HORMONAL REGULATION OF ORN1TIHNE DECARBOXYLASE ACTIVITY AND CENE EXPRESSION IN THE TESTIS, LIVER AND KIDNEY OF RAT.

Thesis submitted to the University of Hyderabad
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Doctor of Philosophy

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DECLARATION

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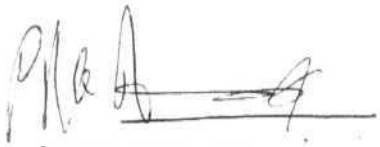
I hereby declare that the work presented in this thesis has been carried by me under the supervision of Prof.P.R.K.Reddy, and that this work has not been submitted for a degree or diploma in this or any other university.



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ABBREVIATIONS

Act. D	Actinomycin D
cAMP	Cyclic Adenosine-3',5'-monophosphate
Cas	Castrated rats
CHX	Cycloheximide
DAG	Diacylglycerol
dCTP	Deoxycytidine 5'triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
FSH	Follicle stimulating hormone
GH	Growth hormone
hCG	Human chorionic gonadotropin
hsp	Heat shock protein
IGF-I	Insulin-like growth factor-I
LB medium	Luria-Bertani medium
LU	Luteinizing honnone
min	Minute(s)
MOPS	Morpholino propane sulfonic acid
ODC	Omithine decarboxylase
PKA	Protein kinase A
PKC	Protein kinase C
PRL	Prolactin
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl Sulfate
SEM	Standard error of the Mean
SSC	Saline sodium citrate
SSPE	Saline sodium phosphate-EDTA
STE	Saline-Tris-EDTA
TE	Tris-EDTA
TP	Testosterone propionate
Tris	Tris-hydroxymethylaminoethane

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Cellular development is a complex process consisting of various intra- and intercellular events involving the induction of specific sequential changes in the cell. Cell differentiation can be regarded as the acquisition by individual cells of various cytological and biochemical features of the differentiated state.

During the last few decades, a large amount of evidence has accumulated implicating the polyamines in the regulation of cell proliferation and differentiation. The polyamines spermine and spermidine and their precursor putrescine occur in most living organisms (Bachrach, 1973). They play an important role in proliferation, are useful markers of cellular growth (Morris, 1974) and neoplasia (Nishioka et *al.*, 1978), and are thought to be associated with several diseases. Cellular polyamine levels and their respective biosynthetic enzymes increase substantially during the early phase of growth and development.

The important characteristic of polyamines is their basic nature, which gives them a high affinity for acidic constituents, and this may be important in determining their physiological actions. It is therefore natural that nucleic acids should figure prominently as targets for polyamines. There are a wide variety of **effects exerted** by polyamines, including the following:

a) **Requirement as growth factors:** Polyamines are required for **optimal growth** in all cells tested and this requirement seems to be absolute. It has been shown that cellular polyamine levels increase progressively as cells traverse the cell cycle from the

G_1 phase to mitosis (Heby *et al.,* 1982).

b). Embryogenesis: During pregnancy, growth of fetus and maternal reproductive tissues is accompanied by increased polyamine levels (Fozard et *al.*, 1980a; Huber and Brown, 1982). Difluoromethylornithine (DFMO), a potent inhibitor of ornithine decarboxylase (and thereby of polyamine biosynthesis) has been shown to have contragestational effects in mice (Fozard et *al.*, 1980a), rat (Fozard et *al.,* 1980b; Reddy and Rukmini, 1981; Slotkin et *al.,* 1983), rabbit (Fozard et *al.,* 1980b) and hamster (Galliani et *al.*, 1983). Polyamines were also shown to play an essential role in the early stage of Xenopus oocyte maturation (Lowkvist et *al.,* 1983).

c). **Organ development:** Polyamines are essential for organ development *in vivo.* In humans, long term DFMO administration was found to inhibit the differentiation of erythropoietic cells (Abeloff et *al.*, 1984). Polyamine deficiency has been shown to produce adverse effects on the secretory activity of the ventral prostate gland (Danzin et *al.*, 1979; Kapyaho et *al.*, 1984; Danzin et *al.*, 1982).

d). **Maintenance of chromosomal integrity:** It has been shown that **polyamine** deprivation causes major chromosomal aberrations in a **polyamine** dependent Chinese hamster ovary (CHO) cell line **(Pohjanpelto** and Knuutila, 1982; 1984).

e) **Regulation of protein synthesis:** It has been postulated by

Panagiotidis et *al.* (1989) that interaction between polyamines, polyamine-synthesising enzymes and S20/L26 and L34 ribosomal proteins may participate in regulation of protein synthesis.

Ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, is one of the most highly regulated enzymes in eukaryotic organisms. ODC is somewhat unigue by virtue of its rapid and dramatic inducibility by a variety of growth stimuli, based on its extremely rapid turnover rate. Another special feature of this enzyme is its regulation by a unique feedback mechanism by its products, involving a specific inhibitory protein known as antizyme (Heller et *al.*, 1976). ODC is present in very minute quantities in cells, but can be induced severalfold by various growth factors, hormones, mitogens and other stimuli. As a result, ODC is one of the most intensively studied enzymes and a complete review is beyond the scope of this thesis. Therefore only some of the salient aspects will be covered.

ODC is a homodimer of 52 to 55 kDa subunits and is quite well conserved in sequence from fungi to humans (Coffino, 1989). The amino-acid sequences of the mouse, human, hamster and rat ODC obtained from the DNA sequences indicate that the protein consists of 461 amino acid residues (Pegg, 1989). All known forms of ODC use pyridoxal phosphate as a cofactor. Mammalian ODCs are strongly dependent on the presence of dithiothreitol or other thiol reducing agents for maximal activity. The enzyme has a pH optimum of 7.0 and the K for L-ornithine is roughly 0.1 mM.

There have been many reports of multiple forms of ODC in mammalian cells, E. Coli, Physarum polycephalum, and T. pyriformis. ODC belongs to a multigene family and it is possible that there are multiple functional genes which could give rise to different forms. Two forms of ODC differing in isoelectric points have been reported in mouse kidney; it is possible that these represent forms having different extents of post-translational modification.

Accumulating evidence indicates that ODC is regulated at the levels of transcription, translation and enzyme degradation. In addition, overproduction of the enzyme occurs rather easily by gene amplification when cells become resistant to an ODC inhibitor, and rapid activation of the enzyme takes place to minor degrees under some conditions.

Transcription: Feinstein et *al.* (1985) showed that rapid induction of ODC activity by nerve growth factor in cultured PC12 pheochromocytoma cells is accompanied by a roughly parallel change in the amount of hybridisable ODC mRNA. It was later shown using nuclear run-on transcription assays that both NGF and epidermal growth factor enhance transcription of the ODC gene (Greenberg *et al.*, 1985). Similarly, androgens induce ODC gene expression and activity in murine kidney, and the phorbol ester TPA also induces ODC transcription.

Translation: A number of stimuli affect ODC at the level of translation. It has been shown by Kanamoto et *al.* (1987) that the

synergistic induction of ODC activity in primary cultured rat hepatocytes caused by glucagon and asparagine was due to a threefold induction of ODC mRNA by glucagon and at least a six-fold stimulation of translation rate caused by asparagine. Stimulation of translational initiation has also been suggested by White et *al.* (1987) for ODC induction in mitogen-activated lymphocytes.

In the case of mouse kidney, androgens cause a25-fold increase in the rate of ODC synthesis (Persson et *al.*, 1984), whereas there was only an 8-20 fold increase in ODC mRNA (Berger et *al.*, 1984). This strongly suggests an involvement of translational stimulation. In rat liver, a large induction of ODC activity both by thioacetamide treatment (Kameji et *al.*, 1984) and by feeding (Kameji et *al.*, 1987) is preceded by only severalfold increases in the activity of polysome-associated ODC mRNA, again suggesting the involvement of a translational mechanism.

In human osteosarcoma cells, Goto et *al.* (1991) suggested that parathyroid hormone regulates ODC activity at the level of translation. In lymphocytes, concanavalin A causes a shift of the ODC message from its untranslated pool into polysomes, suggesting that the increase in ODC activity is in fact due to the stimulation of translational initiation (White et *al.*, 1987). EIF-4E, the least abundant of the translation initiation factors, plays a regulatory role in translation and it has been suggested that cell transformation brought about by eIF-4E overexpression may be caused by relief of suppression of translation of mRNAs

encoding proteins that regulate cell growth. It has been recently suggested that ODC levels are controlled by eIF-4E and that ODC is an essential component of the transformation of 3T3 cells by this factor (Schantz and Pegg, 1994) .

ODC undergoes negative feedback control by polyamines, which induce an increase in the rate of ODC degradation as well as a decrease in the rate of ODC synthesis. It has been observed that the cellular content of ODC mRNA does not change significantly during the rapid ODC decay induced by polyamines (Hayashi, 1989). It is likely therefore, that polyamines inhibit ODC synthesis at the translational level.

Degradation: An extremely rapid turnover rate is one of the fundamental characteristics of eukaryotic ODC and makes the regulation of its synthesis a very effective means of bringing about rapid changes in amount. Also, the turnover rate of ODC itself changes considerably under various conditions, indicating that the process of ODC degradation is also subject to regulation.

Important progress has been made recently with respect to ODC degradation by polyamines. In 1976, Heller *et al.* discovered a protein inhibitor of ODC which is induced by polyamines, is bound to the enzyme, and non-competitively inhibits its activity. This protein was named antizyme. Subsequently, there have been reports of the presence of antizyme in various mammalian tissues, avian tissues, and E. coli, either after treatment with exogenous

polyamines or under physiological conditions. The specificity of action of antizyme suggests that its primary role is to bind to ODC and neutralise its activity. Antizyme seems to be primarily involved in ODC degradation, at least in mammalian cells.

Recently, Li and Coffino (1993) have proposed a model to explain the facilitation of ODC degradation by antizyme. They propose that ODC degradation is a two-step process, wherein ODC and antizyme associate in the first step, stabilising an enzymatically active conformation, with the C-terminus of ODC exposed. The second step is likely to be ATP-dependent, and proteolysis of ODC occurs. Thus, according to this model, the role of antizyme seems to be to render ODC susceptible to proteolysis.

HORMONAL REGULATION OF ORNITHINE DECARBOXYLASE:

A number of hormones enhance ODC activity in their respective target organs, as well as in mammalian cells in culture.

Epidermal growth factor induces a marked but transient increase in ODC activity in cultures of chick embryo epidermis, as well as *in vivo* in mice (Stastny and Cohen, 1970). Studies on the effects of EGF on ODC activity and DNA synthesis in rats during the perinatal period have suggested that EGF has mitogenic effects on neonatal and maternal rat liver (Yamamoto *et al.*, 1993).

The effect of androgens on ODC in various target tissues has been widely studied. In fact, androgen-treated mouse kidney is one of the richest sources of ODC. Testosterone elicits a several hundredfold increase in the ODC level that is generated by changes in both ODC synthesis and turnover (Berger et *al.,* 1984). In the rat, renal ODC mRNA levels were largely unchanged by testosterone deprivation by castration , and subsequent repletion. In contrast to the kidney, ODC expression in the rat ventral prostrate and seminal vesicles is testosterone dependent (Blackshear et al., 1989). Epididymal ODC activity in the rat is also androgen-dependent (de las Heras and Calendra, 1987).

Other steroid hormones also affect ODC activity in various target tissues. Estradiol stimulates uterine ODC activity in immature rats (Lavia et al., 1983). Estrogen stimulates renal ODC activity in male rats and hamsters, but has little effect in male mice (Wing, 1990). progesterone is also thought to play a significant role in the rise of uterine ODC activity in the pregnant hamster (Luzzani et al., 1982).

Single, pharmacological doses of parathyroid hormone, calcitonin, vasopressin, d-aldosterone or L-triiodothyronine were shown to produce a significant increase in ODC activity of rat kidney in hypophysectomised rats (Scalabrino and Ferioli, 1976). A single dose of synthetic salmon calcitonin stimulated ODC activity in brain, liver, kidney, testis and ovary (Nakhla, 1987).

It was shown by Wing and Rillema (1983) that prostaglandins PGE_1, E_2 and I_2 elicit a concentration-dependent stimulation of ODC activity in mammary gland explants from mid-pregnant mice.

PROLACTIN AND ITS ACTION:

The anterior pituitary hormones are included among the major hormones that influence ODC activity. Prolactin (PRL) is known to exist in all vertebrates and more than 85 diverse and distinct functions have been attributed to this hormone. These actions can be broadly divided as follows: i) reproduction and lactation, ii) water and salt balance, iii) growth and morphogenesis, iv) metabolism, v) behavior, vi) immunoregulation, vii) effects on skin and ectoderm. In addition, prolactin is known to have a mitogenic action and has been implicated as a tumor promoter in rat liver. In mammals, PRL is primarily responsible for the development of the mammary gland and lactogenesis. It acts in association with insulin and glucocorticoids to stimulate milk protein gene expression at both the transcriptional and post-transcriptional levels (Guyette *et al.,* 1979).

The biological roles of the high levels of PRL receptors in rat liver are not well understood. Administration of PRL to rats causes hepatic hypertrophy and increases ODC activity (Buckley *et al.,* 1985; Richards 1975). PRL has been shown to stimulate the expression of mRNA for cytosolic PEP carboxykinase in liver of

lactating rats and in primary cultures of hepatocytes (Zabala and Garcia-Ruiz, 1989). Also, PRL induces a factor called synlactin in the liver of pigeons and rats that acts synergistically with PRL to promote the growth of the crop sac or the mammary gland (Nicoll et *al.,* 1985).

Prolactin is also known to regulate the expression of a large number of receptors, including the interleukin-2 receptors on rat splenic lymphocytes (Mukherjee et *al.*, 1990) and nerve growth factor receptors in the β-cell line INS-1 (Scharfmann et *al.,* 1994). PRL and GH have been shown to control β-cell proliferation and insulin production and secretion.

In humans, PRL regulates the gene for a protein known as PRL-inducible protein (PIP). PIP is a secreted glycoprotein whose transcription is regulated by androgens, while PRL has an essentially post-transcriptional (mRNA stabilisation) effect (Murphy et *al.*, 1987b). PIP is present in several human breast cancer cell lines and in benign and malignant tumor biopsies (Murphy et *al.,* 1987a).

Recent evidence has shown that GH and PRL are immunostimulatory factors. Hormone-replacement studies have shown that either of these hormones was able to restore immune function in animal models such as hypophysectomy models or genetic dwarfs. Effects of PRL on lymphocytes have been reported. Russell et *al.* (1984) reported that PRL could increase ornithine decarboxylase activity in peripheral lymphocytes. Antibodies to PRL inhibit lymphocyte proliferation (Hartmann et *al.,* 1989).

Hyperprolactinemia results in a marked suppression of antibody production, delayed type hypersensitivity,, or development of adjuvant-induced arthritis. PRL also regulates lymphocyte growth in hypophysectomised rats and stimulates c-myc expression and DNA synthesis in lymphoid tissues. In the PRL-dependent Nb_2 node T-lymphoma cell line, the immunosuppressive compounds cyclosporine and didemnin B inhibit PRL-stimulated ODC activity and subsequent proliferation (Russell et *al.,* 1987). Similarly, cyclosporine significantly inhibited PRL-stimulated ODC activity in spleen, kidney and adrenal glands in intact rats, and kidney, liver, thymus and adrenal glands in hypophysectomised rats. The major side-effects of the drug cyclosporine being kidney toxicity and depressed renal function, Russell *et al.* (1984) have suggested that kidney failure in response to cyclosporin A may involve the ability to suppress the normal level of protein and RNA synthesis maintained by circulating prolactin.

Endogenous prolactin has been shown to participate in some of the early immunologic events leading to the development of autoimmune diseases (Buskila *et al.,* 1991), such as experimental lupus in B/W mice (McMurray *et al.,* 1991), induced experimental allergic encephalitis (Riskind et *al.,* 1991) and adjuvant arthritis (Jara et *al.,* 1991). It has also been recently reported by Dardenne et *al.* (1994) that cells of the human immune system, and hematopoietic tissues including thymus, bone marrow and peripheral blood express high affinity receptors for PRL. These data, along with those showing that the PRL gene is specifically expressed in human T-cells, suggest that lymphocyte PRL may act

in an autocrine or paracrine fashion in both central and peripheral lymphoid organs.

In 1980, Horrobin reviewed the evidence that prolactin is a regulator of fluid and electrolyte metabolism in mammals. He came to the conclusion that this action of prolactin mainly involves modulation of the actions of other agents. Prolactin receptors are found in mammalian kidneys. PRL modulates renal cyclic AMP formation and that of polyamines, and leads to demonstrable histological changes in the proximal tubules. PRL also seems to be able to cause a prolonged reduction in water, sodium and potassium excretion.

The most important role played by PRL is its control of casein synthesis and regulation of the mammary gland and also its effect in mammary carcinogenesis. When PRL secretion is chronically elevated, as in old female rats and some strains of mice, many pituitary and mammary tumors are present. However, only normal levels of PRL are necessary for development of carcinogen-induced mammary tumors in rats and mice. During reproductive life, PRL secretion is elevated during postpartum **lactation,** galactorrhea, and during chronic administration of neuroleptic drugs. This may result in suppression of gonadotropic hormone secretion and cessation of estrous and menstrual cycles (Meites, 1988). In general, PRL is essential for initiation and maintenance of lactation.

Prolactin-secreting pituitary tumors (prolactinomas) are the

most common type of pituitary tumor in rats, mice and humans, and are associated with an increase in PRL secretion. Chronic administration of estrogens induces pituitary tumors in rats. The principal role of PRL in mammary tumorigenesis is to sensitise the glands to carcinogenic agents by constantly stimulating mammary mitosis.

In 1975, Richards reported an increase in ODC activity in various organs of female rat upon treatment with prolactin. In young male rats an optimal dose of 3 mg per rat induced renal ODC activity which peaked at 2.5 hours after injection. In females, the induction varied with age, being highest in unweaned rats.

In 1980, Levine et *al.* showed that renal ODC activity is located primarily in the medulla, and ODC in both renal medulla and cortex is sensitive to GH and ACTH-stimulation. Hurley et *al.* (1980) injected fetuses *in utero* with 100 μg of various prolactin, GH and placental lactogen (PL) preparations and monitored ODC in liver, heart and brain, at various time intervals. Their results indicated that ovine PL has somatotropic effects in the fetus, and that rat liver ODC becomes responsive to GH and PRL in the perinatal period.

Russell et *al.* (1984) in their studies on one month old female rats, showed that 10^{-7}M PRL resulted in a two-fold elevation of ODC activity within 2h of injection, with peak **activity** at 6h post-injection. The activity then declined by 8h. 10^{-6}M PRL caused maximal stimulation of ODC activity at 6h in

liver and kidney. They concluded that the ability of PRL to induce ODC activity in a variety of tissues in hypophysectomised rats suggests that it may be a direct induction effect and not merely a secondary effect of PRL action. Later, Buckley *et al.* (1986) showed that even a low dose (5.5 mg/kg body weight) was able to stimulate hepatic DNA synthesis in both male and female rats, adult and weanling.

Gonzalez *et al.* (1991) carried out some experiments in PRL-deficient Ames dwarf mice (df/df) to assess the effect of hyperprolactinemia on various functions. The mice were implanted with two pituitaries each, some were castrated, given testosterone propionate (TP) and implanted, some were sham-operated and the last group was castrated and given TP. It was found that hyperprolactinemia produced an increase in ODC activity in seminal vesicle and liver, and also polyamine levels. There was also an enhancement of plasma FSH levels in both intact and castrated, TP-treated mice implanted with pituitaries.

The Nb_2 node lymphoma cell line is a good system in which to study the role of PRL in cell proliferation as it requires PRL for growth. Russell et *al.* (1987) found that PRL to be present in the culture medium for a minimum of 3 to 6 hours to invoke a maximal effect on mitogenesis. They also found that products of the lipoxygenase pathway may contribute to the mechanism of PRL-stimulated mitogenesis. Inhibitors of protein kinase C (PKC) and phospholipase A_2 and C activities blocked the PRL-stimulation of ODC and mitogenesis. This suggested a role for PKC in the

coupling of PRL receptors to the stimulation of ODC activity and mitogenesis in Nb_2 lymphoma cells.

In 1990, Yu-Lee reported that in the Nb_2 lymphoma cell line, the growth-related genes c-myc, ODC, β-actin and a hsp 70 homologue, with rapid but different kinetics, primarily at the transcriptional level. ODC stimulation was maximal 1h after treatment and remained elevated at 4h. ODC mRNA accumulation lagged behind ODC gene transcriptional induction by lh and showed a 5-fold induction at 8h. This was the first report that ODC mRNA levels are regulated by PRL.

Crowe et *al.* (1991) later showed that PRL activates PKC and stimulates growth-related gene expression in rat liver, in a dose-dependent manner. In male rats, ODC mRNA expression was essentially low in liver from control animals , but rapidly increased with time on PRL treatment. ODC mRNA levels also responded in a dose-dependent manner to PRL. PRL administration caused an elevation in liver diacylglycerol levels, which paralleled an increase in particulate-associated PKC activity. Prolactin was shown to play a significant role in the regulation of DNA methylation in the liver and kidney of adult and immature rats (Reddy and Reddy, 1990).

Despite a large amount of work having been carried out on prolactin's effects, its regulation and its mechanism of action, no single mechanism has been pinpointed as yet. It was suggested in 1980 by Rillema that once PRL binds to its plasma membrane

receptor sites, its subsequent actions may involve one or more of **the** following: increased intracellular concentration of potassium **and** decrease of sodium, increased cGMP level, enhanced rate of prostaglandin biosynthesis mediated by a stimulation of phospholipase A_2 activity, and a stimulation of polyamine synthesis. It has also been shown that the actions of prolactin require the presence of calcium ions in the extracellular environment.

In studies on the induction of milk protein gene expression by prolactin, Bayat-Sarmadi and Houdebine (1993) used various protein kinase inhibitors and concluded that a Serine/Threonine kinase which is not protein kinase C , and possibly a tyrosine kinase is involved in transduction of the prolactin message from the receptor to the milk protein gene. Rui et *al.* (1992) showed evidence for rapid tyrosine kinase activation on PRL receptor triggering, and Rillema *et al.* (1992) showed that there is a rapid stimulation of tyrosine kinase activity by PRL in Nb2 lymphoma cells. It was recently reported by David et *al.* (1994) that treatment of Nb2 rat lymphoma cells with PRL activates a latent protein factor, causing it to bind to an enhancer in the interferon regulatory factor 1 gene. This enhancer is required for IFN-γ-activated expression of the gene. In addition, PRL also induced tyrosine phosphorylation of Jak2, a tyrosine kinase required for IFN-γ-activated gene expression.

Many reports have linked various actions of PRL with the activation of protein kinase C. In 1988 Buckley et *al.* found that

PRL activated PKC several hundredfold within minutes of addition to rat liver nuclei. It was also found that hepatic PKC was rapidly translocated to the hepatic membrane, and it was suggested that rapid activation of PKC may be an intermediate step in the hepatotrophic responses stimulated by PRL (Buckley et *al.,* 1987). Subsequently Russell et *al.* (1987) found that in Nb_2 node lymphoma cells, PKC plays a role in the coupling of PRL receptors to the stimulation of ODC activity and mitogenesis. In rat aortic smooth muscle, PRL activated PKC in a dose-dependent manner and this action was blocked by H-7, a PKC inhibitor. The authors suggested that PKC has a role in the signal transduction pathway for PRL action, and this activation may be involved in vascular smooth muscle function.

It was recently found by Meyer et *al.* (1992) that the cell proliferation-linked protein stathmin in Nb_2 lymphoma cells is phosphorylated in response to PRL-stimulation, but PKC does not mediate this response. They have suggested that phosphorylation of stathmin and stathmin-like proteins may mediate some actions of prolactin in these cells.

In 1993, Rao et *al.* found that in Nb_2 lymphoma cells, the PRL receptor is constitutively expressed in both nucleus and membrane/cytosol compartments. Addition of PRL stimulated rapid internalization, and translocation of the hormone to the nucleus; this was ATP-dependent and reversible. Their results indicate that an early event coupled to the mitogenic action of PRL in Nb_2 cells is transport of the hormone to the nucleus during the G_1

and S phases and they suggest that in the nucleus, PRL bound to its receptor may directly influence gene transcription. Recent studies by the same group showed that PRL stimulated rapid tyrosine phosphorylation of mitogen-activated protein (MAP) kinase. The phosphorylated MAP kinase translocated to the nucleus in a manner identical to that found for PRL. They have suggested a possible interactive mechanism whereby PRL, acting via the nuclear PRL receptor, together with MAP kinase, may regulate transcription of genes involved in the proliferative response (Buckley et *al.,* 1994).

Koduri and Rillema (1993) concluded from their studies using cholera and pertussis toxins to study the prolactin-stimulation of lactose synthesis and ODC activity in mouse mammary gland explants, that a G protein, but not $G_{_}$, may be involved in prolactin's mechanism of stimulation.

It has been shown that phospholipase C and arachidonic acid enhance the mitogenic effect of PRL on the Nb_2 cell line, and the data obtained are compatible with a possible involvement of PKC in the PRL-stimulation of mitogenesis (Ofenstein and Rillema, 1987). It was also found by Manni et *al.,* (1986) that the polyamine pathway seems to play an essential role in the expression of autocrine control of tumor growth by PRL in mammary tumor cells in culture.

GROWTH HORMONE AND ITS ACTION:

Growth hormone (GH) is another anterior pituitary hormone that influences ODC levels in various tissues. This hormone plays a central role in the regulation of post-natal mammalian growth. GH has been shown to directly stimulate *in vitro* erythroleukemia cell proliferation (Golde et *al.,* 1978) and synthesis of nucleic acids and proteins (Desai et *al.,* 1973). Many actions of GH, such as on skeletal growth, are indirect and are mediated by insulin-like growth factor (IGF). GH has been shown to directly regulate IGF-I production in liver cells as well as other cells in culture (Mathews et *al.,* 1986). Other direct effects of GH include the regulation of cytochrome $P\text{-}450_{15\beta}$ by liver cells (Toilet et *al.,* 1990), as well as glucose transport and glucose-metabolism in adipocytes.

In preadipocytes, GH is necessary for the initiation of the differentiation program for the cells to become responsive to IGF-I and for its mitogenic effect (Green et *al.,* 1985). Rapid effects of GH in these cells include induction of c-fos and c-jun transcription (Gurland et *al.,* 1990). A similar effect of GH on precursor cells exists in other tissues like adipose tissue, muscle and cartilage.

Prenatal somatic growth seems to be largely independent of GH. Binding of GH to liver membranes from calf, lamb and rat is minimal before birth and increases gradually during the first weeks of postnatal life (Maes et *al.,* 1983; Gluckman et *al.,* 1983).

Recent evidence has shown that GH, like PRL, is involved in immunoregulation. Removal of the pituitary leads to reduced antibody production, diminished skin sensitivity to toxic substances and adjuvant arthritis, and prolonged skin allograft survival (Nagy and Berczi, 1978). Genetic dwarf mice models lacking GH and PRL in their pituitary have atrophied thymus and lymphoid tissue, and a depletion of bone marrow (Baroni, 1967). GH stimulated erythropoiesis in lymphocytes (Golde and Bersche, 1977) . It has also been shown to influence cytotoxic lymphocyte lysis (Snow et *al.,* 1981). The direct production of GH by normal lymphocytes has also been noted (Hartmann et *al.,* 1989).

GH appears to be required for the expression of late differentiation-specific genes, as demonstrated in Ob1771 preadipocytes (Doglio et *al.,* 1986). In the same cells, GH has also been show to trigger IGF-I gene expression, modulate the expression of the lipoprotein lipase gene, transiently increase c-fos gene expression, stimulate the formation of diacylglycerol and to be unable to affect intracellular Ca^{2+} levels (Catalioto et *al.,* 1990) .

Growth hormone was found to stimulate rat hepatic spermidine and putrescine synthesis (Janne et *al.,* 1968), and this was found to be due to GH-stimulation of ODC activity (Janne et *al.,* 1969). This stimulation was independent of the adrenal glands, and maximal **stimulation** occurred at 4h after injection. The stimulation in ODC activity was accompanied by an increase of

more than 100% in RNA polymerase activity.

Subsequently in 1971, Fausto showed that a single injection of GH caused an increase in liver ODC activity, and this effect could be blocked by puromycin and actinomycin D. Ovine GH or hydrocortisone were shown to rapidly increase ODC activity in rat kidney; this activity peaked at 3 to 4 h post-injection and returned to basal levels by 8h (Brandt et *al.,* 1972). Similarly, in 1971, both Richman *et al.* and Russell and Lombardini reported GH-stimulation of hepatic ODC levels. The former group found that though adrenalectomy had no effect on ODC activity, hypophysectomy caused basal ODC activity to decline. Levine *et al.* (1973) showed that GH stimulates adrenal ODC activity, and when given along with ACTH, there is a synergistic stimulation. In neonatal rat brain and liver, bovine GH and PRL stimulated ODC activity. Of these, bPRL had only half the potency of bGH (Roger et *al.*, 1974) .

Sogani et *al.* (1972) showed that GH treatment stimulates hepatic and renal ODC activity in hypophysectomised rats. ODC activity was also stimulated by GH in heart and thymus. Murphy and Brosnan (1976) demonstrated that the greatly increased liver ODC activity observed after GH administration was localised in the cytosol. The effect of stressful stimulation on tissue response to GH was studied in rat liver during forced exertion in rats (Martin et *al.,* 1989). GH was found to cause a 15-fold increase in ODC activity in liver of resting rats; and forced walking of the rats enhanced this effect by about 60% more. The

authors have suggested that tissue hypersensitivity to GH stimulation may be a consequence of forced exertion.

Very little is known about the early events that occur after the binding of GH to its receptor. So far, no second messenger of GH has been positively identified.It has been suggested that a novel tyrosine kinase or kinase activity may be associated with the GH receptor in several cell types, including GH-treated 3T3F442A fibroblasts, human IM9 lymphocytes, rat H-35 hepatoma cells, and freshly isolated rat adipocytes (Anderson, 1992).

It has also been suggested that protein kinase C could be involved in the mechanism of action of GH. GH is able to stimulate phospholipase C activity *in vitro* with the production of inositol triphosphate and diacylglycerol in basolateral membranes of canine kidney (Rogers and Hammerman, 1989).

The stimulation of somatic growth by GH, including growth of long bones, seems to be mediated via stimulation of IGF-I (Behringer et *al.,* 1990). GH appears to be necessary for attainment of normal liver size.

In OB1771 mouse preadipocyte cells, GH has also been shown to stimulate the production of diacylglycerol by means of phosphatidylcholine breakdown, involving a phospholipase C coupled to the GH receptor (Catalioto et *al.,* 1990). In isolated hepatocytes, GH and PRL are able to rapidly stimulate the production of diacylglycerol (Johnson et *al.,* 1990).

Further substantiation of the involvement of PKC is given by **Doglio** *et al.* (1989) who showed that in OB1771 cells, GH stimulates c-fos gene expression by means of protein kinase C activation. This activation was thought by them to be due to a phospholipase C-mediated hydrolysis of glycerophospholipids other than inositol phospholipids. GH is also known to cause acute down-regulation of rat liver somatogenic receptors, in contrast **with** its long-term stimulatory effects (Maiter et *al.,* 1988).

It has been recently shown that in OB1771 cells, c-fos protein is involved in the GH-mediated regulation of transcription of the lipoprotein lipase gene in response to GH (Barcellini-Couget et *al.,* 1993). Anderson (1992) showed that in 3T3-F442A preadipocytes, physiological concentrations of GH induced a rapid and transient activation of mitogen-activated protein kinase (MAP kinase) and S6 kinase. Protein kinase C seems to be involved in the mechanism of action.

HORMONAL REGULATION IN THE TESTIS:

The developing mammalian testis is characterised by a high rate of cellular growth, mainly engaging precursors of somatic cells and early spermatogonia at different time intervals during **testicular** ontogeny. In comparison to other organs, the testis is unique in that it is the only one in which meiotic divisions occur, appearing first during early puberty. The adult testis is also a site of intense mitotic cell proliferation, among the most

rapid known in mammalian tissues. Although testosterone and gonadotropic hormones are required for initiation of spermatogenesis, there are a number of local paracrine factors which also profoundly affect testis function.

While LH secreted by the pituitary is undoubtedly the principal regulator of Leydig cell steroidogenesis, a number of local growth factors are also involved. A testicular analog of GnRH appears to stimulate androgen production by Leydig cells (Hsueh et *al.,* 1981; Sharpe *et al.* , 1982). Specific receptors for insulin and insulin-like growth factors (Handelsman *et al.*, 1985), prolactin, arginine vasotocin-like factor and epidermal growth factor, glucocorticoids and catecholamines (Cooke et *al.*, 1982) have been identified on the surface of Leydig cells. While PRL stimulates testicular steroidogenesis (Catt et *al.,* 1980), most of the latter factors appear to decrease steroidogenesis. The testis of several mammals contains large concentrations of the neurohypophyseal hormones oxytocin and arginine vasopressin (Nicholson et *al.,* 1984; Kasson et *al.,* 1985). ß-endorphin (and other pro-opiomelanocortin- derived peptides) production in rat Leydig cells has been demonstrated and it may facilitate testosterone secretion either directly as an autocrine effect or via intermediates (Bardin, 1984). A number of reports also indicate that prostaglandins can directly inhibit LH-induced steroidogenesis in dispersed rat Leydig cells (Sairam, 1976). FSH and testosterone regulate spermatogenesis through the Sertoli cells (Steinberger, 1971).

Both high gonadotropin and estrogen levels are known to be inhibitory to adult testicular function including LH receptor down-regulation and blockade of steroidogenesis. However, both of these negative responses are missing in fetal and neonatal testis (Pakarinen et *al.,* 1990).

Since ODC activity and gene expression are markers of cell growth and proliferation, the testis is an ideal system in which to study its hormonal regulation. The ontogenesis of ODC mRNA and enzyme levels in testis has been extensively studied. Alcivar et *al.* (1989) found that in mouse testis, ODC mRNA levels increased substantially in enriched populations of pachytene spermatocytes, round spermatids and residual bodies isolated from mature testis, as opposed to low levels observed in prepubertal mouse testis. They also showed the distribution of ODC mRNA in both polysomal fractions prepared from total testis extract, suggesting that ODC is translationally regulated in the mouse testis.

Kaipia et *al.* (1990) showed increasing ODC mRNA levels during prophase of meiosis with highest levels in late pachytene spermatocytes and step 3-5 spermatids. They found three molecular sizes of ODC mRNA, and the relative abundance of these with respect to one another differed in rat and mouse testis, indicating a species difference in the usage of the polyadenylation signals within the ODC gene. They also concluded that the high levels of ODC mRNA in late pachytene spermatocytes and early round spermatids suggest that polyamines may play an important role during late meiosis and early spermatogenesis.

Weiner and Dias (1992) showed that rat testicular ODC mRNA levels began to rise at 21 days of age, reaching maximal levels by 40 days. In contrast, ODC activity decreased with age.

ODC activity in the rat testis is regulated by a number of various hormones and growth factors. FSH and LH have been shown to affect ODC activity, and it was proposed that this action was via cyclic AMP stimulation (Reddy and Villee, 1975). It has been shown that prostaglandins (Madhubala and Reddy, 1980a; 1980b), catecholamines (Madhubala and Reddy, 1981; 1983), gonadotropin releasing hormone (Madhubala and Reddy, 1982), and gonadotropic hormones (Madhubala and Reddy, 1985) stimulate ODC activity in immature rat testis. Similarly, arginine vasopressin (Reddy et *al.,* 1986) was also shown to enhance testicular ODC activity.

Prolactin exerts a marked influence on the gonads, by modulating the effects of gonadotropins (Kelly et *al.*, 1980). Some main actions have been documented: (i) stimulation of the number of LH receptors in the testis during puberty, (ii) stimulation of steroidogenesis, (iii) increase of HDL and LDL binding in the corpus luteum, and (iv) regulation of the growth of ovarian follicles. In humans, hyperprolactinemia caused mainly by pituitary tumors is considered clinically to be one of the major causes of sexual malfunctions such as impaired libido, impotence and male hypogonadism (Hoshino, 1988), leading to infertility.

Zipf et *al.* (1978) found that maintenance of testicular LH receptors is at least partially dependent on PRL and GH; these hormones seem to act at different sites and by different mechanisms. From their studies on the effects of PRL on testicular regression and recrudescence in the golden hamster, Bartke et *al.* (1980) concluded that in contrast to findings in rats and mice, chronic hyperprolactinemia in the male hamster does not inhibit gonadotropin release.

The presence of PRL receptors in the interstitial compartment of the testis shows that PRL can act directly on the testis and testicular steroidogenesis. In hypophysectomised rats, PRL potentiates the effects of LH treatment on testosterone production and spermatogenesis. PRL also regulates the growth and function of male accessory reproductive glands (Bartke et *al.*, 1980). In addition to its effects on testicular LH receptors, PRL can stimulate accumulation of esterified cholesterol and the activities of 36- and 17ß-hydroxysteroid dehydrogenases in the testis (Bartke, 1980).

Dombrowicz et *al.* (1992) suggested that during puberty, PRL stimulates testicular function by promoting multiplication and differentiation of Leydig cells (acting at various steps of steroidogenesis and on tissue responsiveness to LH) and germ cells.

Studies carried out by Park et *al.* (1993) indicate that the rise in FSH after the prolactin-induced suppression of FSH and LH

release after castration requires the involvement of stimulatory factors from the adrenal gland.

AIM AND SCOPE OF THIS STUDY

The vast amount of literature available on ornithine decarboxylase and the polyamines has established their importance in the processes of growth and differentiation. Ornithine decarboxylase is an important cellular marker of proliferation and expression of the ODC gene is one of the early events associated with stimulation by mitogens and growth factors like hormones. Recent reports indicate that expression of the ODC gene is necessary for transformation of some cell types (Auvinen *et al.,* 1992), and it has been suggested that the ODC gene itself may act as an oncogene.

The hormones prolactin and growth hormone are known to exert a mitogenic action on certain tissues, and hyperprolactinemia can lead to the formation of some types of tumours. However, their exact mechanism of action in causing various effects have not been satisfactorily elucidated. It was therefore of interest to study the way in which these hormones affect ODC activity and gene expression in some of their target organs, namely the liver, testis and kidney of the rat.

CHAPTER 2

MATERIALS AND METHODS

CHEMICALS;

Agarose, bromophenol blue, cesium chloride, dithiothreitol (DTT), diethyl pyrocarbonate (DEPC), dextran Sulfate, ethylene diamine tetraacetic acid (EDTA), ethidium bromide, glucose, hyamine hydroxide, 8-hydroxy quinoline, lysozyme, 2-mercaptoethanol, morpholinopropane sulfonic acid (MOPS), L-ornithine, quercetin, quinacrine, ribonuclease, sucrose, sodium **lauryl Sulfate** (SDS), salmon testis DNA, testosterone propionate, tetracycline, Tris base and xylene cyanol FF, were obtained from Sigma Chemical Co., U.S.A. Ovine follicle stimulating hormone, ovine prolactin and bovine growth hormone were gifted by National Hormone and Pituitary Program, Baltimore, and N1ADDK, Bethesda, U.S.A.

Bacto tryptone, Bacto agar, and Bacto yeast extract were obtained from Difco Laboratories, U.S.A. Guanidinium thiocyanate was obtained from Fluka, Switzerland and Bio-Rex RG 501-X8 (20-50) resin was obtained from Biorad Laboratories, U.S.A. Gene Screen transfer membrane was purchased from NEN Research Products, U.S.A. DL-^{14}C-ornithine and nick translation kits (N5500) were obtained from Amersham, U.K. α-^{32}P-dCTP was procured from BARC, India. All other chemicals were obtained locally and were of analytical grade.

ANIMALS;

Rats derived from Wistar strain were used in all experiments. The animals were housed in an air-conditioned room which was maintained at a regimen of 14h light and 10h dark cycles. They

were given water and pellet diet (Hindustan Lever Ltd.) *ad libitum* .

HORMONE TREATMENT;

In the experiments using gonadotropic hormones, FSH and HCG were administered by intratesticular injection of the rats under mild ether anesthesia. The hormones were injected using normal saline as a vehicle, and controls were injected with only saline.

In the castration experiments, adult rats were castrated via the scrotal route under mild ether anesthesia. Testosterone propionate was injected subcutaneously in 0.2 ml of sesame oil.

Ovine prolactin was dissolved in 0.9% NaCl/ 0.1 N NaOH, and the pH was adjusted to 7-8 with HCl. It was injected intraperitoneally. Ovine growth hormone was dissolved in basic saline, pH 11, and the pH was then adjusted to 9.5 with HCl. This was also injected intraperitoneally.

ASSAY OF ODC ACTIVITY;

ODC activity was assayed according to the method of Janne and Williams-Ashman (1972) with a few modifications which were standardised in our laboratory (Reddy *et al.,* 1987). Rats were killed by cervical dislocation. The tissues were dissected out and homogenised in 2 vols of TED buffer (Tris 25 mM, EDTA 0.1 mM, DTT 1 mM, pH 7.4) in a glass homogeniser using a motor-driven Teflon pestle. The homogenates were centrifuged at 25,000 x **g** for 30 min at 4°C, and the resultant

supernatants used for the assay. The assay mixture consisted of 50 µmoles Tris-HCl (pH 7.4), 0.5 µmoles unlabelled ornithine, 2.5 µmoles dithiothreitol, 0.1 µmoles pyridoxal phosphate, 0.2 µCi DL-(^{14}C)-ornithine monochloride (300,000 cpm) and 200 µl of the supernatant from the tissue homogenate in a final volume of 500 µl. The reaction was carried out in glass tubes fitted with rubber stoppers from which glass center wells containing 200 µl of hyamine hydroxide were suspended. The tubes were incubated at 37^{o}C for 60 minutes in a metabolic shaker, after which the reaction was stopped by injecting 0.5 ml of 10% TCA into the tubes through the stoppers. The tubes were re-incubated for a further 30 min to ensure that all the liberated CO_2 was trapped. The center wells were removed and placed in scintillation vials containing 10 ml of Bray's scintillation fluid (4 g PPO, 200 mg POPOP, 60 g naphthalene, 20 ml ethylene glycol and 100 ml methanol made up to 1 litre with 1,4-dioxane). The radioactivity was measured using a Beckman liquid scintillation counter. The ODC specific activity is expressed as pmoles of $^{14}CO_2$ liberated per hour per mg protein. The protein content of each sample was measured according to the method of Lowry *et al.* (1951).

ISOLATION OF LEYDIG CELLS AND SEMINIFEROUS TUBULES:

Leydig cells and seminiferous tubules were isolated from rat testis using the method of Moyle and Ramachandran (1973) with some modifications. Decapsulated testes from six to eight rats were pooled and placed in 50 ml stoppered centrifuge tubes containing Krebs-Ringer bicarbonate buffer (0.5 ml/testis) pH

7.4, 0.1% collagenase, 0.1% bovine serum albumin and 10 mM glucose. Glutathione (reduced) was added to 1 mM. The tubes were incubated at 37°C for 30 min in a metabolic shaker water bath, after which 0.15 M NaCl was added to double the volume of buffer. The tubes were inverted gently and kept at room temperature for 15 minutes. The turbid supernatant, which contained the Leydig cells, was gently aspirated using a syringe fitted with a polypropylene tube. Two ml of 0.15 M NaCl was added to the seminiferous tubules remaining in the centrifuge tube and kept for 5 min at room temperature; then the supernatant was aspirated and pooled with the Leydig cell fraction. This was repeated thrice. The pooled supernatant was filtered through nylon gauze and centrifuged at 100xg for 10 min at room temperature to sediment the Leydig cells.

The seminiferous tubules and Leydig cells were separately homogenized in four volumes of TED buffer in an all glass homogeniser and centrifuged at 25,000xg for 30 min at 4°C. The supernatants obtained were used for the assay of ODC activity.

ISOLATION OF RNA:

RNA was isolated according to the method of Chomczynski and Sacchi (1987). All glassware was treated with 0.1% diethylpyrocarbonate (DEPC) for two hours and then baked at 220°C overnight before use. Reagents were prepared in sterile RNase free water and autoclaved or filter-sterilised. Polypropylene centrifugation tubes, Eppendorf tubes, and micropipette tips were soaked overnight in 0.1% DEPC, dried and autoclaved before use.

The rats were killed by cervical dislocation and the tissue was immediately minced on ice and homogenised (at room temperature) with 10 volumes of Solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) in a glass-Teflon homogeniser, and then transferred to a polypropylene tube. Sequentially, 0.1 vol of 2 M sodium acetate, pH 4, 1 vol of phenol (water saturated), and 0.2 vol of chloroform : isoamyl alcohol mixture(49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was mixed vigorously for 10s and cooled on ice for 15 min Samples were centrifuged at 10,000xg for 20 min at 4°C. After centrifugation, RNA was present in the aqueous phase, while DNA and proteins were present in the interphase and organic phase. The aqueous phase was transferred to a fresh tube, an equal volume of isopropanol was added and the tube placed at -20°C for 2h to precipitate RNA. RNA was pelleted at 10,000 x g for 20 min at 4°C and the resulting RNA pellet was dissolved in 0.3 vol of solution D, and reprecipitated with equal vol of isopropanol for 1h. at -20°C. After sedimentation at 10,000xg for 10 min at 4°C, the RNA pellet was transferred to an Eppendorf tube, washed once with 75% ethanol, vacuum-dried, and dissolved in an appropriate volume of deionised formamide (Chomczynski, 1992). The RNA samples were stored at -70°C until further use.

QUANTITATION OF RNA: The purified RNA from various tissues was estimated spectrophotometrically using a

Shimadzu spectrophotometer. Absorption readings were taken at 260 and 280 nm in a standard 1 cm length quartz cuvette using appropriate blanks. The ratio between the readings at 260 and 280 nm (OD_{260} / OD_{280}) was between 1.8 and 2.0.

PREPARATION OF DOT BLOTS;

30 µg of total RNA were loaded per well of a Schleicher and Schuell minifold SRC 96D dot blotting apparatus. 30 µg of total RNA and sample buffer containing 50% formamide, 0.01 M phosphate buffer and 6.5 M formaldehyde were diluted to a final volume of 40 µl with water, the mixture was incubated at 55°C for 15 min and then immediately chilled on ice. The Gene Screen nylon membrane was soaked in water for 15 min prior to putting it on the manifold. The denatured samples were loaded into the wells, kept for 30 min and then a slight suction was administered for 30 seconds. The membrane was then removed and air-dried before baking at 80°C for 2 h. After baking, the blot was stored in a polythene bag in an airtight container at 4°C until use.

NORTHERN BLOTTING:

Agarose gel electrophoresis of the RNA samples was carried out on a 1% agarose gel containing 2.2 M formaldehyde. 1.5 g of agarose were dissolved in 93 ml of RNase-free water by boiling until clear and cooled to 60°C. Thirty ml 5X formaldehyde gel running buffer [0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8.0] and 27 ml 37% formaldehyde were added. After stirring well, the gel was poured onto a standard slab gel electrophoresis plate fitted

with a comb of required size and allowed to set for at least 45 min RNA samples were denatured as follows: To 30 µg of RNA in 15 µl of formamide, 5.25 µl of 37% formaldehyde and 3.0 µl 5X MOPS were added and the volume made to 30 µl with water. The tubes were incubated at 65°C for 15 min and immediately chilled on ice. 3 µl of sample buffer (50% glycerol, lmM EDTA pH 8.0, 0.25% bromophenol blue , 0.25% xylene cyanol) were added before loading the samples onto the gel. The gel was pre-run at 50 volts for 5 min before loading the samples. Electrophoresis was carried out in 1X formaldehyde gel running buffer at 3-4 volts/cm. until the bromophenol blue front migrated approximately 10 cm. The buffer from each reservoir was collected, mixed and returned to the apparatus after every 1-2 hours during the run. After electrophoresis, the marker lane was excised carefully, the gel was rinsed in water and then incubated in 20X SSC for 45 min at room temperature with gentle agitation. Transfer of the RNA onto Gene Screen nylon membrane was carried out overnight using the capillary blotting technique, with 10 X SSC as the transfer buffer. After blotting, the filter was air-dried and baked at 80°C in a vacuum oven for 2 hours, and stored at 4°C until hybridisation. Equal loading of RNA samples was checked by staining with ethidium bromide.

AMPLIFICATION OF THE ODC PLASMID:

The probe used in all hybridisation experiments was a 1692 bp ODC cDNA insert in pGem 3zf(-) plasmid vector which was a kind gift from Dr. A.E. Pegg. This insert included 59 bp of the 5'-nontranslated region, the entire coding sequence and 240 bases

of the 3'- nontranslated region. The plasmid was amplified after transformation of E. *coli* cells as follows:

Transformation:

E. coli (DH 5a) cells were first made competent. A small clump of cells was transferred from the frozen stock and grown overnight on an LB-agar plate at 37 °C. The next day, a single colony was picked up from the plate and grown in an overnight culture in 10 ml of LB medium (Bacto-tryptone 10 g/1, Bacto-yeast extract 5 g/1, sodium chloride 10 g/1, pH 7.5). Two ml of this culture was then inoculated into 40 ml of LB medium and allowed to grow till its A_{550} was 0.5. The cells were cooled on ice for 15 min and then centrifuged at 875 x g for 12 min at 4°C. The pellet was then suspended in 0.5 volumes of ice cold 50 mM $CaCl_2$ and vortexed briefly. The suspension was kept on ice for 20 min and re-pelleted. After removing the supernatant completely, the pellet was resuspended in 0.1 vol 50 mM $CaCl_2$ and kept on ice for 75 min

Transformation of these competent cells was carried out as follows: to about 20 µl of competent cells in an Eppendorf tube, about 10 ng of the plasmid was added and mixed well. The tube was left for 30 min on ice. The cells were then subjected to heat shock at 42°C by placing the tube in a water bath for 45 seconds. The tube was immediately cooled on ice, and 80 µl of SOC was added at room temperature (SOC is 20 g/1 Bacto-tryptone, 5 g/1 Bacto-yeast extract, 10 mM sodium chloride, 2.5 mM potassium

chloride, 10 mM $MgCl_2.6H_2O$, 10 mM $MgSO_4.7H_2O$, and 20 mM glucose). The tube was shaken at 225 r.p.m. at 37°C for 30 min in a water bath. The tube was then centrifuged, the supernatant discarded, and the pellet re-incubated in 100 µl LB medium for 30 min at 37°C . 100 µl aliquots of the cell suspension were then streaked onto LB antibiotic plates (LB medium containing 15 g/1 bacto-agar and either 35-50 Mg/ml ampicillin or 12.5-15 Mg/ml tetracycline, poured onto petri plates and set). The plates were allowed to dry for 30 min at room temperatureand then incubated at 37°C in a bacterial incubator for 16-18h.

Amplification:

One colony of transformed cells was picked up from the LB ampicillin plate, and added to a tube containing 10 ml of LB medium (containing the same antibiotic). This was grown overnight at 37°C. The next day, 25 ml of the LB-antibiotic medium was inoculated with 0.1 ml of the overnight culture and incubated at 37°C with vigorous shaking till its $O.D._{600}$ reached 0.6 (the late log phase). This 25 ml culture was then inoculated into 500 ml of LB-antibiotic medium which had been pre-warmed to 37°C, and incubated with vigorous shaking for 2.5 hours. Chloramphenicol was then added to a final concentration of 170 mg/1, and the flask was incubated at 37°C with vigorous shaking for a further 12-16 hours.

Harvest and lysis of bacteria:

The cells were harvested by centrifugation at 4000 x g for 10 min at 4°C and the pellet was washed in 100 ml of ice-cold STE

(0.1 M NaCl, 10 mM Tris-Cl pH 7.8, 1 mM EDTA). The pellet was then resuspended in 10 ml of Solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA) containing 5 mg/ml lysozyme and kept at room temperature for 15 min 20 ml of Solution II (0.2 N NaOH, 1% SDS) were then added , the contents of the tube mixed gently by inversion, and allowed to stand on ice for 10 min 15 ml of an ice-cold solution of 5 M potassium acetate (pH 4.8) were then added, mixed by inversion, and kept on ice for 10 min The suspension was centrifuged in a swing-out rotor at 20,000 r.p.m. for 20 min at 4°C. The supernatant was divided between two corex tubes, the plasmid DNA was precipitated by addition of 0.6 vols of isopropanol, and kept at room temperature for 15 min The DNA was recovered by centrifugation at 12,000xg for 30 min at room temperature. The pellet was washed once with 70% ethanol, briefly vacuum dried, and dissolved in 8 ml of TE buffer (pH 8.0.) .

Purification of plasmid DNA:

The closed circular plasmid DNA was then purified by centrifugation to equilibrium on a Cesium chloride-ethidium bromide gradient as described by Sambrook *et al.* (1989). Eight grams of solid cesium chloride were added to 8.0 ml of DNA solution and mixed until dissolved. Then 0.8 ml of ethidium bromide (10 mg/ml) was added for every 10 ml of the solution. This was ultracentrifuged in a Beckman vertical rotor (VTi 80) at 354,000 x g (70,000 r.p.m.) for 5.5h at 20°C. The plasmid band obtained was removed using a syringe and extracted with n-butanol to remove ethidium bromide, dialysed against several changes of TE pH 8.0, at 4°C and precipitated with 0.1 vol 3 M sodium

acetate (pH 5.2) and 2 vols chilled absolute ethanol. The pellet was Vacuum dried and dissolved in TE pH 8.0 at a concentration of 1 µg/µl and stored in aliquots at -20°C until use.

HYBRIDIZATION OF BLOTS:

Nick Translation:

The probe was labelled with ^{32}P-dCTP by nick translation as follows:

The plasmid was incubated for 90 minutes at 16°C with a nicking enzyme (DNase I), ^{32}P-radiolabelled CTP, free unlabelled other nucleotides and DNA polymerase for nick translation. After the reaction, the mixture was passed through a spun column of Sephadex G-50 equilibriated in STE buffer, to separate the free radiolabelled nucleotides. The radiolabelled plasmid was collected in an Eppendorf tube and precipitated with 1 µg carrier DNA, 0.1 vols 3 M sodium acetate , pH 5.2, and 2 vols isopropanol overnight at -20°C. Before use, the labeled plasmid was pelleted down by centrifugation at 12,000xg for 5 min at 4°C, rinsed with 70% ethanol, vacuum dried and dissolved in TE buffer (10 mM Tris, pH 8, 1 mM EDTA). The counts per µl were checked using a liquid scintillation counter.

Just before hybridisation, the probe was denatured by adding 0.1 volume of 3 N NaOH was added, vortex mixed, kept for 5 min at 37°C, mixed again and immediately added to the hybridisation solution and mixed.

Hybridisation:

The pre-hybridisation solution consisted of 5X SSPE, 5% **Sodium** dodecyl **Sulfate,** 30% deionised formamide, 10% dextran **Sulfate** and 100 fig/ml denatured salmon sperm DNA. The blot was **placed** in a polythene bag, 10 ml ofthe above solution was added, all air bubbles removed, and the bag sealed. The blot was pre-hybridised by placing the bag in a shaking water bath at 42°C for at least 6h. The radiolabelled probe was then added along with 100 μg/μl denatured salmon sperm DNA, and the bag re-sealed. Hybridisation was carried out for 18 to 20 hours at 42°C with constant shaking. The blot was then washed with 2X SSPE / 1% SDS twice or thrice for 15 min each at 42°C, then at 55°C for 15 min, and with 1X SSPE / 0.1% SDS at 55°C for 15 min. The blot was then autoradiographed using intensifying screens (Lightning Plus, Dupont, U.S.A.).

All autoradiograms were quantitated by laser densitometry (Zeineh SRL-TRFF, Biomed Instruments Inc., U.S.A.). The data are eexpressed as relative percent of maximal expression.

CHAPTER 3

REGULATION OF ORNITHINE DECARBOXYLASE BY PROLACTIN IN THE KIDNEY AND LIVER OF RAT

Prolactin has been most widely recognised for its lactogenic properties and its role in the regulation of mammary function. However, in the course of studies involving this hormone, it has been shown that PRL has many diverse effects in various tissues. In addition to its mammotrophic properties, it is involved in functions such as growth promotion, osmoregulation, immunomodulation, liver mitogenesis and blood pressure regulation. PRL is an important trophic hormone in rat liver. Administration of PRL induces hepatic ODC activity, DNA synthesis, hypomethylation of DNA and causes hepatomegaly. Mammalian kidneys contain receptors for PRL. Male rat kidneys have been shown to have a higher number of PRL receptors than those of female rats. PRL modulates renal polyamine and cyclic AMP formation. PR1 also triggers the expression of genes which encode growth-related proteins such as insulin-like growth factor and Nb29, a 70 kDa heat shock protein-like molecule.

Prolactin induction can be exerted at multiple levels following PRL-binding to cell surface receptors, but neither the intracellular signalling pathway(s) nor the mechanism is known. It has been demonstrated that induction of milk protein gene expression by prolactin is effected via a serine/threonine kinase and possibly a tyrosine kinase. Various studies have implicated protein kinase C activation as a possible mechanism via which some of the actions of PRL are mediated. Various other mechanisms like stathmin phosphorylation, stimulation of a G protein which is not G_s, and even a direct translocation into the nucleus of a PRL-receptor complex to causing direct stimulation of

transcription, have been suggested.

In this study, an attempt has been made to investigate the mechanism by which PRL influences ornithine decarboxylase **activity** and gene expression in the liver and kidney of rat.

MATERIALS AND METHODS:

The general methods used have been described in Chapter 2. All the experiments in this chapter involved the use of adult male rats of 300-350 g. body weight. The rats were castrated via the scrotal route under mild ether anesthesia, and were used 4 to 5 days after castration.

Ovine prolactin was dissolved just prior to use in 0.9% sodium chloride/ 0.1 N NaOH, and the pH was adjusted to 7.5. The dose of PRL used throughout was 5.5 mg/kg body weight, and injections were administered intraperitoneally in 200 µl of the vehicle. Controls were given an equal volume of vehicle alone.

Actinomycin D was given intraperitoneally at a dosage of 6 mg/kg body weight, dissolved in ethanol/DMSO (1:1). It was injected 1h before administration of the hormone. Cycloheximide was injected intraperitoneally at a dosage of 8 mg/kg body weight, lh before the rats were killed. Quercetin dissolved in ethanol was injected intraperitoneally at a dosage of 10 µmoles or 100 µmoles/kg body weight; 15 minutes before injection of PRL. Quinacrine was injected intra peritoneally at a dosage of 200 µmoles/ kg body weight, 15 minutes before injection of PRL.

The processing of tissue , RNA isolation and hybridisation, and ODC enzyme assay have been described in Chapter II.

RESULTS:

The effect of a single injection of PRL on renal ODC activity is shown in Table 1. The ODC activity increased with time and peaked at 4-6 hours after injection, after which it fell slightly at 7h. The time course of induction of hepatic ODC activity by PRL is given in Table 2. This followed a pattern similar to that shown by renal ODC, except that by 7h the activity had declined to control levels. Also, the basal ODC activity in control groups and the extent of induction by PRL was greater in kidney than in the liver.

Fig. 1 shows the time course of induction of renal ODC mRNA levels in response to PRL, as studied by northern blot hybridisation. ODC mRNA levels peaked at 2h after injection, and were lowered by 6h. An interesting observation was that in orchidectomised rats, the induction of ODC mRNA was higher than in intact rats. At 6h post-injection, ODC mRNA levels were still elevated as compared to intact rats. In subsequent experiments castrated rats treated for 6h with PRL were used. Fig. 2 shows the northern blot of ODC mRNA levels in liver in response to PRL. Two peaks of high mRNA levels were seen, at 1h and 6h after injection. By 7h after injection, the mRNA levels had declined.

The effect of simultaneous injections of testosterone

propionate (TP) and PRL on renal ODC activity is shown in Table 3. At 6h post-injection, the ODC activity was elevated considerably beyond the activity seen in groups treated with PRL or TP alone. The effect of the same treatment on ODC mRNA levels is shown in Fig. 3. It was seen that at 6h after injection, ODC mRNA levels are considerably reduced as compared to those of the groups treated with TP or PRL alone.

Table 4 shows the effect of pre-treatment with actinomycin D, a transcription-blocking agent, on PRL induction of renal ODC activity. It was observed that the ODC activity was totally suppressed by actinomycin D.

The effect of pre-treatment with actinomycin D on PRL induction of renal ODC mRNA levels is shown in Fig. 4. ODC mRNA levels were suppressed by the presence of actinomycin D to about 40% that of groups treated with PRL alone.

The effect of the protein translation inhibitor cycloheximide on ODC activity in PRL-treated kidney is given in Table 5. Rats were given PRL 6h prior to sacrifice and cycloheximide 1h prior to sacrifice. Cycloheximide treatment was seen to completely abolish the induction of ODC activity by the hormone. The same treatment caused an increase in ODC mRNA levels in the group treated with both PRL and cycloheximide (Fig. 5).

The effect of similar cycloheximide treatment on ODC activity in liver is shown in Table 6, where it can be seen that

the activity is totally abolished as in the case of the kidney. Similarly, as shown in Fig. 6, an increase in ODC mRNA levels was seen in the group treated with both PRL and cycloheximide.

Quercetin, a protein kinase C inhibitor, when administered just prior to PRL caused almost total blockage of PRL-induction of renal ODC activity, as shown in Table 7. A lower dose of 10 μM quercetin slightly inhibited ODC activity, whereas the higher dose of 100 μM caused a much higher level of inhibition. However, as shown in Fig. 7, there was no inhibition of ODC mRNA expression by quercetin.

Table 8 shows the effect of quercetin on liver ODC activity. In this case too, pre-treatment with quercetin totally inhibited PRL-induced ODC activity.

Table 9 shows the effect of a phospholipase inhibitor, quinacrine, on the PRL-induced ODC activity. Pre-treatment with quinacrine, like that with quercetin, inhibited the ODC activity. This effect was more pronounced in the liver (Table 10) than in the kidney. In the kidney, quinacrine treatment caused a pronounced increase in PRL-treated ODC mRNA levels (Fig. 9). A similar effect, however, was not observed in the liver (Fig. 10).

DISCUSSION:

The rapid and transient nature of ODC induction by mitogens associates it with growth regulatory phenomena. In any given system in which ODC activity can be made to change, those changes may be dependent to a greater or lesser extent on alterations in mRNA **levels.** In some cases (Feinstein et *al.,* 1985) it was shown that changes in ODC mRNA and enzymatic activity are similar in timing and in extent, whereas in others, the magnitude of induction of activity exceeded that of mRNA (Katz and Kahana, 1987).

Various hormones have been shown to modulate ODC activity in the liver and kidney as well as other tissues. These include parathyroid hormone, calcitonin, vasopressin, d-aldosterone and L-triiodothyronine which increase renal ODC activity (Aragona et *al,* 1976). Prolactin has been shown to increase ODC activity in various tissues including mammary gland, liver, kidney, brain and testis. Prolactin also regulates various cellular functions.

The present study shows that both ODC enzyme activity and mRNA levels are regulated by prolactin in the liver and kidney of male rats. Previous studies have all involved usage of large doses of the hormone (~ 22 mg/kg body weight). In 1986, Buckley et *al* showed that a dose of 5.5 mg/kg body weight was able to **stimulate** rat hepatic DNA synthesis. Subsequently it was shown that the same dose of PRL caused significant hypomethylation of DNA in both **the** liver and kidney of rat (Reddy and Reddy, 1990). **This study** shows that the same low dose of PRL significantly

increases ODC activity and mRNA levels in these organs. Asingle injection of PRL induced both ODC mRNA levels and enzyme activity; however, the extent of induction of activity was by far the greater, and the peak of enzyme activity lagged behind that of the mRNA levels. An interesting observation was that in castrated rats, the basal ODC activity in kidney was higher than that in intact rats.

The extent to which PRL induction of renal ODC activity took place was greater in intact than in castrated rats. However, ODC mRNA levels in PRL-treated castrated rats were higher than in PRL-treated intact rats. This could indicate a post-transcriptional level of regulation in castrated rats. It also suggests a possible antagonistic effect of testosterone on renal ODC induction by PRL. It has been observed by Aragona et *al* (1976) that in rat liver, there is an increased binding of PRL to its receptors after castration, and that this effect is prevented upon treatment with testosterone.

In this study, when castrated rats were injected with testosterone propionate, PRL , or a combination of both hormones (Table 3) it was seen that at 6 h post-injection, groups receiving both hormones had an elevated ODC activity as compared to that of groups treated with either one of the hormones. Examination of the RNA levels showed that these were lowered at 6h in groups given both hormones as compared to those given either hormone alone (Fig. 3). The reason for this is not clear but may possibly be due to some post-transcriptional effect of TP

on PRL-induced ODC.

Pre-treatment with act.D, an inhibitor of transcription, caused a decrease in renal PRL-induced ODC mRNA to about 40% that of its level (Fig.4), and enzyme activity was totally suppressed. This indicates that transcription is at least partially essential for the action of PRL on ODC. actinomycin D has been reported to cause superinduction of ODC in certain systems (Wallon et *al.*,1990); however, no such effect was observed in the experimental model used here.

In kidney and liver, treatment with PRL induced ODC activity and increased mRNA levels. Treatment with the protein synthesis inhibitor cycloheximide caused a complete decline in ODC enzyme activity in both these organs (Tables 5 and 6), indicating that *de novo* protein synthesis is essential for the action of PRL on ODC. The ODC mRNA levels in both these organs were seen to increase slightly upon treatment with cycloheximide plus PRL (Figs. 5 and 6). This is likely to be due to the block in translation.

The signal transduction mechanisms linking prolactin receptors to macromolecular synthesis are poorly understood. Evidence has suggested that the mitogenic signalling enzyme protein kinase C (PKC) is involved in mediating PRL action in several target tissues, including rat liver. The pathway can be described briefly as follows: hormone or mitogen binding to its receptor stimulates the formation of diacylglycerol and inositol

triphosphate from Phosphatidyl inositol bisphosphate via the action of a G protein and the enzyme phospholipase C. Protein kinase C, in the presence of diacylglycerol, causes activation of particular proteins by phosphorylation, leading to the cellular response. The inositol triphosphate formed causes the release of calcium ions from the endoplasmic reticulum, also adding to the cellular response. The inhibitors used in this study, quercetin and quinacrine, inhibit the enzymes protein kinase C and phospholipase C, respectively.

It has been reported that in various tissues, PRL stimulates PKC activity. In cultured mouse mammary tissues, one of the earliest actions of PRL is the stimulation of ODC activity. The PKC inhibitor gossypol has been clearly shown to abolish the stimulatory effect of PRL on ODC activity (Etindi and Rillema, 1987), as well as its effects on lipid synthesis and RNA and casein synthesis. Therefore it is likely that at least some of the actions of PRL may involve protein kinase C.

In this study, quercetin, a PKC inhibitor known to have a minimal effect on RNA synthesis, was used to assess the effects of PKC inhibition on PRL-induced ODC activity in the kidney and liver of male adult rats. It was seen that at 6h after injection, 100 μM quercetin almost completely blocked the induction of ODC activity by PRL in the kidney (Table 7) . In the liver, the same dose of quercetin had an even more dramatic inhibitory effect on ODC activity. This indicated that in kidney and liver, the stimulation of ODC activity by PRL is likely to be via

stimulation of the protein kinase C pathway.

In Nb2 node lymphoma cells, a cell line dependent on externally added PRL for growth, PRL-stimulated ODC activity was inhibited 95% by addition of 100 μM quercetin to the culture. The PKC inhibitors, polymyxin B and tamoxifen also inhibit ODC induction and proliferation in Nb2 node lymphoma cells.

Protein kinase C is known to be activated by diacylglycerols. The latter can be generated by the action of phospholipase C on phospholipids. Crowe et *al.* (1991) showed that in young male rats, PRL stimulated a dose-dependent induction of hepatic c-myc, ODC and β-actin mRNAs at 60 minutes after PRL administration. A dose of 22 mg/kg body weight of PRL increased diacylglycerol levels, followed by an increase in PKC levels.

In rat hepatocytes, it has been shown that PRL-stimulated alterations in PKC activity are preceded by enhanced diacylglycerol generation. In Nb_2 lymphoma cells, quinacrine has been shown to inhibit PRL-stimulated ODC activity by almost 95%. Similarly, in mouse mammary gland explants, quinacrine inhibits ODC induction stimulated by the addition of either phospholipase A_2 or C to the culture.

In this study, an attempt was made to further confirm the possible action of PRL via the protein kinase C pathway. The phospholipase A_2 and C inhibitor, quinacrine, was administered in combination with PRL, to study the effect, if any. As shown in

Tables 9 and 10, the inhibitory effect of quinacrine on PRL-induced ODC activity was more pronounced in the liver than in the kidney. It is possible that in the latter, stimulation of PKC may be only one of the ways in which PRL affects ODC activity.

As mentioned in the introduction, a number of effects of prolactin have been linked to the stimulation of diacylglycerol formation and protein kinase C action. These include hepatotrophic responses stimulated by prolactin, and stimulation of ODC activity and mitogenesis in the Nb_2 node lymphoma cell line. The results show that inhibition of phospholipase C and/or protein kinase C adversely affects the induction of ODC activity in the kidney and liver by PRL *in vivo.* The effect of PRL in enhancing ODC mRNA expression was seen to be more enhanced in the absence of testosterone, i.e. in castrated rats. The expression of PRL receptors have been shown to be regulated by testosterone in liver and kidney of male rats. It has been shown that PRL-binding activity in the kidney is decreased by testosterone treatment. In male rats, sexual maturation caused a decrease in hepatic PRL receptors, and these were restored upon castration. The induction of ODC activity and mRNA expression by PRL was seen to be dependent on both transcription and translation to various extents. Thus, PRL seems to play a role in regulating ornithine decarboxylase in the liver and kidney of rat, and the mechanism whereby it enhances ODC activity and expression seems to be via the formation of diacylglycerol and activation of protein kinase C, as inhibitors of DAG formation and of PKC prevented the stimulation of ODC by prolactin.

TABLE 1

TIME COURSE OF PROLACTIN ACTION ON ODC ENZYME ACTIVITY IN KIDNEY OF ADULT MALE RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)	
1.	Saline	2161 ± 49	(4)
2.	PRL (1h)	2819 ± 161	(4)
3.	PRL (2h)	3020 ± 95	(4)
4.	PRL (4h)	9815 ± 131*	(4)
5.	PRL (6h)	10233 ± 225*	(4)
6.	Cas + Saline	5600 ± 205	(4)
7.	Cas + PRL (6h)	6882 ± 116**	(4)

Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Animals were sacrificed at the indicated times after injection and the ODC activity was estimated. Controls received 200µl of vehicle alone.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* - $p < 0.001$ as compared to group 1.

** - $p < 0.001$ as compared to group 6.

TABLE 2

TIME COURSE OF PROLACTIN ACTION ON LIVER ODC ACTIVITY IN ADULT MALE RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)	
1.	Saline	1502 ± 47	(4)
2.	PRL (1h)	1666 ± 24	(4)
3.	PRL (2h)	1818 ± 140	(4)
4.	PRL (4h)	2403 ± 80*	(4)
5.	PRL (6h)	2238 ± 97*	(4)

Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Animals were sacrificed at the indicated times after injection and the ODC activity was estimated. Controls received 200µl of vehicle alone.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* - $p < 0.001$ as compared to group 1.

TABLE 3

EFFECT OF PROLACTIN ON KIDNEY ODC ACTIVITY OF CASTRATED RATS TREATED WITH TESTOSTERONE PROPIONATE

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	5794 ± 74 (4)
2.	TP (6h)	8881 ± 277* (4)
3.	PRL (6h)	6882 ± 203* (4)
4.	TP + PRL (6h)	12381 ± 180** (4)

Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Testosterone propionate was injected **subcutaneously** in 200µl of sesame oil at a dose of 350 µg/kg body **weight. Animals** were sacrificed at the indicated times **after injection and the** ODC activity was estimated. Controls received 200µl of vehicle alone.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* - $p < 0.001$ as compared to group 1

** - $p < 0.001$ as compared to group 2

TABLE 4

EFFECT OF PRE-TREATMENT WITH ACTINOMYCIN D ON KIDNEY ODC ACTIVITY IN PRL-TREATED CASTRATED RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)	
1.	**Saline**	**5794** ± 74	(3)
2.	PRL (6h)	9048 ± 99*	(3)
3.	Act. D (1h) + PRL(6h)	1146 ± 68**	(3)

Animals **were** castrated 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected **intraperitoneally** at **a** dose of 5.5 mg/kg body weight in 200µl of vehicle. Actinomycin D was dissolved in ethanol:DMSO (1:1) and injected intraperitoneally lh prior to prolactin at a dose of 6 **mg/kg body** weight. Animals were sacrificed 6h after injection of **prolactin** and the ODC activity was estimated. Controls received 200µl of vehicle alone.
Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* - $p < 0.001$ as compared to group 1

** - $p < 0.001$ as compared to group 2

TABLE 5

EFFECT OF TREATMENT WITH CYCLOHEXIMIDE ON KIDNEY ODC ACTIVITY IN PRL-TREATED CASTRATED RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	**Saline**	5794 ± 74 (3)
2.	**PRL**	7848 ± 259* (3)
3.	CHX + PRL	2993 ± 60 (3)

Animals were castrated 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of **vehicle.** Cycloheximide was dissolved in saline and injected intraperitoneally 1h before sacrifice at a dose of 8 mg/kg body **weight.** Animals were sacrificed 6h after injection of prolactin **and the** ODC activity was estimated. Controls received 200µl of vehicle alone.
Results are expressed as Mean ± S.E.M. of 3-4 determinations from **4-5** animals in each group.

* - p< 0.001 as compared to group 1

** - p< 0.001 as compared to group 2

TABLE 6

EFFECT OF TREATMENT WITH CYCLOHEXIMIDE ON LIVER ODC ACTIVITY IN PRL-TREATED CASTRATED RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	1917 ± 28 (4)
2.	PRL	3041 ± 111* (4)
3.	CHX + PRL	2065 - 72 (4)

Animals were castrated 4 days prior to use.Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Cycloheximide was dissolved in saline and injected intraperitoneally 1h before sacrifice at a dose of 8 mg/kg body weight Animals were sacrificed 6h after injection of prolactin and the ODC activity was estimated. Controls received 200µl of vehicle alone.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* - $p < 0.001$ as compared to group 1.

** - $p < 0.005$ as compared to group 2.

TABLE 7

EFFECT OF QUERCETIN, A PROTEIN KINASE C INHIBITOR, ON KIDNEY ODC ACTIVITY IN PRL-TREATED CASTRATED RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	5600 ± 205 (4)
2.	PRL	10800 ± 124* (4)
3.	Quercetin + PRL	6022 ± 218** (4)

Animals were castrated 4 days prior to use. Prolactin was **dissolved** in 0.9% sodium chloride/0.1N NaOH and injected **intraperitoneally at** a dose of 5.5 mg/kg body weight in 200µl of **vehicle.** Quercetin was dissolved in ethanol and injected intraperitoneally at a dose of 300 µmoles/kg body weight 15 **minutes** prior to injection of prolactin. Animals were sacrificed 6h after injection of prolactin and the ODC activity was estimated. Controls received 200µl of vehicle alone.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* - $p < 0.001$ as compared to group 1

** - $p < 0.001$ as compared to group 2

TABLE 8

EFFECT OF QUERCETIN, A PROTEIN KINASE C INHIBITOR ON PRL STIMULATION OF ODC LEVELS IN RAT LIVER

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	1917 ± 28 (3)
2.	PRL (6 h)	3041 ± 112* (3)
3.	Quercetin + PRL	1600 ± 63** (3)

Animals were castrated 4 days prior to use. Prolactin was **dissolved in** 0.9% sodium chloride/0.1N NaOH and injected **intraperitoneally at** a dose of 5.5 mg/kg body weight in 200µl **of vehicle. Quercetin** was dissolved in ethanol and injected **intraperitoneally** at a dose of 300 µmoles/kg body weight 15 **minutes prior** to injection of prolactin. Animals were sacrificed **6h after** injection of prolactin and the ODC activity was **estimated.** Controls received 200µl of vehicle alone. **Results are** expressed as Mean ± S.E.M. of 3-4 determinations from **4-5 animals in** each group.

* - P< 0.001 as compared to group 1.

** - p< 0.001 as compared to group 2.

TABLE 9

EFFECT OF QUINACRINE, A PHOSPHOLIPASE C INHIBITOR, ON KIDNEY ODC ACTIVITY IN PRL-TREATED CASTRATED RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)	
1.	**Saline**	5600 ± 205	(4)
2.	**PRL**	10800 ± 124*	(4)
3.	Quinacrine + PRL	7812 ± 146	(4)

Animals were castrated **4** days prior to use. Prolactin was **dissolved in** 0.9% sodium chloride/0.1N NaOH and injected **intraperitoneally** at a dose of 5.5 mg/kg body weight in 200µl of **vehicle.** Quinacrine was dissolved in ethanol and injected intraperitoneally at a dose of 200 µmoles/kg body weight 15 **minutes** prior to prolactin. Animals were sacrificed 6h after **injection** of prolactin and the ODC activity was estimated. **Controls** received 200µl of vehicle alone.

Results are expressed as **Mean** ± S.E.M. of 3-4 determinations from **4-5 animals in** each group.

* - $p < 0.001$ as compared to group 1.

** - $p < 0.001$ as compared to group 2.

TABLE 10

EFFECT OF QUINACRINE, A PHOSPHOLIPASE C INHIBITOR ON PRL STIMULATION OF ODC IN RAT LIVER

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	1917 +- 28 (3)
2.	PRL (6h)	3041 +- 111* (3)
3.	Quinacrine + PRL	2021 +- 117** (3)

Animals were castrated 4 days prior to use. Prolactin was **dissolved in** 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Quinacrine was dissolved in ethanol and injected intraperitoneally at a dose of 200 µmoles/kg body weight 15 **minutes prior to** prolactin.Animals were sacrificed 6h after injection of prolactin and the ODC activity was estimated. **Controls** received 200µl of vehicle alone.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from **4-5 animals** in each group.

* - **P<** 0.001 as compared to group 1.

* - p< 0.005 as compared to group 2.

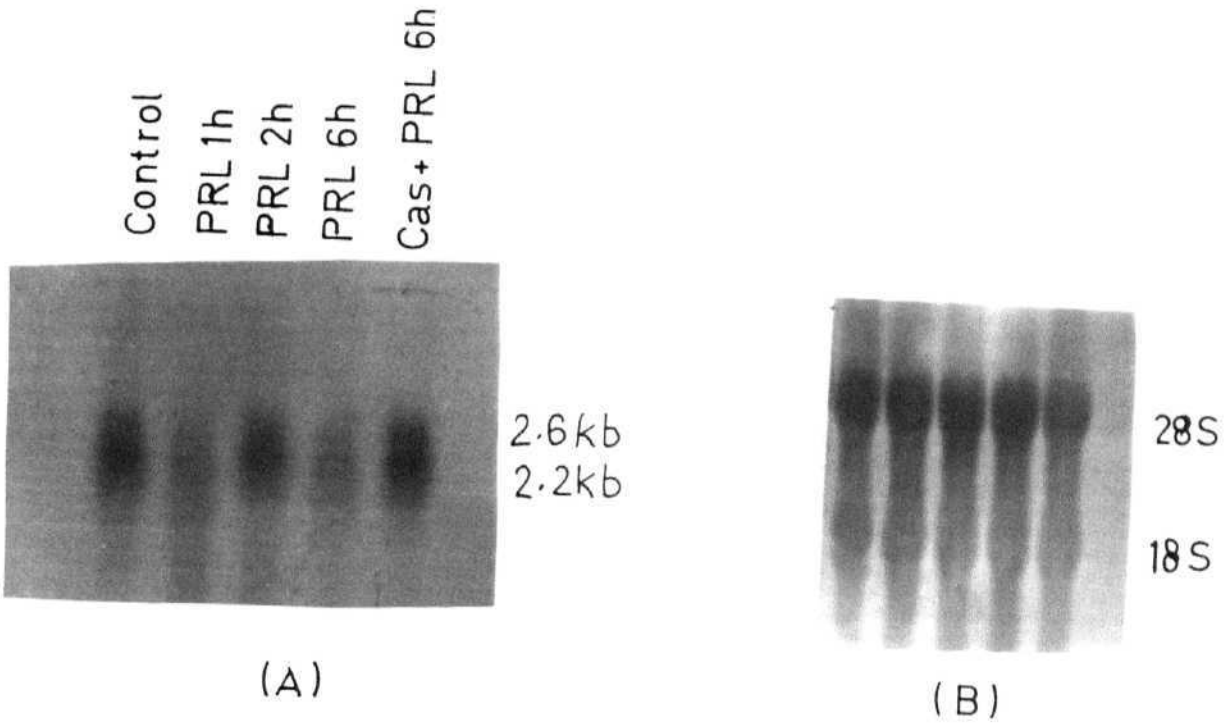
Fig. 1. Time course of prolactin action on ODC expression in the kidney of rat.

Adult rats of 300-350g weight were used. The **rats in** the **castrated group** (Lane 5) were castrated via the scrotal route 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Controls received 200µl of vehicle alone. Animals were sacrificed at the indicated times after injection. RNA isolation and northern analysis were carried out as described in Chapter 2.

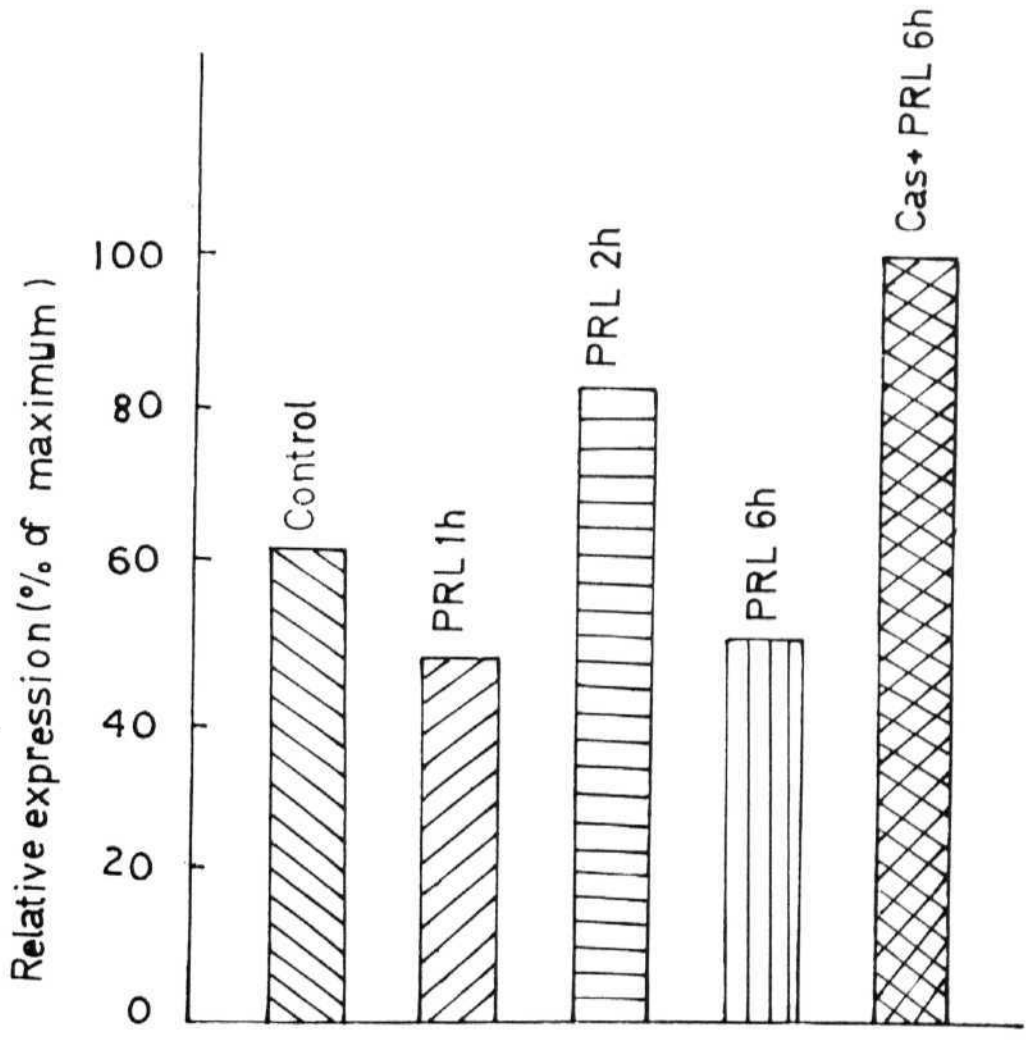
A. Autoradiogram of northern blot showing ODC expression of PRL-treated kidney.

B. Methylene blue stained ribosomal RNA (28S and 18S) of the blot demonstrating equal loading of the samples.

C. Densitometric data of A expressed as relative percent of maximal expression.


Control
PRL 1h
PRL 2h
PRL 6h
Cas+ PRL 6h
2.6kb
2.2kb
28S
18S

(A) (B)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
PRL 1h
PRL 2h
PRL 6h
Cas+ PRL 6h

(C)

Fig. 1

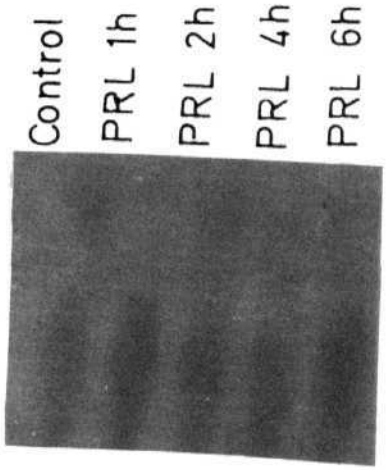
Fig. 2. Time course of prolactin action on ODC expression **in** the **liver of rat.**

Adult rats of 300-350g weight were used. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Controls received 200µl of vehicle alone. Animals were sacrificed at the indicated times after injection. Liver RNA isolation and northern analysis were carried out as described in Chapter 2.

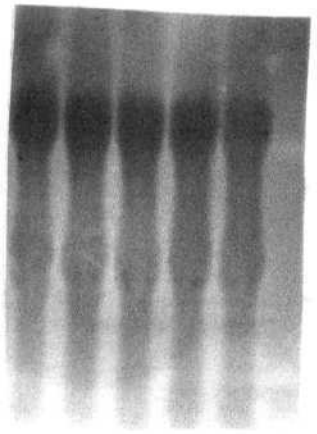
A. Autoradiogram of northern blot showing ODC expression of PRL-treated liver.

B. Methylene blue stained ribosomal RNA (28S and 18S) of the blot demonstrating equal loading of the samples.

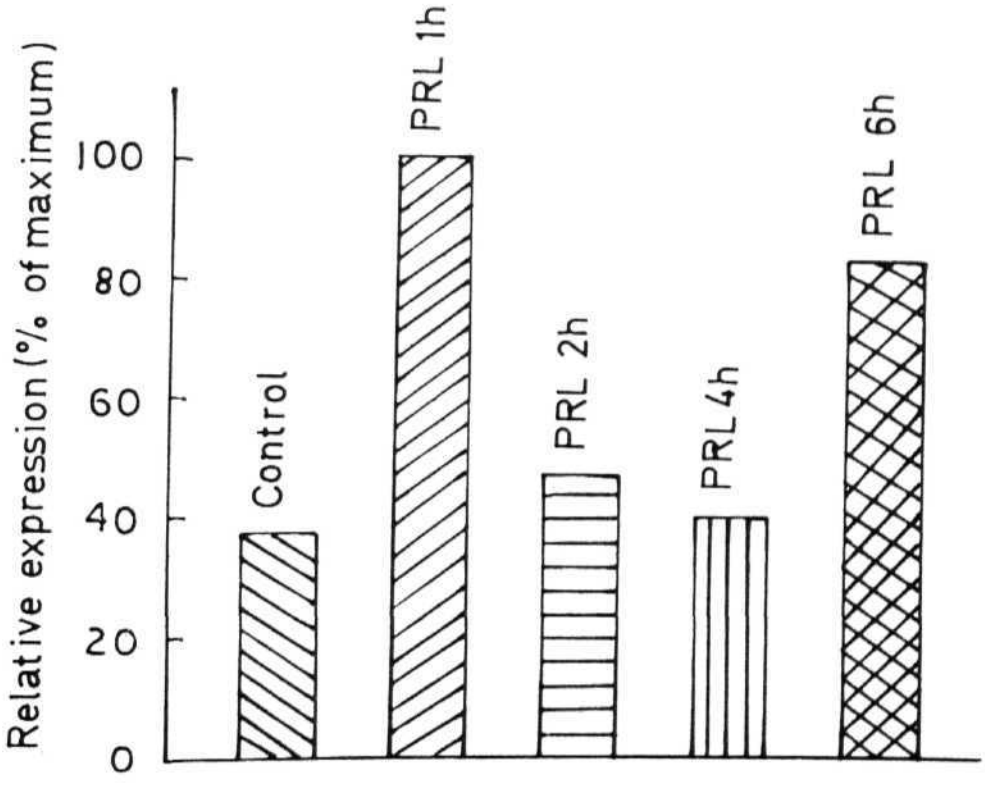
C. Densitometric data of A expressed as relative percent of maximal expression.


Control
PRL 1h
PRL 2h
PRL 4h
PRL 6h

(A)


28 S
18 S

(B)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
PRL 1h
PRL 2h
PRL 4h
PRL 6h

(C)

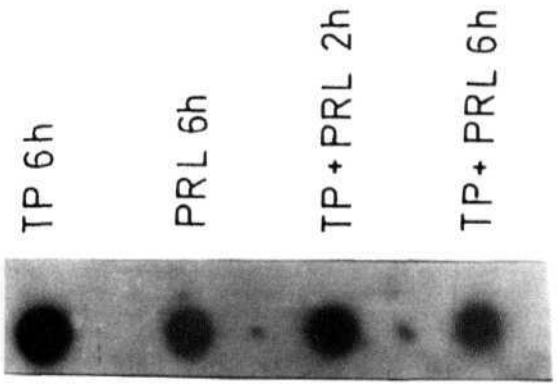
Fig. 2

Fig. 3. Effect of prolactin on kidney ODC expression in rats **treated with testosterone propionate.**

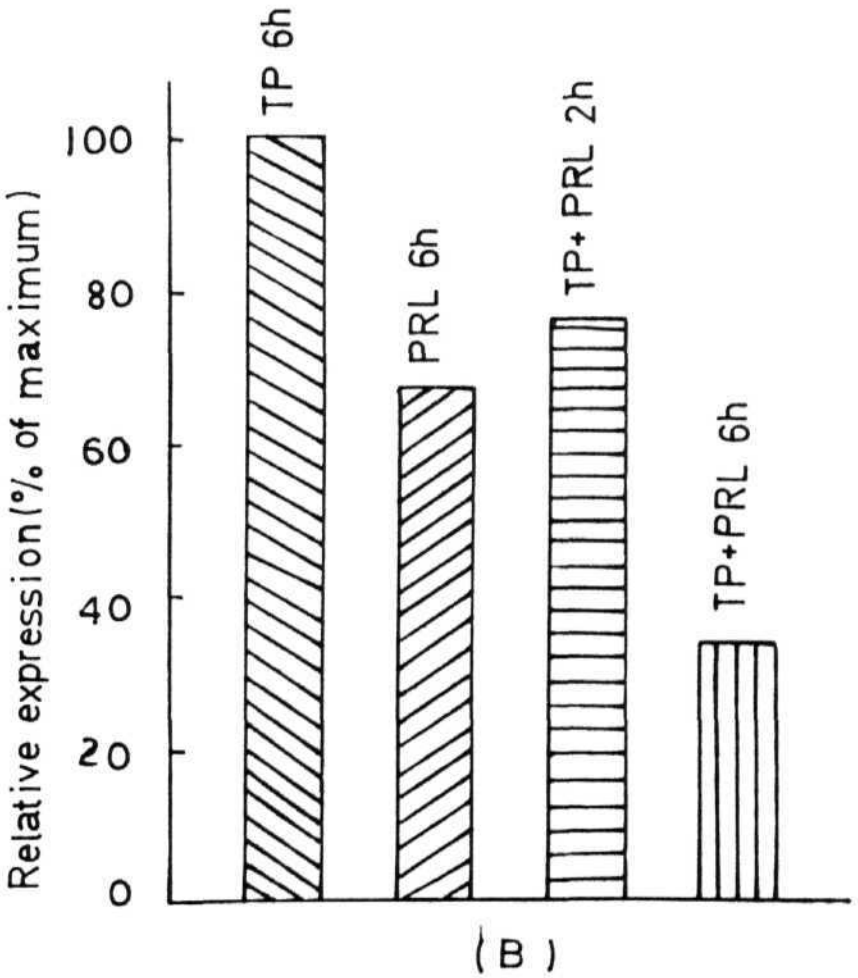
Adult rats of 300-350g weight were castrated via the scrotal route 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Testosterone propionate (TP) was injected subcutaneously in 200µl of sesame oil at a dose of 350 Mg/kg body weight. Animals were sacrificed 6h after injection. RNA isolation and dot blot analysis were carried out as described in Chapter 2.

A. Autoradiogram of northern blot showing the effect of PRL on ODC expression of TP-treated Kidney.

B. Densitometric data of A expressed as relative percent of maximal expression.


TP 6h
PRL 6h
TP + PRL 2h
TP + PRL 6h

(A)


Relative expression (% of maximum)
0
20
40
60
80
100
TP 6h
PRL 6h
TP+ PRL 2h
TP+PRL 6h

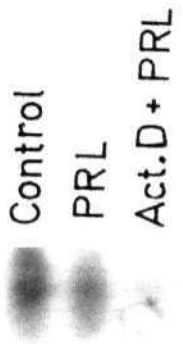
(B)

Fig. 3

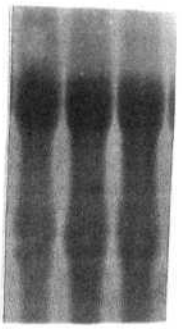
Fig. 4. Effect of pre-treatment with actinomycin D on PRL-induction of ODC expression in kidney of rat.

Adult rats of 300-350g body weight were castrated 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Actinomycin D was dissolved in ethanol:DMSO (1:1) and injected intraperitoneally 1h prior to prolactin at a dose of 6 mg/kg body weight. Controls received 200ml of vehicle. Animals were sacrificed 6h after injection of prolactin. Kidney RNA isolation and northern blot analysis were carried out as described in Chapter 2.

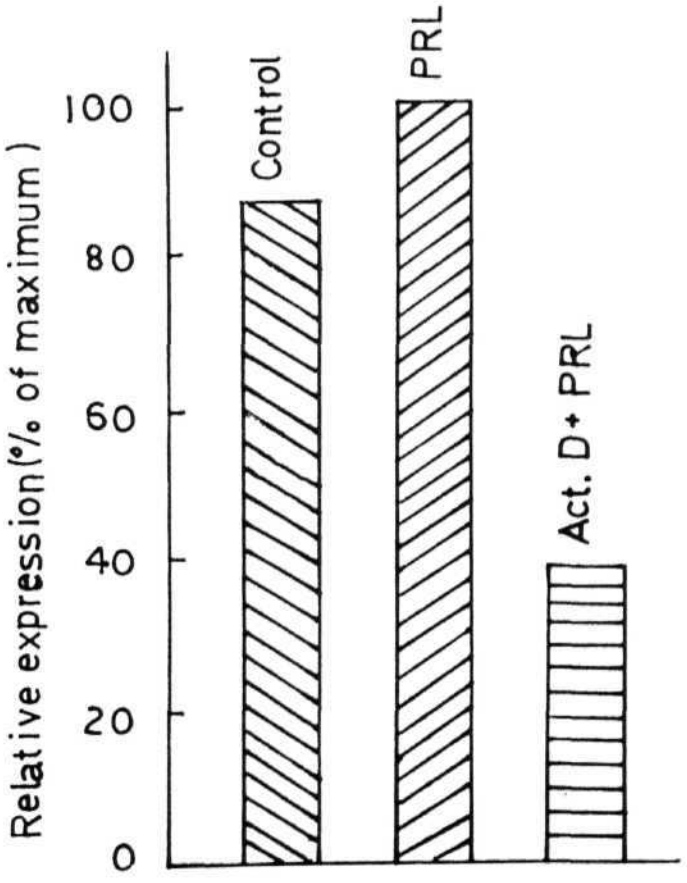
A. Autoradiogram of northern blot showing the effect of pre-treatment with Actinomycin D on ODC expression of PRL-treated liver.
B. Methylene blue stained ribosomal RNA (28S and 18S) of the blot demonstrating equal loading of the samples.
C. Densitometric data of A expressed as relative percent of maximal expression.


Control
PRL
Act.D+PRL

(A)


28 S
18 S

(B)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
PRL
Act. D+PRL

(C)

Fig. 4

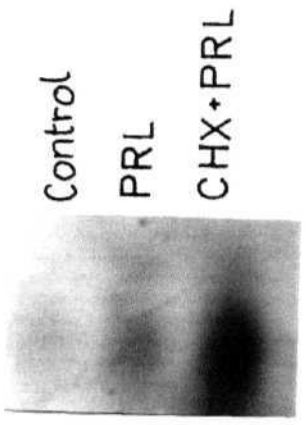
Fig. 5. Effect of cycloheximide on kidney ODC expression in PRL-treated rats.

Animals of 300-350g body weight were castrated 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Cycloheximide was dissolved in ethanol and injected intraperitoneally 1h before sacrifice at a dose of 8 mg/kg body weight. Controls received 200µl of vehicle alone. Animals were sacrificed 6h after injection of prolactin. RNA isolation and northern analysis were carried out as described in Chapter 2.

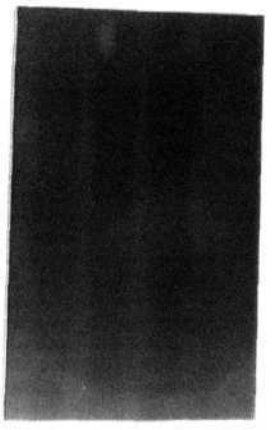
A. Autoradiogram of northern blot showing the effect of treatment with cycloheximide on ODC expression of PRL-treated kidney.

B. Methylene blue stained ribosomal RNA (28S and 18S) of the blot demonstrating equal loading of the samples.

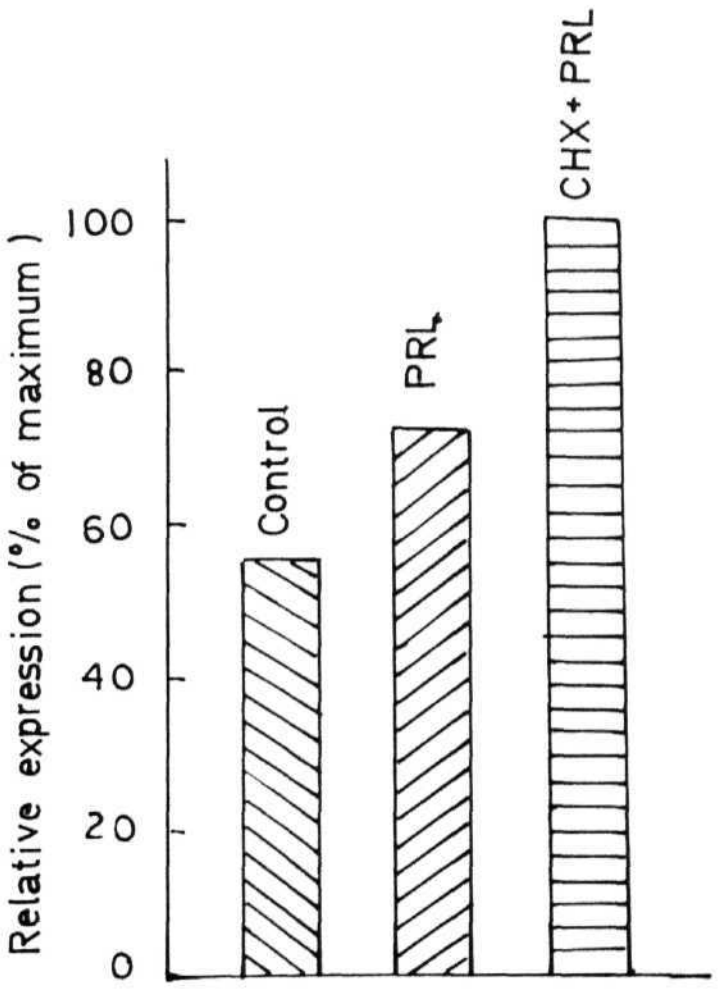
C. Densitometric data of A expressed as relative percent of maximal expression.


Control
PRL
CHX+PRL

(A)


28 S
18S

(B)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
PRL
CHX+PRL

(C)

Fig. 5

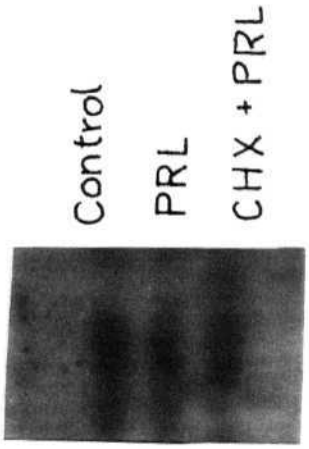
Fig. 6. Effect of cycloheximide on liver ODC expression **in PRL-treated rats.**

Animals of 300-350g body weight were castrated 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Cycloheximide was dissolved in ethanol and injected intraperitoneally 1h before sacrifice at a dose of 8 mg/kg body weight. Controls received 200µl of vehicle alone. Animals were sacrificed 6h after injection of prolactin. RNA isolation and northern analysis were carried out as described in Chapter 2.

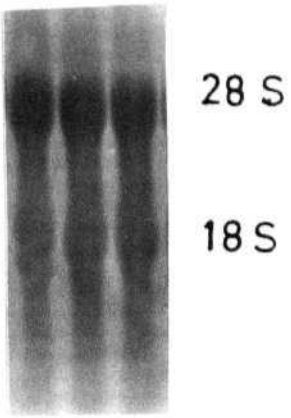
A. Autoradiogram of northern blot showing the effect of treatment with cycloheximide on ODC expression of PRL-treated liver.

B. Methylene blue stained ribosomal RNA (28S and 18S) of the blot demonstrating equal loading of the samples.

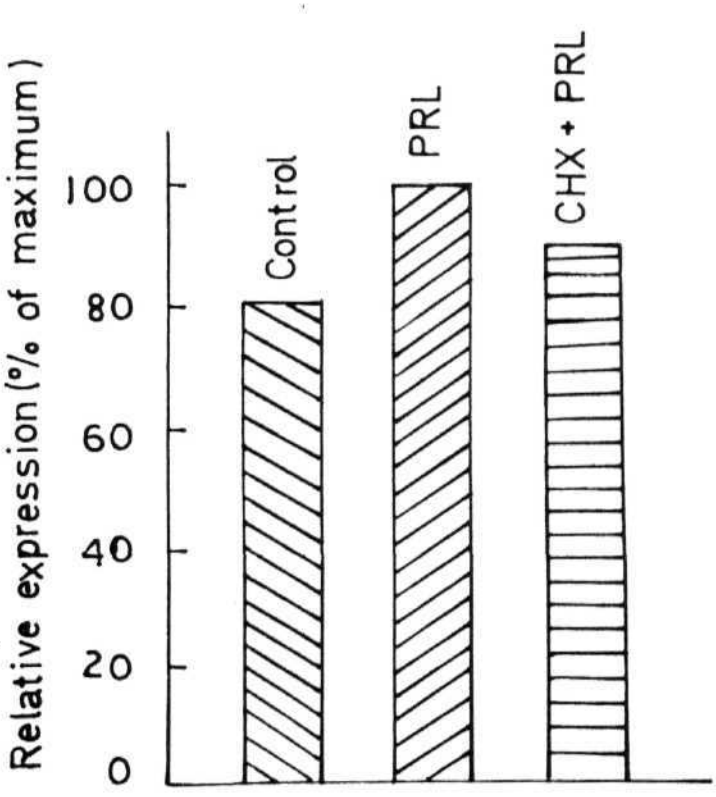
C. Densitometric data of A expressed as relative percent of maximal expression.


Control
PRL
CHX + PRL

(A)


28 S
18 S

(B)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
PRL
CHX + PRL

(C)

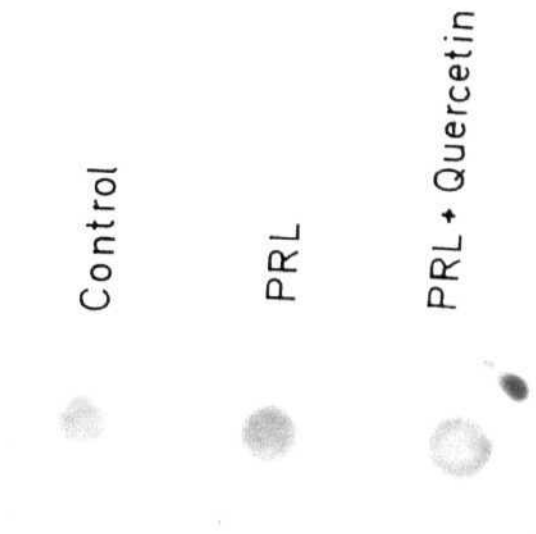
Fig. 6

Fig. 7. Effect of quercetin, a protein kinase C inhibitor, on kidney ODC expression in PRL-treated rats.

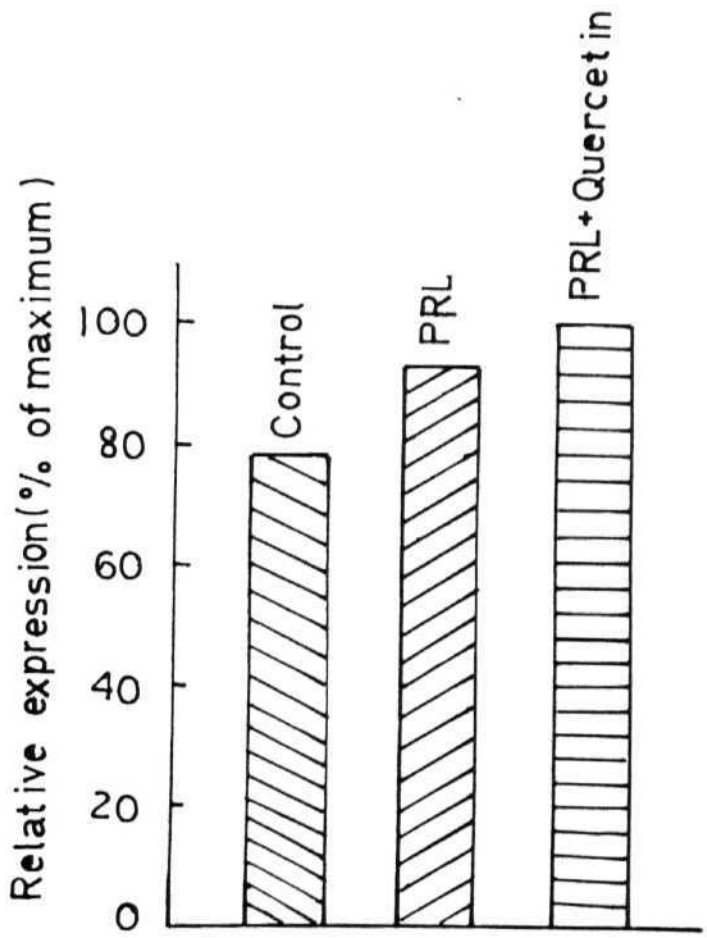
Adult rats of 300-350g body weight were castrated 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight **in** 200µl of vehicle. Quercetin was dissolved in ethanol and injected intraperitoneally at a dose of 300 µmoles/kg body weight 15 minutes prior to injection of prolactin. Controls received 200µl **of** vehicle alone. Animals were sacrificed 6h after injection of prolactin. RNA isolation and dot blot analysis were carried out as described in Chapter 2.

A. Autoradiogram of northern blot showing the effect of treatment with quercetin on ODC expression of PRL-treated kidney.

B. Densitometric data of A expressed as relative percent of maximal expression.


Control
PRL
PRL + Quercetin

(A)


Relative expression(% of maximum)
0
20
40
60
80
100
Control
PRL
PRL+Quercetin

(B)

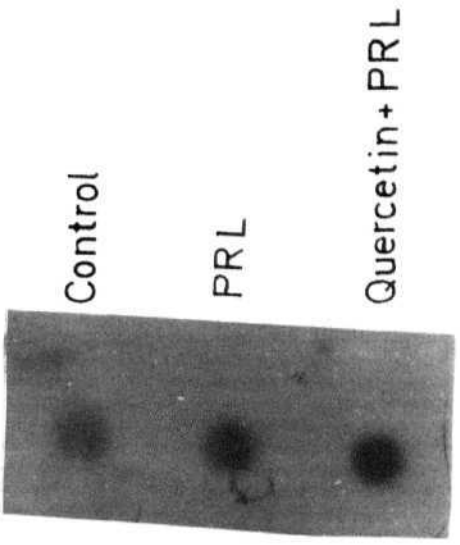
Fiq. 7

Fig. 8. Effect of quercetin, a protein kinase inhibitor, on liver ODC expression in PRL-treated rats.

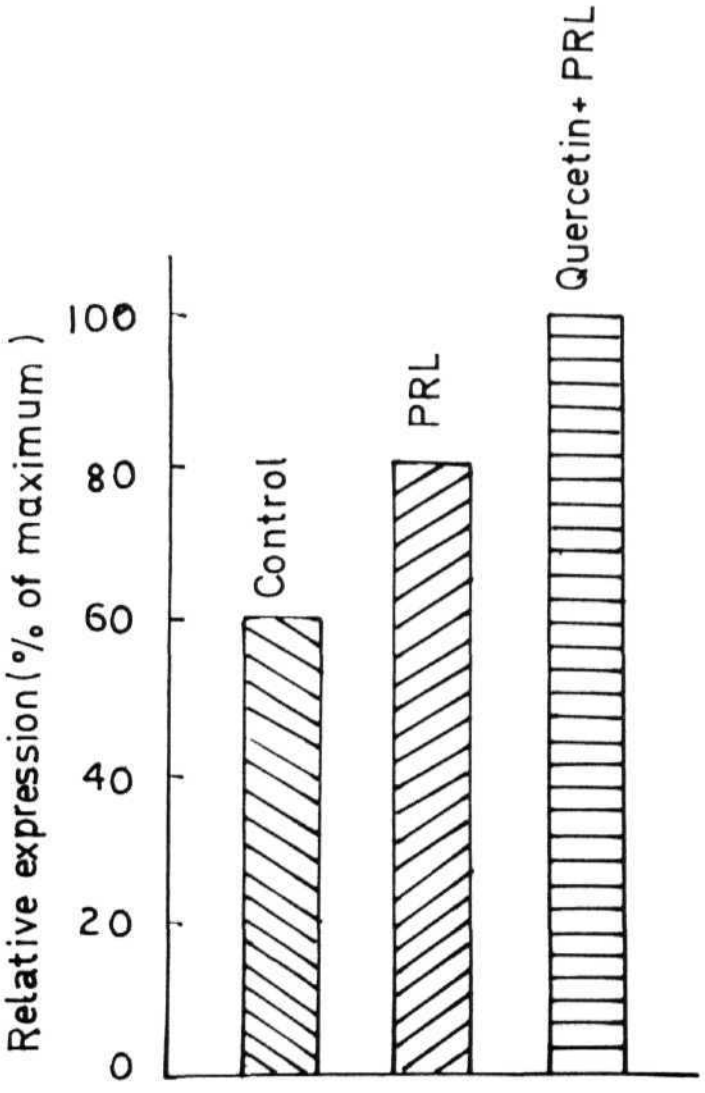
Adult rats of 300-350g body weight were castrated 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Quercetin was dissolved in ethanol and injected intraperitoneally at a dose of 300 µmoles/kg body weight 15 minutes prior to injection of prolactin. Controls received 200µl of vehicle alone. Animals were sacrificed 6h after injection of prolactin. RNA isolation and dot blot analysis were carried out as described in Chapter 2.

A. Autoradiogram of dot blot showing the effect of treatment with quercetin on ODC expression of PRL-treated liver.

B. Densitometric data of A expressed as relative percent of maximal expression.


Control
PRL
Quercetin+ PRL

(A)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
PRL
Quercetin+ PRL

(B)

Fig. 8

Fig. 9. Effect of quinacrine, phospholipase C inhibitor, on kidney ODC expression in PRL-treated rats.

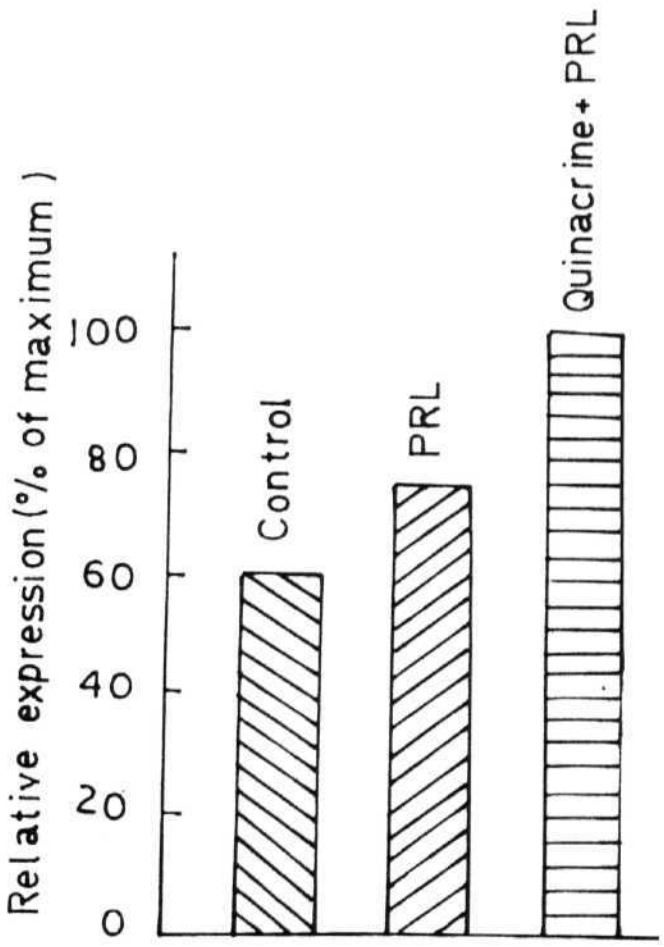
Adult rats of 300-350g body weight were castrated 4 days prior to **use.** Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH **and** injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Quinacrine was dissolved in ethanol and injected intraperitoneally at a dose of 200 mµoles/kg body weight 15 minutes prior to prolactin. Controls received 200µl of vehicle alone. Animals were sacrificed 6h after injection of prolactin. RNA isolation and dot blot analysis were carried out as described in Chapter 2.

A. Autoradiogram of dot blot showing the effect of treatment with quinacrine on ODC expression of PRL-treated kidney.

B. Densitometric data of A expressed as relative percent of maximal expression.


Control
PRL
Quinacrine + PRL

(A)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
PRL
Quinacrine + PRL

(B)

Fig. 9

Fig. 10. Effect of quinacrine, phospholipase C inhibitor, on liver ODC expression in PRL-treated rats.

Adult rats of 300-350g body weight were castrated 4 days prior to use. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight **in** 200µl of vehicle. Quinacrine was dissolved in ethanol and injected intraperitoneally at a dose of 200 mµoles/kg body weight 15 minutes prior to prolactin. Controls received 200µl of vehicle alone. Animals were sacrificed 6h after injection of prolactin. RNA isolation and dot blot analysis were carried out as described in Chapter 2.

A. Autoradiogram **of** dot blot showing the effect of treatment with quinacrine on ODC expression of PRL-treated liver.

B. Densitometric data of A expressed as relative percent of maximal expression.

Control
PRL
Quinacrine + PRL

(A)

Relative expression (% of maximum)
0
20
40
60
80
100
Control
PRL
Quinacrine + PRL

(B)

Fig. 10

CHAPTER 4

REGULATION OF ORNITHINE DECARBOXYLASE BY GROWTH HORMONE IN THE KIDNEY AND LIVER OF RAT

Growth hormone plays a crucial role in the regulation of growth in mammals. It stimulates nucleic acid and protein synthesis. In mouse 3T3-F442A preadipocytes, GH has been shown to promote differentiation, up-regulate vinculin expression, trigger diacylglycerol formation, enhance the rate of glucose uptake and stimulate lipid accumulation. GH exerts important effects on liver function through regulation of the expression of various hepatic proteins. It also participates in the regulation of hepatic low-affinity glucocorticoid binding sites and hepatic prolactin receptors. Induction by GH of c-fos and c-jun transcription has also been observed in preadipocytes. GH, like PRL, has been implicated as an immunomodulator, and direct production of GH by normal lymphocytes has been noted. In addition to these effects, GH stimulates ODC activity in liver, kidney, heart and thymus (Fausto, 1971, Brandt *et al.,* 1972).

The mechanism by which GH induces cell proliferation and differentiation has yet to be clearly elucidated, but GH has been shown to control the expression of a number of proteins thought to be involved in differentiation processes. GH regulates the expression of transcriptional factors, growth factors and also the expression of some growth factor receptors. Thus, GH action may be either direct or mediated by modulation of the action of other growth factors. As yet, no single second messenger mediating the effects of GH has been pinpointed. It has been suggested that a tyrosine kinase may be associated with the growth hormone receptor in certain cell types (Anderson, 1992). The mode of action of GH in regulating ODC activity is not known.

In this study, an attempt has been made to investigate the levels at which GH affects this enzyme.

MATERIALS AND METHODS:

The general methods used have been described in Chapter 2. All the experiments in this chapter involved the use of adult male rats of 300-350 g body weight. The rats were castrated via the scrotal route under mild ether anesthesia, and were used 4 to 5 days after castration.

Bovine growth hormone was dissolved just before use in basic saline solution, and pH was adjusted to 9.5. The dose of GH used throughout was 5.5 mg/kg body weight, and injections were administered intraperitoneally in 200 µl of the vehicle. Controls were given an equal volume of the vehicle alone.

Actinomycin D was given intraperitoneally at a dose of 6 mg/kg body weight, dissolved in ethanol:DMSO (1:1). It was injected 1h prior to the hormone. Cycloheximide was injected intraperitoneally at a dosage of 8 mg/kg body weight, lh before the rats were killed.

RESULTS:

The effect of a single dose of GH on renal ODC activity is shown in Table 11. The ODC activity increased with time and peaked at 4-6 h after administration of GH. The time course of hepatic ODC activity in response to the same dose of GH is given in Table 12. This followed a pattern similar to that of renal ODC

activity.

Fig. 11 shows the time course of induction of renal ODC mRNAlevels in response to a single injection of GH, as shown by northern analysis. It was found that ODC mRNA levels increased at 4-6 h after injection. The hepatic mRNA levels at various time intervals after GH injection were studied by dot blot hybridisation, as shown in Fig. 12. The ODC mRNA levels in liver were also seen to increase and peak at 4-6 h post-injection. The extent of ODC mRNA induction by GH was seen to be much higher in castrated rats than in intact ones.

The effect of simultaneous injections of testosterone propionate (TP) and GH on renal ODC activity is shown in Table 13. At 2h after injection, the ODC activity was higher than that of groups administered only one of the hormones, but the effect seen was not an additive one. By 6h after injection, the ODC activity had declined to control level.

Fig. 13 shows the renal ODC mRNA pattern following simultaneous TP and GH treatment. At 6h after injection, the mRNA level in the groups given TP and GH was reduced when compared to that of the groups given TP alone, but similar to that of the group given GH alone.The effect of pre-treatment with actinomycin D, a transcription inhibitor, on GH-stimulation of ODC activity in the kidney is shown in Table 14. It was seen that the stimulatory action of GH was completely blocked by the presence of actinomycin D. A similar effect was observed in hepatic ODC

activity on pre-treatment with actinomycin D and subsequent GH treatment (Table 15).

Renal ODC mRNA levels stimulated by GH were also seen to be suppressed on actinomycin D and GH treatment, as compared to groups treated with GH alone (Fig. 14).

The effect of blocking protein translation using cycloheximide on GH-stimulation of renal ODC activity is shown in Table 16. It was seen that treatment with cycloheximide completely blocked the stimulation of ODC by GH. Similarly, hepatic ODC stimulation by GH was also blocked by GH, as shown in Table 17.

ODC mRNA accumulation was observed in both kidney (Fig. 15) and liver (Fig. 16) on treatment with a combination of GH and cycloheximide, as compared to groups treated with GH alone.

DISCUSSION:

Very little is known about the early events that occur after the binding of GH to its receptor. As is true for PRL, no second messenger mediating the effects of GH has been positively identified. Some reports have suggested that GH may act via protein kinase C stimulation. It has also been suggested that a novel tyrosine kinase activity may be associated with the GH receptor in certain cell types.

In 1968, Jänne *et al.* reported the stimulation by GH of

spermidine and putrescine synthesis in rat liver, and subsequently established that this was due to GH stimulation of **ODC** activity. This stimulation was independent of the adrenal glands, and maximal stimulation occurred at 4h after injection. Brandt *et al.* (1972) later showed that GH-stimulated ODC activity in kidney peaked at 3-4 h after GH administration and was back to basal levels at 8h.

The present study shows that bGH stimulates both ODC activity and its mRNA expression in the kidney and liver of rat. A single low dose of 5.5 mg/kg body weight was sufficient to have a significant effect at 4 to 6 hours after injection.

Interestingly, it was observed that basal renal ODC activity was higher in castrated rats as compared to intact rats. Also, the extent of ODC mRNA induction by GH was much higher in castrated than intact rats. This suggested the possibility of a **mutual** antagonistic effect of GH and testosterone.

To investigate this possibility, when castrated were administered testosterone propionate (TP) along with GH, it was seen that at 2h post-injection, the ODC activity was higher than that in groups given only one of the hormones. However, this was not an additive effect. At 6h, ODC activity had declined to basal levels. The ODC mRNA levels at 6h after injection of GH and TP was lower than that of the group given TP alone, and similar to **that of** the group given GH alone. The reason for this was not clear **and** does not satisfactorily explain the reason for extent

of ODC mRNA induction by GH being higher in castrated rats. There may possibly be some other factors coming into play here.

Ongoing transcription was seen to be required for the stimulation of ODC by GH, as evinced by the experiments where pre-treatment with actinomycin D was given, followed by GH treatment . GH stimulation of ODC activity as well as mRNA levels in both liver and kidney was reversed completely by actinomycin D pre-treatment. However, as mentioned earlier, there was a discrepancy in the extent to which GH stimulates the ODC activity and mRNA levels, respectively. This indicates that transcription may not be the only mechanism by which GH affects ODC.

Further studies investigated the effect of the protein translation inhibitor cycloheximide, on GH-induction of renal and hepatic ODC. Cycloheximide treatment was shown to block the stimulatory activity of GH, indicating that de *novo* protein synthesis is necessary for the effect of GH on ODC. This was further substantiated by the fact that GH-treated ODC mRNA levels remain unchanged in the presence of cycloheximide.

Recent reports have indicated that GH itself is able to initiate a mitogenic response in a number of tissues *in vivo.* It has been shown to induce transcription of the proto-oncogenes c-myc and fos in the liver and kidney (Murphy et *al.,* 1987a). Further, human GH was shown to stimulate mitogenesis in Nb2 node lymphoma cells, and this effect was enhanced by the tumor promoter TPA (Gertler et *al.,* 1985). Growth hormone has also been

reported to activate mitogen-activated protein kinase in 3T3-F442A preadipocytes (Anderson, 1992).

This study shows that GH induces ODC activity and increases its mRNA expression in both the liver and kidney. The ODC gene is known to be one of those whose expression is enhanced by mitogens, and has been implicated in certain types of carcinogenesis. It has been postulated that the ODc gene itself may act as an oncogene (Auvinen *et al.*, 1992; Moshier *et al.*, 1993). The effect of GH in stimulating ODC mRNA levels is a further reflection of the mitogenic effects of GH, which has earlier been reported to regulate the expression of some growth-regulatory genes and growth factors.. Recent studies have mainly been carried out using human growth hormone. This study demonstrates that even the non-lactogenic bovine GH is capable (at a low dose) of stimulating ODC expression in the kidney and liver of rat. The effect of GH on ODC was seen to involve both transcription and translation. The effect of GH was more pronounced in castrated rats, suggesting that the absence of testosterone may play a role in the action of GH on ODC in liver and kidney.

TABLE 11

TIME COURSE OF GROWTH HORMONE ACTION ON ODC ENZYME ACTIVITY IN KIDNEY OF ADULT MALE RATS

	Treatment	ODC Specific Activity (pmoles $^{14}CO_2$ /h /mg. protein)
1.	Saline	2161 ± 49 (4)
2.	GH (1h)	3445 ± 205* (4)
3.	GH (2h)	3489 ± 353* (4)
4.	GH (4h)	9283 ± 110* (4)
5.	GH (6h)	8924 ± 294* (4)
6.	Cas + Saline	6250 ± 135 (4)
7.	Cas + GH	13872 ± 100** (4)

Growth hormone was dissolved in 0.9% sodium chloride/0.1N **NaOH and injected** intraperitoneally in 200 µl of vehicle at a dose of 5.5 **mg/kg body weight.** Animals were sacrificed at various time **intervals after** injection as indicated and ODC activity **was estimated.**

Results are expressed as Mean ± S.E.M. of 3-4 determinations from **4-5 animals in** each group

* - p< 0.001 as compared to group 1

** - **p< 0.001** as compared to group 6

TABLE 12

TIME COURSE OF GH ACTION ON ODC ACTIVITY IN LIVER OF ADULT MALE RAT

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)	
1.	Saline	1421 ± 37	(4)
2.	GH (1h)	1512 ± 29	(4)
3.	GH (2h)	1638 ± 39	(4)
4.	GH (4h)	2434 ± 24*	(4)
5.	GH (6h)	2754 ± 61*	(4)
6.	Cas + Saline	1917 ± 20	(4)
7.	GH (6h)	3067 ± 77**	(4)

Growth hormone was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally in 200 µl of vehicle at a dose of 5.5 mg/kg body weight. Animals were sacrificed at various time intervals after injection as indicated and ODC activity was estimated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group

* - $p < 0.001$ as compared to group 1

** - $p < 0.001$ as compared to group 6

TABLE 13

EFFECT OF GH ON KIDNEY ODC ACTIVITY OF RATS TREATED WITH TESTOSTERONE PROPIONATE

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	TP (6h)	8881 ± 505 (3)
2.	GH (6h)	13872 ± **140** (3)
3.	TP + GH (6h)	15469 ± 270 (3)
4.	TP + GH (6h)	5300 ± 373 (3)

Animals were castrated **4** days prior to use. Growth hormone was **dissolved in** 0.9% sodium chloride/0.1N NaOH and injected **intraperitoneally in** 200 µl **of** vehicle at a dose **of** 5.5 mg/Kg **body weight.** Testosterone propionate was injected subcutaneously **in** 200 µl **of** sesame oil at a dose of 350 Mg/kg body weight. Controls received 200 µl of vehicle alone. Animals were **sacrificed** at 6hafter injection as indicated and ODC activity was **estimated.**
Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 **animals in** each group
* - p< 0.001 as compared to group 1

TABLE 14

EFFECT OF PRE-TREATMENT WITH ACTINOMYCIN D ON KIDNEY ODC ACTIVITY IN GH-TREATED CASTRATED RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	6250 ± 135 (4)
2.	GH	13872 ± 100* (4)
3.	Act, D + GH	2499 ± 187** (4)

Animals were castrated **4** days prior to use. Growth hormone was **dissolved in** 0.9% sodium chloride/0.1N NaOH and injected **intraperitoneally** in 200 µl of vehicle at a dose of 5.5 **mg/kg body weight.** Actinomycin **D** was dissolved in ethanol/DMSO (1:1) **and injected** intraperitoneally in 100 µl at a dose of 6 **mg/kg body weight.** Controls received 200 µl of vehicle alone. Animals **were** sacrificed at 6h after injection of growth hormone and ODC **activity was** estimated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 **animals** in each group

* - **p< 0.001** as compared to group 1

** - p< 0.001 as compared to group 2

TABLE 15

EFFECT OF PRE-TREATMENT WITH ACTINOMYCIN-D ON GH INDUCTION OF ODC ACTIVITY IN LIVER OF CASTRATED RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)	
1.	**Saline**	1917 +- 20	(4)
2.	**GH (6h)**	3067 +- 77*	(4)
3	**Act.D + GH**	1447 +- 29**	(4)

Animals were castrated 4 days prior to use. Growth hormone was **dissolved** in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally in 200 µl of vehicle at a dose of 5.5 mg/kg **body** weight. Actinomycin D was dissolved in ethanol/DMSO (1:1) **and** injected intraperitoneally in 100 µl at a dose of 6 mg/kg **body weight.** Controls received 200 µl of vehicle alone. Animals **were sacrificed at** 6h after injection of growth hormone and ODC **activity was** estimated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 **animals in** each group

* - $p < 0.001$ as compared to group 1

** - $p < 0.001$ **as** compared to group 2

TABLE 16

EFFECT OF TREATMENT WITH CYCLOHEXIMIDE ON KIDNEY ODC ACTIVITY IN GH-TREATED CASTRATED RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	6250 ± 191 (4)
2.	GH	13872 ± 140* (4)
3.	GH + CHX	3524 ± 233** (4)

Animals were castrated 4 days prior to use. Growth hormone was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally in 200 µl of vehicle at a dose of 5.5 mg/kg body weight. Cycloheximide was dissolved in saline and injected intraperitoneally in 100 µl at a dose of 8 mg/kg body weight. Controls received 200 µl of vehicle alone. Animals were sacrificed at 6h after injection of growth hormone and ODC activity was estimated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group

* - p< 0.001 as compared to group 1

** - p< 0.001 as compared to group 2

TABLE 17

EFFECT OF TREATMENT WITH CYCLOHEXIMIDE ON LIVER ODC ACTIVITY IN GH-TREATED CASTRATED RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	1917 ± 20 (4)
2.	GH	3067 ± 77* (4)
3.	CHX + GH	1237 ± 26** (4)

Animals were castrated **4** days prior to use. Growth hormone was **dissolved in** 0.9% sodium chloride/0.1N NaOH and injected **intraperitoneally** in 200 µl of vehicle at a dose of 5.5 **mg/kg body weight.** Cycloheximide was dissolved in saline and injected **intraperitoneally** in 100 µl at a dose of 8 mg/kg body weight. **Controls received** 200 µl of vehicle alone. Animals were **sacrificed at** 6h after injection of growth hormone and ODC **activity was** estimated.

Results are expressed as **Mean** ± S.E.M. of 3-4 determinations from **4-5 animals in each group**

* - **p< 0.001 as** compared to group 1

** - **p< 0.001** as compared to group 2

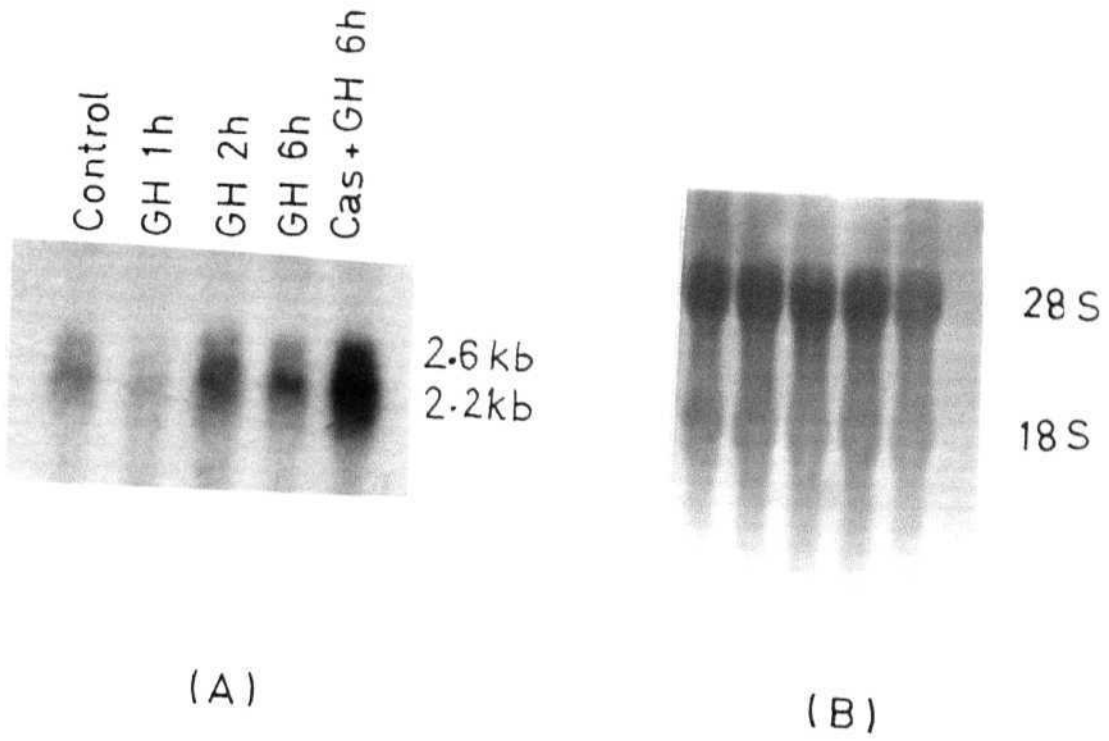
Fig. 11. Time course of GH action on ODC expression in the kidney of rat.

Adult rats of 300-350g weight were used. GH was dissolved in basic saline and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Controls received 200µl of vehicle alone. Animals were sacrificed at the indicated times after injection. RNA isolation and northern analysis were carried out as described in Chapter 2.

A. Autoradiogram of northern blot showing ODC expression of GH-treated kidney.

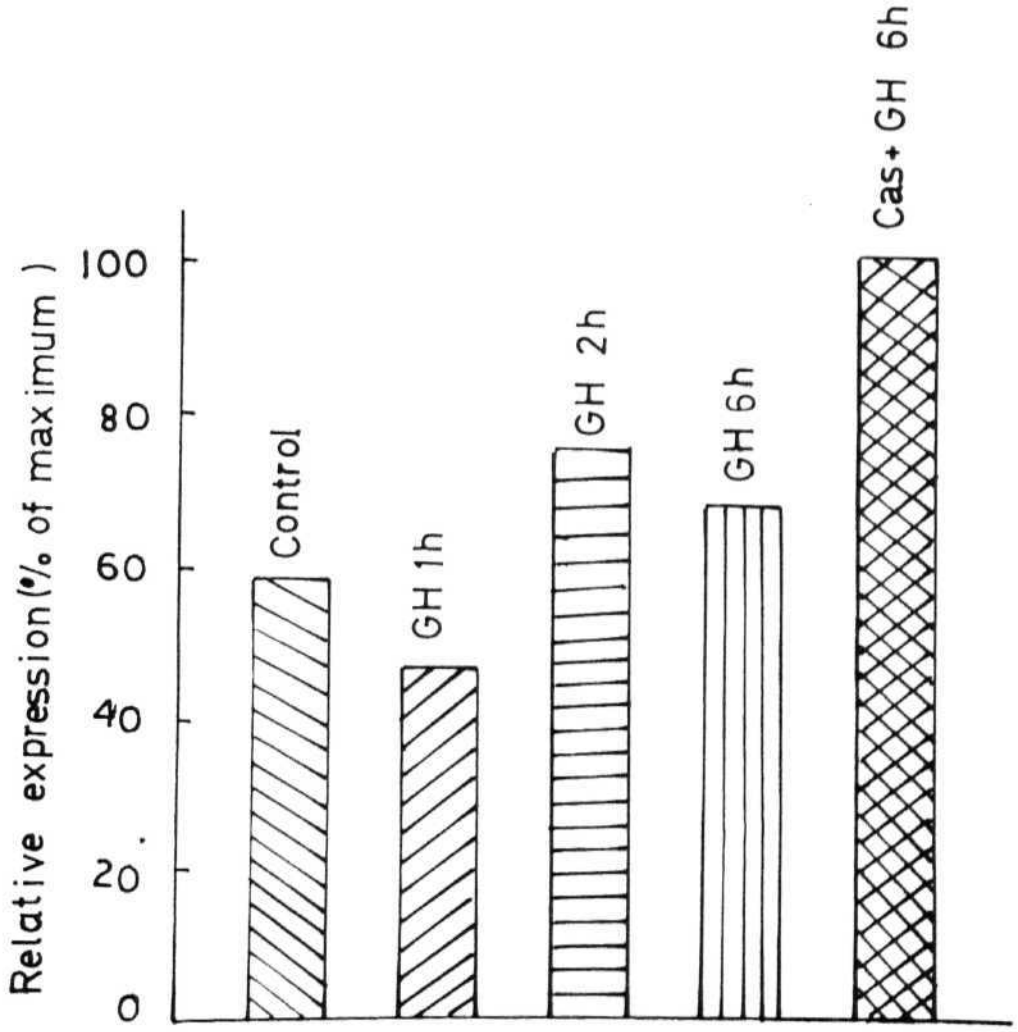
B. Methylene blue stained ribosomal RNA (28S and 18S) of the blot demonstrating equal loading of the samples.

C. Densitometric data of A expressed as relative percent of maximal expression.


Control
GH 1h
GH 2h
GH 6h
Cas + GH 6h
2.6 kb
2.2kb
28 S
18 S

(A)

(B)


Relative expression (% of maximum)
100
80
60
40
20
0
Control
GH 1h
GH 2h
GH 6h
Cas + GH 6h

(C)

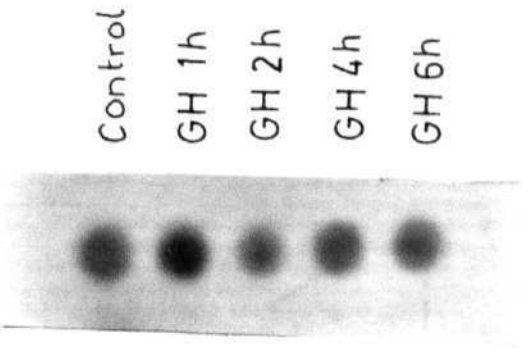
Fig. 11

Fig. 12. **Time** course of GH action on ODC expression in the liver of rat.

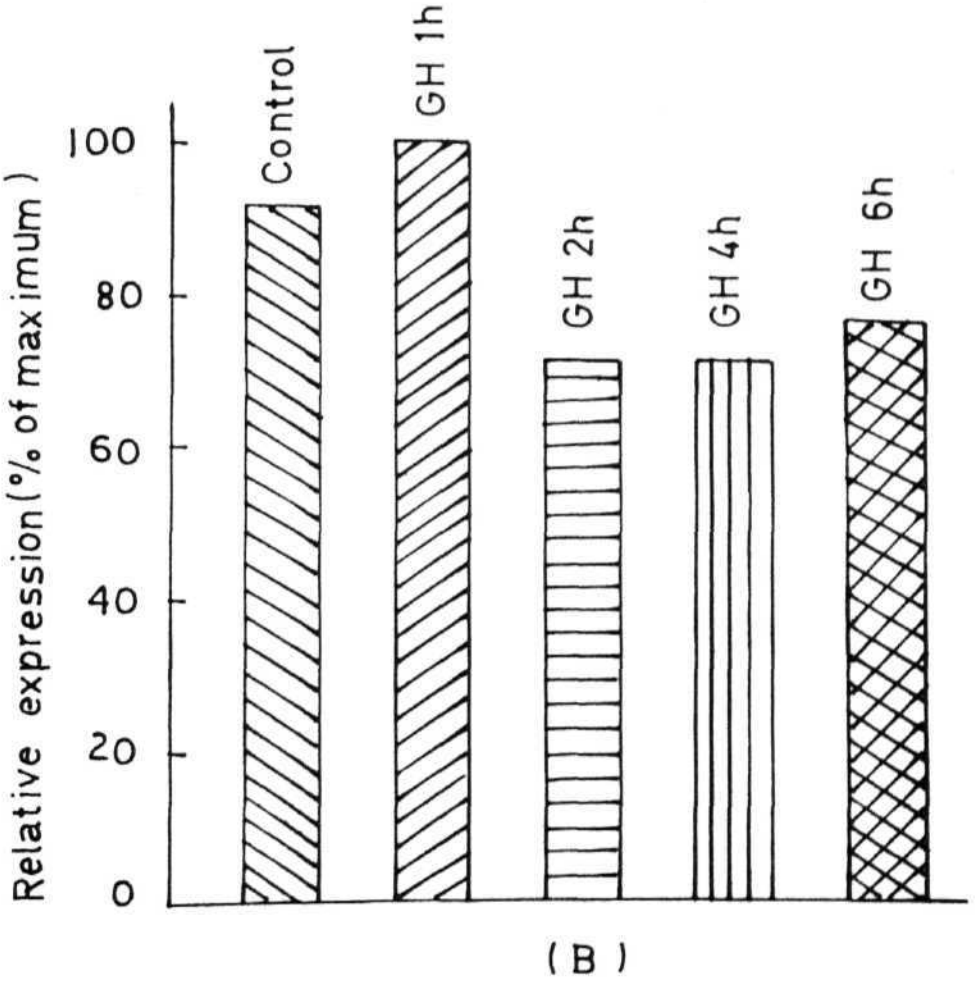
Adult rats of 300-350g weight were used. GH was dissolved in basic saline and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Controls received 200µl of vehicle alone. Animals were sacrificed at the indicated times after injection. RNA isolation and dot blot analysis were carried out as described in Chapter 2.

A. Autoradiogram of dot blot showing ODC expression of GH-treated liver.

B. Densitometric data of A expressed as relative percent of maximal expression.


Control
GH 1h
GH 2h
GH 4h
GH 6h

(A)


Relative expression (% of maximum)
100
80
60
40
20
0
Control
GH 1h
GH 2h
GH 4h
GH 6h

(B)

Fig. 12

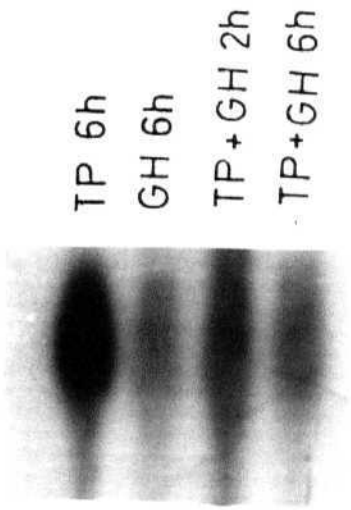
Fig. 13. Effect of prolactin on kidney ODC expression in rats treated with testosterone propionate.

Adult rats of 300-350g weight were castrated via the scrotal route 4 days prior to use. GH was dissolved in basic saline and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Testosterone propionate (TP) was injected subcutaneously in 200µl of sesame oil at a dose of 350 µg/kg body weight. Animals were sacrificed 6h after injection. RNA isolation and northern analysis were carried out as described in Chapter 2.

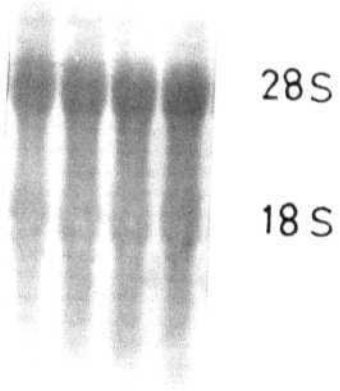
A. Autoradiogram of northern blot showing the effect of PRL on ODC expression of TP-treated Kidney.

B. Methylene blue stained ribosomal RNA (28S and 18S) of the blot demonstrating equal loading of the samples.

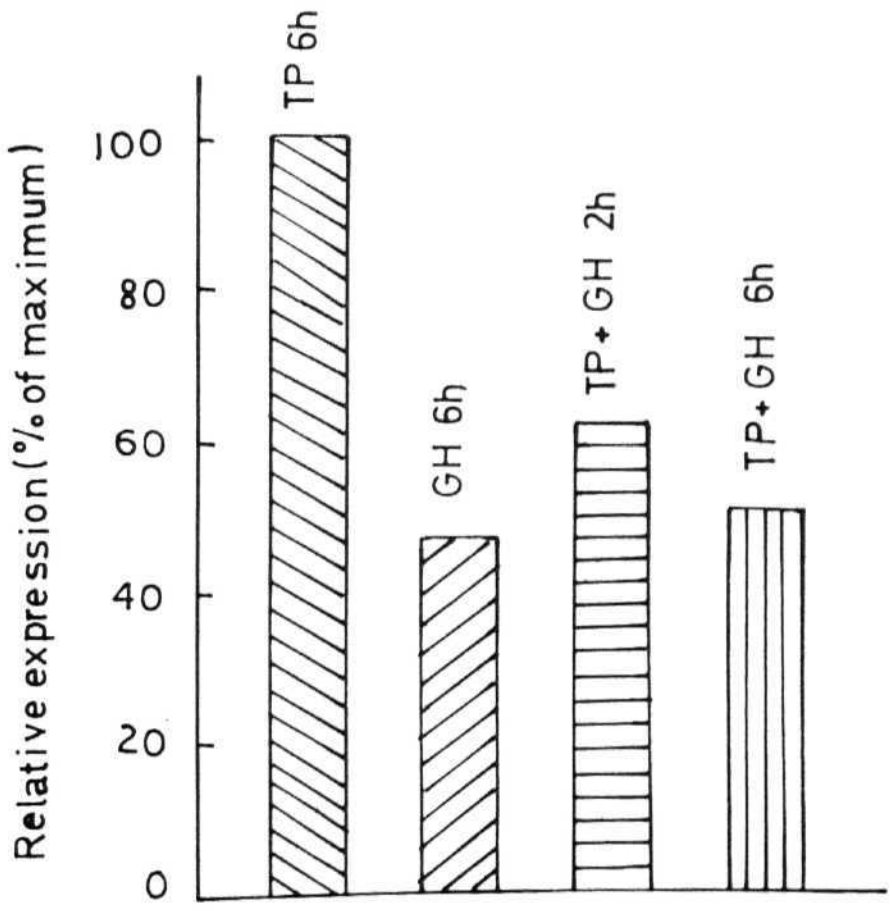
C. Densitometric data of A expressed as relative percent of maximal expression.


TP 6h
GH 6h
TP+GH 2h
TP+GH 6h

(A)


28S
18S

(B)


Relative expression (% of maximum)
0
20
40
60
80
100
TP 6h
GH 6h
TP+GH 2h
TP+GH 6h

(C)

Fig. 13

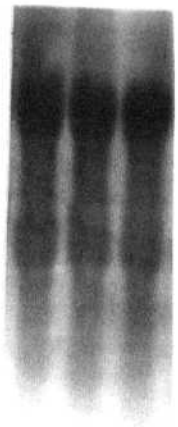
Fig. 14. Effect of pre-treatment with actinomycin D on GH-induction of ODC expression in kidney of rat.

Adult rats of 300-350g body weight were castrated 4 days prior to use. GH was dissolved in basic saline and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Actinomycin D was dissolved in ethanol:DMSO (1:1) and injected intraperitoneally 1h prior to prolactin at a dose of 6 mg/kg body weight. Controls received 200ml of vehicle. Animals were sacrificed 6h after injection of prolactin. Kidney RNA isolation and northern blot analysis were carried out as described in Chapter 2.

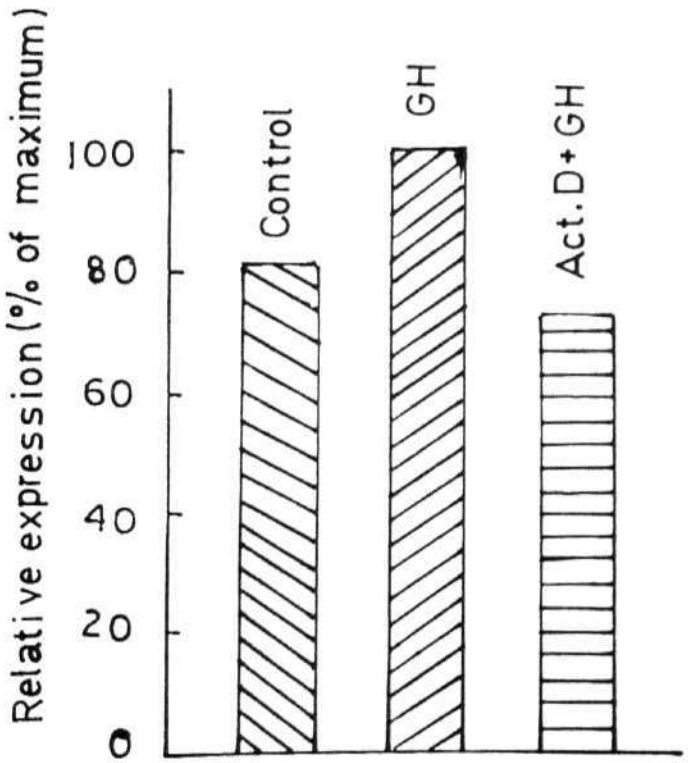
A. Autoradiogram of northern blot showing the effect of pre-treatment with Actinomycin D on ODC expression of PRL-treated liver.
B. Methylene blue stained ribosomal RNA (28S and 18S) of the blot demonstrating equal loading of the samples.
C. Densitometric data of A expressed as relative percent of maximal expression.


Control
GH
Act.+GH
D

(A)


28 S
18 S

(B)


Relative expression (% of maximum)
100
80
60
40
20
0
Control
GH
Act.D+GH

(C)

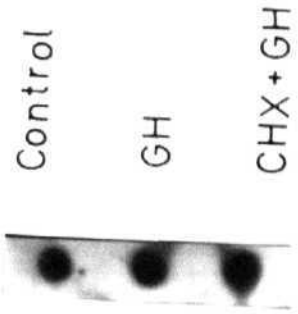
Fig. 14

Fig. 15. Effect of cycloheximide on kidney ODC expression in GH-treated rats.

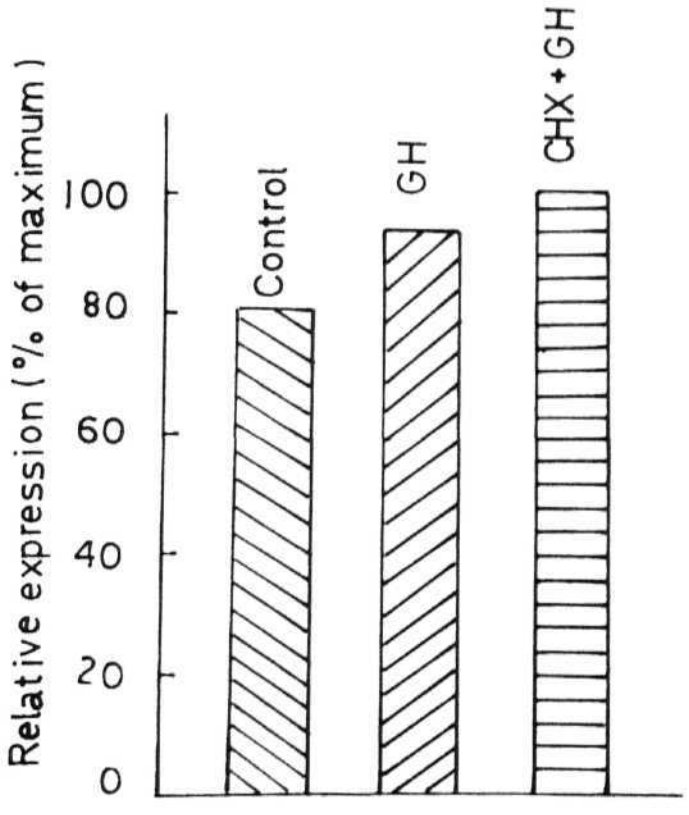
Animals of 300-350g body weight were castrated 4 days prior to use. GH was dissolved in basic saline and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Cycloheximide was dissolved in ethanol and injected intraperitoneally 1h before sacrifice at a dose of 8 mg/kg body weight. Controls received 200µl of vehicle alone. Animals were sacrificed 6h after injection of prolactin. RNA isolation and dot blot analysis were carried out as described in Chapter 2.

A. Autoradiogram of dot blot showing the effect of treatment with cycloheximide on ODC expression of GH-treated kidney.

B. Densitometric data of A expressed as relative percent of maximal expression.


Control
GH
CHX + GH

(A)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
GH
CHX + GH

(B)

Fig. 15

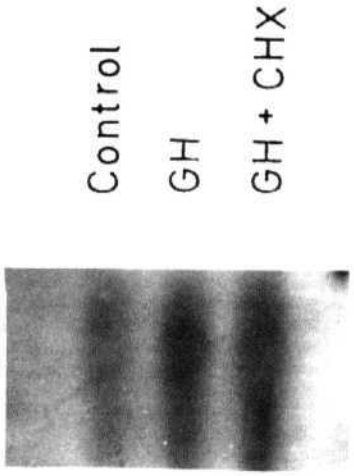
Fig. 16. Effect of cycloheximide on liver ODC expression in GH-treated rats.

Animals of 300-350g body weight were castrated 4 days prior to use. GH was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally at a dose of 5.5 mg/kg body weight in 200µl of vehicle. Cycloheximide was dissolved in ethanol and injected intraperitoneally 1h before sacrifice at a dose of 8 mg/kg body weight. Controls received 200µl of vehicle alone. Animals were sacrificed 6h after injection of prolactin. RNA isolation and northern analysis were carried out as described in Chapter 2.

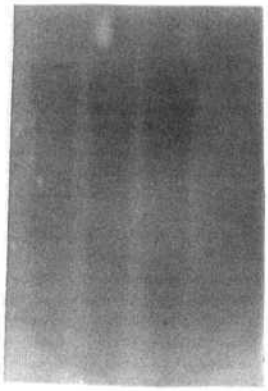
A. Autoradiogram of northern blot showing the effect of treatment with cycloheximide on ODC expression of PRL-treated liver.

B. Methylene blue stained ribosomal RNA (28S and 18S) of the blot demonstrating equal loading of the samples.

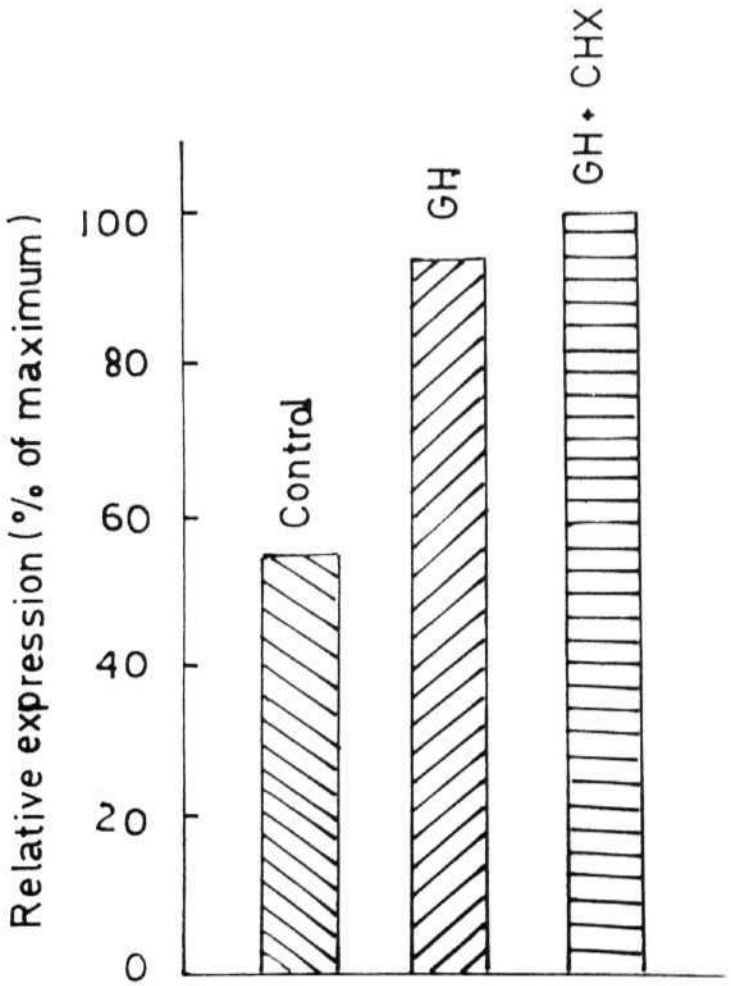
C. Densitometric data of A expressed as relative percent of maximal expression.


Control
GH
GH + CHX

(A)


28 S
18 S

(B)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
GH
GH + CHX

(C)

Fig. 16

CHAPTER 5

HORMONAL REGULATION OF ORNITHINE DECARBOXYLASE IN THE TESTIS OF RAT

Ornithine decarboxylase is an important marker of cellular growth and differentiation. In the testis, this enzyme is modulated by pituitary hormones, gonadotropins and various endocrine and paracrine growth factors such as prostaglandins, catecholamines, and arginine vasopressin (reviewed in introduction). Prolactin plays a major role in the regulation of male reproductive function. The occurrence of testicular receptors for prolactin supports the notion that the testis is a target for this hormone. Maintenance of testicular receptors is at least partially dependent on PRL and GH, which seem to act at different sites. FSH and LH regulate ODC activity in the testis. However, despite numerous studies, the precise mechanisms of action of PRL and GH in eliciting various cellular responses are not clear.

The rat testis is most sensitive to the influence of hormones, especially pituitary hormones, during puberty. The widespread phenomenon of desensitisation to hormone action is thus best studied at this period between immaturity and puberty. In this study the effects of various hormones on testicular ODC have been charted in an attempt to understand more clearly their mode of action in stimulating this enzyme.

MATERIALS AND METHODS:

The general methods have been described in Chapter 2. Immature rats of 19-21 days age or puberal rats of 30 days age were used as indicated in individual experiments.

All hormone solutions were prepared fresh just prior to use.

All intratesticular injections were given in a minimal volume of 5-10 µl of vehicle, under mild ether anesthesia.

Prolactin and GH were given intraperitoneally (5.5 mg/kg body weight) or intratesticularly at the dosages mentioned in specific experiments. hCG in saline was administered subcutaneously at a dosage of 40 I.U. per rat. Quinacrine was given intraperitoneally or intratesticularly, as indicated in specific experiments.

RESULTS:

The ontogenesis of ODC activity in the rat testis is shown in Table 18. The activity was highest at 15 days of age, after which it declined steadily. Fig. 17 shows the testicular ODC mRNA levels at various ages between 15 and 45 days of age. The levels remained more or less steady till 25 days, increased slightly at 30 days, and then showed a sharp increase of almost 50% at 45 days of age (i.e., in the adult rat).

The effect of various hormones that are involved in regulating testis function was studied. Table 19 shows the effect of hCG on ODC activity in immature testis. The effect of hCG administration on ODC mRNA expression in immature rats is shown in Fig. 18. An increase in mRNA levels, studied by northern analysis, was seen at 2h after injection and persisted even at 4h.

Desensitisation of the ODC response to hCG was studied, and the results in terms of ODC activity and mRNA expression are

shown in Table 20 and Fig. 19, respectively. Immature rats were given injections subcutaneously on two consecutive days, and killed 4h after the second injection. Group I was given 40 IU of hCG on Day 1, followed by saline on Day 2. Group II was given saline on Day 1 and hCG on Day 2. Group III was given hCG on both days. It was observed that in Group III, which had been primed with hCG on Day 1, the injection of hCG on Day 2 failed to elicit any increase in ODC mRNA expression, or activity.

The effect of various doses of PRL on testicular ODC activity in 30 day old puberal rats is given in Table 21. The rats were injected intratesticularly with 25 µg, 50 µg or 100 µg PRL per testis, and ODC activity was measured 4h later. It was seen that 25 µg/testis elicited the highest enhancement of ODC activity. A similar result was also seen in immature rats administered the same doses of prolactin (Table 22).

The effect of the phospholipase C inhibitor quinacrine on the prolactin-stimulated testicular ODC activity is shown in Table 23. Table 24 shows the effects of combined treatment with PRL, cAMP and quinacrine, on ODC activity in immature rat testis. Fig. 22. illustrates the ODC mRNA expression pattern for Tables 23 and 24. Although PRL and cAMP both individually enhanced ODC activity, a combination of both did not produce any additive effect. The presence of quinacrine inhibited the enhancement of ODC activity by either PRL or cAMP, and when all three were administered, there was no change in the ODC activity above the control value. In fact, it was depressed to levels below that of the control. The ODC mRNA expression as given in Fig. 22 showed

that when PRL and cAMP were given together, the mRNA levels increased significantly. Though the presence of quinacrine did not much affect PRL-induced ODC mRNA levels, the combination of quinacrine and cAMP caused a drastic increase in mRNA levels.

The effect of quinacrine pre-treatment on the effect of hCG on ODC activity is given in Table 25. It was seen that quinacrine had no effect on the stimulation of ODC activity by hCG.

The effect of quinacrine pre-treatment on enhancement of ODC activity by FSH is given in Table 26, and on ODC mRNA levels in Fig 23. Although the presence of quinacrine slightly lowered mRNA levels, it had no effect on FSH-induced ODC activity.

The effect of various doses of GH on ODC activity in the puberal rat testis is given in Table 27. It can be observed that a dose of 50 μg of GH/testis caused maximal stimulation of ODC activity.

The effect of quinacrine pre-treatment on GH-induced ODC activity was studied by both intraperitoneal (Table 28) and intratesticular injection (Table 28). It was observed that although quinacrine blocked the stimulation of ODC to a certain extent, this was not total.

The effect of quinacrine pre-treatment on GH-induced ODC activity in the testis was also studied after separation of Leydig cells and seminiferous tubules, to observe the effects in these individual fractions (Table 29). It was seen that GH

stimulated ODC in both fractions, and that quinacrine inhibition of this stimulation was partial in seminiferous tubules and total in the Leydig cells.

DISCUSSION:

The functions of the male gonads, both endocrine and reproductive, are mainly regulated by the actions of FSH and LH. However, there are a number of other mediators and local factors that also contribute to the overall maintenance of testicular function. For example, the effect of LH in increasing androgen production actually initiates a cascade of events in which androgens act on peritubular cells to promote the production of a nonmitogenic paracrine factor which acts on Sertoli cells. Sertoli cells also appear to produce FSH-dependent factors that can influence Leydig cell function. Thus, the two major agents known to influence the testis act, to a large extent, through changes in local cellular interactions.

Prolactin (PRL) plays a major role in reproductive function. Recent studies seem to indicate that apart from its role in the stimulation of steroidogenesis via LH, PRL has a direct effect on the testis, which exhibits a trophic response to PRL. In this study, the actions of some of the hormones affecting testicular function on ODC activity and expression have been taken up.

Testicular ODC activity was seen to be highest at 15 days of age, after which it decreased with age, suggesting that ODC activity is negatively regulated. In contrast, the ODC mRNA expression was seen to increase with age. A similar increase in

mouse testis ODC mRNA levels has been reported by Alcivar et *al.* (1989). Thus, the developmental changes in ODC activity are in marked contrast to the changes in ODC mRNA. These results suggest that there is an inhibition of ODC activity seems to be regulated at a post-translational level. It has been previously demonstrated by Alcivar et *al.* (1989) that in the adult mouse testis, ODC mRNA is regulated at the translational level as well. Weiner and Dias (1992) have suggested that in the testis, regulation of ODC at the post-transcriptional level may be an important aspect of spermatogenesis that would allow rapid changes in the level of ODC and polyamines as the need arises. Kaipia et *al.* (1990) conducted a study on stage and cell specific expression of the ODC gene during rat and mouse spermatogenesis, and concluded that the high levels of ODC mRNA in late pachytene spermatocytes and early round spermatids suggest that ODC may play an important role during late meiosis and early spermiogenesis.

The rat testis is most receptive to hormonal influence at the stage between immaturity to puberty. This is the time when the pituitary hormones play a major role. The ODC activity in response to hCG administration was seen to rise significantly and there was an increase (although not exactly corresponding to that in activity) in the ODC mRNA expression as well, at both 2h and 4h after administration of the hormone.

The ODC response to hCG was seen to undergo homologous desensitisation, both at the levels of enzyme activity and mRNA expression. Rats which were given hCG on two consecutive days,

did not show any increase in ODC activity in response to hCG on the second injection. Pakarinen et *al.* (1990) demonstrated that the functional differences of adult and neonatal Leydig cells in rat to high gonadotropic hormone stimulation occur at the level of expression of specific genes, including those of the LH receptor and 17-hydroxylase/17,20-lyase cytochrome P_{450}. LH receptor mRNA is down-regulated in adult rat testis in response to high levels of hCG.

Prolactin plays a significant role in the regulation of male reproductive function. The ability of this hormone to influence the growth and functional integrity of male accessory glands is well-established. In mouse testis, PRL regulates the content of esterified cholesterol. *In vivo* PRL treatment stimulates *in vitro* androgen production by testicular interstitial cells. The occurrence of PRL receptors in the testis supports the idea that the testis is a direct target organ for PRL. Dombrowicz *et al.* (1992) found that during puberty, PRL stimulates testicular function by promoting multiplication and differentiation of Leydig cells and germ cells. It was also reported that the action of PRL in stimulating testicular ODC activity requires an intact hypophysis (de Las Heras and Calandra, 1992). Reports indicate that ODC activity in seminal vesicle of adult rats is regulated mainly by PRL (Gonzalez *et al.,* 1994).

This study indicates that acute treatment with PRL increases ODC activity in the immature and puberal rat testis. Direct intratesticular as well as intraperitoneal injection of PRL elicited this response within a fairly short time, indicating

that this is likely to be a direct effect of the hormone on the testis.

It has been suggested that some of the actions of PRL in its target organs may be mediated via the formation of diacylglycerol and stimulation of protein kinase. The effects of the phospholipase C inhibitor, quinacrine, which prevents the formation of diacylglcerol, when administered in conjunction with PRL, indicate that the stimulation of ODC by PRL in rat testis is mediated via the formation of diacylglycerol. Pre-treatment of rats with quinacrine followed by PRL administration abolished the stimulation of ODC by PRL.

The effect of quinacrine pre-treatment on the stimulation of ODC activity by hCG was shown to have no significant effect. Similarly, treatment with quinacrine slightly lowered mRNA levels but had no effect on ODC activity in response to FSH. This indicates that these hormones may induce ODC only via stimulation of the cAMP pathway, and that the inositol phosphate-protein kinase pathway may not be involved.

Growth hormone has been established to have mitogenic action in some of its target organs. Human growth hormone has been shown to stimulate aromatase activity, associated with a positive effect on germ cell number in the immature hypophysectomised rat testis. In the Nb2 lymohoma cell line, hGH has been shown to have mitogenic effects , and these were enhanced on addition of the tumor promoter 12-*O*-tetradecanoyl-phorbol-13- acetate (TPA) (Gertler *et al.,* 1985).

This study demonstrated that in the puberal rat testis, bGH was able to stimulate ODC activity to a larger extent than PRL, the optimal dose of GH being 50 μg/testis. To examine if this effect of GH was also via the stimulation of diacylglycerol formation, it was examined in the presence of the phospholipase inhibitor, quinacrine. It was observed that although quinacrine blocked the stimulation of ODC by GH, this effect was only partial, indicating that this may not be the only mechanism through which GH acts to increase testicular ODC activity. Effects of GH in other tissues via stimulation of diacylglycerol (DAG) formation have been previously reported. Doglio et *al.* (1989) reported that GH can enhance the formation of diacylglycerol without stimulating inositol lipid breakdown in Ob1771 cells. Rogers and Hammerman(1989) reported that GH can activate phospholipase C in basolateral membranes from canine kidney. Johnson *et al.* (1990) showed that GH stimulates the formation of *sn*-1,2-diacylglycerol in rat hepatocytes, and suggested that production of DAG may be an early signalling event mediated by hormone stimulation of both the GH and PRL receptors.

To further investigate the effects of GH on ODC activity in the testis, the separation of Leydig cells and seminiferous tubules was carried out and it was observed that GH stimulated ODC activity in both these fractions. Quinacrine inhibition of this response was total in Leydig cells, but only partial in seminiferous tubules.

Thus this study on the regulation of testicular ODC showed

that the desensitisation of ODC response to human chorionic gonadotropin involves not only the down-regulation of the enzyme activity, but also that of its mRNA expression. FSH and hCG act to enhance both ODC enzyme activity and the ODC mRNA levels, and this effect does not seem to be mediated via the inositol phosphate pathway, as evinced by the studies using the phospholipase A_2 and C inhibitor, quinacrine. Prolactin and growth hormone, both of which are important for the regulation of testicular function, act to enhance ODC activity during the period between immaturity and puberty. This enhancement seems to be via stimulation of diacylglycerol formation. GH acts to induce ODC both in the Leydig cells and seminiferous tubules, and its action in both these fractions is mediated via DAG formation.

TABLE 18

ONTOGENY OF ODC ACTIVITY IN THE TESTIS OF RAT

Age	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)	
15 days	4310 ± 134	(4)
20 days	3203 ± 222	(4)
25 days	2478 ± 47	(4)
30 days	2259 ± 59	(4)
45 days	1240 ± 24	(4)

Rats of various ages as indicated were sacrificed and **testicular ODC activity was** estimated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 **animals** in each group.

TABLE 19

EFFECT OF hCG ON ODC ACTIVITY IN IMMATURE RAT TESTIS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	3866 ± 101 (3)
2.	hCG (2h)	4662 ± 288* (3)
3.	hCG (4h)	4800 ± 50** (3)

Immature animals of age 19-21 days were used. Human chorionic gonadotropin (hCG) dissolved in saline was injected intratesticularly in 5-10µl of vehicle at a dose of 40 IU/testis under ether anesthesia. Controls received 5-10µl of vehicle alone. Animals were sacrificed at different times after injection as indicated and ODC activity was estimated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* $p < 0.001$ as compared to group 1

** $p < 0.001$ as compared to group 1

TABLE 20

EFFECT OF DESENSITISATION TO hCG ON ODC ACTIVITY IN TESTIS OF IMMATURE RAT

	Treatment		ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)	
	1st day	2nd day		
1.	hCG	saline	3661 ± 80	(4)
2.	saline	hCG	4664 ± 110*	(4)
3.	hCG	hCG	3293 ± 302**	(4)

Immature animals of age 19-21 days were used. Animals were given a first injection of saline or hCG on Day 1, followed by a second injection 24h later, as indicated. Human chorionic gonadotropin (hCG) dissolved in saline was injected intratesticularly in 5-10µl of vehicle at a dose of 40 IU/testis under ether anesthesia. Animals were sacrificed at 4h after the second injection and ODC activity was estimated.as indicated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* $p < 0.001$ as compared to group 1

** $p < 0.001$ as compared to group 2

TABLE 21

EFFECT OF VARIOUS DOSES OF PROLACTIN ON TESTICULAR ODC ACTIVITY IN PUBERAL RATS

	Treatment (μg/testis)	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)	
1.	Saline	1355 ± 12	(4)
2.	PRL (25 μg)	2272 ± 46*	(4)
3.	PRL (50 μg)	1930 ± 66*	(4)
4.	PRL (100 μg)	1325 ± 29	(4)

Puberal male rats of 30 days age were used. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intratesticularly in 10μl of vehicle at various doses as indicated. Animals were sacrificed 4h after injection and ODc activity was estimated. Controls received 10μl of saline alone.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* $p < 0.001$ as compared to group 1

TABLE 22

EFFECT OF VARIOUS DOSES OF PRL ON ODC ACTIVITY IN TESTIS OF IMMATURE RAT

	Treatment	ODC specific activity (pinoles $^{14}CO_2$/ h/ mg protein)	
1.	Saline	1812 ± 47	(4)
2.	PRL (25 μg)	2649 ± 57*	(4)
3.	PRL (50 μg)	2248 ± 25**	(4)
4.	PRL (100 μg)	1749 ± 100	(4)

Immature male rats of age 19-21 days were used. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intratesticularly in 10μl of vehicle at various doses as indicated. Animals were sacrificed 4h after injection and ODc activity was estimated. Controls received 10μl of saline alone.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* $p < 0.001$ as compared to group 1

** $p < 0.001$ as compared to group 2

TABLE 23

EFFECT OF QUINACRINE, **A PHOSPHOLIPASE INHIBITOR,**

ON ODC INDUCTION BY PROLACTIN IN TESTIS OF PUBERAL RATS

	Treatment		ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
	0 time	30 min.	
A:			
1.	Saline	Saline	1176 ± 30 (4)
2.	Quinacrine	Saline	2091 ± 33 (4)
3.	**Saline**	PRL	2234 ± 168** (4)
4.	Quinacrine	PRL	1004 ± 44 (4)
B:			
5.	Saline	Saline	1618 ± 43 (4)
6.	Quinacrine	Saline	1672 ± 15 (4)
7.	**Saline**	PRL	2902 ± 69## (4)
8.	Quinacrine	PRL	1286 ± 34### (4)

Puberal male rats of age 30 days were used. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intraperitoneally (A) at a dose of 5.5 mg/kg body weight or intratesticularly (B) in 10µl of vehicle at a dose of 25µg/testis. Quinacrine was dissolved in sterile water and injected intraperitoneally (A) at a dose of 150 µmoles/kg body weight or intratesticularly (B) at a dose of 20µmoles/testis, 30 minutes prior to injection of prolactin. Animals were sacrificed 4h after injection of prolactin and ODC activity was estimated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* p< 0.001 as compared to group 1
** p< 0.001 as compared to group 3
\# p< 0.001 as compared to group 5
\## p< 0.001 as compared to group 7

TABLE 24

EFFECT OF QUINACRINE ON ODC INDUCTION BY PROLACTIN IN TESTIS OF IMMATURE RAT

	Treatment		ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
	0 time	30 min.	
1.	Saline	Saline	1383 ± 21 (4)
2.	Quinacrine	Saline	1401 ± 33 (4)
3.	Saline	PRL	2655 ± 24* (4)
4.	Quinacrine	PRL	889 ± 66** (4)

Immature male rats **of** age 19-21 days were used. Prolactin was **dissolved** in 0.9% sodium chloride/0.1N NaOH and injected **intratesticularly** in 10µl of vehicle at a dose of 25µg/testis. **Quinacrine** was dissolved **in** sterile water and injected intratesticularly at a dose of 20µmoles/testis, 30 minutes prior **to** injection of prolactin. Animals were sacrificed 4h after **injection** of prolactin and ODC activity was estimated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* $p < 0.001$ as compared to group 1

** $p < 0.001$ as compared to group 3

TABLE 25

EFFECT OF QUINACRINE ON ODC INDUCTION BY hCG

IN TESTIS OF IMMATURE RAT

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	1618 ± 90 (4)
2.	hCG	2952 ± 24* (4)
3.	Quinacrine + hCG	2374 ± 115** (4)

Immature animals of age 19-21 days were used. Human chorionic **gonadotropin** (hCG) dissolved in saline was injected **subcutaneously at** a dose of 40 IU/rat. Quinacrine was dissolved **in sterile water** and injected intratesticularly at a dose of 20μmoles/testis, 30 minutes prior to injection of hCG. Controls **received** 5-10μl of vehicle alone. Animals were sacrificed at **different times after** injection as indicated and ODC activity was **estimated.**

Results are expressed as Mean ± S.E.M. of 3-4 determinations **from 4-5 animals in each group.**

* **p< 0.001 as** compared to group 1

** **p< 0.005 as compared** to group 2

TABLE 26

EFFECT OF QUINACRINE ON ODC INDUCTION BY FSH

IN TESTIS OF IMMATURE RAT

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
1.	Saline	2000 ± 221 (4)
2.	FSH	3547 ± 63* (4)
3.	Quinacrine + FSH	3702 ± 185 (4)

Immature animals of age 19-21 days were used. Follicle-stimulating hormone (FSH) dissolved in saline **was injected intratesticularly** at a dose of 40 µg/testis. Quinacrine **was dissolved** in sterile water and injected intratesticularly at **a dose of** 20µmoles/testis, 30 minutes prior to injection of FSH. **Controls received** 5-10µl of vehicle alone. Animals were **sacrificed at** 4h after injection of FSH and ODC activity **was estimated.**

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 **animals** in each group.

* $p < 0.001$ **as** compared to group 1

TABLE 28

EFFECT OF QUINACRINE ON ODC INDUCTION BY GROWTH HORMONE IN PUBERAL RATS

	Treatment		ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein)
	0 time	30 min.	
A			
1.	Saline	Saline	1176 ± 30 (3)
2.	Quinacrine	Saline	2091 ± 33 (3)
3.	Saline	GH	3011 ± 65* (3)
4.	Quinacrine	GH	1860 - 18 (3)
B			
5.	Saline	Saline	1618 ± 30 (4)
6.	Quinacrine	Saline	1839 ± 54 (4)
7.	Saline	GH	4432 ± 181# (4)
8.	Quinacrine	GH	2503 - 72## (4)

Puberal male rats of age 30 days were used. GH was dissolved in basic saline and injected intraperitoneally (A) at a dose of 5.5 ing/kg body weight or intratesticularly (B) in 10µl of vehicle at a dose of 50 µg/testis. Quinacrine was dissolved in sterile water and injected intraperitoneally (A) at a dose of 150 µmoles/kg body weight or intratesticularly (B) at a dose of 20µmoles/testis, 30 minutes prior to injection of GH. Animals were sacrificed 4h after injection of GH and ODC activity was estimated.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* $p < 0.001$ as compared to group 1
** $p < 0.001$ as compared to group 3
\# $p < 0.001$ as compared to group 5
\#\# $p < 0.001$ as compared to group 7

TABLE 29

EFFECT OF QUINACRINE ON ODC INDUCTION BY GH IN SEMINIFEROUS TUBULES AND LEYDIG CELLS FROM TESTIS OF PUBERAL RATS

	Treatment	ODC specific activity (pmoles $^{14}CO_2$/ h/ mg protein) Seminiferous tubules	Leydig cells
1.	Saline	718 ± 43 (3)	900 ± 51 (3)
2.	GH	1559 ± 36* (3)	1603 ± 49# (3)
3.	Q + GH	1258 - 36 (3)	1055 - 28## (3)

Puberal male rats of age 30 days were used. GH was dissolved in basic saline and injected intraperitoneally at a dose of 5.5 mg/kg body weight. Quinacrine was dissolved in sterile water and injected intraperitoneally at a dose of 150 μmoles/kg body weight, 30 minutes prior to injection of GH. Animals were sacrificed 4h after injection of GH and Leydig cells and seminiferous tubules were separated as described in Chapter II. ODC activity was estimated from each of the fractions.

Results are expressed as Mean ± S.E.M. of 3-4 determinations from 4-5 animals in each group.

* $p < 0.001$ as compared to group 1

** $p < 0.001$ as compared to group 2

\# $p < 0.001$ as compared to group 1

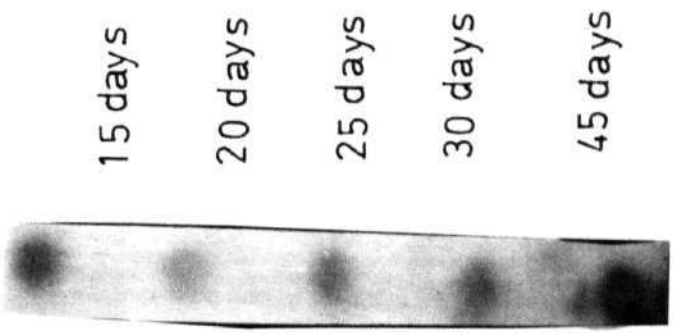
\#\# $p < 0.001$ as compared to group 2

Fig. 17 Ontogenesis of ODC mRNA expression in the testis of rat.

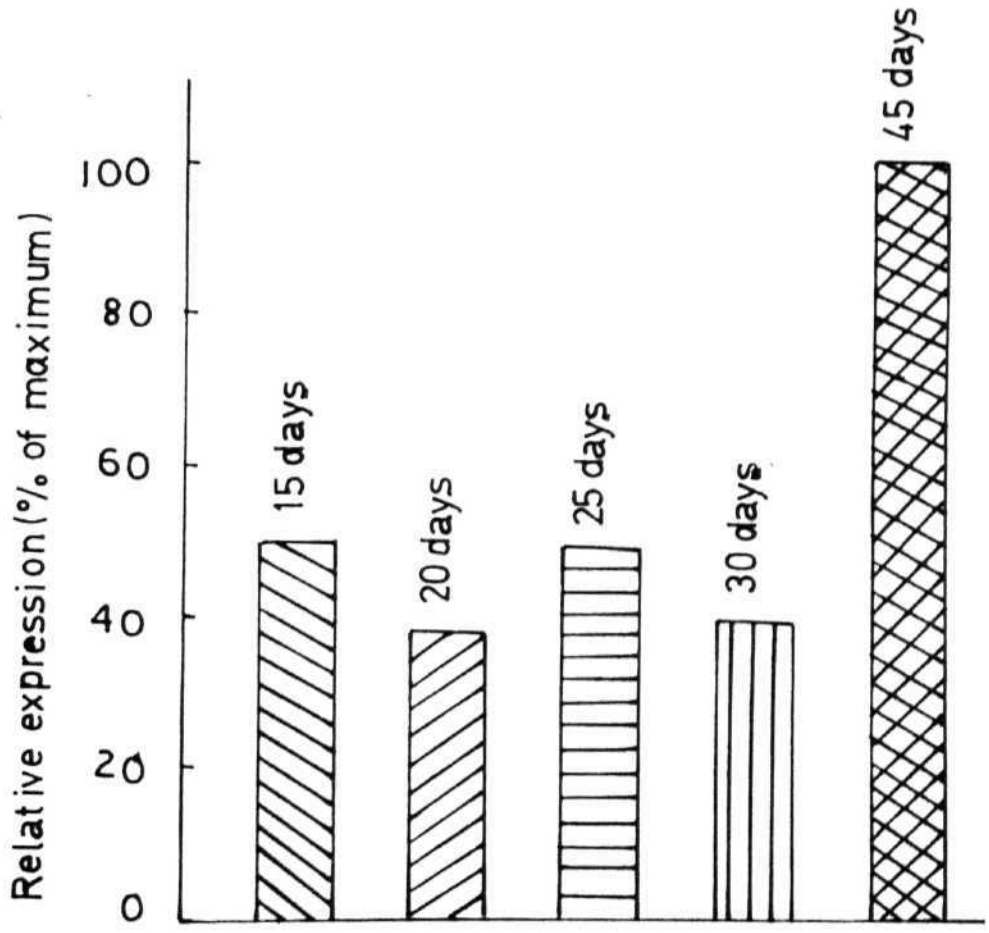
RNA was isolated from the testes of rats of various ages as indicated, and dot blot hybridisation was carried out as described in Chapter II.

A: Autoradiogram of RNA dot blot analysis

B: Densitometric data of A expressed as relative percent of maximal expression.


15 days
20 days
25 days
30 days
45 days

(A)


Relative expression (% of maximum)
0
20
40
60
80
100
15 days
20 days
25 days
30 days
45 days

(B)

Fig. 17

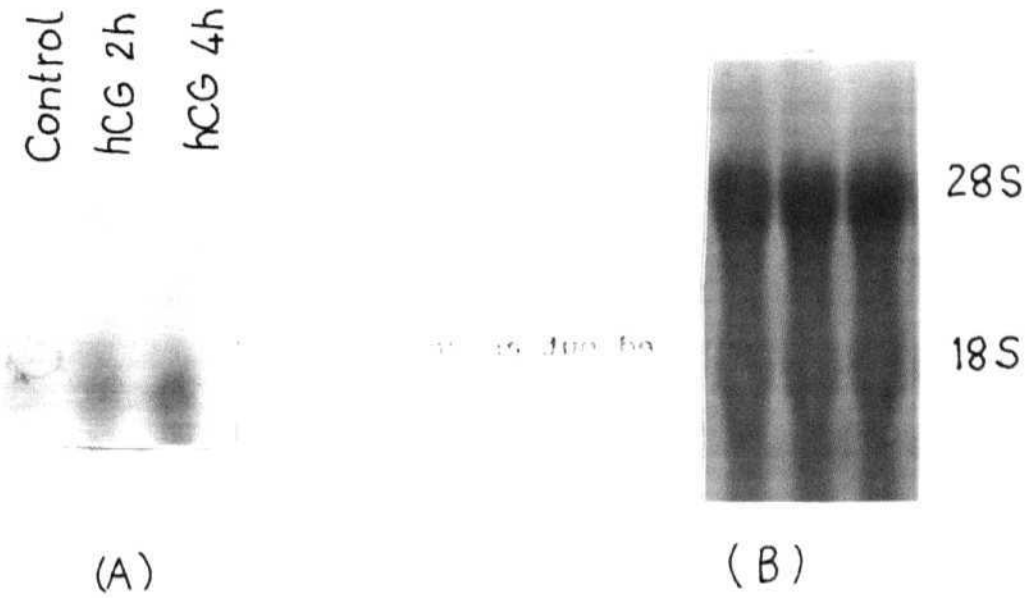
Fig. 18. Effect of human chorionic gonadotropin on **ODC expression in the** testis **of immature rat.**

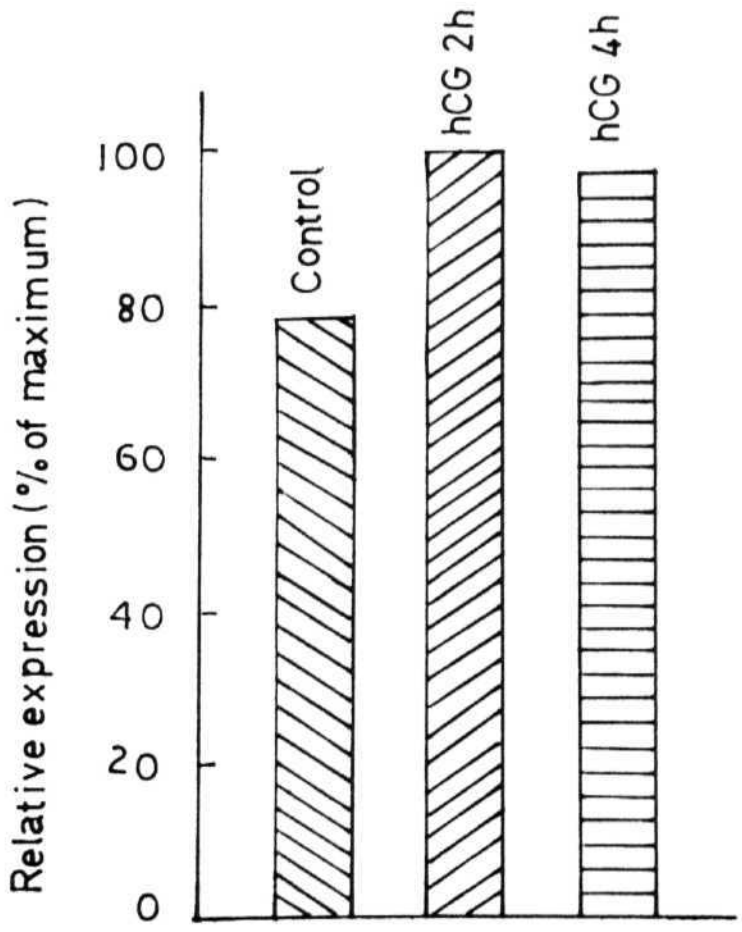
Immature rats were injected subcutaneously with hCG at a dose of 300 I.U./kg body weight in 100µl saline. Controls received 100µl saline. Rats were sacrificed at 2h or 4h after treatment, and RNA was isolated from the testis as described in Chapter 2. Northern analysis **was** carried **out** as described.

A: Autoradiogram of northern blot of RNA from testis of controls and hCG-treated rats.

B. Methylene blue stained ribosomal RNA (28S and 18S) of the, demonstrating equal loading of the samples.

C: Densitometric data of A expressed as relative percent of maximal expression.


Control
hCG 2h
hCG 4h
28S
18S
(A)
(B)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
hCG 2h
hCG 4h

(c)

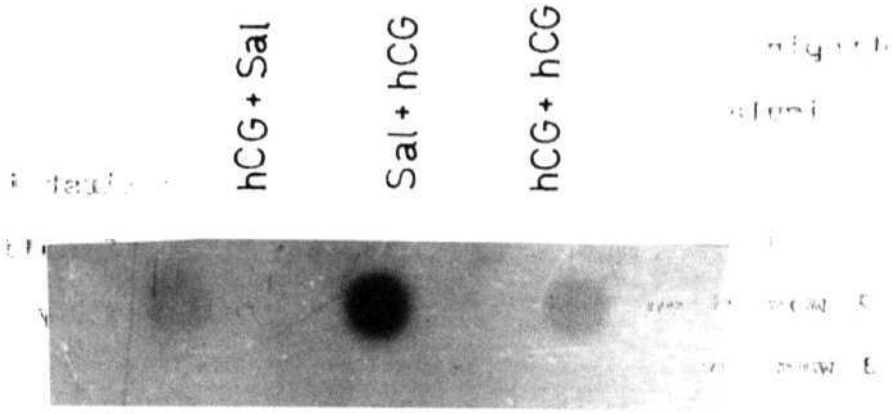
Fig. 18

Fig. 19. Desensitisation of the ODC response to hCG administration in testis of immature rat.

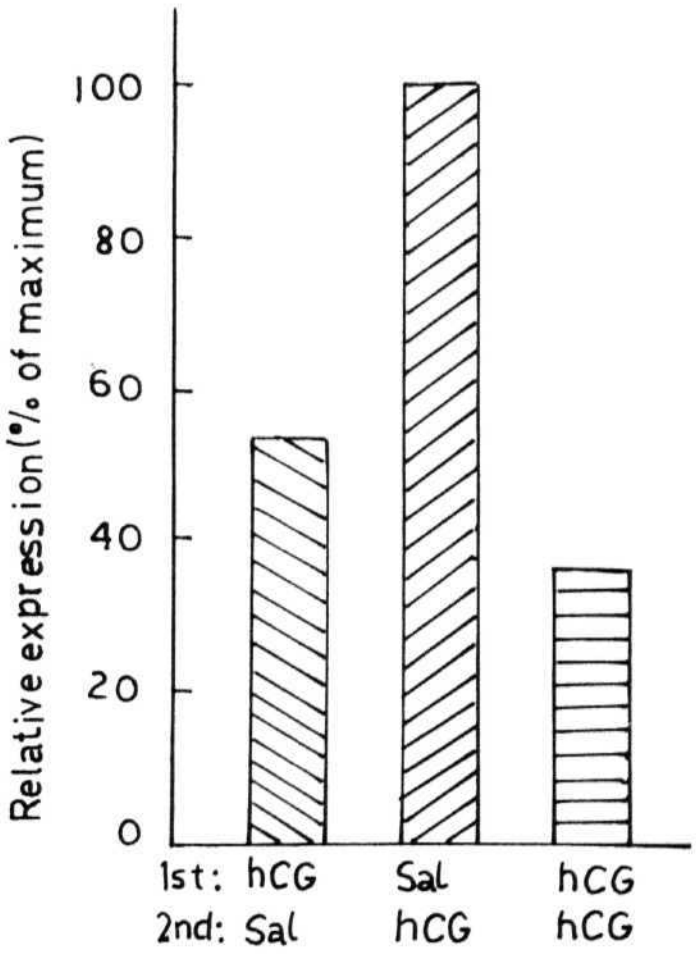
Immature animals of age 19-21 days were used. Human chorionic gonadotropin (hCG) dissolved in saline was injected intratesticularly in 5-10µl of vehicle at a dose of 40 IU/testis under ether anesthesia. Group 1 was given a first injection of hCG on Day 1, followed by a second injection of saline 24h later. Group 2 was given saline on Day 1, followed by hCG on Day 2. Group 3 was given hCG on both days. Animals were sacrificed at 4h after the second injection, RNA was isolated from the testis, and dot blot analysis carried as described in Chapter II.

A: Autoradiogram of dot blot of RNA from testis of various groups of hCG-treated rats.

B: Densitometric data of A expressed as relative percent of maximal expression.


hCG + Sal
Sal + hCG
hCG + hCG

(A)


Relative expression (% of maximum)
100
80
60
40
20
0
1st: hCG
2nd: Sal
Sal
hCG
hCG
hCG

(B)

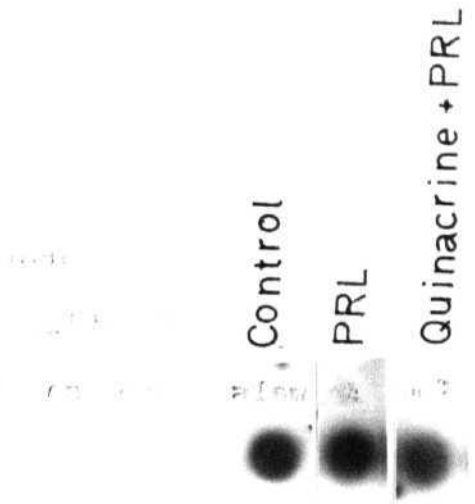
Fig. 19

Fig. 20. Effect of quinacrine on PRL-induction of ODC expression in testis of immature rat.

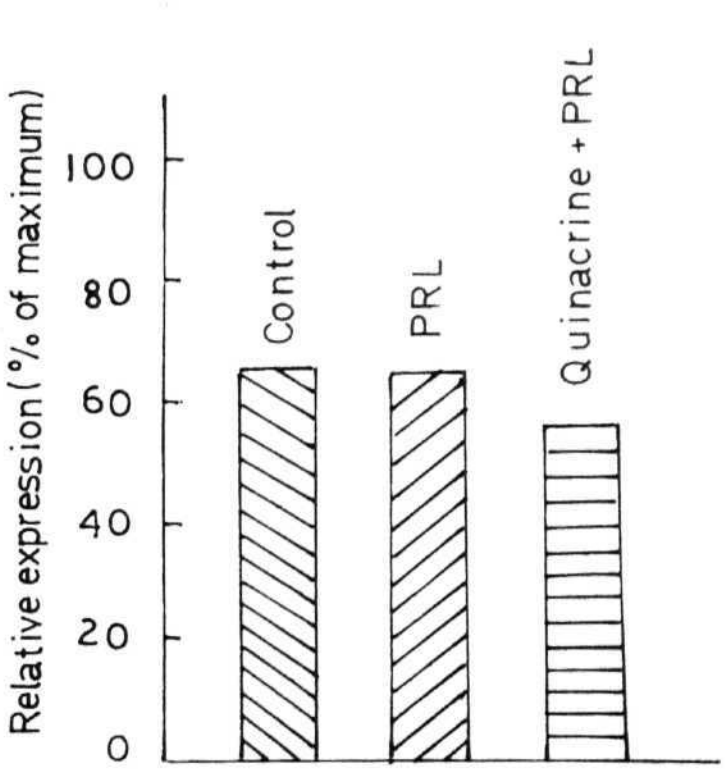
Immature male rats of age 19-21 days were used. Prolactin was dissolved in 0.9% sodium chloride/0.1N NaOH and injected intratesticularly in 10µl of vehicle at a dose of 25µg/testis. Quinacrine was dissolved in sterile water and injected intratesticularly at a dose of 20µmoles/testis, 30 minutes prior to injection of prolactin. Animals were sacrificed 4h after injection of prolactin and RNA isolation and dot blot analysis were carried out as described in Chapter 2.

A: Autoradiogram of dot blot of RNA from testis of various groups of PRL-treated rats.

B: Densitometric data of A expressed as relative percent of maximal expression.


Control
PRL
Quinacrine + PRL

(A)


Relative expression (% of maximum)
0
20
40
60
80
100
Control
PRL
Quinacrine + PRL

(B)

Fig. 20

Fig. 21. Effect of quinacrine on FSH induction of ODC expression **in the testis of immature rat.**

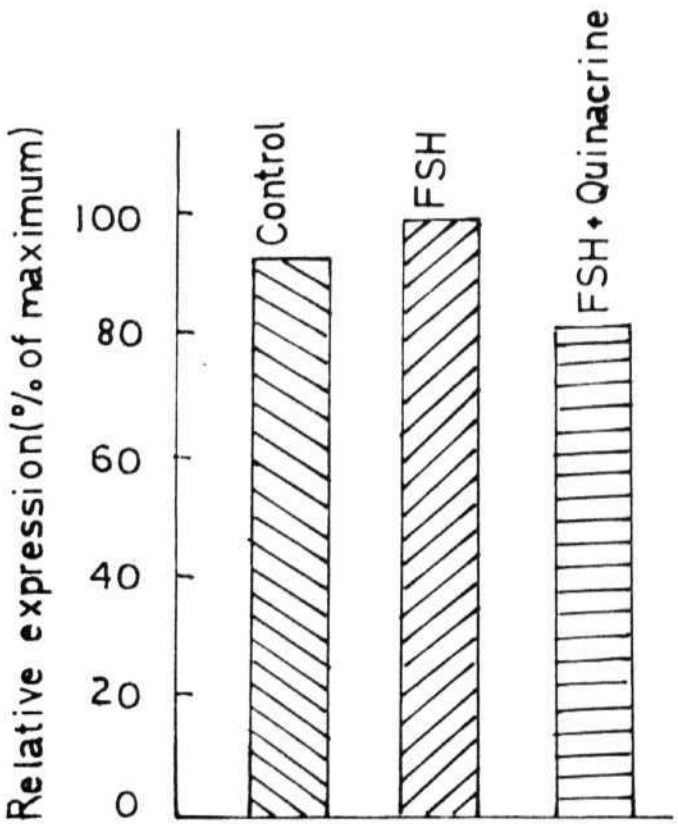
Immature animals of age 19-21 days were used. Follicle-stimulating hormone (FSH) dissolved in saline was injected intratesticularly at a dose of 40 µg/testis. Quinacrine was dissolved in sterile water and injected intratesticularly at **a** dose of 20µmoles/testis, 30 minutes prior to injection of FSH. Controls received 5-10µl of vehicle alone. Animals were sacrificed 4h after injection of FSH . RNA isolation and dot blot analysis were carried out as described in Chapter 2.

A: Autoradiogram of dot blot of RNA from testis of various groups of FSH-treated rats.

B: Densitometric data of A expressed as relative percent of maximal expression.


Control
FSH
FSH + Quinacrine

(A)


Relative expression(% of maximum)
0
20
40
60
80
100
Control
FSH
FSH + Quinacrine

(B)

Fig. 21

SUMMARY

1. Prolactin caused induction of ODC mRNA and enzyme levels in the liver and kidney of adult male rats.

2. **The** extent to which PRL induced ODC mRNA expression was greater in castrated than in intact rats. However, the reverse **was seen in** the case of ODC activity. This suggested a possible **effect** of testosterone on PRL induction of ODC in kidney.

3. The action of prolactin in enhancing renal ODC activity and expression involved the process of transcription.

4. In kidney and liver, treatment with cycloheximide inhibited the action of prolactin in enhancing ODC activity, indicating that *de novo* protein synthesis is necessary for this action of **prolactin.**

5. Inhibition of diacylglycerol formation by the phospholipase C inhibitor quinacrine caused an inhibition of prolactin induction of ODC, indicating that this effect of prolactin may be mediated via this pathway.

6.Further confirmation of the above suggestion was made by the observation that the protein kinase C inhibitor quercetin was **also** able to block the effect of prolactin in inducing ODC. Thus, in the liver and kidney, prolactin seems to act via the inositol phosphate-protein kinase pathway to induce ODC.

7. The effects of growth hormone in the liver and kidney were investigated and this hormone was shown to induce both ODC

activity and expression in these organs of the rat.

8. The effects of growth hormone in inducing ODC mRNA expression were much higher in intact than in castrated rats, indicating an effect of testosterone similar to that seen in the case of prolactin.

9. Ongoing transcription was observed to be necessary for the action of GH on ODC.

10. Cycloheximide treatment confirmed that the effect of GH in enhancing ODC reguired de *novo* protein synthesis.

11. Testicular ODC activity was observed to decrease with the age of the rat, whereas ODC mRNA expression showed an opposite trend.

12. Human chorionic gonadotropin(hCG) enhanced ODC activity and mRNA expression in the testis of immature rat.

13. Desensitisation of the ODC response to hCG was seen to occur at the levels of both ODC activity and mRNA expression.

14. The effect of hCG on ODC was not mediated via diacylglycerol formation.

15. Follicle-stimulating hormone was seen to induce both ODC activity and expression in the immature rat testis.

16. The effect of FSH, like that of hCG, was not mediated via

diacylglycerol formation.

17.PRL stimulated ODC in the testis during the period between immaturity to puberty.

18. The effect of PRL on ODC in the testis was mediated via diacylglycerol formation, as indicated by the use of the phospholipase inhibitor, quinacrine.

19. Growth hormone induced ODC in the rat testis between immaturity and puberty.

20. The effect of GH was observed to occur in both the seminiferous tubules and Leydig cells.

21. The effect of GH on ODC in testis is mediated via stimulation of diacylglycerol formation.

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