



**THE INFLUENCE OF FOREIGN COMPOUNDS
ON TAURINE LEVELS:
AN *IN VIVO* AND *IN VITRO* STUDY**

A

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ABSTRACT

The studies described in this thesis examine the possibility of using changes in levels of urinary taurine as non-invasive markers of liver dysfunction in rats and humans.

The hepatotoxic compounds carbon tetrachloride (CCl_4), thioacetamide, allyl alcohol and galactosamine caused elevations of urinary taurine in rats, at doses which induced liver necrosis. The elevations were correlated with increases in serum transaminase enzymes. However, hydrazine and ethionine administration also increased urinary taurine but neither of these compounds resulted in liver necrosis nor raised serum transaminase enzymes. Conversely, the glutathione depleting hepatotoxic compound bromobenzene, produced liver necrosis but no hypertaurinuria and α -naphthylisothiocyanate resulted in cholestasis and caused a significant hypotaurinuria.

The non-hepatotoxic compounds mercuric chloride and allylamine failed to raise urinary taurine and cadmium chloride only caused slight hypertaurinuria in those animals which developed hepatic steatosis.

Urinary taurine in humans suffering from alcoholic liver disease was significantly raised and was correlated with elevated serum AST and reduced total plasma protein levels.

Other studies demonstrated that urinary creatine was raised by the hepatotoxic compounds CCl_4 , thioacetamide and bromobenzene.

Perturbation of protein synthesis in rats, by treatment with cycloheximide or of glutathione levels by treatment of rats with diethyl maleate, phorone or buthionine sulfoximine, also altered urinary taurine profiles. This suggests that hypertaurinuria is probably enhanced by compounds which inhibit protein and glutathione synthesis and is reduced by compounds which conjugate with glutathione. The contribution made by *de novo* taurine synthesis to CCl_4 -induced hypertaurinuria in rats was investigated using ^{35}S -methionine and ^3H -taurine. Results suggested that the initial hypertaurinuria resulting from CCl_4 administration (0-24 h post-dose) was due to leaked cellular taurine and that an increase in taurine synthesis

contributed to the hypertaurinuria measured 24-48 h after dosing.

The appearance of taurine in the incubation media of isolated rat hepatocytes was an early sign of toxicity and taurine synthesis appeared to be increased in isolated rat hepatocytes challenged with a sub-toxic concentration of CCl_4 .

It was also shown that, in control rats, low levels of taurine in the liver were correlated with low urinary taurine. Animals with low urinary taurine, dosed with CCl_4 , subsequently developed a greater elevation of serum transaminase enzymes than rats with higher levels of urinary taurine. Depletion of liver taurine with administration of β -alanine also resulted in enhanced CCl_4 toxicity. Taurine provided some protection for isolated hepatocytes against CCl_4 and hydrazine toxicity.

These investigations suggest that (a) measurement of urinary taurine could be a useful non-invasive tool for the detection of (i) metabolic perturbations in the liver (ii) direct liver damage by hepatotoxic compounds and (iii) compounds which interfere with protein synthesis (b) lowered levels of taurine in the liver may result in increased toxicity of hepatotoxic compounds (c) taurine may be a useful marker of cytotoxicity in hepatocytes and (d) taurine is cytoprotective, both *in vivo* and *in vitro*.

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ABBREVIATIONS USED IN THE TEXT

ACTH	Adrenocorticotrophin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANIT	α -Naphthylisothiocyanate
AST	Aspartate aminotransferase
BIL	Bilirubin
BSO	Buthionine sulfoximine
BUN	Blood urea nitrogen
CPK	Creatine kinase
CV	Central vein
CytP ₄₅₀	Cytochrome P ₄₅₀
DEM	Diethyl maleate
DMSO	Dimethyl sulfoxide
FFA	Free fatty acids
GES	Guanidinoethyl sulphonate
GSH	Reduced glutathione (γ -glutamyl-cysteinyl-glycine)
GALN	Galactosamine
γ -GT	γ -Glutamyl transpeptidase
H&E	Haematoxylin and eosin
i.p.	intraperitoneal
K+H	Krebs Henseleit (incubation buffer)
LDH	Lactate dehydrogenase
NMR	Nuclear magnetic resonance
OPA	O-phthalaldehyde
PT	Portal tract
PAPS	Adenosine 3'-phosphate-5'phosphate
PAS	Periodic acid Schiff
PALP	pyridoxal phosphate
p.o.	Per os (oral route)
s.c.	Sub-cutaneous
TCA	Trichloroacetic acid
THV	Terminal hepatic venule
TNPSH	Total non-protein sulphhydryls
UDP/UTP	Uridine di/triphosphate
UHQ water	Ultra high quality
VLDL	Very low density lipid

*"...if it was so, it might be; and if it
were so, it would be: but as it isn't, it
ain't. That's logic."*

*So said Tweedledee in "Through the
Looking Glass" by Lewis Carroll.*

Sometimes taurine feels a bit like this.

INTRODUCTION

1.1.

BEGINNINGS

The work described in this thesis is based on an observation made in this department during investigations into the mechanisms of hepatotoxicity of hydrazine.

Proton NMR analysis was used as a tool to identify and quantify the metabolites of hydrazine in urine and plasma. During the course of these studies, Susan Sanins made the observation that urinary levels of taurine were raised in rats dosed with hydrazine (Figure 1.1). Further work demonstrated that other hepatotoxic agents, carbon tetrachloride (CCl_4), thioacetamide and α -naphthylisothiocyanate (ANIT) also elevated urinary levels of this β -amino acid (Sanins *et al.* 1990).

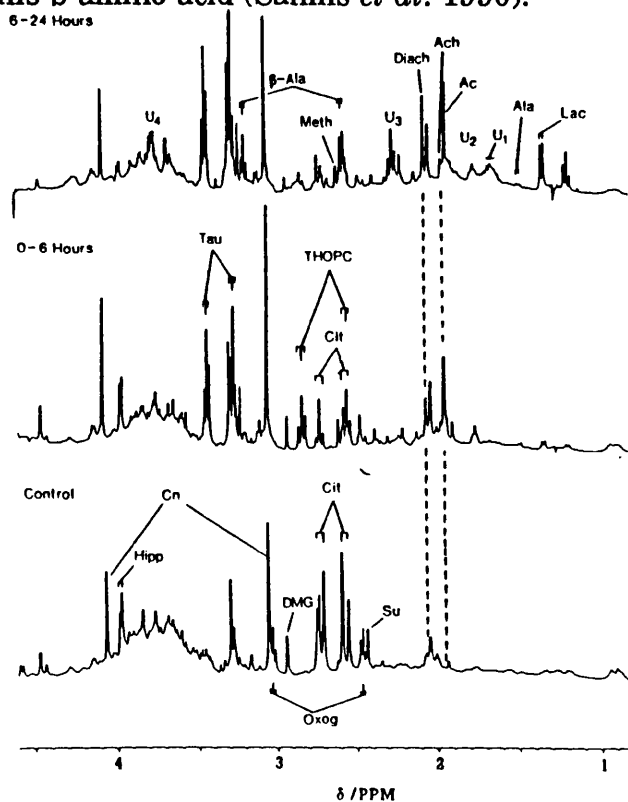


Figure 1.1 400MHz ¹H NMR spectra of the aliphatic region of urine collected 24 h before and 0-6 and 6-24 h after dosing with hydrazine hydrate (60 mg.kg^{-1}); Tau, taurine; β-Ala, β alanine (Taken from Sanins *et al.* 1990)

Hypertaurinuria was reported to be an early sign of toxicity following CCl_4 administration to rats by Cornish and Ryan (1964) and Kocsis *et al.* (1968 and 1975). There are references to other hepatotoxic compounds producing a similar effect, including the elevation of urinary taurine seen after administering colchicine and chlorobenzene to rats (Kostos and Kocsis, 1961 and Kocsis *et al.* 1968). When CCl_4 raised urinary taurine the "extra" taurine was assumed to originate from leaked hepatocellular taurine (Cornish and Ryan, 1964). It was of particular interest therefore, that there was an increase in urinary taurine after hydrazine treatment as this compound causes steatosis, not overt cellular damage (Lamb and Banks, 1979, Scales and Timbrell, 1982 and Haghighi and Honarjou, 1987).

These observations suggested that measurements of urinary taurine might be useful in the early detection of hepatic injury. At present, signs of hepatic damage are usually assessed using a variety of invasive techniques, none of which are able to detect fatty liver reliably.

Therefore, the initial aim of this work was to investigate the possibility of using raised urinary taurine levels as an indicator or marker of liver injury following the administration of hepatotoxic substances.

1.2. Liver as a target organ for toxicity

1.2.1. Central role

The liver is the largest gland in the body, constituting 2 - 5% of an adult's weight and 3% of an adult rat (Jones and Spring-Mills, 1988 and Miyai, 1991). It has a dual blood supply:

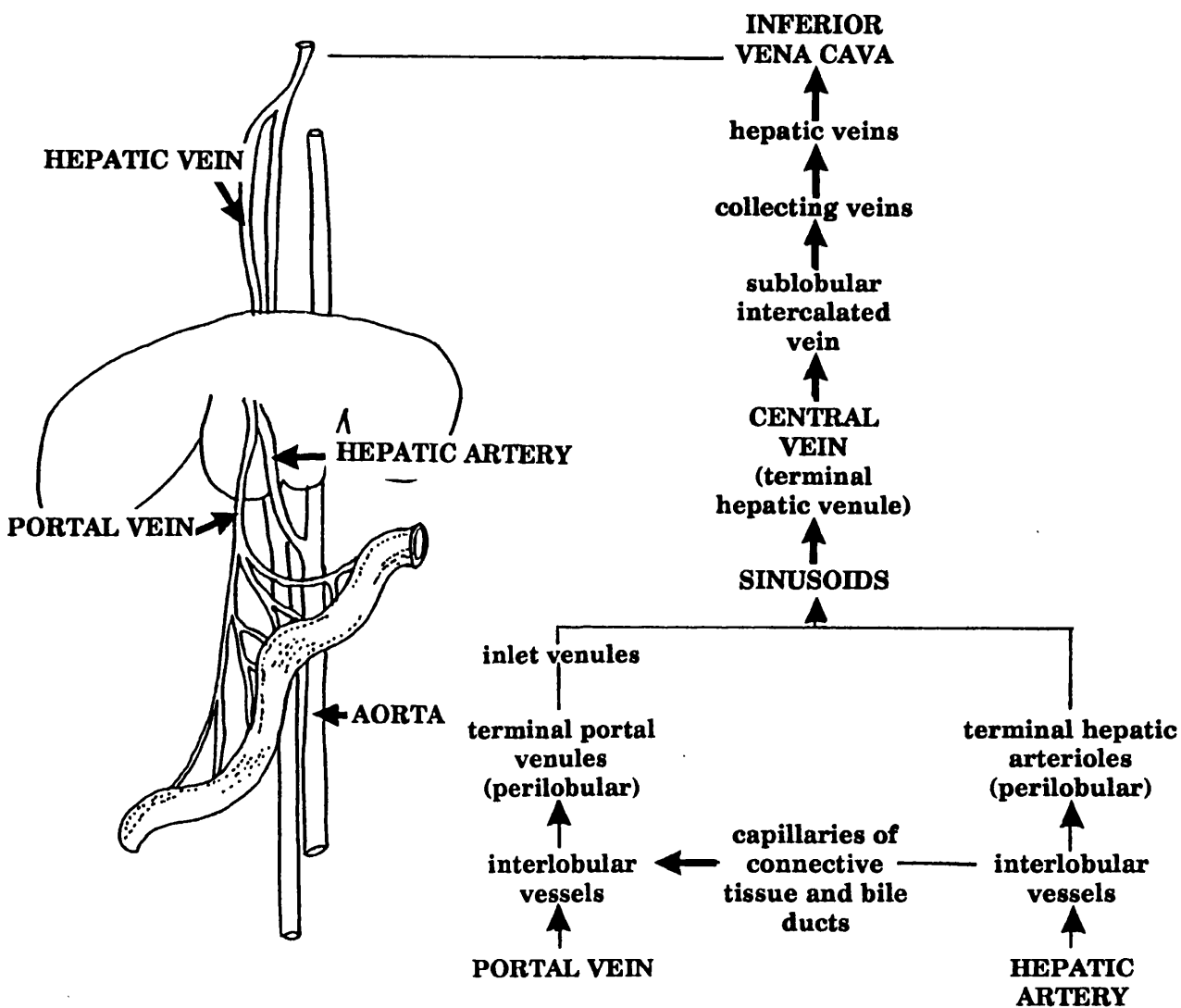
- (a) The hepatic artery, a branch of the coeliac trunk, carries well-oxygenated blood and provides approximately 25% of the afferent blood to the liver.
- (b) The portal vein carries blood that has already passed through the capillary beds of the alimentary tract; spleen and pancreas brings the remaining 75% of the liver's blood supply.

Blood from these two vessels mixes as it passes through branches of the blood vessels and through the liver lobules. Sinusoidal blood is collected by the terminal hepatic venules (central veins) which join to form the intercalated veins and finally the hepatic vein which returns blood to the heart via the inferior vena cava (Figure 1.2).

In this way the liver acts as a guardian situated between the digestive tract and the rest of the body. Because of this interposition, blood entering the liver contains high concentrations of nutrients and potentially toxic compounds which make the liver a target organ for toxicity (Timbrell, 1986), especially in experimental animals. The liver is therefore the main organ for storing nutrients such as glycogen and synthesising important molecules such as plasma proteins from the amino acids which enter via the portal vein. It is also involved in the catabolism and excretion of excess or unwanted molecules such as haemoglobin, excess amino acids and xenobiotics. The production of bile by the liver constitutes its function as an exocrine gland.

Figure 1.2 Over view of hepatic circulation

(Modified from Jones and Spring-Mills, 1988)



1.2.2. Liver morphology

The highest percentage of the liver tissue is made up of hepatocytes (parenchyma), both in cell number and in the volume of space they occupy. In the human liver, approximately 85% of the cells are hepatocytes, the remainder being mainly cells lining the sinusoids. The rat liver has fewer hepatocytes (60%) but these constitute nearly 78% of the liver volume.

The organisation of the hepatic parenchyma (hepatocytes) and microvascular system results in a very efficient system for exchange between the blood and the hepatocytes. The hepatocytes are arranged in plates a single-cell thick with at least two surfaces exposed to the blood. Cells lining the sinusoids are highly porous and unlike capillaries, they are not lined by a continuous basement membrane. The products of hepatocyte metabolism pass into both the blood and bile. This is expressed as the separate compartmentalization of the cell surface of hepatocytes into vascular and biliary spaces; a feature which is altered when isolated hepatocytes are used as a model (Jones and Spring-Mills, 1988 and Miyai, 1991).

In addition, the liver plays an important role in the body's defence against macromolecules and particulate materials such as bacteria. The sinusoid contains two cell types: typical, flattened endothelial cells; and large, fixed macrophages called Kupffer cells (Figure 1.3). These stellate-shaped Kupffer cells occur at various points along the sinusoidal lumen and are thought to be present in greater numbers in the periportal zone (43%) than in the perivenous zone (29%) with 28% lying in the intermediate zone (Bouwens *et al.* 1968).

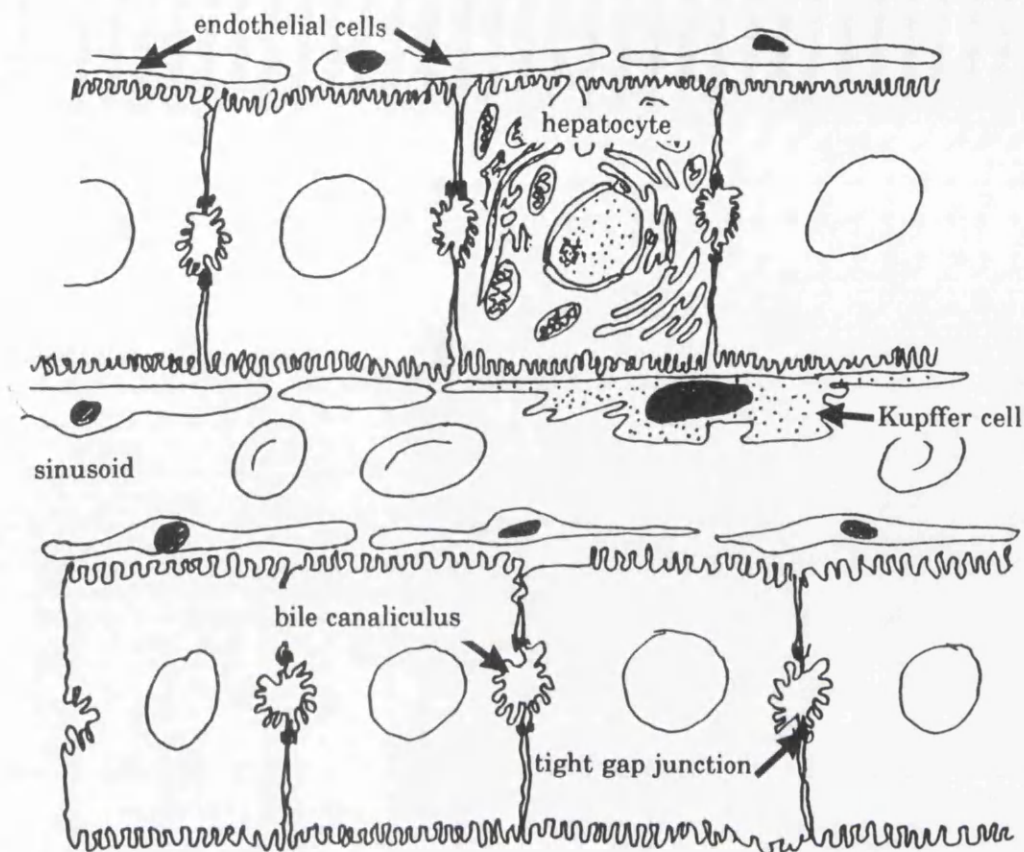


Figure 1.3 Cross section through cords of parenchymal cells and a single sinusoid.

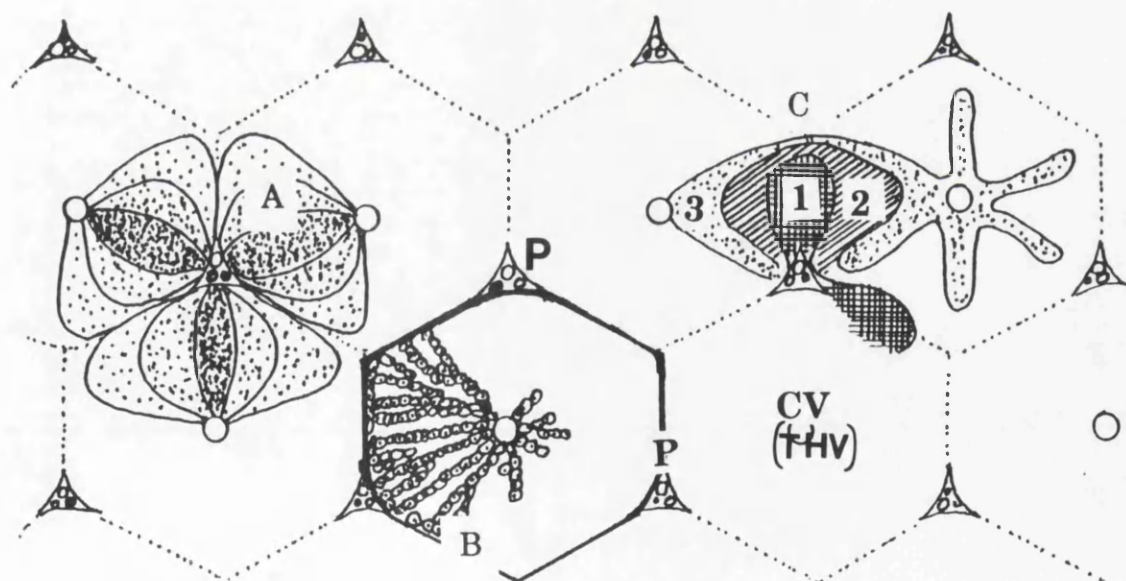


Figure 1.4 Schematic drawing of the three histological units of the liver. A: *Portal lobule*. B: *Hepatic (classical) lobule*. C: *Hepatic acinus*. P: portal tracts or triad, CV: central vein, THV: terminal hepatic venules. 1: periportal zone 1, 2: intermediate zone 2, 3: perivenular zone 3. The perivenular region is formed by the zone 3 of several adjacent acini, forming a star shape.

1.2.3. Organisation of hepatic parenchyma.

Three different concepts have developed to describe both the spatial orientation of the cells in the liver and the functional units within the liver parenchyma. These are the;

- (a) classical lobule as described by Wepfer and Malpighi and refined by Kiernan consisting of a polyhedral prism of tissue with a central vein at the centre.
- (b) portal lobule based on a triangular shape of parenchyma with a portal triad at the centre and central vein at each corner, a concept rarely used.
- (c) hepatic acinus which forms the smallest functional unit of the liver parenchyma and is related to the terminal branches of the afferent circulation (Rappaport, 1979).

The relationship between these differently defined regions in the liver parenchyma is shown in Figure 1.4.

(a) Hepatic lobule.

The hepatic lobule is a polyhedral prism of hepatic tissue. The corners are demarcated by connective tissue or biliary and vascular tissue grouped together to form the portal triads. There is a central vein at the centre of each lobule. The plates of hepatocytes are separated on either side by the narrow vascular spaces or sinusoids.

Blood flows from the portal canals (branches of the portal vein and hepatic artery) into the lobule along the sinusoids to the central vein where the blood is collected and returned to the systemic circulation via the hepatic vein.

Bile flows in the opposite direction, from the parenchyma cells where it is formed, into the bile canaliculi between each hepatocyte and along the interlobular bile ductules to the bile ducts in the portal triads.

(b) Portal lobule

The portal lobule is based on the concept of the direction of bile flow in the liver. For this reason the portal lobule has at its centre the portal triad

with a central vein demarcating the corners of the triangle.

(c) Hepatic acinus

The hepatic acinus is based on the microcirculatory unit of the liver. Rappaport (1979) noticed that the blood supply to each central vein was derived from several different sources. These corresponded to the parenchyma tissue organised around a terminal portal venule and its accompanying hepatic arterioles, bile ducts, lymphatic vessels and nerve fibres. A single acinus lies between the portal tract and the terminal hepatic venules (central vein in the hepatic lobule definition) making a shape similar to a "dumb bell" around the venule and arteriole. The cells adjacent to the terminal afferent vessels are considered to be in zone 1, and those furthest away and nearest the terminal hepatic venules are in zone 3. Zone 2 lies between the two regions. They correspond to the physiological zones in terms of oxygen concentration and nutrients. The cells in zone 1 are in the most favourable position in this respect. Those cells in zone 3, especially where several acini meet (perivenular ring), are the most sensitive to damage.

Hepatic blood flow is an important factor affecting the metabolic activity of the liver (Rappaport, 1979). The extraction of foreign compounds from sinusoidal blood will be influenced by blood flow, which is regulated by the hepatic arterioles (Figure 1.6).

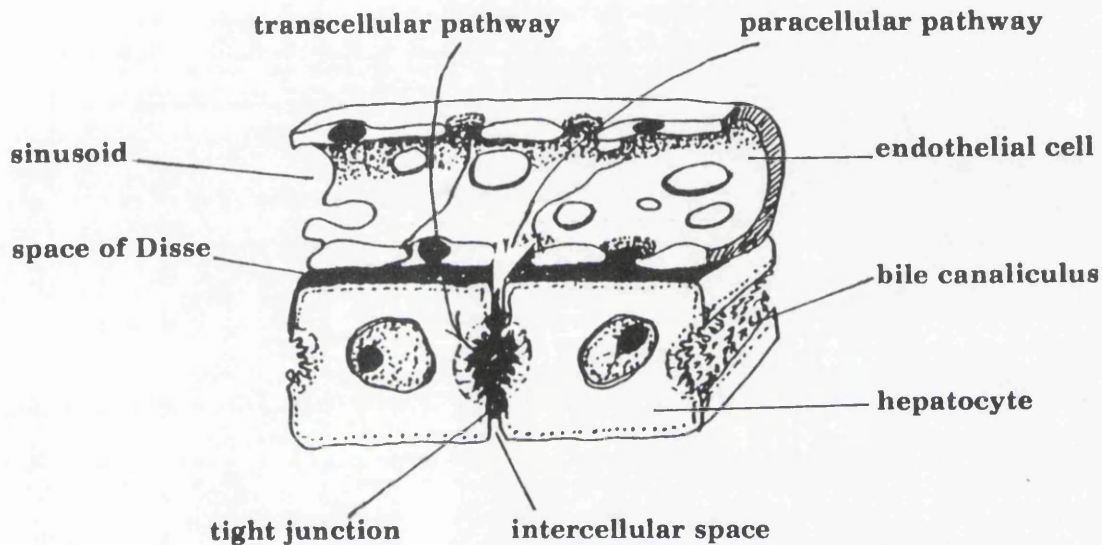
1.2.4. Hepatocytes

The liver parenchyma cells (hepatocytes) are the main functional unit of the liver. They are polygonal with a diameter in the rat of 17 - 23 μm and a volume of 4.77×10^3 - $5.54 \times 10^3 \mu\text{m}^3$. A high proportion of all adult mammalian hepatocytes (30% - 80%) are polyploid and 10 - 25% are binucleate (Figure 1.3).

They have three different cell surfaces. The canalicular surface, intercellular surface where two hepatocytes are next to one another and the sinusoidal surface. The canalicular surface forms the wall of the bile canaliculus and is formed by the apposition of the edges of hemitubular

grooves on the abutting surfaces of neighbouring hepatocytes. This surface is covered with microvilli. The sinusoidal surface is also studded with microvilli and is the site at which metabolite exchanges between hepatocyte and blood take place.

Figure 1.5 A schematic diagram of the hepatocyte and bile canaliculus. Solutes enter the canaliculus from the plasma either via the paracellular pathway (through the intercellular space and the tight junction) or via the transcellular pathway (across the basolateral and canalicular plasma membranes) (Modified from Vore 1991).



The sinusoidal and lateral surfaces differ from the canalicular surfaces in their biochemical composition, attached enzymes and receptors (Evans, 1980 and Maurice *et al.* 1985).

Each hepatocyte has between 800 and 1000 mitochondria (Blouin *et al.* 1977). They are more numerous in hepatocytes of acinar zone 1 than zone 3. These differences may reflect an adaptation to the variation in the metabolic environment within the microcirculation of the liver acinus.

Hepatocytes have an extensive network of endoplasmic reticulum (Miyai, 1991). The rough endoplasmic reticulum (RER) with ribosomes attached is involved in the synthesis of proteins for export and enzymes such as glucose-6-phosphatase. It also plays a role in glycogenesis. The smooth endoplasmic reticulum (SER), which has no ribosomes attached to it, is involved in the metabolism of proteins, carbohydrates and lipids and in the

biotransformation of foreign substances through oxidation, reduction, hydrolysis or conjugation. Through these reactions lipophilic compounds are converted into hydrophilic substances. Other functions of the endoplasmic reticulum include the degradation of steroids and the metabolism of cholesterol, bilirubin, lipid-soluble vitamins and fatty acids, the storage of glycogen and the synthesis of bile acids (DePierre *et al.* 1988).

The endoplasmic reticulum is involved in lipid metabolism. The triglycerides produced in the hepatocyte are not used for energy production or membrane synthesis. Most are exported into the blood as very low-density lipoprotein (VLDL). These VLDL droplets have a coat of apoprotein and cholesterol encasing a droplet of triglyceride. The apoprotein is believed to be produced by the rough endoplasmic reticulum and the lipid component by the smooth endoplasmic reticulum. Some VLDL are released into the blood after packaging into membrane vesicles and the remainder are transported to the Golgi complex where they acquire a carbohydrate component before being released into the blood stream by exocytosis (Jones and Schmucker, 1977). Closely associated with the SER are peroxisomes and lysosomes. Peroxisomes are membrane-bound organelles containing enzymes that catalyse reactions involving hydrogen peroxide. They generate hydrogen peroxide which they use and then destroy by means of catalase detoxifying various molecules such as ethanol and acetaldehyde. In addition they catalyse the β -oxidation of fatty acid to acetyl-CoA which can be utilised in mitochondria. They are known to proliferate after partial hepatectomy and after the administration of the lipid lowering agent, clofibrate. Lysosomes are involved in the autophagic digestion of senescent organelles and inclusions which can be used to provide energy or recycled.

1.2.5. Localization of metabolic activity

The difference in oxygen supply between the cells in zone 1 (periportal) and zone 3 (perivenous) gives rise to biochemical gradients (Figure 1.6.).

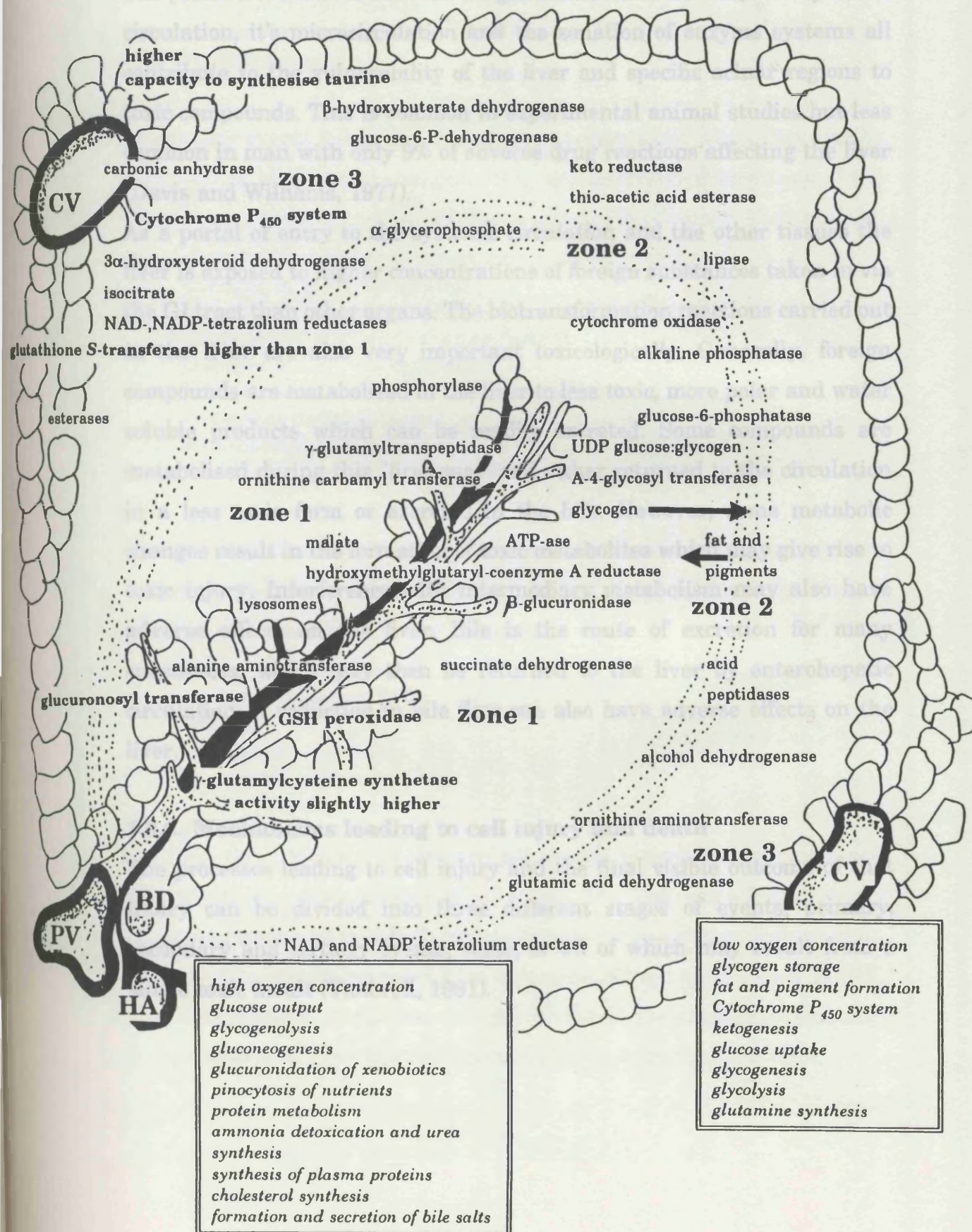
The cells of zone 1 are most aerobic and are well equipped to carry out oxidative energy metabolism, gluconeogenesis, β -oxidation of fatty acids, amino acid catabolism and bile acid secretion. They are also equipped for

the glucuronidation of xenobiotics as these cells have a high concentration of glucuronosyl transferases. Protein synthesis and the formation of plasma proteins may also be carried out to a greater extent in these cells.

The important functions of zone 3 are the storage of glycogen and the formation of fat and pigments. The NADPH and NADH reductase reactions also take place primarily in the cells of zone 3. These examples of zonal specialisation of hepatocytes can be reversed by reversing blood flow (e.g. gluconeogenesis and glycolysis) (Thurman and Kauffman, 1985). However, this is not always the case as the periportal dominance for urea formation remains even when cells are isolated and cultured (Poso *et al.* 1986). Perivenous cells also contain an ample amount of SER with mixed-function oxidases (the cytochrome P₄₅₀ system) whose function can not be changed by altering blood flow. Surprisingly, the protective mechanisms such as glutathione peroxidase and glutathione (GSH) itself are predominant in zone 1. This may explain why the fatty changes are seen commonly in zone 3, where there is less protection from peroxides which would otherwise be detoxified by their reduction by GSH, catalysed by GSH peroxidase.

Interestingly, work by Penttila (1990) has shown a more rapid synthesis of taurine in perivenous hepatocytes and a greater accumulation in these cells. However one of the main functions of taurine in the liver is its conjugation with bile acids which takes place predominantly in periportal hepatocytes. It is possible that the conjugation of bile salts relies not only on hepatic synthesis of taurine but also taurine synthesised in other organs such as the kidney or taken in the diet.

Figure 1.6. Enzyme distribution and metabolic areas in the various zones of the liver acinus according to Rappaport (1979) modified according to Jungermann and Katz (1989) and Penttila (1990).



1.3. Hepatotoxicity

1.3.1 Introduction

The position of the liver between the gastrointestinal tract and the systemic circulation, its microcirculation and the zonation of enzyme systems all contribute to the vulnerability of the liver and specific acinar regions to toxic compounds. This is common in experimental animal studies but less common in man with only 9% of adverse drug reactions affecting the liver (Davis and Williams, 1977).

As a portal of entry to the systemic circulation and the other tissues the liver is exposed to higher concentrations of foreign substances taken in via the GI tract than other organs. The biotransformation reactions carried out in the liver are also very important toxicologically. Generally, foreign compounds are metabolised in the liver to less toxic, more polar and water soluble products which can be readily excreted. Some compounds are metabolised during this "first pass" and either returned to the circulation in a less toxic form or excreted in the bile. However, some metabolic changes result in the formation of toxic metabolites which may give rise to toxic injury. Interference with intermediary metabolism may also have adverse effects on the liver. Bile is the route of excretion for many substances, which may then be returned to the liver by enterohepatic circulation. A reduction in bile flow can also have adverse effects on the liver.

1.3.2. Mechanisms leading to cell injury and death

The processes leading to cell injury and the final visible outcome of that injury can be divided into three different stages of events; primary, secondary and tertiary events, some, or all of which may result from a single toxic insult (Timbrell, 1991).

Primary events induced by toxic compounds can lead to cellular damage and include:

- lipid peroxidation
- covalent binding to macromolecules
- changes in thiol status
- enzyme inhibition
- ischemia

Secondary events follow in the sequence leading to cell death. They may be structural or biochemical changes, many are interrelated and some of the changes are a consequence rather than a cause of the damage. They include:

- changes in membrane structure and permeability
- changes in the cytoskeleton
- mitochondrial damage and inhibition of function
- depletion of ATP and other co-factors
- changes in Ca^{2+} concentration
- DNA damage and poly ADP-ribosylation
- lysosomal destabilization
- stimulation of apoptosis
- damage to the endoplasmic reticulum

Tertiary events are the final, observable manifestations of exposure to a toxic compound. They may be present within a tissue at the same time, or follow on sequentially from one another:

- steatosis (fatty change)
- hydropic degeneration
- blebbing
- apoptosis
- necrosis

The first three tertiary events are potentially reversible, apoptosis and necrosis are two distinct types of cell death. The liver has a high capacity for tissue repair and is able to replace necrotic tissue within a short time. However, the chronic repeated administration of toxic agents such as CCl_4 and thioacetamide stimulates collagen synthesis resulting in irreversible fibrotic lesions in experimental animals similar to cirrhosis in man.

1.3.3. Types of hepatic damage

The various types of liver damage reflect the structure and function of the liver. Viewed simply, they are either the result of the metabolic capabilities of the hepatocyte or involve bile secretion and may be the result of chronic or acute exposure to toxic compounds, they may also occur together or follow an initial injury.

Hepatic injury has been extensively reviewed by Zimmerman (1978), Farber and Fisher (1979), Plaa and Hewitt (1982), Klaassen *et al.* (1986) and Meeks *et al.* (1991). Some important chemically induced hepatic lesions are listed in Table 1.1.

Table 1.1. A histopathological classification of some chemically-induced liver injuries.

TYPE OF INJURY*	AGENTS RESULTING IN INJURY*
ACUTE - CYTOTOXIC Fatty liver (steatosis) macrovesicular	CCl₄, hydrazine, thioacetamide, galactosamine, ethanol, phosphorous, orotic acid, methotrexate
microvesicular	Tetracycline, ethionine , phosphorous
Necrosis periportal (zone 1) centrilobular (zone 3) diffuse	Allyl alcohol, cocaine CCl₄, bromobenzene, thioacetamide Galactosamine
CHOLESTATIC cholangiodestructive canalicular	α-naphthylisothiocyanate (ANIT) Lithocholate
Vascular lesions	pyrrolizidine alkaloids
CHRONIC Cirrhosis	CCl₄, galactosamine, ethanol, aflatoxins, cadmium, chloroform, thioacetamide
Neoplasm	Aflatoxin B ₁ , contraceptive steroids, thioacetamide

* Hepatic injuries denoted in **bold** type are discussed in more detail below and those hepatotoxic agents in **bold** are discussed as examples and used experimentally in the experimental sections of this thesis.

(A) Fatty liver (steatosis)**(i) Pathology**

The accumulation of fat is one of the most common cellular responses of the liver to injury and is normally reversible. It is usually triglycerides that accumulate. They appear as large coalesced drops which may result in the ballooning of hepatocytes and the displacement of the nuclei to one side (macrovesicular). An exception to this occurs when triglycerides accumulate inside lysosomes resulting in small droplet or a foamy appearance (microvesicular) (Dianzani, 1991). Hepatocytes which become swollen as a result of macrovesicular steatosis cause disruption of blood flow through the sinusoids. This may then result in ischemic damage which should not be overlooked when considering later events in the toxicity of compounds such as CCl_4 (Rappaport, 1979).

There are many stages in the formation, mobilisation and metabolism of triglycerides which can be a target for chemical toxicity. For this reason, normal triglyceride metabolism is summarised below.

(ii) Normal triglyceride metabolism in the liver

Fat is absorbed from the intestine in the form of free fatty acids (FFA) which have been released from dietary triglycerides by intestinal lipases. Enterocytes reform the FFA into triglycerides or phospholipids, these are combined with apoproteins to form chylomicrons and released into the lymph. The lymph delivers the chylomicrons to the blood where they are remodelled and reduced in size. Lipoprotein lipase produced by endothelial cells releases FFA from the triglycerides; in this way FFA pass into cells including hepatocytes. Other FFA reach the liver bound to albumin in the blood having been released from adipose tissue (Dianzani, 1991).

FFA are also synthesised inside hepatocytes from acetyl-CoA. This synthesis is the reverse of the β -oxidation process and therefore requires a high $\text{NADPH}_2 : \text{NADP}$ ratio and high levels of ATP.

FFA can now be activated to acetyl-CoA derivatives, transformed to acetyl-carnitines and enter mitochondria or peroxisomes where they can undergo β -oxidation and be used in energy production.

However, the main metabolic fate of the FFA is their inclusion in structural lipids in membranes or in the formation of triglycerides. Triglycerides are transported out of cells as very low-density lipoproteins (VLDL). The different portions of lipoproteins are synthesised in different parts of the cell. The apolipoproteins are produced in the rough endoplasmic reticulum whereas the phospholipids are synthesised either in the smooth endoplasmic reticulum or in the cytosol. The primary synthesis of lipoprotein units from phospholipids and apoproteins (acceptor proteins) occurs in the SER and their final assembly is completed in the Golgi apparatus.

The micelles leave the Golgi region and move towards the plasma membrane. The connection between the plasma membrane is made by microtubules made mainly of tubulin. Part of the tubulin molecule (α -chain) is able to bind to colchicine (Figure 1.7).

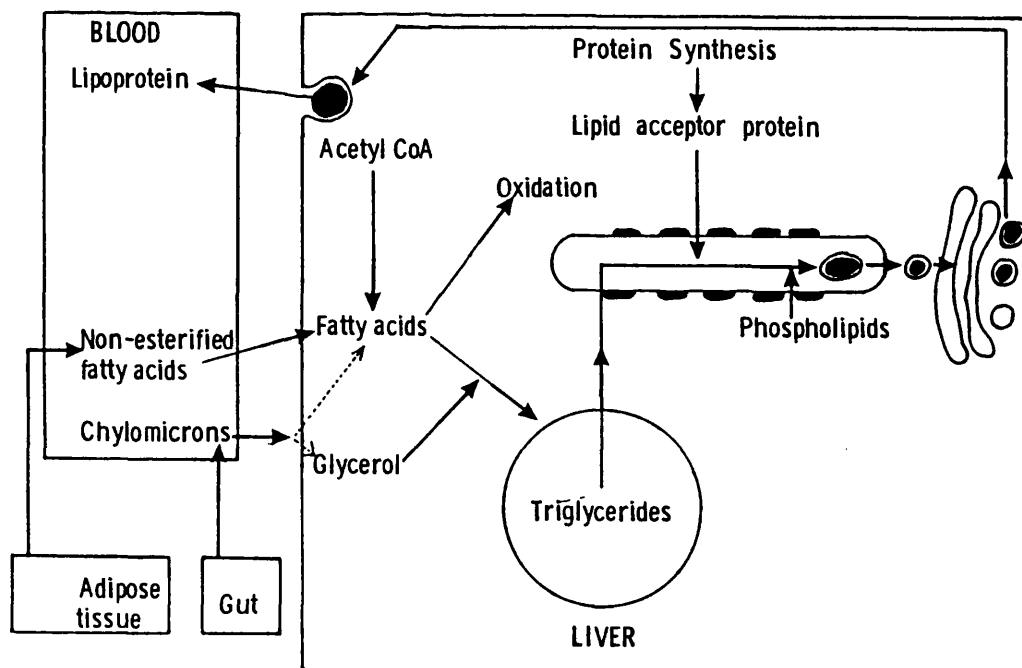


Figure 1.7 Pathways involved in the metabolism and distribution of lipid.
(From Timbrell, 1991)

(iii) Interference with lipid metabolism resulting in steatosis.

1. An increased synthesis of FFA from acetyl-CoA or increased mobilisation from adipose tissue possibly due to an increase in the reduced nucleotide ratio (eg. ethanol) or increased blood flow (barbiturates).
2. Decreased β -oxidation of FFA resulting in raised levels of FFA available for triglyceride formation. This could result from hypoxia (severe cases of anaemia), or a block in the formation of acetyl-CoA or acetyl-carnitine. Choline deficiency reduces carnitine levels, which may partly explain why choline deficiency results in steatosis (Dianzani, 1991).
3. An inhibition of triglyceride transport from the cell may result from many factors such as:
 - (a) a block in the formation of the apoprotein required to form the lipoprotein to transport the lipid out of the cell as VLDL. This could result at any point in the process of protein synthesis: at the point of DNA transcription; during RNA translation (eg. thioacetamide) or at the site of ribosome assembly (eg. tetracycline and puromycin)
 - (b) damage to the smooth endoplasmic reticulum preventing the assembly of the lipoproteins (eg. CCl_4)
 - (c) the alkylation of both nucleic acids and protein by alkylating agents (eg. dimethylnitrosamine)
 - (d) sequestration of ATP which is required for protein synthesis and transportation of the lipoprotein out of the cell (eg. ethionine and CCl_4) (Farber, 1967 and Poli *et al.* 1987).
 - (e) damage to the microtubules involved in transporting lipoproteins out of cells (eg. colchicine) (Zimmerman, 1978).

(B) Cholestasis

(i) Pathology

Cholestasis is the term used to describe the cessation of bile flow. This can be brought about by physical blockage of the bile ducts (extrahepatic cholestasis) or by cessation of bile secretion by the hepatocytes (intrahepatic

cholestasis). Bile acids and bilirubin become elevated in the blood. Serum cholesterol levels are also elevated as are levels of enzymes normally present in the bile such as alkaline phosphatase. Cholestasis results in extensive damage to the parenchyma of the liver if the condition is not reversed (Vore, 1991).

(ii) Normal secretion of bile

Bile is secreted into the bile canaliculus from two adjacent hepatocytes. The hepatocyte membranes form the canalicular membranes and are covered in microvilli (Figure 1.5). The lumen is separated from sinusoidal blood by the intercellular junctions between adjacent cells. The zone of cytoplasm surrounding the canaliculus is devoid of organelles but does contain actin microfilaments which are thought to enhance bile flow by their contractions. Bile is primarily composed of water (97%), inorganic electrolytes and organic solutes such as bile acids, glutathione, cholesterol, phospholipids, bile pigments (mainly conjugated bilirubin), alkaline phosphatase and proteins (Hatoff and Hardison, 1980).

Table 1.2. Bile flow and major components of bile.

	Man	Rat
Flow ($\mu\text{l} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$)	1.5 - 15.4	30 - 50
Na^+ ($\text{mEq} \cdot \text{l}^{-1}$)	132 - 165	157 - 166
K^+ ($\text{mEq} \cdot \text{l}^{-1}$)	4.2 - 5.6	5.8 - 6.4
Cl^- ($\text{mEq} \cdot \text{l}^{-1}$)	96 - 126	96
HCO_3^- ($\text{mEq} \cdot \text{l}^{-1}$)	17 - 55	24
Bile acids (mM)	3 - 45	38
Phospholipids ($\text{mg} \cdot \text{l}^{-1}$)	1400 - 8100	412
Cholesterol ($\text{mg} \cdot \text{l}^{-1}$)	970 - 3200	223

Adapted from Vore, 1991.

The osmolality of bile is similar to plasma and is maintained by the electrolytes rather than the bile salts which form micelles (Wheeler and Ramos, 1960).

Bile acids are formed in the liver from cholesterol. This process is controlled by the reabsorption of bile acids by the liver during enterohepatic circulation. They are conjugated with taurine and glycine in the liver to form bile conjugates with pK_a values of 2 and 4 respectively and are therefore ionised in bile and exist as salts. Taurocholate is the major bile acid in the rat, whereas both taurine and glycine conjugates of primary bile acids and secondary bile acids are found in man (Figure 1.8) (Keele *et al.* 1982).

Uptake of bile acids from portal blood across the basolateral membrane is known to be carrier mediated. This is dependent on a Na^+ gradient which is higher outside the cell than inside and is maintained by Na^+,K^+ -ATPase exchange. This is thought to function primarily in the basolateral part of the plasma membrane (Sztul *et al.* 1987). Transport of bile acids across the hepatocyte is not well understood but is thought to involve a number of binding proteins. The transport of bile acids across the canalicular membrane is an active, carrier mediated process working against a concentration gradient.

A good correlation exists between the rate of bile acid secretion into the bile and the rate of bile flow, suggesting that bile salts are osmotically active. However their micellar structure precludes this activity. Therefore, it is thought that bile flow generated by bile acid secretion (bile-acid dependent flow) is due to the ions which accompany the bile salts into the canaliculi (eg. Na^+). Bile-acid independent flow of bile results from the transport of osmotically active solutes. Chloride and hydrogen carbonate ions are probably involved.

Water moves into the bile osmotically and is believed to be derived from the plasma through the paracellular pathway rather than from the canalicular membrane and across the hepatocyte. In this way bile is able to flow.

Micelles are formed in the bile by the incorporation of bile salts with both cholesterol and phospholipid. Once in the ileum or proximal colon, bacteria

may hydrolyse some of the bile salts back to bile acids and amino acids which are absorbed with unchanged bile salts into the portal blood and returned to the liver (Lack and Weiner, 1967).

(iii) Interference with bile flow resulting in cholestatic injury.

Just as each step in the formation of triglycerides and their transportation out of the hepatocyte is a potential site of interference so it is with the maintenance of bile flow.

Thus cholestasis can result from;

- i. lack of ATP required for the active transport of bile constituents
- ii. direct injury to both the basolateral or canalicular membrane (eg. C-17 alkylated anabolic steroids)
- iii. impairment of bile salt dependent flow by blocking the synthesis or transcanalicular transport of bile acids by anabolic and contraceptive steroids, possibly as a result of their similar structures.
- iv. impairment of bile acid independent flow by inhibition of Na^+, K^+ -ATPase
- v. interference with the formation of normal micelles in the bile, possibly by the inclusion of structurally similar compounds such as lithocholate or alkylated steroids in the micelle
- vi. direct damage to the bile duct epithelium similar to that seen after the administration of α -naphthylisothiocyanate which results in cell debris accumulating in ductules.

Because of the difficulty in assessing cholestasis by histological means and the uncertainty about the mechanisms involved in normal bile flow, it has proved very difficult to define the mechanisms which induce cholestasis (Krell, *et al.* 1987). Indeed it seems likely that cholestasis is not a single molecular disorder but incorporates multiple disturbances which may impinge on some processes of bile formation eventually leading, through a series of events, to depressed bile flow (Kukongviriyapan and Stacey, 1991).

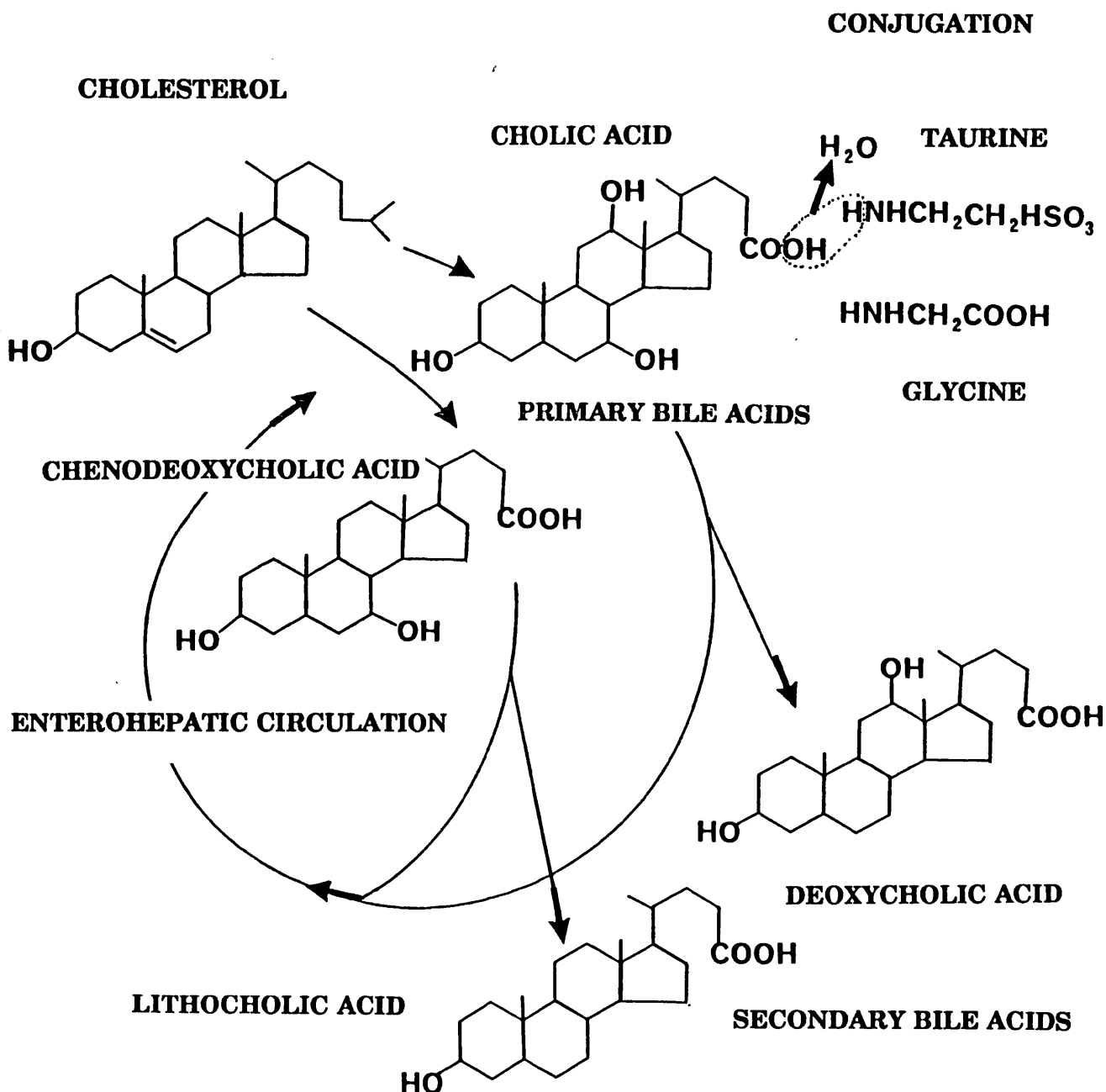


Figure 1.8 BILE ACID SYNTHESIS - primary bile acids (eg. cholic and chenodeoxycholic acid) are synthesised in the liver from cholesterol. They are conjugated with glycine or taurine and enter the bile. Secondary bile acids (eg. lithocholic and deoxycholic acid) are formed in the intestinal tract by bacterial degradation. All bile acids can return to the liver via the enterohepatic circulation where they are conjugated with taurine or glycine. Lithocholate may also be sulphated, increasing the solubility and decreasing its toxicity (Section 1.5.8).

(C) Cirrhosis

There is controversy over the precise definition of cirrhosis (Wight, 1982). It is however, generally accepted that it is a fibrotic lesion throughout the whole liver, where the fibrosis has replaced the normal lobular structure. This results in the development of structurally abnormal nodules. It follows repeated cellular injury such as periods of necrosis, although necrotic tissue is not normally found within the cirrhotic tissue. It is also irreversible and progressive and inevitably fatal in man.

It is difficult to produce a similar model of cirrhosis in animals, although repeated administration of CCl_4 and thioacetamide (Adnani, 1989 and Muller *et al.* 1991) have been used to provide an animal model.

The repeated administration of CCl_4 with phenobarbitone treatment to rats produces splenomegaly, portal hypertension and testicular atrophy and 50% of the animals develop ascites (McLean *et al.* 1969). These lesions are comparable with the human condition. In contrast to true cirrhosis seen in man, the livers from animals treated in this way retain their fibrotic characteristics for at least 70 days after the last treatment, but fail to show progressive cirrhosis (Trivedi and Mowat, 1983).

(D) Necrosis

(i) Pathology

Cellular necrosis is the death of cells within a living tissue and is characterised by hypereosinophilic cells with condensed irregular or pyknotic nuclei (Glaister, 1986). Liver necrosis usually develops in a characteristic region of the acinus for any given compound. It demonstrates very clearly the zonation of metabolising enzymes which result in the production of toxic metabolites. Necrosis is seen most commonly in zone 3, around the central vein. This is sometimes due to the class of enzymes known as the cytochrome P_{450} monooxygenases. These are responsible for metabolising xenobiotics, which may result in metabolites being formed which are more toxic than the parent compound. For example, CCl_4 and bromobenzene both produce necrosis in zone 3 (Mico *et al.* 1983, Waller *et al.* 1983 and Jollow *et al.* 1974). Allyl alcohol produces necrosis around the

portal tracts in zone 1 and galactosamine results in diffuse multifocal necrosis (Rikans, 1989 and Keppler *et al.* 1968).

(ii) Possible mechanisms leading to necrosis.

Early investigations into the mechanisms leading to cell death were concerned with the role of covalent binding of reactive metabolites to macromolecules (Brodie *et al.* 1971) and the destruction of structural membranes by lipid peroxidation as a result of free radical formation (Rechnagel and Glende, 1973).

The production of free radicals during the metabolic activation of CCl_4 , white phosphorous and paraquat have been implicated in their toxicity. Free radicals have an unpaired electron and are very reactive. They can react directly with cellular components or result in the production of the oxygen free radical, super oxide.

Lipid peroxidation is a common result of free radical generation, leading to the destruction of cellular membranes containing unsaturated lipids (Figure 1.9).

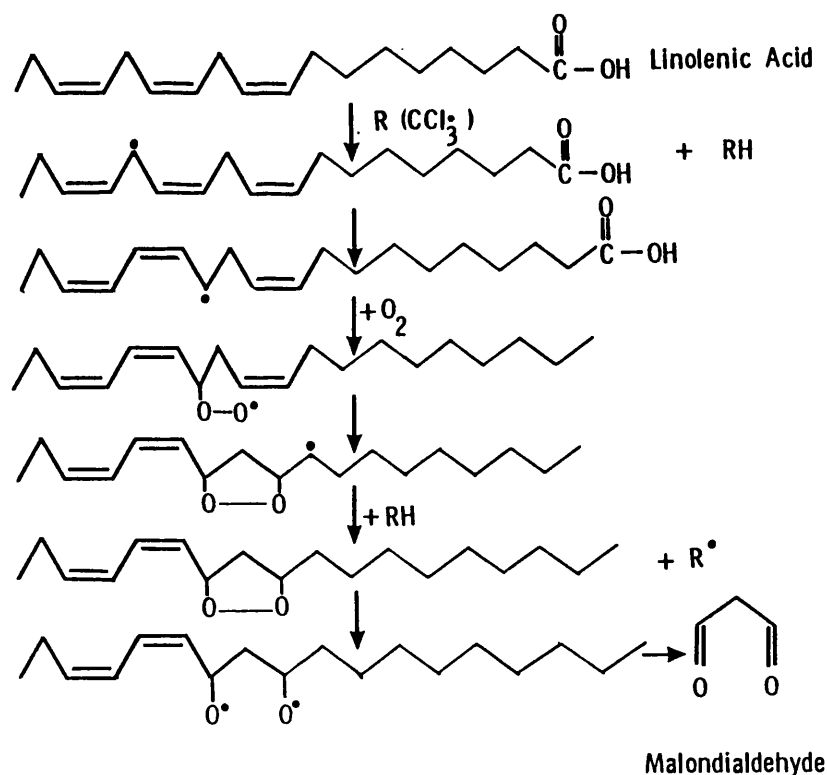
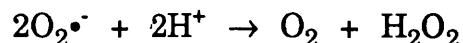


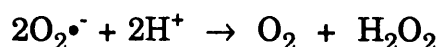
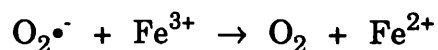
Figure 1.9 Peroxidative destruction of a polyunsaturated lipid initiated by a free radical attack such as by the trichloromethyl radical produced during CCl_4 metabolism.

Oxidative stress can develop when electrons are donated to oxygen to yield superoxide. This may be metabolised to hydrogen peroxide by superoxide dismutase and then to water or protonated to the hydroperoxy radical, $\text{HOO}\cdot$.

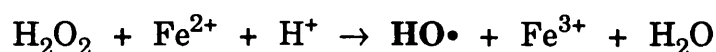


The presence of transition metals can result in the formation of hydroxyl radicals which are very reactive free radicals (Fawthrop *et al.* 1991).

Haber-Weiss reaction:



Fenton reaction:



There are detoxification mechanisms present in cells to remove these radicals. However, these mechanisms may become overwhelmed in the presence of an excess of toxic substances. An excess of superoxide will be produced, GSH and NADPH are depleted and both hydroxyl and singlet oxygen will be formed. This is referred to as oxidative stress and results in DNA and protein damage as well as lipid peroxidation (Timbrell, 1991).

More recent work into mechanisms of cell death have centred around the importance of calcium homeostasis in the cell. It has been recognised for many years that necrotic tissue and ischemic heart muscle have raised levels of Ca^{2+} . This led to the proposal that a rise in cytosolic Ca^{2+} precipitates cell death (Farber, 1981). It was later proposed that the rise in cytosolic Ca^{2+} was due to the failure of the cells to compartmentalise cellular Ca^{2+} by sequestration into the mitochondria and endoplasmic reticulum (Orrenius, 1989). The sustained rise in Ca^{2+} would be expected to activate Ca^{2+} dependent degenerative enzymes such as phospholipases, proteases and endonucleases (Nicotera *et al.* 1989). These would result in membrane damage, impaired enzyme activity and the disruption of the cytoskeleton.

However, cell death is not always associated with a rise in cytosolic Ca^{2+} (Smith *et al.* 1981). Indeed some workers regard the depletion of ATP and subsequent changes in pH as more significant events leading to cell death (Herman *et al.* 1990). At present, attention is focused on both necrosis and apoptosis (programmed cell death) and the role played by the alkylation of both macromolecules and DNA in these processes in the hope of finding a mechanism of toxicity which could result in the final "point of no return" for cells (Corcoran and Ray, 1992).

1.4. Cellular protection

Besides the metabolic transformation of compounds to more polar metabolites for example by conjugation and sulphation (eg. paracetamol) or the conversion of reactive metabolites into more stable ones (eg. epoxides to dihydrodiols) cells possess specific detoxification mechanisms.

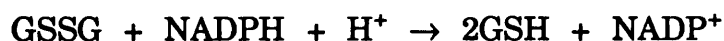
1.4.1. Glutathione

Glutathione is a tripeptide composed of glutamate, cysteine and glycine. It exists in two forms in thiol-reduced (GSH) and disulphide-oxidised (GSSG) forms. It is found in high concentrations (mM) in most tissues and is particularly concentrated in the liver (>7 mM) where it is synthesised (Higashi *et al*, 1983). Probably the most important detoxification mechanisms in cells involve GSH (Kaplowitz *et al*. 1985).

- i. GSH is a specific substrate for the selenium dependent glutathione dependent peroxidase involved in the detoxification of H₂O₂ and organic peroxides.



GSSG is subsequently reduced back to GSH by glutathione reductase at the expense of NADPH:

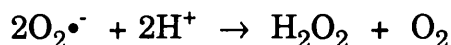


- ii. GSH conjugation can take place by simple chemical reaction with electrophiles or catalysed by one of the glutathione-S-transferases. Some of these reactive metabolites would otherwise have reacted directly with protein sulphydryl groups inactivating enzymes and denaturing structural proteins.

Because GSH has such an important role in cellular defense its depletion can result in increased toxicity of foreign compounds.

1.4.2. Superoxide dismutase and catalase

Superoxide dismutase is present both in the cytosol and mitochondria and is responsible for the removal of superoxide radicals resulting in the production of hydrogen peroxide:



The reactive product, hydrogen peroxide, can be removed by the action of glutathione peroxidase (see above) or by catalase which is present in peroxisomes:



1.4.3. Vitamin E

Vitamin E (α -tocopherol) is found in the phospholipid membranes of various organelles and may be associated with unsaturated fatty acids such as arachidonate (Wills, 1985). Vitamin E scavenges free radicals arising from unsaturated lipids, thus preventing lipid peroxidation. This produces α -tocopherol semi-quinone radicals. The vitamin E can be regenerated by GSH or vitamin C (Dogterom, 1990).

1.5. Hepatotoxic compounds and mechanism of action.

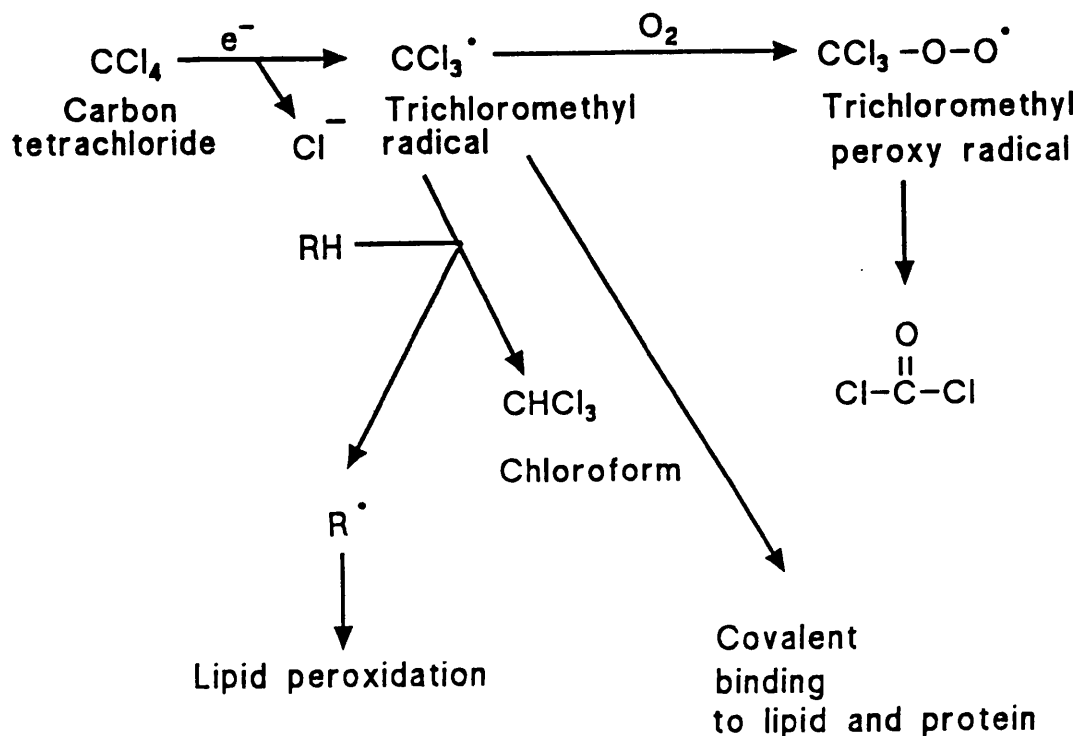
1.5.1. Carbon tetrachloride

Carbon tetrachloride (CCl_4) illustrates a range of toxicological effects resulting from metabolic activation to highly reactive metabolites via the cytochromes P_{450} . The lesions it produces are highly reproducible and dose dependent. From a toxicological point of view it provides both a challenge and insight into the mechanisms leading to cell death. For these reasons carbon tetrachloride is probably the most extensively studied hepatotoxin (Rechnagel, 1973, Slater, 1978 and Dianzani, 1979).

The liver is the major target organ because the toxicity of CCl_4 is dependent on metabolic activation by the cytochromes P_{450} . As the centrilobular (zone 3) region of the liver has the highest concentration of cytochromes P_{450} this is where the damage is greatest.

The hepatotoxic effects are seen histologically as fatty liver, hydropic degeneration of hepatocytes and necrosis beginning around the central veins and spreading as the toxic response increases in severity. Low doses of CCl_4 cause only steatosis and also destroy the cytochromes P_{450} which prevents subsequent doses of CCl_4 from being hepatotoxic. It has been proposed that two forms of P_{450} are responsible for the metabolism of CCl_4 to reactive metabolites (Figure 1.10)

Figure 1.10. The metabolism of carbon tetrachloride



Initially, enzymes of the cytochrome P₄₅₀ 2B family catalyse a one-electron reduction of CCl₄ generating a trichloromethyl radical (CCl₃•) and Cl⁻ which results in the inactivation of the P₄₅₀ 2B. The trichloromethyl radical undergoes a hydrogen ion abstraction to form chloroform (CHCl₃) or becomes further reduced to form dichlorocarbene (:CCl₂). This metabolic route has been proposed to function under conditions of low oxygen tension, similar to those that might be found in zone 3 of the liver acinus (Pohl *et al.* 1984). A second cytochrome P₄₅₀, 2E1 has been proposed for the oxidation of the CCl₃• to form trichloromethyl peroxy radicals (•OOCCL₃) and then phosgene (COCl₂) and electrophilic chlorine (Kadlubar and Hammons, 1987). This requires a concentration of oxygen above 5% and may be the dominant route of metabolism in hepatocytes incubated under an atmosphere of 95% oxygen.

There may not be a single mechanism of hepatotoxicity for the different reactive metabolites of CCl₄. There is evidence for the covalent binding of CCl₄ upon the bioactivation and both phosgene and electrophilic Cl⁻ may react with other macromolecules. The free radicals CCl₃• and •OOCCL₃ readily react with polyunsaturated fatty acids of the endoplasmic reticulum and other hepatocellular membranes to initiate the formation of organic lipid peroxides. Under aerobic conditions these organic peroxy radicals in turn can react with other polyunsaturated fatty acids to perpetuate the series of self-propagating chain reactions (propagation of lipid peroxidation) (Figure 1.9 above). Lipid peroxidation can be detected by the measurement of conjugated dienes within 5 min of CCl₄ administration.

Although CCl₄ intoxication is not marked by a depletion of GSH, a reduction in GSH *in vitro* is found (Berger *et al.*, 1986). This may reflect the inability of isolated cells to regenerate the GSH consumed by free radicals generated during lipid peroxidation (Poli *et al.* 1981) or phosgene-mediated cellular glutathione depletion in tandem with increased covalent binding to macromolecules (Mehendale, 1991).

The pathology of triglyceride accumulation, depression of protein synthesis

and polyribosomal disaggregation cannot be explained entirely on the basis of lipid peroxidation and covalent binding. It was postulated that 4-hydroxyalkenals produced by lipid peroxidation might act as "toxicological messengers" to other parts of the cell. However, experimental results did not bear out the hypothesis as the 4-hydroxyalkenals are rapidly detoxified by GSH (Recknagel, 1983). It was, however, demonstrated that the Ca^{2+} sequestering capabilities of the endoplasmic reticulum were impaired both by covalent binding and lipid peroxidation. A physiologically unacceptable increase in cytosolic free Ca^{2+} could follow resulting in the activation of enzymes such as the membrane bound enzyme phospholipase A_2 , which could disrupt the cytoplasmic membrane (Waller *et al.* 1983).

A disturbance in Ca^{2+} levels may explain the accumulation of intracellular VLDL as Ca^{2+} plays a regulatory role in α -tubulin polymerisation which forms the microtubules needed to transport VLDL out of cells (Recknagel *et al.* 1991).

There are other factors which have been identified as playing a part in the pathological effects of CCl_4 .

For example, triacylglycerol levels rise as a result of depressed activity of acid triacylglycerol lipase and enables triglycerides to accumulate (Kato and Nakazawa, 1987). There is also a direct solvent action of CCl_4 which may be overlooked in the initial stages of toxicity both *in vitro* and *in vivo* where comparable concentrations are found after dosing with CCl_4 (Berger *et al.* 1986 and 1987).

1.5.2. Thioacetamide

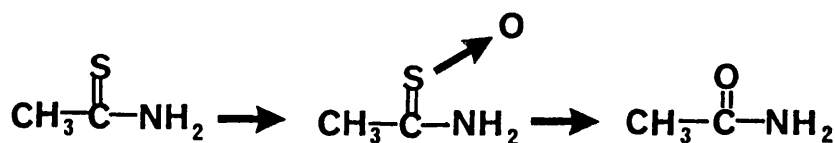
A single dose of thioacetamide leads to the necrosis of hepatocytes in the centrilobular (zone 3) region of the liver. Pre-necrotic changes include the loss of glycogen and acidophilic degeneration. Lipid accumulation also occurs in zone 3, but this is much less striking than the steatosis seen after CCl_4 administration. The endoplasmic reticulum swells and ribosomes become detached from the rough endoplasmic reticulum and as a result, protein synthesis is impaired (Trennery and Waring, 1983). There are also

marked nuclear changes 24 h after administration (Zimmerman, 1978).

Chronic administration of thioacetamide to rats produces bile duct carcinomas and liver cirrhosis (Gupta, 1956, Hunter *et al.* 1977 and Adnani, 1989).

Thioacetamide is rapidly metabolised by S-oxidation to thioacetamide S-oxide by a microsomal mono-oxygenase requiring NADPH and cytochromes P_{450} , which is then further oxidised to acetamide (Timbrell, 1991) (Figure 1.11).

Figure 1.11. S-oxidation of thioacetamide.

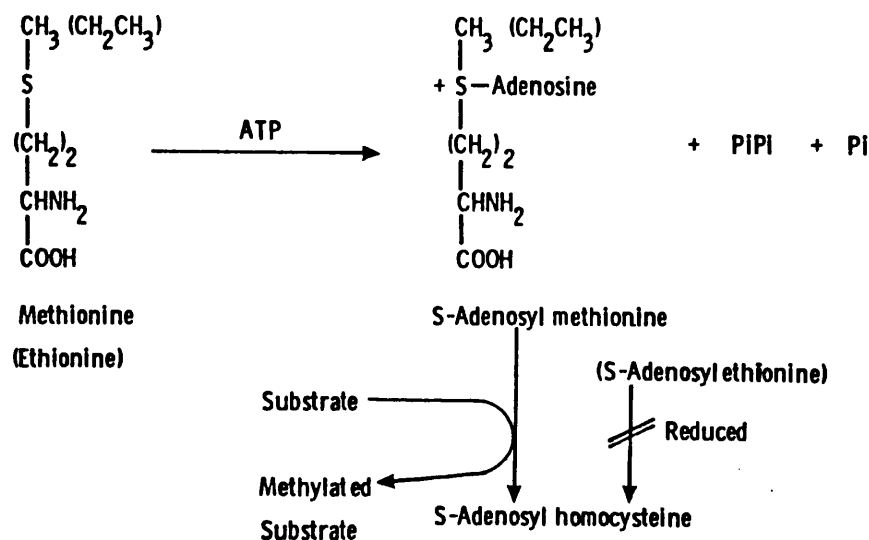


The sulphones are thought to produce hepatotoxic effects, possibly through covalent binding with macromolecules. A major protein adduct of thioacetamide has been demonstrated to be an ϵ -acetyl-lysine product (Dyroff and Neal, 1981 and 1983). The thioacetamide metabolite is thought to interfere with the movement of RNA from the nucleus to the cytoplasm resulting in an increased amount of higher molecular weight RNA in the nucleolus. Although these changes may help to explain the long term effects of thioacetamide toxicity, however, it is more difficult to relate these changes to the development of necrosis. In contrast to CCl_4 toxicity, lipid peroxidation does not appear to be associated with the acute toxicity of thioacetamide, although there are indications that it might be involved in the chronic toxicity (Muller *et al.* 1991). Membrane-stabilizing compounds such as antihistamines are reported to protect against necrosis (Judah *et al.* 1965) indicating that there may be an effect of thioacetamide on membrane integrity and ion flux.

1.5.3. Ethionine

Ethionine is a hepatotoxic analogue of the naturally occurring amino acid, methionine (Figure 1.12) (Timbrell, 1991). Acute doses produce steatosis in the liver and prolonged administration results in liver cirrhosis and hepatic carcinoma (Farber, 1967).

Figure 1.12. Incorporation of ethionine into S-adenosyl ethionine



Ethionine will replace methionine in S-adenosyl methionine to form S-adenosyl ethionine which is relatively inert as far as recycling the adenosyl moiety. This effectively traps adenosyl as S-adenosyl ethionine preventing the reformation of ATP, levels of which drop rapidly. This results in an inhibition of protein synthesis and therefore the apolipoprotein necessary to transport triglycerides out of hepatocytes. Additionally, ethionine causes ethylation of DNA, mRNA, rRNA and tRNA which will also inhibit protein synthesis. As a result, hepatic enzyme synthesis is disrupted affecting intermediary metabolism such as urea and GSH synthesis (McLean 1966 and Glaser and Mager, 1974).

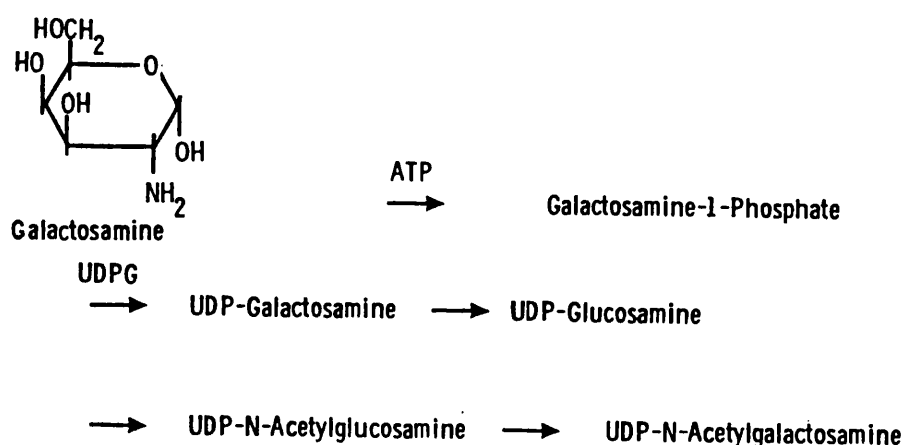
1.5.4. Galactosamine (GALN)

Single doses of galactosamine result in dose dependent hepatic damage which includes ballooning of cells, focal necrosis and periportal

inflammation which resembles viral hepatitis in man (Keppler *et al.* 1968). Fat usually accumulates in all hepatocytes. After repeated doses GALN may also cause chronic progressive hepatitis, cirrhosis and liver tumours.

Galactosamine hepatotoxicity results from its metabolism in the liver and the consequent effect on nucleic acid metabolism. It enters the pathway for galactose metabolism and combines with UDP to form UDP-galactosamine (Figure 1.13). Unlike UDP-glucose, which is produced by galactose metabolism, this cannot serve as a uridylylate donor in the uridylyltransferase reaction.

Figure 1.13. Metabolism of galactosamine.



UDP: uridine diphosphate; UDPG: uridine diphosphate glucose

Therefore, the formation of UDP-hexosamine from GALN is a metabolic "blind alley" which leads to the trapping of uridylylate (Zimmerman, 1978). If the rate at which UDP (and therefore UTP) is trapped, exceeds the rate at which the deficit can be made up various functional and structural defects follow. These include the depression of the synthesis of nucleic acids, membrane structures, glucose, lipids and a marked reduction in glycogen synthesis (Abdul and Mehendale, 1991). Both DNA and RNA synthesis is depressed since uridylylate is a precursor of both. Thus protein synthesis is inhibited.

The abnormal incorporation of hexosamines into membrane glycoproteins or glycolipids and the concomitant decrease in glucose and galactose incorporation may contribute to membrane damage and explain the high

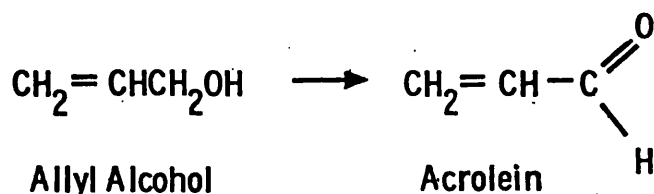
concentrations of calcium found in damaged tissue.

The injury resulting from GALN is more extensive than that produced by the administration of ethionine. It has been proposed that GALN produces hepatitis in experimental animals by a process which is mediated by a leukotriene D₄-dependent mechanism (Tiegs and Wendel, 1988).

1.5.5. Allyl alcohol

The administration of a single dose of allyl alcohol to rats inhibits mitochondrial respiration and protein synthesis and produces necrosis around the portal tracts in zone 1 (Patel *et al.* 1980). Allyl alcohol is oxidized to the toxic intermediate acrolein by liver alcohol dehydrogenase in a NAD⁺-dependent dehydrogenation, to the toxic metabolite acrolein (Figure 1.14).

Figure 1.14. The metabolism of allyl alcohol to acrolein.



It was accepted for sometime that the zonation of necrosis was due to the higher concentration of alcohol dehydrogenase in zone 1 (determined histologically) (Belinsky *et al.* 1984). However, this hypothesis is not supported by the observation that alcohol dehydrogenase activity, (determined microchemically), was greater in zone 3. Further studies have shown similar acinar distribution in all regions. Apparent explanations for these discrepancies are difficult to formulate since conflicting results are reported even in studies using the same methodology (Penttila, 1990). It now seems likely that there is rapid metabolism of allyl alcohol to acrolein in zone 1 and as these cells are the first to be exposed to the allyl alcohol necrosis takes place preferentially in this zone (Penttila, 1988).

The metabolism of allyl alcohol to acrolein is a prerequisite for toxicity. Thus inhibiting alcohol dehydrogenase with pyrazole or inducing aldehyde dehydrogenase with phenobarbitone abolishes allyl alcohol-induced liver damage (Jaeschke *et al.* 1987). Acrolein is a powerful electrophile which

spontaneously reacts with protein and non-protein sulphydryl groups resulting in the rapid depletion of glutathione in the liver. As raised levels of GSH protected the liver the depletion of GSH would seem to be crucial in the toxicity of allyl alcohol. There is also evidence that lipid peroxidation is an important factor in the toxicity of acrolein. This appears to be independent of any redox cycling mechanism and of cytochrome-P₄₅₀ and may result from the destruction of cellular defence mechanisms (Jaeschke *et al.* 1987 and Rikans, 1989).

1.5.6. Bromobenzene

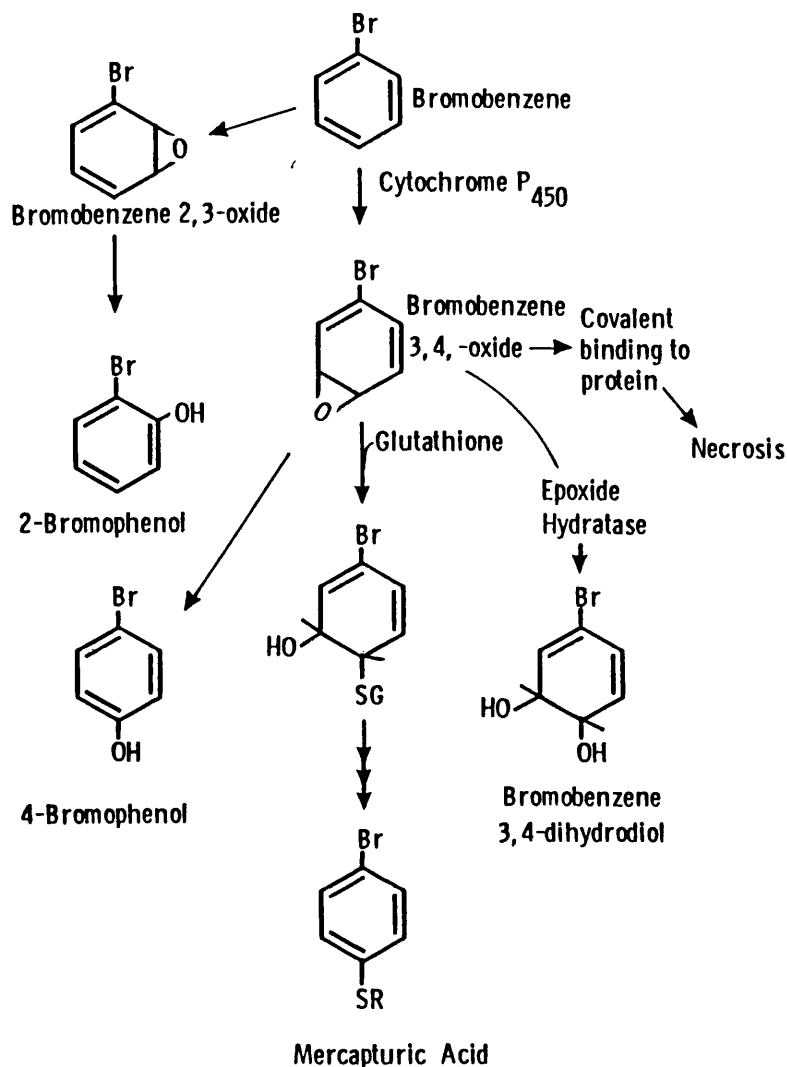
Bromobenzene causes centrilobular necrosis (zone 3) in experimental animals. It can also result in renal and bronchiolar injury. In the context of this thesis, it is important to note that although bromobenzene may alter some aspects of protein synthesis, this is not considered to be a primary cause of toxicity. This is in contrast to CCl₄ which results in a marked inhibition of protein synthesis (Davis *et al.* 1973).

Bromobenzene is another example of a compound which is toxic after metabolic activation to a toxic metabolite (Jollow *et al.* 1973). Metabolic activation is via two cytochrome P₄₅₀ pathways producing a 3,4-bromobenzene oxide (CYP2B) and a 2,3-bromobenzene oxide (CYP1A). The former oxide is believed to be primarily responsible for the hepatotoxicity (Figure 1.15.) (Lau and Zannoni, 1979 Monks *et al.* 1982 and Lindamood, 1991).

The removal of the toxic epoxide relies on conjugation with GSH, catalysed by glutathione-S-transferases. The glutathionyl conjugate is further metabolised and excreted as a mercapturic acid (Recknagel *et al.* 1991).

The toxicity of bromobenzene is only apparent when GSH becomes depleted. Thus a dose threshold for bromobenzene exists (Jollow *et al.* 1974).

The mechanism of bromobenzene-induced necrosis was thought to rely on covalent binding of the toxic metabolites to cellular macromolecules (Reid and Krishna, 1973). However, the correlation between covalent binding and necrosis was later found to be less significant than the correlation between lipid peroxidation and necrosis.

Figure 1.15. The metabolism of bromobenzene

The time course for the development of necrosis and lipid peroxidation was better correlated than covalent binding (Casini *et al.* 1982). It was also found that anti oxidants such as Trolox C were able to reduce both necrosis and lipid peroxidations to very low levels while covalent binding remained unaffected (Casini *et al.* 1985).

It has more recently been observed that mice treated with bromobenzene had raised levels of ALT 24 h after administration, even though there was very little lipid peroxidation (Casini *et al.*, 1987). This suggested that the later stages in the toxicity of bromobenzene might be caused by another factor. It has been suggested that the later toxicity is the result of an impairment in the transportation of Ca^{2+} across cellular membranes as a result of damage to protein thiols. In particular, the ability of mitochondria

to sequester cytosolic Ca^{2+} , a mechanism which can prevent cytosolic Ca^{2+} overload appears to be affected by bromobenzene metabolites (Maellaro, 1990).

1.5.7. α -Naphthylisothiocyanate (ANIT)

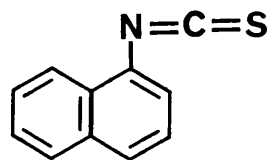


Figure 1.16. Structure of ANIT

Since ANIT was identified by Mazzanti and Lopez (1955) as a compound which produced bile duct proliferation it has been used mainly as an experimental tool for the production of intrahepatic cholestasis and experimental biliary cirrhosis (Zimmerman, 1978). A single dose results in swelling, vacuolization and necrosis of the epithelium of interlobular portal bile ducts, with desquamation of the necrotic debris into the lumens which apparently occlude them. Focal necrosis appears in the parenchyma, which is predominantly in zone 1. Biochemical alterations include hyperbilirubinemia, elevated alkaline phosphatase levels and hypercholesterolemia.

The cholestatic injury was thought to be dependent on the metabolism of ANIT by hepatic, cytochrome P_{450} -dependent mixed-function oxidases (MFO) as phenobarbitone enhanced the toxicity, while SKF 525A reduced it (Roberts and Plaa, 1965 and Plaa, 1970). Capizzo and Roberts (1970) confirmed that ANIT was metabolised in the rat and in 1971 they suggested that the differences in species susceptibility to the cholestatic effects of ANIT might be due to the different metabolising enzyme capabilities in the different species (Capizzo and Roberts, 1971).

The observation was also made that the inhibition of protein synthesis and/or RNA synthesis with compounds such as ethionine, actinomycin D and cycloheximide also reduced the toxicity of ANIT (Indacochea-Redmond *et al.* 1973 and Dahlstrom-King and Plaa, 1989) suggesting that the

synthesis of metabolising enzymes might be important in the toxicity of ANIT. However, the effects of these inhibitors of protein synthesis on the toxicity of ANIT might have been expected to last only as long as they are able to inhibit protein synthesis. This did not appear to be the case. For example, ethionine was effective in reducing ANIT toxicity even when given 24 h before the administration of ANIT. However, it was possible that the protein synthesis inhibitors were acting on specific proteins with a longer turnover time (Dahlstrom-King and Plaa, 1989). Dahlstrom-King and Plaa suggested that the proteins affected were those which acted as cytosolic carriers of bile salts and binding proteins on the canalicular membrane, rather than those involved in metabolic activation of ANIT.

Work by Dahm and Roth, however has led to an alternative suggestion for the toxicity of ANIT. They have pointed out that the compounds used to inhibit or induce MFO activity also alter hepatic glutathione (GSH) and/or GSH *S*-transferase activity. Thus, those which increase MFO activity also increase levels of hepatic GSH and GSH *S*-transferase activity. They confirmed the connection by reducing the toxicity of ANIT by depleting liver GSH with buthionine sulfoximine, diethyl maleate and phorone (Dahm and Roth, 1991). ANIT may reduce the efflux of GSH across the canalicular membrane, thus resulting in an elevation of hepatic GSH but a reduction in that available to be excreted in the bile. Carpenter-Deto *et al.* (1991) have proposed that ANIT forms a reversible GSH-conjugate which is rapidly transported into the bile. This would result in a high concentration of ANIT in the bile ducts where the initial morphological damage appears. It would also explain why parenchymal damage is initially in zone 1 rather than zone 3 which would have been expected if there was formation of a reactive metabolite via the MFO system.

There is ultrastructural evidence that damage to the bile duct lining cells occurs at an earlier time (4 h) and is more severe than damage to hepatocytes after dosing rats with ANIT. Raised γ -glutamyl transpeptidase was also seen after 4 h (Connolly *et al.* 1988). By 8 h after dosing the bile duct cells were clearly damaged, cells were exfoliating and bile ducts became damaged. The parenchymal damage seen 24 h after dosing was

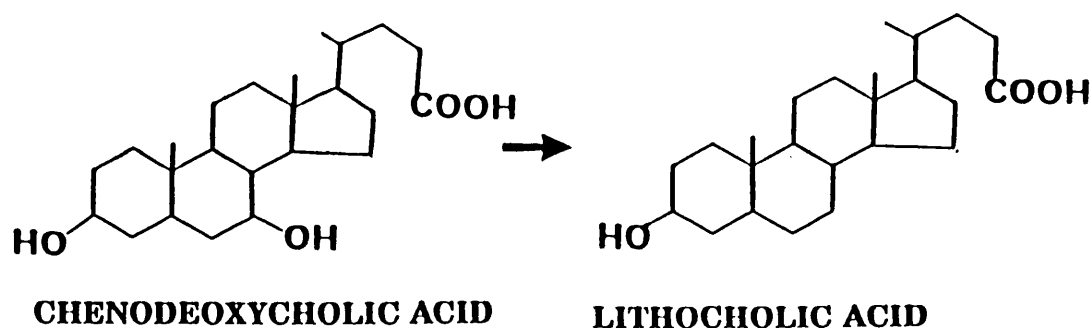
similar to that seen after ligation of the bile duct suggesting that hepatocellular injury is a result of the cholestasis rather than a cause.

The mechanism of ANIT toxicity is, therefore, still not resolved. It may result from the direct action of ANIT, in the bile, on the biliary epithelial cells, with the resultant blockage of the lumen causing the cholestasis. However, the possibility that ANIT is metabolised to a reactive metabolite by the MFO system remains and recent work has implicated neutrophils in ANIT toxicity (Dahm *et al.* 1991b).

1.5.8. Lithocholate

Lithocholate is a secondary bile salt and a natural cholestatic hepatotoxin formed by the action of colon bacteria on primary bile salts (Figure 1.17.)

Figure 1.17. The formation of lithocholate from the primary bile salt chenodeoxycholic acid.



The acute effects of lithocholate administration are a rapid development of cholestasis, dramatic changes in canaliculi and necrosis of hepatocytes. This was thought to be a result of precipitates of lithocholate forming in the canaliculus and changes of bile micelles (Zimmerman, 1978).

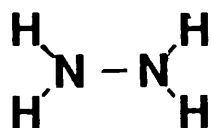
However, work carried out by Kakis *et al.* (1980) and Yousef *et al.* (1984) showed that lithocholate increased the cholesterol content of bile canalicular membranes, which would reduce the fluidity of the membrane and result in a reduced permeability to water and ions, leading to cholestasis.

There is also evidence that cholestatic bile salts act as Ca^{2+} ionophores and may result in a transient increase in cytosolic calcium (Vore, 1991).

Conjugation of lithocholate with taurine prevents cholestasis, increases the solubility of the bile salt and increases bile flow (Gaul, 1989).

1.5.9. Hydrazine

Figure 1.18. The structure of hydrazine.



A single dose of hydrazine to rats, produces a dose dependent accumulation of triglycerides in the livers of experimental animals (Scales and Timbrell, 1982). This may be due to an increase in phosphatidate phosphohydrolase activity which is thought to be involved in the regulation of triglyceride synthesis (Lamb and Banks, 1979 and Haghighi and Honarjou, 1987). However, another reason that there is an accumulation of triglycerides may be the early depletion of ATP which is seen both *in vivo* and *in vitro* after treating rats and isolated hepatocytes with hydrazine (Preece *et al.* 1990b), similar to the ATP depletion seen after treatment with ethionine. These two hepatotoxins are also similar in that they are reported to inhibit protein synthesis (Lopez-Mendoza and Villa-Travino, 1971). Amino acid levels are raised in animals following hydrazine treatment. This may be a result of protein synthesis inhibition, the inhibition of gluconeogenesis or due to the interference of hydrazine in pyridoxal-requiring transamination reactions (Korty and Coe, 1968, Cornish, 1969, and Lopez-Mendoza and Villa-Trevino, 1971).

Hydrazine is a carcinogen which can indirectly cause DNA methylation which may be involved in the carcinogenic process. It also results in a marked hyperglycaemia due to the mobilization of glycogen following the initial toxic insult which is then followed by hypoglycaemia as glycogen stores are depleted and gluconeogenesis is inhibited (Timbrell, 1991). The involvement of lipid peroxidation in the toxicity is uncertain. However, hydrazine does not produce hepatocellular necrosis either at very high doses or after multiple dosing.

1.6. Assessment of liver damage.

Experimental assessment of liver damage uses a selection of different measurements. These include lethality, histological changes observed by light and electron microscopy, chemical changes in the liver and physiological and biochemical tests that measure the functional status or that reflect the type or intensity of hepatic injury (Zimmerman, 1978).

a. Histological analysis of liver injury

Under experimental conditions it is essential to confirm the type of lesion which has been produced, and this can only be done histologically. It also enables qualitative assessment of various biochemical alterations to be made by the use of specific histochemical staining techniques. Quantitation of the degree of injury can be achieved by using a system of scoring the different types of cells identified (Mitchell *et al.* 1973a).

b. Serum enzyme measurements

Determination of hepatic enzymes released into the blood by the damaged liver is one of the most useful methods for assessing hepatic injury. Zimmerman (1978) classified these enzymes into four groups (Table 1.3.)

Table 1.3. Changes in serum enzymes due to hepatic injury, modified from Zimmerman (1978) and Plaa and Hewitt (1989).

ENZYMES	LOCATION	LESION AND COMMENTS
Group I ALP, 5'-NT, γ -GT and LAP	in hepatocyte membranes and bile ducts	markedly increased during cholestasis
Group II a. AST, MDH, LDH and ALD b. ALT, ICDH and GDH c. OCT, SDH and LDH ₅	High concentration in hepatocytes - in cytoplasm and mitochondria (LDH in cytoplasm) - also found in other tissues High concentrations in hepatocytes, located in cytoplasm and mitochondria (GDH in mitochondria) Exclusively in the liver - SDH and LDH ₅ are cytosolic, OCT - mitochondrial	All these enzymes are released during necrotic injury. Enzymes in group (a) are less specific and can reflect damage in other tissues eg. heart. Enzymes in group (c) may be particularly useful for studying agents with unknown hepatotoxic potential.
Group III CPK	Located in heart muscle	If present with AST indicates heart muscle damage rather than liver injury.
Group IV ChE	Synthesised in liver	Reduced serum levels in liver injury

ALP - alkaline phosphatase; 5'-NT - 5'-nucleotidase; LAP - leucine aminopeptidase; γ -GT - γ -glutamyltranspeptidase; AST - aspartate aminotransferase; MDH - malic dehydrogenase; LDH - lactate dehydrogenase; ALD - aldolase; ALT - alanine aminotransferase; ICDH - isocitric dehydrogenase; GDH - glutamate dehydrogenase; OCT - ornithine carbamyl transferase; SDH - sorbitol dehydrogenase; CPK - creatine phosphokinase and ChE - cholinesterase.

c. Biochemical measures of hepatic function

The ability of the liver to synthesise urea, cholesterol and plasma proteins, and to maintain blood glucose and amino acid levels enable dysfunction in these processes to be monitored in the serum. However, these measurements are not sensitive or specific enough to be used in isolation.

d. Hepatic excretory function

The ability of the liver to excrete foreign dyes has been used as a measure of hepatic function for many years. Both sulphobromophthalein (BSP) and indocyanine green (ICG) have been used. Their removal from the blood can be monitored and any reduction in the rate is indicative of reduced excretion into the bile. Raised levels of bile acids and bilirubin similarly indicate depression of hepatic function. These excretory functional tests are used more frequently in human studies (Zimmerman, 1978).

e. Chemical analysis of the liver

Direct chemical analysis of liver tissue can be useful in detecting and quantifying changes in hepatic metabolism. None of the hepatic function tests nor serum enzyme measurements are able to identify the accumulation of triglycerides in the liver but these can be measured directly. Lipid peroxidation can be detected by measuring malondialdehyde, expiration of ethane and pentane and the changes in cytochromes P₄₅₀ or GSH levels and the accumulation of Ca²⁺ may reflect other toxic effects of xenobiotics.

f. Urinalysis

Although an elevation of conjugated bilirubin in urine can be indicative of cholestasis, urinalysis is not normally a useful tool in the detection of liver damage. However, there are pathological conditions which result in the elevation of amino acids in the urine. A group of genetically inherited disorders known collectively as "primary aminoaciduria" such as hyperphenylalaninuria, tyrosinuria, cystinuria and Hartnup disease result either in a specific amino acid overflowing into the urine or a general aminoaciduria (Grant *et al.* 1987). "Secondary aminoacidurias" may affect many amino acids simultaneously. These include acute hepatic failure and acetaminophen poisoning. Milder forms of liver disease such as hepatitis and cirrhosis have been reported to result in a general aminoaciduria and some amino acids such as cystine, taurine, β -aminoisobutyric acid, methylhistidine and methionine were thought to be sensitive to minor liver

damage (Dent and Walshe, 1954). Some of these substances appeared to show greater elevation in the urine than plasma due to their rapid clearance (cystine, taurine and β -aminoisobutyric acid). Ethanolamine appeared to be related to the presence of hepatomas.

g. Summary

There are, therefore, a variety of techniques that can be used to assess liver injury, although no single technique is satisfactory for the detection and quantitation of all forms of injury. A variety of techniques are normally used in conjunction with each other. A major disadvantage of all these methods, however, is the fact that all, except urinalysis, require an invasive technique to be used. They also provide information only at a single time point. The possibility of validating a urinary marker of liver damage is therefore an attractive proposition.

1.7 TAURINE

1.7.1. Introduction

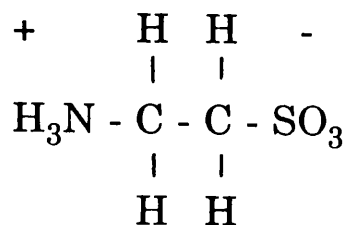
Although taurine was isolated from ox bile (*Bos taurus*) in 1828, interest in this compound only began to flourish after a comprehensive review on taurine was published in 1968, by Jacobsen and Smith. In 1978 the proceedings of the first International Symposium on taurine were published (Ed. Huxtable and Barbeau). Since then, there have been many symposia, numerous reviews and an escalating interest in this ubiquitous molecule covering most aspects of the Biological Sciences, with research being carried out in the Nutritional Sciences, Neurophysiology, Cardiology, Marine Biology and in Free Radical research (Hayes and Sturman, 1981, Chesney, 1985, Wright *et al.* 1986, Gaull, 1989 and Zelikovick and Chesney, 1989 and Huxtable, 1992).

1.7.2. Chemistry

"The biological functions of a compound are an inevitable consequence of its physicochemical properties" (Huxtable, 1982).

Taurine is a β -amino acid with the amino group on the second or β -carbon atom (Figure 1.19). Consequently, taurine is not incorporated into proteins but remains free in the cytosol (Wright *et al.* 1986 and Chesney *et al.* 1978) or bound to cellular structures (Huxtable and Bressler, 1972). It has a sulphonic rather than the carboxyl amino group, with a high acidic dissociation constant.

Figure 1.19. The structure of taurine.



With a pK_a of 1.5, a pK_b of 8.8 and an isoelectric point of 5.16, taurine is zwitterionic over the physiological pH range. This is in contrast to a

significant fraction of carboxylic acids which exist unionised over the same range (Huxtable, 1992). This results in taurine having a high water solubility but low lipophilicity. Consequently, cells such as Hela cells are able to concentrate taurine to 7,000 times the external medium concentration by actively transporting taurine into the cells without expending energy maintaining the concentration gradient (Huxtable, 1992).

1.7.3. Distribution

Taurine is found in millimolar concentrations throughout the animal kingdom and in algae, but appears to be absent from most plants and bacteria (Jacobsen and Smith, 1968). Cellular concentrations in mammals are normally in the range of 1 - 65 mM although plasma concentrations are much lower, in the order of 15 - 100 μM . Tissues which generate oxidants (neutrophils and retina), are rich in membranes (liver) or that are excitable (brain and muscle) have particularly high concentrations (Wright *et al.*, 1986). Liver concentrations of taurine vary more widely than other tissues; 0.6 - 9.2 $\mu\text{mol.g}^{-1}$ (Hirai *et al.* 1987) (Table 1.4).

Table 1.4. The distribution of taurine in some animal tissues.

TISSUE	MAN	RAT	CAT
	$(\mu\text{mol.g}^{-1} \text{ or } \text{ml}^{-1})$		
Plasma	0.08	0.45	0.11
Heart	6	27, 25, 30	14
Muscle	5	7, 15, 24	8
Brain	2	3, 5, 4	2
Testes	-	2	-
Lung	-	11, 13, 17	-
Spleen	-	14, 16, 19	-
Kidney	-	10, 11, 15	-
Liver	2	3, 0.6-9, 2, 7	3

Adapted from Hirai *et al.* 1987, Hayes and Sturman, 1981, Chesney, 1985, Huxtable and Lippincott, 1982.

Up to 50% of the total free amino acids in mammals is taurine, this being

an estimated 60 - 70 g in man and 0.15% - 1% body weight of a rat (Jacobsen and Smith, 1968 and Chesney, 1985). With such high concentrations of taurine in animal tissues, researchers have been endeavouring to find a central role for this molecule. It seems likely that, with the wide phylogenic distribution that it has, like γ -aminobutyric acid (GABA), it will be found to serve many functions (Huxtable, 1992).

1.7.4. Functions of taurine

The following are some of the actions attributed to taurine which were compiled by Huxtable in his recent review (1992).

Table 1.5. Some biological functions of taurine

Cardiovascular system	Retina
Antiarrhythmic	Maintenance of structure and function of photoreceptors, outer segments and tapetum lucidum
Positive inotropy at low calcium	
Negative inotropy at high calcium	
Potentialiation of digitalis inotropy	Liver
Antagonism of calcium paradox	Bile salt synthesis
Hypotensive (central and peripheral)	Xenobiotic conjugation
Retardation of lesion development in calcium overload cardiomyopathy	Reproductive system
Increased resistance of platelets to aggregation	Sperm motility factor
Brain	Muscle
Anticonvulsant and anti-tremor actions	Muscle membrane stabilizer
Modulator of neuronal excitability	General
Maintenance of cerebellar function	Attenuation of hypercholesterolemia
Thermoregulation	Stimulation of glycolysis/glycogenesis
Antiaggressive actions	Cell proliferation and viability
Central regulation of cardiorespiratory functions	Modulation of neurotransmitter and hormone release
Suppression of drinking and eating	Antioxidation
Resistance to anoxia/hypoxia	Regulation of phosphorylation
Altered learning and motor behaviour	

Of these various functions those relating to the protective properties of

taurine are the ones which will be of concern in this thesis.

1.7.5. Cysteine metabolism

Cysteine is incorporated into most proteins, glutathione and CoA and it is also a precursor for taurine. However the concentration of cysteine in tissues is usually maintained at a low level (10 - 100 μ M (Cooper, 1983). These low concentrations may be a protection against the high reactivity of cysteine. Reports of toxicity of cysteine *in vivo* (Sauberlich, 1961 and Olney *et al.* 1971) and *in vitro* (Higuchi, 1963 and Nishiuchi *et al.* 1976) suggest that higher concentrations of cysteine might be cytotoxic. Liver glutathione (GSH) has been suggested as a reservoir for cysteine. This is readily converted back to cysteine when cysteine levels drop (Higashi *et al.* 1983). However, protein synthesis will utilise cysteine preferentially to GSH but when there is a dietary excess of cysteine, simultaneous synthesis of protein and glutathione are supported. Under these circumstances taurine may also be synthesised (Figure 1.20).

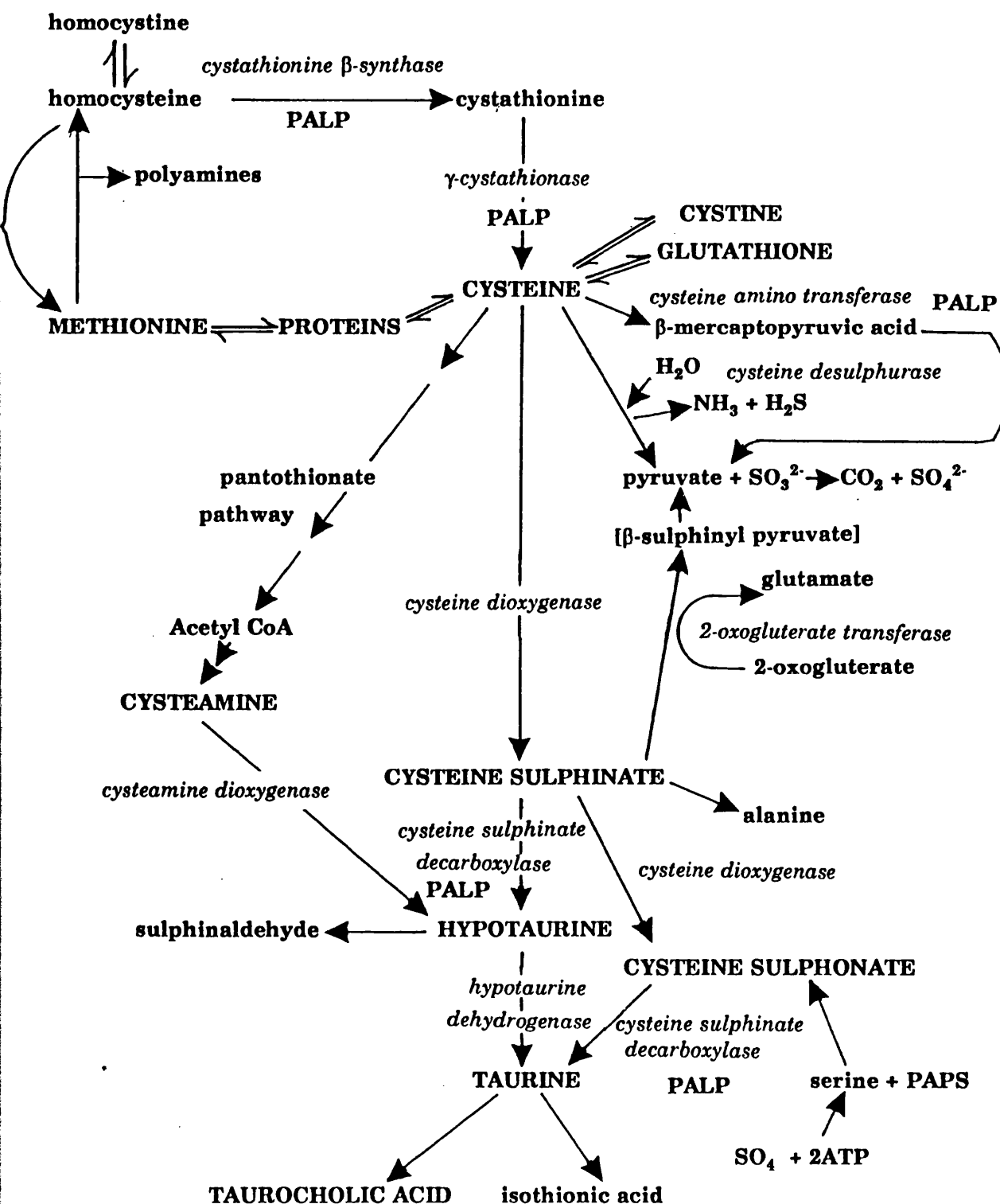
The partitioning of cysteine catabolism between the pathways leading to the final oxidation of sulphur has been the subject of much controversy.

The discrepancy appears to be between those who have worked with *in vitro* systems and those who have examined the problem *in vivo*.

Animal studies have suggested that 30% of cysteine catabolism occurs through the pyruvate pathway and 70% via the taurine pathway (Yamaguchi *et al.*, 1973). Later work by Griffith (1983) was basically in agreement with these results, demonstrating that slightly less cysteine sulfinic acid was transaminated (20%) and more decarboxylated (80%). Thus the taurine pathway seemed to be favoured over the pyruvate pathway.

However, Stipanuk (1979) demonstrated that rats receiving 0.2% cysteine in their diet resulted in less than 1% of the cysteine being converted to taurine and a 2.6% cysteine diet resulted in \approx 9% of cysteine converted to taurine.

Figure 1.20. Metabolic pathways involved in the synthesis of taurine



Modified from Hayes and Sturman (1981), Oja and Kentro (1983), Cooper, (1983) and Zelikovic and Chesney (1989).

[PALP - pyridoxal phosphate]

[PAPS - adenosine 3'-phosphate-5'-phosphosulphate]

Recent work by Yoshida *et al.* (1989) also indicated that cysteine catabolism proceeds preferentially to pyruvate. By decreasing the amount of cysteine administered to rats, sulphate excretion was reduced slightly whereas taurine excretion was reduced to 15% of the starting value. Administration of excess cysteine increased the excretion of both sulphate and taurine but sulphate excretion was increased proportionally more than taurine. They concluded that sulphate was formed from cysteine preferentially to taurine. The difference in these results has not been explained satisfactorily.

In rat hepatocytes, however, 50% of cysteine catabolism has been reported to take place through the cysteine sulphinate *independent* pathway resulting in the production of ammonia and pyruvate via 3-mercaptopyruvate. This may explain why Drake *et al.* (1987) only found that 10% of the cysteine incubated with hepatocytes was converted to hypotaurine and taurine, whereas incubations with cysteine sulphinate resulted in 80% of the cysteine sulphinate being catabolized to taurine. Later work from the same group failed to show any metabolism of cysteine to taurine in rat hepatocytes (Rosa *et al.* 1987).

The marked differences between the proportion of cysteine catabolism resulting in taurine and pyruvate production may be due to differences between isolated cell systems and the whole animal. Alternatively, the method used for assessing the relative importance of the different routes may be responsible for the discrepancies. Griffith and his co-workers used the production of labelled CO_2 to determine the amount of cysteine being metabolised to taurine (Griffith, 1983 and Weinstein *et al.* 1988), whilst Stipanuk and her colleagues measured taurine directly (Rosa *et al.* 1987).

The proportion of cysteine being catabolized to taurine remains unresolved. However, factors such as protein quality and sulphur amino acid levels are known to affect cysteine oxidation by altering the activity of cysteine dioxygenase more than the level of cysteine desulphurase and cystathionase (Stipanuk, 1979).

1.7.6. Taurine synthesis

For many years taurine was considered to be the chemically un-reactive end product of sulphur amino acid metabolism. The only chemical reaction it was known to take part in was the conjugation with bile acids (section 1.3.3.B2). Although this is an important function, only 1% of the body pool of taurine is believed to be involved in bile acid synthesis (Hepner *et al*, 1973). It is also able to conjugate with xenobiotic acids such as clofibrilic acid and 2-naphthylacetic acid (Emudianughe *et al*, 1983) and retinoic acid (Zelikovic and Chesney, 1989). More recent interest has been in the ability of taurine to form taurine chloramine by the chlorination of the amino group with hypochlorous acid (Wright *et al*. 1986). This has been suggested as a method of protecting cells against hypochlorous acid generated in cells such as neutrophils.

The synthetic capacity for taurine varies greatly between species. Generally, herbivorous animals are able to synthesise taurine, whereas, carnivores such as the cat and fox are less able to, as they do not have the necessary enzymes to carry out taurine synthesis. The rat has a remarkably high synthetic capacity for taurine (Worden and Stipanuk, 1985).

Cysteine is the main precursor for taurine although methionine can be metabolised to cysteine by a transulphuration pathway. The sulphur atom is progressively oxidised at the expense of free energy as the oxidation process in animals is not coupled to ATP regeneration.

Several possible pathways exist for the metabolism of cysteine to taurine (Figure 1.20) (Hayes and Sturman, 1981, Cooper, 1983, Zelikovic and Chesney 1983, Oja and Kontro, 1983, Wright *et al*. 1986, Huxtable, 1992).

Metabolic pathways:

- A. Cysteine can be oxidised by cysteine oxidase (cysteine dioxygenase, EC.1.13.11.20) to cysteine sulphinic acid (cysteine sulphinic acid) and then decarboxylated by the pyridoxal phosphate requiring enzyme, cysteine sulphinic acid decarboxylase (CSAD, EC.4.1.1.29) to hypotaurine. Hypotaurine is further oxidised by the poorly characterised enzyme hypotaurine dehydrogenase (hypotaurine :

NAD⁺ oxidoreductase, EC.1.8.1.3.).

- B. An alternative route for cysteine sulphinatate to follow is that to cysteine sulphonatate (cysteate or cysteic acid) via a further oxidation by cysteine sulphinatate dehydrogenase (cysteine dioxygenase EC. 1.13.11.20). Cysteine sulphonatate is then decarboxylated by cysteine sulphonatate decarboxylase (CSAD, cysteic acid decarboxylase), directly to taurine.

Both these pathways (A and B) have a requirement for pyridoxal phosphate.

- C. The other major route proposed for taurine synthesis is via cysteamine. Taurine and hypotaurine have been shown to be derived from cysteamine (Awapara, 1976), but no enzyme has been found to decarboxylate cysteine to cysteamine directly. It is therefore proposed that cysteamine is generated during the biosynthesis of CoA and phosphopantetheine. Pantothenic acid is joined with cysteine to form phosphopantothienoyl-cysteine which is decarboxylated and loses a phosphate group and is finally cleaved to form cysteamine. Cysteamine dioxygenase (E.C. 1.13.11.19) then metabolises the cysteamine to hypotaurine (Oja and Kontro, 1983).

These three pathways are considered to be the most likely routes for the production of taurine. However there appear to be differences between species as well as between organs over the most preferred route. It also seems likely that a great many factors such as the age, sex and nutritional status influence which route is preferred at any one time (Zelikovic and Chesney, 1989).

The key enzyme in these differences appears to be CSAD which is rate limiting in the synthesis of taurine.

CSAD activity is high in the liver and brain but low in the heart. Indeed, there are large differences even between the sexes of the same species such as the 9- to 16-fold differences in the CSAD activity observed in male and female rats, male rats having the higher activity (Worden and Stipanuk,

1985). Worden and Stipanuk also found very little CSAD activity in either guinea pig or adult cat liver. However cat liver was found to contain comparable amounts of taurine to rat liver while guinea pigs had only $0.5 \mu\text{mol.g}^{-1}$ taurine. Female rats also had higher concentrations of liver taurine than males. Clearly, there is no correlation between CSAD activity in the liver and the concentration of taurine found there (Loriette and Chatagner, 1978). Weinstein and Griffith (1987) have shown that CSAD exists as at least five isoenzymes in the liver of the rat and that these appear to be similar to those found in the brain. The presence of only 5% of the activity in female rats was not attributable to there only being one form of the enzyme present.

The relative importance of the two pathways involving cysteine sulphinate (A and B) in taurine synthesis does not appear to have been resolved. The concentration of cysteine sulphonate in mammalian tissues is low. Some studies suggest that there is a greater affinity of CSAD for cysteine sulphinate than cysteine sulphonate, which might explain the reason for the apparent preferred route for cysteine sulphinate through hypotaurine found by Stipanuk and Rotter (1984). However, Weinstein and Griffith reached a different conclusion, which was that there was equal affinity of CSAD for the two substrates (1987).

There is also controversy over the importance of the cysteamine pathway in the formation of taurine.

Vitamin B₆-deficient rats have been shown to reduce their urinary taurine excretion by 50% (Yamaguchi *et al.* 1975). However, these animals continued to excrete some taurine suggesting that although the cysteine sulphinate route had been inhibited, an alternative route was available for taurine production, such as the cysteamine pathway. By measuring the production of taurine in mice treated with the specific CSAD inhibitor, D-cysteine sulfinic acid, Weinstein *et al.* (1988) showed that only a small percentage, if any, of cysteine is converted to taurine via the cysteamine (phosphopantetheine) pathway. The suggestion was made by Griffith and his colleagues that the high levels of taurine found in heart and skeletal muscle (where CSAD activity is negligible) are not due to the cysteamine

pathway but are maintained by uptake of taurine from the plasma. Plasma taurine could be maintained by the CSAD-mediated taurine synthesis in other tissues. Studies by Huxtable and Lippincott (1982) have shown that both dietary and biosynthetic taurine are actively exchanged among tissues. Scandurra *et al.* (1978) believed the importance of the cysteamine pathway may be greater *in vitro*.

- D. A fourth route of metabolism has been proposed which utilises serine and inorganic sulphate. This is known as the inorganic route or PAPS pathway. Inorganic sulphate reacts with ATP to form adenosine 5'-phosphosulphate and 3'-phosphoadenosine 5'-phosphosulphate which in turn reacts with 2-aminoacrylic acid derived from serine to form cysteine sulphonate. This pathway has not been shown to be of any importance even though the enzyme system required is present in the liver. This pathway can be artificially induced by fasting rats or feeding pyridoxal or sulphur-amino acid deficient diets (Robeson and Martin, 1978). It has been suggested that this pathway may operate when other pathways are in some way impaired (Oja and Kontro, 1983).

1.7.7. Taurine metabolism

The possibility that taurine can be further metabolised to isethionic acid has received some attention by workers such as Read and Welty (1962) who measured the synthesis in dog heart slices. Later work by Huxtable and Bressler (1972) and Scandurra *et al.* (1978) in rats, demonstrated the synthesis of isethionic acid but were unable to determine if this had originated from taurine. The suggestion has been made that most catabolism of taurine is carried out by intestinal bacteria resulting in both isethionic acid and free sulphate being formed (Oja and Kontro, 1983).

1.7.8. Regulation of taurine pool size

The total body pool size of taurine appears to be regulated by the kidney (Chesney, 1985). Renal handling of taurine occurs via a group of transport

systems that also interact with other β -amino acids such as β -alanine. The affinity of the carrier for taurine is quite strong, but the capacity of the reabsorption system is low. Transport across the brush border membrane is a single high affinity Na^+ , and Cl^- (Br^-) dependent β -amino acid carrier. Transport across the antiluminal or peritubular membrane appears to involve two carriers, one with a high and one with a low affinity for taurine (Zelikovic and Chesney, 1989).

The renal adaptive response to diet is expressed at the tubular luminal membrane surface. When taurine concentrations are low uptake is increased in response to low taurine concentrations in the cortex; when taurine content of the diet is high, less taurine is reabsorbed. This adaptive response is apparently independent of the plasma concentration of taurine. Tissue concentrations of taurine and brain tissue in particular, appear to be very resistant to changes in taurine concentrations. Only liver concentrations vary significantly. When there is no taurine present in the diet, rats continue to synthesise taurine at the same rate but conserve more taurine via the kidneys (Huxtable and Lippincott, 1982). Taurine is also exchanged between tissues. Organs such as the liver, kidneys and pancreas appear to have a rapidly exchangeable pool of taurine, with a half life of < 1 day. Excitable tissues such as the brain, heart and skeletal muscle have a more slowly exchangeable pool of < 3 days (Sturman, 1973 and Hayes and Sturman, 1981).

Conservation of taurine also takes place during pregnancy in all mammals. The conserved taurine is transferred to the foetus where the synthetic capacity for taurine is low. During the early period of lactation a high taurine concentration is maintained in milk but this declines as the synthetic capacity of the offspring matures. The reabsorption mechanism of taurine across the basolateral surface of renal tubules also takes time to mature, therefore young animals exhibit neonatal "physiologic aminoaciduria" of which a high proportion is taurine (Kuo and Stipanuk, 1984 and Zelikovic and Chesney, 1989).

1.7.9. Urinary taurine

Urinary taurine levels are raised after the ingestion of food, particularly meat and sea food which contain high concentrations of taurine and after cold stress and restraint (Chesney *et al.* 1978). Some types of tissue damage such as severe burns, surgical trauma and radiation are also associated with raised urinary taurine (Turner and Brum, 1964 and Jacobsen and Smith, 1968). The raised levels seen after tissue damage are invariably attributed to the overflow of urinary taurine from damaged tissues (Chesney, 1985). However, work by Kay *et al.* (1957) already demonstrated that in the case of radiation damage it was more likely that increased levels of urinary taurine were due to alterations in sulphur amino acid metabolism. They found that between 1 and 11 h after irradiation levels of sulphate and protein degradation (measured by urea formation) were increased and urinary taurine was elevated 4 fold. Angel and Noon (1959) showed similar effects. They also demonstrated that the time of maximum taurine excretion varied with species, the rat and rabbit had a more rapid increase than either the guinea pig or dog.

Corticosteroids have been shown to increase levels of urinary taurine, probably through their stimulatory effect on cysteine dioxygenase (Yamaguchi *et al.* (1971). Cysteine administration also increases urinary taurine excretion, although the increase only accounts for a small fraction of the cysteine administered (10%) (Awapara, 1955). Yoshida *et al.* (1989) also measured the effects of cysteine administration on taurine levels with a view to using the measurements to determine the nutritional status of sulphur metabolism in mammals.

The idea that urinary taurine may be a useful indicator of protein quality has been explored by Yamaguchi *et al.* (1985) and Hosokawa *et al.* (1988). In these studies rats were fed different protein diets including a gluten diet. They were able to show the dramatic increase in urinary taurine produced in these rats was the result of a deficit of lysine, the first limiting amino acid in this protein source. Without lysine the other amino acids could not be used to synthesise proteins. The excess sulphur amino acids were catabolized to taurine. In soy protein and casein diets the sulphur amino

acids are the limiting amino acids, and consequently animals fed these diets had very low urinary taurine excretion.

The inhibition of taurine synthesis has been demonstrated by the administration of vitamin B₆ deficient diets to rats. The enzymes cystathionase and cysteine sulphinatase decarboxylase are both pyridoxal phosphate dependent enzymes (Figure 1.20). Thus the transsulphuration pathway providing cysteine from methionine and the formation of taurine from cysteine were inhibited. Urinary taurine excretion was greatly reduced (Sturman, 1973). This is believed to be the reason that the hydrazine compound, isoniazid, reduces urinary taurine. Hydrazines are known to inhibit pyridoxine requiring enzymes such as the transaminases (Chesney *et al.* 1978).

Other chemically induced changes in urinary taurine levels have also been documented. Work by Bowden and Goyer (1961) and Goyer *et al.* (1964) demonstrated that the skeletal muscle toxin, plasmocid, the myocardial muscle toxin, isoproterenol and radiation treatment all elevated urinary taurine to a similar extent. They concluded that as they could not detect any loss of taurine from the tissues, it was likely that all three treatments had resulted in an increase in sulphur amino acid oxidation. This increase was compared to the increase seen after colchicine administration to rats (Kostos and Kostos, 1961). The suggestion was made that protein catabolism may have been in some way connected with these observations. There may also be a connection between chemically induced muscle damage and the observation made by Bowden and Goyer (1961) that urinary levels of taurine are raised in some case of muscular dystrophy and polymyositis. Hepatotoxic compounds have also been observed to increase urinary taurine. Thus CCl₄ raises urinary taurine as part of a general aminoaciduria (Dent and Walsh, 1954 and Cornish and Ryan, 1964). These elevated levels were believed to be of the "overflow" type. Kocsis *et al.* (1968 and 1975) used this observation as an early sign of toxicity when assessing the extent to which dimethyl sulphoxide (DMSO) potentiated the effects of the hepatotoxic agents, toluene, benzene and chlorobenzene, all of which elevated urinary taurine. Interestingly, work by Yoshida and Hara (1985) demonstrated that

chlorobenzene resulted in a significant reduction in both hepatic taurine and urinary taurine which was thought to be due to the fact that chlorobenzene depletes glutathione as it is excreted as the mercapturic acid. As GSH and taurine compete for the same precursor, this would explain the reduction of taurine levels.

Sanins *et al.* (1990) demonstrated that the hepatotoxic compounds hydrazine, thioacetamide, α -naphthylisothiocyanate as well as CCl_4 increased urinary levels of taurine. They also demonstrated that allyl alcohol did not result in hypertaurinuria and concluded that this was probably due to the depletion of GSH which is a characteristic of allyl alcohol toxicity (Section 1.5.5.).

It was these observations of elevated levels of urinary taurine following the administration of hepatotoxic compounds which developed into the first investigations in this thesis. The aim being to determine whether changes in urinary taurine could be used to assess liver dysfunction.

1.7.10. The protective properties of taurine

A. Bile salts and osmoregulation

The best understood function of taurine, until recently, was its conjugation with bile acids and xenobiotics in the liver (Emudianughe *et al.*, 1983). Bile salts are either conjugated with taurine or glycine. However, if taurine is available, animals will preferentially use taurine for conjugation. Taurine conjugated bile salts are more soluble and less toxic, than their glycine-conjugated counterparts which may explain why taurine can prevent cholestasis induced by lithocholic acid in guinea pigs *in vivo* (Dorvil *et al.*, 1983). However, these conjugation reactions are a late evolutionary development and involve only a small fraction of the total body taurine (1-2%) in man.

A more general method of cellular protection may simply be a result of the high levels of taurine found in cells. These high cellular concentrations of taurine can be maintained as it is actively taken up into cells through a specific β -amino acid uptake system which is both Na^+ and Cl^- dependent.

However once inside cells its highly lipophobic properties prevent it leaving cells. As a relatively inert molecule, it can accumulate without affecting the metabolism of the cell and facilitate the well documented osmoregulatory role of taurine in marine invertebrates. Since amino acids play an important part in maintaining osmotic potential in all animal cells the relatively high concentration of taurine means that the contribution it makes to osmotic balance is that much greater.

There is also evidence that taurine plays an important role in osmoregulation in the mammalian brain. During hypernatremic dehydration free amino acids accumulate in the brain (a high percentage being taurine) this protects the tissue from water loss (Thurston *et al*, 1980).

Both the conjugation reactions and osmotic properties of taurine can be considered to be protective roles.

B. Taurine deficiency

It is difficult to establish the relative importance of taurine in cytoprotection as most animals conserve taurine very efficiently and are able to synthesise taurine if they do not obtain it from their diet (herbivores). However, the cat is unable to synthesise taurine. For this reason it is possible to deplete cats of taurine by maintaining them on taurine free diets. Indeed it was work carried out on cats that established taurine as an essential amino acid in these animals. Kittens born to mother's deficient in taurine have lower birth weights, grow more slowly than taurine supplemented kittens and have abnormal hind-leg development (Sturman, 1990). Both kittens and adults also suffer from retinal degradation and a decrease in the size of the tapetum lucidum. Cebus and cynomolgus monkeys are also poorly equipped to synthesise taurine and show a reduction in weight gain if they are fed a soya based infant formula devoid of taurine as infants (Hayes *et al*. 1980). Over a 20 week period these monkeys gained 46% and 29%, respectively, less weight than the taurine supplemented controls. They did not however demonstrate any tissue lesions. The authors of this work point out that if comparisons are to be made between these experiments and humans it

must be recognised that the monkey retina is more fully developed at birth than the human retina. In fact a more recent study of work with rhesus monkeys has shown early changes in visual acuity and retinal damage when infant monkeys are fed on human infant formula low in taurine (Sturman, 1990).

The importance of taurine in maintaining normal retinal function has now been recognised in man. Children receiving long-term parenteral nutrition have been shown to develop abnormal electroretinograms which became normal on the addition of taurine to the intravenous solutions (Geggel *et al*, 1985). A similar connection has been made in patients suffering from intestinal bacterial overgrowths. Taurine is not normally degraded in the body, however, bacteria are able to metabolise taurine and may be responsible for the small amount of isethionic acid which is found in urine or result in the conversion of taurine to SO_4^{2-} and NH_4^+ (Sturman *et al*, 1975 and Huxtable and Bressler, 1972). There is a pathological condition which results in both aerobic and anaerobic bacterial overgrowths in the small intestine. Under these conditions it is believed that taurine is degraded and lost from the body, resulting in reduced plasma levels of taurine. Patients with this condition were found to have abnormalities in cone function and pigment epithelium defects, a situation which could also be induced experimentally in rats (Sheika, 1981).

Experimentally induced reductions in retinal taurine levels in rats has proved difficult to achieve. Two conflicting reports have suggested that artificial reductions in retinal taurine using the taurine depleting agent, β -alanine, in the rat result in disruption of photoreceptor structure (Pasantes-Morales *et al*, 1983) while Lake and De Marte (1988) were unable to show an effect of either β -alanine on taurine levels in the retina or effects on the retina itself.

The development of dilated cardiomyopathy in cats is also attributed to taurine deficiency (Pion *et al*, 1987). In addition, myocardial taurine concentrations are raised during congestive heart failure in humans, dogs and rabbits (Huxtable and Bressler, 1974).

The correlation between reduced taurine levels and the development of pathological changes in both the eye and heart suggested that it had a protective role to play in both of these tissues.

Work on the protective role of taurine in the heart has centred on the modulation of calcium levels in the heart. Taurine has the ability to increase calcium levels in the hypodynamic heart but also protects it against calcium overload. It is believed that taurine achieves these modulations in calcium influx by enhancing calcium binding in the sarcolemma by stabilizing the membrane (Kramer *et al.* 1981, Pion *et al.* 1987 and Huxtable, 1987).

The observations of potential clinical manifestations of taurine deficiency have resulted in taurine being described as a "conditionally essential nutrient" (Gaul, 1989).

C. Antioxidant properties

The high concentrations of taurine found in cells which generate oxidants, such as the retina and neutrophils stimulated interest in taurine as a possible antioxidant. It is suggested that taurine reacts with hypochlorous acid produced in neutrophils to form the relatively stable taurochloramine which attenuates the toxicity of hypochlorous acid (Wright *et al.*, 1986 and Huxtable, 1992).

Other antioxidant properties include the reduction of malondialdehyde formation in carbon tetrachloride damaged liver and liver microsomes (Nakashima *et al.*, 1983) although it does not reduce lipid peroxidation induced by Fe^{2+} . It has also been shown to protect dogs against the acute effects of paraquat poisoning (Izumi *et al.*, 1989) which results in superoxide formation and to reduce pulmonary fibrosis produced by bleomycin which is believed to be caused indirectly by the generation of reactive oxygen species (Wang *et al.*, 1991). The prophylactic long term use of taurine has been demonstrated to reduce paraquat and bleomycin damage in hamsters (Gordon, 1991). Another example of the possible antioxidant properties of taurine are suggested by the observations of Gordon *et al.* (1986) who

reported the protection of hamster bronchioles from acute NO₂ damage by including taurine in the drinking water (0.5%).

D. Other protective properties

Investigations into the protective roles of taurine have not been confined to a cytoprotective role as there are also indications that it might be involved in hormonal regulation. Thus, taurine is reported to bind to the insulin receptor (Maturo and Kulakowski, 1988) which may explain the observation that taurine is a potent hypoglycaemic agent and able to prevent the hyperglycaemia induced by streptozotocin in mice *in vivo* (Tokunaga *et al*, 1979).

The protective properties of taurine *in vivo* have led to experimental work being carried out *in vitro* in an attempt to define these cytoprotective mechanisms of taurine. Numerous reports of the protective characteristics of taurine *in vitro* have been published as a result of this experimental work on isolated cell systems.

Studies in lymphoblastoid cells *in vitro* showed that the presence of extracellular taurine at a concentration of 5 mM protected against iron-ascorbate induced calcium accumulation and cytotoxicity. A higher concentration of taurine (20 mM) co-administered with zinc chloride (100 µM) protected lymphoblastoid cells against retinol and retinoic acid toxicity. The protection was believed to result from the stabilization of the membranes, preventing the flux of ions and water. Taurine did not however affect lipid peroxidation in these experiments (Pasantes-Morales *et al*, 1984 and 1985).

However, studies in hepatocytes *in vitro* have shown that taurine exerts a protective effect, decreasing lipid peroxidation due to oxygenation, in Ca²⁺ containing medium (but not Ca²⁺ free medium) (Nakashima *et al*, 1990). Banks *et al* (1990) have shown a protective effect of taurine against ozone exposure in rat alveolar macrophages (*in vitro*). An increase in cytosolic taurine was associated with a delay in more extensive cell damage following ozone exposure. The damage appeared to be lessened in alveolar

macrophages loaded with taurine prior to exposure with ozone.

Clearly, there is evidence for taurine having a protective role in animal tissues, although the mechanism(s) by which it is effective is not completely understood.

Evidence for a protective role for taurine in the liver has been confined to the detoxifying conjugation reactions with the subsequent elimination of conjugates in the bile (Emudianughe *et al*, 1983). The possibility that taurine might have other cytoprotective properties in the liver was therefore of interest and was investigated as part of the studies which follow.

Chapter 2

MATERIALS AND METHODS**2.1 MATERIALS**

Test-Combination Creatinine PAP diagnostic kit (No. 843270) and 2,4,6, tribromo-3-hydroxybenzoic acid were obtained from Boehringer Corp. Ltd., Lewes, East Sussex.

Coomassie Blue Reagent was supplied by Pierce and Warriner, Chester, UK. Methanol (HPLC grade) was provided by Rathburn, Wakeburn, Scotland, UK. Allyl alcohol was supplied by Aldrich Chemical Co., Dorset, UK.

[1,2-³H]Taurine (32 curies.mmol⁻¹) and L-[³⁵S]Methionine (1000 curies.mmol⁻¹) were obtained from Amersham International plc. Bucks. UK.

Aquasol universal cocktail was provided by Du Pont, Biotechnology Systems, D-6072 Drieich, W. Germany.

The following compounds were obtained from Sigma Chemical Co., Dorset, UK:

taurine (cell culture tested)	homoserine	Dowex resins
O-phthalaldehyde (OPA; HPLC grade)		creatinine
sarcosine	sodium arsenite	EGTA
Trypan blue	NADH	pyruvate
ATP	firefly lantern extract	dimethylsulphoxide
L-γ-glutamyle-p-nitroanilide	ammonium sulphamate	cysteine sulphinate
p-nitroaniline	DTNB	Zeolite
sodium metaperiodate	HEPES	bovine serum albumin
Triton	galactosamine	diethylmaleate
buthionine sulfoximine (BSO)	cycloheximide	thioacetamide
α-naphthylisothiocyanate (ANIT) hydrazine hydrate		sarcosine oxidase
4-aminoantipyrine	potassium hexanoferrate II	
Cholic acid (Na salt)	ascorbate oxidase lipase type VII-S	
creatine		
collagenase (No.C-0130, Type I, clostridiopeptidase A EC. 3.4.24.3)		

Other compounds were supplied by BDH Ltd., Pool, Dorset, England and include the following:

Sulphosalicylic acid	mercaptoethanol	glycylglycine
acetic acid	sodium nitrite	boric acid
Carbon tetrachloride	ethionine	phorone
sodium hydroxide (Aristar)		

All remaining compounds were supplied by Sigma or BDH.

2.2

IN VIVO STUDIES

2.2.1 ANIMALS AND TREATMENT

Rats of the Sprague-Dawley strain (Glaxo bred, 220 - 350 g) were used throughout. Animals were acclimatised (6-14 days) in communal cages after arrival and allowed food (rat and mouse maintenance cube diet, Quest Nutrition Ltd, Wingham, Kent, Code 691) and water *ad libitum*. During the different studies, animals were housed either in individual metabolism cages designed to separate and collect faeces and urine (Techmate Ltd., Milton Keynes, UK) or in communal cages, when urine was not being collected.

Animals used in studies were provided with the same diet, in ground form, to minimise the contamination of urine samples when they were housed in metabolism cages and water *ad libitum*. Animals placed in communal cages during the studies were given the same ground diet to minimise variations in urinary taurine levels due to different batches of food. They were kept in a temperature controlled room ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$). Lighting was controlled to give a regular 12 h light-dark cycle (light on 8 am - off at 8 pm).

Animals were dosed between 10.30 and 11.30 am, after a 5 day acclimatization period in the cages. Dosing was by the oral route whenever possible. However, as some compounds were not effective by this route they were given intraperitoneally (ip) or sub-cutaneously (sc). Animals were weighed daily and their food and water intake monitored, as was the general condition of the animals. Urine samples were collected over ice.

2.2.2 POST MORTEM PROCEDURE

Animals were killed between 10.30 am and 2.30 pm whenever possible; those animals from different treatment groups being alternated with one another to minimised variation due to diurnal changes.

Animals were anaesthetized with diethylether and exsanguinated from the abdominal aorta using a 0.8 x 40 mm needle. Blood samples were put into

Microtainers (from Becton Dickinson and Co., Rutherford, N.J. U.S.A.) for separation of serum (centrifuged 4,000 rpm, 15 min, 4°C). Serum samples were stored at -80°C until analyzed. Observations were made of the abdominal and thoracic viscera. The liver was removed immediately and weighed. Approximately 1.0 g of liver from the right lobe was frozen immediately (liquid nitrogen) and stored at -80°C for future taurine, total non-protein sulphhydryls (TNPSH) and triglyceride analysis. The left lobe was divided into two halves and placed in 10.5% (v/v) phosphate buffered formalin (pH 7.2, Pioneer Research Chemicals) for histological processing.

The kidneys, testes and heart were also weighed and placed in fixative for histopathology. The stomach was removed and cut to reveal the gastric mucosa, the contents washed out then it too was fixed.

2.2.3 LIGHT MICROSCOPY

Following fixation, tissues were trimmed and embedded in wax, 3 µm sections were cut and stained with haematoxylin and eosin. Additional sections were stained for glycogen with periodic acid Schiff's (PAS) reagent using diastase as a control. After examination of these sections, tissues were selected for lipid staining. Sections (10 µm) were cut from rapidly frozen, fixed tissues on a cryostat and stained using Oil Red O in triethyl phosphate. Mayer's haematoxylin was used as counter stain. An elastic Van Gieson and reticulin stain were used to show the deposition of collagen and fibrotic tissue in liver sections from animals treated chronically with CCl₄.

BIOCHEMICAL MEASUREMENTS

2.2.4 TAURINE

(A) SAMPLE PREPARATION

(i) Urine

All 24 h rat urine samples were diluted to 25 ml with UHQ water (human samples were treated undiluted) and centrifuged at 2,000 rpm for 10 min to remove hair and food debris. An aliquot (1 ml) was placed onto two previously

prepared ion exchange resins stacked one above the other (Appendix I). The first 1.0 ml of displaced water was discarded and the elution of taurine from the columns was completed using 4.0 ml of UHQ water in 0.5 ml aliquots. Homoserine (4 mM, rat - 1.0 ml, human - 125 μ l) was added to the resulting 4 ml of eluate as internal standard (Durkin *et al.* 1988) to give a final concentration of 0.8 μ mol.ml⁻¹ (rat) or 0.05 μ mole.ml⁻¹ (human) homoserine in the eluate. The eluate was used immediately for taurine measurement or stored at -80°C.

(ii) Liver

Sections of frozen tissue (-80°C) were weighed (0.35 - 0.45 g) into sulphosalicylic acid (4.0 ml, 0.2 M, 4°C) and homogenized using a "Polytron" tissue homogenizer. The homogenate was centrifuged (4,000 rpm, 10 min, 4°C), and 1 ml aliquots of supernatant were extracted for taurine on dual bed ion exchange columns (Appendix I) by elution with 2.5 ml UHQ water (5 X 0.5 ml). Homoserine was added to the 3.5 ml eluate as internal standard (125 μ l, 4 mM).

(iii) Serum

A 200 μ l sample of serum was deproteinated (200 μ l, 0.2 M sulphosalicylic acid, Connolly and Goodman, 1980), mixed, allowed to stand for 5 min (4°C) and centrifuged (4,000 rpm, 15 min). The supernatant was placed onto a dual bed ion exchange column and the first 200 μ l of eluate were discarded. The sediment was resuspended in 0.5 ml UHQ water and centrifuged again. The supernatant was added to the column and the eluate collected along with a further 0.5 ml UHQ water wash. The remaining taurine was eluted from the column (3 X 0.5 ml UHQ water). Homoserine was added as internal standard (100 μ l, 0.4 mM).

(B) ISOLATED HEPATOCYTES

(i) Incubation buffer (medium)

Medium (1 ml) was placed onto a dual bed ion exchange column and taurine eluted (5 x 0.5 ml UHQ water): Homoserine was added as internal standard (40-80 μl , 1 mM).

(ii) Cells and incubation buffer (medium)

A sample (1 ml) of suspended hepatocytes was added to sulphosalicylic acid (0.5 ml, 0.2 M, 4°C). Precipitated protein was removed by centrifugation (11,000 g, 5 min) and an aliquot (1.0 ml) of the supernatant added to a dual bed ion exchange column and taurine eluted with 5 x 0.5 ml UHQ water. Homoserine was added as internal standard (40-80 μl , 1 mM).

(Hirschberger *et al.* 1985)

(C) TAURINE DERIVATISATION AND ADDUCT MEASUREMENT

Taurine was measured as the fluorescent adduct formed with a derivatising solution of σ -phthalaldehyde (OPA) and mercaptoethanol in borate buffer (pH 8-9)(Lindroth and Mopper, 1979. The adduct was chromatographed using reverse phase chromatography on a 30 cm C_{18} $\mu\text{BondaPac}$ column (Stuart *et al.* 1979 and Neidle *et al.* 1988), isocratic elution with phosphate buffer (0.05 M, water/methanol, flow 2 $\text{ml}\cdot\text{min}^{-1}$, 2.5 - 4.0 X1000 psi) and Perkin-Elmer Tridet (fluorometric detection). Early results were obtained using a Waters 501 pump and Perkin-Elmer integrator attached but later data was collected using the Beckman "System Gold" and automatic sample table (Appendix II, method modified from Larson *et al.* 1980).

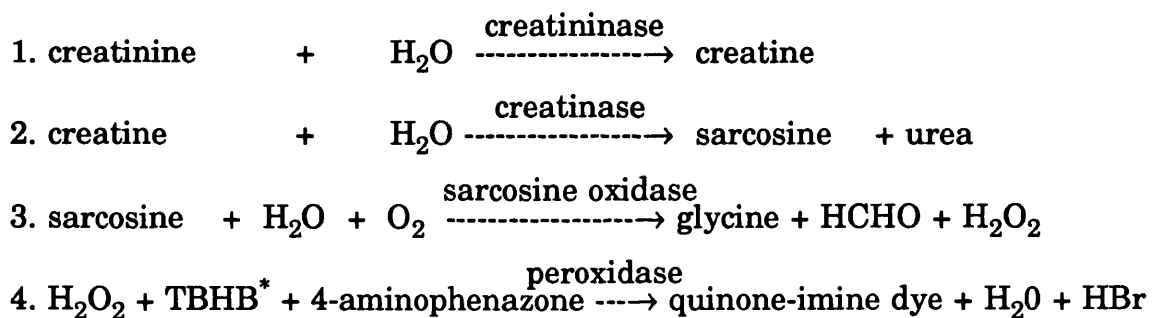
Results were expressed as -

- urinary taurine - $\mu\text{mol}\cdot\text{kg}^{-1}\cdot 24\text{ h}^{-1}$ in rats and $\mu\text{mol}\cdot 24\text{ h}^{-1}$ in humans
- liver taurine - $\mu\text{mol}\cdot\text{g}^{-1}$ wet weight liver or total $\mu\text{mol}/\text{liver}$
- serum taurine - $\mu\text{mol}\cdot\text{L}^{-1}$

2.1.5 CREATININE AND CREATINE

Creatine and creatinine were measured using the Boehringer Test-Combination Creatinine PAP diagnostic kit (No. 843270). This was originally carried out to enable urinary taurine to be expressed as a ratio with creatinine, to "normalize" results as urinary creatinine levels are related to muscle mass. However, some interesting changes in creatine excretion were noted during the treatment of the animals and therefore creatine quantitation was included as part of routine urinalysis.

The assay was an enzymatic colorimetric assay (Siedel *et al.* 1984), whereby creatinine was converted to creatine and then to sarcosine and oxidised to glycine, liberating hydrogen peroxide. This is then measured by its ability to generate a quinone-imine dye. By omitting the conversion of creatinine to creatine, creatine is also measured.



[*TBHB = 2,4,6-tribromo-3-hydroxybenzoic acid]

PROCEDURE

Stored urine (-80°C) was thawed at room temperature and diluted (1+20) with UHQ water and treated according to the manufacturer's schedule (incubated 20°C, 20 min). Results were expressed as $\mu\text{mol creatine or creatinine kg}^{-1} \cdot 24 \cdot \text{h}^{-1}$ (in rats) and $\mu\text{mol} \cdot 24 \cdot \text{h}^{-1}$ in humans, or as a ratio creatine:creatinine.

2.1.6 SARCOSINE

Urine samples which had high creatine concentrations were assayed for sarcosine to ensure that elevated levels were not due to the presence of sarcosine. This was carried out using the same reagent solutions used in the estimation of creatine and creatinine but omitting the enzymes creatininase and creatinase. A standard curve was prepared using sarcosine (40 - 300 $\mu\text{mol.L}^{-1}$).

2.2.7 PROTEIN DETERMINATION IN URINE

Protein was measured in urine as a marker of glomerular damage. Coomassie Brilliant Blue G-250 (Pierce Protein Assay Reagent) was used to estimate protein using 1:250 urine dilution and a micro assay procedure, the absorbence was read at 595 nm and compared to a standard curve prepared using bovine serum albumin (0-20 $\mu\text{g.ml}^{-1}$) (Macart and Gerbaut, 1982).

2.2.8 γ -GLUTAMYL TRANSPEPTIDASE (γ -GT)

γ -GT was measured in urine as a marker of kidney tubular cell damage. It is a membrane bound enzyme and most abundant in kidney tubular cells. It was measured according to a modification of the method by Naftalin (1969) whereby p-nitroaniline was liberated during the transfer of the glutamyl residue from γ -glutamyl p-nitroanilide to the acceptor dipeptide glycylglycine by γ -GT. The amount of p-nitroaniline released was proportional to the activity of enzyme present.

1. L-glutamyl-p-nitroanilide + glycylglycine $\xrightarrow{\gamma\text{-GT}}$ p-nitroaniline + glutamylglycylglycine
2. p-Nitroaniline + HONO \longrightarrow diazo compound
(HONO was formed from NaNO_2 and acetic acid)
3. Diazo compound + N-(1-naphthyl)ethylene-diamine \longrightarrow pink azo dye

Azo dye - λ_{max} 550 nm.

The absorbance was directly proportional to the quantity of γ -GT present. A unit of γ -GT was defined as the activity required to release 1 μ mol of p-nitroaniline at 37°C in 20 min. A standard curve was prepared using p-nitroaniline.

Units were defined as 1 iu = 1 μ mol p-nitroaniline released from L- γ -glutamyl-p-nitroanilide per min at 37°C. (Appendix III).

2.1.9 LIVER TOTAL NON-PROTEIN SULPHYDRYL GROUPS (TNPSH) DETERMINATION

Glutathione (GSH) is an important scavenger of reactive electrophiles and peroxidative agents protecting the liver from these compounds (Jollow *et al.* 1974). Since taurine and GSH share the same precursor, cysteine (Figure 1.20) any perturbation in cysteine or GSH metabolism due to dietary factors or the toxicity of foreign compounds, may affect taurine levels (Higashi *et al.* 1983). Therefore, most *in vivo* studies included the measurement of liver TNPSH as a measure of GSH, (GSH comprises >95% of the TNPSH pool in the rat liver, DeMaster and Redfern, 1987). Determinations were made in liver extracts prepared for taurine analysis (see 2.2.4 A.ii), diluted (50:50 with phosphate buffer, pH 7.4) and assayed (0.5 ml) using a method modified from Ellman (1959) (Appendix IV).

2.2.10 TRIGLYCERIDE DETERMINATION

Liver triglyceride levels were measured in samples removed at *post mortem* and stored at -80°C, using the Butler *et al* (1961) adaption of Van Handel and Ziversmit's method. The method was followed with the following modifications:-

- a. Liver (0.4 g) was homogenized in phosphate buffer (0.1 M, pH 7.0, 4°C) using a Polytron tissue homogenizer.
- b. Saponified samples were prepared in triplicate and unsaponified samples in duplicate.
- c. Unknown triglyceride concentrations were calculated using a standard curve (10-100 μ g commercial corn oil).

d. Sodium arsenite (1.0 M, 0.2 ml).

For details of the method see Appendix V.

2.2.11 SERUM BIOCHEMISTRY

Blood was taken from the abdominal aorta from rats whilst they were under diethyether anaesthesia. Whole blood was put into "Microtainers" (Becton Dickinson) designed to separate serum from whole blood. Samples were allowed to clot at room temperature (45 min) then centrifuged (4,000 rpm, 15 min, 4°C) and frozen (-80°C) until analyzed.

Standard clinical biochemical analysis of serum samples was carried out at Glaxo Research, Ware.

These analyses included the measurement of-

Serum transaminases - aspartate transaminase (AST)

alanine transaminase (ALT)

Alkaline phosphatase (ALP)

Albumin

Total bilirubin

Cholesterol

Total protein

Glucose

Blood urea nitrogen (BUN)

Creatine kinase

Serum creatinine

Triglycerides

Calcium

Phosphate

All samples were assayed using a Hitachi 705 auto-analyzer and the appropriate kit supplied by Boehringer.

2.3. IN VITRO STUDIES

Isolated hepatocytes were used in suspension in an effort to extend and explain some of the *in vivo* data.

2.3.1 ISOLATION OF RAT HEPATOCYTES

The technique used was essentially the two step perfusion method of Moldeus *et al* (1978). It relied on the perfusion of liver *in situ* in an anaesthetized rat with two recirculating balanced salt solutions (Appendix VI). The first was Ca^{2+} free and contained the Ca^{2+} chelating agent, EGTA. This removed the Ca^{2+} that maintains the protein structure of intercellular filaments between desmosomes of the tight junctions between cells (Alberts *et al.* 1983 and Berry *et al.* 1991). The second solution contained collagenase in a solution in which calcium was present (4 mM Ca^{2+}) to enable the collagenase to work effectively. The perfusion with collagenase completed the biochemical separation of the hepatocytes from one another enabling the mechanical separation to be completed.

(A) PERFUSION APPARATUS

Figure 2.1 shows the perfusion apparatus used to enable the isolated liver to be perfused with carbogenated (95% O_2 /5% CO_2) recycling perfusate buffer. The oxygenator had four openings such that perfusate could be pumped into the top and be gassed through a side arm, a controlled flow of buffer was able to leave through the bottom and the excess flowed out to be recycled. The oxygenator also acted as a bubble trap.

The cannula had a filed tip and a shallow groove filed around it 0.4 mm from the end to enable a ligature to be tied securely around the cannula to hold it in the portal vein. Tall-form beakers (250 ml) were used for the perfusate and to hold the cannula on its support. The beaker was held in a water bath at 38-39°C. The fluid in the manometer was adjusted to a height of 40 cm above the bench and the flow of perfusate regulated to 2 drops. sec^{-1} .

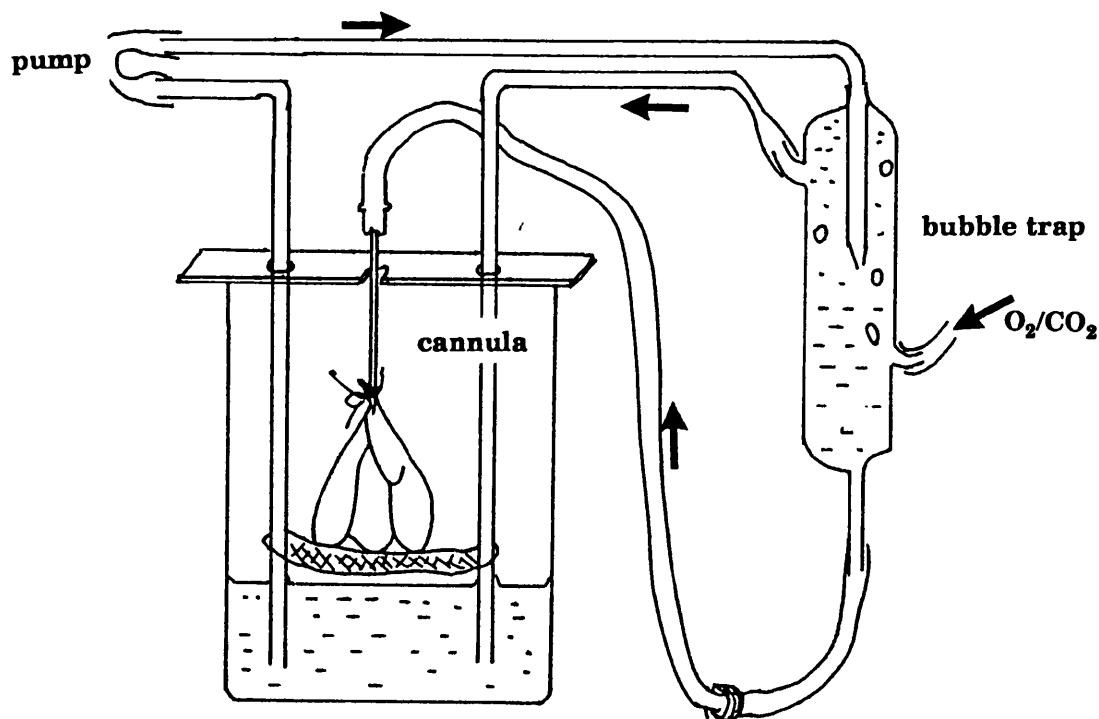


Figure 2.1 Perfusion apparatus used for the perfusion of a rat liver with recirculating balanced salt solutions.

(B) ANIMALS

Male Sprague Dawley rats (Glaxo bred, 240-340 g) were used. Animals were allowed to acclimatize for at least 7 days after arrival and were fed rat and mouse maintenance diet (see 2.2.1) *ad libitum*. Isolation of cells was carried out between 10.30 and 11.30 am.

(C) SURGICAL PROCEDURE

Rats were anaesthetized with ether and the abdominal cavity opened by a "V" shaped incision beginning at the base of the abdomen and extending slightly posterior to the diaphragm. The viscera were deflected to the right to expose the hepatic portal vein. A loose ligature was tied around the portal vein, 1.0 cm below the point of bifurcation into the liver, a small cut was made with a pair of sprung scissors and the cannula inserted. The ligature was secured and the cannula clip opened to clear the liver of blood, at the same time the hepatic artery and vein were cut to prevent excessive swelling of the liver.

(D) PERFUSION AND WASHING PROCEDURE

A pale liver with no blotches was indicative of a successful perfusion. The flow of perfusate was reduced to a slow trickle and the liver was dissected out and transferred to the Hank I reservoir. Perfusion with Hank I was continued for a further 5 min.

Collagenase (50 mg) was added to 100 ml Hank II (containing Ca^{2+} but no albumin) and allowed to settle before mixing. The cannula clip was opened and excess Hank I drained out of the oxygenator until it was almost empty then the rack supporting the liver was transferred to the Hank II buffer. The flow of buffer was adjusted to 2 drops a second and the perfusion continued for 12-15 min until the liver lost its resilience when palpated gently.

The liver was cut from the cannula and put into Krebs-Henseleit + albumin (50 ml, ambient temperature) buffer (Appendix VI) in a shallow glass dish. The capsula was broken and the cells dispersed by gentle "combing" with blunt forceps. The crude suspension was filtered through a polyamide tea strainer into a 100 ml conical flask.

The cells were centrifuged in 2 x 50 ml centrifuge tubes and spun (1 min, 4°C, 250 rpm), the supernatant was aspirated off and the tubes filled with Krebs-Henseleit (K+H, no albumin) and rocked gently to wash the cells. These were spun again, resuspended and washed once more. The final cell pellet was made up to a final known volume and the viability and cell yield estimated using Trypan-blue (see 2.3.2a).

(E) INCUBATION

Cells were incubated (37°C) at a concentration of 2×10^6 cells ml^{-1} K+H (20 ml total volume) in siliconised ("Repellcoat") round-bottomed flasks (100 ml) under a continuous stream of carbogen on a rotary evaporator (30 rpm). Cells were pre-incubated (45 min) before treatment to allow for recovery then treated for 3-4 h.

Alternatively, cells were incubated in sealed 25 ml Erlynmyer flasks fitted with a centre well to allow the delivery of volatile compounds to the cells. These incubations were limited to 45 min and carried out in a shaking water bath (37°C, 30 oscillations. min^{-1}).

2.3.2 ASSESSMENT OF VIABILITY OF ISOLATED HEPATOCYTES

(A) TRYPAN BLUE UPTAKE

Procedure

The initial estimates of isolated hepatocyte viability were measured using the inability of damaged cells to exclude the dye, Trypan blue (0.4%).

Trypan blue - 0.4% in Dulbecco formula A phosphate buffer, was filtered and stored (-20°C) in 5 ml aliquots. This was filtered again (0.22 µm membrane filter) and kept cold (4°C) just prior to use.

An aliquot (50 µl) of cell suspension was mixed with Trypan blue (450 µl) to estimate initial cell density and viability. Cells were counted in a Neubauer haemocytometer. Both total cell and viable cell counts were made in the central gridded area on both sides of the cytometer (0.1 mm³).

Density = viable cells x 10⁵ cells.ml⁻¹ of suspension

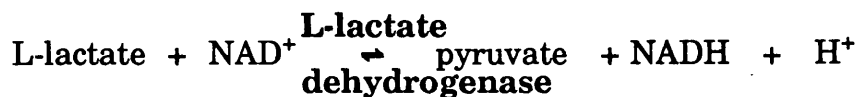
Yield = Density x total suspension vol

Four separate slides were prepared and counted. Preparations showing >87% viability by this method were used and diluted to a concentration of 2 x 10⁶ cells.ml⁻¹ with Krebs-Henseleit Buffer (Appendix VI). Once cells were diluted for incubation, Trypan blue solution and cell suspension were mixed 50:50.

(B) LACTATE DEHYDROGENASE (LDH) LEAKAGE

The activity of the cytosolic enzyme, lactate dehydrogenase, was measured in the suspension medium of hepatocytes as a marker of cell membrane integrity. The method was a modification of Bergmeyer (1965).

The assay is based on the reversible reduction of pyruvate to lactate, the last step of anaerobic glycolysis.



The quantity of enzyme in the medium (20-50 µl) was estimated by measuring the rate of the decrease in optical absorbance at 340 nm when NADH was oxidised to NAD⁺, in a final volume of 3 ml. (Appendix VII).

(C) ATP DETERMINATION

ATP was measured in isolated hepatocytes as a measure of cytotoxicity. The assay was based on the detection of luciferase-linked bioluminescence (Stanley and Williams, 1969) in TCA extracts of hepatocytes, using a firefly lantern extract in the presence of ATP. An aliquot (0.5 ml) of cell suspension was put into ice cold trichloroacetic acid (TCA), frozen (-80°C) and analysed for ATP within a week. Samples were thawed on ice (4°C) and centrifuged (11,000 g, 5 min) to precipitate protein. The supernatant was assayed for ATP using firefly lantern extract. (Appendix VIII).

2.4 STATISTICAL ANALYSIS OF RESULTS

Measurements of urinary taurine and creatine were compared in urinary samples from the same animal, pre-dose values being compared with post-dose values statistical significance was determined using a paired "t" test.

For comparison of a value from a single treated group with a control group (ie. liver taurine concentration) Student's "t" was used.

When one control group was compared to two or more treated groups Dunnett's test for multiple comparisons with a single control (Dunnett, 1964) was used. Analysis of variance was used to analyze the effect of pre-treatment on the dose response to carbon tetrachloride, modified to take into account any interactions between treatments.

Significance was taken as $p < 0.05$ or less, and denoted on figures as -

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$ unless specifically stated otherwise.

URINARY TAURINE AS A MARKER OF LIVER DYSFUNCTION

3.1

INVESTIGATIONS INTO THE EFFECTS OF VARIOUS HEPATOTOXIC COMPOUNDS ON URINARY AND LIVER TAURINE LEVELS IN RATS

3.1.1 INTRODUCTION

The following investigations were carried out to confirm and extend the observations of Sanins *et al* (1990) that single doses of the hepatotoxic compounds hydrazine, carbon tetrachloride, thioacetamide and α -naphthylisothiocyanate (ANIT) resulted in an elevation of urinary taurine. Sanins *et al* (1990) measured taurine by proton NMR and demonstrated that there was an association between the increase in urinary taurine and raised serum AST and ALT levels, except in the case of hydrazine which did not elevate enzyme levels. However, hydrazine resulted in a marked steatosis which was identified by staining liver sections with Oil red O. The elevation of urinary taurine was shown to be dose dependent in the case of hydrazine.

3.1.2. AIM OF STUDY

The aim of the following investigations was to demonstrate the feasibility of using changes in urinary levels of taurine as an indicator of liver dysfunction measuring taurine by the more sensitive and accessible technique of HPLC.

An initial study was carried out to determine the effect of restricting the food intake on urinary taurine levels in rats (male, Sprague Dawley). Sanins *et al* reported an elevation of urinary taurine in rats starved for 24 h but other research has shown that a restricted diet lowers urinary taurine (Chesney, 1985). Clearly, there was a need to establish the effect of reduced

food intake on urinary taurine levels in the strain of rats to be used in these studies as administration of some of these compounds resulted in reduced food intake in the animals. The long term aim of all of the following investigations in this chapter was to establish whether changes in urinary taurine could be used as a marker of liver disfunction over a period of time greater than 24 h. Under Home Office regulations it is not possible to starve animals for longer than 24 h nor was it felt good practice to provide animals with a normal calorific intake in the form of sucrose solution (Jaeschke *et al*, 1987) since this would result in the alteration of levels of endogenous compounds such as glutathione as well as taurine. Removing food from animals may also have resulted in the animals becoming stressed. Increased urinary taurine is reported to occur during various forms of stress associated with the release of ACTH from the pituitary (Hayes and Sturman, 1981).

3.1.3 METHODS

(A) *Effect of restricted food intake on urinary taurine.*

Sixteen male rats (230-275 g) were allocated to 4 groups of 4 animals each and housed individually in metabolism cages. They were provided with food *ad lib* for 24 h. Each group of animals was then given food, estimated from their previous 24 h consumptions to be 100% (25 g), 80% (20 g), 60% (15 g) and 40% (10 g) of the normal intake (23 ± 3 g). Body weight, food and water consumptions and urinary taurine levels were recorded for each animal 24 h before and for 72 h after restricting food intake. The taurine content of the diet was determined using an extraction process the same as that used for tissues.

(B) *Effect of hepatotoxic compounds on levels of urinary taurine.*

Male rats (250-300 g) were housed in individual metabolism cages for 8 days. They were allowed to acclimatize for 3 days before consecutive 24 h

urine collections were made 2 days before and after dosing (-48 → -24 h, -24 → 0 h, 0 → 24 h and 24 → 48 h). In the case of ethionine treatment, this was extended to 72 h.

Dosing was carried out on day 6 between 10.30 and 11.30 am. All dosing was by gavage (p.o.) unless the compound had been found in preliminary studies to be ineffective by that route in which case the compound was administered intraperitoneally (i.p.). Groups of 4-5 rats were given the appropriate vehicle only, as control. All doses were given in a total volume of 2 ml.kg⁻¹. A summary of doses given is shown in Table 3.1.

TABLE 3.1

Hepatotoxic compounds - doses and routes of administration

COMPOUND	ROUTE	DOSE (kg ⁻¹)	VEHICLE	N° OF ANIMALS
CARBON TETRACHLORIDE	p.o.	2 ml	corn oil	5
HYDRAZINE	p.o.	40 mg	saline	3
α-NAPHTHYL-ISOTHIOCYANATE	p.o.	150 mg	corn oil	3
ALLYL ALCOHOL	p.o.	0.1 ml	saline	3
		0.075 ml	saline	4
THIOACETAMIDE	p.o.	150 mg	saline	3
BROMOBENZENE	p.o.	1 ml	corn oil	4
GALACTOSAMINE	i.p.	400 mg	saline	4
		500 mg	saline	4
ETHIONINE	p.o.	800 mg	saline	4
LITHOCHOLATE	p.o.	26 mg	saline	4

For details of dose solutions see Appendix IX

On day 8, 48 h after dosing (72 h after ethionine), animals were anaesthetized and exsanguinated from the abdominal aorta, tissue samples taken and analyzed as described in Section 2.2.2.

3.1.4 RESULTS

(A) *The effect of restricted food intake on urinary taurine excretion in male rats.*

Taurine excretion was reduced in male animals provided with less than 100% of their estimated daily food intake, the differences were significant when the food consumption was 40% of the normal food intake (Table 3.2).

TABLE 3.2 Urinary taurine levels in male rats on restricted diet.

	% TOTAL DIETARY INTAKE			
	100%	80%	60%	40%
MEAN URINARY TAURINE $\mu\text{mol.24}^{-1} \text{ kg}^{-1}$	650 \pm 77	521 \pm 57	474 \pm 50	456 \pm 72*

Values, mean \pm SEM, n = 4; * p< 0.05

(B) *The effects of hepatotoxic compounds on levels of urinary taurine.*

(i) **Clinical signs of toxicity after dosing with hepatotoxic compounds**

All groups of animals showed an increase in bodyweight before dosing. After dosing, animals treated with bromobenzene, ANIT and thioacetamide and the higher doses of galactosamine and allyl alcohol showed a significant weight loss (Figures 3.1, 3.2 and 3.3). This weight loss could be accounted for by the reduction in food consumption by these animals, following dosing. Only two animals showed clinical or histological signs of toxicity due to the lower dose of galactosamine (400 mg.kg⁻¹). Therefore, the clinical and taurine data presented here are from two rats where galactosamine toxicity was demonstrated. One animal treated with 0.1 ml.kg⁻¹ allyl alcohol died 1 h before post mortem, therefore clinical data is presented for the two remaining animals at this dose.

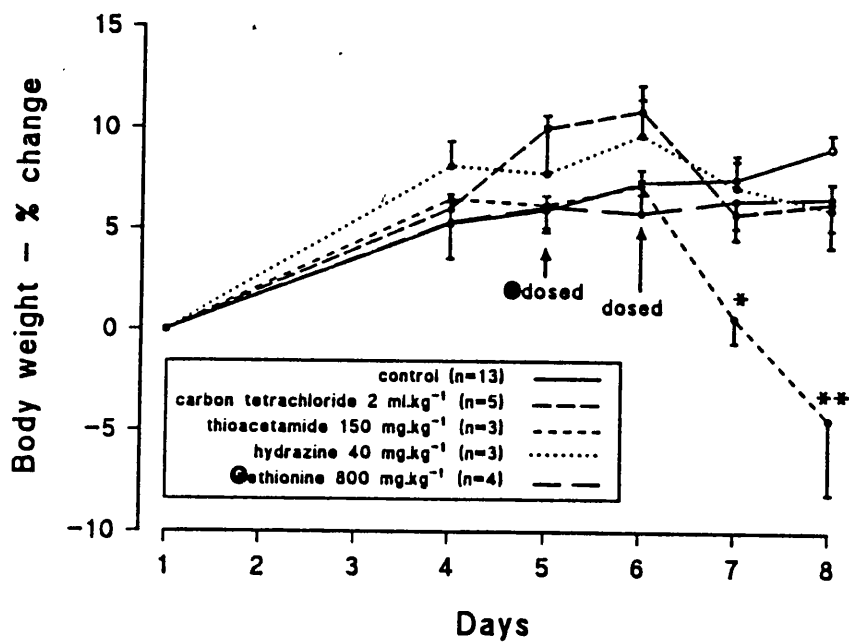


Figure 3.1 Change in body weight (% of starting weight), of rats treated with different hepatotoxic compounds. Number of animals in parentheses. Values, means \pm SEM; * $p < 0.05$, ** $p < 0.01$.

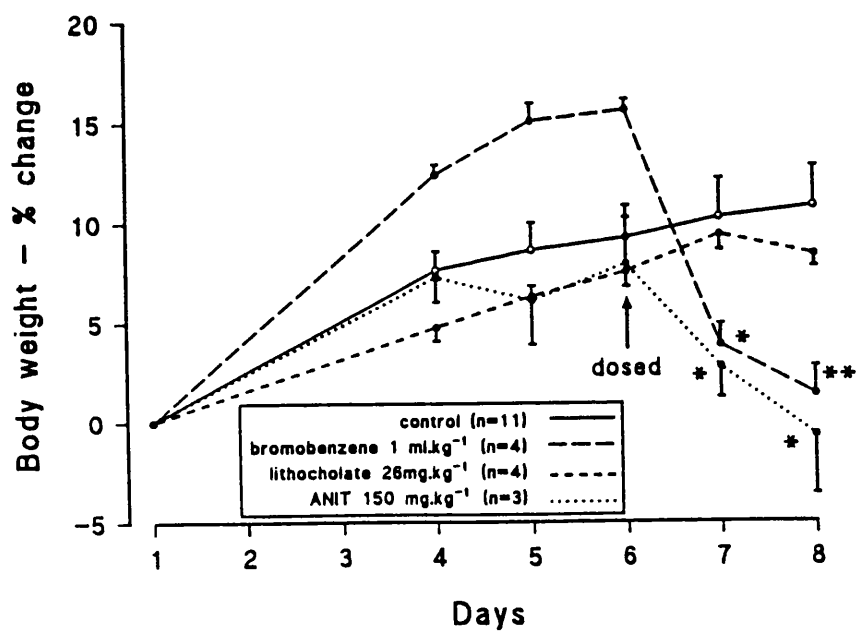


Figure 3.2 Change in body weight (% of starting weight), of rats treated with different hepatotoxic compounds. Number of animals in parentheses. Values, mean \pm SEM; * $p < 0.05$, ** $p < 0.01$.

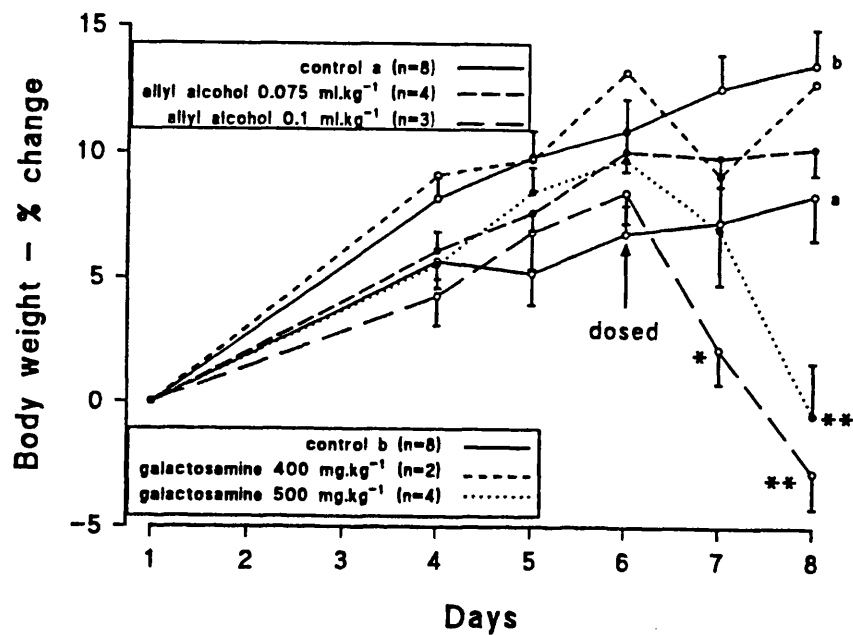


Figure 3.3 Change in body weight (% of starting weight), of rats treated with two different levels of allyl alcohol and galactosamine. Number of animals in parentheses.
Values, mean \pm SEM; ** $p < 0.01$.

Since changes in liver and kidney weight can be indicative of a pathological lesion these were recorded for each of the compounds administered (Table 3.3).

TABLE 3.3 Effect of various hepatotoxic compounds on the mean liver and kidney weight (% body weight) in male rats.

COMPOUND	DOSE (kg ⁻¹)	LIVER % BODY WEIGHT	KIDNEY % BODY WEIGHT
CARBON TETRACHLORIDE^a	2 ml	5.19 ± 0.31**	0.769 ± 0.02
THIOACETAMIDE^b	150 mg	4.25 ± 0.30*	1.034 ± 0.04***
ANIT^b	150 mg	4.24 ± 0.16*	0.877 ± 0.02**
HYDRAZINE^b	40 mg	3.81 ± 0.07	0.820 ± 0.02
LITHOCHOLATE^c	26 mg	3.65 ± 0.07	0.770 ± 0.03
BROMOBENZENE^d	1 ml	4.73 ± 0.10*	0.941 ± 0.03**
ETHIONINE~^e	800 mg	3.76 ± 0.10	0.840 ± 0.01
ALLYL ALCOHOL^f	0.1 ml	4.28 ± 0.27	0.878 ± 0.07
	0.075 ml	4.28 ± 0.15	0.890 ± 0.06*
GALACTOSAMINE^g	500 mg	3.40 ± 0.27	0.830 ± 0.01**
	400 mg	3.28 (n=2)	0.862 (n=2)

Values mean ± SEM for n = 3-5; treatment groups a - h are compared to respective control groups (see Appendix XIII) for the purpose of statistical analysis; * p<0.05, **p<0.01, ***p<0.001; Samples taken for assay 48 h after dosing, except ~ = 72 h.

There was a significant increase in liver weights of animals treated with carbon tetrachloride, thioacetamide, ANIT and bromobenzene. These compounds also resulted in raised liver enzymes in serum taken 48 h after dosing (Table 3.4).

A single acute dose of carbon tetrachloride, thioacetamide, bromobenzene, galactosamine and ANIT, given to male rats, elevated serum transaminase enzymes ALT and AST and the biliary epithelial enzyme ALP in serum. These enzymes were also raised by allyl alcohol (0.1 ml.kg⁻¹). However, as only two animals survived statistical analysis was not possible. Variation in the results from animals treated with a lower dose of allyl alcohol (0.075 ml.kg⁻¹) meant that the increased levels of serum enzymes were not significantly different from control values.

TABLE 3.4 Serum clinical chemistry indicating hepatic damage following treatment of male rats with hepatotoxic compounds.

COMPOUND	DOSE (kg ⁻¹)	ALT iu.L ⁻¹	AST iu.L ⁻¹	ALP iu.L ⁻¹	ALBUMIN g L ⁻¹	TOTAL PROTEIN g L ⁻¹	TOTAL BIL mmol.L ⁻¹
CCl ₄ ^a	2 ml	344 ± 124	1219 ± 360**	1007 ± 201*	35.0 ± 0.5	57.0 ± 1.2	9.3 ± 4.99
Thioacetamide ^b	150 mg	442 ± 71*	1735 ± 97**	766 ± 19	28.5 ± 0.6**	48.8 ± 1.6*	8.9 ± 1.2*
ANIT ^b	150 mg	723 ± 155*	1501 ± 52***	1508 ± 196*	31.8 ± 0.3	62.8 ± 1.3*	177 ± 23*
Hydrazine ^b	40 mg	24 ± 2.6**↓	86.2 ± 7.3	348 ± 48	31.9 ± 0.5	56.4 ± 1.3	1.1 ± 0.39
Lithocholate ^c	26 mg	32 ± 3.8*↓	63.7 ± 1.9	378 ± 44	31.8 ± 0.6	57.0 ± 1.1	1.9 ± 0.09
Bromobenzene ^d	1 ml	173 ± 37*	1185 ± 420*	583 ± 37*	31.3 ± 0.5	51.4 ± 0.9***	10.2 ± 6.4
Ethionine ^e	800 mg	43 ± 7.3	46.9 ± 7.3	501 ± 90	33.0 ± 1.6	54.3 ± 3.7	N.D.
^b Allyl alcohol ^f	0.1 ml# 0.075 ml	429 101 ± 24	991 138 ± 34	995 409 ± 39	26.4 32.4 ± 0.2	56.0 57.0 ± 0.9	3.3 3.8 ± 0.3
^g Galactosamine ^h	500 mg 400 mg#	4295 ± 244* 1363	6479 ± 698*** 1619	730 ± 6** 715	30.9 ± 1.0 28.6	48.0 ± 1.8* 46.9	134 ± 13*** 47

Values - mean ± SEM, n = 3-5, except # where n = 2; treatment groups a - h are compared to respective controls, (see Appendix XIII) for the purpose of statistical analysis; * p< 0.05, ** p< 0.01, *** p< 0.001. Values estimated 48 h after dosing, except ~ = 72 h. N.D. = no data

Total bilirubin and serum cholesterol levels were also significantly raised by ANIT and thioacetamide (Table 3.5) and the higher dose of galactosamine (500 mg.kg⁻¹) elevated total bilirubin levels indicating that these compounds had induced cholestasis, inhibited biliary excretion or caused haemolysis. Ethionine (800 mg.kg⁻¹), CCl₄, bromobenzene and galactosamine significantly reduced serum triglyceride levels (Table 3.5).

TABLE 3.5 Serum cholesterol and triglyceride levels 48 h after dosing rats with different hepatotoxic compounds.

COMPOUND	DOSE (kg ⁻¹)	SERUM CHOLESTEROL mmol.L ⁻¹	SERUM TRIGLYCERIDES mmol.L ⁻¹
CCl ₄ ^a	2 ml	1.62 ± 0.22	0.53 ± 0.03**
THIOACETAMIDE ^b	150 mg	1.57 ± 0.15	0.84 ± 0.08
ANIT ^b	150 mg	4.93 ± 0.37***	0.70 ± 0.09
HYDRAZINE ^b	40 mg	1.68 ± 0.25	0.56 ± 0.16
LITHOCHOLATE ^c	20 mg	1.51 ± 0.05	0.94 ± 0.07
BROMOBENZENE ^d	1 ml	2.07 ± 0.20	0.74 ± 0.11**
ETHIONINE ^e	800 mg	1.59 ± 0.14	0.74 ± 0.08**
^b ALLYL ALCOHOL ^f	0.1 ml# 0.075 ml	3.17 1.72 ± 0.1	1.09 0.93 ± 0.11
^g GALACTOSAMINE ^h	500 mg 400 mg#	0.95 ± 0.18 0.88	0.43 ± 0.13** 0.45

Mean values ± SEM, n = 3-5, except # where n = 2. Treatment groups are a - h are compared to respective control groups (Appendix XIII) for the purpose of statistical analysis; ** p < 0.01 and *** p < 0.001 when compared to control values in same study. Values 48 h post dose except ~ = 72 h post dose.

Neither hydrazine nor lithocholate caused changes in the conventional clinical biochemical measurements made to assess hepatotoxicity although both reduced ALT levels significantly. However, hydrazine *did* induce marked steatosis in the liver which was demonstrated by Oil red O staining.

Table 3.6 Biochemical indications of kidney damage. Serum creatinine and blood urea nitrogen and urinary protein and γ -GT values from rats treated with hepatotoxic compounds.

COMPOUND	DOSE (kg^{-1})	CREATININE $\mu\text{mol.L}^{-1}$	UREA mmol.L^{-1}	PROTEIN $\text{mg.kg}^{-1} 24 \text{ h}^{-1}$	γ -GT $\text{iu.kg}^{-1} 24 \text{ h}^{-1}$
CCl_4^a	2 ml	58.75 ± 0.48	6.67 ± 0.38	43 ± 2.27	10 ± 3.0
Thioacetamide ^b	150 mg	$100.33 \pm 7.26^{*\uparrow}$	$11.48 \pm 0.71^{*\uparrow}$	161 ± 46	131 ± 71
ANIT ^b	150 mg	34.67 ± 3.71	5.80 ± 0.60	48 ± 4.8	10 ± 1.3
Hydrazine ^b	40 mg	43.00 ± 0.00	5.04 ± 0.30	46 ± 8.9	6 ± 0.4
Lithocholate ^c	26 mg	43.00 ± 0.75	4.75 ± 0.26	N.D.	N.D.
Bromobenzene ^d	1 ml	43.75 ± 0.95	5.64 ± 0.30	$48 \pm 7.6^{**\downarrow}$	12 ± 1.5
Ethionine ^e	800 mg	25.4 ± 7.9	5.36 ± 0.77	$46 \pm 8.0^{*\downarrow}$	5 ± 0.7
^b Allyl alcohol ^f	0.1 ml# 0.075 ml	50 49.2 ± 1.7	12.05 4.74 ± 0.66	50 ± 7.1 68 ± 4.7	N.D. 9 ± 1.6
^g Galactosamine ^h	500 mg 400 mg#	48.00 ± 1.00 38	5.11 ± 0.68 4.67	49 ± 5.5 56 ± 8.2	N.D. N.D.

Mean values \pm SEM, n = 3-5, except # where n=2; Treatment groups a - h were compared to their respective control groups for the purpose of statistical analysis - see Appendix XIII; * p< 0.05, ** p< 0.01 compared to appropriate control. Serum creatinine and blood urea nitrogen measured in serum samples taken 48 h after dosing, except ~ = 72 h. Urinary protein and γ -GT measured in 24 h urine sample collected 24 - 48 h post dosing. N.D. = no data

There was a significant increase in the kidney weights relative to body weight in animals treated with thioacetamide, ANIT, bromobenzene the lower dose of allyl alcohol (0.075 ml.kg^{-1}) and the higher dose of galactosamine (500 mg.kg^{-1}), suggesting that these compounds may have caused pathological changes in the kidney.

Levels of serum creatinine and blood urea nitrogen were also measured as raised levels are indicative of kidney damage. Evidence of kidney damage was also supported by urine analysis where elevated levels of urinary protein and the presence of γ -GT were used as additional evidence of a kidney lesion (Table 3.6).

After thioacetamide administration, increased relative kidney weights were accompanied by raised urinary protein and γ -GT levels, an increase in serum creatinine and in blood urea nitrogen. Histological examination of the kidneys confirmed the kidney lesion. However, the other compounds which increased relative kidney weights did not show changes in the clinical biochemistry consistent with kidney damage. This suggested that the relative increase was due to a greater decrease in body weight or other changes, as absolute kidney weights were not increased.

For example: animals dosed with bromobenzene had a significant increase in relative kidney weights but the mean absolute kidney weights for control and bromobenzene treated animals were 2.3 g ($\text{SD} \pm 0.20$) and 2.4 g ($\text{SD} \pm 0.25$) respectively. However, bromobenzene is known to be nephrotoxic as well as hepatotoxic. In this case it is believed that the metabolite 2-bromohydroquinone is formed in the liver and may be transported to the kidney where it contributes to renal damage (Monks *et al*, 1982).

(ii) Histological findings

All the following observations were made on liver samples taken 48 h after dosing (except tissues from ethionine treated animals which were taken 72 h after dosing) using light microscopy and were essentially the same as those reported in the literature.

Carbon tetrachloride

There were necrotic cells and neutrophil/macrophage infiltration around most central veins. Hydropic degeneration of hepatocytes was observed around the necrotic cells or central veins where the necrosis was less marked. Lipid containing vacuoles (detected by staining with Oil red O) were predominantly found in mid-zonal and some periportal hepatocytes. Various stages of mitosis, indicative of regeneration, were seen in hepatocytes throughout the lobules, (Plates 3 & 4).

Thioacetamide

There was extensive necrosis of hepatocytes around the central veins and mild steatosis throughout the remaining parenchyma (Plate 5).

ANIT

Hepatocytes were eosinophilic in all regions and appeared distended. Nuclei were enlarged and there was focal necrosis. Many bile ducts had severely damaged epithelial lining and in some the lumen was occluded with cell debris. There was evidence of localised inflammation (Plate 6).

Hydrazine

Most hepatocytes appeared normal except for those in the midzonal region in which steatosis was confirmed by staining with Oil red O (Plates 7 & 8).

Lithocholate

No abnormality was found in either the liver parenchyma or biliary epithelium. Staining for lipid accumulation and glycogen depletion showed no difference between the treated and the control livers.

Bromobenzene

Most central veins were surrounded by a region of necrotic hepatocytes and there was infiltration of macrophages. There was evidence of slight steatosis around the portal tracts (Plate 9).

Ethionine

Staining with Oil red O showed a marked steatosis in liver sections from ethionine treated rats which appeared to be more concentrated in the perivenous regions (Plates 10 & 11).

Allyl alcohol

The predominant lesion produced was necrosis of the periportal hepatocytes although the lesion was variable and not all the portal tracts developed necrotic regions. Severe necrosis was observed macroscopically around the base of individual lobes. There was some lipid accumulation in a few individual non-necrotic cells (Plate 12).

Galactosamine

Liver sections showed extensive diffuse eosinophilic hepatocytes and regions of diffuse necrosis. There was evidence of bile duct proliferation and in some bile ducts the epithelial cells lining the ducts had become very elongated. Many hepatocytes were undergoing mitosis. There was evidence of fat accumulation in individual cells and in the perivenous hepatocytes (Plates 13 and 14).

(iii) Taurine levels

(a) Urinary taurine

Thioacetamide (150 mg.kg^{-1}) produced an 80% elevation in urinary taurine excretion during the first 24 h following dosing and a significant increase of 254% 24-48 h after dosing (Figure 3.4B). Hydrazine (40 mg.kg^{-1}) resulted in a 200% increase in urinary taurine 24-48 h after dosing (Figure 3.4C). One hydrazine treated rat had a pre-dose value of $29 \text{ } \mu\text{mol.kg}^{-1} \cdot 24 \text{ h}^{-1}$ rising to $290 \text{ } \mu\text{mol.kg}^{-1} \cdot 24 \text{ h}^{-1}$ 24 - 48 h after dosing. Carbon tetrachloride (2 ml.kg^{-1}), galactosamine (500 mg.kg^{-1}) and ethionine resulted in a significant increase in urinary taurine 0-24 h and 24-48 h after dosing (Figures 3.4A and 3.5A & B) which was maintained in the case of ethionine and carbon tetrachloride for a further 24 h and 48 h respectively (Figures

3.22 and 3.44). A lower dose of galactosamine (400 mg.kg^{-1}) resulted in raised levels of urinary taurine in 2/4 animals 0 - 24 and 24 - 48 h following dosing (results not shown).

Changes in urinary taurine following the administration of allyl alcohol were variable (Figure 3.5C). After the highest dose (0.1 ml.kg^{-1}) 2/3 animals showed an increase in urinary taurine from a mean value before dosing of $48.7 \mu\text{mol.kg}^{-1}.24 \text{ h}^{-1}$ to a mean value of $334.3 \mu\text{mol.kg}^{-1}.24 \text{ h}^{-1}$ for the 24 h following dosing. These two animals also had raised serum AST and ALT levels 48 h after dosing. However the third animal had reduced urinary taurine in the same time period despite having elevated levels of serum AST and ALT. Between 24 h and 48 h after dosing all three animals were excreting taurine at the same rate as they had before dosing. Rats treated with a lower dose of allyl alcohol (0.075 ml.kg^{-1}) exhibited **hypotaurinuria** 0-24 h and 24-48 h after dosing the, reduction being significant 24-48 h after dosing.

ANIT resulted in a 70% reduction in urinary taurine during the first 24 h after dosing and an 80% decrease 24-48 h after dosing, both values being significant (Figure 3.6C). Lithocholate resulted in a significant decrease in urinary taurine 0-24 h after dosing although there were no clinical, histological or biochemical observations to indicate that the dose was hepatotoxic (Figure 3.6B). Rats treated with bromobenzene were clearly affected by the dose administered when clinical, histological and biochemical parameters were measured, however, these animals showed no change in levels of urinary taurine (Figure 3.6A).

It should be noted that throughout these studies and those that follow, both urinary and liver taurine levels varied *between* studies as noted previously (Hoskawa, 1988). Data in Appendix II suggest that this was not the result of the technique employed to measure taurine. Despite these variations, results were consistent between repeated studies. Results are discussed with this limitation being recognised. It is notable that variations in serum enzymes *also* demonstrate great variability after hepatic injury.

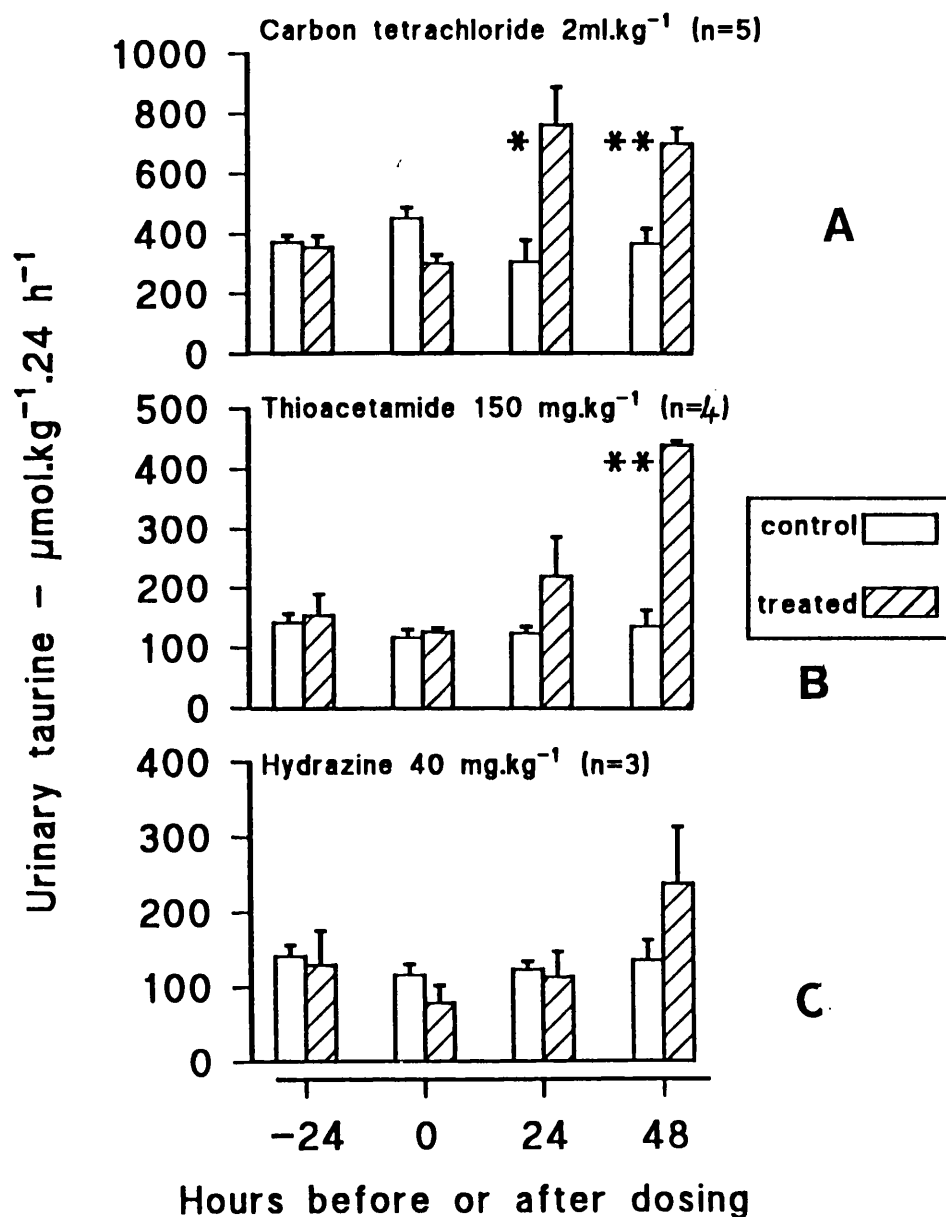
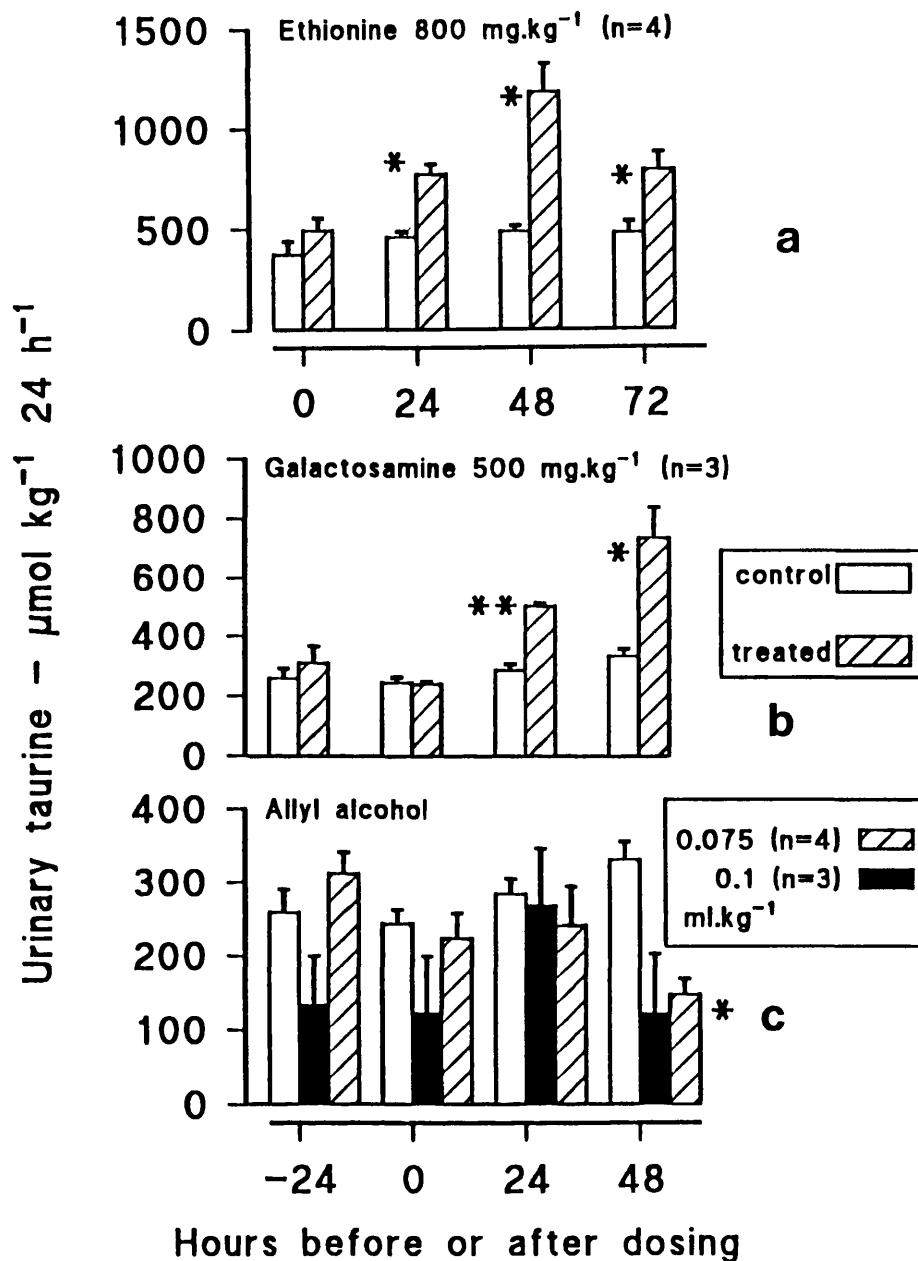


Figure 3.4 Urinary taurine levels in rats treated with CCl_4 , thioacetamide and hydrazine. Each bar represents the total amount of taurine in a complete 24 h urine collection expressed per kg body weight. Animals were dosed after the 0 h collection was made. Number of animals in parentheses. Values, means \pm SEM; * $p < 0.05$, ** $p < 0.01$

**Figure 3.5**

Urinary taurine levels in rats treated with ethionine, galactosamine and two different dose levels of allyl alcohol. Each bar represents the total amount of taurine in a complete 24 h urine collection expressed per kg body weight. Animals were dosed after the 0 h collection was made. Number of animals in parentheses.

Values, means \pm SEM; * $p < 0.05$, ** $p < 0.01$

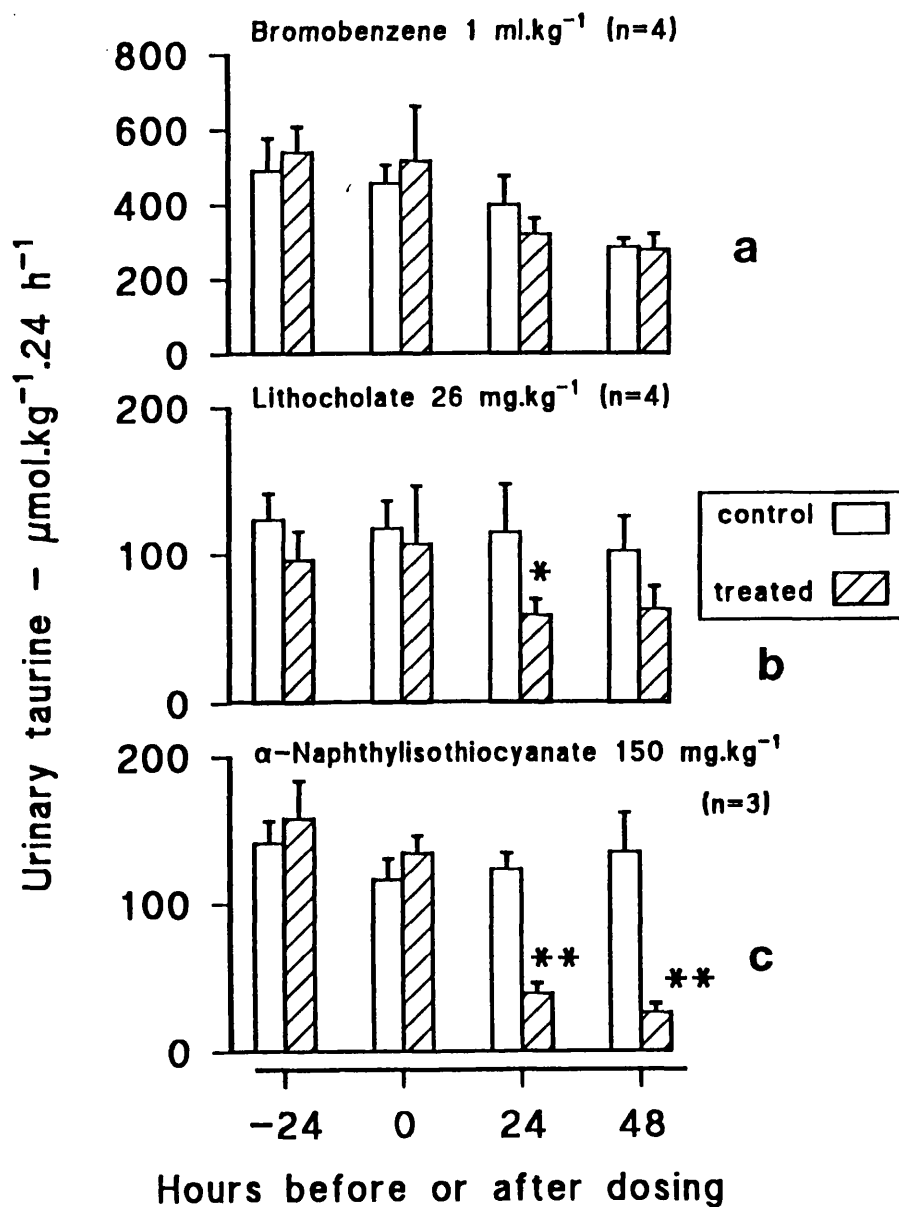


Figure 3.6 Urinary taurine levels in rats treated with bromobenzene, lithocholate and α -naphthylisothiocyanate (ANIT). Each bar represents the total amount of taurine in a complete 24 h urine collection expressed per kg body weight. Animals were dosed after the 0 h collection was made. Number of animals in parentheses.

Values, means \pm SEM; * $p < 0.05$, ** $p < 0.01$

(b) Liver taurine (Figure 3.7)

The concentration of taurine in the livers of control animals was very variable between different studies; mean control values varied between 4.0 (SEM \pm 0.6) and 11.2 (SEM \pm 0.9) $\mu\text{mol.g}^{-1}$ wet weight liver. However, there was less variation between animals within a study.

(See Appendix X for individual study data).

Following dosing with ANIT (48 h) the concentration of taurine in the liver was significantly higher ($p < 0.01$), compared to control animals.

One animal treated with hydrazine raised levels of liver taurine similar to the levels found in animals treated with ANIT and corresponded to the animal with markedly raised urinary taurine.

Allyl alcohol (0.1 ml.kg^{-1}) reduced liver taurine in the two surviving animals to 45 % of the mean control value and a lower dose (0.075 ml.kg^{-1}) reduced liver taurine by 22% when compared to the control values.

Galactosamine also produced a significant reduction in liver taurine levels ($p < 0.01$) and there was a reduction following bromobenzene treatment, but the variation in the control values meant that this was not significant.

There were interesting dose related changes in liver taurine levels 48 h after the administration of ethionine. In two separate studies, doses of 200 mg.kg^{-1} resulted in a significant elevation of liver taurine. Individual animals with high liver levels also had the highest urinary output of taurine 24 - 48 h after dosing. The increase in liver taurine was less after 400 mg.kg^{-1} and was the same as the control values in animals given 800 mg.kg^{-1} , 72 h after dosing (Figure 3.25).

Taurine levels were measured in the livers of animals killed 48 h after dosing with hepatotoxic compounds. The aim was to establish whether changes in urinary levels of taurine could be equated with changes seen in liver taurine levels. The original, working hypothesis and most simple explanation for an increase in urinary taurine was that there was leakage of taurine from cells (hepatocytes) damaged by the hepatotoxic substances. This would have raised serum levels of taurine and would probably have been eliminated via the kidneys.

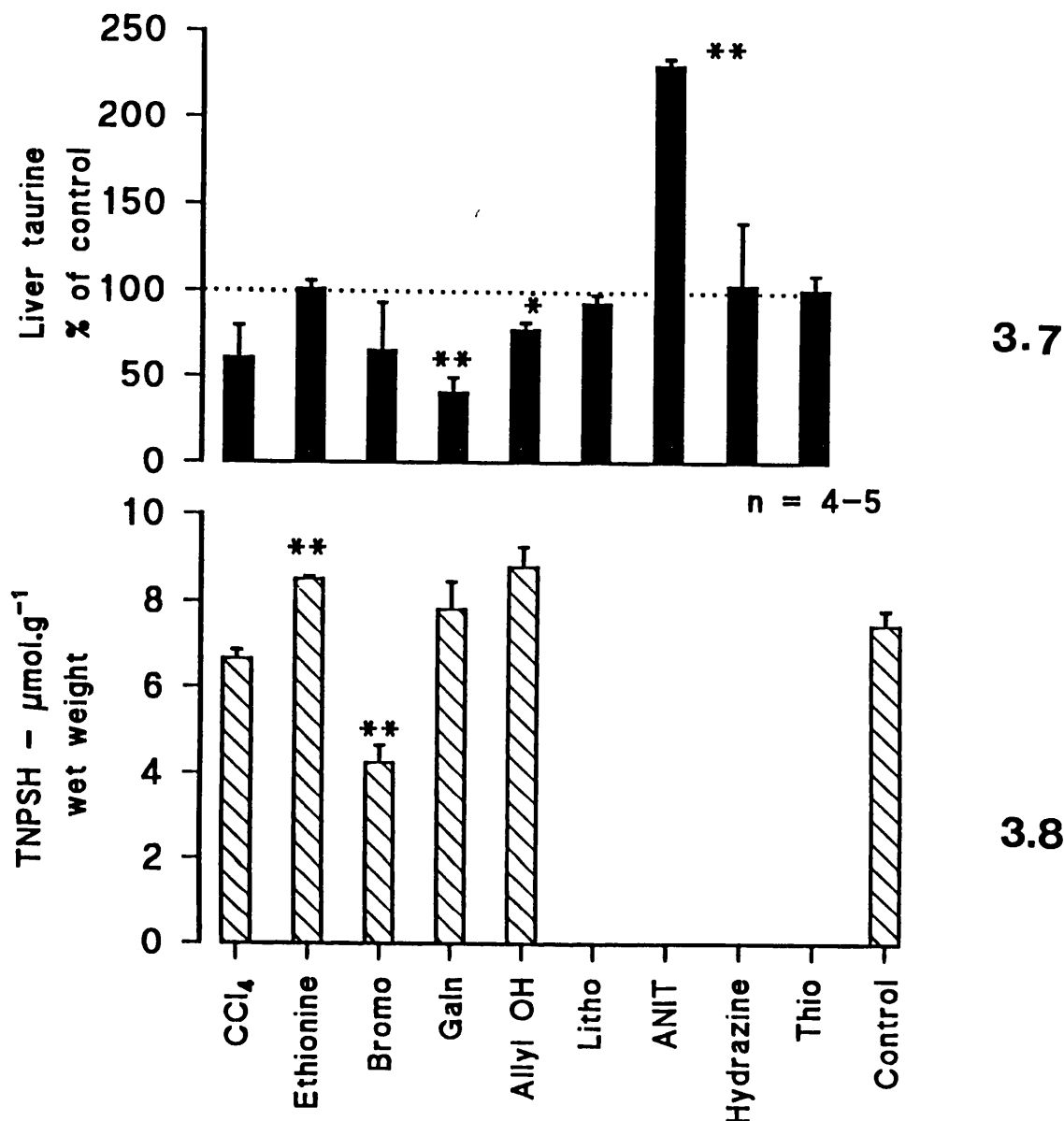


Figure 3.7 Taurine concentration in the liver 48 h after treating rats with various hepatotoxic compounds and 72 h after treatment with ethionine. To enable comparisons to be made between studies, results are expressed as % of control.

Figure 3.8 TNPSH measured in the same liver samples as above. Values are absolute concentrations. No data is presented for lithocholate, ANIT, hydrazine or thioacetamide as these measurements were not made.

Values, mean \pm SEM; * $p < 0.05$, ** $p < 0.01$

Doses/kg body weight: CCl₄, 2 ml.kg⁻¹; ethionine, 800 mg.kg⁻¹; bromobenzene, 1 ml.kg⁻¹; galactosamine, 500 mg.kg⁻¹; allyl alcohol, 0.075 ml.kg⁻¹; lithocholate, 26 mg.kg⁻¹; ANIT, 150 mg.kg⁻¹; Hydrazine 40 mg.kg⁻¹; thioacetamide, 150 mg.kg⁻¹.

If that had been the case, the total amount of liver taurine might have been expected to be reduced by the same amount as the urinary levels of taurine increased.

Thus, when the *difference* between the total amount of *urinary* taurine collected from control and treated animals was calculated (total 48 h pre-dose compared with 48 h total post-dose) and compared with the *difference* between the total amount of *liver* taurine from control and treated animals 48 h after dosing the following figures can be derived (Table 3.7).

Table 3.7. Liver and urinary taurine levels 0 - 48 h after dosing with hepatotoxic compounds.

Compound (ml or mg.kg ⁻¹)	Difference between control and treated animals		% of change in urinary taurine accounted for by difference in liver taurine
	Total 48 h urinary taurine post- dose (μmol)	Total liver taurine 48 h after dosing (μmol)	
CCl ₄ (2 ml)	+239	-21	8.8
Hydrazine (40 mg)	+36	+2	5.0
Thioacetamide (150 mg)	+95	-1	1.0
ANIT (150 mg)	-57	+65	114.0
Galactosamine (500 mg)	+155	-61	40
Bromobenzene (1 ml)	-61	-26	42
Lithocholate (26 mg)	-12	-12	98
Ethionine (200 mg)	+27	+32	118
Allyl Alcohol (0.1 ml)	+34	-16	46.0

Values are means of individual animals and not corrected for animal weight.

These data show that dosing rats with some compounds which produced an elevation of urinary taurine also resulted in a corresponding reduction in liver taurine content (eg. galactosamine and allyl alcohol) but in none of these cases could the total increase in urinary taurine be accounted for by the loss of liver taurine. Indeed, in the case of ethionine where there was an increase in urinary taurine, there was also an *increase* in liver taurine levels.

Conversely, compounds which resulted in a *decrease* in urinary taurine appeared to *reduce* liver taurine (eg. bromobenzene and lithocholate). The exception was ANIT which resulted in animals developing a highly significant hypotaurinuria with a concomitant *increase* in liver taurine, indeed this was the only hepatotoxic compound which induced a change in urinary taurine which *could* be accounted for by an opposite but equal change in liver taurine levels.

Clearly, the hypothesis that elevated levels of taurine after hepatotoxic insult was the result of simple leakage of taurine from damaged hepatocytes was not the only explanation for the altered levels of urinary taurine, although the process of leakage could still be a contributing factor to the raised levels.

The timing of urinary elevation of taurine was also not entirely consistent with the hypothesis that urinary levels of taurine are raised as a result of leaked taurine from damaged hepatocytes. Thus, urinary taurine was maximally elevated 24 - 48 h after dosing with CCl_4 and in a similar time period after thioacetamide and galactosamine although urine samples were not collected any later than 48 h after dosing with the latter two compounds.

The appearance of necrosis at an early stage in the toxicity of galactosamine (3 - 6 h) and in the toxicity of compounds such as thioacetamide and carbon tetrachloride (12 - 30 h) has been reported (Zimmerman, 1978). This might be expected to result in the greatest elevation of urinary taurine in the first 24 h if raised levels of urinary taurine were solely the result of leaked taurine. However the continued elevation of urinary taurine suggested that there was another source of taurine which continued to contribute to the

raised levels. This may have been taurine mobilised from other tissue sources or newly synthesised taurine which may have been produced in the liver and continued to leak from damaged hepatocytes.

A closer examination of urinary taurine levels in the first 24 h after dosing with CCl_4 are discussed in section 3.2. The results in section 3.2 suggest that there is an initial increase in urinary taurine at the same time as hepatocytes are undergoing hydropic degeneration and AST and ALT appear in the serum. This is followed by a second and greater elevation which continues for up to 96 h after treating animals with 2 ml.kg^{-1} CCl_4 . Hypertaurinuria was developed in rats treated with ethionine and hydrazine. This also suggested that an increase in urinary taurine was not solely the result of leaked taurine from necrotic hepatocytes, since neither of these compounds result in overt cell damage as assessed by leaked liver enzymes and histology. Both of these compounds and also, CCl_4 , thioacetamide and galactosamine resulted in fat accumulation in the liver (steatosis). The development of steatosis can be the result of impaired synthesis of lipoproteins which are responsible for the transportation of lipid out of cells, which, in turn, can be the result of impaired protein synthesis (Dianzani, 1991). CCl_4 , thioacetamide, galactosamine, hydrazine and ethionine are known to inhibit protein synthesis and hydrazine is also believed to have an effect on protein flux.

An inhibition of protein synthesis would result in a larger pool of cysteine being available for the synthesis of taurine or the other ubiquitous, cysteine requiring compound, glutathione. If taurine synthesis was indirectly increased by these compounds via raised cysteine levels, it would provide a further explanation for the elevation of urinary taurine in animals treated with these compounds. The raised liver levels of taurine after treatment with ethionine would also support this hypothesis.

Dosing animals with bromobenzene did not raise urinary taurine after a dose which was sufficient to result in hepatic necrosis. The toxicity of this compound relies on its metabolism to the 3,4-epoxide (Jollow *et al*, 1974) and subsequent conjugation with glutathione resulting in glutathione depletion. Glutathione requires cysteine as a precursor and under conditions

when glutathione synthesis is increased as it is after the administration of bromobenzene, cysteine would be reduced, possibly resulting in a reduction of liver taurine.

As the initial studies suggested that glutathione status might be an important factor in determining the extent of taurine synthesis and possibly an indicator of increased levels of cysteine, later studies with hepatotoxic compounds included the measurement of liver total non-protein sulphydryl (TNPSH) as a measure of total liver glutathione 48 h after dosing. The results of these determinations are discussed below.

iv. Liver total non-protein sulphydryls (TNPSH)

Control values for liver TNPSH were found to be consistent between the different studies which were carried out. The effects of CCl_4 , ethionine, bromobenzene, galactosamine and allyl alcohol on liver TNPSH were measured (Fig. 3.8). Of these compounds, only two, bromobenzene and ethionine resulted in liver levels of TNPSH which were significantly different from control levels 48 h (bromobenzene) and 72 h (ethionine) after dosing. (See Appendix X for complete data).

Bromobenzene (1 ml.kg^{-1}) resulted in a reduction in liver TNPSH levels. Jollow *et al* (1974) reported reductions in liver glutathione of 85% after administering 0.2 ml.kg^{-1} of bromobenzene (i.p) with a nadir 5 h after dosing. After 30 h liver levels of GSH were the same as control values and by 36 h liver GSH was elevated to 200% of the starting value due to a rebound synthesis of glutathione. The low values of TNPSH in the rats treated in these studies may have been due to the 5-fold higher dose given to these animals and the different route of administration. Glutathione levels remain low until all the bromobenzene has been metabolised and the larger dose used in this study would have taken longer to be metabolised and thus delayed the rebound synthesis of glutathione. This would explain the low levels of TNPSH found in the livers from animals treated with bromobenzene.

Ethionine (800 mg.kg^{-1} p.o.) resulted in significantly raised liver TNPSH

72 h after dosing. A dose of 400 mg.kg^{-1} also raised TNPSH, but not significantly. Ethionine is reported to lower liver glutathione levels (Glaser and Mager, 1974) at an early stage in its toxicity, with the nadir 5 h after dosing. However, 72 h after dosing the effect of rebound synthesis may still have been apparent. This may have resulted in the dose related increase in TNPSH which was found since the rebound synthesis would occur at a later time after dosing with a higher dose of ethionine than a low dose (Figure 3.26).

v. Urinary creatine and creatinine

Measurements of creatine and creatinine in 24 h urine collections showed no significant changes 0-24 h after treatment, however, 24 - 48 h after treatment there was a significant increase ($p < 0.001$) in urinary creatine from animals treated with carbon tetrachloride, thioacetamide, bromobenzene and the higher dose of allyl alcohol (0.1 ml.kg^{-1} , $p < 0.01$) (Figure 3.9). Creatinuria also developed after dosing with allyl alcohol (0.075 ml.kg^{-1}) and similarly after ANIT treatment ($p < 0.01$ and $p < 0.05$) 24 - 48 h after dosing. However, these elevations in urinary creatine were similar to the significantly increased levels of creatine in urine from rats given restricted food. These were rats which had been provided with a limited daily intake of food to simulate the daily intake of animals given a compound which reduces their food intake. The urinary creatine data from these animals is included for comparison. Hydrazine and galactosamine also elevated creatine in some animals but the increases were not significant.

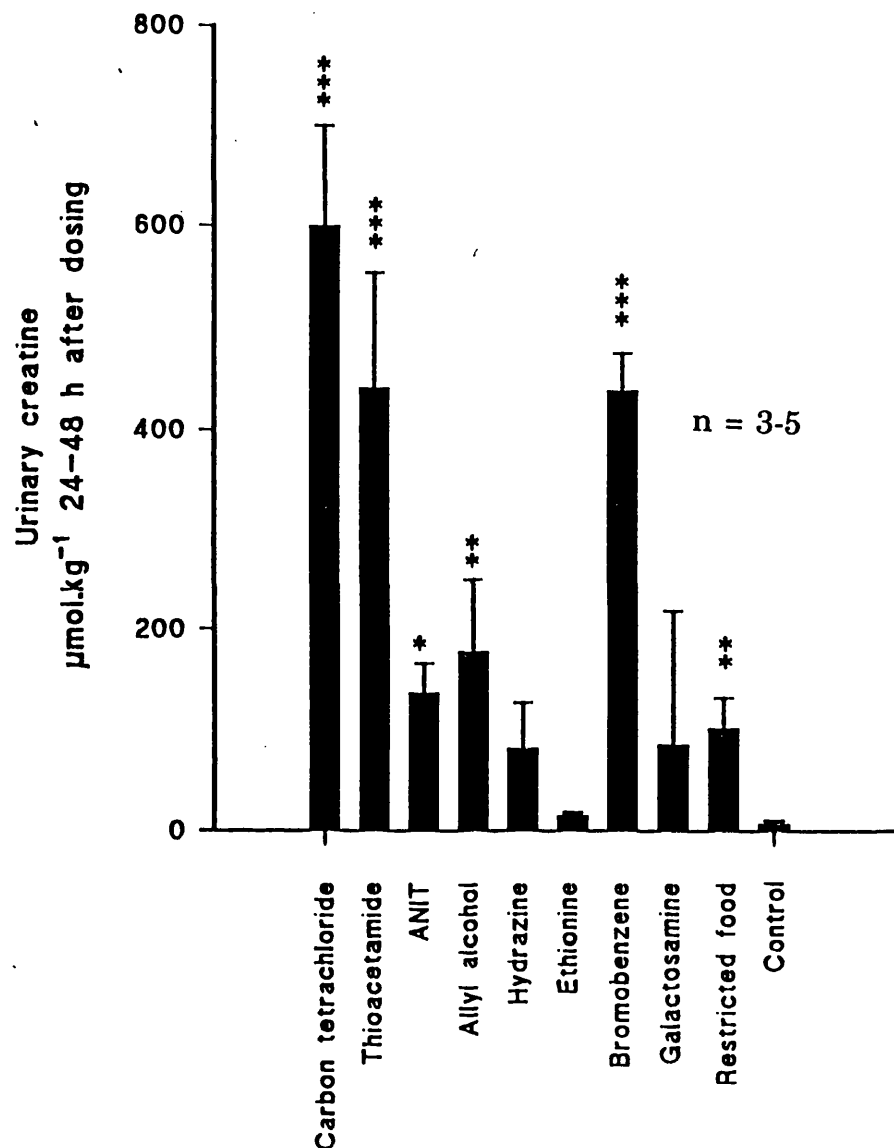


Figure 3.9 Urinary creatine in 24 h urine samples from rats 24 - 48 h after the administration of hepatotoxic compounds.

Values, mean \pm SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Doses/kg body weight: CCl₄, 2 ml.kg⁻¹; ethionine, 200 mg.kg⁻¹; bromobenzene, 1 ml.kg⁻¹; galactosamine, 500 mg.kg⁻¹; allyl alcohol, 0.1 ml.kg⁻¹; lithocholate, 26 mg.kg⁻¹; ANIT, 150 mg.kg⁻¹; Hydrazine 40 mg.kg⁻¹; thioacetamide, 150 mg.kg⁻¹.

Animals on restricted food had a food intake of \approx 25% of control animals during the 48 h experimental period.

3.1.5 DISCUSSION

The data suggest that a variety of compounds considered to be predominantly hepatotoxic, result in altered levels of urinary taurine.

Possible mechanisms for these alterations are discussed below.

(A) Raised levels of urinary taurine.

There are several possibilities which could account for the elevation in urinary taurine seen after dosing rats with CCl_4 , thioacetamide, galactosamine, ethionine, hydrazine and allyl alcohol (0.1 ml.kg^{-1}):-

i. Damage to the kidney leading to reduced uptake. Taurine is extensively reabsorbed by the β -amino acid uptake system (Chesney, 1985) which might be susceptible to damage. However, in these studies, although kidney damage was detected in two thioacetamide treated animals (urinary γ -GT and protein were raised) raised urinary taurine preceded the markers of kidney damage by 24 h. A later study (Section 3.2) has shown that some female rats, treated with CCl_4 , developed kidney damage but excreted less taurine than those animals whose kidney morphology was normal after treatment. Also, a study in which mercuric chloride was administered to rats (Section 3.6) resulted in a significant *reduction* in urinary taurine when kidney damage was induced. Kidney damage induced by other nephrotoxic compounds investigated by Gartland *et al* (1988) did not result in raised urinary taurine. Therefore it seems unlikely that the raised levels of urinary taurine observed were the result of kidney damage.

ii. Reduced food intake and loss of body weight due to the toxicity of hepatotoxic compounds. Reduced food intake has been reported to increase urinary taurine (Sanins *et al*, 1990) although other workers have reported a reduction in urinary taurine as a result of reduced food intake (Chesney, 1984). Although animals treated with carbon tetrachloride, thioacetamide, galactosamine and allyl alcohol suffered a loss of body weight and their food intake was decreased, the data presented here (Table

3.2) and described in later studies (Section 4.1) show that reduced food intake resulted in *reduced* urinary taurine which was greatest 24-48 h after restricting food intake.

iii. An increase in β -alanine synthesis/excretion increasing competition for re-uptake in the kidney. Scriver *et al.* (1976) describe a genetic condition in man, hyper- β -alaninemia, which results in taurinuria as a result of competition in the kidney for the β -amino acid uptake system. Henson *et al.* (1976) reported on the chemically induced increase in β -alanine in the livers of mice treated with either isoniazid or hydrazine. This was believed to be due to the enhanced pyrimidine catabolism. Whether this resulted in an over flow of displaced taurine into the urine was not discussed. Using NMR, however, Sanins *et al.* (1990) did report an increase in urinary excretion of β -alanine in rats treated with hydrazine. They also measured an increase in the urinary levels of taurine in the same urine samples. Thus, an increase in the urinary level of taurine may have been an indirect result of increasing the concentration of β -alanine in the tissues and urine. This may explain why hypertaurinuria is seen after hydrazine treatment when overt cellular damage is not detected.

iv. Leakage of taurine from damaged hepatocytes. It seems likely that the hypertaurinuria observed with some of the compounds studied was due, at least in part, to taurine leaked from damaged hepatocytes. Compounds such as CCl_4 , thioacetamide and galactosamine also elevated AST and ALT in the serum (Table 3.4) which occurs when the cell membrane is damaged or cells become necrotic. Since the liver represents a large pool of taurine (40 - 120 μmol) any leaked into the serum would probably be detected as an elevation in the urine.

Allyl alcohol administration resulted in a pattern of hypertaurinuria which was both variable and different from that observed with other hepatotoxic compounds. When urinary taurine was elevated it was only observed during the first 24 h following dosing (0.1 ml.kg^{-1}). This pattern was also seen after the administration of CCl_4 to female Sprague Dawley rats (section 3.2). The

hepatocellular damage was severe in both cases which suggests that the elevation was due to taurine being lost from damaged cells. However, the elevation was not sustained beyond 24 h in contrast to the elevations of urinary taurine also observed 24 - 48 h after dosing with CCl_4 , thioacetamide and galactosamine. This suggested that CCl_4 , thioacetamide and galactosamine resulted in elevations of urinary taurine which may have been from additional sources, other than damaged hepatocytes.

v. Increased synthesis of taurine. The raised taurine levels found after treating animals with CCl_4 , thioacetamide and galactosamine could not all be accounted for by simple leakage of taurine from damaged cells (Table 3.7). However, these three compounds as well as hydrazine and ethionine induced a marked steatosis in the livers of treated animals. This is consistent with an inhibition of protein synthesis by these compounds. These and other data suggest that there was an increase in taurine synthesis as a result of the inhibition of protein synthesis. Since CCl_4 , thioacetamide and galactosamine also had injured cell membranes (demonstrated by the raised AST and ALT levels) any newly synthesised taurine would have continued to leak from damaged hepatocytes. This may explain why liver levels of taurine in animals treated with these three compounds were reduced. The greatest reduction (50%) was after galactosamine (500 mg.kg^{-1}). This represented only $60 \text{ } \mu\text{mole}$ taurine, in contrast to a total increase in urinary taurine of $556 \text{ } \mu\text{mole}$ (48 h post-dose). The urinary elevation of taurine after ethionine (200 mg.kg^{-1}) was accompanied by an increase in liver taurine levels (72 h post-dose) of 33%. Two experiments were carried out using hydrazine (40 mg.kg^{-1}). In the first, hydrazine resulted in an increase in liver taurine of 30% (48 h after dosing) in 2/3 animals and in the second experiment 1/3 animals (48 h after dosing) had an increase in liver taurine of 75% above control values and corresponded to the individual animal with the greatest increase in urinary taurine. However, the results with this dose of hydrazine were very variable. The remaining animals did not show elevations of liver taurine 48-h after dosing. This might be a reflection of the relatively low dose of

hydrazine used or the fact that changes might have occurred prior to samples being taken. The increase in urinary and liver taurine levels, without an increase in serum AST and ALT could result from an inhibition of protein synthesis and hence perturbation of sulphur amino acid metabolism. Both ethionine and hydrazine alter protein synthesis, possibly resulting in raised intracellular cysteine pools. An excess of cellular cysteine could result in an increase in taurine synthesis, which then overflows into the urine.

In contrast, allyl alcohol did not result in steatosis and is not reported to inhibit protein synthesis. However it is known to deplete hepatic glutathione (GSH) (Jaeschke *et al.* 1987) through its metabolite acrolein. Allyl alcohol results in necrosis of periportal hepatocytes where GSH is found in higher concentrations than in the zone 3, around the central vein (Kaplowitz *et al.* 1985 and Pentilla, 1990b). The effect of hepatic depletion of GSH may lead to more cysteine (a precursor for both taurine and GSH) being converted to GSH rather than taurine (Yoshida and Hara, 1985). This effect would be the opposite to that proposed as a result of inhibited protein synthesis which would raise cysteine levels. Once leakage of taurine from the hepatocytes has taken place, they may be unable to replace it due to cysteine depletion. Although liver levels of taurine were variable between studies the low levels of liver taurine found in rats after allyl alcohol treatment would be consistent with this hypothesis.

(B) Reduced levels of urinary taurine.

Four hepatotoxic compounds did not elevate urinary taurine when administered to rats; bromobenzene (1 ml.kg⁻¹), lithocholate (26 mg.kg⁻¹), ANIT (150 mg.kg⁻¹) and allyl alcohol.

Rats treated with bromobenzene had urinary taurine levels similar to control rats; however lithocholate reduced urinary taurine significantly 0-24 h after dosing and ANIT produced a highly significant reduction 0-48 h after dosing. Allyl alcohol (0.075 ml.kg⁻¹) resulted in a significant reduction in urinary taurine 24-48 h after dosing. The following may account for the reduction of urinary taurine observed.

i. Reduced food consumption. Results presented here show that a reduction in food intake without the administration of cytotoxic compounds reduces urinary taurine (Table 3.2). The amount by which urinary taurine is lowered by reduced food intake will depend on:-

- a) the amount of taurine in the food and
- b) the methionine and cysteine content of the food.

These were calculated by the manufacturer (Quest Nutrition) to be 0.55% of methionine and 0.28% of cysteine by weight. It was estimated, from measurements of taurine concentrations in different food samples, that a rat eating 25 g of ground diet a day would take in between 1 and 80 μmol (up to 0.04% of the diet by weight) of taurine a day depending on the particular "batch" of food supplied. The manufactures listed barley, wheat, maize, middlings, soya extract, fish meal, and yeast amongst the ingredients for the rat maintenance diet. The amounts of each of these ingredients depend on the availability at the time of manufacture of the diet. This would result in variability in taurine concentration since most of the ingredients are of plant origin and the amount of fish meal, for example, included in any given batch of food would affect the taurine concentrations for that particular mixture. Wherever possible animals were maintained on the same stock of food for the duration of the study to reduce variation due to dietary taurine. Most of dietary taurine is reported to be excreted directly into the urine if it is in excess of the animals requirements. Huxtable and Lippincott (1982) reported that rats maintained on a diet providing 0.05 - 0.1% by weight of taurine, resulted in 70% of urinary taurine being derived from the diet and 30% from biosynthesis.

Animals treated with ANIT and bromobenzene showed a significant reduction in their food intake but animals treated with allyl alcohol (0.075 ml.kg^{-1}) and lithocholate did not. The reduction in urinary taurine after dosing with ANIT could be partly accounted for by a reduction in food intake. This reduction in urinary taurine was, however, greater than that seen in animals which had been starved for 24 h. The reduced levels of urinary taurine seen after lithocholate and allyl alcohol (0.075 ml.kg^{-1}) could not be accounted for by the reduction in food intake since this did not occur.

Animals dosed with bromobenzene did not show a reduction in urinary taurine compared to control values. These animals might have been expected to have a reduced level of urinary taurine since bromobenzene (a) depletes glutathione (and therefore increases the demand for cysteine) and (b) resulted in animals eating less food. Indeed the lack of significant taurine reductions in the urine might suggest that there was leakage of taurine from necrotic hepatocytes which offset the reduction of urinary taurine which might have been expected if taurine synthesis was reduced.

ii. Kidney damage. Later studies showed that the administration of mercuric chloride to rats at a dose which caused severe kidney damage results in a significant reduction in urinary taurine (Section 3.6). Kidney damage was not detected after the administration of compounds which either reduced or did not alter urinary taurine levels. Therefore, it seems unlikely that kidney damage was responsible for the reductions in urinary taurine that were seen.

iii. Depletion of glutathione (GSH). Cysteine is a common precursor for both taurine and GSH. Removal of GSH by conjugation with a xenobiotic may result in an increase in GSH synthesis and a reduction in cysteine available taurine synthesis, thus reducing levels of taurine.

The lower concentration of allyl alcohol (0.075 ml.kg^{-1}) produced a mild periportal necrosis and resulted in a decrease in the level of urinary taurine. This may have been the effect of an increase in the synthesis of GSH following its depletion by the metabolite, acrolein.

Bromobenzene also resulted in a slight reduction in urinary taurine although the difference was not significant. There was also a reduction in liver taurine levels, but again values were not significantly different from the control values. However, even 48 h after dosing there was a significant reduction in liver GSH levels after bromobenzene treatment. Therefore, despite the development of centrilobular necrosis, the failure of bromobenzene to result in an elevation of urinary taurine similar to that seen after treating animals with other compounds which cause hepatic

necrosis, could be explained by the depletion of GSH which precedes the development of necrosis. It is also worth noting that, unlike CCl_4 , bromobenzene has not been shown to inhibit protein synthesis, although staining liver sections from animals treated with bromobenzene, with Oil red O, indicated that there was an accumulation of fat around the portal tracts.

iv. Reduced bile flow. Taurocholate is not significantly metabolised when in the intestinal tract (Smith, 1966) and is absorbed in the distal part of the small intestine, without the taurine moiety being cleaved, 95% entering the enterohepatic circulation in man (Balistreri and Shaw, 1987). However, during cholestasis, the concentration of conjugated bile acids in the liver increases resulting in a depression of taurine synthesis (Hardison, 1978). The reduction of urinary taurine seen after treating animals with ANIT may have been the result of this reduced taurine synthesis.

v. Increase in protein synthesis. An increase in protein synthesis without concomitant degradation would reduce the amount of methionine and cysteine available for taurine synthesis. None of the compounds discussed in this section is known to stimulate protein synthesis, although the effect of hydrazine on protein turnover does not seem to be completely understood. However, phenobarbitone is known to increase the rate of protein synthesis. This was used in a later study as a biochemical tool for the induction of the mono-oxygenase system of enzymes responsible for the metabolism of CCl_4 . Urinary taurine levels were found to be decreased in animals treated with 70 mg.kg^{-1} phenobarbitone (i.p. on 4 consecutive days), as would be predicted if an increase in protein synthesis reduced taurine precursors. The results will be discussed in more detail in Section 3.3.

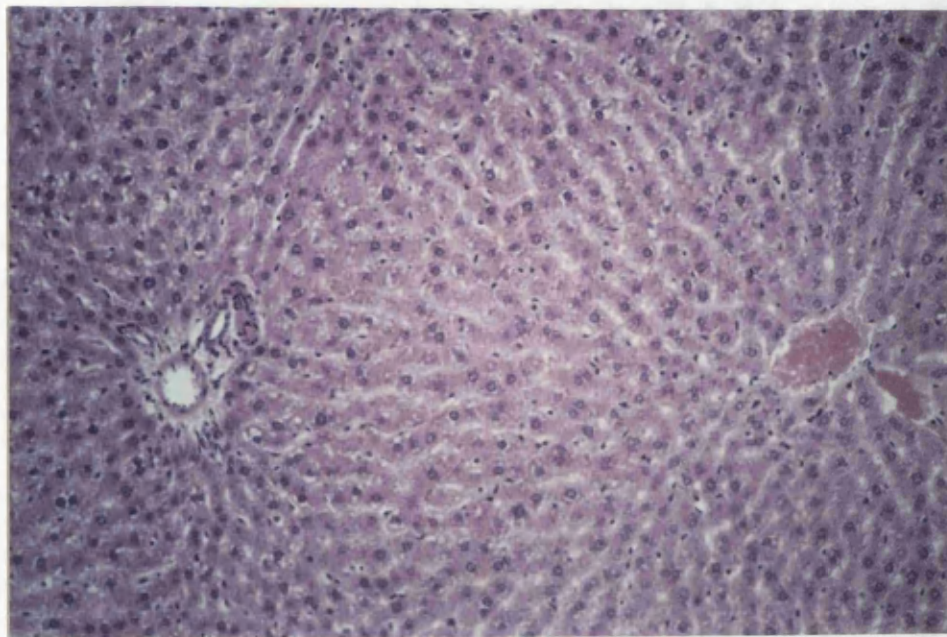


Plate 1. Light micrograph of liver section taken from a control fed male rat. Haematoxylin and eosin stain (H and E). Mag. x160.

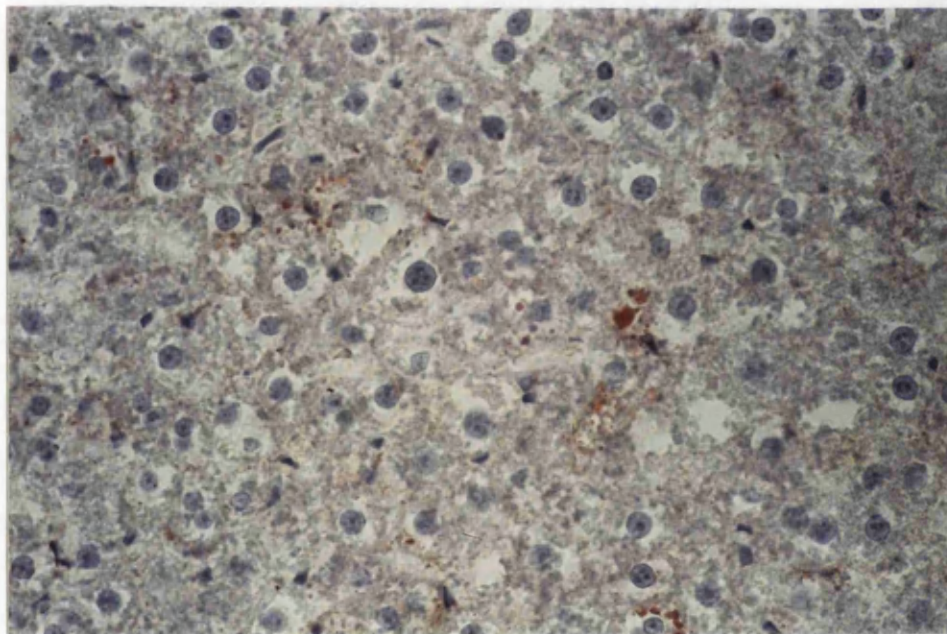


Plate 2. Light micrograph of frozen liver section taken from a control fed male rat. Oil Red O stain. Mag. x 400.

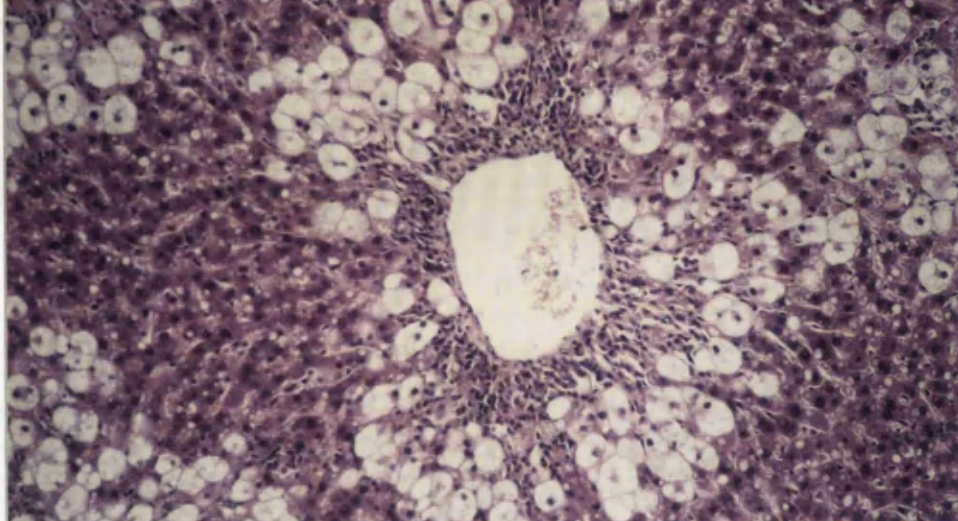


Plate 3. Light micrograph of liver section taken 48 h after dosing a male rat with CCl_4 ($2 \text{ ml.kg}^{-1} \text{ po}$). H and E stain. Mag. $\times 160$

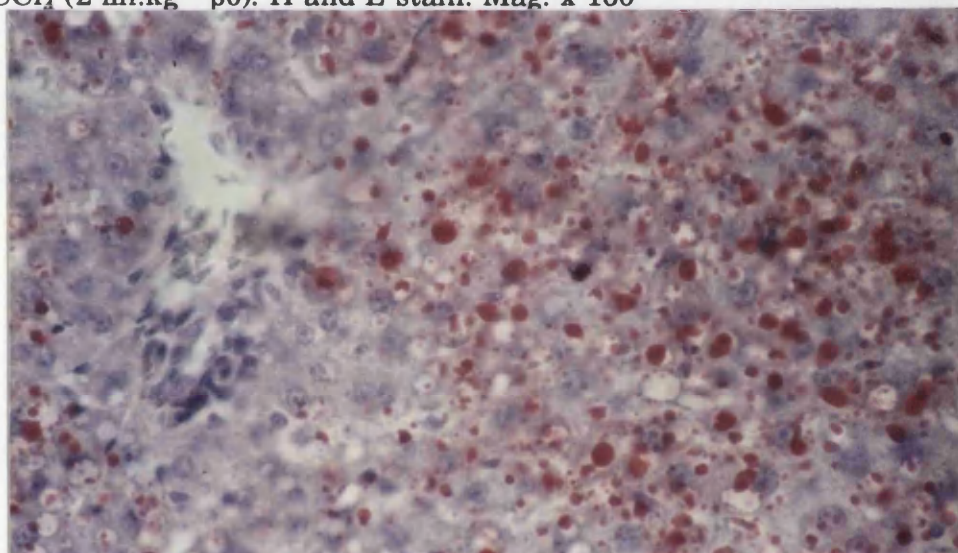


Plate 4. Light micrograph of frozen liver section taken 48 h after dosing a male rat with CCl_4 ($2 \text{ ml.kg}^{-1} \text{ po}$). Oil Red O stain. Mag. $\times 400$

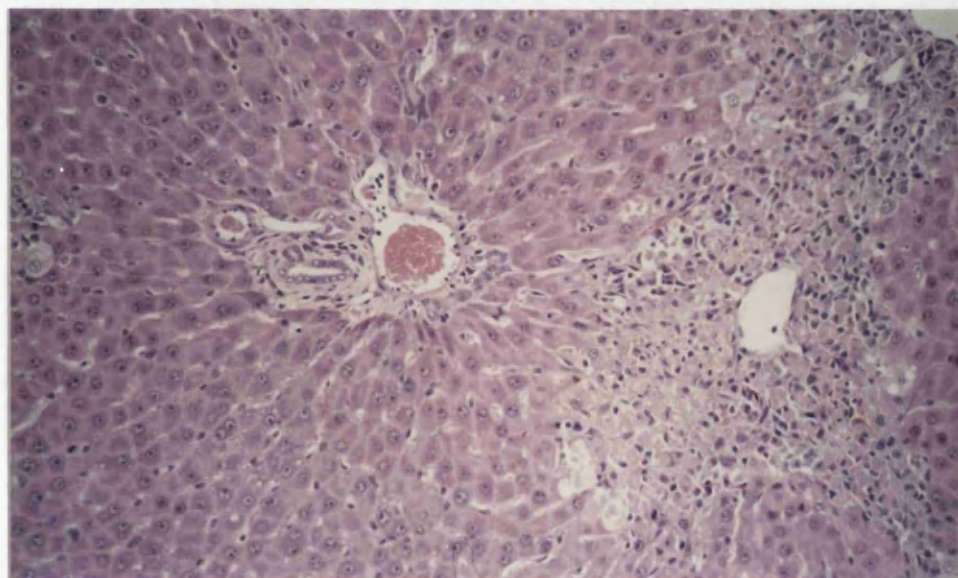


Plate 5. Light micrograph of a liver section taken from a male rat 48 h after dosing with thioacetamide ($150 \text{ mg.kg}^{-1} \text{ po}$). H and E stain. Mag. $\times 160$.

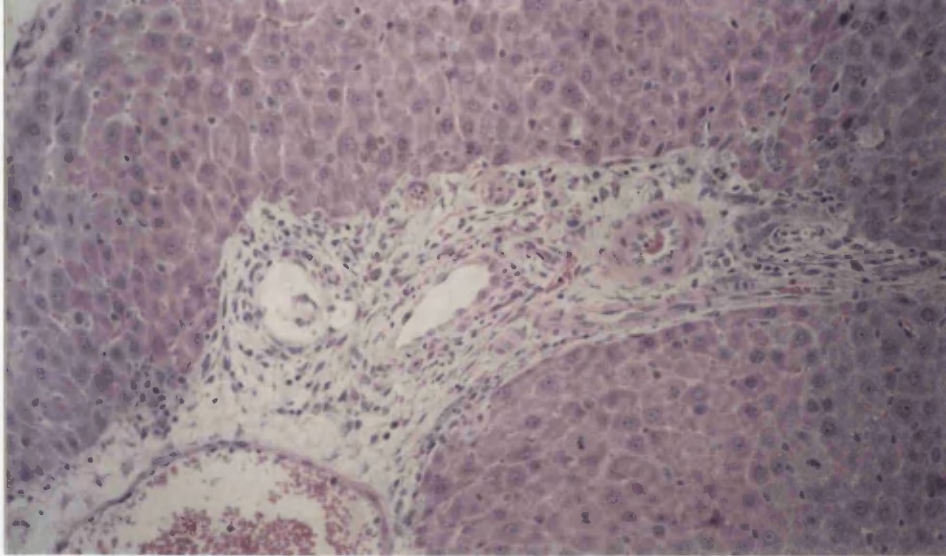


Plate 6. Light micrograph of a liver section taken from a male rat 48 h after dosing with ANIT ($150 \text{ mg.kg}^{-1} \text{ po}$). H and E stain. Mag. x 160.

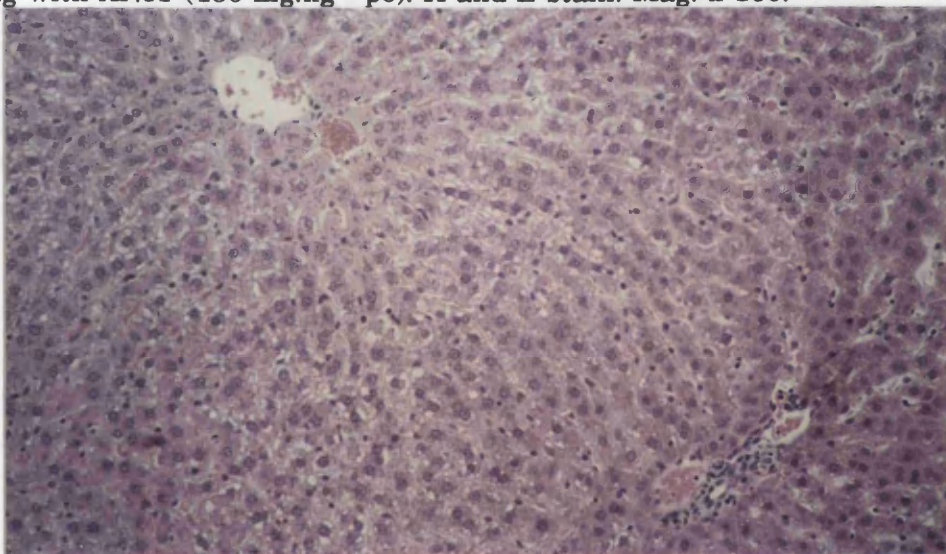


Plate 7. Light micrograph of a liver section taken from a male rat 48 h after dosing with hydrazine hydrate ($40 \text{ mg.kg}^{-1} \text{ free base. po}$). H and E stain. Mag. x 160.

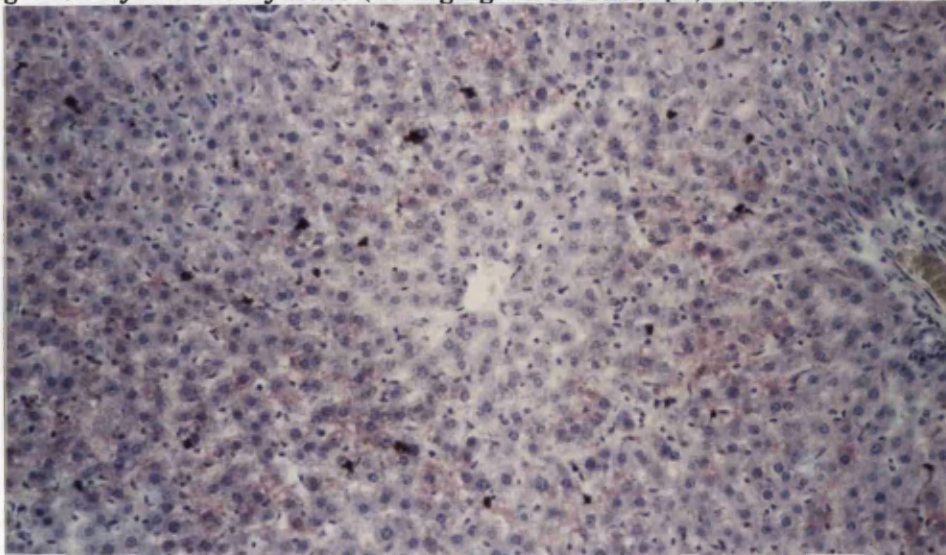


Plate 8. Light micrograph of a frozen liver section taken from a male rat 48 h after dosing with hydrazine hydrate ($40 \text{ mg.kg}^{-1} \text{ free base. po}$). Oil Red O stain. Max. x 160.

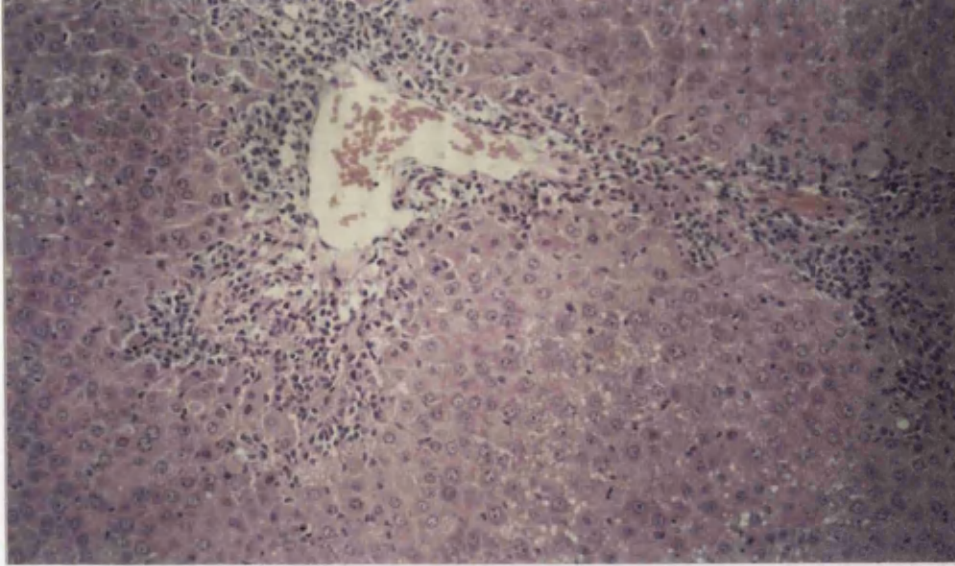


Plate 9. Light micrograph of a liver section taken 48 h after dosing a male rat with bromobenzene ($1 \text{ ml.kg}^{-1} \text{ po}$). H and E stain. Mag. x 160.

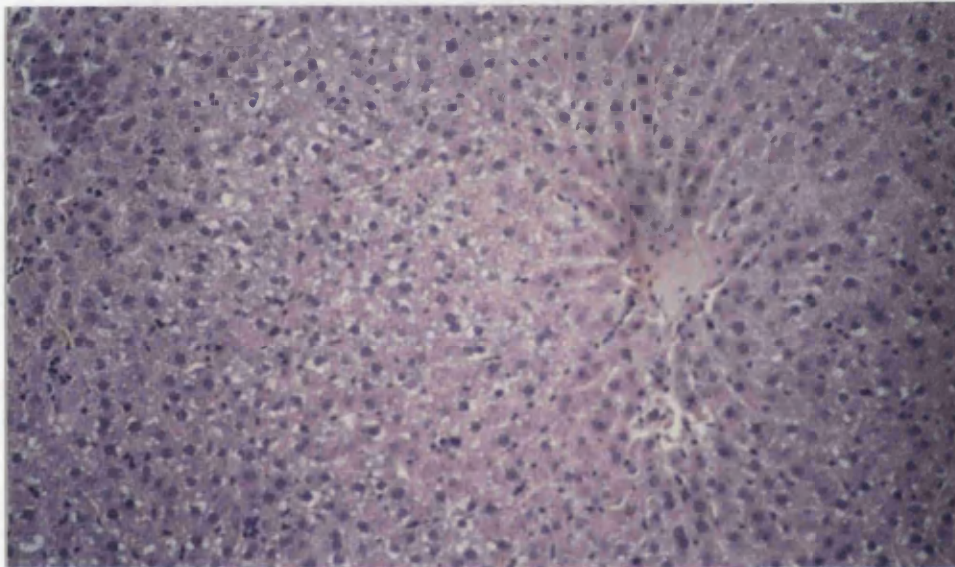


Plate 10. Light micrograph of a liver section taken from a male rat 48 h after dosing with ethionine ($200 \text{ mg.kg}^{-1} \text{ po}$). H and E stain. Mag x 160.

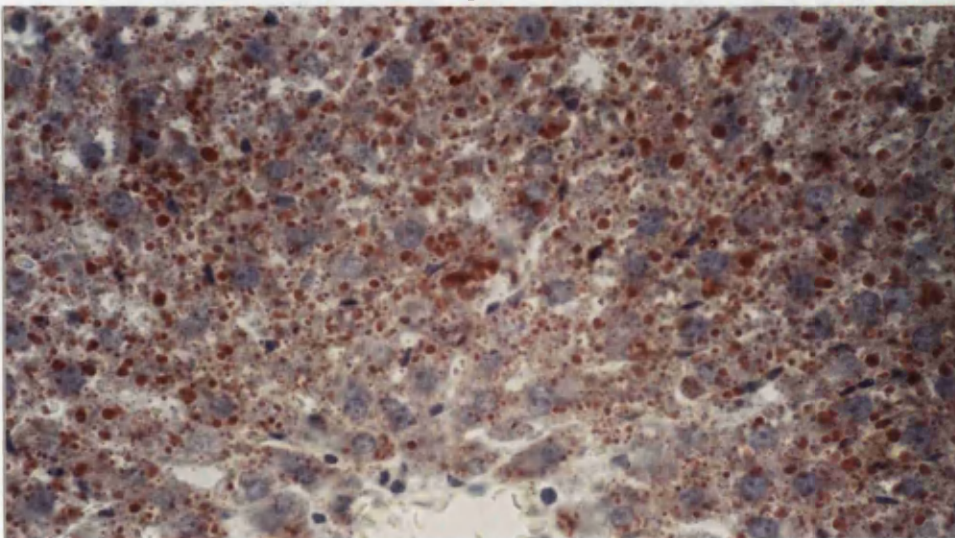


Plate 11. Light micrograph of a frozen liver section taken from a male rat 48 h after dosing with ethionine ($200 \text{ mg.kg}^{-1} \text{ po}$). Oil Red O stain. Mag x400.

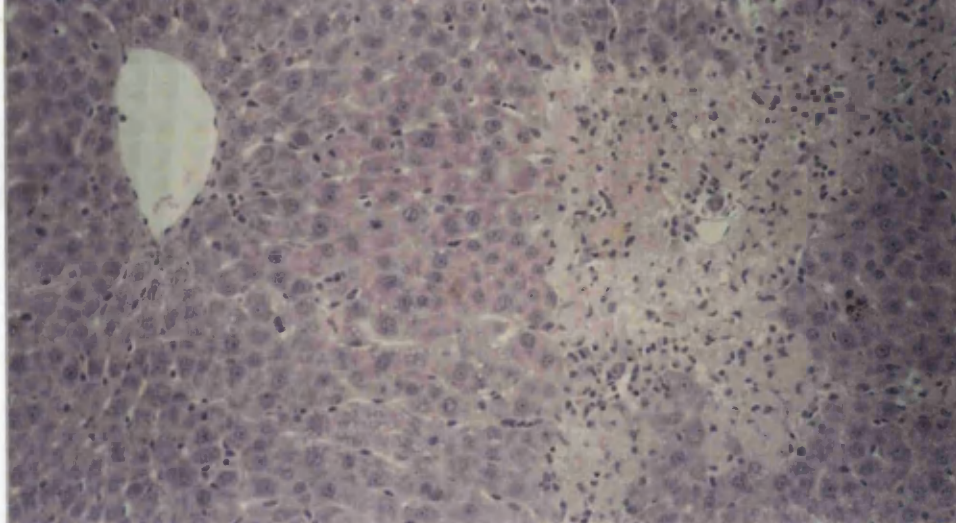


Plate 12. Light micrograph of a liver section taken from a male rat 48 h after dosing with allyl alcohol ($0.1 \text{ ml.kg}^{-1} \text{ po}$). H and E stain. Mag x 160.

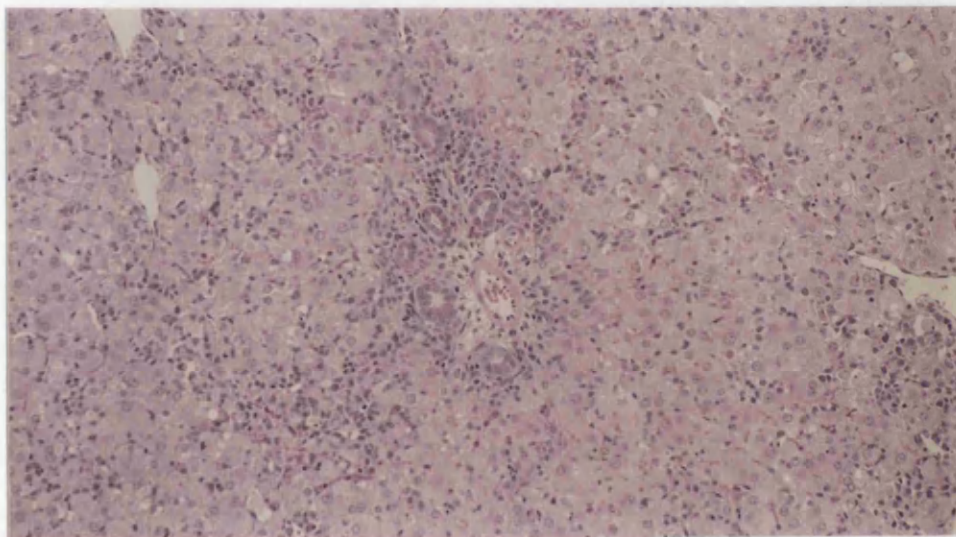


Plate 13. Light micrograph of a section of liver taken from a male rat 48 h after dosing with galactosamine ($500 \text{ mg.kg}^{-1} \text{ ip}$). H and E stain. Mag x 100.

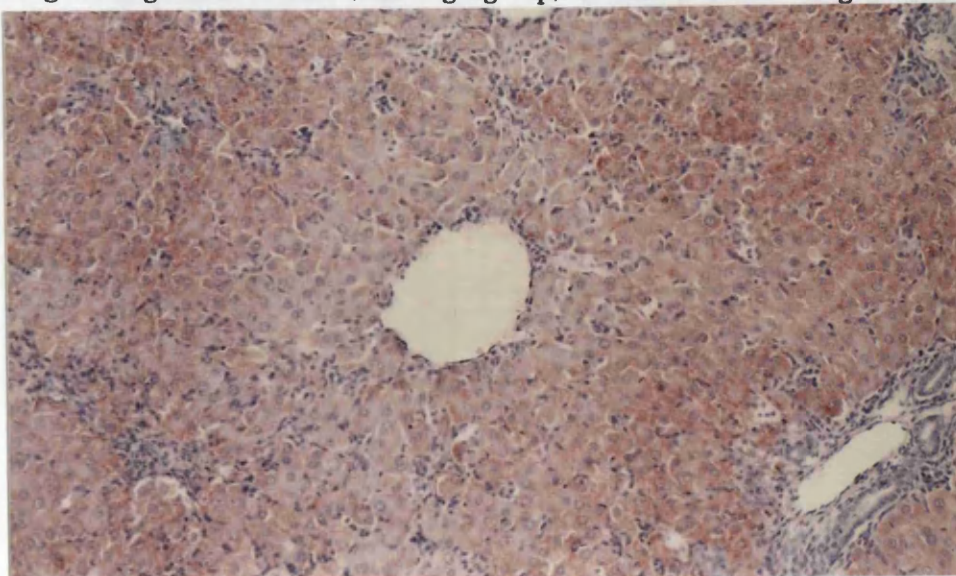


Figure 14. Light micrograph of a frozen liver section taken from a male rat 48 h after dosing with galactosamine ($500 \text{ mg.kg}^{-1} \text{ ip}$). Oil Red O stain. Mag x 100.

3.2

A DETAILED STUDY OF TAURINE LEVELS IN CARBON TETRACHLORIDE-TREATED RATS

3.2.1 AIM OF STUDY

To investigate the development of hypertaurinuria after CCl_4 toxicity in both male and female rats.

3.2.2 INTRODUCTION

The work presented in this section is a detailed study of urinary, liver and serum taurine levels in rats treated with CCl_4 . Carbon tetrachloride was chosen for more detailed investigations because it has been used extensively as a hepatotoxic compound to study the pathogenesis and character of hepatic necrosis and the effects of hepatocyte injury on liver function; it also produces predictable effects. The objective was to equate urinary levels of taurine with the severity and type of hepatic damage produced and to establish whether the observed elevation of urinary taurine in rats treated with CCl_4 was dose related.

3.2.3 METHODS

A. *Effect of CCl_4 on urinary taurine: dose response study.*

Male animals (4 groups of 4 animals each; 265-290 g) were housed in individual metabolism cages for 10 days. Two consecutive 24 h urine collections (48 - 24 h and 24-0 h) were made before dosing on the sixth day. Animals were dosed with CCl_4 (0.5, 1 or 2 ml.kg^{-1} in corn oil 1 ml.kg^{-1} , p.o.). Controls received vehicle only (1 ml.kg^{-1}). Urine was collected every 4 h after dosing for the first 24 h (0 - 4, 4 - 8, 8 - 12, 12 - 16, 16 - 20 and 20 - 24 h post dose), then three further 24 h collections were made (24 - 48, 48 - 72 and 72 - 96 h post dose) before the animals were sacrificed on day 10. Liver, kidneys, testes and blood samples were taken for biochemical and histological examination.

B. *Effect of CCl₄ on serum and liver taurine levels during the 24h period after dosing male rats.*

Male rats (220 - 320 g; 3 per group) were dosed with CCl₄ (2 ml.kg⁻¹; in corn oil 1 ml.kg⁻¹, p.o.). Control groups received vehicle only (1 ml.kg⁻¹). Groups of treated and control animals were killed after 1, 3, 6, 12, 18 and 24 h. Serum, liver and kidneys were taken for analysis of taurine, biochemical parameters and histology as previously described (Chapter 2).

C. *Comparison of the effect of CCl₄ on taurine levels in male and female rats.*

Male (10) and female (10) rats were housed in individual metabolism cages. On the third day 5 animals of each sex were dosed with CCl₄ (2 ml.kg⁻¹; in 1 ml.kg⁻¹ corn oil; p.o.). Control animals were given vehicle only (1 ml.kg⁻¹). Urine collections were made every 24 h, 48 - 24, 24 - 0 h pre-dose and 0 - 24, 24 - 48 h post-dose. Animals were killed 48 h after dosing and serum, liver, kidney and testes were taken for biochemical and histological analysis, as before.

3.2.4 RESULTS

A. *Taurine levels, clinical signs and liver histology in male animals treated with different doses of CCl₄.*

i. Urinary taurine levels.

The effect of different doses of CCl₄ on urinary taurine levels measured at 4-hourly intervals is shown in Figure 3.10. An increase in urinary taurine was observed after doses of 1 or 2 ml.kg⁻¹ with maximum urinary elevation 12-16 h after dosing. When the effect of various doses of CCl₄ on urinary taurine was measured at 24-hourly intervals (Figure 3.11), again hypertaurinuria was detected after doses of 1 and 2 ml.kg⁻¹. This elevated level of taurine was still detectable 4 days after dosing.

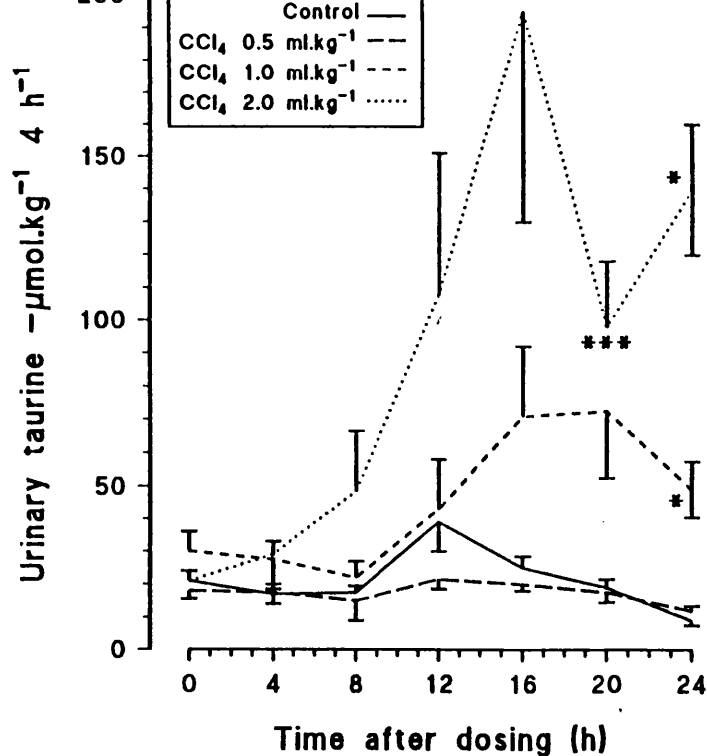


Figure 3.10 Urinary taurine levels in male rats dosed with CCl_4 (0.5, 1 or 2 ml.kg^{-1} , p.o). Values are mean total urinary taurine levels in the 4-hourly collection \pm SEM; Expressed/kg body weight; $N = 4$; * $p < 0.05$, *** $p < 0.001$.

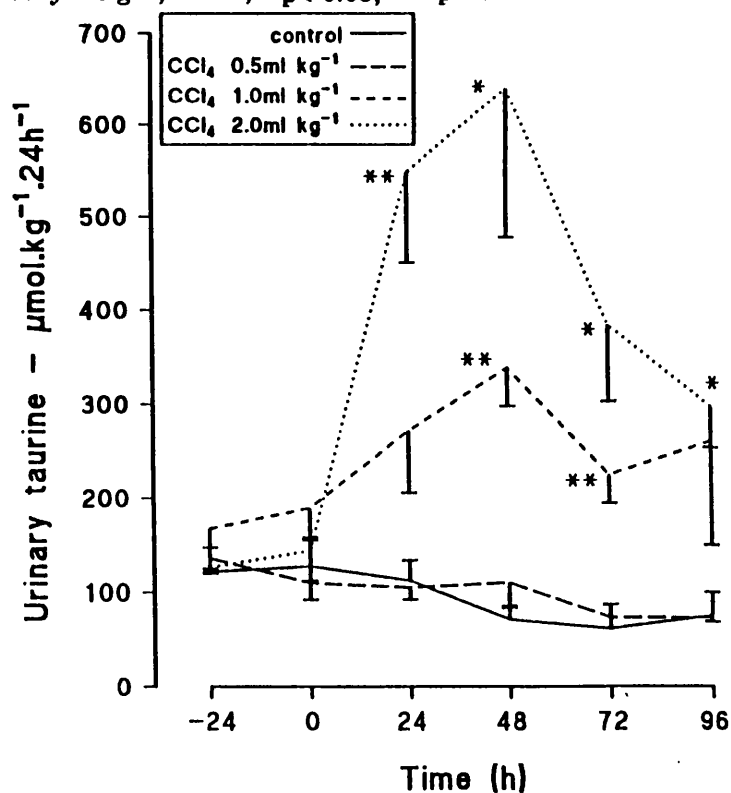


Figure 3.11 Urinary taurine in 24 h urine collections from male rats dosed with CCl_4 (0.5, 1 or 2 ml.kg^{-1} , p.o). Values are mean total urinary taurine levels \pm SEM; Expressed/kg body weight; $N = 4$; * $p < 0.05$, ** $p < 0.01$.

ii. Hepatic taurine levels.

Levels of taurine in the liver 96 h after dosing with CCl_4 (2 ml.kg^{-1}) were significantly lower than control values. After the lower dose of CCl_4 (1 ml.kg_4) total liver taurine was also reduced but not significantly (Figure 3.12).

iii. Urinary creatine.

Urinary creatine was elevated in rats given 1 and 2 ml.kg^{-1} CCl_4 0-72 h after dosing but only significantly 48-72 h after dosing with 2 ml.kg^{-1} (Figure 3.13).

iv. Clinical signs of toxicity.

All groups of animals treated with CCl_4 lost body weight during the 24 h following dosing (Figure 3.14). Liver weights (Table 3.8.) were significantly less 96 h after dosing with 1 ml.kg^{-1} CCl_4 and higher than control values after 2 ml.kg^{-1} but the difference was not significant. Serum AST and ALT levels 96 h after dosing were not significantly elevated above control levels (Figure 3.15), although one animal given 2 ml.kg^{-1} did have elevated levels (ALT - 178 and AST - 212 iu.L^{-1}). All treated animals reduced their food consumption significantly 24 h after dosing (Table 3.8).

v. Histology.

Light microscopy of liver sections 96 h after dosing showed no abnormal tissue from animals treated with 0.5 ml.kg^{-1} CCl_4 . There was no necrosis in the livers from animals given 1 and 2 ml.kg^{-1} CCl_4 although there was extensive fat accumulation around the central veins in both of the highest dose groups which was confirmed using Oil Red O stain (Plates 15 - 18). There was some evidence of repair to the parenchyma in livers from animals treated with 2 ml.kg^{-1} , as there were more hepatocytes undergoing mitosis than in other sections taken from livers of animals receiving the lower doses and there were more macrophages around the central veins.

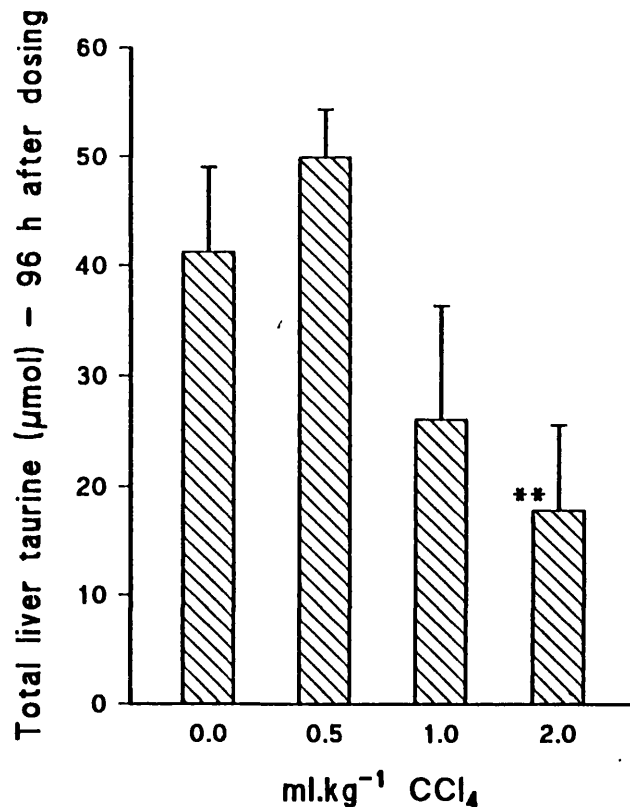


Figure 3.12 Liver taurine (expressed as total) 96 h after dosing with CCl₄ (0.5, 1 or 2 ml.kg⁻¹, p.o). Values are means \pm SEM; N = 4 except 2 ml.kg⁻¹, N = 3.

** p < 0.01.

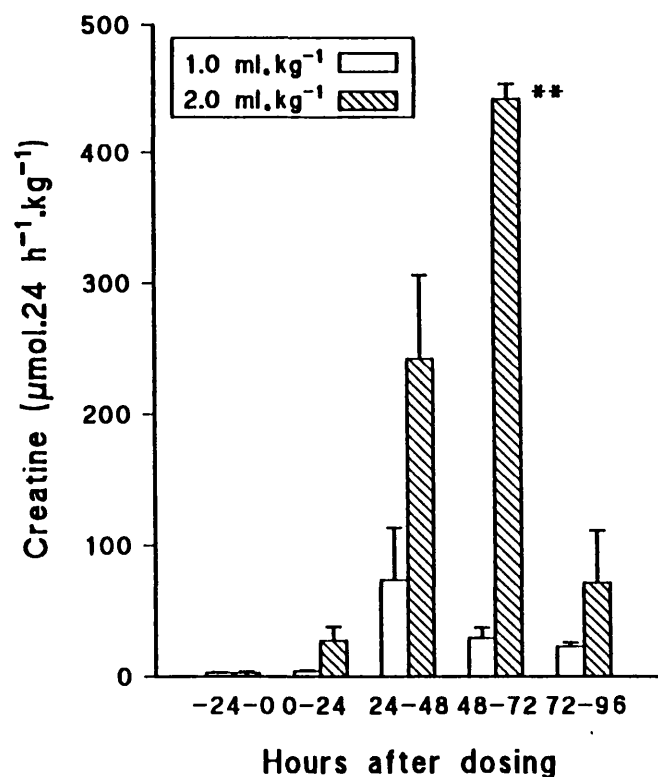


Figure 3.13 Urinary creatine after dosing with CCl₄ (1 or 2 ml.kg⁻¹ p.o). Values mean \pm SEM; N = 3 - 5, results are from more than one experiment; ** p < 0.01.

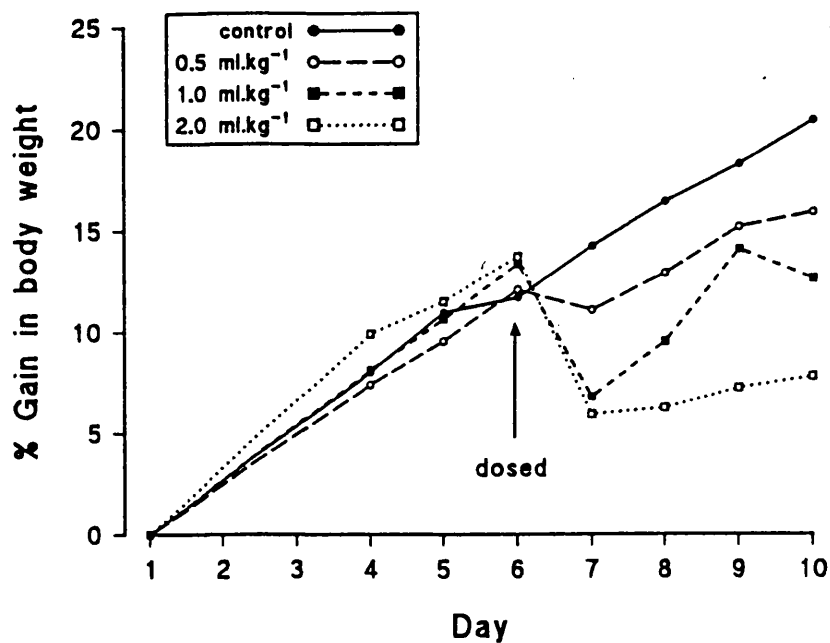


Figure 3.14 % Change in body weight of rats dosed with CCl_4 (0.5, 1 and 2 ml.kg^{-1} , p.o). Rats were dosed on day 6. Values are means of 3-4 animals.

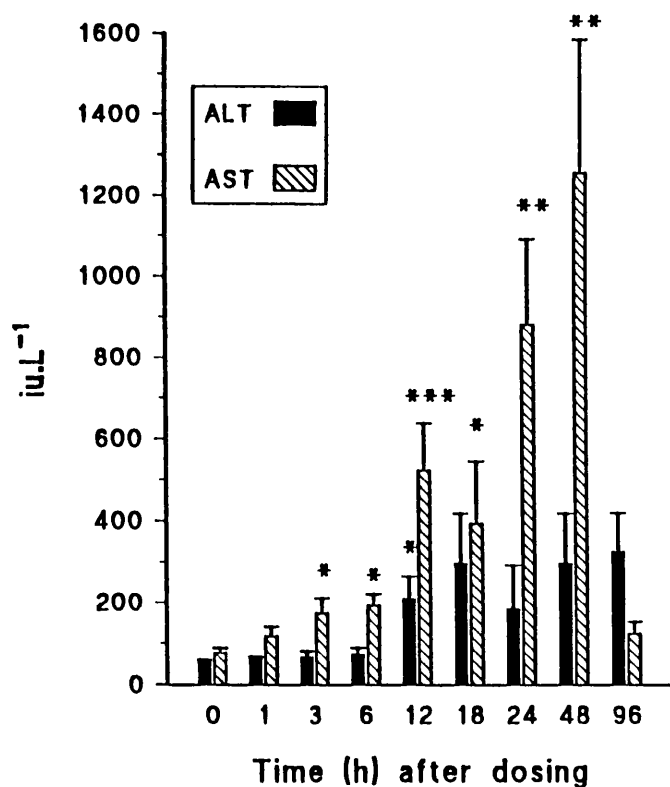


Figure 3.15 Serum ALT and AST after dosing rats with CCl_4 (2 ml.kg^{-1} , p.o) at different times post dose. Values are means \pm SEM; N = 3 - 5; results are from more than one experiment; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to same time control.

Table 3.8 Effect of various doses of carbon tetrachloride on food consumption, liver weight and serum transaminases in male rats

	Dose of CCl ₄ mLkg ⁻¹			
	0	0.5	1.0	2.0
<i>Food consumption (g/rat/day)</i>				
-24 - 0 h pre-dose	25.8 ± 2.6	32.4 ± 3.4	19.7 ± 6.5	29.0 ± 0.8
0 - 24 h post-dose	25.0 ± 1.5	19.3 ± 1.6*	10.8 ± 1.9**	4.4 ± 0.7**
24 - 48 h post dose	28.3 ± 0.6	24.0 ± 1.8	21.2 ± 2.0**	10.4 ± 3.5**
48 - 72 h post-dose	24.7 ± 0.5	23.4 ± 1.0	23.3 ± 3.1	21.3 ± 1.3**
72 - 96 h post-dose	29.1 ± 1.3	25.4 ± 1.5	19.4 ± 4.8	31.0 ± 4.1#
<i>Liver weight (% body weight)</i>				
96 h post-dose	4.07 ± 0.06	3.87 ± 0.09	3.65 ± 0.07*	4.44 ± 0.21#
ALT iu.L ⁻¹ 96 h post-dose	48.0 ± 3.3	41.0 ± 1.1	41.9 ± 2.5	92.0 ± 43.1#
AST iu.L ⁻¹ 96 h post-dose	53.6 ± 8.4	75.8 ± 3.1	70.3 ± 5.6	131.2 ± 40.8#

Values are means ± SEM; N = 4 except # where N = 3; * p< 0.05, ** p< 0.01

B. Effect of $2 \text{ ml.kg}^{-1} \text{ CCl}_4$ on serum and liver taurine levels, clinical chemistry and liver histology in male rats 0-24 after dosing.

i. Serum taurine levels.

Levels of taurine in serum of animals treated with CCl_4 (2 ml.kg^{-1}) remained higher than control values and showed a different profile over the 24 h period following dosing when compared with control levels (Figure 3.16).

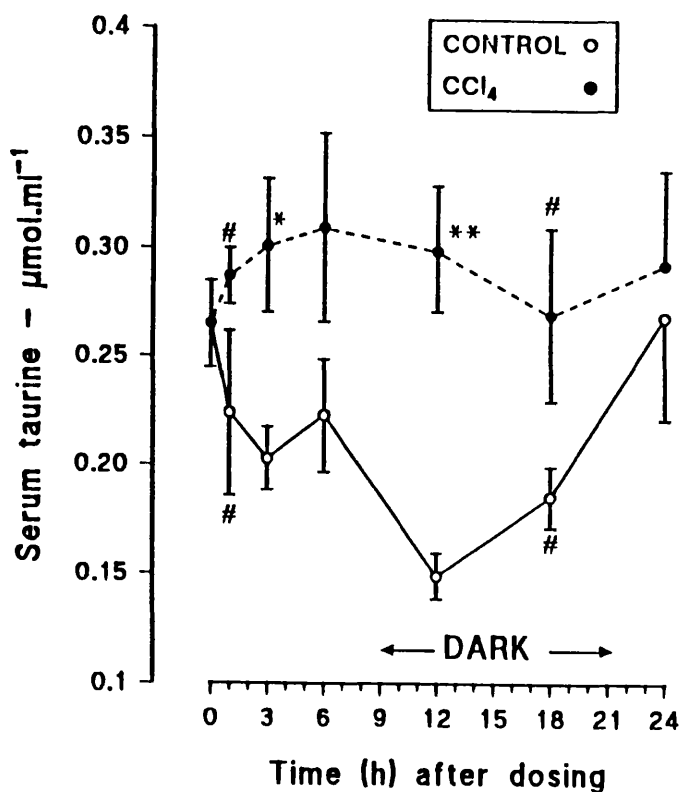


Figure 3.16 Serum taurine levels measured at various times over a 24 h period in control rats and those treated with CCl_4 (2 ml.kg^{-1} , p.o). Values are means \pm SEM from 6-10 male animals except # where $N = 3$. Data from 3 experiments. * $p < 0.05$; ** $p < 0.01$.

ii. Hepatic taurine levels.

Liver weights (% body weight) were significantly elevated 6, 12 and 18 h after dosing (Figure 3.17). The concentration of taurine (as well as total taurine content) in the livers of animals treated with CCl_4 (2 ml.kg^{-1}) were initially elevated above control values but declined over the 24 h period following dosing. The levels showed a different profile from controls (Figure 3.18).

The liver and serum levels of taurine in the control animals showed an interesting variation over a 24 h period which may represent diurnal variation (Figures 3.16 and 3.18). The levels of taurine in the liver were lowest around 5 p.m. (6 h post-dose) and at a maximum around 5 a.m. (18 h post-dose) and a minimum at 11 p.m. (12 h post-dose) and maximum at 11 a.m. (24 h post-dose) in the serum.

iii. Clinical chemistry.

Serum levels of ALT were significantly elevated at 12 h after dosing (Figure 3.15) and serum levels of AST were significantly elevated at 3, 6, 12, 18, 24 and 48 h after dosing. Serum cholesterol values were significantly lower 12 h after dosing (control: $1.55 \pm 0.10 \text{ mmol.L}^{-1}$; treated: $0.82 \pm 0.38 \text{ mmol.L}^{-1}$).

iv. Histology.

Light microscopy of liver sections showed lipid accumulation in hepatocytes around the central veins 3 h after dosing with 2 ml.kg^{-1} CCl_4 , which increased in amount during the 24 h following dosing. Hydropic degeneration of hepatocytes around the central veins was apparent 6 h after dosing and this had progressed to ballooning of cells by 12 h. Many hepatocytes showed eosinophilic cytoplasm after 18 h and by 24 h necrotic tissue had developed around the central veins when there was also extensive fat accumulation in centrilobular and mid-zonal hepatocytes (Plates 19 - 23).

Thus serum taurine was elevated in animals in which histology and serum enzymes indicated that there was hepatic damage.

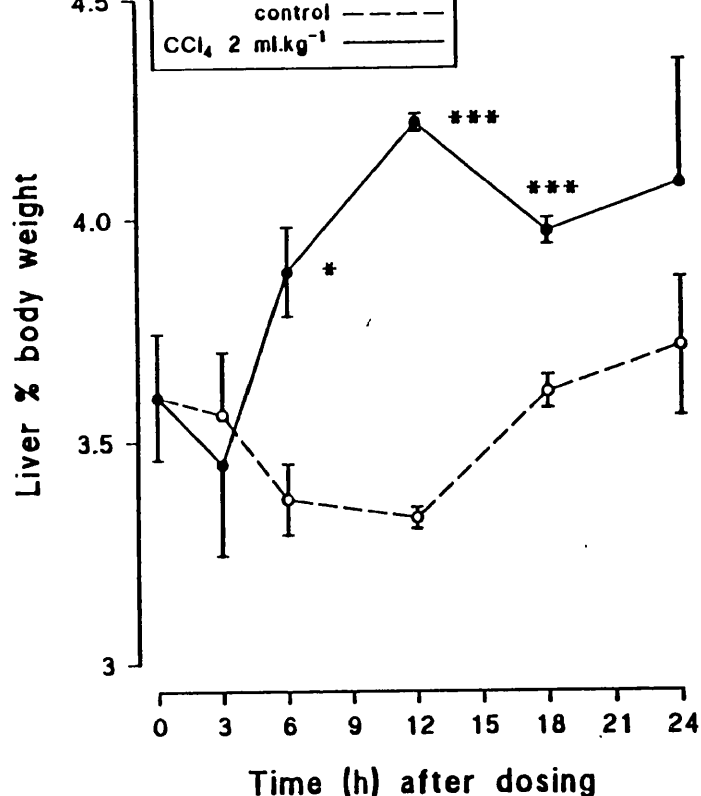


Figure 3.17 Liver weights (% body weight) of rats treated with CCl₄ (2 ml.kg⁻¹) at various times after dosing. Values are means \pm SEM; N = 3; * p < 0.05, *** p < 0.001.

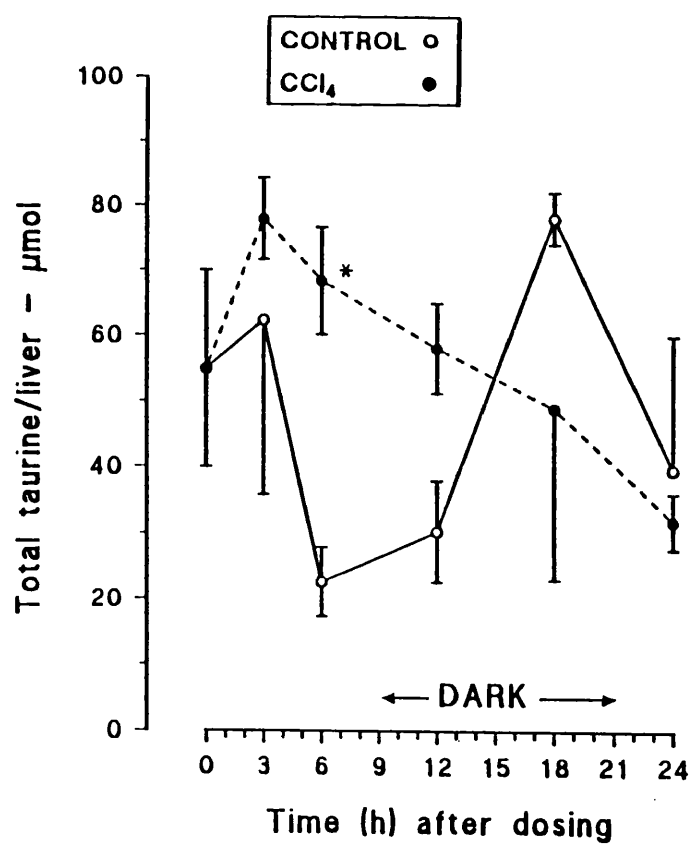


Figure 3.18. Total taurine content of the liver at various times over a 24 h period in control rats and those treated with CCl₄ (2 ml.kg⁻¹, p.o); Values are means \pm SEM; N = 3; * p < 0.05.

C. *A comparison of the effect of 2 ml.kg⁻¹ CCl₄ on urinary taurine levels, clinical signs and liver histology in male and female rats.*

i. Urinary taurine levels.

Table 3.9 shows that urinary taurine was significantly elevated 0-24 h after dosing both male and female rats with CCl₄ (2 ml.kg⁻¹). Levels were 250% of the control values in males and 257% of the control values in female animals. However, in the female animals, the level returned to pre-dose values between 24 and 48 h, whereas hypertauninuria was still observed in male rats 24-48 h after dosing.

ii. Hepatic taurine levels.

Levels of taurine in the liver 48 h after dosing were lower in males and significantly lower in female animals compared to the appropriate controls (Table 3.9).

iii. Serum taurine levels.

Serum levels of taurine were lower in treated male and female animals than in controls 48 h after dosing, but the differences were not significant (Table 3.9).

iv. Clinical indications of CCl₄ toxicity.

Treatment with CCl₄ caused a significant reduction in body weight and there was a significant increase in relative liver weight in both male and female animals (Table 3.9). There was no significant effect on relative kidney weight in either male or female animals after treatment. All treated animals, both male and female, had elevated serum AST levels and all but one female animal had elevated ALT levels in serum taken 48 h after dosing (Table 3.9).

Table 3.9 Effect of 2 ml.kg⁻¹ CCl₄ (p.o.) on liver and kidney weights, serum enzyme levels and urinary taurine levels in male and female rats

	Male		Female	
	Controls	Treated	Controls#	Treated
Liver taurine (μmol.g ⁻¹ wet weight)	8.3 ± 2.4	5.1 ± 2.1	9.1 ± 1.6	5.1 ± 0.8*
Liver weight (% body weight)	3.75 ± 0.07	5.19 ± 0.31*	3.98 ± 1.92	5.26 ± 0.34*
Kidney weight (% body weight)	0.71 ± 0.02	0.77 ± 0.02	0.78 ± 0.02	0.94 ± 0.12
Serum taurine (μmol.ml ⁻¹)	0.33 ± 0.9	0.25 ± 0.04	0.28 ± 0.07	0.23 ± 0.03
Urinary taurine (μmol.kg ⁻¹ 24 ⁻¹ h)	453.0 ± 44.0	302.0 ± 27.8	392.0 ± 53.0	486.0 ± 33.0
0-24 h	307.0 ± 71.4	761.6 ± 125.0*	485.0 ± 60.0	1242.0 ± 157.7**
24-48 h	367.8 ± 50.0	699.8 ± 50.6***	499.8 ± 66.0	567.1 ± 31.0
ALT iu.L ⁻¹	47.2 ± 2.7	123.0 ± 25.2*	43.0 ± 2.3	395 ± 145.5**
AST iu.L ⁻¹	114.8 ± 20.6	331.7 ± 105.9**	107.5 ± 17.0	848.0 ± 29.0**

Liver and blood were taken 48 h after dosing.

Values are means ± SEM; N = 5 except # where N = 4. * p< 0.05, ** p< 0.01, *** p< 0.001.

v. Histology.

Light microscopy of sections of liver taken 48 h after dosing showed lesions which were essentially as reported in Section 3.1.4.ii. However, the lesions were more extensive and severe in female animals than in male animals. Lipid deposition was present throughout the liver, there were fewer ballooned cells and necrosis was more extensive. Most of the hepatocytes had eosinophilic cytoplasm. Inflammation was concentrated around the central veins (Plate 24).

Two animals showed renal changes with lipid accumulation (Plate 25) and enlarged nuclei in the epithelial cells lining the proximal tubules; urinary protein was also raised.

Thus, there was a correlation between the extent of urinary taurine elevation 0 - 24 h after dosing, ALT, AST and the degree of liver damage.

3.2.5 DISCUSSION

The data indicate that urinary taurine was increased in a dose related manner when rats were treated with CCl_4 (Figure 3.19). For the reasons stated earlier (Section 3.1) it seems likely that the hypertaurinuria observed was probably due to damage to the liver. There was also a significant correlation between the AST level (plotted as \log_{10}) and the urinary taurine level (Figure 3.20). Furthermore, the two-fold greater taurinuria seen initially in the female animals is consistent with the greater hepatic damage observed in these animals. The different profile in the female animals with a more rapid return to normal urinary taurine values may reflect differences in the activity of cysteine sulphinate decarboxylase. The activity of this enzyme which catalyses the conversion of cysteine sulphinate to either cysteic acid (cysteine sulphonate) or hypotaurine before their conversion to taurine, is reported to be 16 fold less in female rats than in male rats (Worden and Stipanuk, 1985; Zelikovic and Chesney, 1989). Alternatively, the normal levels of urinary taurine seen 24 - 48 h after dosing female animals may have resulted from the kidney damage which was detected in two of the five animals treated. These animals had

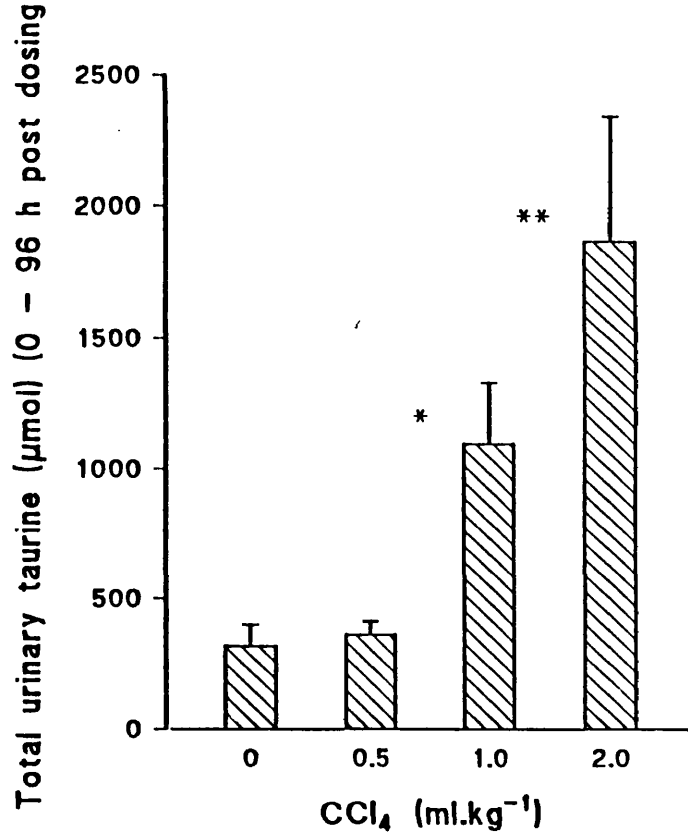


Figure 3.19 Total urinary taurine excreted by rats over the 96 h period following dosing with CCl₄ (0.5, 1 and 2 ml.kg⁻¹, p.o); Values are means \pm SEM; N = 4; * p < 0.05, ** p < 0.01.

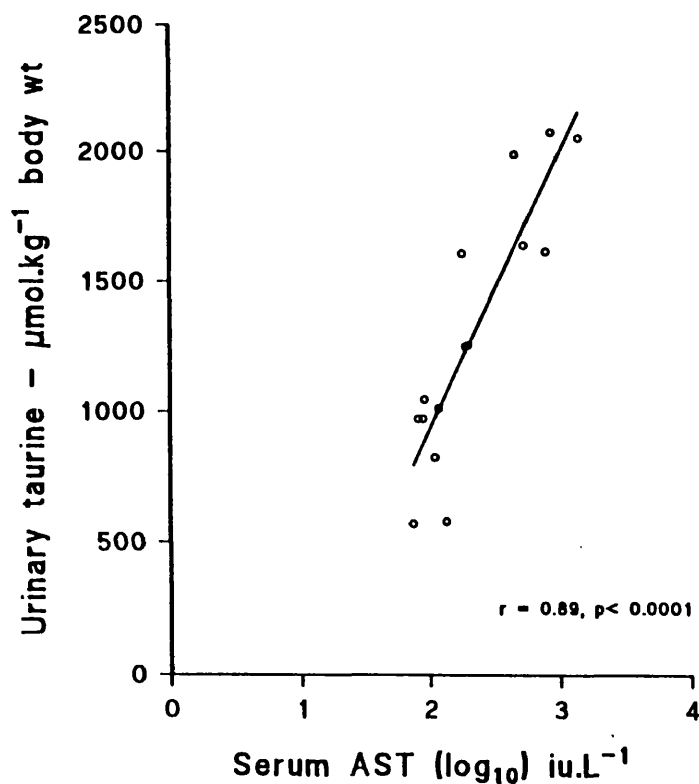


Figure 3.20 Correlation between urinary taurine and serum AST in rats treated with CCl₄ (2 ml.kg⁻¹ p.o). Urinary taurine measurements represent the total excreted in 0 - 48 h. AST was measured in serum 48 h after dosing. Each point is the data from one animal. $r = 0.89$, $p < 0.0001$.

proteinuria (values 2 X and 3 X control values) and lipid accumulation in the kidneys (detected by Oil red O staining). Later investigations (Section 3.6) showed that kidney damage induced by the administration of mercuric chloride to rats caused hypotaurinuria. Therefore, the reduction in urinary taurine 24 - 48 h after dosing female rats could have been due to the development of kidney damage which was not seen in male animals treated with CCl_4 . However, an elevation of serum taurine levels might have been expected if taurine was unable to be excreted via the kidneys and this was not seen. Thus the hypertaurinuria seen with CCl_4 is probably the result of hepatocellular damage and increased permeability, allowing taurine to leak from hepatocytes into the bloodstream. The elevated levels of taurine in serum are consistent with this hypothesis. This would be analogous to the leakage of marker enzymes such as AST and ALT from hepatocytes. This is made possible by the fact that taurine constitutes the major free intracellular amino acid and the male rat liver has a high synthetic capacity for taurine resulting in a large pool. There was, however no significant difference in the serum levels of taurine 48 h after treatment, which is in contrast to the elevations of serum ALT and AST. This was not unexpected in view of the high renal clearance of taurine (Chesney, 1985).

As taurine undergoes active re-uptake in the kidney, the increased amount in the glomerular filtrate may have saturated this uptake system and the excess overspilled into the urine. However, if the taurine was arising simply by leakage from the damaged liver cells, there should be a significant loss of taurine from the liver tissue of the same order as that in the urine to account for this. The data showed that this was not the case. Although the hepatic content of taurine is reduced after dosing, in males this could only account for 8% (after 48 h) and 2% (after 96 h) of the total increase in urinary taurine in that time period. Indeed male animals continued to have raised levels of urinary taurine 96 h after CCl_4 (2 ml.kg^{-1}) despite the fact that damage was diminishing. In the female rats there was a greater loss of hepatic taurine, 25% of which could be accounted for in the urine. Urinary taurine returned to normal levels in female animals 24 - 48 h post

dose which may reflect the greater degree of hepatic damage seen in these livers as well as underlining the lower synthetic capacity of female rats. The data suggested that taurine was being synthesised as a result of the toxic insult either in the liver where it would continue to "overspill" into the urine from the "leaky" hepatocytes or from other tissues, as a result of a perturbation in amino acid metabolism. This could have resulted in raised serum taurine levels above control values 3 - 12 h after dosing and been excreted directly.

A comparison of the profile of liver taurine levels in control and treated animals 24 h following dosing with CCl_4 (2 ml.kg^{-1}) suggest that there was an increase in taurine synthesis. The concentration of taurine (and total liver taurine) was greater than in control livers until 12 - 18 h after dosing. The reduction in liver taurine therefore, coincided with the significant elevation of AST and taurine in the serum, observations that suggest that hepatocytes were leaking enzymes and taurine.

However, a confounding factor may have been the fact that the treated animals ate little food in the 12 h period following dosing. This would have led to a reduction in bile flow which may have resulted in higher liver taurine levels during the first 12 h following treatment. This elevation may be similar to the raised levels of liver taurine seen more markedly after treatment with the cholestatic agent, ANIT (see 3.1).

Serum taurine was significantly raised within 3 h of dosing with CCl_4 and urinary taurine showed signs of elevation at 4 h although the latter was not significant. Thus taurine was probably being lost from the liver before there were overt signs of cellular damage but at a time (within one hour) when carbon tetrachloride is reported to reduce protein synthesis (Rechnagel and Glende, 1973 and Dianzani, 1991). The appearance of hydropic degeneration at 12 h after dosing coincided with the maximum elevation of serum taurine above control levels. The maximum urinary excretion in the first 24 h after dosing occurred slightly later at 12 - 16 h after dosing as would be expected.

The apparent diurnal variation in the taurine levels in liver and serum of control animals may have been the result of food intake. Taurine is conjugated with bile acids and secreted as the conjugate into the duodenum during periods of food ingestion, thus reducing liver taurine content (Hayes and Sturman, 1981). The increase in taurine levels by 5 a.m. may have been the result of increased food intake during the latter part of the dark-phase. Liver glutathione levels were reported by D'Amour and Charbonneau, (1992) to show a similar circadian rhythm in male rats. They also suggest that levels vary with food intake associated with the light cycle. The nadir being 1 h after the beginning of the dark phase, rising to the highest level early in the morning.

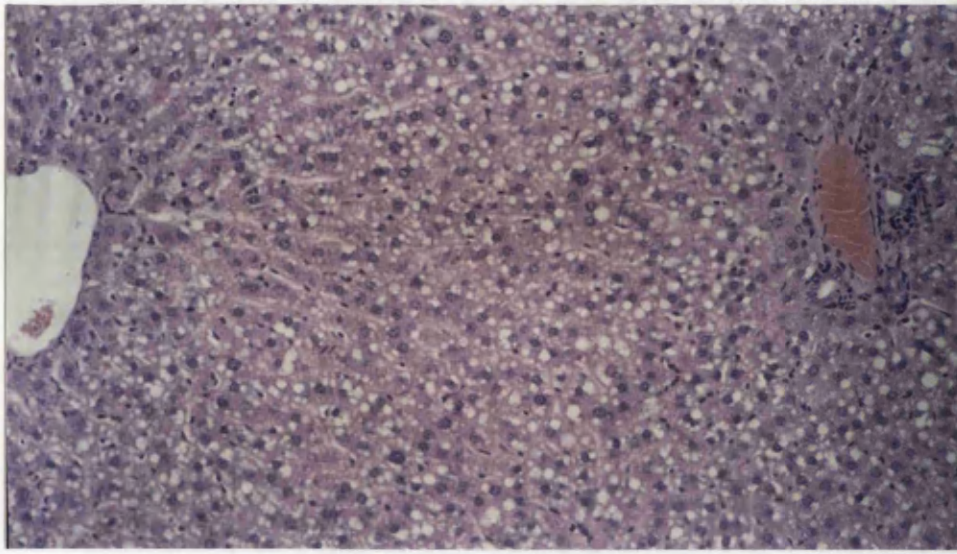


Plate 15. Light micrograph of liver section taken from a male rat taken 96 h after dosing with CCl_4 (2ml.kg^{-1} po). Haematoxylin and eosin stain (H and E). Mag. x 160.

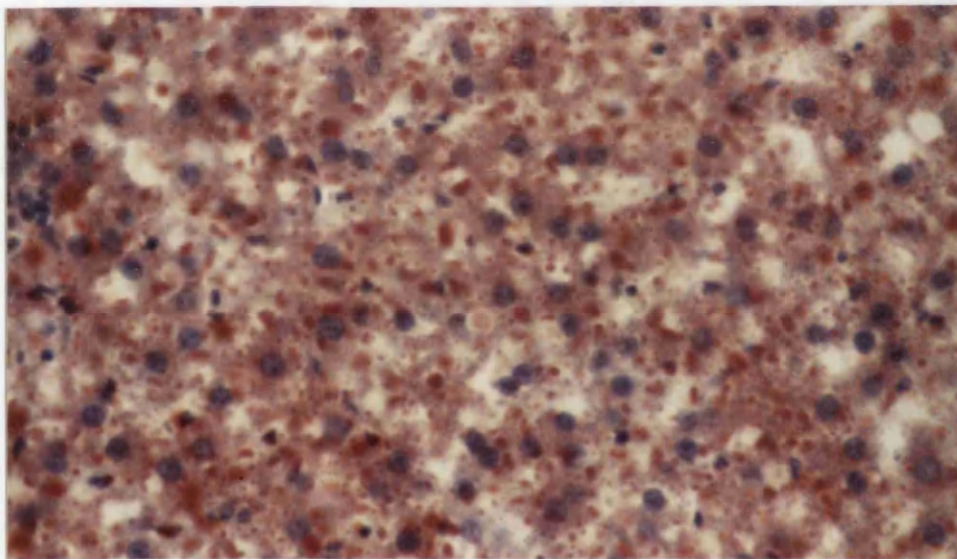


Plate 16. Light micrograph of frozen liver section taken from a male rat 96 h after dosing with CCl_4 (2ml.kg^{-1} po). Oil Red O stain. Mag. x 400.

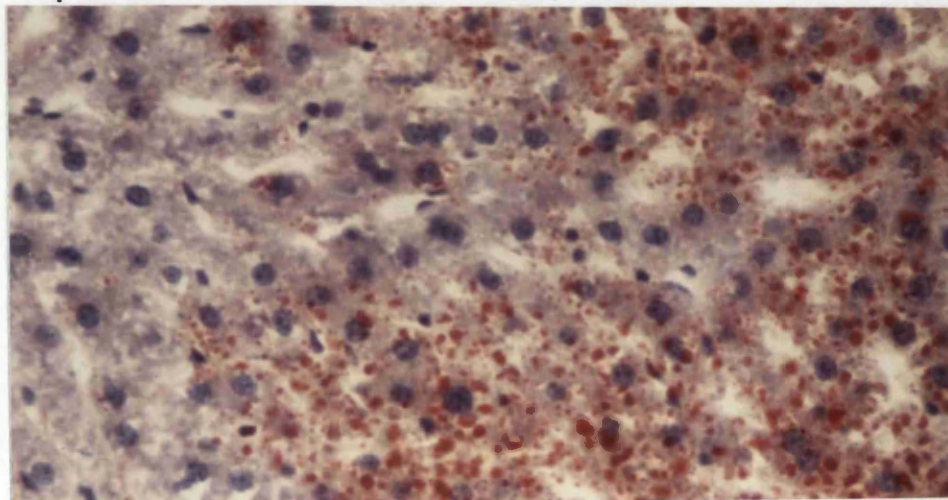


Plate 17. Light micrograph of a frozen liver section taken 96 h after dosing a male rat with CCl_4 (1ml.kg^{-1} po). Oil Red O stain. Mag. x 400

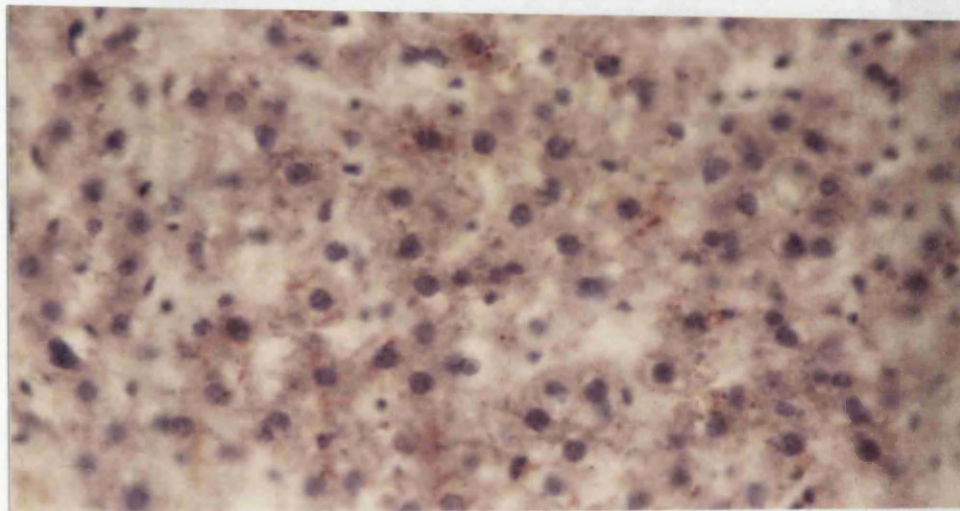


Plate 18. Light micrograph of frozen liver section taken 96 h after dosing a male rat with CCl_4 ($0.5 \text{ ml.kg}^{-1} \text{ po}$). Oil Red O stain. Mag. x 400

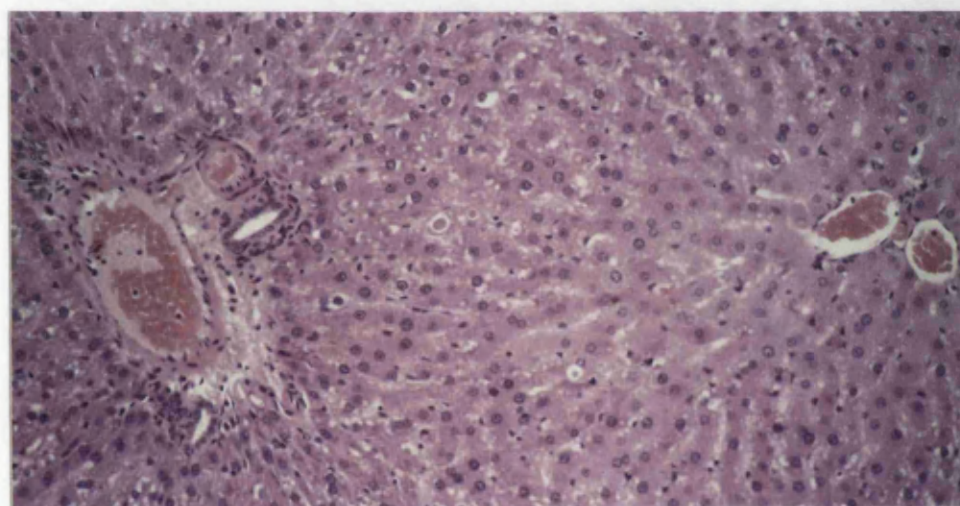


Plate 19. Light micrograph of a liver section taken from a male rat 3 h after dosing with CCl_4 ($2 \text{ ml.kg}^{-1} \text{ po}$). H and E stain. Mag. x 160.

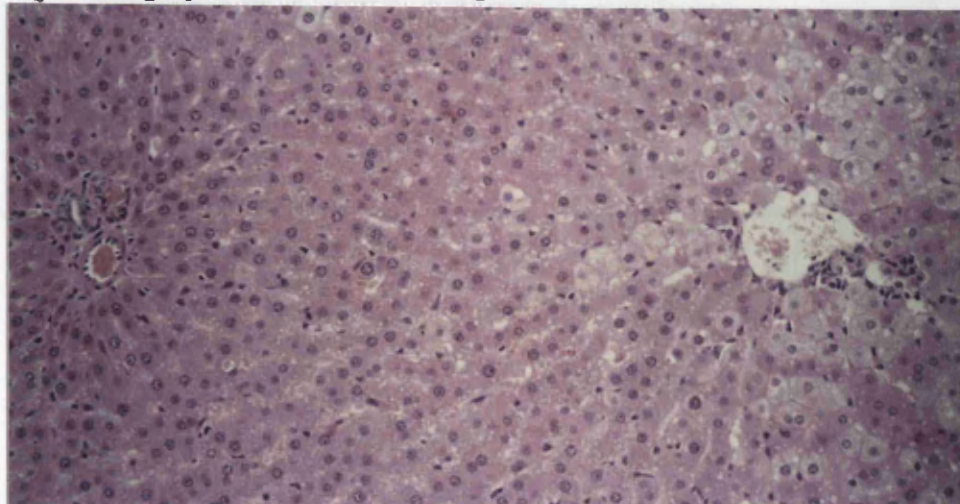


Plate 20. Light micrograph of a liver section taken from a male rat 6 h after dosing with CCl_4 ($2 \text{ ml.kg}^{-1} \text{ po}$). H and E stain. Mag. x 160.

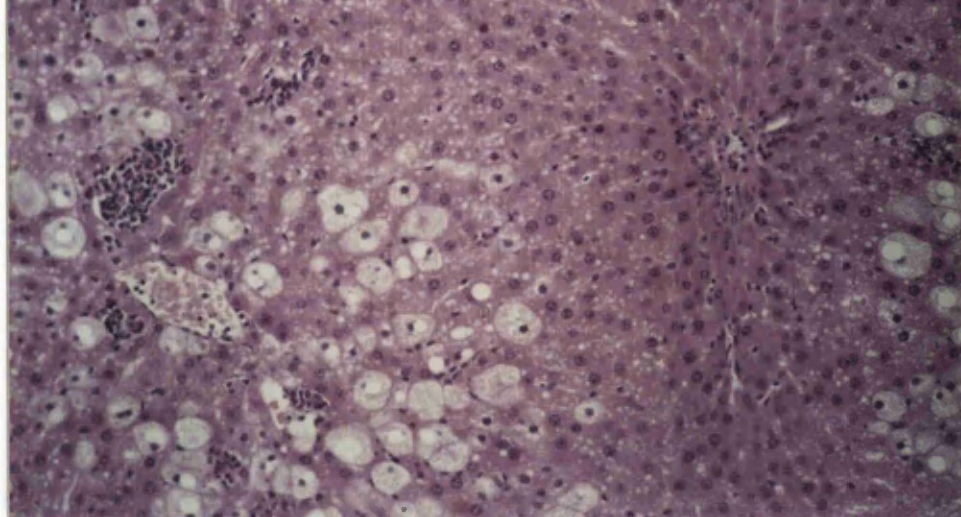


Plate 21. Light micrograph of a liver section taken from a male rat 12 h after dosing with CCl₄ (2ml.kg⁻¹ po). H and E stain. Mag. x 160.

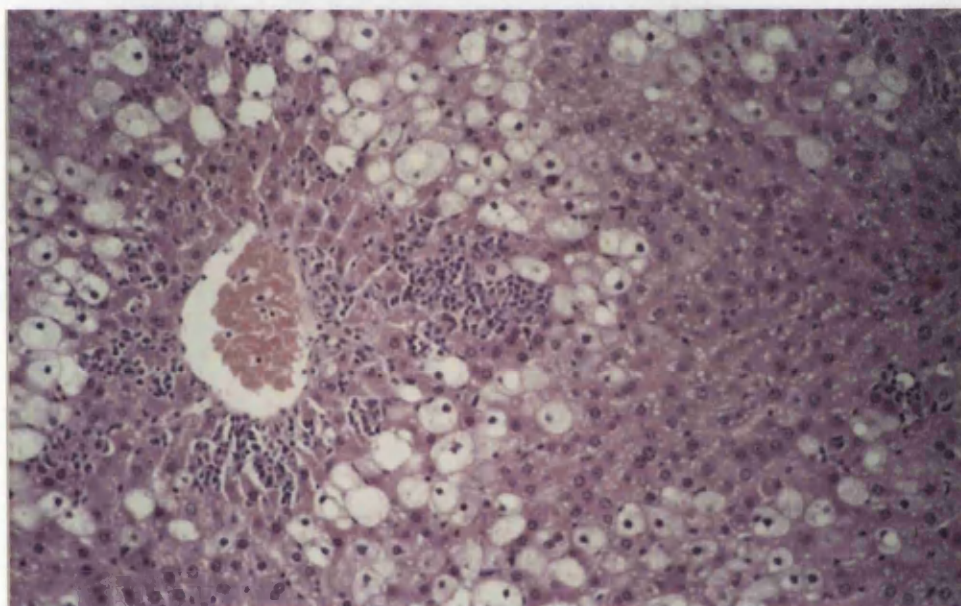


Plate 22. Light micrograph of a liver section taken from a male rat 18 h after dosing with CCl₄ (2 ml.kg⁻¹ po). H and E stain. Mag. x 160.

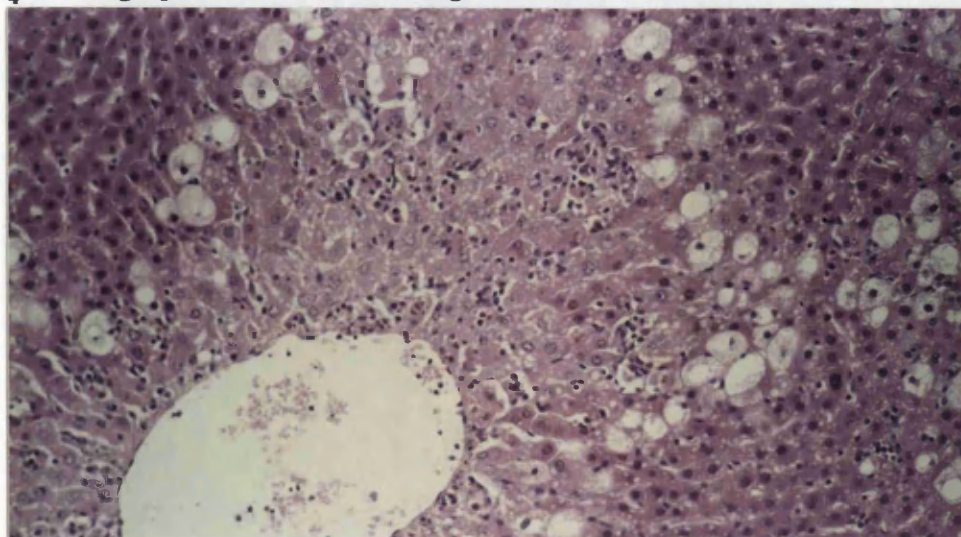


Plate 23. Light micrograph of a liver section taken from a male rat 24 h after dosing with CCl₄ (1 ml.kg⁻¹ po). H and E stain. Mag. x 160.

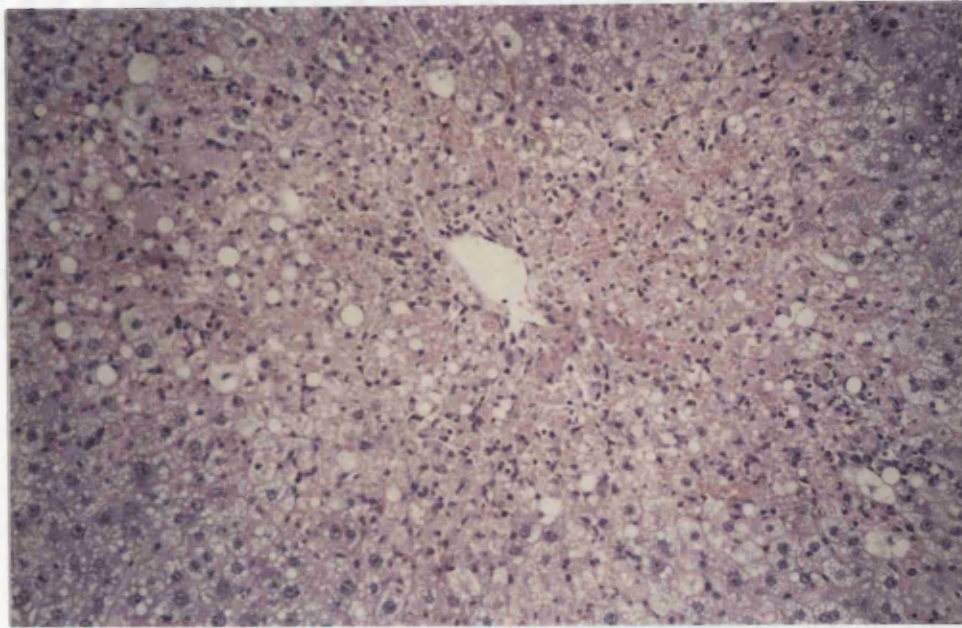


Plate 24. Light micrograph of a liver section taken from a female rat 48 h after dosing with CCl_4 ($2 \text{ ml.kg}^{-1} \text{ po}$). H and E stain. Mag x 160.

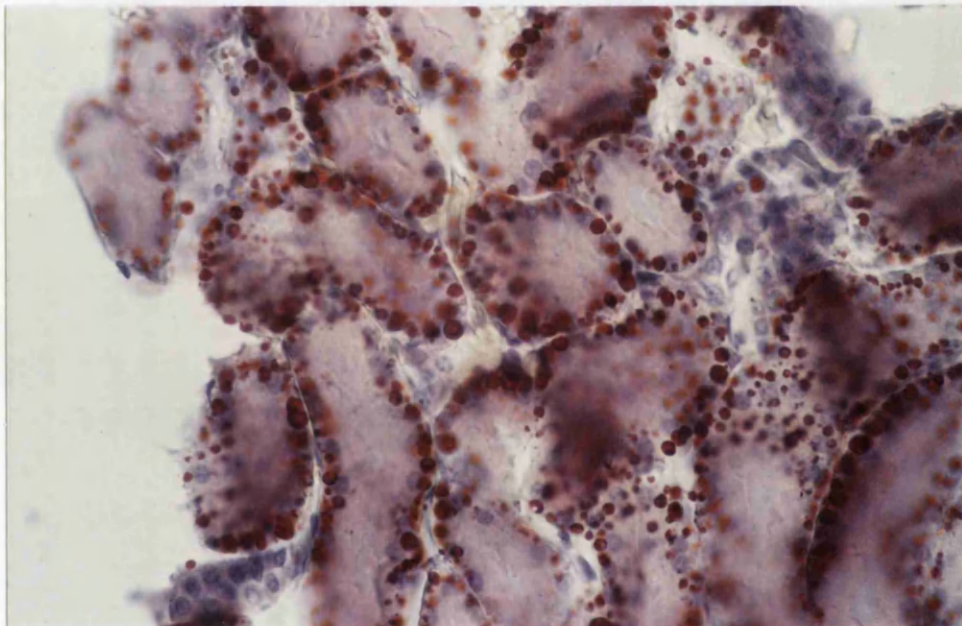


Plate 25. Light micrograph of a frozen kidney section taken from a female rat 48 h after dosing with CCl_4 ($2 \text{ ml.kg}^{-1} \text{ po}$). Oil Red O stain Mag x 400.

3.3

AN INVESTIGATION INTO THE EFFECTS OF SUB-CHRONIC ADMINISTRATION OF CCl_4 ON URINARY TAURINE LEVELS - INDUCTION OF A FIBROTIC/CIRRHOTIC LIVER LESION

3.3.1 AIM OF STUDY

Previous investigations (3.1, 3.2 and later work 3.4) were concerned with the levels of urinary taurine following acute toxicity induced by a single dose of a hepatotoxic compound. The aim of the following investigations was to investigate urinary taurine levels during and after the induction of a sub-chronic pre-cirrhotic fibrotic lesion by multiple dosing with carbon tetrachloride.

3.3.2 INTRODUCTION

Dosing animals with carbon tetrachloride (twice weekly) is known to produce fibrosis and a cirrhotic type of lesion in rats. However, this method of inducing fibrosis/cirrhosis has serious drawbacks (McLean *et al.* 1969) in that it requires a long time to develop the non-reversible lesion, many animals die intercurrently and a proportion of the rats that survive do not develop cirrhosis even after prolonged dosage. For this reason phenobarbitone (Pb) has been used by other investigators to induce the CCl_4 metabolising cytochromes P_{450} (McLean *et al.* 1969, Trivedi and Mowat, 1983), after which much lower doses of CCl_4 are required for a shorter period of time to induce the lesion.

However, as the Pb induction of the P_{450} system relies on DNA-dependent RNA synthesis requiring an increase in protein synthesis (Paine, 1981) it was possible that the Pb might reduce the synthesis of taurine by utilising cysteine for protein synthesis rather than taurine. The reverse effect has been discussed in 3.1, 3.2 and 3.4 as a possible contributory factor in the elevation of urinary taurine following dosing with hepatotoxic compounds known to inhibit protein synthesis. Phenobarbitone has also been reported to increase bile flow by 28% (Levine, 1974). There was therefore the possibility that the treatment of animals with this inducing agent may alter

liver levels of taurine by increasing the amount of taurine conjugated bile salts and thus altering urinary taurine excretion.

A preliminary investigation was therefore carried out to study the effects of Pb on urinary taurine levels.

3.3.3 AN INVESTIGATION INTO THE EFFECT OF PHENOBARBITONE (Na) TREATMENT ON URINARY TAURINE LEVELS IN RATS

3.3.4 METHOD

Two groups of 4 male rats (260 - 330 g) were housed in individual metabolism cages and allowed to acclimatize for 3 days. A 24 h pre-dose urine collection was made and then animals were dosed with phenobarbitone (Na) for 4 consecutive days (70 mg.kg^{-1} , in 2 ml.kg^{-1} saline, i.p.), or with 0.9% saline (2 ml.kg^{-1} , controls). Urine was collected as 24 h collections over a period of 9 days following the first dose of Pb. Animals were killed on day 9 and blood and tissue samples taken for analysis as before.

3.3.5 RESULTS

i. Urinary taurine

Urinary taurine was significantly lower (Figure 3.21) in Pb treated rats than in control animals, 3, 5 and 9 days after they were first dosed. After being in metabolism cages for 11 and 12 days, control animals had significantly lower urinary levels of taurine than in the first urine collection made after animals had been in metabolism cages for 2 days.

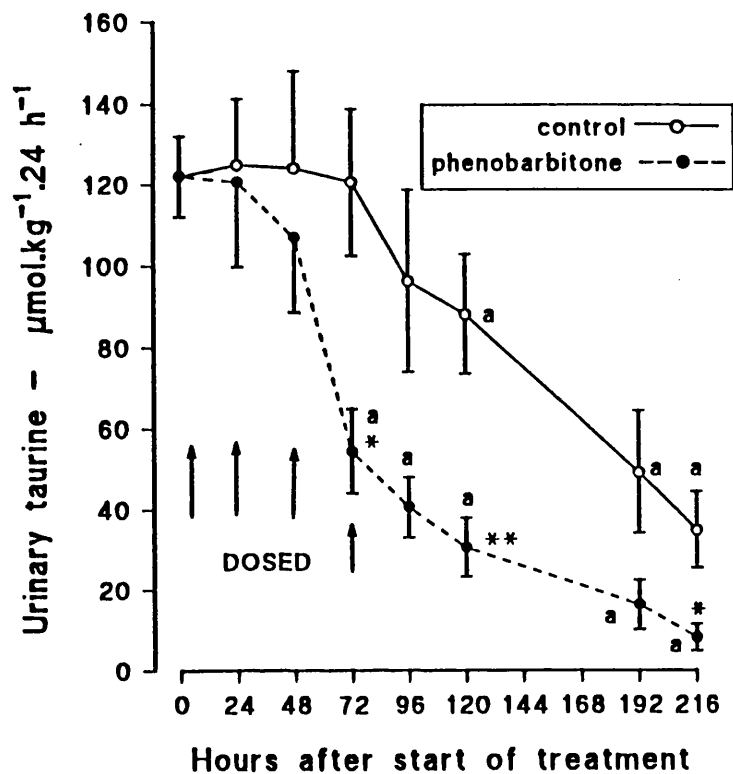


Figure 3.21 Urinary taurine from control rats (male) and rats given phenobarbitone (Na, 70 mg.kg^{-1} , i.p) on four consecutive days. ^a = $p < 0.05$ significantly different from start, * $p < 0.05$, significantly different from same day control

ii. Liver taurine

Livers removed at post-mortem, 6 days after the last dose of Pb was administered, showed an increase in weight above the control when expressed as %body weight, although the difference was not significant. Taurine concentration and total taurine content of livers from treated animals were lower in treated animals than controls (Table 3.10).

TABLE 3.10 **Liver weight and liver taurine after dosing with phenobarbitone (Na)**

TREATMENT	LIVER % BODY WEIGHT	TAURINE $\mu\text{mol.g}^{-1}$ wet wt LIVER
CONTROL	3.63 ± 0.13	5.05 ± 1.39
PHENOBARBITONE	3.86 ± 0.11	4.05 ± 0.08

Values are means \pm SEM, N = 4

iii. Clinical signs of toxicity

There was no difference between weight gain, food consumption or intake of water between the two groups of animals. All blood biochemical measurements in the treated animals were similar to control animals 6 days after the last Pb dose.

3.3.6 DISCUSSION

The aim of this investigation was to measure changes in urinary taurine as a result of Pb treatment and to follow any changes in urinary taurine for a period of 6 days after the last dose. It was not feasible to assess whether the Pb treatment had been effective in increasing protein synthesis 6 days after the last dose of Pb had been given. However, an effect observed by numerous investigators (Levine, 1974) after administration of Pb is an increase in liver weight, which may be 25% or more 24 h after the last dose of Pb. This observation is usually considered to be the result of increased

protein synthesis and possibly decreased protein catabolism. The synthesis of increased protein at the level of transcription involves increased production of mRNA (Zimmerman, 1978 and Paine, 1981). The increase in liver weight seen in this study was only 11% and was not significantly different from the control value. However, the livers were measured 6 days after the last dose of Pb, and may indicate that the level of dosing had been effective.

The reduction of liver taurine was not significant. A reduction in liver taurine concentration might have been expected as an increase in protein synthesis and possibly a reduction in protein breakdown, would probably, result in less cysteine and methionine being available for taurine synthesis. Kaplowitz *et al.* (1983) also demonstrated that phenobarbitone causes a 30% increase in GSH content in the liver and enhanced bile GSH output by 250%. Combining these effects with the reported increase in bile flow after Pb treatment which may have resulted in increased taurine conjugation and loss via bile secretion, the reduction in urinary taurine was not unexpected. Therefore, the method of inducing a cirrhotic type of lesion, by Pb induction, followed by treatment with CCl₄, was, unfortunately, not appropriate in this case.

3.3.7

AN INVESTIGATION INTO THE COURSE OF THE PRE-CIRRHOTIC LESION INDUCED BY MULTIPLE DOSING WITH CCl_4

A pilot study

3.3.8 METHOD

A pilot study was carried out to find an appropriate dosing regime for the induction of a pre-cirrhotic lesion by CCl_4 . It was necessary to establish the number of doses of CCl_4 and the progression of the hepatic liver lesion induced by multiple dosing by killing rats at different times after the beginning of the dosing regime.

Male rats (275 - 320 g) were divided into two groups. Six animals were treated and three were used as controls. They were housed in communal cages and 2 treated animals and 1 control animal were killed at three different time points during the course of the investigation. The livers were taken for histology and sections stained with elastic Van Gieson and reticulin stains to identify fibrotic tissue, Oil red "O" to stain for lipid, periodic acid Schiff to stain glycogen and haematoxylin and eosin. Treated animals were given CCl_4 (1 ml.kg^{-1} , in 1 ml corn oil, p.o.) and controls, corn oil (1 ml.kg^{-1} , p.o.), twice weekly on Tuesday and Friday. Groups of animals (2 treated and 1 control animal) were sacrificed 3 days after dose 4 and 8 and $4\frac{1}{2}$ weeks after dose 9 in order to assess the progress of the lesion and to determine whether it was progressive.

3.3.9 RESULTS

All treated animals showed a reduction in weight gain during the dosing period. The two treated animals which were killed $4\frac{1}{2}$ weeks after the last dose gained weight but did not reach the weight of the control animal (data not shown). The dosing procedure was terminated when one of the treated animals showed signs of kidney damage (blood in the urine). The livers taken from animals given 8 and 9 doses of CCl_4 had a roughened "pig skin"

appearance, even after 4½ weeks "recovery" period. They all showed an increase in fibrotic tissue which did not regress during the recovery period (Plates 26 and 27).

3.3.10 DISCUSSION

The lesion produced was fibrotic, with bridging collagen laid down between periportal tracts. Although the lesion was not progressive as would be expected in a true cirrhotic lesion the fibrosis did not appear to regress over the period studied. The lesion could be best described as "pre-cirrhotic".

Using the information gained in this pilot study on the development of the pre-cirrhotic lesion using carbon tetrachloride a second investigation was carried out to examine the urinary levels of taurine in rats treated in a similar way with sub-chronic administration of carbon tetrachloride.

3.3.11

AN INVESTIGATION INTO URINARY TAURINE LEVELS IN RATS DURING AND AFTER MULTIPLE DOSING WITH CCl₄

3.3.12 METHOD

Ten rats (male, 320 - 345 g) were separated into two groups. They were housed either in communal cages or individual metabolism cages when 24 h urine collections were made. Two control animals were not used for urine collection. The dosing regime followed in this study was similar to that carried out in the pilot study. Times of dosing and urine collections are shown in Table 3.11. All animals were killed 4½ weeks after the last dose of CCl₄.

Table 3.11 Table to show dosing and urine collection times during sub-chronic administration of CCl₄.

Sat	Sun	Mon		Tues		Wed		Thur		Fri
		7 Jan		8		<9>	→	<10>	→	11*
12	13	<14>	→	<15*>	→	<16>	→	<17>	→	18*
19	20	21		22*		23		24		25*
26	27	<28>	→	<29*>	→	<30>	→	<31>	→	1* Feb
2	3	4		5*		6		7		8
9	10	<11>	→	<12*>	→	<13>	→	<14>	→	15
16	17	18		19		20		21		22
23	24	<25>	→	<26>	→	<27>	→	<28>	→	1 Mar
2	3	4		5		6		7		8
9	10	<11>	→	<12>	→	<13>	→	<14>	→	15 P.M.

<X> = animals placed in metabolism cages; → = urine collection;

* = dosing CCl₄ (1.0 ml.kg⁻¹); P.M. = post-mortem, tissues taken for analysis.

3.3.13 RESULTS

i. Clinical signs of toxicity

Animal weight changes are shown in Figure 3.22. Animals treated with CCl_4 had consistently lower body weights compared to control animals, although they were similar in weight just before sacrifice, 4½ weeks after the last dose of CCl_4 . There were no indications of kidney damage measured either by clinical chemistry or by histological examination in any of the animals. All livers from treated animals had a "harder" feel than the control livers and were more difficult to trim for preservation. They had a roughened surface and were significantly heavier ($p < 0.01$) as % body weight than the control livers (Table 3.12). Kidney weights in both treated and control animals were similar.

Table 3.12 Changes in liver and kidney weights, liver taurine and serum enzyme levels after sub-chronic dosing with CCl_4 .

MEASUREMENT	CONTROL (N = 5)	TREATED (N = 5)
Liver weight g	13.22 ± 1.06	15.42 ± 1.04
Liver % body wt	2.86 ± 0.27	3.49 ± 0.32**
Kidney % body wt	0.68 ± 0.01	0.69 ± 0.03
Liver taurine ($\mu\text{mol.g}^{-1}$ wet wt)	4.58 ± 3.2	8.30 ± 1.3*
Total liver taurine (μmol)	59.9 ± 38.4	129.2 ± 27.3*
Serum ALP (iu.L^{-1})	149.0 ± 48.0	244.4 ± 32.0**
Serum ALT (iu.L^{-1})	49.2 ± 11.7	72.3 ± 8.4**

Values means ± SEM; * $p < 0.05$ and ** $p < 0.01$ compared to control values.

ii. Liver taurine

The taurine content in these livers was significantly greater in the treated animals and the total amount of taurine was also significantly greater as the livers in these animals were larger.

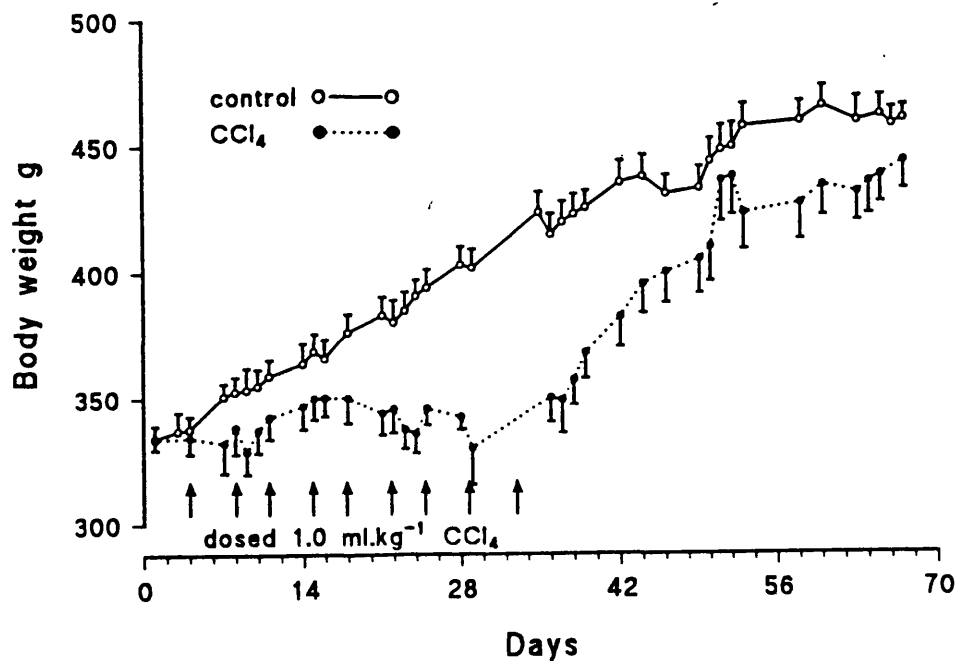


Figure 3.22 Body weights of control and treated rats during the period of multiple dosing with CCl₄ (Study 3.3.12). Each point is the mean weight of 5 animals \pm SEM. Dosing times are indicated by arrows.

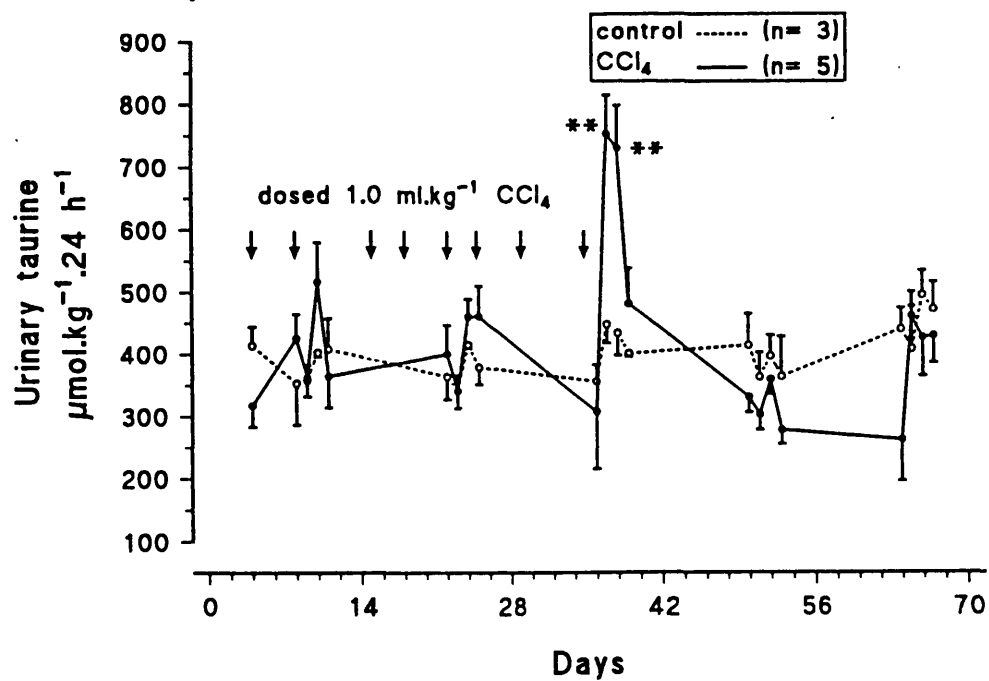


Figure 3.23 Urinary taurine in 24 h urine collections taken at various times during multiple dosing of rats with CCl₄ (Study 3.3.12). Each point represents the mean of 3 (control) or 5 (treated) animals \pm SEM. Arrows show time of dosing

** $p < 0.01$

iii. Urinary taurine

Urine analysis did not show an elevation of taurine during the period of multiple dosing, except in the two 24 h urine collections made after the last dose of CCl_4 . These two collections had significantly raised taurine levels compared to the pre-dose levels and control urinary taurine levels. The administration of this final dose was 6 days after the previous dose (Figure 3.23). Urinary taurine levels in treated animals were generally lower than those in control urine samples during the period following the last dose, although the difference was not significant.

iv. Serum enzyme levels

Serum ALT levels were significantly raised (Table 3.12) 4½ weeks after the final dose of CCl_4 an observation reported by Roos *et al.* (1991) after developing micronodular cirrhosis in rats using chronic exposure to phenobarbital and CCl_4 . Serum ALP values were also significantly higher in the treated animals than the controls (Table 3.12) suggesting that there had been some degree of biliary epithelial damage.

3.3.14 DISCUSSION

The lesion produced by the administration of CCl_4 relies to a large extent on its metabolism to the toxic radical $\text{CCl}_3\cdot$ by P_{450} (Section 1.5.1). This results in the destruction of P_{450} which would make subsequent doses of CCl_4 less toxic via this route as CCl_4 would not be metabolised to the toxic metabolite. This may explain why the elevation of urinary taurine during multiple dosing was less than that seen after a single acute dose of CCl_4 , although the dose used (1.0 ml.kg^{-1}) does not raise urinary taurine levels by more than 50 - 150% (Figures 3.11 and 5.4). However, the development of the fibrotic lesion and raised serum enzyme levels showed that there was toxicity.

It is possible that the repair of damaged parenchyma tissue by the synthesis of collagen resulted in less sulphur amino acids being available for taurine synthesis. However, the higher levels of liver taurine would tend

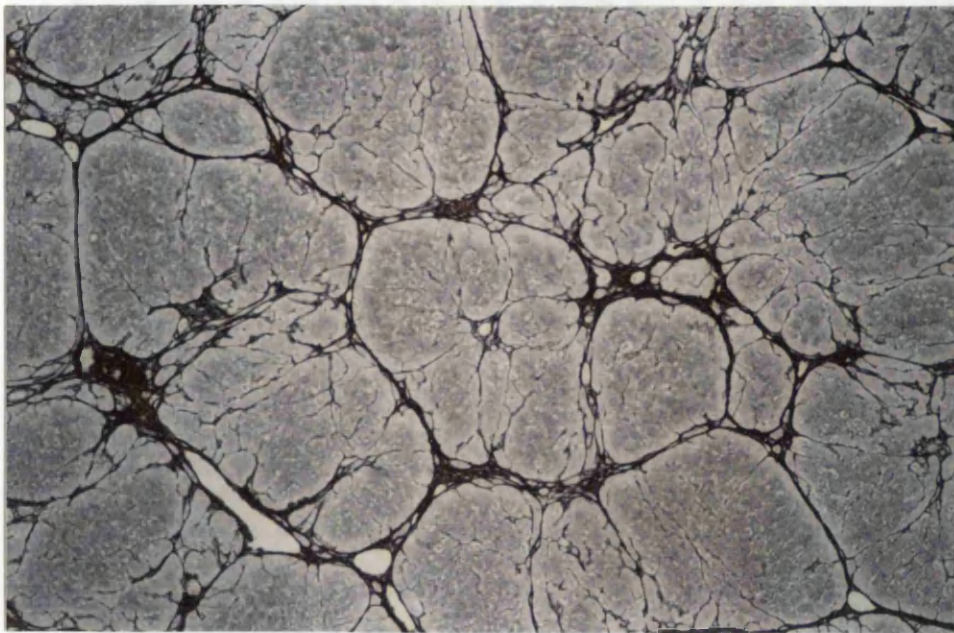


Plate 26. Light micrograph of liver section taken from a male rat 4½ weeks after sub-chronic dosing with CCl_4 (1 ml.kg^{-1} po, 9 doses). Reticulin stain. Mag. x 160.

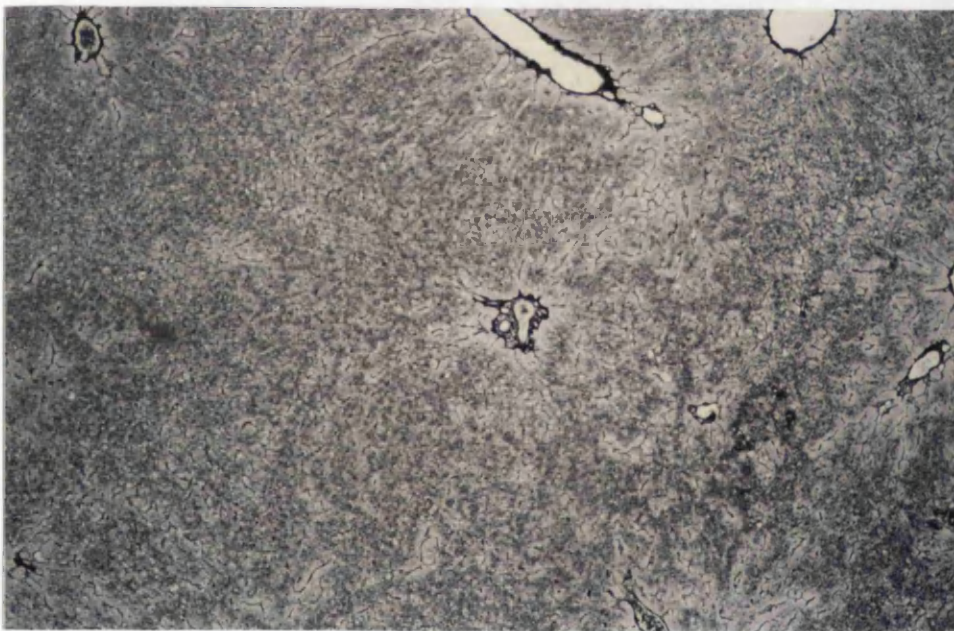


Plate 27. Light micrograph of liver section taken from a control male rat. Reticulin stain. Mag. x 160.

3.4

EFFECT OF ETHIONINE TREATMENT ON TAURINE LEVELS IN MALE RATS - A DOSE RESPONSE STUDY

3.4.1 AIM OF STUDY

To establish whether ethionine results in a dose dependent increase in urinary taurine when it is administered to rats.

3.4.2 INTRODUCTION

The main effect of ethionine toxicity is the accumulation of fat in the liver. This is believed to be the result of protein synthesis inhibition due the depletion of ATP shortly after injection (Diannani, 1991). ATP levels become insufficient for both amino acid and fatty acid metabolism (Section 1.3.3A).

A preliminary study had shown that ethionine (200 mg.kg⁻¹, p.o.) raised urinary taurine in male rats (by 100%), but not significantly and raised the level of liver taurine significantly from 4.4 µmol.g⁻¹ wet weight in control animals to 7.8 µmol.g⁻¹ wet weight in treated rats. Serum cholesterol was the only clinical chemistry measurement which was significantly different from control values (control: 1.6 ± 0.09 mmol.L⁻¹, ethionine: 0.9 ± 0.02 mmol.L⁻¹, p < 0.004).

3.4.3 METHOD

Male rats (280 - 340 g) were divided into groups (4 groups of 4) placed in individual metabolism cages for 7 days and provided with ground diet and water *ad lib*. A 24 h urine collection was made 24 h prior to dosing with ethionine on day 4 (0, 200, 400 and 800 mg.kg⁻¹ in saline, 2 ml.kg⁻¹) and for three 24 h periods following dosing. In previous studies, urine was collected for 48 h following dosing with hepatotoxic compounds and 96 h after CCl₄ administration (Section 3.1 and 3.2). However, the initial study carried out with ethionine showed that urinary taurine was not elevated until 24 - 48h after dosing. Therefore, the collection period was extended for a further 24h and animals were killed as previously described, 72 h after dosing. Tissue

samples were taken for biochemical analysis and histology. Total liver triglyceride levels were measured as a quantitative marker of ethionine fatty liver.

3.4.4 RESULTS

i. Urinary taurine.

Elevation of urinary taurine in rats was dose related and was significantly raised after the administration of ethionine (800 mg.kg⁻¹, p.o.) 0-24, 24-48 and 48-72 h after dosing. Urinary taurine was also significantly raised 24 - 48 after dosing with 200 and 400 mg.kg⁻¹ ethionine (Figure 3.24).

ii. Liver taurine

Liver taurine levels showed the opposite dose response to the levels of urinary taurine after ethionine treatment, the lowest dose resulting in the greatest elevation of liver taurine, 72 h after dosing (Figure 3.25). It should be noted that the control levels of liver taurine were high in this study.

iii. Liver TNPSH

Although liver levels of TNPSH appeared to be raised in a dose dependent manner, the differences from control values were not significant except at the highest dose of ethionine (800 mg.kg⁻¹) (Figure 3.26).

iv. Liver triglycerides

There was fat accumulation in the livers of the control animals, detected by staining with Oil Red O and measured biochemically. The values being 50 - 100% higher than those seen in the control livers in other studies (Table 3.13). Levels of triglycerides were raised by all doses of ethionine (200, 400 and 800 mg.kg⁻¹) in the livers of treated animals.

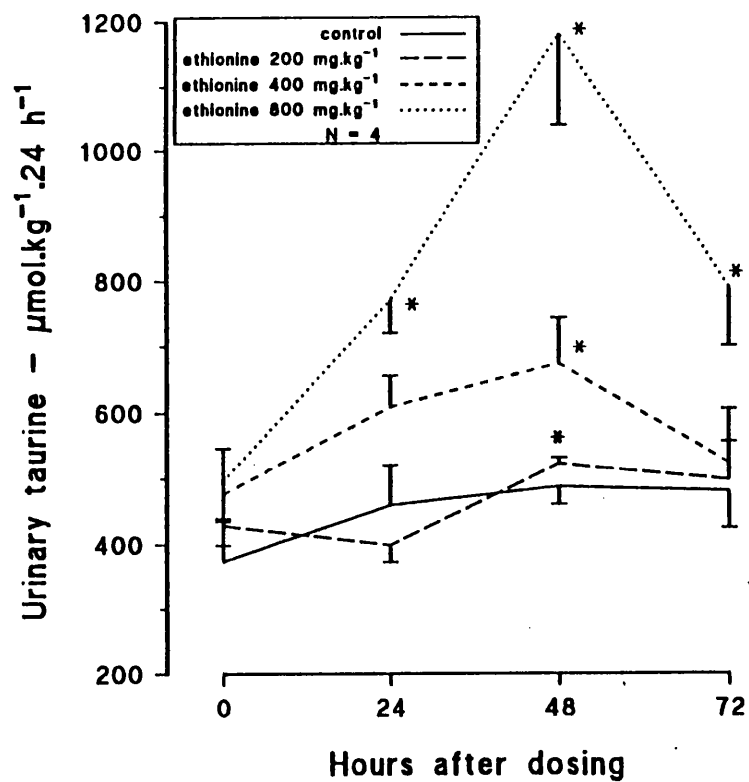


Figure 3.24 Urinary taurine ($\mu\text{mol.kg}^{-1}.\text{24 h}^{-1}$) in rats treated with ethionine (0, 200, 400 or 800 mg.kg^{-1} i.p.); values are means \pm SEM; N = 4; * $p < 0.05$ significantly different from pre-dose value.

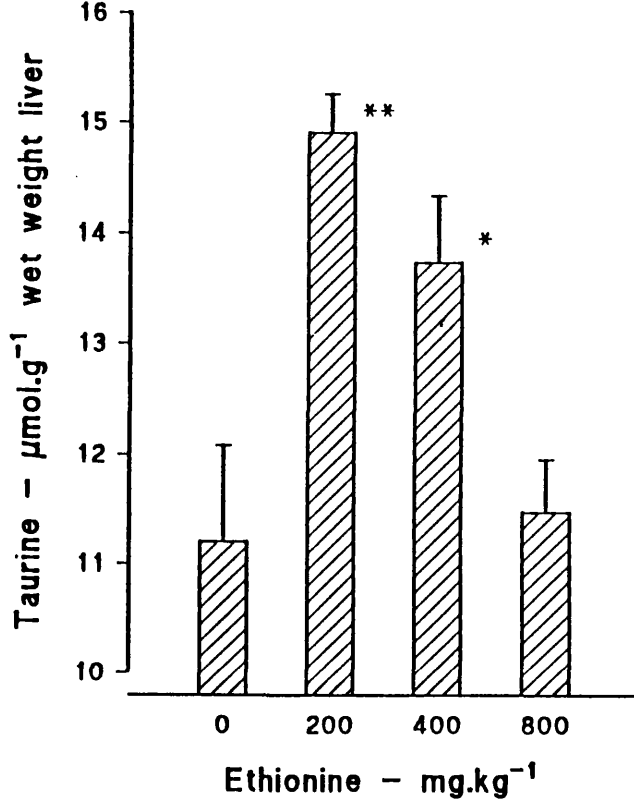


Figure 3.25 Liver taurine concentration ($\mu\text{mol.g}^{-1}$ wet weight) in control rats and rats 72 h after treatment with ethionine (200, 400 or 800 mg.kg^{-1}). Values are means \pm SEM; N = 4; * $p < 0.05$, ** $p < 0.01$.

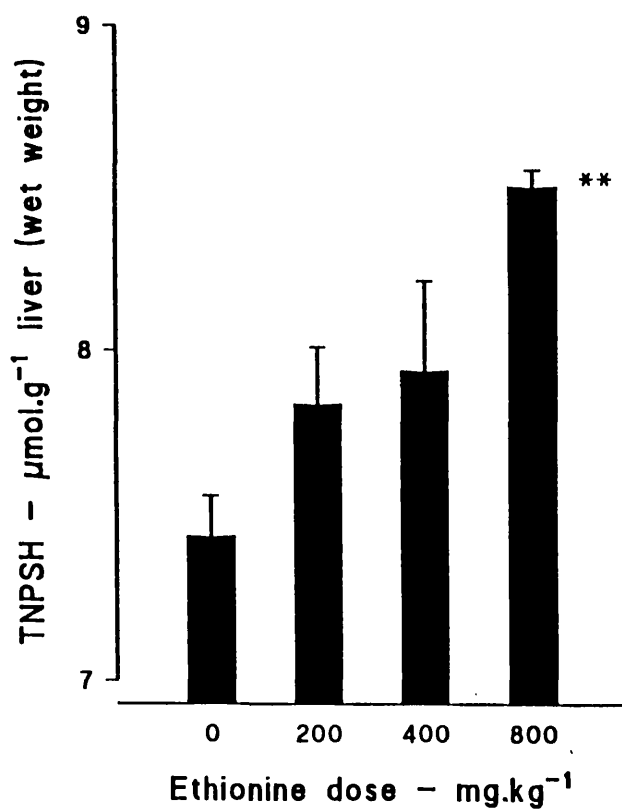


Figure 3.26 Liver TNPSH concentration ($\mu\text{mol.g}^{-1}$ wet weight) in control rats and rats 72 h after treatment with ethionine (200, 400 or 800 mg.kg^{-1}). Values are means \pm SEM; N = 4; ** $p < 0.01$.

Figure 3.13 Liver weight as % body weight and liver triglyceride concentrations 72 h after dosing male rats with ethionine

	Dose ethionine (mg.kg ⁻¹ , p.o.)			
	0	200	400	800
Liver % bodyweight	3.97 ± 0.12	3.77 ± 0.13	3.87 ± 0.04	3.76 ± 0.10
Serum triglycerides (mmol.L⁻¹)	1.42 ± 0.12	1.12 ± 0.21	0.86 ± 0.09*	0.72 ± 0.08**
Liver triglycerides (mg.g⁻¹ wet weight)	14.1 ± 1.1	28.4 ± 2.4**	28.4 ± 3.9*	38.9 ± 6.9*

Values, mean ± SEM; N = 4; * p<0.05, ** p< 0.01

v. Clinical signs of toxicity

Treatment with ethionine did not result in significant differences in weight gain or food intake of any groups of treated animals. Liver weights were not significantly different despite the accumulation of fat in the livers of those animals treated with ethionine (Table 3.13).

vi. Clinical chemistry

There was no elevation of serum enzymes at any dose level of ethionine 72 h after dosing. Serum triglyceride levels were reduced 72 h after treatment of animals with ethionine but serum cholesterol levels were not (Table 3.13). Urinary creatine values were the same as control values in all urine collections.

vii. Histology

Macrovesicular fat vacuoles were present throughout the livers of animals treated with higher doses of ethionine (400 and 800 mg.kg⁻¹) although the hepatocytes immediately around the central veins and portal triads were essentially free of fat. Liver sections from animals treated with 200 mg.kg⁻¹ ethionine showed fat accumulation predominantly in the mid-zonal regions of the liver giving a "halo" effect around the central veins. The livers from control animals which had mild lipid accumulation had lipid vacuoles in the

hepatocytes around the central veins. There were no other abnormalities noted in the liver tissue.

3.4.5 DISCUSSION

The data indicate that urinary taurine is elevated in a dose dependent manner after male rats have been treated with ethionine. Since there was no elevation of serum AST or ALT and no evidence of necrosis in the liver, it seems unlikely that the increased levels of taurine seen in the urine were the result of leakage of taurine from damaged cells. However, the raised taurine levels are consistent with the hypothesis that taurine synthesis might be increased as a result of perturbations in protein metabolism which may have increased the cysteine pool in cells. However, serum samples were not removed for biochemical analysis until 72 h after dosing and any changes in enzyme levels may have diminished. Serum cholesterol was raised after dosing animals with 200 mg.kg⁻¹ in a previous investigation, but not in this study. Again the reason for the difference may be that samples were removed 24 h later in this study. Triglycerides were however lowered in the serum 72 h after dosing which would be expected if the liver was accumulating triglycerides as a result of impaired transportation out of cells (Section 1.3.3A). Liver triglyceride levels were raised (Table 3.13) by all doses of ethionine (72 h post-dose), although the elevation after 200 and 400 mg.kg⁻¹ were similar and 800 mg.kg⁻¹ only resulted in a four fold elevation. Farber (1967) describes an increase of lipid content in the liver 15 to 20 times that of control values 24-48 h after dosing with a return to normal levels 72 - 100 h later.

Urinary taurine was elevated 0 - 24 h after administering ethionine (all doses) but there was a greater elevation 24 - 48 h after dosing. Although necrosis can be induced by chronic administration of ethionine it is not a lesion usually associated with a single acute dose of ethionine and therefore, hepatic necrosis is unlikely to have resulted in an elevation of urinary taurine leaked from damaged hepatocytes. There was no evidence of necrosis in the livers taken from animals 48 h after dosing with 200 mg.kg⁻¹

(Plate 11, page 130) or 72 h after dosing with 200, 400 or 800 mg.kg⁻¹ ethionine.

Ethionine is known to inhibit protein synthesis (Farber, 1967) which has been shown to happen within 2 h of dosing, but returns to normal levels by 4 h after 400 mg kg⁻¹ (Indacochea-Redmond *et al*, 1973). However, inhibition of protein synthesis is believed to be responsible for the protection conferred by ethionine against ANIT induced cholestasis (Dahlstrom-King and Plaa, 1989). This protection is apparent even when ethionine is administered 18 or 48 h *before* animals are treated with ANIT. This suggests that protein synthesis may be impaired by ethionine for 48 h or longer.

An inhibition of protein synthesis would have resulted in the accumulation of methionine and cysteine from dietary sources and protein degradation, which would provide the precursors necessary for both taurine and GSH synthesis, both of which were found to increase in the liver after ethionine treatment in this study but contrary to the results of Glaser and Mager (1974) who showed a decrease in GSH 5 h after treatment with ethionine. However they did not measure GSH at other time points. It is interesting that the maximum elevation of urinary taurine was 24 - 48 h after dosing, which is considerably later than the reported inhibition of protein synthesis by ethionine. However it is at a time when the effects of the protein synthesis inhibition on ANIT induced cholestasis are still apparent.

Taken together, these results suggest that ethionine elevated urinary taurine at a time when there is known to be a maximum elevation of triglycerides and when the effects of inhibited protein synthesis are still apparent. The raised levels of liver taurine and GSH would tend to confirm the increased availability of sulphur amino acid precursors possibly due to a reduction in protein synthesis or an alteration in the turnover of proteins.

3.5

INVESTIGATIONS INTO THE EFFECTS OF MODULATING PROTEIN AND GLUTATHIONE SYNTHESIS ON URINARY TAURINE LEVELS

3.5.1 AIM OF STUDY

To demonstrate that perturbations in protein and glutathione (GSH) synthesis may contribute to the elevations of urinary taurine found after treatment with some hepatotoxic compounds.

3.5.2 INTRODUCTION

The experimental work described in sections 3.1, 3.2 and 3.4 was concerned with the feasibility and validity of assessing hepatic cellular damage by measuring the degree of hypertaurinuria induced by various well documented hepatotoxic agents. It was apparent from the previous results that:

- a. compounds which caused necrosis did not necessarily result in hypertaurinuria despite the fact that serum AST and ALT levels were raised (ie. bromobenzene)
- b. compounds which resulted in liver steatosis (but not necrosis) also caused hypertaurinuria, which was more marked 24 - 48 h after dosing (ie. hydrazine and ethionine)
- c. compounds which caused necrosis and steatosis resulted in the most marked hypertaurinuria (ie. carbon tetrachloride and thioacetamide).

All of the compounds which caused steatosis are reported to inhibit protein synthesis, which is one of the main reasons that fat accumulates in hepatocytes (Sections 1.3.3 and 1.5). Since protein synthesis requires cysteine and methionine, the process would normally reduce the concentration of these amino acids. If, however, protein synthesis is inhibited and protein degradation continues methionine and cysteine levels are likely to increase which would divert cysteine into other metabolic pathways including synthesis of taurine (Section 1.7.6).

Similarly, perturbations of GSH synthesis will alter cysteine levels. Depletion of GSH by compounds such as allyl alcohol and bromobenzene would be likely to reduce cysteine available for taurine synthesis. Conversely, an inhibition of GSH synthesis will increase cellular levels of cysteine and may result in an increased synthesis of taurine.

3.5.3 Compounds used in this study.

- a. **Cycloheximide** - Cycloheximide inhibits protein synthesis by blocking termination of peptide chains. It produces a concomitant reduction in the synthesis of triglycerides and therefore does not result in a marked steatosis (Zimmerman, 1974).
- b. **Buthionine sulfoximine (BSO)** - BSO reduces cellular GSH by inhibiting γ -glutamylcysteine synthetase and therefore prevents the conjugation of cysteine with glutamate to form γ -glutamylcysteine. The latter would normally conjugate with glycine to form GSH (Drew and Miners, 1984).
- c. **Diethylmaleate (DEM)** - GSH is depleted by DEM due to the formation of a conjugate mediated by GSH-transferases (Boyland and Chasseaud, 1967 and Maellaro *et al*, 1990). However, DEM can also result in inhibition of protein synthesis (Costa and Murphy, 1986) and alters the activity of the hepatic microsomal mixed-function oxidase system activity, *in vitro* (Younes et al, 1986).
- d. **Phorone (diisopropylidenacetone)** - Phorone depletes GSH by conjugation in a similar way to DEM. Phorone is not reported to alter protein synthesis but it can be hepatotoxic at doses which are effective at reducing hepatic GSH (Dahm and Roth, 1991).

The hypothesis underlining this study was that cycloheximide and BSO would result in raised levels of urinary taurine as both should raise cysteine levels as a result of perturbations in protein and GSH synthesis respectively. It was predicted that phorone and DEM would lower urinary taurine as both reduce cysteine as a result of increased GSH synthesis in response to the depletion of GSH by these compounds. Phorone might be

expected to be more effective than DEM at reducing cysteine levels and therefore taurine levels, as it is not reported to inhibit protein synthesis.

3.5.4 METHOD

Male rats (245 - 270 g) were divided into groups of 3 - 5 animals and housed in individual metabolism cages for 8 days. After acclimatising for 3 days, two consecutive 24 h urine collections were made (-48 → -24 h and -24 → 0 h pre-dose). Animals were dosed on day six (Table 3.14). Two subsequent 24 h urine collections were made (0 → 24 h, 24 → 48 h). Dosing was carried out between 11.00 and 11.30 am on day 6.

(Treatment of animals with phorone was carried out in a separate experiment. Data is therefore treated separately and compared with the control group treated at the same time as the phorone treatment).

Table 3.14 Doses and routes of administration of cycloheximide and GSH depleting agents

COMPOUND	DOSE mg.kg ⁻¹	ROUTE	VEHICLE (ml.kg ⁻¹)
DIETHYLMALEATE	400	i.p.	2 ml, corn oil
CYCLOHEXIMIDE	2	i.p.	2 ml, 0.9% saline
BUTHIONINE SULFOXIMINE	900	s.c.	4 ml, U.H.Q. water pH to 8.5 with 0.1 M NaOH, warmed
PHORONE	175	i.p.	2 ml, corn oil

(Dosing levels were according to - Mitchell *et al*, 1973b, Gerard-Monnier *et al*, 1992, Higashi *et al*, 1983, Ravindranath and Boyd, 1991, Costa and Murphy, 1986, Younes and Seigers, 1981, Dahm and Roth, 1991, Hardwick, 1991 personal communication).

Animals were killed on day 8 and tissues taken for biochemical measurements and histology as described previously.

3.5.5 RESULTS

i. Urinary taurine

The administration of both cycloheximide and BSO resulted in significant hypertaurinuria in rats 0 - 24 h after dosing (Figure 3.27). The elevation of taurine was sustained in the BSO treated animals for a further 24 h and was significantly elevated compared with control animals (Dunnett's test). Animals dosed with cycloheximide developed a significant hypotaurinuria 24 - 48 h 24 - 48 h after dosing.

The administration of both phorone and diethylmaleate resulted in a significant hypotaurinuria 0-24 h and 24 - 48 h after dosing (Figures 3.27 and 3.28).

ii. Liver taurine

Dosing animals with cycloheximide appeared to reduce the concentration of taurine in the liver 48 h after dosing. The difference was significant from control values ("t" test, $p < 0.05$) but was not significant using Dunnett's test for multiple comparisons (Figure 3.29). None of the GSH depleting compounds resulted in significantly altered hepatic taurine levels 48 h after dosing.

iii. Liver TNPSH

Liver TNPSH content was reduced 48 h after dosing animals with BSO and cycloheximide but values were only significantly different from control values using a "t" test and not Dunnett's test (Figure 3.30). Animals treated with phorone had a significantly higher concentration of TNPSH in the liver, 48 h after dosing.

iv. Liver triglycerides

Liver triglyceride levels were not significantly different from control values 48 h after dosing with any of the compounds used in this study.

v. Urinary creatine

Urinary creatine was elevated significantly in rats treated with diethylmaleate and cycloheximide 24 - 48 h after dosing (Figure 3.31).

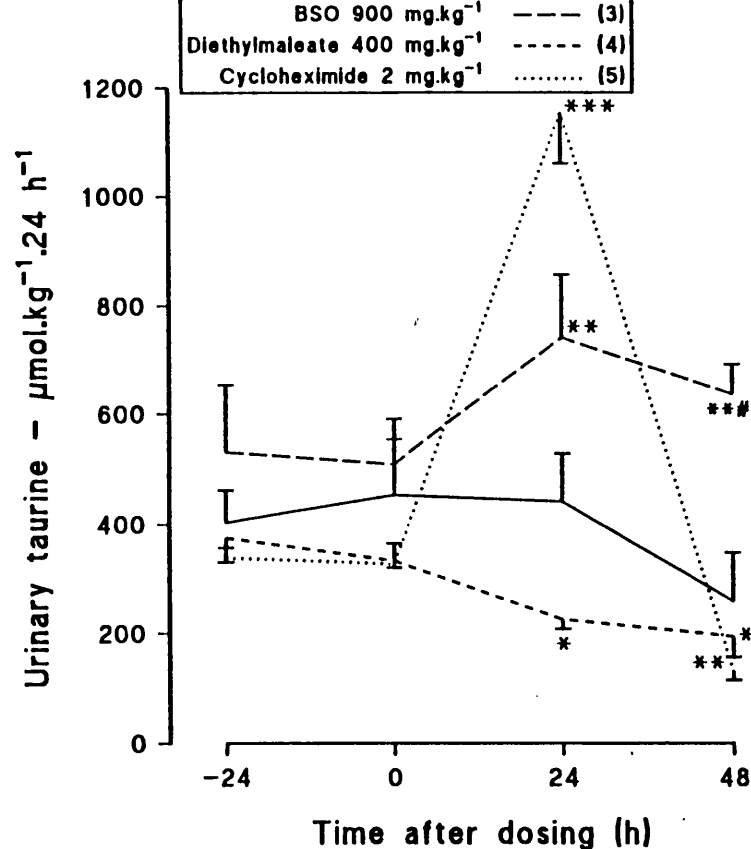


Figure 3.27 Urinary taurine in 24 h urine collections made from control rats and rats treated with BSO (sc), diethylmaleate (ip) and cycloheximide (ip) 48 h before and 48 h after dosing. Values are means \pm SEM; Number of animals in parentheses; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (paired "t" test - comparison made with predose levels); **# $p < 0.01$ Dunnett's test, BSO treated compared to control.

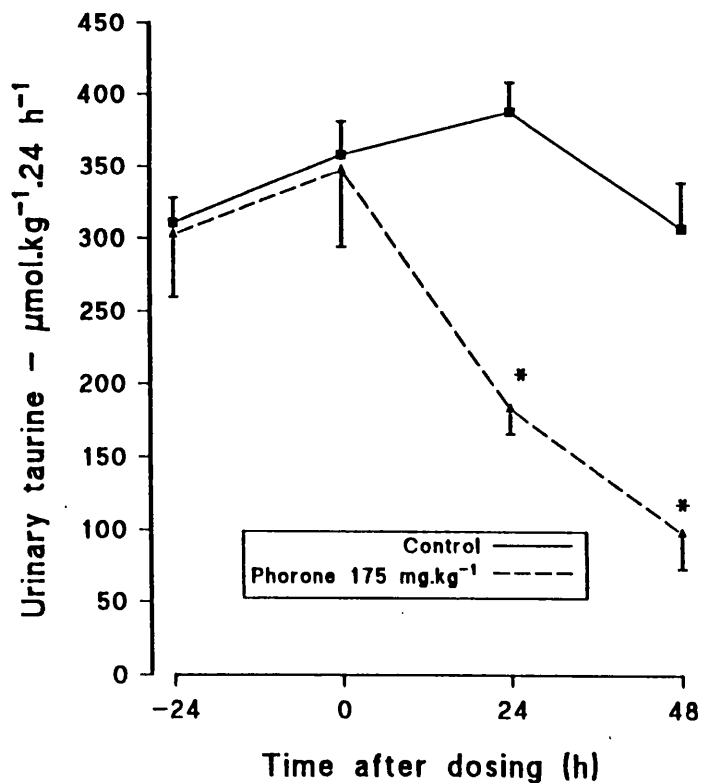


Figure 3.28 Urinary taurine in 24 h urine collections from control rats and treated rats 48 h before and after dosing with phorone (p.o). Values are means \pm SEM; N = 4; * $p < 0.05$.

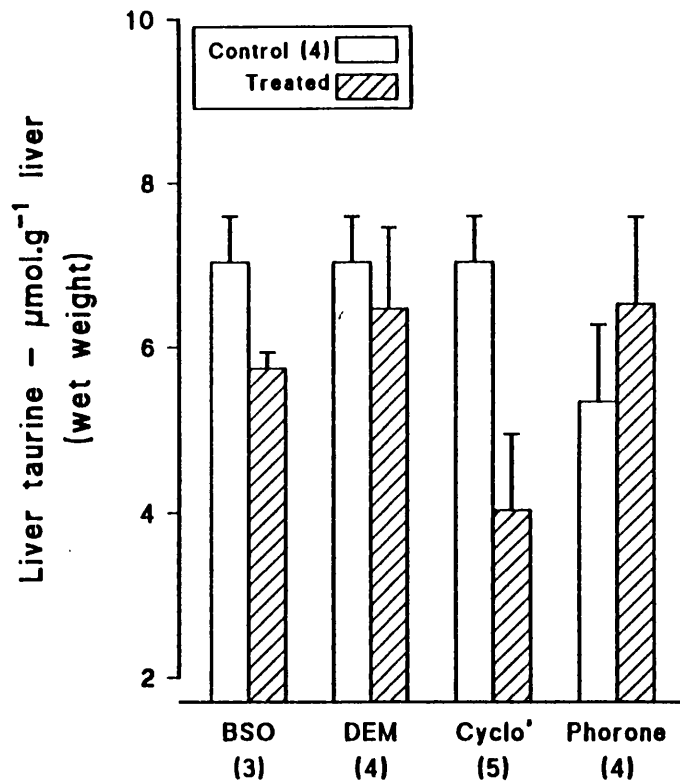


Figure 3.29 Taurine concentration in the liver ($\mu\text{mol.g}^{-1}$ wet weight) 48 h after dosing with BSO, diethylmaleate (DEM), cycloheximide (Cyclo') and phorone. Values are means \pm SEM; N = number in parentheses; No significant difference between the treated and control animals using Dunnett's test. Cycloheximide resulted in significant ($p < 0.05$) taurine reduction using "t" test.

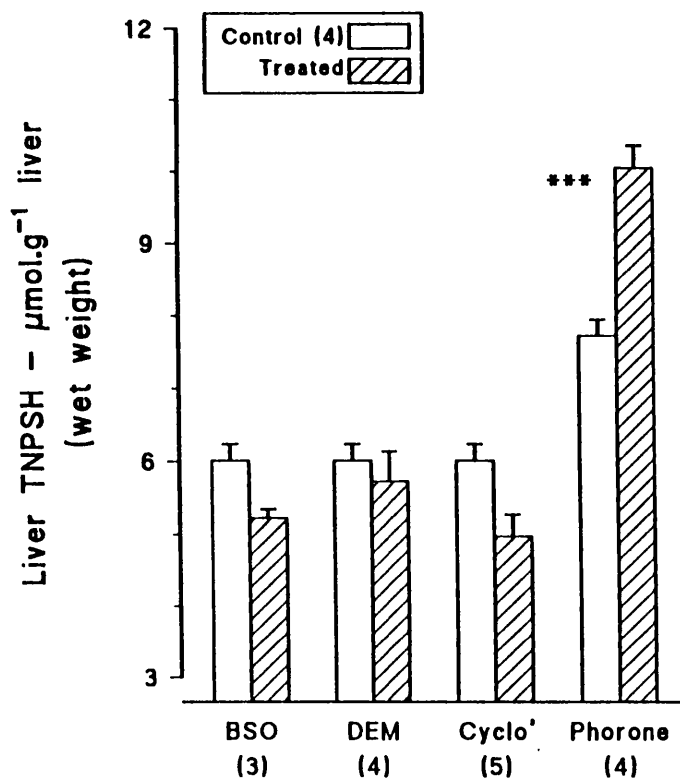


Figure 3.30 Liver TNPSH in the liver ($\mu\text{mol.g}^{-1}$ wet weight) 48 h after dosing with BSO, diethylmaleate (DEM), cycloheximide (Cyclo') and phorone. Values are means \pm SEM; N = number in parentheses; *** $p < 0.001$.

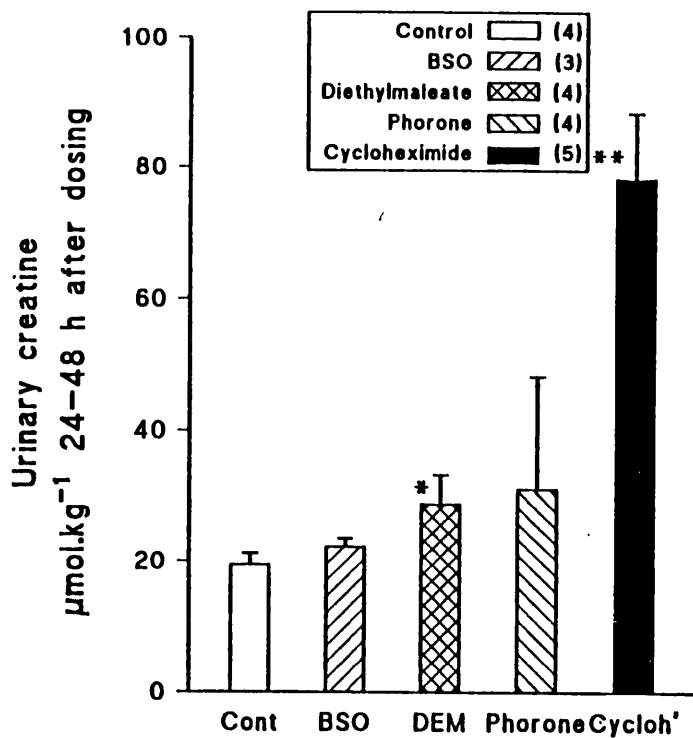


Figure 3.31 Urinary creatine in 24 - 48 h urine collections made after treatment with BSO, diethylmaleate (DEM), cycloheximide (Cyclo') and phorone. Values are means \pm SEM; N = number in parentheses; * $p < 0.05$, ** $p < 0.01$.

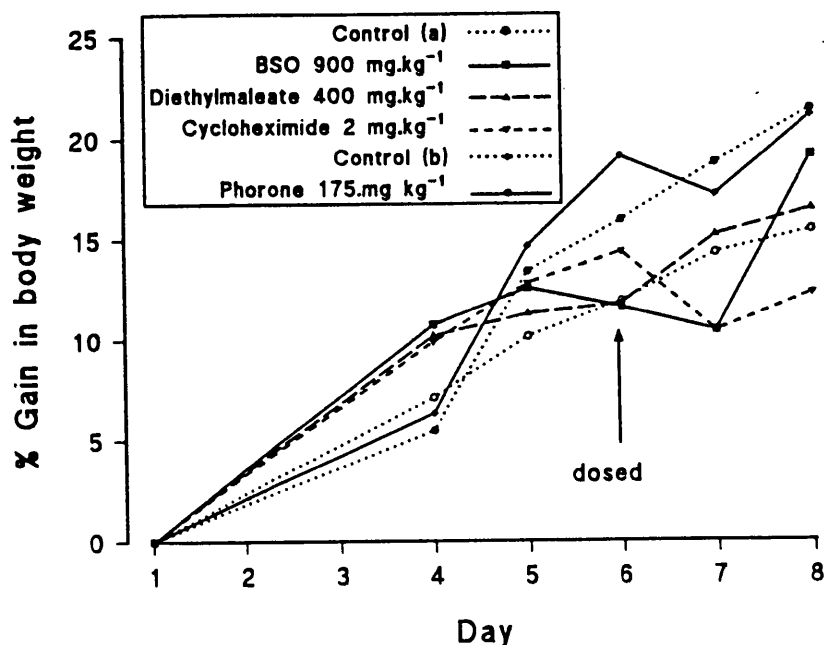


Figure 3.32 % Weight change in control (a) and treated animals before and after treatment with BSO, diethylmaleate (DEM), cycloheximide (Cyclo'); control (b) and phorone. Values are means \pm SEM; N = number in parentheses see figure 3.31 above; There were no significant weight changes after treatment.

vi. Clinical signs of toxicity

None of the animals in this study showed a significant weight loss (Figure 3.32). However, those animals treated with cycloheximide reduced their food intake significantly 0 - 24 h after dosing ($30.0 \pm \text{SD } 3.0$ g pre-dose compared with $8.0 \pm \text{SD } 0.3$ g post-dose) and 24 - 48 h after dosing ($16.6 \pm \text{SD } 3.2$ g).

vii. Clinical chemistry

Serum enzymes were not raised by any of the compounds used in this study. However, both serum ALT and ALP were significantly reduced in the serum from animals treated with cycloheximide (Table 3.15). Treatment of animals with cycloheximide also reduced serum albumin and total serum protein significantly, 48 h after treatment. There was also a reduction in the concentration of protein in the urine from animals dosed with cycloheximide collected 24-48 h after treatment compared to pretreatment values.

viii. Histology

All histology was carried out on tissues taken 48 h after dosing.

a. Cycloheximide - Liver sections taken from animals treated with cycloheximide appeared normal in architecture, although one animal had slight diffuse steatosis which appeared to be microvesicular (Plate 28).

b. BSO - The liver from one animal treated with BSO had infiltrations of macrophages in a few of the periportal regions of the liver possibly indicating that there may have been some necrotic cells in these regions. The remaining tissue was normal in appearance as were the sections take from the other two animals treated with BSO. There was no indication of fat accumulation in any of the sections as indicated by Oil red O staining.

Table 3.15 Serum and urinary levels of proteins and serum enzymes in male rats, 48 h after dosing with cycloheximide and GSH depleting compounds.

Compound	Serum albumin g.L ⁻¹	Serum total protein g.L ⁻¹	Serum total bilirubin μmol.L ⁻¹	Serum ALT iu.L ⁻¹	Serum ALP iu.L ⁻¹	Urinary protein mg.kg ⁻¹ 24-48 h post-dose
Control	33.5 ± 0.8	56.8 ± 2.1	6.7 ± 2.5	67.2 ± 3.0	487 ± 37	53.7 ± 15.1
BSO	32.6 ± 0.2	55.0 ± 0.6	4.8 ± 0.1	67.3 ± 3.0	417 ± 53	66.5 ± 2.3
Diethylmaleate	34.4 ± 0.5	56.7 ± 0.6	4.1 ± 0.8	67.5 ± 5.2	368 ± 31	65.9 ± 4.4
Phorone	33.8 ± 0.2	59.0 ± 0.6	3.7 ± 0.2	69.8 ± 4.7	444 ± 47	N.D.
Cycloheximide	28.3 ± 0.8***	45.8 ± 0.8***	10.5 ± 1.4	49.3 ± 2.9**	219 ± 10***	8.1 ± 1.2***

Values - means ± SEM, N = 3-5, ** p< 0.01, *** p< 0.001, ND - no data.

- c. Diethylmaleate** - Gross pathology showed topical necrosis on one area of the right lobe in one animal. Histology showed that this necrosis was confined mostly to the edge of the liver lobe, although in this one lobe there were necrotic cells in the portal regions of some of the lobules and there was a very marked infiltration of macrophages around many portal tracts (Plate 29). There was no accumulation of fat in any of the sections.
- d. Phorone** - The liver parenchyma appeared normal in all sections taken from animals treated with phorone. However, staining sections with PAS stain showed that there was an unusually high concentration of glycogen in the liver sections taken from three of the four treated animals.

3.5.6 DISCUSSION

a. Cycloheximide.

Although serum samples were not taken until 48 h after dosing rats with cycloheximide (2 mg.kg^{-1}) the significant decrease in both serum total protein and albumin indicated that cycloheximide had probably been effective in inhibiting protein synthesis. The significantly reduced levels of the enzymes ALT and ALP in the serum and the significantly reduced levels of urinary protein would tend to confirm this. It is also possible, however, that cycloheximide interfered with the transportation of serum proteins into the blood stream. However, the fact that the levels of these markers of protein status were still reduced 48 h after dosing, does not indicate the status of protein *synthesis* at the time the tissues were taken for analysis it merely reflects an inhibition of protein synthesis or transportation at some point 0 - 48 h post dosing. Indeed the inhibition of protein synthesis is reported to be effective 2 - 4 h after the administration of cycloheximide (Indacochea-Redmond *et al*, 1973) with protein synthesis remaining low for 18 h after dosing.

Urinary taurine was significantly elevated 0 - 24 h after dosing with cycloheximide despite the fact that the treated animals had a significant

reduction in their food intake which has been demonstrated earlier to lower urinary taurine (section 3.1.4). There was no histological evidence of necrosis and serum transaminase enzymes were significantly reduced rather than raised. The elevation of urinary taurine is therefore consistent with the hypothesis that taurine synthesis had been increased resulting in overflow into the urine rather than elevated as a result of overt cellular damage. It is also possible that there was reduced reabsorption of taurine in the kidney, perhaps due to elevated levels of another β -amino acid such as β -alanine which was not measured. However, the effects of cycloheximide on protein synthesis in the liver are well documented (Dahlstrom-King and Plaa, 1989, Higashi *et al*, 1983, and Zimmerman, 1978). It seems likely, therefore, that the elevation of urinary taurine was a result of this inhibition of protein synthesis. The decrease in the rate of utilisation of both methionine and cysteine as a result of the inhibition leading directly to an increase in taurine synthesis. Indeed, a similar effect on GSH synthesis has been reported by Higashi *et al* (1983) after administration of cycloheximide to rats. They reported an increase in GSH synthesis 4 h after dosing and a maximum 100% increase in liver GSH levels 6 h after dosing. Raised levels of GSH were still apparent 24 h later. This increase in synthesis paralleled the increase in cysteine levels as a result of protein synthesis inhibition. Tracer studies with ^{35}S -cysteine showed that incorporation of cysteine into GSH was stimulated by cycloheximide treatment.

Although protein synthesis is reported to be reduced to 65% of control levels after 18 h (Indacochea-Redmond *et al*, 1973) the effect of reduced protein levels appeared to last for a longer period of time. Thus, urinary taurine was reduced significantly to below pre-dose levels 24 - 48 h after treatment. This reduction would coincide with the time period following recovery from protein synthesis inhibition when protein synthesis would be increasing. However, the increase in protein synthesis was clearly not rapid enough to have made up the deficit of serum proteins by 48 h. The resumption of protein synthesis would reduce cysteine levels for both taurine and GSH synthesis. There would also have been a lack of dietary cysteine 48 h after dosing, as animals had only eaten 40% of their normal

food intake during this time. This would explain the reduction in urinary taurine 24 - 48 h after dosing.

An alternative explanation for the increased levels of taurine might be found in the work of Yamaguchi *et al.* (1971) who showed that cycloheximide inhibited the cysteine mediated induction of cysteine dioxygenase. In this way, any increase in cysteine due to protein synthesis inhibition would not be metabolised via cysteine sulphinic acid to either taurine or pyruvate. It might be expected that under these circumstances the excess cysteine might be metabolised through the cysteamine pathway (1.7C) with the possibility that more cysteine would form taurine as the transamination pathway to form pyruvate would not be favoured.

Measurement of liver taurine and TNPSH 48 h after dosing showed that the levels of both were reduced, although this was not a significant reduction according to Dunnett's test. Any increase in GSH synthesis, as reported by other workers (Higashi *et al.*, 1983) during the 24 h following cycloheximide treatment had probably been abrogated by an increase in protein synthesis. A similar effect would account for the levels of liver taurine.

Despite the fact that protein synthesis had been inhibited by treatment with cycloheximide there was no concomitant rise in the level of liver triglycerides measured biochemically. Only one animal showed a slight steatosis in the liver after staining the liver sections with Oil Red "O". This was an important observation in relation to these studies. The two compounds which induced hypertaurinuria in rats without causing necrosis (ethionine and hydrazine, section 3.1) also induced a marked steatosis. As a result of these studies, it was suggested that compounds which result in hypertaurinuria may do so through the inhibitory effect that they all have on protein synthesis, which, in these cases also results in hepatocytes accumulating fat. However, there was also the possibility that the accumulation of fat by itself or the mechanism underlying the accumulation may have resulted in elevated taurine levels.

The treatment with cycloheximide suggests that the two effects can be separated since urinary taurine was elevated in these animals without

developing steatosis. It also reinforces the hypothesis that the hypertaurinuria which develops in rats treated with some hepatotoxic compounds is either enhanced by the inhibition of protein synthesis (eg. CCl_4) or is a direct result of the inhibition of protein synthesis (eg. hydrazine).

The raised levels of urinary creatine which were found 24 - 48 h after dosing with cycloheximide may have been the result of reduced food intake as these levels (4 X control values) were similar to those found in the urine from rats maintained on a restricted feeding regime (Figure 3.9). However the raised levels may also have been due to the increased levels of amino acids, as a result of the inhibition of protein synthesis (glycine and arginine in particular), which can be metabolised to creatine.

b. Buthionine sulfoximine (BSO)

The administration of BSO (900 mg.kg^{-1}) to rats was reported by Dahm and Roth (1991) to lower GSH levels (measured as TNPSH) by 70%, 24 h after dosing. The concentration of TNPSH was still significantly lower than control values 48 h after dosing, being 60 - 70% of the control values. The measurement of TNPSH in the liver taken from animals 48 h after dosing in the study presented here, were reduced, but not significantly. It is therefore not possible to say how effective the administration of BSO had been in reducing GSH synthesis. The dose used in this study (4 mmol or 900 mg.kg^{-1}) has been reported to reduce liver GSH in rat liver effectively (Ravindranath and Boyd, 1991). Histology also revealed some signs of inflammatory response in the periportal regions suggesting that BSO had resulted in some cellular damage.

Urinary taurine levels were elevated significantly above the pre-dose levels 0 - 24 h after dosing and above control values 24 - 48 h after dosing. Liver taurine levels were reduced but not significantly, 48 h after dosing.

BSO is known to block the synthesis of GSH by inhibiting the enzyme γ -glutamylcysteine synthetase there by reducing GSH levels in the liver and raising the levels of cysteine in plasma (Ravindranath and Boyd, 1991). Providing animals were receiving an adequate supply of methionine and

cysteine in the diet for protein synthesis, this reduction in GSH synthesis would result in an increase in cysteine available for the synthesis of taurine. The animals in this study did not reduce their food intake so their intake of sulphur amino acids was probably in excess of that needed for protein synthesis which would have been diverted to taurine synthesis thus raising levels of urinary taurine.

Diethylmaleate (DEM)

A dose of 400 mg.kg^{-1} (2.3 mmol) of DEM was chosen for this study as $2 - 3 \text{ mmol.kg}^{-1}$ has been reported to deplete GSH maximally without producing side effects such as impairing glycogen metabolism (Krack *et al*, 1980), inhibiting protein synthesis (Costa and Murphy, 1986) and increasing hepatic ornithine decarboxylase activity (Yoshida *et al*, 1988). The depletion of hepatic GSH is reported to be maximal in the liver (12% of control values) between 1 and 2 h after dosing with subsequent increases to 120% of control levels 8, 24 and 48 h after dosing (Gerard-Monnier *et al*, 1992). Cysteine levels were also reported to mirror GSH levels closely.

In the study reported here one animal showed necrosis of the parenchyma around the portal tracts of the left lobe and there was also some necrosis at the edge of the lobe, probably as a result of the i.p. administration of the compound. The serum AST level from this animal was slightly raised above control levels (136 iu.L^{-1} compared to mean control value of 99 iu.L^{-1}).

Urinary taurine levels were significantly reduced 0 - 24 and 24 - 48 h after dosing with DEM. This might be predicted from the reported reduction in cysteine following the administration of DEM in that there would be less cysteine available for taurine synthesis. However, GSH levels are reported to return to pre-dose concentrations 8 h after dosing. The hypotaurinuria was found 24 - 48 h following dosing which may suggest that DEM continues to result in an increase in the incorporation of cysteine into GSH by *de novo* synthesis of GSH. Although the levels of GSH are reported to be similar to control levels, it is possible that DEM continues to deplete GSH at a similar rate to its replenishment by *de novo* synthesis. The levels of cysteine and GSH would then reflect the *net* effect of GSH synthesis and

depletion as a result of a higher turn over due to the continued depletion by DEM. This might explain the continued hypotaurinuria 24 - 48 h post-dosing despite the fact that the concentrations of TNPSH and taurine in the liver 48 h after dosing were the same as control levels in this study.

Phorone

Dahm and Roth (1991) showed that a dose of 125 mg.kg^{-1} of phorone reduced hepatic TNPSH by 88%, 2 h after treatment. Levels returned to normal 6 h after dosing. A dose of 250 mg.kg^{-1} was found to be hepatotoxic in that it raised serum AST levels and reduced bile flow. Gregus *et al* (1988) reported that liver GSH levels returned to control levels 9 h after dosing rats with 275 mg.kg^{-1} , with no concomitant rebound synthesis of GSH. For these reasons a dose of 175 mg.kg^{-1} was chosen. No changes in serum enzymes or other biochemical indicators of hepatotoxicity were found 48 h after dosing. However, staining liver sections with PAS stain showed that the livers from 3/4 animals had more glycogen accumulation than those from control animals. Animals did not reduce their food intake after treatment, so any changes in the levels of taurine were not the result of reduced food intake.

Urinary taurine levels were significantly reduced 0 - 24 h and 24 - 48 h after dosing with phorone, the reduction being more marked than after DEM treatment. The duration of GSH depletion is reported to last longer after phorone treatment than DEM. Phorone is not believed to inhibit protein synthesis, whereas DEM has been shown to inhibit protein synthesis which would tend to counteract the reduction of levels of cysteine caused by GSH depletion (Costa and Murphy, 1986 and Goethals *et al.* 1983).

Interestingly, there was a significant elevation of TNPSH in the liver 48 h after dosing with phorone which does not seem to have been reported in the literature. This suggests that there may have been a delayed rebound synthesis of GSH, which may account for the continued reduction in urinary taurine.

3.5.7 Summary

Despite the variation in urinary excretion between studies, the effect of the hepatotoxic compounds on urinary taurine levels was consistent in repeated studies. Thus, carbon tetrachloride, thioacetamide, hydrazine, ethionine and galactosamine *elevated* urinary taurine when liver injury (necrosis, steatosis or both) was demonstrated by clinical chemistry and/or histology.

An explanation was sought for the observation that bromobenzene and allyl alcohol induced hepatic injury did not result in an elevation of urinary taurine. The depletion of glutathione by these compounds was suggested as contributing to this observation. However, hepatic concentrations of GSH could only be measured in the rats from which urine was collected at the termination of the experiment (usually 48 h after dosing). It is therefore not possible to state that the lack of elevation of urinary taurine levels following bromobenzene and allyl alcohol in these studies was a direct result of GSH depletion although TNPSH levels were reduced 48 h after bromobenzene treatment. Other well documented work has, however, demonstrated a reduction of liver GSH by bromobenzene (Jollow *et al* 1974 and Casini *et al* 1985) and allyl alcohol (Glaser and Mager, 1974 and Jaeschke *et al* 1987). Similar observations were made by Yoshida and Hara (1985 a & b) after the administration of chlorobenzene to rats was shown to deplete GSH and also reduce hepatic taurine levels at both 6 and 24 h after dosing urinary taurine 0 - 24 h post dosing.

Although liver GSH levels were not measured during the collection of urine, the significant *reduction* in urinary taurine in rats treated with diethyl maleate and phorone and significant *elevation* of urinary taurine after buthionine sulfoximine and cycloheximide treatment help to support the view that perturbations in GSH and protein synthesis (ie. by bromobenzene) may contribute to alterations in taurine synthesis and therefore urinary levels of taurine.

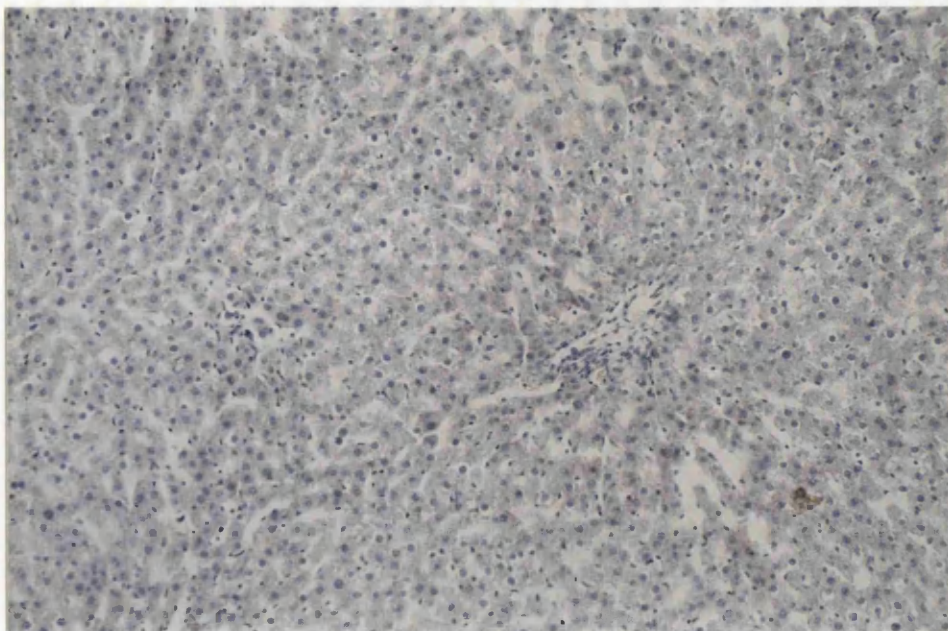


Plate 28. Light micrograph of a frozen section taken from a male rat 48 h after dosing with cycloheximide ($2 \text{ mg.kg}^{-1} \text{ ip}$). There is no evidence of lipid accumulation with Oil red O stain. Mag. x 100.

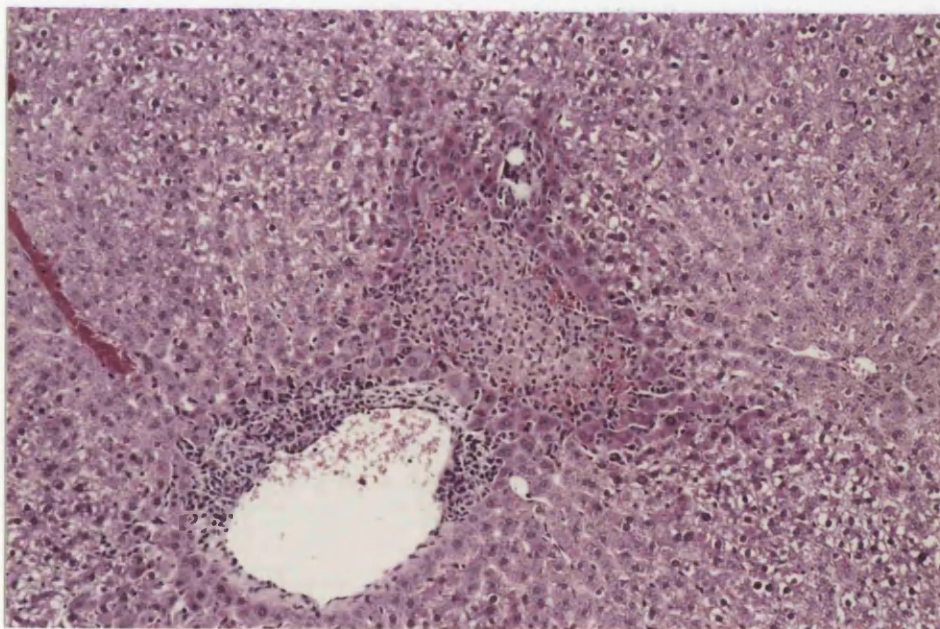


Plate 29. Light micrograph of a liver section from a male rat 48 h after dosing with diethyl maleate ($400 \text{ mg.kg}^{-1} \text{ ip}$) showing an area of necrosis in the liver of one rat. H and E stain. Mag. x 100.

3.6

AN INVESTIGATION INTO THE EFFECT OF NON-HEPATOTOXIC COMPOUNDS ON URINARY TAURINE LEVELS

3.6.1 INTRODUCTION

The studies discussed in Sections 3.1, 3.2 and 3.4 were investigations into the effect of acute administration of hepatotoxic compounds to rats on urinary levels of taurine and creatine. It was apparent from these studies that urinary taurine was elevated by some hepatotoxins, in particular those that caused necrosis and/or steatosis probably as a result of the inhibition of protein synthesis without a perturbation of glutathione (GSH) synthesis. The studies discussed in Section 3.3 and 3.5 supported the hypothesis that an *inhibition* of protein or GSH synthesis may *raise* urinary levels of taurine while an *increase* in protein or GSH synthesis may result in a *reduction* in urinary taurine.

As it was proposed that urinary taurine might be a useful non-invasive marker of liver dysfunction it was important to establish what effect damage to other organs might have on levels of urinary taurine.

3.6.2 AIM OF STUDY

To investigate the effect of different toxins on urinary taurine levels where the primary target organ for damage is *not* the liver.

i. Heart

Heart muscle contains high levels of taurine (30 - 35 mM in rats, Chesney, 1985; Huxtable, 1987) and is reported to be lost from cardiac muscle after acute ventricular ischemia (Crass III and Lombardini, 1977). It seemed likely, therefore, that chemically induced ischemia resulting in necrosis, would result in loss of cardiac muscle taurine which might raise urinary levels of taurine.

Allylamine is primarily a cardio-toxic agent which is toxic after a single dose, via its metabolite, acrolein (Boor *et al*, 1979). This is generated by the oxidative deamination of allylamine *in situ*. Acrolein denatures protein and disrupts nucleic acid synthesis resulting in fibrin like material being deposited in collagen which leads to fibrinoid necrosis (Klaassen *et al*, 1986).

ii. Kidney

As the primary aim of this thesis was to establish whether changes in urinary taurine could be used as an indication of liver dysfunction it was necessary to establish what effect kidney damage might have on the excretion of taurine. Compounds such as carbon tetrachloride and bromobenzene are reported to be nephrotoxic as well as hepatotoxic (Klaassen *et al*, 1986; Lau and Zannoni, 1979). In the studies discussed in section 3.1, two out of three rats treated with thioacetamide (150 mg.kg^{-1}) also developed kidney lesions. It was important, therefore to establish what effect kidney damage might have on urinary taurine levels.

Mercuric chloride is a classic nephrotoxic compound (Haagnum and Pound, 1979 and Nicholson *et al*, 1985) which is known to accumulate rapidly in the kidneys, causing extensive damage to the proximal tubular epithelium and severe renal failure. The particular site of action appears to be the *pars recta* of the proximal tubule. This results in a general inhibition of solute reabsorption from the tubular lumen and in the development of the Faconi syndrome where there is an increase in the excretion of amino acids, glucose, calcium, phosphate, bicarbonate and some low M_r proteins. Therefore, an *increase* in urinary taurine might be expected following an acute dose of mercuric chloride.

iii. Testis

Although the work reported here is predominantly concerned with changes in urinary taurine as a response to the toxic effects of various hepatotoxic compounds, measurements of urinary creatine were also carried out

routinely. This was part of an on-going study in this department into the effect of testicular damage on urinary creatine levels which has been proposed as a useful marker of testicular damage. Gray *et al*, (1990) had found that urinary creatine levels were raised in male rats following the administration of the nephrotoxic agent, cadmium. That creatine was originating from the testis was confirmed in female and orchidectomised male rats which did not develop raised urinary creatine levels following treatment with Cd^{2+} . It was important to establish whether urinary creatine might also be raised if other organs were injured and whether testicular damage resulted in an increase in urinary taurine.

Cadmium chloride. The target organ for cadmium chloride toxicity varies depending on the dose, route of administration and the length of exposure to Cd^{2+} . Whilst there are effects on the lungs, kidney (proximal tubules) and cardiovascular and skeletal systems after long term exposure to low levels of Cd^{2+} (Klassen *et al*, 1986) a single high dose will target other organs. Thus, an acute dose of $1.5 - 3.9 \text{ mg.kg}^{-1}$ or more given i.v. will result in liver damage, measured as elevations in serum transaminase enzymes and histologically (Dudley *et al*, 1982 and 1984). However, a lower dose acute dose, $3.23 - 24 \text{ } \mu\text{mol.kg}^{-1}$, administered intraperitoneally selectively damages the testis producing necrosis as a result of ischemia (Nicholson *et al*, 1989; Gray *et al*, 1990).

3.6.3 METHOD

The investigation into the effects of three different non-hepatotoxic compounds were carried out in two separate investigations. Allylamine was administered in the first study where male rats weighing 225 - 260 g were used and mercuric chloride and cadmium chloride were administered in the second study, where rats weighing 240 - 270 g were used. The procedure in both studies was the same but the results obtained were compared using the control results from the appropriate study.

Animals were put into individual metabolism cages for seven days. Two

24 h urine collections were made on days 3 - 4 and 4 - 5 and animals were dosed on day 5. Two further 24 h urine collections were made after dosing. Groups of 3 - 4 rats each were dosed with; Allylamine ($150 \text{ mg.kg}^{-1} \text{ p.o.}$), cadmium chloride ($1.5 \text{ mg.kg}^{-1} \text{ i.p.}$) or mercuric chloride ($1.4 \text{ mg.kg}^{-1} \text{ i.p.}$).

Control groups were given 0.9% saline i.p. or p.o. (Appendix XI).

[References for dosing levels: Hg^{2+} , Nicholson *et al*, 1985; Gartland *et al*. 1988; Cd^{2+} , Gray *et al*, 1990; Nicholson *et al*, 1990; Allylamine, Boor *et al*. 1979].

Animals were killed 48 h after dosing and tissues taken for histology and biochemical measurements, as before.

3.6.4 RESULTS

Animals treated with all three toxins developed piloerection and were subdued. Their food consumption decreased significantly and they lost weight (Figures 3.33, 3.34 and 3.35).

i. Allylamine

a. *Histology*

Hearts from animals treated with allylamine had areas of neutrophil infiltration, eosinophilic cardiac cells and regions of abnormal myocardium (Plates 30 and 31). Heart weights were slightly raised above control values but not significantly (Table 3.16). Liver weights were slightly reduced, probably due to a reduction in glycogen as these animals ate less than control animals. There was vacuolation in the periportal hepatocytes which stained red with Oil Red O identifying the vacuoles as accumulated fat.

b. *Blood biochemistry*

All blood biochemistry was normal 48 h after dosing except for one animal which had twice the concentration of serum creatine kinase of the control animals (Table 3.18). This animal also had a heavier heart than control animals.

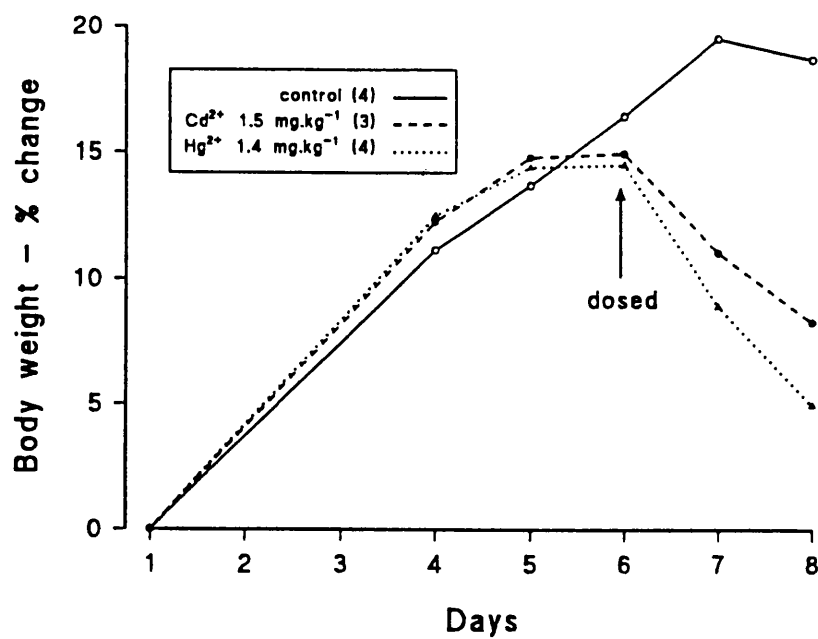


Figure 3.33 %Change in body weight of control rats and rats treated on day 6 with cadmium chloride (1.5 mg.kg⁻¹ i.p) and mercuric chloride (1.4 mg.kg⁻¹ i.p). Values are means; Number of animals in parentheses.

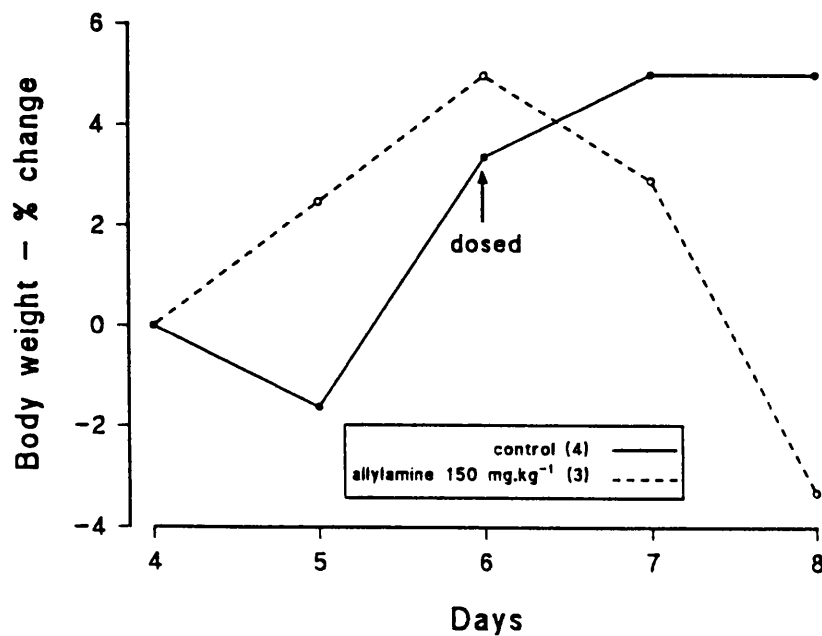


Figure 3.34 %Change in body weight in control rats and rats treated on day 6 with allylamine (150 mg.kg⁻¹ p.o); Values are means; Number of animals in parentheses.

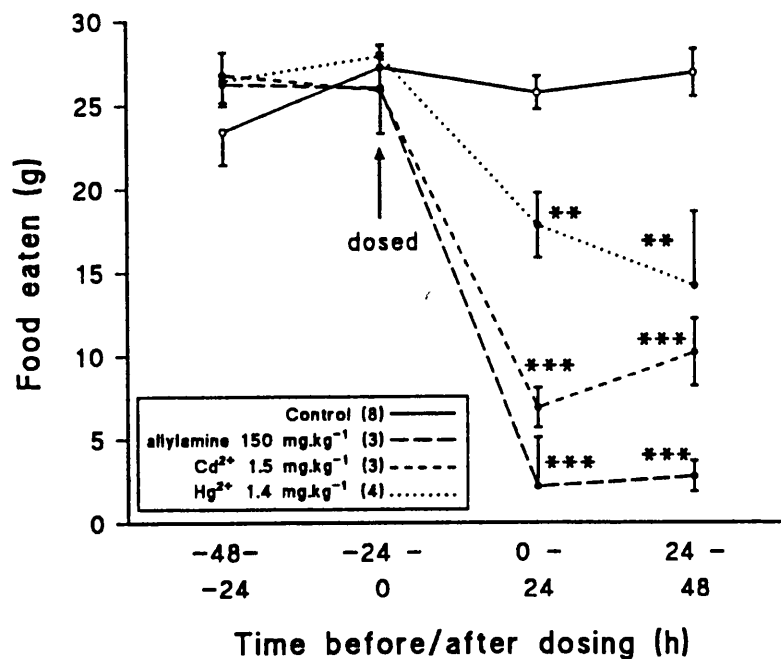


Figure 3.35 Food consumption of control rats and rats treated with allylamine (150 mg.kg⁻¹ p.o), cadmium chloride (1.5 mg.kg⁻¹ i.p) or mercuric chloride (1.4 mg.kg⁻¹ i.p); Values are means \pm SEM; N = number in parentheses; ** p < 0.01, *** p < 0.001 compared to pre-dose food consumption.

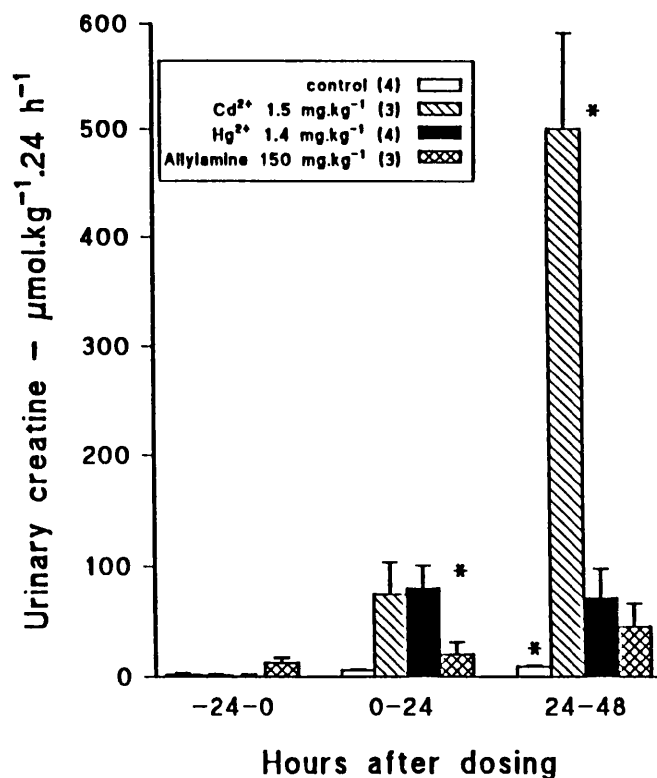


Figure 3.36 Urinary creatinine in 24 h urine collections from control rats and rats treated with allylamine (150 mg.kg⁻¹ p.o), cadmium chloride (1.5 mg.kg⁻¹ i.p) or mercuric chloride (1.4 mg.kg⁻¹ i.p) for -24-0 h before dosing and 0-24 and 24-48 h after dosing; Values are means \pm SEM; N = number in parentheses; * p < 0.05 compared to predose values..

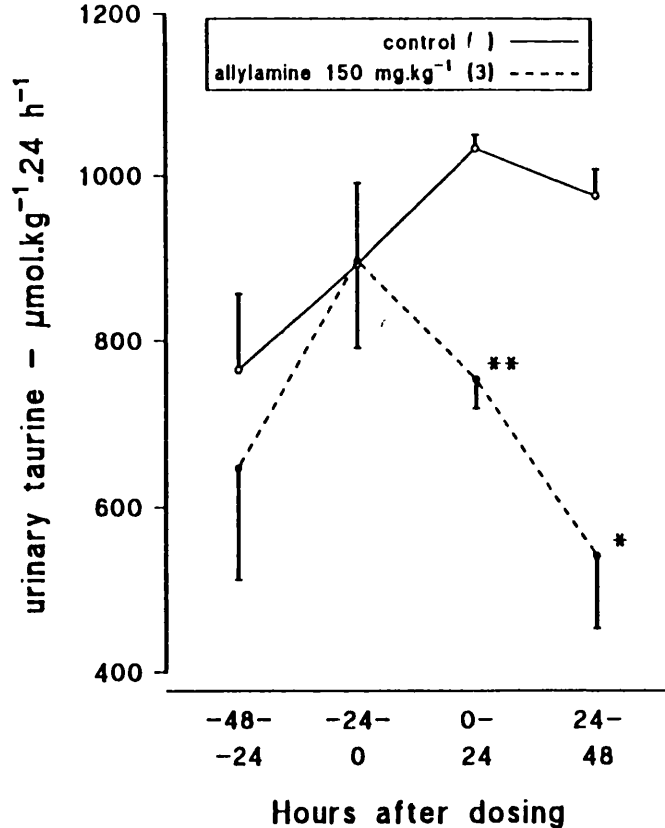


Figure 3.37 Urinary taurine from control rats and rats treated with allylamine (150 mg.kg⁻¹ p.o) in 24 h urine collections made for 48 h before and after dosing. Values are means \pm SEM; N = 3-4; * p < 0.05, ** p < 0.01 treated compared to controls, using "t" test.

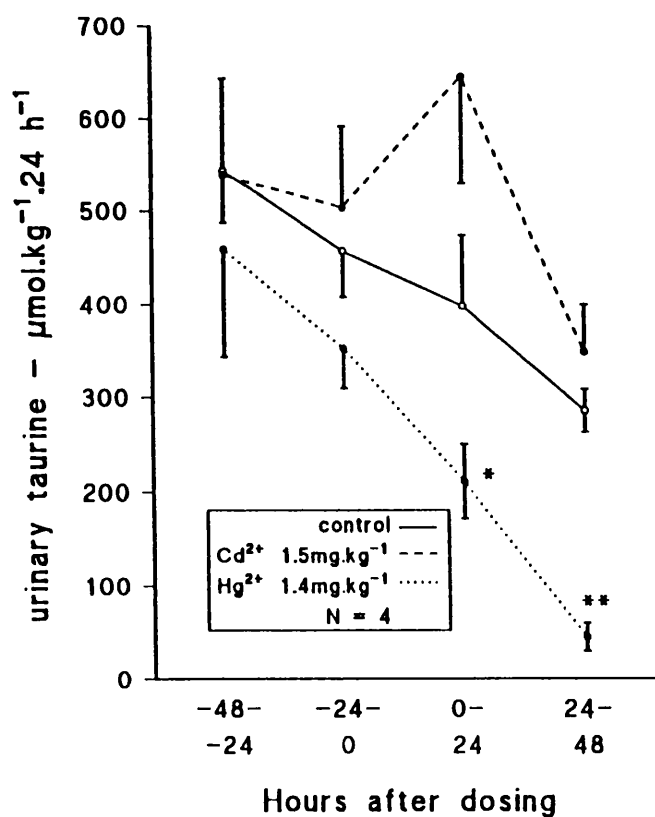


Figure 3.38 Urinary taurine from control rats and rats treated with mercuric chloride (1.4 mg.kg⁻¹ i.p) or cadmium chloride (1.5 mg.kg⁻¹ i.p) in 24 h urine collections made for 48 h before and after dosing. Values are means \pm SEM; N = 4; * p < 0.05, ** p < 0.01 treated compared to controls, using Dunnett's test.

Table 3.16 Relative organ weights, liver taurine and total non-protein sulphhydryls (TNPSH) in male rats 48 h after dosing with allylamine (150 mg.kg⁻¹), Cd²⁺ (1.5 mg.kg⁻¹) and Hg²⁺ (1.4 mg.kg⁻¹)

	CONTROL (A)	ALLYLAMINE	CONTROL (B)	Cd ²⁺	Hg ²⁺
Liver % body wt	4.66 ± 0.25	4.21 ± 0.28	4.33 ± 0.13	4.16 ± 0.17	3.67 ± 0.19*
Kidney % body wt	0.91 ± 0.04	0.93 ± 0.04	0.76 ± 0.02	0.78 ± 0.02	1.36 ± 0.21*
Testes weight (g)	2.47 ± 0.05	2.52 ± 0.09	2.78 ± 0.04	2.38 ± 0.09*	2.65 ± 0.08
Testes % body wt	0.96 ± 0.03	1.05 ± 0.03	0.96 ± 0.02	0.92 ± 0.03	0.97 ± 0.03
Heart % body wt	0.46 ± 0.02	0.49 ± 0.02	N.D.	N.D.	N.D.
Liver taurine (μmol.g ⁻¹ wet wt)	N.D.	N.D.	5.12 ± 1.31	6.87 ± 0.55	6.46 ± 0.44
TNPSH (μmol.g ⁻¹ wet wt)	N.D.	N.D.	7.41 ± 0.47	7.52 ± 0.41	6.77 ± 0.97

Table 3.17 Urinary γ-glutamyl transpeptidase (γ-GT) and protein 0 - 24 h after dosing with allylamine (150 mg.kg⁻¹ p.o.), cadmium chloride (1.5 mg.kg⁻¹ i.p.) and mercuric chloride (1.4 mg.kg⁻¹ i.p.).

	CONTROL (A)	ALLYLAMINE	CONTROL (B)	Cd ²⁺	Hg ²⁺
Urinary protein (mg.24 h ⁻¹ .kg ⁻¹)	66 ± 5.8	59 ± 17.4	89 ± 16	87 ± 13	377 ± 7*
γ-GT, (iu.h ⁻¹ .kg ⁻¹)	7.84 ± 0.34	8.67 ± 0.87	8.97 ± 0.42	10.01 ± 1.57	96.00 ± 10.46**

Values, mean ± SEM, N = 3-4, * p< 0.05, ** p< 0.01, allylamine compared with control(A) and Cd²⁺ and Hg²⁺ compared with control (B).

Table 3.18 Blood biochemistry in male rats 48 h after dosing with allylamine (150 mg.kg⁻¹ p.o.), Cd²⁺ (1.5 mg.kg⁻¹ i.p.) and Hg²⁺ (1.4 mg.kg⁻¹ i.p.).

Serum - clinical chemistry	CONTROL (A)	ALLYLAMINE	CONTROL (B)	Cd ²⁺	Hg ²⁺
ALT iu.L ⁻¹	65.6 ± 5.6	44.3 ± 2.4**	88.6 ± 5.9	85.9 ± 7.2	51.6 ± 2.3**
AST iu.L ⁻¹	103.4 ± 9.2	132.3 ± 46.0	98.1 ± 11.9	214.8 ± 14.2***	164.38 ± 11.4***
Albumin g.L ⁻¹	35.6 ± 0.6	34.6 ± 0.1	33.2 ± 0.6	26.8 ± 1.2**	29.7 ± 1.3
Total protein g.L ⁻¹	63.2 ± 3.1	59.7 ± 2.0	55.8 ± 1.0	46.8 ± 2.3*	52.1 ± 2.4
BUN mmol.L ⁻¹	5.92 ± 0.14	4.71 ± 0.61	5.90 ± 0.42	5.58 ± 0.24	43.45 ± 5.29***
Bilirubin µmol.L ⁻¹	0.85 ± 0.18	1.00 ± 0.12	1.08 ± 0.21	1.53 ± 0.23	8.73 ± 0.31***
Creatinine µmol.L ⁻¹	54.0 ± 1.7	46.3 ± 2.9	44.0 ± 1.6	43.7 ± 1.5	358.8 ± 43.6**
Creatine kinase iu.L ⁻¹	383.8 ± 101.0	461.7 ± 196	N.D.	N.D.	N.D.
ALP iu.L ⁻¹	618.8 ± 73.0	474.0 ± 67.2	551.8 ± 32.2	173.3 ± 6.3**	433.4 ± 54.9

Values, mean ± SEM, N = 3-4, * p< 0.05 ** p< 0.01, *** p< 0.001, allylamine compared to control (A) Student's "t" test, cadmium chloride (Cd²⁺) and mercuric chloride (Hg²⁺) compared with control (B) using Dunnett's test, N.D. = no data.

c. *Urinalysis*

Levels of protein and γ -GT were normal in animals treated with allylamine (Table 3.17). There was a slight elevation of urinary creatine 24 - 48 h after dosing but this was not significant (Figure 3.36). Urinary taurine levels were significantly lower than control values 0 - 24 and 24 - 48 h after dosing (Figure 3.37).

ii. **Cadmium chloride**

a. *Histology*

Three of the four animals, treated with cadmium chloride, showed ischaemic necrosis of the testes (Plates 32 and 33). One animal had normal testicular morphology and did not show any clinical signs of toxicity. The absolute testicular weights of these animals were significantly lower than the control animals and were reduced, but not significantly, when expressed as % body weight (Table 3.16). Liver morphology appeared to be normal but staining with Oil Red O showed that there was an accumulation of fat around the portal tracts the livers of two animals (Plate 34).

b. *Blood biochemistry*

Serum AST values were significantly raised to twice the control values (Table 3.18) and both serum albumin and total serum protein were significantly lower than control values. Serum ALP levels were also significantly lower than control values. All other biochemical parameters were similar to control values.

c. *Urinalysis*

Both urinary protein levels and γ -GT levels were similar to control values. Those animals showing ischemic testicular necrosis had significantly raised urinary creatine levels 24 - 48 h after dosing (Figure 3.36). Two animals had raised urinary taurine levels 0-24 h after dosing. These two animals corresponded to the animals with a marked hepatic steatosis. However, urinary taurine levels were similar to control values 24 - 48 h after dosing (Figure 3.38).

d. *Liver taurine and TNPSH*

Levels of liver taurine and TNPSH were similar to control values 48 h after dosing (Table 3.16).

iii. **Mercuric chloride**

a. *Histology*

Histological examination of the kidneys revealed a marked necrosis of the epithelial cells lining the pars recta of the proximal tubule (Plates 35 and 36). Kidney weights (% body weight) were significantly higher than control values and liver weights were significantly lower, 48 h after dosing (Table 3.16). Liver histology appeared normal, although staining with PAS stain showed that there was less glycogen present than in the control livers. Testicular histology appeared normal.

b. *Blood biochemistry*

Serum levels of ALT were significantly lower than control values and AST levels were raised significantly by 75% (Table 3.18). Serum creatinine was significantly raised 48 h post dosing; an increase which was mirrored by the reduced levels of urinary creatinine 24 - 48 h after dosing. Serum blood urea nitrogen was also raised to 8X control levels.

c. *Urinalysis*

Animals given mercuric chloride showed a marked proteinuria 0 - 48 h post dosing and elevated γ -GT levels 0 - 24 h post dosing (Table 3.17). Urinary creatine was elevated 0 - 48 h post dosing, but not to the same degree as the levels found in cadmium treated animals (Figure 3.36). Urinary taurine was significantly reduced 0 - 48 h post dosing (Figure 3.38).

d. *Liver taurine and TNPSH*

Liver levels of taurine and TNPSH were similar to control values, 48 h after dosing (Table 3.16).

3.6.5 DISCUSSION

i. Heart - allylamine treatment

Dosing rats with the myocardial toxin, allylamine, resulted in damage to myocardial muscle. One animal had raised levels of serum creatine kinase and AST 48 h after dosing. However, urinary taurine was not raised in these animals despite the fact that myocardial lesions were found. This was, perhaps, surprising as a high concentration of taurine is found in heart muscle (Hayes and Sturman, 1981; Huxtable *et al*, 1979). However, as a percentage of body weight the heart constitutes less than 0.5% of the body weight of a rat, ten times less than the liver and any leakage of taurine may have been masked by the day to day variation in urinary taurine levels. One of the contributing factors to the elevation of urinary taurine after liver necrosis may be an increase in taurine synthesis by the liver, possibly as a result of protein synthesis inhibition (Sections 3.3 and 3.5). The heart has a very low synthetic capacity for taurine, as heart tissue has very little cysteine dioxygenase activity (Zelikovic and Chesney, 1989). Tissue levels are maintained by the influx of exogenous taurine into the heart rather than endogenous cardiac biosynthesis (Chubb and Huxtable, 1978). This may result in much lower levels of taurine being seen after necrosis of cardiac muscle than liver tissue where the synthetic capacity for taurine, especially in male rats, is very high (Worden and Stipanuk, 1985). A confounding factor in the use of allylamine to initiate necrosis in cardiac tissue in these studies may be its metabolic conversion to acrolein. Although this takes place specifically in heart muscle the acrolein which is produced could circulate to other organs. As acrolein conjugates with GSH it may have resulted in the depletion of GSH in tissues other than the heart. This could compromise taurine synthesis if cysteine levels were lowered by an increase in the synthesis of GSH. The fat accumulation in the liver suggests that there was an effect on the liver due to the administration of allylamine.

Animals given allylamine also ate less food than control animals (Figure 3.35) which would also have reduced urinary taurine levels.

ii. Testes - cadmium

Although the dose of cadmium chloride administered ($1.5 \text{ mg.kg}^{-1} \text{ Cd}^{2+}$) was judged to be low enough to selectively damage the testes, the organ most susceptible to cadmium, this dose clearly had an effect on the liver. The gross effects on the liver appeared to be confined to hepatic steatosis as there was no increase in serum ALT levels. Serum levels of albumin and total protein were both significantly reduced indicating that protein synthesis had been impaired. This may explain why there was an increase in fat accumulation in the liver as a result of the administration of cadmium. Cadmium has been reported to decrease protein synthesis as a result of the loss of membrane bound ribosomes (Dudley et al, 1984). This would be consistent with the view that lipid accumulates when formation of lipoproteins for transportation out of hepatocytes is impaired.

Taurine excretion was slightly increased (0 - 24 h) in two animals which had hepatic steatosis 48 h after treatment. This is similar to the observation that steatosis induced by hepatotoxins such as hydrazine and ethionine also result in an elevation of urinary taurine. A decrease in protein synthesis would result in more methionine and cysteine being available for taurine synthesis which may explain the slight elevation of taurine in two of the treated animals.

Liver levels of taurine were slightly raised, but not significantly, above control values (Table 3.16). This may indicate a slight increase in taurine synthesis as these animals had reduced their food intake after treatment (Figure 3.35) as the "extra" taurine in the liver and urine would be unlikely to have come from the diet.

This dose of cadmium also resulted in severe testicular lesions. There was a concomitant elevation of urinary creatine 24 - 48 h after dosing which was consistent with previous findings (Gray *et al*, 1990). This creatine is believed to arise directly from damaged Sertoli cells which have a high concentration of creatine.

The reduction in ALP after Cd^{2+} administration (Table 3.18) may have been due to the reduced food intake of these animals as malnutrition has been shown to reduce levels of ALP (Suber, 1989). However rats treated with

mercuric chloride had a similar food intake and did not show this reduction. It seems more likely, therefore, that there was a direct action of Cd^{2+} , possibly by a replacement of Zn^{2+} in the enzyme itself, reducing the activity of the enzyme.

The absence of any elevation of serum creatinine and urea and the lack of γ -GT or raised levels of protein in the urine from these animals confirmed the histological evidence that the kidneys of these animals had not been overtly affected by the dose of cadmium chloride administered.

iii. Kidney - mercuric chloride.

Histology and clinical chemistry confirmed that there was severe renal damage in all the rats treated with mercuric chloride ($1.5 \text{ mg.kg}^{-1} \text{ Hg}^{2+}$). Serum levels of urea, bilirubin and creatinine were all raised, as were urinary levels of γ -GT and protein.

The data presented here show a significant *reduction* in urinary taurine 0 - 24 h and 24 - 48 h after dosing rats with mercuric chloride. This was an unexpected result since the kidney is responsible for the active reabsorption of taurine via a specific β -amino acid uptake system. Indeed, it is recognised that Hg^{2+} toxicity results in aminoaciduria as part of the Fanconi syndrome. However, work carried out by Gartland *et al.* (1988) using various region-specific nephrotoxins also failed to detect any increase in urinary taurine after kidney damage (measured by proton NMR), whereas studies using hepatotoxins did show an elevation in taurine in urine detectable by NMR.

Under normal circumstances, glutathione is removed by the kidney, most being hydrolysed by γ -glutamyltransferase and cysteinylglycine dipeptidase (Kaplowitz *et al.*, 1985). The γ -glutamylcystine formed from transpeptidation, is transported into tubular epithelial cells and intracellular thiol-disulphide exchange liberates cysteine and γ -glutamylcysteine for GSH synthesis. However, mercury has been shown to combine with sulphydryl groups (Klaassen *et al.*, 1986) which may have interrupted the cycle of GSH synthesis by binding sulphydryl groups, less cysteine would have been available for both glutathione and taurine synthesis. It is possible that this

may have contributed to the reduction in urinary taurine seen after Hg^{2+} induced kidney damage.

Damage to kidney tissues might also be expected to result in leakage of cellular taurine directly from the kidney. However, like the heart, the kidney is a small organ and has not been reported to contribute to the synthesis of taurine. Renal concentrations of taurine are 5 - 11.8 $\mu\text{mol.g}^{-1}$ wet weight (Jacobsen and Smith, 1968) and total kidney weight in the animals used in these studies was approximately 3 g. If all the cellular taurine was lost from the kidneys this would only result in an increase of 15 - 33 μmol taurine in total, in the urine, an increase which would be masked by daily variation.

The serum concentration of taurine is usually 100-250 μM . The filtered load in the kidney will only increase substantially if the serum levels are raised, for example, after ingestion of increased levels of taurine, tissue damage and/or increased synthesis. It is possible, therefore, that the reduced levels of urinary taurine indicate a reduction in taurine synthesis. Although it is possible to speculate that this reduction in urinary taurine following Hg^{2+} induced kidney damage was due a reduction in taurine and taurine precursors or an interference with cysteine metabolism, further studies are needed to clarify this unexpected observation. However, the observation that kidney injury does not necessarily result in *raised* levels of urinary taurine and may even lower them, is important as some hepatotoxic compounds cause renal injury (eg. thioacetamide).

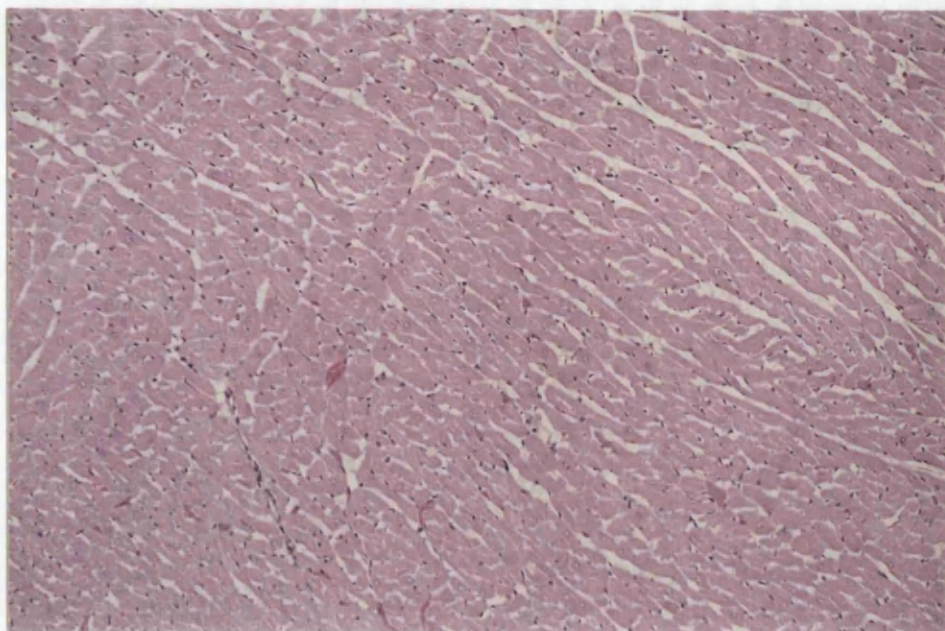


Plate 30. Light micrograph of a section of heart taken from a male control animal. H and E staining. Mag. x 100

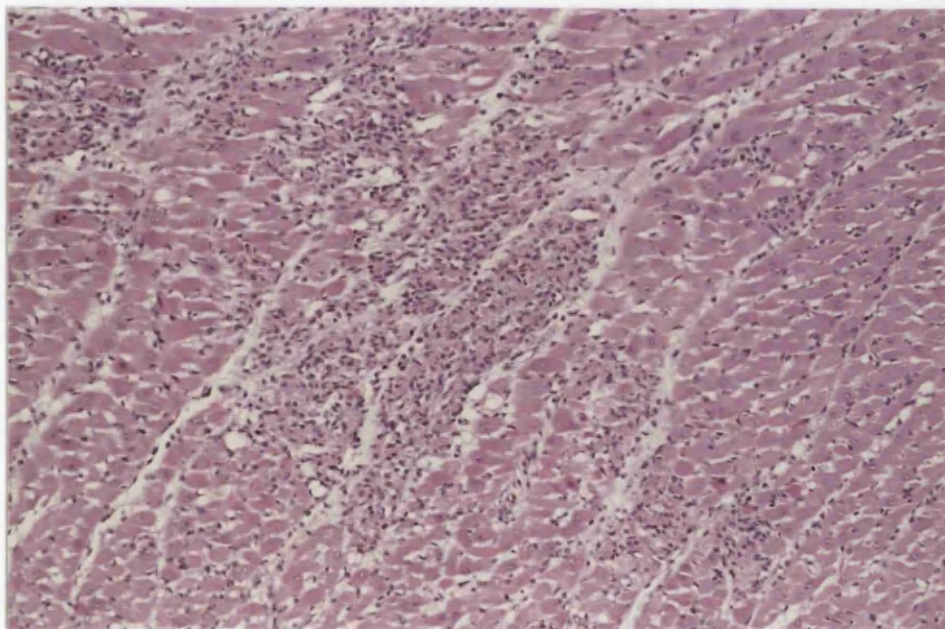


Plate 31. Light micrograph of a section of heart taken from a male rat 48 h after dosing with allylamine (150 mg.kg⁻¹ po). H and E staining. Mag. x 100

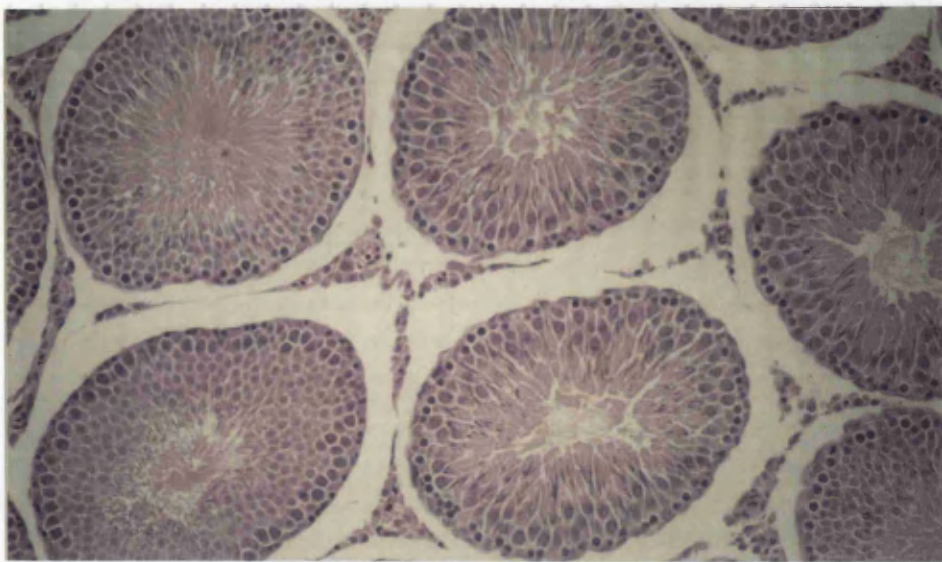


Plate 32. Light micrograph of a section of testes taken from a male control rat. H and E stain. Mag. x 100.

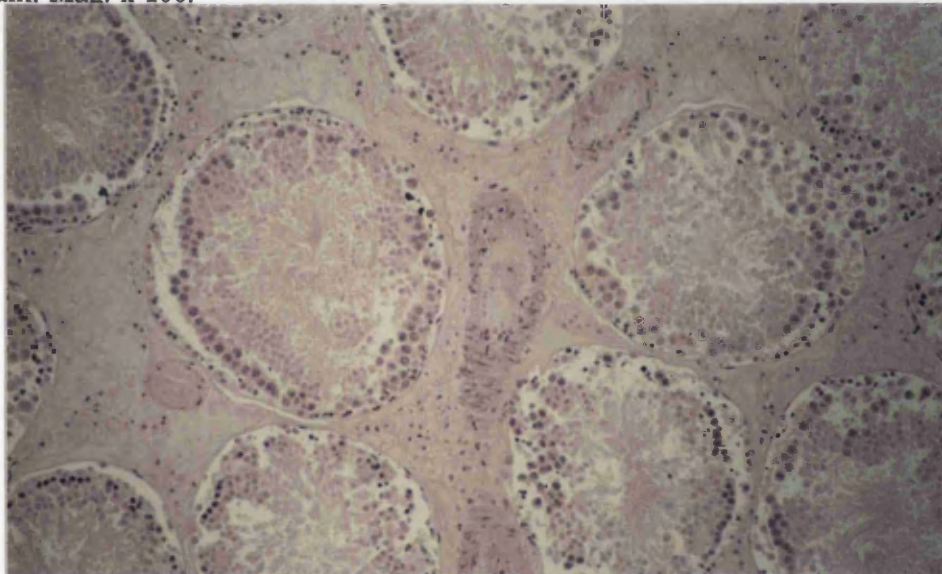


Plate 33. Light micrograph of a section of testes taken from a male rat 48 h after dosing with cadmium chloride ($1.5 \text{ mg.kg}^{-1} \text{ Cd}^{2+}$ ip). H and E stain. Mag. x 100.

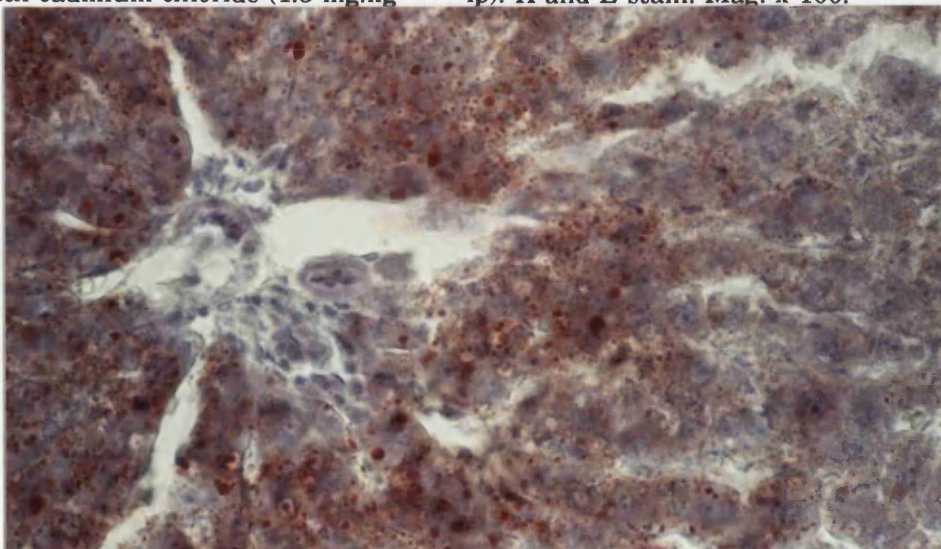


Plate 34. Light micrograph of a frozen section of liver taken from a male rat 48 h after dosing with cadmium chloride ($1.5 \text{ mg.kg}^{-1} \text{ Cd}^{2+}$ ip) which had high urinary taurine levels. Oil Red O stain. Mag. 400

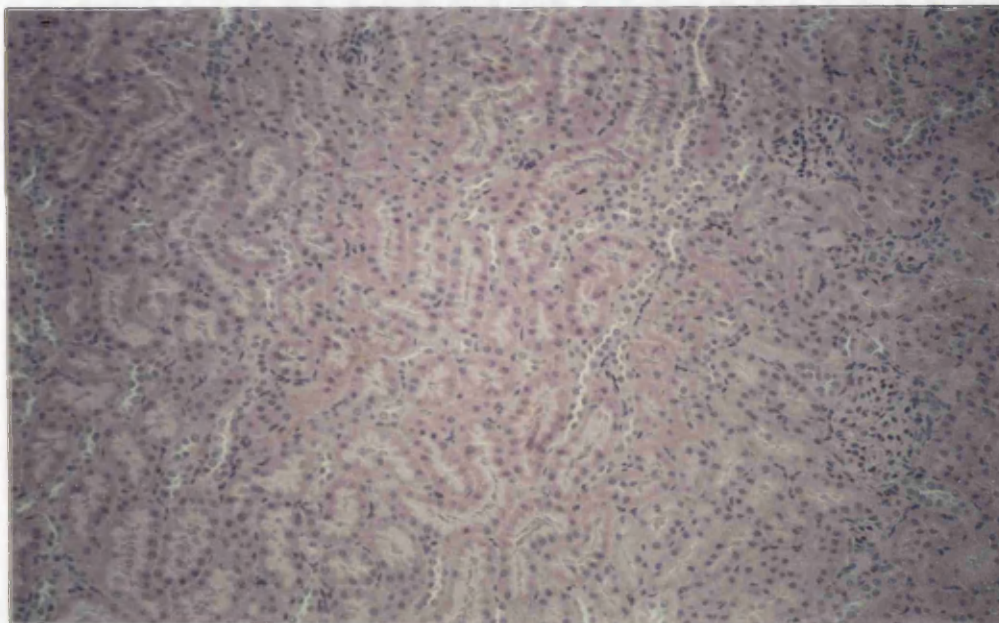


Plate 35. Light micrograph of a section of kidney cortex taken from a control rat. H and E stain. Mag x 160.

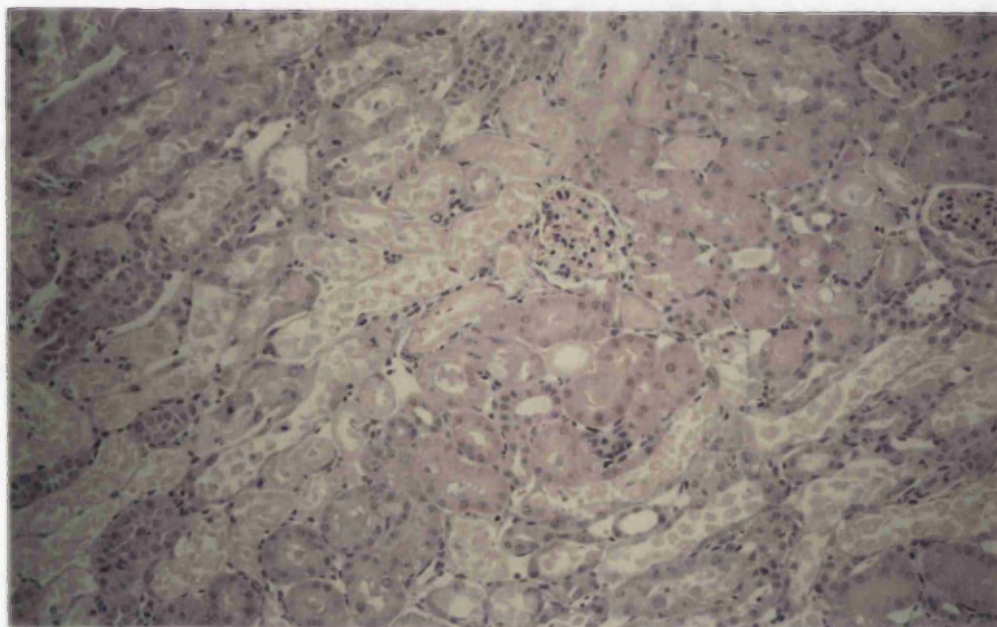


Plate 36. Light micrograph of a section of kidney cortex taken from a rat 48 h after dosing with mercuric chloride ($1.4 \text{ mg.kg}^{-1} \text{ Hg}^{2+} \text{ ip}$). H and E stain. Mag x 160.

DE NOVO SYNTHESIS OF TAURINE

4.1

AN INVESTIGATION INTO THE CONTRIBUTION MADE BY *DE NOVO* SYNTHESIS OF TAURINE TO THE HYPERTAURINURIA FOUND AFTER DOSING RATS WITH CARBON TETRACHLORIDE

4.1.1 INTRODUCTION

The investigations discussed in Chapter 3 were concerned with the feasibility of using an increase in urinary taurine as a marker of liver damage following the treatment of rats with various hepatotoxic compounds. The hypothesis was that necrosis would result in a leakage of intracellular taurine. However, some compounds caused hepatic necrosis without elevating urinary taurine (bromobenzene and allyl alcohol) and others raised urinary taurine levels without causing necrosis (ethionine and hydrazine). It was also apparent that where hepatic necrosis may have contributed to the elevations of urinary taurine observed, it was not possible to account for this loss by a concomitant loss of liver taurine.

The hepatotoxic compounds which did elevate urinary taurine were also those which are known to inhibit protein synthesis (CCl_4 , thioacetamide, galactosamine and ethionine). Those hepatotoxic compounds that did not raise urinary taurine or resulted in hypotaurinuria were those known to increase protein synthesis (phenobarbitone) or indirectly increase glutathione synthesis (allyl alcohol and bromobenzene). Both processes result in the removal of the taurine precursors methionine and cysteine. This suggested that perturbations in taurine synthesis were responsible for some of the changes in levels of urinary taurine which were found in rats treated with these compounds.

The use of diethyl maleate, BSO, phorone and cycloheximide (Section 3.5) tended to confirm the hypothesis that metabolic alterations in taurine precursors were responsible for the marked hypo and hypertaaurinuria seen

after treating rats with some of these compounds. However, the inhibition of protein synthesis tends to be an early event in the sequence of toxic responses to hepatotoxic compounds (Zimmerman, 1978). CCl_4 has been shown to inhibit protein synthesis as early as 5-10 min after dosing and within 6 - 7 h after dosing with ethionine (Dianzani, 1991). The elevation of urinary taurine, however, was maximal 24 - 48 h after dosing with CCl_4 , galactosamine and ethionine which suggests that if the inhibition of protein synthesis contributes to the rise in urinary taurine the effect is delayed. Cycloheximide resulted in a more rapid elevation of urinary taurine which was maximal 0 - 24 h after dosing. If the inhibition of protein synthesis was responsible for the increases in urinary taurine which were observed, it was probably not a simple relationship.

Although these observations suggest that an increase in taurine synthesis might contribute to hypertaurinuria, the evidence is *indirect*. By measuring the incorporation of ^{14}C -L-leucine into proteins it would be possible to *correlate* taurine levels with protein synthesis. However, this is still an indirect method of relating the two processes. Therefore, it was the aim of this study to demonstrate whether there was an increase in taurine synthesis in rats following the toxic insult of CCl_4 .

4.1.2 AIM OF STUDY

To assess *de novo* synthesis of taurine directly by measuring the incorporation of ^{35}S derived from ^{35}S -methionine into taurine after the administration of the hepatotoxic compound, CCl_4 .

4.1.3 PRINCIPLES

At the time of dosing a rat with CCl_4 , the taurine available for excretion into the urine could have been derived from four possible sources:

- (a) taurine taken in directly in the diet
- (b) taurine synthesised by the rat (or gut flora in the rat) from precursors taken in the diet

- (c) taurine synthesised from precursors (methionine and cysteine) resulting from protein catabolism
- (d) Taurine already present in the animal from the mother at the time of birth

(Huxtable and Lippincott, 1982)

In an attempt to determine the contribution made to increased levels of taurine in the urine by leaked cellular taurine and *de novo* synthesis of taurine, the body pool of taurine was labelled (before dosing) by giving rats ^3H -taurine for four days prior to treatment with CCl_4 . After this time tissue levels of ^3H -taurine have been shown to reach an equilibrium with administered ^3H -taurine (Huxtable and Lippincott, 1982).

Whatever the route of biosynthesis, the sulphur atom in taurine, must ultimately be derived from methionine. Sulphur atoms are taken in by mammals mainly as methionine and cysteine in proteins. After digestion and absorption, these sulphur-containing amino acids enter the amino acid pool and are metabolised (Yoshida *et al.* 1989). Methionine was chosen in preference to cysteine as the source of sulphur in these studies in order to include putative pathways of taurine biosynthesis not involving cysteine (Huxtable and Lippincott, 1982). Cysteine is also rapidly metabolised once in the animal (Higashi *et al.*, 1983), possibly before CCl_4 would have had time to affect protein synthesis. Therefore, ^{35}S -methionine was given simultaneously with CCl_4 to enable the metabolism of methionine to taurine to be monitored after CCl_4 administration. It should be possible to measure the contribution made by newly synthesised taurine (labelled with ^{35}S) to the urinary and liver taurine pools after dosing with CCl_4 .

The following assumptions were made:

- i. both ^{35}S -methionine and ^3H -taurine would enter the body pools of methionine and taurine
 - ii. the catabolism of taurine to isethionic acid by intestinal bacteria would be a very small percentage of the total ^{35}S or ^3H -taurine
- (Huxtable and Bressler, 1972)

CCl_4 was chosen as the experimental compound as previous results had indicated that the hypertaurinuria found after dosing rats with CCl_4 may

be the result of an initial leakage of cellular taurine (which could be measured by determining the specific activity of ^3H -taurine) followed by elevated levels due to an increase in taurine synthesis (estimated by the incorporation of ^{35}S into taurine).

A preliminary investigation was carried out to determine the distribution of ^3H -taurine following administration for 4 consecutive days and to establish the degree to which radio labelled taurine equilibrated with endogenous taurine. The % recovery of the total dose in the urine and remaining in the tissues was estimated. It was assumed that most ^3H -taurine would remain un-metabolised as taurine is essentially an end product of sulphur amino acid metabolism with only a small percentage being converted to isethionic acid by intestinal bacteria (Huxtable and Bressler, 1972). Measurement of ^3H would therefore represent the distribution of ^3H -taurine in tissues.

4.1.4 STUDY 1 - An investigation into the distribution of ^3H -taurine after administration to rats for four consecutive days - a pilot study.

4.1.5 METHOD

Four male rats (230 - 245 g) were placed in individual metabolism cages for three days to acclimatize. On days 4, 5, 6 and 7 (11 am) each animal was given a single dose of ^3H -taurine (7.5 μCi in 1 ml UHQ water; specific activity 32 Ci.mmol^{-1} ; i.p.). Each animal received a total of 30 μCi over 4 days.

Food and water intake were monitored and 24 h urine and faecal collections were made over ice.

On day 8, animals were exsanguinated from the abdominal aorta under ether anaesthetic and a post-mortem was carried out. The liver, kidney, heart and brain as well as muscle and serum samples were taken for ^3H -taurine measurements.

Tissues were weighed and homogenised (1:5 w/v) in 0.2 M sulphosalicylic acid (4°C). Tissue homogenates were centrifuged (4,000 rpm, 15 m) and 100 µl of the supernatant added to 3.5 ml Aquasol and ^3H activity counted. Faeces were extracted for taurine in the same way as tissue samples.

4.1.6 RESULTS

The following table (Table 4.1) shows the urinary and faecal levels of ^3H after giving 7.5 µCi ^3H -taurine on four consecutive days. The final column shows the % recovery of the total activity administered.

Table 4.1 Distribution of ^3H activity after treatment with ^3H -taurine.

Total ^3H -taurine - 60441 dpm $\times 10^3$ /rat; N = 4.

SAMPLE	DAY	Total dose dpm $\times 10^3$	Total dpm $\times 10^3$ in sample	% dose recovered
Urine	1	15110	1084 \pm 244	19.05 \pm 1.6
	2	30220	2228 \pm 334	
	3	45330	4140 \pm 630	
	4	60441	4060 \pm 208	
Faeces	2	as above	876 \pm 166	4.85 \pm 0.68
	3		859 \pm 83	
	4		1200 \pm 238	

TISSUE	DAY	Total dose dpm $\times 10^3$	dpm $\times 10^3 \cdot \text{g}^{-1}$ or ml	% dose recovered
Liver	4	60441	123 \pm 6.4	2.42 \pm 0.1
Kidney			187 \pm 1.3	0.77 \pm 0.02
Heart			292 \pm 18.7	0.62 \pm 0.04
Brain			85 \pm 4.5	0.32 \pm 0.02
Muscle			166 \pm 8.5	-
Serum			44 \pm 4.1	-
Mean tissue value			150 \pm 4.1	-
- whole carcass			40283 \pm 1554	66.6 \pm 2.6

Values means \pm SEM; N = 4.

An estimate for the recovery of the total dose was made based on the average activity/g of tissue measured. This was then added to the activity recovered in the urine and the faeces. This approximation was made to ensure that most of the activity administered could be accounted for. Thus, $90.2 \pm 2.9\%$ of the original ^3H was found.

4.1.7 DISCUSSION

Approximately 20% of ^3H administered was recovered in the urine. The liver had the highest % of the dose in the organs which were measured. The tissues investigated indicated that there was uptake of ^3H -taurine into all tissues, although the uptake was greater in some tissues than others. Of those measured it was estimated that the liver and brain had the highest uptake as the specific activity was greatest in these tissues. Serum also had a high specific activity.

4.1.8 Study 2 - An investigation into the urinary and liver levels of pre-formed taurine and taurine produced by *de novo* synthesis after dosing rats with CCl_4 .

4.1.9 METHOD

Eight rats (male, 265 - 290 g from the same stock as those used in the previous pilot study) were housed in individual metabolism cages for three days to acclimatise. A further 8 rats were treated in the same way and used to provide pair fed control animals for the first group of eight animals. All sixteen rats were divided into groups of 4 animals each and paired to provide "pair fed" controls.

The rationale behind the use of pair fed animals in this study was to enable information about the distribution of ^{35}S and ^3H -taurine after dosing with CCl_4 to be related to the direct effect of CCl_4 or the reduction in dietary intake as a result of CCl_4 toxicity. As rats given CCl_4 reduce their intake of food there would be less "cold" taurine and methionine taken into the body pool of these animals. This was likely to result in less dilution of the previously labelled taurine pool and result in a higher specific activity of

^3H -taurine in these rats. The starvation of animals for longer than 24 h was not a viable method of removing this variable from the experiment as it was not permissible to starve animals for longer than 24 h under the Home Office licence employed in these studies. Therefore, the food intake of the CCl_4 and corn oil treated controls was monitored and 2 further groups (treated with ^3H -taurine alone) were subsequently fed the same amount of food as the treated animals. Animal weight, diet intake and water consumption were monitored. They were treated in the following way:

Table 4.2 Dosing regime of rats given ^3H -taurine, ^{35}S -methionine and CCl_4 .

GROUP	^3H -TAURINE (7.5 μCi)	^{35}S -METHIONINE (45 μCi)	CCl_4 (2 ml.kg $^{-1}$)
1	days 1 \rightarrow 4	day 5	Day 5 - no CCl_4
2	days 1 \rightarrow 4	day 5	Day 5 - CCl_4
3	days 1 \rightarrow 4	-	pair fed with group 1 - no CCl_4
4	days 1 \rightarrow 4	-	pair fed with group 2 - no CCl_4

^{35}S -Methionine (1000 Ci.mmol $^{-1}$) was given simultaneously (45 μCi in UHQ water; 1 ml; i.p.) with corn oil (1 ml kg $^{-1}$, p.o.) (Group 1) or CCl_4 (2 ml.kg $^{-1}$, in 1 ml.kg $^{-1}$ corn oil; p.o.) (Group 2). Group 3 was fed diet to correspond to the food intake of animals in Group 1 and those in Group 4 were given similar quantity of diet eaten by group 2. Diet was withheld from Group 4 until 9 h after the dosing time of group 2 as animals dosed with CCl_4 did not resume eating until this time.

Urine collections (24 h) were made throughout the study period.

Animals were exsanguinated from the abdominal aorta under diethyl ether anaesthesia and tissues were taken at post mortem for measurement of ^3H and ^{35}S -taurine. Liver and kidney were also taken for histology.

Tissue and urine samples were extracted for taurine using Dowex resin columns as before (Stipanuk, 1979). Dual counting of ^3H and ^{35}S was carried out by putting 100 μl sample into 3.5 ml Aquasol and counting in

two channels simultaneously. Channels were set at different energy levels to count each isotope with maximum efficiency and least over-lap. Colour, optical and chemical quenching were calculated by the addition of a standard amount of ^3H -taurine and ^{35}S -methionine to each vial and carrying out a second count (Weinstein *et al*, 1988).

Identification of ^3H and ^{35}S -taurine was carried out by collection of the chromatographed adducted taurine in timed fractions from HPLC analysis. The incorporation of ^{35}S into the acid precipitable fraction of liver homogenate was measured by homogenising 0.5 g of liver in sulphosalicylic acid (0.5 ml 0.2 M) and centrifugation of the precipitate (4,000 rpm; 15 min). The precipitated proteins were resuspended and washed three times until the supernatant had no detectable ^{35}S -activity (3 washes). The precipitate was solubilized in NaOH (1 M; 4 ml; 40°C). Aliquots (500 μl) were then counted for ^{35}S -activity.

4.1.10 RESULTS

i. *Body weight* - Rats treated with CCl_4 (group 2) had reduced diet intake and lost body weight (Table 4.4). Weight loss in pair fed animals (group 4) was very similar (Figure 4.1). The two control groups of pair fed animals fed *ad libitum* showed very similar weight gain.

ii. *Histology* - All animals treated with CCl_4 showed hepatic steatosis throughout the liver parenchyma and hydropic degeneration and necrosis around the central veins. Animals "pair fed" with CCl_4 treated rats had microvesicular accumulation of fat in periportal hepatocytes. Staining liver sections with PAS stain indicated that there was a reduction in liver glycogen in both the CCl_4 treated and their pair fed controls, although the reduction was greatest in those animals which were pair fed and not dosed with CCl_4 .

iii. *Serum enzymes and liver weight* - Serum levels of ALT and AST were significantly raised above control values in animals treated with CCl_4 and ALT levels (but not AST levels) were significantly reduced in animals pair

fed with treated animals (Table 4.3). Liver weights (%body weight) were significantly higher in CCl₄ treated animals and significantly lower in animals pair fed with CCl₄ treated animals (Table 4.4).

Table 4.3 Serum enzyme levels 48 h after dosing with CCl₄ (2 ml.kg⁻¹; p.o.) compared with pair fed controls.

GROUP	TREATMENT	SERUM ALT iu.L ⁻¹	SERUM AST iu.L ⁻¹
1	CONTROL	48.1 ± 6.1	72.5 ± 8.3
3	PAIR FED WITH CONTROL	67.3 ± 4.2	82.8 ± 9.2
2	CCl ₄ TREATED	742.8 ± 200.8*	2235.8 ± 433*
4	PAIR FED WITH CCl ₄ TREATED	33.3 ± 3.5***	93.3 ± 9.2

Values - means ± SEM; N = 4; * p< 0.05, *** p< 0.001 (1) compared with (3) and (2) compared with (4), Student's "t" test.

iv. *Urinary taurine* - After dosing with CCl₄, rats excreted significantly more urinary taurine than the control group (group 1) 0 - 24 h and 24 - 48 h after dosing (Figure 4.2 and Figure 4.3 a). Those animals which were pair fed with the CCl₄ treated animals had significantly less urinary taurine than the control groups (1 and 3) 24 - 48 h after limiting the diet available to correspond to the food intake of the CCl₄ treated animals.

There was an increase in the *total* ³H-taurine and ³⁵S-taurine excreted in the CCl₄ treated animals. However, when expressed as dpm.μmole⁻¹ taurine (specific activity) there was no difference in the specific activity of urinary ³H-taurine when values from CCl₄ treated animals were compared with control values (group 1) during the 48 h following dosing (Figure 4.3 c). The specific activity of urinary ³H-taurine from animals "pair fed" with CCl₄ treated animals was twice the activity of the control and CCl₄ treated animals both 0 - 24 and 24 - 48 h after restricting the food intake.

The specific activity of ³⁵S-taurine was significantly higher in control animals (group 1) than in CCl₄ treated animals 0 - 24 h after dosing but significantly higher in treated animals 24 - 48 h after dosing (Figure 4.3 b). Unfortunately there was no ³⁵S-taurine data for "pair fed" control animals.

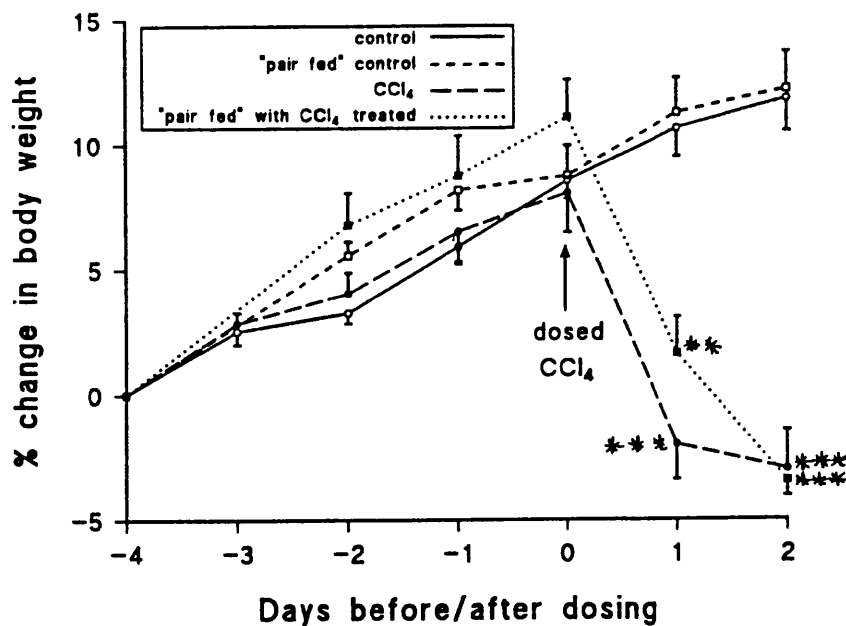


Figure 4.1 % Weight change in control, pair fed control rats, rats treated with CCl₄ (2 ml.kg⁻¹ p.o) and rats pair fed with CCl₄ treated rats. Values are means \pm SEM; N = 4; ** p < 0.01, *** p < 0.001.

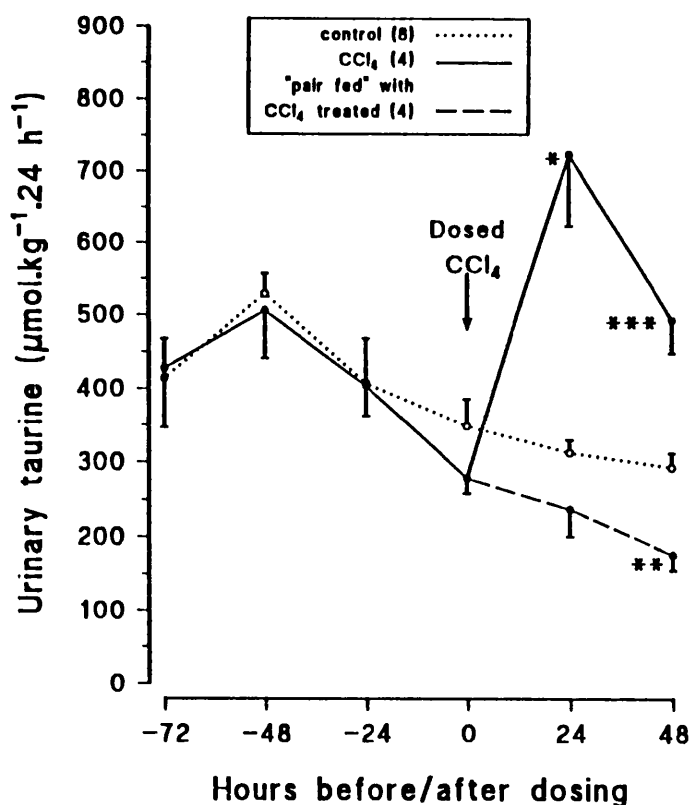


Figure 4.2 Urinary taurine levels (μ mol.kg⁻¹.24 h⁻¹) from control rats (N = 8), rats treated with CCl₄ (2 ml.kg⁻¹ p.o; N = 4) and rats "pair fed" with treated rats (N = 4); * p < 0.05, ** p < 0.01, *** p < 0.001 CCl₄ and "pair fed" with CCl₄ treated (ie. reduced diet) compared to normal fed and "pair fed" control.

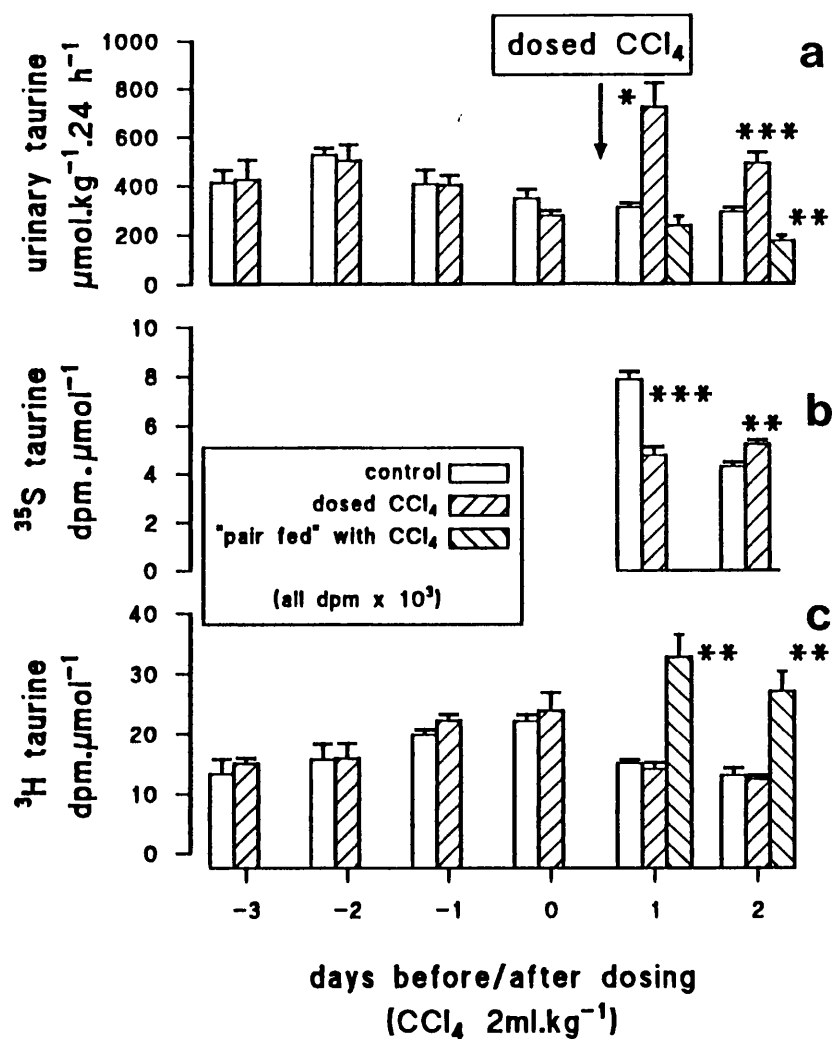


Figure 4.3

- Urinary taurine excretion from control and "pair fed" control rats (N = 8), CCl₄ treated rats (2 ml.kg⁻¹ p.o, N = 4) and rats "pair fed" with treated rats (N = 4). See also figure 4.2.
- Specific activity of urinary ³⁵S-aurine in rats treated with CCl₄(2 ml.kg⁻¹ p.o, N = 4) and control rats (N = 4)
- Specific activity of urinary ³H-aurine in control and control"pair fed" rats (N = 8), CCl₄ treated (2 ml.kg⁻¹, p.o; N = 4) and rats "pair fed" with CCl₄ treated rats (N = 4)

Values are means ± SEM; * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to control values.

Table 4.4 Summary of liver weights, liver taurine concentrations and specific activity of ^3H and ^{35}S -taurine 48 h after dosing rats with CCl_4 and pair feeding groups of rats with treated and control groups.

	control	CCl_4 treated	"pair fed" control	"pair fed" with treated
	Group 1	Group 2	Group 3	Group 4
Diet consumed (g)				
0 - 24 h after dosing	27.8 \pm 1.5	2.9 \pm 1.4***	26.0 \pm 0.7	2.9***
24 - 48 h after dosing	28.0 \pm 1.2	8.4 \pm 2.0***	25.1 \pm 1.0	8.4***
Liver weight (g)	13.0 \pm 0.3	14.3 \pm 0.5	11.6 \pm 0.3	7.5 \pm 0.1***
Liver % body weight	4.12 \pm 0.04	5.38 \pm 0.14***	4.01 \pm 0.08	3.00 \pm 0.05***
Taurine ($\mu\text{mol.g}^{-1}$ liver)	8.30 \pm 1.52	6.76 \pm 0.84	7.40 \pm 0.54	6.63 \pm 0.43
Total taurine ($\mu\text{mol/liver}$)	89.0 \pm 9.7	96.0 \pm 10.9	85.8 \pm 6.4	49.7 \pm 4.1**
^3H -taurine (dpm. μmol^{-1})	7.3 \pm 0.7	12.0 \pm 0.6***	10.1 \pm 2.0	18.3 \pm 0.9**
Total ^3H -taurine (dpm/liver)	701 \pm 36	1146 \pm 11***	852 \pm 160	900 \pm 35
^{35}S -taurine (dpm. μmol^{-1})	2.0 \pm 0.1	3.0 \pm 0.1***	ND	ND
Total ^{35}S -taurine (dpm/liver)	180 \pm 22	310 \pm 39***	ND	ND
^{35}S -acid precipitate (dpm. g^{-1} liver)	100 \pm 8.6	95 \pm 5.9	ND	ND

All dpm values $\times 10^3$; Values mean \pm SEM; N = 4; ** $p < 0.01$, *** $p < 0.001$ compared to control or "pair fed" control, using Student's " t " test; ND = no data.

v. *Liver taurine* - The concentration of taurine in liver tissue, measured as $\mu\text{mol.g}^{-1}$ wet weight was not significantly different in any of the groups of animals. However, the *total* taurine present in the livers from these animals was calculated and those animals "pair fed" with animals treated with CCl_4 were found to have significantly less taurine in their livers when compared with both the treated and control animals (Table 4.4).

The specific activity of ^3H -taurine was higher in CCl_4 treated animals and

their pair fed controls 48 h after treatment and the specific activity of ^{35}S -taurine was significantly higher in CCl_4 treated animals than control animals. The ratio of ^3H -taurine: ^{35}S -taurine was higher in the treated animals but not significantly different from the control animals (4.02 ± 0.26 and 3.69 ± 0.29).

4.1.11 DISCUSSION

The significant body weight loss was probably due to the reduction in diet consumption rather than the treatment with CCl_4 .

It was interesting that the restricted diet of the "pair fed" animals resulted in a significant reduction in serum ALT levels (but not AST) which was reported after hydrazine treatment, a compound known to have anorexic effects (Amenta and Johnson, 1962). The hepatic steatosis was seen in CCl_4 treated animals was consistent with an inhibition of protein synthesis. The slight microvesicular accumulation of fat seen in the animals pair fed with CCl_4 treated animals was probably the result of a reduction in dietary protein available for the production of lipoproteins needed for the transportation of lipid out of cells.

The increase in liver weight of CCl_4 treated animals was probably due to water and lipid accumulation which countered the reduction in glycogen content seen in animals which were "pair fed" with CCl_4 treated animals. The elevation of urinary taurine 24 - 48 h after dosing with CCl_4 was not as marked as might have been expected from previous investigations (Figure 3.4 a and Table 3.9). However, animals "pair fed" with those given CCl_4 showed a significant reduction in urinary taurine 24 - 48 h after the start of the limited feeding regime. This suggests that the difference in urinary taurine between control rats (group 1) and those that were on a restricted diet was the result of reduced dietary excess of taurine and the precursors methionine and cysteine. It also suggests that the increase in urinary taurine after CCl_4 treatment would have been greater if animals had the same dietary intake as untreated control animals.

In order to evaluate the implications of the changes in the specific activities of urinary ^3H and ^{35}S -taurine after treating rats with CCl_4 , a hypothesis was devised to explain possible changes in the specific activities of ^3H and ^{35}S -taurine under various conditions. These hypothetical results were then compared with the values which had been found.

4.1.12 HYPOTHESES

What changes might be expected in the specific activity of ^3H and ^{35}S -taurine after the administration of a hepatotoxic dose of CCl_4 when levels of urinary taurine are increased?

The following table (4.5) suggests four possible factors which may be involved in alterations in urinary taurine levels and suggests what results might be expected after each situation.

Table 4.5 Hypotheses concerning possible causes of alterations in the specific activity of ^3H and ^{35}S -taurine in urinary taurine following CCl_4 treatment and restricted food.

(1) TAURINE LEAKS FROM CELLS - NO CHANGE IN TAURINE SYNTHESIS	(2) INCREASED TAURINE SYNTHESIS	(3) DECREASED TAURINE SYNTHESIS	(4) DECREASED INTAKE OF TAURINE, METHIONINE AND CYSTEINE
^3H -taurine s.a. same as controls	^3H -taurine s.a. reduced	^3H -taurine s.a. increased	^3H -taurine s.a. increased
^{35}S -taurine s.a. lower in treated than controls	^{35}S -taurine s.a. increased	^{35}S -taurine s.a. decreased	^{35}S -taurine s.a. increased

(s.a. = specific activity)

Some assumptions were been made in constructing the different hypotheses. For example, it was assumed that the body pool of taurine is equally labelled with ^3H -taurine, that there is a delay in ^{35}S -methionine metabolism and that only one factor is affected by either CCl_4 treatment or restricted diet at any one time. The hypotheses describe changes in CCl_4 treated animals when compared to control animals.

- (1) If taurine leaks from damaged cells the specific activity of ^3H -taurine might be expected to be similar to control levels, even though there is an increase in the total amount of taurine being excreted (Jacobsen and Smith, 1968). The specific activity of ^{35}S -taurine would be reduced if there was no increase in the synthesis of taurine as it would be diluted by the leaked taurine. This assumes that the animals have a similar intake of cold taurine and precursors to the control animals.
- (2) If there is an increase in taurine synthesis urinary taurine levels should be raised. This will dilute the ^3H -taurine thus reducing the specific activity. At the same time, the specific activity of ^{35}S -taurine should increase.
- (3) A reduction in the total urinary taurine might be expected if taurine synthesis is decreased, but the specific activity of ^3H -taurine would increase as there would be less *de novo* taurine to dilute the ^3H -taurine. The specific activity of ^{35}S -taurine would be decreased by the decrease in synthesis.
- (4) As a high % of urinary taurine is due to overflow from excess taurine taken in in the diet, any reduction in dietary intake of taurine would result in a reduction of urinary taurine. This reduction would have the effect of concentrating the radio-labelled taurine and the specific activity of both ^3H and ^{35}S -taurine would be expected to be raised.

If the changes in specific activity of urinary taurine after CCl_4 treatment are compared with control values without reference to dietary intake of taurine the following conclusions could be drawn -

i. Urinary ^{35}S and ^3H -taurine

Day 1 post CCl_4 treatment - The specific activity of ^3H -taurine in urine was the same in treated animals and control animals but the specific activity of ^{35}S -taurine in urine was lower in treated animals than control animals.

Using the different hypotheses above, this suggests that the predominant

reason for the elevation of urinary taurine 0 - 24 h following treatment was tissue taurine leaking from damaged cells. However this assumption is based on the supposition that the treated and control animals had the same dietary intake of food, which they did not. Those animals which were pair fed with the treated animals had twice the specific activity of ^3H -taurine of the treated animals or control animals (Figure. 4.3 c). A reduction in dietary intake of taurine would have increased the specific activity of urinary ^3H -taurine. Taken together, this suggests that there was a dilution of the ^3H -taurine either by newly synthesised taurine or by the leakage of taurine with a lower specific activity than that excreted by control animals, or both, in the CCl_4 treated animals.

The specific activity of ^{35}S -taurine indicated that there was more *de novo* synthesis of taurine in the control animals than those treated with CCl_4 (Figure. 4.3 b). There may have been a greater real difference between the treated and control animals than was measured as the specific activity of urinary ^{35}S -taurine was probably reduced in the urine of control animals as there was a greater intake of unlabelled taurine in the diet.

Day 2 post CCl_4 treatment - The levels of urinary taurine were significantly higher in CCl_4 treated animals than in control animals but the specific activity of ^3H -taurine was again similar to control values. The specific activity of ^{35}S -taurine was significantly higher in the treated animals than in the control animals.

Using the hypotheses above, these results suggested that there was increased synthesis of taurine as the specific activity of ^{35}S -was increased. However, this should have reduced the specific activity of the ^3H -taurine when compared to control values alone. However, when compared to animals "pair fed" with the CCl_4 treated animals there was effectively half the specific activity of ^3H -taurine that would be expected when dietary intake was compensated for. This suggests, therefore, that there was a dilution effect of the ^3H -taurine with non- ^3H labelled taurine which could not have been derived from the diet as these animals ate very little food. It is possible that the raised levels of urinary ^{35}S -taurine from treated animals

may also may also have been the result of a reduction in unlabelled urinary taurine increasing the specific activity of urinary ^{35}S -taurine, possibly by the reduction in dietary intake of taurine.

ii. Liver taurine 48 h after dosing -

Liver taurine levels were similar in control, CCl_4 treated and "pair fed" animals when taurine was measured as $\mu\text{mol.g}^{-1}$ wet weight of liver (Table 4.4). However animals which had been given limited food ("pair fed" with treated) had half the total liver taurine of treated and control animals. Since the treated animals had the same dietary intake of taurine as the "pair fed animals" this suggested that there was an overall increase in liver taurine after CCl_4 treatment.

The specific activity of both ^3H and ^{35}S -taurine was twice the control values in the CCl_4 treated animals. This may have been due to a concentrating effect due to lack of "cold" dietary taurine as indicated by the results from animals on the restricted feeding regime. However, when the total liver taurine is considered the treated animals had twice the specific activity of ^3H -taurine of both the treated and pair fed animals and twice the ^{35}S -taurine activity of the control animals.

The higher specific activity of liver taurine in both treated and "pair fed" animals may also have been the result of a reduction in bile flow due. If bile flow had been reduced due to the reduction of diet intake, there would have been less turn over of bile salts and a reduction of taurine transported out of the liver as bile salt conjugates.

4.1.13 CONCLUSIONS

The changes in the specific activity of ^3H and ^{35}S -taurine in the urine suggest that *de novo* synthesis of taurine probably contributed to the elevation of urinary taurine seen after dosing rats with CCl_4 , although the results were complicated by the nutritional status of the animals. The results of the specific activity of ^3H -taurine in urine, suggest that there was probably a dilution of ^3H -taurine by unlabelled taurine, but whether this was by newly synthesised taurine (^{35}S labelled) is uncertain. The increase

in the specific activity of ^{35}S -taurine suggests that there was an increase in taurine synthesis following CCl_4 administration. Although the lack of dilution by dietary taurine may have contributed to this higher level in treated animals, "pair fed" animals had half the total liver taurine content of treated animals which suggests that there may have been increased taurine synthesis in treated animals.

These results do however show that there are probably many factors influencing the levels of urinary taurine after CCl_4 treatment, leakage from damaged cells, changes in taurine synthesis, dietary factors and possibly the redistribution of other body pools of taurine.

The incorporation of ^{35}S into liver protein (acid precipitated fraction) was not significantly different in treated animals. This could indicate that there was a rapid incorporation of ^{35}S either as methionine or cysteine into protein before CCl_4 was able to affect protein synthesis or that protein synthesis was not altered by CCl_4 treatment. Alternatively, protein synthesis was restored to control levels 48 h after dosing.

When these results are considered collectively they suggest that there is a leakage of taurine from cells following CCl_4 treatment and there may be an increase in the synthesis of taurine, although the results using ^{35}S -methionine are inconclusive.

Future investigations might include the administration of ^{35}S -methionine at more than one time point as the incorporation of ^{35}S -methionine into proteins may have been very rapid and occurred before CCl_4 had affected the rate of protein synthesis. Once incorporated into proteins, a reduction in protein turn over may have resulted in more taurine precursors being available for taurine synthesis, but they may not have been ^{35}S -labelled. Further investigations would also need to include animals which were "pair fed" and treated with ^{35}S -methionine.

Section 4.2

AN INVESTIGATION INTO TAURINE SYNTHESIS IN ISOLATED RAT HEPATOCYTES CHALLENGED WITH CCl₄

4.2.1 INTRODUCTION

The previous section (4.1) describes investigations into the effects of CCl₄ on taurine levels *in vivo* using radio-labelling of the taurine pool and ³⁵S-methionine to label taurine formed by *de novo* synthesis. The results demonstrated the complexity of the system under investigation, as taurine levels were subject to many influences such as dietary intake of taurine and its precursors, leaked taurine from cells and the status of amino acid metabolism. For *this* reason, studies were continued in isolated rat hepatocytes.

The use of rat hepatocytes both in suspension and as primary cultures have been used extensively as an *in vitro* model for toxicological studies of rat liver. They enable the effects of xenobiotics to be measured at the cellular level when the cells are independent of other physiological influences such as cardiovascular, neural and endocrine effects and in this case eliminate the effect of other tissue sources of taurine (De La Rosa *et al.* 1987). It is also a technique which enables the products of metabolism in the hepatocytes to be analyzed directly (Long and Moore, 1988).

Preliminary studies were carried out to investigate the effects of carbon tetrachloride on taurine levels in isolated hepatocyte suspensions and to correlate any effects with those seen *in vivo* (Santone *et al.* 1988, Gravela *et al.* 1979 and Pohl *et al.* 1983).

4.2.2 METHODS

Isolation of hepatocytes was carried out as described in the Methods chapter (Section 2.3) and was essentially a modification of the liver perfusion technique with collagenase used by Moldeus *et al.* (1978).

Incubation of hepatocytes

Hepatocyte suspensions (20 ml; 2×10^6 cells.ml⁻¹) were pre-incubated for 1 h in rotating 100 ml siliconized flasks at 37°C under 95% O₂:5% CO₂. The viability of the cells was then assessed in each flask using Trypan blue exclusion (Section 2.3.2). Aliquots were taken for measurement of lactate dehydrogenase (LDH) to assess the membrane integrity of the hepatocytes using the method of Bergmeyer *et al.* (1965). Separate aliquots were removed for the measurement of taurine in both the cells and medium (suspension buffer).

CCl₄ was dissolved in a constant volume of dimethyl sulphoxide (DMSO) and added directly to flasks containing the pre-incubated suspensions to give concentrations of 0 mM, 1 mM, 5 mM and 50 mM (Berger *et al.* 1987 and Long and Moore, 1988) in dimethylsulphoxide (DMSO 0.2%). A fifth flask had a single addition of 40 µl suspension buffer. The concentration of CCl₄ (1 mM) had no significant effect on cell viability, measured as Trypan blue exclusion and LDH leakage. A further concentration (5 mM) reduced cell viability by about 20%.

Aliquots of hepatocyte suspension were taken to determine viability, LDH leakage and cellular, medium and total taurine 45 min, 90 min and 135 min after these additions.

Cells for taurine measurement were separated from the medium (1 ml; 11,000 g 2 sec) then resuspended in sulphosalicylic acid (0.5 ml; 0.2 M) and stored at -20°C. A 1 ml aliquot of suspension media was also frozen for later analysis of taurine.

The protein content of the cell pellet was determined using Coomassie Brilliant Blue G-250 and the concentration of taurine was expressed per mg of protein, as a percentage of the starting value. This was necessary as taurine concentrations in control cells varied as much as 10 fold between different preparations.

4.2.3 RESULTS

Cell viability and LDH leakage were not significantly different from control values 135 min after the addition of 1 mM CCl_4 or DMSO. However, there was a 20% reduction in the viability of cells treated with 5 mM CCl_4 after 135 min (Figure 4.4) and a significant leakage of LDH into the medium after 45 min incubation (Figure 4.5). These cells also leaked 60% more taurine into the incubation medium than the control cells after incubation with CCl_4 for 135 min (Figure 4.6). Cells incubated with DMSO alone and 1 mM CCl_4 had a similar concentration of taurine in the medium as the control cells.

Taurine concentrations in the incubation medium of hepatocytes varied between 6 and 70 nmol.ml^{-1} and between 10 and 60 nmol.10^6 cells. The mean diameter of hepatocytes used in these preparations was found to be 20.1 μm , a value similar to those quoted by Berry *et al.* (1991). An estimate of the cellular concentration was made using this measurement to calculate the volume of 10^6 cells. The volume was calculated to be 4.3 μl . Concentrations of taurine were therefore between 2.3 and 14.0 mM which are similar to those found in whole liver. However, the variation in concentrations between preparations made pooling of data difficult. For these reasons the data has been expressed in terms of % change in taurine concentrations after the initial values had been measured. Taurine was expressed per mg of protein.

All the cells treated with 50 mM CCl_4 died within 45 min of incubation and all but 30% of the cellular taurine had leaked into the medium (data not shown). All the hepatocytes except those treated with 50 mM CCl_4 showed an increase in cellular content of taurine above the starting value although the variation in values meant that values were not significant. The greatest increase was in the cells treated with 1 mM CCl_4 after 135 min where there was an increase of 20.6% (Figure 4.7).

Overall, there was a significant increase in total taurine (29%) in the cells and the medium of those cells incubated with 5 mM and 17.5% where the cells were treated with 1 mM CCl_4 (Figure 4.8).

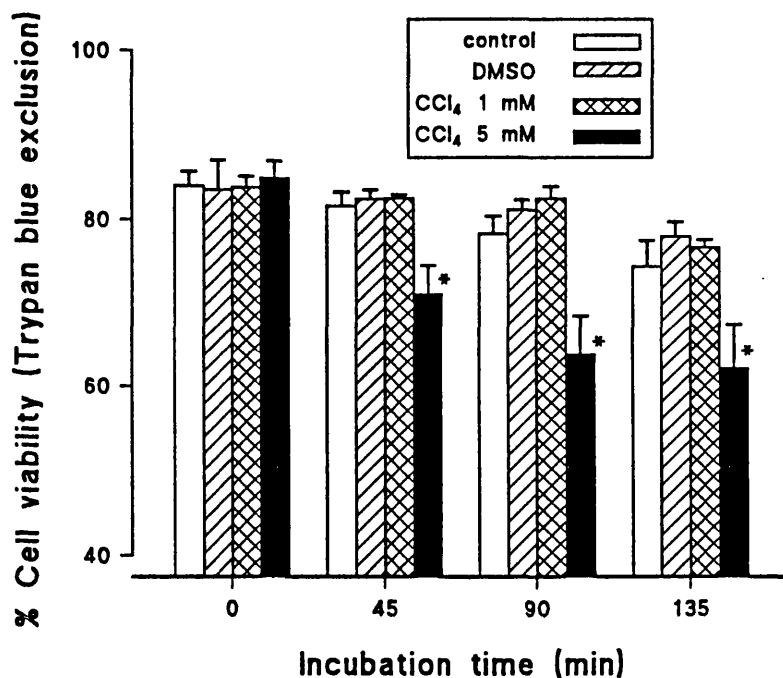


Figure 4.4 The effect of CCl₄ (1 or 5 mM) on the viability of rat hepatocytes in suspension measured by Trypan blue exclusion. Cells were incubated at a density of $2 \times 10^6 \text{ ml}^{-1}$. Values are means \pm SEM; N = 4 different preparations; * p < 0.05 significantly different from controls.

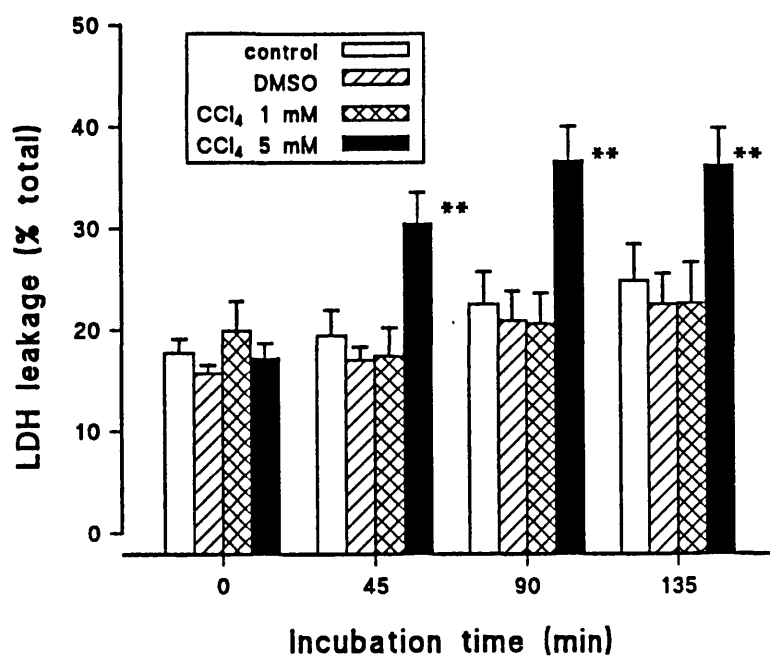


Figure 4.5 The effect of CCl₄ (1 or 5 mM) on the viability of rat hepatocytes in suspension measured by LDH leakage. Cells were incubated at a density of $2 \times 10^6 \text{ ml}^{-1}$. Values are means \pm SEM; N = 4 different preparations; ** p < 0.01 significantly different from controls.

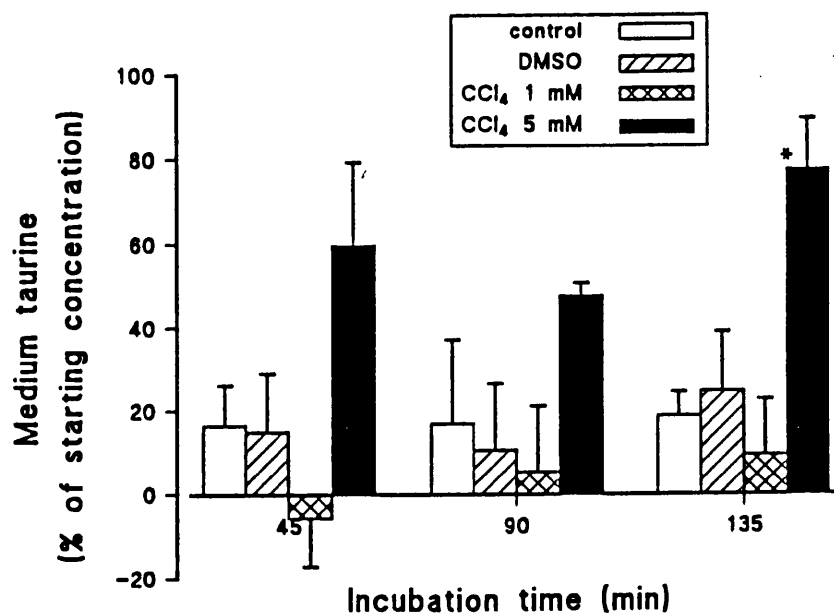


Figure 4.6 Extracellular taurine in the medium (incubation buffer) of isolated rat hepatocytes incubated with CCl₄ (1 or 5 mM). Cells were incubated at a density of $2 \times 10^6 \cdot \text{ml}^{-1}$. Values are means \pm SEM; N = 4 different preparations; * $p < 0.05$ significantly different from controls.

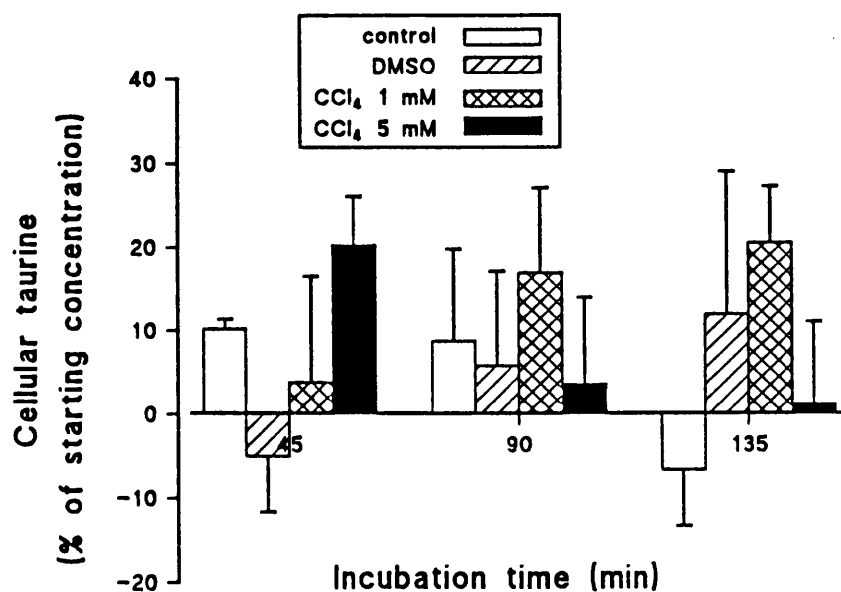


Figure 4.7 Intracellular taurine in isolated rat hepatocytes incubated with CCl₄ (1 or 5 mM). Cells were incubated at a density of $2 \times 10^6 \cdot \text{ml}^{-1}$. Values are means \pm SEM; N = 4 different preparations.

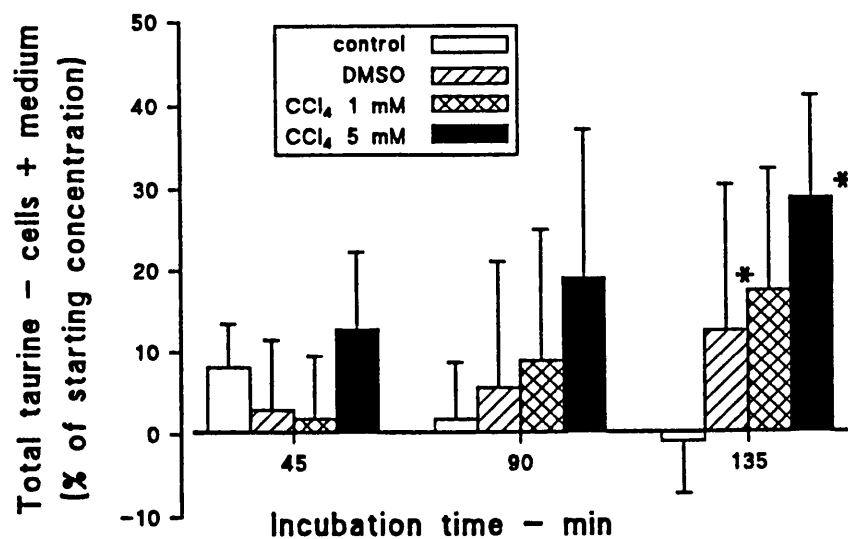


Figure 4.8 Total taurine content of cells and medium (incubation buffer) in isolated rat hepatocytes incubated with CCl₄ (1 or 5 mM). Cells were incubated at a density of $2 \times 10^6 \cdot \text{ml}^{-1}$. Values are means \pm SEM; N = 4 different preparations: * $p < 0.05$ significantly different from controls.

4.2.4 DISCUSSION

When the cytotoxicity of CCl_4 was measured the maximum LDH leakage and uptake of Trypan blue occurred after incubating the cells for 45 - 90 minutes. This may have been due to loss of CCl_4 from the incubation buffer. This was to be expected, since CCl_4 is a very volatile compound and the rotating flasks used were open to the air. It is also possible that the cytotoxicity seen was the result of the direct surface action of CCl_4 on the plasma membrane rather than the result of damage produced by free radicals via metabolism of CCl_4 by cytochromes P_{450} (Berger *et al.* 1986). The results of taurine leakage and synthesis showed a great deal of variability. This may have been due to the levels of taurine in the initial hepatocyte suspensions either as a result of different levels in the livers of the animals selected for hepatocyte isolation or as a function of the perfusion technique itself. Although the concentration of taurine in the cells at the time of isolation was comparable with the concentrations found in whole liver, the process of perfusion may have altered the transportation of taurine into the cells or enabled more to leak out. The different taurine levels in these hepatocytes may have made them more susceptible to the cytotoxicity of CCl_4 . Later experiments demonstrated that additions of taurine to the incubation medium were able to protect cells from the cytotoxic effects of CCl_4 (Section 5.2). The variation in the ability of the different preparations to synthesise taurine once the cells were isolated may reflect the ability of the different preparations to synthesise taurine from precursors.

When all the hepatocytes in suspension died within 45 min of CCl_4 being added to the medium (50 mM CCl_4), all but 30% of the taurine in the cells was able to leak into the surrounding medium, suggesting that the remaining taurine was either bound to macromolecules or cellular components or compartmentalised within cellular organelles. Cells treated with 50 mM taurine showed no changes in total taurine concentration in the cell suspension.

The overall increase in taurine content seen in the suspension medium

135min after the addition of 1 and 5 mM CCl_4 suggests that there was an increase in the synthesis of taurine in response to the leakage of taurine or as a direct response to the toxic effects of CCl_4 , the overall increase being greatest after 5 mM CCl_4 . This could have resulted from metabolic perturbations such as the inhibition of protein synthesis or an increase in protein degradation. This would result in an increase in cysteine and possibly an increase of cysteine catabolism through the cysteine sulphinate decarboxylation pathway as cysteine dioxygenase activity is increased by raised levels of cysteine (Yamaguchi *et al.* 1971). This might increase taurine levels. Cysteine catabolism to pyruvate and sulphate via the desulphuration pathway or by direct transamination to β -mercaptopyruvate may not be favoured in the same way by raised cysteine levels. Drake *et al.* (1987) suggest that cysteine catabolism to pyruvate is favoured *in vitro* in rat hepatocytes under normal circumstances. Treatment with CCl_4 may impair this pathway if, for example, the availability of 2-oxoglutarate was impaired (Figure 1.20).

However, it should be noted that the results of this study are not able to distinguish between taurine which was present in cells at the time of isolation, newly synthesised taurine and taurine which may have been taken up from the surrounding medium after leakage.

These observations are consistent with the observation that CCl_4 results in elevations of urinary taurine after liver damage which may not be simply the loss of cellular taurine, but may also result from an increase in taurine synthesis.

Section 4.3

AN INVESTIGATION INTO TAURINE SYNTHESIS IN ISOLATED RAT HEPATOCYTES CHALLENGED WITH HYDRAZINE

4.3.1 INTRODUCTION

Hydrazine has been shown to increase urinary taurine in a dose dependent manner when administered to rats (Sannins *et al.*, 1990) and the effect was been discussed in this thesis (Section 3.1). In contrast to the effects of CCl_4 , the hepatotoxicity does not result in hepatocyte necrosis but is predominantly manifested as hepatic steatosis. The indications were, therefore, that hydrazine resulted in raised urinary taurine levels as a result of perturbations in sulphur amino acid metabolism and possibly protein synthesis inhibition, rather than leakage from overtly damaged cells. The mechanism underlying the accumulation of triglycerides after hydrazine administration *in vivo* is currently unknown. However, there are indications that a failure in the secretion of triglycerides (Clark *et al.* 1970), inhibition of protein synthesis (Lopez-Mendoza and Villa-Trevino, 1971) or an increase in diglyceride synthesis (Lamb and Banks, 1979; Haghighi and Honarjou, 1987) may all be involved. The key enzyme which regulates triglyceride formation, phosphatidate phosphohydrolase, shows an increase in activity following hydrazine administration. This enzyme is responsible for cleaving phosphatidic acid to give the 1,2-diglyceride. Another mechanism responsible for the production of fatty liver is that manifested by ethionine (Farber, 1967), where the depletion of ATP is responsible. Hydrazine has also been shown to reduce ATP levels both *in vivo* and *in vitro* in the rat and in rat hepatocytes respectively (Preece *et al.* 1990).

The investigations discussed in section 4.2 suggested that there was an increase in taurine synthesis in isolated hepatocytes challenged with a concentration of CCl_4 which caused 20% loss of viability after 135 min incubation. There was a subsequent increase in the concentration of taurine in the medium probably due to the leakage of taurine from dying cells.

The following investigation was carried out using hydrazine in the

incubation buffer of isolated hepatocytes. This hepatotoxic compound was chosen as the mechanism and expression of toxicity of this compound is different from CCl_4 although it also elevates urinary taurine when administered to rats. In addition to the other indicators of toxicity, ATP was also measured in the following study. The depletion of ATP appears to be an early event in the toxicity of hydrazine at concentrations that do not result in significant loss of viability measured by Trypan blue uptake and LDH leakage (Preece *et al.* 1990).

4.3.2 METHOD

Hepatocytes were isolated from fed rats (male; Sprague Dawley; 250 - 300g) between 10.30 am and 11.30, as described before (2.3 and 4.2). Incubations were carried out in 100 ml round bottomed siliconized flasks and gassed with 95% O_2 :5% CO_2 . After 45 min pre-incubation, aliquots of cell suspension were taken for assessment of viability by Trypan blue exclusion, LDH, total taurine, medium taurine and ATP (Section 2.3.2). Hydrazine hydrate (80 mg) was diluted in degassed UHQ water (5 ml) and added to 20 ml of hepatocyte suspension (2×10^6 live cells. ml^{-1}) to give final concentrations of 8, 12, 16 and 20 mM hydrazine (free base). Further aliquots of cell suspension were taken after 1, 2 and 3 h incubation.

4.3.3 RESULTS

There was a dose dependent loss of viability as measured by Trypan blue exclusion which was significant after 2 and 3 h with both 16 and 20 mM hydrazine and after 3 h with 12 mM hydrazine (Figure 4.9). LDH leakage was significantly greater in cells treated with 16 and 20 mM hydrazine after 3 h incubation but not with other concentrations (Figure 4.10). However, ATP depletion preceded these markers of cytotoxicity and after 1 h of incubation with 20 mM hydrazine there was a significant depletion of ATP (Figure 4.11). After 2 and 3 h ATP was significantly depleted with 12, 16 and 20 mM hydrazine in the incubation medium.

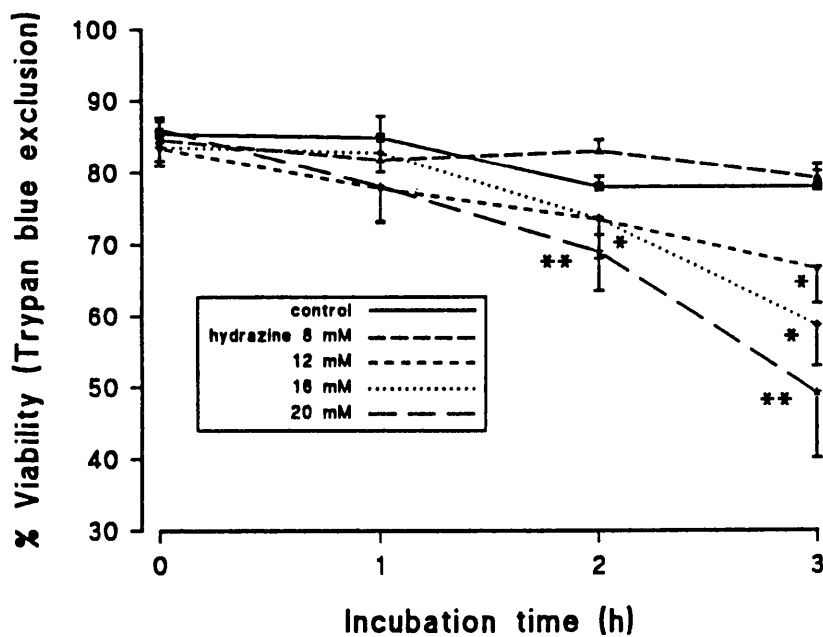


Figure 4.9 The effect of hydrazine (8, 12, 16 or 20 mM) on the viability of rat hepatocytes in suspension measured by Trypan blue exclusion. Cells were incubated at a density of $2 \times 10^6 \text{ ml}^{-1}$. Values are means \pm SEM; N = 3 - 5 different preparations; * $p < 0.05$, ** $p < 0.01$ significantly different from controls.

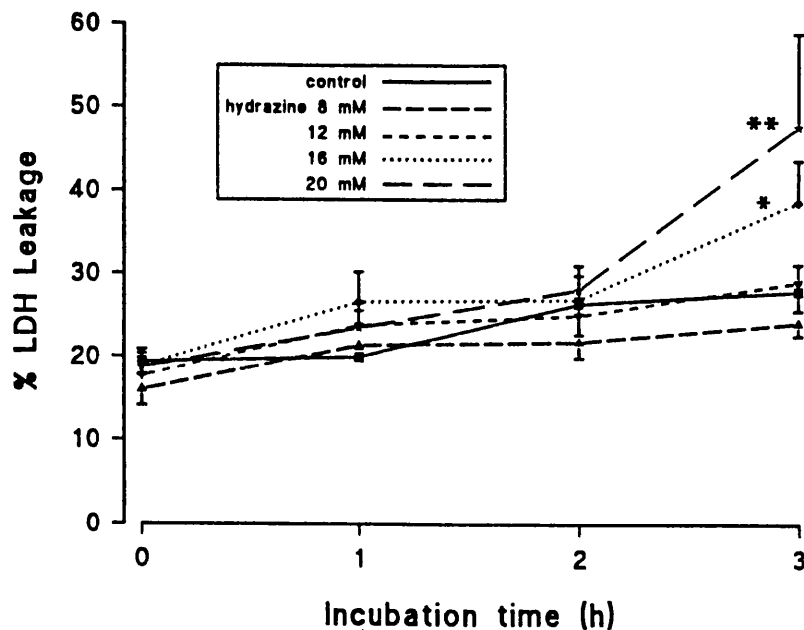


Figure 4.10 The effect of hydrazine (8, 12, 16 or 20 mM) on the viability of rat hepatocytes in suspension measured by LDH leakage. Cells were incubated at a density of $2 \times 10^6 \text{ ml}^{-1}$. Values are means \pm SEM; N = 3 - 5 different preparations; ** $p < 0.01$ significantly different from controls.

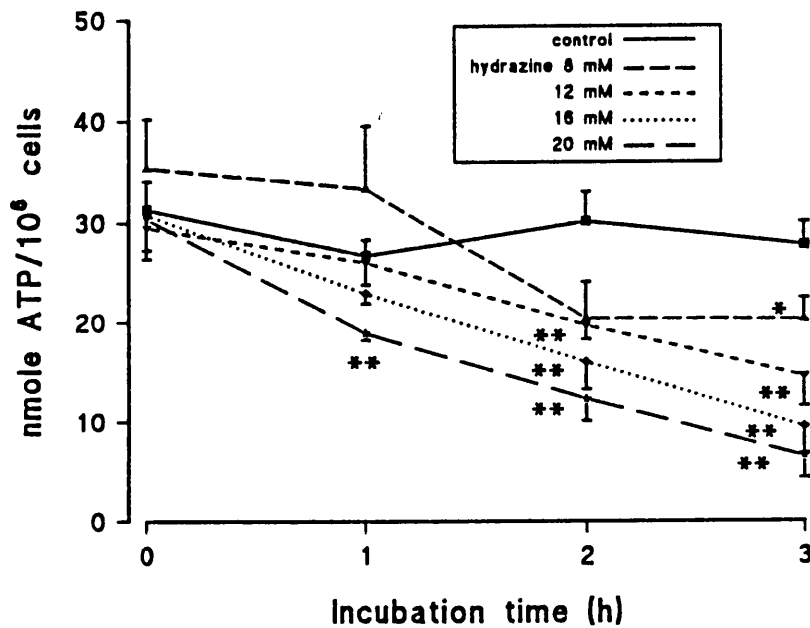


Figure 4.11 The effect of hydrazine (8, 12, 16 or 20 mM) on the concentration of ATP in rat hepatocytes in suspension measured luciferase-linked bioluminescence assay. Cells were incubated at a density of $2 \times 10^6 \cdot \text{ml}^{-1}$. Values are means \pm SEM; N = 3 - 5 different preparations; ** $p < 0.01$ significantly different from controls.

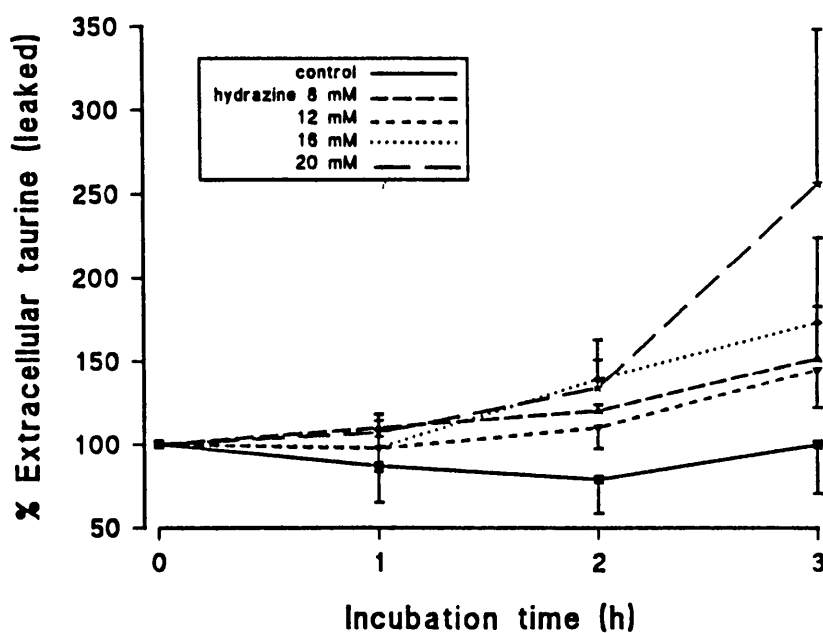


Figure 4.12 Extracellular taurine in the medium (incubation buffer) of isolated rat hepatocytes incubated with hydrazine (8, 12, 16 or 20 mM). Cells were incubated at a density of $2 \times 10^6 \cdot \text{ml}^{-1}$. Values are means \pm SEM; $N = 3 - 5$ different preparations. None of the differences were significant.

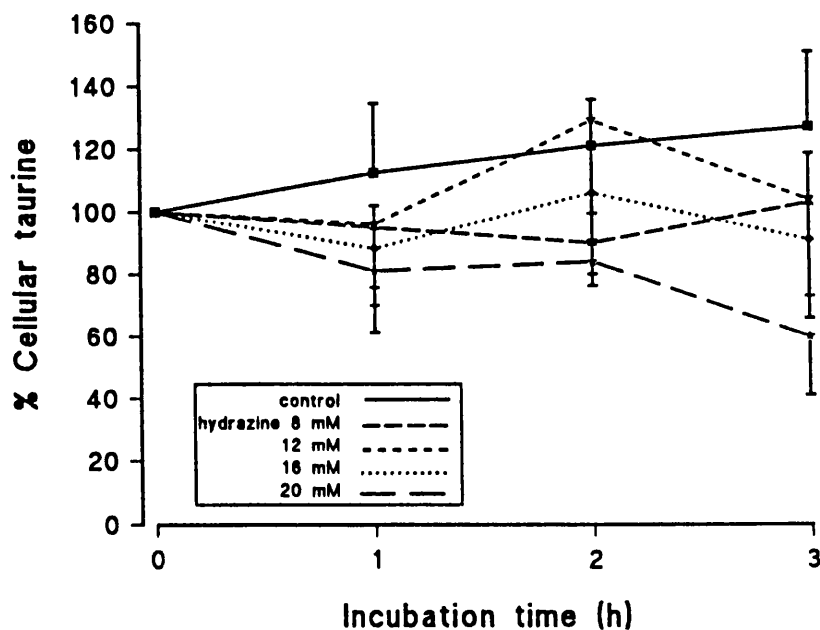


Figure 4.13 Intracellular taurine in isolated rat hepatocytes incubated with hydrazine (8, 12, 16 or 20 mM). Cells were incubated at a density of $2 \times 10^6 \cdot \text{ml}^{-1}$. Values are means \pm SEM; $N = 3 - 5$ different preparations.

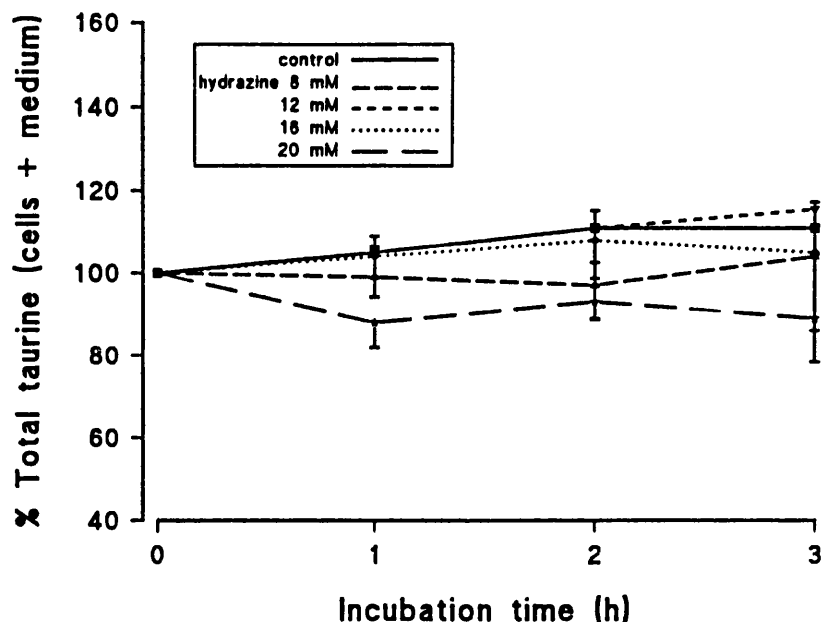


Figure 4.14 Total taurine content of cells and medium (incubation buffer) in isolated rat hepatocytes incubated with hydrazine (8, 12, 16 or 20 mM). Cells were incubated at a density of $2 \times 10^6 \cdot \text{ml}^{-1}$. Values are means \pm SEM; N = 3 - 5 different preparations. No Results were not significantly different.

The levels of taurine both intercellular and extracellular (medium) showed great variation. There appeared to be leakage of taurine into the medium at all concentrations of hydrazine (Figure 4.12) but the differences were not significant. Cellular taurine was reduced below control levels with all concentrations of hydrazine, however, the differences were not significant (Figure 4.13) and there did not appear to be any significant change in the overall concentrations of taurine (cells + medium) suggesting that there had been no increase in the synthesis of taurine after 3 h of incubation with any concentration of hydrazine (Figure 4.14).

4.3.4 DISCUSSION

The cytotoxicity of hydrazine towards isolated hepatocytes was slightly less than reported by Preece *et al* (1990). They showed a reduction in viability to 10% with 20 mM hydrazine, as measured by Trypan blue uptake, after 3 h incubation and a significant reduction in ATP levels after 1 h of incubation with 12, 16 and 20 mM hydrazine. All conditions were the same except that siliconized flasks were used in the experiments presented here. The lack of taurine synthesis after incubating hepatocytes with hydrazine was in contrast to the results *in vitro* after incubating hepatocytes with a sub-toxic concentration of CCl_4 . *In vivo* studies have indicated that an increase in taurine synthesis might give rise to the elevated levels of urinary taurine which are seen after dosing rats with hydrazine (Section 3.1 and Sanins *et al.* 1990).

Hydrazine has been shown to inhibit protein synthesis in isolated cultured hepatocytes at concentrations which are not cytotoxic (0.1 mM) (Ghatineh and Timbrell, 1990). This may be a direct effect on the anabolic pathways leading to protein synthesis or a result of an inhibitory effect on protein degradation. Ammonia and other amines are known to inhibit protein degradation probably affected by their action on the lysosomal pathway for protein degradation (Seglan *et al.* 1980) and it is possible that hydrazine might work in this manner. If this were the case, an inhibition in protein degradation in the absence of an amino acid supplemented medium would not be expected to result in an accumulation of cysteine or methionine which may be diverted for taurine synthesis. Whereas, the accumulation of cysteine and methionine might be expected *in vivo* if protein degradation were reduced with a concomitant reduction in protein synthesis as there would be other sources of amino acids which could accumulate.

However, other work (Lopez-Mendoza and Villa-Trevino, 1971) has shown that there is a direct inhibition of protein synthesis by hydrazine. In this case taurine would be expected to accumulate. It was also possible that hydrazine inhibits the pyridoxal 5'-phosphate (PALP) requiring enzyme (cysteine sulphinate decarboxylase) in the pathway for taurine synthesis.

Hydrazine is known to inhibit other PALP dependent enzymes (Cornish and Wilson, 1968).

A preliminary experiment was conducted to test the hypothesis that hydrazine treated isolated hepatocytes are unable to synthesise taurine, even if precursors are present.

Hepatocytes were isolated from taurine depleted rats (see Section 5.1) and incubated with 2 mM cysteine sulphinate and subsequently challenged with hydrazine. These cells were able to synthesise taurine, at a similar rate to control cells (12 and 16 mM hydrazine) and at a reduced rate with the higher concentration of hydrazine (20 mM). The cellular concentrations of taurine reached were maximal after 2 h at a concentration of approximately 9 mM taurine. Interestingly, there was also an increase in taurine in the medium (incubation buffer) of control cells. This may have been due to leakage of taurine as cellular concentrations increased or the synthesis of taurine in the medium, possibly by leaked enzymes from damaged cells (Figures 4.15, 4.16 and 4.17).

This demonstrated that hydrazine treated isolated hepatocytes were able to synthesise taurine if they were provided with the precursor, cysteine sulphinate, despite the fact that the enzyme involved in the decarboxylation to taurine is a PALP requiring enzyme.

This may be explained by the observation made by Springer *et al.* (1980) that hydrazine increases the activity of another PALP requiring enzyme, ornithine decarboxylase (ODC). It was suggested that the hydrazone formed between the hydrazine and PALP may bind more firmly to ODC than the PALP prosthetic group alone. As the holo form of the enzyme is more resistant to proteolytic degradation than the apo form, hydrazine binding would result in more ODC being present in the holo form. Thus ODC degradation would be reduced, increasing its half life and therefore the activity of ODC. There may be a similar effect on cysteine sulphinate decarboxylase activity, which would explain why isolated hepatocytes were able to synthesise taurine from cysteine sulphinate in the presence of hydrazine.

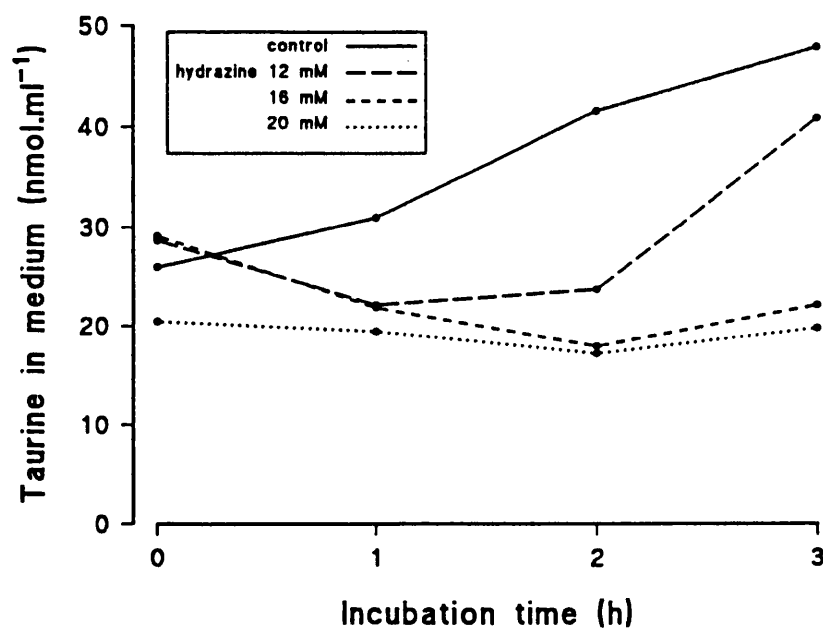


Figure 4.15 Concentration of taurine in medium (incubation buffer) - The effect of hydrazine on taurine synthesis in isolated hepatocytes from rats depleted of taurine by the administration of β -alanine in the drinking water. Cells were pre-incubated with buffer containing cysteine sulphonate (2 mM, 60 min) then hydrazine (12, 16 or 20 mM) was added to the incubation buffer and the cells incubated for a further 3 h. Cell density $2 \times 10^6 \text{ ml}^{-1}$. The results are from a single isolation.

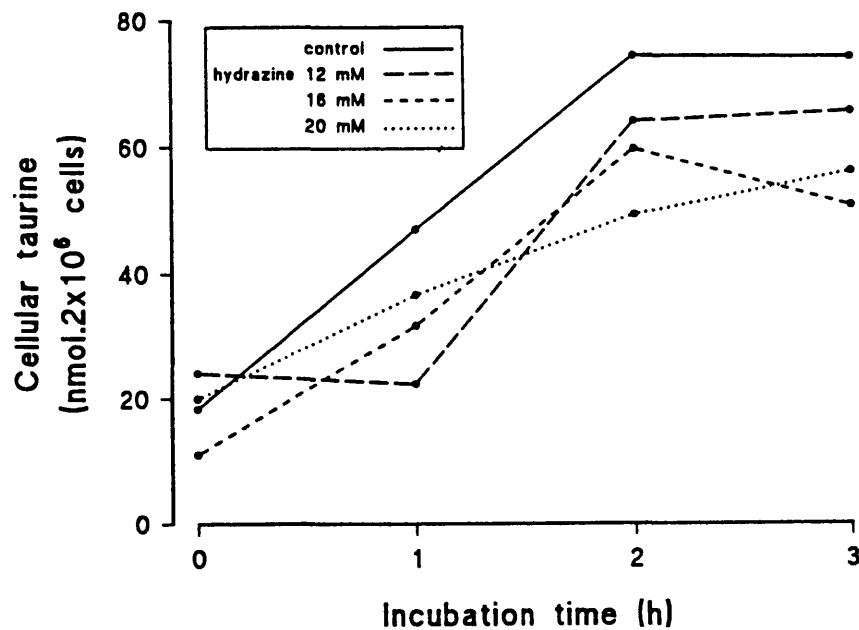


Figure 4.16 Concentration of taurine in hepatocytes from rats depleted of taurine with β -alanine, pre-incubated with cysteine sulphonate, then incubated with hydrazine (12, 16 or 20 mM). Cell density $2 \times 10^6 \text{ ml}^{-1}$. Results are from the same single isolation as Figure 4.15.

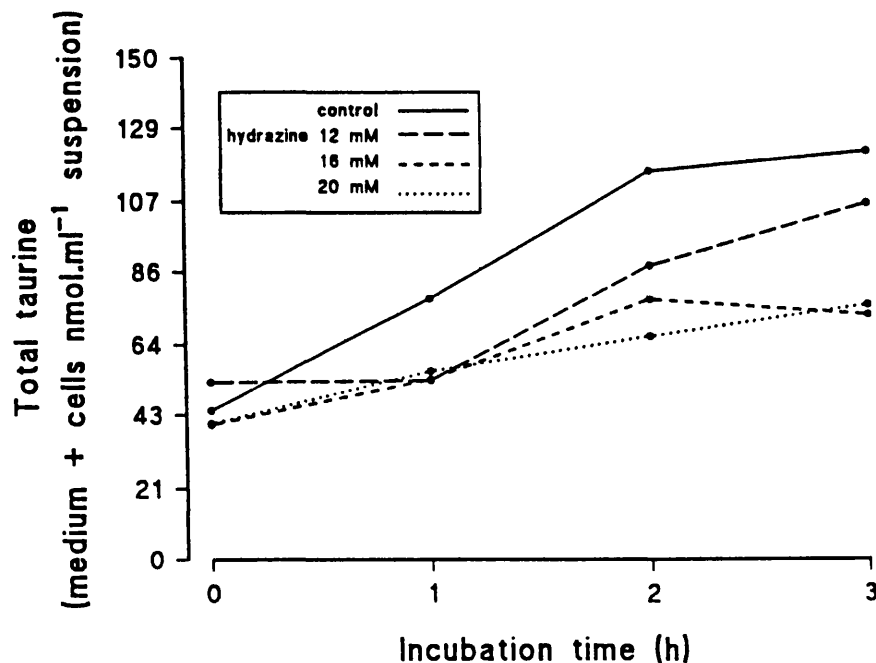


Figure 4.17 Concentration of taurine in hepatocytes and medium from rats depleted of taurine with β -alanine, pre-incubated with cysteine sulphonate, then incubated with hydrazine (12, 16 or 20 mM). Cell density $2 \times 10^6 \text{ ml}^{-1}$. Results are from the same single isolation as Figure 4.15 and 4.16 above.

INVESTIGATIONS INTO THE PROTECTIVE ROLE OF TAURINE **IN VIVO AND IN VITRO STUDIES**

5.1 AN INVESTIGATION INTO THE EFFECT OF REDUCED LIVER TAURINE ON THE HEPATOTOXICITY OF CCl₄, IN VIVO

5.1.1 INTRODUCTION

The belief that taurine has a protective role to play in animal tissues has evolved from the initial observation that taurine depleted cats suffer from retinal defects. It was also recognised that there are particularly high concentrations of taurine in cells where oxidants are generated such as neutrophils and the retina and it was speculated that taurine might function as an anti-oxidant.

During the course of the previous investigations into levels of urinary and hepatic taurine following (Chapters 3 and 4) an hepatotoxic insult with compounds such as CCl₄ two problems arose:

- (a) the toxic response, measured histologically and as elevations of serum AST and ALT and urinary taurine was variable and
- (b) liver taurine levels varied both within groups of animals and between groups of animals in different studies

As the evidence for the protective properties of taurine has been well documented it seemed possible that animals with lower liver taurine concentrations might be more susceptible to the toxic effects of hepatotoxic compounds than animals with higher levels. A parallel could be drawn between this and the effects of chemically induced glutathione depletion and the increased toxicity of bromobenzene (Jollow *et al*, 1974) and acetaminophen (Mitchell *et al*, 1973).

However, a difficulty arose in testing this hypothesis since the levels of liver taurine have been shown to vary greatly from one study to another (1 - 11 mM). This variation could be the result of many factors:

- (a) the diet of the female rats before birth since a high percentage of the body pool of taurine is derived from the dam
- (b) the taurine content of the animal diet before animals are delivered and during the study period

Variation between animals within a study may be a result of:

- (c) the time of the animals last feed before dosing, since both circulating taurine and bile acid conjugation will produce changes in liver taurine levels.

Fortunately, the levels of total non-protein sulphydryls in the liver did not vary in these studies. This suggests that the variability in the toxic response with different hepatotoxic compounds was not the result of gross differences in glutathione concentrations, although there may have been variations within the lobules. It is not practicable to measure liver taurine levels at the time of dosing. However, a correlation was found between liver taurine levels in control animals and their urinary taurine levels for the 24 h prior to liver samples being taken. Thus, animals with high urinary taurine levels tended to have higher liver taurine concentrations. A correlation also appeared to exist between the extent of damage as measured by serum transaminases (AST and ALT) and the *pre-dose* urinary taurine levels. Thus, animals with low pre-dose urinary taurine exhibited a greater increase in serum AST and ALT after dosing with a single bolus of an hepatotoxic compound than those with higher pre-dose urinary levels of taurine (see results 5.1.4 A). This susceptibility to hepatotoxic compounds could be the result of variations in liver taurine. Thus, reducing liver taurine artificially may render animals more susceptible to the toxic effects of compounds such as CCl_4 .

It is possible to manipulate the synthesis of taurine using β -methylene-DL-aspartate which is a potent irreversible inhibitor of cysteine sulphinate decarboxylase responsible for the decarboxylation of cysteine sulphinate to hypotaurine (Griffith, 1983). However, this also inhibits the activity of glutamate-oxaloacetate transaminase which is responsible for the transamination of cysteine sulphinate in the pyruvate pathway. The inactivation of cysteine sulphinate decarboxylase predominates over inactivation of the transaminase *in vivo*. Weinstein and Griffith later demonstrated (1987) the effects of the specific inhibitor β -ethylidene-DL-aspartate which selectively binds to cysteine sulphinate decarboxylase. The inhibition is however less effective than that achieved using the methyl form. A reduction of 60% of the activity of the cysteine sulphinate decarboxylase enzyme being realised in mouse liver. These inhibitors have been used to elucidate the relative importance of the taurine and pyruvate catabolic pathways in the metabolism of cysteine sulphinate. It has not been demonstrated that they are effective in lowering tissue levels of taurine in animals such as the rat which have a large body pool of taurine and high synthetic capacity for taurine.

Attempts to lower taurine by either inhibiting the pyridoxine-dependent enzyme, cysteine sulphinate decarboxylase by providing rats with a pyridoxine deficient diet or giving a diet deficient in taurine does not deplete tissue taurine levels effectively (Sturman, 1973).

By providing cysteine sulphinate with an alternative substrate in the form of D-cysteine sulphinate the enzyme can be inactivated as it binds to the enzyme but is not decarboxylated. Thus, it has been shown to inhibit enzyme activity by >74% *in vitro* (Weinstein and Griffith, 1987) and by similar values *in vivo* in mice (Weinstein *et al*, 1988). In order to block the synthesis of taurine via this route the repeated administration of D-cysteine sulphinate would be required as it is rapidly transaminated to pyruvate. The inhibition of cysteine sulphinate decarboxylase limits the production of taurine via the cysteine sulphinate pathway, but it does not prevent its formation by other routes for example through the phosphopantetheine pathway. However this has been shown to provide only a fraction of the

taurine synthesised *in vivo* (mice) and may be of very little significance (Weinstein *et al*, 1988).

The high synthetic capacity for taurine synthesis and the ability to conserve taurine via the kidney when body taurine levels are low make it difficult to deplete tissues of taurine by interference with the synthetic processes. However, it is possible to deplete the body pool of taurine using structural analogues of taurine which compete for the specific β -amino acid uptake sites into cells. Guanidinoethyl sulphonate (GES) and β -alanine are the most commonly used. GES is more effective at displacing taurine from heart, retina and brain tissue than β -alanine but β -alanine is effective at depleting the liver of taurine to 20% of normal values. The administration of GES has been shown to enhance both paraquat and blomycin toxicity in hamsters lungs. The same study demonstrated that the prophylactic use of taurine reduced the toxicity (Gordon, 1991). However, GES also accumulates in tissues probably because, like taurine, it is not metabolised. It is also suggested that GES reduces tissue taurine levels by interfering with taurine synthesis (Huxtable, 1982). β -alanine however, does not accumulate, probably because it is readily converted to malonic semialdehyde and eliminated as CO_2 (Shaffer and Kocsis, 1981) and does not appear to affect taurine synthesis.

In order to investigate the role of liver taurine in protecting against hepatic damage in this study, the liver taurine was depleted by the administration of the β -alanine. As β -alanine utilizes the same β -amino acid active uptake system in the kidney as taurine (Goldman and Sciver, 1967) it competitively inhibits taurine re-uptake from the tubular fluid and into proximal tubular cells. This results in loss of taurine from the body and hence depletion of taurine in tissues such as the liver. β -alanine will also inhibit taurine uptake into tissues (Paasonen *et al*, 1985; Shaffer and Kocsis, 1981, Lake and Marte, 1988). The levels of taurine are reduced in liver after β -alanine treatment to 20-25% of control but depletion from the brain and retina does not occur and depletion from the heart is less than that from the liver (Shaffer and Kocsis, 1981).

5.1.2 AIM OF STUDY

To show whether reduced liver taurine results in increased toxicity of CCl_4 when administered to rats.

5.1.3 METHODS

A. Retrospective preliminary investigation

Preliminary data on urinary and liver levels of taurine were obtained from control animals used in previous studies. Hepatic taurine levels were compared with urinary taurine levels for the 24 h prior to sacrificing the animals.

Urinary levels of taurine *before* dosing animals with different hepatotoxic compounds (-24 → 0 h) were compared to serum levels of AST and ALT 48 h *after* dosing. Data from animals treated with compounds which elevated levels of serum ALT and AST were used. They were CCl_4 , thioacetamide, galactosamine, allyl alcohol, ANIT and bromobenzene. Data from these animals were used only when serum enzyme evidence of hepatic damage was supported by histological evidence.

Male rats (270 - 320 g) were then used in two separate studies:

B. Taurine depletion studies

a. Study 1.

Animals were divided into 4 groups of 4 rats and placed in individual metabolism cages (Day 0). A 24 h pre-treatment urine collection was made and the groups were then treated as follows;

Group 1 and Group 2 - given β -alanine as a 3% solution in the drinking water for 6 days pre-dosing and for 2 days following dosing.

Group 3 and Group 4 - given tap water throughout the study.

On day 7 - groups 1 and 3 were given corn oil (1.0 ml.kg^{-1} , p.o.).

- groups 2 and 4 were given CCl_4 (1.0 ml.kg^{-1} , in 1 ml corn oil, po).

After β -alanine administration was begun a second 24 h urine was collection made. Animals were then placed into standard communal cages for 48 h after which they were returned to metabolism cages for two 24 h pre-dose urine collections (-48 \rightarrow -24 h; -24 \rightarrow 0 h). Animals in the appropriate groups were then dosed with CCl_4 or vehicle and urine was collected for two further 24 h periods (0 \rightarrow 24 h; 24 \rightarrow 48 h). Administration of β -alanine to the appropriate groups of animals was continued throughout the study.

b. Study 2.

Animals were divided into 2 groups of 15 animals. One group was provided with tap water throughout the experiment and the second with β -alanine (3% in the drinking water) for 6 days before dosing and two days following dosing. Three rats were treated from each group with 0, 0.5, 1.0, 1.5 or 2.0 ml.kg^{-1} CCl_4 in 1.0 ml.kg^{-1} corn oil, p.o. 6 days after the start of β -alanine treatment.

In both studies, animals were killed 48 h after dosing and blood and tissue samples were taken for analysis as described in Chapter 2.

c. Statistical analysis

Urinary levels of taurine and creatine were compared in samples taken from the same animal before and after dosing, each animal acting as its own control. A Student's paired "*t*" test was carried out to determine significance. When two or more treatment groups were compared with a single control group analysis of variance was carried out followed by Dunnett's test to determine significance. Analysis of variance was used with adjustments made for interactions between treatments to determine the effect of treatment with β -alanine on the dose related changes in serum ALT and AST after CCl_4 administration. ALT and AST values were converted to \log_{10} values for the purpose of analysis.

5.1.4 RESULTS

A. Retrospective preliminary investigation

In the retrospective preliminary study, a significant correlation was found between the urinary levels of taurine in untreated control animals (-24 → 0 h) and the liver levels of taurine from the same animals ($r = 0.734$, $p < 0.0001$) (Figure 5.1). There was also a significant negative correlation between the pre-dose levels of urinary taurine (-24 → 0 h) and the serum elevations of ALT ($r = 0.723$, $p < 0.001$) and AST ($r = 0.689$, $p < 0.001$) 48 h after dosing with different hepatotoxic compounds (Figures 5.2 and 5.3)

B. Studies 1 and 2

i. Urinary taurine

Figure 5.4 shows the effect of β -alanine and CCl_4 (1.0 ml.kg^{-1} Study 1) treatment on urinary taurine. It can be seen that urinary taurine increased dramatically after the administration of β -alanine in the drinking water. The urinary level of taurine then declined and remained approximately constant ($779 \pm 14.5 \text{ } \mu\text{mole.kg}^{-1}.\text{day}^{-1}$) for the final 4 days of the experiment in the animals treated with β -alanine alone but significantly higher than that in untreated controls ($317 \pm 11.7 \text{ } \mu\text{mole.kg}^{-1}.\text{day}^{-1}$). Dosing untreated animals with CCl_4 (1.0 ml.kg^{-1}) resulted in an increase in urinary taurine which was significantly higher than pre-dose levels (-24 → 0 h) at 48 h after dosing. In the animals treated with β -alanine and CCl_4 (1.0 ml.kg^{-1}) a significant fall in urinary taurine was observed followed by a sharp rise (Fig 5.4).

ii. Urinary creatine

Levels of urinary creatine are shown in Figure 5.5. Here it can be seen that treatment with CCl_4 (1.0 ml.kg^{-1}) resulted in a significant increase 24-48 h post treatment as observed previously (Waterfield *et al*, 1992). Combined treatment with β -alanine and CCl_4 (1.0 ml.kg^{-1}) caused an earlier (0-24 h) rise in urinary creatine, which was maintained 24-48 h after treatment.

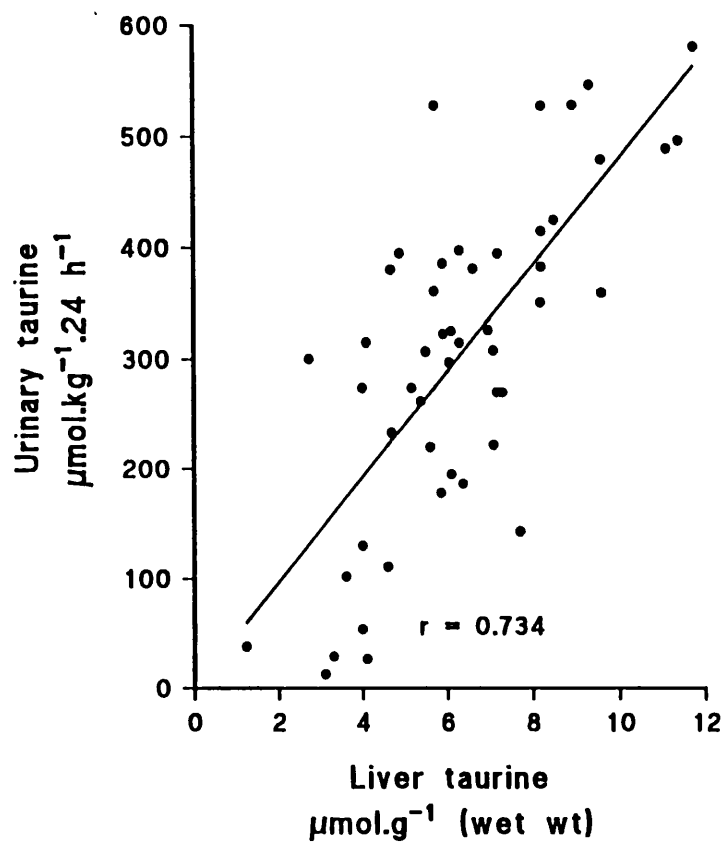


Figure 5.1

The correlation between urinary taurine in untreated rats in urine collected for the 24 h before liver taurine was measured. Each point represents a single animal. $r = 0.734$, $p < 0.0001$

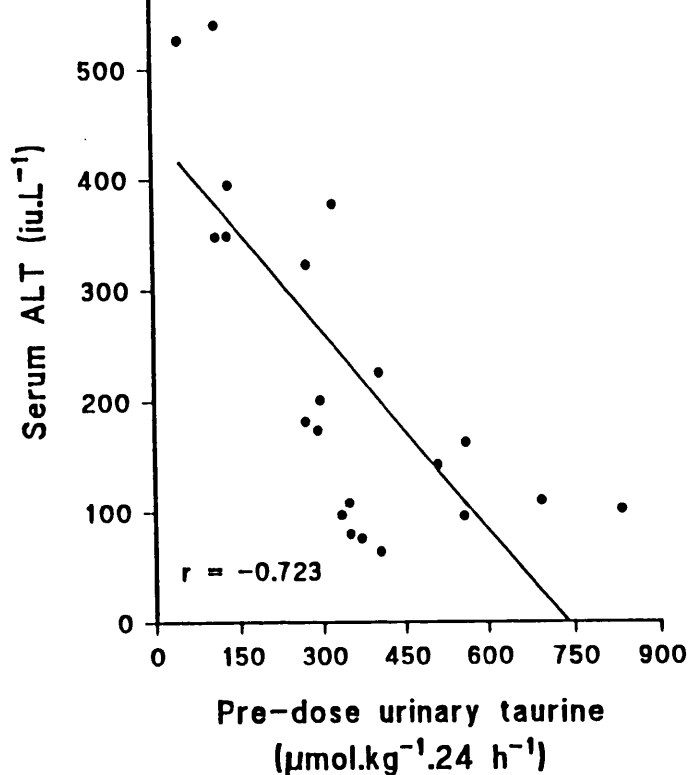


Figure 5.2 Correlation between urinary taurine in 24 h urine collection made before dosing with various hepatotoxic compounds and serum ALT measured 48 h after dosing. Each point represents a single rat. $r = -0.723$, $p < 0.001$.

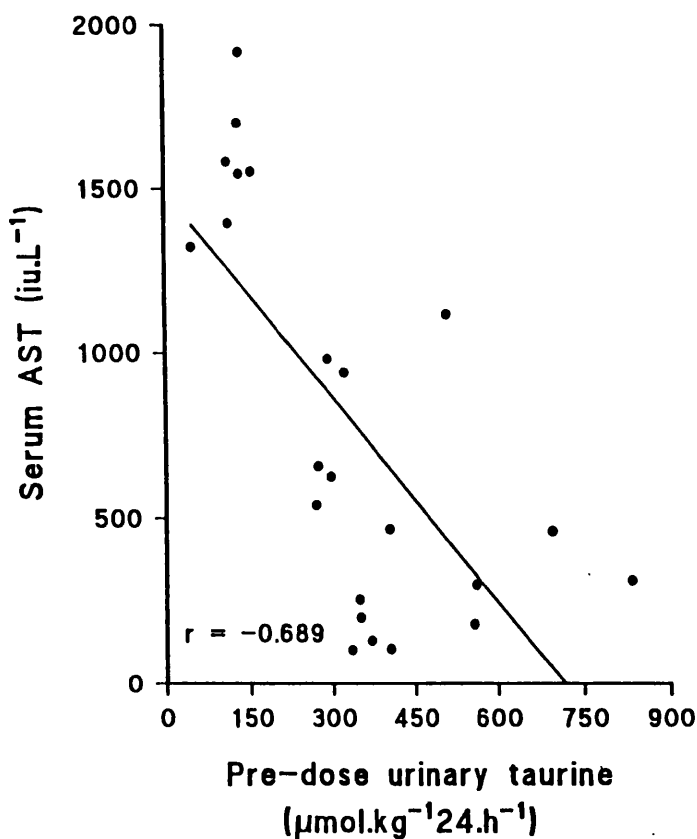


Figure 5.3 Correlation between urinary taurine in 24 h urine collection made before dosing with various hepatotoxic compounds and serum AST measured 48 h after dosing. Each point represents a single rat. $r = -0.689$, $p < 0.001$.

iii. *Liver taurine*

When the liver level of taurine was measured 48 h after dosing this was significantly depleted in β -alanine treated and β -alanine plus CCl_4 treated animals (Figure 5.6). Levels were reduced by β -alanine treatment alone to 20% of the control values. The concentration of taurine in the liver was increased 48 h after dosing with CCl_4 in a dose related manner in animals treated with β -alanine and was significantly higher after 2.0 ml.kg^{-1} .

iv. *Liver TNPSH*

Liver TNPSH levels were significantly higher in β -alanine treated animals than non- β -alanine treated animals 48 h after dosing with CCl_4 (2.0 ml.kg^{-1} , Table 5.1) and significantly higher in β -alanine treated animals after dosing with 2.0 ml.kg^{-1} CCl_4 than in β -alanine treated animals *not* receiving CCl_4 (β -alanine control animals).

v. *Clinical signs of toxicity*

Relative liver weights were raised after dosing with CCl_4 treatment but those receiving β -alanine were not significantly different from those not receiving β -alanine (Table 5.1). However, animals treated with β -alanine and dosed with CCl_4 (2 ml.kg^{-1}) had liver levels significantly raised above control values.

The hepatotoxicity of CCl_4 was determined by measurement of serum AST and ALT (Figures 5.7 and 5.8), histopathology and measurement of liver triglycerides (Table 5.1). Serum ALT was significantly raised 48 h after dosing all animals with 1.0 ml.kg^{-1} CCl_4 but was significantly raised after dosing with 0.5 and 2.0 ml.kg^{-1} CCl_4 only in animals receiving β -alanine (Figure 5.7). Animals receiving β -alanine had a significantly greater dose related increase in ALT than those not treated with β -alanine (ANOVAR). Serum AST was significantly raised after dosing animals (both treated and un-treated with β -alanine) with 0.5 and 1.0 ml.kg^{-1} CCl_4 and in animals receiving β -alanine, after dosing with 2.0 ml.kg^{-1} CCl_4 (Figure 5.8). There was a significant difference between the serum AST levels from animals treated with β -alanine and control animals after dosing with 0.5 and 1.0 ml.kg^{-1} CCl_4 .

TABLE 5.1 Effect of different doses of CCl₄ on total liver non-protein sulphhydryl (TNPSH) and liver triglyceride (TRIG) concentrations and liver weight, with and without β -alanine treatment.
(Combined results of studies 1 and 2).

MEASUREMENT	β -ALANINE TREATMENT	DOSE OF CCl ₄ ml kg ⁻¹ (p.o.)				
		0#	0.5	1.0#	1.5	2.0
LIVER - TNPSH $\mu\text{mol.g}^{-1}$ wet wt	-	7.04 \pm 0.19	6.10 \pm 0.01	7.61 \pm 0.46	7.38 \pm 0.23	6.40 \pm 0.23 ^{**}
	+	6.97 \pm 0.33	7.35 \pm 0.39	8.00 \pm 0.22	8.07 \pm 0.32	8.32 \pm 0.18 ^a
LIVER - TRIG mg.g^{-1} wet wt	-	7.42 \pm 0.82	18.13 \pm 1.42	29.44 \pm 4.48 ^b	17.48 \pm 2.98	28.32 \pm 6.57 ^a
	+	6.28 \pm 1.81	22.64 \pm 5.83 ^b	20.78 \pm 3.71 ^b	19.38 \pm 0.82 ^a	28.77 \pm 4.66 ^b
LIVER % BODY WEIGHT	-	3.76 \pm 0.05	4.18 \pm 0.19	4.03 \pm 0.48	4.06 \pm 0.1	4.55 \pm 0.12
	+	3.79 \pm 0.09	3.92 \pm 0.12	4.03 \pm 0.32	4.23 \pm 0.12	4.67 \pm 0.05 ^a

Values are means \pm SEM; N = 3 except # where N = 7. ^{**} p < 0.01 from same dose, with different β -alanine treatment; Student's "t" test (unpaired).

Using Dunnett's test for multiple comparisons, ^a p < 0.05 and ^b p < 0.01 from 0 ml CCl₄ same β -alanine treatment.

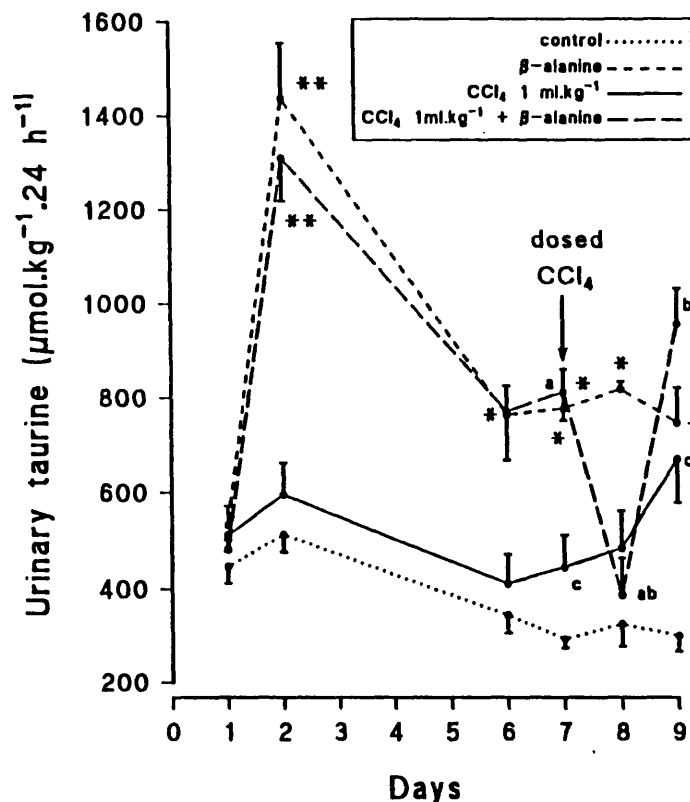


Figure 5.4 Study 1 - Urinary taurine in control rats and rats treated with β -alanine, dosed with CCl_4 (1 ml.kg^{-1} , p.o) and β -alanine treated rats also dosed with CCl_4 (1 ml.kg^{-1} , p.o); Values are means \pm SEM; N = 4; * $p < 0.05$, ** $p < 0.01$ compared to pre-dose levels, a, b, and c $p < 0.05$ compared to same letter urine collection (paired "t" test).

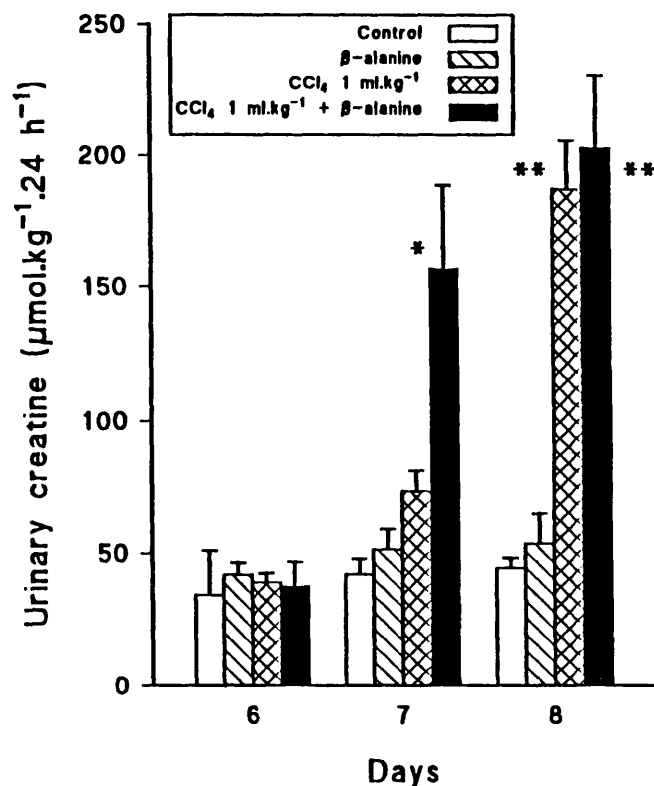


Figure 5.5 Study 1 - Urinary creatine in 24 h urine collections made from rats treated with β -alanine, dosed with CCl_4 (1 ml.kg^{-1} , p.o) and β -alanine treated rats also dosed with CCl_4 (1 ml.kg^{-1} , p.o); CCl_4 dosing was on day 6. Values are means \pm SEM; N = 4; * $p < 0.05$, ** $p < 0.01$ compared to pre-dose values.

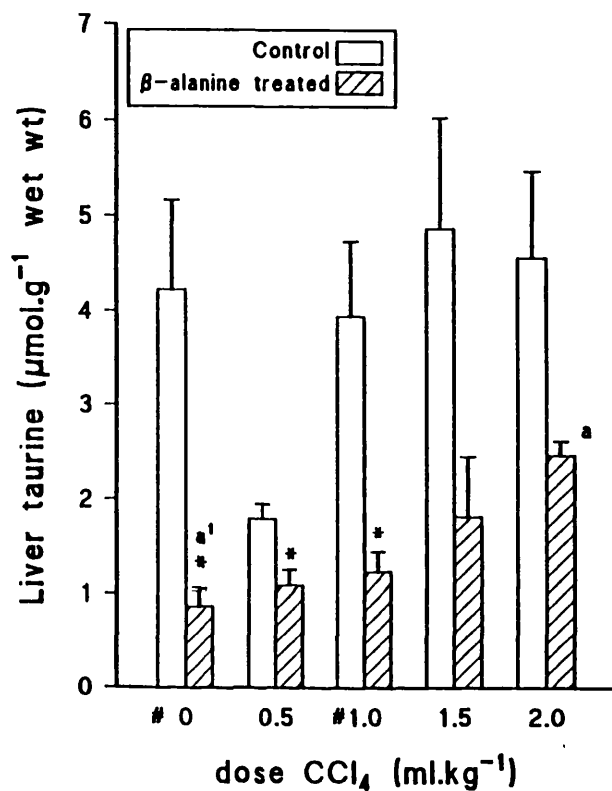


Figure 5.6 Study 1 and 2 combined results - Concentration of taurine in the livers of control rats and animals treated with β-alanine, dosed with CCl₄ (0, 0.5, 1.0, 1.5 or 2.0 ml.kg⁻¹, p.o). Values are means ± SEM; N = 4 or # = 7; * p < 0.05 β-alanine treated compared to non-β-alanine treated (same dose CCl₄); a - significantly different from a¹, p < 0.001.

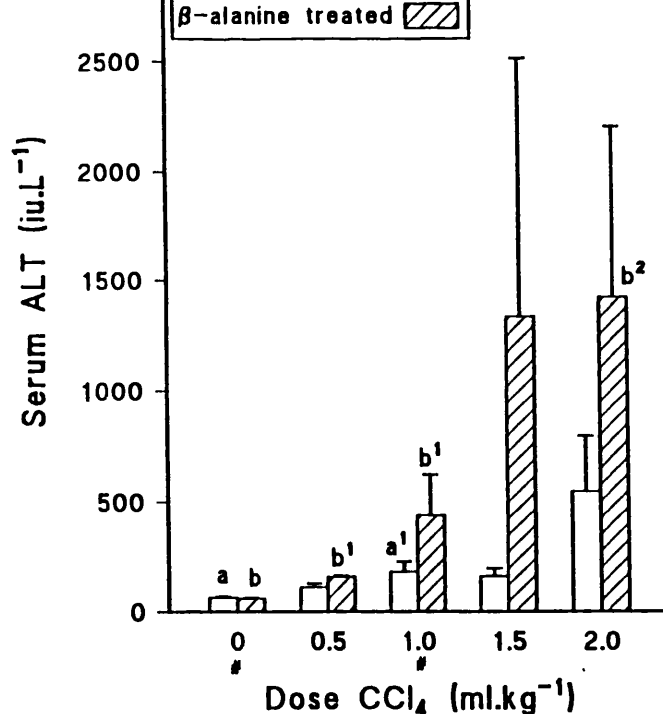


Figure 5.7 Study 1 and 2 combined results - Serum ALT measured in control rats and rats 48 h after dosing with CCl₄, with or without β-alanine treatment. Values are means \pm SEM; N= 4 except # = 7; a¹ significantly different from a, $p < 0.01$, b¹ and b² significantly different from b, $p < 0.001$ and $p < 0.05$ respectively. There was a significant difference between the dose response to CCl₄ with and without β-alanine treatment (ANOVAR), $p < 0.05$.

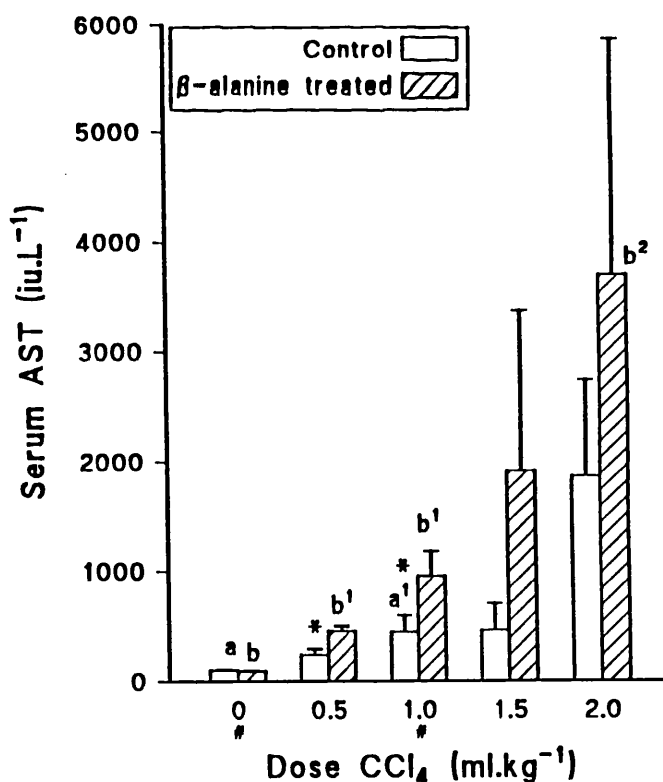


Figure 5.8 Study 1 and 2 combined results - Serum AST measured in control rats and rats 48 h after dosing with CCl₄, with or without β-alanine treatment. Values are means \pm SEM; N= 4 except # = 7; * significantly different with and without β-alanine treatment (same dose CCl₄), a¹ significantly different from a, $p < 0.01$, b¹ and b² significantly different from b, $p < 0.001$ and $p < 0.05$ respectively.

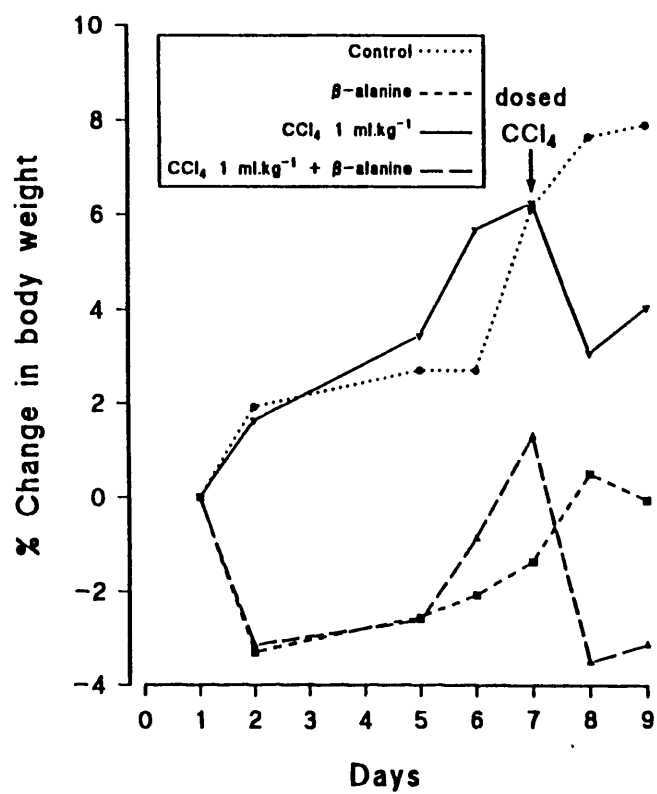


Figure 5.9 Study 1 - % Change in body weight of control rats, rats treated with β -alanine and rats dosed with CCl_4 (1.0 ml.kg^{-1} , p.o) on day 7; Values are means; $N = 4$.

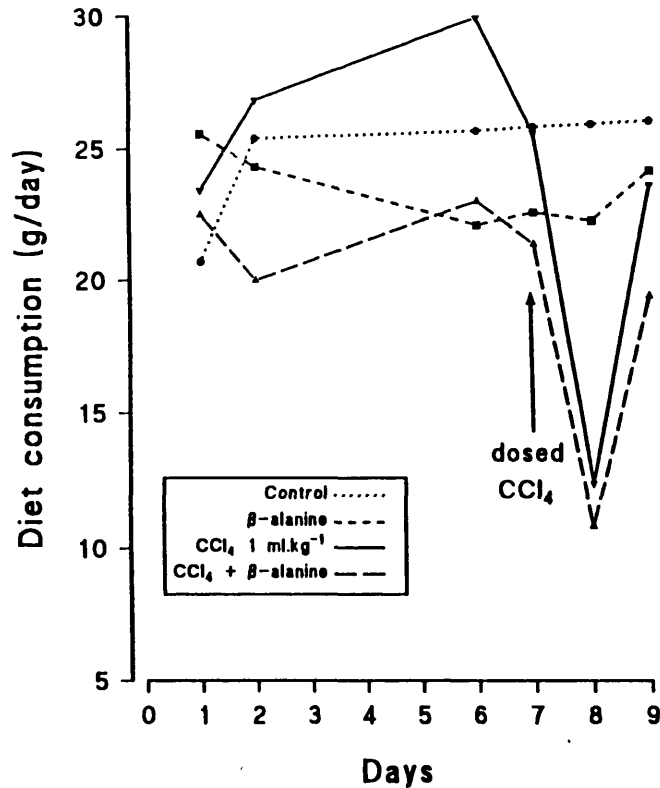


Figure 5.10 Study 1 - Diet consumption of control rats, rats treated with β -alanine and rats dosed with CCl_4 (1.0 ml.kg⁻¹, p.o) on day 7; Values are means; N = 4.

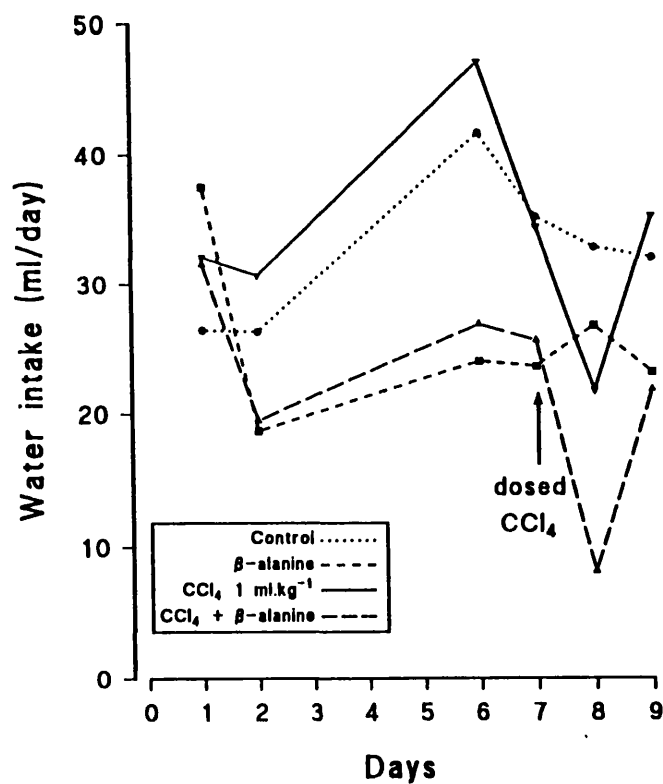


Figure 5.11 Study 1 - Water intake of control rats, rats treated with β -alanine and rats dosed with CCl_4 (1.0 ml.kg⁻¹, p.o) on day 7; Values are means; N = 4.

Histological assessment of liver sections from the animals suggested an increased inflammatory response in those animals treated with β -alanine and CCl_4 (Plates 37 - 46). However after staining frozen sections for lipid using Oil Red O there appeared to be less lipid accumulation in β -alanine plus CCl_4 (1.0 ml.kg^{-1}) treated rats than in those which did not receive β -alanine. Biochemical measurement of liver triglycerides in animals treated with 1.0 ml.kg^{-1} CCl_4 confirmed that there was less accumulation of TRIG in those animals which had also been treated with β -alanine, although the differences were not statistically significant (Table 5.1).

Body weights and food and water consumption are shown in Figures 5.9, 5.10 and 5.11. Body weights of the control animals increased until treatment with CCl_4 whereupon there was a decrease in body weight for the 24 h post dosing. β -Alanine treated animals showed a decrease in body weight for the first 24 h after β -alanine was administered followed by an increase but with subsequent dosing with CCl_4 the body weights were decreased again.

Food intake was slightly decreased by treatment with β -alanine compared with the untreated animals. Dosing with CCl_4 in both β -alanine pretreated and untreated animals resulted in decreased food intake 24 h after dosing with a return towards normal intake at 48 h after dosing. Similarly, the inclusion of β -alanine in the drinking water resulted in a reduced intake of drinking water and all animals dosed with CCl_4 reduced their water intake for 24 h following dosing with CCl_4 .

5.1.5 DISCUSSION

Since urinary taurine in untreated animals is the result of over-spill of taurine from any excess in the diet and synthesised taurine above the needs of the animal, raised levels would be expected when tissue levels are high. The good correlation between liver taurine levels and urinary taurine levels found in the retrospective analysis of the data from previous studies suggested that urinary taurine might be a useful method for monitoring

liver levels of taurine in control animals.

The correlation between urinary taurine prior to dosing with different hepatotoxins and the degree of hepatic damage measured as elevations in serum ALT and AST also suggested that animals with low levels of urinary taurine (and therefore low liver taurine) were more susceptible to the different hepatotoxic compounds. Since animals with low urinary taurine levels had a greater elevation of serum ALT and AST levels.

The results presented confirm previous studies which showed that β -alanine treatment reduced tissue levels of taurine (Mozaffari *et al.*, 1986; Shaffer and Kocsis, 1981; Paasonen *et al.*, 1985). The liver taurine content after 9 days of treatment was reduced to approximately 21% of the control value. However, total non-protein sulphydryl levels in liver were unaffected by β -alanine treatment and as more than 95% of total non-protein sulphydryl are usually considered to be glutathione (DeMaster and Redfern, 1987), this indicated that the treatment with β -alanine did not have a significant effect on glutathione, an important protective agent.

After 6 days of treatment with β -alanine, urinary taurine levels were elevated but constant. This urinary taurine may have been derived from food or resulted from synthesis in the liver which was then displaced from the body pool by β -alanine. However neither source of taurine was sufficient to maintain the liver level of taurine which was significantly depleted.

The decrease in urinary taurine after dosing with CCl_4 in β -alanine treated animals was in contrast to the rise in urinary taurine in those dosed only with CCl_4 in this study and to observations made in previous chapters (see chapters 3 and 4, and published Waterfield *et al.* 1991). This effect was probably due to the reduction in the intake of taurine in the diet as a result of reduced food intake, which occurred immediately after dosing (Figure 5.10). It also suggests that any leakage from the liver as a result of damage was limited as taurine is not present in sufficient quantities in the liver, having been depleted by the β -alanine. Those animals dosed with CCl_4 (1.0

ml.kg⁻¹) alone, showed a significant elevation of urinary taurine 24 - 48 h after dosing, similar to the effect of the same dose described in Chapter 3.2 (Fig. 3.11). The late increase in urinary taurine, 48 h after dosing, may have been due to an increase in taurine synthesis as a result of protein synthesis inhibition, and subsequent leakage, rather than direct cellular damage alone as overt cellular damage is an early event in CCl₄ toxicity.

The dose dependent increase in liver taurine in those animals treated with β-alanine and dosed with CCl₄ which was significant after 2.0 ml.kg⁻¹ suggested that there was an increase in synthesis of taurine in the liver before liver samples were removed for analysis. Although β-alanine would have continued to prevent taurine from being transported into the liver during this time, it is not reported to interfere with the synthesis of taurine in the liver (Huxtable, 1982). If CCl₄ decreased protein synthesis, as suggested in previous studies (Chapters 3 and 4), there may have been increased concentrations of cysteine available for taurine synthesis. This would also explain the significant increase in liver TNPSH after 2.0 ml.kg⁻¹ CCl₄ in the same animals. Liver taurine levels in those animals treated with CCl₄ (but not β-alanine) did not show a dose related increase possibly because the levels of liver taurine would have been maintained both by synthesis *and* by uptake from the circulation since they were not treated with β-alanine.

However, in both groups of animals (β-alanine and non-β-alanine treated), there would probably also have been a loss in liver taurine from damaged hepatocytes, dependent on the severity of damage which may have contributed to the raised levels of taurine seen 24 - 48 h after dosing.

Treatment with β-alanine with subsequent depletion of liver taurine appeared to significantly increase the hepatotoxicity of CCl₄ as determined by enzyme measurements and histopathology. The dose dependent effects of CCl₄ on the elevation of ALT were significantly higher when co-administered with β-alanine. The increase in urinary creatine is also consistent with this increased hepatic damage after 2 ml.kg⁻¹ CCl₄, (Waterfield *et al*, 1992).

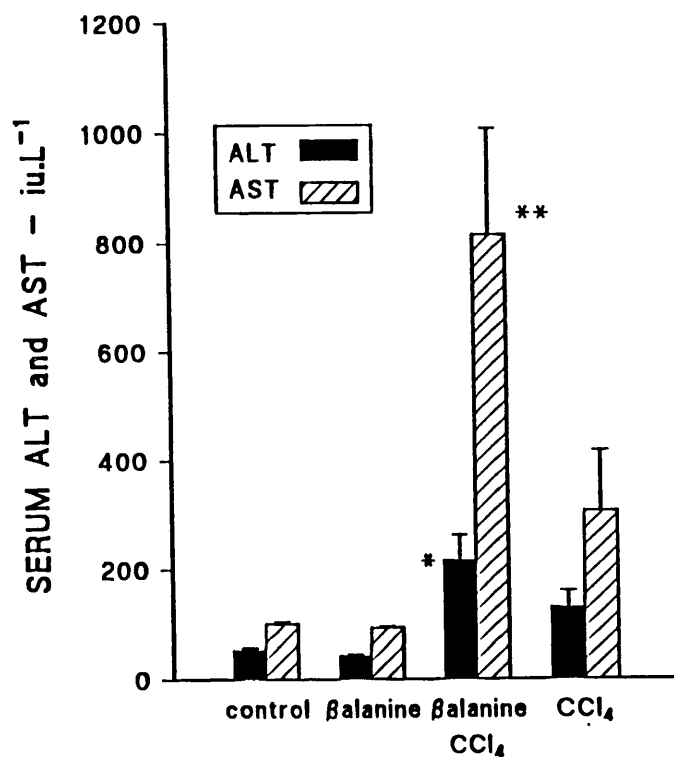


Figure 5.12 Study 1 - Serum ALT and AST in serum from control rats, rats treated with β -alanine and rats dosed with CCl_4 ($1.0 \text{ ml.kg}^{-1} \text{ p.o.}$) with and without β -alanine treatment. Serum was taken 48 h after dosing. Values are means \pm SEM; $N = 4$; * $p < 0.05$, ** $p < 0.01$ significantly different from control.

The dose of CCl_4 (1.0 ml.kg^{-1}) in Study 1 was chosen to give minimal hepatic damage and to lie towards the bottom of the dose response curve rather than near to the top. The treatment with β -alanine appeared to increase the toxicity from minimal to significant hepatic damage, as measured by both serum AST and ALT levels (Figure 5.12) and urinary creatine levels (Figure 5.5).

The results suggest that the taurine in liver may have some protective role, as a reduction in liver levels by β -alanine administration appeared to increase CCl_4 toxicity.

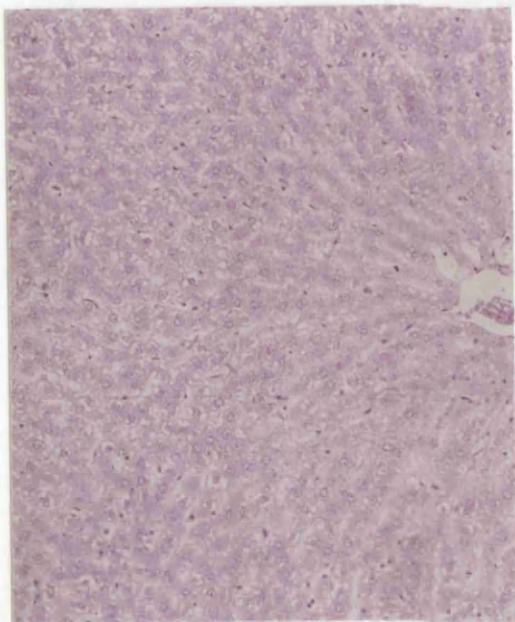
Taurine may be acting in a variety of ways to protect against the hepatotoxicity of CCl_4 . Thus direct interference with the metabolic activation of the CCl_4 or interference with one or more of the subsequent steps might occur. Taurine is known to stabilize membranes (Wright *et al*, 1985), influence calcium levels (Pasantes-Morales *et al*, 1984, 1985) and to counteract water and ion imbalances in cells, all of which may be involved in the toxicity of CCl_4 . The apparent lack of an increase in the triglyceride accumulation caused by β -alanine treatment combined with CCl_4 in contrast to CCl_4 alone, in comparison with the increased hepatic necrosis as indicated by the AST and ALT levels is of interest. It suggests that taurine may not be acting at an early event such as metabolic activation which is involved in both toxic responses but at a later stage in the development of cell damage.

Plates 37 - 46

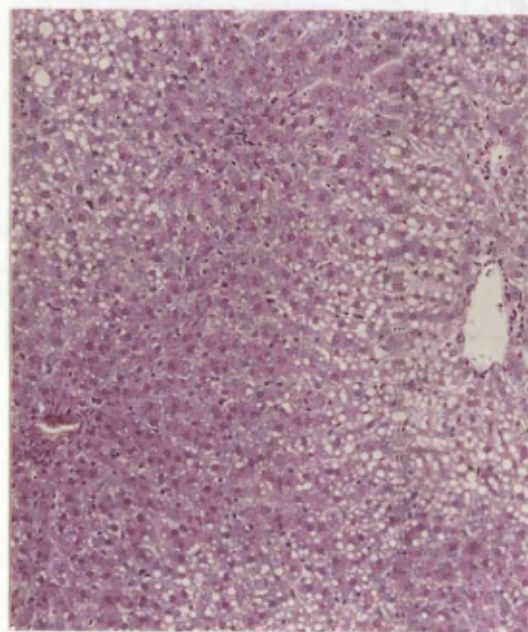
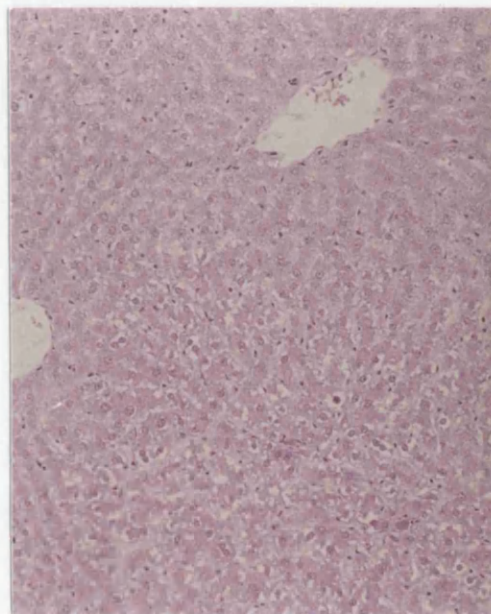
Light micrographs of liver sections taken 48 h after dosing control rats or β -alanine treated rats with CCl_4 (0, 0.5, 1.0, 1.5 or 2.0 ml kg^{-1} , p.o.)

CONTROLS (NO β -ALANINE) **β -ALANINE TREATED**

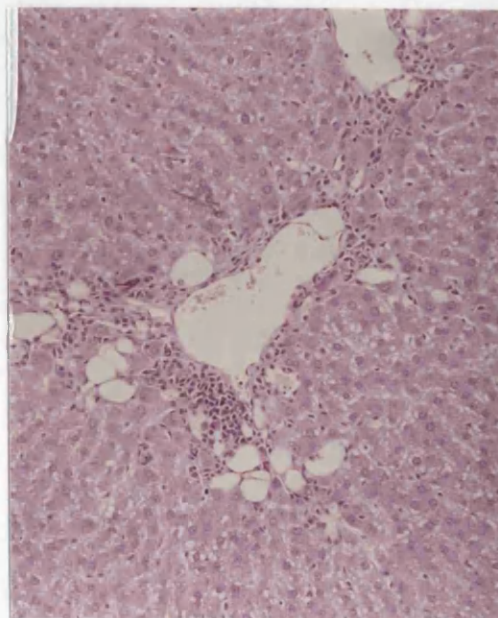
**DOSE
 CCl_4**

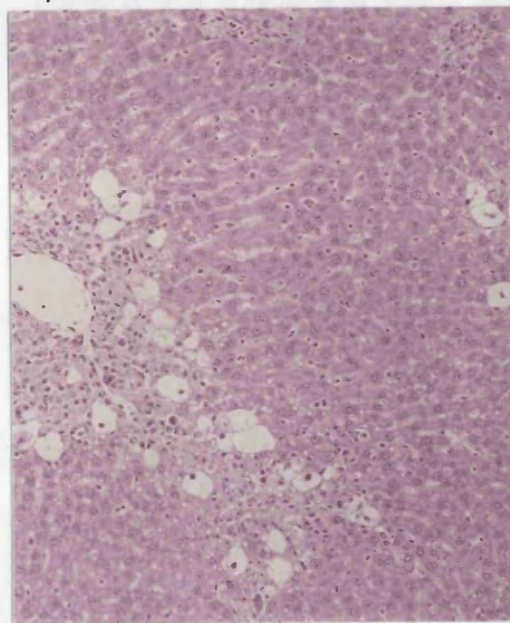
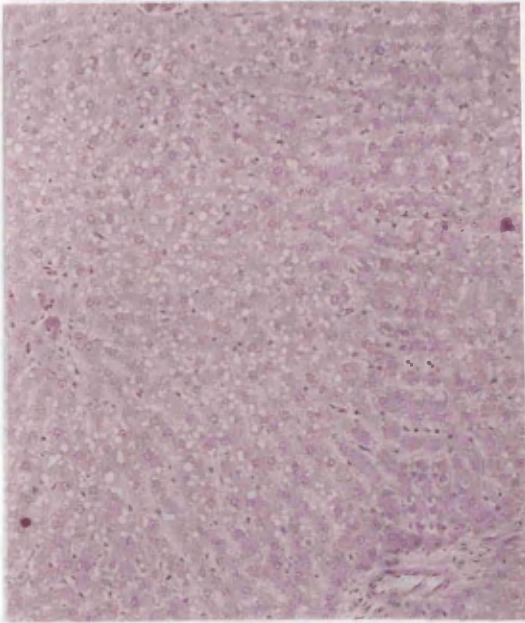
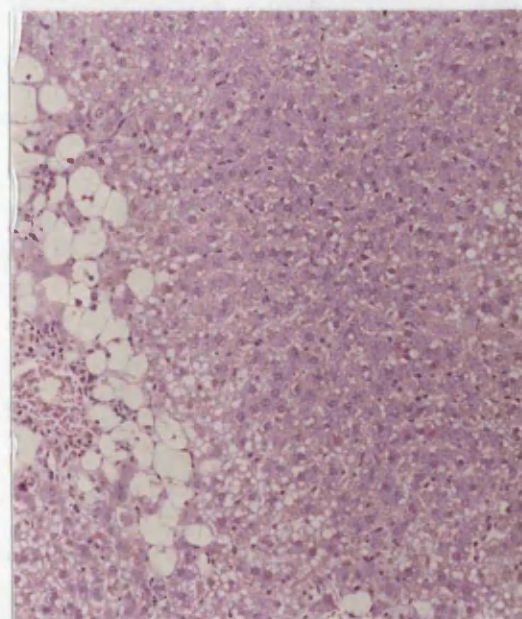
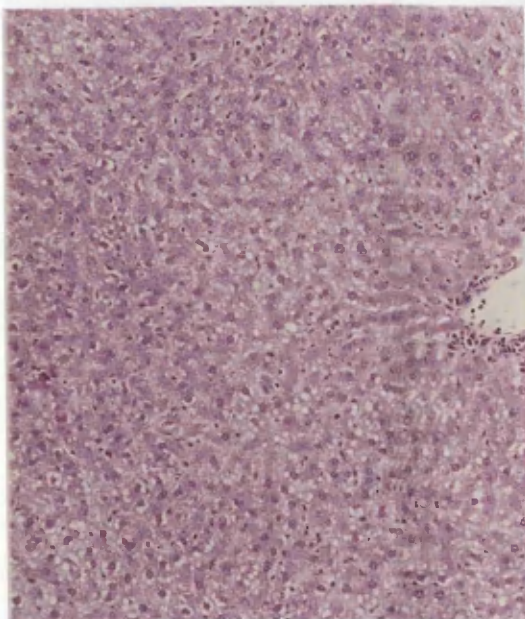
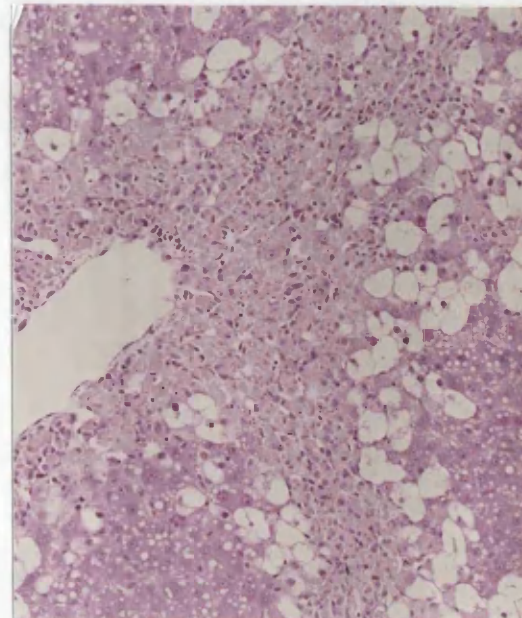
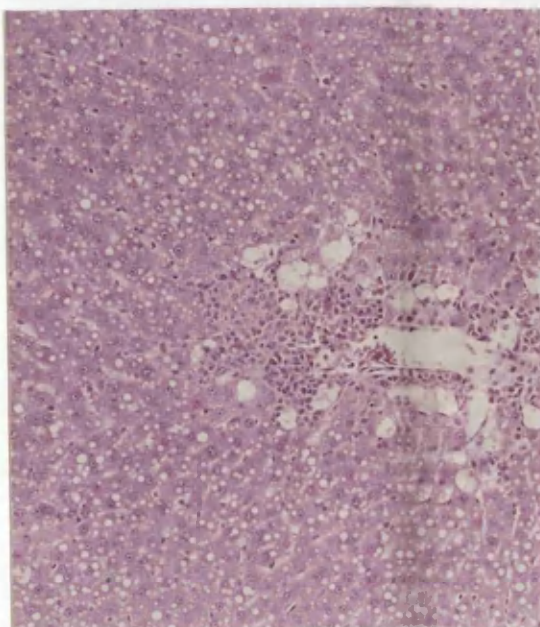


0 ml



0.5 ml



CONTROL (NO β -ALANINE) **β -ALANINE TREATED****DOSE
 CCl_4** **1.0 ml****1.5 ml****2.0 ml**

Section 5.2

AN INVESTIGATION INTO THE PROTECTIVE PROPERTIES OF EXTRACELLULAR TAURINE ON THE CYTOTOXICITY OF CCl₄ AND HYDRAZINE ON ISOLATED HEPATOCYTES IN SUSPENSION

5.2.1 INTRODUCTION

The protective properties of taurine *in vivo* have led investigators to *in vitro* experiments in an attempt to define the cytoprotective mechanisms of taurine. Studies in lymphoblastoid cells *in vitro* (Pasantes-Morales *et al.* 1984 and 1985) demonstrated that the presence of extracellular taurine (5 mM) was able to protect cells against iron-ascorbate induced calcium accumulation and cytotoxicity and 20 mM taurine co-administered with zinc chloride protected lymphoblastoid cells against retinol and retinoic acid toxicity.

Studies in hepatocytes *in vitro* have demonstrated that taurine can exert a protective effect by decreasing lipid peroxidation due to oxygenation, thus preventing cell death due to hypoxia (Nakashima *et al.*, 1990).

The following investigations were carried out to show whether the addition of taurine to the incubation media of isolated hepatocytes could reduce or prevent the cytotoxicity of the two hepatotoxic compounds CCl₄ and hydrazine.

5.2.2 Protection by taurine against LDH leakage from isolated hepatocytes as a result of CCl₄ toxicity

When the cytotoxicity of CCl₄ was investigated in the previous studies (section 4.2), maximum LDH leakage and uptake of Trypan blue occurred after incubating the cells for 45 - 90 min suggesting that CCl₄ was no longer present in the medium. This was to be expected since CCl₄ is a very volatile compound and the rotating flasks used were open to the air. It is also possible that the toxicity of CCl₄ was the result of the direct surface action of CCl₄ on the plasma membrane rather than the result of damage produced

by free radicals via metabolism of CCl_4 by Cyt P_{450} (Berger *et al.* 1986). The following studies were carried out in a closed system under CCl_4 vapour to maintain a similar concentration of CCl_4 throughout the experiments.

5.2.3 METHOD

Hepatocytes were isolated as before and pre-incubated at a concentration of cells of $2 \times 10^6 \text{ ml}^{-1}$ for 1 h in rotating 100 ml round bottomed flasks. An aliquot of cell suspension (2 ml) was then transferred to 25 ml Erlenmyer flasks fitted with a centre well. Taurine dissolved in Krebs Henseleit buffer was added to the cell suspensions to give final concentrations between 0.05 and 20.0 mM with no addition being greater than 200 μl . CCl_4 was added directly to the centre wells (0.0, 7.5, 10 or 15 μl) and allowed to diffuse into the flasks, which were sealed. Flasks were prepared in triplicate. These were placed in an oscillating water bath at 37°C moving at $2 \text{ cycles} \cdot \text{sec}^{-1}$. After 45-50 min the cells were centrifuged at 800 rpm, for 2 min (4°C). The supernatant was removed and stored at 4°C for 24 h and then analyzed for LDH which was expressed as % of that leaked from cells treated with CCl_4 alone (100% leakage). Some cell suspensions were stained with Trypan blue to assess viability but this was not carried out routinely in this study.

An aliquot (200 μl) of cell suspension was removed from flasks at the end of the incubation time and immediately fixed in 1.5 ml 4% formaldehyde:1% gluteraldehyde fixative. The cells were allowed to settle for 24 h then they were post-fixed in osmium tetroxide (1%) in cacodylate buffer (0.75 M, pH 7.4) for 30 min. The cell pellet was dehydrated through 50%, 75% 90% and 100% ethanol and 100% acetone (20 min for each stage). Samples were then air dried on glass cover slips, mounted on brass stubs, splutter coated with gold under vacuum (4 min) and viewed in a JEOL JSM-35 Scanning Electron Microscope at 15 KV.

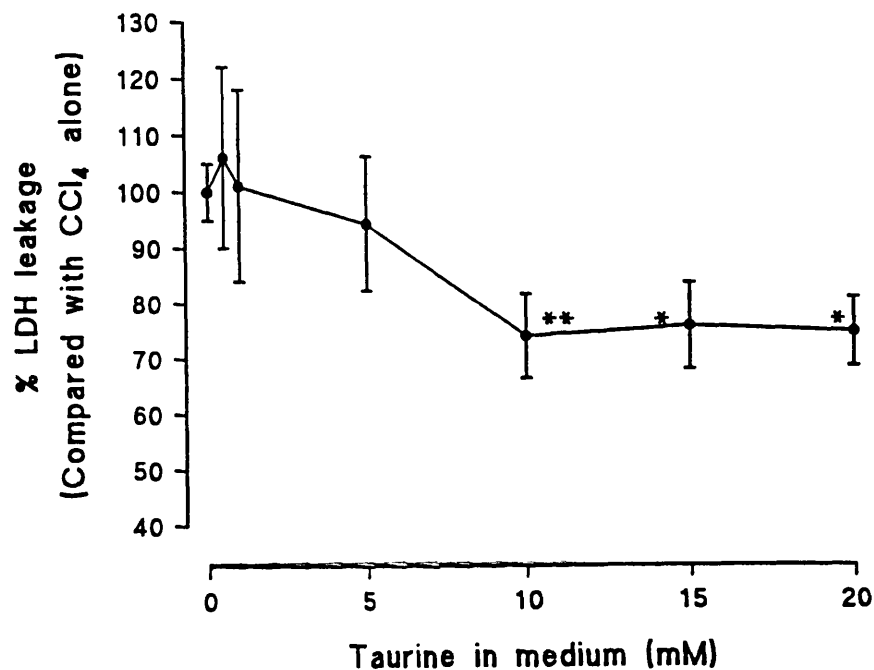


Figure 5.13 Protection of isolated rat hepatocytes in suspension incubated under CCl₄ vapour. LDH was measured as a marker of membrane integrity. Aliquots (2 ml) of cells were incubated in 25 ml Erlenmyer flasks fitted with a centre well containing 15 μ l CCl₄. Taurine was added to the suspensions (0.1 - 20 mM) and cells were incubated for 45 min before samples of the suspension buffer (medium) were taken for LDH measurements. Values are means \pm SEM; N = 5 different hepatocyte preparations; * $p < 0.05$, ** $p < 0.01$ significantly different from cells treated with CCl₄ alone.

5.2.4 RESULTS

Incubating hepatocytes in flasks containing 7.5 μ l and 10 μ l CCl_4 resulted in slight decreases in viability measured by LDH leakage (5% above control cells) and a reduction in viability measured by Trypan blue uptake (5% and 20% below control cells). There was a greater reduction in the viability of cells incubated in flasks containing 15 μ l CCl_4 , (35% measured by LDH leakage and 70% measured by Trypan blue exclusion).

The protective properties of taurine were therefore investigated using hepatocyte suspensions exposed to 15 μ l CCl_4 and taurine over a range of concentrations (0.05 - 20 mM). Extracellular concentrations of taurine below 1 mM failed to show any protection against LDH leakage in hepatocytes treated with 15 μ l of CCl_4 (Figure 5.13). However, 5 mM taurine protected against LDH leakage by 5%, but the effect was not significant whereas, 10, 15 and 20 mM taurine reduced the leakage of LDH by 25% when compared to LDH leaked by 15 μ l CCl_4 alone. The effect seemed to be maximal at 10 mM.

Only preliminary results have been obtained from the scanning electron microscopy of these cells. However, the marked "blebbing" of the hepatocyte plasma membrane as a result of treatment with CCl_4 did not appear to be significantly different from those cells which had shown some protection from LDH leakage by 10 mM taurine.

5.2.5 Protection by taurine against Trypan blue uptake and LDH leakage in isolated hepatocytes as a result of hydrazine toxicity

Hydrazine had previously produced an elevation of urinary taurine *in vivo* (Section 3.1). This did not appear to be as a result of overt cellular damage as no necrosis was observed and serum ALT and AST levels were not elevated. The hepatotoxicity was manifested as development of midzonal steatosis after a single acute dose (40 mg.kg^{-1}). The onset of toxicity of

hydrazine towards isolated hepatocytes was slower than the toxicity of CCl_4 . LDH leakage and Trypan blue uptake is seen after 1 h when hepatocytes are treated with concentrations of 12 mM - 20 mM of hydrazine, with ATP depletion in the cells preceding this (Ghatineh and Timbrell, 1990).

Two separate investigations were carried out with the following aims:-

- a. To show whether the inclusion of taurine in the incubation medium protects hepatocytes from a single toxic concentration of hydrazine.
- b. To reduce the concentration dependent loss of viability in isolated hepatocytes treated with hydrazine using a single effective concentration of taurine.

5.2.6 METHOD

Cells were isolated as before. They were incubated in siliconized round bottomed flasks (100 ml) at a live cell density of $2 \times 10^6 \cdot \text{ml}^{-1}$ (20 ml). Initial viability was $87\% \pm 2\%$. Samples were taken just prior to the addition of taurine and hydrazine then 1, 2 and 3 h after, for measurement of Trypan blue uptake and LDH leakage. An aliquot (0.5 ml) was added to sulphosalicylic acid (0.5 ml, 0.2 M, 4°C) and frozen at -80°C for later analysis of ATP. Both hydrazine hydrate and taurine solutions were prepared in the same way as described in section 4.3 and 5.2.3 respectively. Taurine was added to cell suspensions immediately before the addition of hydrazine.

Preliminary data had shown that concentrations of hydrazine between 12 and 20 mM resulted in a significant increase in Trypan blue uptake into cells after 2 h incubation and a significant increase in LDH leakage with 20 mM hydrazine. A concentration of 24 mM hydrazine was included in the following studies on the protective properties of taurine to enable the effects at a more cytotoxic dose to be investigated.

5.2.7 RESULTS

Hepatocytes incubated with a single cytotoxic concentration of hydrazine (24 mM) and different concentrations of taurine (0, 5, 10 and 15 mM) showed a time dependent loss of viability measured by Trypan blue exclusion, LDH leakage and ATP depletion (Figures 5.14 and 5.15). However, the cytotoxicity of hydrazine measured by dye uptake and LDH leakage was **reduced** by the addition of taurine to the incubation buffer in a dose dependent manner. However, the addition of taurine to the incubation buffer at these concentrations failed to protect the hepatocytes from the hydrazine induced ATP depletion.

The most effective concentration of taurine against loss of cell viability due to the inclusion of 24 mM hydrazine in the medium was 15 mM. This concentration was then used to protect hepatocytes against a range of hydrazine concentrations.

After 3 h incubation, cells treated with 20 and 24 mM hydrazine and 15 mM taurine had significantly less LDH in the incubation medium than those cells incubated with hydrazine alone (Figure 5.17). Cells incubated with 20 and 24 mM hydrazine and taurine also had a significantly increased viability as measured by Trypan blue exclusion compared to cells incubated with hydrazine alone (Figure 5.18). However the reduction in cellular ATP was not affected at any concentration by the inclusion of 15 mM taurine in the medium (5.19).

An interesting observation was made in suspensions of control cells. It was noted that a concentration of 15 mM taurine in the incubation buffer resulted in a significantly higher viability of the cells after 3 h than those incubated without taurine added to the incubation buffer.

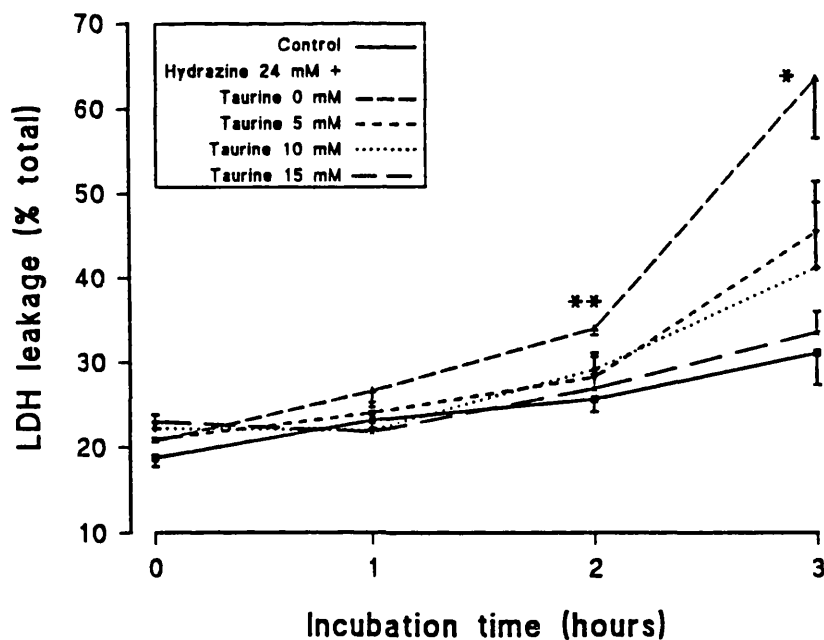


Figure 5.14 Protection of hydrazine treated (24 mM) isolated hepatocytes against LDH leakage using different concentrations of taurine in the incubation buffer (0 - 15 mM). Values are means \pm SEM; N = 4 different incubations; * $p < 0.5$ significantly different from control (no hydrazine).

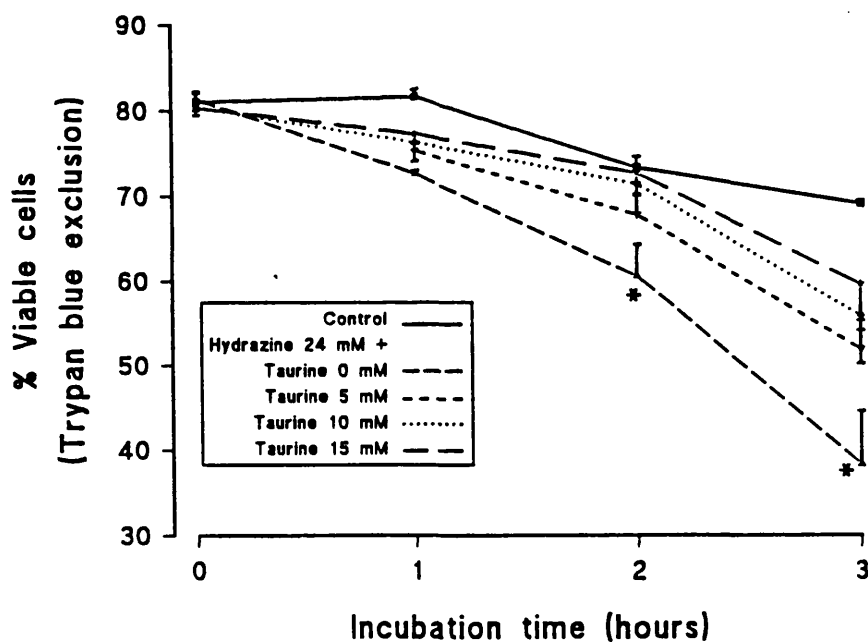


Figure 5.15 Protection of hydrazine treated (24 mM) isolated hepatocytes against Trypan blue uptake using different concentrations of taurine in the incubation buffer (0 - 15 mM). Values are means \pm SEM; N = 4 different incubations; * $p < 0.05$, ** $p < 0.01$, significantly different from control (no hydrazine).

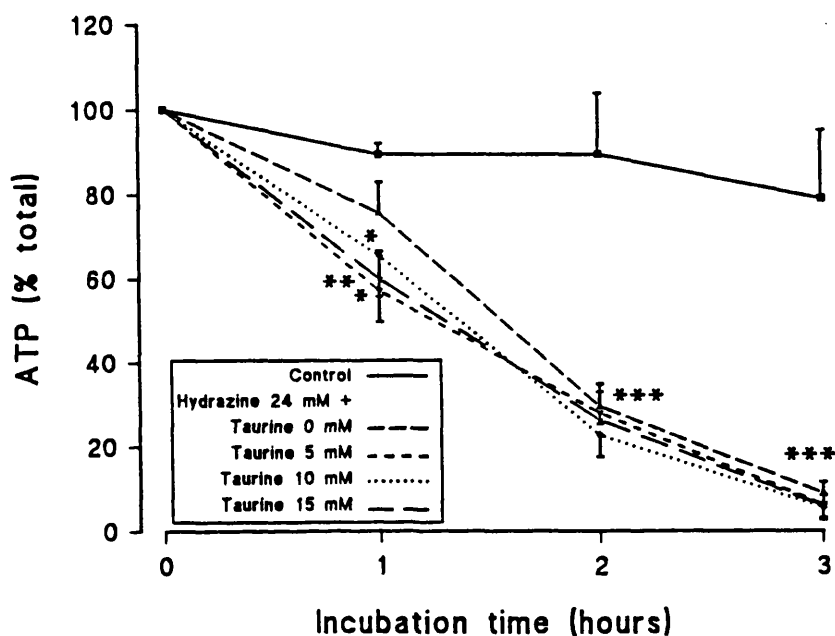


Figure 5.16 Depletion of ATP in hydrazine treated (24 mM) isolated hepatocytes incubated with different concentrations of taurine in the incubation buffer (0 - 15 mM). Values are means \pm SEM; N = 4 different incubations; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different from control (no hydrazine).

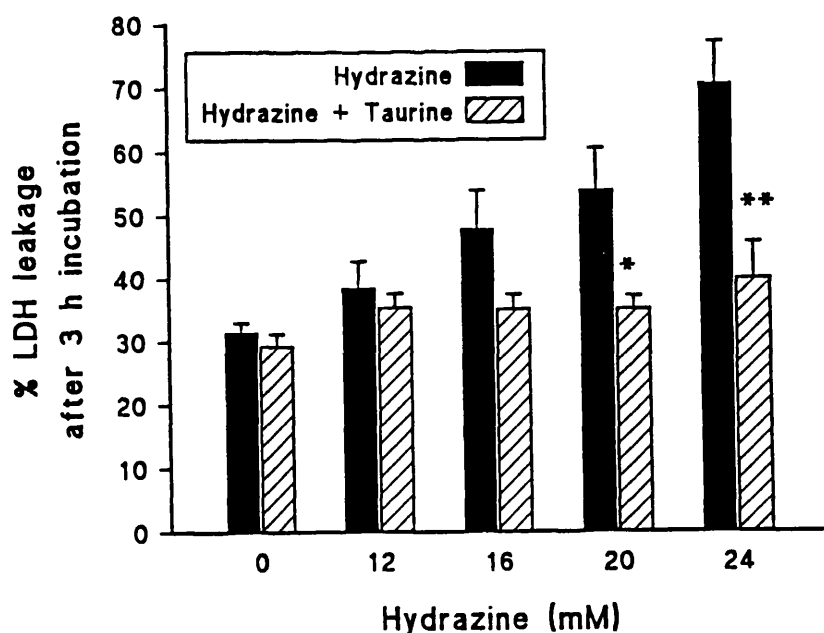


Figure 5.17 Effect of taurine (15 mM) in the incubation buffer on the dose related LDH leakage from isolated hepatocytes treated with hydrazine (0, 12, 16, 20 and 24 mM). Values are means \pm SEM; N = 4 - 7 different incubations; * $p < 0.05$, ** $p < 0.01$ significantly different from same concentration of hydrazine but no taurine.

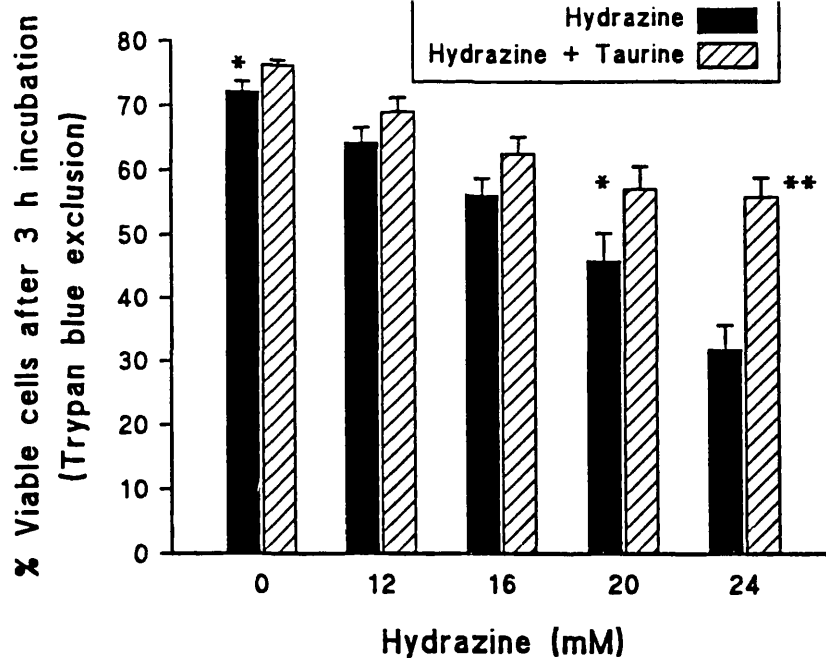


Figure 5.18 Effect of taurine (15 mM) in the incubation buffer on the dose related increase in Trypan blue uptake in isolated hepatocytes treated with hydrazine (0, 12, 16, 20 and 24 mM). Values are means \pm SEM; N = 4 - 7 different incubations; * $p < 0.05$, ** $p < 0.01$ significantly different from same concentration of hydrazine but no taurine.

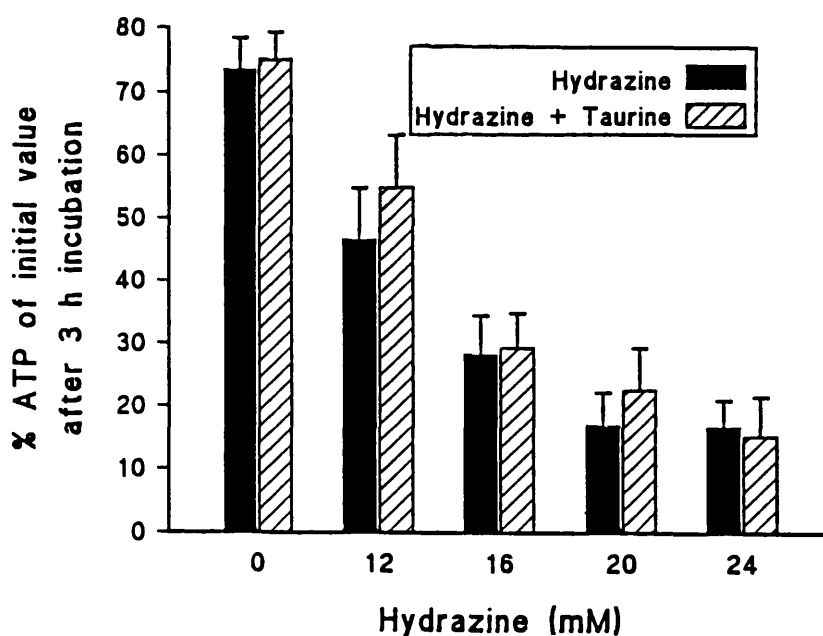


Figure 5.19 Effect of taurine (15 mM) in the incubation buffer on the dose related depletion of ATP in isolated hepatocytes treated with hydrazine (0, 12, 16, 20 and 24 mM). Values are means \pm SEM; N = 4 - 7 different incubations; No significant differences were found between hepatocytes treated with the same concentration of hydrazine with and without taurine inclusion in the buffer.

5.2.8 DISCUSSION

These experiments demonstrated that taurine was able to provide some protection against the cytotoxicity induced by CCl_4 and hydrazine, *in vitro*. The protection of other isolated cell types by taurine has previously been demonstrated by Pasantes-Morales *et al* (1984 and 1985) where similar concentrations of taurine were used to reduce the cytotoxicity of retinol and iron-ascorbate to lymphoblastoid cells. They demonstrated that taurine (5 mM) provided complete protection against the loss of viability of lymphoblastoid cells exposed to 0.2 mM ferrous sulphate and 0.4 mM ascorbate (Trypan blue exclusion). They also showed that taurine (5 mM) reduced the accumulation of Ca^{2+} induced by ferrous sulphate and ascorbate to levels similar to control levels after 30 min incubation. However, this concentration of taurine did not prevent lipid peroxidation induced by these concentrations of ferrous sulphate and ascorbate. Similar work with lymphoblastoid cells exposed to retinol and retinoic acid demonstrated that taurine (20 mM) combined with zinc chloride (100 μM) was able to reduce cell death almost to control levels. Taurine and zinc alone afforded some protection and when combined with α -tocopherol, cell viability was the same as control cells. It was noted that taurine and zinc or taurine and zinc combined with α -tocopherol were able to reduce retinol induced cell swelling after 90 min incubation. The protection was believed to result from the stabilization of the membranes, preventing the flux of ions and water. Taurine did not however affect lipid peroxidation in these experiments. However, incubations for a longer period still resulted in cell death, despite the fact that the cells had not swollen, but remained the same volume as control cells. The additive effects of these three compounds suggested that their mechanisms of protection were different. Taurine has been shown to stabilize membranes, reduce cellular calcium accumulation, remove HOCl by forming the stable taurochloramine and help to maintain the osmotic potential of cells. Thus, any or all of these mechanisms might have been involved in the protection of lymphoblastoid cells.

The mechanism by which taurine was able to protect hepatocytes from both

CCl_4 and hydrazine in the studies presented here may similarly be the result of one or more mechanisms either individually or in a combination. It would appear that the plasma membrane was made less "leaky" by the addition of taurine as both LDH leakage and Trypan blue uptake were reduced by taurine. This may be an effect either on the surface of the plasma membrane or one initiated intracellularly. The observation that a similar reduction in cell death was achieved by using the same concentrations of taurine against two compounds, might suggest that the mechanism of protection may involve one of the fundamental events leading to cell death. This could be the maintenance of membrane integrity, a reduction in membrane permeability, the reduction in ion fluxes or the scavenging of free radicals.

URINARY TAURINE IN HUMAN SUBJECTS

6.1

URINARY TAURINE AND CREATINE IN CONTROL SUBJECTS

6.1.1 Introduction

Glycine, taurine and histidine are the most abundant amino acids in human urine (Soupart, 1959). Much of the early data on urinary excretion of amino acids in human urine utilised paper chromatography and ninhydrin derivatisation. The data published on the analysis of human urine show great variation in urinary taurine levels between individuals. The following data are measurements of urinary taurine from normal individuals:

Evered (1956) -	0.688 - 2.352 mmol.24 h ⁻¹
Pentz <i>et al.</i> (1957) -	1.86 mmol.24 h ⁻¹ (range 0.344 - 2.296)
Southpart (1959) -	0.4 - 1.8 mmol.24 ⁻¹ (men), 0.25 - 1.30 mmol.24 h ⁻¹ (women)
Cusworth and Dent (1959) -	0.056 mmol.L ⁻¹
Sorbo (1961) -	0.372 - 1.12 mmol.24 h ⁻¹
Data and Naryanaswami (1983) -	2.82 mmol.24 h ⁻¹ ± 1.1

Interest in urinary levels of taurine in humans has centred on clinical disorders, such as Friedreich's ataxia, which appear to alter levels of urinary taurine (Barbeau 1978). In Friedreich's ataxia, raised levels of both urinary taurine and β-alanine are believed to be the result of impaired tubular reabsorption.

The data collected from studies in rats indicated that urinary levels of taurine had the potential to be an indicator of liver injury and fatty liver in particular. Creatine was also of interest as raised levels accompanied by raised urinary taurine were indicative of liver necrosis, whereas raised levels of creatine without raised urinary taurine levels indicated testicular damage.

It was of interest therefore, to investigate urinary levels of taurine in human subjects where there was clinical data to suggest that they had liver damage and/or testicular lesions. For this reason taurine excretion was measured in a group of control subjects as a preliminary investigation, both in men and women.

The work was carried out in collaboration with R. Draper at the London School of Pharmacy. Her research is centred on the use of urinary levels of creatine as a marker of testicular damage.

6.1.2 Method

Complete 24 h urine collections were made from volunteers (7 male and 5 female). As the variation in the excretion of urinary taurine between individuals was thought to be partially diet related, two female volunteers made urine collections throughout a 24 h period. The first subject ate chicken at 1 pm but the second subject did not eat meat or fish during the 24 h collection period. Urine samples were kept at 4°C during the collection, then stored in 20 ml aliquots at -20°C until they were analyzed for taurine, creatine and creatinine using the methods described in Chapter 2. Urinary taurine concentrations were lower than in rats, therefore 0.1 ml 1 mM homoserine was added as internal standard to the 4 ml of eluted sample collected from the ion exchange columns.

Urinary taurine and creatine were expressed as a ratio with urinary creatinine ($\mu\text{mol taurine (creatine)}:\mu\text{mol creatinine}$) as well as absolute values.

6.1.3 Results

Mean taurine excretion was higher in the 5 female volunteers than the 7 male volunteers, when expressed as an absolute value and as creatinine corrected values, but there was also greater variation in this group.

female	taurine - 0.719 mmol.24 h ⁻¹ (SD ± 0.892)	taurine:creatinine - 0.0762 (SD ± 0.0753)
male	taurine - 0.595 mmol.24 h ⁻¹ (SD ± 0.289)	taurine:creatinine - 0.0368 (SD ± 0.0148)

Individual values are shown in Figure 6.2.

Creatine values were also higher in females although the results were very variable and were not significantly different from male values even when they were corrected using creatinine excretion.

female	creatinine - 0.543 mmol.24 h ⁻¹ (SD ± 0.591)	creatinine:creatinine 0.0742 (SD ± 0.9077)
male	creatinine - 0.235 mmol.24 h ⁻¹ (SD ± 0.224)	creatinine:creatinine 0.0125 (SD ± 0.0023)

The pattern of urinary excretion of taurine in the female subject excreting the highest total taurine concentration and a female subject excreting one of the lowest values were examined in more detail. Taurine excretion (taurine:creatinine) in these two subjects is shown in Figure 6.1.

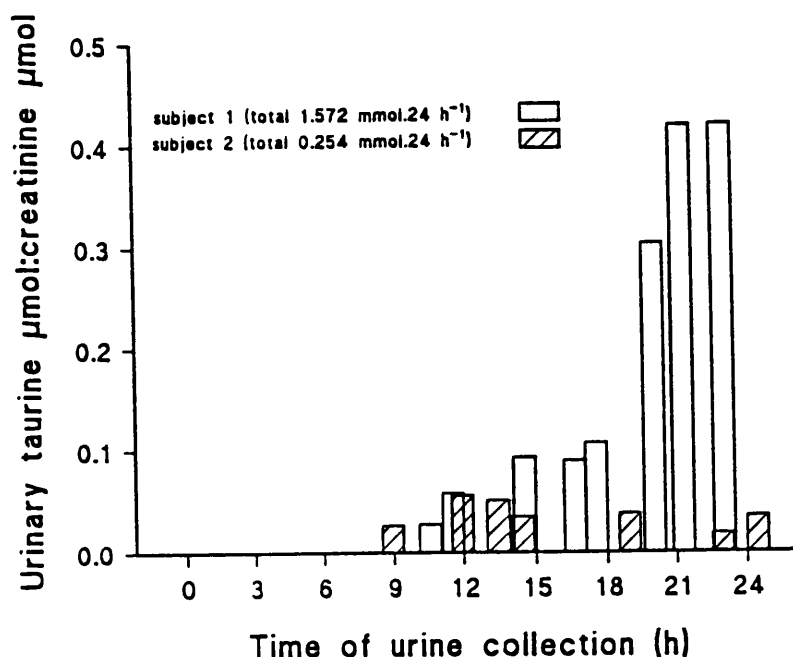


Figure 6.1 Concentration of taurine in spot urine samples taken from two control females the total being a 24 h urine collection. The values are $\mu\text{mol taurine}:\mu\text{mole creatinine}$.

6.1.4 Discussion

The values for taurine excretion in control subjects were within the limits of the published values. Like measurements in the literature they were also very variable. In particular there appeared to be a great deal of variation amongst the female controls. This may have been partly dietary as those with the lowest urinary taurine levels were also people who were vegetarians or who ate little meat. The spot urine samples taken from two females demonstrated the variability of urinary taurine levels throughout the 24 h period. This was due to variations in urinary taurine levels rather than changes in urinary creatinine which were maximal in both subjects between 1 and 6 pm (698 and 592 $\mu\text{mole.h}^{-1}$). Further studies are to be carried out to investigate the variability of urinary taurine in first morning urine samples as the data indicated that this may reflect basal urinary taurine levels which are not raised by overflow of taurine from ingested food.

6.2

URINARY TAURINE AND CREATINE IN PATIENTS WITH ALCOHOLIC LIVER DISEASE

The following study was carried out in collaboration with Dr. D. Sherman at the Institute of Liver Studies, King's College Hospital and R. Draper in the Toxicology Department, School of Pharmacy, University of London.

The aim of this collaboration was to determine whether there was any correlation between the clinical chemistry used to identify alcohol induced liver injury and levels of urinary taurine. Turner and Brum (1964) measured urinary taurine in 24 h urine collections from patients with a variety of acute and chronic diseases, including patients admitted to hospital with acute alcoholism. They reported high levels of urinary taurine at the time of admittance which was independent of dietary intake. These levels declined during the 5 days following admission, often falling below normal levels after 2 weeks. By three weeks levels had returned to normal.

6.2.1 Method

Various liver function tests, histology and serum markers of testicular damage were carried out on 14 male patients (38 - 70 years). The patients had been admitted to King's College Hospital with suspected alcoholic liver damage or infertility which was thought to be alcohol related.

Liver function tests - total protein, albumin, bilirubin, alkaline phosphatase (ALP), AST, γ -GT (γ -glutamyl transpeptidase), serum creatinine. In most cases a histological sample of liver was available.

Indicators of testicular lesion - testicular volume, testosterone, follicle stimulating hormone and luteinizing hormone.

Complete 24 h urine collections were made and frozen in 20 ml aliquots at -80°C. These samples were subsequently analyzed for taurine, creatine and creatinine and correlated with the clinical chemistry provided by Dr. D. Sherman and compared to the results from normal control 24 h urine samples (see 6.1.3 above).

6.2.3 Results

Urinary levels of taurine in patients were higher than male control levels (taurine:creatinine = $0.0794 \pm \text{SD } 0.814$) but similar to female control values (Figure 6.2).

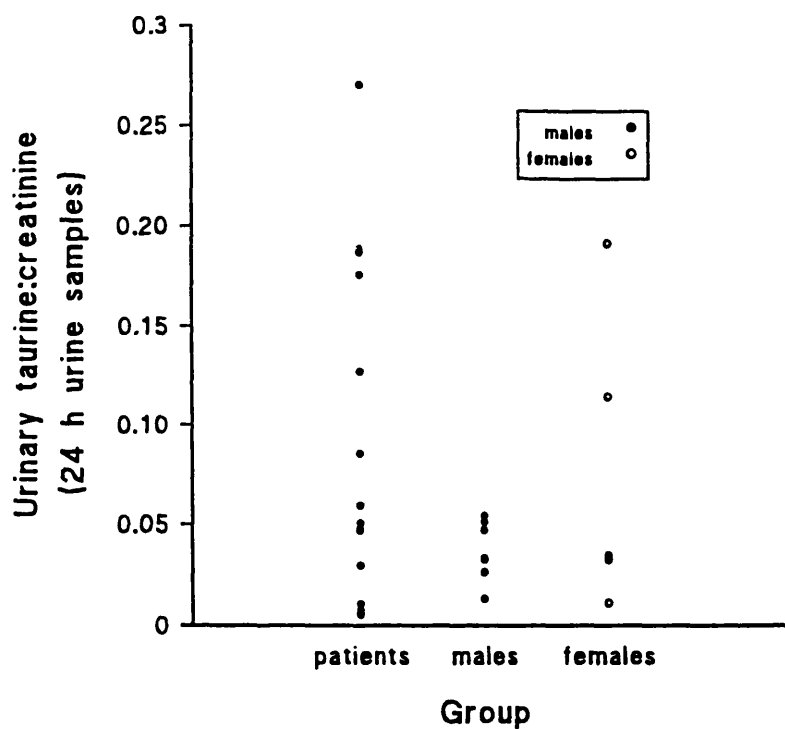


Figure 6.2 Urinary μmol taurine: μmol creatinine in 24 h urine collections from male ($N = 7$) and female control ($N = 5$) subjects and patients ($N = 14$) suffering from alcohol related liver and/or testicular damage. Each point is a value for one individual.

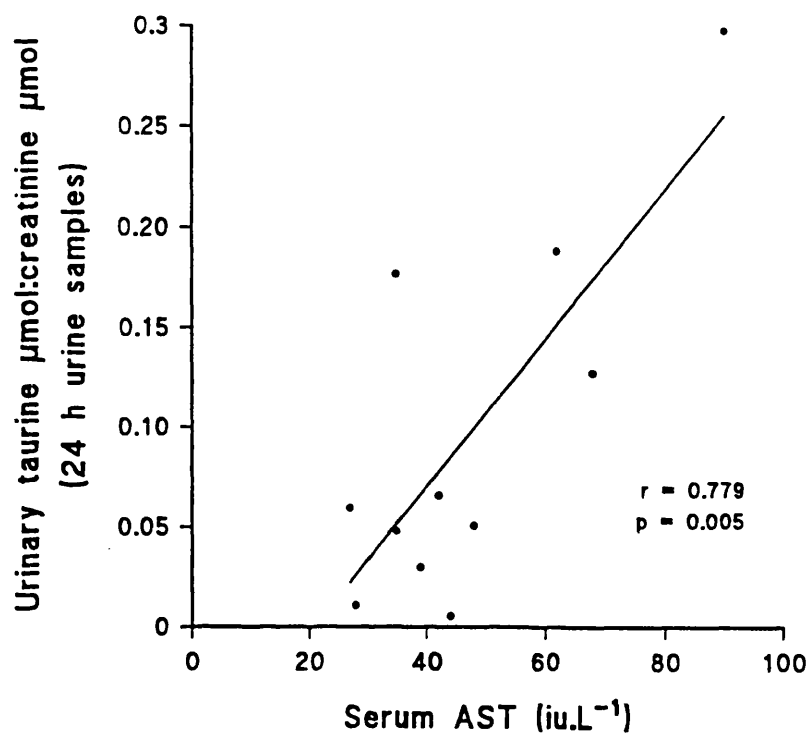


Figure 6.3 Correlation between urinary taurine:creatinine (μmol) and serum AST (iu.L⁻¹). Each point represents data from one individual patient, N = 11. Correlation; $r = 0.779$, significance: $p = 0.005$.

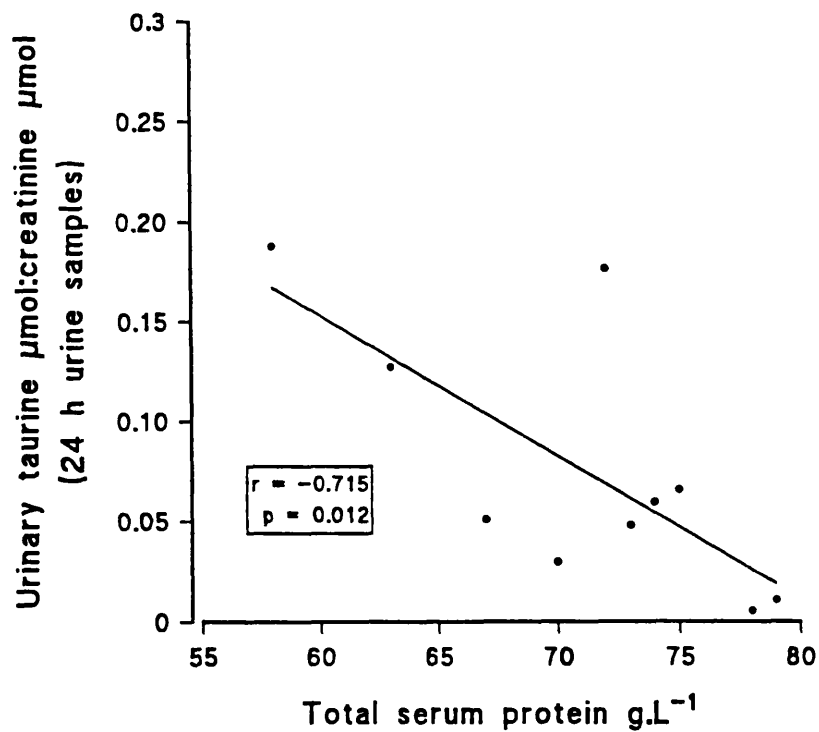


Figure 6.4 Correlation between urinary taurine:creatinine (μmol) and serum total protein (g.L⁻¹). Each point represents data from one individual patient, N = 10. Correlation: $r = -0.715$. Significance: $p = 0.012$.

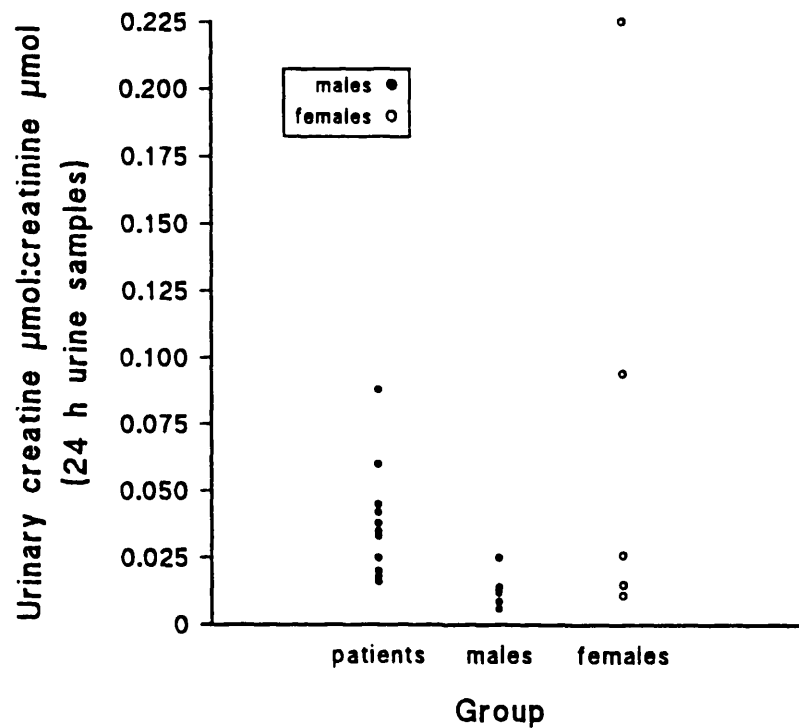


Figure 6.5 Urinary levels of creatine (μmol):creatinine (μmol) in control female ($N = 5$) and males ($N = 7$) and male patients suffering from alcohol related liver and/or testicular injury ($N = 14$). Each point represents data from a single individual.

There was a significant correlation between urinary taurine:creatinine and serum AST levels ($r = 0.779$, $p = 0.005$) and total serum protein ($r = -0.715$, $p = 0.012$) (Figures 6.3 and 6.4). However there were no other significant correlations between urinary taurine and other markers of liver function. Urinary creatine was also higher in male patients than control males and in this case the difference was significant (patients creatine:creatinine $0.0384 \pm \text{SD } 0.0053$ $p < 0.001$) (Figure 6.5). However the only parameter associated with testicular damage was a reduction in urinary creatine (absolute value) which correlated with a reduction in testicular volume ($r = -0.813$, $p < 0.004$). This suggests that the slightly raised levels of urinary creatine compared to controls may be the result of liver injury. The only serum clinical data to correlate well with other serum measurements were serum AST and total serum protein ($r = -0.813$, $p < 0.004$). There were no correlations between the clinical data and other serum parameters used to detect testicular damage.

6.2.4 Discussion

Serum protein levels reflect the degree of protein synthesis taking place in the liver. This is often impaired when there is liver injury and the results presented here suggest that there may be a concomitant rise in urinary taurine when serum protein levels are low. Although taurine levels correlated with serum AST levels there was no correlation with γ -GT, ALP or bilirubin levels. These three markers of liver function are more likely to be raised when there is bile duct damage possibly with cholestasis. Under these circumstances urinary taurine levels might be expected to be reduced (Section 3.1). This might explain why taurine levels were not raised in all patients with alcoholic liver disease as some had raised serum γ GT, ALP and bilirubin levels indicating that they were suffering from cholestasis. Taurine synthesis is increased by the administration of corticosteroids (Yamaguchi *et al* 1971), probably by the induction of cysteine dioxygenase. Corticosteroids are also known to increase the rate of protein catabolism which would increase the levels of both methionine and cysteine available for taurine synthesis. This increase in taurine synthesis could also be

mediated through the action of ethanol as ethanol increases the release of corticosteroids. This would result in an increase in protein catabolism and therefore raise taurine levels which may overspill into the urine. In this way ingestion of alcohol may raise urinary taurine levels when there is no overt cell damage.

Urinary creatine levels were significantly higher in patients than in male controls. This may reflect some degree of liver damage or also be the result of increased protein catabolism (Keel *et al.* 1982) as a result of increased levels of raised of corticosteroids.

Testes have a high concentration of creatine which may be released when they are injured (Gray *et al.* 1990). However, alcohol is known to cause testicular atrophy (Rosenblum *et al.* 1987), which results in reduced serum levels of testosterone. It may be expected therefore, that creatine originating from the testes may be reduced if the volume of the tissue is reduced. There was a significant correlation between a reduction in testicular size in patients and reduced levels of urinary creatine.

FINAL DISCUSSION

7.1

URINARY TAURINE AS A MARKER OF LIVER DAMAGE

Aims of research

- i. to establish a simple, sensitive and reproducible method for measuring taurine in urine;
- ii. to use this method to establish whether raised levels of urinary taurine are indicative of liver dysfunction;

Both of these aims have been achieved.

However there are certain factors which became apparent:

- i. There was variability in urinary taurine particularly between groups of animals. This may have been dietary in origin, stress related or due to other disturbances. For this reason it is probably advisable to include pair fed animals in future studies.
- ii. Urinary taurine levels were not elevated simply as a result of leakage following direct cellular damage.

This supported the suggestion of Kay *et al.* (1957), Goyer and Bowden (1964) and Sanins *et al.* (1990) that elevated levels of taurine, seen after different types of tissue damage, were not simply the result of leakage of intracellular taurine from damaged cells over-spilling into the urine (Zelikovic and Chesney 1989). Thus, the studies described in Chapters 3 and 4 demonstrated that compounds which cause liver necrosis or steatosis, alter GSH levels or interfere with protein synthesis, alter urinary taurine levels. However, urinary taurine is easily measurable and not withstanding these factors, is a useful marker of liver necrosis and steatosis. Raised levels correlate well with serum ALT and AST levels and for CCl₄ and ethionine the increased urinary taurine is dose related. It was also noted in

the research described in this thesis that urinary creatine was indicative of hepatic necrosis. Hence, the determination of the levels of the two urinary markers in the same urine sample provides more information about the possible site of injury of a toxic compound (Table 7.1).

Table 7.1 Summary of the lesions indicated by changes in urinary levels of taurine and creatine.

Taurine/creatine levels in urine	Lesion implicated	Compound
Raised taurine + raised creatine	Liver necrosis possibly with steatosis (due to protein synthesis inhibition)	CCl ₄ , thioacetamide, galactosamine, sometimes allyl alcohol
Raised taurine + no raised creatine	Steatosis, inhibition of protein and/or GSH synthesis	ethionine, hydrazine, cycloheximide, BSO
Taurine not raised + raised creatine	Testicular damage	cadmium chloride
Taurine lowered + no raised creatine	Increased protein/GSH synthesis	phenobarbitone, diethyl maleate, phorone, allylamine
Slightly reduced taurine + raised creatine	Cell necrosis with perturbation of GSH synthesis	allyl alcohol, bromobenzene
Very low taurine + slightly raised creatine	Cholestasis (with concomitant raised liver taurine), kidney damage	ANIT, lithocholate (?), mercuric chloride

Preliminary data and future studies

The clinical use of taurine as a marker of hepatic injury has been investigated in a preliminary study of patients suffering from alcohol related liver disorders. This has shown that there was a significant correlation between urinary taurine and serum AST levels and with serum proteins (Chapter 6). A study of other human subjects is currently being undertaken in a collaboration with Dr. J. Wheeler and Dr. H. Mason at the Health and Safety Executive. Here industrial workers exposed to various solvents have been monitored and tested for different parameters which would indicate liver injury. Preliminary data suggest that these subjects

have a higher concentration of urinary taurine than the unexposed population of control volunteers, even though they do not have an increase in other parameters associated with liver injury.

7.2

SOURCE OF RAISED LEVELS OF URINARY TAURINE

Future work

Inhibition of both protein and GSH synthesis raised levels of urinary taurine. This provided strong evidence that an increase in taurine precursors contributed to the raised levels of urinary taurine after hepatotoxic insult. To show an increase in taurine synthesis more directly, *de novo* synthesis of taurine was measured following the administration of CCl_4 to rats (Section 4.1). The results supported the hypothesis but were not conclusive. An increase in taurine synthesis was shown in isolated hepatocytes incubated with CCl_4 , but not with hydrazine. In order to clarify these investigations the following future studies are suggested:

To correlate protein and taurine synthesis -

- a. Measure ^{35}S and ^{14}C incorporation into proteins and ^{35}S into taurine following the administration of ^{35}S -methionine and ^{14}C -leucine to rats at different time points after dosing with CCl_4 and other compounds which elevate urinary taurine.
- b. Monitor the incorporation of ^3H -leucine into proteins and synthesis of taurine *in vitro* in isolated hepatocytes and primary cultures.
- c. Pre-label proteins and the cysteine and methionine pool *in vivo* with ^{35}S by administering ^{35}S -methionine before the isolation of hepatocytes to enable any increase in taurine synthesis after incubation with test compounds to be measured as an increase in the specific activity of taurine in the hepatocytes. This would take into account taurine synthesised as a result of protein catabolism.
- d. Administer the competitive inhibitor of cysteine sulphinate decarboxylase, D-cysteine sulphinate, to animals at the time of dosing

with CCl_4 to inhibit *de novo* synthesis of taurine (Weinstein and Griffith 1987). If this treatment reduces the increase in urinary taurine produced by CCl_4 and ethionine it would demonstrate that the expected increase in taurine synthesis had been impaired. If the elevation is not reduced it would indicate that the raised levels originate from some other source such as increased mobilization from another pool or an inhibition of uptake in the kidney.

Experimental modifications -

As all the compounds which increased urinary taurine levels are known to inhibit protein synthesis (eg. CCl_4 , thioacetamide, cycloheximide and ethionine) and some may increase protein catabolism (eg. hydrazine) an increase in urinary free sulphate via cysteine transamination or desulphuration to pyruvate (Figure 1.20) might also be expected. It is therefore suggested that the measurement of sulphate in urine from animals treated with hepatotoxic compounds might also indicate if there was an increase in sulphur amino acid catabolism. It might also provide information about the preferred route of cysteine catabolism, for example, after the partial inhibition of cysteine sulphinate decarboxylase with D-cysteine sulphinat. The measurement of urea would similarly provide information about protein catabolism.

The three measurements, **taurine**, **sulphate** and **urea** could be useful indicators of the state of protein metabolism in the body and may provide some insight into the mechanism of toxicity of hepatotoxic compounds.

Further studies into raised urinary taurine levels -

Raised levels of urinary taurine have also been found after exposure of experimental animals to ionising radiation (Angel and Noonan, 1959) and as a result of various forms of stress associated with the release of ACTH (Hayes and Sturman, 1981). The diverse nature of factors shown to increase urinary levels of taurine could be linked by a common component. Factors, such as stress stimulate the release of ACTH from the pituitary and

increase the production of corticosteroids. Corticosteroids increase protein catabolism and would therefore be expected to increase cysteine levels and therefore taurine synthesis. Indeed, the administration of hydrocortisone to rats has been shown to increase taurine synthesis, probably via the increase in cysteine dioxygenase activity, although an increase in cysteine concentration has a similar effect (Yamaguchi *et al.* 1971 and 1975). The liver is also responsible for the degradation of corticosteroids. Therefore, if the liver is injured this process might be impaired and corticosteroids may continue to circulate. A common factor in the elevation of urinary taurine in all of these cases, therefore, may be either the inhibition of protein synthesis or a net increase in the catabolism of proteins, or both. There may also be a relationship between increased levels of urinary taurine seen as a result of stress and changes in the levels of GSH in response to cold-restraint reported by Simmons *et al.* (1990). They demonstrated that stress induced by cold-restraint in mice decreased hepatic GSH levels.

For these reasons it would be interesting to establish whether increased levels of corticosteroids are correlated with increased levels of urinary taurine. Raised levels of urinary taurine might therefore be also indicative of stress (Hayes and Sturman, 1981).

Urinary levels of both taurine and creatine following lesions produced by other hepatotoxic compounds or compounds causing injury to other tissues should also be examined. Goyer *et al.* (1964) demonstrated that raised urinary levels of taurine are found in rats treated with the muscle toxins plasmocid and isoproterenol and suggested that this was also due to an increase in protein catabolism. Taurine levels in spinal cord tissue have been shown to be elevated in mice where a chronic relapsing experimental allergic encephalitis is induced (an animal model of multiple sclerosis) following the subcutaneous injection of spinal cord homogenate and mycobacteria (Preece *et al.* 1990a). It may be found that neuronal toxins such as lead and glutamate also raise urinary levels of taurine as brain tissue has both a high concentration of taurine and is able to synthesise it.

7.3

TAURINE AND PROTECTION *IN VIVO*

The inhibition of protein synthesis as a result of toxic injury to tissue may help to limit cellular injury by increasing the cysteine available for GSH and taurine synthesis. An increase in taurine synthesis as a result of cellular damage could provide additional taurine for cellular protection which may help to limit further cell injury.

It was observed (Chapter 5) that those rats with low urinary levels of taurine also had low levels of liver taurine. These animals were more susceptible to a variety of hepatotoxic compounds as measured by serum levels of AST and ALT. However, it is possible that urinary taurine levels may also reflect levels of precursors. Thus an increase in taurine may be the result of an increase in tissue levels of cysteamine (and therefore cystamine) (Section 1.7.6, Figure 1.20). Cysteamine and cystamine are known to protect against harmful effects of ionizing radiation, galactosamine and CCl_4 toxicity (MacDonald *et al.* 1984 and 1985 and Castro *et al.* 1972). Cysteamine and hypotaurine are believed to be able to scavenge $\bullet\text{OH}$ and HOCl , and it has been proposed that both are more likely to act as antioxidants *in vivo* than taurine (Aruoma *et al.* 1988). Although the pathway via cysteamine is thought to make only a small contribution to taurine levels, the possibility that cysteamine and therefore cystamine levels may be high when taurine excretion is high should be investigated.

However, it was also shown that a reduction of hepatic taurine levels by the displacement of taurine from tissues with β -alanine increased the susceptibility of the liver to toxic insult from CCl_4 indicating that taurine is itself protective (Section 5.1).

The prophylactic use of taurine against CCl_4 toxicity in rats failed to provide protection (Nakashima *et al.* 1983). However, taurine given to rats 12 h *after* CCl_4 intoxication did result in the reduction of lipid peroxidation and Ca^{2+} accumulation. However, we have failed to prevent the toxicity of

CCl_4 in rats conclusively, by the administration of taurine. In some cases animals showed an increased toxic response while others appeared to be completely protected. The timing of the administration of taurine may be crucial to the protective effects. Initial experiments have been conducted to ensure that it is possible to "load" tissues with taurine provided in the drinking water. Early experiments were ambiguous in this respect. Using the dosing regime described by Nakashima *et al.* (1983), liver levels of taurine were not found to be significantly different from control animals after dosing with CCl_4 . However, rats provided with taurine (3 %) in the drinking water have been shown to increase liver levels of taurine significantly (Appendix XII). Similar taurine "loaded" rats will be used in future experiments to determine whether they are protected from the effects of different hepatotoxic compounds.

The question arises as to the effect of reduced levels of liver taurine on the susceptibility of the liver to adverse effects of drugs in humans. It would be of interest to investigate the susceptibility of people with low tissue levels of taurine due to their diet such as vegans. The liver appears to be more susceptible to depletion of taurine than other tissues, both as a result of dietary intake and by artificial reduction by administration of compounds such as guanidinoethyl sulphonate and β -alanine. People with dietary deficiencies including vitamin B_6 deficiency, which can be induced artificially by treatment with isoniazid, may show increased sensitivity to hepatotoxic drugs if liver levels of taurine are reduced.

It has been shown that GSH is reduced by submaximal exercise in rats (Lew and Quintanilha, 1991). However, training rats enables them to maintain GSH levels during similar periods of submaximal exercise. This observation clearly has implications for the detoxification of compounds such as acetaminophen in humans. The effect of exercise on the levels of hepatic taurine would be of great interest. For example, does exercise alter liver taurine levels or the ability of a person to synthesise taurine? Or are tissue levels of taurine reduced by exercise as GSH synthesis is increased

by training?

Future work

It is proposed that studies investigating the effect of depleting liver taurine on the toxic response to various hepatotoxic compounds is continued, particularly the effect on hydrazine toxicity. Hydrazine has not been shown to cause necrosis, despite the fact that it induces steatosis and depletes ATP. However, if liver levels of taurine are low there might be a potentiation of toxicity to the point where necrosis does occur.

The susceptibility of rats to hepatotoxic compounds which have been "loaded" with taurine will also be studied.

7.4

TAURINE AND PROTECTION *IN VITRO*

The *in vitro* experiments carried out as part of the work in this thesis demonstrated the protective effect of taurine against two hepatotoxic compounds, CCl_4 and hydrazine. Subsequently it has also been shown by P. Parnham, working in this laboratory that taurine will also protect isolated hepatocytes against the cytotoxicity of 1,4-naphthaquinone. The degree of protection being similar to that found after CCl_4 and hydrazine toxicity. However, taurine was also able to protect against some loss of ATP which resulted from the quinone toxicity.

At present, the mechanism by which taurine protected isolated hepatocytes in these studies can only be speculated. There are numerous reports of the protective properties of taurine *in vitro* in which taurine has been demonstrated to stabilize membranes, reduce lipid peroxidation, act as an antioxidant and modulate ion fluxes, including Ca^{2+} (Section 5.1.1).

Taurine may stabilize membranes by binding to membrane bound, neutral phospholipids such as phosphatidylcholine and phosphatidylethanolamine, which have similar zwitterionic head groups to taurine. This ensures that the phospholipids remain intact permitting Ca^{2+} binding. In this way

taurine may modulate Ca^{2+} flux (Wright *et al.* 1986). Huxtable *et al.* (1987) also suggested that the interaction of taurine with these phospholipids increases the affinity of Ca^{2+} for high affinity binding sites on acidic phospholipids such as phosphatidylinositol and phosphatidylserine, thus regulating Ca^{2+} transport.

The importance of regulating Ca^{2+} flux has been extensively studied in relation to cell death. Nicotera *et al.* (1989) discussed the role of toxic agents in altering the normal transport mechanisms for Ca^{2+} located in the plasma membrane, endoplasmic reticulum and mitochondria by inhibiting or enhancing influx of extracellular Ca^{2+} . An elevation of cytosolic Ca^{2+} leads to the cytotoxic effects mediated by the Ca^{2+} -dependent degenerative enzymes; phospholipases, proteases and endonucleases (Orrenius *et al.* 1989). Activation of phospholipases or proteases can cause structural or functional injury to other cell components leading to cytotoxicity. Protease activity can result in dissociation of actin microfilaments from, or proteolytic cleavage of vital anchoring proteins in the plasma membrane, leading to blebbing and increased membrane permeability.

Nakashima *et al.* (1990) has shown that taurine decreased the oxygenation-induced lipid peroxidation of hepatocytes and prevented the hypoxia-induced hepatocyte death in a Ca^{2+} -containing medium, but not a Ca^{2+} free medium. It was also shown that taurine could protect hepatocytes from the injury associated with the Ca^{2+} and oxygen paradoxes due to the inhibition of a sudden Ca^{2+} influx into the hepatocytes.

It would be of interest to see whether Ca^{2+} levels were changed in isolated hepatocytes challenged with different hepatotoxins and whether taurine was able to increase the sequestration of Ca^{2+} into mitochondria. If taurine is able to alter Ca^{2+} influx into cells this may reduce the blebbing of hepatocytes when treated with different hepatotoxins. Preliminary work using scanning electron microscopy was inconclusive in establishing whether taurine prevented blebbing of hepatocytes.

All of the protective properties of taurine could be explained if it is considered simply as an important osmolyte. Although this function is not unique to taurine, its inertness, neutrality at physiological pH and high concentration in most animal cells make it ideally suited to this function. Recent work by Pasantes-Morales *et al.* (1991), have shown that taurine is released from human lymphocytes when they are exposed to hyposmotic media. Although other free amino acids were released in response to the rapid cell swelling taurine constituted the highest proportion. The mechanism for this release is not known. This observation may explain the observations made in section 4.2 where there was an earlier release of taurine than LDH from cells treated with both hydrazine and CCl_4 . This may have been an adaptive response to cell swelling rather than release from damaged membranes or dying cells.

Future work

In order to examine further the protection of isolated cells by taurine against hepatotoxins, the following investigations are proposed :

- a. At present it is not known why such high concentrations of taurine are required in the extracellular medium (5 - 15 mM) to protect cells from toxic insult. Although these levels are similar to intracellular levels *in vivo* they are 100 X higher than plasma concentrations. The protective properties of taurine *in vitro* might therefore rely on a high extracellular concentration. In order to discriminate between an intracellular effect and one which is active intracellularly, isolated cells could be "loaded" with taurine and then washed and replaced in taurine free medium before being treated with hepatotoxic compounds.
- b. There are reports that the inclusion of HEPES in the incubation buffer inhibits the uptake of taurine and β -alanine into glial cells (Lieu and Rebel 1990). However, Ohkuma *et al.* (1981 and 1984) and Banks *et al.* (1989) used HEPES in the incubation used to suspend hepatocytes

and alveolar macrophages and type II cells when measuring taurine uptake and did not show any interference. The possible inhibition of uptake of taurine should however be investigated.

- c. Studies investigating the susceptibility of cells prepared from rats depleted of taurine using β -alanine to hepatotoxic compounds, have been carried out as preliminary studies. These studies are being continued. It enables the simultaneous examination of a population of cells derived from one animal to be investigated, both in the taurine depleted state and subsequently provided with taurine in the medium.

REFERENCES

- Al Adnani, M.S. (1989) Differential immunohistochemical localization of cytokeratins and collagen types I and II in experimentally-induced cirrhosis. *J. Path.* **159**, 151-158.
- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1983) *The Molecular Biology of the Cell*, 549-609, NY. and London, Garland Publishing, Inc..
- Amenta, J.S. and Johnson, E.H. (1962) Hydrazine induced alterations in rat liver. *Lab. Invest.* **11**, 956-962.
- Angel, C.R. and Noonan, T.R. (1959) Urinary taurine excretion patterns in rat, rabbit, guinea pig and dog following exposure to whole body X-radiation. *Fed. Proc.* **19**, 353.
- Anzano, M.A., Naebanij, J.O., and Lamb, A.J. (1978) Simplified two-step column-chromatographic determination of taurine in urine. *Clin. Chem.* **24**, 321-325.
- Aruoma, O.I, Halliwell, B., Hoey, B.M., and Butler, J. (1988) The antioxidant action of taurine, hypotaurine and their metabolic precursors. *Biochem. J.* **256**, 251-255.
- Awapara, J. (1955) The taurine concentration of organs from fed and fasted rats. *J. Biol. Chem* **218**, 571-576.
- Awapara, J. (1976) The metabolism of taurine in the animal. In: *Taurine*, 1-20. Edited by Huxtable, R. and Barbeau, A., NY., Raven Press.
- Balisteri, W.F. and Shaw, L.M. (1987) Liver function. In: *Fundamentals of Clinical Chemistry*, 3rd Ed., 729-761. Edited by Tietz, N.W., London., Sanders.
- Banks, M.A., Martin, W.G., Pailles, W.H., and Castranova, V. (1989) Taurine uptake by isolated macrophages and type II cells. *J. Appl. Physiol.* **66**, 1079-1086.
- Banks, M.A., Porter, D.W., Martin, W.G., and Castranova, V. (1990) Effects of *in vitro* ozone exposure on peroxidative damage, membrane leakage and taurine content of rat alveolar macrophages. *Toxicol. Appl. Pharmacol.* **105**, 55-65.
- Barbeau, A. (1978) Taurine and Friedreich's ataxia. In: *Taurine and Neurological Disorders*, 429-440. Edited by Barbeau, A. and Huxtable, R.J., NY., Raven Press.

Belinsky, S.A., Popp, J.A., Kauffmann, F.C., and Thurman, R.G. (1984) Trypan blue uptake as a new method to investigate hepatotoxicity in periportal and pericentral regions of the liver lobule: studies with allyl alcohol in the perfused liver. *J. Pharmacol. Exp. Ther.* **230**, 755-760.

Berger, M.L., Bhatt, H., Combes, B., and Estabrook, R.W. (1986) CCl₄-induced toxicity in isolated hepatocytes: The importance of direct solvent injury. *Hepatology* **6**, 36-45.

Berger, M.L., Reynolds, R.C., and Combes, B. (1987) Carbon tetrachloride-induced morphologic alterations in isolated hepatocytes. *Exp. Mol. Pathol.* **46**, 245-257.

Bergermeyer, H.U., Bernt, E., and Hess, B. (1965) Lactate dehydrogenase (II 2.a.). In: *Methods of Enzymatic Analysis*, 736-743. Edited by Bergmeyer, H.,

Berry, M.N., Edwards, A.M., and Barritt, G.J. (1991) *Laboratory Techniques in Biochemistry and Molecular Biology: Vol 21, Isolated hepatocytes preparation, properties and applications*, Amsterdam, NY., London, Elsevier.

Blouin, A., Bolender, R.P, and Weibel, E.R. (1977) Distribution of organelles and membranes between hepatocytes and non-hepatocytes in the rat liver parenchyma - A stereological study. *J. Cell Biol.* **72**, 441-455.

Boor, P.J., Moslen, M.T., and Reynolds, E.S. (1979) Allylamine Cardiotoxicity: I. Sequence of pathological events. *Toxicol. Appl. Pharmacol.* **50**, 581-592.

Bouwens, L., Baekeland, M., DeZanger, R., and Wisse, E. (1968) Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver. *Hepatology* **6**, 718-722.

Bowden, D.H. and Goyer, R.A. (1962) Drug-induced muscle necrosis with massive taurinuria. *Arch. Path.* **74**, 59-63.

Boyland, E. and Chasseau, L.F. (1967) Enzyme-catalysed conjugations of glutathione with unsaturated compounds. *Biochem. J.* **104**, 95-102.

Brodie, B.B., Reid, W.D., Cho, A.K., Sipes, G., Krishna, G., and Gillette, J.R. (1971) Possible mechanism of liver necrosis caused by aromatic organic compounds. *Proc. Natl. Acad. Sci. USA.* **68**, 160-164.

Butler, W.M., Maling, H.M., Horning, M.G., and Brodie, B.B. (1961) The direct determination of liver triglycerides. *J. Lipid Res.* **2**, 95-96.

Capizzo, F. and Roberts, R.J. (1971) Effect of phenobarbital, chlorpromazine, Actinomycin D and chronic α -naphthylisothiocyanate administration on α -naphthylisothiocyanate- ^{14}C disposition and α -naphthylisothiocyanate-induced hyperbilirubinemia. *J. Pharmacol. Exp. Ther.* **179**, 455-464.

Carpenter-Deyo, L., Marchand, D.H., Jean, P.A., Roth, R.A., and Reed, D.J. (1991) Involvement of glutathione in 1-naphthyl-isothiocyanate (ANIT) metabolism and Toxicity to isolated hepatocytes. *Biochem. Pharm.* **42**, 2171-2180.

Casini, A.F., Giorli, M., Hyland, R.J., Serroni, A., Gilfor, D., and Farber, J.L. (1982) Mechanisms of cell injury in the killing of cultured hepatocytes by bromobenzene. *J. Biol. Chem* **12**, 6721.

Casini, A.F., Maellaro, E., Pompella, A., Ferrali, M., and Comporti, M. (1987) Lipid proxidation, protein thiols and calcium homeostasis in bromobenzene-induced liver damage. *Biochem. Pharm.* **30**, 3689-3695.

Casini, A.F., Pompella, A., and Comporti, M. (1985) Liver glutathione depletion induced by bromobenzene, iodobenzene, and diethylmaleate poisoning and its relation to lipid peroxidation and necrosis. *Am. J. Pathol.* **118**, 225-237.

Castro, J.A., Cignoli, E.C., DeCastro, C.R., and DeFenos, O.M. (1972) Prevention by cystamine of liver necrosis and early biochemical alterations induced by carbon tetrachloride. *Biochem. Pharm.* **21**, 49-57.

Chesney, R.W. (1985) Taurine: It's biological role and clinical implications. *Adv. Pediatr.* **32**, 1-42.

Chesney, R.W., Jax, D.K., Scriver, C.R., and Mohyuddin, F. (1978) Taurine transport in mammalian kidney. In: *Taurine and Neurological Disorders*, 73-93. Edited by Barbeau, A. and Huxtable, R., NY., Raven Press.

Chubb, J. and Huxtable, R.J. (1978) Transport and biosynthesis of taurine in the stressed heart. In: *Taurine and Neurological Disorders*, 161-180. Edited by Barbeau, A. and Huxtable, R.J., NY., Raven Press.

Clark, P.A., Leeder, L.G., Foulds, E.L., and Trout, D.L. (1970) Changes in lipids of rat liver after hydrazine injection. *Biochem. Pharm.* **19**, 1743-1752.

Connolly, A.K., Price, S.C., Connolly, J.C., and Hinton, R.H. (1988) Early changes in bile duct lining cells and hepatocytes in rats treated with α -naphthylisothiocyanate. *Toxicol. Appl. Pharmacol.* **93**, 208-219.

Connolly, B.M. and Goodman, H.O. (1980) Potential sources of errors in cation exchanges chromatographic measurement of plasma taurine. *Clin. Chem.* **26**, 508-510.

Cooper, A.J.L. (1983) Biochemistry of sulphur containing amino acids. *Ann. Rev. Biochem.* **52**, 187-222.

Corcoran, G.B. and Ray, S.D. (1992) The role of the nucleus and other compartments in toxic cell death produced by alkylating hepatotoxicants. *Toxicol. Appl. Pharmacol.* **113**, 167-183.

Cornish, H.H. (1969) The role of vitamin B₆ in the toxicity of hydrazines. *Ann. NY. Acad. Sci.* **166**, 136-145.

Cornish, H.H. and Ryan, R.C. (1964) A study of carbon tetrachloride VI. Aminoaciduria in response to carbon tetrachloride inhalation. *Toxicol. Appl. Pharmacol.* **6**, 96-102.

Cornish, H.H. and Wilson, C.E. (1968) Amino acid levels in hydrazine treated rats. *Toxicol. Appl. Pharmacol.* **12**, 265-272.

Costa, L.G. and Murphy, S.D. (1986) Effect of diethylmaleate and other glutathione depletors on protein synthesis. *Biochem. J.* **36**, 3383-3388.

Crass III, M.F. and Lombardini, J.B. (1977) Loss of cardiac muscle taurine after acute ventricular Ischemia. *Life Sci.* **21**, 951-958.

Cusworth, D.C. and Dent, C.E. (1960) Renal clearances of amino acids in normal adults and in patients with aminoaciduria. *Biochem. J.* **74**, 550-561.

D'Amour, M. and Charbonneau, M. (1992) Sex-related difference in hepatic glutathione conjugation of hexachlorobenzene in the rat. *Toxicol. Appl. Pharmacol.* **112**, 229-234.

Dahlstrom-King, L. and Plaa, G.L. (1989) Effect of inhibition of protein synthesis on cholestasis induced by tauroolithocholate, lithocholate and a manganese-bilirubin combination in the rat. *Biochem. Pharm.* **38**, 2543-2549.

Dahm, L.J., Bailie, M.B., and Roth, R.A. (1991a) Relationship between α -naphthylisothiocyanate-induced liver injury and elevations in hepatic non-protein sulphhydryl content. *Biochem. Pharm.* **42**, 1189-194.

Dahm, L.J. and Roth, R.A. (1991) Protection against α -naphthylisothiocyanate-induced liver injury by decreased hepatic non-protein sulphhydryl content. *Biochem. Pharm.* **42**, 1181-1188.

Dahm, L.J., Schultze, A.E., and Roth, R.A. (1991b) An antibody to neutrophils attenuates α -naphthylisothiocyanate-induced liver injury. *J. Pharmacol. Exp. Ther.* **256**, 412-420.

Datta, S.C. and Naryanaswami, A. (1983) Florometric estimation of taurine in tissue extracts and biological fluids. *Ital. J. Biochem.* **32**, 297-305.

- Davis, D.C., Hashimoto, M., and Gillette, J.R. (1973) Effects of Bromobenzene and carbon tetrachloride on the synthesis and release of proteins by perfused rat liver. *Biochem. Pharm.* **22**, 1989-2001.
- Davis, M. and Williams, R. (1977) Hepatic disorders. In: *Textbook of Adverse Drug Reactions*, 146-172. Edited by Davis, D.M., London, Oxford University Press.
- Day, W.W. and Weiner, M. (1991) Inhibition of hepatic drug metabolism and carbon tetrachloride toxicity in Fischer-344 rats by exercise. *Biochem. Pharm.* **42**, 181-184.
- De la Rosa, J., Drake, M.R., and Stipanuk, M.H. (1987) Metabolism of cysteine and cysteine sulfinic acid in rat and cat hepatocytes. *J. Nutr.* **117**, 549-558.
- DeMaster, E.G. and Redfern, B. (1987) High-performance liquid chromatography of hepatic thiols with electrochemical detection. In: *Methods in enzymology*, 143rd Ed., 110-114. Edited by Jackoby, W.B. and Griffith, O.W., NY, Academic Press.
- Dent, C.E. and Walshe, J.M. (1954) Amino acid metabolism. *Brit. Med. Bull.* **10**, 247-250.
- DePierre, J.W., Anderson, G., and Dallner, G. (1988) Endoplasmic reticulum and Golgi complex. In: *The Liver: Biology and Pathobiology*, 3rd Ed., 165-187. Edited by Arias, I.M., Jackoby, W.B., Popper, H., Scacherler, D., and Shafritz, D., NY, Raven Press.
- Dianzani, M.U. (1979) Reactions of the liver to injury: fatty liver. In: *Toxic injury of the liver, Part A*, 281-332. Edited by Farber, E. and Fisher, M.M., NY, Marcel Dekker.
- Dianzani, M.U. (1991) Biochemical aspects of fatty liver. In: *Hepatotoxicology*, 327-400. Edited by Meeks, R.G., Harrison, S.D., and Bull, R.J., Boston, London, Boca Raton/CRC Press .
- Dogterom, P. (1990) *Cytotoxicity and protein thiol modifications in isolated rat hepatocytes (Academic dissertation)*, "All in" BV - Katwijk.
- Dorvil, N.P., Yoisef, I.M., Tuchweber, B., and Roy, C.C. (1983) Taurine prevents cholestasis induced by lithocholic acid sulfate in guinea pigs. *Am. J. Clin. Nutr.* **37**, 221-232.
- Drake, R., DeLaRosa, J., and Stipanuk, M.H. (1987) Metabolism of cysteine in rat hepatocytes: evidence for cysteine sulphinic acid-independent pathways. *Biochem. J.* **244**, 279-286.

Drew, R. and Miners, J.O. (1984) The effects of buthionine sulfoximine (BSO) on glutathione depletion and xenobiotic biotransformation. *Biochem. Pharm.* **33**, 2989-2994.

Dudley, R.E., Svoboda, D.J., and Klaassen, C.D. (1982) Acute exposure to cadmium causes severe liver injury in rats. *Toxicol. Appl. Pharmacol.* **65**, 302-313.

Dudley, R.E., Svoboda, D.J., and Klaassen, C.D. (1984) Time course of cadmium-induced ultrastructural changes in rat liver. *Toxicol. Appl. Pharmacol.* **76**, 150-160.

Dunnett, C.W. (1964) New tables for multiple comparisons with a control. *Biometrics* **20**, 482-492.

Durkin, T.A., Anderson, G.M., and Cohen, D.J. (1988) High-performance liquid chromatographic analysis of neurotransmitter amino acids in brain. *J. Chromatogr.* **428**, 9-15.

Dyroff, M.C. and Neal, R.A. (1981) Identification of the major protein adduct in rat liver after thioacetamide administration. *Cancer Res.* **41**, 3430-3435.

Dyroff, M.C. and Neal, R.A. (1983) Studies of the mechanisms of metabolism of thioacetamide S-oxide by rat liver microsomes. *Mol. Pharmacol.* **23**, 219-227.

Ellman, G.L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70-77.

Emudianughe, T.S., Caldwell, J., and Smith, R.L. (1983) The utilisation of exogenous taurine for the conjugation of xenobiotic acids in the ferret. *Xenobiotica* **13**, 133-138.

Evans, W.H. (1980) A biochemical dissection of functional polarity of the plasma membrane of the hepatocyte. *Biochem. Phys. Acta* **604**, 27-64.

Evered, D.F. (1956) The excretion of amino acids: A quantitative study with ion exchange chromatography. *Biochem. J.* **62**, 416-427.

Farber, E. (1967) Ethionine fatty liver. *Advan. Lipid Res.* **5**, 119-186.

Farber, E. and Fisher, M.M. (1979) *Toxic Injury of the Liver, Parts A and B*, NY. and Basle, Dekker.

Farber, J.L. (1981) The role of calcium in cell death. *Life Sci.* **29**, 1289-1295.

Fawthrop, D.J., Boobis, A.R., and Davies, D.S. (1991) Mechanisms of cell death. *Arch. Toxicol.* **65**, 437-444.

Gartland, K.P.R., Bonner, F.W., and Nicholson, J.K. (1988) Investigations into biochemical effects of region-specific nephrotoxins. *Mol. Pharmacol.* **35**, 242-250.

Garvin, J.E. (1960) A new method for the determination of taurine in tissues. *Arch. Biochem. Biophys.* **91**, 219-225.

Gaull, G.E. (1989) Taurine in pediatric nutrition. *Pediatrics* **83**, 433-442.

Geggel, H.S., Ament, M., Heckenlively, J.R., Martin, D.A., and Kopple, J.D. (1985) Nutritional requirement for taurine in patients receiving long-term parenteral nutrition. *N. Engl. J. Med.* **312**, 142-146.

Gerard-Monnier, D., Fougat, S., and Chaudiere, J. (1992) Glutathione and cysteine depletion in rats and mice following acute intoxication with diethyl maleate. *Biochem. Pharm.* **43**, 451-456.

Ghatineh, S. and Timbrell, J.A. (1990) Hydrazine toxicity in isolated hepatocytes in suspension and primary culture. *Biochem. Soc. Trans.* **18**, 1217-1218.

Glaser, G. and Mager, J. (1974) Biochemical studies on the mechanism of action of liver poisons. III. Depletion of liver glutathione in ethionine poisoning. *Biochim. Biophys. Acta* **372**, 237-244.

Glazier, J.R. (1986) *Principles of Toxicological Pathology*, London and Philadelphia, Taylor and Francis.

Goethals, F., Krack, G., Deboyser, D., and Roberfroid, D. (1983) Effects of diethylmaleate on protein synthesis in isolated hepatocytes. *Toxicology* **26**, 47-54.

Goldman, H. and Scriver, C.R. (1967) A transport system in mammalian kidney with a preference for β -amino compounds. *Pediat. Res.* **1**, 212-213.

Gordon, R.E. (1991) Taurine prophylaxis against the effects of bleomycin and paraquat: known agents of oxidant and free radical injury (Abstract). *International Taurine Symposium: New dimensions on it's Mechanisms of Action* (Abstract)

Gordon, R.E., Shacked, A.A., and Solano, D.F. (1986) Taurine protects hamster bronchioles from acute NO_2 -induced alterations - A histologic, ultrastructural and freeze-fracture study. *Am. J. Pathol.* **125**, 585-600.

Goyer, R.A., Yin, M.W., and Bowden, D.H. (1964) Taurine excretion and muscle necrosis. *Proc. Soc. Expt. Biol. Med.* **116**, 534-536.

- Grant, G.H., Silverman, L.M., and Christenson, R.H. (1987) Amino acids and proteins. In: *Fundamentals of Clinical Chemistry*, 3rd Ed., 291-345. Edited by Tietz, N.W., London, Sanders.
- Gravella, E., Albano, E., Dianzani, M.U., Poli, G., and Slater, T.F. (1979) Effects of carbon tetrachloride on isolated rat hepatocytes (inhibition of protein and lipoprotein excretion). *Biochem. J.* **178**, 509-512.
- Gray, J., Nicholson, J.K., Creasy, D.M., and Timbrell, J.A. (1990) Studies on the relationship between acute testicular damage and urinary and plasma creatine concentration. *Arch. Toxicol.* **64**, 443-450.
- Gregus, Z., White, C., Howell, S., and Klaassen, C.D. (1988) Effect of glutathione depletion on sulfate activation and sulfate ester formation in rats. *Biochem. Pharm.* **37**, 4307-4312.
- Griffith, O.W. (1983) Cysteine sulfinic acid metabolism altered partitioning between transamination and decarboxylation following administration of β -methylene aspartate. *J. Biol. Chem* **258**, 1591-1598.
- Gupa, D.N. (1956) Nodular cirrhosis and metastasising tumours produced in the liver of rats by prolonged feeding with thioacetamide. *J. Path. Bact.* **72**, 415-425.
- Haagsma, B.H. and Pound, A.W. (1979) Mercuric chloride-induced renal tubular necrosis in the rat. *Br. J. Exp. Pathol.* **60**, 341-352.
- Haghighi, B. and Honarjou, S. (1987) The effects of hydrazine on the phosphatidate phosphohydrolase activity in the rat liver. *Biochem. Pharm.* **36**, 1163-1165.
- Hardison, W.G.M. (1978) Hepatic taurine concentration and dietary taurine as regulators of bile acid conjugation with taurine. *Gastroenterology* **75**, 71-75.
- Hatoff, D.E. and Hardison, W.G.M. (1980) Bile acids modify alkaline phosphatase induction and bile secretion pressure after bile duct obstruction in the rat. *Gastroenterology* **80**, 666-672.
- Hayes, K.C., Stephen, Z.F., and Sturman, J.A. (1980) Growth depression in taurine-depleted infant monkeys. *J. Nutr.* **110**, 2058-2064.
- Hayes, K.C. and Sturman, J.A. (1981) Taurine in metabolism. *Ann. Rev. Nutr.* **1**, 401-425.
- Henson, P.D., Britton, P.S., and Banks, W.L. (1976) Evidence of enhancement of hepatic pyrimidine catabolism following hydrazine or INH treatment to rats. *Proc. 67th Meeting Am. Assoc. Cancer Res.* **17**, 144.(Abstract)

- Hepner, G.W., Sturman, J.A., Hofmann, A.F., and Thomas, P.J. (1973) Metabolism of steroid and amino acid moieties of conjugated bile acids in man III Cholytaurine (taurocholic acid). *J. Clin. Invest.* **52**, 433-440.
- Herman, B., Gores, G.J., Nieminien, A-L., Kawanishi, T., Herman, A., and Lemasters, J.J. (1990) Calcium and pH in anoxic and toxic injury. *Crit. Rev. Toxicol.* **21**, 127-148.
- Higashi, T., Tateishi, N., and Sakamoto, Y. (1983) Liver glutathione as a reservoir of cysteine. In: *Sulfur Amino Acids: Biochemical and Clinical Aspects*, 419-434. Edited by Kuriyama, K., Huxtable, R.J., and Iwata, H., NY., Alan R.Liss Inc..
- Higuchi, K. (1963) Studies on the nutrition and metabolism of animal cells in serum-free media. *J. Infect. Dis.* **112**, 213-220.
- Hirai, T., Ohyama, H., and Kido, R. (1987) A direct determination of taurine in perchloric acid-deproteinized biological samples. *Anal. Biochem.* **163**, 339-342.
- Hirschberger, L.L., De la Rosa, J., and Stipanuk, M.H. (1985) Determination of cysteine sulfinic acid, hypotaurine and taurine in physiological samples by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **343**, 305-313.
- Hosokawa, Y., Niizeki, S., Tojo, H., Sato, I., and Yamaguchi, K. (1988) Hepatic cysteine dioxygenase activity and sulfur amino acid metabolism in rats: possible indicators in the evaluation of protein quality. *J. Nutr.* **118**, 456-461.
- Hunter, A.L., Holcher, M.A., and Neal, R.A. (1977) Thioacetamide induced hepatic necrosis I. Involvement of the mixed function oxidase system. *J. Pharmacol. Exp. Ther.* **200**, 437-448.
- Huxtable, R. and Bressler, R. (1972) Taurine and isethionic acid: distribution and interconversion in the rat. *J. Nutr.* **102**, 805-814.
- Huxtable, R. and Bressler, R. (1974) Elevation of taurine in human congestive heart failure. *Life Sci.* **14**, 1353-1359.
- Huxtable, R.J., and Barbeau, A. (Editors) (1976) *Taurine*, Raven Press.
- Huxtable, R.J. (1982) Physicochemical properties of taurine. In: *Taurine in Nutrition and Neurology*, 1-4. Edited by Huxtable, R.J. and Pasantes-Morales, H., NY. and London, Plenum Press.
- Huxtable, R.J. (1987) From heart to hypothesis - a mechanism for the calcium modulatory action of taurine. *Adv. Expt. Med. Biol.* **217**, 371-387.

Huxtable, R.J. (1992) Physiological actions of taurine. *Physiol. Rev.* **72**, 101-163.

Huxtable, R.J. and Lippincott, S.E. (1982) Sources and turnover of taurine in newborn, weanling and mature rats. In: *Taurine in Nutrition and Neurology*, 23-43. Edited by Huxtable, R.J. and Pasantes-Morales, H., NY. and London, Plenum Press.

Indacochea-Redmond, N., Witschi, H., and Plaa, G.L. (1973) Effect of inhibitors of protein and ribonucleic acid synthesis on hyperbilirubinemia and cholestasis produced by α -naphthylisothiocyanate. *J. Pharmacol. Exp. Ther.* **184**, 780-786.

Izumi, K., Nagata, R., Motoya, T., Yamashita, J., Hirokane, T., Nagata, Y., Satoh, Y., Sawada, Y., Ishibashi, M., Yoshida, H., and Fukuda, T. (1989) Preventative effect of taurine against acute paraquat intoxication in beagles. *Japan J. Pharmacol.* **50**, 229-233.

Jacobsen, J.G. and Smith, L.H. (1968) Biochemistry and Physiology of taurine and taurine derivatives. *Physiol. Rev.* **48**, 424-511.

Jaeschke, H., Kleinwaechter, C., and Wendel, A. (1987) A role of acrolein in allyl alcohol induced lipid peroxidation and liver cell damage in mice. *Biochem. Pharm.* **36**, 51-57.

Jollow, D.J., Mitchell, J.R., Zampaglione, N., and Gillette, J.R. (1974) Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology* **11**, 151-169.

Jones, A.C. and Spring-Milles, E. (1988) The liver and gall bladder. In: *Cell and Tissue Biology - a text book of histology*, 6th Ed., 686-714. Edited by Weiss, L. and Creep, R.O., Urban Schwartzberg.

Jones, A.L. and Schmuckler, D.L. (1977) Current concepts of liver structure as related to function. *Gastroenterology* **73**, 833-851.

Judah, J.D., Ahmed, H.J.Z., and McLean, A.E.M. (1965) Protection provided against carbon tetrachloride and thioacetamide poisoning by strophanthin-G. *J. Path. Bact.* **89**, 619-623.

Kadlubar, F.F. and Hammons, G.J. (1987) The role of cytochrome P₄₅₀ in the metabolism of chemical carcinogens. In: *Mammalian Cytochromes P₄₅₀* Vol 2, 81-130. Edited by Guengerich, F.P., Boston, London, Boca Raton, CRC Press.

Kakis, G., Phillips, L., and Yousef, I.M. (1980) The respective roles of membrane cholesterol and sodium-potassium adenosine triphosphate in the pathogenesis of lithocholate-induced cholestasis. *Lab. Invest.* **43**, 73-81.

- Kaplowitz, N., Aw, T.Y., and Ookhtens, M. (1985) The regulation of hepatic glutathione. *Ann. Rev. Pharmacol. Toxicol.* **25**, 715-744.
- Kaplowitz, N., Eberle, D.E., Petrini, J., Touloukian, J., Corvasce, M.C., and Kuhlenkamp, J. (1983) Factors influencing the efflux of hepatic glutathione into bile in rats. *J. Pharmacol. Exp. Ther.* **224**, 141-147.
- Kato, H. and Nakazawa, Y. (1987) The effect of carbon tetrachloride on the enzymatic hydrolysis of cellular triacylglycerol in adult rat hepatocytes in primary monolayer culture. *Biochem. Pharm.* **36**, 1807-1814.
- Kay, R.E., Early, J.C., and Entenman, C. (1957) Increased urinary excretion of taurine and urea by rats after X-radiation. *Rad. Res.* **6**, 98-109.
- Keel, G.A., Neil, E., and Joels, N. (1982) *Samson Wright's Applied Physiology*, 13th Ed., 432-434, Oxford, NY., Oxford University Press.
- Keppler, D., Lesch, R., Reutter, W., and Decker, K. (1968) Experimental hepatitis induced by D-galactosamine. *Exp. Mol. Pathol.* **9**, 279-290.
- Klaassen, C.D., Amdur, M.O., and Doull, J. (Editors) (1986) *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 3rd Ed., NY., Toronto, London, Macmillan.
- Kocsis, J.J., Harkaway, S., Santoyo, M.C., and Snyder, R. (1968) Dimethyl sulfoxide: interactions with aromatic hydrocarbons. *Science* **160**, 427-428.
- Kocsis, J.J., Harkway, S., and Snyder, R. (1975) Biological effects of the metabolites of dimethyl sulphoxide. *Ann. NY. Acad. Sci.* **243**, 104-109.
- Konikoff, F., Goldman, G., Halpern, Z., Somjen, G.J., and Gilat, T. (1990) Polyamines - potential nucleating factors in bile. *Liver* **10**, 173-176.
- Korty, P. and Coe, F.L. (1968) The effects of hydrazine upon the concentrations of free amino acids of plasma and urine. *J. Pharmacol. Exp. Ther.* **160**, 212-216.
- Kostos, V.J. and Kocsis, J.J. (1961) Effect of colchicine on taurine excretion. *Proc. Soc. Expt. Biol. Med.* **106**, 659-660.
- Krack, G., Goethals, F., Debayser, D., and Roberfroid, M. (1980) Interference of chemicals with glycogen metabolism isolated hepatocytes. *Toxicology* **18**, 213-223.
- Kramer, J.H., Chovan, J.P., and Schaffer, S.W. (1981) Effect of taurine on calcium paradox and ischemic heart failure. *Am. J. Physiol.* **240**, H238-H246.

- Krell, H., Metz, J., Jaeschke, H., Hoke, H., and Pfaff, E. (1987) Drug-induced intrahepatic cholestasis: characterization of different pathomechanisms. *Arch. Toxicol.* **60**, 124-130.
- Kukongviriyapan, V. and Stacey, N.H. (1991) Chemical-induced interference with hepatocellular transport -role in cholestasis. *Chem. Biol. Interactions* **77**, 245-261.
- Kuo, S-M. and Stipanuk, M.H. (1984) Changes in cysteine dioxygenase and cysteine sulfinatase decarboxylase activities and taurine levels in tissues of pregnant or lactating rat dams and their fetuses or pups. *Biol. Neonate* **46**, 237-248.
- Lack, L. and Weiner, I.M. (1967) Role of the intestine during the enterohepatic circulation of bile salts. *Gastroenterology* **52(I)**, 282-287.
- Lake, N. and DeMarte, L. (1988) Effects of β -alanine treatment on the taurine and DNA content of rat heart and retina. *Neurochem. Res.* **13**, 1003-1006.
- Lamb, R.G. and Banks, W.L. (1979) Effect of hydrazine exposure on hepatic triacylglycerol biosynthesis. *Biochim. Biophys. Acta* **574**, 440-447.
- Larsen, B.R., Grasso, D.S., and Chang, S.Y. (1980) A rapid method for taurine quantitation using high performance liquid chromatography. *J. Chromatogr. Sci.* **18**, 233-236.
- Lau, S.S. and Zannoni, V.G. (1979) Hepatic microsomal epoxidation of bromobenzene to phenols and its toxicological implication. *Toxicol. Appl. Pharmacol.* **50**, 309-318.
- Levine, W.G. (1974) Hepatic uptake metabolism and biliary excretion of 7,12-dimethylbenzanthracene in the rat. *Drug. Met. Disp.* **2**, 169-177.
- Lew, H. and Quintanilha, A. (1991) Effects of endurance training and exercise on tissue antioxidative capacity and acetaminophen detoxification. *Eur. J. Drug Metab. Pharmacokinet.* **16**, 59-68.
- Lindamood III, C. (1991) Xenobiotic biotransformations. In: *Hepatotoxicology*, 139-180. Edited by Meeks, R.G., Harrison, S.D., and Bull, R.J., Boston, London, Boca Raton/CRC Press.
- Lindroth, P. and Mopper, K. (1979) High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o-phthalaldehyde. *Anal. Chem.* **51**, 1667-1673.

- Lleu, P.L. and Rebel, G. (1990) Effect of HEPES on the Na⁺ Cl⁻ dependent uptake of taurine and β -alanine by cultured glial cells. Modulation by composition and osmolarity of medium. *Neuropharmacology* **29**, 719-725.
- Long, R.M. and Moore, L. (1988) Biochemical evaluation of rat hepatocyte primary cultures as a model for carbon tetrachloride hepatotoxicity: comparative studies *in vivo* and *in vitro*. *Toxicol. Appl. Pharmacol.* **92**, 295-306.
- Lopez, M. and Mazanti, L. (1955) Experimental investigation on 1-naphthyliso-thiocyanate as a hyperplastic agent of the biliary ducts in the rat. *J. Path. Bact.* **69**, 243-250.
- Lopez-Mendoza, D. and Villa-Travino, S. (1971) Hydrazine-induced inhibition of amino acid incorporation into rat liver protein. *Lab. Invest.* **25**, 68-72.
- Loriette, C. and Chatagner, F. (1978) Cysteine oxidase and cysteine sulfinic acid decarboxylase in developing rat liver. *Experientia* **34**, 981-982.
- Loud, A.V. (1968) A quantitative stereological description of the ultrastructure of normal rat liver parenchymal cells. *J. Cell Biol.* **37**, 27-46.
- Macart, M. and Gerbaut, L. (1982) An improvement of the Coomassie blue dye binding method allowing an equal sensitivity to various fluids: application to cerebrospinal fluid. *Clin. Chim. Acta* **122**, 93-101.
- MacDonald, J.R., Gandolfi, A.J., and Sipes, I.G. (1984) Cystamine modulation of galactosamine-induced hepatotoxicity. *Toxicol. Appl. Pharmacol.* **73**, 551-558.
- MacDonald, J.R., Gandolfi, A.J., and Sipes, I.G. (1985) Structural requirements for the cytoprotective agents in galactosamine-induced hepatic necrosis. *Toxicol. Appl. Pharmacol.* **81**, 17-24.
- Maelaro, E., DelBello, B., Casini, A.F., Comporti, M., Ceccarelli, D., Muscatello, V., and Masini, A. (1990) Early mitochondrial dysfunction in bromobenzene treated mice: A possible factor of liver injury. *Biochem. Pharm.* **40**, 1491-1497.
- Maturo, J. and Kulakowski, E.C. (1988) Taurine binding to the purified insulin receptor. *Biochem. Pharm.* **37**, 3755-3760.
- Maurice, M., Durand-Schneider, A.M., Garbaz, M., and Feldman, G. (1985) Characterization of rat hepatocyte plasma membrane domains by monoclonal antibodies. *Eur. J. Biol.* **39**, 122-129.

McLean, E.K., McLean, A.E.M., and Sutton, P.M. (1969) Instant cirrhosis: An improved method for producing cirrhosis of the liver in rats by simultaneous administration of carbon tetrachloride and phenobarbitone. *Br. J. Exp. Pathol.* **50**, 502-506.

McLean, P. (1966) Effect of ethionine treatment on the activity of enzymes concerned with urea synthesis in the rat liver. *Biochem. J.* **99**, 776-785.

Meeks, R.G., Harrison, S.D., and Bull, R.J. (1991) *Hepatotoxicity*, Boston, London, Boca Raton, CRC Press.

Mehendale, H.M. (1991) Role of hepatocellular regeneration and hepatolobular healing in the final outcome of liver injury: A two stage model of toxicity. *Biochem. Pharm.* **42**, 1155-1162.

Mico, B.A., Branchflower, R.V., and Pohl, L.R. (1983) Formation of electrophilic chlorine from carbon tetrachloride - involvement of cytochrome P₄₅₀. *Biochem. Pharm.* **32**, 2357-2359.

Mitchell, J.R., Jollow, D.J., Potter, W.Z., Davis, D.C., Gillette, J.R., and Brodie, B.B. (1973a) Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J. Pharmacol. Exp. Ther.* **187**, 185-194.

Mitchell, J.R., Jollow, D.J., Potter, W.Z., Gillette, J.R., and Brodie, B.B. (1973b) Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.* **187**, 211-217.

Miyai, K. (1991) Structural organisation of the liver. In: *Hepatotoxicology*, 1-65. Edited by Meeks, R.G., Harrison, S.D., and Bull, R.J., Boston, London, Boca Raton, CRC Press.

Moldeus, P., Hogberg, J., and Orrenius, S. (1978) Chapter. 4. Isolation and use of liver cells. *Methods in Enzymology* **51**, 60-71.

Monks, T.J., Hinson, J.A., and Gillette, J.R. (1982) Bromobenzene and p-bromophenol toxicity and covalent binding *in vivo*. *Life Sci.* **30**, 841-848.

Mozaffari, M.S., Tan, B.H., and Lucia, M.A. (1986) Effect of drug-induced taurine depletion on cardiac contractility and metabolism. *Biochem. Pharm.* **35**, 985-989.

Muller, D., Sommer, M., Kretschmar, M., Zimmerman, T., Buko, V.U., Lukirskaya, O.J., and Dargel, R. (1991) Lipid peroxidation in thioacetamide-induced macronodular liver cirrhosis. *Arch. Toxicol.* **65**, 199-203.

Naftalin, L., Sexton, M., Whitaker, J.F., and Tracy, D. (1969) A routine procedure for estimating serum gamma-glutamyl transpeptidase activity. *Clin. Chim. Acta* **26**, 293-296.

Nakashima, T., Seto, Y., Toshikazu, N., Shima, T., Iwai, Y., Zeizo, K., Okanque, T., and Kashima, K. (1990) Calcium-associated cytoprotective effect of taurine on the calcium and oxygen paradoxes in isolated rat hepatocytes. *Liver* **10**, 167-172.

Nakashima, T., Takino, T., and Kuriyama, K. (1983) Therapeutic and prophylactic effects of taurine administration on experimental liver injury. In: *Sulphur Amino Acids: Biochemical and Clinical Aspects*, 449-459. Edited by Kuriyama, K., Huxtable, R.J., and Iwata, H., NY, Alan R. Liss Inc..

Neidle, A., Banay-Schwartz, M., Sacks, S., and Dunlop, D.S. (1989) Amino acid analysis using 1-naphthylisocyanate as a pre-column high performance liquid chromatography derivatization reagent. *Anal. Chem.* **180**, 291-297.

Nicholson, J.K., Higham, D.P., Timbrell, J.A., and Sadler, P.J. (1989) Quantitative high resolution ^1H -NMR urinalysis studies on the biochemical effects of cadmium in the rat. *Mol. Pharmacol.* **36**, 398-404.

Nicholson, J.K., Timbrell, J.A., and Sadler, P.J. (1985) Proton NMR spectra of urine as indicators of renal damage: Mercury-induced nephrotoxicity in rats. *Mol. Pharmacol.* **27**, 644-651.

Nicotera, P., McConkey, D.J., Dypbukt, J.M., Jones, D.P., and Orrenius, S. (1989) Ca^{2+} -Activated mechanisms in cell killings. *Drug Metab. Rev.* **20**, 193-201.

Nishiuch, Y., Sasaki, M., Nakayasu, M., and Oikawa, A. (1976) Cytotoxicity of cysteine in culture media. *In Vitro* **12**, 635-638.

Ohkuma, S., Tamura, J., Kuriyama, K., and Makainaka, T. (1984) Characteristics of taurine transport in rat hepatocytes in primary culture. *Cell Biochem. Funct.* **2**, 171-177.

Ohkuma, S., Tamura, J., Kuriyama, K., and Takino, T. (1981) Characteristics of taurine transport in freshly isolated rat hepatocytes. *Japan J. Pharmacol.* **31**, 1061-1070.

Oja, S.S. and Kontro, P. (1982) Taurine. In: *Handbook of Neurochemistry*, 3rd Ed., 501-533. Edited by Lajtha, A., NY. and London, Plenum Press.

Olney, J.W., Ho, O.L., and Rhee, V. (1971) Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system. *Exp. Brain Res.* **14**, 61-76.

Orrenius, S., McConkey, D.J., Bellomo, G., and Nicotera, P. (1989) Role of Ca^{2+} in toxic cell killing. *TIPS* **10**, 281-285.

- Paasonen, M.K., Solatunturi, E., and Nieminen, M.L. (1985) Blood platelets: Accumulation and release of taurine. In: *Taurine: Biological Actions and Clinical Perspectives*, 225-233. Edited by Oja, S.S., Ahtee, L., Kontro, P., and Paasonen, M.K., NY., Alan R. Liss..
- Paine, A.J. (1981) Hepatic cytochrome P₄₅₀. In: *Essays in Biochemistry*, 85-126. Edited by Campbell, P.N. and Greville, G.D., Academic Press, London.
- Pasantes-Morales, H., Garcia, J de J., and Olea, R.S. (1991) Hyposmolarity-sensitive release of taurine and free amino acids from human lymphocytes. *Biochem. Pharm.* **41**, 303-307.
- Pasantes-Morales, H., Quesanda, O., Carabez, A., and Huxtable, R. (1983) Effects of taurine transport antagonist guanidinoethane sulfonate and β -alanine on the morphology of rat retina. *J. Neurosci. Res.* **9**, 135-143.
- Pasantes-Morales, H., Wright, C.E., and Gaull, G.E. (1984) Protective affect of taurine, zinc and tocopherol on retinol-induced damage in human lymphoblastoid cells. *J. Nutr.* **114**, 2256-2261.
- Pasantes-Morales, H., Wright, C.E., and Gaull, G.E. (1985) Taurine protection of lymphoblastoid cells from iron-ascorbate induced damage. *Biochem. Pharm.* **34**, 2205-2207.
- Patel, J.M., Wood, J.C., and Leibman, K.C. (1980) The biotransformation of allyl alcohol and acrolein in rat liver and lung preparations. *Drug. Met. Disp.* **8**, 305-308.
- Penttila, K.E. (1988) Allyl alcohol cytotoxicity and glutathione depletion in isolated periportal and perivenous rat hepatocytes. *Chem. Biol. Interactions* **65**, 107-121.
- Penttila, K.E. (1990) Role of cysteine and taurine in regulating glutathione synthesis by periportal and perivenous hepatocytes. *Biochem. J.* **269**, 659-664.
- Penttila, K.E. (1990) *Glutathione biosynthesis and allyl alcohol cytotoxicity in periportal and perivenous rat hepatocytes isolated by digitonin/collagenase perfusion (Academic dissertation)*, University of Kupio.
- Pentz, E.I., Davenport, C.H., Glover, W., and Smith, D.D. (1957) A test for the determination of taurine in urine. *J. Biol. Chem* **228**, 433-445.
- Pion, P.D., Kittleson, M.D., Rogers, Q.R., and Morris, J.G. (1987) Myocardial failure in cats associated with low plasma taurine: A reversible cardiomyopathy. *Science* **237**, 764-768.
- Plaa, G.L. (1970) Hyperbilirubinemia and cholestasis - a different form of liver injury produced in animals. *Essays in Toxicol.* **2**, 137-154.

- Plaa, G.L. and Hewitt, W.R. (1989) Detection and evaluation of clinically induced liver injury. In: *Principles and Methods of Toxicology*, 2nd Ed., 599-628. Edited by Hayes, A.W., NY., Raven Press.
- Pohl, L.R., Schulick, R.D., Highet, R.J., and George, J.W. (1984) Reductive-oxygenation mechanism of metabolism of carbon tetrachloride to phosgene by cytochrome P₄₅₀. *Mol. Pharmacol.* **25**, 318-321.
- Poli, G., Albano, E., and Dianzani, M.U. (1987) The role of lipid peroxidation in liver damage. *Phys. Lipids* **45**, 117-142.
- Poli, G., Cheeseman, K., Slater, T.F., and Dianzani, M.U. (1981) The role of lipid peroxidation in CCl₄-induced damage to liver microsomal enzymes: Comparative studies *in vitro* using microsomes and isolated liver cells. *Chem. Biol. Interactions* **37**, 13-24.
- Porter, D.W., Banks, M.A., Castranova, V., and Martin, W.G. (1988) Reversed-phase high performance liquid chromatography technique for taurine quantitation. *J. Chromatogr.* **454**, 311-316.
- Poso, A.R., Penttila, K.E., Suolinna, E.M., and Lindros, K.O. (1986) Urea synthesis in freshly isolated and in cultured periportal and perivenous hepatocytes. *Biochem. J.* **239**, 263-267.
- Preece, N.E., Bruggen, N.V., Gadian, D.G., Baker, D., Neill, J.K.O., and Turk, J.L. (1990a) ¹H-NMR studies of CNS metabolism in an animal model of multiple sclerosis. *Works in Progress: Society of Magnetic Resonance in Medicine 9th Ann.Sci.Meet.*, 1231.(Abstract)
- Preece, N.E., Ghatineh, S., and Timbrell, J.A. (1990b) Course of ATP depletion in hydrazine hepatotoxicity. *Arch. Toxicol.* **64**, 49-53.
- Rappaport, A.M. (1979) Physicoanatomical basis of toxic liver injury. In: *Toxic injury of the liver. Part A*, 1-57. Edited by Farber, E. and Fisher, M.M., NY, Marcel Dekker.
- Ravindranath, V. and Boyd, M.R. (1991) Effects of modulators of glutathione synthesis on the hepatotoxicity of 2-methyl furan. *Biochem. Pharm.* **41**, 1311-1318.
- Read, W.O. and Welty, J.D. (1962) Synthesis of taurine and isethionic acid by dog heart slices. *J. Biol. Chem* **237**, 1521-1522.
- Rechnagel, R.O. (1983) Minireview: A new direction in the study of carbon tetrachloride toxicity. *Life Sci.* **33**, 401-408.
- Rechnagel, R.O. and Glende Jr., E.A. (1973) Carbon tetrachloride hepatotoxicity: An example of lethal cleavage. *CRC. Crit. Revs. Toxic.* **2**, 263-297.

- Recknagel, R.O., Glende, E.A., and Britton, R.S. (1991) Free radical damage and lipid peroxidation. In: *Hepatotoxicology*, 401-436. Edited by Meeks, R.G., Harrison, S.D., and Bull, R.J., Boston, London, Boca Raton/CRC Press
- Reid, W.D. and Krishna, G. (1973) Centrilobular hepatic necrosis related to covalent binding of metabolites of halogenated aromatic hydrocarbons. *Exp. Mol. Pathol.* **18**, 80-99.
- Rikans, L.E. (1989) Influence of aging on chemically induced hepatotoxicity: Role of age-related changes in metabolism. *Drug Metab. Rev.* **20**, 87-110.
- Roberts, R.J. and Plaa, G.L. (1965) Potentiation and inhibition of α -naphthylisothiocyanate-induced hyperbilirubinemia and cholestasis. *J. Pharmacol. Exp. Ther.* **150**, 499-506.
- Robeson, B.L. and Martin, W.G. (1978) Taurine synthesis from sulfate in the rat induced by dietary zinc (Abs 1711). *Fed. Proc.* **37**, 537.(Abstract)
- Roos, F.J., Zysset, T., and Reichen, J. (1991) Differential effect of biliary and micronodular cirrhosis on oxidative drug metabolism: *in vivo-in vitro* correlations of dextromethorphan metabolism in rats. *Biochem. Pharm.* **41**, 1513-1519.
- Rosenblum, E.R., Gavalier, J.S., and Van Thiel, D.H. (1987) Vitamin A at pharmacologic doses ameliorates the membrane lipid peroxidation injury and testicular atrophy that occurs with chronic alcohol feeding in rats. *Alcohol and Alcoholism* **22**, 241-249.
- Sanins, S.M., Nicholson, J.K., Elcombe, C., and Timbrell, J.A. (1990) Hepatotoxin-induced hypertaurinuria: A proton NMR study. *Arch. Toxicol.* **64**, 407-411.
- Santone, K.S., Acosta, D., Salmon, A., Stavchansky, S.A., and Bruckner, J.V. (1988) The use of primary cultures of postnatal rat hepatocytes to investigate CCl₄ induced cytotoxicity. In: *Liver Cells and Drugs*, 221-233. Edited by Guillouzo, A., Colloque INSERM/John Libbey Eurotext Ltd..
- Sauberlich, H.E. (1961) Studies on the toxicity and antagonism of amino acids for weanling rats. *J. Nutr.* **75**, 61-72.
- Scales, M.D.C. and Timbrell, J.A. (1982) Studies on hydrazine hepatotoxicity. I. Pathological findings. *J. Toxicol. Environ. Health* **10**, 941-953.
- Scandurra, R., Frederici, G., and Dupre, S. (1978) Taurine and isethionic acid production in mammals. *Bull. Mol. Biol. Med.* **3**, 141-147.

- Scriver, C.R., Chesney, R.W., and McInnes, R.I. (1976) Genetic aspects of renal tubular transport: Diversity and topology of carriers. *Kidney Int.* **9**, 149-171.
- Seglan, P.O., Sotheim, A.E., Grinde, B., Gordon, P.B., Schwarze, P.E., Gjessing, R., and Poli, A. (1980) Amino acid control of protein synthesis and degradation in isolated rat hepatocytes. *Ann. NY. Acad. Sci.* **349**, 1-17.
- Shaffer, J.E. and Kocsis, J.J. (1981) Taurine mobilizing effects of beta alanine and other inhibitors of taurine transport. *Life Sci.* **28**, 2727-2736.
- Sheikh, K. (1981) Taurine deficiency and retinal defects associated with small intestine bacterial overgrowth (Abs). *Gastroenterology* **80**, 1363.
- Siedel, J., Mullering, H., and Ziegenhorn, J. (1984) Sensitive color reagent for the enzymatic determination of creatinine. *Clin. Chem.* **30**, 968-969 (Abs.147).
- Simmons, H.F., James, R.C., Hardison, R.D., and Roberts, S.M. (1990) Depression of glutathione by cold restraint in mice. *Toxicology* **61**, 59-71.
- Slater, T.F. (1978) Biochemical studies on liver injury. In: *Biochemical mechanisms of liver injury*, 1-44. Edited by Slater, T.F., London, Academic Press.
- Smith, M.T., Thor, H., and Orrenius, S. (1981) Toxic injury to isolated hepatocytes is not dependent on extracellular calcium. *Science* **213**, 1257-1259.
- Smith, R.L. (1966) The biliary excretion and enterohepatic circulation of drugs and other organic compounds. In: *Progress in drug research*, 300-360. Edited by Jucker, E., Basel and Stuttgart, Bierkauser Verlag.
- Sorbo, B.O. (1961) A method for the determination of taurine in urine. *Clin. Chim. Acta* **6**, 87-90.
- Southpart, P. (1959) Urinary excretion of free amino acids in normal adult men and women. *Clin. Chim. Acta* **4**, 265-271.
- Springer, D.L., Broderick, D.J., and Dost, F.N. (1980) Effects of hydrazine and it's derivatives on ornithine decarboxylase synthesis, activity and inactivation. *Toxicol. Appl. Pharmacol.* **53**, 365-372.
- Stanley, P.E. and Williams, S.G. (1969) Use of the liquid scintillation spectrometer for determining adenosine triphosphate by luciferase enzyme. *Anal. Biochem.* **29**, 381-392.
- Stipanuk, M.H. (1979) Effect of excess dietary methionine on the catabolism of cysteine in rats. *J. Nutr.* **109**, 2126-2139.

- Stipanuk, M.H. and Rotter, M.A. (1984) Metabolism of cysteine, cysteine sulfinic acid and cysteine sulphonate in rats fed adequate and excess levels of sulfur-containing amino acids. *J. Nutr.* **114**, 1426-1437.
- Stuart, J.D., Wilson, T.D., Hill, D.W., and Walters, F.H. (1979) High performance liquid chromatographic separation and fluorescent measurement of taurine, a key amino acid. *J. Liquid Chromatogr.* **2**, 809-821.
- Sturman, J.A. (1973) Taurine pool sizes in the rat: Effects of vitamin B6 deficiency and high taurine diet. *J. Nutr.* **103**, 1566-1580.
- Sturman, J.A. (1990) Taurine deficiency. In: *Taurine: Functional neurochemistry, physiology and cardiology (Progress in Clinical and Biological Research. Vol.351)*, 385-395. Edited by Pasantes-Morales, H., Martin, D.L., Shain, W., and Martin del Rio, R., Wiley-Liss Inc..
- Sturman, J.A., Hepner, G.W., Hufmann, A.L., and Thomas, P.J. (1975) Metabolism of ^{35}S taurine in Man. *J. Nutr.* **105**, 1206-1214.
- Suber, R.L. (1989) Clinical pathology for Toxicologists. In: *Principles and methods of toxicology*, 2nd Ed., 485-520. Edited by Hayes, A.W., NY, Raven Press.
- Sztul, E.S., Biemesderfer, D., Caplan, M.J., Kashgarian, M., and Boyer, J.L. (1987) Localisation of $\text{Na}^+ \text{K}^+$ -ATPase alpha-subunit to the sinusoidal and lateral but not canalicular membranes of rat hepatocytes. *J. Cell Biol.* **104**, 1239-1248.
- Thurman, R.G. and Kauffman, F.C. (1985) Sublobular compartmentation of pharmacologic events (SCOPE): Metabolic fluxes in periportal and pericentral regions of the liver lobule. *Hepatology* **5**, 144-151.
- Thurston, J.H., Hanhart, R.E., and Dirgo, J.A. (1980) Taurine: A role in osmotic regulation of mammalian brain and possible clinical significance. *Life Sci.* **26**, 1561-1568.
- Tiegs, G. and Wendel, A. (1988) Leukotriene-mediated liver injury. *Biochem. Pharmacol.* **37**, 2569-2573.
- Timbrell, J.A. (1986) The liver as a target organ for toxicity. In: *Target Organ Toxicity Vol.I*, 145-173. Edited by Cohen, G.M., Florida, Boca Raton: CRC Press.
- Timbrell, J.A. (1991) Toxic responses to foreign compounds. In: *Principles of Biochemical Toxicology*, 2nd Ed., 193-284. London and Washington, Taylor and Francis.

Tiselius, H-G. and Heuman, R. (1978) A routine method for determination of glycine and taurine distribution in bile acids. *Clin. Chim. Acta* **83**, 49-53.

Tokinaga, H., Yoneda, Y., and Kuriyama, K. (1979) Protective actions of taurine against streptozotocin-induced hyperglycemia. *Biochem. Pharm.* **28**, 2807-2811.

Trennery, P.N. and Waring, R.H. (1983) Early changes in thioacetamide-induced liver damage. *Toxicology Letters* **19**, 299-307.

Trivedi, P. and Mowat, A.P. (1983) Carbon tetrachloride-induced hepatic fibroses and cirrhosis in the developing rat: an experimental model of cirrhosis in childhood. *Br. J. Exp. Pathol.* **64**, 25-33.

Turner, F.P. and Brum, V.C. (1964) The urinary excretion of free taurine in acute and chronic disease, following surgical trauma and in patients with acute alcoholism. *J. Surgery Res.* **4**, 423-431.

Vore, M. (1991) Mechanisms of cholestasis. In: *Hepatotoxicology*, 525-568. Edited by Meeks, R.G., Harrison, S.D., and Bull, R.J., Boston, London, Boca Raton/CRC Press.

Waller, R.L., Glende Jr., E.A., and Rechnagel, R.O. (1983) Carbon tetrachloride and bromotrichloromethane toxicity: Dual role of co-valent binding of metabolic cleavage products and lipid peroxidation in depression of microsomal calcium sequestration. *Biochem. Pharm.* **32**, 1613-1617.

Wang, Q., Giri, S.N., Hyde, D.M., and Li, C. (1991) Amelioration of bleomycin-induced pulmonary fibrosis in hamsters by combined treatment with taurine and niacin. *Biochem. Pharm.* **42**, 1115-1122.

Waterfield, C.J., Turton, J.A., Scales, M.D.C., and Timbrell, J.A. (1991) Taurine a possible urinary marker of liver damage: A study of taurine excretion in carbon tetrachloride-treated rats. *Arch. Toxicol.* **65**, 548-555.

Waterfield, C.J., Turton, J.A., Scales, M.D.C., and Timbrell, J.A. (1992) Urinary taurine and creatine as markers of liver dysfunction. *Hum. Exp. Toxicol.* **In Press**, (Abstract)

Weinstein, C.L. and Griffith, O.W. (1987) Multiple forms of rat liver cysteine sulfinic acid decarboxylase. *J. Biol. Chem.* **262**, 7254-7263.

Weinstein, C.L., Haschemeyer, R.H., and Griffith, O.W. (1988) *In vivo* studies of cysteine metabolism: Use of D-cysteine sulfinic acid, a novel cysteine sulfinic acid decarboxylase inhibitor to probe taurine and pyruvate synthesis. *J. Biol. Chem.* **263**, 16568-16579.

- Wheeler, H.O. and Ramos, O. (1960) Determinations of the flow and composition of bile in the unanesthetized dog during constant infusions of sodium taurocholate. *J. Clin. Invest.* **39**, 161-170.
- Wight, D.G.D. (1982) Cirrhosis. In: *Atlas of Liver Pathology. Vol 4*, 85-93. MTP Press.
- Wills, E.D. (1985) The role of dietary components in oxidative stress in tissues. In: *Oxidative stress*, 197-218. London, Academic Press Inc..
- Worden, J.A. and Stipanuk, M.H. (1985) A comparison by species, age and sex of cysteine sulfinate decarboxylase activity and taurine concentration in liver and brain of animals. *Comp. Biochem. Physiol.* **82B**, 233-239.
- Wright, C.E., Tallan, H.H., and Lin, Y.Y. (1986) Taurine: Biological update. *Ann. Rev. Biochem.* **55**, 427-453.
- Yamaguchi, K., Hosokawa, Y., Niizeki, S., Tojo, H., and Sato, I. (1985) Nutritional significance of cysteine dioxygenase on the biological evaluation of dietary protein in growing rats. In: *Taurine: Biological Actions and Clinical Perspectives*, 23-32. Edited by Oja, S.S., Ahtee, L., Kontro, P., and Paasonen, M.K., Ny., Alan R. Liss..
- Yamaguchi, K., Sakakibara, S., Asamizu, J., and Ueda, I. (1973) Induction and activation of cysteine oxidase of rat liver. II. The measurement of cysteine metabolism *in vivo* and the activation of *in vivo* activity of cysteine oxidase. *Biochim. Biophys. Acta* **297**, 48-59.
- Yamaguchi, K., Sakakibara, S., Koga, K., and Ueda, I. (1971) Induction and activation of cysteine oxidase of rat liver. I. The effects of cysteine, hydrocortisone and nicotinamide injection on hepatic cysteine oxidase and tyrosine trans-aminase activities of intact and adrenalectomized rats. *Biochim. Biophys. Acta* **237**, 502-512.
- Yamaguchi, K., Shigehisa, S., Sakakibara, S., and Hosokawa, Y. (1975) Cysteine metabolism *in vivo* of vitamin B6-deficient rats. *Biochim. Biophys. Acta* **381**, 1-8.
- Yosef, I.M., Lewittes, M., Tuchweber, B., Roy, C.C., and Weber, A. (1984) Lithocholic acid-cholesterol interactions in rat liver plasma membrane fractions. *Biochim. Biophys. Acta* **796**, 345-354.
- Yoshida, M. and Hara, I. (1985a) Variation of cysteine level by chlorobenzene-induced perturbation of glutathione metabolism in rat liver. *J. Nutr. Sci. Vitaminol.* **31**, 69-76.
- Yoshida, M. and Hara, I. (1985b) Composition of urinary metabolites and variation of urinary taurine levels in rats injected with chlorobenzene. *Ind. Health* **23**, 239-243.
- Yoshida, S., Akagi, R., and Ubuka, T. (1989) Sulfate and taurine excretion in rats after L-cysteine administration. *Acta Med. Okayama* **43**, 281-288.

Yoshida, T., Oguro, O., Numazawa, S., and Kuroiwa, Y. (1988) Effect of diethyl maleate on hepatic ornithine decarboxylase. *Toxicol. Appl. Pharmacol.* **92**, 194-202.

Younes, M., Sharma, S.C., and Siegers, C.P. (1986) Glutathione depletion by phorone, organ specificity and effect on hepatic microsomal mixed function oxidase system. *Drug Chem. Tox.* **9**, 67-73.

Younes, M. and Siegers, C.P. (1981) Mechanistic aspects of enhanced lipid peroxidation following glutathione depletion *in vivo*. *Chem. Biol. Interactions* **34**, 257-266.

Zelikovic, I. and Chesney, R.W. (1989) Taurine. In: *New Protective Roles for Selected Nutrients*, 253-294. NY., Alan R.Liss.Inc..

Zimmerman, H.J. (1978) *Hepatotoxicity: The Adverse Effects of Drugs and Other Chemicals on the Liver*, NY., Appleton-Century-Crofts.

APPENDICES

APPENDIX I

PREPARATION OF ION EXCHANGE COLUMNS

All water used was UHQ water

RESINS

Two resins were used for the extraction of taurine; they were washed with 3 volumes of UHQ water, to remove fines.

(a) Dowex-1-X4 (anion exchange, in Cl^- form) 100-200 mesh - washed with 250 ml 1 M HCl then water until pH was above 4.5.

(b) Dowex-50W-X8 (cation exchange, in H^+ form) 100-200 mesh - washed with HCl (500 ml, 4 M, in three washings) then 1 M HCl (250 ml).

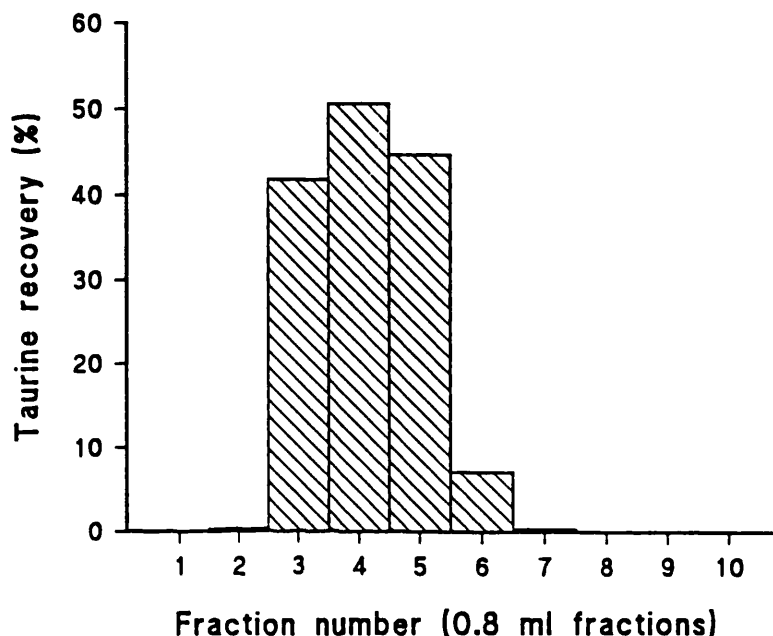
These volumes are for 100 g resin. (Anzano *et al.* 1978 and Sorbo, 1961)

COLUMNS

Stacked columns were prepared by filling individual glass pipettes with 2 ml apparent volume of the resin. An anion exchange column was used directly above a cation exchange column for the extraction of urine samples. They were washed with UHQ water (15 ml) just prior to use. After use they were regenerated by passing NaOH (Aristar, 10 ml, 1 M) down the cation exchange column alone then HCl (12 ml 1 M) down both columns. They were stored in this form (Tiselius and Henman, 1978).

Dual-bed columns for use with more dilute samples (isolated hepatocytes and tissue extracts) were prepared by layering 0.5 ml apparent volume anionic exchange resin directly onto 1.5 ml cation exchange resin. These columns were washed with water (15 ml) before use and regenerated with HCl (12 ml, 1 M) (Garvin, 1960).

The following histogram shows the elution profile of taurine collected in 0.8 ml fractions from stacked ion exchange columns. As a result of this and similar analyses the first 1 ml eluted after applying a sample to the columns was discarded when taurine was extracted from urine samples, and the following 4 ml were collected.



APPENDIX II

HPLC MEASUREMENT OF TAURINE

Mobile phase buffer

The isocratic elution of the derivatized taurine sample required two buffer solutions-

- (i) 12.0 g NaH_2PO_4 (anhyd) in 1950 ml UHQ water (0.05 M) titrated to pH 5.3 with NaOH (5 M "Aristar" NaOH) and made up to 2 L (solution A).
- (ii) 12.0 g NaH_2PO_4 (anhyd) in 500 ml UHQ water (0.05 M) made up to 2.0 L with HPLC grade methanol (solution B). Solution A (430 ml) + solution B (570 ml) were mixed, filtered and degassed using a Millipore vacuum filter (0.22 μm filter). This was the working phase buffer solution (Larsen *et al.* 1980).

Derivatizing solution

40 mg OPA (dissolved in absolute ethanol, 0.8 ml)

40 μ l mercaptoethanol

10 ml borate buffer (0.5 M - 3.1 g boric acid in 90 ml UHQ water

adjusted to pH 10.3 with 5 M Aristar NaOH, made up to 100 ml - prepared weekly).

(Porter *et al.* 1988)

The derivatization solution was kept in a dark bottle at room temperature and used in this form for taurine estimation for urine and tissue samples or diluted 50:50 with UHQ water for taurine estimation from isolated hepatocyte suspensions and media, on the day of preparation.

Taurine in the samples was derivatized by mixing the derivatizing reagent with the ion exchange eluate 50:50 for 1.5 min before the injection of a sample onto the column.

The volume of injection varied with the concentration of taurine in the sample, see below:-

EXTRACT	SAMPLE VOL.(μ l)	TOTAL VOL.INJECTED (μ l)
RAT URINE	5	10
TISSUE	10	20
HUMAN URINE	25	50
ISOLATED HEPATOCYTES	50	100

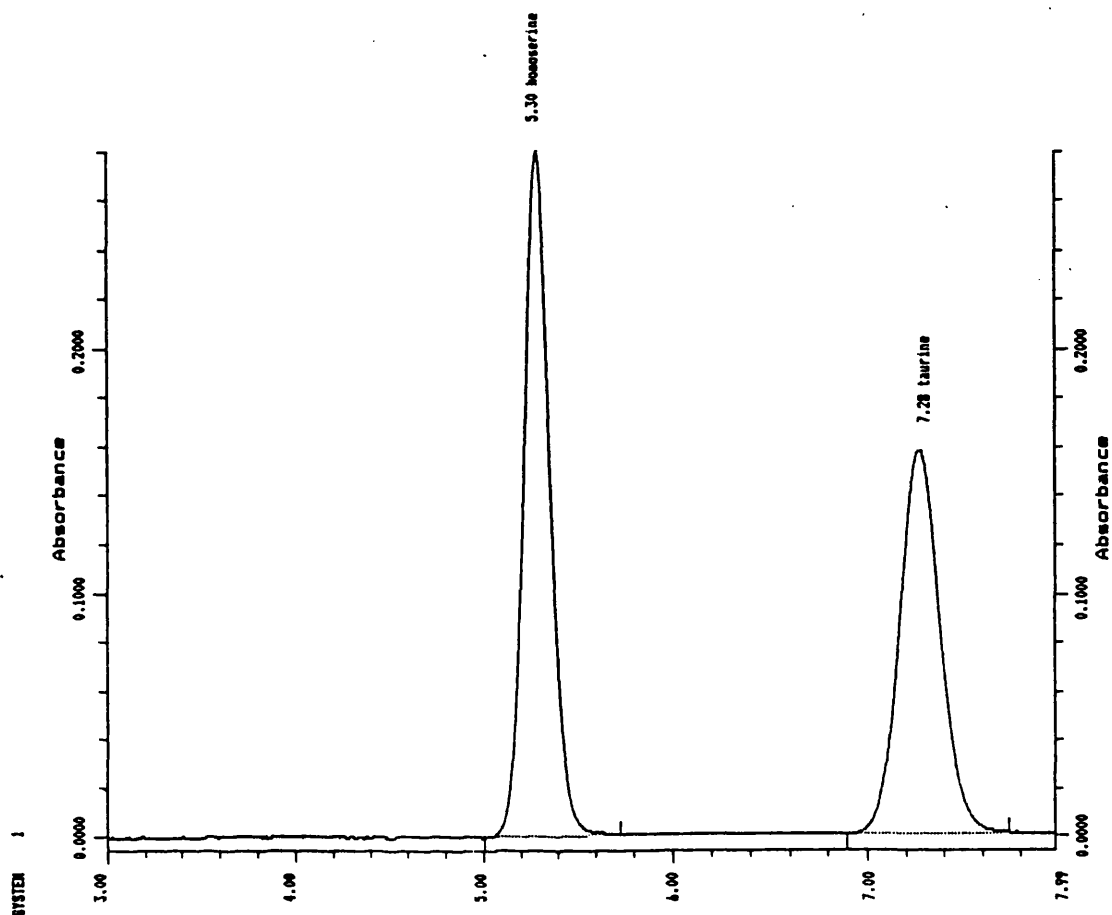
The concentration of taurine in the sample was determined by comparison of the integrated peak area with the integrated peak area of the internal standard (homoserine).

The representative chromatographic profile of taurine extracted from rat urine on stacked Dowex resins and liver taurine extracted on dual bed Dowex resin columns are shown below. The retention time for taurine was usually between 4.5 min and 7.5 min depending on the age of the chromatographic column.

Representative chromatogram of extracted rat urine

NAME CHAN LEV REP TYPE DIRECTORY
 COLLECTION DATA KINSL A 1 0-19 C:\GOLD\SYSTEM\DATA\CAT\HY\LIVER\A
 METHOD RAT-URIN C:\GOLD\SYSTEM\DATA\CAT\HY\A
 SYSTEM 1

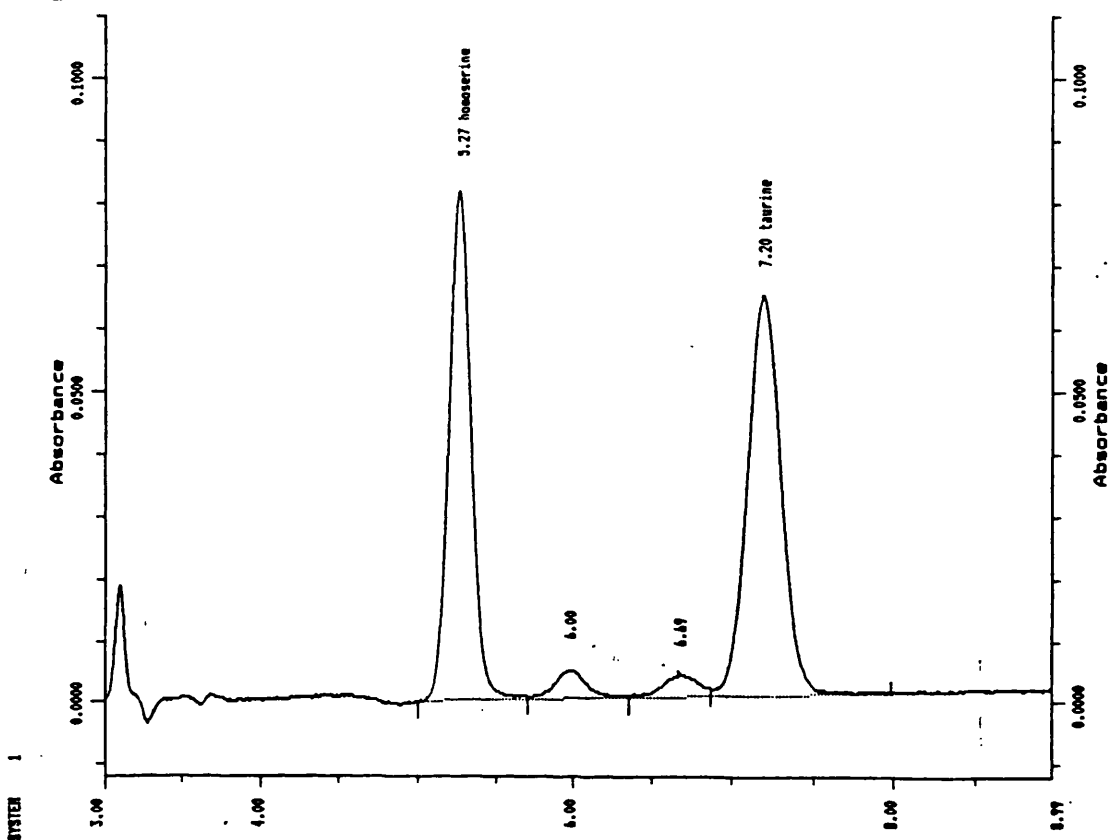
TIME DATE
 INJECTION 15:02:34 18 DEC 1991
 REPORT 15:10:44 18 DEC 1991



Representative chromatogram of extracted liver taurine.

NAME CHAN LEV REP TYPE DIRECTORY
 COLLECTION DATA LHV-111A3 A 1 0-19 C:\GOLD\SYSTEM\DATA\CAT\HY\LIVER\A
 METHOD LIVER-TA C:\GOLD\SYSTEM\DATA\CAT\HY\A
 SYSTEM 1

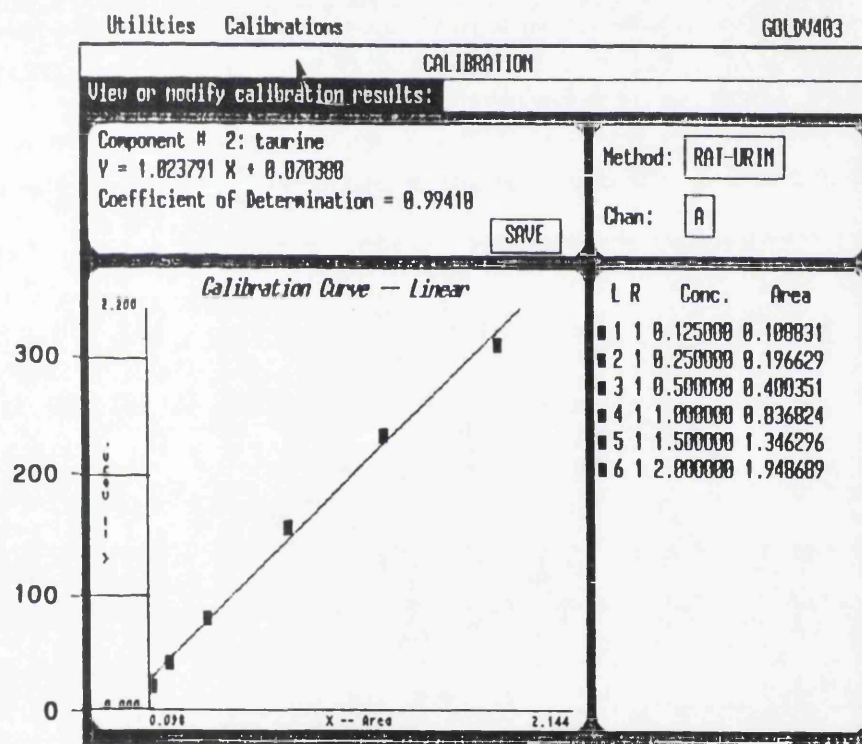
TIME DATE
 INJECTION 2012
 REPORT 2012



The conditions adopted for the sample preparation, derivatisation and chromatography allowed for the rapid determination of taurine with a sensitivity of 50 pmol *on the column* in the initial experiments using a Perkin-Elmer Tridet and 5 pmol *on the column* in later experiments using a Beckman fluorometric detector. The calibration graph was linear in the concentration range of 0 - 1.6 $\mu\text{mol.ml}^{-1}$ with a correlation coefficient of 0.994 - 0.999, (see below).

The following three calibration graphs are for taurine amounts on the column over three different ranges. Numbers in bold are nmol or pmol of taurine *on the column*.

nmol taurine
on the column



Utilities Calibrations

GOLDV403

CALIBRATION

View or modify calibration results:

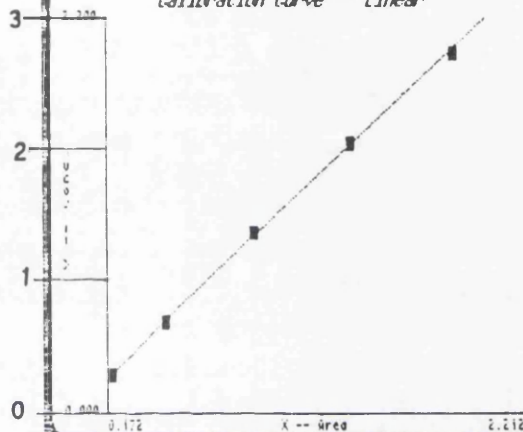
Component # 2: taurine
 $Y = 0.992684 X + 0.020142$
 Coefficient of Determination = 0.99958

Method: LIVER-TA

Chan: A

SAVE

Calibration Curve — Linear



nmol of taurine
on the column

Utilities Calibrations

GOLDV403

CALIBRATION

View or modify calibration results:

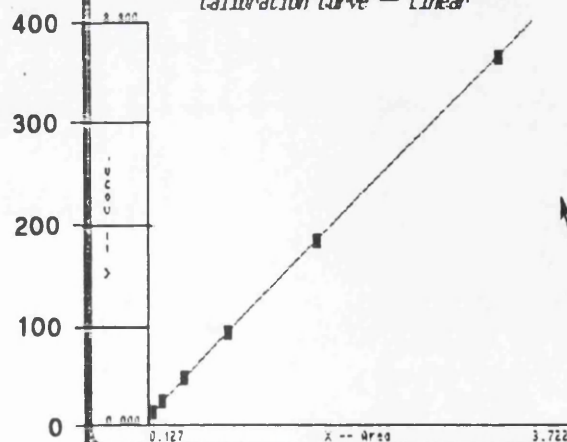
Component # 2: taurine
 $Y = 2.388675 X - 0.00683$
 Coefficient of Determination = 0.99998

Method: HUMAN-UR

Chan: A

SAVE

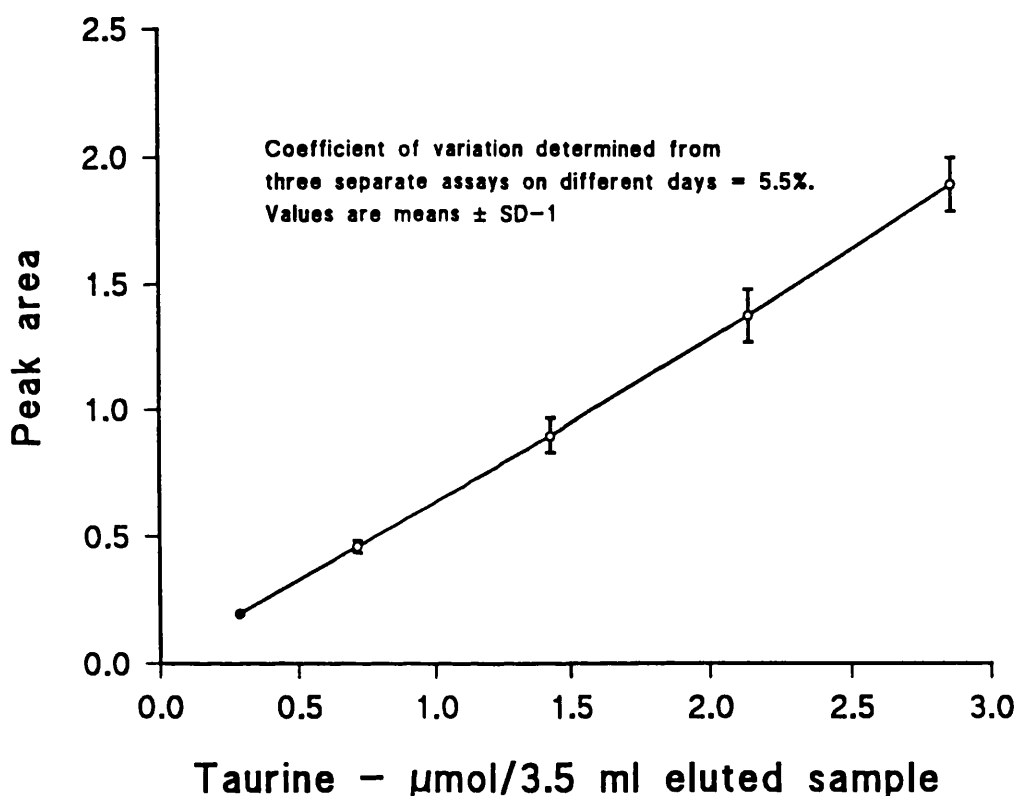
Calibration Curve — Linear



pmol taurine on
the column

The coefficients of variation, determined from 10 duplicates of the same sample were 2% and the inter-batch variation measured from three series was 5.5%, see graph below. The inter-batch variation was found to vary with the life of the column as the retention time was reduced and peaks became less well defined.

Graph to show the variation between calibration graphs made one week apart.



Recovery experiments

Recovery experiments ($N = 8$) were carried out by adding 1 ml of a standard amount of taurine to UHQ water (3 ml + 1 ml homoserine internal standard) and chromatographing the samples. The same standards were added to rat urine, extracted on stacked Dowex resins, eluted with 4 ml UHQ water and had 1 ml homoserine added as internal standard. These were then chromatographed. After subtracting the taurine present in the rat urine at the start, the % recovery of the standards was calculated.

Recovery of taurine from extracted rat urine

Taurine concentration mM	Mean Tau:Hse standards (N=2)	Mean Tau:Hse standards extracted from rat urine (N=2)	% recovery
0.4	2.50	2.47	98.9
0.35	2.07	1.94	94.1
0.3	1.79	1.76	98.3
0.25	1.42	1.44	101.3
0.2	0.83	0.83	100
0.15	0.59	0.61	103.5
0.1	0.39	0.38	96.6
0.05	0.17	0.17	100

Mean % recovery = $99.59 \pm \text{SD } 2.13$

The recovery of liver taurine was carried out in a similar way using the addition of standards to a 1 + 4 liver homogenate instead of urine. The mean % recovery was $94.73 \pm \text{SD } 2.43$.

APPENDIX III

DETERMINATION OF γ -GLUTAMYL TRANSPEPTIDASE

Reagents

1. L- γ -glutamyl-p-nitro anilide (79.5 mg) + glycylglycine (1.645 g) in Tris buffer (125 ml).
2. Acetic acid (10%).
3. Sodium nitrite (0.1%, fresh)
4. Ammonium sulphamate (1%)
5. Naphthylethylenediamine (NED) (0.524%)
6. p-Nitroaniline (126 μM)

Procedure

Aliquots (2.0 ml) of diluted urine samples (24 h sample ----> 25 ml) were dialysed against water (500 ml x 3) for three hours. Samples were then diluted

(1+10) and 10 μ l mixed with glutamyl-p-nitroanilide (0.29 ml) and incubated for exactly 20 min at 37°C the reaction was stopped with acetic acid (1.0 ml). Then the following steps were carried out-

1. sodium nitrite (0.5 ml, mixed, stood 3 min)
2. ammonium sulphamate (0.5 ml, mixed, stood 3 min)
3. naphthylethylene diamine (0.5 ml, mixed, absorbance measured at 550 nm).

A standard curve was prepared using p-nitroaniline (0-126 nmol.ml⁻¹)

APPENDIX IV

MEASUREMENT OF TOTAL NON-PROTEIN SULPHYDRYLS (TNPSH)

Reagents

1. Phosphate buffer (pH, 7.4) - 43.6 g KH₂PO₄ + 900 ml water, pH with NaOH pH 7.4 made up to 1 L.
2. Phosphate buffer (pH 8.0) - 13.6g KH₂PO₄ + 900 ml water, pH with NaOH pH 8.0 made up to 1 L.
3. Sulphosalicylic acid, 0.2 M.
4. DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)], 39.6 mg + 10 ml pH 7.4 buffer.

Standards GSH 0.1 - 1.0 mM (0.2 M sulphosalicylic acid/buffer pH 7.4).

Procedure

1. DTNB (0.05 ml) and phosphate buffer (pH 8.0, 4.5 ml) were added to extracted liver supernatant (0.5) and vortexed.
2. The absorbance was read at 412 nm, after 10 min.
3. The concentration of GSH was calculated from the standard curve and expressed as μ mol.g⁻¹ wet weight of liver and total μ mol/liver.

APPENDIX V

TRIGLYCERIDE DETERMINATION

Reagents

1. Zeolite - activated (dehydrated) 4 h, (or overnight) 125°C.
2. Alcoholic KOH (0.4%) - 2 g KOH dissolved in 5 ml water, made up to 100 ml with redistilled ethanol. Then 10 ml of this stock diluted to 50 ml with 95% ethanol on the day of use.
3. H_2SO_4 (0.1 M)
4. Sodium arsenite (1.0 M).
5. Sodium metaperiodate (0.05 M).
6. Chromotropic acid (0.2%, made fresh every 2-3 weeks) 2 g acid (4,5, dihydroxy-2,7, Naphthalene disulphuric acid) or 2.24 g of Na salt in 200 ml water (4°C). Separately 600 ml conc. H_2SO_4 , added to 300 ml water (4°C). When cooled the acid solutions were mixed and stored in the dark.
7. Phosphate buffer - K_2HPO_4 (0.1 M) and KH_2PO_4 (0.1 M) mixed to pH 7.0
8. Triglyceride standards - 0.5 g of commercial corn oil dissolved in 100 ml chloroform and sealed. This stock was diluted to give a working standard of 0.1 mg.ml^{-1} chloroform at the time of assay.

Extraction of triglycerides (T.G.)

1. An aliquot of liver homogenate (1.0 ml) added to activated Zeolite (4 g) moistened with chloroform (8 ml). Then 12 ml chloroform was added and triglyceride extraction was completed by shaking at intervals for 20 min.
2. The chloroform extract was filtered through coarse, fat free filter paper and the extract sealed tightly.

Procedure

1. Diluted T.G. standards (0.0 ml - 1.0 ml) and T.G. extracts (0.2 - 1.0 ml depending on expected levels of T.G.'s) were pipetted into glass test tubes and the chloroform was allowed to evaporate at 35°C overnight. Five tubes were prepared for each sample (3 saponified, 2 unsaponified).

2. Ethanol (95%, 0.5 ml) was pipetted into 2 tubes (unsaponified) and alcoholic KOH (0.5 ml) into the remaining three tubes (saponified). The tubes were incubated (60°C, 20 min).
3. H_2SO_4 (0.5 ml, 0.1 M) was added and the alcohol evaporated (100°C, 20 min) and the tubes were cooled.
4. Sodium metaperiodate (0.05 M, 0.1 ml) was added and after 10 min sodium arsenite (1.0 M, 0.2 ml), followed by chromotropic acid reagent (0.2%, 5 ml).
5. The samples were incubated (100°C, 30 min in the shade) and the absorbance read at 570 nM. The unsaponified values were subtracted from the saponified samples and the T.G. concentration determined using the standard curve.

APPENDIX VI

SOLUTIONS FOR PREPARATION OF ISOLATED HEPATOCYTES

A. HANK SOLUTIONS -

Stock (x 10 conc) -

The following were made up in 1 L UHQ water

NaCl	80.0 g
KCl	4.0 g
$\text{MgSO}_4 \cdot \text{H}_2\text{O}$	2.0 g
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	0.6 g
KH_2PO_4	0.6 g

Working solutions

Hank x 10 stock	50 ml
water	450 ml
NaHCO_3	1.05 g
HEPES	1.50 g

The solution was gassed with 5% CO_2 /95% O_2 for 2-3 min and divided for -

Hank I (initial perfusate - Ca^{2+} free)

Hank 300 ml
 EGTA 68.4 mg
 Albumin 2.0 g

Hank II (collagenase containing)

Hank 200 ml
 CaCl_2 5.88% 2 ml

(Collagenase 50 mg/100 ml HankII)

Both solutions were adjusted to pH 7.4 with 1 M NaOH.

B. KREBS-HENSELEIT BUFFER -**stock (x 2 conc.)**

a. 16.09% NaCl	80.45 g → 500 ml
b. 1.1% KCl	5.50 g → 500 ml
c. 0.22 M KH_2PO_4	7.49 g → 250 ml
d. 2.74% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	6.85 g → 250 ml
e. 0.12 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	8.82 g → 500 ml
f. 0.97% NaHCO_3	4.85 g → 500 ml

Solutions a - f were mixed

a. 100 ml b. 75 ml c. 12.5 ml d. 25 ml e. 50 ml + 392.5 ml H_2O

These were then gassed with 5% CO_2 /95% O_2 for 10 min before adding - f. 500 ml.

Working solutions -

buffer for incubation of cells (K + H) -

Krebs-Henseleit x 2 stock	150 ml
Water	150 ml
HEPES	1.5 g

This was gassed for 5 min with 5% CO_2 /95% O_2 and 100 ml removed -

buffer for dispersion of cells (K + H + Alb) -

K + H	100 ml
Albumin (bovine)	1.0 g

pH was adjusted to 7.4 with 1 M NaOH

APPENDIX VII

MEASUREMENT OF LACTATE DEHYDROGENASE

Solutions

1. Phosphate buffer, 0.05 M (pH 7.4, 6.8 g KH_2PO_4 L^{-1} , room temperature)
2. Pyruvate, 6.2×10^{-3} M (7.5 mg.10 ml^{-1} buffer, 4°C)
3. NADH. $2\text{H}_2\text{O}$, *ca.* 8×10^{-3} M (65 mg.10 ml^{-1} buffer, 4°C)

Procedure

Cells were separated from the medium by centrifugation (1,000 rpm - 2 min or 11,000 x g - 2 sec) and the medium stored (4°C for up to 4 days) and assayed for LDH activity. The activity in the medium was expressed as a % of the total LDH activity in a sample of cells taken from the same incubation. Cells were stored (4°C) overnight and the LDH release completed by disrupting the cells using a "Soniprep" cell disrupter (4°C, 30 sec, 50% power). Cell debris was pelleted (11,000 x g, 5 min).

1. Phosphate buffer (2710 μl , room temperature) was placed in a 4 ml cuvette.
2. Pyruvate (140 μl) and (100 μl) NADH solution were added and mixed.
3. The reaction was started by the addition of 50 μl of sample (or a suitable volume depending on the LDH activity present). If a different volume was used the difference was made up with buffer.
4. The rate of NADH disappearance was measured at E_{340} (Shimadzu spectrophotometer) and compared with the rate of disappearance in a sample containing total LDH and expressed as a % of the total.

APPENDIX VIII

MEASUREMENT OF ATP IN ISOLATED HEPATOCYTES

Reagents

1. Luciferase-Firefly lantern extract (Sigma FLE-250) was resuspended in water (5 ml) and centrifuged (5 min, 1000 rpm).
2. Buffer - The following were mixed 1:1:1 just before use:-
 - a. 80 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 19.72 g.L⁻¹
 - b. 10 mM KH_2PO_4 1.361 g.L⁻¹
 - c. 100 mM $\text{Na}_2\text{AsO}_4 \cdot 7\text{H}_2\text{O}$ 31.20 g.L⁻¹
 and adjusted to pH 7.4.
3. A range of ATP standards are made 0-40 μM in 10% TCA.

Procedure

Hepatocyte suspensions (0.5 ml, 1×10^6 cells) were pipetted into TCA (20%, 0.5 ml, 4°C) and immediately frozen (-80°C) and analyzed within a week. Samples were centrifuged (11,000 x g, 5 min, 4°C) and a sample of the supernatant or standard (10 μl) added to buffer (2.0 ml). Firefly lantern extract (100 μl) was added to start the reaction. The luciferase-linked bioluminescence produced was measured with a computer-controlled Thorn EMI photon detection system utilising an air cooled photomultiplier (-25°C) for 6 seconds 15 seconds after the start of the reaction.

The sample was compared directly with the standards since they are prepared in the same volumes and values expressed as nmol.ml⁻¹ ATP (1×10^6 cells).

APPENDIX IX

Table to show dosing solutions - all doses given in final volume of 2 ml.kg⁻¹ or as stated

Compound	Dose(s) administered (ml or mg.kg ⁻¹)	Method
Carbon tetrachloride	0.5, 1.0, 1.5 and 2.0 ml	0.5, 1.0, 1.5 or 2.0 ml CCl ₄ in 1 ml corn oil, gave 1.5, 2, 2.5 or 3 ml of dose kg ⁻¹
Thioacetamide	150 mg	150 mg in 0.9% sterile saline
ANIT	150 mg	150 mg in 2 ml corn oil - sonicated in water bath 37°
Hydrazine hydrate	62 µl - to give 40 mg free base	124 µl in 3.5 ml degassed UHQ water adjusted to pH 7.2 with 1 M HCl - made up to 4 ml
Allyl alcohol	0.05, 0.075 and 0.1 ml	volume dissolved in 2 ml 0.9% sterile saline
Ethionine	200, 400 and 800 mg	2 g dissolved in 18 ml 0.9% saline, 6 M HCl added drop wise to dissolve, made up to 20 ml, dose given as 2, 4 or 8 ml.kg ⁻¹ (very difficult to dissolve)
Galactosamine	400 and 500 mg	400 or 500 mg in 2 ml 0.9% sterile saline (ip)
Bromobenzene	1 ml	1ml dissolved in 1 ml corn oil
Lithocholic acid	26 mg	52 mg in 4 ml 0.9% saline + 1 drop BRIJ 35 then sonicated

APPENDIX X

Table to show liver taurine and TNPSH levels 48 h after dosing with various hepatotoxic compounds (except ~ = 72 h after dosing)

Compound	Dose ml or mg kg ⁻¹	Liver taurine		Liver TNPSH
		μmol g ⁻¹	μmol/liver	μmol g ⁻¹
Carbon tetrachloride	1 ml ^a	5.53 ± 0.82	61.5 ± 11.3	7.85 ± 0.67
Thioacetamide	150 mg ^b	4.08 ± 0.34	40.5 ± 6.9	ND
ANIT	150 mg ^b	9.24 ± 0.76**	106.7 ± 13.1*	ND
Hydrazine	40 mg ^b	4.18 ± 1.47	43.7 ± 14.7	ND
Allyl alcohol	#0.1 ml ^b	2.13	26.29	ND
	0.075 ml ^c	6.69 ± 0.52	83.2 ± 7.6	8.81 ± 0.46
Ethionine~	800 mg ^d	11.47 ± 0.48	144.5 ± 4.11	8.11 ± 0.40
Galactosamine	500 mg ^c	3.06 ± 0.25**	24.8 ± 1.36	7.88 ± 0.73
	#400 mg ^f	3.96	35.75	8.39
Bromobenzene	1 ml ^e	3.38 ± 0.47	40.6 ± 9.0	4.25 ± 0.40**
Lithocholate	26 mg ^g	8.40 ± 0.35	110.9 ± 12.2	ND

Controls

a	-	5.98 ± 0.62	63.3 ± 6.3	7.16 ± 0.26
b	-	4.01 ± 0.63	41.98 ± 5.9	ND
c	-	7.40 ± 0.54	85.8 ± 6.35	8.07 ± 0.20
d	-	11.18 ± 0.88	153 ± 14.5	7.40 ± 0.12
e	-	5.12 ± 0.66	66.6 ± 14.9	7.41 ± 0.47
f	-	6.16 ± 0.38	73.7 ± 3.83	6.92 ± 0.55
g	-	8.95 ± 0.56	122.1 ± 8.6	ND

Doses^{a-g} compared with respective controls a-g.

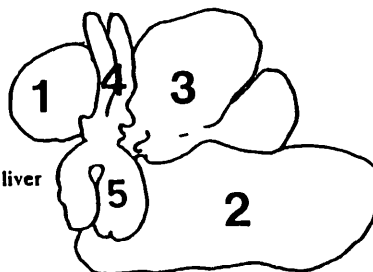
Values mean ± SEM; N = 3-4 except # where N = 2; * p < 0.05, ** p < 0.01;

ND = no data

APPENDIX XI

Doses of non-hepatotoxic compounds used in Section 3.6 were prepared as follows:-

- Allylamine - 150 mg.kg⁻¹ as Cl⁻; 0.908 g in 25 ml flask, 4°C, 8 ml UHQ water added, pH adjusted to 5.75 with 2 M HCl, volume made up to 20 ml, given as 2 ml.kg⁻¹, p.o.
- Cadmium chloride -1.5 mg (13.4 µmol) kg⁻¹, Cd²⁺; 30.5 mg (hydrate) in 20 ml sterile saline, given as 2 ml.kg⁻¹, i.p.
- Mercuric chloride -1.4 mg.kg⁻¹, Hg²⁺; 19 mg in 20 ml sterile saline, given as 2 ml.kg⁻¹ i.p.



APPENDIX XII

Nomenclature used for lobes of rat liver. The liver is viewed from a ventral-posterior position.

Taurine (3%) was administered to rats in their drinking water for 4 days before they were killed and liver samples were taken for analysis of taurine and GSH concentrations as described in Chapter 2. Four animals were used for each treatment and liver lobes were taken and treated separately.

Liver lobe	Concentration of taurine (µmol.g ⁻¹ wet weight)		Concentration of TNPSH (µmol.g ⁻¹ wet weight)	
	-	+ taurine	-	+ taurine
1	1.89 ± 0.16	15.80 ± 1.90***	8.13 ± 0.66	6.95 ± 0.16
2	2.69 ± 0.42	15.29 ± 2.18***	7.37 ± 0.42	6.75 ± 0.08
3	2.18 ± 2.47	14.53 ± 1.89***	7.53 ± 0.14*	6.78 ± 0.24
4	2.47 ± 0.34	15.57 ± 1.10***	7.12 ± 0.36	6.89 ± 0.41
5	4.06 ± 0.92	17.62 ± 2.72***	6.74 ± 0.26	6.65 ± 0.20

Nomenclature used for rat liver lobes: 1 and 4 = right; 2 = left; 3 = median; 5 = posterior or caudate lobes.

Values are means ± SEM, N = 4. *** p < 0.001, all taurine treated significantly different from untreated liver lobes. * p < 0.05 lobe 3 significantly different from lobe 5.

APPENDIX XIII Serum and urinary clinical chemistry of control male rats used in the studies discussed in Chapter 3
Different treatment groups are compared to the relevant control groups in the table below (a - h) for statistical purposes.

Cont. group	Liver % body wt	Kidney % body wt	ALT iu.L ⁻¹	AST iu.L ⁻¹	ALP iu.L ⁻¹	ALB g.L ⁻¹	TOT PROT g.L ⁻¹	TOT BIL mmol. L ⁻¹	CHOL mmol. L ⁻¹	TRIG mmol. L ⁻¹	Serum CREAT μ mol.L ⁻¹	Serum Urea mmol.L ⁻¹	Urine PROT mg.kg ⁻¹ .24 h ⁻¹	Urine γ -GT iu.kg ⁻¹ .24 h ⁻¹
a	3.78 (0.07)	0.713 (0.022)	35 (20)	83 (21)	370 (21)	33.2 (1.7)	56.4 (1.5)	2.4 (0.25)	1.55 (0.18)	1.50 (0.26)	51.0 (3.2)	4.20 (0.20)	56 (3.6)	11 (3.2)
b	3.34 (0.14)	0.767 (0.017)	46 (2.9)	88 (3.8)	397 (42)	32.5 (0.2)	58.1 (0.7)	0.93 (0.46)	1.47 (0.07)	0.73 (0.06)	43.8 (1.1)	4.40 (0.20)	52 (9.2)	5 (2.1)
c	3.47 (0.07)	0.740 (0.020)	39 (2.2)	73 (4.2)	343 (21)	32.9 (0.89)	58.4 (1.5)	1.7 (0.13)	1.58 (0.09)	0.90 (0.11)	44.5 (2.0)	4.71 (0.19)	N.D.	N.D.
d	4.33 (0.13)	0.759 (0.016)	89 (5.9)	98 (12)	551 (32)	33.2 (1.2)	55.8 (1.0)	1.1 (0.20)	1.55 (0.21)	1.27 (0.08)	44.0 (1.6)	5.89 (0.41)	80 (6.5)	4 (2.7)
e	3.97 (0.12)	0.800 (0.020)	47 (7.8)	93 (10)	473 (100)	33.7 (2.8)	56.1 (1.0)	11.2 (0.8)	1.47 (0.07)	1.42 (0.13)	44.0 (1.8)	5.69 (0.41)	65 (2.1)	4 (3.2)
f	4.01 (0.08)	0.780 (0.070)	67 (4.2)	83 (9.8)	506 (81)	34.5 (0.6)	58.8 (1.2)	6.0 (0.9)	1.50 (0.11)	1.37 (0.19)	52.1 (1.9)	5.82 (0.29)	63 (9.0)	7 (3.8)
g	4.00 (0.07)	0.801 (0.010)	65 (3.2)	84 (10)	500 (90)	33.9 (0.6)	58.7 (0.7)	5.0 (0.8)	1.49 (0.10)	1.38 (0.20)	52.0 (1.9)	5.83 (0.30)	64 (7.0)	N.D.
h	4.00 (0.05)	0.772 (0.001)	78 (4.8)	99 (6.4)	481 (34)	32.7 (0.3)	55.2 (0.8)	0.9 (0.21)	1.22 (0.06)	0.95 (0.13)	46.0 (1.9)	5.27 (0.27)	77 (5.1)	N.D.

Values are means \pm (SEM in parentheses); N = 4 - 5; ALT - alanine aminotransferase; AST - aspartate aminotransferase; ALP - alkaline phosphatase; ALB - serum albumin; TOT PROT - total serum protein; TOT BIL - total serum bilirubin; CHOL - serum cholesterol; TRIG - serum triglycerides; Serum CREAT - serum creatinine; Urine PROT - urinary protein; γ -GT - γ -glutamyl transpeptidase.

Hypertaurinuria as a Measure of Hepatic Damage in the Rat after Carbon Tetrachloride Treatment

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Hypertaurinuria has been reported after injury to various tissues¹ and we have previously shown an elevation of urinary taurine using proton NMR, after treatment of rats with various hepatotoxins.² In the present study, we have examined taurine levels in the urine, serum and liver of CCl₄-treated male and female rats and related urinary taurine to serum AST and ALT levels to investigate the usefulness of taurinuria as a marker of liver damage.

Male and female Sprague-Dawley-derived rats (200–250 g) were treated with 2 ml kg⁻¹ CCl₄ in corn oil po. A 24 h urine sample was collected for 48 h before and after dosing. Taurine was measured in the 24 h urine samples and in serum and liver taken at post-mortem (48 h after dosing), using an HPLC method. Pre-column derivatization was carried out with *o*-phthalaldehyde and mercaptoethanol, after an initial purification step using Dowex 50-W and Dowex-1. The fluorescent adduct was measured using a fluorimetric detector, with homoserine as internal standard. Liver and kidney samples were also taken for histological investigation and serum samples were analysed for biochemical parameters, including AST and ALT.

Liver histology from treated animals showed steatosis, hydropic vacuolation and some centrilobular necrosis. All kidney histology was normal. Serum AST levels were elevated above control values 48 h after dosing but ALT levels were only elevated in females. Taurine levels are shown (Table). A significant rise in urinary taurine was seen 24 h after dosing in male and female rats and after 48 h in males. The combined results of the male and female animals showed a significant correlation between urinary taurine and the serum AST levels, ($r = 0.9$, $P < 0.001$). Serum taurine levels were reduced, but not significantly, in both males and females. Hepatic taurine levels were reduced in males and females, the decrease being significant in females ($P < 0.01$). The loss of hepatic taurine could account for approximately 25% of the excess taurine excreted in the urine after treatment with CCl₄.

Table Taurine levels in CCl₄ treated and control rats.

	$\mu\text{mol kg}^{-1} \text{ body wt } 24 \text{ h}^{-1}$ Urine			$\mu\text{mol ml}^{-1}$ Serum	$\mu\text{mol g}^{-1} \text{ wet wt}$ Liver
	0 h pre-dose	24 h post-dose	48 h post-dose	48 h post-dose	48 h post-dose
Male controls	369 ± 35	307 ± 72	368 ± 50	0.33 ± 0.04	8.26 ± 1.07
Male treated		761 ± 125*	700 ± 51**	0.25 ± 0.03	5.08 ± 0.94
Female controls	444 ± 32	485 ± 60#	500 ± 66#	0.28 ± 0.03	9.70 ± 0.79
Female treated		1242 ± 158***	567 ± 66	0.23 ± 0.01	5.10 ± 0.33**

Results: mean ± s.e.m. $n = 5$ (# $n = 4$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

These results suggest that measurement of urinary taurine may be a useful quantitative method for the assessment of hepatic damage, using a non-invasive, continuous sampling technique.

References

- ¹ Chesney RW. Taurine: Its biological role and chemical implications. *Advances in Pediatrics* 1985; 32: 1–42.
- ² Sanins SM, Nicholson JK, Elcombe C & Timbrell JA. Hepatotoxin-induced hypertaurinuria: A proton NMR study. *Archives of Toxicology* (in press).

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Taurine synthesis in isolated rat hepatocytes in suspension exposed to carbon tetrachloride

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Abbreviations used: DMSO, dimethyl sulphoxide; LDH, lactate
dehydrogenase.

We have previously shown that taurine levels are elevated in
the urine of rats after treatment with the hepatotoxin carbon
tetrachloride (CCl_4) [1]. It has been suggested that this is a
result of taurine leakage from dead or damaged cells [2].
However, we found that the amount of taurine excreted into
the urine was greater than that lost from the liver. One hypo-
thesis to account for this observation is that taurine synthesis

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in the liver is increased as a result of the toxic insult. Con-
sequently, isolated rat hepatocytes were used in the following
study to investigate the validity of this hypothesis.

Hepatocytes were isolated from male Sprague-Dawley-
derived rats (250–300 g) using the method of Moldeus *et al.*
[3]. The cells were suspended in Krebs-Henseleit buffer pH
7.4 (20 ml; 2×10^6 cells/ml) and preincubated for 30–45
min. Incubations were carried out in rotating flasks under an
atmosphere of O_2/CO_2 (19:1; v/v) at 37°C . CCl_4 was added
to the suspension (final concentrations: 1 mM and 5 mM) in
dimethylsulphoxide (DMSO) (final concentration 0.08%,
v/v). Control cells and cells exposed to DMSO alone were
also incubated. A 1 ml aliquot of the suspension was
removed from flasks before addition of DMSO or CCl_4 (time
zero), and at 45, 90 and 135 min after the additions. Cells
were separated by centrifugation (11 000 g at 4°C), then
resuspended in sulphosalicylic acid (0.2 M) and the precipi-
tated protein removed by centrifugation. The resultant cellu-
lar extract and incubation buffer were analysed for taurine
after an initial purification and separation using Dowex
anion- and cation-exchange columns. Taurine was deriva-
tized in the purified extracts with *o*-phthalaldehyde and
mercaptoethanol [4]. The fluorescent adduct was separated
and quantified by h.p.l.c. with fluorimetric detection using
homoserine as the internal standard (Waters μ Bondapak C_{18}
column, 30 cm). Total protein was determined using
Coomassie Brilliant Blue G-250 solution, and cellular
taurine was expressed per mg of protein. Lactate dehydroge-
nase (LDH) leakage was determined in the incubation buffer
as a measure of cytotoxicity and cell viability was determined
using Trypan Blue exclusion.

The viability of the hepatocytes was only significantly
reduced by exposure to 5 mM- CCl_4 , but not by the other
treatments after 135 min, whereas LDH leakage, although
raised after 5 mM- CCl_4 , was not significantly different from
the control values. However, the concentration of taurine in
the incubation buffer was significantly elevated by exposure
to 5 mM- CCl_4 after 45 min and continued to increase com-
pared with the control values (Fig. 1). This result suggests
that taurine may be leaking from damaged cell membranes
before significant LDH leakage is observed. Furthermore,
intracellular taurine was also significantly increased above
control values after exposure to 1 mM, but not 5 mM- CCl_4 ,
and there was a significant increase in total taurine (cells plus
medium) after exposure to both 1 mM and 5 mM CCl_4 . It is
suggested that any increase in intracellular taurine which
may have occurred after exposure to 5 mM- CCl_4 may have
been negated by the subsequent loss by leakage into the
buffer, hence contributing to the raised level seen. Taurine
levels were not significantly raised by incubation of cells with
the vehicle, DMSO.

This investigation has demonstrated: (i) a significant
leakage of taurine from hepatocytes *in vitro*; (ii) a significant
increase in taurine in the system *in vitro* (cells and medium)
consistent with an increased synthesis after exposure to CCl_4 ,
and (iii) leakage of taurine before either LDH leakage or
Trypan Blue exclusion are significantly affected.

It is likely that this increased synthesis of taurine after
hepatocyte injury continues to contribute to the taurine

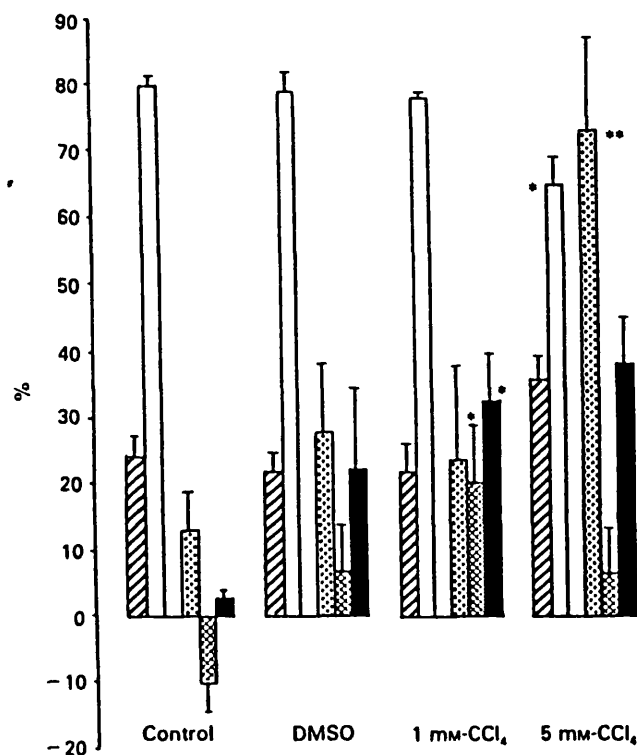


Fig. 1. Lactate dehydrogenase leakage, cell viability and
changes in extracellular, intracellular and total taurine after
exposure of rat hepatocytes to CCl_4 .

Parameters are shown 135 min after the start of the exposure
to CCl_4 . Results are means \pm S.E.M. of three to seven deter-
minations. \square , % of total cellular LDH; \square , % viability of
hepatocytes; \square , % change in taurine in incubation buffer; \blacksquare ,
% change in cellular taurine; \blacksquare , % change in total taurine
(cells and buffer). * $P < 0.05$; ** $P < 0.01$.

leaked from the damaged cells. These results may explain the
increased urinary taurine excretion in rats after CCl_4 -
induced liver damage and why only 25% of the increase in
urinary taurine can be accounted for by taurine lost from the
liver.

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1. Waterfield, C. J., Turton, J. A., Scales, M. D. C. & Timbrell, J. A. (1990) *Human Toxicol.* in the press
2. Cornish, B. H. & Ryan, R. C. (1964) *Toxicol. Appl. Pharmacol.* 6, 96–102
3. Moldeus, P., Hogberg, J. & Orrenius, S. (1978) *Methods Enzymol.* 52, 60–71
4. Larsen, B. R., Grosso, D. S. & Chang, S. Y. (1980) *J. Chromatogr. Sci.* 18, 233–236

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CHANGES IN URINARY AND HEPATIC TAURINE LEVELS IN RATS TREATED WITH
HEPATOTOXINS.

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Hypertaurinuria has been reported after injury to various tissues and we have previously quantified the elevation of taurine in urine after treatment of rats with carbon tetrachloride. In the present study other hepatotoxins were administered to male Sprague Dawley rats (250-280 g). Changes in urinary and hepatic taurine levels were compared to changes in serum AST and ALT and related to liver histology to assess the usefulness of hypertaurinuria as a marker of liver damage. Animals were housed in individual metabolism cages for five days before dosing po. with:- hydrazine (40 mg/kg free base), α -naphthylisothiocyanate (ANIT)(150 mg/kg), thioacetamide (150 mg/kg) or allyl alcohol (0.1 ml/kg) in saline. Taurine was measured in 24 h urine samples collected in the 48 h period before and after dosing using an HPLC method. Serum and liver were taken for biochemical measurements and liver and kidney for histology at post mortem, 48 h after dosing.

Taurine, AST and ALT levels are shown (Table) and expressed as mean \pm s.e.m; n=3 (except \dagger n=4, #n=2). Histology of the liver showed; hydrazine, mild mid zonal steatosis; thioacetamide, centrilobular necrosis and steatosis; ANIT, bile duct epithelial necrosis and cholestasis; allyl alcohol; extensive periportal and mid zonal necrosis.

Treatment	μ moles taurine/kg/ 24 h urine		μ moles taurine/ g wet wt liver	serum enzymes 48 h post dose	
	24 h post dose	48 h post dose	48 h post dose	ALT iu/L	AST
Control \dagger	123 \pm 9	135 \pm 53	4.0 \pm 0.6	46 \pm 3	88 \pm 7
Hydrazine	113 \pm 14	360 \pm 276	4.2 \pm 1.5	24 \pm 3**	86 \pm 7
Thioacetamide	218 \pm 60	439 \pm 7**	4.1 \pm 0.3	442 \pm 72*	1735 \pm 97**
ANIT	39 \pm 7***	25 \pm 6*	9.2 \pm 0.8**	732 \pm 155*	1501 \pm 52**
Allyl alcohol	269 \pm 77	123 \pm 15	#2.1 -	425	991

These results suggest that hypertaurinuria could be a useful quantitative non-invasive method for assessment of hepatic damage when necrosis and/or steatosis are induced, especially if there is continued synthesis of hepatic taurine after leakage from the liver. However, cholestasis appears to result in taurine being retained in the liver.

C.J.W. is grateful for support from Glaxo Group Research Ltd.

Dose Related Changes in Urinary Taurine Levels after Carbon Tetrachloride Administration to Rats

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We have previously reported changes in urinary and liver taurine concentrations in rats after toxic doses of various hepatotoxins.^{1,2} These data suggested that urinary taurine levels may be a useful marker of liver damage. In the following studies, we show a dose related increase in urinary taurine over 96 h following administration of CCl₄ to male rats and compare these changes with liver histology and serum ALT and AST levels.

Male rats (Sprague-Dawley, 265–300 g) were treated with 0.5, 1 or 2 ml kg⁻¹ CCl₄ (po) in corn oil. Taurine concentrations were measured by HPLC in 24 h urine collections taken for 48 h before dosing, every 4 h for the first 24 h, then 24–48 h, 48–72 h and 72–96 h after dosing. Liver and serum were taken at post-mortem for histology and measurement of taurine and serum AST and ALT levels, 96 h after dosing. In separate studies, serum AST and ALT levels were compared with liver histology 1, 3, 6, 12, 18, 24 and 48 h after dosing rats with 2 ml kg⁻¹ CCl₄. The results were correlated with urinary taurine levels found in the previous study.

Results showed a dose related increase in total urinary taurine for 96 h following dosing with CCl₄ (Table).

Table Taurine and serum AST and ALT in CCl₄-treated and control rats

Dose CCl ₄ (ml kg ⁻¹)		Vehicle	0.5	1.0	2.0#
Urinary taurine (µmol kg ⁻¹ 24 h ⁻¹)	-24-0 h	127 ± 32	109 ± 17	189 ± 43	144 ± 27
	0-24 h	112 ± 21	105 ± 12	272 ± 66	587 ± 79**
	24-48 h	71 ± 12	110 ± 26	338 ± 40**	640 ± 140*
	48-72 h	62 ± 25	73 ± 11	225 ± 30**	383 ± 80*
	72-96 h	75 ± 24	73 ± 4	261 ± 110	298 ± 38*
Serum enzymes (iu l ⁻¹)	ALT (12 h)	52 ± 9	ND	ND	213 ± 53*
	AST (12 h)	100 ± 8	ND	ND	653 ± 108*
	ALT (96 h)	48 ± 3	41 ± 1	42 ± 3	92 ± 43
	AST (96 h)	53 ± 8	76 ± 3	70 ± 6	131 ± 40

Results: mean ± s.e.m. n = 4-6 (#n = 3), * P < 0.05, ** P < 0.01, ND no data

Data for the first 24 h following treatment with 1 and 2 ml kg⁻¹ CCl₄ showed that urinary levels of taurine were maximal 12–16 h after dosing at a time when perivenous hepatocytes showed 'ballooning' prior to the development of necrosis at 24 h. Serum AST and ALT levels were significantly elevated 12 h to 48 h after treatment with 2 ml kg⁻¹ CCl₄ but not with any dose after 96 h, whereas urinary taurine remained significantly elevated 72–96 h after giving 2 ml kg⁻¹ CCl₄. Light microscopy of liver sections 96 h after treatment showed perivenous stentosis (all doses) and macrophage infiltration (2 ml kg⁻¹). There was also a significant reduction in liver taurine after 2 ml kg⁻¹ CCl₄. As urinary taurine remains elevated 96 h after treatment, its measurement may be a useful non-invasive, quantitative method for assessing hepatic damage.

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References

- Waterfield CJ, Turton JA, Scales MDC & Timbrell JA. Hypertaurinuria as a measure of hepatic damage in the rat after carbon tetrachloride treatment. *Human and Experimental Toxicology* 1990; 9: 338-339.
- Waterfield CJ, Turton JA, Scales MDC & Timbrell JA. Changes in urinary and hepatic taurine levels in rats treated with hepatotoxins. *Proceedings of Eurotox '90*, Abstract No. 56.

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TAURINE PROTECTS ISOLATED HEPATOCYTES FROM THE CYTOTOXICITY OF HYDRAZINE

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Taurine is believed to protect cells against injury in various ways. These protective properties have been demonstrated in vitro in lymphoblastoid cells and in vivo against CCl₄ hepatotoxicity.

We have previously shown hypertaurinuria in rats after exposure to various hepatotoxins including hydrazine. The data also indicated an increase in taurine synthesis occurred as a response to the exposure. Therefore we have tested the hypothesis that taurine protects isolated hepatocytes against hydrazine toxicity. We have previously shown that hydrazine causes a depletion of ATP in isolated hepatocytes which precedes cytotoxicity.

Hepatocytes were isolated from male Sprague Dawley rats by collagenase perfusion, incubated (2×10^6 cells ml⁻¹) in rotating siliconised flasks under 95%O₂:5%CO₂ in Krebs-Henseleit buffer, containing various concentrations of hydrazine hydrate (12, 16, 20 and 24mM) + taurine (5, 10 and 15mM). Cell viability was assessed using Trypan blue (TB) uptake and lactate dehydrogenase (LDH) leakage. ATP was also measured, using a luciferase-linked bioluminescence assay.

The data showed a dose dependent protection by taurine against TB uptake and LDH leakage induced by hydrazine (24mM) which was significant ($p < 0.01$) with 15mM taurine. Taurine (15mM) shifted the dose response curve for cytotoxicity of hydrazine and reduced the rate of ATP depletion but the difference was not significant. Control cells were also protected by taurine.

The data suggests that taurine protects hepatocytes by stabilizing the plasma membrane, reducing membrane permeability and possibly by moderating ATP depletion. (CW is grateful for support from Glaxo Gp. Research Ltd.)

URINARY TAURINE AND CREATINE AS MARKERS OF LIVER DYSFUNCTION

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We have previously reported the effects of several hepatotoxins on urinary taurine levels [1,2]. The data presented here extends this work and includes levels of urinary creatine, which were measured as part of routine urinalysis.

Male Sprague Dawley rats (270-300 g), housed in individual metabolism cages were treated with a single dose of compound. Urine samples were collected for 48 h before and after dosing. Urinary taurine was measured by pre-column derivatisation with o-phthalaldehyde/mercaptoethanol and separation with hplc. Urinary creatine was measured enzymatically using the Boehringer Test-Combination Creatine PAP diagnostic kit. Post mortems were carried out 48 h after dosing and serum and tissue samples taken for biochemical measurements and histology.

Table: Urinary taurine and creatine, serum enzymes and histology.

Compound	(Dose mg/kg ¹)	¹ Taurine % change	¹ Creatine $\mu\text{mole day}^{-1}\text{kg}^{-1}$	AST lu/L	ALT lu/L	Histology
Control	-	-	7-15	80-105	50-90	-
CCl ₄	3178	+337±21**	600±100***	1219**	299*	HN+++HS+++
Thioacetamide	150	+153±57*	442±112***	1735**	443*	HN++ HS++
ANIT	150	-78±1.5***	136±30*	1501**	723*	HN++
Allyl alcohol	85	+183±121*	178±72**	991*	424*	HN++ HS+
Hydrazine	40	+221±229	81±45	86	23**	HS+
Ethionine	200	+105±32*	15±3	84	37	HS+++
Bromobenzene	1495	-41.7±52	440±36***	1185	174	HN+ HS+
Galactosamine	400 ^b	+69*	11±3	1735*	1299*	HN++ HS+
Allylamine	150	-12±13	45±21	132	49	MN+
Mercuric chloride	1.5 ^b	-69±5*	72±26*	164	51	KN+++
Cadmium chloride	1.5 ^b	-11±8	501±91***	186	87	TN+++ HS++

n = 3-5 (*n = 2), means ± sem. *p<0.05 **p<0.01 ***p<0.001 (paired or unpaired "t" test where appropriate). ¹Total 48 h pre-dose urinary taurine compared to total 48 h post-dose. ¹Urinary creatine 24-48 h post dose. HN = hepatic necrosis, KN = kidney necrosis, MN = myocardial necrosis, TN = testicular necrosis, HS=hepatic steatosis. + mild ++ moderate +++ severe. (ANIT - α -naphthylisothiocyanate).

Urinary taurine was elevated after hepatic damage and/or steatosis, although two hepatotoxins, the cholestatic agent ANIT and the GSH depletor, bromobenzene reduced urinary taurine. The non-hepatotoxins caused a reduction in urinary taurine. Elevation of urinary creatine was generally greatest where there was hepatic necrosis and correlated with the elevation of serum AST and ALT. Only after testicular damage due to CdCl₂ was hypercreatinuria greater than with most hepatotoxins.

Raised urinary taurine levels may result from direct cellular damage and/or interference with sulphur amino acid metabolism or protein synthesis. The results suggest that urinary creatine

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may be the result of direct leakage from damaged cells since it is elevated by compounds that also raise AST and ALT levels. The results indicate that the two measurements (taurine and creatine) are complimentary for the detection of liver damage. If only urinary creatine is raised measurement of serum transaminases may be necessary to distinguish between liver and testicular damage [3]. A slight elevation of urinary creatine is usually associated with decreased food intake [3].

1. Waterfield CJ, Turton JA, Scales MDC & Timbrell JA: Hypertaurinuria as a measure of hepatic damage in the rat after carbon tetrachloride treatment. Human and Experimental Toxicology, 1990; 9:338-339.
2. Waterfield CJ, Turton JA, Scales MDC & Timbrell JA: Changes in urinary and hepatic taurine levels in rats treated with with hepatotoxins. Proceedings of Eurotox '90, 1990. Abstract No. 56.
3. Gray J, Nicholson, JK, Creasy DM & Timbrell JA: Studies on the relationship between acute testicular damage and urinary and plasma creatine concentration. Archives of Toxicology, 1990: 64:443-450.

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INCREASED HEPATOTOXICITY OF CARBON TETRACHLORIDE AS A RESULT OF LIVER TAURINE DEPLETION BY β -ALANINE

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Taurine, a β -amino acid, is reported to have cytoprotective properties *in vitro* (1, 2). We have shown the cytotoxicity of hydrazine (3) and carbon tetrachloride to isolated rat hepatocytes is reduced (by 30%) in the presence of taurine. Our *in vivo* studies have shown i) a significant correlation between urinary taurine and liver taurine concentration ($r=0.734$, $p>0.0001$); ii) a significant correlation between urinary taurine and degree of hepatotoxicity assessed by serum ALT ($r=0.733$, $p<0.001$) and AST ($r=0.689$, $p<0.001$) for various hepatotoxins. These data suggested that reduced liver taurine concentrations may result in increased susceptibility to hepatotoxins in the rat. Therefore the competitive inhibitor of taurine transport, β -alanine, was used in the following studies to reduce tissue levels of taurine.

Male Sprague Dawley rats (270-320 g) were treated with β -alanine (3% in drinking water) for 8 days. On day 6, rats were given carbon tetrachloride (CCl_4) (0, 0.5, 1.0, 1.5 or 2.0 ml kg^{-1} in 1 ml corn oil, p.o.). Rats receiving no β -alanine treatment were treated in the same way with CCl_4 . On day 8 blood was taken from the abdominal aorta for biochemical measurements. Liver samples were taken for histology and measurement of taurine, total non-protein sulphhydryls (TNPSH) and triglycerides (TG). Groups of β -alanine treated and non β -alanine treated animals receiving 0 and 1.0 ml kg^{-1} CCl_4 were housed in individual metabolism cages and taurine was measured in 24 h urine samples collected throughout the 8 day period.

TABLE - Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and liver taurine levels in β -alanine and non β -alanine treated rats measured 48 h after dosing with CCl_4 .

		CCl_4 - ml kg^{-1}				
Treatment		0.0#	0.5	1.0#	1.5	2.0
none	ALT	63 \pm 5 ^a	110 \pm 18	180 \pm 47 ^a	160 \pm 36	546 \pm 284
β -alanine	ALT	50 \pm 3 ^b	158 \pm 6 ^b	437 \pm 185 ^b	1332 \pm 1181	1420 \pm 781 ^b
none	AST	104 \pm 5 ^c	241 \pm 56 ^c	448 \pm 148 ^c	462 \pm 237	1863 \pm 876
β -alanine	AST	96 \pm 2 ^d	459 \pm 42 ^d	955 \pm 220 ^d	1913 \pm 1460	3703 \pm 2161 ^d
none	Tau	4.9 \pm 0.9 ^e	1.8 \pm 0.2 ^e	3.9 \pm 0.8 ^e	4.9 \pm 1.2	4.6 \pm 0.9
β -alanine	Tau	0.9 \pm 0.2 ^e	1.1 \pm 0.2	1.2 \pm 0.2	1.8 \pm 0.6	2.5 \pm 0.1 ^e

Mean \pm SEM, $n=3$ in each group except # where $n=7$; taurine (Tau) $\mu\text{mol g}^{-1}$ wet weight; ALT and AST iu L^{-1} ; ALT and AST values were transformed to Log_{10} for the purpose of analysis.

^a $p<0.05$: no β -alanine treatment compared to β -alanine treatment (Student's "t" test).

$p<0.01$: CCl_4 treated compared to same letter control (CCl_4 , 0 ml kg^{-1}) (Dunnett's test for multiple comparison with single control).

β -alanine treatment significantly increased the toxicity of CCl_4 , measured as raised serum ALT ($p<0.01$, ANOVA with corrections made for interactions between treatments).

Treatment with β -alanine raised urinary taurine by 200% 0-24 h after the start of β -alanine treatment and was maintained at 100% above control values thereafter. Treatment with CCl_4 (1.0 ml kg^{-1}) significantly reduced urinary taurine ($p<0.05$) in β -alanine treated rats 0-24 h after dosing and significantly raised urinary taurine ($p<0.05$) 24-48 h after dosing non β -alanine treated rats. Liver taurine concentration in control rats was reduced to 21% of the normal value. Liver TNPSH were significantly raised ($p=0.0047$) only in animals treated with β -alanine and dosed with 2.0 ml kg^{-1} CCl_4 . There was considerable variation in ALT and AST values after 1.5 and 2.0 ml kg^{-1} CCl_4 . However, serum AST values were significantly higher in β -alanine treated animals than non

β -alanine treated animals 48 h after dosing with 0.5 and 1.0 ml kg⁻¹ CCl₄ and β -alanine treatment significantly increased ALT values above values from non β -alanine treated animals over the dose range of CCl₄ administered (ANOVAR). Biochemical and histological examination of liver tissue showed that CCl₄ caused similar TG and fat accumulation (oil red O staining) but more extensive necrosis in rats which were also treated with β -alanine. These results show that β -alanine can be used to reduce liver levels of taurine despite the high capacity of rat liver for taurine synthesis. The hepatotoxicity of CCl₄ measured by serum AST and ALT levels was increased by β -alanine treatment and tissue necrosis was more extensive in β -alanine treated rats.

1. Pasantes-Morales H, Wright CE & Gaull GE: Taurine protection of lymphoblastoid cells from iron-ascorbate induced damage. *Biochemical Pharmacology*, 1985;34:2205-2207.
 2. Nakashima T, Seto Y, Nakajima T, Shima T, Sakamoto Y, Cho N, Sano A, Iwai M, Kagawa K, Okanoue T & Kashima K: Calcium-associated cytoprotective effect of taurine on the calcium and oxidative paradoxes in isolated rat hepatocytes. *Liver*, 1990;10:167-172.
 3. Mesquita M, Waterfield CJ & Timbrell JA: Taurine protects isolated hepatocytes from the cytotoxicity of hydrazine. *Proceedings of Eurotox '91*, 1991. Abstract No. 149.
- CJW is grateful for support from Glaxo Group Research Ltd.

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ALTERATIONS IN LEVELS OF URINARY TAURINE RESULTING FROM PERTURBATIONS IN GLUTATHIONE SYNTHESIS

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We have reported elevations of urinary taurine in rats dosed with hepatotoxins which cause necrosis and/or fatty liver. However, two hepatotoxins (bromobenzene and allyl alcohol) which deplete reduced glutathione (GSH) by conjugation, did not elevate urinary taurine. This result has been investigated. Male Sprague Dawley rats (245-270 g) in individual metabolism cages were given food and water *ad libitum*. After 48 h, animals were dosed with diethylmaleate (DEM) or phorone which deplete GSH by conjugation, or buthionine sulfoximine (BSO) which reduces GSH by inhibiting γ -glutamyl cysteine synthetase. Taurine was measured in 24 h urine collections (2 pre-dose and 2 post-dose) and liver (48 h post-dose) (Table).

Compound	Dose - mg kg ⁻¹	taurine μ mol kg ⁻¹ 24 h ⁻¹			Liver taurine- μ mol g ⁻¹	Liver GSH- μ mol g ⁻¹
		-24-0h	0-24h	24-48h		
VEHICLE	-	406±62	415±53	283±57	6.1±0.8	7.4±0.4
DEM	400 (ip)	334±13	225±18***	194±37*	6.5±1.0	5.7±0.1
PHORONE	175 (ip)	348±53	183±17*	98±25*	6.5±1.1	10.0±0.3***
BSO	900 (sc)	509±81	742±114*	636±56	5.7±0.2	5.2±0.1

Mean \pm SEM, n=4, * p<0.05, **p<0.01, ***p<0.001, compared to pre-dose level. Reduced urinary taurine was consistent with depletion of cysteine due to GSH synthesis after DEM and phorone treatment, similar to allyl alcohol and bromobenzene. Urinary taurine was raised by BSO probably due to an increase in cysteine for taurine synthesis.

Taurine, a possible urinary marker of liver damage: a study of taurine excretion in carbon tetrachloride-treated rats

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Abstract. Carbon tetrachloride (CCl₄) caused a dose-dependent increase in urinary taurine which correlated with both the histological and biochemical assessment of liver damage. The peak elevation in urinary taurine occurred within the first 48 h after dosing but there was still significant taurinuria 72 and 96 h after the intermediate dose (1 ml.kg⁻¹) and highest dose (2 ml.kg⁻¹), respectively. Levels of taurine in serum were also elevated over the 24 h period following a hepatotoxic dose (2 ml.kg⁻¹) of CCl₄. In contrast, although initially elevated, levels of taurine in the liver declined over the 24 h period following dosing and were significantly lower 96 h after a hepatotoxic dose of CCl₄ (2 ml.kg⁻¹). Male rats showed a different urinary profile for taurine than female rats after dosing with CCl₄. A reduction in food intake seemed to lower urinary taurine levels although these changes were not statistically significant. There was a significant correlation between the level of urinary taurine and the level of serum AST for individual animals given a hepatotoxic dose of CCl₄ (2 ml.kg⁻¹). The data presented suggest that: i) taurine is produced by the liver in response to a toxic insult and subsequent leakage from damaged cells leads to increased levels in the urine; ii) the urinary taurine level may be a useful non-invasive marker of liver damage.

Key words: Carbon tetrachloride – Taurine – Liver damage – Rat

Introduction

Taurine (2-aminoethanesulphonic acid) is a β -amino acid which is not incorporated into proteins but is found in the cytosol of animal cells, in high concentrations (2–30 mM), mostly unbound (Huxtable and Bressler 1972; Chesney et

al. 1978; Wright et al. 1986; Zelikovic and Chesney 1989). In the rat, taurine constitutes the main free amino acid. The diet provides most taurine either directly or by synthesis in the liver and brain from methionine or cysteine via cysteic acid or hypotaurine (Jacobsen and Smith 1968), or via cysteamine in the heart and kidney (Zelikovic and Chesney 1989). The liver and brain are the major organs for the synthesis of taurine which is conjugated with bile acids to form taurocholate in the liver. Taurine is transported from the liver to other organs to maintain tissue levels.

The concentration of taurine in the liver is variously reported as between 2 and 10 mM (Garvin 1960; Mikasa et al. 1980; Yoshida and Hara 1985; Hirai et al. 1987). The highest cellular concentrations of taurine are found in cells rich in membranes (e.g. hepatocytes), or where oxidants are generated (e.g. neutrophils and the retina) or in excitable cells (e.g. cardiac muscle) (Thomas et al. 1985; Wright et al. 1986). These findings have led to the suggestion of a central role for taurine as a protective substance. Taurine stabilises membranes, modulates calcium transport and by the formation of the relatively stable taurochloramine molecule is able to dissipate the toxic effects of HOCl, generated by myeloperoxidases from oxygen radicals. The ability of taurine to conjugate with xenobiotics, retinoic acid and bile salts and its role as a major free amino acid in regulating the osmolality of cells, are also examples of protective functions.

Plasma levels of taurine are regulated by the kidney where the amino acid is reabsorbed in the proximal tubules by a β -amino acid uptake system for which there is competition with other β -amino acids, particularly β -alanine. Excess taurine in the blood results in hypertauroinuria. This has been reported after surgical trauma, X-radiation, muscle necrosis (Kay et al. 1957) and carbon tetrachloride-induced liver damage (Dent and Walshe 1954; Bowden and Goyer 1962; Cornish and Ryan 1964; Goyer et al. 1964; Angel and Noonan 1974) as part of a general amino-aciduria.

Using proton NMR, we have recently shown hypertauroinuria in rats after treatment with several hepatotoxins, including carbon tetrachloride (Sanins et al. 1990). As a

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Table 1. Preparation of fluorescent taurine adduct

Eluate (μ l)	Derivatizing solution (μ l)	Mobile phase buffer (μ l)
Urine 50	50	450
Serum 200	50	0
Tissue 125	100	400

Measurement of taurine. Taurine was measured in the eluates from the ion exchange columns using HPLC with fluorimetric detection (modified from Larsen et al. 1980).

The fluorescent taurine adduct was formed in the different eluates as shown in Table 1. The ratio of eluates: derivatizing solution was dependent on the pH of the eluate and the expected taurine concentration. For example, the serum eluates were too dilute to be further diluted with buffer.

The eluate and derivatizing solution were mixed (30 s), mobile phase buffer added (pH 5.3) and the derivatised sample (10 μ l) injected onto the HPLC column (Waters μ Bondapak C₁₈) exactly 1.5 min after addition of the derivatising solution. Isocratic elution was carried out at a flow rate of 2 ml/min at ambient temperature. Homoserine was eluted first, well separated from taurine which had a retention time between 3 and 4 min (dependent on the characteristics of the column). Adducts were measured using a fluorimetric detector (Tridet, Perkin. Elmer) and integrator. After calibration, taurine concentrations were determined by integration of the peak areas of the eluted homoserine and taurine adducts.

Clinical chemistry

Urinary γ -glutamyl transpeptidase (γ -GT). γ -GT was measured in urine as a marker of kidney tubular cell damage. It was determined by a modification of the method of Naftalin (1969).

Urinary protein. Protein in urine was measured as a marker of kidney damage using Coomassie Brilliant Blue G-250 (Pierce Protein Assay Reagent). Urine was diluted (1:250) and a micro-assay used, the absorbance being measured at 595 nm. Absorbance was compared to a standard curve prepared using bovine serum albumin.

Serum. Serum levels of aspartate transaminase (AST), alanine transaminase (ALT), bilirubin, cholesterol and albumin were measured using the appropriate kits (Boehringer Mannheim GmbH Diagnostica) in an automatic analyser (BM/Hitachi System 705).

Investigations

Effect of restricted food intake on urinary taurine. Sixteen male rats (230–275 g) were divided into four groups, housed individually in metabolism cages, and provided with food and water ad lib for 24 h. Each group (four animals) was then given food, estimated from their previous daily consumption to be 100% (25 g), 80% (20 g), 60% (15 g) and 40% (10 g) of the normal intake (23 ± 3 g). Body weight, food and water consumption were recorded for each animal and urinary taurine levels determined, 24 h before and for 72 h after food intake was controlled. The taurine content of the diet was determined using the same extraction process used for tissues.

Effect of CCl₄ on urinary taurine: dose response study. Male rats (four groups of four animals; 265–290 g) were housed in individual metabolism cages for 10 days. Two consecutive 24 h urine collections (48–24 h and 24–0 h) were made before dosing on day 6. Animals were dosed with CCl₄ (0.5; 1.0 or 2.0 ml.kg⁻¹ in corn oil, 1 ml.kg⁻¹, p.o.). Controls received vehicle only (1 ml.kg⁻¹). Urine was collected every 4 h after dosing for the first 24 h, then three further 24 h collections were made before the animals were sacrificed on day 10. Liver, kidneys and blood samples were taken for biochemical and histological examination.

Effect of CCl₄ on serum and liver taurine levels during the 24 h period after dosing male rats. Male rats (220–320 g; three per group) were

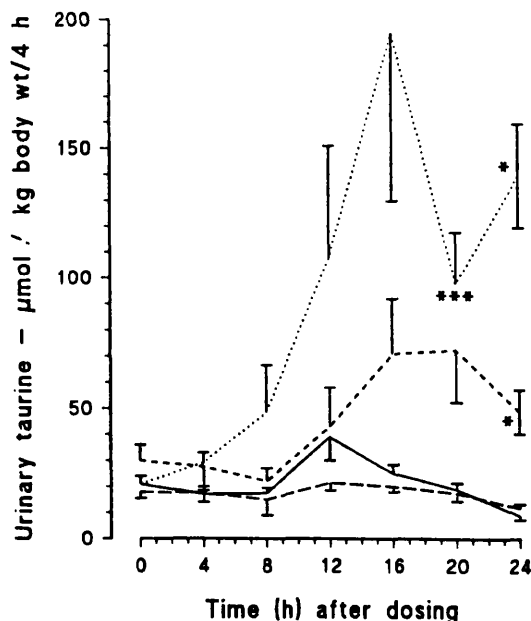


Fig. 1. Effect of various doses of CCl₄ on the levels of taurine in 4-hourly urine samples collected from male rats over the 24-h period after dosing. Control—; 0.5 ml.kg⁻¹—; 1.0 ml.kg⁻¹---; 2.0 ml.kg⁻¹... Values are means \pm SEM from four animals. * $p < 0.05$; *** $p < 0.001$ (comparison with the starting value at time zero using a paired t test)

dosed with CCl₄ (2.0 ml.kg⁻¹; in corn oil, 1 ml.kg⁻¹; p.o.). Control groups received vehicle only (1 ml.kg⁻¹; p.o.). Groups of treated and control animals were killed after 1, 3, 6, 12, 18 and 24 h. Serum, liver and kidneys were taken for analysis of taurine, for determination of biochemical parameters, and for histology, as previously described.

Comparison of effect of CCl₄ on taurine levels in male and female rats. Male (10) and female (10) rats were housed in individual metabolism cages and after three days, five animals of each sex were dosed with CCl₄ (2 ml.kg⁻¹; in corn oil, 1 ml.kg⁻¹; p.o.). Control animals were given vehicle only (1 ml.kg⁻¹; p.o.). Total 24 h urine collections were made every 24 h, for 48 h pre-dose and 48 h post-dose. Animals were killed 48 h after dosing, serum was taken for biochemical analysis, and liver and kidneys were taken for histological examination.

Results

Effect of restricted food intake on urinary taurine excretion in male rats

The average 24 h urinary taurine levels over 3 days of controlled food intake were measured. Reductions in urinary taurine level were observed in rats on reduced food intake but were not statistically significant.

The rat diet contained less than 4 μ g taurine per g.

Taurine levels, clinical signs and liver histology in male animals treated with different doses of carbon tetrachloride

Urinary taurine levels. The effect of different doses of CCl₄ on urinary taurine levels measured at 4-hourly intervals is shown in Fig. 1. An increase in urinary taurine was observed after doses of 1 or 2 ml.kg⁻¹ with maximum excretion 12–16 h after dosing.

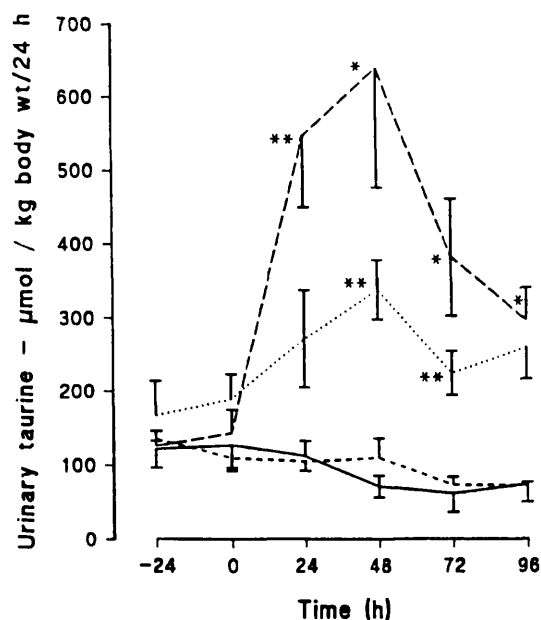


Fig. 2. Effect of different doses of CCl_4 on taurine levels in complete 24 h urine samples from male rats collected over 6 days. Control—; 0.5 ml.kg^{-1} ---; 1.0 ml.kg^{-1} . . . ; 2.0 ml.kg^{-1} —. Values are means \pm SEM from four animals. * $p < 0.05$; ** $p < 0.01$ (comparison with taurine levels before dosing a paired t test)

When the effect of various doses of CCl_4 on urinary taurine was measured at 24-hourly intervals as shown in Fig. 2, again hypertaurinuria was detected after doses of 1 and 2 ml.kg^{-1} . This elevated level of taurine was still detectable 4 days after dosing.

Hepatic taurine levels. Levels of taurine in the liver (μg wet weight) 96 h after dosing with carbon tetrachloride (2 ml.kg^{-1}) were significantly lower ($1.36 \mu\text{mol}$) than control values ($3.62 \mu\text{mol}$). After a lower dose of carbon tetrachloride (1 ml.kg^{-1}) liver taurine was also reduced but not significantly so ($2.4 \mu\text{mol}$).

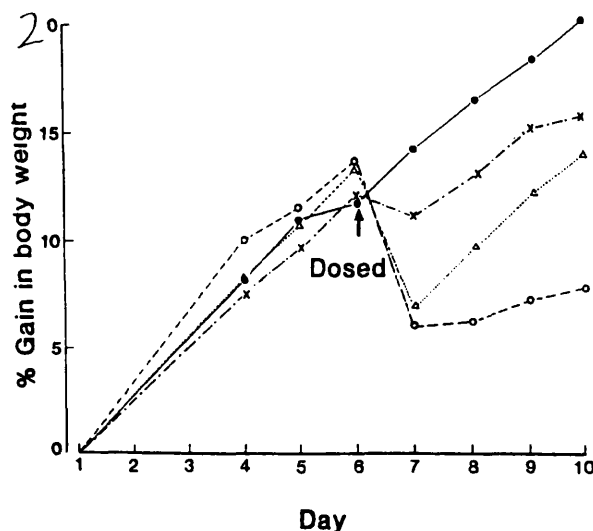


Fig. 3. Effect of various doses of CCl_4 on body weight in male rats. Control ●—●; 0.5 ml.kg^{-1} ×---×; 1.0 ml.kg^{-1} Δ....Δ; 2.0 ml.kg^{-1} ○---○. Points are means from four animals

Clinical signs of toxicity. All groups of animals treated with CCl_4 showed a significant loss of body weight during the 24 h following dosing (Fig. 3). All treated animals reduced their food consumption significantly 24 h after dosing (Table 2). Liver weights (Table 2) were significantly less 96 h after dosing with 1 ml.kg^{-1} CCl_4 , and higher than control values after 2 ml.kg^{-1} although this last difference was not significant.

Serum AST and ALT levels measured 96 h after dosing (Table 2) were not significantly elevated above control levels (apart from one animal given 2.0 ml.kg^{-1} which did have elevated levels: ALT — 178 and AST — 212 iu.l^{-1}).

Histology. Light microscopy of sections of liver taken 96 h after dosing showed no abnormalities in animals treated with 0.5 ml.kg^{-1} CCl_4 . There was no necrosis in the livers

Table 2. Effect of various doses of carbon tetrachloride on food consumption, liver weight and serum transaminases in male rats

	Dose of $\text{CCl}_4 \text{ ml.kg}^{-1}$			
	0	0.5	1.0	2.0
Food Consumption (g/rat/day)				
24–0 h pre-dose	25.8 \pm 2.6	32.4 \pm 3.5	19.7 \pm 6.5	29.0 \pm 0.8
0–24 h post-dose	25.0 \pm 1.5	19.3 \pm 1.6*	10.8 \pm 1.9**	4.4 \pm 0.7**
24–48 h post-dose	28.3 \pm 0.6	24.0 \pm 1.8	21.2 \pm 2.0**	10.4 \pm 3.5**
48–72 h post-dose	24.7 \pm 0.5	23.3 \pm 1.0	23.3 \pm 3.1	21.3 \pm 1.3**
72–96 h post-dose	29.1 \pm 1.3	25.4 \pm 1.5	19.4 \pm 4.8	31.0 \pm 4.1#
Liver weight (% body weight)				
96 h after dose	4.07 \pm 0.06	3.87 \pm 0.09	3.65 \pm 0.07*	4.44 \pm 0.21#
ALT				
IU.l^{-1}				
96 h after dose	48.0 \pm 3.3	41.0 \pm 1.1	41.9 \pm 2.5	92.0 \pm 43.1#
AST				
IU.l^{-1}				
96 h after dose	53.6 \pm 8.4	75.8 \pm 3.1	70.3 \pm 5.6	131.2 \pm 40.8#

* $p < 0.05$; ** $p < 0.01$.

Values are means \pm SEM; $N = 4$ except # where $N = 3$