

IN VITRO INTERACTIONS OF PROLACTIN

WITH HUMAN BREAST CELLS

"IN VITRO INTERACTIONS OF PROLACTIN  
WITH HUMAN BREAST CELLS"

by  
GERALD WALLS

A dissertation submitted to the Faculty of Graduate Studies of  
the University of Manitoba in partial fulfillment of the requirements  
of the degree of

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## ABSTRACT

An attempt was made to establish a mammary cell culture in order to facilitate an investigation into the interaction of PRL with this tissue. Mammary tissue from 10 pregnant and pseudopregnant rabbits were digested with collagenase and the free cells were maintained in culture flasks for 1 to 6 weeks. Fibroblastoid cells usually overgrew the epithelioid cell colonies and were only partially controlled using thimerosal. Four specimens of pleural effusions from women with breast carcinomas were maintained in culture flasks and bottles for 1 to 5 months. Each culture contained four easily identifiable cell types; round, dendritic, spindle-shaped and polygonal cells. The epithelioid, polygonal cells appeared to arise from crowding of round cells and from attached clumps of cells. Insulin and hydrocortisone were not required for cell survival in these cultures. Two malignant human breast cell lines, MDA-MB-157 and MDA-MB-134VII as well as the HBL-100 cell line established from cells found in human milk samples from the healthy, lactating breast were maintained in culture until completion of the studies.

Casein synthesis and ornithine decarboxylase activity were the biochemical end-points used to indicate mammary cell response to PRL. Ovine PRL produced a greater than 2-fold increase in casein synthesis in rabbit mammary explants which had been previously incubated for 3 days at 37°C with insulin and hydrocortisone. In a similar experi-



ment, a slight but significant increase in casein production in HBL-100 cells was stimulated by hPRL at 2 and 12 hours following hormone addition to the culture medium but by 24 hours this effect had ceased. Injections of bGH into female rats increased ornithine decarboxylase activity 58-fold and 6-fold in the liver and kidney respectively but injections of bPRL were without effect in these tissues. Incubation of liver and kidney tissue slices with oPRL at 37°C resulted in significantly increased ornithine decarboxylase activity in the kidney slices but not in the liver slices while incubation with bGH had the opposite effect on enzyme activity in the same tissues. Ornithine decarboxylase activity in rabbit mammary explants, which were maintained in culture for 5 days in the presence of insulin and hydrocortisone, was not affected by the addition of oPRL. Liver explants were maintained in culture one day before treatment with bGH. Ornithine decarboxylase activity in the explants was increased significantly on the first day following hormone addition but not at any subsequent time. However the explants were shown to be non-viable on the fourth day of incubation. The cells and fluid of a pleural effusion from a woman with infiltrating duct mammary carcinoma both contained some ornithine decarboxylase activity. Unfortunately, the casein and ornithine decarboxylase assay systems appeared inadequate as reliable determinations of mammary cell response to PRL.

A more direct method of examining the interaction of PRL with mammary cells was the measurement of  $^{125}\text{I}$ -PRL binding to the cells.

Rabbit mammary tissue was digested over a 4-hour period with 0.1% collagenase and the free cells tested for binding of  $^{125}\text{I}$ -oPRL. Hormone binding occurred at 2 and 4 hours but was only 0.6% and 0.2% respectively, suggesting that treatment with enzyme may have interfered with the process. Less than 1% specific binding of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -hGH also occurred using both untreated human pleural effusion cells and HBL-100 cells. Similar results were obtained when HBL-100 cells growing in culture dishes were exposed to  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins for 2 hours. Growth medium in these culture dishes contained 10% fetal calf serum. Subsequently, as little as 5% of serum was shown to abolish the specific binding of  $^{125}\text{I}$ -hPRL to HBL-100 cells while the effect on insulin binding was negligible. In order to standardize and simplify the binding procedure, HBL-100 cells were homogenized and centrifuged at 15,000Xg and 100,000Xg to give 2 cell fractions used as a source of receptor. The specific binding of  $^{125}\text{I}$ -hPRL to both cell fractions was 0.5% while that of  $^{125}\text{I}$ -Ins was 1%. An increase in the protein concentration of the 100,000Xg fraction was accompanied by an increase in the binding of both hormones. This phenomenon did not occur using the 15,000Xg fraction. The specific binding of  $^{125}\text{I}$ -hGH to this fraction was 3 to 3.5% while that of  $^{125}\text{I}$ -hPRL was less than 1%. This data suggests that, if these 2 hormones occupy the same binding sites in HBL-100 cells as they seem to in the rabbit mammary cells, the PRL tracer itself is somehow incapable of binding to the HBL-100 cell fractions. Specific binding of  $^{125}\text{I}$ -hGH to the 15,000Xg fraction was approximately 3% at

a protein concentration ranging from 1000 to 3500 ug/ml. However, 3 subsequent experiments using the same cell fraction and  $^{125}\text{I}$ -hGH were unsuccessful in duplicating these results.

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## List of Abbreviations

Hormones

GH	growth hormone
PRL	prolactin
Ins	insulin

Prefix denoting species of origin

b	bovine
h	human
o	ovine

Activities

cpm	counts per minute
specific binding	cpm bound (ie. to receptor) that can be displaced by excess hormone
% specific binding	$\frac{\text{cpm specifically bound} \times 100}{\text{total cpm in assay}}$

Units of measure

g	gram
mg	milligram
ug	microgram
ng	nanogram
ml	milliliter
cm	centimeter
mm	millimeter

Miscellaneous

BSA	bovine serum albumin
day 12, day 31, etc.	day of pregnancy
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum

Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ip	intraperitoneal
ORD	ornithine decarboxylase
PBS (CMF)	phosphate buffered saline, calcium and magnesium free
S.D.	standard deviation
TCA	trichloroacetic acid

## STATEMENT OF PURPOSE

The role of PRL in mammary gland physiology is well documented. Two indices of the action of PRL on this organ are increased casein synthesis and increased ornithine decarboxylase activity. Studies on the binding of  $^{125}\text{I}$ -oPRL to rabbit mammary tissue preparations have identified a receptor protein for PRL. Antisera raised against a purified preparation of this receptor blocked the effect of PRL on casein synthesis in rabbit mammary explants in organ culture. The mammary receptor is believed to mediate the action of PRL in this tissue.

The role of PRL in mammary cancer is poorly understood. Rodent models have clarified the role of estrogens resulting in extensive research on the estrogen receptor but they have produced relatively little information about a prolactin receptor. Experimental evidence for such a receptor in human breast cancer is even more scarce. Recently, in vitro culture techniques have led to the development of human mammary tumor cell lines permitting a more direct and flexible means of investigating the problem than was previously possible.

The purpose of this study was to establish a culture of human breast tumor cells and then to characterize the interaction between PRL and these cells in hope of identifying a prolactin receptor.

## INTRODUCTION

The action of PRL on the growth, differentiation and function of the mammary gland is well documented (1). During pregnancy, steroid hormones as well as prolactin, growth hormone, and placental lactogens are necessary for growth and development of the alveolar cell system which is responsible for milk production. In vitro organ culture experiments involving midpregnant mouse mammary explants have elucidated the effects of individual hormones on alveolar cell development and milk secretion (2,3). Milk protein synthesis, measured as  $^3\text{H}$ -leucine incorporation into casein, is dependent on insulin, hydrocortisone and prolactin. Insulin stimulates division of undifferentiated cells while hydrocortisone causes development of rough endoplasmic reticulum, resulting in pre-secretory cells. The combined action of prolactin and insulin are required for various changes in cell morphology such as re-organization of cellular organelles resulting in fully differentiated secretory cells capable of milk protein production. Any of these hormones alone or in pairs are incapable of eliciting casein synthesis (4).

Prolactin exerts its effect on the mammary gland by interacting with binding sites termed 'receptors' which are located on the surface of the secretory cells. Turkington (5) demonstrated that PRL covalently attached to Sepharose beads is biologically active on mouse mammary epithelial cells implying that PRL initiates its action on

the cell membrane as the Sepharose-prolactin complexes presumably did not enter the cells. Binding of  $^{125}\text{I}$ -oPRL to rabbit mammary tissue in vitro and in vivo was reported by Birkinshaw and Falconer (6) and Falconer (7). Autoradiographic studies of these tissue preparations showed that the  $^{125}\text{I}$ -PRL was localized on the surface of the epithelial cell adjacent to capillaries. Shiu et al. (8) identified a prolactin receptor isolated from the mammary glands of pregnant rabbits which was subsequently used in establishing a competitive-binding radioreceptor assay for PRL and other lactogenic hormones. The receptor is specific for PRL as only peptide hormones which have prolactin-like effects in the assay, such as human growth hormone and human placental lactogen, can compete with  $^{125}\text{I}$ -PRL for the receptor sites; a variety of other hormones fail to do so. However, the receptor is not species specific as prolactins from a number of animal species can interact with it. The prolactin binding sites are probably proteins located in the cell membrane as receptor activity is sensitive to trypsin and phospholipase C digestion (9). Recently, a guinea pig antiserum to a purified preparation of this receptor, was shown to effectively block  $^{125}\text{I}$ -PRL binding to its membrane receptors as well as the prolactin-dependent incorporation of  $^3\text{H}$ -leucine into casein in rabbit mammary explants in culture (10). Thus, a prolactin receptor mediates the effect of lactogens in the rabbit mammary gland.

The action of PRL on the mammary gland may be illustrated by recent correlations of prolactin-induced events in this tissue with

intracellular levels of cyclic nucleotides. Incubation of midpregnant mammary gland explants from rats and mice with agents which raise intracellular levels of cyclic AMP suppress the stimulatory effect of PRL on RNA (11,12) and casein synthesis (13). Cyclic GMP has an opposite effect on RNA synthesis but does not have an effect on casein synthesis although it does attenuate the time required for the onset of the stimulatory effect of PRL (12). The two nucleotides are present in the mammary gland in different amounts during pregnancy and lactation; cyclic AMP concentration increases in gestation and then falls at the onset of lactation (14) while cyclic GMP concentration increases 2-fold immediately following parturition (12). These changes coincide with the dramatic increase in binding of  $^{125}\text{I}$ -oPRL to rat mammary tissue slices at parturition (15). Prolactin binding to its receptor and a subsequent involvement of cyclic nucleotides may be part of the same mechanism by which prolactin stimulates casein synthesis in the mammary gland.

Prolactin action on casein synthesis probably involves many mediating systems. Ornithine decarboxylase, a ubiquitous enzyme in animal tissues, is the first step in the synthesis of polyamines spermine and spermidine (16). The concentration of spermidine increases several fold in rat mammary tissue during lactation (17). Oka and Perry reported elevated spermidine levels in mouse mammary explants incubated with prolactin, insulin and hydrocortisone (18). The synergistic action of the 3 hormones enhances the cellular con-



centration of spermidine before the accelerated synthesis of milk proteins occurs. Casein synthesis also results if spermidine is substituted for hydrocortisone but does not occur in the case of prolactin substitution (18,19). Spermidine seems to mimic the steroid effect but not the prolactin effect in the stimulation of milk production. The prolactin effect on casein synthesis in the presence of insulin and hydrocortisone is abolished if an inhibitor of polyamine synthesis is added to the culture system but the simultaneous presence of spermidine overcomes the inhibitory effect of the drug (18). These observations indicate that biosynthesis of spermidine may be an important regulatory step in milk production. A biphasic increase in ornithine decarboxylase activity is associated with spermidine formation (20). The first peak of enzyme activity occurs independently of hormones and is accompanied by a transient increase in cyclic AMP concentration; the second peak depends on both insulin and prolactin. The changes in enzyme activity are rapid and may play an important regulatory role in polyamine biosynthesis. The effects of PRL on ornithine decarboxylase and spermidine in the mammary gland and other tissues (21) suggest that polyamines are possible mediators of prolactin action.

The effectiveness of prolactin action appears to depend on the number and condition of its receptors which are modulated by several physiological and hormonal factors (22-28). Rat liver has been used as a model for these studies and many of the observations are thought to apply to the mammary prolactin receptor. In the rat, there is low

receptor activity in both male and female liver until puberty when there is a significant increase in the number of binding sites in the female only (22). A further increase in receptor number occurs during pregnancy (22). Estrogen ( $E_2$ ) administration (23) and castration (24) increase the number of prolactin receptors in the male liver. In the female, estrogen administration has a similar effect but treatment with testosterone (24) results in a disappearance of prolactin binding sites. Hypophysectomy of the estrogen treated male or the female reduces the number of receptors to low levels (23). These results suggest that estrogen, androgen and prolactin are involved in controlling hepatic prolactin receptors. However, PRL alone seems capable of inducing its own receptors. Pituitary implants under the renal capsule of hypophysectomized, estrogen treated male rats result in hyperprolactinemia and a restoration of prolactin binding sites in the liver (25). Similar results are obtained when PRL alone is administered to hypophysectomized female rats (25,26). Recently, growth hormone and adrenocorticotropin have been added to the list of hormonal factors regulating the hepatic prolactin receptor population (27,28). Therefore, the responsiveness of a tissue to PRL may depend on the level of receptors in the tissue and their physiological state.

The effect of the endocrine environment on mammary tumor growth has been examined extensively (29,30). The model most commonly investigated is the rat mammary tumor induced by administration of the chemical carcinogen, 7,12-dimethylbenzanthracene (DMBA) to female Sprague-Dawley rats (31). Approximately 80% of these tumors are hor-

hormone dependent, that is, they are responsive to endocrine therapy (32). Such therapy usually consists of various ablations, or hormone and drug administration, all of which alter the serum levels of two key hormones, prolactin and estrogen.

Endocrine manipulations which increase serum prolactin concentrations such as median eminence lesions (33-36), drugs (37-39) and estrogen or progesterone treatment (37,40) generally result in enhanced tumor growth and increased tumor number. However, large doses of estrogen inhibit tumor growth (36,41-43). Administration of PRL can overcome the estrogen-induced inhibition of tumor growth (41,43) suggesting that estrogen interferes with the action of PRL at the tumor cell level. Decreasing serum PRL by hypophysectomy (36,37,44) or using ergot drugs (45-48) causes decreased tumor growth or regression. On the basis of this work PRL has been called the most important hormone in mammary cancer (49).

The prolactin stimulus to DMBA-induced rat mammary tumor growth seems to depend on estrogen. Ovariectomy of tumor-bearing rats leads to an inhibition of tumor growth which can be restored with small doses of estrogen (36,50) or ovarian grafts (33,35). Although the administration of PRL to ovariectomized (34,44,50), ovariectomized-adrenalectomized (41,44,50) or ovariectomized-adrenalectomized-hypophysectomized (44) tumor-bearing rats results in progressive tumor growth, this effect is only of brief duration in the absence of steroids (44). Prolactin enhancement of rat mammary tumor growth is more vigorous and more lasting in ovariectomized rats that have received ovarian grafts (35) or in intact animals (34). Prolactin and estrogen both appear to influence

the growth of DMBA-induced rat mammary tumors. Thus a combined therapy of ovariectomy with either pharmacological doses of estrogen or ergocor-nine, a drug which inhibits pituitary secretion of PRL, is the most effective means of inducing tumor regression (51).

The above in vivo studies have been substantiated using in vitro culture techniques. Small slices of DMBA-induced rat mammary tumors in organ culture respond with increased DNA synthesis, measured as increased incorporation of  $^3\text{H}$ -thymidine into DNA, when prolactin is added to insulin-corticosterone-containing medium (52,53). Physiological concentrations of estrogen have no effect on prolactin-stimulated DNA synthesis; pharmacological concentrations inhibit the process (53). In a monolayer culture of rat mammary tumor cells, similar doses of estrogen have the same effect on prolactin-induced cell growth judged as increased total protein (54). At high concentrations PRL counteracts the inhibitory effect of estrogen. Unlike the tissue slices these cells fail to respond to PRL in the absence of estrogen. These studies support the view that the interrelationship of prolactin and estrogen is the most important endocrine role in rat mammary tumorigenesis and it is suggested that the ratio between the two hormones is the key to growth of rat mammary tumor cells (54).

Specific binding sites for prolactin (55-61) and estrogen (55,61-67) have been identified in DMBA-induced rat mammary tumors. The majority of tumors contain both types of receptors. Concentrations of estrogen receptor are higher in hormone-dependent tumors with hormone independent tumors containing little or none (62) although there are some exceptions. On this basis, measurement of estrogen receptor levels in human breast

tumor biopsy specimens is used to predict the success of endocrine ablative procedures (68). Quantitation of prolactin binding capacity of the same tumors may be useful in predicting growth response of tumors to altered serum PRL levels. Kelly et al. (57) demonstrated a correlation between  $^{125}\text{I}$ -PRL binding and rat mammary tumor growth in vivo following prolactin treatment. Those tumors which increased in size in response to PRL also exhibited increased binding of prolactin. Holdaway and Friesen (60) however, did not detect any difference in prolactin binding between hormone-dependent and hormone-independent tumors in untreated rats. Subsequent treatment of the same rats with PRL resulted in decreased prolactin binding by hormone-unresponsive tumors but did not change the binding capacity of hormone-responsive tumors. A similar depression of hormone binding in both tumor groups was elicited using bromoergocryptine, a drug that suppresses pituitary prolactin secretion. Prolactin does not appear to induce its own receptors in hormone-dependent tumors since its administration does not increase the amount of binding; yet it may be necessary for receptor maintenance as suppression of serum PRL levels with bromoergocryptine results in decreased hormone binding in both tumor groups. Taking the two studies (57,60) together it appears that prolactin binding may be a poor indicator of the likelihood of tumor responsiveness to endocrine manipulations.

The relationship between mammary tumor response to endocrine manipulation and the levels of binding sites for prolactin and estrogen is complex. There are many inexplicable instances in which hormone binding is high but cessation of tumor growth or tumor regression occurs

after prolactin administration, or as is sometimes the case, hormone binding is low but tumor growth increases. These anomalies may be explained in part by the effect of one hormone on receptor levels of another. High serum PRL concentrations usually result in increased tumor estrogen binding levels and tumor growth while lower PRL levels produce the opposite effects (66). Treatment of tumor-bearing rats with high doses of estrogen causes a reduction in prolactin binding and inhibition of tumor growth (58). Low doses of estrogen do not seem to affect tumor binding of PRL but do enhance tumor growth (58). It is evident that the ratio of circulating prolactin and estrogen will determine the type and number of receptors in a given tumor. It is possible that some tumors may differ in their sensitivity to the two hormones with the result that one hormone may be more influential on growth than the other. The simple measurement of tumor binding of prolactin or estrogen alone may therefore be insufficient.

Principles adapted from the rat DMBA-induced mammary tumor model have met with little success when applied to human breast cancer. Generally, there is no significant difference in serum PRL levels between healthy patients and those with breast tumors (69-72). Drugs that reduce serum PRL such as L-dopa, ergocornine and bromoergocryptine have been used in an attempt to treat breast cancer patients (69,73-76) but result in few cases of objective tumor regression. It is possible that serum PRL levels are not sufficiently suppressed for tumor remission. However, it is unlikely that an absolute concentration of circulating PRL is the only factor involved. Tissue responsiveness, which may be determined in part by its hormone receptor level must

also be important. Other hormones, especially estrogen, may serve as modulators affecting either or both the serum PRL level and tissue responsiveness. Estrogen receptors have been identified in human mammary tumors (77-81) and their presence indicates a 55 to 60% chance of tumor regression after endocrine ablative therapy (82).

The estrogen receptor assay is accepted as a reliable test to predict the response of human breast tumors to hormone therapy but no such assay exists for prolactin. Salih et al. (83,84) reported the usefulness of a histochemical technique in which the total dehydrogenase activity of the pentose-phosphate shunt, a pathway producing 5 carbon sugars for nucleotide biosynthesis, is employed to demonstrate in vitro hormone sensitivity of human mammary tumors. Fifty biopsy specimens of breast tumors were sliced and incubated 24 hours in the presence of oPRL (84). Improved maintenance of cellular integrity, as judged from histological preparations and growth, as judged by dehydrogenase activity, was shown in 16 explants. A subsequent report (85) states that of 100 specimens, 31 were dependent on PRL with 8 of these showing dependence at low normal concentration of PRL in the culture medium. Sensitivity to such low levels of serum PRL may explain why endocrine ablative procedures, which do not reduce serum prolactin levels to zero, fail to benefit patients with prolactin-responsive tumors. Clinical follow up of results using this method has indicated a correlation between in vitro prolactin dependence and in vivo response to anti-hormonal treatment (85). Unfortunately, other workers (86-88) have not had any success with the assay method. Masters et al. (88) claimed only 10 of 83 tumor explants responded to PRL while Beeby et al. (86) was

unable to show any response at all.

Alternative methods are few in number and do not show clearly that human mammary tumors in vitro are sensitive to PRL. Kleinberg (89) used a sensitive radioimmunoassay specific for human  $\alpha$ -lactalbumin, to measure this milk protein in medium from cultures of breast tumors. Explants of tumor tissue were preincubated at 37°C for 3 days in the presence of insulin and hydrocortisone; and in 4 of 19 cultures  $\alpha$ -lactalbumin was detected. The addition of oPRL to the medium did not increase production of the milk protein in these 4 tumors or 13 others but it did stimulate production in 2 cultures. The addition of human female serum, known to contain  $\alpha$ -lactalbumin, plus oPRL in concentrations greater than physiologic resulted in 2 and 3-fold increases in detectable  $\alpha$ -lactalbumin over that attributable to the serum content. In the same study, serum levels of  $\alpha$ -lactalbumin were lower in women with breast tumors than in healthy, non-lactating women while the serum levels of PRL were similar in both groups. Thus it appears that mammary tissue of women with breast cancer is less sensitive to PRL in vivo than is healthy breast tissue and this difference is reflected in vitro.

Mioduszewska et al. (90) reported a stimulatory effect of oPRL on cell proliferation in 65% of 20 explants of human breast tumors. Measured as the incorporation of  $^3\text{H}$ -thymidine into DNA, Welsch et al. (91) could not demonstrate any effects of a high concentration of oPRL either in the presence of insulin or insulin plus hydrocortisone on DNA synthesis in slices of tumor tissue in organ culture. A more direct approach was attempted by Holdaway et al. (92). Binding of  $^{125}\text{I}$ -hPRL,  $^{125}\text{I}$ -oPRL and other hormones to preparations of human benign and cancerous mammary



tissue was performed. Human prolactin binding in 7 of 34 malignant tissue particulate membrane fractions was significant. Similar binding of the same hormone occurred in ultra-thin cryostat-cut slices of the same 7 tumors. Non-malignant tissue preparations did not bind significant amounts of human prolactin. Binding of oPRL to membrane fractions of tumor tissue was considerably less than that of hPRL. Generally however, the binding was quite low.

Organ cultures of 'normal' human mammary tissue have added very little to the knowledge of prolactin involvement in breast cancer. Prolactin does not seem to be required for explant survival (93,94); in many instances no hormones are required at all (93-96), although when insulin is present, PRL can have a stimulatory effect on cell proliferation (95). In contrast, Flaxman et al. (97) maintained explants of breast tissue from a woman in her sixth month of pregnancy for 3 weeks. Usage of medium without hormones resulted in nearly total lobulo-alveolar degeneration. Replacement with medium containing insulin, hydrocortisone and prolactin did not affect the process; only when the concentration of PRL was increased to a pharmacological level, was an improvement in lobulo-alveolar maintenance observed. This study is particularly interesting because it is very similar to the midpregnant mouse mammary culture system mentioned above but the triple hormone combination in this case was ineffective at physiological levels. Prolactin alone can stimulate DNA synthesis as measured by the incorporation of <sup>3</sup>H-thymidine into DNA but this is not always the case (98). Dilley and Kister (99) reported that human prolactin and human placental lactogen but not ovine prolactin stimulated growth of breast tissue

in culture as judged by mitotic indices in histological sections. Insulin was required for this effect to be manifested. This report suggests a difference between simian and non-simian prolactins in their ability to induce growth in human mammary tissue and is significant when one considers that most of the above-mentioned in vitro studies with 'normal' and malignant mammary tissue employed either ovine or bovine prolactin. It is recalled that the hormone binding study by Holdaway et al. (92) showed considerably less binding of oPRL to breast tumors.

It seems apparent that organ culture methods, which are used in experiments designed to demonstrate in vitro hormone dependence of human mammary tissue, are inadequate and provide inconsistent results. Contributing factors may include the limited amount of breast tissue available, the heterogeneity of cell types within the biopsy specimen and the short-lived nature of the explants in culture. Human mammary cell cultures with stable growth kinetics and of characterized cell morphology are now available (Table 1). Although these cell lines also have disadvantages and cannot of course be used to predict hormone dependence in cancer patients, they do allow for long-term studies involving hormone related events in malignant transformation of the human breast cell.

The majority of human malignant breast cell lines were derived from biopsy specimens (100-103) or pleural effusions (104-107) from women with adenocarcinoma or infiltrating duct carcinoma. Biopsy samples were either cut into small slices from which cells were harvested (100), treated with enzymes (101, 102) or cut into small explants which

eventually gave rise to epithelial monolayers (103). Primary cultures begun in this fashion frequently contained fibroblasts whose growth was later controlled and eliminated. Pleural effusion cells required no treatment prior to culture and rarely contained fibroblasts. Most cell lines arising from primary cultures of malignant breast cells are epithelioid as judged by ultrastructural characteristics and chromosome numbers range from 41 to 95 with a mean of 62. Non-malignant human breast cells have also been maintained in cell culture (108, 109) but only one cell line has been established. This line was initiated with cells collected in milk samples from the healthy lactating breast. These cells are believed to be epithelial and the chromosomal mode is 68 (unpublished data). All cell lines mentioned above have a serum requirement but the majority grow well in the absence of hormones. Insulin appears to be the most important hormonal supplement (102,104-106). There is no requirement for estrogen and prolactin. However, these cell lines have not yet been examined for hormonal effects.

Prolactin clearly plays a leading role in promoting the synthesis of milk proteins in the mammary gland during lactation. Receptors for prolactin have been identified in mammary tissue and are believed to mediate its effect. These receptors are also present in rat mammary tumors but have not yet been fully characterized in human breast tumors. It was the purpose of this study to examine both malignant and non-malignant human mammary cells for 3 effects of PRL known to occur in rodents: a) casein synthesis, b) regulation of orthonine decarboxylase activity and c)  $^{125}\text{I}$ -labelled hormone binding. These 3 pieces of information would provide evidence for a prolactin receptor in human breast tissue.

Table 1

## Published human breast tumor cell lines

<u>Designation</u>	<u>Year</u>	<u>Reference</u>
BT-20	1958	Lasfargues E.Y. and L. Ozzello, J. Nat. Cancer Inst. <u>21</u> , 1131, 1958.
A1Ab	1958	Reed M. and G. Gey, Lab. Invest. <u>2</u> , 638, 1962.
CaMa	1959	Dobrynin Y., J. Nat. Cancer Inst. <u>31</u> , 1173, 1963.
Lev 111	1967	Giraldo G et al., J. Exp. Med. <u>133</u> , 454, 1971.
Sal 111	1967	Giraldo G. et al., J. Exp. Med. <u>133</u> , 454, 1971.
M-1	1968	Martorelli B. et al., Surg. Gynecol. Obstet. <u>128</u> , 1901, 1969.
SK-BR-3	1970	Fogh J. and G. Trempe, in <u>Human Tumor Cells In Vitro</u> , New York, Plenum Press, 1975, pp. 115-159.
734B	1970	Vazquez J. et al., Proc. Am. Assoc. Cancer Res. <u>13</u> , 34, 1972.
MCF-7	1971	Soule H. D. et al., J. Nat. Cancer Inst. <u>51</u> , 1409, 1973.
HBT-3	1971	Bassin R.H. et al., Proc. Soc. Exp. Biol. Med. <u>141</u> , 673, 1972.
HBT-39	1972	Plata E.J. et al., J. Nat. Cancer Inst. <u>50</u> , 849, 1973.
MDA-MB-157	1973	Young R. K. et al., In Vitro <u>9</u> , 239, 1974.
MDA-MB-134	1973	Cailleau R. et al., J. Nat. Cancer Inst. <u>53</u> , 661, 1974.
MDA-MB-157	1974	Cailleau R. et al., J. Nat. Cancer Inst. <u>53</u> , 661, 1974.
MDA-MB-231	1974	Cailleau R. et al., J. Nat. Cancer Inst. <u>53</u> , 661, 1974.
BOT-2	1975	Nordquist R.E. et al., Cancer Res. <u>35</u> , 3100, 1975.
SH-3	1975	Seman G. et al., Cancer <u>37</u> , 1814, 1976.

## MATERIALS AND METHODS

Animals

Pregnant and non-pregnant adult female white New Zealand rabbits; and female Sprague-Dawley rats (135g and 400g) were obtained from North American Laboratory Supply Co. Term-pregnant (day 31) and pseudopregnant (day 12 or day 13) rabbits, which have a similar development of the mammary gland, provided a source of mammary tissue. A single intravenous injection of human chorionic gonadotropin (100 IU) in non-pregnant rabbits induced pseudopregnancy in these animals. The rabbits were killed with nembutal and their ventral sides soaked with 70% ethanol to allow aseptic removal of mammary tissue.

Hormone preparations

All the following hormone preparations were obtained from the NIAMDD division of the National Institutes of Health: human growth hormone (1934D, 2.6 IU/mg); bovine growth hormone (B17, 0.92 IU/mg and B18, 0.81 IU/mg); bovine prolactin (P-B3) and ovine prolactin (P-S-12, 35 IU/mg).

Human prolactin (23 IU/mg) was provided by Dr. I. Worsley of this laboratory.

Hydrocortisone and 17- $\beta$ -estradiol were purchased from Sigma and porcine insulin from Connaught Laboratories.

Hormones used in the in vitro techniques were diluted in the appropriate growth medium. Hormones injected into animals were dissolved and diluted with sufficient sodium chloride to make a 0.9% solution.

### Iodinations

Sodium <sup>125</sup>Iodide was purchased from Amersham Searle and New England Nuclear. All hormones were iodinated as described by Thorell and Johansson (110). Iodinated hormone was separated from the reaction mixture by Sephadex G100 gel filtration and tested for specific binding in a radioreceptor assay (8,9). Tested tracer was aliquoted and stored at -20°C.

Hormone tracers used in the in vitro techniques were diluted in the appropriate growth medium.

### Cell culture supplies

All prepared media, serum, amniotic fluid, L-glutamine, penicillin, streptomycin, fungizone and phenol red were purchased from Grand Island Biological Co., Calgary, Canada. HEPES buffer, gentamycin, glutathione and thimerosal (sodium ethylmercurithiosalicylate) were obtained from Sigma. Trypsin and collagenase were from Worthington Biochemical Corp.

Plastic 25 cm<sup>2</sup> flasks, 75 cm<sup>2</sup> flasks and 60 x 15 mm dishes were purchased from Falcon. Plastic 150 cm<sup>2</sup> flasks and 35 x 10 mm dishes were obtained from Corning. Glass Blake and Povitsky bottles were kindly supplied by Doctor A. M. Wallbank.

Media required for sterility testing such as AC medium, Sabouraud's broth and blood agar plates were also supplied by Doctor Wallbank.

### Rabbit mammary cell culture method

Mammary tissue was aseptically removed and placed in sterile petri dishes containing a washing medium of Medium 199 with 500 U/ml penicillin

and 500 ug/ml streptomycin. Further washings in this medium were usually required to remove blood and hair from the tissue. Muscle and fatty portions were dissected away and the remaining mammary tissue placed in a dry sterile beaker and minced with fine iris scissors. About 4 to 8 g of wet weight tissue was processed in this manner.

Minced tissue was added to 50 or 125 ml sterile Erlenmeyer flasks containing 0.1% to 0.2% collagenase in Medium 199 (10 ml/g wet weight tissue) and placed in a shaker water bath at 37°C for 1 to 2 hours. Digests were filtered through 4 layers of sterile cheesecloth and the filtrates collected in sterile 50 ml tubes. The tubes were centrifuged at 200Xg for 10 minutes at room temperature in an International centrifuge. Pellets were washed once with Medium 199 containing 10 to 30% FCS, 15 mM Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin. These pellets were resuspended in 5 to 10 ml of the same medium and cell counts performed using trypan blue. Cell suspensions were then diluted with medium to concentrations of 0.5 to  $2.0 \times 10^6$  viable cells/ml and added to plastic 25 cm<sup>2</sup> or 75 cm<sup>2</sup> flasks. The flasks were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>-95% air.

### Human pleural effusion

#### 1. Source

Pleural effusions were collected from 3 Caucasian women with mammary carcinoma.

Patient A. This woman, age 52, had a radical mastectomy in 1969 for an infiltrating duct carcinoma. She had a chest wall recurrence in 1971

and radiotherapy was begun. She developed a left pleural metastasis with pleural effusion in January 1975. Chemotherapy was begun March 3, 1975. Subsequent effusions of March 3, and March 8, 1975 yielded 780 ml and 550 ml of fluid respectively.

Patient B. This woman, age 51, had a radical mastectomy for carcinoma of the left breast in January 1969 followed by an oophorectomy in November 1969. Pleural fluid formation occurred and thoracentesis performed in October 1974 produced fluid containing viable malignant cells. Chemotherapy was begun March 13, 1975. Another pleural effusion of March 24, 1975 taken from the right pleural cavity yielded 1100 ml of fluid.

Patient C. This woman, age 46, was nulliparous and premenopausal. She had an ovarian cyst and metastatic liver disease. She had a radical mastectomy in June 1974 for carcinoma of the right breast. In May 1975 she developed a right pleural metastasis with a pleural effusion of June 3, 1975 that contained viable tumor cells. She received no chemotherapy. A subsequent thoracentesis of the right pleural cavity on June 9, 1975 yielded 400 ml of fluid.

## 2. Culture method

Pleural fluids were collected aseptically by thoracentesis into sterile, 500 ml plasma vacuum bottles containing 0.5 - 5 U/ml heparin. Plasma bottles were placed on chopped ice and transported immediately to the laboratory. The pleural effusions were aliquoted to 50 ml plastic tubes and centrifuged at 250Xg in an International centrifuge



for 10 minutes at room temperature. Supernatants were discarded and the pellets washed once with growth medium. Cell counts were performed using trypan blue and the cell suspension then diluted with varying amounts of growth medium before inoculating plastic 25 cm<sup>2</sup> or 75 cm<sup>2</sup> flasks and glass Blake bottles. All cultures were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>-95% air.

### 3. Media

The basic medium consisted of Medium 199 10 to 20% FCS, 15 mM Hepes buffer, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine. Modifications of this medium included the addition of 5 ug/ml 17 β-estradiol, 5 ug/ml hydrocortisone and 20 ug/ml insulin. Three other media including McCoy's 5a, Eagles minimum essential medium (MEM) and Leibovitz's L-15 were also used in various combinations with 10 to 20% FCS, 15 mM Hepes buffer, 50 ug/ml gentamycin, 16 ug/ml glutathione, 5 ug/ml hydrocortisone and 10 ug/ml insulin.

### Human breast cell lines

#### 1. Cell line description

Three human breast cell lines were obtained from the Mason Research Institute, Rockville, Maryland, U.S.A. A description is presented in Table 2. HBL-100 designates cells found in milk samples obtained from normal women who were lactating. MDA-MB-157 and MDA-MB-134VII designate cells derived from pleural effusions of two women with mammary carcinomas. All 3 cell types are aneuploid but cells of the HBL-100 line appear to be normal and non-tumorigenic.

Table 2

## Human breast cell line description

	<u>HBL-100</u>	<u>MDA-MB-157</u>	<u>MDA-MB-134VII</u>
Tissue origin	lactating breast pump milk sample	breast tumor pleural effusion	breast tumor pleural effusion
Diagnosis	normal	medullary carcinoma	infiltrating duct mammary carcinoma
Cell characteristics	epithelial, pile up in heavy growth	scalloped edges, pile up in heavy growth	small, round or spindle-shaped
Chromosomal mode	68	64 - 69	43
Other characteristics	non-tumorigenic in nude athymic mice	tumorigenic in nude athymic mice	

## 2. Culture method

Cells were received in 25 cm<sup>2</sup> plastic flasks filled with growth media. All but 5 ml of media was emptied from the flasks and the remainder subsequently used to maintain initial cultures. Flasks were incubated at 37°C.

All cell lines were initially cultured in plastic 25 cm<sup>2</sup> and 75 cm<sup>2</sup> flasks; and glass Blake and Povitsky bottles. Later, plastic 150 cm<sup>2</sup> flasks were used exclusively.

Confluent cultures were subcultured using either 1:5000 EDTA in PBS(CMF) or 0.25% trypsin in PBS(CMF) and with 10 ug/ml phenol red solution.

## 3. Media

HBL-100. The medium used with these cells consisted of McCoy's 5a, 10% FCS, 15 mM Hepes buffer and 25 ug/ml gentamycin.

MDA-MB-157. The medium employed here was Leibovitz's L-15, 15% FCS, 15 mM Hepes buffer, 50 ug/ml gentamycin, 16 ug/ml glutathione, 10 ug/ml insulin, and 5 ug/ml hydrocortisone.

MDA-MB-134VII. This medium was a modification of the above and consisted of Leibovitz's L-15, 10% FCS, 10% bovine amniotic fluid, 15 mM Hepes buffer, 50 ug/ml gentamycin, 16 ug/ml glutathione and 10 ug/ml insulin.

These 3 media were slightly different from the media supplied with the cultures. Hepes buffer was added to prevent small changes in pH that occurred during medium changes to which cells of the HBL-100 line were very sensitive. Disodium carbenicillin (100 U/ml) was omitted because media with gentamycin alone were found to be equally efficacious.

#### 4. Cell preservation

A frozen stock of cells was prepared from each of the 3 cell lines. Cells were removed from the flasks or bottles with trypsin and centrifuged at 160Xg in an International centrifuge for 10 minutes at room temperature. Pellets were pooled and resuspended in 5 ml of growth medium containing 10% dimethylsulfoxide (DMSO), reagent grade (Fisher). A cell count was performed and the cell suspension diluted with the freezing mixture to  $3 \times 10^6$  viable cells/ml. One ml of the suspension was aliquoted to each 1.2 ml borosilicate glass ampoule (Wheaton Glass). Ampoules were sealed by the pull method using an oxygen-gas mixture and placed in a Revco refrigerator overnight; then stored in a liquid nitrogen refrigerator.

#### Sterility tests and contamination

Prepared media and media from cell cultures were examined for contamination using 3 tests, a) AC medium, b) Sabouraud's broth, and c) blood agar. One ml of medium to be tested was inoculated into 2 sets of tubes containing AC medium and broth; one set was incubated at 24°C and the other set at 37°C. The tubes were left to stand 2 to 3 weeks. A drop of the same medium was applied to the agar plates which were then incubated at 37°C for 3 to 4 days. No change in the appearance of the AC medium or broth and the absence of bacterial colonies on the surface of the plates indicated no growth of bacteria or fungi (yeast).

Bacterial contamination was treated by raising the concentration

of penicillin and streptomycin to levels of 500 to 800 U/ml and 500 to 800 ug/ml respectively. Yeast contamination was controlled by supplementing growth media with 2.5 to 5 ug/ml fungizone.

#### Liquid scintillation counting

Isotopes L-(4,5-<sup>3</sup>H)-leucine (specific activity 5 Ci/m mole) and DL-(1-<sup>14</sup>C)ornithine monohydrochloride (43 to 58 mCi/m mole); protosol; hyamine hydroxide; dioxane; toluene; and omnifluor packets (dimethyl POPOP-PP0) were all obtained from New England Nuclear. Packet contents were dissolved in either dioxane or toluene depending upon whether the material to be counted was soluble in protosol or hyamine hydroxide. Dissolved material and 10 ml of scintillation cocktail were added to a 20 ml glass vial (Wheaton Glass Co.) and counted in a Beckman LS-350 liquid scintillation counter.

Dithiothreitol and pyridoxal-5'-phosphate used in Richards' determination of ornithine decarboxylase activity (21) were purchased from Sigma. Plastic wells and rubber stoppers used in the same assay were obtained from Kontes Glass Co.

#### Rabbit mammary organ culture method

Portions of mammary gland were aseptically removed from day 31 pregnant or day 13 pseudopregnant rabbits and the muscle layers removed. Small pieces of epithelial tissue ranging in weight from 0.7 to 1.4 mg were cut away and washed in Medium 199 containing 500 U/ml penicillin and 500 ug/ml streptomycin. Four such explants were placed on a raft

of siliconized lens paper ( $1 \text{ cm}^2$ ) prepared according to Chen (111). One raft was placed into each 35 x 10 mm plastic culture dish containing 1 ml of Medium 199 with 15 mM Hepes buffer, 50 U/ml penicillin, 50 ug/ml streptomycin, 5 ug/ml fungizone, 10 ug/ml hydrocortisone and 10 ug/ml insulin. All dishes were put into a plastic box fitted with a snap-shut lid and exposed to 5%  $\text{CO}_2$ -95%  $\text{O}_2$  sufficient to maintain the pH at about 7.4 (performed once each subsequent day). The system was closed and incubated at  $37^\circ\text{C}$ .

#### Casein synthesis in rabbit mammary explants in response to oPRL

Mammary tissue explants were prepared from tissue of a day 13 pseudopregnant rabbit and placed into organ culture as described above. All cultures were preincubated 72 hours at  $37^\circ\text{C}$  in medium containing 10 ug/ml insulin and 10 ug/ml hydrocortisone. Then the explants on their rafts were transferred to new dishes containing 1 ml of fresh medium and 1, 10, 100, 1000 and 2000 ng/ml of oPRL. Control cultures received no PRL. The cultures were incubated 72 hours further. Four hours prior to the termination of the incubation 5 uCi of  $^3\text{H}$ -leucine in 0.1 ml of medium was added to all cultures. At the end of this period, the rafts were removed from the culture dishes and the explants blotted on paper towels. The explants were weighed and assayed for casein as described by Juergens et al. (4). Quadruplicate cultures were used throughout.

#### Casein synthesis in human mammary HBL-100 cells in response to hPRL

HBL-100 cells were removed from  $150 \text{ cm}^2$  plastic flasks using 1:5000

EDTA and centrifuged at 160Xg for 10 minutes at room temperature in an International centrifuge. Pellets were resuspended in a growth medium consisting of McCoy's 5a containing 2% FCS, 15 mM Hepes buffer, 25 ug/ml gentamycin, 2.5 ug/ml fungizone, 10 ug/ml hydrocortisone and 10 ug/ml insulin. A cell count was performed using trypan blue. Each 60 x 15 mm plastic culture dish received 4 ml of medium containing  $2 \times 10^6$  viable cells. The cultures were incubated for 72 hours at 37°C in a plastic container fitted with a snap-shut lid. The container was gassed every 24 hours with 5% CO<sub>2</sub>- 95% O<sub>2</sub> sufficient to maintain the pH of the cultures at about 7.4.

At 72 hours 0.1 ml of medium containing 4 ug of hPRL was added to the cultures. Control cultures received 0.1 ml of medium but no PRL. After a further incubation of 2, 12, 24, 48 or 72 hours, 2 ml of medium was removed from the cultures and 10 uCi of <sup>3</sup>H-leucine in 0.1 ml of medium was added to each dish. Incubation with <sup>3</sup>H-leucine was carried out for 4 hours and then terminated by removing the isotopic medium and replacing it with ice-cold PBS(CMF). Cells in each dish were scraped loose with a rubber policeman and assayed for casein as described by Juergens et al. (4). Triplicate cultures were used throughout.

On each of the 3 days subsequent to the addition of PRL, 3 cultures from experimental and control groups were examined by light microscopy for cell attachment to the culture dish surface.

#### ORD activity in rat kidney and liver in response to hormone treatment

##### 1. In vivo hormone injection studies

In 2 separate experiments female rats received intraperitoneal

(ip) injections of either bPRL or bGH. Control animals received ip injections of an equivalent amount of saline. Animals were sacrificed using ether. In both studies, the kidneys and livers were removed, weighed and homogenized in 5 volumes of 0.025M Tris-HCL, 5 mM dithiothreitol, 0.1 mM EDTA, pH 7.3 using a Brinkman polytron. Homogenates were centrifuged at 1500Xg and 4°C for 10 minutes and the supernatants assayed in duplicate according to Richards (21).

## 2. In vitro tissue slices

Two untreated female rats 54 days old were sacrificed using ether and the kidneys and livers removed. The organs were cut into slices 0.5 mm thin on a McIllwain chopper. Tissue slices were placed into glass vials (100 mg wet weight tissue/vial) containing 5 ml of Medium 199 with 1 ug/ml of oPRL or bGH and incubated for 3 hours at 37°C. Controls consisted of tissue slices incubated in medium without hormone. The contents of each vial were blotted on paper towels, weighed and homogenized in 5 volumes of 0.025 M Tris-HCL, 5 mM dithiothreitol, 0.1 mM EDTA, pH 7.3 using a motor driven glass-teflon homogenizer. Homogenates were centrifuged at 1500Xg and 4°C for 10 minutes and the supernatants assayed according to Richards (21).

## ORD activity in rabbit tissues in response to hormone treatment

### 1. Mammary explants in response to oPRL

Mammary tissue was aseptically removed from a day 31 pregnant rabbit and placed into organ culture as described above. On the fifth day of culture, 0.1 ml of growth medium was removed from all cultures



and replaced with 0.1 ml of medium containing oPRL (10 ug/ml). Control cultures received 0.1 ml of fresh medium. At 0.5, 1, 2 and 3 hours following the addition of hormone, media were transferred from the dishes into 25 ml Erlenmeyer flasks containing 0.1 ml of DL-(1-<sup>14</sup>C)ornithine-HCL (5 uCi/ml). Explants were weighed, transferred to new rafts and placed onto the medium in the flasks. The flasks were closed with rubber stoppers each holding a plastic cup and gassed with 5% CO<sub>2</sub>-95% O<sub>2</sub> sufficient to bring the pH to about 7.4. Flasks were incubated 4 hours at 37°C with gentle shaking before adding 0.3 ml hyamine hydroxide to the plastic cups. Immediately, 1 ml of 40% TCA was added to the medium and the flasks allowed to sit 20 to 22 hours at room temperature. The plastic cups and their contents were placed into glass vials containing 10 ml of scintillation cocktail and counted in a Beckman LS-350 counter. In order to destroy the viability of the explants, one half of the rafts transferred to the flasks at 3 hours were first floated on 70% ethanol for 30 minutes.

## 2. Liver explants in response to bGH

Liver tissue was aseptically removed from a day 31 pregnant rabbit and placed into organ culture as described for the rabbit mammary. The following day, after the explants have metabolically stabilized, 0.1 ml of the medium was removed from all cultures and replaced with 0.1 ml bGH (10 ug/ml). Control cultures received 0.1 ml of fresh medium. At 1, 2, 3 and 4 days following the addition of hormone a procedure identical to that described for the mammary explants was followed. However at day 2, all cultures yet to be transferred to flasks were

given a complete medium change for growth medium or growth medium plus bGH. In order to destroy the viability of the explants, one half of the rafts transferred at 4 days were first floated on 70% ethanol for 30 minutes.

#### ORD activity in a human pleural effusion

A pleural effusion from a woman with infiltrating duct mammary carcinoma (patient A described above) was collected by thoracentesis. The specimen was centrifuged at 1500Xg for 10 minutes and the supernatant retained. One ml of packed cells was resuspended in 5 ml of 0.025 M Tris-HCL, 5 mM dithiothreitol, 0.1 mM EDTA, pH 7.3 and homogenized by hand in a 7 ml Potter homogenizer. The homogenate was centrifuged at 1500Xg for 10 minutes. The supernatant and the pleural fluid obtained in the first centrifugation were assayed for ORD activity according to Richards (21). Control samples were pretreated with 40% TCA before incubation in Richards' assay system was begun. Aliquots of pleural fluid and supernatant of the cell homogenate were assayed for protein by the method of Lowry et al. (112).

#### Binding of <sup>125</sup>I-oPRL to rabbit mammary cells

Mammary tissue was removed from a day 31 pregnant rabbit and processed for culture as described above. Four separate batches of minced tissue of equal weight (2 g) were incubated with 0.1% collagenase in Medium 199 containing 10% FCS for 1, 2, 3 and 4 hours at 37°C to achieve different degrees of digestion. The digests were filtered through 4 layers of cheesecloth and collected in 4 separate 50 ml tubes. The tubes were centrifuged at 1500Xg for 10 minutes. Pellets were

resuspended in 5 ml of 0.025 M Tris-HCL buffer pH 7.6 containing 10 mM  $MgCl_2$  and 0.1% BSA, and a cell count was performed before assaying for binding of oPRL. The following assay components were added in order to 12 x 75 mm glass culture tubes: a) 0.2 ml Tris buffer b) 0.1 ml of cell suspension c) 0.1 ml of unlabelled oPRL (10 ug/ml) d) 0.1 ml of  $^{125}I$ -oPRL (117,000 cpm). Control tubes contained no unlabelled hormone; instead they received an extra 0.1 ml of Tris buffer. Duplicate tubes were used throughout. All tubes were incubated for 2 hours at 37°C. Reactions were stopped using 3 ml of cold 0.025 M Tris-HCL buffer pH 7.6 containing 0.1% BSA. The tubes were centrifuged at 1500Xg for 10 minutes, decanted and blotted dry before counting in a Searle gamma counter.

Binding of  $^{125}I$ -oPRL and  $^{125}I$ -hGH to human mammary cells in culture tubes

1. Pleural effusion cells

Pleural effusion cells from a woman with infiltrating duct mammary carcinoma were placed in culture for 2 weeks and then examined for hormone binding. Cells were removed from the culture flasks with 1:5000 EDTA in PBS(CMF) and centrifuged at 160Xg in an International centrifuge for 10 minutes at room temperature. Pellets were resuspended in 0.025 M Tris-HCL buffer pH 7.6 containing 10 mM  $MgCl_2$  and 0.1% BSA; and a cell count was performed using trypan blue. To 12 x 75 mm glass culture tubes, assay components were added in the following order: a) 0.3 ml 0.025 M Tris-HCL buffer pH 7.6 containing 10 mM  $MgCl_2$  and 0.1% BSA b) 0.1 ml unlabelled oPRL (10 ug/ml) or hGH (10 ug/ml) c) 0.1 ml cell suspension ( $10^7$  viable cells/ml) d) 0.1 ml  $^{125}I$ -oPRL (168,000 cpm) or  $^{125}I$ -hGH (115,000 cpm). Control tubes contained no unlabelled hormone; instead an extra 0.1 ml of

Tris buffer was added. Duplicate tubes were used throughout. All tubes were incubated at 37°C for 2 hours in a shaker water bath. Reactions were stopped by adding 3 ml of cold 0.025 M Tris-HCl buffer pH 7.6 containing 0.1% BSA and centrifuging at 1500Xg and 4°C for 10 minutes. Tubes were decanted, blotted dry and the pellets counted in a Searle gamma counter.

## 2. HBL-100 cells

HBL-100 cells were tested in the same manner as the pleural effusion cells except for 2 changes: 1) 0.025 M Tris-HCl buffer pH 7.6 containing 0.1% BSA, 0.2% glucose and 0.01% phenol red was used throughout; 2) unlabelled oPRL and hGH were used at 2 concentrations; 5 ug/ml and 10 ug/ml.

## Binding of $^{125}\text{I}$ -hPRL and $^{125}\text{I}$ -Ins to HBL-100 cells in culture dishes

HBL-100 cells were removed from culture flasks with 1:5000 EDTA or 0.25% trypsin in PBS(CMF) and centrifuged at 1500Xg for 10 minutes. Pellets were resuspended in growth medium and a cell count was performed using trypan blue. Two ml of cell suspension ( $0.5 \times 10^6$  viable cells/ml) were added to 35 x 10 mm plastic dishes and incubated at 37°C for 20 to 25 hours in the presence of 5% CO<sub>2</sub>-95% O<sub>2</sub>. The medium was then aspirated and replaced in order with: a) 0.8 ml growth medium containing various concentrations of FCS b) 0.1 ml unlabelled hPRL (10 ug/ml) or Ins (10 ug/ml) c) 0.1 ml  $^{125}\text{I}$ -hPRL (95,000 to 105,000 cpm) or  $^{125}\text{I}$ -Ins (75,000 to 115,000 cpm). Control dishes contained no unlabelled hormone; instead an extra 0.1 ml of medium was added. Triplicate dishes were

employed. Cultures were incubated further for various time intervals and then the reactions stopped by aspirating the isotopic medium and replacing it with ice-cold PBS(CMF). Cells were removed with a plastic scraper and added to ice-cold 12 x 75 mm glass culture tubes. Residual cells were harvested with a wash of PBS(CMF). The tubes were centrifuged at 1500Xg and 4°C for 10 minutes. Pellets were washed once with cold PBS(CMF) and the tubes decanted, blotted and counted in a Searle gamma counter.

Binding of  $^{125}\text{I}$ -hPRL,  $^{125}\text{I}$ -hGH and  $^{125}\text{I}$ -Ins to HBL-100 cell fractions

1. Preparation of cell fractions

HBL-100 cells reaching confluency in 150 cm<sup>2</sup> plastic flasks were harvested using a rubber policeman and McCoy's 5a medium or PBS (CMF). The cell suspension was centrifuged in 50 ml tubes at 1500Xg and 4°C for 10 minutes. Supernatants were discarded and the pellets resuspended in 3 to 4 volumes of distilled water. Cells were homogenized about 100 strokes in ice-cold Potter homogenizers. The homogenate was frozen at -20°C for 30 minutes. This process was repeated once again and the suspension was homogenized another 100 strokes before diluting 1:2 with 0.5 M sucrose to restore isotonicity. The homogenate was centrifuged at 15,000Xg and 4°C for 20 minutes in a JA-21 rotor using a Beckman JA-21B centrifuge. The supernatant was centrifuged at 100,000Xg and 4°C for 60 minutes in a 50 Ti rotor using a Beckman L5-65 ultracentrifuge. All pellets were resuspended in 0.025 M Tris-HCl buffer pH 7.6 containing 10 mM MgCl<sub>2</sub> and homogenized 50 to 100 strokes. Pellets and supernatants were aliquoted to 12 x 75 mm glass culture tubes and stored at -20°C.

Aliquots of these preparations were taken for protein determination by the method of Lowry et al. (112). Samples were dissolved by boiling 1 to 2 hours in 1N NaOH.

2. <sup>125</sup>I-hormone binding studies

Prior to use, the receptor preparations were homogenized about 50 strokes and kept on ice. To 12 x 75 mm plastic culture tubes the following were added in order: a) 0.3 ml 0.025 M Tris-HCl buffer containing 10 mM MgCl<sub>2</sub> and 0.1% BSA b) 0.1 ml unlabelled hormone (10 to 20 ug/ml) c) 0.1 ml receptor preparation (0.25 to 2.25 mg protein/ml) d) 0.1 ml <sup>125</sup>I-hPRL (85,000 to 100,000 cpm) or <sup>125</sup>I-hGH (80,000 to 95,000 cpm). Two controls were usually employed. One set of tubes contained an extra 0.1 ml of Tris buffer instead of unlabelled hormone. Another set of tubes containing reagents abc and <sup>125</sup>I-Ins (90,000 to 100,000 cpm) served as a second control. Duplicate tubes were used throughout. The tubes were shaken vigorously and allowed to stand 12 to 24 hours at room temperature. Reactions were stopped by adding 3 ml of cold Tris buffer and centrifuging at 1500Xg and 4°C for 10 minutes. Tubes were decanted, blotted dry and the pellets counted in a Searle gamma counter.

## RESULTS

Rabbit mammary cell culture1. Morphology and cell to cell relationships

Ten different mammary tissue preparations were established in culture. Initially, all cells settled onto the flask surface in small groups or clumps. Within 24 hours, the cells became attached and had begun to flatten assuming a spreading appearance. Spreading cells with long thin projections formed a ring around the attached cell clumps. During the first week the outgrowth from these clumps enlarged radially and consisted of an inner ring of polygonal epithelioid cells and a peripheral ring of elongated fibroblastoid cells. In subsequent weeks, the central clump of cells disappeared leaving monolayer patches of epithelioid cells. During this period, small groups of spreading cells were also increasing in number but consisted primarily of spindle-shaped fibroblastoid cells which grew in parallel arrays. In most cases, these cells quickly formed a sheet with a whorling pattern which spread across the entire flask surface thus containing the patches of polygonal cells and restricting their growth.

Figure 1 shows the pleomorphic appearance of cells which had been in culture 31 days. The cells had been subcultured at 7 and 12 days, and were beginning to re-attach. Note the large flattened cytoplasmic area and the granular appearance. At higher densities the 'cobblestone' pattern typical of epithelial cells was seen. However, confluency of epithelioid cells rarely occurred.

## 2. Maintenance of culture

Every 3 to 5 days, 50% of the medium was exchanged for fresh medium and any floating cells were added back. Flasks containing high cell densities received 100% medium changes and floating cells were discarded. In cases where fibroblastoid cell growth was restricting the spreading of epithelioid cells, medium containing 1.5 ug/ml of thimerosal was substituted. Thimerosal was found to effectively destroy fibroblastoid cells at this concentration. However, treatment had to continue several days before any results became apparent. Exposure to thimerosal for periods in excess of one week usually resulted in the destruction of epithelioid cells as well. Therefore, once a culture had become overgrown with fibroblastoid cells, this treatment was useless since it required longer periods of time to have a significant effect.

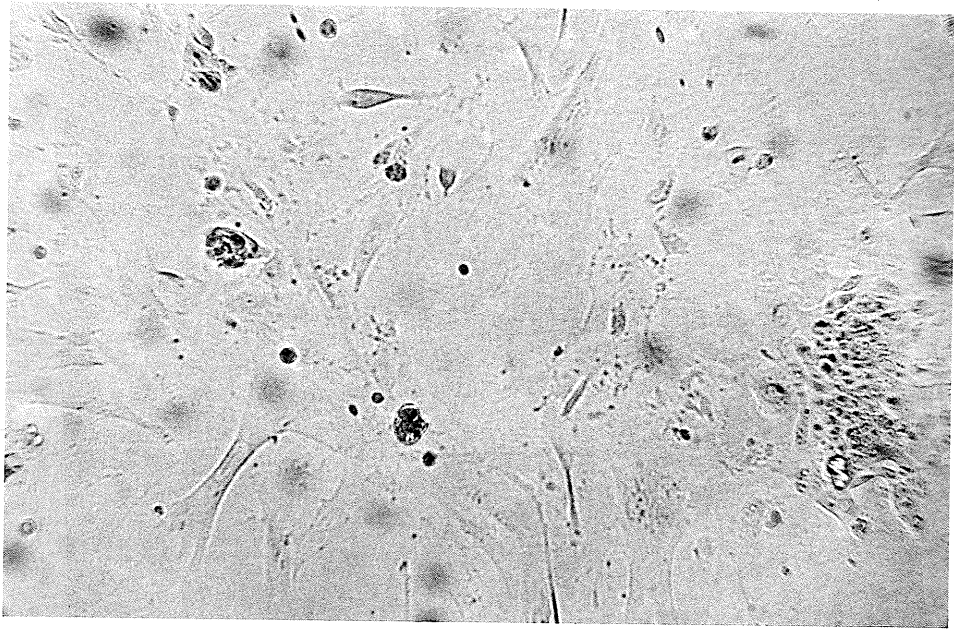
Three separate mammary cell preparations were maintained in vitro for periods of 3 to 6 weeks. After the first 2 or 3 weeks, these cells formed loose monolayers usually consisting of patches of polygonal cells in close approximation to one another, and they were split 1:2 using 0.25% trypsin in PBS(CMF). Then 5 to 7 days thereafter, these cultures were split again. No cultures were passaged more than twice.

## 3. Effect of enzyme digestion

Mammary tissue was digested with 0.1% or 0.2% collagenase for 1 or 2 hours. Cell viability was 92% to 98% and was not changed when either the enzyme concentration or incubation time was doubled. The total numbers of viable cells increased only slightly when 0.2% collagenase



Figure 1. Rabbit mammary cells 31 days after inoculation. This culture was passaged twice at 7 and 12 days. The typical pleomorphic cells with cytoplasmic extensions are shown. A group of smaller polygonal and quite granular cells is located at the right hand side in the photograph; x 100.



and a 2-hour incubation period were employed. There was no correlation between enzyme treatment and longevity of resulting cell cultures. In two cases, partially digested pieces of tissue remaining at the end of the enzyme treatment were resuspended in growth medium, triturated vigorously and placed into plastic culture flasks. These small (about 1 mm<sup>3</sup>) explants resulted in cell cultures which were predominantly epithelioid and which were cultured, but not subcultured, for 5 to 8 weeks.

#### Human pleural effusion cells in culture

##### 1. Cell types present

Pellets of cells obtained from pleural effusions were bloody. Red blood cells were not hemolyzed because it was found that they completely disappeared from culture in 1 to 2 weeks. Leucocytes, lymphocytes and mesothelial cells which were most likely present were not easily identified. However, 4 consistently recurring cell types were easily recognizable in most cultures. These cell types arising in cultures from each of the 3 breast cancer patients are listed in Table 3. It can be seen that dendritic and fibroblast-like cells were present in all cultures and usually survived for longer periods of time than did the round and epithelial-like cells. Only one culture, that arising from patient B, was devoid of epithelial-like cells. This fact may be connected with the relatively short survival time and low viability of cells from this specimen.

The survival times for the individual cell types reflect an order

Table 3

Cells found in pleural effusions from 3 breast cancer patients

	<u>Date</u>	<u>Volume (ml)</u>	<u>Cell viability (%)</u>	<u>Predominant cell types*</u>	<u>Culture survival (months)</u>
Patient A	3/3/75	780	99	†R, E D, F	<1 3-4
	8/3/75	550	95	†E D, F	1-2 4-5
Patient B	24/3/75	1100	75	†R, D, F	<1
Patient C	9/6/75	400	98	†R, D E, F	1-2 2-3
				‡E F, D	1-2 2-3

\* E = epithelial (presumably tumor) cells; R = small, round (epithelial-like) cells;  
D = dendritic cells; F = fibroblast-like cells.

†cells cultured in plastic flasks.

‡cells cultured in glass bottles.

of appearance of these cell types in a culture flask. Initially cultures usually consisted of predominantly round and dendritic cells. Round cells (Fig. 2) were well-attached but not flat and spreading like the dendritic cells. Dendritic cells were large and irregular in shape with several slender processes of various lengths; and usually had pale, poorly defined edges (Fig. 3). Elongated spindle-shaped cells resembling fibroblasts soon became prominent (Fig. 4) and in a few cultures, polygonal cells with lobe-like extensions and large nuclei appeared shortly thereafter. Similar to round cells, these epithelial-like cells had well-defined edges. However, most of these cells existed in clusters rather than as single isolated cells. In these clusters the epithelial-like cells were polygonal and their edges smooth and without projections thus giving a 'cobblestone' appearance (Fig. 5).

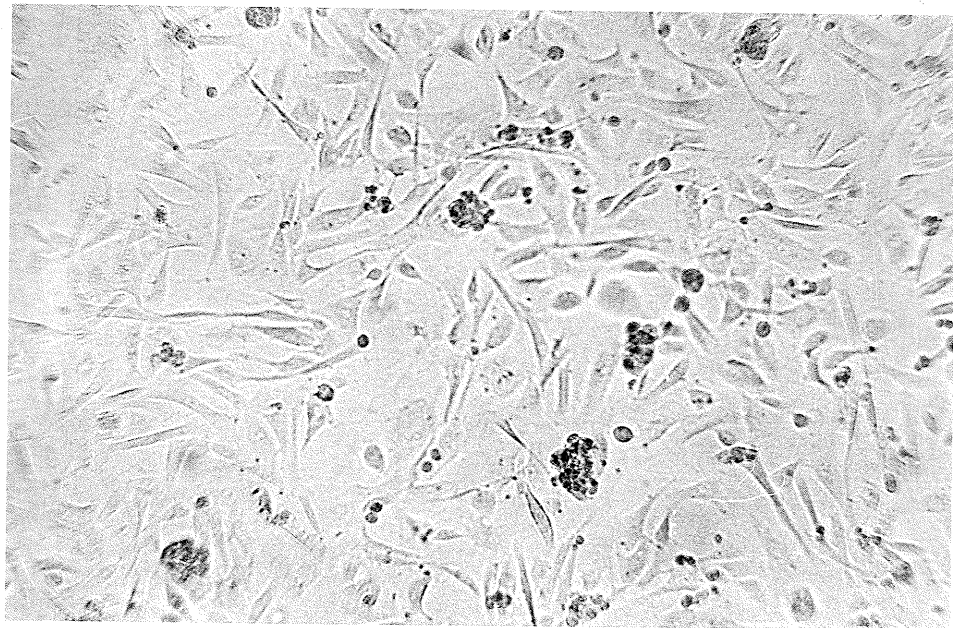
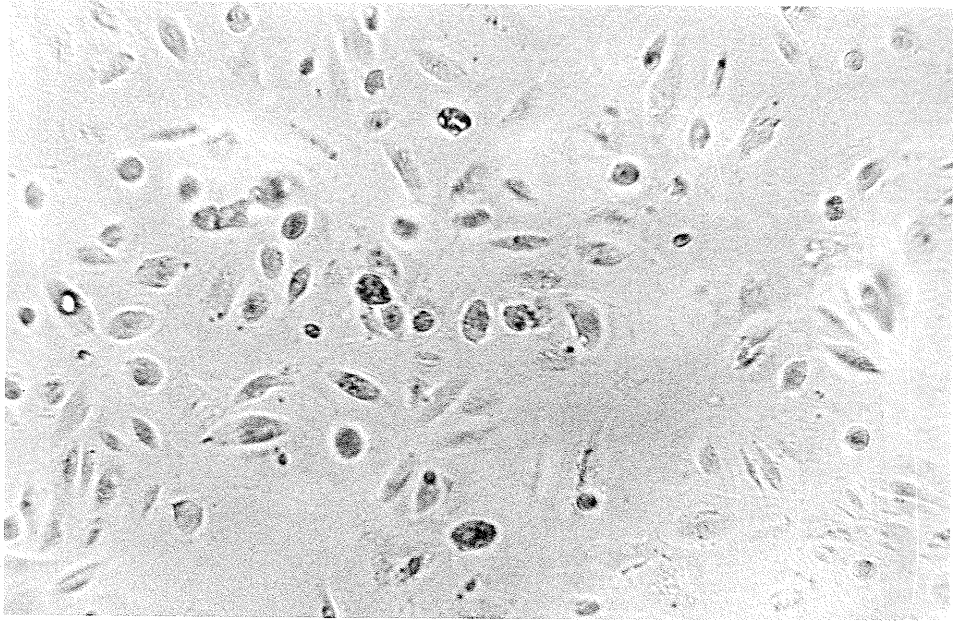
All cells generally had clear cytoplasm although there were periods when the cytoplasm appeared dark and granulated. Such periods may have been critical times for the cultures since it was frequently observed that cells became granular just prior to detachment.

## 2. Cell growth patterns

Inocula consisted of single cells and cell clumps. During the first week in culture, single cells settled onto the flask surface and became well-attached while floating clumps usually required weeks or months to attach themselves. During the second week, red blood cells quickly disappeared. Spindle-shaped (fibroblast-like) and dendritic cells multiplied slowly becoming more round as they filled the flask

Figure 2. Human pleural effusion cells 62 days after inoculation. This culture was passaged twice at 16 and 31 days. Round cells are shown. Most cells are raised and round in appearance; others are slightly elongated as a result of better attachment to the flask surface. The cell borders are smooth and well-defined; x 100.

Figure 3. Human pleural effusion cells 62 days after inoculation. This culture was passaged twice at 36 and 55 days. Dendritic cells, which are most prominent are shown. These cells are well-attached and spreading with several projections one of which is longer than the others. The end of the cell opposite the longest extension is usually relatively large and flat containing the nucleus. Some spindle-shaped and round cells are also seen; x 100.



(Fig. 2). Small groups of round cells grew in until plaques of polygonal (epithelial-like) cells formed at 2 to 17 weeks (Fig. 6). Some piling of these cells occurred. Outgrowths from attached cell clumps also gave rise to epithelial-like cell plaques with peripheral borders of spreading fibroblast-like cells. Plaques of polygonal cells increased in size and at times interconnected to form large expanses of monolayer growth (Fig. 5). Completely confluent monolayers were not produced and cell detachment was common.

All cultures did not exhibit the above growth sequence. Cultures that never reached the stage where cell plaques were formed and others that never passed through any of the intermediate stages of single cell growth were not uncommon. Regressions in which the density of cell growth decreased were also common. Usually single cells and the cell balls in the center of plaques shrivelled up and detached from the flask surface. In some cases, single cells began to increase in number again while in others, the cultures lay seemingly dormant for days before undergoing further regression.

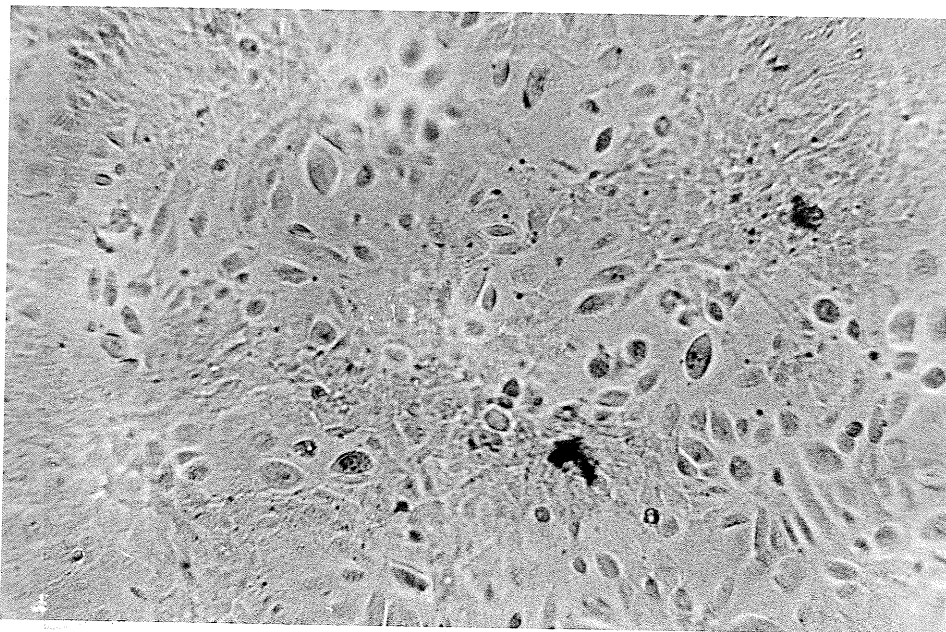
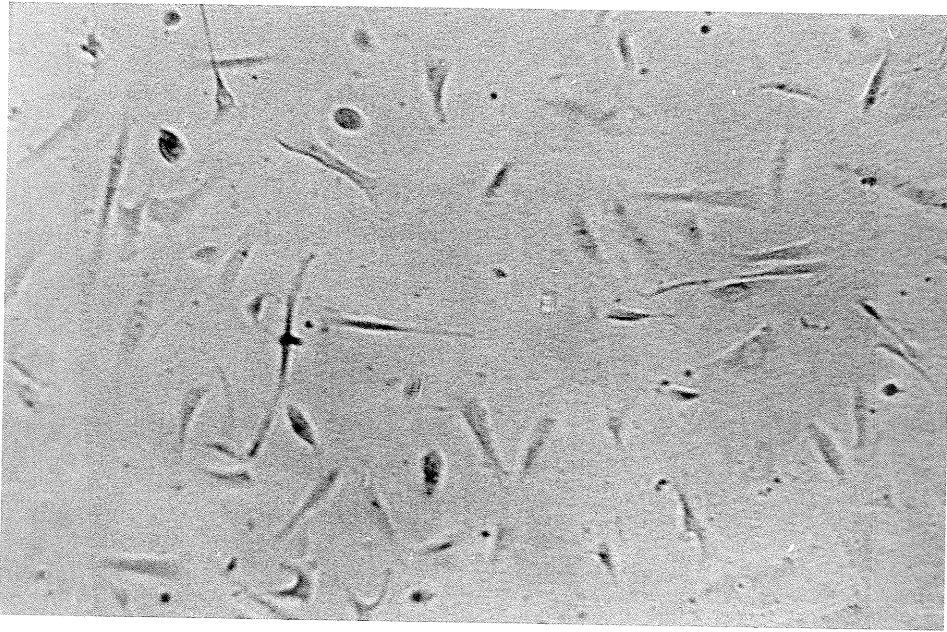
### 3. Cell to cell relationships

Two interesting phenomena occurred in most cultures. The first concerns the initial stages of a culture when cell growth was quite sparse and there were cells lying in close proximity to one another. These cells quickly developed slender projections at many points around their edges with the longest projections being sent off in the direction of neighbouring cells and at times making close contact with these cells (Fig. 7). Such cells resembled the dendritic cells described



Figure 4. Human pleural effusion cells 54 days after inoculation. This culture was passaged twice at 16 and 31 days. Spindle-shaped cells are shown. They are elongated with a slender projection at one or both ends of the cell. Unlike dendritic cells, they are more slender and do not have a flattened end containing a nucleus; x 100.

Figure 5. Human pleural effusion cells 54 days after inoculation. This culture was passaged at 36 days. Clusters of small, polygonal cells loosely interconnected to form an incomplete monolayer are shown. Such clusters arise from crowding of round cells or as outgrowths from attached cell clumps. Large round cells are shown in small groups which lie between and around the epithelioid cell clusters; x 100.



above except that they were flatter and more spreading with many more slender, elongated projections. It was also observed that as these cells filled in an area of the flask and thus came to lie more closely together, they seemed to withdraw or shorten these extensions. As the area continued to increase in density, the cells would crowd and become raised and rounded in shape.

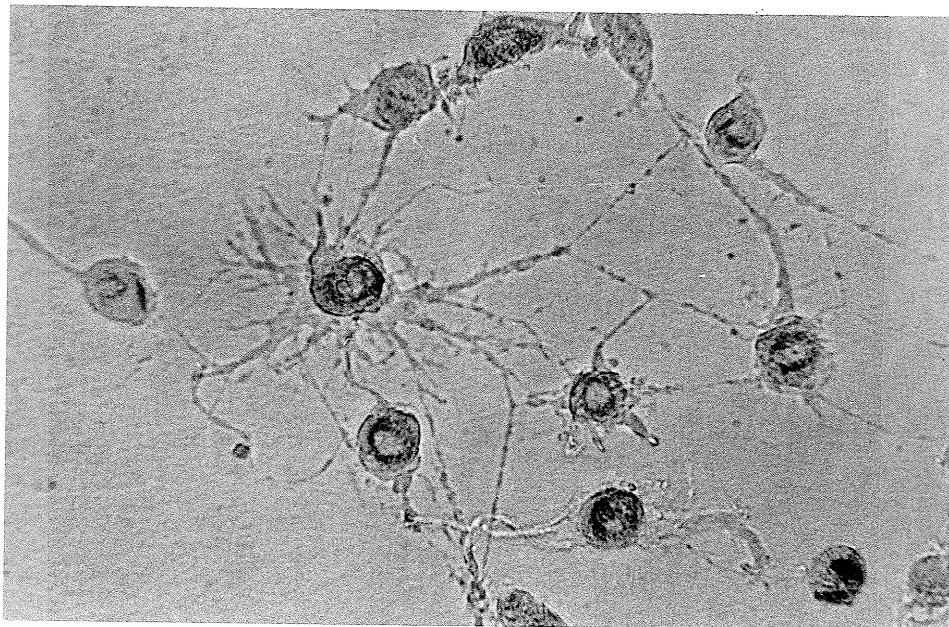
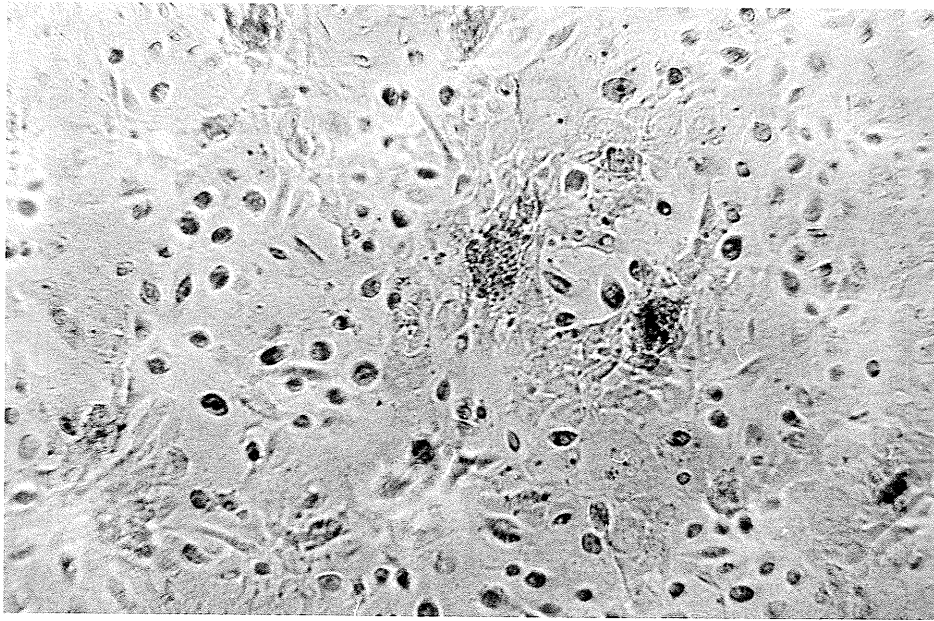
At a later stage in a culture when floating grape-like clumps of round cells had had time to attach a second interesting event occurred; cell outgrowths from these clumps. Initially the outgrowing cells were spindle-shaped fibroblast-like cells. As the outgrowth continued only the outermost border consisted of these cells while the cells lying between them and the inner-most cell clump were polygonal epithelial-like cells (Fig. 8). The cell clumps in the center of the developing plaques of epithelial-like cells diminished as the middle layers increased in size. Cells at the edge of the flattening cell clumps were piled upon one another. In many cases the cell clumps disappeared entirely leaving a monolayer of polygonal cells with a peripheral border of elongated spindle-shaped cells.

#### 4. Characteristics of pleural effusion cell cultures

Table 4 lists the number of cultures obtained from each specimen of pleural fluid and the individual treatment that each received. Cultures of June 1975 were plated with a lower dilution of cells than was used for cultures of March 1975. This procedure resulted in the formation of a monolayer of epithelial-like cells 6 to 7 times more

Figure 6. Human pleural effusion cells 62 days after inoculation. This culture was passaged twice at 36 and 44 days. The formation of 2 plaques of epithelioid cells by round cell crowding is shown. Groups of large round cells grew in and became heavier resulting in polygonal cell plaques. Some piling of these cells occurred and can be seen as 2 dark, raised areas located on top of the cell plaques; x 100.

Figure 7. Human pleural effusion cells 38 days after inoculation. This culture was passaged twice at 19 and 31 days. An unusual group of cells seemingly interconnected by cytoplasmic extensions is shown. All cells in such groups were large and flat with a 'poached-egg' appearance. The nuclei of these cells almost filled the cytoplasm. Such groups were seen only in the initial stages of the culture when cell growth was sparse. As these cells grew in closer together, they withdrew these interconnecting extensions; x 320.



quickly than that observed in the cultures of March 1975. But the cultures of June 1975 had been incubated for one week without disturbance in the presence of Medium 199, 2% FCS, 15 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin which may account for the results. However, a similar situation was seen in some cultures of March 3 when the equivalent of a high dilution of cells was employed. As indicated in table 4, 1 to  $2 \times 10^6$  cells/ml of medium was plated; but 24 hours later, all floating cells and those which were easily shaken loose were transferred to new flasks leaving behind a very sparse population of well-attached, spreading cells. At 3 days the floating cells in the "daughter" flasks were well attached and exceeded in number those cells present in the "mother" flasks; yet both sets of cultures reached the same stage of plaque formation at 3 months. It appears that the effect of high cell dilution was to stimulate cell growth.

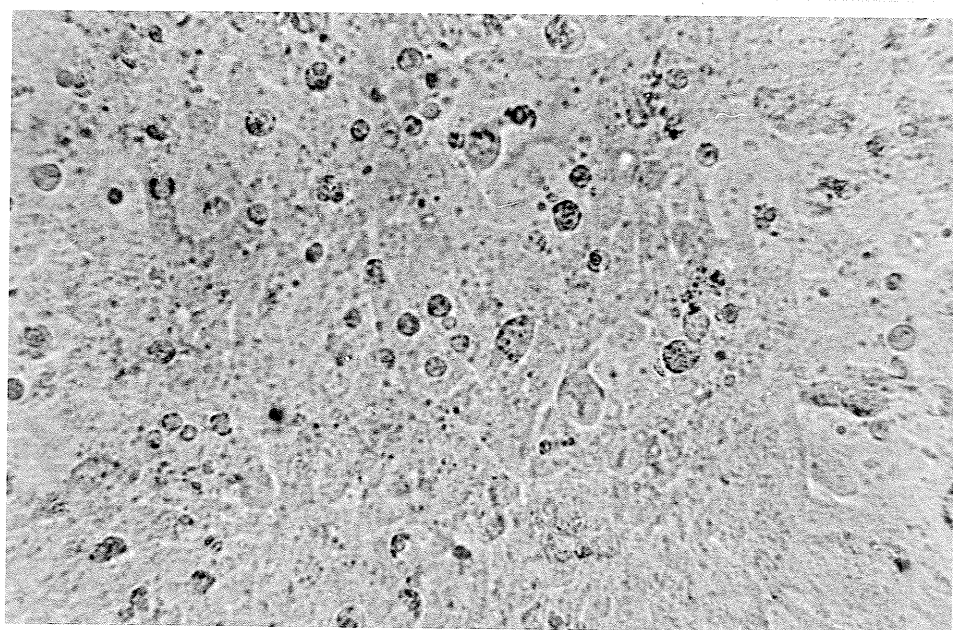
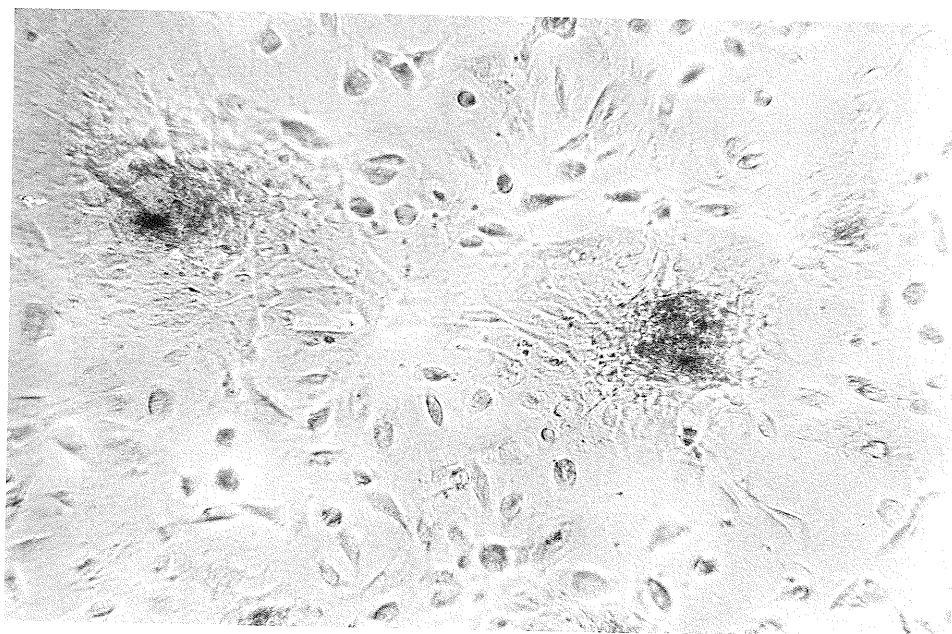
Throughout the course of a culture, free cells were continually shed into the medium. Since these floating cells were added back into the same flask after every change of medium there soon (about 1 to 6 weeks) accumulated many such cells which then were transferred to new flasks. These cultures were usually incubated one week without disturbance and produced the same cell types and growth pattern as described above.

Cultures usually received changes of medium every 3 to 5 days although it was not uncommon for longer periods to be employed. With experience a rule was adopted that unless changes in pH demanded it,

Figure 8. Human pleural effusion cells 78 days after inoculation. This culture was passaged twice at 36 and 44 days. Two epithelioid cell plaques arising from 2 attached cell clumps are shown. A peripheral border of elongated spindle-shaped cells encloses an area of polygonal cells in each plaque. The central clump of cells usually flattened out and disappeared completely to leave a small monolayer patch of epithelioid cells; x 100.

Figure 9. Cells of the human breast cell line HBL-100 after 34 passages. These cells usually grew in small groups rather than as single cells. The cell groups would increase in size and interconnect to form a confluent monolayer. At this stage the cells were quite granular and had poorly defined borders; x 320.







cultures would be left undisturbed for periods of up to 10 days. Different media were used for the various cultures and the most important of these are listed in Table 4. Medium 199 was used throughout the March 1975 cultures and seemed to be adequate for good growth in all but the March 24 culture. The addition of hydrocortisone and insulin to the same cultures for 1 to 2 months did not appear to have any effect on plaque formation and certainly was not necessary for cell survival. Cultures of June 1975 were maintained equally well on a variety of media including Medium 199, MEM, Leibovitz's L-15 and McCoy's 5a, the first two being used most often. All except Medium 199 contained insulin and most contained hydrocortisone as well. Three of the four flask cultures of June 1975 contained many large monolayers of epithelial-like cells in 2 to 3 weeks and were subcultured at a 1:2 dilution using 0.25% trypsin in PBS(CMF). At 13 to 18 day intervals thereafter, these cultures were either split at 1:2 again or transferred to glass bottles. Cell growth in these bottles was no different than in the flasks except that the cells were more easily shaken loose. One bottle culture was subcultured twice at a 1:2 dilution at 3 week intervals. That the presence of hormones in most of the culture media employed with the June 1975 cultures was responsible for their consistent growth is implied but not proven here.

#### Human mammary cell lines

##### 1. HBL-100

These non-malignant cells grew as spreading monolayer patches of polygonal cells which would usually join to form a confluent monolayer

Table 4

Characteristics of individual pleural effusion cell cultures

<u>Date</u>	<u>No. of cultures</u>	<u>Cells/ml x 10<sup>-6</sup></u>	<u>Medium</u>
3/3/75	† 26	1-2	*1,2
8/3/75	† 6	1-2	2, *3
24/3/75	† 1	1	1
9/6/75	† 4 ‡ 2	5 Not done	*3,4,5,6 *5,7,8,9

† cells cultured in plastic flasks

‡ cells cultured in glass bottles

\* medium employed for greater than 50% of the medium changes

1. Medium 199, 10-20% FCS, 15 mM Hepes buffer, 100 U/ml penicillin, 100 ug/ml streptomycin.

2. Medium 199, 10% FCS, 15 mM Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin, 5 ug/ml hydrocortisone, 20 ug/ml insulin.

3. Medium 199, 10% FCS, 15 mM Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin.

4. Leibovitz's L-15, 10% FCS, 10% bovine amniotic fluid, 15 mM Hepes buffer, 16 ug/ml glutathione, 50 ug/ml gentamycin, 10 ug/ml insulin.

5. Minimum essential medium (MEM), 10% FCS, 15 mM Hepes buffer, 50 ug/ml gentamycin, 10 ug/ml insulin.

6. Medium 199, 10% FCS, 15 mM Hepes buffer, 100 U/ml penicillin, 100 ug/ml streptomycin, 5 ug/ml 17- $\beta$ -estradiol, 5 ug/ml hydrocortisone, 20 ug/ml insulin.

7. Leibovitz's L-15, 15-20% FCS, 15 mM Hepes buffer, 50 ug/ml gentamycin, 16 ug/ml glutathione, 5 ug/ml hydrocortisone, 10 ug/ml insulin.

8. McCoy's 5a, 10% FCS, 15 mM Hepes buffer, 50 ug/ml gentamycin, 10 ug/ml insulin.

9. McCoy's 5a, 20% FCS, 15 mM Hepes buffer, 16 ug/ml glutathione, 50 ug/ml gentamycin, 5 ug/ml hydrocortisone, 10 ug/ml insulin.

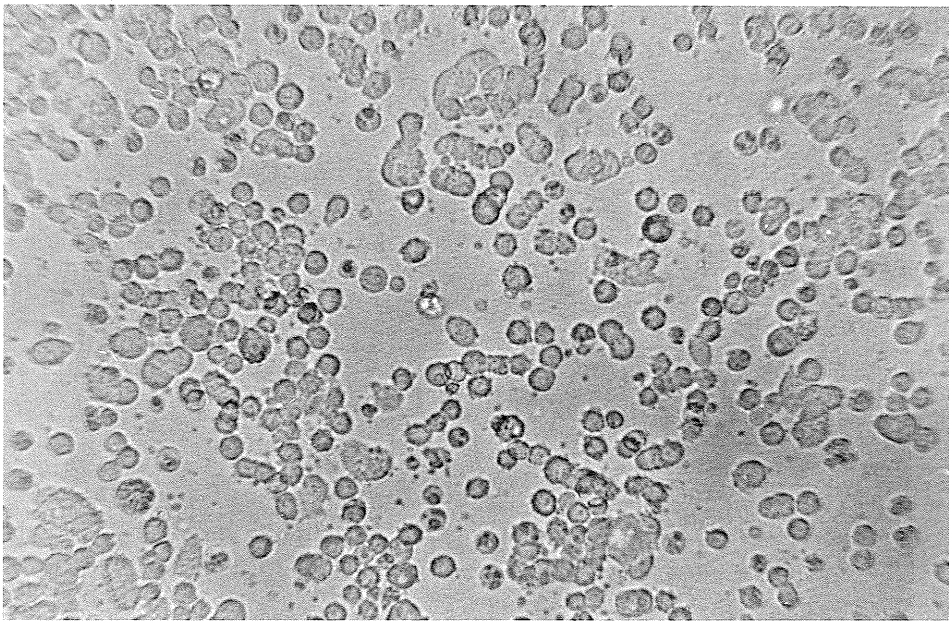
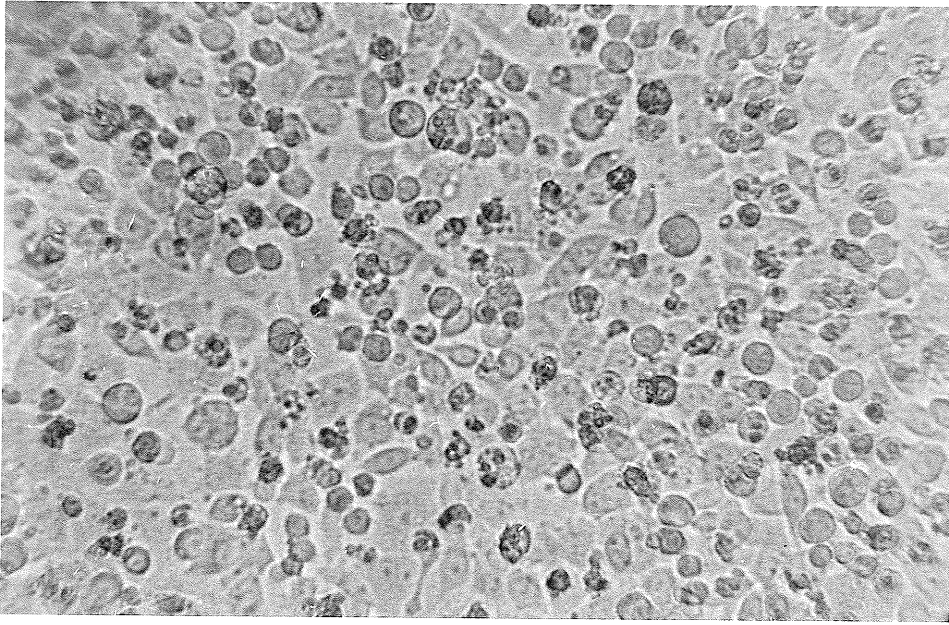
(Fig. 9). However, piling of cells occurred in heavily crowded areas resulting in clumps or thickened edges of the monolayer. These cultures were passaged using 0.25% trypsin in PBS(CMF) or 1:5000 EDTA in PBS(CMF). Trypsin was preferred since the cells seemed to form clumps during the procedure which were difficult to break up even with the enzyme solution and prolonged vigorous pipetting. Resulting cultures containing some of these undigested cell clumps usually did not form complete monolayers but rather scattered, large patches of polygonal cell outgrowths with central clusters of round cells. Dilutions of cell inocula ranged from 1:3 to 1:4 although much higher dilutions could be made thus minimizing the number of cell clumps in each culture. This latter procedure was practically impossible since before a confluent monolayer was formed, a medium change was required and the cells of this line were peculiarly sensitive to any manipulation of medium. Even if the same medium was added back into the flask, cells would begin to round up and detach. Thus changes of medium were avoided and the dilutions of inocula lowered during subculture. Cells cultured in this manner were passaged every 4 to 6 days.

## 2. MDA-MB-157

Cells of this malignant cell line grew as single entities isolated from one another. Cells of this line were much more granular than those of the HBL-100 cell line and they had lobe-like projections along their edges unlike the smooth-edged appearance of the non-malignant cells. Groups of cells soon formed and became more crowded and larger until

Figure 10. Cells of the human breast cell line MDA-MB-157 after 118 passages. These cells grew as single separate entities and populated the flask to the point where confluency was reached. Piling always occurred. These cells were very granular and had well-defined borders. Shown here is a confluent culture; x 320.

Figure 11. Cells of the human breast cell line MDA-MB-134VII after 41 passages. Cells of this line usually grew independently with little contact or together in small groups. These groups and some of the single cells were well-attached and ovoid in shape. Most of the single cells seemed to be just resting on the flask surface and were round; x 320.



interconnecting patches filled the flask (Fig. 10). Confluent monolayers of cells were rare. Much cell piling occurred with clumps of round cells attached loosely to a well-attached underlying layer of cells. These clumps often were shed into the medium and were used to establish new cultures. All cultures were passaged every 6 to 8 days using 0.25% trypsin in PBS(CMF) at dilutions of 1:3. Complete changes of medium were performed each 3 or 4 day interval.

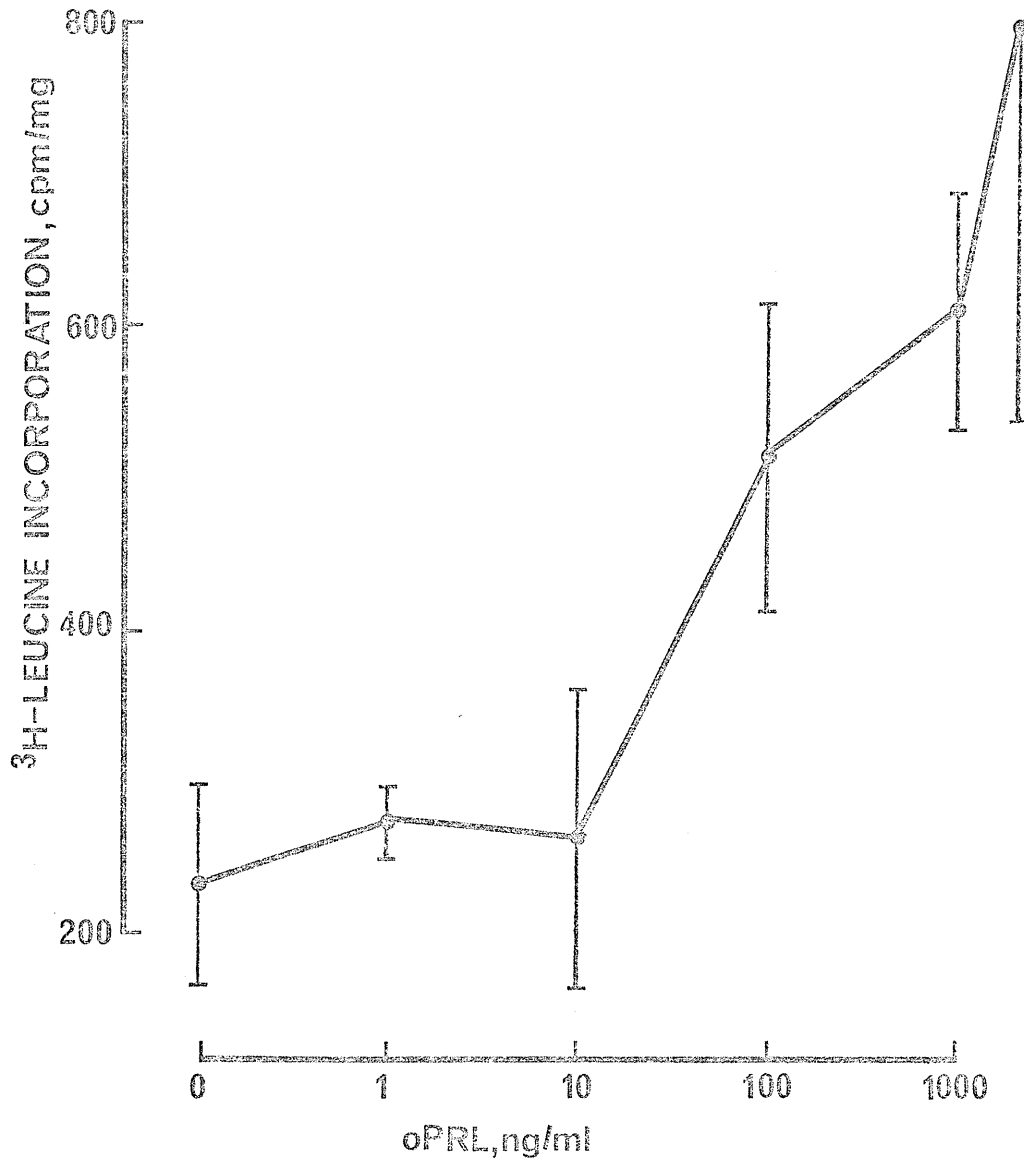
### 3. MDA-MB-134VII

Cells of this malignant cell line usually grew with little cell contact either independently or in small groups (Fig. 11). Unlike those of the other two cell lines, these cells were heterogeneous in both shape and size. They never reached confluency, requiring weeks before even covering the flask surface as scattered isolated clusters. As the cultures became heavier with growth an increasing number of cells detached to form a floating cell population and it was these floating cells which were used to establish most new cultures. Only once 1:5000 EDTA in PBS(CMF) was used to passage this cell line. However, gentle shaking alone would achieve the same results as these cells were never well-attached. As long as 1 to 2 weeks was then required for the majority of cells to re-attach themselves. Medium changes, which were performed every 6 to 10 days, included adding the floating cells back into the flasks.

### Effect of PRL on casein synthesis

#### 1. Rabbit mammary explants

To demonstrate that PRL can stimulate casein synthesis in rabbit



mammary tissue in vitro, mammary explants were incubated with increasing concentrations of oPRL. Figure 12 shows that rabbit mammary tissue in vitro retained its sensitivity to oPRL and was stimulated to synthesize casein. Increases in  $^3\text{H}$ -leucine incorporation into casein was produced by concentrations of oPRL greater than 10 ng/ml. A better than 2-fold increase in casein synthesis was caused by 1 ug/ml of oPRL. Hormone concentrations of 10 ng/ml or less produced no significant change from the control value.

## 2. Human mammary HBL-100 cells

Human mammary HBL-100 cells were originally obtained as cells found in milk samples taken from women who were lactating. It seemed possible that these cells may therefore be capable of milk protein synthesis in response to PRL. These cells were maintained as monolayer cultures in medium containing insulin and hydrocortisone for 3 days and then exposed to hPRL for various time intervals. Table 5 demonstrates that hPRL did stimulate casein production at 2 and 12 hours but by 24 hours this effect had ceased.

Six cultures, 3 containing PRL and 3 without PRL were examined by light microscopy for cell attachment on each day following the addition of hormone. Cell growth appeared the same in all cultures; PRL did not seem to affect cell attachment or morphology. However, the appearance of the cells did change with time. At 24 hours subsequent to hormone addition the cells seemed poorly attached and consisted of 2 types: 1) flat and spreading, and 2) raised and rounded. The cells were still poorly attached at 48 hours but an increase in the well-attached spreading



Table 5

Incorporation of  $^3\text{H}$ -leucine into casein by human mammary cells in response to hPRL. HBL-100 cells were preincubated at  $37^\circ\text{C}$  in medium containing insulin and hydrocortisone. At 72 hours 1  $\mu\text{g}/\text{ml}$  of hPRL was added to the cultures. Control cultures received no PRL. Cultures were further incubated for 2, 12, 24, 48 or 72 hours. Then, half the medium was removed and 5  $\mu\text{Ci}/\text{ml}$  of  $^3\text{H}$ -leucine was added to all cultures. Incubation with  $^3\text{H}$ -leucine was carried out for 4 hours. Each value represents the mean  $\pm$  S.D. of 3 cultures.

<u>Hours</u>	<u><math>^3\text{H}</math>-leucine incorporation into casein (cpm)</u>		<u>Significance</u>
	<u>Control</u>	<u>Prolactin</u>	<u>Student's t-test</u>
2	133 $\pm$ 50	195 $\pm$ 4	p < 0.005
12	146 $\pm$ 44	203 $\pm$ 14	p < 0.005
24	189 $\pm$ 70	213 $\pm$ 7	*NS
48	172 $\pm$ 64	102 $\pm$ 20	NS
72	206 $\pm$ 8	121 $\pm$ 60	p < 0.001

\*NS, not significant

type was noticed. At 72 hours the cells were beginning to detach and the majority were of the raised, rounded type. Floating clumps of cells were present from the start but were not assayed for casein.

#### Effect of bGH on ORD activity in rat kidney and liver

To gain experience with the ORD assay system, an experimental design similar to that described by Russell et al. (113) was employed. Two female rats (135 g) were injected ip with 2 mg of bGH in 0.5 ml of saline and 2 control rats received ip injections of 0.5 ml of saline. All animals were sacrificed 4 hours later. Liver and kidney samples (1.0 g wet weight) were processed in duplicate as described in the Materials and Methods section. Figure 13 shows that in the 2 tissues tested, the ORD activity was significantly elevated in those animals injected with hormone. Enzyme activity was raised 58-fold in the liver and 6-fold in the kidney. The ORD level in the control kidney was 30-fold higher than that of the liver control.

#### Effect of bPRL on ORD activity in rat kidney and liver

To test the effect of PRL on the ORD activity in rat tissues 2 female rats (400 g) were given ip injections of 4 mg of bPRL in 0.5 ml of saline; control animals received ip injections of 0.5 ml of saline. All animals were sacrificed 2 hours later. Liver and kidney samples (0.5 g wet weight) were processed in duplicate as described in the Materials and Methods section. Results of this experiment are presented in Figure 14A. ORD values in both the liver and kidney of rats receiving bPRL were increased above control values. The level of ORD

Figure 13. In vivo effect of bGH on ORD activity in rat kidney and liver tissues. Two female rats (135 g) received ip injections of 2 mg of bGH in 0.5 ml saline. Control animals received injections of saline. All animals were sacrificed 4 hours following treatment. Each value represents the mean  $\pm$  S.D. for 4 determinations.

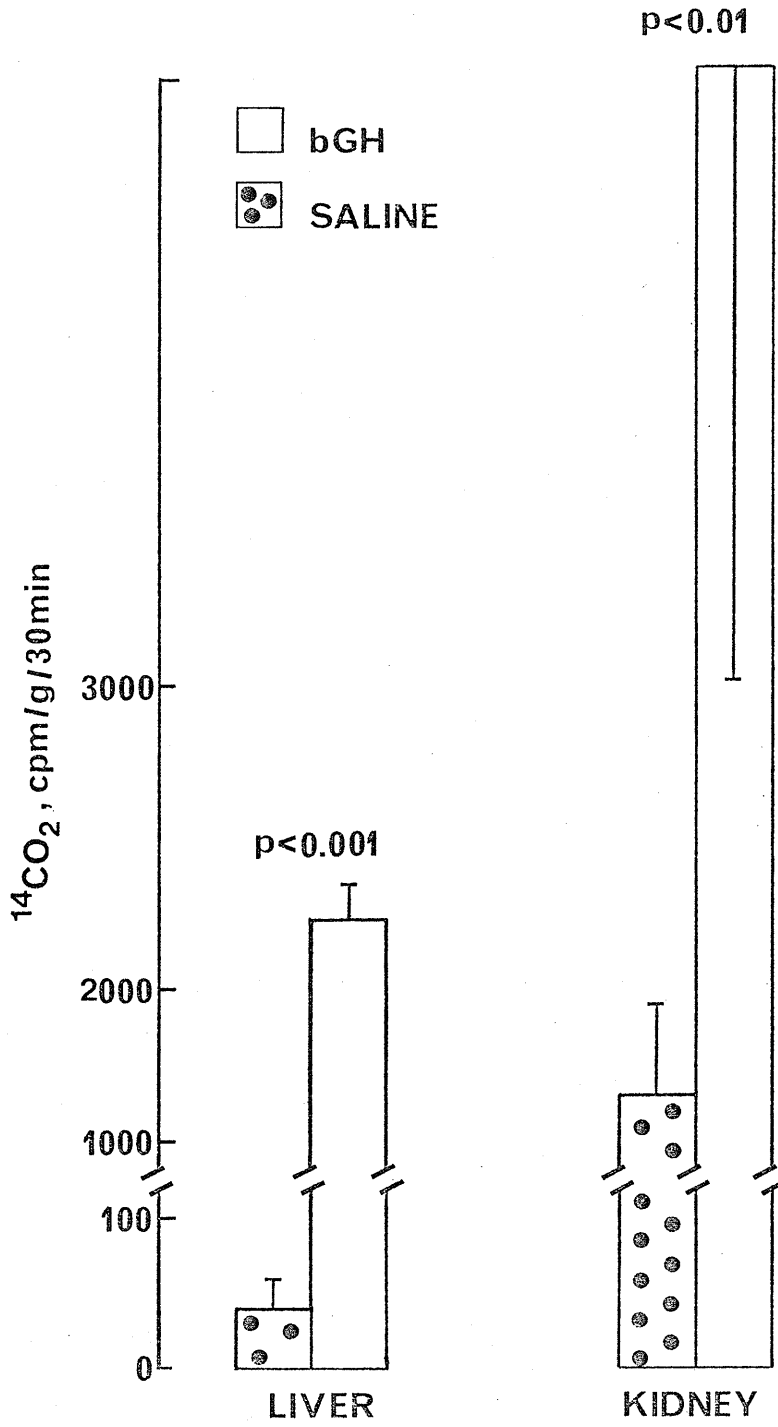
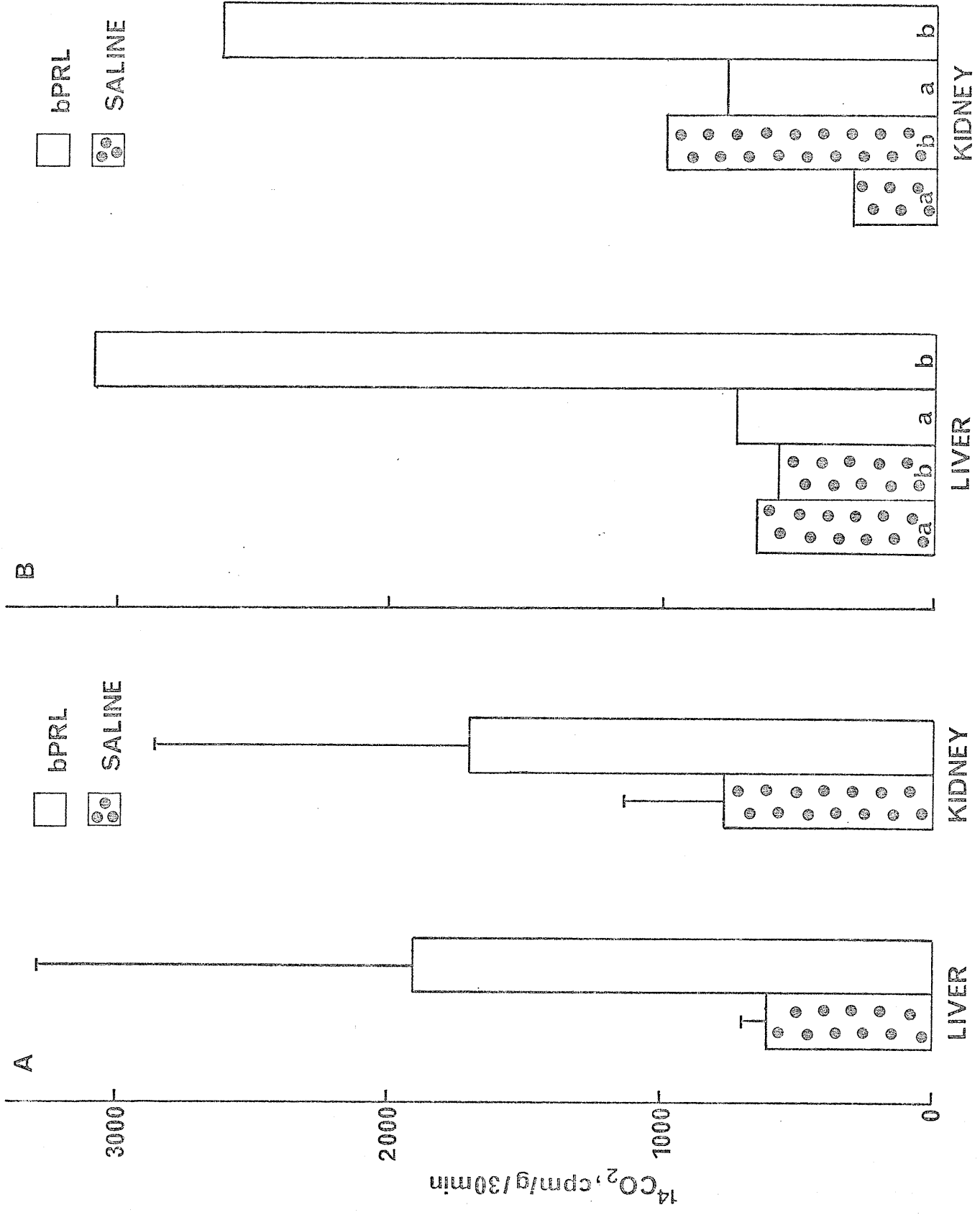


Figure 14A. In vivo effect of bPRL on ORD activity in rat kidney and liver tissues. Two female rats (400 g) received ip injections of 4 mg of bPRL in 0.5 ml saline. Control animals received injections of saline. All animals were sacrificed 2 hours following treatment. Each value represents the mean  $\pm$  S.D. for 4 determinations.

Figure 14B. Data for the 2 control and experimental rats shown in figure 14A are plotted for each animal. This figure shows that the large S.D. evident in figure 14A were responsible for masking any significant increases in ORD activity due to PRL. Each value represents the mean of 2 determinations.



was raised 3-fold in the liver and 2-fold in the kidney. Neither increase was significant using Student's - t statistics.

The large standard deviations evident in Figure 14A were responsible for masking any significant increases in ORD activity due to PRL. Figure 14B. demonstrates that large differences in assay values existed between animal a and animal b in 3 of 4 value comparisons. In each of these 3 comparisons the values for samples from animal a were much lower than those for samples from animal b.

#### ORD activity in untreated rat tissues.

Samples of liver and kidney tissue were removed from 3 untreated female rats (400 g) and assayed for ORD activity as described in the Materials and Methods section. Enzyme values were 3-fold higher in the kidney; a significant ( $p < 0.005$ ) elevation above that found in the liver (data not shown).

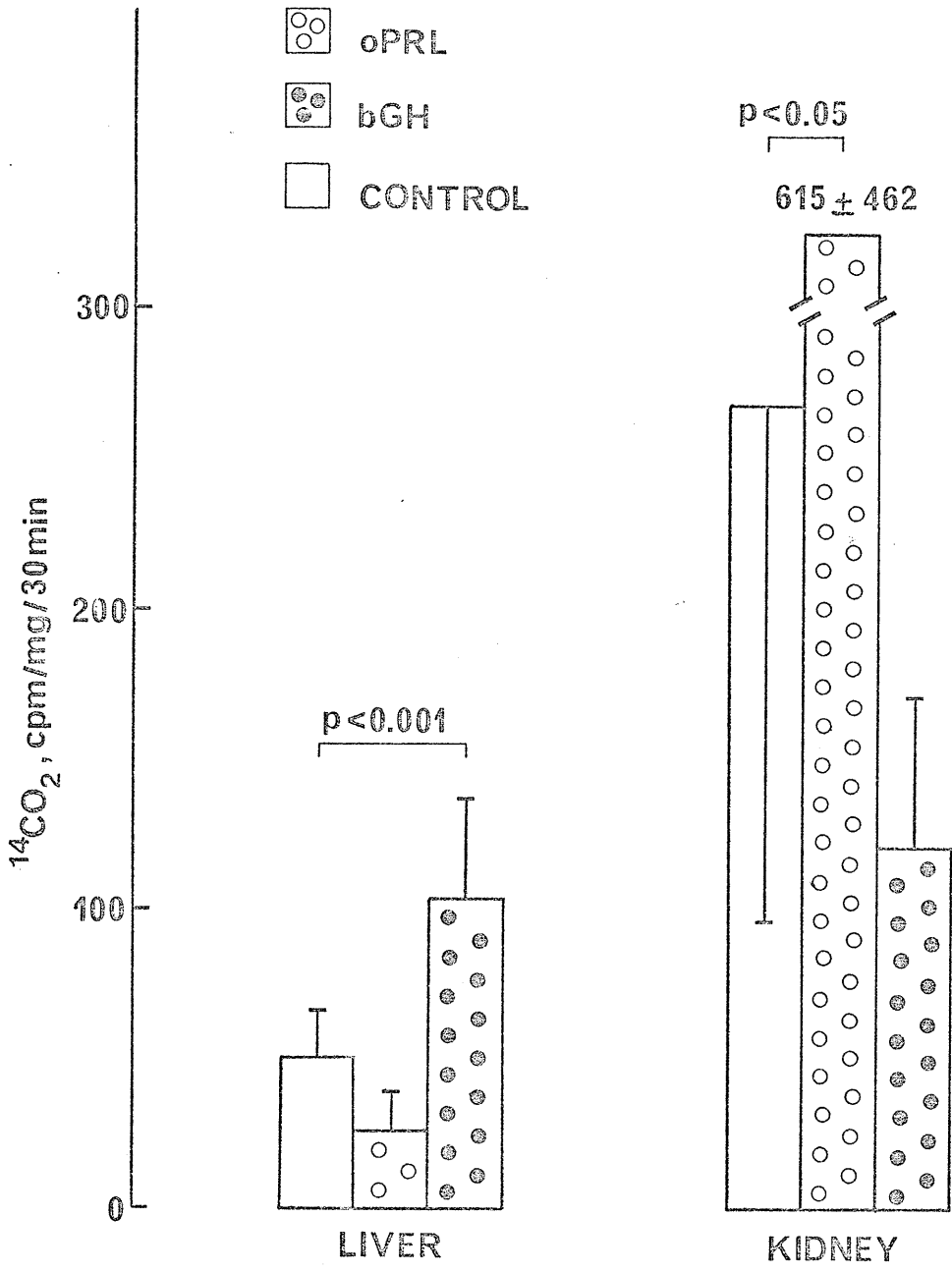
#### ORD activity in rat tissue slices incubated with oPRL and bGH

An in vitro incubation technique was designed as an alternative to the in vivo injection studies. The components of the system were easily controlled and therefore variations in the results seen in the injection studies minimized. Such a system also allowed the use of less hormone. Liver and kidney tissues from two 54 day old untreated female rats were sliced and incubated at 37°C in the presence of bGH and oPRL. Control tissue slices were incubated in the absence of hormone. Further details are given in the Materials and Methods section.

Results using this technique are presented in Figure 15. Slices

Figure 15. In vitro effects of oPRL and bGH on ORD activity in rat kidney and liver. Tissues from 2 female rats (54 days old) were cut into slices and incubated for 3 hours at 37°C in glass vials containing 5 ml of Medium 199 and 1 ug/ml of oPRL or bGH. Control slices were incubated in medium containing no hormones. Each value represents the mean  $\pm$  S.D. of tissues from 12 vials.





of liver incubated with bGH had ORD values significantly elevated above those in the control slices. Incubation of liver slices with oPRL resulted in ORD values not significantly different from the control values. Kidney tissue responded in an opposite manner; oPRL caused a significant increase in ORD activity while bGH produced values not significantly different from those of the control tissue. The control value for the kidney slices was 5-fold higher than that found in the liver slices.

#### Effect of oPRL on ORD activity in rabbit mammary explants

The late-pregnant rabbit mammary gland is a well-known target organ for PRL. The interaction of the hormone with its binding sites in this tissue has been extensively characterized (8,9). Thus, this tissue was examined for its responsiveness, in terms of ORD activity, to oPRL. Explants of mammary tissue were maintained in culture for 5 days and then exposed to 1.0 ug of oPRL for various time intervals before assaying for ORD as in the Materials and Methods section. Control explants were incubated in the absence of hormones.

Table 6 shows the results. No significant differences in ORD activity existed between the 2 groups of explants at any time. Thus, PRL did not seem to stimulate ORD activity in rabbit mammary explants. At 3 hours, pretreatment of some cultures with 70% ethanol clearly interfered with the utilization of the  $^{14}\text{C}$ -ornithine substrate. Those cultures not receiving this treatment had significantly greater ORD activities. This result suggests that at the termination of the incubation, the explants were still viable and that they were permeable to the substrate.

Table 6

Effect of oPRL on ORD activity in rabbit mammary explants. The explants were prepared from mammary gland of a day 31 pregnant animal and placed into organ culture as described in the Materials and Methods section. On the fifth day of incubation at 37°C, 0.1 ml of medium was removed from the culture dishes and replaced with 0.1 ml of medium containing 1 ug of oPRL. Control dishes received 0.1 ml of medium. At various time intervals following hormone addition, media and explants were transferred to flasks containing 0.1 ml of <sup>14</sup>C-ornithine (5 uCi/ml). Some explants were treated for 30 minutes with ethanol before transferring to flasks. Flasks were incubated for 4 hours at 37°C and the reaction terminated with 1 ml of 40% TCA. The flasks remained for 20 to 22 hours at 24°C before assaying. Each value represents the mean ± S.D. of explants from 4 cultures.

<u>Hours</u>	<u><sup>14</sup>CO<sub>2</sub> (cpm/mg wet wt.)</u>		<u>Significance</u>
	<u>Control</u>	<u>Prolactin</u>	<u>Student's t-test</u>
0.5	724 ± 76	683 ± 209	** NS
1.0	936 ± 56	848 ± 178	NS
2.0	669 ± 192	788 ± 50	NS
3.0	640 ± 222	754 ± 51	NS
	*60 ± 6	*45 ± 5	P < 0.005

\* cultures pretreated with ethanol

\*\* NS, not significant

Effect of bGH on ORD activity in rabbit liver explants

In an experiment similar to that described above for the mammary gland, the effect of bGH on ORD activity in the liver from a day 31 pregnant rabbit was examined. Table 7 shows that at day 1 following the addition of hormone ORD levels in the control explants were significantly greater than in those explants that had received bGH. No significant differences between the 2 groups occurred at any other time. On day 4, the ORD values were virtually identical between the 2 groups of cultures. Pretreatment of some cultures with 70% ethanol on day 4 did not significantly alter the utilization of labelled substrate. This was true of both the bGH and control groups suggesting that all explants at day 4 were non-viable.

ORD activity in human pleural effusions

A pleural effusion was collected from a woman with infiltrating duct mammary carcinoma. The cell cytoplasm and pleural fluid were assayed for ORD activity. Table 8 shows that very little  $^{14}\text{CO}_2$  was produced by either of the 2 samples suggesting that there was almost no ORD activity in the cell cytoplasm and pleural fluid. However, the values for the control samples, that is, those samples which were pretreated with 40% TCA before incubation was begun, were significantly lower than the sample values indicating that some ORD activity was present in the 2 samples. No significant difference in this activity existed between the cell cytoplasm and pleural fluid.

Table 7

Effect of bGH on ORD activity in rabbit liver explants. The explants were prepared from the liver of a day 31 pregnant animal and placed into organ culture as described for the rabbit mammary gland in the Materials and Methods. The next day, 0.1 ml of medium was removed from the cultures and replaced with 0.1 ml of medium containing 1 ug of bGH. Control cultures received 0.1 ml of medium. At various time intervals following the addition of hormone, a procedure identical to that described for the mammary explants in the legend to table 5 was carried out. Each value represents the mean  $\pm$  S.D. for explants from 3 cultures.

<u>Days</u>	<u><math>^{14}\text{CO}_2</math> (cpm/mg wet wt.)</u>		<u>Significance</u>
	<u>Control</u>	<u>Growth hormone</u>	<u>Student's t-test</u>
1	295 $\pm$ 34	173 $\pm$ 24	p < 0.005
2	164 $\pm$ 25	125 $\pm$ 8	**NS
3	73 $\pm$ 42	145 $\pm$ 35	NS
4	195 $\pm$ 57	194 $\pm$ 56	NS
	*191 $\pm$ 47	*261 $\pm$ 67	

\* cultures pretreated with ethanol

\*\* NS, not significant

Table 8

ORD activity in human pleural effusions. A pleural effusion from a woman with infiltrating duct mammary carcinoma was obtained by thoracentesis. The pleural fluid and cells were separated by centrifugation. A cell homogenate was prepared and centrifuged at 1500Xg for 10 minutes. The supernatant and pleural fluid were assayed according to Richards (21). Controls were pretreated with 40% TCA before assaying. Each value represents the mean  $\pm$  S.D. from 3 determinations.

	<u><math>^{14}\text{CO}_2</math> (cpm/mg protein)</u>		<u>Significance</u>
	<u>Control (TCA + sample)</u>	<u>Sample</u>	<u>Student's t-test</u>
cell cytoplasm	57.6 $\pm$ 1.8	74.3 $\pm$ 3.8	p < 0.05
pleural fluid	62 $\pm$ 2.7	74.4 $\pm$ 1.4	p < 0.05

#### Binding of $^{125}\text{I}$ -oPRL to rabbit mammary cells

Preparations of mammary tissue from a day 31 pregnant rabbit were digested with 0.1% collagenase in Medium 199 containing 10% FCS for 1, 2, 3 and 4 hours at 37°C. A cell count was performed prior to assaying for binding of  $^{125}\text{I}$ -oPRL. Table 9 shows that over the 4-hour incubation period the number of cells yielded increased. However, the viability of the cells was generally low. Hormone binding occurred using cell preparations that had been incubated with enzyme for 2 and 4 hours; the percent specific binding per  $10^6$  total cells was 0.6% and 0.2% respectively.

#### Binding of $^{125}\text{I}$ -oPRL and $^{125}\text{I}$ -hGH to human cells in culture tubes

##### 1. Pleural effusion cells

Viable, malignant cells from a pleural effusion were incubated in glass culture tubes for 2 hours at 37°C in the presence of  $^{125}\text{I}$ -oPRL

Table 9

Binding of  $^{125}\text{I}$ -oPRL to rabbit mammary cells. Mammary tissue from a day 31 pregnant animal was removed and processed for culture as described in the Materials and Methods. Four batches of tissue were incubated with 0.1% collagenase in Medium 199 containing 10% FCS for various lengths of time at 37°C. The cell suspensions were harvested and assayed for binding of oPRL as described in the Materials and Methods.

Incubation time (hr.)	Percent viability	Cell number ( $\times 10^6$ )	Percent specific binding per $10^6$ total cells
1	15	2.0	0.0
2	26	2.3	0.6
3	19	2.6	0.0
4	17	4.7	0.2

(168,000 cpm) or  $^{125}\text{I}$ -hGH (115,000 cpm). Results of the experiment are presented in Table 10. If 1.0% specific binding is taken as an approximate lower limit of significant binding, there was negligible binding of either hormone to the cells under these conditions.

Table 10

Binding of  $^{125}\text{I}$ -oPRL and  $^{125}\text{I}$ -hGH to human mammary cells in culture tubes. Malignant cells from pleural effusions and non-malignant HBL-100 cells were removed from culture flasks and placed into culture tubes. The tubes were incubated for 2 hours at  $37^{\circ}\text{C}$  in the presence of labelled hormones as described in the Materials and Methods. Each tube contained  $10^6$  cells.

	Conc. of unlabelled hormone (ug/ml)	Percent specific binding	
		<u>o PRL</u>	<u>hGH</u>
pleural effusion	10	0.6	0.3
HBL-100	5	1.1	0.0
	10	0.5	0.0

## 2. HBL-100 cells

Non-malignant cells of the HBL-100 cell line were tested in the same manner except the Tris buffer contained 0.2% glucose and 0.01% phenol red as a pH indicator; the hormones used were  $^{125}\text{I}$ -oPRL (135,000 cpm) and  $^{125}\text{I}$ -hGH (130,000 cpm). As seen in Table 10, there was no detectable binding of  $^{125}\text{I}$ -hGH to these cells. The specific binding of  $^{125}\text{I}$ -oPRL was about 1% when 5 ug/ml of unlabelled hormone was used; but was negligible when the amount of unlabelled hormone was raised to 10 ug/ml.



### Binding of $^{125}\text{I}$ -hPRL and $^{125}\text{I}$ -Ins to HBL-100 cells in culture dishes I

To test the hypothesis that in order to bind hormones, HBL-100 cells required more natural conditions than were offered in the culture tube incubation system, these cells were transferred from culture flasks to culture dishes and then allowed to establish themselves before addition of the hormone tracers. In this experiment, after 20 hours at  $37^{\circ}\text{C}$ , half of the culture dishes received  $^{125}\text{I}$ -hPRL (120,000 cpm) and half received  $^{125}\text{I}$ -Ins (114,000 cpm). Human prolactin was employed since the cells were of human origin. Culture dishes were incubated at  $37^{\circ}\text{C}$  for 0.5, 1 and 2 hours and then the cells were assayed for hormone binding as described in the Materials and Methods section.

The amount of specific binding was  $< 1.0\%$  at all 3 time intervals for both hPRL and Ins (data not shown).

### Effect of FCS on binding of $^{125}\text{I}$ -hPRL and $^{125}\text{I}$ -Ins to HBL-100 cells in culture dishes

The growth medium used in the culture of HBL-100 cells was McCoy's 5a containing 10% FCS. To investigate the effect of FCS on hormone binding, 3 different preparations of McCoy's 5a medium containing 0%, 5% and 10% FCS were employed. After 25 hours at  $37^{\circ}\text{C}$ , culture dishes received a medium change for fresh medium containing the 3 concentrations of FCS. One half of the cultures at each serum concentration received  $^{125}\text{I}$ -hPRL (94,000 to 101,000 cpm) and half received  $^{125}\text{I}$ -Ins (77,000 to 109,000 cpm). All cultures were incubated at  $37^{\circ}\text{C}$  for a further 4 hours before processing the cells as described in the Materials and Methods section.

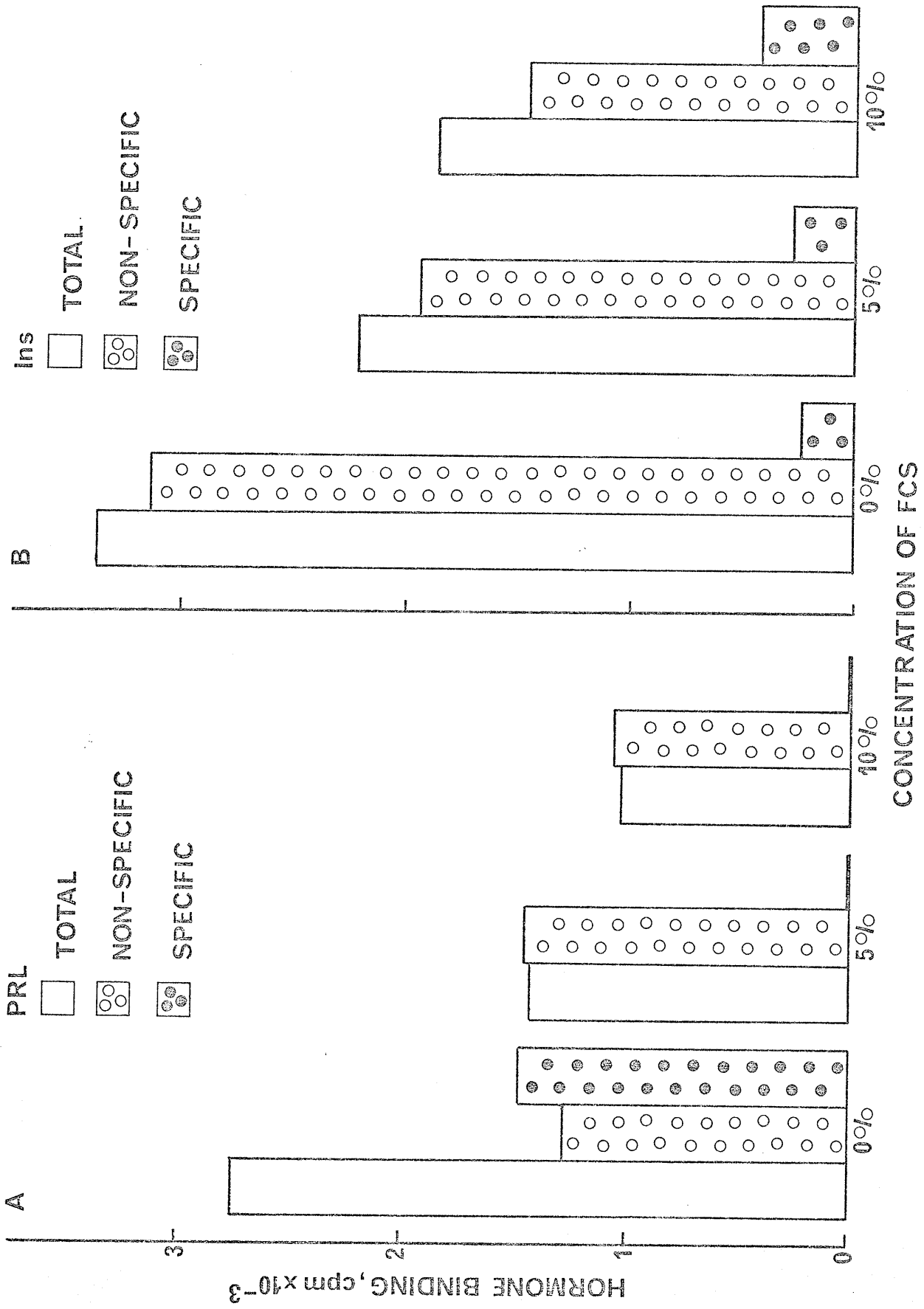


Figure 16 shows the effect of FCS on binding of hPRL and Ins to HBL-100 cells. It is evident that FCS abolished specific binding of hPRL largely by decreasing total binding (Fig. 16 A). But FCS had little effect on the specific binding of Ins (Fig. 16 B); both total and non-specific binding were decreased to about the same extent. In the absence of FCS, the specific binding of hPRL and Ins were 1.6% and 0.3% respectively.

Binding of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins to human mammary HBL-100 cells in culture dishes II

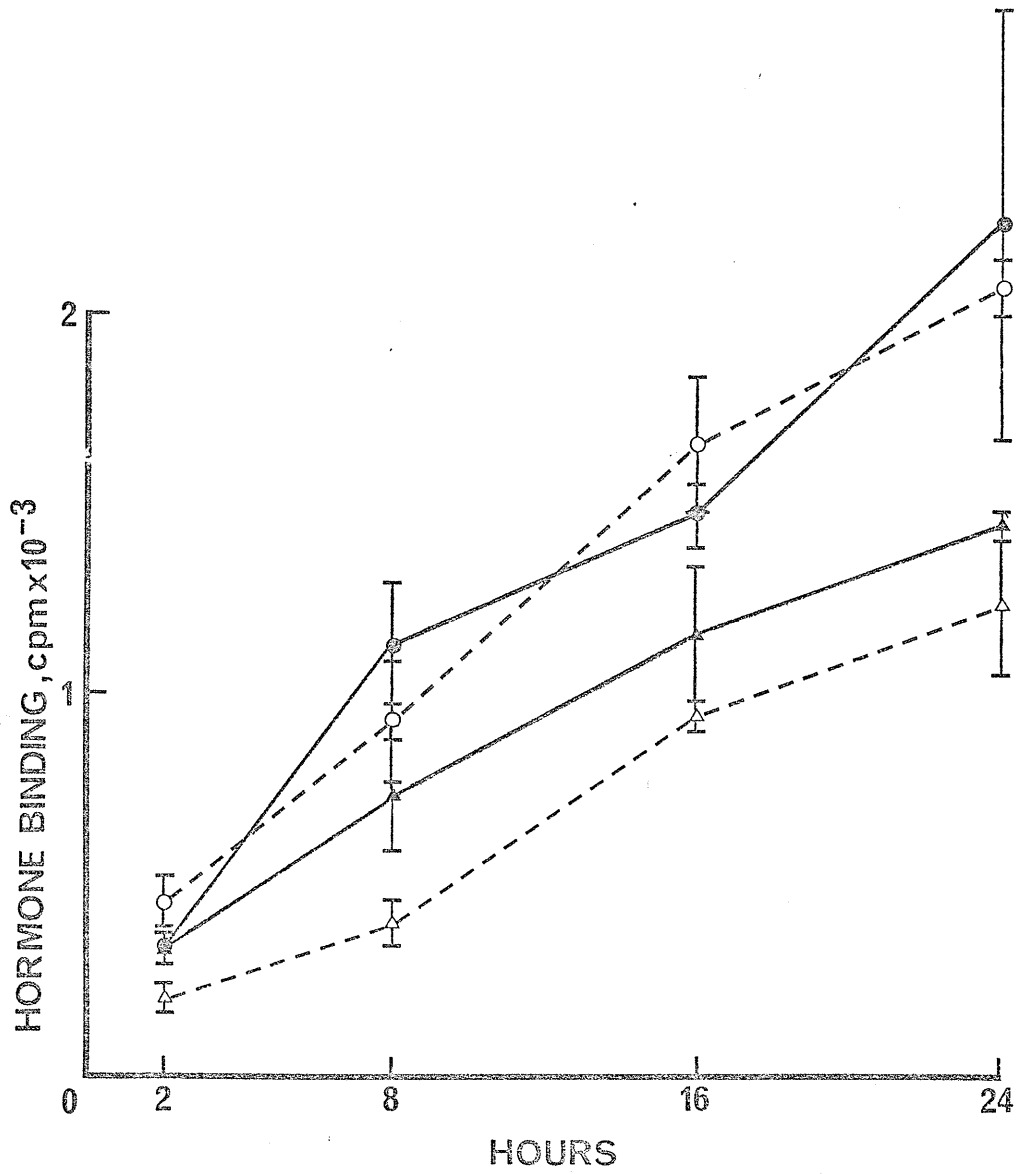
Essentially the same experiment as in I was repeated except no FCS was used and the incubation intervals were extended. After 24 hours at  $37^{\circ}\text{C}$ , all cultures received a medium change for fresh McCoy's 5a medium containing 0.5% BSA instead of serum. One half of the cultures received  $^{125}\text{I}$ -hPRL (88,000 cpm) and the other half  $^{125}\text{I}$ -Ins (99,000 cpm). The dishes were then incubated at  $37^{\circ}\text{C}$  for 2, 8, 16 and 24 hours before processing the cells for counting.

Figure 17 shows both total and non-specific binding of PRL and Ins to the human mammary cells. The total binding of both hormones increased steadily with time but the specific binding was not significant.

Binding of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins to HBL-100 cell fractions

In an effort to standardize and simplify the hormone binding assay procedure, HBL-100 cells were homogenized and various cell fractions obtained by differential centrifugation. The same fraction of several

Figure 17. Binding of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins to HBL-100 cells. The cells were incubated for 24 hours at  $37^\circ\text{C}$  in McCoy's 5a growth medium containing 10% FCS. Then a medium change for fresh medium containing 0.5% BSA but no FCS was performed and the cultures were incubated further for various time intervals in the presence of labelled hormones as described in the Materials and Methods. Both the total ( $\bullet\text{---}\bullet$  hPRL;  $\blacktriangle\text{---}\blacktriangle$  Ins) and non-specific ( $\circ\text{---}\circ$  hPRL;  $\triangle\text{---}\triangle$  Ins) binding are shown. The difference between the 2 values represents specific binding. Each value represents the mean  $\pm$  S.D. of cells from 3 cultures.



different preparations were pooled to provide a stock of receptor. In this experiment, 2 separate preparations of both the 15,000Xg and 100,000Xg pellets which had been prepared one week previously were pooled to give a protein concentration of 650 ug/ml and 300 ug/ml respectively. These pools were assayed for hormone binding as described in the Materials and Methods. Reaction tubes contained 2 ug of unlabelled hormone and  $^{125}\text{I}$ -hPRL (89,000 cpm) or  $^{125}\text{I}$ -Ins (90,000 cpm). Control tubes contained no unlabelled hormones. The reaction was allowed to proceed for 22 hours at room temperature.

Table 11

Binding of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins to HBL-100 cell fractions.

<u>Cell fraction</u>	<u>Specific binding (cpm)</u>	
	<u>hPRL</u>	<u>Ins</u>
15,000Xg pellet (650 ug/ml)	429	1167
100,000Xg pellet (300 ug/ml)	458	946

Table 11 shows that the specific binding of Ins to both cell fractions was approximately twice that of hPRL. The specific binding of each hormone to both cell fractions was about the same and was 1% for Ins and 0.5% for hPRL.

Effect of protein concentration on binding of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins to HBL-100 cell fractions

Separate pools were made of 8 day old 15,000Xg and 100,000Xg receptor preparations. The protein concentration of each pool was 1750 ug/ml and 380 ug/ml respectively. Tris buffer was used to dilute these pools 1:1, 1:2 and 1:4. Reaction tubes contained 2 ug of unlabelled hormone and  $^{125}\text{I}$ -hPRL (92,000 cpm) or  $^{125}\text{I}$ -Ins (95,000 cpm). Control tubes contained no unlabelled hormones. The reaction was allowed to proceed for 22 hours at room temperature.

Table 12

Effect of protein concentration on binding of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins to cell fractions prepared from HBL-100 cells.

Cell fraction	Protein conc. (ug/ml)	Percent specific binding (cpm)	
		hPRL	Ins
15,000Xg pellet	380	0.0	0.8
	750	0.6	0.0
	1750	0.0	0.0
100,000Xg pellet	70	0.1	0.6
	130	0.5	1.6
	380	0.4	2.2

The effect of protein concentration on the binding of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins to cell fractions prepared from HBL-100 cells is shown in Table 12. Better specific binding of both hormones to 100,000Xg pellet

material occurred. In this fraction, an increase in hormone binding accompanied an increase in protein concentration. In the 15,000Xg fraction, Ins binding was seen only at the lowest concentration while that of hPRL occurred only at an intermediate concentration.

Binding of different concentrations of  $^{125}\text{I}$ -hPRL to HBL-100 cell fractions

To determine the effectiveness of different concentrations of  $^{125}\text{I}$ -hPRL in binding to HBL-100 cell preparations, freshly prepared  $^{125}\text{I}$ -hPRL tracer was diluted with Tris buffer to give 3 different concentrations (24,000, 50,000 and 102,000 cpm) and then reacted with 15,000Xg and 100,000Xg receptor pools. The receptor pools were 7 weeks old and had been used once previously. Protein concentrations of the 15,000Xg and 100,000Xg pools were 1450 ug/ml and 250 ug/ml respectively. Unlabelled hPRL was used at a concentration of 20 ug/ml. Accidentally, tubes containing 15,000Xg receptor and 24,000 cpm of tracer received 10 ug of unlabelled hormone. The tubes were allowed to stand at room temperature for 22 hours.

Table 13

Effectiveness of  $^{125}\text{I}$ -hPRL tracer at different concentrations in binding to HBL-100 cell fractions.

<u>Cell fraction</u>	<u>Specific binding of <math>^{125}\text{I}</math>-hPRL (cpm)</u>		
	<u>24,000</u>	<u>50,000</u>	<u>102,000</u>
15,000Xg pellet (1450 ug/ml)	0	132	540
100,000Xg pellet (250 ug/ml)	84	78	490



The low specific binding of  $^{125}\text{I}$ -hPRL at all 3 concentrations is shown in Table 13. However, an increase in specific binding to both cell fractions occurred when the concentration of tracer was raised from 50,000 cpm to 102,000 cpm.

#### Binding of $^{125}\text{I}$ -hGH and $^{125}\text{I}$ -hPRL to an HBL-100 cell fraction

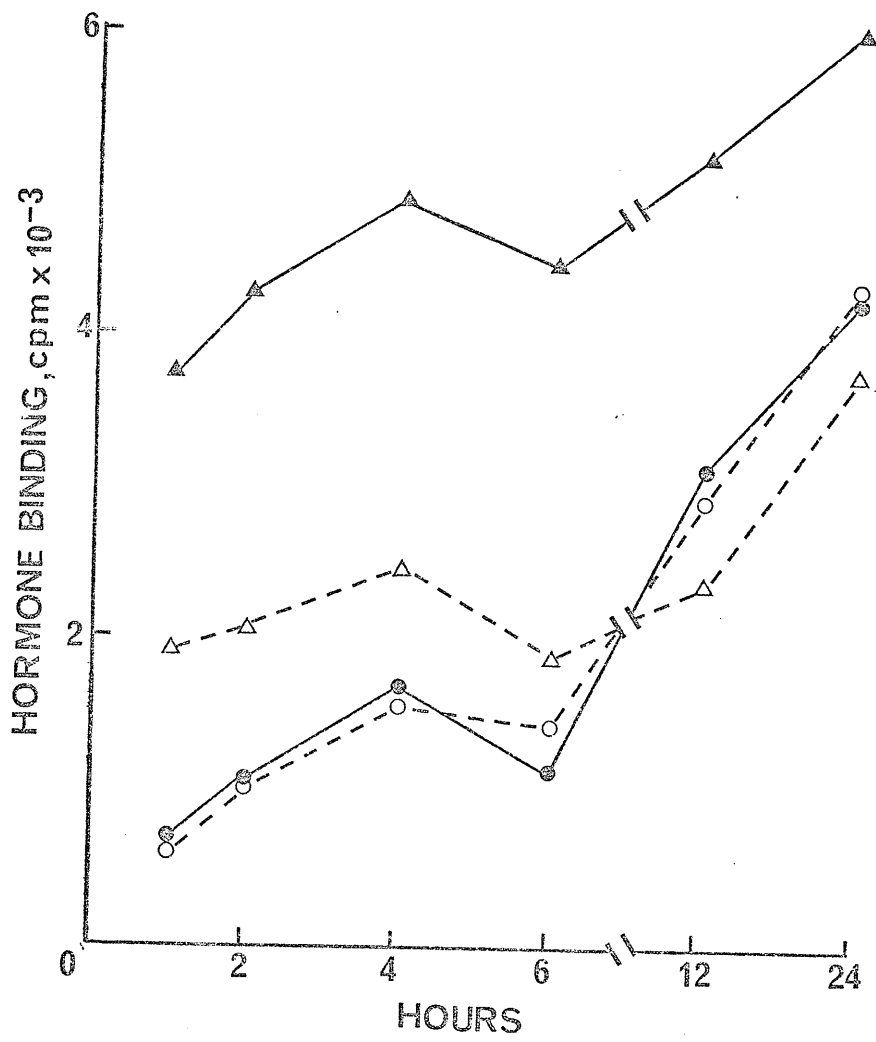
The consistently poor binding of  $^{125}\text{I}$ -hPRL to HBL-100 cell fractions in light of the relatively good  $^{125}\text{I}$ -Ins binding cast some doubt on the binding ability of the prolactin tracer itself. Thus, the specific binding of  $^{125}\text{I}$ -hPRL (85,000 cpm) was compared with that of  $^{125}\text{I}$ -hGH (80,000 cpm). A 15,000Xg receptor pool with a final protein concentration of 2250 ug/ml was made from several preparations less than 6 weeks old. Only 1.0 ug of unlabelled hormone was used to displace the tracer. The tubes were incubated at room temperature for 1, 2, 4, 6, 12 and 24 hours.

Figure 18 demonstrates that the specific binding of  $^{125}\text{I}$ -hGH increased with time until 12 hours and then began to decrease. The specific binding of  $^{125}\text{I}$ -hGH was 3 to 3.5% at 4, 6 and 12 hours while that of  $^{125}\text{I}$ -hPRL was very low with no definite trend over the 24-hour time period.

#### Effect of protein concentration on binding of $^{125}\text{I}$ -hGH to an HBL-100 cell fraction

A receptor pool was made from two 7 day old preparations of 15,000Xg pellet material. Tris buffer was used to dilute the receptor pool. The different dilutions were reacted with  $^{125}\text{I}$ -hGH (83,000 cpm) for 12 hours at room temperature. Unlabelled hGH was used at a

Figure 18. Binding of  $^{125}\text{I}$ -hGH and  $^{125}\text{I}$ -hPRL to a 15,000Xg receptor pool prepared from HBL-100 cells. The total ( $\blacktriangle$ — $\blacktriangle$  hGH;  $\bullet$ — $\bullet$  hPRL) and non-specific ( $\triangle$ — $\triangle$  hGH;  $\circ$ — $\circ$  hPRL) binding for each hormone is shown. The difference between the 2 values represents specific binding.



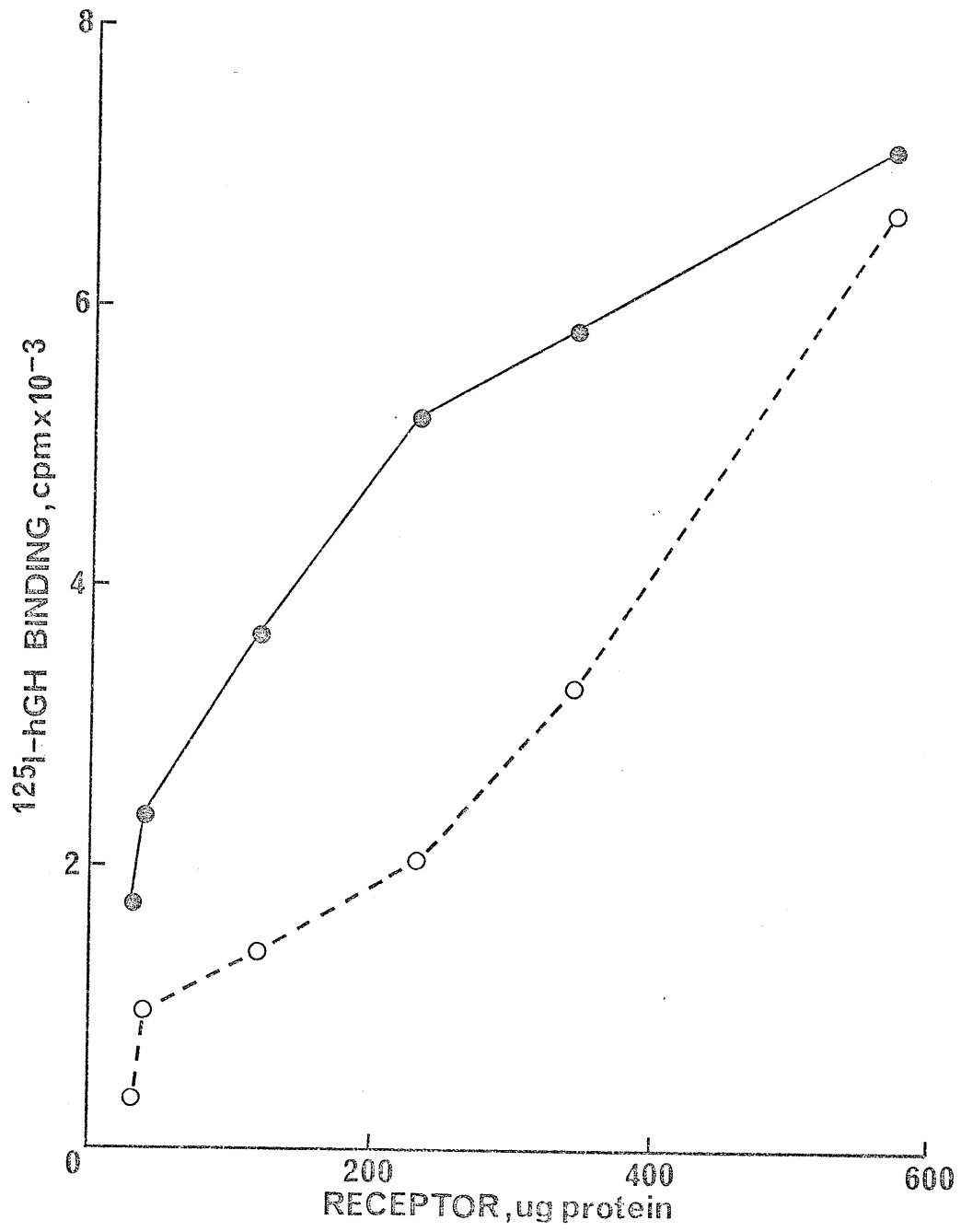
concentration of 10 ug/ml to displace the tracer.

Results are shown in Figure 19. The total and non-specific binding of  $^{125}\text{I}$ -hGH increased as the protein concentration was raised. The specific binding also increased until approximately 1000 ug protein/ml, remained the same until 3500 ug protein/ml and then decreased. The percent specific binding was approximately 3%/100 to 350 ug protein.

#### Lack of reproducibility of $^{125}\text{I}$ -hGH binding to HBL-100 cell fractions

Three subsequent attempts at repeating the above results with  $^{125}\text{I}$ -hGH failed. A different tracer preparation was used in each experiment. In all 3 experiments, the same receptor preparation was tested. This 15,000Xg pool was tested 2, 6 and 14 weeks after being prepared. No specific binding of  $^{125}\text{I}$ -hGH was seen. In one experiment, the same 15,000Xg preparation as used in one of the above successful experiments was also employed. This receptor pool had been in storage at  $-20^{\circ}\text{C}$  for 20 weeks and had been used only once during that period. Again, no specific binding of  $^{125}\text{I}$ -hGH was seen.

Figure 19. Effect of protein concentration on the total (●—●) and non-specific (○---○) binding of  $^{125}\text{I}$ -hGH to a 15,000Xg receptor pool prepared from HBL-100 cells. The difference between the 2 values represents specific binding.



## DISCUSSION

Rabbit mammary cell culture - rationale and problems

Although the purpose of this study was to examine the interaction of PRL with human breast cells, it seemed logical to gain some experience with a cell type known to have receptors for the hormone. Shiu has isolated and purified this prolactin receptor from late-pregnant rabbit mammary tissue (114) and has characterized the binding of  $^{125}\text{I}$ -PRL to it (9). Therefore, pregnant and pseudopregnant rabbit mammary glands were used. This tissue contained much milk indicating the presence of many epithelial cells. Non-pregnant rabbit mammary glands, which were small and consisted primarily of stromal elements, were unsuitable.

Enzymatic digestion of minced mammary tissue resulted in small clusters of free cells and partially digested clumps. Both cell forms gave rise to spreading epithelioid cells which grew out from the central attached cluster. The clusters of free cells eventually gave rise to patches of monolayer growth but such cultures always contained many fibroblast-like cells which often surrounded the epithelioid cell patches thus restricting their growth. Thimerosal was quite effective at destroying fibroblastoid cells but required many days to do so. It was often necessary to treat cultures several times for short intervals so as to protect epithelioid cells which were only slightly less sensitive to the compound. The problem was complicated by the faster growth rate of the fibroblastoid cells. These cells were much less common in cultures initiated with partially digested clumps of tissue; such cultures consisted predominantly of epithelioid cells but growth

was much slower. Only 3 of 10 attempted cell cultures were maintained longer than 3 weeks and none of these cells were passaged more than twice. Cloning may have achieved a homogeneous population of cells for short-term studies but was not performed because of limited time and experience. The degree of difficulty involved in establishing primary cultures of rabbit mammary cells cast some doubt on the usefulness of this approach.

#### Pleural effusions as a source of human breast tumor cells

Human breast tumors usually arise from mammary epithelial cells which line the milk ducts and are the milk-secreting cells. Obtaining pure cultures of epithelial cells, free of connective tissue fibroblasts always accompanying them in biopsy and tumor explants, has been a major difficulty in breast cancer research. Few cell lines have been established directly from solid breast carcinomas because of fibroblast overgrowth, poor cell yield and low viability resulting from treatment with enzymes (115). Pleural effusions contain an abundance of metastatic breast carcinoma cells and few, if any, fibroblasts. No pretreatment with enzymes is required and cells in pleural fluids generally show a viability of 70% or better. For these reasons, pleural effusions were considered as a good source of tumor cells.

#### Heterogeneity of pleural fluid cell types

Many different cell types are present in pleural effusions and most persist for long periods of time in culture (116). Apart from leucocytes and lymphocytes which disappear in less than a month,



3 distinct cell forms are morphologically identifiable in primary cultures: 1) dendritic-like cells which do not appear to divide 2) mesothelial-like cells which are large and irregular becoming more round or ovoid as they slowly fill the flask, and 3) tumor cells, existing either singly or in clumps and which usually multiply 4 to 8 weeks as adherent or floating clumps; sometimes forming spreading plaques on the flask surface. Mesothelial-like and tumor cells share ultrastructural features representative of epithelial cells namely, desmosomes and tonofilaments (116, 117). Tumor cells are distinguishable from mesothelial-like cells by the presence of intracytoplasmic duct-like vacuoles that are frequently found in breast tumor cells (117). Further, the cells identified as being of tumor origin have a high nuclear:cytoplasmic ratio, a well accepted morphologic index of malignancy of cells in vivo (117). A low ratio is typical of mesothelial cells. Dendritic cells have not been characterized.

Initially primary cultures of pleural effusions in our laboratory consisted of 3 cell types: 1) dendritic cells 2) round cells, and 3) spindle-shaped (fibroblastoid) cells; with a predominance of the dendritic and round types. Dendritic cells (Fig. 3) resembled those described by Cailleau (116) except that in some cases, they appeared to multiply slowly. Spindle-shaped cells (Fig. 4) may have been modified tumor cells as in areas of dense growth they were seen to round up and later assume a polygonal shape. Less frequently, dendritic cells also went through the same sequence of events. The round cells (Fig. 2) observed by us were probably comprised of 2 cell types, those that could be compared to the mesothelial-like cells reported by

Cailleau (116) and tumor cells. During the formation of an epithelioid cell plaque, (Fig. 5) it was often impossible to distinguish between those round cells always present and those that seemingly arose from fibroblastoid cells in the process of transformation. Epithelioid cells, which were recognized by their polygonal shape only existed in clusters or plaques. These cells probably represent both spindle-shaped and round cells, which under crowded conditions, assumed a polygonal appearance. Such epithelioid cells may have been of tumor origin since they seemed to exhibit a relatively high nuclear:cytoplasmic ratio. Plaque formation was an event that occurred only in mature cultures and epithelioid tumor cells were not evident in primary cultures.

Free-floating clumps of round cells were present throughout the course of a culture and were used to inoculate new flasks. It was important to incubate the flasks without disturbance for as long as 10 days because the floating clumps did not settle quickly and initially were easily shaken loose. However, once well attached they quickly formed epithelioid cell plaques (Fig. 8). Peripheral cells of these outgrowths resembled fibroblastoid cells but they have been shown by others (116) to be modified epithelial cells since they contain ultrastructural features of carcinoma cells. Thus, the round cells comprising the floating clumps in our cultures are believed to be tumor cells.

#### Growth requirements of pleural effusion cells

Survival times were different for the various cell types in culture (Table 3). Dendritic and fibroblastoid cells were present throughout the

entire culture period. Epithelioid cells developed during the second half of this period. Cailleau (106,116) observed a similar survival pattern for these 3 cell types. Generally, round cells in our cultures exhibited a survival period almost identical to epithelioid cells. It is possible that many round cells were modified tumor cells which would explain their relatively short survival time compared to that of the mesothelial-like cells described by Cailleau (116). Few cultures could be maintained beyond 4 months.

The similarity in survival time between cultures maintained on a variety of media reflects the growth requirements of pleural effusion cells. The basic medium, FCS and hormones were presumed vital to cell function. This was not the case. Table 4 shows the various media employed. Generally, Medium 199 was sufficient for cell growth in all cultures of March 1975. Cultures of June 1975 grew equally well in Medium 199, Eagle's minimum essential medium, McCoy's 5a and Leibovitz's L-15. All media contained 10 to 20% FCS; the amount did not appear critical as cultures of June 1975 survived one week in Medium 199 containing only 2% FCS. Pleural fluid was not added to our cultures although Cailleau (106,116) frequently employed it as a supplement. However, 10% bovine amniotic fluid was used in combination with 10% FCS in Leibovitz's L-15 medium but did not seem to affect cell growth. The effect of hormones on cell growth has not been reported. We found that the presence or absence of hydrocortisone and insulin for 1 to 2 month periods did not appear to have any effect on plaque formation and they were certainly not required for cell survival. Estrogen (17- $\beta$ -estradiol) was also without effect.

Cailleau claimed that the kinds of media employed influence which cell types predominate in culture and their size and shape (116). A medium prepared from 3 complex media mixed together in various amounts and containing FCS, insulin and hydrocortisone was reported to encourage the proliferation of fibroblastoid cells in culture. On the other hand another, more simple medium also with FCS and hormones seemed to favor dendritic cells, mesothelial-like cells and tumor cells. In our cultures, all media seemed equally capable of promoting growth of all cell types.

The lack of cell response to a variety of media used in our study is not surprising since it is possible that pleural fluid cells may be mutants of breast tumor cells. Cells found in pleural effusions have metastasized and adapted themselves to growth in a liquid environment which implies some selection of those cells that are more hardy and active. These cells are therefore capable of adaptation to environments completely different from their initial surroundings. Any small changes that result from switching between media may not present insurmountable difficulties to such cells.

#### Human breast cell lines

Three mammary cell lines of human origin were maintained in culture for 11 months. Two lines were established from pleural effusion specimens taken from women with mammary carcinoma. The MDA-MB-157 and MDA-MB-134VII lines have been fully described elsewhere (105,106). A third line, HBL-100 was derived from milk samples taken from women who were lactating.

Cell growth characteristics were different for malignant and

non-malignant cell types. Malignant cells usually grew independently of one another except under very crowded conditions while non-malignant cells formed patches of cells attached to one another. Growth was more rapid in HBL-100 cell cultures than in those of MDA-MB-157 or MDA-MB-134VII and often reached confluency. Confluency of cell growth was rarely seen in MDA-MB-157 cultures and did not occur in cultures of the MDA-MB-134VII line. Floating single cells and cell clumps were more frequently observed in malignant cell cultures especially in those of MDA-MB-134VII. Free-floating cells were constantly shed into the medium of MDA-MB-157 cultures but in MDA-MB-134VII cultures the opposite process of cell clump settling out occurred. In terms of viable floating cells, failure to achieve confluency and cell growth rate, these malignant cells have much in common with the pleural effusion cells.

Malignant and non-malignant cells have different requirements for growth. All cells had a serum requirement and 10 to 15% FCS was routinely employed; but as a serum supplement, 10% bovine amniotic fluid was also used in MDA-MB-134VII cultures. It is curious that MDA-MB-134VII cells which exist largely in a free-floating form, unlike those of MDA-MB-157 or cells of primary pleural effusion cultures, have an unusually high requirement for serous fluids. Hormones were not used in HBL-100 cultures. Insulin was employed in both malignant cell cultures and hydrocortisone was also added to cultures of MDA-MB-157 cells. In contrast, human pleural effusion cells seemed to grow equally well in the presence or absence of hormones and growth was not inhibited by media containing as little as 2% FCS. However, it is recognized that established cell lines hopefully contain one cell type which has its

own specific requirements while cultures of pleural effusions contain a variety of cell types, each of which may have their own different specific needs.

Effect of PRL concentration on casein synthesis in rabbit mammary explants

Ovine prolactin at concentrations greater than 10 ng/ml stimulated casein synthesis in rabbit mammary explants (Fig. 12). A better than 2-fold increase in casein production was caused by 1 ug/ml of the hormone. The results were not unexpected for 2 reasons: 1) mammary tissue from pseudopregnant rabbits do contain prolactin receptors (8,9) and 2) this tissue is capable of in vitro casein synthesis (10).

The conditions of the assay system employed by Shiu and Friesen (10) were based upon 2 fundamental principles adopted from the mouse mammary gland model. Explants of mouse mammary tissue were found to require a 'priming' with insulin and hydrocortisone before any response to PRL could be seen (118). During this period cellular proliferation increased until 24 hours and then decreased to low levels by 72 hours. Thus a similar preincubation of rabbit mammary explants for 72 hours in the presence of insulin and hydrocortisone was used. Further, the addition of oPRL to the mouse mammary cultures at 72 hours was found to result in casein synthesis but only after a lag period of 8 to 10 hours (119). Shiu and Friesen found that casein synthesis in rabbit mammary explants was stimulated by 1 ug/ml oPRL and that after a similar initial lag period casein production increased until 72 hours (personal communication). Therefore a 6-day incubation period was employed; 3 days in the absence of oPRL followed by 3 days in the presence of the hormone.

Prolactin-induced casein synthesis in human mammary cells in vitro

Human mammary HBL-100 cells appear to be capable of responding to hPRL with casein synthesis. Table 5 shows that casein production was stimulated by hPRL at 2 and 12 hours following addition of the hormone but subsequently ceased by 24 hours. Control values increased slightly with time; this is attributed to the increase in cell number that occurred throughout the culture period. Dilley and Kister (99) reported that insulin stimulates cell replication in human breast explants in vitro and that hPRL in combination with insulin, enhances this effect. Although lacking in precision, light microscopic examination of HBL-100 cells during the course of the experiment following the addition of hormone revealed that hPRL did not seem to affect cell attachment or growth. Thus, in spite of an increased cell number, beyond 12 hours of exposure to PRL, cultures no longer responded with increased casein production.

The interaction between PRL and mammary cells is complex. In vitro studies of midpregnant mouse mammary explants demonstrate that oPRL stimulates both RNA and casein synthesis (119,120). The action of oPRL on RNA synthesis appears to be necessary for the stimulation of casein production since inhibition of RNA synthesis by actinomycin D prevents casein synthesis (119). Prolactin-induced RNA synthesis occurs about 3 to 4 hours after administration of the hormone, while casein synthesis occurs between 8 and 10 hours following exposure to PRL (121). These results suggest that the effect of PRL is to induce de novo synthesis of mRNA specific for casein and then, after a lag period, the synthesis of casein. However, in vivo, this lag period may not

occur. Rosen and Barker (122) reported that during normal development of the rat mammary gland, there is a 12-fold increase in casein mRNA molecules per alveolar cell from day 5 of pregnancy to day 8 of lactation. When measured in a wheat germ cell-free translation system, the casein mRNA activities also increased throughout the same time interval. Thus it appears that biologically inactive mRNA were not being synthesized during pregnancy and later activated at parturition or during lactation. Similarly in the day 14 pseudopregnant rabbit mammary gland the concentration of casein mRNA is about 100 times lower than in the lactating gland (123). Houdebine demonstrated that injection of PRL into pseudopregnant rabbits was followed by an increase in casein synthesis and by a parallel accumulation of casein mRNA as early as 6 hours after hormone administration (123). In the human HBL-100 cells, casein synthesis was also stimulated after 6 hours (2 hours plus 4 hour incubation with  $^3\text{H}$ -leucine) of exposure to hPRL (Table 5), but it is not known whether the effect of PRL results from de novo synthesis of casein mRNA or casein gene expression. The different times required for stimulation of casein synthesis may reflect a difference between the behavior of mammary tissue in vitro and in vivo. However, another perhaps more important consideration is the variation in binding kinetics of PRL to the individual tissue preparations.

Recently, Rillema (124) reported that the interaction of PRL with mouse mammary explants was rapid and stable. As little as 10 seconds of exposure to oPRL at  $0^{\circ}\text{C}$  was required to stimulate RNA synthesis during a subsequent 4-hour incubation at  $37^{\circ}\text{C}$ . This effect of PRL was not diminished by extensive washing for 2 hours at  $0^{\circ}\text{C}$  prior to



incubation at 37°C. Further, a relatively low concentration of oPRL (50 ng/ml) was employed. These results imply that a sustained exposure of mammary tissue to oPRL is not required for the stimulation of RNA synthesis. However, it is conceded that PRL that has interacted with the tissues at 0°C may dissociate at 37°C and be available throughout the 4-hour period. Studies by Shiu and Friesen (9) on the binding of <sup>125</sup>I-oPRL to particulate membrane fractions of rabbit mammary tissue show that the dissociation of oPRL from its binding sites is negligible at 4°C but quickly increases as the temperature is raised to 37°C. Also, the association of <sup>125</sup>I-oPRL to the same membrane fractions appears to be small at 0°C. Thus taking these 2 studies (9,124) together it appears that although binding of radioactive PRL to rabbit mammary tissue preparations appears to be slight at 0°C, the amount bound may provide a sufficient stimulus for RNA synthesis as seen in the mouse mammary explants.

But it is also important to recognize the discrepancy between the findings of Rillema (124) and Shiu and Friesen (9) concerning the rate of the hormone-receptor interaction. Rillema claimed association of oPRL with mammary explants within seconds while Shiu and Friesen demonstrated that the rate of association and dissociation of <sup>125</sup>I-oPRL from mammary membrane fractions was relatively slow requiring hours to equilibrate. In one study (124) only unlabelled oPRL was used and in the other (9), both unlabelled oPRL and <sup>125</sup>I-labelled oPRL were employed. It may be possible that the kinetics of hormone binding were different for the 2 preparations used by Shiu and Friesen since it is conceivable that the enzymatic iodination employed by these workers may have altered

the hormone.

In the rabbit mammary gland, injections of low doses of PRL (12.5 IU) resulted in enhancement of casein mRNA almost to the same extent as seen with high doses (100 IU) but the total cellular RNA was about 3 times lower (123). The effect of these injections was to cause a rapid rise in casein mRNA concentration which occurred within a few hours and then became stabilized until subsequent injections produced further increases in RNA concentration. Plasma levels of PRL were relatively high during stimulation of RNA synthesis and were much lower during the plateau period between injections. Thus, unlike mouse mammary explants, the rabbit mammary gland in situ may require sustained exposure to PRL for stimulation of RNA synthesis.

Casein synthesis in mouse mammary explants was not induced by brief exposures to oPRL at 0°C when subsequently incubated at 37°C for 10 hours (124). However, control cultures incubated at 37°C for 10 hours in the presence of PRL did synthesize casein. Thus, it seems that in the explants stimulation of casein synthesis requires a sustained exposure to PRL. It is possible that a longer exposure period to the hormone is necessary for stimulation of the cell translational machinery. Rabbit mammary tissue in situ also appears to require a sustained exposure to PRL since as the plasma hormone level decreases shortly after injection, it is accompanied by a cessation in casein synthesis (123). Although the effect of PRL on casein mRNA production and casein synthesis in the rabbit are temporally closely related, the 2 events in the mouse mammary explants in culture do not appear to be similarly related.

There is some evidence for the hypothesis that the effect of PRL on RNA and casein synthetic pathways is different in mouse mammary explants. Prostaglandins B<sub>2</sub>, E<sub>2</sub> and F<sub>2</sub> stimulate RNA synthesis in a manner similar to PRL, but have no effect on casein synthesis under the same conditions (125). Indomethacin, an inhibitor of prostaglandin biosynthesis, inhibits both RNA and casein synthesis (125). It appears that prostaglandins play a role in casein production but unlike RNA synthesis, this process may require other mediators as well. Polyamines, notably spermidine, may also participate in casein synthesis. Spermidine levels increase in mouse mammary explants after exposure to prolactin, insulin and hydrocortisone (18,126). This effect occurs before casein synthesis begins and casein production is abolished if an inhibitor to polyamine biosynthesis is added to the culture system (18). Further, spermidine in combination with prostaglandins B<sub>2</sub>, E<sub>2</sub> and F<sub>2</sub> stimulates casein synthesis in a prolactin-like manner (127) indicating that this process requires both prostaglandins and polyamines.

As a final note, it is suggested that there is of course a large difference between casein synthesis in mouse mammary explants; in situ rabbit mammary tissue; and in human mammary cells. Not only are the cell types different but so are the incubation conditions and the prolactin species employed in the individual studies.

#### In vivo effects of bGH and bPRL on ORD in rat tissues

An injection of bGH into young adult female rats stimulated a significant increase in ORD activity in kidney and liver tissue (Fig. 13). At 4 hours following the administration of hormone, ORD levels were

increased about 58-fold in the liver and 6-fold in the kidney demonstrating a greater effect of bGH on the liver enzyme. These results are in keeping with those of others (113,128,129) except that the effect of GH on the kidney is reported to be greater than that on the liver (129). However in this study (129) hypophysectomized female rats (90 to 100 g) were used and it is possible that under these conditions the sensitivity of the 2 target organs to GH may be slightly altered from that in intact animals. ORD levels in kidney and liver samples in the same study were very similar but in our experience with intact untreated rats (135 g) the values in the kidney were 30-fold higher than those in the liver. This effect was age-dependent as ORD levels in the kidney tissue of older animals (400 g) were only 3 to 5 times as great as those found in liver tissue.

ORD activity in liver tissue is also known to be age-related. Russell et al. (113) reported that low dose (1 ug/g) injections of bGH into male weanling (50 to 60 g) and adult (150 to 200 g) rats produced a greater increase in ORD activity in weanlings than in adults. High dose (25 ug/g) injections on the other hand resulted in a further enhancement of ORD activity in both age groups but there was no longer any difference between the 2 groups. Thus, in rat liver, ORD levels are dependent on the age of the animal and the amount of hormone injected.

The stimulation of ORD by GH is time-dependent. Administration of a single dose of bGH results in a marked elevation of ORD activity at 2 hours, which reaches a peak at 4 hours and then rapidly declines to basal levels by 8 hours following the injection (113,128). This

phenomenon suggests a rapid turnover rate of ORD. Using cycloheximide to evaluate the turnover of ORD in GH-treated rats, Russell et al. (113) found a half life for ORD decline of about 15 minutes. Thus it is suggested that ORD plays an important regulatory role in polyamine biosynthesis.

Prolactin has also been shown to induce ORD activity in many rat tissues (21). Similar to GH-treated animals, ORD activity in PRL-treated rat liver and kidney tissues appears to be dependent on time, hormone dose and age. The activity of liver and kidney ORD in female rats (42 days old) receiving an ip injection of oPRL (60 ug/g) seemed to peak at 2.5 hours following hormone administration. Prolactin stimulated ORD activity in the 2 organs to different extents; the kidney responded to lower doses of oPRL than did the liver and at all doses employed, the kidney responded to a much greater extent than did the liver. The response of ORD in both organs to PRL was related to the age of the animal. Generally, the response was greater in younger rats with the greatest ORD activity in the 31 to 33 day age category.

Using older female rats (400 g) we found that an ip injection of bPRL (10 ug/g) did not induce a significant increase in liver and kidney ORD levels at 2 hours following hormone administration (Fig.14A). It is believed that the rats were too old as Richards (21) reports a dramatic reduction in ORD activity in the 2 organs of animals between 33 and 42 days old. Also the species of PRL employed was bovine not ovine and the dose was considerably less than was used by Richards. However, a final consideration is the large variance between the individual response of each animal. Figure 14B demonstrates the extent

of this variation. It is possible that this discrepancy was responsible for masking any significant difference that existed between control animals and those receiving PRL.

#### In vitro effects of bGH and oPRL on ORD in rat tissue slices

The in vivo hormone injection studies do not show that GH or PRL act directly on rat tissues. To this end, slices of female rat liver and kidney were incubated with 1 ug/ml of bGH and oPRL. The effects of each hormone were opposite one another in the same organ. ORD activity in the liver was significantly stimulated to a greater extent in response to GH than to PRL while in the kidney, PRL exerted a more significant effect on ORD than did GH (Fig. 15). The response of ORD activity in the liver to GH was predictable from the in vivo injection study completed earlier (Fig. 13). The effect of PRL on the kidney was also expected as Richards (21) reported a greater increase in ORD levels in this organ after treatment with PRL. Thus, PRL seems to exert its in vivo effect on ORD through direct interaction with the target tissues.

#### In vitro effects of oPRL and bGH on ORD in rabbit tissues

In an effort to show that PRL exerted an effect on ORD in the rabbit mammary gland, explants of this tissue were incubated in the presence of oPRL for various intervals of time. Table 6 demonstrates that explants in culture for 5 days in the presence of insulin and hydrocortisone before exposure to PRL did not exhibit any increase in ORD activity when exposed to PRL for periods of up to 3 hours. This

result cannot be attributed to the non-viability of the explants, because at 3 hours those explants which had been pretreated with ethanol before determining ORD content showed significantly lower ORD levels than did untreated explants. There are 2 possible reasons for the lack of PRL effect. Firstly, it is not known whether  $^{14}\text{C}$ -ornithine is taken up by the mammary explants although it appears that it may be since pretreatment of some explants with ethanol reduces the utilization of this substrate to very low levels. Secondly, the incubation period may have been too short for any noticeable effect to become apparent. In the mouse mammary gland a peak in ORD activity occurs at about 12 hours after exposure to bPRL (20). The resulting increase in spermidine which is dependent on the presence of insulin, hydrocortisone and prolactin occurs before the maximal stimulation of casein synthesis at 48 hours (18,19). Also, in rabbit mammary explants exposure to oPRL for 3 days in the presence of insulin and hydrocortisone was required for maximum casein production to be manifested. Therefore it would have been more informative to have extended the incubation period with PRL in this study.

A similar type of experiment was attempted using rabbit liver explants and bGH. As seen in Table 7, GH did not stimulate ORD activity in liver explants incubated up to 4 days in the presence of the hormone. Pretreatment of some explants with ethanol on day 4 before determination of ORD content did not significantly affect the utilization of the  $^{14}\text{C}$ -ornithine substrate. This fact suggests that all explants at day 4 were non-viable. It is not known how long liver tissue remains viable under culture conditions.

ORD activity in human pleural effusions

Polyamine levels in the urine of patients with malignancies are known to be increased above those in healthy patients (130,131). Therefore it seemed feasible that malignant cells may be producing these elevated levels of polyamines and if this were the case, ORD would almost certainly be involved. To test this hypothesis, both the pleural fluid and the cells of a pleural effusion from a woman with infiltrating duct mammary carcinoma were examined for ORD activity. The ORD levels in both samples were very similar (Table 8). The ORD activities appeared quite low but were significantly higher than in the control samples which had been pretreated with TCA. This result reflects those documented by Lipton et al. (131) in which patients with metastatic breast cancer seemingly have less elevated levels of urinary polyamines.

Binding of  $^{125}\text{I}$ -oPRL to rabbit mammary cells

Cell fractions of mammary tissue from pregnant rabbits are known to bind  $^{125}\text{I}$ -oPRL and this phenomenon has been well documented (8,9). Therefore it seemed possible that the same mammary tissue after enzyme treatment would yield cells capable of binding PRL. However, very little specific binding of  $^{125}\text{I}$ -oPRL to cells resulting from such treatment occurred (Table 9). In comparison, Shiu and Friesen (9) reported about 8% specific binding of  $^{125}\text{I}$ -oPRL to rabbit mammary plasma membrane fractions after incubation at  $37^{\circ}\text{C}$  for 2 hours. Therefore the poor hormone binding to cell digests of rabbit mammary tissue may have resulted from insufficient cell numbers or some



alteration of the actual binding sites.

Table 9 shows that as treatment with collagenase continued cell yield increased but the viability of cells was generally low. Viable cell yield was highest in those preparations exposed to the enzyme for 2 and 4 hours. In spite of a greater number of such cells in the latter preparation less binding occurred. Even if cell viability is not a prerequisite for hormone binding, as it does not appear to be from the studies of Shiu et al. (8,9), it was expected that an increase in binding would accompany the increase in total cell yield over the 4-hour incubation period. Since no such correlation was evident there remained the possibility that the cell binding sites may have been altered during incubation with the enzyme.

Treatment with collagenase may have modified the binding sites for oPRL but this effect would be expected to increase with time. This was not the case. However, it must be noted that 0.1% collagenase was previously used to digest rabbit mammary tissue for cell culture. Exposure to this concentration for 2 hours resulted in cells which were 92 to 98% viable. The enzyme solution employed in the present study was identical except for the addition of 10% FCS. It is doubtful that the presence of FCS reduced the viability of cells (the very reason it was added) but it may have interfered with hormone binding. Although the FCS was not present in the actual assay mixture, it is conceivable that it may have continued to mask the binding sites during this 2-hour period or that it may have altered the binding sites rendering them incapable of interacting with oPRL. In studies yet to be discussed, it was found that as little as 5% FCS completely inhibited the

specific binding of hPRL to human mammary HBL-100 cells.

Binding of  $^{125}\text{I}$ -hormones to human mammary cells

Studies designed to show hormone binding to intact human mammary cells were disappointing. The first approach was to incubate  $10^6$  viable cells in glass culture tubes in the presence of  $^{125}\text{I}$ -oPRL and  $^{125}\text{I}$ -hGH for 2 hours at  $37^\circ\text{C}$ . If 1% specific binding is accepted as the lower limit of significant hormone binding, there was no significant binding of either hormone to either HBL-100 cells or cells from a pleural effusion (Table 10). Holdaway et al. (92) reported about 0.3% specific binding of the non-primate hormones oPRL and bGH to human mammary tumor tissues and proposed that binding of 3 times this level be considered significant. In their system, plasma membrane fractions from normal breast tissue bound less than 0.9% of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -hGH. Only 7 of 34 tumor tissue preparations had specific binding of 0.9 to 4.1%. On the other hand Kelly et al. reported that similar particulate membrane fractions prepared from pregnant rat mammary tissue specifically bound 2 to 3% of the  $^{125}\text{I}$ -oPRL added while the same preparations of DMBA-induced rat mammary tumors from rats pretreated with PRL specifically bound 2 to 34% of the labelled hormone (57). Thus it appears that the binding of  $^{125}\text{I}$ -oPRL is much lower in human normal and malignant mammary tissues than in the same tissues of the rat.

The second approach to hormone binding was to incubate approximately  $10^6$  HBL-100 cells growing in culture dishes with  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins for various periods of time at  $37^\circ\text{C}$ . After incubation for 2 hours, less than 1% specific binding of either hormone occurred (data not shown).

However, the culture medium contained 10% FCS and in order to test the effect of FCS on hormone binding, about  $10^6$  HBL-100 cells were incubated for 4 hours in the presence of  $^{125}\text{I}$ -hPRL,  $^{125}\text{I}$ -Ins and 3 concentrations of serum. Fetal calf serum abolished the specific binding of hPRL (Fig. 16A) but had little effect on the specific binding of Ins (Fig. 16B). This fact suggests that FCS may have masked the binding sites, thus preventing hormone interaction with them. However, when FCS was excluded from the medium and the same experiment described above was repeated, there was no significant specific binding of either  $^{125}\text{I}$ -hPRL or  $^{125}\text{I}$ -Ins to the HBL-100 cells (Fig. 17). It is apparent that under these conditions there is no significant binding of either hormone to HBL-100 cells.

The major criticism of this work is the inadequacy of the assay system itself. The most important consideration here is the extreme sensitivity of HBL-100 cells to small changes in pH and medium changes. Thus when fresh medium was added to the culture system just prior to incubation with the hormones, the cells would usually change appearance from a flat and spreading to a raised and rounded type. Occasionally many cells would detach. Although these floating cells were also included in the assay it was assumed that they introduced some variability into the system. Also, in the absence of FCS, the cells did not seem as well attached to the culture dish surface; yet if FCS were included it interfered with hormone binding. Another point is that it is difficult in such a system to determine any inactivation of hormone that may occur during the incubation period.

#### Binding of $^{125}\text{I}$ -hormones to HBL-100 cell fractions

In an effort to overcome some of the problems discussed above and

to standardize the hormone binding assay procedure, HBL-100 cells were homogenized and 2 cell fractions were obtained by differential centrifugation. Instead of viable cells in culture, these cell fractions were used as a source of substrate for hormone binding in an assay system similar to that described by Shiu et al. (8).

Both  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins bound to both the 15,000Xg (65 ug protein) and 100,000Xg (30 ug protein) pellet. The specific binding of Ins was 1% to both fractions while that of PRL was 0.5% (Table 11). Holdaway et al. (92) reported less than 0.9% specific binding of hPRL and 0.6 to 8.3% specific binding of Ins to a normal human mammary plasma membrane fraction at a comparatively high quantity of protein (500 ug). However, 2 ug of unlabelled hormone was employed whereas only 1 ug was used by Holdaway et al. (92). When different protein concentrations of the cell fractions were assayed, binding of  $^{125}\text{I}$ -hPRL and  $^{125}\text{I}$ -Ins to the 100,000Xg preparation was higher than to the 15,000Xg preparation and an increase in binding accompanied an increase in protein concentration (Table 11). Binding to the 15,000Xg cell fraction was not consistent and each hormone bound to different concentrations of the preparation. Therefore it appeared that the 100,000Xg fraction was richer in terms of the binding sites. This is not surprising since the 15,000Xg fraction was a crude preparation consisting of many cell organelles while the 100,000Xg fraction was thought to contain primarily microsomal material. Although Ins binding to this latter preparation was comparatively high (2.2%) at a low protein concentration (380 ug/ml), the binding of hPRL was low (0.4%).

The consistent low binding of  $^{125}\text{I}$ -hPRL cast some doubt on the

effectiveness of this tracer in these hormone binding studies. To test this effectiveness, increasing concentrations of freshly prepared tracer were reacted with the 100,000Xg and 15,000Xg fractions at protein concentrations of 250 ug/ml and 1450 ug/ml respectively. Table 13 shows that the most effective concentration of  $^{125}\text{I}$ -hPRL for binding to both fractions was 102,000 cpm. At this concentration, the specific binding was similar for both fractions and the actual values were quite close to those seen earlier in Table 11 when 89,000 cpm of  $^{125}\text{I}$ -hPRL was employed. Although these results were consistent there was still little specific binding of the PRL tracer.

Since  $^{125}\text{I}$ -hGH is known to bind well to lactogenic sites in the rabbit mammary plasma membrane fraction (8) it was possible that hGH may also bind to HBL-100 cells. Both hormone tracers were reacted with the 15,000Xg cell fraction at a protein concentration of 2250 ug/ml. The specific binding of hGH was 3% after 4 hours of incubation and increased to 3.5% by 12 hours, while hPRL binding was less than 0.5% throughout the incubation period (Fig. 18). This result clearly indicates that  $^{125}\text{I}$ -hGH is bound to a greater extent than is  $^{125}\text{I}$ -hPRL. The specific binding of  $^{125}\text{I}$ -hGH and  $^{125}\text{I}$ -hPRL to rabbit mammary plasma membrane fractions was 24% and 4%/134 ug protein respectively. Throughout the course of these hormone binding studies, the hPRL tracers usually bound 2 to 6%/100 to 200 ug protein. In contrast,  $^{125}\text{I}$ -oPRL bound approximately 16%/134 ug protein of the same mammary fraction.

These facts and the possibility that hGH and hPRL may bind to the same sites in the HBL-100 cell fractions suggest that  $^{125}\text{I}$ -hGH is a

preferable tracer in hormone binding studies. Figure 19 demonstrates that the specific binding of  $^{125}\text{I}$ -hGH increases as the amount of protein of the 15,000Xg fraction is increased and begins to plateau at about 100 ug. The specific binding of hGH was approximately 3%/100 to 350 ug protein. In the same range of protein concentration the binding of hPRL was only about 0.5% (Tables 11, 12 and 13).

Unfortunately, three subsequent experiments involving  $^{125}\text{I}$ -hGH and the 15,000Xg cell fraction all failed. Since 3 different hormone tracer preparations were tested against the same cell preparation and since the hGH tracers all bound well to rabbit mammary preparations, it is suggested that the cell fractions were responsible for the unsuccessful attempts. However, all cell fractions were prepared by a standard procedure and stored at  $-20^{\circ}\text{C}$  before use. The preparations employed in these 3 experiments remained in storage for the same period of time as those used in the successful studies described above. It seems that fresh preparations may offer the best results but this was not attempted.

#### Possible significance of these studies

Although the purpose of these studies was to provide evidence for a prolactin receptor in human mammary cells there was little more than a hint of this. The binding studies appeared most promising because of the success with the prolactin receptor in the rabbit mammary. However, human mammary tissue appears to bind small amounts of  $^{125}\text{I}$ -hPRL and the sensitivity of the assay system may not be adequate for these studies. On the other hand there may be few binding sites available in

these cells in which case, the low binding of labelled hPRL may represent 'physiological' binding which is sufficient to initiate some cellular event. This is the key to the whole issue, a system is required in which the actual binding of a hormone as well as its effect on metabolic end-points can be examined together.

Culture techniques allow for such studies. Mammary explants that can be maintained in defined media for short intervals are ideal but in the case of the human biopsy specimens are rarely available in large numbers at any one time and are not always taken from the same part of the breast. Therefore these tissues cannot be used to establish a standardized system in which various hormone variables can be investigated. Pleural effusions are a good source of single cells but they consist of malignant cells as well as many other contaminating cell types, which are eliminated in some cases by long-term culture. These cell lines do provide a homogeneous source of malignant breast cells. Another cell line derived from milk samples of the healthy lactating breast provides a source of 'normal' breast cells. The long-term culture of 'normal' and malignant cells of the human breast therefore constitutes a system in which the hormone binding and various modes of hormone action can be systematically studied and compared.

The studies presented here represent an attempt of this nature employing casein synthesis and ornithine decarboxylase activity as metabolic end-points for determining the effect of PRL on human mammary cells. Neither method yielded much information. These techniques worked well with animal tissues but it became increasingly

evident that the human situation was much more complex. Therefore it may be essential to start off such studies using well-known biochemical events shared by all cells such as nucleotide biosynthesis. Binding of  $^{125}\text{I}$ -hormones was employed in an attempt to locate and perhaps quantitate the prolactin binding site. This type of study allowed the use of a standardized preparation of cells but did not provide much insight except that  $^{125}\text{I}$ -hPRL binds in small amounts to human breast cells. It is not known if the enzymatic iodination procedure somehow modifies the hormone so as to alter its activity in binding to the cell preparations. No studies have explored the possibility that labelled and unlabelled hormones have different kinetics of binding.



## SUMMARY

Attempts at establishing cultures of rabbit and human mammary cells were made in an effort to provide a system in which the effect of PRL on casein synthesis and ornithine decarboxylase activity could be studied along with the binding of  $^{125}\text{I}$ -hPRL. These 3 sources of data were to provide evidence for a prolactin receptor in human breast cells. The 2 biochemical end-points proved disappointing, while the binding of hPRL was very low. If a receptor for PRL is present in these cells it is apparent that they are few in number.

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