ADRENERGIC AND SEROTONERGIC FUNCTION IN DNA SYNTHESIS DURING RAT LIVER REGENERATION AND IN HEPATOCYTE CULTURES

THESIS SUBMTTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

by

SUDHA. B.

DEPARTMENT OF BIOTECHNOLOGY

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN - 682 022
KERALA
INDIA

MARCH 1997

CERTIFICATE

This is to certify that the thesis entitled " **Adrenergic and Serotonergic Function in DNA Synthesis during Rat Liver Regeneration and in Hepatocyte Cultures**" is a bonafide record of the research work carried out by Miss.SUDHA. B under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology, and that no part thereof has been presented for the award of any other degree.

COCHIN-22
31/03/1997

Dr. C.S. Paulose
Senior Lecturer
Department of Biotechnology
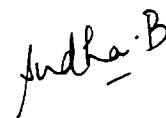Cochin University of Science and Technology

Dr. C .S. P   .   .   .
S+ L.
Coch.. ...   ...
Co.....   ......

DECLARATION

I hereby declare that this thesis entitled "Adrenergic and Serotonergic Function in DNA Synthesis during Rat Liver Regeneration and in Hepatocyte Cultures" has not previously formed the basis of any degree, diploma, assosciateship or other similar titles or recognition.

COCHIN-22.

31/03/1997

SUDHA .B.

(Ph.D. Reg. No. 1410)

ACKNOWLEDGEMENT

ABBREVIATIONS USED IN THE TEXT

Bmax	Binding Maximum
BS	Brain Stem
cAMP	cyclic Adenosine Mono Phosphate
CC	Cerebral Cortex
CNS	Central nervous system
CS	Corpus Striatum
DAG	Diacyl glycerol
EGF	Epidermal Growth Factor
EPI	Epinephrine
FGF	Fibroblast Growth Factor
GABA	γ- aminobutyric acid
Gpp[NH]p	5'- Guanylyl- imidodiphosphate
GTP	Guanosine Triphosphate
HGF	Hepatocyte Growth Factor
5-HIAA	5-Hydroxy Indole Acetic Acid
HPLC	High Performance Liquid Chromatography
HSS	Hepatic stimulatory substance
5-HT	5-Hydroxytryptamine (Serotonin)
5-HTP	5-Hydroxytryptophan
HYPO	Hypothalamus
IGF	Insulin like Growth Factor

IL	Interleukin
IP_3	Inositol triphosphate
IRS	Insulin Receptor Substrate
Kd	Dissociation Constant
K_m	Michaelis Menton constant
LRF	Liver regeneration factor
MAPK	Mitotic Activated Protein Kinase
MHPG	3-methoxy-4-hydroxy-phenyl glycol
NE	Norepinephrine
NF κB	Nuclear factor- kappa-B
8-OH-DPAT	8-Hydroxy-n-dipropylamino tetralin
ODC	Ornithine decarboxylase
p	Level of significance
PH	Partial Hepatectomy
PI	Phosphatidyl Inositol
PI-3K	Phosphatidyl-inositol-3 kinase
PKC	Protein Kinase C
PLC	Phospholipase
PMA	4β-phorbol 12β-myristate 13α-acetate
PS	Phosphatidyl serine
RTK	Receptor Tyrosine Kinase
S.D.	Standard deviation

S.E.M	Standard Error Mean
SRF	Serum response factor
T_3	Tri-iodo-thyronine
T_4	Thyroxine
TGF	Transforming Growth Factor
TK	Thymidine Kinase
TNF	Tumor necrosis Factor
V_{max}	Maximal velocity

CONTENTS

Introduction

INTRODUCTION

The elucidation of mechanisms which control cellular proliferation is a vital step towards understanding the basis of carcinogenesis. In many cell populations, highly differentiated function occurs in mature, non-proliferating cells. These non-dividing cells can be of two types: G_o cells, that are still capable of re-entering the cell cycle and terminally differentiated cells that are destined to die without dividing. The G_o cells which are in a state of quiescence with respect to growth, constantly receive signals from the environment in the form of ion-transport, changes in pH and mitogens (Rozengurt, 1986). These signals are compiled by the resting cells to decide whether to enter into the proliferative phase or not. Recent work on quiescent cells from a wide variety of differentiated cells were shown to trigger normal development to offspring when introduced to enucleated unfertilized egg (Wilmut *et al*, 1997). When G_o cells are triggered to enter into the G_1/S phase by mitogens, a complex series of molecular events occur, which culminate in DNA synthesis (Cantley *et al*, 1991). The G_1 phase of the cell cycle is the functional period during which cells prepare for S phase. Control of cell proliferation in cancer cells is lost mainly due to deregulation of G_1 phase events (Pardee, 1989). Liver regeneration is a unique system to study cellular proliferation and the transition of G_o cells into G_1 and S phase of the cell cycle. A relatively long G_1 period distinguishes adult hepatocytes from other cell types with higher basal proliferative effective and hence, G_1 events probably ensure strict control of hepatocyte proliferative activity (Hunter, 1993).

The adult mammalian liver is predominantly in a quiescent state with respect to cell division. This quiescent state changes dramatically, however, if the liver is injured by toxic, infectious or mechanic agents (Ponder, 1996). Partial hepatectomy (PH) which consists of surgical removal of two-thirds of the liver, has been used to stimulate hepatocyte proliferation (Higgins & Anderson 1931). This experimental model of liver regeneration has been the target of many studies to probe the mechanisms responsible for liver cell growth control (Michalopoulos, 1990; Taub, 1996). After PH most of the

remaining cells in the remnant liver respond with co-ordinated waves of DNA synthesis and divide in a process called compensatory hyperplasia. Hence, liver regeneration is a model of relatively synchronous cell cycle progression *in vivo*. In contrast to hepatomas, cell division is terminated under some intrinsic control when the original cellular mass has been regained. This has made liver regeneration a useful model to dissect the biochemical and molecular mechanisms of cell division regulation. The liver is thus, one of the few adult organs that demonstrates a physiological growth response (Fausto & Mead, 1989; Fausto & Webber, 1994). The regulation of liver cell proliferation involves circulating or intrahepatic factors that are involved in either the priming of hepatocytes to enter the cell cycle (G_o to G_1) or progression through the cell cycle. In order to understand the basis of liver regeneration it is mandatory to define the mechanisms which (a) trigger division, (b) allow the liver to concurrently grow and maintain differentiated function and (c) terminate cell proliferation once the liver has reached the appropriate mass. Studies on these aspects of liver regeneration will provide basic insight of cell growth and differentiation, liver diseases like viral hepatitis, toxic damage and liver transplant where regeneration of the liver is essential. In the present study, $G_O/G_1/S$ transition of hepatocytes re-entering the cell cycle after PH was studied with special emphasis on the involvement of neurotransmitters, their receptors and second messenger function in the control of cell division during liver regeneration.

The role of neurotransmitters, receptors and second messengers as growth regulatory signals in non-neuronal cells has been the focus of recent research (Lauder, 1993). Neurotransmitters stimulate or inhibit cell proliferation in non-neuronal cells by activating receptors coupled to different second messenger pathways (Kluess *et al*, 1991). Serotonin (5-HT) has been found to promote cell proliferation in various cell types. In aortic smooth muscle cells, serotonin induced-mitogenesis was comparable to that of human platelet derived growth factor (Nemeck *et al*, 1986). The serotonin $5-HT_{1C}$ receptor has been reported to function as a proto-oncogene in NIH-3T3 fibroblasts where its expression triggers malignant transformation (Julius *et al*, 1989). The $5-HT_2$ receptors have been shown to mediate cell growth in fibroblasts.(Van Obberghen-Schilling *et al*,

2

1991). The $5HT_2$ receptor has been cloned in the human liver and it has a high degree of homology with that of rat and mouse 5-HT_2 receptors (Bonahus *et al*, 1995). Norepinephrine (NE) has been reported to induce DNA synthesis in primary cultures of rat hepatocytes, acting through the α_1 adrenergic receptor (Cruise *et al*, 1985). Epinephrine has also been shown to stimulate DNA synthesis in cultured hepatocytes through the α_1 adrenergic receptor (Takai *et al*, 1988). The α_1 and β adrenergic receptors have been found to exert positive effects on liver regeneration after PH as antagonists to these receptors inhibit hepatic DNA synthesis (Refnes *et al*, 1992). The basis of growth inhibition may involve decreased activation of Phospholipase β (PLC β) by G protein subunits. This could reduce subsequent generation of diacylglycerol (DAG) and inositol triphosphate (IP3) which in turn may impair regenerative induction of protein kinase C (PKC) and the accumulation of intracellular calcium (Neer, 1995). β adrenergic receptor blockade has been shown to inhibit regenerative induction of adenylyl cyclase, decrease hepatic cAMP concentrations and prevent transcription of cyclin A, a cAMP-inducible gene product necessary for G_1-S transition in hepatocytes (Desdouets *et al*, 1994). The adrenergic receptors activate heterotrimeric G-proteins and several lines of evidence suggest that activation of such G-protein coupled receptors is important in regulating liver regeneration after PH (Diehl & Rai, 1996). Hepatic expression of the stimulating and inhibitory α subunits of G proteins that couple various receptors to their effector targets is differentially regulated during the early prereplicative period. This can modulate the activity of adenylyl cyclase activity that generates the biphasic increase in hepatic cAMP concentrations that occur after PH (Diehl *et al*,1992). Increases in cAMP correlate temporally with increased phosphorylation of the cAMP regulatory element binding protein (CREB) and with increased expression of other cAMP regulated transcription factors such as C/EBPβ and Jun-B that probably influence induction of the cAMP inducible genes in the regenerating liver (Westwick *et al*, 1994). Although α and β adrenergic receptors generally initiate opposing cellular responses, both classes of receptors generate signals that exert the same ultimate effect proliferation.

3

The hepatic sympathetic nervous system has been reported to be important for DNA synthesis during liver regeneration (Morley & Royse, 1981). Kiba *et al* (1995) observed the increase of DNA synthesis in the regenerating liver after lateral lesions of the hypothalamus, which was blocked by hepatic sympathetectomy and vagotomy. The hypothalamus plays a vital role in the integration of neurohormonal function (Oomura & Yoshimatsu, 1984). The hypothalamic adrenergic and serotonergic neurons play an important role in the release of releasing factors from the neurohormonal cells (Brownstein, 1977). The autonomic centres of the hypothalamus are linked to the liver by the autonomic nervous system, which directly innervates the hepatic parenchyma (Nobin *et al*, 1978; Skaaring & Bierring, 1976). The availability of hormones such as insulin glucagon and tri-iodothyronine (T_3) are modulated by catecholamines (Schmelk *et al*, 1980; Silva *et al*, 1983). The possible direct control of pancreatic hormone secretion by adrenergic, noradrenergic and serotonergic neurons was recently demonstrated (Lowey *et al*, 1994). The requirement of these hormones as positive regulators in liver regeneration has been reported by our group and other workers (Mc Gowan *et al*, 1981; Waliuala Mola *et al*, 1996; Tessy *et al*, 1997). Thus, studies on the role of hypothalamus and the sympathetic nervous system in the process of hepatic cell proliferation suggest that the brain can exert a profound influence on liver regeneration either directly or indirectly through endocrine function. However, these studies did not address the role of the brain neurotransmitter receptors and post-receptor mechanisms in the hepatic regenerative response after partial hepatectomy. This work is an attempt to understand the role of the the neurotransmitters, NE and 5-HT and the adrenergic and serotonergic receptors in the brain control of cell division using liver regeneration after partial hepatectomy as an *in-vivo* model for regulated cell proliferation. The present work also focuses on the hepatic adrenergic and serotonergic receptor-mediated mechanisms in-regulation of hepatocyte proliferation.

Research on the molecules that make the brain-body connection is currently emerging into an important discipline (Pennisi, 1997). The molecular links in this network include neurotransmitters, interleukins and hormones., all of which have also been

4

ascertained to be vital in regulating the process of liver regeneration. In this context, liver regeneration is a potentially useful animal model for a holistic study of the interconnections between the nervous, endocrine and immune systems.

The major objectives of this work are:

I) To study the changes in the content and metabolic state of brain neurotransmitters in response to partial surgical resection of the liver.

ii) To assess the alterations in the function of the adrenergic and serotonergic receptors in the hypothalamus (Hypo), brain stem (BS), and cerebral cortex (CC) of hepatectomised rats.

iii) To study the changes in the hepatic adrenergic and serotonergic receptors during the DNA-synthetic phase of liver regeneration after PH in rats.

iv) To investigate the role of norepinephrine and serotonin as potential mitogens during liver regeneration using primary cultures of rat hepatocytes.

iv) To study the changes in the second messengers of the adrenergic and serotonergic receptors of the regenerating liver.

v) To study the NE and 5 HT- receptor-induced changes in protein phosphorylation, during the period of DNA synthesis in the regenerating rat liver

Review Of Literature

REVIEW OF LITERATURE

Liver regeneration after partial hepatectomy is a good model system to dissect the complex multicomponent growth regulatory signals (Michalopoulos, 1990). Despite many years of study of liver regeneration, the detailed nature of the controlling factors that trigger or modulate this phenomenon has only recently begun to be understood. Much progress has been made in the elucidation of the mechanisms involved in this phenomenon by studying control of growth of isolated hepatocytes in primary culture also by studying changing patterns of liver gene expression after PH. Experimentally, liver regeneration can be induced by any acute treatment, surgical or chemical, that will remove or kill a large percentage of hepatic parenchyma. The loss of parenchyma rapidly induces a wave of cell proliferation so that the total mass of the liver is restored to normal. Chemicals such as carbon tetrachloride (CCl_4) will induce necrosis of the central zone of the hepatic lobule. The most preferred approach, however, for inducing liver regeneration is by performing two-thirds PH in rats (Higgins & Anderson, 1931). In this simple surgical procedure, two-thirds of the liver becomes externalised through a small mid-abdominal incision. The externalised portion of the liver is resected. Regeneration of the liver has been demonstrated in most vertebrate organisms and proceeds within 6-8 days in all species examined, including humans (Michalopoulos, 1990). The increased use and success of liver transplantation in clinical medicine have shown that the animal model of liver regeneration correctly reflects the capacity of human liver to regenerate (Van Thiel *et al*, 1989).

Although all cells of the liver participate in regeneration, most studies have focused on the main functional cells of the liver, the parenchymal hepatocytes. These cells constitute the largest portion (80-90%) of the liver cell mass (Daoust & Cantero, 1959). The kinetics of the regenerative response has been well described. The hepatocytes are in the G_o phase and only one in 10,000-20,000 hepatocytes undergo proliferation in the liver at any one time (Bucher & Malt, 1971). DNA synthesis in these cells start within 12-16 hours after two thirds PH and reaches a peak within 22-24 hours. DNA synthesis in the

non-parenchymal cells-endothelial cells, kupffer cells, lipid storing cells and bile ductule cells - starts 24 hours after the DNA synthesis in parenchymal cells (Grisham, 1962). The genesis of hepatocytes during regeneration does not proceed through a stem-cell state. Stem cells are involved in generating hepatocytes only when hepatocytes are totally destroyed as in fulminant hepatitis where hepatic regeneration is impaired or they participate in the formation of hepatocellular carcinomas (Gerber *et al*, 1983; Sell & Dunsford 1989). Alternatively the adult liver, having extensive capacity for maintaining parenchymal cell number through out the life span of the organism, can be viewed as a single lineage stem cell system in which the hepatocyte is the stem cell. Recent data from hepatic cell transplantation experiments in a transgenic mouse model have demonstrated the tremendous growth potential of adult hepatocytes, further supporting the notion of the liver parenchyma as a single lineage stem cell system. (Rhim *et al*, 1994). Hepatocytes appear to be "committed stem cells" that are normally quiescent, but can be activated to produce progeny, whose only differentiation option is hepatocytic (Potten & Loeffler, 1990). Oval cells, the early progeny from the hepatic stem cell compartment are more primitive and purely differentiated than hepatocytes, act as classic stem cells, having multiple differentiation options, including hepatocytes and bile duct epithelial cells (Thorgiersson, 1993). Activation of oval cell proliferation and differentiation occurs only in severe injury as in hepatitis and results in transient re-establishment of a hepatocytic lineage (Grisham & Thorgiersson, 1996; Thorgiersson, 1996). As the oval cells can be infected by hepatitis-B virus at early stage of differentiation, this has significant implications for human hepatocarcinogenesis (Hsia *et al*, 1994; Thorgiersson, 1995).

Biochemical Changes

Early studies have focused on the biochemical changes in hepatic parenchyma during regeneration. Glycogen is depleted and lipids accumulate. The most rapid rate of increase of protein occurs around 36 hours and includes the period when free amino acids are most abundant . Maximal protein synthesis has been observed on the third day. A net increase in RNA is seen by 24 hours and the original total amount is restored by 2-5 days

7

(Bucher, 1963). Ornithine decarboxylase (ODC) shows a biphasic increase after PH (Holtta & Janne, 1972; Mc Gowan & Fausto, 1978). ODC is considered as an early marker of mammalian cell proliferation. Increases in RNA polymerase and DNA polymerase also occur (Lynch & Lieberman 1973). Thymidine kinase (TK), a key regulatory enzyme for DNA synthesis is markedly elevated in the regenerating liver (Bresnick, 1971). Our studies on the kinetic parameters of TK showed an increase in the maximal velocity (V_{max}) during the period of active DNA synthesis in the regenerating liver (Waliaula Mola *et al*, 1996).

Key regulatory Signals

Liver regeneration is the result of a complex interplay of at least two distinct sets of rapidly evolving changes : those elicited by the dramatic metabolic and circulating perturbations imposed by the removal of two thirds of the organ mass (adaptive changes) and those specifically leading to the transitions of liver cells from a quiescent to a replicative state (mitogenic changes). The molecular signals controlling cell division during liver regeneration are becoming rapidly defined. Control of growth in regenerating liver has advanced from elusive serum factors and nutrient effect to identification of entirely new growth factors with apparent liver specificity and establishment of gene expression patterns for growth factors already known. In the recent years, application of the tools of molecular biology has allowed rapid advancement in the knowledge of the mechanisms underlying liver regeneration after PH especially in the areas of growth factors, transcription factors and signal transduction regulators (Fausto & Webber, 1994; Haber *et al*, 1993). Quiescent cells require competence-inducing factors such as platelet-derived growth factor (PGDF), fibroblast growth factor (FGF) and myc oncoprotein to progress into the S-phase (Rozengurt, 1986). Epidermal growth Factor (EGF) and insulin are examples of progression factors which are necessary for the transition from competence phase to S phase. Growth factor-induced changes in gene expression in the quiescent cells is a major part of the mitogenic response. Several mitogen-induced genes such as NF-κB & oncogenes like C-myc, C- fos, C-jun were found to be involved in the

8

regulation of transcription. This indicates that the final stages of the mitogenic stimuli involves the modulation of transcription processes (Lamph, *et al*, 1988).

The key factors involved in liver regeneration was achieved by using hepatocyte cultures in serum free medium. Based on this, growth modulators have been classified as complete hepatocyte mitogens and comitogens (Michalopoulos, 1990). Complete mitogens are substances that are by themselves, in chemically defined media and in the absence of serum, able to stimulate hepatocyte DNA synthesis and mitosis in otherwise quiescent hepatocyte populations. Comitogenic growth factors or growth triggers affect hepatocyte growth positively but in an indirect manner. These substances, do not exert direct mitogenic effects of their own serum free cultures. They enhance the mitogenic effect of growth stimulators and decrease the inhibitory effect of growth inhibitors. Evidence from past literature has demonstrated that during liver regeneration, blood borne factors transmit the mitogenic stimulus to hepatocytes. Grafts of hepatic tissue or transplanted isolated hepatocytes enter into a regenerative activity of their own when the host liver is subjected to two-thirds PH (Jirtle & Michalopoulos, 1982). In animal pairs maintained in parabiotic circulation, hepatectomy of one of the members of the pair results in DNA synthesis in the livers of both partners (Fisher *et al*, 1971). Later studies on liver regeneration identified hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor α (TGF α), acidic fibroblast growth factor (a FGF), and hepatic stimulator substance (HSS) as complete hepatocyte mitogens.

Complete hepatocyte mitogens

Hepatocyte Growth Factor

Hepatocyte Growth Factor, a 150KD polypeptide was isolated from the serum of hepatectomised rats and this was found to stimulate DNA synthesis significantly in cultured hepatocytes and it was also shown to be essential for liver development (Nakamura *et al*, 1984b: Schmidt *et al*, 1995). HGF was also purified from rat platelets and was shown to have growth promoting effect on hepatocytes (Nakamura *et al*, 1986).

9

HGF or Hepatopoietin A (HPTA) was purified from human plasma and characterised as a 69KD protein which was a direct mitogen for hepatocytes (Zarnegar & Michalopoulos, 1989). The human HGF was also cloned and sequenced (Miyazawa et al, 1989). A rapid increase was observed in plasma HGF levels 2 hours after PH and CCl₄ administration in rats (Lindroos et al, 1991) and one of the sources of HGF was suggested to be from extra hepatic sites. HGF activity in the remnant liver was found to increase within 24 hours and the HGF mRNA level increased at 3-6 hours post hepatectomy and peaked at 12 hours (Kinoshita et al, 1991; Zarnegar et al, 1991). Thus, hepatic HGF was suggested to be one of the major early signals that triggered hepatocyte proliferation. The HGF receptor (HGFR) was identified in cultured hepatocytes and liver plasma membranes to be a single class of high affinity receptors. After PH, specific binding of ^{125}I-HGF to the membranes of the remnant liver decreased by 60-70% between 3 and 6 hours and was scanty at 12 h. after hepatectomy. This rapid down regulation was also observed in liver membrane after hepatitis induced by CCl4 (Higuchi & Nakamura, 1991). The HGFR was identified as the C-met protooncogene product (Bottaro et al, 1991). Addition of pure HGF to cultured hepatocytes results in rapid induction of transcription factors such as jun-B and c-fos, which further control gene expression during G1 phase of hepatic growth (Weir et al, 1994).

Epidermal Growth Factor

This prototype mitotic stimulator of most epithelial cells also stimulates DNA synthesis in hepatocytes (McGowan et al, 1981). Vintermyr and Doskeland (1987) described the detailed kinetics of hepatocyte responses to EGF. EGF receptors decrease in hepatocyte cultures. There is also a decrease in affinity for EGF. High affinity receptors rapidly disappear after hepatocyte isolation. Low-affinity receptors, despite the initial decrease are maintained in sufficient numbers and are the only type of EGF receptors present when the mitogenic response is stimulated. This has led to the hypothesis that the low affinity EGF receptors are the true mitogenic EGF receptors (Wollenberg et al, 1989). In vivo, a decline in the number of EGF receptors occurs rapidly, within 8 hours and reaches minimum levels at 40 hours after PH. A parallel

decline is also seen with EGF-dependent tyrosine kinase activity of the receptor (Rubin *et al*, 1982). Within 15 minutes after 2/3 PH, EGF RNA levels increase over ten-fold in the remnant liver and diminish below basal levels prior to the first wave of regenerative cell division. This rapid increase in the EGF RNA levels in the immediate-early phase of liver regeneration point to EGF as an autocrine factor in the prereplicative hepatic growth program (Mullhaupt *et al*, 1994). EGF has been shown to activate the Stat 3, which is a transcription factor complex that pre-exists in the liver (Ruff-Jamison *et al*,1993, 1994). The targets of Stat 3 are c-myc, c-fos and jun which are immediate early genes required for liver regeneration (Taub, 1996). Thus, Stat 3 activation may be one of the mechanisms of EGF mitogenesis in liver regeneration.

Transforming growth factor α

The regenerating hepatocytes have been shown to produce TGF-α and it is a complete hepatocyte mitogen. Secretion of TGF-α by regenerating hepatocytes might constitute an autocrine loop resulting in stimulation of DNA synthesis (Mead & Fausto, 1989). The normal adult rat liver contains TGF-α mRNA (Lee *et al*, 1985). Hepatoma cell lines also synthesise and secrete TGF-α (Luetteke *et al*, 1988). TGF-α shares a high degree of homology with EGF (deLarco & Todaro, 1980) and exerts its effect through binding to cell-surface EGF receptors (Massague', 1983). TGF α has been reported to be a stronger hepatocyte mitogen than EGF (Brenner *et al*, 1989). The production of TGFα by hepatocytes may be the critical step that leads the cells towards DNA synthesis, whereas gene expression changes observed before this point might constitute a primary state in which hepatocytes prepare for DNA synthesis but are not committed to entering into it. Increased levels of TGF-α were observed within 8 hours after 2/3 PH with a peak of expression at 24 hours, followed by a decline and subsequent smaller elevation with a peak at 72 hours. These changes in TGF α gene expression parallel the kinetics of DNA synthesis. TGFα production by hepatocytes might also have a paracrine role, stimulating proliferation of adjacent non parenchymal cells (Michalopoulos, 1990).

Acidic FGF, Hepatopoietn B HPTB, Hepatic stimulatory substance

Acidic FGF is a 16,000KD heparin-binding growth factor which stimulates DNA synthesis. The secretion of acidic FGF by regenerating hepatocytes peaks with DNA synthesis. Non parenchymal cells also produce acidic FGF (Kan *et al*, 1989). HPTB is the another complete mitogen found in the serum of hepatectomised rats, in addition to HPTA. It is smaller than 500D and its properties are those of a glycolipid. HPTB acts in a synergistic manner with EGF and HPTA (Michalopoulos *et al*, 1984). Hepatic stimulatory substance (HSS) has been found to be mitogenic for hepatocytes *in vivo* (La Brecque *et al* 1987). HSS is extracted from neonate and regenerating livers. *In vitro*, it augments the effect of EGF to stimulate hepatocyte DNA synthesis (Fleig & Hoss, 1989).

Growth Inhibitors

These substances have also been defined in primary culture, based on their capability to inhibit EGF mitogenesis.

Transforming Growth Factor-β (TGF-β)

TGF-β found in platelets strongly inhibited HGF and EGF-mediated DNA synthesis in primary cultures of rat hepatocytes (Nakamura *et al*, 1985; Carr *et al*, 1986). TGF-β injection also inhibited the rate of DNA synthesis in the regenerating rat liver after PH (Russell *et al*, 1988). TGF-β mRNA production by non parenchymal hepatocyte first becomes detectable at 4 h, remains at low levels until 18-20 hours and then rises sharply, peaking at 72 hours. It remains at high levels for more than 96 hours (Braun *et al*, 1988; Carr *et al*, 1989)

Interleukin β (IL 1β)

Interleukin β was shown to be a growth inhibitor of cultural hepatocytes. The degree of DNA synthesis inhibition is not as complete as in the case of TGF-β (Nakamura *et al*, 1988).

Comitogenic substances or Growth triggers

The plasticity of growth responses seen during liver regeneration are governed by complete mitogens as well as by comitogenic substances such as neurotransmitters and hormones. Neurotransmitters have been shown to act as modulators of cell division in non-neuronal cells, acting through different second messenger pathways (Lauder, 1993). Stimulation of proliferation is most often associated with activation of G proteins negatively coupled to adenylyl cyclase (G_i), or positively coupled to phospholipase C (PLC), which mediates phosphoinositol (PI) hydrolysis (G_p) or to pertussis-toxin-sensitive pathways (G_O, G_i). In contrast, activation of neurotransmitter receptors positively coupled to cyclic AMP (cAMP) usually inhibits cell proliferation. However, the actual mechanism are more complex than suggested by these correlations and multiple second messenger pathways are involved in the receptor-mediated regulation of cell proliferation(Kleuss *et al*, 1991). Previous studies on the humoral control of liver cell proliferation have suggested that various hormones may act as signals or regulators for stimulating DNA synthesis after PH. . Thyroid hormones and insulin have been reported to be important in regulation of liver regeneration.

Thyroid Hormones

Studies on the role of thyroid hormones in influencing this phenomenon have shown that T_3 can induce proliferative responses after subcutaneous administration in the intact liver (Francavilla, 1984; Tessy *et al*, 1997). The regenerative response of intact liver after subcutaneous T_3 administration is shown to mimic the DNA synthesis pattern induced by 40% hepatic resection (Francavilla *et al* ,1994). Liver cell proliferation can be induced by primary or direct mitogens, without preceding cell loss and the process is defined as direct hyperplasia (Columbano & Shinouzka, 1996). Thus T_3 can act as a mitogen inducing direct hyperplasia. Results from our work indicate that thyroid hormones can influence DNA synthesis during liver regeneration by regulating the activity of thymidine kinase which is a key enzyme for DNA synthesis (Tessy *et al*, 1997). Hypothyroid hepatectomised animals showed significantly lower level of DNA synthesis

than euthyroid counterparts. T_3 treatment of hypothyroid hepatectomised animals caused an additive effect of DNA synthesis. The affinity of thymidine kinase, a key enzyme for DNA synthesis was altered with the thyroid status. The growth associated genes which are expressed during liver regeneration are also expressed due to T_3 administration (Francavilia *et al* ,1994). Thyroid hormone has been shown to decrease the expression of EGF receptor and the EGF receptor levels are subject to regulation by thyroid status (Kesavan *et al*, 1991; Vonderhaar *et al*, 1986). This may be a possible mechanism of mitogenicity of thyroid hormones in the liver as downregulation of EGF receptors are important for the regenerative response.

Insulin and Glucagon

Intravenous infusion of insulin and glucagon into normal adult rats triggered small but significant DNA synthesis in hepatocytes and previous evisceration including pancreatic resection largely suppressed liver DNA synthesis 24 hours after PH in untreated rats but not in animals that received peripheral injections of insulin and glucagon (Bucher *et al*, 1975). Primary cultures of rat hepatocytes could be stimulated to synthesize DNA by EGF in combination with insulin and glucagon (Mc Gowan *et al*, 1981). Insulin and glucagon have been suggested to act synergistically as major regulators of hepatic regeneration (Sato *et al*, 1989). Peripheral infusion of insulin antiserum substantially blocked hepatic DNA synthesis 24 hours after PH in rats (Bucher *et al*, 1978). Cultured hepatocytes degenerate and die in absence of insulin (Michalopoulos, 1990). Tyrosyl phosphorylation of insulin receptor substrate-1(IRS-1) a specific target molecule for insulin β-subunit kinase was strikingly enhanced prior to major wave of DNA synthesis after PH. Phosphatidyl-inositol-3-kinase which is involved in growth pathway was seen to be associated with IRS-1 following tyrosyl phosphorylation *in vivo* (Sasaki, *et al*, 1993). The number of insulin binding sites was significantly increased and the ratio of insulin to glucagon binding was markedly increased after PH in rats. This can lead to increased uptake of insulin resulting in hepatic proliferation (Gerber, *et al*, 1983). Our studies show that the activity of thymidine kinase is regulated by insulin. Streptozotocin-diabetes caused an increase in the maximal velocity, V_{max} of the enzyme (Waliauala Mola *et al*,

1996) after PH. DNA synthesis was also significantly higher in the regenerating liver of diabetic rats. The low levels of insulin in the diabetic conditions are sufficient to promote proliferative responses of the liver cell after PH as observed in our experiments. The diabetic state which does not represent a zero level but a relative deficiency of plasma insulin was reported to promote proliferative response of the liver cell following PH in the early hours of liver regeneration (Nakata *et al*, 1985). Probably, the low levels of insulin sensitises the insulin receptor for its ligand resulting in active hepatic extraction of insulin, thereby promoting DNA synthesis. Suppression of hepatic DNA synthesis in partially hepatectomised rats by exogenous insulin infusions suggest that high plasma levels of insulin are inhibitory for liver regeneration. This correlates well with the observation that plasma insulin levels decline after PH (Leffert *et al*, 1975; Mourelle & Rubaclava,1981). This led to the hypothesis that hypoinsulinemia and hyperglucagonemia is characteristic of enhanced proliferative capacity. Demouzon *et al*, (1995) concluded from their experiments that long term culture with high glucose concentrations increases the amount of insulin receptors and their tyrosine kinase activity. The insulin receptor sensitization as a result of elevated glucose and depleted insulin in the diabetic state may result in increased binding of insulin leading to enhanced proliferation. This receptor sensitization in diabetic state explains our observation of DNA synthesis triggered in diabetic and insulin treated shams even without PH.

Norepinephrine

Addition of NE to cultured hepatocytes has been shown to enhance the mitogenic effect of EGF (Cruise *et al*, 1985). This was strongly antagonised by α_1-adrenergic blocker prazosin, but not by an α_2 antagonist or β adrenergic blocker, indicating that catecholamines interact with the α_1 adrenoreceptor to stimulate DNA synthesis in hepatocytes. Liver has a high relative concentration of α_1 adrenergic receptors (Hoffman *et al*, 1980) and the ability of catecholamines to directly stimulate DNA synthesis through α_1 mediation suggested a significant role of these receptors in the regeneration response. Addition of NE to hepatocytes stimulates Ca^{2+} mobilisation or PI turnover (Exton, 1981) and either or both of these processes was proposed to be involved in the mitogenicity of

NE. The α_1 adrenergic receptor has the potential to stimulate protein kinase C which is a target for tumor promoters such as phorbol esters (Weinstein, 1983). This makes α_1 adrenergic receptor a potential regulator of cell growth and division. NE produced a close dependent inhibition of EGF binding to cultured hepatocytes which was blocked by prazosin. This correlated with the ability of NE to enhance hepatocyte DNA synthesis in the presence of EGF (Cruise et al, 1985 ; 1986). Similar heterologous down regulation of EGF receptors has been shown in other systems where EGF mitogenesis is enhanced. In several murine and human cell lines 12-0-tetradecanoyl-phorbol-13-acetate (TPA) reduced EGF receptor affinity via activation of protein kinase C (Mc Caffrey et al, 1984) Vasopressin, platelet derived growth factor (PDGF) and bombesin all stimulate PI turnover and, like TPA, have been reported to reduce EGF receptor affinity (Brown et al, 1984; Rozengurt et al, 1981). Alternatively, TPA and PDGF have also been demonstrated to reduce EGF receptor numbers, without significant alterations in receptor affinity (Beguinot et al, 1985). Many of the compounds that modulate EGF receptor binding, including NE, stimulate PI metabolism and PKC activation. This suggests that phosphorylation perhaps of the EGF receptor itself, may mediate their effects. Co-internalisation of EGF receptors with those of other ligands has been documented and it has been suggested that heterologous down regulation could be the result of such a mechanism (Wrann et al, 1980). Magun et al, (1980) postulated that a reduction in receptor-mediated degradation of EGF might maintain higher growth factor levels, eventually stimulating more DNA synthesis when EGF concentration is limiting. Cruise and Michalopoulos (1985) reported that NE-stimulated receptor down regulation, although maximal at 1 hour, persisted throughout the first 24 hours of incubation in culture. Thus reduced EGF degradation was a possible mechanism by which NE enhanced EGF-induced DNA synthesis. The α_1-adrenergic receptor blockade by prazosin was also seen to reduce DNA synthesis during liver regeneration in vivo (Cruise et al, 1987). NE also decreased the mito-inhibitory effect of TGF β (Houck et al, 1988), acting through the α_1 adrenergic receptors. Thus, NE was shown to tilt the balance between growth stimulators and growth inhibitors and to act as a trigger for hepatocyte mitogenesis. The α_1 adrenergic receptor is linked to a G protein and stimulates increased activity of

phosphatidyl inositol diphosphate (PIP_2) phosphodiesterase. Stimulation of the α_1-adrenergic receptor triggers increased breakdown of PIP2, resulting in an increase in the cytoplasmic diacylglycerols and inositol 1,4,5-triphosphate. These mediators trigger a cascade of other intracellular events, including activaion of protein kinase C and mobilisation of calcium from intracellular stores (Exton, 1988). Cruise *et al* (1989), demonstrated an uncoupling of the α_1 adrenergic receptor to phosphoinositide turnover and suggested that the uncoupled receptor may be the mediator of interest. The uncoupled α_1 receptor was more potent in antagonising TGF-β mediated inhibition of DNA synthesis. The activation of PKC by diacyl glycerol produced as a result of PIP_2 turnover may cause negative feed back regulation of α_1-adrenergic receptors through its phosphorylation. The uncou;pling of the α_1 receptor was preceded by a drop in hepatic membrane ras p21 content, suggesting an involvement of ras protein in the early events of liver regeneration. Epinephrine also caused a dose-dependent increase in EGF-induced DNA synthesis but the mechanism of action was not apparently mediated by either activation of PKC or Ca^{2+} mobilisation (Takai *et al*, 1988) Three α_1 receptor sub types have been identified. α_{1a}, α_{1b} and α_{1c}, all products of different genes (Lomasney *et al*, 1991). Hepatocytes contain predominantly the α_{1b} - adrenergic receptor subtype (Minneman, 1988). The normal and regenerating rat liver was negative for α_{1a}-receptor m RNA and positive for α_{1b} m RNA characterised by the presence of two bands at 4.0 and 3.2 kb which peaked between 20 and 48 hours after partial hepatectomy (Kost *et al*, 1992). Rat hepatomas lacked the α_{1a} and α_{1b} m RNA and receptor binding, while the human hepatocellular carcinoma cell, line Hep G2 was positive for α_{1a} and α_{1b} message at 4.5 kb but lacked receptor binding. Thus, the presence α_1 adrenergic receptor may be important for preventing deregulation of cell division. Sanae *et al*,(1989), reported that hepatic neoplasms are characterised by an increase in α_2-and β-adrenegic receptors and a concomitant decline in α_1 receptors.

Serotonin

Serotonin has been shown to stimulate DNA synthesis in many non-neuronal cells. In fibroblasts, 5-HT activates phospholipase C, inhibits adenylate cyclase and stimulates

DNA synthesis, acting through the 5-HT $_{4B}$ receptor, coupled to a G_i-protein (Seuwen *et al*, 1988). In vascular smooth muscle cells, mitogenicity involves interaction with 5-HT$_4$ receptors positively coupled to cAMP or with 5-HT$_{1D}$ receptors coupled to a pertussis-toxin-sensitive pathway independent of cAMP (Kavanaugh *et al*, 1988). The 5-HT$_{1C}$ receptor was shown to act as an oncogene for NIH 3T3 cells. Ectopic expression of this receptor in this fibroblast cell line resulted in malignant transformation (Julius *et al*, 1989). Serotonin receptors are also expressed in the liver . Full length clones of the human 5-HT $_{2B}$ receptors had a high degree of homology with the rat 5-HT$_{2B}$ receptors (Bonahus *et al*, 1995).

Vasopressin, angiotensin II and angiotensin III

These hormones also act through receptors that enhance PIP$_2$ turnover, like NE. NE is more potent than these substances in enhancing EGF mitogenesis as well as in decreasing TGF β-mediated DNA synthesis inhibition. Liver regeneration is impaired in rat strains which are congenitally deficient in production of vasopressin (Russell & Bucher, 1983). Vasopressin is secreted in the synapses of the sympathetic nerves of the liver, along with NE thus forming a part of the sympathetic control of liver regeneration (Francavilla *et al*, 1989).

Gamma aminobutyric acid (GABA)

GABA , which is a potent inhibitory neurotransmitter with growth regulatory function, has been shown to inhibit hepatic regeneration after PH (Minuk & Gauthier, 1993). In fulminant hepatic failure in which liver regeneration is impaired, GABA concentrations are elevated (Ferenci et al, 1983). At such high levels of GABA, the levels of insulin-like growth factor-I (IGF-I) and IGF-1 binding protein (IGFBP-I) mRNA expression were lowered and GABA has been proposed to influence the expression of these proteins (Minuk et al, 1995). IGF-I and IGFB-1 are highly expressed in the regenerating liver and have been reported to be important in liver regeneration (Mohn *et al*, 1991b).

Estrogens rise after 2/3 PH, reaching a peak at 24-48 hours and testosterone levels decrease. Tamoxifen given after 2/3 PH blocks hepatic DNA synthesis. (Francavilla *et al*, 1986; 1989). Estrogens added to primary cultures with serum or EGF ehnance mitogenesis (Shi and Yager, 1989).

Sympathetic nervous system in liver regeneration

The autonomic nervous system may co-operatively regulate liver regeneration along with humoral factors. Vagotomy inhibits and delays DNA synthesis and proliferation of liver cells after PH, suggesting an involvement of the parasympathetic nervous system (Kato & Shimazu, 1983). Kiba *et al*,(1994), reported that lesions of the ventromedial hypothalamic nucleus facilitates liver regeneration after PH and this effect was inhibited by vagotomy. The hepatic sympathetic nervous system has been implicated to be important in DNA synthesis during liver regeneration (Morley & Royse, 1981). Kiba *et al*, (1995) also reported the increase of DNA synthesis after lateral lesions of the hypothalamus , which was blocked by hepatic sympathectomy and vagotomy. Chemical sympathectomy has had varying influences on rat liver regeneration. Reserpine, which depletes catecholamine stores, inhibited incorporation of [^{3}H] thymidine into liver DNA at 24 hours post-hepatectomy (Ashirf *et al*, 1974., Cihak *et al*, 1973). 6-Hydroxydopamine (6-OHDA) administration destroys adrenergic nerve terminals and its acute administration has been reported to enhance DNA synthesis in the regenerating liver (Ashrif. *et al*, 1974). This may be explained by false neurotransmitter effects of 6-OHDA or by leaking of NE from damage terminals. More long term-treatment with this compound has been reported to decrease activity in the regenerating liver (Morley & Royse, 1981). Guanethidine which blocks sympathetic neuroeffector functions (Johnson and Manning, 1984) has been shown to depress DNA synthesis in the regenerating liver (Ashrif *et al*, 1974). Thus, *in vivo* studies suggest a role for the sympathetic nervous system in liver regeneration.. The hypothalamus is crucial for co-ordinating neurohormonal responses (Oomura & Yoshimatsu,1984). The the autonomic nervous system links the the hepatic parenchyma

to the autonomic centers in the hypothalamus (Nobin *et al*, 1978). Hence, the hypothalamus and other brain regions may play a crucial role in governing the process of liver regeneration either by direct innervation or by neuro-endocrine regulation or by both. Catecholamines regulate the secretion of hormones necessary for liver regeneration such as insulin, glucagon (Hasegawa *et al*, 1977; Potter *et al*,1977; Schmelk *et al*, 1980)., EGF (Byyny *et al*, 1974., Olsen *et al*, 1984) and T3 (Silva *et al*, 1983).

Two main hypotheses have been advanced relating to the mechanisms triggering liver regeneration (Michalopoulos, 1990). In the first hypothesis, extrahepatic signals such as NE and HGF are generated after 2/3 PH and these transmit a complete mitogenic stimulus to the hepatocytes. A prolonged and sustained stimulation by NE would potentiate the effect of mitogens such as HGF and EGF. The alternate hypothesis suggests that the decisive mitogenic signals for hepatocyte proliferation are derived from the hepatocyte itself. The metabolic changes after acute decrease in the liver mass prime hepatocytes into entering in the G_1 phase of the cell cycle. Further stimulation triggers production of autocrine growth factors that commit hepatocytes into DNA synthesis and force them to make the G1/S transition. However, the two mechanisms suggested above are not mutually exclusive and the mitogenic signals to the hepatocytes is composed of both these processes.

Transcriptional regulation and Signal transduction in liver regeneration

Following rapid intracellular signal transduction in hepatic cells undergoing regeneration, preexisting transcription factors are modified, resulting in their activation. These transcription factors are responsible for activating the transcription of primary or immediate early response genes within minutes after PH in a protein synthesis-independent manner (Almendral *et al*, 1988; Herschman, 1991). Immediate early genes encode proteins that regulate later phases in G_1, including the induction of the delayed-early response genes. Delayed-early response genes are induced within a few hours of hepatectomy, but their transcription requires protein synthesis. As immediate-early genes

20

are induced in a protein synthesis-independent fashion, their transition must be activated by transcription factors that are pre-existing in hepatic cells. The earliest signals triggering the regenerative response activate these transcription factors, which are normally inactive. Within minutes after PH, hepatocytes in the remnant liver undergo a transition from the quiescent G_o state into G_1 phase of the cell cycle. Even though the precise mechanisms responsible for triggering this transition is not known, the enhanced expression of genes occurring within 30 minutes to 2 hours after PH probably mediates G_o/G_1 transition. Many such genes were identified and defined as immediate early genes including the fos and jun family, egr-1, liver regeneration factor-1 (LRF-1), and c-myc (Goyette et al, 1983; Haber et al, 1993). They represent diverse functional classes and include transcription factors, growth factors, signal transduction regulators and other type of proteins (Lau & Nathans, 1987; Mohn et al, 1991a; Zipfel et al, 1989).

The serum response element is found in the promoter regions of c-fos and several other immediate early genes. The serum response factor (SRF) and a cofactor, ets-like protein (ELK-1) which bind to the serum response element are activated by phosphorylation following mitogen stimulation. These transcription factors act in a co-ordinated fashion to activate c-fos gene transcription (Hill & Treisman, 1995). Two transcription factors complexes identified are post hepatectomy factor/nuclear factor- κB (PHF/NF-κB) and Stat 3, that pre-exist in normal liver in an inactive form. They are activated as part of the initial response of the remnant liver following partial hepatectomy (Cressman et al, 1994,1995; Cressman & Taub, 1994; Tewari et al, 1992). Immediate early genes such as I κB-α and KC are potential target genes of PHF/NF-κB. STATs are transcription factors that require tyrosyl phosphorylation before they can translocate to the nucleus and bind to regulatory elements of genes (Sadowski et al, 1993). The targets of Stat 3 are c-myc, c-fos and jun (Taub, 1996). EGF and IL-6 have been shown to activate the Stat 3 (Ruff-Jamison et al, 1993, 1994).The finding of Stat 3 activation in liver regeneration supports the importance of EGF in liver regeneration. IL-6 is reported to be an important mediator of liver regeneration (Fong et al, 1994). Cytokines such as TNF-α,

IL-1 and IL-6 induce both NF-κB and Stat pathways and suggests a common mechanism for the activation of both transcription factor complexes.

Transcription factors induced as immediate early genes in the regenerating liver include fos-jun family, c-myc, the Rel family and LRF-1 (Goyette *et al*, 1984; Hsu *et al*, 1991; Kruijer *et al*, 1986; Thompson *et al*, 1986.). Members of the Jun and Fos families of transcription factors are thought to have a role in activating transcription of delayed-early genes expressed subsequently during the growth response and these transcription factors are important in the G_0 / G_1 transcription. Addition of pure HGF to cultured hepatocytes results in rapid induction of LRF-1, jun-B and c-fos mRNAs. Through the complex interactions among LRF-1, jun B, c-jun and c-fos, control of delayed gene expression may be established during G_1 phase of hepatic growth (Weir *et al*, 1994). In addition, a number of genes are induced as delayed early genes and also as cell cycle specific genes such as cyclins and Histone H3 in S-phase (Albrecht *et al*, 1993).

Immediate early genes also encode growth factors such as EGF and HGF and several of these genes function as proto-oncogenes (Mullhaupt *et al*, 1994; Zarnegar *et al*, 1991). In the regenerating liver, MKP-1 (Sun *et al*, 1993) and PRL-1 (Diamond *et al*, 1994) encode distinct tyrosine protein phosphatases that have been frequently implicated in oncogenic transformation and which are involved in signal trasnsduction.. Examination of the interplay of growth induced and constitutive transcription factors can provide insight into how the liver adapts to the acute loss of mass and maintains its hepatic phenotype during regeneration. Analysis of signal transduction molecules will lead to a greater understanding of specific regulation of growth processes and induction of abnormal hepatic growth that occurs in malignancies.

Ligand activation of receptors with tyrosine kinase activity appears to play an important role in promoting hepatocyte proliferation (Marshall, 1995). EGF, TGFα and HGF bind to this class of receptor. Phospholipase C gamma, phosphatidylinositol-3-kinase (PI-3-K), Src-related tyrosine kinase p59 fyn, GRB2 and ras GAP are among the

downstream signal transducing proteins that have been shown to bind directly to specific sequences surrounding the phosphorylated tyrosine residues of receptor tyrosine kinases (RTKs) (Hill&Treisman, 1995; Heldin, 1995; Marshall, 1995). One of these complexes (Grb2-SOS) interacts with membrane-associated ras and facilitates the exchange of GDP for GTP. This activates ras, which then activates cytosolic raf, which in turn activates another cytosolic kinase, MEK by phosphorylating it. Activated MEK then phosphorylates and activates mitogen activated protein kinase (MAPK) and permits them to translocate to the nucleus (Heldin, 1995; Marshall, 1995). Several potential nuclear targets of the MAPKs have been identified, including growth-regulatory transcription factors, such as Elk-I, C-myc and C/EBPβ (Hill & Treisman, 1995). Phosphorylation of these transcription factors regulates their transcriptional activity and hence modulates expression of their target genes (Hill & Treisman, 1995; Hunter, 1995). Potential cytoplasmic MAPK substrates include the protein kinases that form the cascade that leads to MAPK activation. Phosphorylation of c-raf1 and MEK by MAPK suggests that MAPK kinase cascade itself may be regulated by MAPK, the final kinase in the cascade. Activated MAPK may also phosphorylate other protein kinases such as S6 kinase, which activates and modulates certain protein phosphatases. Phosphatases such as PRL -1 increase in regenerating liver (Diamond et al, 1994). Activated MAPK may also phosphorylate the EGF receptor and phospholipaseA₂ (PLA₂) (Hill & Treisman, 1995; Marshall, 1995; Hunter, 1995). The functional significance of MAPK phosphorylation of EGFR is unclear. MAPK activation of PLA₂ and promotes the release of arachidonic acid which is a well-recognised consequence of many different mitogens and cytokines (Diveeha & Irvine, 1995; Hill & Treisman, 1995).

EGF and HGF increase MAPK phosphorylation in primary hepatocyte cultures (Stolz & Michalopoulos, 1994). The Src-hormology region of activated c-met (the HGF receptor) and the EGFR bind different downstream signal transduction elements (Songyang et al, 1993). Tyrosine kinase pathways appear to regulate not only entry into the initial phases of the cell cycle but also progression through later prereplicative stages and into S phase. In cultured cells, EGF and HGF probably activate PLC gamma and PI 3

kinase because these mitogens increase PIP_2 hydrolysis to IP_3 and DAG (Graziani et al, 1991). DAG, in turn, activates protein kinase C (PKC) and IP_3 facilitates release of Ca^{2+} from intracellular stores (Baffy et al, 1992; Graziani et al, 1991). Increased phosphoinositide hydrolysis and calcium transients have also been documented in the regenerating liver after PH (Bucher, 1991). Although this is consistent with mitogenic activation of RTKS during a hepatic growth response in vivo , G-protein coupled receptors can also activate these responses (Neer, 1995). The relative importance of RTK-initiated signals and other pathways in regulating phosphoinositide hydrolysis and Ca^{2+} release in vivo has not been established.

Receptor activation by several extracellular factors such as growth hormone, prolactin, α and γ interferon, erythropoeitin and 1L-6 activates cytosolic tyrosine kinase, including members of the Janus family (JAK-1, JAK-2) to the receptors' cytoplasmic domains. This receptor-kinase complex then interacts with and activates target SH2-containing cytoplasmic proteins, including transcription factors of the STAT family (Darnell et al, 1994).

Several hepatocyte comitogens such as glucagon, epinephrine, norepinephrine and vasopression bind to plasma membrane receptors that activate heterotrimeric G-proteins. Several lines of evidence suggest that activation of receptors that couple to heterotrimeric G-proteins is important in regulating liver regeneration after PH. The expression of the stimulating and inhibitory α subunits of G proteins that couple various receptors to their effector targets like adenylyl cyclase is differentially regulated during the early prereplicative period in the liver. Thus, the biphasic increase in hepatic cAMP concentrations that occurs after PH correlates temporally with increased phosphorylation of the cAMP regulatory element binding protein (CREB) and with increased expression of other cAMP regulated transcription factors, thus, influencing induction of the cAMP-inducible genes in the regenerating liver (Diehl and Rai, 1996)..Prazosin, an α_1 receptor antagonist and propranolol, a β adrenergic receptor antagonist inhibit hepatic DNA synthesis after PH (Refnes et al, 1992) The basis of growth inhibition may involve

decreased activation of PLCβ by G-Protein submits. This could reduce subsequent generation of DAG and IP$_3$ which in turn may impair regenerative induction of protein kinase C and the accumulation of intracellular calcium (Neer, 1995). β adrenergic receptor blockade has been shown to inhibit regenerative induction of adenylyl cyclase, decrease hepatic cAMP concentrations and prevent transcription of cyclin A, a cAMP-inducible gene product necessary for G1-S transition in hepatocytes (Desdouets *et al.* 1994). Cellular proliferation is tightly regulated by positive and negative regulatory proteins that exert their efforts during different phases of the cell cycle (Hunter, 1993). G$_1$ cyclins are positive regulators that control rate-determining steps during the G$_1$ progression. A relatively long G$_1$ period is characteristic of adult hepatocytes and G1 events probably exert a stringent control of hepatocyte proliferation. G$_1$ cyclins are synthesised during the reentry into the cell cycle and they activate cyclin-dependent kinases which phosphorylate downstream targets that permit eventual entry into the S phase.

Several extra cellular factors such as TGFβ, activins and inhibins have been identified that abort cell cycle progression in hepatocytes (Bever *et al*, 1990; Dubois, 1994; Francavilla *et al*, 1992). These agents are felt to play a crucial role in terminating the regenerative response to PH once recovery of liver mass has been accompanied. In some epithelial cells, TGF β inhibits cellular proliferation largely through its ability to down regulate the activity of cyclin-dependent kinases cdk2 and cdk4 (Koff *et al*, 1993). TGF β decreases the transcription of cdk4 and downregulates cdk2 activity by inactivating cyclin E-cdk2 complexes. These events lead to accumulation of hypophosphorylated tumor suppressor gene Rb (retinoblastoma) and prevent activation of E2F, a transcriptional activator of many S phase genes (Koff *et al*, 1993). Cyclin A, which is essential for the G1/S transition after PH (Hunter, 1993) is known to complex with and activate cdk2 and E2F (Pines, 1993). This may be the signalling pathway that mediates the antiproliferative actions of TGF β in hepatocytes. In addition, in cultured hepatocytes, recombinant TGF β increases the binding activity of C/EBFα, a transcription factor that arrests proliferation in hepatocytes (Rana *et al*, 1995).

The hepatic non parenchymal cells are likely to be a source of TNF α after PH and TNF α and other TNF-inducible cytokines play a critical role in helping hepatocytes escape growth arrest and enter early prereplicative stages of the cell cycle after PH (Akerman *et al*, 1992). The cytokines may activate autocrine or paracrine mechanisms to promote the local release of hepatocyte mitogens in the liver remnant. IL-6 may increase HGF production by hepatic stellate cells by activating IL-6 responsive elements in the regulatory region of the HGF gene (Liu *et al*, 1994). Such events may also help recruit extra hepatic tissues to release factors such as growth regulatory hormones and neurotransmitters that amplify the regenerative response (Matsumoto *et al*, 1992). The ultimate regenerative response is likely to be dictated by the timing with which the various extracellular signals such as growth factors, neurotransmitters and hormones are presented to the hepatocyte.

In the present study, we assessed the neurotransmitter receptor mediated control of cell proliferation using liver regeneration after PH in rats as an *in vivo* model for regulated cell division. The central nervous system mediated adrenergic and serotonergic function in governing hepatocyte division was studied. The mitogenicity of serotonin and norepinephrine and the receptor subtypes involved were investigated in primary cultures of rat hepatocytes. The changes in the hepatic adrenergic and serotonergic receptors and their second messengers were studied during the DNA synthetic period of liver regeneration. NE and 5-HT induced protein phosphorylation during DNA synthetic phase of liver regeneration was also studied

Materials And Methods

MATERIALS AND METHODS

BIOCHEMICALS AND THEIR SOURCES

Biochemicals used in the present study were purchased from SIGMA Chemical Co, U.S.A. All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India.

The following are the list of chemicals purchased from Sigma and used in this study.

Neurotransmitter Standards

(±)Norepinephrine, (-)-Norepinephrine-bitartrate salt, (±)Epinephrine, 5-Hydroxytryptamine, 5-Hydroxytryptophan, 5-Hydroxy Indole Aceticacid, 4-Hydroxy 3-methoxy Phenyl Glycol

Buffer Constituents

Sodium octyl sulfonate, Ethylene Glycol-bis(β-aminoethyl ether)-EGTA, Ethylenediamine tetra acetic acid-EDTA, Benzamidine, Phenylmethyl sulfonyl fluoride(PMSF), Glycylglycine, HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid], Ascorbic acid, Catechol, Pargyline

Neurotransmitter receptor antagonists

Phentolamine, Prazosin, Yohimbine, Propranolol

Chemicals for electrophoresis

Dithiothreitol (DTT), Sodium Dodecyl Sulphate(SDS), β mercaptoethanol,

Coomassie blue R-250, Standard marker proteins (Myosin-205KD, β-Galactosidase-116KD, Phosphorylase-97.4KD, Bovine albumin-66KD, Egg albumin-45KD, Carbonic anhydrase-29KD)

Culture Media and chemicals

Hank's Balanced Salt solution (HBSS), Collagen from rat-tail, William's medium E, Fetal Calf Serum, Insulin, Collagenase type IV

Other Biochemicals

Histone III S, Phosphatidyl serine (PS), Diolein, 4β-phorbol 12β-myristate 13α-acetate (PMA).

The following are kind gifts from JANSSEN LABORATORIES, Belgium

Spiperone and Ketanserin

RADIOCHEMICALS PURCHASED FROM AMERSHAM ENGLAND

1-[7,8-^{3}H]Noradrenaline, specific activity 39.0 Ci/mmol.

[O-methyl-^{3}H]Yohimbine, specific activity 88 Ci/mmol.

5-Hydroxy (G-^{3}H]Tryptamine creatinine sulphate, specific activity 18.4 Ci/mmol.

Furanyl-5-[^{3}H] Prazosin; specific activity 27 Ci/mmol.

RADIOCHEMICALS FROM BHABHA ATOMIC RESEARCH CENTRE (BARC), BOMBAY

[^{3}H] Thymidine (sp.acivity 18Ci/m mole), [γ-^{32}P-ATP] (specific activity 3000Ci/mmole).

ANIMALS

Adult male Sprague Dawley rats weighing 200-300g were used for all experiments. They were fed lab chow and water *ad libitum* and maintained in 12hr light and 12 hr dark cycle.

PARTIAL HEPATECTOMY AND SACRIFICE

Two-thirds of the liver constituting the median and left lateral lobes were surgically excised under light ether anaesthesia according to the method of Higgins and Anderson (1931). Sham operations involved median excision of the bodywall followed by all manipulations except removal of the lobes. All the surgeries were done between 7 and 9 A.M to avoid diurnal variations in responses. After various intervals of surgery the animals were sacrificed by decapitation and the liver was dissected and stored at -70 ^{0}C after immediate freezing in liquid nitrogen.. The brains were rapidly dissected into different regions according to Glowinski and Iversen (1966). The dissection was carried out on a chilled glass plate into hypothalamus (Hypo) , brain stem (BS), and cerebral cortex (CC). These regions were immediately immersed into liquid nitrogen and stored at -70° C for various experiments.

MEASUREMENT OF DNA SYNTHESIS IN LIVER

10 μCi of [^{3}H] Thymidine (BARC, Bombay) (sp.acivity 18Ci/m mole) was injected intraperitoneally into partially hepatectomised rats to study DNA synthesis at 18, 24, 30, 48, 72hrs and 7 days of liver regeneration. [^{3}H] Thymidine was injected 2hrs before sacrifice. DNA was extracted from liver according to Schneider (1957). A 10% Trichloroacetic acid (TCA) homogenate was made lipid free and DNA was extracted from the lipid free residue by heating with 5% TCA at 90 ^{0}C for 15 min. DNA was estimated by diphenylamine method (Burton, 1956). DNA extract was counted in WALLAC (1409)

liquid scintillation counter (LSC) after adding cocktail T containing Triton-X 100. The amount of DNA synthesised was expressed as dpm/mg DNA .

ASSAY OF NEUROTRANSMITTERS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

Brain monoamine concentrations

The monoamines were assayed according to Paulose et al,(1988). Tissues from brain regions were homogenised in 0.4N icecold perchloric acid. The homogenate was centrifuged at 5000xg for 10 minutes at 4^{0}C in Kuboto refrigerated centrifuge and the clear supernatant was filtered through 0.45μm filters and used for HPLC analysis. Norepinephrine (NE), Epinephrine (EPI), 4-Hydroxy-3-methoxy-Phenyl-Glycol (MHPG), 5-Hydroxytryptamine (5-HT), 5-Hydroxytryptophan (5-HTP) and 5-Hydroxyindoleaceticacid (5-HIAA) were determined with High Performance Liquid Chromatography (HPLC) with electrochemical detector (EC) (Shimadzu, Japan) fitted with CLC-ODS reverse phase column of 5 μm particle size, 4.6 mm internal diameter and 25 cm length. The mobile phase consisted of 75 mM sodium dihydrogen orthophosphate, 1 mM sodium octyl sulfonate, 50 mM EDTA and 7% acetonitrile. The pH was adjusted to 3.45 with phosphoric acid, filtered through 0.45 μm filters (Millipore) and deaerated. A Shimadzu model 10AS pump was used to deliver the solvent at a rate of 1 ml/min. The catecholamines were identified by an amperometric detection using an electrochemical detector (Model 6A, Shimadzu, Japan) with a reduction potential of 0.8V, with the range set at 16 and a time constant of 1.5 seconds. Twenty μl aliquots of the acidified supernatant were injected into the system. The peaks were identified by relative retention times compared with standards and quantitatively estimated using an integrator interfaced with the detector. Data from different brain regions during various times of liver regeneration were statistically analysed and tabulated.

Analysis of circulating catecholamines

Plasma catecholamines were extracted from 1ml of plasma and diluted twice with distilled water. To it 50 μl of 5mM sodium bisulphite was added , followed by 250 μl of 1M Tris buffer, pH 8.6. Acid alumina (20mg) was added, shaken in the cold for 20 minutes and was washed with 5mM sodium bisulphite. Catecholamines were extracted from the final pellet of alumina with 0.1 N perchloric acid, mixed well and and 20 μl of filtered sample was analysed for catecholamines as described before.

NEUROTRANSMITTER RECEPTOR STUDIES USING [³H] RADIOLIGANDS

ADRENERGIC RECEPTORS OF BRAIN : ALPHA-1 AND ALPHA-2 ADRENERGIC RECEPTORS

The binding studies were done according to Repaske *et al,*(1987) with slight modifications.

Preparation of the rat brain particulate fraction for alpha-1 and alpha-2 adrenergic receptor binding studies

The tissues used for assay include hypothalamus brain stem and cerebral cortex. The brain tissues were disrupted using a tight fitting teflon glass homogeniser by 10 up and down strokes with on ice for 30 sec. The buffer contained 50 mM Tris HCl, 4 mM $MgCl_2$, 2 mM EGTA, pH 7.6, 10 mM benzamidine and 5 mM phenylmethyl sulfonyl fluoride. The homogenate was centrifuged at 45,000 g in a RP21 rotor in a Hitachi SCP 85 ultracentrifuge for 20 minutes at 4°C. The pellet was washed by resuspension and centrifugation in the above buffer. The final pellet was resuspended in buffer containing 25 mM glycylglycine, 10 mM HEPES, 100 mM NaCl, 2 mM EGTA pH 7.6 and used for determination of [³H]Yohimbine binding. For [³H] Prazosin binding, 50 mM Tris-HCl buffer, containing 10 mM $MgCl_2$, 1 mM EGTA, 0.8 mM ascorbic acid, and 3 mM

catechol, pH 7.4, was used for resuspending the final pellet. Membrane protein was assayed according to the method Lowry *et al*, (1951). Protein concentration of the range of 0.30-0.35 mg were used for the receptor assays in CC., 0.25-0.3 mg for BS and 0.15-0.20 mg for Hypo.

DETERMINATION OF [^{3}H] PRAZOSIN BINDING IN RAT BRAIN PARTICULATE PREPARATIONS

Membrane binding assays were performed in 0.5 ml incubations containing appropriate protein concentrations of particulate preparation of Hypo or BS or CC, 50 mM Tris-HCl, 10 mM MgCl$_2$, 1 mM EGTA, 0.8 mM ascorbic acid, 3 mM catechol, pH 7.4, 0.03 to 3 nM [^{3}H] prazosin with and without 10 μM phentolamine to determine non specific binding. After incubation at 25°C for 30 minutes, the contents of the tubes were rapidly filtered through Whatmann GF/C filters and washed with 10 ml of ice cold buffer containing 50 mM Tris HCl and 10 mM MgCl$_2$, pH 7.4. [^{3}H] prazosin bound to the membranes in the filter was determined by liquid scintillation spectrometry in Wallac LSC

DETERMINATION OF [^{3}H] YOHIMBINE BINDING IN BRAIN PARTICULATE PREPARATIONS

Membrane binding assays were performed in 0.5 ml final incubation volume containing appropriate concentrations of protein of each brain regions. The incubation buffer contained 25 mM glycyl glycine, 10 mM HEPES, 100 mM NaCl, 2mM EGTA, pH 7.6. The concentration of radioligand particulate preparation used varied from 1 to 12.5 nM [^{3}H]Yohimbine with and without 10 μM phentolamine to determine non specific binding and incubated for 90 minutes at 15°C. The incubation was terminated by rapid filtration through GF/C glass fibre filters (Whatmann) using vacuum filtration manifold (Millipore, model 1225) with three washes of 5 ml each of ice cold 25 mM glycylglycine pH 7.6. Hisafe cocktail (Pharmacia) was added to dried filters and kept overnight. The samples were counted in LSC (Wallac)

SEROTONERGIC RECEPTORS OF THE BRAIN

The binding assays were done according to the method of Paulose *et al*, (1985)

Preparation of the rat brain particulate fraction for serotonergic receptor binding studies

The brain regions were homogenised in 50 mM Tris-HCl buffer containing 1 mM PMSF and 1 μM pargyline(pH 8.5) and centrifuged at 45,000xg for 20 minutes. The pellet was washed by recentrifugation and resuspended in the above buffer. The protein concentrations of each brain region was chosen appropriately as described before.

DETERMINATION OF [^{3}H] SEROTONIN BINDING IN RAT BRAIN PARTICULATE PREPARATIONS

Membrane binding assays were performed in 0.5 ml incubations containing the particulate fraction of each brain region, 50 mM Tris-HCl buffer containing 1 mM PMSF and 1 μM pargyline (pH 8.5) and 1 to 12.5nM [^{3}H] 5-HT, with and without excess cold (10 μM)5-HT. The incubation was done at 37^{0}C for 15 minutes. Incubations were stopped by rapid filtration through GF/B filters (Whatmann) with three washes each of 5 ml ice cold 50 mM Tris-HCl pH 8.5. [^{3}H] 5-HT binding to the membranes in the filter was determined by liquid scintillation spectrometry in Wallac LSC

ADRENERGIC AND SEROTONERGIC RECEPTORS OF THE LIVER

Preparation of the rat liver particulate fraction for adrenergic and serotonergic receptor binding studies

Control and regenerating liver were homogenised in 50 volumes of 50mM Tris buffer pH 7.5, containing 0.25M sucrose, 2mM EGTA (TES buffer) and 100μM PMSF. The homogenate was centrifuged at 45,000 x g in a RP 21 rotor in a HITACHI SCP85

ultracentifuge for 30 minutes at 4^0C. The procedure was repeated and the pellet was resuspended in incubation buffer. Protein was estimated method of Lowry *et al,* (1951)

ADRENERGIC RECEPTORS OF THE LIVER

The binding studies were done according Geynet *et al,*(1981) with slight modifications.

DISPLACEMENT ANALYSIS USING [^{3}H] NE USING PROPRANOLOL, PRAZOSIN AND YOHIMBINE

The cold antagonists used for competition binding assays with [^{3}H] NE were propranolol for β adrenergic receptors, prazosin, for α_1 adrenergic and yohimbine for α_2 adrenergic receptors. The incubation buffer contained 50mM Tris-HCl, 10mM MgCl$_2$, 1mM EGTA, 0.8mM ascorbic acid, 3mM catechol, pH 7.4. The assay mixture (0.5ml) contained 0.30-0.33 mg of protein, 50nM of [^{3}H]NE and 10^{-9} to 10^{-3}M of various competing antagonists -prazosin, yohimbine and propranolol in the incubation buffer. The mixture was incubated for 30 minutes at 25^0C. The reaction was stopped by filtering immediately through Whatmann GF/C filters with three washes of buffer containing 50 mM Tris Buffer containing 10mM MgCl$_2$, pH7.4. The filters were dried overnight and counted in Hisafe liquid scintillation cocktail and counted in Wallac 1409 LSC.

ALPHA-1 ADRENERGIC RECEPTORS OF THE LIVER

Alpha-1 adrenergic receptors of the liver were assayed according to the method of Lynch *et al,* (1985)

Determination Of [^{3}H] Prazosin Binding In Particulate Fraction Of Rat Liver

The incubation mixture (0.5ml) contained 50mM Tris-HCl, 10mM MgCl$_2$, 1mM EGTA, 0.8mM ascorbic acid, 3mM catechol, pH 7.4, 0.30 to 0.33 mg protein and concentration of [^{3}H] Prazosin from 0.03nM-3nM. Specifc binding was defined as the difference in radioligand binding in the absence and presence of 10 μM phentolamine. Following a 30 minute incubation at 25^{0}C, samples were rapidly filtered under vacuum through Whatmann GF/C filters by washing with 10 ml of ice-cold buffer containing Tris-HCl buffer, 10 mM MgCl$_2$ pH 7.4. The dried filters were counted in LSC after adding Hi Safe scintillation cocktail.

Displacement of [^{3}H] Prazosin by NE: Effect of GTP analog

Displacement of [^{3}H] Prazosin by cold -(-) NE in the concentration range of 10^{-9} to 10^{-4}M was done with and without 0.1mM GppNHp, basically according to the method described for [^{3}H] NE displacement analysis.

SEROTONERGIC RECEPTORS OF THE LIVER

The serotonergic receptors of the liver were assayed according to the modified procedure of Paulose et al,(1985).

DETERMINATION OF [^{3}H] SEROTONIN BINDING IN PARTICULATE FRACTION OF RAT LIVER

The assay mixture (0.5 ml) contained 50mM Tris-HCl Buffer containg 1μM pargyline ^pH8.5, 0.30-0.33 mg protein, 1nM to 12.5 nM of [^{3}H]serotonin with and without 10 μM of cold serotonin to determine the total and non-specific binding. After

incubation at 37°C for 15 minutes, the contents of the tubes were filtered through Whatmann GF/C filter and washed with 10ml of ice cold Tris-HCl Buffer pH 8.5. The filters were dried and counted in Hi Safe cocktail using LSC.

DISPLACEMENT OF [^{3}H] SEROTONIN WITH SEROTONIN, KETANSERIN AND SPIPERONE.

Serotonergic receptor subtypes were analysed using competing cold ligands including serotonin and the 5HT-2 receptor antagonists ketanserin and spiperone. 10^{-9}M to 10^{-3}M concentrations of the drug were used to perform competitive binding studies with [^{3}H] serotonin..

ANALYSIS OF THE RECEPTOR BINDING DATA

The data was analysed according to Scatchard (1949). The data included both total and nonspecific binding at many concentrations of radioligand and the specific binding was calculated as the difference. Two binding parameters maximal binding (Bmax) and equilibrium dissociation constant (Kd) were derived by linear regression analysis by plotting the specific binding of the radioligand on x axis and bound / free on y axis. This is called a scatchard plot. The maximal binding (B_{max}), which is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant (Kd) of the affinity of the receptors for the radioligand. The Kd is inversely related to receptor affinity or the "strength" of binding.

Displacement Curve analysis

The data of the competitive binding assays are represented graphically with the negative log of concentration of the competing drug on x axis and % of the radioligand bound on the y axis.

36

ISOLATION OF RAT HEPATOCYTES AND PRIMARY CULTURE .

Buffers used for perfusion

A. Ca $^{2+}$-free perfusion buffer : This buffer contained 142 mM NaCl, 6.7 mM KCl, 10 mM HEPES, and 5.5 mM NaOH, pH 7.4. It was made up in sterile triple distilled water and filtered through 0.22 μm filters (Millipore).

B. Collagenase Buffer : This buffer contained 67 mM NaCl, 6.7 mM Kcl, 100 mM HEPES, 4.76 mM CaCl$_2$.2H$_2$O, and 66 mM NaOH pH 7.6. It was made up in sterile triple distilled water. Collagenase type IV (0.05%) (Sigma) was added prior to perfusion and filtered through 0.22 μm filters(Millipore).

Collagen-coating of culture dishes

Sterile rat-tail collagen solution (100μg/ml in 0.1% acetic acid) was added to each 35mm culture dish and spread uniformly. After 2 hrs, the unattached collagen was aspirated out and the dishes were washed thrice with sterile phosphate buffered saline. Finally, sterile Earl's Balanced Salt solution (EBSS, Sigma) was added and the dishes were left in the sterile hood till the seeding of cells.

PROCEDURE FOR HEPATOCYTE CULTURE

Hepatocytes were isolated from adult male Sprague Dawley rats by collagenase perfusion based on the method of Seglen, (1976). The liver was perfused *in situ* with the calcium -free HEPES buffer pH 7.4 and then with Ca $^{2+}$ containing collagenase buffer (pH 7.6). Hepatocytes were dispersed from the perfused liver, filtered through nylon mesh and washed by three centrifugations in EBSS. The final cell pellet was resuspended in William's Medium E. Cell viability was tested by trypan blue exclusion. The hepatocyte preparation having a viability of >90% as assessed by trypan blue exclusion

were chosen for culture. Hepatocytes were plated on rat tail collagen coated dishes at a density of 10^6 cells/ 35mm culture dish in 1ml of William's medium E. Cells were allowed to settle and adhere for three hours in medium supplemented with 10% Fetal Calf Serum, 10^{-7} M Insulin and 50µg/ml gentamycin sulphate. After that the plating media was replaced by serum- free media containing 10ng/ml EGF and 2.5 µCi of $[^3H]$ Thymidine/plate. NE (10^{-8} to 10^{-3}M) , 5-HT(10^{-8} to 10^{-3}M) , prazosin (5×10^{-8}M, 10^{-6}M), propranolol (5×10^{-8}M), yohimbine (5×10^{-8}M), ketanserin (5×10^{-8}M, 10^{-6}M) and spiperone (5×10^{-8}M, 10^{-6}M) were added in the different experiments. The cultures were incubated for 48 hrs at 37^0C in 5% CO_2.

DNA synthesis assays in cultured hepatocytes

DNA synthesis assay was done according to Takai *et al* (1988). After 48 hours of incubation, the cells were washed twice in the cold PBS and cold 10% TCA was added. The hepatocytes were solubilized by incubation at 37^0C for 30 minutes in 1N NaOH and cold 100% TCA was added to the solution to get a final concentration of 15% to precipitate the macromolecules. Then DNA hydrolysed by heating the precipitate at 90^0C for 15 minutes in 10% TCA. DNA synthesis was expressed as dpm of $[^3H]$ thymidine incorporated /mg protein.

PROTEIN KINASE-C (PKC) ASSAY IN CONTROL AND REGENERATING LIVER

Preparation of particulate and cytosolic fractions of rat liver

Control and regenerating liver were homogenised in 20 mM Tris/HCl at pH 7.5 containing 2 mM EDTA and 10 mM EGTA to obtain a 10% homogenate. The

homogenates were centrifuged at 30,000xg for 60 minutes at 4^0C and the supernatant and particulate fractions were used for the enzyme assay.

Enzyme Assays

Protein kinase C assay was done based on the method of Kikkawa *et al*,(1982). Protein kinase-C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}PATP] {3000Ci/mmole} into Histone-III S from calf thymus. The reaction mixture (0.25ml)contained 5μmol of Tris/HCl at pH 7.5, 1.25μmol of magnesium acetate, 50 μg of Histone III S, 2.5 μM to 20 μM [γ-^{32}PATP] (5-10x10^4 cpm/nmol), 10 μg phosphatidyl serine (PS), 0.2 μg of diolein, 125 nmol of $CaCl_2$ and the enzyme preparation to be assayed (40-50 μg protein). Phospholipid and diolein were mixed first in a small volume of chloroform. After chloroform was removed under N_2, the residue was resuspended in 20 mM Tris/HCl, pH 7.5 by sonication and then added to the reaction mixture. After incubation at 30^0C for 3 minutes, the reaction was stopped by adding 25% TCA. The acid precipitible materials were collected by filtration on 0.45 μm membrane filters (Millipore). The filters were dried and counted in LSC with Hi Safe scintillation cocktail. Basal activity which was obtained in the presence of 0.5mM EGTA instead of PS, diolein and $CaCl_2$ was subtracted from the experimental values. PKC activity was expressed as pmoles of ^{32}P transferred per mg protein/minute. Protein was assayed according to Lowry *et al*,(1951).

PROTEIN PHOSPHORYLATION IN CONTROL AND REGENERATING LIVER

Phosphorylation assays in the particulate fraction of control and regenerating liver were done by a modified procedure of Jaiswal *et al*,(1996). For assay of PKC-dependent protein phosphorylation, the reaction mixture (50 μl final volume) consisted of 20 mM Tris/HCl, pH7.5, 5 mM $MgCl_2.6H_2O$, 10 mM DTT, 0.5mM $CaCl_2.2H_2O$ and 10μg PS, and 50 μg protein of particulate preparation. The endogenous, non-specific

phosphorylation was studied in the presence of 2mM EGTA instead of $CaCl_2$ and PS. The compounds, {10^{-5}M concentration each of NE, 5-HT, prazosin, ketanserin and the tumor promoting agent, 4β-phorbol 12β-myristate 13α-acetate (PMA) } were added appropriately to the reaction mixture to study their effect on PKC dependent phosphorylation. After incubation with the various drugs at 0^0 C for 15 minutes, the reaction was started by adding 10 μM ATP containing 1μCi of [γ-^{32}PATP} and further incubated at 30^0C for 2 minutes. The reaction was terminated by adding 15 μl of 4X stop buffer (250 mM Tris/HCl pH 6.8, 8% SDS, 40% glycerol, 20% β mercaptoethanol). The samples were transferred to a boiling water bath for 2 minutes. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel by Laemmli's method (1970). Standard marker proteins were simultaneously electrophoresed. The gels were stained with Coomassie blue R-250, dried on a gel drier (Hoeffer) and autoradiographed on Kodak X-ray films with intensifying screens ,at -70^0C for 24 hours.

STATISTICAL CALCULATIONS.

Results are presented as Mean ± S.E.M. Statistical comparisons were performed by Student's t-test and ANOVA with p≮0.05 being taken as the level of significance (Campbell, 1987).

Results

RESULTS

DNA SYNTHESIS IN THE REGENERATING LIVER .

Tritiated thymidine incorporation into replicating DNA was used as a biochemical index for quantifying liver regeneration. DNA synthesis was negligible in the liver of sham-operated animals. There was a significant increase (p<0.05) in [^{3}H] thymidine incorporation at 18 hours after partial hepatectomy. DNA synthesis peaked at 24 hours after hepatectomy. Elevated levels of DNA synthesis persisted at 30 hours and 48 hours of liver regeneration. DNA synthesis decreased to basal levels by 7 days post hepatectomy (Fig 1)

ADRENERGIC AND SEROTONERGIC RECEPTOR ALTERATIONS IN BRAIN REGIONS AFTER PH.

Adrenergic alterations.

Catecholamine content and turn over.

In the hypothalamus, the levels of NE was significantly higher (p<0.05) at 24 hours and 48 hours of liver regeneration, compared to sham operated animals. The turnover ratio of MHPG/NE was also higher (p<0.05) during this period. While there was no change in the EPI levels, the EPI/NE levels were significantly lower (p< 0.05) at 48 hours of regeneration (Table-1). In the cerebral cortex, NE levels were significantly higher (p<0.05) at 24 hours and 48 hours and the MHPG/NE turnover ratio was also significantly higher (p<0.05) compared to control. The EPI/NE turnover was significantly lower (p<0.05) at 24 and 48 hours of liver regeneration (Table 2). In the brain stem, NE and EPI content was significantly higher (p<0.05) at 24 hours and 48 hours after PH compared to sham-operated animals. The EPI/NE turnover was also higher (p< 0.05) (Table-3).

41

Alpha - Adrenergic receptor kinetics

α_1 adrenergic receptors of the brain regions were assayed by (^{3}H) prazosin binding. α_1 adrenergic receptors of the hypothalamus had a significantly lower maximal binding capacity, (B_{max}) ($p< 0.05$) at 24 hours of regeneration. There was no change in the dissociation constant, Kd (Fig. 2, Table 4). The α_2 adrenergic receptors of the hypothalamus as assayed by (^{3}H) yohimbine binding showed increased B_{max} ($p<0.05$) with no change in Kd at 24 hours of liver regeneration compared to control. (Fig 3, Table 4). The α_1 adrenergic receptors of the cerebral cortex showed no change in B_{max} and a higher Kd ($p< 0.05$), (Fig.4, Table 5) while the α_2 adrenergic receptors exhibited increase in B_{max} and lowered Kd ($p<0.05$) (Fig.5, Table 5). In the brain stem, $\alpha_1$adrenergic receptors showed significantly higher B_{max} and Kd ($p<0.05$) (Fig.6, Table 6). The α_2 adrenergic receptors showed no change in B_{max} and a had significantly higher Kd ($p< 0.05$)(Fig.7, Table-6).

Serotonergic alterations

Serotonin content and turn over

In the hypothalamus 5-HT was significantly higher ($p<0.05$) at 24 and 48 hours of regeneration with a decrease of the turnover of 5-HIAA/5-HT compared to control (Table-7). In the cerebral cortex, 5-HT content was significantly higher ($p<0.05$) and the turnover of 5-HIAA/5-HT was significantly lower ($p<0.05$) at 24 hours of regeneration compared to control. The increase of 5-HT was reversed in 48 hours with a significant increase in the HIAA/5 HT ratio. By 72 hours, the 5 HT turnover was similar to control levels (Table-8). In the brain stem, 5-HT content was significantly higher ($p<0.05$) at 24 hours of regeneration. The turnover HIAA/5-HT and 5HTP/5-HT was also higher which was reversed to control values by 48 hours (Table-9).

Serotonergic receptor kinetics

High affinity serotonin receptors were studied by [^{3}H] serotonin binding. In the hypothalamus the maximal binding, B_{max} was significantly lower (p<0.05) with no change in Kd, at 24 hours of liver regeneration (Fig. 8, Table-4). The B_{max} of [^{3}H] serotonin was significantly lower (p<0.05) in the cerebral cortex with no change in Kd (Fig.9, Table-5). In the Brain stem, B_{max} was significantly higher (p<0.05) at 24 hours of liver regeneration compared to control. There was no change in the Kd (Fig.10,Table-6).

CHANGES IN CIRCULATING CATECHOLAMINE LEVELS

Norepinephrine levels in plasma were significantly higher (p<0.05) in hepatectomised rats after 24 and 48 hours of PH, compared to sham-operated animals. At 72 hours after PH the levels of circulating NE was comparable to that of control rats (Table-10).

CHANGES IN HEPATIC ADRENERGIC AND SEROTONERGIC RECEPTORS DURING LIVER REGENERATION

Adrenergic Receptors

Displacement analysis

The adrenergic receptors on the liver membranes were studied by displacement of [^{3}H] NE by cold adrenergic antagonists, prazosin, propranolol and yohimbine. Prazosin caused a greater displacement of [^{3}H] NE in the low affinity concentration range in the 24 hour regenerating liver compared to control (Fig.11). Propranolol displaced [^{3}H] NE from the adrenergic receptors in the high and low affinity concentration range, in the regenerating liver at 24 hours after PH (Fig.12). In the control liver, yohimbine hardly displaced [^{3}H] NE from adrenergic receptors. However, the degree of displacement of

[^{3}H] NE by yohimbine was higher in the 24 hour regenerating liver (Fig.13). The α_1 adrenergic receptors were studied by scatchard analysis of [^{3}H] prazosin binding to hepatic membranes, during 24 hours of liver regeneration.

α_1 adrenergic receptors

There was a significant decrease (p<0.05) in the maximal binding (B$_{max}$) of [^{3}H] prazosin during the DNA-synthetic phase of liver regeneration. The dissosciation constant Kd did not show any significant change(Table-11, Fig.14). Displacement of [^{3}H] Prazosin by - (-) NE caused a greater displacement of α_1 adrenergic receptors in the high affinity concentration range. When the non-hydrolysable GTP analog, G$_{pp}$ NH$_p$ was present, the displacement curve in control and regenerating liver shifted towards the low affinity concentration range (Fig.15).

Serotonergic Receptors

[^{3}H] 5-HT binding kinetics

Scatchard analysis of [^{3}H] 5-HT binding to liver membranes showed that the B$_{max}$ of serotonergic receptors increased significantly (p<0.05) during the replicative phase. K$_d$ of the serotonin receptors for [^{3}H] 5-HT was significantly higher (p<0.05) in the regenerating liver compared to control (Fig.16, Table-11).

Displacement studies

Cold serotonin caused a significant displacement of [^{3}H] 5-HT in the regenerating liver during the DNA synthetic phase (Fig.17). Ketanserin caused a marked displacement of [^{3}H] 5-HT from its receptors during 24 hours of regeneration compared to control in all concentrations tested (Fig 18). Spiperone displaced [^{3}H] serotonin in the high affinity and

44

low affinity range of concentrations used, during the DNA synthesis phase (Fig 19). Both antagonists caused a shift of the displacement curve to the high affinity concentration range.

DNA SYNTHESIS IN PRIMARY CULTURES OF RAT HEPATOCYTES:

Effect of NE and 5-HT on cultured hepatocytes

Isolated hepatocytes in serum-free culture medium exhibited very low levels of [^{3}H] thymidine incorporation into DNA. Addition of EGF caused a significant increase (p<0.05) in the hepatocyte DNA synthesis. When 5-HT (50μM) or NE (50μM) alone were added to cultured hepatocytes there was no significant increase in the DNA synthesis from basal level. However, addition of 5-HT (1μM) or NE (1μM) to hepatocyte cultures, in presence of EGF and insulin caused a significant increase (p<0.05) in DNA synthesis compared to basal levels and EGF-treated cultures (Table-12, Fig.20).

Dose response of hepatocyte DNA synthesis to NE.

Different doses of NE (5x10^{-9} M to 10^{-4}M) were added to cultured hepatocytes in the presence of EGF (10 ng/ml) and insulin (10^{-7}M). NE at low concentration did not bring about a significant increase in DNA synthesis. A significant increase (p<0.05) was observed with 10^{-6}M NE reaching a maximal effect at 5x10^{-5}M (Fig. 21.)

Effect of adrenergic receptor antagonists on NE-induced DNA synthesis *in vitro*

Adrenergic receptor blockers, prazosin, propranolol and yohimbine (50 nM each) were added to hepatocyte cultures in the presence of EGF (10 ng/ml) and NE (50 nM). Addition of 50 nM NE to EGF treated cultures did not produce a significant elevation of DNA synthesis. However, when 50 nM of prazosin was added, there was a significant reduction (p<0.05) in DNA synthesis. Propranolol and yohimbine did not cause a

significant reduction in DNA synthesis compared to EGF and NE treated cultures. Prazosin (1 μM) was also tested for its inhibition of DNA synthesis elicited by higher concentrations of NE. 50μ M NE, when added to cultures in the presence of EGF, caused a significant increase (p<0.05) in DNA synthesis of hepatocytes than elicited by EGF alone. This increase of DNA synthesis was significantly reduced (p<0.05) when 1 μM prazosin was added to the cultures (Table-13, Fig.22, Fig.25).

Dose dependent response of hepatocyte DNA Synthesis to 5-HT

Varying concentrations of 5-HT (5×10^{-9}M to 10^{-4} M) were added to primary cultures of rat hepatocytes in the presence of rat hepatocytes of fixed concentrations of EGF (10 ng/ml) and insulin (10^{-7}M). Lower concentrations of 5-HT did not cause any significant change in the DNA synthesis compared to EGF-treated cultures alone. However, 10^{-6}M of 5-HT caused a significant increase (p<0.05) in DNA synthesis compared to the EGF induced DNA synthesis. The maximal effect of 5-HT was observed at 5×10^{-5}M of the monoamine (Fig.23)

Effect of serotonergic receptor antagonists on hepatocyte DNA synthesis *in vitro*

Serotonergic receptor blockers ketanserin and spiperone were examined for their ability to block the stimulation of DNA synthesis induced by 5-HT. A concentration of 50nM of 5-HT did not cause a significant increase in the DNA synthesis induced by EGF. Ketanserin (50 nM) inhibited the increase of EGF induced DNA synthesis significantly (p<0.05). Spiperone (50 nM) did not bring about significant reduction in EGF-induced DNA synthesis. A significant (p<0.05) increase of DNA synthesis was elicited by 50 μM 5-HT in the presence of EGF and 50μ M of ketanserin and spiperone caused a significant reduction (p<0.05) in this observed increase (Table 14, Fig .24, Fig.25)

Effect of Epinephrine (EPI) on hepatocyte DNA synthesis

Addition of epinephrine (50 μM) to primary cultures of rat hepatocytes in the presence of EGF (10 ng/ml) caused a significant increase (p<0.05) in DNA synthesis (Table-15).

PROTEIN KINASE C ASSAY IN THE REGENERATING LIVER

Protein Kinase C activity in crude membrane and cytosolic fractions were assayed in the control and in the 24 hour regenerating rat liver. The maximal velocity V_{max} of PKC increased (p<0.05) in membrane fraction of regenerating liver with no change in K_m (Michelis-Menten constant). There was no significant change in the V_{max} and K_m of the enzyme in the cytosolic fraction of the regenerating liver, compared to control (TABLE-16).

NE AND 5-HT INDUCED MEMBRANE PROTEIN PHOSPHORYLATION IN THE 24-HOUR REGENERATING LIVER

The membrane proteins phosphorylated by NE and 5-HT in a PKC-dependent manner was studied in crude liver membrane preparations of the control and regenerating liver. Endogenous PKC-dependent phosphorylation was higher in the 24-hour regenerating liver membrane compared to control. Specifically, there was an enhanced phosphorylation of 29KD and 40KD membrane proteins in the regenerating liver compared to the control. Additional phosphorylation of a 50KD protein and a 59KD protein was seen in the regenerating liver membrane (Plate-2). In the 5-HT treated membrane fraction, the 40KD protein band showed an enhanced phosphorylation compared to the control membrane. While the 50KD protein phosphorylation was slightly higher in the regenerating liver membrane compared to control, the phosphorylation of 59KD protein was markedly enhanced. When Ketanserin was added along with 5-HT, there was a conspicuous decrease in the phosphorylation of the 40KD protein in the control and regenerating liver membrane. The 50KD protein phosphorylation was

eliminated and the 59KD protein phosphorylation decreased proportionately in the control and regenerating membrane fractions (Plate-2). In the membrane fraction incubated with NE, the 29KD and 40KD membrane proteins showed an enhanced phosphorylation in the regenerating liver compared to control. In addition, there was an additional phosphorylation of a 59KD protein in the NE-treated regenerating liver membrane fraction. When prazosin was incubated along with NE, the 29KD protein phosphorylation was markedly reduced in the control and regenerating liver membrane. The 40KD and 59KD protein phosphorylations were eliminated by prazosin treatment in the control and regenerating liver membrane. Incubation of liver membrane fractions with the tumor promoter PMA, showed that the 59KD membrane protein was phosphorylated only in the control liver and the phosphorylation of the 29KD protein was also enhanced in the contol liver compared to the regenerating liver (Plate 3).

TABLE-1

NOREPINEPHRINE CONTENT AND TURNOVER RATIO IN HYPOTHALAMUS OF SHAM-OPERATED AND HEPATECTOMISED RATS (nanomoles/gram wet weight)

Animal status	Hrs after PH	MHPG	NE	EPI	MHPG/NE	EPI/NE
Sham-operated	-	1.50 ± 0.36	1.19 ± 0.32	0.37 ± 0.04	1.98 ± 0.39	0.38 ± 0.07
	24	36.02 ± 5.20	3.45 ± 0.85*	0.33 ± 0.07	10.47 ± 1.60*	0.24 ± 0.05
Hepatectomised	48	251.00 ± 31.00	13.19 ± 1.45*	0.30 ± 0.04	18.88 ± 0.71*	0.02 ± 0.01*
	72	1.44 ± 0.05	0.89 ± 0.10	0.34 ± 0.10	1.63 ± 0.13	0.40 ± 0.04

*$p < 0.05$ compared to control   (Values are Mean ± S.D. of 4-6 separate determinations.)

TABLE-2

NOREPINEPHRINE CONTENT AND TURNOVER RATIO IN CEREBRAL CORTEX OF SHAM-OPERATED AND HEPATECTOMISED RATS (nanomoles/gram wet weight)

Animal status	Hrs after PH	MHPG	NE	EPI	MHPG/NE	EPI/NE
Sham-operated	-	0.25 ± 0.07	0.39 ± 0.09	0.14 ± 0.05	0.70 ± 0.15	0.51 ± 0.09
	24	2.46 ± 0.23	$0.97 \pm 0.09^*$	0.14 ± 0.09	$2.37 \pm 0.17^*$	$0.15 \pm 0.01^*$
Hepatectomised	48	1.71 ± 0.02	$0.81 \pm 0.12^*$	0.14 ± 0.01	$1.88 \pm 0.05^*$	$0.16 \pm 0.05^*$
	72	0.31 ± 0.02	0.43 ± 0.08	0.17 ± 0.04	0.80 ± 0.09	0.41 ± 0.07

*$p<0.05$ compared to control   (Values are Mean ± S.D.of 4-6 separate determinations.)

TABLE-3

NOREPINEPHRINE CONTENT AND TURNOVER RATIO IN BRAIN STEM OF SHAM-OPERATED AND HEPATECTOMISED RATS (nanomoles/gram wet weight)

Animal status	Hrs after PH	MHPG	NE	EPI	MHPG/NE	EPI/NE
Sham-operated	-	16.56 ± 0.90	1.06 ± 0.14	0.15 ± 0.06	15.96 ± 1.37	0.15 ± 0.05
	24	32.31 ± 1.09	2.41 ± 0.12*	2.91 ± 0.13*	14.29 ± 0.63	1.22 ± 0.08*
Hepatectomised	48	92.45± 2.35	6.85 ± 1.07*	2.59 ± 0.08*	14.89 ± 0.29	0.42 ±0.01*
	72	12.88 ± 0.81	1.23 ± 0.15	0.19 ± 0.01	1.85 ± 0.73	0.17 ± 0.01

*$p < 0.05$ compared to control.   (Values are Mean ± S.D. of 4-6 separate determinations)

TABLE-4

ALPHA-ADRENERGIC AND SEROTONERGIC RECEPTOR BINDING PARAMETERS IN THE HYPOTHALAMUS OF SHAM-OPERATED AND HEPATECTOMISED RATS

Animal Status	(^{3}H) PRAZOSIN		(^{3}H) YOHIMBINE		(^{3}H) SEROTONIN	
	B_{max}	Kd	B_{max}	Kd	B_{max}	Kd
Sham-operated	141 ± 4	0.20 ± 0.01	530 ± 21	12.12 ± 0.90	791 ± 12	45.16 ± 0.72
Hepatectomised	$90 \pm 3^*$	0.18 ± 0.02	$1013 \pm 97^*$	12.86 ± 2.56	$483 \pm 8^*$	46.61 ± 2.38

* $p < 0.05$ compared to control. Values are Mean $\pm$ S.D. of 4-6 separate determinations. Crude membrane preparation from hypothalamus of rats 24 hours post-hepatectomy and from sham-operated controls were used for the binding assays..

B_{max}-Binding maximum (femtomoles/mg protein), Kd-Dissociation constant (nM).

TABLE-5

ALPHA-ADRENERGIC AND SEROTONERGIC RECEPTOR BINDING PARAMETERS IN THE CEREBRAL CORTEX OF SHAM-OPERATED AND HEPATECTOMISED RATS

Animal Status	(^{3}H) PRAZOSIN		(^{3}H) YOHIMBINE		(^{3}H) SEROTONIN	
	B_{max}	Kd	B_{max}	Kd	B_{max}	Kd
Sham-operated	313 ± 8	0.23 ± 0.01	426 ± 24	8.63 ± 1.57	225 ± 21	38.03 ± 1.74
Hepatectomised	$317 \pm 6.$	$0.36 \pm 0.01^*$	$668 \pm 36^*$	8.05 ± 0.71	$126 \pm 4^*$	32.69 ± 3.95

$^*p<0.05$ compared to control. Values are Mean $\pm$ S.D. of 4-6 separate determinations. Crude membrane preparation from cerebral cortex of rats 24 hours post-hepatectomy and from sham-operated controls were used for the binding assays..

B_{max}-Binding maximum (femtomoles/mg protein), Kd-Dissosciation constant (nM).

TABLE-6

ALPHA-ADRENERGIC AND SEROTONERGIC RECEPTOR BINDING PARAMETERS IN THE BRAIN STEM OF SHAM-OPERATED AND HEPATECTOMISED RATS

Animal Status	(^{3}H) PRAZOSIN		(^{3}H) YOHIMBINE		(^{3}H) SEROTONIN	
	B_{max}	Kd	B_{max}	Kd	B_{max}	Kd
Sham-operated	261 ± 19	0.14 ± 0.02	$512. \pm 41$	11.41 ± 1.59	621 ± 14	31.25 ± 6.54
Hepatectomised	$433 \pm 30^*$	$0.25 \pm 0.05^*$	524 ± 25	$20.89 \pm 1.77^*$	$984 \pm 12^*$	34.68 ± 1.39

$^*p < 0.05$ compared to control. Values are Mean $\pm$ S.D. of 4-6 separate determinations. Crude membrane preparation from the brain stem of rats 24 hours post-hepatectomy and from sham-operated controls were used for the binding assays.

B_{max}-Binding maximum (femtomoles/mg protein), Kd-Dissosciation constant (nM).

TABLE-7

SEROTONIN CONTENT AND TURNOVER RATIO IN HYPOTHALAMUS OF SHAM-OPERATED AND HEPATECTOMISED RATS (nanomoles/gram wet weight)

Animal status	Hrs after PH	5-HTP	5-HIAA	5-HT	5-HIAA / 5-HT	5-HTP / 5-HT
Sham-operated	-	0.14 ± 0.05	0.76 ± 0.07	0.25 ± 0.02	3.08 ± 0.01	0.54 ± 0.20
Hepatectomised	24	1.24 ± 0.02	3.07 ± 0.10	$1.73 \pm 0.09^*$	$1.71 \pm 0.07^*$	0.752 ± 0.03
	48	1.15 ± 0.06	1.71 ± 0.04	$1.35 \pm 0.05^*$	$1.27 \pm 0.05^*$	0.85 ± 0.10
	72	0.08 ± 0.01	1.02 ± 0.2	0.32 ± 0.04	3.18 ± 0.15	0.24 ± 0.08

* $p < 0.05$ compared to control.   (Values are Mean $\pm$ S.D. of 4-6 separate determinations)

TABLE-8

SEROTONIN CONTENT AND TURNOVER RATIO IN CEREBRAL CORTEX OF SHAM-OPERATED AND HEPATECTOMISED RATS (nanomoles/gram wet weight)

Animal status	Hrs after PH	5-HTP	5-HIAA	5-HT	5-HIAA / 5-HT	5-HTP / 5-HT
Sham-operated	-	0.15 ± 0.02	2.56 ± 0.10	0.60 ± 0.07	2.56 ± 0.10	0.27 ± 0.02
Hepatectomised	24	0.35 ± 0.03	1.49 ± 0.10	$1.34 \pm 0.13^{*}$	$1.05 \pm 0.05^{*}$	0.29 ± 0.03
	48	0.13 ± 0.05	2.48 ± 0.21	0.41 ± 0.17	$5.01 \pm 0.90^{*}$	0.32 ± 0.01
	72	0.17 ± 0.03	2.28 ± 0.09	0.73 ± 0.10	3.61 ± 0.27	0.24 ± 0.01

*$p < 0.05$ compared to control.   (Values are Mean ± S.D. of 4-6 separate determinations)

TABLE-9

SEROTONIN CONTENT AND TURNOVER RATIO IN BRAIN STEM OF SHAM-OPERATED AND HEPATECTOMISED RATS (nanomoles/gram wet weight)

Animal status	Hrs after PH	5-HTP	5-HIAA	5-HT	5-HIAA / 5-HT	5-HTP / 5-HT
Sham-operated	-	0.43 ± 0.02	0.46 ± 0.06	0.62 ± 0.07	0.80 ± 0.05	0.70 ± 0.01
Hepatectomised	24	2.83 ± 0.30	5.09 ± 0.24	$3.02 \pm 0.20^*$	$1.68 \pm 0.03^*$	$1.08 \pm 0.04^*$
	48	0.35 ± 0.01	0.68 ± 0.21	0.49 ± 0.12	0.89 ± 0.10	0.69 ± 0.08
	72	0.29 ± 0.02	0.42 ± 0.04	0.59 ± 0.03	0.78 ± 0.05	0.59 ± 0.10

*$p < 0.05$ compared to control.   (Values are Mean $\pm$ S.D. of 4-6 separate determinations)

TABLE-10

LEVELS OF PLASMA NOREPINEPHRINE

(nanomoles/ml plasma)

Animal status	Hrs after PH	Norepinephrine levels
Sham-operated	-	0.37 ± 0.10
Hepatectomised	24	$1.80 \pm 0.30^*$
	48	$1.55 \pm 0.45^*$
	72	0.28 ± 0.02

*p<0.05 compared to control
Values are Mean $\pm$ S.E.M of 4-6 separate determinations

TABLE-11

ALPHA$_1$. ADRENERGIC AND SEROTONERGIC RECEPTOR BINDING PARAMETERS IN THE LIVER OF SHAM-HEPATECTOMISED AND PARTIALLY HEPATECTOMISED RATS

Animal Status	(^{3}H) PRAZOSIN		(^{3}H) SEROTONIN	
	B$_{max}$	Kd	B$_{max}$	Kd
Sham-operated	57 ± 3	0.14 ± 0.02	463 ± 10	5.22 ± 0.55
Hepatectomised	33 ± 2*	0.11 ± 0.03	2233 ± 58*	20.21 ± 4.70*

*p<0.05 compared to control. Values are Mean ± S.D. of 4-6 separate determinations. Crude membrane preparation from the liver of rats 24 hours post-hepatectomy and from sham-operated controls were used for the binding assays. B$_{max}$-Binding maximum (femtomoles/mg protein), Kd-Dissosciation constant (nM).

TABLE-12

EFFECT OF NE AND 5-HT ON DNA SYNTHESIS OF CULTURED HEPATOCYTES

Experiment	[3H] Thymidine incorporation	
	dpm/mg protein (x10^3)	dpm/10^6 cells(x10^3)
Medium only	23.53 ± 0.68	12.60 ± 0. 51
EGF (10ng/ml)	451.89 ± 7.80*	252.62 ± 52.64*
50μM 5-HT	27.46 ± 0.24	15.13 ± 1.95
50μM NE	26.30 ± 0.76	14.98 ± 0.19
EGF + 1μ M 5-HT	631.80 ± 0.92*	463.74± 1.23*
EGF + 1μM NE	702.38 ±0. 45*	405.22 ± 0. 93*

*P<0.05 compared to respective control
Values are Mean ± S.E.M of 4-6 separate determinations.

TABLE-13

EFFECT OF ADRENERGIC RECEPTOR ANTAGONISTS ON NE- INDUCED DNA SYNTHESIS IN HEPATOCYTE CULTURES

Experiment	[3H] Thymidine incorporation	
	dpm/mg protein (x10^3)	dpm/10^6 cells(x10^3)
Medium only	25.33 $\pm$ 0.68	12.60 $\pm$ 0. 51
EGF (10ng/ml)	451.89 $\pm$ 7.80*	252.62 $\pm$ 52.64*
EGF + 50nM NE	505.35 $\pm$ 8.55*	285.40 $\pm$ 2.75*
EGF + 50nM NE + 50nM prazosin	206.76 $\pm$ 12.16*	187.24 $\pm$ 2.51*
EGF + 50µM NE	879.51 $\pm$ 0.19*	510.59 $\pm$ 2.14*
EGF + 50µM NE + 1µM prazosin	286.92 $\pm$ 10.96*	248.94 $\pm$ 3.28*
EGF + 50nM NE + 50nM propranolol	421.90 $\pm$ 0.40	351.59 $\pm$ 14.90
EGF + 50nM NE + 50nM yohimbine	428.66 $\pm$ 19.33	353.75 $\pm$ 4.57

*$p < 0.05$ compared to respective control.
Values are mean $\pm$ S.E.M. of 4-6 separate determinations.

TABLE-14

EFFECT OF SEROTONERGIC RECEPTOR ANTAGONISTS ON 5-HT INDUCED DNA SYNTHESIS IN CULTURED HEPATOCYTES

Experiment	[3H] Thymidine incorporation	
	dpm/mg protein (x10^3)	dpm/10^6 cells(x10^3)
Medium only	23.53 ± 0.68	12.60 ± 0. 51
EGF (10ng/ml)	451.89 ± 7.80	252.62 ± 52.64
EGF + 50nM 5-HT	448.83 ± 7.74	243.41 ± 4.82
EGF + 50nM 5-HT + 50nM Ketanserin	232.66 ± 3.23*	126.37 ± 4.44*
EGF + 50nM 5-HT + 50nM spiperone	390.20 ± 15.27	216.22 ± 1.35
EGF + 50µM 5-HT	852.40 ± 9.34*	659.84 ± 6.23*
EGF + 50µM 5-HT + 1µM Ketanserine	334.17 ± 14.57*	183.90 ± 2.18*
EGF + 50µM 5-HT + 1µM Spiperone	375.69 ± 9.85*	213.57 ± 1.90*

*P<0.05 compared to respective control.
Values are mean ± S.E.M. of 4-6 separate determinations.

TABLE-15

EFFECT OF EPINEPHRINE ON DNA SYNTHESIS IN CULTURED HEPATOCYTES

Experiment	[3H] Thymidine incorporation	
	dpm/mg protein (x10^3)	dpm/10^6 cells(x10^3)
Medium only	23.53 ± 0.68	12.60 ± 0. 51
Medium + EGF(10ng/ml)	451.89 ± 7.80	252.62 ± 52.64
EGF + 50μM Epinephrine	632.90 ± 8.22*	517.71 ± 5.27*

*p<0.05 compared to control.
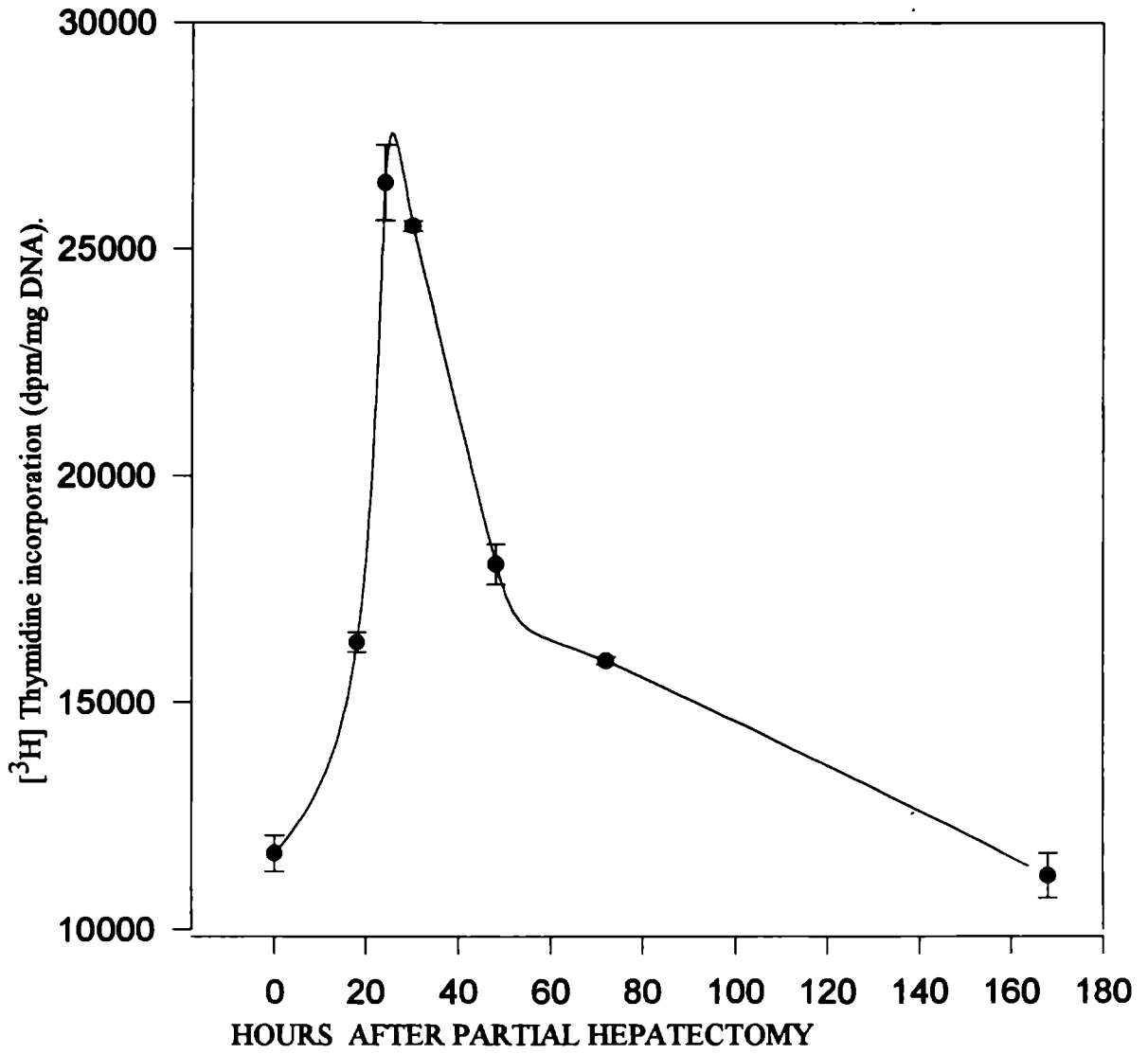Values are mean ± S.E.M. of 3-5 separate determinations.

TABLE-16

KINETIC PARAMETERSOF PROTEIN KINASE C IN

CONTROL AND REGENERATING LIVER OF RAT

	MEMBRANE FRACTION		CYTOSOLIC FRACTION	
	V_{max}	K_m	V_{max}	K_m
Control liver	65.5 ± 2.12	66.73 ± 0.97	42.33 ± 2.34	44.93 ± 0.68
24 hour regenerating liver	$114.68 \pm 4.34^*$	62.75 ± 3.83	47.5 ± 2.8	46.79 ± 3.03

$^*p<0.05$ compared to control

Vmax-Maximal Velocity (Picomoles/min/mg protein) ; Km-Michelis Menten constant,(μ M)

Figure-1

DNA SYNTHESIS IN REGENERATING LIVER .

Values are Mean±S.E.M. of 4-6 separate determinations
Where the error bars are not seen, the standard error of mean is < 2%.

Figure-2

[^{3}H]Prazosin Scatchard in Hypothalamus

Of Control and Hepatectomised Rats
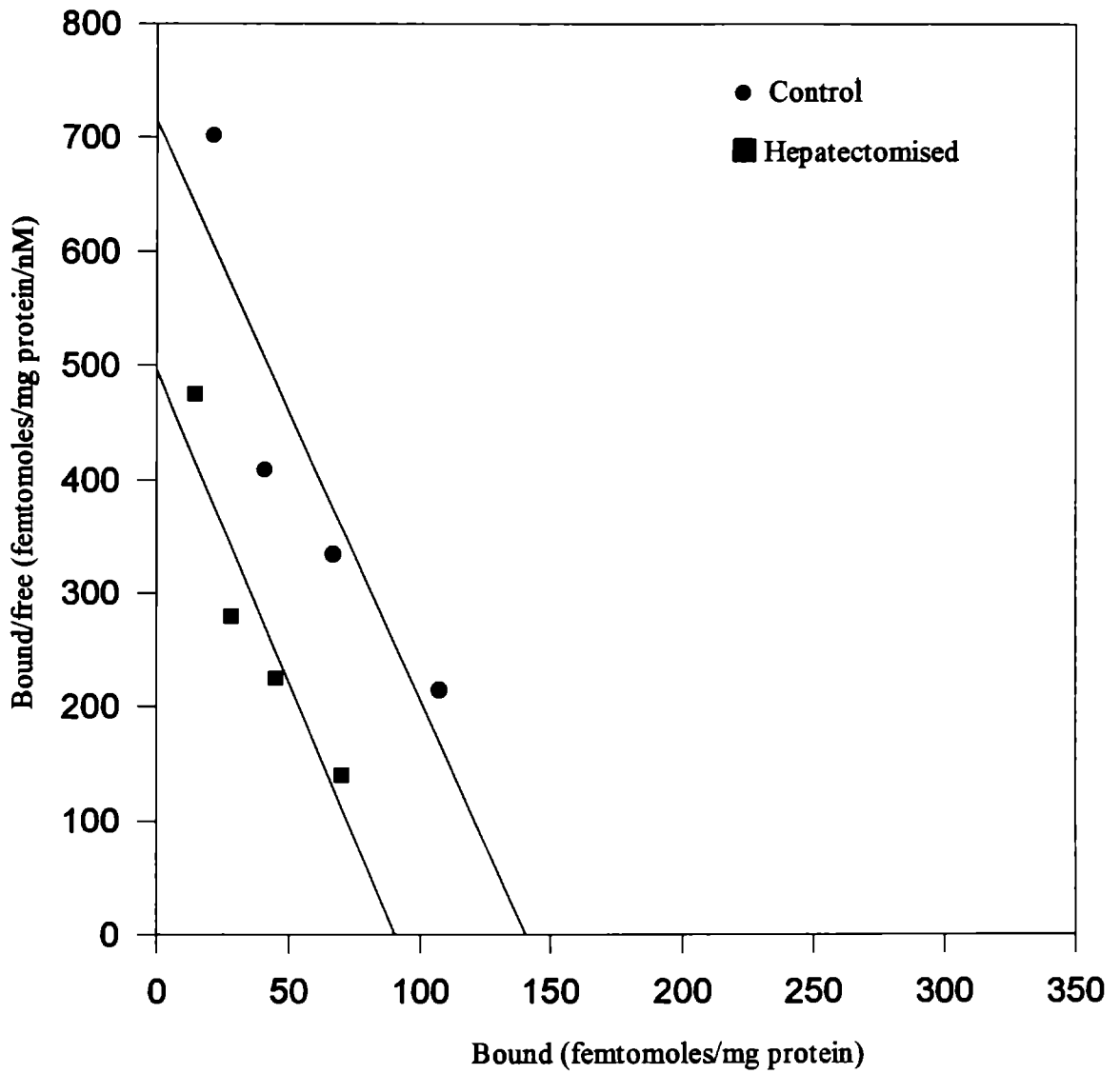
Figure-3

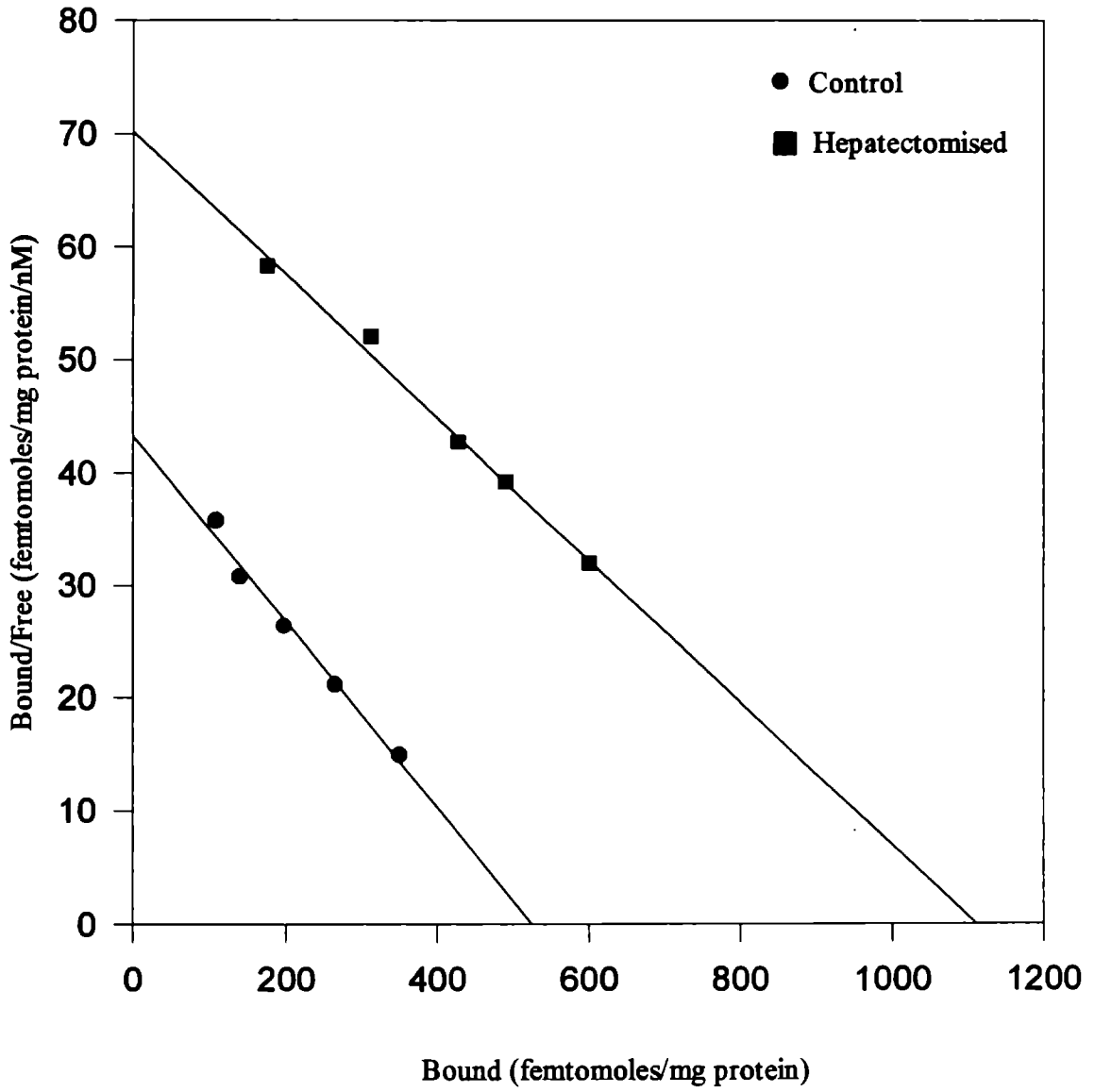## [^{3}H] Yohimbine Scatchard in Hypothalamus

of Control and Hepatectomised Rats

Figure-4

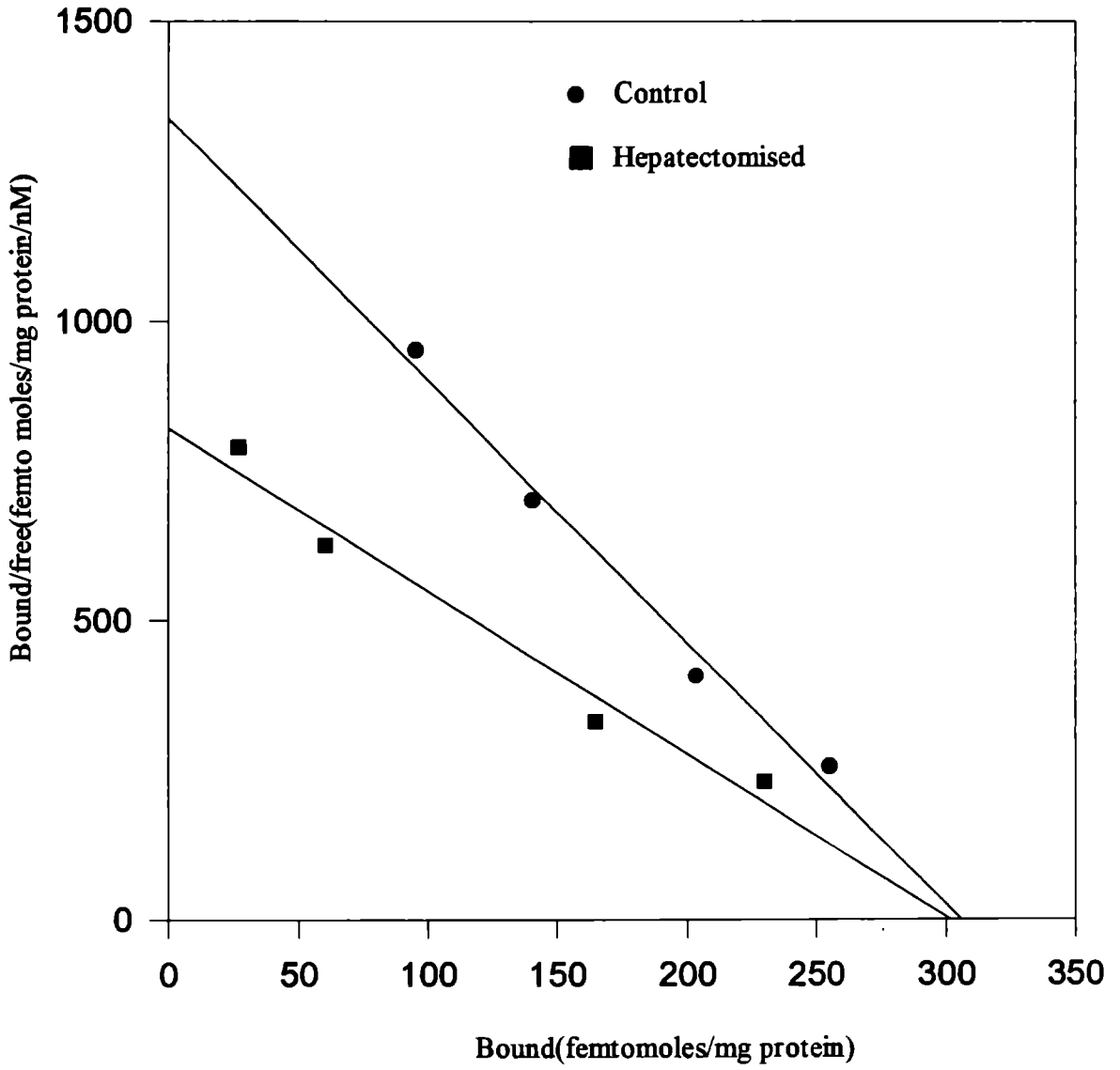## [^{3}H] Prazosin Scatchard in Cerebral cortex
of Control and Hepatectomised Rats

Figure-5

[^{3}H] Yohimbine Scatchard in Cerebral cortex
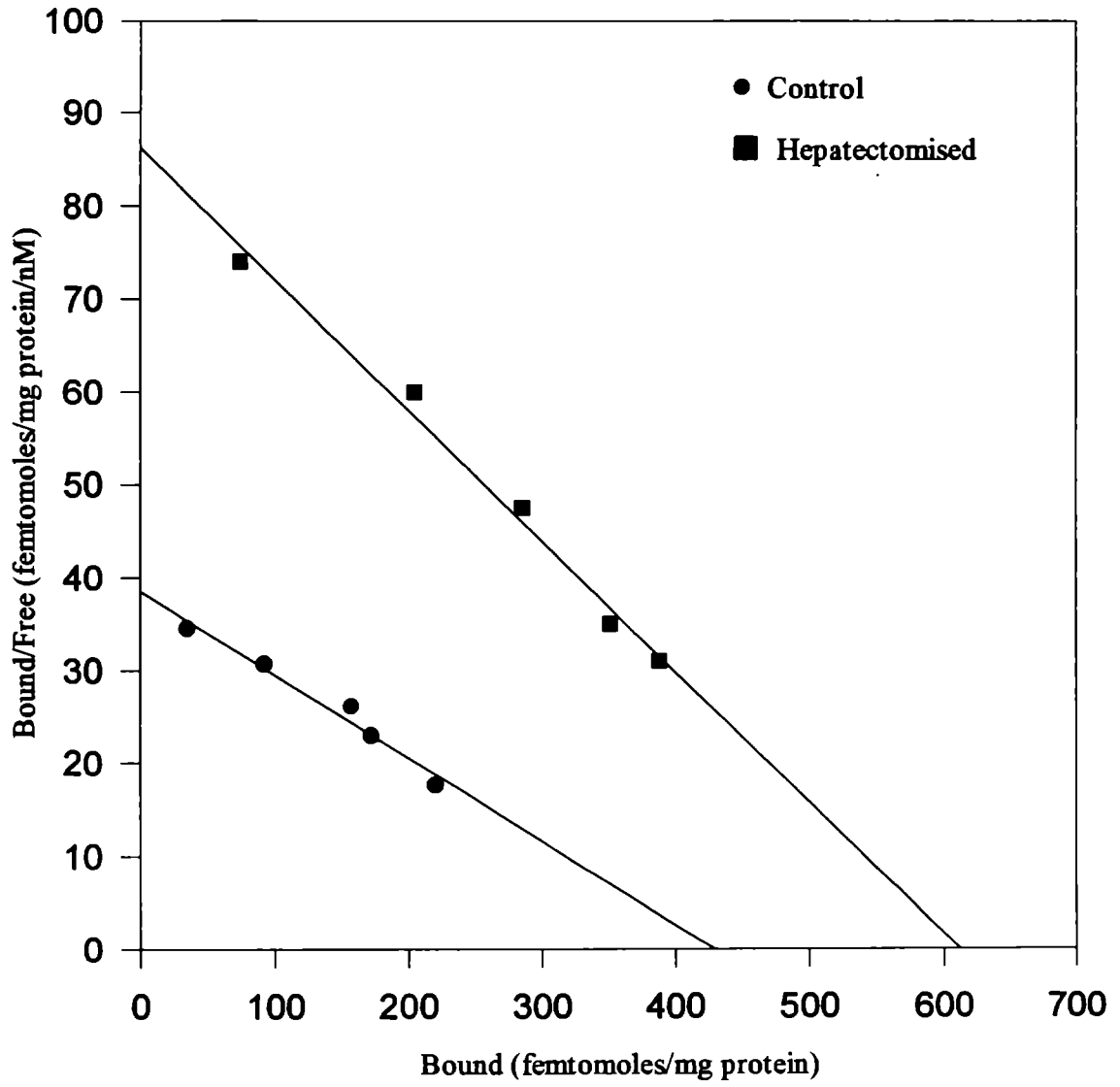
of Control and Hepatectomised Rats

Figure-6

[^{3}H]Prazosin Scatchard in Brain stem
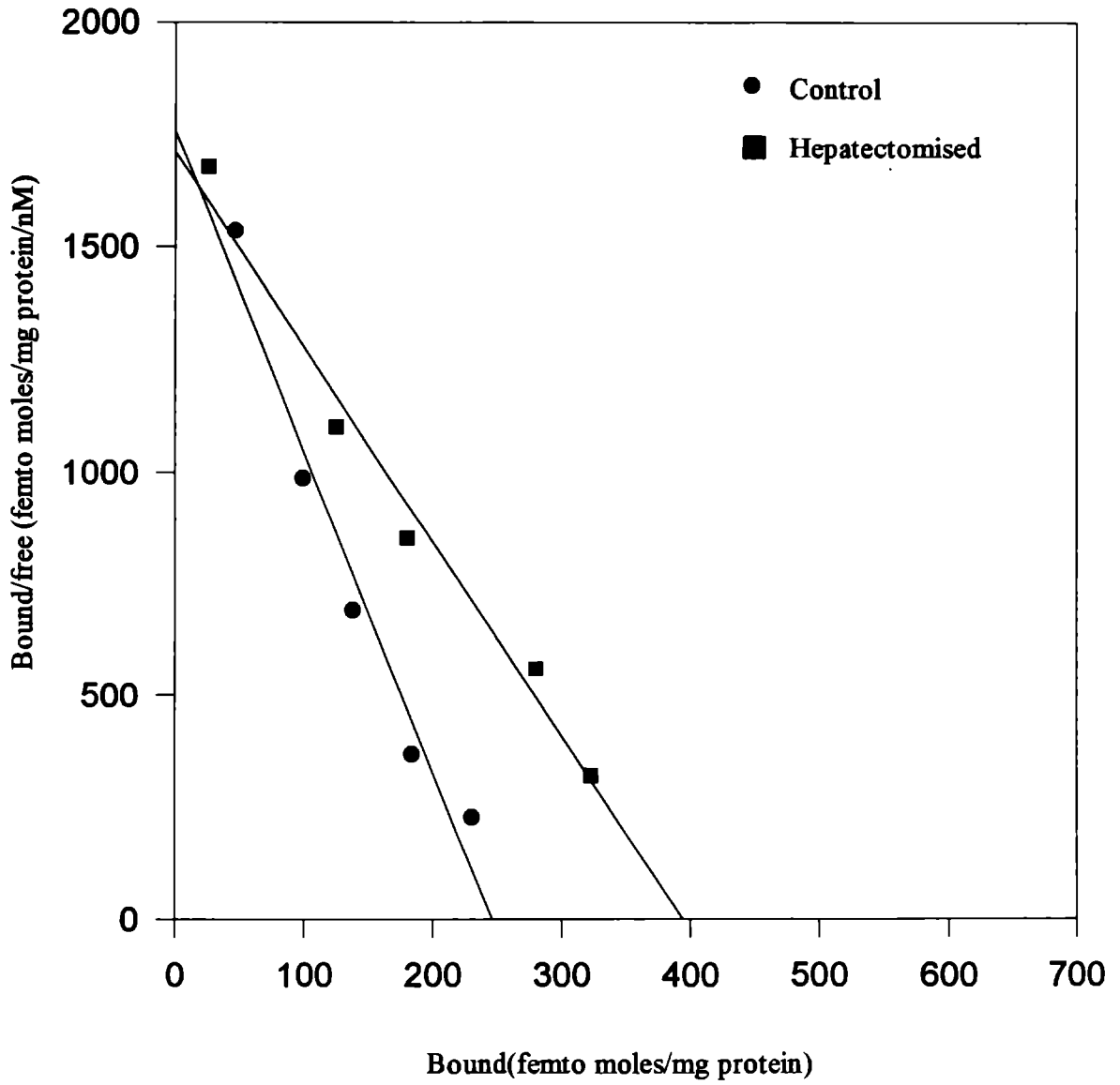
of Control and Hepatectomised Rats

Figure-7

[^{3}H] Yohimbine Scatchard In Brain Stem
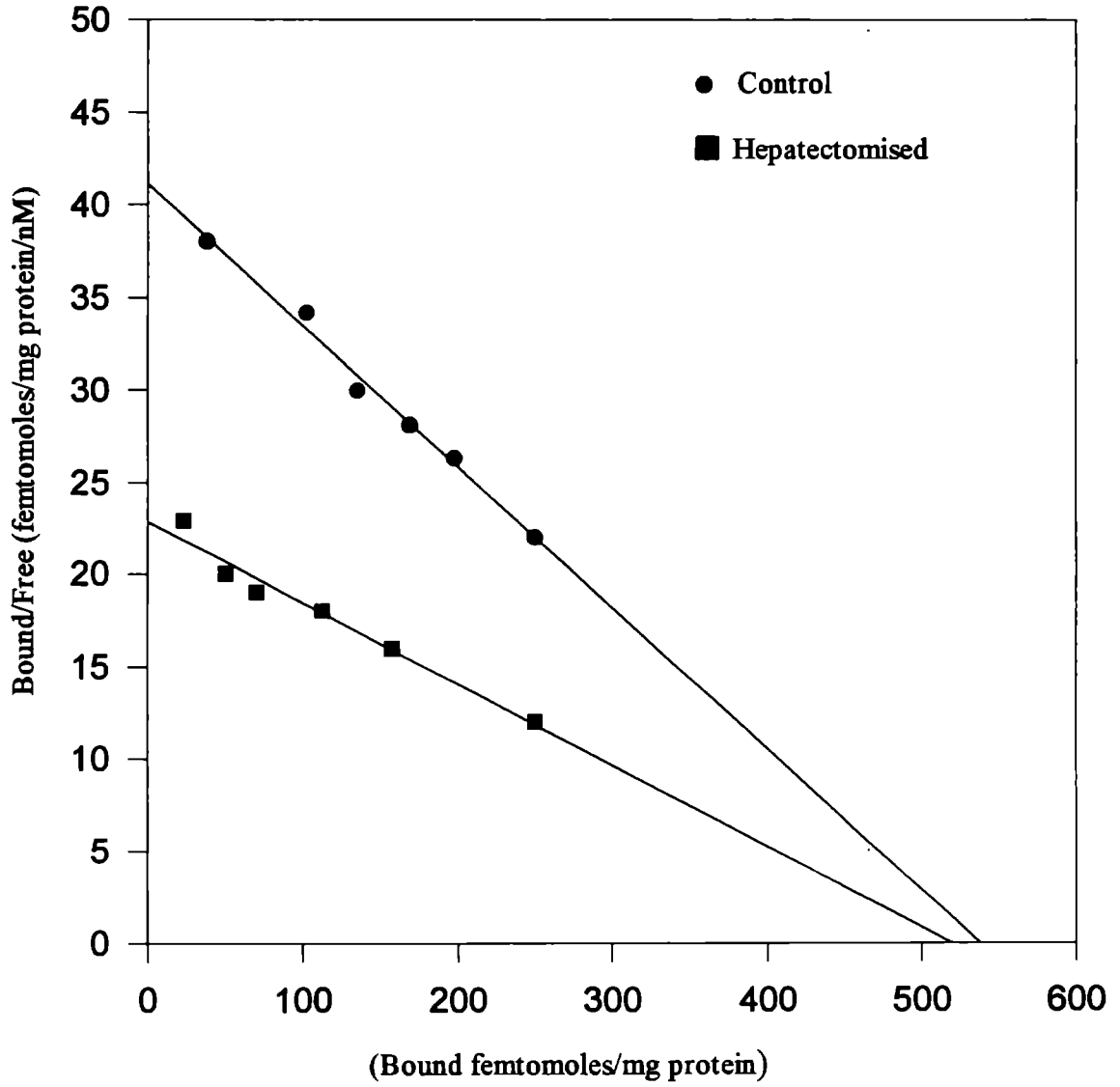
of Control and Hepatectomised Rats

Figure-8

[^{3}H] Serotonin Scatchard in hypothalamus
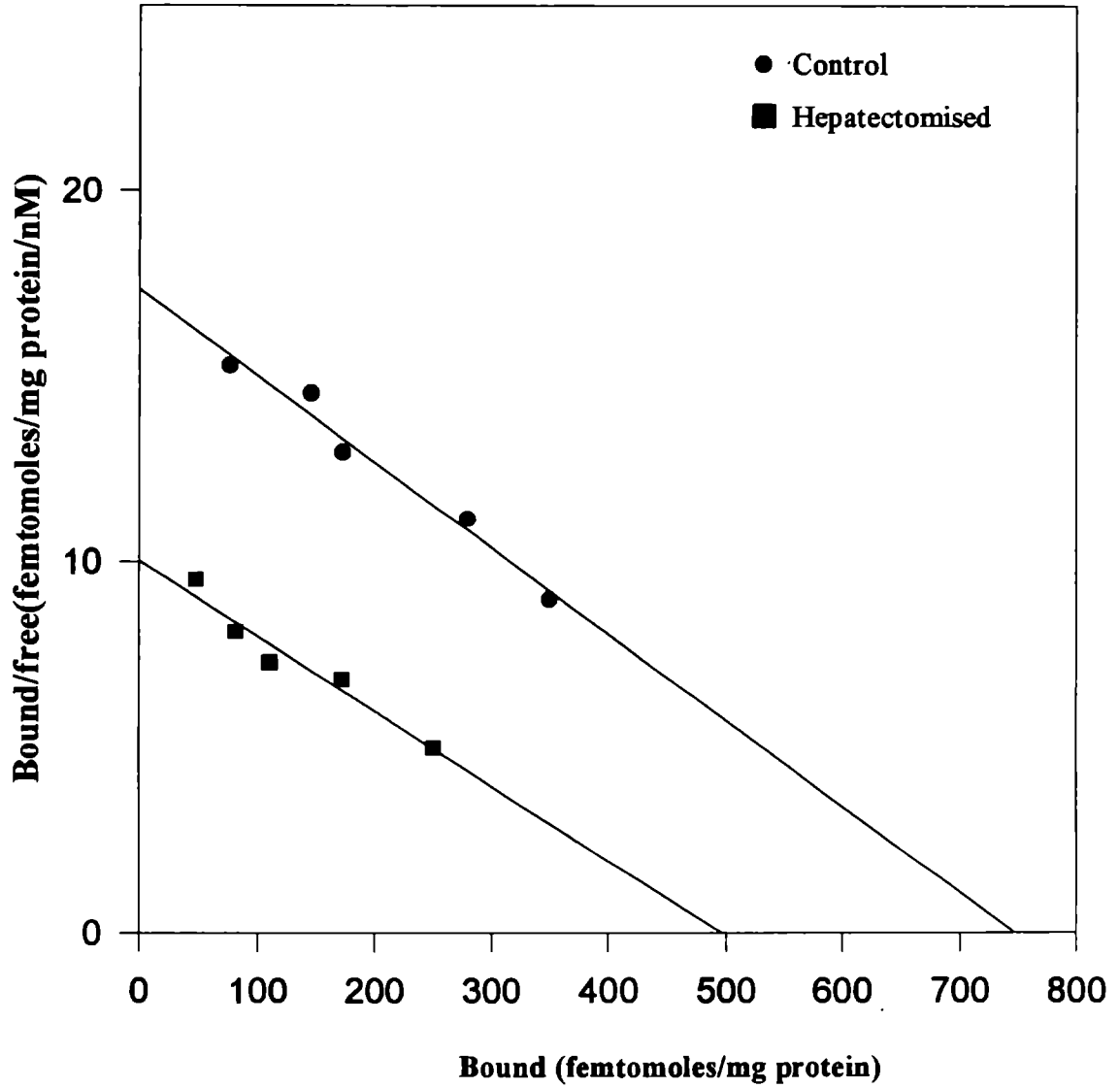
of control and hepatectomised rats.

Figure-9

[^{3}H] Serotonin Scatchard in cerebral cortex
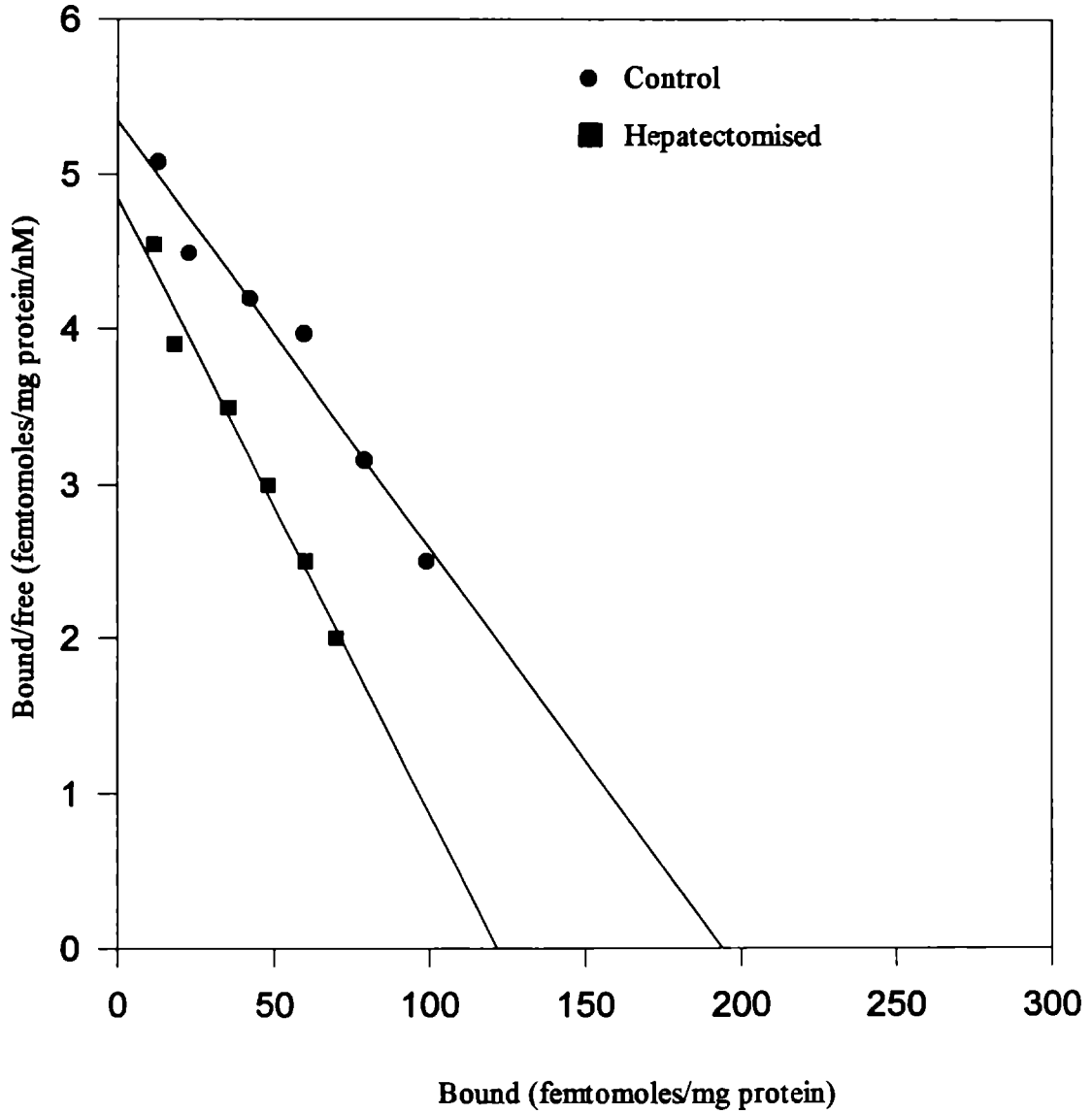
of control and hepatectomised rats.

Figure-10

[^{3}H] Serotonin Scatchard in Brain Stem

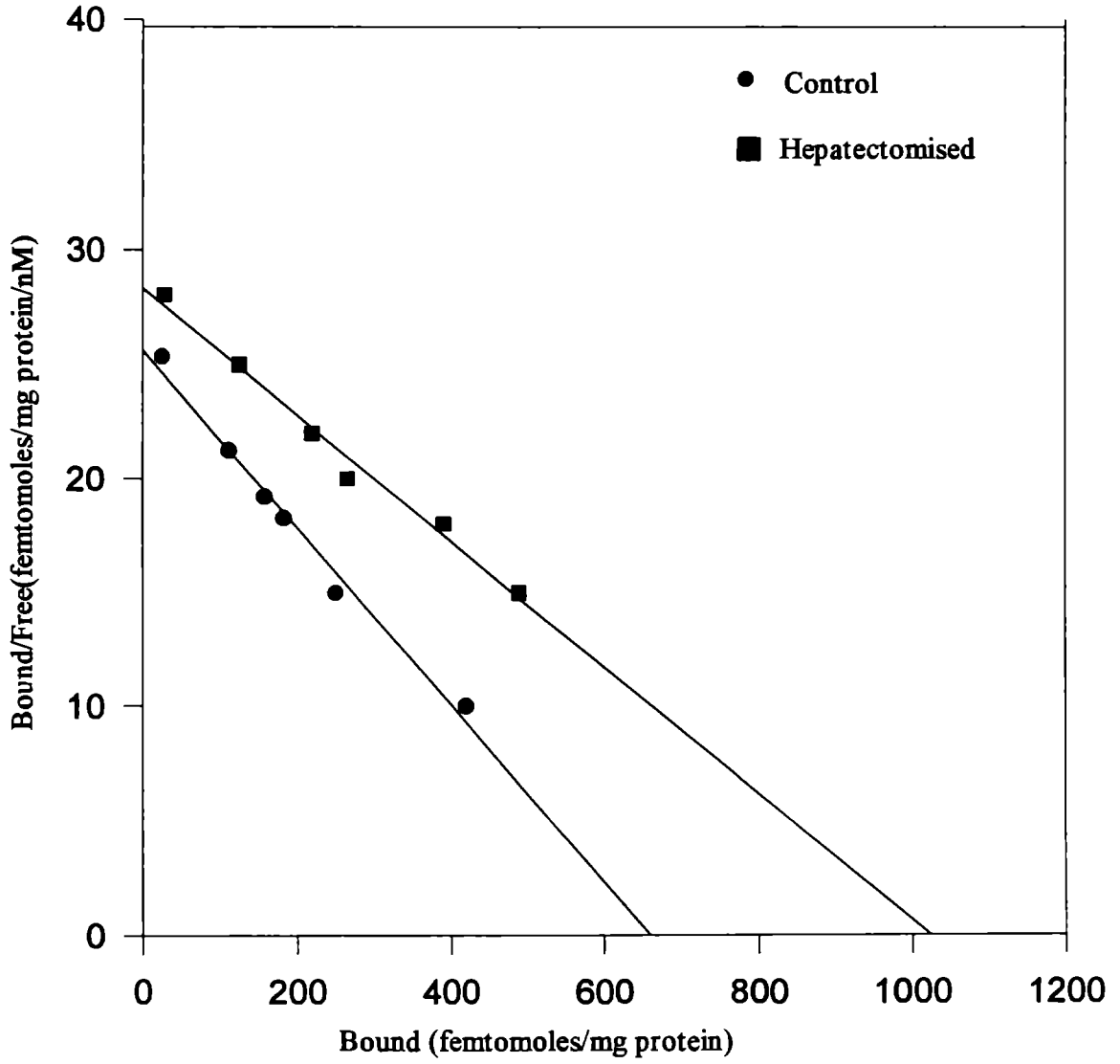of control and hepatectomised rats

Figure-11

DISPLACEMENT OF [^{3}H] NE BY PRAZOSIN

IN LIVER OF CONTROL AND HEPATECTOMISED RATS

Figure-12

DISPLACEMENT OF [3H] NE BY PROPRANOLOL

IN LIVER OF CONTROL AND HEPATECTOMISED RATS

Figure-13

DISPLACEMENT OF [^{3}H] NE BY YOHIMBINE

IN LIVER OF CONTROL AND HEPATECTOMISED RATS

Figure-14

[^{3}H] PRAZOSIN SCATCHARD IN HEPATIC MEMBRANE
OF CONTROL AND HEPATECTOMISED RATS
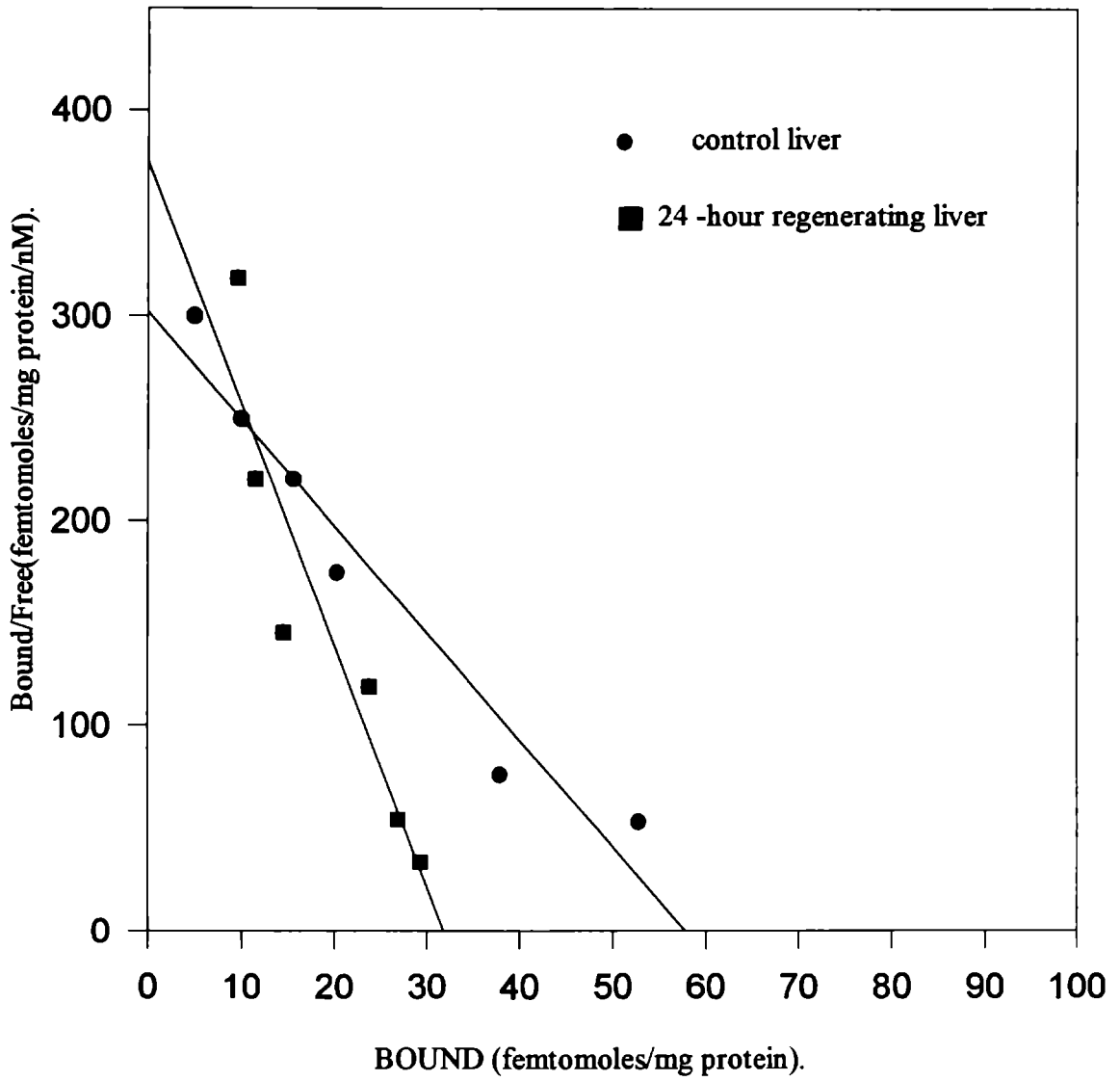
Figure-15

DISPLACEMENT OF [3H] PRAZOSIN BY -(-) NE

IN THE LIVER OF CONTROL AND HEPATECTOMISED RATS:

EFFECT OF GTP ANALOG Gpp[NH]p
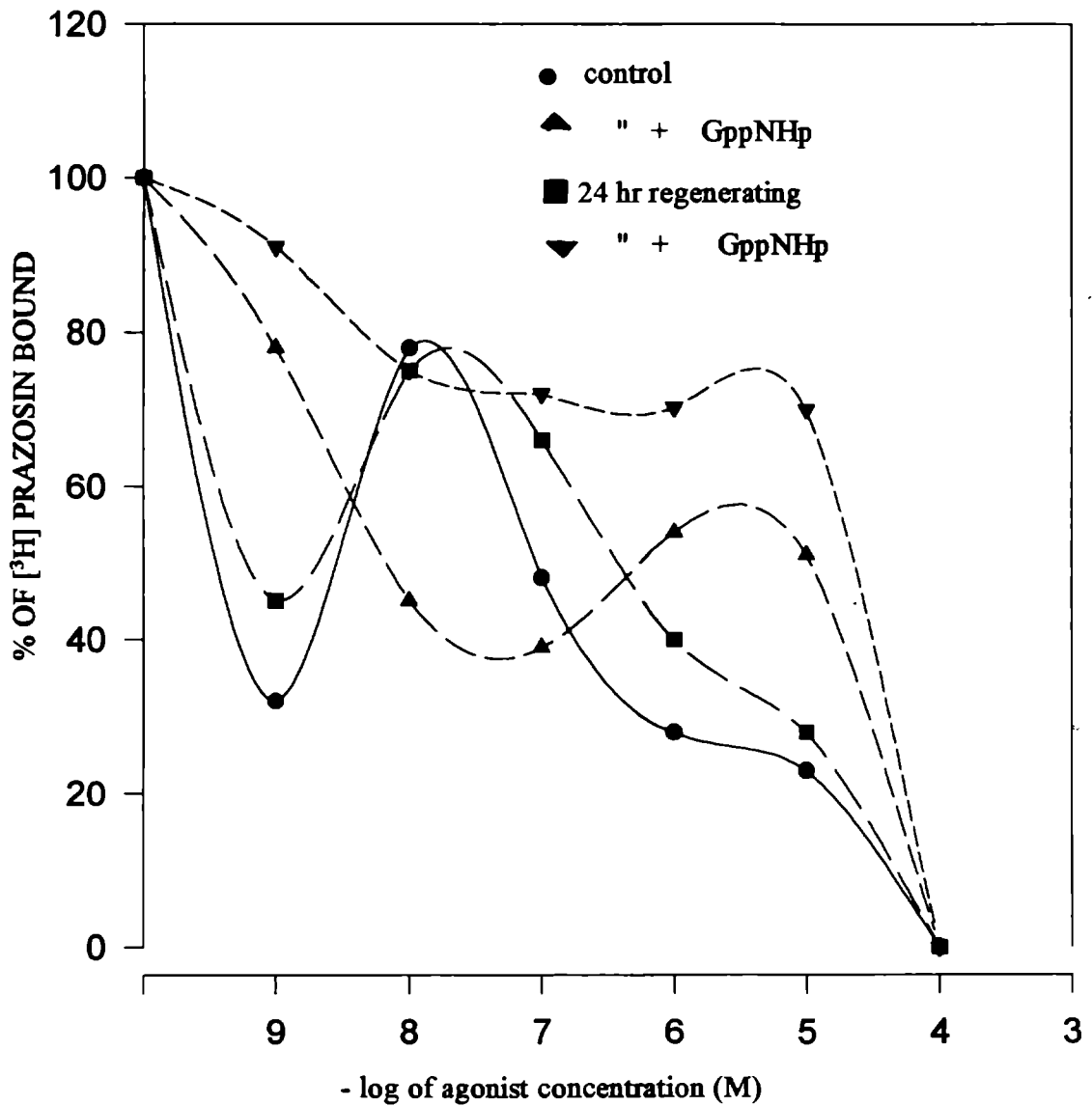
Figure-16

[^{3}H] SEROTONIN SCATCHARD IN THE HEPATIC MEMBRANE

OF CONTROL AND HEPATECTOMISED RATS
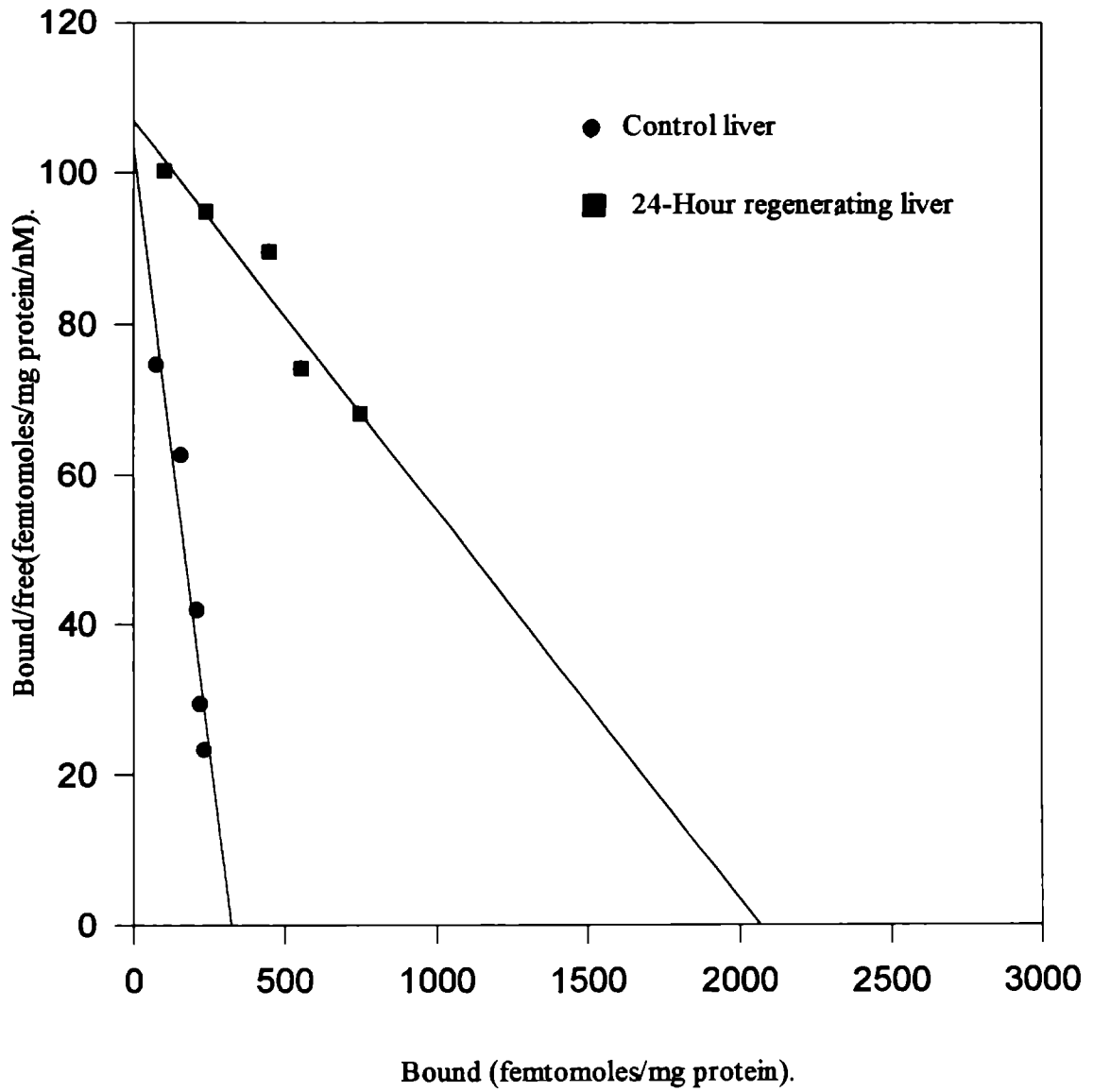
Figure-17

DISPLACEMENT OF [3H] 5-HT BY SEROTONIN

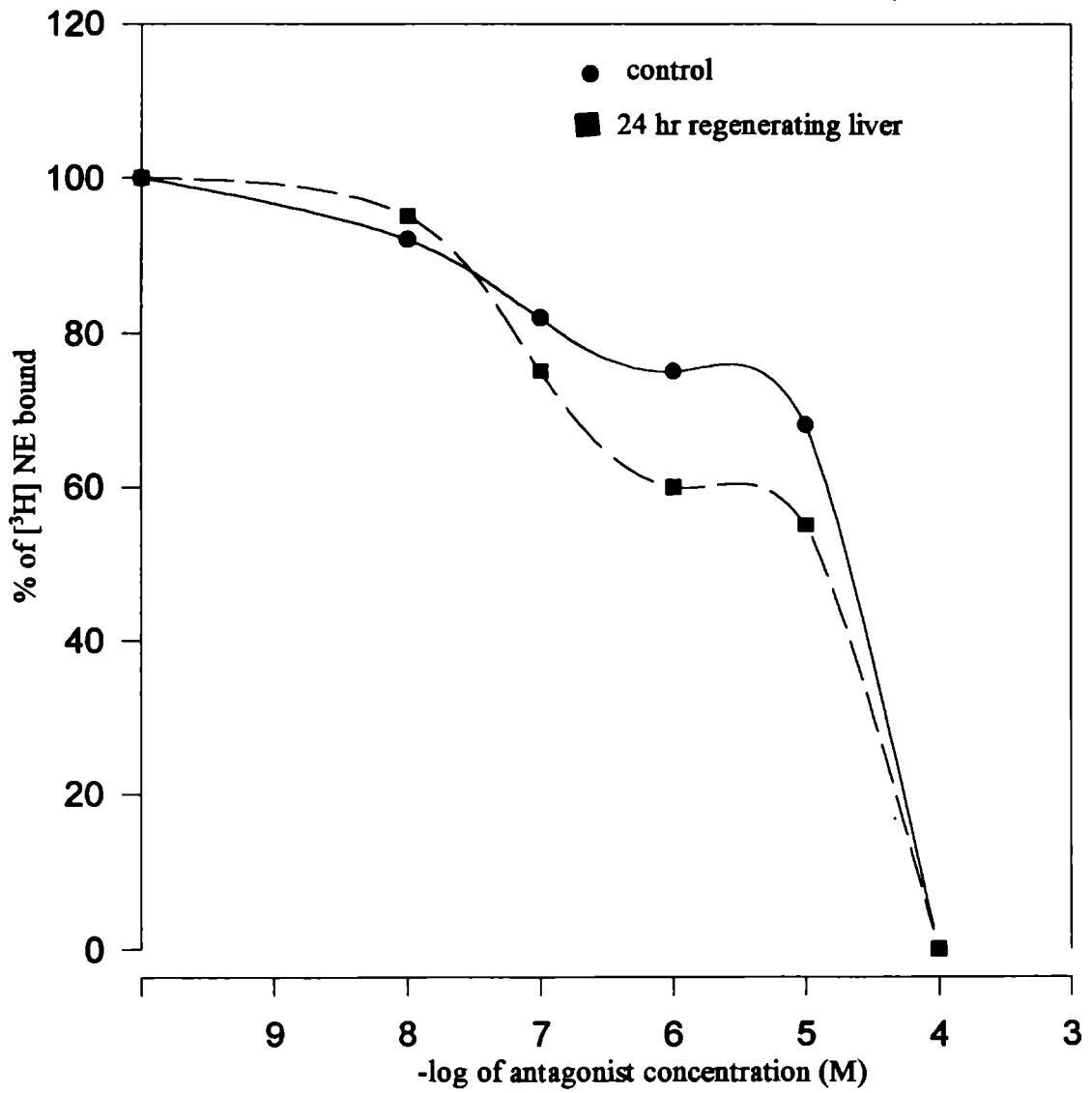**IN LIVER OF CONTROL AND HEPATECTOMISED RATS.**
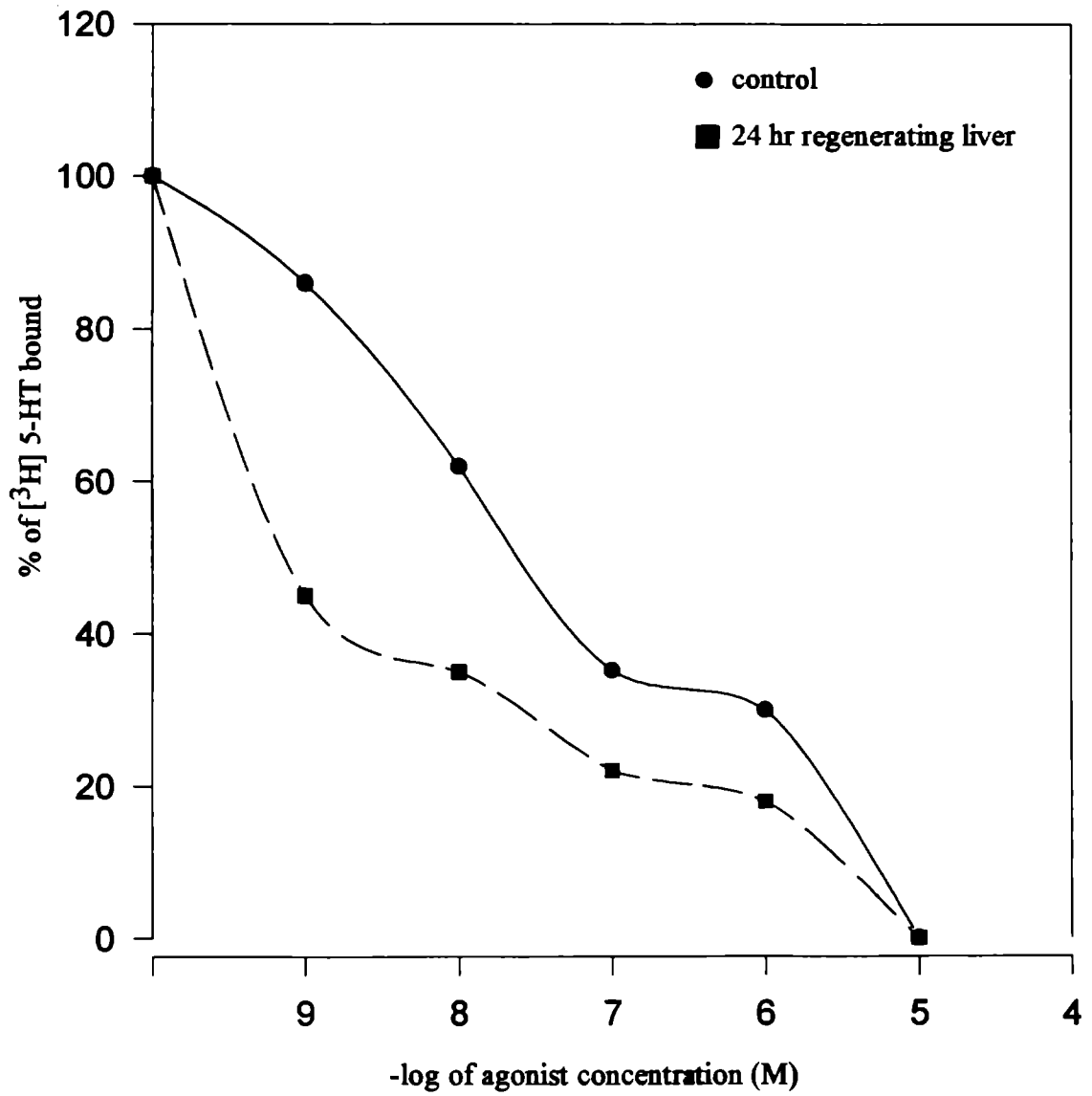
Figure-18

DISPLACEMENT OF [³H] 5-HT BY KETANSERIN
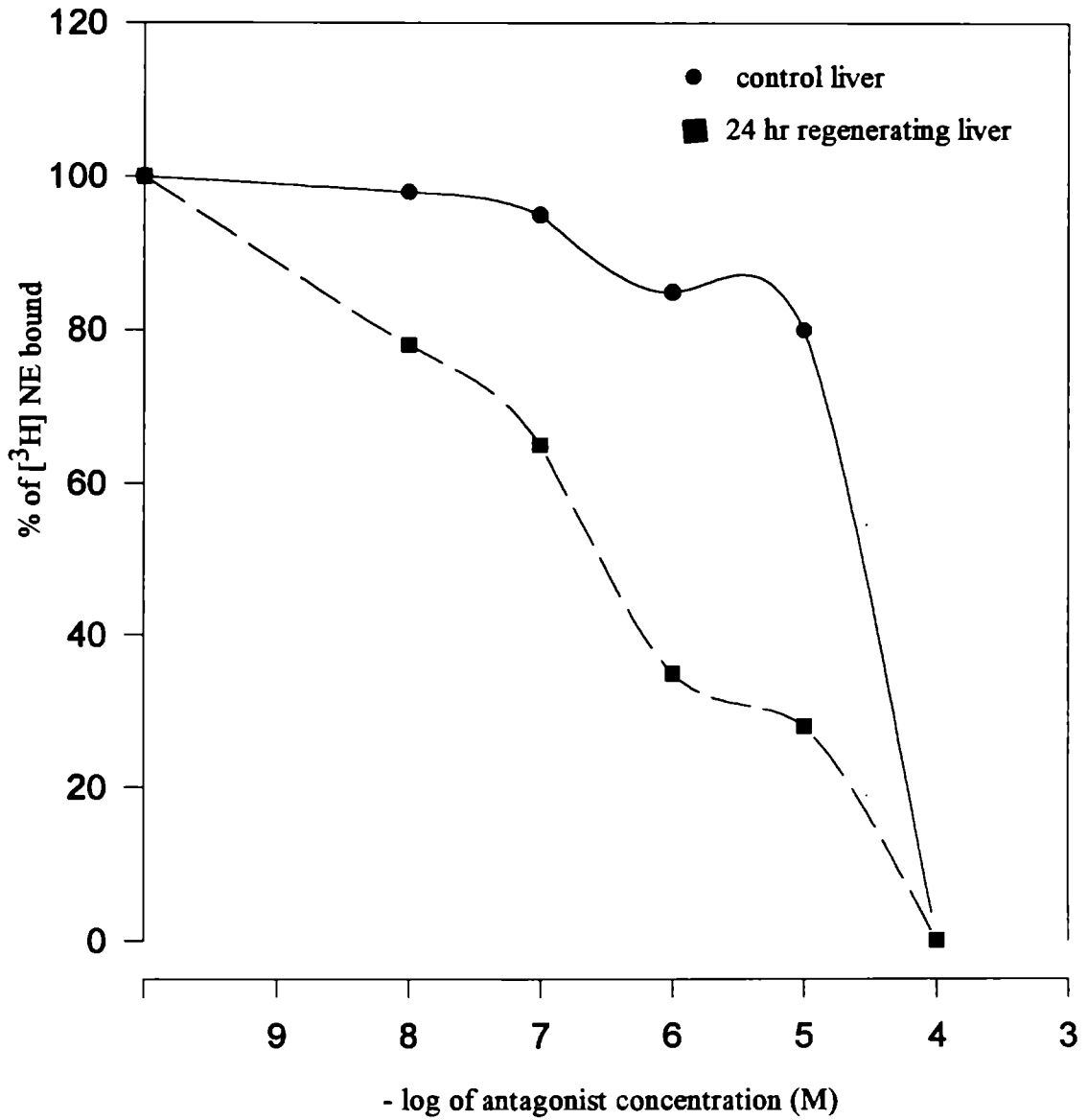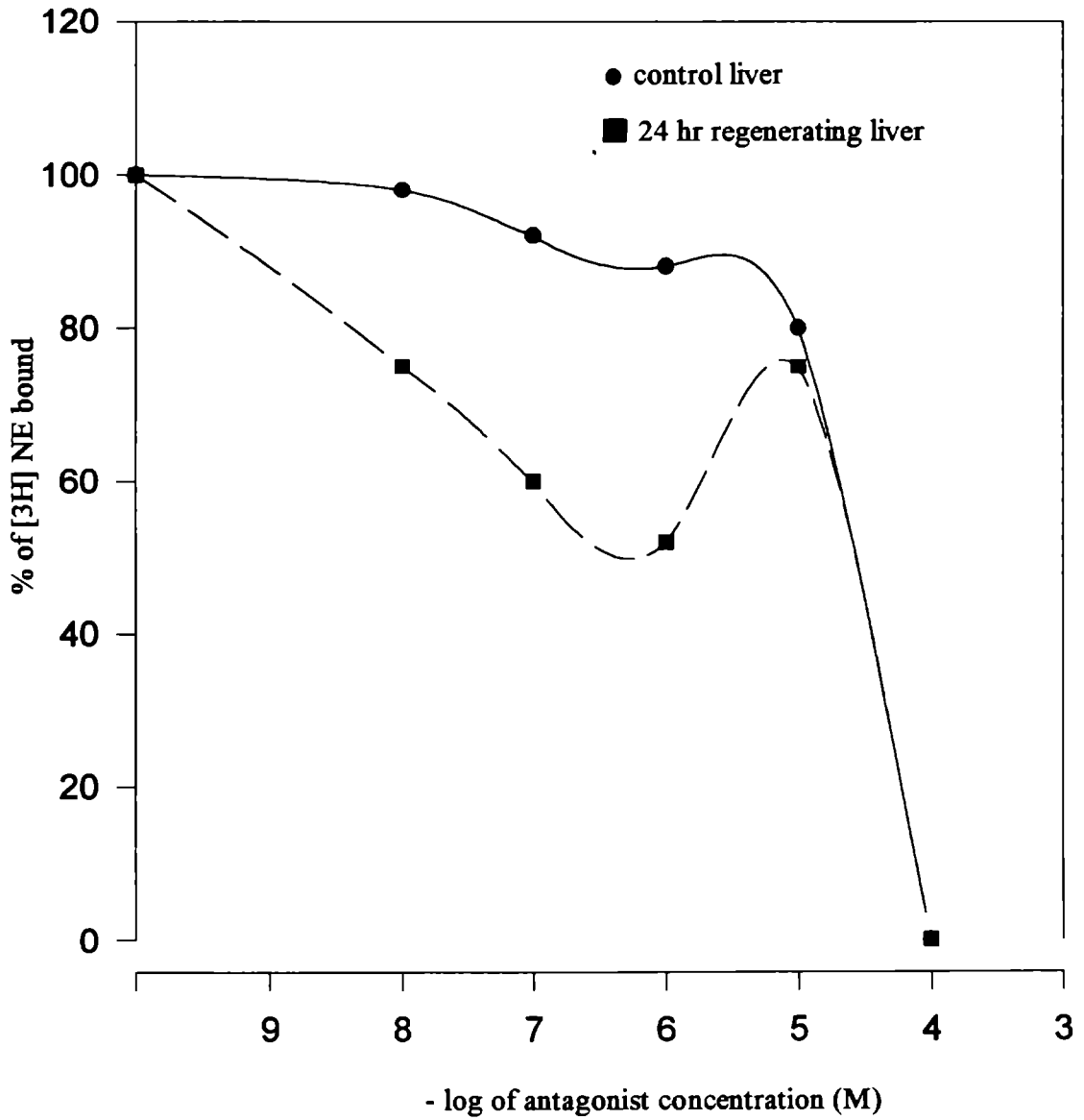
IN LIVER OF CONTROL AND HEPATECTOMISED RATS

- log antagonist concentration (M).
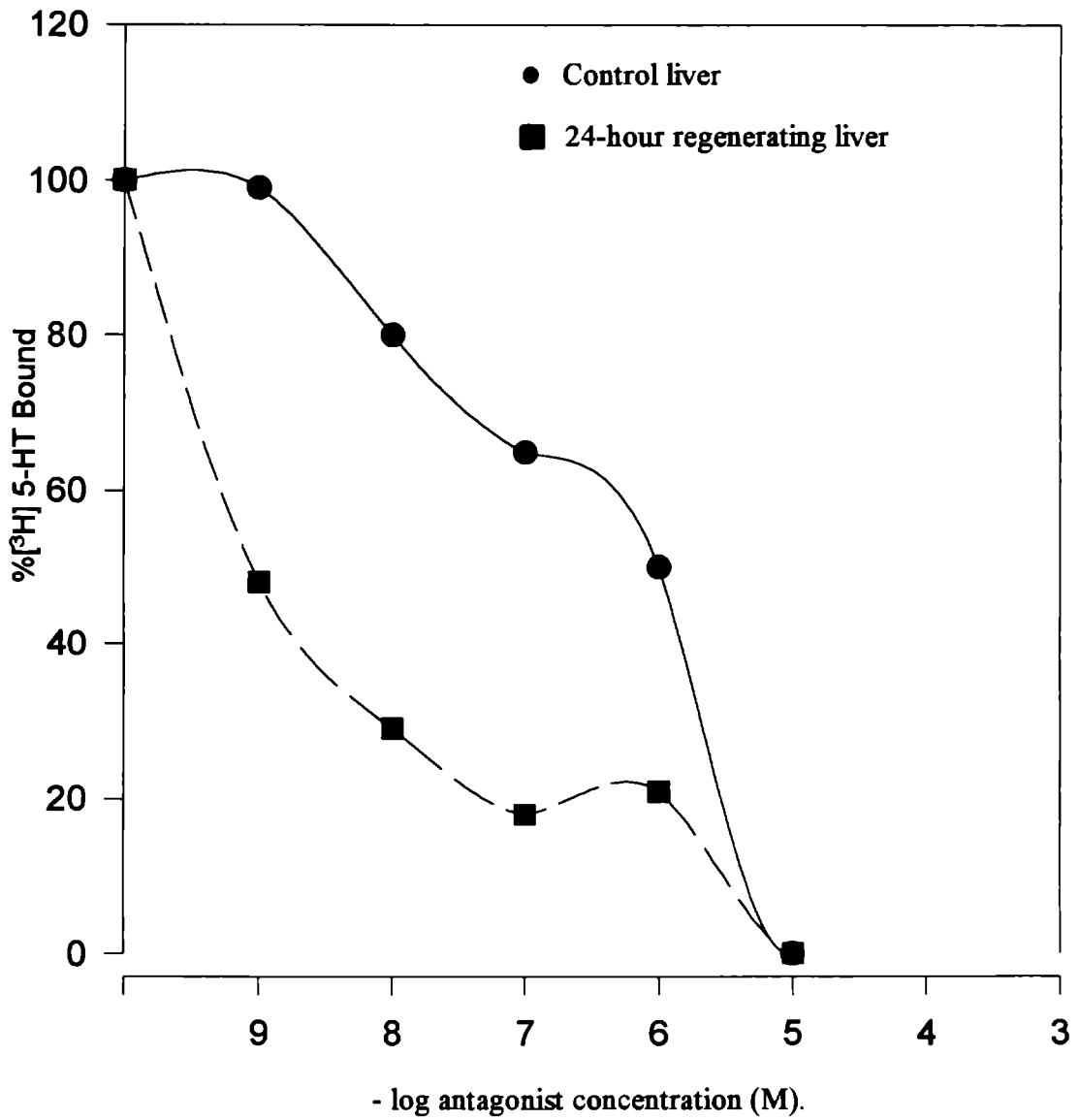
Figure-19

DISPLACEMENT OF [³H]-5HT BY SPIPERONE

IN LIVER OF CONTROL AND HEPATECTOMISED RATS.
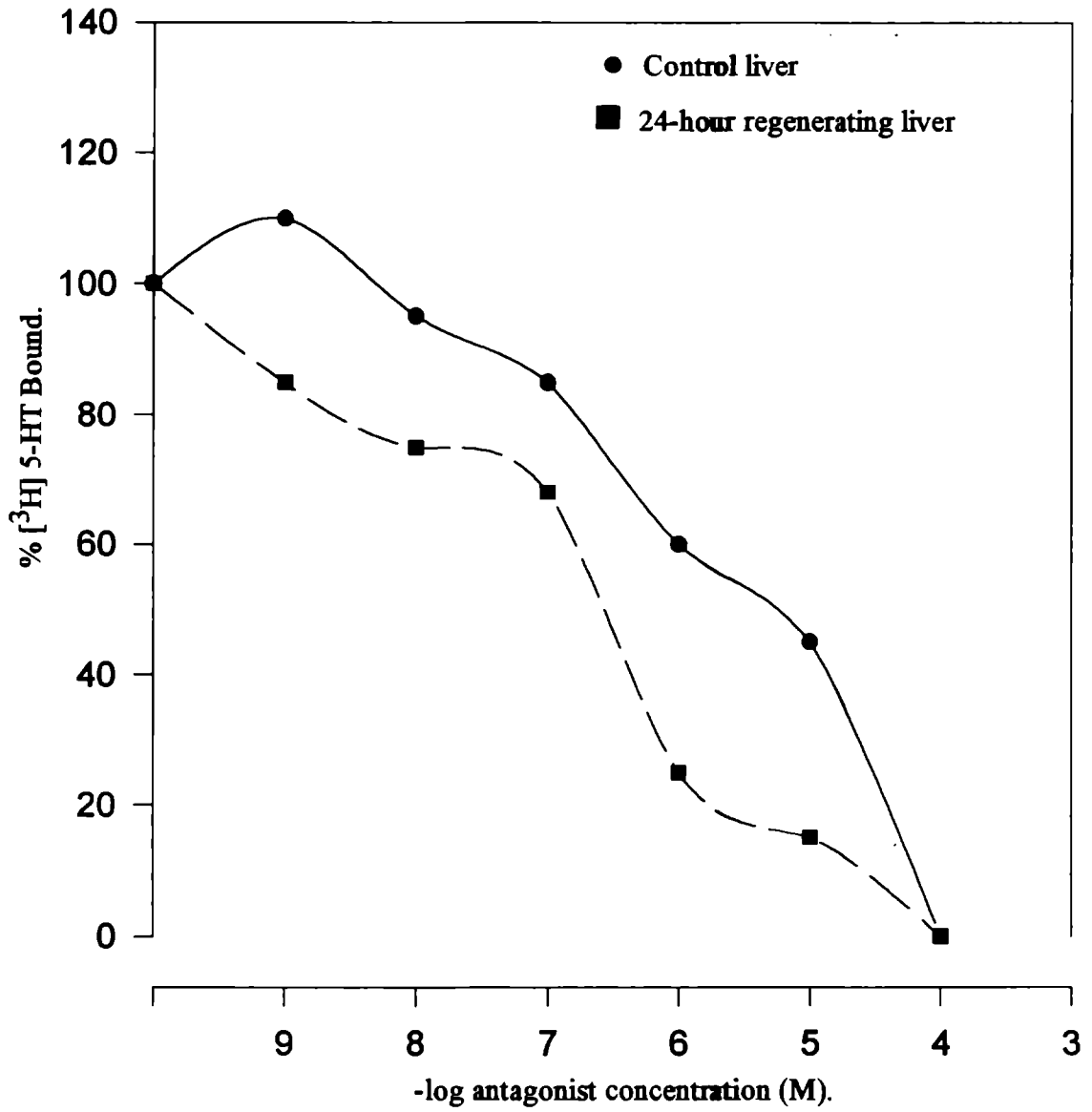
Figure-20

Effect of NE and 5-HT on DNA synthesis of cultured hepatocytes
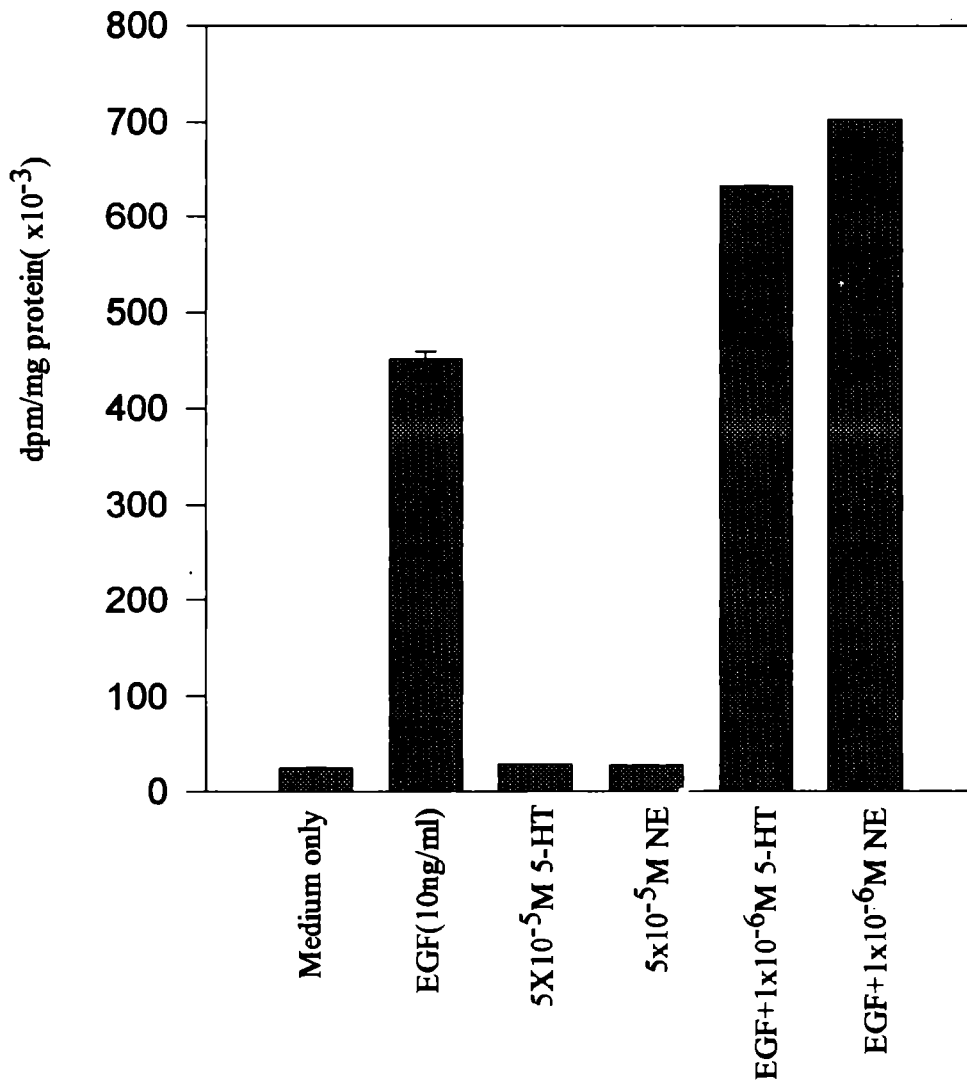
Values are mean ± S.E.M. of 4-6 separate determinations

Where error bars are not visible, standard error of mean is <2%

Figure-21

Dose-dependent response of hepatocyte DNA Synthesis to Norepinephrine.

-log norepinephrine concentration.[M]
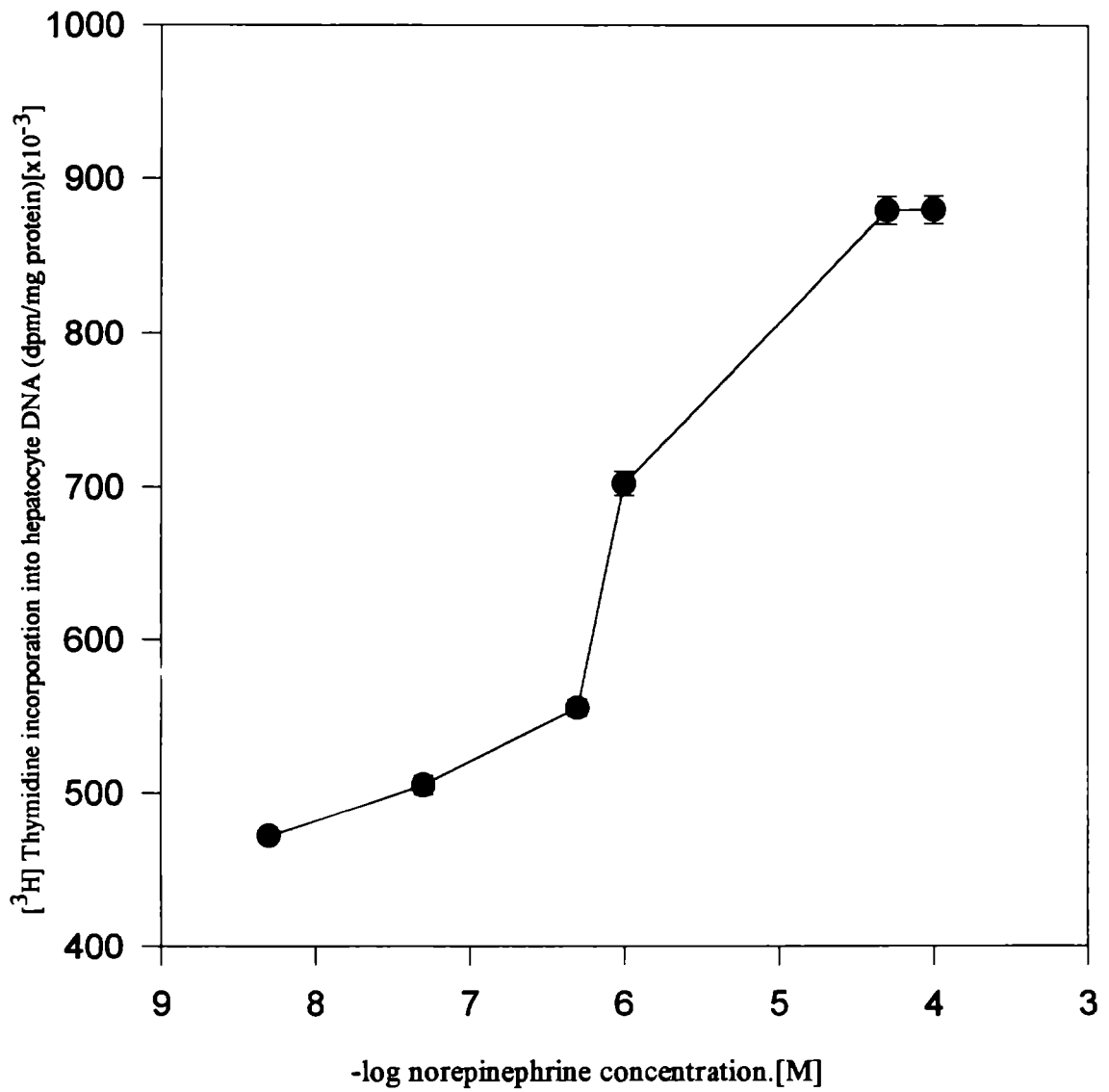
Values are Mean±S.E.M. OF 4-5 Separate determinations.

Where the error bars are not seen, the S.E.M/is <2%.

Figure-22

Effect of adrenergic receptor antagonists on

on NE-induced DNA synthesis *in vitro*
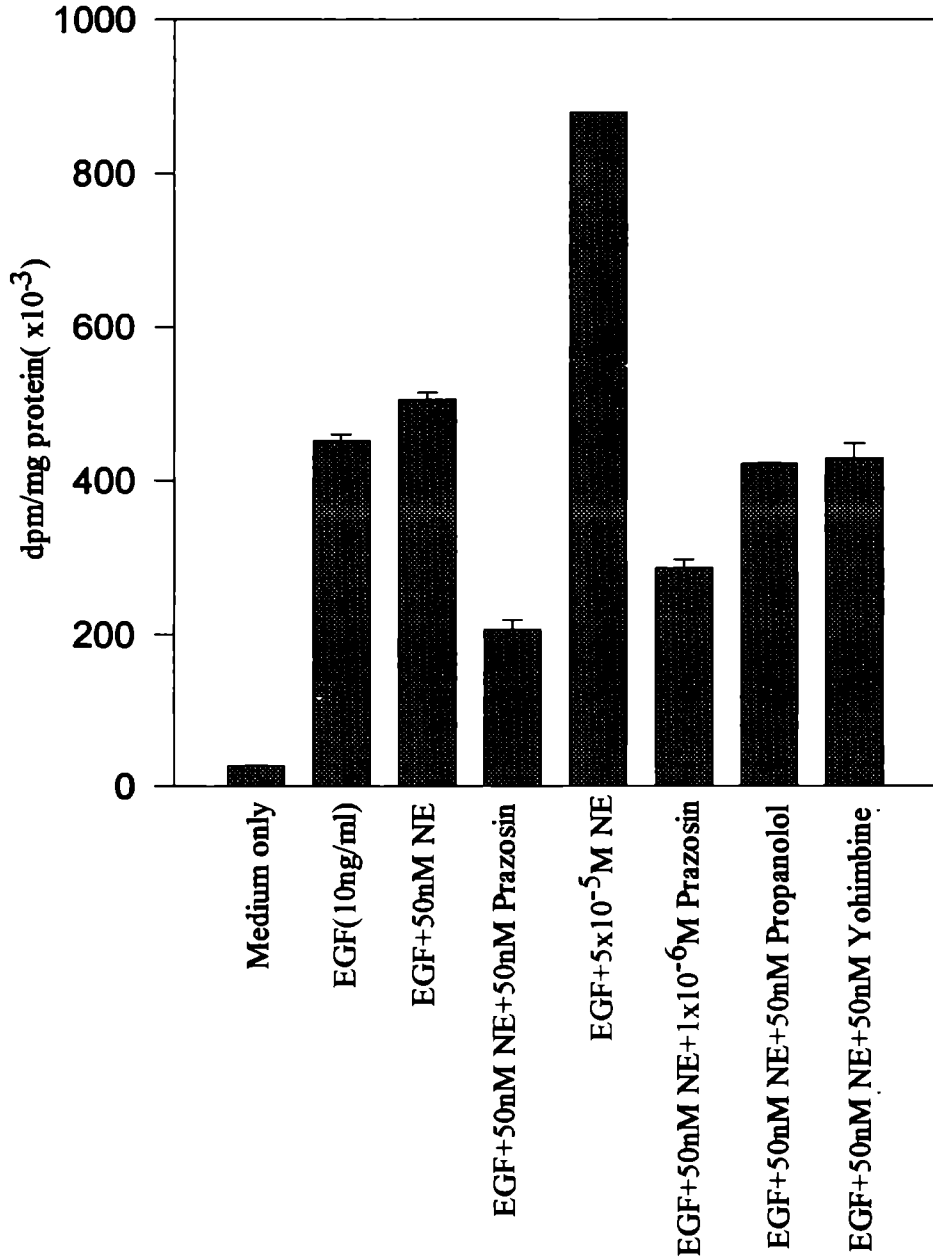
Values are mean±S.E.M. of 4-6 separate determinations

Where the error bars are not visible, standard error of mean is < 2%

Figure-23

Dose-dependent response of hepatocyte DNA synthesis to serotonin.
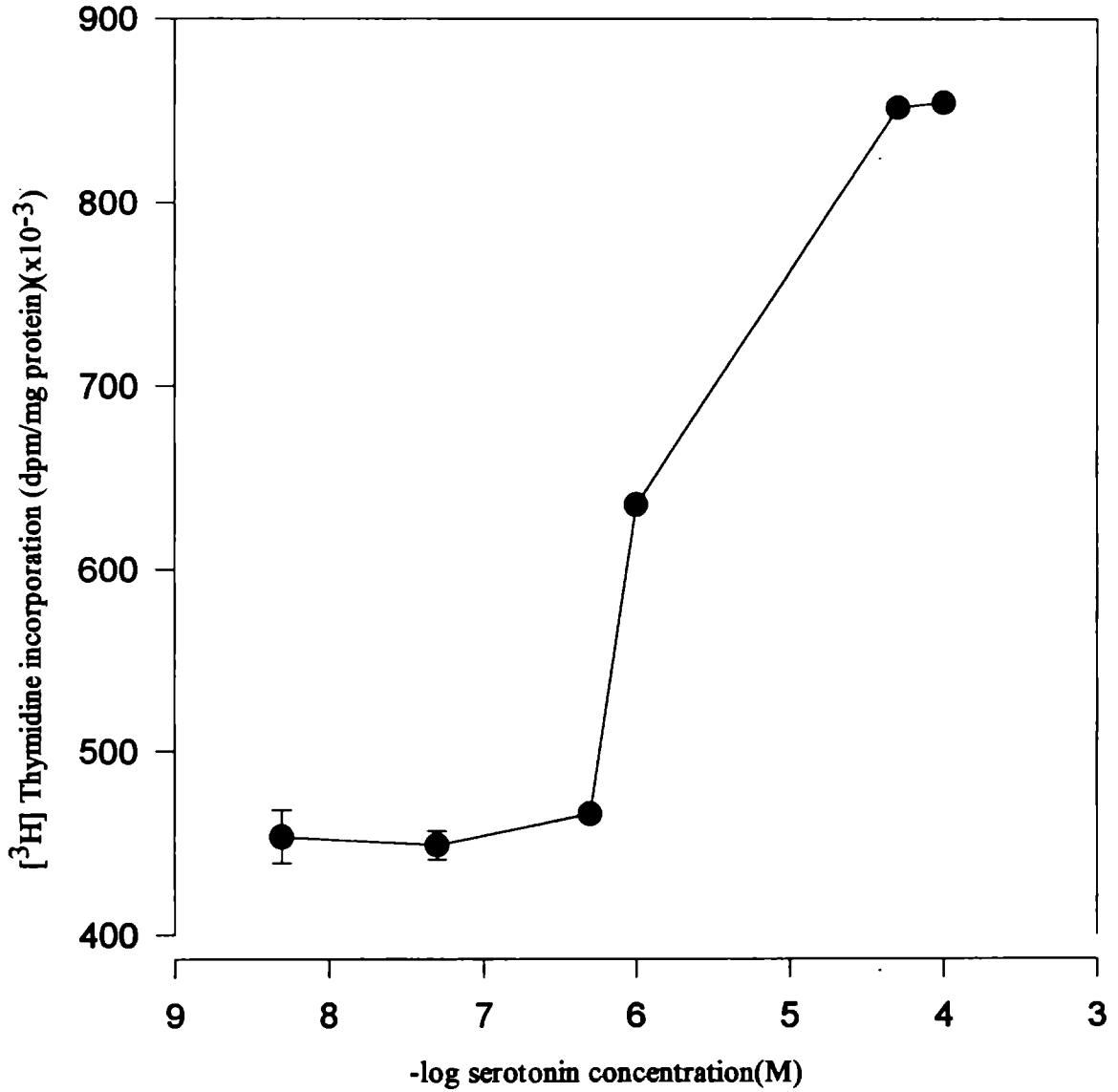
Values are Mean $\pm$S.E.M. Of 4-6 separate determinations.

Where the error bars are not seen the S.E.M. is <2%

Figure-24

EFFECT OF SEROTONIN AND SEROTONERGIC ANTAGONISTS

ON DNA SYNTHESIS IN CULTURED RAT HEPATOCYTES

Values are mean ± S.E.M of 4-6 separate determinations

Where are error bars are not visible, standard error of mean is <2%

Figure-25

Effect of Prazosin and Ketanserin on EGF-induced DNA synthesis in cultured hepatocytes
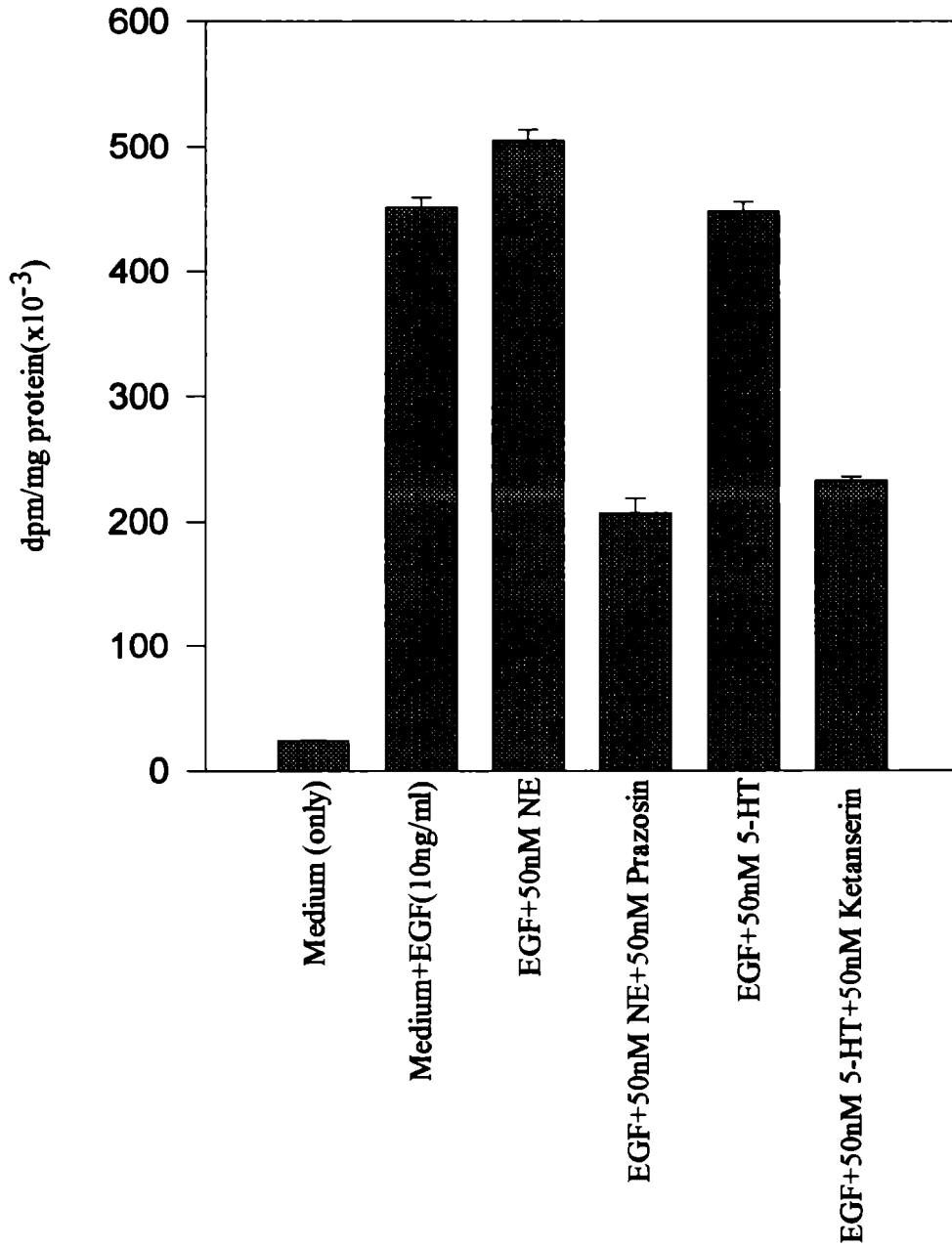
Values are mean ± S.E.M. of 4-6 separate determinations.

Where the error bars are not visible, standard error of mean is <2%

PLATE-1

Phase-contrast micrographs of isolated rat hepatocytes prepared by two-step collagenase perfusion. Preperfusion with a Ca^{2+} free buffer for the removal of Ca^{2+} was followed by perfusion with collagenase buffer. Hepatocytes were separated from non-parenchymal cells by filtration and centrifugation and the viability of the suspension used for culture was assessed by trypan blue exclusion as described in Materials and Methods.
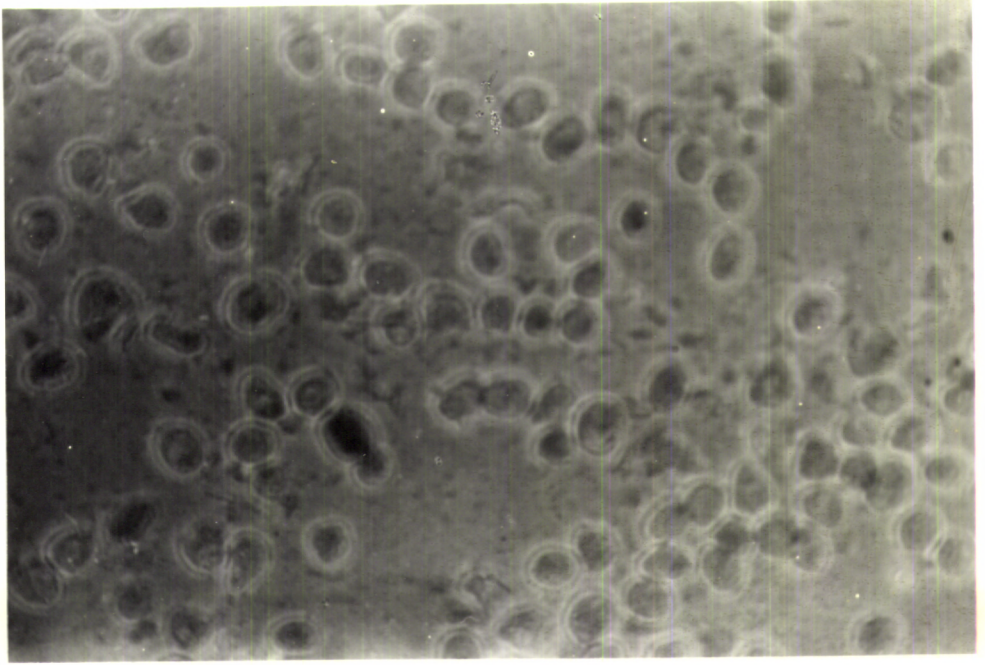
PLATE-2

Autoradiograph of 5-HT induced membrane protein phosphorylation in the control and regenerating rat liver. Liver membrane fractions were incubated with 10^{-5} M concentrations of the drugs and γ-^{32} P-ATP. Sodium dodecyl sulphate, polyacrylamide gel electrophoresis & autoradiography was done as described in Materials and Methods.

Lane-1 endogenous phosphorylation, control liver; *Lane-2* endogenous phosphorylation in regenerating liver; *Lane-3* with 5-HT and ketanserin, control liver; *Lane-4* with 5-HT and ketanserin, regenerating liver, *Lane-5* with 5-HT, control liver; *Lane-6* with 5-HT, regenerating liver. Molecular weight markers used: Myosin-205KD, β-Galactosidase-116KD, Phosphorylase-97.4KD, Bovine albumin-66KD, Egg albumin-45KD and Carbonic anhydrase-29KD

66 KD →

45 KD →

29 KD →

1 2 3 4 5 6
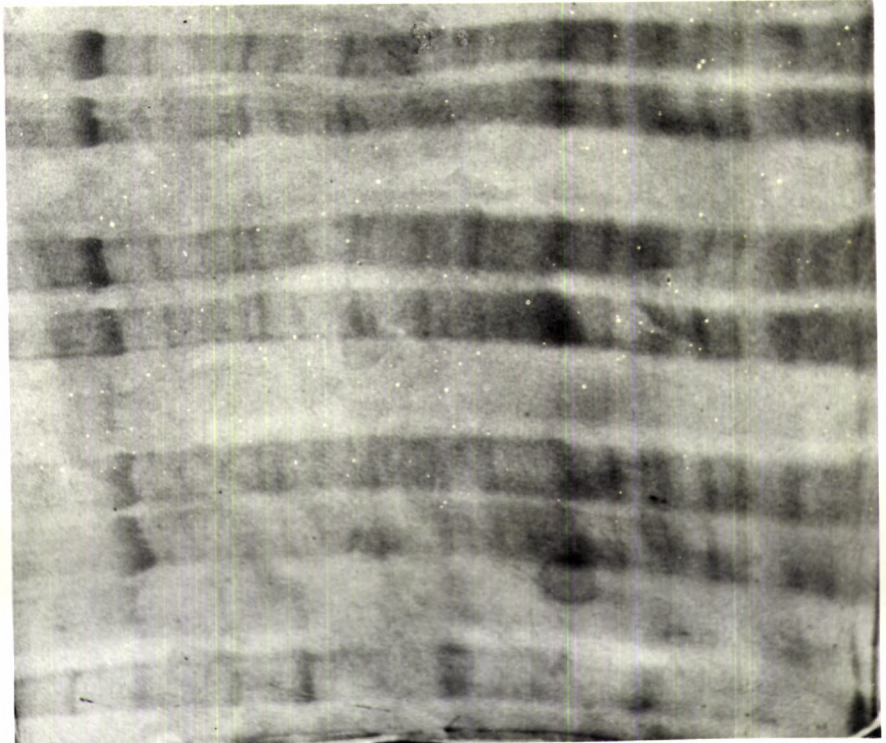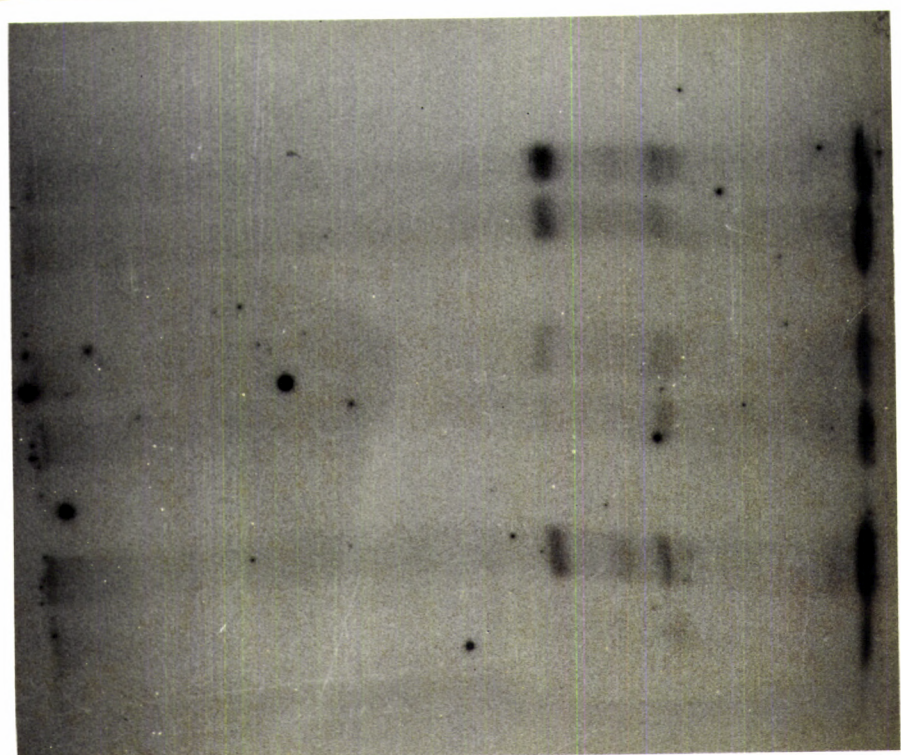
PLATE-3

Autoradiograph of NE-induced membrane protein phosphorylation in the control and regenerating rat liver. Liver membrane fractions were incubated with 10^{-5} M concentrations of the drugs and γ-^{32} P-ATP. Sodium dodecyl sulphate, polyacrylamide gel electrophoresis, autoradiography was done as described in Materials and Methods.

Lane-1 with NE, control liver; *Lane-2* with NE, regenerating liver; *Lane-3* with NE and prazosin, control liver; *Lane-4* with NE and prazosin, regenerating liver, *Lane-5* with PMA, control liver; *Lane-6* with PMA, regenerating liver. Molecular weight markers used: Myosin-205KD, β-Galactosidase-116KD, Phosphorylase-97.4KD, Bovine albumin-66KD, Egg albumin-45KD, Carbonic anhydrase-29KD
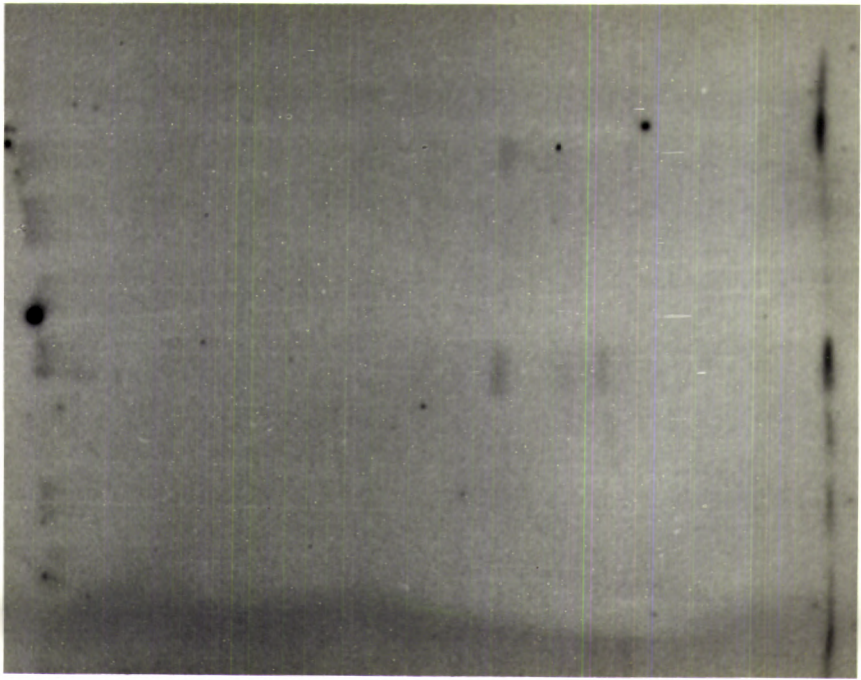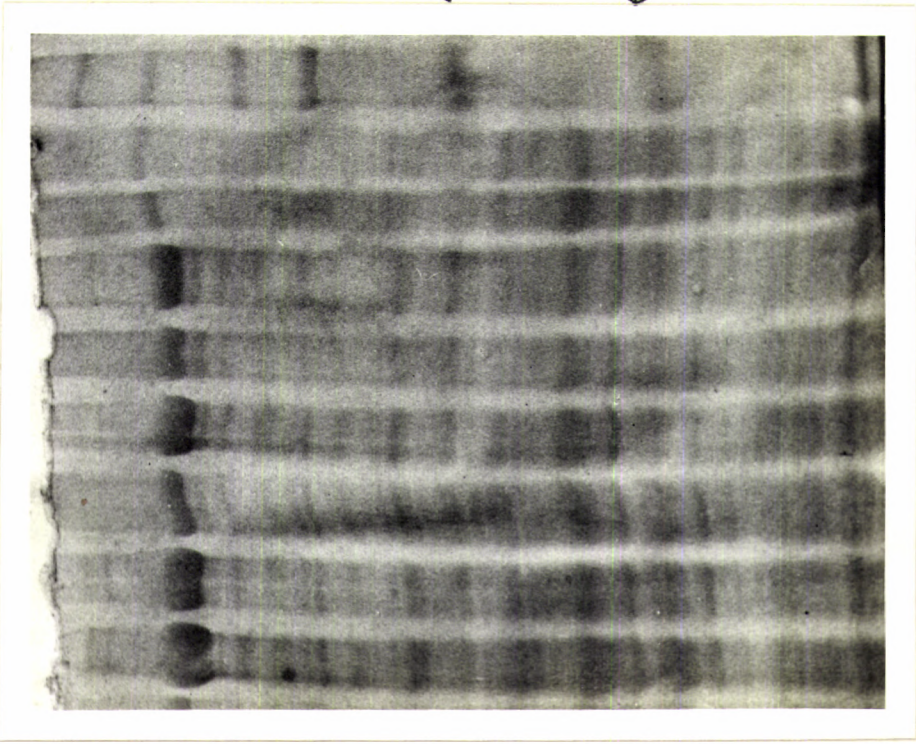
Discussion

DISCUSSION

Time course of DNA Synthesis in the regenerating liver

[^{3}H] thymidine incorporation into hepatic DNA was used to monitor the entry of quiescent hepatocytes into the DNA synthetic phase. DNA synthesis, which was negligible in the intact liver, animals, showed an abrupt rise at 18 hours after partial hepatectomy. The maximal rate of [^{3}H] thymidine incorporation was observed at 24 hours of liver regeneration. DNA synthesis was significantly higher in the liver at 48 hours of regeneraion and reversed to near normal levels by 72 hours after partial hepatectomy. This pattern of DNA synthesis observed by us is concordent with previous reports (Grisham, 1962; Kiba *et al*, 1994). The hepatocytes, which constitute the major part of the liver cell mass are the first to enter the DNA synthetic phase and the 24 hour peak observed corresponds to hepatocyte DNA synthesis. DNA synthesis in the non-parenchymal cells starts after a lag of 24 hours. The [^{3}H] thymidine incorporation observed at 48 hours of hepatectomy indicates DNA synthesis of the non-parenchymal cells. The activity of thymidine kinase, which catalyses phosphorylation of thymidine to thymidylate and that of thymidylate synthetase, responsible for the *de novo* synthesis of thymidylate are also used as an index for liver regeneration (Bresnick *et al*, 1964; Labow *et al*, 1969). We have also reported an increase in the activity of thymidine kinase, a rate-limiting enzyme for DNA synthesis, during liver regeneration (Waliuala Mola *et al*, 1996; Tessy *et al*, 1997)

Brain Neurotransmitters and Receptors in Liver Regeneration

Partial Hepatectomy induced significant changes in the content and turn over of NE and 5-HT in the different brain regions. The NE levels were significantly higher in CC, Hypo and BS at 24 hours and 48 hours of liver regeneration. The increased content of NE in these brain regions closely correlated with the time course of DNA synthesis. After partial hepatectomy, DNA synthesis increases sharply by 18-24 hours and reaches

basal levels by 3 days. The hepatocytes respond first during regeneration and the DNA synthesis in these cells peak at 24 hours. The DNA synthesis of non-parenchymal cells occurs at 48 hours (Michalopoulos, 1990). Thus, the increased content of NE at 24 and 48 hours may be significant in influencing DNA-synthesis of parenchymal hepatocytes and non-parenchymal cells. The turnover of MHG/NE was higher in the hypothalamus at 24 hours and 48 hours after PH and the EPI/NE ratio was also lower at 48 hours indicating an accumulation of NE during these periods. The levels of MHPG (3-methoxy-4-hydroxyphenylglycol) and its sulfate ester is suggested to reflect noradrenergic nervous within the central nervous system (CNS) (Mefford, 1987; Stewart *et al*, 1994). The noradrenergic neurons within specific hypothalamic nuclei and other regions of the CNS have been shown to be associated with insulin function (Bitar *et al*, 1987). The altered noradrenergic activiy in the hypothalamus observed by us may influence liver regeneration through regulation of insulin function. We have reported that in the streptozotocin-diabetic state, there is an increase in the DNA synthesis in regenerating liver. The activity of liver thymidine kinase, a key regulatory enzyme in liver regeneration was also increased in diabetic hepatectomised rats (Waliaula Mola *et al*, 1996). Bitar *et al* (1987) documented that hypothalamic noradrenergic neurons are hyperactive in diabetes. The direct involvement of the hypothalamus in liver regeneration has also been reported. Lesions of the hypothalamic ventromedial nuclei accelerated the increase in hepatic DNA synthesis and raised the peak of thymidine incorporation after PH. These effects were completely inhibited by vagotomy (Kiba *et al*, 1994). The involvement of the sympathetic nervous system in the hypothalamic control of liver regeneration was also confirmed by the observation that lateral hypothalamic lesions increase DNA synthesis during liver regeneration. This effect was blocked by sympathectomy (Kiba *et al*, 1995). Thus, the increased noradrenergic activity of the hypothalamus observed by us can facilitate liver regeneration either directly or by regulating endocrine function.

In the brain stem, NE and EPI content was higher and the synthesis of EPI from NE as indicated by EPI/NE turnover was also higher. In the cerebral cortex increased noradrenergic activity manifested by increased NE levels and an increased MHPG/NE

turnover, indicating an accumulation of NE. The EPI synthesis appeared decreased as the EPI/NE turnover was significantly lower. All these changes were observed at 24 and 48 hours of regeneration when liver DNA synthesis was markedly higher. By 72 hours of PH, there was a reversal of these alterations to near normal levels. Thus, these changes are important signals for triggering the growth process of the liver.

We then addressed the changes in the alpha adrenergic receptors of these brain regions during the period of hepatocyte DNA synthesis, to further confirm the functional significance of these alterations. In the hypothalamus, α_1 and α_2 adrenegic receptors showed reciprocal changes. There was a decrease in the number of α_1 adrenergic receptors, while the α_2 receptors increased 24 hours after PH. There was no change in the affinity of both the receptors. The accumulation of NE may be leading to a down-regulation of the post-synaptic α_1 adrenergic receptors. Geynet et al,(1981) have reported that NE is more potent in recognising α_1 adrenergic receptors. Down-regulation of biological response despite adequate concentration of the activating ligand, may be due to phosphorylation of the adrenergic receptor (Sibley et al, 1984). A similar down regulation of the α_1 adrenergic receptors of the regenerating liver has also been observed in our studies 24 hours post hepatectomy. The down regulation of hypothalamic and hepatic α_1 adrenergic receptors, may be thus significant in the sympathetic regulation of liver regeneration. The autonomic centers of the hypothalamus integrate neurohormonal function through the autonomic nervous system (Oomura and Yoshimatsu, 1984). The hepatic sympathetic nerve (Nobin et al, 1978) can thus co-ordinate the changes in the hypothalamus with those occurring in the regenerating rat liver. The $\alpha 1$ adrenergic receptors of the rat brain are located almost post synaptically and are coupled to PLC and PI turnover. $\alpha 2$ receptors are located pre and post synaptically. They are coupled to adenylate cyclase and, independent of this action, either block Ca^{2+} channels or open K^+ channels (Nicoll et al, 1990). The $\alpha 2$ receptors of the hypothalamus increased 24 hours after PH. This may be due to the increased NE accumulation and a decreased synthesis of EPI. The pre synaptic α_2 receptors are autoreceptors and they regulate the release of NE

from presynaptic nerves into the synaptic cleft. The epinephrine level is thus regulated by these receptors (Mefford, 1987). The increased α_2 adrenergic receptors in the hypothalamus may reflect a feed back regulation of NE release and an increased sensitivity to EPI. Studies using inhibitors of NE synthesis or α-adrenergic blockers have established a stimulatory role for NE in the control of TRH-mediated TSH secretion (Mannisto *et al*, 1979). α-adrenergic stimulation normally increases growth hormone (GH) secretion *in vivo*. GH accelerated hepatic regeneration in the rat by promoting early initiation of the HGF gene expression (Ekberg *et al*, 1992)

In the brain stem, though the NE content was higher 24 and 48 hours post hepatectomy, this was also accompanied by an increase synthesis of EPI. These changes may be the reason for increased α_1 adrenergic receptors with lowered affinity and the lowered affinity of $\alpha2$ adrenergic receptors. These alterations of the α_1 adrenergic receptor may have an important regulatory role through direct innervation, during liver regeneration. The cerebral cortex exhibited an accumulation of NE as observed by an increased MHPG/NE ratio and a lowered rate of EPI synthesis at 24 and 48 hours of liver regeneration. The $\alpha1$ adrenergic receptors showed a desenstisation as evidenced by lowered affinity and the $\alpha2$ adrenergic receptors increased in number and had greater affinity. Accumulation of NE may be the reason for both these changes. An upregulation of post-synaptic $\alpha2$ adrenergic receptors can also occur in response to decreased EPI synthesis.

The 5-HT content was higher in the hypothalamus of hepatectomised rats, with decreased degradation to HIAA, throughout the active period of hepatic DNA synthesis. The high affinity serotonin receptors were decreased as evidenced by a lowered B_{max}. Previous reports have referred to [^{3}H] serotonin as a selective S1 receptor agonist in the high affinity concentration (Paulose & Dakshinamurti, 1985). In the cerebral cortex, 5-HT accumulation was higher at 24 hours of regeneration and this was reversed by 48 hours. The serotonin receptor number decreased at 24 hours after partial hepatectomy. In the

brain stem, though the 5-HT content was high, there was no net change as the increased synthesis was balanced by increased degradation. The serotonin receptor number increased at 24 hours post hepatectomy in the brain stem. Thus, a down regulation of high affinity serotonin receptors invariably occurred in the hypothalamus and the cerebral cortex where there was an accumulation of 5-HT. On the other hand, an increased serotonergic responsiveness was observed in the brain stem. While the former changes may be important in the endocrine regulation of liver regeneration by serotonergic neurons, the latter seems to be significant in regulation by direct innervation. It is also interesting to note that high affinity serotonin receptors exhibited changes that were more or less similar to those of the α_1 adrenergic receptors of the three brain regions. These changes were in general reciprocal to that of α_2 adrenergic receptors.

Neurotransmitters such as 5-HT and γ-aminobutyricacid (GABA) play an important role in regulation of endocrine function (Dakshinamurti *et al*, 1990; Paulose & Dakshinamurti, 1985). The central regulation of thyroid hormone secretion by monoamine neurotransmitters has been reported (Smythe *et al*, 1982). Hypothalamic serotonin content was decreased in hypothyroidism of pyridoxine-deficient rats (Dakshinamurti *et al*, 1984, 1985). Significant changes in the brain serotonin receptors were observed in the hypothyroid state, highlighting the importance of the serotonergic receptors function in regulating the thyroid status (Dakshinamurti *et al*, 1990; Paulose & Dakshinamurti, 1985). Regulation of the release of stimulatory or inhibitory factors by the hypothalamus involves complex neural circuiting in which serotonergic neurons also represent links in the control mechanisms (Dakshinamurti *et al*, 1988). The stimulatory or inhibitory factors of the hypothalamus regulate the secretion of adrenocorticotropic hormone (ACTH), prolactin and thyroid stimulating hormone (TSH). Decreased hypothalamic serotonin content caused a decrease in TSH secretion suggesting that serotonergic neurons stimulate TSH secretion (Dakshinamurti *et al*, 1985; 1986; 1990; Chen & Ramirez., 1981; Smythe *et al*, 1983). Thus, the serotonergic system is essential for maintaining the thyroid function. The mitogenicity of thyroid hormones in liver regeneration has been established by our group and others (Tessy *et al*, 1997, Francavilla

et al, 1994). Hypothyroid hepatectomised animals showed diminished rate of liver DNA synthesis and a decreased activity of thymidine kinase. T_3 treatment reversed this and caused an increase in the affinity of the enzyme. T_3 also acted as a direct mitogen by increasing DNA synthesis and TK activity in the intact liver, reflecting its potential cause direct hyperplasia (Tessy *et al,* 1997).

Thus from our observations, we conclude that the changes in the central adrenergic and serotonergic function may be important in co-ordinating the neurotransmitter-receptor mediated regulation of liver cell proliferation. The alterations in the adrenergic and serotonergic receptors can influence hepatocyte DNA synthesis via direct and indirect mechanisms. Early evidence for the involvement of adrenergic agents in liver regeneration largely support a positive role for catecholamines in hepatic growth, without establishing the receptor type mediating this effect (Morley & Royse, 1981). The involvement of the hypothalamus and the sympathetic nervous system in facilitating liver regeneration was recently documented (Kiba *et al,* 1995). However, the receptor and post receptor mechanisms involved were not addressed. We have studied the changes in the brain alpha adrenergic and serotonergic turnover and receptor binding parameters post-hepatectomy and to our knowledge, this is the first study to focus on this aspect of sympathetic control of liver regeneration. The hypothalamic adrenergic and serotonergic neurons are major components which play an important role in the release of releasing factors from the neurohormonal cells (Brownstein, 1977). The possible direct control of pancreatic hormone secretion by adrenergic noradrenergic and serotonergic neurons was recently demonstrated (Lowey *et al,* 1994). It has been shown that the upper brain region cerebral cortex is well in communication with the lower brain regions (Paulose & Dakshinamurti, 1985). A close association exists between the serotonergic and adrenergic nervous systems (Chen and Reith, 1995; Mongeau *et al,* 1994). Thus, studying the adrenergic receptors along with the serotonergic receptors of the brain will provide an insight into may into the sympathetic regulation of liver regeneration.

Circulating NE Levels Increase during Liver Regeneration

The increased level of of plasma NE may facilitate the mitogenicity of major growth factors such as EGF. Our results and that of others (Cruise *et al*, 1985) from hepatocyte cultures show that higher levels of NE can enhance hepatocyte DNA synthesis induced by EGF. Lindroos *et al*,(1991) demonstrated that the increase in HGF in plasma of hepatectomised rats coincides with levels of plasma levels of NE. Thus, *in vivo*, plasma NE may serve as a strong mitogenic amplifier for major growth factors. The increase in circulating NE may be the consequence of the removal of two-thirds of the liver, as the liver is the primary source of clearance and degradation of circulating catecholamines

Hepatic Neurotransmitter Receptors In Liver Regeneration

Adrenergic Receptor Changes

The α_1 adrenergic antagonist, prazosin caused a shift of the displacement of [^{3}H]NE towards the high affinity concentration range. [^{3}H]catecholamines bind to two classes of binding sites, one with a low binding capacity and high affinity for natural α-adrenergic agonists and the other with a large binding capacity and low affinity for natural α-adrenergic agonists. [^{3}H]NE binds to a finite number of higher affinity catecholamine binding sites in the liver and these binding sites display a high affinity for natural α-adrenergic agonists (Geynet *et al*, 1981). Displacement studies using prazosin indicated that high affinity α_1 adrenergic receptors are involved in the DNA synthetic phase of liver regeneration. Propranolol, a β-adrenergic blocker displaced [^{3}H] NE from higher and low affinity concent ratio ranges and caused a shift of the displacement curve to the high affinity concentration range. Our results indicate an increased expression of β adrenergic receptors during the DNA synthetic phase of liver regeneration. An elevated level of β-adrenergic receptors have been reported in hepatocytes isolated from from regenerating rat liver during the period of DNA synthesis (Sandnes *et al*, 1986). During liver

regeneration, the rat liver acquires an enhanced catecholamine-sensitive adenylate cyclase activity and the hepatocytes show an increased ability to accumulate cAMP (Bronstad & Christoffersen,, 1980). The adrenergic control of hepatic metabolism is converted from a predominantly α_1 type to a β-type mechanism (Huerta-Bahena *et al*, 1983). The catecholamine sensitive adenylate cyclase may be involved in generating the biphasic increase in cAMP in the S phase (Thrower & Ord, 1974). This prereplicative rise in cAMP, with the associated protein kinase forms a complex set of events which triggers the onset of DNA synthesis after PH (Boynton & Whitefield, 1983). The increase in the number of β-adrenergic receptors may be a part of the integrated set of changes including increased portal blood concentration of glucagon and transiently reduced activity of cyclic nucleotide phosphodiesterase which together influence the elevation of intracellular cAMP levels (Leffert *et al*, 1978). Increased number of β-adrenergic receptors have also been found in the early stages of hepatocarcinogenesis (Christofferson & Berg, 1975) Adrenergic receptor involvement in generation of cAMP may be particularly importrant as they are important in conveying signals via the sympathetic nervous system (Morley and Royse, 1981). Increased β-adrenergic responsiveness has been found to result from either an increase in the receptor number, or, alternatively, from a more efficient receptor-cyclase coupling (Riles *et al*, 1984). An increase in β-adrenergic receptors have also been reported in hepatocyte preparations at 48-72 hours after PH and post-receptor changes have been suggested as a basis for the enhanced β-adrenergic influence on hepatic metabolism in regenerating liver (Huerta Bahena *et al*, 1983). The rise of the catecholamine-sensitive adenylate cyclase activity and cAMP response, which develops in hepatocytes during primary monolayer culture has been found to occur in conjunction with both an increase in the β adrenergic receptors and a reduced function of G_i protein (Nakamura *et al*, 1984). The increase in β adrenoreceptors, thus, may be a major factor responsible for the rise in β adrenergic responsiveness during liver regeneration.

Assay of the kinetic parameters of hepatic α_1 adrenergic receptors showed that during regeneration, there is a decrease in the number of receptors with no change of

affinity. Our results are concordent with the findings of Sandnes *et al*, (1986) where a 35% decrease of [³H] Prazosin binding has been reported at 18-24 hour in crude particulate fractions from male rat liver. In hepatocytes isolated from control and regenerating livers, no significant change in the α_1 adrenergic receptors were observed until 48 hours after PH. A down regulation of α_1 adrenergic receptors was observed at 48-72 hours after PH (Cruise *et al*, 1989). Plasma membrane preparations from female rats did not demonstrate reduced binding capacity at 72 hours after PH (Huerta-Bahena *et al*, 1983). Differences in the methodology (whole cells vs. membrane preparations) and the significant sex differences in hepatic adrenergic receptor pathways in the rat (Studer & Borle, 1984) may account for the differences in the observations.

Our results on the adrenergic receptor alterations in the regenerating liver 24 hours after PH show that while α_1 adrenergic receptors decreased $\alpha2$ and β adrenergic receptors increased compared to control liver. Reciprocal changes in the expressin of α_1 and β-adrenergic receptors have been demonstrated to occur in primary cultures of rat hepatocytes. There was an increase in the β adrenergic response and receptor number, while α_1 adrenergic receptors decreased (Kunos *et al*, 1995; Nakamura *et al*, 1984a). The rat ascites hepatoma cell line AH 130 is characterised by an increase in α_2 and β adrenergic receptors and a concomitant decline in α_1 receptors (Sanae *et al*, 1989).

GTP analog caused a decreased in the affinity of α_1 adrenergic receptors to their natural agonist, NE in control and regenerating liver. This indicates a desensitisation of the α_1 adrenergic receptors on G-protein asssociation. In the regenerating liver G protein association also causes a decreased displacement of [³H] Prazosin by -(-) NE. These results further indicate that high affinity α_1 adrenergic receptors are involved in the regenerating liver and G-protein association may precede their downregulation. This is also supported by the decreased B_{max} of [³H] Prazosin binding in liver. The coupling of α_1 adrenergic receptors to PLC occurs through a G protein (Kunos *et al*, 1995) . The association of ras oncoprotein which is a membrane associated G protein, with the α_1

adrenergic receptor has been reported in the regenerating liver. (Cruise *et al,* 1989). Attenuation of biological response despite adequate concentration of activating ligand has been reported for adrenergic receptors and may be the result of receptor phosphorylation and subsequent uncoupling of the receptor (Sibley *et al,* 1984). Hence the downregulation observed by us maybe due to the activation of PKC by diacyl glycerol produced as a result of PIP_2 turnover. This may cause negative feed back regulation of α_1 adrenergic receptors through the phosphorylation of these receptors (Lee-Lundberg *et al,* 1985). Phorbol-12-myristate 13-acetate (PMA) has been shown to cause a downregulation of $\alpha_1$adrenergic receptors in hepatocytes. (Beeler & Cooper, 1995). PMA has been shown to activate PKC (Maloney & Azzi, 1989) and this further supports the role of PKC in receptor desensitisation. A feed back inhibition of α_1 adrenergic receptors seems to be an important part of the α_1-adrenergic regulation of liver cell division. The absence of the α_1 adrenergic receptor m RNA in hepatoma cells and lack of expression of these receptors on the membranes in human hepatocellular carcinoma cell line, Hep G2 (Kost *et al,* 1992) further lends support to the necessity of the α_1 adrenergic receptor to prevent deregualtion of liver cell proliferation. The α_1 adrenergic receptor mediated activation of PKC (Exton, 1988) can also lead to heterologous regulation of major growth factor receptors such as EGFR (Cruise *et al,* 1986). The normal and regenerating liver do not express the α_{1a} receptor subtype while α_{1b} adrenergic receptor is expressed and is involved in the comitogenic response of NE (Kost *et al,* 1992). Expression of the α_{1b} AR gene in the rat liver is controlled by hormonal and developmental factors as well as by conditions associated with hepatocyte dedifferentiation (Rossby & Cornett, 1991). Such regulation has been shown to occur under many conditions including PH (Kunos *et al,* 1995) during primary culture (Ishac et al, 1992), in response to phorbol esters (Hu et al, 1993) and cAMP (Kanasaki *et al,* 1994). In the regenerating liver a transcription factor called Nuclear factor 1 (NF1) activates the transcription of the rat by interacting with its promoter in the α_{1b} AR gene. A decline in the expression of NF1 has been shown to be one of the mechanisms for the downregulation of the transcription and expression of the α_{1b} AR gene during liver

58

regeneration (Gao et al, 1996). Transforming growth factor β (Rossi *et al*, 1988) and protooncogenes like c-myc and Ha-ras (Andres *et al*, 1988; Lubon & Henninghausen, 1988) are known to regulate the activity of NF1. Since Ha-ras protooncogene is activated after PH (Fausto & Mead, 1989) and activation of this gene has been shown to destabilise NF1 mRNAS (Nebl *et al*, 1994), Ha-ras may be one of the factors that down regulates NF1 expression in the regenerating liver. As NE is a strong comitogen for hepatocytes, the decline in α_1 b AR expression in the early stages of the regnerative response may serve to turn off a mitogenic signal and limit the extent of hepatocyte proliferation. A failure to suppress the expression of hepatic α_1 b AR after PH may promote abnormal liver growth and abberant differentiation. The over expression of $\alpha_{1\,b}$ AR induced agonist-dependent focus formation and tumor formation (Allen *et al*, 1991).

The α_2 adrenergic receptor blocker, yohimbine did not cause any significant displacement of [^{3}H] NE in the control liver. At 24 hours of liver regeneration however, there was an increased displacement of [^{3}H] NE in the high affinity concentration range, indicating that α_2 -adrenergic receptors are relatively higher in the regenerating liver compared to the quiescent state. Hoffman *et al* (1980) reported that the liver contains fewer α_2 receptors and the α_1 receptors are more predominant. However, our results from *in vitro* studies showed that yohimbine did not block NE - mediated increase in hepatocyte DNA synthesis. Hence the observed increase in α_2 adrenergic receptors may not contribute directly to mitogenicity but the change on the ratio of α_1 and α_2 adrenergic receptors may be important in triggering DNA synthesis and cell division during liver regeneration.

Serotonergic receptor changes

At 24 hours of liver regeneration , when the DNA synthesis is markedly elevated, there was a significant elevation in the number of hepatic serotonin receptors. These receptors exhibited a decreased affinity for [^{3}H]5-HT. The increase in serotonin receptors during the DNA synthetic phase of liver regeneration was further supported by increased

displacement of [^{3}H]5-HT by cold serotonin. Ketanserin caused a significant displacement of [^{3}H]5-HT in the regenerating liver compared to the control in high and low affinity concentration ranges, indicating an increased presence of the 5-HT$_2$ receptors during the regenerative response. Spiperone caused an increase displacement of [^{3}H]5-HT at higher or, low affinity range of concentrations. The affinity of the 5-HT$_2$ receptors was increased during the period of DNA synthesis as observed by a shift of the displacement curve towards the high affinity concentration range. Studies on the role of neurotransmitters as modulators of hepatocyte division have focused on norepinephrine effects. NE has been shown to antagonise the inhibitory effects of (TGF-β), on DNA synthesis of cultured rat hepatocytes (Houck *et al*, 1988). NE enhanced the mitogenicity of EGF by causing a down-regulation of EGF receptors (Cruise *et al*,1988). Serotonin has been found to promote cell proliferation in various cell types. The 5-HT$_2$ receptors have been shown to mediate the mitogenicity of 5-HT in fibroblasts.(Van Obberghen-Schilling *et al*, 1991). The 5HT$_2$ receptor in the human liver was cloned and it has a high degree of homology with that of rat and mouse 5-HT$_2$ receptors (Bonahus *et al*, 1995). Ketanserin is reported to be a 5-HT$_2$ antagonist. Spiperone can recognise both 5-HT$_1$ and 5-HT$_2$ receptors in general. Receptors antagonised by both these are identified as 5-HT$_2$ receptors (Hoyer *et al*, 1994). The 5-HT$_2$ receptor activates phospholipase C. This receptor activation can lead to increase in phospho-inositide metabolism and hence an increased intracellular Ca^{2+} and possible activation of protein kinase C (de Courcelles *et al*, 1985). Increased release of Ca^{2+} was found to be important for hepatocyte division (Rixon *et al*, 1976; 1989). Protein kinase C, being a target for tumor promoters like phorbol esters, is known to be an important second messenger for cell growth and division (Weinstein,1983). The 5HT$_2$ receptors are present in many tissues and have the potential to activate second messengers required for cell growth and division and hence their role as potential regulators of hepatocyte proliferation merits further study.

The study of hepatocyte proliferation in cultures has several well recognised advantages compared to studies of *in vivo* regeneration. The hepatocytes grow in a controlled environment virtually without interference from other cell types. Growth modulators added to culture medium act directly on the hepatocytes and interference of other factors such as hormones can be excluded. The recognition of key factors involved in liver regeneration was achieved primarily by using hepatocyte cultures in serum-free medium (Michalopoulos, 1990). A large number of viable hepatocytes can be produced by perfusing the rat liver with collagenase (Seglen, 1976). New and refined techniques for separation of specific cell types of the liver have also been developed, like fluorescence-activated cell sorting (FACS), free-flow electrophoresis (FFE), counter flow elutrition (CFE)and isopycnic gradients (Alpini *et al*, 1994)

Isolated hepatocytes in primary culture require insulin in the absence of which they rapidly degenerate within 24-48 hours (Michalopoulos, 1990). Two fundamental requirements needed for eliciting mitotic response in hepatocytes by growth stimuli are proline (Houck & Michalopoulos , 1985) and plating of hepatocytes at low cell density. The entry of G_0 cells into G_1 phase is regulated by cell density and a low cell density may permit cells to enter M phase after DNA synthesis. Primary cultures of rat hepatocytes can be used for liver regeneration studies and the optimal conditions have been described (Michalopoulos *et al*, 1982). Most of the replicating hepatocytes enter into multiple consecutive rounds of DNA synthesis and this replicating system of hepatocytes can be used to investigate the trophic factors that control growth of normal and neoplastic hepatocytes.

Mitogenicity of Norepirephrine and Serotonin

The level of DNA synthesis in rat hepatocytes was minimal when they were

cultured in Williams medium E, which specifically enriches hepatocytes, in the presence of insulin (10^{-7} M). Primary cultures of adult rat hepatocytes retain many liver functions and have been used for biochemical studies on liver function. However, hepatocytes are unable to replicate in such a medium unlike cells such as fibroblasts, despite the fact that these cells easily undergo proliferation after PH. Addition of EGF however, caused a marked increase of DNA synthesis from basal levels. Insulin was present throughout the culture as it is a positive modulator of hepatocyte DNA synthesis, and its absence leads to cell death. Insulin is required for the full magnitude of EGF - stimulated mitogenesis (McGowan et al, 1981, Michalopoulos, 1990). DNA synthesis assay was done 48 hours after plating the hepatocytes in serum-free medium. When EGF is added to cultures of freshly isolated hepatocytes, DNA synthesis does not start for 24 hours. The peak of DNA synthesis occurs from 48-72 hours and hence we chose 48 hours of culture to study the mitogenic effects. The time lag in culture is in contrast to DNA synthesis in liver regeneration, which starts at 12-16 hours and peaks at 24 hours. The difference in time course might reflect repair processes after collagenase perfusion and adaptation of hepatocytes to the in vitro environment. Addition of 5-HT or NE alone (50 µM) did not elicit any significant increase in DNA synthesis. NE has already been reported to be a comitogen for hepatocytes (Cruise et al, 1985). Our results show that though 5-HT is not a hepatocyte mitogen per se, it is comitogenic, enhancing the DNA-synthesis induced by EGF. 5-HT and NE at concentration of 1 µM, added along with EGF caused a significant increase in DNA synthesis from basal levels and also when compared to EGF treated cultures.

Dose dependent induction of DNA synthesis by NE

In order to confirm the effect of NE on the increase in DNA synthesis of EGF-treated cultures, we added increasing concentrations of NE to cultures containing EGF (10ng /ml). Lower concentrations of NE did not elicit a significant response while higher concentrations produced a marked dose-dependent increase in DNA synthesis. Our results are concordant with previous reports (Cruise et al, 1985)

Adrenergic blockers and DNA Synthesis in culture:

The involvement of the adrenergic receptor subtypes of the hepatocytes in the process of stimulation of DNA synthesis by NE was studied by using receptor antagonists. Prazosin, an α_1 adrenergic receptor antagonist, caused a significant reduction in NE-induced DNA synthesis. Prazosin also reduced the DNA synthesis induced by EGF. Thus, the α_1 adrenergic receptor function is required for EGF and NE-mediated DNA synthesis. The α_2 adrenergic receptor yohimbine and β adrenergic receptor blocker, propranolol did not cause significant reduction in the DNA synthesis. Thus, the α_1 adrenergic receptor is directly involved in influencing hepatocyte DNA synthesis. These results are concordent with previously published reports (Cruise *et al*, 1985). NE has been suggested to cause heterologous down regulation of the EGF receptor by downstream phosphorylation events mediated by the α_1-adrenergic receptors (Cruise *et al*, 1986). Downregulation of the EGF receptor has been shown to be important for liver regeneration (Woollenberg et al, 1989). The α_1 adrenergic receptor is also involved in decreasing the DNA -synthesis inhibition produced by TGF β (Houck *et al*, 1988).

5-HT induces a dose-dependent increase of hepatocyte DNA synthesis

The potential of 5-HT to induce DNA synthesis in cultured hepatocytes was further confirmed by using different doses of 5-HT against a single concentration of EGF. Increasing concentrations of 5-HT caused an increase in the DNA synthesis. This is the first report of stimulationof DNA synthesis in primary cultures of rat hepatocytes by 5-HT. The effect of 5-HT on DNA synthesis was comparable to that of NE in cultured hepatocytes 5-HT has been shown to be mitogenic in other non-neural cells including fibroblasts and muscle cells (Nemeck, *et al*. 1986; Seuwen *et al*, 1988).

Serotonergic receptor blockers and hepatocyte DNA synthesis:

The serotonergic receptor subtype involved in mediating the mitogenicity of 5-HT on hepatocyte were studied by using 5-HT receptor antagonists, ketanserin and spiperone. Ketanserin, a 5-HT$_2$ receptor blocker caused a significant reduction in the DNA synthesis at low and high concentrations of 5-HT. Spiperone can recognise both 5-HT$_1$ and 5-HT$_2$ receptors in general and it binds only to 5-HT$_1$ receptors at high affinity concentrations. Lower doses of spiperone was ineffective in reducing DNA synthesis while higher concentrations of the drug caused a significant reduction in 5-HT mediated DNA synthesis. Receptors antagonised by both these drugs are identified as 5-HT$_2$ receptors (Hoyer *et al*, 1992; 1994). These results signify that the 5-HT$_2$ receptors in hepatocytes mediate the DNA synthesis induction by serotonin. The 5-HT$_2$ receptor activates phospholipase C, possibly resulting in increased phosphoinositide metabolism and activation of PKC (de Courcelles *et al*, 1985). PKC is a well recognized second messenger mediating cell division (Weinstein, 1983). The 5-HT$_2$ receptors have been shown to mediate cell growth in fibroblasts (Van Obberghen-Schilling *et al*, 1991).

Our results suggest that serotonin can enhance DNA synthesis in cultured hepatocytes and this is mediated through the 5-HT$_2$ receptor. Receptor kinetics and displacement studies show that serotonin receptors increase during the proliferative phase and that the 5-HT$_2$ receptors are more involved during the DNA synthetic phase. Further studies are required to clarify the exact subtype of the 5-HT$_2$ receptor involved and the mechanisms by which this receptor exerts its regulatory effects on hepatocyte growth and division.

This is the first report of the involvement of 5-HT$_2$ receptor subtype in regulating hepatocyte DNA synthesis. The dynamics of the interaction of 5-HT with EGF and insulin and the molecular signals involved in 5-HT mediated mitogenicity in the liver have to be further investigated. This will lead to the knowledge of regulation of gene expression

during the transition of cells from quiescence to proliferation and will help to elucidate the mechanisms leading to deregulation of the cell cycle.

DNA, synthesis stimulation by epinephrine has already been reported, in the presence of insulin and EGF. This effect of EPI was also reported to be strongly inhibited by prazosin, indicating the involvement of the α_1 adrenergic receptor. But, the mechanism of EPI action did not involve PKC activation or Ca^{2+} mobilisaton. (Takai *et al*, 1988). The integrated function of catecholamines and other putative neurotransmitters explain the activation of second messenger systems influencing the gene expression during the control of cell growth and proliferation.

Protein Kinase C Activation During Hepatic DNA Synthesis

PKC is known to be activated by tumor promoters, making it an important second messenger of many signalling pathways that mediate cell division (Weinstein, 1983). Our studies confirmed that the mitogenicity of NE was mediated by the α_1 adrenergic receptor. We also report that 5-HT stimulates hepatocyte DNA synthesis acting through 5-HT$_2$ receptor. Protein Kinase C was studied as it is the potential second messenger to be activated by these two receptors (de Courcelles *et al*, 1985; Exton, 1988). This enzyme showed an increase in maximal velocity (V_{max})in the regenerating liver membrane. .The activation of this enzyme can lead to downregulation of major growth factor receptors such as EGFR and mitogenic regulators (Cruise *et al*, 1986) such as α_1 adrenergic receptors (Beeler & Cooper, 1995) by phosphorylation. The increased activity of PKC during the DNA synthetic phase of liver regeneration may thus account for decrease in α_1 adrenergic receptors observed by us. Furthermore, this increase in the activity of membrane PKC reflects the enhanced responsiveness of the 5-HT$_2$ receptors and substantiates our finding of increased 5-HT$_2$ receptors during the S phase of the hepatocyte cell cycle.

5-HT₂ Receptors And α_1 Adrenergic Receptor-Mediated Liver Membrane Protein Phosphorylation

Endogenous PKC-dependent phosphorylation was higher in the 24-hour regenerating liver membrane compared to control and this is consistent with our observation of increased activity of PKC in the membrane fraction of the liver. A 40KD protein was specifically enhanced in the PKC-mediated endogenous phosphorylation in the regenerating liver membrane. Interestingly, 5-HT also enhanced the phosphorylation of this 40KD membrane protein in the regenerating liver. Ketanserin, the 5-HT₂ receptor blocker brought about a decrease in the 5-HT induced phosphorylation of this protein. When ketanserin was added along with 5-HT, there was a conspicuous decrease in the phosphorylation of the 40KD protein in the control and regenerating liver membrane. The 5-HT₂ receptor is coupled to phospholipid turnover and induces the activation of phospholipase C (de Courcelles *et al*, 1985). The phosphorylation of a 40KD protein has been reported to be induced by 5-HT in platelets (de Courcelles *et al*, 1984). This 40KD protein was identified as protein kinase C, the second messenger of 5-HT₂ receptor. We postulate that in the membrane of the regenerating liver, serotonin mediates the phosphorylation of protein kinase C, through the 5-HT₂ receptor and this results in the activation of PKC during the DNA synthetic phase. NE also caused the enhanced phosphorylation of the 40KD membrane protein in the regenerating liver and this was blocked by prazosin. This indicates that PKC is also activated by phoshorylation mediated by the α_1 adrenergic receptor. NE also induced the phosphorylation of a 59 KD protein regenerating liver membrane fraction and this was eliminated by prazosin. The α_1 adrenergic receptor has been purified to homogeneity and was found to be a 59KD protein (Graham *et al*, 1982). From our results, it is evident that NE activates PKC and also autophosphorylates. This autophosphorylation of the α_1 adrenergic receptor may be mediated by the activated PKC itself and this can lead to the downregulation of the receptor. Indeed, the decreased number of α_1 adrenergic receptors has been confirmed in our results from scatchard analysis of these receptors in the hepatic membrane. The tumor promoter PMA, induced the phosphorylation of 59KD and 29KD membrane only in the

control liver, indicating that NE and PMA have differential role in growth regulation and cell proliferation.

CONCLUSION

We conclude from our studies that changes in adrenergic and serotonergic function of the hypothalamus and other brain regions form an important part of the sympathetic regulation of liver regeneration. Though many studies implicated the sympathetic nervous system to be an essential part of the regenerative response, the specific neurotransmitters and receptors and their regulatory function were not given emphasis. We have observed an increase in NE and 5-HT content in the CC, BS and Hypo during the period of active DNA synthesis in the regenerating liver. The changes in the content and turnover of NE and 5-HT after PH were reversed to near normal levels by 72 hours. Thus, the time course of these changes closely correlated with that of DNA synthesis in the liver. The functional significance of these changes were further explored by studying the changes in the adrenergic and serotonergic receptors of the brain. The α_1 and α_2 adrenergic receptors of the brain regions showed reciprocal changes. The hypothalamic α_1 adrenergic and high affinity serotonergic receptors exhibited a downregulation, while the α_2 adrenegic receptors were increased. The α_1 adrenergic receptors and high affinity serotonergic receptors showed similar changes in all the three brain regions studied. These alterations of the adrenergic and serotonergic receptors of the brain may govern the regenerative response of the liver through direct innervation or by regulating the availability of major growth factors and hormones. The hepatic α_1 adrenergic receptor were also down regulated during liver regeneration. The PKC-dependent autophosphorylation of the α_1 adrenergic receptor of the regenerating liver was also demonstrated and this accounts for the receptor down-regulation. There was an increased involvement of the serotonin 5-HT$_2$ receptors of the liver during the period of active DNA synthesis in the regenerating liver. Protein Kinase C, the potential second messenger to be activated by the α_1 adrenergic receptor and the 5-HT$_2$ receptor showed an increased activity in the membrane fractions of the regenerating liver. The activation of PKC in the

67

regenerating liver was further confirmed by protein phosphorylation assays. 5-HT induced DNA synthesis in in primary cultures of rat hepatocytes in the presence of EGF and insulin in a dose-dependent manner. The mitogenicity of 5-HT in cultured hepatocytes was seen to be mediated DNA by the 5-HT$_2$ receptor. The ability of 5-HT to stimulate hepatocyte DNA synthesis was comparable to that of NE, which is a known hepatocyte co-mitogen. We also found that the 5-HT$_2$ receptor mediated a direct activation of PKC in the regenerating liver during the DNA synthetic phase of the hepatocyte cell cycle. As the 5-HT$_2$ receptor can activate PKC, a target for tumor promoters, its potential as a regulator of hepatic cell proliferation merits further study. Thus, the adrenergic and serotonergic receptor mediated mechanisms in the brain and regenerating liver may exert a profound influence in regulating the transition of hepatocytes from quiescence to proliferation .

SUMMARY

I) Liver regeneration after partial hepatectomy of rats was used as an *in vivo* model to study controlled cell proliferation.

ii) Primary cultures of rat hepatocytes were used as the *in vitro* system to study liver regeneration.

iii) [^{3}H] Thymidine incorporation into the hepatic DNA was used as the index to study DNA synthesis in the regenerating liver and in cultured hepatocytes. In the regenerating liver, the maximal DNA synthesis was observed at 24 hours after PH.

iv) The role of neurotransmitters, receptors and second messengers in regulatory control of DNA synthesis and cell proliferation was studied *in vivo* and *in vitro*, with emphasis on adrenergic and serotonergic function.

v) In the brain, significant changes were observed in the content and turnover of NE and 5-HT in CC, BS and Hypo during the period of hepatic DNA synthesis. α_1 and α_2 adrenergic receptors of these brain regions showed reciprocal changes. Changes of high affinity serotonin receptors were comparable to α_1 adrenergic receptors. The hypothalamic α_1 adrenergic receptors and serotonergic receptors exhibited a down regulation at 24 hours of liver regeneration.

vi) There was a downregulation of hepatic α_1 adrenergic receptors and an increased involvement of serotonin 5-HT$_2$ receptors in the regenerating liver, during the peak of DNA synthesis.

vii) 5-HT$_2$ receptor involvement in mediating the mitogenicity of 5-HT was confirmed in hepatocyte cultures. Serotonin induced a dose-dependent increase in the DNA synthesis of cultured hepatocytes. The mitogenicity of 5-HT was comparable with that of NE.

viii) PKC, the potential second messenger of both α_1 adrenergic and 5-HT$_2$ receptors showed an increased activity in the regenerating liver membrane, during the period of active DNA synthesis. Membrane protein phosphorylation assays showed an increase in the endogenous PKC-dependent phosphorylation in the regenerating liver

ix) The autophosphorylation of the α_1 adrenergic receptor by a PKC-dependent mechanism was shown and this is the cause of the observed downregulation of these receptors in the regenerating liver.

x) Direct evidence for the phosphorylation and activation of PKC, by the 5-HT$_2$ receptor of the liver was also obtained during the S-phase of the hepatocyte cell cycle. All these results confirm that 5-HT acts as a co-mitogen and can induce DNA synthesis in the regenerating liver after partial hepatectomy. This effect of 5-HT is mediated by the hepatocyte 5-HT$_2$ receptor and at least one of the mechanisms in the mitogenicity of 5-HT in the liver involves the activation of PKC-dependent signal transduction.

References

REFERENCES

Akerman, P.A., Cote, P, Yang, S.Q., Mc Clain, C., Nelson, S, Bagby, G.J., and Diehl, A.M. (1992) Antibodies to tumor necrosis factor-α inhibit liver regeneration after partial hepatectomy. Am.J.Physiol. 263: C579-C585.

Albrecht,J.H., Hoffman, J.S., Kren, B.T., Steer, C.J.(1993) Cyclin and cyclin-dependent kinase 1 m RNA expression in models of regenerating liver and human liver disease. Am. J.Physiol.265, C857-C864.

Allen, L.F., Lefkowitz, R.J., Caron, M.G., and Cotecchia, S.(1991) G.Protein coupled receptor genes as proto-oncogenes: constitutively activating mutation of the α_{1B} - adrenegic receptor enhances mitogenesis and tumorigenicity, Prof. Natl. Acad. Sci. USA 88: 11354-11358.

Almendral, J.M., Somma, D., Mac Donald-Bravo, H., Bruckhardt, J., Perera.J., and Bravo, R. (1988) Complexity of early genetic response to growth factors in mouse fibroblasts. Mol.Cell Biol. 8; 2140-2148.

Alpini M., Phillips O.J., Veroman B., and Larusso N.F. (1994). Recent advances in isolated liver cells. Hepatology 20: 494-514.

Andres, A.C., Van der Valk, M.A., Schonenberger, C.A., Fluckiger, F., Lemeur, M.,. Gerlinger, P., and Groner, B. (1988) Ha-ras and c-myc oncogene expression interferes with morphological and functional differentiation of mammary epithelial cells in double and single transgenic mice. Genes Dev. 2: 1486-1495.

Ashrif S.,Gillespie J.S.,Pollack D. (1974) The effect of drugs on denervation on thymidine uptake into rat regenerating liver. Eur J Pharmacol 29: 324-327.

Baffy, G., Yang, L. Michalopoulos, G.K., and Williamson, L.R. (1992) Hepatocyte

growth factor induces calcium mobilisation and inositol phosphate production in rat hepatocytes.J.Cell. Physiol. 153: 332-339.

Beeler J.F.and Cooper R.H.(1995). Regulation of hepatocyte plasma membrane alpha1-adrenergic receptors by 4beta-phorbol 12-myristate 13-acetate.Biochem.J.305:73-79.

Beguinot, L., Hanover,J.A., Ito,S., Richert, N.D., Willingham,M.C., and Pastan,I.(1985) Phorbol esters induce transient internalization without degradation of unoccupied epidermal growth factor receptors. Proc. Natl. Acad. Sci. U.S.A., 82: 2774-2778.

Bever, H.S., Stanley, M., and Theoglides, A (1990) Tumor necrosis factor α increases hepatic DNAand RNA and hepatocyte mitosis Biochem. Int. 22: 405-410.

Bevilacqua, M., Norbiato, G., Chebat, E., Baldi, E, Bertora, P., Regalia.E , Colella, G., Gennari, L. and Vago, T.(1991). Cancer 67: 2543-2551.

Bitar, M.S., Kouhu, M., and Linnoila, M. (1987) Diabetes-induced changes in monoamine concentrations of rat hypothalamic nuclei. Brain Research 409: 236-42.

Bonahus, D.W; Bach, C; De Souza, A; Salazai, F.H; Matsuoka, B.D; Zuppan, P., Chan, H.W; Eglen, R.M. (1995) The pharmacology and distribution of human 5-hydroxytryptamine 2 B (5-HT 2B) receptor gene products: Comparison with 5-HT 2A and 5-HT 2C receptors Br.J.Pharmacol Jun: 115: 622-628.

Bottaro D.P., Rubin S.J., Faletto D.L., chan A.H.L., Kmiecik T.E., wounde G.F.V and Aaronson S.A.(1991). Identification of hepatic growth factor receptor as the c-met proto-oncogene product. Science 251 : 802-804.

Braun, L., Mead, J.E., Panzica, M., Mikumo, R., Bell, G.I., and Fausto, B. (1988) Transforming growth factor β mRNA increases during liver regeneration: a possibe paracrine mechanism of growth regulation. Proc. Natl. Acad. Sci. U.S.A 85: 1539-1543.

Brenner, D.A., Koch, K.S., and Leffert, H.L.(1989) Transforming growth factor-alpha stimulates proto-oncogene c-jun expression and a mitogenic program in primary cultures of adult rat hepatocytes. DNA 8: 279-285.

Bresnick, E., Mainigi, K.D, Buccino, R. (1971) Deoxythymidine kinase of regenerating rat liver and Escherichia coli Cancer Res. 30: 2502-2506.

Bronstad G. and Christoffersen T.(1980). Increased effect of adrenaline on cyclic AMP formation and positive adrenergic modulation of DNA-synthesis in regenerating hepatocytes. FEBS.Lett. 120:89-93.

Brown, K.D., Blay, J.,Irvine,R.F.,Heslop,J.P.,Berridge, M.J.(1984). Reduction of epidermal growth factor receptor affinity by heterologous ligands:Evidence for a mechanism involving the breakdown of phosphoinositidesand the activation of protein kinase C. Biochem.Biophys.Res. Commun.,123 : 377-384.

Brownstein, M. (1977) Neurotransmitters and hypothalamic hormones in the central nervous system. Fed.Proc., 36, 1960-1963.

Bucher N.L.R.(1963). Regeneration of mammalian liver. Int.Rev.Cytol 15: 245-300.

Bucher N.L.R.and Swaffield M.N.(1975). Regulation of hepatic regeneration in rats by synergistic action of insulin and glucagon. Proc.Natl.Acad.Sci U.S.A. 72 : 1157-1160.

Bucher NLR,Patel U.,Cohen S.(1978). Hormonal factors and liver growth. Adv Enzyme Regul 16: 205-213.

Bucher, N.L.R. (1991) Liver regeneration: an overview J.Gastroenterol. Hepatol. 6: 615-624.

Bucher, N.L.R. and Malt, R.A.(1971) Regeneration of Liver and Kidney, In New England Journal of Medicine, Medical Progress Series, Little Brown Co., Boston, PP 1-76

Bucher,N.L.R., Swaffield, M.N.(1975) Regulation of hepatic regeneration in rats by the synergistic action of insulin and glucagon Proc.Natl. Acad. Sci. USA. 72: 1157-1160.

Burton. K.(1955). A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acids. Biochem. J. 62 :315-323.

Byyny R.L., Orth .D.N., Cohen S.(1974) Epidermal growth factor: effect of androgens and adrenergic agents. Endocrinology 95 : 776-782.

Campbell, J., (1987), In "Statistics for biologists". Cambridge university press.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991). Oncogenes and Signal transduction. Cell, 64: 281-302.

Carr, B.I., Hayashi, I., Bra.. E.L., and Moses, H.L. (1986) Inhibition ofm DNA synthesis in rat hepatocytes by platelet. derived type transforming growth factor. Cancer Res: 46: 2330-2334.

Carr, B.T., Huang, T.M., Itakura, K., Noel, M., and Maiceau.N.(1989) TGFβ gene transcription in normal and neoplastic liver growth. J.Cell, Biochem. 39: 477-487.

Chen Y.F., Ramirez V.D.(1981). Serotonin stimulates thyrotropin-releasing hormone release from super fused rat hypothalamus. Endocrinol. 108 : 2359-2366.

Chen, N.H. and Reith, M.E.A. (1995) Monoamine interactions measured by microdialysis in the ventral fegmental area of rats treated systematically with 8-OH-DPAT J.Neurochem. 64 : 1585-97.

Cihak A.,Vaptzarova K.1.(1973). Decreased synthesis of DNA in regenerating rat liver after administration of resrpine.Br.J.Pharmacol 49: 253-257.

Cohen, G.B., Ren, R., and Baltimor, D.(1995) Modular binding domains in signal transduction proteins cell 80: 237-248.

Columbano, A and Shinozuka, M, (1996) Liver regeneration versus direct hyperplasia. FASEB.J, 10: 1118-1128.

Cotecchia, S., Schwina, D.A., Randall. R.R., Lefkowitz, R.J., Caron, M.G., and Koblika, B.K.(1988) Molecular cloning and expression of the cDNA for the hamster alpha-1 adrenergic receptor. Proc.Natl.Acad. Sci.U.S.A. 85: 7159-7163.

Cressman, D.E., Diamond, R.H., Taub,R. (1995) Rapid activation of stat3 DNA binding in liver regeneration. Hepatology, 21: 1443-1449

Cressman, D.E., Taub, R. (1994) Physiologic turnover of NF-$_k$B by nuclear proteolysis.J.Biol.Chem. 269: 26594-26597.

Cressman. D.E., Greenbaum, I.E., Haber, B.A.,Taub, R.(1994) Rapid activation of PHF/NF$_k$B in hepatocytes, a primary response in the regenerating liver J.Biol.Chem. 269: 30429-30435.

Cruise J.L., Houck, K.A., and Michalopoulos, G.K.(1985). Induction of DNA synthesis in cultured rat hepatocytes through stimuation of α_1 Adrenoreceptor by norepinephrine. Science, 227: 749-751.

Cruise J.L., Muga S.J.,Lee Y.and Michalopoulos G.K. (1989). Regulation of hepatocyte growth: Alpha-1 adrenergic receptor and ras p21 changes in liver regeneration. J.Cell.Physiol. 140:195-201.

Cruise J.L.,Knectle S.J.,Bollinger R.R.,Kuhn C.and Michalopoulos G.(1987).Hepatology 7:1189-1194.

Cruise, J.L. and Michalopoulos, G. (1985) Nerepinephrine and epidermal growth factor: Dynamics of their interaction in the stimulation of hepatocyte DNA synthesis. J.Cell. Physiol. 125: 45-50.

Cruise,J.L.,Knechtle,S.J., Bollinger,R.R.,Kuhn, C., and Michalopoulos, G. (1987) Alpha1-adrenergic effects and regeneration. Hepatology, 7:1189-1194.

Dakshinamurti K., Paulose C.S., Viswanathan M., Siow, Y.L. and Vriend, J. (1990) Thyroid function in pyrodoxine -deficient young rats. Ann. N.Y. Acad. Sci., , 585, 128-144

Dakshinamurti K., Paulose C.S., Viswanathan M.and Sion Y.L.(1988) Neuroendocrinology of pyridoxine deficiency. Neurosci and Behav.Rev. 12:189-193.

Dakshinamurti, K., Paulose C.S., Thilveris J.A. and Vriend, J.(1985) Thyroid function in pyrodoxine deficient young rats. J Endocr. 104: 339-344.

Dakshinamurti, K., Paulose, C.S., and Vriend, J.(1986) Hypothyroidism of hypothalamic origin in pyridoxine dificient rats, J. Endoor, 109: 345-349.

Daoust R.and Cantero A. (1959). The numerical proportions of cell types in rat liver during carcinogenesis by 4-dimethylamino benzamine. Cancer Res 19:757-762.

Darnell, J.E., Kerr, I.M., and Stark, G.R. (1994) Jak-STAT pathways and transcriptional activation in response to interferons and other extracellular signaling. proteins. Science 264: 1415-1421.

De Courcelles, D., Roevens P. and Van Belle H. (1984) FEBS Lett. 171:289-292.
De Courcelles, D., Leysen, J.E., De clerck, F., Van Belle, H., and Janssen. A.P. (1985) Evidence that phospholipid turnover is the signal transducing system coupled to serotonin - S_2 receptor sites.J.Biol. Chem. 260: 7603-7608.

De-Mouzon S., Mrejen C., Alengrin F., Van obberghen E., (1995). Biochem J. 305:119-

124.

Delarco J.E.and Todaro G.J.(1980). Sarcoma growth factors(SGF): Specific binding to epidermal growth factor(EGF)membrane receptors. J.Cell. Physiol. 102:267-277

Denhardt, D.T., Edwards, D.R., and Parfert, C.T.J. (1986). Biochim. Biophys. Acta, 865: 83-125.

Desdouets C.,Drouin C.,Matesic N.,Foulkes N.,Molina C.,Sassone-Corsi P.,Brechot C.and Sobezak-Thepot P.(1994) Cyclin A gene regulation by the cAMP transduction pathway. Hepatology 20:217A(Abstract)

Diamond, R.H., Cressman, D.E., Laz, T.M.,Abrams, C.S., Taub, R.(1994) PRL-1, a unique nuclear protein tyrosine phosphatase, affects cell growth. Mol.Cell. Biol.14, 3752-3762.

Diehl A.H., Yang S.Q., Wolfgang D.,and Wand G.(1992). Differential expression of guanidine nucleotide-binding proteins enhances cAMP synthesis in the regenerating liver. J.Clin .Invest. 89:1706-1712.

Diehl A.M.,Rai R.M (1996) Regulation of signal transduction during liver regeneration FASEB J 10:215-227.

Diehl A.M.,Yang S.Q(1994) Regenerative changes in C/EBPalpha and C/EBPbeta expression modulate binding to the C/EBP site in the c-fos promoter. Hepatology 19: 447-456.

Divecha, N, and Irvine, R.F. (1995) Phospholipid signaling Cell. 80: 269-278.

Duboi R.N. (1994) Activin A and hepatocyte growth regulation, Hepatology 19: 88-790

Ekberg S., Luther M., Nakamura T., Jansson J.O.(1992). Growth hormone promotes

early initiation of hepatocyte growth factor gene expression in the liver of hypophysectomised rats afer partial hepatectomy. J.Endocrinol. 135:59-67.

Exton J.H.(1981) Molecular mechanism involved in α-adrenergic responses. Mol.Cell.Endocrinol 23:233-264.

Exton,J.H.(1988) Role of phosphoinositides in the regulation of liver function. Hepatology. 8: 152-166.

Fabikant, J.I. (1968). The kinetics of cellular proliferation in the regenerating liver. The Journal of Cell Biology 38:

Fausto, N., and Webber, E.M., (1994) Liver regeneration. In: The Liver-Biology and Pathobiology, Third Ed.(Arias, I.M., Boyer, J.L., Fausto, N., Jakoby, W.B., Schachta, D.A., Shafertz, D.A., eds.) pp. 1059-1084, Raven Press, New York.

Fausto,N., and Mead, M.E. (1989) Regulation of liver growth: Proto-oncogenes and transforming growth factors. Lab. Invest. 60: 4-13.

Ferenci, P., Schafer,D.F., Kleinberger,G., Hoofnagle,J.H., Jones,E.A. (1983) Serum levels of gamma-amino butyric acid-like activity in patients with acute and chronic hepatocellular diseases. Lancet 1: 811-814.

Fisher, B., Szuch,P., Levine, M., and Fisher, E.R.(1971). A Portal blood factor as the humoral agent in liver regeneration. Science 171: 575-577.

Fleig, N.E. and Hoss, G., (1989) Partial purification of rat hepatic stimulator substance and characterisation of its action on hepatoma cells and normal hepatocytes. Hepatology, 9: 240-248.

Fong Y.,Helfgott, D.C.Brenner, M.F.(1994) Interleukin-6 blockade impedes recovery

from experimental hepatectomy. Surg.Forum, 49, 190-192.

Francavilla A., Ove P., Vanthiel D.H.,Goetzee M.L.,Wu S.K.,Dileo A.and Staizl
T.E.(1984). Induction of hepatocyte stimulating activity of T3 and appearence of the
activity despite inhibition of DNA synthesis by adriamycin Horm. Metab.Res.16:273-242.

Francavilla A.,Carr B.I.,Azzauore A.,Polimeno L.,Wang Z.,Vanthiel D.H.,Subbotin
V.,Prelich J.G.,Staizl T.E.(1994). Hepatocyte proliferation and gene expression induced
by Triiodothyronine in vivo and in vitro. Hepatology 20:1237-1241.

Francavilla, A., Azzarone, A., Carrievi, G., Scotti-Foglini, C., Zeng, Q.H., and Starzl,
T.E. (1992) Effect on the canine Eck fistula liver of intraportal TGF β alone or with
hepatic growth factors. Hepatology 16: 1267-1270

Francavilla, A., Eagon, P.K., Dileo, A., Polimeno, 1, Panelia, C., Aquilino, A.M.,
Ingrosso, M., Vanthiel, D.H., and Staizl, T.E.(1986) Sex hormone-related functions in the
regenerating male rat liver. Gasteroentenology 91: 1263-1270.

Francavilla, A., Gavaler, J.S., Makowka, Barone, M., Mazzaferro, V., Ambrosino, G.,
Iwatsuki, S., Guglieimi, F.W., Diler, A, Balestrazzi, A., Vanthiel, D.H., and Sta.. ,T.E.
(1989). Estradiol and testosterone levels in patients undergoing partial hepatectomy. A
possible signal for hepatic regeneration. Dig. Dis. Sci. 34: 818-822.

Gao,B., Jiang,L. and Kunos,G. (1996) Transcriptional regulation of α1b adrenergic
receptors (α1b AR) by nuclear factor 1: a decline in the concentration of NF1 correlates
with the downregulation of α1b AR gene expression in regenerating liver Mol.Cell.Biol.
16: 5997-6008.

Gerber, M.A., Thung, S.N., Selin,S., Strohmeyer, F.W., and Ishak, K.G. (1983)
Phenotypic characterisation of hepatic proliferation-Antigenic expression by proliferating

epithelial cells in fetal liver, massive hepatic necrosis. and nodular transformation of the liver. Am.J.Pathol. 110 : 70-74.

Geynet, P., Ferry, N., Borsodi, A. and Hanoune, J. Two distinct α_1 adrenergic receptor sites in rat liver: differential binding of (-)-[^{3}H]norepinephrine, [^{3}H]Prazosin and [^{3}H]dihydroenerocryptine: effects of guanine nucleotides and proteolysis; implication for a two site model of α-receptor regulation. (1981) Eur.J.Pharmacol. 1981 30: 1665-1675.

Glowinski, J. and Iversen, L.L. (1966)Regional studies of catecholamines in the rat brain. The disposition of [^{3}H]norepinephrine [^{3}H] dopa in various regions of the brain. J.Neurochem. 13, 655-669.

Goyette, M., Petropoulos, C.Shanka,P., Fausto., N.(1984) Regulated transcription of c-Ki-ras and c-myc during compensatory growth of rat liver.

Goyettee, M., Petropulos, C.J., Shank, P.R., and Fansto, N. (1983) Expression of cellular oncogene during liver regeneration. Cell Death Diff. 3: 51-59

Graham R.M., Hess H.J. and Homcy C.J.(1982) Biophysical charecteriztion of the purified α_1-adrenergic receptor and identification of the hormone binding subunit. J.Biol. Chem. 257:15174-15181

Graziani, A., Gramaglia, D., Cantley, L.C. and Comaglio, P.M. (1991). The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol-3-kinase.J.Biol. Chem. 266: 22087-22090.

Grisham, J.W.(1962). A morphologic study of doxyribonucleic acid synthesis and cell proliferation in regenerating liver, autoradiography with thymidine-H3 Cancer Res. 22: 842-849.

Grisham, J.W., and Thorgeirsson, S.S.(1996) Liver stem cells,. In The Stem Cell

Handbook (Potten, C.S., ed) Academic Press, New York In press.

Haber, B.A., Mohn, K.L., Diamond, R.M., and Taub, R.(1993) Induction pattterns of 70 genes during nine days after hepatectomy define the temporal cause of liver regeneration J. Clin, Invest. 91: 1319-1326.

Hanley, M.R.(1989) Mitogenic neurotransmitters. Nature 840:97.

Hasegawa K., KogaM.(1977) Induction of liver cell proliferation in intact rats by amines and glucagon. Life Sci 21:1723-1.728

Heldin, C.H. (1995) Dimerization of cell surface receptors in signal transduction. Cell 80: 213-224.

Herschman, H.R. (1991) Primary response genes induced by growth factors and tumor promoters. Ann. Rev.Biochem. 60"281-319.

Higgins, G.M., and Anderson, R.M.(1931) Experimental pathology of the liver: I.Restoration of the liver of the white rat following partial surgical removal. Arch. Path. 12: 186-202.

Higuchi, O., Nakamura,T., (1991) Identification and change in the receptor for hepatocyte growth factor in rat liver after partial hepatectomy or induced hepatitis. Biochem. Biophys. Res. Commun. 176: 599-607.

Hill, C.S. and Treisman, R. (1995) Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 80: 199-212.

Ho, K, Hirose, H., Maryyama, H., Fukumachi, S., Tashiro, Y. and Saruta, T., (1995) Neurotransmitters partially restore glucose sensitivity of insulin and glucagon secretion

from perfused pancreas in streptozotocin-induced diabetic rats. Diabetologia, 38: 1276-84.

Hoffman, B.B., Michel, T., Kilpatrick, D.M., Lefkowitz, R.J., Tolbert, M.E.M.. Gilman, H. and Fain, J.N. (1980) Agonist versus antagonist binding to α-adrenergic receptors, Proc. Natl Acad. Sci. USA, 1980, 78: 4569-4573.

Holtta E. and Janne J.(1972) Ornithine decarboxylase activity activity and the accumulation of putrescine at the early stages of liver regeneration.FEBS Lett. 23:117-121.

Houck K.A. and Michalopoulos. G.(1985) Proline is required for the stimulation of DNA synthesis in hepatocyte cultures by EGF. *In vitro*. 21:121-124.

Houck, K.A., Cruise, J.L., and Michalopoulos, G.(1988) Norepinephine modulates the growth inhibitory effects of Transforming growth factor-Beta in primary rat hepatocyte cultures. J.Cell, Physiol. 135: 551-555.

Hoyer, D Boddeke, M. and Schoeffter, P. Second messenger in the definition of 5-HT receptors in "Molecular Biology, Receptors and functional effects ed. by Fozard, J.R and Saxena P.R. 1992, Birkhausen Verlag bBasel/Switzerland.

Hoyer.D., Clarke, D.E., Fozaid, J.R., Haitig, R.P., Haitins, R. G., Mylechaiane, J.E., Salera, R.P., and Humpherey, P.P.A.(1994). International Union of Pharmacology classification of Receptors for 5-Hydroxytryptamine(Serotonin). Pharmacological reviews 46: 157-203.

Hsia, C.C., Thorgeirsson, S.S.., and Tabor, E.(1994) Expression of hepatitis B surface and core antigens and transforming growth factor in "oval cells' of the liver in patients with hepatocellular carcinoma. Med. Virol.43, 216-221.

Hsu, J.C., Laz, T.M., Mohn, K.L., Taub, R.(1991) LRF-1, a novel leucine-zipper protein

rapidly and highly induced in regenerating liver, Proc.Natl. Acad.Sci.USA 88, 3511-3515.

Hu, Z.W., and B.B.Hoffman, 1993, Cycloheximide induces the α_{1B} adrenergic receptor gene by activation of transcription in DDT, MF-2 smooth music cells. Mol, Pharmacol, 44: 1105-1112.

Huerta-Bahena J., Villalobos-Molina R. and Garcia-Sainz J.A. (1983). Biochim biophys acta 763:112-116.

Hunter, T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling Cell 80: 225-236

Hunter,T (1993) Braking the cell. Cell 75: 839-841.

Ishac, E.J.N.E..Lazar-Wesley, and G.Kunos (1992) Rapid inverse changes in α_{1B} and β_2 - adrenergic receptors and gene transcripts in acutely isolated rat liver cells, J.Cell, Physiol. 152: 79-86.

Jaiswal, A.S., Misra, U.K and Bansal, S.K (1996) Differential activity of protein kinase C in alveolar and peritoneal macrophages Ind. J. Biochem. Biophys. 33: 116-121.

Jirtle, R.L., and Michalopoulos, G. (1982) Effects of partial hepatectomy on transplanted hepatocytes. Cancer Res. 42: 3000-3004.

Johnson E.M jr.,Manning P.T.,(1984) Guanethidine-induced desruction of sympathetic neurons. Int Rev Neurobiol 25:1-37.

Julius, D., Livelli, J.T., Jessell, T.M, Axel, R. (1989) Ectopic Expression of the Serotonin 1C receptor and the triggering of malignant transformation. Science, 244: 1057-1067.

Kan M., Huan J., Mansson P., Yasumitsu H., Carr B., and McKeehan W.(1989) Heparin-

Binding growth factor type 1(acidic fibroblast growth factor): a potential biphasic autocrine ane paracrine regulator of hepatocyte regeneration. Proc.Natl.Acad.Sci U.S.A. 86:7432-7436.

Kanasaki, M., H.Matsubara, S.Murasawa, H.Masaki, Y.Nio, and M.Inada, (1994) cAMP responsive element-mediated regulation of the gene transcription of the a1B adrenergic receptor by thyrotropin.J., Clin. Invest. 94: 2245-2254.

Kato H.and Shimazu T.(1983) Effect of autonomic denervation on DNA synthesis during liver regeneration after partial hepatectomy. Eur.J.Biochem.134:473-478.
Kavanaugh, W.M., Williams, L.C., Ives, M.E. and Coughlin, S.R.(1988) Serotonin-induced DNA synthesis in Vascular smooth muscle cell involves a novel, peprtuss toxin sensisite pathway.Mol. endocrinol. 123: 599-605.

Kesavan, P., Mukhopadhyay, S., Murphy, S., Rengaraju, M., Lazar, M.A., Das, M.(1991) Thyroid hormone decreases the expression of epidermal growth factor receptor. J. Biol.Chem. 266: 10282-10286.

Kiba, T., Tanaka, K., and Inoue, S.(1995) Lateral hypothalamic lesions facilitate hepatic regeneration after partil hepatectomy in rats.P.Flugers Arch. 430: 666-671.

Kiba, T., Tanaka, K., Numata, K., Hoshino, M., and Inoue, S (1994) Facilitation of liver regeneration after PH by ventromedial hypothalamic lesions in rats. P.Flugers Arch. 428: 26-29.

Kikkawa,U., Takai,Y., Minakuchi,R., Inohara,S and Nishizuka,Y (1982) Calcium activated , phospholipid-dependent protein kinase from rat brain J. Biol. Chem. 257: 13341-13348.

Kinoshita, R., Hirao, S., Matsumoto, K and Nakamura, T. (1991) Possible endocrine control by hepatocyte growth factor of liver regeneration after partial hepatectomy.

Biochem. Biophys. Res. Commun. 177: 330-335.

Kluess C., Hescheler J., Ewel C., Rosenthal W., Schultz G.and Wittig B.(1991).Assignment of G protein subtypes to specific receptors inducing inhibition of calcium currents. Nature 353: 43-48.

Koff A., Ohtsuki, M., Polyal, K., Robert, J.M., and Massogine J. (1993) Negative regulation of G-1 n mammalian cells. Inhibition of cyclin E- dependent Kinase by TGF-β Science 260: 536-539.

Kost, D.P., De Frances, M.C., Lee, C., Michalopoulos,G.K (1992) Patterns of alpha-1 adrenergic receptor expression in regenerating and neoplastic hepatic tissue Pathobiology,60: 303-308.

Kruijer. W., Skelly, H., Botteri, F., van der Pullen, H, Barber, J., Leffert, H.(1980) Proto-oncogene expression in regenerating liver is simulated in cultures of primary adult rat hepatocytes, J. Biol. Chem. 261, 7929-7933.

Kunos, G., Ishac, E.J.N., Gao, B., and Jiang, L (1995) Inverse regulation of hepatic α_{1B} and β_2 adreneric receptors , cellular mechanisms and physiological implications, Ann N.Y.Acad, Sci. 757: 261-270.

LaBrecque, D.R., Steele, G., Fogerby, S., Wilson, M., and Barton, J.(1987) Purification and physicochemical characterisation of hepatic stimulator substance. Hepatology 7: 100-106

Laemmli, U.K.(1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature 227: 680-685, 1970.

Lamph W.W., Wamsley P., Sassoni-Corsi P. and Verma I.M (1988) Induction of proto-

oncogene JUN/AP-1 by serum and TPA. Nature 334:629-631.

Lau, L.F., and Nathans, D. (1987) Expression of a set of growth related immediate early genes in BALB/C3T3 Cells: Coordinate regulation with C-fos or C-myc. Proc.Natl. Acad. Sci. USA 84: 1182-1186.

Lauder, J.H.(1993).Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends in neurosci., 16: 233-240.

Lee D.C., Rose T.M., Webb N.R.and Todaro G.J.(1985). Cloning and sequence analysis of a cDNA for rat transforming growth factor-alpha.Nature313,489-491.
Lee-Lundberg, L.M.F., Cotecchia, S., Lomasney, J.W., De Bernarids, J.F., Lefkowitz, R.J., and Caron, H.G. (1985) Phorbol esters promote alpha-1 adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism Proc, Natl. Acad. Sci. U.S.A. 82: 5651-5655.

Leffert, H.L., Alexander,N.M., Faloona,G., Rubaclava,B., Unger,R (1975) Specific endocrine and hormonal receptor changes assosciated with liver regeneration in adult rats Proc. Natl. Acad. Sci. 72: 4033-4036.

Leffert, H.L., and Koch, K.S. (1978) Proliferation of hepatocytes. CIBA Found. Symp. 55: 61-91.

Lindroos, P.M., Zarnegar, R and Michalopoulos, G.K. (1991) Hepatocyte growth factor (Hepatopoeitin A) rapidly increases in plasma before DNA synthesis and liver regeneration stimulated by partial hepatectomy and carbontetrachloride administration Hepatology 13: 743-750.

Liu, Y., Michalopoulos, G.K., and Zarnegar, R.(1994) Structural and functional characterisation of the mouse hepatocyte growth factor gene promoter J.Biol Chem. 269:

Lomasney, J.W. , Cotecchia, S., Lorenz, W., Lenry, W.Y., Schwinn, D.A., Yang-Leng, T.L., Brownstein, M., Lefkowtz, R.J., Caron, M.G.(1991) Molecular cloning and expression of the c DNA for the alpha 1a-adrenergic receptor: The gene for which is located on human chromosome 5. J.Biol, Chem, 266: 6365-6369.

Lowey, A.D., Franklin, M.F. and Haxhiu, M.A. (1994) Central nervous system monoamine cell groups projecting to pancreatic vagal motor neurons: a transneuronal labelling study using pseudorabies virus. Brain Res. 638: 248-60.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, J. Protein measurement with Folin Phenol reagent. J.Biol Chem 1951, 193, 265-75.

Lowry, O.H., Rosserbrough, N.J., Fari, A.L., and Randall, R.J. (1951) Protein measurement with folin Phenol reagent. J.Biol.Chem 193: 265-275.

Lubon, H.and I.Henninghausen, (1988) Conserved region of the rat lactalbumin promoter is a target site for protein binding in vitro Biochem.J.256: 391-396

Luetteke, N.C., Michalopoulos, G.K., Teixidis, J., Gilmore, R., Massagire, J., and Lee, D.C.(1988) Characterisation of high molecular weight transforming growth factor α produced by rat hepaocellular carcinoma cells. Biochemistry 27: 6487-6494.

Lynch C.J., Charest R., Bocckino S.B., Exton J.H. and Blackmore P.F.(1985) Inhibition of hepatic α_1-adrenergic effects and binding by phorbol myristate acetate. J.Biol.Chem 260:2844-2851.

Lynch W and Liberman I.(1973) A DNA polymerase in liver nuclei whose activity rises with DNA synthesis after partial hepatectomy. Biochem.Biophys.Res.Commun. 52:843-849.

Magun,B.E.,Matrisian,L.M.,and Bowden,G.T.(1980) Epidermal growth factor. ability of tumor promoter to alter its degradation, receptor affinity and receptor number.J.Biol.Chem.,255:6373-6381.

Maloney,W.C., and Azzi,A (1989) Control of protein kinase C function by activators and inhibitors In: Molecular basis of membrane associated Diseases. (Azzi A., Diahota Z.and Papu.S eds) Springer Verlag 85-94.

Mannisto P.T., Marvish P., Ranta T., Tuomisto J.(1979) Dual action of adrenergic system on the regulation of thyrotropin secretion in the male rat. Acta Endeocrinol 90:249-258.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80: 179-185.

Massague J.(1983). Epidemal growth factor-like transforming growth factor.I.Isolation, chemical charecterization, and potentiation by other transforming factors from feline sarcoma virus-transformed cells. J.Biol.Chem.258:13606-13613.

Matsumoto K., Taima H., Harmanove M., Kohno S., Kinoshi T. and Nakamura T.(1992).Identification and charecterisation of "injurin" an inducer of expression of gene for hepatocyte growth factor (HGF). Proc.Natl. Acad. Sci. U.S.A. 89:3800-3804.

McCaffrey,P.G.,Friedman,B.,and Rosner,M.R.(1984)Diacylglycerol modulates binding and phosphorylation of epidermal growth factor receptor. J.Biol. Chem., 259:12502-12507.

McGowan J.A. and Fausto N.(1978). Ornithine decarboxylase activity and the onset of deoxyribonucleic acid synthesis in regenerating liver. Biochem.J.170: 123-127.

McGowan, J.A., A.J., and Bucher, N.L.R.(1981).DNA Synthesis in Primary cultures of adult rat hepatocytes in a defined medium: Effects of Epidermal growth factor, Insulin, glucagon & cyclicAMP.J.Cell Physiol. 108: 353-363.

Mead, J.E., and Fausto, N. (1989) Transforming growth factor TGF α may be a physiological regular of liver regeneration by means of an autocrine mechanism. Proc. Natl. Acad. Sci. U.S.A. 86: 1558-1562.

Mefford, I.N. Are there epinephrine neurons in rat brain? (1987)Brain Res. 12: 383-395.

Michalopoulos G., Houck.K.A., Dolan M.L. and Luetteke N.C.(1984). Control of hepatocyte replication by two serum factors. Cancer Res. 44:4414-4419.

Michalopoulos, G., Ciancinlli, H.D., Novotny, A.R., Kligerman, A.D., Strom, S.C., and Jirtle, R.L.(1982). Liver regeneration studies with rat hepatocytes in primary culture. Cancer Res. 42: 4673-4682.

Michalopoulos,G.K (1990) Liver regeneration: molecular mechanisms of growth control. FASEB J. 4: 176-187.

Minneman, K.P. (1988) Alpha-1 adrenergic receptor subtypes, inositol phosphates and sourcesof cell Ca^{2+} Pharmacol. Rev. 40: 87-719.

Minuk, G.Y., and Gauthier,T. (1993) The effect of γ-aminobutyric acid on hepatic regenerative activity following partial hepatectomy in rats. Gasteroenterology 104:217-221.

Minuk, G.Y., Kaita,K., Gauthier,T., Dembinski,T., and Murphy,L.J (1995) Effect of γ-aminobutyric acid (GABA) on hepatic insulin-like growth factor-I (IGF-I) and IGF-I binding protein (IGFBP-I) mRNA abundance following regenerative following partial hepatectomy in rats. Can.J. Physiol.Pharmacol.73: 1546-1551.

Mitchison, J.M.(1971). The Biology of the cell cycle Cambridge Univ. Press, London and New York. PP.1-33.

Miyazawa, K., Tajima, H., Hamanove, M., Kohno, S., Kinoshita, T., and Nakamma, T

(1992) Identification and characterisation of "injurin", an inducer of expression of the gene for hepatocyte growth factor. Proc.Natl, Acad. Sci. U.S.A. 89:3800-3801.

Miyazawa,K., Tsubouchi, H.,Naka, D., Takahashi, K., Okigaki, M., Arakaki, N., Nakayama, H., Hirono, S., Sakiyama, O., Takahashi, K., Gohda, E., Daikuhara,Y., and Kitamura, N., (1989). Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. Biochem. Biophys. Res. Commun, 163: 967-973

Mohn, K.L., Laz, T.M., Hsu, J.C., Melby, A.E., raro, R., and Taub, R.(1991a). The immediate - early growth response in regenerating liver and insulin - stimulted H-35 cells: comparison to serum- stimulated 3T3 cells and identification of 41 novel immediate - early genes. Mol. Cell. Biol.11: 381-390.

Mohn, K.L., Melby, A.E., Tewari,D.S., Laz, T.M., and Taub, R.(1991b).The gene encoding rat insulin-like growth factor-binding protein is rapidly and highly induced in regenerating liver. Mol. Cell. Biol. 11:1393-1401.

Mongeau, R., DeMontigny, C. and Blier, P. (1994) Activation of 5HT$_3$ receptors enhances the electrically evoked release of [^{3}H]NE in rat brain limbic structures. Eur.J.Pharmacol. 256: 269-79.

Morley, C.G.E., and Royse, V.L., (1981) Adrenergic agents as possible regulators of liver regeneration. Int.J.Biochem. 13: 969-973.

Mourelle, M., and Rubaclava, B. (1981) Regeneration of the liver after carbon tetra choloride - Differences in adenylate cyclase and pancreatic hormone receptors. J.Biol. Chem. 256: 1656-1660.

Mullhaupt, B., Feren, A., Fodor, E., and Jones, A (1994) Liver expression of epidermal growth factor RNA. J.Biol. Chem. 269: 19667-19670.

Nakamura, T., Arakaki, R., and Ichihara, A., (1988) Interleukin -1β is a potent growth inhibitor of adult rat hepatocytes in primary culture Exp. cell Res.: 179: 488-497.

Nakamura, T., Nowa, K., Ichihara H.Kaise, N. and Nishino , T.(1987) Purification and subunit structure of hepatocyte growth factor from rat platetlets. FEBS Lett. 224: 311-316.

Nakamura, T., Nowa, K., Ichihara, A (1984b). Partial purification and characterisation of hepatocyte growth factor from serum of hepatectomised rats. Biochem. Biophys. Res. Commun. 122: 1450-1459.

Nakamura, T., Teramoto, H., Ichihara, A (1986) Purification and characterisation of a growth factor from rat platelets for mature parenchymal hepatocytes inprimary cultures. Proc. Natl. Acad. Sci. USA 83: 6489-6493.

Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K., Ichihara, A (1985) Inhibiting effect of transforming growth factor-β on DNA synthesis of adult rat hepatocytes in primary culture. Biochem. Biophys. Res. Commun. 133: 1042-1050.

Nakamura, T., Tomita, Y., Ichihara, A (1983) Density dependent growth control of adult rat hepatocytes in primary culture. J.Biochem. 94: 1029-1035.

Nakamura, T., Tomomura, A., Kato, S., Noda, C., Ichihara,A (1984a) Reciprocal expressions of α_1 and β adrenergic receptors but constant expression of glucagon receptor by rat hepatocytes during development and primary culture J. Biochem. 96: 127-136.

Nakata, R., Tsukamoto, I., Mityoshi, M., and Kojo, S.(1985) Eur.J.Pharmacol. 35: 867-869.

Nebl, G.N.Mermod, and A.C.B.Cato (1994) Post-transcriptional down regulation

expression of transcription factor NFI by Ha-ras oncogene J.Biol.Chem. 269: 7371-7378.

Neer E.J.(1995) Heterotrimer G-proteins: organizers of transmembrane signals. Cell 80:213-221.

Nemeck, G.M., Coughhin, S.R., Handley, D.A., and Moskowitz, M.A. (1986). Stimulation of aortic smooth muscle cell mitogenesis by serotonin. Proc.Natl, Acad. Sci. 83: 674-678.

Nicholl R.A.,Malenka R.C.,Kauer J.A.(1990). Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. Funct. Physiol.Rev 70:513-557. Nobin A.,Baumarten H.G., Flack B.,Ingemansson S., Moghimzadeh E., Rosengren E.(1978). Organization of sympathetic innervation in liver tissue from monkey and man. Cell.Tissue.Res 195:371-380.

Olsen P.S.,Kirkegaard P O.,Poulsen S S.(1984). Adrenergic effect on exocrine secretion of rat submandibular epidermal growth factor. Gut 25:1234-1240.

Oomura, Y., and Yoshimatsu, M (1984) Neural network of glucose monitoring system. J.Auton Nerv. Syst.7: 165-174.

Pardee A.B.(1989). G_1 events and regulation of cell proliferation. Science 246:603-608.

Paulose C.S. and Dakshinamurti, K (1984) Enhancement of high affinity γ-aminobutyric acid receptor binding in cerebellum of pyridoxine deficient rats. Neurosci. Lett. 48: 311-16.

Paulose, C.S. and Dakshinamurti, K (1985) Effect of pyridoxine deficiency in young rats on high- affinity serotonin and dopamine receptors. J.Neurosci. Res., 14: 263-70.

Paulose, C.S., Dakshinamurti, K., Packer, S. and Stephens, N.L.(1988) Sympathetic stimulation and hypertension in pyridoxine deficient adult rat. Hypertension,m1988, 11, 387-91.

Pennisi E.,(1997) Tracing molecules that make brain body connection. Science 275: 930-931.

Pines, J.(1993) Cyclins and cyclin-dependent kinase take your partners Trends Biochem. Sci. 18: 195-197.

Pledger, W.J., Stiles, C.D., Antoniades, H.N., and Scher, C.D., (1978). Proc.Natl. Acad. Sci. U.SA, 75: 2839-2844.

Ponder,P.K.(1996) Analysis of liver development,regeneration,and carcinogenesis by genetic marking studies. FASEB J, 10:673-682.

Potten, C.S., and Loeffler, M., (1990) Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Development 110, 1001-1020.
Potter D.E.,Wilson L.M.,Ellis S.(1977) Changes in plasma glucose and insulin levels induced by adrenergic agents in normal and alloxan-diabetic rats. Pros Soc Exp Biol Med 154:337-340.

Rana B., Xie Y., Mischoulon D., Bucher N.L. and Farmer S.R.(1995). The DNA binding activity of C/EBP transcription factor is regulated in the G_1 phase of the hepatocyte cell cycle J.Biol.Chem. 270:18123-18132.
Rana, B., Xie, Y., Mischoulon, D., Bucher, N.L., and Faimer, S.R., (1995). The DNA binding activity of C/EBP transcription factor is regulated in the G1 phase of the hepatocyte cell cyde J.Biol. Chem. 270: 18123-18132.

Refnes M., Thoresen G.H.,Sandens D.,Dajani O.F.,Dajani L.and Christoffersen T.(1992)Stimulatory and inhibitory effect of catecholamines on DNA synthesisin primary rat hepatocyte cultures: role of alpha-1 and beta adrenergic mechanisms. J.Cell

Physiol.151:164-171.

Repaske, M.G., Nunnari, J.M. and Limbird, L.E. Purification of the α_2 adrenergic receptor from porcine brain using a yohimbine-agarose affinity matrix J.Biol. Chem. 1987, 262(25) 12381-12386.

Rhim, J.A., Sandgren, E.P., Degen, J,L.,Palmiter, R.D., and Brinster, R.L.(1994) Replacement of diseased mouse liver by hepatic cell transplantation. Science. 263, 1149-1152.

Rixon, R.H., Isaacs, R.J., Whitefield, F.J (1989) Control of DNA polymerase α activity in the regenerating liver by calcium and 1 α,25(OH)$_2$D$_3$ J.Cell.Physiol 139: 354-359.

Rixon, R.H., Mac Manus, J.P., and Whitfield, J.F. (1976) The control of liver regeneration by parathyroid hormone and calcium. J. Cell. Physiol., 87: 147-157.

Rossby, S.P., and L.E.Cornett. (1991) Steady state levels of hepatic α1 and $\beta_2$adrenergic receptors and gene transcripts during development of the male rat J.Cell. Physiol,147: 55-61

Rossi, P., G.Karsenty, A.B. Roberts, N.S.Roche, M.Sporn, and B.de Crombrugghe (1988) A nuclear factor 1 binding site mediates the transcriptional activtion of a type 1 collagen promoter by transforming growth factor β Cells 52: 405-414.

Rozengurt E. (1986). Early signals in the mitogenic response. Science 234:161-166.

Rozengurt,E.,Brown,K.D.,and Pettican, P.(1981). Vasopressin inhibition of epidermal growth factor binding to cultured mouse cells. J.Biol.Chem.,256:716-722.

Rubin, R.A., O'Keefe, E.J. (1982) Alteration of epidermal growth factor-dependent phosphorylation during liver regeneration. Proc Natl. Acad.Sci. 79: 776-780.

Ruff-Jamison, S., Zhong, Z., Chem, K, Darnell, Jr. J.E, Cohen, S.(1994) Epidermal growth factor and lipopolysaccharide activate Stat3 transcription factor in mouse liver. J.Biol.Chem. 269: 21933-21935.

Ruff-Jamison, S.,Chem,K.Cohen, S. (1993) Induction by EGF and interferon-Y of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei.Science 261: 1733-1736.

Russel, W.E., and Bucher, N.L.R. (1983) Vasopressin modulates liver regeneration in the Brattleboro rat. Am. J. Physiol, 245: G. 321-G324.

Russel, W.E., Coffey, R.J., Ovellette, A.J., and Moses, H.L.(1988) Type beta transforming growth factor reversibly inhibits the early proliferative response to partial hepatectomy in the rat Prof.Natl, Acad. Sci. U.S.A. 85: 5126-5130.

Russell, W.E.(1988) Transforming growth factor beta TGF inhibits hepatocyte DNA synthesis independently of EGF binding and EGF receptor anto phosphorylation J. Cell. Physiol., 135: 253-261.

Sadowski, H.B., Sherai, K., Darnell, J.E., and Gilman, M.Z (1993) A common nuclear signal transduction pathway activated by growth factor and cytokine receptors, Science 261: 1739-1744.

Sakaue., M., and Hoffman, B.B. (1991). Glucocorticoids induce transcription and expression of the α_{1B} adrenergic receptor gene in DDT_1, MF-2 smooth muscle cells.J.Clin. Invest. 88:384-389.

Sanae F., Miyamoto K.I., Koshiura R.(1989) Altered adrenergic response and specificicity of the receptors in rat ascites hepatoma AH130. Cancer Res 49:6242-6246.

Sandnes, D., Sand, T., Sager, G., Bronstad, G.O., Refs nos.M.R., Gladhang, I.P., Jacobsen, S., and christoffersen, T (1986) Elevated level of β-adrenegic receptors in hepatocytes from regenerating rat liver. Exp. cell.Res. 165: 117-126.

Sasaki, Y., Zhang, X.F., Nishiyama, M., Avruch, J., and Wands, J.R. (1993) Expression and phosphorylation of insulin receptor substrate 1 during rat liver regeneration. J.Biol. Chem. 268: 3805-3808.

Sato,Y., Fujiwara,K., Ogata,I., Tomiya,T., Oka,H (1989) Additive stimulation of hepatic putrescine production by glucagon and insulin after partial hepatectomy in rats. Life Sci. 45: 77-83.

Scatchard, G. (1949) The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51: 660-72.

Schmelck P H,Hanoune J.(1980). The hepatic adrenergic receptors.Molec Cell Biochem 33:35-38.

Schmidt, C., Bladt., F., Goedecko, S., Bunknam V., Zschieschie, W., Sharpe, H., Gheraidi, E., and Birchmeia, C (1995) Scatter factor/Hepatocyte growth factor is essential for liver development. Nature 23: 699-702.

Schmiedeberg, P., Biempica, I., and Czaja, M.J. (1993). Timing of poto-oncogene expression varies in toxin-induced liver regeneration.J.Cell, Physiol, 154: 294-300.

Schneider W.C(1957) Determination of nucleic acids in tissues by pentose analysis. Meth.

Enzymol. (Colowick & Kaplan, eds.,Academic press, NY). vol3, pp680-684.

Schwarz, K.R., Lanier, S.M., Carter, E.A. Homcy, C.J. Graham, R.M. (1988) Rapid reciprocal changes in adrenergic receptors intact isolatedhepatocytes during primary cell culture.Mol. Pharmacol. 27: 200-209.

Seglen, P.O.(1976) Preparation of isolated rat liver cells. Methods in Cell Biology (D.M. Prescott ed. Academic Press, New York) Vol.13, pp. 29-83.

Sell, S., and Ounsford, H.A. (1989) Evidence for the stem cell origin of hepatocellular carcinamo and cholangio carcinoma. Am. J.Pathol. 134: 1347-1363.

Seuwen, K., Magnaldo, I, and Poyssegur, J. (1988).Serotonin stimulates DNA synthesis in fibroblasts acting through 5-HT$_{1B}$ receptors coupled to a G$_i$ Protein. Nature, 335: 254-256.

Shi, Y.E., and Yager, J.E. (1989) Effects of the liver tumor promoter ethinyl estradiol on epidermal growth factor-induced DNA synthesis and epidermal growth factor receptor levels in cultured hepatocytes cancer Res. 49: 3574-3580.

Sibley, D.R., Peters, J.R., Nambi, P., Caron, M.G. and Lefkowitz, R.J.(1984) Desensitisation of turkey erythrocte adenylate cyclase. beta adrenergic receptor phosphorylation is correlated with attenuation of adenylate cyclase activity. J. Biol,.Chem. 259: 9742-9749.

Silva J.E., Larsen P.R. (1983) Adrenergic activation of triiodothyronine production in brown adipose tissue. Nature 305:712-713.

Skaaring, P., and Berring, F. (1976) On the intrinsic innervation of normal rat liver. Cell Tissue Res. 171: 141-155.

Smythe G.A., Bradshaw J.E., Cai W.Y.and Symons R.G.(1982). Hypothalamic serotonergic stimulation of thyrotropin secretion and related brain-hormone and drug ineractions in the rat. Endocrinology 111:1181-1191.

Smythe, G.A, Bradshaw, J.E. and Vining R.F Hypothalamic monoamine control of stress-induced adrenocorticotropin release in the rat. Endocrinology, 1983, 113, 1062-1071.

Songyang Z., Shoelson S.E., Chaudhari M., Cish G., Pauson T., Haser W.G., King F., Roberts T., Ratnofsky S.and Leehleider R.J (1993) SH2 domains recognize specific phosphopeptide sequences. Cell. 72:767-778.

Stewart, J.K., Campbell, T.G., and Gbadebo, T.D., Narasimhachari, N. and Manning, J.W. (1994) Cardiovascular responses and central catecholamines in streptozotocin diabetic rats. Neurochem. Int. 24: 183-89.

Stolz, D.B., and Michalopoulos, G.K. (1994) Comparitive effects of hepatocyte growth factor and epidermal growth factor on mobility, morphology, mitogenesis, and signal transduction of primary rat hepatocytes. J.Cell Biochem. 55: 445-464.

Stromblad, S., and Anderson, G. (1993). The coupling between transforming growth factor α and the epidermal growth factor receptor during rat liver regeneration Exp. Cell. Res. 204: 321-328.

Studer,R.K.,and Borle, A.B.(1982) Differences between male and female rats in the regulation of hepatic glucogenolysis. J.Biol Chem.,257:7987-7993.

Sun,H., Charles, C.H.Lau, L.F.Tonks, N.K.(1993) MKP-1(3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo Cell 75, 487-493.

Takai, S., Nakamura, T., Komi, N., and Ichiharan, I.(1988) Mechanism of stimulation of

DNA synthesis induced by epinephrine in primary culture of adult rat hepatocytes. J.Biochem. 103: 848-852, 198.

Taub, R. (1996) Transcriptional control of liver regeneration FASEBJ. 10: 413-427.

Tessy,T.M , Sudha, B., and Paulose, C. S.(1997) Kinetic parameters of thymidine kinase and DNA synthesis during rat liver regeneration : Role of thyroid hormones. Life Sci. (In Press).

Tewari, M., Dobrzanski, P., Mohn, K.L., Cressman, D.E., Hsu, J.C.Bravo, R.Taub,R.(1992) Rapid induction in regenerating liver of RL/IF-I, an I B that inhibits NH-$_k$B, Rel-p50; and PHF, a novel $_k$B site binding complex. Mol.Cell.Biol. 12, 2898-2908.

Thompson, N.L., Mead, J.E., Braum, I., Goyette, M., Shank, P.R., Fausto, N.(1986) Sequential protooncogene expression during rat liver regeneration Cancer Res. 46, 3111-3117.

Thorgeirsson, S.S.(1993) Commentary, Hepatic stem cells, Am.J.Pathol. 142, 1331-1333.

Thorgeirsson, S.S.(1995) Target cell population in virally associated hepatocarcinogenesis. Proceedings of the 25th Intl. Symposium of the Princess Takamatsu Cancer Research Fund on "Hepatitis C.Virus and its involvement in the Development of Carcinoma" pp. 163-170, Princeton Scientific Publishing Co., Princeton, New Jersy.

Thorgiersson, S.S., (1996) Hepatic stem cells in liver regeneration FASEB J 10: 1249-1256.

Thrower, S., Ord., M.G. (1974) Hormonal control of liver regeneration . Biochem. J., 144: 361-39.

Tsai, W.H., Cruise, J.L. and Hichalopoulos, GK (1989) Blockade of alpha-1 adrenergic receptor inhibits its hepatic DNA synthesis stimulated by tumor promoters Carcinozenesis 10: 73-78.

Van Obberghen-Schilling, E., Vouret-Craviari, V., Haslam, R.J., Chambard, J.C., and Pouyssegur, J.M. (1991) Cloning, functional expression and role in cell growth regulation of a hamster 5-HT$_2$ receptor subtype. Mol.Endocrinol. 5 : 881-889.

Van Thiel D.H.,Gavaler J.S.,Kam I.,Francavilla A.,Polimeno I,Schade P.R.,Smith J.,Diven W.,Penkrot R.J. and Starzl T.E.(1989).Rapid growth of an intact human liver transplanted into a recipient larger than the donor. Gastroenterology 93:1414-1419.

Van, M., Huan,J., Plansson, P., Yassumissu, H., Carr, B., and Mc Kechan.W., (1989) Heparin-binding growth factor): a potential biphasic autocrine and paracrine regulator of hepatocyte regeneration Proc. Natl Acad.Sci.U.S.A. 86: 7432-7436.

Vintermyer, K.O., and Doskeland, S.O. (1987) Cell Cycle paramaters of adult rat hepatocytes in a defined medium. A note on the timing of nucleolar DNA replication. Journal of Cellular Physiol 132: 12-21.

Vonderhaar, B.K., Tang, E., Lyster, R.R., Nascimento,M.C. (1986) Thyroid hormone regulation of epidermal growth factor levels in mouse mammary glands. Endocrinology 119: 580-585.

Waliuala Mola, P., Sudha, B., and Paulose, C.S.(1996). Effect of Insulin on DNA Synthesis and kinetic parameters of Thymidine Kinase during liver regeneration. Biochem.Mol.Biol.Intl., 40: 1067-1075.

Webber, E.M., Godowski, P.J., and Fansto, N.(1994). *In Vivo* response of the hepatocytes to growth factors requires an initial primary stimulus. Hepatology 19: 489-497.

Weinstein, I.B (1983) Protein kinase, phospholipid and control of growth. Nature 302 : 750.

Weir,E., Chen, Q., De Frances, M.C., Bell, A., Taub,R and Zarnegar, R (1994) Rapid induction of mRNAs for liver regeneration factor and insulin-like growth factor binding protein-1 in primary cultures of rat hepatocytes by hepatocyte growth factor and epidermal growth factor. Hepatology 1994 20: 955-960

Westwick J.K., Weitzel C., Minder A., Karin M.,and Brenner D.A.(1994). Tumour necrosis factor α-stimulates AP-1 activity through prolonged activation of C-jun kinase. J.Biol.Chem . 269:26396-26401.

Wilmut I., Schneike A.E., Mcwhir J., Kind A.J., Campbell K.H.S.(1997). Viable offspring derived from fetal and adult mammalian cells. Nature, 385:810-813.

Wollenburg, G.K., Harris, L., Farber, E., and Hanges, M.A. (1989) Inverse relationship between epidermal growth factor induced cell proliferation and expression of high affinity surface epidermal growth factor receptors in rat hepatocytes. Lab. Invest. 60: 254-259.

Wrann, M., Fox, C.F., and Ross, R. (1980) Modulation of epidermal growth factor receptor on 3T3 cells by platelet derived growth factor. Science, 210:1363-1365.

Zarnegar, R., DeFrances, M.C.Kost, D.P., Lindroos, P., Michalpoulos, C.K.(1991) Expression of hepatocyte growth factor mRNA in regenerating rat liver after partial hepatectomy. Biochem, Biophys. Res.Commun. 177, 559-565.

Zarnegar, R., Michalopoulos, G.(1989) Purification and biological characterisation of human hepatopoeitin A., a polypeptide growth factor for hepatocytes. Cancer Res. 49: 3314-3320.

Zippel, P.F, Irving, S.G., Kelby, K. and Siebenlist, U (1989) Complexity of the primary genetic response its mitogenic activation of human T.Cells. Mol.Cell. Biol.9: 1041-1048.

⚓