations were thawed and protein was estimated (Lowery et al., 1951). Preparations containing 400 ug of protein were taken in four tubes. Two of them were subjected to MgCl₂ treatment, while the other two were left as such. These membranes with approximately 100,000 cpm of ¹²⁵I-PRL were then incubated in a final volume of 0.5 ml buffer (Tris 25 mM, MgCl₂ 10mM, BSA 0.1%) in the presence or the absence of excess of unlabelled hormone (1μg). The incubation was performed at 37°C for 1 hr and then left at room temperature overnight. Next day, the reaction was stopped by adding 3 ml of the cold buffer. The tubes were then centrifuged at 1500 g for 30 min. at 4°C to separate the bound ¹²⁵I-PRL from the unbound labelled hormone. After decantation and drying the side of the tubes properly, the pellets were counted in a gamma counter.

Specific binding (SpB) was calculated as the difference between the cpm bound in the absence and the presence of the excess unlabelled hormone and expressed as the percentage of the total radioactivity added in the tube.
RESULTS

Pure plasma membranes were prepared from twenty five different types of breast tissues obtained fresh from surgery operation theatres. We were successful in obtaining the plasma membranes from nineteen cases only. The failure in getting the membranes from the six tissues was either due to excess fat along with the normal breast parenchyma which interfered with the membrane preparation process or due to uncalled technical problems. The plasma membrane thus obtained was tested for the marker enzymes, and was found to be enriched with two positive markers 5' nucleotidase and alkaline phosphatase but the enzyme Glucose 6-phosphatase was absent. Yield of the plasma membrane from the malignant tissue ranged from 0.6 to 0.9 mg protein/g wet tissue while from the benign tissue it was 0.3±1 mg protein/g wet tissue. The yield for normal tissue was the lowest, 0.1 mg protein/g wet tissue. Electron microscopic examination of the fractions showed that they were composed largely of heterogenous vesicles (Fig. 4.1). For further characteristics, electrophoretic profiles of the polypeptides were examined. The pattern obtained by sodium dodecyl sulfate polyacryl amide gel electrophoresis of the three types of the membrane fractions are shown (Fig. 4.2a) in comparison with that of the monkey erythrocyte membrane.

The immunoperoxidase electrophoresis (immunoblotting) of pure plasma membrane of two malignant revealed a sharp band at approximately 25kDa and a diffused band at 29 kDa. (Fig. 4.2b,c).

The iodination mixture that was loaded on the column was collected in 1 ml fraction. The radioactivity in these fractions was measured. Three peaks
Fig. 4.1 Pure plasma membrane fraction of malignant breast tissue: Electron microphotograph x 9900
Fig. 4.2a SDS-Polyacrylamide gel electrophoresis of pure plasma membrane proteins of human breast tissue (including monkey erythrocyte as reference).
Lane i  -  Monkey erythrocyte
Lane ii -  Normal tissue plasma membrane preparation
Lane iii -  Benign  "  "  "  "
Lane iv  -  Malignant  "  "  "  "
            Case I
Lane v   -  Malignant  "  "  "  "
            Case II

Fig. 4.2b & Fig. 4.2c Nitrocellulose immunoblotting of the above two malignant plasma membrane preparation. Immunodetection of PRL with DHSS procedure using anti PRL and subsequent protein staining with DAB. Plasma membrane proteins (Case IV and Case V) were separated on 10% SDS-PAGE gel and electrotransferred onto nitrocellulose paper.

b.  Lane vi  -  Amido black staining
    Lane vii -  Amido black staining

c.  Lane viii -  Immunoblotting
    Lane ix  -  Immunoblotting
Biochemical estimation

of radioactivity were eluted. The first peak represented the damaged PRL aggregate, while the second peak was of the monomeric labelled PRL. The third peak was of free iodine. The second peak was pooled and used for the binding assay. The specific activity was calculated as 35 $\mu$ci/$\mu$g.

The specific binding of crude membranes prepared from 2 out of 5 malignant tissues was above 0.8%. Thus these membranes were considered positive for PRL-R. The specific binding was increased by treating the membranes with MgCl$_2$. No binding was observed with the pure plasma membrane of the above two cases. Specific bindings of lactating rat mammary tissue and rat livers were higher than 0.8%.

No binding was seen in both the cases of fibroadenoma included in the study.

These results indicate that there are specific binding sites of PRL in the malignant breast tissues.
DISCUSSION

According to the technique which we have followed for preparing pure plasma membranes, the cells get ruptured and nuclei and nuclear membranes get largely solubilized. The cell membranes, the largest fragments left in the homogenates, are pelleted by a low speed centrifugation. Microchondria and microsomal fragments that are more dense and smaller in size are separated from these membranes by the sucrose density gradient centrifugation. The only problem faced during the procedure was that of the plasma membrane yield, which was quite poor. This problem was serious in case of normal breast tissue which was full of fatty material. Also due to the awareness of the disease, breast cancer is usually eliminated before its enlargement, and only a small piece is available for the studies. The small fraction of plasma membrane obtained was not sufficient for the binding studies. Yield of the plasma membrane was highest for the malignant and lowest for the normal tissue, possibly because the normal mammary tissue is rich in connective tissue and fat whereas in the malignancy, epithelial cells increase. Positive reaction for the marker enzymes in the test as compared to the control confirmed presence of plasma membrane in the preparation without much contamination by the organelle membranes. Electron micrographs show that purified plasma membrane preparations were mainly composed of plasma membrane vesicles.

The anti human PRL used in immunoblotting were able to stain two bands in malignant pure plasma membrane preparations. The predominant one (25 kDa) may corresponds to PRL found in malignant tumours. The other
diffused band at 29 kDa may be the immuno-reactive variant of PRL. This observation may indicate that some of the plasma membrane receptors of PRL are also occupied by endogenous PRL. This finding agrees with the immunocytochemical results where in some cases membrane of epithelial cells in malignant tissue were also stained.

Results of PRL binding study indicate that breast tumours contain low but measurable levels of PRL binding sites in crude membrane preparations. Such low binding was observed in earlier studies also (Di Carlo et al., 1979, Turcot Lemay and Kelly 1982; Murphy et al., 1984). However, these studies differed from the present study in the percentage of PRL-R positive tumours. Di Carlo et al. (1979) reported 49% positivity for PRL-R in malignant tumours and 30% in benign breast tumours (Di Carlo et al., 1983) while it was only 13% in other studies (Turcot- Lemay et al., 1982). Murphy et al (1984) reported the highest incidence (65%) The results are rather controversial due to the techniques used by these workers. Mainly the technique of membrane preparation, the label used for displacement studies and the interpretation of the results contributes to these differences.

The binding was increased upon treating the tissue with high concentration of MgCl₂ in the present study. This data is in agreement with the findings of Peyrat et al. (1982), where 49% of the tumours were found positive by assaying the free PRL receptors (sp B=1.95±0.15%). However, after the MgCl₂ treatment, 71% of the tumours were PRL-R positive (sp B = 3.17±0.27%). It was suggested that some PRL-R were occupied by the endogenous PRL especially when elevated by stress and or anesthetic drugs.
and due to the irreversible nature of the PRL binding to its receptors (Vander Gugten et al., 1980) such occupied will remain undetected unless an in vitro dissociation procedure is applied. These receptors do not become completely free even under stress during homogenizing (Kelly et al., 1976). The MgCl₂ treatment induces the dissociation of endogenous PRL from the receptors. The detection and measurement of these PRL receptors in the cancerous tissue is necessary for predicting responsiveness to hormonal therapy. The endogenous bound PRL to its receptors was detected immuno-cytochemically as seen in previous chapter.

However, the problem is encountered in ascertaining the stage at which this 'in vitro' desaturation procedure is to be followed, as it could be done at three different stages, viz.

i) during homogenization procedure (Calvo et al., 1981).

ii) before the binding assay (Peyrat et al., 1982).

iii) after incubation with the labelled hormone had been performed (Vander Gugten, 1980).

The extent of binding will be different with all the three cases mentioned above. This treatment also resulted in a loss of about 60-61% of the measurable protein content and no study had taken this fact into account.

It is important to consider the reasons for the presence of this endogenous PRL in the malignant tissue observed in the present study by biochemical as well as immunocytochemical methods. It could not be due to surgical stress per se, as suggested by Peyrat et al. (1982) since no PRL increase was shown in patients treated with the same type of anesthesia who underwent surgery for reasons other than breast cancer by other studies. It
could be possible that breast cancer were associated with the secretion of an unknown factor acting at the hypothalamic-pituitary level to enhance the PRL secretion in the circulation and a portion of the PRL binding sites could be occupied. It could also be possible that the tumor itself secretes PRL and this results in occupancy of some sites in tumors. These data would thus indicate that PRL might be significant in the supporting the growth of breast cancer in vivo, and/or the hyperprolactinemia might be only a consequence of the progression of the disease as increased PRL level 5 years prior to the clinical appearance of the disease was reported (Kwa et al., 1981).

We had prepared both pure and crude membranes from the breast tissue and proceeded for the binding assay. The malignant tissue whose crude membranes were positive for PRL binding sites, their pure plasma membranes preparations were negative. Though the number of specimen studied were less, this finding is in agreement with the reports of the previous workers (Djiane et al., 1981; Posner et al., 1982; Kelly et al., 1983). This was further supported by our immunocytochemical studies in which staining reaction was intracellular as discussed earlier. The negative binding in pure plasma membranes of these is however questionable. In addition, we could not get any PRL binding with the pure plasma membranes of lactating mammary tissue, like the earlier study which reported that in the lactating mammary cell, about 70% of the PRL receptors are intracellular receptors (Djiane et al., 1985). However, we could not verify the presence of PRL receptors intracellularly by the binding assay in the crude membrane preparation due to the scarcity of the lactating breast tissue. The present study differs from the studies of Emane et al. (1986) in this respect.
PRL-R present in cancerous tissue may have a stimulating effect on the tumour. Drugs for removing PRL from the circulation could have some beneficial effects. Hormonal therapy with drugs for reducing PRL deserve a careful controlled clinical trial as the results may vary. Usually, the status of PRL in the body is determined by performing radioimmunoassay. Immunoreactive PRL may not be identical to PRL that is biologically active. Subramanian and Gala (1986) reported three forms of circulating PRL. The two larger forms that were found in the subjects with breast cancer (Subramanian and Gala, 1986) usually remain undetected by the routine radioimmunoassay. On the other hand, small fragments of PRL molecule, detected in women with familial history of breast cancer by a specific assay, were also considered to have some role in induction of breast cancer, especially in sensitive subjects during some period in their life (Love RR and Rose DPO, 1985).

Although this study has succeeded in determining the PRL binding sites both immunocytochemically and biochemically in benign and malignant breast lesions, it could have been more informative if the same tissue were used in both the methods. The logistics of experimental conditions in the present study precluded such investigations.