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

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

by

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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.

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COCHIN-22
31/03/1997
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, associateship or other similar titles or recognition.

COCHIN-22.
31/03/1997

SUDHA .B.
(Ph.D. Reg. No. 1410)
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### ABBREVIATIONS USED IN THE TEXT

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Bmax</td>
<td>Binding Maximum</td>
</tr>
<tr>
<td>BS</td>
<td>Brain Stem</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine Mono Phosphate</td>
</tr>
<tr>
<td>CC</td>
<td>Cerebral Cortex</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CS</td>
<td>Corpus Striatum</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EPI</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>Gpp[NH]p</td>
<td>5'- Guanylyl- imidodiphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-Hydroxy Indole Acetic Acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSS</td>
<td>Hepatic stimulatory substance</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine (Serotonin)</td>
</tr>
<tr>
<td>5-HTP</td>
<td>5-Hydroxytryptophan</td>
</tr>
<tr>
<td>HYPO</td>
<td>Hypothalamus</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin like Growth Factor</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin Receptor Substrate</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation Constant</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis Menton constant</td>
</tr>
<tr>
<td>LRF</td>
<td>Liver regeneration factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitotic Activated Protein Kinase</td>
</tr>
<tr>
<td>MHPG</td>
<td>3-methoxy-4-hydroxy-phenyl glycol</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NF κB</td>
<td>Nuclear factor- kappa-B</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>8-Hydroxy-n-dipropylamino tetralin</td>
</tr>
<tr>
<td>ODC</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>p</td>
<td>Level of significance</td>
</tr>
<tr>
<td>PH</td>
<td>Partial Hepatectomy</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidyl Inositol</td>
</tr>
<tr>
<td>PI-3K</td>
<td>Phosphatidyl-inositol-3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>PMA</td>
<td>4β-phorbol 12β-myristate 13α-acetate</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Abbreviation</td>
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</tr>
<tr>
<td>S.E.M</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Tri-iodo-thyronine</td>
</tr>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine Kinase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis Factor</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal velocity</td>
</tr>
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</table>
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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: \( G_0 \) cells, that are still capable of re-entering the cell cycle and terminally differentiated cells that are destined to die without dividing. The \( G_0 \) 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 \( G_0 \) 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 \( G_0 \) 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 (G₀ to G₁) 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, G₀/G₁/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-HT₁C 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-HT₂ receptors have been shown to mediate cell growth in fibroblasts (Van Obberghen-Schilling et al, 1989).
The 5HT₂ receptor has been cloned in the human liver and it has a high degree of homology with that of rat and mouse 5-HT₂ receptors (Bonahus et al, 1995). Norepinephrine (NE) has been reported to induce DNA synthesis in primary cultures of rat hepatocytes, acting through the α₁ adrenergic receptor (Cruise et al, 1985). Epinephrine has also been shown to stimulate DNA synthesis in cultured hepatocytes through the α₁ adrenergic receptor (Takai et al, 1988). The α₁ 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 (Reijnes 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 G₁-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.
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 sympathectomy 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 (T₃) 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 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
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
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 (CCl₄) 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 G₀ 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 throughout 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
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
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).
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 ¹²⁵I-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, Hepatopoietin 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 (Gi), or positively coupled to phospholipase C (PLC), which mediates phosphoinositol (PI) hydrolysis (Gp) or to pertussis-toxin-sensitive pathways (Go, Gi). 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 T3 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 T3 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 T3 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. T₃ 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 T₃ administration (Francavilla 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 (McGowan 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, Vₘₐₓ 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 α₁-adrenergic blocker prazosin, but not by an α₂ antagonist or β adrenergic blocker, indicating that catecholamines interact with the α₁ adrenoreceptor to stimulate DNA synthesis in hepatocytes. Liver has a high relative concentration of α₁ adrenergic receptors (Hoffman et al, 1980) and the ability of catecholamines to directly stimulate DNA synthesis through α₁ mediation suggested a significant role of these receptors in the regeneration response. Addition of NE to hepatocytes stimulates Ca²⁺ mobilisation or PI turnover (Exton, 1981) and either or both of these processes was proposed to be involved in the mitogenicity of
NE. The $\alpha_1$ 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 $\alpha_1$ 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 $\alpha_1$-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 $\alpha_1$ 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 $\alpha_1$ adrenergic receptor is linked to a G protein and stimulates increased activity of
phosphatidyl inositol diphosphate (P1P2) phosphodiesterase. Stimulation of the α1-adrenergic receptor triggers increased breakdown of P1P2, resulting in an increase in the cytoplasmic diacylglycerols and inositol 1,4,5-triphosphate. These mediators trigger a cascade of other intracellular events, including activation of protein kinase C and mobilisation of calcium from intracellular stores (Exton, 1988). Cruise et al (1989), demonstrated an uncoupling of the α1 adrenergic receptor to phosphoinositide turnover and suggested that the uncoupled receptor may be the mediator of interest. The uncoupled α1 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 P1P2 turnover may cause negative feed back regulation of α1-adrenergic receptors through its phosphorylation. The uncoupling of the α1 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 Ca2+ mobilisation (Takai et al, 1988) Three α1 receptor sub types have been identified, α1a, α1b and α1c, all products of different genes (Lomasney et al, 1991). Hepatocytes contain predominantly the α1b-adrenergic receptor subtype (Minneman, 1988). The normal and regenerating rat liver was negative for α1a-receptor mRNA and positive for α1b mRNA 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 α1a and α1b mRNA and receptor binding, while the human hepatocellular carcinoma cell line Hep G2 was positive for α1a and α1b message at 4.5 kb but lacked receptor binding. Thus, the presence α1 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 α2- and β-adrenergic receptors and a concomitant decline in α1 receptors.

Serotonin

Serotonin has been shown to stimulate DNA synthesis in many non-neuronal cells. In fibroblasts, 5-HT activates phospholipase C, inhibits adenylyl 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$_{1}$ 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 enhance 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 [3H] thymidine into liver DNA at 24 hours post-hepatectomy (Ashirif 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 G1 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 G1, 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
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 G₀ state into G₁ 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 G₀/G₁ 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 coordinated 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 IκB-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 G₀ / G₁ 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 G₁ 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 transduction. 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-1, 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

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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, erythropoietin and IL-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 α1 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 IP₃ 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). G₁ cyclins are positive regulators that control rate-determining steps during the G₁ progression. A relatively long G₁ period is characteristic of adult hepatocytes and G₁ events probably exert a stringent control of hepatocyte proliferation. G₁ 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
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,

**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-^3H]Noradrenaline, specific activity 39.0 Ci/mmol.

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

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

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

**RADIOCHEMICALS FROM BHABHA ATOMIC RESEARCH CENTRE (BARC), BOMBAY**

[^3H] Thymidine (sp.activity 18Ci/m mole), [γ-^32P-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°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 [³H] 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. [³H] 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°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 iced perchloric acid. The homogenate was centrifuged at 5000xg for 10 minutes at 4°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-Hydroxyindoleacetic acid (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 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 MgCl₂, 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 MgCl₂, 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 \[^3H\] 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 \[^3H\] prazosin with and without 10 \(\mu\)M 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. \[^3H\] prazosin bound to the membranes in the filter was determined by liquid scintillation spectrometry in Wallac LSC

**DETERMINATION OF \[^3H\] 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 \[^3H\]Yohimbine with and without 10 \(\mu\)M 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 [3H] 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 [3H] 5-HT, with and without excess cold (10 μM)5-HT. The incubation was done at 37°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. [3H] 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
ultracentrifuge for 30 minutes at 4°C. 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 [$^3H$] NE USING PROPRANOLOL, PRAZOSIN AND YOHIMBINE**

The cold antagonists used for competition binding assays with [$^3H$] NE were propranolol for β adrenergic receptors, prazosin, for α₁ adrenergic and yohimbine for α₂ adrenergic receptors. The incubation buffer contained 50mM Tris-HCl, 10mM MgCl₂, 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 [$^3H$]NE and 10⁻⁹ to 10⁻³M of various competing antagonists - prazosin, yohimbine and propranolol in the incubation buffer. The mixture was incubated for 30 minutes at 25°C. The reaction was stopped by filtering immediately through Whatmann GF/C filters with three washes of buffer containing 50 mM Tris Buffer containing 10mM MgCl₂, 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. Specific 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°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 containing 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 \[^3\text{H}\] 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 \[^3\text{H}\] 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 (B_max) and equilibrium dissociation constant (K_d) 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 (K_d) of the affinity of the receptors for the radioligand. The K_d 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.
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 \(\mu\)m filters (Millipore).

B. Collagenase Buffer: This buffer contained 67 mM NaCl, 6.7 mM KCl, 100 mM HEPES, 4.76 mM CaCl\(_2\cdot2H_2O\), 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 \(\mu\)m filters (Millipore).

Collagen-coating of culture dishes

Sterile rat-tail collagen solution (100\(\mu\)g/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 \textit{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 43 hrs at 37°C in 5% CO2.

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°C 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°C
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°C 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 [$\gamma$-$^{32}$P]ATP (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 [$\gamma$-$^{32}$P]ATP (5-10x10⁴ cpm/nmol), 10 µg phosphatidyl serine (PS), 0.2 µg of diolein, 125 nmol of CaCl₂ 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 N₂, the residue was resuspended in 20 mM Tris/HCl, pH 7.5 by sonication and then added to the reaction mixture. After incubation at 30⁰C for 3 minutes, the reaction was stopped by adding 25% TCA. The acid precipitable 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 CaCl₂ 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 MgCl₂·6H₂O, 10 mM DTT, 0.5mM CaCl₂·2H₂O 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 CaCl₂ 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° C for 15 minutes, the reaction was started by adding 10 μM ATP containing 1μCi of [γ-³²P]ATP} and further incubated at 30° C 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°C 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 [³H] 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).
Alpha - Adrenergic receptor kinetics

\[ \alpha_1 \] adrenergic receptors of the brain regions were assayed by \((^3\text{H})\) prazosin binding. \[ \alpha_1 \] 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, \(K_d\) (Fig. 2, Table 4). The \(\alpha_2\) adrenergic receptors of the hypothalamus as assayed by \((^3\text{H})\) yohimbine binding showed increased \(B_{max}\) \((p < 0.05)\) with no change in \(K_d\) at 24 hours of liver regeneration compared to control. (Fig 3, Table 4). The \(\alpha_1\) adrenergic receptors of the cerebral cortex showed no change in \(B_{max}\) and a higher \(K_d\) \((p < 0.05)\), (Fig 4, Table 5) while the \(\alpha_2\) adrenergic receptors exhibited increase in \(B_{max}\) and lowered \(K_d\) \((p < 0.05)\) (Fig. 5, Table 5). In the brain stem, \(\alpha_1\)adrenergic receptors showed significantly higher \(B_{max}\) and \(K_d\) \((p < 0.05)\) (Fig. 6, Table 6). The \(\alpha_2\) adrenergic receptors showed no change in \(B_{max}\) and a had significantly higher \(K_d\) \((p < 0.05)\) (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, $B_{\text{max}}$, was significantly lower ($p<0.05$) with no change in $Kd$, at 24 hours of liver regeneration (Fig. 8, Table-4). The $B_{\text{max}}$ 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, $B_{\text{max}}$ 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
[³H] NE by yohimbine was higher in the 24 hour regenerating liver (Fig.13). The α₁ adrenergic receptors were studied by scatchard analysis of [³H] prazosin binding to hepatic membranes, during 24 hours of liver regeneration.

**α₁ adrenergic receptors**

There was a significant decrease (p<0.05) in the maximal binding (B_max) of [³H] prazosin during the DNA-synthetic phase of liver regeneration. The dissociation constant Kd did not show any significant change (Table-11, Fig.14). Displacement of [³H] Prazosin by -(-) NE caused a greater displacement of α₁ adrenergic receptors in the high affinity concentration range. When the non-hydrolysable GTP analog, Gpp NH₃ was present, the displacement curve in control and regenerating liver shifted towards the low affinity concentration range (Fig.15).

**Serotonergic Receptors**

**³H] 5-HT binding kinetics**

Scatchard analysis of [³H] 5-HT binding to liver membranes showed that the B_max of serotonergic receptors increased significantly (p<0.05) during the replicative phase. Kd of the serotonin receptors for [³H] 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 [³H] 5-HT in the regenerating liver during the DNA synthetic phase (Fig.17). Ketanserin caused a marked displacement of [³H] 5-HT from its receptors during 24 hours of regeneration compared to control in all concentrations tested (Fig 18). Spiperone displaced [³H] serotonin in the high affinity and
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 \[^3H\] 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 (5×10^{-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 5×10^{-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 (5×10⁻⁹M 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 (10⁻⁷M). Lower concentrations of 5-HT did not cause any significant change in the DNA synthesis compared to EGF-treated cultures alone. However, 10⁻⁵M 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 5×10⁻⁵M 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 $V_{\text{max}}$ 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 $V_{\text{max}}$ and $K_m$ of the enzyme in the cytosolic fraction of the regenerating liver, compared to control.

**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 control liver compared to the regenerating liver (Plate 3).
<table>
<thead>
<tr>
<th>Animal status</th>
<th>Hrs after</th>
<th>PH</th>
<th>MHPG</th>
<th>NE</th>
<th>EPI</th>
<th>MHPG/NE</th>
<th>EPI/NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>-</td>
<td>1.50 ± 0.36</td>
<td>1.19 ± 0.32</td>
<td>0.37 ± 0.04</td>
<td>1.98 ± 0.39</td>
<td>0.38 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>36.02 ± 5.20</td>
<td>3.45 ± 0.85*</td>
<td>0.33 ± 0.07</td>
<td>10.47 ± 1.60*</td>
<td>0.24 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Hepatectomised</td>
<td>48</td>
<td>251.00 ± 31.00</td>
<td>13.19 ± 1.45*</td>
<td>0.30 ± 0.04</td>
<td>18.88 ± 0.71*</td>
<td>0.02 ± 0.01*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1.44 ± 0.05</td>
<td>0.89 ± 0.10</td>
<td>0.34 ± 0.10</td>
<td>1.63 ± 0.13</td>
<td>0.40 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

*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)

<table>
<thead>
<tr>
<th>Hrs after</th>
<th>Animal status</th>
<th>PH</th>
<th>MHPG</th>
<th>NE</th>
<th>EPI</th>
<th>MHPG/NE</th>
<th>EPI/NE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham-operated</td>
<td>-</td>
<td>0.25 ± 0.07</td>
<td>0.39 ± 0.09</td>
<td>0.14 ± 0.05</td>
<td>0.70 ± 0.15</td>
<td>0.51 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>2.46 ± 0.23</td>
<td>0.97 ± 0.09*</td>
<td>0.14 ± 0.09</td>
<td>2.37 ± 0.17*</td>
<td>0.15 ± 0.01*</td>
</tr>
<tr>
<td></td>
<td>Hepatectomised</td>
<td>48</td>
<td>1.71 ± 0.02</td>
<td>0.81 ± 0.12*</td>
<td>0.14 ± 0.01</td>
<td>1.88 ± 0.05*</td>
<td>0.16 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td></td>
<td>0.31 ± 0.02</td>
<td>0.43 ± 0.08</td>
<td>0.17 ± 0.04</td>
<td>0.80 ± 0.09</td>
<td>0.41 ± 0.07</td>
</tr>
</tbody>
</table>

*p<0.05 compared to control (Values are Mean ± S.D. of 4-6 separate determinations.)
<table>
<thead>
<tr>
<th>Animal status</th>
<th>Hrs after PH</th>
<th>MHPG</th>
<th>NE</th>
<th>EPI</th>
<th>MHPG/NE</th>
<th>EPI/NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>-</td>
<td>16.56 ± 0.90</td>
<td>1.06 ± 0.14</td>
<td>0.15 ± 0.06</td>
<td>15.96 ± 1.37</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td>24</td>
<td>32.31 ± 1.09</td>
<td>2.41 ± 0.12*</td>
<td>2.91 ± 0.13*</td>
<td>14.29 ± 0.63</td>
<td>1.22 ± 0.08*</td>
<td></td>
</tr>
<tr>
<td>Hepatectomised</td>
<td>48</td>
<td>92.45 ± 2.35</td>
<td>6.85 ± 1.07*</td>
<td>2.59 ± 0.08*</td>
<td>14.89 ± 0.29</td>
<td>0.42 ± 0.01*</td>
</tr>
<tr>
<td>72</td>
<td>12.88 ± 0.81</td>
<td>1.23 ± 0.15</td>
<td>0.19 ± 0.01</td>
<td>1.85 ± 0.73</td>
<td>0.17 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

*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**

<table>
<thead>
<tr>
<th>Animal Status</th>
<th>(^{3}\text{H} \text{PRAZOSIN})</th>
<th>(^{3}\text{H} \text{YOHIMBINE})</th>
<th>(^{3}\text{H} \text{SEROTONIN})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(B_{\text{max}})</td>
<td>(Kd)</td>
<td>(B_{\text{max}})</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>141 ± 4</td>
<td>0.20 ± 0.01</td>
<td>530 ± 21</td>
</tr>
<tr>
<td>Hepatectomised</td>
<td>90 ± 3(^*)</td>
<td>0.18 ± 0.02</td>
<td>1013 ± 97(^*)</td>
</tr>
</tbody>
</table>

\(^*\)p<0.05 compared to control. Values are Mean \(±\) 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.

\(B_{\text{max}}\)-Binding maximum (femtomoles/mg protein), \(Kd\)-Dissociation constant (nM).
<table>
<thead>
<tr>
<th>Animal Status</th>
<th>(³H) PRAZOSIN</th>
<th>(³H) SEROTONIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B&lt;sub&gt;max&lt;/sub&gt; K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>B&lt;sub&gt;max&lt;/sub&gt; K&lt;sub&gt;d&lt;/sub&gt;</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>313 ± 8</td>
<td>426 ± 24</td>
</tr>
<tr>
<td></td>
<td>0.23 ± 0.01</td>
<td>8.63 ± 1.57</td>
</tr>
<tr>
<td></td>
<td>317 ± 6</td>
<td>668 ± 36&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.36 ± 0.01&lt;sup&gt;*&lt;/sup&gt;</td>
<td>8.05 ± 0.71</td>
</tr>
<tr>
<td>Hepatectomised</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>126 ± 4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.69 ± 3.95</td>
</tr>
</tbody>
</table>

<sup>*</sup>p<0.05 compared to control. Values are Mean ± 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.

<sup>max</sup>-Binding maximum (femtomoles/mg protein), K<sub>d</sub>-Dissociation constant (nM).
TABLE 6

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

<table>
<thead>
<tr>
<th>Animal Status</th>
<th>(^{(3)H}) PRAZOSIN</th>
<th>(^{(3)H}) YOHIMBINE</th>
<th>(^{(3)H}) SEROTONIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(B_{max})</td>
<td>Kd</td>
<td>(B_{max})</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>261 ± 19</td>
<td>0.14 ± 0.02</td>
<td>512 ± 41</td>
</tr>
<tr>
<td>Hepatectomised</td>
<td>433 ± 30(^{*})</td>
<td>0.25 ± 0.05(^{*})</td>
<td>524 ± 25</td>
</tr>
</tbody>
</table>

\(^{*}\)p<0.05 compared to control. Values are Mean ± 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-Dissociation constant (nM).
<table>
<thead>
<tr>
<th>Animal status</th>
<th>5-HTP (nmol/g)</th>
<th>5-HIAA (nmol/g)</th>
<th>5-HT (nmol/g)</th>
<th>5-HTP/5-HT</th>
<th>5-HIAA/5-HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>0.14 ± 0.05</td>
<td>0.76 ± 0.07</td>
<td>3.08 ± 0.01</td>
<td>0.54 ± 0.20</td>
<td>0.752 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>1.24 ± 0.02</td>
<td>3.07 ± 0.10</td>
<td>1.71 ± 0.07</td>
<td>1.27 ± 0.05*</td>
<td>0.85 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>1.15 ± 0.06</td>
<td>1.71 ± 0.04</td>
<td>1.35 ± 0.05*</td>
<td>0.32 ± 0.04</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td>Hepatectomised</td>
<td>0.08 ± 0.01</td>
<td>1.02 ± 0.2</td>
<td>3.18 ± 0.15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05 compared to control. (Values are Mean ± 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)

<table>
<thead>
<tr>
<th>Animal status</th>
<th>Hrs after</th>
<th>5-HTP</th>
<th>5-HIAA</th>
<th>5-HT</th>
<th>5-HIAA / 5-HT</th>
<th>5-HTP / 5-HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>-</td>
<td>0.15 ± 0.02</td>
<td>2.56 ± 0.10</td>
<td>0.60 ± 0.07</td>
<td>2.56 ± 0.10</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>Hepatectomised</td>
<td>24</td>
<td>0.35 ± 0.03</td>
<td>1.49 ± 0.10</td>
<td>1.34 ± 0.13</td>
<td>1.05 ± 0.05*</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.13 ± 0.05</td>
<td>2.48 ± 0.21</td>
<td>0.41 ± 0.17</td>
<td>5.01 ± 0.90*</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.17 ± 0.03</td>
<td>2.28 ± 0.09</td>
<td>0.73 ± 0.10</td>
<td>3.61 ± 0.27</td>
<td>0.24 ± 0.01</td>
</tr>
</tbody>
</table>

*p<0.05 compared to control.  (Values are Mean ± S.D. of 4-6 separate determinations)
<table>
<thead>
<tr>
<th>Animal status</th>
<th>Hrs after PH</th>
<th>5-HP</th>
<th>5-HIAA</th>
<th>5-HT</th>
<th>5-HIAA / 5-HT</th>
<th>5-HTP / 5-HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>-</td>
<td>0.43 ± 0.02</td>
<td>0.46 ± 0.06</td>
<td>0.62 ± 0.07</td>
<td>0.80 ± 0.05</td>
<td>0.70 ± 0.01</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>2.83 ± 0.30</td>
<td>5.09 ± 0.24</td>
<td>3.02 ± 0.20*</td>
<td>1.68 ± 0.03*</td>
<td>1.08 ± 0.04*</td>
</tr>
<tr>
<td>Hepatectomised</td>
<td></td>
<td>\textit{0.35 ± 0.01}</td>
<td>\textit{0.68 ± 0.21}</td>
<td>\textit{0.49 ± 0.12}</td>
<td>\textit{0.89 ± 0.10}</td>
<td>\textit{0.69 ± 0.08}</td>
</tr>
<tr>
<td>48</td>
<td></td>
<td>0.29 ± 0.02</td>
<td>0.42 ± 0.04</td>
<td>0.59 ± 0.03</td>
<td>0.78 ± 0.05</td>
<td>0.59 ± 0.10</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td>\textit{0.29 ± 0.02}</td>
<td>\textit{0.42 ± 0.04}</td>
<td>\textit{0.59 ± 0.03}</td>
<td>\textit{0.78 ± 0.05}</td>
<td>\textit{0.59 ± 0.10}</td>
</tr>
</tbody>
</table>

\*p<0.05 compared to control. (Values are Mean ± S.D. of 4-6 separate determinations)
TABLE-10

LEVELS OF PLASMA NOREPINEPHRINE
(nanomoles/ml plasma)

<table>
<thead>
<tr>
<th>Animal status</th>
<th>Hrs after PH</th>
<th>Norepinephrine levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>-</td>
<td>0.37 ± 0.10</td>
</tr>
<tr>
<td>Hepatectomised</td>
<td>24</td>
<td>1.80 ± 0.30*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.55 ± 0.45*</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.28 ± 0.02</td>
</tr>
</tbody>
</table>

*p<0.05 compared to control
Values are Mean ± S.E.M of 4-6 separate determinations
TABLE-11

ALPHA₁ ADRENERGIC AND SEROTONERGIC RECEPTOR BINDING PARAMETERS IN THE LIVER OF SHAM-
HEPATECTOMISED AND PARTIALLY HEPATECTOMISED RATS

<table>
<thead>
<tr>
<th>Animal Status</th>
<th>(³H) PRAZOSIN</th>
<th></th>
<th>(³H) SEROTONIN</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bₘₐₓ</td>
<td>Kd</td>
<td>Bₘₐₓ</td>
<td>Kd</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>57 ± 3</td>
<td>0.14 ± 0.02</td>
<td>463 ± 10</td>
<td>5.22 ± 0.55</td>
</tr>
<tr>
<td>Hepatectomised</td>
<td>33 ± 2*</td>
<td>0.11 ± 0.03</td>
<td>2233 ± 58*</td>
<td>20.21 ± 4.70*</td>
</tr>
</tbody>
</table>

*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. Bₘₐₓ-Binding maximum (femtomoles/mg protein), Kd-Dissociation constant (nM).
**TABLE-12**

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[(3H)] Thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/mg protein (x10^5)</td>
</tr>
<tr>
<td>Medium only</td>
<td>23.53 ± 0.68</td>
</tr>
<tr>
<td>EGF (10ng/ml)</td>
<td>451.89 ± 7.80*</td>
</tr>
<tr>
<td>50µM 5-HT</td>
<td>27.46 ± 0.24</td>
</tr>
<tr>
<td>50µM NE</td>
<td>26.30 ± 0.76</td>
</tr>
<tr>
<td>EGF + 1µM 5-HT</td>
<td>631.80 ± 0.92*</td>
</tr>
<tr>
<td>EGF + 1µM NE</td>
<td>702.38 ± 0.45*</td>
</tr>
</tbody>
</table>

*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**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[3H] Thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/mg protein (x10^3)</td>
</tr>
<tr>
<td>Medium only</td>
<td>25.33 ± 0.68</td>
</tr>
<tr>
<td>EGF (10ng/ml)</td>
<td>451.89 ± 7.80*</td>
</tr>
<tr>
<td>EGF + 50nM NE</td>
<td>505.35 ± 8.55*</td>
</tr>
<tr>
<td>EGF + 50nM NE + 50nM prazosin</td>
<td>206.76 ± 12.16*</td>
</tr>
<tr>
<td>EGF + 50μM NE</td>
<td>879.51 ± 0.19*</td>
</tr>
<tr>
<td>EGF + 50μM NE + 1μM prazosin</td>
<td>286.92 ± 10.96*</td>
</tr>
<tr>
<td>EGF + 50nM NE + 50nM propranolol</td>
<td>421.90 ± 0.40</td>
</tr>
<tr>
<td>EGF + 50nM NE + 50nM yohimbine</td>
<td>428.66 ± 19.33</td>
</tr>
</tbody>
</table>

*p<0.05 compared to respective control.
Values are mean ± S.E.M. of 4-6 separate determinations.
TABLE-14

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[3H] Thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/mg protein (x10^3)</td>
</tr>
<tr>
<td>Medium only</td>
<td>23.53 ± 0.68</td>
</tr>
<tr>
<td>EGF (10ng/ml)</td>
<td>451.89 ± 7.80</td>
</tr>
<tr>
<td>EGF + 50nM 5-HT</td>
<td>448.83 ± 7.74</td>
</tr>
<tr>
<td>EGF + 50nM 5-HT + 50nM Ketanserin</td>
<td>232.66 ± 3.23*</td>
</tr>
<tr>
<td>EGF + 50nM 5-HT + 50nM spiperone</td>
<td>390.20 ± 15.27</td>
</tr>
<tr>
<td>EGF + 50μM 5-HT</td>
<td>852.40 ± 9.34*</td>
</tr>
<tr>
<td>EGF + 50μM 5-HT + 1μM Ketanserine</td>
<td>334.17 ± 14.57*</td>
</tr>
<tr>
<td>EGF + 50μM 5-HT + 1μM Spiperone</td>
<td>375.69 ± 9.85*</td>
</tr>
</tbody>
</table>

*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**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>[3H] Thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/mg protein (x10^5)</td>
</tr>
<tr>
<td>Medium only</td>
<td>23.53 ± 0.68</td>
</tr>
<tr>
<td>Medium + EGF(10ng/ml)</td>
<td>451.89 ± 7.80</td>
</tr>
<tr>
<td>EGF + 50µM Epinephrine</td>
<td>632.90 ± 8.22*</td>
</tr>
</tbody>
</table>

* p<0.05 compared to control.
Values are mean ± S.E.M. of 3-5 separate determinations.
<table>
<thead>
<tr>
<th></th>
<th>Membrane Fraction</th>
<th>Cytosolic Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control liver</td>
<td>$V_{\text{max}}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td></td>
<td>$65.5 \pm 2.12$</td>
<td>$66.73 \pm 0.97$</td>
</tr>
<tr>
<td>24 hour regenerating</td>
<td>$114.68 \pm 4.34$</td>
<td>$47.5 \pm 2.8$</td>
</tr>
<tr>
<td>liver</td>
<td>$V_{\text{max}}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td></td>
<td>$42.33 \pm 2.34$</td>
<td>$44.93 \pm 0.68$</td>
</tr>
<tr>
<td></td>
<td>$46.79 \pm 3.03$</td>
<td></td>
</tr>
</tbody>
</table>

\( p<0.05 \) compared to control

$V_{\text{max}}$: Maximal Velocity (Picomoles/min/mg protein); $K_m$: Michaelis-Menten constant (\( \mu \text{M} \))
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

$[^3\text{H}]$Prazosin Scatchard in Hypothalamus

Of Control and Hepatectomised Rats

![Graph showing bound/free ratio vs bound (femtomoles/mg protein)](image)
Figure-3

$[3^H]$ Yohimbine Scatchard in Hypothalamus
of Control and Hepatectomised Rats

Bound/Free (femtomoles/mg protein/nM)

Bound (femtomoles/mg protein)
[\(^3\)H] Prazosin Scatchard in Cerebral cortex of Control and Hepatectomised Rats
Figure-5

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

Bound/Free (femtomoles/mg protein/nM)

- Control
- Hepatectomised
Figure-6

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

![Graph showing Scatchard plot for $[^3]$H Prazosin binding to brain stem of control and hepatectomised rats. The x-axis represents bound (femto moles/mg protein) and the y-axis represents bound/free (femto moles/mg protein/nM). The graph includes data points for control (circles) and hepatectomised (squares) groups.]}
Figure-7

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

- **Control**
- **Hepatectomised**
Figure-8

Figure-9

$[^3\text{H}]$ Serotonin Scatchard in cerebral cortex of control and hepatectomised rats.
Figure 10

[3H] Serotonin Scatchard in Brain Stem of control and hepatectomised rats

![Graph showing Serotonin Scatchard plot for control and hepatectomised rats.](image-url)
DISPLACEMENT OF [3H] NE BY PRAZOSIN
IN LIVER OF CONTROL AND HEPATECTOMISED RATS

Figure 11
Figure-12

DISPLACEMENT OF [3H] NE BY PROPRANOLOL
IN LIVER OF CONTROL AND HEPATECTOMISED RATS

- log of antagonist concentration (M)

% of [3H]NE bound

control liver
24 hr regenerating liver
Figure-13

DISPLACEMENT OF [3H] NE BY YOHIMBINE
IN LIVER OF CONTROL AND HEPATECTOMISED RATS

- log of antagonist concentration (M)

% of [3H] NE bound

- control liver
- 24 hr regenerating liver
Figure-14

$[^3H]$ PRAZOSIN SCATCHARD IN HEPATIC MEMBRANE
OF CONTROL AND HEPATECTOMISED RATS

![Graph showing $[^3H]$ PRAZOSIN binding in hepatic membrane of control and hepatectomised rats](image)
Figure 15

DISPLACEMENT OF [3H] PRAZOSIN BY (-) NE
IN THE LIVER OF CONTROL AND HEPATECTOMISED RATS:
EFFECT OF GTP ANALOG Gpp[NH]p

- log of agonist concentration (M)
Figure-16

$[^3\text{H}]$ SEROTONIN SCATCHARD IN THE HEPATIC MEMBRANE
OF CONTROL AND HEPATECTOMISED RATS

![Graph showing Scatchard plot for $[^3\text{H}]$ serotonin binding in control and regenerating liver. The x-axis represents bound/free (femtomoles/mg protein/nM) and the y-axis represents bound (femtomoles/mg protein). Two lines are shown: one for control liver with filled circles and one for 24-hour regenerating liver with filled squares.](image-url)
DISPLACEMENT OF [3H] 5-HT BY SEROTONIN
IN LIVER OF CONTROL AND HEPATECTOMISED RATS.

Figure-17

-log of agonist concentration (M)
%

control
24 hr regenerating liver

% of [3H] 5-HT bound
DISPLACEMENT OF $[^3H]5$-HT BY KETANSERIN
IN LIVER OF CONTROL AND HEPATECTOMISED RATS

- log antagonist concentration (M).

Control liver
24-hour regenerating liver
DISPLACEMENT OF $[^3H]$-5HT BY SPIPERONE IN LIVER OF CONTROL AND HEPATECTOMISED RATS.
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.

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 NE-induced DNA synthesis *in vitro*

![Graph showing the effect of various treatments on DNA synthesis](image)

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 ± S.E.M. Of 4-6 separate determinations.

Where the error bars are not seen the S.E.M. is <2%
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 error bars are not visible, standard error of mean is <2%
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%
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 $\gamma^3$2 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
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 \gamma^{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

\(^3\text{H}\) 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 \(^3\text{H}\) thymidine incorporation was observed at 24 hours of liver regeneration. DNA synthesis was significantly higher in the liver at 48 hours of regeneration and reversed to near normal levels by 72 hours after partial hepatectomy. This pattern of DNA synthesis observed by us is concordant 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 \(^3\text{H}\) 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 (Waliualu Mola et al, 1996; Tessy et al, 1997)

Brain Neurotransmitters and Receptors in Liver Regeneration

Partial Hepatectomy induced significant changes in the content and turnover 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 activity 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 (Waliaaula 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, $\alpha_1$ and $\alpha_2$ adrenergic receptors 
showed reciprocal changes. There was a decrease in the number of $\alpha_1$ adrenergic 
receptors, while the $\alpha_2$ 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 $\alpha_1$ adrenergic receptors. Geynet et al., (1981) have reported 
that NE is more potent in recognising $\alpha_1$ 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 $\alpha_1$ adrenergic receptors of the regenerating liver has also been observed in our 
studies 24 hours post hepatectomy. The down regulation of hypothalamic and hepatic $\alpha_1$ 
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 $\alpha_2$ 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 $\alpha_2$ adrenergic receptors in the hypothalamus may reflect a feedback regulation of NE release and an increased sensitivity to EPI. Studies using inhibitors of NE synthesis or $\alpha$-adrenergic blockers have established a stimulatory role for NE in the control of TRH-mediated TSH secretion (Mannisto et al, 1979). $\alpha$-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 in synthesis of EPI. These changes may be the reason for increased $\alpha_1$ adrenergic receptors with lowered affinity and the lowered affinity of $\alpha_2$ adrenergic receptors. These alterations of the $\alpha_1$ 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 $\alpha_1$ adrenergic receptors showed a desensitisation as evidenced by lowered affinity and the $\alpha_2$ 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 $\alpha_2$ 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 \( \alpha_1 \) adrenergic receptors of the three brain regions. These changes were in general reciprocal to that of \( \alpha_2 \) adrenergic receptors.

Neurotransmitters such as 5-HT and \( \gamma \)-aminobutyric acid (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 heptatectomised animals showed diminished rate of liver DNA synthesis and a decreased activity of thymidine kinase. T₃ treatment reversed this and caused an increase in the affinity of the enzyme. T₃ 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 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 $\alpha_1$ 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 $\alpha$-adrenergic agonists and the other with a large binding capacity and low affinity for natural $\alpha$-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 $\alpha$-adrenergic agonists (Geynet et al., 1981). Displacement studies using prazosin indicated that high affinity $\alpha_1$ adrenergic receptors are involved in the DNA synthetic phase of liver regeneration. Propranolol, a $\beta$-adrenergic blocker displaced $[^3]$H] NE from higher and low affinity concentration ranges and caused a shift of the displacement curve to the high affinity concentration range. Our results indicate an increased expression of $\beta$ adrenergic receptors during the DNA synthetic phase of liver regeneration. An elevated level of $\beta$-adrenergic receptors have been reported in hepatocytes isolated from regenerating rat liver during the period of DNA synthesis (Sandnes et al., 1986). During liver regeneration...
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 $\alpha_1$ type to a $\beta$-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 $\beta$-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 $\beta$-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 important as they are important in conveying signals via the sympathetic nervous system (Morley and Royse, 1981). Increased $\beta$-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 $\beta$-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 $\beta$-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 $\beta$ adrenergic receptors and a reduced function of $G_i$ protein (Nakamura et al, 1984). The increase in $\beta$ adrenoreceptors, thus, may be a major factor responsible for the rise in $\beta$ adrenergic responsiveness during liver regeneration.

Assay of the kinetic parameters of hepatic $\alpha_1$ 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 \[^{[3H]}\text{Prazosin}\] 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 \(\alpha_1\) adrenergic receptors were observed until 48 hours after PH. A down regulation of \(\alpha_1\) 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 \(\alpha_1\) adrenergic receptors decreased \(\alpha_2\) and \(\beta\) adrenergic receptors increased compared to control liver. Reciprocal changes in the expression of \(\alpha_1\) and \(\beta\)-adrenergic receptors have been demonstrated to occur in primary cultures of rat hepatocytes. There was an increase in the \(\beta\) adrenergic response and receptor number, while \(\alpha_1\) 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 \(\alpha_2\) and \(\beta\) adrenergic receptors and a concomitant decline in \(\alpha_1\) receptors (Sanae et al., 1989).

GTP analog caused a decreased in the affinity of \(\alpha_1\) adrenergic receptors to their natural agonist, NE in control and regenerating liver. This indicates a desensitisation of the \(\alpha_1\) adrenergic receptors on G-protein association. In the regenerating liver G protein association also causes a decreased displacement of \[^{[3H]}\text{Prazosin}\] by \(-(-)\) NE. These results further indicate that high affinity \(\alpha_1\) adrenergic receptors are involved in the regenerating liver and G-protein association may precede their downregulation. This is also supported by the decreased B\(_{\text{max}}\) of \[^{[3H]}\text{Prazosin}\] binding in liver. The coupling of \(\alpha_1\) adrenergic receptors to PLC occurs through a G protein (Kunos et al., 1995). The association of ras oncprotein which is a membrane associated G protein, with the \(\alpha_1\)
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 P1P2 turnover. This may cause negative feed back regulation of α1 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 α1 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 α1 adrenergic receptors seems to be an important part of the α1-adrenergic regulation of liver cell division. The absence of the α1 adrenergic receptor mRNA 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 α1 adrenergic receptor to prevent deregualtion of liver cell proliferation. The α1 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 α1a receptor subtype while α1b adrenergic receptor is expressed and is involved in the comitogenic response of NE (Kost et al, 1992). Expression of the α1b 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 α1b 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 α1b AR gene during liver
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 α1 b AR expression in the early stages of the regenerative response may serve to turn off a mitogenic signal and limit the extent of hepatocyte proliferation. A failure to suppress the expression of hepatic α1 b AR after PH may promote abnormal liver growth and aberrant differentiation. The over expression of α1 b AR induced agonist-dependent focus formation and tumor formation (Allen et al, 1991).

The α2 adrenergic receptor blocker, yohimbine did not cause any significant displacement of [3H] NE in the control liver. At 24 hours of liver regeneration however, there was an increased displacement of [3H] NE in the high affinity concentration range, indicating that α2 -adrenergic receptors are relatively higher in the regenerating liver compared to the quiescent state. Hoffman et al (1980) reported that the liver contains fewer α2 receptors and the α1 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 α2 adrenergic receptors may not contribute directly to mitogenicity but the change on the ratio of α1 and α2 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 [3H]5-HT. The increase in serotonin receptors during the DNA synthetic phase of liver regeneration was further supported by increased
displacement of $[^3\text{H}]5$-HT by cold serotonin. Ketanserin caused a significant displacement of $[^3\text{H}]5$-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 $[^3\text{H}]5$-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.
In Vitro Studies

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 G0 cells into G1 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 Norepinephrine 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 concordent 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 $\alpha_1$ adrenergic receptor antagonist, caused a significant reduction in NE-induced DNA synthesis. Prazosin also reduced the DNA synthesis induced by EGF. Thus, the $\alpha_1$ adrenergic receptor function is required for EGF and NE-mediated DNA synthesis. The $\alpha_2$ adrenergic receptor yohimbine and $\beta$ adrenergic receptor blocker, propranolol did not cause significant reduction in the DNA synthesis. Thus, the $\alpha_1$ 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 $\alpha_1$-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 $\alpha_1$ adrenergic receptor is also involved in decreasing the DNA synthesis inhibition produced by TGF $\beta$ (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 stimulation of 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 \( \alpha_1 \) adrenergic receptor. But, the mechanism of EPI action did not involve PKC activation or \( \text{Ca}^{2+} \) mobilisation. (Takai \textit{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 \( \alpha_1 \) 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 \textit{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 \textit{et al}, 1986) such as \( \alpha_1 \) 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 \( \alpha_1 \) 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 α₁ 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 phosphorylation mediated by the α₁ adrenergic receptor. NE also induced the phosphorylation of a 59KD protein regenerating liver membrane fraction and this was eliminated by prazosin. The α₁ 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 α₁ 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 α₁ 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 \( \alpha_1 \) and \( \alpha_2 \) adrenergic receptors of the brain regions showed reciprocal changes. The hypothalamic \( \alpha_1 \) adrenergic and high affinity serotonergic receptors exhibited a downregulation, while the \( \alpha_2 \) adrenergic receptors were increased. The \( \alpha_1 \) 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 \( \alpha_1 \) adrenergic receptor were also down regulated during liver regeneration. The PKC-dependent autophosphorylation of the \( \alpha_1 \) 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 \( \alpha_1 \) 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
regenerating liver was further confirmed by protein phosphorylation assays. 5-HT induced DNA synthesis 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) \(^{31}\)I 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. \(\alpha_1\) and \(\alpha_2\) adrenergic receptors of these brain regions showed reciprocal changes. Changes of high affinity serotonin receptors were comparable to \(\alpha_1\) adrenergic receptors. The hypothalamic \(\alpha_1\) adrenergic receptors and serotonergic receptors exhibited a down regulation at 24 hours of liver regeneration.

vi) There was a downregulation of hepatic \(\alpha_1\) adrenergic receptors and an increased involvement of serotonin 5-HT\(_2\) receptors in the regenerating liver, during the peak of DNA synthesis.
vii) 5-HT₂ 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 α₁ adrenergic and 5-HT₂ 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 α₁ 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-HT₂ 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-HT₂ 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.
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