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CAUSAL ROLES FOR NEUTROPHILS AND GLUTATHIONE IN THE LIVER INJURY CAUSED BY ALPHA-NAPHTHYLISOTHIOCYANATE

presented by

Lawrence J. Dahm

has been accepted towards fulfillment
of the requirements for

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Toxicology

Robert A. Roth
Major professor

Date 2/9/90

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CAUSAL ROLES FOR NEUTROPHILS AND GLUTATHIONE IN THE LIVER INJURY CAUSED BY ALPHA-NAPHTHYLISOTHIOCYANATE

By

Lawrence J. Dahm

A DISSERTATION

Submitted to
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ABSTRACT

Causal Roles for Neutrophils and Glutathione in the Liver Injury Caused by alpha-Naphthylisothiocyanate

by

Lawrence J. Dahm

When administered to rats, alpha-naphthylisothiocyanate (ANIT) causes cholestasis and injury to bile ductular and hepatic parenchymal cells. The mechanism of toxicity is unknown.

An infiltration of neutrophils (PMNs) is associated with the lesion. PMNs cause injury to extrahepatic tissues by releasing toxic oxygen species and degradative enzymes. To determine whether PMNs were involved in ANIT hepatotoxicity, rats were treated with antineutrophil serum (NAS). Administration of ANIT to rats produced cholestasis and elevations in serum of total bilirubin concentration, total bile acid concentration, aspartate aminotransferase activity, and gamma-glutamyltransferase activity. Co-treatment of rats with NAS reduced circulating numbers of PMNs and prevented the cholestasis and elevations in markers of liver injury caused by ANIT. Administration of a combination of superoxide dismutase and catalase coupled to polyethylene glycol to rats caused large elevations of enzyme activities in serum but did not afford protection. These results suggest that PMNs mediate ANIT-induced liver injury by a mechanism which may be independent of toxic oxygen species.

It is commonly believed that ANIT must undergo bioactivation by hepatic, cytochrome P-450-dependent, mixed function oxidases (MFO), since agents

which are inhibitors or inducers of hepatic MFO attenuate or enhance, respectively, ANIT-induced liver injury. Several of these agents alter hepatic glutathione (GSH) content and/or GSH S-transferase activity in a manner to suggest a causal or permissive role for GSH in the pathogenesis. To test the hypothesis that GSH plays a role in ANIT hepatotoxicity, we determined whether agents which decrease hepatic non-protein sulfhydryl (NPSH) content, an indicator of GSH content, afforded protection. Administration of buthionine sulfoximine, diethyl maleate, or phorone to rats decreased hepatic NPSH content and prevented the liver injury caused by ANIT.

In conclusion, results from studies performed in this thesis suggest that both PMNs and GSH are causally involved in ANIT-induced liver injury. Whether or not these components of injury are related remains to be answered. That PMNs are involved is the first evidence to our knowledge of a PMN-dependent component in chemically-induced liver injury. Thus, we may have uncovered a novel mechanism by which ANIT and perhaps other hepatotoxicants act.

To my mom and dad,

for their love and encouragement

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LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
ANIT	alpha-naphthylisothiocyanate
ANOVA	analysis of variance
ARL	adult rat liver
AST	aspartate aminotransferase
BSA	bovine serum albumin
BSO	L-buthionine-S,R-sulfoximine
CAT	catalase
CDC	chenodeoxycholate
CMC	critical micellar concentration
CO	corn oil
CS	control serum
DEM	diethyl maleate
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
FMLP	formyl-methionyl-leucyl-phenylalanine
GCDC	glycochenodeoxycholate
GGT	gamma-glutamyltransferase
GLC	glycolithocholate

GSH	glutathione
HBSS	Hanks' balanced salt solution
hr	hour
i.m.	intramuscular
i.p.	intraperitoneal
KRBB	Kreb's-Ringer bicarbonate buffer
LC	lithocholate
LDH	lactate dehydrogenase
LT	leukotriene
min	minute
MFO	mixed function oxidase
NAS	antineutrophil serum
NPSH	non-protein sulfhydryl
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PMA	phorbol myristate acetate
PMN	neutrophil
RBC	red blood cell
SAL	saline
s.c.	subcutaneous
SOD	superoxide dismutase
TCDC	taurochenodeoxycholate
TLC	taurolithocholate
TSBA	total serum bile acids
WBC	white blood cell

CHAPTER I

INTRODUCTION

Classification of Hepatotoxicants

Agents which cause hepatotoxicity in humans have been classified into two broad categories (Klatskin, 1969). In this classification, agents cause either "toxic hepatitis" or "drug-induced hepatitis". Agents causing "toxic hepatitis" produce a dose-related, reproducible lesion in all exposed individuals after a predictable latent period. In addition, the lesion is reproducible in experimental animals. Agents which cause "drug-induced liver injury" (hepatitis) cause a lesion with a variable, non dose-related histopathology. There is an absence of a temporal relationship from administration of the agent to the onset of liver injury. Only a small fraction of individuals receiving therapy are affected, and the lesions are often not reproducible in experimental animals. Because there are few animal models, mechanisms of "drug-induced liver injury" are poorly understood.

"Drug-Induced Liver Injury"

Agents causing "drug-induced liver injury" may be classified into three main categories, which include cytotoxic, cholestatic, and mixed forms of hepatotoxicity (Zimmerman, 1978). Agents in the cytotoxic class may cause either hepatocellular necrosis or steatosis as exemplified by halothane and tetracycline, respectively. In general, cytotoxic agents cause large elevations in transaminase activities in blood, and they produce a lesion resembling viral hepatitis. Cholestatic agents cause either a cholangiolitic or a canalicular lesion. Cholangiolitic-type agents, i.e., erythromycin estolate and chlorpromazine, cause cholestasis characterized by mild hepatic parenchymal cell injury, modest increases in transaminase activities in blood (< 10-fold), large elevations in blood

alkaline phosphatase activity, and portal inflammatory infiltrates consisting of neutrophils (PMNs), lymphocytes, and eosinophils. The lesion appears to resemble obstructive jaundice. Agents causing canalicular cholestasis, i.e., contraceptive steroids, produce mild hepatic parenchymal cell injury similar to cholangiolitic-type agents. However, there is neither a portal inflammatory infiltrate nor a large rise in alkaline phosphatase activity in blood. Agents which cause a mixed-type of lesion, i.e., sulfonamides, have characteristics of cytotoxic and cholestatic groups of liver injury.

The incidence of "drug-induced liver injury" is low. For example, it occurs in 1-2% of individuals exposed to cholangiolitic-type agents, i.e., erythromycin estolate (Zimmerman, 1978). The incidence of massive, hepatocellular necrosis after halothane anesthesia may be as low as 1 in 22,000-35,000 exposures (Davis *et al.*, 1981). The mortality rate in individuals with "drug-induced liver injury" varies from 50% in cytotoxic injury to less than 1% in cholangiolitic-cholestatic liver injury (Zimmerman, 1978). Chronic, "drug-induced" intrahepatic cholestasis, however, may produce clinical, biochemical, and histologic features of primary biliary cirrhosis, which may be life threatening. Therefore, any type of "drug-induced liver injury" may present a serious health problem, and mechanisms by which suspected agents cause "drug-induced liver injury" are not well understood. Therefore, treatment is largely supportive (Zimmerman, 1978).

"Drug-induced liver injury" has been described as idiosyncratic due to the unpredictable and low incidence of injury. This suggests an unusual host susceptibility rather than intrinsic toxicity of the agent may be an important factor in the development of liver injury. Zimmerman (1978) has proposed that host idiosyncracy may result from drug allergy or from metabolic abnormalities. These are discussed briefly below.

Criteria for an allergic basis as a mechanism of "drug-induced liver injury" include a relatively fixed sensitization period, prompt onset of liver injury upon readministration of the agent, a high incidence of fever, rash, or eosinophilia, and eosinophilic or granulomatous inflammatory lesions in the liver (Zimmerman, 1978). However, an allergic reaction to a drug does not necessarily precipitate liver injury, since certain agents, i.e., penicillin and procainamide, cause allergic reactions and only rarely cause hepatic dysfunction. Acute hepatotoxicity of the agent may target the liver for the allergic reaction. For example, erythromycin estolate and chlorpromazine impair function of the isolated, perfused rat liver and cause toxicity to hepatocytes *in vitro* (Zimmerman *et al.*, 1974; Dujovne, 1975; Kendler *et al.*, 1972; Kendler *et al.*, 1971; Dujovne *et al.*, 1968, Gaeta *et al.*, 1985), and they may cause an hepatic lesion in man suggestive of an allergic reaction (Tolman *et al.*, 1974; Zimmerman, 1978). The requirement for pre-existing liver injury to target the liver for an allergic reaction suggests that "drug-induced liver injury" may be multifactorial in nature. While erythromycin estolate and chlorpromazine cause injury to isolated organ or cell preparations, they do not cause a "drug-induced", cholestatic lesion in animals similar to that in man (Plaa and Priestly, 1977; Fiebiger *et al.*, 1983).

As suggested by Zimmerman (1978), agents causing "drug-induced liver injury" which do not fit the criteria for allergic reactions may cause hepatotoxicity as a result of abnormalities in host metabolism. Individuals sensitive to a particular drug might produce an hepatotoxic metabolite(s) that the majority of the population does not, or they might produce greater amounts of an hepatotoxic metabolite(s). As pointed out by Zimmerman (1978), the basis for the metabolic abnormality might be genetic, or it might result from exposure to other agents which modify the metabolism of the drug in question.

Toxicology of ANIT

ANIT and drug-induced liver injury. Alpha-Naphthylisothiocyanate (ANIT) has been studied widely as a model hepatotoxicant (see Figure I-1 for structure). Chronic treatment of rats with ANIT results in bile duct proliferation and biliary cirrhosis (McClean and Rees, 1958). Acute treatment of rats or mice with ANIT causes cholestatic liver injury (Eliakim *et al.*, 1959; Roberts and Plaa, 1965; Plaa and Priestly, 1977). We are interested in the mechanism of acute ANIT hepatotoxicity as it relates to chemically-induced liver injury. In addition, the study of acute ANIT hepatotoxicity might provide some clues as to how certain agents cause "drug-induced liver injury". Two observations support this proposal. First, ANIT produces a lesion not unlike those agents which cause the cholangiolitic type of "drug-induced liver injury" in humans. For example, ANIT treatment of rats causes cholestasis, mild hepatocellular injury, and a portal inflammatory component, i.e., a lesion which resembles in some respects that in human livers caused by chlorpromazine or erythromycin estolate (Zimmerman, 1978). Second, Roberts and Plaa (1966a) have proposed that co-treatment of animals with a threshold dose of ANIT and a suspected drug might serve as a useful method to uncover cholestatic potential of the drug. Interestingly, certain drugs known to be cholestatic to humans, i.e. acetohexamide and norethandrolone, do potentiate the cholestatic effects of ANIT (Roberts and Plaa, 1966a). Although ANIT-induced liver injury in animals might provide insight about mechanisms of "drug-induced liver injury", it must be emphasized that it is not a model of "drug-induced liver injury". For example, ANIT causes an hepatic lesion in rats which is reproducible and occurs after a predictable latent period.

Species susceptibility to ANIT. There appears to be a species difference in susceptibility to the hepatotoxic effects of ANIT. ANIT causes liver injury

accompanied by cholestasis and hyperbilirubinemia in rats, mice, guinea pigs, and hamsters, although hamsters are much more resistant. Dogs and rabbits are insensitive to the cholestatic effects of ANIT (Indacochea-Redmond and Plaa, 1971; Capizzo and Roberts, 1971). The mechanism for the species difference is not known, although it might relate to differences in metabolism of ANIT (Capizzo and Roberts, 1971).

Hepatotoxicity of ANIT analogs. Becker and Plaa (1965a) examined several analogs of ANIT and determined what structural characteristics of the ANIT molecule might be necessary to elicit hyperbilirubinemia in mice. Alpha-Naphthylisocyanate, the analog in which oxygen replaces the sulfur atom, did not cause hyperbilirubinemia. Therefore, the isothiocyanate moiety appeared to be essential. Alkyl isothiocyanates also did not cause hyperbilirubinemia, indicating that the aryl group was necessary. A planar configuration also appeared to be required, since fluoroscein isothiocyanate, which is aryl but not planar, did not cause hyperbilirubinemia. Phenylisothiocyanate and *B*-naphthylisothiocyanate (BNIT) fit the criteria for cholestatic activity proposed by Becker and Plaa (1965a), i.e., a planar, aryl isothiocyanate . BNIT was inactive in mice and rats, and phenylisothiocyanate caused hyperbilirubinemia in mice, although not in rats (Becker and Plaa, 1965a; Plaa and Priestly, 1977). The reasons for the inactivity of BNIT and the species difference observed with phenylisothiocyanate are unknown. Other aryl isothiocyanates have been shown subsequently to produce cholestatic liver injury, and they include the schistosomicidal drug amoscanate (4-isothiocyano-4'-nitrodiphenylamine) and *p*-phenylenediisothiocyanate (Batzinger *et al.*, 1981; Selye and Szabo, 1972). These agents appear to fit the criteria for cholestatic activity proposed by Becker and Plaa (1965a).

Histopathological Changes in Rat Liver After ANIT Treatment. Liver histopathology following a single administration of ANIT (100-200 mg/kg, p.o.) to rats has been well characterized by light microscopy. Six hours after treatment, portal tracts are mildly edematous and infiltrated with PMNs, mononuclear cells, and eosinophils (McClean and Rees, 1958). At 12 hours, interlobular bile duct epithelial cells are swollen and surrounded by a dense inflammatory cell infiltrate. At 24 hours, the normal cuboidal to columnar epithelium of interlobular bile ducts is swollen or necrotic, and amorphous, cellular debris fill ductular lumens (McClean and Rees, 1958; Goldfarb *et al.*, 1962; Ungar *et al.*, 1962). PMNs are concentrated in the walls of bile ducts (Ungar *et al.*, 1962). Scattered foci of hepatocellular necrosis are observed primarily in periportal regions of the liver (Ungar *et al.*, 1962; Goldfarb *et al.*, 1962; Desmet *et al.*, 1968; Schaffner *et al.*, 1973), although midzonal and centrilobular regions might be affected as well (McClean and Rees, 1958). By 48 hours, the bile duct epithelial cell necrosis appears to be subsiding. Intrahepatic bile ductules appear to increase in number (i.e., bile duct proliferation) around portal areas at 48 hours, although extensive bile duct proliferation is usually associated with chronic ANIT treatment. By 72-96 hours, bile duct epithelial cells have regenerated to line interlobular bile ducts, and the periductular inflammation starts to disappear. The ductular proliferation gradually regresses, and the liver takes on a normal appearance by day 19 (Goldfarb *et al.*, 1962).

Schaffner *et al.* (1973) described ultrastructural changes in the liver after a single administration of ANIT (100 mg/kg, p.o.). At 3 hours, there was dilatation of lamellae of the Golgi zone in hepatocytes and mitochondrial changes including swelling and breaks in the outer mitochondrial membrane. These changes were observed primarily in periportal hepatocytes, although many centrilobular hepatocytes had abnormal mitochondria. The endoplasmic

reticulum and bile canaliculi appeared normal at this time. Epithelial cells of small bile ducts contained luminal surface blebs and breaks in the outer mitochondrial membrane. Endothelial cells of the sinusoid and portal blood vessels, primarily those of hepatic arterioles, were swollen and vacuolated.

All structural alterations observed at 3 hours were more pronounced at 24 hours, except that sinusoidal endothelial cells appeared to return to normal. Additional changes were observed in hepatocytes and bile duct epithelial cells. Hepatocytes showed increased mitochondrial division and dilatation of bile canaliculi, primarily in periportal regions. The smooth endoplasmic reticulum appeared to be increased throughout the lobule, while the rough endoplasmic reticulum was decreased only in periportal regions. Bile duct epithelial cells were necrotic. Hepatic ultrastructure at 48 hours was similar to that at 24 hours. Schaffner *et al.* (1973) noted that alterations in mitochondria and the cell surface of hepatocytes and bile duct epithelial cells were the most striking features of the lesion, and hepatocytes appeared to be affected first.

Recently, Connolly *et al.* (1988) reported that bile duct epithelial cells appeared to be affected before hepatocytes after ANIT treatment (300 mg/kg, p.o.). By 4 hours, bile duct epithelial cells were vacuolated and lacked microvilli. By 6 hours, there was detachment of the nuclear membrane and vacuolization of the endoplasmic reticulum. Very few changes in hepatocytes were observed in the first 6 hours after ANIT treatment. The reasons for the disparate results results between those of Connolly *et al.* (1988) and Schaffner *et al.* (1973) may be related to differences in dosage or unknown factors. However, it is evident from light and electron microscopy studies that bile duct epithelial cells, hepatocytes, and endothelial cells of the sinusoid and portal blood vessels are affected by ANIT.

Functional and toxicological changes after ANIT treatment. The cholestasis in rats after acute treatment with ANIT has received much attention. Sixteen to twenty-four hours after a single oral administration of ANIT, bile flow is reduced (Plaa and Priestly, 1977). There may be complete cessation of bile flow, and it appears to correlate with the necrosis and desquamation of interlobular bile ducts (Ungar *et al*, 1962; Goldfarb *et al*. 1962). Bile flow returns to normal by 4 days and coincides with re-epithelialization of bile ducts (Goldfarb *et al*., 1962).

Although the mechanism of ANIT-induced cholestasis is not clear, several investigators have proposed that mechanical obstruction of bile ducts might occur as a result of desquamated bile duct epithelial cells filling ductular lumens (Ungar *et al*., 1962; Goldfarb *et al*., 1962; Woolley *et al*., 1979). In this proposed mechanism, bilirubin and other biliary constituents might reflux back into sinusoidal blood. Woolley *et al*. (1979) demonstrated that several agents commonly found in bile appeared in blood after ANIT treatment, and they took these observations as evidence for biliary obstruction. These agents included IgA, free IgA secretory component, conjugated bilirubin, and the biliary isozyme of 5'-nucleotidase. Although the hepatocyte plays some role in the handling of each of these agents, i.e., transport of IgA, conjugation of bilirubin, synthesis of IgA secretory component, these investigators proposed that it is unlikely that the appearance of all agents in plasma resulted from a generalized effect of ANIT on hepatocytes. This possibility cannot be dismissed, however.

Other investigators have suggested that ANIT-induced cholestasis is a result of hepatocellular deficits rather than mechanical obstruction of bile ducts. Steiner *et al*. (1963) and Steiner and Baglio (1963) proposed that ANIT-induced cholestasis may be a result of disturbed transport, conjugation, or excretory capacity of hepatocytes. Using whole animals or livers isolated from ANIT-treated rats, others have demonstrated reduced biliary excretion of cholephilic

agents such as bilirubin, bile acids, and bromosulfophthalein (BSP) (Becker and Plaa, 1965b; Roberts and Plaa, 1967; Krell *et al.*, 1982).

Changes in serum or plasma bilirubin concentration appear to coincide with changes in bile flow. For example, hyperbilirubinemia occurs by 24 hours after ANIT treatment, the same time that cholestasis sets in (Goldfarb *et al.*, 1962). Serum bilirubin concentration is maximally elevated at 3 days and returns toward control by 4-5 days when the cholestasis is subsiding (Goldfarb *et al.*, 1962; Leonard *et al.*, 1984). Elevations in serum or plasma bilirubin appear to be composed almost entirely of conjugated bilirubin (Moran *et al.*, 1961; Becker and Plaa, 1965b).

As in the case with cholestasis, hyperbilirubinemia might result from reflux of biliary bilirubin into sinusoidal blood, similar to that which occurs after bile duct ligation. Alternatively, as suggested by Roberts and Plaa (1967), alterations in hepatic bilirubin handling might account for the observed hyperbilirubinemia. For example, the hepatic clearance of exogenously administered bilirubin is reduced in mice and rats treated with ANIT before cessation of bile flow occurs. In these studies, the maximal rate of bilirubin excretion into bile and biliary bilirubin concentration are diminished significantly. In addition, ANIT appears to affect hepatic uptake and storage of bilirubin. For example, the plasma disappearance of an exogenous bilirubin load and hepatic bilirubin concentration are reduced in ANIT-treated, bile duct-ligated mice, when compared to bile duct-ligated control mice. Roberts and Plaa (1968) also demonstrated that ANIT treatment enhances synthesis of bilirubin, an effect which is not linked to erythropoiesis. Taken together, these results suggest that ANIT-induced hyperbilirubinemia might result from enhanced bilirubin synthesis, reflux of biliary bilirubin, and/or hepatocellular deficits in uptake, storage, and excretion of bilirubin.

ANIT treatment of rats also depresses the plasma clearance of the cholephilic anion, BSP, which is a commonly used measure of liver function. BSP is normally taken up by hepatocytes, conjugated to glutathione (GSH), and secreted into bile. ANIT appears to reduces plasma BSP clearance in mice as early as 2 hours after a single administration (Becker and Plaa, 1965b). It does not appear to affect the uptake of BSP by hepatocytes nor the conjugation of BSP to GSH. Alternatively, the reduction in plasma BSP clearance is thought to result from leaky tight junctions at biliary canaliculi (Krell *et al.*, 1982). Presumably, concentration gradients across the canalicular membrane cannot be maintained as a result of the leaky tight junctions, and reduced biliary excretion of BSP ensues.

Acute administration of ANIT to rodents also reduces the activity and content of hepatic MFO. As early as 2 hours after a single treatment of ANIT, there are reductions in the activities of puromycin-N-demethylase, aminopyrine demethylase, and aniline hydroxylase either in hepatic microsomes or the 10,000 x g supernatant fraction (Derr *et al.* 1967; Drew and Priestly, 1976). Inhibition of hepatic MFO activity by ANIT is reflected in prolonged sleep time from hexobarbital or pentobarbital administration and prolonged paralysis time due to zoxazolamine administration (Drew and Priestly, 1976; Buxton *et al.*, 1973). It is unclear whether altered drug metabolism is a result of cytochrome P-450 destruction, since inhibition of MFO activity, prolongation of sleep time from barbiturates, and prolongation of paralysis time from zoxazolamine described above occur within a few hours after ANIT treatment, whereas reductions in cytochrome P-450 content occurs at 12-48 hours (El-Hawari and Plaa, 1979; Gallenkamp and Richter, 1974).

Many of the hepatic functional deficits described above have been used as markers of liver injury in experimental studies employing ANIT. Certain

markers, however, may be intimately related to the mechanism of injury. For example, elevations of hepatic and serum bile acid concentration may have mechanistic significance, since bile acids have detergent-like activity and may be hepatotoxic. ANIT treatment elevates hepatic and serum concentrations of bile acids by approximately 4 and 400-fold, respectively (Schaffner *et al.*, 1973; Leonard *et al.*, 1984). Therefore, as suggested by Connolly *et al.* (1988), bile acids might participate in the liver injury caused by ANIT. On the other hand, it is difficult to imagine how a reduction in hepatic MFO activity might be related to the pathogenesis. Certainly other compounds reduce hepatic MFO activity without causing cholestatic liver injury like ANIT. BNIT, an isomer of ANIT, does not cause cholestatic liver injury yet affects hepatic MFO activity similarly to ANIT (El-Hawari and Plaa, 1979). In addition, rabbits are not susceptible to ANIT-induced liver injury, and they show reductions in hepatic MFO activity (Capizzo and Roberts, 1971; El-Hawari and Plaa, 1979).

Other markers used to assess the liver injury associated with ANIT treatment are indicators of necrosis. These markers appear in blood and include aminotransferases, i.e., aspartate aminotransferase (AST) and alanine aminotransferase (ALT), alkaline phosphatase, 5'-nucleotidase, and gamma-glutamyltransferase (GGT). Elevations in activities of AST and ALT in plasma or serum after ANIT treatment indicate specific damage to hepatocytes in this model (Moran *et al.*, 1961; Leonard *et al.*, 1984). After a single administration of ANIT (150-200 mg/kg, p.o. or i.p.) to rats, ALT activity in plasma or serum is elevated as early as 2 hours, rises to a maximal level by 3 days, and returns to normal by 5 days (Drew and Priestly, 1976; El-Hawari and Plaa, 1979; Leonard *et al.*, 1984). Maximal serum ALT activity after ANIT treatment may be elevated 30-fold (Leonard *et al.*, 1984), although this change is mild compared to a hepatic necrogenic agent such as CCl_4, which can elevate serum ALT activity several

thousand fold. Alkaline phosphatase and 5'-nucleotidase activities in blood have been used widely as an indicator of intrahepatic and extrahepatic bile stasis. After ANIT treatment, activities of 5'-nucleotidase and alkaline phosphatase in plasma or serum are elevated at 18-24 hours and peak at 2-3 days (El-Hawari and Plaa, 1979; Leonard *et al.*, 1984). GGT is localized in bile duct epithelial cells in the liver (Szewczuk *et al.*, 1980), and release of GGT into blood and bile after ANIT treatment appears to correlate with the degree of bile duct epithelial cell necrosis in the ANIT model (Leonard *et al.*, 1984; Connolly *et al.*, 1988; Zafrani *et al.*, 1989). Serum GGT activity peaks approximately 1 day after a single oral dose of ANIT and returns to control by 5 days (Leonard *et al.*, 1984).

Toxicity of ANIT to extrahepatic tissues. Aside from the toxic effects of ANIT on hepatocytes and bile duct epithelial cells in rodents, there have been few other targets reported. ANIT causes toxicity to extrahepatic bile duct epithelium in rats (McClean and Rees, 1958) and the gall bladder in mice (Ungar and Popp, 1972). Another minor alteration observed in rats after acute treatment with ANIT is a transient swelling of kidney tubules (McClean and Rees, 1958). Polyuria, which is thought to occur secondary to polydipsia, is observed in rats treated chronically with ANIT (Tur-Kaspa *et al.*, 1983), although it is not known whether this effect occurs in rats after acute treatment.

Variability associated with ANIT-induced liver injury. A source of variability in the ANIT model is that all animals do not respond to ANIT after acute administration, even when relatively large doses are administered. There are published reports of non-responding animals (Schaffner *et al.*, 1973; Woolley *et al.*, 1979), although the reasons for such have not been addressed. In these studies, the incidence of hyperbilirubinemia in ANIT-treated rats was approximately 75%. It seems unlikely that a problem in administration or some

peculiarity in the treatment regimen might account for the variability, since reports of non-responding animals have come from different laboratories.

Role for bioactivation in ANIT-induced liver injury. Evidence that ANIT undergoes metabolism was reported by Capizzo and Roberts (1970). When ^{14}C-ANIT labelled on the isothiocyanate carbon was administered to rats, $^{14}CO_2$ was detected in expired gases, indicating that the isothiocyanate moiety was metabolized. Furthermore, metabolism of ANIT by hepatic MFO appears to be required for hepatotoxicity, since agents which are inducers or inhibitors of hepatic MFO activity increase or decrease, respectively, the liver injury caused by ANIT. Inducers of hepatic MFO activity, including phenobarbital, 3-methylcholanthrene, and chlorpromazine, potentiate ANIT-induced hyperbilirubinemia in rats and mice (Roberts and Plaa, 1965; El-Hawari and Plaa, 1977). When ANIT-treated mice are killed when the incidence of cholestasis is approximately 40%, i.e., 10 hr after treatment, prior induction of hepatic MFO with phenobarbital and chlorpromazine increases the incidence of cholestasis to 100% (Roberts and Plaa, 1965). In mice treated with phenobarbital or chlorpromazine, co-treatment with an inhibitor of protein synthesis, i.e., actinomycin D or ethionine, prevents the increased incidence of cholestasis (Roberts and Plaa, 1965), suggesting that the enhanced incidence of cholestasis is a result of increased synthesis of hepatic MFO. Connolly *et al.* (1988) have reported that induction of hepatic MFO activity increases the toxicity of ANIT to bile duct epithelial cells.

Agents which are inhibitors of hepatic MFO activity, including piperonyl butoxide, BNIT, disulfiram, diethyldithiocarbamate, and cobaltous chloride, reduce ANIT-induced hyperbilirubinemia (Roberts and Plaa, 1965; El-Hawari and Plaa, 1977; Traiger *et al.*, 1984). The capacity of SKF 525-A to reduce or increase the incidence of cholestasis in mice treated with ANIT corresponds to

the biphasic effects of SKF 525-A on inhibition and stimulation of hepatic MFO activity, respectively (Roberts and Plaa, 1965). In addition to reductions in hyperbilirubinemia and the incidence of cholestasis, hepatic MFO inhibitors reduce bile duct epithelial cell necrosis (Connolly *et al.*, 1988).

Other evidence for bioactivation has been provided by Traiger *et al.* (1984). When rats are administered ^{35}S-ANIT which is labelled on the isothiocyanate moiety, they excrete inorganic sulfate in urine. This result indicates that metabolism of the isothiocyanate moiety occurs, and it confirms the result of Capizzo and Roberts (1970). Traiger *et al.* (1984) demonstrated that agents which increase or decrease ANIT-induced liver injury *in vivo* increase or decrease, respectively, urinary sulfate excretion from ^{35}S-ANIT. For example, pretreatment of rats or mice with phenobarbital, which potentiates the liver injury associated with ANIT (Roberts and Plaa, 1965; El-Hawari and Plaa, 1977), increases urinary sulfate excretion by 300% in the first 12 hours after ANIT treatment. Although the study by Traiger *et al.* (1984) does not provide any information as to the identity of the hypothetical toxic metabolite, it emphasizes the importance of metabolism in ANIT's mechanism of toxicity.

Taken together, results from studies employing inhibitors and inducers of hepatic MFO suggest that ANIT is biotransformed to cause liver injury. Other studies have suggested that unimpaired protein and/or RNA synthesis is required in the pathogenesis of ANIT-induced liver injury, since commonly used inhibitors of protein and RNA synthesis reduce the hyperbilirubinemia associated with ANIT (Indacochea-Redmond *et al.*, 1973, 1974). These agents include cycloheximide, actinomycin D, ethionine, and puromycin. Indacochea-Redmond *et al.* (1973) suggested that these agents reduced ANIT-induced hyperbilirubinemia by inhibiting synthesis of hepatic macromolecules, probably MFO enzymes. However, they reduce the hyperbilirubinemia associated with

ANIT at doses which do not inhibit protein or RNA synthesis (Indacochea-Redmond *et al.*, 1973, 1974), indicating that their mechanism of protection may not relate to effects on protein and/or RNA synthesis. It is not known whether the protection afforded by these agents relates to inhibition of hepatic MFO activity or other effects (Indacochea-Redmond *et al.*, 1973).

Although it is commonly believed that ANIT must undergo bioactivation by hepatic MFO, others have questioned this hypothesis. Using ARL 3 (adult rat liver) cells, Williams (1974) showed that ANIT was toxic to these cells *in vitro*, and the toxicity was not reduced by pretreatment with SKF 525-A. Addition to the culture system of a 10,000 x g supernatant fraction from liver to provide a source of MFO did not enhance the cytotoxicity of ANIT, although it did enhance the toxicity of agents, i.e., CCl_4, dimethylnitrosamine, which require bioactivation. These results suggested that ANIT did not require bioactivation to cause toxicity to epithelial-like cells from adult rat liver *in vitro*, and they called into question the need for bioactivation *in vivo*.

As suggested by Williams (1974), unaltered ANIT may be responsible for some of the toxic effects of ANIT *in vivo*. Others have demonstrated that not all markers of liver injury are affected in the same manner by agents which alter ANIT-induced liver injury *in vivo*. For example, cycloheximide and ethionine prevent the hyperbilirubinemia and cholestasis associated with ANIT, but they do not prevent the early reduction of BSP clearance or prolongation of pentobarbital sleep time (Indacochea-Redmond *et al.*, 1973, 1974). Taken together, these observations suggest that different ANIT species, possibly unaltered ANIT itself, are involved in the injurious process.

Williams (1974) also observed that other isothiocyanates which do not cause cholestatic liver injury *in vivo*, i.e., BNIT, allyl isothiocyanate, fluoroscein isothiocyanate, cause toxicity to ARL 3 cells. As a result of this observation, Plaa

and Priestly (1977) have questioned the appropriateness of the cell culture model to elucidate possible mechanism of ANIT toxicity *in vivo*.

Disposition of ANIT in the rat. Using ^{14}C ANIT labelled on the isothiocyanate carbon, Capizzo and Roberts (1970) examined the tissue disposition and excretion patterns of ^{14}C in rats at various times after ANIT treatment (50-300 mg/kg, p.o.). They showed that approximately 70% of radiolabelled ANIT was absorbed from the gastrointestinal tract within 24 hours and was found widely distributed in body tissues. Plasma concentration of ^{14}C reached a peak at 24 hours, and it remained near this concentration until 72 hours which was the end of the study period. Plasma concentration of ^{14}C from 24-72 hours may have been maintained by redistribution from fat, since the latter tissue showed a large uptake of ^{14}C between 4 and 12 hours and release between 12 to 24 hours. The liver accumulated ^{14}C from ^{14}C-ANIT which was several times that of any other organ examined. ^{14}C concentration in the liver ranged between 2 and 3.5% of the administered dose from 4-72 hours with a maximum at 12 hours. The kidney was the only other organ examined which accumulated a level greater than 0.5% of the administered dose of ANIT. After 72 hours, approximately 80% of the administered dose of ^{14}C from ^{14}C-ANIT was recovered in the urine (40%), expired gases (30%), and feces (10%). It was noteworthy that $^{14}CO_2$ was liberated in expired gases, since it demonstrated that the isothiocyanate moiety of ANIT was metabolized. However, it also indicated that distribution studies using ^{14}C-ANIT labelled on the isothiocyanate carbon do not necessarily give information on the fate of the naphthalene nucleus.

Metabolism of ANIT. After the observation by Roberts and Plaa (1965) that ANIT may undergo bioactivation by hepatic MFO, there has been considerable interest in the metabolism of ANIT. As mentioned earlier, Capizzo

and Roberts (1970) and Traiger *et al.* (1984) obtained evidence that the isothiocyanate moiety of ANIT was metabolized. Further insight into the metabolism of ANIT was provided by studies utilizing dually labelled ANIT (Lock *et al.*, 1974; Skelton *et al.*, 1975). ^{14}C-ANIT (isothiocyanate carbon) and ^{3}H-ANIT (position 4 of naphthalene ring) were mixed to yield an $^{3}H/^{14}C$ specific activity ratio of 6.3, and this mixture was administered to rats (300 mg/kg, p.o.). In blood, the ratio of $^{3}H/^{14}C$ was close to 6.3 a few minutes after dosing. By 30-60 minutes, the ratio dropped to approximately 5.3 and then stabilzed at an $^{3}H/^{14}C$ ratio of 5.8 from 1.5-8 hr. In bile, the ratio of $^{3}H/^{14}C$ remained close to 6.3 for 30 minutes after ANIT administration. Between 1 and 2 hours, the ratio rose to approximately 7.5, and it remained at this value until the end of the study (8 hours). That the $^{3}H/^{14}C$ ratio in blood and bile changed at all indicated that ANIT was metabolized. The decrease in the $^{3}H/^{14}C$ ratio in blood suggested further the presence of a metabolite(s) with predominantly more radiolabel on the isothiocyanate moiety, i.e. a metabolites(s) devoid of the naphthalene ring. This observation is in agreement with those of Capizzo and Roberts (1970) and Traiger *et al.* (1984) who demonstrated that metabolism of the isothiocyanate moiety occurred *in vivo*. In bile, the ratio of $^{3}H/^{14}C$ increased, suggesting the presence of a metabolite(s) with predominantly more label on the naphthylene ring. This observation was interpreted as evidence for a metabolite(s) in bile which contained a modified naphthalene ring only.

Lock *et al.* (1974) examined the effects of cycloheximide, an agent which prevents the hyperbilirubinemia and cholestasis caused by ANIT, on the ratio of $^{3}H/^{14}C$ in blood and bile of ANIT-treated rats. Co-treatment of rats with cycloheximide had little effect on ANIT-induced changes in the $^{3}H/^{14}C$ ratio in blood. However, cycloheximide prevented the ANIT-induced elevation in the $^{3}H/^{14}C$ ratio in bile and maintained it near 6.3 for the 8 hour study. These

results indicated that cycloheximide prevented formation of the proposed biliary metabolite(s) which contains the modified naphthalene ring. Since the $^{3}H/^{14}C$ ratio remained at 6.3, cycloheximide treated rats presumably excreted unaltered ANIT in bile. That the protection afforded by cycloheximide against ANIT hepatotoxicity *in vivo* appeared to be associated with the disappearance of the biliary metabolite(s) suggested to the authors that the biliary metabolite(s) was a toxic metabolite(s) of ANIT. It is unclear how the toxic metabolite of ANIT might cause cholestasis. As suggested by Plaa and Priestly (1977), it might exert its cholestatic effect by affecting canalicular bile formation during biliary excretion. Alternatively, after delivery into the intestinal tract via bile, the toxic metabolite might cause cholestasis somehow subsequent to reabsorption into the portal circulation (Lock *et al.*, 1974). The need for an intact enterohepatic circulation for the development of liver injury after ANIT treatment was reported by Roberts and Plaa (1966b).

Although much attention has focused on bioactivation of ANIT, no toxic metabolite(s) has yet been identified. In studies of the hepatotoxicity and metabolism of ^{14}C-ANIT (isothiocyanate carbon) in various species, Capizzo and Roberts (1971) demonstrated the presence of several urinary ^{14}C metabolites of ANIT by thin layer chromotography. Interestingly, the rabbit, which was the only species tested not susceptible to ANIT-induced liver injury, did not excrete a ^{14}C metabolite in urine like other susceptible species. However, identification of this urinary metabolite was not made. To date, the only known metabolite of ANIT is alpha-naphthylamine which is excreted in bile and urine (Mennicke *et al.*, 1978). It is unknown whether alpha-naphthylamine is involved in ANIT-induced liver injury, although it seems unlikely since it does not apparently produce cholestatic liver injury in rats.

Covalent binding of ANIT and metabolites. There has been much interest in covalent binding of reactive intermediates to tissue macromolecules as a mechanism leading to toxicity. For example, it has been suggested that metabolites of acetaminophen and bromobenzene might cause hepatotoxicity by binding covalently to hepatic macromolecules (Jollow *et al.*, 1973, 1974). The mechanism by which covalent binding causes toxicity is unclear and controversial at present. El-Hawari and Plaa (1977) considered whether covalent binding of ANIT and metabolites to hepatic macromolecules might be a mechanism of toxicity. They observed that ^{3}H from ^{3}H-ANIT (position 4 of naphthylene ring) and ^{14}C from ^{14}C-ANIT (isothiocyanate carbon) were bound to hepatic microsomes when incubated under air without NADPH present, suggesting that metabolism by MFO was not required for binding. However, when NADPH was added under identical conditions, binding of ^{3}H from ^{3}H-ANIT and ^{14}C from ^{14}C-ANIT nearly doubled. This increase in covalent binding could be reduced if microsomes were incubated under N_2 or CO, suggesting that microsomal MFO metabolism enhanced covalent binding to microsomes. These observations indicated that ANIT and metabolites bind covalently to hepatic microsomes.

To determine whether covalent binding might be consistent with ANIT's mechanism of injury, El-Hawari and Plaa (1977) treated naive rats with agents which increase or decrease ANIT-induced liver injury *in vivo*, then prepared hepatic microsomes and determined whether the covalent binding of ANIT and metabolites increased or decreased, respectively. For some agents, including phenobarbital, 3-methylcholanthrene, SKF 525-A, piperonyl butoxide, and cobaltous chloride, the correlation appeared to hold. However, for phenylisothiocyanate, cycloheximide, 16-alpha-pregnenolone carbonitrile, the correlation did not hold. Additional evidence against the covalent binding

hypothesis came from studies examining covalent binding of ANIT metabolites to hepatic microsomes from species with differing susceptibilities to the hepatotoxic effects of ANIT. In a study by Plaa and El-Hawari (1976), covalent binding to hepatic microsomes from hamsters was highest followed by rabbits > dogs > mice > rats. These qualitative and quantitative differences in binding did not correlate with the toxicity *in vivo* in the same species. For example, rabbits and dogs, which are insensitive to the cholestatic liver injury associated with ANIT (Indacochea-Redmond and Plaa, 1971; Capizzo and Roberts, 1971), exhibited more covalent binding to hepatic microsomes than susceptible species. Taken together, these observations suggested that covalent binding of ANIT and metabolites to hepatic macromolecules was probably not involved in the mechanism of liver injury.

A Challenge to the Bioactivation Hypothesis

One characteristic of the ANIT lesion does not fit easily into the bioactivation hypothesis, i.e., the lobular pattern of hepatocellular necrosis. ANIT does not cause a centrilobular necrosis as do other hepatotoxicants that require bioactivation by hepatic MFO. For example, CCl_4, bromobenzene, and acetaminophen are bioactivated by hepatic MFO and cause widespread centrilobular necrosis. ANIT, on the other hand, causes focal necrosis of periportal hepatocytes (Ungar *et al.*, 1962; Goldfarb *et al.*, 1962; Desmet *et al.*, 1968; Schaffner *et al.*, 1973). One possible explanation for this finding is that injury to hepatocytes is a result of toxic biliary products, such as bile salts, released from obstructed or damaged bile ducts (Connolly *et al.*, 1988). In this proposal, hepatic MFO metabolize ANIT to a species which is toxic to bile duct epithelium, and injury to periportal hepatocytes occurs secondary to bile ductular

changes (Connolly *et al.*, 1988). Another explanation suggested by Roberts and Plaa (1966b) is that cycling of a toxic metabolite(s) in the enterohepatic circulation might be required for toxicity. After absorption from the intestinal tract into mesenteric and portal venous blood, the metabolite(s) might cause toxicity to hepatocytes first exposed, i.e., periportal hepatocytes. Either of the two proposed mechansims are consistent with bioactivation of ANIT and might explain the zonal toxicity observed after ANIT treatment. Alternatively, it is possible that ANIT causes liver injury by a novel mechanism which may or may not involve bioactivation. As discussed previously, the results of Williams (1974) suggest that ANIT itself may be involved in hepatotoxicity. Accordingly, the bioactivation hypothesis, at present, might be too simplistic to explain the pathogenesis of the injury.

GSH Involvement in Tissue Injury

Possible role for GSH in ANIT-induced liver injury. Much of the evidence for hepatic MFO involvement in the injurious process was obtained from agents which are inducers or inhibitors of hepatic MFO activity, and it seems possible that these agents share another common action which might explain their effects on ANIT-induced liver injury. As shown in Table I-1, certain agents which alter the liver injury caused by ANIT *in vivo* also affect hepatic GSH content and/or GSH S-transferase activity. These agents include MFO inhibitors, MFO inducers, and agents which affect ANIT hepatotoxicity by unknown mechanisms. Agents which afford protection against ANIT-induced liver injury decrease hepatic GSH content and/or the activity of at least one form of GSH S-transferase, and agents which enhance the liver injury associated with ANIT increase hepatic GSH content and/or the activity of at least one form of GSH S-transferase. Thus,

these various agents affect hepatic GSH status in a manner to suggest a causal role for GSH in ANIT-induced liver injury.

GSH and conjugation to xenobiotic agents. GSH is a tripeptide (L-gamma-glutamyl-L-cysteinyl-glycine) and the major intracellular thiol in mammalian cells. It is thought to be involved in the transport of amino acids into cells, and it participates in reactions that destroy H_2O_2 and organic hydroperoxides (Meister, 1988). GSH is involved in the metabolism of electrophilic compounds, including xenobiotic agents and endogenous compounds, to form mercapturic acids. Conjugation of electrophilic species with GSH may occur non-enzymically or be catalyzed by GSH S-transferases present in the liver and other tissues. In most instances, conjugation of a xenobiotic agent to GSH serves as a detoxification mechanism. For example, bromobenzene is bioactivated to an epoxide metabolite which may lead to hepatocellular toxicity by binding covalently to cellular macromolecules (Jollow *et al.*, 1974). GSH serves as a protective mechanism by conjugating to the epoxide. Depletion of hepatic GSH content with diethylmaleate exacerbates the liver injury caused by bromobenzene (Jollow *et al.*, 1974).

GSH S-conjugates formed in hepatocytes are transported into bile or plasma and are usually excreted in urine as mercapturic acids (see Figure I-2 for pathway). The kidney plays a major role in the metabolism of GSH S-conjugates. Circulating GSH S-conjugates are filtered by the renal glomeruli into tubular lumens, and they may be metabolized to cysteine S-conjugates by the actions of GGT and dipeptidases, which are located on the brush border of proximal tubular cells (Silbernagl *et al.*, 1978; Hughey *et al.*, 1978). Cysteine S-conjugates present in the tubular lumen may be taken up by renal proximal tubular cells, metabolized by N-acetyl transferase, and secreted back into the lumen as mercapturic acids. In some cases, GSH S-conjugates escaping glomerular

filtration may be taken up from blood across the basolateral membrane of renal proximal tubular cells, processed, and excreted into the lumen as mercapturic acids (Lash and Jones, 1984; Anders *et al.*, 1988).

Although the kidney contains the enzymes necessary for metabolism of GSH S-conjugates, these enzymes are located in the hepatobiliary system too. GSH S-conjugates formed in hepatocytes and secreted into bile may be cleaved to their respective cysteine conjugates by actions of GGT and dipeptidases present in epithelial cells of bile ducts and small intestine (Szewczuk et al., 1980; Okajima *et al.*, 1983). In some cases, the liver may N-acetylate cysteine S-conjugates after they are absorbed from the intestinal tract into portal venous blood (Inoue *et al.*, 1984). Alternatively, as described above, circulating cysteine S-conjugates may be filtered by the renal glomeruli and metabolized by epithelial cells in the proximal tubule of the kidney.

GSH requirement in tissue injury: Formation of toxic GSH S-conjugates. As indicated earlier, conjugation of xenobiotic agents with GSH usually serves as a detoxification mechanism. However, certain compounds such as trichloroethylene, 1,2-dichloroethane, and others become toxic to kidney after conjugation to GSH (reviewed by Anders *et al.*, 1988). The GSH S-conjugates of these compounds appear to be selectively toxic to kidney, presumably due, in part, to high renal activity of certain enzymes, i.e., GGT, dipeptidases, cysteine conjugate *B*-lyase, which may be involved in the metabolism of these GSH S-conjugates to their ultimate toxic species (Figure I-2). Two mechanisms have been identified, and each will be discussed briefly below. Certain GSH S-conjugates, i.e., that of 1,2-dichloroethane, or their respective cysteine conjugates form electrophilic episulfonium ions by the internal displacement of the halogen atom by the sulfur atom. The episulfonium ion may react with nucleophilic groups on cellular macromolecules and thereby lead to injury. In

the second mechanism, certain cysteine S-conjugates, i.e., that of trichloroethylene (S-[1,2-dichlorovinyl]-L-cysteine), are metabolized by cysteine conjugate *B*-lyase, which is located in kidney and other tissues, to reactive thiol species. These reactive thiols are presumed to cause toxicity by interfering with mitochondrial function (Anders *et al.*, 1988).

It seems possible that ANIT or a metabolite might form a toxic GSH S-conjugate. Although ANIT is hepatotoxic and not nephrotoxic, many of the enzymes required for metabolism of GSH S-conjugates are present in the biliary and intestinal tracts. For example, epithelia of bile ducts and small intestine contain GGT and dipeptidases (Szewczuk *et al.*, 1980; Okajima *et al.*, 1983), and liver and gut microflora contain cysteine conjugate *B*-lyase (Tateishi *et al.*, 1978; Tomisawa *et al.*, 1984). There is evidence that methyl chloride forms an hepatotoxic GSH S-conjugate (Chellman *et al.*, 1986). To cause toxicity, this GSH S-conjugate requires metabolism by GGT and other enzymes involved in mercapturic acid formation (White *et al.*, 1982; Chellman *et al.*, 1986). That an intact enterohepatic circulation is required for the development of liver injury after ANIT treatment (Roberts and Plaa, 1966b) is consistent with the hypothesis that biliary and gastrointestinal tract mechanisms are needed for ANIT bioactivation.

GSH requirement in tissue injury: Formation of thiol ether leukotrienes. GSH may participate in pathogenic processes by mechanisms other than formation of toxic GSH S-conjugates. For example, GSH is required for the formation of thiol ether leukotrienes from leukotriene A_4 (LTA_4) (see Figure I-3 for pathway). In rats and other species, thiol ether leukotrienes are metabolized by enzymes of the mercapturic acid pathway. LTC_4 may form LTD_4, LTE_4, and N-acetyl-LTE_4 by the actions of GGT, dipeptidases, and N-acetyl transferase, respectively (Hagmann and Keppler, 1988). LTC_4, LTD_4 and LTE_4 comprise slow reacting substance of anaphylaxis, which has been implicated in the acute

respiratory distress syndrome, asthma, and inflammatory disorders (reviewed by Bach, 1983). Recently, thiol leukotrienes have been implicated in cholestasis (Hagmann and Keppler, 1988), liver injury caused by frog virus 3 (Hagmann *et al.*, 1987), and fulminant hepatitis caused by a combination of galactosamine and endotoxin (Tiegs and Wendel, 1988). In the latter study, thiol ether leukotrienes are implicated since depletion of hepatic GSH content and administration of lipoxygenase inhibitors and leukotriene receptor antagonists to mice afford protection against the hepatitis caused by the combination of galactosamine and endotoxin. Administration of the GGT inhibitor AT-125 to mice also prevents liver injury. Since GGT is required in the conversion of LTC_4 to LTD_4, this observation suggests that LTD_4, LTE_4, or N-acetyl LTE_4 are involved in the pathogenesis. LTD_4 appears to be the toxic species, since administration of LTD_4 but not LTE_4 in lieu of endotoxin produces a lesion identical to that caused by galactosamine and endotoxin.

In the galactosmaine/endotoxin model, the exact mechanism by which LTD_4 causes injury is unknown, although it may a cause an ischemia/reperfusion syndrome in the liver (Wendel *et al.*, 1987). For example, formation of thiol ether leukotrienes in the liver might cause vasoconstriction (Krell and Dietze, 1989) and subsequent hypoxia/ischemia. Upon reperfusion, as in other tissues, a burst of toxic oxygen species mediated by xanthine oxidase in liver cells causes liver injury (Marotto *et al.*, 1988; Younes and Strubelt, 1988). The observations that SOD, catalase, allopurinol (xanthine oxidase inhibitor), and iloprost (vasodilator) afford protection in this model support this hypothesis (Wendel *et al.*, 1987).

PMN Involvement in Tissue Injury

Other mechanisms might contribute to ANIT-induced liver injury. For example, activated PMNs may cause tissue injury by releasing reactive oxygen species, degradative enzymes, and other products. The text that follows describes observations which suggest a role for PMNs in ANIT hepatotoxicity. In addition, a brief review of PMN activation and PMN involvement in tissue injury is provided.

PMN activation. PMNs are phagocytic cells which play a central role in host defense against microorganisms. When PMNs come in contact with an invading pathogen, they undergo a respiratory burst and generate O_2^- by a membrane bound NADPH oxidase (Babior, 1978). O_2^- may form H_2O_2 by a reaction catalyzed by superoxide dismutase or by spontaneous dismutation. H_2O_2 and O_2^- may form the potent oxidant ·OH by the iron-catalyzed Haber Weiss reaction. As reviewed by Fantone and Ward (1982), reactive oxygen species as well as other agents released from activated PMNs are bactericidal. For example, PMNs release degradative enzymes, which are located within specific and azurophilic granules in the PMN cytoplasm. Myeloperoxidase, a granular enzyme, catalyzes the reaction of H_2O_2 to hypohalous acids when halides are present. PMNs may also form long-lived oxidants by the reaction of myeloperoxidase with H_2O_2, chloride, and compounds with free amino groups, i.e., taurine. Together, these oxidants and degradative enzymes provide the PMN with an armamentarium against invading pathogens.

Bacteria represent a physiologic stimulus for PMN activation. Other compounds, including physiologic agents as well as xenobiotic agents, may stimulate the respiratory burst in PMNs and, accordingly, have been commonly used in experimental studies *in vitro* to examine mechanisms of PMN activation.

For example, formyl-methionyl-leucyl-phenylalanine (FMLP) is a component of bacterial cell walls that binds to a receptor on PMN plasma membranes and stimulates the catabolism of phosphatidylinositol-4,5-bisphosphate to inositol triphosphate and diacylglycerol. Diacylglycerol in conjunction with phosphatidylserine and Ca^{2+} is thought to activate protein kinase C, which presumably phoshorylates NADPH oxidase to an active form (Tauber, 1987). In FMLP-stimulated PMNs, inositol triphosphate contributes to the mechanism of O_2^- production by acting on endoplasmic reticulum to elevate intracellular free Ca^{2+} concentration. The xenobiotic agent, phorbol myristate acetate, is a commonly used stimulus for PMNs, and it is thought to activate PMNs by mimicking the effects of diacylglycerol on protein kinase C (Tauber, 1987).

Certain agents cause little or no O_2^- release from PMNs, although they alter PMNs such that subsequent stimulation results in enhanced O_2^- release when compared to that of the stimulus alone. This effect is called priming. Subthreshold and threshold concentrations of classic PMN stimuli, such as PMA, calcium ionophores, and FMLP, prime PMNs for O_2^- release (McPhail *et al.*, 1984; Helman-Finkel *et al.*, 1987). Other primers include T-lymphocyte-derived granulocyte-macrophage colony stimulating factor (Gasson *et al.*, 1984; Weisbart *et al.*, 1985), macrophage-derived tumor necrosis factor (Berkow *et al.*, 1987), products from B lymphocytes (Cross *et al.*, 1985), endotoxin (Guthrie *et al.*, 1984), and muramyl dipeptide (Wright and Mandell, 1986). Agents which are chemotactic for PMNs, i.e., FMLP, casein, and the complement fragment C5a, prime PMNs for subsequent O_2^-release (Bender *et al.*, 1983; McPhail *et al.*, 1984; Helman-Finkel *et al.*, 1987). This observation suggests that PMNs which respond to chemotactic agents *in vivo* and inflitrate tissues are are probably primed for oxygen radical release.

Capacity of PMNs to cause tissue injury. Although PMNs are best known for their role in host defense, they may injure host tissue under certain circumstances (Fantone and Ward, 1985) (Figure I-4). Activation of complement *in vivo* in rats by thermal injury to skin or intravenous administration of cobra venom factor causes pulmonary vascular injury (Till *et al*., 1982, 1983). PMN depletion prior to thermal injury or to administration of cobra venom factor attenuates lung injury in these models. Co-treatment with superoxide dismutase (SOD) and/or catalase, which degrade O_2^- and H_2O_2, respectively, also affords protection, suggesting an oxygen radical-mediated mechanism of toxicity in these PMN-dependent models. Other models of PMN-dependent tissue injury include endotoxin-induced lung injury (Brigham and Meyrick, 1984), corneal endothelial cell injury to the eye (Elgebaly *et al*., 1984, ischemia/reperfusion injury to the heart (Romson *et al*., 1983), immune complex injury to the kidney (Johnson and Ward, 1982), and others.

Reactive oxygen species may cause injury by several possible mechanisms. They may oxidize polyunsaturated fatty acids to lipid hydroperoxides and cause structural and functional alterations in the plasma membrane (Mead, 1976). Alternatively, since certain reactive species may cross membranes (Fantone and Ward, 1985), they may act at intracellular sites. For example, it is conceivable that they might oxidize sulfhydryl groups on critical enzymes and lead to toxicity by altering their function. A mechanism of this type has been hypothesized for injury to hepatocytes by intracellularly-generated reactive oxygen species (Di Monte *et al*., 1984a, 1984b). In addition to reactive oxygen species, PMNs may cause tissue injury by releasing degradative enzymes located in cytosolic granules or arachidonic acid metabolites (Figure I-4).

PMN involvement in liver injury. Models of PMN-dependent tissue injury available at present involve extrahepatic tissues, and endothelial cells appear to be a critical target in several models. Presumably, the latter observation is related to the sensitivity of endothelial cells to the toxic effects of reactive oxygen species and/or the proximity of endothelium to the source of oxygen radicals (Fantone and Ward, 1982). When hypothesizing PMN-dependent, oxygen radical-mediated injury to liver, one must consider the protective systems available to hepatocytes. For example, hepatocytes contain millimolar concentrations of reduced GSH, which is required for degradation of H_2O_2 and lipid hydroperoxides via glutathione peroxidase. In addition, the presence of superoxide dismutase and catalase might be expected to degrade PMN-derived O_2^- and H_2O_2, respectively. Some investigators have suggested that these hepatocellular enzymes in conjunction with reduced GSH greatly limit the potential for oxygen radical-mediated injury to liver (Guigui *et al.*, 1988). Consequently, the proposal that PMN-derived oxygen radicals cause liver injury is a topic of controversy.

To date, a very limited number of studies have been performed that address the capacity of activated PMNs to injure liver. There is recent evidence implicating PMNs in the liver injury associated with hypovolemic shock. MAb 60.3, a monoclonal antibody directed to the primary human PMN adherence glycoprotein CD18, afforded protection against liver injury in rabbits caused by hypovolemic shock (Vedder *et al.*, 1989). This observation demonstrates the importance of PMN adherence in this model.

Co-cultures of hepatocytes and PMNs have been used as a model to explore mechanisms of PMN-mediated liver injury. PMNs activated with bacteria, PMA, or opsonized zymosan and incubated with hepatocytes cause the release of hepatocellular enzymes indicative of injury (Holman and Sabo, 1988; Guigui *et*

al., 1988; Mavier *et al.*, 1988). When PMA or opsonized zymosan is used as the stimulus for PMNs, the mechanism of hepatocellular injury depends upon proteinases released from PMNs, but not upon oxygen radicals (Guigui *et al.*, 1988; Mavier *et al.*, 1988). For example proteinase inhibitors such as soybean trypsin inhibitor prevent the leakage of hepatocellular enzymes caused by activated PMNs, whereas a combination of SOD and catalase do not. Presumably, hepatocellular protective mechanisms such as those listed above are able to degrade reactive oxygen species released by PMNs (Guigui *et al.*, 1988).

Involvement of other phagocytic cells in liver injury: Macrophages and Kupffer cells. Although the role of PMNs in liver injury has received very little attention *in vitro* and even less attention *in vivo*, other phagocytic cells, including macrophages and Kupffer cells, have been implicated in liver injury *in vivo*. In one model, intravenous treatment of rats with *Cornebacterium parvum* causes mobilization of macrophages to the liver, and administration of endotoxin 6 days later results in extensive hepatocellular necrosis (Ferluga and Allison, 1978; Arthur *et al.*, 1985). Macrophages isolated from the livers of treated rats are primed for oxygen radical release (Arthur *et al.*, 1986, 1988). O_2^- or possibly some product derived from it appears to be involved in the liver injury *in vivo*, since administration of SOD prior to endotoxin reduces the hepatotoxicity (Arthur *et al.*, 1985).

Activated macrophages accumulate in centrilobular regions of the liver after acetaminophen treatment, although it is not clear whether these macrophages contribute to the injury (Laskin and Pilaro, 1986, Laskin *et al.*, 1986). ElSisi *et al.* (1987) have reported evidence for Kupffer cell involvement in vitamin A potentiation of CCl_4 hepatotoxicity. Reactive oxygen species,

presumably released from Kupffer cells, have been implicated in this model of liver injury.

The studies by Arthur *et al.* (1985) and ElSisi *et al.* (1987) suggest that, under certain circumstances, hepatocellular protective systems might not be able to prevent reactive oxygen species released from phagocytic cells from damaging hepatocytes. Others have shown that O_2^- produced intracellularly in hepatocytes may cause injury. For example, compounds which undergo redox cycling, i.e. menadione and diquat, generate O_2^- in hepatocytes, which may lead to toxicity. Presumably, the mechanisms might relate to lipid peroxidation of membranes or impairment in the function of critical enzymes through oxidation of sulfhydryl groups (Di Monte *et al.*, 1984a, 1984b; Smith, 1987).

Possible involvement of PMNs in ANIT-induced liver injury. Several observations suggest that PMNs might contribute to the pathogenesis of liver injury caused by ANIT. For example, a marked inflammatory infiltrate consisting of PMNs occurs in periportal regions of the liver, and PMNs have been reported to be associated with injured bile duct epithelial cells (McClean and Rees, 1958; Ungar *et al.*, 1962; Goldfarb *et al.*, 1962). McClean and Rees (1958) observed an infiltration of PMNs as early as 6 hr after treatment of rats with ANIT. This observation indicates that PMNs are present in periportal regions of the liver prior to hepatocellular and bile ductular necrosis and suggests that PMNs might play a causal role in the pathogenesis. Other evidence suggesting PMN involvement has been provided by Roth and Hewett (1986, 1990). They observed that ANIT stimulated superoxide anion (O_2^-) (Figure I-5) and *B*-glucuronidase release from rat peritoneal PMNs *in vitro*, suggesting that these potentially injurious products might be involved in the pathogenesis of ANIT hepatotoxicity. Furthermore, they showed that BNIT, a non-cholestatic isomer of ANIT, did not stimulate O_2^- release from PMNs. These observations are

consistent with the hypothesis of PMN involvement in the liver injury caused by ANIT.

Hypotheses and Specific Aims of Thesis Project

The hypotheses tested in this thesis are that PMN-derived oxygen radicals and glutathione are necessary for ANIT to cause liver injury. The specific aims are given below. (1) To determine whether PMN-derived oxygen radicals cause injury to the isolated, perfused rat liver. Since the hypothesis that PMN-derived oxygen radicals cause liver injury is a topic of controversy, it was assessed in a simple model under ideal conditions. (2) To determine whether bile and bile salts stimulate O_2^- release from PMNs *in vitro*. Studies were undertaken to assess whether products released from an ANIT-injured liver might activate PMNs to release oxygen radicals, thereby contributing to liver injury. (3) To determine whether PMNs play a causal role in ANIT hepatotoxicity. This possibility was assessed because PMNs infiltrate periportal regions of the liver after ANIT treatment, and they are associated with necrotic bile duct epithelial cells and necrotic hepatocytes. (4) To determine whether PMN-derived oxygen radicals are involved in ANIT-induced liver injury. This was examined because ANIT stimulates O_2^- release from PMNs *in vitro* (Roth and Hewett, 1986). (5) To determine whether decreased hepatic non-protein sulfhydryl content, an indicator of GSH content, prevents ANIT hepatotoxicity. The rationale for these studies is that agents which alter ANIT hepatotoxicity *in vivo* affect hepatic GSH content and/or GSH S-transferase activity in a manner to suggest a causal or permissive role for GSH in the pathogenesis. (6) To determine whether ANIT-induced elevations in hepatic NPSH content are related to the mechanism of

hepatotoxicity. These studies were initiated because the onset of liver injury after ANIT treatment coincides with elevations in hepatic NPSH content.

Table I-1

Effects of selected agents on ANIT-induced liver injury and hepatic GSH status

Compound	ANIT Injury	Hepatic GSH Content	Hepatic GSH S-transferase Activity[a]
phenobarbital	increase [g]	increase (Kaplowitz *et al.*, 1983)	increase [b,c,d,e] (Kaplowitz *et al.*, 1975)
3-methyl-cholanthrene	increase [g]	increase (Kaplowitz *et al.*, 1983)	increase [b,c,d,e] (Kaplowitz *et al.*, 1975)
SKF 525-A	decrease [g]	decrease (Hoshi *et al.*, 1986)	decrease [b,c,f] (Fromowicz *et al.*, 1987)
piperonyl butoxide	decrease [g]	decrease (James and Harbison, 1982)	
diethyldithio-carbamate	decrease [h]	decrease (Sunderman *et al.*, 1984)	decrease [d] (Younes *et al.*, 1982)
cyanidanol	decrease [i]		decrease [d,f] (Younes *et al.*, 1982)
ethionine	decrease [j]	decrease (Glaser and Mager, 1974)	

[a]Assayed with the following substrates: [b]3,4-dichloronitrobenzene, [c]p-nitrobenzyl chloride, [d]1,2-epoxy-(p-nitrophenoxy)propane, [e]methyl iodide, [f]1-chloro-2,4-dinitrobenzene.
[g]El-Hawari and Plaa, 1977
[h]Traiger *et al.*, 1984
[i]Tajima *et al.*, 1983
[j]Indacochea-Redmond *et al.*, 1973

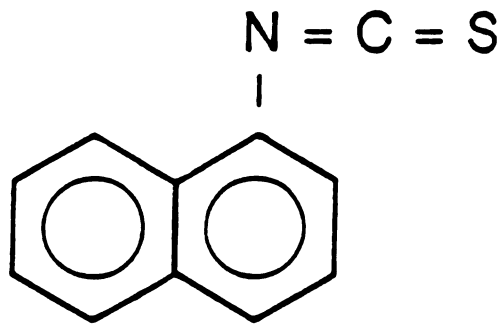


Figure I-1. Structure of ANIT

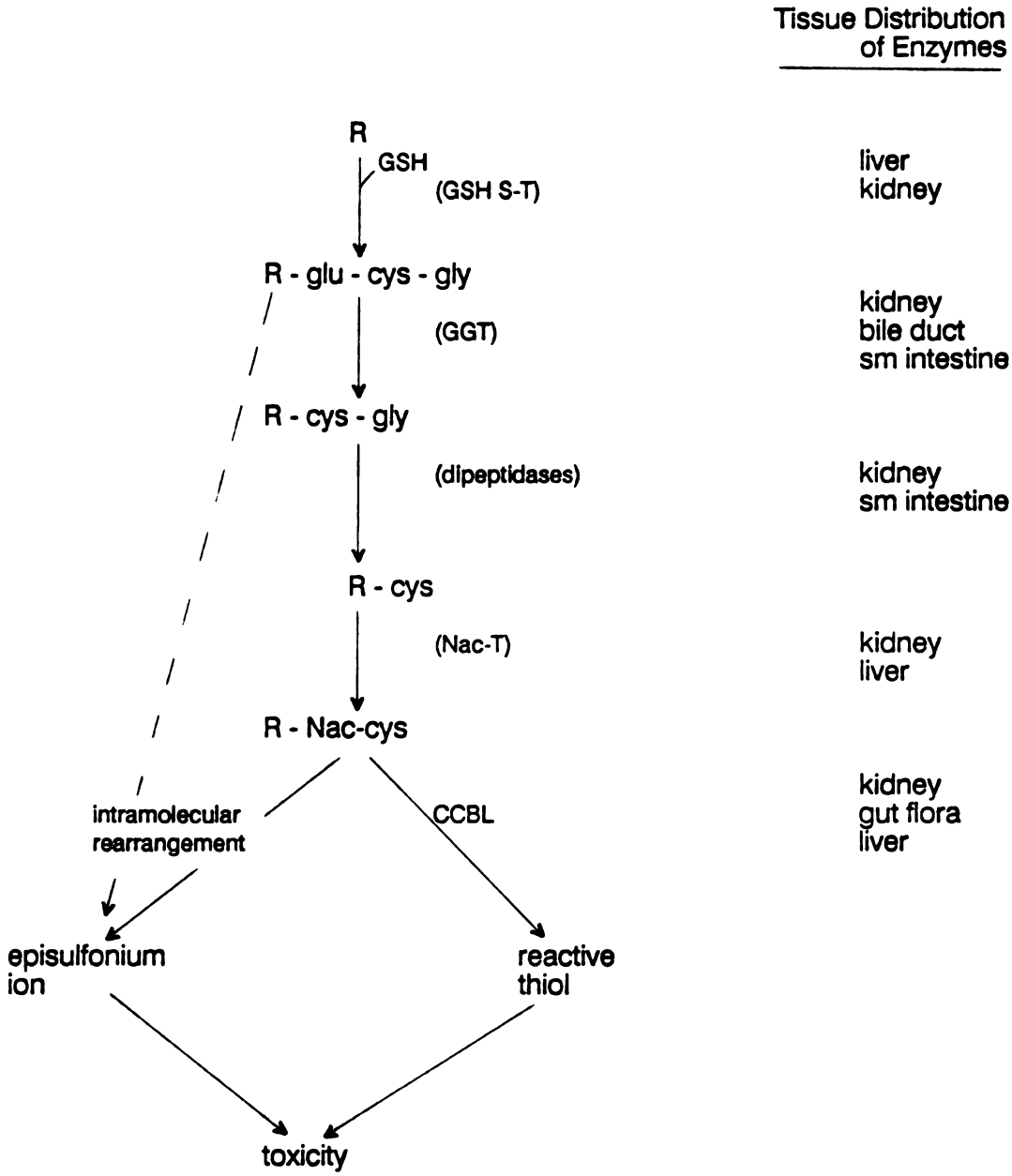


Figure I-2. Pathway of metabolism of toxic GSH S-conjugates. Abbreviations: GSH, glutathione; cys, cysteine; glu, glutamate; gly, glycine; GSH S-T, GSH S-transferase; Nac-T, N-acetyl transferase; GGT, gamma-glutamyltransferase; CCBL, cysteine conjugate B-lyase; sm, small

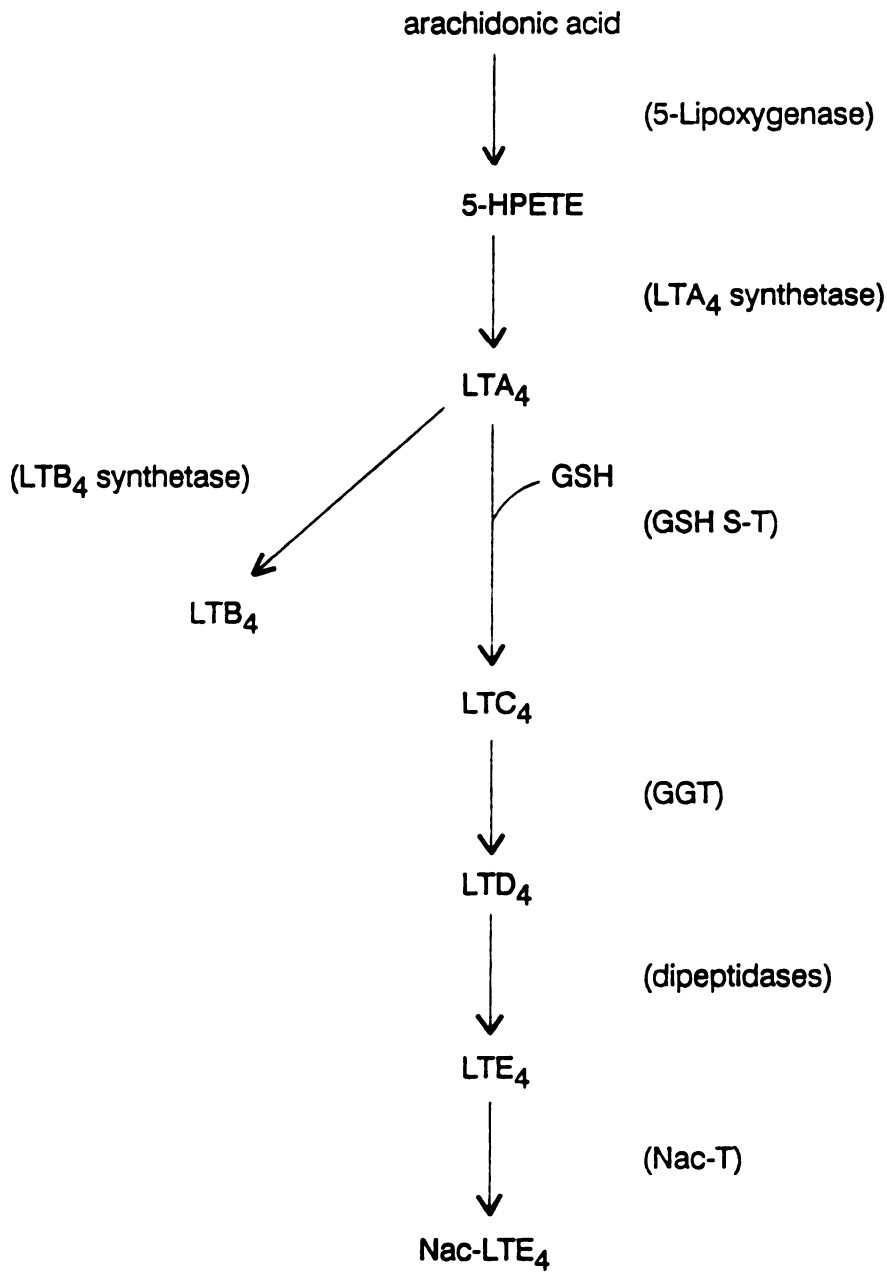


Figure I-3. Pathway for synthesis and metabolism of leukotrienes from arachidonic acid. Abbreviations: 5-HPETE, (5S) -5-hydroperoxy-6,8,11,14-eicosatetraenoic acid. For other abbreviations, see legend to Figure 2.

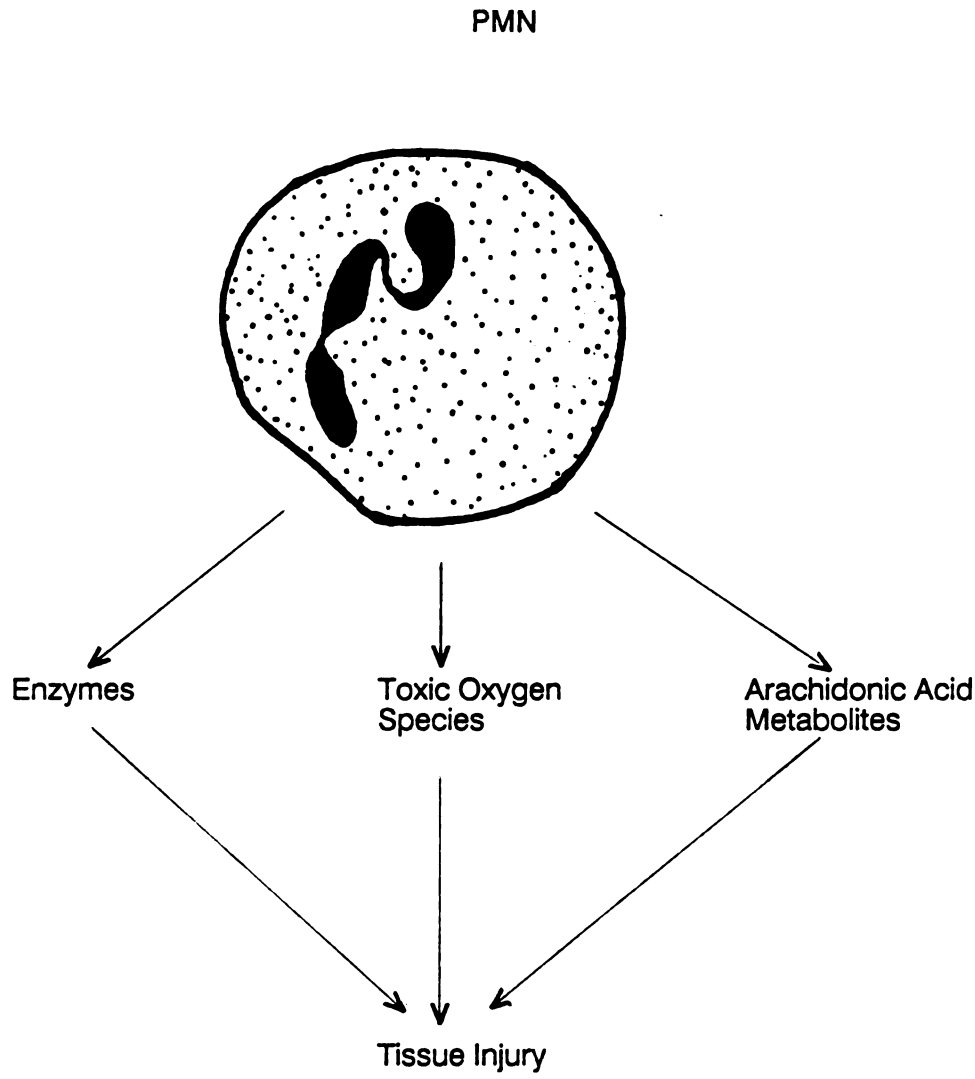


Figure I-4. Injurious products released from activated PMNs

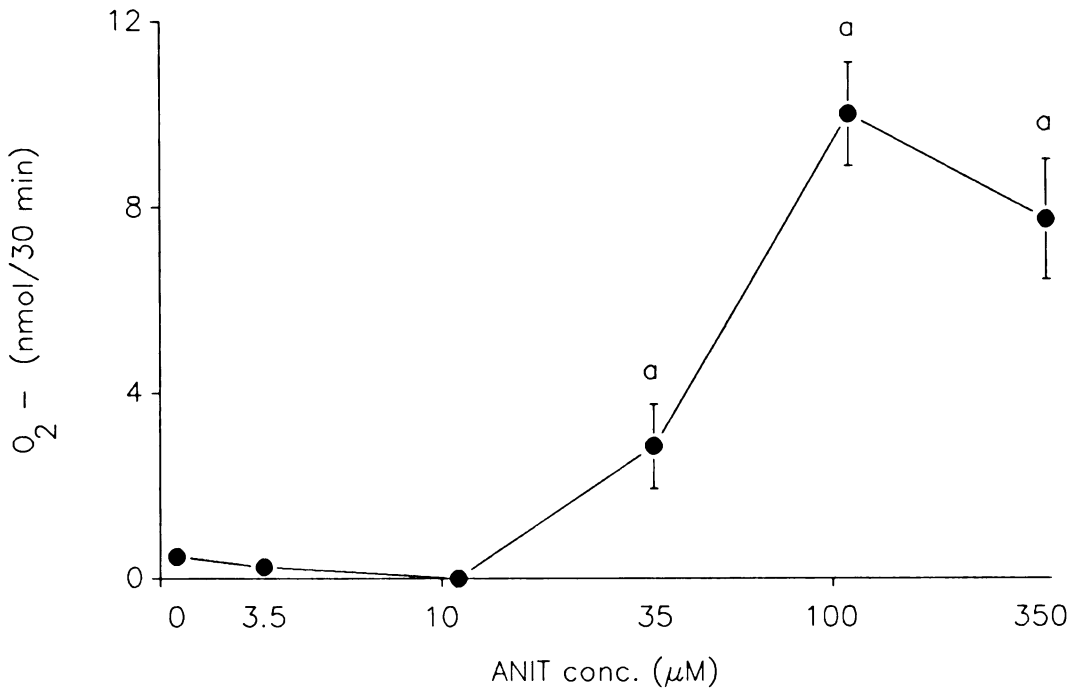


Figure I-5. ANIT stimulated O_2^- release from PMNs in vitro. 2 x 10^6 PMNs were incubated with ANIT or dimethylformamide vehicle for 10 minutes at 37°C. N = 5. [a] Significantly different from vehicle (0 uM ANIT), $p < 0.05$. (Adapted from Roth and Hewett, 1990).

CHAPTER II

ACTIVATED NEUTROPHILS INJURE THE ISOLATED, PERFUSED RAT LIVER BY AN OXYGEN RADICAL-DEPENDENT MECHANISM

INTRODUCTION

PMNs cause injury to extrahepatic organs by mechanisms dependent upon reactive oxygen species. An hypothesis tested in this thesis is that PMN-derived oxygen species are involved in the liver injury caused by ANIT. Whether or not PMN-derived oxygen radicals cause liver injury is a topic of controversy, since hepatocytes contain protective enzymes which would be expected to degrade oxygen radicals. The purpose of this study was to determine whether PMN-derived oxygen species are able to cause liver injury in a simple system under ideal conditions. An isolated, perfused rat liver preparation was used, wherein activated PMNs were perfused.

In ANIT hepatotoxicity, PMN-derived oxygen radicals may mediate the injury, or they might exacerbate a pre-existing lesion. It seems unlikely that PMNs by themselves mediate the injury, since ANIT is toxic to ARL 3 cells, i.e., hepatocytes, in culture under PMN-free conditions. Therefore, using the isolated, perfused rat liver preparation, we explored the latter possibility. Initially, we hypothesized that perfusion of ANIT through livers would cause injury and that addition of PMNs would exacerbate the lesion, presumably via an oxygen radical-dependent mechanism. These studies are not presented, because addition of ANIT to the perfusion medium did not cause liver injury. A second approach was to isolate livers from ANIT-treated rats at a time when liver injury was minimal and perfuse them with activated PMNs. We hypothesized that activated PMNs would enhance liver injury to a greater extent relative to livers isolated from vehicle-treated rats.

METHODS

Materials. ANIT, lithocholic acid, glycogen (Type II from oyster), catalase, and kit 505 for alanine aminotransferase (ALT) activity were purchased from Sigma Chemical Co. (St. Louis, MO). Superoxide dismutase (SOD) was obtained from Diagnostic Data, Inc. (Mountainview, CA). PMA was purchased from LC Services (Woburn, MA). Bovine serum albumin (BSA, fraction V) was purchased from ICN Immunobiologicals (Lisle, IL). All other reagents were of the highest grade commercially available. Polyethylene (PE) tubing was obtained from Clay Adams (Parsippany, NJ).

Animals. Male, Sprague-Dawley rats (CF:CD(SD)BR) (Charles River, Portage, MI) weighing 220-330 g were housed in plastic cages on aspen chip bedding under conditions of controlled temperature (18-21^{o}C) and humidity (55$\pm$5%). A 12 hr light/12 hr dark cycle was maintained. Rats were allowed tap water and rat chow (Wayne Lab Blox, Allied Mills, Chicago, IL) *ad libitum* prior to experimentation.

PMN isolation from rat peritoneum. Male, retired breeder, Sprague-Dawley rats (Charles River) received 25-35 ml of 1% glycogen solution in 0.9% sterile saline, i.p. Four hr later, rats were lightly anesthetized with diethyl ether, decapitated, and exsanguinated. Thirty ml of heparin-treated (1 U/ml), phosphate-buffered saline (PBS, pH 7.4) were injected into the peritoneum. The abdominal wall was opened, and the contents were poured through layered gauze. The peritoneum was washed with another 30 ml of heparin-treated PBS, and the combined contents were spun in a centrifuge at 500 x g for 7 min. The

supernatant was discarded, and the pellet was resuspended in 0.15 M NH_4Cl with 0.01 M $NaHCO_3$ and 0.001 M ethylenediaminetetraacetic acid (disodium salt). After 2 min in this red blood cell lysing solution, PBS was added, and the contents were spun in a centrifuge at 320 x g for 7 min. The supernatant was discarded, and the cells were resuspended in Hank's balanced salt solution (HBSS, pH 7.35). Smears of the cell suspension were prepared and immersed in Wright-Giemsa stain. The percentage of PMNs in the cell preparation and viability were > 95%.

Protocol for perfusion of isolated rat liver. At time zero, chow was removed from rats. Twelve hr later, they were treated with either ANIT (100 mg/kg, p.o.) or an equivalent volume of corn oil vehicle. At 24-30 hr, they were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) in preparation for surgical removal of the liver. Livers isolated from ANIT-treated rats at this time, i.e., 12-18 hr after treatment, show subtle histological changes and are functionally compromised (Plaa and Priestly, 1977).

Livers were isolated and perfused essentially by the method of Miller *et al.* (1973). After a midline laparotomy was performed, the liver was exposed, and the common bile duct was cannulated with PE 10 tubing. Heparin (500 U) was injected into the inferior vena cava, and a blood sample was withdrawn from the same vessel for plasma determination of ALT activity as described below. The portal vein was cannulated with PE 240 tubing, and the liver was perfused with ice cold, heparin-treated (1 U/ml), oxygenated saline. After ligation of the hepatic artery, the thoracic vena cava was cannulated with PE 260 tubing. The inferior vena cava was ligated anterior to the right renal vein. The liver was freed from the rat and transferred to a perfusion chamber maintained at 37^{o}C. It was perfused at an inflow pressure of 16 cm H_20 with an oxygenated (95% O_2/5% CO_2) Krebs-Ringer bicarbonate buffer (KRBB, pH 7.4) containing 1% BSA in a

recirculating system. In the present studies, inflow oxygen content (PO_2) averaged 427 $\pm$ 4 mm Hg (N = 82).

After a 30-50 min pre-perfusion period (stabilization period), a sample of perfusion medium was removed from the reservoir and analyzed for ALT activity as described below. The medium was drained from the apparatus except for a residual amount (26 ml), and 85-92 ml of KRBB containing 4% BSA was added. $4x10^8$ PMNs in a 7-15 ml volume were added to the perfusion system as a bolus approximately 22 cm upstream from the liver. The final perfusion volume was 126 ml and contained 3.0% BSA. In one experiment, SOD and catalase (500 U/ml each) were included in the perfusion medium. Activities of SOD and catalase were 4200 U/mg protein and 2890 U/mg protein, respectively. SOD activity in the medium during perfusion was measured spectrophotometrically by measuring the inhibition of adrenochrome formation from the auto-oxidation of epinephrine as described by Misra (1985). Catalase activity was measured by ultraviolet spectroscopy by monitoring the disappearance of H_2O_2 as described by Beers and Sizer (1952). Livers were perfused for 90 min after addition of PMNs, and the pH of the perfusion medium was kept between 7.3 and 7.4 by adding $NaHCO_3$. Control livers received an equivalent volume of HBSS vehicle in lieu of PMNs. In one experiment, PMNs were stimulated with PMA (31 ng/ml) for 10 min at 37^{o}C before addition to the perfusion system. Control livers received PMA and HBSS in lieu of PMNs. In another experiment, PMNs were stimulated by treatment with PMA (31 ng/ml) for 5 min at 37^{o}C followed by lithocholate (LC, 100 uM) for an additional 5 min before addition to the perfusion system. Control livers received PMA, LC, and HBSS instead of PMNs. In studies employing activated PMNs, PMA was added to the perfusion medium after addition of PMNs such that the final concentration was maintained at 31 ng/ml.

At 90 min, various indicators of liver viability were measured. A sample of perfusion medium was withdrawn from the system for determination of ALT activity. ALT activity (Sigma kit 505) was measured spectrophotometrically by monitoring formation of the phenylhydrazone of pyruvate as described by Reitman and Frankel (1957). It was used as a marker of liver injury, since PMNs contain negligible ALT activity (Guigui *et al.*, 1988). Bile flow was measured and expressed per gram of tissue. At 80-90 min, oxygen consumption was calculated by multiplying the perfusate flow by the inflow-outflow difference in O_2 concentration and was expressed per gram of tissue. Inflow and outflow oxygen concentrations were calculated from pO_2 values measured with an Instrumentation Laboratory model 113-01 blood gas analyzer. At the end of the perfusion, the liver was removed from the apparatus, blotted with gauze, and weighed. Liver weight (wt) was expressed as a percentage of body weight.

Statistical Analysis. Results are expressed as mean $\pm$ S.E. Homogeneity of variance was tested using the F-max test. Log transformations were performed on nonhomogeneous data. Data were analyzed with a mixed design, analysis of variance (ANOVA), completely randomized factorial ANOVA, or Student's t-test, as appropriate. Individual comparisons between treatment means were made with Tukey's omega test (Steel and Torrie, 1980). The criterion for significance was $p < 0.05$.

RESULTS

Effects of PMNs on livers isolated from corn oil-treated rats. A 90 min perfusion of livers receiving HBSS resulted in a small increase in ALT activity in the perfusion medium (Table II-1). The addition of unstimulated PMNs did not further increase ALT activity or change the liver wt/body wt ratio, bile flow, perfusate flow, or oxygen consumption (Table II-1). PMNs caused a transient, i.e. 1-4 min, decrease in perfusate flow when added to the system (data not shown).

PMNs were activated *in vitro* by sequential treatment with PMA and LC since this procedure has been shown to cause > 8-fold release of O_2^- from PMNs compared to PMA treatment alone (Chapter III). The addition of activated PMNs to the system reduced perfusate flow through livers almost completely for approximately 5-15 min (data not shown). Flow returned gradually, and the return appeared to be complete by 10-40 min after addition of PMNs. Activated PMNs caused injury to the liver as indicated by an increase in ALT activity in the medium after 90 min of perfusion (Table II-2). They also slightly increased the liver wt/body wt ratio but did not change bile flow or O_2 consumption.

When PMNs were stimulated with PMA alone and added to the perfusion system, they reduced perfusate flow to nearly the same extent as PMNs stimulated with PMA and LC (data not shown). However, they did not elevate ALT activity in the perfusion medium or increase the liver wt/body wt ratio, although they decreased bile flow by approximately 20% (Table II-3).

Effects of PMNs on livers isolated from ANIT-treated rats. To determine whether PMNs might cause greater injury to chemically-compromised livers, PMNs activated with PMA and LC were perfused through livers isolated from rats treated with ANIT. ANIT was used because the liver injury associated with this hepatotoxicant appears to be mediated, at least in part, by PMNs (Chapter IV). Livers were isolated from ANIT-treated rats when liver injury was minimal. For example, plasma ALT activities in donor rats treated with ANIT were similar to those in donor rats receiving corn oil (compare Table II-4 with Tables II-1, II-2, II-3, and II-5). The addition of activated PMNs to ANIT-compromised livers caused increased leakage of ALT into the perfusion medium. However, the extent of ALT leakage appeared to be similar to that in livers isolated from corn oil-treated rats (compare Tables II-2 and II-4). For example, PMNs activated with PMA and LC caused approximately a 3.7-fold increase in ALT release from livers isolated from corn oil-treated rats when compared to control livers receiving no PMNs. When ANIT-compromised livers were used, they caused approximately a 3.9-fold increase in ALT release. PMNs activated with PMA and LC affected perfusate flow in ANIT-compromised livers in a manner similar to that in uncompromised livers described above.

Effects of SOD and catalase on PMN-mediated liver injury. To determine whether toxic oxygen species released from activated PMNs might mediate the liver injury, SOD and catalase were added prior to PMNs to the perfusion medium of livers from corn oil-treated rats. SOD and catalase activities were elevated in the perfusion medium during the 90 min perfusion, and these agents prevented the PMN-induced elevation in perfusate ALT activity (Table II-5). SOD and catalase did not prevent alterations in perfusate flow caused by the addition of activated PMNs (data not shown).

Table II-1

Effects of unstimulated PMNs on livers isolated from corn oil-treated rats

	Treatment [a]	
	HBSS	PMN
Liver wt/body wt x 100	3.28 ± 0.08	3.20 ± 0.16
Bile flow (ul/hr/g)	74 ± 4	66 ± 3
Perfusate flow (ml/min/g)	8.2 ± 0.4	8.9 ± 0.8
O_2 consumption (ml/hr/g)	2.5 ± 0.2	2.0 ± 0.2
ALT activity (SF units/ml)		
donor rat plasma	18 ± 3	17 ± 2
pre-perfusion medium	3 ± 1	2 ± 1
90-min perfusion medium	10 ± 3 [b]	11 ± 3 [b]

[a]Livers were isolated from fasted rats 12-18 hr after treatment with corn oil (3 ml/kg, p.o.) and were perfused with KRBB containing 1% BSA for a 30-50 min pre-perfusion period. ALT activity was measured in medium at the end of the pre-perfusion period. The medium was changed to KRBB containing 4% BSA, and either PMNs ($4x10^8$)or HBSS were added to the perfusion medium 22 cm upstream from the liver. Markers of liver viability were measured 90 min later. N = 4-5.

[b]Significantly different from respective, pre-perfusion value.

Table II-2

Effects of PMNs activated with PMA and LC on livers isolated from corn oil-treated rats

	Treatment [a]	
	HBSS/PMA/LC	PMN/PMA/LC
Liver wt/body wt x 100	2.76 ± 0.08	3.08 ± 0.11 [c]
Bile flow (ul/hr/g)	58 ± 4	50 ± 5
Perfusate flow (ml/min/g)	7.0 ± 0.4	6.7 ± 0.9
O_2 consumption (ml/hr/g)	2.1 ± 0.2	2.1 ± 0.2
ALT activity (SF units/ml)		
donor rat plasma	15 ± 1	11 ± 1
pre-perfusion medium	2 ± 1	1 ± 0
90 min perfusion medium	15 ± 2 [b]	56 ± 11 [b,c]

[a]Livers were isolated from fasted rats 12-18 hr after treatment with corn oil (3 ml/kg, p.o.) and were perfused with KRBB containing 1% BSA for a 30-50 min pre-perfusion period. ALT activity was measured in medium at the end of the pre-perfusion period. The medium was changed to KRBB containing 4% BSA, and activated PMNs were added to the perfusion medium 22 cm upstream from the liver. PMNs were activated by treatment with PMA (31 ng/ml) for 5 min at 37°C followed by LC (100 uM) for an additional 5 min. Control livers received PMA, LC, and HBSS instead of PMNs. PMA was added to the medium to keep the concentration at 31 ng/ml. Markers of liver viability were measured 90 min later. N = 5-7.

[b]Significantly different from respective, pre-perfusion value.

[c]Significantly different from HBSS/PMA/LC group.

Table II-3

Effects of PMA-activated PMNs on livers isolated from corn oil-treated rats

	Treatment [a]	
	HBSS/PMA	PMN/PMA
Liver wt/body wt x 100	2.84 ± 0.06	3.03 ± 0.12
Bile flow (ul/hr/g)	64 ± 5	52 ± 2 [c]
Perfusate flow (ml/min/g)	7.3 ± 0.4	7.9 ± 0.3
O_2 consumption (ml/hr/g)	2.4 ± 0.1	2.3 ± 0.2
ALT activity (SF units/ml)		
donor rat plasma	17 ± 3	13 ± 1
pre-perfusion medium	2 ± 1	3 ± 1
90 min perfusion medium	16 ± 7 [b]	18 ± 6 [b]

[a]Livers were isolated from fasted rats 12-18 hr after treatment with corn oil (3 ml/kg, p.o.) and were perfused with KRBB containing 1% BSA for a 30-50 min pre-perfusion period. ALT activity was measured in medium at the end of the pre-perfusion period. The medium was changed to KRBB containing 4% BSA, and activated PMNs were added to the perfusion medium 22 cm upstream from the liver. PMNs were activated by treatment with PMA (31 ng/ml) for 10 min at 37°C. Control livers received PMA and HBSS instead of PMNs. PMA was added to the medium to keep the concentration at 31 ng/ml. Markers of liver viability were measured 90 min later. N = 4-6.

[b]Significantly different from respective, pre-perfusion value.

[c]Significantly different from HBSS/PMA group.

Table II-4

Effects of PMNs activated with PMA and LC on livers isolated from ANIT-treated rats

	Treatment [a]	
	HBSS/PMA/LC	PMN/PMA/LC
Liver wt/body wt x 100	2.94 ± 0.09	3.20 ± 0.12
Bile flow (ul/hr/g)	52 ± 5	43 ± 7
Perfusate flow (ml/min/g)	7.4 ± 0.5	6.2 ± 0.3 [c]
O_2 consumption (ml/hr/g)	2.2 ± 0.1	2.0 ± 0.1
ALT activity (SF units/ml)		
donor rat plasma	19 ± 2	16 ± 2
pre-perfusion medium	1 ± 0	1 ± 1
90 min perfusion medium	18 ± 4 [b]	71 ± 15 [b,c]

[a]Livers were isolated from fasted rats 12-18 hr after treatment with ANIT (100 mg/kg, p.o.). Immediately prior to surgical removal of the liver, a blood sample was withdrawn from the inferior vena cava for plasma ALT measurement to assess hepatotoxicity prior to perfusion. Livers were perfused with KRBB containing 1% BSA for a 30-50 min pre-perfusion period. ALT activity was measured in the medium at the end of the pre-perfusion period. The medium was changed to KRBB containing 4% BSA, and either activated PMNs or HBSS were added as described in the footnote to Table 2. Markers of liver viability were measured 90 min later. N = 8-9.

[b]Significantly different from respective, pre-perfusion value.

[c]Significantly different from HBSS/PMA/LC group.

Table II-5

Effects of SOD and catalase on PMN-mediated injury to livers isolated from corn oil-treated rats

	Treatment [a]	
	-	+
Liver wt/body wt x 100	3.17 ± 0.09	2.93 ± 0.13
Bile flow (ul/hr/g)	51 ± 5	51 ± 3
Perfusate flow (ml/min/g)	7.0 ± 0.7	7.8 ± 0.8
O_2 consumption (ml/hr/g)	2.1 ± 0.2	2.1 ± 0.2
ALT activity (SF units/ml)		
donor rat plasma	15 ± 1	19 ± 2
pre-perfusion medium	1 ± 0	2 ± 1
90 min perfusion medium	100 ± 19 [b]	35 ± 10 [b,c]
Catalase activity (U/ml)		
0 min	2 ± 1	455 ± 33 [c]
90 min	6 ± 2	160 ± 20 [c]
SOD activity (U/ml)		
0 min	0 ± 0	603 ± 76 [c]
90 min	4 ± 4	492 ± 48 [c]

[a]Livers were isolated from fasted rats 12-18 hr after treatment with corn oil (3 ml/kg, p.o.). They were perfused as described in the footnote to Table 2 except that SOD and catalase were included in the KRBB containing 4% BSA. + or - refers to the presence or absence, respectively, of a combination of SOD and catalase. SOD and catalase activities were measured in the perfusion medium immediately after addition of PMNs (0 min) and at the end of the perfusion (90 min). Markers of liver viability were measured after 90 min of perfusion. N = 4-5.

[b]Significantly different from respective, pre-perfusion value.

[c]Significantly different from group not receiving SOD and catalase.

SUMMARY

In this chapter, we have shown that activated PMNs cause injury to the isolated, perfused rat liver by a mechanism dependent upon reactive oxygen species. In the next chapter, we consider whether products released from an injured liver might stimulate O_2^- release from PMNs and thereby contribute to liver injury.

CHAPTER III

BILE AND BILE SALTS POTENTIATE SUPEROXIDE ANION RELEASE FROM ACTIVATED, RAT PERITONEAL NEUTROPHILS

INTRODUCTION

In ANIT hepatotoxicity, bile acid concentration in liver and serum are elevated by approximately 4 and 400-fold, respectively. PMNs infiltrate periportal regions of the liver after ANIT treatment, and they would be exposed to high concentrations of biliary products such as bile acids. Studies were undertaken to assess whether biliary products might activate sequestered PMNs to release injurious products and thereby contribute to liver injury. Specifically, the capacity of bile and bile salts to stimulate O_2^- release from PMNs *in vitro* was examined.

In addition, we explored interactions between biliary products and PMNs primed with a threshold concentration of PMA. PMA was used as a priming agent to mimic the activation state of PMNs sequestered in the liver after ANIT treatment. As indicated in Chapter I, PMNs which respond to chemoattractants and infiltrate tissues are primed for O_2^- release. Although ANIT would have been a likely choice to prime PMNs *in vitro*, we did not use it for two reasons. First, PMNs sequestered in the liver after ANIT treatment are probably exposed to priming factors other than ANIT. Therefore, priming of PMNs with PMA, which acts similar to the endogenous PMN activator diacylglycerol, might mimic conditions *in vivo*. Second, ANIT is cytotoxic at concentrations which stimulate O_2^- release from PMNs (Roth and Hewett, 1990). Accordingly, we were concerned that addition of bile and bile salts to the system might cause even greater cytotoxicity to PMNs and mask a potential interaction to enhance O_2^- release.

METHODS

Chemicals. Lithocholic acid, sodium taurolithocholate, glycolithocholic acid, chenodeoxycholic acid, sodium taurochenodeoxycholate, sodium glycochenodeoxycholate, sodium deoxycholate, sodium cholate, cholanic acid, cytochalasin B, dimethylformamide (DMF), and cytochrome C (Type III from horse heart) were purchased from Sigma Chemical Company (St. Louis, MO). Superoxide dismutase (SOD) was obtained from Diagnostic Data Inc. (Mountainview, CA). Dimethyl sulfoxide (DMSO) was purchased from EM Science (Gibbstown, NJ). Petroleum ether was obtained from Mallinckrodt Inc. (Paris, KY). PMA was purchase from LC Services (Woburn, MA). All other reagents were of the highest grade commercially available.

PMA was dissolved in DMSO at 2 mg/ml and stored in 50 ul aliquots under N_2 at -70^{o}C. Shortly before use. an aliquot was thawed and diluted in Hanks'balanced salt solution (HBSS, pH 7.35) to the desired concentration.

Bile collection. Male, Sprague-Dawley rats (CF:CD(SD)BR) (Charles River, Portage, MI) weighing 250-450 g were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After a midline incision was made in the rat, the common bile duct was isolated and cannulated with PE 10 polyethylene tubing. Bile was collected over approximately a 30 min period and placed on ice until use later that day. When the concentration dependence of bile to enhance O_2^- release from primed PMNs was examined, bile was collected over approximately 60 min. Control bile flow was 50-70 ul/g liver/30 min.

Isolation of bile acids from rat bile. Bile was collected for approximately 30 min as described above. A crude fraction of bile salts was isolated as described by Haslewood (1967). Briefly, 10 volumes (1 ml) of 95% ethanol were added to 100 ul of bile, and the mixture was spun in a centrifuge to remove protein, mucin, and bile pigments. The supernatant fluid was decanted, placed into a test tube, and evaporated to dryness in a 37^{o}C water bath. The pellet was washed two times with petroleum ether (1 ml) and after drying under air, it was dissolved in 0.05 M NaOH.

Superoxide anion release from PMNs in vitro. O_2^- release was measured spectrophotometrically by the SOD inhibitable reduction of ferricytochrome C as described by Babior *et al.* (1973). Each assay contained 2x10^{6} PMNs and 80 nmol cytochrome C in 1 ml HBSS. PMNs were isolated from rat peritoneum as described under METHODS in Chapter II. In all assays *in vitro*, duplicate tubes (-SOD tubes) containing PMNs were preincubated with either a priming concentration of PMA (1 or 2 ng/ml) or HBSS vehicle for 5 min at room temperature. This priming concentration of PMA was barely suprathreshold, i.e., it consistently stimulated a small but detectable release of O_2^-. Bile acids or salts (1-100 uM), bile (1:200-1:10 dilution), or respective vehicles were then added, and the assay tubes were incubated at 37^{o}C for 10 min. All bile acids or salts were added to the assay tubes in 1 ul DMF except sodium cholate which was added in 100 ul HBSS. Bile acids extracted from rat bile were added in 5 ul of 0.05 M NaOH. Henceforth, we refer to all species in the incubation mixture as bile salts, since ionized species are favored over protonated species at the incubation pH. An exception to this nomenclature is found in Figure III-3 where the bile acid designation is used, since structures are shown in the protonated form. Control rat bile was diluted with HBSS and added in a 100 ul volume. The reaction was stopped by the addition of 86 U SOD and placing the tubes on ice.

This activity of SOD inhibits O_2^- release from maximally stimulated PMNs. Duplicate tubes (+ SOD tubes) were incubated as above, but received SOD prior to incubation. The samples were spun in a refrigerated centrifuge (0^oC) at 1200 x g for 10 min, and the absorbance of the cell-free supernatant was measuerd at 550 nm. The difference in absorbance between - SOD tubes and + SOD tubes was calculated, and the amount of cytochrome C reduced was estimated using an extinction coefficient of 18.5 cm^{-1} mM^{-1} (Margoliash and Frohwirt, 1959).

Enzyme release from PMNs. Studies were undertaken to determine whether bile salts enhanced granule enzyme release from activated PMNs. Lithocholate, the bile salt most potent in enhancing O_2^- release from PMA-primed PMNs, was used. *B*-glucuronidase, which is located in azurophilic granules of PMNs (Babior and Cohen, 1981), was measured by monitoring the release of phenolphthalein from its glucuronate 18 hours after incubation at 37^oC and pH 4.5 (Fishman *et al.*, 1948). Controls were run to assess whether lithocholate interfered with *B*-glucuronidase activity. A modest inhibition was found only at the highest lithocholate concentration (100 uM), and the data were corrected accordingly.

Briefly, $2x10^6$ PMNs were preincubated with FMLP ($2x10^{-9}$ or 10^{-7} M) or HBSS vehicle for 5 min at room temperature and then incubated with lithocholate (1-100 uM) or DMF vehicle for 10 min at 37^oC. Cytochalasin B (5 ug/ml) was added 10 min prior to incubation at 37^oC. Assay tubes were placed on ice and then spun in a refrigerated centrifuge (0^oC) at 1200 x g for 10 min. Enzyme activity was measured in the cell-free supernatant. Total enzyme release was determined by lysing PMNs at an equivalent concentration in 0.1% Triton X-100 followed by sonication.

Statistical Analysis. All data are presented as mean $\pm$ S.E. Data in Figures III-3, III-4, III-5, and III-6 were analyzed using a completely blocked factorial analysis of variance (ANOVA). Data in Figure III-1 were analyzed by a completely randomized factorial ANOVA. Homogeneity of variance was tested using the F-max test. Log transformations were made on nonhomogeneous data. Individual comparisons between treatment means were made with Tukey's omega test (Steel and Torrie, 1980). The criterion for significance was $p < 0.05$ for all comparisons.

RESULTS

Stimulatory effect of bile on PMNs. The additon of normal rat bile at a final dilution of 1:50 in HBSS was not able to stimulate O_2^- release from rat peritoneal PMNs (Figure III-1). In preliminary experiments, this dilution of bile caused maximal enhancement of O_2^- release from PMA-primed PMNs. To determine whether bile might enhance O_2^- release from activated PMNs, PMNs were primed with a barely suprathreshold concentration of PMA (2 ng/ml). PMA is a commonly used stimulus for PMNs, and it was used to activate PMNs minimally. PMNs preincubated with PMA and then incubated with HBSS, the vehicle for bile, produced 3.4 $\pm$ 0.6 nmol $O_2^-/2x10^6$ cells/10 min. When PMNs were primed with PMA and then incubated with bile (1:50 dilution), O_2^- release was enhanced to 14.4 $\pm$ 2.7 nmol $O_2^-/2x10^6$ cells/10 min (Figure III-1). When the concentration dependence of bile in enhancing O_2^- release from primed PMNs was examined in six samples of bile from different rats, bile with stimulatory actions toward primed PMNs appeared to produce maximal effects at the 1:50 dilution used (Figure III-2).

Not all bile samples were able to potentiate the release of O_2^- from PMA-primed PMNs (Figures III-1 and III-2). However, ethanol extracts of inactive bile samples were able to enhance O_2^- release from PMA-activated PMNs (data not shown), suggesting the presence of factors that interfere with O_2^- release or scavenge O_2^- in these bile samples.

Stimulatory effect of bile salts on PMNs. None of the bile salts surveyed, with the exception of lithocholate, were able by themselves to stimulate O_2^-

release from PMNs. PMNs incubated with lithocholate (32 uM) alone released small, but statistically significant amounts of O_2^- (Fig. III-3).

As in the case with bile, the addition of certain bile salts to PMA-primed PMNs resulted in an enhanced release of O_2^- compared to vehicle controls. Lithocholate, a monohydroxy bile salt, had the greatest effect to enhance O_2^- release from primed PMNs, causing approximately an 8-fold increase in O_2^- release over DMF vehicle (Figure III-3). Lithocholate produced maximal enhancement of O_2^- release from PMA-primed PMNs at concentrations between 10 and 32 uM (Figure III-4). Lithocholate at concentrations as low as 3.2 uM was able to enhance release of O_2^- from primed PMNs.

The dihydroxy bile salts deoxycholate and chenodeoxycholate at a concentration of 100 uM caused more modest enhancement (2-3 fold) of O_2^- release from PMA-primed PMNs (Figure III-3). Higher concentrations of deoxycholate and chenodeoxycholate (1 mM) caused greater enhancement of O_2^- , but they were cytotoxic to PMNs as determined by trypan blue dye exclusion (data not shown). The trihydroxy bile salt, cholate, was not able to enhance O_2^- release from primed PMNs at a concentration of 100 uM (Figure III-3). At concentrations 10-30 fold higher, however, cholate was able to do so (data not shown). For example, the addition of 1 mM cholate to PMA-primed PMNs produced 12.8 $\pm$ 2.2 nmol $O_2^-/2x10^6$ cells/10 min, compared to a PMA control value of 4.6 $\pm$ 0.7 nmol $O_2^-/2x10^6$ cells/10 min. At a concentration of 3 mM, cholate caused even greater enhancement of O_2^-, although it was cytotoxic to PMNs (data not shown). Cholanoate, which is the parent bile salt structure without any hydroxyl (OH) substituents, produced an effect similar to that of the dihydroxy bile salts (Figure III-3).

Effect of bile salt conjugates on activated PMNs. Although free lithocholate caused greater than 9-fold increase in O_2^- release from primed

PMNs compared to DMF vehicle, the glycine and taurine conjugates of lithocholate were not able to enhance O_2^- release from PMA-primed PMNs (Figure III-5). The inability of glycolithocholate and taurolithocholate to potentiate the PMA response was apparently not due to greater cytotoxicity toward PMNs, since PMNs treated with either conjugate excluded trypan blue dye to a similar extent than PMNs treated with lithocholate (data not shown). Higher concentrations of lithocholate conjugates, i.e., > 100 uM, were not tested due to the limited solubility of these conjugates. The glycine and taurine conjugates of chenodeoxycholate were also unable to enhance O_2^- release from PMA-primed PMNs, whereas chenodeoxycholate itself more than doubled the release of O_2^- from PMA-primed PMNs (Fig. III-5). Conjugates of lithocholate or chenodeoxycholate by themselves did not stimulate O_2^- release from PMNs (data not shown).

B-glucuronidase release from PMNs. Lithocholate did not directly stimulate the release of *B*-glucuronidase from PMNs at the concentrations tested (1-100 uM), whereas FMLP in the presence of cytochalasin B caused approximately 39% release (Figure III-6). Lithocholate was unable to enhance *B*-glucuronidase release from FMLP-activated PMNs (Figure III-6).

Figure III-1 Effect of bile on O_2^- release from rat peritoneal PMNs. PMNs ($2x10^6$) were preincubated with either PMA (2 ng/ml) or HBSS for 5 min and then incubated with either normal rat bile (diluted 1:50) or HBSS vehicle for 10 min. Individual data points are plotted, and the means for groups are indicated by the dashed lines. Numbers in parentheses indicate number of samples. [a]Significantly different from group preincubated with HBSS and incubated with HBSS. [b]Significantly different from group preincubated with HBSS and incubated with bile. [c]Significantly different from group preincubated with PMA and incubated with HBSS.

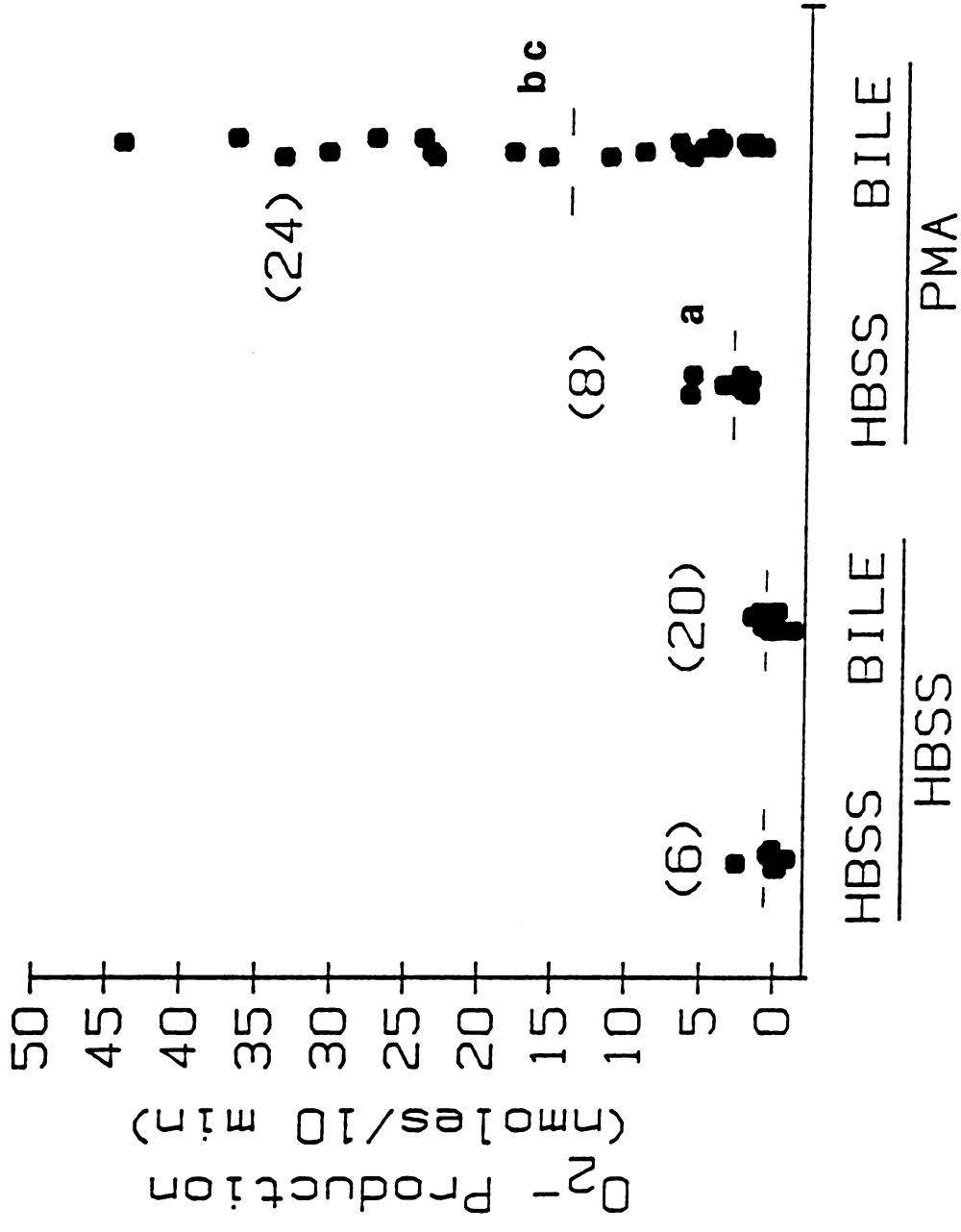

O_2^- Production
(nmoles/10 min)
50
45
40
35
30
25
20
15
10
5
0
(6)
(20)
(8)
(24)
a
bc
HBSS
BILE
HBSS
HBSS
BILE
PMA

Figure III-1

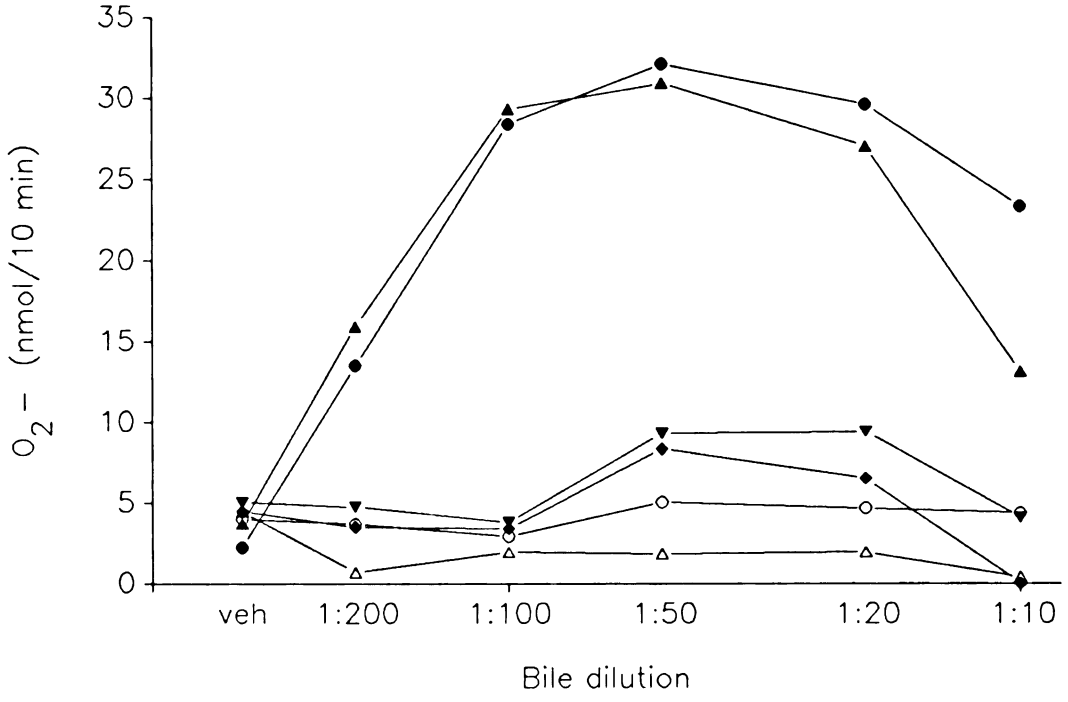


Figure III-2. Concentration dependence of bile-stimulated O_2^- release from primed, rat peritoneal PMNs. PMNs ($2x10^6$) were preincubated with PMA (2 ng/ml) for 5 min and then incubated with bile (1:200-1:10 dilution) or HBSS vehicle (veh) for 10 min. Concentration/response data for 6 different bile samples are plotted individually.

Figure III-3. O_2^- release from rat peritoneal PMNs stimulated with bile salts. PMNs ($2x10^6$) were preincubated for 5 min with either PMA (2 ng/ml) or its vehicle (HBSS) and then incubated with either bile acids/salts or their vehicles for 10 min. In experiments with cholate, the PMA concentration was 1 ng/ml. All bile acids/salts were added in DMF, except cholate which was added in HBSS. N = 3-20. [a]Significantly different from group preincubated with HBSS and incubated with bile acid vehicles. [b]Significantly different from group preincubated with PMA and incubated with bile acid vehicles. [c]Significantly different from group preincubated with HBSS and incubated with bile acids.

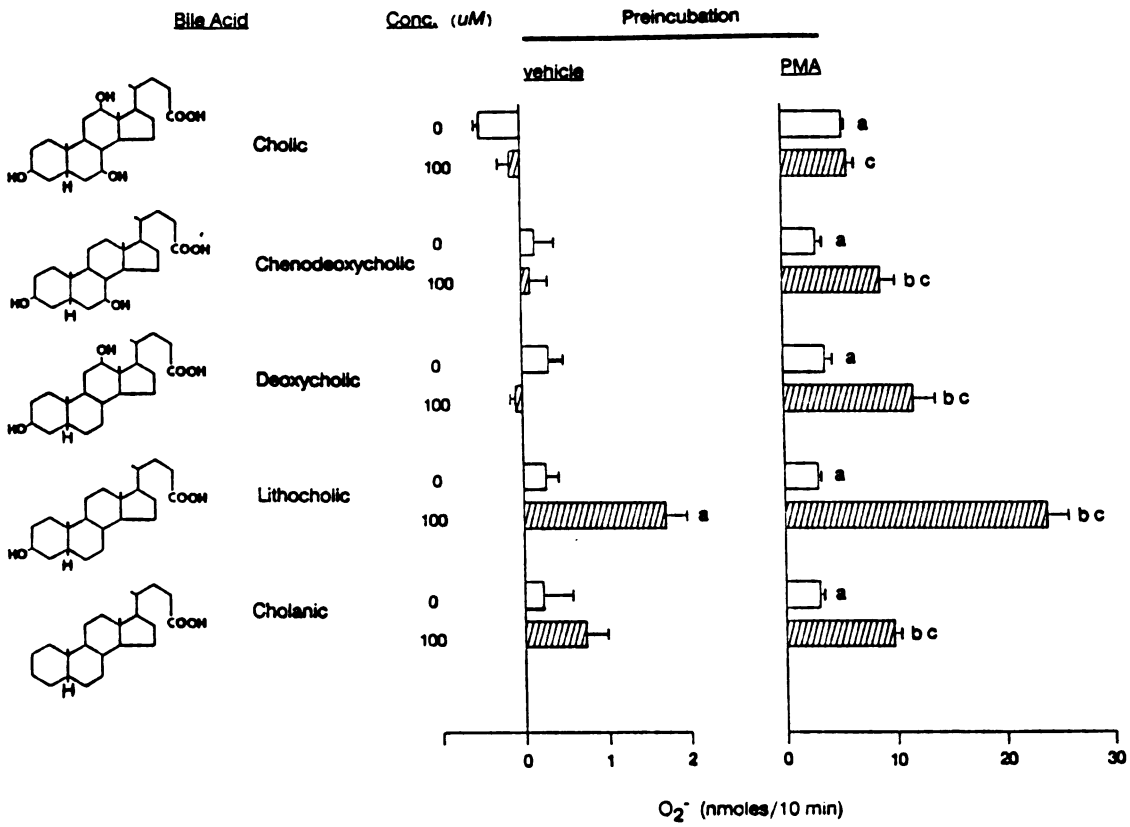

Bile Acid
Conc. (uM)
Preincubation
vehicle
PMA
Cholic
Chenodeoxycholic
Deoxycholic
Lithocholic
Cholanic
0
100
a
c
b c
0
1
2
10
20
30
O2- (nmoles/10 min)

Figure III-3

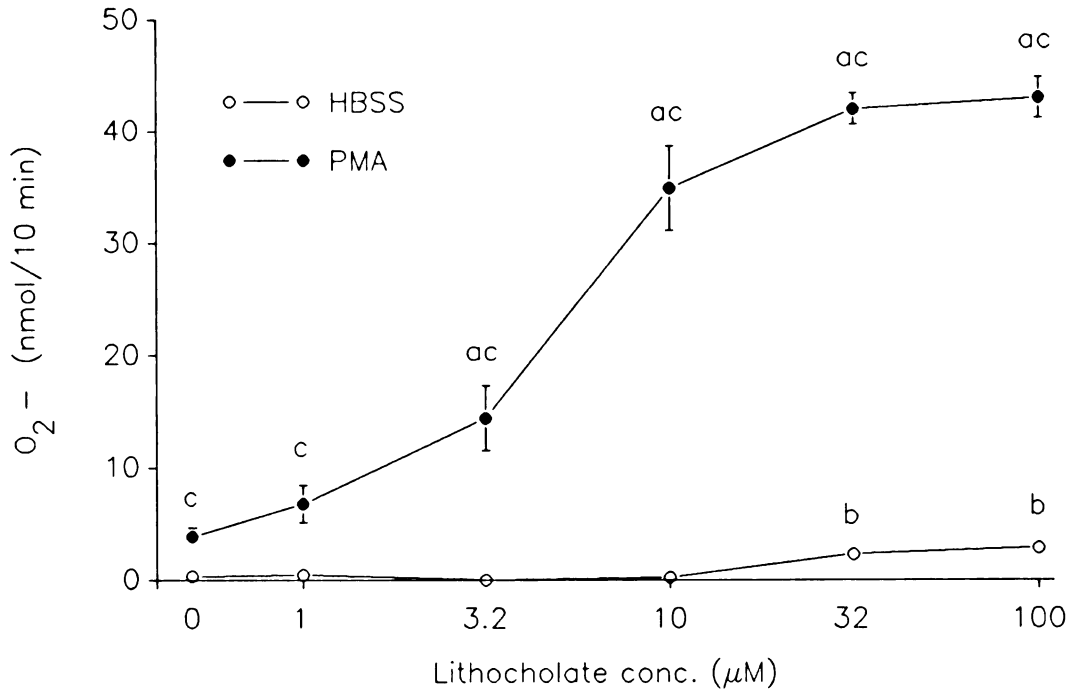
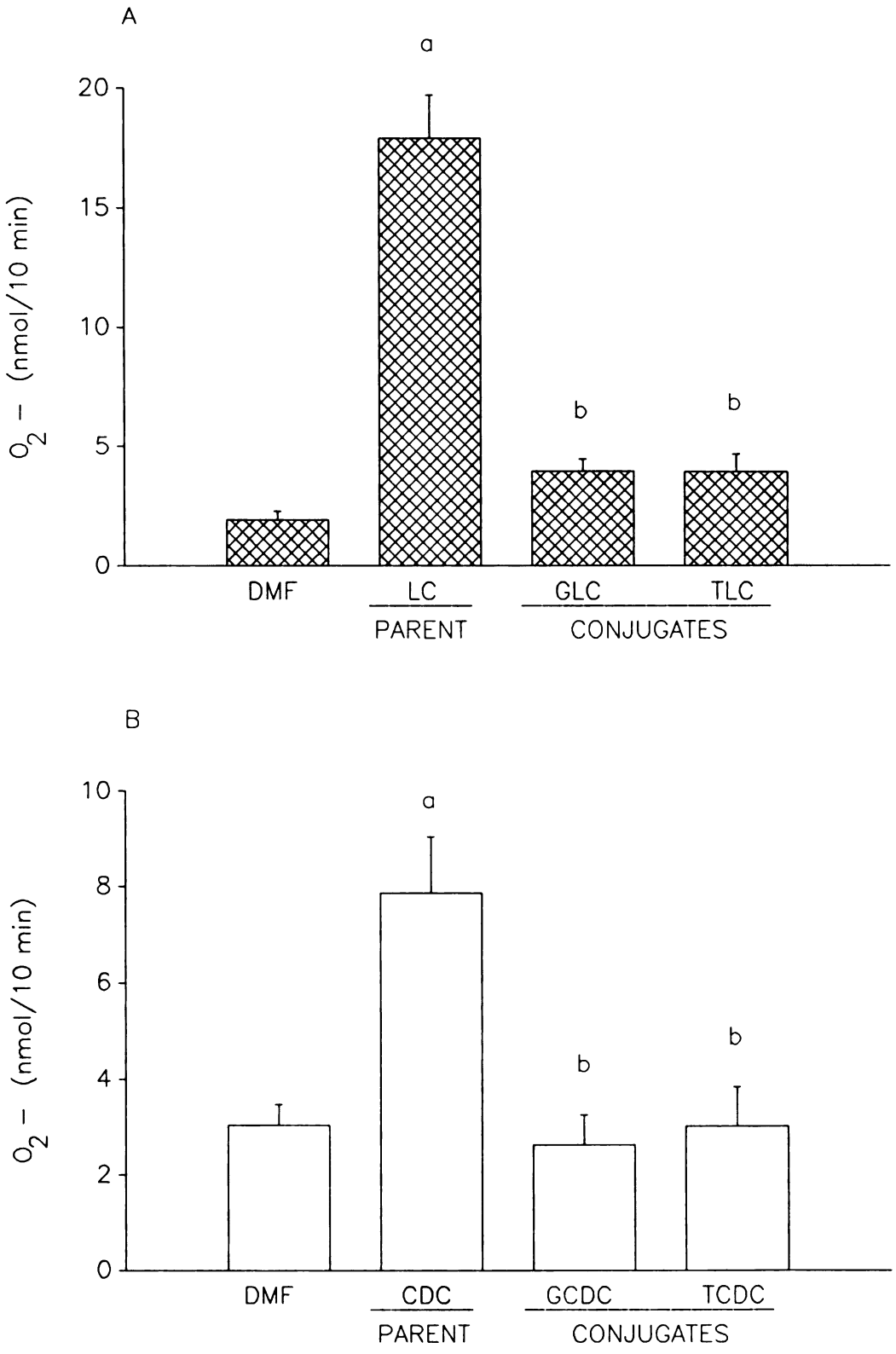


Figure III-4. Concentration dependence of lithocholate-stimulated O_2^- release from rat peritoneal PMNs. PMNs ($2x10^6$) were preincubated with either PMA (1 ng/ml) or HBSS for 5 min and then incubated with either lithocholate (1-100 uM) or DMF for 10 min. Values are mean $\pm$ S.E. for 4 preparations. Points lacking standard error bars have S.E. less than the area covered by the symbols. [a]Significantly different from group preincubated with PMA and incubated with the vehicle for lithocholate (DMF). [b]Significantly different from group preincubated with HBSS and incubated with the vehicle for lithocholate (DMF). [c]Significantly different from group preincubated with HBSS and incubated with an identical concentration of lithocholate.

Figure III-5. Effect of glycine and taurine conjugates (100 uM) of lithocholate (A) and chenodeoxycholate (B) on PMA-primed, peritoneal PMNs. PMNs ($2x10^6$) were preincubated with PMA (2 ng/ml) for 5 min and then incubated for 10 min with parent (free) bile salt, glycine conjugate, taurine conjugate, or DMF vehicle. Abbreviations: LC, lithocholate; GLC, glycolithocholate; TLC, taurolithocholate; CDC, chenodeoxycholate; GCDC, glycochenodeoxycholate; TCDC, taurochenodeoxycholate. N = 8 for lithocholate and N = 4 for chenodeoxycholate. [a]Significantly different from group incubated with DMF vehicle. [b]Significantly different from group incubated with parent bile salt.


A
a
20
15
10
5
0
O_2^- (nmol/10 min)
b
b
DMF
LC
GLC
TLC
PARENT
CONJUGATES
B
a
10
8
6
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O_2^- (nmol/10 min)
b
b
DMF
CDC
GCDC
TCDC
PARENT
CONJUGATES

Figure III–5

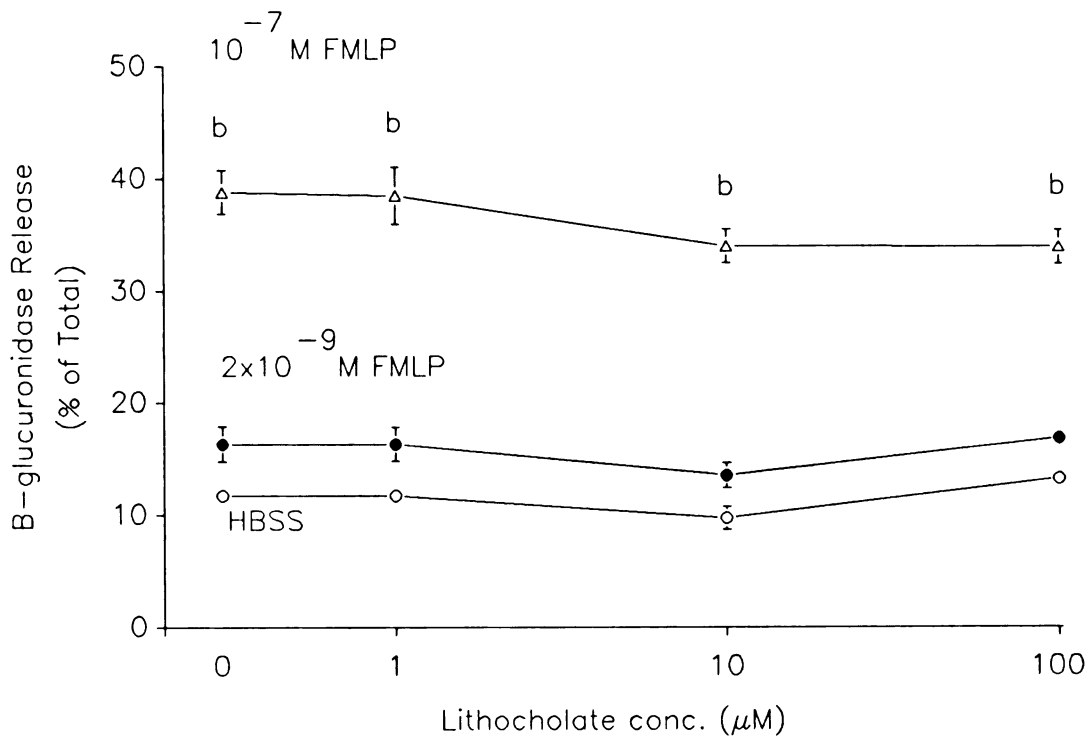

Figure III-6. Effects of lithocholate on FMLP-stimulated B-glucuronidase release. $2x10^6$ PMNs were preincubated with FMLP ($2x10^{-9}$ or 10^{-7} M) or HBSS vehicle for 5 min at room temperature. Lithocholate (1-100 uM) or DMF vehicle was added, and the reactions were carried out for 10 min at 37°C. Cytochalasin B (5 ug/ml) was present in all assay tubes and was added 10 min prior to incubation at 37°C. At the end of the incubation, tubes were placed on ice and spun in a centrifuge at 0°C. B-glucuronidase activity was assayed in the cell-free supernatant. 100% B-glucuronidase release was determined by lysing PMNs at an equivalent concentration in 0.1% Triton X-100 followed by sonication. N = 6. [a] Significantly different from respective group treated with the vehicle for lithocholate (DMF). [b] Significantly different from group treated with the vehicle for FMLP (HBSS) at an identical lithocholate concentration.

SUMMARY

In this chapter, we showed that bile and bile salts had little capacity by themselves to stimulate O_2^- release from PMNs. However, they enhanced O_2^- release from PMA-primed PMNs. The order of activity for O_2^- enhancement by bile salts was monohydroxy (lithocholate) > dihydroxy (deoxycholate and chenodeoxycholate) > trihydroxy (cholate). Free bile salts enhanced O_2^- release from PMA-primed PMNs, whereas glycine and taurine conjugates were inactive. In the next chapter, we examine whether PMNs and PMN-derived oxygen radicals are involved in a model of chemically-induced liver injury characterized by PMN infiltration.

CHAPTER IV

AN ANTIBODY TO NEUTROPHILS ATTENUATES ALPHA-NAPHTHYLISOTHIOCYANATE-INDUCED LIVER INJURY

INTRODUCTION

In Chapter II, we showed that activated PMNs caused liver injury by an oxygen radical-dependent mechanism. The purpose of experiments presented in this chapter is to examine whether PMNs and PMN-derived oxygen radicals are involved in the liver injury caused by ANIT. There are several observations which suggest that PMNs and oxygen radicals are involved. First, PMNs infiltrate periportal regions of the liver after ANIT treatment and are associated with necrotic bile duct epithelial cells and necrotic hepatocytes. Second, ANIT stimulates O_2^- release from rat PMNs *in vitro* (Roth and Hewett, 1986). Third, *B*-naphthylisothiocyanate, a non-hepatotoxic isomer of ANIT, does not stimulate O_2^- release from PMNs *in vitro*.

In studies presented in this chapter, the dose of ANIT was 35 mg/kg, which is lower than those used by most investigators, i.e., > 100 mg/kg. It was chosen because preliminary results indicated that it did not cause complete cholestasis. The dose of ANIT concerned us, since it seemed possible that a PMN-dependent component of injury might be masked if livers were severely compromised.

METHODS

Materials. Decolorizing carbon (NORIT^R) was purchased from the Baker Chemical Co. (Phillipsburg, NJ). ANIT (lot 43F-0528), glycogen (Type II from oyster), L-gamma-glutamyl-*p*-nitroanilide, kit 605-D for total bilirubin determination, and kit 505 for aspartate aminotransferase (AST) activity were purchased from Sigma Chemical Co. (St. Louis, MO). Freund's adjuvants (complete and incomplete) were obtained from Gibco Laboratories (Grand Island, NY). Polyethylene glycol-coupled superoxide dismutase (PEG-SOD), -catalase (PEG-CAT), and -bovine serum albumin (PEG-BSA) were obtained from Enzon, Inc. (South Plainfield, NJ). Antilymphocyte serum was purchased from Accurate Chemical Co. (Westbury, NY). PE polyethylene tubing was obtained from Clay Adams (Parsippany, NJ).

ANIT was decolorized with NORIT^R and recrystallized from hot ethanol prior to use, because we were concerned about impurities in different lots of ANIT. Briefly, approximately 0.75 g ANIT was added to 10 ml of hot 95% ethanol. NORIT^R (0.5-1.0 g) was added, and the contents were filtered into a beaker on ice. After crystals formed, they were transferred to a suction filter apparatus and dried. This procedure did not alter the ability of ANIT to cause liver injury. Consequently, the crude fraction was used in the lymphocyte depletion experiment in this chapter and in experiments shown in chapters V and VI.

Animals. Male, Sprague-Dawley rats (CF:CD(SD)BR) (Charles River, Portage, MI) weighing 250-340 g were housed in plastic cages on corn cob bedding under conditions described under METHODS in Chapter II.

Preparation of polyclonal antibody to rat PMNs. Female, New Zealand White rabbits (1.5-2.0 kg; Bailey's Rabbitry, Dutton, MI) were housed under controlled temperature, humidity, and lighting. 1 x 10^6 PMNs in a 1:1 suspension of HBSS and complete Freund's adjuvant were injected s.c. in a volume of 1 ml into the footpads of rabbits in several sites. PMNs were isolated as desribed under METHODS in Chapter II. Two weeks later, rabbits received 1 x 10^6 PMNs in a 1:1 suspension of HBSS and incomplete Freund's adjuvant i.m. in a 1 ml volume in the dorsum of the rump at 6 sites. Two weeks later, this procedure was repeated. One week later, blood was withdrawn from the central ear artery and allowed to clot for 2 hr at 37^{o}C for collection of antineutrophil serum (NAS). NAS and control serum (CS) obtained from untreated rabbits were heated at 56^{o}C for 45 min to inactivate complement. The effectiveness of NAS in reducing circulating PMN numbers in rats was determined on each batch of NAS collected.

Treatment protocol for depletion of circulating PMNs from rats. Chow was removed from rats just prior to treatment. Rats received NAS or CS (1.5 ml/rat, i.p.) and either ANIT (35 mg/kg, p.o.) or an equivalent volume of corn oil (CO, 2 ml/kg) vehicle 6 hr later. Immediately prior to ANIT or CO administration, rats were warmed under a lamp, and 0.5 ml of blood was collected from the tail into a tube containing 50 ul 3% EDTA. Total white blood cell (WBC) counts were performed with a Coulter Counter Model ZM with Channelyzer (Coulter Electronics Limited, Luton, England). Circulating PMN numbers were determined by multiplying the total WBC count by the percentage of PMNs in a differential leukocyte count of a Wright-Giemsa-stained blood smear. Twelve hr after the initial NAS administration, rats received a second treatment of NAS or CS (1 ml/rat, i.p.). At 24 hr, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed on a heating pad to maintain body temperature. A

midline incision was made in the rat, and the common bile duct was isolated and cannulated with PE 10 polyethylene tubing. Bile was collected for a 30 min period. Bile samples were weighed, and bile flow was calculated assuming a density of 1 g/ml.

A blood sample was taken from the descending aorta for measurements of serum markers of liver injury. Total bilirubin concentration (Sigma kit 605-D) was measured spectrophotometrically by coupling bilirubin to *p*-diazobenzenesulfonic acid to form azobilirubin. Total serum 3 alpha-hydroxy bile acid concentration (TSBA) was determined by measuring the fluorescence of resorfin as described by Mashige *et al.* (1976). GGT activity was measured spectrophotometrically by monitoring the GGT-catalyzed formation of *p*-nitroanilide from L-gamma-glutamyl-*p*-nitroanilide as described by Szasz (1969). AST activity (Sigma kit 505) was measured spectrophotometrically by monitoring formation of the phenylhydrazone of oxalacetate as described by Reitman and Frankel (1957).

Any rat receiving NAS which did not fit the criterion for PMN depletion (i.e., $< 500 \pm 50$ PMN/ul blood) was not included in the study. This degree of PMN depletion suppresses the Arthus reaction and protects rats from lung injury that is PMN-mediated (Johnson and Ward, 1974; Till *et al.*, 1982).

Treatment protocol for reduction of circulating lymphocyte numbers. Rats were fasted 24 hr prior to experimentation and for the remainder of the study. This change in protocol was made because fasting appears to eliminate the non-responsiveness to ANIT (see Chapter VII for discussion). They were given antilymphocyte serum (ALS) or CS (1 ml/rat each) i.v. in the dorsal penis vein six hr prior to treatment with ANIT (35 mg/kg, p.o.). Rats received a second treatment of ALS or CS (1 ml/rat, i.v.) 12 hr after the initial dose. Rats were administered ALS i.v., because we observed subsequent to PMN depletion

experiments described herein that NAS was more effective in reducing circulating PMN numbers by this route of administration. Markers of liver injury were measured 24 hr after ANIT treatment as described above.

Treatment protocol for PEG-CAT/SOD study. Chow was removed from rats just prior to treatment. Rats were treated i.v. in the dorsal penis vein with a combination of PEG-CAT (2000 IU) and PEG-SOD (1000 IU) or with an equivalent amount of protein as PEG-BSA immediately prior to administration of either ANIT (35 mg/kg, p.o.) or an equivalent volume of CO. Twelve hr later, rats were placed under a warming lamp, and a blood sample was taken from the tail for serum measurements of CAT and SOD activities as described under METHODS in Chapter II. Rats were treated with the PEG-CAT/SOD combination or with PEG-BSA (control) after the blood sample was taken. Twenty-four hr after ANIT treatment, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Bile flow and other markers of liver injury were measured as described above. CAT and SOD activities in serum were also measured at this time.

Statistical Analysis. Results are expressed as mean $\pm$ S.E. Homogeneity of variance was tested using the F-max test. Log transformations were performed on nonhomogeneous data. If the variances were homogeneous, data were analyzed using a completely randomized factorial analysis of variance or Student's t-test, as appropriate. Individual comparisons between treatment means were made with Tukey's omega test (Steel and Torrie, 1980). When the variances were nonhomogenous after log transformation of data, data were analyzed with the nonparametric, distribution-free, multiple comparison test (Gad and Weil, 1986). The criterion for significance was $p < 0.05$ for all comparisons.

RESULTS

Effect of NAS treatment on circulating PMN numbers. Administration of NAS to rats reduced circulating PMN numbers 6 hr later to < 500/ul blood, which was the criterion for PMN depletion (Figure IV-1). The NAS treatment protocol would be expected to deplete circulating PMNs for the entire experiment. For example, in a preliminary experiment using the same NAS treatment regimen, circulating PMN numbers were 1224 $\pm$ 300 per ul blood (n = 3) when measured prior to NAS treatment. They were reduced to 130 $\pm$ 82 per ul blood (n = 3) 32-36 hr later, which corresponds to the time rats were killed in the PMN depletion study with ANIT, i.e., 30 hr after initial NAS treatment.

Effect of NAS on ANIT-induced liver injury. Administration of NAS to rats did not cause hepatic injury, since all markers of liver injury were not changed relative to CS controls (Figure IV-2). ANIT treatment caused elevations in serum total bilirubin concentration, total bile acid concentration, and AST and GGT activities (Figure IV-2). Three of 14 rats (21%) receiving ANIT and CS did not exhibit cholestasis or hyperbilirubinemia (data not shown). In these rats, other markers of liver injury were unaffected or elevated slightly. These rats were included in all statistical analyses.

Rats depleted of circulating PMNs with NAS were protected against the hepatotoxic effects of ANIT as indicated by reductions in serum total bilirubin concentration, total bile acid concentration, AST activity, and GGT activity (Figure IV-2). NAS treatment afforded complete protection against these markers of liver

injury with the exception of total bile acid concentration, for which a partial protection was observed.

Rats treated with ANIT and CS appeared to have reduced bile flow, although the decrease was not statistically significant (Figure IV-3). Bile flow in rats receiving NAS and ANIT was near control values. In a followup PMN depletion study in which ANIT significantly reduced bile flow, co-treatment of rats with NAS prevented the cholestasis (data not shown). For example, bile flow in rats receiving ANIT and CS was 13 $\pm$ 4 ul/g liver/30 min (N = 7), whereas it was 40 $\pm$ 6 ul/g liver/30 min (N = 11) in rats treated with ANIT and NAS. In this followup study, a treatment protocol identical to that indicated for ALS administration was used. NAS afforded protection against serum total bilirubin concentration, serum total bile acid concentration, and GGT activity (data not shown), which confirmed our earlier observations. NAS administration tended to reduce the elevation of serum AST activity caused by ANIT, although the decrease was not statistically significant (data not shown).

Effects of ALS on ANIT-induced liver injury. Products of peripheral lymphocytes may produce cholestasis in rats (Mizoguchi *et al.*, 1981, 1986; Marbet *et al.*, 1984). Administration of NAS to rats caused a 20-25% reduction in circulating lymphocyte numbers (data not shown). Although this decrease was not statistically significant, it seemed possible that NAS might have afforded protection against ANIT-induced liver injury by an effect on lymphocytes. Administration of ALS to rats reduced circulating lymphocyte numbers by approximately 65% 7 hr after treatment (data not shown) but did not afford protection against the liver injury caused by ANIT (Table IV-1). On the contrary, ALS administration enhanced the hyperbilirubinemia caused by ANIT (Table IV-1).

Effect of PEG-CAT/SOD on ANIT-induced liver injury. Since ANIT stimulates O_2^- release from rat PMNs *in vitro* (Roth and Hewett, 1986), we determined whether agents which degrade toxic oxygen species afford protection against the liver injury caused by ANIT. Rats treated with ANIT and PEG-BSA vehicle had serum elevations in total bilirubin concentration, total bile acid concentration, AST activity, and GGT activity (Figure IV-4). They tended to have reduced bile flow, although the decrease was not statistically significant (Figure IV-5). Three of 11 rats (27%) receiving ANIT and PEG-BSA did not respond to the hepatotoxic effects of ANIT (data not shown). As in the PMN depletion experiment, these rats were included in statistical analyses.

Administration of the PEG-CAT/SOD combination to rats caused large elevations in CAT and SOD activity in serum throughout the experiment (Table IV-2), although it did not reduce the elevation caused by ANIT in any marker of liver injury (Figure IV-4). PEG-CAT/SOD administration did not appear to have any effect on bile flow in ANIT-treated rats (Figure IV-5).

The PEG-CAT/SOD combination had minimal effects on markers of liver injury in CO-treated rats, although serum GGT activity was slightly elevated, and bile flow was decreased slightly (Figures. IV-4 and IV-5, respectively).

TABLE IV-1

Effects of antilymphocyte serum (ALS) on ANIT hepatotoxicity

	Treatment[a]	
	CS	ALS
Bile flow (μl/g liver/30 min)	3±3	11±1
Serum total bilirubin conc. (mg/dl)	2.4±0.1	3.2±0.2[b]
Serum AST activity (SF units/ml)	351±44	301±31

[a]Rats were treated with ANIT (35 mg/kg, p.o.) and either ALS or CS as described under METHODS. Markers of liver injury were measured 24 hr after ANIT treatment.

[b]Significantly different from group treated with CS.

TABLE IV-2

Serum CAT and SOD activities after PEG-CAT/SOD administration to rats

		Treatment[a]			
		CO		ANIT	
		PEG-BSA	PEG-CAT/SOD	PEG-BSA	PEG-CAT/SOD
CAT activity (IU/ml)	12 hr	10±3	108±5[c]	11±2	106±4[e]
	24 hr	14±6	142±10[b,c]	33±7[b]	142±8[b,e]
SOD activity (IU/ml)	12 hr	13±3	60±4[c]	13±4	86±4[d,e]
	24 hr	14±1	123±8[b,c]	12±2	119±6[b,e]

[a]Rats were treated i.v. with a combination of PEG-CAT (2000 IU) and PEG-SOD (1000 IU) immediately prior to ANIT (35 mg/kg, p.o.) or CO vehicle and again 12 hr later. n=7-11.

[b]Significantly different from respective 12 hr group.

[c]Significantly different from group treated with CO and PEG-BSA.

[d]Significantly different from group treated with CO and PEG-CAT/SOD.

[e]Significantly different from group treated with ANIT and PEG-BSA.

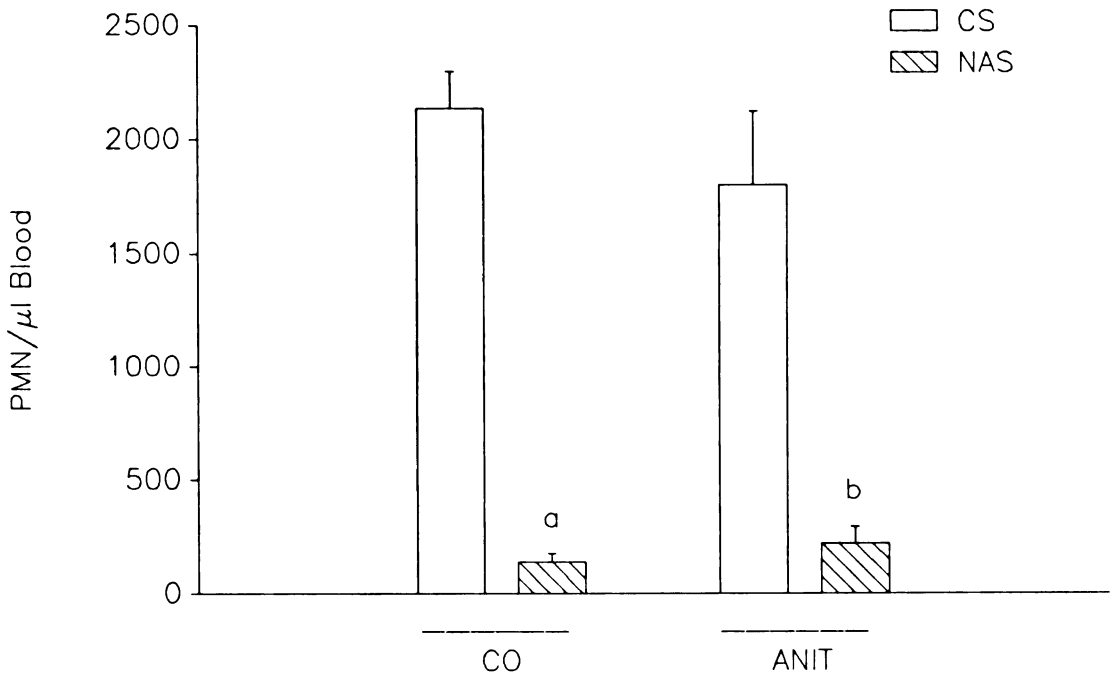

Figure IV-1. Effect of NAS on circulating PMN numbers. Rats were treated with NAS or CS (1.5 ml/rat, i.p.) 6 hr prior to treatment with ANIT (35 mg/kg, p.o.) or an equivalent volume of corn oil (CO) vehicle. Circulating PMN numbers were determined immediately prior to treatment with ANIT. Rats received a second treatment of NAS or CS (1 ml/rat, i.p.) 12 hr after the initial administration. N = 4-11. [a] Significantly different from CO/CS group. [b] Significantly different from ANIT/CS group.

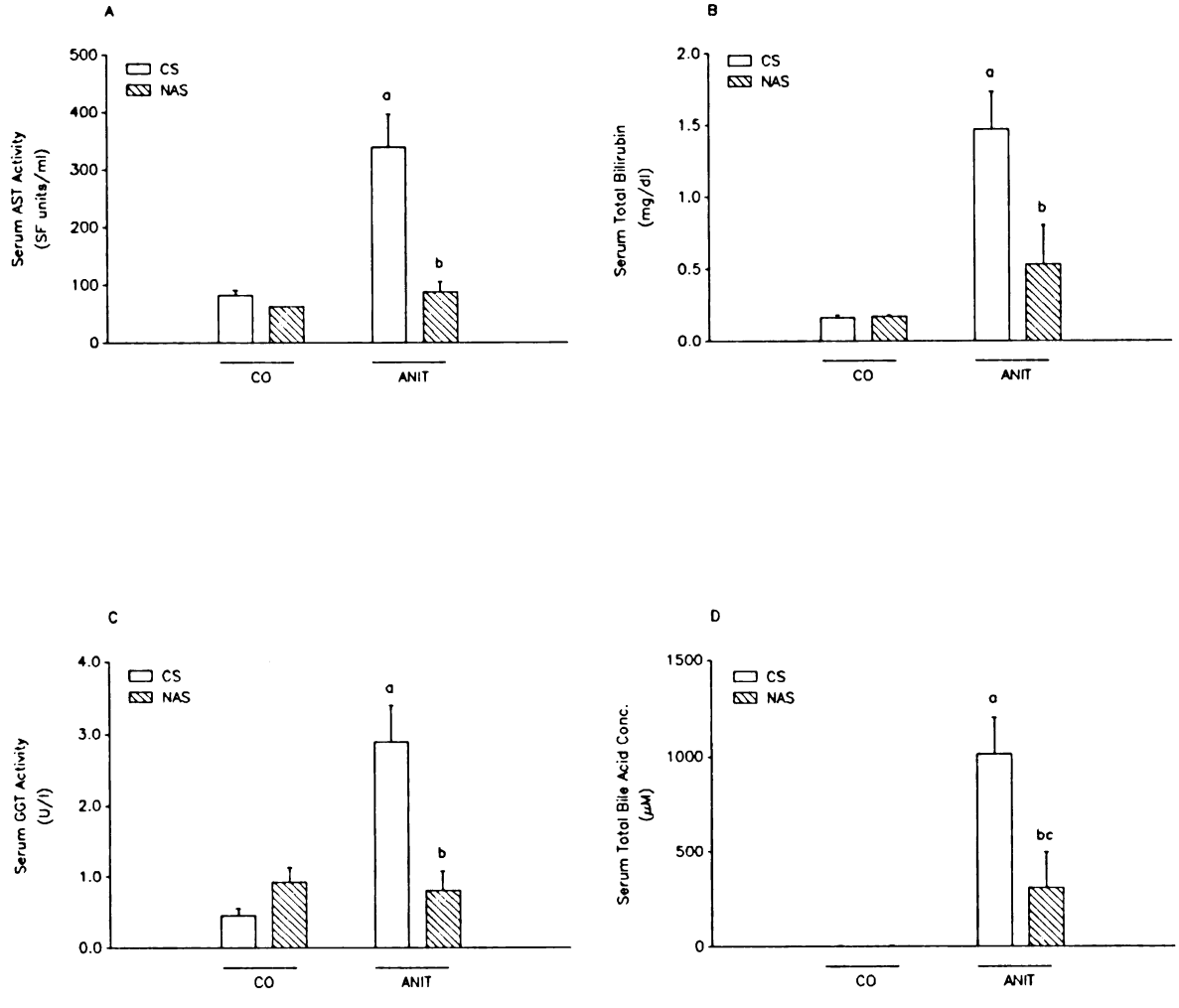

Figure IV-2. Effect of NAS on ANIT-induced elevations in serum AST activity (panel A), total bilirubin concentration (panel B), GGT activity (panel C), and total bile acid concentration (panel D). Treatment regimen is described in the legend to Figure. IV-1. Markers of liver injury were measured 24 hr after treatment of rats with ANIT or CO. N = 4-11. [a] Significantly different from CO/CS group. [b] Significantly different from ANIT/CS group. [c] Significantly different from CO/NAS group.

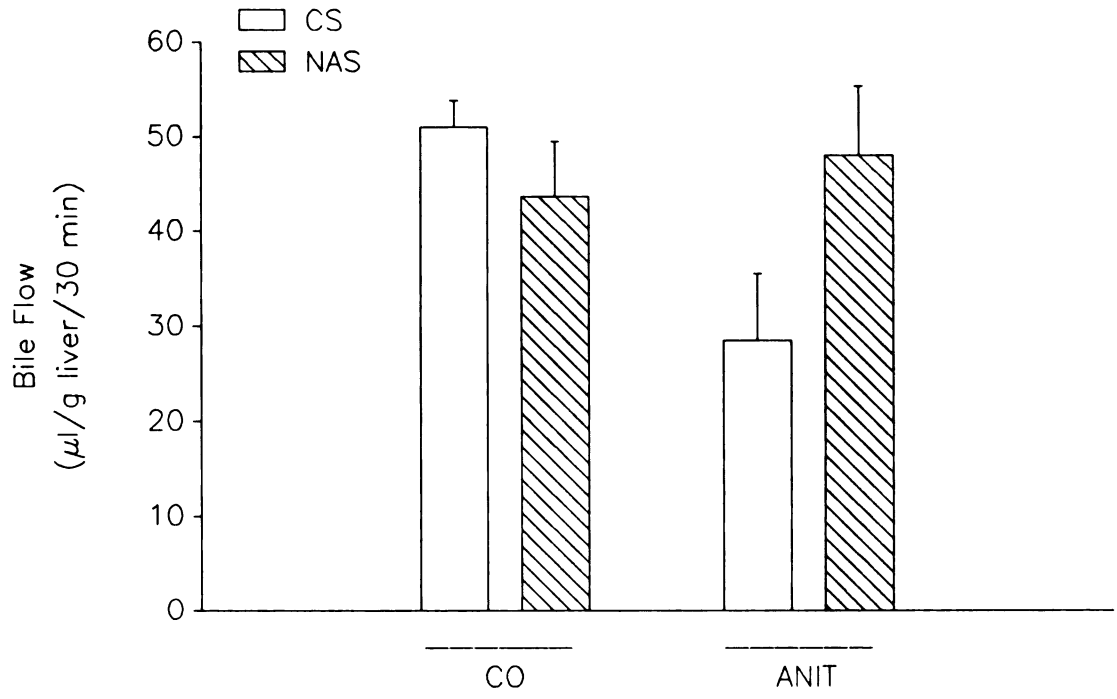

Figure IV-3. Effect of NAS on bile flow. Treatment regimen is described in the legend to Figure IV-1. Bile flow in rats anesthetized with sodium pentobarbital (50 mg/kg, i.p.) was measured 24 hr after treatment with ANIT or CO. N = 4-11. There were no statistically significant differences among groups.

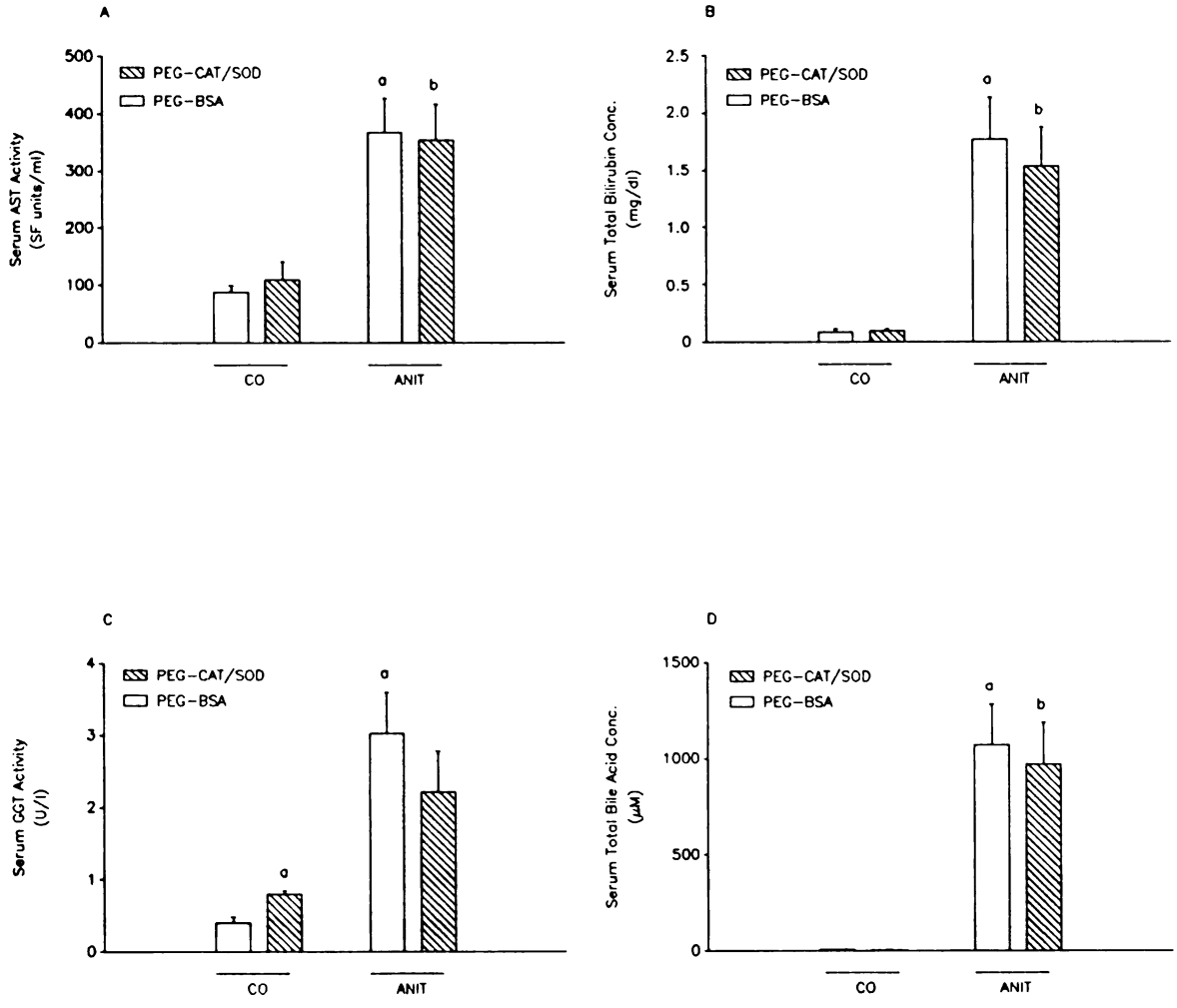

Figure IV-4. Effect of PEG-CAT/SOD on ANIT-induced elevations in serum AST activity (panel A), total bilirubin concentration (panel B), GGT activity (panel C), and total bile acid concentration (panel D). Rats were treated with a combination of PEG-CAT (2000 IU) and PEG-SOD (1000 IU) or equivalent protein concentration as PEG-BSA i.v. immediately prior to ANIT (35 mg/kg, p.o.) or equivalent volume of CO. Rats received a second treatment of PEG-CAT/SOD or PEG-BSA 12 hr after the initial administration. Markers of liver injury were measured 24 hr after ANIT or CO treatment. N = 4-11. [a] Significantly different from group treated with CO and PEG-BSA. [b] Significantly different from group treated with CO and PEG-CAT/SOD.

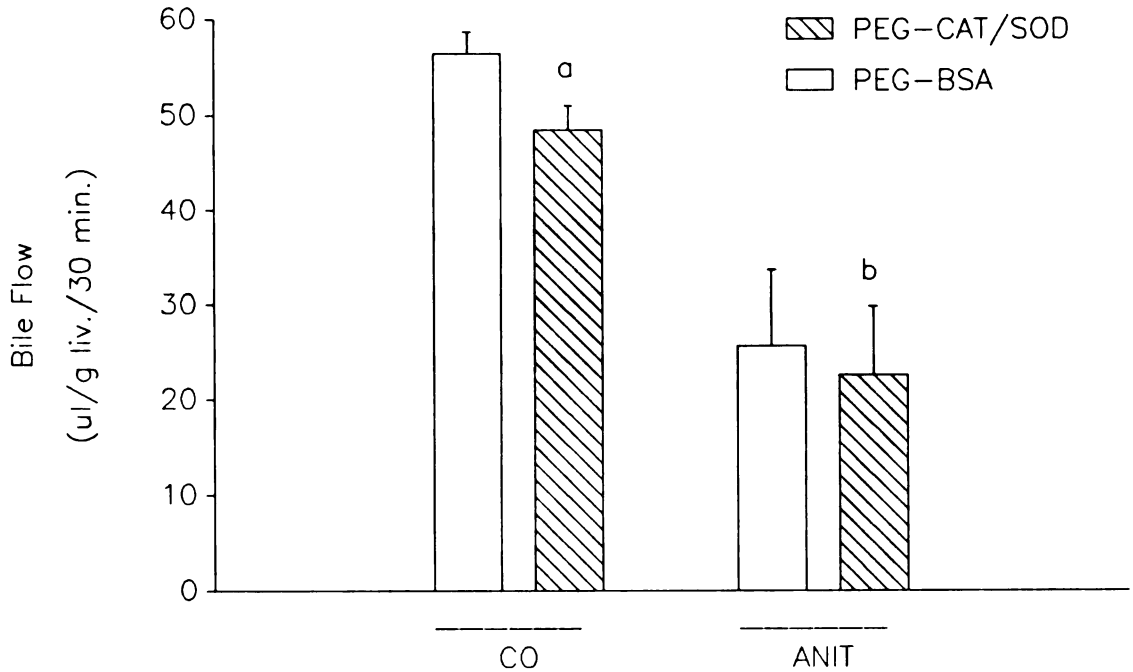

Figure IV-5. Effect of PEG-CAT/SOD on bile flow. Treatment regimen is described in legend to Figure IV-4. Bile flow in rats anesthetized with sodium pentobarbital (50 mg/kg, i.p.) was measured 24 hr following treatment with ANIT or CO. N = 4-11. [a] Significantly different from group treated with CO and PEG-BSA. [b] Significantly different from group treated with CO and PEG-CAT/SOD.

SUMMARY

In this chapter, we showed that PMNs play a causal role in ANIT-induced liver injury by a mechanism which may not involve PMN-derived oxygen radicals. In the next chapter, we examine whether glutathione plays a role in the liver injury caused by ANIT.

CHAPTER V

DECREASED HEPATIC NON-PROTEIN SULFHYDRYL CONTENT AFFORDS PROTECTION AGAINST ALPHA-NAPHTHYLISOTHIOCYANATE-INDUCED LIVER INJURY

INTRODUCTION

It is thought that ANIT must undergo bioactivation by hepatic MFO to cause liver injury, since agents which are inhibitors or inducers of hepatic MFO activity attenuate or enhance ANIT-induced liver injury, respectively. These agents also affect hepatic GSH content and/or GSH S-transferase activity in a manner to suggest a causal or permissive role for GSH in the pathogenesis (see Table I-1). In this chapter, we tested the hypothesis that GSH was involved by determining whether agents which decrease hepatic GSH content afford protection against ANIT hepatotoxicity.

In studies presented in this chapter and the subsequent one, the dose of ANIT was 100 mg/kg. This dose was larger than that used in Chapter IV, i.e., 35 mg/kg and was used in an attempt to eliminate the incidence of non-responding rats, which was approximately 25%.

METHODS

Materials. ANIT (lot 43F-0528), reduced GSH, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), L-buthionine-S,R-sulfoximine (BSO), kit 605-D for bilirubin determination, and kit 505 for aspartate aminotransferase (AST) activity were purchased from Sigma Chemical Co. (St. Louis, MO). Diethyl maleate (98%) and phorone (93%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were of the highest grade commercially available. PE 10 polyethylene tubing was purchased from Clay Adams (Parsippany, NJ).

Animals. Male, Sprague-Dawley rats (CF:CD(SD)BR) (Charles River, Portage, MI) weighing 210-290 g were housed in plastic cages on aspen chip bedding under conditions described under METHODS in Chapter II.

Treatment protocols to decrease hepatic non-protein sulfhydryl content. Rats were fasted 24 hr prior to experimentation and for the remainder of the study to prevent changes in hepatic GSH content from food consumption. At time zero, they were treated with BSO (890 mg/kg, i.p.) or an equivalent volume of saline (SAL) vehicle. Rats were given either ANIT (100 mg/kg, p.o.) or an equivalent volume of corn oil (CO, 3 ml/kg) vehicle 2.5 hr later. At 12 hr, they received a second treatment of either BSO or SAL. Hepatic non-protein sulfhydryl (NPSH) content was measured 24 hr after ANIT treatment as an indicator of GSH content. In a separate experiment, DEM (642 mg/kg, 1:1 in CO, i.p.) or an equivalent volume of CO vehicle was given to rats 30 min prior to either ANIT (100 mg/kg, p.o.) or an equivalent volume of CO vehicle (3 ml/kg). In another experiment, rats were treated with phorone (125 mg/kg, 1:3 in CO,

i.p.) or an equivalent volume of CO vehicle 30 min prior to ANIT (50 mg/kg, p.o.) in CO (16.7 mg/ml). In a followup study, rats were treated with phorone (250 mg/kg, 1:3 in CO, i.p.) or an equivalent volume of CO vehicle 30 min prior to either ANIT (100 mg/kg, p.o.) or an equivalent volume of CO (3 ml/kg). In either naive or CO-treated rats, decreases in hepatic NPSH content were confirmed 2.5 and 2 hr after treatment with DEM or phorone, respectively.

Twenty-four hr after ANIT treatment, bile flow, serum total bilirubin concentration, total bile acid concentration, GGT activity, and AST activity were measured as described under METHODS in Chapter IV. NPSH content in liver was measured by a modification of the method of Ellman (1959) as described by Costa and Murphy (1986). Briefly, sulfhydryl groups react with DTNB at pH 8.0 to produce the *p*-nitrothiophenol anion which is detected spectrophotometrically at 412 nm. NPSH content was calculated from a standard curve of reduced GSH.

Treatment protocol to determine whether decreased hepatic NPSH content delayed the onset of injury. In one study, rats were fasted 24 hr prior to experimentation and for the remainder of the study. All received ANIT and either BSO or SAL vehicle as described above. Half of the rats were killed 24 hr after ANIT treatment, similar to the BSO study described above. The other half received no additional BSO after the second treatment and were killed 48 hr after ANIT treatment. Markers of liver injury and hepatic NPSH content were measured 24 hr and 48 hr after ANIT treatment as described above.

A separate study was performed with DEM to determine whether DEM afforded complete protection or delayed the onset of injury. Rats were fasted 24 hr prior to experimentation and for the remainder of the study. They were treated with either DEM or CO vehicle 30 min prior to ANIT as described above, and several markers of liver injury were measured 48 hr later.

Statistical Analysis. Results are expressed as mean $\pm$ S.E. Homogeneity of variance was tested using the F-max test. Log transformations were performed on nonhomogeneous data. If the variances were homogeneous, data were analyzed using Students t-test or a completely randomized factorial analysis of variance, as appropriate. Individual comparisons between treatment means were made with Tukey's omega test (Steel and Torrie, 1980). When the variances were nonhomogeneous after log transformation of data, data were analyzed as described under METHODS in Chapter IV. The criterion for significance was $p < 0.05$ for all comparisons.

RESULTS

Effects of decreased hepatic NPSH content on markers of liver injury 24 hr after ANIT treatment. Administration of ANIT to rats caused cholestasis and elevations in serum total bilirubin concentration, total bile acid concentration, AST activity and GGT activity (Figure V-1). All rats responded to ANIT in this experiment and all others presented in this chapter as well as Chapter VI. Treatment of rats with ANIT increased hepatic NPSH content nearly 2-fold when compared to vehicle controls (Figure V-1). Co-treatment of rats with BSO decreased hepatic NPSH content by 70% 24 hr after ANIT treatment and prevented the cholestasis and elevations in markers of liver injury caused by ANIT (Figure V-1). BSO treatment had minimal effects on markers of liver injury in rats treated with CO, although it caused a slight reduction in bile flow (Figure V-1).

Co-treatment of rats with DEM also prevented the cholestasis and elevations in markers of liver injury 24 hr after ANIT treatment (Table V-1). DEM treatment had minimal effects on markers of liver injury in rats treated with CO. In a preliminary experiment in CO-treated rats, DEM administration decreased hepatic NPSH content by approximately 56% 2.5 hr later, but NPSH returned to control levels by 6 hr (data not shown). Hepatic NPSH content was not measured between 2.5 hr and 6 hr.

Phorone treatment (125 mg/kg) afforded partial protection against ANIT-induced elevations in serum total bilirubin concentration and AST activity, although it did not affect the cholestasis and elevation in serum GGT activity

(Table V-2). In a separate experiment in naive rats, this dose of phorone decreased hepatic NPSH content by 88% 2 hr after treatment. As in the case with DEM, hepatic NPSH content returned to control by 6 hr (data not shown). A larger dose of phorone (250 mg/kg, i.p.) decreased hepatic NPSH content by > 80% for at least 7 hr in naive rats (data not shown), and it afforded near-complete protection against ANIT-induced elevations in serum total bilirubin concentration, total bile acid concentration, and GGT activity (Table V-3). However, this dose of phorone was hepatotoxic, as indicated by a reduction of bile flow (Table V-3) and an elevation of serum AST activity, i.e., > 1000 SF units/ml (data not shown) in rats receiving phorone and CO. The low dose of phorone (125 mg/kg) did not appear to be hepatotoxic, since serum AST activity was not elevated in rats receiving phorone alone (data not shown).

Effects of BSO administration on liver injury 48 hr after ANIT treatment. Rats treated with ANIT had reduced bile flow and elevations in serum total bilirubin concentration and GGT activity at 24 hr and 48 hr (Figure V-2). Co-treatment of rats with BSO decreased hepatic NPSH content at 24 hr, and it prevented the cholestasis and elevations in serum total bilirubin concentration and GGT activity (Figure V-2). These results confirm those described above and shown in Figure V-1.

Although BSO administration prevented the cholestasis and elevations in serum total bilirubin concentration and GGT activity 24 hr after ANIT treatment, markers of liver injury were elevated by 48 hr. Rats treated with ANIT and BSO had values for bile flow, serum total bilirubin concentration, and serum GGT activity similar to those of rats treated with ANIT and the SAL vehicle (Figure V-2). The onset of liver injury by 48 hr coincided with a return of hepatic NPSH content (Figure V-2).

Effects of DEM administration on liver injury 48 hr after ANIT treatment. A reduction in bile flow and elevations in serum total bilirubin concentration and AST activity occurred in rats 48 hr after ANIT treatment (Table V-4). The protection by DEM seen 24 hr after ANIT treatment (Table V-1) was not apparent at 48 hr (Table V-4).

TABLE V-1

Effects of DEM on liver injury 24 hr after ANIT treatment

	Treatment[a]			
	CO		ANIT	
	CO	DEM	CO	DEM
Bile flow (μl/g liver/30 min)	54±2	55±5	0±0[b]	54±2[c]
Serum total bilirubin conc. (mg/dl)	0.2±0	0.2±0	2.7±0.2[b]	0.4±0.1[c]
Serum AST activity (SF units/ml)	138±9	156±15	498±63[b]	201±26[c]
Serum GGT activity (U/l)	0±0.1	0.2±0.1	5.0±0.4[b]	0.6±0.2[c]
Serum total bile acid conc. (μM)	4±0	4±0	1388±91[b]	54±22[c]

[a]Rats were treated with either DEM (642 mg/kg, i.p.) or CO vehicle 30 min prior to either ANIT (100 mg/kg, p.o.) or CO vehicle. Markers of liver injury were measured 24 hr after ANIT treatment. N = 4-7.

[b]Significantly different from CO/CO group.

[c]Significantly different from ANIT/CO group.

TABLE V-2

Effects of phorone (125 mg/kg, i.p.)on markers of liver injury 24 hr after ANIT treatment

	Treatment[a]	
	CO	Phorone
Bile flow (μl/g liver/30 min)	3±2	7±2
Serum total bilirubin conc. (mg/dl)	3.3±0.2	2.1±0.2[b]
Serum AST activity (SF units/ml)	705±59	354±38[b]
Serum GGT activity (U/l)	5.1±0.4	5.0±0.2

[a]Rats were treated with either phorone or CO vehicle 30 min prior to ANIT (50 mg/kg, p.o.). Markers of liver injury were measured 24 hr after ANIT treatment. N = 5-6.

[b]Significantly different from CO group.

TABLE V-3

Effects of phorone (250 mg/kg, i.p.) on markers of liver injury 24 hr after ANIT treatment

	Treatment[a]			
	CO		ANIT	
	CO	Phorone	CO	Phorone
Liver wt/body wt x 100	2.73±0.13	4.23±0.13[b]	3.70±0.09[b]	4.34±0.09[c]
Bile flow (μl/g liver/30 min)	64±9	3±10[b]	3±1[b]	21±4
Serum total bilirubin conc. (mg/dl)	0.1±0	0.4±0.1[b]	3.6±0.3[b]	0.5±0.1[c]
Serum GGT activity (U/l)	0.2±0.1	0.7±0	4.5±0.3[b]	1.5±0.2[c]
Serum total bile acid conc. (μM)	16±2	42±3	1400±42[b]	68±21[c]

[a]Rats were treated with either phorone or CO vehicle 30 min prior to either ANIT (100 mg/kg, p.o.) or CO vehicle. Markers of liver injury were measured 24 hr after ANIT treatment. N = 3-7.

[b]Significantly different from CO/CO group.

[c]Significantly different from ANIT/CO group.

TABLE V-4

Effects of DEM on markers of liver injury 48 hr after ANIT treatment

	Treatment[a]	
	CO	DEM
Bile flow (μl/g liver/30 min)	2±1	3±1
Serum total bilirubin conc. (mg/dl)	5.4±0.7	7.1±0.4
Serum AST activity (SF units/ml)	2053±274	1396±165

[a]Rats were treated with either DEM (642 mg/kg, ip.) or CO vehicle 30 min prior to ANIT (100 mg/kg, p.o.). Markers of liver injury were measured 48 hr after ANIT treatment. N = 5-6.

Figure V-1. Effects of BSO on ANIT-induced changes in hepatic NPSH content (panel A), bile flow (panel B), serum total bilirubin concentration (panel C), serum AST activity (panel D), serum GGT activity (panel E), and serum total bile acid concentration (panel F). Rats were fasted prior to treatments. At time 0, they received either BSO (890 mg/kg, i.p.) or SAL vehicle and either ANIT (100 mg/kg, p.o.) or CO vehicle 2.5 hr later. They received a second treatment of either BSO or SAL at 12 hr. Hepatic NPSH content and other markers of liver injury were measured 24 hr after ANIT or CO treatment. N = 4-7. [a]Significantly different from group treated with CO and SAL. [b]Significantly different from group treated with ANIT and SAL. [c]Significantly different from group treated with CO and BSO.

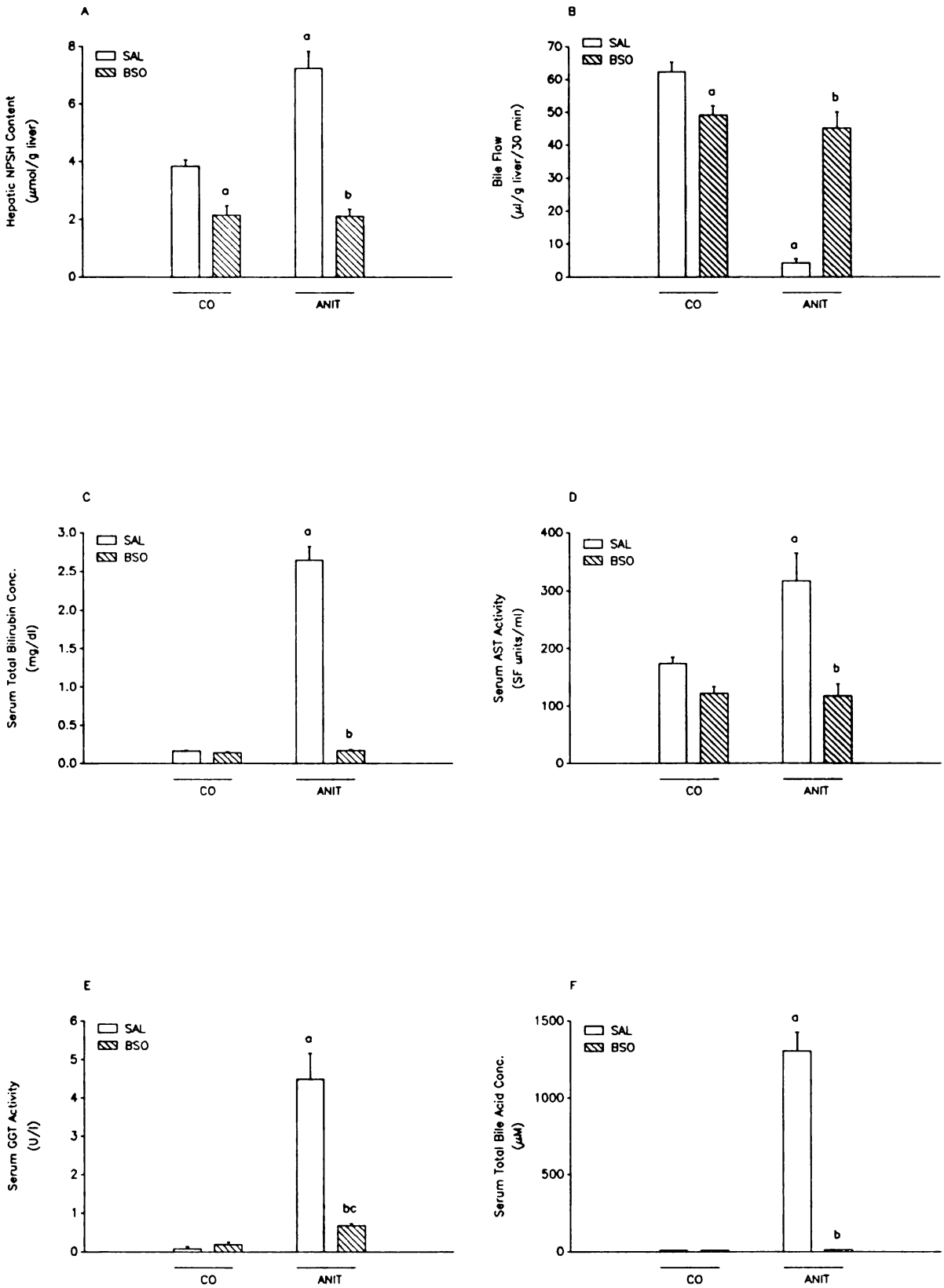
A
Hepatic NPSH Content (μmol/g liver)
SAL
BSO
a
a
a
b
CO
ANIT
B
Bile Flow (μl/g liver/30 min)
SAL
BSO
a
a
b
CO
ANIT
C
Serum Total Bilirubin Conc. (mg/dl)
SAL
BSO
a
b
CO
ANIT
D
Serum AST Activity (SF units/ml)
SAL
BSO
a
b
CO
ANIT
E
Serum GGT Activity (U/l)
SAL
BSO
a
bc
CO
ANIT
F
Serum Total Bile Acid Conc. (μM)
SAL
BSO
a
b
CO
ANIT

Figure V–1

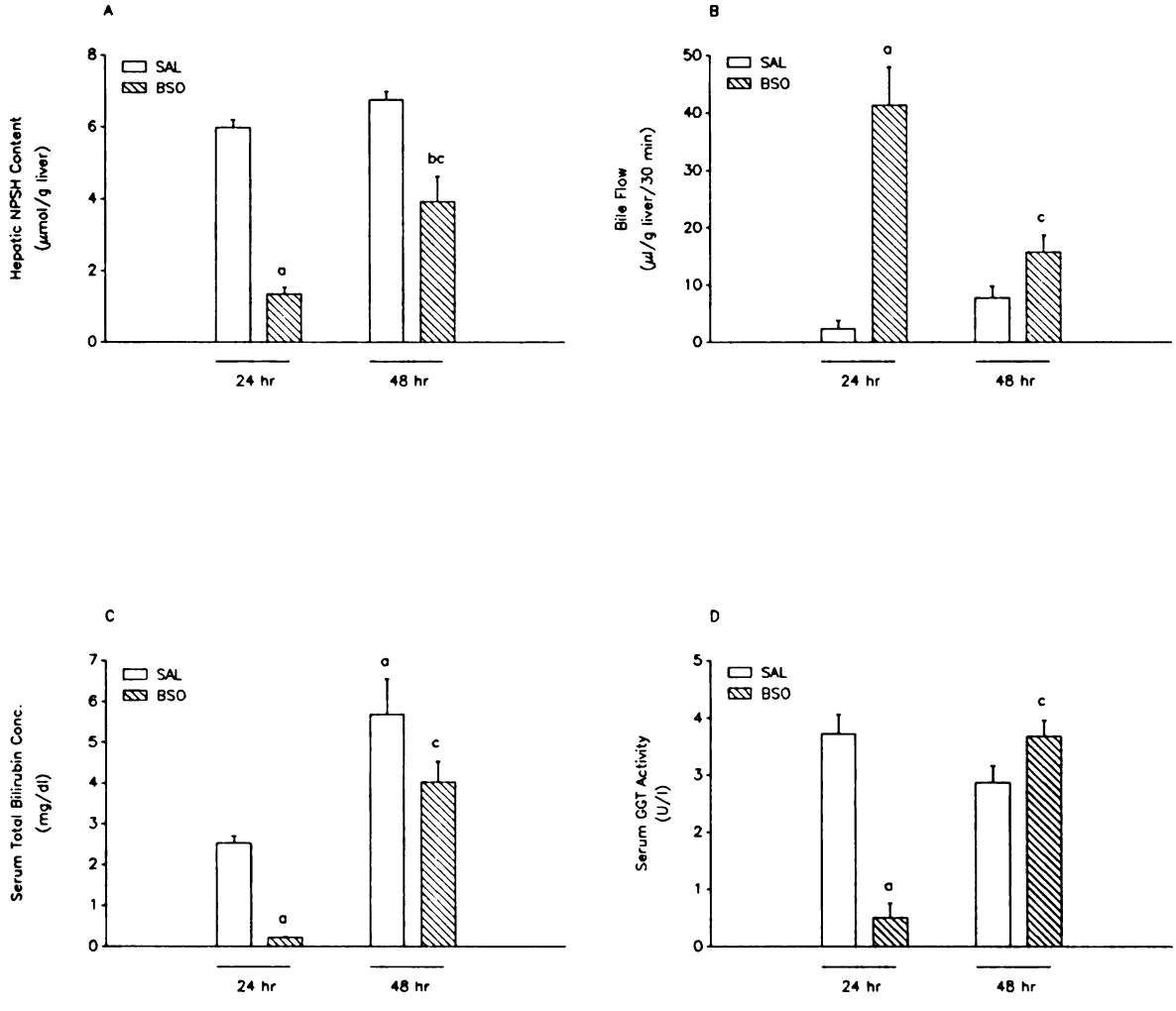

Figure V-2. Effects of BSO on hepatic NPSH content (panel A), bile flow (panel B), serum total bilirubin concentration (panel C), and serum GGT activity (panel D) 24 hr and 48 hr after ANIT treatment. Rats were fasted prior to treatments, which are described in the legend to Figure V-1. N = 5-8. [a]Significantly different from group treated with CO and SAL. [b]Significantly different from group treated with ANIT and SAL. [c]Significantly different from group treated with CO and BSO.

SUMMARY

In this chapter, we showed that GSH may play a causal or permissive role in ANIT hepatotoxicity, since agents which decrease hepatic NPSH content, an indicator of GSH content, afford protection. In the next chapter, we examine whether changes in hepatic NPSH content after ANIT treatment are related to the mechanism of liver injury.

CHAPTER VI

RELATIONSHIP BETWEEN ALPHA-NAPHTHYLISOTHIOCYANATE-INDUCED LIVER INJURY AND ELEVATIONS IN HEPATIC NON-PROTEIN SULFHYDRYL CONTENT

INTRODUCTION

In chapter V, we observed that ANIT caused a 2-fold elevation of hepatic NPSH content 24 hr after treatment. Since this increase occurred when liver injury was evident and since NPSHs appear to play a causal or permissive role in ANIT hepatotoxicity, we determined whether this increase in hepatic NPSH content was related to the mechanism of injury. Two approaches were taken to answer this question. First, *B*-naphthylisothiocyanate (BNIT), a non-hepatotoxic isomer of ANIT, was administered to rats to determine whether it increased hepatic NPSH content. If the elevation of hepatic NPSH content observed after ANIT treatment were related to the mechanism of injury, BNIT should not elevate hepatic NPSH content. Second, the effect of bile duct ligation on hepatic NPSH content was determined, because it was possible that the cholestasis caused by ANIT elevated hepatic NPSH content. In addition, we characterized the time course for changes in hepatic NPSH content after ANIT treatment.

METHODS

Materials. ANIT (lot 43F-0528), reduced GSH, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), kit 605-D for bilirubin determination, and kit 505 for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were purchased from Sigma Chemical Co. (St. Louis, MO). *B*-naphthylisothiocyanate (BNIT) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were of the highest grade commercially available. PE 10 polyethylene tubing and surgical staples were purchased from Clay Adams (Parsippany, NJ). Ethilon^{R} surgical suture was obtained from Ethicon (Somerville, NJ).

Animals. Male, Sprague-Dawley rats (CF:CD(SD)BR) (Charles River, Portage, MI) weighing 220-320 g were housed in plastic cages on aspen chip bedding under conditions described under METHODS in Chapter II.

Treatment protocol in studies with ANIT and BNIT. Rats were fasted 24 hr prior to experimentation and for the remainder of the study. They were treated with ANIT (100 mg/kg, p.o.), BNIT (100 mg/kg, p.o.), or an equivalent volume of corn oil (CO, 3 ml/kg) vehicle. At 12 or 24 hr after treatment, bile, serum total bilirubin concentration, GGT activity, and AST activity were measured as described under METHODS in Chapter IV. Serum ALT activity was measured as described under METHODS in Chapter II. Hepatic NPSH content was measured as described under METHODS in Chapter V.

Treatment protocol for bile duct ligation study. Rats were fasted 24 hr prior to experimentation and for the remainder of the experiment. They were

anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and a midline incision was made. The common bile duct was isolated and ligated at two sites with 3-0 surgical silk. In sham-operated, control rats, the bile duct was isolated but not ligated. Abdominal muscles were closed with 4-0 Ethilon^{R}, and the skin was closed with surgical staples.

At 6-48 hr after surgery, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A blood sample was taken from the descending aorta for determination of serum total bilirubin concentration to confirm cholestasis.

Statistical Analysis. Results are expressed as mean $\pm$ S.E. Homogeneity of variance was tested using the F-max test. Log transformations were performed on nonhomogeneous data. If the variances were homogeneous, data were analyzed using Student's t-test or a completely randomized analysis of variance, as appropriate. Individual comparisons between treatment means were made with Tukey's omega test (Steel and Torrie, 1980). When the variances were nonhomogeneous after log transformation of data, data were analyzed as described under METHODS in Chapter IV. The criterion for significance was $p < 0.05$ for all comparisons.

RESULTS

Effects of ANIT on hepatic NPSH content and markers of liver injury. ANIT administration to rats did not change hepatic NPSH content up to 12 hr after treatment when compared to CO controls (Figure VI-1). Bile flow and serum values for total bilirubin concentration, ALT activity, and GGT activity were also unchanged 12 hr after ANIT treatment (Figure VI-2). By 20-24 hr, however, hepatic NPSH content was elevated (Figure VI-1). Likewise, ANIT-treated rats exhibited cholestasis and had elevations in serum total bilirubin concentration and ALT and GGT activities at 24 hr (Figure VI-2). ANIT treatment of rats caused cholestasis and elevated serum markers of liver injury as early as 18 hr after treatment (data not shown). Thus, cholestasis and liver injury appeared to be associated temporally with the elevation in hepatic NPSH content.

Effects of BNIT on hepatic NPSH content and markers of liver injury. BNIT is a non-hepatotoxic isomer of ANIT (Becker and Plaa, 1965a; El-Hawari and Plaa, 1979), and it was used to assess whether ANIT-induced changes in hepatic NPSH content were linked to hepatotoxicity. Administration of BNIT to rats at a dose equivalent to that of ANIT (100 mg/kg, p.o.) did not cause cholestasis or elevate serum markers of liver injury 24 hr later (Table VI-1). However, BNIT treatment did elevate hepatic NPSH content (Table VI-1). Hepatic NPSH content was not measured at any time points prior to 24 hr.

Effects of bile duct ligation on hepatic NPSH content. To determine whether the cholestasis caused by ANIT treatment might have elevated hepatic NPSH content, an extrahepatic cholestasis was produced by bile duct ligation.

Elevations in serum total bilirubin concentration by 6 hr after bile duct ligation in rats confirmed cholestasis (Figure VI-3A). Ligation of the common bile duct caused elevations in hepatic NPSH content compared to sham-operated controls starting 12 hr after ligation (Fig. VI-3B). Hepatic NPSH content as well as total bilirubin concentration remained elevated for the duration of the study (48 hr).

TABLE VI-1

Effects of BNIT on hepatic NPSH content and markers of liver injury

	Treatment[a]	
	CO	BNIT
Hepatic NPSH content (μmol/g)	3.3±0.1	4.7±0.2[b]
Bile flow (μl/g liver/30 min)	63±5	71±7
Serum total bilirubin conc. (mg/dl)	0.12±0.01	0.13±0.02
Serum AST activity (SF units/ml)	174±21	184±21
Serum GGT activity (U/l)	N.D.	N.D.

[a]Rats were treated with either BNIT (100 mg/kg, p.o.) or CO vehicle. Hepatic NPSH content and markers of liver injury were measured 24 hr after treatment with BNIT or CO. N.D. = not detectable. n = 4-8.

[b]Significantly different from CO group.

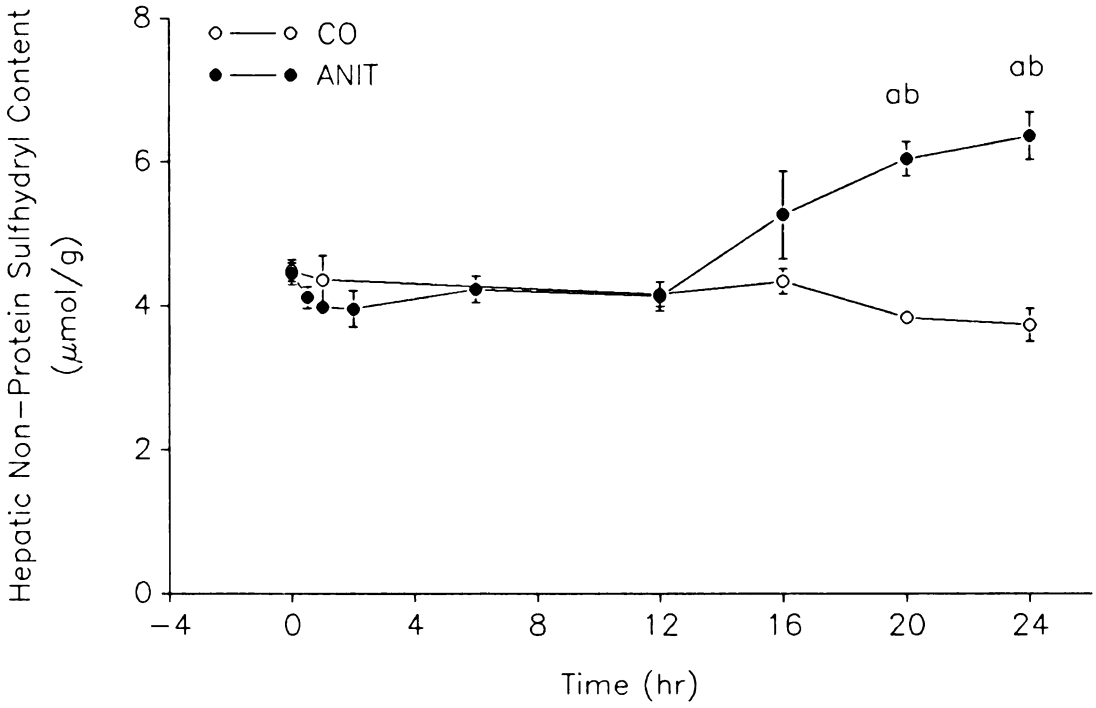

Figure VI-1. Effect of ANIT on hepatic NPSH content. Rats were fasted prior to treatment with either ANIT (100 mg/kg, p.o.) or an equivalent volume of CO. Hepatic NPSH content was measured 0-24 hr later. N = 3-4. [a]Significantly different from CO group at the same time point. [b]Significantly different from 0 hr time point.

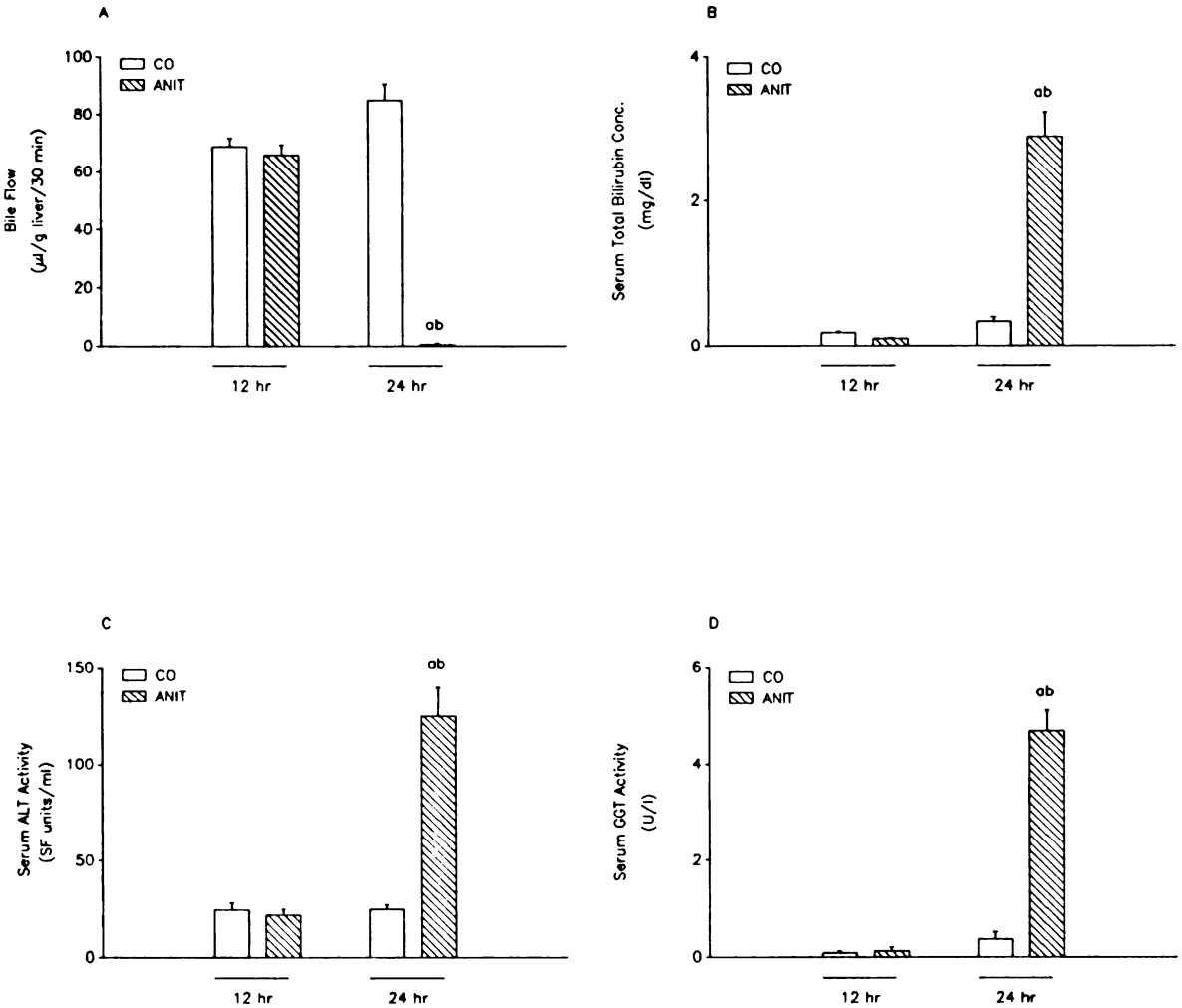

Figure VI-2. Association of liver injury after ANIT treatment with changes in hepatic NPSH content. Rats were fasted prior to treatment with either ANIT (100 mg/kg, p.o.) or an equivalent volume of CO. At 12 or 24 hr, bile flow (panel A) and determinations of serum total bilirubin concentration (panel B), ALT activity (panel C), and GGT activity (panel D) were made. N = 3-4. [a]Significantly different from group treated with CO at 12 hr. [b]Significantly different from group treated with CO at 24 hr.

Figure VI-3. Effect of bile duct ligation (BDL) on hepatic NPSH content. Rats were fasted prior to BDL or sham operation, and serum total bilirubin concentration (panel A) and hepatic NPSH content (panel B) were measured 6-48 hr later. N = 4-6. [a]Significantly different from sham-operated group at same time point.

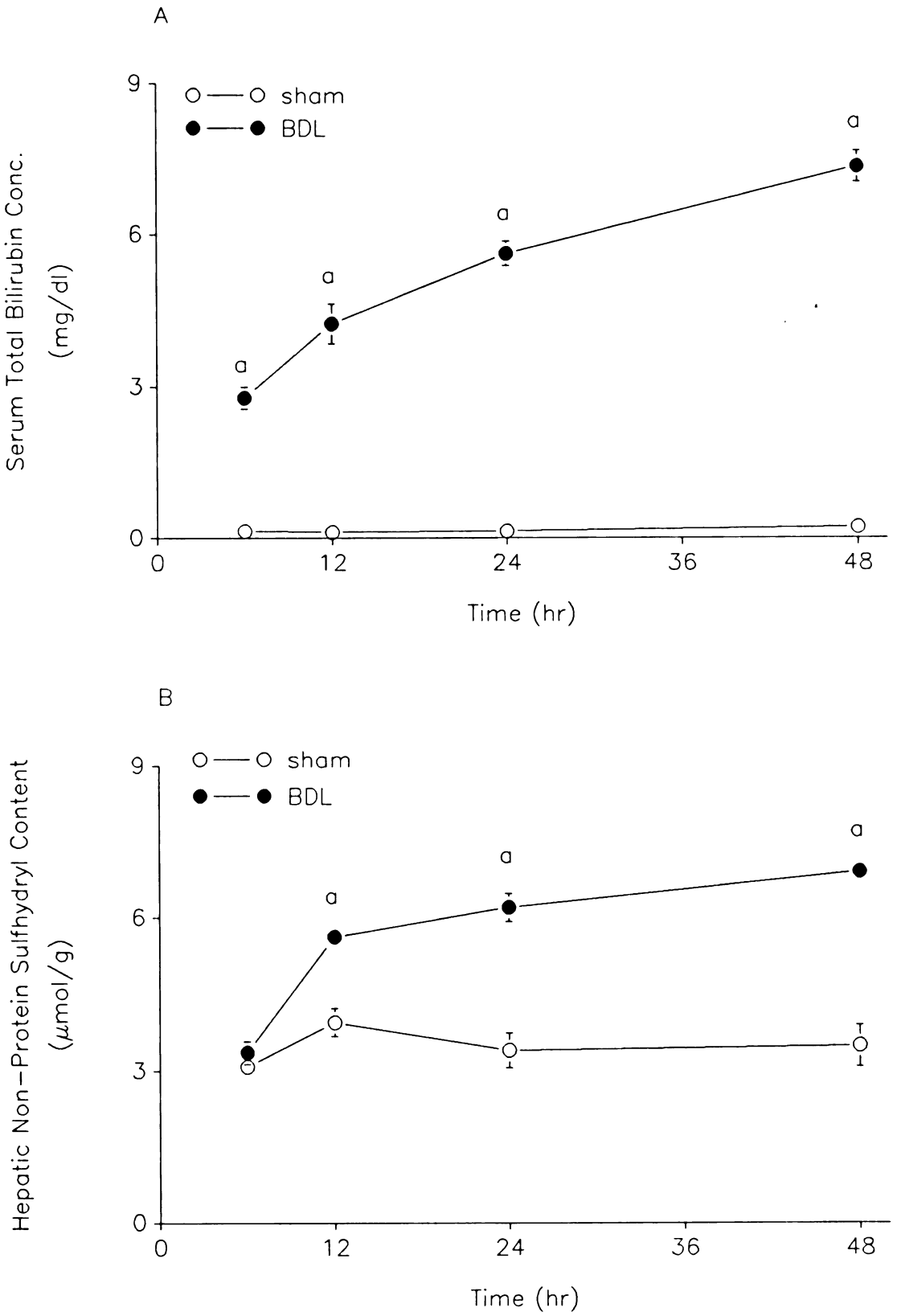
A
sham
BDL
Serum Total Bilirubin Conc.
(mg/dl)
9
6
3
0
a
a
a
a
0
12
24
36
48
Time (hr)
B
sham
BDL
Hepatic Non-Protein Sulfhydryl Content
(μmol/g)
9
6
3
0
a
a
a
0
12
24
36
48
Time (hr)

Figure VI-3

SUMMARY

In this chapter, we provided evidence that the elevation of hepatic NPSH content after ANIT treatment is not related to the mechanism of injury, although it coincides with the onset of liver injury. In the next chapter, the results of Chapters II-VI are discussed and integrated.

CHAPTER VII

DISCUSSION

I. Capacity of PMN-Derived Oxygen Species to Cause Liver Injury

Activated PMNs caused modest injury to the isolated, perfused rat liver as indicated by elevated ALT activity in the perfusion medium. That a combination of SOD and catalase prevented the increase in ALT activity in the perfusion medium suggests that PMN-derived oxygen species were involved in the injury. PMNs were activated by treatment with PMA and the bile salt, lithocholate, since this procedure has been shown to cause a much greater release of O_2^- from rat PMNs than other commonly used stimuli (ChapterIII; Dahm and Roth, 1990). Activated PMNs were added to the perfusion system when maximal release of O_2^- from PMNs occurred (Dahm and Roth, 1990), and liver injury was evident 90 min later. It is unclear which toxic oxygen species is/are involved in the injury, since the combination of SOD and catalase would be expected to degrade O_2^- and H_2O_2, as well as prevent ·OH formation by the iron-catalyzed Haber Weiss reaction. Further studies are required to determine which species is/are involved in PMN-mediated liver injury.

We assessed whether livers isolated from ANIT-treated rats were more sensitive to the liver injury caused by activated PMNs. We hypothesized that pre-existing liver injury would increase the sensitivity of the liver to the injurious effects of activated PMNs. ANIT was used to compromise livers, because PMNs appear to play a causal role in the pathogenesis in this model (Chapter IV). Consequently, livers were isolated from ANIT-treated rats at a time when they show functional changes, i.e., decreased BSP clearance, and subtle histological changes (Plaa and Priestly, 1977). However, there was little, if any,

hepatocellular necrosis in these livers, since serum ALT activity was not elevated in donor rats. ANIT compromised livers were not more sensitive to PMN-derived oxygen radicals. These results suggest that functional hepatic deficits do not predispose the liver to oxygen radical-dependent injury. However, we did not test further whether a greater degree of pre-existing liver injury might increase the sensitivity of the liver to the injurious effects of PMN-derived oxygen radicals.

Although activated PMNs caused injury to the isolated perfused rat liver by a mechanism dependent upon oxygen radicals, others have demonstrated that PMNs cause toxicity to cultured hepatocytes by an oxygen radical-independent mechanism (Guigui *et al.*, 1988; Mavier *et al.*, 1988). When PMNs were activated with PMA or opsonized zymosan and co-cultured with hepatocytes, the injury to hepatocytes was dependent upon proteinases but not toxic oxygen species (Guigui *et al.*, 1988; Mavier *et al.*, 1988). The apparent insensitivity of hepatocytes to toxic oxygen species was attributed to the presence of protective systems such as glutathione peroxidase, SOD, and catalase (Guigui *et al.*, 1988).

The reasons for the disparate results may relate to differences in preparations. For example, we used an isolated, perfused rat liver, which has an intact architecture. We observed that activated PMNs caused a transient cessation of hepatic perfusion, suggesting that they might have caused liver injury secondary to hypoxia. In livers isolated from fasted rats and perfused with a PMN-free medium, hypoxia causes liver injury by a mechanism which appears to be mediated by toxic oxygen species generated intracellularly in hepatocytes by xanthine oxidase (Bradford *et al.*, 1986; Marotto *et al.*, 1988; Younes and Strubelt, 1988). The protection afforded by SOD and catalase in our study is consistent with this suggestion, since either enzyme prevents hypoxia-induced injury to the isolated, perfused rat liver when included in the perfusion medium

(Younes and Strubelt, 1988). The mechanism of protection is not well understood, although it may reflect degradation of intracellular oxygen radicals, since SOD can enter hepatocytes by pinocytosis (Kyle *et al.*, 1988). Therefore, in our studies, it is not known whether the protection afforded by SOD and catalase reflects degradation of toxic oxygen species generated intracellularly, extracellularly, or both.

One way to test whether xanthine oxidase in hepatocytes is a source of injurious oxygen radicals in our system is to treat donor rats with allopurinol and/or include it in the perfusion medium to determine whether it prevents ALT leakage. Allopurinol is an inhibitor of xanthine oxidase and affords protection against hypoxia induced liver injury (Marotto *et al.*, 1988; Younes and Strubelt, 1988).

The cause of the reduction in perfusate flow through the liver is not clear. It does not appear to be mediated by PMN-derived oxygen species, since the combination of SOD and catalase did not alter the reduction in flow caused by activated PMNs. Activated PMNs release vasoconstrictor agents (Bach, 1983), and it is possible that vasoconstriction occurred in the liver. For example, activated rat PMNs may release thiol ether leukotrienes (Orange *et al.*, 1967), and these may cause vasoconstriction in the rat liver (Krell and Dietze, 1989). Alternatively, PMNs aggregate when activated, and it is possible that aggregates of PMNs occluded sinusoids and blocked perfusate flow. Either vasoconstriction or mechanical obstruction of flow might produce local tissue hypoxia and thereby cause the observed rise in ALT activity in perfusion medium.

Certain observations suggest that hypoxia might not be involved in PMN-mediated injury to the isolated rat liver. For example, when PMNs were activated with PMA and perfused through livers, they affected perfusate flow in a manner similar to PMNs activated with the PMA/LC combination. However, PMA-

activated PMNs did not elevate ALT activity in the perfusion medium. Also, Younes and Strubelt (1988) demonstrated that hepatic oxygen consumption after hypoxia/reoxygenation was lower than that in livers perfused with an oxygenated medium. In our studies, hepatic oxygen consumption after 90 min of perfusion with activated PMNs was not different than that in control livers, suggesting that hypoxia did not occur to any great extent. We cannot dismiss the possibility, however, that localized tissue hypoxia accounted for the modest degree of liver injury caused by activated PMNs.

II. Bile and Bile Salts Potentiate O_2^- Release from Activated, Rat Peritoneal PMNs

During ANIT-induced cholestasis, biliary products may reflux into sinusoidal blood, and PMNs sequestered in periportal regions of the liver would come in contact with potentially high concentrations of bile salts and other products. We hypothesized that biliary products would stimulate oxygen radical release from sequestered PMNs and thereby contribute to the pathogenesis of ANIT-induced liver injury. We found that normal rat bile did not activate PMNs *in vitro* as measured by O_2^- release. However, the addition of bile to PMNs which were primed with PMA enhanced O_2^- release. PMA was used as a priming agent to mimic the activation state of PMNs sequestered in the liver after ANIT treatment. As indicated in Chapter I, PMNs which respond to chemoattractants and infiltrate tissue are primed for O_2^- release.

Not all bile samples were able to enhance O_2^- release from PMA-primed PMNs. Bile samples which enhanced O_2^- release from primed PMNs might contain higher concentrations of PMN activators, such as bile salts, endotoxin, or leukotrienes (LTs), than inactive bile samples. Endotoxin may be excreted in bile

(Mathison and Ulevitch, 1979), and it enhances O_2^- release from PMA-primed PMNs (Guthrie *et al.*, 1984). Likewise, LTs are excreted in bile (Appelgren and Hammarstrom, 1982), and certain LTs stimulate O_2^- release from PMNs (Serhan *et al.*, 1982; Sumimoto *et al.*, 1984). Alternatively, bile samples unable to enhance O_2^- release from PMA-primed PMNs might contain factors which interfere with O_2^- release or scavenge O_2^-. The observation that ethanol extracts of inactive bile samples enhanced O_2^- release from primed PMNs supports the latter hypothesis.

Bile salts, with the exception of lithocholate, could not directly stimulate O_2^- release from PMNs. Lithocholate incubation alone caused small but statistically significant release of O_2^- from PMNs. These results are in contrast to those of Cohen and Chovaniec (1978), who observed that deoxycholate, at similar concentrations as those in our study, stimulated O_2^- release from elicited, guinea pig PMNs. Although the reasons for the disparate results are not entirely clear, they might relate to differences in species and/or elicitation procedures. Cohen and Chovaniec (1978) elicited guinea pig peritoneal PMNs 18 hr following casein treatment, whereas rat peritoneal PMNs were harvested 4 hr after glycogen administration in our study.

Although bile salts had little capacity by themselves to stimulate O_2^- release from PMNs, several were found to enhance the release of O_2^- from PMA-primed PMNs similar to bile. Rossi *et al.* (1980) reported that deoxycholate could enhance O_2^- release from PMA-activated PMNs at concentrations approximately 10-fold higher than those employed in our study. Bile salt conjugates of glycine and taurine, the major forms of bile salts within bile, were not able to enhance O_2^- release from PMA-primed PMNs. These data are consistent with the observations of Graham *et al.* (1967) that conjugation reduced PMN respiration stimulated by deoxycholate. These results suggest that factors other than bile

salts, e.g. LTs, might account for the stimulatory effect of bile toward primed PMNs. However, the concentration of total conjugated bile salts in rat bile is approximately 23 mM (Mroszczak and Riegelman, 1972). Therefore, the concentration of conjugated bile salts in bile at the dilution (1:50) which maximally enhances O_2^- release from primed PMNs would be > 400 uM. If conjugated bile salts at a concentration near 400 uM enhance O_2^- release from primed PMNs, then bile salts might account for the stimulatory effect of bile toward primed PMNs. Whether or not conjugated bile salts at concentrations near 400 uM are active toward primed PMNs to release O_2^- remains to be determined.

The mechanism by which bile salts exert their effects on the PMN is not known. Bile salts able to enhance O_2^- release from primed PMNs may perturb the plasma membrane of PMNs. Membranes isolated from PMA-primed PMNs release an enhanced amount of O_2^- when incubated with deoxycholate, apparently by decreasing the latent portion of NADPH oxidase activity (Rossi *et al.*, 1980; Bender *et al.*, 1983). Bile salts have also been reported to hemolyze red blood cells, presumably by perturbing plasma membranes (Berliner and Schoenheimer, 1938; Kappas and Palmer, 1963). Interestingly, the degree by which bile salts enhance O_2^- release from PMA-primed PMNs, i.e., monohydroxy (lithocholate) > dihydroxy (deoxycholate and chenodeoxycholate) > trihydroxy (cholate) is similar to the order for red blood cell hemolysis. These observations support the contention that bile salts enhance O_2^- release from PMA-primed PMNs by altering PMN plasma membranes.

The degree of hydroxylation influenced the ability of bile salts to enhance O_2^- release from activated PMNs. The order of activity for O_2^- enhancement as described above appears to relate to the hydrophobicity of the bile salt. For example, the monohydroxy bile salt, lithocholate, is more hydrophobic and more

active toward PMNs than cholate, which has three hydroxyl groups on the hydrophobic nucleus. Interestingly, glycine and taurine conjugation of bile salts, which decreases hydrophobicity of the molecule (Carey and Small, 1972), also reduces activity toward primed PMNs. Hydrophobic characteristics may enable the bile salt to bind to or become embedded in the plasma membrane, thereby perturbing the membrane.

However, cholanoate is devoid of any hydroxyl substituents and is more hydrophobic than lithocholate, yet it is less active toward primed PMNs. Furthermore, bile salt analogs which would be expected to be more hydrophobic by virtue of less hydrophilic side chains, such as etiocholanolone, were not active toward PMA-primed PMNs (Dahm *et al.*, 1988) While hydroxylation of the hydrophobic nucleus and alteration of the hydrophilic side chain reduce the ability of the bile salt to enhance O_2^- release from PMA-primed PMNs, the reasons for these effects might relate to factors other than hydrophobicity of the molecule. From the data presented, one cannot rule out a receptor-mediated effect. The classic shape of the concentration/response curve for lithocholate lends support to such an effect.

Like other detergents, bile salts form micelles at a critical micellar concentration (CMC). Bile salt micelles might perturb membranes by solubilizing membrane components (Jorgensen and Skou, 1971; Philippot and Authier, 1973). The concentrations of bile salts employed (100 uM) were at least an order of magnitude below their respective CMCs, although incubation conditions, such as the presence of Na^+, may have reduced the CMCs of the bile salts tested (Carey and Small, 1972). Other interactions suggest that micellar interactions were not responsible for the enhancing effect of bile salts on O_2^- release. Conjugation of free bile salts with glycine or taurine has little effect on CMC (Roda *et al.*, 1983), but the glycine and taurine conjugates of

lithocholate and chenodeoxycholate were not able to enhance O_2^- release from primed PMNs. Lithocholate, the bile salt with the greatest stimulatory effect toward primed PMNs, may not form micelles until temperatures exceed 50°C (Small and Admirand, 1969) which is above the 37°C incubation temperature used in the present study.

The interaction between bile salts and primed PMNs that results in the release of enhanced amounts of O_2^- and possibly other injurious species might have relevance to ANIT-induced liver injury. For example, since PMNs appear to play a causal role in ANIT hepatotoxicity (Chapter IV), it is conceivable that biliary products might exacerbate the injury by enhancing the release of injurious products from PMNs, i.e., oxygen radicals (Figure VII-1). In support of this proposal, serum total bile acid concentration after ANIT treatment may approach 2 mM, and local concentrations in periportal regions of the liver may be even higher. However, whether or not bile salts in bile would be the species to interact with PMNs in this scenario is questionable, since free bile salts are the active species to enhance O_2^- release and since bile salts refluxing from bile to blood would be expected to be conjugated. It is not known, however, what effect ANIT treatment has on conjugating capacity of the liver. If increased amounts of free bile acids were produced as a result of decreased conjugation, then bile salts might be the species to enhance O_2^- release from sequestered PMNs.

That administration of PEG-CAT/SOD affords no protection against ANIT hepatotoxicity (Chapter IV) does not support a role for oxygen radicals in the pathogenesis. Therefore, although it is possible that bile salts and other biliary products enhance O_2^- release from sequestered PMNs *in vivo*, this might have little relevance to the mechanism of injury. However, biliary products might enhance the release of other injurious products from PMNs and thereby contribute to PMN-dependent injury in the ANIT model. As demonstrated in

Chapter III and by Dahm and Roth (1990), lithocholate does not enhance the release of granule enzymes, i.e., *B*-glucuronidase and lysozyme, from primed PMNs. This observation indicates that an interaction between bile salts and PMNs to release granule enzymes probably does not operate in the ANIT model. Whether or not biliary products enhance the release of other injurious PMN products remains to be determined.

III. Role of PMNs and Oxygen Radicals in ANIT Hepatotoxicity

Reduction of blood PMN numbers by NAS treatment was associated with protection against the liver injury caused by ANIT. NAS treatment protected against bile duct epithelial cell injury as well as hepatocellular injury as reflected in changes in serum GGT and AST activities, respectively. In a followup PMN depletion study in which ANIT treatment significantly reduced bile flow, co-treatment of rats with NAS prevented the cholestasis (data not shown). These results suggest that blood PMNs play a role in causing or exacerbating ANIT hepatotoxicity, and, to our knowledge, they represent the first evidence implicating PMNs in chemically-induced liver injury *in vivo*. Thus, PMN involvement in chemically-induced liver injury may be a novel mechanism by which ANIT and perhaps other toxicants act (see Figure VII-1).

We have repeated the neutrophil depletion experiment reported herein and obtained similar results (data not shown). For example, NAS prevented ANIT-induced elevations in serum total bilirubin concentration, total bile acid concentration, and GGT activity. As indicated above, NAS prevented ANIT-induced cholestasis. In this followup study, serum AST activity was reduced in rats receiving ANIT and NAS, although it was not statistically significant.

In two other experiments in which NAS reduced circulating PMN numbers below the criterion for depletion, protection against ANIT hepatotoxicity was not observed. In these experiments, changes were made in the treatment protocol which might have masked or eliminated the PMN component of injury. In one study, rats receiving ANIT and NAS were killed 48 hr after ANIT treatment. In PMN depletion experiments which afforded protection, rats were killed 24 hr after ANIT treatment. One explanation, which is consistent with these observations, is that PMN depletion merely delays the onset of injury. Presumably, protection afforded by NAS at 24 hr would not be evident at 48 hr. In the second experiment, rats were warmed repeatedly under a lamp for short periods of time, i.e., 30-40 min, after ANIT treatment for collection of blood samples to confirm reductions in circulating PMN numbers. In experiments which demonstrated a protective effect of NAS on ANIT-induced liver injury, rats were not warmed after ANIT treatment. Since decreases in body temperature in rats reduce ANIT-induced liver injury (Roberts and Plaa, 1966b), it is conceivable that repetitive heating for short periods of time might have enhanced the ANIT-induced liver injury. Thus, any protection afforded by NAS in studies employing repetitive heating might have been masked. These explanations for the variability of protection afforded by NAS are clearly tentative, and other unknown factors might be involved.

Ruwart *et al.* (1984) observed that treatment of rats with 16,16-dimethyl-prostaglandin E_2 reduced the inflammation in periportal regions of the liver caused by ANIT and afforded protection against the liver injury. Although the mechanism of protection is unknown, prostaglandin E_2 is known to inhibit PMN function *in vitro* and reduce inflammation *in vivo* (Lehmeyer and Johnston, 1978; Fantone *et al.*, 1982). It seems possible that 16,16-dimethyl-prostaglandin E_2

afforded protection against ANIT-induced hepatotoxicity by inhibiting PMN function.

Since we used a polyclonal antibody, it seemed possible that NAS afforded protection nonspecifically by reducing some other type of blood cell. NAS administration to rats decreased circulating lymphocyte numbers by 20-25%, although the decrease was not statistically significant (data not shown). Inasmuch as products of peripheral lymphocytes may produce cholestasis in rats (Mizoguchi *et al.*, 1981, 1986; Marbet *et al.*, 1984), it seemed possible that an antilymphocyte effect of NAS might account for the protection against ANIT-induced liver injury. In a preliminary study, administration of antilymphocyte serum to rats reduced circulating lymphocyte numbers by approximately 65% 7 hr after treatment but did not afford protection against ANIT-induced liver injury. These results suggest that NAS was not acting via an effect on lymphocytes and supports further the interpretation that PMNs play an important role in ANIT-induced liver injury.

To determine whether toxic oxygen species elicited from PMNs might mediate the injury associated with ANIT (see Figure VII-1), PEG-CAT and PEG-SOD were administered to rats. Native enzymes were not used since they have very short half lives in the circulation of rats, i.e. < 10 min, whereas PEG-coupled enzymes have half lives on the order of hours (Till *et al.*, 1983). CAT was included since H_2O_2, the dismutation product of O_2^-, is the injurious species in certain models of PMN-dependent tissue injury (Johnson and Ward, 1981). Also, the PEG-CAT/SOD combination should inhibit formation of hydroxyl radical, another potentially injurious toxic oxygen species, by destroying O_2^- and H_2O_2 required for the iron-catalyzed Haber Weiss reaction. The doses of PEG-CAT and PEG-SOD employed were greater than those which afford protection in other models of PMN-dependent injury to the lung (Till *et al.*, 1983). The PEG-

CAT/SOD combination caused large elevations in SOD and CAT activities in serum but did not afford protection. Thus, although ANIT stimulates the release of O_2^- from PMNs *in vitro* (Roth and Hewett, 1986), our results *in vivo* do not support a role for PMN-derived oxygen species in the hepatotoxicity caused by ANIT.

Results obtained in the ANIT model regarding involvement of oxygen radicals are different from those obtained in the isolated, perfused rat liver. For example, in the latter system, a combination of SOD and catalase prevented the injury caused by activated PMNs. One possible explanation is that PMN-derived oxygen radicals mediate injury to the isolated organ preparation, whereas other PMN-derived products cause injury in the ANIT model (see below). A second possibility is that, while PMN-mediated injury to the isolated rat liver is dependent upon oxygen radicals, PMNs might not be the only source of these oxygen species (see above). For example, activated PMNs might cause injury to livers by occluding perfusate flow and causing an ischemia/reperfusion syndrome. Therefore, PMN-derived oxygen radicals might not be involved in either model of PMN-dependent liver injury. Alternatively, the PEG-CAT/SOD experiment may be inconclusive (see below for discussion). Consequently, PMN-derived oxygen radicals may be involved in both models.

That PEG-CAT/SOD failed to afford protection suggests that PMN-derived oxygen species are not involved in the mechanism of ANIT hepatotoxicity, but it does not rule out this possibility completely. For example, using co-cultures of PMNs and hepatocytes, Guigui *et al.* (1988) demonstrated the importance of close contacts between these cell types when studying PMN-mediated cytotoxicity to hepatocytes. Therefore, it is possible that PEG-coupled enzymes could not reach sites of oxygen radical attack on hepatocytes. Another possible explanation for the failure of PEG-CAT/SOD to afford protection is that changes

in the treatment protocol masked the PMN-dependent component of liver injury, and thereby masked the oxygen radical-dependent component. For example, as indicated earlier, we have been unable to demonstrate protection of NAS against ANIT-induced liver injury in studies in which rats were placed under a warming lamp after ANIT treatment. Since rats were placed under a warming lamp in studies employing PEG-CAT/SOD to obtain blood from the tail to confirm circulating enzyme activities, it is possible that we did not observe any protection by PEG-CAT/SOD because the PMN-dependent component was reduced.

Another possible explanation for the failure of PEG-CAT/SOD to afford protection is that, while the doses were larger than those needed to prevent PMN-dependent lung injury (Till *et al.*, 1983), they might not have been large enough to prevent liver injury. For example, 10,000 IU/kg or 40,000 IU/kg of PEG-SOD or PEG-CAT, respectively, prevented Kupffer cell dependent, oxygen radical-mediated injury in a model of vitamin A potentiation of CCl_4 hepatotoxicity (ElSisi *et al.*, 1987). In these studies, vitamin A increased serum ALT activity 19-fold relative to CCl_4 treatment alone, and PEG-CAT or PEG-SOD administration reduced this elevation by > 85%. A lower dose of PEG-SOD (6000 IU/kg) reduced the elevation of serum ALT activity by approximately 44%. Lower doses of PEG-CAT (< 40,000 IU/kg) and SOD (< 6000 IU/kg) were not used. Since the doses of PEG-CAT and PEG-SOD in our studies were approximately 6550 IU/kg and 3275 IU/kg, respectively, it is possible that they were not large enough to prevent liver injury.

These explanations for the lack of protection by PEG-CAT/SOD *in vivo* are possible, although the most straightforward interpretation is that PMN products other than toxic oxygen species might be involved in ANIT-induced hepatotoxicity. ANIT stimulates release of *B*-glucuronidase from PMNs *in vitro* (Roth and Hewett, 1986), suggesting that degranulation products released from

PMNs might be involved. As indicated above, proteinases released from PMNs have been implicated in hepatocellular injury *in vitro* (Guigui *et al.*, 1988; Mavier *et al.*, 1988). Further work is needed to clarify the role of toxic oxygen species as well as other possible mediators released by PMNs.

IV. Causal or Permissive Role for GSH in ANIT Hepatotoxicity

GSH may play a causal or permissive role in ANIT-induced liver injury, since decreased hepatic NPSH content, a measure of GSH content, afforded protection. The strongest evidence for GSH involvement is that provided by BSO. BSO blocks GSH synthesis by inhibiting gamma-glutamylcysteine synthetase (Griffith and Meister, 1979). Administration of BSO to animals causes a decrease in GSH content in liver and other tissues with a high GSH turnover, i.e. kidney. The advantage of BSO as a means to decrease hepatic GSH content is that it is rather specific and apparently does not affect hepatic MFO activity or enzymes involved in phase II metabolism (Drew and Miners, 1984; Sun *et al.*, 1985; White *et al.*, 1984). Therefore, the protection afforded by BSO against ANIT-induced liver injury is likely related to its effects on GSH.

Although BSO afforded protection when markers of liver injury were measured at 24 hr after ANIT treatment, injury was evident by 48 hr. Since the onset of injury appeared to coincide with a return of hepatic NPSH content, these data support a role for GSH in causing injury. Presumably, the liver injury occurs as hepatic NPSH content increases to a critical level and triggers an unidentified, GSH-dependent process leading to toxicity.

Protection against ANIT-induced liver injury with DEM or phorone provide additional support for GSH involvement in ANIT's mechanism of toxicity. DEM

and phorone are alpha, beta-unsaturated carbonyl compounds which decrease hepatic GSH content by conjugating to GSH (Plummer *et al.*, 1981). Both compounds afford protection against ANIT hepatotoxicity. In naive rats or those treated with CO, these agents decrease hepatic NPSH content for only 2-6 hr after treatment, suggesting that hepatic GSH is somehow utilized in the first few hours after ANIT administration. However, since hepatic NPSH content was not measured in rats treated with ANIT and these agents, it is not known whether ANIT alters the time course for depletion of hepatic NPSH content.

DEM and phorone are effective in decreasing hepatic NPSH content, but their use as experimental tools is complicated by their other biological effects. For example, DEM alters hepatic MFO activity *in vitro* (Anders, 1978), and it decreases body temperature (Costa and Murphy, 1986), reduces cytochrome P-450 content (Yoshida *et al.*, 1988), and causes a transient choleresis (Barnhart and Combs, 1978) when administered *in vivo*. Phorone itself is hepatotoxic to rats at doses close to those needed to deplete hepatic NPSH content. Thus, interpretation of results of experiments employing these agents is complicated by their other effects. However, taken together, the observations that BSO, DEM, and phorone afford protection against ANIT-induced liver injury support a causal or permissive role for GSH in the pathogenesis.

Certain agents which are MFO inducers or inhibitors alter hepatic GSH status (Table I-1), suggesting that their effects on ANIT-induced liver injury might result, at least in part, from changes in GSH status. It is tempting to speculate that other manipulations that alter ANIT injury act via an effect on GSH. For example, Indacochea-Redmond *et al.* (1973) reported that unimpaired protein synthesis may be required in the mechanism of injury, since protein synthesis inhibitors such as ethionine, actinomycin D, and cycloheximide afforded protection. Glaser and Mager (1974) demonstrated that ethionine decreased

hepatic GSH content. This raises the possibility that ethionine and perhaps other protein synthesis inhibitors might afford protection against ANIT hepatotoxicity by a mechanism involving a decrease in hepatic GSH.

The mechanism by which GSH is involved in ANIT-induced liver injury is presently unknown. Certain nephrotoxicants and hepatotoxicants, which require GSH to cause injury, form toxic GSH S-conjugates (see Chapter I). It is possible that ANIT or a metabolite might form a toxic GSH S-conjugate (see Figure VII-1). That ANIT injures bile duct epithelium seems consistent with this hypothesis, since this is a tissue with high activity of GGT (Szewczuk *et al.*, 1980) necessary in processing GSH S-conjugates.

A hypothetical scheme for involvement of a toxic GSH S-conjugate in ANIT hepatotoxicity is shown in Figure VII-2. After an oral dose of ANIT to rats. ANIT or a metabolite might be conjugated to GSH in the liver and secreted into bile. A cysteine S-conjugate of ANIT (or metabolite) might be formed by the actions of GGT and dipeptidases, which are located in bile ductular and small intestinal epithelial cells (Szewczuk *et al.*, 1980; Okajima *et al.*, 1983). Upon reabsorption from the intestinal tract, the putative cysteine S-conjugate might undergo intramolecular rearrangement to form an episulfonium ion, thereby causing hepatotoxicity. Since the reactivity of episulfonium ions varies with structure (Anders *et al.*, 1988), an episulfonium ion formed from a putative cysteine S-conjugate of ANIT (or metabolite) might travel from intestine to liver to cause injury. However, it must be emphasized that formation of an episulfonium ion from the putative cysteine S-conjugate is hypothetical, since the chemistry of the ANIT conjugate might not favor formation of this species. Alternatively, cysteine conjugate *B*-lyase in gut microflora might metabolize the cysteine S-conjugate to a toxic thiol, which might cause hepatotoxicity when it reaches hepatocytes after reabsorption from the intestinal tract into portal blood. It is unclear whether a

toxic thiol produced in the intestine would survive transit to the liver to exert hepatotoxicity, although it might depend on the reactivity of the thiol. Alternatively, since hepatocytes contain cysteine conjugate *B*-lyase, a reactive thiol might be produced there to cause injury. In all of these hypothetical pathways, the anatomical distribution of enzymes needed for metabolism of GSH S-conjugates in the liver and gastrointestinal tract is consistent with the requirement for an intact enterohepatic circulation (Roberts and Plaa, 1966b). In addition, these hypothetical pathways are consistent with the periportal hepatotoxicity associated with ANIT, since, after enterohepatic cycling, periportal hepatocytes would be exposed first to a toxic GSH S-conjugate of ANIT (or metabolite).

It is unfortunate that knowledge of the metabolism of ANIT is limited. For example, it is not known whether ANIT or a metabolite forms a GSH conjugate *in vivo*. After administration to rats, ANIT does not lower hepatic GSH content (El-Hawari and Plaa 1977; Chapter VI) as do other agents which conjugate to GSH in the liver (Plummer *et al.*, 1981), suggesting that perhaps GSH conjugation might not occur. However, other isothiocyanates, such as benzyl isothiocyanate and allyl isothiocyanate, conjugate with GSH and ultimately form mercapturic acids that are detectable in urine (Brusewitz *et al.*, 1977; Mennicke *et al.*, 1983). An interesting finding is that conjugation of isothiocyanates to GSH is a reversible reaction, unlike reactions of other electrophilic compounds (Drobnica *et al.*, 1977). Accordingly, GSH S-conjugation may serve as a novel mechanism to transport isothiocyanates to extrahepatic sites to cause toxicity after liberation of free isothiocyanate (Bruggeman *et al.*, 1986). Mennicke *et al.* (1978) reported that ANIT administration to rats did not result in formation of a mercapturic acid in urine or bile. This observation does not rule out the formation of a GSH S-conjugate of ANIT, since mercapturic acids are generally end products of GSH

S-conjugate metabolism, and ANIT might conjugate with GSH to cause toxicity without ever forming a mercapturate. Further work is needed to clarify any role of GSH in the metabolism of ANIT.

GSH might be involved in ANIT-induced liver injury by mechanisms other than formation of a toxic GSH S-conjugate. For example, GSH is required in the formation of thiol ether leukotrienes, which have been implicated in liver injury (see Chapter I). We can only speculate how a thiol ether leukotriene might be involved in the cholestatic liver injury caused by ANIT. Since administration of thiol ether leukotrienes intravenously to guinea pigs causes plasma extravasation around bile ducts (Hua *et al.*, 1985), it is conceivable that edema might lead to cholestasis by exerting pressure on the walls of bile ducts and occluding bile flow. Interestingly, ANIT causes periportal edema in rats (Ungar *et al.*, 1962; Goldfarb *et al.*, 1962). Alternatively, since thiol ether leukotrienes cause vasoconstriction in the rat liver (Krell and Dietze, 1989), they might produce an hepatic ischemia/reperfusion syndrome similar to that observed after administration of galactosamine and endotoxin to mice (Wendel *et al.*, 1987; see Chapter I). However, the periportal hepatotoxicity associated with ANIT is inconsistent with a reperfusion injury. For example, the hepatic lobule has a decreasing oxygen gradient from periportal to centrilobular regions, and one would expect the greatest injury to occur in centrilobular regions (Bradford *et al.*, 1986). It is possible that thiol ether leukotrienes cause hepatotoxicity by mechanisms other than effects on the vasculature.

The source of putative hepatotoxic thiol ether leukotrienes in liver injury is also unknown. In the galactosamine/endotoxin model in which LTD_4 is the toxic species (Tiegs and Wendel, 1988; see Chapter I), it was suggested that white blood cells, possibly PMNs, might be the source. Activated rat PMNs release thiol ether leukotrienes (Orange *et al.*, 1967), which is consistent with this

suggestion. If PMNs are the source of hepatotoxic thiol ether leukotrienes in the galactosamine/endotoxin model, then the reason that hepatic GSH depletors afford protection against the liver injury might relate to their non-specificity. For example, these agents, i.e., DEM and phorone, might decrease sources of GSH required for formation of thiol ether leukotrienes by PMNs, in addition to decreasing hepatic GSH content, and thereby prevent liver injury.

If thiol ether leukotrienes are involved in ANIT hepatotoxicity, then it seems possible that PMNs are the source, as suggested by Tiegs and Wendel (1988). That PMN depletion affords protection against ANIT-induced liver injury is consistent with this proposal. This scheme brings together the PMN- and GSH-dependent components of liver injury associated with ANIT and is shown in Figure VII-1.

V. Relationship Between ANIT-Induced Elevations in Hepatic NPSH Content and Liver Injury

In Chapter V, we observed that hepatic NPSH content was elevated after ANIT treatment, and this increase appeared to be associated with the onset of liver injury. Changes in hepatic NPSH content were not the result of any nutritional differences between groups, since all rats were fasted throughout the study. The elevation in hepatic NPSH content probably reflects an increase in GSH content, since the latter is the major soluble thiol in mammalian cells. However, it is possible that sulfhydryl-containing compounds other than GSH might increase in liver after ANIT treatment and contribute to the elevated NPSH content.

The observation that ANIT elevates hepatic NPSH content does not by itself implicate GSH in the mechanism of toxicity. However, since this elevation

was associated temporally with ANIT-induced liver injury, and GSH appeared to be involved in the mechanism of injury (Chapter V), we determined whether the elevation of hepatic NPSH content was linked to the development of liver injury.

Two lines of indirect evidence suggest that the elevation in hepatic NPSH content is unrelated to the mechanism of toxicity. The first evidence was the result of the study with BNIT. BNIT has some effects on the liver that are similar to those of ANIT, but it is not hepatotoxic (Becker and Plaa, 1965a; El-Hawari and Plaa, 1979). For example, acute administration of BNIT to rats alters hepatic mixed function oxidase content and activity similar to ANIT, but it does not affect markers of liver injury as does ANIT (Becker and Plaa, 1965a; El-Hawari and Plaa, 1979). As expected, BNIT administration to rats did not cause cholestasis or elevate any marker of liver injury. However, BNIT elevated hepatic NPSH content 24 hr after treatment. Since BNIT was not hepatotoxic and still caused an elevation in hepatic NPSH content, it seems unlikely that the elevated NPSH content after ANIT treatment is critical to the mechanism of toxicity.

A second line of evidence which suggests that elevated hepatic NPSH content is not involved in the mechanism of ANIT toxicity comes from the bile duct ligation experiment. Serum total bilirubin concentration was greatly elevated by 6 hr after bile duct ligation, although hepatic NPSH content did not increase until after 6 hr. This suggests that elevated hepatic NPSH content is a result of cholestasis and is not in any way causal. Although bile duct ligation probably does not reflect completely the liver injury caused by ANIT, it does produce some similar pathological changes in the liver (Steiner *et al.*, 1963; Ungar *et al.*, 1962), and ANIT is thought to cause cholestasis in part by obstruction of bile ductules (Goldfarb *et al.*, 1962; Woolley *et al.*, 1979). Therefore, certain changes in the liver after bile duct ligation might be applicable to the ANIT model.

Since both reduced and oxidized glutathione are transported from hepatocytes into bile (Sies *et al.*, 1983), it seems possible that cessation of bile flow, i.e. cholestasis, might cause hepatic glutathione content to increase. One might expect a similar time course for increases in hepatic NPSH content after cholestasis caused by bile duct ligation and ANIT treatment, if ANIT-induced cholestasis was caused solely by biliary obstruction. However, the lag from the onset of cholestasis to the elevation in NPSH content after bile duct ligation is not observed with ANIT, suggesting that ANIT probably elevates hepatic NPSH content by mechanisms in addition to cholestasis. As indicated above, there is evidence for mechanical obstruction of bile ductules after ANIT treatment. However, ANIT also injures hepatocytes and alters the secretion of cholephilic agents, i.e., bromosulfophthalein, bilirubin, and bile acids, into bile (Becker and Plaa, 1965b; Roberts and Plaa, 1967; Krell *et al.*, 1982). Therefore, hepatocellular changes after ANIT treatment probably contribute to increases in hepatic NPSH content.

VI. Variability of Response in the ANIT Model

As indicated in Chapter I, published reports indicate that approximately 25% of ANIT-treated rats do not develop hyperbilirubinemia. In the experiments shown in Chapter IV, 24% (6/25) of rats receiving ANIT alone did not develop hyperbilirubinemia, indicating that the incidence of non-responders in our studies agree with other reports. The criterion for hyperbilirubinemia in our studies is a serum total bilirubin concentration > 0.4 mg/dl when measured 24-48 hr after ANIT treatment. This value was chosen, since total serum bilirubin concentrations in control rats do not exceed this value.

The reasons for non-responsiveness to ANIT are not known, although results from experiments in this thesis suggest that fasting rats prior to ANIT administration eliminates this phenomenon. For example, in experiments *in vivo* in which rats were fasted, 64 out of 65 rats receiving ANIT (25-100 mg/kg, p.o.) alone developed hyperbilirubinemia. There are at least two possible explanations for the effect of fasting. One explanation is that food in the stomach delays the absorption of ANIT. Presumably, varying amounts of food present in the stomach cause the variable response to ANIT. In this proposal, one would expect that rats would not actually be non-responders. For example, if monitored for > 24 hr, they might develop hyperbilirubinemia as greater amounts of ANIT are absorbed. Since our studies employing fed rats and ANIT (35 mg/kg, Chapter IV) were terminated at 24 hr, we cannot make a definite conclusion regarding this explanation. Using a similar dose of ANIT as in our studies, i.e., 45 mg/kg, Desmet *et al.* (1968) and Krstulovic *et al.* (1968) have provided evidence supporting this proposal. For example, they observed that bile flow and serum bilirubin concentration were not reduced 24 hr after ANIT treatment. However, after a second treatment with ANIT at 24 hr, rats exhibited cholestasis and hyperbilirubinemia within 12-24 hr.

A second explanation for the effects of fasting on the incidence of ANIT injury relates to GSH metabolism. For example, fasting increases hepatic GGT activity (Tateishi *et al.*, 1974), an enzyme required for degradation of GSH S-conjugates as well as formation of thiol ether leukotrienes. Since we proposed that either of these two mechanisms might be involved in the pathogenesis of ANIT-induced liver injury (see above), an increase in hepatic GGT activity by fasting would be expected to increase formation of the toxic species. Presumably, this would increase the incidence of liver injury associated with ANIT.

The results of Desmet *et al.* (1968) and Krstulovic *et al.* (1968) demonstrate that rats are not true non-responders to ANIT and that low doses of ANIT appear to delay the onset of liver injury beyond 16-24 hr. In regard to the latter point, when doses of ANIT exceeding 150 mg/kg are used, plasma ALT activity is elevated by 2 hr and bile flow is reduced at 16-24 hr (Drew and Priestly, 1976; Plaa and Priestly, 1977; El-Hawari and Plaa, 1979). However, at lower doses of ANIT, i.e., 45-80 mg/kg, bile flow does not decrease until 18-36 hr, and plasma ALT activity appears to be elevated sometime between 18 and 24 hr (Desmet *et al.*, 1968; Fukumoto *et al.*, 1980). We have also observed this delay to onset of cholestatic liver injury in fed rats at low doses of ANIT, i.e., 35 mg/kg.

ANIT reportedly causes dose-dependent, cholestatic liver injury (Plaa and Priestly, 1977). However, this phenomenon of delayed onset of injury with low doses of ANIT may influence the dose/response relationship. For example, if rats are treated with varying doses of ANIT and are killed at 24 hr, those given large doses will all exhibit cholestatic liver injury by 24 hr. Rats given low doses might not respond at all, or, alternatively, if injury is starting to occur, some rats might respond while others do not. In this way, an "apparent" dose-response relationship is demonstrated, which is dependent upon the time rats were killed. If the delay in onset to injury is eliminated, then a clear dose-response relationship to ANIT may not be evident. For example, fasting appears to eliminate "non-responders" to ANIT, presumably by making all rats respond within 24 hr to ANIT. When hyperbilirubinemia data from all of our ANIT studies using fasted rats were examined, there was not a graded dose-response relationship (Figure VII-3). It appeared to be an "all or none" response. Perhaps this unusually steep dose/response curve may provide further clues regarding ANIT's mechanism of toxicity. In addition, future studies might determine the

mechanism by which fasting affects the incidence of ANIT-induced cholestatic liver injury.

VII. Major Contributions of Thesis Project

In this thesis, we have made several important observations, which contribute to the area of hepatotoxicology. First, we have shown that PMNs play a causal role in ANIT-induced liver injury. This is the first evidence, to our knowledge, that PMNs mediate chemically-induced liver injury and may represent a novel mechanism by which other hepatotoxicants act. Second, we have provided evidence supporting a novel role for GSH in bioactivation of hepatotoxicants. GSH usually serves as a protective mechanism to hepatocytes by conjugating with electrophilic xenobiotic agents. Third, we have demonstrated that biliary products, such as bile salts, interact with PMNs to release O_2^-, suggesting that they may be physiological and/or pathophysiological PMN activators.

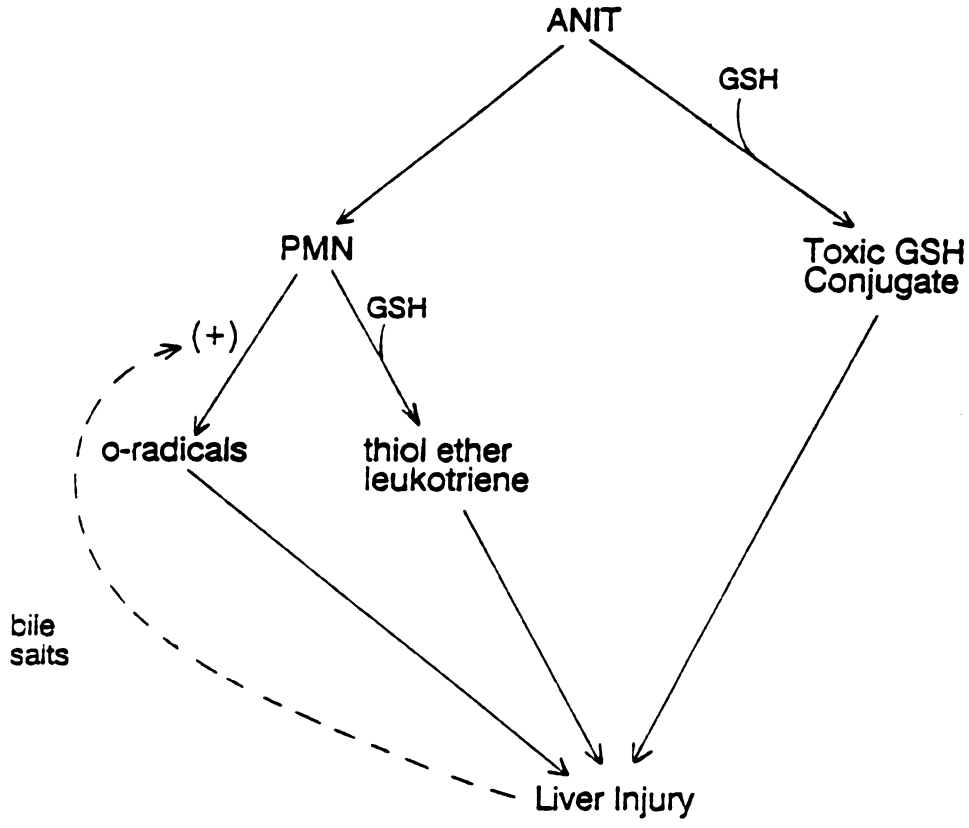

Figure VII-1. Diagram of possible mechanisms of ANIT hepatotoxicity

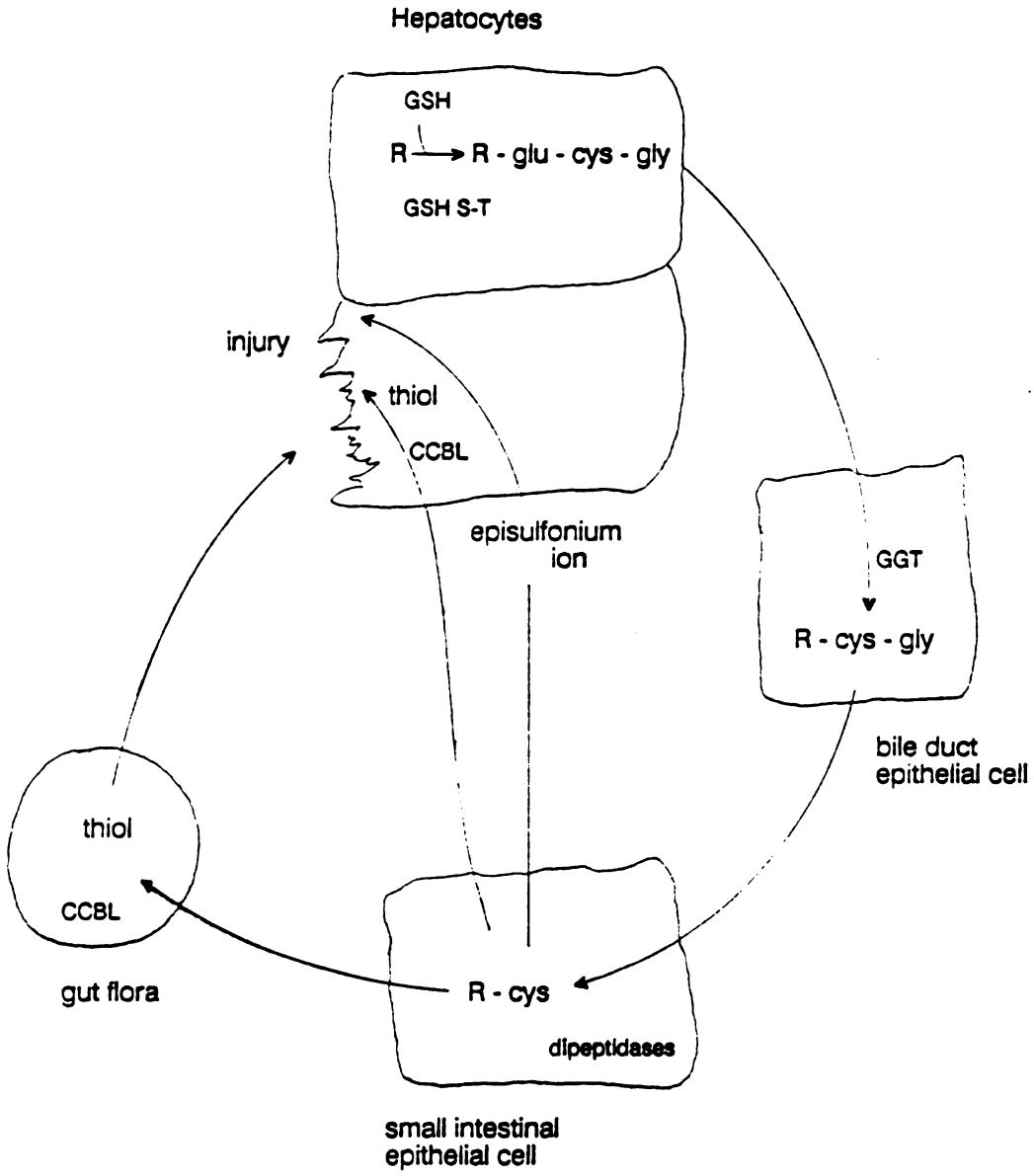

Figure VII-2. Metabolism of a putative GSH S-conjugate of ANIT or ANIT metabolite. R = ANIT or ANIT metabolite. Enzymes: GSH S-T, glutathione S-transferase; GGT, gamma-glutamyltransferase; CCBL, cysteine conjugate B-lyase

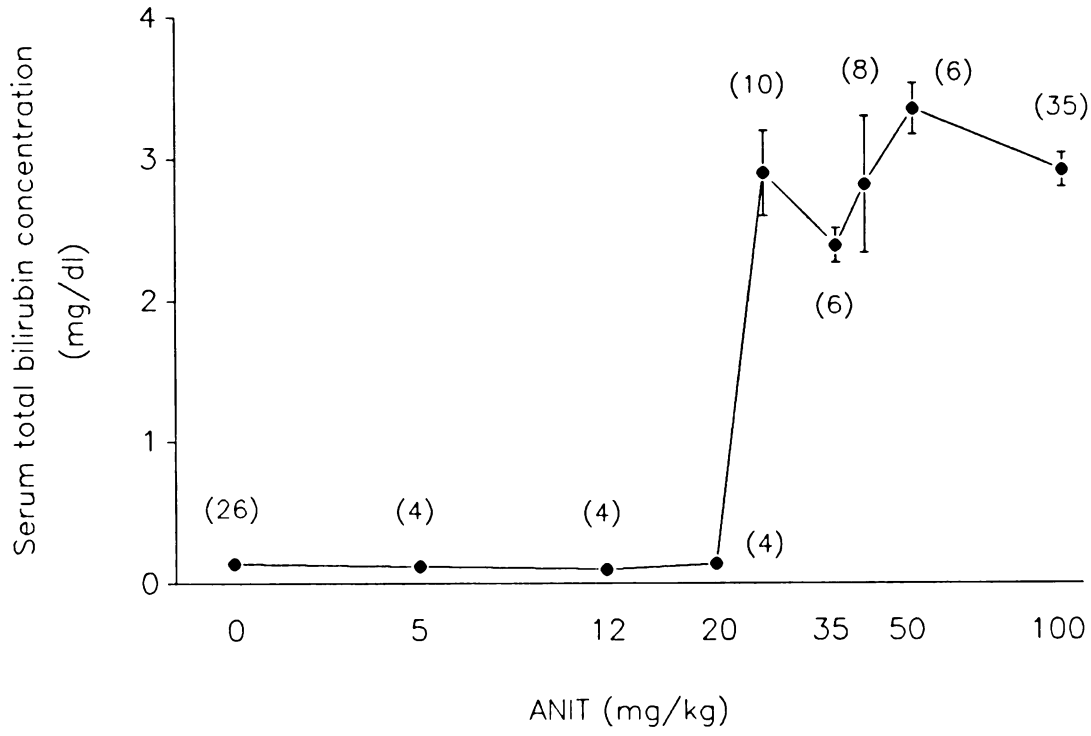

Figure VII-3. Dose/response relationship for ANIT-induced hyperbilirubinemia in fasted rats. Rats were fasted for 24 hr prior to either ANIT (5-100 mg/kg, p.o.) or CO vehicle (0 mg/kg) and remained off chow thereafter. Rats were killed 24 after treatment, and total serum bilirubin was measured as described under METHODS in Chapter IV. Data are pooled from all ANIT studies employing fasted rats. In most cases, rats received a vehicle, i.e., CS, saline, or CO along with either ANIT or CO. Numbers in parentheses indicate number of samples. Points lacking S.E. bars have S.E. less than the area covered by the symbols.

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