

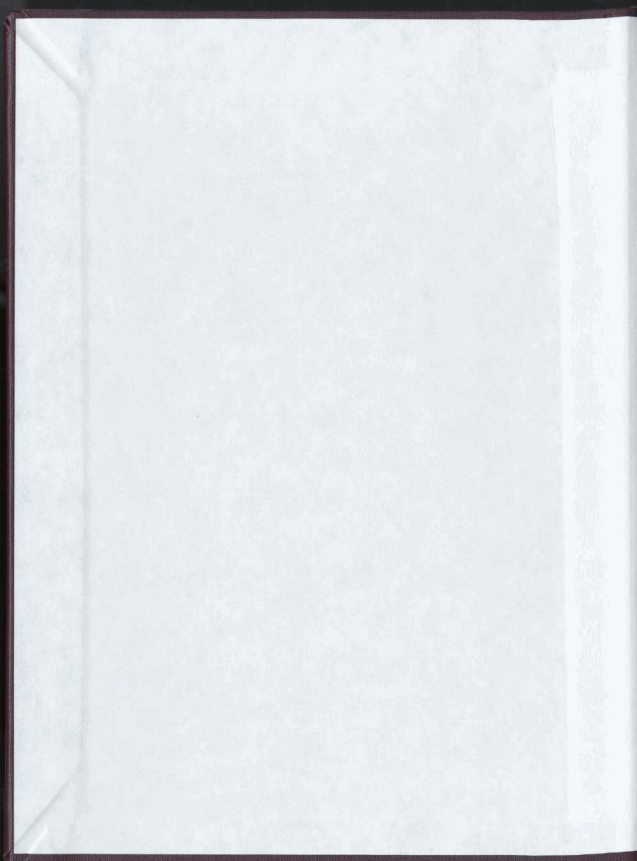
STUDIES OF THE PANCREATOTOXICITY
OF 1-CYANO-2-HYDROXY-3-BUTENE

CENTRE FOR NEWFOUNDLAND STUDIES

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MAURICA MAHER



Studies of the Pancreatotoxicity
of 1-cyano-2-hydroxy-3-butene

by

© Maurica Maher

A thesis submitted to the School of Graduate
Studies in partial fulfillment
of the requirements for the degree of
Master of Science

Toxicology

Memorial University of Newfoundland

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Abstract

A racemic mixture of (R)- and (S)-1-cyano-2-hydroxy-3-butene (CHB) was synthesized using 1,3-butadiene as a starting compound. The effects of CHB on pancreatic non-protein thiol and glutathione S-transferase activity were investigated in adult male rats when administered orally in olive oil at a dose of 200 mg/kg. At 4 hours after dosing, pancreatic non-protein thiol content was depleted but rebounded at 24 hours to 3 times control values. At 120 hours after dosing, pancreatic non-protein thiol levels were still elevated over control levels. Hepatic non-protein thiol levels were greater than control levels at 4 hours after dosing, but were not significantly different from control values at 24 and 120 hours after dosing. The pattern of pancreatic non-protein thiol changes with synthetic CHB resembles that reported with the natural (S)-isomer of CHB.

Glutathione S-transferase activity was decreased at 4 hours following CHB treatment in the rat pancreas, and elevated at 24 hours. Hepatic glutathione S-transferase

activity was not altered by CHB treatment.

The effect of increasing intraduodenal doses of CHB (25-200 mg/kg) on rat pancreatic juice secretion and bile secretion was investigated in conscious rats. CHB was administered intraduodenally and caused a stimulation in pancreatic juice secretion followed by a return to control values at the lower doses. At higher doses, CHB causes the same early stimulation, but is followed by a depression of pancreatic juice flow. All doses of CHB administered resulted in a dose-dependent decrease of protein concentration in pancreatic juice.

CHB caused the pancreata of treated rats to become oedematous, a condition also seen in certain forms of acute pancreatitis. The severity of the oedema was dose-dependent and could be visually detected. The exudate collected from these pancreata had protein concentrations comparable to plasma.

Radioactive CHB (^{14}C) was synthesized and administered to rats intraduodenally that were also fitted with biliary fistulae. CHB was found to be excreted over 8 hours in the bile and urine, with higher amounts excreted in the urine. Little ^{14}C was detected in the pancreas and kidneys, but did accumulate in the adipose tissue (7.7%) and in the liver (5.5%).

These results indicate that synthetic CHB is a powerful and selective pancreatotxin and the present data are not

incompatible with a glutathione-mediated mechanism, as is known to occur with other unsaturated nitriles.

Key Words

1-cyano-2-hydroxy-3-butene

pancreas

liver

detoxication

glutathione

glutathione S-transferase

nitrile

protein

bile

metabolites

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Abbreviations

CHB	1-cyano-2-hydroxy-3-butene
OZT	oxazoline-2-thione
SCN ⁻	thiocyanate
CHEB	1-cyano-2-hydroxy-3,4-epithiobutane
GSH	reduced glutathione
GSH-T	glutathione S-transferase
GSSG	oxidized glutathione
NP-SH	non-protein thiol
DTNB	5',5'-dithiobis-(2-nitrobenzoic acid)
CDNB	1-chloro-2,4-dinitrobenzene

Dedication

This thesis is dedicated to the memory of
Dr. James A. Barrowman

and

to Tabatha

Chapter 1

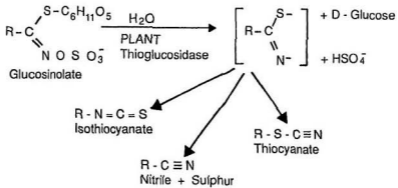
Introduction

1.1. Glucosinolates

Glucosinolates are compounds whose breakdown products are responsible for the pungent flavouring of cultivated plants such as turnip and cabbage (Fenwick *et al*, 1983). Most glucosinolate-containing plants belong to the *Cruciferae* family, many of which are major commercial crops. A variety of crucifers are used as pasture, forage, and silage for livestock and belong to the genus *Brassica* (Tookey *et al*, 1980). Mustard, broccoli, cabbage, cauliflower, turnip, and brussel sprouts are all commonly consumed *Brassica* plants. It is interesting to note that ancient cultivation of *Brassica* crops was performed to use the plants for medicines (Fenwick *et al*, 1983).

Glucosinolates exist as anionic salts in plants usually associated with potassium (Figure 1-1). They occur in all parts of the plant. An accompanying enzyme system is present which can hydrolyse glucosinolates, but is physically separated from the glucosinolate itself in the

**Figure 1-1: Glucosinolates
and their breakdown products**



HYDROLYSIS OF GLUCOSINOLATES
AND FORMATION OF POTENTIAL TOXINS

(For CHB : R = CH₂ = CH - CHOH - CH₂-)

plant. The enzyme was called myrosin or myrosinase (Daxenbichler *et al*, 1966) in early investigations, but the recommended name is thioglucoside hydrolase (EC 3.2.3.1.) (Tookey *et al*, 1980). This thioglucosidase has been identified in the leaf, stem, and roots of plants containing glucosinolates (Fenwick *et al*, 1983).

Thioglucosidase hydrolyses glucosinolates whenever the plant containing them is wet and crushed. The products of this hydrolysis are glucose, bisulfate ion, and an organic component (Figure 1-1). This remaining organic portion usually forms a nitrile with the loss of sulfur, or intramolecular rearrangement may occur yielding thiocyanates and isothiocyanates (VanEtten *et al*, 1969a). Another rearrangement may result in the formation of cyclic compounds such as oxazoline-2-thiones (OZT) and epithionitriles. Which aglucon product is formed depends on factors such as pH, the presence of Fe^{2+} ions, and the presence of an epithiospecifier protein (Tookey *et al*, 1980). The R- group of the glucosinolate itself may contain a cyclic organic moiety, as is found in indole glucosinolates.

1.1.1 Beneficial Effect of Cruciferous Diets

Early work by Wattenberg (1971) showed that cabbage, broccoli, and brussel sprouts added to rat diet caused a significant increase in the activity of benzo[a]pyrene

hydroxylase in rat intestine. This enzyme and other aryl hydroxylases are Phase I metabolizing enzymes which transform aryl hydrocarbons into more water soluble compounds, by a monooxidation reaction that inserts an atom of oxygen between a carbon and a hydrogen. Further work in this area by Loub *et al* (1975) showed that the inducing chemicals in these vegetables were glucosinolate derivatives, indole-3-carbinol, indole-3-acetonitrile, and 3',3'-diindolylmethane, the most potent of these being indole-3-carbinol. These three derivatives induced benzo[a]pyrene hydroxylase activity in the liver and small intestine. This work was supported by the studies of Pantuck *et al* (1976). The same three glucosinolate derivatives tested were found to increase the intestinal metabolism of four xenobiotics (hexobarbital, phenacetin, 7-ethoxycoumarin, and benzo[a]pyrene) with indole-3-carbinol again being the most powerful inducer.

Cruciferous diets are known to decrease the incidence of colon and rectal cancer. Graham *et al* (1978) reported that the risk of colon cancer in males and females decreases with amount of cabbage, brussel sprouts, and broccoli consumed. These are all cruciferous vegetables containing glucosinolates.

Stoewsand *et al* (1978) found that feeding cauliflower to rats enhanced hepatic xenobiotic metabolizing enzymes. The cauliflower diet also resulted in reduction of fatty

livers produced by dietary polybrominated biphenyls (PBB) and reduced the toxic effects of aflatoxin B₁. Both PBB's and aflatoxin B₁ are well known carcinogens.

Glutathione S-transferases (GSH transferases) in the liver were found to be induced by feeding weanling rats brussel sprouts (Godlewski *et al*, 1985). Both a glucosinolate-containing extract from brussel sprouts and the remaining residue (not containing glucosinolates) also induced GSH transferases. This information suggested that other compounds besides glucosinolates in cruciferous vegetables may play a role in some detoxication mechanisms.

Diets supplemented with cauliflower, kale, and brussel sprouts given to mice were found to induce the activity of ethoxycoumarin O-deethylase activity in the mouse liver (Bradfield *et al*, 1984). This enzyme is responsible for the deethylation of 7-ethoxycoumarin, a powerful carcinogen, by making it more water soluble and hence more readily excreted. Cauliflower and brussel sprouts diets did not induce the activity of mouse hepatic aryl hydrocarbon hydroxylase, but did induce the activity of mouse hepatic GSH transferases. The authors pointed out that increases in enzymic activity also occur as a response to toxic stress, and that the effective compounds in the vegetables may be toxins themselves.

Two kinds of cabbage (White and Savoy-type) fed to rats in 25% of the diet were found to significantly increase

deethylation of ethoxyresorufin (ERR: a carcinogen, McDannell et al (1988)).

1.1.2. Glucosinolate Breakdown Products

As previously mentioned, many crucifers are of important economic value. One of these is rapeseed, which is derived from varieties of *Brassica napus* and *Brassica campestris*. Oil is extracted from the seed and the remaining meal is used to feed livestock. However, it can only be fed in limited amounts because it contains toxic substances as described below (Tookey et al, 1980).

Rapeseed meals treated to yield products high in nitriles or goitrin ((S)-5-vinyl-OZT) cause decreased weight gain in rats and chicks (Srivastava et al, 1975). Rats are more sensitive to the nitrile-rich diet than chicks, and have pale enlarged kidneys. Turkeys fed rapeseed products for 16 weeks developed sclerosis of the liver showing multiple focal and/or solitary hepatocytic necroses preceding fibrosis of the liver (Umemura et al, 1977). The authors proposed that glucosinolates and their hydrolytic products are the likely cause for the diseased livers. Martland and Butler (1984) found that feeding rapeseed meal to laying hens resulted in liver hemorrhage, reticulolysis, thyroid enlargement, and other biochemical changes. Both high and low glucosinolate containing meals tested caused these problems.

Goitrin, found in rapeseed, was found to be thyroid-suppressing. Goitrin and its enantiomer (*R*)-5-vinyl-OZT are both antithyroid agents derived from glucosinolates having a side chain of (*R*)- and (*S*)-2-hydroxy-3-butene. These compounds act by inhibiting the uptake of iodine by the thyroid and by interfering with thyroxine secretion (Astwood et al, 1949).

Thiocyanate ions (SCN^-), also derived from glucosinolate hydrolysis, are antithyroid agents as well. They prevent the accumulation of iodine by the thyroid by inhibiting uptake and by causing the discharge of iodine from the gland (VanderLaan and VanderLaan, 1947).

Isothiocyanates also cause changes in thyroid function. Langer and Stolc (1963) found that allylisothiocyanate, a natural mustard oil component, significantly depressed radioiodine uptake by the rat thyroid. It was later determined that metabolic detoxication of isothiocyanates, such as allylisothiocyanate, involves the release of the thiocyanate ion from the molecule which is believed to be responsible for the thyroid inhibition of these compounds (Wood, 1975).

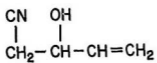
A substantial body of literature describes the deleterious effects of glucosinolates given in animal feeds including the important antithyroid effects of OZT compounds, thiocyanates, and isothiocyanates (Tookey et al, 1980; Fenwick et al, 1983).

1.1.3. Nitriles from Crucifers

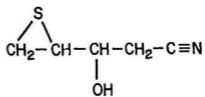
Nitriles obtained from cruciferous plants have also been investigated with respect to toxic effects. Abyssinian kale (*Crambe abyssinica*) contains a high level of (S)-2-hydroxy-3-butenyl glucosinolate at 80-97 mg/gm of plant (Daxenbichler et al, 1965). A mixed nitrile extract obtained from *Crambe* seed and fed to rats caused death at 0.2% by weight of the diet. At 0.1% of the total diet, rats had decreased weight gain along with liver and kidney lesions (VanEtten et al, 1969b). Daxenbichler et al (1968) previously showed that the mixed nitrile extract from *Crambe* meal contained 2S-1-cyano-2-hydroxy-3,4-epithiobutanes (erythro- and threo- forms), abbreviated to CHEB, and (S)-1-cyano-2-hydroxy-3-butene, abbreviated to CHB (Figure 1-2). Both of these compounds have been investigated for target organ toxicity and mode of action.

Nishie and Daxenbichler (1980) investigated the teratogenicity of glucosinolates and some of their derivatives including CHEB and CHB. Both compounds were administered daily to rats starting on day 8-9 of gestation by subcutaneous injection (s.c), and continued until 11-12 days after the beginning of treatment. Neither was found to be teratogenic, but at the highest doses tested (95 mg/kg body weight for CHEB, 150 mg/kg for CHB) both caused significant decreases in fetal weight (sc dose). CHB also resulted in a significant increase in occurrence of resorbed

Figure 1-2: 1-cyano-hydroxy-3-butene (CHB) (1)
and
1-cyano-2-hydroxy-3,4-epithiobutane (CHEB) (2)



(1)



(2)

fetuses. Both compounds caused depressed weight gains (s.c. dose) and increased adrenal weights in pregnant rats (s.c. dose). Pregnant rats treated with CHB had decreased liver weights and hepatic necrosis (s.c. dose of 175 mg/kg). CHEB was nephrotoxic to both pregnant female and male rats causing increased kidney weights (s.c. dose of 95 mg/kg).

The effect of feeding diets containing 75, 150, and 300 mg/kg CHEB to male rats was examined by Gould *et al* (1980). All diets caused decreased weight gain in the animals. Livers of CHEB treated animals had dose-related bile-duct hyperplasia and fibrosis. All rats fed the highest dose had severe liver damage and nephrocytomegaly. Three rats out of six fed the highest dose of CHEB had pancreatic acinar cell karyomegaly.

The nephrotoxicity of CHEB was further examined by Gould *et al* (1985). Rats gavaged with CHEB had increased serum urea nitrogen and creatinine and decreased glomerular filtration rates. Histology of kidneys showed necrosis and coarse vacuolation of proximal tubular epithelial cells. The pars recta (S₃ segment) of the proximal tubule was the initial target of CHEB. More extensive lesions were observed with larger doses and/or longer times of CHEB treatment.

The damage to rat organs caused by CHEB is discussed here as CHEB is similar in structure to CHB, the compound investigated in the present work. Wallig *et al* (1988)

studied the effect of natural (*S*)-CHB prepared from *Crambe abyssinica* on rats by gavaging them with 200 mg/kg CHB. They found upon examining organs by light microscopy that only the pancreas of treated animals was damaged. Some pancreata of treated animals were enlarged and oedematous after 2 days. Seen in all pancreata of treated rats were decreased zymogen granules and fragmentation of acinar cells. The authors stated that these and other changes seen in the pancreata of CHB treated rats were indicative of both necrosis and apoptosis (programmed cell death). Urinary thiocyanate excretion was monitored to determine if cyanide release from CHB was the cause of the pancreatic damage observed. Cyanide is detoxified in the liver by the enzyme rhodanase to yield thiocyanate, which is much less toxic than cyanide (Sipes and Gandolfi, 1986). Results showed that low molar amounts of thiocyanate were excreted in proportion to the molar dose of CHB administered and not enough to cause pancreatic lesions which in any case are not a feature of cyanide toxicity. These results show that natural CHB has a selective pancreatotoxic effect in rats either directly or as some metabolite other than cyanide.

Further work by Wallig and Jeffery (1990) showed that a single oral dose of CHB (200 mg/kg) reduced glutathione (GSH) content in the pancreas of rats until six hours after dosing. After this time GSH content of the pancreas increased and remained elevated up to 96 hours after CHB

treatment. GSH is involved in the detoxication of various xenobiotics, conjugating foreign compounds to make them available for excretion in the bile and urine. The authors reported that ultrastructural and histological changes seen in the pancreata of CHB treated rats were indicative of apoptosis and not necrosis. The GSH content of the liver was also determined after CHB dosing, and there was a slight depletion in GSH at 1, 6, and 24 hours after dosing followed by elevations of GSH at 48 and 72 hours. No hepatic lesions were found in the livers of treated rats at the 200 mg/kg dose used.

Ahmed and Farooqui (1982) determined that the toxicity of aliphatic nitriles is not dependent on cyanide release, but the degree of toxicity depends on the degree of unsaturation of the compound. Nitriles studied included acrylonitrile, acetonitrile, and fumaronitrile.

Silver et al (1982) examined the metabolism of nitriles *in vivo* to cyanide. They determined showed intraperitoneal injections of unsaturated nitriles resulted in less thiocyanate excretion as compared to that observed after oral administration of the same nitriles. Overall, the thiocyanate excretion associated with dosing of unsaturated nitriles was lower than that of saturated nitriles. The authors indicated that their results suggested that a number of factors are important in the release of cyanide from nitriles including the position of double bonds, route of

administration, length of the carbon chain, and presence of substitutions at the α -carbon.

The mechanism of pancreatotoxic action of CHB is not known. As discussed above, cyanide release from CHB is not thought to be a major factor in its toxicity.

1.2. Metabolism of Xenobiotics

Xenobiotics are compounds that are foreign to biological systems and are often harmful to living cells. Xenobiotics include compounds found in nature, pollutants, and other chemicals such as drugs. The majority of these compounds are lipophilic. Exposure to xenobiotics can occur through dermal absorption, inhalation, or ingestion.

To cope with harmful xenobiotic exposure, organisms have developed a number of metabolic pathways which act on xenobiotics so they may be excreted by regular routes. As organisms are exposed to a variety of chemicals, they possess a number of different enzymes which are involved in the metabolism of xenobiotics (Armstrong, 1987).

Lipophilic compounds pose the greatest threat to organisms as they can become widely distributed throughout the organism in lipid stores and membranes. Furthermore, they tend to remain in these lipid stores for long periods of time. To be excreted from the organism, they must therefore be transformed to more hydrophilic molecules which can be readily eliminated (Armstrong, 1987).

Biotransformation reactions take place mainly in the liver (Chhabra and Eastin, 1984), but other organs such as the brain, kidneys, adrenals, small intestine, and the pancreas have xenobiotic metabolism capability.

Biotransformation can occasionally result in the inadvertant retention of xenobiotics by causing the formation of more reactive metabolites which react with endogenous macromolecules (Caldwell, 1985).

There are two classes of biotransformation reactions, known as phase I and phase II reactions. Phase I reactions modify the molecular structure of the xenobiotics by oxidation and reduction reactions. These reactions are carried out by a number of related hemoproteins, notably the cytochrome P-450 mono-oxygenase system. Other oxidative reactions are carried out by aldehyde and alcohol dehydrogenases (Laitinen and Walkins, 1985).

Phase II reactions involve the addition of endogenous substrates which are hydrophilic and increase the solubility of the compound, enabling it to be excreted in the bile or urine.

There are seven major phase II reactions of xenobiotics occuring in mammals. These are glucuronic acid, sulfuric acid, glutathione, and amino acid conjugations, and also methylation, acetylation, and epoxide hydration reactions (Caldwell, 1985; Sipes and Gandolfi, 1986).

Only GSH conjugation will be discussed here as it has

been implicated in the detoxication of unsaturated aliphatic nitriles.

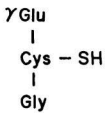
1.2.1. Glutathione and Glutathione S-Transferase

GSH is a tripeptide composed of L-glutamate, L-cysteine, and glycine (Figure 1-3). GSH exists in 2 forms, as a thiol reduced molecule (GSH) and in a disulfide oxidized (GSSG) form. The ratio of GSH:GSSG in the liver is maintained at 250:1. It is found in nearly all tissues of aerobic organisms and is usually found in millimolar concentrations (Kaplowitz *et al*, 1985). GSH is present mainly in the cytosol of cells and is the most abundant sulfhydryl in animal tissues (Chasseaud, 1979).

One of the highest concentrations of GSH in the rat is found in the liver (approximately 172 mg/100g tissue). A substantial amount of GSH is also found in the pancreas (approximately 56 mg/100g tissue) (Boyland and Chasseaud, 1969).

The GSH molecule is of biological importance because it is a specific substrate for GSH transferases (GSH-T), a group of enzymes that play an important role in the detoxication of foreign compounds (Kaplowitz *et al*, 1985). GSH transferases (a family of isoenzymes) conjugate nucleophilic GSH to lipophilic compounds that have an electrophilic center. Many of these conjugations occur nonenzymatically, but the action of GSH transferases

**Figure 1-3: Glutathione
(γ -glutamyl-cysteinyl-glycine)**



increases the rate of the reaction (Chasseaud, 1979).

GSH transferases bind the lipophilic compounds and GSH by lowering the pK_a (9.3) of GSH which results in the ionization of GSH to GS^- and H^+ . The GS^- ion is more nucleophilic than GSH and is more likely to bind to the electrophilic center of the bound substrate molecule (Keen *et al*, 1976). The resulting compound is a thiol ether.

Different GSH transferases are responsible for the conjugation of GSH with different classes of lipophilic compounds. The isozymes are specific with respect to organic groups in the electrophilic substrates such as alkyl, aryl, and epoxy groups (Mannervik *et al*, 1983). GSH transferases can conjugate suitable substrates that are both endogenous and exogenous, but a major function is their role in detoxifying reactive molecules that may react with biological molecules such as proteins and DNA.

In addition to their enzymic activity, GSH transferases are important in functioning as intracellular ligands. For example, ligandin or Y protein of the hepatocyte is important in binding and intracellular transport of bilirubin (Ketterer *et al*, 1988).

Xenobiotics may be reactive as the intact forms, or they may be activated following phase I biotransformation. GSH transferases are one of a number of detoxifying phase II enzyme systems responsible for detoxifying such molecules. Some reactive molecules are known to initiate carcinogenesis

and mutagenesis. Therefore the ability of GSH transferases to detoxify reactive electrophiles is of vital importance, as is the case concerning the detoxication of acetaminophen where small amounts of a reactive quinone imine are potentially very injurious (Ketterer, 1988).

Other detoxifying reactions of GSH transferases involve their ability to act as GSH peroxidases and detoxify hydroperoxides. Hydroperoxides are harmful to tissues (Mosialou and Morgenstern, 1989), forming GSSG which is later reduced by GSH reductase. Hydroperoxides which are formed from lipids during oxidative stress can propagate free radical formation within cells, and have been implicated in carcinogenesis (Ketterer, 1988).

1.2.2. Mercapturic Acid Formation

After glutathione conjugation occurring in the liver, the metabolites are transported into plasma and bile (Anders et al, 1988). The enzyme γ -glutamyltransferase (EC 2.3.2.2.) catalyses the removal of the glutamyl portion of the conjugate. Glycine is then removed from the molecule by cysteinyl glycine dipeptidases (EC 3.4.13.6. and EC 3.4.11.2.), leaving a cysteine S-conjugate (a thiol ether). Both processing of plasma GSH and cysteine S-conjugates is carried out primarily in the kidney, which metabolises GSH conjugates as described above. Cysteine S-conjugates are acetylated by *N*-acetyltransferase (EC 2.3.1.80.) to form

mercapturic acids which are water-soluble and excreted in the urine (Wood, 1970; Anders et al, 1988) (Figure 1-4).

1.3. Pancreatotoxins

The pancreas, although primarily an endocrine and exocrine functioning organ, is capable of a number of biotransformation reactions (Scarpelli, 1989). These reactions can both protect it from xenobiotic damage or cause harm by creating more reactive metabolites, as also seen in the liver and other organs.

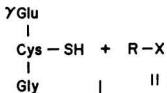
Toxins that have selective toxicity for the pancreas are not common, and hence CHB is a very interesting compound because of its selective pancreatotoxicity. There is no commonality among pancreatotoxins. Pancreatotoxins cause acinar cell necrosis and other injuries, depending on the intensity, duration, and frequency of exposure. Pancreatotoxins include alcohols (such as ethyl and allyl alcohol), amino acids and their derivatives (i.e. azaserine), sulfonamides, and estrogens (Scarpelli, 1989).

Carcinoma of the exocrine pancreas was fifth among cancers as a cause of death in the United States in 1983 (Wiebkin et al, 1984). The pathogenesis of this disease is not known, but several pancreatic carcinogens are currently being investigated. Compounds known to induce pancreatic cancer include the nitrosamines *N*-nitrosobis(2-oxopropyl)amine (Kokkinakis, 1983) and *N*-Nitroso-2,6-

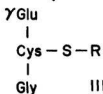
Figure 1-4: Mercapturic Acid Formation

dimethylmorpholine (Scarpelli *et al*, 1982), which are carcinogenic to the hamster pancreas. Azaserine, an amino acid derivative, is a rat pancreatic carcinogen, and has been extensively studied by Longnecker and his colleagues (Longnecker *et al*, 1981). It is not known if CHB is carcinogenic.

I Glutathione

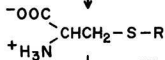


II Reactive Electrophile



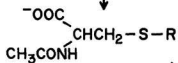
III Glutathione Conjugate

hydrolysis



IV Cysteine Conjugate

acetylation



V Mercapturic Acid

1.4. Objectives of the present study

In view of the evidence quoted above that the natural (*S*)- enantiomer of 1-cyano-2-hydroxy-3-butene is a pancreatic toxin in rats, the following objectives for the present study were developed.

1. To determine the effect of a synthetic 50:50 enantiomeric mixture of CHB on pancreatic and hepatic non-protein thiol concentrations.
2. To determine the inducibility of pancreatic and hepatic glutathione *S*-transferase by synthetic CHB.
3. To determine the effect of synthetic CHB on pancreatic juice secretion and on the protein concentration of pancreatic juice.
4. To investigate the effect of synthetic CHB on bile secretion and bile acid concentration.
5. To examine the excretion of [¹⁴C] synthetic CHB in the rat and its possible accumulation by the pancreas.

Chapter 2

Materials and Methods

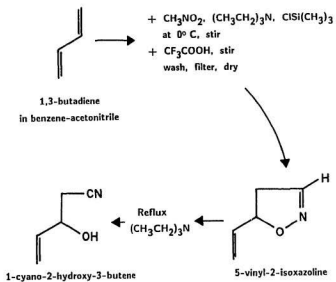
2.1. Synthesis of CHB

CHB was derived synthetically as a racemic mixture of (*R*)- and (*S*)-CHB as described by Das and Torsell (1984), with some slight modification. 1,3-Butadiene is the starting compound used in the synthesis, which is performed in 2 steps (Figure 2-1). The first step produces 5-vinyl-2-isoxazoline, which is then used to produce CHB. All chemicals were obtained from the Aldrich Chemical Company Inc. (WS,USA) and were of the highest purity available.

2.1.1. Synthesis of 5-vinyl-2-isoxazoline

Approximately 5.2 g of 1,3-butadiene was dissolved in 48 mL of benzene:acetonitrile (2:1). At 0°C 4.4 g of nitromethane, 7.0 g of triethylamine, and 7.8 g of chlorotrimethylsilane were added to the 1,3-butadiene in benzene:acetonitrile. The mixture was stirred for 3 hours at 0°C, then at room temperature for 3 days, at which point

Figure 2-1: Synthesis of CHB



0.5 mL of trifluoroacetic acid was added. The mixture was stirred at room temperature for another 24 hours. The final mixture was an orange-brown solution containing a light brown precipitate. The precipitate was filtered and washed with 10 mL of benzene and then with 30 mL of water. The filtrate was then dried with anhydrous magnesium sulphate, filtered, and excess solvents were evaporated from the remaining filtrate. The filtrate was 5-vinyl-2-isoxazoline, a dark orange-brown liquid.

2.1.2. Synthesis of CHB from 5-vinyl-2-isoxazoline

5-vinyl-2-isoxazoline (1.8 g) was refluxed with 4.0 g of triethylamine in an oil bath at 100°C for one hour. The excess triethylamine was evaporated and the mixture distilled *in vacuo*. At a pressure of 6 mmHg CHB had a boiling point of approximately 63°C and distilled as a pale yellow liquid. Approximately 1-1.5 g of CHB was obtained from each synthesis. Structure of CHB was confirmed by [¹H] NMR (CDCl₃). The spectrum showed that the compound was essentially pure, contaminated slightly by acetonitrile.

2.2. Non-Protein Thiol and Glutathione S-Transferase Induction

2.2.1. Animals

Male Sprague-Dawley rats weighing 235 to 280 g were purchased from Canadian Hybrid Farms (NS, Canada). The rats were housed in plastic rectangular Nalgene cages with removable lids lined with sawdust (Beta-Chip, Charles River, St. Constant, PQ) (3 or 4 rats/cage). Animals were maintained under a standard 12 hour photoperiod, at 23°C, and 40% relative humidity. The animals were provided with food (Purina Rat Chow #5012) and tap water *ad libitum* until the day of sacrifice.

2.2.2. Treatment with CHB

Synthetic CHB was emulsified in olive oil to a final concentration of 100 mg CHB/mL olive oil. Rats received CHB in oil intragastrically using a 5 French infant naso-gastric feeding tube (Medi-Craft Limited, ON, Canada). The dose of CHB in oil received was 200 mg/kg body weight (2.1 mmol/kg) which was approximately 0.5 mL. Control animals were dosed with olive oil alone (0.5 mL). The animals were lightly anaesthetized just prior to dosing with diethyl ether.

Animals used in the time course experiment were sacrificed at 0 hour and at 4, 24, and 120 hours after

dosing. Six animals were used for each treatment group and the 0 hour group was used as a control.

2.2.3. Cell Fractionation and Protein Precipitation

Rats were sacrificed by exsanguination from the abdominal aorta under diethyl ether anaesthesia. The abdominal cavity was flooded with chilled 0.9% NaCl (saline) solution. Approximately 500 mg of liver and the entire pancreas were removed and placed in ice-cold saline. The pancreas was trimmed of remaining fat, major blood vessels, and connective tissue. Both the liver and pancreas were blotted between two layers of No.2 filter paper (Whatman) under a 200 g weight for 15 seconds to remove surface saline. Approximately 200 mg of hepatic tissue (from the bottom right lobe of the liver) and 400 mg of pancreatic tissue from each animal were taken and placed into beakers containing 25 mL of cold Tris buffer (50 mM Tris with 0.25 M sucrose, pH 7.5) for GSH-T and protein assays. For homogenization, samples were poured into 30 mL glass homogenization tubes (Wheaton, USA).

For determination of non-protein thiol (NP-SH) approximately 100 mg of hepatic tissue and 200 mg of pancreatic tissue from each animal was taken and placed in a test tube containing 5.0 mL of 0.02 M EDTA. For homogenization, samples were poured into 10 mL glass

homogenization tubes (Wheaton, USA).

All tissues were homogenized at 0-4°C using a teflon pestle attached to an electric 3/8" drill (Black and Decker). The pestle was moved up and down nine times for homogenization of pancreatic tissue, and six times for homogenization of liver tissue at a pestle speed of 1200 rpm. The vertical movement and rotation of the pestle results in a tearing and mincing of the tissue and agitation of the cells generating a homogenate composed of diluted cell cytoplasm, intracellular particles, and some unbroken cells.

The GSH-T/protein homogenates were then transferred to Beckman polycarbonate centrifuge bottles 1"×3.5" and centrifuged for 60 minutes at 105,000 $g_{(uv)}$ in a Beckman L5-65 type H ultracentrifuge (35,000 rpm, 50.2 Ti rotor, 4°C). Two 3 mL samples were placed in 7 mL glass scintillation vials, one vial placed in ice for protein assays, the other stored at -70°C until assayed for GSH-T activity. After samples were removed for protein assays, the first vial was also stored at -70°C. Duration of storage never exceeded 7 days.

The NP-SH homogenates were used immediately for the NP-SH assay.

2.2.4. Principle of Protein Assay

Estimation of sample protein concentration was executed using the Bio-Rad protein assay kit, a dye-binding assay (Bio-Rad, ON). The assay is based on the differential colour change of a dye, in this case Coomassie Blue G-250, in response to various protein concentrations. The absorbance maximum of this dye shifts from 465 nm to 595 nm when binding to protein occurs (Pierce and Suelter, 1977), and this increase is monitored spectrophotometrically.

2.2.4.1. Preparation of Dye Reagent

The dye reagent was provided as a five-fold concentrate which was diluted with deionized water and filtered through a No.1 Whatman filter paper into a glass beaker just prior to use. The reagent was newly prepared every time the assay was performed and kept at room temperature until used.

2.2.4.2. Protein Standard

The protein standard used was Bio-rad Standard I, lyophilized bovine gamma globulin which was reconstituted with 20.0 mL of deionized water. The final concentration of this standard was 1.4 mg/mL. It was stored at 4°C for up to 60 days.

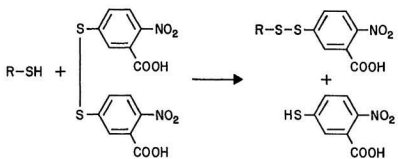
2.2.4.3. Protein Assay Method

Volumes of 10, 20, 40, 60, 80, and 100 μL of the standard protein solution were added to 4.5 mL polystyrene disposable cuvettes. This yielded final protein concentrations of 5.82, 11.6, 23.0, 34.2, 45.3, and 56.2 μg protein/mL after 2.5 mL of diluted Bio-rad reagent was added using an Eppendorf repeater pipetter. The standards and unknown samples were mixed by gentle inversion. Samples were allowed a 5 minute equilibration period and then were read at 595 nm in a Gilford 250 spectrophotometer against a blank containing only diluted reagent. The absorbance readings of the known concentrations were used to construct a standard curve (protein concentration versus absorbance). The curve was then used to determine protein concentration of the unknown samples. A new standard curve was constructed each time the assay was performed, and all assays were done in triplicate.

2.2.5. Principle of Non-Protein Thiol Assay

GSH is the major free thiol in most living cells and thus GSH levels were determined using the non-protein sulfhydryl assay based on the method of Ellman (1958) and modified by Sedlak and Lindsay (1968) (Figure 2-2). Ellman reported that 5',5'-dithiobis-(2-nitrobenzoic acid) (DTNB) is reduced by 1 mole of sulfhydryl group to form 1 mole of

Figure 2-2: The reaction of
5',5'-dithiobis-(2-nitrobenzoic acid)
(DTNB) with a sulfhydryl group to form
2-nitro-5-mercaptobenzoic acid



2-nitro-5-mercaptobenzoic acid. The resulting nitromercaptobenzoic acid anion has an intense yellow colour, and its absorbance is measured spectrophotometrically. By creating a standard curve of the concentration of GSH versus absorbance, determination of NP-SH concentrations in unknown samples is possible.

2.2.5.1. Standard Preparation

A standard curve of GSH concentration versus absorbance was constructed using concentrations of 1 to 10 ($\times 10^{-5}$) M of GSH (Sigma, USA). The GSH was dissolved in deionized water. The GSH was added to 0.4 M Tris buffer at pH 8.9 in a test tube to yield a final volume of 6.0 mL, then 0.1 mL of 0.01 M DTNB in absolute methanol was added. The tube was inverted to mix the contents, then the contents were transferred to a cuvette and the absorbance read at 412 nm within 5 minutes of the DTNB addition. Buffer with DTNB added served as a blank.

2.2.5.2. Determination of NP-SH Groups in Samples

After homogenization in 0.02 M EDTA, the 5 mL homogenates were placed in 15 mL test tubes, and 5 mL of 10% TCA was added to each tube to precipitate the protein present. The tubes were allowed to stand for 15 minutes at room temperature with intermittent shaking, then centrifuged

for 20 minutes at 3000 g in a Clay Adams (NJ, USA) Dync II Centrifuge. Two mL of the supernatant was added to a test tube containing 4.0 mL of 0.4 M Tris buffer at pH 8.9. Then 0.1 mL of a 0.01 M solution of DTNB in absolute methanol was added, the contents mixed and transferred to a polycarbonate cuvette. The absorbances of the cuvettes were read at 412 nm within 5 minutes of the addition of DTNB against a reagent blank containing no homogenate.

2.2.6. Principle of Glutathione-S-Transferase Activity Assay

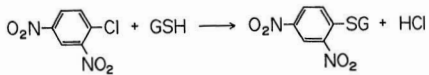
The assay technique used was the method reported by Habig et al (1974) as modified by Kraus and Kloft (1980). The assay determines GSH-T activity by measuring the change in absorbance as a GSH-substrate conjugate is formed by the enzyme.

The substrate used in this assay is 1-chloro-2,4,-dinitrobenzene (CDNB) and is the most common substrate used for this assay. CDNB in ethanol has a slight yellow colour, but when conjugated to GSH, the product is a light milky white solution (Figure 2-3). The greater the activity of GSH-T present in the test sample, the greater the change in absorbance will be during the assay.

2.2.6.1. GSH-T Activity Assay Method

The buffer used in the assay was 10 mM phosphate buffer

Figure 2-3: The reaction of
1-chloro-2,4-dinitrobenzene (CDNB)
with GSH to form a glutathione conjugate



(pH 7.4) containing 1.1 mM magnesium chloride, kept warm at 37°C. The volume of liver cytosol assayed was 20 μ L and the volume of pancreatic cytosol assayed was 100 μ L. A Gilford 250 spectrophotometer containing a 4 chambered water-jacketed cuvette holder was used for this assay, equilibrated at 37°C.

Phosphate buffer was pipetted into the cuvettes placed in the spectrophotometer, then the appropriate volume of sample was added to the cuvettes, with the fourth acting as a blank, containing no sample. Buffer was added to yield a final volume of 3 mL in all cuvettes taking into account additions of GSH and CDNB later. 100 μ L of GSH in water was added to all cuvettes (made up to yield a final concentration of 1 mM in 3 mL). The samples were incubated at 37°C in the spectrophotometer for 5 minutes, then 50 μ L of CDNB in ethanol was added (made up to yield a 1 mM solution in 3 mL) to each cuvette and the contents of each cuvette mixed.

The absorbance change was measured at 340 nm and recorded on a Gilford 6050 chart recorder for 3 minutes after the CDNB addition. The average of the three enzyme activities was calculated using the extinction coefficient for CDNB as $9.6 \text{ mM}^{-1}\text{cm}^{-1}$ and expressed as nmol GSH conjugated/min/mg protein.

2.3. The Effect of CHB on Pancreatic Juice and Bile Secretion

2.3.1. Animals

Male Sprague-Dawley rats weighing 235 to 325 g were obtained from Canadian Hybrid Farms (NS, Canada) and maintained as previously described.

2.3.2. Surgery

2.3.2.1. Anaesthesia

Diethyl ether vapour was used to induce and maintain anaesthesia. The rat was placed in a glass jar containing cotton soaked with ether, and the jar covered with a heavy plexiglas lid. After anaesthesia had been achieved the rat was removed. A 150 mL beaker containing gauze soaked with ether was used as a mask to maintain anaesthesia during the surgery.

2.3.2.2. Bile Duct Cannulation

The rat was placed in the supine position and the abdomen shaved with an electric shaver. The abdomen was opened by a midline laparotomy incision 3.5 cm long using a No.10 blade.

The duodenum was deflected to expose the common bile

duct and the duodenal head of the pancreas. The common bile duct was cannulated at the ampullae of Vater with polyethylene tubing (PE 10, Clay Adams, O.D.=0.61 mm, I.D.=0.28 mm) measuring 18 cm long. The cannula was inserted into a small incision in the duct and secured with 2 silk sutures (Ethicon, 4-0 silk). This cannula collects a mixture of bile and pancreatic juice but after cannulation of the common bile duct above the pancreas it collects pure pancreatic juice (Figure 2-4).

The common bile duct above the pancreas was also cannulated by inserting another 18 cm long PE 10 tube into the duct. This cannula was used to collect bile.

Both cannulae were exteriorized through stab wounds in the side of the abdomen.

2.3.2.3. Duodenal Cannulation

A 5 French infant naso-gastric tube, 45 cm long, was placed in the duodenum approximately 8-10 mm distal to the pylorus (Figure 2-5). The cannula was inserted into a small incision and secured with a cyanoacrylate tissue adhesive. The tubing was exteriorized through a stab wound in the right flank of the abdomen. To ensure that administered doses are quantitatively delivered to the duodenal lumen and as a check against leakage, approximately 0.5 mL of 0.9% NaCl was infused through the cannula.

Figure 2-4: Bile Duct Cannulation

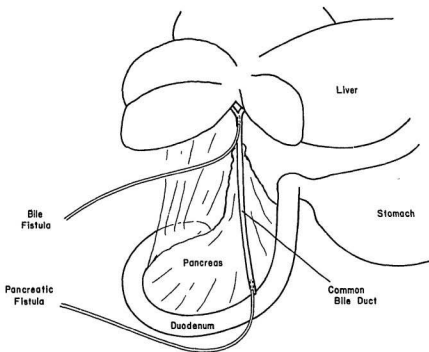
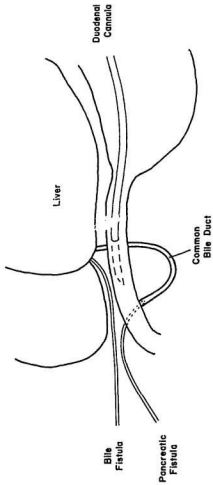


Figure 2-5: Duodenal Cannulation



2.3.2.4. Closure

The abdominal organs were returned to their proper positions and the muscle layers and skin closed separately with a continuous silk suture (Ethicon, 4-0 silk).

2.3.2.5. Post-operative housing and sacrifice

The animals were secured in Bollman restraint cages immediately after surgery as this provides humane immobilization to prevent access to the cannulae. A saline-glucose solution (0.9% NaCl containing 5% glucose) was infused into the duodenal cannula at a rate of 2.5 mL/hr during recovery (approximately 24 hours) using a Harvard pump. Food and water were withheld and the animals kept at a temperature of 23°C with a 12 hour photoperiod.

After the experiment, animals were anaesthetized by diethyl ether and sacrificed by exsanguination from the abdominal aorta. Animals that did not have constant pancreatic juice and bile secretion on the first post-operative day were eliminated from the study.

2.3.3. Test Dosages and Administration

CHB was emulsified in 1 mL volumes of olive oil at the following doses: 25, 50, 100, 150, and 200 mg/kg body weight. Each test group for each dose contained six rats. Experiments were performed on the first post-operative day (

approximately 20 hours after surgery). At zero time, the saline-glucose infusion was stopped and the appropriate dose was administered as a pulse through the duodenal cannula (volume approximately 0.5 mL). Approximately 0.5 mL of saline (0.9% NaCl) was then passed through the cannula ensuring that the test dose had been delivered into the duodenum. The intraduodenal glucose-saline infusion was then restarted at a rate of 3 mL/hr with the infusion pump.

2.3.4. Pancreatic Juice and Bile Sample Collections

Pancreatic juice and bile were collected in preweighed plastic tubes and glass vials, respectively. Both were collected at half hour intervals for seven hours. Control samples of pancreatic juice and bile were collected at one hour and 30 minutes before administration of the test dose.

Tubes and vials were re-weighed to determine the mass of pancreatic juice and bile collected. Both pancreatic juice and bile were assumed to have a density of 1 g/mL.

2.3.5. Determination of Protein Concentration in Pancreatic Juice

Protein concentration in collected pancreatic juice was determined using the Bio-rad assay previously described. As pancreatic juice has a very high protein concentration, each sample was diluted five times for the assay with deionized

water.

2.3.6. Principle of Bile Acid Concentration Assay

The major bile acids are taurine and glycine conjugates of cholic acid, chenodeoxycholic acid, deoxycholic acid, and lithocholic acids. In the determination of bile acid concentrations, the bile acids are first oxidized to 3-oxo bile acids by 3 α -hydroxysteroid dehydrogenase (3 α -HSD) along with the reduction of NAD to NADH. NADH is then oxidized to NAD with the reduction of nitro blue tetrazolium (NBT) to formazan by the enzyme diaphorase (Mashige et al, 1981). Formazan has a maximum absorbance at 530 nm and the intensity of the colour produced is directly proportional to the concentration of bile acids present.

2.3.6.1. Preparation of Reagents

Reagents were supplied in the Bile Acids Kit (Sigma, USA). Two types of reagents were used in this assay. Reagent A contained NAD (2.5 mmol/L), NBT (0.61 mmol/L), diaphorase (625 units/L), and buffer (pH 7.0). Reagent B contained 3 α -HSD (1250 units/L).

Reagents A and B were reconstituted with the appropriate amount of water. Test reagent was prepared by combining 4 mL of Reagent A with 1 mL of Reagent B. Blank reagent was prepared by combining 4 mL of Reagent A with 1

2.3.6.3. Calculations

The concentration of bile acids in each sample was determined by the following equation:

bile acid concentration =
(AST - ASB/ACT - ACB) (concentration of calibrator).

In this equation, AST = absorbance of sample with test reagent, ASB = absorbance of sample with blank reagent, ACT = absorbance of calibrator with test reagent, and ACB = absorbance of calibrator with blank reagent.

2.3.7. Determination of Pancreatic Water Content and Protein Concentration of Pancreatic Exudate

The pancreas of each control and treated animal was removed, blotted for 20 seconds with a 200 g weight between filter paper (Whatman, No.2), and a portion weighed. This portion was then dried to constant weight at 100°C (approximately 48 hours), then reweighed. The percent water of each portion was then calculated.

As the pancreata from animals treated with 200 mg/kg CHB were the most oedematous, as much exudate was removed from these pancreata as possible with an Eppendorf pipette and kept at 4°C until the samples could be analyzed for protein concentration. Protein determination was carried out by the method described in Section 2.2.4.

mL of deionized water.

Other reagents used were the Bile Acids Calibrator (bile acids in calf serum at a concentration of 100 $\mu\text{mol/L}$) and the Bile Acids Stop Reagent (1.33 mol/L phosphoric acid) (Sigma, USA).

2.3.6.2. Assay Procedure

The test and blank reagents were warmed to 37°C. A series of labelled test tubes were made: calibrator, calibrator blank, sample, and sample blank. Into the appropriate tubes 0.2 mL of either Bile Acids Calibrator or bile sample was pipetted. At 30 second intervals 0.5 mL of the test reagent was added to the calibrator and sample labelled tubes, and 0.5 mL of the blank reagent was added to the calibrator blank and sample blank labelled tubes. Tube contents were mixed immediately and incubated to 37°C for 5 minutes. After 5 minutes, the enzyme reaction was stopped with the addition of 0.1 mL Bile Acids Stop Reagent which maintains the colour for at least 1 hour. The final solutions were pipetted into microcuvettes (capacity of 1 mL) and the absorbance read at 530 nm. All assays were done in duplicate and the average of 2 absorbances used for calculations of the bile acid concentration in the bile samples.

2.4. Distribution of Radioactive CHB in the Rat

2.4.1. Animals

Male Sprague Dawley rats weighing 290 to 310 g were obtained from Canadian Hybrid Farms (NS, Canada) and were maintained as described previously.

2.4.2. Synthesis of [¹⁴C] CHB

The synthesis of [¹⁴C] CHB followed the same procedure previously described for the synthesis of the compound except that [¹⁴C] nitromethane (3.34 mCi/mol; New England Nuclear, MA, USA) was used having a specific activity of 1.2×10^8 dpm. The synthesis yielded approximately 1 g of [¹⁴C] CHB having a specific activity of 6.76×10^4 dpm/mg.

2.4.3. Surgery

Anaesthesia, surgery, and post-operative housing were performed in the same manner as previously outlined, establishing duodenal and biliary cannulae in the animals. In a preliminary experiment, a pancreatic fistula was also established in the animals.

2.4.4. Sacrifice

Rats were lightly anaesthetized with ether and killed by exsanguination from the abdominal aorta. The abdominal

organs were flooded with ice-cold saline (0.9% NaCl). Pancreas, liver, kidney, and the epididymal fat pads were removed and placed in saline until weighed.

2.4.5. Radioactive Test Meal Composition

The radioactive meal consisted of CHB in olive oil to give a total dose of 100 mg/kg of which 5 mg/kg was [^{14}C] CHB. Emulsification of the test meal was achieved by inversion and vigorous mixing. Three aliquots of the test meal were removed for scintillation counting. The average specific activity of the test meal administered was 309355 dpm/g of meal.

2.4.6. Test Meal Administration

The experiments were done on the first post-operative day. The test meal was drawn up into a 1cc tuberculin syringe and weighed. The saline-glucose infusion was interrupted and the meal given as a pulse through the duodenal cannula. The cannula was then cleared with 0.5 mL of saline (0.9% NaCl) ensuring that the test meal had been delivered to the duodenum. The rat was returned to the glucose-saline intraduodenal infusion at a rate of 3 mL/hr by the Harvard infusion pump.

2.4.7. Sample Collections

Bile was collected every 15 minutes for the first hour of the experiment, and then hourly for a total of 8 hours. Urine was collected as one sample for eight hours. Preliminary experiments demonstrated that virtually no radioactivity was present in the pancreatic juice, so in the final experiment no pancreatic juice was collected.

Bile was collected in pre-weighed glass vials and the vials were reweighed to determine bile weight. The bile weight was used as the bile volume, assuming a specific gravity of 1.000 g/mL.

Urine was collected in pre-weighed plastic medicine cups and the cups were reweighed to determine urine weight which was taken to be equivalent to urine volume assuming a specific gravity of 1.000 g/mL.

2.4.8 Tissue Samples

At the time of sacrifice (8 hours after commencement of sample collection) the liver, pancreas, kidneys, and adipose tissue (epididymal fat pads) were removed from each animal and placed in saline (0.9% NaCl). The kidneys and pancreas were trimmed of fat. All tissues were blotted to remove excess saline and weighed. A sample weighing 100 to 120 mg of each tissue was placed in a tared glass vial, its weight recorded, and 5 mL of NCS tissue solubilizer

(Amersham, IL, USA) added to each vial. Samples were left until solubilized (2 to 4 days) and shaken at least twice daily. The total body weight of adipose tissue was calculated, knowing that adipose tissue constitutes 7.08% of body weight, and this figure was used to determine the radioactivity in the total adipose tissue in the body (Caster et al, 1956).

2.4.9. Liquid Scintillation Counting

100 μ L of bile or urine was combined with 10 mL of Ready Value liquid scintillation cocktail (Beckman, CA, USA) for direct scintillation counting by a Beckman LS 8100 scintillation counter. 600 μ L of each solubilized tissue was combined with 10 mL of liquid scintillation cocktail and counted in the same fashion. Each sample was done in duplicate and counted for 10 minutes or to the 2% two sigma error. The cpm were corrected for quenching.

2.5. Statistical Methods Used

All results were expressed as mean \pm standard error of the mean (S.E.).

Control and experimental groups were compared using the Student's t-test. Significance levels are stated in Chapter 3, Results.

Chapter 3

Results

3.1. Effect of a Synthetic, Racemic Mixture of (S)- and (R)-1-cyano-2-hydroxy-3-butene on Non-protein Thiol (NP-SH) Levels and Glutathione S-Transferase (GSH-T) Activity in the Pancreas and Liver of the Rat

This study was undertaken in order to determine the effect of CHB on pancreatic and hepatic NP-SH content at various times after CHB administration. Also investigated was the activity of GSH-T in pancreatic and hepatic tissue after CHB treatment.

3.1.1. Pancreatic and Hepatic NP-SH

The results show that in the rat the NP-SH content of the pancreas and liver was affected by CHB treatment. Figures marked with an asterisk or cross denote a significant difference using the following code: *($p < 0.001$), **($p < 0.005$), +($p < 0.01$), and ++($p < 0.05$).

The NP-SH content of the pancreas at 4 hours after administration of 200 mg/kg CHB are significantly decreased

($p < 0.05$) by 53% as compared to control pancreatic levels (Figure 3-1). NP-SH content was measured in $\mu\text{mol/mg}$ soluble protein. Also seen in Figure 3-1 is a significant increase in NP-SH of 260% ($p < 0.001$) at 24 hours after dosing. This increase in NP-SH remains even after 120 hours following treatment at 36% over control levels ($p < 0.005$).

Figure 3-2 shows the NP-SH content of liver at 4, 24, and 120 hours after CHB treatment. At 4 hours following dosing the NP-SH content had decreased significantly by 33% ($p < 0.05$). The NP-SH content at 24 hours is increased over the content at 4 hours but is not significantly greater than control values. At 120 hours there is no significant difference in NP-SH content when compared to control levels.

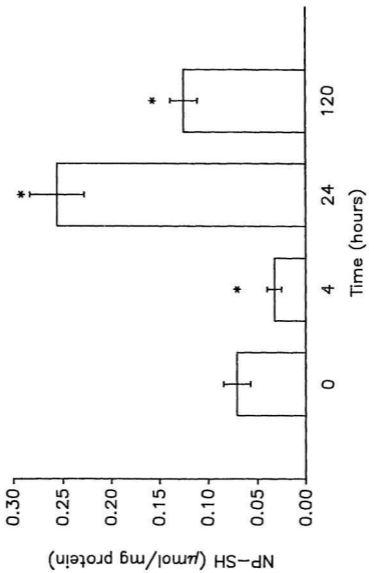
3.1.2. Pancreatic and Hepatic GSH-T Activity

The effect of CHB on pancreatic GSH-T activity (nmol CDNB conjugated/min/mg protein) followed a similar pattern as was seen with NP-SH content (Figure 3-3). At 4 hours following CHB administration, GSH-T activity had fallen significantly by 33% ($p < 0.001$). The GSH-T activity at 24 hours after dosing was significantly increased over control values by 46% ($p < 0.01$). At 120 hours after dosing the GSH-T activity of the pancreas was not significantly different from control activity.

CHB did not significantly GSH-T activity in the liver at any time examined after dosing (Figure 3-4).

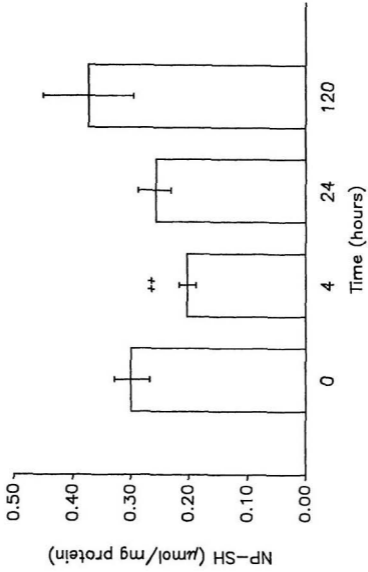
**Figure 3-1: Effect of CHB on NF-SH
content in rat pancreas**

Each bar represents the mean [\pm s.e.]
n=6 at 0hr, 4hr, and 120 hr
n=5 at 24 hr



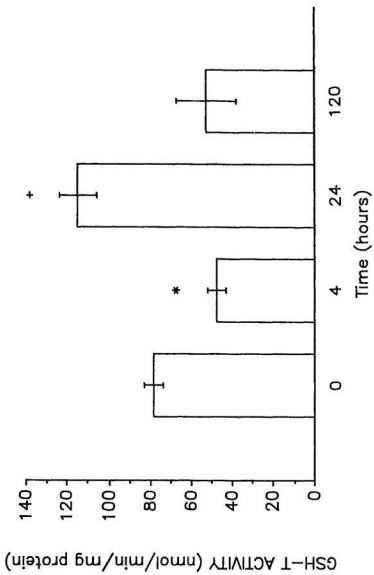
**Figure 3-2: Effect of CHB on NP-SH
contents of rat liver**

Each bar represents the mean [\pm s.e.]
n=6 at 0hr, 4hr, and 120 hr
n=5 at 24 hr



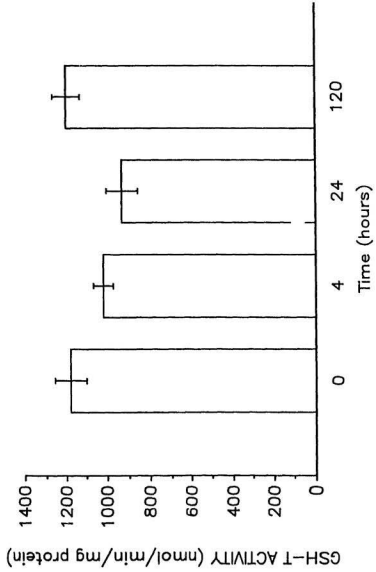
**Figure 3-3: Effect of CHB on GSH-T activity
in rat pancreas**

Each bar represents the mean \pm s.e.
n=6 at 0 hr, 4 hr, and 120 hr
n=5 at 24 hr



**Figure 3-4: Effect of CHB on GSH-T activity
in rat liver**

Each bar represents the mean [\pm s.e.]
n=6 at 0hr, 4hr, and 120 hr
n=5 at 2^h hr



3.2. Effect of Increasing Doses of CHB on Pancreatic Juice Secretion and Protein Concentration

This study was performed to determine the effect of CHB in increasing doses from 25 mg/kg to 200 mg/kg on pancreatic juice secretion in conscious restrained rats and on the protein concentration of the pancreatic juice.

3.2.1. Pancreatic Juice Secretion

Figure 3-5 shows the volume of pancreatic juice secreted from control animals over 6 hours after an intraduodenal bolus dose of olive oil only (approximately 0.5 mL). The volume of juice secreted per 30 minute periods remains essentially constant for the duration of the experiment.

The effect of 25 mg/kg CHB on rat pancreatic juice secretion is seen in Figure 3-6. Pancreatic juice secretion seems to decrease slightly at 0.75 hours then increase to reach a peak at 2.75 hours after CHB dosing, but no volumes are significantly different from the control volumes.

Rat pancreatic juice secretion is significantly decreased at 0.75 hours after dosing with 50 mg/kg CHB by 34% (Figure 3-7). The figures illustrating the results from these experiments denote significant difference with these symbols above the standard error bars: *($p < 0.001$), **($p < 0.005$), +($p < 0.01$), and ++($p < 0.05$). After 0.75 hours, the volume of pancreatic juice collected per 30 minute

period increased steadily and reached a peak at 2.25 hours where it is increased over controls by 45%. The volume of pancreatic juice secreted remained significantly elevated at 2.75 hours, then steadily approached control volumes for the duration of the experiment.

The volume of pancreatic juice secreted by rats dosed with 100 mg/kg CHB does not decrease but does significantly rise by 1.25 hours following treatment (Figure 3-8). Juice secretion reached a peak at 1.75 hours, a 41% increase over control volumes. After this time the volumes collected start to decrease steadily, approaching normal values by 6.25 hours.

Figure 3-9 shows the effect of 150 mg/kg CHB on rat pancreatic juice secretion. The volume secreted per 30 minute period increases to reach a maximum at 1.25 hours which is a 53% increase over control volumes. After this time it steadily decreases to reach a significant decrease at 6.25 hours which is only 45% of the control volumes. The low volumes seen from 2.75 hours to 5.75 hours are consistently low, yet are not significant due to the large inter-animal variation.

The greatest effect on pancreatic juice secretion was observed at the highest dose administered, 200 mg/kg. At this dose there was a significant increase in pancreatic juice volume collected per 30 minute period by 0.75 hours after dosing (Figure 3-10). A maximum secretion of volume

**Figure 3-5: Effect of olive oil on rat
pancreatic juice secretion**

Each point represents the mean [\pm s.e.]
n=6 for all points

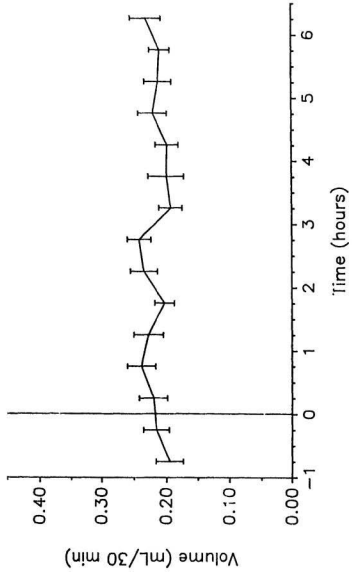


Figure 3-6: Effect of 25 mg/kg CHB on rat
pancreatic juice secretion

Each point represents the mean [\pm s.e.]
n=6 for all points

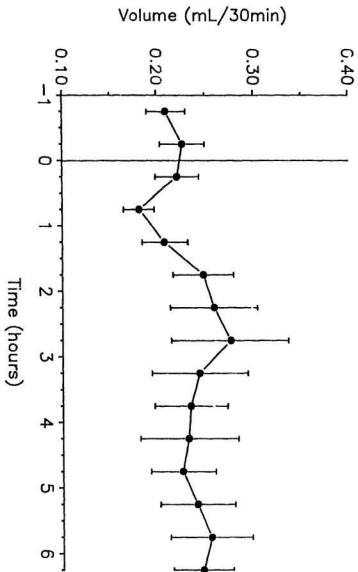
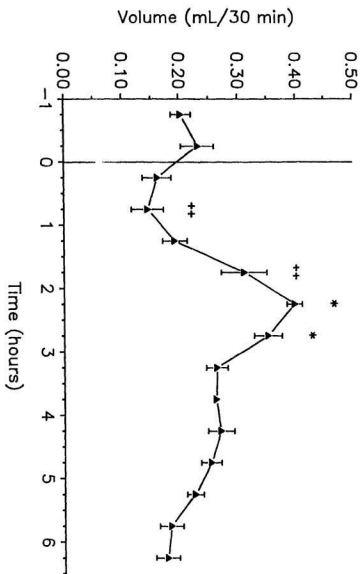


Figure 3-7: Effect of 50 mg/kg CHB on rat
pancreatic juice secretion

Each point represents mean (\pm s.e.)
n=6 for all points



**Figure 3-8: Effect of 100 mg/kg CHB on rat
pancreatic juice secretion**

Each point represents the mean [\pm s.e.]
n=6 for all points

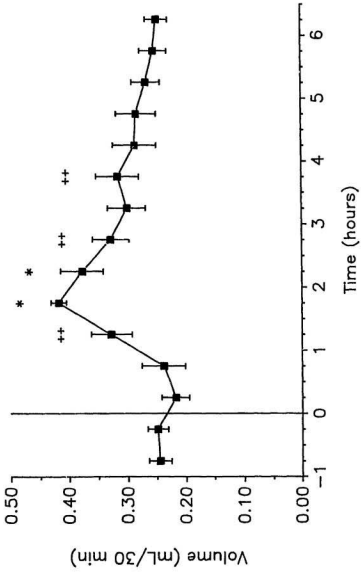
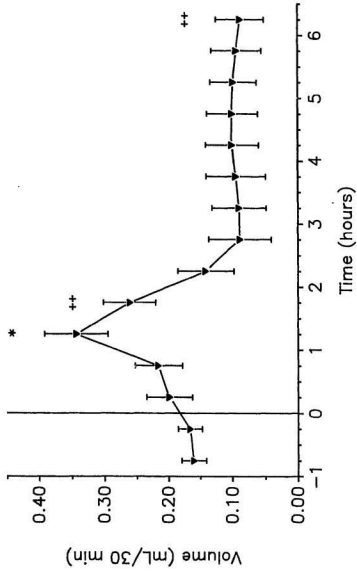
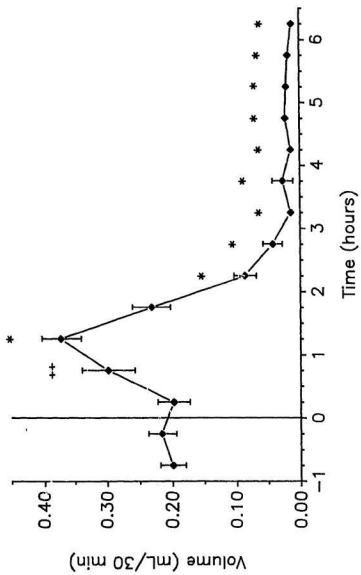


Figure 3-9: Effect of 150 mg/kg CHB on rat
pancreatic juice secretion

Each point represents the mean [\pm s.e.]
n=6 for all points





**Figure 3-10: Effect of 200 mg/kg CHB on rat
pancreatic juice secretion**

Each point represents mean [\pm s.e.]
n=6 for all points

was observed at 1.25 hours which was a 45% increase over control volumes. After 1.25 hours, volumes decreased rapidly and by 2.25 hours the volume was 59% lower than control volumes. From this point volume collections remained very low and flow was almost zero during the time periods from 3.25 hour to 6.25 hours.

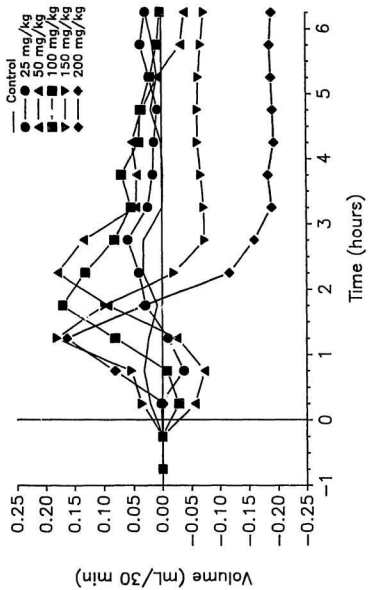
Figure 3-11 shows all the normalized data obtained in this experiment on one graph for comparison purposes. This graph, although complex, illustrates the dose-effect relationship of CHB on pancreatic juice. It can be seen that an initial stimulation is followed by a return to normal flows at the low doses, and depressed flows at the two largest doses. Also, it can be seen that the initial stimulation occurs earlier with increasing doses of CHB.

3.2.2. Protein Concentration of Pancreatic Juice

Figure 3-12 shows the protein concentration of pancreatic juice from animals dosed intraduodenally with olive oil only (bolus dose). The protein concentrations obtained were approximately the same from animals treated with olive oil throughout the experiment.

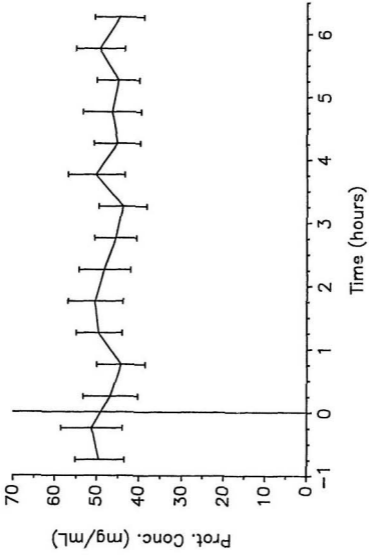
The 25 mg/kg dose of CHB caused some variation in the protein concentration of pancreatic juice with time (Figure 3-13). At 1.25 hours after dosing the protein concentration was significantly decreased from control values by 11% ($p < 0.05$). This decrease in protein concentration was not

Figure 3-11: Effect of increasing doses
of CHB on rat pancreatic juice secretion with time
(using normalized data)



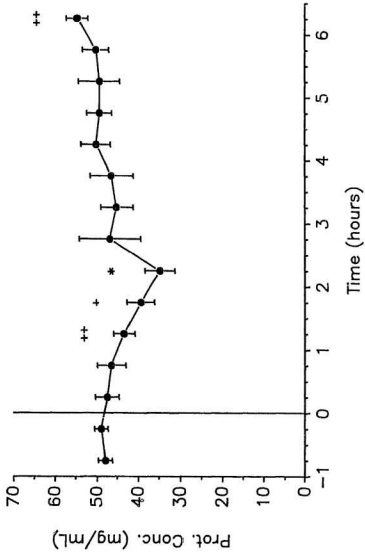
**Figure 3-12: Effect of olive oil on protein
concentration of rat pancreatic juice**

Each point represents the mean [\pm s.e.]
n=6 for all points



**Figure 3-13: Effect of 25 mg/kg CHB on protein
concentration of rat pancreatic juice**

Each point represents the mean [\pm s.e.]
n=6 for all points



prolonged and reached a minimum at 2.25 hours after dosing and was 28% lower than control levels ($p < 0.001$). After this time point protein concentration was not significantly different from controls until 6.25 hours after CHB treatment when the protein concentration of the juice was increased over controls by 11% ($p < 0.05$).

The 50 mg/kg dose of CHB resulted in a decrease in the protein concentration of the pancreatic juice secreted after 1.25 hours following CHB treatment (Figure 3-14). A minimum protein concentration was reached at 2.75 hours when it was decreased by 52% as compared to control protein concentration ($p < 0.001$). After this time the protein concentration of the pancreatic juice began to increase fairly steadily for the duration of the experiment. At 6.25 hours the protein concentration was still significantly decreased as compared to the control values by 18% ($p < 0.01$).

Figure 3-15 illustrates the dramatic effect of 100 mg/kg CHB on protein concentration of rat pancreatic juice. The protein concentration is significantly decreased by 1.75 hours after CHB administration by 45% ($p < 0.005$). After this time all pancreatic juice collected had protein concentrations significantly lower than control values ($p < 0.001$). At 6.25 hours the protein concentration was decreased by 72% ($p < 0.001$) as compared to control values. At this dose not all pancreatic juice collected from each

**Figure 3-14: Effect of 50 mg/kg CHB on protein
concentration of rat pancreatic juice**

Each point represents the mean [\pm s.e.]
n=6 for all points

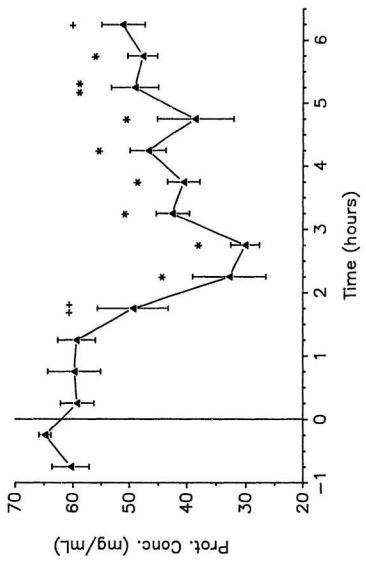
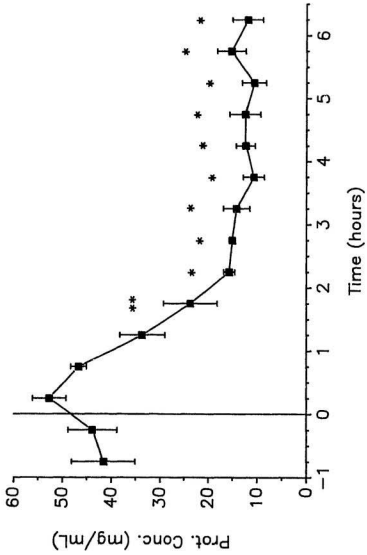


Figure 3-15: Effect of 100 mg/kg CHB on protein concentration of rat pancreatic juice

Each point represents mean [\pm s.e.]
n=6 at all points except
n=5 at 4.25 hr, 5.25 hr, and 5.75 hr



animal could be assayed for protein concentration as there was not enough sample available for testing due to decreased volumes. As a result, not all the points have a sample size of 6, and the sample sizes are indicated in the figure caption.

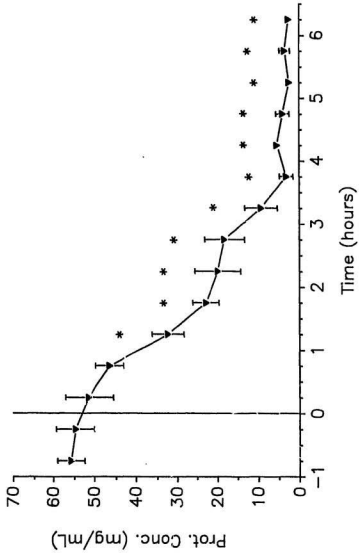
The effect of 150 mg/kg CHB is much the same as that of 100 mg/kg on protein concentration of rat pancreatic juice (Figure 3-16). The protein concentration is significantly decreased earlier with the higher dose at 1.25 hours after dosing by 41% ($p < 0.001$). Protein concentrations of samples continue to decrease and after 3.25 hours remain lower than 10 mg/mL, a decrease of over 82%. Sample sizes for each time point are indicated in the figure caption.

The largest dose tested, 200 mg/kg, caused a significant decrease in protein concentration of pancreatic juice by 0.75 hours after dosing of 23% ($p < 0.05$) (Figure 3-17). After 1.75 hours all pancreatic juice collected had protein concentrations less than 10 mg/mL, and after 3.75 hours protein concentrations were less than 5 mg/mL.

Figure 3-18 shows a comparison graph of all doses using normalized data. As the doses of CHB increased, the protein concentration of pancreatic juice decreased more rapidly and did not recover to approach normal values.

Figure 3-16: Effect of 150 mg/kg CHB on protein concentration of rat pancreatic juice

Each point represents mean [\pm s.e]
n=6 at all points except
n=5 at 3.25 hr;
n=3 at 3.75 hr, 5.25 hr, 4.75 hr
5.75 hr, and 6.25 hr;
n=2 at 4.25 hr



**Figure 3-17: Effect of 200 mg/kg CHB on protein
concentration of rat pancreatic juice**

Each point represents the mean [\pm s.e]
n=6 for all points except
n=5 at 2.75 hr;
n=3 at 3.25 hr to 5.25 hr;
n=2 at 5.75 hr;
n=1 at 6.25 hr

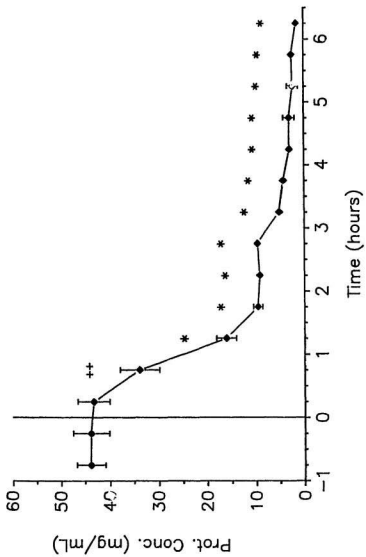
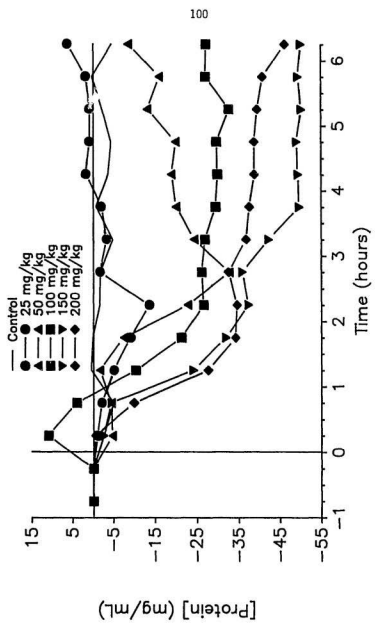


Figure 3-18: Effect of increasing doses of CHB
on protein concentration of rat
pancreatic juice with time
(using normalized data)



3.3. Effect of Increasing Doses of CHB on Bile Secretion and Bile Acid Concentration

The purpose of this experiment was to observe the effect of CHB in increasing doses from 25 mg/kg to 200 mg/kg on bile secretion in rats. Bile collected from animals treated with 200 mg/kg CHB was also assayed for bile acid concentration (n=5).

3.3.1. Bile Secretion

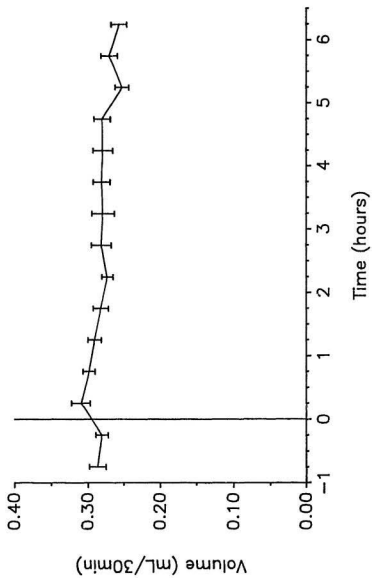
Bile secretion in rats treated with an intraduodenal bolus dose of about 0.5 mL olive oil only (the vehicle) is constant throughout the experiment (Figure 3-19).

The smallest dose of CHB given to rats, 25 mg/kg (Figure 3-20), caused a significant increase in bile secretion, reaching a maximum at 0.25 hours after CHB treatment which was 45% greater than control values ($p < 0.005$). The bile secretion remained significantly higher than control secretion until 2.75 hours after dosing, after which the bile volumes are approximately the same as control volumes.

The 50 mg/kg dose of CHB caused a significant increase in bile secretion immediately after dosing (Figure 3-21). Bile secretion reached a maximum at 0.75 hours after dosing when it was 71% greater than control bile volumes ($p < 0.001$). After this time, bile volumes decreased and although they remained significantly greater than control volumes, they

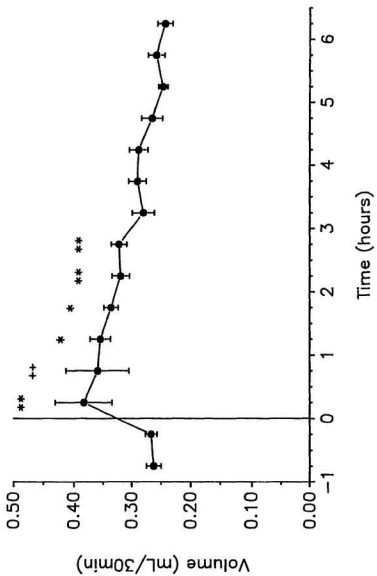
**Figure 3-19: Effect of olive oil on bile
secretion in the rat**

Each point represents the mean [\pm s.e.]
n=6 for all points



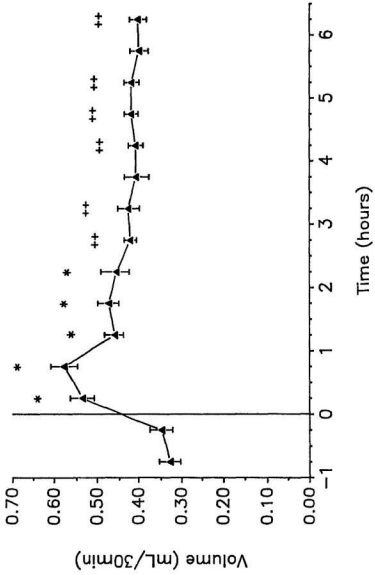
**Figure 3-20: Effect of 25 mg/kg CHB on bile
secretion in the rat**

Each point represents the mean [\pm s.e.]
n=6 for all points



**Figure 3-21: Effect of 50 mg/kg CHB on bile
secretion in the rat**

Each point represents the mean [\pm s.e.]
n=6 for all points



began to approach control values by the end of the experiment.

After dosing with 100 mg/kg CHB (Figure 3-22), rat bile secretion increased sharply, reaching a maximum at 0.25 hours which was 102% greater than control bile volumes ($p < 0.001$). The bile volumes remained significantly greater than control volumes while slowly decreasing for the duration of the experiment. At 6.25 hours, bile volume is still significantly greater than control levels by 43% ($p < 0.001$).

Figure 3-23 shows the effect of 150 mg/kg CHB on rat bile secretion. Bile volume increased by 151% immediately after dosing ($p < 0.001$). After this time point the bile volumes decreased sharply until 1.75 hours after dosing. The volumes then steadily decreased from 3.25 hours until the end of the experiment, at which time bile volume secretion was still greater than control volumes by 29% ($p < 0.01$).

Figure 3-24 shows the dramatic effect of 200 mg/kg CHB on rat bile secretion. At 0.25 hours after dosing, bile secretion is increased by 175% as compared to control volumes ($p < 0.001$). After this time bile secretion decreased throughout the experiment and by 5.75 hours is significantly decreased by 44% ($p < 0.05$). At the end of the experiment, 6.25 hours, bile volume has decreased by 54% ($p < 0.01$).

Figure 3-25 is a composite graph showing the dose-

Figure 3-22: Effect of 100 mg/kg CHB on bile
secretion in the rat

Each point represents the mean [\pm s.e.]
n=6 for all points

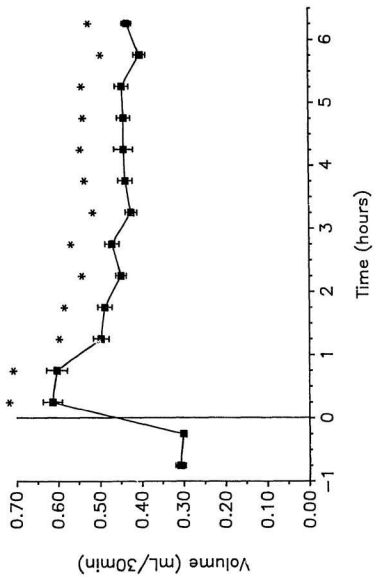
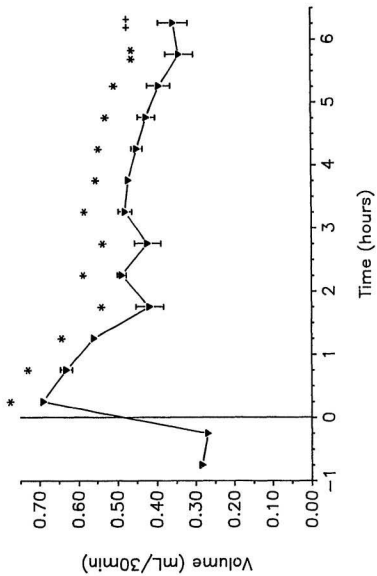


Figure 3-23: Effect of 150 mg/kg CHB on bile
secretion in the rat

Each point represents the mean [\pm s.e]
n=6 for all points



**Figure 3-24: Effect of 200 mg/kg CHB on bile
secretion in the rat**

Each point represent the mean [\pm s.e.]
n=6 for all points

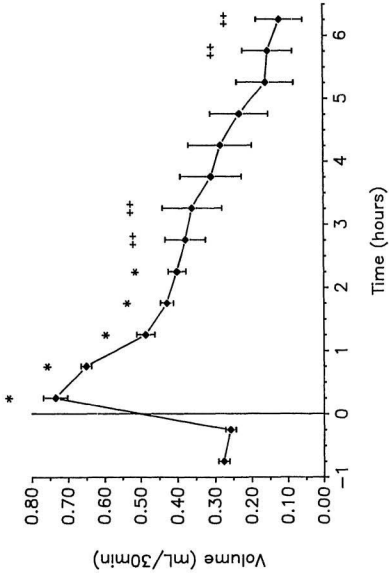
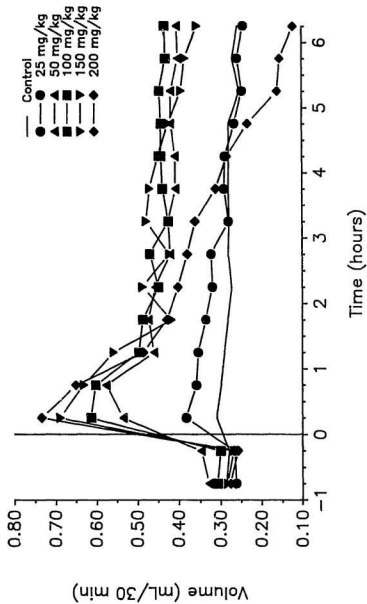


Figure 3-25: Effect of increasing doses of CHB
on rat bile secretion with time



effect of the increasing amount of CHB on rat bile secretion. As the dose increases, there is a proportional increase in bile secretion by 0.25 hours after CHB treatment with a late depression of bile flow at the largest dose.

3.3.2. Bile Acid Concentration

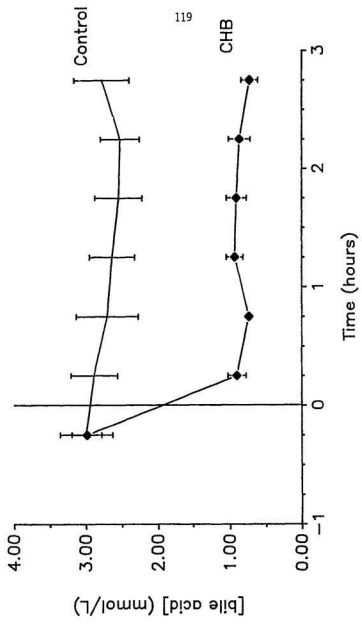
Bile acid concentration of bile obtained for control animals treated with olive oil and that of bile from animals treated with 200 mg/kg CHB were compared until 2.75 hours after dosing (Figure 3-26). Bile acid concentration remained constant for control animals, but by 0.25 hours after dosing, bile acid concentration of treated animals had fallen by 70% as compared to control concentration ($p < 0.001$). For all time points examined, bile acid concentration remained below control values by 69-70% (for all points $p < 0.001$).

3.4. Pancreatic Water Content

The pancreas from each animal in the experiment was dried to constant weight to obtain the water content of each pancreas (Figure 3-27). The pancreata obtained from animals treated with 25 mg/kg and 50 mg/kg CHB did not have increased water content as compared to control pancreata. The water content of the three higher doses was increased as compared to control pancreata. Pancreata from animals treated with 100 mg/kg was increased by 3% ($p < 0.01$).

**Figure 3-26: Effect of 200 mg/kg CHB on
bile acid concentration in rat bile
up to 2.75 hr after dosing**

Each point represents the mean [\pm s.e.]
n=4 for control animals
n=5 for CHB treated animals



**Figure 3-27: Effect of increasing doses of CHB
on pancreatic water content**

Each bar represents mean [\pm s.e.]
n=6 for all doses

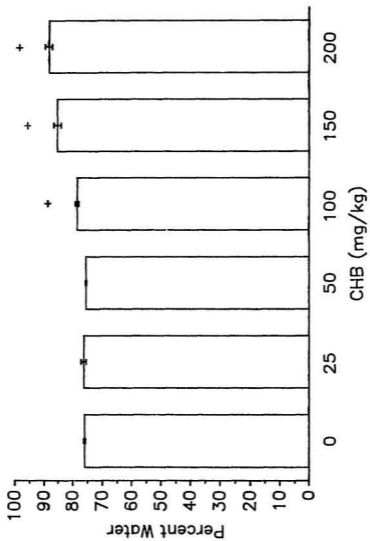
Pancreata obtained from animals treated with 150 mg/kg and 200 mg/kg CHB had significantly more water than control pancreata by 12% and 16%, respectively ($p < 0.01$). Samples of the oedematous fluid analyzed for protein concentration contained an average of 77.9 mg protein/mL exudate. Figure 3-28 is a photograph of 2 pancreata from control animals and 2 pancreata from treated animals. The oedema in the 2 treated pancreata is very obvious and striking.

3.5. Disposition of Oral CHB in the Rat

3.5.1. Radioactivity in the Bile and Urine

This study was performed to determine the general pattern of excretion of [^{14}C] CHB or its metabolites in the bile and urine of the rat.

Figure 3-29 shows the percentage of radioactivity administered as [^{14}C] CHB that was excreted rapidly in the bile. Within the first hour after administration almost 5% of the radioactivity was excreted in the bile. Substantial amounts of the radiolabel was found in the bile by 15 minutes after dosing, pointing to rapid intestinal absorption of CHB. The excretion of radioactivity in the bile reached a peak at 0.50 hours after treatment, after which it slowly decreased. The total percentage of [^{14}C] radioactivity excreted in the bile was 20.6% over 8 hours. Figure 3-30 shows the cumulative excretion of radioactivity



**Figure 3-28: Pancreata from control animals and
from treated animals**

Pancreata at upper and lower left from
control animals
Pancreata at upper and lower right from animals
treated with 200 mg/kg CHB (intraduodenally)

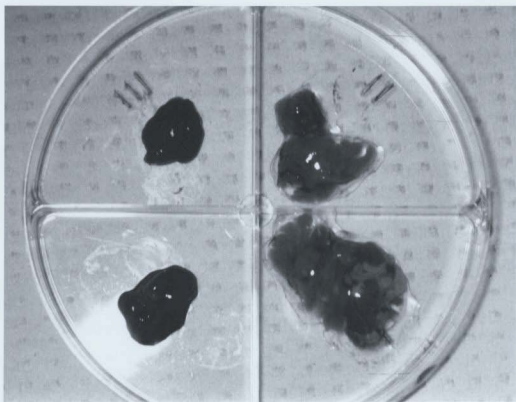


Figure 3-29: Radioactivity recovered in rat bile
for 8 hours after dosing with [¹⁴C] CHB

Each point represents the mean [\pm s.e.]
n=4 for all points

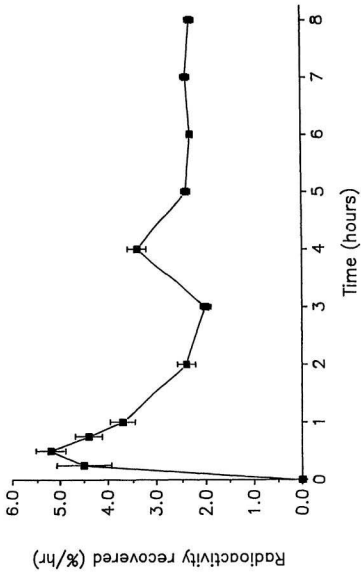
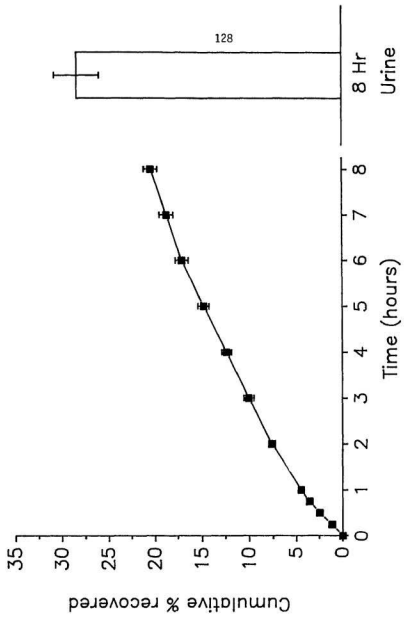


Figure 3-30: Cumulative radioactivity recovered
in bile and urine for 8 hours
after [¹⁴C] CHB dosing

Each point represents the mean [\pm s.e.]
n=4 for all points



in the bile up to eight hours after [^{14}C] CHB treatment.

The total radioactivity excreted in the urine at eight hours after [^{14}C] CHB administration is 28.4% (Figure 3-29).

3.5.2. Radioactivity in the Liver, Pancreas, Kidney, and Adipose Tissue

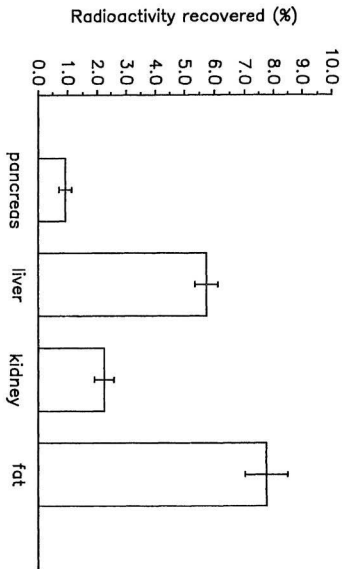
The highest percentage of radioactivity recovered in an organ eight hours after [^{14}C] CHB administration was in the adipose tissue, being 7.7% (Figure 3-31). The total amount of adipose tissue present in each animal was estimated by the method discussed in Chapter 2, Methods and Materials. The liver had the next highest radioactive count at 5.7%. The kidneys and the pancreas contained the lowest amounts of radioactivity at 2.3% and 0.9%, respectively.

Radiolabel accumulated on a per gram basis was as follows: pancreas, 9099 dpm/g (s.e. \pm 2024); liver, 69417 dpm/g (s.e. \pm 7748); kidney, 17526 dpm/g (s.e. \pm 3984); and fat, 10051 dpm/g (s.e. \pm 2649).

After 8 hours, the total amount of radioactivity accounted for (compared to amount given) was 65%.

**Figure 3-31: Radioactivity recovered in rat
organs 8 hours after dosing**

Each bar represents the mean [\pm s.e.]
n=4 for all points
with [^{14}C] CHB



Chapter 4

Discussion

4.1. Effect of CHB on Non-protein Thiol and Glutathione S-Transferase in the Liver and Pancreas

Synthetic racemic CHB administered orally to rats (200 mg/kg) was found to significantly decrease non-protein thiol (NP-SH) content in the pancreas at 4 hours after treatment. This result was in agreement with those presented by Wallig and Jeffery (1990), where pancreatic GSH was decreased 2 hours after an oral dose of natural (S)- CHB (200 mg/kg). At 24 hours after CHB treatment, pancreatic NP-SH content was significantly increased. This was also in agreement with results published by Wallig and Jeffery (1990). At 120 hours following CHB treatment, pancreatic NP-SH was still significantly greater than control values. results published by Wallig and Jeffrey (1990) showed that at 96 hours after CHB treatment, pancreatic GSH was also still elevated as compared to control values.

GSH transferase activity in the pancreas followed a

similar pattern to NP-SH content changes in the pancreas. At 4 hours following CHB treatment GSH transferase activity was decreased, and at 24 hours following treatment it was increased as compared with control values.

It is not known if the depletion of NP-SH followed by elevation is in direct response to toxic insult. However, this decrease in pancreatic NP-SH content followed by the elevation observed may indicate the consumption of pancreatic GSH by conjugation to CHB or a metabolite of the toxin. This sustained effect on pancreatic GSH is unusual. Irreversible loss of GSH occurs in the case of mercapturic acid formation. When this occurs, resynthesis of GSH takes place by cells to replenish the contents of the cells (Igwe, 1986). The rebound effect seen following depletion suggests an increased production of GSH in response to the large loss due to toxic insult.

The liver is known to have this type of response to toxins such as acetaminophen and butylated hydroxytoluene. These are both metabolized in the liver to reactive intermediates and produce a decline in hepatic GSH levels followed by a rebound within a few hours after treatment (McMurty et al, 1978; Nakagawa et al, 1984).

CHB treatment resulted in a decrease in liver NP-SH content at 4 hours following administration, but this decrease was not as large as that observed in the pancreas.

At 24 and 120 hours after treatment, hepatic NP-SH was not significantly different from controls. This result, combined with the finding that 200 mg/kg CHB did not alter hepatic GSH transferase activity, suggests that the liver is resistant to CHB at this dose and manages to detoxify the compound or possibly uses other detoxifying systems not present in the pancreas. However, it is not known if CHB can result in liver necrosis at a later time with this dose. The depletion of hepatic NP-SH seen at 4 hours may indicate the conjugation of CHB to GSH, as xenobiotics that conjugate to GSH cause an initial depletion of the molecule due to the high concentration of GSH transferases in the liver (Black and Howerton, 1984).

Because of the profound effect of CHB on pancreatic NP-SH, it has been suggested that CHB or its metabolites are especially toxic to the pancreas, or that they accumulate in the pancreas (Wallig and Jeffery, 1990). Black and Howerton (1984) suggested that the GSH enzyme pathway was a major biotransformation route for the pancreas. To protect against pancreatic damage, the cells may conjugate toxic metabolites to GSH to be ultimately converted to mercapturic acids and excreted. It is possible that in the process of GSH conjugation to CHB that a more toxic metabolite may be formed.

As discussed in Chapter 1, the release of cyanide is

considered unlikely to be responsible for the effects observed in the pancreas after CHB treatment. There is evidence suggesting that unsaturated nitriles may be metabolized via a GSH mediated pathway (Silver *et al*, 1982).

4.2. GSH and Nitrile Metabolism

The effect of CHB on glutathione levels in the liver and pancreas was investigated in this work, and also by Wallig and Jeffery (1990), as previously discussed. Three other unsaturated nitriles (acrylonitrile, allylnitrile, and fumaronitrile) have also been shown to significantly decrease GSH content of the liver after oral dosing (Ahmed and Farooqui, 1982). This phenomenon is thought to be associated with GSH conjugation to a xenobiotic as a method of detoxication, or in response to lipid peroxidation.

Acrylonitrile is one unsaturated aliphatic nitrile that has been extensively studied, as it is a widely used compound in the plastics industry. Short term experiments with acrylonitrile by Szabo *et al* (1977) showed a time and dose dependent decrease in liver (and other organ) content of GSH. Chronic administration of acrylonitrile resulted in a dose dependent increase in hepatic GSH levels which may be indicative of a precancerous biochemical alteration. Cote *et al* (1984) studied the acute effects of acrylonitrile on tissue GSH concentrations in rat, mouse, and hamster. They

reported that acrylonitrile decreased GSH concentration in brain, liver, lung, and kidney in all species with the rat being the most sensitive to acrylonitrile.

Two types of mercapturic acids have been isolated and identified in urine after acrylonitrile administration (van Bladeren *et al*, 1981), showing that acrylonitrile is conjugated to GSH and the conjugate is then metabolized to mercapturates.

Another compound structurally similar to CHB, 3,4-epithiobutanenitrile, was found to be metabolised mainly via the glutathione pathway by Brocker *et al* (1984). Unsaturated nitriles, including CHB, are probably metabolized by a GSH conjugation pathway.

CHB is unique in its effect on pancreatic NP-SH content and induction of pancreatic GSH transferases. It is unlikely that the increase in pancreatic NP-SH is in response to lipid peroxidation caused by CHB or a metabolite, as no increase in pancreatic or hepatic GSSG content was observed by Wallig and Jeffery (1990) after CHB treatment. More formal exclusion of this possibility could be carried out by measurement of indices of lipid peroxidation such as ethane exhalation.

A decrease in cellular GSH does not cause cell death in itself. Cell death may be due to the presence of reactive metabolites which are not conjugated due to unavailability

of GSH (Mitchell et al, 1985). For example, oxidation products may accumulate in the cell, causing toxic injury to the cell.

4.3. Effects of CHB on Pancreatic Juice Secretion and Protein Concentration

The loss of zymogen granules seen in histology of pancreatic tissue after CHB treatment prompted this investigation of the effects of CHB on pancreatic juice (Wallig and Jeffrey, 1990).

Pancreatic juice is secreted partially by the acinar cells of the exocrine pancreas and contains enzymes (i.e. proteolytic zymogens, lipase, phospholipase A₂, and amylase) (Rinderknecht, 1986). These enzymes are secreted in a fluid of low bicarbonate concentration of about 25 mM. The ducts of the pancreas secrete a solution with a high bicarbonate concentration of about 140 mM. The main pancreatic duct delivers the pancreatic juice to the small intestine. Enzymes make up virtually all the protein in pancreatic juice (Solomon and Grossman, 1982). The pancreas has an extremely high rate of export enzyme protein synthesis.

Pancreatic juice from acinar cells is secreted in response to the endogenous stimulants cholecystokinin (stimulates pancreatic enzyme secretion), acetylcholine (stimulates secretion of enzymes from acinar cells), and

secretin (stimulates secretion of bicarbonate from ductal cells). The role of pancreatic juice enzymes is to hydrolyse proteins, triglycerides, and starch into peptides, fatty acids and monoglycerides, and small saccharides which can then be further metabolized and absorbed by the enterocytes (Rinderknecht, 1986). These enzymes are therefore essential for the absorption of nutrients by the small intestine.

Orally administered CHB was found to cause an increase in pancreatic juice volume over a 30 minute period followed by a return to control volumes for the 25, 50, and 100 mg/kg doses. The higher doses of CHB (150 and 200 mg/kg) also resulted in increases in pancreatic juice volume which then fell below control volumes that did not return to normal values. Pancreatic juice secretion in animals given olive oil only had constant pancreatic juice secretion.

The protein concentration of pancreatic juice collected from control animals remained constant throughout the experiment. CHB treatment caused a decrease in the protein concentration of pancreatic juice. At the 25 and 50 mg/kg doses there appeared to be a recovery of protein concentration by the end of the experiment. In the case of the three largest doses there was no recovery observed and protein concentration remained significantly decreased at the end of the experiment.

The effect of CHB on pancreatic juice secretion and its protein concentration may represent an early stimulation of pancreatic secretion and zymogen granule discharge followed by an inhibition of export protein synthesis. This could account for the absence of zymogen granules seen in pancreatic tissue after CHB treatment (Wallig *et al*, 1988).

It is not known how larger doses of CHB inhibit pancreatic secretion. It may interfere with the action of the stimulatory hormones acetylcholine, cholecystokinin, and secretin. It is also possible that the biosynthesis of enzymes within the acinar cell may be inhibited by CHB and/or its metabolites, which would lead to a decrease in pancreatic juice secretion of protein. Inhibition of protein synthesis could take place at many levels within the cell, for example during the transcription of DNA, RNA processing, and mRNA translation.

Inhibition of pancreatic enzyme secretion and synthesis may provide an explanation for decreased weight gain observed in animals fed rapeseed meals and nitrile extracts from *Crambe* meals (Srivastava *et al*, 1975; VanEtten *et al*, 1969b). With a reduction of pancreatic enzyme availability, the digestion of protein, carbohydrate, and fat will be curtailed and malabsorption and malnutrition would be the result.

The unique, selective effect that CHB has on the

pancreas and its function deserves further investigation. Inhibition of pancreatic secretion induced by CHB could be countered by treatment with stimulant hormones such as secretin to observe any beneficial effect of extra hormones. The effect of repeated dosing of CHB at low levels on pancreatic structure and function needs to be investigated. Also of interest would be the effect of CHB on radioactive amino acid incorporation into export enzymes of the pancreas.

4.4. Effect of CHB on Bile Secretion and Bile Acid Concentration

Bile is secreted by hepatocytes in the bile canaliculi and by the intrahepatic bile ducts. It is the exocrine secretion of the liver and contains organic and inorganic compounds. Biliary secretion provides an essential pathway for the flow of lipids, vitamins, drugs (and other xenobiotics) and their metabolites, bile pigments, and bile salts (Erlinger, 1982).

Bile consists of electrolytes and organic solutes. The electrolytes found in rat bile are sodium, potassium, chloride, and bicarbonate ions (Klaassen, 1971). The major organic solutes in bile are conjugated bile acids, phospholipids, cholesterol, and bile pigments (Erlinger, 1982). The concentration of bile acids in rat bile ranges

from 8-25 mM (Klaassen, 1971).

Bile acids are synthesized from cholesterol by hepatocytes to the two primary bile acids, cholic and chenodeoxycholic acid. The secondary bile acids, deoxycholic and lithocholic acid, are produced by bacteria in the intestine from the primary bile acids. Bile acids are secreted as conjugates with taurine and glycine which makes them more water soluble and more readily absorbed by the ileum (Sernka and Jacobson, 1983).

Bile salts are of great importance in the absorption of fat by the small intestine. Triglycerides are broken down by pancreatic lipase into fatty acids and monoglycerides. The long chain fatty acids and monoglycerides are then incorporated into a shell of bile salts to form a micelle. The micelle transports its contents to the microvilli of the intestine where the lipid contents are released from the micelle and are absorbed by passive transport. Micellar contents also include cholesterol, lethicin, and fat-soluble vitamins. After the absorption of the fatty acids, monoglycerides, cholesterol, and phospholipids, the bile salts are mostly retained in the lumen, where they may form new micelles or are absorbed actively by the ileum and returned to the liver via the portal vein in an enterohepatic circulation (Sernka and Jacobson, 1983; Solomon and Grossman, 1982).

Bile also functions as an excretory route for endogenous compounds that may be toxins, such as steroid hormones, and exogenous compounds such as drugs or their metabolites (Coleman, 1987).

All doses of CHB significantly increased bile secretion immediately after dosing for all doses. The smallest dose of CHB (25 mg/kg) caused only a small increase in bile secretion, but the rest of the doses (50-200 mg/kg) resulted in a dose dependent transient choleresis seen for 2 hours following CHB treatment. The 50, 100, and 150 mg/kg doses of CHB resulted in an elevation of bile secretion that remained high for the duration of the experiment, whereas the 200 mg/kg dose caused bile flows to fall towards zero after the initial choleresis.

Bile acid concentration of bile obtained from rats treated with 200 mg/kg CHB was found to be severely depressed after dosing. The concentration of bile acid in control animals was found to be only 2.5-3 mmol/L, but this was probably due to the interruption of the enterohepatic circulation of bile salts (Nilsson et al, 1988).

The mechanism by which CHB stimulates bile flow is not known. Bile flow is either bile acid-dependent, generated by the biliary secretion of bile acids, or bile acid-independent, generated by inorganic ion transport (Erlinger, 1982). The stimulation of bile secretion

following CHB administration is probably bile acid-independent due to the low output of bile acid in the bile after treatment.

Bile acid-independent bile flow is not fully understood. It is thought that sodium via $\text{Na}^+\text{-K}^+$ ATPase and bicarbonate transport may be involved in this type of bile flow (Strange, 1984). Both endogenous and exogenous compounds have been found to alter bile acid-independent flow, such as insulin and phenobarbitol (Okolicsanyi et al, 1986).

It would be worthwhile to examine the effect of CHB on these bile acid independent mediators. CHB may cause an increase in the activity of liver plasma membrane $\text{Na}^+\text{-K}^+$ ATPase activity and in doing so increase bile acid independent flow; this would be an interesting avenue of investigation. It is also possible that CHB or its metabolites could cause an increase in bile flow through osmotic effects resulting from their presence in bile.

4.5. Pancreatic Oedema in CHB-treated Animals

Oedema is the accumulation of abnormally large amounts of fluid in the intracellular spaces in the body. It occurs when the rate of net fluid filtration into extravascular spaces is greater than the rate of extravascular fluid drainage by the lymphatics (Grega and Svensjo, 1984).

Increased permeability of gastrointestinal microvessels is thought to be the cause of interstitial oedema associated with gastrointestinal disease. Pancreatic oedema can be caused by a number of factors, with acinar cell injury being a common initiator (Granger and Barrowman, 1984). Exudation of extravascular fluid is commonly seen in acute pancreatitis, although the fluid is lost to areas away from the pancreas, instead of being confined to the pancreas, as is seen in CHB poisoning. It is believed to be due to a diffuse increase in capillary permeability, but the mechanism is unknown (Steer, 1986).

The oedema induced by CHB treatment was found to increase in severity with increased dosage, as indicated by the water content of the pancreas increasing with CHB dosing. This increase in the severity of the pancreatic oedema could also be observed visually. The pancreatic exudate was determined to be very protein rich in nature, suggesting that there is a large increase in the macromolecular permeability of the pancreatic microcirculation.

The identity and amounts of these proteins present in pancreatic exudate has yet to be determined. Also of interest is the possibility of pancreatic enzymes being present in the exudate, and in the lymph and blood vessels as is seen in pancreatitis.

4.6. Disposition of CHB

Radioactive [^{14}C] CHB was administered intraduodenally to rats and was found to be excreted in the bile and the urine, with more radioactivity being excreted in the urine. As CHB is a lipophilic compound, it must be biotransformed into a water-soluble molecule to be excreted in both the urine and the bile, probably through the GSH pathway. The criteria are not known which result in a xenobiotic being excreted in the bile or the urine. Generally, however, small xenobiotic molecules are eliminated preferentially in urine while larger molecules are chiefly eliminated in bile. Chemicals with molecular weights of 350 are excreted well into the bile (Gregus and Klaassen, 1986). CHB metabolites conjugated to GSH, or their corresponding mercapturic acids would be good candidates for biliary excretion, based on their molecular weights (approximately 400 g/mol).

The radioactive metabolites found in the bile and urine have not been identified at this time. They could be isolated by gas-liquid chromatography in a method similar to that used by Brocker et al (1984), then their chemical structures identified by the use of mass spectrometry. This could confirm the hypothesis that CHB is conjugated to GSH and further metabolized to a mercapturic acid which would be eliminated in the urine.

As CHB is a lipophilic compound, it is not surprising

that it accumulates in the adipose tissue. The storage of toxicants in the body fat may serve as a protective mechanism, but there is the danger of a release of considerable amounts of chemicals into the bloodstream in the case of sudden mobilization of fat for energy. CHB may also be stored in the fat as metabolites, and this is another topic for investigation.

CHB or its metabolites were also found to be still present in the liver after 8 hours, but not in great amounts. The liver is the primary organ of metabolism of xenobiotics, so it would be expected to contain some radioactivity.

The kidneys did not accumulate radioactivity in large amounts but the small amount of the radioactivity present suggests participation by the kidneys in the metabolism and elimination of a proportion of the CHB.

The pancreas contained the least percentage of radioactivity of the organs examined and pancreatic juice did not contain significant [^{14}C]. On a dpm/g basis the ratio of radioactivity in the liver to the pancreas was expected to be approximately 1:12 (the liver of the adult rat is approximately 12 grams, while the pancreas is approximately 800-900 milligrams). However, the data show that this ratio is actually 1:7.6 (69417 dpm/g in the liver and 9099 dpm/g in the pancreas). This suggests that the

pancreas accumulated a 1.5 times greater concentration of [¹⁴C] on a per gram basis as compared to the liver. This may contribute to the toxic effect of CHB on the pancreas.

The percentage of radioactivity administered that was accounted for from the bile, urine, and organs tested was 65%. The remaining 35% of the radioactivity could probably be accounted for in other organs such as the brain, lungs, muscle, bone, and spleen and possibly as unabsorbed compound.

If the metabolites in the bile and urine could be identified, perhaps they could be synthesized and administered to pancreatic cell cultures to observe their effect on acinar cell structure and function.

Much work remains to be done to determine the mechanism by which CHB causes the cell damage and oedema observed in the pancreas. Of particular interest are the effects of chronic dosing of the compound on the rat pancreas. The effects of CHB on endocrine pancreatic function deserve investigation as does the mechanism of the remarkable increase in the permeability of the pancreatic microcirculation which is evident from the formation of an exudate of such high protein concentration. CHB therefore offers the opportunity for studying the ability of the pancreas to handle a toxic insult and for creating models of acute and chronic pancreatic injury.

Conclusions

Synthetic (R)- and (S)- 1-cyano-2-hydroxy-3-butene (CHB) causes a decrease in pancreatic non-protein thiol in adult male rats 4 hours after treatment, followed by a rebound over control levels at 24 hours, and remains elevated at 120 hours after dosing. Hepatic non-protein thiol was also decreased at 4 hours after CHB administration, but not altered at 24 and 120 hours. Glutathione S-transferase activity followed a similar pattern as observed with non-protein thiol in the pancreas, but was not altered at 120 hours. No changes were observed in hepatic glutathione S-transferase activity following CHB dosing. Further investigation is required to determine if CHB is conjugated to glutathione as a means of detoxication. Also of interest is the possibility that CHB induces cytochrome P-450 in the pancreas.

CHB caused the secretion of pancreatic juice in the rat to be stimulated by lower doses of the compound followed by a return to normal secretion volumes. Higher doses of CHB

Radioactive CHB was found to be excreted in the bile and the urine of rats. It was accumulated in the adipose tissue of the rat, and was found in lesser amounts in the liver and then the kidney. Little radioactivity was detected in the pancreas, indicating that CHB is not accumulated in the pancreas. The radioactive metabolites in the bile and urine need to be isolated, and then the chemical structure can be determined to confirm or deny the hypothesis that CHB is metabolised by a glutathione-mediated pathway.

caused stimulation of juice flow, but this was followed by a depression of flows. A dose-dependent decrease in protein concentration of pancreatic juice was observed after dosing. The mechanism by which CHB inhibits pancreatic juice secretion is not known, but it may interfere with the action of stimulatory hormones, and/or inhibit the biosynthesis of pancreatic enzymes. Both of these possibilities deserve examination.

Bile secretion was stimulated in a dose-dependent fashion following CHB treatment. At the highest dose tested, 200 mg/kg, the stimulation in bile flow was followed by a severe depression and almost stopped by the end of the experiment. Bile acid concentrations in bile collected from animals treated with 200 mg/kg CHB were decreased as compared to bile from control animals, showing that the choleresis observed was not bile acid-dependent. The effect of CHB on bile acid-independent secretion mediators needs to be investigated to possibly determine the cause of the choleresis.

CHB treatment resulted in the pancreata of rats to become oedematous. This pancreatic exudate was found to be very protein rich. It would be of considerable interest to determine the identity of these proteins and the relative amounts of each in the exudate to examine how CHB has effected the pancreatic microcirculation.

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