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The effects of ethanol on putrescine and GABA metabolism in the liver

by

Ganlu Lou

A Thesis

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MASTER OF SCIENCE

Department of Pharmacology and Therapeutics
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FACULTY OF GRADUATE STUDIES

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THE EFFECTS OF ETHANOL ON PUTRESCINE AND GABA METABOLISM IN THE LIVER

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GANLU LOU

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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Abstract

The mechanisms responsible for ethanol-associated inhibition of liver regeneration following partial hepatectomy remain unclear. Previous data indicate that ethanol-induced decreases in hepatic putrescine levels and enhancement of hepatic gamma-aminobutiric acid (GABA)ergic activity play important pathophysiologic roles. Of interest is the fact that GABA is a product of putrescine metabolism via either diamine oxidase (DAO) or monoamine oxidase (MAO) enzyme activity. Whether ethanol alters the process of polyamine to GABA metabolism remains to be determined. Thus the purpose of this study was to determine the effects of ethanol on hepatic polyamine metabolism following partial hepatectomy.

Hepatic DAO, MAO and GABA transaminase (GABA-T), the enzyme responsible for GABA metabolism, and levels of putrescine, GABA and other polyamines (spermidine and spermine) were determined in the livers of adult male Sprague-Dawley rats (200-250 g) which had been pretreated with a dose of either ethanol (5g / kg) to inhibit hepatic regeneration or equal volumes of saline by gastric gavage 1 h prior to a 70% partial hepatectomy or sham surgery. Rats were then sacrificed in groups (N= 4-9/ group) at various times (0-48 hrs) post partial hepatectomy. Enzymatic activities were determined by radiochemical techniques and the levels of polyamines and GABA by high pressure liquid chromatograghy (HPLC).

Hepatic DAO activities in acute ethanol exposed rats were consistently increased post partial hepatectomy when compared to saline exposed controls. Statistical significance was achieved at 48 h [ethanol vs. saline at 48 h; 260±128 vs 135±35 pmoles / mg protein / h ($p<0.05$)]. Hepatic MAO activities were essentially identical in the two groups. Hepatic putrescine levels exhibited a biphasic pattern in the ethanol exposed group, i.e. slightly

decreased hepatic putrescine levels during the earlier period, (6-12 h), while increased during the later period (24- 48 h) post-partial hepatectomy, [ethanol vs. saline at 48 h; 15.2±2.3 vs 8.8±1.5 nmoles /g liver ($p<0.05$)]. Hepatic GABA levels were also significantly increased in the ethanol exposed group at 48 h post partial hepatectomy [ethanol vs. saline at 48 h; 859±168 vs 383±97 nmoles /g liver ($p<0.01$)]. GABA-T activities were increased and decreased significantly at 6 h and 48 h respectively, [ethanol vs. saline at 6 h; 40±1.7 vs 30±1.4 and 48 h; 21±2.9 vs 32±5.5 pmoles / mg protein / h ($P<0.05$ and 0.01 respectively)]. Ethanol exposure had no consistent effects on hepatic spermidine or spermine levels.

The results of this study provide further support for the hypothesis that the antiregenerative effects of ethanol on the liver may be mediated by increased hepatic conversion of putrescine to GABA and inhibition of GABA metabolism in a post-partial hepatectomy model of hepatic regeneration in rats.

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Chapter I
Introduction

A series of studies have documented that excess ethanol exposure impairs hepatic regeneration following partial hepatectomy and toxin-induced liver injury (Leevy and Chen 1979, Orrego et al., 1981, and Diehl et al., 1988). Although the precise mechanism(s) is still under investigation, recent data have implicated disturbances in polyamine and GABA homeostasis. Putrescine, a simple polyamine, is believed to be essential for hepatic regeneration (Luk 1986). Several studies by Diehl et al., (1990), Tanaka et al., (1991) and Minuk et al., (1993) revealed that acute and chronic ethanol exposure suppresses the hepatic accumulation of putrescine and further retards hepatic regenerative activity. Of interest in these studies is the fact that ornithine decarboxylase (ODC), the enzyme responsible for converting ornithine to putrescine, remained unchanged by ethanol exposure. Moreover, decreased hepatic putrescine levels by ethanol are unlikely to be explained by either decreasing putrescine uptake and synthesis or increasing putrescine conversion to other polyamines such as spermidine and spermine, or increasing putrescine excretion into bile (Diehl et al., 1992). Thus, the most likely explanation for ethanol-induced decreases in hepatic putrescine levels is enhancement of enzymatic pathways from putrescine to GABA in the liver.

GABA, a potent inhibitory amino acid neurotransmitter in the brain, has growth regulatory properties. GABA-mediated impairment of liver regeneration includes inhibition of restitution of liver mass, DNA and protein synthesis, ODC activity and insulin-like growth hormone (IGF)-1 and IGF binding protein-1 mRNA expression (Minuk et al., 1992, 1993). In addition, GABA markedly attenuates the increase in hepatic putrescine levels in

response to partial hepatectomy (Minuk et al 1991). A possible explanation for GABA's inhibitory effect on hepatic putrescine is suggested by the hyperpolarization of hepatocyte membranes which is mediated via GABA receptor sites (Minuk et al., 1987). As a result of the cell membranes being hyperpolarized, putrescine, a strongly cationic protein, is retained within the cytoplasm where oxidation of GABA occurs.

Oxidation of hepatic putrescine is catalyzed by diamine oxidase (DAO). DAO is thought to be involved in the modulation of putrescine levels in growing and regenerating tissues. Alternative pathways of GABA synthesis from putrescine involve acetylation of putrescine and further oxidation by monoamine oxidase (MAO). Although acute ethanol exposure has been reported to induce DAO (Sessa et al., 1984) and but not MAO (Kennedy et al., 1992) activity during regeneration, these experiments were performed in isolation and did not take into consideration substrate concentrations, alternative pathways or regulative feedback loops. With respect to GABA catabolism, GABA transaminase (GABA-T) is the only known catabolic enzyme for GABA and facilitates its conversion to the end products of carbon dioxide and water.

The primary objectives of this study were to document the effects of acute ethanol exposure on putrescine/GABA metabolic pathways and possibly identify safe and effective therapeutic options that could eventually be applied to patients with alcohol-induced liver injury.

Chapter II

Literature Review

2.1. Alcoholic Liver Disease

2.1.1. Epidemiologic and Clinical Features

Approximately 5% of the population in Canada are alcoholics (Depew WT. 1984). Of these, 10 to 20% go on to develop liver disease. Alcohol-induced liver disease ranks fourth as the cause of death in individuals between the ages of 35 and 55. The majority of these deaths relate to acute alcoholic hepatitis, cirrhosis and liver cancer.

2.1.2. Pathogenesis of Alcoholic Liver Disease

It is generally agreed that the pathogenesis of alcoholic liver disease involves multiple processes (Lieber 1988). Based on recent literature, a few of the most relevant pathogenic factors are discussed below.

2.1.2.1. Nutritional Factors

Originally, it was widely accepted that nutritional disturbances and deficiencies played an important role in the pathogenesis of alcoholic liver disease. Primary malnutrition was considered a result of long-term, excessive consumption of alcohol (Lieber 1990). While secondary malnutrition was thought to result from alcohol-induced changes in gastro-intestinal digestion or absorption. The fact that alcoholic liver disease can also occur in well nourished individuals argues against nutritional deficiencies being a principal cause in the development of alcoholic liver disease.

2.1.2.2. Toxicity of Alcohol and Its Metabolite, Acetaldehyde

The liver is the site of ethanol metabolism. However, it is not ethanol itself, but rather, acetaldehyde, a product of ethanol metabolism that is thought to induce hepatotoxicity. Although the cellular mechanisms of intrinsic cytotoxicity of acetaldehyde remain unclear, acetaldehyde-mediated oxidative stress leading to lipid peroxidation, immunostimulation and acetaldehyde-adduct formation have been documented (Nordmann et al., 1992).

Thus, the major mechanisms for the toxic effects of acetaldehyde on the liver include the followings. Acetaldehyde promotes the formation of toxic oxygen species, alters the structure and function of mitochondria due to interactions with phospholipid, depletes antioxidants through interactions with glutathione (GSH) (Videla et al., 1982, Speisk et al., 1985) and vitamin E, stimulates immune-mediated cell injury, and forms adducts with proteins (Jennett et al., 1990), lipids and DNA (Rajasinghe et al., 1990).

2.1.2.3. Ethanol-Related Oxidative Stress

There is an increasing body of evidence which suggests that generation of excess amounts of free radicals constitutes an important factor accounting for the hepatotoxicity of ethanol. The levels of hepatic glutathione, a potent antioxidant, were dramatically reduced following acute and extended exposure to ethanol (Videla et al., 1982). A similar experiment was carry out by Koch et al., (1991), who found that the depletion of the lipid-soluble antioxidant vitamins in ethanol-treated rats lead to the presence of induced oxidative stress. Furthermore, elevations in lipid peroxidation following ethanol treatment have been observed in the liver (Uysal et al., 1989). These findings provide additional support to the hypothesis that ethanol can induce hepatic injury through a mechanism

involving free radical induced lipid peroxidation. However, the benefits of antioxidants in alcoholics have yet to be determined.

2.1.2.4. Associated Immune Alterations

That immune mechanisms are involved in the liver cell injury and the fibrosis stage of alcoholic liver disease is suggested by the fact that alcohol-induced hepatitis and cirrhosis are characterized by infiltration of the liver with a variety of inflammatory cells and the fact that in some cases, the inflammatory process persists despite withdrawal of the alcohol. Moreover, a number of researchers have observed specific immunological changes in chronic alcoholic liver disease. For example, studies by Spinozzi et al., (1991) reported depressed T-cell function in alcoholic patients with cirrhosis, whereas, Khoruts et al ., (1991) found that serum levels of tumor necrosis factor (TNF- alpha) and interleukin-1 are increased in patients with alcoholic hepatitis. These results seem to indicate a potential role played by the immune system in the pathogenesis of alcoholic liver disease. However, it remains unclear as to whether such findings represent a cause or effect of the liver injury.

2.1.2.5. Acetaldehyde-Protein Adducts

Acetaldehyde, a highly reactive metabolite of alcohol, interacts with proteins and other cell structures to form stable covalent adducts. Such acetaldehyde adducts may serve as neoantigens, eliciting an immunologic response (Israel et al., 1986) which could, in part, be responsible for causing the cell injury associated with excessive alcohol consumption. The acetaldehyde adducts could also interfere with hepatocyte function either by preventing tubulin polymerization (Smith et al., 1989) or by affecting key enzyme activity

(Solomon 1987). However, the functional consequences of these adducts in vivo are not well understood.

2.1.2.6. Other Factors

The duration of alcohol use and amount of intake are important factors in determining the point at which liver disease develops in individual alcoholics (Lelbach 1972). In addition, other factors such as gender, age and genetics also affect the outcome. Specifically, women seem to be more susceptible to liver damage from alcohol than men (Morgan and Sherlock 1977, Tuyns, 1984) This is thought to be due to gender differences in ethanol metabolism and perhaps, sex hormone levels. Also, an age-related decrease in the rate of ethanol metabolism has been observed, increasing the potential toxicity in older individuals (Hahn and Burch 1983). Finally, under certain circumstances, the role of genetics appears to determine an individual's susceptibility to the development of alcoholic liver disease (Ingelman-Sundberg et al., 1993).

2.1.2.7. Types of liver injury

Excess alcohol consumption typically leads to three types of liver injury which include; fatty liver, alcoholic hepatitis and cirrhosis. Often, these types of liver damage overlap. Fatty liver is the most common and mildest form of the three. It is considered to result from decreased fatty acid oxidation and the promotion of fatty acid synthesis. In most instances fatty liver is asymptomatic, nonprogressive, and fully reversible with abstinence.

Alcoholic hepatitis is a more advanced, potentially dangerous form of liver disease with extensive hepatic inflammation that may be similar in severity to viral or toxin-induced hepatitis. If the initial injury is not severe and further exposure to alcohol does not occur,

the hepatitis is reversible. Whereas, if the injury is severe (but not fatal) and alcohol exposure continues, the hepatitis is likely to progress to cirrhosis.

Alcoholic cirrhosis results from repeated attacks of alcoholic hepatitis with subsequent fibrosis. The condition is irreversible. Although alcoholic cirrhosis is the end stage of alcoholic liver disease, five-year survival rates can be 80-90% if subjects stop drinking before serious complications of cirrhosis (decompensated disease) develop (Cartithhers 1992).

2.1.2.8. The Liver's Response to Alcohol Induced Injury

By the time most patients with alcoholic liver disease present to their physicians, alcohol consumption has ceased and hepatic inflammation is already established. Thus, the liver's only remaining is response to undergo a regenerative process. It has been demonstrated by Frank et al., 1979 and Wands et al., 1979 that alcohol interferes with the regenerative process which further contributes to the pathogenesis of alcoholic hepatitis and cirrhosis. Indeed, the balance between hepatocyte injury and liver regeneration determines the outcome of alcoholic liver disease. Thus, the understanding of alcohol's effect on hepatic regeneration is at least of equal importance and in terms of therapy, perhaps more important than our understanding of alcohol-induced hepatic injury.

2.1.3. Treatment of Alcoholic Liver Disease

In the past, treatment for alcoholic liver disease was focused on nutritional supplementation and control of alcohol consumption. However, this "traditional" approach has met with only limited success. More recent approaches have shifted the emphasis of treatment to hepatoprotection. The beneficial effect of hepatoprotective agents such as

prostaglandins on alcoholic liver disease in animals is encouraging but preliminary. Moreover, whether the results can be transferred to humans who tend not to take these medications until under hospital supervision needs to be assessed.

2.1.3.1. Glucocorticoids

Glucocorticoids are immunosuppressive agents that are mainly used in the treatment of severe acute alcoholic hepatitis. Results from controlled trials (Helman et al., 1971, Porter et al., 1971) and Carithers et al., 1989) suggest that glucocorticoids are of value in reducing the short-term mortality of these patients, especially in individuals with alcoholic hepatitis and encephalopathy. Whether long-term survival is also improved has yet to be determined.

2.1.3.2. Propylthiouracil

Propylthiouracil has also been suggested for the treatment of alcoholic liver disease. The rationale for using this agent is based on the fact that the hypermetabolic state of the liver induced by ethanol is similar to that seen systemically in hyperthyroidism (Orrego et al., 1979). Early uncontrolled clinical trials (Orrego et al., 1979) suggested that propylthiouracil was benificial in treating moderate alcoholic hepatitis. A more recent long- term, controlled study of survival in outpatients with alcoholic liver disease reported that propylthiouracil reduced mortality in the treated group (Orrego et al., 1987). However, drop-out rates in the study were high and the beneficial effect of propylthiouracil in hospitalized patients with acute alcoholic hepatitis has not been demonstrated.

2.1.3.3. Proly-4-hydroxylase (P4H) Inhibitors and S-adenosylmethionine (SAM)

Proly-4-hydroxylase (P4H) inhibitors and S-adenosylmethionine (SAM) are still in the early stages of investigation, however, preliminary results are encouraging. Proly-4-hydroxylase (P4H) inhibitors inhibit the progression of alcoholic hepatitis to fibrosis and prevent further deterioration of established fibrosis. The mechanism appears to involve the blockage of the hydroxylation of proline, a step which is essential for triple helix and collagen formation. SAM is required for the synthesis of glutathione, an antioxidant that protects cells from oxidative stress and injury. The administration of exogenous SAM may thereby decrease oxidative stress and injury in the liver.

2.1.3.4. Colchicine

Colchicine, has been proposed more for the treatment of alcoholic cirrhosis than alcoholic hepatitis. Its antiinflammatory and antifibrogenic effects are intended to decrease the inflammatory infiltrate and inhibit the release of collagen and proinflammatory substances. Colchicine also prevents collagen synthesis (Harris and Krane 1971) and the secretion of procollagen (Ehrlich et al., 1974). Although theoretically these properties of colchicine appear to be promising, the results of clinical trials have been disappointing (Trinchet et al., 1989). Thus, at present, colchicine has not been recommend for the routine management of alcoholic liver disease.

2.1.3.5. Enhanced Regeneration

To date, attempts to stimulate hepatic regenerative activity in alcoholic liver disease have been limited. Insulin and glucagon therapy was not effective (Bird et al., 1991, Trinchet et al., 1992) and the beneficial effects of putrescine (Nishiguchi et al., 1990) and GABA receptor antagonists (Minuk et al., 1996) remain confined to animal studies. The recovery process from alcohol-induced injury depends on not only the degree of liver damage

produced but the ability of hepatic regenerative activity as well. Efforts for future treatment of this disease should be aimed at an approach to reverse alcohol-induced inhibition of hepatic regeneration, because the majority of hepatocyte injury may have occurred before patients are hospitalized.

2.2. Hepatic Regeneration

Awareness of liver regeneration can be traced back to more than two thousand years. The Greek god, Zeus, who punished Promotheus for his disobedience in revealing the secret of fire to mankind, chained him to a rock and all day long let an eagle peck pieces of his liver. But the most cruel aspect of the punishment was that the liver grew back by night, so that his torture could go on without end. Decades of work have enriched our knowledge, but many features of the regenerative process remain unclear. For example, the stimuli sensed by the liver in response to a reduced liver mass are unknown. Moreover, the precise growth promoters responsible for maintenance of the regenerative process have yet to be determined. Finally, even less is known about the growth inhibitors that prevent the growth of extensively injured and cirrhotic livers.

2.2.1. Basic Characteristics of Liver Regeneration

The resting hepatocyte is a highly differentiated cell. Its proliferative activity is very low in normal adult livers of animals and humans (Bucher 1963). However, healthy liver is capable of very rapid regeneration in response to partial hepatectomy (Higgin and Anderson 1931) or chemical toxins (Recknagel 1967). The process is much slower in diseased livers (Gerber et al., 1983).

2.2.1.1. Response to Partial Hepatectomy

Liver regeneration after resection of part of the liver (standard: 68-70 %) is largely characterized by functional cell hyperplasia (increase in the number of cells) rather than hypertrophy (enlargement of the cells) which plays a minor role and occurs earlier in the process. The hyperplasia is driven by an increase in DNA synthesis which can be measured by tritiated thymidine incorporation into DNA. During the prereplicative phase (0-12 hours post partial hepatectomy), the basal rate of DNA synthesis does not change. At this point, hepatocytes move from G_0 to G_1 in the cell cycle. During the replicative phase (12-36 hours post partial hepatectomy), DNA synthesis is markedly increased (S phase of the cell cycle) and reaches a maximum at 24 hours. The process of regeneration is generally completed in seven to fourteen days in rats, less in mice and more in humans (Higgins and Anderson 1931). The synchronous features of the process suggest that liver growth after partial hepatectomy follows a well controlled and regulated pattern and is not autonomous. No evidence exists for the differentiation of epithelial cells into hepatocytes (Tatematsu et al., 1984).

2.2.1.2. Response to Chemical Toxins

The regenerative process in response to hepatocyte damage caused by hepatoxins such as CCl_4 is more complicated as cell injury and regeneration occur side by side. In this setting, the pre-proliferative period of the regenerative response is longer (in rats 24 hours) and time to peak proliferative is later (in rats 48 hours) than in the response to partial hepatectomy. In addition, proliferating characteristics are more prominent in hepatic nonparenchymal cells than with partial hepatectomy. The latter finding raises the possibility that a specific "wound hormone" is released from the damaged cells.

2.2.2. Growth Control Factors Related to Liver Regeneration

2.2.2.1. Growth Factors

Hepatocytes are constantly influenced, in a balanced way, by growth stimulators and inhibitors which regulate liver regeneration. Many such factors have been and continue to be identified. Among these are hepatocyte growth factor (HGF), epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) which stimulate hepatocyte proliferation. In addition, factors such as transforming growth factor-β (TGF-β), and GABA have inhibitory effects on the regenerative process.

2.2.2.1.1. Growth Stimulators

HGF was first identified and purified as a potent liver mitogen by Nakamura et al., (1987). Subsequent studies reveal that it is produced mainly by mesenchymal cells, such as fat storing or Ito cells, Kupffer cells, and endothelial cells in response to liver injury and exerts its biological effects on hepatocytes by endocrine or paracrine mechanisms (Noji et al., 1990, Maher, 1993). Elevated circulating levels of HGF after partial hepatectomy (Lindroos et al., 1991) or CCl_4 injection (Webber et al., 1993) indicate that the factor may act as an inducer of compensatory DNA synthesis in the regenerative process. This is further supported by the observation that increased blood levels of HGF correlate inversely with subject survival in patients with fulminate liver failure (Shiota et al., 1995). More recently, it was reported that exogenous human HGF stimulates hepatic regeneration by increasing hepatic putrescine levels and DNA synthesis (Fujiwara et al., 1994).

EGF, is another important stimulator of hepatocyte proliferation. Bucher et al., (1978) reported that whole EGF can serve as a complete mitogen by itself to stimulate DNA synthesis in vivo, its effect is greatly enhanced in combination with insulin or glucagon. These findings are supported by the results of in vitro studies by Francavilla et al., (1986). More recently, Mullhaupt et al., (1994) observed that synthesis of EGF mRNA and peptide increases in regenerating liver shortly after partial hepatectomy. Thus, it is conceivable that EGF functions as a hepatocyte mitogen but the significance of its role related to other mitogens in liver regeneration needs to be further evaluated.

Transforming growth factor (TGF-α) is also mitogenic for hepatocytes, perhaps because TGF-α and EGF share the same receptor. The role of TGF-α in liver regeneration attracted attention when it was reported that regenerating livers actively produce TGF-α in an autocrine and paracrine fashion. Moreover increased levels of TGF-α were observed following partial hepatectomy and these changes of TGF-α expression coincided with DNA replication. Therefore, TGF-α is most likely to be a physiologic mediator of liver regeneration through stimulation of DNA synthesis in response to cell injury (Mead and Fausto 1989).

That partial hepatectomy results in an increase in other circulating growth promoters such as norepinephrine (Cruise et al., 1985), insulin and glucagon (Bucher and Swaffield 1975) suggest that these hormones may also act to promote the growth of hepatocytes.

2.2.2.1.2. Growth Inhibitors

In contrast to TGF-α, TGF-β is a potent inhibitor of hepatocyte proliferation. There is strong evidence that TGF-β inhibits hepatocyte DNA synthesis in vitro (Carr et al., 1986

I.) and in vivo (Russell et al., 1988). That TGF-β mRNA increases significantly in the liver after partial hepatectomy and the peaks lasting beyond the maximum DNA synthesis period (Jakowlew et al., 1991) would be consistent with the hypothesis that TGF-β functions as a repressor to prevent uncontrolled proliferation during liver regeneration. TGF-β is produced by nonparenchymal cells of the liver, particularly endothelial cells (Braun et al., 1988). TGF-β appears to function via a paracrine mechanism, that is, the factor released by liver endothelial cells, acts on hepatocytes.

Overall, TGF- α and TGF-β appear to act in opposite ways, as stimulator and inhibitor of DNA synthesis during liver regeneration. The balance between the effects of these two factors and presumably others may provide a mechanism to control the induction and cessation of regenerating hepatocytes. At present, it is still difficult to assess the importance of changes in hepatic growth factors to liver regeneration, because there is no direct evidence that these substances alter DNA synthesis when infused into the circulation of normal or regenerating livers.

GABA, a potent inhibitory amino acid neurotransmitter in brain, has growth regulatory properties. On the basis of carefully controlled metabolic pathways, it is assumed that GABA might play a role in regulating hepatic regenerative activity. The role of GABA during liver regeneration will be discussed in the section entitled *GABA and liver regeneration.*

2.2.2.2. Proto-oncogene Expression

Proto-oncogene induction has been proposed as the fundamental process for the initiation and progression of cellular proliferation. Their perceived importance is largely based on

the fact that these growth regulatory genes have been conserved through evolution. It is now evident that the regulation of liver regeneration is associated with the orderly expression of certain protooncogenes after partial hepatectomy in rats (Thompson et al., 1986). The activation of c-fos and c-myc is one of the first responses detected after partial hepatectomy which reflects the entry of hepatocytes into the cell cycle (G_0 to G_1). The products of these proto-oncogenes bind to DNA and regulate the expression of other genes required for liver regeneration. Enhanced expression of both c-Ha-ras and c-raf alter the transduction of growth factor signaling (Weinberg, 1985). Trauma associated with partial hepatectomy induces the release of tumor necrosis factor (TNF-α) which is capable of activating early proto-oncogenes, and thereby initiating hepatic regeneration. However, the release of TNF-α is local and regeneration is observed in all parts of the liver. Thus, the association between TNF-α, proto-oncogene expression and liver regeneration remains unclear.

2.2.2.3. Other Events

It has been noted that there is an association between the heat shock response and reaction to cellular stress. Carr et al., (1986) observed increased expression of the heat shock gene in regenerating livers subjected to a variety of stimuli i.e. trauma and hepatotoxins. However, the precise effect of the heat shock gene on liver regeneration needs to be elucidated.

Electrical depolarization of the liver occurs in the immediate period following partial hepatectomy (Zhang et al., 1995). This event may be responsible for the transition of the cell from G_0 to G_1. Since DNA is negatively charged and putrescine, a potent growth promoter, is positively charged during the depolarization associated with regeneration,

putrescine and other polyamines may move into the negatively charged nucleus. In the nucleus, they initiate DNA replication and stabilize DNA and mRNA which permits cells to proliferate.

2.2.3. Gene Expression and Biochemical Changes during Liver Regeneration

During liver regeneration, the increase in DNA synthesis results in an increase in the expression of certain genes and proteins which are responsible for the biochemical changes that are required for the liver to adapt to regenerating demands.

2.2.3.1. Gene Expression during Liver Regeneration

Church and McCarthy (1967) found that some genes were only expressed in regenerating liver and not in resting livers. However, detailed studies by Grady et al., (1981), Scholla et al., (1980) and Fausto (1984) have shown that there are no "new" mRNAs detected during liver regeneration, but rather ncreased concentrations of total RNA and mRNA (Atryzek and Fausto 1979). These findings indicate that most of the changes in gene expression in the regenerating liver involve quantitative modulations rather than the expression of "new" genes that are silent in the resting liver.

2.2.3.2. Biochemical Changes during Liver Regeneration

During the pre-replicative phase of liver regeneration, many metabolic changes occur (Fausto 1984), which are thought to be associated with the synthesis of DNA. Specifically, metabolites of the urea cycle increase after partial hepatectomy because the liver remnant has less functional capacity for their removal. Amino acids such as ornithine and lysine also accumulate and may contribute to the induction of DNA replication (Ferris and Clark 1972, Ord and Stocken 1972). Other metabolic changes during liver regeneration include

an increase in ornithine decarboxylase (McGowan and Fausto 1978), Na/K-ATP activity, and elevations in cAMP levels (Boynton and Whitfield 1983).

2.3. The Effects of Alcohol, Polyamines and GABA on Liver Regeneration

2.3.1. Alcohol and Liver Regeneration

Alcohol causes both cytotoxic and fibrogenic effects on the liver. Often, these effects are already present prior to patients seeking medical care. Alcohol also causes inhibition of hepatic regeneration in response to injury. This effect is still operative at the time of patient presentation. Thus, considerable interest should be focused on the effects of alcohol on hepatic regeneration.

The possibility that ethanol has an inhibitory effect on hepatic regeneration was suggested by the following observations; 1) With excess alcohol exposure, decreased restoration of liver mass is consistently observed in the early post-partial hepatectomy period (Diehl et al., 1988, Orrego et al., 1981). 2) Liver blood flow which may act as a regulating factor contributing to restoration of liver mass, is significantly altered by long-term ethanol exposure (Lieber, 1988). 3) Both Frank et al., (1979) and Wands et al., (1979) showed that acute and chronic administration of ethanol reduces radioactive thymidine incorporation into DNA following partial hepatectomy, which is a sensitive indicator of DNA synthesis and liver regeneration. 4) Determination of mitosis by autoradiography is also used to assess liver regeneration. When cell cycle changes were evaluated after partial hepatectomy, there was a reduction both in the prevalence of mitotic figures (Diehl et al., 1988) and in the magnitude and duration of peak DNA synthesis (Mendenhall et al., 1987) which indicate a decrease or delay in the proportion of

cells undergoing mitosis. 5) The effects of ethanol on RNA and protein synthesis have also been documented. Pösö and Pose (1981) reported that RNA synthesis in the liver remnant and in nuclei is reduced following ethanol intoxication. Using an ethanol-sensitive hepatoma cell line, Higgins (1987) found that there is a reduction in cellular RNA associated with decreased proliferation after short-term in vitro exposure to ethanol. Finally, a report by Renis et al., (1975) showed that ethanol inhibits overall protein synthesis in hepatic organelles after acute and chronic ethanol administration, which indicates that the decreased production of enzymes and structural proteins contribute to the functional and structural heterogenicity of cell changes in response to ethanol-related hepatic injury.

Taken together, excess ethanol exposure appear to significantly inhibit the restitution of liver mass, liver blood flow, DNA synthesis, prevalence of mitotic figures, and RNA and protein synthesis, all of which are important components of hepatic regeneration.

The mechanism whereby ethanol achieves these effects is unclear. Emerging evidence suggests that ethanol impairs the signal transduction mediated by hormones and growth factors in response to cell injury. This effect may also contribute to disordered hepatic regeneration in experimental animals and alcoholic patients during ethanol consumption. An example of this effect is that when adrenergic stimuli were administed, ethanol pretreatment prevented DNA synthesis in cultured hepatocytes (Carter and Wands 1985). It is conceivable that ethanol desensitized the hepatocytes to the trophic effects of the adrenergic stimuli through a signal transduction process. Diehl et al., (1992) demonstrated that chronic ethanol administration interrupted postreceptor activation of adenylate cyclase due to disturbed G protein expression and function which significantly inhibit

hepatic accumulation of cyclic AMP after partial hepatectomy. Cyclic AMP-dependent signals are not only important regulators of cellular proliferation and differentiation (Boynton and Whitfield 1988), by promoting the transition of hepatocytes from G_0 to G_1 (Thoresen et al., 1990), but also modulate liver cell growth and function during hepatic regeneration (Diehl et al., 1992). Therefore, ethanol-induced desensitization of signal transduction, particularly by the cyclic AMP-dependent pathway, may partly explain the inhibitory effects of ethanol on liver regeneration.

Cytokine-mediated signaling is also necessary for orderly hepatic regeneration. Inflammatory cytokines such as tumor necrosis factor (TNF)- α, interleukin-1 (IL-1) and IL-6 are involved in cell to cell communication during liver regeneration triggered by partial hepatectomy or toxic liver injury (Cornell 1989, Akerman et al., 1992). Substantial evidence supporting a role for TNF-α in these communications has been provided by Akerman et al., (1993) who reported that chronic ethanol exposure inhibits DNA synthesis and hepatocyte proliferation after partial hepatectomy after pretreatment with antibody to tumor necrosis factor. These data suggest that TNF-α may act as a regulator of hepatic regenerative growth through cytokine-mediated signaling mechanisms. Therefore, it is likely that any alteration in cytokine release or cellular responsiveness to cytokines will disturb liver cell proliferation and influence the rate and /or extent of hepatic regeneration.

As mentioned above, despite much effort to describe the process, the precise mechanisms responsible for ethanol-associated inhibition of liver regeneration are poorly understood. Moreover, any theory must take into account the lack of correlation between blood ethanol levels and liver regenerative activity, induction of hepatic microsomal enzymes and

decreased production of insulin and glucagon which are also important for cell replication (Wands et al., 1979). Furthermore, ethanol-related impairment of liver regeneration does not appear to be explained by altered transcription of protooncogenes such as C-fos, C-myc, and C-Ha-Ras (Diehl et al., 1990).

Although a number of factors are considered to be important for the regulation of liver cell proliferation, few are essential. One group of agents that is considered essential are the polyamines. Thus, the role and significance of polyamines in the setting of alcohol-impaired hepatic regeneration warrant further consideration.

2.3.2. Polyamines and Liver Regeneration

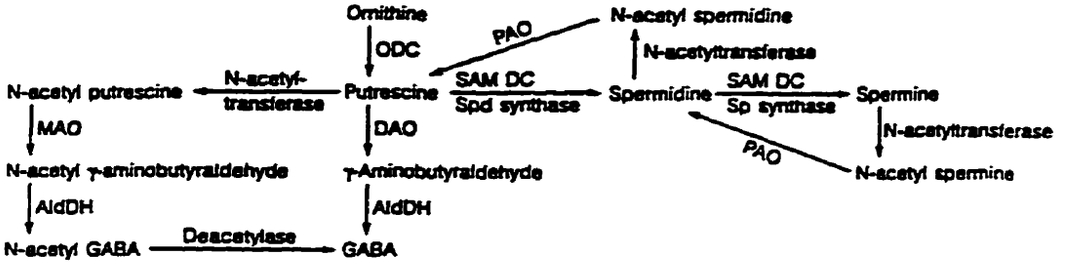
The polyamines including spermidine, spermine and their precursor, putrescine are widely distributed in almost all tissues, and have been thought to play an essential role in cell growth and differentiation (Tabor and Tabor 1984). The content of intracellular polyamines closely correlates with normal and pathological cell proliferation, both DNA and protein synthesis and may also affect the expression of genes which regulate cell division (Luscher and Eisenman 1988).

2.3.2.1. Polyamine Biosynthesis in the Liver

The metabolism of putrescine in mammals (Fogel 1986) is summarized in Figure 1. The key step in the synthesis of the principal polyamine, putrescine is the decarboxylation of ornithine. This step is controlled by ornithine decarboxylase (ODC), the first and rate limiting enzyme of the pathway (Tabor and Tabor 1984). A minor pathway for the production of putrescine is via the acetylation of spermidine and oxidation of N^1-acetylspermidine (Matsui et al., 1981).

The conversion from putrescine to spermidine and spermine occurs in the liver by the actions of aminopropyltransferases, the processes are catalyzed by spermidine synthase and spermine synthase respectively. The source of these propylamine groups is produced by the decarboxylation of S-adenosylmethionine which is catalyzed by S-adenosylmethionine decarboxylase. The latter enzyme is activated by putrescine and inhibited by spermidine and spermine.

Fig. 1. A short survey of the metabolism of putrescine in mammals. AldDH = Aldehyde dehydrogenase; MAO = monoamine oxidase; PAO = polyamine oxidase; SAMDC = S-adenosylmethionine decarboxylase



2.3.2.2. The Role of Polyamines during Liver Regeneration

Many studies have demonstrated the importance of polyamines in hepatic regeneration (Poso and Pegg 1982, Sato and Fujiwara. 1988). There is general agreement that polyamines are essential for the regenerative process. Luk (1986) reported that blockage of ornithine decarboxylase (ODC), the rate limiting enzyme for polyamine synthesis, by difluromethylornithine (DFMO), an irreversible inhibitor of ODC, completely retards rat hepatic regeneration following partial hepatectomy. DFMO prevents the increase in putrescine levels and further inhibits DNA and protein synthesis during regeneration. The impairment in cell proliferation can be largely overcome by putrescine supplementation. Subsequent work by Nishigushi et al., (1990) provided additional support, in that they showed that exogenous putrescine reverses D-galactosamine-induced suppression of liver regeneration. Further evidence are the observations by Minuk et al., (1991) and Marchesini et al., (1992) that hepatic and plasma levels of putrescine and spermidine significantly increase following stimulation of liver regeneration by partial hepatectomy in rats and humans respectively.

2.3.2.3. The Regulation of Polyamine Production

As would be expected of such a key enzyme, induction and inhibition of ODC are under extensive but rapid control mechanisms. Activity is regulated by many hormones, growth factors, tumor promoters, some drugs and after partial hepatectomy. For example, EGF which is known to stimulate DNA synthesis in vitro and in vivo, increases hepatic putrescine production after partial hepatectomy by inducing the content and expression of ODC mRNA (Nagoshi et al., 1991). Yet despite these controlling factors, the $T_{1/2}$ of ODC is the shortest of all enzymes.

2.3.3. GABA and Liver Regeneration

It is universally accepted that GABA is a potent inhibitory amino acid neurotransmitter in the central nervous system. However, since GABA was detected in several tissues of many species, including human, by various techniques (Erdō 1986), its role is now thought not to be restricted to the CNS. Over the past two decades, much effort has been expended on elucidating the functions of peripheral GABA. The results of these efforts suggest that GABA possesses growth inhibitory properties (Boggust and Nakib 1986; Gilon et al., 1987). In the liver, GABAergic innervation and the ability to synthesize and metabolize GABA is not only present, but altered during hepatic development and function (Minuk et al., 1984, 1987, and 1993). It is also likely that GABA plays a inhibitory role in alcohol-induced inhibition of hepatic regeneration (Minuk et al., 1996).

2.3.3.1. GABA Biosynthesis and degradation in the Liver

The contribution of glutamate-derived GABA to the total GABA pool in the liver is considered to be insignificant, due to the relatively low hepatic concentrations of glutamate decarboxylase (GAD). Thus, the predominant pathway of GABA formation in the liver is via diamine oxidase (DAO) (Fogel 1986).

DAO catalyzes the direct oxidative deamination in the conversion of putrescine to gamma-aminobutyraldehyde which is then further oxidized to GABA, by aldehyde dehydrogenase. GABA itself is ultimately oxidized to carbon dioxide and water via the Krebs' cycle. Alternative pathways of GABA formation from putrescine involve acetylation of putrescine by N-acetyltransferase. Subsequent steps comprise the oxidative deamination of monoacetylputrescine by monoamine oxidase (MAO), the oxidation of the aldehyde product to N-acety-GABA, and then deacetylation to GABA.

The degradative pathway of GABA is thought to be under the control of GABA transaminase (GABA-T) which is the predominant route for GABA catabolism in the liver. In fact, GABA-T seems to be the most important catabolic enzyme responsible for GABA metabolism. It deaminates GABA to succinyl semialdehyde from which the end products, carbon dioxide and water are formed. The liver contains relatively high concentrations and activity of GABA-T (White and Sato 1978). There may be an alternative pathway for GABA metabolism in which GABA is converted directly to spermidine.

2.3.3.2. GABA Transport and Receptor in the Liver

It is likely that both intrinsic biosynthesis and an active uptake system may contribute to the presence of GABA in the liver. Bondy and Harrington (1978) were the first to describe specific binding of radiolabelled GABA to liver microsomal fractions. Sixteen years later, Minuk et al., (1984) identified and characterized a specific GABA transport system in the liver. The system, which was present on the surface of isolated hepatocytes, was time- and sodium-dependent as well as saturable and demonstrated competitive inhibition. The system is thought to be involved in hepatic GABA clearance from the circulation as significant elevations in serum GABA concentrations occur in the setting of hepatitis, cirrhosis and partial hepatectomy which may contribute to the neurological disorder of hepatic encephalopathy.

On the basis of similar ligand-receptor binding assays and electrophysiologic responses, a specific, sodium-independent, bicuculline-sensitive GABA receptor site was also identified on the surface of isolated rat hepatocytes by Minuk et al., (1987). The receptor system

was found to possess biochemical and pharmacological properties similar to those of GABA receptors in the brain. Moreover, the physiologic effect of activating the receptor system by GABA and muscimol, a specific GABA receptor agonist, produced marked hyperpolarization of the resting membrane potential of hepatocytes and this effect was prevented and completely reversed by bicuculline, a specific GABA receptor antagonist.

It should be noted, however, that the evidence suggesting the presence of GABA transport and receptor systems in the liver are mainly indirect, their respective genes have yet to be cloned in hepatocytes. Moreover, the possible functional relevance of the two systems and physiological and pathophysiological role of GABA in the liver are much debated.

2.3.3.3. The Role of GABA during Liver Regeneration

The possibility that GABA might play a role in influencing hepatic regenerative activity was based on several observations. Firstly, elevated serum GABA levels are observed in patients with acute and chronic liver failure, settings where severe inhibition of hepatic regeneration often occurs (Ferenci et al., 1983, Minuk et al., 1985) and the sera from patients with fulminant hepatic failure inhibit DNA synthesis of regenerating hepatocytes (Gove et al., 1982). Secondly, exogenous GABA inhibits the growth of rapidly developing tissues and tumours (Sobue and Nakajima 1977, Boggust et al., 1986, Gilon et al., 1987). Thirdly, GABA is a putrescine derivative, thus, the inhibitory effect of GABA may be via a negative feedback mechanism to decrease further putrescine production.

Subsequent studies have provided strong support for an inhibitory role of GABA in hepatic regeneration. Exogenous GABA significantly inhibits restitution of liver mass and

protein synthesis by the liver following partial hepatectomy (Minuk and Gauthier 1993), hepatic DNA synthesis (Minuk et al., 1992), inhibits peak insulin-like growth hormone (IGF)-1 and IGF binding protein-1 mRNA expression (Minuk et al., 1990), prevents the depolarization that occurs in the early regenerative period (Minuk et al., 1987, 1997), attenuates the increase in hepatic putrescine levels that normally occurs after partial hepatectomy and interferes with hepatic ODC activity (Lapinjoki 1983, Minuk et al., 1991). Of interest, supplemental administration of putrescine reverses the regenerative activity to normal in GABA-treated rats (Minuk et al., 1993). These findings provide some insight into how GABA exerts its inhibitory effects on liver regeneration.

2.4. Alcohol/putrescine/GABA-interactions

As mentioned above, putrescine is thought to be essential to the hepatic regenerative process. Substantial evidence has demonstrated that ethanol inhibits the accumulation of putrescine, which further impairs the hepatic regenerative response (Diehl et al., 1990, Tanaka et al., 1991,1993). Exogenous putrescine restores ethanol-associated inhibition of liver regeneration (Diehl et al., 1990). GABA seems to exert its inhibitory effect in a similar way (Minuk et al., 1993).

Of note, ethanol significantly increases serum GABA levels (Bannister et al., 1988) and decreases GABA transport mRNA (Gong et al., unpublished data). Ethanol also potentiates GABA receptor activity (Varga and Kunos 1992). More recently, our laboratory demonstrated that ciprofloxacin, an antimicrobial with GABA receptor antagonist properties, completely prevented the inhibitory effects of acute ethanol administration on the regenerative process in rats. Thus, these findings suggest that there

may be an association between GABA's hyperpolarization, decrease in hepatic putrescine levels and the impairment of hepatic regeneration (Minuk et al., 1995). Because both ethanol and GABA decrease hepatic putrescine levels, and both exert similar inhibitory effects on hepatic regeneration, we assumed that the inhibitory effect of ethanol in the liver may be via GABA-mediated mechanisms.

Chapter III

Hypothesis

The antiregenerative effect of ethanol on the liver is mediated by increased hepatic conversion of putrescine (a hepatic growth promoter) to GABA (a hepatic growth inhibitor)

Chapter IV

Objectives of the Study

4.1. Objectives

The overall objective of this study was to investigate the effects of acute ethanol exposure on the putrescine metabolic pathway and in the process to identify possible therapeutic options that could eventually be applied to patients with alcohol-induced liver disease.

4.2. Specific Objectives

To document:

1. The effects of ethanol on putrescine catabolism and GABA synthesis by measuring DAO and MAO activity.

2. The effect of ethanol on GABA catabolism by measuring GABA-T activity

3. The effect of ethanol on hepatic GABA and polyamine levels in regenerating livers.

Chapter V

Experimental Design and Methods

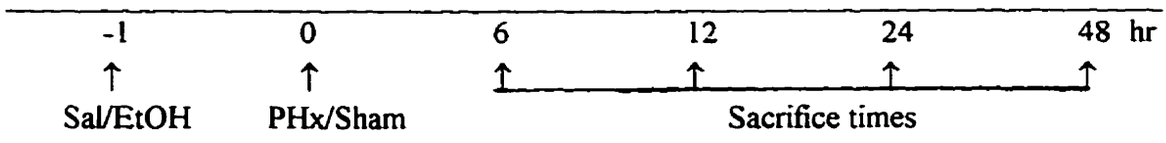
5.1. Animals

Adult male Sprague-Dawley rats (200-250 g) were obtained from Charles River Canada. The animals were housed in a temperature-and light-controlled room. A minimum of five animals were used in each study group.

5.2. Surgery

After fasting overnight , rats received either a single dose of alcohol (5 g / kg) or an equal volume of saline by gastric gavage. One hour later, a partial (70 %) hepatectomy which was performed under light ether anesthesia between 9 and 12 AM as described by Higgins and Anderson (1931). In sham operations, the rats were laparotomized, and the livers were gently manipulated. The rats were killed at 6, 12, 24 and 48 hours post partial hepatectomy. The remnant liver was immediately isolated and frozen with liquid nitrogen. The liver tissue was stored at -70°C for determining diamine oxidase, monoamine oxidase activity and GABA transaminase activity and intracellular GABA and polyamine levels.

5.3. Experimental protocol



5.4. GABA Synthesis Enzyme Activity

5.4.1. *Diamine Oxidase (DAO) Activity Determination*

5.4.1.1. Enzyme Preparations

After thawing, the tissues were homogenized 1:10 (w / v) in 0.01 M Na^+ / K^+ phosphate buffer at pH 7.4 containing 0.25 M sucrose in a glass-teflon homogenizer, and the homogenate was centrifuged at 100,000 × g for 60 min. The pellets (microsomes) were resuspended in 0.01 M Na^+ / K^+ phosphate buffer at pH 8.5, divided into several parts and stored at -70°C for use as enzyme sources.

5.4.1.2. Reagents

- Phosphate buffer (0.01 M , pH 8.5) Na_2HPO_4 and KH_2PO_4 (sigma).
- Putrescine dihydrochloride solution (1.5 mM) : 12.7 mg were dissolved in 50 ml distilled water as a stock solution. The solution was diluted 10-fold with 0.01 M Na / K phosphate buffer (pH 8.5) to give a final concentration 10 µmol/L.
- Putrescine-1,4- ^{14}C dihydrochloride (Radiochemical Center, Amersham) : Specific activity 109 mCi / mmol, 50 µCi / ml corresponding to 0.46 µmol of putrescine. The content of 0.2 ml (10 µCi) was dissolved in 10 ml of the solution of unlabelled putrescine as described above. A specific activity of 1 µCi / ml = 2.2 × 10^6 dpm / ml was obtained.
- $HClO_4$ 70 % : 7 ml were dissolved in water to a total volume of 50 ml.
- Strong alkaline buffer: 1000 ml stock solution saturated with solid $NaHCO_3$ at room temperature was prepared. A 2 N NaOH solution was added in 200 ml of the clear supernatant to reach pH 12.2.

5.4.1.3. Isotope Assay

DAO activity was assayed by the radiochemical method of Okuyama and Kobayashi (1961) as described by (Kusche et al., 1973). The steps of the incubation and extraction procedure for DAO are described in Table 1.

Two different blanks were used in order to ensure that the radioactive products extracted into toluene were formed by the enzymatic reaction: (1) Enzyme blank: The enzyme was inactivated by boiling for 3 min before the addition of the substrate. (2) Reagent blanks: Phosphate buffer was added to the incubation mixture instead of enzyme. The DAO activity was described as pmol / mg in 60 min.

Table 1.

Incubation and extraction procedure for determining DAO activity

	volume (ml)	final composition of the mixture
(1) incubation procedure:		
Na / K PO_4 buffer (pH 8.5)	0.60	0.01M
Enzyme: liver microsomes	0.10*	
Mix & bring the solution to 37°C, start with		
Substrate: ^{14}C-putrescine & cold Putr	0.05	10 μM ≈10^5 dpm
Mix & allow to react, stop after 60 min		
Perchloric acid	0.20	0.2M
(2) Extraction procedure:		
Strong alkaline buffer (pH 12.2)	1.0	pH 10.0
Toluene	6.0	
Shake 4 min, centrifuge 2 min		
(3) Scintillation counting:		
An aliquot of organic phase	5.0	
scintillation fluid	5.0	

5.4.2. Monoamine Oxidase (MAO) Determination

5.4.2.1. Enzyme preparations

After thawing, the tissues were homogenized 1:10 (w / v) in 0.1 M Na / K phosphate buffer at pH 7.2 in a glass-teflon homogenizer, and centrifuged at 10,000 × g for 20 min. The pellets (mitochondria) were resuspended in the same phosphate buffer, divided into several parts and stored at -70°C for use as enzyme sources.

5.4.2.2. Reagents

- Phosphate buffer (0.1 M , pH 7.2) Na_2HPO_4 and KH_2PO_4 (Sigma, St. Louis).
- tryptamine dihydrochloride solution (2.5 mM) : 48.4 mg were dissolved in 50 ml 0.1 M phosphate buffer to give a final concentration of 0.5 mM.
- ^{14}C labelled tryptamine dihydrochloride (The Radiochemical Center, Amersham) Specific radio-activity 55 mCi / mmol, 50 µCi / ml corresponding to 0.9 µmol of tryptamine. The content of 0.1 ml (5 µCi) was dissolved in 10 ml of the solution of unlabelled tryptamine as described above. A specific radio-activity of 0.5 µCi / ml = 1.1×10^6 dpm / ml was obtained.
- 2M citric acid .
- Toluene: ethylacetate (1:1).

5.4.2.3. Isotope Assay

MAO activity was assayed by the radiochemical method of Tipton (1985). The steps of the incubation and extraction procedure for MAO are described in Table 2.

Enzyme and reagent blanks were used as for the DAO assay. MAO activity was described as nmol / mg in 30 min.

Table 2.

Incubation and extraction procedure for determining MAO activity

	volume (ml)	final composition of the mixture
(1) incubation procedure:		
Na / K PO_4 buffer (pH 7.2)	0.30	0.1 M
Enzyme: liver mitochondrias	0.10*	
Mix & bring the solution to 37°C, start with		
Substrate: ^{14}C-tryptamine & cold Try	0.10	0.5 mM $\approx 10^5$ dpm
Mix & allow to react, stop after 30 min		
citric acid	0.20	2.0M
(2) Extraction procedure:		
Toluene-ethylacetate	6.0	
Shake 4 min, centrifuge 2 min		
(3) Scintillation counting:		
An aliquot of organic phase	5.0	
scintillation fluid	5.0	

5.5. GABA Transaminase (GABA-T) Activity

5.5.1. Enzyme Preparations

After thawing, the liver tissues were homogenized in 4 vol of 0.1 M sodium phosphate buffer at pH 7.35 in a glass-teflon homogenizer, and centrifuged for 30 min at 130,000 × g (Beckman 70.1 Ti) at 0 °C. Pellets of the homogenates were resuspended in 50 mM Tris-HCl buffer (pH 8.4) with 0.5 % (w / v) Triton X-100, 20 mM mercaptoethanol and 0.3 mM pyridoxal phosphate, divided into several parts and stored at -70°C for use as an enzyme source.

5.5.2. Reagents

- Tris-HCl buffer (50 mM , pH 8.4) with 0.5 % (w / v) Triton X-100, 20 mM mercaptoethanol and 0.3 mM pyridoxal phosphate
- GABA solution (8 mM) : 41 mg was dissolved in 50 ml distilled water to give a final concentration of 2 mM.
- ^{3}H labelled GABA (Amersham) : Specific radio-activity 88.4 Ci / mmol, 1 mCi / ml corresponding to 0.01 μmol of GABA . 25 μl (25 μCi) was dissolved in 10 ml of the unlabelled GABA solution. Thus, a specific radio-activity of 2.5 μ Ci / ml = 5.5×10^{6} dpm / ml was obtained.
- 40 mM NADP
- 10 mM Succinate
- 20 mM α- ketoglutarate
- 8% trichloroacetic acid.

5.5.3. Column Chromatography

A Dowex-50 w column, of particle size 100-200 u, was used to separate the principle product, succinate, from ^{3}H-GABA. The ionic exchange resin was swelled with distilled water. A glass column (0.5×3 cm) was packed by the open flow procedure, and was allowed to equilibrate overnight with 0.1 N HCl solution. The column was loaded with 0.2 ml of sample and eluted with distilled water. Fractions (5 ml) were collected, and aliquots (1 ml) of each fraction were added to scintillator fluid to determine radioactivity.

GABA-T activity was determined by the method of Hall and Kravitz (1967). The steps of the incubation and separation procedure are described in Table 3. Enzyme and reagent blanks were used as for the DAO assay. GABA-T activity was described as nmol / mg in 30 min.

Table 3.

Incubation and extraction procedure for determining GABA-T activity

	volume (ml)	final composition of the mixture
(1) incubation procedure:		
Tris-HCl buffer (pH 8.4)	0.075	50 mM
10 mM Succinate	0.010	0.5 mM
40 mM NADP	0.010	2 mM
20 mM α- ketoglutarate.	0.010	1 mM
Enzyme: liver homogenate	0.045*	0.1 mg protein
Mix & bring the solution to 37°C, start with		
Substrate: ^{3}H-GABA & cold GABA	0.050	2 mM $\approx 1.6\times10^{5}$ dpm
Mix & allow to react, stop after 30 min		
Trichloroacetic acid	0.05	0.4M
(2) Extraction procedure:		
Dowex-50 w column		
to separate GABA & product		
by washing the column with water	5.0	
(3) Scintillation counting:		
An aliquot of eluate solution	1.0	
scintillation fluid	10.0	

5.6. Tissue GABA and Polyamine Determinations

5.6.1. GABA Determinations

5.6.1.1. Chemicals

GABA and 1,7-diaminoheptane were obtained from Sigma. HPLC-grade acetonitrile from Fisher. All other chemicals were analytical-reagent grade.

5.6.1.2. Chromatographic Equipment

GABA levels were measured by high-performance liquid chromatography (HPLC) using the method described by Ming (1988) . Analyses were performed on a LiChrosorb RP-18 column (25cm × 0.4 cm I.D.) filled with a 5- μm C18 reverse-phase packing (Merck). Detection was accomplished using a fluorescence detector (Waters) set at excitation wavelength 340 nm, emission 540 nm. The signal was recorded by a computing integrator (Nelson).

5.6.1.3. Sample Preparation

After thawing, the liver tissues were homogenized in 4 vol of 0.1 M sodium phosphate buffer at pH 7.35 in a glass-teflon homogenizer, and centrifuged for 30 min at 130,000 × g (Beckman 70.1 Ti) at 0 °C. The supernatants, deproteinized with 1.5 volumes of 0.2 M perchloric acid and mixed with 100 nmol of 1,7-diaminoheptane as an internal standard, were centrifuged at 8000 × g for 15 min. Dried aliquots of the supernatants were adjusted to pH 9.5-10.5 with 1.5 M sodium carbonate buffer and dansylated with 1.5 volumes of dansyl chloride (15 mg /ml acetone). Derivatization was carried out at 70 °C for 15 min in a water bath. The mixture was then passed through a Sep Pak (5 C_{18}) column. Dansylated GABA was eluted with 60 % acetonitrile and evaporated to dryness. The

residue was dissolved in a mobile phase and filtered through 0.22- μm filters for HPLC analyses. The same procedure was followed for an external standard which was prepared from a stock solution consisting of GABA (5×10^{-4} M) in 0.2 M perchloric acid. Known amounts of standard were spiked into liver homogenates for validation.

5.6.1.4. Chromatography

A gradient system with buffer A (0.1% trifluoroacetic acid, TFA) and buffer B (100 % acetonitrile) was used. The samples were eluted with a linear gradient of 20 -75% solvent A in B. Pumps was set to deliver the mobile phase through the analytical column at a flow-rate of 1.0 ml / min. The gradient program is reported in Table 4.

Table 4. Composition of the gradient

	Elution time (min)	Percentage of solvent	
		A	B
	0.0	80	20
	13.0	25	75
End time	15.0		
Equilibration time	5.0		

5.6.2. Determination of Polyamines

5.6.2.1. Chemicals

Putrescine, spermidine, spermine and 1,7-diaminoheptane were obtained from (Sigma) and HPLC-grade acetonitrile from (Fisher). All other chemicals were analytical-reagent grade.

5.6.2.2. Chromatographic Equipment

Polyamine levels were measured by HPLC using the method described by Desidrio (1987) . The HPLC system was as described above.

5.6.2.3. Sample Preparation

After thawing, liver tissues were homogenized in 4 vol of 0.1 M sodium phosphate buffer at pH 7.35 in a glass-teflon homogenizer, and centrifuged for 30 min at 130,000 × g (Beckman 70.1 Ti) at 0 °C. The supernatants (0.2 ml) were deproteinized with 1.0 volume of 0.2 M perchloric acid mixed with 25 nmol of 1,7-diaminoheptane as the internal standard and centrifuged at 8000 × g for 15 min. The supernatants (0.2 ml) were adjusted to pH 9.5-10.5 with 1.5 M sodium carbonate buffer and dansylated with 1.5 volumes of dansyl chloride (15 mg /ml acetone). Derivatization was carried out at 70 °C for 15 min in a water bath. The dansyl amines were extracted with benzene and the mixture was centrifuged to form two phase systems, the organic phase was then transferred and evaporated to dryness. The residue was dissolved in methanol and filtered through 0.22- μ m filters for HPLC analysis. The same procedure was followed for the external standard, which was prepared from a stock solution for each amine (putrescine, spermidine and spermine) at 5×10^{-4} M in 0.2 M perchloric acid. Known amounts of the standards were spiked into liver homogenates for validation.

5.6.2.4. Chromatography

A gradient system with buffer A (1.2 M disodium hydrogenphosphate) and buffer B (80 % acetonitrile and 20 % 1.2 M disodium hydrogen phosphate) was used. Samples were eluted with a gradient of 40 -99% solvent A in B which was interrupted and followed by two isocratic periods of 6 and 8 min, respectively. The pumps were set to deliver the mobile phase through the analytical column at a flow-rate of 1.5 ml / min. The gradient program is as reported in Table 5.

Table 4. Composition of the gradient

	Elution time (min)	Percentage of solvent	
		A	B
	0.0	60	40
	5.5	27	73
	11.5	27	73
	16.0	1	99
End time	24.0		
Equilibration time	8.0		

5.7. Statistical analysis

The data are expressed as mean± SD of five to nine animals. Significance of differences between groups was determined by one-way analysis of variance (ANOVA) and where appropriate, Student's *t* test with Bonferoni's adjustment for multiple comparisons.

Chapter VI

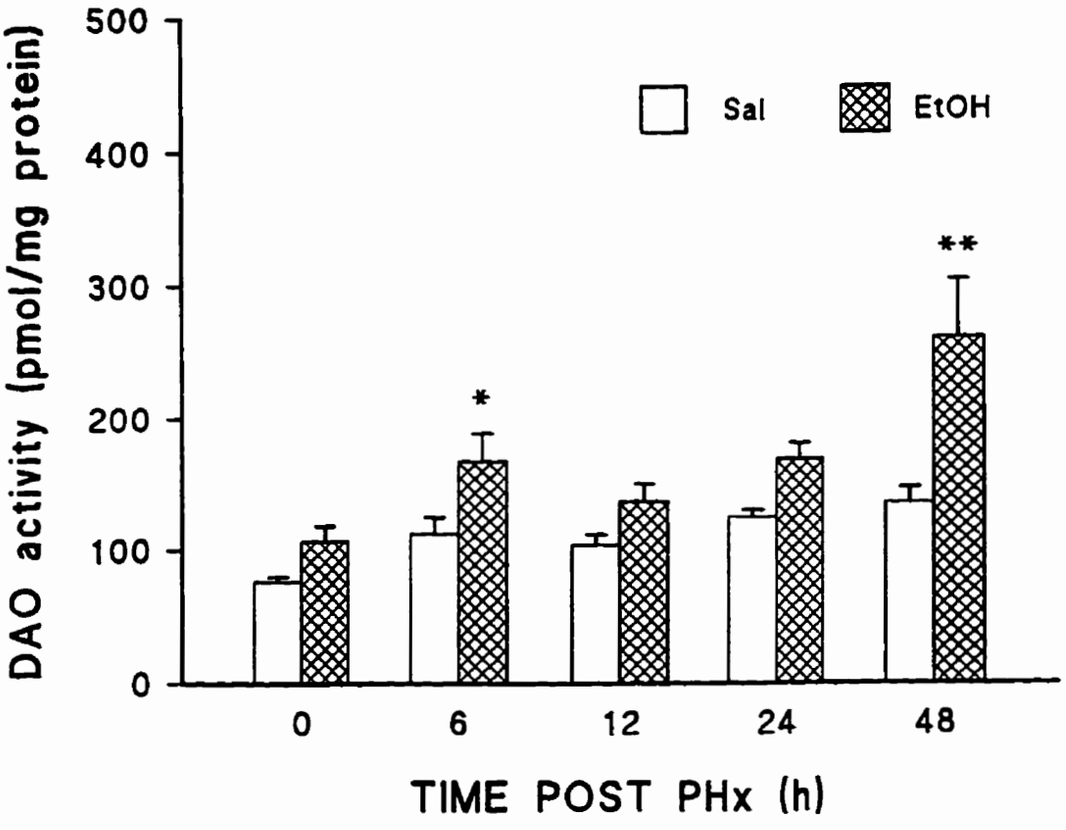
Results

6.1. Effect of ethanol on hepatic DAO activity in rats

As shown in Figure 1, in general, DAO activity was relatively unaltered post partial hepatectomy in saline- treated controls. In ethanol-treated rats, DAO activity was consistently higher than in the saline-treated group. The maximum difference occurred at 48 h when DAO activity in ethanol-treated rats was approximately twice that of the saline treated controls. Statistical significance was achieved at 6 h ($P<0.05$) and at 48 h ($P<0.01$).

Figure 6.1. Hepatic diamine oxidase (DAO) activity (pmol / mg protein / h) in ethanol-treated vs. control rats after partial hepatectomy. Tissue was obtained from rats that were treated with a single dose of alcohol (5 g / kg) or an equal volume of saline by gastric gavage. Partial hepatectomy was conducted 1 hour later. Tissue from ethanol treated and control rats were assayed concurrently as described under Methods. Each point represents the mean±SD of data in nine rats from two separate but similar experiments. *$P < 0.05$ at 6 h and **$P<0.01$ at 48 h vs. controls.

Fig. 6.1. DAO Activity in Regenerating Liver



6.2. Effect of ethanol on hepatic MAO-A activity in rats

The results of hepatic MAO-A activity in control and ethanol-treated rats post partial hepatectomy are provided in Figure 2. MAO-A activity was essentially identical in saline- and ethanol-treated groups at each time interval studied.

Fig. 6.2. MAO Activity in Regenerating Liver

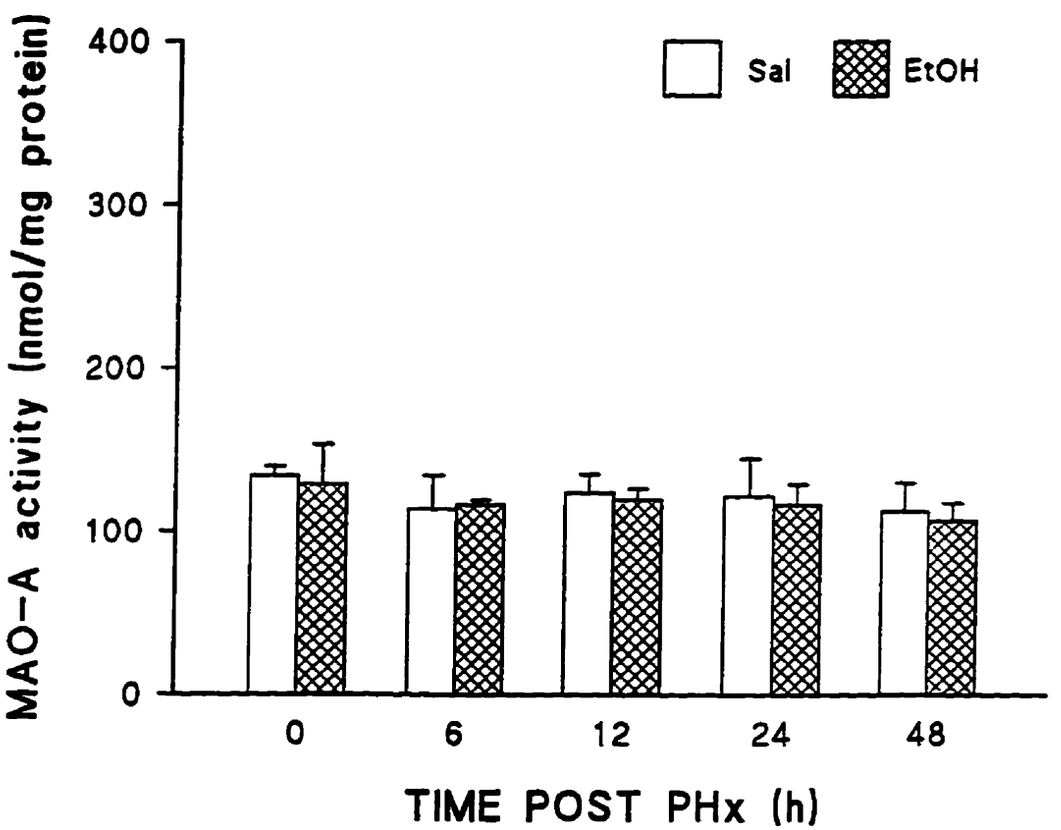


Figure 6.2. Hepatic monoamine oxidase (MAO) activity (nmol / mg protein / h) in ethanol-treated vs. control rats after partial hepatectomy. Tissue was obtained as described in Methods Each point represents the mean±SD of data in five rats.

6.3. Polyamine (putrescine, spermidine and spermine) determinations

HPLC profiles of a standard of dansylated polyamines (250 pmol of each compound) and rat liver tissue extract are shown in Fig. 6.3. Satisfactory separation and symmetrical peak shapes of dansyl derivatives of putrescine, spermidine, spermine and internal standard (1,7-diaminoheptane) were achieved with the retention times of 13.5 min, 17.6 min, 21.6 min and 15.7 min respectively. The HPLC elution profiles obtained from rat liver tissue samples were very similar to that of the standards. The standard curves were prepared by adding known amounts of each compound to 0.2 M perchloric acid over the range 10 to 50 ng / ml. The quantification was based on the ratio between peak areas of the compounds to be determined to that of the internal standard.

Putrescine levels were measured in order to determine if the ethanol-induced increases in DAO activity were associated with reduced hepatic putrescine and other polyamine levels. As shown in fig. 6. 4. putrescine levels peaked biphasically after partial hepatectomy with the early peak occurring at 6 h followed by a gradual decrease until the second peak occurred at 48 h. In the early post partial hepatectomy period (0-12 h), putrescine levels were lower in ethanol-treated vs. saline-treated but the differences were not significant. By 24 h, putrescine levels in ethanol-treated rats were no longer lower than saline-treated controls and indeed, were significantly higher when they reached their maximum at 48 h ($P<0.01$).

Because putrescine is the substrate for spermidine and spermine formation, levels of these polyamines were also measured. The results are provided in Figures 6. 5. and 6. 6. respectively. Hepatic spermidine levels gradually increased after partial hepatectomy in both saline and ethanol-treated groups. There were no differences in spermidine levels

between the two groups. As shown in Fig. 6. 6., neither partial hepatectomy nor ethanol exposure significantly affected the hepatic concentrations of spermine.

Figure 6.3. HPLC profiles of a standard of dansylated polyamines (A) and rat liver tissue extract sample (B). Chromatography conditions: Separation was performed on Beckman HPLC system with a RP-C 18 column (SPE, 5 μm, 25cm × 0.4 cm I.D.). The mobile phase was a mixture of 1.2 mM Na_2HPO_4 and acetonitrile with a solvent gradient at flow rate 1.5 ml/min. A fluorescence detector was used with the wavelength set at Ex 340nm and Em 540 nm.

Fig. 6.3 HPLC Profiles of a Standard of Dansylated Polyamines and Rat Liver Tissue Extract

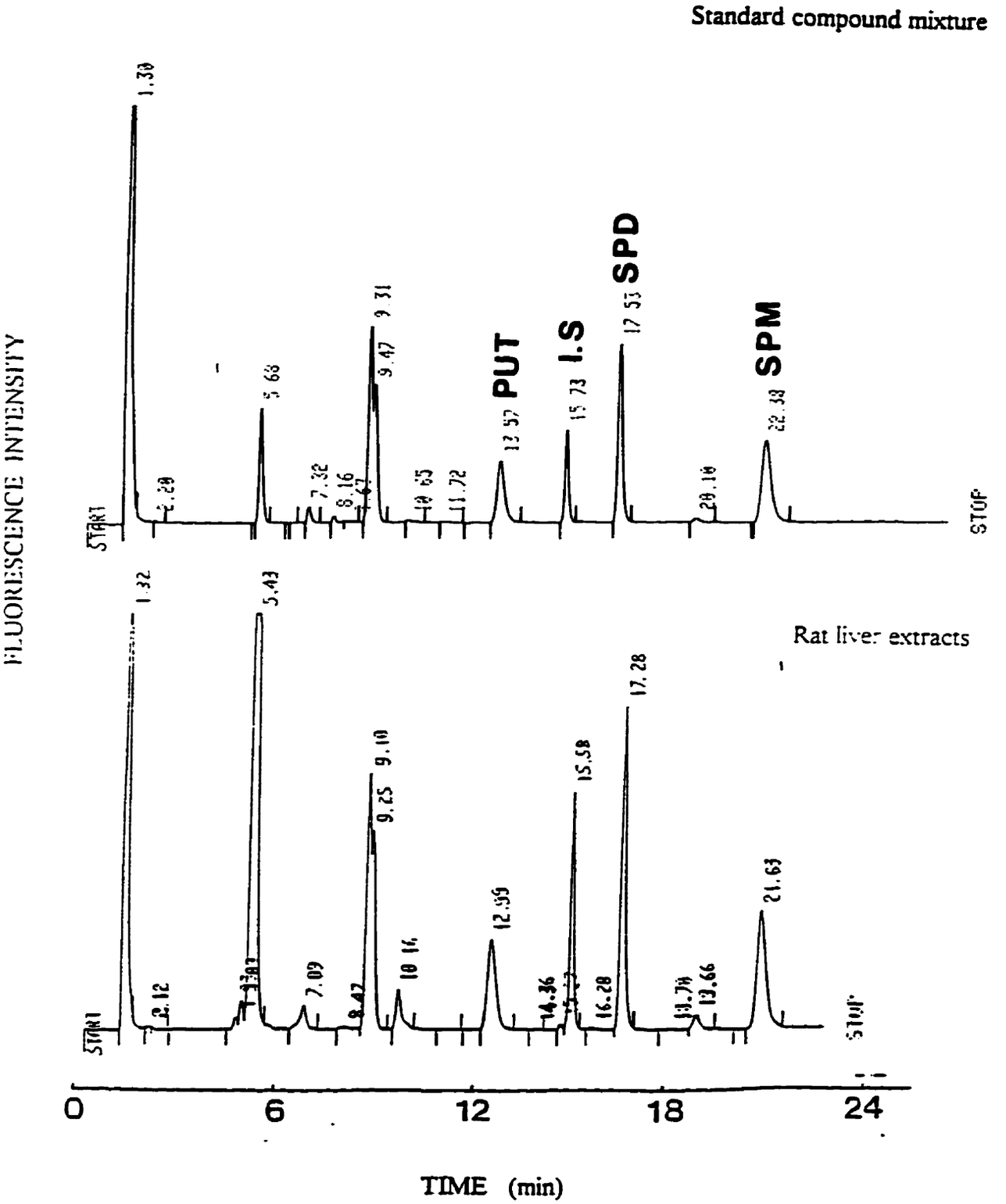


Figure 6.4. Hepatic putrescine concentrations (nmol/g liver) in saline- and ethanol-treated rats after partial hepatectomy. Tissue was obtained as described in Methods. Dansylated putrescine was measured in acid-precipitated liver extracts using the HPLC method as described in Methods. 1,7-diaminoheptane was used as an internal standard and all samples were analyzed in duplicate. Each point represents the mean±SD of data in five to six rats. * $P<0.05$ at 48 hours vs. ethanol-treated rats.

Fig. 6.4. Hepatic Putrescine Concentrations in Regenerating Liver

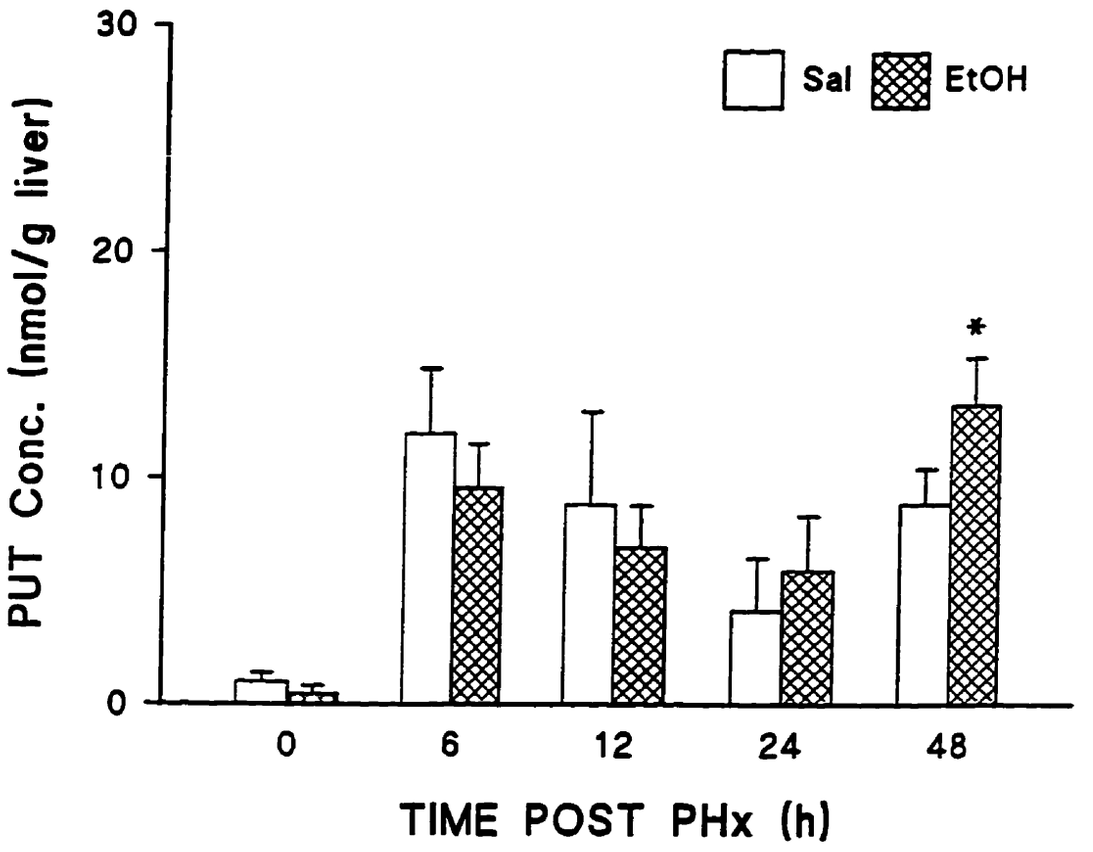


Fig. 6.5 Hepatic Spermidine Concentrations in Regenerating Liver

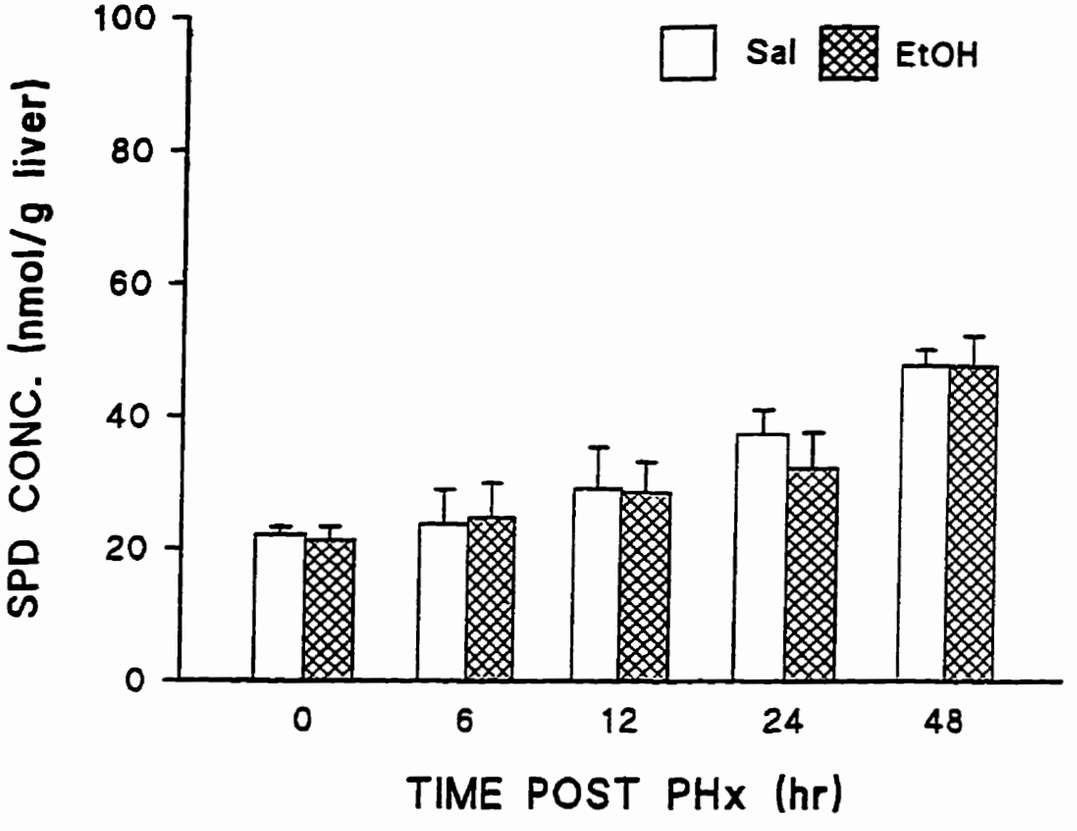


Figure 6.5. Hepatic spermidine concentrations (nmol/g liver) in saline- and ethanol-treated rats after partial hepatectomy. Dansylated spermidin was used as described Method. Each point represents the mean±SD of data in five to six rats.

Fig. 6.5 Hepatic Spermine Concentrations in Regenerating Liver

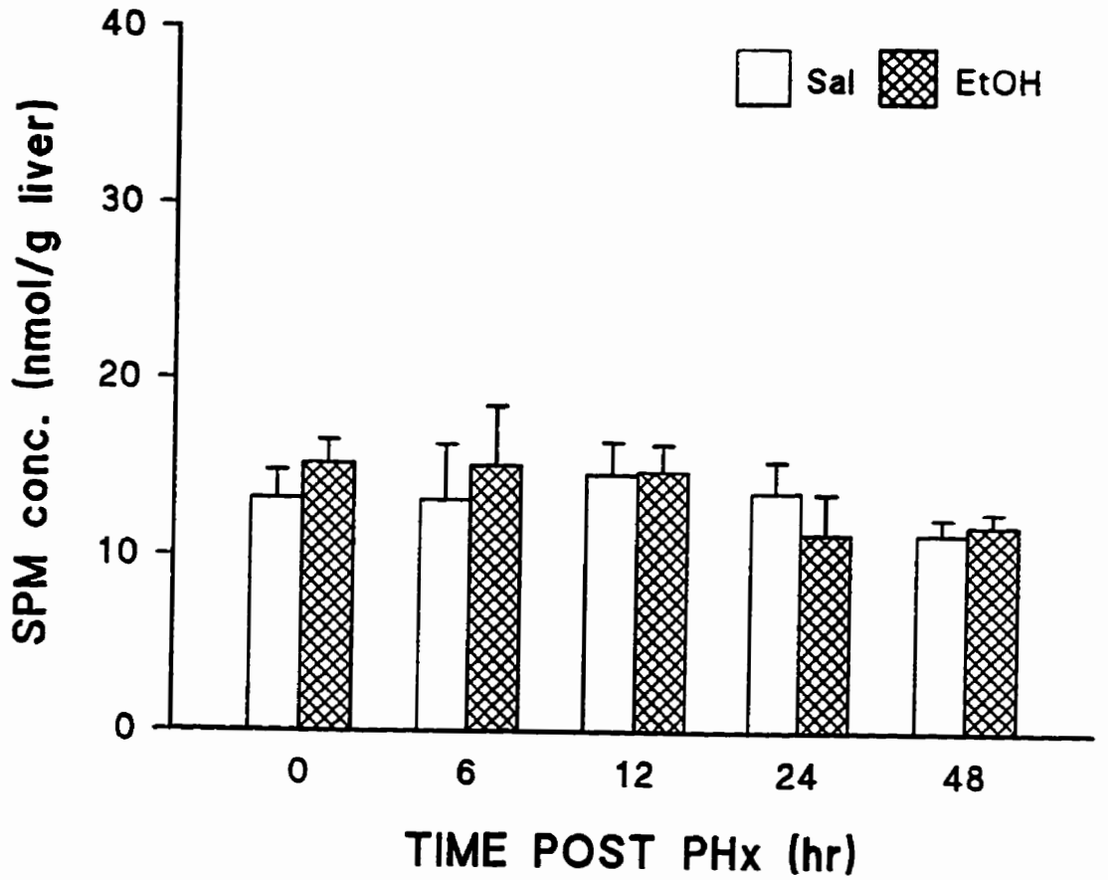


Figure 6.6. Hepatic spermine concentrations (nmol/g liver) in saline- and ethanol-treated rats after partial hepatectomy. Dansylated spermines were used as described in Methods. Each point represents the mean±SD of data in five to six rats.

6.4. GABA determinations

Figure 6. 7. (A) represents chromatograms of a standard of dansylated GABA (10 μmol / ml) and 6.7. (B) that of rat liver tissue extracts. GABA was well separated from other peaks with a retention time of 6.62 min. Peak height plotted against concentration was linear over a range from 3.13 to 50 μg / ml. Sample concentrations were determined from the chromatograghic GABA to the internal standard peak area ratio from a standard curve prepared concurrently with the samples.

As shown in figure 6. 8., GABA levels were significantly increased following partial hepatectomy in saline treated controls when compared to the values in resting livers at time 0. Ethanol exposure was associated with lower GABA levels at 12 h ($P<0.01$) and higher levels at 48 h ($P<0.01$) post partial hepatectomy.

Figure 6. 7. HPLC chromatogram of a standard of dansylated GABA (A) and rat liver tissue extract sample (B) on a Beckman HPLC system with a RP-C 18 column (SPE, 5 μ m, 25cm × 0.4 cm I.D.). The mobile phase was a mixture of 0.1% trifluoroacetic acid (TFA) and acetonitrile with a solvent gradient at flow rate 1.0 ml/min. A fluorescence detector was used with the wavelength set at Ex 340 nm and Em 540 nm.

Fig. 6.7 HPLC Chromatogram of a Standard of Dansylated GABA and Rat Liver Tissue Extract

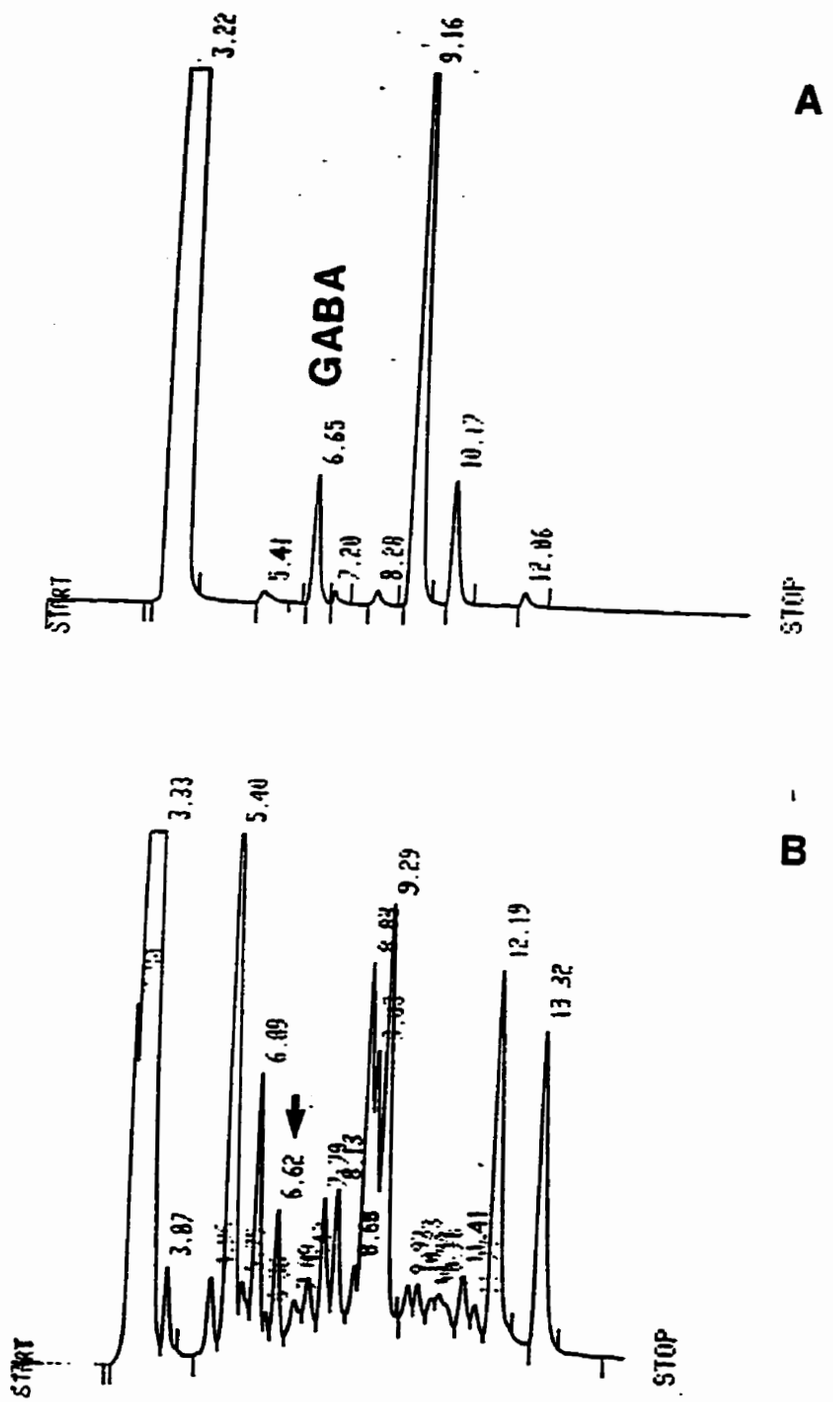
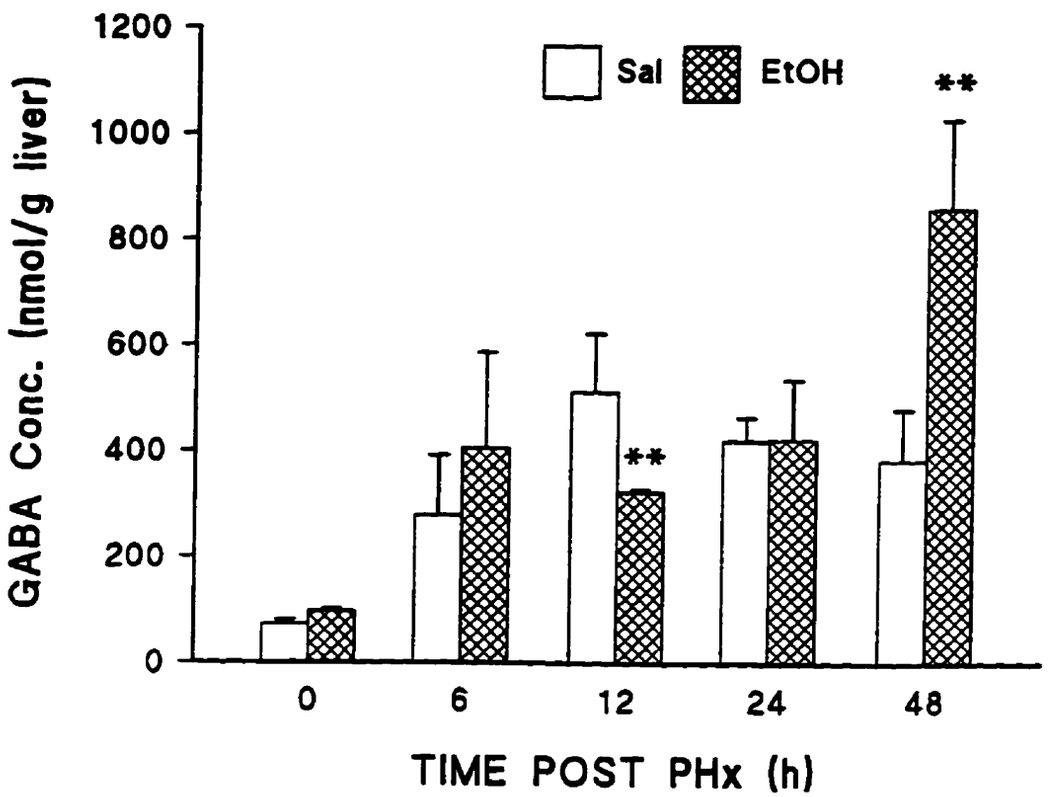


Figure 6. 8. Hepatic GABA concentrations (nmol/g liver) in saline- and ethanol-treated rats after partial hepatectomy. Tissue was obtained as described in Method. Dansylated GABA were measured in acid-precipitated liver extracts using a HPLC method described in Methods. 1,7-diaminoheptane was used as an internal standard and all samples were analyzed in duplicate. Each point represents the mean±SD of data in five to six rats. $P<0.01$ at 12 and 48 h vs. ethanol-treated rats.

Fig. 6.8. Hepatic GABA Concentrations in Regenerating Liver



6.5. Effect of ethanol on hepatic GABA-T activity in rats

In the liver, GABA-T is the most important and perhaps only catabolic enzyme responsible for GABA metabolism. Thus, GABA-T activity is also relevant to hepatic GABA levels. GABA-T activity profiles following sham surgery and partial hepatectomy are provided in Figure 6. 9. GABA-T activities were similar in sham operated nonregenerating livers whether treated with saline or ethanol. While in regenerating livers, an increase in GABA-T activity occurred over the first 12 h in both saline and ethanol treated-groups. Maximum GABA-T activity occurred at 12 h. Ethanol significantly increased GABA-T activity at 6 h ($P< 0.01$) and decrease at 48 h ($P<0.01$).

Fig. 6.9. GABA-T Activity in Regenerating Liver

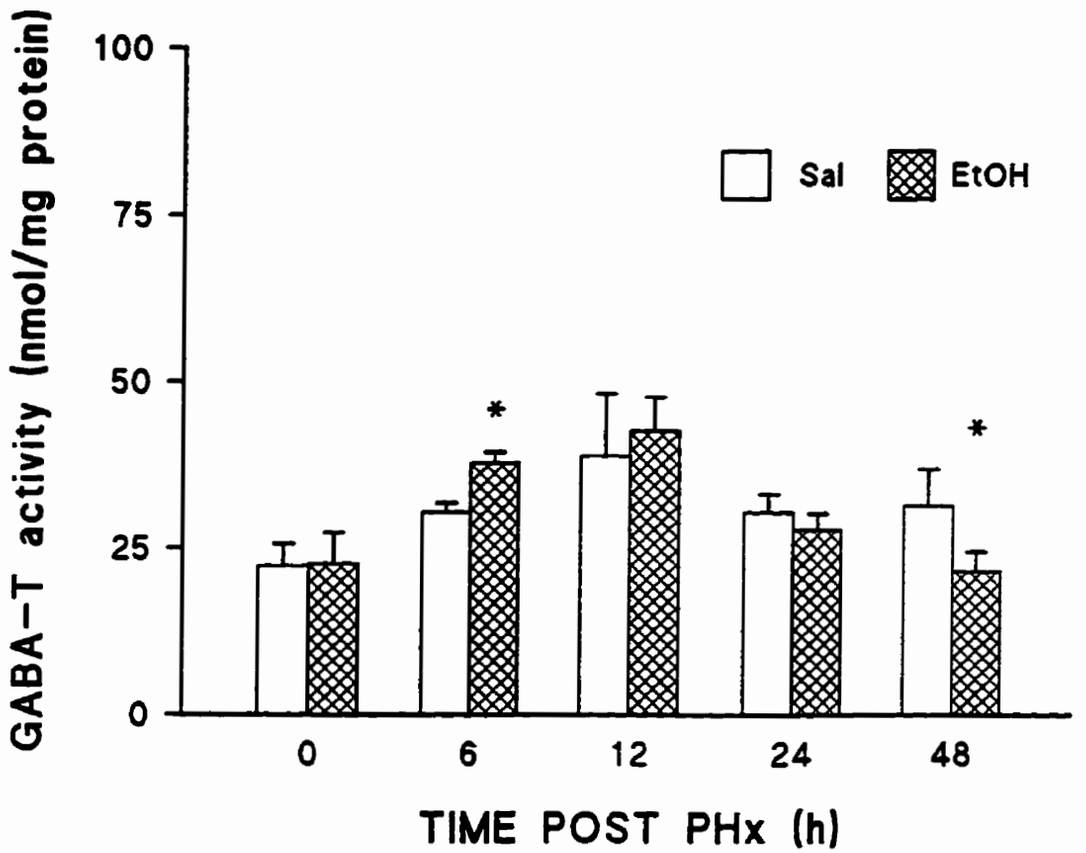
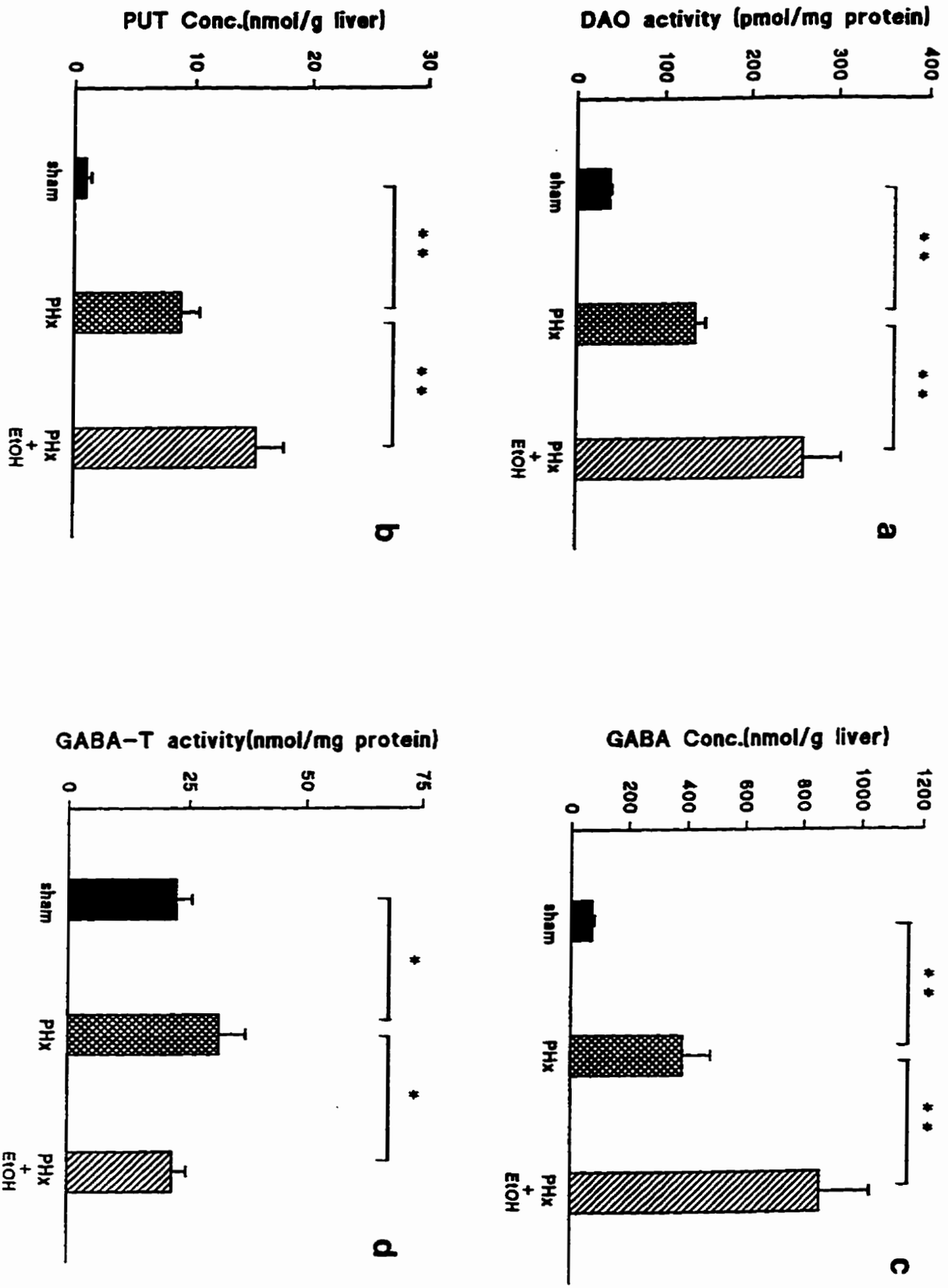


Figure 6. 9. Hepatic GABA transaminase (GABA-T) activity (nmol / mg protein / h) in saline- and ethanol-treated rats after partial hepatectomy. Tissue was obtained as described in Method. Each point represents the mean±SD of data in four to six rats. $P<0.01$ at 6 h and 48 h.

Figure 6.10. summarizes the effects of ethanol on DAO, putrescine, GABA and GABA-T activity at 48 h post partial hepatectomy. Panel (A) shows that partial hepatectomy was associated with a significant induction of DAO activity ($P<0.01$) and the induction was intensified in ethanol-treated rats vs contol rats ($P<0.01$). Panel (B) shows that hepatic putrescine levels were increased at this time interval ($P<0.01$) and ethanol did not suppress the increase as expected. Indeed, hepatic putrescine levels in ethanol-treated rats were significantly higher than in saline-treated controls. Panel (C) provides the results of hepatic GABA levels at 48 h post partial hepatectomy. As with putrescine levels, GABA levels were significantly increased in saline-treated controls compared to baseline and further increased by ethanol administration. The results of hepatic GABA-T in saline- and ethanol treated rats post partial hepatectomy are provided in panel (D). Compared to saline-treated controls, ethanol administration significantly decreased GABA-T activity. Taken together, The above findings suggest that ethanol increases DAO- mediated conversion of putrescine to GABA but decreases GABA metabolism by GABA-T activity. The source of additional putrescine in the liver at 48 h post-partial hepatectomy is unclear.

Fig. 6.10. Summarized effects of ethanol on DAO activity (A), putrescine (B) and GABA (C) concentrations and GABA-T activity (D), 48 h after partial hepatectomy. Sham surgery was performed as a control. * $P<0.05$, ** $P<0.01$


DAO activity (pmol/mg protein)
0
100
200
300
400
sham
PHx
PHx + EtOH
**
**
a
PUT Conc.(nmol/g liver)
0
10
20
30
sham
PHx
PHx + EtOH
**
**
b
GABA Conc.(nmol/g liver)
0
200
400
600
800
1000
1200
sham
PHx
PHx + EtOH
**
**
c
GABA-T activity(nmol/mg protein)
0
25
50
75
sham
PHx
PHx + EtOH
*
*
d

Chapter VII

Discussion

The present study was undertaken to determine whether ethanol enhances the metabolism of growth promoting polyamines such as putrescine to the growth inhibitor, GABA, during the post-partial hepatectomy period. Such an effect might contribute to the inhibitory effects of ethanol on hepatic regeneration. Despite several reports of ethanol-associated effects on hepatic putrescine levels (Diehl et al., 1990, Tanaka et al., 1991) or DAO (Sessa et al., 1984) and MAO activity, few studies have been devised to monitor the complete metabolic pathway in the regenerating period. The results of the present study strongly suggest that ethanol's inhibitory effects on hepatic regeneration are more likely to be related to enhanced suppression (increased GABA) than attenuated stimulation (decreased putrescine).

7.1. DAO in Relation to Putrescine Metabolism

7.1.1. Response of DAO to Ethanol Exposure

The results of the present study indicate that a single ethanol administration (5 g /kg) causes a slight increase in hepatic DAO activity over the initial 24 h post partial hepatectomy followed by a significant increase at 48 h. Other than the present report, only one other study (Sessa et al., 1984) has documented the effects of acute ethanol exposure on hepatic DAO activity. In that study, a 3 fold increase in DAO activity was obtained at 6 h after ethanol intubation. Although our increase in DAO activity was much less pronounced (approximately 30%), the different dosages of ethanol used in the two studies

(5g / kg vs 2g /kg) may in part explain these differences. Unfortunately, the investigators in the Sessa study did not document further changes in DAO activity beyond 6 h.

The mechanism whereby ethanol stimulates DAO activity is unclear. Previously reported increases in DAO mRNA abundance following ethanol exposure support that increased possibility (Sessa et al., 1984). That such increases are prevented by pyrazole, an inhibitor of alcohol dehydrogenase is not only in keeping with this possibility but also serves to implicate products of ethanol metabolism rather than ethanol per se. However, an indirect effect via other hormones or neurotransmitters such as those from the adrenal gland can not be excluded.

7.1.2. Possible Links between GABA and DAO Activity in the Liver

The pathway of putrescine metabolism to GABA catalyzed by DAO appears to prevail in the liver (Tabor and Tabor 1984). The results of this study showed that hepatic GABA levels were highest in ethanol-exposed rats at 48 h post partial hepatectomy which corresponds to the onset of the inhibitory process that follows rapid liver growth. It is unlikely that the increase in GABA levels at 48 h reflect alternative pathways of GABA synthesis. To date, only three alternative pathways have been described; 1) oxidation of monoamines, 2) decarboxylation of glutamate and 3) a recently described spermine to GABA shunt. The first of these possibilities (MAO metabolism) is unlikely for reasons that will be discussed below. The second, glutamate decarboxylase is also unlikely. While active in the brain, glutamate decarboxylase activity is limited in the liver and is not known to be induced by ethanol. The third, the spermine/GABA shunt has only been identified in the brain and the absence of changes in hepatic spermine levels argues against the presence of the shunt in the liver. Moreover, our findings are in keeping with data from other

peripheral organs such as the gastrointestinal system and adrenal gland where GABA synthesis is almost exclusively derived from putrescine via DAO-mediated oxidative deamination. The effects of DAO inhibitors on putrescine metabolism to GABA in the mouse liver has been studied previously (Seiler and Eichentopf 1975) and the results were analogous to experiments carried out with the rat adrenal gland which revealed that aminoguanidine, a potent inhibitor of DAO not only eliminated the synthesis of radioactive GABA from labeled putrescine, but also depleted endogenous GABA from the adrenals (Caron et al., 1988).

7. 2. MAO-A in Relation to Putrescine Metabolism

As mentioned above, another catabolic pathway for putrescine involves MAO. In the brain, MAO ultimately degrades the monoacetyl derivative of putrescine to GABA. In our study, acute ethanol exposure did not modify MAO activity. These results are in keeping with those of Kennedy et al., 1992, who reported that ethanol intake had no effect on hepatic MAO activity. Although a role for MAO in hepatic GABA synthesis is supported by a report that pargyline, a MAO inhibitor, decreases hepatic metabolism of putrescine to GABA (Seiler and Eichentopf 1975), it should be noted that pargyline also affects DAO activety (Crabbe and Bardsley 1974) and therefore, the results can not be considered specific for MAO.

7. 3. Response of Hepatic Putrescine to Ethanol Exposure

Hepatic polyamines levels, especially putrescine, correlate with DNA synthesis and proliferation (Pösö and Pegg 1982). Our results revealed that, following partial hepatectomy, hepatic putrescine levels significantly increased (5-10 fold) beyond baseline but that the maximum increase occurred earlier (6 h) than had been reported by others

(Luk 1986. Tanaka et al., 1991). The reason for the different patterns of putrescine elevation is unclear. All studies employed the same model of partial hepatectomy. Diets, which may also influence hepatic polyamine levels (Minuk et al., 1990) were consistent as were the ages and weights of the experimental animals. The recent recognition that polyamines migrate to the nucleus following partial hepatectomy raises the possibility that differences in nuclear versus cytoplasmic versus total polyamine determinations may be relevant. Of note is the fact that ethanol only decreased putrescine levels during the initial period following partial hepatectomy and then, only to a limited extent. That observation is in agreement with previous reports by Tanaka et al., (1991, 1993). Unfortunately, in the Tanaka study the effect of ethanol on putrescine was not documented beyond 4 hours post partial hepatectomy.

The lower putrescine levels at 6 h in ethanol exposed rats relative to saline exposed controls can, in part be explained by ethanol-induced enhancement of DAO activity at this time period. The ethanol-induced effect on putrescine metabolism might not only be caused by ethanol-induced enhancement of DAO activity but also inhibition of ODC activity. On the other hand, the transient delay in putrescine synthesis associated with ethanol exposure appears to, in part, explain the lower putrescine levels at the earlier stage of post partial hepatectomy. The reason why our results do not agree with the significant decreases in hepatic putrescine levels reported by Nakajima et al., (1990) may relate to the fact that the latter group employed female rats only and ODC activity is influenced by sex hormones levels.

That putrescine levels were higher in ethanol-treated rats at 24 and 48 h post partial hepatectomy further attests to the fact that ethanol's inhibitory effect on hepatic

regeneration is not via decreases in hepatic putrescine levels. Why putrescine levels were higher in the ethanol-exposed group at 48 h is unclear. A number of possibilities need to be considered. For example, ethanol depolarizes hepatocyte membranes which would result in increased putrescine migration from the cytoplasm to the negatively charged nucleus. Because we measured total rather than cytoplasmic polyamine levels as was the case in other studies, the apparent increase in ethanol-exposed rats we report may reflect a large contribution of nuclear putrescine to the total putrescine levels. Secondly, ethanol may have augmented the late peak in ODC activity. Partial hepatectomy induces two peaks in ODC activity, one at 4 and another at 12 h post surgery (Tanaka et al 1988). Ethanol-induced inhibition of ODC activity has been observed as early as 4 h (Tanaka et al., 1991), and as late as 12 h post surgery (Minuk et al., 1991). Because hepatic regenerative activity has been reported to be delayed rather than inhibited by ethanol, it is conceivable that suppressed ODC activity rebounded later in the post partial hepatectomy period (48 h). Thirdly, it is possible that some of the increase in hepatic GABA levels at 48 h due to increased DAO and decreased GABA-T might have resulted in bi-directional enzyme activity and hence, putrescine synthesis. Fourthly, spermidine acetyltransferase (SAT) might have also played a role. This is another important enzyme in the pathway of hepatic polyamine metabolism. Spermidine is first acetylated by SAT and further oxidized to putrescine by polyamine oxidase. Thus, SAT promotes the production of putrescine by a reverse route from spermidine to putrescine. The lower levels of spermidine at this time interval in ethanol-exposed rats tend to support this possibility. Obviously additional studies in this area including polyamine compartmentalization and actual ODC and SAT determinations are warranted.

7.4. Response of Hepatic Spermidine and Spermine to Ethanol Exposure

Our current results have shown that the spermidine and spermine in the liver after acute treatment with ethanol are unchanged with the exception of a transient decrease in hepatic spermidine levels at 24 h post partial hepatectomy. A previous study reported by Tanaka et al., (1993) indicating that no significant changes occurs in hepatic spermidine or spermine levels after acute exposure to ethanol is keeping with our findings.

That few changes occurred in hepatic spermidine and spermine levels in ethanol-treated rats was not unexpected. Spermidine and spermine both serve as storage forms of the polyamines, while putrescine appears to be the more active growth-regulator because of its relatively short biological half-life and the rapid turnover of ODC (Synder et al., 1970). Previous studies support that putrescine, rather than spermidine or spermine, is more important for liver regeneration. Specifically, Nishiguchi et al., (1986) showed that administration of interferon suppressed the stimulation of DNA synthesis in the liver induced by partial hepatectomy by inhibiting the accumulation of putrescine without affecting spermidine and spermine levels. The impairment in DNA synthesis was reversed by exogenous putrescine. In contrast, Higaki et al., (1994) reported that methylglyoxal bis (guanylhydrazone) (MGBG), a reversible inhibitor of S-adenosylmethioornithine decarboxylase (SAMD), that causes an accumulation of putrescine and a decrease in spermidine and spermine levels, completely inhibited HGF-induced DNA synthesis. The inhibitory effect of MGBG was overcome by the addition of exogenous spermidine and spermine, further indicating that an increase in spermidine and spermine rather than putrescine, is essential for DNA synthesis. The role of spermidine and spermine in DNA synthesis and cell proliferation need to be further studied.

7. 5. Response of Hepatic GABA to Ethanol Exposure

The liver contains endogenous GABA and its synthesizing and degradating enzyme i.e. DAO and GABA-T respectively, however, hepatic GABA synthesis is not continuous. It is activated only at certain times in response to hepatic development or function. Our findings confirm that GABA levels are significantly increased following partial hepatectomy when compared to the resting liver which is in agreement with previous observations (Minuk 1991).

Ethanol exposure significantly increased hepatic GABA levels at 48 h which corresponds to the time of termination of the rapid growth phase following partial hepatectomy. It remains unclear which of the numerous means of maintaining GABA homeostasis that include GABA uptake systems, biliary excretion pathways, GABA synthesizing enzymes and GABA metabolizing enzymes, was responsible for the changes in hepatic GABA levels observed. That DAO activity was highest and GABA-T activity lowest at 48 h suggests that a combination of increased synthesis and decreased metabolism may have contributed to the findings.

Previous studies documenting that GABA uptake is enhanced at 48 h post partial hepatectomy suggest that increased uptake might also contribute to the findings. Excretion of GABA into bile has not been determined in the post partial hepatectomy period. Finally, it must be kept in mind that GABA tissue determinations can be influenced by the technique of sample preparation which makes it difficult to accurately document tissue GABA levels.

One of the mechanisms by which ethanol exerts its inhibitory effects on the CNS is via the GABA receptor complex. The relationship was first suggested by Simson et al., (1991)

who demonstrated that ethanol potentiates GABA-mediated inhibitory effects in the CNS. It has also been shown that acute ethanol exposure increases brain GABA concentrations (Sytinsky et al., 1975). Moreover, activation of the GABAergic system may be involved in the pathogenesis of hepatic encephalopathy because elevated serum GABA levels and altered GABA receptor levels were observed in patients with largely alcohol-induced hepatic encephalopathy (Baraldi and Zenoroli 1982, Ferenci et al., 1983 and Bannister et al., 1988). In addition, Varga and Kunos (1992) reported that ethanol inhibits baroreflex activity through potentiation of the actions of endogenous GABA on the cardiovascular control center of the CNS. These results raise the possibility that ethanol's inhibitory effects on hepatic regeneration may be mediated by GABA receptor activation.

The major finding of the present study is that ethanol increases DAO activity with concomitant increases in hepatic GABA levels at the specific time interval when inhibition of hepatic regeneration is under way. Although relatively little is known about the role GABA plays in hepatic function and disease, it appears reasonable to assume that increased GABA levels and/or GABAergic activity has some role to play in ethanol-induced inhibition of hepatic regeneration. Our findings, combined with previous work by Diehl et al., (1990), who found that ethanol-induced decreases in hepatic putrescine levels were responsible for the inhibition of liver regeneration and data by Minuk and Gauthier (1993), who found that elevated serum GABA concentrations interfere with hepatic regeneration following partial hepatectomy, further support the hypothesis that the antiregenerative effects of ethanol on the liver may be mediated by increased hepatic conversion of putrescine to GABA rather than a depletion of putrescine per se.

7. 6. GABA-T in Relation to Hepatic GABA Levels

The liver is probably the major site for GABA catabolism outside the CNS, containing the highest GABA-T activity of all non-CNS tissues studied to date. (Ferenci et al., 1981). As discussed above, ethanol has been shown to alter GABAergic activity in both the CNS and peripheral non-neuronal tissues. The levels and metabolism of GABA are affected by ethanol in a variety of ways. GABA-T is thought to be the only enzyme responsible for GABA metabolic elimination in the liver and thereby might play a role in regulating hepatic GABA levels. Over the past few years, there have been studies on brain GABA-T activity after acute exposure to ethanol, indicating both unchanged (Sutton and Simmonds, 1973; Sytinsky et al., 1975; Sherif et al., 1994) and decreased (Hakkinen and Kulonen, 1979) activity.

In the present study, the effects of ethanol on hepatic GABA-T activity in regenerating livers were studied for the first time. These results revealed that ethanol induces the activity of GABA-T at 6 h post partial hepatectomy followed by a significant decrease at 48 h. The later may contribute to the elevation in hepatic GABA levels at this time period. The ethanol-induced accumulation of hepatic GABA within the hepatocytes can in part be explained by the impairment of hepatic GABA catabolism.

In summary, these data suggest that the inhibitory effect of ethanol on liver regeneration appears to result more from an increase in hepatic GABA rather than a decrease in hepatic putrescine levels.

Chapter VIII

Conclusions

The effects of ethanol on hepatic polyamine metabolism following partial hepatectomy were studied in male rats and the results can be summarized as follows:

1). Acute ethanol exposure increased hepatic DAO activity at 48 h following partial hepatectomy.

2). Acute ethanol exposure had no effect on hepatic MAO-A activity.

3). Hepatic putrescine levels exhibited a biphasic pattern following ethanol administration, i.e. a slight decrease in hepatic putrescine levels during the early period post partial hepatectomy, while increased during the later period. The decreased levels at the earlier stage may be more relevant to inhibition of liver regeneration following ethanol exposure.

4). Ethanol altered GABA-T activity after partial hepatectomy (slight increase early, decrease late).

5). Hepatic GABA levels were significantly increased by ethanol during the later period of partial hepatectomy. This increase can be attributed in part to an increase in DAO activity but also a decrease in GABA-T activity at the same time period.

6). Ethanol had no consistent effect on hepatic spermidine and spermine levels.

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