THE POSSIBLE HEPATOPROTECTIVE ROLE OF TAURINE: AN IN VIVO AND IN VITRO STUDY

A Thesis submitted by
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ABSTRACT

The experiments described in this thesis aimed to examine the possible protective role of taurine against the hepatotoxic effects caused by two cholangiodestructive compounds, α-naphthylisothiocyanate (ANIT) and 4,4'-diaminodiphenylmethane (DAPM).

Studies investigated the protective effect of taurine in vivo and in vitro, using respectively male Sprague-Dawley rats and freshly isolated rat hepatocyte suspensions. Control animals fed a normal diet and tap water were investigated for their response after being dosed with ANIT and DAPM. When liver taurine levels were raised by giving taurine in the drinking water, rats were slightly less susceptible to ANIT- and DAPM-induced hepatotoxicity than control animals. Two taurine uptake inhibitors (β-alanine and guanidinoethane sulphonate (GES) were given in the drinking water to decrease liver taurine levels. Both compounds were effective in decreasing the liver levels of taurine. β-alanine treated animals were more susceptible, to a certain extent, than control animals to the hepatotoxic effects of both compounds tested. Conversely, GES did not increase rat susceptibility to either of the toxicants. When the time-course of DAPM-induced hepatotoxicity was studied, taurine pretreatment to rats shifted the development of hepatotoxicity to an earlier time point (12-18 hours) by a mechanism that appears to be connected with an increased bile flow. This might explain the slight protection observed when a later time point (24 hours) was assessed.

A different approach to modulate liver taurine levels was taken by feeding a semi-synthetic, taurine-free, 15% or a 30% casein diet to rats. Although animals fed the higher content casein diet showed a meaningful difference in urinary taurine excretion throughout the study, their liver levels at the time of dosing were comparable to their counterparts fed the 15% casein diet. Taurine urinary excretion seemed to be the path used by the rat to eliminate the excess of sulphur in the diet. Animals fed these diets and dosed with ANIT or DAPM were shown to be less susceptible than animals fed a normal
diet when serum biochemical parameters were assessed but the lack of correlation with the histopathological evaluation leads to the conclusion that these protective effects should be further investigated and should be regarded with caution.

The cytoprotective properties of taurine against the cytotoxic effects of ANIT and DAPM were also tested. Both compounds caused a depletion of intracellular GSH and ATP. Cells isolated from β-alanine treated animals showed lower levels of taurine but they were not more susceptible to ANIT than hepatocytes isolated from control rats. When taurine was added to hepatocytes isolated from control or β-alanine treated animals no protection was afforded. Cysteinesulphinate, a taurine metabolic precursor, added to hepatocytes in suspension afforded a slight protective effect against ANIT cytotoxicity.

These studies have shown that taurine afforded some hepatoprotection against tested toxicants.
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<tr>
<th>Abbreviation</th>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ANIT</td>
<td>α-naphthylisothiocyanate</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine triphosphate nucleoside</td>
</tr>
<tr>
<td>BAIF</td>
<td>Bile-acid independent bile flow</td>
</tr>
<tr>
<td>BSO</td>
<td>Butoximine sulphoxide</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>CSAD</td>
<td>Cysteine sulphinate decarboxylase</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>DAPM</td>
<td>4,4'-diaminodiphenylmethane</td>
</tr>
<tr>
<td>DC-Na</td>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>DEM</td>
<td>Diethyl maleate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetracetic acid</td>
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<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FAA</td>
<td>Free amino acids</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GES</td>
<td>Guanidinoethane sulphonate</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione, reduced form</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione, oxidized form</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl-phosphatidyl inositol-anchored proteins</td>
</tr>
<tr>
<td>γ-GT</td>
<td>γ-Glutamyl transpeptidase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HEPES</td>
<td>(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])</td>
</tr>
<tr>
<td>3α-HSD</td>
<td>3α-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>K+H</td>
<td>Krebs Henseleit (incubation buffer)</td>
</tr>
<tr>
<td>LDL-R</td>
<td>Low-density lipoprotein receptor</td>
</tr>
<tr>
<td>MFO</td>
<td>Mixed-function oxidases</td>
</tr>
<tr>
<td>Na/K*-ATP</td>
<td>Na⁺, K⁺-activated adenosinetriphosphate</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetyl-L-cysteine</td>
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<tr>
<td>NAD</td>
<td>β-Nicotinamide adenine dinucleotide (NAD)</td>
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<tr>
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<td>β-Nicotinamide adenine dinucleotide phosphate (NADH)</td>
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<tr>
<td>NADPH</td>
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</tr>
<tr>
<td>NDI</td>
<td>N-decylidazol</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>5'-NT</td>
<td>5'-Nucleotidase</td>
</tr>
<tr>
<td>OPA</td>
<td>o-phthaldehyde</td>
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<tr>
<td>OTC</td>
<td>L-2-oxothiazolidine-4-carboxylate</td>
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<tr>
<td>PAPS</td>
<td>Adenosine 3'-phosphate-5'-phosphate</td>
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<tr>
<td>PALP</td>
<td>Pyridoxal phosphate</td>
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<tr>
<td>PMA</td>
<td>12-O-tetradecanoyl-phorbol-13-acetate</td>
</tr>
<tr>
<td>P.o.</td>
<td>Per os (oral route)</td>
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<tr>
<td>RAW 264.7</td>
<td>Murine macrophage cell line</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAMe</td>
<td>S-adenosyl-L-methionine</td>
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<tr>
<td>SBA</td>
<td>Serum bile acids</td>
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<tr>
<td>SKF-525-A</td>
<td>Diethylaminoethyl-2,2-diphenyldervate</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>TNPSH</td>
<td>Total non-protein sulphydryls</td>
</tr>
<tr>
<td>UHQ water</td>
<td>Ultra high quality</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoproteins</td>
</tr>
<tr>
<td>2-VP</td>
<td>2-vynlypyridine</td>
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"... if one believes,
  it might be,
  but you shouldn't just disbelieve,
  'cos nothing is as simple as it seems."

  Anon.
CHAPTER 1
INTRODUCTION

1.1. Preliminary remarks

It would be inequitable, if the reader of this thesis was not first introduced to the work previously done in this department by my predecessors. Some years ago Susan Sanins made the observation, while using proton nuclear magnetic resonance (NMR) analysis, that the urinary levels of the β-amino acid taurine were elevated in rats dosed with hydrazine. Work carried out later, showed that other hepatotoxicants, such as carbon tetrachloride (CCl₄) and α-naphthylisothiocyanate (ANIT) also raised this sulphur containing amino-acid in the urine (Sanins et al., 1990).

More recently, Catherine Waterfield committed her experimental work to investigate the possible relationship between hypertaurinuria and the hepatotoxic effects caused by several xenobiotics, when administered to the rat. During the course of these studies, it was proposed that urinary taurine could be used as a non-invasive marker of hepatic injury and dysfunction (Waterfield et al., 1991; Waterfield et al., 1993d). Moreover, it was observed that compounds that alter protein synthesis or sulphur amino acid metabolism also exhibit changes in urinary taurine (Waterfield et al., 1993b). A significant correlation was also established for urinary taurine levels and liver taurine concentration in control animals before dosing. After rats were dosed with several hepatotoxicants, a significant negative correlation was obtained when the serum transaminase levels were plotted against the pre-dosing urinary levels of taurine, suggesting a hepatoprotective function for taurine (Waterfield et al., 1993c).

Additionally, when rats were treated with β-alanine, they showed hypertaurinuria and a marked depletion of the liver taurine levels. These animals were more susceptible to a single exposure to CCl₄ than vehicle dosed controls (Waterfield et al., 1993e).

A parallel was successfully drawn between the possible hepatoprotective
effects of taurine and the partial cytoprotection observed when isolated hepatocytes were exposed to hydrazine, 1,4-naphthoquinone and CCl₄ in the presence of taurine (Waterfield et al., 1993a).

The findings described above, were not part of a extensive comprehensive study aimed to study the specific effect that the changes in levels of taurine might exert in the establishment of liver damage. Therefore, the initial aims of these studies were to investigate, using an in vivo and an in vitro approach, the possible hepatoprotective properties of taurine.

1.2. The liver as a target organ for toxicity

1.2.1. Liver gross anatomy, function and central role

The liver is the largest mass of glandular tissue in the body and also its largest internal organ. It weights 1.2 to 1.5 kg in the human adult, accounting for 2 - 5% of its body weight and 3% of an adult rat body weight. Located in the right upper quadrant of the abdomen it has a dual blood supply. First, the portal vein carries venous blood from the capillary bed of the digestive tube, pancreas and spleen. The portal vein supplies approximately 75% of the blood volume that reaches the liver. Second, the hepatic artery, which is a branch of the coeliac trunk, conveys well-oxygenated blood into the liver accounting for approximately 25% of its afferent blood supply. Both vessels enter the liver at the porta hepatis and arborize throughout the hepatic parenchyma (Balistreri and Rej, 1994). Within the liver, the distributing branches of the portal vein and hepatic artery supplied the sinusoidal capillaries (sinusoids) with blood. These sinusoids are in intimate contact with hepatocytes and provide for the exchange of substances between blood and the liver cells. Despite the dual source at the entrance gate, blood exiting the liver may arise from several afferent sources and blood that entered the liver through a given afferent vessel may be randomly drained out via one of the two hepatic veins (right or left). The right and left hepatic veins enter into the inferior vena
cava near the right atrium.
The liver has both endocrine and exocrine functions. The endocrine liver secretions are released towards the sinusoidal blood that also supplies the liver parenchymal cells with oxygen and nutrients. These secretions include substances secreted by the liver cells (i.e. albumin, lipoproteins, prothrombin and glycoproteins). Glucose is also released by hydrolysis of glycogen stored in the liver cells. The liver has its exocrine function in producing and secreting bile that contains degraded waste products to be delivered to the intestine for disposal and also bile salts that help fat digestion.
Positioned between the digestive system and the rest of the body, the liver acts as a guardian preventing the remaining body tissues being exposed to many potential toxic substances, particulate and other foreign material entering the whole blood stream from the portal circulation (Timbrell, 1991).

After primary uptake of material by the liver, a versatile metabolic apparatus for degradation and biotransformation is available in intracellular organelles such as lysosomes and endoplasmic reticulum to render them harmless, but it is often overwhelmed by such materials and/or substances and damaged. Liver injury caused by chemicals has been recognised as a toxicological problem for over 100 years. The incidence of liver injury varies within animal species, time of exposure and nature of the chemical agent involved and often is not related to a simple or single mechanism of hepatic degeneration or alteration of liver function (Plaa, 1991).

1.2.2. Liver morphology

The highest percentage of liver tissue is constituted of parenchymal cells (hepatocytes). Hepatocytes are large polygonal cells measuring around 30 μm in each dimension. They account for about 80% of the liver cell population in the human (Ross et al., 1995) and in experimental animals the average lifespan is 150 days (Balistreri and Rej, 1994). The rat liver has fewer hepatocytes (60%) but these constitute nearly 78% of the liver volume.
At birth, the liver parenchymal cells are arranged in two-cell-thick plates (muralium duplex) which, after the age of 5 years are converted into a one cell thick layer (muralium simplex). The disposition of hepatocytes in single-cell-thick plates surrounded on either side by sinusoidal blood (Figure 1.1.) renders a very efficient system for exchange between blood and hepatocytes. It also builds a sponge-like wall-work of hepatocellular plates that anastomoses at different angles and surrounds the hepatic spaces occupied by the sinusoids (Desmet, 1994).

**Figure 1.1.** Schematic illustration of a liver cell plate. Single-cell-thick liver plate (top panel) is separated from the sinusoidal blood on either side by sinusoidal lining cells. Lining cells have large fenestrations in the cytoplasm which allow free access of plasma to Disse space. The bottom panel shows an enlargement of the hemicanaliculus shown above. Canalicular membranes of adjacent hepatocytes are joined by tight junctions (from Van Dyke et al., 1989).
The sinusoids are lined by a characteristic and specific set of cells that include sinusoidal epithelial cells, Kupffer cells, perisinusoidal Ito cells and Pit cells (Desmet, 1994). The sinusoidal endothelial cells show discontinuous basal lamina that is absent in large areas. This discontinuity of the endothelium is evident in two forms: Large *fenestrae* (present in the endothelial cells) and large *gaps* (present between neighbouring endothelial cells). This fenestration of the endothelium increases in size and number from the periportal to the pericentral area.

The Kupffer cells are the resident macrophages of the liver. They represent 29% of the sinusoidal cells in the liver and constitute approximately 80 to 90% of all the fixed macrophages in the body. With a stellate shape and being attached to the endothelium provides them the possibility of migrating along its lumen. The major function of Kupffer cells is acting as a waste receptacle for all kinds of damaged, altered, senile cells and also foreign material such as bacteria and circulating tumour cells. This is accomplished primarily through the process of phagocytosis. Upon activation they also secrete an array of products with potent biological effects including cytokines, superoxide anion, hydrogen peroxide and proteases that influence neighbouring cells or other immunocompetent cells in the vicinity or thereby recruited by chemotaxis (Desmet, 1994; Decker, 1990; Laskin, 1990). The presence of red cell fragments and iron in the form of ferritin and hemosiderin implicated a role in iron metabolism (Balistreri and Rej, 1994).

Perisinusoidal cells, Ito cells or fat-storing cells are located between the sinusoidal endothelial cell and the hepatocyte in the perisinusoidal space (Space of Disse) and they constitute 20% of the hepatic sinusoidal cells. With a resemblance of fibroblasts, these cells have been shown to store high quantities of vitamin A localized in intracellular lipid droplets.

Pit cells are large granular lymphocytes and they represent the 3% of hepatic sinusoidal cells with natural killer activity, playing a role against viral infection and tumour growth (Desmet, 1994; Laskin, 1990).
1.2.3. Structural and functional organization of the liver

As indicated above the liver is constituted of hepatocytes, blood vessels and sinusoidal capillaries between plates of hepatocytes. The cell mass is traversed by two tracts of connective tissue that branch within the liver. The wider one represents the portal tract that carries afferent blood vessels, bile ducts, lymphatics and nerves. The narrower one surrounds tributaries of draining hepatic veins. The two tracts interdigitate but do not connect, with this disposition the ends of afferent vessels are kept apart from hepatic vein tributaries by hepatic parenchyma and sinusoids.

There are several ways to describe the organization of these structural elements in order to understand the major functional relationship of the liver. As a result, three major, different concepts have been developed to describe the histological and the functional units within the liver and exhaustively dissected in recent textbooks and reviews (Miyai, 1991; Desmet, 1994; Ross et al., 1995; Rappaport, 1979).

These are the: hepatic (classic) lobule, portal lobule and hepatic acinus.

i. The classical hepatic lobule concept was first described during the seventeenth century by Wepfer and Malpighi and subsequently refined by Kieman as the unit of liver tissue, with portal tracts in its periphery and the hepatic tributary vein in the centre (the concept of a centrilobular vein) into which the sinusoids drain. The six sided polyhedral prism of hepatic tissue appears well defined only in a few species (e.g. pig) defined by interlobular septa of connective tissue (Figure 1.2.). According to this concept, the blood flows from the peripheric portal triads (branches of the portal vein and hepatic artery) into the lobule towards 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 parenchymal cells where it is formed, into the bile canaliculi between each hepatocyte and along the intralobular bile ductules to the bile ducts in the portal triads.
Figure 1.2. Schematic drawing of the three histological units of the liver. A- Classical hepatic lobule; B- Portal lobule; C- Hepatic acinus; P- Portal tracts or triad; CV- central vein; 1- periportal zone; 2- intermediate zone; 3- perivenous region

ii. The portal lobule idea was proposed by Mall after other authors described a prevalent lobulation in the seal liver in which liver cell plates and sinusoids radiate from the portal canals. Impressed by this observation, Mall proposed "his" portal lobule as the basic structural unit of the liver with the portal triad at the centre and draining hepatic vein at the periphery demarcating the vertices of a triangle (Figure 1.2.). The concept emphasises the direction of bile flow. According to this idea, bile flows from the periphery to the centrally located bile duct. Although this concept allows the liver to be compared with other exocrine glands, it has been criticized for overstating the excretory function of the liver. Others argue that it does not describe the smallest functional unit within the liver since the vascular supply to the portal lobule can be considered preterminal (Miyai, 1991).

iii. The Hepatic acinus or Liver acinus concept arose after hepatic microcirculation studies performed by Rappaport et al (1979). They proposed
the simple hepatic acinus as the smallest functional unit of the liver. According to this concept the parenchymal tissue in the liver acinus is organized around the terminal portal venule and its accompanying hepatic arterioles, bile ducts, lymphatic vessels and nerve fibres. The sinusoids drain towards two or more peripheric terminal hepatic venules (denominated central vein in the hepatic lobule concept) (Figure 1.2.). This three-dimensional berry-shaped hepatic acinus lies at the end of the terminal vascular and biliary stalk (Figure 1.3.). According to its proximity to the terminal afferent vessels, the hepatocytes are described as being arranged in three concentric elliptical zones surrounding the axis formed by the afferent vessels.

**Figure 1.3.** The liver acinus concept is a functional interpretation of liver organization. Parenchymal cells are arranged in three elliptical concentrical areas surrounding the axis formed by the afferent vessels. Zones marked 1, 2 and 3 are supplied with blood that is most oxygenated and rich in nutrients in zone 1 and least so in zone 3; C.V.- central vein; P.T.- portal triad (from Ross et al., 1995).
The zone 1 is composed of the cells adjacent to the terminal afferent vessels. They are the first to receive nutrients and xenobiotics and they are also in contact with well-oxygenated blood. They are the first to show morphological changes if bile duct occlusion occurs but also the last to die if circulation is impaired, and the first to regenerate.

The cells in zone 3, situated at the distal end of the system (or at the microcirculation periphery), farthest from the axis and closest to the terminal hepatic vein are the first to suffer from ischemic necrosis in situations of reduced perfusion (Figure 1.3.). They receive blood that has already exchanged gases and metabolites with cells in other zones and they are the last to respond to toxic insult and bile stasis.

Lying in between one can find zone 2 that has no sharp boundaries. It is regarded as a transitional region between zone 1 and 3 having intermediate morphological and responses characteristic (Figure 1.3.). Its dimension changes in response to the hemodynamic state within the acinus. (Rappaport, 1979).

Several other areas of hepatic tissue have been proposed as the functional unit, based both on the microvascular framework and hepatic angioarchitecture or on the bile secretory function (Desmet, 1994).

Despite the fact that human liver has no anatomically clear-cut definable units and can be defined as a "indivisible continuum", different parenchymal "territories" and its associated functions will be undoubtedly connected in the future with yet to define angioarchitectural "units" as they will determine regional differences in blood flow (expressing distinct oxygen gradients and nutrient concentration) and they might also delineate areas with different metabolic engagements.

1.2.4. Liver parenchymal heterogeneity

Liver parenchymal cells (hepatocytes) are the main functional unit of the liver. Hepatocytes are large polygonal cells measuring between 15 and 30 µm
in each dimension; human hepatocytes have an average diameter of 25 µm (Miyai, 1991). They constitute about 80% of the cell population in the liver. The hepatocyte nuclei are large and spherical and occupy the centre of the cell. A large proportion of the adult mammalian liver cells are polyploid (30-80%) and many are binucleated. In the rat, hepatocytes have a diameter of 17-23 µm and a volume of $4.77 \times 10^5 - 5.54 \times 10^5 \text{ µm}^3$.

With a superficial first glance through the light microscope, the liver parenchyma appears to be homogeneous. However, closer analysis and the use of histochemical, ultrastructural and morphometric techniques revealed a prominent heterogeneity (Desmet, 1994).

Hepatocytes are positioned between two different environments, the plasma and the bile. This means that the functions carried out in the two fronts are distinct and that the hepatocyte surface is polarized or asymmetric (Hubbard et al., 1994).

1.2.4.1. Hepatocyte surface polarity

Hepatocytes have three different cell surfaces. Because of its multipolarity, it is also common to find a hepatocyte with several surfaces in a given section of the liver tissue. The proportion of surface area allocated to each domain has been determined stereologically, to be in the rat liver: canalicular or apical surface, $680 \text{ µm}^2 (13\%)$, intercellular or lateral surface, $780 \text{ µm}^2 (15\%)$ and the sinusoidal surface or basal plasma membrane, $3800 \text{ µm}^2 (72\%)$.

The canalicular surface forms the wall of the bile canalculus and is formed by the apposition of the edges of hemitubular grooves of the abutting surfaces of neighbouring hepatocytes (Miyai, 1991). The surface of the bile canalculus is studded with microvilli and the canalicular lumen is sealed by the tight junction (Figure 1.1.). The specialized functions of this type of membrane surface includes the transport of bile acids and products of detoxication across the membrane bilayer into the bile, release/transport of lipids at the canalicular front and delivery of secretory immunoglobulin A to bile via fusion.
of transport vesicles to the apical surface (Hubbard et al., 1994).

The sinusoidal surface or basal plasma membrane is also studded with microvilli (this amplifies its effective surface area sixfold if compared with hypothetical smooth counterparts) and is the specialized area where exchanges between the sinusoidal blood and hepatocyte occur (i.e. transport of small molecules across the membrane like amino acids, glucose, bile acids).

Figure 1.4. Hepatocyte ultrastructure. (From Balistreri and Rej, 1994).
The lateral surface is closely approximated to an adjoining hepatocyte through a desmosome, gap junction and studlike protrusions and their corresponding indentations (Figure 1.4.). The lateral surface shares most of the function with the sinusoidal surface and additionally is specialized for cell attachment and cell to cell communication. Functionally the existence of gap junctions appears to be very important in providing the channels to intercellular exchange of ions and small molecular weight materials. Control and maintenance of normal cellular homeostasis appears to be the major function of gap junctions in adult hepatocytes. Both physical and chemical insult of the liver alters gap junction communication. The blockage of this intercellular communication through these "small canals" by toxic agents either directly or indirectly interferes with the transfer of low molecular weight materials between the cells. When one or more cells dies through either cytolethal toxicity or apoptosis, the gap junctions in contiguous cell suffer a down-regulation (closing down). This is believed to be a protective mechanism acting in favour of the viable cells from the transfer of toxic metabolites or ions (such as calcium) from the dying cell. The drawback of this important mechanism is that preneoplastic cells are isolated from the suprainimposed growth regulatory control by the adjacent cell and they proliferate and grow (Mehendale et al., 1994).

In analogy to the epithelial cells, the sinusoidal and lateral membranes are also denominated basolateral surface. Basolateral surface differs from the canaliculi surface in its biochemical membrane composition, enzymes attached and by expressing different receptors. For example Na⁺/K⁺-ATPase, the insulin receptor, the low density lipoprotein receptor (LDL-R), epidermal growth factor receptor (EGF-R) are basolateral membrane proteins. In opposition Mg²⁺-ATPase, alkaline phosphatase, aminopeptidase N, Glycosylphosphatidyl inositol anchored (GPI) and proteins such as 5'-nucleotidase (5'-NT) are confined to the apical or canalicular surface (Desmet, 1994; Hubbard et al., 1994).

Hepatocyte surface polarity is established very early in life and is preserved
in periods of regrowth. This characteristic is also maintained while the cell synthesises, delivers, removes, recycles or degrades one of the several constituents of the plasma membrane domain (Hubbard et al., 1994).

### 1.2.4.2. Hepatocyte zonation and metabolic differences

Hepatocytes from the periportal and perivenous zones of the liver parenchyma differ in their enzyme content and subcellular structures. Thus, different metabolic capacities and functional differentiation is in agreement with the concept of metabolic zonation that proposes a functional specialization of two different zones of the hepatic parenchyma based on carbohydrate metabolism and other metabolic pathways (Jungermann and Katz, 1989).

Each hepatocyte has between 800 and 1000 mitochondria. They vary in size, number, shape and enzymatic characteristics according to their position in the hepatic acinus. Periportal hepatocytes appear to be better equipped than their perivenous counterparts for the final oxidation of energy substrates such as glucose, amino acids and fatty acids that initially are degraded to acetyl-CoA. Periportal hepatocytes contain a greater volume of mitochondria (20% mitochondria vol/cell vol) when compared to perivenous counterparts (12%). Moreover mitochondria from acinar zone 1 are larger (0.95 μm³), thicker (0.56 μm) and shorter (3.5 μm) than the more numerous mitochondria present in acinar zone 3 (respectively 0.41 μm³, 0.32 μm, 5.0 μm) (Jungermann and Katz, 1989).

These differences reflect an adaptation to the variation in the metabolic environment due to different oxygen composition and microcirculation within the liver acinus. Zonal distribution of toxicity caused by xenobiotics in the liver has been known for a long time.

The difference in oxygen supply between cells in zone 1 (periportal) and zone 3 (perivenous) gives rises to biochemical gradients (Figure 1.5.).

While the cells in zone 1 are most aerobic are well equipped to carry out oxidative energy metabolism, gluconeogenesis, β-oxidation of fatty acids,
amino acid catabolism and bile acid excretion. Glucoronidation of xenobiotics is another function that these cells perform as they have a high concentration of glucoronosyl transferases. According to the model of metabolic zonation, based on enzyme distribution, the disposal of xenobiotics is catalyzed preferentially in the perivenous zone (Jungermann and Katz, 1989). In the adult animal, many reactions involved with the metabolism of xenobiotics are located in the SER. The NADPH and NADH reductase catalysed reactions also take place primarily in hepatocytes located in zone 3.

Work done by Pentilla (1990) has shown that perivenous hepatocytes synthesise taurine more rapidly and these cells also accumulate this amino acid to a greater extent than perivenous hepatocytes. Interesting, one of the main functions of taurine in the liver is it's conjugation with bile acids which predominantly occurs in periportal hepatocytes. It was suggested that periportal hepatocytes might utilize taurine coming from the diet, from the enterohepatic circulation or from perivenous hepatocytes to conjugate bile acids (Pentilla, 1990).

Several metabolic differences can occur within the liver parenchyma that are linked with the peculiar oxygen and nutrient distribution. Thus, during an absorptive phase the perivenous hepatocytes take up glucose and incorporate it into glycogen, and degrade it to lactate. The periportal cells take up lactate formed in perivenous hepatocytes and convert it via glycogenesis to glycogen. During the postabsorptive phase, the periportal cells release glucose generated from glycogen reserves whereas perivenous hepatocytes release lactate formed mainly from glycogen (Jungermann and Katz, 1989).

Hepatocytes have an extensive network of endoplasmic reticulum. The rough endoplasmic reticulum (RER) with ribosomes attached to the cytoplasmic side of the membrane is related to the synthesis of protein for export and enzymes that play a role in metabolic processes such as glucose-6-phosphatase (Miyai, 1991).
Figure 1.5. Enzyme distribution and metabolic areas in the various zones of the liver acinus according with Rappaport, 1979, Jungermann and Katz, 1989 and; Pentilla, 1990 (used with permission from Waterfield, 1992).
The smooth endoplasmatic reticulum (SER), which is contiguous with the membrane of RER but has no ribosomes attached to it, is involved in the metabolism of proteins, carbohydrates and lipids and in the biotransformation of foreign substances. Reflecting the complexity of their function, both RER and SER are well developed in the hepatocytes. RER forms several proteins for export (e.g. albumin and other plasma proteins). Other functions of the endoplasmic reticulum include the degradation of steroids and the formation and metabolism of cholesterol, lipid-soluble vitamins and fatty acids. Most steroids and lipid-soluble drugs and chemicals are metabolized into more water-soluble compounds by the microsomal mixed-function oxidase system (e.g. cytochrome P<sub>450</sub> and NADPH cytochrome c reductase) situated primarily in the SER.

Another important function of the endoplasmic reticulum is its role on lipid metabolism. Thus, triglycerides synthesised from fatty acids and glycerol in the hepatocyte are not used for energy production or membrane synthesis or repair. Most are exported into the blood as very low-density lipoprotein (VLDL).

1.3. Bile duct epithelial cells, bile and bile flow

1.3.1. Bile duct epithelial cells and the biliary tree

The other type of epithelial cells that compose the liver are biliary epithelial cells. Although biliary epithelial cells account only for 3-5% of the liver cell population in comparison with liver parenchymal cells (60%), they form a complex three-dimensional network of interconnecting tubular structures. These cells, also denominated cholangiocytes, line the lumen of finer and larger branches of intrahepatic and extrahepatic portions of the bile draining system which not only conducts and delivers the bile into the intestine but also participates actively in absorptive and secretory processes (Desmet, 1994; Alpini et al., 1994).

In contrast to hepatocytes, cholangiocytes are lined by a basement membrane
and express several molecular markers that are absent in their parenchymal epithelial cell-like counterparts, such as cytokeratins 7 and 19, carcinoembryonic antigen and γ-glutamyl-transpeptidase. Cholangiocytes have fewer mitochondria, less extensive endoplasmic reticulum and showed a more developed network of intermediate cytoskeletal filaments when compared to hepatocytes (Desmet, 1994).

Cholangiocytes exhibit some morphological heterogeneity, tending to be cuboidal in small ducts and columnar in large ducts. Ultrastructural studies revealed that cholangiocytes contain microvilli, tight junctions and many other types of vesicles which together constitute anatomical characteristics typical of transporting epithelia. Thus, it is certain that this type of cell is involved in the absorption of water, carbohydrates, proteins and under certain experimental conditions even bile acids (Alpini et al., 1994).

The intrahepatic biliary ductal system is formed by an interconnecting duct tubular structure of different size but lined by cholangiocytes. Anatomically, the intrahepatic biliary system is divided into ductules and ducts (small, intermediate and large). The smaller ones, ductules (cholangioles, lumen diameter <20 μm), connect the orifice or canals of Hering (represent areas between the hepatocyte canalicular domain and the ductules) with the interlobular bile duct. The interlobular ducts (lumen diameter 20-100 μm) drain into the septal ducts (lumen diameter >100 μm) their confluence will originate intermediate ducts (lumenal diameter 400-800 μm) and finally its anastomosis will lead to the formation of the large intrahepatic bile ducts (lumenal diameter >800 μm), left and right (Alpini et al., 1994).

1.3.2. Biliary excretion, bile composition and bile flow

Since ancient civilizations were established, the liver and bile have been considered to be important in determining the temperament and health status of the body. In Hypocratic medicine, bile was one of the four cardinal humours (blood, phlegm, yellow bile from the liver and black bile from the
stomach) though to regulate the health condition of an individual. Since then, bile excretion and composition was the subject of countless studies that led to the concept that bile is a physiological secretion necessary for the digestion of fat and also an excretory product containing cholesterol and bile pigments (Klaassen and Watkins III, 1984).

The secretion of bile is one of the major functions of the liver, constitutes one unique characteristic of this organ and represents one of its major activities. Bile is an excretory and a secretory fluid with a complex composition that varies accordingly with the nutritional status of the individual (Coleman, 1987). Thus, this solution is the only excretory pathway for water-soluble catabolic products of two important body constituents (cholesterol and haemoglobin), the former is converted into a group of amphipathic molecules denominated bile salts as well as forming the backbone of other liver-derived potentially toxic endogenous metabolites (e.g. steroid hormones); the latter first converted to biliverdin and excreted as such or reduced to bilirubin, and excreted into bile. Bile is also a secretion, since its major organic component, the bile acids, function as amphipathic molecules facilitating fat digestion and lipid absorption. Moreover, in some species, bile contains high concentrations of bicarbonate that might help to neutralize the acid gastric content in the duodenum, and may also contain polymeric immunoglobulin A to help protect the biliary and upper intestinal tracts from infection (Coleman, 1987; Hofmann, 1989).

Bile serves also an excretory route for xenobiotic elimination. Thus, amphipathic molecules that are not eliminated in the urine due to lipophilicity that enhances binding with albumin, decreasing the glomerular filtration are likely to be eliminated via the bile. Heavy metals, in general extensively bound to plasma protein that reduces kidney elimination, use the bile as the exit gate from circulation. Another group of compounds that use the bile as elimination route are bulky antigen complexes (Hofmann, 1989).

Hepatic elimination of xenobiotics is part of a vital role played by the liver. Its anatomical position, as mentioned before, is particularly advantageous for
removing toxicants from the portal blood after being absorbed in the gastrointestinal tract. A distinctive characteristic of the liver parenchymal cell is its direct contact with chemicals present in the sinusoidal blood without the shielding presence of vascular tissue. Therefore, xenobiotics can be extracted and/or biotransformed the first time they pass through the liver (first-past effect or presystemic hepatic elimination) and excreted into the bile or enter the systemic circulation and be excreted by the kidney.

On the other hand, chemicals can be transformed by the liver to more toxic metabolites that re-entering the blood might increase their toxicity. In this circumstance an increase in the presystemic elimination increases the toxicity of a chemical. However there is a large interindividual variation in the first-pass effect due to different hepatic metabolic activity and singular amounts of a given chemical might enter the systemic circulation in different individuals (Klaassen and Watkins III, 1984).

Most xenobiotics for which biliary excretion is a preferential route of elimination usually include those with a bile to plasma ratio greater than 1.0 (10 to 1000). One has to consider that biliary excretion of endogenous and exogenous compounds requires transport into and out of the liver. Thus, one compound may penetrate the sinusoidal membrane by passive diffusion and enter the bile by an active process or vice-versa or even undergo active transport across both sinusoidal and canalicular membranes. The liver metabolizes many endogenous and exogenous compounds, upon uptake, frequently by oxidative reaction followed by conjugation pathways that yield substrates, particularly glucuronide and glutathione conjugates, suitable for secretion into bile. The discovery of ATP-dependent mechanisms that regulate the transport of S-glutathione and glucuronide conjugates in canalicular membranes clearly unveil an important number implications in toxicology (Vore, 1993). Thus, the biliary excretion of S-glutathione conjugates of several toxicants might be considered as an initial step in the process of inducing toxic effects in other tissues (Monks et al., 1990).

Several factors were listed as able to influence biliary excretion of compounds (Klaassen and Watkins III, 1984). These include their physicochemical
characteristics, such as polarity and molecular size as well as the extent of plasma protein binding.

Biological factors such as variation between species, sex, age, nutritional status and pregnancy are other factors that control biliary excretion of xenobiotics. The pharmacological factors implicated in the biliary excretion of xenobiotics are the chemicals that interfere with the synthesis of various metabolizing enzymes, such as microsomal enzyme inducers; chlorinated compounds and hepatotoxicants in general decrease the hepatic excretory function. Liver injury generally produces deleterious effects on hepatic excretory function.

Another important factor to consider in biliary excretion of chemicals is its enterohepatic circulation and the factors that might influencing the enterohepatic cycle. Numerous chemicals, biotransformed or not, are secreted into the bile and deposited into the intestinal lumen to later be reabsorbed by the intestine into the portal blood and taken up by hepatocytes. Although this process enables the conservation of important endogenous substances, such as bile acids and vitamins it also allows drugs and xenobiotics to be in direct contact with liver.

Bile is primarily composed of water (97%), inorganic electrolytes (Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻, HCO₃⁻) and organic solutes such as bile acids, glutathione, cholesterol, phospholipids, bilirubin and proteins. Bile composition varies among species and upon the physiological and nutritional status of the animal at the time of collection. Table 1.1. gives a range of concentrations of the major components found in the bile of studied species.

In bile, electrolytes are generally present at concentrations similar to those found in the plasma. Its major cation is Na⁺, and bile acids, Cl⁻ and HCO₃⁻ functioning as balancing anions (Vore, 1991a). The osmolarity of the bile is usually equivalent to that of plasma and varies accordingly.
Bile salts are anions in the bile and are water-soluble products of cholesterol metabolism. In most vertebrates, one can distinguish two mechanisms and anatomical sites of bile acid biosynthesis. The first involves de novo synthesis in the hepatocyte from cholesterol and traditionally are denominated primary bile acids, containing two or three hydroxy groups. The second occurs during their enterohepatic circulation at the distal portion of the intestine where bacterial enzymes modify their hydroxyl substituents (Figure 1.6.). The most important of these modifications is a 7-dehydroxylation to form a class of bile acids that might be denominated as 7-deoxy bile acids and that are also called secondary bile acids. Dehydroxylation only occurs at the 7 position via the formation of a labile 3-oxo, Δ⁴-Δ⁶ resonating intermediate (Hoffman, 1994).

Table 1.1. Major components of bile in different species

<table>
<thead>
<tr>
<th></th>
<th>Man</th>
<th>Rat</th>
<th>Dog</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow (μl.min⁻¹.kg⁻¹)</td>
<td>1.5-15.4</td>
<td>30-150</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Na⁺ (mEq.l⁻¹)</td>
<td>132-165</td>
<td>157-166</td>
<td>141-230</td>
<td>145-156</td>
</tr>
<tr>
<td>K⁺ (mEq.l⁻¹)</td>
<td>4.2-5.6</td>
<td>5.8-6.4</td>
<td>4.5-11.9</td>
<td>3.6-6.7</td>
</tr>
<tr>
<td>Cl⁻ (mEq.l⁻¹)</td>
<td>96-126</td>
<td>94-98</td>
<td>31-107</td>
<td>77-99</td>
</tr>
<tr>
<td>HCO₃⁻ (mEq.l⁻¹)</td>
<td>17-55</td>
<td>22-26</td>
<td>14-61</td>
<td>40-63</td>
</tr>
<tr>
<td>Bile acids (mM)</td>
<td>3-45</td>
<td>8-25</td>
<td>16-187</td>
<td>6-24</td>
</tr>
<tr>
<td>Phospholipids (mg.l⁻¹)</td>
<td>1400-8100</td>
<td>412</td>
<td>1620</td>
<td>58</td>
</tr>
<tr>
<td>Cholesterol (mg.l⁻¹)</td>
<td>970-3200</td>
<td>223</td>
<td>250</td>
<td>15</td>
</tr>
<tr>
<td>Osmolarity (mOsmol.l⁻¹)</td>
<td>332</td>
<td>293</td>
<td>312</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from (Klaassen and Watkins III, 1984; Vore, 1991a; Erlinger, 1988).
Figure 1.6. Structures of major bile acids. Primary bile acids (e.g., cholic and chenodeoxycholic acid) are those biosynthesised in the liver from cholesterol. They are conjugated with glycine or taurine and enter the bile. Secondary bile acids (e.g., deoxycholic, lithocholic and ursodeoxycholic acid) are those formed by bacterial biotransformation of primary bile acids. All bile acids can return to the liver via the enterohepatic circulation.
The variation observed correlates well with the diet. Thus, herbivores (except bovines) conjugate primarily dihydroxy or monohydroxymonoketo bile acids with glycine, whereas carnivores use taurine to conjugate trihydroxy bile acids. Omnivores and bovines show significant amounts of both types (Klaassen and Watkins III, 1984; Hoffman, 1994). The conjugation of bile acids with taurine and glycine results in a pKa of 2 and 4 respectively.

To understand canalicular bile formation it is necessary to comprehend the mechanisms generating bulk movement of water into the bile canaliculi. These include filtration, vesicular transport and active transport of certain solutes that led to passive water flow. Another mechanism thought to be involved is the extrusion of materials by exocytosis (Klaassen and Watkins III, 1984).

The leading force in bile secretion is generally accepted to be that generated by the active transport of osmotically active agents into a confined space and the resulting passive movement of water until osmotic equilibrium is reached (Diamond and Tormey, 1966).

The pathways by which water and ions enter the biliary tree are of importance for the understanding of the mechanisms and regulation of bile formation and diagrammatically exemplified in Figure 1.7.

The uptake of bile acids across the sinusoidal membrane is carrier-mediated and appears to be dependent of the activity of Na⁺/K⁺-ATPase located in the basolateral membrane. Other transport mechanisms for bile acids across the sinusoidal membrane include intercellular binding and translocation and once translocated, bile acids are secreted across the canalicular membrane against an electrochemical and concentration gradient. The secretion of bile acids across the canalicular membrane is carrier-mediated, saturable and appears to be specific (i.e. other organic anions such as bilirubin and bromosulfophtalein do not exert competitive inhibition).
The good correlation existing between the bile acid secretory rate into bile and bile flow led to the assumption that bile acids acting as osmotic active solutes were responsible for a portion of the bile flow - bile acid dependent bile flow. However, bile acids exist in micellar form in bile and their osmotic
activity is generally less than that of non-associated molecules; thus their osmotic activity has been attributed to the accompanying counterions (e.g. sodium).

Nevertheless, canalicular bile is not produced completely by the osmotic properties of bile acids, since in the absence of bile secretion bile flow is not completely abolished. The portion of bile flow independent of bile secretion is so-called bile acid-independent bile flow (BAIF) and may account for approximately 50-70% of total bile flow depending on the species (Handler et al., 1994).

Transport of osmotically active organic and inorganic solutes into the canalicular space have been proposed to account for the subsequent movement of water. Thus the secretion of sodium, chloride or bicarbonate into the canalicular space are generally accepted as mechanisms of BAIF formation of bile (Klaassen and Watkins III, 1984). Glutathione has also been postulated to be a significant driving force in the generation of BAIF (Ballatori and Troung, 1989).

In addition to canalicular transport, substances may enter the bile via paracellular pathway. Indeed, the bulk of fluid movement into the bile is thought to occur primarily across the junctional complex from the blood to the bile via the paracellular pathway rather than across the sinusoidal membrane and then from the hepatocyte through the canalicular membrane-transcellular pathway (Vore, 1991a). The tight junctional complexes between hepatocytes act as permeability barriers preventing regurgitation of biliary components into the vascular space. However, these junctional complexes are relatively "leaky" to macromolecules, such as horseradish peroxidase (Lowe et al., 1985).

Other processes, such as pinocytosis followed by transcytosis and exocytosis may mediate the transport of other plasma components (i.e. proteins) to the bile (Coleman, 1987).
Finally, but not less important, once canalicular bile is formed it can be further modified by the biliary epithelial cell (Figure 1.7.). Although not fully understood, the biliary epithelia seems to be involved in the active reabsorption of glucose (Lira et al., 1992).

1.4. Hepatotoxicity

The particular position of the liver between the gastrointestinal tract and the systemic circulation, together with its microcirculation and partition of enzyme systems in different acinar zones, all contribute to the vulnerability of this organ to different toxic compounds. The liver is the primary site of biotransformation, followed by the lung, kidney, intestinal flora, skin and endocrine glands (Lindamood III, 1991). Biotransformation of xenobiotics is not a detoxifying process on its own, but a mechanism for converting poorly excretable lipophilic compounds to more readily excretable water-soluble molecules. These reactions carried out in the liver, may enhance the toxicity of the metabolized compounds. Many electrophilic reactive species are chemically reactive with basic cellular constituents, such as proteins, RNA, DNA. Fortunately, foreign compounds are generally metabolized in the liver to less toxic more polar and water-soluble compounds which can reach the systemic circulation and be readily excreted by the kidney. Another route of elimination is the excretion to the bile. Although the bile is route of excretion for several compounds, they can return to the liver via enterohepatic circulation and cause liver damage or they can cause reduction of bile flow that is associated with hepatotoxic effects caused by several compounds.

1.4.1. Mechanisms leading to cell injury and death

The processes leading to cell injury and finally to cell death can be divided into three different stages of events; primary, secondary and tertiary, 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
- change in thiol status
- enzyme inhibition
- ischaemia

**Secondary** events follow in the sequence leading to cell death. Structural or biochemical change occur, many of them interrelated and some of the changes are a consequence rather than a cause of the damage;

- membrane structure and permeability alterations
- changes in the cytoskeleton
- mitochondrial damage and inhibition of function
- depletion of ATP and other co-factors
- alterations on Ca\(^{2+}\) homeostasis
- DNA damage and poly ADP-rybosylation
- lysosomal disturbance
- stimulation of apoptosis
- damage to the endoplasmic reticulum

**Tertiary** events are those observable at the final stages. They may occur together within a tissue or they may happen sequentially:

- steatosis/fatty change
- hydropic degeneration
- blebbing
- apoptosis
- necrosis

All of these except the last two are potentially reversible (Timbrell, 1991). Apoptosis and necrosis are two distinct and differentiated types of cell death. Briefly, **apoptosis** or programmed cell death occurs in both physiological and pathological conditions and is a process in which cells die in a controlled manner in a response to a stimuli, following an innate programme (Fawthrop *et al.*, 1991). It helps the organism to keep a balanced cell number, in a variety of organs where circumstances impose that excessive cell numbers have to be eliminated. An imbalance between mitosis and its counterpart
apoptosis is now believed to be one the major mechanistic changes that mediate the development of cancer (Oberhammer and Roberts, 1994). Several morphological changes, such as the loss of contact with neighbouring cells, widespread plasma and nuclear membrane blebbing, chromatin condensation, fragmentation and aggregation of oligonucleotides at the periphery near the nuclear membrane and cellular organelles gathering with normal appearance for mitochondria as the most striking morphological aspects of apoptosis (Fawthrop et al., 1991; Oberhammer and Roberts, 1994).

In contrast to apoptosis, necrosis causes morphological abnormalities in the mitochondria with enlargement and sometimes disruption of the structure, swelling of the endoplasmic reticulum, appearance of vacuoles and accumulation of fluid in the cytoplasm, plasma membrane blebbing and pyknotic nuclear chromatin that may become fragmented.

The liver has a remarkable ability to overcome injury from physical or chemical agents in an attempt to enhance the survival of its cells and to preserve its role in the body. The mechanisms underlying this capacity are divided into two classes. First are the endogenous biochemical defence mechanisms that enable the liver to prevent injury after a noxious toxic insult; and second the biological response intended to overcome injury, by promoting hepatocellular regeneration and tissue recovery (Mehendale, 1991).

1.4.2. Cholestasis

The term cholestasis is derived from the Greek word chole that means gall or bile and stasis that means stagnation. Cholestasis is referred to the impairment, stagnation or cessation of bile flow caused by the mechanical obstruction of the biliary tract or absence of bile formation by the liver. As a consequence, there is retention in the liver cell and in the blood of biliary constituents (Phillips et al., 1986; Hofmann, 1989; Vore, 1991a).

Cholestasis is one of the main manifestations of hepatotoxicity induced by
drugs and many chemicals may produce an interruption of bile flow as their only or primary action (Plaa and Priestly, 1977). Cholestasis is thus a very broad term, which includes morphological, biochemical and clinical features. To consider the mechanisms underlying cholestasis, one has to establish, as close as possible, the site of obstruction to bile flow. Classifications have been devised to address the anatomic site of bile stagnation but due to the array of morphological and clinical conditions associated with cholestasis, it is believed that multiple factors are involved (Phillips et al., 1986).

Succinctly, the stagnation of bile flow can be due either to physical obstruction of the biliary tree, as is termed extrahepatic cholestasis or due to a decrease in the secretion of bile by the hepatocyte, denominated intrahepatic cholestasis.

Extrahepatic cholestasis is relatively easy to understand since it is caused by blockage or mechanical obstruction of the extrahepatic bile duct. This can be caused by bile duct stones, sclerosing cholangitis, carcinoma of the pancreas, of the biliary tree and also parasites (Phillips et al., 1986; Klaassen and Watkins III, 1984).

The possibility to distinguish extra- from intrahepatic cholestasis based on morphological features alone is not currently accepted, based on the observation that some of the morphological changes occurring in intrahepatic cholestasis are the result rather than the cause of the stagnation of bile (Vore, 1991b).

On the other hand, the intrahepatic cholestasis concept is less susceptible to be accurately defined without considering the morphoanatomical basis underlining the blockage, by biliary material from damaged bile ducts, of the intrahepatic bile ducts and small ductules (Phillips et al., 1986).

Recently the pathophysiological changes occurring during the biochemical processes required to the formation of bile, namely those involved in chemically induced intrahepatic cholestasis, received more attention and emphasis in investigations (Vore, 1991a; Handler et al., 1994).
Investigations on experimental intrahepatic cholestasis mechanisms suggested that several different functional alterations may be of relevance, and virtually all organelles in the liver cell can be conceived as being involved in bile secretion and hence, theoretically, in bile secretory disorders.

Several mechanisms were proposed to be involved in cholestasis, such as:

**Impairment in sinusoidal uptake;** considering that it is at the sinusoidal cell membrane that the uptake of bile acids, bilirubin and other components that end up in the bile occurs, alterations at the sinusoidal membrane level may lead to interference with the hepatocellular transport process and decreased bile flow. Examples are the decrease in bile acid uptake caused by ethinyl estradiol (i.e. decreasing Na⁺/K⁺ ATPase activity and abolishing Na⁺-dependent uptake of taurocholate in basolateral membrane vesicles) (as reviewed in Vore, 1991a) and the inhibition of the uptake of taurocholate by rat hepatocytes isolated from cyclosporin A-treated animals (Erlinger, 1988) leading to the hypothesis that the raised levels of individual serum bile acids were caused by the effects of cyclosporin-A on the hepatocellular uptake and accumulation (Reichen and Simon, 1988; Azer and Stacey, 1994).

**Disruption of tight junctions** caused by compounds that cause alteration at the contiguous membrane leading to permeabilization of the bilo-sinusoidal barrier with subsequent diffusion of osmotically active solutes from the bile to the plasma through the tight junctions is believed to be one of the mechanisms of ANIT-induced cholestasis (Krell et al., 1987; Vore, 1991a).

**Alterations in membrane lipids and membrane fluidity** caused by increased incorporation of cholesterol into the phospholipid membrane bilayer has been proposed as a mechanism of lithocholate-induced cholestasis (Phillips et al., 1986; Vore, 1991b). However, other compounds such as estrogens (and their glucuronide conjugates) are believed to alter the membrane fluidity by promoting binding to membrane phospholipids and thus altering the mobility of bile acid carriers and/or other functions associated with the generation of bile flow (Vore, 1991b).

**Alterations at the canalicular membrane** affecting transport mechanisms
and altering permeability were implicated in intrahepatic cholestasis (Reichen and Simon, 1988). Alterations in the network of microfilaments and microtubules especially present at the pericanalicular region were implicated in several mechanisms of chemically-induced cholestasis since they are believed to be important for maintenance of cell shape, motility and secretion of several substance, including lipoproteins, proteins and bile (Vore, 1991a; Phillips et al., 1986; Vore, 1991b). Colchicine (and other Vinca alkaloids) inhibits tubulin polymerization into microtubules disrupting various vesicle-mediated transport pathways believed to be important in the transcellular flux and biliary excretion when a bile salt load is present but not under physiological conditions (Vore, 1991a; Vore, 1991b).

Phalloidin, a bi-cyclic heptapeptide derived from Amanita phalloides, after being taken up by the hepatocyte disrupts the "toned contraction" of the microfilaments present in the pericanalicular ectoplasm postulated to facilitate canalicular bile flow by means of contraction of the canalicular wall (Phillips et al., 1986). Cytochalasin B also causes cholestasis by a mechanism believed to be involved with the dilation of canaliculi and loss of microvilli (Phillips et al., 1986).

The accumulation of F-actin in the pericanalicular area has also been implicated recently with compounds causing hepatocellular cholestasis by a mechanism believed to be involved with the disruption of the microfilament network critical in canalicular normal function (Thibault et al., 1993; Roman and Coleman, 1994).

Compounds that cause alterations in the metabolism (i.e. alterations in the conjugation rate, metabolic activation and inhibition, hypoxia and pH) or that are likely to disturb the haemodynamic condition of the liver (i.e. redistribution of blood flow and microvascular alterations) are also believed to be involved in the induction of intrahepatic cholestasis.

The complexity of mechanisms by which toxicants can cause cholestasis, together with the pathophysiological alterations observed in hepatocytes and biliary epithelia during the cholestatic process led to the development of
numerous techniques in vivo, ex vivo and in situ to evaluate the hepatobiliary function recently reviewed by Handler and coworkers (1994).

More recently, studies using isolated rat hepatocyte couplets have been developed aiming to understand how the canalicular function might be affected during oxidative stress and/or disruption of calcium homeostasis (Stone et al., 1994; Wilton et al., 1993).

1.5. Hepatotoxic compounds tested and mechanisms of action

1.5.1. α-naphthylisothiocyanate (ANIT)

ANIT (for chemical structure see Appendix VI) has been long used as a model for studying intrahepatic cholestasis (Roberts and Plaa, 1965). Although its mechanism of toxicity has not yet been established, several promising reports have been produced over the past ten years. A single oral dose of ANIT produces cholestasis, hepatocellular and biliary epithelial cell necrosis and bile duct obstruction in rats (Plaa and Priestly, 1977). It was initially reported that ANIT needed metabolic activation to exert its toxicity, since pretreatment with phenobarbital or with chlorpromazine markedly increased its toxicity (Plaa and Priestly, 1977). Conversely, pretreatment with SKF-525A (Plaa and Priestly, 1977) and N-decylimidazole (NDI) (Traiger et al., 1985) blocked the hepatotoxic response in rats. Furthermore, metabolic activation via a cytochrome P-450-dependent S-oxidation pathway was proposed by Traiger and co-workers as the initial step in the metabolism of ANIT (Traiger et al., 1984; Traiger et al., 1985). It was observed by Dahm and Roth (1991) that the agents used as MFO inhibitors and inducers may alter ANIT-induced liver injury by a mechanism other than interfering with hepatic MFO activity. Thus, agents that afforded protection against ANIT-induced liver injury decrease hepatic GSH content and/or at least one form of GSH S-transferase, whereas, agents that enhance the liver injury caused by ANIT increased hepatic GSH content and/or at least one form of GSH S-transferase (Dahm and Roth, 1991). The treatment of rats with BSO, an
agent that blocks the synthesis of GSH, protects the animals against ANIT-induced liver injury. A similar outcome was observed when other agents (DEM and phorone) which affect the levels of NPSH were tested (Dahm and Roth, 1991). Without ruling out a role for MFO in ANIT-induced hepatic injury, they have proposed a causal or permissive role for GSH in ANIT-induced liver injury (Dahm and Roth, 1991). An elevation of hepatic NPSH was found to be associated with the onset of liver injury, relating the impairment of the hepatic glutathione turnover (i.e. efflux of glutathione across the sinusoidal and canalicular membranes of the hepatocytes) with the possible mechanism by which ANIT-induces hepatotoxicity (Dahm et al., 1991a).

In isolated hepatocytes, ANIT forms an unstable GSH conjugate (GS-ANIT), causing a depletion of intracellular GSH with a concomitant increase in extracellular GSH. These observations supported the hypothesis that a GS-ANIT conjugate is formed intracellularly, is subsequently exported and decomposes into GSH and ANIT (Carpenter-Deyo et al., 1991). GSH conjugates are avidly secreted into bile. It has been proposed that the conjugation and further release of ANIT from a GS-ANIT conjugate may explain why the biliary epithelial cells are targets for ANIT toxicity in vivo (Carpenter-Deyo et al., 1991). A preliminary report by the same authors outlines the importance of the presence of a GS-ANIT conjugate in the bile for the onset of ANIT-induced cholestasis (Jean et al., 1994). Recently, it has been shown that ANIT induced an elevation of biliary glutathione with concomitant concentration of ANIT in the bile (Jean et al., 1995) . Furthermore, the same work shows an ANIT bile:plasma ratio greater than 1, up to 12 hours after dosing.

The accumulation of ANIT in the bile happens before the onset of cholestasis. It was proposed that due to the instability of the ANIT-glutathione conjugate and lipophilic nature of ANIT upon release, ANIT would be expected to be partitioned into the surrounding cells and/or bind to cellular constituents. Glutathione would be expected to be excreted or degraded into its constituent amino acids. It seems clear that ANIT concentrates in the bile upon release
from a glutathione conjugate and this plays a crucial role in the onset of the mechanism of cholestasis.

The primary event in the onset of ANIT-induced cholestasis was proposed to be a dysfunction of the tight junction complexes, with concomitant increased permeability in the diffusion barrier between the biliary and sinusoidal spaces (Kan and Coleman, 1986; Lowe et al., 1985; Krell et al., 1987). A later study showed that the morphological changes in the liver seen after ANIT treatment are secondary to bile duct obstruction resulting from desquamation of damaged biliary epithelium (Connolly et al., 1988). Kossor and co-workers have recently established a temporal relationship for the hepatobiliary and morphologic changes observed in rats following ANIT administration. Thus, it was proposed that ANIT-induced cholestasis is characterized by several distinct time related events; first, the onset of cholestasis is preceded or accompanied by changes in hepatocellular function and tight junction permeability and secondly, a more profound phase of hepatotoxicity is closely related to a combination of biliary cell damage, bile obstruction and hepatocellular dysfunction (Kossor et al., 1993b).

In accordance with this temporal relationship pattern, pathophysiological changes at a later and more profound phase of ANIT-induced cholestasis might be related to the neutrophil involvement featuring the ANIT-induced hepatotoxicity (Dahm et al., 1991b). They have shown that depletion of circulating neutrophils attenuates the injury caused by ANIT in the rat.

ANIT also activates PMN in vitro to release superoxide in a dose dependent manner (Roth and Hewett, 1990). However superoxidase dismutase and catalase failed to alter markers of liver injury or afford protection when injected before ANIT-dosing. This ruled out a direct involvement of PMN-released oxidative species in ANIT-induced hepatotoxicity (Dahm et al., 1991b). PMN challenged with ANIT also releases proteinases (Roth and Hewett, 1990), and the latter have been implicated in hepatocellular injury caused in vitro by PMN towards isolated hepatocytes (Guigui et al., 1988; Ganey et al., 1994; Mavier et al., 1988).

Another blood component, the platelets, were recently implicated as a
contributor to the expression of ANIT-induced hepatotoxicity, inasmuch as platelet depletion affords protection from ANIT-induced liver injury (Bailie et al., 1994b). The mechanism by which PMN contribute to ANIT-induced hepatotoxicity remains to be fully elucidated. Despite this, a hypothesis formulated in a recent review outlined the possibility of ANIT initiating the changes in liver homeostasis that will lead to release of PMN chemoattractants or possibly a direct effect of ANIT on PMN or priming PMN by increasing its responsiveness to secondary stimuli (Mehendale et al., 1994). According to the authors, stimulated neutrophils release toxic products that injure nearby hepatic parenchymal cells and bile duct epithelial cells (Mehendale et al., 1994).

1.5.2. 4,4’-diaminodiphenylmethane (DAPM)

DAPM (for chemical structure see Appendix VI) is a primary aromatic amine that is used extensively in a variety of industrial synthetic processes. DAPM is an important intermediate used in the production of several chemical products. It is an intermediate in the production of isothiocyanates, rigid polyurethane foam, epoxy resins and polymers. The industrial use of these compounds as insulation devices, wire coating, floor coverings, aircraft and spacecraft parts, curing agents (i.e. production and maintenance of electricity power generators) and in the production of elastomers, quiana nylon and other epoxy resin composite materials increase the potential risk of human exposure and intoxication (Bailie and Roth, 1992; Peterson et al., 1991; Kanz et al., 1992; Bailie et al., 1994a; Bailie et al., 1993).

Some of these products are in daily contact with the unaware human population since they are used in a panoply of medical equipment such as artificial dialysis devices, intra-aortic balloons, vascular grafts, potting material for plasma separators, ventricular assist devices, etc. Their high elasticity and tensile strength associated with a low density make these resins a valuable material for orthopaedic and odontological implants. Furthermore, risk of direct contact for the general population was apparent
when DAPM was shown to leach from medical devices during gamma-ray or autoclave sterilization (Shintani and Nakamura, 1989). The hepatotoxic effects of DAPM in humans came to light in the Epping jaundice incident in 1965 in which 84 people became ill, some of them seriously, with jaundice after having eaten bread made from flour accidentally contaminated with DAPM during transit in a lorry (Kopelman et al., 1966).

This jaundice outbreak has been followed and despite level of exposure, the follow up study suggested that there is no obvious link between current health status of survivors and the poisoning episode (Hall et al., 1992). One case of biliary cancer and possibly two cases of retinopathy do not provide, according to the authors, conclusive evidence of long term health sequelae. The relevance of the findings described in this follow up study to those occupationally exposed to DAPM is doubtful since the route of exposure was different as it was the dose and time of exposure. Nevertheless, high levels of DAPM metabolites in the urine were ascertained from urine collected in a cohort of power generator workers but no evidence of cancer hazard was found (Selden et al., 1992). However the authors emphasise that several limitations in the cohort, such as size, age and cancer latency hampered a definitive risk assessment and that dermal absorption should be highlighted in the future studies.

More recently, methodology to determine DAPM (and metabolites) in the plasma and urine was devised to better assess the occupational exposures which air monitoring would underestimate (Peterson et al., 1991; Sabbioni et al., 1994; Skarping and Dalene, 1995).

DAPM causes an array of histological and pathophysiological modifications to the liver of exposed animals. In rats, short-term oral administration causes necrotizing cholangitis with periportal necrosis (Gohlke and Schmidt, 1974), while subcronic exposure elicits inflammatory cell infiltration, severe bile duct proliferation and portal fibrosis/cirrhosis (Fukushima et al., 1979). Long-term exposure was associated
with liver tumours and hepatic lesions consisting of midzonal focal necrosis associated with marked leukocytic infiltrates (Schoental, 1968). Chronic exposure to rodents also indicated that DAPM is carcinogenic (Weisburger et al., 1984; Lamb et al., 1986).

Kanz and collaborators (1992) characterized the histological lesions of DAPM after a single acute dose of DAPM (250 mg.kg⁻¹) in the rat. They have shown that DAPM causes an early and selective toxic injury to epithelial cells of the major bile ducts associated with altered biliary function and bile constituents. Hepatic parenchymal injury was reported to be present at a later stage of DAPM-induced hepatotoxicity.

More recently, Bailie et al (1993) characterized the acute hepatotoxicity of DAPM in the rat, implying a dose- and time-related change in the markers of liver injury with a threshold dose between 25 and 75 mg.kg⁻¹. Metabolic activation via the cytochrome P⁴⁵⁰ monooxygenase system seemed to be required for DAPM to exert its toxicity. This study also urged the positive identification of DAPM metabolites.

DAPM oxidized metabolites, namely azo and azoxy compounds, were identified in vitro from microsomal preparations of rabbit liver (Kajbaf et al., 1992).

In a preliminary report, Kanz and Wang (1994) suggested that bile is the route of exposure of primary target cells (biliary epithelial cells) to DAPM or its metabolite(s) in the rat.

Another characteristic of DAPM-induced hepatotoxicity is an inflammatory response, consisting of moderate to large multifocal areas of polymorphonuclear leukocyte and mononuclear cell infiltration (Bailie et al., 1993; Kanz et al., 1992).

This phenomenon is evident at an early time point that precedes the onset of explicit hepatic dysfunction and necrosis in DAPM-induced liver damage (Kanz et al., 1992; Bailie et al., 1993).

Despite the fact that in situ neutrophil action has recently been implied as a contributory factor in the hepatic tissue damage exerted by other compounds (Hewett et al., 1992; Dahm et al., 1991b), Bailie et al (1994a) have
recently ruled out the direct leukocyte involvement in DAPM-induced hepatotoxicity.

1.6. Taurine

1.6.1. Introduction

First discovered in 1827 in the bile of the ox (*Bos taurus*), scientific interest in taurine was dormant for 140 years. It was only after a comprehensive review published by Jacobsen and Smith (1968) that taurine interest blossomed among those inquisitive enough to devote time to this unknown and ubiquitous amino acid. Since then, several International Symposia have been held and reviews published, fostering attention on this molecule. These covered interests in virtually all fields of biological sciences, ranging from the nutritional to the neuroscience field of research (Huxtable and Barbeau, 1978; Hayes and Sturman, 1981; Chesney, 1985; Zelikovic and Chesney, 1989; Chesney, 1987; Huxtable, 1986; Kendler, 1989; Gaull, 1989; Sturman, 1993; Wright *et al.*, 1986; Huxtable, 1992).

1.6.2. Physicochemical properties

Taurine, 2-aminoethanesulphonic acid, is a β-amino acid with a molecular mass of 125.1 daltons. It is a colourless compound, readily soluble in water (10.48 g.100 ml⁻¹, 25°C) (Jacobsen and Smith, 1968) with the amino group residing on the second or β-carbon atom relatively to a sulphonic rather than a carboxylic acid group (Figure 1.8.).

\[ \text{H}_2\text{N}^\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{SO}^\cdot_3 \]

*Figure 1.8. Chemical structure of taurine*

The sulphonate group confers stronger acidic properties to this molecule comparatively to carboxylate groups. With a dissociation constant (pKₐ=1.5)
equivalent to that observed for a mineral acid, such as hydrochloric acid, and a more acidic amine function (pKₐ=8.8) than other β-amino acids (β-alanine, pKₐ=3.6 and pKₐ=10.2), taurine is almost a complete zwitterionic molecule over the physiological pH range (Huxtable, 1992). This results in taurine having a high water solubility and low lipophilicity. Consequently, diffusion through lipophilic membranes is slow for taurine when compared to other carboxylic amino acids. This impermeability of biological membranes to taurine may be the underlying reason why high concentration gradients are maintained across membranes for this amino acid but not for other structural analogues (e.g. β-alanine), which show much higher non-saturable uptake values (indicating membrane diffusion) (Huxtable and Sebring, 1986; Huxtable, 1992).

In contrast to other amino acids, taurine forms less stable metal complexes with various transition metals. This diminishes one important feature of the biological activity of the dipole ions formed by the amino acids which is metal complexation. Therefore, despite a significant biological effect on calcium and zinc binding in the living systems, a direct contribution between taurine and metal seems to be minimal. The low stability constants obtained for taurine-metal complexes are the result of the sulphonate ion being a poor ligand (Wright et al., 1986). The failure to chelate, unlike most other amino acids, earned taurine the true designation "free" amino acid present in tissues (Gaull, 1989).

1.6.3. Biochemistry and distribution

Taurine, by its unique characteristics, has been considered the biochemically inert end product of methionine and cysteine catabolism. It is not used for energy production nor as a source of inorganic sulphate or organic sulphur and is not translationally incorporated into proteins, remaining free in the cytosol (Wright et al., 1986; Gaull, 1989).

To maintain such high concentration gradients, taurine is transported by a unique β-amino acid transport system, with high-capacity and high affinity,
unique β-amino acid transport system, with high-capacity and high affinity, Na⁺ dependent and responsive to other osmotic substances, such as glucose. With an extremely high intra to extracelluar gradient, maintained due to its hydrophillic properties taurine fulfils the requirements of an osmoregulator (Huxtable, 1992).

Taurine is found in the millimolar concentration range throughout the animal kingdom (except for protozoans). Invertebrates, namely marine arthropods, molluscs and crustacea, show in general high taurine concentrations (Jacobsen and Smith, 1968; Huxtable, 1992). In plants its presence appears to be sporadic (marine algae) and when found the concentration is low (nmol/g wet tissue). Generally, taurine concentrations are high in species having cells lacking rigid cell walls and low or absent in species where the cellular content is surrounded by a rigid cell wall (Huxtable, 1992).

### Table 1.2. Taurine concentrations in some mammals tissues

<table>
<thead>
<tr>
<th>Tissue (µmol.g⁻¹ or ml⁻¹)</th>
<th>Liver</th>
<th>Heart</th>
<th>Lung</th>
<th>Brain</th>
<th>Kidney</th>
<th>Muscle</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0.3-2</td>
<td>6</td>
<td>1-5, 11</td>
<td>2-4</td>
<td>1.4-1.8</td>
<td>2.5</td>
<td>0.08</td>
</tr>
<tr>
<td>Monkey</td>
<td>3-16</td>
<td>11-17</td>
<td>-</td>
<td>2.8</td>
<td>-</td>
<td>4-20</td>
<td>0.01-0.02</td>
</tr>
<tr>
<td>Cat</td>
<td>3-10</td>
<td>14,</td>
<td>-</td>
<td>2.3</td>
<td>3-6</td>
<td>4.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Dog</td>
<td>7.2</td>
<td>10</td>
<td>-</td>
<td>1.3, 2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>1-10</td>
<td>22-25</td>
<td>6-11</td>
<td>2-9</td>
<td>8-10</td>
<td>13-17</td>
<td>0.45</td>
</tr>
<tr>
<td>Mouse</td>
<td>-</td>
<td>43</td>
<td>-</td>
<td>2.5, 9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>-</td>
<td>9-12</td>
<td>-</td>
<td>1.0</td>
<td>1-1.8</td>
<td>9.2</td>
<td>-</td>
</tr>
</tbody>
</table>

In mammals, taurine is generally ubiquitously distributed. Cellular concentrations present in tissues are typically in the range of micromole per gram of wet tissue whereas plasma and other body fluids contain much lower concentrations (10-100 μM) (Wright et al., 1986; Huxtable, 1992). Taurine is present in higher concentrations in tissues where oxidants are generated (retina and neutrophils), are rich in membranes (liver) or that are excitable (brain and muscle). Liver taurine concentration vary more widely than in other organs.

The percentage of taurine accounting for the free amino acid pool in the intracellular content could be up to 60% (Huxtable, 1992), making taurine by far the most abundant free amino acid in tissues. In a human adult, 12 to 18 g. (100-150 mmol) of its body weight is taurine (Zelikovic and Chesney, 1989), whereas in the rat 1% of its dry body weight is taurine (Chesney, 1985). The high taurine concentration present in tissues, prompted investigators to find a role other than bile acid conjugation.

1.6.4. Functions of taurine

The oldest known function associated with taurine has been the conjugation with bile acids bile in the liver to form bile salts crucial for normal fat absorption in the intestine (Zelikovic and Chesney, 1989). Many other functions were discovered and can be associated with an interest to find new biological roles for this amino acid. These newly discovered functions for this molecule were compiled by Huxtable, in a recent review, that outlines the most important biological functions attributed to taurine (1992).
Table 1.3. Some biological functions of taurine

<table>
<thead>
<tr>
<th>Cardiovascular system</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiarrythmic</td>
<td>Anticonvulsant and anti-tremor actions</td>
</tr>
<tr>
<td>Positive inotropy at low calcium and negative inotropy at high Ca(^{2+}) levels</td>
<td>Modulator of neural excitability</td>
</tr>
<tr>
<td>Potentiation of digitalis inotropy</td>
<td>Maintenance of cerebellar function</td>
</tr>
<tr>
<td>Antagonism of calcium paradox</td>
<td>Thermoregulation</td>
</tr>
<tr>
<td>Hypotensive (central and peripheric)</td>
<td>Antiaggressive action</td>
</tr>
<tr>
<td>Retardation of lesion development in calcium overload cardiomyopathy</td>
<td>Central regulation of cardiorespiratory functions</td>
</tr>
<tr>
<td>Increased resistance of platelets to aggregation</td>
<td>Suppression of drinking and eating</td>
</tr>
<tr>
<td></td>
<td>Resistance to anoxia/hypoxia</td>
</tr>
<tr>
<td></td>
<td>Altered learning and motor behaviour</td>
</tr>
<tr>
<td>Retina</td>
<td></td>
</tr>
<tr>
<td>Maintenance of structure and function of photoreceptors, outer segments and tapetum lucidum</td>
<td></td>
</tr>
<tr>
<td>Liver and hepatobiliary system</td>
<td></td>
</tr>
<tr>
<td>Bile salt synthesis and conjugation</td>
<td>Modulation of neurotransmitter and hormonal release</td>
</tr>
<tr>
<td>Xenobiotic conjugation</td>
<td>Attenuation of hypercholesterolemia</td>
</tr>
<tr>
<td>Increased lipid and cholesterol solubility</td>
<td>Stimulation of glycolysis/glycogenesis</td>
</tr>
<tr>
<td>Reproductive system</td>
<td>Cell proliferation and viability</td>
</tr>
<tr>
<td>Sperm motility factor</td>
<td>Antioxidation</td>
</tr>
<tr>
<td></td>
<td>Regulation of phosphorylation</td>
</tr>
</tbody>
</table>

Adapted from (Huxtable and Sebring, 1986; Huxtable, 1992).

As shown above, taurine has been implicated to exert biological functions that might afford protection in highly-developed living organisms. Of the protective properties of taurine, those related to the liver are to be of concern in this dissertation.

1.6.5. Taurine metabolism

The only metabolic reaction that has been acceptably documented in which
taurine participates is conjugation with bile acids in the liver (Hayes and Sturman, 1981). Although this function is rather important, only 1% of the taurine body pool is believed to be involved in bile acid synthesis (Hepner et al., 1973).

Earlier reports, frequently describe isethionic acid (ISA) and inorganic sulphate as taurine catabolic products in mammals (Jacobsen and Smith, 1968). More recent work has minimized the importance of the former reaction (Fellman et al., 1978) while other literature, despite acknowledging that ISA occurs in the brain in rather small amounts, ruled out the quantitative importance that isethionic acid formation might have in the metabolic route of taurine (Huxtable, 1986). Another suggestion made is that further catabolism of taurine is the result of intestinal bacteria, this positively yielding isethionic acid and sulphate (Oja and Kondro, 1983).

Mammals are only capable of sulphur oxidation, not reduction. Therefore, reduced sulphur in the form of sulphur-containing amino acids (methionine and cysteine) is an essential component of the diet.

1.6.5.1. Taurine biosynthesis

Taurine is derived from methionine, an essential amino acid, and cysteine, a non essential amino acid. Besides being a taurine precursor, cysteine is also incorporated into most proteins, glutathione and CoA. However the concentration of cysteine in tissues is usually maintained at a low level (10 - 100 μM (Cooper, 1983). Several reports outline the toxicity of cysteine both in vivo and in vitro and liver glutathione (GSH) has been suggested as a reservoir for cysteine, since GSH is readily converted back to cysteine when cysteine levels drop (Higashi et al., 1983). Cellular cysteine levels are controlled mainly by the reactions using cysteine - protein and GSH synthesis and cyst(e)ine catabolism. In general, the synthetic processes will utilize cysteine preferentially to synthesise GSH but when there is a dietary excess of cysteine, simultaneous synthesis of protein and glutathione are supported. Cysteine used for protein synthesis is balanced by cyst(e)ine released by
protein degradation (Weinstein et al., 1988).

Taurine biosynthesis can be streamed via several processes linked with the enzymatic oxidation and conversion of sulphur amino acids. The sulphur atom is progressively oxidized at the expense of free energy that is wasted in animals, as they are unable to couple it to ATP synthesis (Huxtable, 1992). Several pathways were proposed to exist for the metabolism of methionine and cysteine to taurine (Figure 1.9.).

Taurine synthesis results in the liver predominantly via the formation and decarboxylation of cysteinesulphinate (Bagley and Stipanuk, 1994).

Thus, cysteine can be oxidised by cysteine dioxygenase to cysteine sulphinate and then it can follow two pathways both requiring pyridoxal phosphate:

A. Cysteine sulphinate can be further decarboxylated by cysteine sulphinate decarboxylase (CSAD) to yield hypotaurine. Hypotaurine is finally oxidised to taurine, by hypotaurine dehydrogenase (hypotaurine: NAD\(^+\) oxidoreductase)

B. Cysteine sulphinate can follow an alternative route and be oxidised to cysteine sulphonate, under catalysis of cysteine dioxygenase, and finally cysteine sulphonate is decarboxylated by cysteine sulphonate decarboxylase to yield taurine.

Alternatively, taurine synthesis from cysteine can occur via the formation of cysteamine. Taurine and hypotaurine have been shown to be derived from cysteamine (Awapara, 1979), but no enzyme has been found to catalyse the decarboxylation of cysteine directly to cysteamine. It was therefore proposed that cysteamine is generated during the biosynthesis of CoA and phosphopantetheine. Pantothenic acid is joined with cysteine to form phosphopantothenoyl-cysteine which is decarboxylated and loses a phosphate group and is finally cleaved to form cysteamine. Cysteamine dioxygenase then metabolises the cysteamine conversion to hypotaurine (Oja and Kondro, 1983). Although, this pathway was described to account for the formation of taurine, Weinstein and co-workers (1988) showed that only a small percentage, if any, of cysteine is converted to taurine via the cysteamine pathway.
Figure 1.9. Metabolic pathways involved in the synthesis of taurine; O-enzyme requiring pyridoxal phosphate - PALP; PAPS - adenosine 3’-phosphate-5’-phosphate. Modified from (Hayes and Sturman, 1981; Oja and Kondro, 1983; Waterfield et al., 1993b; Kaye et al., 1990; Zelikovic and Chesney, 1989).
A fourth route of metabolism has been proposed which utilizes serine and inorganic sulphate. This is known as the inorganic route or PAPS pathway. Inorganic sulphate reacts with ATP to form adenosine 5'-phosphate and 3'-phosphoadenosine 5'-phosphosulphate which in turn reacts with 2-aminoacrylic acid derived from serine to form cysteine sulphonate. It has been suggested by Oja and Kondro (1983) that this pathway may operate when other pathways are in some way impaired.

Although until recently some discrepancies were found between results from those that have worked with in vitro systems and those who have examined the problem in vivo, these seemed to have succumbed when methodological problems were resolved.

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 data from Griffith's group (1988; 1983) was in agreement with these results, demonstrating that slightly less cysteine sulphinate was transaminated (20%) and more decarboxylated. Thus the taurine pathway seemed to be favoured over the pyruvate pathway.

Although, cyst(e)ine may be catabolized in mammalian liver by both cysteinesulphinate-dependent and cysteinesulphinate-independent pathways (Weinstein et al., 1988; Griffith, 1983; Drake et al., 1987) to yield pyruvate and inorganic sulphate, only the former gives rise to taurine production (Bagley and Stipanuk, 1994).

Thus, the partitioning of cysteine catabolism between the formation of cysteinesulphinate and metabolism by the cysteinesulphinate-independent pathway is thought to be important in the regulation of taurine synthesis from cysteine.

Indeed, cysteine concentration was found to have a major effect on partitioning the cysteine sulphur to taurine in rat isolated hepatocytes (Stipanuk et al., 1992b). The same authors concluded that this increased conversion to taurine in response to an increased availability of cysteine was
primarily due to increased saturation of cysteine dioxygenase, the enzyme that catalyses the conversion of cysteine to cysteine sulphinate, with substrate. Furthermore, the cysteine dioxygenase activity plays a key role in the conversion rate of cysteine to taurine in freshly isolated rat hepatocytes (Stipanuk et al., 1992a) as it does in the in vivo situation (Hosokawa et al., 1988).

It was also shown that formation of glutathione, sulphate and taurine all increased considerably on a molar basis as cysteine concentration was increased in the hepatocyte incubation medium. Moreover, low cysteine availability favoured its utilization for glutathione, as this essential metabolite serves as a reservoir for cysteine (Higashi et al., 1983) and as a vehicle for cysteine delivery upon cleavage of the circulating glutathione by the membrane bound and externally located γ-glutamyltranspeptidase (Richman and Meister, 1975). When cysteine availability was increased the partitioning of cysteine metabolism favoured its catabolism to sulphate and taurine (Stipanuk et al., 1992b).

Stipanuk et al (1992a; 1992b) also proposed that taurine, when the supply of cysteine is limited, may come from dietary intake and conversely in the presence of high concentrations of cysteine, taurine formation and excretion may be an important route for the disposal of excess sulphur from methionine and cysteine.

More recently the same group of researchers concluded that the cysteine catabolic enzyme in the liver responds very differently to excess protein than to excess methionine or cysteine in a given diet. They found that cysteine dioxygenase seems to respond most strongly to excess sulphur amino acid intake whereas cysteinesulphinate decarboxylase activity seems to be clearly affected by an excess of protein intake (Bagley and Stipanuk, 1994; Bagley and Stipanuk, 1995).

Thus, rats fed a low protein diet supplemented with sulphur amino acids have increased cysteine dioxygenase activity that was paralleled by a greater total of catabolism products (taurine + sulphate) from cysteine. The same
authors have also shown that taurine production expressed as a percentage of total cysteine catabolism, when [\(^{35}\)S]-cysteine was added to freshly isolated rat hepatocytes, was significantly greater in hepatocytes isolated from rats fed a diet with excess of methionine or cysteine than for those hepatocytes isolated from animals fed the basal diet (Bagley and Stipanuk, 1995). The same study also found that L-methionine and L-cysteine supplementation of the diets had similar effects on cysteine dioxygenase activity, with a trend for a greater effect if was added methionine rather than cysteine.

As there are ways to increase the synthesis of taurine it is also possible to manipulate taurine synthesis in an attempt to decrease its levels in different organs. There are several ways to manipulate the synthesis of taurine:

a) using \(\beta\)-methylene-DL-aspartate, an irreversible inhibitor of cysteine sulphinate decarboxylase accountable for the decarboxylation of cysteine sulphinate to hypotaurine (Griffith, 1983). However, this compound also inhibits the transamination reaction catalysed by glutamate-oxolacetate transaminase (ALT) which is responsible for the conversion of cysteine sulphinate to pyruvate.

b) using \(\beta\)-ethyldene-DL-aspartate, which selectively binds to cysteine sulphinate decarboxylase yielding a potent specific inhibitory effect (Weinstein and Griffith, 1987). Although both compounds have been shown to reduce taurine liver levels in the mouse, their effectiveness to do so in the rat is questionable since the rat has a large taurine body pool associated with a high synthetic capacity (Griffith, 1983). Moreover, the inhibition of cysteine sulphinate decarboxylase limits the production of taurine via the cysteine sulphinate pathway, but it is ineffective in preventing its formation by other routes, such as the phosphopantetheine pathway.

c) providing cysteine sulphinate decarboxylase 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. Although it has been shown to inhibit the enzyme activity in vitro by >74% (Weinstein and Griffith, 1987) and by similar values in vivo in the mouse (Weinstein et al., 1988), it has a drawback as in order to block the synthesis of taurine, repeated administrations are required due to its rapid transamination to pyruvate.

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. Guanidinoethane sulphonate (GES) and β-alanine are the most commonly used (for chemical structures see Appendix VI).

Although GES is more effective at displacing taurine from the heart, retina and brain than β-alanine (Huxtable et al., 1979), the latter is more effective at depleting the taurine levels in the liver and gastrocnemius muscle (Shaffer and Kocsis, 1981). However, GES also accumulates in tissues probably because, like taurine it is not metabolised. It was also suggested that GES reduces tissue taurine levels by interfering with taurine synthesis (Huxtable, 1982). Indeed, GES has been shown to reduce the activity of cysteine decarboxylase and cysteine dioxygenase in the liver (Ide and Murata, 1994).

On the other hand, β-alanine does not accumulate probably because it is readily converted to malonic semialdehyde and eliminated as CO₂ (Shaffer and Kocsis, 1981) and does not appear to affect taurine synthesis. β-alanine utilizes the same β-amino acid active uptake system in the kidney as taurine (Goldman and Scriver, 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 (Shaffer and
1.6.6. Taurine body pool, turn over and excretion

Metabolic studies have suggested a multicompartment model for taurine metabolism with a total body pool of 100-150 mmol (12-18 g) in human species (Sturman et al., 1975) and 2-3 mmol in the rat (Sturman, 1973). The largest taurine pool is present in the skeletal muscle, accounting for almost 75% of total taurine pool (Sturman et al., 1975). Other organs or tissues with a high content of taurine are the heart, pineal and pituitary glands, brain, adrenals, retina and lung (Kendler, 1989).

The kidney appears to regulate the total body pool of taurine (Chesney, 1985). Thus, depending on an adaptative response modulated by a differential reabsorption in the proximal tubule, the kidney responds to fluctuations in dietary taurine by conserving this amino acid during the times of need and excretes it during times of surplus, controlling the total taurine body pool (Gaull, 1989; Trachtman et al., 1993).

The Na\(^+\) and Cl\(^-\)-dependent β-amino acid transporter responsible for taurine reabsorption is located in the proximal tubule brush border membrane (Chesney, 1985). Recently cDNA for the sodium chloride ion dependent taurine transporter was cloned in the basolateral membrane of Madin-Darby canine cells (MDCK) and was shown to be responsive to hypertonicity (Uchida et al., 1992). Taurine is reabsorbed from the renal filtrate by the action of a group of transport systems that also interact with other β-amino acids (e.g. β-alanine) and GABA. Despite a strong affinity, the reabsorption capacity of taurine by this system is lower for taurine (90-95%) when compared to other amino acids (97-99%) (Chesney, 1985). For this reason taurine is the main amino acid in rodent urine and a major amino acid in human urine (Jacobsen and Smith, 1968).

Conservation of taurine also takes place during pregnancy in all mammals. Taurine is conserved and transferred to the foetus hence its synthetic capacity is limited. During the early period of lactation a high taurine concentration...
is maintained in the milk but a decline is observed towards the time where the offspring mature. This might be due to an undeveloped renal tubular transport system present at an early time in life in the pups that for most species is revealed as an aminoaciduria in the earlier period of their lives (Zelikovic and Chesney, 1989).

The turn-over of taurine in the body varies from organ to organ. Thus, parenchymal organs such as liver, kidney and pancreas exchange rapidly with injected radioactive taurine showing a half-life for taurine pool in these organs of less than 1 day; whereas taurine in tissues such as the heart, skeletal muscle and brain exchange taurine slowly, with a half-life of less than three days (Spaeth and Schneider, 1974a; Hayes and Sturman, 1981).

Urinary taurine excretion is increased after several insults and/or situations, such as radiation, severe burns, surgical trauma, acute alcohol intake (Kay et al., 1957; Jacobsen and Smith, 1968) and disorders that cause amino aciduria generally produce hypertaurinuria (Kendler, 1989). Although raised urinary taurine levels observed after tissue damage were invariably attributed to the overflow of taurine to urine proceeding from damaged tissue (Chesney, 1985), it was also demonstrated that in the case of radiation damage the occurring hypertaurinuria was more likely to be due to alterations in sulphur amino acid metabolism (Kay et al., 1957).

The idea that urinary taurine could serve as an indicator of protein quality has been explored by several investigators (Hosokawa et al., 1988). In these studies, rats were fed diets with different protein contents. Depending upon the absence of the limiting amino acid, animals were not able to synthesise proteins and the excess of sulphur amino acids was catabolized to taurine. The dramatic increase of urinary taurine was attributed to the absence of lysine in a gluten diet, and cysteine and methionine in a soy protein or casein diets (Hosokawa et al., 1988).

When rats were fed a vitamin B₆ deficient diet, an inhibition of taurine synthesis was observed. The enzymes cystathionine and cysteine sulphinate
decarboxylase are both pyridoxal phosphate dependent enzymes (Figure 1.9.),
thus the formation of taurine via the transsulphuration pathway was
inhibited and a concomitant decrease in the urinary taurine levels was
observed (Sturman, 1973; Yamaguchi et al., 1975).
A similar observation was also reported by Bowden and Goyer (1962)
following cardiac muscular necrosis induced by 8-(3-diethylaminoproplamineno)-6-methoxyquinoline ("Plasmocid").
Hepatotoxic compounds were shown to raise urinary taurine levels. Thus,
carbon tetrachloride increased taurine in the urine (Waterfield et al., 1990;
Waterfield et al., 1991; Waterfield et al., 1993d) and it was early proposed
that the phenomenon was part of a general aminoaciduria (Cornish and Ryan,
1964).
Yoshida and Hara (1985b) demonstrated that chlorobenzene resulted in a
significant reduction in both hepatic taurine and urinary taurine which was
though 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, cysteine, this would explain the reduction of taurine levels.
It was shown by several investigators that compounds that deplete GSH did
not increase the urinary levels of taurine (Sanins et al., 1990; Yoshida and
Hara, 1985a).

1.6.7. The protective properties of taurine

1.6.7.1. Bile salts conjugation and detoxication

Bile salts play an important role in digestion and absorption of fat in
mammals, especially during the early neonatal period. They act on the
surface of the emulsified lipid droplet that contains triglycerides and increase
their water solubility. In addition they also promote the action of a lipase
contained in human milk (Chesney, 1985).
The comparative relationship of bile acid conjugation with taurine and lipid
metabolism is of interest to man and other mammals, since metabolism of
cholesterol appears to be associated with the ratio glycine/taurine (G/T) in bile.
Consequently, herbivores such as the rabbit and guinea pig, whose normal diets do not include cholesterol, present a higher G/T ratio and a low level of hepatic taurine synthesis. On the other hand omnivores, such as the rat, monkey and man, which ingest considerable amounts of cholesterol, have considerably larger pools of taurochenodeoxycholic acid and lower G/T ratios than herbivores (Hayes and Sturman, 1981).
The best understood function of taurine, until recent years, was its property to conjugate bile acids and xenobiotics in the liver (Emudianughe et al., 1983). Cumulative evidence showed that taurine may serve as a conjugator and detoxifier of various toxic endogenous compounds (Wright et al., 1986).
Lithocholate and some of its conjugates are known to induce intrahepatic cholestasis in a variety of experimental animals (Yousef et al., 1981). Lithocholic acid sulphate when administered to guinea pigs and rats, caused cholestasis and pathological changes in the liver. Administration of taurine in the drinking water prevents the cholestasis and the resulting liver damage (Dorvil et al., 1983; Hunter and Grimble, 1994).
The monohydroxylated bile salts, secondary bile salts, are formed by degradation of the primary bile salts by the bacterial flora of the gut. Thus, during enterohepatic circulation of bile acids, some of the major primary bile acids undergo bacterial degradation to yield deoxycholic and lithocholic acids. One of the advantages of bile acid conjugation is that it results in a smaller proportion of secondary bile acids in the pool, equivalent to less potential toxic material (Wright et al., 1986). Taurine serves this function well.

Both hamster and man obtain taurine principally from the diet, and hepatic taurine conjugation of bile acids in these species accounts for 30% to 35% of the total bile acid pool (Gaull, 1989). Glycine and taurine bile salts show different physicochemical properties, with taurine, conjugates being more water soluble and less toxic than their glycine counterparts (Hoffman and Roda, 1984).
Taurine has been given to patients with acute hepatitis and a reduction in the glycine:taurine ratio was observed in association with reduction of bilirubin and total bile acids. Taurine administration also reduced the icteric period when compared to controls. In another study taurine was used together with ursodeoxycholate in the treatment of gallstone formation. The underlying idea was that the ursodeoxycholic acid tauroconjugate is more soluble than its glycine conjugate, thus increasing the probability of dissolving cholesterol and other nonpolar lipids (Igimi and Carey, 1981).

There are several pieces of evidence for the detoxifying properties of taurine, such as conjugation with retinol in the liver to form retinotaurine (Skare et al., 1982). Taurine has an amino group that is able to react with organic carboxylic acids and form peptide-like linkages. These properties were used to identify several taurine conjugates in the ferret (Emudianughe et al., 1983).

Taurine properties to detoxify endogenously produced toxins, have also been claimed as secondary bile acids are detoxified by conjugation with taurine (Wright et al., 1986).

Numerous xenobiotics are metabolized and conjugated with taurine in a variety of species (Huxtable, 1992). Other examples include the prostaglandin E₂ analogue, trimoprostil (Kolis et al., 1986) and 2-(4-chlorophenyl)thiazol-4-yl acetic acid (fenclozic acid), the latter excreted into rat bile as a tauroconjugate in a dose dependent manner (Bradbury et al., 1981).

Recently, a taurine conjugate of the widely used nonsteroidal anti-inflammatory drug, Ibuprofen was isolated in human urine by tandem mass spectrometry (Shirley et al., 1994).

Taurine can be included with sulphate, glucuronic acid and glutathione as a conjugation substrate for xenobiotics, acting by increasing polarity, water solubility and, in most cases, facilitating the clearance of the xenobotic (Huxtable, 1992).
1.6.7.2. Taurine deficiency

Taurine is a component of the dietary regimen in most omnivores and most mammals efficiently preserve taurine. Others, such as herbivores, despite their inability to obtain it from the diet, are able to synthesize this amino acid. Several animal models were investigated and clinical data was also collected from humans and evaluated in the past two decades.

The cat is appreciably, the most widely used animal model for studying taurine deficiency. The cat has low levels of activity of cysteine sulphinate decarboxylase, the enzyme that limits the rate of taurine biosynthesis, and is, therefore dependent on the dietary source to maintain its body pools (Sturman, 1993).

When cats were fed a diet deficient in taurine, the result was a malfunctioning of several systems including the visual system with a decrease of visual acuity, abnormal electroretinograms and degeneration of retina and tapetum lucidum (Hayes et al., 1975). The reproductive system was also affected resulting in spontaneous resorption, abortions and abnormal ontogeny of the surviving offspring. Another system where alterations were reported is the immune system, where changes in the leukocyte proportions and function were observed together with alteration of spleen function and morphology (Schuller-Levis et al., 1990).

The cardiovascular system is one of the more affected by taurine deficient diets, in which myocardial failure (dilated cardiomyopathy) is a common feature (Sturman, 1993).

The importance of taurine in maintaining normal retinal function has been identified in man. Children on long-term parenteral nutrition have been shown to develop abnormal electroretinograms (ERG) which became normal with addition of taurine to the intravenous solutions (Geggel et al., 1985).

Rats made taurine-deficient by treatment with GES showed abnormal electroretinograms and changes in the retinal structure. Intravenous infusion of taurine decreased the retinal damage and improved the ERG abnormalities in a dose-dependent manner (Shimada et al., 1992).
1.6.7.3. Osmoregulation

For a long time, taurine was suggested to be involved in osmoregulatory functions or to maintain osmotic balance in marine animals (Krogh, 1939). More recently, Huxtable wrote the statement "Taurine meets the requirements for a biologically perfect osmoregulator almost ideally". The reasons given were its transport being carried out by a system unique to β-amino acids and that this transport system is responsive to ionic changes (Na+/Cl' dependent) and also to other osmotic substances such as glucose. These reasons taken together with the fact that taurine is a rather lipophobic molecule generates conditions for high intra- to extracellular concentrations. These can be maintained without the expenditure of energy, sparing other metabolically important amino acids (Huxtable, 1992).

Therefore, a primary osmoregulatory function proposed for taurine helps to explain why this amino acid has an ubiquitous presence in the animal kingdom but it is absent (or present in low amounts) in the walled cells of the bacterial and plant kingdoms (Huxtable, 1992). As a relatively small inert molecule, taurine can accumulate without causing a significant effect on the metabolism of the cell and facilitate the well documented osmoregulatory role for taurine in marine invertebrates. Taurine constitutes the major intracellular osmolyte in the hepatocytes of the little skate (Raja erinacea) (65 mM) and is selectively released after cell swelling (Ballatori and Boyer, 1992).

Osmoregulation in isolated hepatocytes and perfused livers of the elasmobranch little skate is mediated by the uptake or release of intracellular taurine. When challenged by a hypotonic stimuli, the perfused livers release all of the taurine into the sinusoidal perfusate, this effect is reversed by restoring isotonicity. The same authors localized a receptor in the basolateral membrane and it appears to require a sustained intracellular concentration of ATP to maintain its function (Ballatori et al., 1994). Isolated rat liver when perfused with a hypotonic solution showed a significant increase in its mass and a 1.7 to 14-fold increase of taurine in the
perfusate, respectively when the hypotonic stress was set to -50 mosM or -100 mosM. This release was specific for taurine since no other amino acid followed such pattern under these conditions (Brand et al., 1994).

Evidence is also available for the role of taurine in osmoregulation in the mammalian brain. Thus, during sustained hypernatremic dehydration free amino acids were reported to accumulate in the brain with a high percentage being identified as taurine (Thurston et al., 1980). Later, Trachtman and co-workers (1990) obtained direct evidence that taurine works as a cerebral osmoprotective molecule limiting brain cell shrinkage during chronic hypernatremic dehydration.

Further experiments confirmed that taurine is also involved in hyponatremic states. It was concluded that taurine is an osmoregulatory molecule in cerebral and extracerebral tissues during severe hyponatremia. Furthermore, the authors speculated that reductions in the brain cytosolic taurine content might play an important role in the long-term cell volume regulatory response to hypoosmotic states (Trachtman et al., 1990).

Recently, a study revealed that marked changes in taurine transport occurred in cortex slices chronically exposed to a hypernatremic solution. These changes were associated with accurate cell volume maintenance and were reversed slowly following the return to normonatremia (Law, 1995).

Taurine unequivocally plays an important role in brain osmoregulation and considering the turnout for this area of research, important discoveries can be predicted for near future.

Human lymphocytes were other cell type used in the study of hyposmolarity-sensitive release of intracellular molecules, namely free amino acids (Pasantes-Morales et al., 1991). Pasante-Morales and collaborators (1991) started investigations aimed at the involvement of free amino acids (FAA) in the mechanisms of cell volume adjustment. Taurine accounts for 65% of the total FAA pool in human lymphocytes with a concentration around 35mM. It was postulated that non-electrolyte solutes, especially free amino-acids, may
be important to compensate a lymphocyte intrinsic anion deficit in respect to
cations. Furthermore they have shown that human lymphocytes possess a
hyposmolarity-sensitive release of FAA, especially of the most abundant one,
taurine. This process is now believed to contribute to the volume regulatory
decrease in lymphocytes (Jesus-Garcia et al., 1991).

1.6.7.4. Cardioprotection and the "calcium story"

The high content of taurine in mammalian heart tissue has long been
reported (Jacobsen and Smith, 1968). These high levels are maintained at the
expense of ATP in an energy requiring process (Huxtable et al., 1981).
Taurine levels in left ventricular myocardium from patients that had died of
congestive heart failure were double the levels of those who perished due to
other heart pathology (Huxtable and Bressler, 1974). Although the
physiological role of taurine in the heart remains undefined several reports
have mentioned this amino acid and its various actions in this tissue.

Studies conducted in humans led authors to conclude that taurine improves
the overall haemodynamics in patients with congestive heart failure
(McBroom and Welty, 1977).
Young adult male patients with borderline hypertension had their systolic
and diastolic blood pressure reduced by taurine treatment when compared to
placebo-treated controls.
Taurine has been shown to act in experimental models, reducing the $\text{Ca}^{2+}$
overload and the resultant necrotic lesions in the cardiomyopathic hamster
(McBroom and Welty, 1977) and to counteract isoprenaline-induced $\text{Ca}^{2+}$
accumulation and myocardial necrosis in chicks (Ohta et al., 1986). Later, the
same authors, suggested that the protective mechanism was based on several
factors such as the suppression of lipoperoxide formation, inhibition of
phospholipid degradation and reduction of calcium overload in the hearts of
taurine treated chicks (Ohta et al., 1988).
Azuma and collaborators (1987) showed that taurine protects against the
cardiac cell damage induced by isoproterenol and Adriamycin, through a mechanism also believed to be mediated by the reduction of the myocardial calcium overload.

The observations that cats fed a taurine deficient diet developed cardiomyopathy and that this condition was reversible upon dietary supplementation with taurine (Pion et al., 1987) were paralleled by other studies where it has been shown that the dilated cardiomyopathy observed in the fox is associated with dietary deficiency of taurine and this disease is more likely to occur in young animals with low hepatic cysteinesulphinate decarboxylase (Moise et al., 1991).

Interaction between taurine and membrane phospholipids has become a field of extensive research. The direct effects exerted by taurine on phospholipid components in membranes may modify other lipid-dependent phenomena, including the operation of ion channels, the regulation of membrane bound enzymes and protein phosphorylation processes as suggested by Huxtable (1987).

The theory that has been proposed as the unified hypothesis for taurine action encloses a triad of compounds. Thus, taurine, phospholipids and calcium were proposed by Huxtable and Sebring (1983) to interact in a way that weak ion-ion forces were developed between taurine and the positively charged group of neutral phospholipids. This interaction increases the negatively charged phospholipid region, thereby enhancing the affinity of the phosphate moiety for cations, such as calcium.

Another theory was proposed by Chovan and co-workers (1980) who maintained that the initial step in the action of taurine involves an interaction with a protein, upon the discovery of two taurine-binding proteins on cell membranes prepared from either rat or dog heart.

Recently, it has been demonstrated that taurine inhibits the myocardial phospholipid methyltransferase, an enzyme that catalyses the methylation of phosphatidyl ethanolamine to phosphatidyl choline in the heart sarcolemmal membrane (Hamaguchi et al., 1991). The methylation of phosphatidyl ethanolamine to phosphatidyl choline was reported to play an important role
in the regulation of a number of calcium transporters, such as the sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchanger. Taurine by inhibiting the methyltransferase reaction may exert a cardioprotective effect once it prevents the loss in Na\(^+\)-Ca\(^{2+}\) activity and also outlines its possible regulatory effect in heart calcium homeostasis (Hamaguchi et al., 1991; Schaffer and Azuma, 1992).

1.6.7.5. Antioxidant properties

The high concentration of taurine found in cells which generate oxidants, such as retina (50-70 mM) and neutrophils (50 mM) stimulated great interest in taurine as a possible antioxidant (Green et al., 1991; Sturman, 1993). Neutrophils are known to elicit a respiratory burst response to extracellular stimuli culminating in the extracellular release of several oxidant species including superoxide, hydrogen peroxide, hypochlorous acid and possibly hydroxyl radicals (Weiss et al., 1983; Samuni et al., 1988). Evidence suggested that taurine reacts with hypochlorous acid produced by neutrophils yielding taurochloramines (Grisham et al., 1984a; Thomas et al., 1985). The formation of these relatively stable taurochloramines is believed to attenuate the toxicity of hypochlorous acid (Wright et al., 1986; Huxtable, 1992).

The subcellular location of taurine and its precursor, hypotaurine, in human neutrophils may shed some light on the antioxidant action of both compounds. Thus, it was observed that hypotaurine is converted to taurine under conditions in which hydroxyl radicals are expected to be generated via a Fenton reaction (Green et al., 1991). The antioxidant role of hypotaurine and taurine or its direct involvement in the formation of taurochloramines or other oxidants reaching the cytosol in activated neutrophils is not fully understood. When lymphoblastoid cells were exposed to an iron-ascorbate mixture, taurine (1-10 mM) protected against the cytotoxic effect of the mixture. A maximal taurine effect was obtained at a concentration of 5 mM. It was suggested that taurine may affect factors associated with membrane damage occurring during peroxidative processes. However, the presence of taurine does not decrease lipid peroxidation under condition where cell
viability is preserved. The oxidising system acts by interfering with membrane stability causing formation of crenations and protrusions (blebbs). It was proposed that the effect of taurine on cell swelling and distortion might be mediated by an effect on calcium permeability through the injured membranes (Pasantes-Morales et al., 1985).

Apart from hypotaurine, other similar compounds such as the amino acids glycine and β-alanine showed only a slightly protective effect and cysteine was ineffective.

In another in vitro system, red blood cells were protected by taurine from osmotic shock and oxidative damage resulting in haemolysis (Koyama et al., 1992).

Due to its ability to react with and thereby remove hypochlorous acid to form taurochloramines, taurine attenuates the DNA damage caused by aromatic amino compounds in vitro (Kozumbo et al., 1992). Taurine is believed to protect neutrophils against the toxic effects of the hypochlorous acid itself generated in situ (Wright et al., 1985) and also decreases the oxidation of haemoglobin in vitro by neutrophil activity (Grisham et al., 1984b).

The long lived, low molecular weight, hydrophilic oxidizing agents mono-N-chloroamines (RNHCl) formed by the reaction of hypochlorous acid with ammonia (NH₄⁺), taurine, α-amino acids and peptides, accumulate in the extracellular medium. These RNHCl derivatives showed low cytotoxicity but reacted with ammonia to yield the lipophilic oxidizing agent monochloramine (NH₂Cl) that has bactericidal, cytotoxic and cytolytic properties (Grisham et al., 1984a).

It was shown that taurine protects neutrophils and other cells against oxidative attack and damage by acting as a trap for hypochlorous acid and by competing with the endogenous NH₄⁺ for reaction with hypochlorous acid (Grisham et al., 1984a).

When the fate of these oxidants was studied in vitro, evidence for a role of taurine in the oxidative toxicity of neutrophils towards erythrocytes was showed by Thomas and co-workers (1985). Under physiologic conditions the
Chlorination of taurine by neutrophils and the subsequent uptake and reduction of the anionic taurochloramines by erythrocytes prevents the accumulation of oxidants and may exert a protective effect in blood cells, plasma components and tissues against oxidative toxicity (Thomas et al., 1985).

Recently it was shown in vitro using the cat lung epithelium cell line - AKD, that taurine converts HOCl/OCl⁻ to chloramines, which are less cytotoxic than HOCl/OCl⁺, but these chloramines are likely to be taken up by the lung epithelial cell through an anion transport mechanism and subsequently induce cytotoxicity. In this model, lung epithelial cell injury is decreased under conditions that favour the formation of the less toxic taurine monochloramines, such as an excess molar ratio of taurine to the HOCl/OCl⁻ and a alkaline pH. Conversely, in an acidified milieu the protective role of taurine in the lung epithelial cells against the myeloperoxidase-derived oxidants is significantly impaired (Cantin, 1994).

High levels of taurine and hypotaurine are also found in parts of the male and female reproductive system where it is suggested that hypotaurine acts as an antioxidant being oxidized to taurine in the process (Holmes et al., 1992; Huxtable, 1992). The high concentration of hypotaurine in the male reproductive tract is believed to function as an antioxidant, protecting the highly unsaturated membranes of spermatozoa. Upon ejaculation, sperm changes from an anaerobic to an aerobic environment and is subject to a considerable oxidative stress, as superoxide anion is formed aerobically. A recent review integrating the concepts of hypotaurine acting as antioxidant, proposed that the millimolar concentration of hypotaurine found in the ejaculate and oviductal fluid may serve to protect the highly unsaturated membranes of sperm from the high oxygen tension found in the female genital tract (Huxtable, 1992). Moreover, it was proposed that hypotaurine is enzymatically oxidised to taurine via the formation of a disulphone that may serve as a hydroxyl radical trap.
Using isolated rat alveolar macrophages, Banks et al (1990) showed that after ozone exposure, taurine is increased in the cytoplasm of challenged cells. If the increase in cellular taurine is an event associated with a delay in cellular damage, as the cell preserves its membrane integrity, it was also observed that the intracellular taurine concentrations fall along with all indicators of cellular viability. This sequence of events observed was postulated to be consistent with a proposed antioxidant role for taurine.

The same authors showed that when intracellular taurine in rat alveolar macrophages reaches 100 µM (similar to plasma concentration in the rat) a significant decrease in cytotoxicity was observed and it occurs along with alleviated biochemical effects of ozone exposure that include decreased lipid peroxidation, slower loss of ATPase activity and leakage of glutathione and protein (Banks et al., 1992).

Several workers have shown that taurine has in vivo protective properties against models of oxidant injury.

Thus, Gordon et al (1986) described the use of dietary taurine to protect hamster lung epithelium from acute nitrogen dioxide (NO₂) injury. The prophylactic administration of taurine (0.5% solution) has proved to be effective in reducing inflammation and morphological changes in the lung induced by NO₂. Later the same group described that taurine given in the drinking water prior to dosing prevented the morphopathological alteration in the lung observed after paraquat and bleomycin administration (Gordon et al., 1992).

Testing the effectiveness of taurine in the prevention of inflammation and fibrosis caused by bleomycin and paraquat, they were able to obtain qualitative evidence for the effect of taurine in protecting the early oedema and inflammatory response. It was speculated that taurine may interact with oxidants or free radicals directly or it might stabilize the plasma membrane hampering lipid peroxidation. It might interact or bind to the plasma membrane of the lung epithelium and prevent the release of lipid peroxidation products that act as chemotactic factors for the recruitment of inflammatory cells. These elicit an inflammatory response, migration and
proliferation of fibrocytes responsible for the development of fibrosis (Gordon et al., 1986; Gordon et al., 1992).

Other authors also have shown that taurine alone or given with niacin, a B vitamin, ameliorates pulmonary fibrosis occurring after administration of bleomycin in hamsters (Wang et al., 1991). They have speculated that an antioxidant effect and/or a membrane stabilization effect were responsible for the combined effect of the prophylactic treatment, resulting in decreased malondialdehyde, superoxide dismutase and calcium content, indicating a reduced toxic effect. Moreover they proposed that taurine and/or niacin, regardless of the mechanism, offered a novel therapeutic approach in the prevention of chemically-induced pulmonary fibrosis (Giri and Wang, 1992).

Later it was shown that taurine/niacin blocked the formation of collagen-cross links, a process believed to be actively involved in the development of lung fibrosis, preventing the deposition of collagen in the lungs of bleomycin treated hamsters (Blaisdell et al., 1994). More recently, Giri and co-workers (1994) demonstrated that dietary supplementation with taurine and niacin offered almost complete protection against the lung fibrosis caused by a multidose exposure to bleomycin in the hamster model. The beneficial effect of the combination of taurine and niacin against lung induced fibrosis was attributed to the decreased influx of neutrophils into various compartments of the lung as well as the availability of taurine to trap HOCl. Moreover, intracellular oxidation and calcium rise induced by bleomycin in isolated rat alveolar macrophages were inhibited if exposed cells were pretreated with various concentrations of taurine (Bhat et al., 1994). The authors claimed that preloading the cells with taurine might provide protection against a rapid intracellular calcium rise that seems to precede the oxidative process caused by the bleomycin-Fe^{2+}-complex that leads to fibrosis.

Other reports claimed for taurine/niacin the attenuation of lung fibrosis caused by drugs such as amiodarone, an antiarrhythmic drug (Wang et al.,
1992) and cyclophosphamide, a cytotoxic and immunosuppressive drug (Venkatesan and Chandrakasan, 1994). Both authors indicate the antioxidant and anti-lipid peroxidative properties of taurine play a key role in the possible mechanisms of protection.

Taurine has also been shown to protect dogs (Izumi et al., 1989) and rats (Nagata et al., 1991) against the acute effects of paraquat poisoning. The continuous infusion of taurine appears to change the pharmacokinetics of paraquat, prolonging its circulation time and inhibits its accumulation in target organs.

Recently, the chemical reactivity of taurine and its potential antioxidant physiological role was addressed by Ogasawara and co-workers (1993) in a new perspective. Exhorting the study of aldehydes as oxidants generated in geriatric disorders, such as diabetes or senility, they have studied the chemical reaction \textit{in vitro} of taurine with these noxious agents. They were able to show that taurine reacts with glucose and other aldehydes to a greater extent than other related amino acids (glycine, \(\alpha\)- and \(\beta\)-alanine). Furthermore, the taurine-glucose reaction product showed an antioxidative effect on peroxidation of liposomes as a model of biomembrane.

\textbf{1.6.7.6. Hepatoprotection}

The hepatoprotective effects obtained by the modulation of liver taurine or by increasing the taurine levels in isolated hepatocytes were poorly explored comparatively with the protective effects tested in other organs or using other compounds.

Nevertheless, the hepatic cytoprotective effects of taurine were investigated in galactosamine-induced hepatic necrosis (MacDonald \textit{et al.}, 1985). These authors reported a therapeutic effect exerted by taurine when injected 12 hours after rats were challenged with galactosamine. Despite reducing the galactosamine-induced necrosis, taurine treatment was not able to reduce the
hepatic calcium accumulation. It was suggested that taurine could enhance the intracellular sequestration of calcium without interfering with the measured total liver calcium content.

Later it was shown that the concomitant administration of taurine may promote the effect with cystathionine against acetaminophen-induced necrosis by increasing the half-life of cyst(e)ine. It was also proposed that taurine could exert its effect by interfering with the catabolism of cysteine and promoting the intrahepatic synthesis of glutathione, by indirectly increasing the availability of cysteine in the liver (Kitamura et al., 1989). Interestingly, the same studies revealed that taurine alone did not afford any protective activity against acetaminophen-induced liver injury.

When 3% taurine solution was given orally for three days prior to dosing with CCl$_4$, Nakashima and co-authors (1983) reported an increase in hepatic lipid peroxidation. However when 10% taurine was administered orally 12, 16 and 20 hours after dosing it did protect the liver against carbon tetrachloride induced lipid peroxidation as appraised by malondialdehyde production and reduced accumulation of calcium (Nakashima et al., 1982). Upon data presented the same authors proposed that devising the possible involvement of a timing factor for determining the therapeutic efficacy of taurine should be considered as a priority.

Conversely, depletion of the hepatic taurine levels by pretreatment with $\beta$-alanine significantly increased the hepatotoxicity of carbon tetrachloride in male rats (Waterfield et al., 1993e).

1.6.7.7. Other protective properties

Investigations into the protective roles of taurine have broadened in the last decade, embracing new areas as taurine was found to be involved with the normal function of several organs and/or systems. An attempt to establish cytoprotective mechanisms for taurine led the way and several reports of the protective characteristics have been published since.
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 (Kulakowski and Maturo, 1984) which may explain the observation made by Tokinaga et al (1979) that reported taurine as a potent hypoglycaemic agent able to prevent streptozotocin-induced hyperglycaemia in mice.

These studies were extended to the clinical use of taurine. Thus, taurine supplementation is starting to be regarded as a potential tool to overcome certain diseases or physiopathological status. Taurine supplementation in the diet of young patients suffering from cystic fibrosis was proposed to be an aid in improving their nutritional status and helps decrease the degree of steatorrhoea observed in these patients (Carrasco et al., 1990).
CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

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

- Taurine (cell culture tested)
- Sodium arsenite
- Albumin, bovine
- ATP
- 3α-HSD
- Sodium deoxycholate
- Diaphorase (EC 1.1.1.50)
- 2-mercaptoethanol
- Firefly lantern extract
- GSH
- Collagenase (C-0130, type I, clostridiopeptidase A, EC 3.4.24.3)
- Glutathione reductase (EC 1.6.4.2.)

- Homoserine
- Trypan blue
- EGTA
- NADPH
- ANIT
- ALP 10 (alkaline phosphatase kit)
- L-cysteinesulphinic acid
- 5-sulphosalicylic acid
- Methyl isothiopseudourea
- O-phthalaldehyde (OPA; HPLC grade)

Methanol (HPLC grade) was supplied by Rathburn, Wakeburn, Scotland.
Bilirubin diagnostic test kit (No. 123919) and γ-GT diagnostic test kit (MRP2) were obtained from Boehringer Mannheim, UK.
AST (Unimate 3) and ALT (Unimate 3) kits were purchased from Roche Diagnostic Systems, Welwyn Garden City, UK.
Polythene tubing was supplied by Laboratoire Portex SA, Berck-sur-Mer, France.
Microtainer serum collection tubes were obtained from Becton Dickinson, Rutherford, NJ, USA.
Anaesthetic diethylether B.P. was purchased from Rhône-Poulenc Ltd., Manchester, UK.
Pentobarbitone sodium B.P.(Vet) was supplied by RMB Animal Health Ltd., Dagenham, UK.
Other compounds were purchased by BDH Ltd., Poole, England and included the following:
magnesium sulphate 7-hydrate mercaptoethanol sodium nitrite
sodium hydroxide (Aristar) boric acid acetic acid
potassium dihydrogen orthophosphate trichloroacetic acid
Silicone Treatment "Repelcoat", water repellant sodium arsenate 7-hydrate
DAPM and 2-vinylpyridine was obtained from Aldrich

All remaining compounds were supplied by Sigma or BDH with the highest grade available.

2.1.1. Synthesis of guanidino ethanesulphonate (GES)

Taurine (90 g) was mixed with methyl isothiopseudourea (150 g) in concentrated ammonium hydroxide (180 ml) on a round bottomed 500 ml flask. A water refrigeration system was adapted to the top of the flask. The mixture was heated (60°C, 18 hours) in a oil bath in the fume cupboard (methane thiol is generated in this reaction). After this period the mixture was cooled and allowed to crystallize. The crystals formed were filtered and recrystallized from water four times in order to remove all impurities (Huxtable et al., 1979). Crystals were dried using an oven (80°C, 45 min) and weighed until there was no difference between three consecutive weighing. The yield was 79.5 g (55 %). The taurine concentration in the synthesized product was shown by HPLC to be insignificant (8.0×10⁻⁴%). The presence of GES was verified by infra-red spectra, NMR, MS-FAB and elemental analysis. The melting point was determined to be 285°C.
2.2. Methods

2.2.1. In Vivo studies

2.2.1.1. Animals and treatment

Rats of the Sprague-Dawley strain (Glaxo bred, 80-225 g) were used throughout. Animals were left to acclimatise in communal cages after arrival (6 or more days) and allowed food (SDS R+ME, rat and mouse expanded diet, Witham, Essex) and tap water ad libitum. During all studies, animals were housed in individual metabolism cages (Techmate Ltd., Milton Keynes, UK) in order to allow the collection of urine and faeces separately. Rats were housed in communal cages when urine was not being collected or 48 hours exposure to the test compound was investigated.

Animals were kept in a temperature controlled room (20°C ± 2°C). Lighting was controlled to give a regular 12 h light-dark cycle exposure (light on 07:00 - off 19:00). Animals were weighed daily and general condition monitored. Food and water or other solution intake was recorded as was urine excretion volume. Urine samples were collected over ice when taurine was to be measured.

Rats were dosed between 09:00 and 12:00, after which food was removed until they were sacrificed. Dosing was by oral route (p.o.-gavage).

2.2.1.2. Post mortem procedure

Animals were killed between 09:00 and 13:00, in order to minimize diurnal variation of taurine and TNPSH liver levels.

Animals were anaesthetized with diethylether and killed by exsanguination from the abdominal aorta. Blood samples were collected using a 19G needle attached to a 10 ml syringe and after the needle was removed and discharged, blood was passed into microtainers for separation of serum (by centrifugation in a MSE microcentaur - microcentrifuge at 13,000 rpm, 5min).
Serum samples were stored at 4-8°C until analyzed, except aliquots for bilirubin determination (stored at room temperature for less than 3 hours) and serum bile acids and taurine determination (stored at -20°C wrapped in aluminium foil, until analyzed).

The abdominal and thoracic viscera were observed in order to determine if any abnormality was present. The liver was then excised, cleaned of blood and weighed. The distal portion of the median lobe was cut and placed in 10.5% (v/v) phosphate buffered formalin (pH 7.2, Pionner Research Chemicals, Colchester, UK.) for histology processing. The remainder of the liver was immediately frozen (liquid nitrogen) and stored at -80°C for future analysis for taurine and total non-protein sulphydryls (TNPSH).

The kidneys were also removed and weighed; the right kidney was halved and placed in fixative for histopathology. The remainder was also frozen (liquid nitrogen) and stored at -80°C.

The thymus was excised, weighed and placed in fixative for histopathologic assessment.

2.2.1.3. Bile duct cannulation

The surgical procedure used for bile duct cannulation was a modified version of operations already described elsewhere.

Briefly, male Sprague-Dawley rats were anaesthetized with pentobarbital (70 mg.kg⁻¹; i.p.). After rats were insensible to external stimuli, a small cut was made in the lower abdomen, skin layers and muscle were separated using blunt, curved scissors. An incision into the abdominal cavity was made via a 3cm long cut along the mid-line from the lower abdomen to the xyphoid cartilage. The bile duct was located, isolated and the connective tissue removed from both sides of the duct for 2-3 cm, using forceps. A ligature was applied (untied) closer to the distal end of the bile duct; backed by a scalpel handle, the duct was "nicked" (but not cut) using fine scissors, and cannula polythene tubing pushed gently into the duct approximately 2 cm towards the proximal end and secured in place by the ligature. Body temperature was
followed throughout with a rectal thermometer, and maintained at 37°C by using a heat lamp. Body fluids levels were maintained by periodic injection of saline to the peritoneal cavity.

Bile samples were collected into foil covered pre-weighed Eppendorf tubes.

2.2.1.4. Light microscopy

After fixation, tissues were trimmed and embedded in paraffin, 3μm sections were cut and stained with haematoxylin and eosin. Tissues were mounted in glass slides and examined by light microscopy.

2.2.2. In vitro studies

2.2.2.1. Isolation of rat hepatocytes

The technique employed was basically the two step perfusion method of Moldeus et al. (1978). It involves a perfusion of liver in situ to an anaesthetized rat with two recirculating balanced salt solutions (Appendix II).

The first solution used to perfuse the liver was Ca²⁺ free and contained a Ca²⁺ chelating agent, EGTA, which removes the Ca²⁺ that maintains the protein structure of intracellular filaments between desmosomes of the tight junctions between hepatocytes (Berry et al., 1991). The second solution contained collagenase in a solution in which calcium was present (4mM Ca²⁺) in order to promote the action of collagenase.

The perfusion with collagenase completed the biochemical separation of the hepatocytes from one another enabling the mechanical separation to be completed.

A) animals

Male Sprague-Dawley rats (Glaxo bred, 210-290 g) were used. Animals were allowed to acclimatize in the animal house for at least 6 days after arrival and were given SDS R+ME diet (see 2.2.1.1) and water ad libitum. Isolation
of hepatocytes was carried out between 11:00 and 12:00 hours. Food was removed at 09:00 in order to minimize differences in liver levels of taurine and TPNSH due to food intake shortly before the surgical procedure was performed.

B) surgical procedure

Animals 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 abdominal viscera were shifted up and to the left to expose the portal vein. A loose ligature was placed underneath the portal vein, about 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 into the portal vein. The ligature was tied and secured around the shallow groove present in the cannula in order to hold it inside the portal vein. The hepatic artery and vein were cut to allow the blood and perfusate to flow out of the liver, preventing excessive swelling. The liver was dissected out, removed from the animals abdominal cavity and placed into the Hank I reservoir suspended by the portal vein.

C) perfusion apparatus, perfusion and washing procedure

i) perfusion apparatus

An apparatus was setted to perfuse the liver with carbogenated (95% O₂:5% CO₂) recycling buffer. The oxygenation system, had four openings such that the perfusate could be pumped into the top and gassed through a side opening. A controlled buffer outflow was obtained from the bottom opening and regulated to 2 drops.sec⁻¹ at the tip of the cannula. The excess of buffer was removed to the Hank I beaker from the side-top aperture. The oxygenator also acted as a bubble trap. The pump (Watson-Marlow 502S, Falmouth, UK.), working at 65% of its maximal capacity, provided a continuous flow of buffer to the oxygenator system in order to obtain such a pressure in the manometer equivalent to a height of water, 40 cm above the bench. The
waterbath (Grant JB2, Barrington, UK.) was regulated to 38-40°C in order to obtain 37°C at the tip of the cannula.

**ii) perfusion**

The liver was perfused *in situ* until dissected out of the rat abdominal cavity and suspended by the portal vein for more 5 min. Collagenase (50 mg) was added to 100 ml Hank II (with Ca²⁺) and allowed to settle before mixing. The cannula clip (which regulates the flow) was opened and the 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 the buffer was reestablished and adjusted to 2 drops a second and the perfusion continued for 12-15 min until the liver lost its resilience when palpated gently.

**iii) washing procedure**

The liver was cut from the cannula into Krebs-Henseleit+albumin (50 ml, room temperature) in a shallow glass dish. The Gilsson's capsula was then broken using a comb and the cells gently dispersed in the solution. The cell suspension was filtered through a polyamide tea strainer into 2 × 25 ml centrifuge tubes. Cells were 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 25 ml with K+H and the viability and cell yield determined using Trypan-blue dye exclusion.

**D) incubation**

Cells were incubated (37°C) at a concentration of $2 \times 10^6$ cells.ml⁻¹ (15 ml total volume) in siliconised ("Repelcoat") round-bottomed flasks (100 ml) under a continuous stream of carbogen on a rotary evaporator (30 rpm). Cells were pre-incubated (60 min) with buffer, or several concentrations of taurine or cysteine sulphinate, before treatment. Different doses of toxic compound were added to the medium and incubated for 3 hours.
2.2.2.2. Assessment of viability of isolated hepatocytes

A) Trypan blue uptake
The viability of isolated hepatocytes was assessed by the ability of undamaged 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 (0.22 µm membrane filter) and kept cold (4°C) just prior to use.

i) initial estimation of hepatocyte viability and yield
An aliquot (50 µl) of cell suspension was mixed with Trypan blue (450 µl) to calculate the initial cell density and viability. Cells were counted in a Neubauer haemocytometer. Both total and viable cell counts were performed in the central gridded area on both sides of the cytometer (0.1 cm²).

\[
\text{Density} = \text{viable cells} \times 10^6 \text{ cells.ml}^{-1} \text{ of suspension}
\]
\[
\text{Yield} = \text{Density} \times 25 \text{ (total suspension volume)}
\]

Five separate slides were prepared and counted. Preparations showing ≥ 87% viability by this method were used and diluted to a concentration of $2 \times 10^6$ cells.ml⁻¹ with K+H. Once cells were diluted for incubation, Trypan blue and cell suspension were mixed in even proportions (50 µl: 50 µl).

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.

\[
\text{L-lactate} + \text{NAD}^+ \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+
\]
The amount of enzyme in the medium (50 µl) was estimated by measuring
the rate of the decrease in absorbance at 340 nm when NADH was oxidized to NAD$^+$ in a final volume of 3 ml (Appendix III).

2.3. Biochemical measurements

2.3.1. Taurine

2.3.1.1. Sample preparation

A) Urine

All 24 hour rat urine samples were diluted to 25 ml with UHQ water and centrifuged at 4,000 rpm for 10 min in order to remove hair and food debris. Two aliquots (5 ml) were stored at -80°C until analyzed. Each aliquot was diluted 1:10 with UHQ water and 25 µl was placed onto previously prepared dual bed ion exchange resin columns (Appendix I), the first 25 µl were discharged. Taurine was eluted with 4 ml of UHQ water in 1.0 ml aliquots. Homoserine (100 µl, 100 µM) was added to the eluate as internal standard (Durkin et al., 1988) to give a final concentration of 2.44 nmol.ml$^{-1}$ homoserine in the eluate. The eluate was used immediately for taurine measurement or stored at -80°C.

B) Liver

Frozen sections from different liver lobes were weighed (0.4 - 0.6 g) into sulphosalicylic acid (4.0 ml, 0.2 M, 4°C) and homogenized using a tissue homogenizer (Polytron®, Kinematica, Luzern, Switzerland). The homogenate was centrifuged (4,000 rpm, 10 min, 4°C), duplicate aliquots of supernatant (100 µl) were placed in dual bed ion exchange resin columns and the first 100 µl discharged. Taurine was eluted with 4 ml UHQ water (4 × 1ml). Homoserine was added as internal standard (100 µl, 100 µM).
C) Serum

A 100 μl sample of serum was deproteinated (100 μl, 0.2 M SSA, (Connoly and Goodman, 1980), mixed and centrifuged (13,000 rpm, 5 min). 50 μl of the supernatant was placed onto a dual bed ion exchange column and the first 50 μl of the eluate were discharged. Taurine was eluted with 4 ml UHQ water (4 × 1 ml) and stored at -80°C until analyzed. Homoserine was added as internal standard (100 μl, 100 μM).

D) Isolated hepatocytes

i- Incubation buffer (medium)
An aliquot (100 μl) of cell suspension supernatant (see 2.2.2.2.D) was placed into dual bed ion exchange column and the first 100 μl of the eluate discharged. Taurine was eluted with 4 ml (4 × 1 ml) UHQ water. Sample was frozen at -80°C until analyzed. Homoserine was added as internal standard (100 μl, 100 μM).

ii- Cells and incubation buffer (total taurine)
An aliquot (500 μl) of cell suspension was added to 250 μl SSA 0.2 M and centrifuged (11,000 rpm, 5 min) to precipitate proteins. The supernatant was collected and stored at -80°C until eluted for taurine analysis. An aliquot (100 μl) of the supernatant was placed in a dual bed ion exchange column and the first 100 μl of the eluate discharged. Taurine was eluted with 4 ml (4 × 1 ml) of UHQ water. Homoserine (100 μl, 100 μM) was added as internal standard and samples analyzed for taurine content (Waterfield, 1994).

2.3.1.2. Taurine derivatization and adduct measurement

Taurine was measured using the adduct formed with a derivatizing solution of 6-phthaldehyde (OPA) and mercaptoethanol in borate buffer (pH 10.3). The adduct was chromatographed using reverse phase high performance liquid chromatography (HPLC-RP) on a LiChrosphor® 100 RP-18 (5 μm) (Merck 50943) column, using isocratic elution with phosphate buffer (0.05 M,
water:methanol, flow 2 ml.ml⁻¹, 2.5 - 3 ×1000 psi) and Beckman (157 Fluorescence Detector) detector (Waterfield, 1994). Data was collected using a Beckman "System Gold" and sample table automatic injection.

Results were expressed as:

- **urinary taurine** - μmol.kg⁻¹.24h⁻¹
- **liver taurine** - μmol.g⁻¹ wet liver weight
- **serum taurine** - μmol.ml⁻¹
- **taurine in isolated hepatocytes** - nmol.10⁶ cells (medium, total, cellular).

### 2.3.2. ATP determination

ATP was measured in isolated hepatocytes as an indicator of cytotoxicity. The assay was based on the detection of luciferase-linked bioluminescence (Stanley P.E. and Williams, 1969) in TCA extracts, using firefly lantern extract in the presence of ATP.

#### 2.3.2.1. In isolated hepatocytes

An aliquot (500 μl) of cell suspension, was centrifuged (800 rpm, 1 min) and the excess of supernatant removed by aspiration. 250 μl of TCA 1M + EDTA 10 mM was added to the pellet, vortexed and kept at -80°C until analyzed (less than 1 month). Samples were thawed on ice (4°C) and centrifuged (11,000 g, 5 min) to precipitate protein. The supernatant was assayed for ATP content using firefly lantern extract. (Appendix III).

#### 2.3.2.2. In the liver

Liver (0.5 - 0.6 g) was placed into TCA (10%, 4°C). Tissue homogenization was carried out immediately and the homogenate centrifuged (4,000 rpm, 5min, 4°C). The supernatant was divided into 1 ml aliquots and frozen at -80°C until analyzed (less than 1 month). (Appendix III).
2.3.3. Total non-protein sulphydryl (TNPSH)

Determination of TNPSH as a measure of GSH (GSH comprises more than 95% of TPNSH pool in rat liver, (DeMaster and Redfern, 1987) was performed in isolated hepatocytes and liver extracts.

2.3.3.1. Reduced glutathione in isolated hepatocytes

Reduced glutathione was measured using a fluorometric method based on the specific reaction between GSH and OPA at pH 8.0 yielding a highly fluorescent product (method modified from Hissin and Hilf, 1976).

A 250 µl aliquot was centrifuged (800 rpm, 1 min) and the supernatant discarded. TCA (750 µl, 6.5%) was added to the pellet and vortexed. Samples were frozen at -20°C until analyzed (less than 1 week).

2.3.3.2. TNPSH in the liver

An aliquot (2 ml) of the liver homogenate (see 2.3.1.1.-A) was diluted 1:1 with phosphate buffer (pH 7.4). The mixture (500 µl) was assayed for TPNSH using the reaction between DTNB and the sulphydryl group on the GSH molecule, yielding a coloured compound which was measured at 412 nm (modified from (Ellman, 1959) (Appendix III).

2.3.4. Determination of GSSG in isolated hepatocytes

Glutathione disulphide was measured in isolated hepatocytes by a method modified from Griffith (1980). The method is based on reactions described by equations i), ii) and iii). Briefly, glutathione disulfide is reduced by glutathione reductase in the presence of NADPH to form GSH. The newly formed GSH reacts with DTNB to yield GS-5-thio-2-benzoate measured
spectrophotometrically at 412 nm for a minute. In order to overcome the natural or artefactual oxidation of GSH to GSSG, this method uses 2-vynylpyridine (2-VP) as a -SH group blocker. The drawback of using of 2-VP as a sulphhydryl group blocker is that its effectiveness is around 85-95% but on the other hand it does not inhibit glutathione reductase, saving some cumbersome purification steps. It also enables the spectrophotometer to be readjusted to zero, prior to the moment when glutathione reductase is added, deducting the absorbance accounting for the residual -SH groups (5-15%) that had reacted with DTNB.

The general scheme of reactions for the method is:

i). \[ \text{GSH} + \text{GSSG} + 2\text{-VP} \rightarrow \text{GS-VP} + \text{GSSG} + \text{GSH} \]
(sample) (85-95%) (5-15%)

ii). \[ \text{GSH} + 4\text{-DTNB} \rightarrow \text{GS-5-thio-2-benzoate} \]

\[ \text{GSH reductase} \]

iii). \[ \text{GSSG} \rightarrow 2 \text{GSH} + \text{DTNB} \rightarrow \text{GS-5-thio-2-benzoate} \]

\[ \text{NADPH} \]

\[ \text{NADPH} \]

The concentration of glutathione disulphide in the sample was determined using a GSSG calibration curve run in parallel.

**2.3.5. Serum bile acids**

Serum enzymes were inactivated by heating, and bile acids were converted to 3-oxo bile acids with 3α-HSD with the concomitant reduction of NAD$^+$ to NADH. The hydrogen of NADH generated is then transferred by diaphorase to resazurin, yielding the fluorophore, resorphin. The fluorescence of resorphin measured is proportional to the amount of bile acids present in the sera (Mashige et al., 1976). An aliquot (100 μl) of serum was mixed with Tris/HCl buffer (2 ml, pH 9.0) and heated in a waterbath (30 min, 67°C). After cooling to room temperature, 3α-HSD (0.2 ml, 0.012 U) and NAD/diaphorase solution (0.5 ml; 6 mg.ml$^{-1}$/0.05 mg.ml$^{-1}$ in phosphate buffer
pH 7.4) were added to the samples. Samples were mixed and resazurin (0.2 ml, 100 μM) added. After samples were mixed and left waiting for 1 hour at room temperature, fluorescence was read at 560/580 nm (excitation/emission) using a Perkin-Elmer LS-3 Fluorescence Spectrometer. Serum bile acid concentration was determined using sodium deoxycholate (DC-Na) as a standard.

2.3.6. Serum biochemistry

2.3.6.1. Total bilirubin

Bilirubin was determined in the serum using a commercial kit from Boehringer Mannheim, UK. The determination is based on a colorimetric method where bilirubin present in the specimen is coupled with diazotized suphanilic acid in the presence of caffeine, yielding a coloured compound that is measured spectrophotometrically at 578 nm (Jendrassik et al., 1938).

2.3.6.2. Gamma-Glutamyltransferase (γ-GT)

Serum γ-GT activity was determined using a commercial kit purchased from Boehringer Mannheim, UK. The determination is based on a kinetic colourimetric method where L-γ-glutamyl-3-carboxy-4-nitranilide in the presence of glycylglycine is converted by the enzyme γ-glutamyltransferase into 5-amino-2-nitrobenzoate. The rate of formation of the product is spectrophotometrically monitored at 405 nm (Szasz and Persijn, 1974).

2.3.6.3. Aspartate aminotransferase (AST) and Alanine aminotranferase (ALT)

The determination of AST and ALT in the serum was made using a commercial kit obtained from Roche Products Ltd, UK. The kinetic U.V. method is based on the rate of NADH oxidation to NAD⁺ that is
spectrophotometrically monitored at 340 nm, according with the IFCC recommendations (International Federation of Clinical Chemistry (IFCC), 1986).

2.3.6.5. Alkaline phosphatase (ALP)

The determination of ALP was performed using a kit commercially available from Sigma Chemical Co, UK. The method is based on the serum ALP property to hydrolize p-nitrophenyl phosphate to p-nitrophenol at alkaline pH. The p-nitrophenol formed shows an absorbance at 405 nm. The rate of increase in absorbance is proportional to the ALP activity in the serum and is monitored spectrophotometrically (Bowers and McComb, 1966).

2.3.7. Statistical analysis of the results

The analytical statistical approach adopted to compare different groups was that proposed by Gad and Weil (1989). Thus, all different groups in comparison were first evaluated for their homogeneity of variances (using Bartlett's test). If the variances were found to be homogeneous, analysis of variance (ANOVA) was carried out. Upon a significant result in the ANOVA, Dunnett's t-test was run comparing a given pair of groups.
If variances were found to be heterogeneous by Bartlett’s test, and the data in analysis was parametric, three different strategies were used:
1) a Student t-test (unpaired and two-tailed) was run if the variances of the groups in comparison were homogeneous (F test non-significant);
2) If the variances were heterogeneous (F test significant) but the groups contained an equal number of data, a modified Student’s t-test was used;
3) if the F test was significant (heterogeneous variances) and $N_1 \neq N_2$ a Cochran’s t-test was used to compare two given groups.

A probability of less than 0.05 (p<0.05) was chosen as the criterion of significance.
CHAPTER 3

INVESTIGATIONS INTO THE ROLE OF LIVER TAURINE LEVELS ON ANIT-INDUCED HEPATOTOXICITY

3.1. Introduction

The following experiments were carried out to investigate what effect the modulation of liver taurine levels might have on α-naphthylisothiocyanate (ANIT)-induced cholestasis and liver damage.

ANIT has been long used as a model for studying intrahepatic cholestasis (Roberts and Plaa, 1965). Although its mechanism of toxicity has not yet been established, several promising reports have been produced over the past ten years. A single oral dose of ANIT produces cholestasis, hepatocellular and biliary epithelial cell necrosis and bile duct obstruction in rats (Plaa and Priestly, 1977).

Concern for developing an effective clinical treatment for patients that suffer from cholestasis and hyperbilirubinemia occurring either sporadically or as an inconvenient response to certain drugs has been present for a long time in the mind of scientists and physicians. Di Padova et al. (1985) showed that pretreatment of rats with SAMe was associated with a protective effect against cholestasis induced by a single dose of ANIT (100 mg.kg⁻¹). Other authors have also shown that SAMe pretreatment and treatment protects against both cholestasis and tissue damage induced by ANIT (Nanno et al., 1987). Moreover, exogenous SAMe has been shown to promote the transulphuration pathway, activating cystathionine synthetase (Finkelstein et al., 1975), favouring the synthesis of glutathione and other sulphated compounds (i.e. taurine) via homocysteine and cysteine as was summarized by Kaye et al. (1990). Recently, Angelico et al. (1994) showed that cirrhotic patients treated orally with SAMe, showed an enhanced bile salt conjugation with taurine and some improvement in the
biochemical markers of liver damage was observed. A protective effect against ANIT-induced toxicity was also claimed for cysteine, cysteamine and cystamine since they have been effective in reducing the hyperbilirubinemia induced by ANIT (El-Hawari and Plaa, 1977). It is conceivable to accept that cysteine, cysteamine and cystamine possibly are converted to GSH and taurine via the transsulphuration pathway. Since the protective effect of GSH in the ANIT-induced cholestasis has been ruled out (Dahm and Roth, 1991), it was interesting to investigate the effect of the modulation of liver taurine levels on the onset of ANIT-induced cholestasis and hepatotoxicity.

On the other hand, taurine depletion has been proved to exacerbate the hepatotoxicity of CCl₄ in rats (Waterfield et al., 1992). Although rats are a very efficient species for synthesizing taurine, depletion of taurine liver levels can be achieved by pretreating animals with β-alanine (6 days) or GES (10 days) in the drinking water (Huxtable, 1982; Huxtable et al., 1979; Shaffer and Kocsis, 1981). Taurine levels in the liver in rats can also be raised by giving them a 3% taurine solution as their drinking solution for 6 days (Nakashima et al., 1983).

Both strategies were carried out in order to establish the susceptibility of rats to ANIT-induced cholestasis and hepatotoxicity when taurine depleted or "boosted" liver levels were achieved. An array of biochemical and histological tests were performed in order to compare the possible differences in ANIT-susceptibility that treated animals might exhibit when compared to control animals that had received tap water.

3.2. Methods

Sprague-Dawley male rats (Glaxo bred, 130-270 g at the beginning of the experiments) were used throughout these studies.
3.2.1. Section I. Effect of liver taurine depletion on ANIT-induced liver injury: comparison with control animals.

In each individual experiment, animals were divided into 4 groups of 4 rats each and placed in individual metabolism cages. Food and water or other solution was allowed *ad libitum* until dosing. After dosing, rats were deprived of food for a 24 hour period, after which they were killed as described in Chapter 2.

a. **Control animals** were allowed a *normal diet ad libitum* for 6 days after which they were dosed with vehicle (corn oil, 2 ml.kg⁻¹) or with ANIT (100 mg.kg⁻¹ in corn oil, 2ml.kg⁻¹) orally by gavage. Food was removed after dosing. From the start of the experiments, *tap water* was provided *ad libitum*, until animals were killed by exsanguination from the abdominal aorta under ether anaesthesia (see Chapter 2) 24 hours after dosing.

b. **β-alanine treated animals** were supplied a *normal diet ad libitum* for 6 days after which they were dosed with corn oil (2 ml.kg⁻¹) as vehicle or ANIT (100 mg.kg⁻¹ in corn oil) orally by gavage. Food was removed after dosing. Rats were given a 3% *β-alanine solution in tap water* as drinking solution *ad libitum* until the end of the experiment, as mentioned above for control animals.

c. **GES treated animals** were provided with a *normal diet ad libitum* for 10 days after which they were dosed with corn oil (2 ml.kg⁻¹) as vehicle or ANIT (100 mg.kg⁻¹ in corn oil) orally by gavage. Food was removed after dosing. Animals were supplied a 1% *GES solution in tap water* as drinking solution *ad libitum* throughout the experimental period, until termination performed as described above.

Results from 4 different experiments were combined for control animals (n=16), while results for β-alanine treated animals were obtained from one experiment (n=4, vehicle dosed animals) or from two separate sets of experiments (n=8 for ANIT-dosed rats). Results shown for GES treated
animals were collected from a single experiment.

3.2.2. Section II. Effects caused by augmentation of liver taurine levels in ANIT-dosed animals: comparison with ANIT-dosed controls.

a. **Control animals** - the methods used here were the same as for section I, since the same animals were used as controls for experiments described in sections I and II.

b. **Taurine treated animals** were allowed a *normal diet ad libitum* for 6 days after which they were dosed with corn oil (2 ml.kg⁻¹) as vehicle or ANIT (100 mg.kg⁻¹ in corn oil) orally by gavage. Food was removed after dosing. Rats were given a 3% *taurine solution in tap water* as drinking solution *ad libitum*, until termination by exsanguination from the abdominal aorta under ether anaesthesia (see Chapter 2) was performed 24 hours after dosing. The data presented for these groups of rats was collected in one set of experiments for the vehicle dosed animals (n=4) and from two different set of experiments for the ANIT-dosed taurine treated animals (n=7).

3.2.3. Section III. Investigation into the effects caused by intraperitoneal taurine dosing to depleted animals prior- and post-ANIT dosing.

Twenty Sprague-Dawley rats were randomly divided into 5 groups of 4 animals each and housed in metabolism cages for the length of the study. Animals were fed a *normal diet* for 6 consecutive days. A 3% *β-alanine solution in tap water*, was provided as drinking solution. Food was removed after dosing with vehicle (corn oil, 2 ml.kg⁻¹) or ANIT (100 mg.kg⁻¹, p.o.). After dosing, the β-alanine drinking solution was replaced by tap water supplied *ad libitum*. Animals received saline (sterile NaCl 0.9%, filtered through a 0.22 μm pore filter; 2 ml.kg⁻¹) or a taurine solution (5 mmol.kg⁻¹ in saline) intraperitoneally one hour before and six hours after dosing with ANIT or vehicle. Except rats in group 1 that were killed one hour after having received
saline, every other single animal in the remaining groups (2 to 5) received a
different treatment as follows:

- Group 1- saline only
- Group 2- ANIT + saline
- Group 3- ANIT + taurine
- Group 4- vehicle + taurine
- Group 5- vehicle + saline

Animals were killed twenty-four hours after dosing with vehicle or ANIT as
described in Chapter 2.

3.2.4. Statistical analysis

Urinary levels of taurine were averaged for the 48 hours before dosing and
this figure compared with the 24 hour post-dose value for the same animal.
A Student’s paired t-test was carried out to determine the level of
significance. Groups were considered significantly different if p<0.05.
The analytical statistical approach adopted was that described in Chapter 2.
3.3. Results

3.3.1. Section I. Effect of liver taurine depletion on ANIT-induced liver injury: comparison with control animals.

3.3.1.1. Percentage change in the body weight

An increase in the body weight of the animals was observed, regardless of treatment, throughout the study (Figure 3.1.). An exception was found in day 2 for one of the groups treated with β-alanine. Control animals showed a higher percentage body weight gain than their counterparts given β-alanine or GES for the first two days. After the second day of treatment, changes in the percentage of body weight were similar in all the groups.

After animals had been dosed with vehicle or ANIT and deprived of food for a 24 hour period, they lost between 8.61% to 11.96% of their body weight (Fig.3.1). No significant difference was found between vehicle and ANIT dosed animals.

![Graph showing percentage change in body weight](image)

**Figure 3.1.** % Change in body weight of control (32), β-alanine (12) and GES (8) treated rats. Animals were dosed with ANIT (100mg.kg⁻¹, p.o.) or vehicle on day 6 or 10 (as indicated). Values are means±SEM;N=(n);
3.3.1.2. Food and water (β-alanine or GES solution) intake

Food and water consumption are shown in Figures 3.2. and 3.3. Animals treated with β-alanine and GES ate less than controls (water given animals) during the period of the study (Figure 3.2.). Similarly, the inclusion of β-alanine or GES in the drinking water resulted in a reduced intake of drinking solution compared to control animals given tap water (Figure 3.3.). No significant difference for food or water (β-alanine, GES) consumption was found for animals receiving similar pretreatment. All animals dosed with ANIT (b) drank significantly less (p<0.001) than those dosed with vehicle (a), except those given β-alanine in the drinking water (Figure 3.3.).

![Figure 3.2](image)

**Figure 3.2.** Food consumption of control(32), β-alanine(12) and GES(8) treated animals. Values are means ± SEM; N=(n).

3.3.1.3. Serum biochemistry and changes in organ weight

The measurements described herein were performed to evaluate clinical signs
of hepatotoxicity.

A) Bilirubin

Serum bilirubin levels were significantly elevated ($p<0.01$) in all ANIT-dosed groups, regardless of treatment, when compared to the respective vehicle dosed groups. Rats treated with β-alanine and dosed with ANIT showed a statistically significant increase ($p<0.05$) in serum bilirubin levels when compared to the ANIT-dosed control group (Figure 3.4.). GES treatment did not produce a significant change in the serum bilirubin levels when vehicle or ANIT-dosed animals were compared with corresponding control groups.

![Figure 3.3. Daily water, 3% β-alanine or 1% GES solution intake. Rats were dosed on day 6 or 10 (as indicated). Values are means ± SEM; N=control(16), β-alanine(4-8), GES(4). a,b indicates the groups dosed respectively with vehicle or ANIT (100 mg.kg$^{-1}$).** $p<0.01$ significantly different from vehicle dosed groups (a) by a Student's t-test.](image-url)
Figure 3.4. Serum bilirubin measured in control (12), β-alanine (4-8) and GES (4) treated animals 24 hours after dosing with vehicle (corn oil, p.o.) or ANIT (100 mg.kg⁻¹, p.o.). Values are means±SEM; N=(n); ** p<0.01 when compared to corresponding vehicle dosed group by a Cochran's test; ♦ p<0.05 when compared to ANIT-dosed control group by a Dunnett's t-test after ANOVA.

B) Serum γ-glutamyltranspeptidase (γ-GT)

Rats dosed with ANIT showed a significant increase in serum γ-GT levels when compared to vehicle dosed animals (Figure 3.5.). In ANIT dosed animals, β-alanine treatment significantly increased γ-GT levels when compared to ANIT-dosed control group (Figure 3.5.).
Figure 3.5. Serum γ-GT for control(16), β-alanine(4-8) and GES(4) groups. Rats were dosed with vehicle or ANIT as described under methods. Values are means ± SEM; N=(n);* p,0.05 and ** p<0.01 when compared to the respective vehicle dosed group by a Cochran's t-test. ♦♦ p<0.01 when compared to ANIT-dosed control group by a Dunnett's t-test after ANOVA.

C) Aspartate transaminase (AST) and Alanine transaminase (ALT)

Control and β-alanine treated groups dosed with ANIT, showed a statistically significant (p<0.01) increase for both transaminases when compared to groups dosed with vehicle (Table 3.1.) . Although animals treated with GES and dosed with ANIT showed an increment in their transaminase levels when compared to vehicle dosed rats, the rises were not statistically significant when compared by a Cochran's t-test (Table 3.1.).
D) Liver TNPSH

Liver TNPSH levels were elevated (p<0.01) in control and GES treated animals, 24 hours after dosing with ANIT when compared to vehicle dosed rats. No significant differences were found when different treatments were compared to the control group (water alone) for the levels of TNPSH in the liver (Figure 3.6.).

E) Liver taurine

Liver taurine levels were significantly depleted (p<0.05) in β-alanine and GES treated groups when compared to the control group. Liver taurine levels did not suffer any significant alteration 24 hours after animals received ANIT when compared to vehicle dosed groups, regardless of treatment employed (Figure 3.7.).

F) Liver weight

Liver weight (percent of animal body weight) was raised significantly (0.001<p<0.05) in all ANIT-dosed groups when compared to the respective vehicle dose group. When β-alanine or GES treated rats, after dosing with vehicle or ANIT, were compared to control animals dosed similarly no
significant difference was found (Figure 3.8.).

**Figure 3.6.** Liver TNPSH levels in control (16), β-alanine (4-8) and GES (4) treated animals 24 hours after dosing with vehicle or ANIT. Values are means ± SEM; N=(n); ** p<0.01 when compared to respective vehicle dosed group by a Student's t-test.

**Figure 3.7.** Liver taurine levels for control(16), β-alanine(4-8) and GES(4) treated rats after 24 hours exposure to vehicle or ANIT (100 mg.kg⁻¹.p.o.). Values are means ± SEM; N=(n); *p<0.05, ** p<0.01 when compared to the respective control animal dosed groups by a Dunnett's t-test after ANOVA.
Figure 3.8. Liver weight (percent of body weight) for control(16), β-alanine(4-8) and GES(4) treated rats after 24 hours exposure to vehicle or ANIT (100 mg.kg⁻¹,p.o.). Values are means ± SEM; N=(n); * p<0.05, **** p<0.001 by a Student's t-test when compared to the respective vehicle dosed group.

G) Kidney percentage of body weight
No statistically significant difference was found for kidney weight (percent of body weight) either for animals dosed with ANIT or for those dosed with vehicle, when compared to respective controls. No significant differences were found between control and treated groups when dosed with vehicle or ANIT.

3.3.1.4. Urinary taurine
The urinary taurine levels were monitored in control and GES treated rats at the beginning of the treatment. Animals to be dosed with vehicle and those to be dosed with ANIT formed two different groups. When monitored at day 2,3 and 4 of treatment, GES had increased dramatically the urinary taurine levels (1372 ± 169.0 μmol.kg⁻¹.day⁻¹) when compared to those animals given tap water (369.2 ± 27.5 μmol.kg⁻¹.day⁻¹) in the two groups to be dosed with vehicle. This striking difference was also observed for the groups to be dosed with ANIT (GES group 1283 ± 68.2 compared to control 378.8 ± 25.2). Figure 3.9. shows the effect of GES and ANIT on urinary taurine levels when given
to rats. Thus, GES significantly increases the urinary taurine levels (values for individual animals were averaged for a 48 hours period before dosing) when compared to control animals (Figure 3.9.). These differences remain after dosing either with vehicle or with ANIT (Figure 3.9.). Dosing GES treated animals with vehicle or ANIT did not cause a significant change in the urinary taurine when compared to pre-dose urinary taurine levels. When control animals were dosed with ANIT their urinary taurine levels decreased significantly (p<0.001), whereas control rats dosed with vehicle showed only a slight decrease in the urinary taurine levels when compared to their pre-dosing levels of taurine (Figure 3.9.).

![Figure 3.9. Taurine concentration in the urine of rats given water (control) or a 1% GES solution (GES) as drinking solution. Values are means ± SEM for control(16) or GES(4). Urinary taurine excretion per kilogram body weight per day was averaged for each individual animal 48 hours before dosing. After rats were dosed with vehicle or ANIT (100 mg.kg⁻¹, p.o.) urinary taurine was measured (after dosing); N=(n); **** p<0.001 by a paired two-tailed t-test.](image)

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3.3.1.5. Histological features
Control animals, 24 hours after being dosed with vehicle showed a periportal area with normal appearance; intact bile duct structures and surrounding parenchyma showing no signs of damage; no signs of glycogen since animals had been starved (plate 3.1). A high magnification photograph shows a large bile duct with lining biliary epithelial cells easily identifiable by their cuboidal appearance with a large nucleus:cytoplasm ratio (plate 3.2.).

Plate 3.1. Periportal and central regions of liver from animal dosed with vehicle. Magnification (x 100).

Animals showed a depletion in their liver taurine levels after being treated with β-alanine or GES in the drinking water. No signs of biliary epithelial cells injury nor periportal parenchymal damage were observed 24 hours after these animals were dosed with vehicle (β-alanine, plate 3.3).
Plate 3.2. High magnification (× 400) photograph of a large bile duct from a control animal, 24 hours after being dosed with vehicle.

Plate 3.3. Large periportal area of liver from an animal treated with β-alanine for 7 days. Normal appearance of bile duct cells and bile duct morphology. Magnification (× 100).
Twenty-four hours after rats were dosed with ANIT (100 mg.kg\(^{-1}\)), several histopathological alterations accompanied the rise in the serum markers. Thus, bile ducts showed the most pronounced changes, with widespread necrosis and desquamation of biliary epithelial cells. A moderate to severe oedema was present in all large portal areas. The majority of the large bile ducts were clearly damaged or even destroyed, showing virtually no lining cells, with the lumen containing amorphous material and/or plugs of mucus (plate 3.4.). Focal necrosis was present in areas surrounding the periportal areas. An increased neutrophilic infiltration was evident in areas surrounding the bile duct and necrotic periportal areas. Other inflammatory cells were also present in the swollen areas surrounding the periportal space (plate 3.5).

![Plate 3.4. Photomicrograph of a periportal triad of an animal dosed with ANIT (100 mg.kg\(^{-1}\)). Magnification (x 100).](image)

Animals treated with β-alanine and dosed with ANIT (100 mg.kg\(^{-1}\)) showed no distinctive extent of biliary epithelial damage when compared to control animals exposed to an equal dose of toxic compound. Nevertheless the extent
of oedema and inflammatory infiltration in the areas surrounding the periportal space were somewhat larger (Plate 3.6.).

Plate 3.5. Photomicrograph of periportal triad showing a massive neutrophilic infiltration and necrotic parenchymal cells. Magnification (x 400).

Plate 3.6. Large periportal area from an animal treated with β-alanine and dosed with ANIT (100 mg.kg\(^{-1}\)). The bile duct is destroyed with some bile duct lining cells scattered among exudate material and cellular infiltrate. Some small bile ductules remain present without visible disruption.
Treatment with GES did not cause any increment on the hepatotoxicity of ANIT when assessed histologically. Accordingly, a similar extent of bile duct and periportal parenchymal damage was observed 24 hours after 100 mg.kg\(^{-1}\) ANIT when compared to control animals (plate 3.7.).

Plate 3.7. Liver periportal area from an animal treated with GES, twenty-four hours after being dosed with ANIT (100 mg.kg\(^{-1}\)). Bile duct space without lining cells, mild to moderate periportal inflammation and oedema. Magnification (x 40)

3.3.2. Section II. Effects caused by augmentation of liver taurine levels on ANIT-dosed animals: comparison with ANIT-dosed controls.

3.3.2.1. Percentage change in the body weight

Treatment of rats with 3% taurine in the drinking water did not affect the daily variation in body weight when compared to control animals. Although small variations were seen throughout the study when taurine treated animals were compared to rats given tap water (Figure 3.10a.).
3.3.2.2. Food, water and 3% taurine solution intake

Different treatments did not affect significantly the amount of food ingested by each group. Rats given 3% taurine drank more than control animals (figure 3.10c.). This difference was observed throughout the study. Moreover, rats given taurine in the drinking water also excrete more urine than control animals (data not shown). After dosing with vehicle (a) animals treated with taurine showed a reduced consumption of drinking solution when compared to vehicle dosed control rats (Figure 3.10c.). A reduced drinking solution intake was also seen when control rats dosed with vehicle were compared to ANIT-dosed animals. Conversely, no significant effect of ANIT-dosing was detected between different treatments, for the amount of drinking solution ingested (Figure 3.10c.).

3.3.2.3. Serum biochemistry and organ weight changes

A) Bilirubin

Serum bilirubin levels for animals treated with taurine and dosed with ANIT (43.0 ± 7.58) were similar to those obtained for control ANIT-dosed rats (40.05 ± 5.35). No data was obtained for taurine treated animals when dosed with vehicle.

B) γ-GT

Rats treated with taurine and dosed with ANIT showed a statistically significant increase in their serum γ-GT levels when compared to vehicle dosed animals (p<0.01). No difference was found between control rats dosed with ANIT compared to taurine treated animals (Figure 3.11a.).
Figure 3.10. Body weight change (a), food (b) and water (or 3% taurine) intake (c) for control (32) and taurine (8) treated animals. Values are means ± SEM; N=(n): a-vehicle dosed rats; b-ANIT dosed rats; *** p<0.005 when compared to a by a Student's t-test.
C) **AST and ALT**

Both taurine treated and control animals showed a rise in serum transaminase levels 24 hours after dosing with ANIT, when compared to vehicle dosed group (Figure 3.11b. and 3.11c.). Although taurine treated rats showed a smaller increase in their serum transaminase levels, these differences were not statistically significant when compared to ANIT-dosed controls (Figure 3.11b. and 3.11c.).

D) **Liver TNPSH**

Liver TNPSH levels were significantly elevated in control animals dosed with ANIT when compared to vehicle dosed animals (Figure 3.11d.). Taurine treated animals dosed with ANIT showed no statistical significance for liver TNPSH levels when compared to rats dosed with vehicle (figure 3.11d.). Taurine treatment did not significantly alter liver TNPSH levels.

E) **Liver taurine**

Taurine levels in the liver were elevated when taurine treated rats were compared to control animals (given tap water). No significant difference was found between rats dosed with vehicle compared to ANIT-dosed animals for both treatments (Figure 3.11e.).

F) **Liver weight**

Liver weight (percent of body weight) was raised in ANIT-dosed rats when compared to vehicle dosed animals, regardless of the treatment (Figure 3.11f.). Different treatments did not affect significantly the liver weight in vehicle or ANIT-dosed rats (Figure 3.11f.).
Figure 3.11. Serum biochemistry and liver weight for control (16) and taurine (4-7), 24 hours after dosing with vehicle or ANIT (100 mg.kg\(^{-1}\), p.o.). Values are means± SEM; N=(n); * p<0.05, ** p<0.01 and *** p<0.005 when compared to vehicle dosed group by a Student's or Cochran's t-test; ♦♦ p<0.01 by a Duncan's t-test after ANOVA when compared to vehicle dosed group.
G) Kidney weight

No significant differences in kidney weight were found either due to different treatments or due to dosing with ANIT (data not shown).

3.3.2.4. Histological features

Taurine treated animals showed no evidence of hepatic injury twenty-four hours after dosing with vehicle (plate 3.8).

When taurine treated animals were challenged with ANIT similar histological features were present as in control animals. Large bile ducts were disrupted with a small number or virtually no bile duct cells lining the ducts. The extent of periportal parenchymal involvement appears to be generally smaller when compared to control animals exposed to a similar dose of ANIT. However, a marked inflammatory response with neutrophils and other inflammatory cells parallel that observed for control animals (plate 3.9.).

Plate 3.8. Liver photomicrograph of an animal treated with taurine, twenty-four hours after dosing with vehicle. Magnification (x 100).
Plate 3.9. Photomicrograph of a liver periportal area from taurine treated animal, 24 hours after dosed with ANIT (100 mg.kg\(^{-1}\)). Magnification (x 100).

3.3.3. Section III. Investigation into the effects caused by intraperitoneal taurine dosing to depleted animals prior- and post-ANIT dosing.

3.3.3.1. Daily change in the body weight

Animals lost body weight after the first day of treatment with β-alanine (as previously was observed - Figure 3.1). No significant differences were found between different groups treated with β-alanine for the period prior to dosing with ANIT or vehicle. After animals were dosed with ANIT or vehicle and with saline or taurine again no significant differences were found when groups were compared (Figure 3.12a.).
Figure 3.12. Percentage change in body weight (a), food (b) and \( \beta \)-alanine solution intake (c) in animals allocated to 5 different groups. Values are means ± SEM; N=4.
3.3.3.2. Food and β-alanine solution consumption

All groups consumed similar amounts of food and similar amounts of 3% β-alanine solution during the experimental period (Figure 3.12b. and 3.12c.). A drop in consumption of β-alanine solution was observed after dosing for all animals.

3.3.3.3. Serum biochemistry and organ weight changes

All figures concerning the parameters measured in this experiment are represented in table 3.2.

A) Bilirubin

Serum bilirubin levels were statistically significantly elevated when taurine depleted animals were dosed with ANIT. The pre-treatment with taurine (5 mmol.kg⁻¹) or saline did not afford significant protection against the rise of serum bilirubin levels in ANIT-dosed animals (table 3.2).

B) γ-GT

No statistically significant differences were detected among comparable groups (table 3.2.). However, rats dosed with ANIT (groups 2 and 3) showed a rise in serum γ-GT levels when compared to vehicle dosed animals (groups 4 and 5).

C) AST and ALT

Animals dosed with ANIT showed a statistically significant increase in their serum transaminases levels when compared to vehicle dosed animals, for the same treatment (table 3.2.).
D) Liver TNPSH

In animals dosed with ANIT a significant increase in their TNPSH levels was observed when compared to vehicle dosed rats for the same treatment (table 3.2.).

Food deprivation in vehicle dosed animals (groups 4 and 5), caused a statistically significant drop in their TNPSH levels when compared to normal fed rats killed before dosing with vehicle (group 1).

E) Liver taurine

Treatment with β-alanine for 6 days had reduced effectively the levels of liver taurine in rats supplied a normal diet, as observed for group 1 (table 3.2.). When fasted animals were dosed intraperitoneally with taurine (5 mmol.kg⁻¹; groups 3 and 4) their levels of liver taurine rose, independently of using ANIT or vehicle, when compared to rats dosed similarly but pre-treated with saline (groups 2 and 5).

Animals pre-treated with saline and dosed with vehicle (group 5) showed a statistically significant increase in liver taurine levels when compared to rats equally pre-treated with saline but dosed with ANIT (group 2).

F) Liver weight

The only statistically noticeable change in the liver weight was found between animals being deprived of food (group 4 and 5) when compared to normal fed animals (group 1). Although not statistically significant rats dosed with ANIT (groups 2 and 3) showed an increase in their liver weight when compared to respective vehicle dosed controls (groups 4 and 5 - table 3.2.).

G) Kidney weight

No significant changes in kidney weight were observed for any of the groups studied (table 3.2.).
### Table 3.2. Effect of direct taurine treatment on the toxicity induced by ANIT. Rats were depleted of taurine in the liver by pre-treatment with a 3% β-alanine solution in the drinking water. Animals were dosed with saline or taurine (5 mmol.kg⁻¹) 1 hour before and 6 after been dosed with vehicle or ANIT (100 mg.kg⁻¹) Values are means ± SEM; N=4; * p<0.05; ** p<0.01; *** p<0.005; **** p<0.001 (significantly different when compared with group number inside brackets by a Student’s t-test); ♦ p<0.05; ♦♦ p<0.01; ♦♦♦♦ p<0.001 (significantly different from group mentioned inside brackets by a Dunnett’s t-test after ANOVA).

<table>
<thead>
<tr>
<th>Parameters dose with</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>bilirubin</td>
<td>not determined</td>
<td>30.01±6.25 ****(5)</td>
<td>27.8 ± 8.75</td>
<td>3.65 ± 0.8</td>
<td>3.14 ± 0.45</td>
</tr>
<tr>
<td>γ-GT</td>
<td>0.48 ± 0.09</td>
<td>3.14 ± 0.72</td>
<td>2.51 ± 0.74</td>
<td>0.77 ± 0.16</td>
<td>1.7 ± 1.19</td>
</tr>
<tr>
<td>AST</td>
<td>22.7 ± 2.6</td>
<td>79.9 ± 14.1 *(5)</td>
<td>85.4 ± 11.9 ***(4)</td>
<td>25.3 ± 3.2</td>
<td>24.4 ± 0.84</td>
</tr>
<tr>
<td>ALT</td>
<td>28.3 ± 3.3</td>
<td>35.2 ± 5.5 *(5)</td>
<td>37.5 ± 7.9 *(4)</td>
<td>11.3 ± 1.2</td>
<td>15.7 ± 1.7</td>
</tr>
<tr>
<td>Liver TNPSh</td>
<td>8.41 ± 0.36</td>
<td>8.93 ± 0.89 ♦(5)</td>
<td>8.59 ± 0.43 ♦(4)</td>
<td>6.4 ± 0.15 ♦♦(1)</td>
<td>6.81 ± 0.48 ♦(1)</td>
</tr>
<tr>
<td>Liver taurine</td>
<td>1.15±0.29 ♦♦♦♦(4)</td>
<td>1.89±0.43 ♦(5)</td>
<td>8.77 ± 1.15 ♦♦♦♦(2)</td>
<td>6.03 ± 0.47 ♦(3)</td>
<td>3.3 ± 0.69 ♦(1,4)</td>
</tr>
<tr>
<td>Liver % bwt.</td>
<td>4.25 ± 0.09</td>
<td>3.75 ± 0.09</td>
<td>3.83 ± 0.09</td>
<td>3.01 ± 0.11 ♦(1)</td>
<td>3.32 ± 0.07 ♦(1)</td>
</tr>
<tr>
<td>Kidney % bwt.</td>
<td>0.90 ± 0.025</td>
<td>0.90 ± 0.013</td>
<td>0.85 ± 0.03</td>
<td>0.93 ± 0.03</td>
<td>0.92 ± 0.02</td>
</tr>
</tbody>
</table>
3.3.3.4. Histological features

Animals treated with β-alanine for 6 days showed no alteration in liver morphology prior to dosing. Their bile ducts were intact and no signs of liver parenchymal damage was present. Moreover, since these animals were not starved, glycogen can be observed in the liver parenchymal cells (plate 3.10.).

Plate 3.10. Photograph showing a periportal area of an animal treated for 6 days with β-alanine in the drinking water.

Dosing β-alanine treated animals, with taurine intra-peritoneally (1 hour before and 6 hours after), prior to dosing with ANIT did not cause significant differences in their liver histological features when compared to control animals. Despite the fact that liver taurine levels were back to normal twenty-four hours after dosing (table 3.2.), the histological signs of ANIT-induced hepatotoxicity were present. As shown in plate 3.11., complete destruction of bile duct structure, with a marked inflammatory infiltration
was present. Fibrotic tissue is observed surrounding what was formerly a bile duct structure.

Plate 3.11. High magnification (x 400) photomicrograph from a periportal area of a rat treated with β-alanine for 6 days. The animal was dosed intraperitoneally with taurine 1 hour before and 6 hours after it had been dosed with ANIT (100 mg kg⁻¹).

3.4. Discussion

This investigation of the possible role of taurine in ANIT-induced hepatotoxicity was based on the modulation of the liver taurine level during the experiment. However, several factors must be considered since they might influence other important liver protective agents (e.g. glutathione) or their precursors. Since depleting (β-alanine or GES treatment) or increasing the liver taurine level (taurine treatment) was based on animal daily intake of solutions given ad libitum, one aspect which needs to be addressed is the food and water (or other treatment solution) intake and their relationship to body weight gain. Generally, animals given β-alanine or GES ate and drank less
than control animals given tap water prior to ANIT dosing, although the percentage body weight gain was not dramatically different from control animals (except for β-alanine treated animals for the first 48 hours). It has been reported previously that β-alanine treated animals (3% w/v in the drinking water) ate and drank less than for a control period (Shaffer and Kocsis, 1981) than control animals given tap water and a similar decrease in body weight was reported for the first 24 hours after β-alanine treatment (Waterfield et al., 1993e). The difference in the percentage change in body weight is likely to be due to the reduced intake of food (and β-alanine solution) but could be caused by another physiological adaptative response not detected in the parameters measured.

The liver taurine levels were reduced to approximately 44% and 15% of the control values after treatment with β-alanine and GES respectively. The results presented here are in accordance with previous studies which have shown that treatment with β-alanine reduces tissue levels of taurine (Shaffer and Kocsis, 1981; Mozaffari et al., 1986; Waterfield et al., 1993e) as does the treatment of rats with a 1% solution of GES (Huxtable et al., 1979; Huxtable, 1982; Mozaffari et al., 1986). Furthermore, animals treated with taurine (3% w/v in the drinking water) showed an increase of approximately 91% in their liver taurine level, close to those previously reported (Nakashima et al., 1983).

Total non-protein sulphydryl levels (TNPSH) in the liver were unaffected by all treatments and as more than 95% of TNPSH are usually considered to be glutathione (DeMaster and Redfern, 1987), this indicates that all treatments used to modulate liver taurine levels did not alter liver GSH levels. Glutathione has been long recognised as an important liver protective agent (Nakashima et al., 1983; Meister, 1992; Bray and Taylor, 1994; Kretzschmar and Klinger, 1990; Kaplowitz et al., 1985; Droge et al., 1994) and directly implicated in the onset of ANIT-induced hepatotoxicity (Carpenter-Deyo et al., 1991; Dahm and Roth, 1991; Dahm et al., 1991a; Jean et al., 1994; Jean et al.,
1995). Thus, it was crucial to substantiate that liver TNPSH levels were not altered either due to treatment or after dosing due to disparate food intake. Since it has been proposed that fasting prevents diurnal variation of hepatic TNPSH (Dahm et al., 1991a), animals were fasted after dosing with ANIT, ensuring that further variation of TNPSH levels was not linked with a different pattern of food intake. It has also been reported that β-alanine treatment alone has no significant effect on TNPSH levels (Waterfield et al., 1993e) and it was confirmed in these studies that TNPSH levels were normal in β-alanine treated animals before dosing (Table 3.2.)

Treatment with β-alanine which depleted liver taurine, appeared to significantly increase the extent of ANIT-induced intrahepatic cholestasis as determined by serum bilirubin and serum γ-GT activity when compared to control ANIT-dosed rats. Although, no marked difference was found when biliary epithelial cells damage was histologically evaluated between control and β-alanine animals, serum γ-GT has been shown to be the most specific indicator of bile duct injury (Leonard et al., 1984; Zafrani et al., 1989). Hyperbilirubinemia was reported to occur at the time of onset of ANIT-induced cholestasis (Roberts and Plaa, 1965; Plaa and Priestly, 1977; Schaffner et al., 1973). These biochemical parameters might quantitatively assess an extent of damage overlooked by a non-extensive histological evaluation.

Thus, taurine depletion can be regarded as a possible contributing cause for the extended damage observed in ANIT-dosed β-alanine treated rats.

Although the depletion of liver taurine levels by β-alanine appears to play a role in ANIT-induced cholestasis, when liver taurine levels were effectively reduced by GES treatment no alteration in the parameters measured was observed when compared to ANIT-dosed control animals.

GES is effective in depleting taurine levels in all tissues (Shaffer and Kocsis, 1981; Huxtable, 1982; Huxtable et al., 1979), and as in those studies no gross changes in animal behaviour or obvious signs of organ morphopathologic
alteration were observed.

It was hypothesized by Huxtable et al. (1979), that either taurine serves a nonfunctional role and therefore altering the levels of a biological component that has no function will produce no effect or only a small fraction is functional and this fraction is unaltered by GES treatment or guanidinosulphonate can act as a taurine agonist and can functionally replace taurine (Huxtable et al., 1979). Since the first and third hypothesis are not currently accepted (Huxtable, 1992), one is tempted to try the second, admitting that GES might alter the percentage of taurine present in the liver derived from de novo synthesis (Huxtable, 1982). Recently, it was shown that GES decreased the activities of enzymes involved in the synthesis of taurine in the rat. Thus, GES reduces the activity of cysteinesulphinate decarboxylase and cysteine dioxygenase in the liver without affecting the activity of the former in the kidney or brain (Ide and Murata, 1994).

It seems that GES affects specifically the amount of taurine synthesized in the liver since rats fed a taurine-free diet showed depleted levels in other organs such the brain and the kidney (Huxtable, 1982; Huxtable et al., 1979). Rats fed a taurine-free diet possibly rely on their liver taurine synthesising capacity to maintain cellular concentration elsewhere in the body. If an impaired synthetic capacity in the major contributor organ fails to provide enough taurine in the bloodstream to be taken up by other organs, a taurine depletion will be observed in non-hepatic tissues (Ide and Murata, 1994).

As GES, as opposed to $\beta$-alanine, accumulates intracellularly and does not appear to be metabolized (Shaffer and Kocsis, 1981; Ide and Murata, 1994). Moreover, it was shown that animals treated with GES show a different pattern of bile acid conjugation (De La Rosa and Stipanuk, 1985). Thus, an increase close to 10-fold in the ratio of glycine- to taurine-conjugated bile acids was reported. This change occurred with a concomitant 30% decrease in the proportion of bile acids being conjugated with taurine without modification in the bile acid turnover.
An inhibition of taurine transport obtained from the diet should result in a higher proportion of taurine being synthesized in the liver. Taken together this might explain why depletion of the liver taurine levels using GES exerts a different effect than β-alanine in ANIT-induced toxicity. Thus, in the light of these results, by no means conclusive, a small amount of functionally protective taurine present, named as "depot taurine" by Huxtable and Lippincott (1982), might answer some questions since this amount is tenaciously held for three months in deprived animals and has possibly been synthesized in the liver.

It was reported by Waterfield and coauthors, that reduction of liver taurine levels in rats by β-alanine treatment increases carbon tetrachloride (CCl₄) toxicity (Waterfield et al., 1993e). Although, CCl₄ is known to cause toxicity by a different mechanism than ANIT, a significant rise in serum ALT and AST was shown in taurine depleted animals when compared to CCl₄-dosed control rats.

In ANIT-dosed animals, neither scattered necrotic areas nor major damage in the hepatic parenchymal cells were detected. The dose of ANIT used in these studies was smaller than that shown to cause moderate hepatocellular damage with a marked rise in the serum biochemical parameters of liver injury (Waterfield et al., 1993d). Moreover, a massive increase in serum transaminase activities was not observed and studies using similar doses of ANIT reported comparable serum transaminase levels (Leonard et al., 1984; Dahm et al., 1991b; Dahm and Roth, 1991).

When taurine treated animals were dosed with ANIT, their serum transaminase levels were smaller than, although not statistically significant, those obtained from control animals receiving the same challenge. These data correlate with the histopathological analysis of liver slices. Taurine treatment seemed to slightly protect hepatic parenchyma in the areas surrounding the
portal tracts.

In animals with depleted liver taurine levels, the liver taurine levels were increased by dosing the animals with taurine one hour before and six hours after dosing with ANIT. No differences were found either in the biochemical parameters measured or in the histological features when compared to saline dosed rats. It could be concluded that taurine does not exert a direct protective effect on early stages of ANIT-induced toxicity in animals previously depleted of this amino acid.

The onset of ANIT-induced cholestasis has been proposed to be due to hepatocanalicular dysfunction (Kossor et al., 1993b) and closely related to an increased hepatocellular and/or biliary cell tight junction permeability prior to the onset of cholestasis (Kan and Coleman, 1986; Connolly et al., 1988; Krell et al., 1987). In contrast, ANIT-induced cholestasis in vitro in the isolated perfused rat liver was due exclusively to hepatocanalicular exposure without evidence for increased tight junction permeability and bile duct obstruction (Kossor et al., 1993a). The same authors proposed that these in vivo/in vitro differences might rely on different hepatic exposure, PMN activation and potential cytotoxic effects on biliary epithelium. In this likely scenario, leaky tight junctions would favour the regurgitation of bile acids to the sinusoidal blood, exposing hepatocytes to mounting cytotoxic concentrations of bile acids.

Wang and Stacey (1990) showed that ANIT causes elevation in serum bile acids at doses that have no effect on serum liver enzymes or liver histology. Furthermore, increased junctional permeability does not correlate with changes in serum AST, that otherwise indicate increased liver damage (Kan and Coleman, 1986).

Moreover, an increase in serum bile acids conjugated with taurine was reported in animals dosed with ANIT (particularly taurocholate) (Wang and Stacey, 1990). Similar findings were described by Fannin and Thompson
They observed a significant increase in tauroconjugates, after they had exposed precision-cut liver slices to ANIT, over a 5 to 7 hour period. Polymorphonuclear neutrophils (PMNs) may come in contact with bile salts at an earlier stage of cholestasis or during chemical insult. In vitro, bile salts have been shown to potentiate superoxide release in primed rat PMNs (Dahm et al., 1988). ANIT has also been shown to stimulate superoxide release in PMNs (Roth and Hewett, 1990). More recently it has been shown that cotreatment of rats with antineutrophil serum reducing the circulating PMN numbers, prevented ANIT-induced cholestasis and attenuated other markers of liver injury (Dahm et al., 1991b).

It is known that hydrophobic bile acids cause cholestasis and hepatocellular necrosis (Heuman et al., 1991). The same authors showed that tauroursodeoxycholate protects against cholestasis and hepatocellular necrosis caused by more hydrophobic bile acids. Another report claimed prevention of cholestasis and hepatoprotective action for a taurine ursodeoxycholate conjugate in the rat (Tsukahara et al., 1993). A recent editorial describing the hepatoprotective properties of ursodeoxycholate also associates its effects with taurine conjugation (Heuman, 1993).

More recently protection against ANIT-induced cholestasis in mice was shown using a taurine conjugate of ursodeoxycholate (Kinbara et al., 1993).

It has been known for a long time that the hepatic taurine concentration is the major determinant for the proportion of bile acids conjugated with taurine in the rat (Hardison and Proffitt, 1977) and man (Hardison, 1978). Taurine treated rats were shown to dramatically increase the proportion of primary bile acids, especially β-muricholate, conjugated with taurine, whereas those of taurochenodeoxycholate and taurodeoxycholate decrease (Yan et al., 1993). The same study reported that the proportion of the cholestatic taurochenodeoxycholate in the bile is inversely related to the liver taurine levels (Yan et al., 1993).

Hydrophilic tauroconjugates are less toxic to hepatocytes and taurine conjugation of either lithocholate or chenodeoxycholate markedly reduces the
ability of these hydrophobic bile salts to enhance superoxide anion release \textit{in vitro} from primed activated PMNs (Dahm \textit{et al.}, 1988).

Several pieces of evidence suggest that taurine treatment might contribute to reducing ANIT-induced hepatotoxicity. Although speculative, one can hypothesise that taurine treated animals will preferentially conjugate the surplus of bile acids present in sinusoidal blood, after leaking from damaged tight junctions, with taurine. Hence, these taurine bile acid conjugates will be less likely to elicit the inflammatory response. Furthermore, tauroconjugates are less hepatotoxic and these could result in the slight protective effect observed for taurine treated animals when serum ALT levels were compared with control ones. Histological evaluation also shows a milder periportal necrosis in taurine treated animals when compared to control rats.

This possible protective role of taurine in ANIT-induced hepatotoxicity should be investigated further with different doses and considering more than one time point. Quantitative and qualitative analysis of serum bile acids should be performed to investigate the possible correlation of the presence of tauroconjugates with the onset of hepatotoxicity.

From current studies the only conclusion that can be drawn, is that taurine does not prevent the initial onset of cholestasis in the rat. Since the onset and progression of ANIT-induced cholestasis in the rat is believed to be characterized by several distinct events, divided into two pathophysiologically distinct phases (Kossor \textit{et al.}, 1993b), taurine might only interfere with the later phase of cholestasis, thus its effects might not be seen in the first 24 hours.

Taurine excretion follows an interesting pattern in the 24 hours after control animals have been dosed with ANIT. A massive decrease in the urinary taurine content was noticed after animals were dosed with ANIT (averaging 62.2%) whereas in control animals dosed with vehicle only a small decrease was observed (averaging 10.8%). If starvation is the factor most likely to
account for the observed drop in urinary taurine excretion in control animals
dosed with vehicle, the same reason is not likely to explain the abrupt fall in
urinary taurine excretion in ANIT-dosed animals. The liver is one of the main
organs in taurine synthesis in the rat. Evidence is presented herein that
serum taurine levels were not decreased when starved control animals dosed
with vehicle (0.383 ± 0.094 µmol.ml⁻¹) were compared to similarly starved
control animals dosed with ANIT (0.426 ± 0.091 µmol.ml⁻¹). Therefore the
liver is the biggest contributor to taurine synthesis in the rat it is not likely
that ANIT-induces an impairment in taurine synthesis.

Despite a drastic reduction in the taurine provided by the food (due to
starvation) the reduced amounts of taurine in the urine cannot be totally
accounted by the reduction in food intake. Thus, impairment of bile flow and
decreased enterohepatic recirculation of taurine after cleavage of conjugated
bile acids in the gut, can be another explanation for the reduced urinary
taurine levels.

One might also speculate that taurine that might have been used for
conjugating a rising amount of free bile acids present in the hepatocyte
(resulting from leaky tight junctions) will be unavailable to be excreted by the
kidney.

This idea is supported by the fact that 24 hours after ANIT exposure, animals
showed a raised serum bile acid concentration associated with a slight
increase in the liver taurine content when compared to control animals. This
increase was massive (± 200%) 48 hours after exposure to ANIT with urinary
taurine levels still decreased (Waterfield et al., 1993d). Therefore, taurine
might be retained in the liver at an initial phase of ANIT-induced cholestasis
as suggested by evidence that taurine conjugates free bile salts.

Taurine could play a role in ANIT-induced cholestasis, but the data presented
herein are too inconclusive to ascertain its protective function.
CHAPTER 4

INVESTIGATIONS INTO THE ROLE OF LIVER TAURINE LEVELS ON DAPM-INDUCED HEPATOTOXICITY

4.1. Introduction

Due to similarities in the hepatotoxicity between ANIT and 4,4'-methylenedianiline (4,4'-diaminodiphenylmethane, DAPM), it was thought that DAPM might provide a good model for the study of possible protective effects of taurine when rat liver taurine levels were modulated before animals were challenged with this cholangiodestructive agent. DAPM causes a vast array of histological and pathophysiological modifications to the liver of exposed animals.

In rats, short-term oral administration causes necrotizing cholangitis with periportal necrosis (Gohlke and Schmidt, 1974), while subcronic exposure elicits inflammatory cell infiltration, severe bile duct proliferation and portal fibrosis/cirrhosis (Fukushima et al., 1979). Long-term exposure was associated with liver tumours and hepatic lesions consisting of midzonal focal necrosis associated with marked leukocytic infiltrates (Schoental, 1968). Chronic exposure to rodents also indicated that DAPM is carcinogenic (Weisburger et al., 1984; Lamb et al., 1986).

Kanz and collaborators (1992) characterized the histological lesions of DAPM after a single acute dose of DAPM (250 mg.kg⁻¹) in the rat. They have shown that DAPM causes an early and selective toxic injury to epithelial cells of the major bile ducts associated with altered biliary function and bile constituents. Hepatic parenchyma injury was reported to be present at a later stage of DAPM-induced hepatotoxicity.

More recently, Bailie et al (1993) characterized the acute hepatotoxicity of DAPM in the rat, implying a dose- and time-related change in the markers of liver injury with a threshold dose between 25 and 75 mg.kg⁻¹. Metabolic activation via the cytochrome P₄₅₀ monooxygenase system seemed to be
required for DAPM to exert its toxicity. This study also urges the positive identification of DAPM metabolites. DAPM oxidized metabolites, namely azo and azoxy compounds, were identified in vitro from microsomal preparations of rabbit liver (Kajbaf et al., 1992).

In a preliminary report, Kanz and Wang (1994) suggested that bile is the route of exposure of primary target cells (biliary epithelial cells) to DAPM or its metabolite(s) in the rat.

Another characteristic of DAPM-induced hepatotoxicity is an inflammatory response, consisting of moderate to large multifocal areas of polymorphonuclear leukocyte and mononuclear cell infiltration (Bailie et al., 1993; Kanz et al., 1992). This phenomenon is evident at an early time point that precedes the onset of explicit hepatic dysfunction and necrosis in DAPM-induced liver damage (Kanz et al., 1992; Bailie et al., 1993).

Despite the fact that in situ neutrophil action has recently been implied as a contributory factor in the hepatic tissue damage exerted by other compounds (Hewett et al., 1992; Dahm et al., 1991b), Bailie et al. (1994a) have recently ruled out the direct leukocyte involvement in DAPM-induced hepatotoxicity.

This set of experiments was planned to: First, establish a possible dose-dependent relationship for DAPM-induced hepatotoxicity. Second, characterize the biochemical and histological changes caused by different doses of DAPM and its possible relationship with liver, serum and urinary taurine levels. Third, investigate the effects caused by the modulation of liver taurine in DAPM-induced hepatotoxicity.

4.2. Methods

Sprague-Dawley male rats (Glaxo bred, 130-270 g at the beginning of the experiments) were used throughout these studies.
4.2.1. Treatment for control animals

In each individual experiment, animals were randomly divided into 4 groups of 4 rats each and placed in individual metabolism cages. Food and drinking solution was allowed *ad libitum* until dosing time. After dosing, rats were deprived of food for a 24 hours period, after which they were killed as described under materials and methods (Chapter 2).

a. **Control animals** were allowed a *normal diet ad libitum* for 6 days after which they were dosed with vehicle (35% ethanol solution; v/v; 0.8 ml.kg⁻¹) or with DAPM (50, 75, 100, 250 or 400 mg.kg⁻¹ in 35% ethanol solution (v/v) orally by gavage. Food was removed after dosing. From the start of the experiments, *tap water* was provided *ad libitum*, until termination by exsanguination from the abdominal aorta under ether anaesthesia (see Chapter 2) was performed 24 hours after dosing.

4.2.2. Treatment for animals other than controls

a. **β-alanine treated animals** were supplied a *normal diet ad libitum* for 6 days after which they were dosed with a 35% aqueous ethanol solution (0.8 ml.kg⁻¹) as vehicle or DAPM (50, 75 and 100 mg.kg⁻¹ in vehicle) orally by gavage. Food was removed after dosing. Rats were given a *3% β-alanine solution in tap water* as drinking solution *ad libitum* until they were culled by abdominal aorta exsanguination under ether anaesthesia.

b. **GES treated animals** were provided with a *normal diet ad libitum* for 10 days after which they were dosed with a 35% aqueous ethanol solution (v/v) (0.8 ml.kg⁻¹) as vehicle or DAPM (50, 75 and 100 mg.kg⁻¹ in 35 % ethanol) orally by gavage. Food was removed after dosing. Animals were supplied a *1% GES solution in tap water* as drinking solution *ad libitum* throughout the experimental period, until termination as performed as described above.
c. Taurine treated animals were allowed a normal diet ad libitum for 6 days after which they were dosed with a 35% ethanol solution (v/v; 0.8 ml.kg\(^{-1}\)) as vehicle or DAPM (50, 75 and 100 mg.kg\(^{-1}\) in corn oil) orally by gavage. Food was removed after dosing. Rats were given a 3% taurine solution in tap water as drinking solution ad libitum, until termination by exsanguination from the abdominal aorta under ether anaesthesia (see Chapter 2) was performed 24 hours after dosing.

4.2.3. Statistical analysis

Urinary levels of taurine were averaged for the 48 hours before dosing and this figure compared with the 24 hour post-dose value for the same animal. A Student’s paired t-test was carried out to determine the level of significance. Groups were considered significantly different for p<0.05. The analytical statistical approach adopted to compare different groups was the one described under Chapter 2.
4.3. Results

4.3.1. Effects caused by different doses of DAPM in control animals

4.3.1.1. Serum biochemistry and changes in organ weight

The measurements described herein were performed to evaluate biochemical and clinical signs of hepatotoxicity.

A) Bilirubin

Serum bilirubin levels were significantly elevated (p<0.01) in all tested doses of DAPM when compared to the respective vehicle dosed control group. No further increase in serum bilirubin levels was observed for DAPM doses higher than 100 mg.kg\(^{-1}\) (Figure 4.1a.).

B) Serum γ-GT

Animals dosed with DAPM (50, 75 and 100 mg.kg\(^{-1}\)) showed a significant rise (p<0.01) for serum γ-GT levels when compared to vehicle dosed animals (Figure 4.1.). For DAPM doses higher than 100 mg.kg\(^{-1}\) no statistically significant differences were found when compared to vehicle dosed controls.

C) Serum AST and ALT

Rats dosed with DAPM showed a statistically significant rise in AST and ALT for all doses tested. A plateau shaped dose-response curve was observed for both serum markers of liver injury. No significant increment in the serum transaminase levels was observed for doses higher than 100 mg.kg\(^{-1}\) DAPM (Figure 4.2.).
Figure 4.1. (a) Serum bilirubin levels and (b) Serum γ-GT levels were monitored in vehicle dosed animals (8) and in animals dosed with 50 (3), 75 (3), 100 (4-8), 250 (4) and 400 (3) mg.kg\(^{-1}\) DAPM, p.o. Values are means±SEM; N=(n); ♦♦ p<0.01 when compared to vehicle dosed group by Dunnett’s t-test after ANOVA.

D) Liver TNPSH

Liver TNPSH levels were elevated (p<0.01) in animals 24 hours after dosing with 100 mg.kg\(^{-1}\) of DAPM when compared to vehicle dosed control rats. No
statistically significant difference was found for any other tested dose (Figure 4.3.).

![Graph of AST and ALT levels vs. DAPM dose]

**Figure 4.2.** Serum transaminase levels for animals dosed with vehicle or DAPM (50(3), 75(3), 100(8), 250(4) and 400(3) mg.kg\(^{-1}\), p.o.). Values are means±SEM; N=(n); * p<0.05, ** p<0.01 when compared to vehicle dose animals by a Cochran's t-test.

**E) Liver taurine**

Liver taurine levels were elevated 24 hours after all tested doses of DAPM, the rise in the liver taurine levels was statistically significant (p<0.05) only for 100 mg.kg\(^{-1}\) DAPM when compared to vehicle dosed animals (Figure 4.3.).

**F) Serum bile acids**

The serum bile acid levels showed a statistically significantly rise for all tested DAPM doses higher than 50 mg.kg\(^{-1}\) when compared to vehicle dosed rats. This serum parameter follows a similar plateau pattern after increasing
doses of DAPM. Once more, 100 mg.kg\(^{-1}\) DAPM appears to be the dose that produces the maximal rise in the serum bile acids (Figure 4.4.).

*Figure 4.3.* Liver TNPSH (a) and liver taurine levels (b), 24 hours after animals have been dosed with vehicle (8) or after been challenged with DAPM 50(3), 75(3), 100(8), 250(4) and 400(3) mg.kg\(^{-1}\). Values are means ± SEM; N=(n); ♦, ♦♦ respectively p<0.05 and p<0.01 when compared to respective vehicle dosed group by a Dunnett’s t-test after ANOVA.
G) Serum taurine levels

Serum taurine levels were significantly elevated (p<0.05) for 400 mg.kg⁻¹ DAPM when compared to vehicle dosed animals. All other DAPM tested doses (except 50 mg.kg⁻¹) caused a slight increase in the serum taurine levels (Figure 4.5.).

H) Liver weight

Liver weight (percent of animal body weight) was raised significantly (p<0.01) in all DAPM-dosed groups (except for 50 mg.kg⁻¹ group) when compared to the vehicle dose animals (Figure 4.6.).

![Graph showing serum bile acids levels](image)

**Figure 4.4.** Serum bile acids levels observed 24 hours after rats were dosed with DAPM (50(3), 75(3), 100(8), 250(4) and 400(3) mg.kg⁻¹, p.o) or vehicle. Values are means±SEM; N=(n); *p<0.05, ** p<0.01 after Cochran t-test when compared to vehicle dosed animals.
Figure 4.5. Serum taurine levels for vehicle dosed animals (8) and for rats dosed with DAPM (50 (3), 75 (3), 100(8), 250(4), 400(3) mg.kg\(^{-1}\),p.o.). Values are means ± SEM; N=(n); *p<0.05 when compared by a Cochran t-test with vehicle dosed group.

Figure 4.6. Liver weight (percent of body weight) for vehicle (8) dosed animals and for DAPM dosed animals (50(3), 75(3), 100(8), 250(4) and 400(3) mg.kg\(^{-1}\),p.o.). Values are means ± SEM; N=(n); ♦♦ p<0.01 when compared to the vehicle dosed group by a Dunnett's t-test after ANOVA.
4.3.1.2. Urinary taurine

The urinary taurine levels were monitored for the 48 hours prior to dosing (predosing), represented in fig 4.7. as open bars, and for the twenty-four hours after dosing (post-dose) plotted in the fig 4.7., as hatched bars.

After rats have been dosed with vehicle a decrease in the urinary taurine levels was observed. This decrease was also noticed after animals have been exposed for a 24 hour period to 50 mg.kg\(^{-1}\) or 100 mg.kg\(^{-1}\) DAPM. When rats were dosed with 250 mg.kg\(^{-1}\) DAPM a similar level of urinary taurine was found when compared to that observed prior to dosing. Although not statistically significant, rats dosed with the highest DAPM tested showed a rise in their urinary taurine levels compared to the amount excreted before being dosed (Figure 4.7.).

![Figure 4.7. Urinary taurine concentration in rats dosed with DAPM (50(3), 100(8), 250(4) and 400(3) mg.kg\(^{-1}\), p.o.) or vehicle (8). Values are means ± SEM; N=(n); Urinary taurine excretion per kilogram body weight per day was averaged for each individual animal 48 hours before dosing.](image-url)
4.3.1.3. Histological features

Control animals showed normal liver histology 24 hours after being dosed with vehicle. Thus, bile ducts are easily identifiable in the portal triad by their distinctive cuboidal epithelial cells. Bile ductules are also present in the periphery of the portal triad adjacent to the hepatic parenchyma (Plate 4.1.).

Plate 4.1. Light photomicrograph of the liver of control rat, 24 hours after being dosed with vehicle. Magnification × 100

Twenty-four hours after control animals were dosed with DAPM, moderate
to severe alterations were present in the periportal triads and hepatic parenchyma. Thus, the main bile ducts were denuded of epithelial cells or the few cells lining the duct were necrotic and detaching from the basal membrane. No typical bile duct structures were present and the "bile duct space" was generally recognizable by the irregular shape of the underlying interstitial tissue. Moderate to marked oedema was present in all large portal triads associated with fibrin exudate and neutrophil infiltration. Necrotic parenchymal cells were found extending out towards the portal triads in a oval or pear shaped and sometimes involving midzonal areas. Generally, these hepatic parenchymal lesions did not surround the large portal triads. Hepatic parenchymal necrotic foci were also observed, frequently associated with small portal triads found at the edge of the liver lobe.

These multifocal lesions consisted of inflammatory cells, prominently neutrophils, that appeared more numerous in areas of necrotic hepatocytes than within injured portal triads, associated with haemorrhage.

Plate 4.2. Liver photomicrograph of a control animal dosed with DAPM (100 mg.kg⁻¹). Magnification x 100
Plate 4.3. Photomicrograph of rat liver, 24 hours after DAPM (100 mg.kg\(^{-1}\)). Magnification x 100. Area magnified shows a necrotic foci with cellular infiltration and signs of haemorrhage.

Plate 4.4. Photomicrograph of a high magnification (x 400) liver necrotic area from an animal that had being dosed with DAPM (100 mg.kg\(^{-1}\)).
4.3.2. Effects of liver taurine modulation on DAPM-induced hepatotoxicity

4.3.2.1. Percentage change in the body weight

Different treatments used to modulate taurine liver levels did not cause a significant change on the daily variation of body weight. As observed before (see Chapter 3), β-alanine treated animals showed a decrease in their body weight at the beginning of the experiment (Figure 4.8a.). Neither taurine nor GES treatment affected the body weight gain of animals submitted to such treatments. After animals were dosed with vehicle or DAPM a striking decrease in their body weight was observed. There was no significant difference for body weight variation after dosing, between different treatments (Figure 4.8a.).
Figure 4.8. Comparative effect in the % change body weight, food and water (β-alanine, GES and taurine) consumption caused by different treatments aimed to modulate liver taurine levels; Values are means ± SEM for N=(4-32).
4.3.2.2. Food, water (or other solution) intake

Different treatments affected the amount of food and drinking solution ingested by the animals. Thus, animals treated with β-alanine ingested less food and drank less 3% β-alanine solution throughout the experiment when compared to control animals (Figure 4.8b. and 4.8c.).

Animals treated with taurine in the drinking water, did not show any appreciable difference for food intake, but a reduction in drinking solution intake is noticeable when compared to control animal values (Figure 4.8b. and 4.8c.).

Rats given 3% GES as the drinking solution, drank more than control animals and their intake of food was also higher than that observed for control rats (Figure 4.8b. and 4.8c.).
4.3.2.3. Serum biochemistry and organ weight changes

A) Bilirubin

Serum bilirubin levels for animals treated with β-alanine, GES or taurine did not show any statistically significant difference from those found for control animals when dosed with vehicle or 50 mg.kg\(^{-1}\) DAPM. Rats treated with β-alanine and dosed with DAPM showed a rise (p<0.01) in their serum bilirubin levels when compared to vehicle dosed ones (Figure 4.9a.). Treatment with GES significantly decreases serum bilirubin levels (p<0.005) when compared to control animals given tap water after exposure to 75 mg.kg\(^{-1}\) DAPM for 24 hours (Figure 4.9a.).

B) γ-GT

All animal groups, regardless treatment, shown a statistically significant rise in their serum γ-GT levels after dosing with DAPM. Different treatments caused erratic patterns when compared to control animals. Thus, control animals dosed with 50 mg.kg\(^{-1}\) DAPM exhibit a smaller increase (p<0.001) in their serum γ-GT levels when compared to rats treated with either β-alanine or taurine (Figure 4.9b.). Animals treated with GES also showed an increase in their γ-GT levels after being dosed with 50 mg.kg\(^{-1}\) DAPM, although not statistically significant. Higher doses of DAPM (75 and 100 mg.kg\(^{-1}\)) did not cause any statistically significant effect when animals treated with β-alanine, GES or taurine were compared to control animals. Rats treated with GES showed a smaller rise in their γ-GT levels when compared to control animals after similar doses of DAPM (Figure 4.9b.).
Figure 4.9. Serum bilirubin (a) and serum γ-GT levels (b) for control (0(8); 50(3); 75(3); 100 (4-8)), β-alanine (all doses (4)), GES (all doses (4)) and taurine (all doses (4)) treated animals, 24 hours after exposed to DAPM. Values are means ± SEM; ***, **** p<0.005 and p<0.001 by a Student's t-test when compared to animal control dosed with similar dose of DAPM.; a,b -p<0.05 and p<0.01 respectively when compared by a Student's or Cochran t-test with vehicle dosed group.; c,d - p<0.05 and p<0.01 respectively when compared to vehicle dosed group by a Dunnett's t-test after ANOVA.
C) AST and ALT

Animals treated with GES always showed a smaller increase in their serum transaminase levels when compared to control rats given the same dose of DAPM. These differences were statistically significant at all doses for ALT and at 50 mg.kg\(^{-1}\) of DAPM for AST (Figure 4.10a and 4.10b.). Similar results were found for taurine treated animals. As can be observed in figure 4.10., when one compares control rats dosed with DAPM and taurine treated animals given the same dose of the hepatotoxicant, the latter group showed a smaller increase in their serum transaminase levels. Moreover, the only dose of DAPM that cause a statistically significant rise in ALT levels on taurine treated animals when compared to vehicle dosed controls was 100 mg.kg\(^{-1}\) of DAPM.

On the other hand, rats treated with β-alanine showed higher serum transaminase levels when compared to control animals. These differences were found to be statistically significant (p<0.05) only for serum AST levels, after 100 mg.kg\(^{-1}\) DAPM when compared to control animals (Figure 4.10a. and 4.10b.).

D) Serum bile acids

All DAPM tested doses (except DAPM 50 mg.kg\(^{-1}\) for GES treatment) caused a statistically significant rise in the serum bile acid levels, notwithstanding treatment employed, when compared to vehicle dosed control animals. Taurine treated rats dosed with the higher dose of DAPM showed a statistically significant (p<0.01) rise in their serum bile acid levels when compared to control animals challenged with similar dose of the toxicant (Figure 4.11a.).
Figure 4.10. Serum AST (a) and ALT (b) levels for control (line plotted from values presented in Fig. 4.2.), β-alanine (all doses (4)), GES (all doses (4)) and taurine (all doses (4)) treated animals, 24 hours after exposed to DAPM. Values are means ± SEM for N=(n); *,**, and **** are respectively p<0.05, p<0.01 and p<0.001 by a Student’s t-test when compared to control animals given the same dose of DAPM; ♦, ♦♦ are respectively p<0.05 and p<0.01 by a Dunnett’s t-test after ANOVA when compared to control animals dosed with same dose DAPM.; a,b -p<0.05 and p<0.01 respectively when compared by a Student’s or Cochran t-test with the respective vehicle dosed group.; c,d - p<0.05 and p<0.01 respectively when compared to vehicle dosed group by a Dunnett’s t-test after ANOVA.
E) Serum taurine

Serum taurine levels measured in taurine treated animals were higher than those measured in the serum of control animals. These differences were statistically significant after 50 and 100 mg.kg\(^{-1}\) DAPM. Serum taurine levels were decreased in taurine treated animals after being dosed with DAPM (100 mg.kg\(^{-1}\)) when compared to animals treated with taurine after been dosed with vehicle (Figure 4.11b.). Treatment of animals with β-alanine and GES yield an opposite effect. Thus, decreased serum taurine levels were observed for β-alanine and GES treated rats. These lower serum taurine levels observed for taurine depleted animals are statistically significant when compared to those obtained for control animals after exposure to 100 mg.kg\(^{-1}\) of DAPM (Figure 4.11b.).

F) Liver TNPSH

Liver TNPSH levels were significantly elevated in GES treated animals when compared to control animals dosed with DAPM (50 and 75 mg.kg\(^{-1}\)). Taurine treated animals after being dosed with 50 mg.kg\(^{-1}\) DAPM showed a significant (p<0.05) decrease in their TNPSH levels in the liver when compared to vehicle dosed animals (Figure 4.12a.). Treatment of rats with β-alanine does not seem to interfere with TNPSH liver levels when compared to control animals for any tested dose of the toxic. When the β-alanine treatment was compared, no significant change between DAPM dosed animals and vehicle dosed rats was observed (Figure 4.12a.).
Figure 4.11. Serum bile acids (a) and serum taurine levels (b) for control (0(8), 50(3), 75(3), 100(8)), β-alanine (all doses (4)), GES (all doses (4)) and taurine (all doses (4)) treated animals; Values are means ± SEM; N=(n); *,**, and **** are respectively p<0.05, p<0.01 and p<0.001 by a Student's t-test when compared to animal control dosed with similar dose of DAPM; ♦♦ p<0.01 by a Dunnett's t-test after ANOVA when compared to control animals dosed with same dose DAPM; a,b -p<0.05 and p<0.01 respectively when compared by a Student's or Cochran t-test with vehicle dosed group.
G) Liver taurine

Taurine levels in the liver were significantly elevated (p<0.01) when taurine treated rats were compared to control animals, 24 hours after receiving vehicle. No statistically significant difference was found between rats dosed with vehicle compared to DAPM-dosed animals, either for control rats or other treatments employed (Figure 4.12.b.). Although without statistical significance, taurine treated animals have shown a consistent decrease in their taurine liver levels for increasing doses of DAPM. Animals treated with β-alanine or GES showed a statistically significant decrease in their liver levels of taurine when compared to control rats dosed with the same dose of DAPM (Figure 4.12b.).

H) Liver weight

Liver weight (percent of body weight) was raised in all DAPM-dosed animal groups when compared to vehicle dosed ones, regardless of the treatment (exception made for control and β-alanine treated rats dosed with 50 mg.kg⁻¹ DAPM) (Figure 4.12c.). Different treatments did not affect significantly the liver weight in vehicle dosed rats. For all DAPM doses, only taurine treated rats after dosing with 50 mg.kg⁻¹ showed a significant (p<0.01) increase in their liver weight when compared to control animals exposed to a similar dose of the hepatotoxicant (Figure 4.12c.).
Figure 4.12. Liver parameters measured for control, β-alanine, GES and taurine treated animals 24 hours after exposed to DAPM (50(3-4), 75(3-4), 100(4-8) mg.kg⁻¹) or vehicle(4-8). Values are means± SEM; N=(n); * p<0.05 or ** p<0.01 when compared to control dosed group; ♦♦ p<0.01 by a Duncan's t-test after ANOVA when compared to control dosed group. a,b respectively p<0.05 and p<0.05 by a Cochran or Student's t-test when compared to correspondent vehicle dosed group; c,d respectively p<0.05 and p<0.01 by Dunnett's t-test after ANOVA when compared to correspondent vehicle dose group.
4.3.2.3. Histological features

Treatment of animals with β-alanine increased their susceptibility to DAPM when compared to control animals. Histopathologically this effect appeared evident at all doses tested, with extended hepatic parenchymal damage in β-alanine treated rats. Moreover, rats treated with β-alanine and dosed with 50 mg.kg\(^{-1}\) DAPM showed fewer or no biliary cells lining the large bile duct and an extended hepatocellular necrosis (plate 4.6.) whereas in control or GES treated animals a slightly preserved a bile duct structure (plate 4.7. and 4.8., respectively) can be observed, although biliary epithelial cells are going necrotic and exfoliating to the lumen of the duct (plate 4.9.).

Plate 4.6. Photomicrograph of β-alanine treated rat liver, 24 hours after being dosed with DAPM (50 mg.kg\(^{-1}\)). Absence of biliary epithelial cells lining the large bile duct, bile ductules still present and intact. Necrosis is extending outwards the portal triad associated with inflammatory cell infiltration, fibrosis and oedema. Magnification (x 100).
Plate 4.7. Photomicrograph of control rat liver slide, 24 hours after being dosed with DAPM 50 mg.kg⁻¹. Magnification (x 100).

Plate 4.8. Photomicrograph of a GES treated rat liver slide, 24 hours after the animal was dosed with DAPM (50 mg.kg⁻¹). Magnification (x 100).
Plate 4.9. High magnification (x 400) photomicrograph from a control rat liver 24 hours after it has been dosed with DAPM (50 mg.kg\(^{-1}\)). Bile duct structure remains present with biliary epithelial cells (BEC) lining in the duct. Some BEC are clumping outside the bile duct structure after being disrupted from the bile duct among amorphous material and inflammatory cells.

Taurine treated animals showed a somewhat smaller extent of hepatic parenchymal damage associated with the all around cholangitis observed for DAPM (75 and 100 mg.kg\(^{-1}\)) dosed rats. Differences observed for serum biochemistry (namely, transaminases) between control animals and other treated groups was not fully corroborated by the extent of parenchymal damage for doses of DAPM higher than 50 mg.kg\(^{-1}\).
4.4. Discussion

The first set of results presented in this chapter were obtained from experiments aiming to establish a possible dose-response relationship for DAPM-induced liver injury. Indeed, similar experiments were described in a paper by Bailie et al. (1993), that observed a dose dependence for DAPM-induced hepatotoxicity. Interestingly, a similar pattern in the DAPM dose-response curve was obtained in both studies for the commonly measured markers of liver damage.

Animals receiving DAPM (50-400 mg.kg\(^{-1}\)) showed a dose-dependent elevation in all measured serum parameters (i.e. bilirubin, \(\gamma\)-GT, alanine and aspartate transaminases and bile acids). The maximal effect appears to occur at a dose of DAPM around 100 mg.kg\(^{-1}\) (Fig 4.1., 4.2. and 4.4.). This observation is in accordance with Bailie and co-workers results (1993) that had also proposed a threshold dose for toxicity between 25 and 75 mg.kg\(^{-1}\) DAPM. Although such a lower dose (25 mg.kg\(^{-1}\)) was not considered in the studies presented here, 50 mg.kg\(^{-1}\) DAPM produced a significant effect when serum biochemical alterations and histological changes were assessed as a sign of hepatotoxicity. Histological findings reported here are in accordance with those previously described for DAPM hepatotoxicity in the rat (Kanz et al., 1992; Bailie et al., 1993).

Thus, after 24 hours exposure, the histological features common to all doses tested were: 1. Damage to the main bile duct epithelial cells with destruction of virtually all epithelial cells lining the main bile ducts. Bile ductules appeared to be less affected by DAPM. 2. Multifocal lesions in the hepatic parenchymal cells were observed in the periportal area but not normally surrounding the totality of the periportal tract. The hepatocellular necrosis, when present, was extended outwards from the periportal tracts in an oval- or pear-shape involving midzonal regions of hepatic lobules. Moderate to severe infiltration of inflammatory cells, mainly neutrophils, and haemorrhage were associated with the necrotic parenchymal cells. These hepatocellular lesions occurred more frequently in small portal triads found
at the edge of the liver lobe. The feature mentioned above was not reported by any of the authors cited here.

The work published had reported histological changes in the liver, but none had addressed the possible changes in hepatic TNPSH or liver taurine levels after DAPM exposure. No specific trend was observed for the levels of TNPSH or taurine in the liver in this study. Nevertheless, a significant rise in TNPSH and taurine liver levels was noted after control animals received 100 mg.kg\(^{-1}\) DAPM. This dose was found to produce the maximal effect in control animals. An interesting pattern was observed when serum taurine, liver taurine and urinary taurine were considered altogether.

Liver taurine levels of fasted control animals (5.49 ± 0.38) showed no significant difference relative to fed animals (5.30 ± 0.86), therefore the small decrease in urinary taurine levels observed for control animals (Fig 4.7) is likely to be caused by fasting, due to a reduced amount of taurine and/or precursors being taken up. Twenty-four hours after being dosed with DAPM, animals showed increased liver taurine levels and this increment was significant at 100 mg.kg\(^{-1}\) DAPM. Since biochemical and histological signs of cholangitis were present and cholestasis was described to be present at this time (Bailie et al., 1993), the impaired or absent bile flow will certainly contribute to the observed rise in liver taurine. Thus hypothetically, impaired or absent taurine enterohepatic recirculation will reduce taurine in the serum affecting the amount of taurine excreted in the urine. On the other hand, when hepatocellular necrosis occurs it is likely that taurine will leak from damaged hepatocytes into sinusoidal blood and hypertaurinuria is an expected outcome (Waterfield et al., 1991; Waterfield et al., 1993d). Data shown in figures 4.2, 4.5 and 4.7 are in accordance with the idea that when cholestasis is present with mild toxic insult to hepatic parenchymal cells (smaller AST and ALT levels) post-dose urinary taurine levels were reduced. As hepatocellular damage becomes more evident (higher DAPM doses), and the levels of taurine rise in the urine it overrides the cholestatic effect of
DAPM. This conclusion is corroborated by a significant increase in the serum taurine levels (for 400 mg.kg\(^{-1}\) DAPM) with a concomitant decrease in the liver taurine levels (when compared to levels observed for 100 mg.kg\(^{-1}\) DAPM).

Since these initial experiments confirmed a previous report that 100 mg.kg\(^{-1}\) DAPM is the dose that induces the maximal detected response in the animal strain used (Bailie et al., 1993), it was thought that possible exacerbation or mitigation of DAPM-induced hepatotoxicity should be tried at a submaximal effective dose.

When liver taurine levels were depleted in the rat by β-alanine treatment, increased susceptibility to DAPM-induced hepatotoxicity was observed. When liver taurine depletion was achieved by treatment of rats with guanidinoethane sulphonate (GES) a reduction in the rise of serum transaminases levels was observed for almost all DAPM-doses when compared to control animals. Although this difference was observed, there was no major histological difference between the extent of hepatic damage caused by DAPM in GES treated animals when compared to control rats exposed to a similar dose of DAPM.

Animals treated with taurine, showed a significant increase in serum and liver levels of taurine. Moreover, a reduction in the extent of hepatocellular damage was observed histologically, consistent with significantly lower ALT levels when compared to control (non-taurine treated) animals.

Taken together, the modulation of liver taurine levels seems to affect at some stage the toxic effect exerted by DAPM.

Since less is known about DAPM compared with ANIT, a widely used model of intrahepatic cholestasis, drawing a parallel between the mechanism of action of both agents may be speculative. Nevertheless, the speculation instigated by these results might present a working hypothesis for future research.
DAPM was shown to undergo P₄₅₀ monooxygenase-mediated metabolism in rat liver (Bailie et al., 1993). The same authors also proposed that a bioactivation and detoxication pathway may coexist and compete for the same substrate, DAPM, that at higher doses may saturate the bioactivation pathway, regardless of the action of the detoxication route (Bailie et al., 1993). This explains why no significant increment in the dose-response curve is observed for doses higher than 100 mg.kg⁻¹ DAPM.

Although, DAPM metabolites in the rat are yet to be identified as the cause of liver toxicity, an earlier report by Kanz and Wang (1994) outlined the presence of a DAPM proximate toxicant in the bile. Thus, diverting bile from DAPM-treated animals through the common bile duct of untreated ones, they were able to show biliary epithelial cell necrosis in the bile duct of naive animals. These studies also indicated that bile is the route of exposure to DAPM and/or metabolites.

If a parallel could be drawn between DAPM and ANIT, one would expect that DAPM follows a similar mechanism of biliary concentration (Jean et al., 1995), with an accumulation of DAPM in the bile.

DAPM was shown to interfere with glutathione levels in the bile at an early stage of DAPM-induced cholestasis, causing a 100% increase during a choleretic period (Kanz et al., 1992). Hepatic cells in vivo readily secrete glutathione conjugates into bile (Wahllander and Sies, 1979) possibly by a carrier-mediated transport system present in the canalicular membranes that might favour the secretion of xenobiotic glutathione S-conjugates (Inoue et al., 1984). Glutathione itself could exert a choleretic effect by increasing the bile acid-independent bile flow (Ballatori and Troung, 1989). Thus, it is possible that DAPM or a metabolite followed this route of excretion into the bile. The initial augmentation in bile flow could favour this pathway of elimination, concentrating DAPM/metabolites at the target site.

Thus, bile duct lining cells will be primarily affected and at a later stage the damage will be extended to hepatic parenchymal cells as reported (Kanz et al., 1992).
In the likely scenario, the leakiness of the bile duct will allow a surplus of bile acids to leak into the periportal space. Inasmuch as bile acids themselves are hepatotoxic or are able to elicit the oxidative burst in polymorphonuclear neutrophils primed with 12-O-tetradecanoyl-phorbol-13-acetate (PMA) (Dahm et al., 1988), it is important per se to consider the hypothesis that bile acids could modulate the onset of an immuneresponse. This is consistent with the in situ marked inflammatory response present during the initial stage of DAPM-induced hepatotoxicity.

Recent work by Bailie et al (1994a) claimed no direct contribution for the involvement of the large number of leukocytes infiltrating the liver observed in DAPM-induced hepatotoxicity. Thus, a polyclonal antibody against rat neutrophils was effective in reducing circulating cells as well as preventing hepatic infiltration. However, this did not afford protection from DAPM-induced liver injury. A similar result was obtained in the same work when panleukopenia induced by cyclophosphamide, ruled out the involvement of other leukocytes in the DAPM-induced hepatic damage.

In previous preliminary work, the same authors presented a different story, reporting that pentoxyphilline, a compound known to inhibit the release of cytokines (IL-1 and TNFα) by macrophages (Sullivan et al., 1988; Zabel et al., 1989), markedly reduced all biochemical markers of hepatotoxicity (Bailie and Roth, 1992). Since it has been proved that resident liver macrophages (Kupffer cells) are involved in chemical-induced hepatotoxicity by other compounds (Laskin and Pilaro, 1986; Laskin et al., 1986; Edwards et al., 1993; Przybocki et al., 1992), there is the possibility that cell mediated toxicity might be involved in DAPM-induced hepatotoxicity.

Liver taurine modulation has shown possibilities as a useful tool to study the hepatotoxic effects caused by DAPM.
5.1. Introduction

It was observed that taurine treatment could ameliorate the hepatotoxic effects caused by DAPM twenty-four hours after animals were dosed (Chapter 4). Therefore it was felt that the investigation of the possible effect of liver taurine modulation on the onset of DAPM-induced hepatotoxicity might provide some insight into the mechanism by which taurine exerts its effect. The results described in this chapter were collected from experiments designed to evaluate the potential effect that liver taurine modulation might exert on the onset of DAPM-induced hepatotoxicity.

Very few studies had described the pathophysiologic changes occurring in the liver during the onset of DAPM-induced toxicity (Kanz et al., 1992; Bailie et al., 1993) and despite some efforts to describe the acute effects of DAPM exposure, little is known regarding its effects on liver taurine and GSH levels at early time points.

Once more the DAPM dose chosen was 100 mg.kg\(^{-1}\), since it was the dose that caused significant differences between taurine or \(\beta\)-alanine treated rats dosed with DAPM when compared to control animals.

Furthermore, it was thought to be important to investigate DAPM-induced hepatotoxicity 48 hours after dosing and its effects on hepatic taurine and GSH levels.
5.2. Methods

Sprague-Dawley male rats (Glaxo bred, 210-280 g at the beginning of the experiment) were used throughout these studies.

Data presented in this chapter was collected from several experiments, as follows:

a. Animals were divided into 6 groups of 4 rats each and placed in individual metabolism cages for 6 days. Half of the groups were given tap water ad libitum and the other half a 3% taurine solution ad libitum, as drinking solution. Food was provided ad libitum until dosing. All rats were dosed with DAPM (100 mg.kg\(^{-1}\)) and the food was removed after dosing. Animals were killed 6, 12 and 18 hours after dosing with DAPM.

b. Animals were divided into 5 groups of 4 animals each and placed into metabolism cages for 6 to 8 days. All rats were provided with food ad libitum until dosing. Animals used as controls were assigned to three groups, one to be used as a non-dosed control group, another to be dosed with vehicle and a third to be dosed with DAPM (100 mg.kg\(^{-1}\)). All control animals were given tap water ad libitum until the bile duct cannulation was performed. The β-alanine treated group was provided with a 3% β-alanine solution as drinking solution ad libitum throughout the experiment; rats were dosed with 100 mg.kg\(^{-1}\) DAPM and the bile duct was cannulated 4 hours after dosing. Taurine treated animals were given a 3% taurine solution ad libitum until the bile duct was cannulated. This surgical procedure was carried out 4 hours after animals were dosed with 100 mg.kg\(^{-1}\) DAPM. Bile flow was measured for 2 hours, as described in Chapter 2. Rats were killed by abdominal aorta exsanguination 6 hours after dosing with DAPM.

c. Twelve animals were randomly divided into 3 groups of four animals each and caged in metabolism cages. Control, β-alanine and taurine groups were treated as above. All rats were dosed with 100 mg.kg\(^{-1}\) of DAPM, bile duct cannulation was performed 8 hours after animals were dosed and the
bile collection lasted for two hours. Animals were sacrificed 10 hours after
dosing.

d. Data for the twenty-four hour time point was already presented in
chapter 4 and were included herein to add an extra data point in the time-
course study.

e. Animals exposed for 48 hours to DAPM were divided into 4 groups of
4 animals each and placed in communal cages. All groups were allowed food
ad libitum until dosing. Control animals were given tap water ad libitum
throughout the experiment. β-alanine treated rats were provided with a 3%
β-alanine solution in tap water ad libitum, as drinking solution, until killed.
Taurine treated animals were given a 3% taurine solution in tap water as
drinking solution, until killed as described in Chapter 2.
Food was removed from all groups after dosing with DAPM (100 mg.kg⁻¹) or
vehicle. In order to comply with the 1986 Animal Act Regulations, food supply
was resumed after an initial 24 hour period of fasting.

5.3. Results

5.3.1. Serum and liver parameters for control animals dosed with
vehicle.

The following table shows the measured parameters for control animals at
different time points after being dosed with vehicle (35% ethanol solution).
Table 5.1. Serum parameters: bilirubin and bile acids (SBA) (µmol.l⁻¹); γ-GT, AST, ALT and ALP (iu.l⁻¹); taurine (µmol.ml⁻¹). Liver parameters: TNPSH and taurine (µmol.g⁻¹ wet wt). Values are means ± SEM; N=(n).

<table>
<thead>
<tr>
<th>Measured parameter</th>
<th>means ± SEM (number of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 hours</td>
</tr>
<tr>
<td>Serum bilirubin</td>
<td>3.26 ± 0.16 (3)</td>
</tr>
<tr>
<td>Serum γ-GT</td>
<td>1.41 ± 0.50 (3)</td>
</tr>
<tr>
<td>Serum AST</td>
<td>33.8 ± 5.45 (3)</td>
</tr>
<tr>
<td>Serum ALT</td>
<td>17.0 ± 1.31 (3)</td>
</tr>
<tr>
<td>Serum ALP</td>
<td>108.2 ± 9.22 (3)</td>
</tr>
<tr>
<td>Serum bile acids</td>
<td>22.6 ± 0.70 (3)</td>
</tr>
<tr>
<td>Liver TNPSH</td>
<td>4.53 ± 0.27 (4)</td>
</tr>
<tr>
<td>Liver taurine</td>
<td>3.47 ± 1.01 (4)</td>
</tr>
<tr>
<td>Serum taurine</td>
<td>0.291 ± 0.042 (3)</td>
</tr>
</tbody>
</table>

5.3.2. Serum and liver parameters measured in animals other than vehicle dosed controls

5.3.2.1. Serum bilirubin

Serum bilirubin levels steadily increased in DAPM dosed animals until the last time point measured (48 hours) for all different treatments (Figure 5.1a.). Animals treated with taurine showed an earlier rise (12 hours time point) in the serum bilirubin level when compared to control animals (Figure 5.1a.). Despite slight variations among different treated groups when compared to control, no statistically significant difference was found for each time point considered.
5.3.2.2. Serum γ-GT

Serum gamma-glutamyltranspeptidase activity was increased for most of the time-course in all groups. However, 48 hours after exposure to DAPM, animals showed a decreased activity of γ-GT in the serum. β-alanine and taurine treated groups showed an earlier rise (respectively at 6 and 12 hours) in their serum levels of γ-GT when compared to control animals, although sustained up to 24 hours after dosing. These increases were not statistically significant (Figure 5.1b.).

5.3.2.3. Serum transaminases

In control animals, serum transaminase levels were elevated after rats were dosed with 100 mg kg⁻¹ DAPM and their values increased with time (Figures 5.2a. and 5.2b.) when compared to vehicle dosed animals for the same time point (table 5.1.). Animals treated with taurine showed a statistically significant increase in their serum transaminases levels at 12 hours after dosing when compared to control rats (Figures 5.2a. and 5.2b.). This increase was still present 18 hours after DAPM, although without statistical significance. The trend was modified after 24 hours. At this time point (as shown in Chapter 4) taurine treated animals presented statistically significant reduced levels of both serum transaminases (Figures 5.2a. and 5.2b.). Animals treated with β-alanine, showed an increase in their serum transaminases levels that was present 10 hours after dosing. This tendency remained present 24 hours after challenge with DAPM. Two-days after β-alanine treated animals were dosed their ALT values were elevated (p<0.05) when compared to control animals dosed with the hepatotoxicant. When the bile duct was cannulated and the enterohepatic circulation interrupted the levels of both serum transaminases (10 hours after animals were dosed with DAPM) were diminished compared with those observed 6 hours after exposure to DAPM (Figure 5.2.).
Figure 5.1. Serum bilirubin (a) and serum gamma-glutamyltransferase (b) levels for control (4-8), β-alanine (4) and taurine (4-8) treated animals after being dosed with DAPM (100 mg.kg⁻¹). Values are means±SEM; N=(n).
Figure 5.2. Serum AST (a) and ALT (b) levels for control (4-8), β-alanine (4) and taurine (4-8) treated animals, at different time points after being dosed with DAPM (100 mg.kg⁻¹). Values are means ± SEM; N=(n); * p<0.05 by a Student's t-test when compared to control animals for the same time point; ♦, ♦♦ respectively p<0.05 and p<0.01 by a Dunnett's t-test after ANOVA when compared to control animals for the same time point.
5.3.2.4. Serum alkaline phosphatase

Serum alkaline phosphatase activity was increased in all treated groups 48 hours after dosing with DAPM. No statistical significance was found when different treatments were compared to control animals, at any time point considered (Figure 5.3.).

![Graph showing serum alkaline phosphatase activity](image)

**Figure 5.3.** Serum alkaline phosphatase activity measured in samples taken from control (4-7), β-alanine (4) and taurine treated animals (4-8) at several time points after being dosed with DAPM (100 mg.kg⁻¹). Values are means ± SEM; N=(n).

5.3.2.5. Serum bile acids

Serum bile acids levels were elevated, in control animals, 6 hours after DAPM exposure. Throughout the experiment, β-alanine treated animals showed a rise in their serum bile acids levels when compared to control animals. A similar tendency was shown by taurine treated animals, despite only starting 10 hours after dosing with DAPM. For the last two time points assessed this rise in serum bile acids levels was statistically significant when compared to control animals dosed with 100 mg.kg⁻¹ DAPM (Figure 5.4.).
Figure 5.4. Serum bile acids levels in control (4-8), β-alanine (4) and taurine (4-8) treated animals. Values are means ± SEM; N=(n); ** and ♦♦ p<0.01 respectively by a Student's t-test and Dunnett's t-test after ANOVA when compared to control animals for the same time point.

5.3.2.6. Liver TNPSH

Animals treated with β-alanine or taurine prior to DAPM showed an increase in liver TNPSH levels, up to the 18 hour time point, when compared to control dosed animals. This increase was statistically significant for the 10 hours time point (Figure 5.5a.).

Liver TNPSH levels were raised in all groups 24 hours after exposure to DAPM. This tendency was unaltered at the 48 hours time point (Figure 5.5a.).
Figure 5.5. Liver TNPSH (a) and taurine levels (b) and serum taurine levels (c) in control (4-8), β-alanine (4) and taurine (4-8) treated animals at various time points after receiving DAPM (100 mg.kg⁻¹). Values are means ± SEM; N=(n); *, ** respectively p<0.05 and p<0.01 by a Student’s t-test; ♦♦♦♦♦ respectively p<0.05 and p<0.01 by a Dunnett’s t-test after ANOVA.
5.3.2.7. Liver taurine

Liver taurine levels were significantly raised in taurine treated animals at every time point up to 18 hours after dosing with DAPM when compared to the respective control animal group (Figure 5.5b.). This difference was absent at 18 and 24 hours after DAPM. At 48 hours after dosing taurine treated animals showed an increase in their liver taurine levels when compared to control animals.

Animals treated with β-alanine had lower liver taurine levels at all time points, except for 48 hours after dosing, when compared to control animals (Figure 5.5b.).

5.3.2.8. Serum taurine

Serum taurine levels were significantly raised in taurine treated animals, at all time points assessed, when compared to control animals (Figure 5.5c.). Taurine treated animals showed a steady decrease in their serum taurine levels after dosing with DAPM until the 18 hour time point.

Animals treated with β-alanine presented lower serum taurine levels for all time points evaluated when compared to control animals (Figure 5.5c.).

5.3.3. Urinary taurine excretion

Urinary taurine excretion was monitored at various time points after control animals were dosed with DAPM. Values for individual animals challenged with DAPM or vehicle were compared to those found in a pre-dosing period. This pre-dosing collection was performed in a 2×24 hours consecutive period and the urinary taurine excretion was calculated as μmol.kg⁻¹.day⁻¹. For the purpose of comparing different time points after exposure to DAPM, all data was normalized to μmol.kg⁻¹.hour⁻¹. No statistically significant difference was found for the urinary taurine excretion in rats challenged with DAPM when compared to their pre-dose values. A peak in urinary taurine excretion for
DAPM-dosed rats was observed at 12 hours after exposure (Figure 5.6.). This possible transient rise in urinary taurine excretion was neither present at 18 nor at 24 hours after the DAPM challenge.

**Figure 5.6.** Urinary taurine excretion for control animals (4-8) before being dosed with DAPM (average for 48 hours) and at different time points after they received DAPM (100 mg.kg<sup>-1</sup>). Values are means ± SEM; N=(n).

**Figure 5.7.** Urinary taurine excretion for control animals, measured at several time points after dosing with DAPM (100 mg.kg<sup>-1</sup>). An estimation for the 24 hour value was plotted as hatched bars. Values are means ± SEM for 4 to 8 individual rats.
5.3.4. Bile flow

Bile flow was measured in control, β-alanine and taurine treated animals at different time points after rats were exposed to DAPM (Figure 5.8.). For the initial time points assessed (4 to 6 hours) a comparison of the bile flow was also established between naive control animals and vehicle dosed rats (Figure 5.8. - upper window). Vehicle dosing did not interfere with bile flow rate, since no significant difference was found when bile flow in naive control animals was compared to control animals, 4 to 6 hours after being dosed with vehicle. When control animals were dosed with DAPM (100 mg.kg⁻¹), although they have shown a decrease in bile flow when compared to control rats dosed with vehicle, this difference was not significant for the time points assessed.

Animals treated with β-alanine did not show a significant alteration in their bile flow rate when compared to control rats dosed with a similar dose of the toxicant.

On the other hand, taurine treated animals showed a rise in their bile flow rate, although not statistically significant, five hours after being dosed with DAPM when compared to DAPM-dosed control animals. This trend remained present throughout the period of time for which the bile flow rate was evaluated.
Figure 5.8. Bile flow measured in control (4), β-alanine (3) and taurine (3-4) treated animals at different time points after being dosed with DAPM (100 mg.kg⁻¹). Upper window; bile flow measured in naive rats (3) and in control animals (3) at several time points after being dosed with vehicle. Values are means ± SEM; N=(n).
5.3.5. Histological features

Throughout the time-course study, multiple histological alterations were observed in the livers of rats dosed with DAPM. The histopathological features appeared to correlate well with the serum biomarkers of hepatotoxicity. When bile flow was measured, its absence or dramatic decrease was associated with extensive damage to the bile duct structures.

Thus, six hours after animals being dosed with vehicle no histological alterations were found in the liver (Plate 5.1.) when compared to naive (non-vehicle dosed) animals.

Plate 5.1. Photomicrograph (x 100) of the liver of a control animal six hours after being dosed with vehicle. The large bile duct structure is intact and the hepatic parenchyma shows a normal appearance.

Animals dosed with DAPM (100 mg.kg\(^{-1}\)), regardless of treatment, started to show evidence of biliary epithelial cell damage six hours after dosing. In animals where bile flow rate was measured, a dramatic decrease or absence of bile flow was
associated with complete bile duct destruction (Plate 5.2.). At this time point, the establishment of DAPM-hepatotoxicity as assessed histologically seemed to be variable. Large bile duct structures are still present, although some biliary cell necrosis could be observed (Plate 5.3.). Some large portal triads showed clear signs of bile duct damage, biliary epithelial cells are extruding towards the lumen of the duct and some are scattered among fibrotic tissue outside the bile duct (Plate 5.4.). At this stage mild inflammatory infiltration is present in the periportal area. Moreover, in the few necrotic foci found, clearly no evident sign of neutrophil infiltration was present (Plate 5.5), contrasting with a massive neutrophilic infiltration at later time points.

Plate 5.2. Photomicrograph of a portal triad showing mild oedema and total absence of a bile duct. Magnification (x 100).
Ten hours after animals were dosed with DAPM, the liver histological features were similar to those observed at 6 hours. Necrotic foci in hepatic parenchyma were rare at this time point and when present were confined to an area proximate to the edge of the lobe. The major histological alteration was a disruption of the bile ducts (mainly the large bile ducts) with BEC necrosis and cellular infiltration surrounding the duct and within the periportal triad.

Plate 5.3. High magnification (x 400) photomicrograph of a bile duct showing earlier signs of disruption. Some biliary epithelial cells (BEC) are becoming necrotic and beginning to separate from each other and from the basement membrane. A few BEC are aggregating outside the bile duct.
Plate 5.4. Photomicrograph of a large portal triad showing damaged bile ducts with few biliary epithelial cells (BEC) lining the duct. BEC are necrotic and scattered among the fibrotic tissue that surrounds the bile duct structure. Moderate oedema is present. Magnification (x 100).

Twelve hours after animals were challenged with DAPM, biliary epithelial cells lining the main bile duct are destroyed and absent from the bile duct structures still recognizable by the irregular shape of the underlying interstitial tissue. Some bile ducts showed a few BEC lining the duct. Total bile duct destruction is present for all the main bile ducts, although small portal triads showed damaged bile duct structures without complete disruption. No distinctive pattern of histological damage was observed between control and taurine treated animals after receiving DAPM.

Eighteen hours after rats were dosed with DAPM, bile duct destruction was a common feature for all slides assessed, regardless of the treatment.
animals whereas control animals did not show an involvement of the parenchymal cells at this time point.

Plate 5.5. High magnification (x 400) photomicrograph showing hepatic parenchymal necrotic cells with haemorrhage associated but with few inflammatory cells.

Forty-eight hours after animals were dosed with DAPM a large regeneration process is evident in the periportal areas. A large majority of the bile ducts are undergoing a regeneration process, the bile duct structure is newly formed with biliary epithelial cells present in the majority of bile duct structures (Plate 5.6.). Periportal oedema, with inflammatory infiltration and fibrotic tissue remains at this stage. A limited number (2-5) of necrotic foci were detected in the hepatic parenchyma associated with haemorrhage and
this stage. A limited number (2-5) of necrotic foci were detected in the hepatic parenchyma associated with haemorrhage and inflammatory infiltrate. Another common feature at this time point is a massive cellular infiltration of macrophages in nearby areas showing bile duct reorganization. Bile duct hyperplasia could be considered to be present in several cases, since several bile ducts are being formed within a periportal space whereas one is normally present.

Plate 5.6. Photomicrograph of a rat liver, 48 after being dosed with DAPM (100 mg.kg⁻¹). Bile ducts are present despite a necrotic area with cellular infiltration and oedema evident. Magnification (x 100).
5.4. Discussion

Evidence for a possible protective action of taurine against DAPM-induced hepatotoxicity was presented in the last chapter. When a time-course study was performed, a rise in the serum markers of hepatic parenchymal damage occurred at an earlier time point for taurine treated animals than for control animals dosed with the same dose of the toxicant (Figure 5.2.). It appeared that animals treated with taurine before dosing with DAPM, were less susceptible to a certain extent, to the DAPM-induced hepatotoxicity when the toxic effects were assessed 24 hours after dosing. When the onset of DAPM-induced hepatotoxicity was evaluated at time points earlier than 24 hours, a different pattern was observed. Thus, taurine treated animals showed higher levels in all measured biochemical markers of hepatotoxicity at 12 hours after rats were dosed with DAPM when compared to control animals (Figures 5.1. and 5.2.). Despite a statistically significant rise in serum ALT and AST for taurine treated animals when compared to control rats, no histological parenchymal damage was found at this time point (12 hours) for both groups. Although the rise in the serum markers of hepatotoxicity persists eighteen hours after taurine treated animals were dosed with DAPM, it was not statistically significant when compared to control rats. On the other hand, signs of parenchymal damage were observed for taurine treated rats whereas no parenchymal involvement was observed in control animals at this time point.

Relative to control rats, the data is in accordance with that published by Bailie et al (1993) which reported a significant rise in the ALT, γ-GT and bilirubin, 12 hours after rats were given a similar dose of DAPM. For earlier time points (4-8 hours) the same authors reported subtle changes in the histological appearance of the portal region. However, Kanz and collaborators (1992) showed an earlier rise in serum parameters after DAPM dosing.
DAPM was reported to exert an immediate choleretic effect in the rat and this effect lasted for about 1 hour after animals were dosed. The same work reported a decrease of 60% in the bile flow rate four hours after dosing with a significant rise in serum ALT and bilirubin; in this study a higher dose (250 mg.kg\(^{-1}\)) of DAPM was used (Kanz et al., 1992).

Results presented in figure 5.8. showed a small decrease in bile flow when DAPM-dosed control rats were compared to vehicle-dosed controls. One of four animals dosed with DAPM showed total absence of bile flow five hours after being dosed with DAPM. In this particular case, liver histological assessment showed that all main bile ducts were disrupted and denuded of biliary epithelial cells. In opposition, those animals that retained bile flow showed milder but significant evidence of biliary duct damage. Total absence of bile flow was not observed up to ten hours after animals were dosed with DAPM (100 mg.kg\(^{-1}\)).

The hypothesis of competing detoxication/bioactivation pathways proposed by Bailie and coworkers (1993) partially explains the different pattern in bile flow and onset of DAPM-induced hepatotoxicity found for different doses of DAPM. Thus, the higher dose used by Kanz and collaborators (1992) might saturate the bioactivation pathway overriding the action of a detoxifying route for DAPM metabolism. This explains the similarity of results for lower doses of DAPM (100 mg.kg\(^{-1}\)) when data present herein is compared to that shown by Bailie et al (1993), and an earlier establishment of the cholestatic effects when a higher dose of DAPM was used (Kanz et al., 1992).

Taurine treated animals showed a higher bile flow rate, although not statistically significant, when compared to control rats exposed to a similar dose of DAPM. Taurine treatment was also shown to have choleretic effect in guinea-pigs (Dorvil et al., 1983). The lack of a taurine treated group dosed with vehicle does not allow an optimal comparison to be established, since one would not know if taurine treated animals intrinsically have high bile flow rate at the time of DAPM-dosing. It seems unlikely that DAPM alone caused
a rise in the bile flow rate in taurine treated rats whereas in control animals no significant effect was observed for a similar time of exposure. Thus, it is possible to conclude that taurine treatment might promote bile flow. Several pieces of evidence support this hypothesis: First, taurine levels in the liver were increased after taurine treatment (Figure 5.5.); Second, hepatic taurine concentration is the major determinant of the proportion of bile acids conjugated with taurine (Hardison and Proffitt, 1977); Third, taurocholate increases bile flow rate by promoting an electrolyte secretion (Spaeth and Schneider, 1974b).

If one of the possible effects of taurine is acting by increasing the bile flow rate in the rat, it is reasonable to suggest that more DAPM and/or its metabolite(s) would be more readily available at the target site (biliary cell epithelium). This might explain why taurine treated animals showed a shift towards an increment in hepatotoxicity at an earlier time point when compared to control animals or β-alanine treated rats. Furthermore, the smaller increase in serum AST and ALT (Figure 5.2.) together with slightly lower SBA levels (Figure 5.4.) observed in bile duct cannulated animals (10 hours) compared with those observed after 6 hours of exposure suggests the possibility that the enterohepatic circulation of DAPM or a metabolite might act as a contributing factor for DAPM-induced hepatotoxicity.

Urinary taurine was monitored for animals other than the ones submitted to the bile cannulation procedure during the time course study and compared to the pre-dosing excretion levels. Six hours after animals were dosed with DAPM, the total amount of taurine excreted for this period was lower than the averaged pre-dosing 24 hour period. When an estimation for a 24 hour urinary taurine excretion level was made for animals exposed to DAPM for 6 hours, similar urinary taurine levels were obtained for the pre- and post-dosing periods (Figure 5.7.). Twelve hours after dosing, animals showed an increase in their urinary taurine levels either when excretion was estimated for 24 hours or calculated hourly (Figures 5.6 and 5.7.). However, when the amount of taurine excreted hourly (or estimated to be excreted for a 24 hour period) was assessed 18 hours after dosing with DAPM, no difference was
found when compared with the pre-dosing levels. It seems that during the period that precedes the onset of cholestasis and hepatocellular damage the urinary taurine levels were unaltered. Furthermore, the increase in urinary taurine excretion registered between 6 and 12 hours after rats were dosed with DAPM coincides with a slight decrease in values for serum and liver taurine. Hypothetically it is between these time points that parenchymal damage is established and leaky or damaged hepatocyte membranes will certainly contribute to an increased amount of taurine being excreted in the urine. This hypothesis is supported by the rise in the serum markers of hepatotoxicity at 12 hours and thereafter shown in figure 5.2. and corroborated by data previously presented by Bailie and coworkers (1993).

It was shown recently that hypertaurinuria can be used as a marker of liver damage (Waterfield et al., 1991; Waterfield et al., 1993d; Waterfield et al., 1993c).

Despite the fact that urinary taurine excretion was not evaluated for taurine treated animals, it is likely that the urinary taurine excretion had also increased during the period considered above. This probable increase in the urinary taurine levels was paralleled by a marked decrease in the liver and serum taurine levels (Figure 5.5b and 5.5c.). Although, one can argue that the decrease in both liver and serum taurine levels was caused by a reduction of taurine intake from the drinking solution and/or food, it would not explain the drastic reduction in the liver taurine levels ($8.57 \pm 0.29$) observed for taurine treated animals dosed with DAPM when compared to taurine treated animals dosed with vehicle ($16.5 \pm 3.10$), 24 hours after dosing. Moreover both animal groups consumed a similar volume of 3% taurine (w/v) drinking solution during the fasting period.

The bile duct regeneration observed at 48 hours was associated with the presence of several foci of parenchymal necrosis and massive inflammatory cell infiltration near to small portal triads. Clumps of inflammatory cells were observed close to newly formed bile ducts.
Forty-eight hours after animals were dosed with DAPM, some serum markers of hepatotoxicity (AST, γ-GT and serum bile acids) showed a decrease when compared to values obtained at twenty-four hours after dosing, while others remained higher or even showed an increase (bilirubin, ALT and ALP) compared to the 24 hours time point. This can be explained by the different half-lives shared between different markers, for instance AST has a half-life of 6 to 14 hours dependent on its origin (respectively, mitochondrial or cytoplasm) whereas ALT has reported half-life of 50 hours (Moss and Henderson, 1994). Another explanation may be that biliary obstruction was proved to stimulate ALP production by the liver, accounting for much of its production and release in hepatobiliary disease (Moss and Henderson, 1994) and bile duct obstruction and/or destruction maintains high serum bile acids levels. On the other hand, γ-GT an enzyme located on the exterior surface of the hepatocytes (Moss and Henderson, 1994) and mainly in the biliary epithelial cells lining the bile ducts (Parola et al., 1988; Leonard et al., 1984), was shown to be decreased 48 hours after DAPM dosing. This is in accordance with the bile duct regeneration observed.

These time course studies were planned to establish if taurine treatment causes any change in the onset of DAPM-induced hepatotoxicity. Although, for presentation purposes all time points were plotted in the same figure, one has to bear in mind that data was collected from different experiments, as stated above under methods. This might have influenced the pattern of onset of DAPM-induced hepatotoxicity as assessed by serum biochemical markers or histologically. Although the experiments were planned carefully to avoid misleading results, it was impossible to perform all of them at once.

Taurine treatment shifts the onset of DAPM-induced hepatotoxicity to an earlier time point. This might be the answer for the slight protection observed at 24 hours for taurine treated animals when compared to control rats. It is advisable for future studies using similar approaches that a follow up for a longer period is performed and if possible all groups are combined in the same experiment.
CHAPTER 6
STUDY OF THE EFFECT OF DIETS WITH LOW & HIGH CASEIN CONTENTS ON TAURINE LIVER LEVELS AND ITS INFLUENCE IN ANIT- AND DAPM-INDUCED HEPATOTOXICITY

6.1. Introduction

In the previous chapters it was described how the modulation of liver taurine levels might affect the hepatotoxicity caused by two cholangiolytic agents.

Although all treatments used to modulate liver taurine levels have produced the previously reported and desired effects (i.e. depletion or increase in the taurine liver levels), the modulation of taurine levels in the liver was primarily achieved by means other than affecting only the taurine biosynthetic metabolic pathway. Several reports in recent years, suggested that modifying dietary taurine content might exert an effect on established or induced hepatobiliary dysfunctions, such as that developed during long-term parenteral nutrition (Guertin et al., 1993).

Since it was proposed that one of the possible mechanisms by which taurine might confer protection is by its active modification in the pattern of bile acid conjugation (Ide and Sugano, 1991), animals were fed a modified diet specially designed to increase their liver taurine levels. One requisite of this diet was to contain no taurine, in order to ascertain that possible higher levels of taurine obtained in the liver were the result of synthesis.

6.2. Methods

Sprague-Dawley male rats (Glaxo bred, 80-150 g at the beginning of the experiments) were used in these studies. Rats were randomly divided into 6 groups (3 groups - 15% casein diet; 3 groups - 30% casein diet) of four animals each and placed in metabolism cages for an initial period of ten days.
After one day in communal cages, isolation was resumed until animals were sacrificed. Data presented in this chapter resulted from separate experiments performed using special diets. Food and tap water were provided *ad libitum* and its consumption monitored throughout the experiments. Food was removed after dosing. Food provided in these studies was a special diet purchased from Harlan-Teklad, Blackthorn, Bicester, U.K. Dietary composition was planned to ascertain that taurine was absent from the diet. The diet manufacturer guarantees that taurine is removed while casein is isolated from an animal source (milk). To verify this, a diet extract was prepared and the percentage of taurine present in both experimental diets was determined to be 0.0025%. Normal diet contains approximately 0.054% of taurine (over 20-fold more).

Two different types of diet were used:
- TD 93218 containing 15% *casein* and supplemented with 0.225% methionine.
- TD 93258 containing 30% *casein* and supplemented with 0.45% methionine. Detailed diet composition is given in Appendix IV.

Body weight gain was monitored throughout the experiment as well as urinary taurine excretion.

In the first experiment performed, each group of animals was fed a different type of diet and dosed either with DAPM (50 mg.kg⁻¹) or vehicle. The two remaining groups were used as non-dosed controls and were killed at dosing time. In a second experiment, rats were dosed with DAPM (75 or 100 mg.kg⁻¹) or ANIT (100 mg.kg⁻¹). Food was removed for all groups after dosing. Animals were killed 24 hours after dosing.

The statistical analysis of the results was performed as described under materials and methods (Chapter 2) with an exception taken for the analysis of urinary taurine levels before and after dosed with ANIT and DAPM where a two tailed paired t-test was used.
6.3. Results

6.3.1. Percentage change in body weight

The percentage change in animals body weight was evaluated throughout the experiment. Different diets supplied to animals did not alter significantly their weight gain. Animals showed a daily body weight gain of approximately 4 to 7 g. At day eleven, when rats were placed in communal cages, the percentage change in body weight was almost nil. A normal body weight gain was observed from the twelfth day until the dosing day. As food was removed, twenty-four hours after being dosed animals lost between 5 and 10 percent of their pre-dose body weight (Figure 6.1.).

![Graph showing the percentage change in body weight of animals given two different diets.](image)

**Figure 6.1.** Weight variation as percent of body weight in animals given two different diets. Values are means ± SEM; N= 12-24.
6.3.2. Food and water intake

Food intake rose from an initial (day 1) value around of 7-8 g.24 hr⁻¹.100g⁻¹ body weight to 12-13 g.24hr⁻¹.100g⁻¹ observed at days 2 and 3. Afterwards, a decline in the slope accounting for food consumption was observed (Figure 6.2a.). Although, no significant difference could be found, animals fed the 15% casein diet ate slightly more than those given a 30% casein diet (Figure 6.2a.).

A similar pattern in the slope of the curve was noticed for water intake in rats fed these two different diets. Furthermore, rats fed a 30% casein diet drank significantly more water (for several time points) than those on a 15% casein diet (Figure 6.2b.). After dosing, animals fed the highest casein content diet showed a decrease in their water intake. In contrast, animals fed a 15% casein diet, appeared to increase their water intake.
Figure 6.2. Food consumption (a) and water intake (b) in rats given different diets. Values are means ± SEM; N=12-24.

6.3.3. Serum biochemistry and liver measurements

Biochemical parameters measured in the serum and liver of animals fed a 15% or 30% casein diet were compared to those found for animals fed a normal diet and described in Chapters 3 and 4.

Non-dosed animal groups were killed at the same time the other groups were dosed with vehicle or DAPM. The different dietary casein content did not alter the levels normally observed for the measured parameters (Table 6.1.).
### Measured parameters

<table>
<thead>
<tr>
<th>Measured parameters</th>
<th>Dietary casein content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15%</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>3.39 ± 1.37</td>
</tr>
<tr>
<td>γ-GT</td>
<td>0.96 ± 0.22</td>
</tr>
<tr>
<td>AST</td>
<td>13.92 ± 10.15</td>
</tr>
<tr>
<td>ALT</td>
<td>4.12 ± 1.86</td>
</tr>
<tr>
<td>ALP</td>
<td>108.40 ± 9.05</td>
</tr>
<tr>
<td>Liver TNPSH</td>
<td>8.52 ± 0.32</td>
</tr>
<tr>
<td>Liver taurine</td>
<td>4.00 ± 4.53</td>
</tr>
<tr>
<td>SBA</td>
<td>40.00 ± 31.3</td>
</tr>
<tr>
<td>Serum taurine</td>
<td>0.313 ± 0.054</td>
</tr>
</tbody>
</table>

Table 6.1. Serum and liver parameters measured in rats fed a special diet for 21 days and killed at the time of dosing. Serum parameters: bilirubin and bile acids (SBA) (µmol.l⁻¹); γ-GT, AST, ALT and ALP (iu.l⁻¹); taurine (µmol.ml⁻¹). Liver parameters: TNPSH and taurine (µmol.g⁻¹ wet wt). Values are means ± SD for 4 animals in each group.

#### 6.3.3.1. Serum bilirubin

Animals fed a 15% casein diet showed a statistically significant (p<0.01) smaller rise in their serum bilirubin levels when compared to animals fed a normal diet equally dosed with ANIT (100 mg.kg⁻¹). Conversely, animals fed a diet containing 30% casein have shown no difference when compared to control animals dosed with ANIT (Figure 6.3a.).

It can be also observed in figure 6.3a. that rats fed both special diets are less affected than control animals (for doses of DAPM up to 75 mg.kg⁻¹). The only statistically significant difference was observed between control animals and those fed a 30% casein diet after DAPM (75 mg.kg⁻¹).

After animals were dosed with 100 mg.kg⁻¹ DAPM, control animals showed lower serum bilirubin levels than rats fed a 15% or 30% casein containing diet (Figure 6.3a.).
6.3.3.2. Serum γ-GT activity

Animals fed either special diet were less susceptible to ANIT or DAPM as assessed by a rise on their serum γ-GT levels than control animals fed a normal diet and dosed with a similar dose of the toxicant (Figure 6.3b.). Rats fed a 15% casein diet were less affected by DAPM or ANIT, as assessed by the rise in serum γ-GT levels, than 30% casein diet fed animals when compared to control animals fed a normal diet.

6.3.3.3. Serum transaminase activity

A dose-dependent increase in the serum transaminase activity was observed for DAPM-dosed rats, regardless of the diet that animals were fed. Animals fed a 15% casein diet showed a statistically significantly lower serum AST activity when compared to control animals after dosing with ANIT or DAPM (Figure 6.4a.). Animals fed the higher content casein diet were less susceptible (p<0.05) to ANIT than rats fed a normal diet, as assessed by serum AST activity. This difference was not observed for DAPM challenged animals, where the higher content casein diet had a similar effect only at 50 mg.kg\(^{-1}\) DAPM. For higher doses of this hepatotoxicant, AST levels in the serum were similar or even higher when compared to control animals (Figure 6.4a.). Serum ALT activity followed a similar pattern, with the difference that the high content casein diet provided some hepatoprotection when compared to control animals fed a normal diet given 50 mg.kg\(^{-1}\) DAPM (figure 6.4b.).
Figure 6.3. Serum bilirubin (a) and γ-GT (b) levels for control rats consuming a normal diet (12-15, ANIT(A); 3-8, DAPM (D)), rats given a 15% casein diet (4(A);3-4(D)) and animals fed a 30% casein diet (4(A);4(D)), 24 hours after dosing with ANIT (100 mg.kg⁻¹) or DAPM (0, 50, 75 and 100 mg.kg⁻¹). Values are means ± SEM; N=(n). * , ** are respectively p<0.05 and p<0.01 by a Student’s t-test when compared to rats fed a normal diet and dosed with the same dose of the toxicant; ♦ , ♦♦ are respectively p<0.05 and p<0.01 by a Dunnett’s t-test after ANOVA when compared to animals fed a normal diet and dosed with same dose of the toxicant; a , p<0.05 when compared by a Student’s or Cochran’s t-test with vehicle dosed group.; c,d ♦ p<0.05 and p<0.01 respectively when compared to vehicle dosed group by a Dunnett’s t-test after ANOVA.
6.3.3.4. Serum ALP

DAPM caused a dose-dependent increase in serum ALP activity (Figure 6.5). When control rats were dosed with DAPM (75 mg.kg^-1), they showed a statistically significant increase in their serum ALP activity when compared...
to 15% or 30% casein diet fed counterparts (Figure 6.5).

6.3.3.5. Serum bile acids

As observed for all other serum parameters, bile acid levels increased dose-dependently after DAPM (Figure 6.6a.). Despite a smaller serum bile acids level, rats on a 15% casein diet did not show any statistically significant difference when compared to control animals receiving a similar dose of DAPM (Figure 6.6a.).

Animals fed a normal diet and dosed with ANIT showed a statistically significant (p<0.05) higher level of serum bile acids when compared to animals fed a 15% casein diet (Figure 6.6a.). There is no statistically significant difference between control animals fed a normal diet and rats fed a diet containing 30% casein after being dosed with ANIT.

Figure 6.5. Serum alkaline phosphatase activity for animals fed a normal diet (3), a 15% casein diet (3-4) or a 30% casein diet (3-4) and exposed ANIT or DAPM for a 24 hour period. Values are means ± SEM; N=(n); ♦♦ p<0.01 when compared by a Dunnett's t-test after ANOVA with rats fed a normal diet and exposed to a similar dose of DAPM.
Figure 6.6. Serum bile acids (a) and serum taurine (b) levels for rats fed a normal diet, a 15% casein diet or a 30% casein diet, twenty-four hours after being dosed with ANIT or DAPM. Values are means ± SEM; N=(n); * p<0.05 by a Student's t-test when compared to rats fed a normal diet and dosed with the same dose of the toxicant; ♦ p<0.05 by a Dunnett's t-test after ANOVA when compared to animals fed a normal diet and dosed with same dose of the toxicant; α, β, respectively p<0.05 and p<0.01 when compared by a Student's or Cochran t-test with vehicle dosed group.

6.3.3.6. Serum taurine

Serum taurine levels were similar after either control animals or those fed a special diet were exposed for 24 hours to 100 mg.kg\(^{-1}\) ANIT. Animals fed 15% or 30% casein diet showed higher serum taurine levels than
control animals, after being dosed with DAPM or vehicle. This difference is statistically significant for rats fed a 30% casein when compared to those fed a normal one, after 50 and 100 mg.kg\(^{-1}\) of DAPM (Figure 6.6b.).

**6.3.3.7. Liver TNPSH**

Liver total non-protein sulphydryl levels were elevated in control animals after being dosed with ANIT and DAPM (or vehicle) when compared to rats fed a special diet (Figure 6.7a.). These levels were statistically significantly raised in control animals dosed with ANIT, DAPM (50 mg.kg\(^{-1}\)) or vehicle when compared to rats fed either a 15% or a 30% casein diet and which received the same dose of the toxicant.

**6.3.3.8. Liver taurine**

After being dosed with DAPM (75 or 100 mg.kg\(^{-1}\)), rats on a 30% diet showed a rise in their liver taurine levels when compared to animals fed a normal diet (Figure 6.7b.). Taurine levels in the liver were slightly decreased in rats fed 15% casein diet after being dosed with ANIT or DAPM (or vehicle) when compared to animals fed a normal diet. Rats fed a higher diet content of casein showed similar or increased liver taurine levels when compared to control animals fed a normal diet. Control animals and those fed a 15% casein diet showed a rise (p<0.05) in their taurine liver levels when compared to vehicle dosed ones (Figure 6.7b.).

**6.3.3.9. Liver weight**

Liver weight (as percentage of body weight) increased dose-dependently up to 75 mg.kg\(^{-1}\) DAPM in all groups (figure 6.7c.). No statistically significant differences were found when rats fed a special diet were compared to control animals fed a normal diet.
Figure 6.7. Liver TNPSH (a), liver taurine (b) and liver weight (percent of body weight) (c) for animals fed a normal diet, 15% casein diet or a 30% casein diet, 24 hours after being dosed with ANIT (100 mg.kg⁻¹) or DAPM (0, 50, 75 and 100 mg.kg⁻¹). Values are means ± SEM; N=(n); ♦, ♦♦ p<0.05 and p<0.01, respectively, by a Dunnett's t-test after ANOVA when compared to rats fed a normal diet and dosed with a similar dose of toxicant. a,b respectively, p<0.05 and p<0.01 when compared by a Student's or Cochran t-test with vehicle dosed group; c,d - respectively p<0.05 and p<0.01, when compared to vehicle dosed group by a Dunnett's t-test after ANOVA.
6.3.4. Urinary taurine

Daily monitoring of urinary taurine excretion was performed for all animals fed both 15% casein and 30% casein diet. With the exception observed of day 19 all differences were statistically significant for urinary taurine excretion between different diet treatments. Throughout the study, animals fed a higher content casein diet excreted significantly (0.001<p<0.05) more taurine than those rats fed a 15% casein diet (Figure 6.8.). At the beginning of the experiment rats fed a higher content casein diet showed a urinary taurine excretion 7 to 10 fold higher than animals on a 15% casein diet. This difference in the amount of taurine excreted between the two groups was smaller at the time of dosing, but remained in average 2-3 fold higher (Figure 6.8.).

Figure 6.8. Urinary taurine excretion for animals fed a 15% casein diet or a 30% casein diet until the time of dosing with ANIT or DAPM. Values are means ± SEM, * p<0.05 by a Cochran's t-test when compared to rats fed a 15% casein diet; α not statistically significant when compared to 15% casein diet animal group by a Cochran's t-test. For all other time points, a level of significance of 0.001 was obtained either by Student's or Cochran's t-test (as appropriate) when 30% casein fed rats were compared to animals given a 15% casein diet.
Since animals were provided different diets, it was necessary to investigate if ANIT or different doses of DAPM exerted an influence on the urinary taurine excretion pattern after dosing.

When the percentage change in urinary taurine was plotted for all doses of hepatotoxicant used, one observes that animals fed a 30% casein diet showed a decrease (observed as a negative percentage) in their urinary taurine excretion levels (Figure 6.9.). Conversely, rats fed a 15% casein diet showed an increase in their percentage change in urinary taurine excretion after DAPM (all doses). In contrast, rats fed a 15% casein diet and dosed with ANIT showed a decrease in their urinary taurine levels (Figure 6.9.).

![Figure 6.9. Effect of the diet on the percentage change in urinary taurine excretion.](image)

Individual rat urinary taurine excretion levels were averaged for a period of 96 hours before dosing and compared with the value obtained, 24 hours after rats have being dosed with ANIT or DAPM. Values are means ± SEM for 3-4 rats.** p<0.01 when compared to 30% casein diet by a two-tailed paired Student's t-test.
When urinary taurine pre-dosing and post-dosing levels were compared for the different diets tested, a statistically significant decrease using a paired t-test was observed for all doses tested in rats fed a 30% casein diet (Figure 6.10.). Rats fed a 15% casein diet showed a significant difference (p<0.05) when dosed with DAPM (50 mg.kg⁻¹) but no statistically significant changes neither for all other DAPM doses nor for ANIT (100 mg.kg⁻¹) were found when pre- and post-dosing urinary taurine levels were compared.

![Figure 6.10. Daily urinary taurine excretion for animals dosed with ANIT or DAPM. Urinary taurine levels were averaged for the 96 hours before dosing and quantified for the 24 hours after rats being dosed with either the toxicant or vehicle. Values are means ± SEM for 3-4 animals; *, ** are respectively p<0.05 and p<0.01 by a paired t-test when compared to the same diet treatment before dosing.](image)

**6.3.5. Histological features**

At the time of dosing, animals fed either a 15% casein diet or a 30% casein diet showed a normal hepatic parenchyma and portal tracts, featuring respectively normal glycogen content and intact bile duct structures (Plate
Twenty-four hours after animals were dosed with vehicle, no visible alterations in the bile ducts were observed in the liver histological preparations assessed, notwithstanding the diet tested. Nevertheless, visible fat deposits or fat droplets were observed at the periphery of the liver lobe in some animals (Plate 6.2.). This histological alteration was not associated with different diets used.

When both animal groups were challenged with the lowest dose of DAPM (50 mg.kg⁻¹), a diverse histopathological response was observed. Some animals showed complete bile duct destruction and associated periportal necrosis or necrotic foci scattered within the hepatic parenchyma, whereas others showed no signs of parenchyma involvement and only mild to moderate bile duct disruption with biliary epithelial cells still lining the duct. Another histopathological feature not common to all animals was a mild to moderate steatosis. Animals fed a 15% casein diet showed the smallest hepatocellular involvement but were more affected by fat accumulation, whereas rats fed a
30% diet showed an extended involvement of the hepatic parenchyma but did not feature a marked “fatty” change when compared to vehicle dosed controls.

Plate 6.2. Microphotograph (× 40) of a rat liver fed a 15% casein diet, 24 hours after being dosed with vehicle. Mild steatosis is observed at the edge of the liver lobe.

Plate 6.3. Liver photomicrograph (× 100) from a rat fed a 15% casein diet and dosed with DAPM (75 mg·kg⁻¹). Moderate steatosis is observed mostly surrounding the portal areas.
Animals dosed with 75 or 100 mg.kg\(^{-1}\) DAPM, showed bile duct disruption and biliary epithelial cell damage. The extent of damage of the bile duct structure was variable, ranging from moderate to complete destruction of the bile duct. Once again, animals fed a 15% casein diet and dosed with DAPM (75 mg.kg\(^{-1}\)) showed a marked inflammatory response, hepatocellular damage and moderate to severe steatosis (Plate 6.3 and 6.4.).

Plate 6.4. High magnification photomicrograph (x 400) of a rat liver dosed with DAPM (75 mg.kg\(^{-1}\)) showing a portal triad without a defined bile duct and with severe steatosis surrounding the portal vessels. Marked accumulation of inflammatory cells can be observed with no signs of parenchymal necrosis.

The extent of steatosis seemed to be associated with the presence or absence of necrotic foci, since rats that present necrotic foci scattered in the liver parenchyma did not have such marked steatosis (Plate 6.5.). Interestingly, serum biochemical markers measured were unaltered for these rats, when compared to vehicle dosed controls.
Plate 6.5. Photomicrograph (x 100) of a rat liver showing a necrotic foci extending from the portal triad towards the parenchyma. Mild steatosis is also observed.

Plate 6.6. Photomicrograph (x 100) from a large portal triad of an animals fed a 15% casein diet, 24 hours after being dosed with DAPM 75 mg.kg⁻¹. Mild inflammation is present with disruption of the large blood vessel.
Another interesting histological alteration observed in these animals was a necrotising vasculitis seen in some of the branches of the portal vein (Plates 6.6 and 6.7.)

Plate 6.7. High magnification microphotograph (x 400) from the same blood vessel shown above. A disruption in the endothelium is observed.

Animals fed a 30% casein diet and dosed with DAPM (75 mg.kg\(^{-1}\)) showed an extended parenchymal necrosis, with several small haemorrhagic foci of necrosis associated with small portal triads with higher frequency in the periphery of the lobe (Plate 6.8). Biliary epithelial cell (BEC) damage was variable, ranging from complete obliteration of some large bile ducts to BEC exfoliation and necrosis with accumulation of debris and fibrotic tissue,
present in others.

Animals, regardless of the diet treatment, challenged with the higher dose of DAPM (100 mg.kg\(^{-1}\)), showed a complete disruption of the large bile ducts associated with inflammation and fibrosis of the portal triads. Several haemorrhagic necrotic foci, infiltrated with macrophages and/or neutrophils were observed in the hepatic parenchyma or extending outwards from the portal tracts. Animals given the higher content casein diet showed hepatic parenchymal damage to a larger extent than those given a 15% casein diet (Plate 6.9.).

Animals dosed with ANIT (100 mg.kg\(^{-1}\)) showed biliary epithelial cell necrosis

Plate 6.8. Photomicrograph (x 100) of peripheric area in the liver showing four necrotic foci. These parenchymal alterations appeared to be associated with small portal triads regarding its distance to the central vein.
with periportal neutrophil infiltration, 24 hours after being dosed. Large bile ducts were not totally disrupted in animals fed the 15% casein diet, some BEC remained lining the bile duct structure. Moderate to marked steatosis was observed, mostly at the periphery of the lobe and surrounding the small portal triads (Plate 6.10.).

Plate 6.9. Photomicrograph (x 100) of a portal triad showing a completely disrupted bile duct without BEC. A small foci of necrosis with some inflammatory cells is observed in the parenchyma.

Conversely, animals fed a higher content casein diet showed no signs of steatosis, although biliary epithelial cell damage and massive neutrophilic infiltration was observed in the portal tracts. The extent of inflammation, cellular infiltration and bile duct disruption is more evident in this group.
than in rats fed a 15% casein diet and dosed with a similar dose of ANIT (Plate 6.11.).

Plate 6.10. Photomicrograph (x 100) from the liver of a rat fed a 15% casein diet, 24 hours after being dosed with ANIT (100 mg.kg\(^{-1}\)). No signs of parenchymal damage are observed and the bile duct structure still intact. Moderate steatosis is observed involving the surrounding parenchyma.
Plate 6.11. Photomicrograph (x 100) of a rat liver fed a 30% casein diet, 24 hours after being dosed with ANIT (100 mg.kg$^{-1}$). No major signs of steatosis is observed when compared to plate 6.10, but the bile duct is completely disrupted without BEC. Moderate to marked inflammatory infiltration is also observed.

6.4. Discussion

When animals were given a diet with different casein contents (15 or 30%), no statistically significant differences were found for body weight gain or food consumption, throughout the studies. Rats fed the higher content casein diet drank significantly more water than animals given the 15% casein diet. In
parallel, the volume of urine excreted was higher for animals given the 30% casein diet (data not shown) and these animals also showed significantly higher urinary taurine levels (Figure 6.8.). Since there is ample evidence that the kidney regulates the whole body homeostasis of taurine (Chesney, 1985) and taurine can act as an osmolyte (Huxtable, 1992), it is possible that increased excretion of water is accompanied by an increased amount of taurine being excreted by the kidney. The increment in the volume of urine being excreted may lead to a higher water intake and it may explain why rats fed a 30% casein diet drank more water than those given a 15% casein diet.

None of the diets tested in these studies caused a statistically significant alteration in the serum parameters measured (Table 6.1.), when compared to values obtained for control animals fed a normal diet (data shown in Appendix V), at the time that animals were dosed with vehicle. None of the diets was effective in increasing taurine liver levels when compared to levels obtained for animals fed normal diet. Although rats fed a higher content casein diet showed a slight increase in liver taurine when compared to 15% casein fed animals, this difference was not significant. Rats fed both diets showed a normal variation in their liver taurine levels at the time of dosing. Thus, 15% casein fed animals have values ranging from 0.896 to 10.75 μmol.g⁻¹ liver weight; whereas 30% casein diet fed animals showed a range for taurine liver levels between 2.317 to 8.44 μmol.g⁻¹ liver weight. Total non-protein sulphydryls were at a higher level at the time of dosing in animals fed either a 15% or a 30% casein diet (table 6.1.) than in control animals fed a normal diet (Appendix V). Hosokawa and collaborators had reported similar hepatic glutathione values (9.40 ± 0.32 and 9.82 ± 0.14) for rats fed respectively a 16% and a 24% casein diet (1988). Recently, another publication reported a value for liver glutathione content of approximately 8.5 μmol.g⁻¹ after rats were fed a 20% casein diet supplemented with 0.3% DL-methionine (Goto et al., 1993). The surplus of methionine present in these diets can be metabolically converted to cysteine via the transsulphuration
pathway and increase the amount of cysteine available in the hepatic parenchymal cell. Methionine was shown to be a better source of cysteine for glutathione production than cysteine provided as such in studies using rat hepatocytes in primary culture (Stein et al., 1987).

Recently, Stipanuk and co-workers in a series of publications, proposed that the availability of cysteine plays a major role in the regulation of cysteine metabolism (Stipanuk et al., 1992b; Stipanuk et al., 1992a; Stipanuk et al., 1984). Thus, when the availability of cysteine is lower, this amino-acid is efficiently used for glutathione synthesis, with little loss of cysteine by catabolism to taurine or sulphate. Conversely, as cysteine availability increases and glutathione accumulates in the hepatocyte, the activity of γ-glutamylcysteine synthetase would be inhibited (Richman and Meister, 1975) and more cysteine will be partitioned to the catabolic pathway, resulting in production of sulphate and taurine (Stipanuk et al., 1992b).

In these studies, both diets tested had a percentage of sulphur amino acids derived from the diet similar or even higher (15% casein = 0.705%; 30% = 1.41%) than those reported by Hosokawa et al (1988), 0.73-0.82% for a 18% casein diet, to produce a saturation of the hepatic glutathione pool. Thus, it is likely that the excess of sulphur amino acids in the diets accounts for the increased hepatic glutathione levels observed for both diets tested in these studies.

Furthermore, when rats fed these semi-synthetic diets were dosed with vehicle and deprived of food for a 24 hour period, their hepatic glutathione levels decreased to values significantly lower than those found for control animals fed a normal diet after being submitted to a similar treatment (Fig.6.7a.). This indicates that the surplus of sulphur amino acids supplied in the diet was essential to maintain the high glutathione levels in the liver.

Throughout the study, urinary taurine levels of animals fed the 30% casein diet were also markedly elevated when compared to rats given a 15% casein diet. This may be due to the fact that the amount of sulphur amino acid
content in a diet is directly associated with urinary excretion of taurine and sulphate (Yoshida et al., 1989). Although sulphate excretion was not measured in these studies it is likely to be elevated since previous reports showed that rats fed a 25% casein diet (Yoshida et al., 1989) or isolated rat hepatocytes metabolized cysteine to taurine and to a higher extent to sulphate (Stipanuk et al., 1992a).

Cysteine dioxygenase catalyses the oxygenation of the thiol group present in cysteine to yield cysteine sulphinate. The latter undergoes a further decarboxylation to yield hypotaurine and finally taurine is formed by the catabolic action of hypotaurine dehydrogenase. It was shown in isolated rat hepatocytes that both cysteine availability and cysteine dioxygenase activities play key roles in determining the rate of conversion of cysteine to taurine (Stipanuk et al., 1992a). In intact animals, a similar role for cysteine dioxygenase activity was proposed by Hosowaka et al (1988). These authors found that hepatic cysteine dioxygenase activity was 23- times higher in rats fed 30% casein as in animals given 10% casein diet. A proportional increase in urinary taurine was also reported. Stipanuk and co-workers proposed that taurine formation and excretion may be an important route of disposal when excessive sulphur is provided in the diet and in opposition, when a limited amount of cysteine is supplied, the diet may be the major source of taurine under such conditions (Stipanuk et al., 1992a).

In previous chapters the increase of liver taurine levels was achieved by giving taurine to animals in their drinking water. The studies described here were designed to investigate the possible protective effect of de novo synthesised taurine on the hepatotoxicity of ANIT and DAPM. Since the tracing residues of taurine present in the semi-purified diets used in these studies were insignificant and given the evidence that the half-life of injected S\textsuperscript{35}-taurine was shown to be shorter than the length of the experiments described here (Awapara, 1957; Sturman, 1973), one can expect that rats synthesized the taurine present in the liver at the end of these
Despite the ineffectiveness of the tested diets in raising liver taurine levels, animals fed the lower content casein diet (15%) and dosed with ANIT (100 mg.kg\(^{-1}\)) showed statistically significant lower levels for all serum markers of hepatotoxicity when compared to control animals dosed with a similar dose of the toxicant. The smaller rise in the serum markers of hepatotoxicity observed for this group of animals was well corroborated by the histological assessment. Thus, animals fed a 15% casein diet were less susceptible to ANIT-induced hepatotoxicity than control animals fed a normal diet. Rats fed a diet containing 30% casein were as susceptible to the hepatotoxic effects induced by ANIT as the animals fed a control diet. Histological assessment provides evidence that rats fed a 30% casein diet were similarly affected as animals fed a normal diet and dosed with ANIT. Despite the fact that serum transaminase levels were significantly decreased in rats fed the 30% casein diet when compared to animals fed a normal diet these lack of support from the histological data where parenchymal cell damage, inflammation and cellular infiltration was not different. These contradictory observations were exacerbated when data from the DAPM-dosed animals were compared. Thus, when animals were dosed with DAPM (50 mg.kg\(^{-1}\)) the extent of parenchymal damage was not followed by a similar rise in serum transaminase levels. Despite there being no significant difference between the hepatocellular damage, assessed histologically, between rats fed a 15% or a 30% casein diet when compared to animals fed a normal diet, if one had evaluated simply the serum parameters, hepatoprotection from the 15 or 30% casein might have been claimed. When higher doses of DAPM were used, the trend in the levels of serum transaminases did not change for rats fed a 15% casein diet despite the apparent parenchymal damage. The lack of correlation between the rise in
serum transaminase levels and the presence of necrotic foci seems to be associated in these experiments with the presence of moderate or severe steatosis.

Recently it has been observed that cycloheximide, a compound that causes steatosis also affected the serum levels of ALT and caused hypertaurinuria (Waterfield et al., 1993b). These effects were proposed to be associated with an inhibition of protein synthesis. Indeed, cycloheximide has been shown to inhibit significantly the incorporation of $^{14}$C-leucine into liver proteins and to protect against ANIT-induced hyperbilirubinemic response when administered before dosing with the toxicant (Indacochea-Redmond et al., 1973).

Interestingly, animals fed a 15% casein diet showed raised urinary taurine levels after dosing with all doses of DAPM along with histological features of steatosis. In contrast, animals fed a 30% casein diet showed milder signs of steatosis and despite histological signs of parenchymal necrosis along with a decrease in their urinary taurine levels.

Taken together these observations lead to the hypothesis that the possible inhibition of protein synthesis after dosing 15% casein fed rats with DAPM might reduce AST and ALT levels in the hepatocyte, contributing to a smaller rise in the serum levels upon hepatic damage. Indeed, it was observed that rats fed a 30% casein diet and dosed with 100 mg.kg$^{-1}$ DAPM showed milder signs of steatosis and the hepatic damage was associated with a significant rise in the serum transaminases (Figure 6.4.b).

Moreover the possibility of a direct interference of DAPM in the assay of the serum transaminase was considered, but from the list of compounds supplied by the manufacturer known to interfere with assay, DAPM or DAPM-like compounds were not included.

These studies provide further evidences that one should be cautious in defining hepatoprotection based solely on the assessment of data from serum parameters. Without the histological assessment these studies might have
lead to the conclusion that both diets to a certain extent afford protection against ANIT- and DAPM-induced hepatotoxicity.

Unfortunately, all previous nutritional studies conducted in the rat to determine the extent of cysteine catabolism to taurine or sulphate using diets with different protein contents and several sulphur supplements were performed either in freshly isolated hepatocytes or when performed in vivo no histological assessment was carried out.

In the cat, the presence of a high taurine content in the diet affected protein concentration and lipid metabolism in the liver (Cantafora et al., 1991). It was suggested that the high content of taurine in the diet increases the conversion of free fatty acids (FFA) into triglycerides, but did not affect nor the secretion of very low density lipoproteins (VLDL) nor the rate of lipolysis (Cantafora et al., 1991). The same study concluded that the liver of taurine-supplemented cats, expanded the lipid stores either by a mechanism involving reduction of lipolysis and/or increasing the synthetic activity equilibrating the pool of fatty acids among triglycerides, cholesteryl esters and FFA.

It would be interesting from my point of view to examine whether low or high levels of protein or sulphur amino acid supplementation cause similar effects in the rat liver, and if so how they might affect protein synthesis and possibly enzyme levels in the hepatocyte and in the serum.

It would be interesting to investigate if an effective modulation of the liver taurine levels by feeding animals with diets with different protein levels and different sulphur amino acid supplementation might exert any change in the susceptibility of animals to different hepatotoxicants.
CHAPTER 7
IN VITRO STUDIES

7.1. Introduction

These studies were planned to assess the possible protective role of taurine against the cytotoxic effects caused by ANIT and DAPM. Moreover, an attempt to draw a parallel between the toxic effects observed when animals were exposed to the hepatotoxicants and the effects caused by the toxicants in vitro was evaluated.

The protective effects of taurine in vivo have led investigators to design in vitro experiments in an attempt to outline the cytoprotective mechanisms of taurine. Pasantes-Morales and co-workers (1985) demonstrated that the presence of extracellular taurine (5 mM) protects lymphoblastoid cells in vitro against iron-ascorbate induced calcium accumulation and cytotoxicity. Taurine (20 mM) co-administered with zinc chloride protected lymphoblastoid cells against retinol and retinoic acid cytotoxicity (Pasantes-Morales et al., 1984).

Using isolated hepatocytes, Nakashima and collaborators (1990) have demonstrated that taurine exerts a protective effect by decreasing lipid peroxidation due to oxygenation and preventing the hypoxia-induced hepatocyte death in a calcium-containing medium, but failing to do so in a calcium-free medium. Moreover, taurine protective effects were extended in these experiments to calcium- and oxygen-paradox allegedly by preventing calcium influx into hepatocytes (Nakashima et al., 1990).

More recently, Waterfield and co-authors (1993a) provide evidences that taurine (15 mM) partially protects rat isolated hepatocytes against the cytotoxic effects caused by hydrazine, 1,4-naphthoquinone and carbon tetrachloride when assessed by means of trypan blue uptake and LDH leakage. ATP depletion caused by 1,4-naphthoquinone was also shown to be ameliorated in the presence of taurine, but not for hydrazine.
The studies described in this chapter were planned and carried out aiming to investigate whether the addition of taurine to the incubation media of isolated hepatocytes could reduce the cytotoxicity of the two hepatotoxicants ANIT and DAPM. Furthermore, for ANIT other strategies were taken to investigate the possible cytoprotective role of taurine. Thus, hepatocytes were isolated from rats depleted of taurine (β-alanine treated) and their susceptibility to ANIT tested and compared to cells isolated from non-treated animals.

Although concentrations of taurine added to the incubation buffer were much higher than those observed in normal plasma, similar concentrations were showed to be effective in isolated cells to give protection against other toxicants (Pasantes-Morales et al., 1984; Nakashima et al., 1990; Waterfield et al., 1993a).

Taurine or its percursor, cysteinesulphinic acid were also added to the incubation buffer of taurine depleted cells and the susceptibility tested for different doses of ANIT.

7.2. Methods

Hepatocytes were isolated from male Sprague-Dawley rats (210-285 g) using the two step perfusion method of Moldeus et al (1978) as described in Chapter 2. After initial viability was assessed, if over 85%, aliquots of a cell suspension (15 ml, 2 x 10^6 cells.ml^-1) in Krebs-Henseleit Buffer were placed in round-bottomed 100ml flasks and rotated slowly under a continuous carbogen stream (37°C; 95%O_2 : 5% CO_2).

In all experiments, isolated hepatocytes were incubated for an initial period of one hour either with buffer (control) or taurine dissolved in Krebs-Henseleit buffer to give a final concentration of 15mM. After this incubation period, hepatocytes were exposed to different concentrations of ANIT solubilized in DMSO (0.49%).

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Animals were treated with β-alanine (3% in the drinking water) for 6 to 8 days in order to decrease the hepatic taurine content. After this period, hepatocytes were isolated as described above. After isolation, hepatocytes were incubated under the conditions described above with buffer, taurine (15 mM) or cysteinesulphinate (15 mM) and exposed to different concentrations of ANIT or vehicle (DMSO).

In separate experiments taurine depleted hepatocytes were incubated with several concentrations of taurine or cysteinesulphinate (0.5, 5, 10, 15 mM) and its effects on cell viability, taurine synthesis (only cysteinesulphinate incubated cells), GSH and ATP levels monitored for a period of three hours.

For all experiments aliquots of the cell suspension were taken from each flask, hourly, after the incubation period, to determine several parameters: Trypan Blue uptake, LDH leakage (only for DAPM), GSH, GSSG (only for DAPM experiments), ATP and taurine as described in Chapter 2 and Appendix III.

7.3. Results

7.3.1. Section I. Studying the role of taurine and its percursor, cysteinesulphinate on the cytotoxic effects caused by ANIT.

7.3.1.1. Cells isolated from non-treated animals

7.3.1.1.1. Cell viability

A time and dose dependent decrease in cell viability was observed, as assessed by Trypan Blue exclusion, for all ANIT doses tested (Table 7.1.).
<table>
<thead>
<tr>
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<tr>
<td><strong>DMSO</strong></td>
<td>75.7±4.84</td>
<td>71.4±5.85</td>
<td>71.6±5.1</td>
<td>67.8±5.09</td>
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<tr>
<td><strong>0.5mM ANIT</strong></td>
<td>73.6±3.29</td>
<td>69.8±5.37</td>
<td>67.9±6.0</td>
<td>65.0±6.03</td>
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<td><strong>0.75mM ANIT</strong></td>
<td>72.4±4.3</td>
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<td>57.7±6.7</td>
<td>51.3±6.70</td>
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<td><strong>1.0mM ANIT</strong></td>
<td>75.9±5.7</td>
<td>63.5±7.2</td>
<td>52.5±9.5</td>
<td>42.3±12.1</td>
</tr>
<tr>
<td><strong>1.25mM ANIT</strong></td>
<td>75.0±3.6</td>
<td>62.8±6.1</td>
<td>46.3±18</td>
<td>31.8±20.3</td>
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<tr>
<td><strong>1.5mM ANIT</strong></td>
<td>75.9±2.21</td>
<td>38.2±24</td>
<td>20.5±26</td>
<td>9.8±16.8</td>
</tr>
</tbody>
</table>

Table 7.1. Cell viability assessed by Trypan Blue exclusion. Values are means±SD for seven different experiments.

When taurine was added to the incubation buffer, the cytotoxic effect caused by ANIT was not altered (Table 7.2.) when compared to cells incubated with buffer alone (Table 7.1.).

<table>
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<th>3</th>
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<tr>
<td><strong>DMSO</strong></td>
<td>79.0±7.34</td>
<td>73.3±5.2</td>
<td>69.8±1.86</td>
<td>72.4±2.91</td>
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<td><strong>0.5mM ANIT</strong></td>
<td>79.5±3.31</td>
<td>71.9±2.5</td>
<td>73.1±5.31</td>
<td>72.0±5.13</td>
</tr>
<tr>
<td><strong>1.0mM ANIT</strong></td>
<td>76.8±3.65</td>
<td>64.5±5.3</td>
<td>61.3±9.51</td>
<td>48.2±24.8</td>
</tr>
<tr>
<td><strong>1.25mM ANIT</strong></td>
<td>79.2±2.26</td>
<td>59.8±8.0</td>
<td>48.3±20.7</td>
<td>30.1±26.1</td>
</tr>
<tr>
<td><strong>1.5mM ANIT</strong></td>
<td>75.8±1.73</td>
<td>43.8±23.0</td>
<td>33.6±29.7</td>
<td>9.43±16.3</td>
</tr>
</tbody>
</table>

Table 7.2. Cell viability for isolated hepatocytes incubated with 15 mM taurine and exposed to several doses of ANIT. Values are means±SD for three different experiments.
For the last time point assessed, cell viability (as a percentage) was plotted against the range of ANIT doses used (Figure 7.1.). No significant differences were found between isolated hepatocytes when incubated with or without taurine for the same dose.

Figure 7.1. Comparison of cell viability (trypan blue exclusion) for different doses of ANIT at last time point assessed. Values are means±SEM for 3-7 different experiments. There is no statistically significance between different treatments.

7.3.1.1.2. Glutathione

When the GSH level was measured in isolated hepatocytes promptly after the isolation procedure was finished it was 23.84±2.88 nmol GSH/10^6 cells (n=3). This level dropped by 33.7% after one hour incubation with buffer. A gradual decrease in the intracellular GSH level was also observed over the time course of the experiment for the control group. ANIT caused a significant depletion of GSH at concentrations greater than 1.0 mM after 2 hours (Figure 7.2.). Cells exposed to the highest dose of ANIT showed a significant depletion of GSH after 1 hour when compared with the control (DMSO dosed cells).
The only dose of ANIT which did not cause any statistically significant difference on GSH levels when compared to DMSO alone throughout the experiments is 0.5mM ANIT.

![Bar graph showing the effect of different concentrations of ANIT on GSH levels.](image)

**Figure 7.2.** Effect of different concentrations of ANIT on GSH levels. Values are means±SEM for three different experiments. *, ** are respectively p<0.05 and p<0.01 when compared by a Dunnett's t-test with DMSO exposed hepatocytes.

When the reduced glutathione levels were measured (9.6±1.8 nmol/10^6 cells) one hour after incubation with 15mM taurine a 58.6% decrease was reported compared with the starting value (23.8±2.8 nmol/10^6 cells).

This difference remained until the last time point, when hepatocytes incubated with taurine and exposed to DMSO and 0.5 mM ANIT were compared to hepatocytes incubated with buffer alone and exposed to similar doses of the toxicant.

Despite the GSH levels of isolated cells incubated with taurine were significantly lower than those measured in hepatocytes incubated with buffer alone, this seems to be caused by an interference in the method (see discussion) since the extent of GSH depletion caused by ANIT is similar.
7.3.1.1.3. ATP

When isolated hepatocytes were exposed to ANIT, a dose and time depletion of ATP levels in isolated hepatocytes incubated with 15 mM taurine was observed (Figure 7.3.). As was observed for GSH levels, one hour after being exposed to the toxicant (top dose) isolated cells showed a statistically significant depletion of ATP. Two hours after exposure, cells exposed to doses higher than 1.0 mM ANIT showed a significant depletion in their ATP levels when compared to DMSO exposed cells.

No comparison can be established for isolated cells incubated with buffer alone since ATP levels were not quantified in these experiments.

Figure 7.3. ATP content in isolated hepatocytes incubated with 15 mM taurine after being exposed to different doses of ANIT. Values are means±SEM for three different experiments.* and ** are respectively p<0.05 and p<0.01 when compared to DMSO dosed group by a Dunnett's t-test after ANOVA.

7.3.1.1.4. Taurine

Total taurine levels were determined, promptly after isolation, to be 46.2±27.2 nmol/10⁶ cells (n=3). After one hour incubation with buffer a drop in the total taurine levels was observed. Nevertheless, the level had not decreased over
3 hours more than 6.51±8.3 nmol/10^6 cells at the highest dose of ANIT. There was an increase in total taurine levels of 0.8±6.4 nmol/10^6 cells for DMSO exposed cells.

7.3.1.2. Hepatocytes isolated from β-alanine treated animals.

Hepatocytes were isolated from rats that had been treated with β-alanine (3% in drinking water) for 6 to 8 days. The aim of this treatment was to deplete hepatocytes of taurine so that the biochemical indicators of cytotoxicity caused by ANIT could be compared with those obtained from hepatocytes with normal taurine levels.

After β-alanine treatment, isolated hepatocytes showed an initial total taurine level which had decreased to 9.4±5.3 nmol/10^6 cells, being significantly different from normal cells (46.2±27.2 nmol/10^6 cells; p<0.05, Student's t-test).

7.3.1.2.1. Cell viability

As can be seen in table 7.3., cell viability for hepatocytes isolated from β-alanine treated rats when incubated with buffer and exposed to several doses of ANIT did not change significantly when compared with normal cells (hepatocytes isolated from non-treated animals) for the same time point (Table 7.1.).

To assess the effect of taurine and cysteinesulphinate on ANIT-induced cytotoxicity in hepatocytes isolated from β-alanine treated animals, both compounds were added to the incubation buffer in a concentration of 15mM. Thus, after one hour incubation with 15 mM taurine or cysteinesulphinate, isolated cells were exposed to several concentrations of ANIT or vehicle (DMSO) and the cell viability assessed at each time point by Trypan Blue exclusion (Table 7.4.).
<table>
<thead>
<tr>
<th>Dose ANIT \ TIME (h)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>75.8±4.47</td>
<td>73.4±3.85</td>
<td>70.9±5.18</td>
<td>69.3±5.23</td>
</tr>
<tr>
<td>0.25mM ANIT</td>
<td>76.9±3.58</td>
<td>69.9±8.17</td>
<td>70.6±6.3</td>
<td>66.5±5.9</td>
</tr>
<tr>
<td>0.5mM ANIT</td>
<td>76.0±3.94</td>
<td>69.2±6.11</td>
<td>67.5±6.75</td>
<td>64.8±5.87</td>
</tr>
<tr>
<td>0.75mM ANIT</td>
<td>75.7±5.68</td>
<td>66.8±6.65</td>
<td>58.9±11.6</td>
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<tr>
<td>1.0mM ANIT</td>
<td>77.3±5.67</td>
<td>64.1±9.08</td>
<td>42.1±26.4</td>
<td>28.5±29.2</td>
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<td>1.25mM ANIT</td>
<td>77.1±1.52</td>
<td>54.9±18.5</td>
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<tr>
<td>1.5mM ANIT</td>
<td>76.2±2.97</td>
<td>43.2±18.2</td>
<td>14.7±13.9</td>
<td>8.5±13.18</td>
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</table>

**Table 7.3.** Cell viability assessed by Trypan Blue exclusion after incubation with buffer and different doses of ANIT or vehicle at different time points. Values are mean±S.D. for 5-12 different experiments.
Table 7.4. Results shown are percentage cell viability for different treatments. Values are mean±S.D. for 4-12 different experiments in 15mM taurine added and 3 experiments for 15mM cysteinesulphinate incubated cells.
No statistically significant difference was observed when hepatocytes isolated from β-alanine treated animals and incubated with buffer were compared with taurine or cysteinesulphinate incubated hepatocytes for similar doses of ANIT throughout the experiment.

However, cells incubated with cysteinesulphinate did appear to be less susceptible to the cytotoxic effects of ANIT (0.75 - 1.5 mM), when the last time point was assessed, in comparison to buffer incubated hepatocytes (Figure 7.4.).

![Figure 7.4. Effect of different concentrations of ANIT on cell viability of taurine depleted isolated hepatocytes after three hours exposure. Cells incubated with buffer alone or with 15 mM taurine or 15 mM cysteinesulphinate in the buffer. Values are means±SEM for 4-12 different experiments.](image_url)

7.3.1.2.2. Glutathione

A 17% drop in the initial concentration (18.72±4.92 nmol/10^6 cells) of reduced glutathione was observed when hepatocytes isolated from β-alanine treated animals were incubated for one hour with buffer alone.
ANIT causes a dose and time dependent decrease in the concentration of GSH (Figure 7.5a.) when rat hepatocytes isolated from β-alanine treated animals and incubated with buffer alone were exposed to the toxicant. One hour after exposure to the top dose of the toxicant in use, isolated cells showed a significant depletion of GSH levels. This decline was statistically significant for doses higher than 1.0 mM ANIT, two hours after exposure.

When hepatocytes isolated from β-alanine treated animals were incubated with taurine (15 mM) for one hour, before any ANIT was added, a significant drop in their GSH levels was observed. Once more this effect seemed to be related with an interference in the method rather than a direct effect of taurine.

After one hour of exposure to doses higher than 1.0 mM ANIT, isolated cells showed a statistically significant depletion of GSH when compared to DMSO exposed hepatocytes.

No statistically significant difference could be found when GSH levels from hepatocytes isolated from β-alanine treated animals were compared with those obtained from cells isolated from non-treated rats at similar concentrations of ANIT (Figure 7.2 and Figure 7.5a.).

Hepatocytes isolated from β-alanine treated rats and incubated with 15 mM cysteinesulphinate showed a depletion of GSH similar to that observed for cells incubated with buffer alone after being exposed to ANIT (Figure 7.5c.).

The direct effect of several concentrations of taurine and cysteinesulphinate on the GSH levels of isolated hepatocytes was also investigated. No statistically significant differences were found until the last time point evaluated. Three hours after incubation with 10 mM and 15 mM taurine and 15 mM cysteinesulphinate, cells isolated from β-alanine treated animals showed a statistically significant decrease of GSH when compared to cells incubated with buffer alone (Figure 7.6.).
Figure 7.5. Effect on GSH levels caused by ANIT in hepatocytes isolated from β-alanine treated animals. Values are means±SEM for hepatocytes incubated with (a) buffer alone (n=6); (b) 15 mM taurine (n=4) and (C) 15 mM cysteinesulphinate (n=3). * and ** are respectively p<0.05 and p<0.01 by a Dunnett's t-test after ANOVA when compared to DMSO exposed group.
Figure 7.6. Effect of taurine (a) and cysteinesulphinate (b) on GSH levels of hepatocytes isolated from β-alanine treated animals. Different concentrations of taurine and cysteinesulphinate were added to the incubation buffer and compared with cells incubated with buffer alone. Values are means ± SEM for three different experiments. * and ** are respectively p<0.05 and p<0.01 when compared with buffer incubated cells at the same time point by a Dunnett's t-test after ANOVA.

7.3.1.2.3. ATP

For cells isolated from β-alanine treated animals, the ATP level after the isolation procedure was completed was 6.42±0.54 nmol/10^6 cells (n=3). This is almost half of the initial value observed for cells isolated from non-treated animals (11.77±3.17 nmol/10^6 cells). However, this difference was not statistically significant after 1 hour of incubation with buffer (0 time point).
As previously observed for normal cells, ANIT causes a statistically significant (p<0.01) depletion of ATP in cells isolated from β-alanine treated animals except for those cells exposed to the lowest concentration of the toxicant in use. For these cells as for those exposed to DMSO alone, there was an increase in ATP levels over the time course of the experiment (Figure 7.7a. and 7.7b).

**Figure 7.7.** Levels of ATP measured in cells isolated from β-alanine treated animals after incubation with buffer (a) and 15 mM taurine (b) and exposed to DMSO (vehicle) or ANIT. Values are means±SEM for three different experiments; **p<0.01 by a Dunnett’s t-test after ANOVA when compared to DMSO dosed cells.
Cells isolated from β-alanine treated animals were depleted of ATP more rapidly than cells isolated from non-treated animals when exposed to ANIT (1.25 and 1.5 mM) for a period of one hour. This rapid decline in ATP content observed in both groups, when cells were exposed to ANIT, was not correlated to a similar extent of loss of cell viability. When taurine was added to the incubation buffer, no statistically significant change was observed in ATP levels for all concentrations of ANIT tested when compared to cells with buffer alone.

7.3.1.2.4. Taurine

Hepatocytes isolated from β-alanine treated animals showed a taurine level after isolation of 5.81±3.4 nmol/10^6 cells. These cells incubated with cysteinesulphinate showed a statistically significant increase (p<0.01) in taurine when compared to cells incubated with buffer alone, for all ANIT concentration at the last time point (4 hours after incubation, 3 hours after exposure to ANIT). The change in total taurine was always higher than 20 nmol/10^6 cells for all ANIT concentrations when cysteinesulphinate was added to the incubation buffer (Figure 7.8.).

In order to determine what would be the effect of different cysteinesulphinate concentrations in the synthesis rate of taurine, a range of concentrations of the precursor was added to taurine depleted hepatocytes. A significant increment in taurine was observed for all doses of cysteinesulphinate added to the incubation buffer when compared with cells incubated with buffer alone. As is shown in Figure 7.9c., hepatocytes isolated from β-alanine treated animals were able to synthesise taurine at a similar rate regardless of the
concentration of cysteinesulphinate present in the incubation buffer. The amount of taurine synthesised by the hepatocytes does not seem to be dose related with the cysteinesulphinate concentration, but it is time related (Figure 7.9b.).

![Graph showing change in total taurine content over the experiment](image)

**Figure 7.8.** Change in total taurine content over the experiment when cells isolated from β-alanine treated rats were exposed to different doses of ANIT and incubated with buffer alone or 15 mM cysteinesulphinate. Hepatocytes incubated with cysteinesulphinate showed a statistically significant increase in their taurine levels when compared to cells incubated with buffer alone.
Figure 7.9. Levels of taurine: in the supernatant (a), intracellular (b) and total taurine (combined value - c) when taurine depleted hepatocytes were incubated with different concentrations of cysteinesulphinate. Values are means±SEM for three different experiments.
7.3.2. Section II. Studying the role of taurine on DAPM-induced cytotoxicity in isolated hepatocytes.

7.3.2.1. Cell viability

In this set of experiments the viability of isolated hepatocytes was assessed by means of Trypan blue exclusion and LDH leakage. A dose and time dependent loss of viability was observed when hepatocytes were exposed to DAPM, compared to hepatocytes exposed to DMSO (control vehicle) (Figure 7.10a and 7.11a).

When taurine (15 mM) was added to the incubation media no protection was observed against the top dose of DAPM (5 mM), apart from the 1 hour time point. However, taurine afforded slight protection against 4 mM DAPM, at all time points considered, when compared to cells incubated with buffer alone (Figure 7.10a). The difference was statistically significant when Trypan blue was used to assess cell viability, but statistical significance was acheived for LDH leakage only at the last time point.

This effect has a parallel on LDH leakage since in hepatocytes incubated with taurine show a smaller rise in their LDH values than cells incubated with buffer alone.
Figure 7.10. Percentage of cell viability as assessed by Trypan blue exclusion. Hepatocytes were incubated for one hour with buffer (a) or 15 mM taurine (b). After this period several concentrations of DAPM or DMSO (vehicle) were tested. Values are means ± SEM for three different experiments. * and ** are respectively p<0.05 and p<0.01 by Student's t-test when compared to similar time point for cells incubated in buffer without taurine.
Figure 7.11. Protection of isolated rat hepatocytes in suspension. Effect of taurine on cell viability expressed as percentage of total LDH leakage. Buffer (a) or 15 mM taurine (b) was added to the cells and after one hour they were exposed to DMSO (vehicle) or DAPM. Values are means ± SEM for three different experiments. * p<0.05 by a Students t-test, when compared to the cells incubated with buffer without taurine.
7.3.2.2. Reduced glutathione (GSH)

After isolation, hepatocytes showed a GSH level of 38.4 ± 9.7 nmol/10^6 cells. DAPM caused a rapid drop in GSH levels in isolated hepatocytes for all concentration tested. This effect is visible, at least, after the first hour of exposure. Taurine has no effect in preventing this drop despite its slight protective properties. Moreover, when hepatocytes were incubated with taurine for one hour - 0 time -(prior to DAPM dosing) a marked drop in GSH levels was already present (Figure 7.12.) when compared to cells incubated with buffer alone. This reduction in GSH levels for hepatocytes incubated with taurine and exposed to DMSO is statistically significant after one and two hour of exposure, when compared to the GSH levels of cells incubated with buffer (Figure 7.12.).

Taurine in the medium interferes with the assay used to quantify GSH, thus this drop can not be assumed as a direct effect of taurine. Furthermore and depending on the extent of the interference, taurine might uphold GSH levels in cells exposed to DAPM corroborating a possible protective effect.

7.3.2.3. Oxidised glutathione (GSSG)

When hepatocytes were isolated, the level of GSSG was 1.95 ± 0.9 nmol/10^6 cells. One hour after hepatocytes were incubated either with buffer or taurine, GSSG levels were raised to values arround 7 nmol per million cells. After isolated cells were exposed to DAPM, a statistically significant rise in GSSG was observed for 1 and 2 mM DAPM when compared to DMSO dosed cells at all time points considered (Figure 7.13.). On the other hand, DAPM concentrations that cause rapid cytotoxicity (4 and 5 mM) did not give rise to higher GSSG levels. There is no difference in the extent of GSSG formation between taurine treated cells or buffer added hepatocytes for any of the time points considered.
Figure 7.12. Cellular glutathione content for isolated rat hepatocytes incubated after incubated with buffer (a) or 15 mM taurine (b). Hepatocytes were exposed to DAPM or DMSO for a 3 hour period. Values are means ± SEM for three different experiments. * p<0.05 by a Student's t-test when compared to buffer added cells for the same dose and time point.
Figure 7.13. Oxidized glutathione concentration for isolated rat hepatocytes after being added of buffer (a) or 15 mM taurine (b) and exposed to DAPM or DMSO. Values are means ± SEM for three different experiments.
7.3.2.4. ATP

After hepatocytes were isolated, ATP levels were 37.44 ± 15.5 nmol/10⁶ cells (mean±SD). When isolated cells were incubated with buffer for one hour prior to exposure to DAPM, no significant alteration of the ATP levels was observed (Figure 7.14a). On the contrary, a statistically significant drop in the ATP levels after one hour was observed when cells incubated with taurine (15 mM) were compared with those incubated with buffer alone (Figure 7.14b). This initial effect was no longer present two hours after taurine was added to isolated cells (1 hour time point). Moreover, taurine incubated hepatocytes where cells viability was preserved above a certain level showed a ATP content similar if not higher (4 mM DAPM - 1 hour) than buffer incubated cells.

DAPM causes a dose and time dependent depletion of ATP regardless of the presence of taurine. Nevertheless, cells incubated with taurine showed a slower depletion of ATP than hepatocytes incubated with buffer alone. This difference assumed statistical significance at the last time point assessed for a dose of 4 mM DAPM (Figure 7.14b.).
Figure 7.14. Effect of DAPM on ATP depletion in isolated hepatocytes. Isolated cells were incubated with buffer alone (A) and with 15 mM taurine (B). A dose related depletion of ATP in isolated hepatocytes dosed with DAPM was observed. Values are means ± SEM for three different experiments. * and ** are respectively p<0.05 and p<0.01 by a Student’s t-test when compared to cells incubated with buffer (A).
7.4. Discussion

Contrasting with data published by Waterfield and co-authors (1993a), showing that taurine provides some protection against the cytotoxic effects of carbon tetrachloride, hydrazine and 1,4-naphthoquinone in isolated hepatocytes, these experiments provided little evidence that taurine when added directly to the incubation buffer was able to protect isolated rat hepatocytes against the cytotoxic effects induced by ANIT or by DAPM. ANIT is known to deplete GSH in a dose dependent manner with the formation of a ANIT-GS intermediate. It has also been reported that the loss of cell viability was not related to an increase in lipid peroxidation (Carpenter-Deyo et al., 1991). Despite the fact that in these studies the dose of ANIT necessary to produce cytotoxicity and to deplete GSH in isolated hepatocytes was higher (approximate 2-fold) than that mentioned by the author cited above, a dose relation can also be established between loss of cell viability and depletion of intracellular GSH.

When taurine (15 mM) was added to hepatocytes, it did not afford protection against loss of viability or ATP depletion caused by ANIT. This depletion of ATP seemed to be closely associated with loss of cell viability.

Pasantes-Morales et al (1984) using taurine concentrations ranging from 5 to 20 mM, were able to protect human lymphoblastoid cells against the cytotoxic effects of retinol and iron-ascorbate, claiming the cell membrane stabilizing properties and reduction of accumulation of calcium, promoted by taurine as the possible mechanism of protection. When combined with zinc and α-tocopherol, taurine afforded complete protection against retinol induced cell swelling and loss of cell viability, but these effects appeared to be unrelated to lipid peroxidation.

To investigate the effect of taurine depletion on ANIT-induced cytotoxicity in rat hepatocytes, cells were isolated from β-alanine treated animals. Despite the statistically significant depletion of taurine promptly after isolation, no significant difference in cell viability, GSH and ATP depletion was found
when these cells were challenged with ANIT compared to cells isolated from normal rats.

These results taken together with those obtained from normal cells when exposed to ANIT suggested that the initial levels of taurine present in hepatocytes might not interfere with the mechanism of ANIT-induced cytotoxicity.

When the cellular levels of taurine in depleted cells were replenished with this amino acid by adding it to the incubation medium, no significant change in the cytotoxic effects induced by ANIT nor in the depletion of GSH or ATP were achieved.

![Graph](image)

**Figure 7.15.** Effect of taurine and its precursor cysteinesulphinate on GSH depletion and cell viability, 3 hours after hepatocytes isolated from β-alanine treated rats were exposed to different concentrations of ANIT. Data are means ± SEM for 4-12 separate experiments. *p<0.01 by a Dunnett's t-test after ANOVA when compared to respective DMSO dosed controls.
In order to assess if cysteinesulphinate (15 mM) might afford protection against ANIT-induced cytotoxicity in taurine depleted cells, it was added to the incubation medium. As can be observed in figure 7.15., cysteinesulphinate provides slight protection from the cytotoxic effects caused by ANIT when compared with cells incubated with buffer alone. This effect seemed to be mediated or at least follow a smaller depletion of glutathione. When cysteinesulphinate is added to isolated hepatocytes, it is rapidly metabolized to taurine, as recently reported by Stipanuk et al (1992a). Data from figure 7.8. shows a significant increase in the taurine concentration for cells incubated with cysteinesulphinate when compared to hepatocytes incubated with buffer alone. This observation leads to the hypothesis that taurine synthesis inside the cell might be more important than its presence outside (in the incubation medium) when the protective properties of taurine are considered.

Cysteine sulphinate is the major metabolic precursor of hypotaurine and taurine, thus any of the intermediates in the pathway may be cytoprotective; they may also modulate the intracellular GSH levels, exerting a protective effect against ANIT-induced cytotoxicity.

Therefore, the slight protective effect when cysteinesulphinate was added to the incubation medium, might be due to the ability to maintain GSH levels perhaps above a threshold level for a longer period of time.

An observation made in the course of the experiments was that reduced glutathione was statistically significantly decreased in isolated hepatocytes, one hour after incubation with 15 mM taurine when compared to cells incubated with buffer alone. As can be observed in figure 7.16., taurine above a certain concentration in the sample to be assayed interferes with the arbitrary fluorescence units measured in the reaction of GSH with OPA. It is known that taurine directly reacts with OPA forming a highly fluorescent adduct (Larsen et al., 1980). This reaction is the same utilized in the method that chromatographically quantifies taurine and that was used in these studies (Waterfield, 1994).
is also possible that above a certain concentration, taurine changes the stability of the highly fluorescence product yielded by the reaction of GSH with OPA.

For the reasons expressed above, the measurements of GSH in the presence of 15 mM of taurine are certainly incorrect, but when expressed in a percentual basis of the initial value measured in the presence of the same concentration of taurine they might be suitable for comparison.

**Figure 7.16.** Interference observed in the method for measuring GSH, when GSH (750 ng or 32.5 nmol.ml\(^{-1}\)) was added to several concentrations of taurine in the medium.

When the potential cytoprotective effect of taurine was tested against DAPM-induced cytotoxicity, no clear cut evidence of protection was acheived.
In contrast to ANIT, for which a mechanism of action has been proposed, the cytotoxic effects of DAPM have never been tested in isolated hepatocytes. A dose- and time-dependent decrease in cell viability was observed with a rapid glutathione depletion in all groups regardless of the dose. On the other hand, oxidized glutathione levels rose in those groups that attained cell viability above a certain level at the last time point assessed.

When taurine was added to the incubation medium a statistically significant smaller drop in cell viability (assessed by means of trypan blue exclusion) was observed at all time points for 4 mM DAPM when compared to hepatocytes incubated with buffer. A similar effect was observed after the first hour of exposure to 5 mM DAPM. However, no significant differences were found between the groups mentioned above when LDH leakage was monitored (except for 4 mM, third hour). No major differences between the two different groups for the parameters assessed were found. The slight protective effect of taurine observed for trypan blue uptake lacks conclusive support from the other parameters measured and should be regarded with caution.

Although taurine has been proposed as a protective molecule against the cytotoxic effect of several compounds in different types of isolated cells, none of these toxicants were reported to cause a sharp depletion of GSH levels when taurine was able to afford protection. The evidence that taurine confers protection against cell death, caused by other compounds, by mechanisms such as the maintenance of membrane integrity, reduction of ion fluxes or even by scavenging free radicals but fails to provide sustained protection for ANIT and DAPM, leads to the assumption that perhaps taurine is not able to protect against all types of cell damage namely when GSH depletion is involved.
8.1. Introduction and aims of the research

The studies described here aimed to investigate the potential hepatoprotective effects of taurine when the rat was exposed to two different cholangiodestructive hepatotoxicants. Another goal was to investigate in parallel studies the possible cytoprotective effect of taurine when added to freshly isolated hepatocytes before challenging them with the same toxicants. The possible correlation between the hepatotoxicity observed in vivo, by means of assessing biochemical parameters of liver damage and liver histology, and the effect caused by the two toxicants in vitro on isolated hepatocytes was also one of the aims of the methodological approach. Thus, the leakage of cytoplasmic enzymes and the preservation of membrane integrity as well as the maintenance of ATP and GSH levels were investigated.

The importance of changing the levels of taurine in the hepatocyte both in vivo and in vitro and its possible relation with the toxicity caused by the compounds used in these studies were the main objectives in this research project.

Liver taurine levels were modulated directly, by adding taurine to the drinking water in order to increase the liver taurine levels or by adding taurine uptake inhibitors (β-alanine or GES) to decrease the taurine levels in the liver.

The other approach was an indirect attempt to modulate the taurine levels in the liver by feeding rats with a taurine-free diet but containing different amounts of casein that will provide metabolic precursors for taurine.
8.2. Effects of the direct modulation of liver taurine levels on ANIT- and DAPM-induced hepatotoxicity.

Taurine given in the drinking water to rats increased levels in the liver and was found to exert a slight protective effect against ANIT-induced hepatotoxicity, as judged by a smaller rise in serum transaminase levels and by a reduction in the extent of parenchymal damage caused by ANIT.

Conversely, when animals were depleted of taurine by giving them β-alanine in the drinking water, they were more susceptible to ANIT toxicity assessed by the rise in the serum levels of γ-GT and bilirubin. In contrast, when GES depleted liver taurine levels to an even greater extent prior to exposure to ANIT, animals did not show increased susceptibility. Currently, no explanation for this is proposed but the evidence reported recently that GES reduces significantly the liver activity of two key enzymes of the taurine biosynthetic pathway (Ide and Murata, 1994), may lead to the hypothesis that the taurine obtained by the diet and that synthesised in the liver may serve different functions in the hepatocyte. The reported change in the ratio of glycine:taurine bile acid conjugation - augmented by almost 10-fold after GES treatment - together with the fact that a 30% decrease in the amount of bile acids conjugated with taurine was observed in the rat (De La Rosa and Stipanuk, 1985) may also play a role in ANIT-induced hepatotoxicity.

It was also noticed from the studies described in this thesis that taurine treated animals showed a smaller periportal polymorphonuclear leukocyte infiltration than control animals dosed with ANIT. These observations were paralleled by the fact that taurine treated animals showed a smaller increase in their serum transaminases when compared to control animals dosed with similar dose of ANIT.

It is known that both ANIT and bile acids stimulate neutrophils (primed or not) to produce and release oxygen species that are believed to contribute to hepatic injury (Roth and Hewett, 1990; Dahm et al., 1988). It has also been
proposed that the fact that bile salts have been shown to potentiate the release of superoxide from activated PMN \textit{in vitro} (Dahm \textit{et al.}, 1988) might underlie some contribution of these bile components to activation of PMN \textit{in vivo} (Roth and Hewett, 1990). Moreover, it was shown that taurine possess anti-inflammatory properties and the response to its presence in a \textit{in vitro} system is believed to be mediated by the modulation of HOCl production (McLoughlin \textit{et al.}, 1991). The same authors were able to demonstrate that both reactive oxygen species (ROS) and LTB\textsubscript{4} levels were reduced when taurine was added to the \textit{in vitro} system. More recently, it has been shown that taurine chloramine inhibits the synthesis of nitric oxide and the production of TNF-\alpha in RAW 264.7 cells and it may play an important role in modulating the levels of these important cytotoxic mediators (Park \textit{et al.}, 1993; Schuller-Levis \textit{et al.}, 1994). Furthermore, it has been shown that taurochloramine decreases the production of tissue-damaging inflammatory mediators by interfering with the transcriptional and translational events that lead to the release of inflammatory mediators and may thus act as a physiological modulator and/or suppressor of nitric oxide and TNF-\alpha production (Park \textit{et al.}, 1995).

In the cat, an animal more vulnerable to taurine depletion, it was demonstrated that lung macrophages and PMN isolated by bronchoalveolar lavage from taurine deprived animals produced more superoxide anion in response to PMA than cats fed a taurine supplemented diet (Schuller-Levis and Sturman, 1992). An increase of taurine levels in the alveolar cells was associated with cats fed a taurine supplemented diet. It was also hypothesised that taurine functions to prevent terminal activation and release of cytotoxic mediators by lung macrophages (Schuller-Levis and Sturman, 1992). Indeed, more recently Bhat \textit{et al} (1994) proved it using alveolar macrophages harvested from Sprague-Dawley rats after exposure to bleomycin.

A similar approach as described above for ANIT was used to assess which effects the modulation of the liver taurine levels might exert on the
hepatotoxic effects induced by DAPM. Thus, rats pre-treated with β-alanine were more susceptible to the hepatotoxic effects induced by DAPM than control animals. Although, GES depleted liver taurine levels to a greater extent than β-alanine, rats pre-treated with GES were not more susceptible to the toxic effects of DAPM. When rats were treated with taurine a protective effect was found for some of the doses of DAPM tested when compared to control animals.

When the time course of DAPM-induced hepatotoxicity was studied it was found that liver taurine "loading" caused earlier damage from DAPM exposure, assessed by means of serum biochemistry and histologically (Chapter 5). This effect could be related to an alteration in the bile flow that might influence the presence of DAPM or DAPM metabolite in the bile or by altering the enterohepatic circulation of DAPM/metabolite. Indeed, when the bile duct was cannulated and the enterohepatic circulating interrupted the levels of the serum biochemical markers of hepatic damage at 10 hours after rats were dosed with DAPM were diminished compared with those observed 6 hours after exposure to DAPM.

Proposals for future work

In order to further explore the potential role of taurine in ANIT- and DAPM-induced hepatotoxicity and to address the mechanism(s) by which the modulation of liver taurine levels influence the development and the recovery from DAPM-induced hepatotoxicity the following aspects should addressed:

a. Investigate how the modulation of liver taurine levels might influence and to what extent, the presence of bile acids conjugated with taurine in the serum and in the bile at different time points after exposure to ANIT. Investigate the effect of different liver taurine levels on the presence of glutathione in the bile and its relation with bile flow after exposure to ANIT. Serum biochemistry and histopathological assessment should be carried out concomitantly in order to enable the possible correlation of changes in the bile
acid pattern in the serum and bile with the alteration in the liver parameters of hepatotoxicity. Liver content of taurine and TNPSH should also be followed during the time course of ANIT-induced hepatotoxicity.

b. Since it was observed by Waterfield et al (1993d) that ANIT caused a massive increase in the serum transaminase levels 48 hours after exposure to a single dose, it will be interesting to investigate if the slight protective effects afforded by increasing liver taurine levels observed 24 hours after exposure to ANIT remained present at later time points (e.g. 48 and 72 hours) and how the modulation of liver taurine levels might affect hepatocellular regeneration at later time points.

c. Investigate if the modulation of liver taurine levels influences the presence of free bile acids and/or the pattern of bile acid conjugation in the serum and bile of DAPM dosed animals when compared to control animals.

d. Extend the exposure of animals to DAPM upto one week (72 hours, 4 days and 7 days) in order to evaluate if the modulation of liver taurine levels may influence the recovery from biliary and hepatocellular damage.

e. Identify DAPM/metabolite(s) in the bile and establish a possible temporal relation between its appearance and alterations in bile flow and other bile parameters. Investigate the effect that the modulation of liver taurine levels might have on bile flow and if it interferes with the disposition of DAPM/metabolites into the bile. Establish a possible temporal relationship between the changes in bile flow caused by different liver levels of taurine with the alteration in bile constituents and the presence of DAPM/metabolites and explore how this alteration might correlate with the establishment of hepatotoxicity.

f. It would be interesting, although speculative, to investigate the effect produced by the modulation of liver levels of taurine on PMN and/or
macrophages isolated from the liver of taurine treated animals and how these particular cells behave upon PMA, bile acid or ANIT stimulus in respect to the release of chemical mediators that can act directly on hepatic parenchymal cells or elicit a response from circulating blood cells.

8.3. Effect of the indirect modulation of liver levels of taurine in the rat on ANIT- and DAPM-induced hepatotoxicity.

When rats were fed diets containing different amounts of casein and methionine, the objective was to increase the levels of taurine in the liver by \textit{de novo} synthesis and to correlate a possible increase of the liver taurine levels with the susceptibility to ANIT- and DAPM-induced hepatotoxicity. Although the first objective was not achieved, as the liver taurine levels in the rats fed both diets were comparable to control animals fed a normal diet, it was observed that the animals fed a 15% casein diet were less susceptible to ANIT than control animals. Discrepancies were found between the serum parameters measured and the liver histopathological assessment for animals showing marked steatosis.

In recent work Bagley and Stipanuk (1994; 1995) concluded after a series of experiments by feeding different diet to rats, that freshly isolated hepatocytes showed a more sustained increase in cysteine dioxygenase activity with more taurine production from cysteine when diets containing a high level of sulphur amino acids were provided, whereas cysteinesulphinate decarboxylase activity seemed to respond more strongly to excess protein intake.

The indirect modulation of liver taurine levels using a dietary approach offers a considerable challenge when one deals with the rat species as the working model. The rat is a very good taurine synthesiser and it is very difficult to deplete the rat of taurine by suppressing solely this amino-acid from the diet. Nevertheless, it would interesting to further investigate which effect the rise in the liver taurine levels would have on the hepatotoxicity exerted by other compounds. The role of N-acetyl-L-cysteine (NAC) and L-2 oxythiazolidine-4-
carboxylate (OTC) has been extensively investigated with respect to their properties to increase cysteine levels in the hepatocyte and provide the rate limiting percussor for glutathione synthesis. Recently, it has been shown that the utilization of NAC and OTC by the rat isolated hepatocyte is limited by their rate of uptake and conversion to cysteine (Banks and Stipanuk, 1993). Furthermore, when OTC was administered into the stomach of rats the levels of cysteine increased in the serum and in the liver up to 8 hours after administration (Yamada et al., 1995). When the excretion of taurine and sulphate were quantified in the urine of these animals it was found they accounted for as much as 40% of the total of OTC administered.

Proposals for future work

Although both 15% and 30% casein diet used in these studies were ineffective to increase the liver levels of taurine, the approach of raising the liver taurine levels by means of de novo synthesis of this amino acid may lead to a new approach in the potential protective action of taurine against toxicants.

a. It would be advisable to investigate if the 15% and/or the 30% casein diets affect the lipid metabolism in the liver and how it might correlate with the levels of taurine in the liver and to the susceptibility of animals to different toxicants.

It will be important to establish if the presence of steatosis and/or interference with protein synthesis would interfere with the level of enzymes in the hepatocyte and/or in the serum of naive and exposed animals or if the discrepancies observed for the histological versus serum parameters are due to a direct interference of DAPM or any metabolite in the serum with the assay.

b. Considering that sulphur amino acid supplementation favoured the formation of taurine from cysteine in the rat and that rats fed a low casein diet (10%) supplemented with 10g of L-methionine/Kg for two weeks showed
a raised activity of cysteine dioxygenase in the liver but maintained cysteine
decarboxylase activity at normal levels (Bagley and Stipanuk, 1995), it would
be very interesting to: first, examine whether a similar approach will be a
more effective way to increase liver taurine levels than the one undertaken
in the studies described in Chapter 6; second, if liver taurine levels are
successfully raised, evaluate whether this approach could afford protection
against the hepatotoxic effects caused by several compounds.

Another approach will be to investigate whether the combined effects of
feeding rats a semi-synthetic diet and giving them NAC or OTC will increase
the liver levels of taurine and how they might correlate with a change in the
hepatotoxicity induced by chemicals.

c. It would be also interesting to investigate whether the hepatotoxic effects
casted by chemicals could be ameliorated by inhibiting the synthesis of
 glutathione from cysteine with buthionine sulphoximine (BSO).
In the presence of NAC or OTC, as precursors, cysteine levels will be boosted
by the inhibition of γ-glutamylcysteine synthetase using BSO. It will be
important to determine if taurine levels are increased in the liver and the
effect of this on hepatotoxicity.

8.4. How the modulation of taurine levels in isolated hepatocytes
might influence their susceptibility to ANIT and DAPM.

ANIT and DAPM caused a time- and dose-dependent decrease in cell viability
when hepatocytes isolated from control animals were exposed to the toxicants.

The cytotoxic effects were paralleled by a decrease in the ATP and GSH
content. DAPM caused a more rapid depletion of GSH than ANIT. When
hepatocytes isolated from β-alanine treated animals were exposed to ANIT no
significant difference was found compared to cells isolated from control animals.
When taurine (15 mM) was added to the incubation medium and cells challenged with ANIT and DAPM, no significant difference was detected for ANIT. Although, cells incubated with taurine showed slight protection against the cytotoxicity of DAPM this effect was only significant at one of the top doses when cell viability, LDH leakage and ATP levels were compared to isolated cells incubated with buffer.

It was also suggested from the analysis of data shown in chapter 7, that the addition of cysteinesulphinate to the incubation medium could ameliorate the cytotoxic effect of ANIT either by modulation of taurine synthesis or by affecting the cysteine catabolism pathway and thus interfere with the intracellular levels of glutathione shown to be depleted by this toxicant.

**Proposals for future work:**

**a.** It would be interesting to investigate if the same approach as described above will be effective in the protection against the cytotoxic effect of DAPM and to what extent.

**b.** It would be important to further explore the potential of cysteinesulphinate in protecting rat isolated hepatocytes when exposed to toxicants. Also important is the relationship between taurine synthesis and its distribution between the intra and extracellular compartments in relation to protection.

**c.** The modulation of the cysteine catabolism pathway would be another way to address the question. When rats were fed a low content casein diet (10%) supplemented with 1.5 g DL-methionine/Kg their cysteinesulphinate decarboxylase activity was 7 to 12-fold higher than when rats were fed higher casein content diets, respectively 30 and 60% (Bagley and Stipanuk, 1994). Moreover, hepatocytes isolated from the rats fed the low content casein diet produced 3 to 7-fold more taurine from cysteinesulphinate than cells isolated from their counterparts fed respectively a 30% and a 60% casein diet.

Thus, using a low casein content diet could also provide a good working model
to maintain the levels of cysteinesulphinate decarboxylase in the hepatocyte. This would allow the evaluation of the effect of addition of cysteinesulphinate to the medium and its transformation to taurine on the susceptibility of isolated cells to different toxicants.
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APPENDICES

Appendix I

Preparation of ion exchange columns
All water used in these procedures 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, was first washed with 250 ml 1 M HCl and then with water until pH was above 4.5

(b) Dowex-50W-X8 (cation exchange, in H⁺ form) 100-200 mesh, was washed initially with HCl (500 ml 4 M, in three washings) and later with 1 M HCl (250 ml).
The volumes used are for 100 g of resin.

COLUMNS

Dual-bed columns for multi-use propose (isolated hepatocytes, tissue extracts and dilute urine) were prepared by layering 0.5 apparent volume of the 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, 1M).
Appendix II.

BALANCED SALT SOLUTIONS FOR PREPARATION OF ISOLATED HEPATOCYTES

A. HANK SOLUTIONS - Stock (x 10 conc) -

The following were made up in 1 L UHQ water

\[
\begin{align*}
\text{NaCl} & \quad 80.0 \, \text{g} \\
\text{KCl} & \quad 4.0 \, \text{g} \\
\text{MgSO}_4\cdot\text{H}_2\text{O} & \quad 2.0 \, \text{g} \\
\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O} & \quad 0.6 \, \text{g} \\
\text{KH}_2\text{PO}_4 & \quad 0.6 \, \text{g}
\end{align*}
\]

Working solutions

\[
\begin{align*}
\text{Hank x 10 stock} & \quad 50 \, \text{ml} \\
\text{Water} & \quad 450 \, \text{ml} \\
\text{NaHCO}_3 & \quad 1.05 \, \text{g} \\
\text{HEPES} & \quad 1.50 \, \text{g}
\end{align*}
\]

The solution was gassed with 5%CO\_2/95%O\_2 for 3-5 min and divided for:

**Hank I** (initial perfusate - Ca\(^{2+}\) free)

\[
\begin{align*}
\text{Hank} & \quad 300 \, \text{ml} \\
\text{EGTA} & \quad 68.4 \, \text{mg} \\
\text{Albumin} & \quad 2.0 \, \text{g}
\end{align*}
\]

**Hank II** (collagenase containing)

\[
\begin{align*}
\text{Hank} & \quad 200 \, \text{ml} \\
\text{CaCl}_2\, 5.88\% & \quad 2 \, \text{ml}
\end{align*}
\]

(collagenase 50 mg/100 ml Hank II)
Both solutions (Hank I and Hank II) were adjusted to pH 7.4 with 1 M NaOH.

B. KREBS-HENSELEIT BUFFER -

stock (× 2 conc.)

a. 16.09% NaCl 80.45 g → 500 ml
b. 1.1% KCl 5.50 g → 500 ml
c. 0.22 M KH₂PO₄ 7.49 g → 250 ml
d. 2.74% MgSO₄·7H₂O 6.85 g → 250 ml
e. 0.12 M CaCl₂·2H₂O 8.82 g → 500 ml
f. 0.97% NaHCO₃ 4.85 g → 500 ml

Solutions a - f were mixed in the following proportions: a. 100 ml; b. 75 ml; c. 12.5 ml; d. 25 ml; e. 50 ml and 392.5 ml of water added. These were then gased with 5%CO₂/95% O₂ for 10 min before adding solution f. 500 ml.

Working solutions -

buffer for incubation of cells (K + H) -

Krebs-Henseleit × 2 stock 150 ml
Water 150 ml
HEPES 1.5 g

This was gassed for 5 min with 5% CO₂/95% O₂ and a 100 ml aliquot removed

- buffer for dispersion of cells (K + H + Alb) -

K + H 100 ml
Albumine (bovine) 1.0 g

pH was adjusted to 7.4 with 1 M NaOH.
Appendix III

DETERMINATION OF TOTAL NON-PROTEIN SULPHHYDRYLs (TNPSH)

Reagents
1. Phosphate buffer (pH 7.4) - 43.6 KH$_2$PO$_4$ + 900 ml water, pH with NaOH to pH 7.4 made up to 1 L.
2. Phosphate buffer (pH 8.0) - 13.6 KH$_2$PO$_4$ + 900 ml water, pH with NaOH pH 8.0 made up to 1 L.
3. Sulphosalycylic acid, 0.2 M
4. DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] - 39.6 mg + 10 ml pH 7.4 phosphate buffer

Standards GSH 0.1 - 1.0 mM (0.2 sulphosalycylic acid/ phosphate 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 ml) 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 μmol.g$^{-1}$ wet weight of liver.

MEASUREMENT OF LACTATE DEHYDROGENASE

Solutions

1. Phosphate buffer, 0.05 M (pH 7.4, 6.8 g KH$_2$PO$_4$ L$^{-1}$, room temperature)
2. Pyruvate, 6.2×10$^{-3}$ M (7.5 mg.10ml$^{-1}$ buffer, 4°C)
3. NADH.2H$_2$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) 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 percentage 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 (30 sec, 50% power). Cell debris was pelleted (11,000 ×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.
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 finally expressed as a % of the total.

MEASUREMENT OF ATP IN ISOLATED HEPATOCYTES

Reagents

1. Luciferase-Firefly lantern extract (Sigma FLE-250) was resuspended in 5 ml UHQ water and centrifuged (5 min, 1000 rpm).
2. Buffer - The following were mixed 1:1:1 just before use:
   a. 80 mM MgSO₄·7H₂O 19.72 g.L⁻¹
   b. 10 mM KH₂PO₄ 1.361 g.L⁻¹
   c. 100 mM Na₂AsO₄·7H₂O 31.20 g.L⁻¹
   and adjust to pH 7.4

Procedure

Hepatocyte suspension (0.25 ml, 2 × 10⁶ cells) were placed into TCA (20%, 0.5 ml, 4°C) and immediately frozen (-80°C) and analyzed within a week. Samples were centrifuged (11,000 × g, 5 min, 4°C) and a sample of the supernatant or standard (10 μl) added to buffer (2.0 ml). Firefly latern extract (100 μl) was
added to start the reaction. The luciferase-linked bioluminescence produced was measured with a computer-controled Thorn EMI photon detection system utilising an air cooled (-25°C) photomultiplier for 6 seconds, 15 seconds after the reaction was started.
The sample was compared directly with the standards since they are prepared using similar volumes and values expressed as nmol.ml⁻¹ ATP per million of cells.
Appendix IV

Semi-synthetic diets used in Chapter 6 studies.

TD93218

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, high methionine (g/Kg)</td>
<td>300.0</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>4.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>274.52</td>
</tr>
<tr>
<td>Corn Strach</td>
<td>270.0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>60.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
</tr>
<tr>
<td>Vitamin Mix, Teklad (40060)</td>
<td>10.0</td>
</tr>
<tr>
<td>Ethoxyquin (antioxidant)</td>
<td>0.01</td>
</tr>
<tr>
<td>Mineral Mix, Ca-P defic (TD79055)</td>
<td>13.37</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic CaHPO$_4$</td>
<td>10.6</td>
</tr>
<tr>
<td>Calcium Carbonate, CaCO$_3$</td>
<td>7.0</td>
</tr>
</tbody>
</table>

TD93258

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, high methionine (g/Kg)</td>
<td>150.0</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>2.25</td>
</tr>
<tr>
<td>Sucrose</td>
<td>274.52</td>
</tr>
<tr>
<td>Corn Strach</td>
<td>422.25</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>60.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
</tr>
<tr>
<td>Vitamin Mix, Teklad (40060)</td>
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</tr>
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<td>10.6</td>
</tr>
<tr>
<td>Calcium Carbonate, CaCO$_3$</td>
<td>7.0</td>
</tr>
</tbody>
</table>

According with the manufacturer specifications, diet TD93258 is based on TD93218 with the reduction in casein compensated with an equivalent increase in starch. Minor adjustments in calcium and phosphorus contents were made to compensate their removal from the diet with high casein content (casein includes modest amounts of calcium and phosphorus).
Appendix V.

Values for serum and liver measured parameters in control animals, fed a normal diet and before dosing with vehicle.

<table>
<thead>
<tr>
<th>Measured parameters</th>
<th>mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum bilirubin</td>
<td>2.46 ± 6.01</td>
</tr>
<tr>
<td>Serum γ-GT</td>
<td>0.77 ± 0.19</td>
</tr>
<tr>
<td>Serum ALP</td>
<td>91.6 ± 13.1</td>
</tr>
<tr>
<td>Serum AST</td>
<td>19.2 ± 3.4</td>
</tr>
<tr>
<td>Serum ALT</td>
<td>12.0 ± 4.3</td>
</tr>
<tr>
<td>Serum bile acids</td>
<td>13.7 ± 5.1</td>
</tr>
<tr>
<td>Serum taurine</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>Liver taurine</td>
<td>5.30 ± 1.72</td>
</tr>
<tr>
<td>Liver TNPSH</td>
<td>4.98 ± 1.12</td>
</tr>
</tbody>
</table>

Serum and liver parameters measured in rats fed a normal diet for 6 days and killed at the time of dosing. Serum parameters: bilirubin and bile acids (SBA) (μmol.l⁻¹); γ-GT, AST, ALT and ALP (iu.l⁻¹); taurine (μmol.ml⁻¹). Liver parameters: TPNSH and taurine (μmol.g⁻¹ wet wt). Values are means ± SD for 3-4 animals.
Appendix VI.

\[
\begin{align*}
\text{N=C=S} \\
\alpha\text{-naphthylisothiocyanate} \\
\text{H}_2\text{N} \quad \text{H} \quad \text{C} \quad \text{H} \\
\text{H} \quad \text{H} \\
4,4'\text{-diaminodiphenylmethane} \\
\text{H}_3\text{N}^+\text{CH}_2\text{CH}_2\text{CO}_3^- \\
\beta\text{-alanine} \\
\text{H}_3\text{N}^+ \quad \text{C} \quad \text{NH} \quad \text{CH}_2\text{CH}_2\text{SO}_3^- \\
\text{Guanidinoethane sulphonate}
\end{align*}
\]
Not all Rats are Resistant to Cocaine Hepatotoxicity.

S.J. Charles,* H. Partridge, S. Cottrell & C.J. Powell, DH Department of Toxicology, St. Bartholomews Hospital Medical College, London ECI 7ED.

Cocaine hepatotoxicity has been well documented in humans, however rats are reported to be resistant (Perrino et al 1987; Evans & Harbison 1978; Rauckman et al 1982). Previous studies in rats have been limited to one strain or dose level, and so this study was conducted using four different strains of rat and two dose levels of cocaine, and included pretreatment with phenobarbitone (PB), a known sensitizer to cocaine in mice, in an attempt to obtain a definitive answer to the question of rat susceptibility to cocaine hepatotoxicity.

Groups of 4 male Sprague-Dawley (W), Fischer 344 (F344) and Wistar-Kyoto (WKY) rats (Olac Ltd. U.K.), received either saline, cocaine alone at 40 or 60 mg/kg or the same two doses of cocaine preceded by PB (80 mg/kg). PB+ saline was administered ip for 3 consecutive days, followed the next day by a single ip dose of saline or cocaine (40 or 60 mg/kg) with post mortem 24 hours later. Four rats from 3 of the different strains were susceptible to the acute (probably CNS) effects of cocaine, with mortalities occurring shortly after cocaine administration.

The levels of liver enzymes in the plasma were measured as a marker of liver damage. In WKY rats, 40 or 60 mg/kg cocaine alone caused significant elevations in levels of alanine aminotransferase (ALT) and α-hydroxybutyrate dehydrogenase (α-HBDH, P <0.05), and in combination with PB produced significant elevations of ALT (P<0.05). The group of F344 rats treated with PB and 60 mg/kg cocaine had elevated levels of aspartate aminotransferase (AST). Lactate dehydrogenase (LDH), ALT and α-HBDH and the group of CD rats given only 60 mg/kg cocaine had elevations of AST and ALT. These increases were not statistically significant, as they were attributable to only 3 animals, 2 F344 rats and a single CD rat. 2-fold elevations in LDH were observed in both F344 rats, while AST was elevated 10-fold in 1 F344 rat and 5-fold in the CD rat. Therefore, despite the apparent resistance of the majority of rats, certain individuals were susceptible to cocaine hepatotoxicity. Histopathology showed that 2/3 CD rats given 60 mg/kg cocaine developed degeneration and necrosis in centrilobular hepatocytes, despite only 1 of these animals having high plasma enzyme levels. Histological liver damage was not observed in any other rat strain given cocaine either with or without PB pretreatment, despite raised plasma enzyme levels in 2 F344 rats. It appears therefore that PB may protect against cocaine-induced liver damage in CD rats. Biochemical analysis of liver microsomes revealed that in animals with elevated plasma enzyme levels, total cytochrome P-450 content was reduced by up to 50%, while cytosolic glutathione S-transferase activity was increased by up to 100%, and reduced glutathione levels were marginally elevated.

This evidence suggests that CD rats are the most sensitive to cocaine, i.e. having died immediately after 60 mg/kg cocaine and 2 more having liver lesions 24 hours later, one of these also displaying elevations in plasma enzyme levels and changes in biochemical parameters. In addition, PB protected CD rats from the histological manifestation of liver damage, as when PB was given prior to cocaine, no hepatic lesions were observed. In mice, PB pretreatment shifts the site of cocaine-induced necrosis across the lobule. Centrilobular hepatocytes which are damaged after treatment with cocaine alone, are protected from necrosis following pretreatment with PB and peripheral portal cells become the targets for cocaine-induced damage (Charles & Powell 1992). It appears unlikely that this phenomenon occurs in rats. As cocaine induced hepatic necrosis in CD rats, elevated plasma enzymes in occasional F344 rats and caused centrilobular glycogen depletion and steatosis in some WKY rats, the following order of sensitivity to cocaine hepatotoxicity can be assigned: CD > F344 > WKY > W.


Effects of Liver Taurine Changes on α-Naphthylisothiocyanate (ANIT) Hepatotoxicity.

V. Seabra* & J.A. Timbrell, Toxicology Department, School of Pharmacy, University of London, 29 Brunswick Sq, London WC1N 1AX, U.K.

Taurine, a β-amino acid has been recently reported to protect against cholestasis and liver damage caused by lithocholic acid sulphate in guinea pigs (Dervil et al, 1983). Recently, Waterfield et al (1992) reported that depletion of liver taurine by β-alanine treatment increases the toxicity of multiple chemicals in rats. We have investigated the effect of taurine on ANIT hepatotoxicity. ANIT damages primarily the biliary epithelial cells causing a rise of bilirubin and y-GT in serum. y-GT has been described as a specific marker of bile duct damage in rat liver (Leonard et al, 1984). Male Sprague Dawley rats (170-230g) were divided into 6 groups, individually caged in metabolism cages and treated either with tap water (groups 1 and 2), β-alanine (3 and 4) (3% in drinking water) or taurine (5 and 6) (3% in drinking water) and given food ad lib 6 days. On day six, rats were dosed with ANIT (100 mg/kg) in corn oil (50 mg/ml), p.o. or vehicle. Food was removed after dosing. After 24-h, blood was taken from the abdominal aorta for biochemical measurements. Liver samples were taken for histology and total non-protein sulphydryl (TNPSH). The liver tissues showed that ANIT causes bile duct damage with some periportal necrosis and neutrophil infiltration in all groups. The extent of damage was increased in β-alanine treated animals but taurine treatment doesn’t show any difference when compared to control, indicating that β-alanine treatment decreases liver taurine and increases bile duct damage measured histologically and biochemically (bilirubin and y-GT).

V. Seabra was supported by JNICT (Portugal) - Programa Ciência.

Hum. Exp. Toxicol. 12, 566 (1993)
**P10/02**

**CHOLESTATIC COMPOUNDS INDUCE F-ACTIN ALTERATION IN VITRO AND IN VIVO.**

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It has been shown that cholestatic compounds (CC) by impact on hepatocytes induced F-actin alteration in isolated rat hepatocyte couplets (IRHC) (1). The aim of this study was to assess if this phenomenon occurs with CC inducing lesions of biliary cells: methylene dianiline (MD) and α-naphtylisothiocyanate (ANIT).

**Methods:** IRHC were isolated from non treated SD rats and incubated with MD or ANIT or from pretreated rats, with phalloidin (PH) as a positive control. Serum bilirubin levels were estimated. F-actin distribution in IRHC was assessed by staining with FITC-phalloidin and measurement of the canalicular area fluorescence/total couplet fluorescence ratio (CF/TF) by scanning laser cytometry.

**Results and Discussion:**

<table>
<thead>
<tr>
<th></th>
<th>In vitro treatment</th>
<th></th>
<th>In vivo treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>controls PH MD ANIT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF/TF</td>
<td>1.61±0.21</td>
<td>2.31±0.21*</td>
<td>1.59±0.19</td>
</tr>
<tr>
<td>Bilirubin (mg/L)</td>
<td>3±1</td>
<td>40±20*</td>
<td>59±33*</td>
</tr>
<tr>
<td>CF/TF</td>
<td>1.43±0.28</td>
<td>2.93±0.29*</td>
<td>1.99±0.11*</td>
</tr>
</tbody>
</table>

(means±SD, n=80 IRHC from 4 isolations, *P<0.001, variance analysis). Compared to PH, inducing a direct toxicity on hepatocytes, MD and ANIT, inducing a predominant toxicity on biliary cells did not provoke pericanalicular F-actin accumulation in **in vitro** IRHC. By contrast IRHC isolated from pretreated rats showed a significant pericanalicular F-actin accumulation in all cases.

**Conclusion:** This suggests that abnormalities of F-actin is a general key mechanism in cholestasis. **In vitro** and **ex vivo** models are complementary tools to reveal this action, according to the type of injured liver cells.


**Key Words:** Cholestasis - Isolated rat hepatocyte couplets - F-actin - Scanning laser cytometry

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**P10/03**

**COMPARISON OF TOXIC EFFECTS CAUSED BY TWO BILE DUCT TOXICANTS**

V. Seabra and J.A. Timbrell, Toxicology Dept., School of Pharmacy, University of London, U.K.

**Introduction** The aim of this study was to examine the effect of α-naphthylisothiocyanate (ANIT) and 4,4′-diaminodiphenylmethane (DAPM) two known bile duct toxicants(1) on parameters of toxicity. Previous studies have shown that hepatic damage causes an increase in urinary taurine(2).

**Methods** Male rats (S.D.; 190-300 g) were kept in individual metabolism cages and urine collected over ice. After 6 days, animals were treated with ANIT (100 mg.kg⁻¹) in corn oil or DAPM (100, 250 and 400 mg.kg⁻¹) in 35% ethanol or vehicle. Food was removed after dosing. After 24 h, animals were sacrificed and blood and liver removed for assessment of biochemical and histological parameters.

**Results and Discussion** ANIT and DAPM caused a rise in serum bilirubin, γ-GT, AST, ALT (p<0.01) when compared to controls. Urinary taurine significantly decreased in animals dosed with ANIT (p<0.001) whereas for DAPM, the lowest dose caused a decrease and the highest doses an increase in urinary taurine when compared to control animals. Histology showed that although both toxicants causes bile duct destruction, DAPM also caused periporal and focal liver necrosis. ANIT and DAPM at a similar dose caused a rise in the TNPSH liver levels (p<0.01) while higher doses of DAPM did not cause any change. Biochemical and histological parameters of liver damage correlated for both compounds. Changes in serum enzymes plotted against percent change in urinary taurine for individual animals were differently correlated for each compound.

**Conclusions** These results suggest that when only bile duct damage occurred urinary taurine was decreased whereas if hepatocytes were also damaged there was a shift in the urinary taurine towards increased excretion.

**References**


**Key Words**

DAPM, ANIT, urinary taurine, liver damage

V. Seabra is supported by JNICT - Programa Ciência - Portugal

Pharmacol. & Toxicol. 73 (Suppl.II), 109 (1993)
The polymer curing agent, methylene diaminodiphenylmethane (DAPM), rapidly causes cholestasis and injury to biliary epithelial cells (BEC) in the liver and common bile duct of rats. Our objective was to determine if the proximate toxicant was present in bile. Male SD rats (- 350 g) had biliary cannulas positioned under pentobarbital anaesthesia and were given DAPM (250 mg/kg) po in 35% alcohol. Bile from DAPM-treated rats was directed through the common bile duct of untreated rats via inflow and outflow cannulas for 4 hr. Control rats had similar cannulas in their common bile ducts through which bile from vehicle-treated rats flowed. Cholestasis and BEC injury in the common bile duct of rats receiving DAPM-bile or control bile was assessed in a minimum of 4 cross-sections using Color Assisted Computer Image Analysis. Sections were imaged at 40X and the number of necrotic cells per 100 cells determined by point counting. Necrotic cells were defined as eosinophilic cells containing nuclei in karyolysis, pyknosis or karyorrhexis. Percent necrosis in the common bile ducts of control rats was < 1% compared to > 16% in rats receiving DAPM-bile. These results indicate that bile is the route of BEC exposure to DAPM proximate toxicant(s) and suggest a new method for assessing the mechanism of biliary DAPM/metabolite injury to BEC.

Previous studies indicate that during manganese-bilirubin (Mn-BR) induced cholestasis, molecular organisation of the bile canalicular membrane (BCM) is altered. The purpose of the present study was to evaluate lipid composition and fluidity of BCM during cholestasis. Cholesterol and phospholipid content were investigated after isolation of hepatic membranes in male Sprague-Dawley rats. To induce cholestasis, Mn (4,5 mg/kg, i.v.) was given 15 min before BR (25 mg/kg, i.v.). The rats were killed 60 min after BR injection. Liver cell plasma membranes were isolated into enriched fractions by differential centrifugation. Lipids were extracted and measured colorimetrically. To assess fluidity, membranes were incubated in vitro with fluorescent probes (DPH and TMA-DPH). Results indicate a marked increase (about 3-fold) in cholesterol incorporation followed by a decrease in BCM fluidity after Mn-BR treatment. Thus, the hypothesis that altered lipid composition and membrane fluidity are involved in Mn-BR cholestasis appears supported (Funded by the Medical Research Council).
TOXICITY OF α-NAPHTHYLISOTHIOCYANATE IN ISOLATED HEPATOCYTES: POSSIBLE ROLE OF TAURINE

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Abstract—Exposure of isolated rat hepatocytes to α-naphthylisothiocyanate (ANIT) results in time and dose-dependent decreases in glutathione (GSH), ATP and cell viability. When taurine (15 mM) was added to cells no protection was afforded and a drop in the initial levels of GSH was also found. Prior treatment of rats with β-alanine (3% in drinking water) did not increase ANIT toxicity, although taurine levels significantly dropped in cells. When 15 mM cysteinesulfinate was added to taurine depleted cells, some protection was afforded with a smaller drop in GSH than for cells incubated with buffer or taurine. The taurine pathway may thus play a protective role in ANIT cytotoxicity in isolated hepatocytes.

INTRODUCTION

α-Naphthylisothiocyanate (ANIT) has been used as a model to study intrahepatic cholestasis in vivo and more recently, its cytotoxicity has been studied in isolated hepatocytes (Carpenter-Deyo et al., 1991). El-Hawari and Piaa (1977) showed that pretreatment of rats with sulfhydryl compounds such as cysteine, cystamine and cysteamine protected animals against ANIT-induced hyperbilirubinemaia. Recently, Stipanuk et al. (1992) showed that the metabolism of cysteine to taurine is highly dependent on the availability of cysteine and on cysteine dioxygenase activity, which is responsible for the first metabolic step in the conversion of cysteine to taurine by the cysteinesulfinate-dependent pathway.

It has also been shown that taurine protects isolated hepatocytes against the cytotoxic effects of hydrazine, 1,4-naphthoquinone and carbon tetrachloride (Waterfield et al., 1993a) and against the calcium and oxygen paradoxes when added directly to isolated hepatocytes (Nakashima et al., 1990).

Furthermore, there is evidence that depletion of liver taurine, through administration of a 3% β-alanine solution, in rats increases their susceptibility to carbon tetrachloride (Waterfield et al., 1993b) and to ANIT (Seabra and Timbrell, 1993).

Carpenter-Deyo et al. (1991) showed that ANIT depletes intracellular glutathione (GSH) by promoting its transport or leakage, or by forming an intermediate glutathione–ANIT conjugate.

Consequently, we have examined the effects of ANIT in isolated hepatocytes and studied the protective role of taurine and its precursors against the cytotoxicity of ANIT.

Abbreviations: ANIT = α-naphthylisothiocyanate; CS = cysteinesulfinate; DMSO = dimethyl sulfoxide; GSH = glutathione; TCA = trichloroacetic acid.

MATERIALS AND METHODS

Male Sprague–Dawley rats (210–285 g) obtained from Glaxo Group Research were allowed to acclimatize for up to 6 days. Rats were given food and tap water (or 3% β-alanine in tap water) ad lib. for a 6–8-day period prior to use. Hepatocytes were prepared by the method of Moldeus et al. (1978).

Cell viability was assessed by trypan blue exclusion and was required to be at least 87%. Cells were placed in siliconized round-bottomed flasks (100 ml), suspended in Krebs–Hensenleit buffer at a viable cell concentration of 2 x 10⁶/ml (15 ml) and gassed throughout the experiment with 95% O₂/5% CO₂.

After an initial incubation period of 60 min with buffer, 15 mM taurine or 15 mM cysteinesulfinate (CS), ANIT [0–1.5 mM in dimethyl sulfoxide (DMSO), 0.49%] was added to the suspension. ANIT was obtained from Sigma Chemical Co. Ltd (Poole, Dorset, UK).

Parameters measured

Cell viability. This was assessed by measuring the uptake of trypan blue by damaged cells.

Intracellular GSH. Aliquots of cell suspension (0.25 ml) were taken from each flask at different time points, centrifuged (800 rpm, 1 min) and the supernatant discarded. The intracellular content of GSH was measured in a trichloroacetic acid (TCA; 6.5%) homogenate by a modified method of Hissin and Hilf (1976).

ATP. Aliquots (0.75 ml) of cell suspension were centrifuged (800 rpm, 1 min) and the supernatant discarded. 0.25 ml TCA (1 M) plus EDTA (10 mM) was added to the cells, which were vortexed and the solutions kept at −80°C until they were analysed for ATP. ATP content was measured using the detection of luciferase-linked bioluminescence in the TCA extracts as described by Stanley and Williams (1969).
Fig. 1. Effect of different concentrations of ANIT after 3 hr on GSH, ATP and cell viability in isolated rat hepatocytes. Data are means ± SE for 4–7 experiments. *Significantly different from DMSO (P < 0.01) by Dunnett’s t-test after ANOVA. ♦ Significantly different from cells incubated with buffer (P < 0.05) by Student’s t-test.

Fig. 2. Effect of different concentrations of ANIT on GSH and cell viability after 3 hr in hepatocytes isolated from β-alanine treated rats. Data are means ± SE for 4–12 experiments. *Significantly different from DMSO (P < 0.01) by Dunnett’s t-test after ANOVA.
**Role of taurine in ANIT toxicity in hepatocytes**

Taurine. This was measured in suspension buffer or in hepatocytes by HPLC as described by Waterfield (1992) modified from Larsen et al. (1980).

**RESULTS**

Hepatocytes exposed to ANIT showed dose and time dependent decreases in cell viability, GSH and ATP. The vehicle (DMSO) or 0.25 mM ANIT caused no change in these parameters (data not shown).

Incubation with 15 mM taurine had no significant effect on cytotoxicity and ATP depletion.

A drop in the initial values for GSH (23.8 ± 2.8 nmol/10⁶ cells) was observed after pre-incubation with buffer (33.7%) and 15 mM taurine (58.6%) in the absence of ANIT. The level of GSH was significantly lower in taurine-treated cells compared with controls ($P < 0.05$, Student's $t$-test) at all time points for DMSO and 0.5 mM ANIT [data not shown, except for 3 hr (Fig. 1)].

Total taurine levels were constant during the experiments for all concentrations of ANIT (data not shown). At the last time point (3 hr) no significant differences were found when buffer or taurine were added to isolated cells (Fig. 1).

When hepatocytes isolated from $\beta$-alanine-pretreated rats were used, a significant decrease ($P < 0.05$, Student's $t$-test) in the initial total taurine concentration was found compared with cells from non-pretreated rats (data not shown).

ANIT cytotoxicity was not affected by pretreatment of animals with $\beta$-alanine despite lower levels of taurine in cells from those animals. After 3 hr of exposure no significant differences were found between cells from control (Fig. 1) and pretreated animals (Fig. 2).

Adding taurine to the incubation buffer had no effect on cytotoxicity or depletion of GSH by ANIT.

When CS was added to the incubation buffer loss of viability and depletion of GSH at 3 hr was less than in control cells (Fig. 2). Cells incubated with CS were able to synthesize taurine (30 nmol/10⁶ cells) over a period of 3 hr, whereas those exposed to buffer alone synthesized only 4 nmol/10⁶ cells (data not shown).

**DISCUSSION**

This study has shown that ANIT causes concentration and time dependent depletion of GSH and ATP and loss in cell viability in isolated hepatocytes. Moreover, a temporal relationship between lactate dehydrogenase leakage and GSH depletion prior to cell death has been reported by Carpenter-Deyo et al. (1991).

No protective effect of taurine was found against ANIT toxicity, unlike that recently reported by Waterfield et al. (1993a) for other compounds. This is perhaps due to the initial depletion of GSH caused by adding taurine to the cells, which might cause increased toxicity of ANIT by depleting GSH at an earlier time point.

When the percentage cell viability was plotted against the percentage GSH depletion a significant negative correlation ($r = -0.95, P < 0.01$) was found for all experiments, except for those cells incubated with CS.

When CS is added to isolated hepatocytes, it is rapidly metabolized to taurine, as recently reported by Stipanak et al. (1992). We have also shown a significant increase ($P < 0.01$) in taurine content in hepatocytes after incubation with CS. The synthesis of taurine inside the cell may be more important for protection than its presence outside.

CS is the major metabolic precursor of hypotaurine and taurine; thus any of the intermediates in this pathway may be cytoprotective. Also they may modulate intracellular GSH levels, and thus exert a protective effect. Therefore, the protective effect found for CS against ANIT cytotoxicity might be due to the ability to maintain GSH levels perhaps above a threshold level for a longer period of time.

In conclusion, the taurine metabolic pathway may play a protective role in ANIT cytotoxicity in isolated hepatocytes. The mechanism by which it affords protection needs to be addressed in future work.

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**REFERENCES**


REVIEW
The In Vivo and In Vitro Protective Properties of Taurine

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(Received 28 June 1994)

Abstract—1. Taurine is a ubiquitous, free amino acid found in mammalian systems.
2. The biological functions of taurine are unclear.
3. Various in vivo data suggest that taurine has a variety of protective functions and deficiency leads to pathological changes.
4. Depletion in rats of taurine increases susceptibility to liver damage from carbon tetrachloride.
5. Susceptibility to a variety of hepatotoxicants correlates with the estimated hepatic taurine level.
6. In vitro data suggest that taurine can protect cells against toxic damage.
7. Taurine protects isolated hepatocytes against carbon tetrachloride, hydrazine and 1,4-naphthoquinone but not against allyl alcohol, α-naphthylisothiocyanate (ANIT) or diaminodiphenyl methane (DAPM) cytotoxicity.
8. The mechanisms of protection are unclear but may include modulation of calcium levels, osmoregulation and membrane stabilization.

Key Words: Taurine, cytoprotection, susceptibility, hepatotoxicity

INTRODUCTION
Taurine is a β-amino acid found in all tissues of most animal species (Fig. 1). It is not incorporated into proteins and is in fact the most abundant free amino acid in many tissues (Huxtable, 1992). Taurine is taken in via the diet in carnivores and omnivores, but is also synthesized from cysteine or methionine present in the diet (Fig. 1). The rate of synthesis varies within tissues and between species, with rats being relatively proficient compared to humans (Worden and Stipanuk, 1985), whereas cats are completely unable to synthesize taurine (Huxtable, 1992). There are various known and proposed roles for taurine. Conjugation of bile acids is a well known function, but this only accounts for a small proportion of the body pool of taurine in humans and other mammals. Proposed functions include removal of hypochlorous acid in tissues where oxidants are generated such as neutrophils and the retina, modulation of calcium levels, maintenance of osmolality and stabilization of membranes (Huxtable, 1992).

Although the liver is the primary site of taurine synthesis it is also the organ which shows the most variation in concentration (4–11 μmol/g wet weight in rat liver; Waterfield, 1994) and even within the liver, taurine levels vary between the zones of the lobule (Pentilla, 1990). This variation in the level of taurine in the liver may be an important determinant of susceptibility to toxic responses and is in contrast to the important protective agent glutathione (GSH), levels of which, although zonal, remain fairly constant in the liver. Taurine levels in heart (25–30 mM) and lung (11–17 mM) are higher than liver but the highest levels of taurine are found in the neutrophil where the cytosolic concentration is 50 mM (Green et al., 1991) and the retina (50–70 mM) (Sturman, 1993).

A factor contributing to variation in taurine levels between individuals is dietary supply of taurine and its precursors. Hence blood levels of taurine have been found to be low in humans receiving parenteral nutrition (Geggel et al., 1985). Synthesis is determined by availability of cysteine and methionine, which will be affected by various factors such as GSH synthesis and protein synthesis as well as by diet. One of the enzymes involved in the synthesis of
taurine, cysteine dioxygenase (Fig. 1), may be induced by sulphur amino acids present in diet leading to increased synthesis of taurine (Hosokawa et al., 1988).

There are other factors which may influence the tissue levels of taurine. For example we have recently shown that treatment of rats with β-agonist drugs (salbutamol and clenbuterol) will lower urinary and liver levels of taurine (Carvalho et al., 1994; Waterfield et al., 1994a). In humans also salbutamol treatment lowers urinary taurine levels (Waterfield et al., 1994b; Waterfield et al., unpublished observations) and plasma taurine levels are reduced in patients receiving chemotherapy (Desai et al., 1992). Gut flora may also be a factor in modulating taurine levels as there is enterohepatic circulation of taurine via conjugation of bile salts, cleavage of conjugates in the gut and possible metabolism of released taurine by gut bacteria. The bacterial degradation of taurine is believed to contribute to low taurine levels in cats (Hickman et al., 1992).

Studies in various species in vivo have shown that taurine is essential in certain aspects of mammalian development and that low levels of taurine...
are associated with various pathological lesions, especially if deficiency occurs during development (Sturman, 1993). Thus taurine deficiency in cats and hamsters leads to cardiomyopathy and in kittens, retinal degeneration and growth retardation (Sturman, 1990, 1993). The myocardial failure is reversed by dietary supplementation with taurine and taurine has also been shown to be effective in the treatment of congestive heart failure in humans (Azuma et al., 1983).

The administration of taurine has been demonstrated to provide protection both in vitro and in vivo in various tissues and organs. Our recent studies have been directed at protective effects in the liver.

**IN VITRO PROTECTIVE EFFECTS**

Studies in human lymphoblastoid cells demonstrated that taurine will protect against retinol and retinoic acid induced damage when added to the medium (5–20 mM) (Pasantes-Morales et al., 1984). Thus taurine (20 mM) reduced the toxicity measured as loss of viability, whereas other amino acids including β-alanine and glycine had less effect; cysteine reduced viability. Taurine in combination with zinc reduced cytotoxicity and also cell swelling to a greater extent (Pasantes-Morales et al., 1984). When lymphoblastoid cells were exposed to an Fe/ascorbate mixture, taurine (1–10 mM) protected against the toxicity, with complete protection at the optimum concentration of 5 mM, but it did not decrease lipid peroxidation in this system. It was suggested that ion accumulation may be affected and indeed it was shown that taurine (5 mM) decreased the accumulation of calcium in the cells exposed to the Fe/ascorbate system (Pasantes-Morales et al., 1985). Apart from hypotaurine, the immediate metabolic precursor of taurine, other similar compounds such as the amino acids glycine and β-alanine showed only a slight protective effect and cysteine was ineffective. In another in vitro system, red blood cells were protected against both osmotic shock and oxidant induced haemolysis by taurine (Koyama et al., 1992).

Due to its ability to react with and thereby remove hypochlorous acid (a potent oxidising agent) to form a relatively stable taurochloramine, taurine attenuates the DNA damage caused by aromatic amino compounds in vitro (Kozumbo et al., 1992). Taurine is also believed to protect neutrophils against the toxic effects of the hypochlorous acid itself generated in situ (Wright et al., 1985) and will also decrease the oxidation of haemoglobin in vitro by neutrophil activity (Grisham et al., 1984). High levels of taurine and hypotaurine are also found in parts of the male reproductive system where it is suggested that hypotaurine acts as an antioxidant being oxidized to taurine in the process (Holmes et al., 1992).

Using isolated rat alveolar macrophages, Banks et al., (1990, 1992) showed that preincubation in taurine (0–500 μM) significantly decreased the toxicity and ameliorated the biochemical effects of ozone exposure including lipid peroxidation, loss of ATPase and leakage of glutathione.

Studies in hepatocytes in vitro have demonstrated that taurine can exert a protective effect by decreasing lipid peroxidation due to hyperoxygenation, thus preventing cell death (Nakashima et al., 1990).

We have shown recently that taurine protects against the cytotoxicity of some, but not all hepatotoxic compounds evaluated in isolated rat hepatocytes (Waterfield et al., 1993a; Waterfield and Timbrell, unpublished observations).

In our studies with isolated hepatocytes in suspension, taurine, added to the incubation buffer protected against the toxicity of three different toxicants: hydrazine, carbon tetrachloride and 1,4-naphthoquinone. The protection was dependent on the concentration of taurine (Fig. 2). Depletion of ATP was observed as a response to both hydrazine and 1,4-naphthoquinone exposure. However in the case of hydrazine, although taurine protected against...
cytotoxicity, measured by lactate dehydrogenase leakage and Trypan Blue uptake it did not protect against ATP depletion (Waterfield et al., 1993a) (Fig. 3). The protection afforded by taurine against the cytotoxicity of these three compounds was not complete in the case of 1,4-naphthoquinone even at the highest concentration of taurine used (15 mM). At the highest concentration of naphthoquinone (200 μM), there was no protection at all. A significant protective effect was, however, observed at a taurine concentration of 5 mM for 1,4-naphthoquinone (100 μM) for loss of viability and ATP depletion. With carbon tetrachloride and hydrazine a concentration of 10 mM taurine was necessary to show significant protection against loss of viability. In carbon tetrachloride exposed hepatocytes taurine concentration and leakage increased in response to the exposure (Waterfield et al., 1991).

However, taurine did not protect hepatocytes against 2-naphthylisothiocyanate (Seabra and Timbrell, 1994a) (Fig. 4) or allyl alcohol toxicity (Waterfield and Timbrell, unpublished observations) (Fig. 5). The metabolic precursor of taurine, cysteine sulphinate, did however protect against 2-naphthylisothiocyanate toxicity in hepatocytes depleted of taurine (Seabra and Timbrell, 1994a) although again taurine itself did not (Fig. 4). It has been shown that taurine is synthesized in isolated hepatocytes from precursors such as cysteine and cysteine sulphinate (Stipanuk et al., 1992) and also N-acetylcysteine and hypotaurine (Waterfield and Timbrell, unpublished observations). The protection afforded against allyl alcohol toxicity by N-acetylcysteine (Waterfield and
Timbrell, unpublished observations) is almost certainly due to an increased production of glutathione or N-acetylcysteine itself, rather than taurine, as the precursors cysteine sulphinate and hypotaurine, as well as taurine itself, were not protective (Waterfield and Timbrell, unpublished results). However, levels of taurine in freshly isolated hepatocytes vary widely (1-118 nmol/10^6 cells) (Waterfield, Seabra and Timbrell, unpublished observations) whereas levels of glutathione do not. This variation may be an important factor in the variability in susceptibility to cytotoxic compounds. The reason for the loss during isolation is not known at present.

**IN VIVO PROTECTIVE EFFECTS**

Various workers have shown that taurine has protective properties in vivo in several different organs, particularly the lungs, heart and liver.

For example Gordon et al. (1986) administered taurine prophylactically to hamsters in the drinking water (0.5% solution). They found this completely protected hamsters against inflammation and morphological changes in the lungs caused by exposure to nitrogen dioxide. The same group also observed that taurine administered in the drinking water (1%) prior to dosing reduced the pathological changes in the lungs of hamsters caused by administration of paraquat and bleomycin (Gordon et al., 1992).

Other workers have shown that taurine gives partial protection against pulmonary fibrosis caused by bleomycin in hamsters (Wang et al., 1991). The protection was greater when taurine and niacin were given together and various biochemical markers such as malondialdehyde, superoxide dismutase and calcium content were decreased indicating a reduced toxic effect (Giri and Wang, 1992). The authors speculated that an antioxidant effect and/or membrane stabilization were responsible.

Taurine has also been shown to be protective against cardiotoxicity. For example in studies by Ohta et al. (1986) and Azuma et al. (1987) taurine administered orally both prior to and during dosing with isoprenaline protected against damage in chick hearts. Thus both myocardial necrosis and biochemical changes such as ATP depletion, loss of creatine kinase activity and increasing calcium levels were ameliorated by taurine. Similarly taurine was protective in adriamycin induced cardiotoxicity and indicators of oxidative stress such as malondialdehyde and depletion of glutathione were normalized by taurine treatment (Azuma et al., 1987).

Apart from the lungs and heart, in vivo protective effects have also been reported in the liver. Thus Macdonald et al. (1985) found that galactosamine induced hepatic necrosis was decreased from 48 to 25% when taurine (1.5 mmol/kg) was administered 12 hr after dosing with galactosamine. The accumulation of calcium was only slightly decreased however. It was suggested that although taurine may not change total calcium content, it may alter the intracellular sequestration of the ion, in particular its uptake into mitochondria (Macdonald et al., 1985).

Nakashima et al. (1982) found that taurine administered, 12, 16 and 20 hr after dosing protected the liver against carbon tetrachloride induced lipid peroxidation as measured by malondialdehyde production and reduced calcium accumulation. Preincubation of liver microsomes in vitro with very high concentrations of taurine (70 mM) reduced lipid peroxidation due to carbon tetrachloride. However, pretreatment of rats with taurine actually increased lipid peroxidation after carbon tetrachloride administration (Nakashima et al., 1983). Waterfield and Timbrell (unpublished observations) have also observed that rats pretreated with taurine administered in the drinking water (3% for days prior to dosing) were more susceptible to carbon tetrachloride induced liver damage (Table 1). Conversely depletion of taurine by prior administration of β-alanine significantly increased the hepatotoxicity of carbon tetrachloride in male rats (Waterfield et al., 1993c).

Similarly depletion of liver taurine by prior pretreatment of rats with β-alanine increased the toxicity of both 2-naphthylisothiocyanate (ANIT) and diamino

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**Table 1. Serum biochemistry 48 hr after dosing rats with CCl4 (1 ml.kg^-1), with or without taurine pretreatment**

<table>
<thead>
<tr>
<th>Serum parameter</th>
<th>Control</th>
<th>CCl4</th>
<th>Taurine</th>
<th>CCl4 + Taurine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP IU.1^-1</td>
<td>552 ± 48</td>
<td>758 ± 94*</td>
<td>669 ± 87</td>
<td>816 ± 132</td>
</tr>
<tr>
<td>ALT IU.1^-1</td>
<td>86 ± 5</td>
<td>153 ± 21</td>
<td>83 ± 2</td>
<td>479 ± 141</td>
</tr>
<tr>
<td>AST IU.1^-1</td>
<td>103 ± 3</td>
<td>611 ± 97</td>
<td>130 ± 18</td>
<td>1290 ± 422</td>
</tr>
<tr>
<td>T-Pro g.l^-1</td>
<td>58 ± 0.9</td>
<td>56 ± 1.0</td>
<td>56 ± 0.4</td>
<td>56 ± 0.7</td>
</tr>
<tr>
<td>T-Bil μmol/l^-1</td>
<td>7.0 ± 1.0</td>
<td>5.8 ± 0.7</td>
<td>6.4 ± 1.0</td>
<td>6.8 ± 1.0</td>
</tr>
<tr>
<td>TRIG mmol/l^-1</td>
<td>1.24 ± 0.14</td>
<td>0.57 ± 0.04*</td>
<td>1.11 ± 0.14</td>
<td>0.85 ± 0.17</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, *P < 0.05, control compared with CCl4 alone; ALP (alkaline phosphatase); ALT (alanine transaminase); AST (aspartate transaminase); T-Pro (total protein); T-Bil (total bilirubin); TRIG (triglycerides).
diphenylmethane (DAPM) to the bile duct and surrounding hepatic parenchyma (Seabra and Timbrell, 1993, 1994b) (Fig. 6). Despite these results pretreatment of rats with taurine administered in the drinking water did not offer any significant protection against the bile duct toxicity of either ANIT or DAPM as indicated by serum bilirubin and γ-glutamyl transferase levels (Fig. 6). However, serum enzyme indicators of parenchymal cell damage, (aspartate transaminase and alanine transaminase) were reduced in the taurine treated group (Seabra and Timbrell, 1993 and unpublished observations) (Fig. 6). These data therefore suggest that the level of taurine in the liver may be critical with perhaps a threshold level for protection as is the case with glutathione. Thus if the taurine level is below a threshold, susceptibility will be increased. In support of this concept of a critical threshold level, we have found a highly significant correlation between extent of liver damage in rats due to various hepatotoxic chemicals and predicted liver taurine concentration estimated from the urinary taurine prior to dosing (Waterfield et al., 1993a) (Figs 7 and 8). This observation has important implications for susceptibility to hepatotoxicity both in animals and humans. However, it seems that raising the liver level by prior administration of taurine may not necessarily decrease and may even increase toxicity depending on the compound and its site of action. There is currently no explanation for this observation.

Taurine has a well established role in bile acid conjugation and the availability of amino acids such as taurine and glycine controls the rate of bile flow; taurine conjugates tend to increase the rate. Dorvil et al. (1983) observed that pretreatment of guinea pigs with taurine protected against the hepatotoxicity of sulpholithocholate, a cholestatic agent which reduces bile flow in rats. The taurine pretreatment resulted in normal bile flow and prevented histopathological damage after sulpholithocholate was administered. Similarly pretreatment of rats with taurine increased bile flow after dosing with diaminodiphenylmethane (DAPM) when compared with non-pretreated dosed animals (Seabra and Timbrell, unpublished observations).

As well as protecting against the pathological damage caused by toxic compounds in lung, heart and liver, taurine has also been reported to modulate the biochemical effects of compounds. In rats taurine and hypotaurine have been found to influence blood glucose and insulin levels (Kulakowski and Matura, 1984) and increase glycogen synthesis. Pretreatment of mice with taurine protected against streptozotocin induced hypoglycaemia (Tokunaga et al., 1979). Cysteine and methionine were also protective.

Fig. 6. Effect of pretreatment of rats with β-alanine or taurine on the toxicity of α-naphthylisothiocyanate (ANIT) or diamino diphenylmethane (DAPM). Markers of toxicity: (a) serum alanine transaminase (ALT); (b) serum aspartate transaminase (AST); (c) serum bilirubin; (d) serum γ-glutamyl transferase (γ-GT). Control rats ○; β-alanine pretreated ■; taurine pretreated □. Doses used were ANIT: 100 mg/kg; DAPM: 100 mg/kg. Results are means ± SEM, n = 4–12 (ANIT); n = 4–8 (DAPM). * P < 0.05; ** P < 0.01; *** P < 0.001, significant difference from control using Dunnetts t-test. (a) P < 0.05; (b) P < 0.01; (c) P < 0.001, significant difference from ANIT or DAPM treatment alone using Duncans t-test. Data from Seabra and Timbrell, 1993, 1994b.
However, all three treatments were found to raise the level of taurine in the pancreas. Taurine was observed to decrease hyperglycaemia, suppress the fall in insulin in the pancreas and reduce the morphological changes (necrosis, coagulation of cytoplasm, loss of nuclei in β-cells) in the pancreas caused by streptozocin. It was suggested that taurine plays a role in the function and integrity of pancreatic β-cells.

All these data and other observations not cited suggest that taurine has various protective effects in vivo.

MECHANISMS OF PROTECTION

Currently the mechanism(s) underlying the various protective effects of taurine are not known with certainty but various hypotheses have been made. Proposed mechanisms include the modulation of Ca²⁺ levels (Azuma et al., 1987; Schaffer and Azuma, 1992), membrane stabilization and maintenance of osmolarity. Study of these mechanisms has been carried out in various different cell and tissue types. Therefore the mechanism may only be relevant to that particular system. For example, the heart has been studied extensively. Thus in the heart taurine protects against myocardial injury in several models of Ca²⁺ overload induced heart failure including that induced by drugs and hypoxia. Thus taurine may protect cells including myocardial cells by modulating calcium levels, possibly modulating the handling of calcium by the sarcolemma and mitochondria. Taurine treatment will certainly reduce myocardial calcium content in the cardiomyopathic hamster and this treatment leads to protection against myocardial injury (Schaffer and Azuma, 1992). However, taurine also modulates phospholipid metabolism in two ways. One is through the phosphoinositide pathway, which might influence the release of calcium from intracellular stores via inositol trisphosphate (IP₃). The other is the methylation of phosphatidylethanolamine. The phosphatidylethanolamine produced by this reaction may also influence calcium levels via Ca²⁺ transporters, notably the Na⁺-Ca²⁺ exchanger. The loss of this exchanger activity and associated loss of myocardial function are restored by taurine (Schaffer and Azuma, 1992).

Taurine is an osmotically active substance which may prevent intracellular volume changes resulting from alterations in plasma osmolality (Schaffer and Azuma, 1992). In myocardial ischaemia there is an increase in cellular water in the myocardium accompanied by a loss of taurine from myocytes. Taurine will also reduce cell and tissue swelling as a result of exposure to toxic compounds. This may be related to its ability to modulate calcium levels, such as decreasing calcium release from mitochondria and uptake into the cell. This in turn may be due to its effects on membranes. It has been postulated that taurine could interact with membrane proteins such as phospholipid methyltransferase in a similar way.
to phosphatidylethanolamine. This could lead to changes in membrane function by distortion of the membrane proteins and the lipid bilayer. Hence this type of alteration or others in cell membrane function such as in charge distribution could allow changes in the intracellular concentrations of ions such as calcium. The ability to modulate cellular ion concentrations and cell volume regulation may underlie the protective effects of taurine. The interactions with membranes however could also lead to stabilization as well as changes in permeability to or transport of particular ions. The protection against compounds such as streptozotocin could be via membrane stabilization. It was suggested that taurine binds to the membrane at or near the insulin receptor (Kulakowski and Maturo, 1984).

Our recent preliminary studies in hepatocytes have confirmed studies in other cell types that taurine is rapidly released from hepatocytes as a result of external changes in osmolarity (Waterfield and Timbrell, unpublished observations). We have also shown that taurine leaks from cells in response to exposure to toxic compounds but it is not currently clear whether this is simply leakage due to increased permeability of the membrane or a specific transport process for taurine (Waterfield et al., 1991).

Taurine is known to react with and detoxify hypochlorous acid generated by neutrophils from myeloperoxidase, hydrogen peroxide and chloride during the oxidative burst. This is a protective function which involves formation of stable taurochloramine and is believed to be the reason for the high levels of taurine (50 mM) found in neutrophils. It has been suggested that this process does not involve free hypochlorous acid but an enzyme intermediate. Hence provided that sufficient taurine is present the neutrophil is protected against hypochlorous acid (Marquez and Dunford, 1994). The removal of oxidants such as hypochlorous acid may reduce lipid peroxidation. However, the reduction of lipid peroxidation which has been reported could also be due to its ability to alter polyunsaturated fatty acids in the cell membrane. The ability of taurine to act as a direct antioxidant has been reported to be low however (Aruoma et al., 1988).

Taurine also has a protective role in the liver in the conjugation of foreign organic acids and bile acids (Huxtable, 1992). However, its protective role in the liver in relation to toxic compounds which are not conjugated is not yet understood. Similarly the mechanism underlying its protective role in the lung against oxidants is not clear but again may be due to its ability to remove hypochlorous acid (Sturman, 1993). Alternatively the specific uptake system for taurine in the lung (Lewis et al., 1990) may play a role in its protective action in this tissue.

**SUMMARY AND CONCLUSIONS**

Taurine quite clearly has a number of protective functions which have been observed both *in vitro* and *in vivo*. These protective functions have been demonstrated in a variety of cell types and organs from different mammalian species. However, the mechanism underlying these protective effects is not clear. There could be several mechanisms or the underlying basis may be linked to one crucial event such as maintaining calcium homeostasis. It seems likely, however that taurine is acting in several ways, by reacting with hypochlorous acid, by modulating calcium levels and by osmoregulation. Much further work is required in order to determine the bases for the protective effects of taurine and its importance in susceptibility to toxicity.

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