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Generation and expression of halothane derived protein adducts in the guinea pig liver

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The University of Arizona, 1993
GENERATION AND EXPRESSION OF HALOTHANE DERIVED PROTEIN ADDUCTS
IN THE GUINEA PIG LIVER

by

Alan Perry Brown

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

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DEDICATION

This dissertation is dedicated to my late father, Stephen Samuel Brown, who allowed me the freedom to develop into my own person.
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ABSTRACT

The volatile anesthetic halothane can be bioactivated in the liver to the reactive intermediate, trifluoroacetyl chloride, which is capable of covalently modifying liver protein. The product of this reaction is trifluoroacetyl-\(\text{N}-\text{\(\epsilon\)}\)-amino lysine, which can act as a foreign epitope in altering both protein immunogenicity and antigenicity. Protein adduct formation appears to be responsible for the development of both an acute and an immune-mediated hepatotoxicity. The goal of this research project was to detect, quantify, and characterize the formation of protein adducts in the guinea pig liver, following exposure to halothane. This species provides the most accurate animal model for halothane hepatitis to date.

An *in vitro* liver slice system was used to study the conditions for the production of protein adducts during halothane exposure. Covalent binding to slice protein occurred in a linear fashion over the time course of exposure, and was concentration dependent. Oxidative metabolism of halothane was required for adduct production. Adduct formation occurred to specific and identifiable proteins. The majority of the protein adducts in the liver slice were localized to cytosolic glutathione-S-transferase (GST). GST can be released from the liver slice, transporting the adduct to the extracellular environment.

Guinea pigs were anesthetized with halothane to compare the results obtained *in vitro*, with what occurs in the whole animal. Covalent binding
to liver protein occurred predominately in the microsomal fraction. The protein adducts identified in the guinea pigs corresponded to those seen in liver slices. GST was identified as a target for the acid chloride intermediate in the liver of these animals. Covalent binding to cytosolic protein was dependent on liver glutathione content. A specific relationship between adduct formation to cytosolic protein and glutathione concentration was further demonstrated using an in vitro bioactivation system. GST may catalyze the reaction between the electrophile and glutathione. Liver glutathione content appears to mediate the degree and selectivity of covalent binding to target proteins. The development of halothane induced hepatotoxicity may be related to the interactions between its reactive intermediate, glutathione, and GST.
Drug Hypersensitivity

Adverse drug reactions pose a major clinical problem in modern medicine today. While the majority of negative responses to therapeutic agents can be attributed to their pharmacological actions, metabolic pathways resulting in direct toxicity, or the administered dose, a more difficult problem to assess is idiosyncratic or hypersensitivity reactions to drug administration. These adverse reactions appear to be mediated by the immune system and may be responsible for 10-20% of all adverse drug reactions (Park and Kitteringham, 1990). Idiosyncratic reactions are potentially life threatening, and at the present time are totally unpredictable.

Drug induced hypersensitivity responses are characterized by several key factors. The patient exhibiting this syndrome must have had either a prior exposure to the compound or be under continuous therapy for at least a week. This initial exposure creates a sensitization to the drug. Upon re-exposure or re-challenge with the compound, an immediate adverse reaction ensues. Clinical features of drug hypersensitivity are expressed by skin rash, fever, eosinophilia, tissue or organ damage, and appear to be dose independent (Uetrecht, 1992).

Most drugs of small molecular weight are not capable of initiating an immune response. Known as haptens, they must first be covalently bound to
a protein and then presented to the cells of the immune system to provoke a response (Fig. 1). This carrier protein may be either endogenous or foreign in nature (Parker, 1982). For a drug-protein conjugate to be sufficiently immunogenic, the hapten must create an antigenic determinant capable of B lymphocyte recognition. A portion of the carrier protein must also be associated with the major histocompatibility complex (MHC) of an antigen presenting cell in order to be presented and recognized by T lymphocytes (Coleman, 1990). This initial process is known as immune sensitization. Sensitization involves either the humoral or cellular branches of the immune system. Following a secondary exposure to the compound resulting in antigen formation, the immune system launches a response with the subsequent development of hypersensitivity. A humoral immune response results in antibodies generated against the hapten-protein complex. A cellular response will produce cytotoxic lymphocytes against the antigenic determinants (Park, et al, 1987).

Most therapeutic agents, in themselves, are not capable of forming a covalent bond with protein, the classic exception being the penicillins (Park and Kitteringham, 1990). Therefore, biotransformation of the drug to a reactive intermediate capable of covalent binding is required. The degree of covalent binding of a drug or metabolite to a carrier protein is very important. As the hapten density on a carrier protein increases, so does the antigenicity or recognizability of the complex. The degree of hapten density determines whether the hapten itself or the carrier protein is more immunodominant (Utrecht, 1992).
Figure 1. Drug Induced Hypersensitivity
Drug hypersensitivity reactions can be categorized into four subtypes based on the classification of Gell and Coombs (Park and Kitteringham, 1990).

Type I (immediate or anaphylactic) reactions result from the interaction of a drug-protein antigen with a specific IgE antibody on the surface of mast cells or basophils. The result is the release of chemical mediators such as histamine and leukotrienes into the extracellular environment. Clinical features include erythema, urticaria, angioedema, gastrointestinal disturbances, bronchospasm, pulmonary edema, and hypotension.

Type II (cytotoxic) reactions are the result of the recognition of drug-protein antigens by specific IgG or IgM antibodies on the surface of a cell. The cell is destroyed either by cytotoxic leukocytes or by complement fixation. Type II reactions may produce organ or tissue specific necrosis.

Type III (immune complex) reactions involve the formation of circulating immune complexes from specific antigen and either IgG or IgM antibodies. These complexes can deposit in the vasculature or renal glomeruli, resulting in tissue damage due to complement fixation or recruitment of phagocytic cells.

Type IV (delayed hypersensitivity) reactions are cell mediated and
usually occur in the skin. Recognition of drug-protein antigens by specific T lymphocytes results in the release of various lymphokines. These lymphokines attract infiltrating mononuclear cells, which produce inflammation and tissue damage.

Halothane Hepatitis

Halothane (CF₃CHClBr) is a volatile anesthetic which was first introduced into clinical practice in 1956 and shortly became the anesthetic of choice for general anesthesia. Halothane gained widespread use due to its advantages of potency, lack of flammability, and general ease of administration. However, by 1958 several cases of postoperative liver necrosis appeared (Ray and Drummond, 1991). The incidence of "unexplained" liver damage, or halothane hepatitis, became more prevalent, precipitating concern within the medical community.

The apprehension surrounding the use of halothane resulted in the National Halothane Study conducted by the National Institute of Health. The study reviewed the occurrence of fatal hepatic necrosis occurring within 6 weeks of anesthesia at 34 medical centers in the United States from 1959 to 1962. The report, which was published in 1969, reviewed 250,000 cases of halothane anesthesia and concluded that the incidence of unexplained fatal hepatic necrosis following the use of halothane was as rare as 1:35,000 (Strock and Strunin, 1985; Martin, 1992). Further epidemiological studies reported the incidence of halothane induced liver damage between 1:6000 to
1:20,000 cases of anesthesia. In spite of the inconsistent data, hepatic
damage resulting from halothane anesthesia is rare in occurrence with a
complex epidemiological history (Ray and Drummond, 1991).

What became apparent was that two distinct types of liver damage occurred
following halothane anesthesia. The first type is a mild hepatotoxicity
resulting in a slight disturbance of liver function. These patients
experienced fever, nausea, vomiting, and lethargy. There is a mild rise
in the liver enzymes alanine aminotransferase and aspartate
aminotransferase in the serum. This acute hepatotoxicity is noted in
approximately 25% of patients receiving halothane and has a good prognosis
(Martin, 1992; Neuberger, 1990). The second form of liver toxicity is
more serious and can lead to severe hepatic necrosis with subsequent
liver failure. This form of hepatic damage has been termed "halothane
hepatitis" and can result in a 50% lethality rate.

Halothane hepatitis is consistently characterized by several clinical
features. Patients with halothane hepatitis exhibit gastrointestinal
upset, fever, jaundice, eosinophilia, and a dramatic rise in liver
transaminase levels in the serum. These events occur within two weeks of
anesthesia. The most noticeable histological features of halothane
hepatotoxicity is centrilobular necrosis. Severe cases of halothane
hepatitis can result in massive confluent liver necrosis (Ray and
Drummond, 1991). These patients present circulating IgG antibodies
reactive towards liver protein antigens. Patients most susceptible to
this syndrome tend to be middle aged, obese females. One of the hallmark factors involved with the development of halothane hepatitis is repeated exposures to this anesthetic. Other potential risk factors include a susceptibility to allergy and genetic predisposition (Martin, 1992). Halothane hepatotoxicity has been noted to occur in pairs of closely related women, demonstrating a familial predisposition for toxicity (Hoft, et al, 1981). The evidence surrounding the development of halothane hepatitis suggests that this syndrome is an immune mediated drug hypersensitivity reaction.

**Halothane Biotransformation**

Approximately 20% of the administered dose of halothane undergoes biotransformation, occurring predominately in the liver. Halothane is bioactivated by the liver cytochrome P-450 system along a reductive or an oxidative route, depending on the oxygen tension (Fig. 2) (Lind, et al, 1990). Reductive metabolism results in the addition of an electron on the molecule, followed by the loss of a bromide ion. The radical intermediate can rearrange, producing either 2-chloro-1,1,1-trifluoroethane or 2-chloro-1,1-difluoroethylene plus fluoride ion. The radical intermediate may covalently react with lipids, resulting in lipid peroxidation (Martin, 1992). Oxidative metabolism of halothane produces the reactive intermediate, trifluoroacetyl chloride. This intermediate can hydrolyze with water producing trifluoroacetic acid, which is readily excreted in the urine (Sakai, et al, 1991). The acid chloride can also react with lysine residues of proteins, producing trifluoroacetyl-N-ε-amino-lysine.
Figure 2. Halothane Biotransformation
This moiety can not only alter the protein's function, but can also change the immunogenicity and antigenicity of the carrier protein. The result is a drug-protein adduct capable of eliciting an immunological response directed against the liver. At the present time, the exact cytochrome P-450 isozyme(s) responsible for halothane biotransformation has not been identified. However, current evidence suggests that cytochrome P-450IIIE1 plays a major role in halothane metabolism, along with other volatile anesthetics (Kharasch and Thummel, 1991; Tsutsumi, et al, 1990).

**Immune Hypersensitivity**

The possibility that an immune mediated hypersensitivity reaction may be responsible for halothane hepatitis became more apparent following numerous serological studies. In 1978, Vergani and co-workers demonstrated that leukocytes from halothane hepatitis patients were sensitized or reactive towards liver antigens from halothane exposed rabbits (Vergani, et al, 1978). This group later reported that circulating antibodies from halothane hepatitis patients were reactive to surface membrane antigens of hepatocytes from halothane exposed rabbits (Vergani, et al, 1980). These initial studies suggested that halothane hepatitis patients were immunologically responsive towards halothane induced liver antigens. Patients exposed to halothane but who did not develop liver dysfunction were not immunologically sensitized.

Serological studies have consistently shown that patients clinically
diagnosed as having halothane hepatitis contain circulating IgG antibodies reactive towards the trifluoroacetyl moiety (Bird and Williams, 1989). There appears to be a mixed population of antibodies with varying epitope specificities amongst patients. These antibodies appear to be either hapten specific or recognize epitopes on the carrier proteins (Hastings, et al, 1991; Martin, et al, 1990). It is postulated that these anti-drug and anti-liver protein antibodies mediate a Type II drug hypersensitivity response against the liver.

Halothane Protein Adducts/Neoantigens

Currently, numerous halothane derived protein adducts have been identified in the livers of rats, rabbits, and guinea pigs following exposure to halothane (Pohl, et al, 1989; Hubbard, et al, 1989; Roth, et al, 1988). These adducts are comprised of the trifluoroacetyl group covalently bound to lysine residues on proteins. Termed neoantigens, these adducts may alter the immunogenicity and antigenicity of the carrier protein, resulting in stimulation and elicitation of an immune response. Protein adduct formation appears to predominate in the liver, which is the major site of halothane metabolism.

Pohl and co-workers have isolated and identified numerous neoantigens from the livers of rats exposed to halothane via intraperitoneal injection. These proteins range in molecular weight from 54-100 kDa as determined by electrophoresis, and appear to be highly localized in the microsomal compartment or endoplasmic reticulum of the hepatocyte.
Several of these neoantigens were isolated and partially characterized. A 100 kDa protein neoantigen has been identified as endoplasmin, an endoplasmic reticulum glycoprotein (Thomassen, et al, 1991). This protein binds calcium and appears to be localized in the lumen of the endoplasmic reticulum. A 63 kDa protein, recently identified as calreticulin, is a target for adduct formation (Butler, et al, 1992). Calreticulin is a major calcium binding protein present in the lumen of the endoplasmic reticulum. Several liver microsomal enzymes appear to become neoantigenic following halothane exposure. The enzymes; 59 kDa carboxylesterase, 57 kDa protein disulfide isomerase, and a 54 kDa cytochrome P-450 are all targets for the halothane intermediate (Satoh, et al, 1989; Martin, et al, 1989; Satoh, et al, 1985). Liver autopsy samples from halothane hepatitis patients have demonstrated the expression of antigenically altered proteins with molecular weights of 100, 76, and 57 kDa (Kenna, et al, 1988).

Protein adduct formation in the liver is not isolated to hepatocytes. Kupffer cells prepared from livers of rats exposed to halothane have exhibited trifluoroacetylated protein adducts (Christen, et al, 1991a). Recently, extrahepatic sites of protein adduct formation have been detected, although to a lesser extent. Protein adducts have been identified in both heart and kidney tissue, following halothane exposure (Huwyler and Gut, 1992; Huwyler, et al, 1992). This may be due to the presence of functional cytochrome P-450 isozymes capable of halothane biotransformation in these tissues.
Although rats produce trifluoroacetylated protein adducts following halothane exposure, a pathological response related to halothane hepatitis is not evident. At the present time, neither the neoantigens responsible for eliciting an immune response, nor the mechanism for presentation of these drug-protein conjugates to the immune system, have been identified.

Other Volatile Anesthetics
Currently, concern is mounting that anesthetic induced immune mediated hepatotoxicity is not limited to halothane, but may occur in rare instances with other halogenated anesthetics (Brunt, et al, 1991). Both enflurane (CHF₂-O-CF₂CHFCI) and isoflurane (CHF₂-O-CHC1CF₃) are volatile anesthetics which are commonly used today. These compounds may undergo oxidative bioactivation by the liver cytochrome P-450 system to produce reactive intermediates capable of acylating protein (Christ, et al, 1988a). Exposure in rats to either isoflurane or enflurane results in the expression of liver microsomal protein adducts, the greatest extent occurring with the latter. The degree of adduct formation appears to be dependent on the relative extent of metabolism of these compounds. Approximately 2% of administered enflurane and 0.2% of isoflurane is metabolized, versus 20% of administered halothane (Christ, et al, 1988b). The potential exists for cross-reactivity or cross-sensitization between protein adducts produced by these anesthetics resulting in hypersensitivity.
Animal Models of Halothane Hepatotoxicity

Numerous attempts have been made by various researchers to develop an animal model for halothane hepatitis utilizing the rat. Unfortunately, these models required excessive pretreatment and manipulation of the animals such as enzyme induction, or hypoxia for development of toxicity (Ray and Drummond, 1991). Hepatic necrosis following halothane anesthesia depended on either triiodothyronine, isoniazid, or phenobarbital pretreatment (Rice, et al, 1987; Berman, et al, 1983; Uetrecht, et al, 1983). Additionally, animals pretreated with phenobarbital required hypoxic conditions during halothane exposure for the development of liver necrosis. The experimental conditions did not adequately represent the clinical setting nor the pathological outcome.

Recently, an animal model of halothane toxicity has been developed and described utilizing the guinea pig. Unlike the rat, guinea pigs require no such pretreatment as liver enzyme induction or hypoxia for the development of liver necrosis following in vivo exposure to halothane (Lind, et al, 1992a). Guinea pigs anesthetized with halothane produce a pathological lesion similar to that seen in humans (Lunam, et al, 1989). The development of halothane induced hepatic necrosis in guinea pigs appears to be influenced by a variety of factors such as age, gender, strain, and heredity (Lind, et al, 1989a; Lind, et al, 1987; Lunam, et al, 1986). Guinea pigs generate anti-trifluoroacetyl-lysine reactive antibodies, along with liver protein-adducts, following inhalation exposure to halothane (Hubbard, et al, 1989; Siadat-Pajouh, et al, 1987).
Covalent binding of a halothane intermediate to liver protein is dependent on oxidative metabolism, and has been implicated as a mechanism for acute hepatotoxicity in the guinea pig (Lind, et al, 1990). The guinea pig, therefore, appears to provide the most representative animal model for both the acute and immune mediated toxicities following halothane anesthesia.

Liver Slices
Toxicological studies involving whole animals present certain limitations in the study of organ or tissue specific toxicity, due to the large number of animals required. Mechanisms of toxicity may be difficult to assess in the whole animal due to limitations in experimental manipulation, along with hormonal, physiological, and pharmacokinetic differences between the animals. In addition, in vivo exposure to radiolabeled compounds or materials in limited supply may be prohibitive due to the excessive costs involved.

In vitro models such as isolated hepatocyte suspensions and isolated perfused livers have been used in the past to study volatile anesthetic toxicity (DiRenzo, et al, 1985). Unfortunately, these model systems present certain limitations. Hepatocyte preparations routinely utilize collagenase digestion in the isolation and preparation process. This can result in damaged cellular membranes, disruption of cellular contact, and loss of stratification. Hepatocyte suspensions present a disruption of the functional heterogeneity of the liver. In isolated perfused liver
model systems, only short term experiments were possible and a large number of animals were required (Ghantous, et al, 1990a; Azri, et al, 1990).

A novel in vitro system utilizing precision cut liver slices provides an alternative model for the study of xenobiotics and hepatotoxicity in the liver (Smith, et al, 1987). Unlike other in vitro hepatocyte systems, liver slices provide the maintenance of tissue integrity, cellular diversification, identity, and stratification. Large numbers of liver slices can be rapidly and easily prepared from a single organ providing a multiple range of experimental conditions to be examined from a common source. Liver slices retain their biochemical functions and have been used to study the biotransformation and toxic potential of a wide variety of xenobiotics. Species variation in metabolism and toxicity can also be investigated using liver slices (Azri, et al, 1990).

Precision cut liver slices prepared from male Hartley guinea pigs have proved useful in studying the biotransformation and hepatotoxic potential of various volatile anesthetics (Ghantous, et al, 1990a). The biotransformation of halothane has been demonstrated in guinea pig liver slices (Ghantous, et al, 1990b). The route of biotransformation is dependent on the oxygen tension present in this in vitro model system. Reductive metabolism of halothane resulting in the production of fluoride ion is maximal under low oxygen tension (2.5% O₂). Oxidative metabolism increases and is greatest when the O₂ concentration is 95%, resulting in
trifluoroacetic acid formation. Under these conditions, reductive metabolites are no longer detectable in the media. Exposure to halothane under oxidative conditions results in a loss of intracellular K⁺ content. This decrease in liver slice viability upon exposure to halothane is both concentration and time-dependent (Ghantous, et al, 1990c). Exposure to halothane also results in a decrease in protein synthesis and secretion in the slice (Ghantous, et al, 1992). Liver slices prepared from male Hartley guinea pigs provide a model system for studying both the bioactivation and toxicity of halothane.

**Statement of Purpose**

Halothane can be bioactivated in the liver to a reactive intermediate, which is capable of acylating liver proteins. These protein adducts appear to play a role in both the acute and immune mediated toxicities, resulting from halothane exposure. Neither the protein adducts responsible for initiating an immune response, nor the mechanism for the elicitation of a hypersensitivity reaction have been described. Alternatively, the covalent modification of critical protein targets may result in the acute hepatotoxicity noted with halothane. These protein targets have yet to be identified.

The initial hypothesis of this research project is that the guinea pig can be used as an animal model for the study of protein adduct formation in the liver, following halothane exposure. Adduct formation to specific and identifiable proteins can be described with various techniques. The
covalent modification of these proteins can result in novel epitopes of varying antigenicity. The analysis of protein targets in the hepatocyte, susceptible to acylation, will provide a better understanding of the generation of halothane hepatotoxicity.

Specific questions addressed in this research project were the following:

1. Can guinea pig liver slices bioactivate halothane to a reactive intermediate capable of covalently modifying liver protein?

2. Which proteins undergo covalent modification in the liver slice?

3. Which protein(s) are major targets for halothane derived reactive intermediates in the liver slice?

4. Can protein adducts produced in the liver slice be released or secreted into the extracellular environment?

5. Does glutathione play a role in regulating the covalent binding of a halothane derived reactive intermediate to target protein?

6. Do guinea pigs exposed to halothane under in vivo conditions produce covalently bound adducts to specific and identifiable proteins?

7. How do the results obtained in vitro compare with those in the
whole animal under \textit{in vivo} conditions?
MATERIALS AND METHODS

Guinea Pigs
An outbred strain of adult male Hartley guinea pigs (600-800 g) were used for all of the studies. Currently an animal model for halothane hepatotoxicity has been developed in these guinea pigs (Lind, et al, 1992a). These animals were purchased from Sasco Incorporated (Omaha, NE). The guinea pigs were housed in hanging cages with a 12 hr light/dark cycle and given food (Wayne Chow, cabbage) and water ad libitum.

In Vitro Liver Slice Exposure
After the animals were sacrificed by cervical dislocation, their livers were removed and placed in cold Krebs-Henseleit buffer (27.6 g NaCl, 18.0 g glucose, 8.0 g NaHCO₃, 1.44 g KCl, 1.7 g CaCl₂⋅H₂O, 1.18 g MgSO₄⋅7H₂O, 0.52 g KH₂PO₄, 4 liters H₂O, pH 7.4, 4°C). Cores were taken (1 cm in diameter) from various areas of the liver lobes using a stainless steel corer and drill press and slices (30-35 mg wet weight, 250-300 μm thick) were prepared using a plastic Krumdieck tissue slicer (Smith, et al, 1987). The liver slices were prepared in Krebs-Henseleit buffer gassed with 95% O₂/5% CO₂. Liver slices were incubated in 20 ml glass scintillation vials (3 per vial) on stainless steel mesh cylinders circumscribed with two wheels. Each vial contained 1.6 ml Krebs-Henseleit buffer (6.9 g NaCl, 4.5 g glucose, 2.0 g NaHCO₃, 0.425 g CaCl₂⋅2H₂O, 0.36 g KCl, 0.295 g MgSO₄⋅7H₂O, 0.13 g KH₂PO₄, 1 l H₂O, pH 7.4) supplemented with 1 mM each of basal medium Eagle-vitamins, basal medium Eagle-amino acids,
and L-glutamine along with 50 μg/ml gentamycin (Gibco, Grand Island, NY). Vials were gassed with 95% O₂/5% CO₂, placed on a heated vial rotator in an acrylic box, and allowed to incubate at 37° C (Ghantous, et al, 1990b).

After a 1 hr equilibration period, either halothane (Abbott Laboratories, North Chicago, IL or Halocarbon Laboratories, North Augusta, SC), deuterated halothane, or 1-¹⁴C-halothane (New England Nuclear, Boston, MA) was injected through a Teflon septa cap onto a circular filter paperwick (diameter 0.7 cm) and allowed to vaporize in the vial. The deuterated halothane was previously synthesized in the laboratory to greater than 99% purity (Lind, et al, 1989b). Radiochemical purity of the ¹⁴C-halothane was initially determined by the manufacturer using gas liquid chromatography to be greater than 99%.

Partitioning of anesthetic occurred between the media and air space resulting in a constant equilibrium media concentration which was determined by gas chromatography (Ghantous, et al, 1990b). An initial media concentration of 1.7 mM halothane (8 μl injected halothane) was used. The concentration was then lowered to 1.0 mM halothane (4 μl injected halothane) in subsequent investigations to decrease potential toxicity over the incubation period (Ghantous, et al, 1990c).

Control and exposed slices were taken at various time points over the 12 hr incubation period and either sonicated in water to produce a whole liver cell homogenate or used to prepare microsomes and cytosol. Liver
slice proteins present in the incubation media were precipitated and collected.

Isolation of Microsomal and Cytosolic Protein from Liver Slices
Approximately 30-40 liver slices were first homogenized with a Dounce homogenizer in 50 mM Tris, 1.15% KCl, pH 7.4, 4° C. The homogenate was then passed through glass wool and centrifuged at 10,000 g for 20 min. The supernatant fraction was collected and centrifuged at 220,000 g for 45 min. The microsomal pellet was re-homogenized in the Tris/KCl buffer and the protein was concentrated upon precipitation using 80% ethanol (ice cold). The protein pellet was then collected and reconstituted in approximately 0.200 ml of Tris/KCl buffer.

In order to isolate cytosolic protein, ammonium sulfate (0.66 g/ml) was added to the 220,000 x g supernatant fraction followed by mixing on a rotary shaker for 30 min. This resulted in a protein precipitate which was pelleted upon centrifugation at 10,000 g for 15 min. The protein pellet was reconstituted in Tris/KCl buffer and dialyzed overnight against the same buffer. Protein from the dialysate was then concentrated using an 80% ethanol precipitation. All of the above procedures were carried out at 4° C.

Isolation of Protein Released into the Incubation Media
Protein present in the incubation media was solely derived from the liver slices. No exogenous proteins were present in any incubations. Proteins
were precipitated from the incubation media upon the addition of ammonium sulfate, 0.66 g/ml media. Protein was pelleted upon centrifugation at 10,000 g for 15 min and reconstituted in Tris/KCl buffer.

**Detection of Protein Adducts to Liver Slice Proteins**

Liver slices were initially exposed to $^{14}$C-halothane followed by analysis of covalent binding of a radiolabeled metabolite to total liver slice protein. Detection and identification of specific target protein in the liver slice undergoing covalent modification was accomplished using several techniques based on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins can be resolved in a polyacrylamide gel matrix due to their denatured molecular mass upon addition of an electric field. Low molecular weight proteins will migrate faster than high molecular weight proteins, resulting in separation and resolution of proteins from a complex mixture. Electrophoretically resolved proteins can be visualized in the gel by staining with Coomassie blue R-250 dye. Specific liver proteins trifluoroacetylated by a halothane intermediate can be identified using anti-hapten antibodies in Western immunoblot analysis. Proteins containing a covalently bound radioactive metabolite can be identified by autoradiography or fluorography of the acrylamide gel. Alternatively, sequential horizontal sections can be made down the gel and the radioactivity of those sections can be determined by scintillation counting as a quantitative measure of covalently bound radioactivity.
Radiolabeled Covalent Binding Assay to Total Slice Protein

Liver slices (3 per vial) were exposed to a media concentration of either 1.0 or 1.7 mM $^{14}$C-halothane (0.5 µCi) for up to 12 hr. At various time points, liver slices were removed and sonicated in 0.5 ml water using a Kontes cell disrupter (Vineland, NJ). Total liver proteins were precipitated and washed with 2.5 ml 100% ice cold ethanol, to remove unreacted halothane and lipid, followed by centrifugation at 3500 rev/min to produce a protein pellet. The supernatant fraction was removed and the pellet was sonicated again in 2.5 ml 100% ethanol followed by re-centrifugation. This procedure was repeated and the protein pellet was resuspended in 1.0 ml 5% trichloroacetic acid followed by centrifugation at 3500 rev/min. The supernatant fraction was removed and the procedure repeated twice, to extract unbound metabolites. The protein pellet was dissolved overnight in 0.5 ml 1 M NaOH. Radioactivity of the protein solution was then analyzed using a liquid scintillation counter (Beckman Instruments, Fullerton, CA). The protein content of the dissolved pellet was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). Covalent binding was expressed in nmole equiv/mg protein.

BCA Protein Assay

Protein solutions or homogenates were diluted from 1:10 to 1:40 in distilled water depending on approximate protein content. Fifty parts Bicinchoninic acid solution (Pierce or Sigma) was mixed with 1 part of a 4% copper sulfate solution (Pierce or Sigma). One hundred microliters of diluted protein was added to 2.0 ml of the above solution and heated at
60° C for 30 min. After heating, the colored product was cooled to room temp and the absorbance at 562 nm was determined on a Gilford spectrophotometer. Bovine serum albumin standards (0.2 to 1.2 mg/ml) were included for construction of a standard curve.

**Protein Gel Electrophoresis**

Liver proteins were present in either particulate homogenates (microsomal proteins, whole liver cell mixtures, etc.) or dissolved in solutions (cytosol). Proteins were first precipitated with 80% ice cold ethanol, pelleted by centrifugation, and then reconstituted in water. Protein content was then determined using the BCA protein assay followed by dilution with water resulting in a 4.0 - 8.0 mg/ml solution. Protein samples were diluted 1:4 in a reducing buffer containing 10% glycerol, 5% β-mercaptoethanol, 0.0025% bromophenol blue (w/v), and 2% sodium dodecylsulfate in 62.5 mM Tris-HCl buffer followed by heating at 100° C for 4.5 min. Proteins were electrophoretically resolved using 12% polyacrylamide minigels with a 4% stacking gel (gel thickness 0.75 mm) at 200 volts for 40 min at room temp in a Mini-PROTEAN II Cell equipped with a Model 200/2.0 power supply (BIO RAD, Richmond, CA). The electrophoretic cell contained 500 ml of running buffer (25 mM Tris base, 192 mM glycine, pH 8.3). Final protein content per lane varied between 15-30 μg depending on experimental conditions. Molecular weight standards ranging from 97.4 - 14.4 kDa were obtained (BIO RAD). Proteins were visualized using Coomassie blue R-250 stain (BIO RAD) in a 0.12% (w/v) solution containing 50% methanol, 40% water, and 10% glacial acetic acid. Gels were destained
first in a 50% methanol, 40% water, 10% glacial acetic acid solution for 1 hr followed by a 5% methanol, 8% glycerol, 80% water, 7% glacial acetic acid solution overnight.

Western Immunoblot Analysis
Immediately following gel electrophoresis, proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA) overnight at 30 volts in 4° C using a Mini Trans Blot Cell and Model 200/2.0 power supply (BIO RAD). The transfer cell was filled with transfer buffer containing 25 mM Tris base, 192 mM glycine, 15% (v/v) methanol, pH 8.2 - 8.3. Following transfer, the membranes were washed with 100 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.4 (TTBS). Proteins were assayed for TFA adducts using rabbit antitrifluoroacetylated rabbit serum albumin antibodies diluted 1:100 in TTBS. Bound rabbit antibodies were detected using biotinylated goat anti-rabbit antiserum diluted in TTBS followed by an avidin-biotin-peroxidase complex in TTBS (Vector Laboratories, Burlingham, CA). All antibody incubations were for 30 min at room temp on a rotary shaker. The peroxidase substrate diaminobenzidine tetrahydrochloride/NiCl₂ (Sigma) was then incubated with the membranes (1 ml 40 mg/ml diaminobenzidine, 0.25 ml 80 mg/ml NiCl₂, 0.15 ml 3% H₂O₂, 50 ml 100 mM Tris, pH 7.4). Molecular weight standards were biotinylated using biotinyl-N-hydroxysuccinimide ester (Sigma) according to the method of Della-Penna, et al, (1986) and included in the gel electrophoresis. These molecular weight markers transferred onto the PVDF membrane along with the other proteins and were subsequently recognized by
the avidin-biotin-peroxidase complex.

An alternative Western immunoblot protocol was later implemented which proved to be more sensitive in detecting trifluoroacetylated proteins while decreasing non-specific recognition of other transferred proteins. Following electrophoresis, proteins were electrophoretically transferred to PVDF blotting membranes at 70 volts for 2 hr at 4°C. Following transfer, lanes containing molecular weight standards were cut from the PVDF membranes and the blots are washed in 10 mM Tris, 0.9% NaCl, pH 7.4, 4°C. The blots containing the molecular weight standards were then stained with amido black dye for visualization. The membranes were first stained for 5-10 min in a 0.1% amido black 10B, 25% methanol, 10% glacial acetic acid, 65% H₂O solution. The PVDF blots were then destained in 45% methanol, 48% H₂O, 7% glacial acetic acid. The remaining membranes were blocked with 2.5% casein, 10 mM Tris, 0.9% NaCl, 0.02% thimerosal, pH 7.4 for 1 hr at 4°C. The blots were washed and incubated with the primary antibody: rabbit IgG anti-TFA-RSA diluted 1:100 in 0.5% casein, 10 mM Tris, 0.9% NaCl, 0.02% thimerosal, pH 7.4 buffer (Casein/Tris) overnight at 4°C. Afterwards the primary antibody solution was decanted and the membranes were washed prior to addition of the secondary antibody. The blots were incubated with affinity purified peroxidase labeled goat anti-rabbit IgG Fc fragment specific antibodies (Organon Teknika Corp., West Chester, PA) diluted 1:200 in casein/Tris overnight at 4°C. Following incubation with the secondary antibody, the membranes were washed extensively and developed using the peroxidase substrate
diaminobenzidine/NiCl₂ as described above.

Generation of Anti TFA-RSA Antibodies

Polyclonal rabbit antibodies were generated against trifluoroacetylated rabbit serum albumin (TFA-RSA) to detect trifluoroacetylated proteins in Western immunoblot analysis. These IgG class antibodies were initially produced in male New Zealand White rabbits according to the method of Callis, et al, (1987).

Synthesis of TFA-RSA

Rabbit serum albumin (Sigma), 500 mg in 50 ml H₂O, was placed in a 3-neck round bottom flask. Ethyl thioltrifluoroacetate (Pierce), 750 µl, was slowly added to the RSA while simultaneously maintaining the pH at 10 with 1 N KOH. The reaction was allowed to proceed at room temp. Following completion of the reaction, the trifluoroacetylated rabbit serum albumin (TFA-RSA) was removed and dialyzed extensively against water for 2 days at 4°C. Following dialysis, total protein content of the conjugate was determined and the ratio of free lysine to TFA conjugated lysine was assessed (Kakade and Lienen, 1969). Protein bound fluorine was determined by the covalently bound fluorine assay.

Immunization Schedule

Rabbits were initially immunized with 2 mg TFA-RSA emulsified in 0.5 ml H₂O, 1.5 ml complete Freunds adjuvant (Difco Laboratories, Detroit, MI) by intradermal and intramuscular injection (1 ml in each site). Immunized
rabbits were then boosted 4 wk later with 2 mg TFA-RSA emulsified in 0.5 ml H$_2$O, 1.5 ml incomplete Freund's adjuvant (Difco Laboratories) by i.m. injection followed by a repeat boost after 2 wk. Two wk following the last booster immunization, the rabbits were exposed to 1% halothane in 80% O$_2$ (balanced N$_2$) via inhalation for 2 hr. The inhalation exposure to halothane was repeated 14 days later. Three days following the last halothane exposure, the rabbits were sacrificed via sodium pentobarbital injection and blood was collected by cardiac puncture. Clot formation was inhibited by the presence of heparin and plasma was collected after centrifugation of the blood.

An alternative method of polyclonal antibody generation was later developed which produced more specific rabbit anti-TFA-RSA antibodies. Male New Zealand White rabbits were immunized as previously described against TFA-RSA. The rabbits received a booster immunization of TFA-RSA 3 wk post-primary immunization followed by a second booster immunization 1 wk later. The rabbits were sacrificed 3 days following the last boost and plasma was collected as above.

Due to problems with non-specific recognition of liver proteins in Western immunoblot analysis by the rabbit anti-TFA-RSA plasma, the IgG antibody proteins were subsequently purified (McKinney and Parkinson, 1987). The resulting antibody preparations demonstrated high antibody titer against trifluoroacetylated protein (TFA-RSA) with decreased recognition of the carrier protein (RSA). Rabbit anti-TFA-RSA plasma was diluted four fold
with 60 mM acetate buffer, pH 4.0 and adjusted to pH 4.5 with 1 M NaOH. Caprylic acid (n-octanoic acid, Sigma) was slowly added to the solution, 25 μl per ml diluted material, and stirred for 30 min at room temp. The mixture was then centrifuged at 10,000 g for 30 min at 4° C to remove precipitated protein and the supernatant fraction was collected. Concentrated PBS (10X, 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄, 2 g KH₂PO₄, 0.744 g EDTA, 1 l H₂O, pH 7.4) was added, 10 parts supernatant to 1 part PBS, and the pH was adjusted to 7.4. The solution was cooled to 4° C and ammonium sulfate, 0.277 g/ml, was added followed by mixing for 30 min. The slurry was centrifuged at 5000 g for 15 min at 4° C. The pelleted IgG proteins were collected, reconstituted in ten fold diluted PBS (0.5 ml per ml starting volume), and dialyzed extensively overnight against diluted PBS at 4° C.

Antibody Assay
Antibodies reactive with trifluoroacetylated albumin were detected by an enzyme linked immunosorbent assay (ELISA) from an adaptation of Hastings, et al, (1991). TFA-RSA, 1 μg in 100 μl PBS pH 7.4, was absorbed overnight in 96 well polystyrene microtiter plates (Costar, Cambridge, MA) at 4° C. Rabbit serum albumin was included as a control to assay antibody recognition of the carrier protein. The plates were first washed with 0.5% Tween-20 in PBS, pH 7.4 (PBS-Tween) followed by blocking with 0.5% casein, 10 mM Tris, 0.9% NaCl, 0.02% thimerosal, pH 7.4 (casein/Tris), 150 μl per well for 1 hr at 37° C. The casein/Tris solution was then decanted and the wells were washed with PBS-Tween. The primary antibodies were
diluted from 1:100 to 1:400,000 in casein/Tris and were loaded in triplicate, 100 µl/well, followed by incubation overnight at 4° C. Next the plates were washed with PBS-Tween and bound antibody was detected by incubating the plates with goat anti-rabbit IgG peroxidase conjugate antibodies (Organon Teknika-Cappel, Malvern, PA) diluted 1:1000 in casein/Tris for 1 hr at 37° C. Following incubation with the secondary antibody, the plates were washed first with PBS-Tween then PBS, followed by development with the peroxidase substrate o-phenylenediamine (Sigma). The plates were incubated with 100 µl/well of 20 mg o-phenylenediamine, 20 µl 30% H₂O₂, 12.15 ml 0.1 M citric acid, 12.85 ml 0.2 M Na₂HPO₄, 25 ml H₂O for 20 min at room temp in the dark. The peroxidase-substrate reaction was terminated with the addition of 50 µl/well of 0.17% hydrofluoric acid, 6 mM NaOH. The absorbance at 492 nm was read using a Skatron EAR 340 microtiter plate reader (Skatron, Sterling, VA). Antibody titers were determined as the reciprocal of the dilution producing an absorbance of 0.100 and were typically between 200,000 and 400,000 with TFA-RSA as the antigen.

Detection of Covalently Bound Radioactivity to Liver Proteins

Gel Dissection Technique
A semi-quantitative measure of covalently bound radioactivity to distinct liver proteins can be determined. Liver proteins from slices exposed to ¹⁴C-halothane are first resolved by SDS-PAGE. Immediately following electrophoresis, sequential horizontal sections (0.5 cm thick) were made
down the acrylamide gel and the sections were placed in glass scintillation vials with 1 ml 1 M NaOH and left overnight at room temp. Following neutralization with 2 N HCl, radioactivity was determined by liquid scintillation counting. Molecular weight standards were used to calibrate the gels.

**Autoradiography and Fluorography of Radiolabeled Proteins**

To detect the adducts to individual proteins separated electrophoretically, the acrylamide gel was dried onto filter paper using a gel drying apparatus. The dried gel was then overlaid with Kodak X-OMAT AR x-ray film (Eastman Kodak, Rochester, NY) for 1 month at -80° C. Fluorographic detection of radiolabeled proteins using 2,5-diphenyloxazole (Aldrich, Milwaukee, WI) as a fluor was according to the method of Bonner and Laskey, (1974). Fluorography using Kodak X-OMAT AR x-ray film was carried out at -80° C for 3 wk. The x-ray film was developed using an automatic processor and compared to the original gel.

**Isolation and Identification of Cytosolic Proteins**

**Chromatographic Resolution of Cytosolic Proteins**

Cytosolic protein (9 mg) from liver slices exposed to 14C-halothane for 6 hr were applied to a Sephadex G-75 (Pharmacia LKB Biotech., Piscataway, NJ) gel filtration column (45 x 1.5 cm i.d.). Proteins were eluted off the column with 0.02 M KH₂PO₄ buffer, pH 7.45 at 4° C with a flow rate of 4 ml/hr. Column fractions were monitored for the presence of
radioactivity by scintillation counting and the absorbance at 280 nm using a Beckman DU-7 spectrophotometer. The fractions containing the greatest amount of radioactivity (fractions 43-45, 50-56) were individually prepared in sample buffer for electrophoresis. SDS-PAGE analysis determined that a 27 kDa protein was partially purified to the greatest extent in fraction #56. This preparation was then used for amino acid analysis.

Protein Sequence Analysis
The partially purified 27 kDa protein was prepared in sample buffer and heated at 37° C for 10 min prior to SDS-PAGE. Electrophoresis was carried out as previously described. Immediately following gel electrophoresis, proteins were electrophoretically transferred to PVDF blotting membranes at 70 volts for 2 hr at 4° C. Following transfer, the blots were washed extensively with H₂O and stained with Coomassie blue R-250 dye (0.10 g in 60 ml H₂O, 40 ml methanol). The blotting membranes were then destained in a 50:50 methanol:H₂O solution and allowed to dry. The 27 kDa protein was excised from the membrane and frozen at -80° C prior to sequence analysis. Automated N-terminal amino acid sequence analysis by Edman degradation was performed by Matthew Williamson of The Protein Sequencing Facility, Dept. of Biology, University of California, San Diego. Amino acid analysis was carried out using an Applied Biosystems Model 470 A Sequenator equipped with a Model 120 On-Line HPLC for detecting phenylthiohydantoin-amino acids. Data was collected and processed using a Perkin-Elmer 7500 computer.
IN VITRO MICROSONAL/CYTOSOLIC PROTEIN INCUBATIONS

Preparation of Microsomes and Cytosol
Livers were removed from male Hartley guinea pigs (600-800 g) killed by cervical dislocation and rinsed in 50 mM Tris, 1.15% KCl, pH 7.4, 4° C. Livers were homogenized (25% w/v) in the same buffer with a Dounce homogenizer. The homogenate was centrifuged at 10,000 g for 20 min and the supernatant fraction was collected. The supernatant fraction was centrifuged at 220,000 g for 45 min resulting in a microsomal pellet. The pellet was re-homogenized in the Tris/KCl buffer and the microsomes were flushed with N₂ and frozen immediately at -80° C. The supernatant fraction was centrifuged again at 220,000 g for 45 min producing the soluble cytosolic fraction. Cytosol was dialyzed extensively against 50 mM Tris, 1.15% KCl, pH 7.4 at 4° C to remove endogenous glutathione. Cytosol and dialyzed cytosol were stored at -80° C. All of the above steps were carried out on ice or at 4° C. Microsomal cytochrome P-450 content was determined by the method of Omura and Satoh (1964).

Glutathione Content Determination
Glutathione concentration of the cytosol and dialyzed cytosol was determined by the method of Standeven and Wetterhan (1991). Cytosol, 0.3 ml, was added to 0.7 ml of a trichloroacetic acid solution (5% TCA, 0.1 N HCl, 1 mM EDTA, 4° C) to precipitate protein. The protein was pelleted by centrifugation at maximum speed on a Sorval table top centrifuge. After centrifugation, 0.1 ml of supernatant fraction was added to 1.9 ml of 1%
sodium citrate buffer, pH 7.0, followed by addition of 0.5 ml 1 mM 5,5'-dithio-bis-2-nitrobenzoic acid (Sigma) in citrate buffer. A glutathione standard curve was prepared in the trichloroacetic acid solution (2 - 0.2 mM). After incubation at room temp for 15 min, the absorbance at 412 nm was determined. Glutathione was not detected in the dialyzed cytosol.

Incubation System
Microsomal and cytosolic protein (8 mg protein each) were incubated together in 2 ml 50 mM Tris, 1.15% KCl, pH 7.4 buffer in glass scintillation vials under 95% O₂ at 37° C. An NADPH generating system containing 0.5 mM NADP (Sigma), 2.75 mM D-glucose-6-phosphate (Sigma), 0.25 mM MgCl₂-6H₂O, and 1 U glucose-6-P₀₄-dehydrogenase (Sigma) was included in all incubations except controls. Reduced glutathione (Sigma) at a concentration of either 1.0 or 5.0 mM was included in some experimental groups. After a 15 min pre-incubation, either halothane (Halocarbon Laboratories) or ¹⁴C-halothane (New England Nuclear, 0.1 - 0.3 μCi) was injected through a Teflon septa cap and allowed to vaporize off a filter paper wick. A constant medium concentration of 0.6 mM halothane was determined by gas chromatography. After a 90 min incubation, the reaction was terminated by the addition of 8 ml 12.5 mM sucrose, 8.8 mM CaCl₂-2H₂O, 5.5 mM MgCl₂-6H₂O, 4° C resulting in precipitation of the microsomal protein (Baker, et al, 1973). The microsomal protein was pelleted by centrifugation at 2000 g for 10 min. Cytosolic protein was precipitated from the supernatant fraction by 5% trichloroacetic acid followed by centrifugation.
**Covalent Binding Assay**

Microsomal protein was washed first with 80% ice cold ethanol followed by centrifugation to pellet protein. The supernatant fraction was removed and the protein pellet was sonicated in 3 ml 5% trichloroacetic acid to remove any unbound metabolites. Protein was pelleted upon re-centrifugation and the TCA wash was repeated three times. Cytosolic protein was washed with 5% trichloroacetic acid as above. Protein pellets were dissolved in 1 ml 1 M NaOH and an aliquot was taken for protein determination (BCA protein assay). Covalently bound radioactivity was determined by liquid scintillation counting.

**Covalently Bound Fluorine Assay**

Microsomal and cytosolic protein were washed to remove any unbound metabolites as stated above. The protein pellets were reconstituted in 2 ml H<sub>2</sub>O and an aliquot was taken for protein determination (BCA protein assay). Protein bound fluorine was determined as described in the following section.

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**IN VIVO HALOTHANE EXPOSURE**

**Inhalation Exposure to Halothane**

Halothane exposures were carried out in a 180 l plexiglass exposure chamber equipped with a circulating fan (Lind, et al, 1990). The floor of the chamber was equipped with two 20 watt heaters to maintain body temperatures near normothermia. Male Hartley guinea pigs were placed in
the chamber and exposed to 1% halothane (Halocarbon Laboratories) in 40% O₂ (balanced N₂) with a flow rate of 6 l/min using a Vernitrol Vaporizer (Ohio Medical Products, Madison WI) for a total of 4 hr. Halothane concentrations were monitored at regular intervals by gas chromatography using a Varian 1440 Gas Chromatograph equipped with a 5% SE-30 column on Chromosorb W (5 ft by 1/8 in i.d.) and a thermal conductivity detector. Column temp was 75°C, injection temp 175°C, detector temp 180°C, and helium was the carrier gas with a flow rate of 30 ml/min. Oxygen concentration was measured with an Instrumentation Laboratories Model 408 polarographic oxygen electrode (Instrumentation Laboratories, Lexington, MA). The animals were terminated 10 hr after anesthesia by cervical dislocation and the livers were removed.

Liver glutathione content was depleted in guinea pigs to determine if glutathione acts as a protectant against acylation of protein following exposure to halothane. Guinea pigs were pretreated with 1.6 g/kg d,l-buthionine-S,R-sulfoximine (Schweizerhall Inc., Piscataway, NJ) via intraperitoneal injection 24 hr prior to 1% halothane exposure. Buthionine sulfoximine, a gamma-glutamyl cysteine synthetase inhibitor, has previously been demonstrated to deplete liver glutathione by up to 85% in the guinea pig (Lind, et al, 1992b). The buthionine sulfoximine was prepared in 10 ml 1 M NaOH/kg animal wt, at pH 8.5.

Intraperitoneal Exposure to Halothane
Guinea pigs were injected intraperitoneally with 10 mmoles halothane/kg in
a 20% corn oil solution. The animals were sacrificed 15 hr later by cervical dislocation and the livers were removed (Christ, et al, 1988b).

**Preparation of Liver Microsomes and Cytosol**

Following cervical dislocation of the animals, livers were removed and rinsed in 50 mM Tris, 1.15% KCl, pH 7.4, 4°C. Livers were homogenized (25% w/v) in the same buffer with a Dounce homogenizer. The homogenate was centrifuged at 10,000 g for 20 min and the supernatant fraction was collected. The supernatant fraction was centrifuged at 220,000 g for 45 min resulting in a microsomal pellet. The pellet was re-homogenized in the Tris/KCl buffer and frozen at -80°C. The supernatant fraction was centrifuged again at 220,000 g for 45 min producing the soluble cytosolic fraction. The cytosol was also frozen at -80°C. All of the above steps were carried out on ice or at 4°C.

**Western Immunoblot Analysis**

Liver microsomal and cytosolic protein from control and halothane exposed animals were prepared in SDS-PAGE sample buffer. Cytosolic proteins (30 μg protein/lane) were resolved electrophoretically in 12% polyacrylamide minigels with a 4% polyacrylamide stacking gel at 200 volts for 40 min. Microsomal protein (30 μg protein/lane) were separated in a 10% polyacrylamide minigel at 160 volts for 1 hr. Electrophoretic transfer to PVDF membranes and Western immunoblot analysis was as previously described.
**Covalently Bound Fluorine Assay**

Liver microsomal and cytosolic proteins from control and halothane exposed animals were precipitated with 80% ice cold ethanol and pelleted by centrifugation. Protein pellets were sonicated in 3 ml 5% trichloroacetic acid to remove any unbound metabolites followed by centrifugation. The 5% TCA wash was repeated twice, the final protein pellets were reconstituted in H₂O, and an aliquot was taken for protein determination (BCA Protein Assay). The protein homogenate was transferred to Pyrex screw top test tubes, frozen at -80°C, and lyophilized. Bound organic fluorine was determined using the sodium fusion technique (Soltis and Gandolfi, 1980; Lind, et al, 1990). Sodium metal (20-25 mg) was added to the lyophilized protein followed by heating in a Bunsen burner flame. After cooling, 1 ml H₂O, 50 μl methanol, and 50 - 100 μl glacial acetic acid were added to each sample to acidify the solution. The supernatant fraction was collected and mixed 50:50 with TISAB (Orion Research, Boston, MA) and directly analyzed for fluorine content with an Orion specific fluorine ion electrode (Model 94-09 fluorine electrode, Model 96-09 combination fluoride electrode, Orion Research Microprocessor Ionalyzer-901). Fluorine content was determined using NaF standards. Covalently bound fluorine was normalized to total protein content and expressed as nmols F/mg protein.

**Isolation of Glutathione-S-Transferase**

Liver cytosol, containing approximately 150 mg protein, was loaded on a Sephadex G-75 gel filtration column (60 cm x 3 cm i.d.). Protein was
eluted through the column with 0.01 M KH$_2$PO$_4$ buffer, pH 7.4 at 4° C. The flow rate was between 10-15 ml/hr. Fractions were collected every 20 min using a Gilson automated fraction collector and were later analyzed for the absorbance at 280 nm. Selected fractions were prepared in SDS-PAGE sample buffer and resolved electrophoretically in 12% polyacrylamide mini gels. Resolved proteins were visualized by staining with Coomassie blue R-250 dye. Fractions containing glutathione-S-transferase were pooled together and dialyzed overnight against 4 l 10 mM Tris, pH 7.8 at 4° C.

After dialysis, the protein solution was loaded onto a S-hexyl-glutathione affinity column. Non-specifically bound protein was eluted off the column with 10 mM Tris, 2 mM dithioerythritol (Sigma), pH 7.8 at 4° C. The flow rate was between 10-15 ml/hr. Specifically bound protein was eluted from the column with the above buffer containing 0.2 M NaCl and 8 mM glutathione. Fractions were collected every 20 min using an automated fraction collector and the absorbance at 280 nm was determined. The fractions containing the specifically bound protein were pooled together and examined by electrophoresis for the presence of glutathione-S-transferase. The affinity chromatography procedure is an adaptation of the method of Jensson, et al, (1985).

Any residual trifluoroacetate was removed from the pooled fractions by the addition of 0.5% acetic acid followed by dialysis overnight against 4 l of H$_2$O at 4° C. After dialysis, the protein solution was frozen, lyophilized to dryness, and assayed for covalently bound fluorine.
Preparation of S-hexyl-glutathione Affinity Chromatography Column

Affi-Gel 15 activated affinity support, 25 ml (BIO RAD), was washed with approximately 400 ml H₂O at 4° C. S-hexyl-glutathione, 500 mg (Sigma), was dissolved in 75 ml 100 mM MOPS, pH 9.0 and then mixed for 4 hr with the Affi-Gel at 4° C. After mixing, the gel support was washed with 100 ml 100 mM MOPS, pH 8.0, 4° C and the solution was decanted. The gel was then mixed with 5 ml of 1 M ethanolamine-HCl (prepared in 100 mM MOPS, pH 8.0) for 1 hr at 4° C. The gel was then packed into a glass chromatography column (25 cm x 1 cm i.d.) and washed with 100 mM MOPS, pH 8.0 at 4° C.

Glutathione-S-Transferase Enzyme Assay

Fractions containing glutathione-S-transferase were either prepared in 0.1 M phosphate, pH 7 at 4° C or dialyzed against that buffer overnight at 4° C. Separate solutions of 2 mM glutathione and 2 mM 1-chloro-2,4-dinitrobenzene, CDNB, (Sigma) were prepared in 0.1 M phosphate, pH 6.5 at room temp. The protein samples were diluted (approximately 1:10-30) in the phosphate buffer, pH 6.5 at room temp, and 0.05 ml was then mixed with 0.475 ml each of glutathione and CDNB and immediately placed in a 1 cm cuvette. Enzyme activity was assayed as the change in absorbance at 340 nm/min, measured every 10 sec for 1 min. The diluted protein sample plus buffer only was used for a background sample (blank). The molar extinction coefficient of the glutathione conjugate is 9.6/mM/cm. One unit of enzyme activity (U) is defined as the amount of enzyme catalyzing the formation of 1 µmol product/min (Habig, et al, 1974).
Statistics

Unless otherwise stated, experiments involving liver slices were prepared from approximately 10-15 guinea pigs, producing 60-90 liver slices per animal. Approximately 30-40 liver slices from each animal were combined to produce sufficient microsomal and cytosolic protein for analysis. Results obtained for the separate experimental animals were consistent and representative data is presented. Experiments involving the microsomal/cytosolic incubation system were carried out using livers from a total of 4 animals. The in vivo halothane studies utilized 8 experimental guinea pigs. Statistical tests applied to the data were ANOVA (analysis of variance) and the student's t test.
Chapter 1: Generation and Detection of Protein Adducts in Liver Slices Incubated with Halothane.

Liver slices were exposed to $^{14}$C-halothane to determine if bioactivation would result in a reactive intermediate capable of covalently binding to protein. Slices exposed to either 1.0 or 1.7 mM $^{14}$C-halothane (media concentration) in a 95% $O_2$ : 5% $CO_2$ atmosphere produced covalently bound adducts to liver protein (Fig. 3). Covalent binding of a halothane intermediate was observed at 1 hr of incubation and increased linearly through 12 hr upon exposure to 1.0 mM halothane. Covalent binding was seen to be concentration dependent. Adduct formation was approximately twice as great with the higher concentration of halothane throughout the 12 hr incubation period.

Rabbit polyclonal antibodies reactive towards trifluoroacetylated proteins were used to identify protein targets that had been trifluoroacetylated. Western immunoblot analysis of proteins from liver slices exposed to 1.7 mM halothane demonstrated the formation of five protein adducts, recognized by rabbit anti-TFA-RSA antibodies (Fig. 4). This halothane medium concentration was chosen because it produced the greatest amount of covalent binding. Trifluoroacetylated epitopes increased progressively from 1 to 12 hr of incubation, where presentation was greatest. These protein adducts have molecular weights of 97, 62, 57, 54, and 51 kDa. The
Figure 3.
Covalent binding of a $^{14}$C-halothane reactive intermediate to guinea pig liver slice proteins. Guinea pig liver slices were exposed to either 1.0 or 1.7 mM (media concentration) $^{14}$C-halothane (0.5 $\mu$Ci) in 95% O₂:5% CO₂. Covalent binding is expressed in n mole equiv/mg protein. N = 24-30 slices from three animals. Values are the mean ± standard error of the mean.
Figure 4. Western immunoblot analysis of proteins from guinea pig liver slices exposed to halothane. Liver slices were incubated for 1, 6, and 12 hr with 1.7 mM halothane. Whole liver cell proteins (30 μg protein/lane) were resolved in a 12% polyacrylamide gel and assayed for protein adduct formation using rabbit anti-trifluoroacetylated rabbit serum albumin antibodies. Control liver slice proteins from the same animal were used for comparison (C). A progressive development of proteins recognized by the anti-hapten antibodies were seen in the halothane exposed liver slices but not in the control. Protein-adduct formation was greatest at 12 hr where proteins of molecular weights (M_r) ranging from 51-97 kDa were recognized.
presence of these adducts in halothane exposed liver slices was contrasted by their absence in the control liver slices. The progressive development of these protein adducts correlates well with the linear increase seen in covalent binding (Fig. 3) over the same 12 hr time course. Several proteins of varying mass are consistently recognized by the antibodies in both control and halothane exposed liver slices. This non-specific recognition of proteins is due to the presence of epitopes recognized by either the polyclonal rabbit antibodies or the goat anti-rabbit secondary antibodies. Therefore, comparisons were always made between the profile of proteins recognized in the experimental groups with those from controls.

These results indicate that covalent binding of a halothane reactive intermediate to normal liver proteins correlates with the formation of these antigenically altered proteins. The presence of the trifluoroacetyl group, derived from trifluoroacetyl chloride, covalently bound to these proteins is suggested due to the recognition by antibodies specific for this epitope.

To determine if the oxidative route of halothane biotransformation is responsible for protein adduct formation, deuterated halothane can be substituted for the later. Deuterated halothane is more resistant to oxidative metabolism than halothane, due to the increased bond strength between carbon and deuterium atoms (Sipes, et al, 1980 and Lind, et al, 1989b). Therefore exposure to deuterated halothane should result in a
decrease in trifluoroacety chloride formation with a decrease in covalent binding to protein. Liver slices were exposed to 1.7 mM deuterated halothane for up to 12 hr (Fig. 5). The result was a decrease in detection of proteins recognized by the anti-TFA-RSA antibodies. This implies that deuterated halothane exposure results in a decrease in reactive intermediate production and subsequent hapten formation.

Liver slices were exposed to $^{14}$C-halothane in order to correlate the presence of protein adducts as recognized using antibodies (anti-TFA-RSA) with the presence of covalently bound radioactivity. Liver slices were exposed to 1.7 mM $^{14}$C-halothane for 12 hr and whole liver cell proteins were resolved by SDS-PAGE (0.25 mg protein with specific activity of 900 dpm/mg protein) followed by gel dissection and radioactivity counting. Greater than 80% of detectable radioactivity was covalently bound to proteins in the 20-30 kDa range of the polyacrylamide gel (Fig. 6). An equivalent amount of microsomal protein from these liver slices did not demonstrate detectable radioactivity significantly above background.

Whole liver cell proteins were prepared from slices exposed to $^{14}$C-halothane at various time points to determine if adduct formation to protein in the 20-30 kDa region is time-dependent. Covalent binding of a halothane intermediate to whole liver cell protein in the 20-30 kDa range was seen to increase in a linear fashion over the 12 hr incubation period (Fig. 7). The linear increase in acylation of target protein in
Figure 5.
Western immunoblot analysis of proteins from guinea pig liver slices exposed to deuterated halothane. Liver slices were incubated for 1, 6, and 12 hr with 1.7 mM deuterated halothane (d-halothane). Whole liver cell proteins (30 μg protein/lane) were resolved in a 12% polyacrylamide gel and assayed for protein adduct formation using rabbit anti-TFA-RSA antibodies. Control and halothane exposed liver slice protein, from the same animal and incubated for 12 hr, were included for comparisons of protein-adduct formation (C and H respectively).
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**d-Halothane**
Figure 6.
Resolution of protein adducts in guinea pig liver slices exposed to $^{14}$C-halothane. Whole liver cell proteins from slices exposed to 1.0 mM $^{14}$C-halothane for 12 hr were separated by SDS-PAGE (0.25 mg protein with specific activity of 900 dpm/mg protein). Horizontal sections were made in the gel and the radioactivity in those sections was determined by scintillation counting. The majority of the radioactivity was localized in the 20-30 kDa region of the gel.
Figure 7.
Linear increase in covalent binding of a $^{14}$C-halothane intermediate to 20-30 kDa molecular weight protein in guinea pig liver slices. Whole liver cell proteins from slices exposed to 1.7 mM $^{14}$C-halothane for 1, 6, and 12 hr were separated by SDS-PAGE. Horizontal sections were made in the gel and the radioactivity in those sections was determined by scintillation counting. The radioactivity localized in the 20-30 kDa region of the gels was reported. Each data point represents 10-20 liver slices produced from two guinea pig livers.
the 20-30 kDa range correlates with the increase in covalent binding to whole liver slice protein (Fig. 3).

Autoradiographic analysis was used to determine which protein(s) in the liver slice contain the covalently bound radiolabel derived from 14C-halothane. Whole liver cell protein from slices exposed to 1.7 mM 14C-halothane for 12 hr were separated by SDS-PAGE and stained with Coomassie blue dye prior to autoradiography (25 µg protein with specific activity of approximately 2.6 dpm/µg). Covalently bound radioactivity was localized to a protein(s) of apparent molecular weight of 27 kDa (Fig. 8). The radiolabel derived from halothane was not detected in any other molecular weight region. These results indicate that the 27 kDa protein(s) is a primary target for the reactive intermediate, trifluoroacetyl chloride, derived from halothane in the liver slice. Other protein sites for radiolabeled covalent binding may exist, but are below the limits of detection. (Specific activity of the radiolabel was approximately 32 pmole halothane equivalents per dpm.)

Subcellular fractionation of guinea pig liver resulting in microsomal and cytosolic fractions, followed by SDS-PAGE analysis, demonstrates that the 27 kDa protein(s) resides predominately in the cytosolic protein fraction (Fig. 9). If the 27 kDa protein(s) is the principal site for adduct formation, this may account for the lack of detectable radiolabel to microsomal protein from the slices exposed to 14C-halothane.
Figure 8.
Autoradiography of whole liver cell proteins from liver slices exposed to $^{14}$C-halothane. Liver slices were incubated for 12 hr with 1.7 mM $^{14}$C-halothane. Proteins were separated by SDS-PAGE and stained with Coomassie blue dye (lane 1) prior to autoradiography (lane 2). The autoradiogram demonstrates the presence of covalently bound radioactivity to a single protein band (2). This radiolabeled protein correlates with the 27 kDa protein(s) seen in lane 1.
Figure 9.
Localization of 27 kDa protein in microsomes and cytosol from guinea pig liver. Microsomal and cytosolic proteins from a guinea pig liver were separated by SDS-PAGE and stained with Coomassie blue dye. The 27 kDa protein(s) is present predominately in the cytosol of the liver (C) as compared to the microsomal compartment (M).
The cytosolic protein fraction was prepared from liver slices exposed to $^{14}$C-halothane to determine if a 27 kDa protein(s) becomes covalently modified by a radioactive intermediate. Cytosolic protein prepared from liver slices exposed to 1.0 mM $^{14}$C-halothane for 12 hr and separated by SDS-PAGE (0.24 mg protein with specific activity of approximately 340 dpm/mg protein) indicates the majority of the covalently bound radiolabel occurs to protein(s) in the 20-30 kDa range (Fig. 10). The electrophoretically separated cytosolic proteins were stained with Coomassie blue dye prior to fluorographic analysis for radiolabel detection. The results indicate that the $^{14}$C-halothane intermediate(s) is covalently bound to two proteins with apparent molecular weights of 27 and 26 kDa (Fig. 11). These proteins co-migrate closely together during electrophoresis. Covalent binding of a $^{14}$C-halothane derived reactive intermediate occurs predominately to proteins of molecular weights 26-27 kDa located in the cytosol.

Organically bound fluorine was quantitated as a non-radiochemical method to confirm the presence of halothane derived adducts to cytosolic protein. Liver slices were exposed to 1.7 mM halothane for 12 hr and the cytosolic protein was assayed for covalently bound fluorine. Covalent binding of a halothane intermediate to cytosolic protein was detected and quantitated as $2.04 \pm 1.10$ nmole F/mg protein (value is the mean ± std dev, N = 30-40 slices from three animals).

Proteins in solution can be separated based on their native, undenatured
Figure 10. Resolution of protein adducts in cytosol from liver slices exposed to $^{14}$C-halothane. Cytosolic protein from liver slices exposed to 1.0 mM $^{14}$C-halothane for 12 hr were separated by SDS-PAGE. Horizontal sections were made in the gel and the radioactivity in those sections was determined by scintillation counting. The majority of the radioactivity was localized to proteins in the 20-30 kDa region of the gel.
Figure 11.
Fluorography of cytosolic proteins from liver slices exposed to $^{14}$C-halothane. Liver slices were exposed to 1.0 mM $^{14}$C-halothane for 12 hr. Cytosolic protein was separated by SDS-PAGE and stained with Coomassie blue dye (lane 1) prior to fluorography (lane 2). The fluorogram demonstrates the presence of covalently bound radioactivity to two closely migrating protein bands present in the cytosol (lane 2). These radiolabeled proteins correlate to the 27 and 26 kDa proteins seen in the Coomassie stained gel (lane 1).
molecular mass by gel filtration chromatography. Cytosol from liver slices exposed to 1.0 mM 14C-halothane for 6 hr was applied to a Sephadex G-75 gel filtration column in order to partially resolve the proteins by molecular weight (Fig. 12). The majority of the protein eluted from the column between fractions 40 and 50. Protein present in these fractions typically correspond to molecular weight proteins of approximately 80 kDa and higher. The majority of the protein bound radioactivity eluting from the Sephadex column occurred by fraction #53 and correlated with the presence of a 27 kDa cytosolic protein(s) as determined by SDS-PAGE analysis (Fig. 13). The 27 kDa protein was present in a partially purified condition in column fraction #56.

In order to determine the possible identification of the 27 kDa protein, NH₂-terminal sequence analysis was performed. The column fraction containing the partially purified 27 kDa protein was prepared in sample buffer for electrophoresis. Following SDS-PAGE under denaturing conditions, this protein was subsequently transferred to a PVDF blotting membrane for N-terminal analysis. A total of ten cycles by automated Edman degradation analysis yielded the NH₂-terminal amino acid sequence (Table 1). The NH₂-terminal sequence of the 27 kDa cytosolic protein had 100% sequence homology with guinea pig glutathione-S-transferase b. (Sequence analysis was done at the University of California, San Diego).

Drug-protein adducts released or secreted from a cell upon exposure may provide a route for presentation of altered epitopes to the immune
Figure 12.
Resolution of cytosolic protein by Sephadex G-75 chromatography. Cytosol from $^{14}$C-halothane exposed liver slices was applied to a Sephadex G-75 column and eluted with 0.02 M $\text{KH}_2\text{PO}_4$ buffer, pH 7.45. Proteins were eluted from the column with a flow rate of 4 ml/hr and collected in 0.7 ml fractions. Both protein content (Abs$_{280}$ nm) and covalently bound radioactivity were compared.
Figure 13.
Electrophoretic profile of the Sephadex G-75 column fractions. Protein in the fractions containing peak radioactivity eluted from the Sephadex column were individually resolved by SDS-PAGE and stained with Coomassie blue dye. The fraction containing the partially purified 27 kDa protein (fraction #56) was used for N-terminal sequence analysis. This protein was transferred to a PVDF blotting membrane, excised from the blot, and sequenced by automated Edman degradation.
Table 1:
Comparison of the NH₂-terminal amino acid sequence of the 27 kDa cytosolic protein with guinea pig glutathione-S-transferase b (*).

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<td>3.</td>
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* Data from Kamei, Oshino, Hara (1990).
system. The in vitro liver slice system provides a tool for determining what proteins are released or secreted during the course of incubation. Because no exogenous proteins were added in any experiment, all proteins present in the media are derived solely from the liver slice.

Liver slices were exposed to 1.7 mM halothane for 3 hr and the incubation media was collected and pooled together from either halothane or control slices. Protein was precipitated from the incubation media and resolved by electrophoresis (Fig. 14). Numerous proteins were released into the media from both halothane exposed and control slices as early as 3 hr of incubation. Proteins of a 27 kDa molecular weight were released into the media in a high concentration by both control and halothane exposed liver slices.

The incubation media was pooled together and assayed for the presence of glutathione-S-transferase activity, released from either control or 1.7 mM halothane exposed liver slices at 6 hr of incubation. Enzyme activity (U) was assayed using glutathione and 1-chloro-2,4-dinitrobenzene as substrates, and defined as the amount of enzyme catalyzing the production of 1 μmol product/min (Habig, et al, 1974). Specific activity of GST, present in the media, was determined as 0.49 ± 0.23 and 0.38 ± 0.17 U/mg protein for control and halothane exposed liver slices respectively (values are the mean ± std dev, N = 45 liver slices from 3 animals). No difference was noted between the control and halothane exposed liver slices. Glutathione-S-transferase was released by the liver slices into
Figure 14.
Liver slice proteins released into the incubation media. Liver slices were incubated for 3 hr with 1.7 mM halothane and proteins released into the media were collected. Proteins were resolved by SDS-PAGE and stained with Coomassie blue dye. A 27 kDa protein(s) was released into the incubation media by both control (C) and halothane exposed slices (H).
the extracellular environment.

Liver slices were exposed to 1.0 mM $^{14}$C-halothane for 12 hr and the incubation media was collected to determine if protein adducts produced in the liver slice can be released into the media. Resolution of the media proteins by SDS-PAGE (0.24 mg protein with specific activity of approximately 470 dpm/mg protein) followed by gel dissection and scintillation counting, demonstrated that the majority of the radioactivity was covalently bound to proteins in the 24-30 kDa range (Fig. 15). The electrophoretically resolved media proteins were stained with Coomassie blue dye prior to fluorographic analysis (Fig. 16). The results indicated that a $^{14}$C-halothane intermediate was localized to a 27 kDa protein(s). A faint band of protein bound radioactivity was observed in the 100 kDa region of the gel, as depicted by fluorographic analysis. The presence of the trifluoroacetyl group covalently bound to the 27 kDa protein was implied by Western immunoblot analysis (Fig. 17). Protein adducts of 27, 43, and 61 kDa were recognized by anti-TFA-RSA antibodies. The 27 kDa protein was recognized by the anti-hapten antibodies to the greatest extent.

The majority of the protein adducts in the incubation media appear to be localized to a 27 kDa protein. Radiochemical detection of the other protein adducts appears to be below the limits of detection. These results show that protein adducts produced in the liver slice during halothane exposure can be subsequently released into the incubation media.
Figure 15.
Resolution of incubation media proteins from liver slices exposed to $^{14}$C-halothene. Liver slices were exposed to 1.0 mM $^{14}$C-halothon for 12 hr. Proteins released into the incubation media were collected and resolved by SDS-PAGE. Horizontal sections were made in the gel and the radioactivity in those sections was determined by scintillation counting. The majority of the radioactivity was localized to proteins in the 24-30 kDa region of the gel.
Figure 16. Fluorography of incubation media proteins from liver slices exposed to $^{14}$C-halothane. Liver slices were exposed to 1.0 mM $^{14}$C-halothane for 12 hr. Protein released into the incubation media was separated by SDS-PAGE and stained with Coomassie blue dye (lane 1) prior to fluorography (lane 2). The fluorogram demonstrates the presence of covalently bound radioactivity to a single protein band (lane 2). This radiolabeled protein correlates with the 27 kDa protein(s) seen in the Coomassie stained gel (lane 1).
Figure 17.
Western immunoblot analysis of incubation media proteins from liver slices exposed to halothane. Liver slices were exposed to 1.0 mM halothane for 12 hr. Proteins released into the media from control (lane 1) and halothane exposed (lane 2) slices were collected and assayed for protein adduct formation using anti-TFA-RSA antibodies. Protein adducts of molecular weights ranging from 27-61 kDa were recognized by the anti-hapten antibodies in the halothane exposed media relative to the control. The 27 kDa protein was recognized to the greatest extent.
To confirm the presence of halothane derived protein adducts in the incubation media, organically bound fluorine was quantitated. Liver slices were exposed to 1.7 mM halothane for 12 hr and protein present in the media was collected and assayed for covalently bound fluorine. Covalent binding of a halothane intermediate to protein present in the media was detected and quantitated as $4.04 \pm 3.69$ nmole F/mg protein (value is the mean ± std dev, N = 30-40 slices from three animals).
Chapter 2: Bioactivation of Halothane to a Reactive Intermediate in a Microsomal/Cytosolic Protein Incubation System.

An in vitro incubation system was used to bioactivate halothane to a reactive intermediate capable of acylating either microsomal or cytosolic protein. This model system provided a tool for studying the bioactivation of halothane under varying glutathione concentrations. The glutathione content was varied to determine what role this peptide plays in mediating acylation to target protein.

A microsomal/cytosolic incubation system was used to bioactivate 0.6 mM $^{14}$C-halothane (media concentration) to a reactive intermediate capable of covalently modifying protein. Bioactivation of halothane was dependent on the presence of cytochrome P-450 (0.9 nmole Cyt P-450/mg protein) and a NADPH generating system. Covalent binding of a halothane intermediate to microsomal protein was dependent on the glutathione concentration (Fig. 18). Protein adduct formation was greatest when glutathione (GSH) was absent and decreased with increasing GSH concentration. Covalent binding to cytosolic protein was also altered by the GSH concentration present in the incubation (Fig. 19). Adduct formation to cytosolic protein was greatest with 0.4 mM GSH present in the incubation.

Due to the lack of available $^{14}$C-halothane for radiochemical detection of adduct formation, halothane was substituted in later studies. The presence of a covalently bound halothane intermediate to protein was
Figure 18.
Effect of glutathione concentration on covalent binding of a $^{14}$C-halothane intermediate to microsomal protein in an in vitro bioactivation system. Glutathione (GSH) concentration was varied (0-5 mM) in a microsomal/cytosolic incubation system in the presence of 0.6 mM $^{14}$C-halothane (0.1 - 0.3 μCi). Covalent binding is expressed as nmole equiv/mg protein. Values are the mean ± sem, N = 9 incubations from 2 animals.
Figure 19.
Effect of glutathione concentration on covalent binding of a $^{14}$C-halothane intermediate to cytosolic protein in an in vitro bioactivation system. Glutathione (GSH) concentration was varied (0-5 mM) in a microsomal/cytosolic incubation system in the presence of 0.6 mM $^{14}$C-halothane (0.1 - 0.3 μCi). Covalent binding is expressed as nmole equiv/mg protein. Values are the mean ± sem, N = 9 incubations from 2 animals.
determined using the covalently bound fluorine assay. Covalent modification of microsomal protein was not seen to be altered significantly in the presence of varying glutathione concentrations (Fig. 20). This was in contrast to the previously reported data. Covalent binding to cytosolic protein was dependent on glutathione concentration (Fig. 21). Adduct formation to cytosolic protein was greatest with 0.3 mM GSH and decreased significantly when GSH was absent or in high concentration (5 mM). The degree of covalent binding to cytosolic protein was approximately 10 fold less than to microsomal protein, following incubations with halothane. This contrasts with the results obtained using $^{14}$C-halothane, where adduct formation was approximately equal.

Cytosolic protein from a microsomal/cytosolic incubation exposed to 0.6 mM $^{14}$C-halothane was resolved by SDS-PAGE prior to fluorographic analysis for radiochemical detection (38 µg protein with specific activity of approximately 0.5 dpm/µg protein). Covalently bound radioactivity was localized to a single protein band which correlated with a 27 kDa protein(s) (Fig. 22). Western immunoblot analysis of the cytosolic protein indicated the presence of the trifluoroacetyl group covalently bound to proteins of 27 and 22 kDa (Fig. 23). The 27 kDa protein was recognized to the greatest extent by the anti-TFA-RSA antibodies. This protein appears to be the major target for trifluoroacetylation to cytosolic protein in the microsomal/cytosolic incubation system.
Effect of glutathione concentration on covalent binding of a halothane intermediate to microsomal protein in an in vitro bioactivation system. Glutathione (GSH) concentration was varied (0-5 mM) in a microsomal/cytosolic incubation system in the presence of 0.6 mM halothane. Covalent binding is expressed as nmole F/mg protein. Values are the mean ± sem, N = 10 incubations from 2 animals.
Figure 21. Effect of glutathione concentration on covalent binding of a halothane intermediate to cytosolic protein in an \textit{in vitro} bioactivation system. Glutathione (GSH) concentration was varied (0-5 mM) in a microsomal/cytosolic incubation system in the presence of 0.6 mM halothane. Covalent binding is expressed as nmole F/mg protein. Values are the mean ± sem, N = 10 incubations from 2 animals.
Figure 22.
Fluorography of cytosolic protein covalently bound by a $^{14}\text{C}$-halothane intermediate. Cytosolic proteins from a microsomal/cytosolic incubation system were resolved by SDS-PAGE and stained with Coomassie blue dye (lane 1). The polyacrylamide gel was impregnated with the fluor 2,5-diphenyloxazole and dried onto filter paper (lane 2) prior to fluorographic analysis. The fluorogram (lane 3) demonstrates the presence of covalently bound radioactivity to a single protein band. This radiolabeled protein correlates with the 27 kDa protein(s) seen in lane 1.
Figure 23. Western immunoblot analysis of cytosolic protein from a microsomal/cytosolic incubation system. Cytosolic protein from a control (lane 1) and a 0.6 mM ¹⁴C-halothane exposed incubation (lane 2) were assayed for protein adduct formation using anti-TFA-RSA antibodies. Protein adducts of 22 and 27 kDa were recognized in the halothane exposed cytosol (lane 2) but not in the control (lane 1). The 27 kDa protein was recognized to the greatest extent.
Chapter 3: In Vivo Exposures to Halothane in Guinea Pigs.

Guinea pigs were exposed to halothane in vivo to compare the results obtained in vitro with those in the whole animal. Guinea pigs were exposed to 1% halothane in 40% O₂ (balanced N₂) for 4 hr. Animals were sacrificed 10 hr post exposure and the livers were removed for determination of protein adduct formation. These conditions have resulted in maximal covalent binding of a halothane intermediate to liver tissue macromolecules (Lind, et al, 1990). Covalent binding of halothane biotransformation intermediates to cytosolic and microsomal proteins were determined by the covalently bound fluorine assay. By 10 hr post-exposure, the vast majority of protein adducts were localized to the microsomal fraction (Fig. 24). Covalently bound adducts to cytosolic proteins were detected, although to a lesser degree (1.99 vs 13.18 nmole F/mg protein respectively).

Liver glutathione content was depleted in guinea pigs to determine if this protein thiol protects against covalent modification of proteins by halothane reactive intermediates. Guinea pigs were pretreated with buthionine sulfoximine 24 hr prior to halothane exposure. Buthionine sulfoximine has previously been demonstrated to deplete liver glutathione by up to 85% without causing hepatotoxicity or altering halothane biotransformation (Lind, et al, 1992b). The animals were exposed to 1% halothane in 40% O₂ (balanced N₂) for 4 hr and sacrificed 10 hr after exposure. Covalent binding of a halothane intermediate to microsomal
Figure 24.
Covalent binding of a halothane intermediate to guinea pig liver protein following an in vivo exposure to halothane. Guinea pigs were exposed to 1% halothane in 40% O₂ for 4 hr by inhalation. Liver microsomal and cytosolic protein fractions were produced and assayed for covalently bound fluorine. Covalent binding is expressed as nmole F/mg protein. Values are the mean ± sem, N = 3-5 animals, * indicates statistical significance vs control (p < 0.0005) by unpaired t test.
protein was not altered by glutathione depletion (Fig. 25). However, covalent binding to cytosolic proteins increased upon glutathione depletion as compared to non-depleted animals (1.99 vs 3.32 nmole F/mg protein respectively) (Fig. 26). This data suggests that glutathione depletion results in increased susceptibility of cytosolic proteins for adduct formation.

Western immunoblot analysis was used to identify specific protein adducts in the livers of animals exposed to halothane. The liver microsomal fraction from guinea pigs exposed to 1% halothane presented two unique proteins. Protein adducts of approximately 53 and 57 kDa were recognized by the anti-hapten antibodies (Fig. 27). To compare adduct formation resulting from separate routes of halothane exposure, guinea pigs were exposed to 10 mmole halothane/kg by intraperitoneal injection and sacrificed at 15 hr. This protocol was based on the work of Christ, et al, 1988. These guinea pigs expressed the same microsomal protein adducts as described above (Fig. 27).

Western immunoblot analysis of liver cytosolic proteins from animals exposed to 1% halothane revealed several unique proteins. Protein adducts of 58 and 61 kDa were recognized by the anti-hapten antibodies in the exposed animals but not in the control (Fig. 28). The 58 kDa protein was present in all of the exposed animals and was recognized to the greatest extent. A 27 kDa protein(s) was not recognized by the antibodies as unique in the exposed animals with respect to the controls.
Figure 25.
Covalent binding of a halothane intermediate to guinea pig liver microsomal protein following an in vivo exposure. Liver glutathione was depleted in guinea pigs prior to a halothane exposure by buthionine sulfoximine pretreatment (BSO). Animals were exposed to 1% halothane in 40% O₂ for 4 hr. Liver microsomal protein was produced and assayed for covalently bound fluorine. Covalent binding is expressed as nmole F/mg protein. Background fluorine has been subtracted (0.329 nmole F/mg protein). Values are the mean ± sem, N = 3 animals.
Figure 26.
Covalent binding of a halothane intermediate to guinea pig liver cytosolic protein following an in vivo exposure. Liver glutathione was depleted in guinea pigs prior to a halothane exposure by buthionine sulfoximine pretreatment (BSO). Animals were exposed to 1% halothane in 40% O₂ for 4 hr. Liver cytosolic protein was produced and assayed for covalently bound fluorine. Covalent binding is expressed as nmole F/mg protein. Background fluorine has been subtracted (0.343 nmole F/mg protein). Values are the mean ± sem, N = 3 animals.
Figure 27.
Western immunoblot analysis of guinea pig liver microsomal proteins following an in vivo exposure to halothane. Microsomal proteins were resolved by SDS-PAGE and assayed for adduct formation using anti-TFA-RSA antibodies. Lanes 1-3 = 1% halothane in 40% O₂, lanes 4, 5 = 10 mmol/kg halothane/kg i.p., lane C = control. Protein adducts of 53 and 57 kDa were recognized in the halothane exposed guinea pigs.
Figure 28. Western immunoblot analysis of guinea pig liver cytosolic protein following an in vivo exposure to halothane. Cytosolic proteins were prepared from guinea pigs exposed to 1% halothane in 40% O₂ for 4 hr (lanes 1, 2, 3) and assayed for adduct formation using anti-TFA-RSA antibodies. Cytosol from a control animal (C) was included for comparison. Protein adducts of 58 and 61 kDa were recognized in the halothane exposed animals.
Guinea pigs injected with 10 millimoles halothane/kg expressed protein adducts of 58 and 61 kDa in the liver cytosol (Fig. 29). The 58 kDa protein was recognized to the greatest extent by the anti-TFA-RSA antibodies. This 58 kDa protein appears to be a highly antigenic protein adduct in the liver cytosol following an in vivo exposure. A 27 kDa protein(s) was not recognized by the antibodies as unique in the exposed animals with respect to the control.

Guinea pig liver microsomal proteins from glutathione depleted and 1% halothane exposed animals demonstrated the formation of several protein adducts recognized by anti-TFA-RSA antibodies. Protein adducts of 53, 58, and 62 kDa were seen in the halothane exposed animals (Fig. 30). The 53 and 62 kDa proteins were recognized to the greatest extent. These protein adducts may be the same as those previously seen in the microsomal fraction of guinea pigs exposed to halothane in vivo (Fig. 27).

Guinea pig liver cytosolic proteins from the glutathione depleted and 1% halothane exposed animals demonstrated the formation of protein adducts recognized by the anti-TFA-RSA antibodies. A protein adduct of 58 kDa was recognized in all of the halothane exposed animals whereby a 68 kDa protein was present in only two experimental animals (Fig. 31). This 68 kDa protein was not seen in the non-glutathione depleted animals. This protein adduct appears to be unique due to the fact that the apparent molecular weight is significantly different from those previously described. Once again, a 27 kDa protein(s) was not distinguished as
Figure 29.
Western immunoblot analysis of guinea pig liver cytosolic protein following an intraperitoneal exposure to halothane. Cytosolic proteins were prepared from guinea pigs exposed to 10 mmoles halothane/kg via ip injection (lanes 1, 2) and assayed for adduct formation using anti-TFA-RSA antibodies. Cytosol from a control animal (C) was included for comparison. Protein adducts of 58 and 61 kDa were recognized in the halothane exposed animals.
Figure 30.
Western immunoblot analysis of guinea pig liver microsomal protein following an in vivo exposure to halothane. Microsomal proteins were prepared from glutathione depleted guinea pigs exposed to 1% halothane in 40% O₂ for 4 hr (lanes 1-3). Microsomes from control animals were included (lanes 4, 5) for comparison. Protein adducts of 53, 58, and 62 kDa were recognized by the anti-TFA-RSA antibodies in the halothane exposed animals but not in the controls.
Figure 31.
Western immunoblot analysis of guinea pig liver cytosolic protein following an in vivo exposure to halothane. Cytosolic proteins were prepared from glutathione depleted guinea pigs exposed to 1% halothane in 40% O₂ for 4 hr (lanes 1-3). Cytosol from control animals were included (lanes 4, 5) for comparison. Protein adducts of 58 and 68 kDa were recognized by the anti-TFA-RSA antibodies in the halothane exposed animals but not in the controls.
unique by the anti-hapten antibodies between the control and exposed animals.

Guinea pig liver glutathione-S-transferase has been shown to be the major site for protein adduct formation in the liver slice, following exposure to halothane. Western immunoblot analysis has not identified this protein, with apparent molecular weight of 27 kDa, as a target for covalent modification in the livers of guinea pigs exposed in vivo to halothane. Glutathione-S-transferase was isolated from the livers of guinea pigs exposed to 1% halothane in 40% O₂ for 4 hr, and assayed for covalently bound fluorine to detect the presence of protein adducts. This enzyme was isolated from liver cytosol by Sephadex G-75 gel filtration chromatography followed by affinity chromatography on a S-hexyl-glutathione affinity column (Table 2). This procedure resulted in the isolation of the glutathione-S-transferase isozymes (the majority with molecular weights of 25 kDa) as determined by SDS-PAGE (Fig. 32). The lower molecular weight determination was more accurately determined using a 15% polyacrylamide gel rather than a 12% gel. Covalently bound fluorine to glutathione-S-transferase was detected and quantitated as 4.7 ± 1.6 nmole F/mg protein (value is the mean ± sem, N = 3 animals). No detectable protein bound fluorine was seen with control glutathione-S-transferase.

Guinea pig liver cytosol from control and halothane exposed animals was assayed for glutathione-S-transferase enzyme activity to determine if a
Table 2.
Purification Scheme for Glutathione-S-Transferase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (ml)</th>
<th>[Protein] mg/ml</th>
<th>Specific Activity U/mg protein</th>
<th>Total Activity U</th>
<th>% Yield</th>
<th>Fold Purif.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>6</td>
<td>26.4</td>
<td>6.40</td>
<td>1015</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Seph G-75</td>
<td>23</td>
<td>1.4</td>
<td>23.94</td>
<td>782</td>
<td>77</td>
<td>3.7</td>
</tr>
<tr>
<td>S-Hexyl-GSH</td>
<td>20</td>
<td>0.7</td>
<td>39.89</td>
<td>576</td>
<td>57</td>
<td>6.2</td>
</tr>
</tbody>
</table>

One unit of enzyme activity (U) is defined as the amount of enzyme catalyzing the formation of 1 μmol product per min under the assay conditions (Habig, et al, 1974).
Figure 32. Electrophoretic resolution of isolated glutathione-S-transferase isozymes from guinea pig liver. Glutathione-S-transferase (GST) was isolated from the cytosolic fraction of the livers of guinea pigs exposed to 1% halothane in 40% O₂ for 4 hr. The isozymes of GST were electrophoretically resolved in a 15% polyacrylamide gel and stained with Coomassie blue dye (Lane 1).
change occurred upon halothane exposure. Liver cytosol was prepared from animals exposed to 1% halothane in 40% O₂ for 4 hr and assayed for activity using glutathione and 1-chloro-2,4-dinitrobenzene as substrates (Habig, et al, 1974). Enzyme activity (U) is defined as the amount of enzyme catalyzing the production of 1 μmol product/min. Specific activity of GST was determined as 3.39 ± 0.08 and 3.34 ± 0.06 U/mg protein for control and halothane exposed animals respectively (values are the mean ± std dev, N = 3 animals). No difference in glutathione-S-transferase activity was noted between the control and experimental group.
DISCUSSION

Drug induced hypersensitivity reactions present unique problems due to their idiosyncratic nature, low incidence, and potentially serious outcomes. Immunologically mediated responses to chemical exposure represent a complex field of chemical toxicology and clinical medicine. Presently, our knowledge of the etiology of these processes is limited, due to the difficulty in reproducing the clinical pathology and outcome in animal models (Park, et al, 1990; Uetrecht, 1992). To understand the mechanisms of drug and chemical induced toxicity, we must first describe the biological fate of xenobiotics in the organism, along with the physiological responses elicited upon exposure.

In order for an immune hypersensitivity reaction to a foreign compound or drug to occur, appropriate immune sensitization to the foreign substance must take place. A small molecular weight compound such as a drug or metabolite must be covalently bound to a protein carrier and presented to the immune system. The compound or hapten can thus be recognized as a novel epitope with subsequent cellular or humoral immune responses directed towards it (Coleman, 1990). Following repeat presentation or exposure to the sensitized immune system by the hapten-protein complex, a hypersensitivity response can be elicited involving either the humoral or cellular components of the immune system (Park, et al, 1987).

The anesthetic halothane can be oxidatively biotransformed by the hepatic
cytochrome P-450 system to produce the reactive intermediate, trifluoroacetyl chloride. This intermediate has the potential to covalently bind to lysine residues of native liver proteins to produce trifluoroacetyl-N-ε-amino-lysine. This drug-protein or hapten-protein conjugate may be involved in eliciting an adverse immune hypersensitivity response against the liver (Martin, 1992). Alternatively, protein adduct formation may also precipitate an acute hepatotoxicity (Lind, et al, 1990). Dual toxicities may result from the covalent modification of liver proteins.

Numerous halothane derived protein adducts have been identified in the livers of rats, rabbits, and humans following exposure to halothane (Kenna, et al, 1992; Roth, et al, 1988; Kenna, et al, 1988). Extrahepatic sites of protein adduct formation have also been identified (Huwyler, et al, 1992). However, at the present time, neither the protein adducts responsible for eliciting an immune response, nor the mechanism for presentation of these drug-protein conjugates to the immune system, have been described (Brown, et al, 1992). In addition, the covalent modification of critical protein targets may result in the acute hepatotoxicity noted with halothane (Lind, et al, 1990). These proteins have yet to be identified.

Initially when this research project was undertaken, the goal was to utilize an in vitro liver slice system as a tool for studying the bioactivation of halothane with the hope that protein adduct formation
would occur. Liver slices were chosen due to their capacity to bioactivate halothane in an in vitro system similar physiologically to an intact organ (Ghantous, et al, 1990b). The liver slice system allows the manipulation of experimental conditions, along with a more precise analysis of the time course of events to be described. This system is advantageous since $^{14}$C-halothane can be used efficiently and economically as a tool in describing protein adduct formation. This research expanded into a microsomal/cytosolic protein incubation system followed by studies in vivo, in an attempt to correlate and expand upon the findings described using liver slices.

Guinea pig liver slices determined the conditions required for the bioactivation of halothane to a reactive intermediate, the extent of adduct formation, and whether covalent binding correlates with the formation of antigenically altered proteins (Brown, et al, 1991). Liver slices incubated with $^{14}$C-halothane demonstrated that halothane is bioactivated to a reactive intermediate capable of covalently modifying liver protein. Adduct formation increased in a linear fashion and correlated with the development of antigenically altered proteins. Five distinct protein adducts, recognized by anti-trifluoroacetylated rabbit serum albumin antibodies (anti-TFA-RSA), were seen by 12 hr of incubation. These protein adducts have the molecular weights of 97, 62, 57, 54, and 51 kDa. The presence of a covalently bound trifluoroacetyl group was inferred due to the recognition of these proteins by the anti-hapten antibodies. The time course of adduct presentation produced in vitro
correlates well with previous in vivo studies. Guinea pigs exposed to 1% halothane for 4 hr presented TFA antigen by 6 hr and maximal expression by 12 hr post exposure, as determined by immunohistology (Hubbard, et al, 1989).

Liver slices were exposed to deuterated halothane to determine if oxidative metabolism is a prerequisite for the generation of a reactive intermediate capable of covalently modifying protein. Deuterium substitution on the halothane molecule renders this compound more resistant to oxidative metabolism, due to the increased strength of the deuterium-carbon bond (Lind, et al, 1989). Past in vivo studies have shown that oxidative biotransformation of halothane is a prerequisite for covalent binding to liver protein, with subsequent development of liver injury in guinea pigs (Lind, et al, 1990). Liver slices exposed to deuterated halothane produced a decreased amount of detectable protein adducts reactive with the anti-TFA antibodies, confirming the importance of oxidative metabolism.

The protein adducts identified in the liver slices appear to be consistent with those previously reported in the rat, which were identified with antibodies reactive towards trifluoroacetylated proteins. A 100 kDa protein neoantigen has been identified as endoplasmin, an endoplasmic reticulum glycoprotein (Thomassen, et al, 1991). The 97 kDa protein adduct seen in the liver slice may be related to this protein. The 59 kDa protein is currently believed to be a microsomal carboxylesterase
glycoprotein (Satoh, et al, 1989). Recently, a 64 and a 57 kDa guinea pig carboxylesterase has been reported (Hosokawa, et al, 1990). The possibility exists that either the 62 or the 57 kDa proteins seen in the guinea pig liver slices are related to the same carboxylesterase. The difference in molecular weight may be due to species variability for this protein. Alternatively, the 62 kDa protein seen in the liver slices may be similar to the 63 kDa protein, calreticulin, identified as a target for trifluoroacetylation in the rat (Butler, et al, 1992). The 57 kDa protein adduct seen in this study may also be the 57 kDa protein disulfide isomerase isolated from liver microsomes of halothane exposed rats (Martin, et al, 1989). The 54 kDa protein seen in the guinea pig may be related to the rat liver 54 kDa neoantigen which has been determined to be a cytochrome P-450 isozyme (Satoh, et al, 1985).

Liver slices were exposed to 14C-halothane to correlate the presence of protein adducts as recognized using antibodies (anti-TFA-RSA) with covalently bound radioactivity. Radiolabeled halothane provides direct chemical evidence for protein adduct formation. The presence of the trifluoroacetyl group of halothane covalently bound to protein has been inferred by past researchers due to antibody recognition of various proteins. The resolution of radiolabeled proteins by SDS-PAGE, followed by autoradiography or fluorography provides an alternative to antibody recognition of protein adducts. Antibody recognition of trifluoroacetylated proteins may be limited due to variance in antibody specificity, variable epitopes of the carrier protein, and cross
reactivity with native protein (Christen, et al, 1991b; Martin, et al, 1992). Furthermore, the use of radiolabeled halothane facilitates the purification and subsequent identification of target proteins undergoing covalent modification.

Liver slices were exposed to $^{14}$C-halothane under a 95% O$_2$ atmosphere for up to 12 hr. Covalent binding of a halothane intermediate occurred predominately to liver slice protein in the 20-30 kDa range and increased in binding over the 12 hr exposure (Brown, et al, 1992). These results are in contrast to the antibody recognition of trifluoroacetylated liver slice proteins, which occurred in the 51-97 kDa molecular weight region. Paradoxically, the majority of covalently bound adducts, containing the radiolabel derived from halothane, occurred to lower molecular weight proteins.

The results obtained with $^{14}$C-halothane suggest that a distinction exists between the absolute degree of adduct formation and the degree of antigenicity of a specific protein, as recognized by the anti-hapten antibodies. Covalent modification of high molecular weight proteins in the liver slice appear to produce highly antigenic novel epitopes, which are reactive with the anti-hapten antibodies. However, the actual amount of covalently bound adducts to these proteins may be substantially less when compared to alternate protein sites in the cell. The immunogenicity of trifluoroacetylated proteins appear to be more dependent upon the structural features of the specific proteins than on the level of TFA

Greater than 80% of the detectable radiolabel was localized to proteins of cytosolic origin with molecular weights of 26 and 27 kDa. The 27 kDa protein appears to be a major cytosolic protein as evidenced by its predominance in the cytosolic fraction (Brown, et al, 1992). Purification of the 27 kDa protein followed by N-terminal sequence analysis of the first ten amino acids showed 100% sequence homology with guinea pig glutathione-S-transferase b (Kamei, et al, 1990).

Glutathione-S-transferases (GST) are cytosolic proteins of ubiquitous nature. These enzymes catalyze the conjugation of glutathione with electrophilic compounds as a cellular defense mechanism in dealing with xenobiotics and reactive intermediates. Various isozymes of GST are found in the liver cytosol and constitute as much as 5% of total cytosolic protein (Boyer, 1989; Vos and Van Bladeren, 1990). GST is localized predominately in the centrilobular region of the liver lobule, which is also the primary site of halothane bioactivation (Redich, et al, 1982). GST are dimers with native molecular weights of approximately 50 kDa. Various subunits exist in the guinea pig with molecular weights of 23.5 - 25.7 kDa (Oshino, et al, 1990). GST b is the major isozyme of glutathione-S-transferase in the guinea pig liver and is a homodimer with subunits of 25.7 kDa. This isozyme has a near neutral isoelectric point and has 80% sequence homology with the rat GST 3-3 (Yb) isozyme. Guinea pig GST b is therefore a mu class isozyme (Kamei, et al, 1990).
Glutathione-S-transferases have been demonstrated to be targets for covalent binding by xenobiotics such as bromobenzene, benzo(α)pyrene, phenol, and ethacrynic acid (Schelin, et al, 1983; Aniya, et al, 1988; Wallin and Morgenstern, 1990; Yamada and Kaplowitz, 1980). These enzymes are also targets for inactivation by halogenated ethylenes such as 1,1-dichloroethylene and ethylene dibromide (Moslen and Reynolds, 1985; Ivanetich, et al, 1984). GST appears to be the major target for trifluoroacetylation in the liver slice following exposure to halothane.

In past studies, researchers had focused on the microsomal fraction as the primary site for halothane derived protein adduct formation in the liver. Recently, however, other investigators have identified the cytosolic compartment to be a subcellular site for protein adducts. Rats exposed to halothane by inhalation produce numerous trifluoroacetylated cytosolic proteins (Harris, et al, 1991; Harris, et al, 1992). Due to the limits of detection for radiolabeled proteins, other trifluoroacetylated proteins were not detected. Protein targets of higher molecular weight, specifically in the 50-100 kDa range, may become acylated to a lower extent or are present in the cell in a sufficiently low concentration such that covalent modification by the 14C-halothane intermediate is below the limits of detection (approximately 2 dpm/μg protein as determined by electrophoretic techniques). That previous researchers have reported microsomal proteins as the major targets for trifluoroacetylation may be due to species variation, routes of exposure to halothane, experimental
protocol, or antibody specificity (Kenna, et al, 1988). Animals pretreated with enzyme inducers such as phenobarbital or isoniazid prior to halothane exposure may present an artificial elevation of cytochrome P-450 isozymes and microsomal proteins, resulting in preferential targeting of those proteins (Kenna, et al, 1990).

The time course of analysis following a single exposure to halothane may be important for the detection of various protein adducts. Proteins which become trifluoroacetylated exhibit various halflives in the hepatocyte as demonstrated in the rat following intraperitoneal exposure to halothane (Kenna, et al, 1990). Protein adducts may become preferentially degraded or released from the hepatocyte and thereby exhibit heterogeneous expression. A decrease in TFA adducts over time may be due to endogenous deacetylase activity, normal protein turnover, and proteases specific for chemically modified proteins (Christen, et al, 1991b). It is possible that following a single exposure to halothane, cytosolic protein adducts undergo a more rapid turnover as compared to the microsomal adducts. Therefore the time of analysis for detection of these adducts may be important. Liver slices are exposed continuously to halothane over a 12 hr period, whereby halflives of these protein adducts may not be a factor in expression.

Patients undergoing halothane anesthesia can release hepatic glutathione-S-transferase into the circulation as early as 3 hr post anesthesia (Hussey, et al, 1988). The mechanism for enzyme leakage is believed to be
loss of hepatocellular integrity. Release of this enzyme from hepatocytes is also seen following exposure to bromobenzene and carbon tetrachloride (Aniya and Anders, 1985; Lee, et al, 1991). Glutathione-S-transferase may become trifluoroacetylated and subsequently released from the hepatocyte following halothane exposure. This may account for the absence of this protein adduct detected in previous in vivo studies. The release of protein adducts into the extracellular environment may provide a mechanism for immune recognition of trifluoroacetylated proteins (Roth, et al, 1988).

The enclosed in vitro slice system provides a tool for determining what proteins are released or secreted from the liver slice during the course of incubation. Because no exogenous proteins were added in any experiment, all proteins present in the media are derived solely from the liver slice. Liver slices exposed to halothane over a 12 hr period produced and released protein adducts into the incubation media. The majority of these adducts were localized to a 27 kDa protein(s). This protein(s) is most likely glutathione-S-transferase. Enzymatically active GST was present in the incubation media, by 6 hr, following release from the liver slices. Glutathione-S-transferase is covalently modified in the cytosol during halothane exposure. Because GST is readily released from the hepatocyte upon chemical exposure, this protein can transport the bound adduct into the extracellular environment. To a lesser extent, other protein adducts of higher molecular weight can act in a similar fashion. This mechanism may provide a route for transporting hapten-
protein conjugates to the external environment whereby immune recognition may occur.

Glutathione (GSH) is a tripeptide (L-gamma-glutamyl-L-cysteinyl-glycine) containing a free thiol group and is the most abundant nonprotein sulfhydryl present in the cell. GSH is present throughout the tissues of the body but is most heavily concentrated in the liver, where it exists predominately in the cytosol in 5-10 mM concentration. This compound plays a key role in maintaining cellular homeostasis. Many of glutathione's functions are due to the reactive thiol present on the cysteine residue. GSH provides a mechanism of detoxification of electrophilic compounds and reactive intermediates produced in the cell or upon chemical exposure. GSH is responsible for reducing protein disulfide bonds and maintaining the thiol status in the cell. Organic peroxides can be reduced by GSH with catalysis by glutathione reductase. GSH serves as the major store of cysteine and provides the transport of this amino acid between organs (Deleve, 1990).

The possibility exists that glutathione may react with trifluoroacetyl chloride as a detoxification mechanism during halothane exposure. Acyl chlorides are highly electrophilic and should readily react with an available thiol group (Liebler, et al, 1988). Glutathione may act to mediate the degree of covalent binding to protein by trifluoroacetyl chloride and therefore serve as a defense for halothane hepatotoxicity. This hypothesis has been promoted by the work of Lind and co-workers.
Guinea pigs depleted of liver glutathione by buthionine sulfoximine pretreatment and exposed to halothane, produced an increase in protein adduct formation with subsequent increased hepatotoxicity (Lind, et al, 1992b). Buthionine sulfoximine pretreatment did not alter halothane metabolism or cause toxicity by itself. Recently, an animal model for fatal halothane hepatotoxicity has been developed in guinea pigs with depleted liver glutathione content, following buthionine sulfoximine pretreatment (Lind, et al, 1992c).

An in vitro incubation system, based on the liver slice apparatus, was used to bioactivate halothane to a reactive intermediate capable of acylating protein. Both microsomal and cytosolic protein were made available as potential targets for the intermediate. The glutathione content was varied to determine if this peptide can interact with the reactive intermediate and mediate the covalent modification of protein. Covalent binding of a halothane intermediate to cytosolic protein was highly dependent on the glutathione concentration. Adduct formation was greatest with a low GSH concentration (0.3-0.4 mM) and decreased with increasing concentration or when GSH was absent. This data suggests that glutathione interacts with a reactive intermediate and mediates acylation of cytosolic protein in a concentration specific manner. A 27 kDa protein was identified as the major target for trifluoroacetylation in the cytosolic protein fraction. The results obtained regarding microsomal protein were conflicting. Incubations using 14C-halothane demonstrated that radiolabeled binding to microsomal protein was dependent on the GSH
concentration. When halothane was substituted for the radiolabeled compound, the covalently bound fluorine assay was used to quantify adduct formation. Covalent binding to microsomal protein, as expressed as nmole F/mg protein, was not influenced by the GSH concentration. This discrepancy in data cannot be explained.

The relationship between glutathione (GSH) concentration and adduct formation to cytosolic protein appears to be highly specific and suggests that GSH is required for targeting these proteins for covalent modification. This may be due to the enzymatic conjugation of GSH with a reactive intermediate by glutathione-S-transferase(s). Glutathione-S-transferase mediated enzymatic catalysis of GSH with electrophilic compounds is more predominate at low GSH concentrations (Rollins and Buckpitt, 1979). With increasing GSH concentrations, rapid nonenzymatic detoxication can predominate (Coles, et al, 1988). It is possible that the low GSH concentrations (0.3-0.4 mM) present in the in vitro incubation system induce glutathione-S-transferase to become more enzymatically active. This may result in the selective targeting of the enzyme for covalent modification by the halothane reactive intermediate, trifluoroacetyl chloride.

A major target for adduct formation in the microsomal/cytosolic incubation system was a 27 kDa cytosolic protein. This protein may be glutathione-S-transferase. Together with the results obtained using liver slices, the data suggests that GST catalyzes the reaction of the acid chloride,
derived from halothane, with glutathione and in the process becomes trifluoroacetated. Recently, a lysine residue has been identified in the active site of glutathione-S-transferase. This amino acid is in close proximity to the bound glutathione molecule and may interact with the carboxylate group of the glycine part of GSH (Widersten, et al, 1992; Reinemer, et al, 1992). This lysine residue may be a preferred target for the acid chloride, providing a mechanism for the specific targeting of the transferase for acylation.

An alternate mechanism for the selective targeting of glutathione-S-transferase for adduct formation may be proposed due to a non-cytochrome P-450 mediated pathway. Direct conjugation of glutathione with halothane, or catalysis by the transferase, may produce trifluoroacetaldehyde, capable of forming a shiff base with lysine. This reaction may be similar to that described between dichloromethane and glutathione, which results in formaldehyde formation (Monks, et al, 1990). However, no data in the literature supports this hypothesis or demonstrates the ability of halothane to react with nucleophiles without cytochrome P-450 activation. Halothane also does not appear to be a glutathione-S-transferase substrate (Ivanetich, et al, 1988).

Guinea pigs were exposed to halothane by inhalation to compare and contrast the events occurring in the whole animal, with the results obtained using in vitro methodology. Guinea pigs anesthetized with 1% halothane for 4 hr produced covalently bound adducts to both microsomal
and cytosolic proteins, as determined with the covalently bound fluorine assay. The majority of the adduct formation occurred to microsomal protein targets. This is contrasted to what occurred in liver slices, where cytosolic proteins were the primary target. However, the extent of covalent modification to liver cytosolic protein was the same in both liver slices and the whole animal (2.04 and 1.99 nmole F/mg protein respectively).

Western immunoblot analysis, utilizing anti-TFA-RSA antibodies, was used to identify antigenically altered proteins produced in the livers of animals exposed in vivo. Protein adducts of 57 and 53 kDa were detected in the microsomal fraction and proteins of 61 and 58 kDa were identified in the cytosolic fraction. These antigenically recognized protein adducts correspond to those previously described in liver slices. Recently, a 58 kDa liver cytosolic protein was identified as a target for trifluoroacetylation in rats exposed to halothane by inhalation (Harris, et al, 1991).

The anti-hapten antibodies did not demonstrate that glutathione-S-transferase becomes antigenically altered in vivo. This however, is inconclusive evidence as to whether GST is a target for acylation under the experimental conditions examined. The presence of the trifluoroacetyl group bound to this protein may result in an antigenically weak epitope, poorly recognized by the antibodies in Western immunoblot analysis. This may be due to a low degree of acylation to the individual peptides of the
transferase or due to the structural features of the carrier molecule. An alternative methodology was employed to assay for the presence of the adduct to this protein. Cytosolic glutathione-S-transferase was isolated and purified from the livers of animals exposed to 1% halothane for 4 hr. Using the covalently bound fluorine assay, the adduct derived from halothane was identified bound to GST (4.7 nmole F/mg GST or 1.57 nmole TFA equiv/20 nmole GST). Stoichiometric calculations determined that 0.08 mole equivalents of a halothane intermediate are bound per mole GST in the experimental conditions examined.

Liver glutathione content was depleted in guinea pigs prior to a halothane exposure to determine if a change in acylation of protein would occur. Covalent binding of a halothane intermediate to microsomal proteins was not altered by glutathione depletion. However, GSH depletion produced a 67% increase in adduct formation to cytosolic proteins. This should not be surprising because the vast majority of cellular GSH resides in the cytosolic compartment, where synthesis occurs. The microsomal fraction, derived from the endoplasmic reticulum, contains very little glutathione and the majority is in the oxidized form (GSSG) (Hwang, et al, 1992). Therefore, buthionine sulfoximine pretreatment of the guinea pigs should deplete predominately the cytosolic pool of GSH, thereby making cytosolic proteins more susceptible to an acylating intermediate. This data suggests that covalent modification of cytosolic protein by a halothane intermediate is dependent on liver glutathione concentration. These results correlate well with the data obtained with the in vitro
bioactivation system, where covalent modification of cytosolic protein was highly dependent on the GSH concentration present in the incubation.

The data produced in this research project can be used to postulate the events occurring in the hepatocyte during halothane exposure (Fig. 33). Halothane is bioactivated by the cytochrome P-450 system, located on the cytosolic face of the endoplasmic reticulum, to the reactive intermediate trifluoroacetyl chloride (Brown and Black, 1989; Kenna, et al, 1992). This reactive species can covalently modify proteins associated with the endoplasmic reticulum. The acid chloride can also covalently react with cytosolic proteins. A major target for the intermediate in the cytosol is glutathione-S-transferase. GST can be released from the hepatocyte upon loss of membrane integrity, subsequently transporting the adduct to the external environment. The release of trifluoroacetylated proteins, such as GST, from the cell to the extracellular environment may provide a route for presentation of these novel epitopes to immunocompetent cells. This may provide a mechanism for immunological sensitization to trifluoroacetylated proteins. Glutathione-S-transferase may also provide a role as a "sink" for reactive intermediates, thereby protecting other proteins from acetylation. GST may act as a cellular defense mechanism in preventing the covalent modification of proteins vital for cell homeostasis.

Glutathione plays a role in cellular detoxification by trapping the acid chloride intermediate in the cytosol. Depletion of glutathione
Figure 33.
Summary of the events occurring in the hepatocyte during halothane exposure. Abbreviations used are; CYT = cytosol, CYTP450 = cytochrome P-450, ER = endoplasmic reticulum, GSH = glutathione, GST = glutathione-S-transferase, H = halothane, PM = plasma membrane, TFA = trifluoroacetyl, TFACL = trifluoroacetyl chloride.
concentration results in increased protein adduct formation with increased hepatotoxicity. Enzymatic catalysis of GSH with the acid chloride may be mediated by glutathione-S-transferase. This interaction between enzyme and substrate may provide a mechanism for the specific targeting of the transferase for covalent modification. The reaction between GSH and trifluoroacetyl chloride may result in the formation of a S-trifluoroacetyl-glutathione conjugate, which may be present for a short period of time.

Future efforts will be needed to expand upon the data and ideas presented in this dissertation project. The possibility that a S-trifluoroacetyl-glutathione (S-TFA-GSH) conjugate exists was suggested in this study. Previous experimental evidence suggests the existence of this intermediate. Liebler and co-workers synthesized and identified S-(2-chloroacetyl)-glutathione, a putative intermediate of 1,1-dichloroethylene (Liebler, et al, 1988). If a S-TFA-GSH conjugate exists in the hepatocyte, then a transfer of the trifluoroacetyl group to lysine residues on proteins could occur. Glutathione-S-transferase, which has a high affinity glutathione binding site, may be preferentially targeted by such a conjugate. The existence of a lysine residue in the glutathione binding site provides supporting evidence for the selective targeting of the transferase by a halothane reactive intermediate (Widersten, et al, 1992). This selective targeting of GST has actually been demonstrated, with glutathione conjugates of chlorinated benzoquinones (Van Ommen, et al, 1991). To resolve this research question, an attempt can be made to
synthesize and purify S-trifluoroacetyl-glutathione. Successful synthesis of the conjugate may allow studies involving transfer of the trifluoroacetyl group to protein targets such as GST to be undertaken.
REFERENCES


