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THE ROLE OF ENDOTOXINS IN HALOTHANE-ASSOCIATED LIVER INJURY

THE UNIVERSITY OF ARIZONA M.S. 1982

# THE ROLE OF ENDOTOXINS IN HALOTHANE-ASSOCIATED LIVER INJURY

by

Richard Charles Lind

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In Partial Fulfillment of the Requirements
For the Degree of

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THE UNIVERSITY OF ARIZONA

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This thesis has been approved on the date shown below:

I. Glenn Sipes

I. GLENN SIPES
Professor, Pharmacology & Toxicology

Dec 10, 1982
Date

To my parents, Helen Hanson and the memory of Esther Hanson for all the love, support and guidance they have given me in my endeavors.

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## ABSTRACT

Since endotoxins, lipopolysaccharides (LPS), have been implicated as a causative factor in the development of hepatic necrosis in rats, the role of LPS in the halothane-hypoxia (HH) model of hepatic damage in male Sprague-Dawley rats was investigated. When injected intravenously immediately post halothane anesthesia, a subnecrotic dose of LPS (0.5 mg/kg E. coli 026:B6) was found to potentiate HH-induced hepatic necrosis by several fold. Pretreatment of the animals with lactulose prior to exposure to halothane significantly reduced the hepatic damage normally seen from HH. A possible mechanism of LPS-induced potentiation was indicated by changes in hepatic calcium levels at 24 hr post treatment. Thus it was demonstrated that the rat HH model increases the susceptibility to hepatic damage from subnecrotic amounts of LPS, that endogenous LPS may play a role in HH-induced hepatic necrosis, and that the mechanism of LPS-induced potentiation may be due to an LPS-related membrane dysfunction.

# INTRODUCTION

Within three years of its introduction in the mid-1980's, halothane ($CF_3CBrClH$), a volatile halogenated ethane, became the world's most widely used operating room inhalation anesthetic (Vaughan, Sipes and Brown, 1978). Although it expressed excellent properties for use in general anesthesia, a sporatic incidence of post-anesthetic fulminant hepatic failure soon became associated with the use of halothane as the inhalation anesthetic during surgery (Brody and Sweet, 1963; Summary of the National Halothane Study, 1966). The incidence of this "halothane hepatitis" appears to be approximately one in every seven to thirty thousand administrations, proving fatal in about 50% of the cases (Strunin, 1977), while a mild transient hepatic damage is seen in up to 20% of patients repeatedly anesthetized with halothane (Davis et al., 1980). Of course, the number of sub-clinical cases of mild liver damage following any halothane anesthesia in unknown and may be much higher in incidence. Attempts to reproduce this halothane induced hepatic necrosis in animals has proven only moderately successful. Several animal models, all utilizing rats, produce an acute centrilobular hepatic lesion after exposure to halothane, although none of them progress to a fulminant hepatitis as in the serious human cases (Harper et al., 1982a; Wood et al., 1980; Mclain et al., 1979; Sipes and Brown, 1976).

Carbon tetrachloride ($CCl_4$) is another organohalogen that also produces hepatic necrosis. Its mechanism of necrotic action in animals has been intamately linked with endotoxins, lipopolysaccharides (LPS),

that are toxic moieties derived from gram negative bacteria residing in the lower large intestine (Nolan, 1981). The bacterial LPS are continually absorbed through the intestinal wall and travel via the portal system to the liver, where they are removed and detoxified by the Kupffer cells lining the sinusoids which act as the body's primary defense to these toxins (Nolan et al., 1977). In cases of severe hepatic failure in humans, such as alcoholic cirrhosis and viral hepatitis, LPS reaching the systemic circulation have been incriminated in observed complications such as fever, hypotension, the hepato-renal syndrome, and intravascular coagulation among others (Nolan, 1981). Thus, LPS could well be involved in both the halothane-induced transient acute hepatic damage in humans and animal models as well as in the rare progressive fulminant halothane hepatitis observed in humans.

## Metabolic Pathways of Halothane

Originally, halothane was considered to be refractory to metabolism, until reports in 1964 demonstrated an increased urinary excretion of inorganic bromide ion in rats and humans after halothane anesthesia and trifuluoracetic acid (TFA) in the urine of rabbits exposed to the anesthetic (Stier, 1964a,b; Stier et al., 1964). Soon thereafter, by utilizing tissue slices and subcellular fractions, it was shown that halothane is metabolized primarily in the liver by the microsomal cytochrome P-450 mixed-function oxidase system (Van Dyke, 1966; Van Dyke and Chenoweth, 1965). Two pathways of metabolism an oxidative and a reductive, are now recognized with the oxidative pathway being by far the major route (Figure 1) (Sipes, Gandolfi and Brown, 1981). TFA,

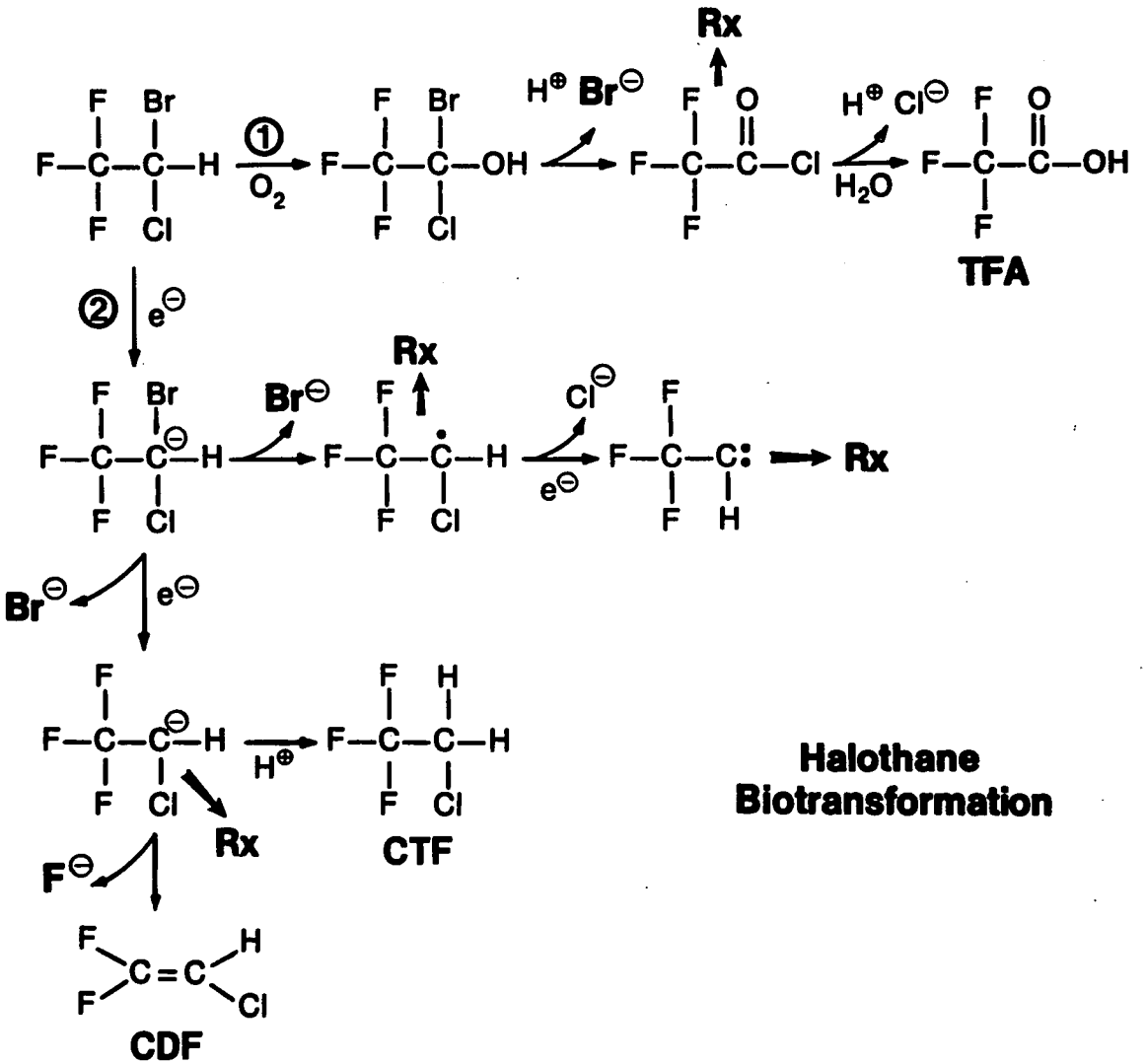


Figure 1. Proposed scheme of halothane biotransformation. -- (Bentley et al., 1982).

bromide ion and chloride ion are liberated via the oxidative route, while reductive metabolism primarily produces the volatile metabolites chlorotrifluoroethane (CTFE) and chlorodifluroethylene (CDFE) and fluoride ion (Sipes et al., 1981). Bromide ion, is also liberated by the reductive pathway, although vastly greater quantities are produced by the oxidative route and thus it is not possible to distinguish whether the bromide ion is liberated by the reductive cleavage of the C-Br bond or from rearrangement following oxidation at the C-H bond (Sipes et al., 1981). Only the CDFE metabolite has ever exhibited any tendency for further metabolism, with defluorination being demonstrated in vivo and by hepatic microsomes in vitro, but neither it nor any of the other identified metabolites have been proven to possess any hepatotoxic action in themselves at the expected concentrations produced by halothane anesthesia (Maiorino et al., 1981). In vivo production of all these metabolites has been shown in both animals and humans undergoing halothane anesthesia (Maiorino et al., 1980; Sharp, Trudell and Cohen, 1979; Mukai et al., 1977). Both the oxidative and reductive pathways produce reactive intermediates in vivo that covalently bind to hepatic subcellular macromolecules, althogh reducing the oxygen tension during exposure to halothane and thus promoting the reductive pathway of metabolism greatly enhances this binding (Widger et al., 1976).

## Clinical Manifestations of Halothane Hepatitis

As previously mentioned, the use of halothane as a general operating room anesthetic has been associated with a sporatic incidence

of severe post anesthesia hepatic complications; and a more prevalent, milder transient hepatic damage. Both forms of liver dysfunction primarily occur in persons who have received repeated halothane anesthesia (Davis et al., 1980), although multiple exposures to halothane are not necessary for expression of the fulminant hepatitis, as several women have developed the disease after a single anesthesia with halothane (Hoft et al., 1981). Halothane has been directly linked to the fulminant hepatitis by the demonstration within victims of circulating antibodies which bind to the surface of rabbit hepatocytes that have been altered by halothane (Vergani et al., 1980). This has led to the view that the mild form of hepatic damage may be due to a manifestation of direct toxic injury while the rare severe lesion involves an additional immune component (Davis et al., 1980). The onset of halothane-implicated fulminant hepatitis is generally delayed until several days after anesthesia with jaundice reaching its maximum within one week (Moult and Sherlock, 1975). The disease primarily strikes overweight middle-aged to elderly women, but it has even been recently observed in a four-year old girl as well (Lewis and Blair, 1982).

## Factors Influencing Halothane Hepatitis

Genetic, physical and environmental factors all could contribute to both of the observed forms of halothane-implicated hepatic damage. Different strains of rats have been reported to display differing levels of reductive metabolism of halothane as well as varying degrees of hepatotoxicity from exposure to the anesthetic (Gourlay et al., 1981). A recent report cites antibody proven post-halothane associated

hepatitis in three pairs of closely related women, all of whome were Mexican-Indian or Mexican-Spanish in ancestry (Hoft et al., 1981).

The physical state of obesity has been shown to cause an increased level of biotransformation of volatile halogenated anesthetics as compared to non-obese persons (Miller, Vaughan and Gandolfi, 1980; Bentley, Vaughan and Gandolfi, 1982; Gourlay et al., 1980). Halothane associated hepatitis is known to primarily strike obese persons and indeed the previously mentioned Mexican-Indian and Mexican-Spanish women were all either overweight or markedly obese.

Work-related exposure to halothane in the environment of the operating room has led to cases of operating room technicians and anesthesiologists developing a sensitivity to halothane that caused liver dysfunction even at low background levels of the anesthetic (Newberger et al., 1981). Again, as in the case of the halothane-sensitive anesthesiologists, circulating antibodies to halothane-altered rat hepatocyte membranes were demonstrated. Also, the degree of halothane metabolism during anesthesia could be increased by induction of hepatic drug metabolizing enzymes through alcohol ingestion, drug therapy or exposure to certain environmental pollutants. In animal models of halothane-induced liver necrosis, hepatic enzyme induction by either the environmental pollutants, polychlorinated biphenyls or the drug phenobarbital is used in order to obtain hepatic necrosis from halothane exposure (Sipes and Brown, 1976; McLain et al., 1979). Polychlorinated biphenyls are widespread in our environment being formerly used as wax extenders, in heat transfer systems, as microscope immersion

oil, and incarbonless copy paper and are still utilized in electrical capacitors and transformers (Hutzinger, Safe and Zitko, 1974), making these known inducers of hepatic enzymes (Alvares, Bickers and Kappas, 1973) readily available for human exposure. Ethanol consumption, a common practice in our society, can also induce hepatic drug metabolizing enzymes (Ariyoshi and Takabatake, 1970).

Enhanced metabolism of halothane in rats along the reductive pathway via hepatic enzyme induction and reduced atmospheric oxygen tension during halothane exposure has been implicated as the source of the animal model hepatic lesion through binding of reactive reductive metabolic intermediates to subcellular macromolecules (Sipes et al., 1981). In animals, upper abdominal surgery during halothane anesthesia has been shown to reduce hepatic arterial blood flow by 34% (Gelman, 1976) and to cause greater hepatic injury as compared to peripheral surgery (Harper et al., 1982b). In fact, halothane anesthesia alone has been shown to decrease hepatic blood flow (Ross and Daggy, 1981; Benumof et al., 1976). Within the microenvironment of the liver, a reduction in the oxygen-supplying arterial blood flow, due to halothane anesthesia and/or the type of surgery being performed, would lead to areas of lesser oxygen tension and consequently promote the reductive pathway of halothane metabolism.

## Animal Models of Halothane Associated Liver Injury

The first reported animal model to consistently produce a hepatic lesion in rats from exposure to halothane involved dosing the animals with 500 mg/kg polychlorinated biphenyls four days prior to a

2 hr, 1% halothane exposure in a 99% oxygen atmosphere (Sipes and Brown, 1976). Unfortunately, polychlorinated biphenyls, as well as being potent enzyme inducers, are hepatotoxins in themselves and their use in such high amounts makes this model less than realistic, although they have been suggested as a possible contributing factor in one case report of halothane hepatitis (Zander, 1979).

On the basis of reported enhanced covalent binding of halothane equivalents to liver macromolecules under reduced oxygen tension (Widger et al., 1976), McLain and coworkers (1979) developed the halothane-hypoxia (HH) model of hepatic damage in male Sprague-Dawley rats (Sipes et al., 1981). The HH model calls for prior induction of hepatic enzymes with phenobarbital, followed by a 2 hr exposure to 1% halothane in a 14% oxygen atmosphere (McLain et al., 1979). The necrosis caused by these conditions is centrilobular in nature and reaches a maximum at 24-48 hr post exposure, with a rapid return towards normal hepatic physiology occurring by 72 hr (Jee, Sipes and Brown, 1980). This rapid time course makes the HH model unlike the halothane-associated progressive fulminant hepatitis seen in humans although it may have parallels with the more often occurring transient minor hepatic damage after human halothane anesthesia (Sipes et al., 1981; Davis et al., 1980).

Two other models of halothane-induced liver necrosis in rats that cause a more severe lesion than the HH model have also been reported. One involves triodothyronine pretreatment and causes hepatic damage upon exposure to 1% halothane regardless of the oxygen content of the atmosphere during exposure (Wood et al., 1980). The major

drawback to this model is that it demonstrates high serum glutamate pyruvate transaminase levels immediately after halothane anesthesia which return to normal values by 24 hr post exposure, making it even more dissimilar than the HH model from any observed human condition.

The other reported means of inducing hepatic necrosis with halothane calls for overnight fasting as well as phenobarbital induction prior to halothane anesthesia in an 8% $O_2$ atmosphere (Harper et al., 1982a; Van Dyke, 1981). Hepatic damage from both enflurane and isoflurane anesthesia is reported to occur under these conditions (Harper et al., 1982a). Both of these anesthetics do not induce any hepatic damage in the other reported models, nor do they form free radicals in vivo which has been associated with the hepatotoxic actions of halothane (Plummer et al., 1982; Wood et al., 1980; Sipes et al., 1981). Thus, the HH model seems the most realistic in terms of causing hepatic damage from an action of halothane and for this reason it is the animal model employed for the studies contained herein.

## Endotoxins

Endotoxins are lipopolysaccharides (LPS) unique to gram-netative bacteria, constituting an integral part of their trilaminar outer membrane (Shands, 1965). Usually released upon lysis of the cell, LPS can also escape from intact bacteria that have not undergone autolysis (Ecker, 1917). LPS posess a wide compositional and structural diversity depending on the species of bacteria from which they originate and yet they express very similar biological activities. The general structure of LPS is that of an amphipathic molecule consisting of a hydrophobic

lipid region known as lipid A and a covalently bonded, often branched, hydrophillic heteropolysaccharide chain, which is subdivided into the core and O-specific chains (Fig. 2). The O-specific chains, which are made up of repeating units of oligosaccharides, give LPS its antigenic properties and are what differ the most among various species of bacteria leading to the many known serotypes. Less structural diversity is seen in the polysaccharide core and lipid A portions of the molecule. But, even among the same bacteria, the fatty acid composition of lipid A can be altered by changing the bacterial growth-media composition (Luderitz, Galanos and Rietschel, 1982). The molecular weight of LPS from S. typhimurium is approximately 10,300 (Romeo, Girard and Rothfield, 1970). However, LPS tend to form aggregates of 1 to 20 x $10^6$ molecular weight in solution due to the hydrophobic nature of the lipid portion of the molecule (Morrison and Leive, 1975).

## Methods of LPS Extraction from Bacteria

Although many methods have been developed involving a wide variety of solvents for the extraction of LPS from bacteria, there are two procedures that are most often employed. One involves the use of 0.5 N trichloroacetic acid (Boivin and Mesrobeanu, 1935), while the other utilizes the tendency for LPS to remain in the water phase of a phenol-water mixture (Palmer and Gelough, 1940; Westphal, Luderitz, and Bister, 1952). In each method other bacterial components are also extracted. Trichloroacetic acid removes phospholipid, lipoprotein and protein along with LPS, while the phenol-water method, which gives the highest yields of LPS, also readily extracts nucleic acids from the bacteria. The

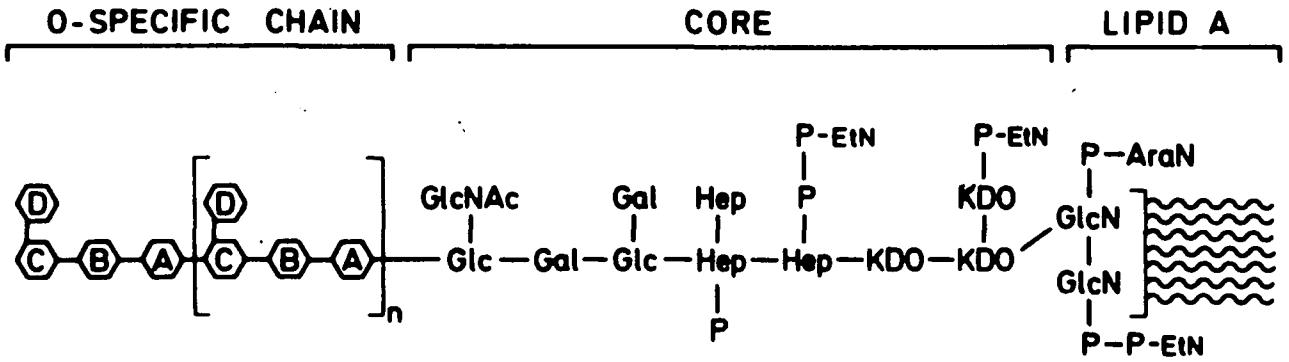


Figure 2. General structure of lipolysaccharide. -- A-D, sugar residues; Glc, D-glucose; Gal, D-galactose; GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycero-D-manno-heptose; KDO, 2-keto-deoxy-D-mannooctonate; AraN, 4-amino-L-arabinose; P, phosphate; EtN, ethanolamine; ∿∿, hydroxy- and nonhydroxy fatty acids (Luderitz et al., 1982).

phenol-water extracted LPS is the easier to purify of the two, requiring several ultracentrifugations in order to remove the lighter nucleic acids which remain in the supernatant.

## Detection of LPS

There exist several means of assaying for LPS, all of which have serious problems that limit their reliability in detecting LPS *in vivo* and ease of use in the laboratory. The most widely employed assay for LPS is the Limulus Amebocyte Lysate (LAL) test, which involves the clotting of extracts from the amebocyte blood cells of horseshoe crabs (Bang, 1956). Since the indication of the presence of LPS in this test is the formation of a gelatinous clot, serial dilutions of a sample must be carried out in order to estimate the quantity of LPS present. A more quantitative and sensitive adaption of the LAL assay, utilizing a chromogenic substrate, has been designed based on the proteolytic activity of the clotting enzyme. This assay involves the cleavage of p-nitroaniilide from a synthetic peptide chain by LAL that has been activated in the presence of LPS with the liberated p-nitroanilide being measured spectrophotometrically (Iwanaga et al., 1978). Each of these assays will give a positive response in the presence of any bacteria or pyrogen, thus contamination of samples is an easy source of false positive results (Stumacher, Kovnat and McCabe, 1973). Inhibitory substances to the LAL enzymes are known to occur in plasma, making necessary dilution and heating of plasma samples to inactivate these inhibitors (Berg, Nausley and Riegle, 1979) or a time consuming chloroform extraction to remove them (Levin et al., 1970). Human blood has

been shown to inactivate LPS response in the chromogenic substrate assay at a rapid and variable rate, demonstrating a need to immediately cool or freeze any blood samples to be assayed (Thomas et al., 1981).

Radioimmune assays have been developed for LPS but are specific for the bacterial serotype(s) to which the antibodies have been produced and thus would not be able to detect LPS from other bacteria as would be required in a general situation (Liebowitz, Vladutiu and NOlan, 1979).

Also available is the enterobacterial common antigen passive hemaglutination test which detects the presence of circulating antibodies to LPS or bacteria (Kunin, 1963). Of course, with this assay an individual must have had systemic exposure to bacteria or LPS from bacteria for sufficient time to build a detectable antibody titer (Malkamki, 1981).

A promising means of detecting LPS in biological samples is the gas chromatographic-mass spectrometric analysis for the unique bacterial fatty acid found in LPS, B-hydroxymyristic acid (Miatra et al., 1981). The great advantage of a gas chromatographic analysis is that the serum factors influencing biochemical assays for LPS would not affect the quantity of fatty acid present and consequently a more direct measurement of the amount of LPS in a sample would be possible. The need for a mass spectrometer is a major drawback to this assay, but it seems likely that a modified fatty acid analysis by gas chromatography could lead to a simpler, useable assay for LPS.

## Fate of LPS

LPS originating from bacteria in the lower G.I. tract are activily absorbed through the intestinal wall (Nolan et al., 1977), causing a constant state of endotoxemia in the portal vein that has been detected in approximately 50% of normal human patients tested (Prytz et al., 1976). Upon reaching the liver, LPS is removed from the blood by the Kupffer cells which make up 89-90% of the total reticulo-endothelial system (Wiznitzer et al., 1960). Exogenously introduced LPS, not captured by the KUpffer cells has been shown to also be degraded by circulating complement and esterases as well as by liver parenchymal cells (Miatra et al., 1981; May, Kane and Frank, 1972; Skarnes, 1970).

## Biological Effects of LPS

A myriad of host responses have been attributed to the infusion of LPS. Among these are fever, hypotension, complement activation, lymphocyte activation, leukocytosis, dissemination of intravascular coagulation, abortion, hyperglycemia followed by hypoglycemia, hypo-ferremia, interferon production and Sanarelli-Schwartzmann reactions (Milner, Rudback and Ribi, 1971). Some of these LPS effects, such as fever, hypotension, abortion, and Schwartzmann reactions, are felt to be mediated by an LPS-induced activation of macrophages and their sub-sequent release of prostaglandins (Schade and Reitschel, 1982).

Sensitivity to LPS and its various effects differ widely among species, strains, and even individuals (Milner et al., 1971). Differences in sensitivity among species can be as great as 100,000 fold

and even individuals among a highly inbred group of animals can show a several fold difference in susceptibility to LPS (Milner et al., 1971). Certain primates such as baboons and vervets are very insensitive to LPS, mice, rats, and guinea pigs have medium sensitivity, while rabbits and dogs are highly sensitive (Luderitz et al., 1982). One interesting anomaly in the effects of LPS is that rats and mice exhibit a temperature depression rather than the fever, which is seen in all other species after the infusion of LPS (Milner et al., 1971). Of all animals studied though, humans are the most sensitive to LPS with fever being induced from the administration of as little as 0.1 microgram LPS (Wolff, 1973). This extreme sensitivity to LPS pyrogenicity, which may be due to the fact that man's reticulo-endothelial system phagocytic ability is quite low as compared to animals, severely limits studies into other biological effects of LPS. Only an increase in leukocytosis and plasma cortisol levels can be noted in humans at these low dosage levels (Wolff, 1973).

The organ that shows the greatest effects from LPS is the one primarily responsible for detoxification, the liver. Within one hr of the infusion of LPS in mice, liver glycogen is markedly depleted, hepatocytes contain vacuoles and mitochondrial degeneration has occurred (Levy, Slusser and Ruebner, 1968). Diffuse small foci of necrosis developed within 4 hr. LPS also causes activated lymphocytes to invade the liver. In fact, a four-fold increase in their numbers was noted in the livers of rabbits at 24 hr post dosing (Likovsky and Konickova, 1977). At least some of the hepatotoxic effects of LPS are probably due to a direct action as binding sites for LPS have been demonstrated on

the plasma membranes of rabbit hepatocytes (Ramadori, Hopf and Meyer, 1979) and metabolites of LPS have been shown in the bile of rats after infusion with the compound (Miatra et al., 1981). On the other hand, the glycogenic effects of LPS on the liver is probably a mediated one, since in vitro exposure of isolated rat hepatocytes to LPS showed no effects on glycogenolysis (Filkins and Buchanan, 1977). Hepatic calcium levels are also affected by LPS with a significant increase being shown within 4 hr by LPS infusion into mice (Fritz and Keppler, 1982). Another effect of LPS on the liver is to cause a decrease in activity of the mitochondrial enzyme systems, which has been shown to be direct effect of LPS through the use of primary liver cell cultures (McGivney and Bradley, 1979). In vivo work has also shown an LPS induced drop in the drug metabolizing ability of the hepatic microsomal P-450 mixed function oxidase enzymes (Mannering et al., 1980).

## LPS Involvement in Dietary-Induced Cirrhosis

Luckey, Reynicrs and Gyorgy (1954) found that rats fed the necrogenic diet of Himsworth, which is lacking in tocopherol and utilizes yeast as the sole source of protein, died of massive liver necrosis in only 35 days, while germ-free animals on the same diet exhibited no signs of hepatic damage. Choline deficient diets willalso cause hepatic necrosis in rats, but this action was shown to be prevented by the chronic administration of the nonabsorbed antibiotic, neomycin, although the animals still developed fatty livers (Gyrogy, 1954; Rutenberg et al., 1957). It was further demonstrated that including exogenous LPS in the drinking water containing neomycin

caused the return of the hepatic necrosis normally seen in unprotected animals on the choline-deficient diet (Broitman, Gotllieb and Zamcheck, 1964). These studies combined with evidence of the choline deficient diet increasing susceptibility in rats to hepatotoxicity from normally innocuous doses of injected LPS (Nolan and Vilayat Ali, 1968), strongly indicate the involvement of LPS derived from intestinal bacteria in the development of dietary induced cirrhosis.

## Role of LPS in Direct Hepatotoxicity

$CCl_4$ is a well known hepatotoxin. It was first shown over 40 years ago that orally administered sulfonamide drugs protect against $CCl_4$-induced liver necrosis and death in rats (Leach and Forbes, 1941). It was then found that the less absorbed the antibiotic, the greater the protection from $CCl_4$ poisoning (Wilson, Ludec and Arnold, 1950). Again, as in the choline-deficient diets, hepatic necrosis was prevented but not fatty vacuolization within the hepatocytes. The antibiotic, polymyxin B, which has been shownto physically disrupt the structure of LPS by electron microscopy (Lopes and Inniss, 1969), also protected rats from $CCl_4$ induced hepatic necrosis after intraperitoneal injections 16 hr and 2 hr prior to $CCl_4$ dosing (Nolan and Leibowitz, 1978). In the same study, gentamicin sulfate, which has an anti-bacterial spectrum similar to Polymyxin B, but not the anti-LPS action, did not protect against hepatic injury. Tolerance to LPS, induced by a series of daily injections of steadily increasing amounts of LPS, has also been found to decrease the hepatotoxicity of $CCl_4$ in rats (Nolan and Ali, 1973). Another treatment that has exhibited a protective action

against $CCl_4$ is the feeding to rats of the anion exchange resin, cholestyramine, for several days prior to dosing with $CCl_4$ (Bioulac et al., 1981). The resin has been shown by the uptake of radiolabeled LPS to bind very strongly to the bacterial moiety. Pretreatment of LPS solutions with cholestyramine effectively reduces their subsequent toxicity in mice (Nolan et al., 1975).

The hepatotoxin that has been the most intimately linked with LPS in its mechanism of action is galactosamine. Upon metabolism in liver, galactosamine causes a biochemical injury to hepatocytes through the loss of uracil nucleotides (Faber and El Mofty, 1975). Liehr et al. (1978) have accrued evidence to postulate the following steps that then lead to liver cell necrosis. The galactosamine causes the degranulation of mast cells in the peritoneum and intestine causing a histaminemia that induces an edema of the colonic wall allowing increased LPS absorption. The LPS is then absorbed in sufficient amounts to decrease the clearing ability of the Kupffer cells and systemic endotoxemia results. The systemic LPS activates the complement system and the activated complement subsequently binds to the damaged hepatocytes causing lysis of the cells. These steps were well proven through the use of colectomized rats and genetically complement-deficient mice, neither of which developed necrosis from galactosamine, while both exhibited the biochemical defect.

## Lactulose as an Antiendotoxin

Lactulose, 4-O- -D-galactopyranosyl-D-fructrofuranose, is a nontoxic synthetic disaccharide, which when taken orally is poorly

absorbed and leads to an alteration of the colonic flora by causing a decrease in stool pH through release of the acidic by-products lactic acid, acetic acid and formic acid from bacteria metabolism of the molecule (Merrell, 1979). When a 10% solution is used as drinking water for 8 days prior to galactosamine dosing, lactulose virtually prevents the hepatic necrosis in the rat galactosamine model (Liehr et al., 1980). Continuous intravenous infusion of a dilute solution of lactulose after administration of galactosamine also prevents hepatic necrosis (Liehr and Heine, 1981). This effect, combined with *in vitro* data in the same report that showed that lactulose will inhibit the formation of LPS-induced clotting in the LAL assay, provides evidence that lactulose has a direct antiendotoxin effect as well as its ability to alter gut flora by colonic pH reductions when administered via a parental route. Clinically, lactulose has long been accepted as an agent to reduce the gut-derived bacterial by-products that lead to the hepatic failure complication known as portal-systemic encephalopathy (Bircher et al., 1966) and has also recently been shown to effectively reduce endotoxemia that was detected in a group of patients suffering from acute viral hepatitis (Magliulo et al., 1981).

## The Role of LPS in Clinical Complications of Hepatic Failure

Studies suggest that in cases of severe liver dysfunction, such as alcoholic cirrhosis, crypto-genetic cirrhosis, and viral hepatitis, intrahepatic venous shunts occur so that portal blood, containing LPS absorbed from the intestine, is not exposed to Kupffer cells and thus not cleansed of these toxins (Liehr and Grun, 1979). It follows that

LPS can then reach the systemic circulation where its presence has been correlated with a variety of the complications of hepatic failure such as fever (Nolan and Ali, 1974), intravascular coagulation and the hepato-renal syndrome (Wilkinson et al., 1974). High titers of antibodies to intestinal microbes have been found in persons suffering liver diseases, suggesting that bacterial antigens not sequestered by the diseased liver contribute to the often observed complication, hyperglobulinemia (Triger, Alp and Wright, 1972). It has also been observed that the vascular tone and cardiac output in sick cirrhotics resembles that seen in patients with septic shock, implicating endotoxemia in the vascular abnormalities of liver disease (Siegel, Greenspan and Cohen, 1968).

Unfortunately, even though there are many published reports that endotoxemia occurs in hepatic failure (Caridis et al., 1972; Nolan and Ali, 1974; Wilkinson et al., 1974; Prytz et al., 1976; Magliulo et al., 1981), others have reported finding either no evidence of endotoxemia in cirrhotic patients (Fulenwider et al., 1980), or no correlation between shock and death from gram negative bactermia and a positive result in an assay for LPS (Stumacher et al., 1973). These discrepancies are probably due to the assay for LPS involved in the studies; the LAL assay with its previously mentioned concomitant problems in detecting LPS in vivo.

## SPECIFIC AIMS

Halothane is a widely used operating room inhalation anesthetic that has been implicated in post-anesthesia hepatic damage which can progress to a fulminant hepatitis that often results in death. One hypothesis is that the binding of reactive metabolites of halothane to subcellular macromolecules causes transient minor damage, while the production of antibodies to the halothane-altered hepatocyte membranes leads to the fulminant form of the disease (Sipes et al., 1981; Davis et al., 1980). A model to consistently produce hepatic damage in rats has been developed by inducing their hepatic microsomal enzymes with phenobarbital and then exposing the rats to 1% halothane under reduced oxygen tension (14% $O_2$) for 2 hr.

Endotoxins, lipopolysaccharides (LPS), originating from the cell walls of the gram-negative bacterial flora of the lower intestine, are potentially systemic toxins. The reticulo-endothelial system of the liver is the major means of detoxification of LPS arriving from the intestines via the portal vein. LPS have been shown to be intrinsically involved in the acute hepatotoxic actions of $CCl_4$ and galactosamine in rats.

Thus, the role of LPS in anesthetic-associated liver injury in rats was tested by:

1. Determining if exogenously introduced LPS would potentiate the rat halothane-hypoxia model.

2. Determining if the antiendotoxin agent lactulose could alter the degree of damage produced in the halothane-hypoxia model.
3. Demonstrating a possible mechanism of the potentiation caused by LPS in the halothane-hypoxia model by measuring the phagocytic ability of the reticuloendothelial system after halothane anesthesia and by measuring the influx of calcium ion into the livers of rats after treatment with LPS, halothane, or both.

## MATERIALS AND METHODS

### Chemicals

Halothane (Fluthane) and enflurane (Ethrane) were obtained from Ayerst Laboratories (New York, NY) and Ohio Medical Products (Madison, WI). Reference 1% halothane and 2% enflurane precision gas mixtures diluted in nitrogen were purchased from Matheson Gases (Cucamonga, CA). Nitrogen and oxygen were supplied by Liquid Air Inc. (Tucson, AZ). Sodium phenobarbital was purchased from the Mallinkrodt Company (Paris, NY). Albumin protein standard stock solution, lipolysaccharide; E. coli 026-B6 trichloroacetic acid extracted, lot # 129C-0512 and KE. coli 026-B6 phenol-water extracted, lot # 111F-4023, and serum glutamate-pyruvate transaminase kits were all obtained from Sigma Chemical Company (St. Louis, MO). Lactulose was obtained as the commercial preparation of 66% lactulose syrup, Chronulac, from Merrell Dow Pharmaceuticals (Cincinnati, OH). Shellac-free Pelikan Fount India Ink (Pelikan AG, Hanover, W. Germany) was suspended in Knox Unflavored Gelatin Englewood Cliffs, NJ). Calcium standards were prepared from reagent grade calcium chloride supplied by the J. T. Baker Chemical Company (Phillipsburg, NJ). Lanthanum oxide was obtained from the Eastman Kodak Company (Rochester, NY). Reagent grade nitric and hydrochloric acids were both purchased from the J. T. Baker Chemical Company (Phillipsburg, NJ). Hydrogen peroxide (30%) was obtained from Matheson Coleman and Bell (Norwood, OH). Sodium heparin, Panheparin, Abbott Laboratories (North Chicago, IL), 1000 units/ml, was

the anticoagulant of choice. All buffers were made using reagent grade or better chemicals and deionized reverse-osmosis water. Double distilled water was kindly provided by the Department of Surgical Biology, Arizona Health Science Center.

## Animals

Male, Sprague-Dawley rats (100-200 gm) were obtained from either Hilltop Laboratories (Hilltop, PA) or from the Division of Animal Resources of the Arizona Health Sciences Center as F-1 offspring of Hilltop parents. The rats were housed in pairs in stainless steel cages in an isolated room with an independent air supply. A 12-hr light/dark cycle and a temperature of 22°C was maintained. The rats were allowed standard laboratory rat chow and water ad libitum. They were then utilized upon reaching a weight of 250 to 400 gm.

## Animal Induction

The hepatic cytochrome P-450 drug metabolizing enzymes were induced by two meghods. Except for the experiments involving lactulose administration, the rats received a single intraperitoneal (IP) injection of 100 mg/kg sodium phenobarbital followed by five days of 1 mg/ml sodium phenobarbital in their drinking water. The rats were then allowed 24 hr on untreated water prior to utilization in experiments. An alternate method of induction was used in the lactulose experiments, due to the necessity of administering lactulose in the drinking water. Thus these animals received IP injections of 80 mg/kg sodium phenobarbital for three days with the animals ready for experimentation two

days after the last injection. All fasted animals were removed from food 16 hr prior to further treatment and allowed food ad libitum immediately after treatment.

## Inhalation Exposure Conditions

Animals were exposed to hypoxia (8% $O_2$ or 14% $O_2$ in inspired air), 1% v/v halothane at 14% $O_2$, or 2% v/v enflurane at 14% or 21% $O_2$ in a 180L plexiglass chamber equipped with a circulating fan, inlet and outlet ports, and a sampling port. Gas flow through the chamber was maintained at 6 L/min. An Ohio MOdel 700 Polarographic Oxygen Monitor was used to monitor chamber oxygen levels during exposure to the anesthetics. The anesthetics and gases were delivered by an Airco Vernitrol vaporizing system using compressed gas cylinders of nitrogen and oxygen. Animals were introduced into the chamber after it was brought to exposure conditions. Exposures to anesthetics were for 2 hr during which time anesthetic concentrations were monitored by taking air samples with a Precision Sampling 5cc gas-tight syringe approximately every 20 min. The samples were analyzed on a Varian 1400 gas chromatograph equipped with a 5 ft 10% SE-30 column and a thermal conductivity detector. Matheson compressed gas reference standards of 1% halothane or 2% enflurane were injected at the same time and peak heights used to calculate exposure chamber anesthetic gas concentrations.

## Lipopolysaccharide Administrations

E.coli 026-B6 trichloroacetic acid extracted LPS, Sigma lot # 129C-0512, proven in the thesis to be the more efficacious form was used in all experiments. In the experiment to prove efficacy of

extraction type E. coli 026-B6 phenol-water extracted LPS, Sigma lot # 111F-4023 was also employed. The LPS solutions were made up in 0.9% saline at concentrations to allow a constant dosage volume of 1 ml/kg whether the doses were IP or IV. IV dosings were all done via the tail vein.

## Monitoring of Body Temperature

The alterations in the core temperatures of the rats brought about by IV doses of 0.5 and 1.5 mg/kg LPS were monitored over a hr period during which the rats were restrained in plexiglass holders. This was accomplished using Yellow Springs Instrument Company Series 400 rectal probes attached to a YSI Model 46 TVC Telethermometer.

## Lactulose Administration

The commercial 66% lactulose syrup was diluted with tap water to a 10% solution and used as drinking water for the rats for 7 days prior to their exposure to halothane-hypoxia (Liehr et al., 1980). Daily consumption of the 10% lactulose solution by each pair of rats was monitored by using volumetrically calibrated water bottles.

## Liver Microsomal Membrane Isolation

In order to test the degree of enzyme induction occurring during pretreatment with 10% lactulose water, the drug metabolizing microsomal membranes were obtained from the animals' livers. Rats were induced by the three IP phenobarbital injections (80 mg/kg), while receiving 10% lactulose drinking water or tap water. At termination by cervical dislocation, the livers were immediately perfused via the portal vein

with cold 0.05 M Tris-HCl, 1.15% KCl, pH 7.4 and portions of the livers homogenized in a Dounce hand homogenizer in a 3-1 v/w ratio of cold Tris buffer. To remove cellular debris, the resultant homogenates were centrifuged in a Sorvall RC2-B refrigerated centrifuge at 1,000 x g, 12,000 x g and 27,000 x g for 10 min at each force. The supernatants were then centrifuged at 162,000 x g for 45 min in a Beckman L-5-55 ultracentrifuge in order to obtain the microsomal pellets which were resuspended in cold Tris buffer and frozen at -70°C until analysis.

## Microsomal Protein Content

A portion of each of the frozen microsomal suspensions was thawed and the protein content was determined by the Biuret Method (Gornall, Bardwill and David, 1949). Duplicate 50 and 100 microliter aliquots of each sample were dissolved in 0.1 N NaOH to produce a total volume of 1 ml to which 2 ml of Biuret solution was then added. Sigma albumin stock solution (100 mg/ml) was used to produce a standard curve (Fig. A.1). All samples were measured for absorbance at 540 nm after 30 min on a Gilford Stasar II spectrophotometer using distilled water as a blank. Reagent blanks as well as turbidity blanks for each concentration of microsomes were also measured for absorbance at 540 nm and the values subtracted from those of the microsomal samples.

## Microsomal P-450 Analysis

Frozen microsomal suspensions were thawed on ice and aliquots (1 ml) were diluted to 5 ml with Tris buffer in order to carry out analysis for cytochrome P-450 content by the carbon monoxide difference spectra method of Omura and Sato (1964). A few mg of sodium dithionite

was dissolved in the microsomal mixture. One half of this was used as a reference while the other half was carefully bubbled with carbon monoxide and used in the sample position. These were scanned from 500 nm to 400 nm on a dual-beam Beckman ACTA III Spectrophotometer. This procedure was carried out in triplicate on each sample to assure optimum conditions. Microsomal cytochrome P-450 enzyme levels were then calculated from the following equation:

$$\frac{\text{O.D. @ 450nm x 1000}}{91\text{mM}^{-1} \text{ x [protein]}} = \frac{\text{nmoles P-450}}{\text{mg microsomal protein}}$$

Carbon Clearance by the Reticulo-Ekdothelial System

The reticulo-endothelial systems of phenobarbital induced rats and rats exposed to halothane-hypoxia (HH) were evaluated for their ability to clear small carbon particles from the blood by a modification of the procedure of Biozzi, Benacerrat and Halpern (1953). Shellac-free Pelikan Fount India Ink was centrifuged at 12,000 x g for 15 min to remove the larger carbon particles. Triplicate aliquots (1 ml) of the supernatant were then lyophilized in preweighed tubes in order to estimate the carbon content. The ink was then diluted with water with the addition of sufficient gelatin to produce a colloidal suspension of 40 mg/ml carbon in 2% gelatin. Rats injected with up to 160 mg/kg IV of this solution showed no adverse reactions. After being given several hours to recover from the anesthesia of the HH exposure, the rats were injected with the colloidal carbon suspension (80 mg/kg, IV) via the tail vein. Blood samples were collected from the retro-orbital plexus

using a 20 microliter glass capillary pipets that had been prerinsed with heparin solution (1000 units/ml) and dried. Samples were taken just prior to infusion of the colloidal carbon and then every 5 min for 25 min. The collected samples were dissolved in 2 ml of a hemolyzing solution of 0.1% $Na_2CO_3$ with any remaining clots being disrupted by sonification with a Branson Model 200 Sonifier. The phenobartibal induced rats were treated in a similar manner and alternated with the HH-exposed animals. The disappearance of the carbon from the blood was measured by absorbance of the samples at 600 nm using the preinjection samples as the blank. Since absorbance is directly proportional to concentration, the exponential decrease was plotted as the log (absorbance x 100) vs time. The slopes of the resultant straight lines were used to compare the clearing ability of the reticulo-endothelial system.

## Evaluation of Hepatotoxicity

Following cervical dislocation of the rats, blood was drawn into a heparinized syringe from the inferior vena cava just anterior of the liver. Plasma was obtained by centrifuging the blood at 1000 x g for 10 minutes at room temperature and was stored at $4^{\circ}C$ until glutamate-pyruvate transaminase (GPT) levels expressed in Wroblewski-LaDue (W-L) units, could be measured using Sigma 55-10P kits (Sigma Technical Bulletin No. 55-UV). After the rats were terminated, liver sections (1-2 mm thick) were taken and fixed in a solution of 10% phosphate buffered formalin for preservation until subsequent histological processing. The remaining liver tissues were frozen on dry ice and

stored at -15°C. The formalin fixed liver sections were then processed by the Histology Laboraory of the Division of Animal Resources, Arizona Health Sciences Center, utilizing standard techniques to produce 7 micron thick slides stained with hematoxylin and eosin for the histological assessment of damage. Evaluation of the degree of necrotic damage in each hepatic histological sample was carried out through measuring the inflammatory response by recording the incidence of infiltration of polymorphic neutrophils (PMN) in a 10 x 10 grid at 40X. In each sample, this was done for 6 randomly chosen fields with the average value being reported as the percent incidence of PMN infiltration.

## Hepatic Tissue Calcium Levels

The following method of measuring tissue calcium levels is a modification of the procedure provided by W. Nichols (1982). All glassware used in this assay was soaked in 6N HCl for at least three days prior to use and then rinsed with double distilled water. Two gm of stored liver tissue were homogenized in 4 ml of double distilled water in a Dounce hand homogenizer. Duplicate aliquots (1 ml) of the homogenates were transferred into oven-dried preweighed 30 ml beakers and then frozen and lyophilized for 24 hr after which the beakers were reweighed to determine the dry weight of the homogenized tissue. The dried samples were dissolved in 5 ml of concentrated nitric acid and then oxidized with 30% hydrogen peroxide (5 ml). These dissolved samples were then heated to dryness on a hotplate adjusted to 150° to 200°C with only a few mg of residue remaining in the beakers. At this point, 10 ml of 0.5% lanthanum trichloride in 0.1 N HCl, produced by reacting

lanthanum oxide with concentrated HCl and diluting with double distilled water, was added to each beaker. This solution was transferred after overnight storage to 5 ml plastic snap-cap tubes. Any particulate matter was removed by centrifugation at 1,000 x g for 15 minutes and the supernatant carefully decanted into another 5 ml plastic snap-cap tube. These solutions were analyzed for calcium content on a Perkin-Elmer 305A Atomic Absorption unit using stnadards of 1 to 5 ppm calcium prepared from calcium chloride dissolved in the 0.5% lanthanum chloride solution.

## RESULTS

### LPS-Induced Temperature Changes

The IV administration of LPS has been reported to result in a decrease in body temperature in rats (Milner et al., 1971). In this study, a fall of approximately $1^{o}C$ with the 0.5 mg/kg LPS dose and up to a $2^{o}C$ drop with the 1.5 mg/kg LPS dose was observed (Figure 3). The onset of these temperature decreases generally did not occur until 10 to 20 min post injection and did not reach a maximum until 60 to 80 min after administration. A second, lesser temperature decrease was also noted, occurring between 3 to 4 hr after injection.

### Potentiation of the HH Model with LPS

LPS proved to be a potent potentiator of the hepatic damage caused by the HH model as measured by plasma GPT levels 24 hr post treatment. Whether administered prior to HH (Fig. 4 and 5), immediately post HH (Table 1), by an IP route (Fig. 4), or by an IV route (Fig. 5), LPS administration caused a several fold increase in 24 hr plasma GPT over HH-only treated animals.

### Determination of the Best Dose and Route of Administration of LPS to Potentiate the HH Model

To best determine the dose and route of administration of LPS for potentiation of the HH model, rats received a range of doses of LPS either IP or IV immediately prior to hypoxia (14% $O_2$) or HH. The dosing with LPS just prior to treatment in the exposure chamber was done

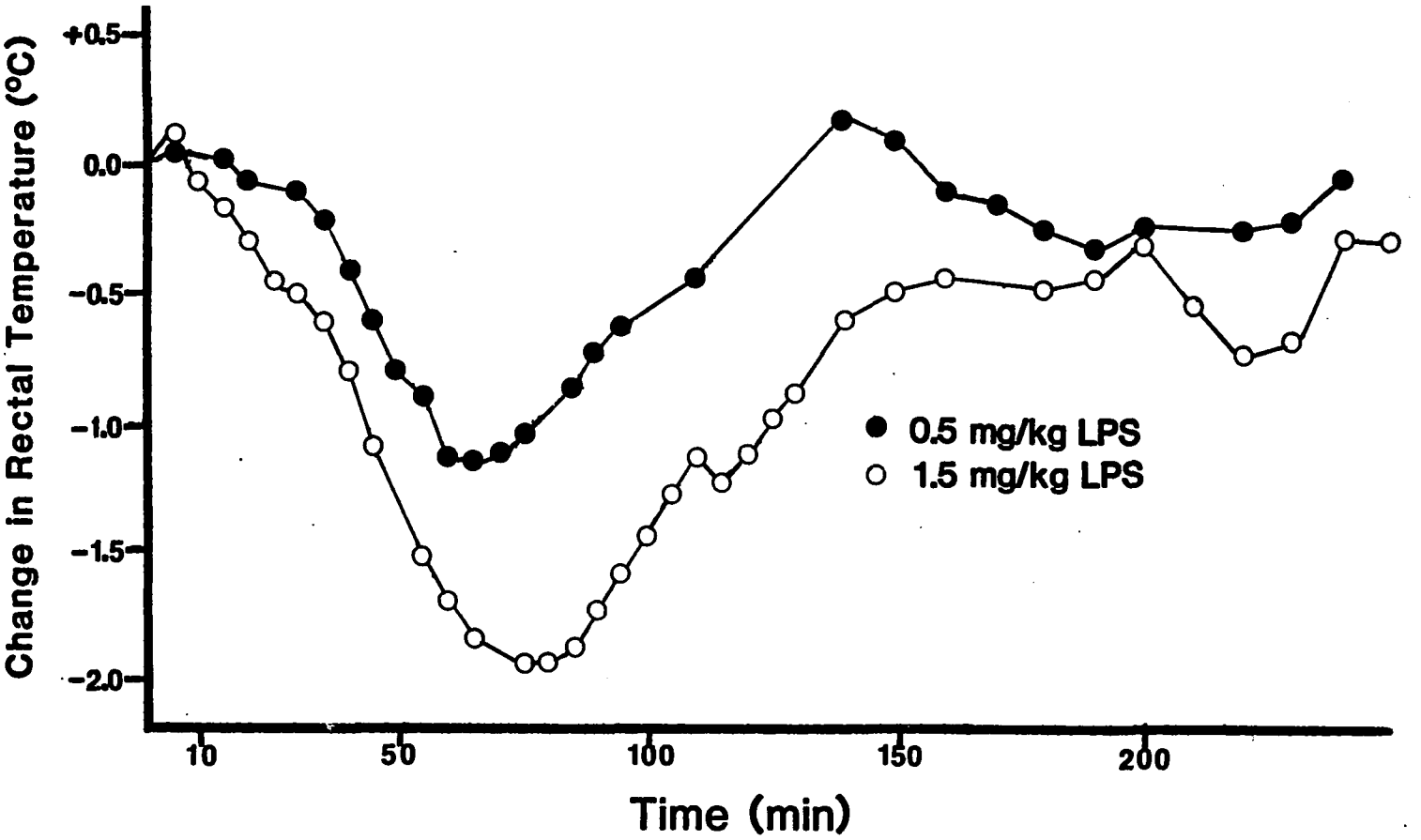


Figure 3. Changes in rectal temperature from the IV infusion of 0.5 mg/kg LPS (●) and 1.5 mg/kg LPS (o). -- Curves are representative from single animals at each dose.

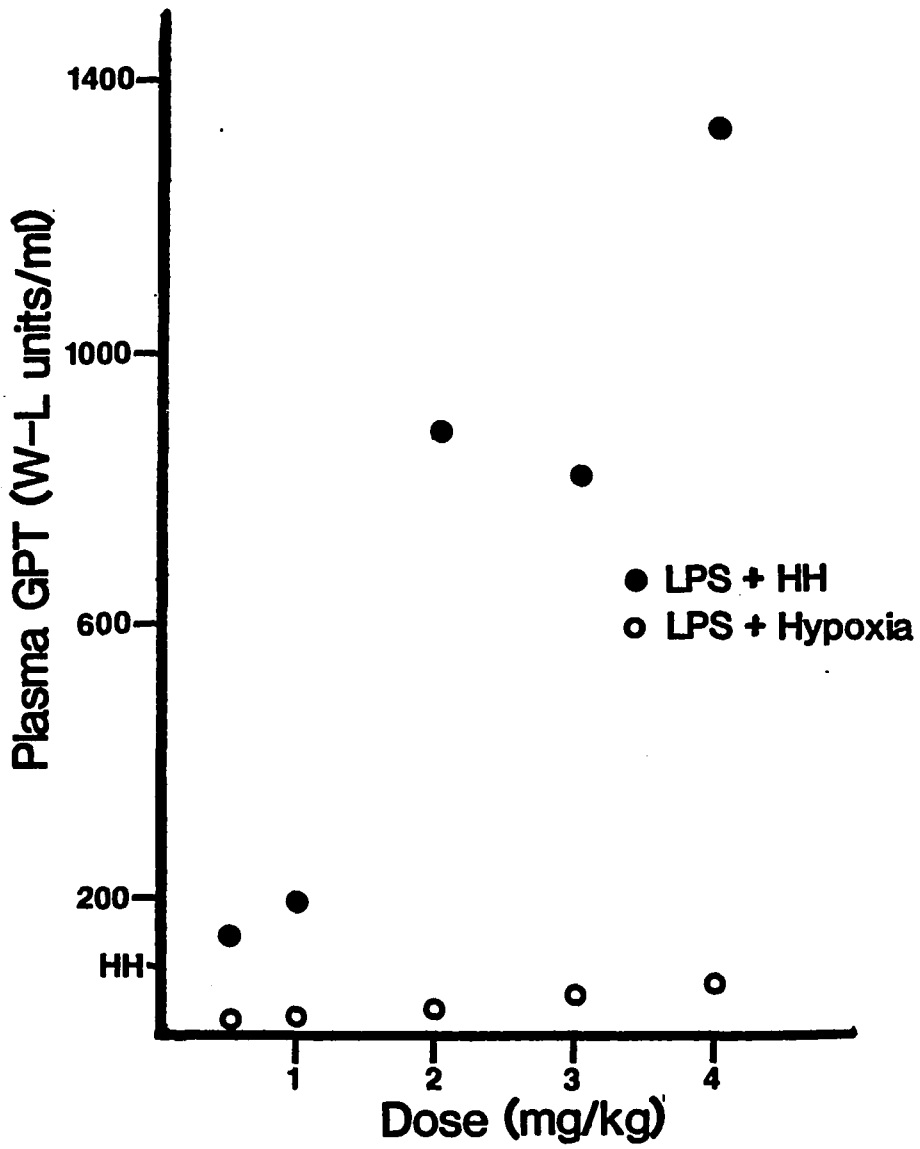


Figure 4. Dose-response potentiation of IP LPS on HH. -- 24 hr plasma GPT levels resulting from the administration of LPS IP immediately prior to hypoxia (14% $O_2$) (o) or HH (•). Error bars not shown for clarity (n = 3).

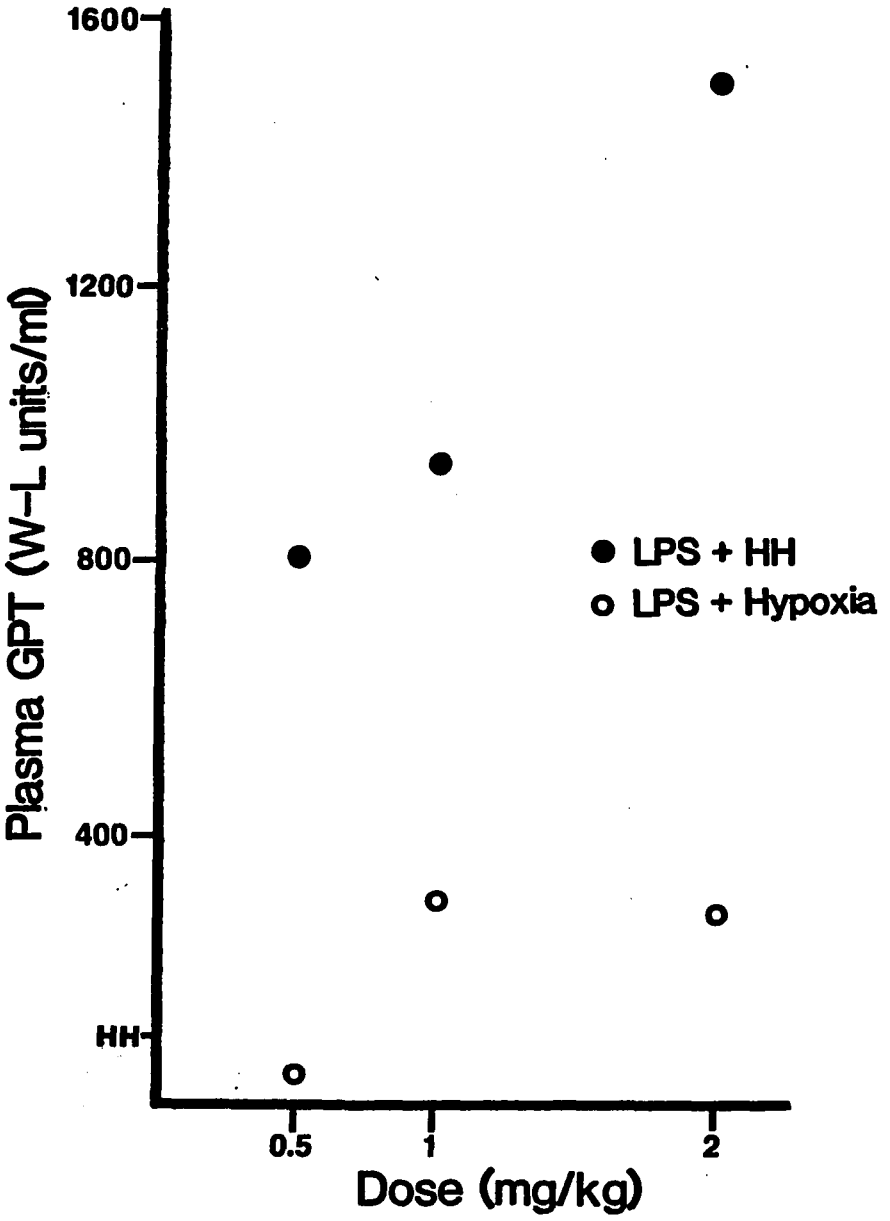


Figure 5. Dose-response potentiation of IV LPS on HH. -- 24 hr plasma GPT levels resulting from the administration of LPS IV immediately prior to hypoxia (14% $O_2$) (o) or HH (●). Error bars not shown for clarity (n = 3).

Table 1. Effect on 24 hr plasma GPT levels from the intravenous infusion of TCA or phenolic LPS immediately post hypoxia or HH.

| Treatment | n | GPT[a] (W-L units/ml) |
|---|---|---|
| HH[b] + TCA LPS[c] | 4 | 998±532[+] |
| HH + Phenolic PLS | 6 | 321±229[+] |
| Hypoxia[d] + Phenolic LPS | 5 | 20±4 |
| HH | 21 | 100±66 |

[+] $p < 0.02$

[a] $\bar{x} \pm$ S.D.

[b] 1% halothane, 14% $O_2$, 2 h4

[c] 0.5 mg/kg, IV

[d] 14% $O_2$, 2 hr

due to the reported LPS potentiation of the galactosamine model of hepatic necrosis in rats in which LPS was administered at the same time as the galactosamine (Liehr et al., 1978). When administered IP and followed by hypoxia, LPS caused a rise in 24 hr plasma GPT only in doses of 3 mg/kg or greater (Fig. 4), while the IV route led to elevated 24 hr plasma GPT levels at doses of only 1 mg/kg (Fig. 5). Either route of administration proved to be effective in potentiating the hepatic damage in the HH model as measured by 24 hr plasma GPT values (Fig. 4 and 5). Due to its fairly consistent potentiation of the HH model, while showing little or no damage in hypoxia controls, the 0.5 mg/kg LPS IV dose was chosen for future experiments. Since LPS is reported to decrease P-450 metabolism (Egawa and Kasai, 1979), and the interest was in seeing LPS effects after the halothane-induced hepatic injury had occurred, LPS was subsequently administered immediately after hypoxia or HH.

## Toxicity of TCA vs Phenol-Extracted LPS

Two preparations of LPS, resulting from different methods of extraction, are commercially available; TCA and phenolic. Neither is pure LPS, the TCA extract often being contaminated with bacterial proteins, lipoproteins and phospholipids, while the phenolic LPS can have high amounts of nucleic acid contamination. Most often in literature no mention is made of the type LPS used, and when it is, it is the TCA-extracted type. Therefore, the efficacy of each of these types of LPS in potentiating the HH model was tested. The rise in 24 hr plasma GPT, resulting from the IV infusion of TCA-extracted LPS

(0.5 mg/kg) into rats immediately post HH, was three times greater than that caused by the same dose of the phenolic form of LPS (Table 1). Thus the TCA-extracted LPS is a much stronger potentiator of the hepatic damage caused by the HH model. On this basis, the TCA-extracted LPS was the type used in all experiments reported herein.

Time Course of LPS Induced Potentiation of the HH Model

The plasma GPT levels were measured and liver histologies were evaluated for rats receiving 0.5 mg/kg LPS immediately post hypoxia (14% $O_2$) or HH up to 72 hr post treatment. In the hypoxia + LPS treated animals both plasma GPT and percent PMN infiltration into the liver began to rise 2 hr post treatment, peaked by 12 hr post and then returned to near normal values by 48 hr post treatment (Fig. 6). The HH + LPS rats had elevated hepatic percent PMN infiltration values by 2 hr post treatment which continued a steady rise until 48 hr post and then began a rapid decline towards normal values. By 48 hr most areas of coagulative necrosis that had occurred appeared "old," in that the coagulated areas stained more darkly and phagocytosis was fairly well progressed, but in approximately 30% of the samples newly occurring extensive areas of "fresh" coagulative necrosis was noted. No such areas were seen in the 72 hr post samples, although the low N value of 4 at this time point may have caused the phenomenon to be easily missed. The plasma GPT values for the HH + LPS treated rats began a very rapid rise after the 2 hr post-treatment time point, peaking at 12 hr post and remaining elevated until 48 hr after which the values fell rapidly towards normal. Although large, the drop in the plasma GPT

Figure 6. Time course of plasma GPT (——) and hepatic percent PMN infiltration (---) in hypoxia (14% $O_2$) exposed (o) and HH treated (●) rats after the intravenous infusion of LPS (0.5 mg/kg) immediately post exposure. -- Error bars now shown for clarity. N = 4 unless otherwise indicated ( ). ★ plasma GPT values statistically significant ($p < 0.005$) from hypoxia exposed animals. 24 hr HH + LPS plasma GPT values not significantly different from 12 and 48 hr.

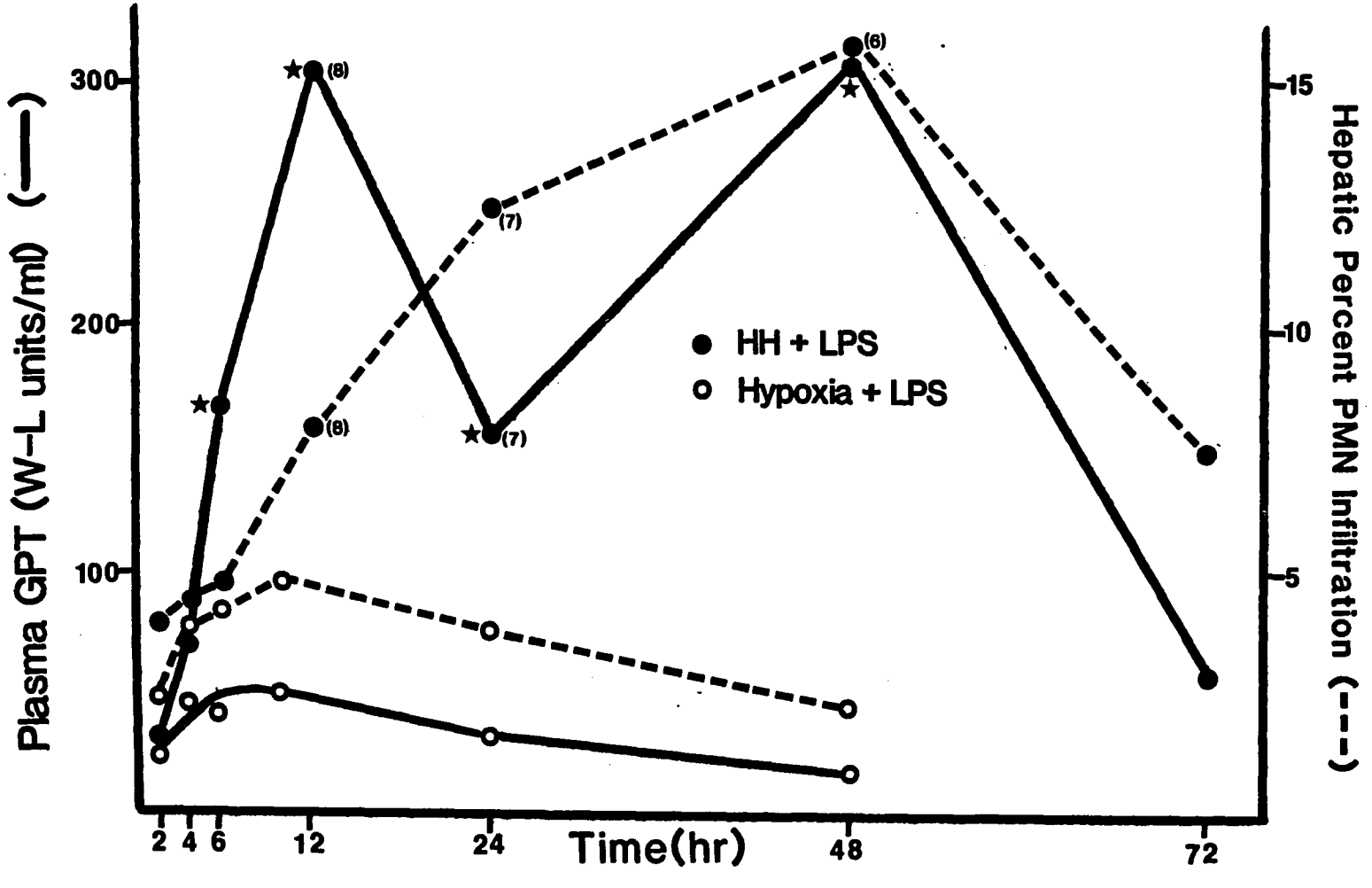


Figure 6. Time course of plasma GPT (—) and hepatic percent PMN infiltration (---) in hypoxia (14% $O_2$) exposed (o) and HH treated (•) rats after the intravenous infusion of LPS (0.5 mg/kg) immediately post exposure.

values at 24 hr did not prove significantly different from the 12 and 48 hr levels due to the sizeable standard deviations at each time point.

## Time Course for the Potentiation of HH by LPS

The ability of LPS to potentiate the hepatic damage caused by HH at various times post anesthetic exposure was evaluated by administering LPS up to 72 hr post HH. The animals were all killed 24 hr post LPS administration with the HH-only animals being killed at 48 hr post HH. Potentiation, as measured by plasma GPT levels, occurred up to the 48 hr post HH + LPS injection time point with means of the GPT levels at the 12, 24, and 48 hr injection time points being approximately double those of the HH-only group at 48 hr (Table 2). All of the values for LPS injection times proved significantly different ($p < 0.025$) from the HH-only plasma GPT except for the 12 hr post time point.

## Effect of Lactulose on HH-Induced Hepatic Damage

Since lactulose has been shown to possess antiendotoxin activity and protective effects against hepatic necrosis caused by galactosamine (Liehr et al., 1980), the ability of it to protect against halothane-associated liver injury was tested.

The consumption of 10% lactulose drinking water over a 7 day period by the rats exposed to HH was at an average rate of 6.3±0.3 gm lactulose/kg/day, while the three lactulose treated rats used as sources of liver microsomes had an intake of 6.0±0.4 gm lactulose/kg/day. Because the lactulose was in the drinking water, hepatic enzymes were induced by three daily injections of 80 mg/kg sodium phenobarbital, starting 4 days prior to exposure. Three rats that received no

Table 2. Effect on 24 hr plasma GPT levels from the intravenous infusion of LPS at various times post HH.

| Treatment | Time[a] (hr) | n | GPT[b] (W-L units/ml) |
|---|---|---|---|
| HH[c] | 48[d] | 14 | 94±72 |
| HH+LPS[e] | 0 | 4 | 272±312[+] |
| | 12 | 4 | 186±192 |
| | 24 | 4 | 192±111[+] |
| | 48 | 4 | 196±139[+] |
| | 72 | 4 | 24±12[+] |

[+] $p < 0.025$ vs HH

[a] time post HH for injection of LPS, sacrifices 24 hr post injection

[b] $\bar{x} \pm$ S.D.

[c] 1% halothane, 14% $O_2$, 24 hr

[d] sacrificed 48 hr post HH

[e] 0.5 mg/kg, IV

lactulose and the three lactulose-treated rats that were used as sources of microsomes were terminated on the same day as the HH exposure. Their hepatic microsomes were isolated and hepatic cytochrome P-450 enzyme levels measured as outlined in the Methods. All other animals were terminated 24 hr post exposure.

The animals treated with lactulose and phenobarbital and the group treated with phenobarbital only exhibited similar levels of hepatic microsomal cytochrome P-450 enzymes (1.05±0.35 nanomoles cytochrome P-450/mg microsomal protein), thus demonstrating that lactulose administration did not affect enzyme induction.

Lactulose administration for 7 days prior to halothane exposure proved an effective means of decreasing the hepatic damage produced by halothane in the hypoxia model. There was a three-fold decrease in the 24 hr plasma GPT levels and a 50% reduction in PMN infiltration into hepatic tissue at 24 hr as compared to the HH-only values (Table 3).

Hepatic Calcium Levels after Treatment with LPS, HH and HH+LPS

Due to the work of Fritz and Keppler (1982), which demonstrated increased calcium ion influx into the hepatocytes of female mice within 4 hr of the administration of 0.3 mg/kg LPS (S. Minnesota R 595), it was decided to evaluate the effect of our treatment regimens on the concentration of hepatic calcium. Livers were chosen at random from animals that were treated with phenobarbital only; with phenobarbital, hypoxia and LPS (0.5 mg/kg, IV); with phenobarbital and HH; and with phenobarbital, HH and LPS. All samples were 24 hr post final treatment, except for the phenobarbital-only livers which came from animals that

Table 3. Prevention of HH-induced hepatic damage by lactulose pretreatment.

| Treatment | n | GPT[a] (W-L units/ml) | Percent PMN Infiltration |
|---|---|---|---|
| Hypoxia[a] | 6 | 22±4 | 0.5 |
| Hypoxia + LPS[c] | 10 | 30±9 | 2.9 |
| HH[d] | 21 | 100±66 | 9.8 |
| HH + LPS | 9 | 419±300[+] | 19.0[+] |
| Lactulose[e] + HH | 8 | 31±17[+] | 4.4[+] |

[+] $p < 0.01$ vs HH

[a] $\bar{x}$ ± SD, 24 hr post treatment

[b] 14% $O_2$, 2 hr

[c] 0.5 mg/kg IV

[d] 1% halothane, 14% $O_2$, 2 hr

[e] 10% in drinking water, 7 days prior to HH

were terminated 48 hr after removal of the phenobarbital. Thus these animals were killed at a time point equivalent to other treatment groups' removal from phenobarbital.

Significant increases in hepatic calcium levels above the phenobarbital-only control samples were noted in each treatment group with HH causing a rise of 14%; a subnecrotic (GPT = 32±11) dose of LPS, 30%; and HH+LPS, 63% (Table 4). It would appear that the affect of the two treatments, HH and LPS infusion, are additive in respect to the increases in hepatic calcium levels while being synergistic in raising plamsa GPT (Table 4). No correlation was noted between the individual hepatic calcium levels and plasma GPT values in any of the groups.

## Carbon Clearance by the Reticulo-Endothelial System

Halothane has been reported to inhibit human leukocyte chemotaxis *in vitro* by Moudgil, Allan and Russell (1977) and *in vivo* by Hill, Stanley and Lunn (1977). Both a reduction in human leukocyte phagocytic ability and bacterial killing have been induced *in vitro* with halothane by Cullen (1974) and Welch (1981), respectively, although Nunn, Sturrock and Jones (1979) reported no effect from halothane *in vitro* on human leukocyte phagocytosis. To help evaluate the possibility that decreased RES function from HH exposure may be the mechanism behind the demonstrated LPS potentiation, the phagocytic ability of the Kupffer cells which comprise approximately 80 to 90% of the RES was evaluated *in vivo*, after HH. This was accomplished by measuring the disappearance of colloidal carbon from the blood using a modification of the method of Biozzi et al. (1953).

Table 4. Effect of LPS on liver calcium levels following HH.

| Treatment | n | Liver Calcium[a] (ppm) | GPT[a] (W-L units/ml) |
|---|---|---|---|
| Phenobarbital[b] | 4 | 139±7[+] | -- |
| HH[c] | 6 | 159+11[+] | 119±66 |
| Hypoxia + LPS[d] | 6 | 180±13[+] | 32±11 |
| HH + LPS | 6 | 226±47[+] | 414±274 |

[+] all groups differ by $p < 0.01$

[a] $\bar{x} \pm$ SD, 24 hr post treatment

[b] phenobarbital induction, 48 hr post treatment

[c] 1% halothane, 14% $O_2$, 2 hr

[d] 14% $O_2$, 2 hr, 0.5 mg/kg LPS IV, immediately post

Four phenobarbital-induced control rats and four HH treated rats were injected with 80 mg/kg colloidal carbon via the tail vein over a 7 hr period after the HH exposure. Blood samples were collected every 5 min after injection for a 25 min period. The decrease in absorbance of the blood due to the removal of the carbon particles was plotted as log (absorbance x 100) vs time (first-order kinetics). The resulting clearance factors (K), (-slope x 1000), were identical for the two groups including the values obtained for one animal from each group which had part of its dose of colloidal carbon lost subcutaneously (Table 5).

## Enflurane+LPS

Hepatic damage in humans following enflurane anesthesia has been reported in the literature (Danielwitz et al., 1980; Ona, Patanella and Ayub, 1980; Tsang, 1975; Denlinger, Lecky and Nahrwold, 1974). In order to determine whether or not enflurane anesthesia would increase the susceptibility of the liver to damage from LPS, phenobarbital induced rats were exposed to 1.5-2.0% enflurane for 2 hr at 14% or 21% $O_2$ and were injected with 0.5 mg/kg LPS IV immediately post exposure (see Methods). A group of phenobarbital-induced rats received LPS only. The plasma GPT at 24 hr post anesthesia exhibited no difference between the three groups (Table 6). since there was no indication of hepatic damage as per an elevation in plasma enzyme levels, liver samples were not subsequently processed for histological evaluation.

Table 5. Effect of HH on hepatic phagocytic ability.

| Treatment | Animal # | $K^a$ ($min^{-1}$) |
|---|---|---|
| Induction[b] | 1 | 29.7 |
| | 2 | 30.1 |
| | 3 | 24.5 |
| | 4 | 49.1[d] |
| | $\bar{x} \pm$ SD | 33.4±0.8 |
| HH[c] | 1 | 25.0 |
| | 2 | 43.6[d] |
| | 3 | 31.8 |
| | 4 | 29.1 |
| | $\bar{x} \pm$ SD | 32.4±8.0 |

[a] Clearance factor (first order elimination) for colloidal carbon (80 mg/ml)

[b] Phenobarbital induction only

[c] 1% halothane, 14% $O_2$, 2 hr

[d] Part of carbon dose lost subcutaneously

Table 6. Effect of LPS[a] on enflurane hepatotoxicity.

| Treatment | n | GPT[e] (W-L units/ml) |
|---|---|---|
| Hypoxia[b] | 5 | 26±6 |
| Enflurane + Hypoxia[c] | 5 | 28±5 |
| Enflurane + Normoxia[d] | 5 | 25±5 |

[a] 0.5 mg/kg, IV, immediately post treatment

[b] 14% $O_2$, 2 hr

[c] 2% enflurane, 14% $O_2$, 2 hr

[d] 2% enflurane, 21% $O_2$, 2 hr

[e] $\bar{x} \pm$ SD, 24 hr post treatment

## Induction, Fasting, and Severe Hypoxia (8% $O_2$) Plus LPS Administration

The anesthetic animal model reported by Harper et al. (1982a), and Van Dyke (1981), in which phenobarbital induced rats were fasted overnight and then exposed to halothane, enflurane, or isoflurane at 8% $O_2$ for 2 hr, caused massive hepatic necrosis at 24 hr. Previous experience in our laboratory with these exposure conditions had shown some degree of liver toxicity to animals exposed to the hypoxia only; plasma GPT were slightly elevated (43±18 W-L units) at 24 hr (Table 7). This, combined with the fact that the conditions proved fatal to about 30% of the rats treated with the exposure conditions only (no anesthetic), led to an attempt to prove that the conditions caused a compromised liver state that increases the susceptibility to hepatic damage from LPS infusion.

Groups of phenobarbital induced rats were either allowed food or fasted overnight followed by exposure to 8%, 14%, or 21% $O_2$ for 2 hr then injected with 0.5 mg/kg LPS IV. Two groups of noninduced animals were also fed or fasted, exposed to 8% $O_2$ for 2 hr and then injected with LPS. All were killed 24 hr later.

The data summarized in Table 7 clearly show that the animals that were induced, fasted overnight, and exposed to 8% $O_2$ for 2 hr were much more susceptible to hepatic damage from LPS than animals treated with any other condition. The mean 24 hr plasma GPT value is approximately ten times higher in the induced, fasted, 8% $O_2$ + LPS animals as the other fasted groups and the degree of PMN infiltration into the liver over two times as great.

Table 7. The effect of intravenous LPS on 24 hr plasma GPT and hepatic percent PMN infiltration levels after phenobarbital induction, fasting and hypoxia.

| Induction[a] | Fed[b] | Oxygen Tension[c] | LPS[d] | n | GPT[e] (W-L units/ml) | Percent PMN infiltration |
|---|---|---|---|---|---|---|
| + | + | 8% | + | 6 | 40±22 | 5.2 |
| + | - | 8% | + | 5 | 220±144[+] | 11.0[+] |
| + | + | 14% | + | 4 | 23±8 | 1.4 |
| + | - | 14% | + | 5 | 26±9 | 3.3 |
| + | + | 21% | + | 6 | 26±14 | 3.0 |
| + | - | 21% | + | 6 | 24±5 | 4.3 |
| + | - | 8% | - | 7 | 43±18 | - |
| - | + | 8% | + | 5 | 22±6 | 2.8 |
| - | - | 8% | + | 8 | | 4.8 |

[+] $p < 0.01$ vs all other conditions

[a] + = phenobarbital induction, - = noninduced

[b] + = food ad libitum, - = fasted overnight

[c] oxygen condition for 2 hr prior to LPS infusion

[d] 0.5 mg/kg, IV

[e] $\bar{x} \pm SD$

All three conditions, induction, fasting, and 8% $O_2$, appear to contribute to the observed increase in susceptibility to LPS. The role of induction is demonstrated by the fact that toxicity parameters are significantly less for the noninduced, fasted, 8% $O_2$+LPS group than the induced animals at the same conditions. The contributions of fasting to the increased susceptibility to LPS is evident in that fasted animals displayed a higher percent PMN infiltration over fed animals at each oxygen level, although the differences are not significant ($p < 0.10$). The exposure to 8% $O_2$ for 2 hr is also a factor, demonstrated by the highest PMN infiltration and GPT levels for the induced, fed, 8% $O_2$, LPS group over the other oxygen conditions. In this case, only the percent PMN infiltration differences proved significantly different ($p < 0.02$).

# DISCUSSION

Lipolysaccharides (LPS), derived from bacteria residing in the lower intestine, have the potential to play a role in both of the observed forms of halothane-associated liver damage in humans; the transient mild hepatic dysfunction and the fulminant progressive hepatitis. As in the case of many hepatotoxic agents (Nolan, 1981), intravenous infusion of LPS into rats significantly potentiated the acute hepatic necrosis produced by the halothane-hypoxia model. LPS could also be involved in the fulminant, progressive form of halothane hepatitis, contributing to the demise of a victim via its causation of a myriad of hepatic-failure-related systemic complications as has been observed in other forms of severe liver dysfunction such as alcoholic and crypto-genetic cirrhosis and viral hepatitis (Nolan and Ali, 1974; Wilkinson et al., 1974).

In creating the rat model of LPS-induced potentiation of hepatic necrosis caused by halothane, several factors had to be determined: the halothane animal model to use in order to initiate hepatic damage, the dose of LPS, its route of administration and time of injection in relation to the halothane exposure, and the extraction-type of LPS to employ. As outlined in the Introduction, there are several animal models that cause hepatic necrosis in rats, involving different pretreatments and halothane exposure conditions. Due to its lesser severity of conditions to cause liver necrosis and its similarities to the acute hepatic

damage observed in humans after halothane anesthesia, the halothane-hypoxia (HH) model of McLain, Sipes and Brown (1979) was used in these experiments.

Both IP and IV routes of administration of LPS were found to potentiate hepatic necrosis in the HH model as shown by increases in 24 hr post treatment plasma GPT levels. The observed IP administered LPS potentiation of HH demonstrates ready absorption of LPS from the peritoneum, although the 0.5 mg/kg LPS IV regime was decided upon due to the more consistent 24 hr plasma GPT levels produced by the IV route, the lack of significant hepatic damage in hypoxia-exposed animals injected with the 0.5 mg/kg IV dose, an attempt to avoid other side effects of LPS by administering as low a dose as possible and the reported use of the IV route of administration in the LPS-potentiated model of galactosamine-induced hepatic necrosis (Liehr et al., 1978).

Since LPS administration is known to reduce hepatic microsomal cytochrome P-450 enzyme levels (Mannering et al., 1980) and the metabolism of halothane by these enzymes is implicated in its hepatotoxic actions (Sipes et al., 1981), it was decided to inject the LPS immediately after halothane exposure in order to observe its effects on a supposedly already damaged liver.

Of the two commerically available and commonly used forms of LPS, the TCA-extracted type proved much more efficacious in potentiating the HH model, causing an increase of over 3 fold in 24 hr plasma GPT levels as compared to the potentiation caused by the same dose of phenolic-extracted LPS. Thus, a dose of 0.5 mg/kg TCA-extracted LPS IV

immediately post HH was decided upon as the model of LPS-induced potentiation of hepatic damage caused by halothane anesthesia in male Sprague-Dawley rats.

The 0.5 mg/kg IV dose of LPS proved biologically active in that it caused a body temperature drop in the rats of approximately $1^{o}C$ within 60 min, although a higher dose (1.5 mg/kg) led to a greater temperature change ($2^{o}C$). The infusion of 0.5 mg/kg LPS into rats after exposure to 2 hr of hypoxia (14% $O_2$) also caused another physiological response in that a large increase in the numbers of macrophages was observed within the livers at 24 hr post treatment. This is consistent with other reported work which demonstrated a four-fold increase in activated lymphocytes into the livers of LPS treated rabbits at 24 hr (Likovsky and Konickova, 1977). Thus the employed dose of LPS showed a strong but not miximal induction of physiological responses while only creating a slight rise in plasma GPT at 24 hr in animals exposed to hypoxia only.

When the LPS is injected immediately post HH, the plasma GPT are found to be generally increased by two to four fold and the degree of hepatic PMN infiltration by two to three fold as compared to HH controls at 24 hr post exposure. This LPS-created potentiation can be produced by infusing the LPS up to 48 hr post halothane anesthesia but not at 72 hr post. This is compatible with the previously reported time course of HH induced hepatic damage in rats (Jee, Sipes and Brown, 1980), in which their parameters of liver damage peaked by 48 hr with a rapid return towards normal values by 72 hr post HH. This makes sense

in that a liver which is well into a repair phase and returning to normal function would be less susceptible to LPS-induced damage.

The plasma GPT levels in the HH-only model are reported to not begin to rise significantly until 12 hr post HH and peak at 24 to 48 hr post (Jee et al., 1980). In the LPS-potentiated HH model the infusion of LPS immediately after HH led to a rapid rise in plasma GPT beginning after only 2 hr and peaking by 12 hr with these levels remaining elevated until 48 hr post. Since the rise and peak in both plasma GPT and hepatic PMN infiltration in hypoxia+LPS treated animals also occurs in the same 2 to 12 hr time frame, the more rapid rise in plasma GPT observed in the LPS-potentiated HH vs the HH-only time course is probably due to the effects of the LPS or an LPS-mediated mechanism.

Histological evaluation of liver tissue from the HH+LPS treated animals at 48 hr post treatment revealed newly generated hepatic coagulative necrosis amongst areas of older damage in 30% of the animals. This fresh necrosis could be the result of small pockets of ischemia forming due to the disruption of local architecture and subsequent loss of perfusion caused by the initial coagulative lesion. Thus, the possibility is raised of primary acute damage perpetuating itself into a chronic lesion in the HH+LPS model of potentiation, although it is not known whether this occurrence is unique to the HH+LPS lesion or merely the result of the extent of damage regardless of the source.

This potentiation of the animal model of halothane hepatotoxicity by LPS could well apply to a human situation where the liver has been injured during surgery by the abberant metabolism of halothane and thus

made more susceptible to damage by LPS. Any surgical procedure causing an increased absorption of LPS from the lower intestine could conceivably potentiate an otherwise minor hepatic injury. LPS has been implicated in irreversible hemorrhagic shock where the sever hypotension involved has led to a necrosis of the intestinal mucosa allowing increased absorption of LPS (Sanford and Noyes, 1958). The release of histamine by mast cells in the intestine and peritoneum, causing an edema of the colonic wall, has been linked with increased levels of LPS absorption in the chain of events leading to the hepatotoxic action of galactosamine (Liehr et al., 1978). Therefore, a surgical procedure resulting in either hypotension to the lower intestine or the release of histamine in the peritoneum could enhance the absorption of LPS and thus potentiate any hepatic damage that may have been caused by halothane. This is especially significant considering that abdominal surgery and halothane anesthesia have both been shown to reduce hepatic blood flow (Harper et al., 1982b; Ross and Daggy, 1981; Benumof et al., 1976; Gelman, 1976) which could decrease oxygen levels within the liver enhancing the reductive route of metabolism of halothane and consequently the degree of hepatic damage caused by the anesthetic as seen in the HH rat model (Sipes et al., 1981).

Fasting, a common practice prior to and after surgery may well increase both the numbers of bacteria within the intestine and the areas which they inhabit, making greater amounts of LPS available for absorption. This increase in bacteria has been demonstrated in mice fasted for 48 hr which led to greater numbers of coliforms inhabiting

the jejunum-ileum and cecum (Tannock and Savage, 1974). There also exists a possibility that LPS could be involved in the initiation of the production of the observed circulating antibodies to halothane-altered rabbit hepatocytes in human cases of fulminant halothane hepatitis. LPS are implicated in the liver necrosis caused by other hepatotoxins (Nolan, 1981), as well as halothane, as supported by the data contained herein. This necrotic action involves the infiltration of macrophages into the damaged areas followed by the phagocytosis of dead cells. The macrophages could then process halothane-altered macromolecules for subsequent antibody production by lymphocytes as is often the case involving antigenic substances (Walter, 1977). Additional amounts of LPS beyond those normally present increase the degree of necrosis and numbers of macrophages within the liver and thus could cause an increase in the amounts of altered-tissue processed for subsequent antibody production. Halothane-altered macromolecules can also be available to serve as antigens by reaching the general circulation as is supported by the work of Gandolfi et al. (1981) which shows the presence of C14-halothane equivalents bound to high molecular weight phospholipid entities in the urine of rats treated with C14-halothane plus hypoxia.

A subsequent production of antibodies against these halothane-bound liver macromolecular "antigens" could lead to an auto-immune response against the hepatocytes upon the next exposure to halothane that again produced halothane-altered macromolecules on the hepatocyte membrane. The degree of initial damage, a possible potentiation of the

damage by LPS, as well as a person's idiosyncratic tendency to produce autoimmune antibodies could all be factors in the levels of antibody formed. The three pairs of closely related women, reported by Hoft et al. (1981), who developed the fulminant hepatitis after halothane anesthesia and exhibited the circulating hepatocyte membrane antibodies, demonstrate some of the previously mentioned physiologic factors on several levels. All were overweight or markedly obese, which could have led to increased bio-transformation of the halothane (Bentley et al., 1982). Each pair were related as sisters or cousins and were all of Mexican-Indian or Mexican-Spanish ancestry. This raises the possibility of genetic tendency for abberant metabolism of halothane and/or autoimmune antibody formation. The reported six year old girl (Lewis and Blair, 1982) and anesthesiologists (Newberger et al., 1981) who developed a sensitivity to halothane and demonstrated the circulating hepatocyte membrane antibody also, lend further support to the idea of an idiosyncratic tendency to produce autoimmune antibodies.

In the fulminant form of halothane hepatitis, as in the case in other forms of severe liver dysfunction, normally absorbed LPS could escape sequestration by the liver, leading to circulating levels of the toxin (Wilkinson et al., 1974). The resulting endotoxemia would then cause many of the LPS-attributed hepatic failure complications such as fever, hepato-renal syndrome, intra-vascular coagulation and hyper-globulinemia (Nolan, 1981). In this situation a regime of orally administered lactulose, which has been shown to reduce LPS levels in the blood of persons suffering from viral hepatitis (Magliulo et al., 1981) may prove beneficial in managing the LPS-implicated complications. This

treatment may also help prevent further damage to a severely compromised liver that has been rendered susceptible by its diseased state to normally innocuous levels of continually absorbed endogenous LPS.

Lactulose has been shown to be an antiendotoxin both in vivo and in vitro (Liehr et al., 1980). In rats, the hepatotoxic action of galactosamine, has been strongly linked to endogenously absorbed LPS (Liehr et al., 1978). This hepatotoxicity can be virtually eliminated by either a 7 day regime of 10% lactulose drinking water prior to galactosamine administration (Liehr et al., 1980) or by intravenous administration of lactulose after the injection of galactosamine (Liehr and Heine, 1981). In vitro, lactulose has directly inhibited the Limulus amebocyte lysate assay for LPS (Liehr et al., 1980). The protective action afforded from the hepatotoxic effects of HH by a 7 day prior administration of 10% lactulose drinking water indicates a role of endogenous-LPS in the rat HH model of hepatotoxicity. The several fold decrease in both plasma GPT levels and degrees of inflammatory response, as compared to HH controls at 24 hr, is strong evidence of this protective action since lactulose did not affect the levels of induction of hepatic cytochrome P-450. $CCl_4$ is another hepatic damaging organohalogen which has been shown to form free radicals in vivo as has halothane (Plummer et al., 1982). $CCl_4$ has had its hepatotoxic actions in animals inhibited by prior treatment with colonic flora altering nonabsorbed antibiotics (Wilson et al., 1950), LPS-binding cholestyramine anion exchange resin (Bioulac et al., 1981), and the specific antiendotoxin Polymyxin B (Nolan and Leibowitz, 1978). This work lends further

support to the possibility of endogenously derived LPS contributing to the degree of hepatic necrosis caused by the HH model.

On the other hand, enflurane, another halogenated inhalation anesthetic which has been implicated in post surgical hepatic damage in humans (Danilewitz et al., 1980; Ona et al., 1980), has never proven to be hepatotoxic to rats exposed to it under the same experimental conditions as the HH model (Plummer et al., 1982; data unpublished). Even the infusion of LPS after anesthesia with enflurane in either a 14% $O_2$ or 21% $O_2$ atmosphere in phenobarbital induced rats failed to show any sign of a hepatotoxic action by enflurane that would leave the liver more susceptible to LPS-induced necrosis.

Although halothane has been reported to reduce both the phagocytic ability and bacterial killing power of human leukocytes in vitro (Cullen, 1974; Welch, 1981), HH exhibited no effect on the ability of the reticulo-endothelial system to phagocytize carbon particles in vivo in the rat. The Kupffer cells of the liver, which comprise 80 to 90% of the reticulo-endothelial system, are well known to be the primary line of defense against LPS (Nolan, 1981). The fact that HH did not depress the clearing capacity of the Kupffer cells indicates that possible HH-induced laterations in these macrophages' phagocytic ability is not a mechanism in either the demonstrated potentiation of HH-induced hepatic necrosis by LPS or in the initiation of the initial HH lesion where prevention of necrosis with the antiendotoxin lactulose has implicated LPS involvement. Of course, the effect of HH on the ability of the Kupffer cells to detoxify LPS was not investigated as neither was the possibility of an LPS-induced release from KUpffer cells of any locally

toxic factors such as lysozymes, either of which could play a role in the HH or HH+LPS models.

What does shed some light on possible mechanisms of LPS potentiation is its effect on liver calcium levels. A subnecrotoc dose of LPS raised 24 hr hepatic calcium levels by 30%, which is more than twice the 14% increase above controls caused by HH alone. This is especially significant considering that only livers from animals demonstrating hepatic damage from HH, i.e., 24 hr plasma GPT from 45 to 220 W-L units/ml, were analyzed. The opening of ion channels, allowing an influx of calcium into hepatocytes, has been whown to occur within 4 hr of LPS administration (Fritz and Keppler, 1982). Evidence exists that would indicate that either a direct or an indirect action of LPS could cause this alteration in membrane permeability. Supporting a direct action of LPS, is the fact that LPS binding sites have been shown on the surface of rabbit hepatocyte plamsa membranes (Ramadori et al., 1979). The lipid portion of LPS is known to interact with the hydrophobic portion of membranes, altering their lipidic composition organization and decreasing their fluidity (Davies, Stewart-Tull and Jackson, 1978). This has been hypothesized as the mechanism behind the LPS-induced inhibition of some membrane-bound enzyme systems (Utili et al., 1982). It seems reasonable then to suggest that the membrane fluidity alterations caused by LPS could lead to a change in membrane permeability, allowing the observed enhanced influx of calcium ions.

Other work would seem to indicate an indirect mechanism via the release or activation of endogenous substances by LPS in causing

membrane changes. The release of prostaglandins by LPS-activated macrophages is known to mediate many of the physiological effects of LPS, such as fever, lethality, shock, Schwartzmann reactions and abortion (Shade and Rietschel, 1982). In the galactosamine model of hepatic damage, the development of endotoxemia is correlated with the loss from the blood of complement, which is activated by LPS, and the appearance of hepatic necrosis (Liehr et al., 1978). Complement is also shown to accumulate in the areas of liver cell necrosis. In the same report, complement deficient mice did not develop the galactosamine hepatic lesion although they did manifest the hepatocyte uracil nucleotide deficiency and the endotoxemia common to the galactosamine model. Each of these possibilities lends credence to the indirect mechanism of LPS influence on the permeability of hepatocyte membranes.

Perhaps the answer is a ciombination of direct and indirect effects, as seen in LPS-induced lysis of platelets, where it has been shown that LPS binds to the surface of the platelet via its lipid A region and the polysaccharide chain of the LPS provides the surface for the assembly of complement pathway components leading to subsequent platelet lysis (Morrison et al., 1981). Thus, in the situation of infused LPS, the binding of LPS to hepatocyte membranes could provide the sites for the assembly of complement, which the LPS has activated, and subsequently distrub membrane function.

Regardless of whether a direct or indirect mechanism of LPS action is involved, lPS alone induces a sufficient change in membrane permeability that the flow of calcium ions into the hepatocytes is

greater than that observed inlivers damaged by HH. Meanwhile, the plasma GPT in LPS-treated animals rise to only a fraction of the level of those exposed to HH. In animals treated with LPS after HH, a rapid rise in plasma GPT is observed after only 2 hr as compared to the 12 hr time delay before increased plasma GPT is seen in the HH-only model. This more rapid loss of cytosolic enzyme may well be due to a leaky plasma membrane caused by an LPS-induced increase in membrane permeability combined with damage already incurredby HH, leading to the observed augmented 24 hr hepatic calcium and plasma GPT. Thus the hepatocyte membrane becomes so permeable from these combined effects than an enhanced eflux of cytosolic contents and influx of extracellular calcium occurs with the resultant increase in cell death and subsequent necrosis observed in the LPS-potentiated model of HH. Another possibility is that HH damages the hepatocyte's intracellular mechanisms for detoxifying LPS and thus causes the observed potentiation in the HH + LPS model. In this case, the increased calcium ion and plasma GPT changes would be secondary to cell death resulting from internal damage.

LPS may prove useful in demonstrating less obvious altered functional states of the liver. An increased susceptibility to LPS-induced hepatic necrosis was observed in rats resulting from the combination of phenobarbital enzyme induction, fasting, and exposure to 8% $O_2$ for 2 hr. This illustrates a mechanism of severely compromised liver function resulting from experimental conditions contributing to the massive liver damage caused by halothane, isoflurane and enflurane under these conditions as reported by Harper et al. (1982a) and Van Dyke

(1981). The increased susceptibility to hepatic damage by exogenous LPS would also indicate a mechanism of greater sensitivity to endogenous LPS as a factor in the severe hepatic necrosis obtained with various anesthetics under these conditions. These results also present a useful application of LPS in future liver research as an indicator of compromised liver function. A procedure or chemical may well alter liver function to a sufficient extent such that hepatocytes are left highly susceptible to any further insult. Yet no obvious sign of toxicity such as elevated plasma GPT or visible pathological changes would manifest themselves. A resulting increased susceptibility to hepatic damage from normally subnecrotic doses of LPS would be a quick, simple indicator through elevated plasma GPT, of such compromised functional states.

These studies not only show that the hepatic damage induced by HH can be potentiated by subnecrotic doses of exogenous LPS but also imply that endogenous LPS absorbed fromthe lower intestine may be involved in the initial HH-induced necrosis as indicated by the protective action of the antiendotoxin lactulose. The LPS-induced potentiation f the HH model was possible until 48 hr post the anesthetic exposure which is consistent with the reported time course of the HH-induced hepatic lesion which shows a rapid return towards normal serum enzyme levels and liver histology after 48 hr. Although the HH+LPS-potentiated model demonstrated a more rapid rise and ulitmately higher levels of plasma GPT than HH, repair of the hepatic damage seemed well under way by 72 hr as in the HH model.

No damage was noted from the same dose of LPS to the livers of animals exposed to anesthetic concentrations of enflurane in mildly hypoxic and normoxic atmospheres. Rats exposed to the 2 hr of 8% $O_2$ after prior enzyme induction and overnight fasting, which has been shown to increase the hepatotoxicity of halothane and make enflurane and isoflurane hepatotoxic, were found to be much more susceptible to hepatic damage from a normally subnecrotic dose of LPS. This demonstrates not only that the experimental conditions are responsible for the hepatic necrosis caused by the anesthetics in this animal model, but also that LPS can be used to demonstrate otherwise undetected compromised liver function states via increased susceptibility to hepatic damage from LPS.

The mechanism of potentiation of HH by LPS proved not to be due to any HH-induced decrease in the phagocytic ability of the Kupffer cells that normally clear LPS from the blood, but LPS-induced increases in calcium levels within the liver would tend to indicate a cellular membrane alteration caused by LPS as a source of the observed potentiation.

# APPENDIX A

## STANDARD CURVES FOR CHEMICAL ASSAYS

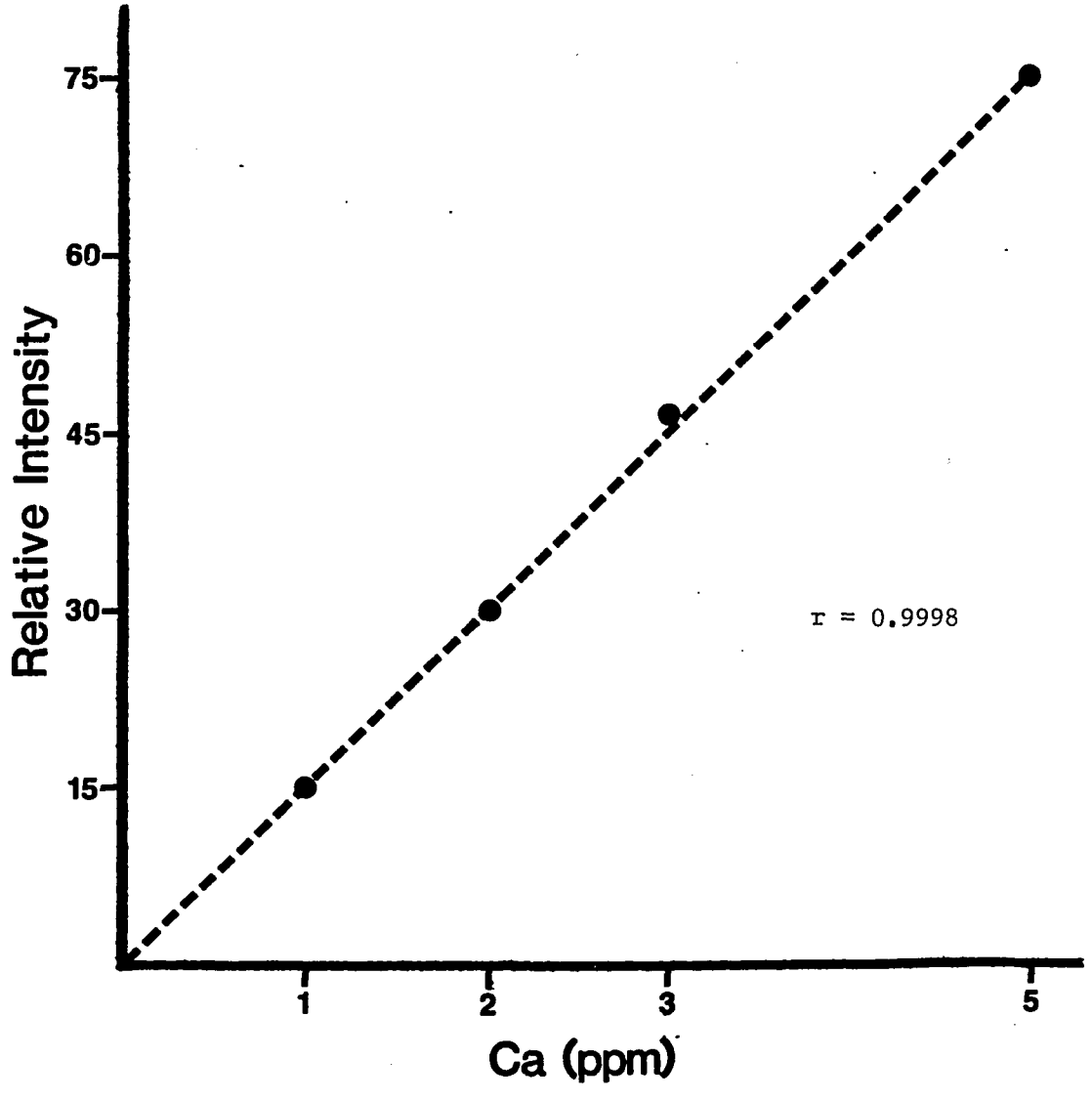


Figure A.1. Standard curve for calcium atomic absorption assay.

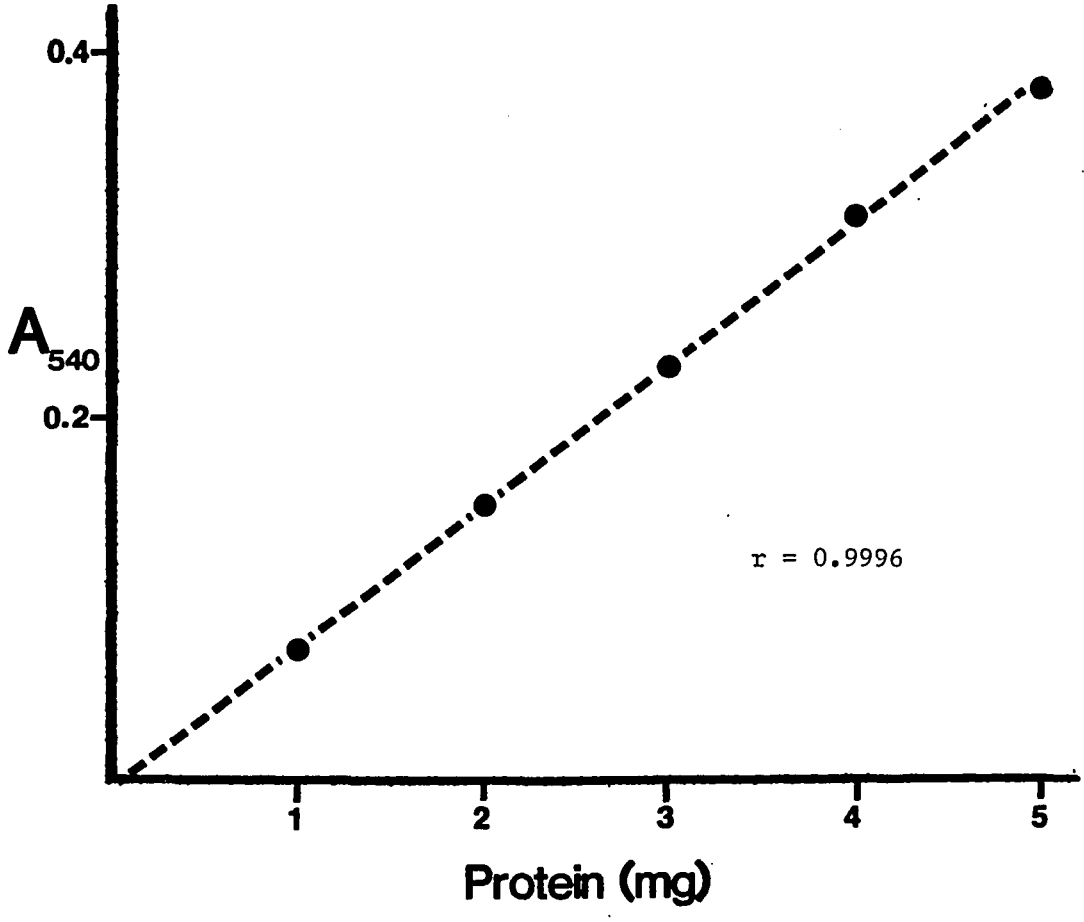


Figure A.2. Standard curve for Biuret protein assay.

## REFERENCES


Alvares, A. P., Bickers, D. R., Kappas, A. (1973): Polychlorinated biphenyls: A new type of inducer of cytochrome P-448 in the liver. Proc. Nat. Acad. Sci. 70:1321.

Ariyoshi, T. and Takabatake, E. (1970): Drug metabolism in ethanol induced fatty liver. Life Sciences 9:361.

Bang, F. B. (1956): A bacterial disease of LImulus polyphemus. Bull. Johns Hopkins Hosp. 98:325.

Bentley, J. B., Vaughan, R. W., Gandolfi, A. J. (1982): Halothane metabolism in obese versus nonobese patients. Anesthesiology 57:132.

Benumof, J., Bookstein, J., Saidman, L., Harris, R. (1976): Diminished hepatic arterial flow during halothane administration. Anesthesiology 45:454.

Berg, J. N., Nausley, C. A., Riegle, L. (1979): Heat extraction of animal plasma in preparation for endotoxin testing with Limulus amebocyte lysate test. Am. J. Vet. Res. 40:1048.

Bioulac, P., Despuyoos, L., Bedin, C., Iron, A., Saric, J. and Balabaud, C. (1981): Decreased acute hepatotoxicity of carbon tetrachloride and bromobenzene by cholestyramine in the rat. Gastroenterol. 81:520.

Biozzi, G., Benacerrat, B., Halpern, B. N. (1953): Quantitative study of the granulopectic activity of the reticuloendothelial system. II. Brit. J. Exp. Path. 34:441.

Bircher, J., Müll34, J., Guggenheim, P., Haemmerli, U. P. (1966): Treatment of chronic portal-systemic encephalopathy with lactulose. Lancet 1:890.

Boivin, A. and Mesrobeanu, L. (1935): Recherches sur les endotoxines des bacteries. Rev. J. Immunol. 1:553.

Bradley, S. G. (1979): Cellular and molecular mechanisms of action of bacterial endotoxins. Ann. Rev. Microbiol. 33:67.

Brody, G. L. and Sweet, R. B. (1963): Halothane anesthesia as a possible cause of massive hepatic necrosis. Anesthesiology 24:29.

Broitman, S. A., Gotllieb, L. S., Zamcheck, N. (1964): Influence of neomycin and ingested endotoxin in the pathogenesis of choline deficiency cirrhosis in the adult rat. J. Exp. Med. 119:633.

Caridis, D. T., Woodruff, P. W. H., Reinhold, R. B., Fine, J. (1972): Endotoxaemia in man. Lancet 1:1381.

Cullen, B. F. (1974): The effect of halothane and nitrous oxide on phagocytosis and human leukocyte metabolism. Anesth. Analg. 53:531.

Danilewitz, M. D., Braude, B. M., Bloch, H. M., Botha, J., Kew, M. C. (1980): Acute hepatitus following enflurane anaesthesia. Br. J. Anaesth. 52:1151.

Davies, M., Stewart-Tull, D. E. S., Jackson, D. M. (1978): The binding of lipopolysaccharide from E. coli to mammalian cel membranes and its effect on liposomes. Biochim. Biophys. Acta 508:260.

Davis, M., Eddleston, A. L. W. F., Neuberger, J. M., Vergani, D., Mieli-Vergani, G., Williams, R. (1980): Halothane hepatitis. N. Engl. J. Med. 303:1123.

Denlinger, J. K., Lecky, J. H., Nahrwold, M. L. (1974): Hepatocellular dysfunctionwithout jaundice after enflurane anesthesia. Anesthesiology 41:86.

Ecker, E. E. (1917): The pathogenic effect and nature of a toxin produced by B. parathyphosus B. J. Infect. Diseases 21:541.

Egawa, K. and Kasai, N. (1979): Endotoxic glycolipid as a potent depressor of the hepatic drug-metabolizing enzyme systems in mice. Microbiol. Immunol. 23:87.

Farber, J. L. and El Mofty, S. (1975): The biochemical pathology of liver cell necrosis. Am. J. Path. 81:237.

Filkins, J. P. and Buchanan, B. J. (1977): In vivo vs in vitro effects of endotoxin on glycogenolysis, gluconeogenesis and glucose utilization. Proc. Soc. Exp. Biol. Med. 155:216.

Fritz, M. M. and Keppler, D. O. R. (1982): LIver calcium and endotoxin actions. Naturwissenschaften 69:147.

Fulenwider, J. T., Sibley, C., Stein, S. F., Evatt, B., Nordinger, B. M., Ivey, G. L. (1980): Endotoxaemia of cirrhosis: An observation not substantiated. Gastroenterol. 73:539.

Gandolfi, A. J., Lind, R. C., Sipes, I. G., Brown, B. R., Pohl, L. R. (1981): Profile of urinary radioactivity following exposure to $^{3}H/^{14}C$ halothane in the hypoxic model. Toxicologist 1:367.

Gelman, S. I. (1976): Disturbance in hepatic blood flow during anesthesia and surgery. Arch. Surg. III:881.

Gornall, A. G., Bardwill, C. J., David, M. M. (1949): Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751-756.

Gourlay, G. K., Adams, J. F., Cousins, M. J., Hall, P.H. (1981): Genetic differences in reductive metabolism and hepatotoxicity of halothane in three rat strinas. Anesthesiology 55:96.

Gourlay, G. K., Adams, J. F., Cousins, M. J. Sharp, J. H. (1980): Time course of formation of volatile reductive metabolites of halothane in humans and an animal model. Br. J. Anaesth. 52:331.

Gyorgy, P. (1954): Antibiotics and liver injury. Ann. N. Y. Acad. Sci. 57:925.

Halothane, Summary of the National Halothane Study. (1966): JAMA 197:775.

Harper, M. H., Collins, P., Johnson, B., Eger, E. I., Biava, C. (1982a): Hepatic injury following halothane, enflurane and isoflurane anesthesia in rats. Anesthesiology 56:14.

Harper, M. H., Collins, P., Johnson, B. H., Eger, E. I., Biava, C. G. (1982b): Postanesthetic hepatic injury in rats; influence of alterations in hepatic blood flow, surgery, and anesthesia time. Anesth. Analg. 61:79.

Hill, G. E., Stanley, T. H., Lunn, J. K. (1977): Neutrophil chemotaxis during halothane and halothena-$N_2O$ anesthesia in man. Anesth. ANalg. 56:696.

Hoft, R. H., Bunker, J. P., Goodman, H. I., Gregory, P. B. (1981): Halothane hepatitis in three pairs of closely related women. New Eng. J. Med. 304:1023.

Hutzinger, O., Safe, S., Zitko, V. (1974): The Chemistry of PCBs. CRC Press, Cleveland. p. 8.

Iwanga, S., Morita, T., Horada, T., Nakamura, S., Niwa, M., Takada, K., Kimura, T., Sakaibara, S. (1978): Chromogenic substrates for horseshoe crab clotting enzyme. Haemostasis 7:183.

Jee, R., Sipes, I. G., Brown, B. R., Jr. (1980): Factors influencing halothane hepatotoxicity in the rat hypoxic model. Tox. Appl. Pharm. 52:267.

Kunin, C. M. (1963): Separation, characterization and biological significance of a common antigen in Enterobacteriaceae. J. Exp. Med. 118:565.

Leach, B. E. and Forbes, J. C. (1941): Sulfonamide drugs as protective agents against carbon tetrachloride poisoning. Proc. Soc. Exp. Biol. Med. 48:361.

Liebowitz, A. I., Vladutiu, A. O., Nolan, J. P. (1979): Quantitation of circulating endotoxin in rats by a solidjphase radioimmuno-assay. Clin. Chem. 25:68.

Levin, J., Poore, T. E., Zauber, N. P., Oser, R. S. (1970): Detection o of endotoxin in the blood of patients with espsis due to gram negative bacteria. N. Eng. J. Med. 283:1313.

Levy, E., Slusser, R. J., Ruebner, B. H. (1968): Hepatic changes produced by a single dose of endotoxin in the mouse. Am. J. Pathol. 52:477.

Lewis, R. B. and Blair, M. (1982): Halothane hepatitis in a young child. Brit. J. Anaesth. 54:349.

Liehr, H., Englisch, G., Rasenack, U. (1980): Lacutose- a drug with antiendotoxin effect. Hepato-gastro-enterol. 27:356.

Liehr, H. and Grun, M. (1979): Endotoxins in liver disease. From Progress in Liver Diseases VI, H. Popper and F. Schaffner, eds., Grune and STratton, Inc., New York, p. 313.

Liehr, H., Grun, M., Seelig, H. P., Seelig, R., Reutter, W., Heine, W. D. (1978): On the pahtogenesis of galactosamine hepatitis: Indications of extrahepatocellular mechanisms responsible for liver cell death. Virchows ARch. B Cell Path. 26:331.

Liehr, H. and Heine, W. D. (1981): Treatment of endotoxemia in galactosamine hepatitis by lactulose administered intravenously. Hepato-gastroenterol. 28:296.

Lopes, J. and Inniss, W. E. (1969): Electron microscopic effect of polymyxin on E. coli lipopolysaccharide. J. Bacteriol. 100:1128.

Luckey, T. D., Reynicrs, J. A., Gyorgy, P. (1954): Germ-free animals and liver necrosis. Ann. N.Y. ACad. Sci. 57:923.

Lüderitz, O., Galanos, C., Rietschel, E. T. (1982): Endotoxins of gram-negative bacteria. Pharmac. Ther. 15:383.

McGivney, A. and Bradley, S. G. (1979): Action of bacterial endotoxin and lipid A on mitochondrial enzyme activities of cells in culture and subcellular fractions. Infect. and Immun. 25:664.

McLain, G. E., Jr., Sipes, I. G., Brown, B. R., Jr. (1979): An animal model of halothane hepatotoxicity: Role of microsomal enzyme induction and hypoxia. Anesthesiology 51:321.

Magliulo, E., Cruciani, M., Dietz, A., Torre, D., Armignacco, O., Bernardi, R., Brumana, N. (1981): Endotoxaemia in acute viral hepatitus. Hepato-gastroenterol. 28:299.

Maiorino, R. M., Gandolfi, A. J., Sipes, I. G. (1980): Gaschromatographic method for halothane metabolites, trifluoroacetic acid and bromide, in biological fluids. J. ANalyt. Toxicol. 4:250.

Maiorino, R. M., Sipes, I. G., Gandolfi, A. J., Brown, B. R., Lind, R. C. (1981): Factors affecting the formation of chlorotrifluoroethane and chlorodifluoroethylene from halothane. Anesthesiology 54:383.

Malkamaki, M. (1981): Antibodies to the enterobacterial common antigen: standardization of the passive hemagglutination test and levels in normal human sera. J. Clin. Microbiol. 13:1074.

Mannering, G. J., Renton, K. W., Azhary, R., Deloria, L. B. (1980): Effects of interferon-inducing agents on hepatic cytochrome P-450 drug metabolizing systems. Ann. N.Y. Acad. Sci. 350:314.

May, J. E., Kane, M. A., Frank, M. M. (1972): Host defense against bacterial endotoxemia-contribution of the early and late components of complement to detoxification. J. Immunol. 109:803.

Merrell Dow Pharmaceuticals, Inc. (1979): Chronulac (lactulose) syrup. From the product information insert. The Dow Chemical Company, Cincinnati, Ohio.

Miatra, S. K., Rachmilewitz, D., Eberle, D., Kaplowitz, N. (1981): The hepatocellular uptake and biliary excretion of endotoxin in the rat. Hepatology 1:401.

Miller, M. S., Vaughan, R. W., Gandolfi, A. J. (1980): The disposition of enflurane in obese patients. J. Pharmacol. Exp. Therap. 215:292.

Milner, K. C., Rudback, J. A., Ribi, E. (1971): General characteristics of endotoxins. In: Microbial Toxins Volume IV, ed. S. J. Ajl, A. Cicgler, S. Kadis, T. C. Montie and G. Weinbaum, Academic Press, New York, NY, p. 6.

Morrison, D. C. and Leive, L. (1975): Fractions of lipopolysaccharide from E. coli 0111-B4 prepared by two extraction methods. J. Biol. Chem. 250:2911.

Morrison, D. C., Oades, Z. G., DiPietro, L. (1981): Endotoxin-initiated membrane changes in rabbit plateltes. In Pathophysiological Effects of Endotoxins at the Cellular Level J. A. Majde and R. J. Person (eds.), Alan R. Liss, Inc., New York, NY, p. 48.

Moudgil, G. C., Allan, R. B., Russell, R. J. (1977): Inhibition by anaesthetic agents, of human leucocyte locomotion towards chemical attractants. Br. J. Anaesth. 49:97.

Moult, P. J. A. and Sherlock, S. (1975): Halothane-related hepatitis. Q. J. Med. 44:99.

Mukai, S., Morio, M., Fujii, K., Hanaki, C. (1977): Volatile metabolites of halothane in the rabbit. Anesthesiology 47:248.

Newberger, J., Vergani, D., Mieli-Vergani, G., Davis, M., Williams, R. (1981): Hepatic damage after exposure to halothane in medical personnel. Br. J. Anaesth. 53:1173.

Nichols, W. (1982): Digestion of tissue for calcium measurement by atomic absorption. Personal Communication from Department of Surgical Biology, Arizona Health Sciences Center, Tucson, AZ.

Nolan, J. P. (1981): Endotoxin, reticuloendothelial function and liver injury. Hepatology 1:458.

Nolan, J. P. and Ali, M. V. (1973): Endotoxin and the liver. II Effect of tolerance on carbon tetrachloride-induced injury. J. Med. 4:28.

Nolan, J. P. and Ali, M. V. (1974): Endotoxemia in liver disease. Lancet 1:999.

Nolan, J. P., Hare, D. K., McDevitt, J. J., Vilayal Ali, M. (1977): In vitro studies of intestinal endotoxin absorption I. Gastroenterol. 72:434.

Nolan, J. P. and Leibowitz, A. I. (1978): Endotoxin and the liver. III. Modification of acute carbon tetrachloride injury by polymyxin B - an antiendotoxin. Gastroenterol. 75:445.

Nolan, J. P., McDevitt, J. J., Goldmann, G. S. (1975): Endotoxin binding by charged and uncharged resins. Proc. Soc. Exp. Biol. Med. 149:766.

Nolan, J. P. and Vilayat Ali, M. (1968): Endotoxin and the Liver I, Toxicity in rats with choline deficient fatty livers. Proc. Soc. Exp. Biol. Med. 129:29.

Nunn, J. F., Sturrock, J. E., Jones, A. J. (1979): Halothane does not inhibit human neutrophil function in vitro. Br. J. Anaesth. 51:1101.

Omura, T. and Sato, R. (1964): The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239:2370.

Ona, F. V., Patanella, H., Ayub, A. (198)): Hepatitis associated with enflurane anesthesia. Anesthe. Analg. 59:146.

Palmer, J. W. and Gerlough, T. D. (1940): A simple method for preparing antigenic substances from the typhoid bacillus. Science 92:155.

Plummer, J. L., Beckwith, A. L., Bastin, F. W., Adams, J. F., Cousins, M. J. and Hall, P. (1982): Free radical formation in vivo and hepatotoxicity due to anesthesia with halothane. Anesthesia 57:160.

Prytz, H., Holtz-Christensen, J., Korner, B., Liehr, H. (1976): Portal venous and systemic endotoxemia in patients with cirrhosis. SCand. J. Gastroenterol. 11:857.

Ramadori, G., Hopf, U., Meyer, K. H. (1979) Binding sites for entotoxic lipopolysaccharide on the plasma membrane of isolated rabbit hepatocytes. Acta Hepato-Gastroenterol. 26:368.

Romeo, D., Girard, A., Rothfield, L. (1970): Reconstitution of a functional membrane enzyme system in a monomolecular film. J. Molec. Biol. 53:475.

Ross, W. T., Jr. and Daggy, B. P. (1981): Hepatic blood flow in phenobarbital-pretreated dats during halothane anesthesia and hypoxia. Anesth. Analg. 60:306.

Rutenberg, A. M., Sonnenblick, E., Koven, I., Aprahamian, H. A., Reiner, L., Fine, J. (1957): The role of intestinal bacteria in the development of dietary cirrhosis in rats. J. Exp. Med. 106:1.

Sanford, J. P. and Noyes, H. E. (1958): Studies of the absorption of E. coli endotoxin from the gastrointestinal tract of dogs in the pathogenesis of "irreversible" hemorrhagic shock. J. Clin. Invest. 37:1425.

Schade, U. and Reitschel, E. T. (1982): The roleof prostaglandins in endotoxic activities. Klin. Wochenschr. 60:743.

Shands, J. W. (1965): Localization of somatic antigen on gram-negative bacteria by electron microscopy. J. Bact. 90:266.

Sharp, J. H., Trudell, J. R., Cohen, E. N. (1979): Volatile metabolites and decomposition products of halothane in man. Anesthesiology 50:2.

Siegel, J. H., Greenspan, M., Cohen, J. D. (1968): The prognostic implications of altered physiology in operations for portal hypertension. Sufg. Gynecol. Obstet. 126:249.

Sipes, I. G. and Brown, B. R., Jr. (1976): An animal model of hepatotoxicity associated with halothane anesthesia. Anesthesiology 45:622.

Sipes, I. G., Gandolfi, A. J., Brown, B. R. (1981): Halothane-associated hepatitis: evidence for direct hepatotoxicity. In Drug Reactions and the Liver, M. Davis, J. M. Tredger and R. Williams (eds.) Pitman Medical Limited, London, pp. 219-231.

Skarnes, R. C. (1970): Host defense against bacterial endotoxemia: mechanism in normal animals. J. Exp. Med. 132:300.

Stier, A. (1964a): Trifluoracetic acid as metabolite of halothane. Biochem. Pharmacol. 13:1544.

Stier, A. (1964b): Frage der stabilitat von halothan im stoffwechsel. Naturwissenschaften 51:65.

Stier, A., Alter, H., Hessler, O., and Relder, K. (1964): Urinary excretion of bromide in halothane anesthesia. Anesth. Analg. 43:723.

Strunin, L. (1977): The Liver and Anesthesia, W. B. Saunders Company, London.

STumacher, R. J., Kovnat, M. J., McCabe, W. R. (1973): Limitations of the usefulness of the limulus assay for endotoxin. N. Engl. J. Med. 288:1261.

Tannock, G. W. and Savage, D. C. (1974): Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. Infect. Immun. 9:591.

Thomas, L. M., Sturk, A., Kahle, L. H., tenCate, J. W. (1981): Quantitative endotoxin determination in blood with a chromogenic substrate. Clin. Chim. Acta. 116:63.

Triger, D. R., Alp, M. H., Wright, R. (1972): Bacterial and dietary antibodies in liver disease. Lancet 1:60.

Tsang, H. S. (1975): Jaundice after enflurane anesthesia. Med. J. 148:393.

Utili, R., Di Donato, A., Draetta, G., Paolisso, G., ABernathy, C. O., Illiano, G. (1982): Endotoxin inhibits the fluoride-stimulated adenylate cyclase activity of rat liver plasma membranes enriched with bile canaliculi. Experimentia 38:831.

Van Dyke, R. A. (1966): Metabolism of volatile anesthetics. III. Induction of microsomal dechlorinating and ether-cleaving enzymes. J. Pharmacol. Exp. Ther. 154:364.

Van Dyke, R. A. (1981): Effects of fasting on liver toxicity following exposure to enflurane, isoflurane and halothane. Pharmacologist 23:212.

Van Dyke, R. A., Chenoweth, M. B. (1965): The metabolism of volatile anesthetics II. In vitro metabolism of methoxyflurane and halothane in rat liver slices and cell fractions. Biochem. Pharmacol. 14:603.

Vaughan, R. W., Sipes, I. G., Brown, B. R. (1978): Role of biotransformation in the toxicity of inhalation anesthetics. LIfe Sciences 23:2447.

Vergani, D., Mieli-Vergani, G., Alberti, A., Neuberger, J. M., Eddleston, A. L. W. F., Davis, M., Williams, R. (1980): Antibodies to the surface of halothane-altered rabbit hepatocytes in patients with severe halothane-associated hepatitis. New Eng. J. Med. 303:66.

Walter, J. B. (1977): The immune response. From An Introduction to the Principles of Disease. W. B. Saunders Company, Philadelphia, p. 154.

Welch, W. D. (1981): Halothane reversibly inhibits human neutrophil bacterial killing. Anesthesiology 55:650.

Westphal, C., Luderitz, O., Bister, F. (1952): Uber die extraktion von bacterien mit phenol/wasser. 2 Naturf. 76:148.

Widger, L. A., Gandolfi, A. J., Van Dyke, R. A. (1976): Hypoxia and halothane metabolism in vivo. Release of inorganic fluoride and halothane metabolite binding to cellular constituents. Anesthesiology 44:197.

Wilkinson, S. P., Gazzard, B. G., Arroyo, V., Moodie, H., Williams, R. (1974): Relation of renal impairment and haemorrhagic diathesis to endotoxaemia in fulminant hepatic failure. Lancet 1:521.

Wilson, J. W., Ludec, E. H., Arnold, L. E. (1950): Protective action of sulfa drugs against $CCl_4$ poisoning in mice. Fed. Proc. 9:349.

Wiznitzer, T., Better, N., Rachlin, W., Atkins, N., Frank, E. D., Fine, J. (1960): In vivo detoxification of endotoxin by the reticulo-endothelial system. J. Exp. Med. 112:1157.

Wolff, S. M. (1973): Biological effects of bacterial endotoxins in man. J. Infect. Dis. 128:5259.

Wood, M., Berman, M. L., Harbison, R. D., Hoyle, P., Phythyon, J. M., Wood, A. J. J. (1980): Halothane-induced hepatic necrosis in triiodothyronine-pretreated rats. Anesthesiology 52:470.

Zauder, H. L. (1979): A possible interaction of PCB and halothane in man. Anesthesiology 51:95.