PROLACTIN ACTION AND MECHANISM OF ACTION IN NB2 LYMPHOMA CELLS

by

Harry Paul Elsholtz

A Thesis
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in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

Department of Physiology
Faculty of Medicine
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ABSTRACT

Prolactin (PRL) stimulates proliferation of a lymphoma cell line (Nb2) derived from an estrogenized male rat. In order to elucidate the cellular mechanisms which regulate or mediate PRL-stimulated mitogenesis, a series of studies on 'early' responses to PRL were conducted. Firstly, the role of polyamines in PRL-dependent growth was examined. Ornithine decarboxylase (ODC) activity reached a maximum 6 to 8 hr after addition of PRL to stationary Nb2 cells. This increase was associated with passage of cells through the G_1 phase of the cell cycle. Similar increases in ODC activity were also triggered under non-growth conditions, when high density cultures were transferred to fresh medium. This indicated that rapid induction of ODC was not necessarily involved in mediating PRL-stimulated cell growth. But, difluoromethylornithine (DFMO, an ODC inhibitor) slowed the growth of Nb2 cells, indicating that polyamine synthesis was required for PRL-stimulated cell proliferation. Polyamines (spermidine, spermine) were more potent than diamines (putrescine, cadaverine) in restoring normal growth to DFMO-inhibited cultures. The Nb2-SP cells, which proliferate in the absence of PRL, were more resistant to DFMO than PRL-dependent Nb2 cells. This suggests that the loss of responsiveness of Nb2 cells to PRL involves an alteration in polyamine metabolism. In a second set of experiments, the effect of PRL on cell protein phosphorylation was investigated. Two proteins, Mr 33,000 (pp33) and 19,000 (pp19), which became phosphorylated following PRL stimulation were examined. The first, pp33, co-sedimented with the particulate cell fraction and was detectable only under reducing conditions when ana-

lyzed by SDS-polyacrylamide gel electrophoresis. It was most highly phosphorylated 1 to 3 hr after PRL stimulation. This phosphoprotein had a pI > 6.7 and associated with the ribosomal fraction suggesting that it was ribosomal protein S6. The 19K phosphoprotein appeared in the cytosol fraction and was unaffected by reducing agents. Maximal phosphorylation of pp19 occurred 7 to 9 hr after PRL stimulation. The identity of pp19 was not determined. Prolactin stimulated the incorporation of ^{35}S-methionine into 33K and 19K proteins, suggesting that synthesis and phosphorylation of pp33 and pp19 could be regulated by PRL. Thirdly, the ability of anti-PRL receptor antibodies to mimic PRL action in Nb2 cells was tested. Divalent F(ab')$_2$ fragments stimulated ^{32}P uptake and incorporation in a PRL-like manner whereas monovalent Fab' fragments had no effect. However, if anti-Fab' antiserum was added to cells preincubated with anti-receptor Fab', phosphate uptake/incorporation was significantly ($P < 0.01$) stimulated. This supported an earlier study (ref. 115) suggesting that 'cross-linking' of PRL receptors is important for hormone action. Fourthly, the role of cell membrane phospholipids in Nb2 cell growth was examined using phospholipase C (Clostridium). Enzyme treatment exhibited a small inhibitory effect on non-growing cells but enhanced (30%) the growth of PRL-stimulated cells. Phospholipase C, therefore, did not activate biochemical pathways mediating PRL action, but triggered alternate processes involved in cell growth. Lastly, the ability of Nb2 cells to retain a 'PRL-like signal' following hormone withdrawal was studied. Cells exposed to PRL for 10 min, then incubated without hormone

and in the presence of anti-PRL antiserum were able to incorporate ^{32}P at an enhanced rate for several hours. Therefore, PRL may act by triggering a sustained alteration in cell membrane structure or by generating an intracellular 'messenger' which stimulates protein phosphorylation.

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LIST OF ABBREVIATIONS

Hormones and Growth Factors

EGF - epidermal growth factor
FGF - fibroblast growth factor
GH - growth hormone
LH - luteinizing hormone
PDGF - platelet-derived growth factor
PRL - prolactin
PTH - parathyroid hormone
TSH - thyroid-stimulating hormone

Enzymes

ATPase - adenosine triphosphatase
DNAase - deoxyribonuclease
ODC - L-ornithine decarboxylase
RNAase - ribonuclease

Reagents and Chemicals

cAMP - adenosine 3',5'-cyclic monophosphate
cGMP - guanine 3',5'-cyclic monophosphate
dbcAMP - dibutyryl adenosine 3',5'-cyclic monophosphate
dbcGMP - dibutyryl guanine 3',5'-cyclic monophosphate
DFMO - DL-alpha-difluoromethylornithine
DMBA - 7,12-dimethylbenzanthracene
EDTA - ethylenediaminetetraacetate
EGTA - ethyleneglycol-bis(beta-amino ethyl ether)N,N,N',N'-tetra-acetate
methyl GAG - methylglyoxal-bis-guanyl-hydrazone
SDS - sodium dodecyl sulfate
TCA - trichloroacetic acid

Miscellaneous

FCS - fetal calf serum
HS - horse serum
MW - molecular weight
PBS - phosphate-buffered saline
pI - isoelectric point
SDS-PAGE - SDS-polyacrylamide gel electrophoresis

INTRODUCTION

The action of PRL on target tissues requires the initial interaction of hormone with specific receptors on the cell surface. Such receptors for PRL have been detected in literally dozens of tissues, from mammary gland and brain to muscle and hair follicles, and in virtually all vertebrates ranging from teleosts to man. As shown in a review by Nicoll (1), over 80 different actions of PRL have been catalogued in a broad range of species and tissues. The mechanism of PRL action, however, has been examined in few tissues. It is therefore unclear whether the initial intracellular events which follow PRL binding and lead to hormone action are the same in various target tissues, or if the interaction of PRL with its receptor generates species- and tissue-specific signals which mediate the actions of PRL. To date, the mammary gland has served almost exclusively as the model for investigating PRL action, with the stimulation of milk protein synthesis being the major effect studied. Other effects of PRL on the mammary gland that have been examined include the action of PRL on lipid and RNA metabolism, lactose synthesis and ion transport.

Studies using the mammary gland model may have caused certain generalizations to be made about the mechanism of PRL action. For example, numerous reviews and textbooks of endocrinology now list PRL with those hormones that do not stimulate adenylate cyclase or enhance intracellular levels of cAMP, the most studied and possibly best understood second messenger system of hormones acting at the cell surface. This 'conclusion' is derived primar-

ily from studies on PRL action in the mammary gland (2) and may prove not to be valid in other tissues. In the case of another hormone, vasopressin, elevation of cAMP levels via adenylate cyclase stimulation is considered to be the classic mode of action in the kidney (3). Yet in the liver, another target tissue, in which vasopressin lowers fatty acid synthesis and stimulates glucose release, the hormone does not activate adenylate cyclase or increase cAMP concentration (4a, 4b). Thus, while the mammary gland is an important physiological site of PRL action and has been instrumental in the study of PRL-induced lactogenesis (milk production) there is need for additional studies on the mode of PRL action in other target tissues. The numerous and diverse actions of this hormone warrant such investigations.

This Introduction reviews studies on the mammary gland that have contributed to our present knowledge of the mechanism of PRL action. Certain studies in other peptide hormone systems are also described in relation to the action of PRL. The last section of the Introduction outlines proposals for investigating the action and mechanism of action of PRL in a lymphoma cell line.

A. Mechanism of PRL Action in the Mammary Gland

A variety of endogenous substances have been shown to mediate or support the actions of polypeptide hormones. These include cyclic nucleotides, polyamines, prostaglandins and both monovalent and divalent cations. Modification of cellular lipids and proteins by specific methylation or phosphorylation steps have been implicated in polypeptide hormone action as well. The effects of these agents and reactions have been examined in the

mammary gland to clarify what role they might play in the mediation of PRL action. Comprehensive reviews on this subject have been written by Shiu and Friesen (5, 6) and Rillema (7).

a) Cyclic Nucleotides

Despite the fact that PRL does not stimulate adenylate cyclase and cAMP does not mimic PRL action (2, 8, 9), there may be some involvement of cAMP in mediating the effect of PRL. Turkington et al (8) have shown that the hormone does cause an increase in cAMP-dependent protein kinase activity in mammary gland organ cultures. The availability of these enzymes, then, rather than the concentration of intracellular cAMP may be the critical factor in mediating certain actions of PRL in the mammary gland. Since cAMP and its analogs have in fact been found to inhibit some actions of PRL in mammary tissues (9, 10), there is a possibility that some effects of PRL are brought about by the depression of intracellular levels of cAMP. According to a model proposed by Rillema (7), PRL may accomplish this by activating cAMP phosphodiesterase, possibly by elevating the level of cGMP within the mammary cell (11).

Unlike cAMP, cGMP has been implicated as a mediator of PRL action in the mammary gland. Lactation which in rodents is marked by high circulating levels of PRL, is accompanied by an increase in concentrations of cGMP within the mammary gland (12). Cyclic GMP has been shown to stimulate RNA synthesis in cultured explants from mouse mammary gland in a PRL-like manner (9) and also enhances RNA synthesis in nuclei isolated from lactating rat mammary glands (13). While cGMP does not appear to enhance the synthesis of casein in vitro , Matusik and Rosen (14) have

demonstrated that cGMP can increase levels of casein mRNA in cultured rat mammary explants. This effect is however of considerably lower magnitude than that observed in the presence of PRL, indicating that cGMP alone cannot mediate PRL action on casein mRNA levels. If cGMP is indeed a physiological mediator of PRL-induced lactogenesis, then additional factors are required to potentiate its effect.

b) Prostaglandins and Phospholipases

Studies from several laboratories have provided evidence that prostaglandins, particularly those of the '2' series, may be required for PRL to act on the mammary gland. Rillema (15 - 17) has shown that prostaglandins B_2, E_2, $F_{2\alpha}$ and arachidonic acid have PRL-like effects on RNA synthesis in the mouse mammary gland. In his studies, indomethacin, which inhibits the membrane-associated enzyme complex prostaglandin synthetase, attenuated both PRL- and arachidonic acid-induced RNA synthesis, suggesting that synthesis of prostaglandins is a necessary step in PRL action. However, since prostaglandins alone did not stimulate casein synthesis it was clear from his work that other factors were required to elicit the complete response to PRL. Such factors are discussed in section A(c).

Additional evidence suggesting that prostaglandins mediate certain actions of PRL is that phospholipase A_2, an enzyme which converts plasma membrane phospholipids into potential precursors of prostaglandin synthesis, is activated by PRL in membrane preparations of the mouse mammary gland (18). Inhibitors of phospholipase A_2 activity (eg. quinacrine) block

the stimulatory action of PRL on RNA and casein synthesis in mammary gland explants (19). Furthermore, when phospholipase A_2 is added exogenously to cultured mouse mammary tissues, it mimics the effect of PRL on RNA synthesis (20). This effect is blocked by indomethacin -- consistent with the view that PRL-stimulated phospholipase A_2 activity initiates the synthesis of prostaglandins which then promote RNA synthesis. Casein synthesis in mammary gland cultures, however, is unaffected by phospholipase A_2. Thus, while phospholipase A_2 and various prostaglandins may be important in PRL-stimulated RNA synthesis in the mammary gland, they cannot account for the stimulatory action of PRL on milk protein synthesis. Several investigators have shown that polyamines may, in part, be responsible for this latter effect of PRL.

c) Polyamines

Polyamines and diamines constitute a ubiquitous class of intracellular cations affecting a range of physiological functions in virtually all cell types. These molecules have been shown to stimulate cell growth, stabilize whole cells, membranes and subcellular particles, associate with nucleic acids, affect protein synthesis and stimulate or inhibit the activity of various enzymes (21, 22). Numerous hormones have been shown to stimulate ODC (22, 23) which, because of its low or negligible basal activity in most mammalian tissues, is considered to be the rate-limiting enzyme in polyamine synthesis. Anabolic responses in target tissues generally involve the induction of ODC and subsequent synthesis of the polyamines spermidine and spermine. A 1972 study reporting increased levels of polyamines in

lactating mammary glands of rats (24) therefore triggered a number of investigations of the role that these molecules might play in PRL-stimulated lactogenesis.

Oka and Perry (25) and Rillema (26) demonstrated that PRL causes an increase in ODC activity in cultured mammary explants, an effect that is dependent on RNA synthesis (25). Prolactin-stimulated induction of ODC has also been reported recently in primary cultures of DMBA-induced mammary tumors (27). In mammary explants, the effect of PRL on ODC may be an intermediate step in the stimulation of casein synthesis. Addition of spermidine to cultured mammary tissues increases the rate of casein synthesis in the presence of agents that enhance RNA production such as cGMP, prostaglandins, arachidonic acid, or phospholipase A_2 (28, 29). Furthermore, when methyl GAG (a potent inhibitor of spermidine synthesis) is added to cultures, the stimulatory effect of PRL on milk protein synthesis is abolished (29). Although spermidine mimics the effects of PRL on casein production qualitatively, it cannot generate a response of similar magnitude to that seen in the presence of hormone (29). The mechanism of spermidine action is thus obscure; it seems possible, however, that polyamines support rather than mediate the action of PRL on casein synthesis. Matusik and Rosen (30) have shown that PRL induces synthesis of casein mRNA rapidly (within one hour) in organ cultures of rat mammary gland. This effect therefore precedes the stimulatory action of PRL on ODC activity (26) and subsequent spermidine synthesis. It also precedes PRL-enhanced uptake of polyamines (31). Furthermore, addition of spermidine

to mammary explant cultures does not promote the accumulation of casein mRNA (14, 32). These results suggest that polyamines have a post-transcriptional site of action, possibly enhancing casein synthesis by their effects on ribosomes, tRNA binding to ribosomes or tRNA nucleotidyl transferases (21). Whether the effects of polyamines on protein synthesis in the mammary gland are truly selective for milk protein synthesis has not yet been resolved.

d) Ions

Prolactin alters ion fluxes in mammary gland slices, stimulating retention of K^+ and reducing the concentrations of intracellular Na^+ and Cl^- (33). This effect, which may be important for establishing the ionic concentrations of milk, could be related to the osmoregulatory action of PRL in lower vertebrates (1). Falconer et al (33) have proposed that PRL-induced changes in Na^+/K^+ concentrations may mediate certain actions of the hormone in the mammary gland. In support of their hypothesis they have shown that ouabain, a specific inhibitor of Na^+,K^+-ATPase, abolishes PRL stimulation of lipogenesis in rabbit mammary explants. Prolactin effects on casein and RNA synthesis were unaffected by the inhibitor, however. In the laboratory of Houdebine and Djiane (34) ouabain did in fact block PRL-stimulated synthesis of both casein and casein mRNA in rabbit mammary cultures. Because the drug also depleted PRL receptors in the cultures, however, the investigators concluded that intracellular Na^+/K^+ ratios may not be directly involved in PRL action. Rather, ouabain may exert its effect non-specifically by acting on PRL receptors. Since a direct activation of Na^+,K^+-

ATPase by PRL has not yet been demonstrated in the mammary gland, the role of this enzyme in mediating PRL action remains obscure. Interestingly, in isolated erythrocyte membranes high concentrations of PRL significantly inhibit Na^+,K^+-ATPase activity (35), an effect inconsistent with that predicted in the mammary gland.

Calcium has been implicated widely as a mediator of hormone action, acting in general via the troponin C-like regulatory molecule calmodulin to regulate intracellular functions including cytoskeletal organization and protein kinase activity. Little evidence currently supports a role for this ion as a mediator of PRL action. Rillema (7) has reported that EGTA, a calcium chelator, completely blocks the stimulatory action of PRL on RNA and casein synthesis in mouse mammary gland in vitro. Addition of calcium to EGTA-treated explants restored the responses to PRL; addition of other divalent ions like magnesium or manganese had no effect. While this study implies that calcium is necessary for certain PRL effects it does not demonstrate a mediatory role for the ion. Contrary to the work of Rillema are studies by Houdebine (35) indicating that removal of calcium from the medium of cultured rabbit mammary explants had little effect on PRL action; nor did calcium ionophore A23187 enhance or mimic the effect of PRL.

Reports demonstrating direct involvement of calmodulin in PRL action have not yet emerged. Houdebine (unpublished observations described in ref. 37) states that trifuoperazine, a calmodulin inhibitor, did not influence casein synthesis in

mammary explants. Whether the calcium-binding molecule affects other PRL actions has not been determined.

e) Cytoskeletal Elements

The cytoskeletal system, composed of microtubules, microfilaments and intermediate filaments, is closely associated with the cell surface and has thus been implicated in transferring hormonal information from plasma membrane receptors to intracellular loci (38). Houdebine and Djiane (39) examined the effect of microfilament- and microtubule-disrupting agents (ie. cytochalasin B and colchicine, respectively) on PRL-induced casein synthesis in mammary tissues. In their study cytochalasin B suppressed casein production only slightly in the presence of PRL and did not interfere with the PRL-induced accumulation of casein mRNA. Because the drug non-specifically reduced total protein synthesis in the mammary cultures, the authors concluded that an intact microfilament system was not required for PRL-dependent casein gene activation. Colchicine, on the other hand, markedly inhibited PRL stimulation of both casein and casein mRNA production, suggesting that destabilization of microtubules could prevent PRL-induced gene expression. The apparent specificity of colchicine action in the mammary gland has been demonstrated in vivo where the inhibitor specifically prevented the effect of PRL on casein gene transcription without suppressing hormone-enhanced synthesis of 28S rRNA (39, 40). Interestingly, in pseudopregnant rabbits injection of colchicine appeared to block the action of PRL on the rate of casein gene transcription completely while inhibiting only partially the accumulation of casein mRNA (41). In other

words, colchicine interfered primarily with PRL-stimulated induction of casein gene with little effect on PRL enhancement of casein mRNA half-life (an effect described by Guyette et al, 42).

The mechanism by which colchicine inhibits PRL-induced casein gene expression is still unclear. Recently, the role of microtubules was questioned by a study which demonstrated that griseofulvin (an antimitotic agent which disrupts microtubule structure) had no effect on PRL action (43). The authors of the study suggested that colchicine may prevent PRL induction of the casein gene by acting on the plasma membrane rather than on the microtubule system. They found that ^{3}H-colchicine binds specifically to plasma and Golgi membranes in the mammary gland, an interaction that is inhibited by excess unlabeled colchicine but not appreciably by lumicolchicine, an analog which does not bind to tubulin. Thus, colchicine may associate with tubulin-like components of the plasma membrane -- blocking PRL/receptor-dependent generation of 'second messengers'.

Colchicine has been shown to inhibit internalization of PRL in vivo. Posner et al (44) injected radioiodinated PRL into rats and monitored incorporation of the label into hepatic Golgi and plasmalemma elements. Colchicine prevented the accumulation of PRL in Golgi but not plasmalemma fractions indicating that translocation of PRL into the cell had been decreased whereas cell surface binding of hormone had not. The colchicine effect displayed some specificity in that the internalization of insulin was not impaired significantly by the drug (44). Whether the effect of colchicine on internalization of PRL-receptor complexes

is related to its inhibitory effect on PRL-stimulated lactogenesis is not known. The role of internalized PRL molecules in mediating hormone action in the mammary gland is examined next.

f) Hormone Internalization and Intracellular Binding Sites

The plasma membrane receptors of many polypeptide hormones are not only responsible for ligand recognition but also for directing bound hormone molecules into the cell (45, 46). At present, the purpose of hormone internalization is not fully understood but the process does appear to cause desensitization of target cells by decreasing the number of cell surface receptors and facilitates hormone degradation via Golgi- or lysosome-dependent mechanisms (45, 46). Because internalization is not an immediate consequence of hormone binding (requiring up to 30 minutes), rapid hormonal effects such as modification of cell membrane phospholipids or activation of ion transport systems are unlikely to be the result of this event. However, delayed hormone responses like gene induction, cell differentiation or mitogenesis may (at least in theory) be effected by processing of internalized hormone-receptor complexes. Some support for this hypothesis has been found in EGF-responsive cell lines (47). In recent years numerous investigations have reported the presence of polypeptide hormone receptors at intracellular loci including Golgi regions (48, 49), endoplasmic reticulum (49, 50), and nuclei (50, 51), a finding that also supports the possibility of intracellular sites of action for internalized polypeptide hormones.

Receptor-mediated internalization and processing of PRL in

mammary tissues has been studied in several laboratories including our own. Shiu (52) has shown that human mammary tumor cell lines bind and degrade PRL, the rate of degradation correlating closely with the number of PRL receptor sites in each cell type. Agents inhibiting lysosomal enzyme activity attenuate proteolysis of bound PRL, indicating an intracellular locus of degradation. Costlow and Hample (53) have demonstrated in another way that PRL is internalized by cultured rat mammary tumor cells. Receptor-bound PRL becomes resistant to removal by pH 3 and is degraded when cells are incubated with hormone at 37°C. If cells are first energy depleted with inhibitors of oxidative phosphorylation, however, pH 3 treatment strips bound PRL from the cells in intact form such that receptor-binding capacity is retained (53). Thus, internalization of PRL appears to be an ATP-dependent process. By using this approach the investigators were also able to demonstrate that lysosomotropic agents block degradation of internalized PRL specifically without inhibiting hormone internalization (53).

But what is the purpose of internalization and degradation of PRL? Nolin and colleagues (54, 55), using Bouin's fixed mammary tissues, have demonstrated with immunoperoxidase staining that PRL is incorporated into cells and accumulates at the periphery of the nucleus. They found that estrogen-induced lactational failure in rats was accompanied at first, by an inability of milk secretory cells to translocate PRL to nuclei, later by failure to internalize PRL and ultimately failure to recognize PRL (as determined by binding studies). Thus there appears to

be a functional relationship between PRL internalization and action. Such an hypothesis seems to be supported by in vitro studies in rabbit mammary explants, in which synthesis of casein, casein mRNA and DNA are enhanced by PRL while lactogen receptor levels are depressed (down-regulated) by the hormone (56). The physiological dose-dependent increase in both a) internalization of receptor-hormone complexes and b) hormonal response suggested a functional relationship between the two events.

Reports by Mittra (57, 58) have suggested a biological role for processing or degradation of PRL. He found that proteolytic cleavage followed by reduction of native PRL produced a 16,000 dalton peptide which stimulated DNA synthesis and mitosis in mammary cells in vivo, whereas native PRL (an effective stimresponse (58). The cleaved form of PRL is apparently secreted from the pituitary (57) while reduction (which generates the 16 K fragment) occurs at a more distal site possibly in the mammary gland. It is still unclear how the 16 K moiety might interact with mammary cells to trigger mitogenesis. Since local subcutaneous injections of 16 K fragment increased the mitotic index of mammary cells near the injection site (58), the peptide appears to act directly on the mammary gland and not by stimulating release of mitogen from a peripheral tissue. While the action of 16 K fragment on the mammary gland is interesting, the peptide is probably not an intracellular mediator formed by the degradation of internalized bound PRL, as native PRL cannot mimic the effect of the peptide in the mammary gland (58).

The discovery of intracellular polypeptide hormone receptors

has led to speculation that these sites may mediate actions of internalized hormone molecules (50). Receptors for PRL have been detected in the Golgi elements of both liver (48) and mammary gland (59). The presence of intact (immunoreactive) PRL inside mammary (55) and ovarian (60) cells has been observed as well, strengthening the proposal that PRL achieves certain effects by binding to intracellular sites. Scatchard analysis has revealed that Golgi receptors have a higher binding affinity than plasma membrane receptors (48, 61); thus a physiological role might be expected. At present, however, no such role has been established. PRL receptors in the Golgi regions may be newly synthesized and en route to the cell surface; on the other hand they may have been internalized either through down-regulation or merely by a cellular scavenger process.

There are several lines of evidence suggesting that PRL does not require an intracellular site of action but exerts its effect at the cell surface. Firstly, inhibition of lysosomal enzymes which degrade internalized PRL does not prevent hormone action in the mammary gland. Chloroquine and ammonium chloride effectively inhibit degradation of internalized PRL by mammary cells (52) without affecting PRL-enhanced synthesis of casein and casein mRNA in rabbit mammary explants (39). On the other hand they do inhibit down-regulation of PRL receptors in mammary gland cultures (62). This latter effect would suggest that internalization of hormone-receptor complexes (which appears to be the mechanism involved in down-regulation (45)) is not a prerequisite for PRL action on casein synthesis. Furthermore, receptor clustering

on the cell surface, an early event in hormone-induced down-regulation is also inhibited in some systems by ammonium chloride (63). Such an effect has not been demonstrated on PRL receptors in mammary cells but it does question the importance of receptor aggregation in PRL-stimulated casein production. In summary, then, the presence of PRL and PRL receptors inside target cells suggests an intracellular site of action for PRL (or a fragment thereof) while the effects of inhibitors of hormone internalization and degradation support the view that PRL action is restricted to the cell surface.

g) Anti-receptor Antibodies

Antibodies to hormone receptors have been used by numerous investigators to demonstrate the obligatory role of plasma membrane receptors in mediating hormone action. They have been of particular importance in systems where synthetic hormone agonists and antagonists are not readily available. In 1976 Shiu and Friesen (64) reported that antiserum raised against partially purified PRL receptors from rabbit mammary gland could block PRL binding to its receptor in explants of mammary tissues. Furthermore, the antiserum prevented both PRL-stimulated incorporation of ^{3}H-leucine into casein and transport of ^{14}C-aminoisobutyric acid. The study also revealed, however, that in the absence of PRL the anti-receptor antibodies could partially trigger the same responses that were blocked when hormone was present (64). These experiments provided the first direct evidence that PRL binding sites in the mammary gland were true receptors, necessary for mediating PRL action.

In vivo studies have shown that if lactating rats are

treated with anti-PRL receptor antibodies weight of the pups is significantly reduced (65), suggesting that milk production is impaired by the inhibition of PRL binding. Administration of the antisera to female rats also alters ovarian histology, the number of corpora lutea being increased. Thus anti-receptor antibodies appear able to block the luteolytic action of PRL in rats. Recently Dusanter-Fourt et al (66) have reported that administration of gamma-globulins from an anti-PRL receptor serum induces synthesis of casein and casein mRNA in pseudopregnant rabbits, thus mimicking the action of PRL in vivo. Moreover, progesterone, a physiological inhibitor of PRL action in the mammary gland, also abolished the antibody effect. No inhibition of PRL action by the gamma-globulins was reported. While the similarities between actions of PRL and anti-receptor antibodies in vivo are interesting there may be some doubt as to the validity receptor antisera often contain high concentrations of PRL such that crude immunoglobulin preparations (proteins precipitated in 30-35% ammonium sulfate) also contain substantial quantities of the hormone. This contaminating PRL could generate the hormone-like responses. Thus, in studies where PRL-like effects are attributed to anti-receptor antibodies, investigators should first establish that immunoglobulin fractions are PRL free. The PRL molecules could readily be removed by adsorption to an anti-PRL antibody affinity column.

Djiane et al (67) have also reported that anti-PRL receptor antibodies mimic PRL action in rabbit mammary gland explants. As

in earlier studies (64), the antibodies inhibited PRL-enhanced synthesis of casein and also DNA synthesis but triggered the same responses in the absence of hormone. As with PRL, colchicine blocked the antibody effects while chloroquine and ammonium chloride did not (67). More recently, antibodies to PRL receptors have been reported to mimic the stimulatory effect of PRL on PRL binding activity in mammary tumor explants (68) and in rat liver cells cultured in suspension (69).

In agreement with the inhibitor studies discussed in this section, studies with anti-receptor antibodies would indicate an extracellular site of action for PRL. Since immunoglobulins differ greatly in structure from PRL but elicit the same responses, the PRL receptor -- to which both ligands bind, would appear to be of greater importance than the structure of the ligand in triggering a lactogenic or mammogenic effect. Thus, PRL may be required only for binding to the receptor to initiate the sequence of events necessary for transmission of the hormonal message (66 - 69).

However, these data are still subject to criticism. Three of the above mentioned studies (64, 67, 68) demonstrated qualitative similarities between PRL and anti-receptor antibody action, but in all cases the magnitude of response to hormone was greater than that to anti-receptor antibodies. Why such quantitative differences exist remains speculative. Firstly, it may be that the interaction between PRL and its receptor causes a conformational change in the receptor leading to <u>maximal</u> liberation of membrane-associated second messengers. Anti-receptor antibodies, by binding to different regions of the

PRL binding site, may modify the receptor's tertiary structure suboptimally -- thereby impairing the release of second messengers. Secondly, the polyclonal antisera used in the above-mentioned studies may consist of stimulatory and inhibitory antibodies, the latter partially neutralizing stimulation by the former by binding to cell surface proteins near the PRL receptor. The development of a library of monoclonal antibodies to the PRL receptor may yield both antibodies which are only inhibitory and those which have PRL-like potency. Thirdly, while studies with lysosomotropic agents suggest that PRL degradation does not mediate hormone effects, it cannot be precluded that a fragment(s) of PRL released by the action of specific cell membrane proteases (ie. "prolactinases") augment(s) PRL receptor-dependent events. Similarly, PRL or fragments of the hormone may become covalently conjugated to prosthetic groups which then impart biological activity to the molecule(s). The recent demonstration that EGF-stimulated tyrosine kinase phosphorylates human GH (70), a lactogenic hormone, gives credence to this possibility. Of course antibodies may yet be found (either monoclonal or polyclonal) which mimic PRL action on casein and DNA synthesis in an equipotent manner. Anti-insulin receptor antibodies, for example, are known to invoke insulin-like responses of the same magnitude as that hormone (71). Until such antibodies are developed, however, post-receptor involvement of the PRL molecule cannot be ruled out completely.

While anti-receptor antibodies have provided invaluable clues to the mechanism of action of PRL and other hormones, the

exact nature of antibody action is still unclear. It is generally accepted that anti-receptor antibodies bind to cell surface receptors and induce clustering or patching of antibody-receptor complexes in the plasmalemma. The release or activation of intracellular mediators of hormone action is thought to depend on this process. Kahn et al (71) first demonstrated the importance of clustering in insulin-responsive adipocytes. Monovalent Fab' fragments from anti-insulin receptor antibodies were able to bind to the insulin receptor but could not mimic insulin action on glucose oxidation, an activity intrinsic to divalent $F(ab')_2$. Insulin-like activity was restored to Fab' fragments by cross-linking them on the adipocyte surface with an anti-$F(ab')_2$ antiserum. Similarly, submaximal stimulation of adipocytes by insulin was enhanced by the addition of cross-linking anti-insulin antibodies. Schechter et al (72) have shown that cell surface events are also important for action of EGF which like insulin induces rapid aggregation of receptors after binding. Cyanogen bromide cleavage of EGF generated a fragment which was virtually devoid of mitogenic activity but which retained substantial binding activity (72). When this fragment was preincubated with cells, addition of bivalent anti-EGF antibodies restored receptor clustering and biological activity (72). More recently Schreiber et al (73) have shown that Fab' fragments from monoclonal anti-EGF receptor antibodies bound to or near the receptor binding site and stimulated the EGF receptor-sensitive protein kinase but did not induce receptor clustering or DNA synthesis. Again, cross-linking with anti-Ig antibodies generated an EGF-like response. Studies with monoclonal antibodies in

other polypeptide hormone systems including TSH (74) and LH (75) have strongly supported the view that the hormone molecule is not required to act at an intracellular site but serves only to trigger receptor aggregation which then plays a crucial role in generating the biological response.

However, antibodies that mimic hormonal effects do not always exert their actions in the "classic style" described above. Schreiber et al (73) showed that antibodies which recognized the EGF receptor but not the binding site, could still enhance DNA synthesis in an EGF-like manner if cross-linking "second" antibodies were added. Likewise in insulin-sensitive cells, antibodies to plasma membrane antigens which did not even interact with insulin receptors were able to mimic hormone action (76). In this study insulin receptors may have been clustered indirectly and nonspecifically by the aggregating effect of the antibodies on other cell surface antigens.

The concept of hormone-induced receptor aggregation leading to a hormonal response has not been supported by some investigators. In one study, done in Steiner's laboratory (77), Fab' fragments derived from a stimulatory anti-insulin receptor antiserum were unable to increase ^{14}C-glucose incorporation into hepatic glycogen when cross-linking antibodies were added to hepatocyte cultures. Glucose transport in adipocytes, however, was stimulated by this treatment (77). Jarett et al (78) showed that receptor aggregation occurs in the natural state of certain tissues but not others. Using monomeric ferritin-labeled insulin, an equipotent hormone conjugate, they also showed (with

electron microscopy) that receptor occupation does not alter distribution of receptors in plasma membranes (79, 80). This questions the biological significance of receptor clustering reported by others. Lyen (81) has suggested that splitting of anti-receptor antibodies into Fab' fragments alters conformation of the latter such that binding activity is retained but biological activity is abolished. Addition of "cross-linking" antibodies may then mimic hormone action by merely restoring the original conformation of Fab' fragments.

Although antireceptor antibodies are thought to act at the cell surface, evidence has been presented to the contrary. Carpentier et al (82) demonstrated that radiolabeled anti-insulin antibodies were internalized by lymphocytes and distributed in a manner similar to that found for insulin. An intracellular site of action for antireceptor antibodies has recently been identified. In a provocative study in Goldfine's laboratory (83), anti-insulin receptor antibodies were shown to mimic the direct effects of insulin on nuclei from rat liver. Both insulin and the antibodies had biphasic dose-dependent effects on dephosphorylation of proteins in nuclear envelopes. The antibody effect was additive to that of insulin suggesting that both ligands exerted their actions through similar mechanisms. Very little is known of how nuclear receptors for polypeptide hormones mediate hormone action. However, the discovery of biologically relevant sites in the nucleus opens a new and exciting area of investigation in endocrinology.

h) Putative Second Messengers

A 'factor' that appears to mediate PRL action on the beta-casein gene was described by Teyssot et al (84) in 1981. The investigators reported that incubation of PRL with microsomal membranes from rabbit mammary gland liberated a 'second messenger' which stimulated production of beta-casein mRNA in isolated mammary cell nuclei. Action on the beta-casein gene appeared to be specific in that transcription of the 28S rRNA gene was not affected by the factor. Other lactogenic hormones like human GH and ovine placental lactogen which bind to PRL receptors triggered release of the membrane-associated factor whereas other hormones could not. The PRL-dependent factor seemed to act on the mammary casein gene specifically since casein mRNA was not synthesized when the factor was incubated with nuclei from liver or reticulocytes. Specificity appeared also to exist at the cell membrane level; only microsomal fractions of tissues bearing PRL receptors (liver, ovary, and adrenal gland but not heart, lung, or muscle) liberated the factor in response to PRL.

Some characteristics of the 'mediator' have been reported by Teyssot et al (85). The factor is heat-stable but sensitive to trypsin, suggesting a peptide nature. Sephadex G-25 chromatography indicates that the factor has a molecular weight of 1000. Its generation from microsomal membranes is temperature-dependent with no release at 4°C, and a more rapid release at 37°C than at 20°C. Antibodies to the PRL receptor which mimic hormone action in mammary explants also liberate the factor from membranes. Interestingly, the antibodies are about as effective as PRL (9-

fold vs. 10-fold stimulation) in triggering release of the 'mediator' (85), a similarity not seen previously when synthesis of casein was examined in mammary explants (67). The authors (85) have also reported that colchicine prevents PRL-stimulated release of the factor when incubated with membranes but does not interfere with 'mediator-enhanced' synthesis of casein mRNA in isolated nuclear preparations. This finding is compatible with the view that colchicine inhibits PRL action at the cell membrane level and not by disrupting the structure of intracellular microtubules. Recently, butyrate has also been found to specifically block release of the factor from mammary membranes (86). Generation of the putative PRL mediator appears to be specific for membrane 'type'. Hepatic plasma membranes but not Golgi membranes produce the factor when incubated with PRL even though both bind hormone specifically (85). As mentioned in section A(f), this might suggest that Golgi sites are newly synthesized and 'immature' -- capable of recognizing PRL molecules but not yet coupled to an effector system. Conversely, the sites could be internalized cell membrane receptors having undergone partial inactivation in Golgi regions such that only the capacity to bind PRL has been retained. In other studies the putative mediator has been found to stimulate casein mRNA synthesis in intact mammary epithelial cells indicating an apparent ability to enter and activate target cells (87). The mechanism of action of the factor on gene transcription is unknown. In a recent study, Houdebine et al (88) have found that various phosphatase inhibitors added to isolated nuclei completely suppress stimulation of beta-casein gene transcription by the putative mediator. They suggest,

therefore, that the 'mediator' exerts its effect on gene expression by triggering dephosphorylation of mammary nuclear proteins. The non-specific actions of some phosphatase inhibitors, however, warrants cautious interpretation of this data.

The potential role of small molecular weight peptides as mediators of hormone action was first proposed for insulin. Larner et al (89, 90) demonstrated that extracts from insulin-treated muscle inhibited cAMP-dependent activation of purified protein kinase. Since those initial studies numerous others have appeared, verifying that a factor produced in insulin-responsive cells or membranes from target tissues mimics insulin action in cell-free systems (91 - 94) and intact cells (95). The insulin 'mediator' appears to share certain properties with the PRL 'mediator'. It, too, has a low molecular weight, estimates ranging from 1000 - 3000 daltons, is heat-stable, and can be generated not only by insulin but also by ligands that mimic insulin action, ie. anti-receptor antibodies and concanavalin A (96). It has been suggested that the insulin and PRL 'mediators' may be the same entity (87, 88); however, too little information is currently available to support such a view. The putative second messenger of PRL has been shown to exert only one specific action in one system -- that of increasing beta-casein mRNA synthesis in isolated mammary nuclei (84). The insulin 'mediator' has been shown to trigger a variety of hormone-like responses which, with one possible exception (97), occur in extra-nuclear subcellular fractions (90, 91, 93). The observation that insulin 'mediator' stimulates nuclear RNA synthesis

(97), is inconsistent with the finding that PRL 'mediator' does not promote a generalized increase in RNA synthesis (84). Furthermore, the peptide nature of the insulin second messenger has not been established, as protease sensitivity of the mediator is observed by some investigators but not others (96). Kiechle et al (98) have postulated that the insulin 'mediator' may in fact be a group of phospholipids. At present, the peptide nature of the PRL 'mediator' has not yet been demonstrated by studies other than those of Houdebine and colleagues.

Purification of these mediators of hormone action has proven difficult. Larner (96) has listed numerous problems including instability of the factor, discrepancies regarding proteolysis of the factor, the apparent existence of multiple mediators, low concentrations of mediator in hormone-treated tissues and co-purification of non-specific stimulators or inhibitors (eg. metals) with the hormone-sensitive species.

Existence of the PRL 'mediator' proposed by Teyssot et al (84) has not been confirmed by other investigators and thus it remains the subject of controversy. No characterization of a partially purified 'mediator' has been reported by the French team. Studies in Rosen's laboratory (99) are currently underway in an effort to substantiate (or refute) the earlier studies.

B. PRL and the Immune System

Immunoglobulins in milk help to protect neonates against a number of infectious diseases. These antibodies, primarily IgA, are secreted by plasma cells localized in mammary tissues. As reviewed by Lamm (100a) and recently by Bienenstock et al

(100b), studies with mice have shown that the plasma cells originate from lymphoid tissue of the gut. During pregnancy and lactation migration of lymphoblasts to the mammary gland is enhanced dramatically (101) and this effect can be mimicked by injection of PRL into virgin female mice (102). The effect of PRL is specific for IgA-producing plasma cells, but the mechanism by which 'homing' to the mammary gland is initiated is not understood. Nevertheless, the phenomenon may be of clinical interest. Alm et al (103) have recently reported the presence of a primary IgA-secreting non-Hodgkin lymphoma in the breast of an elderly woman. Since bilateral breast lymphomas are not uncommon (104), it is possible that an altered mammary microenvironment promotes homing of certain transformed lymphoid cells to the breast. Such an alteration in mammary tissue may be induced by PRL. Of additional interest are case reports of patients in which Burkitt lymphomas form bilateral lesions in breast tissue, particularly during pregnancy and lactation (105). In one patient, lactation was followed by a complete regression of the Burkitt tumor (105). Although it is not known whether PRL, per se, was responsible for the appearance and/or proliferation of Burkitt cells in the breasts of these patients, several human lymphomas examined in our laboratory have been found to bind PRL and human GH specifically, suggesting a direct action of lactogenic hormones on some human lymphoid cells. Others (106) have reported that PRL-binding activity in lymphocytes increased dramatically in a patient during a bout with infectious mononucleosis. A loss of binding activity accompanied disappearance of clinical symptoms. These authors speculated that infection with

Epstein-Barr virus may have triggered the elevated levels of PRL-binding and that a similar viral effect may be responsible for the migration of Burkitt lymphoma cells to the human mammary gland (106). Support for this hypothesis would indeed be of great interest but has not yet appeared in the literature.

Studies by Berczi and colleagues have suggested an immunoregulatory role for PRL in rats. They reported that both hypophysectomized and bromocryptine-treated animals were unable to develop contact dermatitis in response to dinitrochlorobenzene (107). Normal reaction to the chemical was restored by daily injections of PRL while combined treatment with other pituitary hormones was ineffective (107). Hypophysectomy also caused impaired antibody responses (both IgM and IgG) to sheep red blood cells but again, administration of PRL restored immunocompetence (108). The effects of PRL on cell-mediated and humoral immunity are indeed striking in these studies and similar findings by other investigators would strongly support the view that PRL acts as an immunoregulatory hormone.

C. Nb2 Lymphoma Cells

a) History

That PRL promotes the growth of certain rat lymphomas was first reported by Noble and colleagues (109, 110) at the University of British Columbia. Ten months after implantation of an estrogen pellet into a male Nb rat, a lymphoma (later labeled Nb 2 node lymphoma) arose which grew most rapidly when transplanted into rats bearing a pituitary tumor graft. Suspension cultures of the cells grew readily in the presence of serum from estrogen-

treated rats (which were hyperprolactinemic) but did not respond to serum from hypophysectomized animals (109). Gout et al (110) established that PRL, specifically, stimulated proliferation of Nb 2 node lymphoma cells (Nb2 cells) and that hormone concentrations as low as 10 pg/ml had a detectable effect on growth. Studies with Nb2 cells in our laboratory (111) aided in developing the most sensitive bioassay for PRL-like hormones yet available. A wide range of non-lactogenic hormones and growth factors were found to be inactive in stimulating proliferation of Nb2 cells (111), indicating that the assay is highly specific. The Nb2 cell bioassay has been used in clinical studies in our lab to measure physiological concentrations of both PRL and GH in human serum (112 - 114).

b) PRL Receptors

The marked responsiveness of Nb2 cells to PRL suggests that the cells may provide a useful model for studying the mechanism of PRL action. Binding studies in our lab have shown that there are about 12,000 PRL receptors per Nb2 cell (115). Only lactogenic hormones compete effectively with radioiodinated PRL for the sites. Antibodies to the rabbit mammary PRL receptor bind to PRL receptors of Nb2 cells and exert both stimulatory and inhibitory effects on cell growth (115), indicating that the antigenic structure of these receptors is similar to PRL receptors in other target tissues. Furthermore, studies using chemical cross-linking reagents to covalently couple ^{125}I-PRL to Nb2 cell receptors have suggested a molecular weight of about 37,000 for the binding subunit (J.P. Hughes, unpublished). This value is in agreement

with estimates for binding subunits of PRL receptors in mammary gland and liver (116, 117). Scatchard analysis of PRL binding in intact Nb2 cells has suggested that Nb2 PRL receptors have a dissociation constant (K_D) of 6.5×10^{-11} M (115), about 4 - 5 times lower than that of hepatic or mammary receptors. In studies with Triton X-100-solubilized PRL receptors (unpublished), however, I have been unable to confirm major differences between the binding affinities of receptors from Nb2 cells and rat liver. Both K_Ds were approximately 4×10^{-10} M. It is possible that hormone binding studies with viable cells at 37°C may not represent true equilibrium conditions.

Thus, while PRL stimulates many different physiological events (1), PRL receptors have not been shown to vary greatly, either physico-chemically or immunologically, in a variety of target tissues. It is reasonable to expect, of course, that tissue or species variations may become apparent with further purification of the receptor, application of recombinant DNA techniques to cloning of the receptor gene(s) and development of specific monoclonal antibodies to various determinants on or near the receptor. Nevertheless, even in a given tissue -- mammary gland, for example, PRL/receptor interaction causes numerous effects including stimulation of ion and amino acid transport, lipogenesis, RNA and DNA synthesis, activation of gene transcription and stabilization of specific mRNAs. How PRL initiates these different responses at the receptor is not known. The suggestion by Teyssot et al (84), that a PRL receptor-associated 'second messenger' affects only casein gene induction, requires the question of how PRL exerts its effect on other cellular

functions to be readdressed more precisely with reference to subcellular locus. In contrast to the 'mediator' of PRL action, cAMP mimics several actions of hormones like glucagon or epinephrine in target cells, thereby fulfilling the role of a second messenger more completely. Future work by Houdebine and colleagues may reveal whether their previously described factor (84) is indeed active at only one intracellular site.

c) Current Investigation

The primary objective of this study is to identify post-receptor events involved in and possibly mediating the PRL dependent growth of Nb2 cells. Understanding the mechanism of PRL-stimulated mitogenesis in lymphoma cells is of value not only because of a potentially important role of PRL in immunoregulation (107, 108), but because PRL also stimulates this process in other target tissues. In vitro, PRL promotes DNA synthesis in both normal and neoplastic mammary tissue (118). In vivo, biopsies of human breast tumors transplanted into athymic nude mice respond to lactogenic hormones with an increase in DNA synthesis (119) and the growth of several experimental mammary tumors is dependent on PRL (118). A mitogenic role for PRL has also been demonstrated in other tissues such as pigeon crop sac (1) and possibly pancreatic islet cells (120). The highly specific response and exquisite sensitivity of Nb2 cells to lactogenic hormones suggests that these cells may be ideal for studying the mechanism of PRL-induced mitogenesis. Furthermore, because of the multi-functional nature of PRL, examining PRL-dependent post-receptor events in Nb2 cells may provide greater insight into the

mechanism of action of PRL in "extra-mammary" target tissues. The proposed studies are outlined briefly below and are described further in the Discussion section.

Firstly, the functional role of ornithine decarboxylase and polyamines is examined in the Nb2 lymphoma cell line. Polyamines are thought to play a role in PRL-stimulated lactogenesis in the mammary gland (section A.c). Studies by Richards et al (121) have shown that PRL triggers putrescine (1,4-diaminobutane) synthesis in Nb2 cells, suggesting that diamines and polyamines may be required for PRL action in these cells. In this investigation the effect of PRL on ornithine decarboxylase activity in Nb2 cells is examined as is the role of polyamines in growth of PRL-dependent and -independent Nb2 cells. Secondly, the potential role of protein phosphorylation in PRL action is studied. Although phosphorylation has been shown to be a major regulatory mechanism for a number of hormones and growth factors (122), little is known of the role that phosphoproteins play in PRL action. Polyacrylamide gel electrophoresis is used in this study to examine whether PRL promotes or inhibits the incorporation of ^{32}P into specific Nb2 cell proteins. Regulation and subcellular localization of PRL-dependent phosphoproteins is examined. Thirdly, the effects of antibodies to PRL receptors are evaluated using the Nb2 cell line. As detailed in section A.g, cross-linking or aggregation of cell surface hormone receptors may be crucial to hormone action and evidence supporting this concept, already suggested by Shiu et al (115), is investigated further in the present study. Fourthly, the role of phospholi-

pases in PRL-stimulated growth of Nb2 cells is tested. Section A.b discusses the evidence for involvement of these enzymes in PRL-dependent lactogenesis. Since phospholipases have also been implicated in lymphocyte activation (123), proliferation of Nb2 lymphoma cells may depend on PRL-enhanced activity of the enzymes. Fifthly, and lastly, the hypothesis is tested that PRL interaction with Nb2 cells generates a 'signal' which mediates hormone action. In earlier studies I used the methodology of Teyssot et al (84) to determine whether a PRL-generated cell membrane factor could trigger ornithine decarboxylase induction and mitogenesis in Nb2 cells. However, such a factor was not detected. In the present study, the ability of Nb2 cells to retain a PRL-like signal following brief exposure to hormone is examined. As a final note, certain assays used in these studies (eg. stimulation of ornithine decarboxylase or ^{32}P incorporation in Nb2 cells) may be useful in rapid detection of lactogenic hormone activity. Thus, they could offer an advantage over the currently used Nb2 cell growth assay which requires three days (111). Advantages and drawbacks of the new assays are discussed.

MATERIALS AND METHODS

A) Materials

Hormones ; human GH (hGH; 79-7-23H, 2.2 IU/mg, prepared in our laboratory), ovine PRL (oPRL; NIH-P-S12, 35 IU/mg), ovine GH (oGH; NIH-GH-S11, 0.56 IU/mg; NIH-GH-0986C), porcine insulin (Connaught Research Laboratories, Toronto: 24 U/mg).

Antisera : antisera to hormones were prepared in our laboratory by H. Cosby; guinea pig anti-mammary PRL receptor antisera, $F(ab')_2$ and $F(ab')$ fragments were generously provided by Dr. R. Shiu, rabbit anti-guinea pig $F(ab')_2$ antiserum was purchased from Cappel Laboratories (Westchester, PA).

Medium, sera, and antibiotics : culture supplies were obtained from Gibco Canada (Burlington, Ontario); horse serum was purchased from Gibco or from Flow Laboratories (McLean, VA).

Electrophoresis reagents : acrylamide, N,N'-methylene-bis-acrylamide, 2-mercaptoethanol, N,N,N',N'-tetramethylenediamine, ammonium persulfate, sodium dodecylsulfate and bromophenol blue were obtained from Bio-Rad (Richmond, CA); marker proteins were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Enzymes : phospholipase C (C. perfringens and B. cereus), phospholipase A_2 (Naja naja) were purchased from Sigma Chemical Co. (St. Louis, MO); phospholipase D was a gift from Dr. J. N. Kanfer (Univ. of Manitoba); proteinase K (BRL: 20 mA U/mg), deoxyribonuclease (Worthington Biochemical Corp., Freehold, NJ: 2029 U/mg) and ribonuclease A (Sigma: 85 KU/mg) were provided by Dr. R. Matusik.

Miscellaneous Reagents: ^{35}S-methionine (600 -1500 Ci/mmol; New England Nuclear, Boston, MA), alpha-difluoromethyl ornithine (a generous gift from Merrell-Dow, Cincinnati, OH); A23187 (provided by Dr. K. Wrogeman, Univ. of Manitoba); demecolcine (Colcemid; Gibco), L-ornithine, putrescine, spermidine, spermine, cadaverine, chloroquine, N^6,O^2-dibutyryladenosine 3':5'-cyclic monophosphate, N^2,O^2-dibutyrylguanosine 3':5'-cyclic monophosphate, indomethacin, cycloheximide and puromycin (Sigma); other reagents (Fisher Scientific Co., Pittsburgh, PA).

B) Cell Culture

Suspension cultures of Nb2 lymphoma cells were maintained in 75 cm^2 tissue culture flasks (Corning, Corning, NY) or in spinner flasks (one liter, Bellco Glass, Inc.) essentially as described by Gout et al (110). Fischer's medium (for leukemic mouse cells) containing FCS (10%) or 1 ng/ml PRL, HS (10%), 2-mercaptoethanol (10^{-4} M), penicillin (50 U/ml), and streptomycin (50 ug/ml) was used to stimulate cell growth maximally (doubling time approx. 20 hr). Stationary cultures were obtained by removing FCS (or PRL) from the medium. An incubation atmosphere of 5% CO_2 - 95% air (37°C) was used for cell culture.

A cloned Nb2 cell line (Nb2-11C) was developed in our laboratory (H. Cosby) and used in the current investigation. Growth chararacteristics were comparable to those of the original PRL-dependent cell line.

A spontaneously proliferating Nb2 variant (Nb2-SP) arose following frequent passaging of the original cell line. The growth rate of these cells in the absence of PRL was similar to

that of normal Nb2 cells maximally stimulated by the hormone (doubling time approx. 20 hr). Nb2-SP cells had PRL-binding sites (not shown) but did not respond significantly to lactogen concentrations 10-fold greater (ie. 10 ng/ml) than those used in stimulation of PRL-dependent Nb2 cells.

C) Measurement of Cell Growth

Growth of Nb2 cells was quantitated according to the bioassay of Tanaka et al (111) with minor modifications. Growing cells were transferred to 'non-growth' medium (ie. without FCS) for 20 to 24 hr. Cells were collected by centrifugation (500 x g) and resuspended in fresh medium (without FCS) at a concentration of $1 - 2 \times 10^5$ cells/ml. Two ml aliquots were pipetted into 35 mm tissue culture dishes (Falcon Plastics, Los Angeles, CA). Samples to be assayed for growth-promoting activity were added to the dishes in a volume of 0.05 - 0.10 ml. Following a 3-day incubation period, the contents of each dish were added to 8 ml Isoton (Fisher Scientific Co., Pittsburgh, PA) and cell number was determined using a Coulter Counter (Coulter Electronics Inc., Hialeah, FL). Each sample was assayed in triplicate.

D) Ornithine Decarboxylase Assay

ODC activity was determined by measuring the release of $^{14}CO_2$ from L-(1-^{14}C) ornithine (50 - 60 mCi/mmol; New England Nuclear). Cell extracts were prepared by sonication (30 sec) of Nb2 cells in 0.5 ml 5mM Na_2HPO_4 / 5mM NaH_2PO_4, 0.1 mM EDTA, 2 mM dithiothreitol (pH 7.4) on crushed ice. After 30 min centrifugation at 90,000 x g (type 25 rotor, Beckman Instruments Inc.,

Palo Alto, CA) supernatants were mixed with pyridoxal 5'-phosphate and added to polypropylene tubes (17 x 100 mm, Falcon Plastics). L-ornithine (0.55 mCi/mmol) was added to the enzyme / cofactor mixture and tubes were capped with a rubber stopper / plastic well assembly (Kontes Glass Co., Vineland, NJ). Incubations were conducted at 37°C in the presence of 0.22 uCi L-(1-^{14}C) ornithine (0.8 mM ornithine) and 0.26 mM pyridoxal 5'-phosphate in a final volume of 0.5 ml. Production of $^{14}CO_2$ was linear with protein concentration and time (for up to 4 hr). The reaction was terminated by injection of 0.5 ml 50% trichloroacetic acid and $^{14}CO_2$ was collected on glass fibre filters (GF/C, Whatman, Clifton, NJ) located in the plastic wells and saturated with 100 ul hyamine hydroxide (New England Nuclear). Filters were then placed into scintillation vials, 5 ml Aquasol-2 (New England Nuclear) was added, and precipitated ^{14}C was quantitated with a LKB-Rackbeta beta counter. Background radioactivity was determined by incubating the same reaction mixture without cell extract. All samples were assayed in triplicate. Results were expressed as pmol CO_2 /60 min/10^6 cells.

E) Phosphorylation Assay

Incorporation of ^{32}P-orthophosphate (carrier-free, New England Nuclear) by Nb2 cells into acid-insoluble precipitates was measured. Prior to assay, cells were cultured without PRL or human GH for 20 to 24 hr, then transferred to phosphate-free Fischer's medium containing 10% horse serum; (dialysed serum, which inhibited growth of Nb2 cells was not required for adequate incorporation of ^{32}P). Cells (2 x 10^6 /ml) were preincuba-

ted at 37°C in the presence or absence of PRL (plus/minus various agents) for 60 min. ^{32}P (100 - 200 uCi/ml) was then added to the cells for 2 hr (assay volume = 1.0 ml). At the end of the incubation the cell suspension was added to 0.5 ml cold phosphate-buffered saline (pH 7.4). Cells were centrifuged for 15 sec in a microfuge (Beckman). The cell pellet was dissolved in 0.1 ml 2% SDS/ 10% glycerol/ 2.5% 2-mercaptoethanol/ 0.002% bromophenol blue and heated for 5 min at 100°C. Following centrifugation (12,000 x g, 5 min) supernatants were subjected to polyacrylamide gel electrophoresis (see below) or were acid-precipitated to determine the amount of ^{32}P incorporated. Duplicate aliquots of the supernatant were spotted onto GF/C filters (Whatman) and then precipitated and washed extensively in 10% cold TCA. Acid-insoluble ^{32}P was quantitated using a LKB-Rackbeta counter.

F) SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (124a) on a vertical slab gel unit (Bio-Rad) with 0.75 mm-thick gels. Samples prepared as described above were applied (equal dpm per track) to a 4% stacking gel/9% resolving gel and electrophoresed at 20 mA/gel. Gels were fixed in 30% ethanol/7% acetic acid, dried and autoradiographed on Kodak XAR-5 film (Eastman Kodak, Rochester, NY). Molecular weight markers: phosphorylase b (94,000), albumin (68,000), ovalbumin (43,000), trypsin inhibitor (20,100), alpha-lactalbumin (14,400).

In 2-D gel electrophoresis (124b), isoelectric focusing was carried out in the first dimension and SDS-gel electrophor-

esis in the second dimension. Following ^{32}P-labeling, Nb2 cells were dissolved and dissociated with 8 M urea, 5% Triton X-100 and 1% 2-mercaptoethanol (in Tris-HCl, pH 7.0). Samples were layered onto ampholine (pH 3.5 - 10, LKB) containing urea-polyacrylamide gels in 6 mm glass tubes. Isoelectrofocusing was carried out at 600V for 18 hr; pH gradients were determined by cutting gels into 5 mm sections, placing each section into 1 ml 0.1% NaCl (2 hr) then reading the pH using a standard glass combination electrode. Isoelectrofocused gels containing ^{32}P-labeled samples were dialyzed by immersion in 10 ml 2% SDS/ 10% glycerol/ 2.5% 2-mercaptoethanol/ 0.002% bromophenol blue (2 hr). SDS-PAGE in the second dimension was carried out as described above, using 9% polyacrylamide slab gels. Agarose (1%) was used to seal the tube gel in position atop the slab gel. Following electrophoresis and drying, 2-D gels were autoradiographed on XAR-5 film (Kodak).

G) Sucrose Gradients

^{32}P-labeled extracts from control and PRL-stimulated Nb2 cells were analyzed on 10 - 30% sucrose gradients to determine whether PRL-responsive phosphoproteins were associated with ribosomal subunits. According to studies by M. Cobb (Yeshiva University, personal commun.), 40S ribosomal subunits will band near the center of 10 - 30% sucrose gradients following 8 hr centrifugation at 40,000 rpm (SW 41 rotor). Labeled Nb2 cells (10^7) were treated with Triton X-100 (1%) for 10 min with intermittent vortexing (on ice). Polarizing microscopy was used to confirm that cells had lysed. Nuclei were removed by centrifugation at 2200 x g (10 min) and a 400 ul aliquot of the crude extract was incubat-

ed with 100 ul of 1 mM puromycin/ 2.5 M KCl/ 5 mM $MgCl_2$ / 100 mM NaF/ 20 mM Tris-HCl, pH 7.4 for 30 min at 37°C. Mixtures were centrifuged at 10,000 x g for 20 min and aliquots (300 ul) of the supernatant were applied to linear 10 - 30 % sucrose gradients (1 M KCl/ 10 mM $MgCl_2$ / 40 mM Tris-HCl, pH 7.5). Gradients were centrifuged at 39,000 rpm (SW 40 rotor) for 8 hr and fractionated (20 fractions). Prior to SDS-PAGE fractions were first dialyzed against 10 mM Tris-HCl (24 hr) to remove KCl and then vacuum-dried (approx 8 hr) and dissolved in SDS-containing cocktail. SDS-PAGE was performed as described above.

H) Statistics

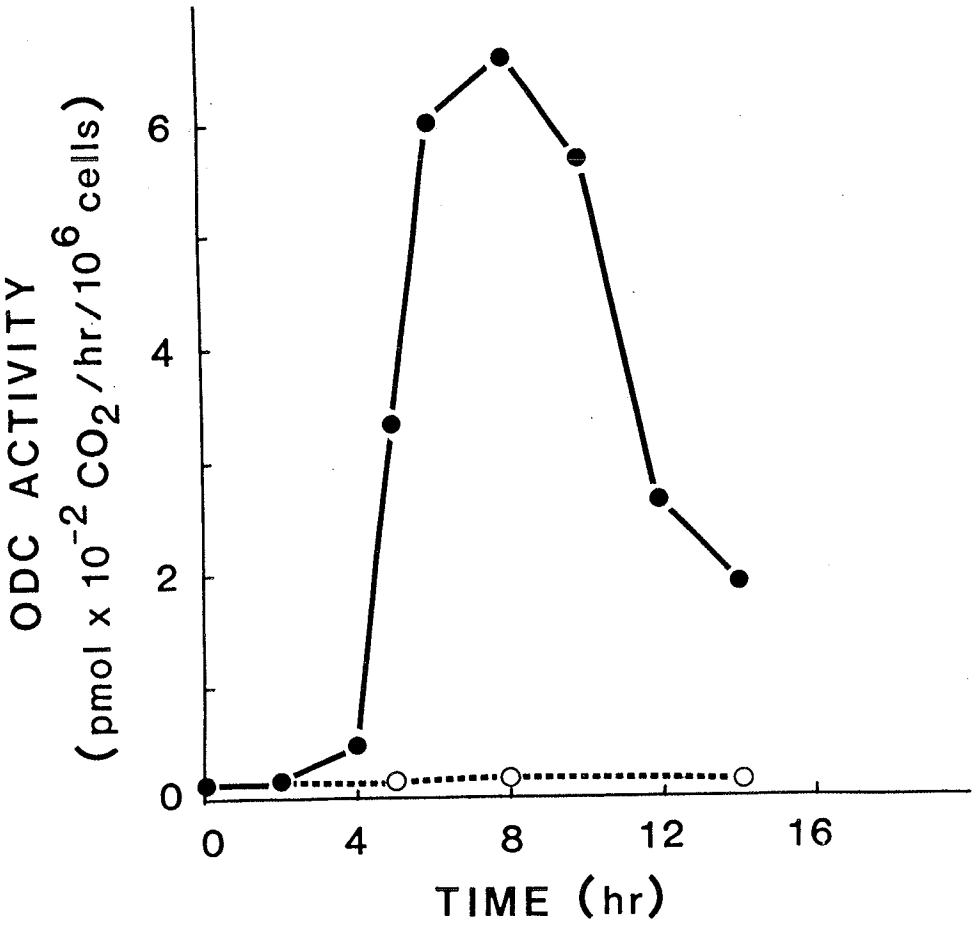
Statistical analysis was performed using the 'Student' t-distribution.

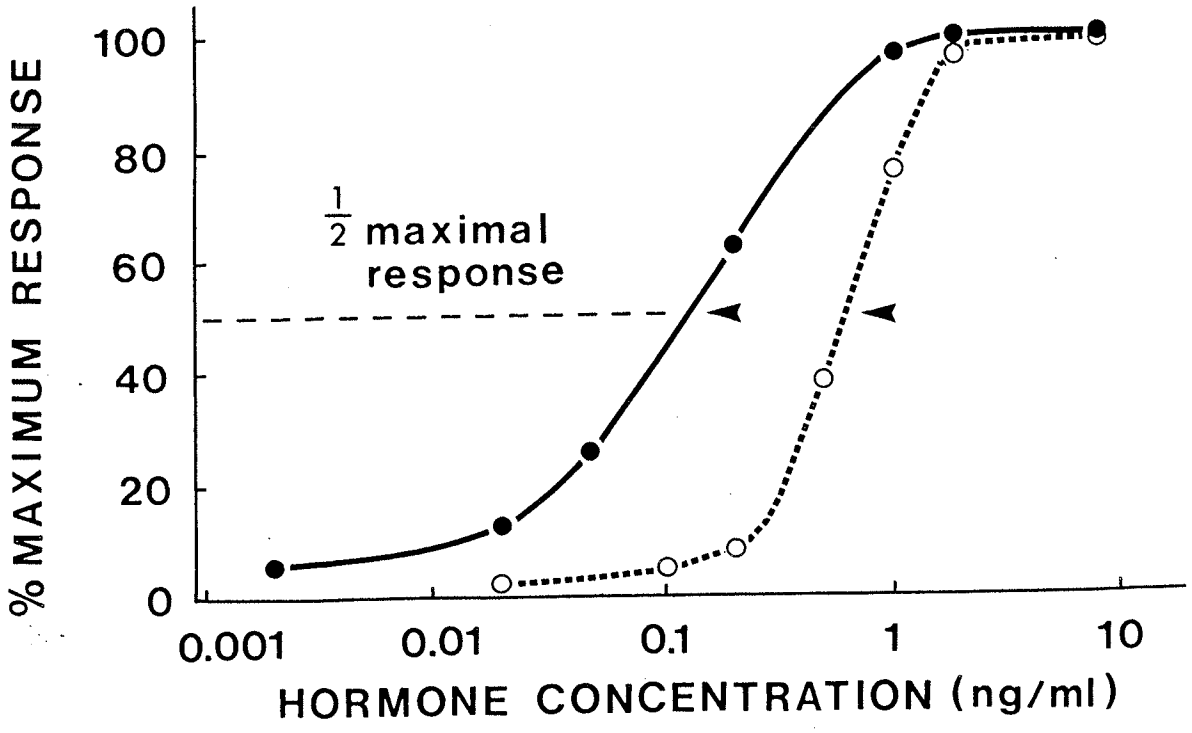
RESULTS

A) Lactogen-dependent stimulation of ornithine decarboxylase

The effect of hGH (a lactogen) on ODC induction, an 'early' mitogenic response in Nb2 cells is shown in figure 1. Enzyme activity which was non-detectable in unstimulated controls increased 20 to 100 fold in the presence of hGH. Ovine PRL had similar effects (not shown). Increased activity was first seen at about 4 hr after addition of hGH and reached a maximum 6 to 8 hr following exposure to the hormone. The peak of lactogen-stimulated ODC activity decreased 8 to 10 hr after hGH addition. In other experiments (not shown), a second peak of ODC activity was found to occur in Nb2 cells 18 to 20 hr following stimulation with hGH. The second peak of enzyme activity was sustained during the logarithmic growth phase of the cells. This biphasic pattern of ODC induction was reported earlier by Richards et al (121).

The magnitude of the lactogen-stimulated increase in ODC activity was dependent on hormone concentration. Figure 2 demonstrates the dose-relationship between lactogen-induced ODC activity and cell growth. Concentrations of hGH as low as 20 pg/ml stimulated proliferation of Nb2 cells, while higher concentrations (100 pg/ml) were required to significantly ($p < 0.01$) stimulate ODC. However, the dose-response curves of cell growth and ODC activity converged such that maximal rate of cell growth occured at about 1 ng/ml hGH and maximal ODC activity occurred at 2 ng/ml hormone. Human GH caused half-maximal increases in ODC activity at a concentration of 600 pg/ml (30 pM) and half-maximal stimulation of growth at 120 pg/ml (6 pM).


ODC ACTIVITY
(pmol x 10^{-2} CO_2/hr/10^6 cells)
0
2
4
6
0
4
8
12
16
TIME (hr)


% MAXIMUM RESPONSE
0
20
40
60
80
100
½ maximal response
0.001
0.01
0.1
1
10
HORMONE CONCENTRATION (ng/ml)

In order to define more clearly the relationship between ODC activity and lactogen-stimulated growth of Nb2 cells a number of agents were tested for their ability to stimulate both ODC and mitogenesis. Consistent with numerous studies that have demonstrated a correlation between enhanced ODC levels and cell growth, Nb2 cell proliferation appeared to be closely linked to increased ODC activity. Lactogenic hormones and anti-PRL receptor antibodies, but not other hormones or pharmacological agents (eg. dibutyryl cyclic nucleotides), were able to stimulate both responses (not shown).

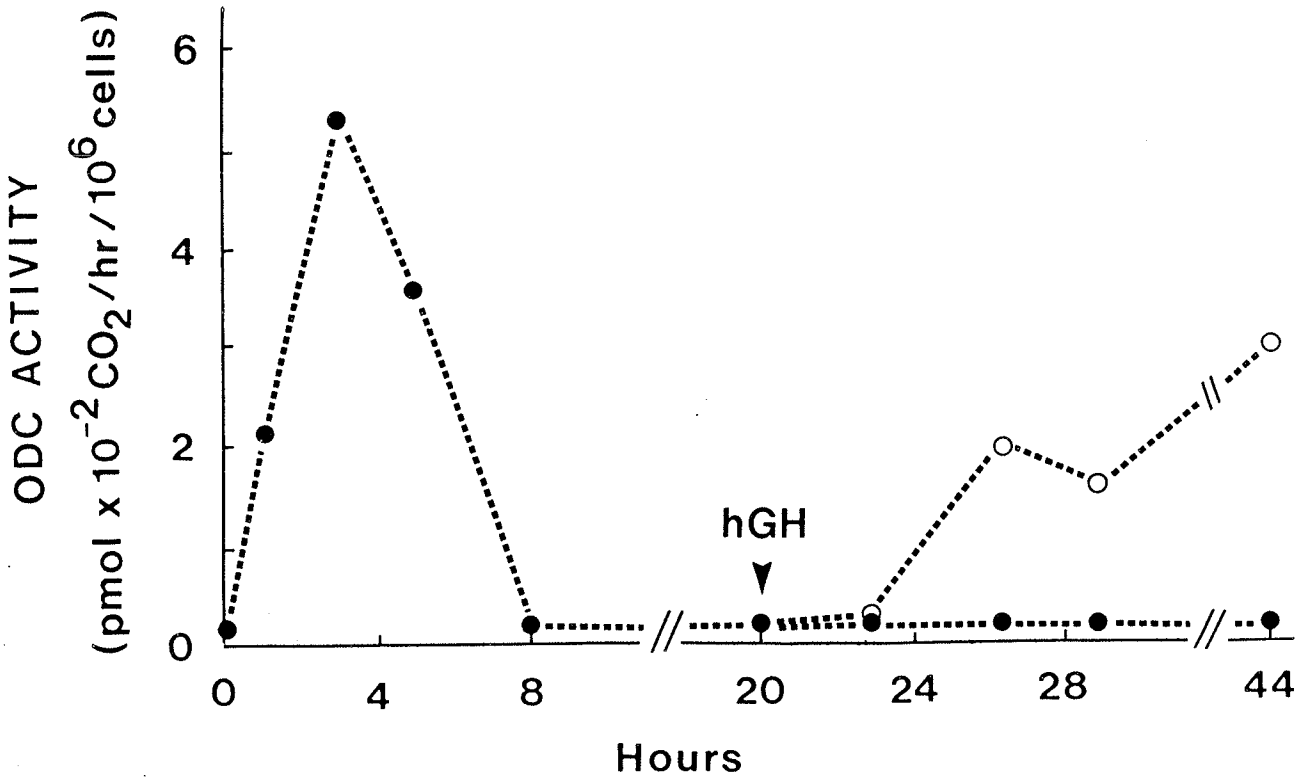
B) Lactogen-independent stimulation of ornithine decarboxylase

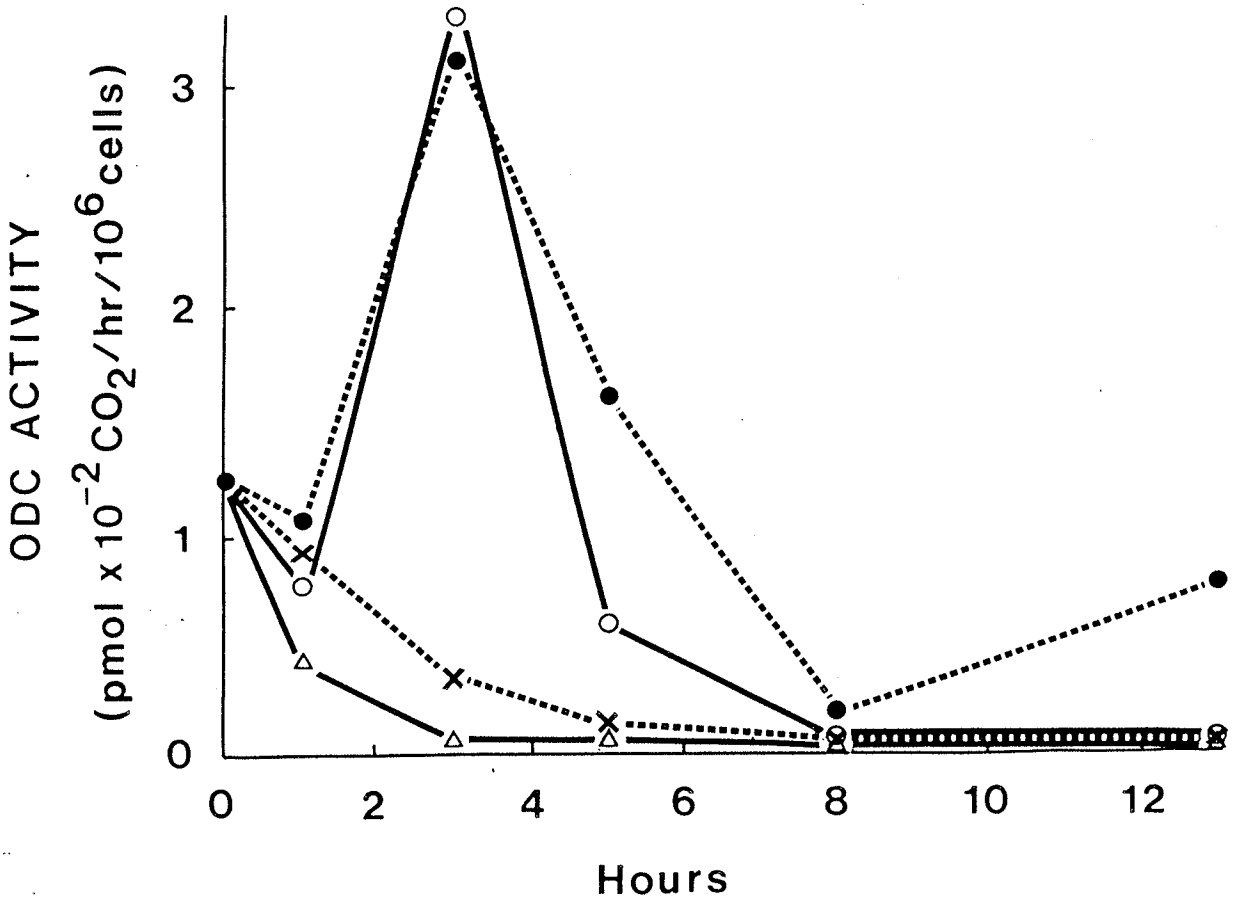
A dissociation between ODC induction and cell growth was demonstrable. Figure 3 shows that ODC activity was low (abscissa: Hours = 0) in Nb2 cells having reached a growth plateau ($1.5 - 2.0 \times 10^6$ cells/ml). Transfer of these cells to medium which was supplemented only with horse serum (and thus unable to sustain growth of Nb2 cells; see ref. 110) caused a marked and rapid increase in ODC activity. Unlike the ODC induction associated with mitogenic stimulation of Nb2 cells, the activation of enzyme activity following transfer of cells to fresh medium was both rapid (latency period less than 2 hr) and transient, with ODC activity returning to near-basal levels within 8 hr of transfer (figure 3 vs. figure 1). Following the peak in ODC activity, enzyme levels in these cells remained low but responded to mitogenic stimulation by hGH with a delayed but prolonged increase in ODC (figure 3).

Figure 4 demonstrates that stimulation of ODC activity by

transfer of high-density cultures to fresh medium was a lactogen-independent event. Fresh medium containing hGH elicited a response of similar magnitude to that of medium without hGH. Furthermore, conditioned medium (obtained from 4-day old cultures of Nb2 cells) containing lactogenic hormone was unable to stimulate ODC. An inhibitor of protein synthesis, cycloheximide (10 uM), blocked lactogen-independent activation of ODC (figure 4); the effect of the drug on lactogen-dependent induction of ODC was the same, and is therefore not shown. De novo synthesis of enzyme may be required for both 'types' of ODC stimulation even though the latency periods which precede the increases in ODC activity differ in length (figure 1/ figure 3 vs. figure 4).

Lactogen-independent induction of ODC may not be strictly related to the concentration of cells following resuspension in fresh medium. Table 1 shows that when Nb2 cultures having reached a growth plateau were resuspended at different cell densities in fresh medium, the magnitude and time-course of ODC induction was similar over a range of 0.6 - 2.4 x 10^6 cells/ml. At 3 hr after transfer (but not at 2 hr) sparse cultures (0.6 x 10^6 cells /ml) had higher ODC levels than did denser cultures (1.2 or 2.4 x 10^6 cells/ml); however the differences were small and not statistically significant. The inability of culture-conditioned medium to effectively increase ODC activity (Table 1) indicates that lactogen-independent induction of ODC was not merely due to mechanical agitation (ie. centrifugation, resuspension) of Nb2 cells, but rather was stimulated by component(s) present in fresh medium which contained serum.


ODC ACTIVITY
(pmol x 10^{-2} CO$_2$/hr/10^6 cells)
6
4
2
0
hGH
0
4
8
20
24
28
44
Hours


ODC ACTIVITY
(pmol x 10^{-2} CO$_2$/hr/10^6 cells)
3
2
1
0
0
2
4
6
8
10
12
Hours

	Density of Nb2 Cell Cultures ($\times 10^{-6}$ cells/ml)			Time (hr)
	0.6	1.2	2.4	
Control	—	0.15	—	0
Conditioned Medium	—	0.18	—	2
	—	0.20	—	3
Fresh Medium	0.72	0.78	0.75	2
	1.38	1.26	1.13	3

Table 1. Lactogen-independent stimulation of ODC in Nb2 cells: effect of cell density on magnitude of response. Nb2 cells from dense cultures (1.4×10^6 cells/ml) were transferred to conditioned Fischer's medium (from 3-day old cultures) or to fresh medium at different cell densities. The times shown indicate the number of hrs after transfer of cells. Medium-stimulated increases in ODC activity are expressed as pmol ($\times 10^{-2}$) CO_2 produced/hr/10^6 cells.

C) Role of ornithine decarboxylase and polyamines in growth

Difluoromethyl ornithine (DFMO), an irreversible inhibitor of ODC was used to determine the importance of ODC in PRL-stimulated cell growth of Nb2 cells. Figure 5 demonstrates that growth of Nb2 cells was inhibited by DFMO with decreased rates of growth being apparent by day 3 of culture. The DFMO (0.2 - 5 mM) caused about 60% inhibition of growth by the fourth day. On the other hand, figure 6 shows that 0.2 mM DFMO inhibited lactogen-dependent induction of ODC by nearly 80% and 1 mM DFMO caused greater than 90% inhibition. Thus, growth of Nb2 cells was more resistant to DFMO at higher concentrations than the effect of the drug on ODC activity would have predicted.

That the effect of DFMO on PRL-stimulated mitogenesis was due specifically to inhibition of ODC is shown in figure 7. Addition of the enzyme product putrescine to DFMO-inhibited cells completely restored normal growth rate, indicating that even high concentrations of DFMO were not toxic to Nb2 cells. Figure 8 demonstrates that 1 uM putrescine was sufficient to reverse the inhibition by DFMO on cell growth while as little as 10 nM putrescine elicited a detectable response. The polyamines spermidine and spermine also restored growth of DFMO-inhibited Nb2 cells and were significantly ($p<0.001$) more potent than putrescine at concentrations below 1 uM (figure 8); half-maximal restoration by both polyamines was 0.16 uM as compared to 0.25 uM for the diamine putrescine. Another diamine, cadaverine (product of lysine decarboxylation), was relatively ineffective in promoting growth of Nb2 cells inhibited by DFMO. Even at 10 uM, cadaverine was only

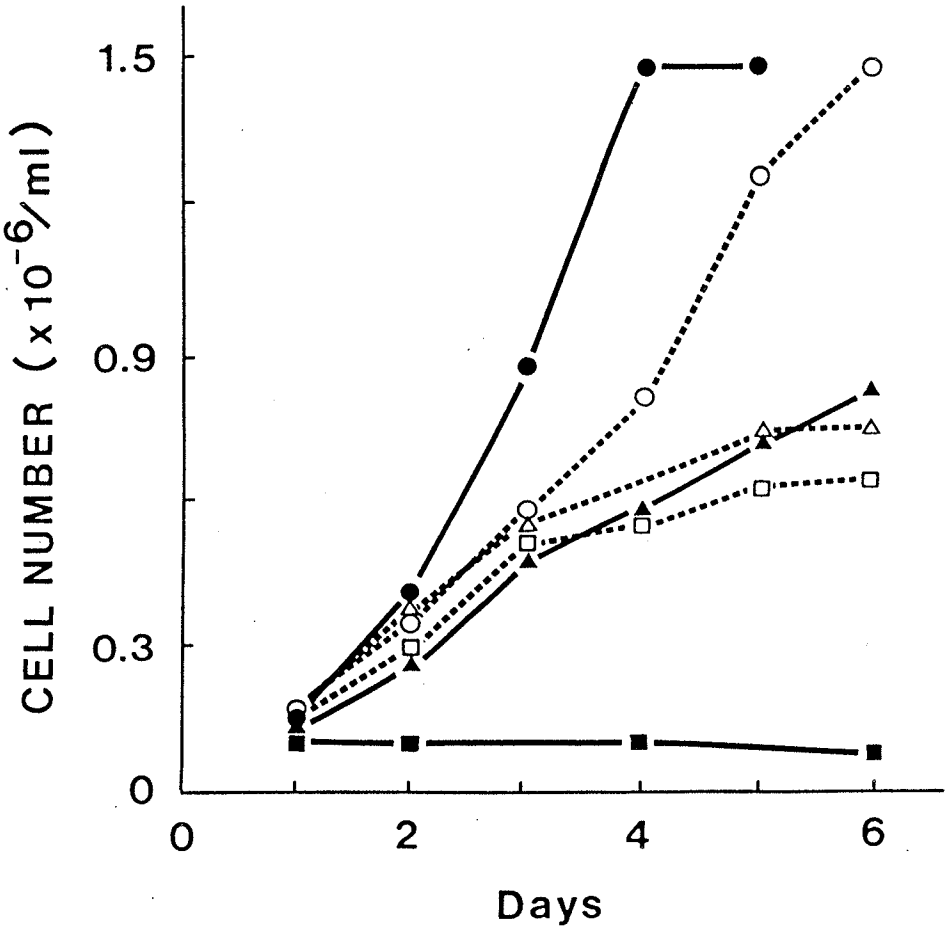
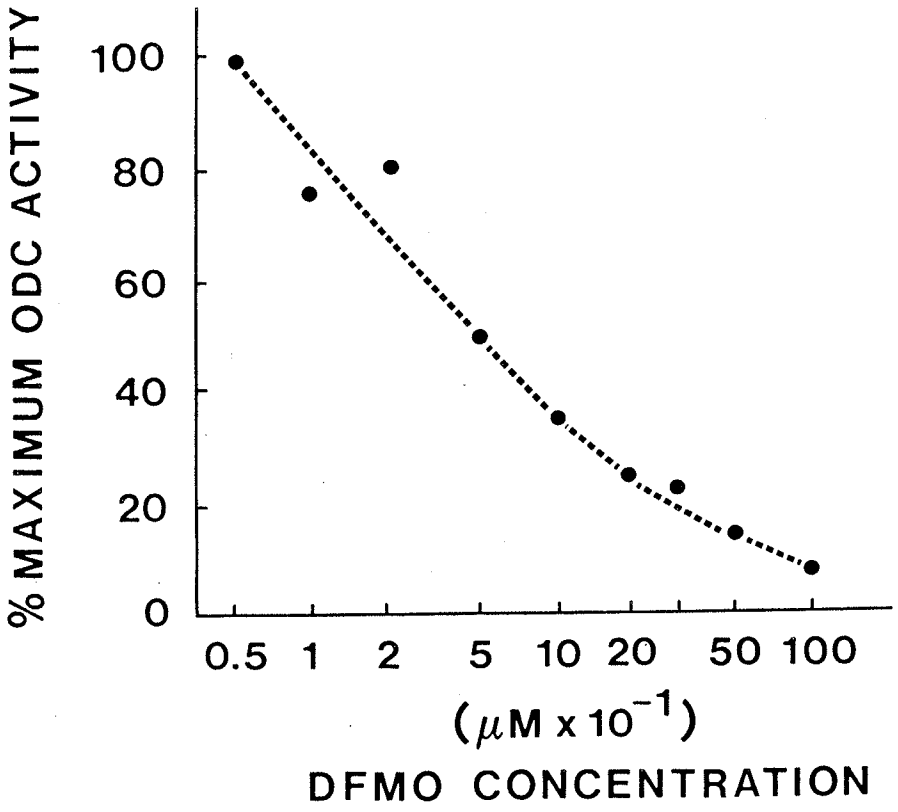
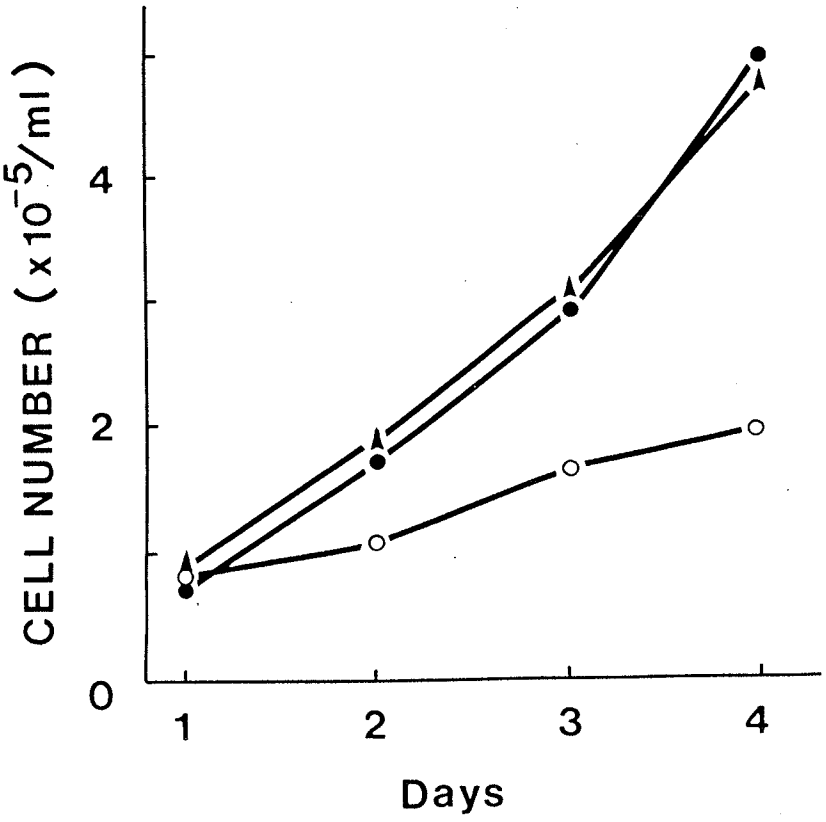
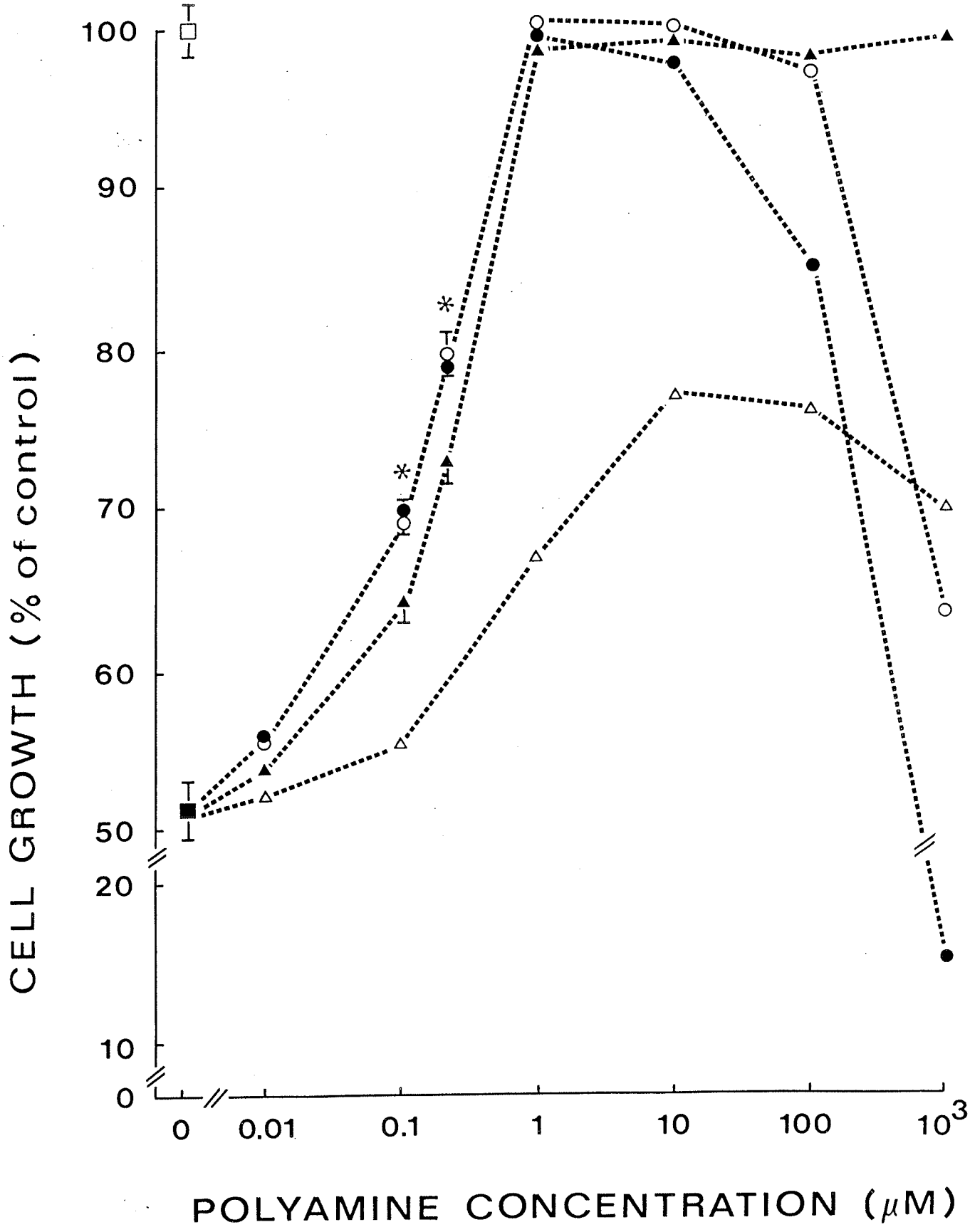


Figure 5. Effect of DFMO on proliferation of Nb2 cells. DFMO was added to 75 cm^2 flasks of Nb2 cell cultures stimulated to grow by hGH (2 ng/ml). Cell number was determined daily by counting (Coulter) one ml aliquots from each flask. Control, no DFMO (closed circles); 50 uM DFMO (open circles); 200 uM DFMO (closed triangles); 1 mM DFMO (open triangles); 5 mM DFMO (open squares); no DFMO, no hGH (closed squares).


% MAXIMUM ODC ACTIVITY
100
80
60
40
20
0
0.5
1
2
5
10
20
50
100
(μM x 10⁻¹)
DFMO CONCENTRATION


CELL NUMBER (x10⁻⁵/ml)
4
2
0
1
2
3
4
Days

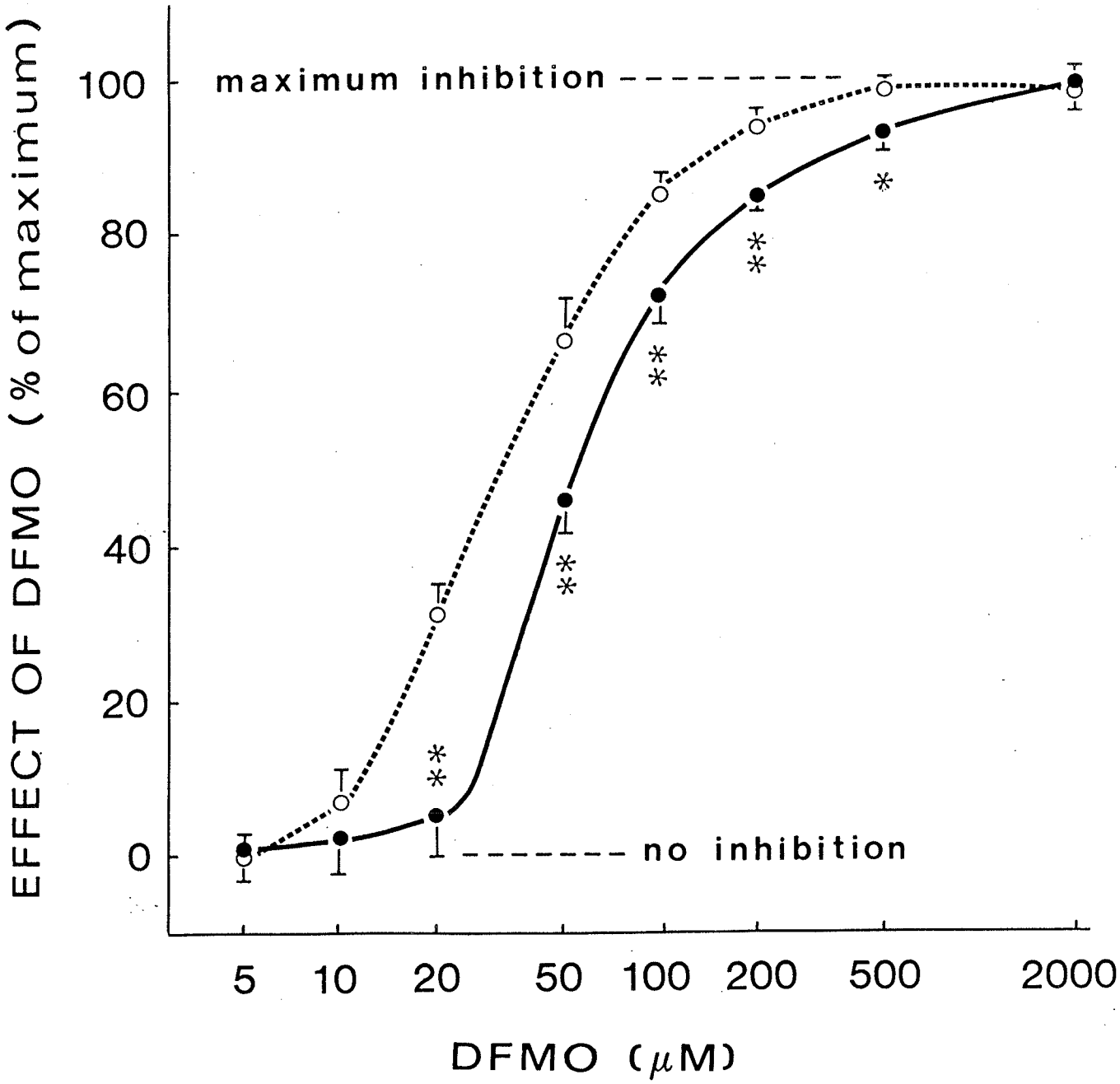

CELL GROWTH (% of control)
100
90
80
70
60
50
20
10
0
0
0.01
0.1
1
10
100
10^3
POLYAMINE CONCENTRATION (μM)
*
*

about 50% as potent as putrescine, spermidine, or spermine at 1 uM. Higher concentrations of cadaverine inhibited growth. Putrescine, spermidine, and spermine varied greatly in their effects on Nb2 cell growth at higher concentrations. Putrescine (1 mM) did not interfere with growth at all while spermidine (1 mM) reduced maximal cell growth by almost 40%. Spermine was the most toxic to Nb2 cells at 1 mM, completely preventing cell proliferation.

The effect of DFMO on the growth of an Nb2 variant (Nb2-SP), which proliferates in the absence of lactogenic hormones, was examined and compared to the effect of the drug on PRL-dependent cells. Figure 9 shows that PRL-dependent cells were more sensitive to DFMO than were their spontaneously proliferating counterparts. While Nb2-SP cells were almost unaffected by 20 uM DFMO, growth of PRL-dependent cells was inhibited 30% at this concentration. Half-maximal suppression of Nb2 and Nb2-SP cell growth occurred at 31 uM and 56 uM DFMO, respectively.

D) Lactogen-stimulated uptake and incorporation of phosphate

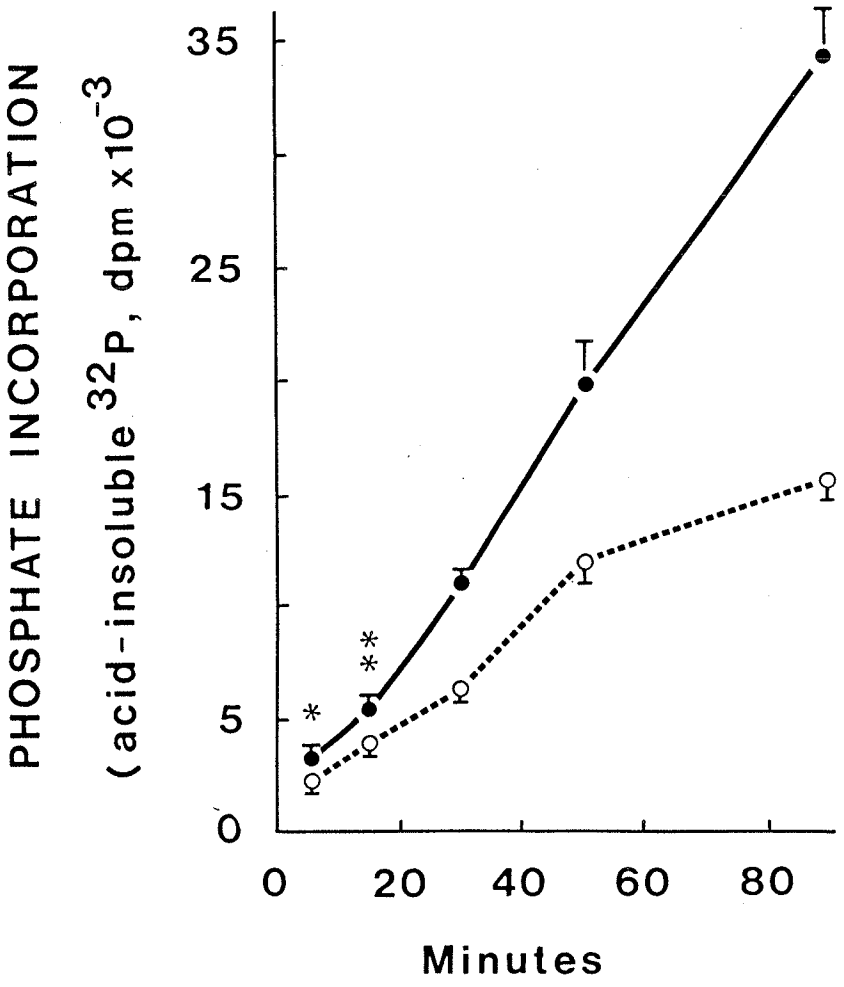
When ^{32}P-orthophosphate was added to Nb2 cells that had been preincubated with hGH (10 ng/ml) for 60 min, the rate of phosphate transport into cells and/or incorporation of phosphate into TCA-insoluble biomolecules was markedly enhanced (figure 10). Significant increases above the basal rate of uptake/incorporation were detectable less than 20 min after addition of ^{32}P (80 min after stimulation with lactogenic hormone). After 90 min of incubation with ^{32}P, cells in the presence of hGH had incorporated approximately twice the amount of phosphate of unstimulated cells.


EFFECT OF DFMO (% of maximum)
100
80
60
40
20
0
maximum inhibition
no inhibition
5
10
20
50
100
200
500
2000
DFMO (μM)

Sonicates of PRL-stimulated, ^{32}P-labeled Nb2 cells were digested with various enzymes in order to characterize the phosphorylated acid-insoluble precipitates. Table 2 shows that the proteolytic enzyme proteinase K was most effective in degrading ^{32}P-labeled TCA-precipitable material. Treatment with RNAse or phospholipase C also caused a decrease in the amount of acid-insoluble ^{32}P while incubation with DNAse had little effect.

The results of Table 2 provide only an indication of the biochemical nature of the ^{32}P-labeled precipitates. Use of high enzyme concentrations to facilitate complete digestion magnified the problem of contamination in the commercial grade enzyme preparations. Thus it is likely that the numerical estimates presented in Table 2 are too high. As summation of the degradative effects of the four enzymes exceeds 100% (total TCA-insoluble dpm ^{32}P), this indeed appears to be the case. RNAse, DNAse and phospholipase C were used as provided by the manufacturer. However, proteinase K was 'purified' prior to use by preincubating the enzyme -- allowing time for proteolytic inactivation of other enzymes which may contaminate the commercial protease preparation. Although "72% degradation by proteinase K" (Table 2) may include some degradation of non-protein ^{32}P-containing molecules, most of the effect of the protease preparation would appear to be specific for proteins. This view was supported by SDS-PAGE analysis of proteinase K-digested extracts of ^{32}P-labeled cells (not shown). A dramatic increase in low molecular weight radioactivity was seen, with a concomitant disappearance of higher molecular weight radioactive bands.

Because the marked effect of PRL on phosphate uptake/incor-

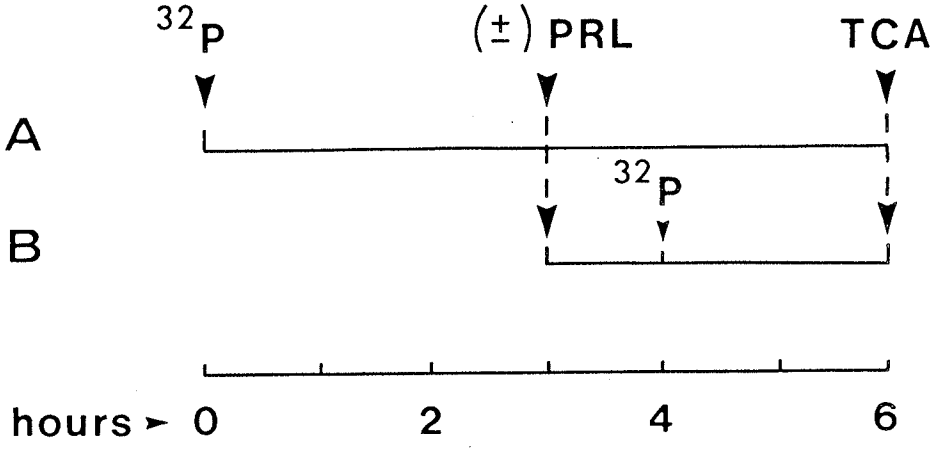


Enzyme	% Decrease in Acid-insol. ^{32}P
none	0
Proteinase K	72
RNase	36
Phospholipase C	26
DNase	8

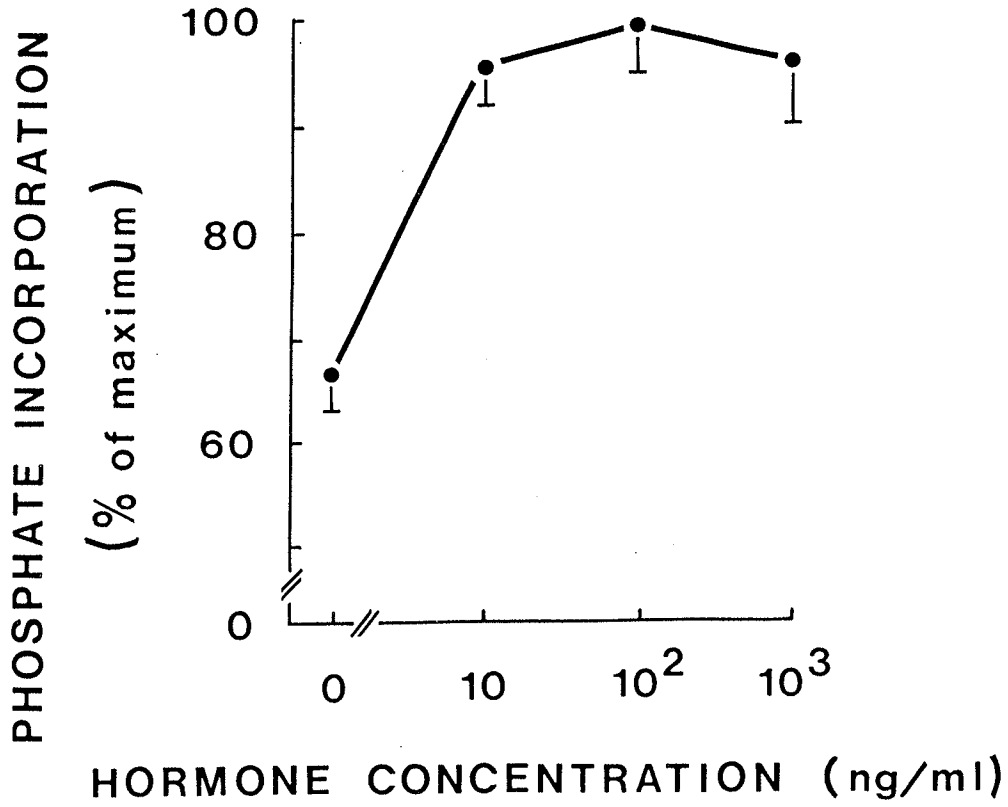
poration was observed in cells not preincubated with ^{32}P (figure 10), it was necessary to establish whether PRL acted primarily on phosphate transport or on post-transport events (egs. stimulation of kinase activity, enhancement of substrate synthesis). In the absence of PRL, incorporation of ^{32}P by stationary Nb2 cells reached a plateau after a 3 to 4 hr incubation (not shown). Addition of PRL at this time caused a 38% increase in ^{32}P incorporation over a 3 hr period (Table 3A). The magnitude of this stimulatory effect, however, was much lower than that observed in cells first preincubated with hormone, then labeled with ^{32}P (Table 3B, figure 10). The result suggests that PRL stimulates both uptake and incorporation of phosphate by Nb2 cells but that the effect on uptake is the major one.

Figure 11 shows the effect of PRL concentration on ^{32}P incorporation by Nb2 cells preincubated with isotope. Stimulation by the hormone was essentially maximal at 10 ng oPRL/ml. In cells which were not preincubated with ^{32}P, this concentration of PRL triggered only a half-maximal increase in phosphate uptake and incorporation (figure 12). A hormone concentration of more than 100 ng/ml was required for maximal stimulation. PRL-dependent increases in phosphate incorporation may thus be more sensitive to hormone concentration than PRL-dependent stimulation of phosphate uptake .

As phosphorylation reactions in many cells are regulated by a variety of hormones, the effect of several hormones on phosphate uptake and incorporation by Nb2 cells was examined. Estrogen, which either directly or indirectly (by its effect on serum PRL levels) triggered development of the Nb2 node lymphoma

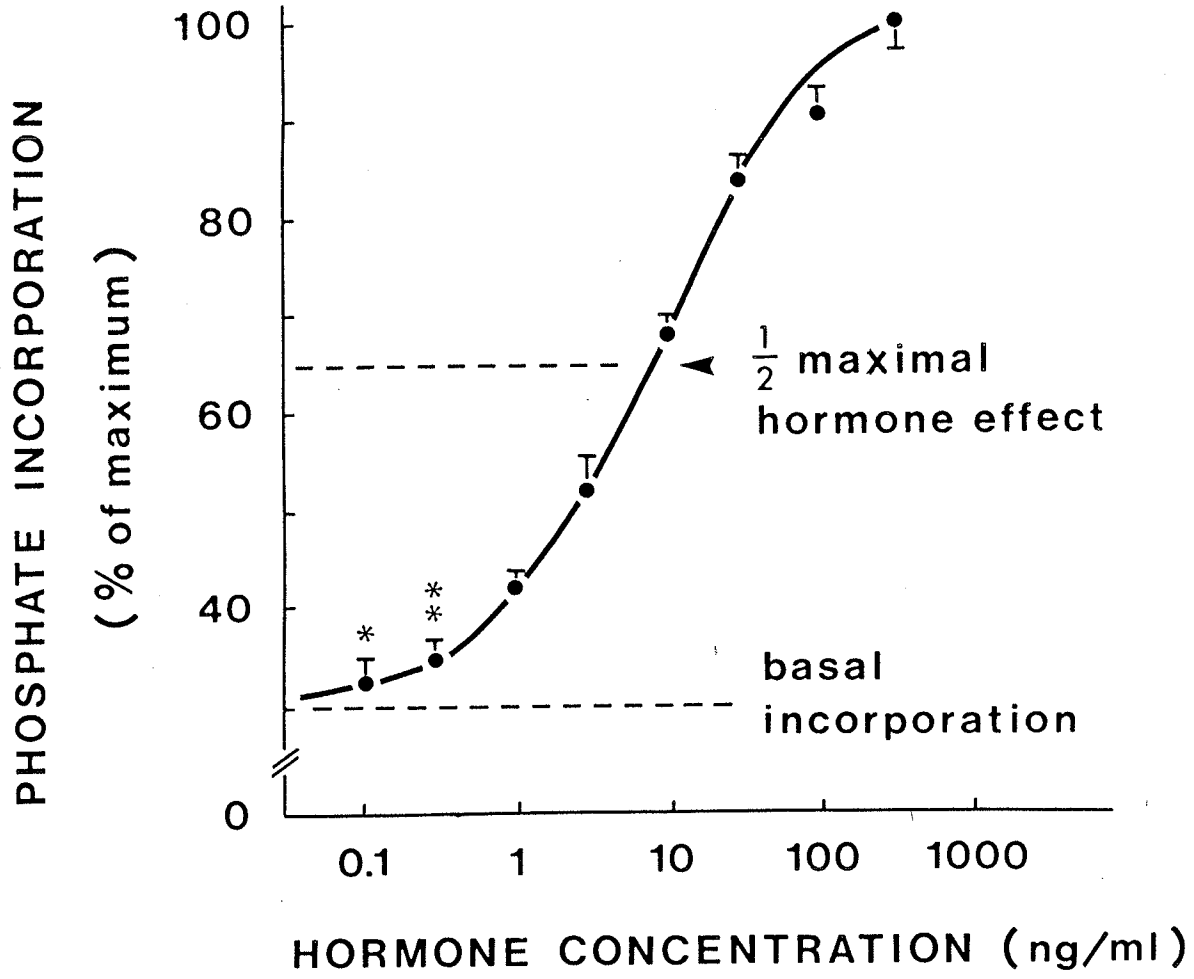


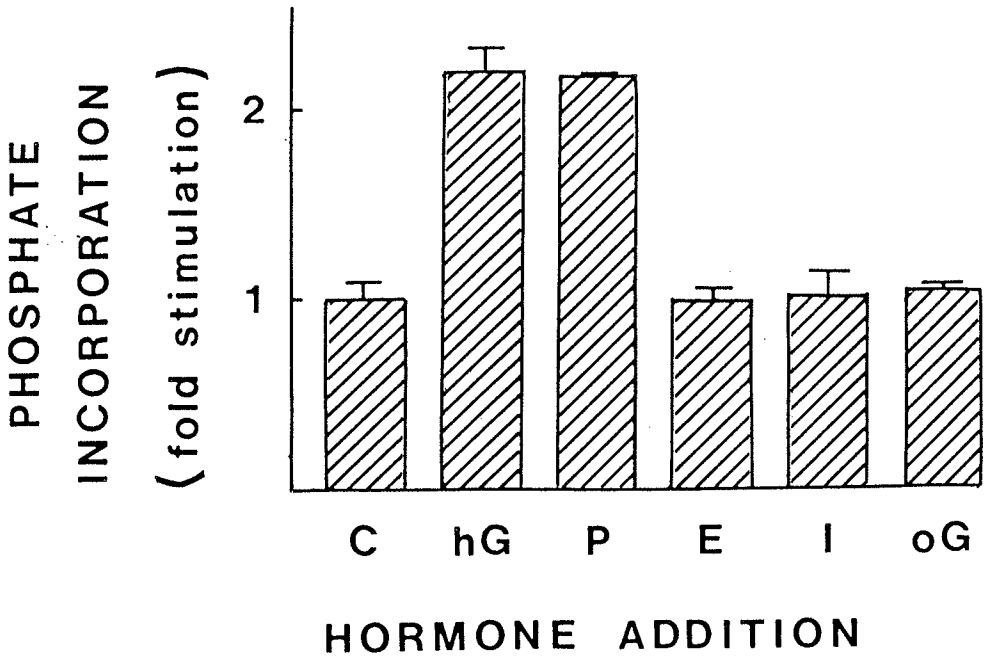
	-PRL	+PRL	% INCREASE
A	13341	18352	38
B	6349	16060	153

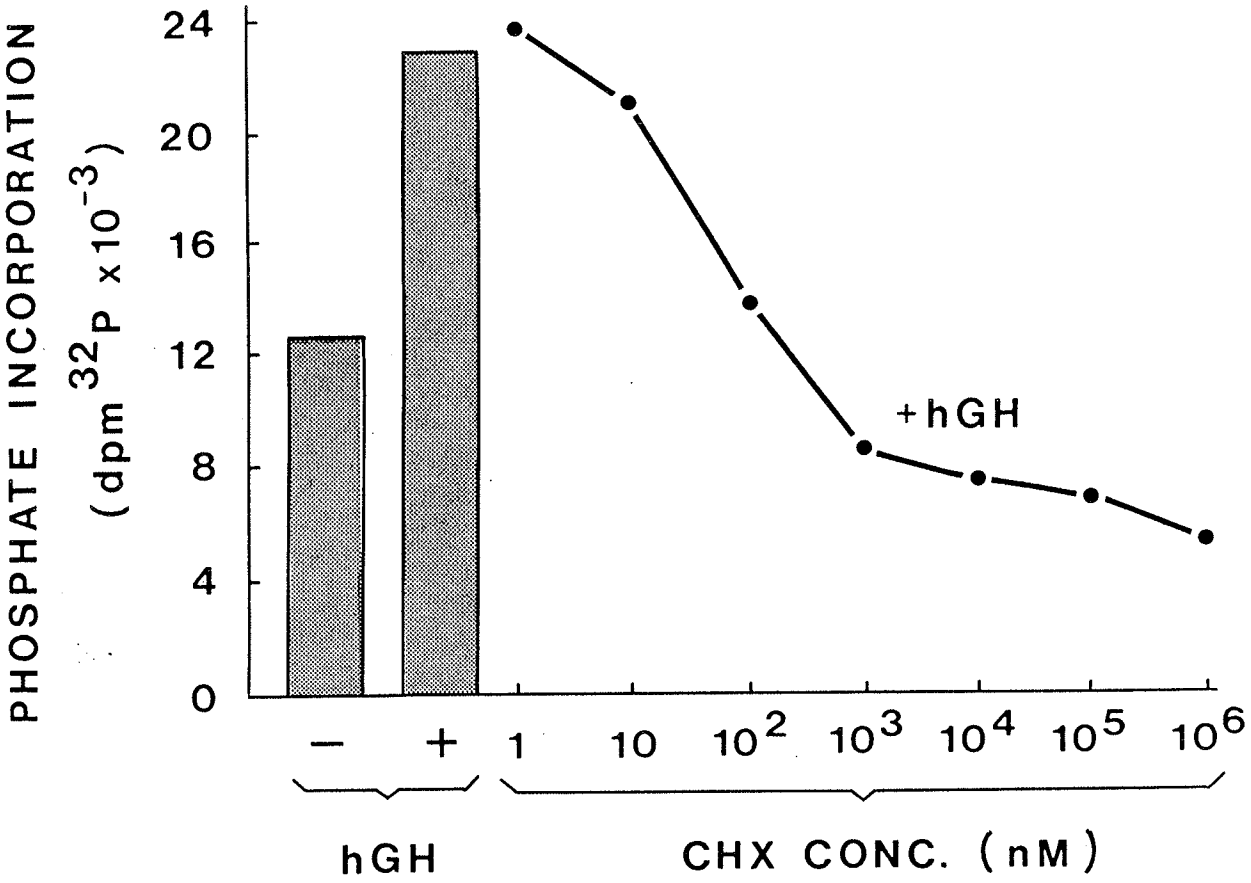


(109), insulin, which regulates a plethora of phosphorylation/dephosphorylation reactions in many cell types, and ovine GH, which is structurally related to both hGH and oPRL but is not lactogenic, were tested at both physiological (not shown) and pharmacological (figure 13) concentrations. No significant changes in phosphate uptake and incorporation occurred in the presence of these non-lactogenic hormones. Furthermore, one-dimensional SDS-PAGE did not reveal qualitative changes in the phosphorylation of Nb2 cell phosphoproteins following exposure of cells to estradiol, insulin or oGH (not shown).

To clarify the mechanism of lactogen-stimulated transport and incorporation of phosphate by Nb2 cells, the effects of several pharmacological agents were examined. Figure 14 demonstrates that cycloheximide, an inhibitor of protein synthesis, prevented hGH-stimulated uptake and incorporation of phosphate. At 10 nM cycloheximide a 20% decrease in the hormone effect was seen; greater than 90% inhibition occurred in the presence of 100 nM cycloheximide. Higher concentrations of the drug decreased the uptake and incorporation of phosphate below that measured in unstimulated controls, but even 1 mM cycloheximide did not block uptake and incorporation entirely (figure 14). Dibutyryl cAMP at 10 uM did not affect either PRL-stimulated or basal uptake and incorporation of phosphate; at higher concentrations of dbcAMP a decrease in ^{32}P incorporation was seen in the presence or absence of hormone (Table 4) suggesting that inhibition was non-specific. Unlike dbcAMP, dbcGMP had little effect on phosphate uptake or incorporation over a 100-fold range of concentration. While dbcGMP did not significantly


PHOSPHATE INCORPORATION
(% of maximum)
100
80
60
40
0
*
**
½ maximal
hormone effect
basal
incorporation
0.1
1
10
100
1000
HORMONE CONCENTRATION (ng/ml)


PHOSPHATE
INCORPORATION
(fold stimulation)
2
1
C
hG
P
E
I
oG
HORMONE ADDITION



Agent	Conc.	^{32}P-orthophosphate Incorporation (% of control ± SEM) Unstimulated	Stimulated
dbcAMP	10 uM	90.0 ± 2.6	96.8 ± 6.9
	1 mM	47.5 ± 2.4(c)	44.9 ± 1.6(c)
dbcGMP	1 uM	107.1 ± 4.5	108.4 ± 5.0
	10 uM	111.2 ± 3.2(a)	102.1 ± 2.3
	100 uM	106.0 ± 4.0	100.6 ± 0.6
	1 mM	104.0 ± 2.6	97.0 ± 3.2
A23187	150 nM	85.2 ± 1.6(b)	86.9 ± 5.9
	1500 nM	81.1 ± 0.5(b)	66.1 ± 1.3(c)

alter incorporation of ^{32}P in lactogen-stimulated cells, it caused a small (11%) but significant increase (at 10 uM) in phosphate incorporation by stationary cells (Table 4). At a concentration of 0.15 uM the calcium ionophore A23187, like dbcAMP, inhibited both basal and PRL-stimulated uptake and incorporation of phosphate by Nb2 cells (13 to 15% decrease). When present in the medium at a 10X greater concentration, however, A23187 caused only a 19% decrease in basal ^{32}P incorporation but inhibited PRL-stimulated incorporation of ^{32}P by 34% (Table 4). This effect suggests that the ionophore may specifically inhibit the action of PRL.

E) SDS-PAGE of Nb2 cell phosphoproteins: regulation by PRL

In an attempt to identify very early actions of PRL on a target tissue, Nb2 cells which had been pre-labeled with ^{32}P-orthophosphate (2 to 3 hr) were stimulated briefly (30 sec to 30 min) with oPRL or hGH. Although no increase in TCA-precipitable ^{32}P was measurable within these time periods (unlike ^{32}P incorporation by cells preincubated with PRL, figure 10), SDS extracts of the cells were electrophoresed on polyacrylamide gels (7 to 15%) and autoradiograms were examined for PRL-induced alterations in protein phosphorylation. However, no consistent changes were detectable within a molecular weight range of 10,000 to 200,000 (not shown).

PRL-induced changes in protein phosphorylation were demonstrable in Nb2 cells after somewhat longer exposure to hormone, particularly when cells were stimulated with PRL prior to ^{32}P-labeling. Figure 15 compares PRL-stimulated phosphorylation

in cells treated first with PRL and then labeled with ^{32}P (Tracks A and B) to phosphorylation in cells 'equilibrated' with ^{32}P and then stimulated with PRL (Tracks C and D). As seen on one-dimensional gels, two proteins of relatively low molecular weight became phosphorylated in the presence of oPRL (10 ng/ml) -- the first had a molecular weight of 33,000 (pp33), migrating slightly above the carbonic anhydrase marker; the second had a molecular weight of 19,000 (pp19), migrating just below trypsin inhibitor. Enhanced phosphorylation of pp19 was evident in cells that were preincubated with ^{32}P (Track D) as well as in cells that were not (Track B). On the other hand, phosphorylation of pp33 was apparent only in cells that were first incubated with PRL and subsequently labeled with ^{32}P.

Migration of pp33 and pp19 during SDS-PAGE was examined under reducing and non-reducing conditions. Figure 16 shows that while pp33 was visible under reducing conditions (Track B) omission of 2-mercaptoethanol during solubilization of cells resulted in disappearance of the band (Track D). Under non-reducing conditions, migration of pp19 was unaffected (Tracks C and D).

Subcellular localization of pp33 and pp19 is shown in figure 16 (Tracks E - H, reducing conditions). Phosphoprotein 33 was present in the 10,000 x g pellet of PRL-stimulated cells (Track F) but a PRL-enhanced ^{32}P-containing band also appeared in the 10,000 x g supernatant at (or near) MW 33,000 (Track H). Incomplete sedimentation of membrane fragments may account for the presence of pp33 in both subcellular fractions. Phosphoprotein 19 was visible only in the 10,000 x g supernatant (Tracks G and H), suggesting that it is a cytosolic protein. As in figure 15,

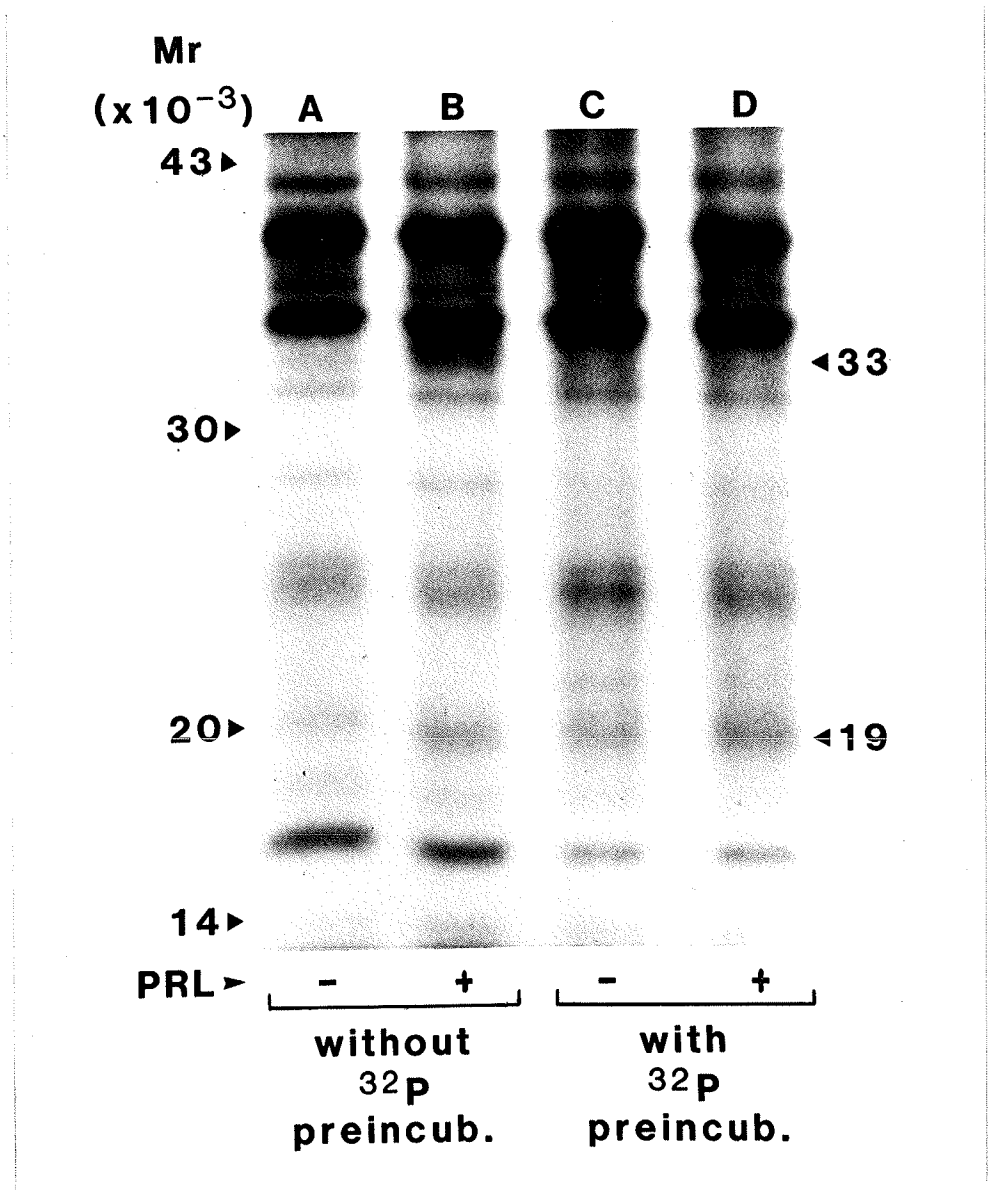


Figure 15. SDS-PAGE of ^{32}P-labeled Nb2 cell proteins: different effects of PRL on cells preincubated and cells not preincubated with ^{32}P. Suspensions of stationary Nb2 cells were stimulated with oPRL (10 ng/ml) and labeled with ^{32}P as described in the legend to Table 3 (diagram). Tracks A and B represent Method B; C and D -- Method A (p. 55). Cells were dissolved in 2% SDS/10% glycerol/2.5% 2-mercaptoethanol. Heat-treated (100°C) extracts were electrophoresed on 9% polyacrylamide gels and autoradiographed on X-ray film.

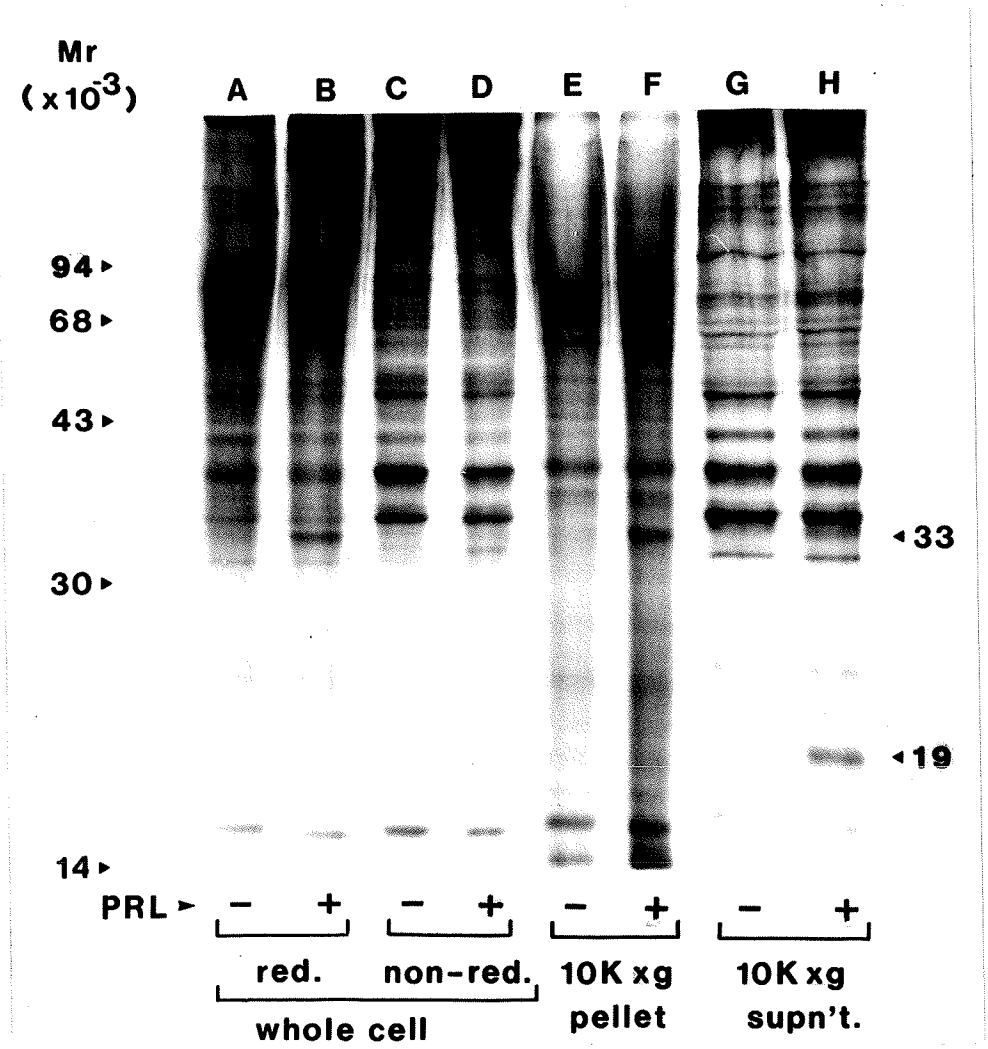


Figure 16. SDS-PAGE of ^{32}P-labeled Nb2 cell proteins: a) subcellular localization of PRL-sensitive phosphoproteins; b) effect of non-reducing conditions on PRL-sensitive phosphoproteins. Stationary Nb2 cells were preincubated with oPRL (10 ng/ml) 60 min and labeled with ^{32}P for 2 hr. Cells were then treated in one of the following ways: a) dissolved in 2% SDS/10% glycerol/2.5% 2-mercaptoethanol; b) dissolved in 2% SDS/10% glycerol; c) sonicated 30 sec in PBS, centrifuged at 10,000 x g (5 min), mixed (supernatant) or dissolved (pellet) in cocktail to yield 2% SDS/10% glycerol/2.5% 2-mercaptoethanol (final concentration). Solubilized extracts were heat-treated and analyzed on 9% gels. Only tracks C and D represent non-reduced conditions.

pp19 was phosphorylated in the absence of PRL (figure 16, Track G) but appeared to contain more ^{32}P in the presence of hormone (Track H). Phosphoprotein 33, on the other hand, appeared not to be phosphorylated unless PRL was present (Track E vs F).

In order to determine the mechanism involved in PRL-stimulated phosphorylation of pp33 and pp19, the effect of PRL on synthesis of 33K and 19K dalton proteins was examined. Cells preincubated with PRL for 60 min and incubated with ^{35}S-methionine for 2 hr exhibited enhanced incorporation of label into acid-insoluble material (not shown). The stimulatory effect of PRL was concentration-dependent; a 20% increase in ^{35}S-methionine incorporation was seen at a PRL concentration of 10 ng/ml and a 31% increase occurred at 100 ng/ml. Figure 17 shows SDS-PAGE of total Nb2 cell proteins labeled with ^{35}S-methionine in the absence or presence of oPRL. Prolactin promoted synthesis of a number of proteins (*) of different molecular weight: 25K, 27K, 30K, 45K, 74K and 94K. Prolactin also enhanced the synthesis of proteins having molecular weights 33K and 19K, suggesting that the apparent stimulation of pp33 and pp19 phosphorylation by PRL may be due, either in part or solely, to an increase in substrate synthesis.

Phosphoproteins from Nb2 cells were examined by two-dimensional gel electophoresis (dimension one: isoelectric focusing, dimension two: SDS-PAGE) to characterize pp33 and pp19 more completely. The autoradiograms shown in figure 18A and 18B exposure to PRL. Figure 23 demonstrates the changes in phosphate uptake and incorporation that occur at early and late stages of the PRL-stimulated mitogenic response. Cells from PRL-stimulated

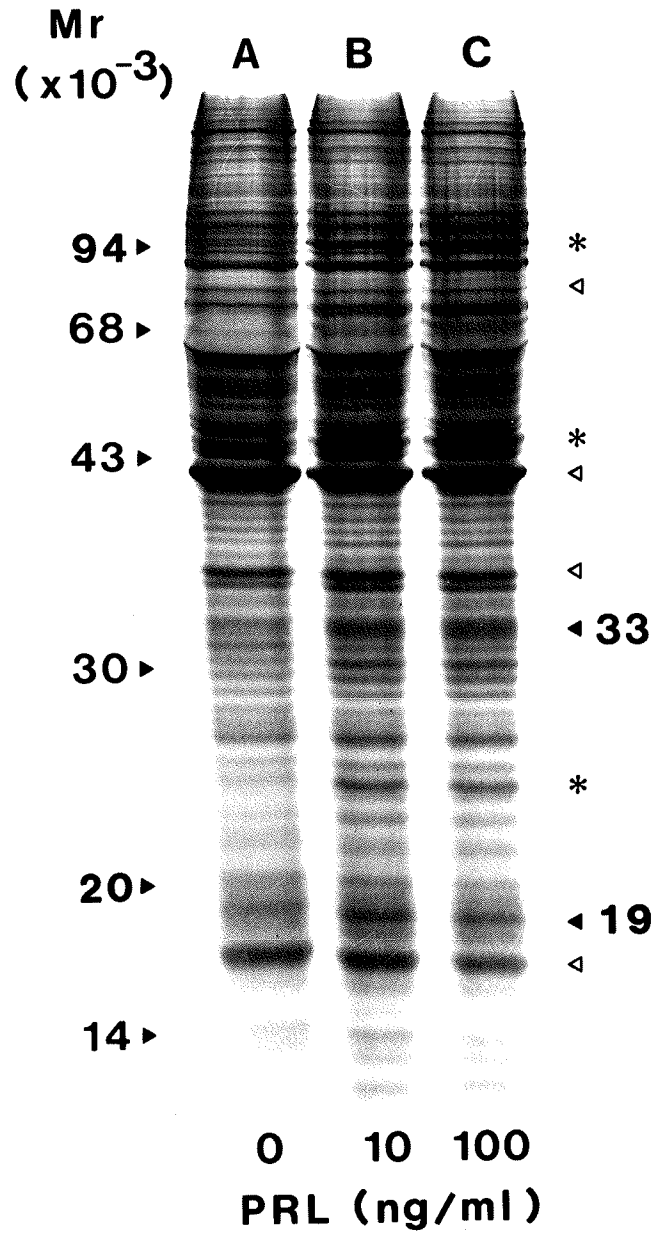
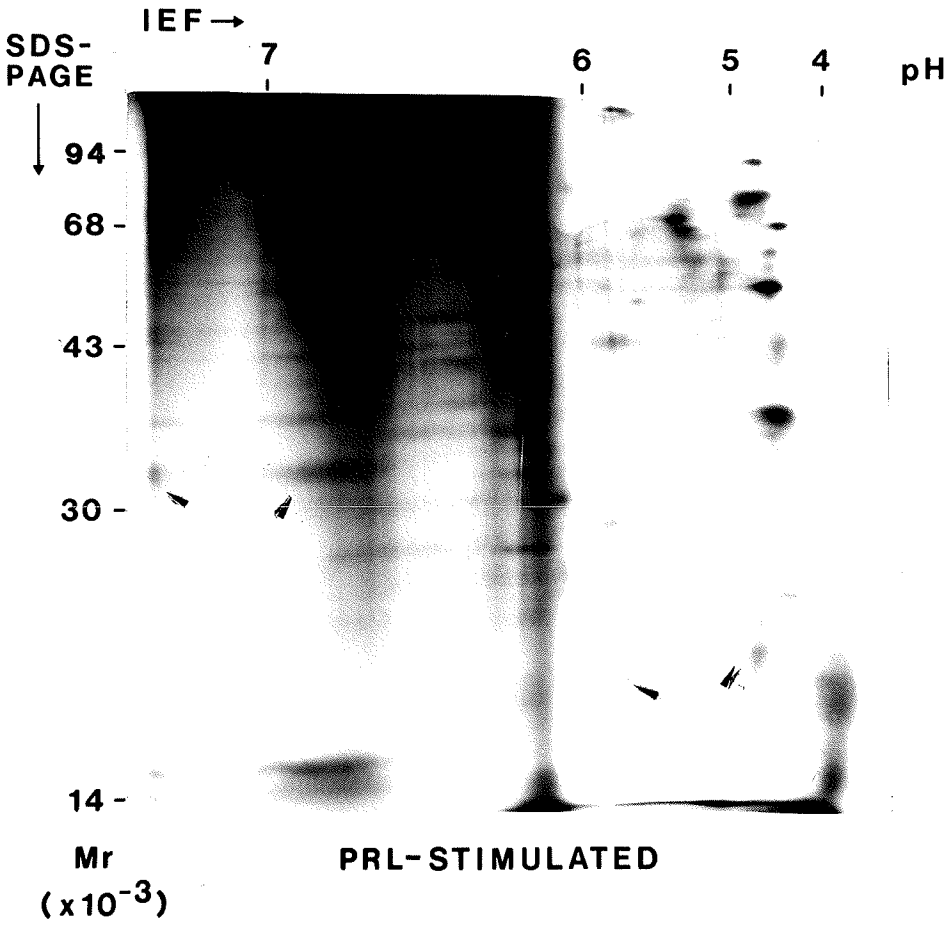


Figure 17. Analysis of ^{35}S-methionine labeled proteins from PRL-stimulated Nb2 cells by SDS-PAGE. Nb2 cells were stimulated with oPRL for 60 min and labeled with ^{35}S-methionine (50 uCi/10^6 cells/0.5 ml) for 2 hr. ^{35}S-labeled cells were prepared for SDS-PAGE as previously described for ^{32}P-labeled cells. Equal dpm ^{35}S were applied per track (internal standards ◁ ; PRL-enhanced proteins *).

compare ^{32}P-labeled proteins from stationary and PRL-stimulated Nb2 cells. A number of hormone-regulated phosphoproteins not readily detectable by one-dimensional analysis were visible on two-dimensional gels (egs. 38K/ PI 4.5, 78K/ PI 4.9, 70K/ PI 5.3, 15K/ PI 6.8). One phosphoprotein (80K/ PI 6.1) was more prominent in control than in PRL-stimulated cells suggesting that PRL may also trigger dephosphorylation and/or decreased synthesis of certain phosphoproteins in Nb2 cells. Phosphoprotein 33 was not sharply resolved in the first dimension but had a PI > 6.7. Identification of pp19 was difficult because several acidic PRL-stimulated phosphoproteins were visible on two-dimensional gels in the MW range 19 to 22,000. However, a PRL-sensitive phosphoprotein which focused at pH 5.8 and had a MW very near 19,000 may be pp19.

The 33,000 dalton phosphoprotein of Nb2 cells (pp33) shared a number of characteristics with S6 -- a protein (MW 30 to 33,000) of the small ribosomal subunit; S6 becomes rapidly phosphorylated in a number of cell types following mitogenic stimulation (see Discussion). In order to determine whether pp33 from Nb2 cells is S6, nuclei-free extracts from PRL-stimulated (^{32}P-labeled) Nb2 cells were centrifuged on 10 to 30 % sucrose gradients and fractions of the gradients were analyzed by SDS-PAGE. The top gradient fractions (1 to 3) contained the majority of ^{32}P-labeled bands (not shown). A number of these bands, however, were also apparent in subsequent fractions (figure 19), probably a result of gradient disruption and mixing during fractionation. Phosphoprotein 33 appeared in fraction 7 (but not fractions 5 and 9) of the gradient containing extracts from PRL-stimulated cells

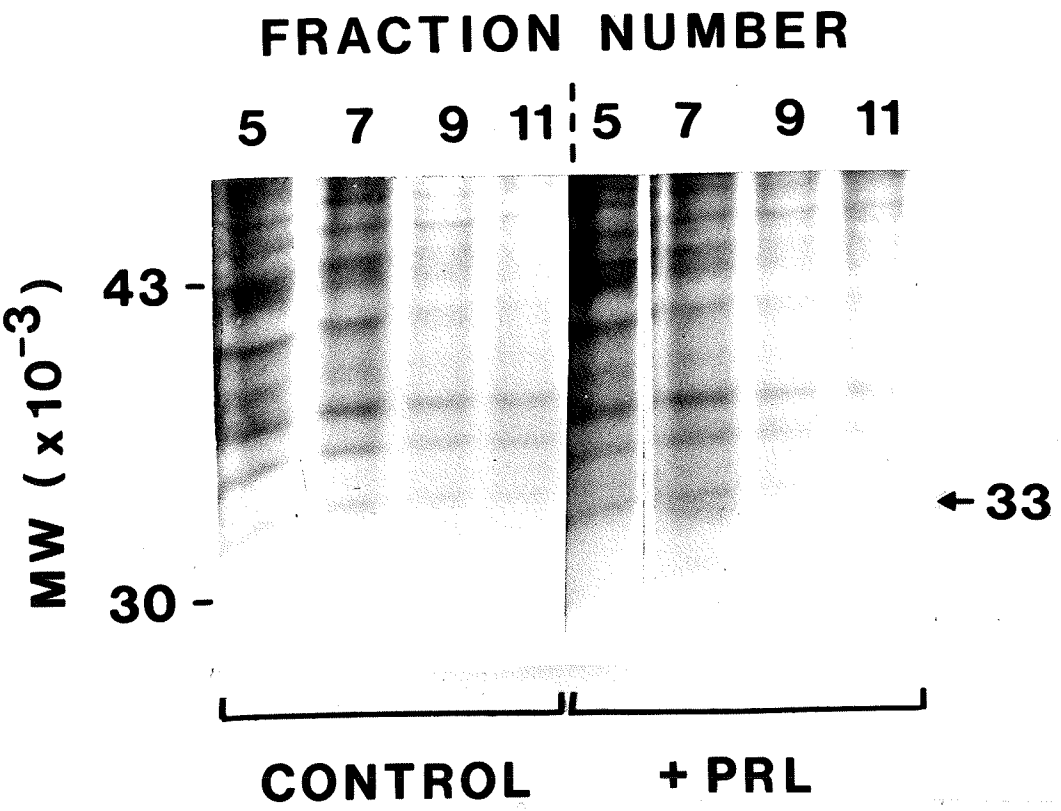

IEF →
SDS-
PAGE
7
6
5
4
pH
94 -
68 -
43 -
30 -
14 -
Mr
(x10-3)
PRL-STIMULATED

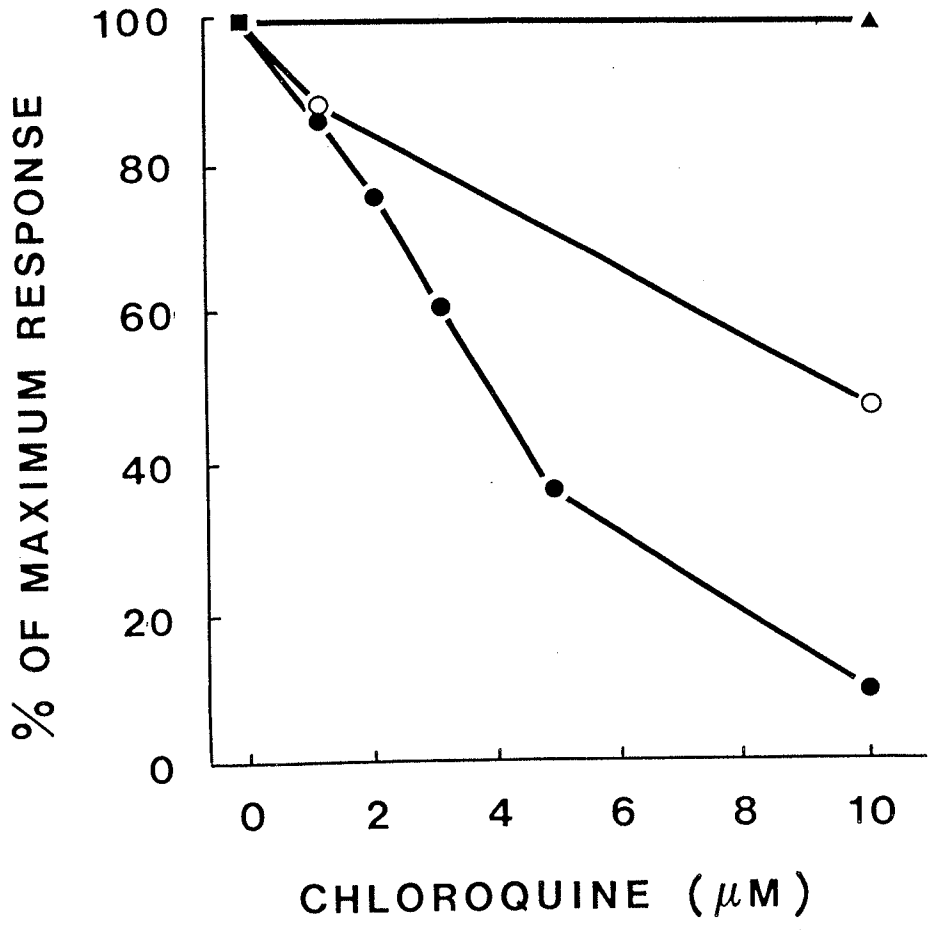
(figure 19). Fractions 6 and 8 were not examined by SDS-PAGE. The sedimentation of pp33 to a near-central position on a linear 10 to 30 % sucrose gradient (under the centrifugation conditions described in Materials and Methods) is consistent with that of the 40S ribosomal subunit (124c). This data therefore suggests that the PRL-responsive phosphoprotein, pp33, is ribosomal protein S6.

F) Effects of chloroquine and colcemid on PRL action in Nb2 cells

Both chloroquine and colchicine have been used extensively to investigate the mechanism of PRL action in the mammary gland (discussed in Introduction). In this study, chloroquine inhibited certain effects of PRL on Nb2 cells, but not others. Figure 20 demonstrates that chloroquine (10 uM) did not interfere with PRL-stimulated uptake and incorporation of phosphate, but caused about 50% inhibition of ODC induction and 90% inhibition of cell proliferation. In stationary cultures, concentrations of chloroquine greater than 2 uM caused a decrease in cell number over a 3 day period (not shown), suggesting that the inhibitory effect of the drug may be related to its cytotoxicity rather than to its blockage of PRL action. This view is supported by the observation (figure 20) that chloroquine 'inhibits' most strongly the effects of PRL measured after prolonged exposure of cells to the drug (ie. ODC induction, cell growth).

Colcemid (demecolcine) is an antimitotic drug structurally related to colchicine. The latter has been shown to block PRL-induced synthesis of casein and casein mRNA in the mammary gland (39,40). Although examining the effect of colcemid on PRL-stimu-

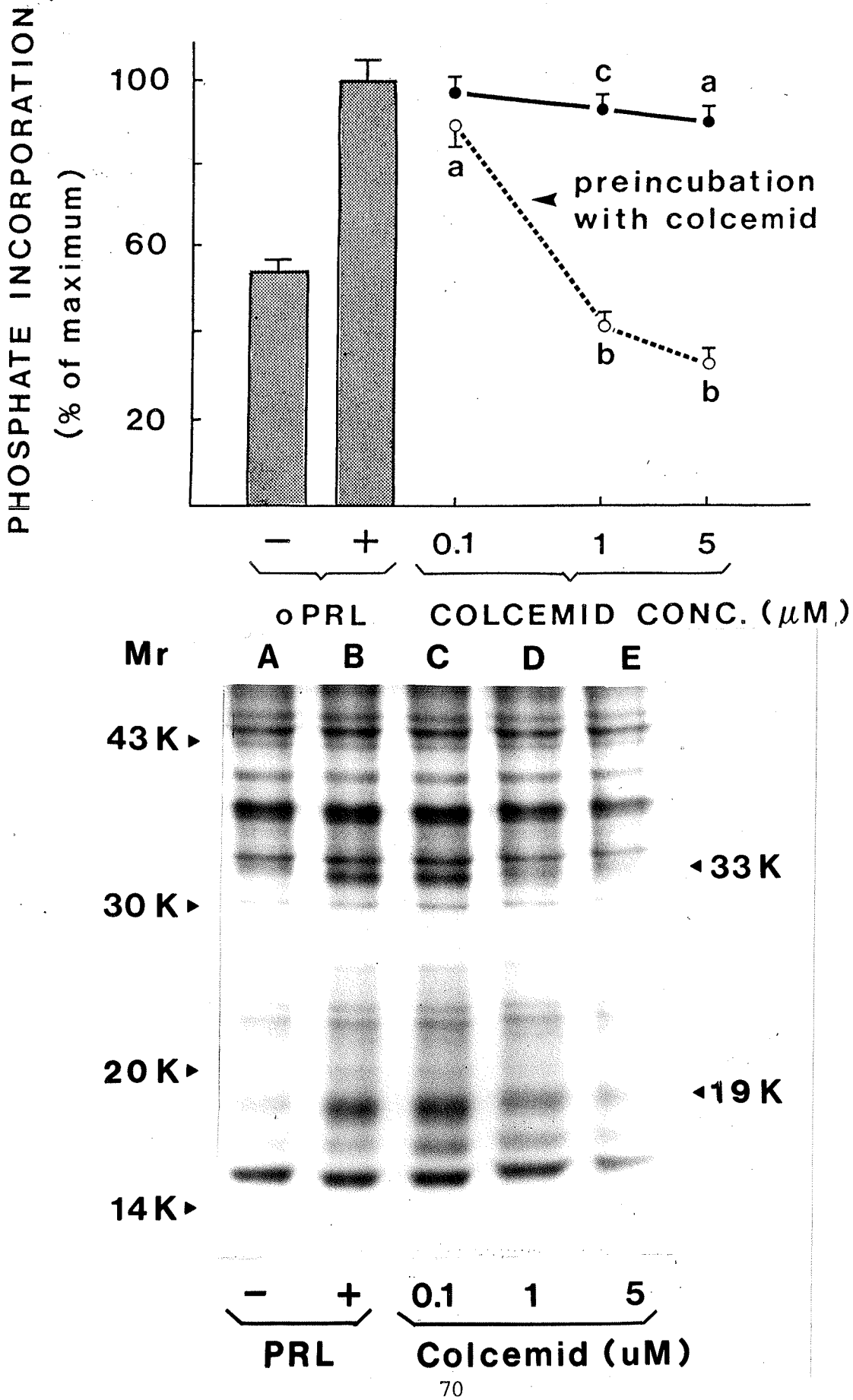

FRACTION NUMBER
5 7 9 11 5 7 9 11
MW (x10^{-3})
43
30
33
CONTROL
+PRL


100
80
60
40
20
0
% OF MAXIMUM RESPONSE
0 2 4 6 8 10
CHLOROQUINE (μM)

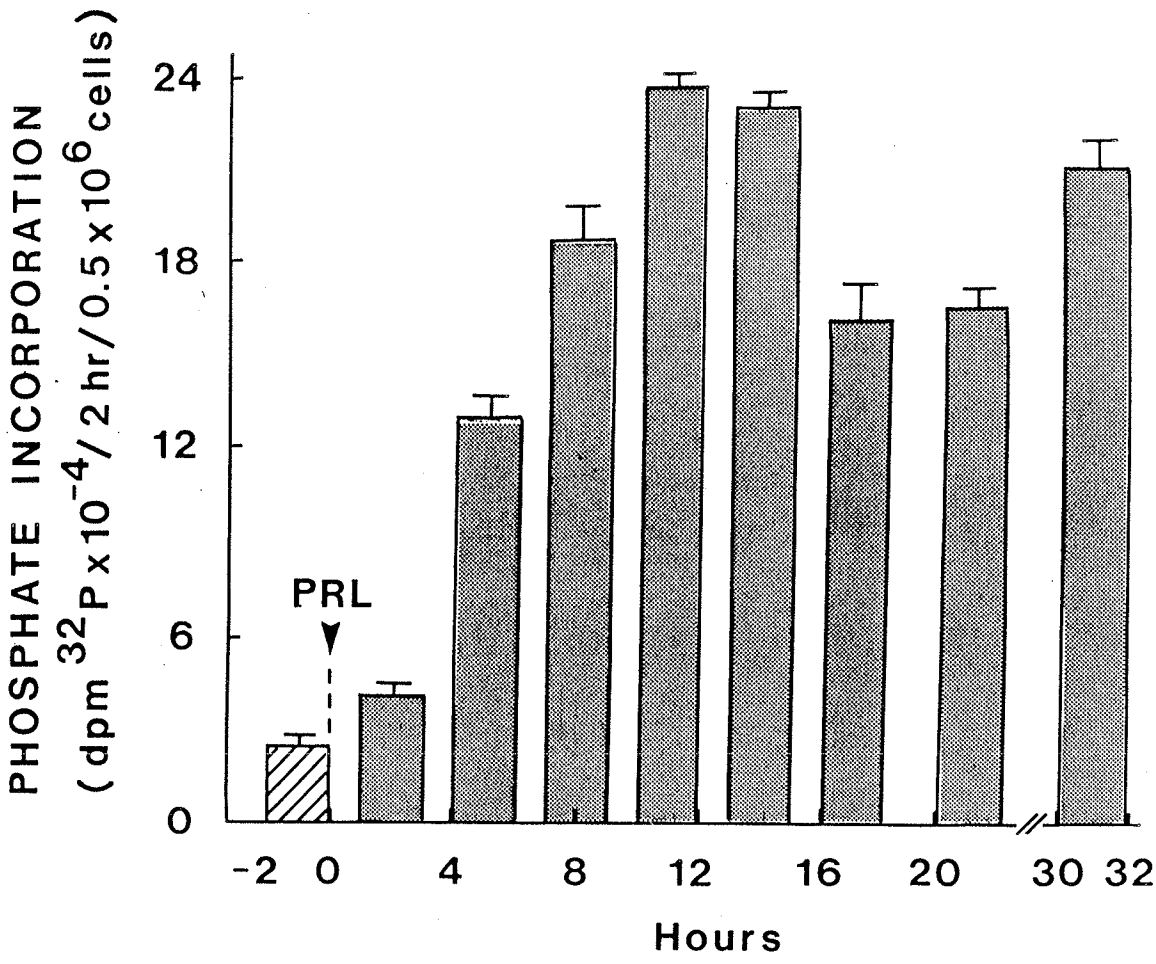
lated mitogenesis would be meaningless because of its inhibition of cytokinesis, the effect of the drug on an early PRL-dependent event -- phosphate uptake and incorporation -- was investigated. In Nb2 cells not preincubated with colcemid, the agent (0.1 to 5 uM) caused a small but dose-dependent inhibition of PRL-stimulated ^{32}P incorporation (figure 21). However, when Nb2 cells were preincubated (4 hr) with colcemid (0.1 to 5 uM) there was a dramatic decrease in ^{32}P incorporation -- below basal levels found in stationary cells (figure 21). This indicated that at least part of the inhibitory effect was non-specific. To establish whether colcemid inhibited certain PRL actions specifically, SDS-PAGE of ^{32}P-labeled extracts of Nb2 cells was performed. Colcemid (1 to 5 uM) decreased ^{32}P incorporation into numerous bands (compared to PRL-stimulated controls) when SDS gels were equalized according to protein quantity (not shown). When gels were equalized according to radioactivity (dpm ^{32}P), phosphorylation of most bands was unchanged by colcemid (figure 22); however, phosphorylation and/or synthesis of pp33 and pp19 was impaired, as the quantity of ^{32}P in these bands was selectively decreased. These findings suggest that colcemid inhibits both basal and PRL-stimulated transport and incorporation of phosphate but also specifically blocks the effect of PRL on certain phosphoproteins.

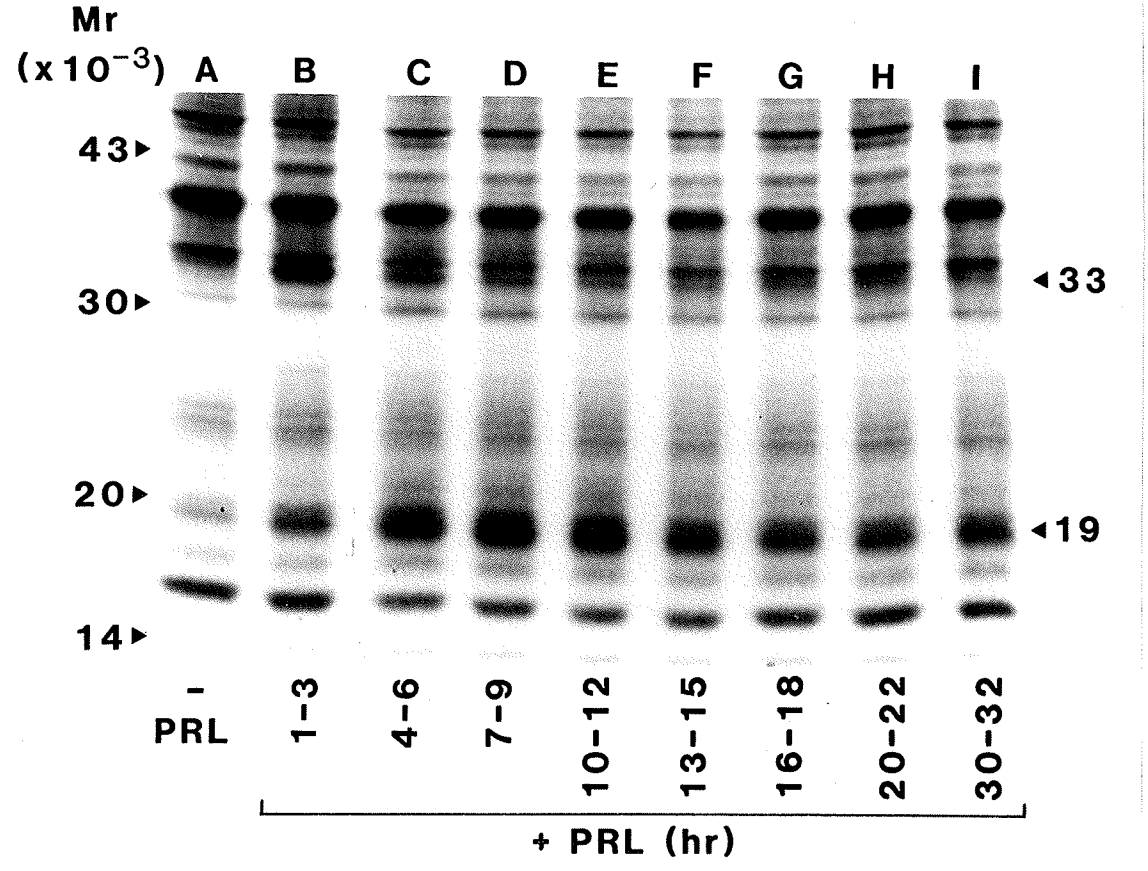
G) Uptake and incorporation of phosphate during the Nb2 cell cycle: regulation of pp33 and pp19

Earlier experiments examined uptake and incorporation of phoshate and phosphoprotein regulation in Nb2 cells after a 3 hr


PHOSPHATE INCORPORATION
(% of maximum)
100
60
20
c
a
a
preincubation
with colcemid
b
b
–
+
0.1
1
5
oPRL
COLCEMID CONC. (μM)
Mr
A
B
C
D
E
43K
30K
20K
14K
33K
19K
–
+
0.1
1
5
PRL
Colcemid (uM)

cultures were labeled for 2 hr periods at regular intervals. The net rate of phosphate uptake and incorporation continued to increase for 10 to 12 hrs following addition of PRL to stationary Nb2 cells (figure 23). At peak levels, uptake and incorporation of phosphate in activated cells was more than 9-fold greater than in unstimulated controls. Incorporation of ^{32}P decreased to about 70% of maximum between 16 to 22 hrs after addition of PRL but was maintained at a high level in growing cultures (figure 23). Qualitative changes in protein phosphorylation occurring during the Nb2 cell cycle (at the same intervals designated in figure 23) were analyzed by SDS-PAGE. As observed on autoradiograms of one-dimensional gels, phosphorylation of most proteins remained remarkably constant throughout the cell cycle (figure 24). The intensity of the phosphorylated proteins, pp33 and pp19, varied however. Phosphoprotein 33, which was not readily detectable in unstimulated cells was induced and/or phosphorylated maximally 1 to 3 hr after exposure of cells to PRL. The intensity of pp33 diminished after this early peak but the phosphoprotein remained conspicuous during later stages of the cell cycle (figure 24). Regulation of pp19 phosphorylation (and/or synthesis) differed from that of pp33. Phosphoprotein 19, which was present in stationary cells, became more intensely phosphorylated after a 1 to 3 hr exposure of cells to PRL but maximal phosphorylation first occurred 4 to 9 hr following PRL addition. The decrease in intensity of pp19 was more gradual than that of pp33 and it remained prominent throughout the cell cycle.


PHOSPHATE INCORPORATION
(dpm $^{32}P \times 10^{-4}$/2 hr/0.5 x 10^6 cells)
24
18
12
6
0
PRL
-2
0
4
8
12
16
20
30
32
Hours


Mr
(x 10^{-3})
A
B
C
D
E
F
G
H
I
43
30
20
14
33
19
-
PRL
1-3
4-6
7-9
10-12
13-15
16-18
20-22
30-32
+ PRL (hr)

H) Effect of anti-PRL receptor antibodies on (1) growth and (2) phosphate uptake and incorporation by Nb2 cells

Antibodies to the rabbit mammary gland PRL receptor were used to investigate PRL action in Nb2 lymphoma cells. Studies on antibody-stimulated growth confirmed earlier work in our lab by Shiu et al (115). During a 3-day culture period, whole antiserum stimulated cell growth about 2.5-fold above controls, a final antiserum concentration of 0.1% generating a near-maximal response (Table 5b). The magnitude of the antibody effect was about 50% of that elicited by 5 ng hGH/ml (Table 5a). Divalent $F(ab')_2$ fragments from anti-PRL receptor antiserum also stimulated growth of Nb2 cells; the stimulatory effect was almost maximal at a protein concentration of 5.5 ug/ml (Table 5c). Unlike $F(ab')_2$, monovalent F(ab') fragments had almost no effect on cell growth.

Studies in other systems have demonstrated that biologically inactive anti-receptor F(ab') fragments can be "activated" by antibodies to the F(ab') fragments. This effect may be due either to cross-linking of F(ab') fragments on the surface of target cells or to antibody-induced changes in F(ab') conformation (discussed in Introduction). The effect of anti-PRL receptor $F(ab')_2$ and F(ab') on phosphate uptake and incorporation was investigated; anti-F(ab') antiserum was tested for the ability to restore biological activity to F(ab') fragments. Figure 25 (bar c) shows that $F(ab')_2$ fragments (5 ug protein/ml) significantly stimulated phosphate uptake and incorporation. This concentration of $F(ab')_2$ was less than 20% as potent as PRL in promoting ^{32}P incorporation (figure 25, bar b: this value represents only a half-maximal increase in PRL-stimulated ^{32}P incorporation, see figure 12). The F(ab') fragments (5 ug protein/ml) were unable to

A. No addition 11.0
hGH 52.3

B.

	Antiserum Concentration (%) 0.1	1.0
Anti-mammary membrane antiserum (10-2b)	10.2	11.7
Anti-mammary PRL receptor antiserum (7-13b)	25.7	27.4

C.

		Ig Concentration (ug protein/ml) 0.2	0.5	1.1	2.7	5.5
$F(ab')_2$	(10-2b)	11.0	11.0	10.9	10.5	10.6
	(7-13b)	13.3	17.5	20.1	23.9	25.8
F(ab')	(10-2b)	11.0	10.9	11.4	11.0	10.7
	(7-13b)	10.9	11.2	11.8	12.3	12.9

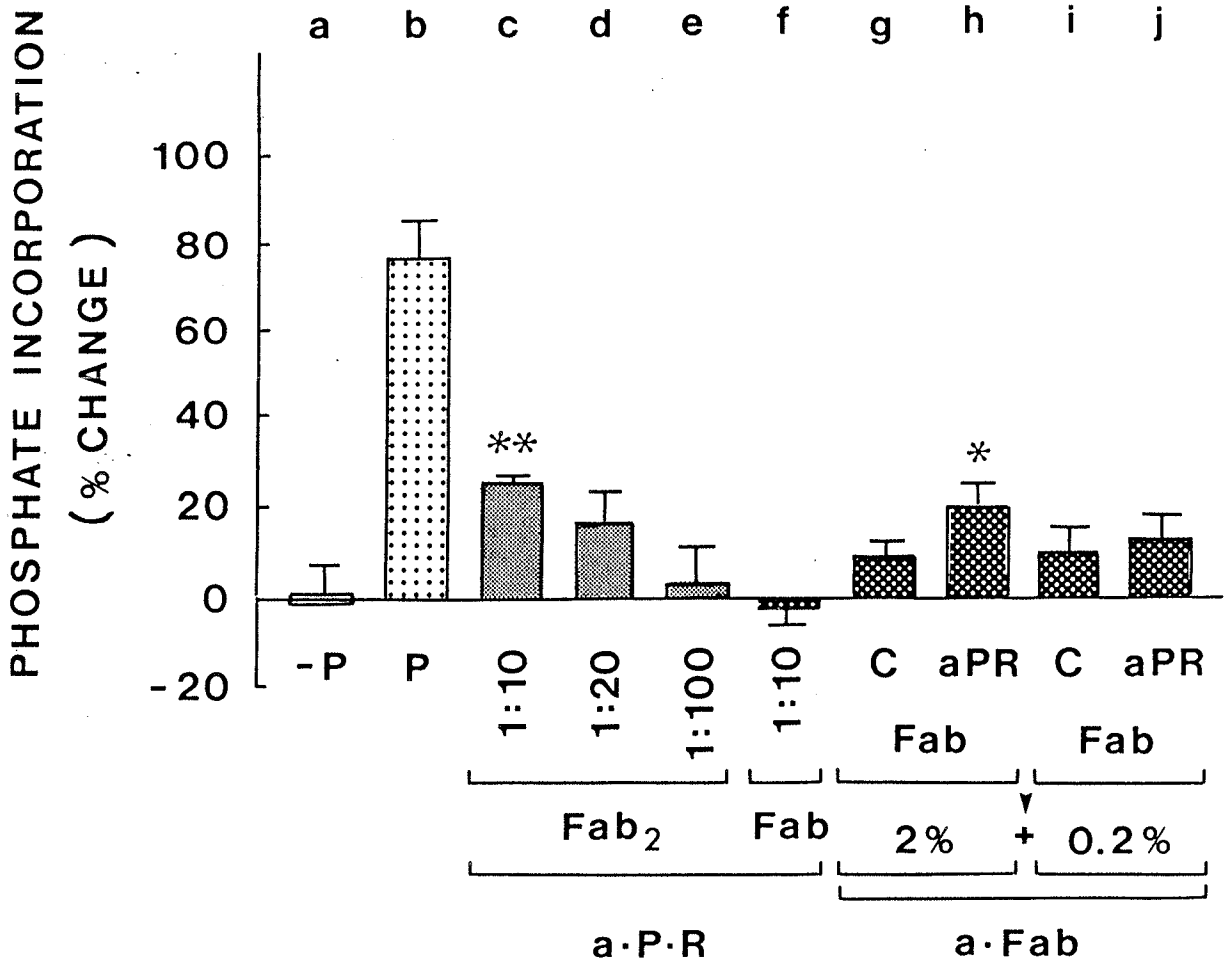
Table 5. Effects of an anti-PRL receptor serum and $F(ab')_2$ and F(ab') derivatives on proliferation of Nb2 cells.

A. Controls - Nb2 cells (10^5 cells/ml) were cultured for 3 days +/- hGH (5 ng/ml).

B. Stationary cultures of Nb2 cells (10^5 cells/ml) were incubated with control antiserum to crude rabbit mammary membranes (10-2b) or with antiserum to partially purified PRL receptor from rabbit mammary gland (7-13b).

C. Stationary cultures of Nb2 cells (10^5 cells/ml) were incubated with control $F(ab')_2$ and F(ab') fragments or with $F(ab')_2$ and F(ab') fragments isolated from anti-PRL receptor antiserum.

Each value (cell number x 10^{-4} /ml) represents the mean of duplicate determinations.


a b c d e f g h i j
PHOSPHATE INCORPORATION
(% CHANGE)
100
80
60
40
20
0
-20
**
*
-P
P
1:10
1:20
1:100
1:10
C
aPR
C
aPR
Fab
Fab
Fab2
Fab
2%
+
0.2%
a·P·R
a·Fab

enhance phosphate uptake and incorporation (figure 25, bar f). When anti-F(ab') antiserum (2%) was added to Nb2 cells previously incubated with anti-receptor F(ab'), a small but significant stimulatory effect on ^{32}P incorporation was generated (figure 25, bar h). In the presence of anti-F(ab') antiserum a small stimulatory effect was also found in negative controls (figure 25, bars g and i), but this was significantly lower ($p < 0.01$) than the effect of anti-receptor F(ab') fragments (figure 25, bar g or i compared to bar h).

I) Phospholipase C and stimulation of Nb2 cell proliferation

A role for phospholipases in PRL-stimulated lactogenesis has been suggested; also, phospholipase C has been implicated in the activation of lymphocytes (discussed in Introduction). These findings suggest that phospholipase C may be involved in the PRL-dependent mitogenic response of Nb2 cells. Early effects of phospholipase C on Nb2 cells were demonstrable. While higher concentrations of enzyme were inhibitory, low levels (0.01 U/ml) stimulated both basal and PRL-enhanced ^{32}P incorporation by 15 - 20% (figure 26). The stimulatory effect of phospholipase was not related to the concentration of PRL; maximally stimulated uptake and incorporation of phosphate (100 ng oPRL/ml) was increased to the same degree (in the presence of enzyme) as the basal level of incorporation in stationary cells (not shown).

Surprisingly, the effect of phospholipase C on Nb2 cell mitogenesis appeared very dependent on PRL concentration (figure 27). In unstimulated cultures, phospholipase C (0.01 to 0.03 U/ml) caused a small decrease in cell number during a 3 day period,

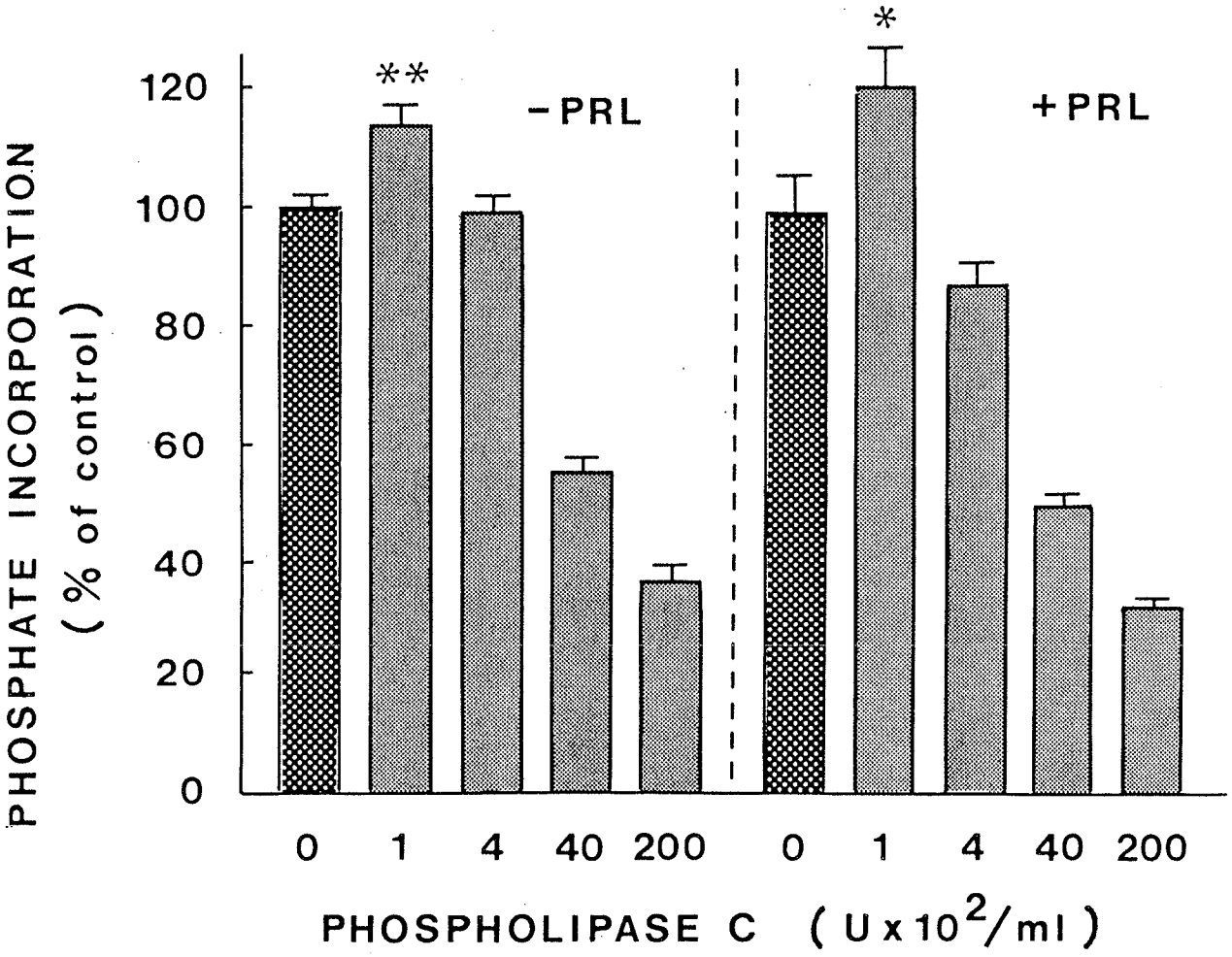
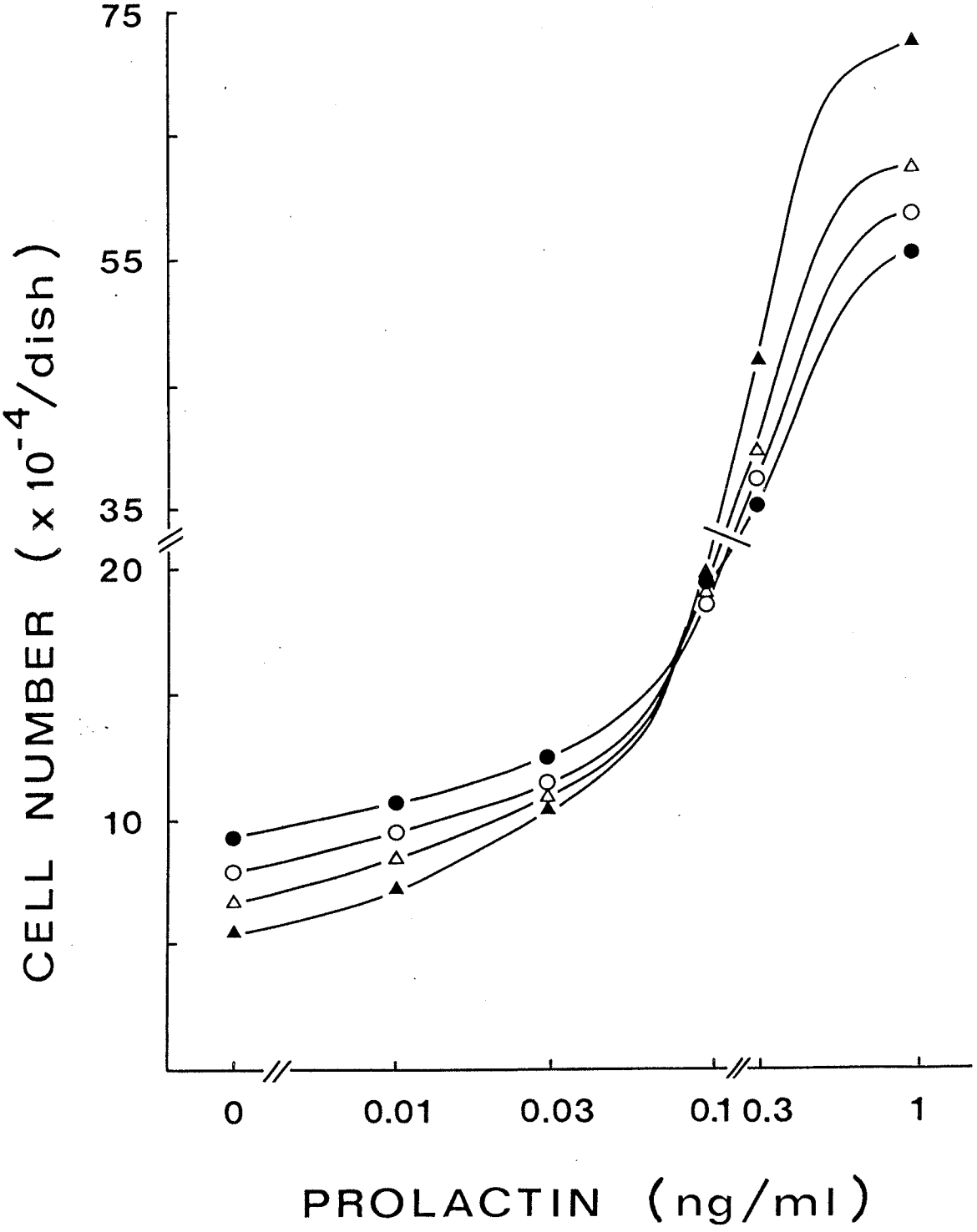


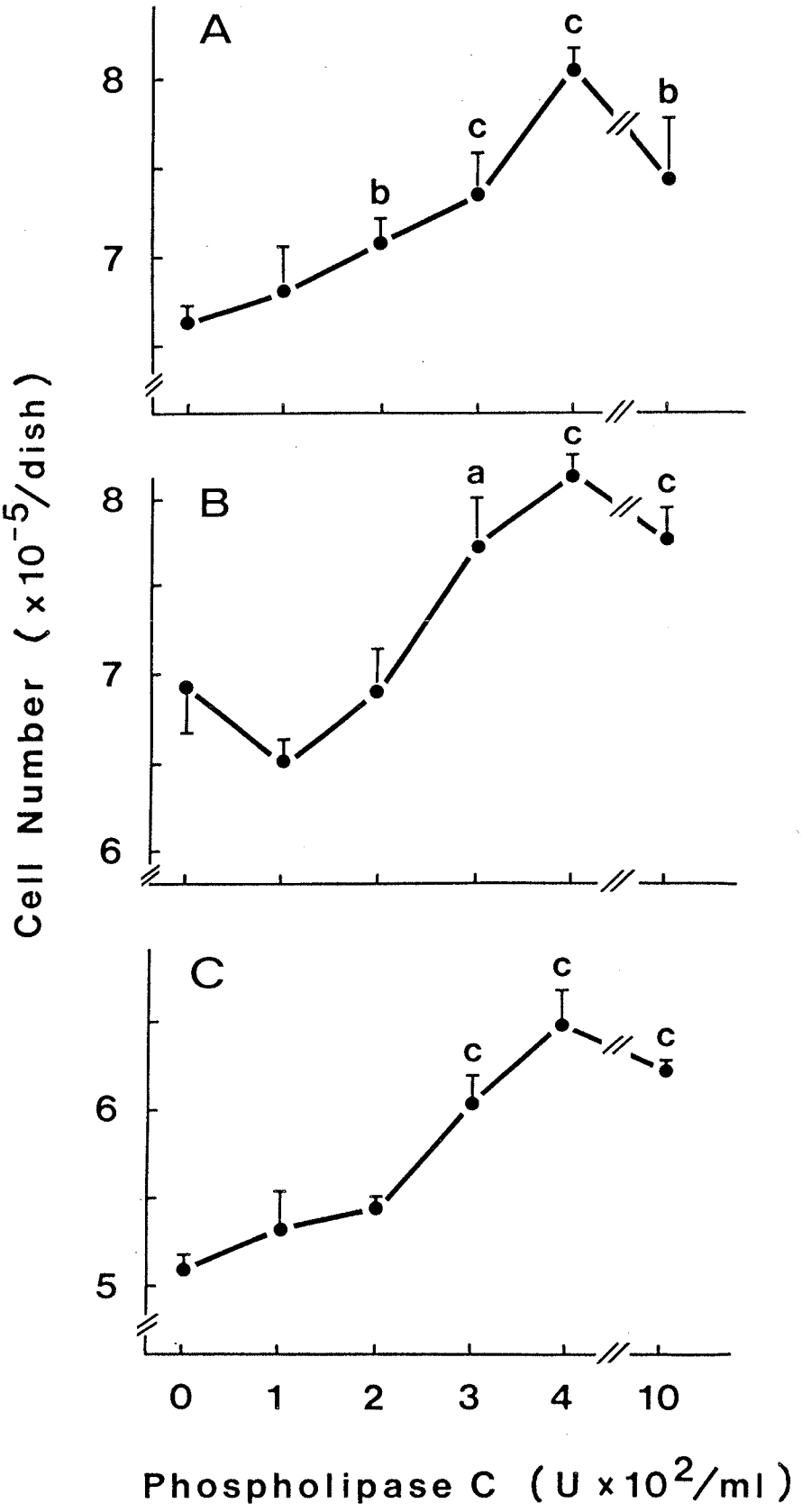
Figure 26. Effect of phospholipase C on basal and PRL-stimulated uptake/incorporation of phosphate. Phospholipase C +/- oPRL (10 ng/ml) was added to stationary Nb2 cells for 60 min. Cells were incubated with ^{32}P for 2 hr. Each bar represents the mean of four determinations. Dark bars - controls. * $P < 0.005$ compared to positive control. ** $P < 0.001$ compared to negative control.

while in moderately stimulated cultures (100 pg oPRL/ml) the enzyme had no appreciable effect. Conversely, in cells maximally stimulated by PRL, phospholipase C too, was maximally stimulatory, enhancing cell proliferation by about 30% (figure 27). Phospholipase C, therefore, altered (or improved) the standard curve of the Nb2 cell bioassay by lowering the background cell count slightly while increasing the cell count of highly stimulated cultures.

In figure 28, the comparative effect of phospholipase C on growth of: (A) stationary Nb2 cells stimulated by PRL, (B) growing Nb2 cells, and (C) growing PRL-independent (Nb2-SP) cells, is demonstrated. The stimulatory effect of phospholipase C was similar in all three cases; an enzyme concentration of 0.04 U/ml stimulated cell growth maximally and the magnitude of the phospholipase C effect ranged from a 20% increase in cell number (figure 28, A) to a 25% increase (figure 28, C). These results demonstrate that the effect of phospholipase C on Nb2 cell growth was additive to that of PRL. Furthermore, PRL was not strictly required for phospholipase C-enhanced growth; however, only cells which were already in a growing phase were further stimulated by the enzyme.

To determine if the continual presence of phospholipase C was necessary for a stimulatory effect on growth, stationary Nb2 cells were incubated with the enzyme, washed and then stimulated with PRL. Proliferation was significantly enhanced by exposing cells to phospholipase C prior to stimulation with PRL (figure 29). As shown in figure 29, sensitivity of Nb2 cells to phospholipase C concentration was not affected by previous exposure to


75
55
35
20
10
CELL NUMBER (x10^{-4}/dish)
0
0.01
0.03
0.1
0.3
1
PROLACTIN (ng/ml)


A
B
C
8
7
6
5
a
b
c
Cell Number (x10^{-5}/dish)
0
1
2
3
4
10
Phospholipase C (U x10^{2}/ml)

the enzyme. The ability of phospholipase C treatment to enhance the 'potential' of Nb2 cells to proliferate suggests that a long-term alteration in cell metabolism or structure may be triggered by the enzyme.

J) Mediators of PRL action: retention of PRL-like "signal" by Nb2 cells following withdrawal of hormone

The mechanism of PRL action was investigated by determining whether continual presence of hormone was required for a biological effect to be elicited in Nb2 cells. Briefly, stationary cells were first stimulated with PRL, then transferred to PRL-free medium; ^{32}P was added to the cells and incorporation of label was measured (figure 30, diagram). Complete removal of PRL following the first incubation was achieved by: 1) dilution of PRL by transfer of cells, and 2) addition of anti-oPRL antiserum to the cells. Because only 10 ng PRL/mg was used to stimulate the cells, even a 1:50 dilution of hormone would effectively reduce its concentration to the limit of detection (see figure 12). Anti-oPRL antibodies were added, however, to ensure neutralization of any remaining PRL molecules. In addition, as shown in figure 30 (diagram), cells were incubated for 30 min without PRL and in the presence of antibody before ^{32}P was added. Figure 31 demonstrates the kinetics of oPRL-antibody complex formation. At an antibody dilution of 1:10,000, maximal precipitation of PRL occurred within 60 min at 37°C (figure 31B). However, at the dilution of antibody used experimentally (ie. 1:100) formation of antibody-hormone complexes was very rapid, the reaction being essentially complete within 10 sec (figure 31A).

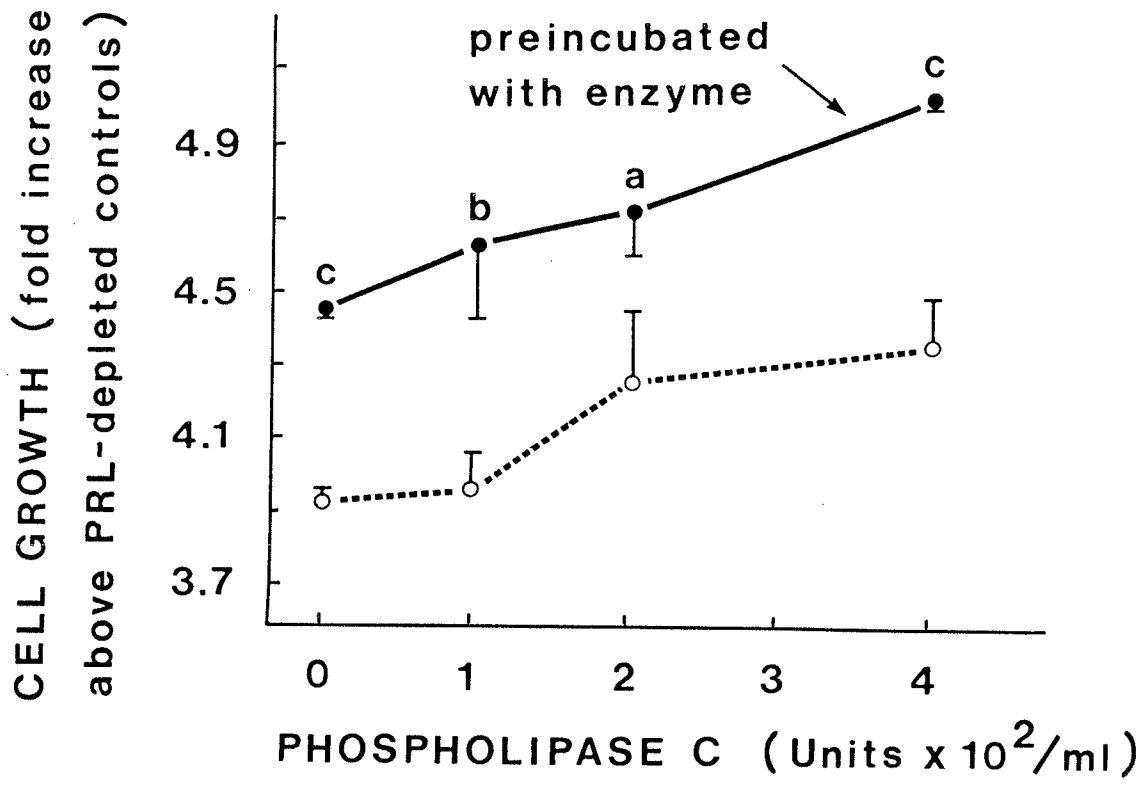
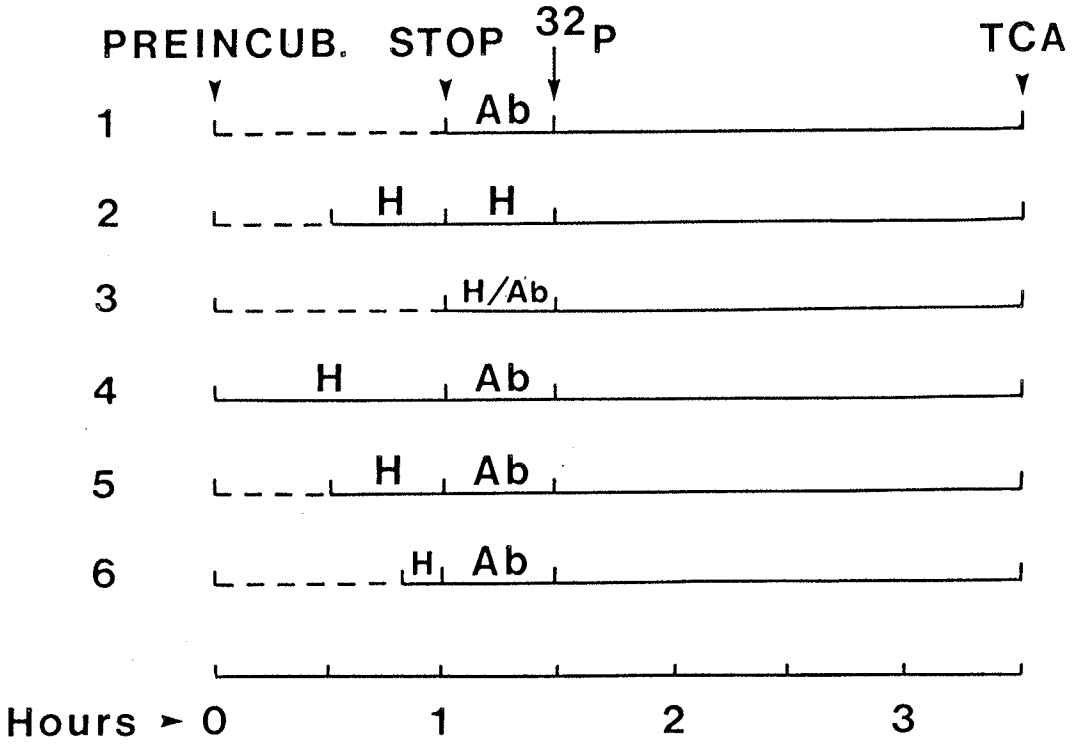


Figure 29. Effect of preexposure to phospholipase C on subsequent growth rate of Nb2 cells. Nb2 cells were preincubated without (open symbols) or with (closed symbols) 0.04 U phospholipase C /ml for 16 hr prior to stimulation with oPRL (1 ng/ml). Cell number was measured after 3 days of culture. Each point represents the mean of 3 determinations ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ compared to controls not pretreated with enzyme.



H = 10 ng oPRL/ml

Ab = anti-oPRL antiserum (in excess)

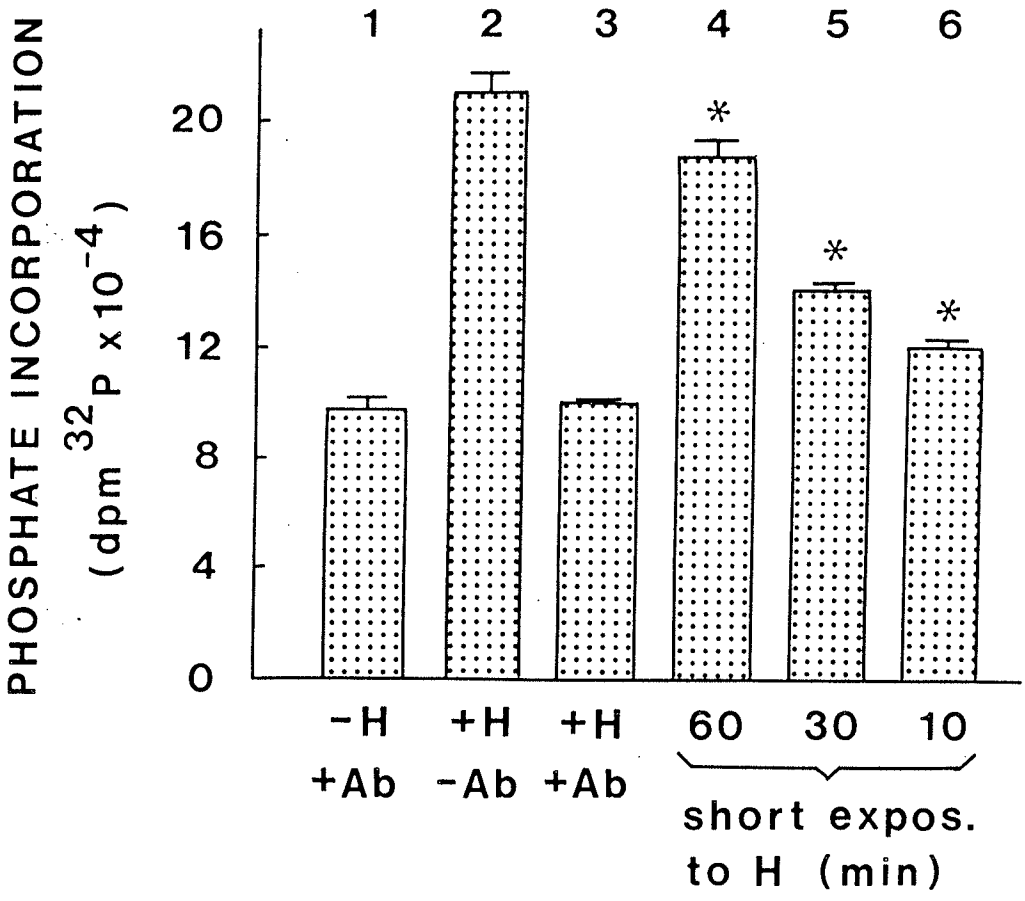
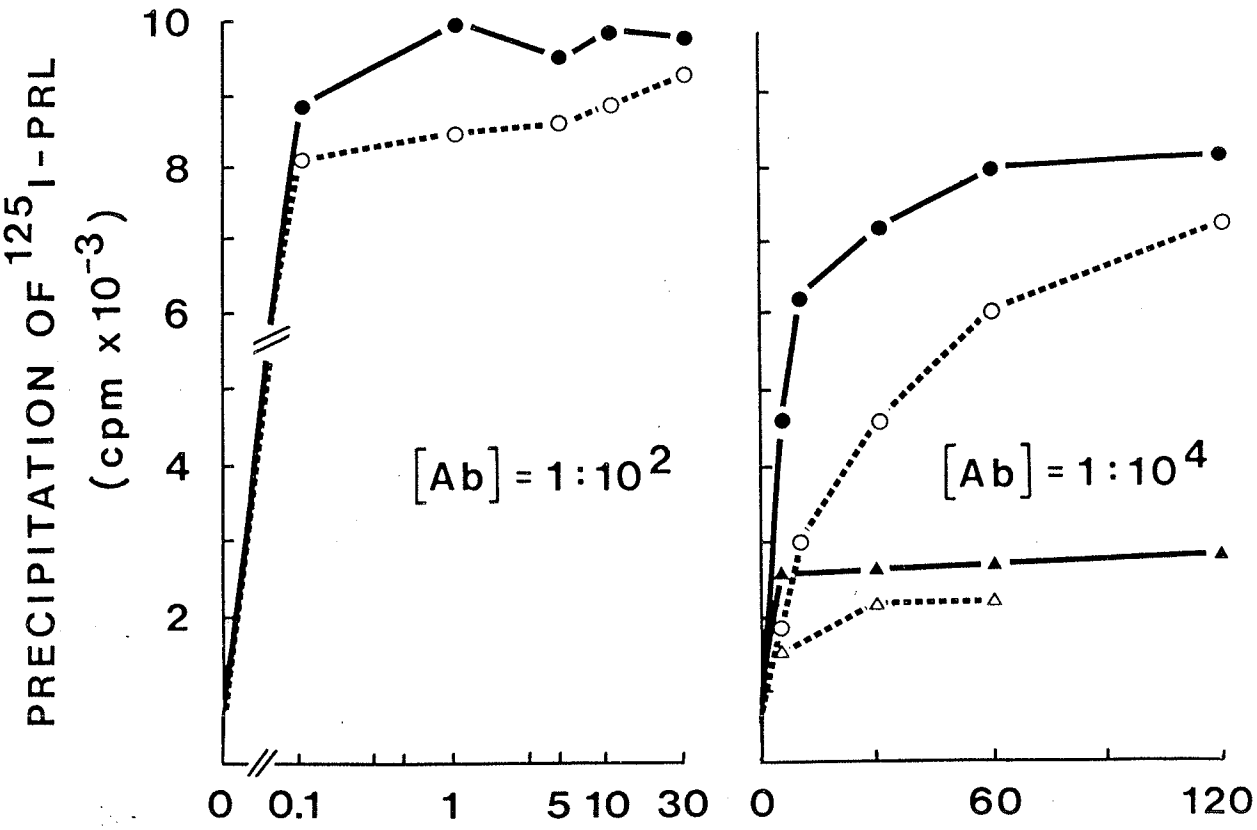
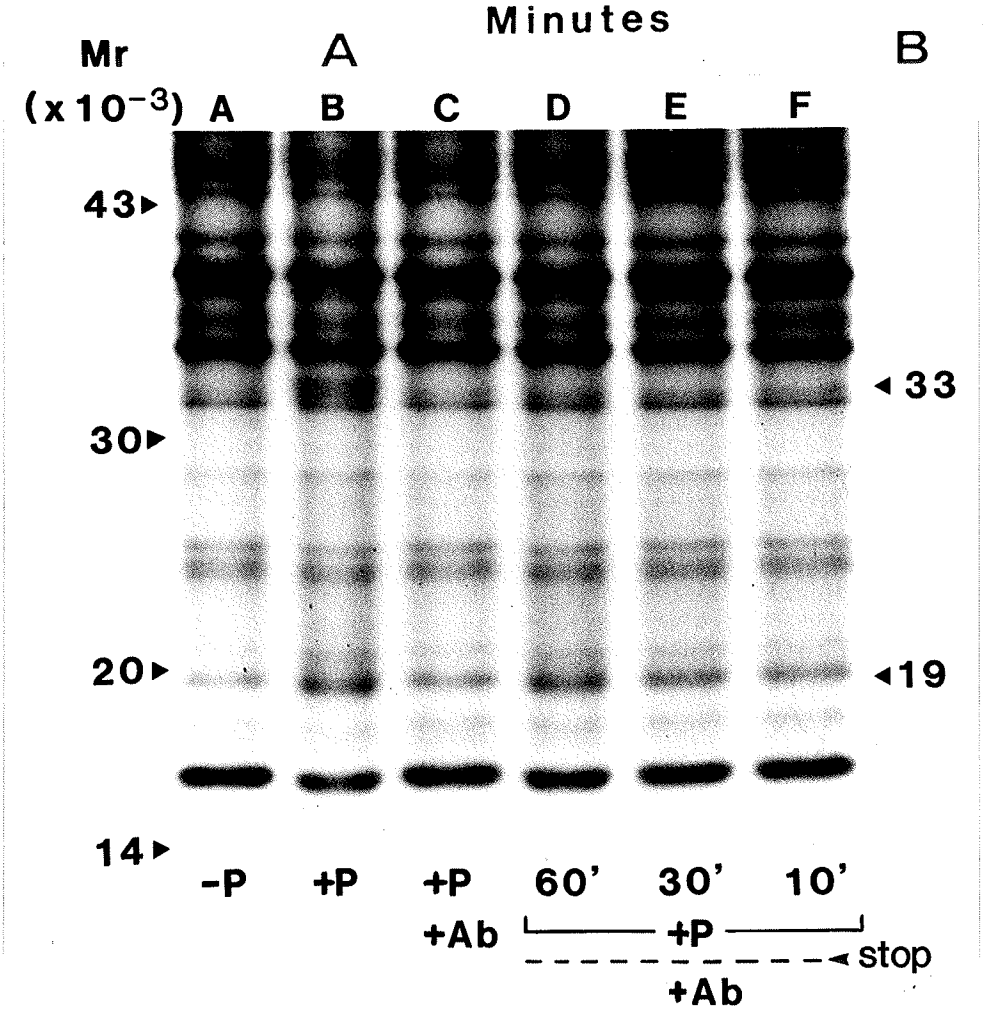


Figure 30 (bottom) demonstrates the effect of hormone withdrawal (after a 10 to 60 min preincubation with Nb2 cells) on ^{32}P incorporation. In cells exposed to PRL for 60 min (bar 4), uptake and incorporation of phosphate was nearly double that of unstimulated cells (bar 1) or cells exposed to PRL and anti-hormone antiserum simultaneously (bar 3). The magnitude of the PRL 'signal' was proportionate to the length of exposure to PRL, but even in cells preincubated with hormone for 10 min only (bar 6), phosphate uptake and incorporation was significantly enhanced ($P < 0.001$).

The effect of PRL withdrawal on lactogen-sensitive phosphoproteins was also studied. The autoradiogram shown in figure 32 demonstrates that pp33 was phosphorylated only in cells continually stimulated by PRL (track B). Even after a 60 min exposure to hormone, enhanced phosphorylation of pp33 was not detectable if PRL was subsequently removed from the medium. Stimulation of pp19 phosphorylation, on the other hand, did not require continual presence of PRL. Withdrawal of hormone after a 60 min preincubation with Nb2 cells had almost no inhibitory effect on subsequent phosphorylation of the protein (figure 32, track D). Exposure of cells to PRL and anti-PRL antibodies simultaneously resulted in complete suppression of phosphorylation of both pp33 and pp19 (track C).


PRECIPITATION OF ^{125}I-PRL
(cpm x10^{-3})
10
8
6
4
2
[Ab] = 1:10^2
[Ab] = 1:10^4
0
0.1
1
5
10
30
0
60
120
Minutes


Mr
(x10^{-3})
A
B
C
D
E
F
A
B
43
30
20
14
33
19
-P
+P
+P
60'
30'
10'
+Ab
+P
stop
+Ab

DISCUSSION AND CONCLUSIONS

A. Ornithine Decarboxylase Induction in Nb2 Cells: Role of Polyamines in Proliferative Growth.

a) Lactogen-dependent and -independent induction of ODC.

As shown in a previous study (121), ODC activity was undetectable in Nb2 cells that were cultured without PRL for 18 to 24 hr. The low level of ODC activity paralleled the non-proliferative state of Nb2 cells under these culture conditions. Mitogenic stimulation of the cells by hGH (figure 1) or PRL caused a dramatic increase in enzyme levels, a response which precedes lactogen-dependent synthesis of DNA (121). In Nb2 cells, therefore, stimulation of ODC activity by lactogenic hormones is similar to that of short-term lymphocyte cultures stimulated to grow by phytohemagglutinin (125) or concanavalin A (126). Stimulation of adenosylmethionine decarboxylase activity (an enzyme required for spermidine synthesis) also has been observed in both mitogen-activated lymphocytes (125) and Nb2 cells (121). In the present study induction of ODC by hGH was blocked completely by 10 uM cycloheximide (not shown) suggesting an important role for protein synthesis in this lactogen-dependent effect. Using lymphocyte cultures, others have found that undisturbed synthesis of rRNA is also required for proliferation-associated increases in ODC activity (127).

Activation of ODC in stationary Nb2 cells was sensitive to low lactogen concentrations. In a previous investigation (121), 20 ng oPRL/ml was used to stimulate activity of the enzyme in Nb2 cells; the present study demonstrates that a 10-fold lower concentration of hGH (which was equipotent to oPRL-NIH-P-S12 (not

shown)) was sufficient to trigger a maximal response (figure 2). This concentration of hGH (ie. 2 ng/ml) was only about 2-fold greater than the hormone concentration needed for maximal growth stimulation. Interestingly, at the lower concentrations of lactogen (20 to 200 pg/ml) the growth response of cells was 7 to 10 times more sensitive to lactogenic stimulation than was ODC. The physiological significance of these non-parallel response curves is not clear. Shiu et al (115) have demonstrated that PRL receptors of Nb2 cells are saturated 50% at a hormone concentration of 1.5 ng/ml. This concentration is several times greater than that required for half-maximal stimulation of ODC activity, suggesting that 'spare receptors', not necessary for ODC activation, may be present in these cells. However, an even smaller number of receptors appear to be required for mediating lactogen-induced growth (115).

Lactogen-dependent induction of ODC was demonstrable in cells which had been cultured without PRL; lactogen-independent ODC activity was stimulated in cells which had been cultured with PRL but had reached a growth plateau. Because the onset of lactogen-dependent ODC activation was slower, it is possible that the cellular mechanisms mediating the two 'types' of ODC activation are different. The sensitivity of lactogen-independent ODC stimulation to cycloheximide (figure 4) suggests that both mechanisms (see above) require synthesis of protein. However, Canellakis et al (128) have observed that ODC activation in some types of non-growing cells does not require synthesis of RNA (ie. actinomycin D resistant). The longer lag phase that precedes ODC

activation in PRL-stimulated cells may, in part, be due to a requirement for de novo synthesis of RNA.

The stimulus for lactogen-independent activation of ODC appears to be a factor, possibly a nutrient, present in fresh culture medium. Several observations support this view. Firstly, as shown in figure 4 and Table 1, rapid increases in ODC activity were not due to mechanical stimuli such as centrifugation or resuspension of Nb2 cells, since conditioned (spent) medium did not trigger a response. Secondly, increasing or decreasing the concentration of cells in fresh medium did not appreciably alter the magnitude of the ODC response (Table 1), indicating that rapid 'release' of cells from density inhibition was not of primary importance in activating ODC. Thirdly, the presence of lactogenic hormones in fresh medium did not augment the ODC response; thus, this mechanism of ODC activation was completely independent of PRL. These findings strongly suggest that lactogen-independent stimulation of ODC under non-growth conditions was triggered by nutritional or trophic factors present in fresh serum-containing medium. Amino acids may be partly responsible for the stimulatory effect. In non-growing neuroblastoma cells cultured in salts-glucose solutions, ODC activity is elevated markedly by the addition of asparagine (128). Decreased levels of extracellular Na^+, K^+, and Mg^{2+} have also been shown to enhance ODC activity (128); however, under the experimental conditions used in the present study, variations in cation concentration would have been nearly undetectable.

Richards et al (121) have demonstrated that PRL-initiated mitogenesis in Nb2 cells is preceded by a dramatic increase in

ODC activity during the G_1 phase of the cell cycle. The present data suggest that this early peak of ODC activity triggered by PRL is not directly involved in mediating lactogen-dependent mitogenesis. First of all, an acute increase in ODC activity was demonstrable under non-growing conditions indicating that enhanced ODC activity and mitogenesis are dissociable cellular responses. Secondly, low concentrations of hGH (10 to 20 pg/ml) which stimulate cell growth significantly after a 3 day culture period did not elicit a measurable increase in ODC activity (figure 2). Thirdly, in other studies (not shown), addition of putrescine (1 uM to 1 mM) to stationary Nb2 cells -- to mimic the effect of elevated ODC levels -- did not generate a mitogenic response. And lastly, if PRL was removed from culture medium after a 12 hr incubation with Nb2 cells, the cultures failed to grow (not shown), even though this period of time was sufficient for early increases in ODC activity to occur. Interestingly, this result also suggests that PRL does not act as a 'competence' factor, as do PDGF or FGF, but rather may stimulate 'progression' of Nb2 cells through the cell cycle, in a manner similar to EGF and somatomedin C in other cell types (129a).

In EGF-responsive cells, Yarden et al (129b) showed that a number of 'early' responses associated with mitogenesis could be elicited by the cyanogen bromide cleaved form of the mitogen. However, while this analog bound to EGF receptors, stimulated membrane protein phosphorylation and Na^+,K^+-ATPase activity, and activated ODC in an EGF-like manner, it was non-mitogenic. As discussed in the Introduction, divalent anti-EGF antibodies re-

stored mitogenic activity to the analog (72). In the Nb2 cell system, non-mitogenic PRL (or hGH) analogs which trigger early responses have not been developed. Recently, Aston and Ivanyi (129c) have shown that a subtilisin-cleaved 15K fragment of hGH retains the ability to bind to hGH receptors of IM9 lymphocytes, and stimulates mitogenesis of Nb2 cells in an hGH-like manner (60 to 70% activity). In the future, similar analogs or fragments of lactogenic hormones may become an important tool for dissecting early cellular responses that accompany lactogen-dependent mitogenesis from those that mediate it.

b) Dependency of Nb2 cell proliferation on polyamines.

Despite numerous studies which have demonstrated the importance of ODC and polyamines in proliferative growth of cells (130 - 133), the role of the polyamine biosynthetic pathway in hormone or serum-stimulated mitogenesis has still not been clearly defined. Although it is probably true that most anabolic cellular responses (eg. proliferation, differentiation) are associated with increases in ODC activity, such responses may not always be dependent on ODC activity. For example, in both Friend erythroleukemia cells (134, 135) and PTH-stimulated chondrocytes (136), differentiation and mitogenesis are preceded by activation of ODC. However, differentiation is prevented by inhibitors of ODC activity whereas cell proliferation is not (134 - 136). Conversely, in murine embryonal carcinoma cells, proliferation is halted following inhibition of ODC activity while differentiation is actually induced by a reduction in ODC levels (137).

The role of ODC is even more complex in vivo . Bartolome et

al (138) have shown that inhibition of cardiac ODC activity does not prevent normal growth of the rat heart or triiodothyronine-induced cardiac hypertrophy. Surprisingly, however, the hypertrophic effect of isoproteronol on the heart is significantly attenuated when ODC activity is decreased (138). Others (139) have demonstrated that in rats, hematopoietic production of blood elements (leukocytes, erythrocytes, platelets) is suppressed by administration of DFMO for a 5 week period -- an effect that is completely reversed by administration of putrescine. But paradoxically, DFMO stimulates proliferation of hematopoietic cells when administered for a shorter time period (5 to 12 days) (139, 140). The stimulatory effect of DFMO is also blocked by putrescine, suggesting that under these conditions increased ODC activity attenuates cell proliferation. In vivo , neoplastic cells also vary in their response to inhibition of ODC activity. The growth of some cell types, such as L1210 leukemic cells (141) and renal adenocarcinoma cells (142) is decreased by DFMO while growth of others, such as Wilms' tumor cells (142), is not affected despite a major reduction in intracellular putrescine and spermidine levels. It is clear from these studies that the precise role of ODC in cell growth and differentiation is not yet well-defined and may in fact vary, depending on tissue, species, and experimental conditions.

In the present study DFMO was used to evaluate the importance of ODC in PRL-stimulated Nb2 cell growth. Briefly, DFMO acts as an alternate substrate to ornithine; during decarboxylation of this analog, a highly unstable intermediate is formed that alkylates ODC at or near its catalytic site, resulting in

inactivation of the enzyme (143a). The irreversible nature of this inhibitor, as well as its specificity and low cytotoxicity, have made it an important tool for investigating the role of ODC and polyamines in cellular functions. As shown in figures 5 and 6, DFMO inhibited PRL-stimulated induction of ODC in Nb2 cells and caused a dose-dependent decrease in cell proliferation over a 3 - 4 day culture period. The inhibitory effect of DFMO was completely prevented by simultaneous addition of putrescine, spermidine or spermine to cell cultures (figures 7 and 8). These studies clearly demonstrate that, while the early activation of ODC by PRL may not be requisite for a mitogenic response, ODC-dependent synthesis of putrescine and polyamines is critical for normal growth of Nb2 cells in the presence of PRL.

Rillema and Cameron (143b) have reported recently that DFMO does not block PRL-stimulated biosynthesis of casein and lipids in mouse mammary gland explants. On the other hand, inhibition of spermidine synthesis prevented PRL action. The investigators suggested that early increases in mammary ODC activity, observed 2 to 4 hr after exposure of explants to PRL, are not of critical importance for stimulation of these lactogen-dependent responses. In this study (143b), de novo synthesis of putrescine was effectively inhibited by DFMO; however, ODC activity (and hence putrescine synthesis) was demonstrable in mammary explants before treatment with PRL. Thus, because endogenous levels of putrescine would not have been affected by DFMO, spermidine (and casein) synthesis may have been able to continue unimpaired for a period of time (16 hr in 143b). It is possible that, in vivo ,

during lactation, prolonged inhibition (several days) of PRL-stimulated ODC activity in the mammary gland would indeed block the lactogenic effects of the hormone. In short-term mammary explant cultures, however, the physiological role of ODC in PRL-dependent lactogenesis may be more difficult to evaluate.

The relative effectiveness of putrescine, spermidine, spermine and cadaverine in restoring normal growth to DFMO-inhibited Nb2 cells was examined. At concentrations below 1 uM, spermidine and spermine were more active in stimulating growth of such cultures than was putrescine (figure 8). One explanation may be that the larger polycations spermidine and spermine are required for maintaining a normal rate of DNA synthesis, whereas putrescine is not required directly but serves only as a biosynthetic precursor for these polyamines. At low concentrations (< 1 uM), insufficient quantities of putrescine may be converted into spermidine or spermine to restore optimal growth. This possibility is supported by studies in rat embryo fibroblasts (144). When growth of these cells is inhibited by methylglyoxal bis(guanylhydrazone), which blocks spermidine and spermine synthesis, the addition of either spermidine or spermine enhances cell proliferation, whereas putrescine cannot substitute for these polyamines. However, in rat mammary gland explants cultured in hypotonic medium, partial prevention of hormone-stimulated DNA synthesis by an ODC inhibitor is reversible by addition of putrescine only -- spermidine, spermine and cadaverine have no stimulatory effect (145). Whether this 'direct' effect of putrescine on DNA synthesis is a tissue-specific phenomenon or possibly dependent on culture conditions has not been determined.

Cadaverine partially restored growth of PRL-dependent Nb2 cells in the presence of DFMO, but was considerably less effective than the other polyamines (figure 8). Mammalian ODC decarboxylates lysine to yield cadaverine, but because the Km for lysine (9.2 mM) is much greater than for ornithine (0.09 mM), lysine decarboxylation would presumably be negligible under physiological conditions (146). Nevertheless, in cells depleted of putrescine by DFMO, cadaverine can be utilized during growth as a spermidine synthase substrate (146) or as a precursor for aminopropyl derivatives of cadaverine; these replace the natural polyamines in maintenance of growth (147) (albeit at a slower rate). In some cells, like transformed mouse fibroblasts (146), high (mM) concentrations of cadaverine restore growth completely. This was not observed in Nb2 cells, however; cadaverine had an inhibitory effect on growth at higher doses. In general, at high concentrations, polyamines (but not putrescine) inhibited growth of Nb2 cells (figure 8), the magnitude of inhibition being proportional to the molecular size of the polyamine. The degradation of polyamines by serum amine oxidase may account for these cytotoxic effects. Oxidation of spermine and spermidine by this enzyme generates toxic products including NH_3 and H_2O_2 (148).

The present investigation demonstrates (for the first time, to my knowledge) that loss of hormonal dependence in a tumor cell can be associated with an increase in resistance to ODC inhibitors (figure 9). Several explanations can be suggested for the alteration in polyamine metabolism that accompanies a loss of sensitivity to PRL. Firstly, the autonomous variant (Nb2-SP) may have a

reduced requirement for polyamines -- lower levels of putrescine being adequate for normal cell growth. Secondly, transport of DFMO into Nb2-SP cells may be reduced resulting in less inhibition. Thirdly, DFMO may be metabolized or degraded more rapidly in Nb2-SP cells than in normal PRL-dependent cells. Fourthly, a DFMO-resistant isoenzyme of ODC may be expressed in Nb2-SP (but not Nb2) cells. Fifthly, synthesis of ODC may be enhanced in Nb2-SP cells; higher concentrations of DFMO would then be required to inhibit ODC activity sufficiently to decelerate proliferation of the variant. At present, evidence for only the last possibility has been found. Ornithine decarboxylase was 3 - 4 fold higher in exponentially growing Nb2-SP cells compared to PRL-responsive controls (not shown). Interestingly, a similar increase in ODC activity was reported in 3T3 mouse fibroblasts following viral transformation (149). In this study, ODC was purified from normal and SV40-transformed cells and physicochemical properties (eg. molecular weight, kinetic parameters, degradation) of the two enzymes was compared. No evidence for an altered form of ODC in transformed cells was found, however, and the investigators concluded that increased specific activity of ODC in crude extracts of transformed cells was the result of increased enzyme biosynthesis (149). Recently, McConlogue and Coffino (150) have developed DFMO-resistant variants of the S49 lymphoma cell line and have demonstrated (using ^{35}S-methionine labeling) that increased synthesis of ODC accompanies and probably accounts for DFMO-resistance.

To summarize, PRL stimulates ODC activity in stationary Nb2 cells -- an early pleiotypic response associated with

mitogenesis. However, large and rapid increases in ODC activity are also demonstrable in Nb2 cells under non-growth conditions, indicating that this cellular response is not directly involved in mediating PRL-stimulated cell growth. Inhibition of ODC activity by DFMO decreases the rate of PRL-dependent cell growth; thus ODC and polyamine biosynthesis are necessary to support proliferation of Nb2 cells. Lastly, loss of PRL-dependence by Nb2-SP cells is associated with increased resistance to DFMO, an alteration which is probably due to enhanced ODC synthesis in the autonomous variant.

B. Effect of PRL on Phosphate Uptake and Incorporation

Mitogenic stimulation of Nb2 cells by lactogenic hormones triggered an enhanced net rate of ^{32}P-orthophosphate uptake, as determined in cells not 'equilibrated' with ^{32}P (figure 10, Table 3). PRL-stimulated incorporation of ^{32}P into macromolecules was also demonstrable when cells were first 'equilibrated' with the isotope (figure 11, Table 3). Studies in other cell culture systems have demonstrated that stimulation of growth causes an enhanced rate of phosphate uptake (151) while attainment of confluency selectively suppresses transport of this ion (152). Increased phosphate transport, therefore, like ODC activation, appears to be part of the pleiotypic response of Nb2 cells to PRL. Investigation of phosphate uptake in proliferating Ehrlich ascites tumor cells has suggested that this process is carrier-mediated, associated with Na^+ and H^+ transport but not directly dependent on cellular ATP levels (153).

Unlike mitogenesis and ODC activity, uptake of phosphate was readily measurable in unstimulated Nb2 cells. This basal level of phosphate transport served as a useful control when examining the inhibitory effects of pharmacological agents on PRL-stimulated uptake and incorporation of phosphate. In previous studies (H. Cosby, unpublished), various agents were added to control and PRL-stimulated Nb2 cultures in an effort to determine which biochemical pathways may be important in PRL-induced mitogenesis. Although a number of reagents inhibited the effect of PRL on cell proliferation, it was difficult to determine whether this inhibition was specific -- directed at hormone-dependent pathways which regulate mitogenesis, or non-specific -- preventing cell growth because of cytotoxic side effects. In the presence of certain agents, cell number in non-growing cultures decreased over 3 days; however, this was the result of cell death or lysis, and therefore could not serve as a sensitive indicator of non-specific drug effects. The specific and non-specific actions of dbcAMP, dbcGMP and calcium ionophore A23187 on phosphate uptake and incorporation were therefore determined (table 4). Dibutyryl cAMP did not appear to inhibit PRL-stimulated effects on phosphate metabolism specifically since an inhibitory effect of similar magnitude occurred in control Nb2 cells. In the presence of A23187 (1.5 uM), both basal and PRL-enhanced uptake/incorporation of phosphate were inhibited, but the inhibitory effect in unstimulated cells was only about half that observed in stimulated cells. This may suggest that calcium ions (and possibly other divalent metal ions) specifically oppose the stimulatory effect of PRL on phosphate uptake or on utilization of phosphate in

macromolecular synthesis. Contrary to this observation, Wrogeman et al (154) showed that both concanavalin A and A23187 (1.5 uM) elicited an increase in chemiluminescence in rat thymocyte preparations -- a response which is thought to be an early event in cell activation. It is possible, of course, that other (as yet unexamined) effects of PRL in Nb2 cells can be mimicked by A23187. As discussed in the Introduction, the role of calcium in PRL-stimulated lactogenesis has not been determined; both stimulatory (or permissive) (7) and inhibitory (35) actions have been reported.

A small but significant increase in phosphate uptake and incorporation occurred in the presence of 10 uM dbcGMP (table 4). Concentrations lower or higher than 10 uM did not produce a significant stimulatory effect. Also, no significant stimulation by dbcGMP was observed in PRL-stimulated cells, suggesting that this cyclic nucleotide derivative did not act synergistically with PRL. Rather, cGMP may play a role in mediating this PRL-dependent event. The effect of butyric acid on phosphate uptake and incorporation was not assessed, and thus it is not entirely clear if the stimulatory effect of dbcGMP is a cyclic nucleotide-dependent event. In mammary gland explants, Matusik and Rosen (30) demonstrated that casein mRNA levels were elevated 1.3 to 2.3-fold in the presence of dbcGMP (10 uM). This PRL-like effect was not mimicked by butyric acid. As was observed in Nb2 cells, the stimulatory action of dbcGMP in the mammary gland represented only a fraction of the maximum response that could be elicited by PRL (30). In Nb2 cells therefore, cGMP may be

involved in mediating some PRL-dependent responses but interaction of the cyclic nucleotide with other regulatory molecules would be necessary for maximal generation of these responses.

a) PRL-regulated phosphoproteins in Nb2 cells

Nb2 lymphoma cells were examined for PRL-specific phosphoproteins which might be involved in the mitogenic response to the hormone. It has become clear that protein phosphorylation and dephosphorylation are major mechanisms for regulating the activity of enzymes and consequently the responses of cells to external stimuli (rev. in 122,156,157). Activation of peripheral blood lymphocytes by various mitogenic agents leads to enhanced phosphorylation of numerous proteins (158), suggesting that Nb2 cells may also respond to PRL stimulation in this manner.

During the G_1 phase of the cell cycle, which lasts about 10 hr in PRL-activated Nb2 cells (121), ^{32}P was incorporated largely into proteins, as evidenced by degradation of most of the ^{32}P-labeled, TCA-precipitable material with protease treatment (table 2). Addition of ^{32}P to Nb2 cells stimulated with PRL for 30-60 min caused enhanced labeling of numerous protein bands compared to controls (not shown, SDS-PAGE: equal ug protein/ track). However, this effect was due primarily to enhanced transport of phosphate in PRL-stimulated cells (figure 10, table 3) and did not reflect hormone effects on kinase activity or synthesis of protein substrates. When equal dpm ^{32}P were applied to SDS gel tracks, the appearance of two phosphorylated proteins was observed in PRL-stimulated cells in the MW range 14 to 92 kilodaltons (figure 15). Both phosphoproteins pp33 and pp19 were more readily detectable in cells stimulated with PRL prior to ^{32}P-label-

ing. This effect may have been due, in part, to increased background labeling in cells preincubated with ^{32}P.

Characterization and regulation of the PRL-sensitive phosphoproteins were examined with reference to subcellular localization, disulfide bonding, isoelectric point, biosynthesis, effects of inhibitors, expression during the Nb2 cell cycle, and dependency on continual exposure of cells to PRL. A table summarizing differences and similarities of the PRL-dependent phosphoproteins is presented in this Discussion.

The 33 K phosphoprotein was localized primarily in the particulate fraction of sonicates from Nb2 cells, while pp19 was clearly in the soluble fraction (figure 16); thus, PRL appears to regulate phosphorylation of both membrane-associated and cytosolic proteins in these cells. Electrophoretic migration of pp19 was similar under both reducing and non-reducing conditions, suggesting that in the physiological state disulfide bonding may not be required for activity of this phosphoprotein. Conversely, pp33 was detectable only under reducing conditions, but because higher molecular weight bands (of which pp33 may be a component) were not visible under non-reducing conditions (figure 16), it was unclear whether this phosphoprotein forms disulfide bridges with other Nb2 cell proteins.

Prolactin enhanced synthesis of a number of low MW, cellular proteins within 3 hr of exposure to stationary Nb2 cells (figure 17). Of particular interest was the synthesis of 19 and 33 kilodalton proteins in the presence of hormone, which suggested that de novo synthesis of pp33 and pp19 may be stimulated by PRL.

Both synthesis and phosphorylation of pp33 and pp19 may therefore act as regulatory foci during PRL stimulation.

b) Phosphorylation of ribosomal protein S6 by PRL

Initiation of a mitogenic response in cells is typically accompanied by an enhanced rate of protein synthesis (159). This metabolic change involves a large shift of inactive ribosomes and also mRNA to polysomal elements (159, 160). The phosphorylation of ribosomal protein S6 (primarily on serine residues) has been observed in various cells following treatment with growth factors and the phosphoprotein has been implicated in the regulation of protein synthesis (161). In recent years, major contributions to our understanding of S6 function have been made by Thomas and coworkers (159, 162). They have shown, firstly, that a close temporal relationship exists between the extent of S6 phosphorylation and the onset of protein synthesis (159). Secondly, concentrations of inhibitors which block S6 phosphorylation also inhibit protein synthesis (162). Thirdly, those 40S ribosomal subunits in which S6 phosphorylation is maximal are preferentially incorporated into polysomes (159). And lastly, S6 is situated in the tRNA-mRNA binding site of the 40S subunit, next to the large ribosomal subunit (161); in this position S6 could regulate ribosomal affinity for mRNA. Thus, while enhancement of protein synthesis by phosphorylated forms of S6 have not been demonstrated directly, there is much circumstantial evidence in support of this function.

In the Nb2 cell system, the effect of PRL on phosphorylation of S6 ribosomal protein was examined. The observation that mitogenic hormones stimulated phosphorylation of a 33 K protein in

Nb2 cells strengthened the argument that PRL regulated the phosphorylation of S6, which has a similar MW. During Nb2 cell fractionation, pp33 was localized in the particulate fraction. Ribosomal protein S6 also sediments in the particulate fraction (163, 164), due to its association with intracellular membranes. Two dimensional gel analysis of Nb2 cell phosphoproteins provided additional evidence that pp33 might be S6. The pI of pp33 was greater than 6.7, consistent with previous reports on the basic nature of S6 (163). Lastly, sucrose gradient analysis of phosphoproteins from Nb2 cells (post-nuclear fraction) revealed a 33 K phosphoprotein near the mid-region of the gradient -- the approximate position to which 40S particles migrate (124). Because this phosphoprotein was present only in Nb2 cells stimulated with PRL (figure 19) it could be identified as pp33. These findings suggest that PRL stimulates phosphorylation of ribosomal protein S6 in Nb2 cells.

Earlier studies in mouse mammary gland explants (8) demonstrated that phosphorylation of several ribosomal proteins is enhanced in the presence of PRL. However, because explants were not preincubated with ^{32}P prior to stimulation with PRL and because samples for electrophoresis were equalized with reference to tissue weight rather than ^{32}P (8), the apparent stimulatory effect on phosphorylation may have been due to enhanced phosphate uptake. It is thus unclear whether PRL directly triggered phosphorylation of these proteins; but since PRL also activated protein kinase activity in mammary explants (8), specific phosphorylation of cellular proteins might be expected. Turkington et al

(8) reported that phosphorylation of ribosomal proteins in mammary explants was maximal at 16 hr after addition of PRL to the medium. On the other hand, in Nb2 cells maximal phosphorylation of pp33 was achieved 1 to 3 hr after hormone stimulation (figure 24), during which time activation of protein synthesis was also observed (figure 17). Because increased protein synthesis accompanies stimulation of lactogenesis, PRL might also be expected to enhance phosphorylation of ribosomal protein S6 in the mammary gland.

C. Effects of Chloroquine and Colcemid on PRL-dependent Phosphate Metabolism.

Lysosomotrophic agents such as chloroquine, and microtubule disrupting drugs (colchicine) have been used to investigate the mechanism of PRL-stimulated lactogenesis (39 - 41). The effects of these agents on PRL action in Nb2 cells was therefore examined. Because chloroquine was toxic, inhibiting growth and ODC activation in Nb2 cells (figure 20), it was difficult to assess whether lysosome-dependent proteolysis of internalized ligand-receptor complexes was important for PRL action. However, chloroquine did not inhibit the early stimulatory effect of PRL on phosphate uptake and incorporation (figure 20). Cytotoxic effects of the drug were probably not significant during this short incubation period. Thus, in Nb2 cells (as in mammary gland explants; 39) degradation of internalized PRL/receptor complexes in lysosomes appears not to be required for mediation of hormone action.

Colcemid exhibited both non-specific and specific effects on

phosphate metabolism in PRL-stimulated Nb2 cells. The non-specific effect was apparent in cells preincubated with colcemid in which PRL-enhanced phosphate uptake and incorporation was suppressed below basal levels (figure 21). Since microtubule-disrupting drugs are known to bind tubulin components of the plasma membrane, the inhibitory effect of colcemid may have been due to impairment of phosphate transport across the cell membrane. In the mammary gland, colchicine is thought to inhibit PRL-stimulated casein synthesis specifically, because synthesis of total proteins is not significantly affected by the drug (39). However, microtubule-disrupting drugs also have non-specific inhibitory effects in the mammary gland. Colchicine (and colcemid) inhibit both PRL-stimulated and basal synthesis of DNA in mammary explants (165), an effect probably caused by non-specific inhibition of thymidine uptake (166). Thus, in both Nb2 and mammary cells, the use of microtubule-disrupting agents for examining the mechanism of PRL action may be restricted to those cellular responses which are inhibited specifically by the drugs.

Phosphorylation of pp33 and pp19, the PRL-responsive proteins in Nb2 cells, appeared to be specifically inhibited by colcemid (figure 22). However, the mechanism of this inhibition is not clear. In the mammary gland, colchicine inhibition of PRL action was thought to result from the failure of PRL 'second messengers' to be released when the drug was bound to tubulin-containing plasma membranes (85). But there are alternative explanations as well. Studies with anti-PRL receptor antibodies have suggested that receptor interaction (or 'cross-linking') is important for PRL-stimulated mitogenesis of Nb2 cells

(115). It is therefore possible that colcemid restricts the lateral mobility of PRL receptors in the plasma membrane or sterically hinders receptor cross-linking by binding to tubulin molecules near the receptors. Future studies will determine whether tubulin-binding drugs can specifically block PRL activation of ODC in Nb2 cells. Betel and Martijnse (166a) and others (166b) have reported that drugs which affect microtubule integrity do not inhibit lymphocyte activation; thus, inhibition of Nb2 cell activation by colchicine might indicate some specificity of the tubulin-binding agents toward PRL receptor containing tissues, as has been suggested by studies in the mammary system (39 - 41).

D. Effects of PRL Receptor Antibodies on Phosphate Metabolism.

The present investigation confirmed earlier studies by Shiu et al (115) which demonstrated that anti-receptor antibodies had PRL-like effects on Nb2 cell growth (table 5). In that study (115), 1% antiserum mimicked the mitogenic action of PRL but was less potent than PRL. This study shows that 0.1% antiserum was almost as effective as 1.0% antiserum in stimulating cell growth. It is therefore unlikely that antiserum (or $F(ab')_2$) concentration can account for the lower potency of anti-receptor antibodies compared to PRL. As discussed in the Introduction, quantitative differences in PRL and anti-receptor action may be accounted for in a number of ways: firstly, the mechanism by which each interacts with PRL receptors may differ, secondly, stimulatory and inhibitory antibodies may be present in the polyclonal anti-receptor serum, and thirdly, PRL molecules may act at an additional, post-receptor site in the target cell.

Anti-PRL receptor antibodies also mimicked early PRL effects in Nb2 cells. Divalent $F(ab')_2$, but not monovalent F(ab') fragments, stimulated ^{32}P incorporation in stationary cells. If anti-F(ab') antiserum was added to Nb2 cells preincubated with monovalent F(ab') fragments, a small but significant increase in phosphate uptake and incorporation was generated (figure 25). This effect was similar to that reported by Kahn et al (71), in which anti F(ab') antibodies 'restored' biological activity to monovalent anti-insulin receptor F(ab') fragments, suggesting that cross-linking of receptors was a necessary event in hormone action. Despite these studies, the possibility cannot be discounted that anti-F(ab') antibodies elicit a biological response by altering the conformation of inactive anti-receptor F(ab') fragments.

This study therefore supports earlier work from our laboratory (115) as well as studies from other laboratories (71 - 75) suggesting that 'hormonal information' is intrinsic to the hormone receptor and can be released into the cell by non-hormonal stimuli.

E. Phospholipase C and Proliferative Growth of Nb2 Cells.

The hypothetical role of membrane lipids in hormone action has often been restricted to the provision of a fluid matrix in which receptors and other membrane proteins can interact with each other, leading to generation of an intracellular hormone signal. It is becoming apparent, however, that lipids also have a more dynamic role in hormone action -- regulating ligand binding, acting as cofactors for the functional receptor and serving

as regulators for the coupling between receptors and effector systems (167). In addition, the work of Mitchell (168) and Farese (169) has contributed greatly to an understanding of the role of phosphatidylinositol, a membrane lipid, in mediating the action of certain hormones.

Dave et al (170 - 172) have demonstrated that PRL binding in liver membranes is dependent on the lipid environment. Both PRL (170) and prostaglandin I_2 (171) caused a concomitant increase in membrane fluidity and hormone binding, an effect which was prevented by the prostaglandin synthesis inhibitor, indomethacin (172). The investigators suggested that the number of PRL receptors in target tissues could be regulated by the effect of PRL on membrane microviscosity; synthesis of specific prostaglandins was a necessary intermediate event. How prostaglandins alter membrane fluidity is not yet clear. Others (173) have shown that phospholipase A_2, an apparently important enzyme in PRL-stimulated synthesis of prostaglandins (7), causes a decrease in fluidity of hepatic membranes.

Because exogenous phospholipases can alter the properties of plasma membranes, they have been used to investigate the role of membrane phospholipids in hormone action. In general, studies have focussed on the importance of phospholipids in hormone-receptor interaction, but because the effects of phospholipases vary considerably, depending on the hormone system studied, no common function for membrane phospholipids has been found (167). In studies of PRL receptors, treatment of mammary (174) or hepatic (175) membranes with phospholipase C caused a decrease

in binding activity, suggesting that phospholipid moieties were required for PRL-receptor interaction.

In recent years, phospholipase C has become of special interest because of its ability to hydrolyze phosphatidylinositol, a minor lipid component of the cell membrane, into inositol phosphates and diacylglycerol. The latter activates the Ca^{++}-dependent enzyme, protein kinase C. It is thought (176) that protein kinase C may mediate the action of hormones that trigger phosphatidylinositol turnover, and that diacylglycerol may act as the 'second messenger' in these systems. Although phospholipase C from Clostridium perfringens has a broad specificity range (177) but with little or no effect on phosphatidylinositol, it has been shown to enhance diacylglycerol production and activate protein kinase C when added exogenously to platelets (178) and pancreatic cells (179). The demonstration by Kuramoto et al (123) that phospholipase C activates ODC in lymphocytes, suggested that an effect of this enzyme on Nb2 cell mitogenesis might be demonstrable.

In the present study, phospholipase C stimulated phosphate uptake and incorporation in stationary cells in a PRL-like manner (figure 26), but did not stimulate cell growth. Thus, although increased incorporation of phosphate is characteristic of Nb2 cells mitogenically stimulated by PRL, this effect (like ODC activation) can be dissociated from the growth response under certain conditions.

Exogenous phospholipase C did enhance proliferation of PRL-stimulated cells (figures 27 - 29). Light microscopy did not reveal any morphological changes in Nb2 cells grown in the pres-

ence of the enzyme (not shown).

Several aspects of these data (figures 27 - 29) deserve comment. First of all, it is unlikely that phospholipase C triggered the release or production of PRL 'second messengers', since, a) it had no stimulatory effect on cell growth in the absence of hormone, and b) the additive stimulatory effect of the enzyme could not be mimicked by increasing the concentration of PRL. Phospholipase C, therefore, potentiates the mitogenic action of PRL. Secondly, the effect of phospholipase C probably involves a direct action on the cell. When phospholipase C was removed from cultures pretreated with the enzyme, cells retained the 'potential' for enhanced growth upon exposure to PRL (figure 29). This makes the possibility unlikely that phospholipase C (or a contaminant in the commercial enzyme preparation) was activating a serum factor(s) which could synergistically promote the growth of PRL-stimulated cells. Lieberman (180) has recently isolated a novel mitogenic protein from 3T3 cell plasma membranes which is released by mild trypsin treatment. In Nb2 cells, phospholipase C may activate or liberate a membrane protein capable of augmenting the mitogenic response of the cells. Thirdly, the 'phospholipase C effect' is probably not due to a contaminant in the enzyme preparation (Sigma type I). Kuramoto et al (123) compared the effects of Sigma type I phospholipase C and purified phospholipase C (> 100-fold purification vs. type I) on ODC activation in lymphocytes. When compared on an activity basis (ie. equal U/ml), the stimulatory effect of crude and purified enzyme was identical. Furthermore, in the present study trypsin was unable

to generate a phospholipase C-like response (not shown), indicating that this protease was not significantly affecting growth of Nb2 cells. Fourthly, although phospholipase C from Clostridium has been shown to activate protein kinase C (178, 179), alternative explanations for the stimulatory effects of this enzyme should be considered. Takahashi et al (177) have observed that Clostridium phospholipase C shows a broad specificity toward various phospholipids, but particularly to phosphatidylcholine and sphingomyelin -- the two major phospholipids in the external monolayer of the plasma membrane. Thus, exogenous phospholipase C (Clostridium) may exert its hydrolytic effect primarily on these phospholipids. Changes in either membrane fluidity or cholesterol: phospholipid ratios (181) could affect the conformation of individual PRL receptors or alter receptor-receptor interaction; such alterations could, in turn, favor an amplified mitogenic signal. As mentioned above, activation or release of a growth-enhancing protein could also account for the phospholipase effect; changes in membrane fluidity could regulate the activation or release of the factor. Fifthly, prostaglandins may not account for the phospholipase C-enhanced growth of Nb2 cells. Unlike phospholipase A, phospholipase C does not convert membrane phospholipids into fatty acyl precursors (ie. arachidonic acid) for prostaglandin synthesis. Lastly, the ability of phospholipase C to stimulate the growth of Nb2-SP cells indicates that the presence of PRL, per se, was not a requirement for the synergistic effect of the enzyme. However, since both Nb2 and Nb2-SP cells contain PRL receptors, an effect of phospholipase C involving these receptors cannot be ruled out. It is unclear at

present whether the PRL receptor of Nb2-SP cells is required for mitogenesis. While growth of this variant may be completely independent of PRL, it is conceivable that in Nb2-SP cells the receptor is permanently 'coupled' to a membrane effector system which continually transmits mitogenic signals into the cell. In this case, phospholipase C could enhance growth of the variant by altering the microenvironment of PRL receptors in the membrane.

F. Retention of a Hormone 'Signal' by Nb2 Cells Incubated Briefly with PRL.

Nb2 cells may provide a useful model for studying the mechanism of PRL-stimulated DNA synthesis and mitogenesis. In the present study, Nb2 cells were exposed to PRL for periods ranging from 10 to 60 min, incubated in the absence of hormone and then examined for a residual stimulatory effect on phosphate uptake and incorporation -- an early event in PRL-dependent mitogenesis.

As shown in the results (figure 30) even a 10 min exposure of Nb2 cells to PRL was sufficient for triggering a PRL-like effect following hormone withdrawal. Antibodies which effectively neutralized free PRL (figure 31) did not block the PRL-like 'signal' retained by the cells. This result indicates that the stimulatory effect was not due to residual PRL in the medium but to a PRL-induced cellular alteration. It is possible that the 'signal' was due to intact PRL molecules associated tightly with cell surface receptors and hence, not accessible to hormone antibodies. However, Shiu et al (115) have shown that cell-associated ^{125}I-PRL is cleared rapidly from Nb2 cells, both by dissociation and by degradation of the bound hormone. In the present

study, Nb2 cells briefly stimulated with PRL were incubated for 30 min (37°C) with anti-PRL antibodies prior to the addition of ^{32}P (figure 30). Thus, most of the intact, cell surface-bound PRL would have dissociated from the cells or been inactivated intracellularly. Two possible explanations for the retention of a PRL 'signal' are considered here. Firstly, PRL may rapidly alter the structure of cell membranes, allowing for a faster rate of phosphate uptake. This membrane alteration could involve, a) the perturbation of specific lipids (possibly near the receptor) or, b) the activation of phosphate carriers (153) within the cell membrane. An action of this type may not require a PRL 'second messenger' at all, but could be a direct consequence of either PRL-receptor interaction or receptor 'capping'. Secondly, PRL may trigger the release of a receptor-associated factor that (either directly or indirectly) increases membrane permeability to phosphate ions, activates specific protein kinases or prolongs the half-life of intracellular phosphoproteins.

An early effect of PRL on protein phosphorylation, and one that could trigger subsequent actions leading to mitogenesis, may be phosphorylation of the PRL receptor. Receptors for insulin (182), EGF (183, 184) and PDGF (185) (all growth-promoting hormones), undergo hormone-induced phosphorylation. They also possess protein kinase activity with specificity for tyrosine residues. Because the Nb2 PRL receptor triggers mitogenesis, it may also function via this mechanism. Tyrosine phosphorylation may prove to be an important regulatory mechanism for growth control, but at present it is incompletely understood (186). For instance, certain EGF analogs and anti-receptor F(ab') fragments stimulate

tyrosine phosphorylation but not growth. Also, growth factor concentrations that stimulate tyrosine phosphorylation maximally are often 10-fold greater than those needed for maximal stimulation of mitogenesis. Furthermore, tyrosine phosphorylation is rapid -- often reaching reaching a maximum in several minutes; competence to synthesize DNA, on the other hand, often requires exposure to growth factor for several hours. Isolation and functional characterization of proteins which are phosphorylated by receptor kinases should provide a better understanding of the biological relevance of tyrosine phosphorylation.

In Nb2 cells, the PRL-dependent phosphoproteins pp33 and pp19, responded differently to hormone withdrawal (figure 32). Clearly, pp33 phosphorylation was dependent on the continual presence of PRL, while pp19 was phosphorylated if Nb2 cells were exposed to PRL for at least 30 to 60 min. This may indicate that pp33 has a shorter half-life than pp19 or is preferentially (compared to pp19) dephosphorylated by cellular phosphatases. An alternative explanation may be that PRL transmits various 'signals' into Nb2 cells -- some 'long-lived' and others 'short-lived'. Because of their intrinsic lability or rapid rate of enzymatic degradation, the latter would require the continual presence of hormone. Thus, pp33 may be a site of action for 'short-lived signals' while pp19 is acted on by PRL 'signals' retained after hormone withdrawal. While the role of pp19 is unknown, it is interesting that in PRL-stimulated cells the time course of pp19 phosphorylation (figure 24) parallels the induction of ODC activity. It is conceivable that pp19 may regulate specific cell

functions such as ODC activation, during the G_1 phase of the cell cycle.

Servely et al (187) and Houdebine (165) have examined whether exposure of mammary explants (and isolated cells) to PRL produces a 'signal' that can stimulate casein synthesis following withdrawal of hormone. These studies showed that a 6 hr exposure of explants and a 2 to 6 hr exposure of epithelial cells to PRL permitted synthesis of casein or casein mRNA for one day after hormone removal. However, unlike Nb2 cells, incubation of mammary explants or cells with anti-PRL antibodies abolished the 'signal' generated by PRL. The investigators suggested that a small amount of PRL firmly bound to its receptors was necessary to generate the 'intracellular relays' which activated casein genes (187), and that the 'relay' was essentially unstable (165). In view of their other studies, showing that PRL can trigger release of a stable 'factor' which induces casein gene transcription in isolated nuclei (84, 85), it is somewhat suprising that antibodies to PRL were able to neutralize the PRL 'signal' (165, 187). It might be suggested that in intact cells, cytoplasmic factors rapidly degrade the 'second messenger'; thus, PRL must be continually present for casein mRNA synthesis to be stimulated. However, Servely et al (87) have also reported that the 'factor' stimulated casein gene when added to the culture medium of mammary epithelial cells (24 hr). From this it would seem that activity of the 'factor' is not affected by serum or by cellular enzymes. The precise nature of this putative second messenger, therefore, awaits further characterization.

G. Bioassays for Lactogenic Hormones

The Nb2 cell growth assay is a remarkably sensitive and specific bioassay for PRL-like hormones (111). The present study may provide a means for improving the assay. Small quantities of phospholipase C (0.03 - 0.04 U/ml) in the culture medium enhanced growth of lactogen-stimulated cells but caused a slight decrease in cell number in unstimulated cultures (figure 27). Phospholipase C, therefore, 'extends' the linear portion of the standard growth curve.

One drawback of the growth assay is the length of time required for its completion (3 days). As shown in this investigation, both ODC activation and stimulation of phosphate uptake and incorporation are early cellular events in PRL-stimulated mitogenesis. Since the phosphorylation assay lasts only 3 hr, it is probably the most rapid bioassay for PRL-like hormones currently available. Both phosphorylation and ODC induction exhibit the same hormone specificity as mitogenesis and may thus be useful for rapid detection of lactogenic hormones in biological fluids. Furthermore, these assays could be helpful in monitoring the bioactivity of lactogenic hormones during hormone purification.

Richards et al (121) first proposed that ODC induction in Nb2 cells could serve as a bioassay for PRL and related hormones. While this assay (figure 2) may be more sensitive to hormone than phosphorylation (figure 12), it requires large quantities of cells (compared to the growth or phosphorylation assays) and would be too tedious to perform with large numbers of samples. Furthermore, ODC activation (maximum at 6 to 8 hr) is a later res-

ponse to PRL than is phosphorylation (3 hr).

The phosphorylation assay, on the other hand, is both rapid and simple to perform. It can be adapted to large numbers of samples and requires fewer than 10^5 cells per assay point. In addition, intra- and inter-assay variation is small (SD < 5% and 10%, respectively). The drawback of this assay is its lower sensitivity to lactogenic hormones relative to the growth assay, although this problem can be greatly minimized by reducing the reaction volume. In this study, a convenient volume of 1 ml was used in the phosphorylation assay; by reducing the assay volume from 1 ml to 0.1 or 0.2 ml, the assay sensitivity could be improved by more than 5-fold. Under these conditions, however, it would be necessary to examine the effect of increased sample concentration on ^{32}P incorporation. In conclusion, lactogen-dependent phosphorylation in Nb2 cells may be used as a convenient bioassay for PRL-like hormones, particularly when a rapid assay system is required.

COMPARISON OF PRL-RESPONSIVE PROTEINS pp33 AND pp19

	pp33	pp19
1. Subcellular Fraction	particulate (ribosomal)	soluble
2. Sensitive to 2-mercaptoethanol	+	-
3. Phosphorylated in non-stimulated cells	-	+
4. Inhibited by colcemid	+	+
5. Time of peak phosphorylation after PRL exposure	1 - 3 hr	7 - 9 hr
6. Continual presence of PRL necessary for phosphorylation	+	-
7. Isoelectric point	> 6.7	4.6, 5.7 ?
8. Possible identity	ribosomal protein S6	?

REFERENCES

1. Nicoll, C.S. (1974). Handbook of Physiology, Sec. 7: Endocrinology, Vol. IV, part 2. Bethesda, Maryland. Am. Physiol. Soc., pp. 253 - 292.

2. Majumder, G.C. and Turkington, R.W. (1971). J. Biol. Chem. 246: 2650 - 2657.

3. Roy, C., Guillon, G. and Jard, S. (1976). Biochem. Biophys. Res. Commun. 72: 1265 - 1270.

4a. Kirk, C.J. and Hems, D.A. (1974). FEBS Lett. 47: 128 - 131.

4b. Keppens, S. and de Wulf, H. (1975). FEBS Lett. 51: 29 - 32.

5. Shiu, R.P.C. and Friesen, H.G. (1980). Ann. Rev. Physiol. 42: 83 - 96.

6. Shiu, R.P.C. (1980). Prog. Reprod. Biol. 6: 97 - 112.

7. Rillema, J.A. (1980). Fed. Proc. 39: 2593 - 2598.

8. Turkington, R.W., Majumder, G.C., Kadohama, N., MacIndoe, J.H. and Frantz, W.L. (1973). Recent Prog. Horm. Res. 29: 417 - 449.

9. Rillema, J.A. (1975). Horm. Metab. Res. 7: 45 - 49.

10. Sapag-Hagar, M., Greenbaum, A.L., Lewis, D.J. and Hallowes, R.C. (1974). Biochem. Biophys. Res. Commun. 59: 261 - 268.

11. Smith, R.D. and Rillema, J.A. (1975). Proc. Soc. Exp. Biol. Med. 150: 763 - 765.

12. Sapag-Hagar, M. and Greenbaum, A.L. (1974). FEBS Lett. 46: 180 - 183.

13. Kleczkowska, D. and Chomczynski, P. (1976). Bull. Acad. Pol. Sci., Ser. Sci. Biol. 24: 189 - 193.

14. Matusik, R.J. and Rosen, J.M. (1980). Endocrinology 106: 252 - 259.

15. Rillema, J.A. (1975). Nature 256: 466 - 467.

16. Rillema, J.A. (1975). Prostaglandins 10: 307 - 312.

17. Rillema, J.A. (1976). Endocrinology 99: 490 - 495.

18. Rillema, J.A. and Wild, E.A. (1977). Endocrinology 100: 1219 - 1222.

19. Rillema, J.A. (1979). Prostaglandins Med. 2: 155 - 160.

20. Rillema, J.A. and Anderson, L.D. (1976). Biochem. Biophys. Acta 428: 819 - 824.

21. Janne, J., Poso, H. and Raina, A. (1978). Biochem. Biophys. Acta 473: 241 - 293.

22. Tabor, C.W. and Tabor, H. (1976). Ann. Rev. Biochem. 45: 285 - 306.

23. Goyns, M.H. (1982). J. Theor. Biol. 97: 577 - 589.

24. Russel, D.H. and McVicker, T.A. (1972). Biochem. J. 130: 71 -76.

25. Oka, T. and Perry, J.W. (1976). J. Biol. Chem. 251: 1738 - 1744.

26. Rillema, J.A. (1976). Endocr. Res. Commun. 3: 297 - 305.

27. Frazier, R.P. and Costlow, M.E. (1982). Exp. Cell Res. 138: 39 - 45.

28. Rillema, J.A. (1976). Biochem. Biophys. Res. Commun. 70: 45 - 49.

29. Rillema, J.A., Linebaugh, B.E. and Mulder, J.A. (1977). Endocrinology 100: 529 - 535.

30. Matusik, R.J. and Rosen, J.M. (1978). J. Biol. Chem. 253: 2343 - 2347.

31. Kano, K. and Oka, T. (1976). J. Biol. Chem. 251: 2795 - 2800.

32. Houdebine, L.M., Devinoy, E. and Delouis, C. (1978). Biochimie 60: 735 - 741.

33. Falconer, I.R., Forsyth, I.A., Wilson, B.M. and Dils, R. (1978). Biochem. J. 172: 509 - 516.

34. Houdebine, L.M. and Djiane, J. (1980). Biochimie 62: 433 - 440.

35. Gopalakrishnan, N., Ramaswang, S., Padmanabha Pillai, N., Ranganathan, S. and Ghosh, M.N. (1980). Experientia 36: 1423 - 1425

36. Houdebine, L.M. (1981). Biol. Cell 40: 129 - 141.

37. Houdebine, L.M. (1983). Annales d'Endocrinologie 44: 85 - 100.

38. Zor, U. (1983). Endocr. Rev. 4: 1 - 21.

39. Houdebine, L.M. and Djiane, J. (1980). Molec. Cell. Endocr. 17: 1 - 15.

40. Teyssot, B. and Houdebine, L.M. (1980). Biochem. Biophys. Res. Commun. 97: 463 - 473.

41. Teyssot, B. and Houdebine L.M. (1981). Eur. J. Biochem. 117: 563 - 568.

42. Guyette, W.A., Matusik, R.J. and Rosen, J.M. (1979). Cell 14: 1013 - 1023.

43. Houdebine, L.M., Ollivier-Bousquet, M. and Djiane, J. (1982). Biochimie 64: 21 - 28.

44. Posner, B.I., Verma, A.K., Patel, B.A. and Bergeron, J.J.M. (1982). J. Cell Biol. 93: 560 - 567.

45. Catt, K.J., Harwood, J.P., Aguilera, G. and Dufau, M.L. (1979). Nature 280: 109 - 116.

46. King, A.C. and Cuatrecasas, P. (1981). N. Engl. J. Med. 305: 77 - 88.

47. Fox, C.F. and Das, M. (1979). J. Supramol. Struct. 10: 199 - 214.

48. Posner, B.I., Josefsburg, Z. and Bergeron, J.J.M. (1979). J. Biol. Chem. 254: 12494 - 12499.

49. Mitra, S. and Rao, C.V. (1978). Arch. Biochem. Biophys. 191: 331 - 340.

50. Goldfine, I.D. (1981). Biochim. Biophys. Acta 650: 53 - 67.

51. Yankner, B.A. and Shooter, E.M. (1979). Proc. Natl. Acad. Sci. 76: 1269 - 1273.

52. Shiu, R.P.C. (1980). J. Biol. Chem. 255: 4278 - 4281.

53. Costlow, M.E. and Hample, A. (1982). J. Biol. Chem. 257: 9330 - 9334.

54. Nolin, J.M. and Witorsch, R.J. (1976). Endocrinology 99: 949 - 958.

55. Nolin, J.M. and Bogdanove, E.M. (1980). Biol. Reprod. 22: 393 - 416.

56. Djiane, J., Houdebine, L.M. and Kelly, P.A. (1982). Endocrinology 110: 791 - 795.

57. Mittra, I. (1980). Biochem. Biophys. Res. Commun. 95: 1750 - 1759.

58. Mittra, I. (1980). Biochem. Biophys. Res. Commun. 95: 1760 - 1767.

59. Djiane, J., Houdebine, L.M. and Kelly P.A. (1981). Proc. Soc. Exp. Biol. Med. 168: 378 - 381.

60. Dunaif, A.E., Zimmerman, E.A., Friesen, H.G. and Frantz, A.G. (1982). Endocrinology: 110: 1465 - 1471.

61. Kelly, P.A., Djiane, J. and Leblanc, G. (1983). Proc. Soc. Exp. Biol. Med. 172: 219 - 224.

62. Kelly, P.A., Djiane, J. and De Lean, A. (1980). Prog. Reprod. Biol. 6: 124 - 136.

63. Maxfield, F.R., Willingham, M.C., Davies, P.J. and Pastan, I. (1979). Nature 277: 663 - 665.

64. Shiu, R.P.C. and Friesen, H.G. (1976). Science 192: 259 -261.

65. Bohnet, H.G., Shiu, R.P.C., Grinwich, D. and Friesen, H.G. (1978). Endocrinology 102: 1657 - 1661.

66. Dusanter-Fourt, I., Djiane, J., Houdebine, L.M. and Kelly, P.A. (1983). Life Sci. 32: 407 - 412.

67. Djiane, J., Houdebine, L.M. and Kelly, P.A. (1981). Proc. Natl. Acad. Sci. 78: 7445 - 7448.

68. Edery, M., Djiane, J., Houdebine, L.M. and Kelly, P.A. (1983). Cancer. Res. 43: 3170 - 3174.

69. Rosa, A.A.M., Djiane, J., Houdebine, L.M. and Kelly, P.A. (1982). Biochem. Biophys. Res. Commun. 106: 243 - 249.

70. Baldwin, G.S., Grego, B., Hearn, M.T.W., Knesel, J.A., Morgan, F.J. and Simpson, R.J. (1983). Proc. Natl. Acad. Sci. 80: 5276 - 5280.

71. Kahn, C.R., Baird, K.L., Jarett, D.B. and Flier, J.S. (1978). Proc. Natl. Acad. Sci. 75: 4209 - 4213.

72. Schechter, Y., Hernaez, L., Schlessinger, J. and Cuatrecasas, P. (1979). Nature 278: 835 - 838.

73. Schreiber, A.B., Libermann, T.A., Lax, I., Yarden, Y. and Schlessinger, J. (1983). J. Biol. Chem. 258: 846 - 853.

74. Valente, W.A., Vitti, P., Yavin, E., Rotella, C.M., Grollman, E.F., Toccafondi, R.S. and Kohn, L.D. (1982). Proc. Natl. Acad. Sci. 79: 6680 - 6684.

75. Podesta, E.J., Solano, A.R., Attar, R., Sanchez, M.L. and Vedia, L.M.Y. (1983). Proc. Natl. Acad. Sci. 80: 3986 - 3990.

76. Pillion, D.J., Carter-Su, C.A., Pilch, P.F. and Czech, M.P. (1980). J. Biol. Chem. 255: 9168 - 9176.

77. Baldwin, Jr., D., Terris, S. and Steiner, D.F. (1980). J. Biol. Chem. 255: 4028 - 4034.

78. Jarett, L., Schweitzer, J.B. and Smith, R.M. (1980). Science 210: 1127 - 1128.

79. Jarett, L., and Smith, R.M. (1977) J. Supermol. Struct. 6: 45 - 59.

80. Lyen, K.R., Smith, R.M. and Jarett, L. (1982) J. Receptor Res. 2: 523 - 530.

81. Lyen, K.R. (1983). Diabetologia 24: 304 - 305.

82. Carpentier, J.L., Van Obberghen, E., Gorden, P. and Orci, L. (1979). Diabetes 28: 345.

83. Purrello, F., Burnham, D.B. and Goldfine, I.D. (1983). Science 221: 462 - 464.

84. Teyssot, B., Houdebine, L.M. and Djiane, J. (1981). Proc. Natl. Acad. Sci. 78: 6729 - 6733.

85. Teyssot, B., Djiane, J., Kelly, P.A. and Houdebine, L.M. (1982). Biol. Cell 43: 81 - 88.

86. Martel, P., Houdebine, L.M. and Teyssot, B. (1983). FEBS Lett. 154: 55 - 59.

87. Servely, J.L., Teyssot, B., Houdebine, L.M., Delouis, C., Djiane, J., and Kelly, P.A. (1982). FEBS Lett. 148: 242 - 246.

88. Houdebine, L.M., Djiane, J., Teyssot, B., and Kelly, P.A. (1983). Reprod. Nutr. Develop. 23(2B): 403 - 410.

89. Larner, J., Huang, L.C., Brooker, G. Murad, F. and Miller, T.B. (1974). Fed. Proc. 33: 261.

90. Larner, J., Huang, L.C., Hazen, R., Brooker, G. and Murad, F. (1975). Diabetes 24: 394.

91. Seals, J.R. and Czech, M.P. (1981). J. Biol. Chem. 256: 2894 - 2899.

92. Kiechle, F.L., Jarett, L., Kotagal, N. and Popp, D.A. (1981). J. Biol. Chem. 256: 2945 - 2951.

93. McDonald, J.M., Pershadsingh, H.A., Kiechle, F.L. and Jarett, L. (1981). Biochem. Biophys. Res. Commun. 100: 857 - 864.

94. Saltiel, A.R., Siegel, M.I., Jacobs, S. and Cuatrecasas, P. (1982). Proc. Natl. Acad. Sci. 79: 3513 - 3517.

95. Zhang, S.R., Shi, Q.H. and Ho, R. (1983). J. Biol. Chem. 258: 6471 - 6476.

96. Larner, J. (1982). J. Cycl. Nuc. Res. 8: 289 - 296.

97. Horvat, A. (1980). Nature 286: 906 - 908.

98. Kiechle, F.L., Strauss, J.F.,III, Tanaka, T., and Jarett, L. (1982). Fed. Proc. 41: 1082.

99. Rodgers, J.R., Yu-Lee, L.Y. and Rosen, J.M. (1983). 65th Endocr. Soc. Ann. Mtg. (Abst.# 569). p. 223.

100a. Lamm., M.E. (1976). Adv. Immunol. 22: 223 - 290.

100b. Bienenstock, J., Befus, D., McDermott, M., Mirski, S. and Rosenthal, K. (1983). Fed. Proc. 42: 3213 - 3217.

101. Weisz-Carrington, P., Roux, M.E. and Lamm, M.E. (1977). J. Immunol. 119: 1306 - 1309.

102. Weisz-Carrington, P., Roux, M.E., McWilliams, M., Phillips-Quagliata, J.M. and Lamm, M.E. (1978). Proc. Natl. Acad. Sci. 75: 2928 - 2932.

103. Alm, P., Brandt, L. and Olsson, H. (1983). Virchows Arch. 399: 355 - 360.

104. Schouten, J.T., Weese, J.L. and Carbone, P.P. (1981). Ann. Surg. 194: 749 - 753.

105. Mann, R.B. and Berard, C.W. (1977). Lancet # 8028: 84.

106. Karmali, R.A. and Horrobin, D.F. (1977). Lancet # 8039: 654.

107. Nagy, E. and Berczi, I. (1981). Allergy 36: 351 - 357.

108. Berczi, I., Nagy, E., Kovacs, K. and Horvath, E. (1981). Acta Endocr. 98: 506 - 513.

109. Noble, R.L., Beer, C.T. and Gout, P.W. (1980). Cancer Res. 40: 2437 - 2440.

110. Gout, P.W., Beer, C.T. and Noble, R.L. (1980). Cancer Res. 40: 2433 - 2436.

111. Tanaka, T., Shiu, R.P.C., Gout, P.W., Beer, C.T., Noble, R.L. and Friesen, H.G. (1980). J. Clin. Endocr. Metab. 51: 1058 - 1063.

112. Cowden, E.A., Friesen, H.G., Gout, P.W., Beer, C.T. and Noble, R.L. (1980). Clin. Res. 28: 695A.

113. Rowe, R.C., Cowden, E.A., Tolis, G. and Friesen, H.G. (1981). Clin. Invest. Med. 4: 12B.

114. Tanaka, T., Shishiba, Y., Gout, P.W., Beer, C.T., Noble, R.L. and Friesen. H.G. (1983). J. Clin. Endocr. Metab. 56: 18 - 20.

115. Shiu, R.P.C., Elsholtz, H.P., Tanaka, T., Friesen, H.G., Gout, P.W., Beer, C.T., and Noble, R.L. (1983). Endocrinology 113: 159 - 165.

116. Hughes, J.P., Simpson, J.S.A. and Friesen, H.G. (1983). Endocrinology 112: 1980 - 1985.

117. Haeuptle, M.T., Aubert, M.L., Djiane, J. and Kraehenbuhl, J.P. (1983). J. Biol. Chem. 258: 305 - 314.

118. Shiu, R.P.C. (1980). Prog. Reprod. Biol. 6: 97 - 112.

119. McManus, M.J., Dembroske, S.E., Pienkowski, M.M., Anderson, J.J., Mann, L.C., Schuster, J.S., Vollwiler, L.L. and Welsch, C.W. (1978). Cancer Res. 38: 2343 - 2349.

120. Maiti, B.R., Chakraborty, S. and Boral, A. (1982). Arch. Histol. Jap. 45: 449 - 452.

121. Richards, J.F., Beer, C.T., Bourgeault, C., Chen, K. and Gout, P.W. (1982). Molec. Cell. Endocr. 26: 41 - 49.

122. Greengard, P. (1978). Science 199: 146 - 152.

123. Kuramato, A., Otani, S., Matsui, I. and Morisawa, S. (1983). FEBS Lett. 151: 233 - 236.

124a. Laemmli, U.K. (1970). Nature 227: 680 - 681.

124b. Cabral, F. and Schatz, G. (1979). Meth. Enzymol. 56: 602 - 613.

124c. Rosen, O.M., Rubin, C.S., Cobb, M.H. and Smith, C.J. (1981). Cold Spring Harb. Symp. 8: 771 - 781.

125. Kay, J.E. and Cooke, A. (1971). FEBS Lett. 16: 9 - 12.

126. Fillingame, R.H. and Morris, D.R. (1973). Biochemistry 12: 4479 - 4487.

127. Kay, J.E. and Lindsay, V.J. (1973). Exp. Cell Res. 77: 428 - 436.

128. Canellakis, E.S., Viceps-Madore, D. Kyriakidis, D.A. and Heller, J.S. (1979). Curr. Top. Cell. Reg. 15: 155 - 202.

129a. O'Keefe, E.J. and Pledger, W.J. (1983). Molec. Cell. Endocrinol. 31: 167 - 186.

129b. Yarden, Y., Schreiber, A.B. and Schlessinger, J. (1982). J. Cell Biol. 92: 687 - 693.

129c. Aston, R. and Ivanyi, J. (1983). EMBO J. 2: 493 - 497.

130. Mamont, P.S., Duchesne, M.C., Grove, J. and Bey, P. (1978). Biochem. Biophys. Res. Commun. 81: 58 - 66.

131. Oredsson, S., Anehus, S. and Heby, O. (1980). Biochem. Biophys. Res. Commun. 94: 151 - 158.

132. Bartholeyns, J. and Koch-Weser, J. (1981). Cancer Res. 41: 5158 - 5161.

133. Luk, G.D., Goodwin, G., Marton, L.J. and Baylin, S.B. (1981). Proc. Natl. Acad. Sci. 78: 2355 - 2358.

134. Gazitt, Y. and Friend, C. (1980). Cancer Res. 40: 1727 - 1737.

135. Gazitt, Y. (1981). Cancer Res. 41: 2959 - 2961.

136. Takigawa, M., Hiroshi, I., Takano, T. and Suzuki, F. (1980). Proc. Natl. Acad. Sci. 77: 1481 - 1485.

137. Schindler, J., Kelly, M. and McCann, P.P. (1983). Biochem. Biophys. Res. Commun. 114: 410 - 417.

138. Bartolome, J., Huguenard, J. and Slotkin, T.A. (1980). Science 210: 793 - 794.

139. Luk, G.D., Sharkis, S.J., Abeloff, M.D., McCann, P.P., Sjoerdsma, A. and Baylin, S.B. (1983). Proc. Natl. Acad. Sci. 80: 5090 - 5093.

140. Niskanen, E., Kallio, A., McCann, P.P. and Baker, D.G. (1983). Blood 61: 740 - 745.

141. Prakash, N.J., Schechter, P.J., Grove, J. and Koch-Weser, J. (1978). Cancer Res. 38: 3059 - 3062.

142. Kingsnorth, A.N., McCann, P.P., Diekema, K.A., Ross, J.S. and Malt, R.A. (1983). Cancer Res. 43: 4031 - 4034.

143a. Metcalf, B.W., Bey, P., Danzin, C., Jung, M.J., Casara, P. and Vevert, J.P. (1978). J. Am. Chem. Soc. 100: 2551 - 2553.

143b. Rillema, J.A. and Cameron, C.M. (1983). Proc. Soc. Exp. Biol. Med. 174: 28 - 32.

144. Rupniak, H.T. and Paul, D. (1978). J. Cell. Physiol. 94: 161 - 170.

145. Inoue, H. and Oka, T. (1980). J. Biol. Chem. 255: 3308 - 3312.

146. Pegg, A.E., Hibasami, H., Matsui, I. and Bethell, D.R. (1981). Adv. Enzym. Reg. 19: 427 - 451.

147. Alhonen-Hongisto, L., Seppanen, P., Holtta, E. and Janne, J. (1982). Biochem. Biophys. Res. Commun. 106: 291 - 297.

148. Bachrach, U. (1970). Ann. N.Y. Acad. Sci. 171: 939 - 959.

149. Weiss, J.M., Lembach, K.J. and Boueck, Jr., R.J. (1981). Biochem. J. 194: 229 - 239.

150. McConlogue, L. and Coffino, P. (1983). J. Biol. Chem. 258: 8384 - 8388.

151. Cunningham, D.D. and Pardee, A.B. (1969). Proc. Natl. Acad. Sci. 64: 1049 - 1056.

152. Weber, M.J. and Edlin, G. (1971). J. Biol. Chem. 246: 1828 - 1833.

153. Wehrle, J.P. and Pedersen, P.L. (1982). J. Biol. Chem. 257: 9698 - 9703.

154. Wrogeman, K., Weideman, M.J., Peskar, B.A., Staudinger, H., Rietschel, E.T. and Fischer, H. (1978). Eur. J. Immunol. 8: 749 - 752.

155. deleted.

156. Cohen, P. (1982). Nature 296: 613 - 620.

157. Ingebritsen, T.S. and Cohen, P. (1983). Science 221: 331 - 338.

158. Chaplin, D.D., Wedner, H.J. and Parker, C.W. (1980). J. Immunol. 124: 2390 - 2398.

159. Thomas, G., Martin-Perez, J., Siegmann, M. and Otto, A. (1982). Cell 30: 235 - 242.

160. Rudland, P. (1974). Proc. Natl. Acad. Sci. 71: 750 - 754.

161. Martin-Perez, J., Thomas, G., Novak-Hofer, I. and Thomas, G. (1983). Cell Biol. Intl. Rep. 7: 501 - 502.

162. Thomas, G., Siegmann, M., Kubler, A.M., Gordon, J. and

Jimenez de Asna, L. (1980). Cell 19: 1015 - 1023.

163. Freedman, S.D. and Jamieson, J.D. (1982). J. Cell Biol. 95: 909 - 917.

164. Chambard, J.C., Franchi, A., Le Cam, A. and Pouyssegur, J. (1983). J. Biol. Chem. 258: 1706 - 1713.

165. Houdebine, L.M. (1980). Eur. J. Cell. Biol. 22: 755 - 760.

166a. Betel, I. and Martijnse, J. (1976). Nature 261: 318 - 320.

166b. Cuthbert, J.A. and Shay, J.W. (1983). J. Cell. Physiol. 116: 127 - 134.

167. Loh, H.H. and Law, P.Y. (1980). Ann. Rev. Pharmacol. Toxicol. 20: 201 - 234.

168. Michell, R. (1975). Biochem. Biophys. Acta 415: 81 - 147.

169. Farese, R.V. (1983). Endocr. Rev. 4: 78 - 95.

170. Dave, J.R., Brown, N.V. and Knazek, R.A. (1982). Biochem. Biophys. Res. Commun. 108: 193 - 199.

171. Dave, J.R. and Knazek, R.A. (1980). Proc. Natl. Acad. Sci. 77: 6597 - 6600.

172. Dave, J.R. and Knazek, R.A. (1981). Endocr. Soc. Meet. Cincinnati, OH, Abstr. no. 553.

173. Liu, M.S., Ghosh, S. and Yang, Y. (1983). Life Sci. 33: 1995 - 2002.

174. Shiu, R.P.C. and Friesen, H.P. (1974). Biochem. J. 140: 301 - 311.

175. Silverstein, A.M. and Richards, J.F. (1979). Biochem. J. 178: 743 - 751.

176. Nishizuka, Y. (1983). Trends Biochem. Sci. 8: 13 - 16.

177. Takahashi, T., Sugahara, T. and Ohsaka, A. (1981). Meth. Enzymol. 71: 710 - 725.

178. Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K. and Nishizuka, Y. (1980). Biochem. Biophys. Res. Commun. 97: 309 - 317.

179. Tanigawa, K., Kuzuya, H., Imura, H., Taniguchi, H., Baba, S., Takai, Y. and Nishizuka, Y. (1982). FEBS Lett. 138: 183 - 186.

180. Lieberman, M.A. (1983). J. Cell. Physiol. 114: 73 - 76.

181. Hoover, R.L., Dawidowicz, E.A., Robinson, J.M. and Karnovsky, M.J. (1983). J. Cell Biol. 97: 73 - 80.

182. Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M. and Kahn, C.R. (1982). Nature 298: 667 - 669.

183. Cohen, S., Carpenter, G. and King, L. (1980). J. Biol. Chem. 255: 4834 - 4842.

184. Cohen, S., Ushiro, H., Stoscheck, C. and Chinkers, M. (1982). J. Biol. Chem. 257: 1523 - 1539.

185. Cooper, J.A., Bowen-Pope, D., Raines, E., Ross, R. and Hunter, T. (1982). Cell 31: 263 - 273.

186. Hunter, T. and Cooper, J.A. (1983). Cell Biol. Intl. Rep. 7: 505 - 506.

187. Servely, J.L., Teyssot, B., Houdebine, L.M., Delouis, C. and Djiane, J. (1982). Biochimie 64: 133 - 140.