POTENTIAL ROLE OF ACTIVATED NONPARENCHYMAL CELLS IN ACETAMINOPHEN-INDUCED POTENTIATION OF HEPATOTOXICITY

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WRIGHT

POTENTIAL ROLE OF ACTIVATED NONPARENCHYMAL CELLS IN ACETAMINOPHEN-INDUCED POTENTIATION OF HEPATOTOXICITY.

by
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ABSTRACT

Title of Dissertation: Potential Role of Activated Nonparenchymal Cells in Acetaminophen-Induced Potentiation of Hepatotoxicity.

Patrice B. Wright, Doctor of Philosophy, 1991

Dissertation directed by: Leon Moore, Professor
Department of Pharmacology

A role for macrophages in the manifestations of acute chemically-induced hepatic necrosis has been hypothesized, but evidence to prove this has been limited. Acetaminophen has been shown at both necrogenic and non-necrogenic doses to stimulate an inflammatory cell infiltration into the liver (Dixon *et al*. 1971, 1975; Walker *et al*. 1980, 1983; and Laskin and Pilaro, 1986). It was the goal of this project to determine if the presence of these activated inflammatory cells in the liver after non-necrogenic doses of acetaminophen alters the hepatotoxicity of prototype hepatotoxicants. The studies presented here show that pretreatment of rats with non-necrogenic doses of acetaminophen increased the susceptibility of rats to hepatic injury induced by allyl alcohol, bromobenzene, carbon tetrachloride, 1,1-dichloroethylene, and thioacetamide. Acetaminophen-pretreatment also increased the lethality induced by allyl alcohol and 1,1-dichloroethylene.

In vivo, hepatic glutathione levels were decreased twenty-four hours after acetaminophen administration. Yet,

acetaminophen-induced glutathione depletion cannot fully explain acetaminophen-induced potentiation of hepatotoxicity in these studies. Administration of dextran sulfate and gadolinium chloride, compounds which suppressed the function of the reticuloendothelial system, protected animals from acetaminophen-induced potentiation of allyl alcohol toxicity. *In vitro*, nonparenchymal cells from control or acetaminophen-pretreated rats cocultured with hepatocytes did not mimic the *in vivo* observations. These cocultures were resistent to allyl alcohol at doses which were cytotoxic to hepatocytes cultured alone. Hepatocytes cultured in media conditioned by nonparenchymal cells from acetaminophen-pretreated rats were more sensitive to allyl alcohol than hepatocytes cultured in media from control-pretreated rats. The conditioned media studies appear to support the hypothesis that activated nonparenchymal cells increase susceptibility of hepatocytes to an allyl alcohol insult. These data agree with the *in vivo* studies which indicated that activation of nonparenchymal cells by acetaminophen has a role in acetaminophen-induced potentiation of hepatotoxicity.

DEDICATION

To my loving husband, Bob

Acknowledgements

To my husband Bob-
For his love, support, and encouragement throughout the past five years, without him I could have never gotten through the hard times.

To my parents-
For their love, support, and encouragement throughout all the stages of my life. It was with their guidance I am able to achieve the things that I have.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

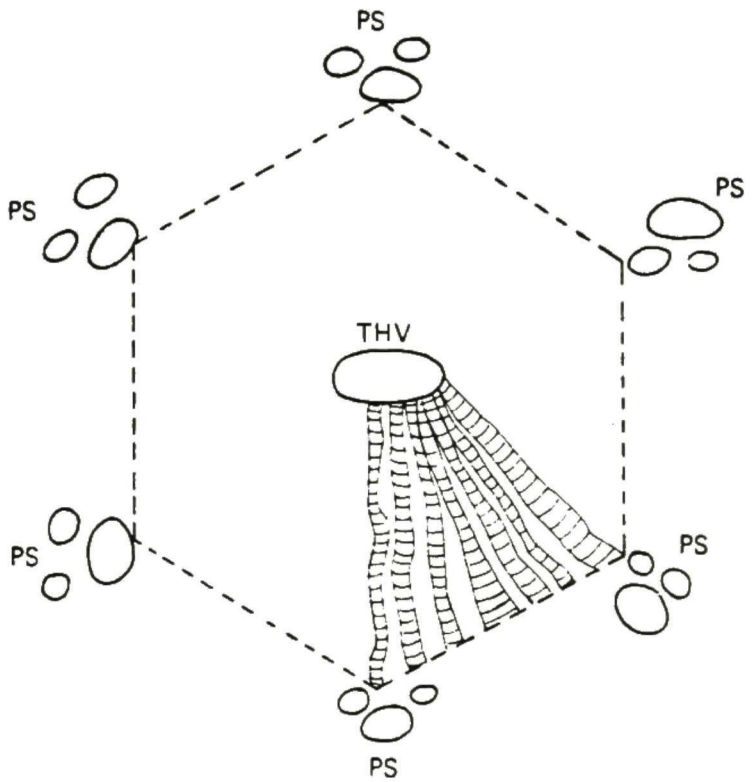
The Functional Structure of the Liver

The liver is one of the largest organs of the body. It performs a significant vascular function, regulates metabolism and homeostasis, and acts as a secretory organ. The classical manner of describing the functional unit of the liver is the hepatic lobule. The hepatic lobule consists of a hepatic vein in the center surrounded by six portal triads at each point of a hexagon (Figure 1A). A portal triad consists of a hepatic artery, portal vein and bile duct. This is the model from which the morphological classification of hepatotoxicant action originated (Plaa, 1986). Toxicants are classified as centrilobular (affecting the central zone of the lobule) or periportal (affecting the outer zone of the lobule). Toxicants may also be characterized as midzonal (affecting the area between the centrilobular and periportal regions) or diffuse (showing no zonal pattern) (Zimmermann, 1982).

An alternative model to the hepatic lobule is the hepatic acinus (Figure 1B). The hepatic acinus is described as a microcirculatory unit with a group of cells nourished by a vascular axis containing a terminal portal venule, hepatic arteriole, bile duct, lymphatic vessels and nerves (Rappaport, 1975). Within the acinus there are cords of hepatocytes. One side of the hepatocyte borders a sinusoid, one side a bile duct canaliculi and the remaining sides border adjacent hepatocytes. The hepatocytes are grouped

Figure 1: Functional Structure of the Liver. Schematic drawings of the current concepts of the functional structure of the liver. A) The hepatic lobule with central vein or terminal hepatic venule (THV) in center and portal triads (PS) at each point of the hexagon (Plaa, 1986). B) The hepatic ascinus with the vascular axis in the center (Plaa, 1986). Zone 1 is the area of highest oxygen tension and Zone 3 is the area of lowest oxygen tension.

A.



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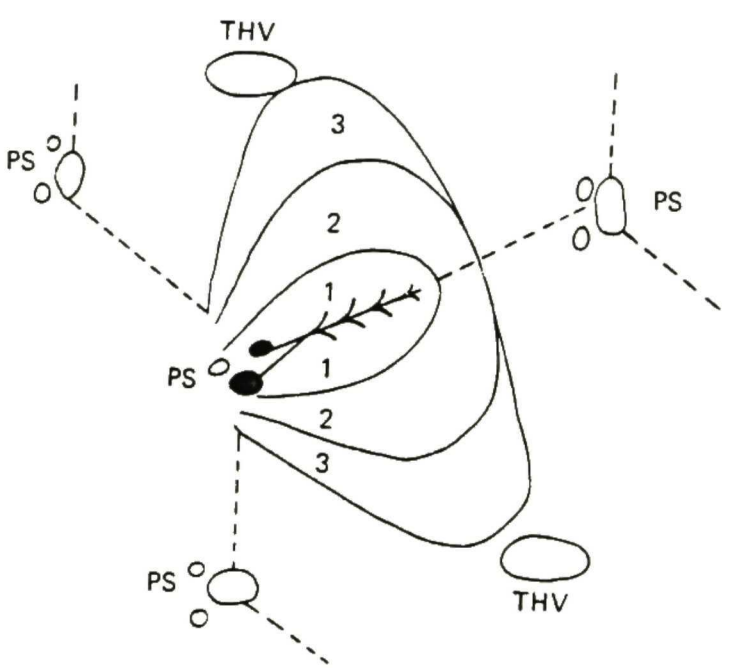


around sinusoids which drain from the portal tract into hepatic venules. There is a metabolic zonation from periportal (Zone 1) to perivenous (Zone 3) due to a gradual reduction in oxygenation of blood (Plaa, 1986). The hepatic lobule will consist of parts of many ascini. The hepatic ascinar unit is used to understand hepatic physiology and the hepatic lobule is used in classification of hepatotoxicants.

The Cellular Composition of the Liver

The liver is composed of two major cell classifications: parenchymal and nonparenchymal. Parenchymal cells are the hepatocytes; nonparenchymal cells (NPCs) are the sinusoidal cells (endothelial cells, Kupffer cells, fat-storing cells, and pit cells) and the bile duct epithelial cells (Table 1). Hepatocytes comprise 60-70% of the cells in the liver. They are 20-30 μm in diameter and are polyhedral shaped cells with ruffled membrane projections. Hepatocytes which contain lipid droplets and an abundance of endoplasmic reticulum and mitochondria have gap and tight junctions between cells (Bruni and Porter, 1965). The functions of hepatocytes include synthesis and secretion of plasma proteins; carbohydrate, lipid and amino acid metabolism; drug metabolism; bile formation; and elimination of nitrogen components after urea synthesis (Steinberg, 1987).

TABLE 1

CELLULAR COMPOSITION OF THE LIVER

Parenchymal Cells	60-70%
Nonparenchymal Cells	
Endothelial Cells	13-17%
Kupffer Cells	8-10%
Fat Storing Cells	5-7%
Pit Cells	0.8-1%
Bile Duct Epithelial Cells	2-3%

---

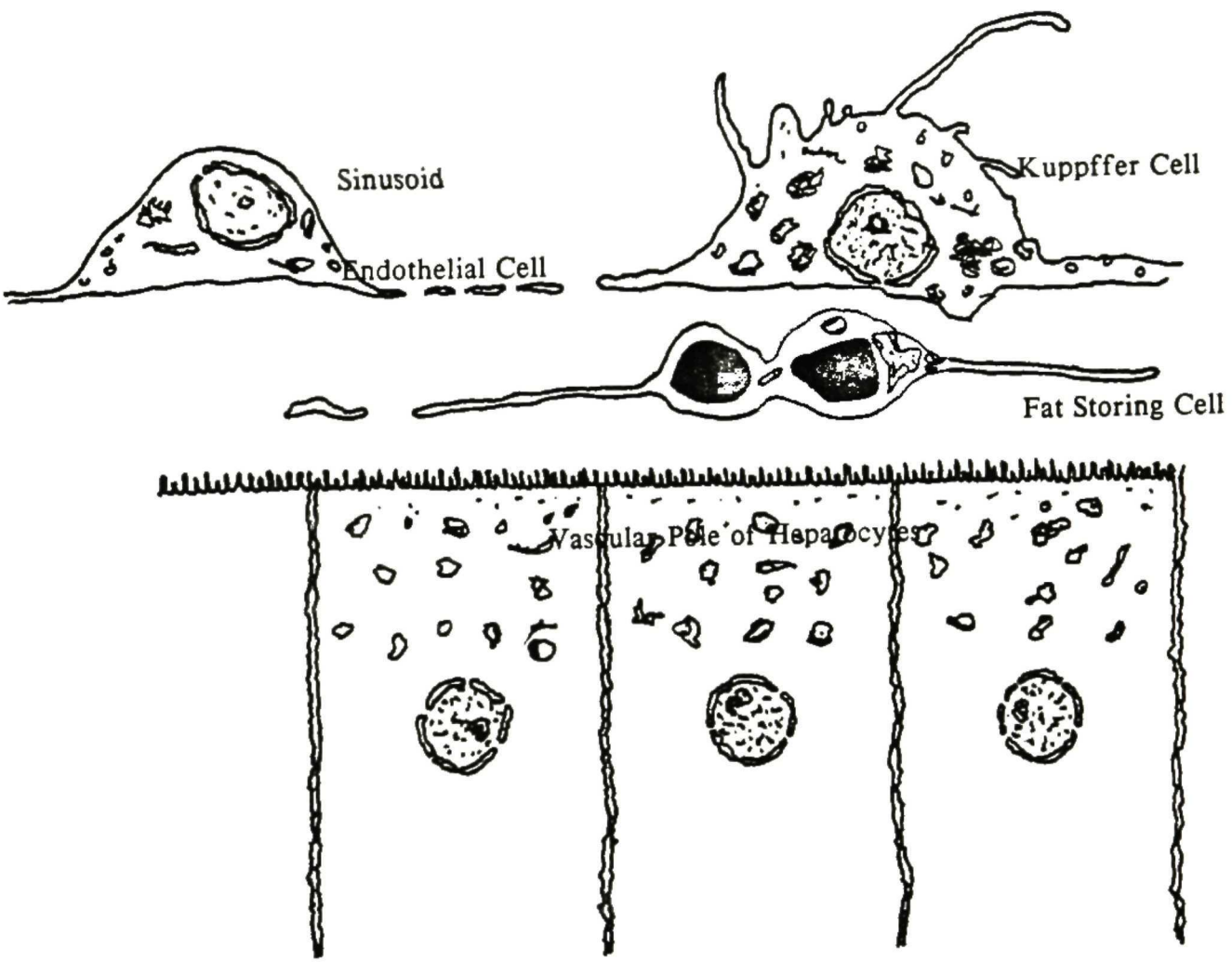
[a] The cells of the liver are broken into parenchymal and nonparenchymal. Data is expressed at percent of total cells in the liver (Laskin, 1990).

The sinusoidal cells of the liver are the cells which line the blood-filled sinusoids between the portal tract and the hepatic venule (Figure 2). There are four types of sinusoidal cells; endothelial cells, Kupffer cells, fat storing cells, and pit cells (Wisse *et al.*, 1989).

Endothelial cells comprise 13-17% of cells in the liver. Morphologically these are cells with slender extended processes. Endothelial cells have pores and fenestra which allow regulated and direct contact of parenchymal cells with plasma and its solutes. Endothelial cells are important in lipoprotein homeostasis and metabolism (Horiuchi *et al.*, 1989). These cells have high activities of lysosomal enzymes and a large endocytic capacity. These cells have been shown to endocytose many exogenous and endogenous particles such as small latex beads, colloidal carbon, denatured albumin, glycoproteins, lipoproteins, and immune complexes (Brouwer, 1988). Endothelial cells release a variety of cytokines to aid in the functional coordination of all cell types in the liver. These include of IL-1, IL-6, interferon and reactive oxygen and nitrogen intermediates (Wisse *et al.*, 1989).

Kupffer cells comprise 8-10% of the total cells in the liver. These cells are usually located in the lumen of the sinusoids and are anchored to endothelial cells by long cytoplasmic processes. These are the major phagocytic cells of the liver and 43% of these cells are located in the

Figure 2: The Hepatic Sinusoidal Unit. Endothelial cells, Kupffer cells, and Fat-storing cells of the hepatic sinusoid and their relationship to hepatocytes within the sinusoidal structure. Blood flows from the portal triads to the terminal hepatic vein through the sinusoids. Endothelial cells and Kupffer cells line the sinusoids.


Sinusoid
Endothelial Cell
Kuppffer Cell
Fat Storing Cell
Vascular Pole of Hepatocytes

periportal region to phagocytize particles in blood before the blood enters the acinus. These cells have numerous functions; phagocytosis of particulate matter, detoxification of endotoxin, regulation of the microcirculation in the sinusoid, metabolism of lipoproteins, secretion of mediators to regulate hepatic function, processing of antigens, and mediation of immune responses in the liver (Jones and Summerfield, 1988).

Fat-storing cells comprise 5-7% of the total cells in the liver. These cells are located in the perisinusoidal space between endothelial cells and hepatocytes. Fat-storing cells are responsible for collagen synthesis and also the storage and metabolism of vitamin A (Brouwer, 1988).

Pit cells are few in number and are not well characterized. Pit cells are large granular lymphocytes, the natural killer cells in the liver. They are located in the endothelial lining and are thought to play a role in tumor cytotoxicity (Bouwens and Wisse, 1989 and Kaneda *et al.*, 1989).

Bile duct epithelial cells comprise 2-3% of cells in the liver. These cells line the bile ducts and produce a distinct basement membrane for the bile ducts (Laskin, 1990). Each of the cell types in the liver perform a specific function. Yet, normal hepatic physiology is dependent upon the regulated communication and coordination of both parenchymal and NPCs.

The Reticuloendothelial System and Tissue Injury

The reticuloendothelial system (RES) consists of monocytes, tissue macrophages and endothelial cells. In the liver, the RES cells are the resident Kupffer cells and sinusoidal endothelial cells. The major function of these cells is to control the substances to which the liver is exposed. This function is very important as a defense against infection. This is demonstrated by two agents which suppress RES function; dextran sulfate (DS) and gadolinium chloride ($GdCl_3$). DS has been shown to decrease phagocytosis in macrophages (Souhami, 1981; and Das *et al.*, 1987). Inducing RES blockade with DS has been shown to increase infection in the liver (Hahn, 1974). $GdCl_3$ has been shown to decrease the clearance of exogenously administered carbon from the circulation (Lazar, 1973). It has also been shown to specifically decrease uptake of red blood cells in the liver while increasing their uptake in other organs (Husztik *et al.*, 1980). Using monoclonal antibodies and peroxidase staining it has been shown that $GdCl_3$ is cytotoxic specifically to Kupffer cells in the liver (Koudstaal *et al.*, 1990). $GdCl_3$ induced Kupffer cell suppression has been shown to decrease the clearance of infectious organisms from the blood (Lazar, 1976).

While the RES is important in protection of an rganism it can also be responsible for direct or indirect tissue injury. Injury may be caused as a direct result of

secretory products, phagocytosis or cell mediated cytotoxicity as in the case of tumor cells or microbial infections (Tracey, 1983). Alternatively, RES can be involved in tissue injury indirectly or secondary to inflammatory reactions as in fungal and parasitic infections, rheumatoid arthritis, Crohn's Disease, silicosis and asbestosis, and emphysema (Tracey 1983).

Nonparenchymal Cell Activation in Hepatic Injury

RES-induced tissue injury depends on activation of macrophages and endothelial cells. Activation is defined as the acquisition of competence to mediate or complete a complex function. Macrophages are normally subject to many stimulatory and suppressive signals. In altered homeostasis, for example inflammation, these signals modulate the activation or suppression of these cells. Activated macrophages have increased secretion of inflammatory mediators, increased receptors for inflammatory mediators, increased ability for the presentation of antigen, and increased reactive oxygen and hydrogen peroxide production (Adams and Hamilton, 1884; and Matsuo *et al.*, 1985). Cytokines can activate endothelial cells to have altered morphology, membrane receptor pattern, and increased cytotoxic ability (Pober, 1988; and Decker *et al.*, 1989). Cytokines also increase reactive oxygen radical production and monocyte chemoattractant secretion from endothelial

cells (Matsubara and Ziff, 1986 and Rollins *et al.*, 1990). Endothelial cells release nitric oxide in response to the potent immune activator, lipopolysaccharide (Salvemini *et al.*, 1989).

The RES may have a role in liver injury. This is demonstrated in the interaction of the hepatic RES with gut-derived endotoxin or lipopolysaccharide. RES cells of the liver are the first cells to encounter blood born endotoxins. One of their main functions is to clear blood of endotoxin. If this is not done, an endotoxemia may ensue (Nolan and Camara, 1982). *In vitro*, hepatocyte protein synthesis, is inhibited by the presence of Kupffer cells and lipopolysaccharide. Mediators of this response include heat labile, soluble substances liberated by the Kupffer cells (West *et al.*, 1986) which include nitric oxide (Billiar *et al.*, 1989A). Cocultures of sinusoidal cells and hepatocytes in the presence of lipopolysaccharide have increased collagenase activity (Kashiwazaki *et al.*, 1986). Other evidence of RES-induced liver injury comes from *in vivo* administration of carbon and silica particles, substances which stimulate Kupffer cell phagocytosis, stimulate mitotic activity in hepatocytes of mice (Bradfield *et al.*, 1980). This increased mitotic activity is indicative of the liver replacing injured cells. Vitamin A, a substance which activates the resident Kupffer cells in the liver, has been shown to potentiate the hepatotoxicity of carbon

tetrachloride (el Sisi, 1987). This mechanism of potentiation has been attributed to the release of reactive oxygen species from activated Kupffer cells upon carbon tetrachloride insult to hepatocytes.

Chemically-Induced Hepatic Injury

Since the liver has a major role in the regulation of metabolism and homeostasis in the body, disruptions of the organ can have many physiological effects which include, altered biotransformation, altered hormone metabolism, altered salt and water balance, alterations of blood coagulation and hepatic encephalopathy. The liver is specialized for enzymatic processes of xenobiotic metabolism, and one of its primary functions is to receive and process chemicals absorbed from the gastrointestinal tract before distribution in the general circulation. Many xenobiotics are lipophilic. These compounds remain in plasma or within cells until they are metabolized to more water soluble compounds. Metabolism is accomplished by Phase I enzymes, dehydrogenases, reductases, oxidases, and monooxygenases of hepatocytes. These enzymes expose or add functional groups by oxidation, reduction or hydrolysis of the xenobiotic. These reactions increase the water solubility of the compound or permit the Phase II conjugation reactions which utilize the body's established excretion mechanisms (reviewed by Klaassen, 1986).

Xenobiotics metabolized by the Phase I enzymes of the liver are sometimes more reactive and deleterious to the tissue than the parent compound. When exposure to a toxic metabolite exceeds the ability of the liver to detoxify it, hepatic injury will ensue. The injury can take many forms, lipid accumulation, cirrhosis, neoplasia, cholestasis, or degenerative processes leading to cell death.

Chemically-induced cell death has been recognized to occur in one of two ways, apoptosis or coagulative necrosis (Reviewed by Boobis *et al.*, 1989). Apoptosis is a normal turnover of cells or programmed cell death in which the cytoplasm condenses, nuclear alterations are observed, microvilli and junctions disappear. This process requires energy and an endogenous endonuclease (Wyllie, *et al*, 1980). Coagulative necrosis is a histopathological definition of a pattern of cell death. It is characterized by contiguous remnants of cells with eosinophilic cytoplasm and pycnotic nuclei. The cells lie in the extracellular space and have been expelled from cell plates (Popper, 1988). An understanding of the morphological characteristics of xenobiotic/chemically induced liver injury requires further analysis of the mechanism. Details of the mechanism may lead to effective methods of treatment or prevention of injury.

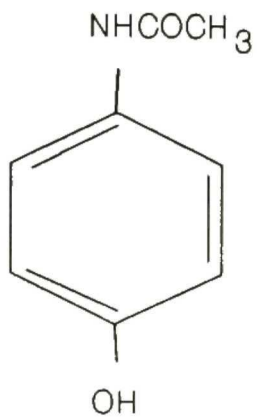
Model Hepatotoxicants Studied in Chemically Induced Injury

Chemically-induced liver injury is an effect of agent and exposure. Many agents have been used to study this phenomenon. Allyl alcohol (AA, 2-propen-1-ol, Figure 3) is an alcohol used in the manufacture of allyl compounds, resins, and plasticizers. It is a very volatile solvent. AA is metabolized in the liver by alcohol dehydrogenase to the reactive metabolite acrolein which requires glutathione for detoxification (Belinsky *et al.*, 1986.). Acrolein produces periportal necrosis by a mechanism involving oxygen and peroxidation of cellular lipids (Reid, 1972; Badr *et al.*, 1986; and Jaeschke *et al.*, 1987) (Table 2).

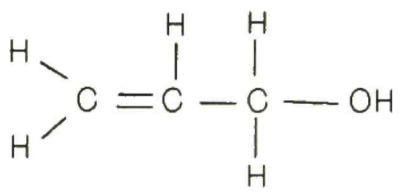
Bromobenzene (BB, Figure 3) is used in organic synthesis as a solvent and as an additive in motor oils. BB requires bioactivation by the cytochrome P450 system (Jollow *et al.*, 1974). The mechanism of hepatotoxicity involves lipid peroxidation (Casini, et al., 1987). It produces centrilobular necrosis and also requires glutathione for detoxification (Reid *et al.*, 1971) (Table 2).

Carbon tetrachloride (Tetrachloromethane, Figure 3) is an organic solvent used in dry cleaning, as a solvent for fats and oils, and as a starting material in the synthesis of many organic compounds. CCl_4 requires metabolism to a reactive metabolite by the cytochrome P450 mixed function oxidase system and causes centrilobular necrosis. It produces injury by inducing lipid peroxidation of cellular

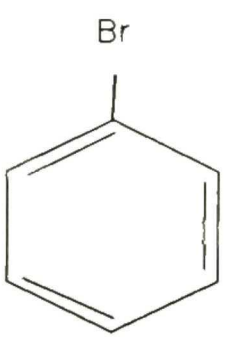
Figure 3: Chemical Structure Of Prototype Hepatotoxicants.



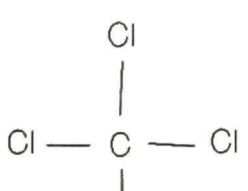
Acetaminophen



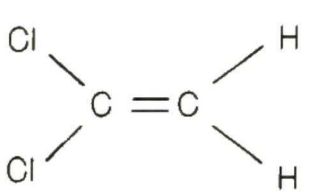
Allyl Alcohol



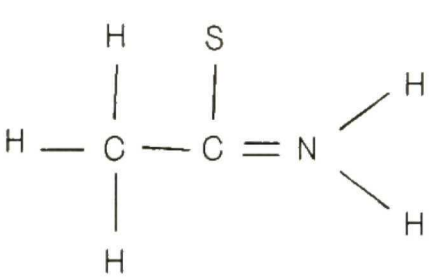
Bromobenzene



Carbon Tetrachloride



1,1 Dichloroethylene



Thioacetamide

TABLE 2

CHARACTERISTICS OF MODEL HEPATOTOXICANTS[a]

	GSH Depletion	Lipid Peroxidation	Centrilobular Necrosis	P450 Metabolism
AA	+	+	+	-
APAP	+	+	+	+
BB	+	+	+	+
CCl_4	-	+	+	+
DCE	+	-	+	+
TA	+	+	+	+

[a]This table indicates the similarities and differences of the most well defined toxicological characteristics of the challenge hepatotoxicants used in these studies.

components (Recknagel *et al.*, 1977 and Younes *et al.*, 1985) and disrupting calcium homeostasis (Recknagel, 1989; Long *et al.*, 1986a, 1986b, 1988) (Table 2).

1,1-Dichloroethylene (vinylidenechloride, DCE, Figure 3) is a volatile liquid monomer used in the manufacture of copolymeric plastics. DCE is bioactivated by the cytochrome P450 monooxygenase system (Reynolds *et al.*, 1975). It produces centrilobular necrosis and requires glutathione for detoxification (Reynolds, *et al.*, 1980). DCE does not induce lipid peroxidation (Reynolds *et al.*, 1975) but does induce aberrations in hepatic Na^{+}, K^{+}, and Ca^{+2} (Reynolds, *et al.*, 1975). DCE has been shown to inhibit the liver endoplasmic reticulum pump (Moore, 1982). Early mitochondrial damage and ion perturbation is involved in the evolution of DCE induced hepatotoxicity (Reynolds, *et al.*, 1980; and Jaeger, *et al.*, 1977) (Table 2).

Thioacetamide (TA, Ethanethioamide, Figure 3) was described as a centrilobular hepatotoxicant (Gupta, 1956). The mechanism of action remains unclear but P450 bioactivation is required (Hunter, et al., 1977) and plasma membrane damage has been reported (Nikolaev, *et al.*, 1986) (Table 2).

Many of the compounds have been classified as hepatotoxicants. These compounds have been characterized but few have been as well defined as acetaminophen (N-acetyl-p-aminophenol, APAP, Figure 3). APAP is a

commonly used analgesic and antipyretic agent. APAP is safe when used in therapeutic regimens, but outside the therapeutic window, it is a potent hepatotoxicant (Black, 1984).

To understand the mechanism by which APAP causes hepatotoxicity, the rat and mouse have been used as models. APAP has been found to cause centrilobular necrosis which leads to coagulative necrosis in both models. This damage can lead to severe hepatic congestion and death (Mitchell *et al.*, 1973; Dixon *et al.*, 1975; Walker *et al.*, 1980; and Dixon *et al.*, 1971). Inflammatory cell infiltration into the necrotic area has been reported and evidence has been presented for the disruption of sinusoidal structure in both mice and rats (Dixon *et al.*, 1975; Walker *et al.*, 1983; Walker *et al.*, 1980; and Dixon *et al.*, 1971; and Laskin *et al.*, 1986).

Evidence to date suggests that APAP hepatotoxicity is a consequence of a complex sequence of events. The precipitating event in the hepatotoxicity has been attributed to generation of a reactive intermediate by cytochrome P450 metabolism in hepatocytes. APAP is removed from the body either by conjugation with sulfate or glucuronide or by metabolism by the cytochrome P450 mixed function oxidase system in the liver (Mitchell *et al.*, 1976). Normally, only a small percentage of APAP is metabolized by the cytochrome P450 system. However, at high

doses, as the sulfate and glucuronide conjugation pathways become saturated, more APAP is shunted to the cytochrome P450 system where it is bioactivated to a highly reactive metabolite, *N*-acetyl-benzoquinoneimine (NAPQI)(Mitchell *et al.*, 1973 and Dahlin *et al.*, 1984). This reactive metabolite is detoxified in the liver by glutathione and excreted by the kidney as mercapturic acid. When glutathione stores are depleted, the metabolite binds to hepatocellular macromolecules. This binding has been linked to hepatocellular damage (Potter *et al.*, 1973; Jollow *et al.*, 1973; and Mitchell *et al.*, 1973). The exact mechanism by which hepatocellular necrosis occurs remains unclear but generation of a reactive metabolite and its protein binding (Boobis *et al.*, 1989), generation of oxygen radicals (Tirmenstein and Nelson, 1990), mitochondrial damage (Esterline *et al.*, 1989), disruption of calcium homeostasis (reviewed by Orrenius *et al.*, 1989), and presence of inflammatory cells (Laskin and Pilaro, 1986) are all hypothesized to have a role in the manifestation of toxicity.

In 1973, four landmark papers in toxicology, linking metabolism and covalent binding of APAP to hepatotoxicity, were published (Jollow *et al.*, 1973; Mitchell, *et al.*, 1973a,b; and Potter *et al.*, 1973). Using histopathology and autoradiographic data, a correlation between the extent of tissue binding and tissue injury was observed. A more

recent study has shown that binding to specific proteins rather than overall protein covalent binding to be a critical factor in the hepatotoxic response (Beierschmitt, 1989). APAP arylated two major groups of proteins in the liver *in vivo*. Arylation was specific and selective (Bartolone *et al.*, 1987). These proteins were located in the plasma membrane and mitochondrial fraction (Pumford *et al.*, 1990 A,B). Immunochemical localization has shown this binding selectively in the centrilobular region of the necrotic liver (Bartolone *et al.*, 1989, and Roberts *et al.*, 1991). Birge *et al.*, have shown that a comparison of mouse and human liver *in vitro*, in culture and *in vivo* revealed that in both species, protein arylation is selective and specific for two major species of 58 and 130 kDa (Birge *et al.*, 1990). These data suggest that covalent binding of the reactive metabolite of APAP to proteins is an important event in the manifestation of hepatotoxicity.

While protein binding of APAP has been shown to correlate with necrosis, convincing evidence has been presented that oxidative stress also contributes to cell death. In isolated hepatocytes, APAP cytotoxicity has been shown to be associated with the generation of both superoxide and hydrogen peroxide (Gerson *et al.*, 1985, Kyle *et al.*, 1987, and Farber *et al.*, 1988). Until recently, oxidative stress and lipid peroxidation were thought not to play a role in toxicity *in vivo* (Smith and Mitchell, 1985;

and Younes and Sieger, 1985). However, Tirmenstein and Nelson (1990) have reported data from *in vivo* studies which suggests a transient oxidative stress is produced by APAP. This oxidative stress was hypothesized to be from an APAP-stimulated conversion of xanthine dehydrogenase to the oxidase form which leads to an increase in activated oxygen species (Tirmenstein and Nelson, 1990). The evidence for a transient increase of oxidative stress has been further strengthened by experiments using liposome encapsulated superoxide dismutase (Nakae *et al.*, 1990A). Superoxide dismutase protected animals from APAP-induced hepatotoxicity only when administered one hour before or up to 0.5 hours after APAP administration (Nakae *et al.*, 1990B). Therefore, APAP-induced hepatotoxicity induces oxidative stress in animals which contributes to the necrosis.

The appearance of APAP-induced hepatotoxicity has been associated with the disruption of normal hepatocellular function. Mitochondrial dysfunction has been shown to be an important feature in APAP hepatotoxicity. In mice, electron microscopy revealed mitochondrial changes associated with hepatotoxic doses of APAP (Placke *et al.*, 1987). APAP-protein adducts have been found in the mitochondrial fraction of liver isolated from hepatotoxic mice (Pumford, *et al.*, 1990 A,B). Biochemically, APAP has been shown to disrupt mitochondrial respiration in mice (Meyers *et al.*, 1988), rats (Esterline and Ji, 1989), and in isolated

hepatocytes (Burcham and Harman, 1990). Both the parent compound and the metabolite, NAPQI, contributed to mitochondrial disruption at the level of the electron transport chain with the NAPQI changes being irreversible (Esterline *et al.*, 1989). The metabolic effects of this mitochondrial alteration were increased glucose release from glycogen into the cytoplasm and inhibition of oxygen uptake in mitochondria which alters normal energy balance of hepatocytes (Itinose *et al.*, 1989).

Intracellular calcium levels are normally rigorously maintained at low concentrations. Disruption of calcium homeostasis has a role in APAP-hepatotoxicity. Hepatotoxic doses of APAP, in mice, increased total liver calcium as early as two hours after administration (Corcoran *et al.*, 1987). APAP alkylation of the plasma membrane of hepatocytes inhibited the ability of the liver to regulate intracellular calcium levels by inhibiting a calcium stimulated, magnesium ATPase (Tsokos-Kuhn *et al.*, 1988). An increase of intracellular calcium correlates well with loss of cell viability. Increases in intracellular calcium levels can cause perturbation of cytoskeletal organization, phospholipase activation, protease activation, endonuclease activation, and impaired mitochondrial function (Orrenius *et al.*, 1989). Any or a combination of these cellular perturbations may be involved in APAP hepatotoxicity.

APAP, at non-necrogenic doses, causes a centrilobular accumulation of mononuclear cells in the liver. By biochemical and functional characteristics, these cells were activated macrophages (Laskin and Pilaro, 1986, 1988). Sinusoidal endothelial proliferation has also been noted under these conditions (Laskin, 1990). It is postulated that the presence of these inflammatory cells, at doses of APAP which cause necrosis, may exacerbate the hepatotoxicity induced by APAP by releasing cytokines, reactive oxygen and nitrogen species.

Exactly which mechanism or combination of mechanisms induces cell death remains unclear. Metabolism of APAP to a reactive compound is an important requirement for APAP hepatotoxicity. Covalent binding of this metabolite results in cell death. Death of cells involves disruption of mitochondrial function, disruption of calcium homeostasis and generation of an oxidative stress. APAP also stimulates infiltration and accumulation of monocytes, proliferation of sinusoidal endothelial cells, and activation of these cells in the centrilobular region of the liver which may also play a role in APAP-induced hepatic necrosis.

Hypothesis and Specific Aims

While a role for macrophages in the manifestations of acute chemically-induced hepatic necrosis has been hypothesized, evidence to prove this has been scarce.

APAP has been shown at both necrogenic and non-necrogenic doses to stimulate an inflammatory cell infiltration into the liver (Dixon *et al.* 1971, 1975; Walker *et al.* 1980, 1983; and Laskin and Pilaro, 1986). It was the goal of this project to determine if the presence of these inflammatory cells in the liver after non-necrogenic doses of APAP alters the hepatotoxicity of prototype hepatotoxicants. To accomplish this goal both *in vivo* and *in vitro* models were examined.

In vivo, a two-stage model of hepatotoxicity was developed. In the first-stage, activation of nonparechymal cells (NPCs) was induced in the liver by single, oral, nonnecrogenic dose of APAP. In the second-stage, injury of hepatocytes was induced by the administration of prototype hepatotoxicants. Using the *in vivo* model, three specific aims were examined. Experiments were performed to characterize the response of prototype hepatotoxicants in APAP-pretreated rats. Behavioral, biochemical, and histological alterations induced *in vivo* by APAP in male rats which correlate with the appearance of hepatotoxicant potentiation were examined as possible explanations for APAP-induced potentiation. It was determined if the suppression of the reticuloendothelial system *in vivo* by dextran sulfate or gadolinium chloride could alleviate APAP-induced potentiation of allyl alcohol hepatotoxicity.

To specifically implicate NPCs in APAP-induced potentiation of hepatotoxicity, an *in vitro* coculture system was used. The dose response curve of allyl alcohol in isolated hepatocytes was determined in the presence or absence of macrophages and endothelial cells isolated from APAP-pretreated or control-pretreated rats. These experiments were done to determine if coculture of activated NPCs and hepatocytes could model *in vivo* hepatotoxicity and be used as a model to study APAP-induced potentiation of hepatotoxicity.

MATERIALS AND METHODS

In Vivo Experiments

Experimental Protocol for Animal Use

Male, Sprague-Dawley rats (Taconic Farms, Germantown, NY), weighing 200-300 g, were used in these experiments. The animals were housed in groups of four, maintained, before and throughout the experiments, on a 12 hour light/dark cycle, fed a standard lab chow (Chesapeake Feed, Beltsville, MD.), and allowed food and water *ad libitum*. Animals were allowed to acclimate to the animal facility for at least six days prior to use.

Animals were weighed daily while in the study. To examine the response of prototype hepatotoxicants in APAP-pretreated rats and to characterize the biochemical and histological changes induced by APAP, rats were pretreated orally with APAP or with a 20% Tween 80 vehicle (Sigma, St. Louis, MO). APAP was administered at a volume of 3 ml/kg at the doses indicated in figure legends. Twenty-four hours after the administration of APAP or vehicle, rats were either killed or challenged with a prototype hepatotoxicant. These compounds were AA, APAP, BB, CCl_4, DCE and TA. AA (Aldrich, Milwaukee, WI), CCL_4 (Fisher, Columbia, MD) and DCE (Aldrich, Milwaukee, WI) were given intraperitoneally in a corn oil vehicle. BB (Fisher, Columbia, MD) was given intraperitoneally in a sesame oil vehicle and TA (Aldrich,

Milwaukee, MI) was given intraperitoneally in a 0.9% saline vehicle. APAP was given intraperitoneally in 20% Tween 80. Animals were injected intraperitoneally at a volume of 6 ml/kg with hepatotoxicant doses indicated in the figure legends. Depending on the experiment, animals were killed four or twenty-four hours after challenge with the prototype hepatotoxicant (Figure 4).

To study the effect of glutathione depletion on the hepatotoxicity of prototype toxicants, rats were pretreated with diethylmaleate (DEM) (0.6 ml/kg) or with a sesame oil vehicle. Animals were injected intraperitoneally at a volume of 6 ml/kg. Rats were challenged 45 minutes after DEM with prototype hepatotoxicant. Animals were killed four or twenty-four hours after challenge with prototype hepatotoxicant (Figure 5).

To study the effects of RES suppression in APAP-induced potentiation, rats were anesthetized by inhalation of methoxyflurane (Metofane^{R}, Pitman-Moore, Washington Crossing, PA) to a surgical plane III anesthesia. Tails were wiped with 70% alcohol followed by a gauze pad dipped in warm water. Either a 0.9% saline vehicle, DS (15 mg/kg) (Pharmacia, Uppsala, Sweden) or $GdCl_3$ (5 mg/kg) (Aldrich, Milwaukee, WI) were injected intravenously at a volume of 6 ml/kg. One hour after injection of the RES suppressant, APAP was administered orally at 1.6 g/kg. Twenty-four hours after APAP administration, rats were challenged

Figure 4: Time Lines Describing Relationships Between APAP-Pretreatment and Hepatotoxicant Challenge. Rats were pretreated at time zero. Twenty-four hours later, the point at which nonparenchymal cell accumulation was observed, animals were challenged with hepatotoxicant. Panel A and B demonstrate the differences in the time course of the experiments. When AA and DCE were challenge hepatotoxicants, animals were sacrificed at four hours due to increased number of deaths after this point.

A.


HOURS
0
APAP
Pretreatment
24
AA or DCE
Challenge
28
Sacrifice

B.

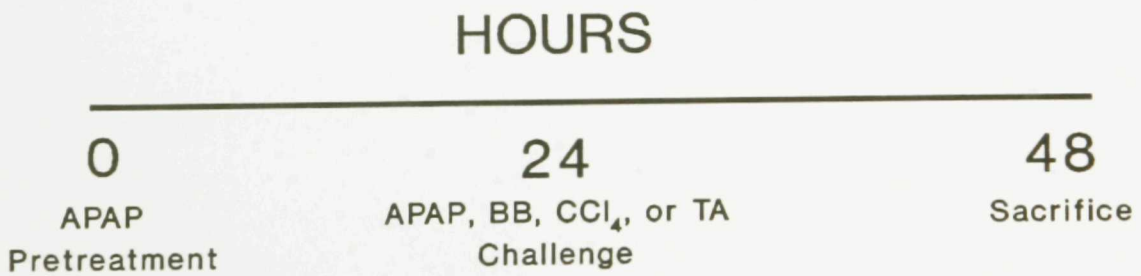
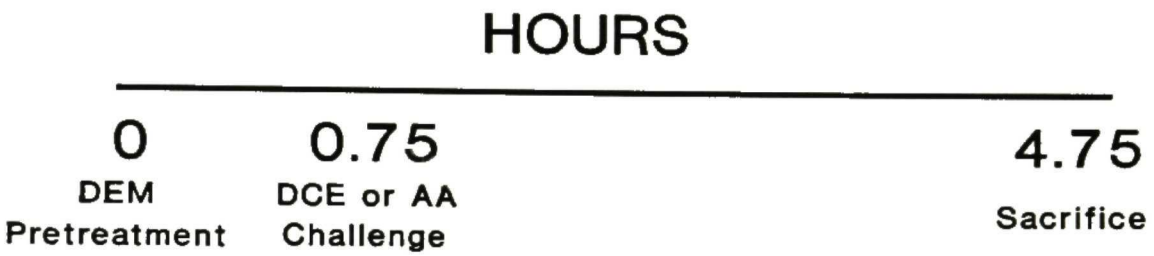
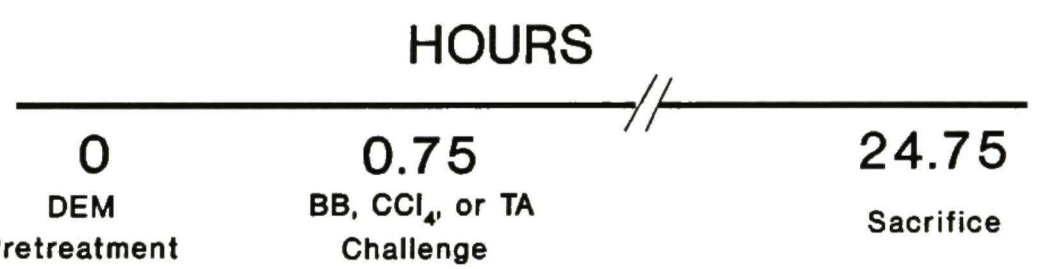

HOURS
0
APAP
Pretreatment
24
APAP, BB, CCl_4, or TA
Challenge
48
Sacrifice

Figure 5: Time Lines Describing Relationships Between DEM-Pretreatment and Hepatotoxicant Challenge. Rats were pretreated with DEM at time zero. Forty-five minutes later, animals were challenged with hepatotoxicant. Panel A and B demonstrate the differences in the time course of the experiments.

A.


HOURS
0
DEM
Pretreatment
0.75
DCE or AA
Challenge
4.75
Sacrifice

B.


HOURS
0
DEM
Pretreatment
0.75
BB, CCl_4, or TA
Challenge
24.75
Sacrifice

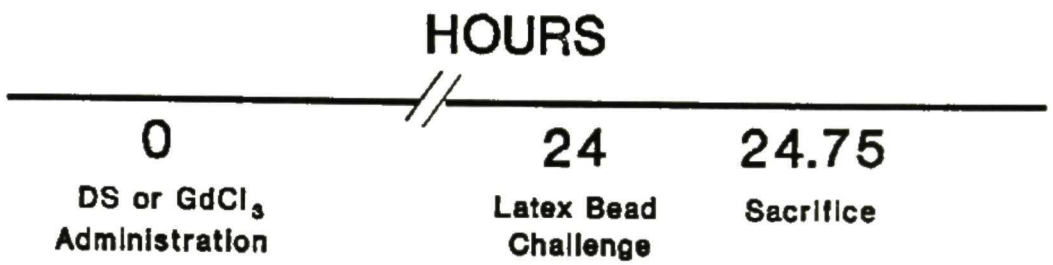
with 1 μm latex beads in 0.9% saline or intraperitoneally with AA (0.4 μmol/kg) in corn oil or vehicle alone at a volume of 6 ml/kg. Forty-five minutes after beads or four hours after the AA challenge rats were killed (Figure 6).

After all experiments except the latex bead clearance studies, animals were sacrificed by decapitation. Blood was collected in a 12x75 glass tube and centrifuged at 1000 g in a cold room in an IEC clinical centrifuge. Serum was harvested for transaminase or APAP blood level determinations. For latex bead clearance studies, ten minutes before sacrifice, rats were anesthetized with pentobarbital (78 mg/kg). Forty-five minutes after injection of the beads, a mid-line incision was made. One and a half ml of blood was drawn from the inferior vena cava into a Vaccutainer^{R}, containing EGTA.

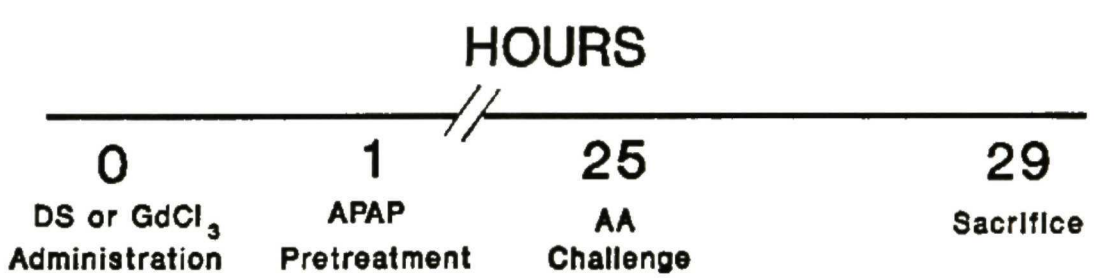
The abdominal cavities of the rat sacrificed by decapitation, were opened with a midline incision. Gross abnormalities were observed, the liver and/or stomach removed, weighed, and processed as per the experimental protocol. When necessary, liver samples were collected for histopathology by slicing small sections from the center of the liver with a tissue-slicer blade (Thomas Scientific, Philadelphia, Pa), and fixed in 4% w/v para-formalin (Kodak,Rochester, NY) in 0.15 M phosphate buffer. The embedding, sectioning, and staining of liver samples in intravenously para-formalin was

Figure 6: Time Lines Describing Relationships Between RES Suppressant-Pretreatment and Hepatotoxicant Challenge. A. Time line for latex beads experiments. At time zero, rats were pretreated with DS or $GdCl_3$. Twenty-four hours later, rats were challenged with the latex beads and sacrificed forty-five minutes later. B. Time lines for RES suppressant-pretreatment and hepatotoxicant challenge. At time zero, rats were pretreated with DS or $GdCl_3$. One hour later rats were pretreated with APAP. Twenty-four hours later, rats were challenged with AA and sacrificed four hours later.

A.


HOURS
0
DS or GdCl3 Administration
24
Latex Bead Challenge
24.75
Sacrifice

B.


HOURS
0
DS or GdCl3 Administration
1
APAP Pretreatment
25
AA Challenge
29
Sacrifice

performed by the diagnostic services group at USUHS. Briefly, livers were embedded in paraffin, cut to 5 μm thickness, and stained with hematoxylin and eosin. Hematoxylin stains the nuclei of cells and eosin stains the cytoplasm.

For glutathione (GSH) and alcohol dehydrogenase (ADH, EC 1.1.1.1) determinations, liver samples were cooled in 0.25 M sucrose. For GSH, one-half gram of liver was weighed and homogenized with a polytron (Brinkman Instruments, Westbury, NY) in 5 ml of 5% trichloroacetic acid. The homogenate was centrifuged at 900 x g for ten minutes in an IEC clinical centrifuge. The nonprotein-supernatant was decanted and used for GSH determinations. For ADH determinations, two g of liver were weighed and homogenized in 10 ml of 0.25 M sucrose for six strokes at 800 rpm using a Potter-Elvejhem tissue grinder. The homogenate was centrifuged for ten minutes at 4°C at 1475 g. The supernatant from this spin was centrifuged at 4°C at 12100 x g. The supernatant from this fraction containing the cytosol and microsomes and was assayed for ADH.

Protocol for Biochemical Analysis

In *in vivo* experiments, hepatotoxicity was measured as release of alanine aminotransferase (ALT) from hepatocytes into the blood. Hepatocytes have a high activity of ALT. When cells become necrotic and lose their membrane integrity, cytosolic enzymes spill into the serum (Plaa and Hewitt, 1984). ALT was measured in serum by the optimized standard

method recommended by the German Society for Clinical Chemistry (1970, 1971) with a clinical test kit (Sigma, St. Louis, MO). This assay is based on the coupled reactions:

2-oxyglutarate + *l*-alanine --ALT--→ *l*-glutamate + pyruvate

pyruvate + NADH --LDH--→ lactate + NAD^+.

ALT catalyzes the transfer of the amino group from alanine to 2-oxyglutarate to yield glutamate and pyruvate. Pyruvate is then reduced to lactate in the presence of lactate dehydrogenase (LDH, EC 1.1.1.27) with simultaneous oxidation of reduced nicotinamide adenine dinucleotide (NADH). The reaction mixture consists of *l*-alanine (963mM), phosphate buffer pH 7.4 (96 mM), NADH (0.217 mM), LDH (1444 U/L), and 2-oxyglutarate (17 mM). The reaction was begun by adding 20 µl of serum and the oxidation of NADH followed for 10 minutes at 340 nm using a Gilford spectrophotometer. ALT activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol NAD^+/L per minute under these assay conditions. The activity was be calculated from the formula provided in Appendix B.

GSH, the most abundant low molecular weight thiol in cells (Meister, 1981), was assayed as acid soluble non-protein sulfhydryls using 5,5'-dithio-bis-2-nitrobenzoic acid (Sigma, St. Louis, MO)(Ellman, 1959). Non-protein sulfhydryls in the supernatant of the liver homogenate or GSH standards (100, 300, or 1000 µg/ml)(Sigma, St. Louis, MO) in 5% trichloroacetic acid were mixed with 0.1 M phosphate buffer

at pH 8.0 and Ellman's reagent. Ellman's reagent reacts with sulfhydryl groups to form an intensely colored anion, 5-thio-2-nitro-benzoic acid. After ten minutes, the absorbance was read at 412 nm in a Sargent-Welch 6-550 spectrophotometer and GSH concentration in the liver homogenate was calculated from a standard curve.

Serum APAP levels were determined by the method of Walberg (1977) using a kit supplied by Sigma. In this procedure, serum proteins were precipitated with 3% trichloroacetic acid. This mixture was centrifuged at 900 x g in a clinical IEC centrifuge for ten minutes. The supernatant, which contains unmetabolized APAP, was decanted. The supernatant or APAP standards (20, 40, 60 mg/dL) were reacted with nitrous acid to form 2-nitro-5-acetamidophenol that assumes a deep yellow color in alkaline medium. After thirty minutes, the absorbance was read at 430 nm and serum APAP concentrations calculated from a standard curve.

Alcohol dehydrogenase activity was assessed in liver cytosol based on the reaction

Ethanol + NAD^+ --ADH---→ Acetaldehyde + NADH + H^+.

ADH was assayed using ethanol as a substrate in the presence of phosphate buffer (0.15 M) pH 7.4, semicarbazide (2.2 M), GSH (300 mM) and NAD^+ (27.7 mM) (Sigma, St. Louis, MO). The increase in absorbance at 340 nm was monitored and ADH activity was expressed as U/mg protein. ADH activity was calculated from the equation in appendix B. Protein was

determined using Folin-Ciocalteu's phenol reagent and calculated from a standard curve using bovine serum albumin as a standard (Shakin, 1969).

To document RES suppression, the clearance of latex beads was assessed (Shiratori, *et al.*, 1989). Whole blood, 0.5 ml, was mixed with 0.5 ml of 0.1% Na_2CO_3 to lyse red blood cells. Extracellular beads were counted using a hemacytometer and the number of beads/ml calculated. Increased number of beads in the blood is interpreted as decreased clearance by the RES.

Pathological Evaluation of Hepatic Necrosis

Pathological evaluation of livers was conducted by Experimental Pathology Laboratories (Herndon, Va.). Slides were evaluated without the pathologist's knowledge of treatment protocol. The slides were scored for incidence of non-necrogenic changes and zonal necrosis as follows:

1-minimal,
2-slight/mild,
3-moderate,
4-moderately severe,
5-severe/high.

In Vitro Experiments

Isolation of Hepatic Nonparenchymal Cells

Rats were administered either APAP (1.6 g/kg) or a 20% Tween 80 vehicle 24 hours prior to cell isolation. Hepatic macrophages and endothelial cells (NPC) were isolated

from control or APAP-pretreated rats by the method described by Laskin *et al.*, 1988.

Rats were anesthetized with pentobarbital at a dose of 78 mg/kg. A midline incision was made, the liver isolated, and perfused through the portal vein with 100 ml of Hanks Balanced Salt Solution (HBSS) containing EGTA (0.5 mM) at pH 7.4 at 20 ml/minute. The solution was warmed in a water bath set at a temperature to allow the solution to be 37°C when it enters the rat. At the completion of the HBSS perfusion, HBSS was replaced with sterile Liebovitz's L-15 media (Flow, McClean, VA) containing protease type XIV (2.2 U/ml, EC 3.4.24.-) (Sigma, St. Louis, MO) and collagenase type IV (104 U/ml, EC 3.4.24.3) (Sigma, St. Louis, MO), and the liver was perfused with 180 ml of perfusion media at a rate of 20 ml/minute. Liebovitz's L-15 media was used for cell isolation for its ability to buffer in free gas exchange with the atmosphere (Liebovitz, 1963). At the end of the perfusion, the liver was excised. It was combed and digested for 8 minutes at 37°C with protease type XIV (8.8 U/ml) in a total volume of L-15 media of 100 ml. Deoxyribonuclease I (848 U/ml, EC 3.1.21.1) (Sigma, St. Louis, MO) was added and the incubation continued for 10 minutes. After the digestion, the cell suspension was filtered through a 70 μm tissue sieve into two conical, polypropylene 50 ml centrifuge tubes (Corning, Corning, NY) and was centrifuged at 50 x g for five minutes in an IEC clinical centrifuge to remove hepatocytes which were

in the pellet. The NPCs in the supernatant were recovered by centrifugation at 300 x g for five minutes (Dupont/Sorvall RC-5 centrifuge in an HS-4 rotor). This cell pellet, containing NPCs, was washed with L-15 media four times at 300 x g for 5 minutes.

The cells were then resuspended in 5 ml of L-15 media and split into two polystyrene 15 ml conical tubes (Corning, Corning, NY). Red blood cells were removed from the NPCs by a 17.5% metrizamide gradient. The gradient was formed by mixing 3.5 ml of 30% metrizamide (Sigma, St. Louis, MO) in Geys balanced salt solution with the cell suspension and 1 ml of L-15 media was carefully layered over the cell suspension. The cell suspension was centrifuged at 700 x g for 20 minutes at 4°C without a brake. Mononuclear phagocytes and endothelial cells were located at the interface of the two layers. The cells were washed twice with L-15 media.

At this point, an aliquot of cells was counted and checked for viability using a hemacytometer and Trypan blue (Sharpe, 1988). This tested the integrity of the plasma membrane. Damage to the plasma membrane has been suggested to be a critical step in the sequence of events leading to cell death (Farber *et al.*, 1975). Equal volumes of cell suspensions and 0.2% Trypan blue in Krebs-Henseleit buffer was mixed and incubated for five minutes. Viable cells will not take up the dye; therefore the percentage of viable cells was calculated using a hemacytometer. Viability of NPC

preparations was always above 85%.

Cells were plated on 24 well plates (Costar, Cambridge, MA) coated with collagen. Collagen coating was done by layering a sterile solution of 2 ml of collagen (0.04 mg/ml) (Vitrogen^R, Collaborative Research, Bedford, MA) onto the plates for two hours prior to use. The cells were cultured in Williams E media (Flow/ICN, Costa Mesa, CA) supplemented with 10% Serum Plus (Hazelton, Lexton, KS); penicillin-streptomycin (50 μg/ml) (Flow/ICN, Costa Mesa, CA); glutamine (5 μg/ml) (Flow/ICN, Costa Mesa, CA); insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 μg/ml) (ITS^R Premix, Collaborative Research, Bedford, MA); HEPES (10 mM); and $CaCl_2$ (1 mM) and kept at 37°C in a humidified, 5% CO_2 incubator.

NPCs were confirmed microscopically by using non-specific peroxidase staining. The NPC population from both control and APAP treated rats were stained. Peroxidase staining was performed by the method of Page and Garvey (1979). The reaction mixture contained sucrose (0.2 M), 50 mM Tris HCl, HCl (0.04 N), 0.02% H_2O_2 and diaminobenzidine (3 mM) (Sigma, St. Louis, MO). This mixture was unstable and was prepared immediately before use and kept dark and at 4°C. One million cells were resuspended in 1 ml of reaction mixture in 15 ml conical tubes and incubated at 37°C for 30 minutes in a shaking water bath. After the incubation, the cells were centrifuged at 300 x g and resuspended in Williams-E media

with 10% fetal bovine serum (Flow, Inc., Costa Mesa, CA). The cells were placed in IEC cytobuckets (Needham Hts., MA) and recovered by centrifugation at 300 x g onto microscope slides. The slides were then fixed in 0.2% glutaraldehyde in phosphate buffered saline. The cells were examined by bright field microscopy. Kupffer cells have dark brown granules in endoplasmic reticulum and the nuclear envelope in a clear cytoplasm; other non-parenchymal cells fail to stain with the exception of erythrocytes which stain uniformly dark brown. Contaminating parenchymal cells were recognized by their characteristic morphology and overall light brown staining.

Isolation of Hepatocytes

Hepatocytes were isolated using modifications of the methods described by Bissell and Guzelian (1980) and Seglen (1976). Control rats were anesthetized with pentobarbital (78 mg/kg). The liver was perfused through the inferior vena cava with 200 ml of an oxygenated wash out buffer at pH 7.4 at 42°C at a rate of 20 ml/minute. This buffer contained NaCl (117 mM), KCl (6.7 mM), HEPES (10 mM), and glucose (11.5 mM). After the washout buffer, the liver was perfused at a rate of 15 ml/minute with 300 ml of an oxygenated collagenase buffer that contained the wash out buffer with $CaCl_2$ (1mM) and collagenase (106 units/ml) (Worthington Biochemical Corporation, Freehold, NJ) at pH 7.5. At the completion of the perfusion, the liver was carefully

cut out and washed gently in a petri dish to dislodge the cells into 30 ml of media. The suspension was filtered through 250 μm nylon mesh, and the filtrate centrifuged at 50 x g for 2 minutes. The supernatant was aspirated. The cells were washed again in 30 ml of media and centrifuged for 2 minutes. The cell pellet was resuspended at a cell concentration necessary for the experiment. Cells were cultured in Williams E media supplemented with 10% Serum Plus (Hazelton, Lexton, KS), penicillin-streptomycin (50 μg/ml), glutamine (5 μg/ml), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 μg/ml), HEPES (10 mM), and $CaCl_2$ (1 mM) and kept at 37°C in a humidified, 5% CO_2 incubator.

Biochemical Analysis of Cytotoxicity

AA was used as the model hepatotoxicant in the *in vitro* studies. AA was diluted in culture media, sterilized with a 0.22 μm syringe filter, and assessed for cytotoxicity in doses from 0 to 1000 nM. Hepatocytes (2.5 x 10^5 cells/cm^2), NPCs (5 x 10^6 cells/cm^2), or cocultures of both cell types were treated with AA. Two hours later, cytotoxicity was assessed as release of hepatocyte cytosolic enzymes into the media. The endpoints tested in the dose response curves for AA were the percent of the total alanine aminotransferase (ALT, EC 2.6.1.2), aspartate aminotransferase (AST, EC 2.6.1.1), and lactate dehydrogenase (LDH) released into the media. An aliquot of 200 μl of media was assayed.

Total enzyme activity in cells was assessed by scraping cells off the plate with a policeman into 2 ml of HBSS. Cells were sonicated and the enzyme activity determined. Cytotoxicity is expressed as percent enzyme released from total and calculated as [{(Activity in Media)/(Activity in Media + Activity in Sonicate)} x 100].

ALT activity was determined as described earlier except 200 µl of media or 50 µl of sonicate were used instead of serum. AST was measured by the optimized standard method recommended by the Scandinavian Society for Clinical Chemistry and Clinical Physiology and the International Federation of Clinical Chemistry (1974, 1978) with a clinical test kit (Sigma, St. Louis, MO). This assay is based on the coupled reactions:

2-oxyglutarate + *l*-aspartate-AST---→*l*-glutamate + oxalacetate

oxalacetate + NADH-MDH---→*l*-Malate + NAD^+.

AST catalyzes the transfer of the amino group from aspartate to 2-oxyglutarate to yield glutamate and oxalacetate. The oxalacetate is then reduced to malate in the presence of malate dehydrogenase (MDH, EC 1.1.1.37) with simultaneous oxidation of NADH. The reaction mixture contains *l*-aspartate (160 mM), NADH (0.26 mM), 2-oxyglutarate (12 mM), 600 U MDH/L, and buffer to pH 7.8. The reaction is started by mixing 1 ml of reagent and 200 µl media or 50 µl of sonicate, the oxidation of NADH is followed for ten minutes at 340 nm using a Gilford spectrophotometer. AST activity is defined as the

amount of enzyme that catalyzes the formation of 1 μmol NAD^+/L per minute under these assay conditions. The activity was calculated from the formula provided in Appendix B.

LDH was determined by the method described by Wroblewski and LaDue, 1955 in serum free media. This assay measures the disappearance of NADH according to the reaction:

pyruvate + NADH --LDH→ lactate + NAD^+.

The rate of disappearance of NADH over time is proportional to the amount of enzyme added. The reaction was initiated by mixing 0.15 M phosphate buffer, pH 7.4, containing NADH (100 μg/ml) with 100 μl culture media or 50 μl sonicate. This mixture was incubated for 20 minutes. Then 0.1 ml of sodium pyruvate (2.5 mg/ml) was added and changes in the absorbance followed at 340 nm for ten minutes. LDH activity was determined at room temperature and the specific activity was expressed as units/ml of media. A unit is defined as the amount of enzyme that catalyzes the reduction of 1 μmol NADH/L per minute. The activity was calculated from the formula in Appendix B.

Coculture of Hepatocytes and Nonparenchymal Cells

In order to test the hypothesis that activated NPCs potentiate hepatotoxicant action, a coculture system was developed. Hepatic NPCs from either control- or APAP-pretreated (1.6 g/kg) rats were isolated and cultured as described above. Eighteen hours later, hepatocytes were

isolated from a control rat and cocultured with NPCs. Hepatocytes were added to NPCs cultures in 100 μl aliquots of $5x10^6$/ml media. NPCs were plated 2.5 x 10^6, 1.5 x 10^6, 8 x 10^5 cell/cm^2 so that hepatocytes would be exposed to ratios of 10:1, 5:1, and 3:1 (NPCs:hepatocytes). Cocultures were allowed to equilibrate for two hours before manipulation. At this time point, the cocultures were administered AA and used two hours later for cytotoxicity assessment.

Cultured Hepatocytes in Nonparenchymal Cell-Conditioned Media

NPC-conditioned media (NPC-CM) was prepared by culturing NPCs from control and APAP-pretreated rats at 5 x 10^5 cells/cm^2 on 24 well plates for eighteen hours. Media from NPCs was decanted and centrifuged at 300 x g to remove any cells. Freshly isolated hepatocytes were plated with the NPC-conditioned media at 2.5 x 10^5 cells/cm^2. After culture for two hours, a dose response curve to AA-induced cytotoxicity was obtained with the hepatocytes cultured in NPC-conditioned media from control- or APAP-pretreated rats.

Statistical Analysis

Data was expressed as mean ± SE. When only two experimental groups were compared, a Student's *t* test was performed to determine statistical significance (Duncan *et al.*, 1977). When more than two groups were being compared an analysis of variance was performed (von Fraunhofer and Murray,

1976). If the variances being compared were significantly different, a Newman-Keuls test which identified statistically significant differences between experimental groups was performed (Snedecor and Cochran, 1980).

RESULTS

APAP-Induced Alterations in the Liver

APAP, in doses up to 2.4 g/kg, produced no significant increase in serum ALT activity in fed rats, twenty-four hours after administration (Figure 7). Histologically, APAP (1.6 g/kg) produced an accumulation of macrophages into the centrilobular region of the liver in male rats (Figure 8-b), which agreed with the findings of Laskin and Pilaro (1986) in female rats. Figure 8-b shows mononuclear cell infiltration and sinusoidal dilation in the livers of APAP-pretreated rats. In all but one animal, minimal necrosis was present up to 28 hours after the administration of APAP (Table 3). No increases of serum ALT activity were observed for up to 72 hours after APAP administration (Figure 9). Therefore, APAP, does not induce hepatic necrosis, under these experimental conditions.

APAP-Induced Potentiation of DCE and AA Toxicity

Rats were pretreated orally with APAP (1.2 g/kg) and challenged twenty-four hours later with DCE. APAP-pretreated rats challenged with DCE had increased biochemical (Figure 10) and histological (Figure 11D) evidence of necrosis four hours after DCE administration. In APAP-pretreated animals, DCE induced extensive centrilobular necrosis with diffuse congestion. Congestion of the livers was extensive and was demonstrated by the increase of liver weight normalized to body weight

Figure 7: Serum ALT Activity 24 Hours after APAP-Pretreatment. Twenty-four hours after APAP, rats were sacrificed and serum samples collected for ALT determinations. Data expressed as mean ± SE. n=6.

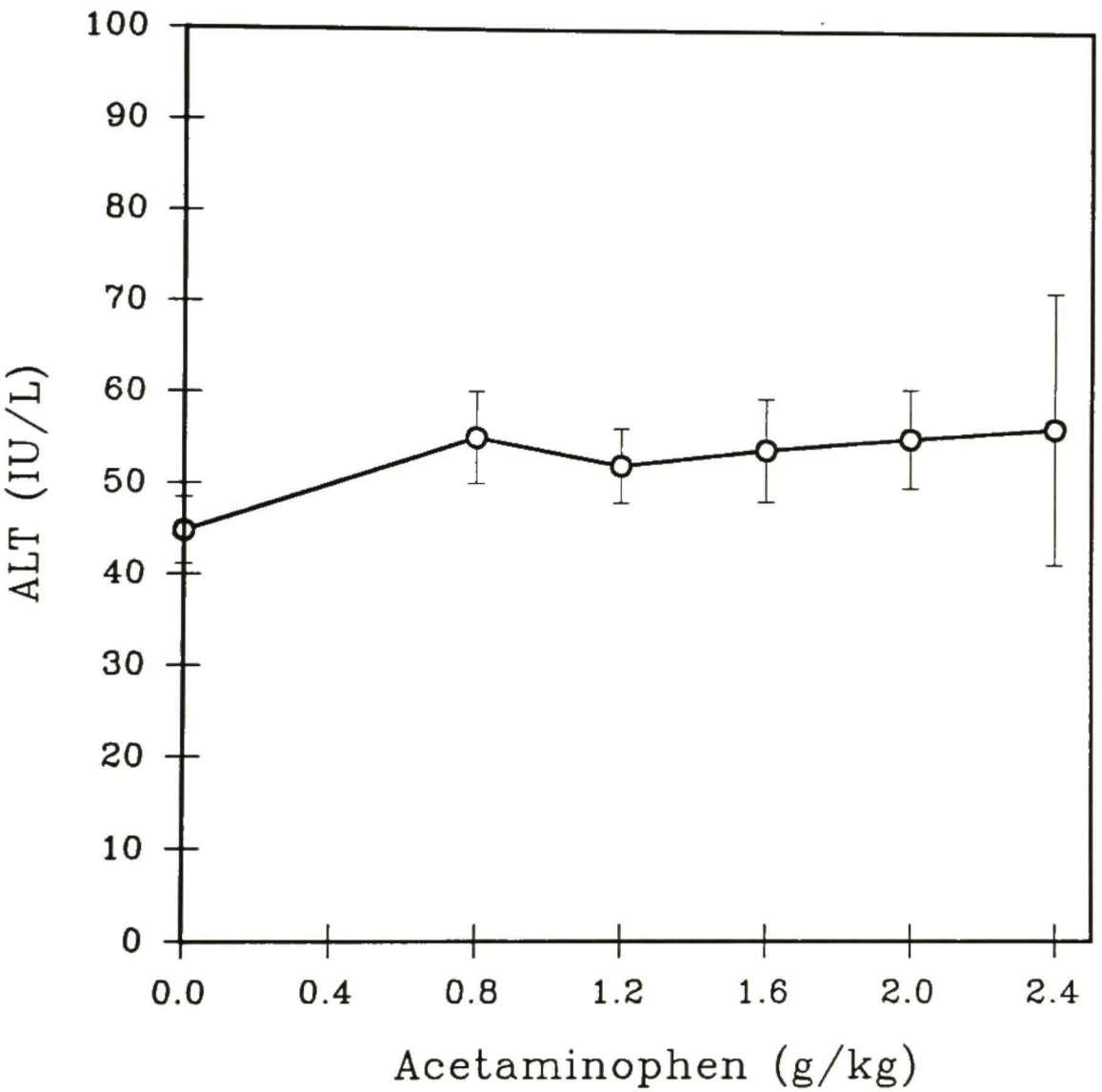
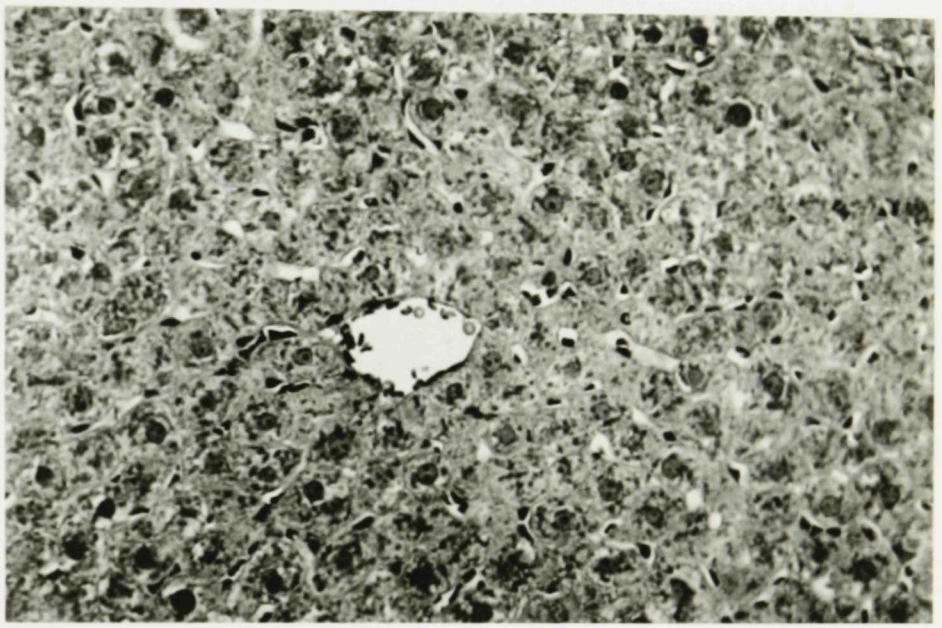

100
90
80
70
60
50
40
30
20
10
0
ALT (IU/L)
0.0
0.4
0.8
1.2
1.6
2.0
2.4
Acetaminophen (g/kg)

Figure 8: Histopathology of Liver from Control- or APAP-Pretreated Rats. Hematoxylin and eosin stains of formalin fixed livers from **A**) control and **B**) APAP-pretreated (1.6 g/kg) rats twenty-four hours after APAP administration. In **B** there is dilation of sinusoids and mononuclear cell infiltration. Photographed at 400x.

A



B

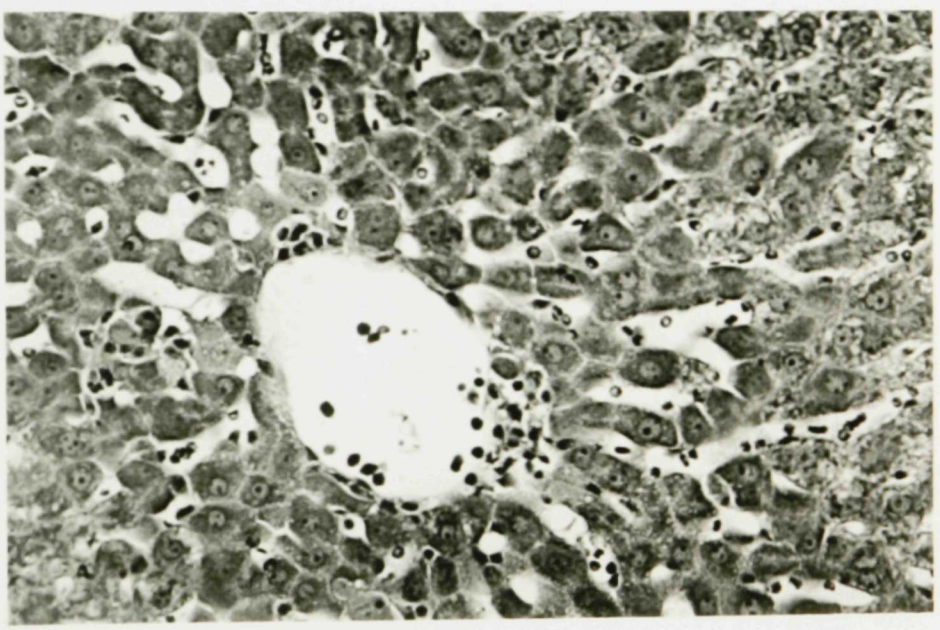


TABLE 3

FREQUENCY OF NECROSIS SCORES 28 HOURS AFTER ACETAMINOPHEN (1.6 g/kg)[a]

	Necrosis Score		
	0	**1**	**2**
Frequency	8	2	1

[a]Hematoxylin and eosin stained sections of liver from APAP-pretreated (1.6 g/kg) rats twenty-four hours after APAP administration were scored for incidence and severity of centrilobular necrosis. The slides were scored for incidence of non-necrogenic changes and zonal necrosis as follows: 0-none, 1-minimal, or 2-slight/mild.

Figure 9: Serum ALT Activity 24, 48, and 72 Hours after APAP-Pretreatment. APAP (0, 1.2, or 1.6 g/kg) was administered to rats. The animals were sacrificed at 24, 48, or 72 hours and serum samples collected for ALT determinations after administration. Data expressed as mean ± SE. $n \geq 4$.

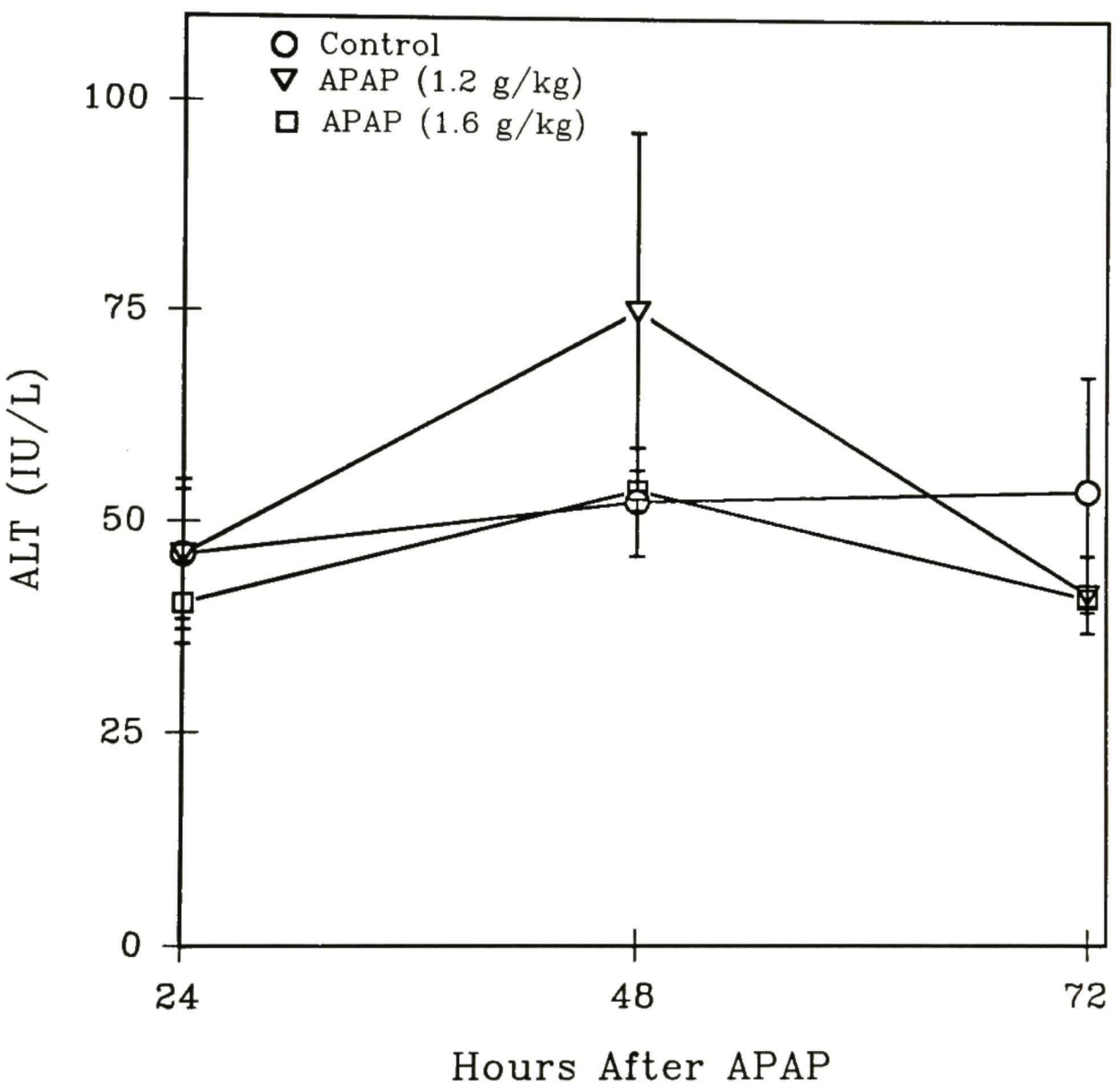

Control
APAP (1.2 g/kg)
APAP (1.6 g/kg)
100
75
50
25
0
ALT (IU/L)
24
48
72
Hours After APAP

Figure 10: Serum ALT Activity in Control- and APAP-Pretreated Rats after DCE. Rats were pretreated with APAP (1.2 g/kg) and challenged 24 hours later with DCE (0, 0.38, 1.25, or 2.5 μmole/kg). Four hours after DCE challenge the animals were sacrificed and serum samples collected for ALT determination. Data expressed as mean ± SE. * $p < 0.05$ as compared to control-pretreated group at the same dose of DCE. $n \geq 7$.

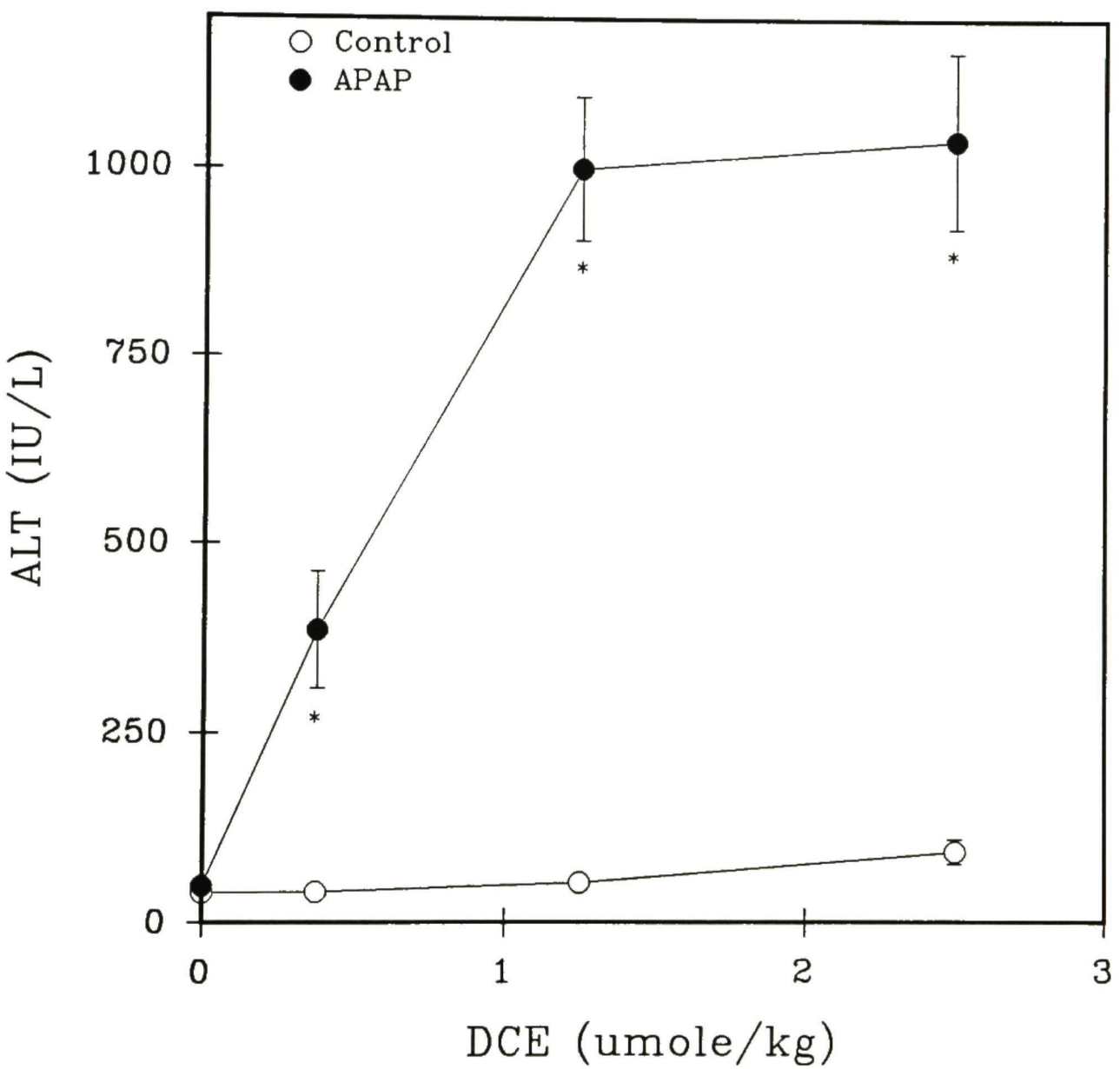
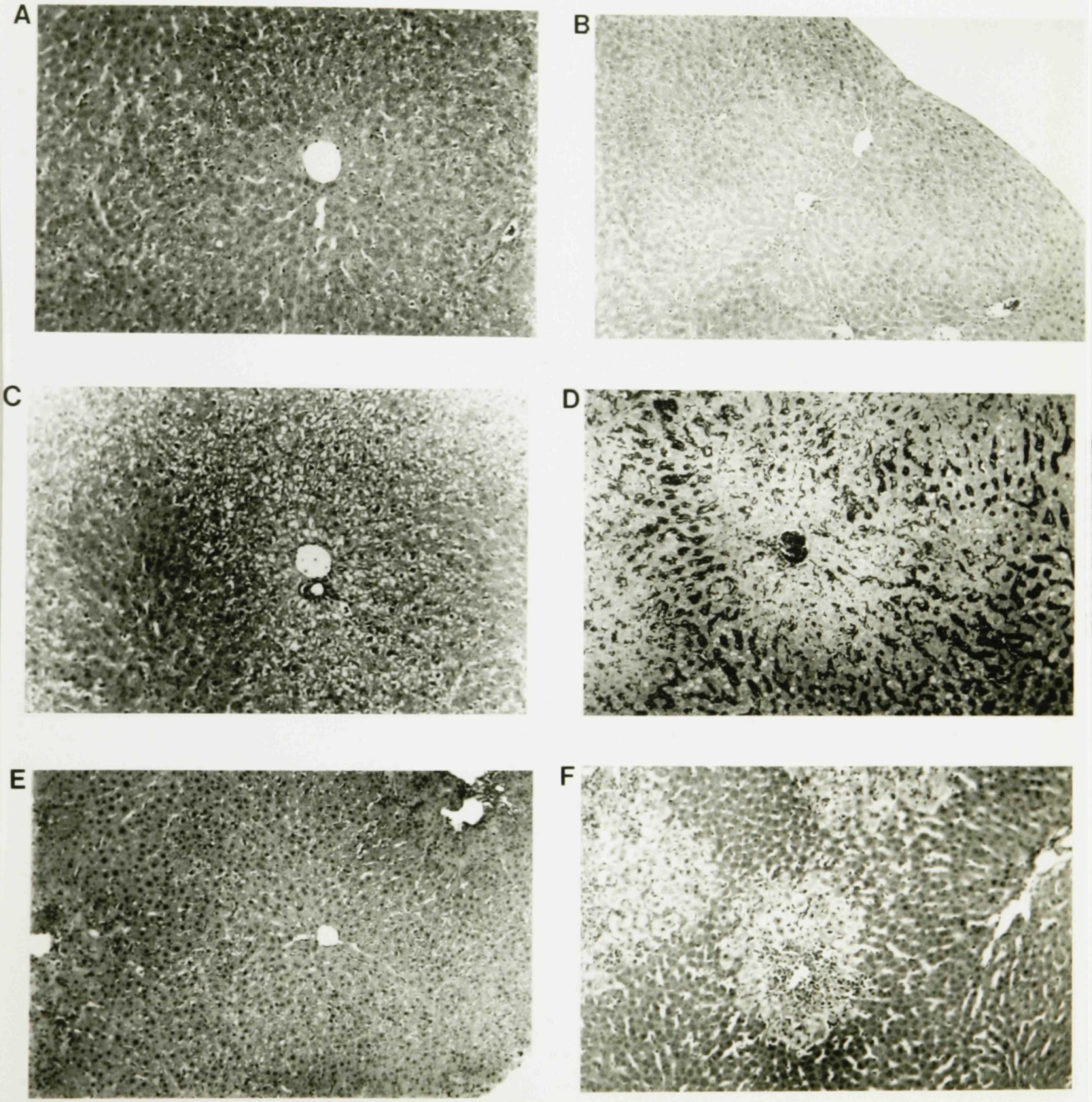

Control
APAP
1000
750
500
250
0
ALT (IU/L)
0
1
2
3
DCE (umole/kg)
*
*
*

Figure 11: Hematoxylin and Eosin Stained Liver Sections From Control- and APAP-pretreated Rats Challenged with AA or DCE. Twenty-four hours after pretreatment, rats were challenged as control, DCE (0.2 μmole/kg) or AA (0.03 umole/kg) and killed 4 hours after challenge. **A**) control, **B**) APAP, **C**) DCE, **D**) APAP and DCE, **E**) AA, and **F**) APAP and AA. Photographed at 100x. Note in **D**, congestion of the sinusoids with red blood cells. Note in **F**, centrilobular necrosis and marked inflammatory cell infiltration.


A
B
C
D
E
F

(Figure 12). Elevations of serum ALT activity (Figure 10) roughly correlated with severity of hepatic necrosis.

It was hypothesized that increasing the dose of APAP might increase the responsiveness of the animals to APAP-induced potentiation. To assure that this hypothesis was true, the dose response of APAP-induced potentiation was studied using the same time line as the initial experiments. A sigmoidal dose response curve was obtained (Figure 13). The dose of APAP was increased to 1.6 g/kg for further experiments in an attempt to decrease the number of animals needed for experiments.

To determine if APAP-induced potentiation pertained only to DCE, other model hepatotoxicants were examined. APAP-pretreated (1.6 g/kg) rats challenged with AA had increased biochemical (Figure 14) and histological (Figure 11-f) evidence of necrosis four hours after AA administration. AA, alone, produced periportal necrosis (Reid, 1972). In APAP-pretreated rats, AA induced periportal necrosis and congestion in fifty percent of animals and centrilobular necrosis and congestion in fifty percent of animals. This observation was an example of the inherent variability with which toxicants affect these animals.

APAP-pretreatment also increased the lethality of AA and DCE (Tables 4 and 5). When APAP-pretreated rats died after AA or DCE challenge, they appeared to be in the

Figure 12: Liver Weights of Control- and APAP-Pretreated Rats after DCE Challenge. Rats were pretreated with APAP (1.2 g/kg) and challenged 24 hours later with DCE (0, 0.38, 1.25, or 2.5 μmole/kg). Four hours after DCE challenge the animals were sacrificed and the liver was removed, blotted dry and weighed. Data expressed as mean ± SE. * $p < 0.05$ as compared to control-pretreated group at the same dose of DCE. $n \geq 6$.

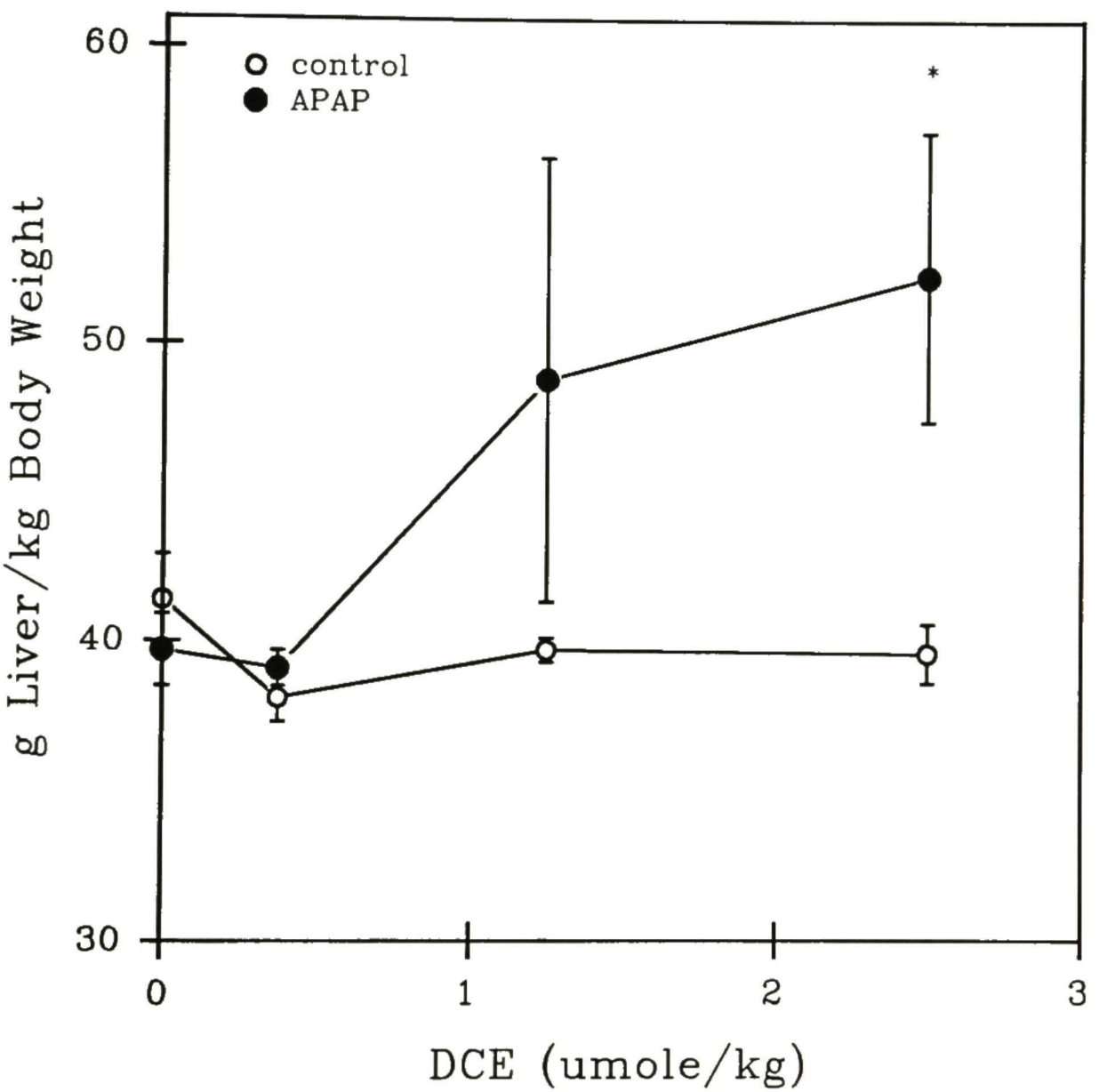

60
50
40
30
control
APAP
*
g Liver/kg Body Weight
0
1
2
3
DCE (umole/kg)

Figure 13: Dose Response of APAP-Induced Potentiation After DCE Challenge. Serum ALT activity in Control- and APAP-pretreated rats after DCE. Rats were pretreated with APAP up to 2.4 g/kg and challenged 24 hours later with DCE (2.5 μmole/kg. Four hours after DCE challenge the animals were sacrificed and serum samples collected for ALT determination. Rats pretreated with APAP (1.2 g/kg) and challenged with DCE had serum ALT activity 428% of control-pretreated rats. Rats pretreated with APAP (1.6 g/kg) and challenged with DCE had serum ALT activity 1740% of control-pretreated rats. * $p < 0.05$ as compared to control-pretreated group at the same dose of DCE. Data expressed as mean ± SE. n=6.

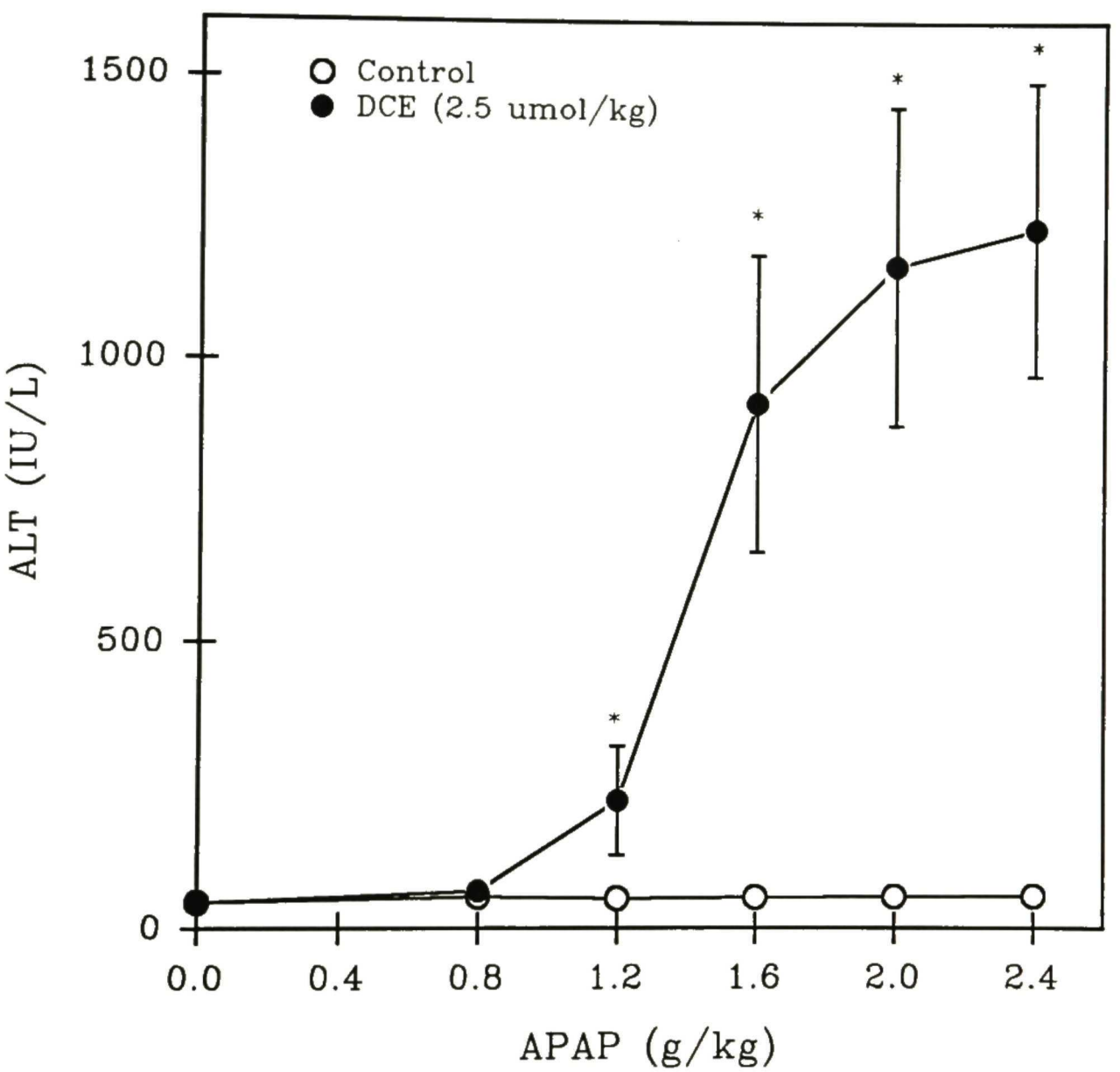

Control
DCE (2.5 umol/kg)
ALT (IU/L)
1500
1000
500
0
0.0
0.4
0.8
1.2
1.6
2.0
2.4
APAP (g/kg)
*
*
*
*

Figure 14: Serum ALT Activity in APAP-Pretreated Rats after AA Challenge. Twenty-four hours after 1.6 g/kg APAP-pretreatment, rats were challenged with AA (0, 0.4, or 0.8 μmole/kg). Four hours after AA challenge, the animals were sacrificed and serum samples collected for ALT determinations. Rats pretreated with APAP and challenged with AA (0.4 μmole/kg) had serum ALT activity 1850% of control-pretreated rats. Data expressed as mean ± SE. * $p \leq 0.05$ as compared to control-pretreated group. # $p \leq 0.05$ as compared to control-challenged group. $n \geq 4$.

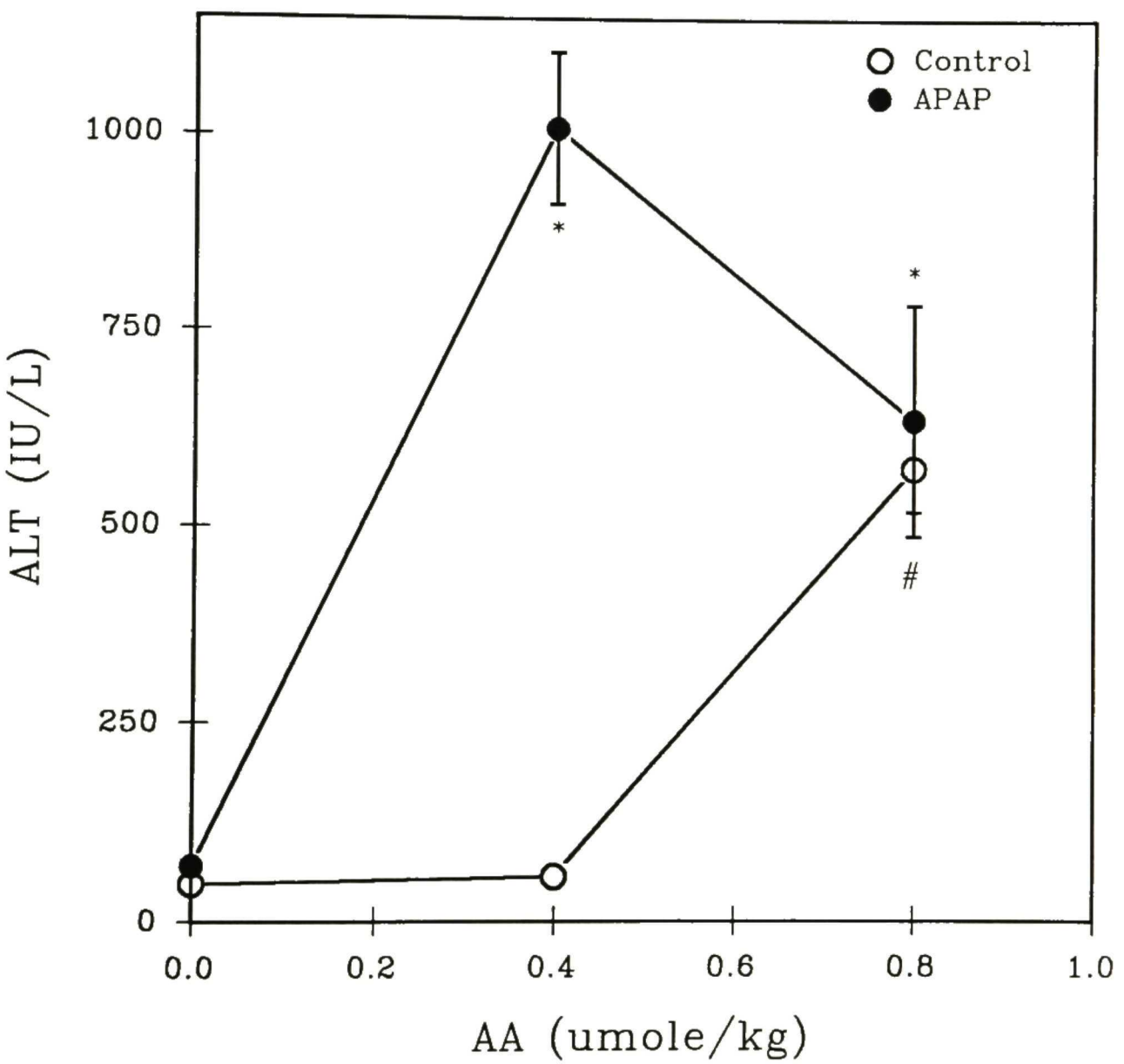

Control
APAP
1000
750
500
250
0
ALT (IU/L)
0.0
0.2
0.4
0.6
0.8
1.0
AA (umole/kg)
*
*
#

TABLE 4

LETHALITY IN APAP-PRETREATED RATS CHALLENGED WITH AA

Control-Pretreated	Deaths
AA (0.0 μmole/kg)	0/6
AA (0.4 μmole/kg)	0/6
AA (0.8 μmole/kg)	0/6
APAP-Pretreated	
AA (0.0 μmole/kg)	0/6
AA (0.4 μmole/kg)	0/7
AA (0.8 μmole/kg)	4/8

[a]Twenty-four hours after APAP-pretreatment (1.6 g/kg), rats were challenged with AA (0, 0.4 or 0.8 μmol/kg). Rats were observed for four hours after treatment and the incidence of lethality recorded.

TABLE 4

LETHALITY IN APAP-PRETREATED RATS CHALLENGED WITH DCE

Control-Pretreated	Deaths
DCE (0.0 μmole/kg)	0/5
DCE (2.5 μmole/kg)	0/5
APAP-Pretreated	
DCE (0.0 μmole/kg)	0/5
DCE (2.5 μmole/kg)	4/5

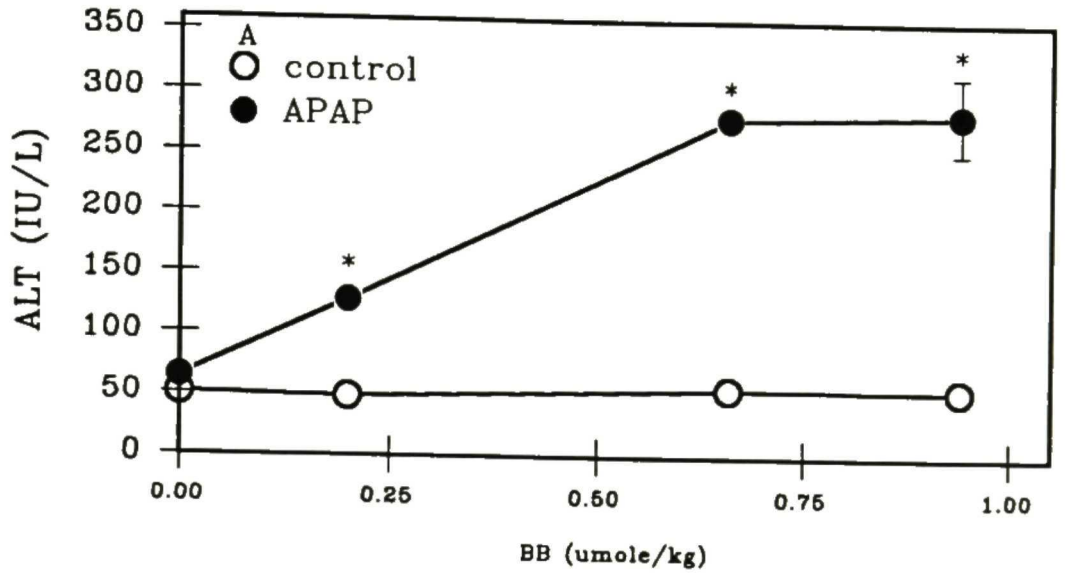
[a]Twenty-four hours after APAP-pretreatment (1.6 g/kg), rats were challenged with DCE (0 or 2.5 μmole/kg). Rats were observed for four hours after challenge and the incidence of lethality recorded.

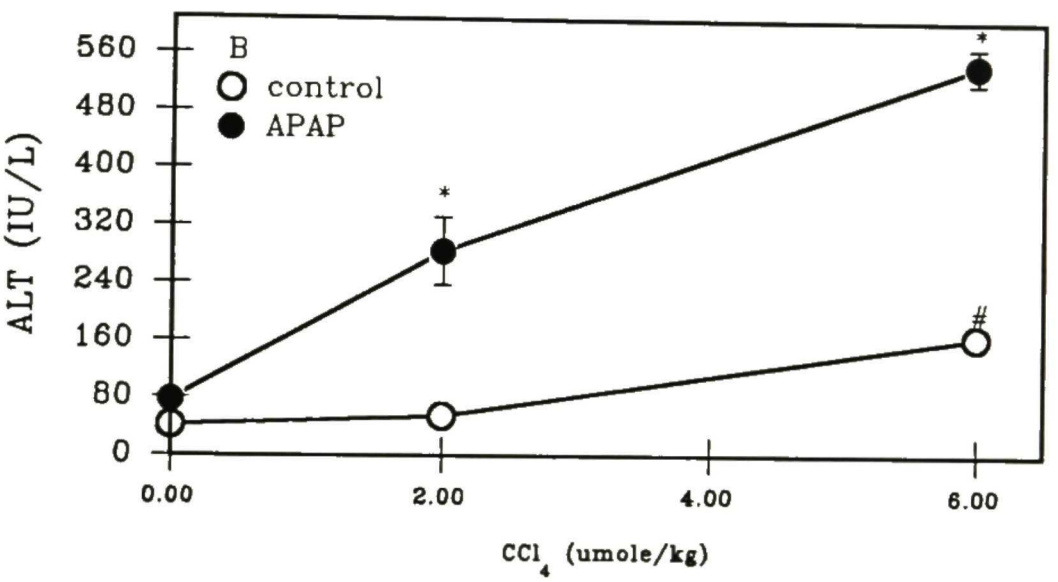
advanced stages of cardiovascular shock within four hours after challenge hepatotoxicant administration. The ears, paws, and tail of the rats were pale. The affected rats were cold to the touch and bled sparingly when killed for tissue and blood collection. In the case of DCE, congestion of the liver in APAP-pretreated rats is probably responsible for the hypovolemia. The APAP-pretreated rats challenged with AA had minimally congested livers (Figure 11-f) and a bloody ascites. The formation of the bloody ascites is probably responsible for the hypovolemic shock induced by AA.

APAP-Induced Potentiation of BB, CCl_4, and TA Hepatotoxicity

Rats were pretreated with APAP and challenged with APAP, BB, CCl_4, or TA to determine the scope of this phenomenon. APAP-pretreated rats challenged with BB and CCl_4, at doses that normally do not produce necrosis, had increased serum ALT activity twenty-four hours after challenge (Figure 15-A,B). There was no significant difference between control and APAP-pretreated rats when challenged with non-necrogenic doses of TA but doses that do produce necrosis were exacerbated (Figure 15-C). In the case of APAP, there was no increase in serum ALT activity after a second dose of APAP (1.6 g/kg)(Table 6). It is concluded from this data that APAP-induced potentiation of hepatotoxicity pertains to a wide range of toxicants. However, APAP-pretreatment did not sensitize the rat to

Figure 15: Serum ALT Activity in APAP-Pretreated Rats after Challenge with BB, CCl_4, or TA. Rats were pretreated with APAP (1.6 g/kg), twenty-four hours after challenge the animals were sacrificed and serum samples collected for ALT determinations. A) BB $n \geq 5$, B) CCl_4 $n \geq 9$, and C) TA $n \geq 4$. Rats pretreated with APAP and challenged with BB (0.94 μmole/kg) had serum ALT activity 514% of control-pretreated rats. Rats pretreated with APAP and challenged with CCl_4 (6.2 μmole/kg) had serum ALT activity 474% of control-pretreated rats. Rats pretreated with APAP and challenged with TA (0.13 mmol/kg) had serum ALT activity 136% of control-pretreated rats. Data expressed as mean ± SE. * $p \leq 0.05$ as compared to control-pretreated group. # $p \leq 0.05$ as compared to control-challenged group.


A
control
APAP
ALT (IU/L)
350
300
250
200
150
100
50
0
0.00
0.25
0.50
0.75
1.00
BB (umole/kg)
*


B
control
APAP
ALT (IU/L)
560
480
400
320
240
160
80
0
0.00
2.00
4.00
6.00
CCl4 (umole/kg)
*
#

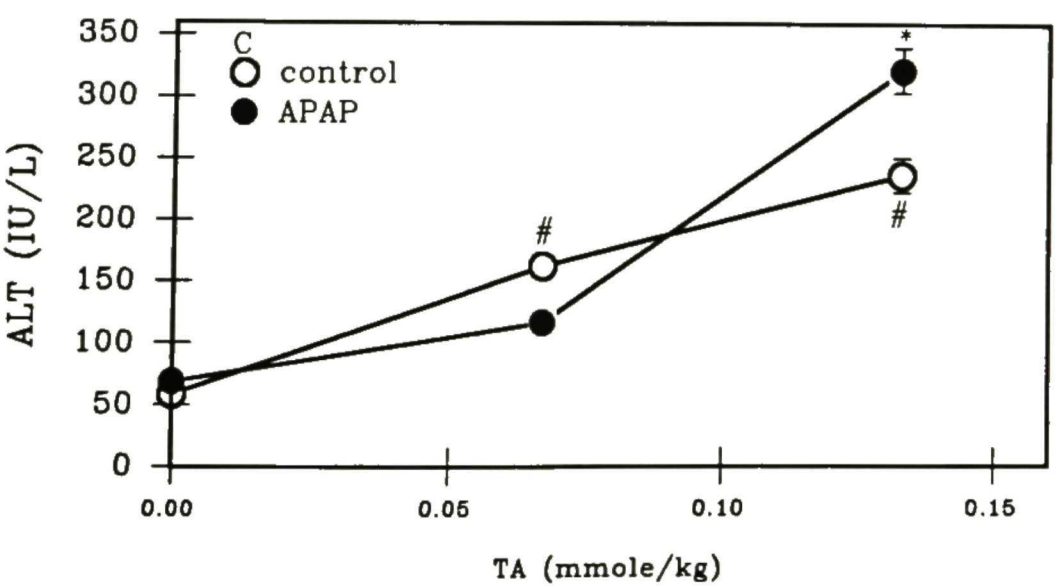

C
control
APAP
ALT (IU/L)
350
300
250
200
150
100
50
0
0.00
0.05
0.10
0.15
TA (mmole/kg)
*
#

TABLE 6

SERUM ALT LEVELS IN APAP-PRETREATED RATS CHALLENGED WITH ACETAMINOPHEN

	ALT (IU/L)
Control-Pretreated	
Control	50.5±1.6
Acetaminophen	53.8±2.7
APAP-Pretreated	
Control	64.8±3.6
Acetaminophen	80.2±10.9

[a]Rats were pretreated with APAP and challenged twenty-four hours later with APAP (1.6 g/kg) i.p. Twenty-four hours later animals were sacrificed and serum samples collected for ALT determinations. n = 4.

subsequent APAP challenge.

Mechanism of APAP-Induced GSH Depletion

To begin to determine the reason or reasons APAP potentiates hepatotoxicity, some of the toxic but non-necrogenic effects of APAP in rats were investigated. When the initial experiments with APAP and DCE (Figure 10) were performed, glutathione (GSH) levels were determined in the liver. It was found that GSH levels were not only depressed in DCE challenged rats, as expected, but also in APAP-pretreated controls (Table 7).

To explain this GSH depletion twenty eight hours after APAP, it was administered to rats; stomach weights (Figure 16) and GSH levels (Figure 17) were monitored for time points up to 24 hours. Stomach weights of APAP treated rats did not decrease during the daytime hours as did the controls (Figure 16). This did not appear to be due to increased food consumption because the rats given APAP (1.2 or 1.6 g/kg) were observed to be lethargic, unresponsive to external stimuli, and did not eat during daytime hours. Twenty-four hours after APAP-pretreatment, rats have lost weight (Table 8). This confirms the observation that the rats did not eat or absorb nutrients. APAP inhibited emptying of gastric contents.

GSH levels in APAP-pretreated rats are significantly decreased two hours after APAP administration and are only 65% of control twenty four hours after APAP administration

TABLE 7

GLUTATHIONE IN APAP-PRETREATED RATS CHALLENGED WITH DCE[a]

		GSH (mg/g liver)
Control-Pretreatment		
Control	2 hrs.	2.18±0.13
	4 hrs.	1.92±0.09
DCE	2 hrs.	1.22±0.09#
	4 hrs.	0.40±0.13#
APAP-Pretreatment		
Control	2 hrs.	1.24±0.10*
	4 hrs.	1.23±0.16*
DCE	2 hrs.	1.23±0.19
	4 hrs.	0.11±0.10#

[a]Rats were pretreated with APAP (1.2 g/kg) and challenged twenty-four hours after pretreatment with DCE (2.5 μmole/kg). Two or four hours after DCE administration, rats were sacrificed and liver samples were taken for glutathione determinations. Data expressed as mean ± SE. $n \geq 6$. * $p \leq 0.05$ as compared to control-pretreated group at same time point. # $p \leq 0.05$ as compared to control within the same pretreatment group at same time point.

Figure 16: Twenty-four Hour Time Course of Changes of Stomach Weight in APAP-Pretreated Rats. At time zero rats were pretreated with APAP at 0 (control), 1.2 g/kg, or 1.6 g/kg. At the specified time points animals were sacrificed, stomachs excised and trimmed of fat, and weighed. Data normalized to body weight and expressed as mean ± SE. $n \geq 6$. * $p \leq 0.05$ as compared to control-pretreated group at same time point.

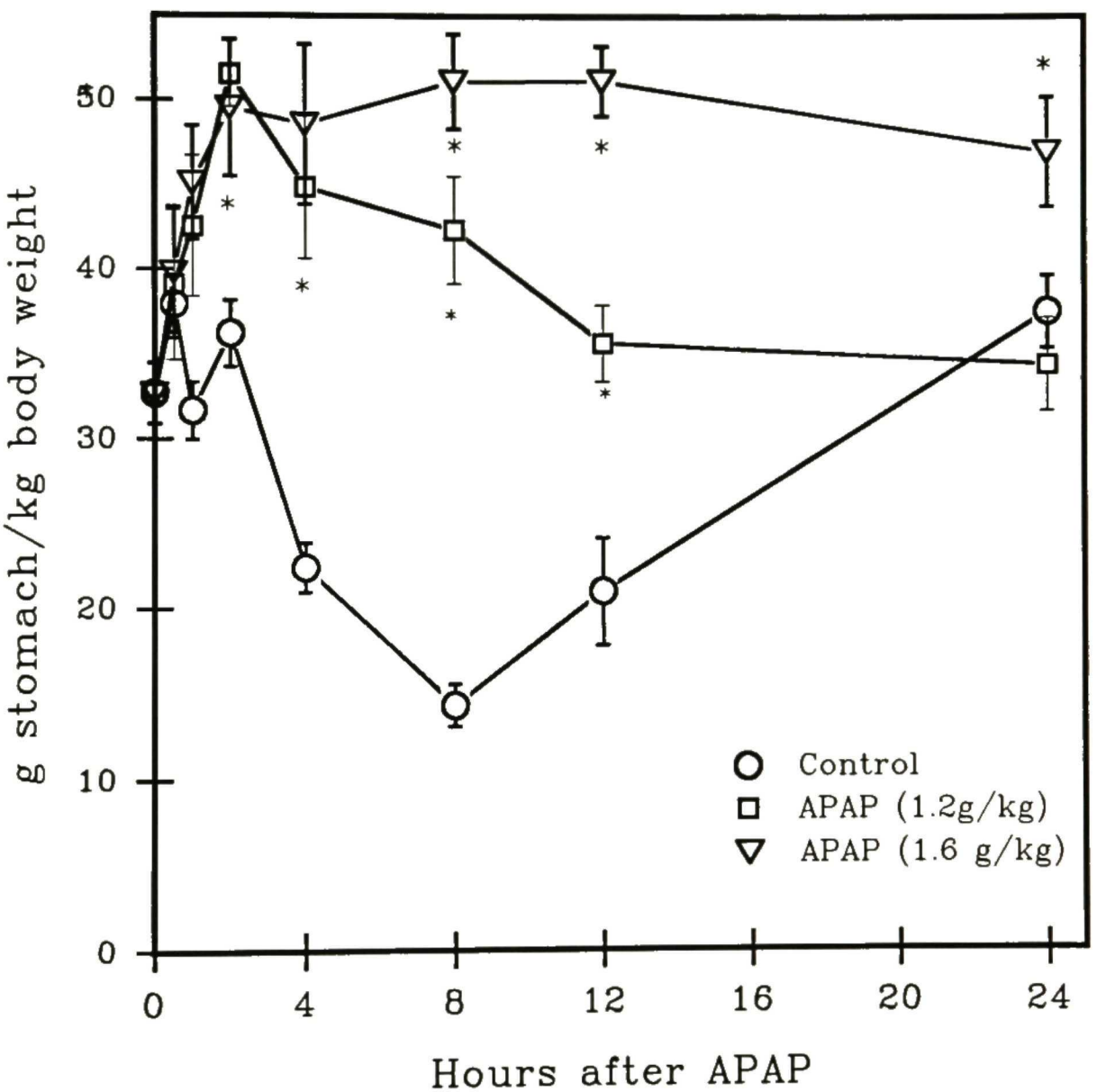

g stomach/kg body weight
0
10
20
30
40
50
0
4
8
12
16
20
24
Hours after APAP
Control
APAP (1.2g/kg)
APAP (1.6 g/kg)

Figure 17: Twenty-four Hour Time Course of Hepatic Glutathione Changes in APAP-Pretreated Rats. At time zero rats were pretreated with APAP at 0 (control), 1.2 g/kg, or 1.6 g/kg. At the specified time points animals were sacrificed, livers excised and trimmed of fat, and assayed for glutathione. Data expressed as mean ± SE. $n \geq 6$. * $p \leq 0.05$ as compared to control-pretreated group at same time point.

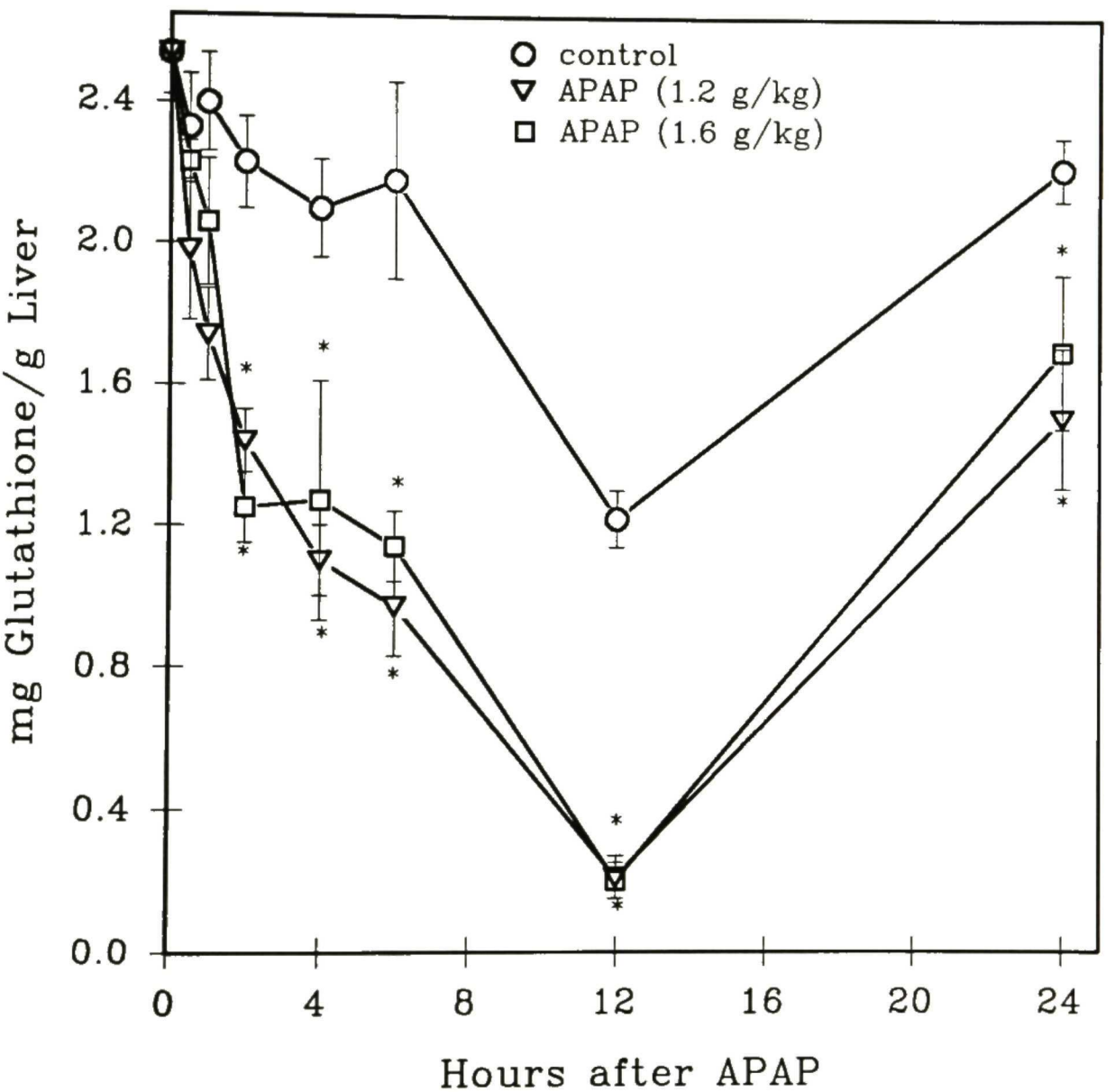

control
APAP (1.2 g/kg)
APAP (1.6 g/kg)
mg Glutathione/g Liver
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0
4
8
12
16
20
24
Hours after APAP

TABLE 7

BODY WEIGHTS OF RATS GIVEN APAP BEFORE AND 24 HOURS AFTER ADMINISTRATION[a]

	Before	After
Control	250±4g	256±4g
APAP (1.2 g/kg)	245±4g	234±4g*
APAP (1.6 g/kg)	242±3g	235±3g*

[a]Rats were weighed before and twenty-four hours after APAP-pretreatment (1.2 or 1.6 g/kg). $n \geq 10$. * $p \leq 0.05$ as compared to control-pretreated group at same time point.

(Figure 17). Depressed glutathione levels may be present for two reasons; decreased availability of necessary precursors for synthesis and/or presence of APAP in the blood. Since rats pretreated with APAP are not absorbing nutrients subsequent to decreased presentation of nutrients, as indicated by a constant stomach weight during daytime hours (Figure 16), decreased availability of precursors of synthesis of glutathione seems likely. APAP blood levels were monitored for 24 hours after administration. Twenty-four hours after dosing, low levels of APAP were present in the serum (Figure 18). Therefore depressed glutathione levels twenty-eight hours after APAP administration, are most likely due to a combination of decreased availability of precursors for synthesis and the presence of unmetabolized APAP in blood.

Effect of DEM-Induced GSH Depletion on Hepatotoxicants

To examine the effect of decreased GSH levels on the hepatotoxicity of AA, BB, CCl_4, DCE, and TA, diethylmaleate (DEM) was used. DEM alkylates GSH molecules and decreases overall levels without deleterious effects on cells (Plummer *et al.*, 1981). Rats were treated with DEM (0.6 ml/kg) to deplete GSH, sacrificed 45 minutes later, and GSH levels determined (Table 9). DEM depleted hepatic GSH to 35% of control. After DEM administration, rats were administered AA, BB, CCl_4, DCE, or TA. DEM-pretreatment had no effect on BB, CCl_4, or TA induced hepatotoxicity (Figure 19). DEM-

Figure 18: Twenty-four Hour Time Course of Serum APAP Levels. At time zero rats were pretreated with APAP at 0 (control), 1.2 g/kg, or 1.6 g/kg. At the specified time points animals were sacrificed and blood samples collected for APAP determinations. Data expressed as mean ± SE. $n \geq 6$.

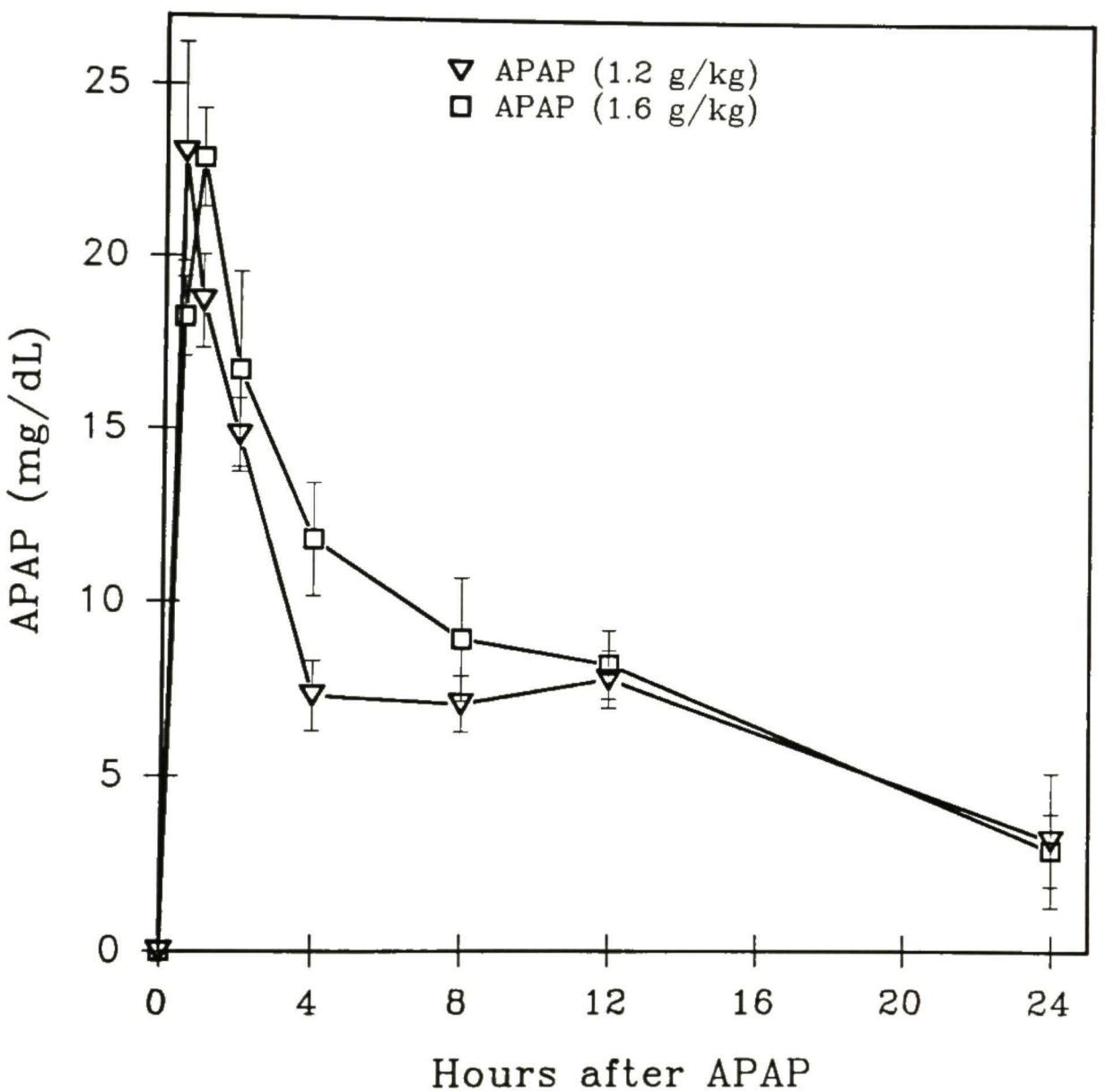

APAP (1.2 g/kg)
APAP (1.6 g/kg)
APAP (mg/dL)
25
20
15
10
5
0
0
4
8
12
16
20
24
Hours after APAP

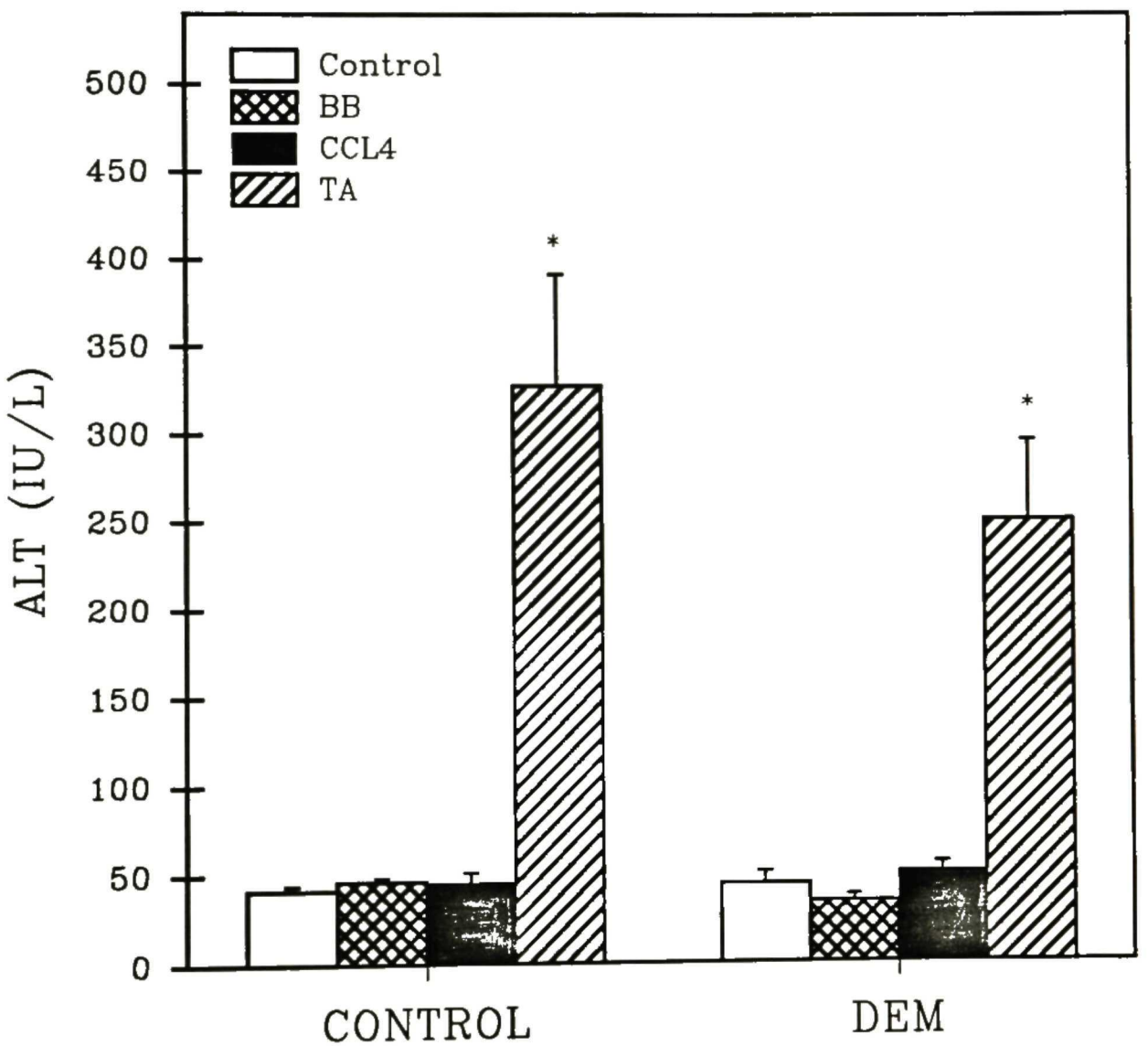
TABLE 9

HEPATIC GLUTATHIONE LEVELS IN RATS AFTER DEM

Control	1.64±0.22
DEM	0.60±0.19*

[a]Rats were treated either with the sesame oil or DEM (0.6 ml/kg). Forty-five minutes after treatment, animals were sacrificed by decapitation and liver samples taken for glutathione determination. Data points expressed as mean ± SE and n = 6.

Figure 19: Serum ALT Activity in DEM-Pretreated Rats Challenged with BB, CCl_4, or TA. Rats were pretreated with DEM (0.6 ml/kg) and challenged 45 minutes later with BB (0.94 μmole/kg), CCl_4 (2 μmole/kg), or TA (1.3 mmole/kg). Animals were sacrificed by decapitation 24 hours after challenge and serum sample collected for ALT determinations. Data points expressed as mean ± SE and $n \geq 4$. * $p \leq 0.05$ as compared to control within respective pretreatment group.


Control
BB
CCL4
TA
ALT (IU/L)
500
450
400
350
300
250
200
150
100
50
0
*
*
CONTROL
DEM

pretreatment did potentiate AA and DCE hepatotoxicity (Figure 20). Serum ALT activity was 1850% and 1740% of control in APAP-pretreated rats (Figure 10, 14) challenged with AA and DCE as compared to 654% and 720% of control in DEM-pretreated rats challenged with AA and DCE (Figure 20). DEM did not potentiate the lethality of AA or DCE. Since DEM depleted GSH to a greater extent than did APAP, it was concluded that APAP-induced GSH depletion cannot be the sole cause of the potentiation of hepatotoxicity.

Effect of RES Suppression on APAP-Induced Potentiation

To determine if activated NPCs are a component of APAP-induced potentiation of hepatotoxicity, the effects of two agents known to inhibit RES activity were used, DS and $GdCl_3$. If normal RES function of the liver is essential for APAP to potentiate hepatotoxicity, then administration of these agents should alleviate APAP-induced potentiation of hepatotoxicity.

Using the clearance of latex beads from the serum, it was documented that DS and $GdCl_3$ decreased RES function in this experimental system (Figure 21). DS and $GdCl_3$ were used to disrupt RES function one hour prior to APAP administration. AA was the challenge hepatotoxicant of choice in these experiments because of the interaction between the immune system and cytochrome P450 system (Peterson and Renton, 1984, 1986A,B). AA causes periportal necrosis and is metabolically activated by alcohol

Figure 20: Serum ALT Activity in DEM-Pretreated Rats Challenged with AA or DCE. Rats were pretreated with DEM (0.6 ml/kg) and challenged 45 minutes later with AA (0.4 μmole/kg) or DCE (2.5 μmole/kg). Animals were sacrificed four hours after challenge and serum samples collected for ALT determinations. DEM-pretreated rats challenged with AA had serum ALT activity 654% of control-pretreated rats. DEM-pretreated rats challenged with DCE had serum ALT activity 720% of control-pretreated rats. Data expressed as mean ± SE. $n \geq 4$. * $p < 0.05$ as compared to control within respective pretreatment group.

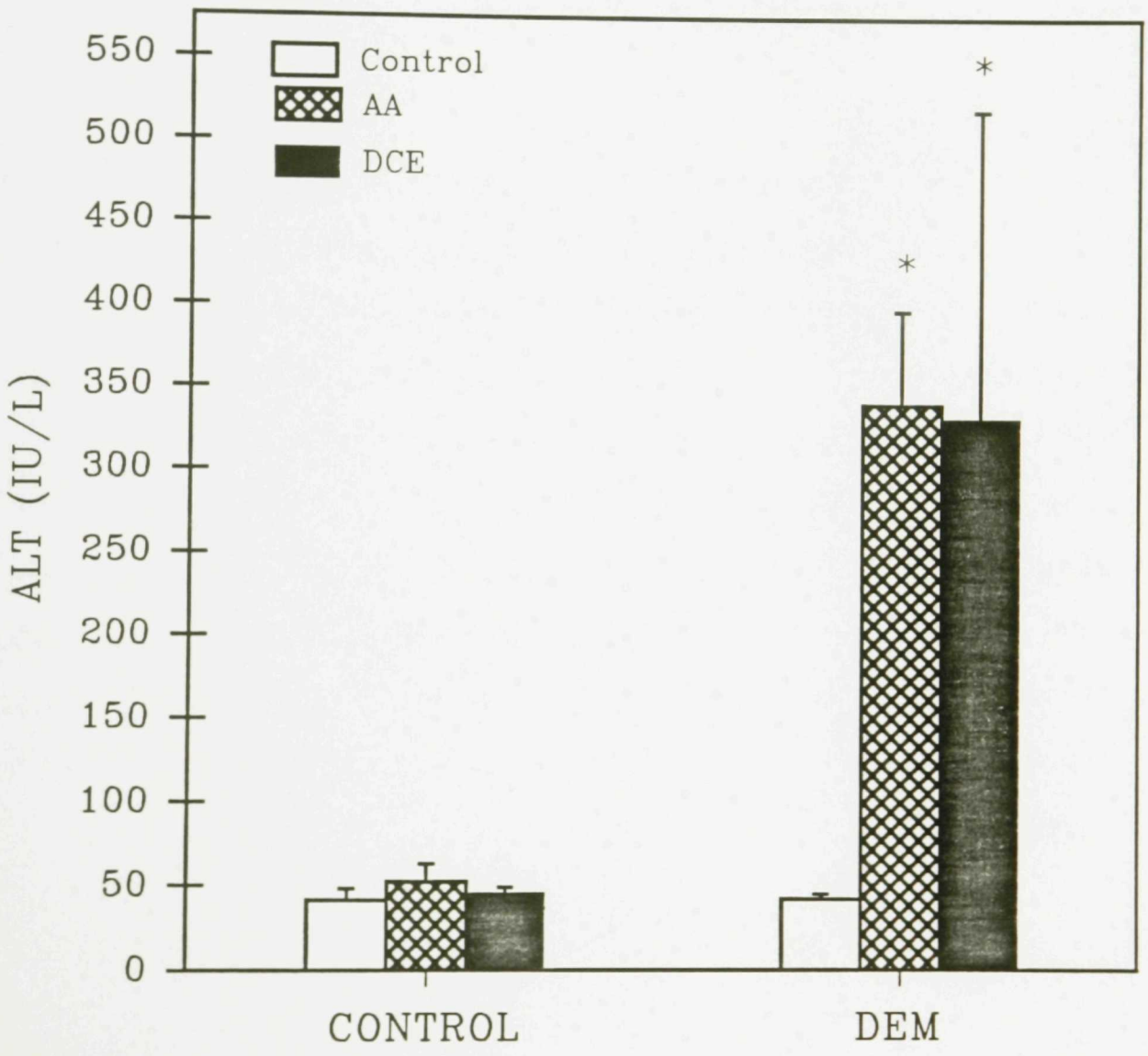
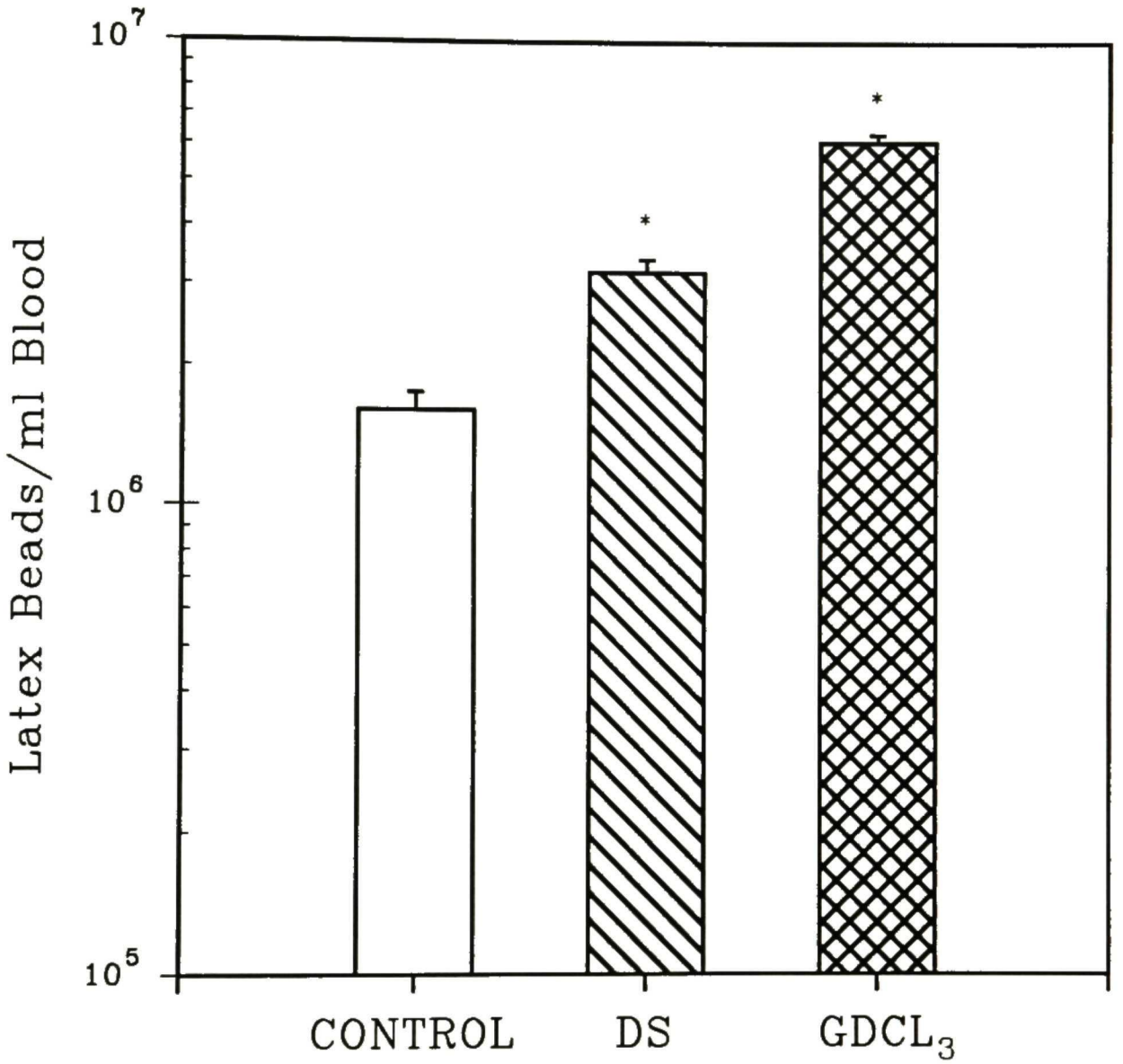

Control
AA
DCE
ALT (IU/L)
550
500
450
400
350
300
250
200
150
100
50
0
*
*
CONTROL
DEM

Figure 21: Clearance of Latex Beads in Rats Treated with DS or $GdCl_3$. Rats were pretreated intravenously with DS (15 mg/kg), $GdCl_3$ (5mg/kg), or 0.9% saline vehicle. Twenty-four hours later rats were injected intravenously with 2 x 10^9 1 μm latex beads in 0.9% saline. Forty minutes later, rats were anesthetized with pentobarbital (78 mg/kg). Five minutes later, blood was withdrawn from the inferior vena cava. Red blood cells were lysed with 0.1% Na_2CO_3 and beads in blood outside cells counted using a hemacytometer. Data expressed as mean ± SE. n = 3. * $p < 0.05$ as compared to control.


10^7
10^6
10^5
Latex Beads/ml Blood
*
*
CONTROL
DS
$GDCL_3$

dehydrogenase not the cytochrome P450 system (Reid, 1972). In rats pretreated with DS and APAP, the hepatotoxicity of AA was not potentiated (Figure 22). When $GdCl_3$ was used to disrupt Kupffer cell function in APAP-pretreated rats, the potentiated increase in serum ALT activity produced by AA challenge was decreased (Figure 23). It should be noted that the extent to which APAP potentiated AA in these experiments is lower than in the initial experiments. These animals were anesthetized and injected with saline to serve as controls. It was speculated that this stress may have induced immunosuppression. This would agree with the hypothesis that activated NPCs are involved in APAP-induced potentiation.

The hepatotoxicity of AA is dependent upon generation of the reactive metabolite acrolein by alcohol dehydrogenase (ADH)(Reid, 1972). The effect of DS and $GdCl_3$ on the activity of ADH was examined to exclude an interaction between the RES suppressants and AA metabolism. DS and $GdCl_3$ did not have a significant effect on ADH activity (Figure 24). DS and $GdCl_3$ alleviate potentiation of AA hepatotoxicity in APAP-pretreated rats, without grossly affecting metabolism.

DS and $GdCl_3$ were examined for their effects on APAP-induced changes in body weight and of GSH depletion. APAP-pretreatment decreased body weight of animals twenty-four hours after administration (Table 8). DS and $GdCl_3$ administration did not alter the APAP-induced decrease in

Figure 22: Serum ALT Activity in DS+APAP-Pretreated Rats. Rats were anesthetized with methoxyflurane and injected intravenously with either a saline vehicle (Control Pretreated) or DS (15 mg/kg)(DS Pretreated). One hour later, animals were pretreated with APAP (1.6 g/kg). Twenty-four hours after APAP dosage, rats were challenged with AA (0.4 μmole/kg). Four hours after AA, rats were sacrificed and serum samples harvested for ALT determinations. Data expressed as mean ± SE. $n \geq 4$. * $p < 0.05$ as compared to DS-pretreated group.

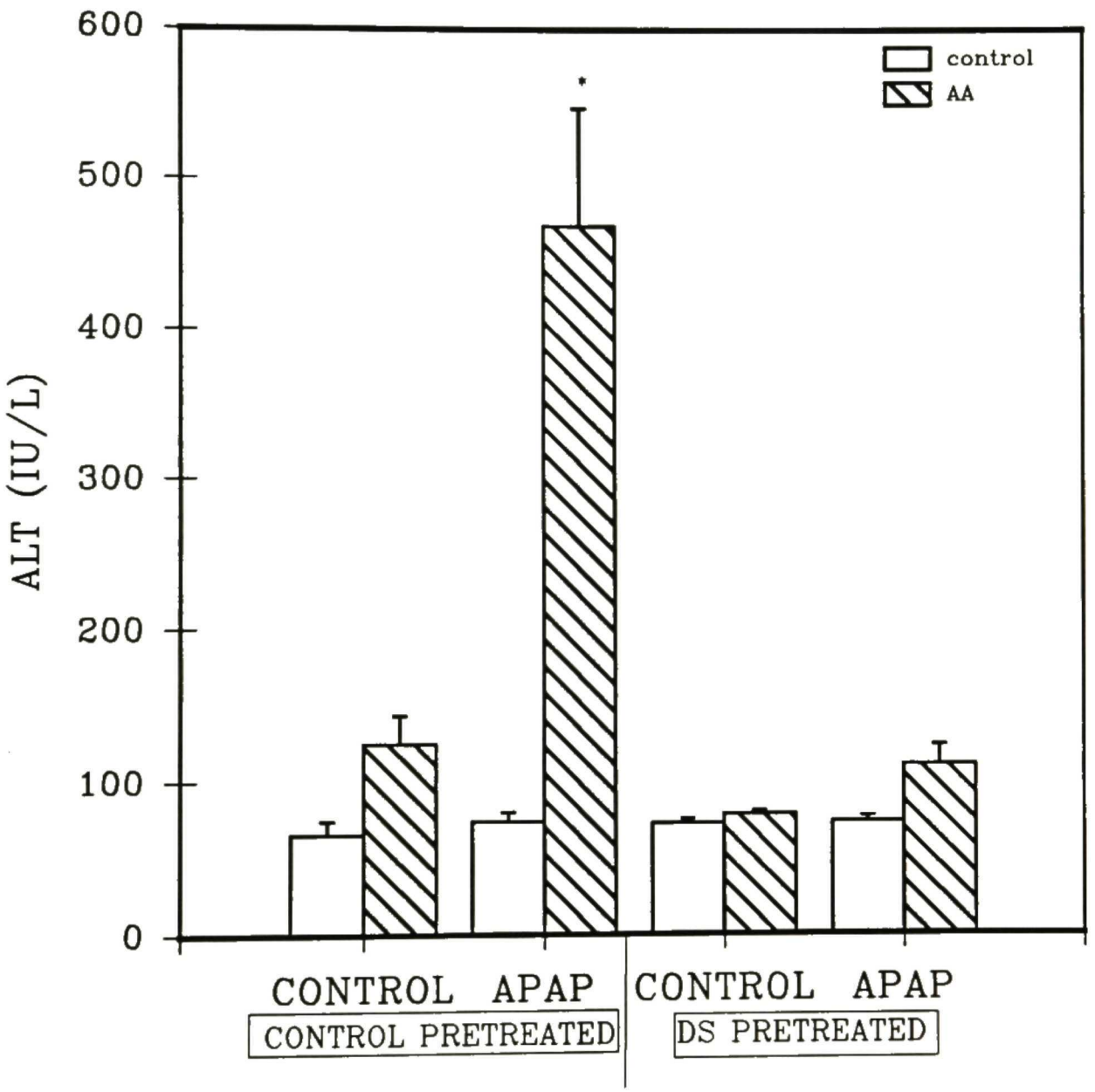

600
500
400
300
200
100
0
ALT (IU/L)
control
AA
*
CONTROL APAP CONTROL APAP
CONTROL PRETREATED
DS PRETREATED

Figure 23: Serum ALT Activity in $GdCl_3$+APAP-Pretreated Rats Challenged with AA. Rats were anesthetized with methoxyflurane and injected intravenously with either saline vehicle (Control Pretreated) or $GdCl_3$ (5 mg/kg) ($GdCl_3$ Pretreated). One hour later, rats were pretreated with APAP (1.6 g/kg). Twenty-four hours after APAP-pretreatment, rats were challenged with AA (0.4 μmole/kg). Four hours after AA, rats were sacrificed and serum samples were harvested for ALT determinations. Data expressed as mean ± SE. $n \geq 4$. * $p \leq 0.05$, significantly different from control-pretreated, APAP-pretreated control, and control-pretreated AA group and # $p \leq 0.05$, significantly different from non-$GdCl_3$, APAP-pretreated AA group.

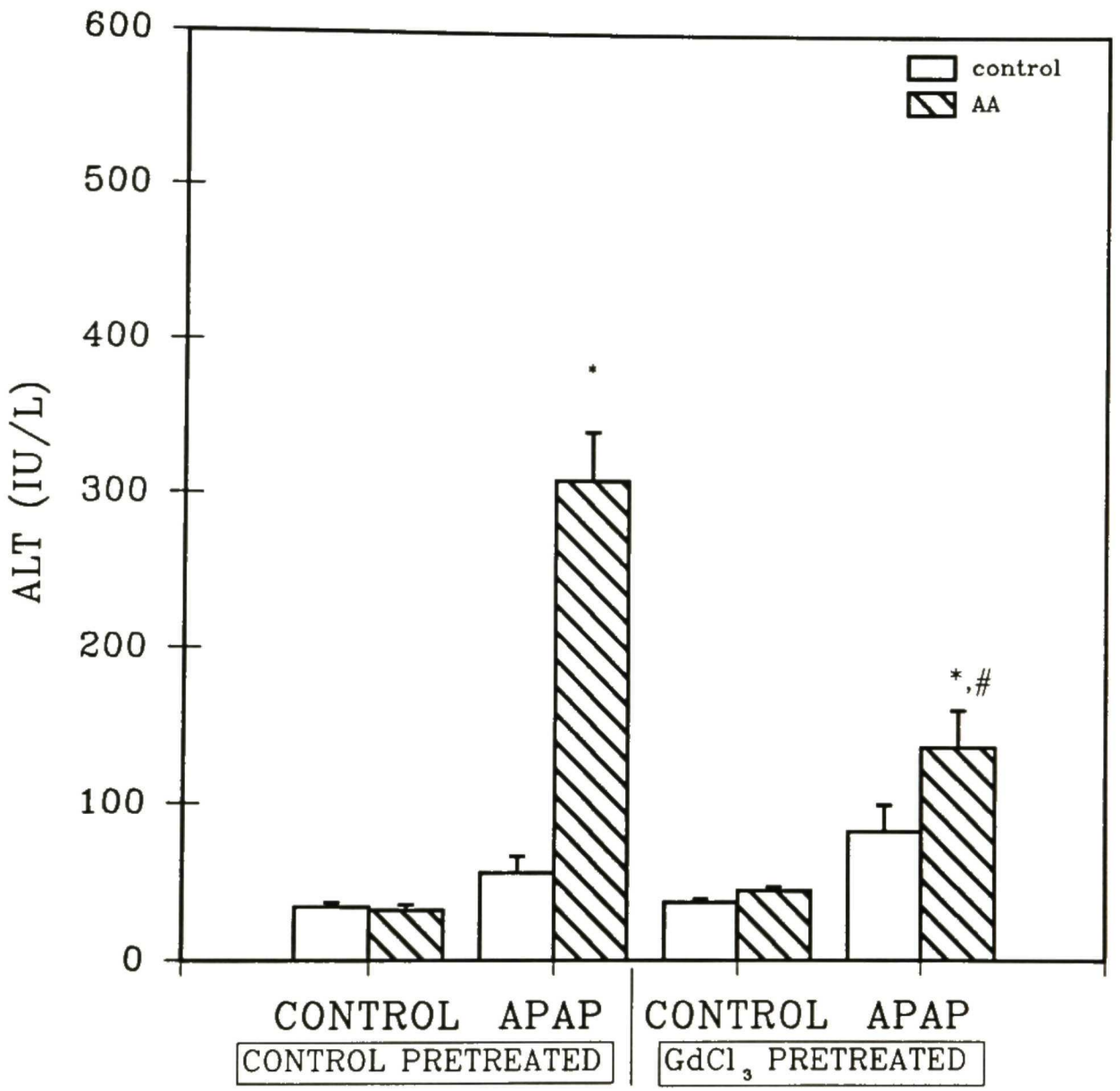
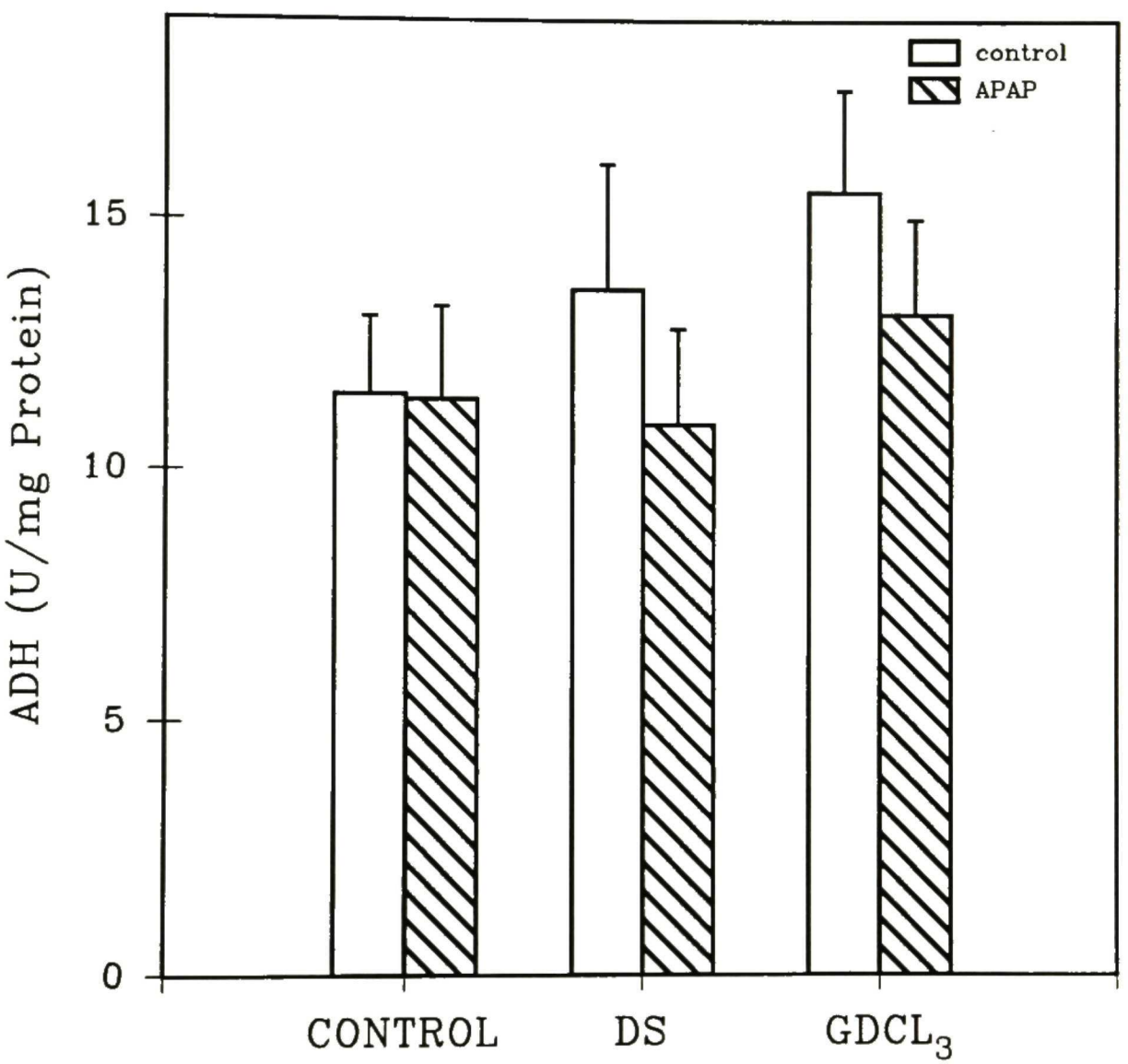

600
500
400
300
200
100
0
ALT (IU/L)
control
AA
*
*,#
CONTROL APAP
CONTROL PRETREATED
CONTROL APAP
GdCl3 PRETREATED

Figure 24: Hepatic Cytosolic ADH in APAP-Pretreated Rats Treated with RES Suppressants. Rats were anesthetized with methoxyflurane and injected intravenously with either saline vehicle (control), DS (15 mg/kg) or $GdCl_3$ (5 mg/kg). One hour later, animals were pretreated with APAP (1.6 g/kg). Twenty-four hours after APAP pretreatment, rats were sacrificed and cytosol was prepared by differential centrifugation. Data expressed as mean ± SE. $n \geq 5$.

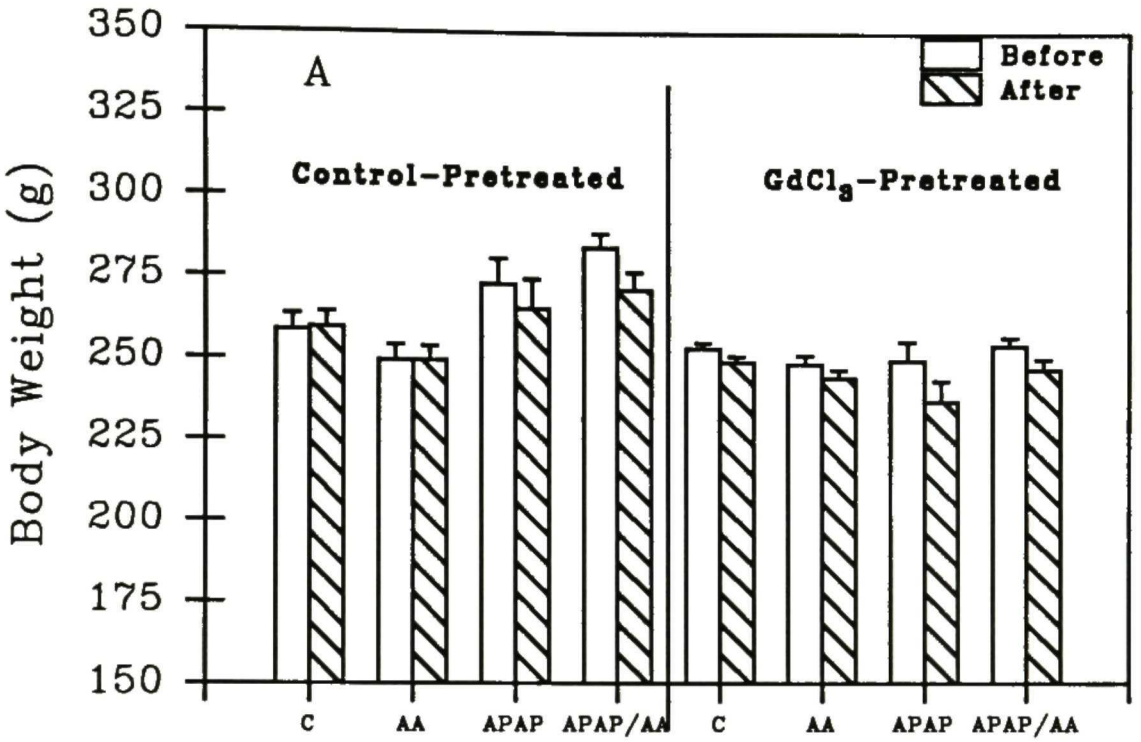

control
APAP
ADH (U/mg Protein)
15
10
5
0
CONTROL
DS
GDCL3

body weight 24 hours after APAP administration (Figure 25). DS had no effect on GSH time course profiles (Figure 26). $GdCl_3$ itself decreased GSH levels that were decreased even further upon APAP administration (Figure 26). The effect of APAP administration on GSH levels in both DS- and $GdCl_3$-pretreated rats indicates that DS and $GdCl_3$ did not effect generation of the reactive metabolite after APAP metabolism. These results indicate that intact RES function is necessary for the ability of APAP to potentiate the hepatotoxicity of AA.

Use of an *In Vitro* System to Study APAP-Induced Potentiation

A coculture system was set up to determine if activated NPCs directly affect hepatocyte viability upon toxicant challenge. This model consisted of isolating hepatic NPCs from control- or APAP-pretreated rats and culturing them for eighteen hours. Hepatocytes were isolated from a naive rat and cultured with the NPCs isolated the previous day. NPC yield from rats pretreated with APAP was 2.7 x greater than that from control rats (Figure 27). When these cells were stained for peroxidase, the populations from control and APAP-pretreated rats are 60% positive (Table 10), indicating a constant percentage of these cells are macrophages. Since there is an increase in yield of both macrophages and endothelial cells from these livers, it was hypothesized that both cell types could have a role in this phenomenon. After coculture of hepatocytes

Figure 25: Body Weight Changes in DS-APAP- and $GdCl_3$-APAP-Pretreated Rats. Rats were weighed before and twenty-four hours after A) DS (15 mg/kg) and 1.6 g/kg APAP-pretreatment or B) $GdCl_3$ (5 mg/kg) and 1.6 g/kg APAP-pretreatment. Data expressed as mean ± SE. n = 5.


A
Before
After
Control-Pretreated
GdCl3-Pretreated
Body Weight (g)
350
325
300
275
250
225
200
175
150
C
AA
APAP
APAP/AA
C
AA
APAP
APAP/AA

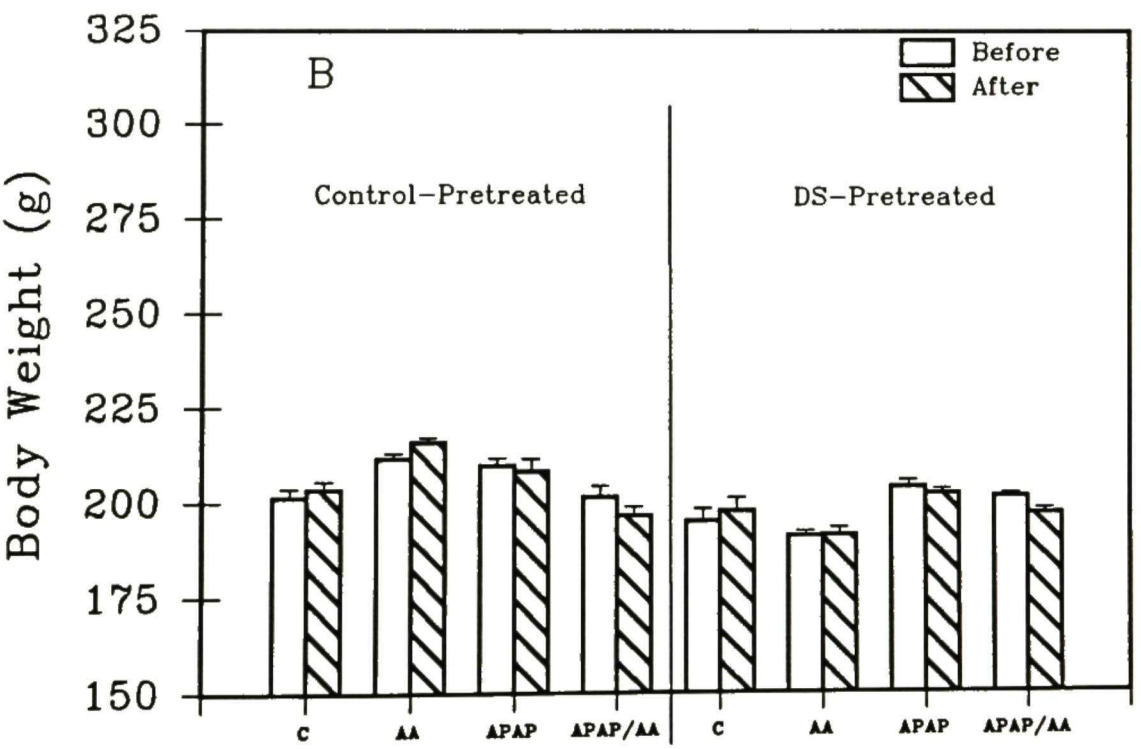

B
Before
After
Control-Pretreated
DS-Pretreated
Body Weight (g)
325
300
275
250
225
200
175
150
C
AA
APAP
APAP/AA
C
AA
APAP
APAP/AA

Figure 26: Twenty-four Hour Time Course of Hepatic Glutathione Levels in DS- and $GdCl_3$-Pretreated Rats Challenged with APAP. Rats were injected intravenously with either a saline vehicle (Control Pretreated), DS (15 mg/kg), or $GdCl_3$ (5 mg/kg). One hour later (time zero), animals were pretreated with APAP (1.6 g/kg). At the specified time points animals were sacrificed, livers specimens were excised, and assayed for glutathione. Data expressed as mean ± SE. $n \geq 6$. * $p \leq 0.05$ as compared to control-pretreated rats. # $p \leq 0.05$ as compared to control RES inhibited rats.

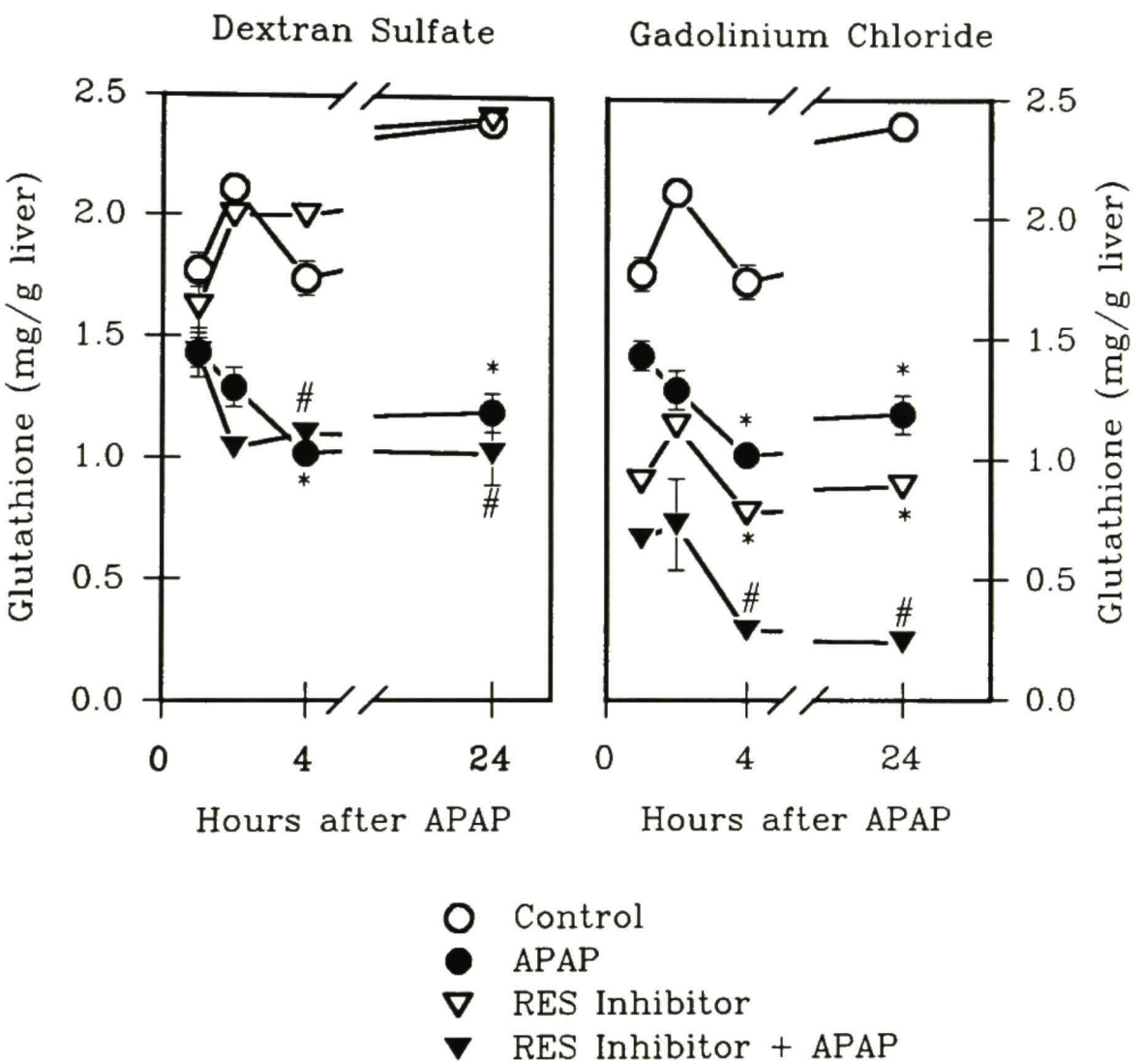

Dextran Sulfate
Gadolinium Chloride
Glutathione (mg/g liver)
2.5
2.0
1.5
1.0
0.5
0.0
0
4
24
Hours after APAP
Control
APAP
RES Inhibitor
RES Inhibitor + APAP

Figure 27: Hepatic NPC Yield from Control and APAP-Pretreated Rats. Hepatic macrophages and endothelial cells were isolated and counted. Data expressed as mean ± SE. n = 6. * $p \leq 0.05$.

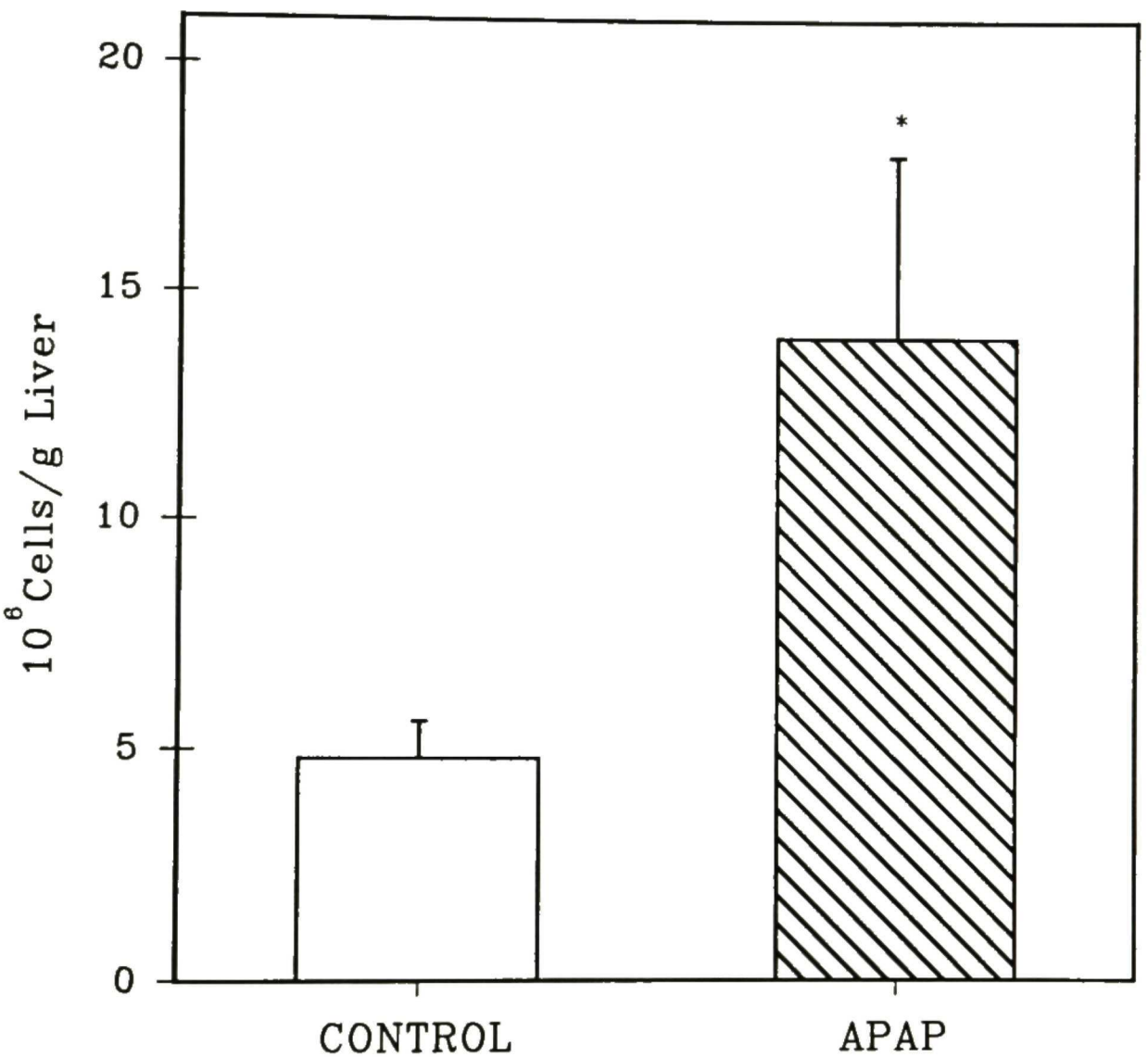

20
15
10
5
0
10^6 Cells/g Liver
*
CONTROL
APAP

TABLE 10

PEROXIDASE POSITIVE CELLS FROM CONTROL- OR APAP-PRETREATED RATS[a]

	% Total Cells Positive
Control	60.0±6.5
APAP (1.6 g/kg)	65.7±7.3

[a]Hepatic NPC cells were isolated and stained for nonspecific peroxidase. Data expressed as % of total cells staining. n=4.

with NPCs for two hours, response to toxicant challenge was determined.

AA is the toxicant of choice for these experiments. AA causes periportal necrosis and is metabolically activated by alcohol dehydrogenase not cytochrome P450 (Reid, 1972). Cytochrome P450 levels in hepatocytes are dependent upon inclusion of dexamethasone in the culture media (Guzelian *et al.*, 1979). Since macrophages are inhibited in the presence of dexamethasone and their effects on hepatocytes in coculture are inhibited by dexamethasone (Keller *et al.*, 1986), it was not included in the media.

Hepatocytes were cultured alone and the dose response curves for AA were generated (Figure 28) using leakage of the enzymes LDH, ALT and AST as the index of cytotoxicity. Since the percent of total enzyme released was the greatest with LDH and ALT, it was concluded that these enzymes are more sensitive indicators of hepatotoxicity in isolated hepatocytes.

NPCs have a high activity of LDH but very little AST or ALT (Figure 29). Therefore, in the coculture system, it was initially decided that ALT leakage from hepatocytes was to be assessed. Cytosolic LDH leakage into media was assessed in cultures of NPCs in response to 75 or 1000 nM AA to determine if AA is cytotoxic to these cells (Figure 30). It was determined that AA, at these doses, was innocuous to these cells. It should be noted that a difference between control- and APAP-NPCs was demonstrated by increased basal

Figure 28: Dose Response to AA in Isolated Hepatocytes. Hepatocytes were isolated from a naive rat. Cells were treated with AA two hours after plating. Two hours after treatment, cells were harvested for ALT, AST, and LDH determinations. Data expressed as mean ± SE. n = 4. * $p \leq 0.05$ as compared to AA (0 nM).

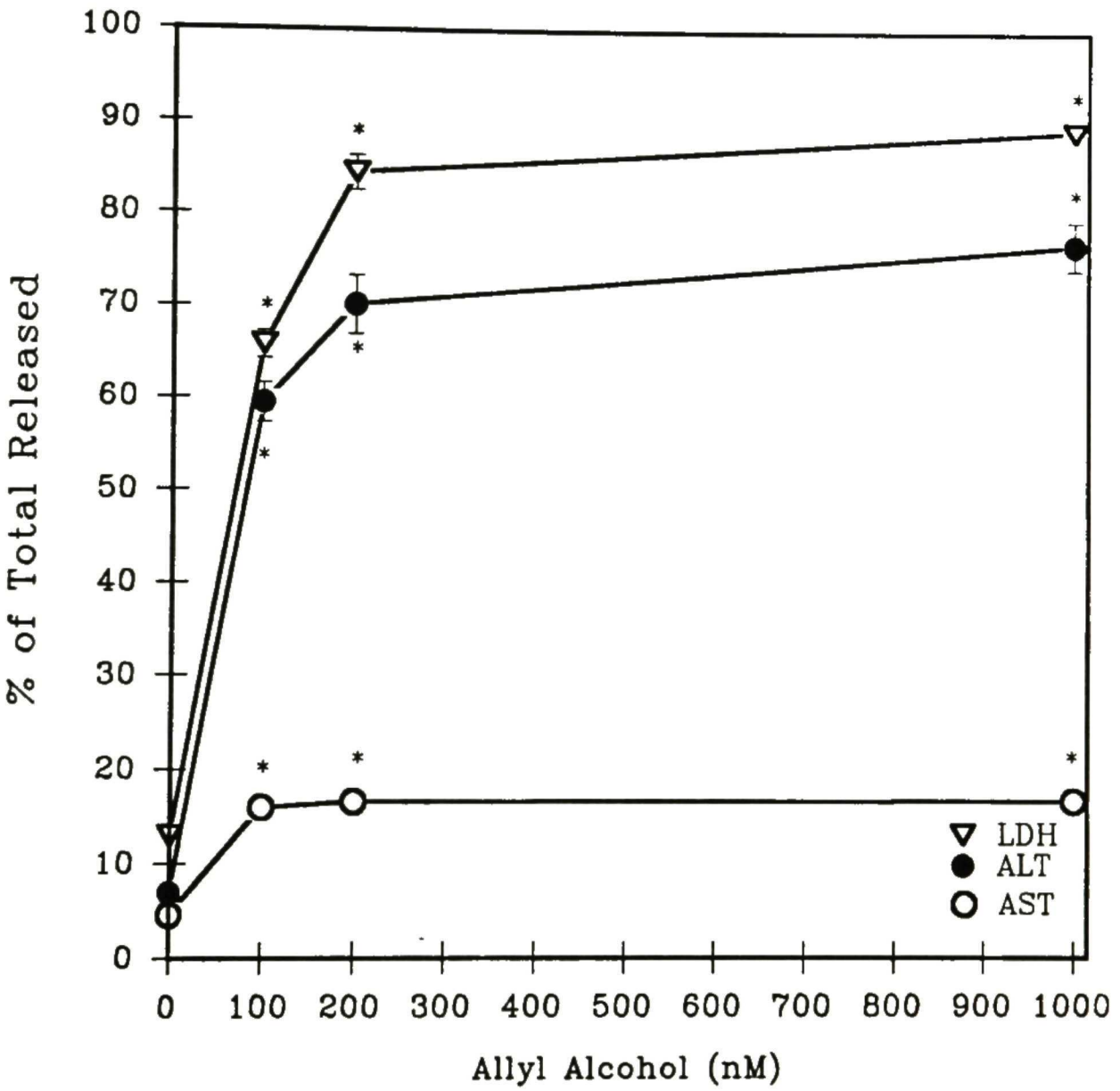

100
90
80
70
60
50
40
30
20
10
0
% of Total Released
0
100
200
300
400
500
600
700
800
900
1000
Allyl Alcohol (nM)
LDH
ALT
AST

Figure 29: Cytosolic Enzymes in NPCs. NPCs were isolated and cultured for eighteen hours. Cells were then scraped; sonicated; and ALT, AST, and LDH assayed in sonicate. Data expressed as mean ± SE. n = 4. * $p \leq 0.05$ as compared to control-NPCs.

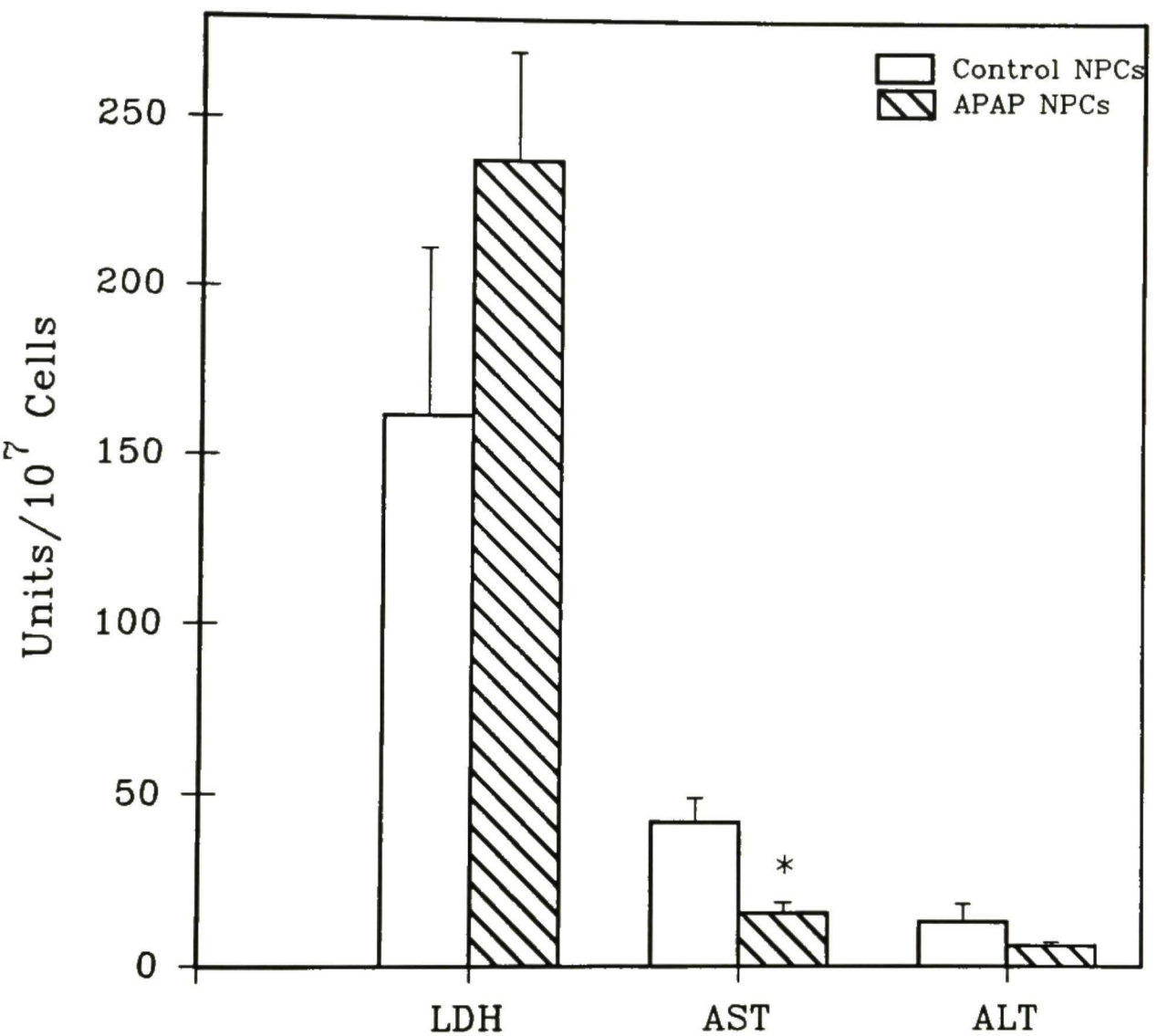
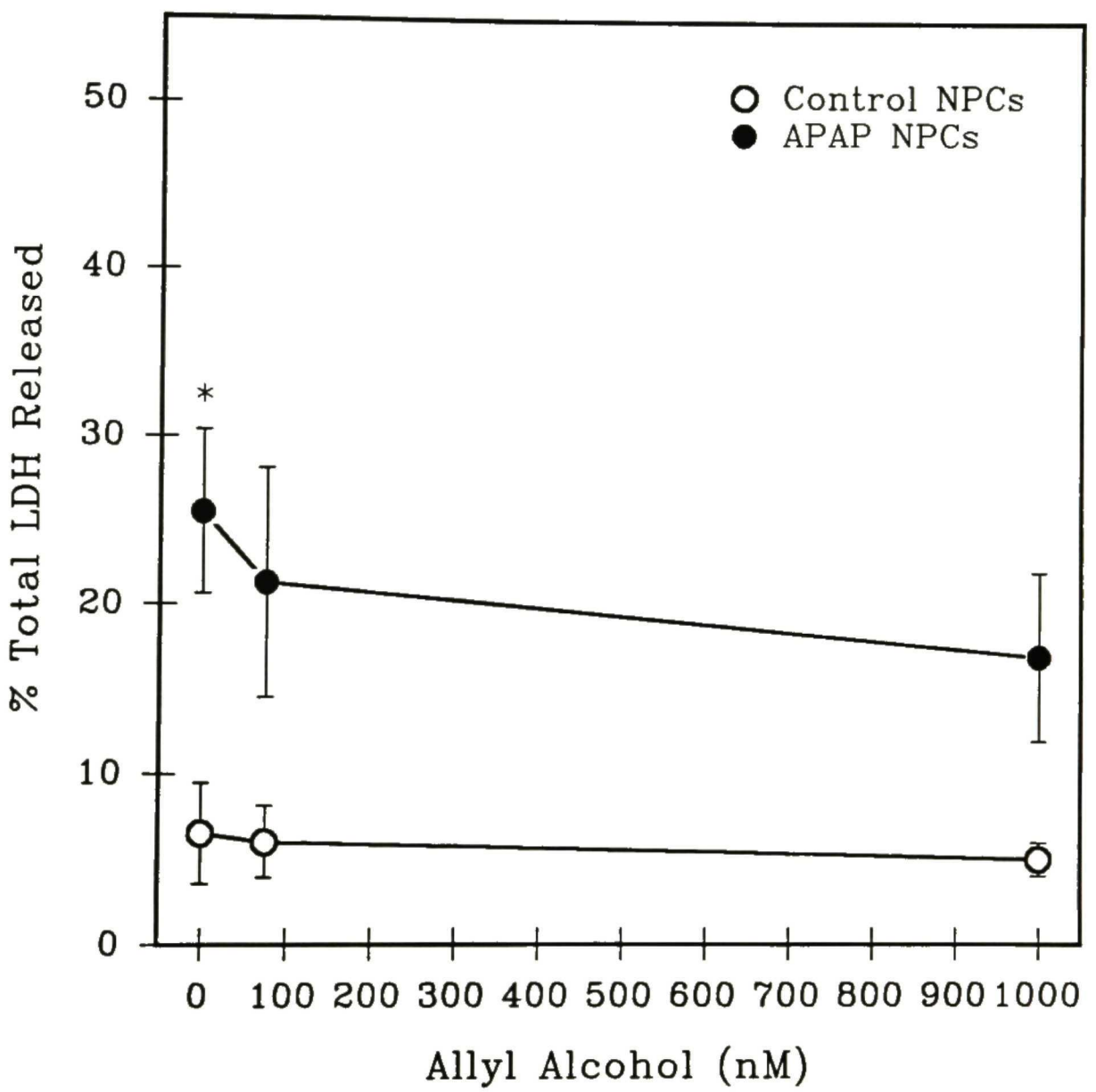

Control NPCs
APAP NPCs
Units/10^7 Cells
250
200
150
100
50
0
*
LDH
AST
ALT

Figure 30: Release of LDH from NPC in Response to AA. Cells were isolated and cultured for eighteen hours. Cells were then treated for two hours with AA. The media and sonicate were assayed for LDH activity. Data expressed as mean ± SE of percent of total activity released. Total LDH activity/10^6 cells in Control-NPC was 175 $\pm$ 55 and total activity in APAP-NPCs was 304 $\pm$ 45. n = 4. * $p \leq 0.05$ as compared to control NPCs at the same dose.

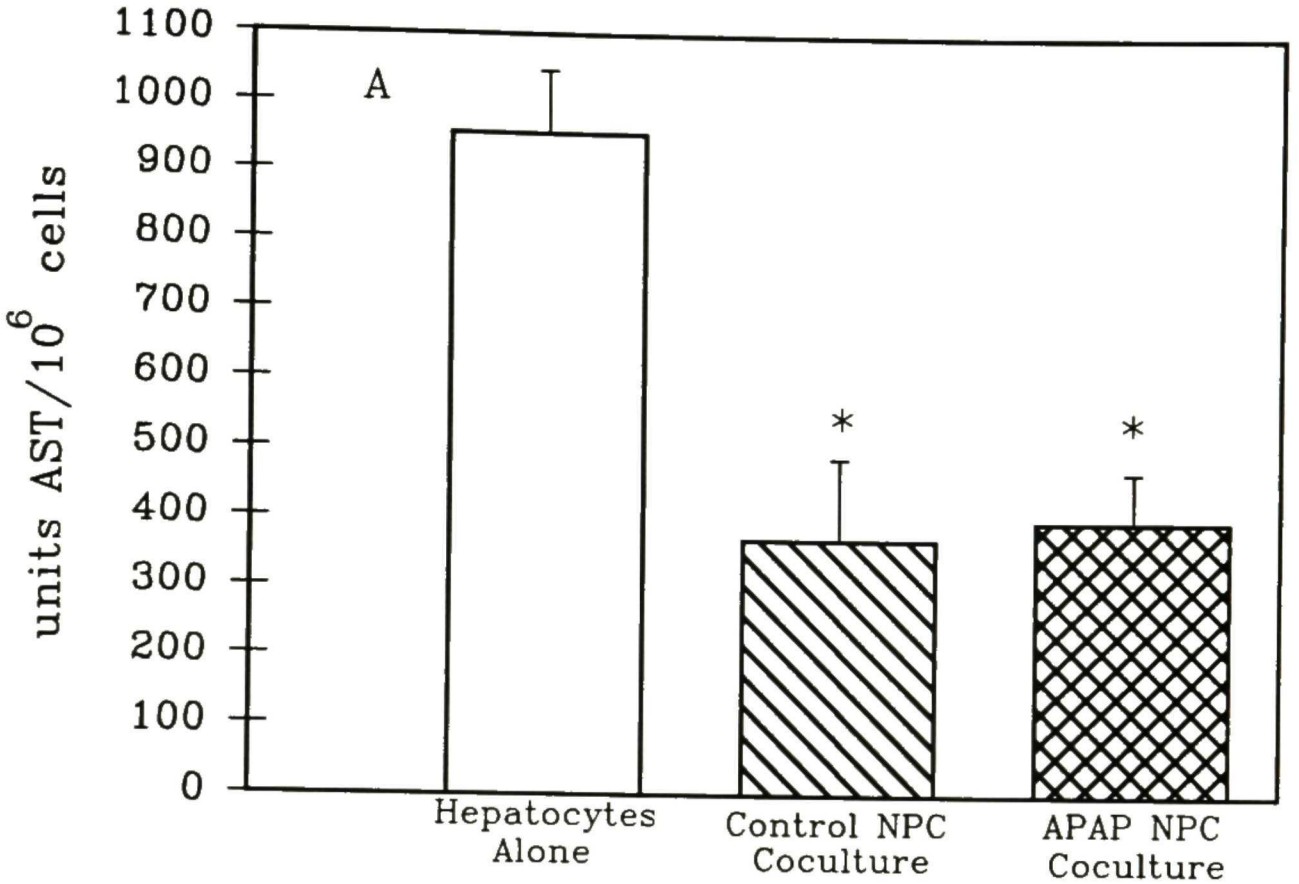

Control NPCs
APAP NPCs
% Total LDH Released
50
40
30
20
10
0
*
0
100
200
300
400
500
600
700
800
900
1000
Allyl Alcohol (nM)

levels of LDH release in APAP-NPCs (Figure 30).

NPCs and hepatocytes were cocultured ratios of 10:1, 5:1, or 3:1. In cultured hepatocytes there was some base line release of ALT and AST at AA (0 nM)(Figure 28). In hepatocytes cocultured with NPCs, base line ALT release or any ALT activity in media of cocultures in response to AA could be not detected. This phenomenon was also found in a study of cytotoxicity of Kupffer cells to hepatocytes in response to lipopolysaccharide (Billiar *et al.*, 1989B). Therefore, AST was the enzyme used to assess cytotoxicity. AST activity was significantly decreased in hepatocytes cocultured with NPCs as compared to those cultured alone (Figure 31). The doses of AA chosen to assess cytotoxicity did not elicit any AST release (Figure 32). In fact, doses of AA that released the majority of the ALT from hepatocytes cultured alone (1000 nM), did not injure hepatocytes cocultured with NPCs. The effect of coculture of hepatocytes with either control-NPCs or APAP-NPCs, on cytosolic enzyme activity and cytotoxicity, demonstrated the intricate interactions between NPCs and hepatocytes.

To determine if the effect of coculture on cytotoxicity in hepatocytes was due to the NPCs or mediators secreted by the NPCs, cytotoxicity of AA was determined in hepatocytes cultured in NPC-conditioned media (NPC-CM). Hepatocytes cultured in media conditioned by NPCs from Control- or APAP-pretreated rats acquired characteristics of both the *in vivo* and coculture models. In these experiments

Figure 31: Total AST and ALT Activity in Hepatocytes in NPC:Hepatocyte cocultures at a 10:1 Ratio. NPCs were isolated and cultured for eighteen hours. Hepatocytes were isolated and cocultured with the NPCs for four hours. A) total AST activity/ 10^6 cells and B) total ALT activity/ 10^6 cells. Data expressed as mean ± SE. n=4. * $p \leq 0.05$ as compared to hepatocytes alone.


A
units AST/10^6 cells
1100
1000
900
800
700
600
500
400
300
200
100
0
*
*
Hepatocytes Alone
Control NPC Coculture
APAP NPC Coculture

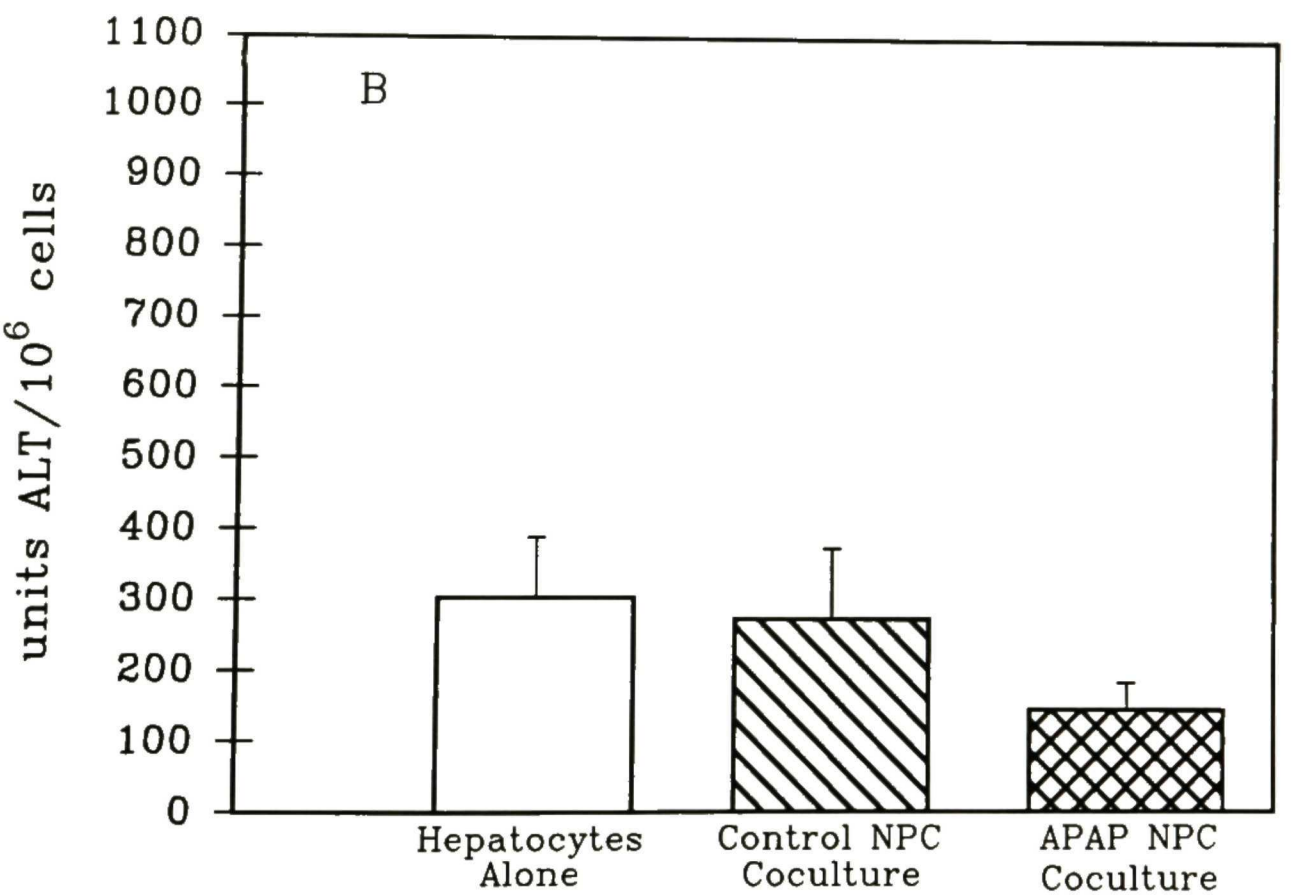
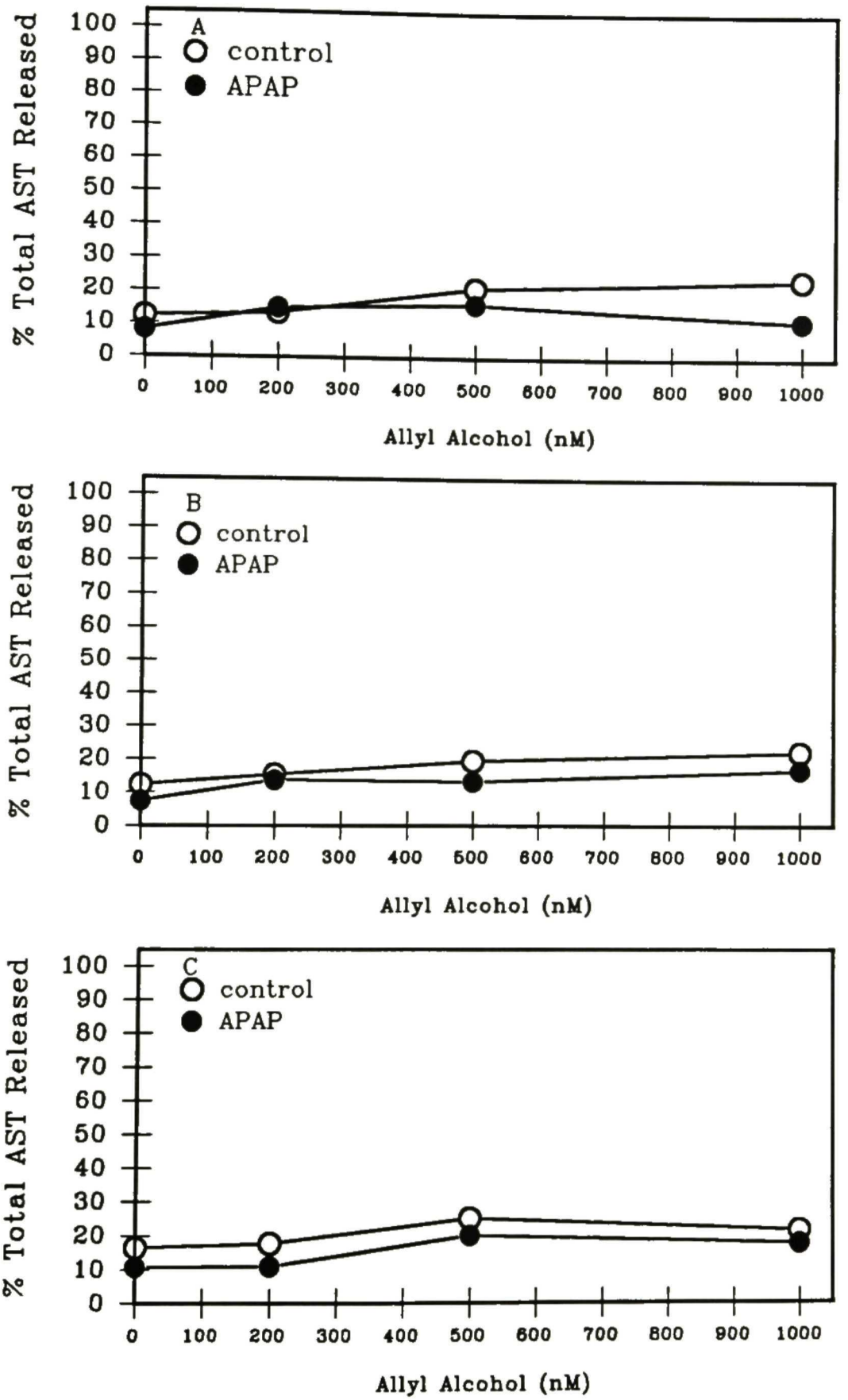
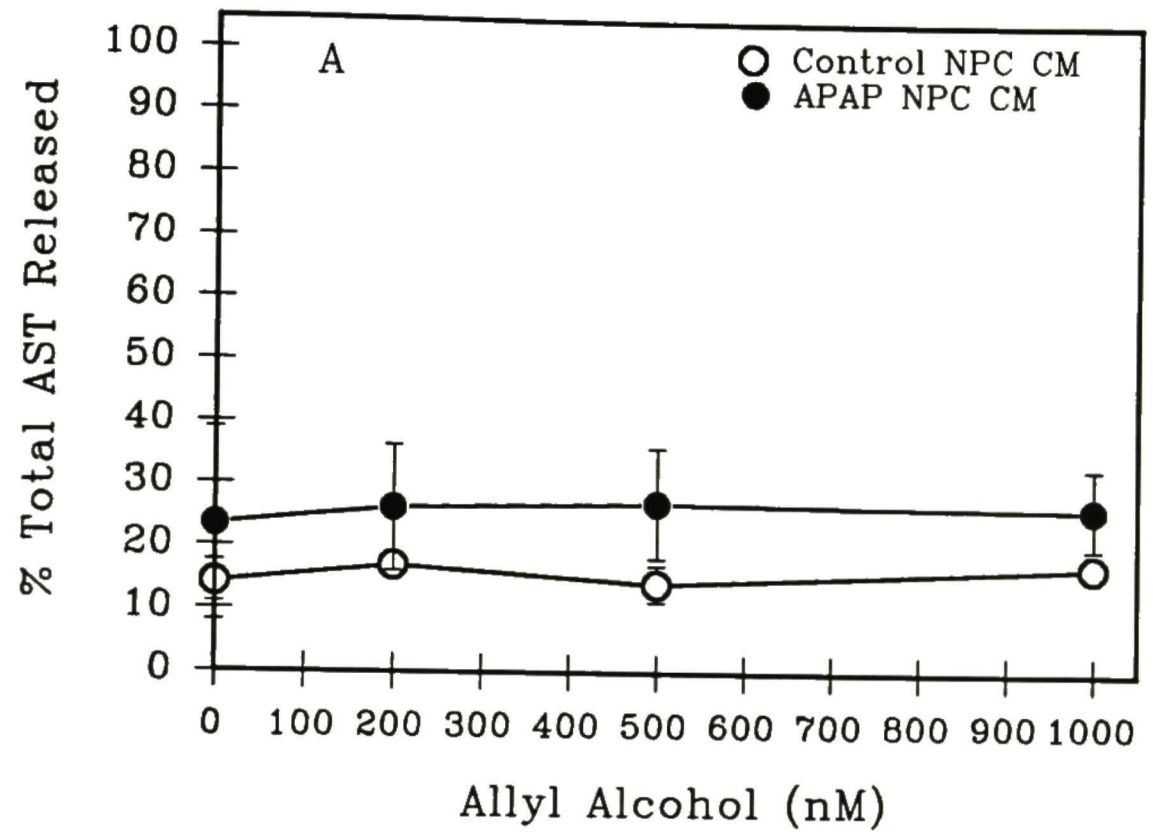

B
units ALT/10^6 cells
1100
1000
900
800
700
600
500
400
300
200
100
0
Hepatocytes Alone
Control NPC Coculture
APAP NPC Coculture

Figure 32: Dose Response of AA in Cocultures of NPCs and Hepatocytes. A) 10:1 ratio, B) 5:1 ratio, and C) 3:1 ratio. NPC were isolated and cultured for eighteen hours. The next day hepatocytes were isolated and cocultured with the NPCs for two hours. Cocultures were challenged with AA (0, 200, 500, or 1000 nM). Two hours later cells were harvested and assayed for AST activity. Data expressed as mean of percent of total activity released. AST activity/10^6 cells in AA (0 nM) cocultures was A) control-433 $\pm$ 124 and APAP-441 $\pm$ 72, B) control-348 $\pm$ 62 and APAP-405 $\pm$ 94, C) control-387 $\pm$ 77 and APAP-432 $\pm$ 76. n=4.


A
control
APAP
% Total AST Released
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
700
800
900
1000
Allyl Alcohol (nM)
B
control
APAP
% Total AST Released
Allyl Alcohol (nM)
C
control
APAP
% Total AST Released
Allyl Alcohol (nM)

ALT and AST release from hepatocytes cultured in NPC-CM was similar to release from hepatocytes cultured alone. ALT was the sensitive indicator of cytotoxicity (Figure 33). NPC-CM from NPCs from either control- or APAP-pretreated rats had decreased AST activity compared to hepatocytes alone with no effect on ALT, which take on characteristics of NPC-hepatocyte cocultures (Figure 34). Hepatocytes cultured in NPC-CM from control rats were not sensitive to AA. Hepatocytes cultured in NPC-conditioned media from APAP-pretreated rats were sensitive to AA and had statistically increased ALT activity compared to the same doses in hepatocytes cultured in Control-NPC-CM (Figure 33).

Figure 33: Culture of NPC-Conditioned Media with Hepatocytes. NPC were isolated and cultured for eighteen hours. Media was retrieved and freshly isolated hepatocytes were cocultured with the NPC-Conditioned Media (NPC CM) for two hours. Cocultures were treated with AA (0, 200, 500, or 1000 nM). Two hours later cells were harvested and assayed for ALT and AST activity. Data expressed as mean ± SE of percent of total activity released. AST activity/10^6 cells in AA (0 nM) cocultures was Control-NPC-CM, 522 ± 127 and APAP-NPC-CM, 452 ± 118. Total ALT activity/10^6 cells in 0 nM AA cocultures was Control-NPC-CM, 375 ± 42 and APAP-NPC-CM, 269 ± 70. n=4. * $p \leq 0.05$ as compared to Control-NPC at same dose.


A
Control NPC CM
APAP NPC CM
% Total AST Released
100
90
80
70
60
50
40
30
20
10
0
0 100 200 300 400 500 600 700 800 900 1000
Allyl Alcohol (nM)

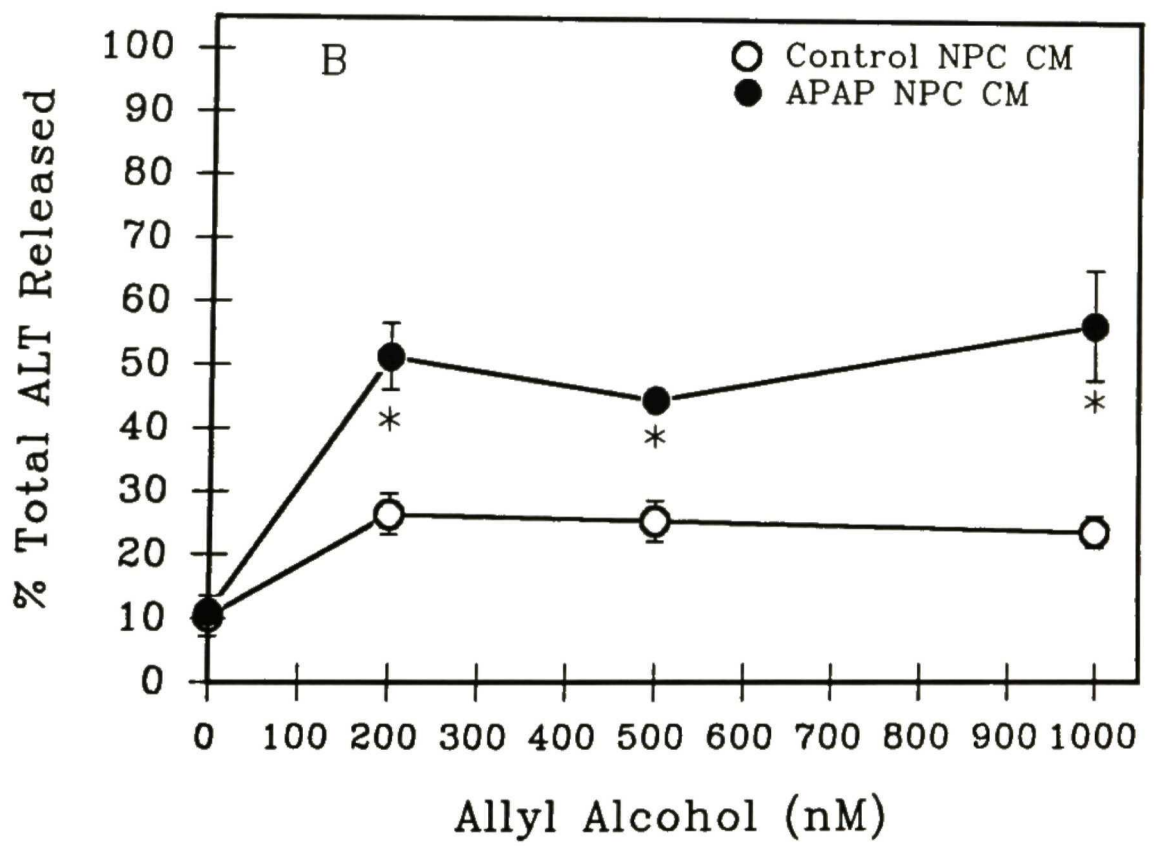
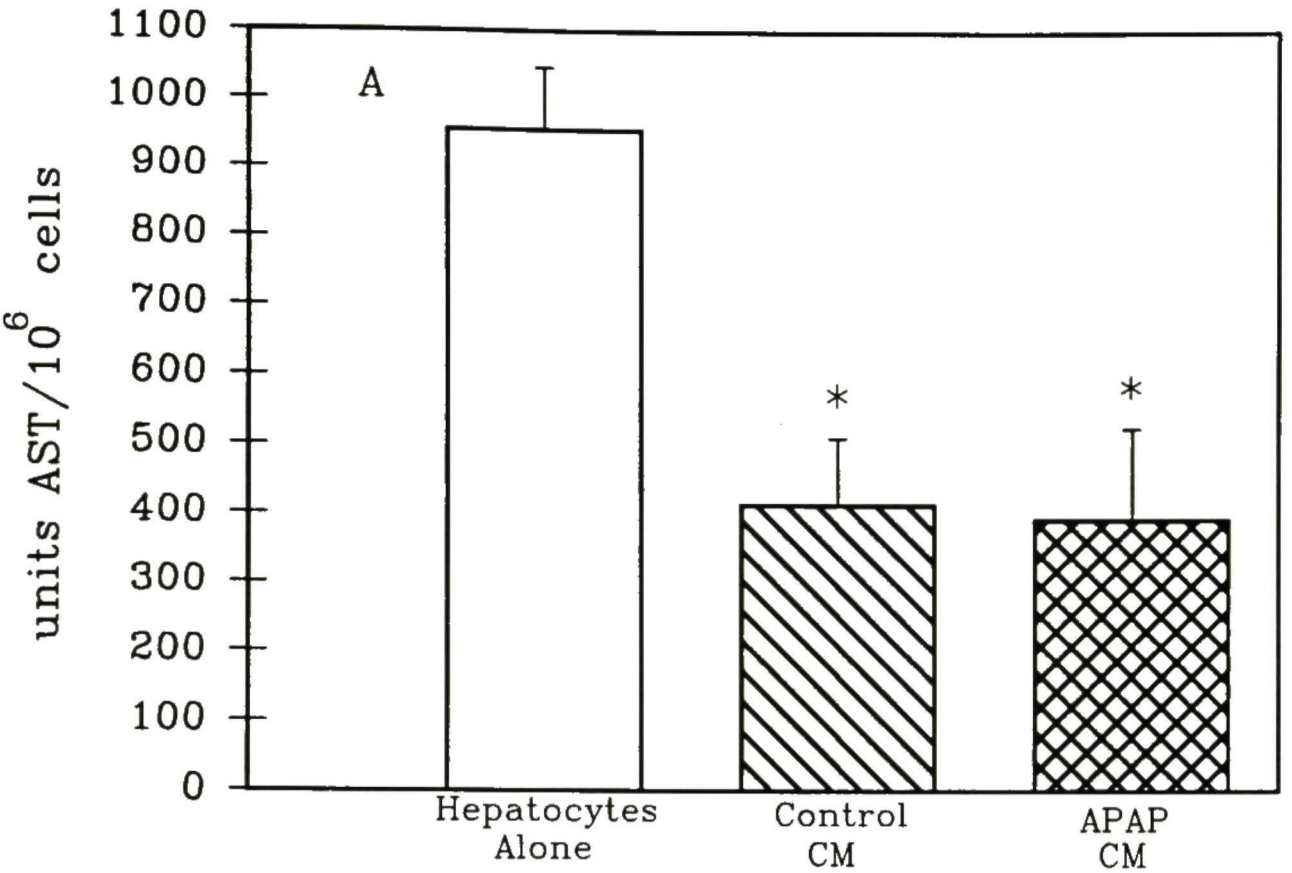
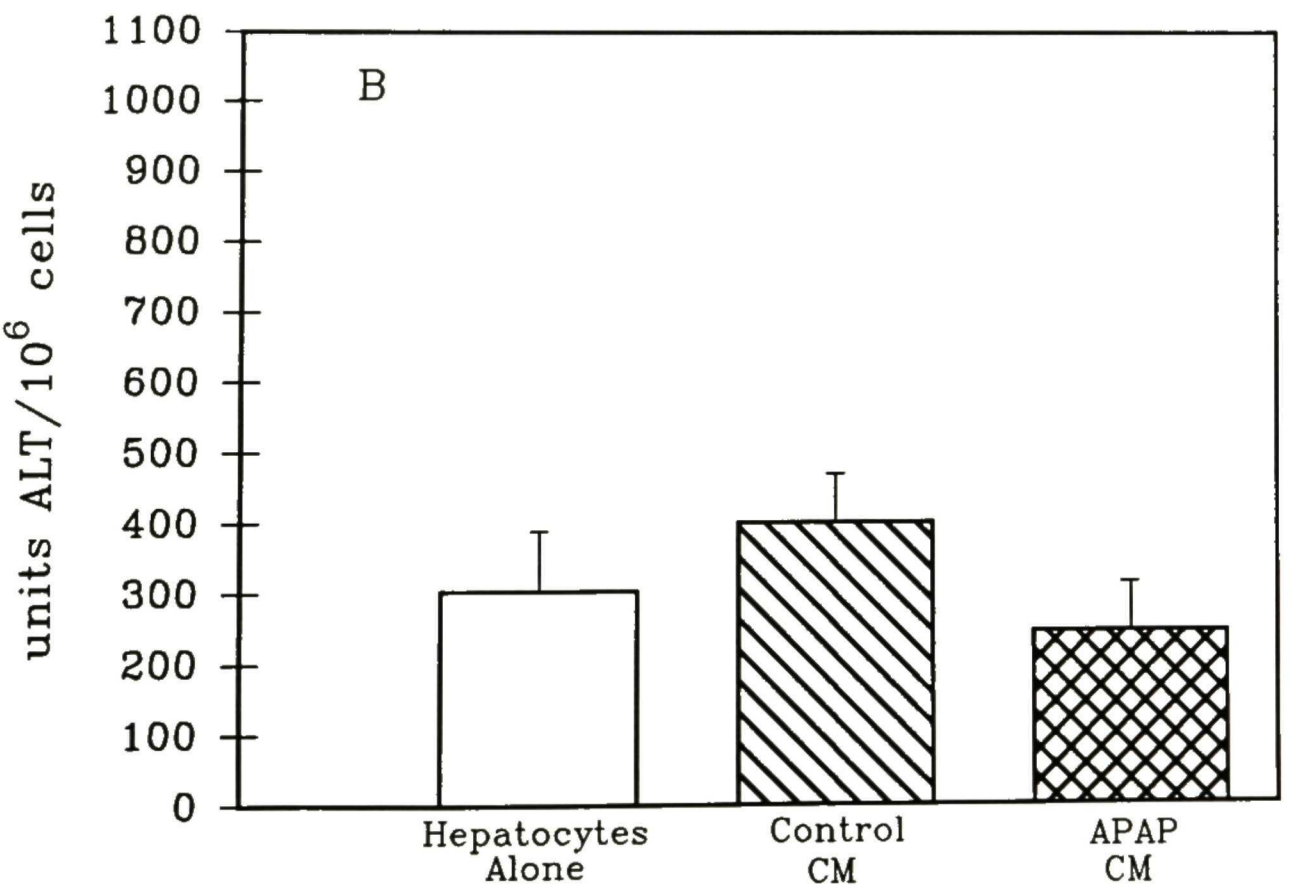

B
Control NPC CM
APAP NPC CM
% Total ALT Released
100
90
80
70
60
50
40
30
20
10
0
*
*
*
0 100 200 300 400 500 600 700 800 900 1000
Allyl Alcohol (nM)

Figure 34: Total AST and ALT Activity in Hepatocytes Cultured with NPC-Conditioned Media. NPCs were isolated and cultured for eighteen hours. The next day hepatocytes were isolated and cultured with the NPC-Conditioned Media for four hours. Data expressed as mean ± SE. n=4. * $p \leq 0.05$ as compared to hepatocytes alone.


A
units AST/10^6 cells
1100
1000
900
800
700
600
500
400
300
200
100
0
*
*
Hepatocytes Alone
Control CM
APAP CM


B
units ALT/10^6 cells
1100
1000
900
800
700
600
500
400
300
200
100
0
Hepatocytes Alone
Control CM
APAP CM

DISCUSSION

APAP-Induced Potentiation of Hepatotoxicity

To examine the possibility that activation of NPCs increases susceptibility of rats to hepatotoxicity, a two-step model of hepatotoxicity was developed. In the first step, NPC activation was induced by pretreatment of rats with non-necrogenic doses of APAP. In the second step, twenty-four hours after APAP-pretreatment, rats were challenged with the hepatotoxicants AA, APAP, BB, CCl_4, DCE, or TA. The hepatotoxicants used to challenge integrity of hepatocytes in APAP-pretreated rats were selected to include a spectrum of chemical structures (Figure 3), properties, and toxicological characteristics (Table 2). AA was selected to include an agent that produces necrosis localized in the periportal region and is not activated to a reactive metabolite by the cytochrome P450 system (Reid, 1972). CCl_4 was selected because its hepatotoxic characteristics are well defined and because the reactive metabolite does not deplete GSH (Recknagel, 1989); all other compounds deplete hepatic GSH. DCE was selected because it clearly does not produce lipid peroxidation (Jaeger *et al.*, 1973); all other compounds used induce lipid peroxidation. TA was selected because it is not a halogenated hydrocarbon but is a compound activated by cytochrome P450 (Chiele and Malvaldi, 1984 and Hunter *et al.*, 1977). As assessed by serum ALT activity and histopathology, APAP lowered the

threshold dose for cell injury and death induced by AA, BB, CCl_4, and DCE. APAP-pretreatment did not decrease the threshold dose of toxicity for TA but did accentuate hepatotoxic doses.

The potentiation of AA and DCE toxicity differed from that of BB, CCl_4, and TA. APAP reduced the dose of AA and DCE that was acutely lethal and the rats died of what appeared to be hypovolemic shock. The shock induced by AA was due to the formation of a bloody ascites. The toxicity of AA is attributed to its metabolite acrolein (Jaeschke *et al.*, 1987) and a bloody ascites is characteristic of acrolein toxicity (Beauchamp *et al.*, 1985). The mechanism of the formation of this ascites is undetermined at the physiological level. Therefore, it would be difficult to postulate why the threshold dose for formation of the ascites was decreased in APAP-pretreated rats. After DCE challenge, hepatic congestion was responsible for the symptoms of shock. The severe congestion of the liver in APAP-pretreated rats challenged with DCE, may be explained by three events; APAP-induced sinusoidal dilation (Figure 8), DCE-induced perturbation of sinusoidal architecture, and DCE-induced disruption of hepatocyte function (Reynolds *et al.*, 1980). DCE causes a retraction of the hepatocyte borders that form spaces within the hepatic cords. A combination of these events could lead to a disruption of the liver sinusoid structure rendering the sinusoids unable to regulate blood flow.

APAP-induced potentiation of BB, CCl_4, and TA toxicity was restricted to increases of serum enzyme activity and histopathological evidence (data not shown) of hepatic necrosis. Congestion of the liver or other gross alterations were not evident in these animals. APAP-induced potentiation of BB, CCl_4, and TA confirmed that this phenomenon pertained to a wide range of hepatotoxicants with different characteristics. All of the challenge compounds effect hepatocytes differently yet APAP-pretreatment potentiates their hepatotoxicity. It is interesting to note that APAP did not potentiate a second dose of APAP. It is possible that the changes induced by the challenge dose of APAP did not sensitize hepatocytes to the activated NPCs as did the other challenge hepatotoxicants. Some substance is added to this explanation by the observation that in the rat, APAP induced this accumulation of NPCs in the liver in the absence of necrosis.

Role of APAP-Induced Glutathione Depletion in Potentiation

To begin to explain the APAP-induced potentiation of hepatotoxicity, changes induced by APAP in the liver of these animals that may be responsible for the potentiation were examined. The most obvious biochemical alteration was GSH depletion twenty-four hours after APAP administration, the time of toxicant challenge. This observation was relevant because GSH is important for the detoxification of reactive molecules in the liver. All of the challenge

hepatotoxicants except CCl_4 are detoxified by GSH (Table 2). The GSH depletion observed after APAP may be the summation of two effects: first, the presence of APAP in the blood and second, decreased gastric emptying. Twenty-four hours after APAP administration, low levels of APAP were detected in the blood. This may be metabolized by P450 to the reactive metabolite which is detoxified by GSH. The second effect was APAP-induced inhibition of gastric emptying over the twenty-four hour period after administration and the subsequent reduction in nutrient absorption. If rats are not absorbing nutrients, GSH synthesis will not occur. The effects of APAP on gastric emptying may be related to the observation that APAP has been shown to decrease prostaglandin formation in rat gastric mucosa up to 17 hours after administration (Van Kolfschoten, *et al.*, 1981). This demonstrates that APAP has long lasting local effects in the gastric mucosa which may be involved in the inhibition of gastric emptying.

To examine if GSH depletion could account for the APAP-induced potentiation, rats were pretreated with a compound that is not hepatotoxic but that depletes GSH, DEM. The dose of DEM employed depletes GSH to 35% of control compared to only 65% of control in APAP-induced GSH depletion. It was found that DEM-induced GSH depletion did potentiate AA or DCE hepatotoxicity but did not potentiate the hepatotoxicity of the other challenge toxicants. Potentiation of AA and DCE induced by DEM-pretreatment was

less than that produced by APAP. It was concluded that while GSH depletion may have a role in the observed potentiation, it does not fully explain the phenomenon.

RES Suppressants and APAP-Induced Potentiation

Since GSH depletion could largely be discounted as an explanation of APAP-induced potentiation, the effect of RES suppressants on APAP-induced potentiation was examined. DS and $GdCl_3$ were used as the RES suppressants. DS precipitates blood proteins and acts as an RES suppressant by exhausting the ability of the RES cells to function leaving them refractory to activating stimuli (Walton, 1954, and Saba, 1970). DS will effect all populations of RES cells in all organs which contains these types of cells. $GdCl_3$ is cytotoxic to Kupffer cells (Koudstaal, *et al.*, 1990). The mechanism by which $GdCl_3$ acts remains unclear but it is hypothesized to involve Kupffer cells recognizing $GdCl_3$ as calcium (Husztik *et al.*, 1980). In these experiments, RES suppression was documented by decreased clearance of latex beads from the blood. Clearance of latex beads from the blood is a function shared by all of the RES cells in the animal, but the liver is the predominant site for phagocytosis (Jones and Summerfield, 1988). Therefore, RES cells in the liver are the most important contributors to the clearance of the latex beads.

AA was the challenge hepatotoxicant of choice for the RES suppression studies. RES activation decreases

cytochrome P450 levels (Peterson and Renton, 1984, 1986A,B). AA is metabolized by ADH not by the cytochrome P450 system (Reid, 1972). DS alleviated the ability of APAP to potentiate the hepatotoxicity of AA. $GdCl_3$-APAP-pretreatment did potentiate the hepatotoxicity of AA but not to the same degree as in APAP-pretreated rats.

To determine if RES-suppressant treatment effected AA metabolism, ADH activity was assayed. No significant changes in ADH activity were observed in DS- and $GdCl_3$-pretreated rats challenged with APAP. It was concluded that DS and $GdCl_3$ are not effecting the ability of the liver to metabolize AA.

To insure that the RES suppression effects of DS and $GdCl_3$ were responsible for protection, the effects of RES suppressants on the APAP-induced profile of GSH depletion were examined. DS and $GdCl_3$ did not effect the patterns of decrease in body weight after APAP-pretreatment. This indicates that DS and $GdCl_3$ did not effect the manner in which the animals respond to APAP, with regards to anorexia and gastric emptying. In DS-pretreated rats, the time course of GSH levels over twenty-four hours after APAP administration was not altered when compared with APAP alone. $GdCl_3$, alone, decreased hepatic GSH and upon APAP-pretreatment GSH was depressed even further.

The observation that DS-pretreatment inhibited the ability of APAP to potentiate AA hepatotoxicity supports the hypothesis that a functional RES is required for APAP to

potentiate hepatotoxicity. The observation that $GdCl_3$ did not completely protect against APAP-induced potentiation can be explained in two ways. $GdCl_3$ pretreatment decreased GSH levels which were depressed even further upon APAP-challenge. AA is dependent on GSH for detoxification of the reactive metabolite. This extreme decrease in GSH levels may explain the hepatotoxicity of AA observed in $GdCl_3$-APAP pretreated rats. The second possible explanation is that the APAP-induced macrophage accumulation and activation includes both resident Kupffer cells and monocytes which have migrated from the blood into the liver. DS affects all populations of RES cells in rats and therefore affects both the Kupffer cells and monocytes activated by APAP. $GdCl_3$ is cytotoxic only to Kupffer cells; therefore endothelial cells and monocytes may be activated by APAP and effect hepatocytes upon AA challenge.

Evidence has been presented here that agrees with the original hypothesis for the experiments. Data have been presented which agree with previous findings of Laskin *et al.* (1986) that APAP causes an infiltration and activation of NPCs in the liver. It has been demonstrated that pretreatment of rats with agents which suppress or inhibit NPCs decreased the severity of APAP-induced potentiation. The increase in activated NPCs in the sinusoids could lead to a locally increased concentrations of reactive oxygen species, nitric oxide, cytokines, and enzymes that are toxic or lethal to hepatocytes (Bradfield and Souhami, 1980;

Matsuo *et al.*, 1985; Kashiwazaki *et al.*, 1986; Billiar *et al.*, 1989B & C; and Decker, 1990).

Coculture of Nonparenchymal Cells with Hepatocytes

To define a role for NPCs in APAP-induced potentiation of hepatotoxicity, an attempt was made to demonstrate the phenomenon *in vitro*. NPCs from control-or APAP-pretreated rats were isolated. In each of these cell populations about 60% of the cells were positive for non-specific peroxidase. Since only macrophages stain positive for peroxidase and the cell yield from APAP-pretreated rats was 2.7x greater than that from control-pretreated rats, this can be interpreted as showing that in APAP-pretreated rats there is an increase in both endothelial cells and macrophages. Both endothelial cells and macrophages were cocultured with freshly isolated hepatocytes.

NPCs from control- or APAP-pretreated rats were cocultured with hepatocytes at three different ratios of NPCs to hepatocytes. In the cocultures, the pattern of total cytosolic AST and ALT activity, the majority of which is contained in hepatocytes, was different from that of hepatocytes cultured alone. In hepatocytes cultured alone, the most sensitive cytosolic enzyme to assess cytotoxicity was ALT but in the cocultures the only enzyme which could be measured was AST. In the cocultures, ALT is either being degraded or the activity is inhibited by something in the

media. AST activity in cocultures of NPCs and hepatocytes was significantly decreased when compared to enzyme activity in equivalent cultures of hepatocytes alone. Despite the coculture ratio, cytotoxicity to hepatocytes was not induced by AA.

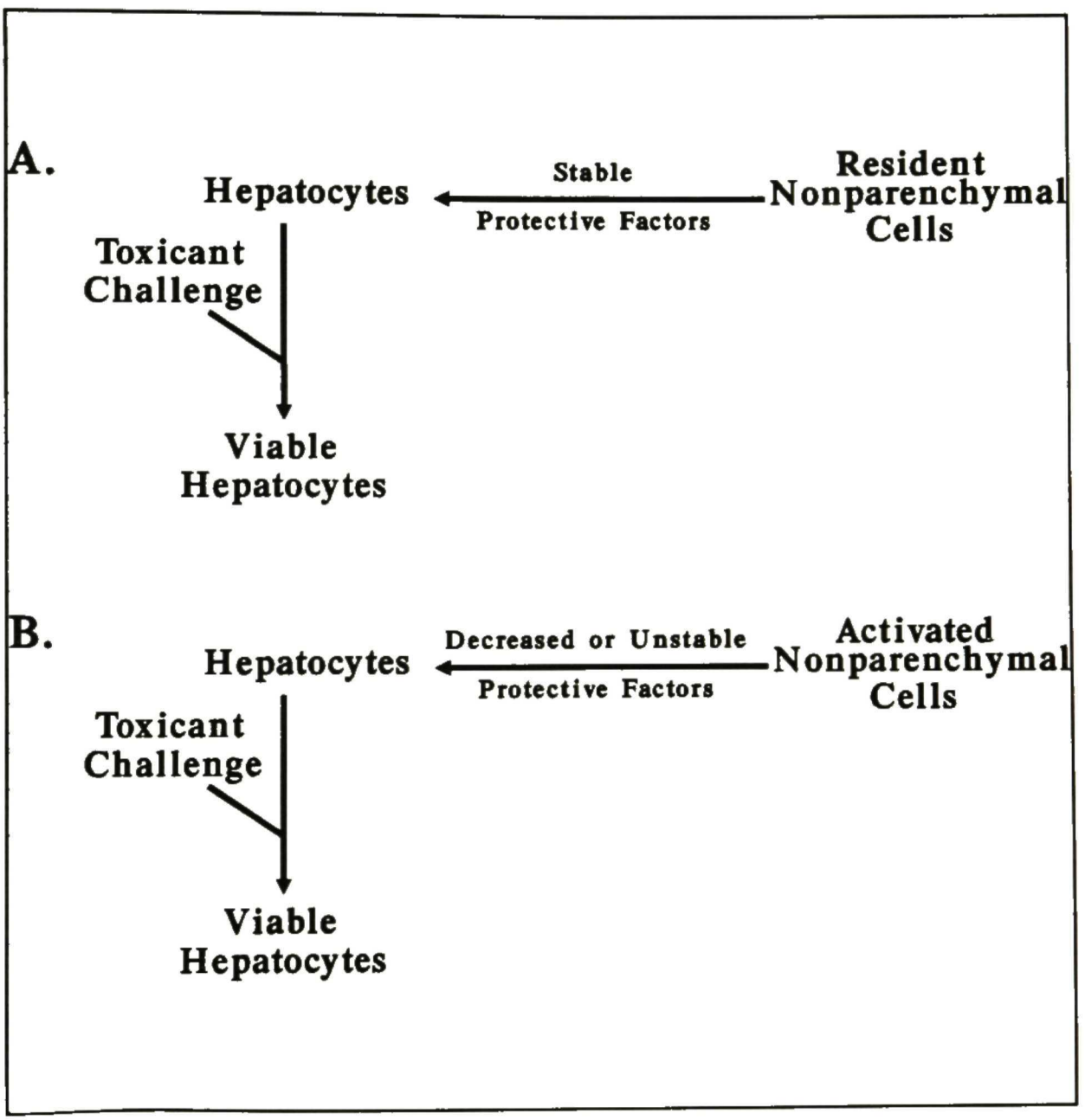
To determine if the observations made in coculture were direct effects of NPCs or effects of mediators secreted by these cells, hepatocytes were cultured with NPC-CM. NPC-CM was prepared by culturing NPCs from Control- or APAP-pretreated rats for eighteen hours. Hepatocytes were cultured in NPC-CM and cytotoxicity of AA was assessed. It was observed that hepatocytes cultured with NPC-CM acquired characteristics from both the *in vivo* and coculture situations. The changes in cytosolic enzyme pattern mimicked the changes observed in the coculture system. The sensitivity of hepatocytes to AA in the presence of NPC-CM mimicked the *in vivo* situation. Hepatocytes cultured in APAP NPC-CM were more sensitive to AA than those cultured in control NPC-CM. These data suggest that the effects of NPCs on cytosolic enzyme activity can be carried by conditioned media to hepatocytes. Studies on the effects of NPC coculture on cytotoxicity to hepatocytes have not been published, but CM from NPCs has been shown to have a stimulatory effect on hepatocytes as indicated by increased protein synthesis (Kuiper *et al.*, 1988) and increased glucose output (Casteleijn *et al.*, 1988 A,B).

Proposed Working Model of *In Vitro* Data

The results of the coculture experiments do not support the initial hypothesis that APAP-induced activation of NPCs has a role in potentiation of hepatotoxicity. The CM experiments do support this hypothesis. This conflicting data merits further examination to determine if this could be valid model to study APAP-induced potentiation of hepatotoxicity. A model could be suggested from a comparison of the outcome of the coculture and conditioned media and hepatocyte culture experiments (Figure 35). In this model, NPCs from Control- or APAP-pretreated rats produce factors which are protective to hepatocytes. The protective factors produced by control NPCs are stable (Figure 35A). The protective factors produced by APAP-NPCs are unstable in culture or are produced at a low concentration (Figure 35B) and constant secretion is required to protect hepatocytes from AA insult. This was indicated by the lack of the cytotoxicity of AA on hepatocytes in coculture with NPCs and Control-NPC-CM. Upon AA challenge in APAP-NPC-CM, hepatocytes were sensitized to AA because the protective factors were not present or were present at lower concentrations. This model does assume the *in vitro* data is not an experimental artifact and it also assumes that insult to hepatocytes is not involved in the protective process.

To test this model, release of protective factors from NPCs (Figure 35) would need to be examined. This could

Figure 35: Proposed Model of *In Vitro* Protection of Hepatocytes from Cytotoxicity of AA in Coculture with Nonparenchymal Cells. A) Stable factors are released from Control-NPCs. B) Unstable factors or a lower concentration of stable factors are released from APAP-NPCs.


A.
Hepatocytes
Stable
Protective Factors
Resident Nonparenchymal Cells
Toxicant Challenge
Viable Hepatocytes
B.
Hepatocytes
Decreased or Unstable
Protective Factors
Activated Nonparenchymal Cells
Toxicant Challenge
Viable Hepatocytes

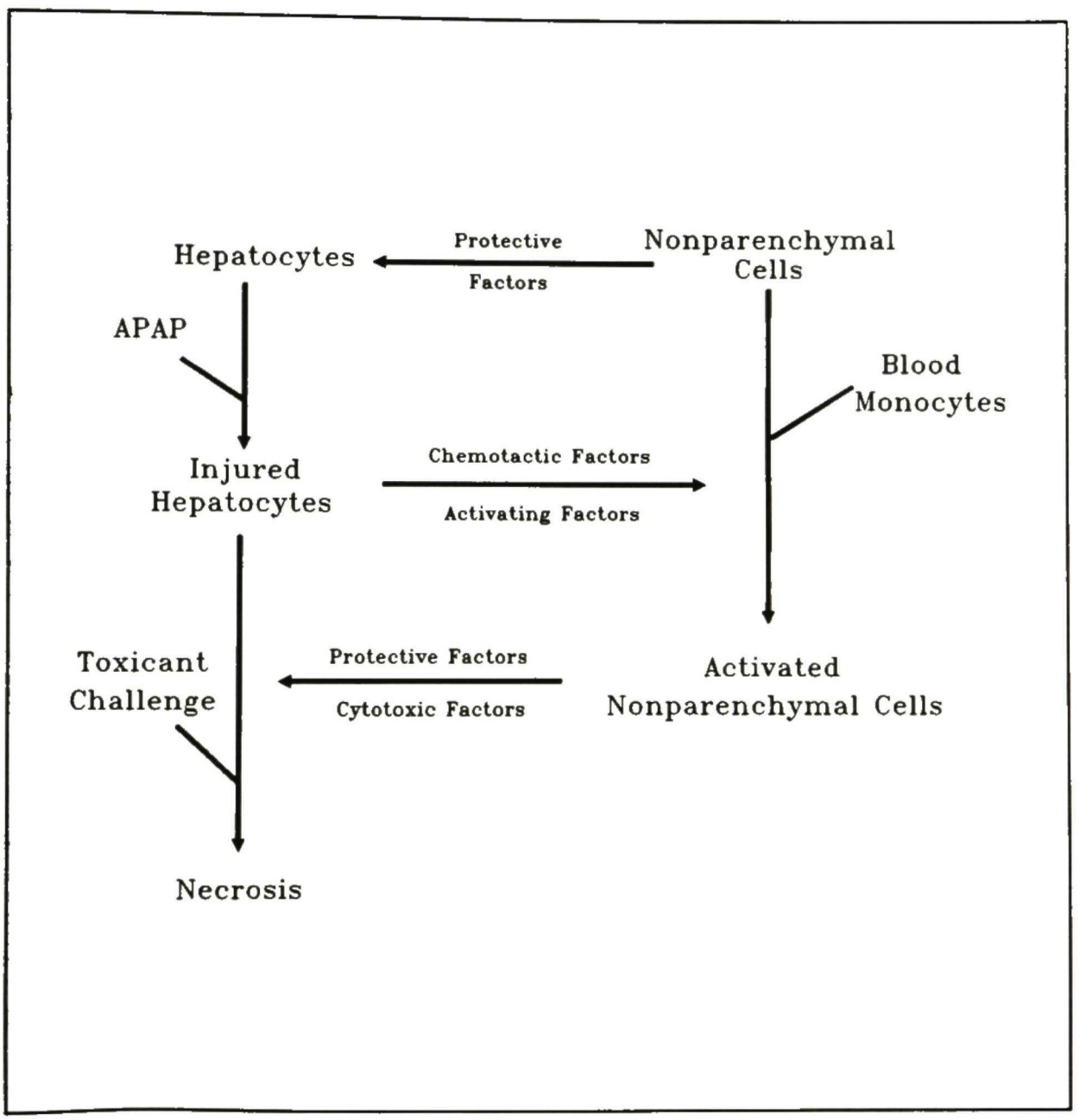
be investigated by freeze/thawing NPC-CM or treating the NPC-CM with trypsin after it has been conditioned by the NPCs. Aspirin, 5,6-dehydroarachodonic acid, or dexamethasone could be added to cocultures or media as it in conditioned by NPCs to inhibit release of the factors. Aspirin will inhibit the formation of prostanoids and 5,6-dehydroarachodonic acid will inhibit the formation of lipoxygenase products. Dexamethasone will generally inhibit the NPCs and suppress their response to stimuli. A control of fresh media should be included in these manipulations. The cocultures or hepatocytes cultured in CM that have undergone these treatments would be tested for cytotoxicity of AA to determine if protection has occurred. If hepatocytes are not protected from AA cytotoxicity, these manipulations of the media will determine the stability of the factors and give insight on their nature. The next step will be to identify the factor or factors responsible for these effects. Available antibodies to known cytokines and lipid mediators could be added to cocultures and conditioned media and hepatocytes cultures in an attempt to inhibit the protective effect of NPCs. If the antibody experiments are unsuccessful, an attempt to purify the factor or factors would be made. Finally, after the factor or factors have been identified, they could be added to cultures of hepatocytes to determine if protection from cytotoxicity can be mimicked.

This model addresses the differences between CM experiments and coculture experiments. Activated and resident NPCs have been shown to have different patterns of release of immunological mediators. Many of the modulators have higher activity in activated cells as compared to resident cells (Decker *et al.*, 1989; Decker, 1990). All of the mediators examined in the literature are increased in activated cells. All molecules released by these cells have not been examined and it is possible that some of these mediators increase at the expense of other mediators. Pursuing this model may lead to identification of factors which may protect cells from chemically-induced liver injury which may also have applications in other organs.

Proposed Working Model of APAP-Induced Potentiation

A working model of APAP-induced potentiation in Figure 36 is proposed from the evidence presented *in vivo* and *in vitro*. In this model, resident NPCs normally secrete factors which aid hepatocytes in defense against injury. One interpretation of the *in vitro* experiments presented here suggest release of protective factors from NPCs. Upon APAP-pretreatment, an alteration in hepatocytes releases chemotactic factors which recruit blood monocytes into the liver and activating factors which activate the monocytes and NPCs (Laskin and Pilaro, 1986 and Laskin, 1990). The activated NPCs influence hepatocytes' response to toxicant administration. Alternatively, the activated-NPCs secrete

Figure 36: Proposed Model of APAP-Induced Potentiation of Hepatotoxicity from *In Vivo* and *In Vitro* Data.


Hepatocytes
Protective
Factors
Nonparenchymal
Cells
APAP
Blood
Monocytes
Injured
Hepatocytes
Chemotactic Factors
Activating Factors
Toxicant
Challenge
Protective Factors
Cytotoxic Factors
Activated
Nonparenchymal Cells
Necrosis

less of the protective factors or disrupt the balance of normal communication and mediators which could also sensitize hepatocytes to injury. *In vivo*, a role for NPCs in this model is validated by the experiments in which RES suppression was induced with the subsequent inhibition of potentiation of AA hepatotoxicity.

To document this model an isolated perfused liver system in which sinusoidal structure is intact would have to be studied. If it is found that APAP-pretreatment can potentiate AA hepatotoxicity in the isolated perfused liver then this system could examine changes in release of factors or known immunological mediators in the liver after APAP-pretreatment or upon toxicant challenge. To identify changes in factor release RIAs, ELIZAs, or bioassays for known cytokines and lipids mediators would be done on effluent fluid from the perfused liver. The first step would be to identify a change in factor or factors release in control- or APAP-pretreated rats. The next step would be to perfuse the liver with toxicant to determine if factor release could be influenced by the presence of AA. Release of cytosolic enzymes into the effluent could also be measured to correlate a change in mediator release after APAP-pretreatment or AA challenge in APAP-pretreated rats with the appearance of cytotoxicity. If the factors can be identified which correlate with the appearance of hepatotoxicity, the scenario could be set up in a perfused

liver system using these factors in a control liver to attempt to mimic potentiation of a hepatotoxic response.

Summary

It was the goal of this project to determine if activated NPCs were a factor in APAP-induced potentiation of hepatotoxicity. Data has been presented *in vivo* which suggest this to be true. Data from *in vitro* experiments give preliminary evidence that APAP-NPCs may influence hepatocytes response to toxicant insult. These data suggest models to be tested to determine if NPCs are releasing mediators which influence hepatotoxicity. These data support the initial hypothesis that to study the role of activated macrophages and NPCs in the manifestation of hepatotoxicity, a two step model of inducing hepatotoxicity is necessary.

REFERENCES

Adams, D. O. and T. A. Hamilton. 1984. The Cell Biology of Macrophage Activation. Annual Reviews in Immunology. 283-318.

Badr, M. Z., S. A. Belinsky, F. C. Kauffman and R. G. Thurman. 1986. Mechanism of Hepatotoxicity due to Allyl Alcohol: Role of Oxygen and Lipid Peroxidation. Journal of Pharmacology and Experimental Therapeutics. 238: 1138-1142.

Bartolone, J. B., S. D. Cohen and E. A. and Khairallah. 1989. Immunohistochemical Localization of Acetaminophen-bound Liver Proteins. Fundamental and Applied Pharmacology. 13: 859-862.

Bartolone, J. B., K. Sparks, S. D. Cohen and E. A. Khairallah. 1987. Immunochemical Detection of Acetaminophen-bound Liver Proteins. Biochemical Pharmacology. 36: 1193-1196.

Beauchamp, R. O., D. A. Andjelkovich, A. D. Kligerman, K. T. Morgan and H. D. Heck. 1985. A Critical Review of the Literature on Acrolein Toxicity. CRC Critical Reviews in Toxicology. 14: 309-335.

Beierschmitt, W. P., J. T. Brady, J. B. Bartolone, D. S. Wyand, E. A. Khairallah and S. D. Cohen. 1989. Selective Protein Arylation and the Age Dependency of Acetaminophen Hepatotoxicity in Mice. Toxicology and Applied Pharmacology. 98: 517-529.

Belinsky, S. A., M. Z. Badr, F. C. Kauffman and R. G. Thurman. 1986. Mechanism of Hepatotoxicity in Periportal Regions of the Liver Lobule Due to Allyl Alcohol: Studies on Thiols and Energy Status. Journal of Pharmacology and Experimental Therapeutics. 238: 1132-1137.

Billiar, T. R., R. D. Curran, D. J. Stuehr, M. A. West, B. G. Bentz and R. L. Simmons. 1989A. An l-Arginine-Dependent Mechanism Mediates Kupffer Cell Inhibition of Hepatocyte Protein Synthesis IN VITRO. Journal of Experimental Medicine. 169: 1467-1472.

Billiar, T. R., R. D. Curran, M A. West, K. Hoffman, and R. L. Simmons. 1989B. Kupffer Cell Cytotoxicity to Hepatocytes in Coculture Requires L-Arginine. Archives of Surgery. 124: 1416-1421.

Billiar, T. R., R. D. Curran, D. J. Stuehr, F. K. Ferrari and R. L. Simmons. 1989C. Evidence that Activation of Kupffer Cells Results in Production of

L-Arginine Metabolites that Release Cell-associatedIron and Inhibit Hepatocyte Protein Synthesis. Surgery. 106: 364-372.

Bissell, D. M. and P. S. Guzelian. 1980. Phenotypic Stability of Adult Rat Hepatocytes in Primary Monolayer Culture. In Differentiation and Carcinogenesis in Liver Cell Cultures, ed. C. Borek and G. M. Williams, 85-98. New York: The New York Academy of Sciences.

Black, M. 1984. Acetaminophen Hepatotoxicity. Annual Reviews in Medicine. 35: 577-593.

Boobis, A. R., D. J. Fawthrop and D. S. Davies. 1989. Mechanisms of Cell Death. Trends in the Pharmacological Sciences. 10: 275-280.

Bouwens, L. and E. Wisse. 1989. Hepatic Pit Cells Have Natural Cytotoxic (NC) Activity Against Solid Tumor-Derived Target Cells. In Cells of the Hepatic Sinusoid, Volume 2. ed. E. Wisse, D. L. Knook, and K. Decker, 215-220. Netherlands: Kupffer Cell Foundation.

Bradfield, J. W. B. and R. L. Souhami. 1980. Hepatocyte Damage Secondary to Kupffer Cell Phagocytosis. In The Reticuloendothelial System and The Pathogenesis of Liver Disease, ed. H. Liehr and M. Grun. New York: Elsevier/North Holland Biomedical Press.

Brouwer, A., E. Wisse and D. L. Knook. 1988. Sinusoidal Endothelial Cells and Perisinusoidal Fat-Storing Cells. The Liver: Biology and Pathobiology, ed. I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, D. A. Shafritz, 665-682. New York: Raven Press.

Bruni, C. and K. R. Porter. 1965. The Fine Structure of the Parenchymal Cell of the Normal Rat Liver. American Journal of Pathology. 46: 691.

Burcham, P. C. and A. W. Harman. 1990. Mitochondrial Dysfunction in Paracetamol Hepatotoxicity: IN VITRO Studies in Isolated Mouse Hepatocytes. Toxicology Letters. 2229: 37-48.

Casini, A. M., P. A. Ferrali and M. Comporti. 1987. Lipid Peroxidation, Protein Thiols, and Calcium Homeostasis in Bromobenzene-Induced Liver Damage. Biochemical Pharmacology. 36: 3689-3695.

Casteleijn, E. K., John van Rooij, Henri C. J. Kamps, Jan A. A. Kaster, and T. J. C. van Berkel. 1988A. Hormonal Control of Glycogenolysis in Parenchymal

Liver Cells by Kupffer and Endothelial Liver Cells. Journal of Biological Chemistry. 263: 2699-2703.

Casteleijn, E., J. Kuiper, H. C. J. Van Rooij, J. F. Koster and T. J. C. Van Berkel. 1988B. Conditioned Media of Kupffer and Endothelial Cells Influence Protein Phosphorylation in Parenchymal Liver Cells. Biochemical Journal. 252: 601-605.

Chieli, E. and G. Malvaldi. 1984. Role of Microsomal FAD-Containing Monooxygenase in the Liver Toxicity of Thioacetamide S-Oxide. Toxicology. 31: 41-52.

Corcoran, G. B., B. K. Wong and B. L. Neese. 1987. Early Sustained Rise in Total Liver Calcium During Acetaminophen Hepatotoxicity in Mice. Research Communications in Chemical Pathology and Pharmacology. 58: 291-305.

Dahlin, D. C., G. T. Miwa, A. H. Lu and S. D. Nelson. 1984. N-Acetyl-p-Benzoquinone Imine. A Cytochrome P450-Mediated Oxidation Product of Acetaminophen. Proceedings of The National Academy Of Science. 81: 1327-1331.

Das, N., M. K. Basu and M. K. Das. 1987. Suppression of Liver Uptake of Orally Fed Liposomes by Injection of Dextran Sulfate 500. Bioscience Reports. 7: 465-470.

David, H. and P. Reinke. 1987. The Concept of the Perisinusoidal Functional Unit of the Liver: Importance to Pathological Processes. Experimental Pathology. 32: 193-224.

Davidson, D. G. D. and W. N. Eastman. 1966. Acute Liver Necrosis Following Overdose of Paracetamol. British Medical Journal. 2: 497-499.

Decker, T., M. L. Lohmann-Matthes, U. Karck, T. Peters and K. Decker. 1989. Comparative Study of Cytotoxicity Tumor Necrosis Factor and Prostaglandin Release after Stimulation of Rat Kupffer Cells, Murine Kupffer Cells, and Murine Inflammatory Liver Macrophages. Journal of Leukocyte Biology. 45: 139-146.

Decker, K. 1990. Biologically Active Products of Stimulated Liver Macrophages (Kupffer Cells). European Journal of Biochemistry. 192: 245-261.

Dixon, M. F., J. Nimmo and L. F. Prescott. 1971. Experimental Paracetamol-Induced Hepatic Necrosis:

A Histopathological Study. Journal of Pathology. 103: 225-229.

Dixon, M. F., B. Dixon, S. R. Aparicio and D. P. Loney. 1975. Experimental Paracetamol Induced Hepatic Necrosis: A Light and Electron Microscope and Histochemical Study. Journal of Pathology. 116: 17-29.

Duncan, R. C., R. G. Knapp, and M. C. Miller. 1977. Introductory Biostatistics for the Health Sciences. New York: John Wiley and Sons.

Ellman, G. C. 1959. Tissue Sulfhydryl Groups. Archives of Biochemistry and Biophysics. 82: 70-77.

Esterline, R. L. and S. Ji. 1989. Metabolic Alterations Resulting From The Inhibition of Mitochondrial Respiration By Acetaminophen IN VIVO. Biochemical Pharmacology. 38: 2390-2392.

Esterline, R. L., S. D. Ray and S. Ji. 1989. Reversible and Irreversible Inhibition of Hepatic Mitochondrial Respiration by Acetaminophen and its Toxic Metabolite, N-acetyl-benzoquinoneimine (NAPQI). Biochemical Pharmacology. 38: 2387-2390.

German Society for Clinical Chemistry. 1970. Standardization of Methods for the Estimation of Enzyme Activities in Clinical Chemistry. Z. Klin. Chem. Klin. Biochem. 8: 658-660.

German Society for Clinical Chemistry. 1971. Recomenadations for a Uniform Temperature for the Estimation of Enzyme Activities in Clinical Chemistry. Z. Klion. Chem. Klin. Biochem. 9: 464-465.

Farber, J. L. and S. K. El-Mofty. 1975. The Biochemical Pathology of Liver Cell Necrosis. American Journal of Pathology. 81: 237-250.

Gerson, R. J., A. Casini, D. Gilfor, A. Serroni and J. L. Farber. 1985. Oxygen-Mediated Cell Injury in The Killing of Cultured Hepatocytes by Acetaminophen. Biochemical and Biophysical Research Communications. 126: 1129-1137.

Gupta, D. N. 1956. Acute Changes In The Liver After Administration of Thioacetamide. Journal of Pathological Bacteriology. 72: 183-192.

Guzelian, P. S., R. F. Diegelmann, R. G. Lamb and H. J. Fallon. 1979. Effects of Hormones on Changes in Cytochrome P-450, Prolyl Hydroxylase, and Glycerol

Phosphate Acyltransferase in Primary Monolayer Cultures of Parenchymal Cells from Adult Rat Liver. The Yale Journal of Biology and Medicine. 52: 5-12.

Hahn, H. 1974. Effects of Dextran Sulfate 500 on Cell-Mediated Resistance to Infection with Listeria Monocytogenes in Mice. Infection and Immunity. 10: 1105-1109.

Horiuchi, S., K. Takata, A. T. M. A. Rahim, A. Miyazaki, N. Araki and Y. Morino. 1989. Lipoprotein Receptors of Sinusoidal Liver Cells. In Cells of the Hepatic Sinusoid, Volume 2, ed. E. Wisse, D. L. Knook, and K. Decker, 99-104. Netherlands: Kupffer Cell Foundation.

Hunter, A. L., M. A. Holscher and R. A. Neal. 1977. Thioacetamide-Induced Hepatic Necrosis. I. Involvement of The Mixed Function Oxidase System. Journal of Pharmacology and Experimental Therapeutics. 200: 439-448.

Husztik, E., G. Lazar and A. Parducz. 1980. Electron Microscopic Study of Kupffer-Cell Phagocytosis Blockade Induced By Gadolinium Chloride. British Journal of Experimental Pathology. 61: 624-630.

Itinose, A. M., M. Leiko Doi Sakuno and A. Bracht. 1989. Metabolic Effects of Acetaminophen: Studies in the Isolated Perfused Rat Liver. Cell Biochemistry and Function. 7: 263-273.

Jaeger, R. J., R. B. Connolly and S. D. Murphy. 1973. Diurnal Variation of Hepatic Glutathione Concentration and Its Correlation with 1,1-Dichloroethylene Inhalation Toxicity in Rats. Research Communications in Chemical Pathology and Pharmacology. 6: 465-471.

Jaeger, R. J., L. G. Shoner and L. Coffman. 1977. 1,1-Dichloroethylene Hepatotoxicity: Proposed Mechanism of Action and Distribution and Binding of 14C-Radioactivity Following Inhalation Exposure in Rats. Environmental Health Perspectives. 21: 113-119.

Jaeschke, H., C. Kleinwaechter and A. Wendel. 1987. The Role of Acrolein in Allyl Alcohol-induced Lipid Peroxidation and Liver Cell Damage in Mice. Biochemical Pharmacology. 36: 51-57.

Jollow, D. J., J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie. 1973. Acetaminophen-Induced Hepatic Necrosis. II. Role of Covalent Binding IN VIVO. Journal of

Pharmacology and Experimental Therapeutics. 187: 195-202.

Jones, E. A. and J. A. Summerfield. 1988. Kupffer Cells. In The Liver: Biology and Pathobiology, ed. I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, D. A. Shafritz, 683-704. New York: Raven Press.

Kaneda, K., Y. Natsuhara, S. Oka, K. Uehara, I. Yano and K. Wake. 1989. IN VIVO Defensive Role of Pit Cells in the Liver. In Cells of the Hepatic Sinusoid, Volume 2, ed. E. Wisse, D. L. Knook, and K. Decker, 221-223. Netherlands: Kupffer Cell Foundation.

Kashiwazaki, K., M. S. Hibbs, J. M. Seyer, C. L. Mainardi and A. H. Kang. 1986. Stimulation of Interstitial Collagenase in Co-cultures of Rat Hepatocytes and Sinusoidal Cells. Gastroenterology. 90: 829-36.

Keller, G. A., M. A. West, F. B. Cerra and R. L. Simmons. 1986. Macrophage-Mediated Modulation of Hepatocyte Protein Synthesis: Effects of Dexamethasone. Archives of Surgery. 121: 1199-1205.

Klaassen, C. D. 1986. Distribution, Excretion, and Absorption of Toxicants. In Toxicology: The Basic Science of Poisons, 33-63. New York: Macmillan.

Koudstaal, J., F. W. J. Dijkuis and M. J. Hardonk. 1990. Selective Depletion of Kupffer Cells in Rats by Gadolinium Chloride. In Cells of the Hepatic Sinusoid, Volume 3, In Press.

Kuiper, J., E. Casteleyn and T. J. C. van Berkel. 1988. Regulation of Liver Metabolism by Intracellular Communication. Advances in Enzyme Regulation. 27: 193-203.

Kyle, M. E., S. Miccadei, D. Nakae and J. L. Farber. 1987. Superoxide Dismutase and Catalase Protect Cultured Hepatocytes From The Cytotoxicity of Acetaminophen. Biochemical and Biophysical Research Communications. 149: 889-896.

Laskin, D. L. 1990. Nonparenchymal Cells and Hepatotoxicity. Seminars in Liver Disease. 10: 293-304.

Laskin, D. L. and A. M. Pilaro. 1986. Potential Role of Activated Macrophages in Acetaminophen Hepatotoxicity. I. Isolation and Characterization of Activated Macrophages From Rat Liver. Toxicology and Applied Pharmacology. 86: 204-215.

Laskin, D. L., A. M. Pilaro and S. Ji. 1986. Potential

Role of Activated Macrophages in Acetaminophen Hepatotoxicity. II. Mechanism of Macrophage Accumulation and Activation. Toxicology and Applied Pharmacology. 86: 216-226.

Laskin, D. L., A. A. Sirak, A. M. Pilaro and J D. Laskin. 1988. Functional and Biochemical Properties of Rat Kupffer Cells and Peritoneal Macrophages. Journal of Leukocyte Biology. 44: 71-78.

Lazar, G. 1973. The Reticuloendothelial-Blocking Effect of Rare Earth Metals in Rats. Journal of the Reticuloendothelial Society. 13: 231-237.

Lazar, G. 1976. Reticuloendothelial Blockade with Rare Earth Metals, The Role of Reticuloendothelial Activity in Hepatopathies and Intravascular Coagulation. The Reticuloendothelial System in Health and Disease, ed. S. M. Reichard, M. R. Escobar, and H. Friedman, 427-433. New York: Plenum Press.

Liebovitz, A. 1963. The Growth and Maintenance of Tissue-Cell Cultures in Free Gas Exchange with the Atmosphere. American Journal of Hygiene. 78: 173-180.

Long, R. M. and L. Moore. 1986A. Inhibition of Liver Endoplasmic Reticulum Calcium Pump by CCl_4 and Release of a Sequestered Calcium Pool. Biochemical Pharmacology. 35: 4131-4137.

Long, R. M. and L. Moore. 1986B. Elevated Cytosolic Calcium in Rat Hepatocytes Exposed to Carbon Tetrachloride. Journal of Pharmacology and Experimental Therapeutics. 238: 186-191.

Long, R. M. and L. Moore. 1988. Biochemical Evaluation of Rat Hepatocyte Primary Cultures as a Model for Carbon Tetrachloride Hepatotoxicity: Comparative Studies IN VIVO and IN VITRO. Toxicology and Applied Pharmacology. 92: 295-306.

Matsubara, T. and M. Ziff. 1986. Increased Superoxide Anion Release from Human Endothelial Cells in Response to Cytokines. Journal of Immunology. 137: 3295-3298.

Matsuo, S., A. Nakagawara, K. Ikeda, M. Mitsuyama and K. Nomoto. 1985. Enhanced Release of Reactive Oxygen Intermediates by Immunologically Activated Kupffer Cells. Clinical and Experimental Immunology. 59: 203-209.

McClain C. J., J. Holtzman, J. Allen, J. Kromhout and S. Shedlofsky. 1988. Clinical Features of Acetaminophen Toxicity. Journal of Clinical Gastroenterology. 10: 76-80.

Meister, A. 1981. Metabolism and Functions of Glutathione. Trends in Biochemical Sciences. 6: 231-234.

Meyers, L. L., W. P. Beierschmitt, E. A. Khairallah and S. D. Cohen. 1988. Acetaminophen-induced Inhibition of Hepatic Mitochondrial Respiration in Mice. Toxicology and Applied Pharmacology. 93: 378-387.

Mitchell, J. R., W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie. 1973. Acetaminophen-Induced Hepatic Necrosis. I. Role of Drug Metabolism. Journal of Pharmacology and Experimental Therapeutics. 187: 185-194.

Mitchell, J. R., S. D. Nelson, S. S. Thorgeirsson, R. J. McMurtry and E. Dybing. 1976. Metabolic Activation: Biochemical Basis for Many Drug-induced Liver Injuries. In Progress in Liver Disease, ed. H. Popper and F. Schaffner. 259-279. New York: Grunete and Stratton.

Moore, L. 1982. 1,1-Dichloroethylene Inhibition of Liver Endoplasmic Reticulum Pump Function. Biochemical Pharmacology. 31: 1463-1465.

Nakae, D., Y. Kazuhiko, H. Yoshiji, T. Kingasa, H. Maruyama, J. L. Farber, and Y. Konishi. 1990. Liposome-encapsulated Superoxide Dismutase Prevents Liver Necrosis Induced by Acetaminophen. American Journal of Pathology. 136: 787-795.

Nakae, D., H. Yoshiji, K. Yamamoto, H. Maruyama, T. Kingasa, T. Yoshinori, A. Densa and Y. Konishi. 1990. Influence of Timing of Administration of Liposome-encapsulated Superoxide Dismutase of its Prevention of Acetaminophen-Induced Liver Cell Necrosis in Rats. Acta Pathologica Japonica. 40: 568-573.

Nikolaev, V., M. Kerimova, E. Naydenova, S. Dimov, G. Savov and E. Ivanov. 1986. The Effect of Thioacetamide on Rat Liver Plasma Membrane Enzymes and its Potentiation by Fasting. Toxicology. 38: 203-208.

Nolan, J. P. and D. S. and Camara. 1982. Endotoxin and Liver Disease. In Sinusoidal Liver Cells, ed. D. L. Knook and E. Wisse, 371-386. Netherlands: Elsevier Biomedical Press.

Orrenius, S., D. J. McConkey, G. Bellomo and P. D. Nicotera. 1989. Role of Calcium in Toxic Cell Killing. Trends in the Pharmacological Sciences. 10: 281-285.

Page, D. T. and J. S. Garvey. 1979. Isolation and Characterization of Hepatocytes and Kupffer Cells. Journal of Immunological Methods. 27: 159-173.

Peterson, T. C. and K. W. Renton. 1984. Depression of Cytochrome P450-Dependent Drug Biotransformations in Hepatocytes After the Activation of the Reticuloendothelial System by Dextran Sulfate. Journal of Pharmacology and Experimental Therapeutics. 229: 299-304.

Peterson, T. C. and K. W. Renton. 1986A. Role of Lymphocytes, Macrophages and Interferon in the Depression of Drug Metabolism by Dextran Sulfate. Immunopharmacology. 11: 21-28.

Peterson, T. C. and K. W. Renton. 1986B. Kupffer Cell Factor Mediated Depression of Hepatic Parenchymal Cell Cytochrome P450. Biochemical Pharmacology. 35: 1491-1497.

Plaa, G. L. and W. R. Hewitt. 1984. Detection and Evaluation of Chemically Induced Liver Injury. In Principles and Methods of Toxicology, ed. A. W. Hayes, 407-446. New York: Raven Press.

Plaa, G. L. 1986. Toxic Responses of the Liver. In Toxicology: The Basic Science of Poisons, 33-63. New York: Macmillan Press.

Placke, M. E., G. L. Ginsberg, D. S. Wyand and S. D. Cohen. 1987. Ultrastructural Changes During Acute Acetaminophen-Induced Hepatotoxicity in the Mouse: A Time And Dose Study. Toxicologic Pathology. 15: 431-438.

Pober, J. S. 1988. Cytokine Mediated Activation of Vascular Endothelium. American Journal of Pathology. 133: 426-433.

Popper, H. 1988. Hepatocellular Degeneration and Death. In The Liver: Biology and Pathobiology, ed. I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, D. A. Shafritz, 1087-1104. New York: Raven Press.

Portman, B., I. C. Talbot, D. W. Day, A. R. Davidson, I. M. Murray-Lyon and R. Williams. 1975. Histopathological Changes in the Liver Following Paracetamol Overdose: Correlation with Clinical and

Biochemical Parameters. Journal of Pathology. 117: 169-181.

Potter, W. Z., D. C. Davis, J. R. Mitchell, D. J. Jollow, J. R. Gillette and B. B. Brodie. 1973. Acetaminophen Induced Hepatic Necrosis. III. Cytochrome P450-Mediated Covalent Binding IN VITRO. Journal of Pharmacology and Experimental Therapeutics. 187: 203-210.

Proudfoot, A. T. and N. Wright. 1970. Acute Paracetamol Poisoning. British Medical Journal. 3: 557-558.

Pumford, N. R., D. W. Roberts, R. W. Benson, and J. A. Hinson. 1990. Immunochemical Quantitation of 3-(Cystein-S-yl)Acetaminophen Protein Adducts in Subcellular Fractions Following A Hepatotoxic Dose of Acetaminophen. Biochemical Pharmacology. 40: 573-579.

Pumford, N. R., J. A. Hinson, R. W. Benson and D. W. Roberts. 1990. Immunoblot Analysis of Protein Containing 3-(Cystein-S-yl)acetaminophen Adducts in Serum and Subcellular Liver Fractions from Acetaminophen-Treated Mice. Toxicology and Applied Pharmacology. 104: 521-532.

Rappaport, A. M. 1975. Liver Architecture and Microcirculation. In Alcoholic Liver Pathology, ed. Khanna, Isreal, and Kalant, 43-67. Toronto: Addiction Research Foundation.

Recknagel, R. O., E. A. Glende and A. M. Hruszkewycz. 1977. Chemical Mechanisms in Carbon Tetrachloride Toxicity. In Free Radicals in Biology, 97-132. New York: Academic Press.

Recknagel, R. O., E. A. Glende, J. A. Dolak and R. L. Waller. 1989. Mechanisms of Carbon Tetrachloride Toxicity. Pharmacology and Therapeutics. 43: 139-154.

Reid, W. D., B. Christie, G. Krishna, J. R. Mitchell, J. Moskowitz and B. B. Brodie. 1971. Bromobenzene Metabolism and Hepatic Necrosis. Pharmacology. 6: 41-55.

Reid, W. D. 1972. Mechanism of Allyl Alcohol Induced Hepatic Necrosis. Experientia. 15: 1058-1061.

Reynolds, E. S., M. T. Molsen, S. Szabo, R. J. Jaeger and S. D. Murphy. 1975. Hepatotoxicity of Vinyl Chloride and 1,1-Dichloroethylene. American Journal of Pathology. 81: 219-236.

Reynolds, E. S., M. T. Moslen, P. J. Boor and R. J. Jaeger. 1980. 1,1-Dichloroethylene Hepatotoxicity. American Journal of Pathology. 101: 331-344.

Roberts, D. W., T. J. Bucci, R. W. Benson, A. R. Warbritton, T. A. McRae, N. R. Pumford and J. A. Hinson. 1991. Immunochemical Localization and Quantification of the 3-(Cystein-S-yl)-acetaminophen Protein Adduct in Acetaminophen Hepatotoxicity. American Journal of Pathology. 138: 359-371.

Rollins, B. J., T. Yoshimura, E. J. Leonard and J. S. Pober. 1990. Cytokine-activated Human Endothelial Cells Synthesize and Secrete a Monocyte Chemoattractant, MCP-1/JE. American Journal of Pathology. 136: 1229-1233.

Rumack, B. H. and H. Matthew. 1975. Acetaminophen Poisoning and Toxicity. Pediatrics. 55: 871-876.

Saba, T. M. 1970. Physiology and Physiopathology of the Reticuloendothelial System. Archives of Internal Medicine. 126: 1031-1052.

Salvemini, D., R. Korbut, E. Anggard and J. R. Vane. 1989. Lipopolysaccharide Increases Release of a Nitric Oxide-like Factor from Endothelial Cells. European Journal of Pharmacology. 171: 135-136.

Scandanavian Society for Clinical Chemistry and Clinical Physiology. 1974. Recomended Methods for the Determination of Four Enzymes in the Blood. Scandanavian Journal of Clinical Laboratory Investigation. 33: 291.

Seglen, P. O. 1976. Preparation of Isolated Rat Liver Cells. Methods in Cell Biology. 9: 29-83.

Shatkin, A. J. 1969. Colorimetric Reactions for DNA, RNA, and Protein Determinations. Fundamental Techniques in Virology, ed. K. Habel and N. P. Salzman, 231-237. New York: Academic Press.

Shiratori, Y., H. Teraoka, S. Matano, K. Matsumoto, K. Kamii and M. Tanaka. 1989. Kupffer Cell Function in Chronic Ethanol-Fed Rats. Liver. 9: 351-359.

Smilkstein, K., Gary L. Kenneth and B. H. Rumack. 1988. Efficacy of Oral N-Acetylcysteine in the Treatment of Acetaminophen Overdose. New England Journal of Medicine. 319: 1557-1562.

Smith, C. V. and J. R. Mitchell. 1985. Acetaminophen Hepatotoxicity IN VIVO is Not Accompanied by Oxidant Stress. Biochemical and Biophysical Research

Communications. 133: 329-336.

Snedecor, G. W. and W. G. Cochran. 1980. Statistical Methods. Ames: The Iowa State University.

Souhami, R. L., H. M. Patel and B. E. Ryman. 1981. The Effect of Reticuloendothelial Blockade on the Blood Clearance and Tissue Distribution of Liposomes. Biochimica et Biophysica Acta. 674: 354-371.

Steinberg, P., W. M. Lafranconi, C. R. Wolf, D. J. Waxman, F. Oesch, and T. Friedberg. 1987. Xenobiotic Metabolizing Enzymes are not Restricted to Parenchymal Cells in Rat Liver. Molecular Pharmacology. 32: 463-470.

Tirmenstein, M. A. and S. D. Nelson. 1990. Acetaminophen induced Oxidation of Protein Thiols. Journal of Biological Chemistry. 265: 3059-3065.

Tracey, D. E. 1983. Macrophage-Mediated Injury. In The Reticuloendothelial System: A Comprehensive Treatise, ed. N. R. Rose and B. V. Siegel, 77-101. New York: Plenum Press.

Tsokos-Kuhn, J. O., H. Hughes, C. Smith and J. Mitchell. 1988. Alkylation of the Liver Plasma Membrane and Inhibition of the Calcium ATPase by Acetaminophen. Biochemical Pharmacology. 37: 2125-2131.

Walberg, C. B. 1977. Determination of Acetaminophen in Serum. Journal of Analytical Toxicology. 1: 79-80.

Walker, R. M., W. J. Racz and T. F. McElligot. 1980. Acetaminophen Induced Hepatotoxicity in Mice. Laboratory Investigation. 42: 181-189.

Walker, R. M., W. J. Racz, and T. F. McElligott. 1983. Scanning Electron Microscopic Examination of Acetaminophen-Induced Hepatotoxicity and Congestion in Mice. American Journal of Pathology. 113: 321-330.

Walton, K. W. 1954. Investigation of the Toxicity of a Series of Dextran Sulphates of Varying Molecular Weight. British Journal of Pharmacology. 9: 1-14.

West, M. A., G. A. Keller, B. J. Hyland, F. B. Cerra and R. L. Simmons. 1986. Further Characterization of Kupffer Cell/Macrophage-Mediates Alterations in Hepatocyte Protein Synthesis. Surgery. 100: 416-423.

Williams, R. 1973. Paracetamol Hepatotoxicity in Man. Drugs and The Liver, ed. W. A. Sickinger and K.

Gerok, 337-345. New York: Stuttgart.

Wisse, E., A. Geerts, L. Bouwens, H. Van Bossuyt, K. Vanderkerken and F. Van Goethem. 1989. Cells of the Hepatic Sinusoid ,Anno 1988. In Cells of the Hepatic Sinusoid Volume 2, ed. E. Wisse, D. L. Knook, and K. Decker, 1-9. Netherlands: Kupffer Cell Foundation.

Wroblewski, F. and J. S. LaDue. 1955. Lactic Dehydrogenase Activity in Blood. Proceedings of the Society of Experimental Biology and Medicine. 90: 210-213.

Wyllie, A. H., J. F. R. Kerr and A. R. Currie. 1980. Cell Death: The Significance of Apoptosis. International Reviews of Cytology. 68: 251-306.

Younes, M. and C. P. Siegers. 1985. The Role of Iron in the Paracetamol and CCl4-Induced Lipid Peroxidation and Hepatotoxicity. Chemico-Biologico Interactions. 55: 327-334.

Zimmerman, H. J. 1982. Chemical Hepatic Injury and Its Detection. In Toxicology of the Liver, ed. G. L. Plaa and W. R. Hewitt, 1-46. New York: Raven Press.

APPENDIX A

ABBREVIATIONS

AA	Allyl Alcohol
ADH	Alcohol Dehydrogenase
ALT	Alanine Aminotransferase
APAP	Acetaminophen
AST	Aspartate Aminotransferase
BB	Bromobenzene
CCl_4	Carbon Tetrachloride
CM	Conditioned Media
DCE	1,1-Dichloroethylene
DEM	Diethylmaleate
DS	Dextran Sulfate
GBSS	Gey's Balanced Salt Solution
$GdCl_3$	Gadolinium Chloride
GSH	Glutathione
HBSS	Hank's Balanced Salt Solution
LDH	Lactate Dehydrogenase
L-15	Liebovitz's L-15 Media
MDH	Malate Dehydrogenase
NAD	Nicotinamide Adenine Dinucleotide (oxidized form)
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NPCs	Nonparenchymal Cells
NPC-CM	Nonparenchymal Cell Conditioned Media
RES	Reticuloendothelial System
TA	Thioacetamide

APPENDIX B

Equation for calculation of ADH, ALT, AST and LDH activity:

activity= {(AV)/(edtv)}x1000; where

A = change in absorbance

V = total assay volume

1000 = converts mmol NADH to μmol NADH

e = extinction coefficient (6.22×10^{2} $mmol^{-1}$ mm^{-1})

d = path length (10mm)

t = change in time (10 minutes)

v = volume of media added.