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INVESTIGATION OF THE MECHANISMS INVOLVED IN CYLINDROSPERMOPSIN TOXICITY: HEPATOCYTE CULTURE AND RETICULOCYTE LYSATE STUDIES.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The aim of this study was to determine the extent to which protein synthesis inhibition, lowered glutathione (GSH) levels and toxin metabolism contribute to the toxicity of cylindrospermopsin. Both hepatocyte cultures and reticulocyte lysates were utilized as *in vitro* tools of investigation.

Cylindrospermopsin was purified from *Cylindrospermopsis raciborskii* extracts by high performance liquid chromatography (HPLC). The toxin (93% purity) was identified by UV absorbance maximum at 262 nm and mass spectral analysis of the M + H ion (416 *m/z*) by HPLC-MS/MS (HPLC coupled to tandem mass spectrometry).

Cylindrospermopsin (IC_{50} =120 nM) was three times more potent than cycloheximide (IC_{50} = 368 nM) for the inhibition of protein synthesis in reticulocyte lysates. Cylindrospermopsin was effective immediately upon addition to lysates, arresting the elongation stage of translation. The potency and nature of inhibition was reproduced in hepatocyte culture (IC_{50} ~ 200 nM) and could not be reversed, displaying behaviour similar to that of the irreversible inhibitor emetine.

In cultured hepatocytes 1-5 μM cylindrospermopsin caused significant cytotoxicity (52 – 82% cell death) after 18 hr incubation. GSH levels were extensively depleted by all toxic concentrations, to 14% and 6% of controls for 1 and 5 μM cylindrospermopsin respectively at 18 hr. Such GSH depletion preceded the loss of cell viability. Although the antioxidant capacity of the cells was compromised by the depletion of GSH, further investigation did not reveal a role for oxidative damage in the toxicity process. The lipid peroxidation product malondialdehyde (MDA) did not increase above controls in cylindrospermopsin treated cells and inhibition of glutathione reductase (GSSG-Rd) activity with 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) did not alter the toxicity of cylindrospermopsin.

Protein synthesis inhibition was not correlated to cytotoxicity in hepatocytes. Furthermore, inhibition of cytochrome P450 (CYP450) activity with proadifen or

ketoconazole alleviated the toxicity of cylindrospermopsin, but not the effects on protein synthesis.

These findings imply that the inhibition of protein synthesis by direct action of the toxin cannot be considered a primary cause of hepatocyte cell death over an acute time frame. CYP450-derived metabolites may play a crucial role in cytotoxicity, and the toxicity process does not appear to involve oxidative damage.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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PUBLICATIONS

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Froscio, S.M., Humpage, A.R., Burcham, P.C. and Falconer, I.R. (2002). Dissociation of Cylindrospermopsin-induced Protein Synthesis Inhibition from Acute Toxicity in Mouse Hepatocytes. In preparation.

Other Work Published During This Candidature Not Reported in This Thesis

Humpage A.R. Hardy S.J. Moore E.J. Froscio S.M. and Falconer I.R. (2000) Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. *Journal of Toxicology and Environmental Health* Part A. 61(3):155-165.

Falconer, I.R., Hardy, S.J., Humpage, A.R., Froscio, S.M., Tozer, G.J. and Hawkins, P.R. (1999). Hepatic and Renal Toxicity of the Blue-Green Alga (Cyanobacterium) *Cylindrospermposis raciborskii* in Male Swiss Albino Mice. *Environmental Toxicology*. 14, 143-150.

ABBREVIATIONS

ANOVA	analysis of variance
ATP	adenosine triphosphate
BCNU	1,3-bis (chloroethyl)-1-nitrosourea
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CHEX	cycloheximide
CI	confidence interval
cm	centimeter
CYN	cylindrospermopsin
deoxy-CYN	deoxycylindrospermopsin
7-*epi*-CYN	7-*epi*-cylindrospermopsin
CYP450	cytochrome P450
DEM	diethylmaleate
DMEM/F12	1:1 mix of Dulbecco's modified Eagle's medium and Ham's F12 nutrient mixture
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
ε	extinction coefficient
EGTA	ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
g	gram or gravity
γ-GCS	γ-glutamylcysteine synthetase
GSH	reduced glutathione
GSH-Px	glutathione peroxidase
GSSG	oxidized glutathione
GSSG-Rd	GSSG reductase
GSTase	glutathione-S-transferase
HBBS	Hank's buffered salts solution
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
4-HNE	4-hydroxynonenal
HPLC	high performance liquid chromatography
HPLC-MS/MS	HPLC coupled to tandem mass spectrometry

hr	hour(s)
i.p.	intraperitoneal
IC_{50}	inhibitory concentration, 50%.
kg	kilogram
L	litre
LD_{50}	dose likely to kill 50% test animals
LDH	lactate dehydrogenase
LPS	lipopolysacharride
M	Molar
µM	micromoles per litre
MDA	malondialdehyde
4-MP	4-methyl pyrazole
mg	milligram
min	minute(s)
ml	milliliter
mm	millimeter
mRNA	messenger RNA
MSDS	material safety data sheet
MQ H_2O	Milli Q water
m / z	mass to charge ratio
N	normal
NAPQI	*N*-acetyl-*p*-benzoquinone imine
ng	nanogram
nm	nanometer
nM	nanomoles per liter
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PCA	perchloric acid
PCV	pyrocatechol violet
PDA	photodioarray
PPG	RNA ribonucleic acid
rRNA	ribosomal RNA
s	second(s)
SE	standard error
t-BH	*tert*-butyl hydroperoxide
TCA	trichloroacetic acid
TMOP	1,1,3,3-tetramethoxypropane

U	units
U/L	units per litre
UV	ultraviolet
VIS	visible

1 GENERAL INTRODUCTION

Adverse human health effects associated with exposure to cyanobacterial toxins are of increasing concern. Widespread reports of cyanobacterial blooms and recognition of toxins in water supplies have prompted toxicological investigations to understand the risks associated with each toxin. Cylindrospermopsin is emerging as threat in many areas of the world. This thesis investigates the mechanisms by which cylindrospermopsin induces toxicity. The Introduction provides a background to cyanobacteria, their toxins and their impact on human health. The existing knowledge of cylindrospermopsin toxicology is discussed and the current areas of investigation are detailed.

1.1 Cyanobacteria, Bloom Formation and Toxin Production

1.1.1 Biology of Cyanobacteria

The cyanobacteria comprise a large group of prokaryotic photoautotrophs (Campbell, 1990) which were originally, and still commonly referred to, as blue-green algae. They exhibit a combination of properties found in algae and bacteria. The cellular organization of cyanobacteria is prokaryotic, characterized by the lack of membrane bound organelles. Their principle mechanism of energy metabolism is photosynthesis, and the mechanism of photosynthesis employed (utilization of chlorophyll-*a* and liberation of oxygen as a byproduct) is plant-like and differentiates cyanobacteria from other phototrophic bacteria.

The cyanobacteria are a common component of aquatic ecosystems, widely distributed in freshwater and marine environments. Environmental adaptations aid in their survival. Many species of cyanobacteria possess gas vesicles that enable buoyancy regulation, providing an advantage over other phytoplankton. Environmental stimuli affect cell density allowing cyanobacteria to adjust their vertical position in the water column and find a niche

suitable for their survival and growth (Mur *et al.*, 1999). In addition, some cyanobacteria have the ability to fix molecular nitrogen providing them with a competitive advantage under nitrogen limiting conditions. Nitrogen-fixing heterocysts are commonly produced by filamentous cyanobacterial species (Fay, 1983). The heterocyst reduces atmospheric nitrogen and provides it to all the cells in the filament, leaving the photosynthetic cells the task of acquiring carbon (Wolk *et al.*, 1994). In addition, some non-heterocystous cyanobacteria also possess the ability to fix nitrogen.

Since the physiological properties of cyanobacteria vary between different species, the occurrence and dominance of cyanobacterial species in a particular water body is dependent upon the environmental conditions. A sequence of dominant species may be seen in a single water body during an annual cycle (Mur *et al.*, 1999).

The cyanobacteria are known to produce a variety of secondary metabolites, the function of which is not clear, although it has been suggested that they have a protective effect functioning as allelochemicals (Carmichael, 1992). Interest in these metabolites has risen since many have been found to be biologically active, in both a beneficial (antibiotics and antitumour compounds) and detrimental (toxins) manner for humans.

1.1.2 Cyanobacterial Blooms

Domination of a waterbody with a single (or a few) cyanobacterial species is commonly termed a cyanobacterial bloom and refers to both scum-forming species and those that are dispersed in the upper water layer. In addition colonies of benthic cyanobacteria can grow to form large mats on the sediments. Conditions that influence the formation of a bloom include nutrient availability, water temperature, light intensity and water turbidity and flow (Mur *et al.*, 1999). In Australia, documented accounts of scums or discoloured water consistent with cyanobacterial blooms refer back to the mid 1800s (Codd *et al.*, 1994). In the present day, cyanobacterial blooms are becoming more frequently reported. Eutrophication of waterways through agricultural run-off, sewerage and erosion is considered the likely explanation for the increasing numbers of blooms. It is however

recognized that a combination of favourable environmental factors (mentioned above) are required for bloom formation (Steinberg and Hartmann, 1988) and the presence of cyanobacteria does not necessarily indicate eutrophic conditions.

1.1.3 Toxin Production

Survey data compiled by Sivonen and Jones (1999) from around the world, (26 references from 18 countries) reports the frequency of toxin occurrence in freshwater blooms as 59%. While this estimate has some countries under-represented, it signifies the global nature of the problem and extent to which toxins are produced.

Laboratory studies have shown that environmental factors (temperature, nutrients, light intensity) can influence toxin production three to four fold (Sivonen and Jones, 1999) but do not explain larger variations in toxin production by different strains of the same species grown under identical culture conditions. Research is actively involved in the identification of the genes and enzymes involved in cyanotoxin production (Dittman *et al.*, 1997; Schembri *et al.*, 2001; Wilson *et al.*, 2000) and understanding their regulation (Kaebernick *et al.*, 2000; Kaebernick and Neilan, 2001)

1.2 Cyanobacterial Toxins

Descriptions of the different types of cyanotoxins, their structures and characteristics of their toxicity and occurrence, has been extensively reviewed and constantly updated with current research (Carmichael, 1988; Carmichael, 1992; Codd and Poon, 1988; Falconer, 1998; Kuiper-Goodman *et al.*, 1999; Ressom *et al.*, 1994; Sivonen and Jones, 1999). Many common genera of cyanobacteria produce toxins including *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis, Microcystis, Nodularia* and *Oscillatoria* (*Planktothrix*). Examples of some of the most commonly produced toxins are provided here grouped on the basis of the type of injury caused; hepatotoxins, neurotoxins and cytotoxins. The lipopolysaccharides or

endotoxins are considered separately since they are common to all cyanobacteria and may have important implications for exposure to cyanobacterial extracts.

1.2.1 Hepatotoxins

The hepatotoxins have been the most extensively researched cyanotoxins. Their primary site of action is the liver, where they cause structural and hepatocyte damage.

Microcystins

Microcystins were first isolated from *Microcystis aeruginosa* and since then have been isolated from other species of *Microcystis* in addition to other genera of cyanobacteria including *Anabaena, Nostoc, Oscillatoria* (*Planktothrix*) and *Anabaenopsis*. The general structure for the microcystins is shown in Figure 1.1. More than 60 structurally related microcystins have been identified (molecular weights ranging from ~800-1100 Daltons), which differ primarily in 2 L-amino acids and methylation/demethylation of the MeAsp and Mdha groups (Dawson, 1998). The LD_{50} for the common analogue microcystin LR = 50 μg kg^{-1} (i.p. mouse). Other analogues vary in toxicity. Sivonen and Jones (1999) provide a review of toxicities reported in the literature for different microcystins.

Mice poisoned with microcystin show symptoms as soon as 15 min after intraperitoneal injection and morphological changes to the liver are apparent in 30 min (Falconer *et al.*, 1981). After poisoning of large animals, the symptoms shown include weakness, variable heart rate, muscle twitchings and heavy breathing. Internal damage manifests as a swollen mottled liver, edema of the body cavities and widespread small hemorrhages (Jackson *et al.*, 1984). Microcystins are not directly cell permeant due to their hydrophilic nature, thus their specificity for the liver is due to their requirement for cellular uptake by a mechanism similar to the bile acid transport system (Eriksson *et al.*, 1990a; Falconer *et al.*, 1992; Runnegar *et al.*, 1981; Runnegar and Falconer, 1986). Hepatotoxicity results from the inhibition of protein phosphatases type 1 and type 2A which are important in regulation of the cell

structure (Eriksson *et al.*, 1990b). Inhibition of protein phosphatases type 1 and 2A also provides a molecular basis for tumour promotion due to their involvement in the regulation of cell metabolism (Cohen and Cohen, 1989).

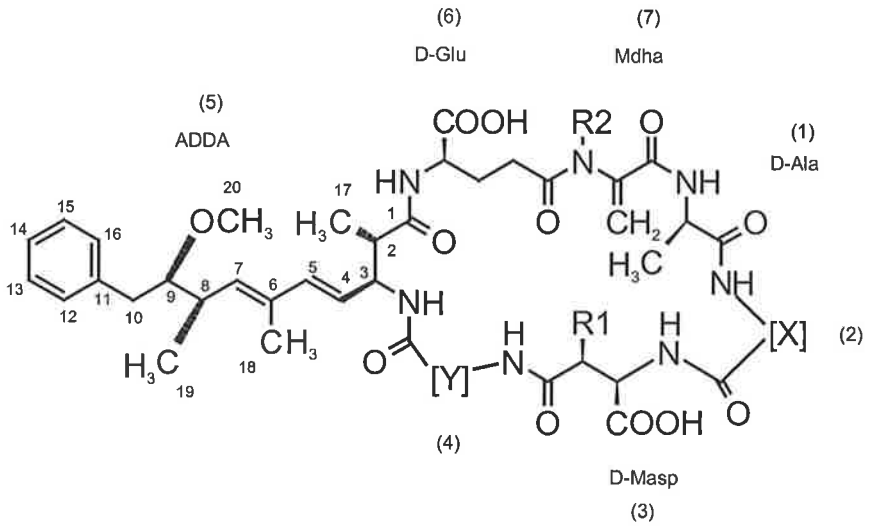


Figure 1.1 General structure for the microcystins. The variable amino acids are represented by X and Y. (in Microcystin-LR, X = L-Leucine (L) and Y=L-Arginine (R)).

Nodularin

Nodularin produced by *Nodularia spumigena* is structurally similar to microcystin (see Figure 1.2). Its molecular weight is 824 Daltons. It has a LD_{50} of 60 μg kg^{-1} (i.p., mice) very similar to microcystin and has also been shown to be an inhibitor of protein phosphatases 1 and 2A (Ressom *et al.*, 1994).

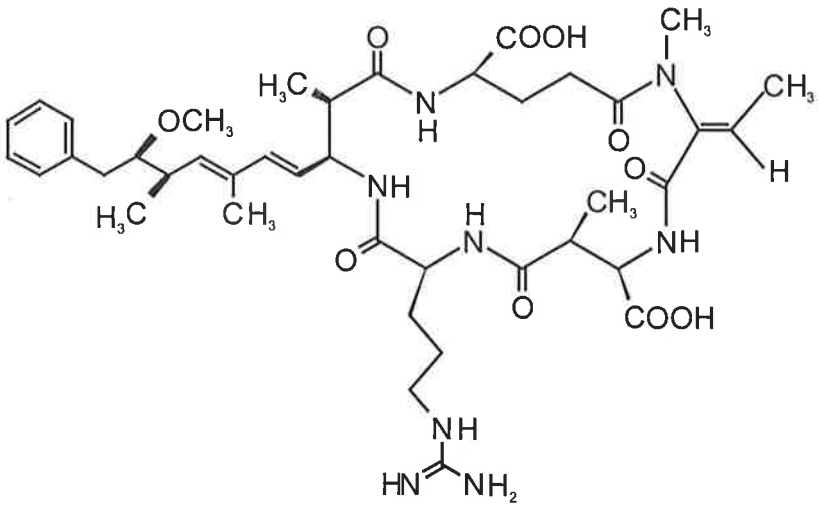


Figure 1.2 Structure of nodularin.

1.2.2 Neurotoxins

Anatoxin-a and Anatoxin-a(s)

The neurotoxins are the most rapidly acting cyanobacterial toxins. The first neurotoxin characterised was anatoxin-a from *Anabaena flos-aquae* (Devlin *et al.*, 1977). The structure of this alkaloid toxin (molecular weight 165 Daltons) is shown in Figure 1.3. The LD_{50} is 200 μg kg^{-1} (i.p., mouse) (Carmichael *et al.*, 1990). The toxin is a neuromuscular blocking agent. It is a nicotinic (cholinergic) agonist that binds to acetylcholine receptors. Mice poisoned with a lethal dose show symptoms of laboured breathing within a few (2-3) min. This is followed by progressive limb paralysis, abdominal breathing, convulsions and death due to respiratory arrest within 15 min (Skulberg *et al.*, 1992). Most reports of cyanobacteria containing anatoxin-a are associated with waterblooms of *Anabaena flos-aquae* or other related *Anabaena* species, but the toxin can also be produced by different genera of cyanobacteria including *Aphanizomenon*, *Oscillatoria* (*Planktothrix*) and *Cylindrospermum* (Sivonen *et al.*, 1989).

Also isolated from *Anabaena* is the neurotoxin anatoxin-a(s), so called due to the excessive salivation observed upon administration to laboratory mice. It is structurally unrelated to anatoxin-a (see Figure 1.4) and has a molecular weight of 252 Daltons. It is 10 times more lethal than anatoxin-a which equates to an LD_{50} of 20 μg kg^{-1} (i.p., mouse) (Carmichael, 1992). At the LD_{50} the typical post dose survival time for mice is 10 - 30 min. It is a potent inhibitor of acetylcholinesterase (Mahmood and Carmichael, 1986; Mahmood and Carmichael, 1987). Symptoms of poisoning with anatoxin-a(s) include marked salivation, muscular weakness, convulsions and death due to respiratory failure (Mahmood *et al.*, 1988).

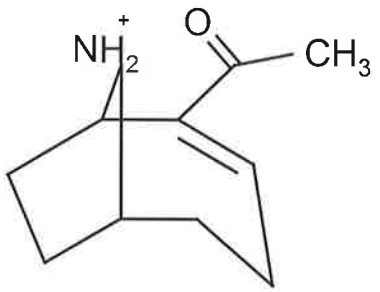


Figure 1.3 Structure of anatoxin-a.

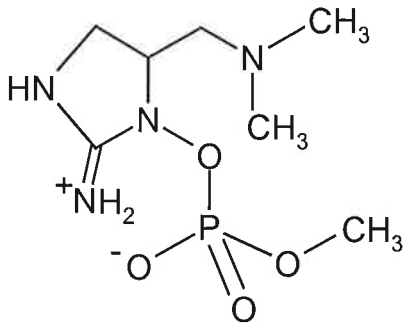


Figure 1.4 Structure of anatoxin-a(s).

Saxitoxins

The saxitoxins are another group of alkaloid neurotoxins. They were first described in cyanobacteria as aphantoxins and subsequently shown to be identical to the paralytic shellfish poisons (PSPs) produced by marine dinoflagellate species (Alam *et al.*, 1978; Ikawa *et al.*, 1982). The PSPs have long been associated with dinoflagellate 'red tides'. These toxins can be accumulated in the tissues of mussels, clams and oysters which are consumed by humans. The saxitoxins include a range (at least 19 have been reported) of hydroxylated and sulfated analogues of the parent compound shown in Figure 1.5. They are fast acting neurotoxins that inhibit nerve conduction by blocking sodium channels. The LD_{50} of saxitoxin is 10 $\mu g\ kg^{-1}$ (i.p. mouse). The toxicity of related analogues varies (Humpage *et al.*, 1994). Saxitoxins have been identified in the cyanobacterial species *Aphanizomenon flos-aquae*, *Anabaena circinalis*, *Lyngbya wollei* and *Cylindrospermopsis raciborskii* (Sivonen and Jones, 1999).

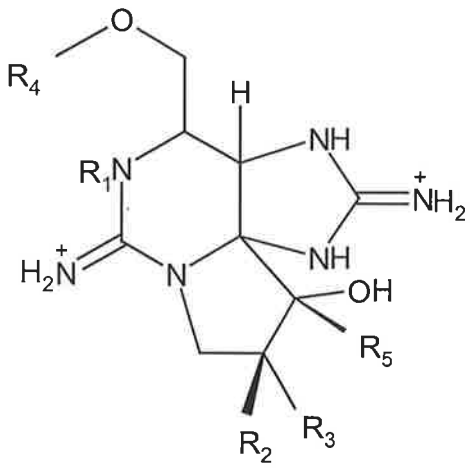


Figure 1.5 General structure of the saxitoxins. The variable chemical groups are indicated. R_1 = H or OH, R_2 = H or SO_3^-, R4 = H, $CONH_2$ or $CONHSO_3^-$ and R5 = H or OH.

1.2.3 Cytotoxins

Cylindrospermopsin

The alkaloid toxin cylindrospermopsin (Figure 1.6) consists of a tricyclic guanidine group attached to a hydroxymethyl uracil moiety. Its molecular weight is 415 Daltons. The LD_{50} is 2100 μg kg^{-1} at 24 hr and 200 μg kg^{-1} at 5-6 days (i.p., mice) (Ohtani *et al.*, 1992). It was first isolated from *Cylindrospermopsis raciborskii* (Ohtani *et al.*, 1992) and subsequently identified in the cyanobacterial species *Umezakia natans* (Terao *et al.*, 1994) and *Aphanizomenon ovalisporum* (Banker *et al.*, 1997; Shaw *et al.*, 1999). Although it is primarily hepatotoxic, non specific toxicity is observed in a range of other organs including the kidneys, gastrointestinal tract, lungs and thymus. Unlike the microcystins, cylindrospermopsin does not inhibit protein phosphatases types 1, 2A and 3 (Ohtani, 1992). While cylindrospermopsin has been demonstrated to be a potent inhibitor of protein synthesis (Terao *et al.*, 1994), the mechanism(s) of toxicity are still being elucidated and are discussed in detail later in this Chapter.

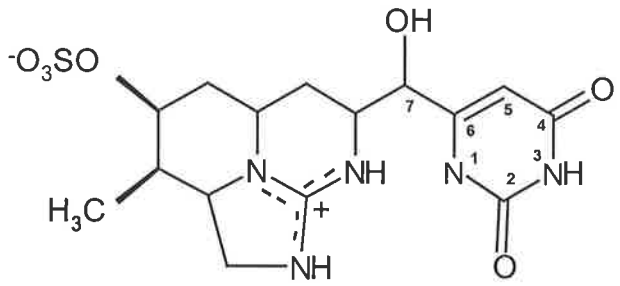


Figure 1.6 Structure of cylindrospermopsin.

To date only one analog of cylindrospermopsin has been isolated from cyanobacteria, deoxycylindrospermopsin, and this has been determined to be non-toxic (Norris *et al.*, 1999). For deoxycylindrospermopsin the hydroxyl group is lost from the uracil bridge (position C-7 of Figure 1.6). One isomer, 7-epicylindrospermopsin has been identified. It is the C-7 epimer of cylindrospermopsin (Banker *et al.*, 2000) and has been demonstrated to retain toxicity (Banker *et al.*, 2001).

1.2.4 Lipopolysaccharide Endotoxins

The lipopolysaccharides (LPS) or endotoxins are regarded as irritant toxins. Distinct from the metabolite toxins detailed above, the LPS are an integral part of the cell wall. Common to all Gram negative bacteria, which include cyanobacteria, LPS can illicit allergic and toxic responses in animals and humans. Upon isolation cyanobacterial LPS have demonstrated lower biological activity than LPS from other Gram negative bacteria such as Salmonella (Keleti and Sykora, 1982; Raziuddin *et al.*, 1983).

1.3 Cyanobacterial Toxins and Human Health

Cyanobacterial toxins have been reported as the cause of illness or death for wildlife and livestock consuming contaminated water for over 100 years (Codd *et al.*, 1994; Ressom *et al.*, 1994). Evidence linking adverse human health effects to exposure to cyanobacterial toxins has emerged from epidemiological studies, largely in retrospect with subsequent toxicological investigation. The most likely routes of human exposure to cyanobacterial toxins are via contaminated recreational or drinking waters. In addition, one incident is recorded where inadequately treated water containing cyanobacterial toxins was used to treat renal dialysis patients with devastating effects.

1.3.1 Recreational Water Exposure

In the United Kingdom in 1989, 10 Army recruits who had been ordered to swim and "Eskimo roll" their canoes in a lake with a bloom of toxic *Microcytsis* presented with symptoms of intoxication - headache, vomiting, diarrhoea and blistered mouths (Turner *et al.*, 1990). The 2 most severely affected recruits developed pneumonia and required hospitalization.

Increased incidence of flu symptoms, skin rashes, eye and ear irritations have also been observed after recreational exposure to cyanobacteria. The results of this epidemiological study (852 participants) demonstrated the symptoms were correlated with increased duration of water contact and cyanobacterial cell density (Pilotto *et al.*, 1997). The symptoms were not correlated with the presence of hepatotoxins, and the authors suggest that they may be related to allergenicity of the cyanobacterial cells, although the contribution of hepatotoxins to allergic type reactions cannot be discounted.

1.3.2 Exposure via Drinking Water

Incidents of human exposure to toxins in drinking water have occurred sporadically throughout the world. The following two examples occurred in Australia.

In 1979, an outbreak of hepatoenteritis at Palm Island, (QLD, Australia) afflicted 150 people, mainly children, with most requiring hospitalization (Byth, 1980). The illness occurred a few days after treatment of the water supply reservoir with copper sulfate to disperse a dense bloom of cyanobacteria (Bourke *et al.*, 1983). All affected had been exposed to the contaminated water supply. A toxigenic strain of *Cylindrospermopsis raciborskii* was subsequently isolated from the water supply reservoir and implicated as the causative agent (Hawkins *et al.*, 1985). A detailed description of the "Palm Island Mystery Disease" is provided in Section 1.4 as it is linked to cylindrospermopsin exposure.

In the second incident, an extensive bloom of *Microcystis aeruginosa* occurred in the water supply of Armidale, NSW (Australia) in 1981. Many complaints were received about the unpleasant odour and taste to the water, and the bloom was dispersed with copper sulphate. Subsequent epidemiological investigations revealed a correlation between the termination of the *Microcystis* bloom in the water supply and elevated levels of the liver enzyme γ-glutamyl transferase in human plasma, providing evidence of subacute liver damage in the human population consuming the contaminated water (Falconer *et al.*, 1983).

In addition to acute effects, it has been suggested that chronic exposure to microcystins in drinking water can favour the growth of hepatocellular carcinomas (Yu, 1995). The hepatocellular carcinoma rate in China, one of the highest in the world, is associated with the prevalence of the hepatitis B virus and consumption of the dietary carcinogen aflatoxin. In addition, on a regional basis it has also been associated with the source of drinking water. Yu (1995) reported that the use of deep well water for drinking was associated with a lower cancer mortality rate compared to the those that used pond or ditch waters for drinking. These shallow surface waters have been found to contain microcystins, and due to their action as protein phosphatase inhibitors, and hence potential tumour promoting activity, have been proposed as being responsible for the increased incidence of hepatotocellular carcinoma. Tumour promotion by microcystins in drinking water has been demonstrated in experimental animals (Humpage *et al.*, 2000b).

1.3.3 Other routes of Exposure

In a disastrous event, a severe outbreak of hepatitis and the resultant death of 60 patients at a Brazilian haemodialysis center in 1996 was attributed to the presence of the cyanobacterial toxins in the dialysis water (Jochimsen *et al.*, 1998; Pouria *et al.*, 1998). Microcystins were found both in the liver and sera of afflicted patients. In a recent report, concentrations of cylindrospermopsin were found, some 10 times higher than microcystin, in the carbon filters obtained from the dialysis center (Carmichael *et al.*, 2001). The source was traced back to inadequately treated water from a reservoir prone to domination by cyanobacteria.

1.4 The Palm Island Mystery Disease Linked to Cylindrospermopsin Exposure

The Palm Island mystery disease occurred in 1979 as an illness afflicting 138 children and 10 adults of the Palm Island community. Most patients required hospitalisation. Symptoms first presented as a hepatitis-like illness with malaise, vomiting, enlarged and tender liver, headache, fever and constipation. Urine analysis revealed the presence of protein, sugars, ketones and sometimes blood. The later phase of the illness demonstrated symptoms of severe kidney dysfunction. One to three days following onset, 69% of the patients required intravenous therapy to correct an electrolyte imbalance, and in the most severe cases, for the reversal of hypovolemic and acidotic shock. Profuse diarrhoea accompanied the later phase. The illness lasted between 4 and 26 days and all recovered (Byth, 1980).

The original report by Byth (1980) revealed that at the time of the epidemic that there had been low rainfall, the water level in the Palm Island dam (Solomon Dam) was low and the reticulated water had an unpleasant taste and odour for 2 months prior to the illness. It was also reported that 1ppm copper sulphate was added to the dam for treatment of a dense algal bloom, and that five days after the addition of the copper sulphate, the first patient with the illness was seen (Bourke *et al.*, 1983). Epidemiological investigation revealed that households connected to the reticulated water supply from Solomon Dam were affected by the illness while others receiving alternative supplies were spared, indicating the water as the source of the illness. The suggestion of algal intoxication came from the realization that treatment of algal bloom on the Solomon Dam would have lysed the cyanobacterial cells and released any toxins into the water (Bourke *et al.*, 1983; Hawkins *et al.*, 1985). However, toxic cyanobacteria were not isolated at the time of the incident and this suggestion of algal intoxication was disputed on the basis that the symptoms of algal intoxication previously described in the literature could not explain all the features of the Palm Island illness. An alternative explanation of copper sulphate poisoning was proposed, as copper itself is a cumulative poison (Prociv, 1987; Prociv, 1992). The subsequent isolation of a toxic strain

of *Cylindrospermopsis raciborskii* from the water supply on Palm Island (Hawkins *et al.*, 1985), and the isolation of the toxin cylindrospermopsin (Ohtani, 1992) from this cyanobacterium revealed that the chemical nature of this toxin was different to other cyanobacterial toxins which had been described. Furthermore, toxicological studies with *C.raciborskii* and purified cylindrospermopsin support the theory that this toxin was capable of producing the diverse symptoms seen on Palm Island.

1.5 Cylindrospermopsin Toxicology

1.5.1 *In vivo* Studies

Acute Effects

Experimentally-induced poisoning of laboratory mice with *Cylindrospermopsis raciborskii* extract or purified cylindrospermopsin has revealed that the characteristics of the poisoning are hepatonecrosis with extrahepatic lesions of variable location and severity. Illustrating delayed toxicity, the LD_{50} of purified cylindrospermopsin (i.p., mice) is 2100 μg kg^{-1} at 24 hr, and 200 μg kg^{-1} at 5-6 days (Ohtani *et al.*, 1992). Prolonged effects were also seen after dosing mice i.p. with *C.raciborskii* extract although some discrepancy in toxicity based on the known cylindrospermopsin content was observed. The extract LD_{50} was equivalent to a cylindrospermopsin dose of 286 μg kg^{-1} at 24 hr and 176 μg kg^{-1} at 7 days (Hawkins *et al.*, 1997). Similar values for extract LD_{50} based on toxin content were reported by Falconer *et al.* (1999).

Damage to the liver is reported consistently in the literature as dose dependent, centrilobular necrosis at lower doses and more severe and generalised necrosis at higher doses (Hawkins *et al.*, 1985; Hawkins *et al.*, 1997; Falconer *et al.*, 1999). Characteristic vacuolation of the cytoplasm of hepatocytes, due to the accumulation of fat droplets has also been consistently described (Falconer *et al.*, 1999; Terao *et al.*, 1994). Damage to other organs has been variable. Apart from the liver, Hawkins *et al.* (1985; 1997) report damage to the kidneys, small intestine, and lungs of mice treated with cell extract, while Terao *et al.*

(1994) reported kidney, thymus and heart damage by purified cylindrospermopsin. The severity of the extrahepatic lesions is also variable. For example, kidney damage has been reported as mild, restricted to the proximal tubular epithelium (Hawkins *et al.*, 1997; Hawkins *et al.*, 1985). In contrast, Falconer *et al.* (1999) illustrate that kidney damage is a significant contributor to morbidity for some *C.raciborskii* extracts, causing proximal tubule necrosis, increase in the diameter of tubule lamina and deposition of proteinaceous material in the distal tubules.

In addition to dosing mice via intraperitoneal injection, oral dosing studies have been carried out to mimic the route of exposure through drinking water. Both Seawright *et al.* (1999) and Falconer *et al.* (1999) demonstrated that the characteristics of toxicity (fatty liver, hepatonecrosis, variable extrahepatic lesions) were also recorded after oral dosing with *C.raciborskii* extracts. Both studies indicated that the oral LD_{50} is at least 25-fold higher than that observed for intraperitoneal injection. Consistent with laboratory findings, examination of a calf poisoned after drinking water from a farm dam contaminated with a toxic *C.raciborskii* bloom showed typical symptoms of cylindrospermopsin intoxication. Histological examination of the liver revealed areas of extensive necrosis while damage to the heart, gall bladder and small intestine were recorded (Saker *et al.*, 1999).

Discrepancy between the toxicity observed with *C.raciborskii* extracts and that expected from the cylindrospermopsin content (see quoted LD_{50} for extract and purified toxin above), coupled with the observation that *C.raciborskii* extracts of the same strain, but grown at different times also produce variable results (Falconer *et al.*, 1999) suggests that more than one toxin may be produced by the cyanobacterium. As described in Section 1.2 numerous analogs of other toxins (microcystins and saxitoxins) are produced by cyanobacteria which vary in toxicity. To date the only other cylindrospermopsin analog isolated, deoxycylindrospermopsin, has been reported to be non-toxic (Norris *et al.*, 1999). The isomer 7-epicylindrospermopsin retains toxicity, although it has only been found as a minor component in cyanobacterial extracts in comparison to cylindrospermopsin (Banker *et al.*, 2000). Other suggestions proposed which may account for this variability include mouse sensitivities and synergistic effects from other cyanobacterial cell components (Hawkins *et al.*, 1997).

Supporting a role for metabolic activation of the toxin in the liver, suppression of cytochrome P450 activity *in vivo* with piperonyl butoxide has been demonstrated to increase the survival of Quackenbush mice dosed i.p. with 200 – 800 μg kg^{-1} cylindrospermopsin (Norris *et al.*, 2002). However, this protective effect was not observed in C57/B16 mice.

Repeated Administration

Increased susceptibility of mice to repeated oral doses of cylindrospermopsin in *C.raciborskii* extracts has been observed (Falconer and Humpage, 2001). Mortality occurred with two doses equivalent to 60% of minimum oral lethal dose despite a 2 week recovery period. This suggests the residual effects of the toxin may be more severe than expected from single dose toxicology. Consequently injury from chronic exposure to cylindrospermopsin may be an important consideration with this toxin. However, repeated dosing of mice over 14 days with low doses of cylindrospermopsin (1 μg $kg^{-1}day^{-1}$ (i.p.) or 10 μg $kg^{-1}day^{-1}$ (oral)) was reported to produce no adverse effects (Shaw *et al.*, 2000).

Genotoxicity

The occurrence of a DNA adduct in mouse liver has been tentatively reported after i.p. administration of cylindrospermopsin in *C.raciborskii* extracts (Shaw *et al.*, 2000). The chemical nature of the adduct and the mechanism(s) involved in its formation are yet to be described.

Carcinogenicity

A recent study explored the potential for cylindrospermopsin to initiate tumours. Mice were treated up to 3 times orally with *C.raciborskii* extract containing cylindrospermopsin followed by the tumour promoter TPA (O-tetrdecanoylphorbol 13-acetate). From 53 mice treated with extract, 5 of those displayed evidence of neoplastic processes (3 frank tumours) in contrast to none observed in the 27 controls (Falconer and Humpage, 2001). The number

of animals used in this trial was insufficient to provide statistical evidence of tumour initiation but indicates a potential risk for carcinogenesis and requires further study.

1.5.2 *In vitro* Studies

Cylindrospermopsin has been demonstrated to be a potent inhibitor of protein synthesis in a cell-free reticulocyte lysate (Terao *et al.*, 1994).

In primary hepatocyte culture the depletion of cellular glutathione (GSH) precedes the loss of cell viability (Runnegar *et al.*, 1994). Subsequent investigation revealed that this was due to the inhibition of GSH synthesis (Runnegar *et al.*, 1995b). Furthermore Runnegar *et al.* (1995b) also demonstrated that pretreatment of the hepatocytes with the cytochrome P450 (CYP450) inhibitor α-naphthoflavone could partially protect against the cylindrospermopsin-induced fall in GSH and toxicity. This indicates that CYP450-mediated modification of the toxin was likely and that the metabolites generated may be more toxic than the parent compound. The uptake of cylindrospermopsin into primary rat hepatocytes can be facilitated both by the bile acid transporter system and passive diffusion (Chong *et al.*, 2002).

Genotoxicity

The introduction of DNA strand breaks and whole chromosome loss in a human lymphoblastoid cell line after cylindrospermopsin exposure have been demonstrated by the cytokinesis-block micronucleus assay (Humpage *et al.*, 2000a). Cylindrospermopsin concentration-dependently increased the frequency of micronuclei in binucleated cells. Multimicronucleated cells were also induced. Fluorescent labelleling of centromeric DNA sequences indicated that many of the micronuclei induced by cylindrospermopsin contained at least one centromere, indicating whole chromosome loss. The results of this study suggest that cylindrospermopsin acts via two distinct mechanisms to induce the cytogenetic damage. Micronucleus production results from strand breaks that occur that at the level of the DNA,

while whole chromosome loss is likely to be due to disruption of kinetochore or spindle function.

1.6 Cylindrospermopsin Structure –Activity Relationship

The presence of the pyrimidine uracil, the guanidine and the sulphate group within the structure of cylindrospermopsin (Figure 1.6) provide several potentially reactive sites. Biological activity has been linked to the hydroxymethyl uracil, as the absence of the hydroxyl group on the uracil bridge results in loss of toxicity (Norris *et al.*, 1999). In addition, studies with chemically synthesized cylindrospermopsin and its derivatives demonstrated that the sulphate group was not necessary for biological activity or entry into primary hepatocytes (Xie *et al.*, 2000).

Modified uracil analogues are known to have affinity for nucleic acids when their structural orientation allows (Friedberg, 1985). This may provide an explanation for the interference of cylindrospermopsin with ribosomal protein synthesis, which is dependent upon the function of RNA. Potential incorporation or intercalation of the 6-hydroxylmethyluracil group of cylindrospermopsin into DNA could also provide a basis for DNA damage described in the micronucleus assay in Section 1.5.2.

1.7 Investigating the Mechanisms Involved in Cylindrospermopsin Toxicology

Although it is convenient to discuss the possible mechanisms involved in cylindrospermopsin toxicity as discrete entities, it is most likely that a number of pathways contribute to cell death. The relative importance of each of the known toxic events is discussed.

1.7.1 Protein Synthesis Inhibition

The protein synthetic system can be disrupted at a number of different levels: gene expression, transcription, mRNA processing, translation or through post-translational modifications (Hershey *et al.*, 1986). We can conclude from the work by Terao *et al.* (1994) in reticulocyte lysates (an exogenous mRNA-dependent system) that cylindrospermopsin interferes with the translation of mRNA into protein on the ribosomes. The authors also related this observation to ultrastructural damage caused to the protein synthesis machinery of the liver *in vivo*. Both cylindrospermopsin and the protein synthesis inhibitor cycloheximide were reported to cause ribosome dissociation from the endoplasmic reticulum. In addition, the fatty liver or steatosis consistently observed by many researchers as a response to cylindrospermopsin injury is consistent with impairment of the protein synthetic system. Triglycerides are accumulated as their transport out of the cell via lipoproteins is disrupted by a block in protein synthesis affecting lipoprotein production (Dianzani, 1991).

Clearly triglyceride accumulation in hepatocytes illustrates how cellular functions are impaired as a consequence of the inhibition of protein synthesis. Numerous enzymes and functional proteins would be similarly affected. It is well established that following protein synthesis inhibition, DNA synthesis and then RNA synthesis is inhibited (Olsnes and Pihil, 1997). Continued disturbance of the normal metabolic functions of the cell, leads to loss of

cellular viability. Consistent with a slow deterioration of cell function cylindrospermopsin toxicity is notably delayed. *In vivo*, mortality can occur within 24 hr at high doses. At lower doses, the effects of the toxin are extended to 5-6 days. Progressive changes in tissue injury have been observed over 4–144 hr (Falconer *et al.*, 1999). It is also noted that protein synthesis inhibition affects all cell types, providing a plausible explanation for the extrahepatic effects caused by cylindrospermopsin.

However, the extent to which inhibition of protein synthesis contributes to cylindrospermopsin toxicity, particularly hepatotoxicity, is not clear. Plaa (1991) lists a number of hepatotoxins including the protein synthesis inhibitors cycloheximide, puromycin and emetine which have been reported to produce fatty liver upon acute administration *in vivo* without inducing necrosis. Similarly, although Terao *et al.* (1994) describe the onset of fat accumulation followed by massive necrosis after *in vivo* administration of cylindrospermopsin to mice (5-6 day LD_{50} dose), cycloheximide treated mice (at a notably high dose) accumulated fat without observation of the necrotic phase. Furthermore, the fat accumulation was far more profuse in cylindrospermopsin treated mice, with fat droplets accumulating over time (2-4 days) then fusing together to form fat lakes and depressing space for cellular organelles. Terao *et al.* (1994) suggested that the pathogenesis of the fatty liver induced by cylindrospermopsin more closely resembled that produced by xenobiotics known to cause free radical mediated injury, such as carbon tetrachloride (CCl_4). CCl_4 is highly steatogenic and induces necrosis (Dianzani, 1991). It also impairs protein synthesis. However, its toxicity and triglyceride accumulation are due to the *in vivo* formation of a trichloromethyl radical which binds to cellular lipids and proteins (Recknegel *et al.*, 1989). The haloalkylation of apolipoproteins prevents their secretion, leading to a rapid onset of triglyceride accumulation. Thus the protein synthesis inhibition contributes to the triglyceride accumulation only in the latter stages. This illustrates how different mechanisms in addition to protein synthesis inhibition can coexist to contribute to fat accumulation.

When investigated *in vitro* it is found that cells survive for many hours after the cessation of protein synthesis. Treatment of primary hepatocytes with cycloheximide for 20 hr inhibits 90% of the protein synthesis and impairs the uptake of extracellular polyamines by 75% without any effect on cell viability (Martin *et al.*, 1990). The absence of cytotoxicity in hepatocytes exposed to ethionine for 20 hr has also been described

(Waterfield *et al.*, 1998). Ethionine, a methionine analogue is highly steatogenic *in vivo*. It is an alklyating agent and impairs protein synthesis secondary to ATP depletion and inhibition of RNA synthesis. In contrast, after exposure of hepatocytes to cylindrospermopsin, cytotoxicity develops within 16-18 hr (Runnegar *et al.*, 1994). The extent of protein synthesis inhibition in these cells was not measured.

Given that cylindrospermopsin toxicity at lower doses *in vivo* is delayed, the confirmation that it was a potent protein synthesis inhibitor provided some explanation for this delayed onset of toxicity. Undoubtedly the impairment of protein synthesis influences cylindrospermopsin toxicity. However it does not appear to fully explain both the extent of triglyceride accumulation and cell toxicity *in vivo*. For example, cytotoxicity observations *in vitro* reveal hepatocyte death earlier than could be expected from protein synthesis inhibition alone. It is also conceivable the mechanisms of toxicity differ over time, with continued protein synthesis inhibition becoming more significant over extended periods, while playing a less substantial role at earlier times.

1.7.2 Glutathione Depletion

Glutathione (GSH) is the most abundant low molecular weight thiol in mammalian cells (Fahey and Newton, 1983). It is recognized as the cell's major antioxidant defense mechanism, participating in redox reactions and thioether formation (Figure 1.7). GSH prevents the oxidation of –SH groups in proteins and participates in the detoxication of electrophilic intermediates such as epoxides, or peroxides and free radicals generated through xenobiotic metabolism (Moldeus and Jernstrom, 1983). In these reactions GSH can serve as a nucleophile forming conjugates catalyzed by GSH-*S*-transferase, as in the case for the hepatotoxin Aflatoxin B_1-GSH conjugation described in Section 1.7.3. Alternatively, in reactions catalyzed by GSH peroxidase it acts as a reductant, and is oxidized to its disulfide (GSSG). These reactions reduce toxic peroxides, produced either endogenously or from exogenous sources (Jones and Kennedy, 1983). For example, *tert*-butyl hydroperoxide (*t*-BH) is metabolized by GSH peroxidase in isolated hepatocytes, leading to a marked decrease in GSH content and concomitant increase in GSSG (Latour *et al.*, 1999).

Runnegar *et al.* (1994) first established that GSH depletion played a role in cylindrospermopsin toxicity in hepatocyte culture, demonstrating profound GSH depletion well prior to the onset of cell death, and illustrated that treatment with propargylglycine (PPG) to reduce cell GSH, further predisposed the cells to toxic effects. Further investigation revealed that the GSH depletion by cylindrospermopsin was due to a block in GSH synthesis, exhibiting effects similar to those produced by a known inhibitor, buthionine-sulfoximine (BSO) (Runnegar *et al.*, 1995b). There was no detection of increased utilization of GSH, as would be expected under severe oxidant stress. In addition, GSH conjugates were not detected.

In vivo, the 7 day survival rate of mice pretreated with BSO and diethylmaleate (DEM) to deplete hepatic GSH prior to dosing with 200 μg kg^{-1} of cylindrospermopsin was not significantly different from the control group (Norris *et al.*, 2002). It is worth noting that the survival rates after cylindrospermopsin treatment were 5/13 and 9/14 for the BSO/DEM pretreated and non-pretreated mice respectively. The effect of such preteatment on higher cylindrospermopsin doses was not tested.

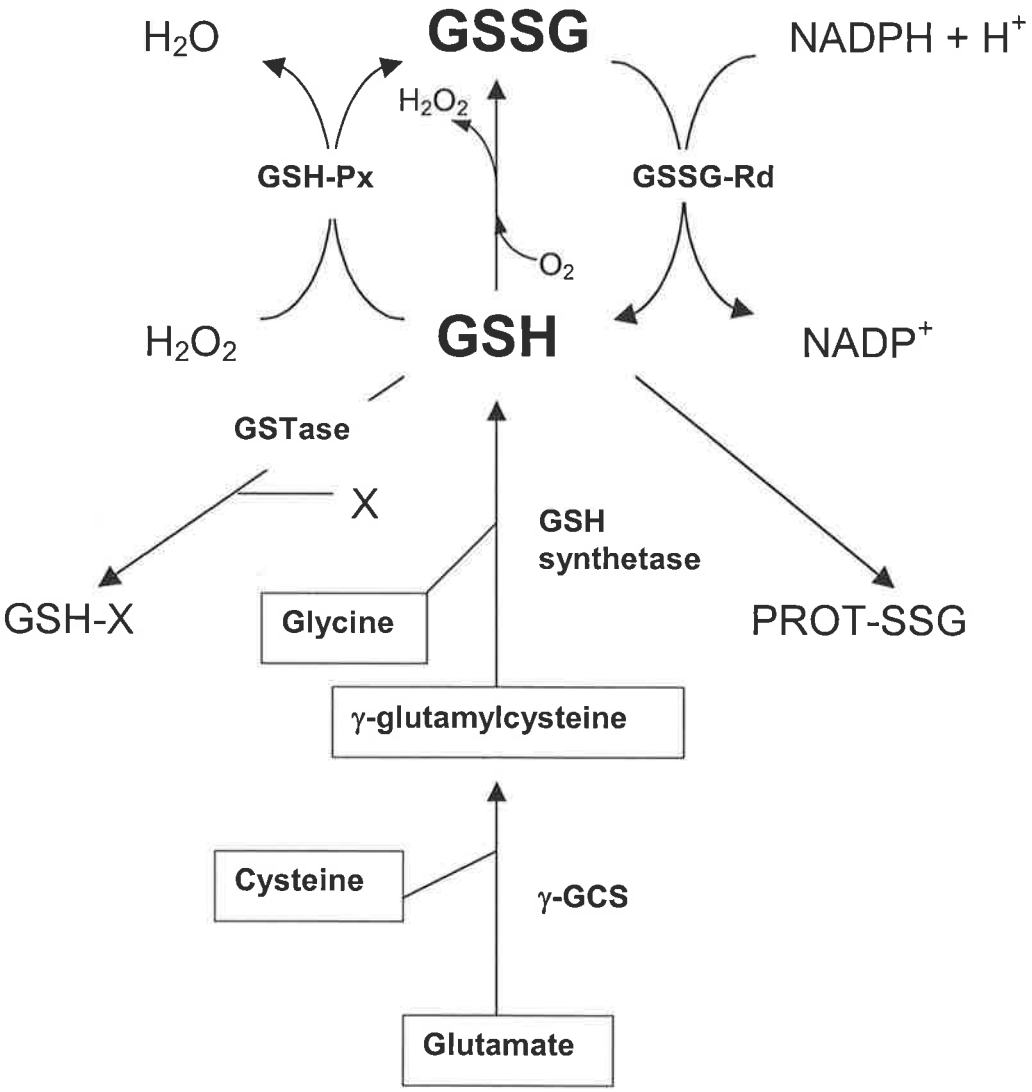


Figure 1.7 An outline of the metabolism and function of glutathione (GSH). GSH-Px = glutathione peroxidase; GSSG-Rd = glutathione reductase; GSTase = glutathione *S*-transferase; γ-GCS = γ-glutamylcysteine synthetase. X = compounds that react with glutathione to yield conjugates. Adapted from Kodavanti and Mehendale (1991).

Glutathione Synthesis

The enzymatic synthesis of GSH from its constituent amino acids, L-glutamate, L-cysteine and L-glycine, involves two ATP-requiring steps. The first step is the formation of γ-glutamylcysteine from glutamate and cysteine (Figure 1.7). It is catalyzed by γ-glutamylcysteine synthetase. In the second step, GSH synthetase catalyzes the formation of a peptide bond between γ-glutamylcysteine and glycine (Figure 1.7). The first step is rate limiting. It is regulated by negative feedback inhibition by GSH and the availability of the precursor cysteine (Griffith, 1999). The inhibition of GSH synthesis by buthionine-sulfoximine results from inhibition of γ-glutamylcysteine synthetase (Griffith and Meister, 1979). Is not known whether cylindrospermopsin inhibits γ-glutamylcysteine synthetase or GSH synthetase. Runnegar *et al.* (1995) also established that precursor availability (cysteine) and ATP levels were not affected by cylindrospermopsin treatment.

Consequences of Glutathione Depletion

The depletion of GSH renders the cell susceptible to oxidative attack, both by xenobiotics and reactive oxygen species generated as a by-product of normal cellular functions. Endogenous sources of reactive oxygen species in hepatocytes include mitochondrial respiration and cytochrome P450 activity (Puntaruli and Cederbaum, 1998). When protective mechanisms are overwhelmed, damage ensues. Lipid peroxidation is often associated with severe GSH depletion. Reactive oxygen species attack polyunsaturated lipids, extracting a hydrogen from the methylene bridge between double bonds. Membrane lipids, containing large amounts of polyunsaturated fatty acid side chains, are the target of such attack (Horton and Fairhurst, 1987). The chemistry is complex, but once lipid peroxidation has been initiated it propagates by autocatalysis, terminating either when nonradical products are formed or by the action of antioxidants (Halliwell and Gutteridge, 1999). The membrane damage leads to loss of membrane function and integrity, while the products of lipid peroxidaton form protein and DNA adducts, all contributing to cytotoxicity (Recknagel *et al.*, 1991). Lipid peroxidation products with this potential include a number of unsaturated aldehydes, such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and

acrolein (Esterbauer, 1982). A wide range of toxins are known to produce peroxidative decomposition of liver lipids. Such damage is a critical component of the hepatocyte injury that accompanies CCl_4 poisoning. The carbon-centered trichloromethyl radical (a liver metabolite) couples with O_2 to form the trichloromethylperoxy radical which initiates lipid peroxidation. Demonstrating the importance of these events, antioxidants can reduce the damage (Recknegel *et al.*, 1989). As previously noted, Terao *et al.* (1994) remarked that latter stages of cylindrospermopsin pathology *in vivo* resembled that of free-radical mediated injury induced by CCl_4. One theory is that following GSH depletion, reactive oxygen species are overproduced, producing damage and eventually cell death. The possibility that lipid peroxidation is inherently involved in cylindrospermopsin toxicity remains to be explored.

1.7.3 Bioactivation

Detoxication enzymes have evolved as mechanisms for converting xenobiotics to a form that facilitates their rapid elimination, usually via increased polarity. In addition to detoxication the reactions involved can result in the bioactivation of a xenobiotic to a more hazardous metabolite. Electrophilic reactive species can be generated which are capable of reacting with the cellular constituents, protein, RNA and DNA, and thus providing mechanisms for cytotoxicity (Nelson and Harvison, 1987) and carcinogenicity (Kadlubar and Hammons, 1987; Miller and Miller, 1981). Many toxic chemicals do not produce their detrimental effects in the form in which they are consumed, but after bioactivation to reactive metabolites.

The liver is the primary site of biotransformation reactions, followed by the lung, kidney and intestine (Lindamood, 1991). The enzymes involved are divided into two classes of reactions, Phase I (oxidation, reduction, hydrolysis) or Phase II reactions (conjugation). Phase I reactions often produce suitable substrates for Phase II reactions.

Phase I Reactions

Cytochrome P450 oxidation

The enzyme system playing the predominant role in Phase I bioactivation of xenobiotics is the cytochrome monooxygenase P450 (CYP450) enzyme system, a family of haemoprotein enzymes, where different isoforms have different substrate specificities (Gangolli and Phillips, 1993). A recent review by Guengerich (2001) details the CYP450 reactions related to chemical toxicity illustrating the wide variety of reactions catalyzed. By far the most common CYP450 reactions involve oxidations including carbon hydroxylation, heteratom oxygenation, heteratom release (dealkylation), and epoxidation. For example, the hepatotoxicity and hepatocarcinogenicity of the dietary carcinogen aflatoxin is associated with its CYP450 oxidation to a highly reactive epoxide that can form adducts with DNA, RNA and protein (Guengerich, 1992). Less commonly, reduction reactions, ring formations or ring expansions are also catalyzed by CYP450 enzymes.

Other Phase I reactions

The flavin-containing monooxygenases are also active in xenobiotic metabolism, participating in the oxygenation of nitrogen and sulfur compounds (Ziegler, 1980). Epoxide hydrolyase activities are also present in close proximity to CYP450 in the endoplasmic reticulum, reflecting their role in detoxication of electrophilic epoxides generated by CYP450 (Lindamood, 1991). While most Phase I enzymes are localized in the endoplasmic reticulum where lipophilic substrates partition, cytosolic enzymes such as alcohol dehydrogenase also participate in biotransformation reactions.

Phase II Reactions

Conjugation

Examples of conjugation reactions include glucuronidation, methylation, sulfation, amino acid conjugation and glutathione conjugation. Conjugations such as glutathione conjugation (mediated by glutathione *S*-transferase) are recognised as playing an important role in the detoxification of reactive intermediates produced by CYP450 enzyme reactions. For example, following CYP450 mediated bioactivation of aflatoxin B_1, the reactive epoxide aflatoxin B_1-9-9-oxide is then able to undergo phase II conjugation with glutathione (Hinson and Kadlubar, 1988; Ketterer *et al.*, 1988). This conjugation prevents the reactive epoxide binding to DNA, playing a key role in cellular defense. The GSH conjugate is excreted.

Bioactivation of Cylindrospermopsin

Several lines of evidence point toward the involvement of CYP450 derived metabolites in cylindrospermopsin toxicity. *In vivo,* liver damage is consistently reported as originating within the centrilobular region of the hepatic lobule (refer to Section 1.5.1). Pathological lesions are commonly described as being centrilobular - involving cells surrounding the centrilobular vein, or peripheral - involving cells in the periportal region of the lobule. CYP450 enzymes are present at higher concentrations in the cells of the centrilobular region than of the periportal region (Gooding *et al.*, 1978) and hence more susceptible to the toxic effects of CYP450 metabolites. Consisitent with this observation, activity of the CYP450 enzyme system was subsequently demonstrated to be important for development of cylindrospermopsin toxicity both *in vitro* in hepatocyte culture (Runnegar *et al.*, 1995b) and *in vivo* in Quackenbush mice (Norris *et al.*, 2002). Furthermore cylindrospermopsin is more toxic to primary rat hepatocytes than to the KB epithelial carcinoma cell line (Chong *et al.*, 2002) or to HeLa cell types (Shaw *et al.*, 2000). It has been suggested that these differences in toxicity may be explained on the basis of lower or absent CYP450 activity in the cell lines.

On the other hand, *in vivo* toxicology studies show that cylindrospermopsin produces effects in a variety of organs less likely to metabolize the toxin than the liver. The activity (albeit lower) of cylindrospermopsin to cell types other than hepatocytes has also been demonstrated *in vitro*. The possibility exists that cylindrospermopsin is active both in the form in which it is consumed, and after bioactivation to a more toxic metabolite in the hepatocytes of the liver.

Protein synthesis inhibition by cylindrospermopsin was first demonstrated in reticulocyte lysates. It is known that erythrocytes (mature reticulocytes) are a cell type deficient in the CYP450 system (Guengerich and Shimada, 1991). This indicates that protein synthesis inhibition by cylindrospermopsin in reticulocyte lysates is likely to occur without prior bioactivation of the toxin. The question is then raised; does protein synthesis inhibition occur independently of metabolite mediated toxicity in hepatocytes? Studies with hepatocyte cultures have linked toxicity and GSH depletion with CYP450 dependent metabolite formation (Runnegar *et al.*, 1995b), but the role of metabolites in protein synthesis inhibition has not been explored.

1.8 Project Rationale

Cylindrospermopsin toxicology appears complex. An important lesion is within the liver. Although a number of events have been explored, their progression leading to hepatotoxicity is not well understood. Questions have been raised concerning the extent to which protein synthesis inhibition contributes to cylindrospermopsin toxicity; the importance of oxidative stress in mediating hepatotoxicity; and whether biotransformation products are involved in the toxic process. The primary aim of the work described in this thesis was to clarify which processes are critical to toxicity.

The experimental protocols used in this work involved the use of *in vitro* mammalian cell models and purified cylindrospermopsin. Primary hepatocytes in culture were shown to be a suitable tool for elucidating the mechanisms of hepatocyte cell toxicity induced by cylindrospermopsin and was the model of choice in this project also. To investigate protein synthesis inhibition in greater detail a reticuloctye lysate was utilized. The work was motivated by two broad hypotheses:

- protein synthesis inhibition is not solely responsible for cylindrospermopsin toxicity in hepatocytes, and
- cylindrospermopsin-derived metabolites produce effects other than protein synthesis inhibition that contribute to toxicity.

The specific aims of the work described in this thesis were:

- To purify cylindrospermopsin from cyanobacterial extracts (Chapter 2).

- To assess the potency and mechanism by which cylindrospermopsin inhibits protein synthesis. This was investigated *in vitro* using reticulocyte lysates (Chapter 3).

- Using a primary hepatocyte culture model, to investigate the mechanisms involved in cylindrospermopsin toxicity. This involved investigating the contribution of protein synthesis inhibition to hepatocyte cell death (Chapter 4); Investigating the role of glutathione dyshomeostatsis and lipid peroxidation in toxicity (Chapter 5); and assessing the involvement of CYP450 metabolites in protein synthesis inhibition and toxicity (Chapter 6).

2 PURIFICATION OF CYLINDROSPERMOPSIN

2.1 Introduction

In order to effectively evaluate the toxicological properties attributable to cylindrospermopsin a purified sample was required. While recent advances have led to the chemical synthesis of active cylindrospermopsin (Xie *et al.*, 2000), to date, cylindrospermopsin used in experimental protocols has been purified from cyanobacterial cell extracts (Banker *et al.*, 1997; Banker *et al.*, 2001; Humpage *et al.*, 2000a; Runnegar *et al.*, 1994; Runnegar *et al.*, 1995b; Terao *et al.*, 1994). This has been achieved by High Performance Liquid Chromatography (HPLC) with or without prior clean-up of the extract by flash chromatography and/or size exclusion chromatography. Upon purification, a mass of 415 Daltons and UV absorbance maximum at 262 nm are used to confirm the identity of the toxin (Ohtani *et al.*, 1992). The UV absorbance maximum of cylindrospermopsin at 262 nm reflects the presence of the uracil group in the toxin structure. Since potential nucleoside contaminants also exhibit similar UV absorbance spectra, Harada *et al.* (1993) suggest monitoring the ratio of the UV absorbance maxima at 200 and 262 nm for confirmation of cylindrospermopsin (2:1 ratio respectively). Eaglesham *et al.* (1999) utilized the characteristic fragmentation patterns of cylindrospermopsin on HPLC-MS / MS (HPLC coupled to tandem mass spectrometry) for their identification and quantification procedure. Estimations of purity of the toxin have been carried out using nuclear magnetic resonance (NMR) analyses (Harada *et al.*, 1993).

The procedures used for purification and identification of cylindrospermopsin have also led to the identification of cylindrospermopsin-related compounds including the non-toxic analog deoxycylindrospermopsin (deoxy-CYN) (Norris *et al.*, 1999) and the toxic enantiomer 7-*epi*-cylindrospermopsin (7-*epi*-CYN) (Banker *et al.*, 2001). Deoxy-CYN is known to elute from a reverse-phase HPLC column at approximately double the retention time of cylindrospermopsin, and can be identified by it's mass, 399 Daltons, and characteristic

HPLC-MS / MS fragmentation pattern (Norris *et al.*, 1999). The 7-*epi*-CYN is reported to elute at a shorter retention time than cylindrospermopsin by reverse-phase HPLC and is distinguished from cylindrospermopsin by optical rotation (Banker *et al.*, 2000). Both compounds are reportedly present in cyanobacterial extracts as a minor fraction in comparison to cylindrospermopsin.

This chapter describes the extraction, HPLC purification and subsequent characterization of cylindrospermopsin from lyophilized *Cylindrospermopsis raciborskii* culture for use in the current research project.

2.2 Materials and Methods

2.2.1 Materials

All solvents used were of HPLC grade. *Cylindrospermopsis raciborskii* strain AWT205 was obtained as lyophilized culture from Dr Peter Hawkins of AWT EnSight, Sydney, Australia. A cylindrospermopsin standard, characterised by HPLC-MS / MS (Eaglesham *et al.*, 1999) was used as reference material.

2.2.2 Preparation of *Cylindrospermopsis raciborskii* Culture Extracts

Following the procedure described by Hawkins *et al.* (1997), a 10 mg/ml *C.raciborskii* extract was prepared in 5% acetic acid by repeated ultrasonication (6 x 20 sec, 50W) using a Braun Labsonic 1510 Sonicator. The extract was clarified by centrifugation in a benchtop microfuge (14,000 g, 10 min) and filtered through a 0.2 µm Minisart® filter unit (Sartorius, Germany) in preparation for loading onto the HPLC.

2.2.3 HPLC Purification of Cylindrospermopsin from *C.raciborskii* Extracts

The HPLC method employed for cylindrospermopsin analysis was essentially as described by Harada *et al.* (1993). The HPLC system consisted of a LC1150pump (GBC), a series 1100 UV-VIS detector (Hewlett Packard) and a manual injector. The *C.raciborskii* extract was applied to a μBondapak C18 reverse phase preparative column (7.8 mm x 300 mm, 10 μm particle size) with C18 guard column. Samples were eluted with isocratic 5% methanol at a flow rate of 2 ml/min and UV detection at 262 nm. Fractions were collected in accordance with the retention time of the cylindrospermopsin standard.

Alternatively, cylindrospermopsin was purified on a HPLC system which consisted of a Waters 600 Controller, 996 photodioarray (PDA) detector and a manual injector. In this case extracts were applied to an Activon GoldPak Exsil™ C18 preparative column (20 mm x 250 mm, 5 μm particle size), eluted at 2 ml/min in 5% methanol with monitoring at 262 nm. Fractions were collected and processed as described above. Eluate containing cylindrospermopsin was lyophilized and re-suspended in MilliQ water prior to characterization.

2.2.4 HPLC Analysis of Purified Cylindrospermopsin

Characterization of purified cylindrospermopsin was carried out on an analytical column (Alphabond C18 300 mm x 3.9 mm, 10 μm, Alltech), on either of the two HPLC systems detailed in Section 2.2.3. Isocratic 5% methanol was used at a flow rate of 0.75 ml/min to elute the samples and peaks were detected at 262 nm.

A standard curve was constructed with cylindrospermopsin reference material and samples were quantified using the linear regression of HPLC peak area vs quantity of cylindrospermopsin (Figure 2.1).

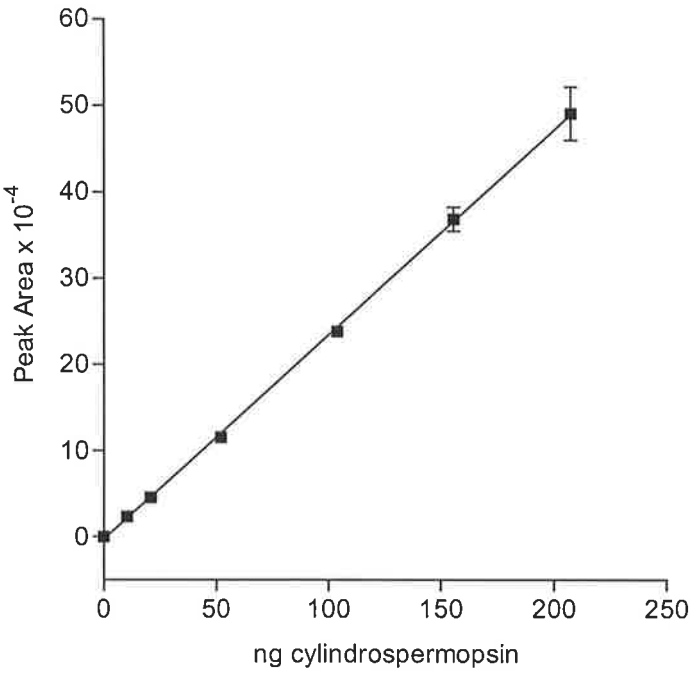


Figure 2.1 Representative standard curve for cylindrospermopsin determination by UV-HPLC. Peak area (mean ± SE, n = 3) was monitored at 262 nm. Linear regression, slope = 49260, Y intercept = -3289, $r^2 = 0.98$.

2.2.5 HPLC-MS / MS Analysis of Cylindrospermopsin

HPLC-MS / MS analysis of cylindrospermopsin was carried out by Queensland Health Scientific Services as described by Eaglesham *et al.* (1999). Transition of the cylindrospermopsin M + H ion (416 *m / z*) to the 194 *m / z* fragment was monitored for quantification. The 194 *m / z* fragment is $(M\text{-}C_5H_6N_2O_3)\text{-}SO_3 + H^+$ (Norris *et al.*, 1999).

2.3 Results

Cylindrospermopsin was purified from the *C.raciborskii* culture in several batches. Details are provided for the batch purified 'May2000' and are representative of the results obtained in each instance. HPLC analysis of the *C.raciborksii* extract revealed several peaks absorbing at 262 nm (Figure 2.2). Collection of the fraction containing cylindrospermopsin was sufficient to purify the toxin to a single HPLC peak (Figure 2.3(a)) with UV spectrum exhibiting the characteristic absorbance at 262 nm and 2:1 ratio of 200 and 262 nm peaks (Figure 2.3(b)). As detailed in Table 2.1 the HPLC estimate of toxin purity was 93% and similar cylindrospermopsin quantifications were obtained by both HPLC and HPLC-MS/MS. The yield of toxin obtained was 0.1% of the total *C.raciborskii* dry weight, and 24% of the theoretical toxin content based upon HPLC-MS / MS analysis of the extract.

***C.raciborskii* extract**	
Dry weight	1.2 g
Toxin content (HPLC-MS / MS analysis)	4µg CYN/mg dry wt
Total toxin present	4.8 mg
Cylindrospermopsin yield	
Total CYN yield	1.15 mg
Yield as % *C.raciborskii* dry weight	0.1%
Yield as % of expected toxin content	24%
Cylindrospermopsin analyses	
Quantification - HPLC	125 µM
Quantification – HPLC-MS / MS	152 µM
Purity estimate by HPLC	93%
DAD scan: ratio of 200 nm to 260 nm peak	2:1

Table 2.1 Details of cylindrospermopsin yield and analysis for toxin batch 'May2000'.

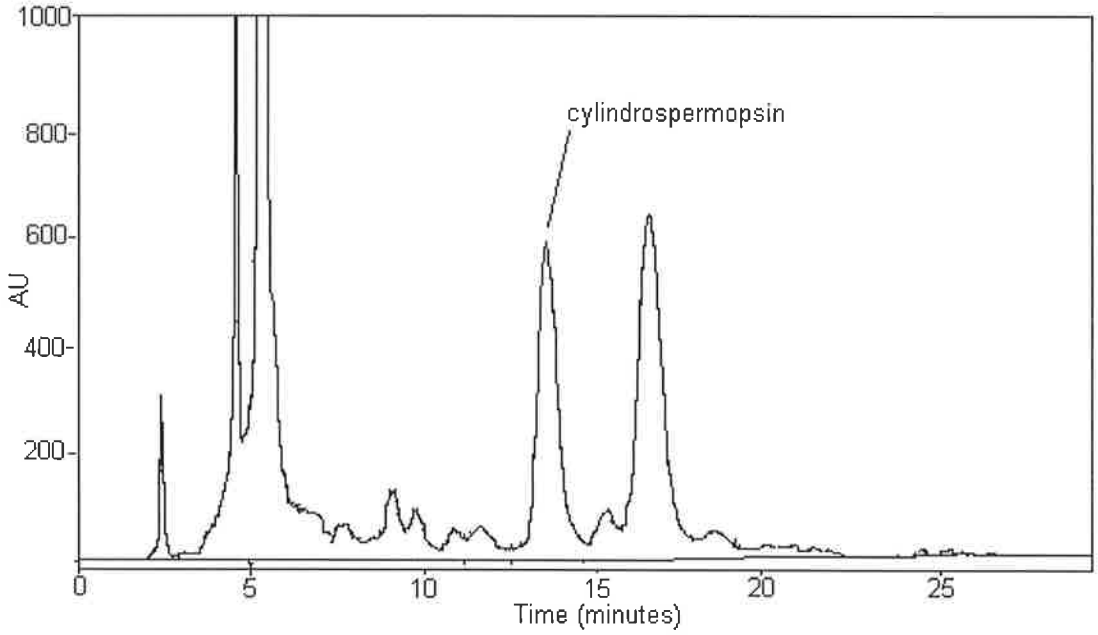


Figure 2.2 HPLC chromatogram (262 nm) illustrating separation of the *C.raciborskii* extract on the preparative column. The cylindrospermopsin peak at 13.47 min is indicated.

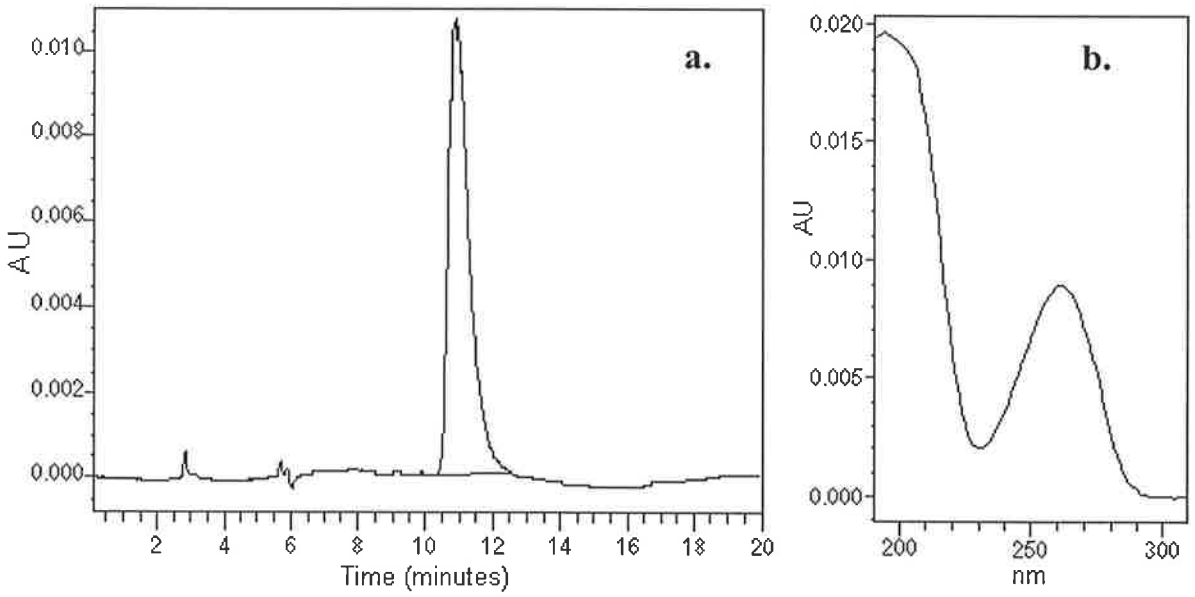


Figure 2.3. Analysis of purified cylindrospermopsin (a) HPLC chromatogram (262 nm) of the purified cylindrospermopsin sample and (b) the UV spectra (200-300 nm) of the peak.

2.4 Discussion

The HPLC purification procedure from *C.racibroskii* extract provided toxin samples identified as cylindrospermopsin by UV absorbance maxima and mass spectra analyses. The material was assessed to be of high purity by HPLC. Low product yields restricted purity estimation by alternative methods such as Nuclear Magnetic Resonance (NMR) imaging. Harada *et al.* (1993) records that NMR analysis of purified cylindrospermopsin exhibiting a single HPLC peak reveals the presence of amino acid (phenylalanine and tyrosine) and nucleoside (cytidine and uridine) contaminants. Similarly, the presence of the amino acid phenylalanine has also been noted from other HPLC purified cylindrospermopsin samples from our laboratory when sufficient material has been available for NMR analysis (personal communication, Dr A Humpage). While the presence of such contaminants cannot be excluded from the current preparation, these substances are largely inert in toxicological terms and would not be expected to contribute to cytotoxicity.

Previously reported yields of cylindrospermopsin from dry weight of cyanobacterial culture range from 0.09% (Harada *et al.*, 1993) and 0.1% (Banker *et al.*, 2000) to 0.5% (Ohtani *et al.*, 1992). A similar yield to Harada *et al.* (1993) and Banker *et al.* (2000) was obtained by the procedure used in this study. However only a quarter of the toxin present in the extract was recovered. Enhanced recovery may be achieved by improvement of the chromatography. To date efforts to define a column type and mobile phase composition which enhances the retention and hence separation of this toxin have been hampered by its hydrophilic nature and resulted in varying conditions used by different researchers. Alternatively, synthetic cylindrospermopsin may provide a pure source of the toxin in the future.

3 INHIBITORY EFFECTS OF CYLINDROSPERMOPSIN ON CELL-FREE PROTEIN SYNTHESIS: POTENCY AND MODE OF ACTION

3.1 Introduction

The synthesis of protein from mRNA (translation) can be carried out *in vitro* using a variety of cell-free extracts (Olliver and Boyd, 1998; Olliver *et al.*, 1998). Of these, reticulocyte lysates have found many applications in studying the eukaryotic process of translation and understanding its regulation (Clemens, 1984; Jackson and Hunt, 1983; Olliver and Boyd, 1998). The potency and mechanism of action of various protein synthesis inhibitors including cycloheximide (Obrig *et al.*, 1971), pactamycin and emetine (Lodish *et al.*, 1971) have been elucidated in this system. More recently reticulocyte lysates have been used to dissect effects on translation by compounds with potential therapeutic value (Ahuja *et al.*, 2000; SirDeshpande and Toogood, 1995).

Terao *et al.* (1994) reported that cylindrospermopsin was a potent inhibitor of protein synthesis with complete inhibition of globin synthesis in reticulocyte lysates observed at 50 ng ml^{-1} (120 nM cylindrospermopsin). Although comparisons were made to cycloheximide *in vivo* these observations cannot be related to their relative potencies as translation inhibitors. Terao *et al.* (1994) compared the extent of fat accumulation in the liver (a common response to inhibition of protein synthesis) after administration of cylindrospermopsin and cycloheximide. As noted in Chapter 1, mechanisms other than protein synthesis inhibition can coexist to contribute to fat accumulation. These include the alkylation of lipoproteins, effects on intrahepatic lipid (triglyceride) metabolism, or increased arrival of precursors from outside the hepatocyte (Dianzani, 1991). Hence the relative potencies of the translation

inhibitors remains unknown. There have been no further investigations into the effects of cylindrospermopsin on translation.

The process of translation is complex and there are many sites at which a toxin could interact (Hershey *et al.*, 1986). Translation can be separated into three stages, initiation, elongation and termination. Briefly, the first phase, *initiation* is the joining of the 40S and large 60S subunits of the ribosome, mRNA, and initiation factors to form an initiation complex (Merrick *et al.*, 1986). *Elongation* is the major phase of protein synthesis, involving the sequential addition of amino acids to the peptide chain. It involves the cyclic repetition of aminoacyl-tRNA binding to the ribosome, formation of a peptide bond between two adjoining amino acids, and the movement of the ribosome along the mRNA (Mathews and van Holde, 1991). Protein elongation factors are involved, and energy in the form of guanosine triphosphate is required. Upon reaching a stop codon, translation is *terminated.* Release factors facilitate the dissociation of the protein synthesis machinery. The newly synthesized peptide and the tRNA are released, the ribosome dissociates into its subunits and mRNA is also released. The protein synthesis machinery can then be used in the next round of translation.

The kinetics of amino acid incorporation after the addition of inhibitors to reticulocyte lysates can be used to readily distinguish whether a toxin interferes with initiation or elongation (Clemens, 1984). Upon addition to an active system, elongation inhibitors are capable of immediately arresting translation. In contrast an initiation inhibitor must wait for the ongoing translation to complete and the protein synthesis machinery to dissociate before associating with it. They inhibit the formation of the initiation complex, and prevent the next round of translation. Thus during study of the time course of toxin effects, a lag phase is seen. Initiation inhibitors include pactamycin (Lodish *et al.*, 1971) and pyrocatechol violet (Huang and Grollman, 1973). Elongation inhibitors include emetine and cycloheximide (Jimenez, 1976; Willingham *et al.*, 1981).

The aims of this Chapter were to (1) assess the potency of cylindrospermopsin in comparison to a known protein synthesis inhibitor; and (2) investigate the mechanism by which cylindrospermopsin interrupts translation (ie. at initiation or elongation stage).

3.2 Materials and Methods

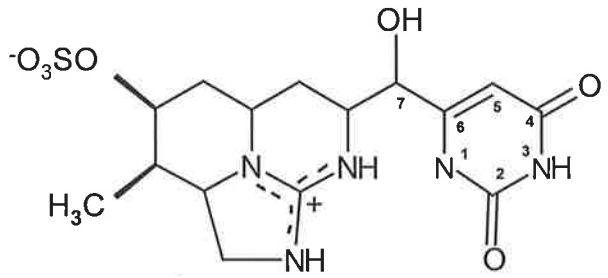
3.2.1 Protein Synthesis Inhibitors

Cylindrospermopsin (CYN) was purified as described in Chapter 2. Pyrocatechol violet (PCV) and cycloheximide (CHEX) were both obtained from Sigma-Aldrich (St Louis, MI, USA). The structures of the inhibitors are shown in Figure 3.1.

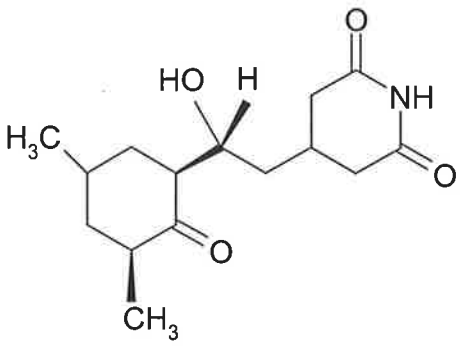
3.2.2 Translation Procedure

In vitro protein synthesis (translation) was performed using commercially prepared rabbit reticulocyte lysate obtained from Promega Corporation (Madison, WI, USA) with minor adaptation of the manufacturers instructions. Translation mixes (25 µl or 50 µl) were prepared on ice, and comprised 70% rabbit reticulocyte lysate, 20 µM unlabelled amino acids and 3.5 µCi [^{3}H]-L-Leucine, (140 Ci/mmol, NEN™ Life Sciences, Boston, MA, USA). RNasin® Ribonuclease inhibitor (Promega) was added at a final concentration of 1 unit / µl. All reactions were made up to volume using sterile Milli Q water. Reactions were preincubated for 5 min at 30°C prior to the initiation of translation.

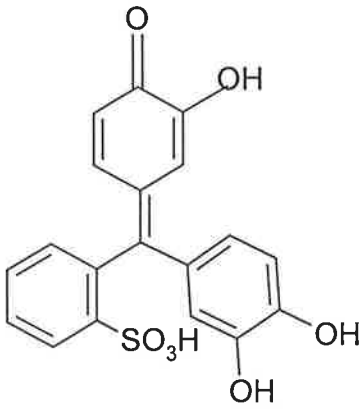
Luciferase mRNA (Promega) was denatured for 3 min at 65°C to destroy local regions of secondary structure and increase the efficiency of translation. The denatured mRNA was cooled briefly on ice prior to use. Each translation reaction was initiated by the addition of 40 µg / ml mRNA. Reactions were incubated at 30°C for the duration of the experiment. A negative control (no mRNA) was processed with each experiment.



cylindrospermopsin



cycloheximide



pyrocatechol violet

Figure 3.1 Structures of protein synthesis inhibitors.

3.2.3 Post Translation Analysis

Protein synthesis was measured by determining the extent of [^{3}H]-Leucine incorporation into trichloroacetic acid (TCA) insoluble material as described by Clemens (1984). Samples (5 µl) of the incubation mix were withdrawn and pipetted onto 2.2cm^2 Whatman No1. Filter paper. The filter papers were washed successively for 15 min each in (1) ice-cold 5% TCA, (2) 5% TCA at 90°C, and (3) 5% TCA at room temperature. The filter papers were then washed for ~1 min each in absolute ethanol and then acetone before they were allowed to dry. 40 µl of 10% hydrogen peroxide was spotted on to each sample to prevent colour quenching during scintillation counting. Dried filter papers were placed in 3 ml Ready Protein+™ (Beckman Coulter, CA, USA) scintillation fluid. Samples were counted for 5 min in a Beckman LC3801 Scintillation counter. The dpm response was calculated based upon a predefined quench curve using Beckman quench standards.

3.2.4 Potency – Concentration Response Curves

Three independently purified and quantified cylindrospermopsin stocks were used to construct a protein synthesis inhibition concentration-response curve in reticulocyte lysates. Similarly, 3 independently prepared cycloheximide stock solutions were used to construct a concentration-response curve. The lot number of reticulocyte lysate remained constant throughout. Serial toxin dilutions were prepared in Milli Q H_2O. For each sample tested, 2.5 µl of toxin was added to a 25 µl translation reaction. The reaction mix was pre-incubated for 5 min at 30°C in the presence of the toxin prior to initiation of the translation reaction with mRNA. Samples were incubated for 30 min at 30°C. At the time of completion, 5 µl samples were withdrawn and processed for incorporation of [^{3}H]-Leucine into trichloroactetic acid (TCA) insoluble material as described in Section 3.2.3. Each translation reaction was assayed in duplicate. Assay results were reported as % protein synthesis inhibition in toxin treated samples with respect to the control (Milli Q water). The control reaction was taken to represent 100% protein synthesis, that is, 0% inhibition.

3.2.5 Mode of Action- Time Course Experiments

Optimization

Trial time-course experiments with control translation reactions revealed that incorporation of [^{3}H]-Leucine was linear for approximately 15 min after the initiation of translation with mRNA (data not shown). Following this time, the rate of [^{3}H]-Leucine incorporation decreased dramatically (data not shown). Supplementation of the reaction mix with cold L-Leucine (unlabelled) to increase the final concentration of L-Leucine to equal that of other amino acids in the reaction mix (20 μM), failed to prolong the duration of [^{3}H]-Leucine incorporation. To compensate, time course experiments were carried out within a 15 min time period during which amino acid incorporation and hence translation was linear.

Time Course Experiments

Stock solutions of protein synthesis inhibitors were prepared in sterile Milli Q. water. Translation reactions (50 μl) were prepared as described in Section 3.2.2. Translation was initiated by the addition of mRNA, and 5 μl samples were taken at 2 min intervals for t = 0, 2, 4 min. Immediately after the 4 min sample was taken, inhibitors (or MilliQ water) were added to translation reactions to give a final concentration of 1 mM pyrocatechol violet, 10 μM cycloheximide or 10 μM cylindrospermopsin in the reaction mix. Samples were taken at 2 min intervals until the end of the incubation period. All samples were processed for incorporation of [^{3}H]-Leucine into trichloroactetic acid (TCA) insoluble material as described in Section 3.2.3.

3.2.6 Statistical Analysis

Graph Pad Prism version 3.00 for Windows, (GraphPad Software, SanDiego, CA, USA) was used for all statistical tests and graphing.

3.3 Results

3.3.1 Potency

Both cylindrospermopsin and cycloheximide displayed sigmoidal concentration response curves (variable slope) for inhibition of protein synthesis in reticulocyte lysates (Figure 3.2). The inhibition displayed by the two toxins was not in parallel. As illustrated, and further detailed in Table 3.1, cylindrospermopsin (IC_{50} =120 nM) was at least three times more potent than cycloheximide (IC_{50} = 368 nM). The potency of cylindrospermopsin was further exaggerated at levels of inhibition greater than the IC_{50} reflecting the steep concentration response curve. The slope factor for cylindrospermopsin was 5 times that of cycloheximide (3.0 and 0.6 respectively). Despite different potencies and slope factors, both toxins elicited the same maximal response, completely abolishing protein synthesis.

3.3.2 Mode of Action

The protein synthesis inhibitors pyrocatechol violet and cycloheximide were chosen due to their selective inhibition of the initiation and elongation steps of protein synthesis respectively. Their responses were compared to the effects of cylindrospermopsin. Upon addition to an active translation system, cylindrospermopsin produced immediate inhibition of translation (Figure 3.3), confirming that it inhibits elongation. Cycloheximide also caused immediate inhibition. In contrast, a 2 min lag phase was observed before the effects of pyrocatechol violet became evident. These results are consistent with pyrocatechol violet allowing the completion of ongoing translation before blocking *de novo* protein synthesis.

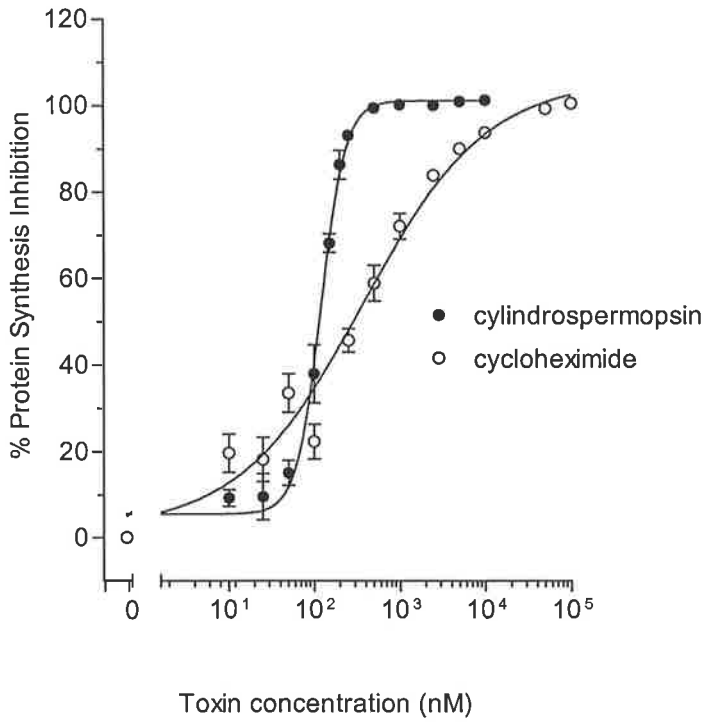


Figure 3.2 Sigmoid concentration response curves for inhibition of protein synthesis by cylindrospermopsin (R^2=0.98) and cycloheximide (R^2 = 0.94) in reticulocyte lysates. Data are mean ± SE, n ≥ 3.

	cylindrospermopsin	cycloheximide
IC_{50} (95% C.I.)	120 nM (110-130)	368 nM (316-626)
Slope	3.0	0.6

Table 3.1 Parameters of the protein synthesis inhibition concentration response curves. Comparison of toxin potency and curve slope factors. Data are mean ± SE, n ≥ 3.

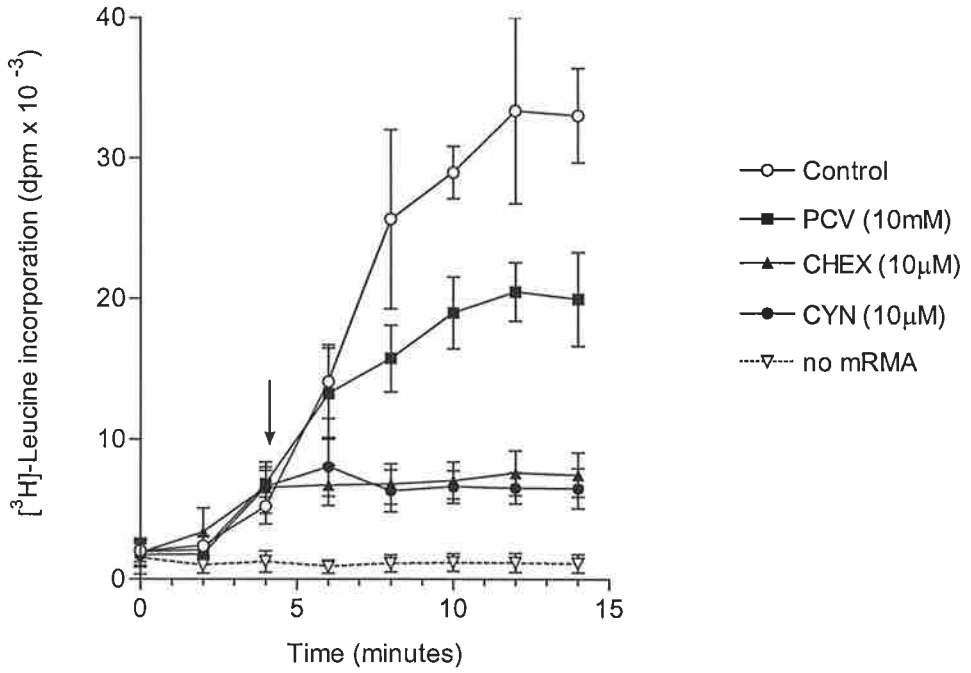


Figure 3.3 The effect of cylindrospermopsin (CYN), cycloheximide (CHEX) and pyrocatechol violet (PCV) on translation in reticulocyte lysates. The reaction was initiated by the addition of mRNA (t = 0) and incubated at 30°C. After 4 min incubation inhibitors were added (arrow). At each time point 5 µl aliquots were removed for post translation analysis. Data are mean ± SE, n = 3.

3.4 Discussion

Potency reflects both the affinity (for a receptor) and efficacy (ability to cause response) of a toxin. In this study cylindrospermopsin was demonstrated to have greater potency in arresting translation than the well known protein synthesis inhibitor cycloheximide. While we cannot conclude if cylindrospermopsin has a higher affinity or efficacy on the ribosomes (or both) from the data obtained in this study, comparison of their chemical structures (Figure 3.1) may provide some explanation. The activity of cycloheximide is related to the nitrogenous glutarimide ring in the structure, related glutarimide antibiotics are also active protein synthesis inhibitors (Obrig *et al.*, 1971). The similarity of the glutarimide ring and position of hydroxyethyl in cycloheximide to the hydroxymethyl uracil of cylindrospermopsin is noted (Figure 3.1). Given the biological role of uracil, it is not surprising that this would exhibit both higher affinity and efficacy at the ribosomes than cycloheximide's glutarimide structure, thereby explaining its steep concentration response curve. As mentioned previously, toxicological studies point toward the hydroxymethyluracil being an important contributor to cylindrospermopsin toxicity.

While both cylindrospermopsin and cycloheximide inhibit the elongation step in translation, it is possible that the toxins mediate their inhibition through different sites. Inhibition of polypeptide elongation can either result from the inhibition of aminoacyl-tRNA delivery, inhibition of peptidyltransferase (enzyme involved in peptide bond) or inhibition of translocation (movement of the ribosome along the mRNA) (Hershey *et al.*, 1986; Lodish, 1976). Cycloheximide is known to bind to the 60S ribosome subunit and prevent translocation (Jimenez, 1976). Further investigation into the mechanism by which cylindrospermopsin interferes with the elongation step will require a detailed biochemical analysis with fractionated reticulocyte lysate components.

4 INHIBITION OF PROTEIN SYNTHESIS BY CYLINDROSPERMOPSIN IN HEPATOCYTE CULTURE

4.1 Introduction

To explore the role of protein synthesis inhibition in cylindrospermopsin hepatotoxicity primary hepatocyte culture was employed as an *in vitro* model. The capacity of isolated hepatocytes in monolayer culture (primary hepatocytes) to maintain normal liver-specific functions over short term incubations provides a valuable research tool. They have been used extensively for investigating the mechanisms of xenobiotic hepatotoxicity (Rauckman and Padilla, 1987), and have been demonstrated as a suitable model for studying the mechanisms of cylindrospermopsin induced injury (Runnegar *et al.*, 1994; Runnegar *et al.*, 1995b). Within our laboratory, *in vivo* cylindrospermopsin toxicology studies have been carried out using Swiss Albino mice (Falconer *et al.*, 1999; Falconer and Humpage, 2001). To maintain consistency Swiss Albino mice were also chosen for the isolation of hepatocytes in this study.

A number of observations on cylindrospermopsin-induced protein synthesis inhibition are described in this Chapter. Continuous exposure experiments were conducted to determine the concentration-response and time frame for protein synthesis inhibition in relation to hepatocyte cell death. While providing information on the relationship between the two parameters, these experiments also provide the basis for further hepatocyte culture work resported in subsequent Chapters.

A short–term exposure experiment was also employed to determine whether damage to the protein synthetic system induced by cylindrospermopsin could be reversed following toxin removal. For this experiment the response of cylindrospermopsin was compared to cycloheximide and emetine, established reversible and irreversible inhibitors of protein synthesis respectively. Recovery of protein synthesis after removal of cycloheximide has been demonstrated in number of cell types including HeLa cells (Liao *et al.*, 1976), primary

rat hepatocytes (Helenek *et al.*, 1982) and rat pancreas cells (Garcia-Barrado *et al.*, 2001). In contrast, the effects of emetine are prolonged. After 30 min exposure of HeLa cells to emetine and its removal, protein synthesis did not recover over the subsequent 2 hr incubation period (Liao *et al.*, 1976). Similarly, the irreversible nature of the protein synthesis inhibition induced by emetine has also been demonstrated in other cell types including rat macrophages (Antoni *et al.*, 1986).

Finally, the effect of cylindrospermopsin on protein synthesis in hepatocytes was compared to the effect in a cell-free system (reticulocyte lysate).

4.2 Materials and Methods

4.2.1 Materials

Cylindrospermopsin was purified as described in Chapter 2. Cycloheximide and emetine dihydrochloride were obtained from Sigma Aldrich (St Louis, MI, USA).

4.2.2 Animals

Swiss Albino mice were bred and supplied by the Adelaide University Animal Services Facility. Animals were housed in the Medical School Animal House, Adelaide University. Mice were kept in groups of animals up to 8 per cage, and were fed laboratory ration L103 (Ridley Agriproducts, Pty Ltd) and water *ad libitum*. They were maintained on a 12 hr light/dark cycle at 19 - 23°C.

Animal Ethics Approval

Approval was obtained from the Adelaide University Animal Ethics Committee (Ethics number M/76/98) prior to the commencement of experiments using mice.

4.2.3 Preparation and Culture of Hepatocytes

Sources of chemicals, along with solution recipes and media compositions used for the isolation and culture of hepatocytes are detailed in Appendix 1. Solutions were sterilized by autoclaving or passage through a 0.2 μm Minisart® filter unit (Sartorius, Germany). Glassware was sterilized by autoclaving. Aseptic techniques were used throughout.

Hepatocytes were isolated from adult male Swiss Albino mice (30-35 g) by a modification of the two-step collagenase perfusion procedure introduced by Seglen (1972, 1976). The perfusion was retrograde via the inferior vena cava as is recommended for mouse given the small size of the liver (Berry *et al.*, 1991). The perfusion tubing was flushed with 70% ethanol followed by sterile M.Q. H_2O and then primed with the first perfusion medium in preparation for use. Perfusion media were pre-equilibrated in a 37°C water bath. The surgical instruments and the surgery area were sprayed with 70% ethanol prior to the commencement of the procedure.

Mice were administered 50 U (50 μl) of the anticoagulant heparin (CSL Ltd, Australia) and 100-150 μl of the sedative Nembutal® (60 mg/ml, Rhone Merieux Australia Pty Ltd) in a single intraperitoneal injection. Upon anaesthesia, the abdomen was moistened with 70% ethanol and the abdominal cavity opened from pubis to sternum, along the midline. Incisions were made perpendicular to the midline cut, to expose the abdominal cavity. Using forceps the subhepatic inferior vena cava was isolated and freed from the underlying connective tissue. Two silk threads were then passed underneath and loosely knotted around the vein. The vena cava was cannulated, secured into position with one thread, and then ligated distally with the second thread. The thoracic vena cava was occluded and the portal vein severed.

The liver was perfused with Ca^{2+}-free Krebs-Henseleit medium for 5 min at 4 ml/min. Collagenase perfusion in Ca^{2+} supplemented medium then followed for 6-8 min, until the liver leaked liquid through the capsule.

Upon completion, the gall bladder was removed, the liver excised and placed into ~20 ml of Krebs-Henseleit wash medium. Work then proceeded in a laminar flow hood. The liver was transferred into clean wash medium (~20 ml). The hepatic epidermis was opened with scissors and the hepatocytes were gently scraped into the medium with a spatula. The cell suspension was passed through a layer each of 200 µm and 100 µm (pore size) nylon mesh and into a 50 ml centrifuge tube. The mesh was washed with media until the total volume reached 45 ml. The suspension was centrifuged at 50 x g for 2 min at room temperature. The supernatant was aspirated and the pellet resuspended in 45 ml of wash medium. The centrifugation and wash was repeated twice. If required a 200 µl sample was taken prior to the final centrifugation for determination of Trypan Blue exclusion and cell counts as described below. After the final wash, the pellet was resuspended in an appropriate volume of DMEM/F12 supplemented with 1% BSA, 50 U/ml of Penicillin, 50 µg/ml Streptomycin, 100 nM Insulin and 1 µM Hydrocortisone. Cells (~5 x 10^6) in 1.5-2 ml medium were plated on 36 mm collagen-coated dishes and incubated at 37°C, 5% CO_2. Two to three hours after plating, the medium was removed, cells washed with 2 ml Hanks Buffered Salts Solution (HBSS) to remove dead, unattached cells and 2 ml DMEM/F12 supplemented as described above was added. Incubation continued at 37°C, 5% CO_2 in the presence of cylindrospermopsin or other test chemicals or appropriate vehicle (control).

Collagen Coating of Cell Culture Dishes

The substrata for monolayer hepatocyte culture was provided by coating cell culture dishes (Sarstedt, Australia) with rat tail collagen. Type I collagen from rat tail tendons was purified essentially as described in Berry *et al.* (1991). Rat tails were obtained frozen from the Department of Clinical and Experimental Pharmacology, Adelaide University. The tails were thawed, cleaned with 70% ethanol and the skin removed. Each tail was cut into 3-4 cm lengths and the collagen fibres removed using forceps. The fibres were placed into 0.1%

acetic acid and stirred overnight at 4°C. The dissolved collagen then was dialysed against 1 mM HCl, 3% chloroform overnight at 4°C. The purified collagen was lyophilized and re-suspended as 10 mg/ml stock in sterile M.Q. H_2O as required. Just prior to plating the collagen stock was diluted to 2.5 mg/ml in 20% ethanol. Dishes were coated with 15-20 μg collagen /cm^2 by spreading an appropriate volume (200 μl for 36 mm dish) over the plate.

Alternatively purified Type I rat tail collagen was obtained from Sigma-Aldrich (St Louis, MI USA). Collagen was dissolved in 0.1 M glacial Acetic Acid (1 mg/ml) and used to coat cell culture dishes at 10 μg collagen /cm^2 after 1:1 dilution with 50% ethanol.

Inclusion of ethanol in the collagen coating procedures markedly improved the evenness of plate coverage. After coating, dishes were dried in the laminar flow hood and UV irradiated 20 min for sterilization prior to use.

Cell Counts and Assessing the Integrity of the Cell Preparation.

For cell counts, cells were examined under the light microscope and counted using a haemocytometer. Conversion of counts to number of cells/ml provided an estimate of the number of cells loaded onto each dish.

For a rapid assessment of cellular integrity, Trypan Blue exclusion was determined as detailed by Berry *et al.* (1991). 10 μl 2% Trypan Blue in 0.15 M NaCl was diluted in 100 μl of cell suspension. After a 1 min incubation, cells excluding or stained with Trypan Blue were counted. Cell preparations routinely exhibited 70%-80% Trypan Blue exclusion prior to seeding. While higher exclusion rates would have been desirable, subsequent observation of the cell culture revealed that >90% of the cells loaded were attached to the cell culture dish and the hepatocytes remained viable for at least 18 hr by assessment of LDH leakage.

Cell morphology assessments were made by phase contrast microscopy (Olympus CK2 microscope) after attachment to collagen (2-3 hr incubation). Suitable hepatocyte preparations displayed spherical or oval cells exhibiting distinct, highly refractile cell

membranes, with obvious contrast between the cytoplasm and nucleus. Conversely, damaged cell preparations were typified by irregularly shaped cells of granular appearance.

4.2.4 Lactate Dehydrogenase Leakage Assay

Cellular viability was assessed by measuring the amount of lactate dehydrogenase (LDH) released from the hepatocytes into the cell culture medium. At designated time points aliquots of cell culture medium were taken to assay for LDH leakage. At the end of the incubation, the total volume removed was replaced with DMEM/F12. The hepatocyte monolayer was scraped into the medium to produce a cell suspension, and 200 μl was transferred to a microcentrifuge tube. An equal volume of Triton X-100 (1% v/v) was added to lyse the cells before mixing via agitation over the next 30 min. The mix was centrifuged for 1 min at 10,000 g, and the resultant supernatant used to assay total LDH.

LDH was assayed as described by Bergmeyer (1983). To a 1.5 ml cuvette, 1.2 ml of Tris/L-lactate Buffer (112 mM Tris, 56 mM L-Lactate, 170 mM KCl, pH 9.3) was added, followed by 50 µl of 172 mM β-NAD and 100 μl sample. The increase in absorbance at 340 nm was followed for 1 min in a Hitachi U-2000 Spectrometer. The LDH activity in each sample was calculated as U/L, where U is defined as the enzyme required to produce 1µmol NADH per minute. Results were expressed as LDH activity (U/L) in the medium as a percentage of the total LDH activity (U/L) in cells plus medium.

4.2.5 Protein Synthesis Assay

Incorporation of [^{3}H]-Leucine into trichloroacetic acid (TCA) precipitate was used to determine the extent of protein synthesis by minor modification of the procedure described by Freshney (1994). Hepatocytes were incubated with 1 μCi [^{3}H]-Leucine (140 Ci/mmol, NEN™ Life Sciences, Boston, MA, USA) per ml of medium. At the appropriate time the medium was removed, cells were washed with HBSS (2 ml), and then 500 μl ice-cold 10% TCA was added. The monolayer was scraped into the TCA, transferred to a microcentrifuge tube and centrifuged at 5,000 g for 5 min. The supernatant was removed and the 10% TCA wash repeated twice on the pellet. After the final wash, pellets were digested in 250 μl of 0.3 M NaOH at 37°C for ~1hr or until digestion was complete. Next, 200 μl of the cell digest was transferred to 5 ml of Ready Protein+™ (Beckamn Coulter, CA, USA) scintillation fluid. Samples were counted for 5 min in a Beckman LC3801 Scintillation counter. The disintegrations per minute (dpm) response was calculated based upon a pre-defined quench curve using Beckman quench standards. The remaining 50 μl of cell digest was retained for DNA quantification (Section 4.2.6). Results were expressed as dpm/μg DNA. For t=0 reading, hepatocytes were treated with [^{3}H]-Leucine on ice. A negative control (no [^{3}H]-Leucine) was also processed with each experiment to determine background dpm counts. For toxin-treated hepatocytes, [^{3}H]-Leucine was added at the same time as the toxin.

4.2.6 DNA Quantification Assay

DNA was quantified using the method of Labarca and Paigen (1980). This assay utilizes the enhancement of fluorescence observed when Hoechst 33258 binds to DNA. The original buffer was modified to accommodate samples in 0.3 M NaOH, and allow quantification in a 96 well microtitre plate.

The assay buffer consisted of 0.2 M Tris, 0.2 M NaCl, 1 mM EDTA and 1 mg/ml H33258 pH 7.4. Samples (5 µl) were added to 195 µl of buffer and mixed with a pipette tip. Samples were analysed on a fluorescence microplate reader with excitation λ = 356 nm and emission λ = 458 nm or as near to the wavelength maxima as possible. The assay was optimized on the following fluorescence microplate readers: Biolumin 960 (Molecular Dynamics); Perkin Elmer Luminescence Spectrometer LS50B with Perkin Elmer plate reader; POLARStar Galaxy (BMG Lab Technologies). Each data set was completed on one microplate reader. Calf Thymus DNA (Boehringer Mannheim, Germany) in 0.3 M NaOH was used to construct a standard curve (Figure 4.1). Standards were run on each day of analysis in triplicate. Samples were run in duplicate.

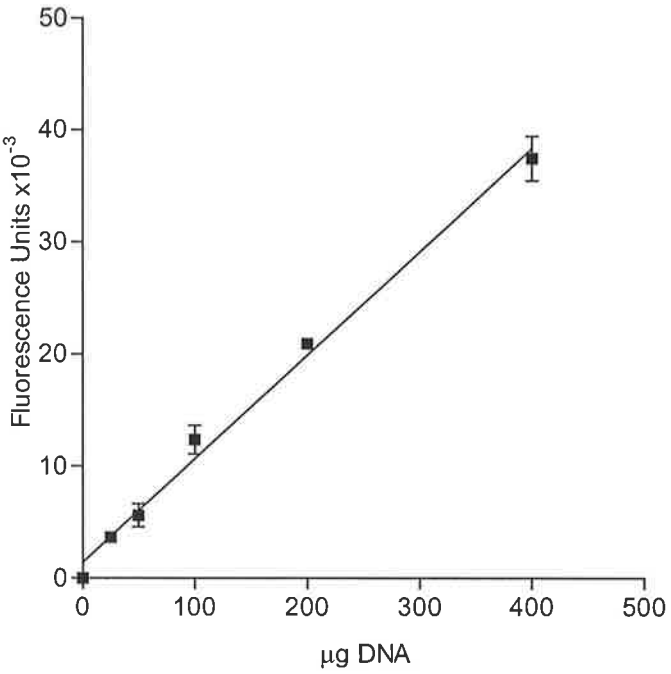


Figure 4.1 DNA standard curve. Hoechst 33258 fluorescence was measured at 460 nm (excitation was at 355 nm) using a POLARstar Galaxy (BMG Lab Technologies) microplate reader. Linear regression, slope = 0.092, Y intercept = 0.077, $r^2 = 0.98$. Data are mean ± SE, n = 3.

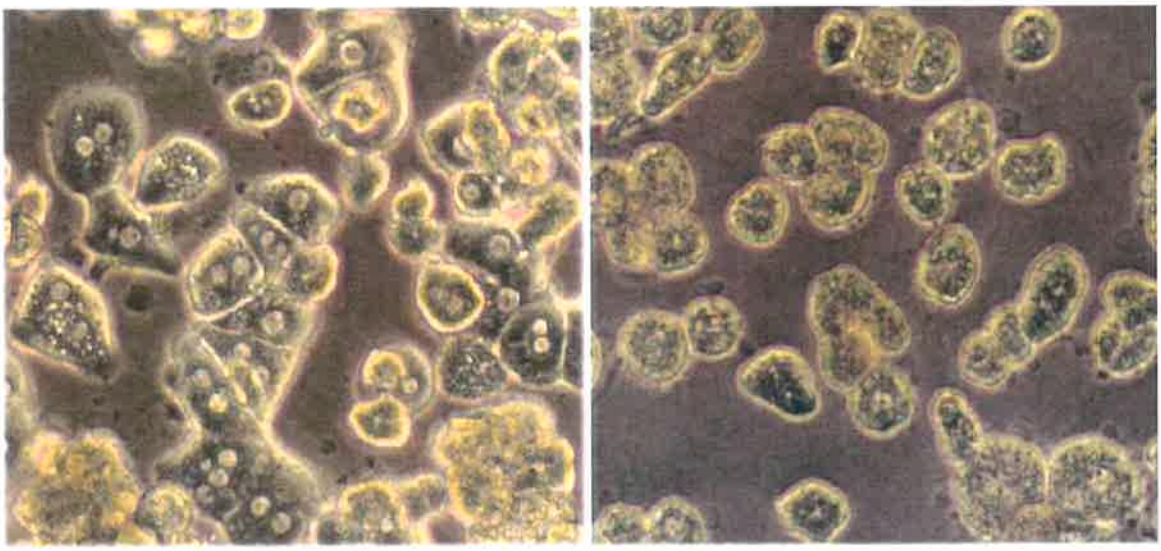
4.2.7 Statistical Analysis

Graph Pad Prism version 3.00 for Windows, (GraphPad Software, SanDiego, CA, USA) was used for all statistical tests and graphing. Analysis of data was by 1 way analysis of variance (ANOVA) at each time point. If a significant variance ratio was noted, the data were further analysed by Dunnett's test for comparison to control only, or Tukey's test if comparison to control and other experimental groups was required. Statistical significance was accepted at $P < 0.05$.

4.3 Results

4.3.1 Hepatocyte Morphology after Cylindrospermopsin Treatment

Cylindrospermopsin exposure produced concentration and time dependent alterations in hepatocyte morphology. Treatment with 5 μM toxin caused progressive rounding of hepatocytes from 12-18 hr until most displayed the characteristics shown in Figure 4.2. Although rounded, hepatocytes did not form a smooth sphere. The cell membrane was often irregular and the cytoplasm was granular in appearance. Most hepatocytes were still attached to the substratum. Occasionally a few hepatocytes appeared unaffected. In contrast, by 18 hr in culture the control hepatocytes were well attached and spread out on the substratum, forming connections with adjacent hepatocytes. The cell membrane was smooth, cytoplasm was clear and the nuclei were visible.



a. **b.**

Figure 4.2 Morphology of (a) control and (b) 5 μM cylindrospermopsin treated hepatocytes after 18 hr incubation at 37 °C, 5% CO_2. Cells were visualized by phase contrast microscopy using an Olympus CK2 microscope. Magnification X 250.

4.3.2 Effect of Cylindrospermopsin on Cell Viability

Exposure to cylindrospermopsin caused concentration dependent loss of hepatocyte viability as assessed by LDH leakage. A significant increase in LDH leakage at 18 hr was observed after treatment of hepatocytes with a concentration of 1 μM or greater (Figure 4.3). 1 μM toxin resulted in 52 ± 8% LDH leakage (27% increase above control) while 5 μM caused 82 ± 8% LDH leakage (57% increase above control).

4.3.3 Time Course for LDH Leakage after Exposure to Cylindrospermopsin

The time course for LDH leakage after cylindrospermopsin exposure illustrated a delayed onset of toxicity (Figure 4.4). LDH leakage from hepatocytes exposed to 5 μM cylindrospermopsin (the highest test concentration) increased after 10 hr of exposure to the toxin. Up to and including this point the LDH levels closely resembled the controls.

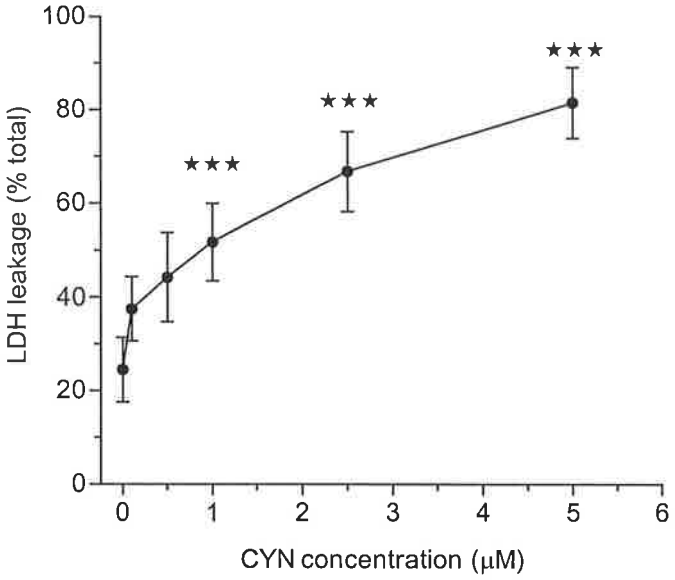


Figure 4.3 Lactate dehydrogenase (LDH) leakage from hepatocytes exposed to cylindrospermopsin (CYN) for 18 hr. Values represent mean ± SE of 4 independent experiments. The data were analysed by one-way ANOVA (repeated measures) followed by Dunnett's test. Significant differences from control are indicated as ★★★ ($p<0.001$).

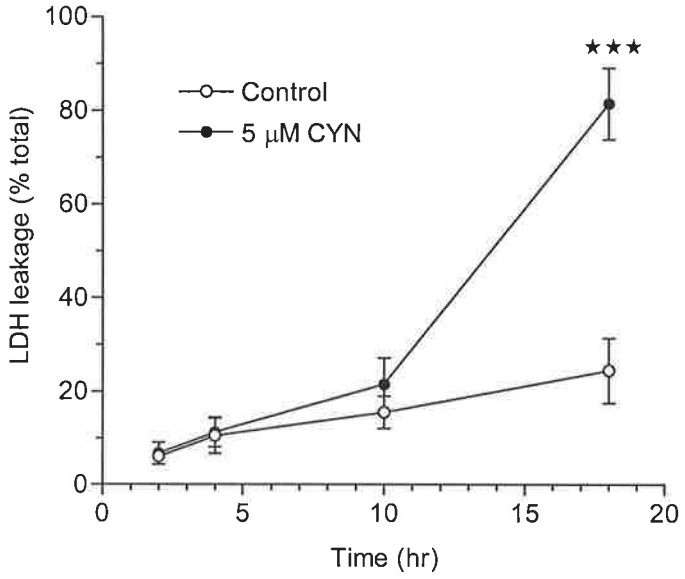


Figure 4.4 Time course for lactate dehydrogenase (LDH) leakage from hepatocytes exposed to 5 μM cylindrospermopsin (CYN). Values represent mean ± SE of 4 independent experiments. The data were analysed by one-way ANOVA (repeated measures) followed by Dunnett's test. Significant difference from control at the defined time point is indicated ★★★($p<0.001$).

4.3.4 Effect of Cylindrospermopsin on Protein Synthesis

Protein synthesis in hepatocytes was expressed as [^{3}H]-Leucine incorporation per μg DNA. Significant inhibition of protein synthesis was observed after treatment of hepatocytes for 18 hr with a concentration of 0.5 μM cylindrospermopsin or greater (Figure 4.5). The extent of inhibition over the 10-fold concentration range 0.5-5 μM varied only slightly, with these concentrations producing 74% - 88% inhibition compared to the control respectively. Protein synthesis after treatment with 0.1 μM cylindrospermopsin was similar to the control.

4.3.5 Time Course for Protein Synthesis Inhibition after Cylindrospermopsin Exposure

The time course in Figure 4.6 illustrates that protein synthesis inhibition induced by cylindrospermopsin occurred early in the 18 hr incubation period. For concentrations 0.5-5 μM cylindrospermopsin, inhibition was maximal by 4 hr, so that no further protein synthesis occurred during the subsequent 4-18 hr period. This was confirmed statistically by linear regression. No significant deviation from zero was observed for protein synthesis over this period for the 0.5-2.5 μM treatments. The insert in Figure 4.6 shows the linear regression data for cells treated with 0.5 μM cylindrospermopsin over 4-18 hr ($r^2 = 0.002$). Although 5 μM cylindrospermopsin inhibited protein synthesis to the greatest extent at all time points, linear regression revealed a significant deviation from zero ($r^2 = 0.72$, data not shown). This can be explained on the basis of DNA quantifications. A significant decline in cellular DNA occurred over time for the 5 μM treated group. At 18 hr the DNA quantification was 4.67 ± 1.15 μg DNA/plate in contrast to 8.5 ± 1.73 μg DNA/plate for the controls.

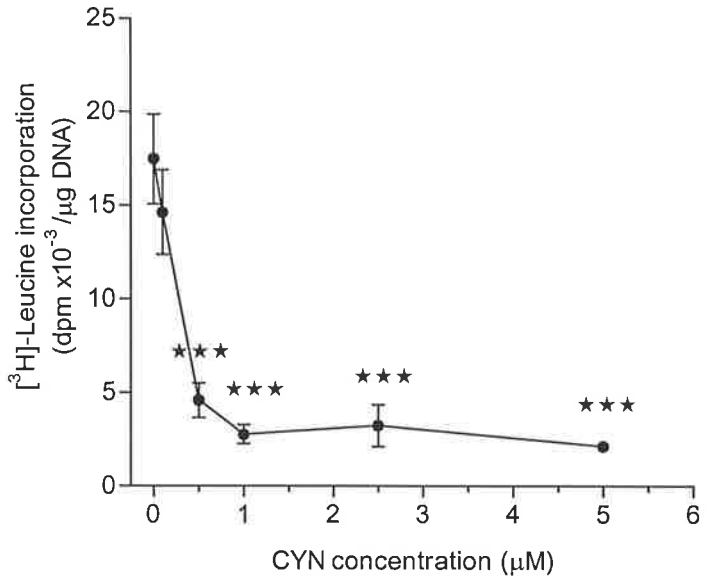


Figure 4.5 Inhibition of protein synthesis in hepatocytes exposed to cylindrospermopsin (CYN) for 18hrs. Values represent mean ± SE of 3-4 independent experiments. The data were analysed by one-way ANOVA followed by Dunnett's test. Significant differences from control are indicated as ★★★($p<0.001$).

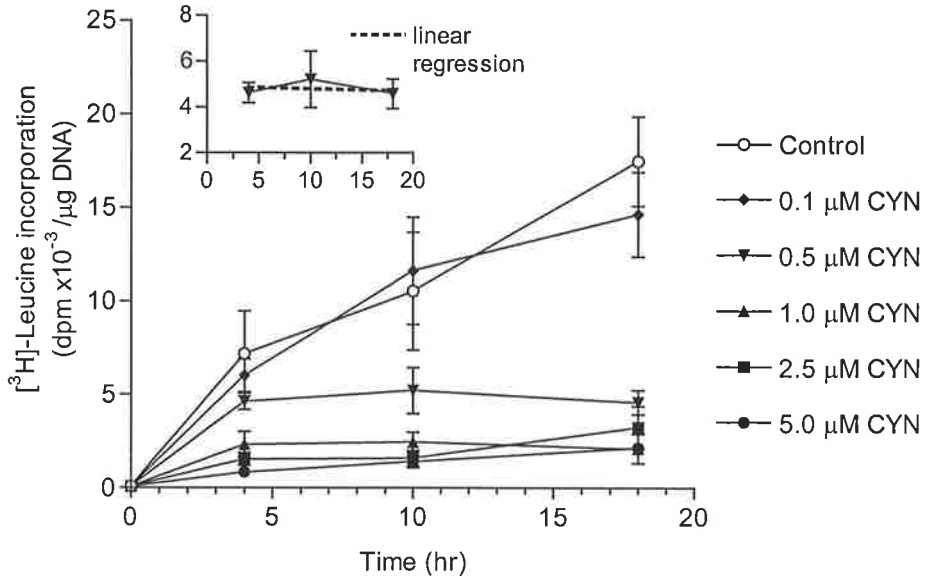


Figure 4.6 Time course for the inhibition of protein synthesis in hepatocytes by cylindrospermopsin (CYN). Values represent the mean ± SE of 3-4 independent experiments.

4.3.6 Is the loss of Hepatocyte Viability a Result of Protein Synthesis Inhibition?

Comparison of the concentration response and time course graphs for LDH leakage and protein synthesis after cylindrospermopsin exposure (Sections 4.3.2 to 4.3.5), reveals that significant inhibition of protein synthesis occurred at a non toxic cylindrospermopsin concentration of 0.5 μM, (compare Figure 4.3 to Figure 4.5) and well prior to the onset of LDH leakage produced by 1-5 μM concentrations of cylindrospermopsin (compare Figure 4.4 to Figure 4.6). Overlaying the 18 hr concentration response curves, expressed as a percentage control viability or protein synthesis, clearly illustrates the unrelated nature of the two cellular responses (Figure 4.7). As shown, 0.5-5 μM toxin extensively inhibit protein synthesis, all maximally for at least 14 hr (refer to the time course in Figure 4.6), while causing a clearly graded response to loss of cellular viability.

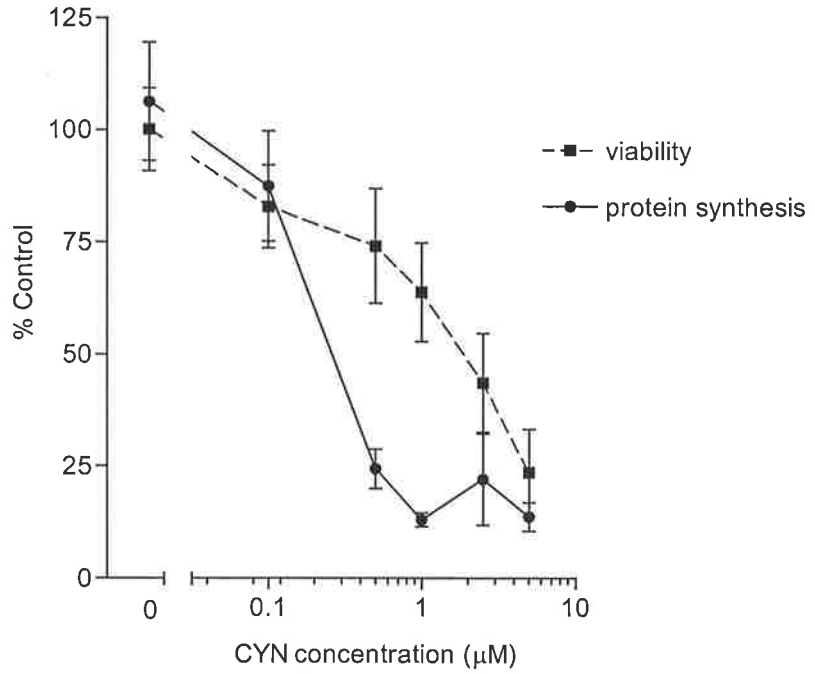


Figure 4.7 Loss of viability and protein synthesis in hepatocytes exposed to varying concentrations of cylindrospermopsin (CYN) for 18 hr. All values are mean ± SE of 3-4 independent experiments.

4.3.7 Irreversible Inhibition of Protein Synthesis after Short-Term Exposure to Cylindrospermopsin

Exposure of hepatocytes to cylindrospermopsin (5 μM), cycloheximide (10 μM) or emetine (10 μM) for 1 hr inhibited protein synthesis by 70 ± 11%, 91 ± 3.6% and 94 ± 1.7% respectively (mean ± SE of 3 experiments). Following removal of the toxin and washing of hepatocytes (twice with phosphate buffered saline (PBS)), protein synthesis was followed for 2 hr in fresh culture medium. At the end of the incubation period, both cylindrospermopsin and emetine treated cells had not regained the ability to carry out protein synthesis (Figure 4.8). They were both significantly different from the control (1 way ANOVA, followed by Tukey's test at 120 min, $p<0.001$) and cycloheximide pre-treated hepatocytes ($p<0.01$). In contrast, after removal of cycloheximide, hepatocytes regained the ability to carry out protein synthesis at a similar rate observed for the controls (no significant difference). There was no significant difference between cylindrospermopsin and emetine treated hepatocytes.

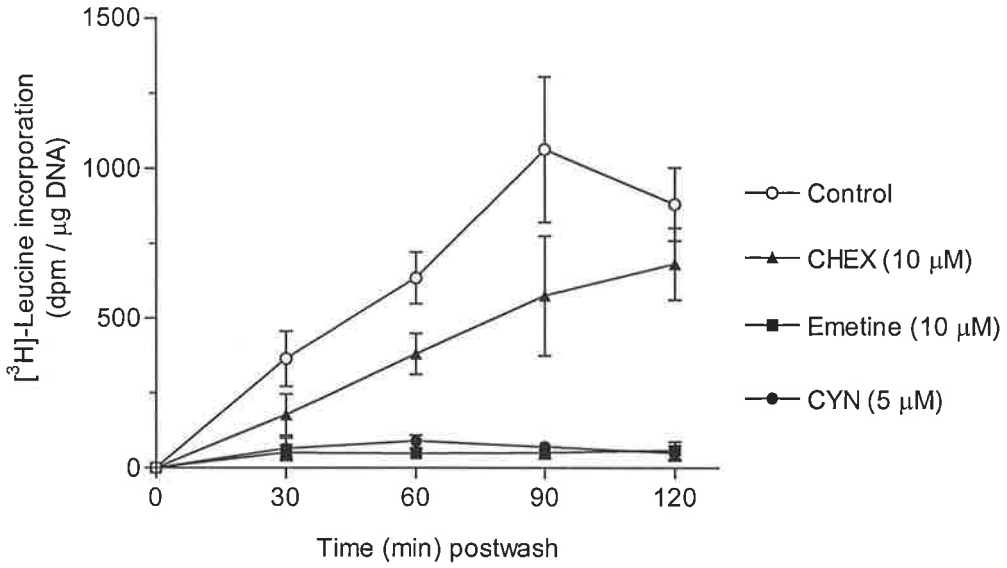


Figure 4.8 Protein synthesis in hepatocytes after toxin removal. After 1hr incubation with cylindrospermopsin (CYN), cycloheximide (CHEX) or emetine, hepatocytes were washed twice with PBS, and the culture medium replaced. Values are mean ± SE for 3 independent experiments. The data were analysed by 1 way ANOVA followed by Tukey's test at 120 min and showed significant differences between control and CYN or emetine treated hepatocytes ($p<0.001$), and between CHEX and CYN or emetine treated hepatocytes ($p<0.01$).

4.3.8 Inhibition of Protein Synthesis in Hepatocytes and Reticulocyte Lysates: Comparison of Concentration-Response Curves.

Since 0.5-5 μM cylindrospermopsin was found to maximally inhibit protein synthesis in hepatocytes after 4 hr (Figure 4.6), the concentration-response curve was re-adjusted to exclude the protein synthesis occurring prior to this time point. The extent of protein synthesis from 4-18 hr was calculated for each treatment and expressed as a percentage of the control. Protein synthesis inhibition in the reticulocyte lysate was analysed as detailed in Section 3.3.1.

Comparison of the inhibition of protein synthesis in a hepatocyte (cellular) and reticulocyte lysate (cell-free) model illustrates the similarity of the concentration-response curves (Figure 4.9). Both displayed a steep concentration-response curve and a similar effective concentration range. The IC_{50} for protein synthesis inhibition in hepatocyte culture was ~200 nM, approximately 2 fold that determined in the reticulocyte lysate (120 nM).

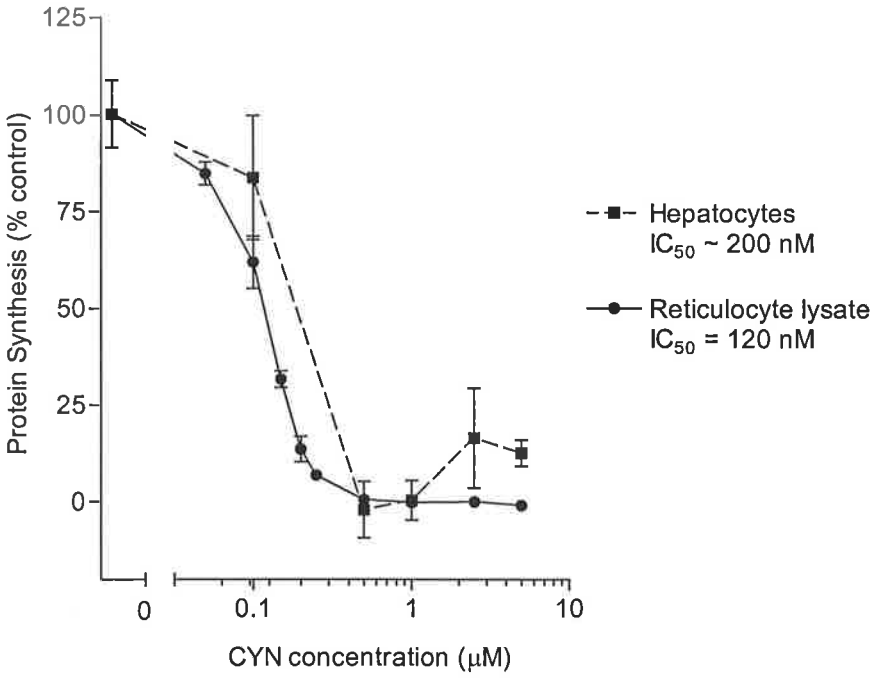


Figure 4.9 Inhibition of protein synthesis by cylindrospermopsin (CYN) in a cellular (hepatocyte) vs cell-free (reticulocyte lysate) model. All values are mean ± SE for 3-4 experiments. The reticulocyte lysate IC_{50} was calculated from the sigmodal concentration response curve (variable slope) as described in Chapter 3. The hepatocyte IC_{50} was estimated from the available data.

4.4 Discussion

The concentration-response and time course for LDH leakage from mouse hepatocytes exposed to cylindrospermopsin in this study was similar to that described by Runnegar *et al.* (1994) in rat hepatocytes. Following the establishment of toxicity parameters, it was demonstrated that protein synthesis inhibition in hepatocytes was a sensitive, early indicator of cellular responses to cylindrospermopsin, occurring at non-toxic cylindrospermopsin concentrations and well prior to the onset of toxicity at higher concentrations. The protein synthesis inhibition occurred over a remarkably similar concentration range to that observed in reticulocyte lysates and could not be reversed following removal of the toxin.

Supportive of the hypothesis that protein synthesis inhibition is not solely responsible for cylindrospermopsin toxicity in hepatocytes (see General Introduction, Chapter 1) was the observation that cylindrospermopsin caused a concentration-dependent increase in LDH leakage at 18 hr over a concentration range eliciting maximal inhibition of protein synthesis (an effect that was fully evident from at least 4hr). This reveals that the two parameters are not influenced in parallel. If protein synthesis is inhibited to the same extent in hepatocytes exposed to 0.5 μM cylindrospermopsin and higher concentrations, other cylindrospermopsin-induced effects must contribute to the toxic process in order to produce the concentration-dependent response. It was considered unlikely that the hepatocyte cell death over the time frame investigated was due to protein synthesis inhibition and that the difference in toxicity observed over the tested concentration range was merely a time-delay effect, depending upon how rapidly protein synthesis was completely inhibited within the first few hours of toxin exposure.

Characteristic of the protein synthesis inhibition by cylindrospermopsin in hepatocytes was the delayed response occurring within the first 4 hr of exposure to the toxin. The extent of delay was concentration dependent. This may be a reflection of the mechanism of uptake of the toxin. Transport of chemicals across the hepatocyte plasma membrane can occur directly by diffusion or via membrane transporters. Active uptake is mediated by distinct transporters; bile salt and anionic transporters that are either Na+-dependent or Na+-independent and

organic cation transporters (Lecureur *et al.*, 2000). The broad multi substrate specificities of anion transport systems also allows the entry of some xenobiotics (Frimmer and Ziegler, 1988; St-Pierre *et al.*, 2001). To date, only one paper has explored the potential entry routes of cylindrospermopsin into hepatocytes or other cell types. Chong *et al.* (2002) demonstrated that partial protection against cylindrospermopsin toxicity in rat hepatocytes was afforded by coincubation with high concentrations of bile acid salts, the normal substrate for the bile acid transporter system. This protection however, was not maintained over time. This suggested that the bile acid transporter system plays some role in cylindrospermopsin transport into hepatocytes, but that another uptake route also contributes. The subsequent incubation of the epithelial carcinoma cell line KB with cylindrospermopsin resulted in cell death despite the lack of the bile acid transporter in this cell type. The conclusion reached was that passive diffusion is a likely mechanism of uptake by cylindrospermopsin into hepatocytes and other cell types. The current observations of protein synthesis inhibition in this Chapter are consistent with passive diffusion as the entry mechanism of cylindrospermopsin into hepatocytes. The rationale for this is discussed below.

The process of active uptake is rapid, and works against a concentration gradient, accumulating the toxin within the cell. The active uptake of microcystin, facilitated by an anion transporter system similar to the bile acid transporter system (Runnegar *et al.*, 1995a), demonstrates these attributes. It has been shown that after incubation of hepatocytes with radiolabelled microcystin (17 nM) for 60 min, the intracellular microcystin concentration reaches 0.9 μM, 80 times the original medium concentration (Runnegar *et al.*, 1991). Other researchers have reported that the uptake of radiolabelled microcystin into rat hepatocytes reaches a plateau within 5-6 min (Eriksson *et al.*, 1990a). In contrast, the ultimate result of passive diffusion is that the concentration of diffusing substance is in equilibrium on both sides of the membrane, and the rate of transport is proportional to the concentration difference. As shown, the effective concentration range for protein synthesis inhibition by cylindrospermopsin was remarkably similar in hepatocytes to that obtained in the cell-free reticulocyte lysate. If cylindrospermopsin was actively taken up and concentrated in hepatocytes, it would have been expected that the effective concentration in hepatocyte culture was lower when compared to the cell-free system. Furthermore, since the rate of transport by passive diffusion is proportional to the concentration difference across a

membrane, higher concentrations on one side of the membrane will reach a defined concentration on the other side more rapidly than that obtained by a lower dose. If we consider 0.5 μM cylindrospermopsin the minimum concentration required to completely inhibit protein synthesis in hepatocytes, incubation of cells with 5 μM toxin will reach an internal threshold concentration of 0.5 μM and achieve total inhibition of protein synthesis more rapidly than in cells incubated with e.g. 1 μM cylindrospermopsin. Thus the concentration-dependent inhibition of protein synthesis within the first 4 hr of exposure to cylindrospermopsin can be explained on the basis of passive diffusion.

While both the conclusions reached above are indirect, they suggest passive diffusion as the major mechanism of entry into hepatocytes, and support the observations of Chong *et al.* (2002). Further conclusive information may be obtained in the future using radiolabelled cylindrospermopsin in hepatocyte culture.

While cylindrospermopsin may enter hepatocytes by diffusion, clearly it does not readily diffuse out. Removal of cylindrospermopsin after a short term (1 hr) exposure did not allow protein synthesis to recommence. The behaviour of cylindrospermopsin was similar to emetine, a known irreversible inhibitor of protein synthesis. Association of cylindrospermopsin with the protein synthesis machinery may also be irreversible. The results presented in Chapter 3 in reticulocyte lysate demonstrated that cylindrospermopsin potently inhibited protein synthesis, and differences between the concentration-response curve to that of the reversible inhibitor cycloheximide were noted. However, irreversible effects may extend beyond protein synthesis inhibition. Runnegar *et al.* (1995b) showed that after exposure of hepatocytes to cylindrospermopsin for 12 hr, continued presence of the toxin was not required during the late phase (12-17 hr) of toxicity. While extensive damage may have occurred by 12 hr that was unable to be reversed, another possibile explanation is that cylindrospermopsin derived metabolite(s) remain intracellular after removal of the parent toxin, thereby prolonging the toxin's effects. This could explain both the irreversible protein synthesis inhibition and results demonstrated by Runnegar *et al.* (1995b).

The results described in this Chapter have raised a number of issues for further investigation. If protein synthesis inhibition cannot be considered the primary cause of

cytotoxicity in hepatocytes over an acute time frame, what other factors contribute to the cell death? Chapter 5 investigates the role of oxidative stress in cylindrospermopsin toxicity.

Futhermore, the similarity of cylindrospermopsin's protein synthesis potency in metabolically active hepatocytes and metabolically inert reticulocyte lysate indirectly suggests that the protein synthesis inhibition may be due to cylindrospermopsin itself, and independent of the action of potential (unidentified) metabolite(s) in hepatocytes. This is further investigated in Chapter 6.

5 THE ROLE OF OXIDATIVE STRESS IN CYLINDROSPERMOPSIN-INDUCED TOXICITY. HEPATOCYTE CULTURE STUDIES

5.1 Introduction

Oxidative stress can be defined as a disturbance in the prooxidant-antioxidant balance in favour of the former with potential for injury (Sies, 1997). For a number of chemicals, oxidative stress plays a critical role in the mechanism of toxicity. Xenobiotics can induce an oxidative stress by a number of mechanisms including: overproduction of reactive oxygen species such as the superoxide anion radical ($O^{\cdot}_2$-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$); production of radical metabolites (e.g. carbon tetrachloride derived radicals); weakening cellular antioxidant defence mechanisms; or a combination of these events (Recknagel *et al.*, 1991). The antioxidant defence mechanisms used to counteract reactive species include Vitamin E, superoxide dismutase, catalase and the GSH dependent systems. As discussed in Chapter 1, the most crucial intracellular antioxidant defence mechanism is dependent upon GSH (refer to Figure 1.7) and cylindrospermopsin treatment is known cause its depletion in hepatocytes via inhibition of GSH synthesis (Runnegar *et al.*, 1994; Runnegar *et al.*, 1995b). Decreasing or overwhelming the antioxidant defence mechanisms provides potential for the oxidative deterioration of membrane lipids, which can be initiated by some radical species. Due to the destructive nature and toxicity of end products, lipid peroxidation is often proposed as a mechanism of toxicity (Horton and Fairhurst, 1987). Lipid peroxidation products with toxic potential include a number of unsaturated aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (Esterbauer, 1982). In many cases oxidative stress caused by GSH depletion results in lipid peroxidation (Recknagel *et al.*, 1991).

This chapter investigates the consequences of cylindrospermopsin-induced GSH depletion in hepatocyte cultures. It is hypothesized that following decline in GSH levels, enhanced lipid peroxidation occurs producing toxic products that amplify the toxic process. Malondialdehyde production, a commonly used measure of lipid peroxidation (Jentzsch *et al.*, 1996; Liu *et al.*, 1997; Poli and Gravela, 1982), was assayed after toxin exposure. Furthermore, if reactive oxygen species and oxidative mechanisms are involved in cylindrospermopsin toxicity, the activity of the glutathione peroxidase / glutathione reductase (GSH-Px / GSSG-Rd) enzyme system (see Figure 1.7) would be important in protecting against the toxicity. In order to investigate the role of this enzyme system in the toxicity process, 1,3-bis (chloroethyl)1-nitrosourea (BCNU) was used to inhibit GSSG-Rd activity and further compromise the cells ability to cope with oxidative stress. Inhibition of GSSG-Rd activity in hepatocytes is known to potentiate the toxicity of compounds that induce damage via oxidative mechanisms. Examples of such chemicals include the analgesic acetaminophen (paracetamol) (Adamson and Harman, 1993); the herbicide diquat (Tsokos-Khun, 1988) and the aryl halide bromobenzene (Coleman *et al.*, 1990).

In this study, *tert*-butylhydroperoxide (*t*-BH) was used as a positive control as it induces oxidative damage. The onset of *t*-BH cytotoxicity in hepatocytes is preceded by GSH depletion and extensive lipid peroxidation (Jewell *et al.*, 1986; Masaki *et al.*, 1989).

5.2 Materials and Methods

5.2.1 Materials

Glutathione (GSH) and *tert*-butyl hydroperoxide (*t*-BH) were obtained from Sigma-Aldrich (St Louis, MI USA). 1,3-bis (chloroethyl)-l-nitrosourea (BCNU) was obtained from Bristol Laboratories (NJ USA). Butylated hydroxytoluene (BHT) was obtained from ICN Biochemicals (OH, USA). Cylindrospermopsin was purified from *C.raciborskii* extracts as described in Chapter 2.

5.2.2 Preparation of Cultured Hepatocytes

The isolation and culture of Swiss Albino mouse hepatocytes for use in this study was as detailed previously in Section 4.2.3.

5.2.3 Measurement of Toxicity

Toxicity was measured by the leakage of lactate dehydrogenase (LDH) from hepatocytes as detailed in Section 4.2.4.

5.2.4 Glutathione Determination

Glutathione (GSH) levels were determined based on the fluorometric method of Hissin and Hilf (1976). This method utilizes the reaction of GSH with the fluorescent reagent *o*-phthalaldehyde. The original buffer was modified to allow quantification by assay in a 96 well microplate.

To process hepatocyte monolayers for the GSH assay, the cell culture medium was first removed and the cells washed with HBSS (2 ml). 500 μl of 3% perchloric acid (PCA) was added to each 36 mm dish. The monolayer was scraped into the PCA, transferred to a microcentrifuge tube and centrifuged at 5000 g for 5 min to pellet the cells. The supernatant was removed for GSH determination while the pellet was retained for DNA quantification as described in Section 4.2.6.

The assay buffer consisted of 0.2 M Tris, 0.2 M NaCl and 1 mM EDTA (pH 8.0). To maintain pH at 8.0 in the above buffer system, PCA samples were neutralised with NaOH (16 μl of 2.5 M NaOH to every 100 μl 3% PCA sample). 200 μl of Buffer, 50 μl of NaOH treated PCA sample and 10 μl of 1mg/ml *o*-phthalaldehyde stock prepared in methanol were loaded sequentially into microplate wells. The reaction mix was incubated at room temperature for 20 min and then fluorescence determined at excitation $\lambda = 340$ nm, emission $\lambda = 420$ nm on a Biolumin 960 (Molecular Dynamics) microplate reader. The GSH content of the cells was expressed as ng GSH /μg DNA. On each day of analysis, a GSH standard curve was constructed using reduced GSH (Figure 5.1). Standards were analysed in triplicate. Samples were analysed in duplicate.

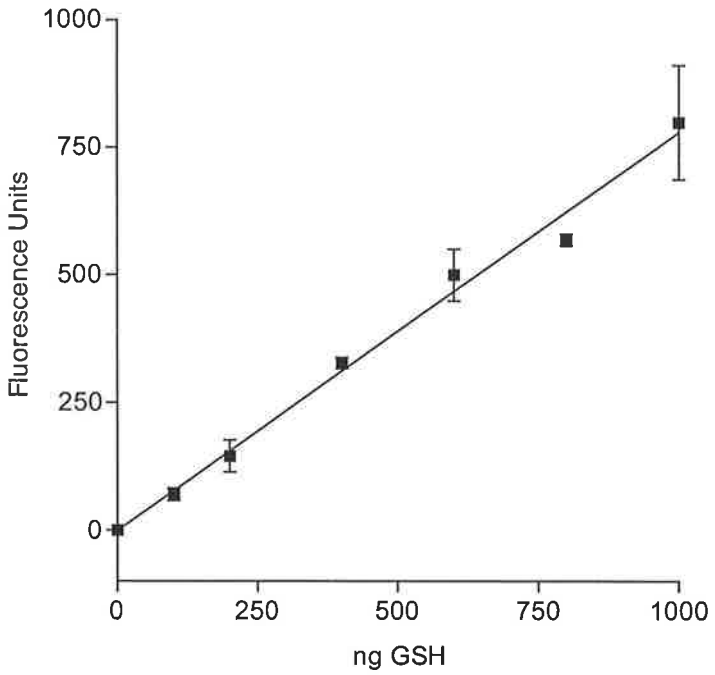


Figure 5.1 Representative glutathione (GSH) standard curve. Fluorescence was monitored at 420 nm (excitation was at 340 nm). Data are mean ± SE, n = 3. Linear regression, slope = 0.78, Y intercept = -1.3, $r^2 = 0.93$.

5.2.5 Malondialdehyde Assay

The lipid peroxidation product malondialdehdye (MDA) was assayed using the BIOXYTECH® LPO-586™ kit obtained from OXIS International, Inc (Portland, OR, USA). The procedure was carried out with minor modification of the manufacturer's instructions. The assay is based on the reaction of N-methyl-2-phenylindone with MDA to yield a chromophore that absorbs strongly at 586 nm (Gérard-Monnier *et al.*, 1998).

To determine cellular MDA levels in hepatocyte culture, the cell culture medium was removed and the hepatocyte monolayer was washed with 20 mM Tris Buffer (pH 7.4). Cells were scraped from the bottom of the dish using a cell scraper (costar®, MA, USA) and into 100 µl 20 mM Tris Buffer containing 5 mM butylated–hydroxytoluene (BHT) to prevent further sample oxidation. To provide adequate sensitivity in MDA assay, the contents of three 36 mm hepatocyte dishes were pooled for each sample. Samples were sonicated on ice for 3 x 20 s at 50W using a Braun Labsonic 1510 Sonicator. Samples were then centrifuged at 3000 g for 5 min. The supernatant was used for MDA determination as described below. The remaining sample (mostly pellet) was precipitated in 2 washes of 10% TCA and digested in 0.3 M NaOH for DNA quantification as previously described in Section 4.2.6. Results were expressed as ng MDA/ µg DNA for each sample.

For assay, a 300 µl sample or standard was added to 650 µl of 2.5 mM N-methyl-2-phenylindole in a mixture of acetonitrile/methanol (3:1). The reaction was started by the addition of 150 µl of 37% HCl (12 N). Samples were vortexed briefly, and then tubes were capped and incubated for 60 min at 45°C. The samples were then clarified by centrifugation (15,000 g, 10 min), transferred to a 1 ml cuvette before the absorbance at 586 nm was measured against a blank (20 mM Tris buffer). The contribution of the hepatocyte sample to A_{586} was negligible (sample blank).

Due to the instability of MDA itself, the diethylacetal 1,1,3,3-tetramethoxypropane (TMOP) was used as the source of MDA. It is hydrolysed to MDA during the 60 min incubation at 45°C in the presence of HCl. TMOP standards were prepared in 20 mM Tris

Buffer to generate a standard curve of 0.6-9 μM TMOP final concentration in the reaction mix (Figure 5.2). The slope of the observed standard curve was within 5% of that expected based on the molar extinction coefficient (ϵ) of MDA (110,000) and was used to calculate the concentration of MDA in each sample. A buffer blank was included with each assay set.

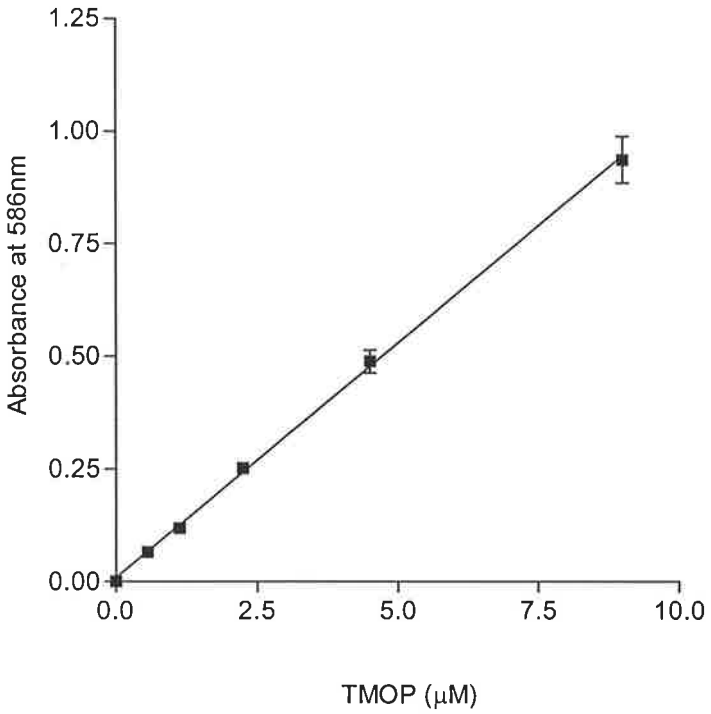


Figure 5.2 Malondialdehdye standard curve generated with the diethylacetal standard, 1,1,3,3-tetramethoxypropane (TMOP). Data are mean ± SE, n = 3. Linear regression, slope = 0.10, Y intercept = 0.0078, $r^2 = 0.99$.

5.2.6 Inhibition of GSSG-Rd Activity

Glutathione disulphide reductase (GSSG-Rd) activity was inhibited by treatment of hepatocytes with 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) as described by Adamson and Harman (1989). BCNU was dissolved in ethanol. Hepatocytes were exposed to 100 μM BCNU (0.5% ethanol) for 30 min followed by a 2 hr recovery incubation in BCNU-free media. Cells were then exposed to cylindrospermopsin or *t*-BH for the required incubation period. Vehicle (0.5% ethanol) treated hepatocytes were used as the control.

5.2.7 Statistical Analysis

Graph Pad Prism version 3.00 for Windows, (GraphPad Software, SanDiego, CA, USA) was used for all statistical tests and graphing. Analysis of data was by 1 way analysis of variance (ANOVA) at each time point. If a significant variance ratio was noted, the data were further analysed by Dunnett's test for comparison to control only or Tukey's test if comparison to control and other experimental groups was required. Where two experimental groups only were present a t-test was used to analyse the data. Statistical significance was accepted at $P < 0.05$.

5.3 Results

5.3.1 Glutathione Depletion

Glutathione (GSH) depletion in hepatocytes exposed to cylindrospermopsin was both concentration and time dependent. Significant depletion of GSH at 18 hr was observed after treatment of hepatocytes with a dose of 1 μM cylindrospermopsin or greater (Figure 5.3). Extensive GSH depletion was observed over the concentration range 1-5 μM. After 18 hr, mean GSH levels were reduced to 14% and 6% of control levels (176 ± 78 ng GSH/ μg DNA) by 1 μM and 5 μM cylindrospermopsin treatments respectively (Figure 5.3). The concentrations of cylindrospermopsin inducing significant GSH depletion correspond closely to those concentrations causing significant elevation of LDH levels at the same time point (refer to Figure 4.3).

The time course for GSH depletion in cells treated with 5 μM cylindrospermopsin is shown in Figure 5.4. Over the 18 hr incubation period, mean control GSH levels were maintained within 130-210 ng GSH/ μg DNA. In contrast, for 5 μM cylindrospermopsin treated hepatocytes, GSH was maintained at control levels up until 4 hr only. Following this time, the GSH level progressively decreased to the very low levels evident at 18 hr. A significant reduction in GSH compared to controls was observed after 10 hr in culture. As observed previously at 10 hr, no toxicity (LDH leakage) was evident at this time point (refer to Figure 4.4).

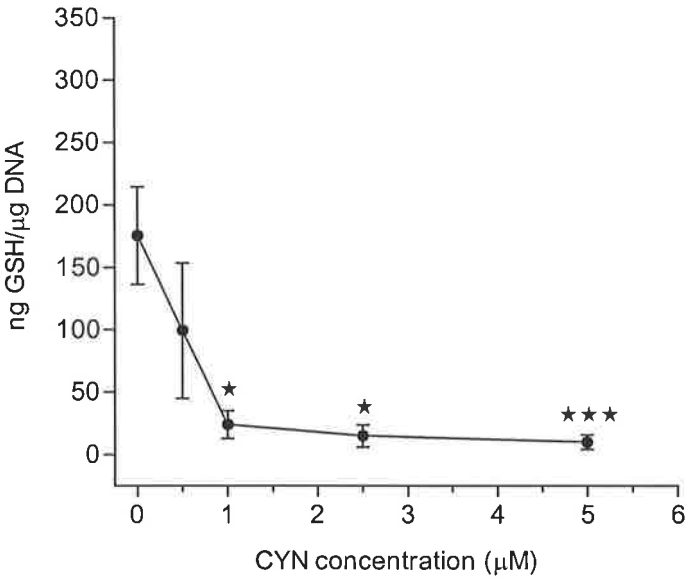


Figure 5.3 Concentration response for glutathione (GSH) depletion in hepatocytes exposed to cylindrospermopsin (CYN) for 18hrs. Values represent mean ± SE of 4 independent experiments. The data were analysed by one-way ANOVA (repeated measures) followed by Dunnett's test. Significant differences from control are indicated as ⋆ ($p<0.05$) or ⋆⋆⋆ ($p<0.001$).

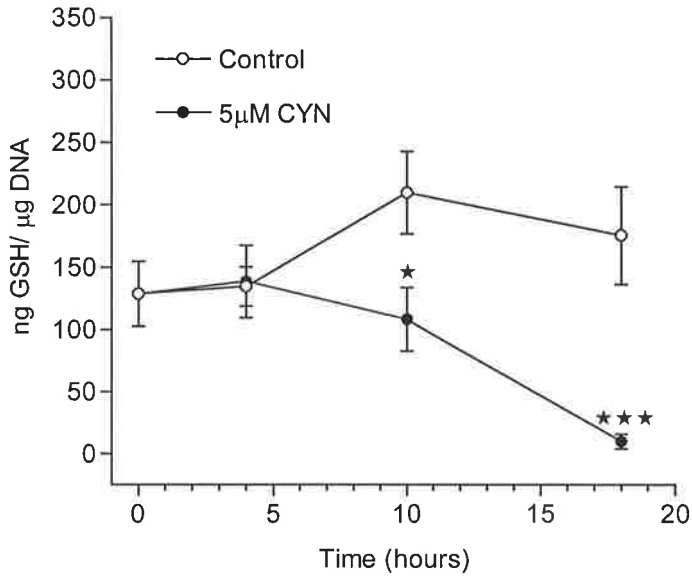


Figure 5.4 Time course for glutathione (GSH) depletion in hepatocytes exposed to 5µM cylindrospermopsin (CYN). Values at 18 hrs are mean ± SE of 4 independent experiments. All other values are mean ± SE of 5 independent experiments. The data were analysed by one-way ANOVA (repeated measures) followed by Dunnett's test. Significant differences from respective control are indicated as ⋆ ($p<0.05$) or ⋆⋆⋆ ($p<0.001$).

5.3.2 Analysis of Lipid Peroxidation Products

As demonstrated in the previous section, significant GSH depletion occurs in hepatocytes exposed to 5 μM cylindrospermopsin for 10 hr, prior to the onset of LDH leakage. To implicate lipid peroxidation products as a cause, rather than a result of cell death, it was required to investigate the extent of lipid peroxidation prior to the onset of LDH leakage. Exposure of hepatocytes to 5 μM cylindrospermopsin for 12 hr was chosen as an appropriate concentration and time point for malondialdehyde (MDA) measurement. At this time, GSH levels in cylindrospermopsin treated hepatocytes were expected to be <50% of the control and declining. No LDH leakage was expected. The effect of BCNU pre-treatment on MDA formation was also examined at this time point.

After exposure of hepatocytes to 5μM cylindrospermopsin for 12 hr, MDA levels were comparable to control levels (Figure 5.5a). A similar response was observed for hepatocytes pretreated with BCNU prior to 5μM cylindrospermopsin exposure. In contrast, treatment of hepatocytes with 0.25-1 mM *t*-BH for 30 min demonstrated a concentration-dependent increase in MDA to 3 fold the control levels (Figure 5.5b). This increase in MDA was observed without significant loss of cellular integrity (Table 5.1). The MDA levels in control hepatocytes analyzed either after 30 min or 12 hr incubation remained comparable, 58 ± 48 and 68 ± 15 pmoles MDA/ μg DNA respectively (compare controls in (b) and (a) of Figure 5.5).

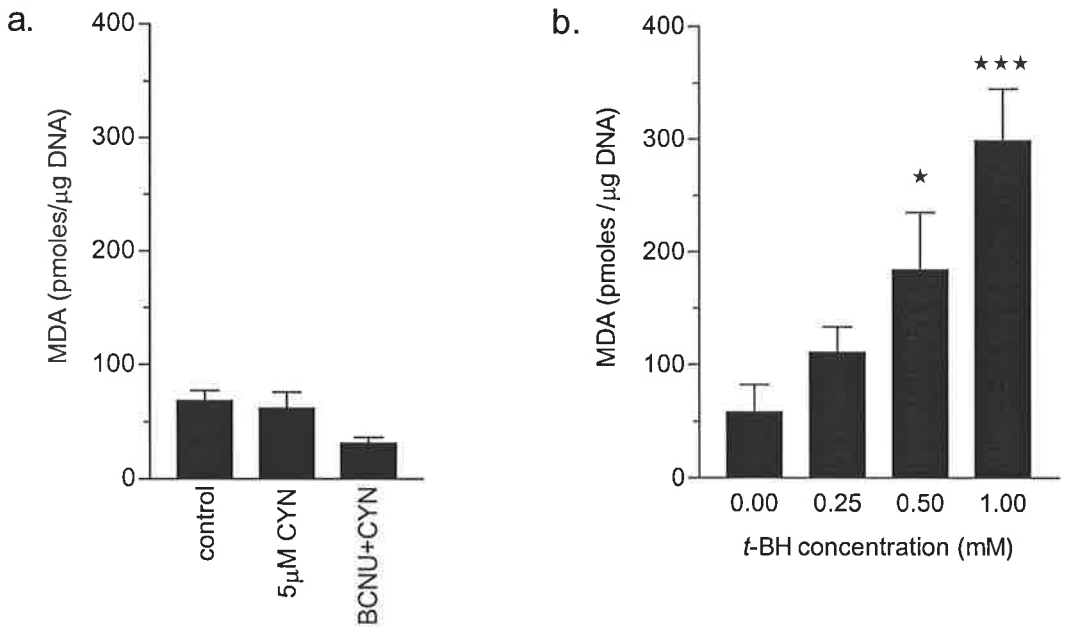


Figure 5.5 Malondialdehyde (MDA) levels in control and toxin treated hepatocytes. (a) After exposure to 5µM cylindrospermopsin (CYN) for 12 hr with or without BCNU pretreatment (100 µM, 30 min). Values are mean ± SE for 3 independent experiments (b). Generation of MDA after 30 min exposure to 0.25-1 mM *t*-BH. Values are mean ± SE of 4 independent experiments. Data was analysed by 1 way ANOVA (repeated measures) followed by Dunnett's test. Significant differences from control are indicated as ⋆ ($p<0.05$) or ⋆⋆⋆ ($p<0.001$).

Treatment	LDH leakage (% total)
Control (untreated)	7 ± 1.2
0.25 mM *t*-BH	21 ± 6.2
0.5 mM *t*-BH	17 ± 2.0
1 mM *t*-BH	15 ± 3.8

Table 5.1 LDH leakage from hepatocytes exposed to 0-1 mM *tert*-butyl hydroperoxide (*t*-BH) for 30 min. Values are mean ± SE from 3 independent experiments.

5.3.3 Effect of GSSG-Rd Inhibition on Toxicity

In order to exclude the possibility that lipid peroxidation mediated effects after cylindrospermopsin treatment remained undetected in Section 5.3.2 due to either (1) the measurement of MDA at one time point only (12 hr) or (2) the instability of free MDA over a prolonged incubation period, an alternative approach was adopted. The effect of GSSG-Rd inhibition (BCNU pretreatment) on the development of cylindrospermopsin toxicity (LDH leakage) was investigated over 12-18 hr. Although the effects of BCNU are reportedly irreversible over a 24 hr period in mouse hepatocytes (Adamson and Harman, 1989), the effectiveness of BCNU pretreatment over 12-18 hr period was first confirmed in the current hepatocyte culture system. The positive control *t*-BH was used.

Effect of GSSG-Rd Inhibition on *t*-BH Toxicity

The effect of GSSG-Rd inhibition on the development of *t*-BH toxicity was assessed 12 hr post BCNU pretreatment. As shown in Figure 5.6, BCNU pretreatment significantly enhanced toxicity of *t*-BH. The inhibition of GSSG-Rd resulted in a more rapid onset of *t*-BH toxicity over time, significantly different from control at both 30 and 60 min. This confirms that inhibition of GSSG-Rd potentiates toxicity of a compound when oxidative damage is involved in the toxicity process. In addition, it confirms that the effects of BCNU remain 12-13 hr after the initial 30 min treatment to achieve GSSG-Rd inhibition. This time frame is critical for the development of cylindrospermopsin toxicity.

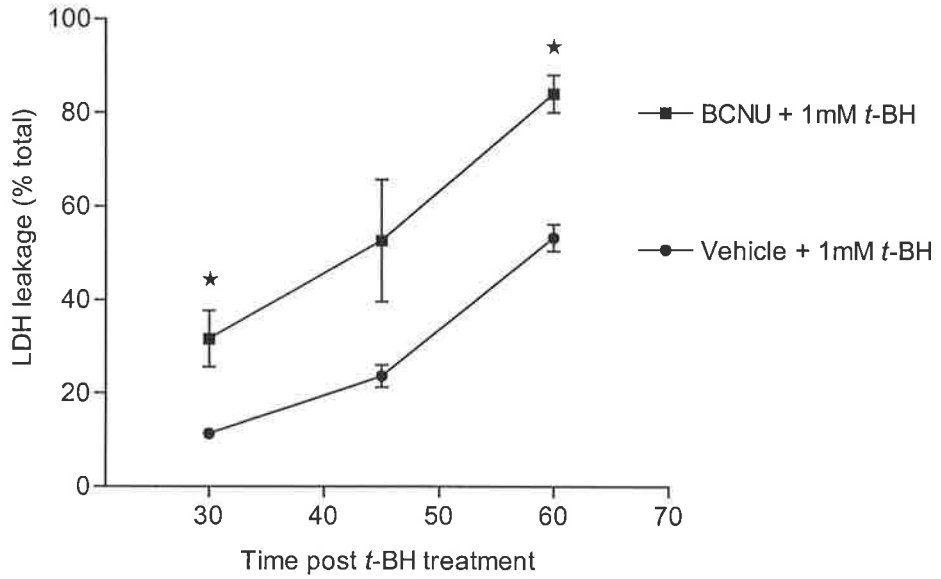


Figure 5.6 Potentiation of *tert*-butyl hydroperoxide (*t*-BH) toxicity in hepatocytes by inhibition of GSSG-Rd with BCNU. Hepatocytes were pretreated with 100 μM BCNU or vehicle for 30 min followed by a 2 hr recovery period. After 12 hr incubation, each treatment group was challenged with 1 mM *t*-BH and toxicity development assessed by LDH leakage over 30-60 min. Values are mean ± SE of 3 independent experiments. Data was analysed by paired t-test (two tailed). Significant differences are indicated as ⋆ ($p<0.05$).

Effect of GSSG-Rd inhibition on the Development of CYN Toxicity

The inhibition of GSSG-Rd by BCNU pretreatment did not affect the development of cylindrospermopsin toxicity (Figure 5.7). Treatment with 5 μM cylindrospermopsin (BCNU or vehicle pretreatment) significantly elevated LDH levels at 18 hr from the BCNU or vehicle controls (all $\star$ $P < 0.05$, one-way ANOVA followed by Tukey's test). However, the slight increase in LDH leakage after 12 hr exposure to 5μM cylindrospermopsin in BCNU pre-treatment hepatocytes was not significantly different from the cylindrospermopsin control by ANOVA ($P > 0.05$). No significant difference in 5 μM cylindrospermopsin toxicity was observed between BCNU and vehicle pretreated hepatocytes over the 14-18 hr period (one-way ANOVA). The BCNU treatment alone did not induce toxicity over the 18 hr incubation period.

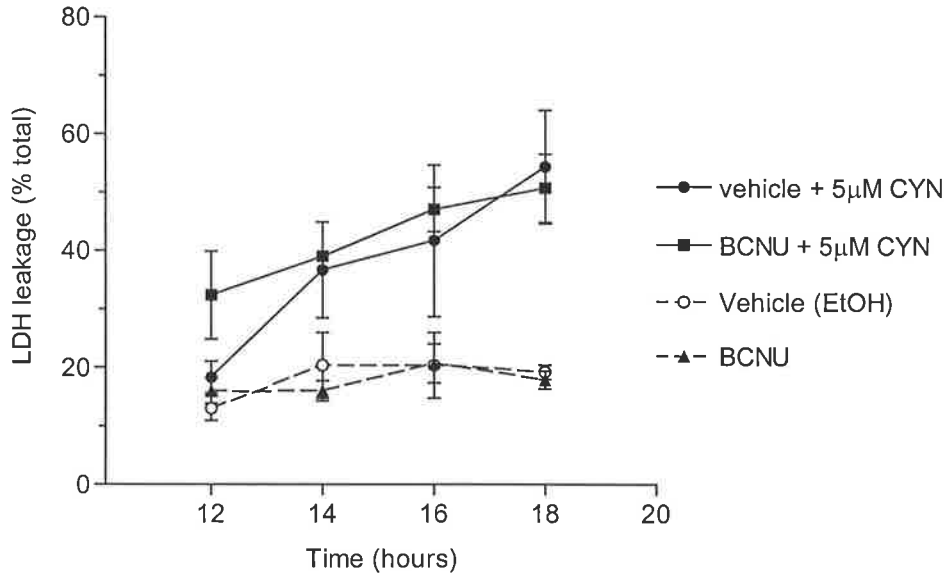


Figure 5.7 Effect of GSSG-Rd inhibition on LDH leakage from hepatocytes treated with 5 μM cylindrospermopsin (CYN). Hepatocytes were pretreated with BCNU to inhibit GSSG-Rd activity or vehicle for 30 min followed by a 2 hr recovery period prior to exposure to CYN. The development of CYN toxicity was followed from 12-18 hr post exposure. Values are mean ± SE of 3 independent experiments.

5.4 Discussion

The depletion of GSH in cylindrospermopsin treated rat hepatocytes prior to the onset of LDH leakage (Runnegar *et al.*, 1994) led to the suggestion that oxidative damage may be involved in its mechanism of toxicity. In the current study oxidative damage was assessed by determining the extent of lipid peroxidation following the fall in GSH levels and investigating any involvement of the GSH-Px / GSSG-Rd enzyme system in protecting against cylindrospermopsin toxicity. Consistent with the results of Runnegar *et al.* (1994) cylindrospermopsin produced clear time and concentration-dependent GSH depletion in mouse hepatocytes. All toxic concentrations of cylindrospermopsin caused severe GSH depletion, and significant depletion occurred prior to the onset of LDH leakage. Despite the loss of GSH caused by cylindrospermopsin treatment, lipid peroxidation products did not increase over control levels. Also, compromising the cells ability to cope with oxidative stress by inhibiting GSSG-Rd activity did not alter this response. Furthermore the GSSG-Rd inhibition did not affect the development of cylindrospermopsin toxicity over an extended period. These results rule out a major role for lipid peroxidation and oxidative stress in cylindrospermopsin toxicity. In contrast, lipid peroxidation products were readily detected after treatment of hepatocytes with *t*-BH, and its toxicity was potentiated by GSSG-Rd inhibition.

Runnegar *et al.* (1994) also demonstrated that lowering cell GSH with the transsulfuration inhibitor propargylglycine (PPG), further potentiated GSH depletion induced by cylindrospermopsin and predisposed the hepatocytes to toxic effects. While these results illustrate that GSH plays some role in protecting against cylindrospermopsin induced cell death, the results presented in this Chapter, using BCNU to inhibit GSSG-Rd activity, indicate that the protective role of GSH is not likely to involve it's use during GSSG-Px mediated reactions.

Despite the presence of endogenous initiating species, lipid peroxidation is minimized under normal circumstances due to a range of antioxidant defence mechanisms. A severe depletion of GSH will not necessarily result in the onset of lipid peroxidation. Some

researchers suggest that there is a threshold level below which GSH concentrations must fall before large increases in lipid peroxidation and cell injury occurs. Yet the capacity for the hepatocyte to cope with severely depleted cytosolic GSH stores for extended periods without the onset of lipid peroxidation has been demonstrated. At non-toxic concentrations, diethyl maleate (DEM) can deplete GSH in isolated hepatocytes to below limit of detection (<5% control) for 5 hr without the onset of lipid peroxidation and cell death (Tiermenstein *et al.*, 2000). DEM conjugates directly to GSH without the requirement for metabolic activation.

Alternatively it has been suggested that depletion of the mitochondrial pool of GSH is required for the onset of lipid peroxidation. In the hepatocyte, cytoplasmic GSH constitutes approximately 85% of the total, while 15% is found in the mitochondria. The mitochondrial GSH pool is distinct from that in the cytoplasm with respect to its synthetic rate, turnover and chemical sensitivity (Meredith and Reed, 1982). In isolated rat hepatocytes, the time of onset of MDA production and LDH leakage after treatment with BCNU and adriamycin corresponds to the time of depletion of the mitochondrial GSH pool to less that 10% of the control (Meredith and Reed, 1983). Extensive depletion of the cytoplasmic GSH levels with BCNU alone did not cause MDA production and LDH leakage if the mitochondrial GSH pool was maintained. Similarly, protection of the mitochondrial GSH pool may explain the lack of lipid peroxidation at non-toxic concentrations of DEM despite the severe depletion of cytosolic GSH (Tiermenstein *et al.*, 2000).

In other cases, lipid peroxidation occurs when GSH levels are only partially compromised and may be explained by the formation of xenobiotic-derived radicals that can initiate the peroxidation process. During the progression of *t*-BH toxicity (utilized as a positive control in this study), the oxidation of GSH to GSSG poses an oxidative stress, but it is generation of a *tert*-butyl alkoxyl radical which is implicated as initiating the lipid peroxidation process (Masaki *et al.*, 1989). Leal *et al.* (1998) report a linear increase (0-10 min) in the lipid peroxidation product MDA after treatment of isolated rat hepatocytes with 1 mM *t*-BH. This occurred prior to the onset of toxicity, and GSH levels were approximately 50% of the control during this period. Following the onset of lipid peroxidation the GSH levels declined further and toxicity developed. As previously cited, the trichloromethyl peroxy radical generated through the CYP450 dependent metabolism of carbon tetrachloride is associated with the

onset of lipid peroxidation, yet causes only a minimal reduction in GSH levels (Recknegel *et al.*, 1989).

Chemicals such as paraquat and diquat that increase endogenous reactive oxygen species by redox cycling can also induce lipid peroxidation. Treatment of hepatocytes with paraquat has been shown to decrease GSH levels to approximately 50% of control, followed by a significant elevation of lipid peroxidation and onset of cell death (Sugihara *et al.*, 1995). In some cases compounds that cause redox cycling may only induce lipid peroxidation to a limited extent in hepatocyte culture and in the absence of extensive cytotoxicity until the GSH defence against oxidative stress is compromised. Tsokos-Khun (1988) reports that cytotoxicity of diquat in isolated rat hepatocytes was not observed unless GSSG-Rd activity was inhibited by BCNU treatment.

As described above, the conditions that result in lipid peroxidation vary for different chemicals. A fall in GSH will not necessarily result in lipid peroxidation, although depletion of the mitochondrial pool of GSH may increase this possibility. However, a fall in GSH accompanied by other circumstances such an increase in endogenous reactive oxygen species or generation of radicals via xenobiotic metabolism poses a serious threat to the cell (Recknagel *et al.*, 1991). Although it seemed possible that metabolites generated via the CYP450 dependent metabolism of cylindrospermopsin (subject of Chapter 6) might generate oxidative stress, the present data indicates that this scenario appears unlikely. It would be expected that a putative radical metabolite capable of initiating lipid peroxidation would have increased MDA levels and/or produced enhanced toxic effects after the inhibition of GSSG-Rd activity.

In conclusion, oxidative stress seems to play only a minor role in cylindrospermopsin toxicity in hepatocytes. A loss of GSH clearly occurs in cylindrospermopsin treated cells, but this does not appear to result in elevated levels of lipid peroxidation. However, GSH loss may exaggerate other toxin / metabolite effects. Aside from antioxidant properties discussed, GSH has been reported to be involved in a number of other cellular functions, often where glutathionylated proteins are involved (Sies, 1999). The combination of toxic events induced by cylindrospermopsin is discussed further in Chapter 7 of this thesis.

6 THE ROLE OF CYLINDROSPERMOPSIN DERIVED METABOLITES IN PROTEIN SYNTHESIS INHIBITION AND TOXICITY IN HEPATOCYTE CULTURE

6.1 Introduction

As discussed in Chapter 1, the involvement of CYP450 derived metabolites may explain the hepatotoxicity of cylindrospermopsin, but does not adequately account for its effects in cell types less likely to metabolize the toxin. These observations suggest that cylindrospermopsin is active both unmetabolised and after bioactivation to reactive metabolite(s) in the hepatocyte. To date these events have not been distinguished.

The activity of cylindrospermopin in the reticulocyte lysate indicates that protein synthesis inhibition is likely to occur without prior bioactivation of the toxin. While the CYP450 metabolism of cylindrospermopsin in hepatocytes could potentially generate metabolite(s) capable of inhibiting protein synthesis, current results suggest that the inhibition may be explained by direct action of the toxin and is independent of CYP450 mediated effects. As shown in Chapter 4, protein synthesis inhibition is of similar potency and nature in hepatocyte culture to that described in reticulocyte lysate, despite the different metabolic capabilities of the two systems. Furthermore, the protein synthesis inhibition does not appear to be directly related to the onset of toxicity. Therefore this aim of this Chapter is to investigate the role of metabolites in both protein synthesis inhibition and toxicity in hepatocyte culture. It is hypothesized that inhibition of CYP450 activity in hepatocyte culture will attenuate the toxicity of cylindrospermopsin but will not prevent the cylindrospermopsin-induced protein synthesis inhibition.

Since evidence exists for the CYP450 modification of cylindrospermopsin in hepatocytes (Runnegar *et al.*, 1995b), exploring this hypothesis for toxicity is the primary focus of the Chapter. However it is also considered that bioactivation of cylindrospermopsin may not be entirely limited to CYP450 activity. A number of other metabolizing enzymes are found in the liver which may also catalyse bioactivation reactions (refer to Section 1.7.3). One puzzling aspect about the CYP450-dependent oxidation of cylindrospermopsin is the suitability of this toxin as a substrate for CYP450 modification. Given that cylindrospermopsin is hydrophilic and the CYP450 enzymes are known to be primarily located within the hydrophobic membrane of the endoplasmic reticulum, a question of bioavailablity is raised. Cylindrospermopsin would seem a more suitable substrate for cytosolic enzyme modification. The activity of cylindrospermopsin is known to be related to the hydroxymethyl uracil group in the toxin's structure (Figure 1.6). Considering then the hydroxymethyl uracil moiety as a potential site for modification, the hydroxyl group on the uracil bridge would be the site most likely to be readily oxidized. This type of oxidation reaction can be carried out by CYP450 enzymes (Guengerich, 2001), and can also be facilitated by cytosolic alcohol dehydrogenase activity (Bosron and Li, 1980). Thus it is possible that alcohol dehydrogenase plays a role in oxidizing cylindrospemopsin.

To explore these hypotheses a number of metabolic inhibitors were employed in hepatocyte culture experiments. CYP450 activity was inhibited with the widely used inhibitors proadifen (SKF525A) and ketaconazole. These inhibitors were chosen as they were known to inhibit a broad range of CYP450 isoforms. Their effectiveness was first confirmed in pilot studies using acetaminophen (paracetamol) as a positive control. It is well established that the hepatoxicity of acetaminophen is associated with its oxidation by CYP450 to from the toxic reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Dahlin *et al.*, 1984; Holme *et al.*, 1984). In addition, 4-methylpyrazole (4-MP) was used to inhibit alcohol deydrogenase activity, with allyl alcohol used as the positive control (Ohno *et al.*, 1985).

6.2 Materials and Methods

6.2.1 Materials

Proadifen hydrochloride (SKF-525A), 4-methyl pyrazole (4-MP), acetaminophen and allyl alcohol were obtained from Sigma-Aldrich (St Louis, MI, USA). Ketaconazole was obtained from Janssen Pharmaceuticals (Beerse, Belgium). Cylindrospermopsin was purified from *C.raciborskii* extracts as described in Chapter 2.

6.2.2 Preparation of Cultured Hepatocytes

The isolation and culture of Swiss Albino mouse hepatocytes for use in this study was as detailed previously in Section 4.2.3.

6.2.3 Measurement of Toxicity

Toxicity was measured by the leakage of lactate dehydrogenase (LDH) from hepatocytes as detailed in Section 4.2.4. To examine the effect of the inhibitors on cylindrospermopsin-induced LDH leakage, toxin concentrations of 2.5 μM and 5 μM were chosen as these caused extensive LDH leakage in earlier studies (Chapter 4). LDH leakage was measured 22 hr after cylindrospermopsin treatment.

6.2.4 Protein Synthesis Assay

The extent of protein synthesis in hepatocytes was measured by the incorporation of [^{3}H]-Leucine into trichloroacetic acid (TCA) precipitate as detailed previously in Section 4.2.5. As detailed in Chapter 4, extensive protein synthesis inhibition occurs at lower cylindrospermopsin concentrations that required to induce toxicity. Therefore to examine the effect of biotransformation inhibitors on cylindrospermopsin-induced protein synthesis inhibition, 0.5 μM and 1 μM concentrations were chosen to avoid excess toxin. Protein synthesis inhibition was measured 22 hr after cylindrospermopsin treatment.

6.2.5 Inhibition of CYP450 Activity

CYP450 activity was inhibited by treatment of hepatocytes with proadifen or ketoconazole. Stock solutions of proadifen were prepared in cell culture medium, while ketoconazole was initially dissolved in methanol. The final concentration of methanol in culture media did not exceed 0.5%. Hepatocytes were incubated with 50 μM proadifen or 50 μM ketoconazole for 30 min to prior to treatment with cylindrospermopsin or acetaminophen.

6.2.6 Inhibition of Alcohol Dehydrogenase Activity

Alcohol dehydrogenase activity was inhibited by treatment of hepatocytes with 4-methyl pyrazole (4-MP). 4-MP was prepared in cell culture medium immediately prior to use. Hepatocytes were incubated with 100 μM 4-MP for 30 min prior to treatment with cylindrospermopsin or allyl alcohol.

6.2.7 Statistical Analysis

GraphPad Prism version 3.00 for Windows, (GraphPad Software, SanDiego, CA, USA) was used for all statistical tests and graphing. Analysis of data was by 2-way analysis of variance (ANOVA). When a significant interaction between inhibitor treatment and cylindrospermopsin response was noted, Bonferroni's post-test was used to compare mean response for each cylindrospermopsin concentration. Statistical significance was accepted at $P < 0.05$.

6.3 Results

6.3.1 Validation of Model

Pilot studies indicated that both 50 μM proadifen and 50 μM ketoconazole inhibited CYP450 activity. This was confirmed using the positive control acetaminophen. LDH leakage in hepatocytes challenged with 1 mM acetaminophen for 6 hr was 50%. Pretreatment of hepatocytes with 50 μM ketaconazole for 30 min reduced the acetaminophen-induced LDH leakage to 8% (values represent mean of 2 independent experiments, data not shown). Similar results were obtained with 50 μM proadifen. The inhibitor concentrations employed did not cause cause appreciable cytotoxicity to hepatocytes over the time frame required to assess cylindrospermopsin toxicity. LDH leakage from hepatocytes treated with 50 μM ketoconazole alone for 24 hr was 27% (mean of 2 independent experiments). Similar results were obtained with 50 μM proadifen (data not shown).

Pilot studies indicated that 100 μM 4-MP inhibited alcohol dehydrogenase activity. This was verified using the positive control allyl alcohol. LDH leakage from hepatocytes challenged with 250 μM allyl alcohol for 2 hr was 73 ± 8.7 % (mean ± SE of 4 independent experiments, data not shown). Pretreatment of hepatocytes for 30 min with 100 μM 4-MP prior to allyl alcohol reduced LDH leakage to 11 ± 1.3 % (significantly different from controls by t-test, data not shown). Incubation of hepatocytes with 100 μM 4-MP alone for 24 hr did not cause appreciable cytotoxicity as assessed by LDH leakage. This indicates that concentration of 4-MP employed is appopriate for use over the time frame required to assess cylindrospermopsin toxicity.

6.3.2 Effect of CYP450 Inhibitors on Cylindrospermopsin-Induced Toxicity.

As shown in Figure 6.1, pretreatment with proadifen to inhibit CYP450 activity significantly alleviated cylindrospermopsin induced LDH leakage. Exposure to 2.5 μM cylindrospermopsin alone resulted in 67 ± 5 % LDH leakage at 22 hr, while pretreatment with proadifen prior to 2.5 μM toxin reduced the LDH leakage to 30 ± 7% (Figure 6.1). Similarly for the 5 μM cylindrospermopsin treatment group, exposure to toxin alone resulted in 77 ± 4% LDH leakage at 22 hr, while pretreatment with proadifen reduced the LDH leakage to 45 ± 10%. LDH leakage from the untreated control or proadifen alone was 29 ± 7 % for each treatment group (Figure 6.1).

Inhibition of CYP450 activity with ketoconazole also significantly attenuated cylindrospermopsin induced LDH leakage (Figure 6.2). Exposure to 2.5 μM cylindrospermopsin produced 65 ± 8 % LDH leakage at 22 hr, and was reduced to 35 ± 4% by pretreatment with ketoconazole (Figure 6.2). Exposure to 5 μM cylindrospermopsin resulted in 77 ± 4% LDH leakage while pretreatment with ketoconazole reduced the LDH leakage to 40 ± 4 %. LDH leakage from the vehicle control or ketoconazole alone was 29 ± 9 % and 30 ± 5% respectively (Figure 6.2).

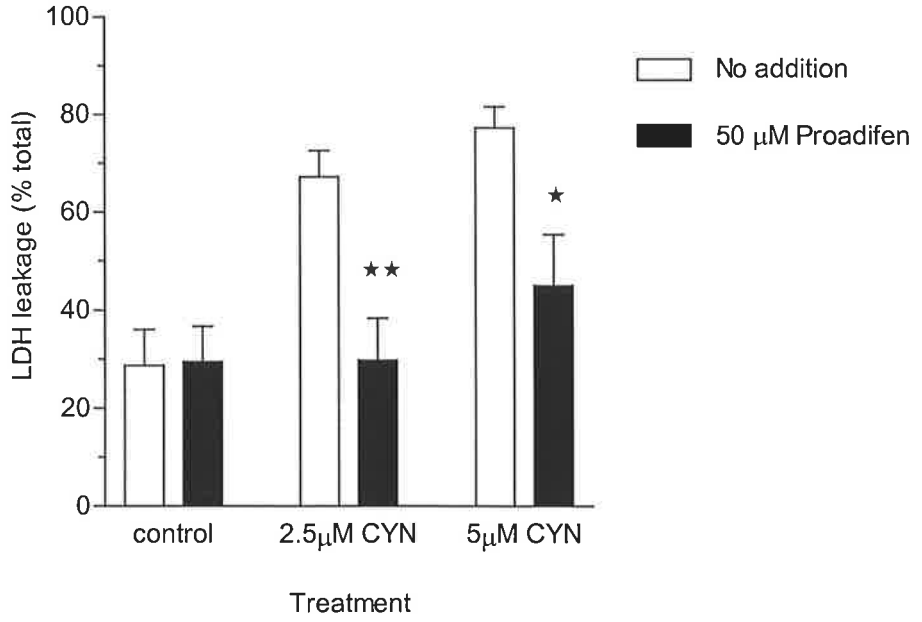


Figure 6.1 The effect of proadifen on LDH leakage from hepatocytes treated with cylindrospermopsin (CYN). Hepatocytes were pretreated with 50 μM proadifen for 30 min prior to CYN (2.5 or 5 μM) for 22 hr. Values represent mean ± SE of 4 independent experiments. The data was analysed by 2-way ANOVA followed by Bonferroni's test. Within each treatment group, a significant difference from the untreated control is indicated as★($p<0.05$) or★★($p<0.01$).

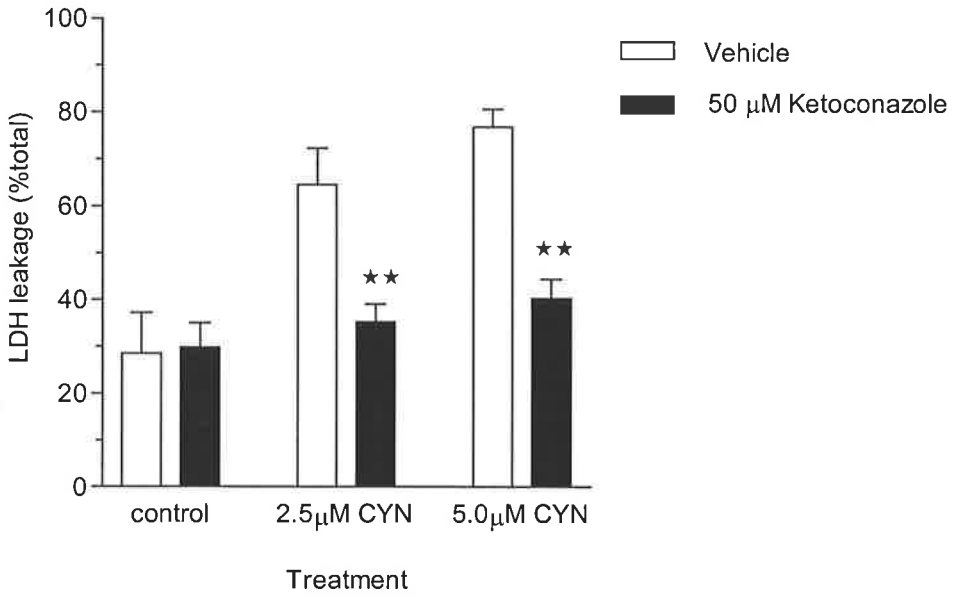


Figure 6.2 The effect of ketoconazole on LDH leakage from hepatocytes treated with cylindrospermopsin (CYN). Hepatocytes were pretreated with 50μM ketoconazole for 30 min prior to CYN (2.5 or 5 μM) for 22hr. Values represent mean ± SE of 3-4 independent experiments. The data was analysed by 2-way ANOVA followed by Bonferroni's test. Within each treatment group, a significant difference from the vehicle control is indicated as★★($p<0.01$).

6.3.3 The Effect of CYP450 Inhibitors on Cylindrospermopsin-Induced Protein Synthesis Inhibition.

In contrast to the effect of proadifen on cylindrospermopsin-induced LDH leakage (Section 6.3.2), this CYP450 inhibitor did not significantly alter the inhibition of protein synthesis (Figure 6.3). Treatment with 0.5 μM and 1 μM cylindrospermopsin alone for 22 hr diminished protein synthesis to 25% and 18 % of the untreated control level (3403 ± 489 dpm / μg DNA) respectively (Figure 6.3). The extent of protein synthesis in the proadifen pretreament plus 0.5 μM or 1 μM cylindrospermopsin was 29% and 24% of the inhibitor control level (3251 ± 330 dpm/ μg DNA) respectively (Figure 6.3). The extent of protein synthesis after 22 hr incubation with proadifen alone was similar to the untreated control.

In addition, while ketoconazole attenuated cylindrospermopsin induced LDH leakage (Section 6.3.2) it also did not significantly interfere with the inhibition of protein synthesis (Figure 6.4). The extent of protein synthesis after remaining after treatment with 0.5μM and 1μM cylindrospermopsin alone for 22 hr was 26% and 14 % of the vehicle control (3604 ± 726 dpm / μg DNA) respectively (Figure 6.4). The extent of protein synthesis in the ketoconazole preatreament plus 0.5μM and 1μM cylindrospermopsin was 46% and 31% of the inhibitor control level (3921 ± 330 dpm/ μg DNA) respectively (Figure 6.4). The effects were not statistically significant ($P > 0.05$).

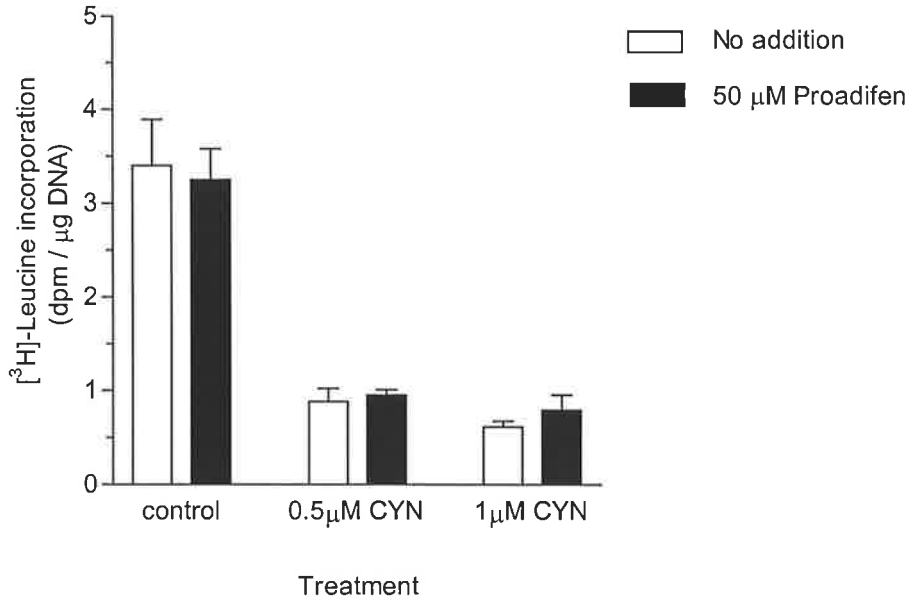


Figure 6.3 The effect of proadifen on the inhibition of protein synthesis in hepatocytes treated with cylindrospermopsin (CYN). Hepatocytes were pretreated with 50 μM Proadifen for 30 min prior to CYN (0.5 or 1 μM) for 22 hr. Values represent mean ± SE of 3-4 independent experiments.

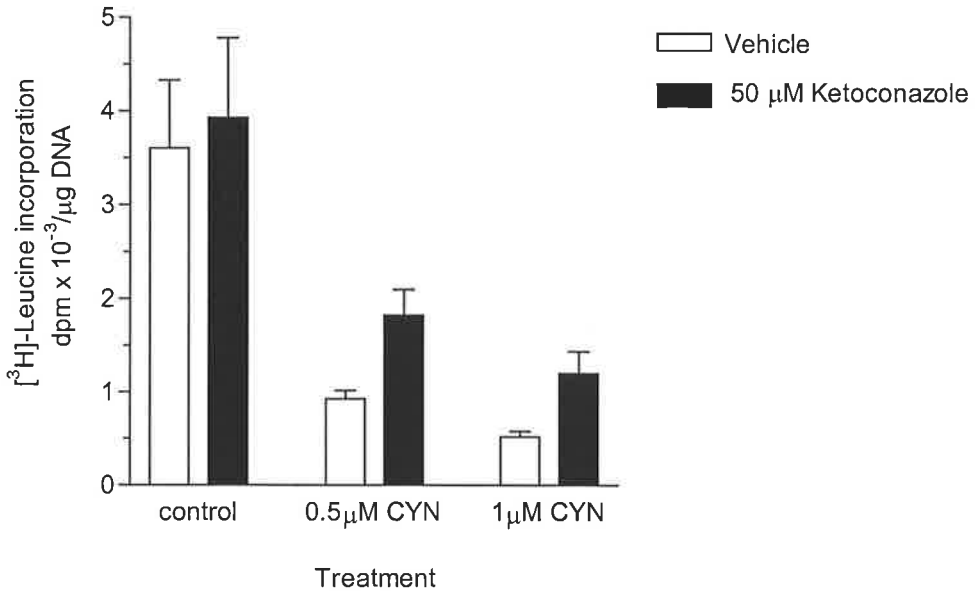


Figure 6.4 The effect of ketoconazole on the inhibition of protein synthesis in hepatocytes treated with cylindrospermopsin (CYN). Hepatocytes were pretreated with 50 μM Ketoconazole for 30 min prior to CYN (0.5 or 1 μM) for 22 hr. Values represent mean ±SE of 3-4 independent experiments.

6.3.4 The Effect of 4-Methyl pyrazole on Cylindrospermopsin-Induced Toxicity

As shown in Figure 6.5 pretreatment of hepatocytes with 4-methylpyrazole (4-MP) to inhibit alcohol dehydrogenase activity did not significantly affect cylindrospermopsin induced LDH leakage. The LDH leakage from 2.5 μM cylindrospermopsin treated hepatocytes was 69 ±11 and 68 ± 7.6 % with and without 4-MP pretreatment respectively. LDH leakage from 5 μM cylindrospermopsin treated hepatocytes was 73 ± 9.3 and 79 ± 5.8 % with and without 4-MP pretreatment respectively.

6.3.5 Effect of 4-Methyl pyrazole on Cylindrospermopsin-Induced Protein Synthesis Inhibition.

Protein synthesis inhibition after treatment with 0.5μM and 1μM cylindrospermopsin was not significantly affected by pretreatment with 4-methylpyrazole (4-MP) (Figure 6.6) The extent of protein synthesis after treatment with 0.5μM and 1μM cylindrospermopsin alone for 22 hr was 28% and 20 % of the untreated control (3071 ± 508 dpm / μg DNA) respectively. The extent of protein synthesis after 4-MP preatreament plus 0.5μM and 1μM cylindrospermopsin was 35% and 18% of the inhibitor control level (2745 ± 398 dpm/ μg DNA) respectively.

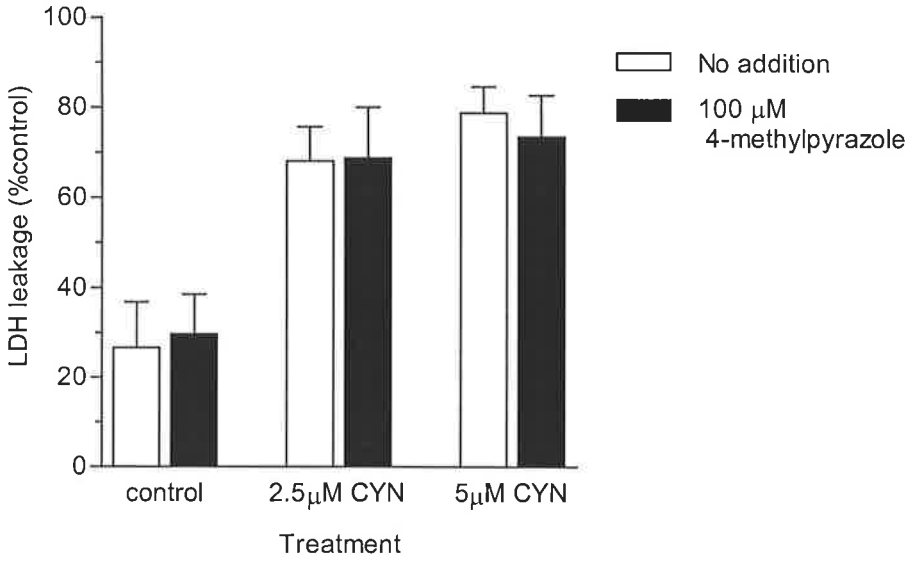


Figure 6.5 The effect of 4-methylpyrazole (4-MP) on LDH leakage from hepatocytes treated with cylindrospermopsin (CYN). Hepatocytes were pretreated with 100 μM 4-MP for 30 min prior to CYN (2.5 or 5 μM) for 22 hr. Values represent mean ± SE of 3 independent experiments.

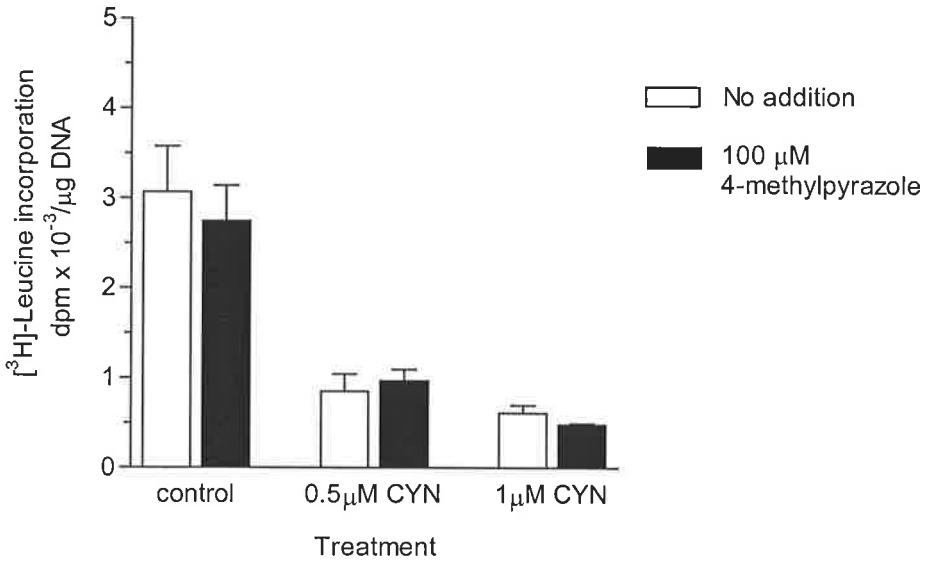


Figure 6.6 The effect of 4-methylpyrazole (4-MP) on the inhibition of protein synthesis in hepatocytes treated with cylindrospermopsin (CYN). Hepatocytes were pretreated with 100 μM 4-MP for 30 min prior to CYN (0.5 or 1 μM) for 22 hr. Values represent mean ± SE of 3-4 independent experiments.

6.4 Discussion

The results presented clearly demonstrate that inhibition of CYP450 activity in mouse hepatocytes attenuates the toxicity of cylindrospermopsin. However, while reducing toxicity, the CYP450 inhibitors did not have a significant effect on the inhibition of protein synthesis, supporting the hypothesis that this event occurs due to the direct effect of unmetabolised toxin. This provides the first clear evidence that protein synthesis inhibition and CYP450 mediated toxicity can be distinguished into two discrete events during cylindrospermopsin exposure. In contrast to the effects of the CYP450 inhibitors, inhibition of alcohol dehydrogenase activity with 4-MP did not affect toxicity or protein synthesis inhibition. Thus, the hypothesis that cylindrospermopsin could be oxidized by this enzyme into toxic metabolites is rejected.

With respect to the metabolism of cylindrospermopsin, the current results support previous reasearch of Runnegar *et al.* (1995b) implicating the involvement of CYP450 derived metabolite(s) in the mechanism of toxicity in hepatocytes. A number of different CYP450 inhibitors have been shown to attenuate the toxicity of cylindrospermopsin. Aside from proadifen and ketoconozole in this study, Runnegar *et al.* (1995b) demonstrated the effectiveness of α-napthoflavone in rat hepatocytes and noted that cimetidine produced a similar response (data not shown in cited report). The reactions mediated by CYP450 enzymes have been well characterized and inhibitors can be used as a useful tool for determining the CYP450 isoforms involved in toxin biotransformation. The major CYP450 enzymes (in humans) involved in the oxidation of xenobiotics include 1A1, 1A2, 2A6, 2B6, 2C, 2D6, 3E1 and 3A proteins (Shimada *et al.*, 1994). It is however difficult to draw any conclusions about the potential CYP450 isoforms involved in cylindrospermopsin bioactivation. The inhibitors used in this study were known to inhibit a wide range of CYP450 isoforms and were also used at relatively high concentrations to increase the coverage of the CYP450 isoforms inhibited. For example, at low concentrations ketoconazole is known to inhibit CYP3A, the major CYP450 isoform in liver tissue (accounting for 30% of the total CYP450 protein). At higher concentrations ketoconazole also inhibits CYP2C,

CYP2D and CYP2E enzymes (Baldwin *et al.*, 1995; Eagling *et al.*, 1998). Furthermore much of the information on CYP450 isoform-inhibitor interactions has been derived from human liver samples. It is also noted that differences in inhibitor selectivity and/or differences in the CYP450 isoforms responsible for toxin metabolism can occur between different species (Bogaards *et al.*, 2000).

Although the present work confirms that metabolic activation is involved in the cytotoxicity of cylindrospermopsin in hepatocytes, further work is required to clarify the chemical nature of the metabolites involved in cell damage, and identify the critical cellular targets. It is well established that CYP450 bioactivation can give rise to electrophilic species capable of reacting irreversibly with tissue nucleophiles (Guengerich and Shimada, 1991). Since Runnegar *et al.* (1995b) linked both the GSH depletion and toxicity to CYP450 metabolite formation, it could be speculated that a reactive metabolite is capable of inhibiting the enzyme(s) involved in GSH synthesis by forming a protein adduct. The formation of protein adducts would not be limited to one enzyme and could provide a mechanism of toxicity as described for other toxins. It has been proposed that acetaminophen hepatotoxicity is mediated by the covalent binding of the electrophilic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) to critical proteins (Albano *et al.*, 1985). Numerous functional proteins have been identified as the target of NAPQI adduct formation (Qui *et al.*, 1998).

In the absence of CYP450 derived metabolites (in cell types other than hepatocytes), protein synthesis inhibition may play a more crucial role in the development of cytotoxicity. It has been clearly demonstrated that this event can occur without bioactivation of the toxin. Since cells can survive for extended periods in the absence of protein synthesis (refer to Section 1.7.1) it would be expected that cytotoxicity due to protein synthesis inhibition alone occurs over a different time frame to that described in hepatocyte culture. It has consistently been noted that the cytotoxicity of cylindrospermopsin in metabolism-incompetent cells requires exposure over more extended periods than that needed to produce toxicity in hepatocytes. As shown by Chong *et al.* (2002), incubation of primary rat hepatocytes with ≥ 2 μM (800 ng/ml) cylindrospermopsin reduced cell viability to less than 25 % in 48 hr. In the same study, similar cylindrospermopsin concentrations did not reduce cell viability in the KB epithelial carcinoma cell line until 72 hr. These observations reinforce the conclusions

reached on the basis of the present work, that while cylindrospermopsin itself is a potent inhibitor of protein synthesis, acute cytotoxicity in hepatocytes occurs via bioactivation-dependent events.

7 GENERAL DISCUSSION

The aim of the work reported in this thesis was to investigate the mechanisms by which cylindrospermopsin induces liver toxicity. The research focused on investigating the roles of protein synthesis inhibition, GSH depletion and toxin metabolism in the development of cytotoxicity *in vitro* in cultured mouse hepatocytes. The research addressed a number of questions arising about the extent to which each of these factors contributed to the toxicity process. In addition, the inhibitory effects of the toxin on protein synthesis were also investigated in the cell-free reticulocyte lysate, thus allowing assessment of its potency and mode of action and providing a comparison to its effects on protein synthesis in the cellular model. HPLC purified toxin was used throughout.

Mechanisms of Cytotoxicity *in vitro*.

Cylindrospermopsin was demonstrated to be a potent inhibitor of protein synthesis causing an immediate cessation of translation upon its addition to reticulocyte lysates. The inhibitory potency and nature of the concentration-response curve obtained was reproduced in hepatocyte culture, but did not correlate with the concentration-response curve for cytotoxicity at 18 hr (Chapter 4). This suggests that events other than protein synthesis inhibition are required to induce toxicity in hepatocytes. The subsequent observation that inhibition of CYP450 activity in hepatocyte culture could alleviate cylindrospermopsin induced toxicity, but not the effects on protein synthesis (Chapter 6), demonstrated a dissociation of the two events and implicates the involvement of metabolites in the cytotoxicity process, but not in the impairment of protein synthesis. Thus the results support the hypotheses proposed in Chapter 1; that protein synthesis inhibition is not solely responsible for hepatocyte cell death and that formation of CYP450-derived metabolites produces effects other than the impairment of protein synthesis.

Cylindrospermopsin has earlier been shown to block GSH synthesis in rat hepatocytes, lowering the GSH levels prior to the onset of toxicity (Runnegar *et al.*, 1994; Runnegar *et al.*, 1995b). The current work also demonstrated that all toxic concentrations of cylindrospermopsin depleted GSH, and that the depletion occurred well prior to the onset of toxicity in mouse hepatocytes (Chapter 5). It was assumed that this event would occur independent to protein synthesis inhibition. A number of observations indirectly support this assumption. Firstly, the time course experiments in Chapter 4 and Chapter 5 demonstrate that both protein synthesis inhibition and GSH depletion respectively are evident by 4 hr after treatment with 5 μM cylindrospermopsin. The similar timing of events indicates that GSH depletion is not likely to be simply a consequence of protein synthesis inhibition. Furthermore, a literature search failed to find evidence of adverse effects of protein synthesis inhibitors on GSH levels in hepatocyte culture. In fact, exposure of primary rat hepatocytes to cycloheximide for 8 hr at concentrations impairing protein synthesis has been observed to increase GSH levels 50% above the control level (Sanchez *et al.*, 1997). Secondly, although not investigated in this study, the work of Runnegar *et al.* (1995b) suggests that GSH depletion, at least in part, is mediated by CYP450 derived metabolites. Clearly, the present study has demonstrated a dissociation of CYP450 metabolite mediated events from protein synthesis inhibition (Chapter 6).

Chapter 5 investigated the consequences of cylindrospermopsin-induced GSH depletion in hepatocyte culture. It focused on the antioxidant properties of GSH, proposing that oxidative damage was a significant contributor to cell death after cylindrospermopsin exposure. Despite the possibility that oxidative stress would be imposed by GSH depletion increasing the susceptibility of the cells to oxidative events, the data did not indicate that elevated oxidative damage accompanied the toxicity process. Lipid peroxidation was not elevated following the fall in GSH levels and the GSH-Px / GSSG-Rd enzyme system did not appear to protect against cylindrospermopsin toxicity. It was concluded that the extent of GSH depletion produced by cylindrospermopsin was not of a sufficient magnitude to result in enhanced generation of reactive oxygen species, and that potential metabolite(s) generated through the CYP450 dependent metabolism of the toxin (Chapter 6) did not generate oxidative stress.

The results described in Chapter 6 along with the reported findings of Runnegar *et al.* (1995b) imply that bioactivation plays a key role in cylindrospermopsin toxicity in hepatocytes, via the production of reactive metabolites. One function of GSH is to detoxify reactive intermediates formed via enzymatic oxidation of xenobiotics, such as electrophiles (epoxides, quinones, cations) or peroxides and free radicals (Moldeus and Jernstrom, 1983). As described in Chapter 1, in these reactions GSH may serve as nucleophile forming conjugates, or as a reductant generating GSSG (see also Figure 1.7). The nature of the metabolite specie(s) produced during the metabolism of cylindrospermopsin is unknown, however results presented in Chapter 5 do not suggest a role for the generation of a free radical species and/or the use of GSH as a reductant. One scenario that has not been adequately explored is the potential role of GSH conjugation for detoxication of cylindrospermopsin metabolites.

To date, conjugates of cylindrospermopsin metabolites with GSH have not been isolated. Runnegar *et al.* (1995b) utilized HPLC to search for GSH derivatives in the culture media of cylindrospermopsin treated hepatocytes, but did not find evidence of extra peaks which may have been GSH conjugates. Because of the cylindrospermopsin's potent effects on GSH levels in hepatocytes, the total extent of GSH loss exceeds that which could be expected to occur through GSH conjugation to toxin / metabolite alone (assuming 1:1 conjugation stoichiometry). Runnegar *et al.* (1995b) notes that the total extent of GSH depletion in rat hepatocytes is 10 fold that of the total cylindrospermopsin content (at ≤ 5 nmoles toxin). Calculation of molar equivalents from the results in Chapter 5 show a similar response. For example, the total equivalent of 2 nmoles of cylindrospermopsin per cell culture dish resulted in 16 nmoles total loss of GSH.

Although electrophilic metabolites generated through the CYP450 dependent modification of cylindrospermopsin might only make a minor contribution to oxidative stress, electrophilic speices may well explain the toxicity in hepatocytes. Electrophiles are capable of reacting with tissue nucleophiles including GSH, protein, DNA and RNA. If GSH plays a role in the detoxication by conjugation to the metabolite(s), this could explain why the toxicity occurs when GSH levels are depleted. The loss of GSH due the toxin's effects on its re-synthesis, reduces its protective capacity, allowing the metabolites to target nucleophilic

sites on cellular macromolecules. The production of covalently bound adducts e.g. protein adducts, might lead to the disruption of normal cellular functions and the onset of toxicity. In addition, the impairment of protein synthesis would be likely to exaggerate the loss of cellular integrity particularly during the later stages when the GSH levels are severely depleted and the electrophilic attack becomes more prevalent.

The conjugation of GSH to reactive metabolites has been demonstrated as an important detoxication mechanism for a number of toxins. For example, the reactive epoxides produced from the metabolism of aflatoxin are readily conjugated to GSH (Johnson *et al.*, 1992; Raney *et al.*, 1992). Similarly acetaminophen toxicity involves a loss of GSH and diminished ability to detoxicate the *N*-acetyl-*p*-benzoquinone imine (NAPQI) metabolite (Hinson and Kadlubar, 1988; Moldeus and Jernstrom, 1983). At lower concentrations of acetaminophen, NAPQI is effectively detoxicated by GSH. At higher concentrations, the GSH is depleted and the metabolite(s) are able to target other cellular nucleophilic components (protein, DNA, RNA). Significant amounts of acetaminophen metabolite-protein binding does not occur until hepatic GSH levels are reduced by approximately 80%.

It is also noted that the loss of GSH affects the overall thiol status in the cell, resulting in the loss of protein-SH groups. Such destabilization of proteins has been reported to be involved in oxidative stress response (Freeman *et al.*, 1997), and may also play a role in the complex nature of cylindrospermopsin toxicology. The protection of these vulnerable protein-SH groups by alternative reducing agents such as dithiothreitol may aid in maintaining cell structure and function, and cause some delay in the toxicity. Dithiothreitol can provide a protective effect and delay the onset of acetaminophen toxicity in mouse hepatocytes that is independent of the replenishment of GSH stores (Rafeiro *et al.*, 1994).

Relationship of Current Results to Cylindrospermopsin Toxicity *in vivo*

The characteristics of acute cylindrospermopsin toxicology *in vivo* are a hepatic lesion with a range of effects at extrahepatic sites of variable location and severity (see Chapter 1). The toxicity is both concentration and time dependent. The LD_{50} of purified cylindrospermopsin (i.p., mice) is 2100 μg kg^{-1} at 24 hr, and 200 μg kg^{-1} at 5-6 days (Ohtani *et al.*, 1992).

The present confirmation of a role for metabolism in cylindrospermopsin toxicity *in vitro* (Chapter 6) provides insight into its acutely hepatotoxic nature *in vivo*. However, investigation of the importance of metabolite production in the overall toxicology *in vivo* in mice has produced inconsistent results. Norris *et al.* (2002) report that *in vivo* administration of piperonyl butoxide (to inhibit CYP450 activity) prior to 200 μg kg^{-1} (5-6 day LD_{50}) cylindrospermopsin significantly increased the survival rate of Quackenbush mice over 7 days. This response was maintained with 400 and 800 μg kg^{-1} doses of the toxin (data not shown in cited report). However, it was also noted that in C57/B16 mice the protective effect was not confirmed (data not shown in cited report). These differences may be related to altered metabolic capacities of the different strains of mice. It is known that considerable variation in CYP450 isoforms can occur between different species, as well as individuals of the same species resulting in altered metabolism of the same compound (Bogaards *et al.*, 2000; Park *et al.*, 1995).

It is not clear how extensively the direct effects of the toxin contribute to the toxicology *in vivo*, particularly at lower doses and over extended time frames. The extrahepatic lesions may be related to the protein synthesis inhibition (and other events) occurring in the absence of CYP450 activation. As cylindrospermopsin is suggested to enter cells via diffusion (Chapter 4) it is not restricted by its mechanism of entry to cell types with particular transport mechanisms. Consistent with entry into a number of cell types, Norris *et al.* (2001) report that after *in vivo* administration of radiolabelled cylindrospermopsin to mice the toxin can be found primarily in the liver, but also in the kidneys and other organs to a much lesser extent.

A notable finding in this thesis was that the protein synthesis inhibition was irreversible in hepatocytes (Chapter 4). As a consequence, long term effects may be expected *in vivo*. Norris *et al.* (2001) report that most of the radiolabelled cylindrospermopsin administered to mice is excreted within the first 12 hr (73%), primarily in the urine. However, up to 23% of the total radiolabelled cylindrospermopsin could be retained in the liver of some mice at 48 hr. A lesser amount was retained in the kidneys. Over a longer time frame, repeated administration of cylindrospermopsin *in vivo* to mice has been shown to increase susceptibility to the toxin even after a two week recovery period (Falconer and Humpage, 2001). Some of the long term toxicity may result from the irreversible effects on protein synthesis. The effects of the irreversible protein synthesis inhibitor emetine are long term. The material safety data sheet (MSDS) (Sigma-Aldrich) for emetine reveals that after *in vivo* administration of the toxin it can be still found in animal tissues three months after exposure (specific references to literature were not given). Emetine is also known to persist in the human body for extended periods and chronic exposure has resulted in fatality (Schiff *et al.*, 1986).

In addition, the production of electrophilic metabolites resulting in covalently bound adducts may also contribute to the delayed recovery of cylindrospermopsin in mice as observed by Norris *et al.* (2001). These researchers reported the presence of protein-bound cylindrospermopsin (radiolabelled) in the liver after administration of the alkaloid to mice. Extraction with methanol yielded a more hydrophilic compound than cylindrospermopsin itself, suggesting the formation of a metabolite. This was not found in other tissues (e.g. kidneys). Certainly the formation of covalently bound metabolite adducts may present more than long term toxic effects. Depending on the nature of the adducts formed, a carcinogenic risk may also occur. Preliminary studies have indicated formation of a DNA adduct *in vivo* (Shaw *et al.*, 2000), while other research has reported a potential risk of carcinogenisis after *in vivo* exposure of mice to the toxin (Falconer and Humpage, 2001).

However, while the production of electrophilic metabolites via CYP450 bioactivation of the toxin provides a mechanism for adduct formation, another consideration is that cylindrospermopsin itself may produce genotoxic effects. Interestingly, cylindrospermopsin produced cytogenetic damage in the micronucleus assay carried out using a lympoblastoid

cell line (Humpage *et al.*, 2000a). It is expected that this cell line has limited metabolic potential, particularly with respect to CYP450 activity. While cylindrospermopsin modification may not limited to CYP450 oxidation, it is also known that cylindrospermopsin itself is biologically active and this may also need to be addressed with respect to genotoxic and potential carcinogenic risk.

Future Directions

The toxicology of cylindrospermopsin has now reached a very interesting phase and future developments will be of crucial value in assessing the risks associated with exposure to this toxin.

The results presented in this study highlighted the importance of CYP450 metabolite(s) formation for the development of cylindrospermopsin-induced cytotoxicity in hepatocytes. Future identification of toxic cylindrospermopsin metabolites and their subcellular targets is likely to improve the understanding of cylindrospermopsin toxicology. Clarifying any interaction of electrophilic CYP450 derived metabolites with macromolecules will provide a mechanism of toxicity, and also indicate whether a genotoxic, and potentially carcinogenic risk results from adduction of the cellular genetic material. This is of crucial interest in understanding risk assessment for exposure to cylindrospermopsin. Following the identification of protein / DNA adducts, the use of hepatocyte culture and CYP450 inhibitors would readily confirm whether metabolic activation is required for the generation of these products.

As described in this thesis, much of the experimental work has been carried out using rodent models. Given the interspecies variability in the metabolism of xenobiotics, the identification CYP450 derived products of cylindrospermopsin metabolism and the confirmation that they are also relevant in human tissues will be of interest. The future identification of cylindrospermopsin adducts will not only assist in toxicity and carcinogenicity assessment, but may also provide biomarkers for human exposure.

APPENDIX A

Media, Buffers and Solutions Used for Hepatocyte Culture

A1-1 Media and chemical sources

Analytical grade chemicals were used, other reagents and media were purchased as cell culture tested products.

Chemical / Product	Source
BSA (fatty acid free)	Sigma-Aldrich, St Louis, MI, USA Trace Biosciences Ltd, New Zealand
$CaCl_2$	(BDH) Merck Pty Ltd, Vic, Australia
Collagenase (type IV for hepatocyte isolation)	Sigma-Aldrich, St Louis, MI, USA
DMEM/F12 (1:1 Mixture)	Sigma-Aldrich, St Louis, MI, USA
DMSO	ICN Biochemicals Inc, Ohio, USA
EGTA	Sigma-Aldrich, St Louis, MI, USA
Glucose	ICN Biochemicals Inc, Ohio, USA
HEPES (cell culture tested)	Sigma-Aldrich, St Louis, MI, USA
HBSS	Sigma-Aldrich, St Louis, MI, USA
Hydrocortisone (cell culture tested)	Sigma-Aldrich, St Louis, MI, USA
Insulin (cell culture tested)	Sigma-Aldrich, St Louis, MI, USA
KCl	ICN Biochemicals Inc, Ohio, USA
KH_2PO_4	(BDH) Merck Pty Ltd, Vic, Australia
$MgSO_4.7H_2O$	(BDH) Merck Pty Ltd, Vic, Australia
NaCl	ICN Biochemicals Inc, Ohio, USA
Na_2HCO_3	(BDH) Merck Pty Ltd, Vic, Australia
Penicillin/Streptomycin solution	Sigma-Aldrich, St Louis, MI, USA
Phenol Red	ICN Biochemicals Inc, Ohio, USA

A1-2 Stock Solutions

(i) 10X Modified Krebs-Henseleit Buffer

Chemical	Grams/Litre
NaCl	67.3
KCl	4.0
KH_2PO_4	0.54
Na_2HPO_4	0.43
$NaHCO_3$	21.8
HEPES	47.6
BSA	10.0
Glucose	10.0
Phenol Red	0.1

Make up to 1 Litre with M.Q. H2O.

Adjust pH to 7.3.

Filter sterilize. Store at 4°C.

(ii) 10X Magnesium Sulphate Solution (8 mM)

Dissolve 2.0 g of $MgSO_4.7H_2O$ in 1 L M.Q. H_2O. Autoclave. Store at room temperature.

(iii) 1000X Calcium Chloride Solution (1 M)

Dissolve 14.7 g $CaCl_2.2H_2O$ in 100 ml of M.Q. H_2O. Autoclave. Store at room temperature.

(iv) 10X EGTA Solution (1 mM)

Dissolve 0.38 g of EGTA in ~80 ml of 1X Krebs-Henseleit. Adjust pH to 7.3. Make up to 100 ml. Filter sterilize. Store at room temperature.

(v) 200X Insulin Solution (20 µM)

Dissolve 3.5 mg of Insulin in 35 ml 0.9% NaCl. Filter sterilize. Aliquot into microcentrifuge tubes. Store at -20°C.

(vi) 1000X Hydrocortisone Solution (1 mM)

Dissolve 2 mg of hydrocortisone in 5 ml of DMSO. Store 200 µl aliquots at -80°C.

(vii) BSA Solution (10%)

Dissolve 1 g BSA (fatty acid free) in 10 ml of DMEM/F12. Adjust pH to 7.3. Filter sterilize.

(viii) Penicillin/Streptomycin Antibiotic Stock

Obtained as 10,000U Penicillin and 10 mg Streptomycin / ml in 0.9% NaCl. Store at –20°C.

A1-3 Perfusion Media

(i) 1X Modified Krebs-Henseleit Buffer

Make up 1X Modified Krebs-Henseleit Buffer on the day of use. 300 ml is sufficient for liver perfusion of one mouse. Dilute as follows:

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30 ml 10X Modified Krebs-Henseleit Buffer
30 ml 10X $MgSO_4.7H_2O$ stock solution
240 ml M.Q. H_2O

Adjust pH to 7.3. Filter Sterilize.

(ii) First Perfusion Medium (Ca^{2+}-free perfusion)

To 1X Modified Krebs-Henseleit Buffer add:

0.5 ml 100X EGTA stock solution
0.25 ml 200X Insulin stock
0.25 ml Penicillin/Streptomycin stock
Make up to 50 ml with 1X Krebs-Henseleit Buffer

Equilibrate at 37°C in a water bath.

(iii) Second Perfusion Medium (Collagenase perfusion)

To 1X Modified Krebs-Henseleit Buffer add:

0.05 ml of 1000X $CaCl_2$ stock solution

0.25 ml 200X Insulin stock

0.25 ml Penicillin / Streptomycin stock

Make up to 50 ml with 1X Krebs-Henseleit Buffer

Equilibrate at 37°C in a water bath.

Immediately prior to use add 8 mg of collagenase powder.

(iv) Wash Medium

To 1X Krebs-Henseleit Buffer add:

0.2 ml of 1000X $CaCl_2$ stock solution

1 ml of 200X Insulin stock

1 ml Penicillin / Streptomycin stock

Make up to 200 ml with 1X Krebs-Henseleit Buffer

A1-4 Buffers

(i) 10X Phosphate Buffered Saline (PBS)

Chemical	Grams/Litre
NaCl	80
KCl	2.0
KH_2PO_4	2.4
Na_2HPO_4	14.6

Dissolve in ~900 ml M.Q. H_2O.

Adjust pH to 7.4.

Make up to 1 L with M.Q. H_2O. Store at room temperature.

For 1X PBS working solution, dilute 10X stock 1:9 with M.Q. H_2O.

Check pH is 7.4. Store at 4°C

A1-5 Cell Culture Medium

(i) Dulbecco's Modified Eagles Medium/Nutrient Mixture F-12 Ham (DMEM/F12 1:1 Mixture).

Obtained as sterile solution which contains 15 mM HEPES pyridoxine and sodium bicarbonate. Without L-glutamine. Substitutes pyridoxine hydrochloride for pyridoxal hydrochloride. Since the DMEM/F12 ordered was supplied without L-glutamine, it was added at the standard concentration of 0.35 g/L immediately prior to use.

Cell culture supplements:

Supplement 100 ml of DMEM/F12 as follows:

	Final Concentration
10 ml BSA (10% stock).	1%
0.5 ml Penicillin/Streptomycin	50U/ml Penicillin, 50 µg/ml Streptomycin
0.5 ml Insulin (200X stock)	100 nM
0.1 ml Hydrocortisone (1000X stock).	1 µM

Check pH 7.3. Re-adjust if necessary. Filter sterilize.

APPENDIX B

Journal Publication

Cell-Free Protein Synthesis Inhibition Assay for the Cyanobacterial Toxin Cylindrospermopsin

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Froscio, S.M., Humpage, A.R., Burcham, P.C. & Falconer, I.R. (2001) Cell-free protein synthesis inhibition assay for the cyanobacterial toxin cylindrospermopsin.
Environmental Toxicology, v. 16(5), pp. 408-412

NOTE:
This publication is included on pages 122-126 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://doi.org/10.1002/tox.1050

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