ACETONE POTENTIATION OF 1,1,2-TRICHLOROETHANE HEPATOTOXICITY

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To my family

whose support and encouragement over the years have been

instrumental in bringing these efforts to fruition

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ABSTRACT

A variety of alcohols and ketones have been demonstrated to potentiate the hepatotoxicity of a number of hepatotoxins. The mechanism by which this potentiation occurs has remained obscure. To investigate possible mechanisms of potentiation an animal model was developed utilizing the commonly used industrial ketone, acetone, and a halogenated hydrocarbon hepatotoxin, 1,1,2-trichloroethane, in male Sprague-Dawley rats. An oral dose of 0.5 ml/kg was found to be the most effective single dose for potentiating 1,1,2-trichloroethane hepatotoxicity when administered 16-18 hours prior to the toxin. The potentiation effect was found to be greatest at or near the threshold dose of 1,1,2-trichloroethane induced hepatotoxicity. Acetone pretreated rats displayed significantly lower hepatic reduced glutathione levels two hours after all doses of 1,1,2-trichloroethane compared to controls. This suggested that acetone pretreatment may enhance the bioactivation of 1,1,2-trichloroethane to an electrophilic reactive intermediate. In vitro microsomal incubations demonstrated that hepatic microsomes from acetone pretreated rats are more active in producing metabolites of ^{14}C -1,1,2-trichloroethane that are covalently bound to microsomal proteins and lipids. In vivo experiments also suggest that the potentiation of 1,1,2-trichloroethane hepatotoxicity by acetone pretreatment is due, at least in part, to increased bioactivation of 1,1,2-trichloroethane to a reactive intermediate.

INTRODUCTION

Man is exposed to a wide variety of chemicals in the food he eats, the water he drinks, and the air he breathes. The source of these exposures may be from environmental contamination, the workplace, or the home. In contrast to the human situation, animal toxicity studies are usually designed to test the toxicity of single agents. Because of this discrepancy between real life exposures and the laboratory setting, the extrapolation of animal toxicity data to the human condition is extremely difficult.

Toxic interactions between different chemicals in animals are well documented (Casarett, 1975). Despite the recognition of many toxic interactions, the different mechanisms by which an exposure to one or more chemicals may affect the toxic response to other chemicals are not well known or understood. In order to more accurately assess and predict toxicities of both new and existing compounds it is important that we understand how these toxicities may be altered by prior, coincident, or subsequent exposures to other chemicals. Toxic interactions need to be more fully understood and closely defined so that they may be considered and incorporated into toxicologic risk assessments and other regulatory decision making processes.

Potentiation is one toxic interaction that deserves special attention, because it results in a net toxicity greater than would be expected if no interaction occurred. Casarett (1975) defines potentiation as a toxic interaction in which coincident exposure to more than

one toxin results in a net toxic effect greater than that expected if the individual toxicities were simply additive.

Alcohols and ketones are widely used industrial chemicals that are among the chemicals that have been shown to potentiate both the renal and hepatic toxicity of other known toxins. Cornish and Adefuin (1967) tested the ability of a series of aliphatic alcohols to potentiate the hepatotoxicity of carbon tetrachloride in acute inhalation exposures. Methanol, ethanol, isopropanol, and sec-butyl and tert-butyl alcohol were all found to have considerable capacity for potentiating carbon tetrachloride hepatotoxicity, while n-propyl, n-butyl, i-butyl, and amyl alcohol were much less effective. Cornish and Adefuin (1967) and Traiger and Plaa (1971) found that the potentiation effect was greatest when the alcohols were administered 16-18 hours prior to carbon tetrachloride exposure.

Ethanol is metabolized in the liver to acetaldehyde and then to acetate by alcohol dehydrogenase and aldehyde dehydrogenase, respectively (Lieber, 1973). Traiger and Plaa (1972) used the alcohol dehydrogenase inhibitor, pyrazole, to demonstrate that ethanol was the chemical entity responsible for the potentiation effect, as the ethanol metabolite had no ability to potentiate carbon tetrachloride hepatotoxicity. Isopropanol, a more potent potentiator than ethanol, and its metabolite acetone were both shown to have the ability to potentiate carbon tetrachloride hepatotoxicity (Traiger and Plaa, 1972, 1973). Similarly, 2-butanol and its primary metabolite, 2-butanone, have been shown to have hepatotoxic potentiating capabilities (Traiger and Bruckner, 1976). 1,3-butanediol, which is metabolized to the two major endogenous

ketone bodies, β -hydroxybutyrate and acetoacetate, will potentiate carbon tetrachloride hepatotoxicity in male rats (Hewitt and Plaa, 1979).

The uniqueness of a ketone moiety for eliciting the potentiation of chloroform hepatotoxicity was demonstrated using the structurally related compounds mirex (dodecachlorooctahydro-1,3,4-metheno-2H-cyclobuta-[c,d] pentalene) and kepone (Chlodecone; decachlorooctahydro-1,3,4-metheno-2H-cyclobuta-[c,d] pentalen-2-one). Administration of kepone 18 hours prior to a chloroform challenge was found to markedly potentiate the hepatotoxicity of chloroform, while a similar administration of mirex had no effect on the animals' subsequent response to the chloroform challenge (Hewitt et al., 1979).

Metabolic ketosis, or elevated blood levels of endogenous ketones (β -hydroxybutyrate, acetoacetate, and acetone), is the result of production of ketone bodies by the liver at rates which overwhelm the ability of peripheral tissues to utilize them as energy yielding substrates (Searcy, 1969). Ketosis will occur in metabolic states where the supply of carbohydrate intermediates is inadequate and the rate of fat mobilization accelerated, as occurs in uncontrolled diabetes mellitus (Harper, 1977).

Alloxan and streptozotocin are drugs known to produce diabetes, and hence ketosis, in experimental animals. Alloxan or streptozotocin induced diabetes have been shown to potentiate the hepatotoxicity of a number of chemicals (Hanasono, Cote and Plaa, 1975; Hanasono, Witschi and Plaa, 1975). This potentiation may be reversed by insulin treatment. It is not clear whether elevated ketone levels are responsible for the potentiation of hepatotoxicity of known hepatotoxins as seen in drug

induced diabetes. It remains possible, however, that diabetes mellitus represents a clinical condition in which elevated ketone levels may increase the likelihood of sustaining hepatic injury upon exposure to hepatotoxic agents in the workplace or the environment.

There is evidence to suggest that toxic interactions should be considered in establishing safe exposure limits for workers, as toxic potentiation has been documented in the workplace. One such incident involving an alcohol or ketone potentiation of toxicity occurred in an isopropanol packaging plant (Folland et al., 1976). Fourteen workers became sick after carbon tetrachloride was accidentally used as a cleaning solvent, four of whom required hospitalization for hepatitis and renal failure. Elevated levels of isopropanol and its metabolite, acetone, were found in expired air samples obtained from employees working nearest to the packaging line. Workers from this area of the plant were found to have a higher illness rate due to the toxic effects of the carbon tetrachloride exposure than workers in other areas of the plant. A subsequent study of the plant conditions existing at the time of the incident led the investigators to conclude that isopropanol exposure had a significant adverse effect on the response of workers to toxic insult by carbon tetrachloride and that multiple solvent exposures should be considered in determining allowable exposure limits for solvents known to be toxic.

Alcohols and ketones have been shown to potentiate the hepatotoxicity of a variety of hepatotoxins other than carbon tetrachloride. Ethanol pretreatment may enhance the hepatotoxicity of acetaminophen,

thioacetamide, and allyl alcohol as well as carbon tetrachloride in mice (Strubelt, Obermeier, and Siegers, 1978). In the same study ethanol pretreatment did not affect the toxicity of bromobenzene, phalloidin, α -amanitin, or praseodymin. Chloroform, 1,1,2-trichloroethane, and trichloroethylene were found to be more hepatotoxic to mice when the toxin was administered 18 hours after isopropyl alcohol or acetone (Traiger and Plaa, 1974). Chloroform hepatotoxicity has been demonstrated to be potentiated by kepone (Hewitt et al., 1979). Chloroform, 1,1,2-trichloroethane, and galactosamine induced hepatotoxicities are potentiated by drug induced diabetes, as is carbon tetrachloride hepatotoxicity (Hanazono, Cote, and Plaa, 1975; Hanazono, Witschi, and Plaa, 1975).

Considerable research has gone into the identification of alcohols, ketones, and physiologic conditions of metabolic ketosis that may potentiate the hepatotoxic response to a wide variety of hepatotoxins. Despite this effort there is little evidence to suggest a common mechanism by which this toxic potentiation may occur.

An attempt to elucidate a mechanism by which alcohols and ketones potentiate the hepatotoxicity of known hepatotoxins requires a knowledge of the nature and mechanism of toxicity of the hepatotoxins. Among the hepatotoxins whose toxicities have been found to be potentiated by alcohols or ketones, carbon tetrachloride, chloroform, 1,1,2-trichloroethane, trichloroethylene, thioacetamide, and acetaminophen all cause a centrilobular necrosis of the liver (Gupta, 1956; Rouiller, 1963; Browning, 1965), while D-galactosamine causes a focal liver necrosis (Decker and Keppler, 1972) and allyl alcohol a periportal necrosis (Rouiller, 1963).

The known toxins that produce an exclusively centrilobular necrosis are all metabolized in the liver to known or presumed toxic metabolites (Recknagel, 1967; Ilett et al., 1973; Carlson, 1973; Van Duuren and Banerjee, 1976; Ammon et al., 1967; Mitchell, Jollow, Potter, Davis, et al., 1973). The following is a proposed sequence of events leading to toxic cell death for the centrilobular toxins. After entering the circulation the toxin is metabolized by the enzymes of the endoplasmic reticulum of hepatocytes to one or more reactive intermediates which may then react with macromolecules within the hepatocyte; this interaction of the reactive intermediate with cellular macromolecules leads to some irreversible alteration in cellular integrity and the cell dies. The results of many studies support this proposed biochemical chain of events. Experimental labeling of hepatotoxins with carbon-14 has shown that ^{14}C -labeled toxins and/or their metabolites may covalently bind to cellular proteins, lipids, and nucleic acids, in both in vivo and in vitro experiments (Jollow et al., 1973; Uehleke and Werner, 1975; Ilett et al., 1973; Banerjee and Van Duuren, 1979).

Further evidence for a link between xenobiotic metabolism, binding of reactive intermediates to tissue macromolecules, and hepatocellular necrosis was presented in a classic series of papers on acetaminophen-induced hepatic necrosis (Mitchell, Jollow, Potter, Davis et al., 1973; Mitchell, Jollow, Potter, Gillette et al., 1973; Jollow et al., 1973; Potter et al., 1973). Pretreatment of male rats with phenobarbital to induce the cytochrome P-450 xenobiotic metabolizing enzymes resulted in increased covalent binding of ^{14}C -labeled metabolites of acetaminophen to microsomal proteins, both in vivo and in vitro.

Coupled with this increased binding in the phenobarbital treated rats was a marked potentiation of acetaminophen-induced centrilobular necrosis. Thus, the mechanism by which phenobarbital enhances acetaminophen hepatotoxicity is increased biotransformation of acetaminophen to reactive electrophilic intermediates which, in turn, leads to enhanced covalent binding to cellular macromolecules and necrosis.

One plausible mechanism by which alcohols and ketones may potentiate the toxicity of some hepatotoxins is the effect they may have on xenobiotic metabolism. Ethanol has been reported to enhance the metabolism of a number of xenobiotics in vivo (Lieber, 1973). Anders (1968) has reported marked enhancement of the microsomal hydroxylation of aniline in the presence of acetone in in vitro incubations. There is evidence for enhanced microsomal metabolic activity, as measured by increased N-demethylation of dimethylnitrosamine, in hepatic microsomes prepared from acetone or isopropanol pretreated rats and mice (Sipes, Stripp et al., 1973; Sipes, Slocumb and Holtzman, 1978). These two studies provide further evidence for the link between xenobiotic metabolism and covalent binding of ^{14}C -labeled intermediates to cellular macromolecules by demonstrating increased covalent binding of both carbon tetrachloride and dimethylnitrosamine to microsomal proteins following isopropanol or acetone pretreatment. An orally administered dose of acetone has been shown to enhance the in vivo O-deethylation of phenacetin, without affecting aniline hydroxylation, or ethylmorphine, aminopyrine, and benzphetamine N-demethylation (Kitada, Kamataki and Kitagawa, 1978).

There are several important points about alcohol or ketone potentiation of xenobiotic induced hepatotoxicity that must be considered. First, a variety of hepatotoxins that produce centrilobular, periportal, or focal necrosis have been shown to be more toxic to animals that have been pretreated with alcohols or ketones. However, not all hepatotoxins are potentiated by prior exposure to alcohols or ketones. The second major point is that a wide variety of alcohols and ketones are capable of potentiating the toxicity of model hepatotoxins in experimental animals. A third point is that there is no convincing evidence for a common biochemical mechanism for this potentiation phenomena. There is, however, reason to believe that an effect on xenobiotic metabolism may account, at least in part, for the potentiation effect. The majority of the hepatotoxins now known to be susceptible to potentiation by alcohols and ketones are centrilobular toxins that are believed to be metabolized to reactive intermediates in the liver. Certain alcohols and ketones are known to enhance selected microsomal pathways of xenobiotic metabolism, both in vivo and in vitro, and hence may elicit the hepatotoxic potentiation response by simply contributing to the production of more reactive intermediates. This link has not been clearly established between any particular alcohol/ketone and hepatotoxin in an in vivo situation.

In order to initiate an investigation of the mechanism of alcohol/ketone potentiation of hepatotoxicity it was necessary to develop an animal model with a known hepatotoxin and a suitable alcohol or ketone.

Proposed Research

Acetone was selected as the model ketone to be employed in this study (Figure 1).

Figure 1. Acetone Structure.

Acetone is a volatile, highly flammable liquid, used as a solvent for fats, oils, waxes, resins, rubber, plastics, lacquers, varnishes, and rubber cements. It is used in the manufacture of methyl isobutyl ketone, mesityl oxide, acetic acid, diacetone alcohol, chloroform, iodoform, bromoform, explosives, rayon, photographic films, isoprene, in storage of acetylene gas, and as a component of commercial paint and varnish removers. Acetone has a molecular weight of 58.08, a density of 0.788 grams/ml, a boiling point of 56.5°C, a melting point of -94°C, and a flash point of -20°C (Merck Index, 1976). The oral LD₅₀ in rats is 10.7 ml/kg (Smyth et al., 1962).

The criteria for selecting acetone as the model potentiating agent for this study include proven efficacy in eliciting the potentiation response, ease of administration, and relevancy in terms of human occupational exposure. Oral pretreatment of rats with 1.0 ml/kg of acetone has been shown to potentiate the hepatotoxicity of a carbon tetrachloride challenge dose administered 18 hours later (Traiger and

Plaa, 1972, 1973). The hepatotoxicity of chloroform, 1,1,2-trichloroethane, and trichloroethylene are potentiated by a single oral dose of 2.5 ml/kg of acetone administered 18 hours prior to the organohalogen in mice (Traiger and Plaa, 1974). Single oral doses of 2.5 ml/kg of acetone have also been shown to increase the in vitro N-demethylation of dimethylnitrosamine and the in vitro covalent binding of ^{14}C -carbon tetrachloride metabolites to hepatic microsomal proteins when the microsomes are prepared from livers of rats sacrificed 16-18 hours after acetone treatment (Sipes et al., 1973). Single intraperitoneal injections of 1.3 ml/kg of acetone will also enhance the in vitro N-demethylation of dimethylnitrosamine and the covalent binding of ^{14}C labeled dimethylnitrosamine metabolites to hepatocellular proteins in mouse hepatic microsomes prepared from mice sacrificed 16 hours after the acetone administration (Sipes et al., 1978).

The use of acetone as a potentiating agent is advantageous in that it can elicit dramatic potentiating effects on toxicity and biotransformation after a single oral dose. Single administrations reduce animal handling and minimize trauma to the experimental animals.

Acetone production in the United States is exceeded by only 39 other chemicals in terms of quantity produced, with 2.06 billion pounds produced in 1974 (Chemical and Engineering News, 1975). The quantity of acetone produced in this country and its many commercial uses may combine to yield a substantial risk for toxic potentiation to occur in persons that may have exposure to both acetone and compounds with hepatotoxic properties.

The selection of the hepatotoxin to be used for the animal model of acetone potentiation of hepatotoxicity was also an important consideration in designing a study to investigate the mechanism of alcohol/ketone potentiation. The chlorinated hydrocarbon 1,1,2-trichloroethane was chosen for this study (Figure 2).

Figure 2. 1,1,2-Trichloroethane Structure.

The two carbon organohalogen 1,1,2-trichloroethane (also known as ethane trichloride, β -trichloroethane, 1,2,2-trichloroethane, or vinyl trichloride) is a nonflammable liquid with a molecular weight of 133.42, a density of 1.442 grams/ml, and a boiling point of 113-114°C (Merck Index, 1976). This compound is used in the United States as an intermediate in the production of vinylidene chloride, as a solvent, and as a component of adhesives. As an adhesive component 1,1,2-trichloroethane is approved by the United States Food and Drug Administration for articles intended for use in packaging, transporting, or holding food (United States Food and Drug Administration, 1977).

1,1,2-trichloroethane is not known to occur as a natural product, yet the compound has been found in the drinking water supplies of 5 U.S. cities (Coleman et al., 1976), in other drinking water samples at less than 0.1-8.5 $\mu\text{g/liter}$ (Safe Drinking Water Committee, 1977), and as one constituent of 75 million kilograms of chlorinated aliphatic hydrocarbons dumped at sea (Rosenberg, Grahn and Johansson, 1975).

The oral LD₅₀ of 1,1,2-trichloroethane in rats is 835 mg/kg body weight (Smyth et al., 1969) and the intraperitoneal LD₅₀ in mice is 500 mg/kg body weight (Klaasen and Plaa, 1966). 1,1,2-trichloroethane is a central nervous system depressant, and is both hepatotoxic and nephrotoxic (Gehring, 1968; Plaa, Evans and Hine, 1958; Klaasen and Plaa, 1966). The hepatotoxic effect is a centrilobular necrosis (Klaassen and Plaa, 1966). There is no reported evidence of embryotoxicity or teratogenicity for the compound. 1,1,2-trichloroethane is not mutagenic in the Ames test (Rannug, Sundvall and Ramel, 1978). The compound does produce a hepatocellular carcinoma in susceptible mice, but not in rats (National Cancer Institute, 1978). Low concentrations of 1,1,2-trichloroethane have a narcotic effect on humans. Human exposure may produce irritation of the eyes and mucous membranes of the respiratory tract, and causes erythema and cracking of the skin. Long term human exposure may result in chronic gastric symptoms, fat deposition in the kidneys, and lung damage (Hardie, 1964).

According to a 1974 National Occupational Hazard Survey, workers that are exposed to 1,1,2-trichloroethane are primarily employed by blast furnaces and steel mills, in telephone communications, or in the engineering and scientific instrument manufacturing industry (National Institute of Occupational Safety and Health, 1977a). The threshold limit value set by the Occupational Safety and Health Administration for inhalation exposure to 1,1,2-trichloroethane is 10 parts per million or 45 mg/cubic meter in workplace air as an 8 hour time weighted average for any work day (National Institute of Occupational Safety and Health, 1977b).

There were several reasons for selecting 1,1,2-trichloroethane as the model hepatotoxin for a mechanistic study of acetone potentiation of hepatotoxicity. The hepatotoxicity of 1,1,2-trichloroethane has already been shown to be potentiated when acetone is administered as a pretreatment (Traiger and Plaa, 1974). In vitro microsomal incubations with ^{14}C -labeled 1,1,2-trichloroethane produces more covalently bound ^{14}C -labeled adducts to microsomal proteins and lipids than equimolar incubations with ^{14}C -labeled carbon tetrachloride (Gandolfi, MacDonald and Sipes, 1979). This property may be exploited in attempting to assess binding of ^{14}C labeled reactive intermediates to hepatic proteins and lipids in vivo.

Unlike the more thoroughly studied centrilobular toxin carbon tetrachloride, 1,1,2-trichloroethane is known to form glutathione conjugates in the liver (Yllner, 1971). Assessment of hepatic glutathione of both acetone pretreated and control animals receiving a subsequent dose of 1,1,2-trichloroethane may then provide another measure of the in vivo metabolism of 1,1,2-trichloroethane. The in vivo binding of ^{14}C -labeled metabolites to hepatic proteins and lipids and the assessment of hepatic glutathione levels should provide two means of determining the effect of acetone pretreatment on in vivo metabolism of a known hepatotoxin.

1,1,2-trichloroethane has the added advantage of producing large increases in hepatic damage as a function of relatively small increases in administered dose (Plaa et al., 1958). A steep dose response curve for hepatic damage should allow for the selection of a critical dose range where the effect of acetone potentiation on toxicity

is maximized relative to untreated controls. This critical dose range may then be employed to assess the effect of acetone pretreatment on in vivo metabolism in subsequent experiments designed to test the role of metabolism in acetone potentiation of toxicity.

Statement of the Problem

Despite the amount of research that has been done in identifying chemical agents that may potentiate the hepatic toxicities of known hepatotoxins and in the identification of toxins that may be potentiated, definitive evidence for a mechanism by which potentiation occurs is severely lacking. To characterize the mechanism of alcohol/ketone potentiation an animal model needs to be developed using a model ketone or alcohol and a model toxin that can be manipulated to yield information about potentiation mechanisms.

MATERIALS AND METHODS

Experimental Animals

Male, Sprague-Dawley rats, weighing between 175 and 400 grams, were used for all experiments. The rats were obtained from Hilltop Laboratories (Chatsworth, CA) or as F₁ generation males of Hilltop parents from the Division of Animal Resources of the Arizona Health Sciences Center. After arrival at the Division of Animal Resources the animals were maintained in an isolated room with an independent air source. The rats were housed in stainless steel wire cages on a fourteen hours of dark to ten hours of light cycle at a constant temperature of 22°C. Prior to any experimental manipulations the rats were allowed at least one week adaptation time. During this adaptation period the animals were observed for general appearance, health, and normal weight gain. Utilization of an isolated room with an independent air source insured freedom from inadvertent exposure of the rats to enzyme inducing agents such as solvents, smoke, or bark bedding (Conney and Burns, 1962; Fouts, 1963). Prior to experimentation the rats were fed a standard laboratory chow and allowed water ad libitum.

Chemicals

Technical grade 1,1,2-trichloroethane, the highest quality grade commercially available, was purchased from J. T. Baker Co. (Phillipsburg, NJ). Uniformly labeled ¹⁴C-1,1,2-trichloroethane was obtained from California Bionuclear Corporation (Sun Valley, CA). Radiochemical purity, determined by gas chromatography, was reported to be 99%.

Chemical purity of the radiolabeled compound was checked by gas-liquid chromatography in our laboratory and found to be 99% pure. Spectral grade carbon tetrachloride was purchased from Mallinckrodt Chemical Works (St. Louis, MO). Sesame oil and pesticide grade acetone were obtained from Fisher Scientific Company (Fair Lawn, NJ). Albumin stock solution, β,γ -dipalmitoyl- α -lecithin, nicotine adenine dinucleotide phosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, reduced glutathione, sulfosalicylic acid, and serum glutamate-pyruvate transaminase kits were all obtained from Sigma Chemical Company (St. Louis, MO). Ellman's reagent (5,5' dithiobis-2-nitrobenzoic acid) was purchased from K and K Laboratories (Irvine, CA). Betaphase^R scintillation cocktail was purchased from Westchem Products (San Diego, CA). All buffers employed were made in the laboratory using distilled deionized water and chemicals of reagent grade or better. All other chemicals were of reagent grade or better.

Acetone Administration

Pesticide grade acetone was diluted in distilled deionized water and administered by gastric intubation as a final volume of 10 ml/kg body weight. The individual doses of acetone that were administered ranged between 0 and 3.5 ml/kg of body weight. Acetone was always administered between 4 and 6 P.M. to minimize possible diurnal variation in the potentiation response. Following acetone dosing the rats became lethargic and anorexic. As a consequence both acetone treated and corresponding control rats were fasted for 16 hours after the acetone dosing and prior to any further experimental manipulation.

Water was allowed ad libitum during the fasting period. Both acetone treated and control rats exhibited the same percent weight loss following 16 hours of fasting.

Hepatic Toxicity Studies

Both control and acetone treated rats were administered 1,1,2-trichloroethane as an intraperitoneal (i.p.) injection in a sesame oil vehicle. The doses of 1,1,2-trichloroethane ranged from 0 to 2.0 mmoles/kg of body weight. Appropriate dilutions were made so that the total administered volume of 1,1,2-trichloroethane and vehicle was 1 ml/kg of body weight. The 1,1,2-trichloroethane was administered immediately following the 16 hour fasting period. Following administration of the toxin, control and acetone treated animals were returned to their original cages and allowed food and water ad libitum until the time of sacrifice.

At selected time intervals the rats were killed by cervical dislocation and blood samples were drawn by cardiac puncture. This method allowed for the recovery of between 2 and 8 ml of whole blood per animal. Serum was separated by centrifugation of the blood samples at 1,000 xg for 10 minutes and then stored at 4°C. The serum glutamate-pyruvate transaminase activity was measured within 24 hours after sample collection and the results were expressed as Karmen units (Sigma Technical Bulletin No. 55-UV).

Liver sections (1-2mm thick) were taken from each animal at the time of sacrifice and fixed in a 10% solution of formalin buffered with phosphate for subsequent histological processing and examination. The

remainder of the livers were quick frozen in plastic specimen bags between two blocks of dry ice and stored at -70°C for subsequent analysis of reduced glutathione content.

Histological Techniques

Liver sections were fixed for at least 48 hours in buffered formalin. Some sections were fixed in Carnoy's solution to preserve cellular glycogen (Luna, 1968). Following fixation of the excised tissue the sections were processed by the Histology Laboratory of the Division of Animal Resources, Arizona Health Sciences Center. Standard techniques were employed in the processing and preparation of tissue sections for microscopic examination. Formalin fixed sections were slide mounted, stained with eosin, and counter stained with hematoxylin (H&E staining) for histological assessment of hepatic damage. The sections fixed in Carnoy's solution were stained using Best's carmine method to demonstrate the presence of intracellular glycogen (Luna, 1968). Hepatic H&E sections were examined at 400X and scored quantitatively for the extent of necrosis. Twenty microscopic fields were examined for each liver section and nine reference points in each field scored as corresponding to a necrotic hepatocyte, a normal hepatocyte, or neither (Mitchell, Jollow, Potter, Davis et al., 1973). Histological scores for necrosis ranged from 0 = no necrotic cells to 5 = necrotic cells comprising more than 40% of the total hepatocytes examined for a given section. Some formalin fixed liver sections were stained to show fat content by both oil red O and osmium tetroxide techniques (Luna, 1968).

Hepatic Reduced Glutathione Content

Reduced glutathione of rat livers was estimated by a modification of the method of Sedlak and Lindsay (1968). Approximately 2 grams of frozen (-70°C) liver tissue was homogenized per 6 ml of cold 0.05 M Tris pH 7.4 buffer containing 10^{-3} M disodium ethylenediaminetetraacetic acid (EDTA) in Dounce hand homogenizers using a tight fitting pestle. A 0.5 ml aliquot of the homogenate was diluted with 4.5 ml of additional cold buffer. To precipitate sulfhydryl containing proteins 200 μl of 5% sulfosalicylic acid was added and the samples centrifuged at 3,000 RPM for 15 minutes in a Sorvall GLC-2B tabletop centrifuge. One ml of the resulting supernatant was added to 4 ml of 0.2 M Tris pH 8.9 buffer containing 10^{-2} M EDTA. One hundred microliters of Ellman's reagent (99 mg of 5,5' dithiobis-2-nitro-benzoic acid/25 ml of absolute methanol) was added, the sample vortexed, and the absorbance at 412 nm read within 5 minutes on a Gilford Stasar III spectrophotometer. A second 0.5 ml aliquot of the original homogenate was similarly diluted, but without the addition of the Ellman's reagent. This sample served as a turbidity blank, as turbidity was frequently encountered in this assay.

A standard curve was prepared fresh daily using reduced glutathione dissolved in cold 0.05 M Tris pH 7.4 buffer containing 10^{-3} M EDTA. Standards were treated and diluted by the same procedure described for the samples (see Appendix A for standard curve).

Preparations of Subcellular Fractions

In Vitro Experiments

Hepatic microsomes and cytosol were prepared from livers of acetone treated and control rats immediately following the 16 hour fasting period. Microsomes and cytosol from these rats were used to assess the covalent binding of ^{14}C -1,1,2-trichloroethane to proteins and lipids in in vitro incubations. These animals were killed by cervical dislocation and their livers were immediately perfused with 0.05 M Tris pH 7.4 buffer containing 1.15% KCl (w:v) via the portal vein prior to excision.

In Vivo Experiments

Hepatic microsomes and cytosol were prepared from the frozen (-70°C) livers of rats dosed with ^{14}C -1,1,2-trichloroethane. Microsomes and cytosol from these rats were used exclusively for the assessment of the extent of covalent binding of ^{14}C label to cellular proteins and lipids in an in vivo situation.

Subcellular Fraction Preparation

The livers were homogenized in three volumes (w:v) of ice-cold 0.05 M Tris pH 7.4 buffer containing 1.15% KCl (w:v) in a Dounce hand homogenizer. The homogenates were centrifuged in a Sorvall RC2-B refrigerated centrifuge for 10 minutes at 1,000 X g, 10 minutes at 12,000 X g, and 10 minutes at 27,000 X g. The supernatant was poured through a glass wool filter to remove visible lipid residues and the pellet was discarded. The supernatant was then centrifuged for 40

minutes in a Beckman 50.2 TI fixed angle rotor at 50,000 rpm (162,000 X g) on a Beckman L8-55 preparative ultracentrifuge to precipitate the microsomal membranes. The cytosol containing supernatant was decanted and frozen at -70°C. The microsomal pellet was resuspended in the cold Tris-KCl buffer and precipitated again by a repetition of the ultracentrifugation step. The resulting supernatant was discarded and the microsomal pellet resuspended in the cold Tris-KCl buffer. Microsomes prepared for in vitro experiments were either used immediately or frozen at -70°C. Prior to use in in vitro experiments, cytosolic fractions were dialyzed overnight at 4°C in cellulose dialysis tubing (Sigma Chemical Company, St. Louis, MO) in two buffer changes of 2 liters each of 0.05 M Tris pH 7.4 buffer. Dialysis was performed to remove residual glutathione and to retain glutathione-S-transferase activity (Johnson, 1966). The resulting dialyzed cytosol contained approximately 30 mg of protein/ml.

Covalent Binding of ^{14}C -1,1,2-Trichloroethane
to Cellular Macromolecules In Vitro

The in vitro metabolism of ^{14}C -1,1,2-trichloroethane to reactive intermediates capable of covalently binding to cellular macromolecules was assessed using techniques described by Gandolfi, MacDonald, and Sipes (1979, 1980). All incubations were performed in 2 ml total incubation volumes in 12 ml glass screw cap incubation tubes. All incubations included 100 μl of a NADPH generating system, while different incubations consisted of microsomes (5 mg of microsomal protein), reduced glutathione (1 mM), or dialyzed cytosol (3 mg cytosolic protein)

separately or in combination in 0.05 M Tris pH 7.4 buffer containing 1.5% KCl (w:v). The NADPH generating system consisted of 5 mg nicotine adenine dinucleotide phosphate, 15 mg glucose-6-phosphate, 50 μ l of 0.1 M MgCl_2 , and 50 μ l of glucose-6-phosphate dehydrogenase (100 units/ml) per ml of Tris-KCl buffer. The complete incubation mixtures were prepared and kept on ice. Each incubation vessel was flushed 4 times with pure oxygen with 5 minutes allowed for equilibration of the head-space in each closed vessel between flushes. Finally, 2 μ l of ^{14}C -1,1,2-trichloroethane containing 2 μ moles of 1,1,2-trichloroethane and 1 μ Ci total radioactivity in an ethanol carrier was added to each sample and the vessel resealed. Incubations were conducted at 37°C in a Dubnoff metabolic shaking incubator. Complete incubation mixtures, prior to the addition of the 1,1,2-trichloroethane, were boiled for 15 minutes in a water bath to serve as blanks to determine the extent of non-enzymatic binding of ^{14}C label to cellular macromolecules. All incubations were stopped by the addition of 8 ml of ice cold ethanol to precipitate the microsomal proteins and the isolation of the constituent macromolecules proceeded from that point.

Covalent Binding of ^{14}C -1,1,2-Trichloroethane
To Cellular Macromolecules In Vivo

Following the 16 hour fasting period acetone treated and control rats were dosed i.p. with 1,1,2-trichloroethane in a sesame oil vehicle. Each animal received a total dosage volume of 1 ml/kg of body weight containing unlabeled 1,1,2-trichloroethane, sesame oil, and approximately 10 μ Ci of ^{14}C -labeled 1,1,2-trichloroethane. The total dose of

1,1,2-trichloroethane was 0.5, 1.2, or 1.7 mmole/kg of body weight. The specific activity of the administered 1,1,2-trichloroethane ranged from 45 to 160 DPM/nanomole in different experiments.

Several precautions were taken to minimize human exposure to the ^{14}C -1,1,2-trichloroethane or its metabolites. The rats were dosed in a high flow hood to vent away any volatile radioactivity. Following careful i.p. administration of the ^{14}C -1,1,2-trichloroethane the animals were housed in solid bottom cages containing wood shavings as bedding. The cages were kept in the high flow hood to allow venting of any volatile radioactivity exhaled by the rats (Yllner, 1971). The rats were killed in the hood by cervical dislocation two or four hours after dosing. The livers were excised immediately without perfusion and quick frozen in plastic specimen bags between two blocks of dry ice. Carcasses, bedding, and excreta were all sealed in plastic bags and turned over to the Radiation Safety Office at the Arizona Health Science Center for disposal.

The frozen livers were maintained at -70°C until processed for macromolecular binding determinations. Livers from these animals were carefully homogenized in buffer and assayed for reduced glutathione content, as previously described. Whole liver homogenate, microsomes, and cytosolic fractions were prepared from these livers and proteins and lipids isolated from these fractions.

Macromolecule Isolation

In vitro incubations with ^{14}C -1,1,2-trichloroethane substrate were stopped by the addition of 8 ml of ice-cold ethanol. In in vivo ^{14}C binding experiments 8 ml of ice-cold ethanol was added to aliquots of whole liver homogenate, microsomal suspension and cytosol. Following addition of the ethanol the samples were centrifuged for 15 minutes in a Sorvall GLC-2B tabletop centrifuge at 3,000 RPM. (All subsequent centrifugation steps are identical.) The supernatant was decanted and transferred to a 50 ml glass beaker. Four ml of CHCl_3 :ethanol (1:3) was added to the pellet and the sample heated at 70°C for 20 minutes in a water bath. The sample was centrifuged, 4 ml of cold ethanol added and recentrifuged. This supernatant was combined with the first supernatant in the appropriate beaker and together they constituted the extractable lipid fraction.

Lipids

The beakers containing the lipid fraction were placed in a vented hood and allowed to evaporate to dryness, effectively removing any unbound volatile radioactivity. The lipids were re-extracted from the beakers by two 2 ml rinses with chloroform, one 2 ml rinse with methanol, and one 2 ml rinse with water that had been equilibrated with chloroform:methanol (2:1). Each sample was vortexed, centrifuged and the aqueous layer removed by aspiration. The organic, lipid containing, layer was washed with 1 ml of 0.05 N NaOH to remove any unbound non-volatile ^{14}C metabolites that may have remained. Each sample was then vortexed, centrifuged, the basic aqueous layer removed by aspiration,

and 1 ml aliquots removed for liquid scintillation counting. Aliquots for scintillation counting were evaporated to dryness in individual vials prior to the addition of the counting fluor to remove chloroform from the sample. Aliquots taken for lipid phosphate analysis (200 μ l) were evaporated to dryness in open test tubes. Lipid phosphate was detected colorimetrically using a modification of the method of Chen, Toribara and Warner (1956). β,γ dipalmitoyl- α -lecithin, 4.8% phosphorus by weight, was the phospholipid used to establish standard curves for the assay (see Appendix). Covalent binding to lipids of ^{14}C containing reactive intermediates of 1,1,2-trichloroethane metabolism was quantified as nanomoles of ^{14}C label bound/ μ mole of lipid phosphorus.

Proteins

The pellet that remained after the lipids were extracted was washed extensively to remove any unbound radioactivity. The first wash of the pellet was with 4 ml of 0.05% trichloroacetic acid (w:v). The sample was centrifuged and the wash discarded. The pellet was then washed three times with 4 ml methanol:ether (3:1), with vortexing and centrifugation of each sample between washes. The final wash of the pellet was with 2 ml acetone. Following vortexing and centrifugation the acetone was discarded and the samples evaporated to dryness in a 37°C water bath. The remaining protein pellet was dissolved in 2 ml of 1 N NaOH by warming overnight at 37°C in a water bath. The dissolved protein content was determined by a biuret method (Gornal, Bardawill and David, 1949) using bovine serum albumin as the protein standard (Appendix). ^{14}C radioactivity was determined by liquid scintillation counting.

Covalent binding of ^{14}C containing reactive intermediates of 1,1,2-trichloroethane metabolism to proteins was quantified as nanomoles of ^{14}C label bound/mg protein.

Liquid Scintillation Counting

^{14}C radioactivity covalently bound to proteins and lipids was quantified using a Beckman 8100 liquid scintillation counter. Lipid samples were evaporated to dryness in Betavials (Westchem Products, San Diego, CA) and resuspended in Betaphase scintillation fluor (Westchem Products, San Diego, CA). Aliquots of proteins dissolved in base were neutralized by an equal volume of 1 N perchloric acid to stop chemiluminescence, and then suspended in Betaphase^R. Other samples containing ^{14}C radioactivity were treated in a manner that insured solubility of the sample in the counting fluor and allowed a high degree of counting efficiency.

Quench curves for the betaphase counting fluor were prepared using ^{14}C -toluene in betavials. All samples were counted for 10 minutes. The random coincidence monitoring feature of the Beckman LSC 8100 was employed to verify that recorded counts represented true disintegration events.

Amino Acid Adduct Profiles

Profiles of the covalently bound amino acid adducts resulting from the in vivo bioactivation of ^{14}C -1,1,2-trichloroethane were obtained for both fasted control and acetone pretreated animals. Hepatic proteins from the microsomal subfraction of rats dosed with

1.2 or 1.7 mmole ^{14}C -TCEA/kg were isolated as previously described. Following an acetone wash of the protein pellets they were evaporated to dryness and given to Dr. Richard M. Maiorino for resolution of the amino acid adduct profiles (Maiorino et al., 1980). The method of resolution involves Pronase digestion of the proteins, ultrafiltration through a Diaflo exclusion membrane (UM-05) using a stirring filtration cell (Amicon Corp., Lexington, MA), lyophilization of the ultrafiltrate, and dissolution in 0.3 N HCl prior to chromatographic resolution of the adducts. Aliquots (100 μl) of the dissolved amino acids and amino acid adducts were applied to a constant flow, high efficiency DC-4A cation exchange resin column. O-phthalaldehyde was added to the column eluant to form fluorescent derivatives of the amino acids which were detected by passing the derivatized eluant through an Aminco fluorescent detector (Silver Springs, MD) monitored at 455 nm. The column eluant was collected as 0.25 ml fractions which were assessed for ^{14}C -radioactivity by liquid scintillation counting. The profiles were plotted as nanomoles of ^{14}C adduct per mg protein eluted from the column as a function of retention time.

RESULTS

Time Course of 1,1,2-Trichloroethane Induced Hepatic Damage

An intraperitoneal dose of 1.75 mmoles of 1,1,2-trichloroethane (TCEA)/kg causes marked increases in SGPT activity and extensive coagulative necrosis of the liver by 24 hours after dosing. This toxic dose was selected to characterize the time course for the development of TCEA induced hepatic lesion (Fig. 3). Two hours after TCEA was administered SGPT activities were still at control levels and there was no evidence of cellular damage. By six hours after dosing there was a slight elevation of SGPT activity and some necrotic cells were present in the centrilobular regions of the sectioned livers. All animals appeared normal at this time. By twelve hours after TCEA administration the rats were very lethargic and some of them appeared ungroomed and did not react normally to handling or other stimuli. These behavioral changes and changes in appearance coincided with average SGPT activities of more than 6,000 Karmen units and areas of confluent coagulative necrosis comprising more than 50% of the liver sections. The necrosis was centrilobular in orientation as undamaged hepatocytes were only found in periportal areas. Hepatic damage was still extensive 24 hours after TCEA dosing, but by 48 hours SGPT activities were returning towards normal, the animals had resumed normal grooming, and histology showed fewer necrotic cells. Furthermore, liver sections taken at this time

Figure 3. Time course of TCEA induced hepatic lesion development.
 -- Extent of necrosis and SGPT activity.

showed numerous mitotic figures indicating that hepatic repair processes were active by this time (Robbins, 1974).

Some of the animals' kidneys had an abnormally yellow appearance 24 hours after TCEA administration. Histologic examination of kidney sections taken from these animals indicated some necrotic damage to the proximal tubules. This condition only appeared in some animals and only at the 24 hour time point.

Although the degree of hepatic damage was maximized 12 hours after TCEA administration, as assessed by both SGPT measurements and histological evaluation, the damage was still extensive after 24 hours with substantially less variation in hepatotoxic indices. As a consequence, all subsequent assessments of hepatotoxic damage caused by TCEA were made 24 hours after dosing.

Elevation of SGPT activity was found to correlate ($r = .8028$) with the extent of necrosis seen in individual animals (Fig. 4). Due to this correlation, SGPT activity was the principal measure of hepatic damage used in most of the experiments.

Acetone Potentiation of 1,1,2-Trichloroethane Hepatotoxicity

The extent of hepatocellular damage caused by 1.25 or 1.75 mmole/kg TCEA was greatly affected by the quantity of acetone administered as a pretreating dose (Fig. 5). Acetone was administered as a single oral dose of between 0 and 3.5 ml/kg. In addition to TCEA, the more thoroughly studied acetone potentiated hepatotoxin, carbon tetrachloride (CCl_4 , 1.0 mmole/kg) was administered i.p. in a sesame oil vehicle as a positive control.

Figure 4. Correlation of SGPT activity and the extent of necrosis.
-- $r = .8028$.

Figure 5. Acetone dose-response curve for SGPT activity for TCEA and CCl₄.

An acetone dose of 0.5 ml/kg was found to potentiate the greatest increase in SGPT activity caused by either dose of TCEA. Pretreating doses of acetone greater than 0.5 ml/kg did not potentiate the toxicity of 1.25 mmoles/kg TCEA and resulted in SGPT activities significantly less than those in untreated controls receiving 1.75 mmoles/kg TCEA. The hepatotoxicity of carbon tetrachloride was also markedly potentiated by an acetone pretreating dose of 0.5 ml/kg.

These results established a pretreatment dose of 0.5 ml of acetone/kg as the most effective single oral dose for potentiating TCEA hepatotoxicity. As a result an acetone dose of 0.5 ml/kg was used for all subsequent development of the rat model for acetone potentiation of TCEA hepatotoxicity.

Dose-Response for the Hepatotoxicity of
1,1,2-Trichloroethane with and
without Acetone Pretreatment

The hepatotoxic dose-response relationships were established for TCEA administered i.p. to fasted control rats and rats pretreated with 0.5 ml of acetone/kg by assessing SGPT activity 24 hours after TCEA administration (Fig. 6). SGPT activities were found to increase dramatically in fasted control rats as the administered dose of TCEA increased from 1.25 to 1.5 mmoles/kg.

It was anticipated that the steepness of the dose-response curve would provide a sensitive tool for demonstrating an all or none potentiation of hepatotoxicity by acetone, in which a critical dose of TCEA could be found at which all acetone treated animals would show a potentiated hepatotoxicity and fasted controls would show minimal toxicity. It was

Figure 6. TCEA dose-response curve for SGPT activity for control rats (●-●) and rats receiving 0.5 ml/kg acetone as oral pre-treatment (□). --★ equals highest SGPT within acetone group.

found, however, that at several different dose levels some animals were potentiated by acetone while others were not.

Although no critical dose of TCEA could be determined at which all acetone pretreated animals were potentiated, those animals that did show a potentiation response exhibited dramatic elevation of SGPT activity and extensive necrosis in hepatic tissue sections.

Histology

Completely normal hepatic architecture is seen in fed control rats (Fig. 7). Sixteen hours of fasting produces a characteristic reversible hydropic vacuolization of hepatocytes in control animals (Fig. 8) that has also been shown by Hewitt et al. (1980). These vacuoles do not contain any glycogen or fats. Twenty-four hours of re-feeding is not sufficient to reverse this transient vacuolization (Fig. 9). Rats that have been treated with 0.5 ml of acetone/kg, fasted for sixteen hours, and allowed to feed ad libitum for an additional twenty-four hours display the same vacuolization (Fig. 10). as control rats. When fasted control rats received an i.p. dose of 1.25 mmoles of TCEA/kg no necrosis was observed after twenty-four hours (Fig. 11). In contrast, many rats receiving 0.5 ml of acetone/kg as a pretreatment exhibited extensive coagulative necrosis of the liver twenty-four hours after a dose of 1.25 mmoles of TCEA/kg (Fig. 12). The necrosis was always centrilobular in orientation, often with a slight amount of accompanying hemorrhage. Necrotic areas typically displayed prominent pyknotic and karyolytic nuclei with a homogeneous eosinophilia of the non-nuclear cellular material. Hydropic changes marked

Figure 7. H&E stain of a liver section from a fed control rat.

Figure 8. H&E stain of a liver section from a control rat fasted for 16 hours.

Figure 9. H&E stain of a liver section from a control rat fasted for 16 hours then allowed food for 24 hours.

Figure 10. H&E stain of a liver section from an acetone pretreated rat (0.5 ml/kg p.o.) fasted for 16 hours and then allowed food for 24 hours.

Figure 11. H&E stain of a liver section from a fasted control rat 24 hours after administration of 1.2 mmoles of TCEA/kg i.p. -- SGPT activity = 32 Karmen units. No evident necrosis.

Figure 12. H&E stain of a liver section from an acetone pretreated rat (0.5 ml/kg p.o.) 24 hours after administration of 1.2 mmoles of TCEA/kg i.p. -- SGPT activity = 2992 Karmen units. Extensive coagulative centrilobular necrosis.

by so called "balloon cells" characteristically delineated the margin of necrotic areas.

Hepatic Glutathione Content

It has been demonstrated by Yllner (1971) that 1,1,2-trichloroethane forms mercapturic acids which are end products of glutathione conjugations. To assess the possible role of metabolism and reduced glutathione (GSH) involvement in the expression of TCEA hepatotoxicity, the hepatic levels of GSH were assessed in different control rats and in acetone pretreated and fasted control rats at different times after TCEA administration.

Control rats allowed food and water ad libitum had a hepatic GSH content of 5.64 ± 0.26 μ moles/g of liver (wet weight) (Table 1). The 16 hour fasting period caused a 30% reduction in hepatic GSH content in both acetone treated and fasted control rats. Acetone treatment did not cause any depletion of GSH beyond the 30% depletion caused by 16 hours of fasting.

The extent of GSH depletion caused by several different doses of TCEA was examined in fasted control rats and in rats pretreated with 0.5 ml/kg acetone two hours after TCEA administration (Table 1). The GSH levels were found to be significantly different ($p < 0.025$) between the acetone pretreated rats and the fasted controls at all TCEA doses examined. The differences in hepatic GSH levels between either fasted controls or acetone pretreated rats given different doses of TCEA were insignificant.

Table 1. Hepatic GSH level ($\mu\text{mole/g liver} \pm \text{S.E.}$).

TCEA dose ($\mu\text{mole/kg}$)	Fed Control	Acetone (0.5 ml/kg)	Fasted Control
0	5.64 ± 0.26	3.86 ± 0.32	4.14 ± 0.19
0.75		$2.38 \pm 0.24^*$	3.05 ± 0.32
1.25		$2.57 \pm 0.19^*$	3.59 ± 0.45
1.75		$2.46 \pm 0.16^*$	3.59 ± 0.38

* Significantly different from fasted controls $p < .025$.

A time course study of GSH depletion by TCEA was conducted at a TCEA dose of 1.75 mmol/kg (Fig. 13). After TCEA administration the rats were allowed food ad libitum. Hepatic concentrations of GSH were found to be maximally depleted 2 hours after TCEA administration. By twelve hours after TCEA administration hepatic GSH concentrations were higher than those seen in normal fed control rats. Hepatic levels of GSH were found to remain at these higher levels as long as 48 hours after TCEA dosing. This was not surprising as similar results have been reported by Mitchell, Jollow, Potter, Davis et al. (1973) for GSH depletion by acetaminophen.

Covalent Binding of ^{14}C -1,1,2-Trichloroethane
to Microsomal Macromolecules In Vitro

In order to more thoroughly examine the effect of acetone pretreatment on metabolism of TCEA, in vitro incubations were performed utilizing hepatic microsomes from acetone treated and fasted control rats. ^{14}C -labeled TCEA is biotransformed in vitro to one or more reactive intermediates that can covalently bind to microsomal proteins and lipids (Gandolfi et al., 1979). In these incubations the extent of binding of ^{14}C label from ^{14}C -TCEA to proteins and lipids was used as a measure of metabolism and bioactivation of TCEA by the microsomes.

The covalent binding of ^{14}C label was found to be linear for 30 minutes to both microsomal proteins (Fig. 14) and lipids (Fig. 15). Acetone pretreatment of the rats resulted in significantly higher levels ($p < 0.025$) of ^{14}C label covalently bound to proteins and lipids after 10, 20, and 30 minutes of incubation.

Figure 13. Time course of GSH depletion by 1.75 mmoles of TCEA/kg.

Figure 14. Time course of covalent binding of ^{14}C -TCEA to microosomal proteins in vitro.

Figure 15. Time course of covalent binding of ^{14}C -TCEA to microosomal lipids in vitro.

The inclusion of GSH (1 mM) in incubations resulted in an 80% reduction of the amount of ^{14}C -label covalently bound to microsomal proteins and lipids for both the acetone pretreated and fasted control groups (Figs. 16 and 17). When 3 mg of dialyzed cytosolic protein was included in the incubations the amount of ^{14}C label covalently bound to protein was less per mg protein, as was the amount of ^{14}C label covalently bound to lipid. When both dialyzed cytosolic protein (3 mg) and GSH (1 mM) were included in microsomal incubations the amount of covalently bound ^{14}C label is reduced by 46% to proteins in incubations from acetone pretreated rats, by 13% to proteins in incubations from fasted control rats, and by 85% to lipids in both groups compared to incubations containing only microsomes and cytosol. Incubations of ^{14}C TCEA with 3 mg of dialyzed cytosolic protein resulted in some covalent binding of ^{14}C label to proteins that was not altered by pretreatment of the rats with acetone. The inclusion of GSH (1 mM) in cytosolic incubations caused a 42% reduction of covalent binding of ^{14}C -label to cytosolic proteins in both groups of microsomes.

The degree of non-enzymatic binding of ^{14}C -label to microsomal protein and lipid was assessed by performing incubations with heat denatured microsomes and ^{14}C -TCEA. The levels of non-enzymatic covalent binding in 30 minutes were found to 12% of control to proteins and 4% of control to lipids for microsomes from both acetone pretreated rats and fasted control rats.

Figure 16. Covalent binding of ^{14}C -TCEA to protein in vitro in thirty minutes under different incubation conditions. -- See text.

Figure 17. Covalent binding of ^{14}C -TCEA to lipid in vitro in thirty minutes under different incubation conditions. -- See text.

Covalent Binding of ^{14}C -1,1,2-Trichloroethane
to Hepatic Macromolecules In Vivo

In order to assess the bioactivation of TCEA in vivo, ^{14}C -labeled TCEA was administered i.p. to rats and the covalent binding of ^{14}C -label to proteins quantified two and four hours after administration. Covalent binding of ^{14}C label to proteins was quantified in whole liver homogenate, hepatic microsomes and cytosol. GSH content of the livers was also assessed to determine the extent of GSH depletion by TCEA.

Acetone pretreatment resulted in increased covalent binding of ^{14}C -TCEA to proteins two hours after the administration of 1.7 mmol/kg TCEA (Table 2). There was no significant difference in the extent of binding found between the acetone pretreated and fasted control animals at any other doses of TCEA or any other time points. There was, however, a significant difference in the GSH content of the livers two hours after TCEA administration between acetone pretreated and fasted control animals at all doses of TCEA tested (Table 1).

There was significantly more radioactivity recovered in the acid soluble fraction of hepatic cytosol from the acetone pretreated animals two hours after administration of 1.2 or 1.7 mmol/kg TCEA (Table 3). This difference was not evident by four hours after TCEA administration.

Preliminary work elucidating profiles of amino acid adducts formed when TCEA is administered to rats indicates that acetone pretreatment may alter both the type of adduct formed and the relative amounts of different adducts isolatable from microsomal proteins (Fig. 18).

Table 2. ^{14}C Covalently bound to protein in vivo (nanomoles/mg protein).

TCEA Dose (mmoles/kg)	Tissue Fraction	2 hrs.		4 hrs.	
		Acetone	Control	Acetone	Control
0.5	Homogenate	1.46±0.40	1.33±0.04		
	Microsomes	2.10±0.08	1.43±0.12		
	Cytosol	1.29±0.04	1.51±0.10		
1.2	Homogenate	2.78±0.12	2.52±0.20	7.62±0.31	6.00±0.26
	Microsomes	8.14±0.70	6.32±0.88	9.63±0.49	9.42±0.38
	Cytosol	3.84±0.48	3.14±0.40	3.66±0.12	3.50±0.32
1.7	Homogenate	2.98±0.17	1.55±0.04	8.25±0.21	6.71±0.29
	Microsomes	9.26±1.06	4.08±0.37	11.58±0.64	10.37±0.71
	Cytosol	4.36±0.83	1.54±0.12	4.78±0.19	4.64±0.50

Table 3. TCEA fragments in acid soluble fraction of hepatic cytosol (nanomoles $\pm$ S.E.).

TCEA Dose (mmoles/kg)	2 hrs.		4 hrs.	
	Acetone	Control	Acetone	Control
0.5	3084 $\pm$ 240	3703 $\pm$ 187		
1.2	10881 $\pm$ 455	8272 $\pm$ 536	9067 $\pm$ 632	8473 $\pm$ 702
1.7	5058 $\pm$ 80	4690 $\pm$ 108	10370 $\pm$ 879	10516 $\pm$ 416

Figure 18. Amino acid adduct profile from the hepatic microsomal proteins of fasted control (.....) and acetone pretreated (—) rats (0.5 ml/kg p.o.) 2 hours after receiving 1.7 mmol of ^{14}C -TCEA/kg i.p.

DISCUSSION

The ability of a variety of alcohols and ketones to potentiate the hepatotoxicity of known toxins in experimental animals has been well documented (Cornish and Adefuin, 1967; Traiger and Plaa, 1974; Traiger and Bruckner, 1976; Strubelt et al., 1978; Hewitt et al., 1979). The mechanism of this toxic potentiation has been the subject of much speculation, but experimental data supporting a biochemical or physical mechanism has been very scarce.

The mechanism of potentiation of hepatotoxicity has been postulated to be either non-specific membrane changes making the cell more susceptible to toxic injury (Curtis, Williams and Mehandale, 1979) or increased bioactivation of hepatotoxins to reactive intermediates (Maling et al., 1975; Strubelt et al., 1978; Curtis et al., 1979). Induction of the cytochrome P-450 mixed function oxidase system has been proposed as a possible mechanism of toxic potentiation by some ketones (Curtis et al., 1979). The best existing evidence for a mechanism of potentiation is the increased covalent binding of ^{14}C -labeled carbon tetrachloride and chloroform to hepatic proteins and lipids in vivo following ethanol or isopropanol treatment (Maling et al., 1975).

The intent of this study has been to determine a generalized mechanism of toxic potentiation in an animal model utilizing 1,1,2-trichloroethane (TCEA) as the hepatotoxin and acetone as the model potentiating agent.

TCEA has been shown previously to have a very steep dose-response curve for hepatotoxicity (Plaa et al., 1958). Traiger and Plaa (1974) demonstrated that TCEA hepatotoxicity could be potentiated by a single oral dose of 2.5 ml/kg of acetone in mice without changing the centrilobular nature of the necrogenic toxicity. Although it was hoped that, with a steep dose-response curve for hepatotoxicity and a proven potentiation of hepatotoxicity by acetone, a dose range of TCEA could be found at which TCEA alone would produce minimal hepatotoxic damage and acetone pretreated animals would all display a potentiated toxicity, no TCEA dose was found that was both non-hepatotoxic in all fasted control animals and hepatotoxic in all acetone treated animals. Animals that did exhibit an acetone potentiated toxicity, however, displayed dramatic increases in SGPT activity and hepatocellular necrosis over those seen in fasted controls.

A single oral dose of 0.5 ml/kg of acetone was established as the optimum dose for eliciting the hepatotoxic potentiation response for TCEA. This dose is 5 fold less than the dose of acetone used to produce this response to TCEA and other hepatotoxins in previous reports in the literature (Traiger and Plaa, 1974; Maling et al., 1975). There has now been one other report that doses in the range of 0.5 ml/kg of acetone will potentiate carbon tetrachloride (CCl_4) hepatotoxicity (Hewitt et al., 1980). This is consistent with the finding that potentiation of CCl_4 hepatotoxicity is nearly maximized by 0.5 ml/kg of acetone (Fig. 3). Doses higher than 0.5 ml/kg of acetone have also been used to demonstrate enhancement of xenobiotic metabolism by acetone pretreatment for both in vivo and in vitro experiments (Sipes et al., 1973; Maling et al.,

1975; Sipes et al., 1978). An acetone dose of 0.5 ml/kg was employed in all experiments in this study as this dose was found to be the optimum dose for potentiating TCEA hepatotoxicity.

The time course of TCEA induced hepatic lesion development, with the greatest amount of damage observed between twelve and twenty-four hours after dosing, was consistent with those of other organohalogen hepatotoxins. Similar time courses have been reported for carbon tetrachloride by Recknagel (1967) and for chloroform and halothane (Ilett et al., 1973; Jee et al., 1980).

In order to characterize some of the mechanistic aspects of acetone potentiation it was necessary to understand the nature of TCEA's ability to cause hepatic damage. It has been shown that reactive toxic metabolites of a number of hepatotoxins, including the organohalogen hepatotoxins, bind covalently to tissue macromolecules. This has been shown to occur in vivo with carbon tetrachloride (Slater, 1966; Recknagel, 1967), chloroform (Ilett et al., 1973), and acetaminophen (Jollow et al., 1973). Covalent binding of reactive intermediates to proteins and lipids occurs well in advance of the cellular necrosis that appears at later time points after administration of the toxin to experimental animals. ¹⁴C-Chloroform, for example, exhibits maximal covalent binding to hepatic proteins by 6 hours after administration while hepatocellular necrosis is maximized at later time points (Ilett et al., 1973). Microsomal incubations with ¹⁴C-TCEA have shown that TCEA can be bioactivated to a reactive intermediate which covalently binds to cellular macromolecules (Gandolfi et al., 1979). It is therefore believed that TCEA causes hepatic centrilobular necrosis by

the same mechanism as the other, more thoroughly studied, centrilobular hepatotoxins.

It is believed that covalent binding of reactive intermediates to cellular macromolecules leads to some irreversible alteration in cellular integrity that leads to eventual necrosis and cell death because covalent binding of radiolabeled toxins to cellular macromolecules occurs well in advance of the release of cytosolic enzymes by the cell and cell death (Jollow et al., 1973; Maling et al., 1975). The whole sequence of biochemical and physical events leading from bioactivation of a compound to a reactive intermediate that can covalently bind to tissue macromolecules to the death of the cell is not known and is currently the subject of much speculation, debate, and research.

Alterations of xenobiotic metabolism may be a critical factor in the mechanism of acetone potentiation of TCEA hepatotoxicity. The role of cytochrome P-450 mediated metabolism in the generation of reactive intermediates capable of binding to tissue macromolecules has been clearly demonstrated in vivo by the effect of known cytochrome P-450 inducers and inhibitors on the subsequent levels of covalent binding (Ilett et al., 1973; Mitchell, Jollow, Potter, Davis et al., 1973). Phenobarbital induction of cytochrome P-450 results in more covalently bound ^{14}C -labeled chloroform adducts to cellular proteins than in uninduced control animals. If animals are treated with piperonyl butoxide, a known inhibitor of cytochrome P-450 mediated metabolism (Anders, 1968), less covalent binding of $^{14}\text{C-CHCl}_3$ is observed than is seen in controls (Ilett et al., 1973). This effect of metabolic

induction and inhibition has also been demonstrated in vivo for covalent binding of ^{14}C and ^3H -acetaminophen to protein (Jollow et al., 1973).

Covalent binding of ^{14}C label to macromolecules in in vitro systems has been demonstrated with many compounds (Uehleke and Werner, 1975; Sipes, Krishna and Gillette, 1977; Sipes et al., 1978; Gandolfi et al., 1979). Comparative amounts of covalent binding seen in in vitro incubations seems to correlate relatively well with the known toxicities of a number of organohalogens (Gandolfi et al., 1979). Compounds with a greater known hepatotoxic potential exhibited a greater degree of binding to microsomal macromolecules. This supports the contention that bioactivation to a reactive intermediate capable of binding to tissue macromolecules and their subsequent binding are essential initiating events in the production of many chemically induced liver injuries.

The sequence of events leading to cell death in TCEA induced hepatic necrosis have been found to be somewhat more complicated than the more thoroughly studied organohalogen hepatotoxin, CCl_4 . Unlike CCl_4 , TCEA induced hepatotoxicity seems to have a more sharply delineated threshold dose for toxicity, above which hepatic damage can occur (Plaa et al., 1958). This dose dependent phenomena contrasts with the fact that in in vitro incubations with ^{14}C -TCEA more ^{14}C label is covalently bound to proteins and lipids than in incubations with equimolar amounts of ^{14}C - CCl_4 (Gandolfi et al., 1979).

The threshold nature of TCEA hepatotoxicity and the seeming discrepancy between TCEA's high ability to yield a binding species in vitro and its lesser capacity for causing in vivo toxicity relative to CCl_4 is explained by the ability of TCEA to be metabolized to

glutathione conjugates. Yllner (1971) identified mercapturic acid metabolites of ^{14}C -TCEA that derive from GSH conjugates in mice. Furthermore, the inclusion of 1 mM GSH in microsomal incubations of ^{14}C -TCEA and ^{14}C - CCl_4 yielded an 80% reduction in covalent binding in ^{14}C -TCEA incubations, but did not affect the level of binding in ^{14}C - CCl_4 incubations (Gandolfi et al., 1980). This participation of GSH conjugation as a detoxification mechanism for TCEA metabolism is similar to the routes of metabolism seen in other threshold hepatotoxins, such as acetaminophen and chloroform (Mitchell, Jollow, Potter, Davis et al., 1973; Mitchell, Jollow, Potter, Gillette et al., 1973; Jollow et al., 1973; Potter et al., 1973; Brown, Sipes and Sagalyn, 1974).

The concept of a threshold toxicity, where a critical dose exists above which normal cellular mechanisms for detoxification are overwhelmed, has been most clearly demonstrated for acetaminophen (Mitchell, Jollow, Potter, Davis et al., 1973; Mitchell, Jollow, Potter, Gillette et al., 1973; Jollow et al., 1973; Potter et al., 1973). GSH conjugation is presumed to be a protective detoxifying pathway of acetaminophen metabolism when sulfation and glucuronidation conjugation reactions become saturated by an excess of acetaminophen. In the presence of excess acetaminophen cytochrome P-450 mediated metabolism will occur and electrophilic reactive intermediates are formed. When the excess of reactive intermediates is large enough, hepatic stores of GSH are depleted and acetaminophen metabolites begin to bind to tissue macromolecules. Furthermore, acetaminophen induced hepatic damage is only seen when doses large enough to cause hepatic GSH depletion are administered.

Glutathione is now known to have a number of important functions in the liver. GSH has three detoxifying functions (Orrhenius and Jones, 1978). GSH can act as a source of reducing equivalents reducing peroxides generated in the liver preventing the initiation of destructive peroxidative chain reactions (Chance et al., 1978; Orrhenius and Jones, 1978). GSH can act as a cofactor in aldehyde and α -ketoaldehyde oxidation and GSH is an important nucleophile in drug conjugation reactions (Orrhenius and Jones, 1978; Mitchell, Jollow, Potter, Gillette et al., 1973).

Tateishi and Higashi (1978) have presented evidence for the existence of at least two distinct pools of glutathione in the liver; a labile pool with a rapid turnover that acts as a source of cysteine for protein synthesis when other conditions are satisfied and cysteine availability is rate limiting, and a stable pool with a long half-life that is not readily affected by the metabolic or nutritional state of the liver. The existence of two hepatic glutathione pools is consistent with the fact that even high doses of TCEA will not deplete hepatic GSH stores below 2 or 3 μ moles/g of liver.

The hepatic levels of GSH seem to be critical to the expression of TCEA induced hepatotoxicity. Products of TCEA metabolism form GSH conjugates (Yllner, 1971) as well as covalently bind to tissue macromolecules (Gandolfi et al., 1979). TCEA is also known to cause lipoperoxidation in vitro (Klaassen and Plaa, 1969) reducing the amount of GSH immediately available for conjugation reactions.

In the present study of TCEA hepatotoxicity, GSH was found to significantly inhibit covalent binding of TCEA intermediates to

microsomal proteins and lipids in vitro. Like acetaminophen, TCEA induced hepatic damage was only seen at doses of TCEA that caused a significant depletion of GSH within 2 hours of administration. This evidence is strongly supportive of the supposition that the threshold nature of TCEA hepatotoxicity is attributable to and dependent upon depletion of hepatic stores of GSH before toxicity can occur.

Acetone has been demonstrated to potentiate the hepatotoxicity of TCEA in this and other studies. Acetone is also known to enhance the metabolism of a number of xenobiotics, both in vivo and in vitro (Anders, 1968; Sipes et al., 1973; Kitada et al., 1978; Sipes et al., 1978). The effect of acetone pretreatment on TCEA metabolism and bioactivation was assessed by measuring TCEA induced depletion of hepatic GSH and by the covalent binding of ^{14}C -TCEA to macromolecules in microsomal incubations and in vivo.

Overnight fasting of both acetone treated (0.5 ml/kg) and control rats caused a 35% reduction in hepatic GSH levels relative to fed controls. This effect of fasting is consistent with previously reported values (Tateishi and Higashi, 1974). Administration of TCEA to the fasted animals caused a further decrease in hepatic levels that was significantly greater in the acetone treated animals at all doses examined (Table 1). This evidence suggests that in the presence of acetone pretreatment more intermediates capable of reacting with hepatic GSH are produced, causing a greater net depletion of GSH (Table 1).

In vitro studies lend further support to the contention that acetone enhances bioactivation of TCEA. Hepatic microsomes prepared from the livers of acetone treated and fasted control rats display

different capabilities of generating reactive species from ^{14}C -TCEA that can bind covalently to proteins and lipids. Acetone pretreatment results in significantly more covalent binding to microsomal protein and lipid over thirty minutes of incubation. Similar results have reported by Sipes et al. for the covalent binding of ^{14}C - CCl_4 (1973) and ^{14}C -dimethylnitrosamine (1978) in the presence of acetone pretreatment.

Information from the in vivo studies utilizing ^{14}C -TCEA as a tracer along with unlabeled TCEA to follow covalent binding is not conclusive. Among the cellular subfractions examined, the covalent binding of ^{14}C label is highest in the microsomal subfraction of the liver. This would be expected if reactive intermediates are indeed generated by cytochrome P-450 mediated metabolism, as cytochrome P-450 is localized in the smooth endoplasmic reticulum. Hepatic GSH concentrations are significantly less and the amount of ^{14}C label covalently bound to proteins is significantly higher in the acetone pretreated rats receiving 1.7 mmol/kg TCEA two hours after dosing. Four hours after TCEA administration the amount of covalently bound ^{14}C label is higher in both acetone pretreated and fasted control groups than after two hours, but there is no difference between the two groups. This suggests the possibility that acetone pretreatment may have some effect on the initial rates of TCEA bioactivation, but may not alter the total amount of a dose that will eventually be bioactivated. Further work needs to be done to elucidate the time course effect that acetone may have on ^{14}C covalent binding to tissue macromolecules.

Preliminary studies of the amino acid adducts formed by the covalent binding of reactive intermediates of TCEA to proteins indicate that acetone pretreatment may have qualitative as well as quantitative effects on the amount and types of adducts formed. This is an area that is just being developed and will require much more work before definitive statements can be made.

These studies suggest that acetone pretreatment of rats results in a subsequent enhanced ability of the hepatic mixed function oxidases to bioactivate TCEA to reactive intermediates that are capable of reacting with GSH and covalently binding to hepatic proteins and lipids. Increased bioactivation has been shown to enhance toxicity of many toxins including CCl_4 and acetaminophen (Garner and McClean, 1969; Mitchell, Jollow, Potter, Davis et al., 1973) as well as TCEA (Carlson, 1973).

The evidence of greater depletion of hepatic GSH by TCEA, increased bioactivation of TCEA in in vitro systems, and preliminary evidence for possible increases in bioactivation of TCEA in vivo in the presence of acetone pretreatment suggests that the potentiation of TCEA hepatotoxicity by acetone is attributable, at least in part, to increased bioactivation of TCEA to a reactive intermediate species. The reactive intermediate may in turn form GSH conjugates or initiate lipo-peroxidation, depleting hepatic GSH stores, leaving the cell at risk for other forms of oxidative damage and allowing reactive intermediates to covalently bind to cellular constituents.

Evidence for increased bioactivation of TCEA does not preclude the possibility of other mechanisms contributing to the toxic

potentiation response of acetone pretreatment. Acetone or other alcohol/ketones may cause some potentiation of hepatotoxicity by some non-specific membrane effects in the cell, making the cell more susceptible to injury. These effects should be examined in this model or another model of alcohol/ketone potentiation of hepatotoxicity. It is possible that perturbation of cellular membranes by alcohols or ketones may account for the potentiation response by altering the membranes around the xenobiotic metabolizing enzymes. Kinetic studies should be performed to determine if the K_m or V_{max} of TCEA metabolism is altered by acetone pretreatment. The preliminary differences in the amino acid adduct profiles of ^{14}C -TCEA bound to hepatic proteins in vivo should be examined more closely between acetone pretreated and control animals.

APPENDIX A

STANDARD CURVES FOR BIOCHEMICAL ASSAYS

Figure A.1. Standard curve for GSH assay. — $r = .9992$.

Figure A.2. Standard curve for biuret protein assay. -- $r = .9998$.

Figure A.3. Standard curve for lipid phosphate assay. -- $r = .9996$.

List of Selected Abbreviations

°C	degrees Celsius
CCl ₄	carbon tetrachloride
CHCl ₃	chloroform
DPM	disintegrations per minute
EDTA	disodium ethylenediaminetetraacetic acid
g	gravity or grams
GSH	reduced glutathione
H&E	hematoxylin and eosin
i.p.	intraperitoneal
kg	kilogram
M	molar
μCi	microcurie
μl	microliter
μmole	micromole
ml	milliliter
mm	millimeter
mM	millimolar
mmole	millimole
N	normal
nm	nanometer
NADPH	nicotine adenine dinucleotide phosphate
p.o.	per os
RPM	revolutions per minute
SGPT	serum glutamate pyruvate transaminase
TCEA	1,1,2-trichloroethane
(w:v)	weight to volume

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