# TUMOUR PROMOTION BY THE CYANOBACTERIAL TOXIN MICROCYSTIN

A thesis submitted to the University of Adelaide in fulfilment of the requirements of the degree of PhD

in

the Department of Clinical and Experimental Pharmacology of the University of Adelaide

by

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November 1997

## ERRATA

Page ix, lines 4 to 5: "However, the dose-dependent response has shifted to the left...." should read: "However, the effective concentrations were about 10-fold lower."

Page 2, line 8: "....most genera have developed...." should read: "some nitrogen-fixing genera have developed....."

Page 2, line 15: "....which has allowed the cyanobacteria...." should read: "....which has allowed some cyanobacteria...."

Page 2, line 19: "....gas vacuoles are produced in response....." should read: "....gas vacuoles are produced in these species in response....."

## ABSTRACT

The microcystins are a range of cyclic heptapeptide hepatotoxins produced by a number of common freshwater cyanobacteria. They have been shown to be tumour promoters in mouse skin and rat liver. There is also strong epidemiological evidence of their involvement in the high rates of primary liver cancer in certain areas of China due to the consumption of cyanobacterially contaminated drinking water.

The effects of the microcystins are believed to be due to their potent inhibition of protein phosphatases 1 and 2A, enzymes which are intimately involved in the maintenance of cellular homeostasis, and in the regulation of the cell cycle. The Ki for the interaction of microcystin and these enzymes is in the range 0.1 - 1.0 nM. Microcystin binds to the active site and can form a covalent attachment there. Other toxins which target these enzymes, but which do not interact covalently, include nodularin, okadaic acid, the calyculins, and cantharidin. Microcystin and nodularin also differ from the others just mentioned in that they require active uptake by a transport system related to that responsible for bile acid uptake. This confers a high degree of liver specificity, and also means that a high intracellular concentration of toxin can be achieved at relatively low exposure levels.

The present study examined the tumour promoting effects of the microcystins through two independent means. First, a long term *in vivo* dosing study was performed in which cyanobacterial extract containing a range of microcystins was given in drinking water to mice which had been previously treated with the tumour initiator N-nitroso-N-methylurea by gavage. The aim of this study, given that liver cancer is not a major form of neoplasm in western populations, was to determine whether microcystins could promote non-hepatic tumour growth, in particular in the upper intestine. Morbid animals were killed and a careful post-mortem examination was performed. Liver, duodenum and any other affected tissues were examined histologically and tumour growth was quantified using image analysis techniques. No significant differences were found between the two microcystin-treated groups and the control group in terms of survival or degree of development of tumours, nor in the type of tumour engendered.

Second, the effects of pure microcystin-LR were examined in cultured primary hepatocytes from immature mice. The cells were isolated by collagenase perfusion from C3H/HeJ mice and grown in conditions under which proliferation occurred without mitogenic stimulation. Previous studies have used Epidermal Growth Factor (EGF) to induce proliferation in the hepatocytes in culture, however, in this system EGF masked the effects of microcystin-LR. Therefore unstimulated cells were exposed to the toxin. Effective concentrations of toxin were lower in this system than in others which have been reported.

Microcystin-LR was selectively toxic to hepatocytes in the range 1.0 - 100.0 nM. Cellular uptake of tritium-labelled toxin was progressively lost during five days in culture. Therefore, further studies were performed over three days of toxin exposure. A number of outcomes were seen depending upon the toxin concentration and dosing regime used. In an experiment where cells were exposed to a single addition of toxin, 1.0 nM microcystin-LR induced effects consistent with a stimulation of cytokinesis, whereas a concentration of 10.0 nM microcystin-LR induced an inhibition of cytokinesis. Cell-selective toxicity at this higher concentration also caused the enrichment of a more proliferatively active cohort of cells. A reduced rate of cell death, possibly due to interference with the apoptotic process, was also detected at 1.0 and 3.0 nM toxin.

Microcystin-LR did not promote an EGF-like proliferative response. In all other experiments, cells were exposed to multiple changes of medium containing microcystin-LR over three days in order to maintain a more constant exposure. Similar effects to those seen in the single-exposure experiment were observed. However, the dose-response was shifted to the left by a factor of approximately 10. Thus, a stimulation of cytokinesis occurred at picomolar concentrations, whereas an inhibition of this process was apparent at 1.0 nM toxin. Cells surviving a first exposure to 10.0 nM microcystin-LR remained insensitive to further additions of the toxin, instead proliferating at a rate greater than the untreated control.

The interaction of microcystin-LR with the apoptosis inhibitor nafenopin was explored. This compound generally minimised the effect of microcystin-LR at all concentrations. This finding suggests that at picomolar concentrations, microcystin-LR may have had an inhibitory effect on apoptosis, whereas at selectively toxic (nM) concentrations of microcystin-LR, the toxin may have been inducing an apoptotic response in some cells.

The cellular toxicology of microcystin-LR was compared with that of okadaic acid. There was no evidence that okadaic acid had any effect on cytokinesis. Instead okadaic acid inhibited DNA synthesis in a purely dose-dependent manner. The dose-response curves for cell death and DNA synthesis inhibition were of similar shape. However, that for cell death was in the low nM region, whereas that for DNA synthesis inhibition occurred at concentrations about ten-fold lower. This was not the case with microcystin-LR, where DNA synthesis was unaffected in cells resistant to microcystin-LR toxicity. Therefore, the extent of $^{3}$H-thymidine labelling of DNA in cells exposed to okadaic acid decreased with increasing toxin concentration, whereas the labelling of DNA in cells exposed to microcystin-LR did not directly correlate with toxin concentration. This suggests that either the differing affinities of these toxins for the protein phosphatases led to different growth regulatory outcomes, or that microcystin-LR exerted a selective pressure on the hepatocyte population such that less toxin-sensitive, proliferatively active cells were favoured.

To further explore the possible effects of microcystin-LR on the various hepatocyte sub-populations, flow cytometric methods were developed in order to be able to differentiate between cells based on their DNA content, that is their ploidy, which directly correlates with the level of differentiation. To validate these techniques, nuclear (DNA) and cytoplasmic (protein) dual fluorescent staining was used to monitor changes in ploidy and the rate of cellular division, respectively, in a mitogen-stimulated hepatocyte population. The patterns of effects seen in these studies compared well with those reported by other workers using different experimental approaches. These techniques were then applied to a study of the cell cycle effects of microcystin-LR. It was found that the acute toxicity of a single dose of microcystin-LR was only minimally selective on the basis of ploidy at concentrations below 10.0 nM, the approximate $EC_{50}$ for acute toxicity in this system. After three days' exposure to microcystin-LR, using the multiple-addition protocol outlined above, the number and proportion of binuclear cells increased with toxin concentration up to 1.0 nM, above which a reversion to control levels was seen. Average nuclear ploidy followed a similar pattern. These results suggest that up to 1.0 nM microcystin-LR, proliferating binuclear cells accumulated in the hepatocyte population. At 10 nM microcystin-LR, where cell-selective acute toxicity occurs, this effect was not seen in the hepatocyte population surviving the first addition of the toxin. Therefore, the cells affected by microcystin-LR at lower concentrations are also those preferentially killed by the toxin at higher doses.

Overall, the results from these hepatocyte culture experiments support the hypothesis that the actions of microcystin-LR are not the same in all hepatocytes in the population. This selectivity

may be due to the different rates of uptake of the toxin by the various sub-populations of cells within the hepatocyte population, and also to variations in their cell cycle status at the time of exposure. Therefore, one way in which this toxin might promote tumour growth is to selectively kill the more toxin-sensitive cells in the liver causing a regenerative response in the remaining population. Furthermore, tumourigenic cells are generally diploid, tend to express fewer differentiated characteristics than normal cells, and so are also likely to be less sensitive to microcystin toxicity. Hence, they are likely to obtain a growth advantage during toxin exposure. These effects are likely to play a role at higher exposure levels. When cells were exposed to a single addition of 1.0 nM microcystin-LR, or multiple additions of the toxin at pM concentrations, a promotion of cytokinesis appeared to occur in a significant proportion of the population. This would have the effect of lowering the average ploidy of the hepatocyte population and therefore, possibly, its level of differentiation. Lower ploidy cells, lacking the genetic redundancy of their higher ploidy counterparts, are perhaps more susceptible to the effects of mutagens. An inhibition of apoptosis, also suggested by observations made at these concentrations, could facilitate the fixation of mutations in the genome of affected cells.

## 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.*

*I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.*

Andrew Raymond Humpage
November 1997

SIGNED DATE.....5/11/97.....

## PUBLICATIONS IN SUPPORT OF THIS THESIS

Falconer, I. R. and Humpage, A. R. (1996). Tumour promotion by cyanobacterial toxins. *Phycologia* **35**, 74-79.

Humpage, A. R. and Falconer, I. R. (1998). Microcystin-LR effects on cytokinesis, ploidy, and apoptosis in cultured hepatocytes. In preparation.

Humpage, A. R. and Falconer, I. R. (1998). Effect of microcystin-LR on population dynamics in proliferating cultured hepatocytes, as determined by flow cytometry. In preparation.

## OTHER WORK PUBLISHED DURING THIS CANDIDATURE BUT NOT REPORTED IN THIS THESIS

Hawkins, P. R., Chandrasena, N. R., Jones, G. J., Humpage, A. R. and Falconer, I. R. (1997). Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. *Toxicon* **35**, 341-6.

Kwok, F., Humpage, A. and Cheng, C. H. K. (1996). Phosphatidylinositol Phosphate 5-Kinase Purification and Inhibition Studies.*Preparative Biochemistry and Biotechnology* **26**, 1-19.

## ACKNOWLEDGMENTS

Thanks must go, first and foremost, to my supervisors, Professor Ian Falconer, Dr. Phil Burcham, and Dr. Dennis Steffensen, whose immense goodwill and considerable string-pulling enabled this project to go ahead, and whose delicate guidance has allowed me to bring it to fruition.

However, the completion of this project is also due in no small part to Sandy Dickson who, whilst my supervisors were keeping my mind together, was nourishing body and soul with "meals on wheels" and considerate understanding. Thanks Sandy.

Others who have contributed stimulating discussions and often timely advice include Joanna Rositano, Dr. Rod Irvine, Gordon Crabb, and Dr. Stephen Hardy. May there be many more in the future.

Finally, to the staff and students of the Departments of Clinical and Experimental Pharmacology and of Microbiology and Immunology of the University of Adelaide, as well as to the people at the Australian Water Quality Centre: Thanks for all those acts of selfless good nature without which the world would grind to a halt.

## ABREVIATIONS USED IN THIS THESIS

| | |
|---|---|
| 2N | diploid |
| 2x2n | binucleate tetraploid |
| 2x4n | binucleate octaploid |
| 4N | tetraploid |
| 8N | octaploid |
| ADDA | 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid |
| ALT | alanine amino transferase |
| AMP | adenosine 5'-monophosphate |
| ANOVA | analysis of variance |
| AP-1 | activator protein-1 |
| Asp | aspartic acid |
| AST | aspartate amino transferase |
| ATP | adenine 5'-triphosphate |
| ATPase | ATP-dependent kinase |
| AU | absorbance units |
| BSA | bovine serum albumin |
| $Ca^{2+}$ | calcium |
| CAK | cdk activating kinase |
| cAMP | cyclic AMP |
| cdc2 | cell division cycle kinase-2 |
| cdk | cyclin-dependent kinase |
| cDNA | complementary DNA |
| CFSE | 5-(6-)-carboxyfluoresceindiacetate, succinimdyl ester |
| cm | centimetre |
| CRE | cAMP response element |
| Cys | cysteine |
| DEN | diethylnitrosamine |
| Dha | dehydroalanine |
| DMBA | demethylbenzanthracine |
| DMEM/F12 | 1:1 mix of Dulbecco's Modification of Eagle's Medium and Hank's F12 medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| dpm | disintegrations per minute |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |
| EGTA | ethylene glycol-bis[Baminoethyl ether]-N,N,N'N'-tetraacetic acid |
| FCS | foetal calf serum |
| FU | fluorescence units |
| g | gram or gravity |
| Gln | glutamine |
| HBSS | Hank's buffered salts solution |
| HCC | hepatocellular carcinoma |

| | |
|---|---|
| HEPES | N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid |
| HPLC | high performance (pressure) liquid chromatography |
| hr | hours |
| I1, I2 | inhibitors of PP1 |
| ip | intraperitoneal |
| ITS | Insulin-Transferrin-Selenium medium additive (Sigma) |
| ITS/100 | ITS at 1% of recommended concentration |
| $K^+$ | potassium |
| kDa | kilodalton |
| kg | kilogram |
| L | litre |
| $LD_{100}$ | dose likely to kill 100% of test animals |
| $LD_{50}$ | dose likely to kill 50% of test animals |
| LDH | lactate dehydrogenase |
| Ln | natural logarithm |
| LPS | lipopolysaccharide |
| M | moles per litre |
| Masp | N-methylaspartic acid |
| MC | microcystin |
| µCi | microCurie |
| MCYST | microcystin |
| Mdha | N-methyldehydroalanine |
| mg | milligram |
| µg | microgram |
| $Mg^{2+}$ | magnesium |
| min | minutes |
| ml | millilitre |
| µl | microlitre |
| MLCK | myosin light chain kinase |
| µM | micromoles per litre |
| mM | millimoles per litre |
| MPF | maturation promoting factor |
| MQ.H2O | milli-Q water |
| mRNA | messenger RNA |
| MTD | maximum tolerated dose |
| $Na^+$ | sodium |
| NAF | nafenopin |
| ng | nanogram |
| nM | nanomoles per litre |
| NMU | N-nitroso-N-methylurea |
| NSW | New South Wales |
| PBS | phosphate buffered saline |
| PCC | premature chromosome condensation |
| PCNA | proliferating cell nuclear antigen |
| pg | picogram |
| PI | propidium iodide |
| PM | post mortem |
| pM | picomoles per litre |

| | |
|---|---|
| PP | protein phosphatase |
| PSI | photosystem I |
| PSII | photosystem II |
| PSP | paralytic shellfish poison |
| Rb | retinoblastoma (protein) |
| Rb-P | phosphorylated Rb-protein |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| SD | standard deviation |
| SDH | sorbitol dehydrogenase |
| SEM | standard error of the mean |
| Ser | serine |
| SHE | Syrian hamster embryo |
| SPE | solid phase extraction |
| t=0 | initial conditions |
| TCA | trichloroacetic acid |
| TFA | trifluoroacetic acid |
| Thr | threonine |
| TNF-α | tumour necrosis factor-α |
| TPA | 12-O-tetradecanoylphorbol-13-acetate |
| TRE | TPA response element |
| Tyr | tyrosine |
| UK | United Kingdom |
| USA | Untied States of America |
| wrt | with respect to |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 Cyanobacterial ecology and the occurrence of toxicity

### 1.1.1 General cyanobacterial physiology and biochemistry, and factors influencing bloom formation

For many years these organisms were called blue-green *algae,* however it is now considered more correct to refer to them as cyano*bacteria* since in fact they are photosynthetic prokaryotes (of the phylum *Cyanophyta*). They have bacterial cell walls, the deposition of peptidoglycans into which can be inhibited by penicillin (Wolk, 1973). Likewise, they lack a defined nucleus and chloroplasts.

Cyanobacteria are however photosynthetic. They have the typical arrangement of pigments and related proteins into two photosystems (PSI and PSII), situated in a thylakoid membrane. They lack chlorophyll b, but do possess chlorophyll a, which resides at the centre of the photosystem II complex. The thylakoid membranes are arranged mainly around the periphery of the cell (Wolk, 1973). The phycobilisome complex, which gathers light for PS-II, is almost unique to the cyanobacteria - eukaryotic red algae being the only other organisms known to possess such a structure (Tandeau de Marsac and Houmard, 1993). It contains unique light gathering pigment molecules called phycoerythrin, phycoerythrocyanin, and phycocyanin, which have absorption maxima at 565nm, 568nm and 615-620nm, respectively. In most cyanobacteria phycocyanin constitutes the major component and it is this which, when mixed with the chlorophyll a, gives

these organisms their typical blue-green colouration. The efficiency of light harvesting and energy transfer of the phycobilisome to photosystem II is almost 100%, aided by a process called complementary chromatic adaptation, present in many cyanobacteria, whereby the relative quantities of the component pigments are altered in response to changes in the quality of incident light (Tandeau de Marsac and Houmard, 1993; Reviewed in Kehoe, *et al.*, 1994).

Many of these organisms are also capable of nitrogen fixation, and because the nitrogenase complex is extremely sensitive to rapid and irreversible inactivaton by the oxygen produced by photosynthesis, most genera have developed specialised cells called heterocysts to isolate the nitrogen fixation process. Heterocyst development has been used extensively as a model system to study the molecular biology of cellular differentiation processes in cyanobacteria (see Buikema and Haselkorn, 1993; Wolk, 1996, and references therein for reviews). Some nitrogen fixing genera do not produce heterocysts however, and the method of protection of the nitrogenase complex in these cells is not well understood, although a temporal separation of the two processes is thought to be involved (Fay, 1992).

Another adaptive feature which has allowed the cyanobacteria to prosper is the ability to position themselves in the water column to their best advantage, especially in calm conditions when vertical mixing is minimised. Practically no irradiation above 600 nm penetrates further than 4 metres in natural waters (Boulter, *et al.*, 1974 quoted in Tandeau de Marsac and Houmard, 1993). Proteinaceous intracellular gas vacuoles are produced in response to decreases in light intensity, and it is these which are used to regulate the buoyancy of the cell (Oliver and Walsby, 1984; Damerval, *et al.*, 1991) These structures are not unique to the cyanobacteria (Walker, *et al.*, 1984). Nevertheless, they appear to be one of the main reasons why blue-green algae can

form thick scums on the leeside of lakes and rivers and so pose such a threat to drinking livestock (Falconer, 1988).

There is still discussion as to the relative contributions of different environmental factors to the growth of large cyanobacterial blooms. Nevertheless, phosphorus seems to be the nutrient whose variation in natural systems best correlates with the occurrence of bloom events (Lukatelich and McComb, 1986). The increasing levels of this nutrient in waterways due to agricultural fertiliser run-off and sewage discharges has no doubt lead to the increasing incidence of blooms. Given adequate nutrients, as in culture conditions, light availability has been shown to be the greatest effector of both cell growth and toxin production, with low light intensities giving rise to low cell growth but high toxin production per cell, and visa versa (Watanabe and Oishi, 1985; Sivonen, 1990).

### 1.1.2 Widespread nature of the toxic bloom phenomenon

Toxicity in freshwater cyanobacteria has been reported throughout the world (see Carmichael, 1992 for a list of reported occurrences). Systematic surveys of cyanobacterial toxicity have been carried out in the former East Germany (Henning and Kohl, 1981), the Netherlands (Leeuwangh, *et al.*, 1983), Scandinavia (Berg, *et al.*, 1986), Finland (Sivonen, *et al.*, 1989b; Sivonen, *et al.*, 1990; Ekman-Ekebom, *et al.*, 1992), the United Kingdom (Codd, *et al.*, 1989; Pearson, 1990; Codd and Beattie, 1991), and Australia (Baker and Humpage, 1994). Ekman-Ekebom *et al.*, (1992), Pearson (1990), and Baker and Humpage (1994) contain the most comprehensive data sets and report the incidence of toxicity in blooms to be 44%, 68%, and 42% respectively. These surveys all concentrated on bloom forming planktonic species and it is probable that toxic benthic species are under-represented in the data. That benthic organisms can be of importance is shown

by reports of dog deaths due to a neurotoxic benthic *Oscillatoria* species (Edwards, *et al.*, 1992), and by evidence of hepatotoxin production by benthic cyanobacterial mats, sometimes in conjunction with neurotoxic symptoms in exposed animals (Mez, *et al.*, 1997).

The report by the National Rivers Authority, UK (Pearson, 1990) listed 25 cyanobacterial species which were implicated world wide in toxic events by their presence in toxic blooms. Others have suggested a number closer to 40 species (Scott, 1991). However, conclusive evidence of toxin production by use of axenic cultures exists for far fewer species. In Australia, the evidence so far suggests that only four species produce toxins in significant quantities. These are *Anabaena circinalis*, *Microcystis aeruginosa*, *Nodularia spumigena* and *Cylindrospermopsis raciborskii*. A range of toxins are produced (Baker and Humpage, 1994).

### 1.1.3 Epidemiological evidence for human toxicity

Epidemiological evidence for the correlation of blue-green algal blooms and human illness has come for a number of sources (for reviews see Schwimmer and Schwimmer, 1968; Falconer, 1989; Hunter, 1991; Volterra, 1993; and Yu, 1995).

Recreational use of water bodies can bring people into close contact with potentially harmful blooms. Skin rashes and dermatitis appear to be regular occurrences in such patients (Cohen and Reif, 1953; Carmichael, *et al.*, 1985; Codd and Bell, 1985; Soong, 1993). Others have suggested a link with hay-fever-like and/or respiratory (asthma-like) symptoms (Heise, 1949; Heise, 1951; McElhenney, *et al.*, 1962; Mittal, *et al.*, 1979; Billings, 1981). A number of these investigators have demonstrated positive reactions in skin tests or bronchial provocation tests in challenged patients, indicating an allergic aetiology rather than the action of toxins.

Other reported symptoms occurring after recreational contact include gastroenteritis, some showing the presence of cyanobacterial cells in the stools of patients (Dillenberg and Dehnel, 1960; Billings, 1981), and atypical pneumonia (Turner, *et al.*, 1990), where a toxin-mediated aetiology is more probably indicated. The consumption of fish from bloom infested lagoons, and of the fish livers in particular, has been implicated in the occurrence of a syndrome known as Haff Disease, from which fatalities have occurred(Berlin, 1947).

Infestation of drinking water has lead to cases of poisoning in consumers. Cases involving gastrointestinal symptoms range from Rhodesia (Zilberg, 1966) to Clark Air Base in the Philippines (Dean, *et al.*, 1972; Dean and Jones, 1972) and Sewickley, Pennsylvania (Lippy and Erb, 1976). The presence of endotoxins in the water supply in Washington DC in 1974, as shown by a positive *Limulus* lysate test, appears to have been the cause of a number of episodes of endotoxicosis in dialysis patients. Forty-nine reactions in 23 patients were documented over a four week period during which there were high cyanobacterial cell counts in the water supply. The cases subsided when the counts went down (Hindman, *et al.*, 1975). Poisoning of dialysis patients with more tragic consequences has occurred recently in Portugal (Araujo, F., personal communication) and Brazil (Azevedo, 1997; Carmichael, *et al.*, 1997). In the latter event 131 people developed symptoms including visual disturbances, nausea and vomiting, with many of them having a common syndrome associated with painful liver enlargement, liver haemorrhage or liver failure. Fifty-five subsequently died.

Human hepatotoxicoses linked with consumption of contaminated drinking water have been observed in two cases from Australia. In the first, in 1979, 139 children, as well as a number of adults, were stricken with an acute hepatitis. Other symptoms included loss of protein, sugars, and

ketones in the urine, severe electrolyte imbalance, and bloody urine and diarrhoea. As a result of hospitalisation and intensive care all survived and recovered within a month (Byth, 1980). A bloom of *Cylindrospermopsis raciborskii* on the water supply reservoir had been lysed with copper sulphate just prior to the outbreak (Bourke, *et al.*, 1983; Hawkins, *et al.*, 1985). Recently, a previously unknown general cytotoxin was isolated and characterised from material collected from this reservoir (Ohtani, *et al.*, 1992). The second case, reported by Falconer *et al,* in 1983, described time-based correlations between blooms of toxic cyanobacteria in the local water reservoir (Malpas Dam) and increases in liver enzymes in the plasma of the population of the city of Armidale, NSW, who drank water supplied from the infested reservoir. Enzyme levels in the blood of people from comparable rural towns drinking from different water supplies, did not show this correlation. The enzyme showing best correlation with the bloom event was γ-glutamyl transferase, which is known to be a sensitive indicator of microcystin poisoning in test animals (Falconer, *et al.*, 1994).

The possibility that the presence of toxic cyanobacteria in the drinking water may enhance the growth of human tumours has recently been supported by studies in China. It has been known by the authorities there for some time that certain areas of the country have very high incidences of hepatocellular carcinoma (HCC), even by Chinese standards, and that this increased risk was associated with the use of pond or ditch water for drinking (Su, 1979). More recently, microcystin has been found to be present in higher concentrations in pond and ditch water in high HCC areas than in well water in low HCC areas (Zhang, *et al.*, 1991; Yu, 1995). Furthermore, partially hepatectomised, aflatoxin-$B_1$ initiated F-344 rats have been given pond-ditch water residuals to drink for 8 months. After this time liver sections from this group, but not those of animals given well water, showed increased numbers of abnormal pre-cancerous cell foci when

stained for γ-glutamyl transferase (Zhou and Yu, 1990, quoted in Yu, 1995), a phenomenon thought to be indicative of cancerous cellular transformation.

## 1.2 Cyanobacterial toxins

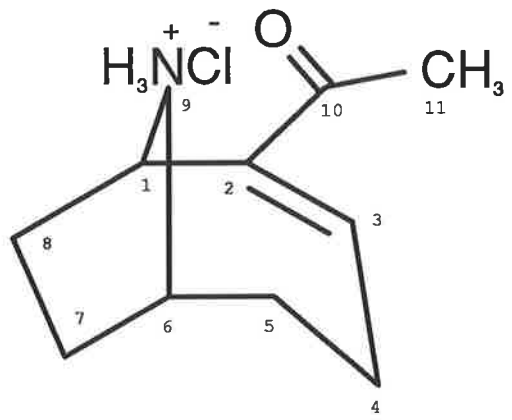
Cyanobacterial toxins in drinking water fall into a number of classes based on their site of action and symptoms, *viz.*, neurotoxins, hepatotoxins, and lipopolysaccharide (LPS) endotoxins. The neurotoxins include anatoxin-a, anatoxin-a(s), and the saxitoxin group of paralytic shellfish poisons. The hepatotoxins include the microcystins, nodularin and cylindrospermopsin. The LPS endotoxins remain largely uncharacterised. A number of toxins are also produced by marine cyanobacteria (examples include lyngbyatoxin and aplysiatoxin) but these will not be discussed in this review.

### 1.2.1 Neurotoxins

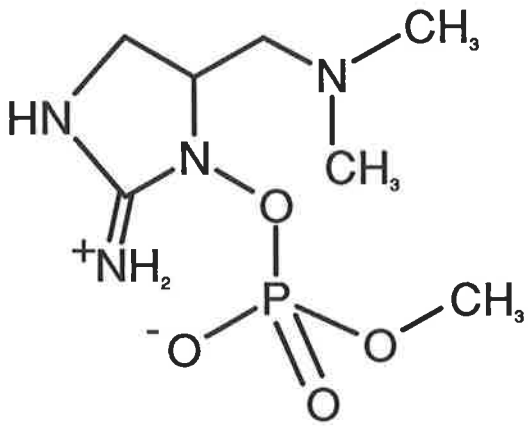
Anatoxin-a was originally found in an isolate of *Anabaena flos-aquae* from Canada (Devlin, *et al.*, 1977). It has since been found elsewhere in this species and also in *Anabaena circinalis* (Sivonen, *et al.*, 1989a; Stevens and Krieger, 1991), *Anabaena planctonica* (Bruno, *et al.*, 1994); *Aphanizomenon* sp., *Cylindrospermum* sp., and *Oscillatoria* sp. (Sivonen, *et al.*, 1989a) and in a benthic *Oscillatoria* sp. (Edwards, *et al.*, 1992). It is a small alkaloid, 2-acetyl-9-azabicyclo[4,2,1]non-2,3-ene (Huber, 1972) (Figure 1.1) which acts as a post-synaptic cholinergic depolarising agent (Carmichael, *et al.*, 1979). Typical symptoms include muscle fasciculations, loss of muscle coordination, gasping, convulsions, and death by respiratory arrest within minutes of the onset of symptoms (Carmichael and Gorham, 1974). In the isolated rat phrenic nerve-

hemidiaphragm preparation, the toxin causes complete block of muscle twitch at concentrations greater than 25μM (4μg $ml^{-1}$) (Carmichael, *et al.*, 1979).

**Figure 1.1** - Structures of anatoxin-a and anatoxin-a(s)




Anatoxin-a hydrochloride




Anatoxin-a(s)

The $LD_{50}$ by intraperitoneal injection in mice is 200μg $kg^{-1}$ (Carmichael, *et al.*, 1990). An analogue of anatoxin-a, possessing an ethyl rather than a methyl group on the ketone carbon (C10), called homoanatoxin-a, has been isolated from a strain of *Oscillatoria formosa*. Its toxicity and mode of action are similar to anatoxin-a (Skulberg, *et al.*, 1992; Lilleheil, *et al.*, 1997). Anatoxin-a was not detected by gas chromatography/electron capture detection analysis in any of 92 Australian samples (33 field samples and 59 cultured isolates) (Baker, *et al.*, 1993; Baker and Humpage, 1994).

Anatoxin-a(s) has also been isolated from *A. flos-aquae* (Carmichael and Gorham, 1978; Mahmood and Carmichael, 1986), but appears to be less widely distributed than anatoxin-a, all occurrences having been reported from the USA (Carmichael, 1992). It is a potent acetylcholinesterase inhibitor (Mahmood and Carmichael, 1986; Mahmood and Carmichael, 1987), having an $LD_{50}$ (ip mouse) of 20μg $kg^{-1}$ (Carmichael, *et al.*, 1990). Symptoms are typical of an anticholinesterase, that is, marked salivation, lachrymation in mice or chromodacryorrhoea in rats, urinary incontinence, muscular weakness, fasciculation, convulsion, and defecation, followed by death due to respiratory failure (Carmichael, *et al.*, 1990). Chemically, anatoxin-a(s) is the phosphate ester of a cyclic N-hydroxyguanidine (Matsunaga, *et al.*, 1989) (Figure 1.1), which exhibits a mechanism of inhibition of acetylcholinesterase similar to the organophosphate pesticides (Carmichael, *et al.*, 1990). The symptoms caused by anatoxin-a(s) in animals distinguish it from any cyanobacterial toxicity seen in Australia (Runnegar, *et al.*, 1988; Baker, *et al.*, 1993; Baker and Humpage, 1994).

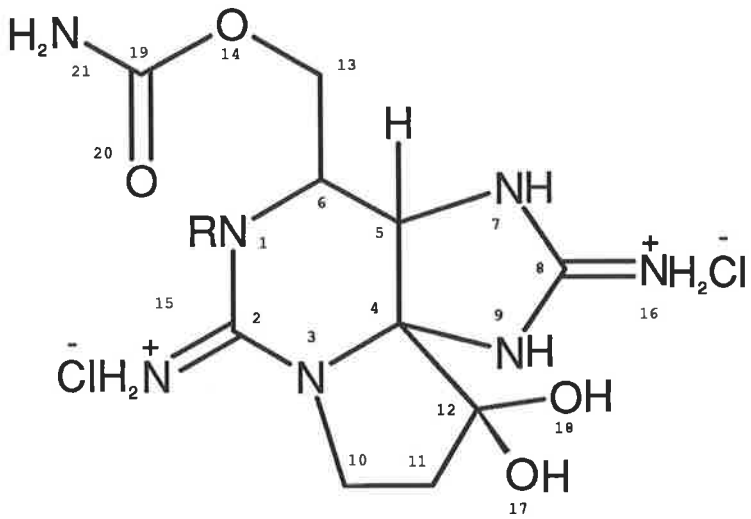
The "aphantoxins" were isolated from a bloom of *Aphanizomenon flos-aquae* which caused the death of 6 tons of fish in Kezar Lake, New Hampshire, USA, in 1966, the toxin having been released from the cells by copper sulphate treatment (Sawyer, *et al.*, 1968). Similar blooms occurred throughout the 1960s (Sasner, *et al.*, 1984). The electrophysiological and chemical properties of the toxins were found to be similar to the paralytic shellfish poison (PSP), saxitoxin (Jackim and Gentile, 1968; Sawyer, *et al.*, 1968). Purified toxins were shown to be saxitoxin and substances similar to other known PSPs (Alam, *et al.*, 1978). Neosaxitoxin was later identified and saxitoxin confirmed (Ikawa, *et al.*, 1982; Sasner, *et al.*, 1984) in a strain isolated from a small farm dam, Durham, New Hampshire (Carmichael, 1982). The biosynthesis of saxitoxin analogues has been studied in *Aphanizomenon flos-aquae* strains isolated from this outbreak (Shimizu, *et*

*al.*, 1984). The blooms of toxigenic *Aphanizomenon flos-aquae* all occurred in New Hampshire between 1966 and 1980 and appear to have subsided since then (Carmichael, *et al.*, 1990).

The production of PSPs by Australian bloom and cultured material has now been demonstrated (Humpage, *et al.*, 1993; Humpage, *et al.*, 1994). The gross symptoms of PSP toxicity seen in mouse bioassay are indistinguishable from those of anatoxin-a, since both cause complete block of muscle activation, anatoxin-a due to its action at the neuromuscular synapse, and the PSPs due to their effects on neuronal signal conductance. However, in the isolated desheathed amphibian sciatic nerve only the PSPs caused a time dependent decrease in peak height and rate of rise of the compound action potential. This was the first indication as to the identity of the Australian neurotoxins. The PSPs are the only class of neurotoxins to be identified in Australia thus far (Baker and Humpage, 1994). Animal deaths due to neurotoxic *A. circinalis* poisoning have been reported previously (Runnegar, *et al.*, 1988), although only recently were PSP toxins confirmed as the causative agents (Negri, *et al.*, 1995). The bioaccumulation of these toxins in the freshwater mussel *Alathyria condola* has also been demonstrated (Negri and Jones, 1995).

The PSPs constitute a range of hydroxylated and sulphated analogues of the parent compound, saxitoxin (Figure 1.2). They pose a significant public health threat in the marine environment where they are produced by a number of "red tide" dinoflagellate species, and can be accumulated in the tissues of filter feeders such as mussels, clams and oysters (Kao, 1993), hence the generic term. A bacterial source of the toxins, isolated from a cultured toxigenic dinoflagellate, has also been reported (Kodama, *et al.*, 1989). They are potent sodium channel blockers, and as a result are extremely neurotoxic, saxitoxin having an $LD_{50}$ (ip mouse) of 10$\mu g\ kg^{-1}$. Other analogues vary greatly from this figure however (Oshima, *et al.*, 1989).

**Figure 1.2** - Structures of saxitoxin and neosaxitoxin




R = H; saxitoxin dihydrochloride
R = OH; neosaxitoxin dihydrochloride

As many as six *Anabaena* neurotoxins were distinguished on symptomological grounds from Canadian isolates (Carmichael and Gorham, 1978). However, only anatoxin-a and anatoxin-a(s), have ever been characterised from these samples. In Finland, only 13 of 30 neurotoxic bloom samples showed evidence by gas chromatography/mass spectrometry of anatoxin-a production (Sivonen, *et al.*, 1989a). In two of these 13, the quantity of anatoxin-a found was not enough to account for the mouse toxicity observed. Ekman-Ekebom *et al,.* (1992) noted a correlation between the dominance of blooms in some Finnish lakes by *A. flos-aquae* and *A. lemmermannii* and the presence of an unidentified neurotoxin. Presence of anatoxin-a correlated with *A. circinalis* dominance. PSP's were not found (Sivonen, K., personal communication).

Overseas, simultaneous production of both neurotoxins and hepatotoxins has been found in *A. flos-aquae* (Al-Layl, *et al.*, 1988; Sivonen, *et al.*, 1989a; Harada, *et al.*, 1991) and *Anabaena planctonica* (Bruno, *et al.*, 1994). In Australia, both neurotoxic (May and McBarron, 1973) and hepatotoxic (McBarron, *et al.*, 1975) symptoms in test animals have been attributed to *A. circinalis*. A report on the 1991 River Darling *A. circinalis* bloom (Bowling, 1992) also described both neurotoxic and hepatotoxic symptoms in mouse bioassays. Similarly, subacute hepatotoxicity was seen in both neurotoxic and non-toxic samples of *A. circinalis* during the latest cyanobacterial survey of the Murray-Darling Basin (Baker, *et al.*, 1993; Baker and Humpage, 1994). Whether these findings indicate the presence of a contaminating hepatotoxin producer, or the concurrent production of both toxins by the one species, is unknown at present.

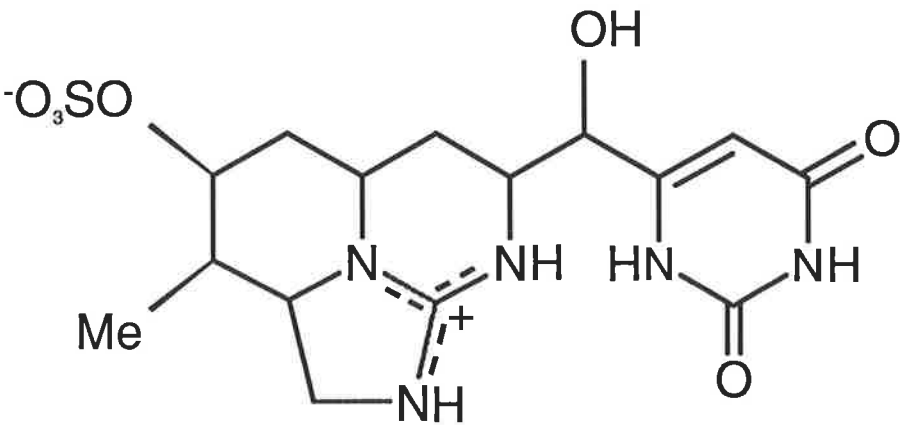
### 1.2.2 Hepatotoxins

Acute hepatotoxicity is the most frequently reported toxicosis involving cyanobacteria (Carmichael, 1992). Three classes of freshwater cyanobacterial hepatotoxin are known: cylindrospermopsin, nodularin, and the microcystins.

As mentioned above, cylindrospermopsin has recently been isolated (Ohtani, *et al.*, 1992) from a strain of *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju cultured from bloom material implicated in one of the worse recorded cases of human poisoning by cyanobacterial toxins (Bourke, *et al.*, 1983). Cylindrospermopsin is a novel alkaloid toxin in which a guanidinyl group is linked to an hydroxyuracil (Figure 1.3). Its $LD_{50}$ (ip) in mice is 2.1 mg/kg after 24 hours and 0.2 mg/kg after 5-6 days (Ohtani, *et al.*, 1992). It is a general cytotoxin and hence its effects are particularly apparent in those tissues, such as the liver and kidneys, with high blood flow. The mechanism of action of cylindrospermopsin involves protein synthesis

inhibition (Terao, *et al.*, 1994). However, it is likely to be extensively metabolised in the liver as indicated by glutathione synthesis inhibition and evidence for the involvement of the P450 system (Runnegar, *et al.*, 1994; Runnegar, *et al.*, 1995d), and so cylindrospermopsin itself may not be the active agent. There has been marked variability in the toxicology of cultured isolates of *C. raciborskii*, but it is unsure at this stage whether this signifies the presence of other toxins, or differences in the metabolism of cylindrospermopsin due either to inter-individual variability between test animals or the presence of modifying substances in the cellular extracts (Hawkins, *et al.*, 1997).

**Figure 1.3** - Structure of cylindrospermopsin

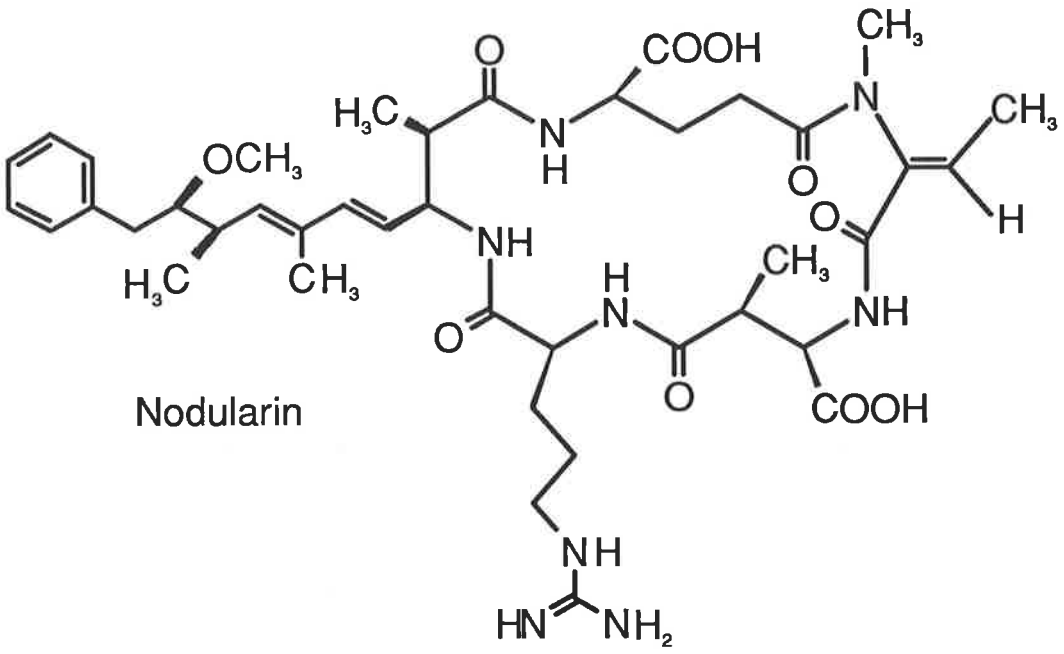



Cylindrospermopsin

Nodularin (nodularin-R, for arginine) is a cyclic pentapeptide produced by *Nodularia spumigena* (Rinehart, *et al.*, 1988), with considerable structural similarity to the microcystins (see Figures 1.4 and 1.5). A similar compound, originally called "Motuporin", was isolated from a marine sponge (de Silva, *et al.*, 1992). Motuporin has now been given the more systematic name

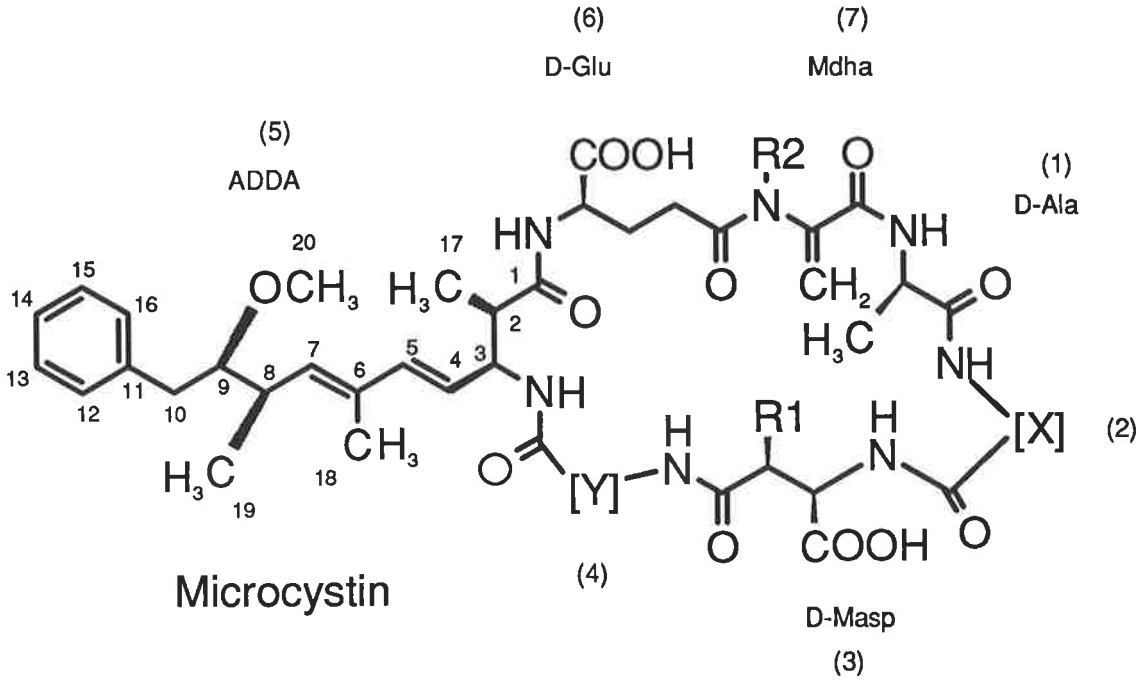
nodularin-V (for valine), and three other variants have also been identified (Quinn, *et al.*, 1996). The general toxicology of nodularin is similar to that of microcystin and will be discussed with that compound below.

**Figure 1.4** - Structure of nodularin-R




The microcystins occur as a range of cyclic heptapeptide analogues, of which more than 40 are now known (See Figure 1.5). All are comprised of five relatively invariant D-amino acids plus two L-amino acids (marked X and Y in the depiction below) which are more variable between analogues.

**Figure 1.5** - Generic structure of the microcystins




The method for the naming of the toxins is based upon the use a two letter suffix to denote these two variable L-amino acids (Carmichael, *et al.*, 1988). Thus, a microcystin (MCYST) with leucine and arginine at these positions would be termed MCYST-LR, whereas MCYST-RR has arginine at both. Additional notation has been found necessary to designate those toxins in which the residues at positions 3 or 7 (R1 and R2 in the figure), methylaspartate (Masp) and N-methyldehydroalanine (Mdha), respectively (see Figure 1.5), are demethylated, giving rise to aspartic acid at position 3 (D-Asp$^3$) and dehydroalanine at position 7 (Dha$^7$), respectively. Therefore, the doubly demethylated MCYST-RR is designated [D-Asp$^3$, Dha$^7$]-MCYST-RR. The novel amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA) at position 5 has been shown to be absolutely required for toxicity (Dahlem, 1989). Furthermore, isomerization at C-8 of ADDA to the *cis* configuration also negates toxicity (Harada, *et al.*, 1990). Most other analogues exhibit $LD_{50}$'s (ip mouse) of about 60-70 μg/kg with the exception

of MCYST-RR and the D-Asp$^3$ and Dha$^7$ derivatives, which have $LD_{50}$'s of about 200-250 μg/kg (Carmichael, 1992). In rats, the $LD_{50}$ (ip) for MCYST-LR is 122 μg/kg in the fed animal, but 72 μg/kg in fasted rats (Miura, *et al.*, 1991). Oral toxicity in mice is approximately 30 - 100-fold lower than by the ip route (Falconer, *et al.*, 1994; Fawell, *et al.*, 1994).

### 1.3 **Toxicological studies into the effects of the microcystins**

Animals exposed to a lethal dose of microcystin-containing material exhibit muscular weakness and lethargy, pallor of mucous membranes and extremities, some muscle twitching, and laboured breathing (McBarron and May, 1966; Elleman, *et al.*, 1978). The most noticeable pathologic finding is a grossly enlarged liver (Elleman, *et al.*, 1978). Furthermore, the blood content of the liver increases considerably by 45 min after injection of a lethal dose (Falconer, *et al.*, 1981). Death occurs between a few hours and a few days after exposure due to hypovolaemic shock as the animal haemorrhages into this organ (Falconer, *et al.*, 1981). Liver histology shows a progressive loss of sinusoidal architecture, scattered hepatocyte necrosis, and congestion with blood (Elleman, *et al.*, 1978). Species differences have been observed in the order of progression of histologic effects. Whereas in mice the initial effect is seen at the periphery of the lobule, but quickly spreading throughout (Falconer, *et al.*, 1981), in rats the initial focus is centrilobular (Ashworth and Mason, 1946). In sheep, a widespread coagulative necrosis has been described (McBarron and May, 1966). With chronic dosing, mononuclear infiltration, cirrhosis, and Kupffer-cell hyperplasia are seen (Elleman, *et al.*, 1978; Hermansky, *et al.*, 1990). With low doses, numerous mitotic figures are seen (Elleman, *et al.*, 1978). Increases in serum LDH, SDH, and ALT are usually found (Elleman, *et al.*, 1978; Falconer, *et al.*, 1981; Hermansky, *et al.*, 1990; Mereish, *et al.*, 1991). In the isolated perfused rat liver, 0.3-0.5 μg/ml microcystin-LR in the

perfusate for 60 min caused stimulation of glycogenolysis, liver engorgement, and cessation of bile flow. Electron micrographs of liver show dilation of bile canaliculi and the space of Disse, loss of sinusoidal endothelial lining, and decreased hepatocyte intercellular contacts. Concomitant with these changes is an influx of erythrocytes (Falconer, *et al.*, 1981; Pace, *et al.*, 1991). The hepatocyte plasma membrane is seen to remain intact, although internal membranes such as the endoplasmic reticulum and the nuclear membrane are damaged (Falconer, *et al.*, 1981).

Intravenous or intraperitoneal exposure of an animal to radiolabelled microcystins has shown that between 20% and 90% of the toxin is sequestered by the liver (the variation perhaps explained by the route of exposure, and the use of mice, or fasted or fed rats by different workers), with the rest distributed amongst the kidneys, blood, urine, gut contents and faeces (Falconer, *et al.*, 1986; Runnegar, *et al.*, 1986; Brooks and Codd, 1987; Robinson, *et al.*, 1991c). The half-life in blood, after an initial rapid redistribution into the extracellular compartment, is about 42 min (Falconer, *et al.*, 1986). The liver fraction is stable for at least 6 days (Robinson, *et al.*, 1991a; Robinson, *et al.*, 1991c), and is tightly bound to mainly cytosolic elements with between 80% and 90% of this bound to cytosolic protein (Hooser, *et al.*, 1991). Biotransformation products of administered radiolabelled microcystin have been detected chromatographically in both *in vivo* (Robinson, *et al.*, 1991c) and *in vitro* (Robinson, *et al.*, 1991a) experiments, but these products were not characterised. That microcystins are metabolised in the liver is suggested by results showing that induction of microsomal enzymes prior to toxin exposure reduced the acute toxic response (Brooks and Codd, 1987). Glutathione and cysteine conjugates of Mdha, along with sulphated conjugates of ADDA, have since been detected using immuno-affinity chromatography (Kondo, *et al.*, 1996).

Studies with isolated hepatocytes have demonstrated that the toxin is taken up by active transport, and that this is inhibited by bromosulfophthalein, cholate, deoxycholate, taucholate, rifampicin, and silymarin (Runnegar, *et al.*, 1981; Eriksson, *et al.*, 1990a; Runnegar, *et al.*, 1991; Thompson and Pace, 1992; Mereish and Fajer, 1993; Runnegar, *et al.*, 1995a). These findings suggest that the multispecific anion transport system (or "bile acid transporter") is the likely means of hepatic uptake. This would explain the hepatoselective toxicity of these compounds. However, the characteristics of the microcystin transporter do not match any of the known bile acid transporters, and so some uptake of the toxin seems to be due to as yet uncharacterised transporters (Runnegar, *et al.*, 1995a). Similarly, chicken intestinal enterocytes, which must take up the toxins from an oral dose, have been shown to exhibit typical toxic effects and to be protected by compounds which are substrates for the bile acid transporter (Falconer, 1993). In the whole animal, membrane-active antioxidants such as vitamin E and silymarin were partially protective against microcystin-LR intoxication, whilst blockers of the bile uptake system, such as cyclosporin-A and rifampin were highly protective (Hermansky, *et al.*, 1991). It should be noted, however, that the above experiments have almost invariably used microcystin-LR, the most readily available pure toxin standard, but which is relatively hydrophilic. Other analogues, containing for example leucine, valine, or phenylalanine in place of arginine in microcystin-LR, are considerably more hydrophobic and might be expected to passively diffuse across the plasma membrane more easily(Craig, *et al.*, 1993).

Apart from their direct effects on hepatocytes, the microcystins have also been shown to elicit expression of tumour necrosis factor-α (TNF-α) in spleen and peritoneal macrophages, suggesting that some of their effects may be secondary to macrophage activation (Nakano, *et al.*, 1991). Consistent with this hypothesis, these workers found that prior injection of anti-TNF-α was partially protective against liver damage and prevented mouse death. This observation might

explain the discrepancy in enzyme loss between reports from *in vivo* dosing experiments, where serum LDH, ALT, and AST have been shown to rise markedly (Elleman, *et al.*, 1978; Falconer, *et al.*, 1981), and *in vitro* experiments with isolated hepatocytes, where enzymes are not initially lost and Trypan Blue integrity is maintained even with marked blebbing (Runnegar, *et al.*, 1981; Boe, *et al.*, 1991). Hence, hepatocyte leakage and death *in vivo* may be secondary to activation of the reticulo-endothelial cells rather than being a primary toxic effect. An alternative mechanism is that cells are ruptured due to hydrostatic and shearing forces as the liver becomes engorged with blood.

It has also been suggested that primary hepatocytes (and other cell types) become apoptotic (i.e., enter programmed cell death) rather than becoming necrotic upon exposure to microcystin or okadaic acid (Boe, *et al.*, 1991), which would therefore entail a regulatory, rather than a purely toxic, mechanism. However, the evidence presented by these workers differs from the conventional view of apoptosis in two important ways - lack of DNA fragmentation and lack of a requirement for protein synthesis (Bursch, *et al.*, 1994; Wyllie, 1994) - whilst the remaining criteria cited (condensation of chromatin, nuclear membrane convolution, internal membrane and organelle rearrangement without effect on mitochondrial volume, plasma membrane blebbing with loss of fine structure, loss of cell-cell adhesion and rounding up of cells in monolayer, and cell condensation leading to lower cell volume) can all be explained by the known toxic effect of disruption of the cytoskeleton. These authors noted that only with prolonged exposure did the cells in culture become necrotic and allow entry of Trypan Blue. However, it might be expected that long-term disruption of intracellular structure, even in the absence of macrophages, would lead to metabolic dysfunction and necrotic cell death. The above considerations do not of course preclude a regulatory effect, perhaps at concentrations well below those necessary for morphological effects to be seen, which would have consequences for cell cycle regulation.

The mutagenic potential of the microcystins has been investigated in the *Salmonella typhimurium* and *Bacillus subtilis* bacterial systems (Runnegar and Falconer, 1982; Repavich, *et al.*, 1990). The toxin was not found to be mutagenic in these systems, although there was evidence of a clastogenic effect in human lymphocytes (Repavich, *et al.*, 1990). However, the neurotoxins anatoxin-a(s) and neosaxitoxin, also purified from toxic cyanobacteria, were almost as effective as the purified hepatotoxin. Therefore these results must be interpreted with some caution.

Tumour promotion following exposure of animals to microcystins has also been demonstrated. In the mouse skin model, mice initiated by topical application of the carcinogen dimethylbenzanthracine (DMBA) and given *Microcystis* extract in their drinking water exhibited increased numbers and weights of tumours when compared to control groups drinking either tap water or nontoxic *Anabaena* extract (Falconer and Buckley, 1989). It is important to note that the oral route of exposure was used in this study indicating that, notwithstanding the supposed liver specificity of the microcystins (Matsushima, *et al.*, 1990), the toxin must still have had an influence in the cells of the skin to significantly promote tumour growth there. Whether this involves macrophage stimulation, direct toxin action, or some other mechanism, is unknown. Altered foci of cells expressing the placental isozyme of γ-glutamyl transferase have been reported in the livers of F-344 rats which were 2/3 hepatectomised, treated with the carcinogen diethylnitrosamine (DEN), and then given ip injections of purified microcystin-LR (Nishiwaki-Matsushima, *et al.*, 1992). As noted previously, partially hepatectomised, aflatoxin-$B_1$ initiated F-344 rats given residuals from Chinese pond-ditch water to drink for 8 months, also showed increased numbers of abnormal foci when stained for γ-glutamyl transferase (Zhou and Yu, 1990,

quoted in Yu, 1995). These pond and ditch waters are known to be subject to *Microcystis* blooms and to contain measurable quantities of microcystins (Zhang, *et al.*, 1991; Yu, 1995).

## 1.4 Mechanism of action of microcystin-group toxins

Other natural toxins known to have actions similar to the microcystins, and which have been used almost interchangeably, are okadaic acid and the calyculins (Fujiki and Suganuma, 1993). Okadaic acid, although first isolated from a marine brown sponge *Halichondria okadai* (Tacibana, *et al.*, 1981), is the parent compound of a number of substituted analogues (dinophysistoxins) actually produced by a dinoflagellate, *Dinophysis fortii* (Murata, *et al.*, 1982). When these compounds become bioconcentrated in the tissues of filter feeders they can cause human diarrhetic shellfish poisoning. The calyculins (A-H) were isolated from another marine sponge, *Discodermia calyx*, and shown to have toxic properties in starfish oocytes and a leukaemia cell line (Kato, *et al.*, 1988). Both types of compound have been shown to compete with microcystins for protein phosphatase binding sites (MacKintosh, *et al.*, 1990; Suganuma, *et al.*, 1990; Yoshizawa, *et al.*, 1990). Because okadaic acid and calyculin A are more hydrophobic than microcystin-LR and hence can cross the cellular membrane by passive diffusion, they have been more widely used in the study of the intracellular actions of this group of toxins. Nevertheless, these findings have direct relevance to the study of microcystin toxicity and so will be included, when relevant, in this review.

Phosphorylation/dephosphorylation of strategic proteins plays a major role in the regulation of every aspect of cellular metabolism. The hydroxyl groups of critically placed serine, threonine, or tyrosine residues in the target protein are the usual sites of phosphorylation. Such reactions are a simple and elegant means by which protein structure, or access to catalytic or allosteric binding

sites, can be modified. The protein kinases and phosphatases which carry out these reactions generally have specificity for either serine and threonine, or for tyrosine (Barritt, 1992). They are often the subjects of phosphorylatory regulation themselves, leading to the formation of cascades of linked enzymes. This allows for the rapid amplification of a regulatory signal, but also for subtle modification of the original signal by tissue or cell cycle specific effectors. An example is the well known cascade of phosphorylations and dephosphorylations linking hormone binding to receptors at the cell surface with the breakdown of glycogen by glycogen phosphorylase (Barritt, 1992). The serine/threonine protein phosphatases are particularly sensitive to inhibition by a group of toxins which includes the microcystins, calyculins and dinophysistoxins and will therefore be discussed more fully below.

### 1.4.1 Serine/threonine protein phosphatases

The serine/threonine protein phosphatases (PPs) are a ubiquitous class of enzymes occurring in organisms as diverse as mammals, fruit flies, starfish, yeast, and higher plants (Cohen and Cohen, 1989; Mumby and Walter, 1993). The protein phosphatases from mammalian tissues have been classified empirically, based on their substrate specificity, requirement for divalent cations, and their susceptibility to inhibition by a number of agents (Cohen, 1989; Cohen, 1991). The primary distinction is between those which preferentially dephosphorylate the ß subunit (type 1 PPs) or the α subunit (type 2 PPs) of phosphorylase kinase. Furthermore, only PP1 is susceptible to inhibition by two small heat- and acid-stable proteins called inhibitors 1 and 2 (I1 and I2, respectively). The PP2 class can be further subdivided based upon the requirement for divalent cations: PP2A, like PP1, is at least partially active towards most substrates in the absence of divalent cations, whereas PP2B and PP2C have an absolute requirement for $Ca^{2+}$ and $Mg^{2+}$, respectively. A third class of PP (PP3) has recently been described which has a preference for the β subunit of phosphorylase

kinase, no requirement for divalent cations, but is stimulated by I2 and, in contrast to the other PPs, appears to be an integral membrane-bound enzyme (Honkanen, *et al.*, 1991). Two other protein phosphatases have recently been identified in cDNA libraries, but little is known of their physiological roles as yet (Brewis, *et al.*, 1993; Chen, *et al.*, 1994). PP1, PP2A, PP3 are inhibited by the microcystin-group toxins, as were PP4 and PP5 when generated in a bacterial expression system.

The protein phosphatases must play a key role in cellular metabolism, judging by their broad distribution and the high degree of homology of structure and action seen between diverse species. For example, the predicted sequences of PP2A, derived from cDNA libraries from *Brassica napus*, Alfalfa, rabbit, and *Drosophila*, have been shown to be highly conserved (Cohen, *et al.*, 1990). Similarly, the inhibition of PP1 by mammalian peptide inhibitors 1 and 2 (I1 and I2), and the inhibition of PP1 and PP2A by okadaic acid, are virtually universal findings (Cohen and Cohen, 1989). *Paramecium* is the only example so far found of an organism expressing an okadaic acid resistant PP2A-like activity(Cohen, *et al.*, 1988).

PP1 exists as a 37 kDa catalytic subunit, $PP1_C$ (Tung and Cohen, 1984), which associates *in vivo* with one of a number of regulatory subunits. For example, the G subunit has high affinity for glycogen and directs the active catalytic subunit to this intracellular site (Stralfors, *et al.*, 1985). In rabbit skeletal muscle extracts, using physiological assay conditions, PP1 constitutes 85-90% of the phosphorylase phosphatase and 65-70% of the glycogen synthetase phosphatase activities, the remainder being of the PP2A type (Ingebritsen, *et al.*, 1983; Alemany, *et al.*, 1984). Similarly, an M subunit is believed to be involved in the specific binding of PP1 to myofibrils in skeletal muscle (Chisholm and Cohen, 1988b), and PP1M is thus thought to be the major myosin P-light chain phosphatase in these tissues (Chisholm and Cohen, 1988a). A cell-cycle dependent nuclear

localisation of PP1 has been observed: the enzyme is mainly found in the cytoplasm during $G_1$- and S-phases, but accumulates in the nucleus during $G_2$ to reach a peak during mitosis when it appears to be bound to the chromatin (Fernandez, *et al.*, 1992). I2 levels also vary considerably during the cell cycle, rising towards the end of $G_1$, dropping during S-phase, and then rising again during $G_2$ and M-phases. The majority of PP1 activity is associated with the particulate fraction of the cell (Cohen, 1989), presumably in association with site-specific regulatory subunits. The remaining PP1 catalytic subunits are to be found inactive in the cytosol bound to I2 (Vandenheede, *et al.*, 1981; Yang, *et al.*, 1981). Phosphorylation of I2 by glycogen synthase kinase-3 causes dissociation of I2 from $PP1_C$ and activation of the latter (Hemmings, *et al.*, 1982; Resink, *et al.*, 1983). Purified cdc2 kinase can phosphorylate PP1 catalytic subunit and I2 *in vitro*, whether alone or complexed. The activation of the PP1.I2 complex appears to correlate best with phosphorylation of I2 (Villa-Moruzzi, 1992). In contrast, phosphorylation of I1 by cAMP-dependent protein kinase promotes its inhibitory activity against PP1 (Huang, *et al.*, 1976). Both inhibitors are widely distributed in different tissues and different organisms (Cohen, 1989).

PP2A is composed of a catalytic subunit of 36 kDa, $PP2A_C$ (Tung, *et al.*, 1984), plus associated regulatory subunits A, and B or B'. A number of different electrophoretic species have been characterised based on these associations, and these have been ascribed to the following formulations: $PP2A_0$, $A.B'.C_2$ ; $PP2A_1$, $A.B.C_2$ ; $PP2A_2$, A.C; and $PP2A_C$, the catalytic subunit C alone (Cohen, *et al.*, 1988). Subunits B (55 kDa) and B' (54 kDa) are distinct proteins as judged by peptide mapping (Tung, *et al.*, 1985). The association of A with C appears to be a prerequisite for the binding of B or B' (Imaoka, *et al.*, 1983). PP2A is generally a cytosolic enzyme, although a chromatin-associated form has also been identified (Jakes, *et al.*, 1986).

In rabbit skeletal muscle cDNA libraries, two $PP1_C$ clones and two $PP2A_C$ clones have been identified, designated PP1α, PP1β, PP2Aα, and PP2Aβ, respectively (Berndt, *et al.*, 1987). The two PP1 clones are probably encoded by the same gene, with either different promoters or different post-transcriptional splicing regimes, or both, giving rise to separate mRNAs (Cohen, 1989). The two PP2A clones arise from different genes, since even though the predicted amino acid sequences are 97% identical, the nucleotide sequence is only 82% the same, with the 3' coding regions being completely different (Cohen, 1989). As already mentioned, the sequences of these clones from a wide variety of sources are virtually identical. In fact, expression of the mammalian PP1 encoded by PP1α cDNA in a mutant *Aspergillus* strain lacking this activity completely restores the normal *Aspergillus* phenotype (Doonan, *et al.*, 1991).

### 1.4.2 Inhibition of serine/threonine protein phosphatases by microcystin-group toxins

PP1 and PP2A are both highly sensitive to specific inhibition by a number of naturally occurring toxins including okadaic acid, the calyculins, and the microcystins (Bialojan and Takai, 1989; Suganuma, *et al.*, 1990; Yoshizawa, *et al.*, 1990). $IC_{50}$ concentrations against the catalytic subunits of PP1, PP2A, and PP2B for okadaic acid are in the ranges of 10 nM, 0.1 nM, and 5 μM, respectively, using glycogen phosphorylase as substrate (Cohen, 1991). Microcystin-LR, by comparison, was found to be apparently equally effective against the catalytic subunits of both PP1 and PP2A, with an $IC_{50}$ concentration of 0.1 nM when measured in the same assay system. The $IC_{50}$ for PP2B was 1000-fold higher. (MacKintosh, *et al.*, 1990). Honkanen *et al,* 1990, however, reported $IC_{50}$ values against PP1 and PP2A of about 80 nM and 0.3 nM, respectively, for okadaic acid, and of 1.7 nM and 0.04 nM, respectively, for microcystin-LR. In other assay systems, somewhat different results have been obtained (Bialojan and Takai, 1989; Suganuma, *et*

*al.*, 1992) depending upon the substrate used, on the relative activity of the PPs towards the substrate, and on the relative concentrations of the PPs and the inhibitor. The binding of microcystin in particular to these enzymes is so tight that it is effectively titrated out if the enzyme concentration is not reduced to below that of the inhibitor (Honkanen, *et al.*, 1990; MacKintosh, *et al.*, 1990). Therefore, assumptions about which PP is involved in a particular cellular process based on the inhibitory concentration of toxin should be treated with caution. A true estimate of the $IC_{50}$ concentrations is considered to be attained when further dilution of the enzyme ceases to result in further reduction of the apparent $IC_{50}$ (MacKintosh, *et al.*, 1990). For microcystin inhibition of PP1 and PP2A, these $IC_{50}$'s are about 0.5-1.5 nM and 0.1-0.4 nM, respectively (Honkanen, *et al.*, 1990; MacKintosh, *et al.*, 1990). The inhibition by okadaic acid, at least, has been shown to be either non-competitive or mixed, depending upon the substrate used (Bialojan and Takai, 1989). The $IC_{50}$'s for the inhibition of PP3 by okadaic acid and by microcystin-LR are about 5 nM and 0.3 nM, respectively (Honkanen, *et al.*, 1991). The presence of okadaic acid, I1, or I2 in the reaction mixture interferes with the binding of microcystin-LR or okadaic acid to $PP1_C$ (MacKintosh, *et al.*, 1990; Picking, *et al.*, 1991), indicating that they compete for the same site. Recently, it has been suggested that the catalytic subunit of PP2A is phosphorylated and inactivated by an unidentified autophosphorylation-activated protein kinase, and that the presence of microcystin-LR enhances the phosphorylation of $PP2A_C$ apparently by blocking an autodephosphorylation activity of the phosphatase (Guo and Damuni, 1993).

Microcystins can bind covalently to the active sites of PP1 and PP2A (Goldberg, *et al.*, 1995; Mackintosh, *et al.*, 1995; Runnegar, *et al.*, 1995c). Microcystin-LR binds to PP-1 catalytic subunit in such a way that it interacts with three critical regions of the active site: the metal binding site, the hydrophobic groove, and the edge of the C-terminal groove near the active site.

The N-methyldehydroalanine is in close proximity to the S atom of Cys 273 and forms a covalent bond with it (Goldberg, *et al.*, 1995). In motuporin and the nodularins, on the other hand, there is a shorter distance between the ADDA side chain, which anchors the toxin to the hydrophobic cleft of the phosphatase, and the N-methyldehydrobutyrine moiety. This latter moiety also has a different alignment, relative to both the enzyme and the rest of the toxin molecule, than does the N-methyldehydroalanine of microcystin-LR. Therefore, it is considered unlikely that these latter toxins can form covalent linkages with the phosphatases (Bagu, *et al.*, 1997).

### 1.4.3 Effects on the hepatocyte cytoskeleton

As stated above, characteristic changes in cell morphology occur upon treatment of cultured cells with microcystin. These include loss of fine microvillar surface texture and marked blebbing, but these occur without loss of membrane integrity as shown by maintenance of Trypan Blue exclusion and retention of intracellular enzymes such as alanine amino transferase (Runnegar, *et al.*, 1981; Runnegar and Falconer, 1982). Thirty nM caused deformation of 50% of cells after a 20 minute incubation, with 1 μM causing 95% deformation after this time. When exposed to 10 μM toxin, 50% of cells were deformed by between 1 and 5 minutes and 94% by 20 minutes (Runnegar, *et al.*, 1981).

This loss of structural integrity of hepatocytes is due to disruption of the cytoskeleton upon exposure to low μM intracellular concentrations of microcystin. This effect is not due to the depolymerisation of filamentous actin (F-actin), since the ratio of F-actin to G (globular)-actin does not change (Runnegar and Falconer, 1986; Eriksson, *et al.*, 1989), although the F-actin filaments are clearly disrupted (Falconer and Runnegar, 1987a; Falconer and Yeung, 1992) as are

the smooth endoplasmic reticulum and Golgi (Eriksson, *et al.*, 1989). Rather, the disruption is due to specific depolymerisation of the intermediate filament cytokeratins concomitant with their becoming hyperphosphorylated (Eriksson, *et al.*, 1989; Falconer and Yeung, 1992; Ohta, *et al.*, 1992). Desmoplakin phosphorylation at the desmosome is the first visible sign of this process, with collapse of the intermediate filament cytoskeleton following soon after (Toivola, *et al.*, 1997). Microtubule disaggregation also appears to be an early, and sometimes prior, response, at least in hepatocytes (Wickstrom, *et al.*, 1995; Khan, *et al.*, 1996). Microtubule-dependant vesicle movement is inhibited in rat hepatocytes at lower concentrations (50 nM microcystin) (Hamm-Alvarez, *et al.*, 1996).

These toxins have been shown to cause the potent and specific inhibition of protein phosphatases 1 and 2A (Eriksson, *et al.*, 1990b; Yoshizawa, *et al.*, 1990) leading to an increase in the overall level of protein phosphorylation in the effected cell. The disruption of the cytoskeleton is accompanied by a hyperphosphorylation of the cytokeratin intermediate filaments (Falconer and Yeung, 1992; Ohta, *et al.*, 1992). Cytokeratin polymerisation is known to be regulated during the cell cycle by the phosphorylation state of the constituent subunits (Bershadsky and Vasiliev, 1988; Phillips and Satir, 1988). It is unknown whether the cytoskeletal elements themselves are direct substrates for the protein phosphatases or whether one or both are acting to regulate the activity of a more specific phosphatase(s). Hamm-Alvirez *et al* (1996) present data to suggest that PP2A is involved in regulation of microtubule-dependant vesicle movement, but that PP1 and PP2A have opposing roles in the regulation of microtubule structure so that okadaic acid inhibition of PP2A causes microtubule disruption but that inhibition of both PP's by microcystin balances out so that no effect on microtubule structure is seen. In the sea urchin, PP1 has been shown to regulate the phosphorylation state of a protein (p62) component of the mitotic apparatus, an

action which directly correlates with microtubule disassembly during mitosis (Johnston, *et al.*, 1994).

Similar effects on cytoskeletal structure have been found using other, cell permeant, protein phosphatase inhibitors. Higher concentrations of okadaic acid and dinophysistoxin-1 (low μM) were required than calyculin-A (10 nM) to cause cytoskeletal disruption in BHK-21 cells (Eriksson, *et al.*, 1992). As with microcystin, intermediate filaments and microtubules, but not actin microfilaments, were affected, with the intermediate filament vimentin being the apparent primary target of hyperphosphorylation. Upon withdrawal of calyculin A, but not the other toxins, the effected cells recovered their normal appearance within a few hours, indicating that phosphatase inhibition *per se*, for 20 minutes at least, was not lethal. These affective toxin concentrations were consistent with their relative inhibitory activities against PP1 and PP2A and so it was suggested that a PP1 is the enzyme involved in maintenance of intermediate filament structure. However, in rat hepatocytes, okadaic acid at only 30 nM induced complete intermediate filament disruption (Blankson, *et al.*, 1995) suggesting that differences between cell-type as well as between toxin specificity for protein phosphatases might play a part in observed cellular sensitivity (for example, see Khan, *et al.*, 1995).

Microcystin and okadaic acid may also have more subtle and specific (ie non-PP-mediated) effects on cytoskeletal elements. For example, Hayakawa and Kohama (1995) present evidence that the actin-activated ATPase activity of skeletal muscle myosin is stimulated by okadaic acid but inhibited by microcystin-LR, and that these actions are due to direct interaction of the toxins with the head unit ATPase active site.

1.4.4 Other cellular effects.

Other cellular effects reported have included activation of phosphorylase a without changes in cAMP levels (now explainable in terms of PP1 inhibition; (Cohen, 1989)), $Ca^{2+}$ homeostasis disruption, and glutathione depletion (Falconer and Runnegar, 1987a; Runnegar, *et al.*, 1987). These latter two effects were not found by Eriksson *et al* (1989) who reported no effect on intracellular $Ca^{2+}$ or thiols. Glutathione and cysteine adducts of the Mdha moiety, along with sulphated conjugates of ADDA, have since been identified as *in vivo* metabolites of microcystin-LR and -RR (Kondo, *et al.*, 1996). However, since these compounds were only detectable after immuno-affinity chromatography using an anti-microcystin-LR monoclonal antibody, this might indicate that they are not produced efficiently in the mouse or rat liver. Falconer and Runnegar (1987) found that intracellular calcium levels in isolated rat hepatocytes were immediately increased in response to 2.5 and 5 μM toxin. Eriksson *et al* (1989) on the other hand, found that 4 μM toxin was without effect on intracellular calcium levels. The toxin used by Falconer and Runnegar was microcystin-YM whereas that used by Eriksson was microcystin-LR, and so it is possible that this may account for the discrepancy. For example, if these toxins had preferential affinity for different transporters they might be taken up at different rates, or if the transporters were linked to different intracellular delivery systems (vesicular transport or carrier proteins) then the site of intracellular delivery could be different.

Microcystin-YM was shown to cause an influx of extracellular calcium in isolated rat thymocytes (Falconer and Runnegar, 1987a). However, calyculin-A, tautomycin and okadaic acid, had an inhibitory effect on $Ca^{2+}$ entry across the plasma membrane in rat parotid acinar cells (Tojyo, *et al.*, 1995). Purified microcystin-LR was without effect on ionic transport processes in

tilapia (*Oreochromis mossambicus*). Instead, inhibitory effects on ion transport including whole-body $Ca^{2+}$ fluxes and P-type ATPases of the gill were found to be due to other cytotoxic substances in the cyanobacterial extract used (Bury, *et al.*, 1996). Therefore, it seems likely that the effects of microcystins and other protein phosphatase inhibitors on cellular calcium homeostasis are not only toxin, but also cell, dependent.

A significant increase in both phosphate and potassium effluxes from hepatocytes at exposure levels far lower than those inducing morphological changes (0.1 and 1.0 nM microcystin, respectively) have also been reported (Eriksson, *et al.*, 1987). It is possible that the effect on potassium ion movement may be related to that seen on the $Na^+$, $K^+$-ATPase of the gill membranes of the carp (Zambrano and Canelo, 1996), which is thought to be due to inhibition of a phosphatase activity specific for this ion pump, although of course, in this latter case an inhibition of transport is induced. An alternative mechanism for this potassium ion efflux may involve the formation of ion channels in the plasma membrane by microcystin (Petrov, *et al.*, 1991). For example, nodularin, via a similar pore-forming mechanism, was found to increase transmembrane conductance of potassium ions through a biological membrane at concentrations of 20-200 ng/ml (Spassova, *et al.*, 1995). It should be noted that okadaic acid may have similar ion transporting capabilities but via a totally different mechanism, namely complexation with the ions and passive diffusion through the membrane (Blaghen, *et al.*, 1997). As yet however, this has only been shown in a non-biological model system.

Golgi disassembly was reported to occur in hepatocytes after treatment with 500 nM okadaic acid, but not after microcystin treatment up to 500 nM (Hamm-Alvarez, *et al.*, 1996), an effect linked to the okadaic acid (hence PP2A) specific disassembly of acetylated microtubules. However, Eriksson *et al* (1989) noted the loss of Golgi stacks in rat hepatocytes treated with 1

μM microcystin-LR. In an *in vitro* system comprising mitotic Golgi fragments including vesicles, membrane tubules, and cisternal remnants, 10 μM microcystin had marked effects on Golgi apparatus dynamics (Rabouille, *et al.*, 1995). Differing effects were seen depending on whether microcystin was present during the disassembly process, in which case reassembly was inhibited, or present only during reassembly, in which case only cisternal stacking was inhibited. Okadaic acid was not used, but other phosphatase inhibitors (EGTA and β-glycerophosphate) were also found to be effective. In this system, microtubules and microfilaments do not appear to be involved since nocodazol and cytochalasin-B had no effect.

### 1.4.5 Role of PP1 and PP2A in cell cycle regulation

The use of inhibitors of PP1 and PP2A, particularly okadaic acid, since the late 1980s has lead to the discovery of their importance in the regulation of normal cellular function and their involvement in neoplastic processes. For example, Haystead *et al,* 1989 used okadaic acid to induce phosphorylation of proteins involved in a variety of metabolic processes in cells, including a demonstration that the effects of insulin on glucose transport in adipocytes could be mimicked by okadaic acid. In that same year, it was shown that expression of PP2Aα and PP2Aβ mRNAs was up-regulated in hepatocellular hyperplastic nodules or carcinomas initiated with 2-amino-3-methylimidazo[4,5-f]quinoline, and in NIH 3T3 cells transformed by the introduction of activated *c-raf*, *ret-II*, or *Ki-ras* oncogenes (Nagao, *et al.*, 1989). Furthermore, the morphology of raf and ret-II transformants returned to normal in the presence of okadaic acid (Sakai, *et al.*, 1989). Therefore, inhibition of the PP's appeared likely to have a role not only in the acute toxicity of these toxins, but also in their more long-term tumour promotional effects.

Further evidence for this was soon forthcoming. Unidentified microtubule associated proteins became labelled with $^{32}P$ in meiotic oocytes as microtubule spindle/kinetochore interactions were disrupted by okadaic acid treatment (Depennart, *et al.*, 1993). This was linked to premature chromosome condensation and nuclear envelope breakdown (Dyban, *et al.*, 1993). Similarly, in BHK21 Golden Hamster fibroblasts synchronised in early S-phase exposed to okadaic acid, mitosis specific events such as chromosome condensation, the production of MPM-2 antigens (phosphorylated cdc2 recognition motifs), dispersion of nuclear lamins and the appearance of mitotic asters were induced (Yamashita, *et al.*, 1990). In the latter case at least, these characteristics disappeared again upon further incubation, indicating that mitosis was aborted presumably because S-phase was not completed (Yamashita, *et al.*, 1990). Microinjection of either PP1 or PP2A into the nucleus of cells in $G_1$ caused inhibition of entry into S-phase, whereas microinjection into S-phase cells did not stop progression of the cell cycle (Alberts, *et al.*, 1993a). However, there are cell cycle differences in regulation between mammalian species since premature mitosis is relatively easily inducible in some species, for example hamster cells, compared to mouse or human cells (Steinmann, *et al.*, 1991).

In myeloid leukemic cells, okadaic acid induces cell cycle arrest at the $G_2$/M boundary and entry into apoptosis at sub-toxic doses (10 nM) (Ishida, *et al.*, 1992). Similarly in primary rat hepatocytes, okadaic acid caused an inhibition of DNA replication at low levels, with 20 nM causing approximately 50% decrease in labelling index in EGF-stimulated cells (Boe, *et al.*, 1991). Interestingly, microcystin had no effect on DNA synthesis at these levels in this system. The authors suggested that a differential effect of the two toxins on protein phosphatases 1 and 2A could be responsible for these differences (Mellgren, *et al.*, 1993). Obviously, the effects of species, cell-type, transformation state, and type and concentration of toxin can all have marked effects upon the outcome of an experiment.

More detailed studies have been carried out using mainly okadaic acid since microcystin, which should provide a useful comparison, cannot be taken up by most cellular systems (Eriksson, *et al.*, 1994). Much of this work has been done in yeast or *Xenopus* models, although much of this knowledge is now been found to be comparable with the more complex mammalian system. These studies have highlighted interactions between the protein phosphorylation and specific intracellular regulators, for example, the cell division cycle kinase p34, the tumour suppressor proteins p53 and the retinoblastoma (Rb) protein, the products of the c-*jun* and c-*fos* genes, and with products of transforming-virus genomes of the polyomavirus, papillomavirus, and adenovirus types. The evidence for these interactions will be reviewed in the following sections.

#### 1.4.5.1 The cell cycle dependent protein kinases

The eukaryotic cell cycle is regulated by a series of protein phosphorylation events determined by the sequential expression of regulatory subunits (cyclins) of the cyclin dependant serine/threonine protein kinases (cdk's) cdc2 (so-called in fission yeast; homologues are cdc28 in budding yeast and cdk1 in humans), cdk2, cdk4, and cdk6 (Moreno and Nurse, 1990). The elements of the mammalian system are introduced in Figures 1.6 and 1.7 and in the following paragraphs. The cell cycle dependent interactions of these basic elements, and the involvement of protein phosphorylation, are described in more detail in the subsequent sections.

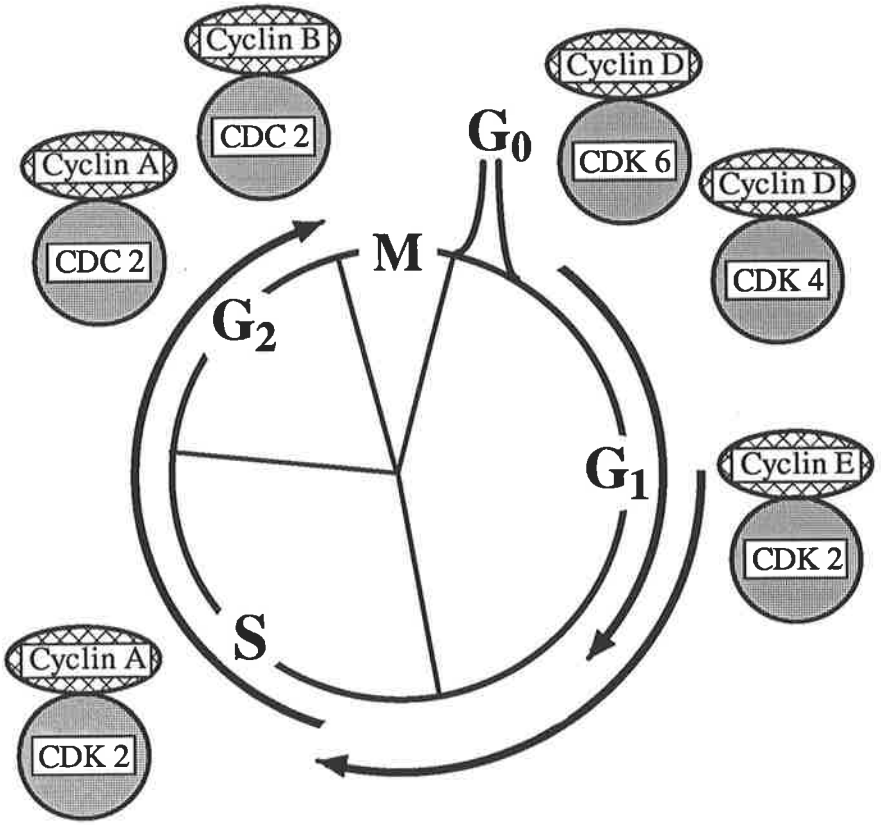



**Figure 1.6** - Diagram summarising the sequential nature of cdk/cyclin interactions during the cell cycle. In early G1, cyclins D1, D2, and D3 associate with cdk's 4 and 6, but as the cell progresses through G1, the activity of these complexes wanes, to be replaced by cdk2/cyclin E, whose activity reaches a maximum at the G1/S boundary. During S-phase cyclin A replaces cyclin E as the regulator of cdk2 activity, but then after S-phase, in G2, cyclin A is found to be associated with the cdc2 kinase, until by the G2/M boundary this in turn is replaced by cyclin B. See text for more detail. (Adapted from Sidle, 1996).

The cdk must associate with a cyclin for activity and the rate of this association is mainly controlled through regulation of cyclin levels during the cell cycle. Each cdk has a particular sub-set of cyclins with which it can bind. The cdk's are all regulated by similar influences, as shown in Figure 1.7.

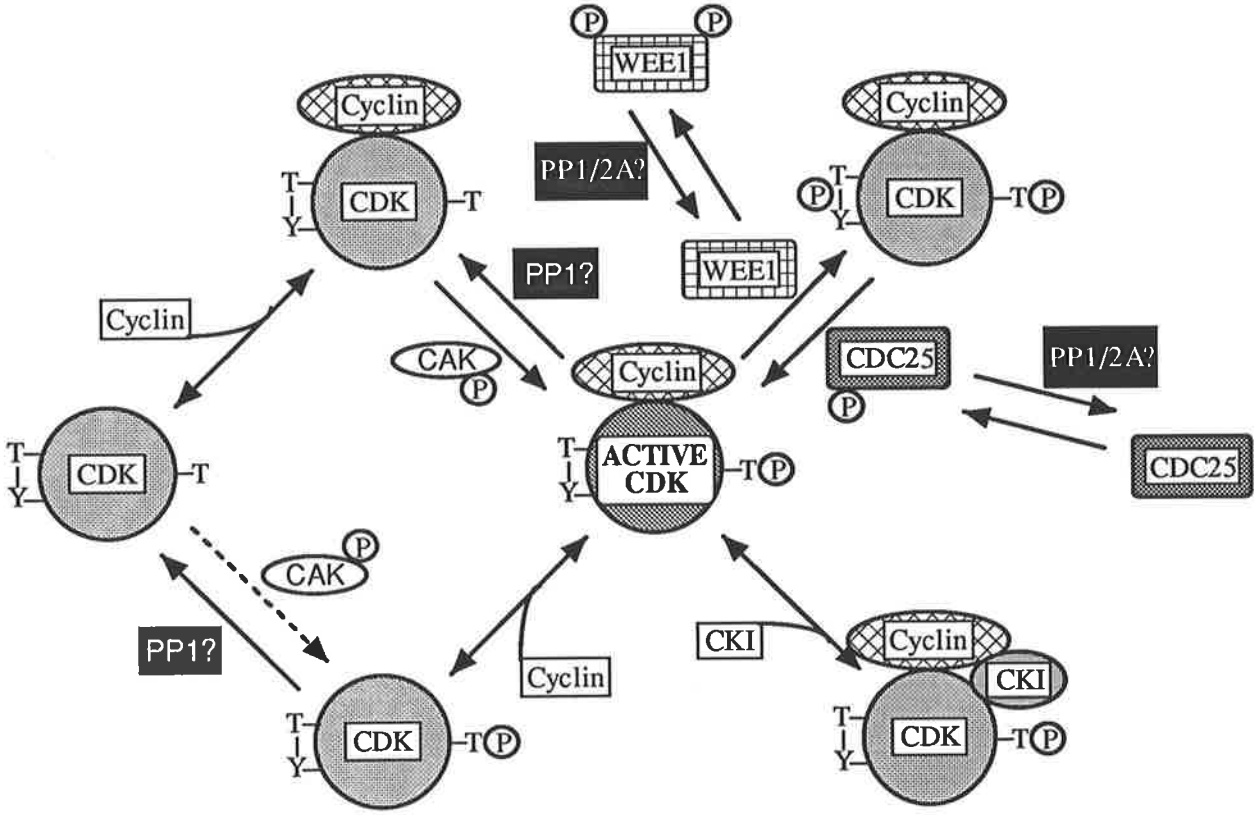



**Figure 1.7** - Generic features of the regulation cdk activity highlighting the involvement of protein phosphorylation in this process. The cdk must bind to a cyclin for activity and the levels of these vary with the cell cycle. The cdk must also be phosphorylated on an activating threonine, a reaction catalysed by cdk-activating kinase (CAK), which is itself regulated by phosphorylation. There is mounting evidence that PP1 acts in a cdk-inhibitory role by dephosphorylating the threonine phosphorylated by CAK. The Wee1 kinase catalyses an inactivating phosphorylation of a second threonine residue and possibly also a tyrosine. This kinase is counter-balanced by the dual specificity cdc25 phosphatase. Both Wee1 and cdc25 are also regulated by phosphorylation and there is evidence that PP1 and/or PP2A are involved in the dephosphorylation of these enzymes. A number of inhibitory peptides (CKI) can also associate with the complex. See text for details. (Adapted from Morgan, 1995).

The cdk/cyclin complexes, and possibly the cdks alone, are phosphorylated on a single specific threonine (Thr-160 or 161, depending on species), this being required for activation. The enzyme responsible for this conversion is CAK (cdk-activating kinase = cdk7 plus cyclin H). It appears that this activity is not itself regulated via transcription or association since immunoprecipitable activity is present throughout the cell cycle, but it does possess a threonine phosphorylation site

(Thr-170 in human cdk7) analogous to that of other cdks. A protein phosphatase, possibly PP1, negatively regulates this activating cdk phosphorylation step (Morgan, 1995).

The cdk/cyclin complex, even when phosphorylated on Thr-160/161, can be inhibited by further phosphorylations on specific threonine (Thr-14) and tyrosine (Tyr-15) residues. At least the tyrosine phosphorylation is catalysed by the *wee1* kinase, which itself is subject to inhibitory phosphorylations. Both the threonine and tyrosine residues on the cdk are dephosphorylated by a dual specificity phosphatase called cdc25. This phosphatase is activated by a dephosphorylation step. Both the wee1 and cdc25 dephosphorylations may involve PP1 and/or PP2A (Morgan, 1995).

A number of inhibitory agents have been identified which can also influence cdk/cyclin activity (Sherr and Roberts, 1995). These vary in their specificities for the cdks, and further details are given in the relevant sections below.

Known substrates of the cdk's include the Rb family of proteins and their E2F cofactors, p53, histone H1, lamins, nucleolin, myosin light chain, and RNA polymerase II (Barritt, 1992; Levine, 1993; Sidle, *et al.*, 1996). The protein products encoded by the oncogenes c-*abl* and c-*src* are also phosphorylated in a cell cycle dependent manner by the cdc2 kinase (Kipreos and Wang, 1990; Shalloway, *et al.*, 1992).

#### 1.4.5.1.1 Regulation of G1 and the commencement of DNA synthesis

Mammalian cells are sensitive to various extracellular factors (growth factors, anti-proliferative agents, differentiation inducers, spatial cues) until late G1, after which time they are committed to

the cell cycle if supplied with factors supporting viability. It has been suggested that the G1 cyclins mediate this interaction of growth factors and or suppressors with the regulation of cell cycle progression (Sherr, 1993). Progression through G1 is regulated by expression of these cyclins (D1, D2, and D3) and their association with cdk6 and cdk4. Later in G1 as the cell nears the G1/S transition, cdk2/cyclinE becomes the dominant regulatory complex (Sherr, 1994).

The cdk4/cyclin D complexes are the targets of a number of other regulatory influences. These include the retinoblastoma protein Rb and the related proteins p107 and p130, the tumour suppressor p53, and a number of small inhibitor proteins. The D cyclins, but not cyclins E or A, contain a consensus sequence which mediates binding to Rb and p107, thus directing the cdk4 and cdk6 to these proteins. Unlike most cdks, cdk4/D and cdk6/D have higher kinase activity towards Rb than histone-H1 (Sherr, 1994). This sequence also occurs in the SV40 T antigen, adenovirus E1A and human papilloma virus E7 proteins and binding of these factors to Rb results in its inactivation. Phosphorylation of Rb, or binding of viral proteins, leads to its dissociation from the E2F transcription factor which therefore can cause transcription of various genes whose products are necessary for entry into S-phase (Sidle, *et al.*, 1996).

Higher eukaryotes express a number of inhibitors with broad specificity for cdks called $p21^{Cip1}$, $p27^{Kip1}$ and INK4. Some anti-mitotic signals (eg DNA damage) induce further expression of these inhibitors above basal levels. This can cause arrest almost anywhere in the cell cycle, suggesting that at least some are not cdk-specific (Heichman and Roberts, 1994). In particular, the expression of the $p21^{Cip1}$ universal cdk inhibitor is regulated by p53 (Sherr, 1994) as well as other transcription regulators (Sherr and Roberts, 1995). Thus increased p53 activity, eg in response to DNA damage, suppresses Rb phosphorylation and release of E2F via inhibition of cdk4/cyclin D and cdk6/cyclin D. If E2F levels do rise whilst p53 is high, the cell enters apoptosis (Wu and

Levine, 1994). DNA tumour viruses get around these controls by inactivating both suppressor proteins.

PP1 and/or PP2A appear to be involved in the regulation of the phosphorylation state of the Rb and p53 proteins, and so these interactions are discussed in more detail in a later section (Section 1.4.5.2). The usurpation by tumour viruses of this regulatory process is discussed in Section 1.4.5.4.

Cdk2, in association with cyclin E, is the kinase most implicated as being involved in regulating the G1/S checkpoint (Heichman and Roberts, 1994). Unlike the D cyclins, cyclin E is synthesised periodically during the cycle so that maximum levels of cdk2/E activity occur at the G1/S boundary (Sherr, 1994). Once the cell enters S-phase, cyclin E is degraded and cdk2 associates with cyclin A(Sherr, 1994).

#### 1.4.4.1.2 Regulation of the G2/M transition

Cyclin B is first synthesised during S-phase and accumulates in complexes with cdc2 as cells approach the G2/M boundary. Phosphorylation of Thr-161 on cdc2 may stabilise cyclin B binding and is required for subsequent activation of the enzyme. Phosphorylations on Thr-14 and Tyr-15 within the cdc2 ATP binding site inhibit the enzyme throughout S-phase and G2. Dephosphorylation of these residues leads to activation of the enzyme (Sherr, 1993). Site-specific mutation of these sites to preclude phosphorylation leads to premature mitosis. Phosphorylation of Tyr-15 at least is mediated by *wee1* kinase. Mutation of *wee1* in fission yeast causes premature mitosis, double mutation causes a grossly premature mitosis phenotype (King, *et al.*, 1994). Other kinases may also act on these phosphorylation sites (Kornbluth, *et al.*, 1994). *Wee1* is itself

phosphorylated by *Nim1* (aka *Cdr1*) causing its inhibition and thus allowing activation of the mitotic cdk. This may provide a means of response to nutritional status and other stimuli (King, *et al.*, 1994). *Wee1* is also phosphorylated, on different sites to those targeted by *Nim1*, during mitosis. This is probably part of the positive feedback loop occurring as the cell enters mitosis.

The dephosphorylation of Tyr-15 and Thr-14 is performed by the cdc25 dual specificity phosphatase. Cdc25 is itself closely regulated with peak activity as the cell enters mitosis, then activity dropping again as the cell exits mitosis (Kumagai and Dunphy, 1992). Cdc2/cyclin B phosphorylates and activates cdc25, forming another arm of the positive feedback loop at G2/M. PP1 activity can inhibit cdc25, and the activity of this nuclear phosphatase transiently drops as the cell enters mitosis (Izumi, *et al.*, 1992; Walker, *et al.*, 1992). A truncated PP1 thought to be lacking an inhibitory phosphorylation site was not a substrate for the mitotic kinase (whereas the wild-type was) and blocked mitotic progression unless excess cdc25 was also present (King, *et al.*, 1994). PP1 activity is also regulated by inhibitor 2 which accumulates during mitosis (Brautigan, *et al.*, 1990). Therefore, it may be that this PP1 acts as a repressor of cdc25 activation of the cdc2/cyclin B mediated initiation of mitosis.

The inhibition of cdc2 kinase activity in mitotic mouse fibroblast extracts by cAMP and ATP is mimicked by the addition of I1 or okadaic acid (Hohmann, *et al.*, 1993). Phosphorylation of I1 by cAMP-dependent protein kinase (PKA) promotes its inhibitory activity against PP1 (Huang, *et al.*, 1976). Therefore, Hohmann *et al,* (1993) suggest that PKA activates I1, thus leading to the inhibition of one or more PP1 isoenzymes, and allowing phosphorylation and inactivation of cdc2.

PP2A can also dephosphorylate cdc25 *in vitro* (Izumi, *et al.*, 1992). Furthermore, in interphase *Xenopus* egg extracts, cdc25 dephosphorylation is inhibited by okadaic acid but not by I2 (Clarke,

*et al.*, 1993). Inhibition of PP2A by okadaic acid causes premature entry into mitosis in *Xenopus* (Felix, *et al.*, 1990). Microinjection of okadaic acid into *Xenopus* oocytes causes a rapid burst of phosphorylation and an activation of MPF (Maturation Promoting Factor, ie, cdc2/cyclin B) which occurs with or without addition of cAMP and is not susceptible to protein synthesis inhibitors. These events lead to the breakdown of the nuclear envelope, depolymerisation of lamin, and condensation of the chromosomes, although mitotic spindles are not formed (Rime, *et al.*, 1990). However, it has since been shown *in vivo*, again in *Xenopus*, that PP2A cannot act upon cdc2 Tyr-15 phosphorylation via cdc25 when cdc2/cyclin B is already activated (Lee, *et al.*, 1994). Instead it appears to act upon Thr-161 phosphorylation to negatively regulate the action of the mitotic kinase (Lee, *et al.*, 1994). Inactivation of the H1 kinase activity of cdc2 kinase occurs after M-phase, once cyclin degradation has begun, by dephosphorylation of Thr-161. This dephosphorylation does not occur in the presence of okadaic acid (Lorca, *et al.*, 1992). However, genetic evidence was cited by these authors to indicate that PP1 rather than PP2A is involved. Cyclin degradation itself was also found to be negatively regulated by okadaic acid-sensitive phosphatase(s) (Lorca, *et al.*, 1991).

The above effects of okadaic acid should in theory lead to a stimulation of cell growth. However, the *cdc2* gene itself has also been shown to be positively regulated by PP2A, an action which is inhibited by okadaic acid. Therefore, in this way, okadaic acid should impose a negative influence on cell growth (Jaramillo-Babb, *et al.*, 1996). These authors suggest that these apparently contradictory actions of PP2A may be applied at different points in the cell cycle, aiding the coordination of the cell cycle. Perhaps contributing to this close regulation of PP2A during the cell cycle is the finding that the C-terminus of the PP2A catalytic subunit is transiently methylated during S-phase in HL-60 cells (Zhu, *et al.*, 1997).

Therefore, it may be that both of these protein phosphatases are involved in different modes of regulation of the cdc2/cyclin B kinase.

It now appears likely that another form of inhibition of cdc2/cyclin B occurs via inhibitory proteins such as p21, although the mechanism for this is yet to be properly defined (King, *et al.*, 1994).

1.4.5.1.3 Mitosis

Three phases of cdc2/cyclin B regulation can be identified through G2 and M-phase: (1) Cyclin accumulation with inhibition of activity until prophase leading to the sudden activation of kinase activity to begin M-phase, (2) ubiquitin-mediated breakdown of cyclin B during anaphase, and (3) switch off of the ubiquitin system before the next cell cycle (King, *et al.*, 1994).

Activation of cdc2/cyclin B results in phosphorylation of a wide variety of mitosis-specific target proteins and leads to the reorganisation of the cytoskeleton for division and the condensation of the chromosomes at the metaphase plate (Tiwari, *et al.*, 1996). As these processes end, cdc2/cyclin B activates the "destruction box"-dependent, ubiquitin-mediated, cyclin degradation system and so causes its own inactivation. The "destruction box" is a motif common to a number of target proteins, including cyclin B, which are removed at this time. The remaining steps of cell division, including sister chromatid separation and cytokinesis occur without active cdc2/cyclin B being present. However, continued presence of active cdc2/cyclin B does not inhibit sister chromatid separation, a process which appears instead to be instigated upon activation of the cyclin degradation system (King, *et al.*, 1994). Once this proteolytic system is

activated there do not appear to be any other regulatory steps which might halt sister chromatid separation. Cytokinesis, however, does appear to require the prior inactivation of cdc2/cyclin B (Holloway, *et al.*, 1993; Surana, *et al.*, 1993).

PP2A1 (the A.B.C complex) seems to be the phosphatase that dephosphorylates a number of cyclin-dependent kinase substrates *in vivo* in both amphibian and mammalian cells (Ferrigno, *et al.*, 1993). It is suggested that the A and B subunits that are associated with PP2Ac in PP2A1 accelerate the dephosphorylation of these substrates, while suppressing the dephosphorylation of most other proteins.

1.4.5.1.4 Sequential control of S-phase and M-phase.

As just described, whether a cell undergoes S-phase or M-phase depends upon the type and state of the cdk/cyclin complex present. However, during meiosis there are two successive mitoses without an intervening S-phase. This appears to be achieved, in yeast at least, by maintaining cdc2/cyclin B in an active form after the first round of mitosis (Nurse, 1994). In contrast, in the cells of certain Drosophila tissues, numerous rounds of endoreduplication occur without intervening mitoses. These cells appear to lack cyclin B (Lehner and O'Farrell, 1990). It seems therefore that the cdc2/cyclin B complex identifies a cell as being in G2. Hence loss of this complex causes a yeast cell to re-enter S-phase without undergoing M-phase, suggesting that the cell is reacting at the end of S-phase as though it has been reset back to G1 (Nurse, 1994).

Since PP1 is likely to be involved in the timing of exit from S-phase and entry into M-phase, and since PP1 activity also appears to be required for DNA replication (Walker, *et al.*, 1992) it was postulated by these authors that PP1 is involved in the signalling pathway which ensures that

DNA synthesis is complete before M-phase is begun. However, it has since become clear that cdc2/cyclinB itself inhibits the formation of prereplicative RP-A complexes on double-stranded DNA (RP-A is a multi-subunit single-stranded DNA binding protein involved in the formation of the replicative apparatus) (Adachi and Laemmli, 1994). Therefore how PP1 is likely to be involved in this S-phase/M-phase coordination is still unclear. However, Murphy *et al* (1995) have shown, in an *in vitro* model of nuclear reassembly and DNA synthesis derived from *Xenopus* eggs, that PP2A activity was in fact necessary for correct assembly of the pre-synthesis complex at the replication fork, and that this assembly was inhibited by 250 nM microcystin-LR. Higher concentrations of the toxin prevented the assembly of both nuclear lamina and the nuclear envelope, perhaps suggesting that multiple phosphatase activities were present with different sensitivities to microcystin-LR.

A further level of regulation of the sequencing of these events is imposed by the putative "licensing factor" which marks the sites of future replication forks (Blow, 1993). Old licensing factor is destroyed at the beginning of each round of DNA synthesis and is renewed after mitosis is complete and before nuclear re-assembly (Blow, 1993). Therefore, this process also seems to dictate a limit of one S-phase per cycle on the cell.

In yeast, although cdc2/cyclin B inactivation may be required to allow the cyclin protease system to be turned off, this is not sufficient. It also appears that expression of G1 cyclins is required (Amon, *et al.*, 1994), thus ensuring the correct sequence of events occurs. No data on mammalian systems is available at this time.

Yet another aspect of the coordinated regulation DNA synthesis and mitosis is the nuclear versus cytoplasmic localisation of cdc2/cyclin B. Dephosphorylation of the nuclear cdc2/cyclin B

complex is normally required for M-phase to begin, but defects in nuclear transport and integrity (RCC1 mutant, Dasso, 1993) can allow premature entry into M-phase (Nurse, 1994), possibly due to entry into the nucleus of activated cytoplasmic cdc2/cyclin B kinases which might normally be involved in other cytoplasmic processes. Similarly, in *S. pombe*, Nim1 is cytoplasmically located whereas its substrate, wee1, is primarily nuclear. Introduction of Nim1 to the nucleus led to an advancement of M-phase (Wu, *et al.*, 1996). Therefore, there are numerous interacting regulatory systems involved in coordinating the phases of the cell cycle. Protein phosphatases are certainly involved but their role is ill-defined as yet.

1.4.5.1.5 Cytokinesis

Cytokinesis involves the movement of pre-existing actin filaments from a wide distribution throughout the inner surface of the plasma membrane to focal bundles which then form the contractile ring. This contractile ring then shrinks to divide the cell. Myosin-II appears to be the biochemical engine which drives this contraction process (Reviewed in Pollard, *et al.*, 1990). There is increasing evidence that this process, along with other cytoskeletal functions, are regulated by a family of small GTP-binding proteins, designated Rho (Boivin, *et al.*, 1996). Kishi *et al* (1993) have presented evidence that the process of furrow formation in *Xenopus* embryos, preliminary to cell division, is inhibited by a Rho/GDP dissociation inhibitor. Nuclear division was not inhibited. (Kishi, *et al.*, 1993). How these Rho proteins are regulated during the cell cycle is unknown.

In *S. pombe*, cytokinesis is dependent only on the initiation of mitosis rather than its completion (Minet, *et al.*, 1979) but mitosis is not dependent upon cytokinesis, since cells lacking functional genes necessary for cytokinesis form elongated multinucleate cells (Fankhauser, *et al.*,

1993). Okadaic acid, alone but more so in conjunction with deletion of one of the two fission yeast PP2A genes, gave rise to an accumulation of binucleate cells (Kinoshita, *et al.*, 1993). This inhibition of cellular division was reversible upon removal of the toxin. Therefore, okadaic acid-sensitive phosphatases are required to enable cytokinesis in this organism. In budding yeast, where the key cell cycle regulatory kinase, cdc28, associates sequentially with G1 cyclins and later with G2 cyclins, control of cortical actin arrangement through the cell cycle was determined by the type of cyclin bound to cdc28 (Lew and Reed, 1993). In *Xenopus*, the myosin-II regulatory light chain is phosphorylated by the cdc2 kinase, inhibiting its ATPase activity, and thus cytokinesis (Satterwhite, *et al.*, 1992). Therefore, the cytokinetic process relies upon the phosphorylation state of a number of its key components, and phosphatase inhibition by okadaic acid appears to inhibit this process. However, whether these effects are due to inhibition of the deactivation of cdc2, or dephosphorylation of myosin II light chain, or some mixture of these plus other effects, is not yet known.

#### 1.4.5.1.6 The hepatocyte cell cycle

The hepatocyte cell cycle can differ in a number of important ways from the system just described. After DNA synthesis, hepatocytes from rodents and humans, but not cats or guinea pigs, are capable of an acytokinetic mitosis (Brodsky and Uryvaeva, 1977), as shown in Figure 1.8.

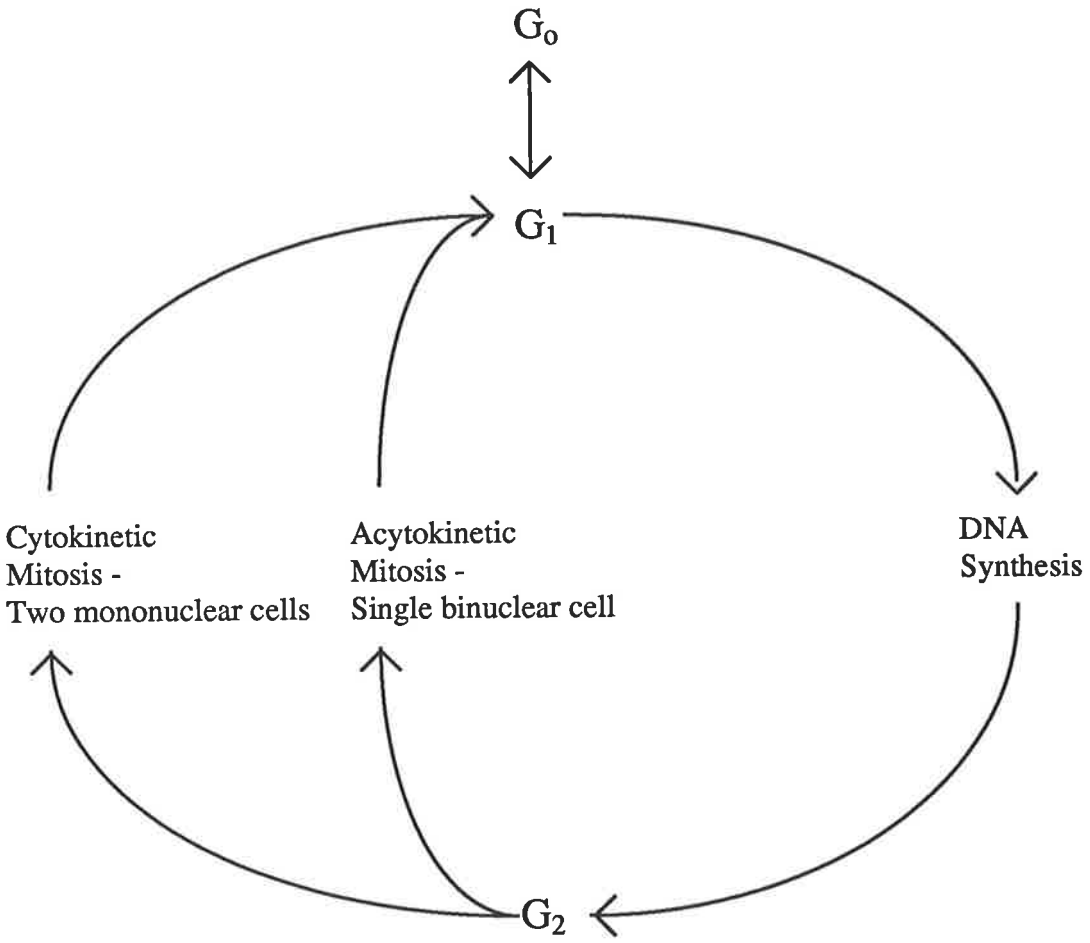



**Figure 1.8** - The mouse hepatocyte cell cycle. Rodent and human hepatocytes can undergo either a normal cytokinetic mitosis or an acytokinetic mitosis leading to the formation of a binucleate cell. Further rounds of the cell cycle can lead to higher degrees of polyploidy. See text for further discussion.

An acytokinetic mitosis leads to the production of a single binuclear daughter cell. After a subsequent round of DNA synthesis, a normal cytokinetic mitosis usually occurs, producing two mononuclear daughter cells each containing twice the DNA content of the original mononuclear cell. Thus, if a diploid (2N) hepatocyte undergoes acytokinetic mitosis, a binuclear tetraploid (2x2N) hepatocyte is produced, and after the next S-phase followed by a cytokinetic mitosis, two mononuclear tetraploids (4N) are formed.

How this comes about, in terms of the biochemical mechanisms just described, is still unknown. However, independent control of the rates of breakdown of cyclin B and of the inactivation of cdc2 kinase might be one way in which the cell could exercise this level of control over the sequence of S- and M-phases in these cells. As noted above, activation of the "destruction box" proteolytic system is sufficient to initiate sister chromatid separation (and therefore, presumably, karyokinesis), whereas cytokinesis requires inactivation of the cdc2/cyclin B complex. Therefore, if the cell could activate the mitotic proteolytic system but still protect cyclin B and inhibit cdc2 dephosphorylation on Thr-160/161, then karyokinesis but not cytokinesis could occur and so the acytokinetic first round of the hepatocyte cycle could occur. However, if active cdc2/cyclin B remains present then further DNA synthesis should be blocked due to its actions at the RP-A complex. Therefore, to allow the second round of the hepatocyte cycle to occur, this active cdc2/cyclin B must be excluded from the nucleus until the end of S-phase, whilst its continued presence in the cytoplasm could prevent cytokinesis. Once the second round of the cycle is complete then both arms of the cdc2/cyclin B inactivation process could occur, returning the cell to a normal $G_0$ state.

#### 1.4.5.2 Tumour suppressor gene products

The nuclear phosphoprotein known as p53 is a tumour suppressor gene product intimately involved in regulating the transition in the cell cycle from $G_1$ to S phases (for reviews see Lane and Benchimol, 1990; Levine, 1993). In response to DNA damage, p53 induces cell cycle arrest and/or apoptosis via both transcriptional and non-transcriptional control of the activity of a range of regulatory effectors (Yan, *et al.*, 1997). It is a substrate for cdc2 kinase (Levine, 1993). It occurs in minute quantities in normal tissues but can be greatly increased in tumorous cells, mainly due to mutations which increase the half-life of the p53 protein from 6-20 min to 4-6 hr (Lane and

Benchimol, 1990). Most cancer causing mutations occur within certain critical domains which have been highly conserved during evolution, the homology between human and *Xenopus* genes in these regions being 90-100% (Levine, 1993). Mutations in p53 are the most common genetic finding in cancers (Hollstein, *et al.*, 1991). They usually lead to a loss of function, which can sometimes be dominant in heterozygotes over normal p53, but they may also gain alternative functions (Levine, 1993). The sites of such mutations are often found to be tissue or tumour specific. For example, 8 of 16 hepatocellular carcinomas examined from Qidong province in China had base substitutions at the third base pair of codon 249, 7 of which were G to T transversions. Similarly, 4 of 10 HCCs from southern Africa had G to T transversions, 3 at codon 249. In contrast, none of 6 substitution mutations in 22 Japanese HCCs were at codon 249. Aflatoxin $B_1$ and hepatitis B are known to be risk factors in China and in Africa. Mutagenesis experiments with aflatoxin $B_1$ have demonstrated a preference of this mutagen for the induction of base substitutions, principally G to T transversions in specific sequences (Hollstein, *et al.*, 1991). It has been proposed that screening for p53 mutations might be a reasonable means of early detection of many types of cancer, especially those showing familial predisposition, and could also be used as a method for identifying the tissue of origin of metastases (Harris, 1993).

Inhibition of PP1 and PP2A by okadaic acid leads to hyperphosphorylation of a suite of cellular and nuclear proteins, including p53, Rb, and the EGF receptor (Guy, *et al.*, 1992). The rate of phosphorylation of p53 in primary human fibroblasts in the presence of okadaic acid was 8 times that seen without the toxin (Yatsunami, *et al.*, 1993). p53, labelled with $^{32}P$ *in vivo* and then immunoprecipitated as a complex with SV40 large T antigen, is a substrate for PP2A *in vitro* (Scheidtmann, *et al.*, 1991). The lone catalytic subunit, $PP2A_C$, was most active, followed by the AC form, with the ABC form being least active. Three of six major phosphopeptides of p53, as determined by 2D electrophoretic mapping, were specifically dephosphorylated. Inhibition of

phosphatase activity in Balb/C 3T3 cells by a 6 hour exposure to 500 nM okadaic acid led to a transcription-independent induction of apoptosis (Yan, *et al.*, 1997). Therefore, the phosphorylation state of p53 is likely to be a key factor determining its interactions with both genomic and non-genomic targets (Lohrum and Scheidtmann, 1996; Milczarek, *et al.*, 1996) and therefore whether a cell continues in the cell cycle, arrests, or enters apoptosis.

Like p53, Rb is a tumour suppressor gene product, first discovered because a mutated inactive form was found to cause the rare retinoblastoma. Related factors, called p107 and p130, have also been identified. Also like p53, these factors are nuclear phosphoproteins regulated via phosphorylation/dephosphorylation rather than transcription (Levine, 1993; Sidle, *et al.*, 1996). In non-cycling cells, Rb forms a complex with members of the E2F family of transcription factors. E2F binds preferentially to the hypophosphorylated form (Sidle, *et al.*, 1996). When released, these E2F proteins bind to specific E2F-responsive promoter elements leading to increased transcription of cell cycle specific genes (Chellappan, *et al.*, 1991), for example, N-*myc* (Hara, *et al.*, 1993). Phosphorylation of Rb on specific serine or threonine residues is controlled during the cell cycle by the actions of various cyclin/cdk complexes. Rb-P is found in high concentrations during S-, $G_2$-, and M-phases, and then in late mitosis a specific phosphatase reactivates Rb (Buchkovich, *et al.*, 1989; Ludlow, *et al.*, 1990).

PP1 and/or PP2A appear to be involved in the regulation of the Rb phosphorylation state since, *in vitro*, Rb phosphorylated by cdc2 kinase serves as a substrate for both phosphatases (Alberts, *et al.*, 1993b). However, Rb phosphorylation has also been reported to be blocked by okadaic acid (Kim, *et al.*, 1993; Schonthal and Feramisco, 1993). Other workers have shown that this is a cell cycle specific effect. Rb dephosphorylation begins in anaphase and continues through to the next $G_1$. The phosphatases responsible for dephosphorylation of Rb at $G_1$ are not okadaic acid

susceptible. However, the presence of okadaic acid leads to an accumulation of cells in $G_2$/M and at this point an overall increase in the phosphorylation of Rb is seen (Van Dolah and Ramsdell, 1992). Cell extracts prepared from synchronised cells in late mitosis are capable of dephosphorylating Rb *in vitro*, with anaphase extracts being more active than pro- or metaphase extracts. This Rb phosphatase activity is inhibited by okadaic acid and by I1 and I2, suggesting that PP1 is the responsible activity (Ludlow, *et al.*, 1993). This Rb-specific PP1 activity may be mediated by a 110-kDa protein thought to promote the binding of PP1 to Rb during exit from mitosis (Nelson, *et al.*, 1997; Nelson and Ludlow, 1997).

#### 1.4.5.3 The Activator Protein-1 (AP-1) transcription factor

Activator Protein-1 (AP-1) is a nucleoprotein complex first identified as the transcription factor mediating cellular responses to the phorbol ester tumour promoters (Angel, *et al.*, 1987). Its activity is rapidly and transiently induced in response to various growth factors, and these signals are mediated by protein kinase C, protein kinase A and many oncogene products (Jehn and Osborne, 1997). AP-1 recognises a number of closely related sites on DNA called "TPA response elements" (TREs) and "cyclic AMP response elements" (CREs) which occur in the promoter or enhancer regions of many cellular genes (MacGregor, *et al.*, 1990). AP-1 has also recently been shown to be involved in the apoptotic process (Jehn and Osborne, 1997).

The protein products of the c-*fos* and c-*jun* oncogene families (eg, c-Fos, FosB, Fra-1, and c-Jun, JunB, JunD, respectively) associate to form the AP-1 transcription factor (Schonthal, 1992). The Jun proteins can associate as homodimers, whereas the Fos proteins can only associate with Jun proteins as heterodimers (Thevenin, *et al.*, 1991). Fos is a substrate for a numbers of different kinases, including protein kinase A, protein kinase C, and cdc2 (Abate, *et al.*, 1991), and this

post-translational modification, along with regulation of the expression of the various AP-1 subunits themselves and their cellular localisation, is thought to be involved in targeting the transcription factor to either proliferative or apoptotic genes, as required (Jehn and Osborne, 1997).

Keratinocytes exposed to 120 nM okadaic acid showed a marked increase in c-jun mRNA and protein levels by 6 hours, and this was correlated with increased phosphorylation of serine residues in c-jun (Peng, *et al.*, 1997). The kinetics of this process are different to the effect of the phorbol esters since high levels of *c-fos* and *c-jun* transcription were seen within minutes of exposure to TPA (as a result of translocation of AP-1 to TRE's within these genes) and these decreased within an hour, whereas okadaic acid induced a slower increase over several hours (Angel, *et al.*, 1987; Thevenin, *et al.*, 1991; Holladay, *et al.*, 1992). The induction of AP-1 formation by TPA was found to be independent of protein synthesis, involving instead the phosphorylation of previously formed cytoplasmic proteins and their translocation to the nucleus (Angel, *et al.*, 1987). Since the TRE is known to be present in the *c-jun* promoter region (Thevenin, *et al.*, 1991), it may be that increased phosphorylation of pre-existing c-jun leads to transactivation of its own expression. Okadaic acid enhancement of AP-1 phosphorylation increased its affinity for the TRE (Rosenberger and Bowden, 1996; Peng, *et al.*, 1997). Interestingly, in contrast to these high concentrations of okadaic acid, which in SHE cells caused the induction of c-fos and led to apoptosis, 0.16 nM toxin induced DNA synthesis without any effect on c-fos levels (Afshari, *et al.*, 1994). Therefore, the choice between induction of proliferation or apoptosis depends upon the level of exposure of the cell to protein phosphatase inhibition.

1.4.5.4 Tumour viruses

Since resting cells are far more abundant than actively dividing cells in an adult host, viruses are much more likely to infect these cells, even though they lack the nucleotide precursors, polymerase, ligase, topoisomerase, helicase, etc., which many viruses need to replicate their genome. Therefore, a number of DNA tumour viruses express protein products which specifically bind to, and interfere with the function of, a number of regulatory proteins, causing release the cell from $G_0$. For example, the simian virus (SV40) large T antigen can bind p53, Rb, and a 300 kDa nuclear protein on specific sites (Levine, 1993). In primate cells infected with SV40, murine p53 could block the large T antigen from binding to DNA polymerase $\alpha$ and thus replication of the viral genome whereas human p53 could not. Mutant murine p53 which could not bind large T could not produce these effects. Murine p53 binds large T more strongly than primate p53 does, perhaps making it insensitive to a cell cycle dependent regulatory switch. SV40 cannot infect mouse cells (Lane and Benchimol, 1990 and refs therein). Similarly, adenovirus E1A protein binds to the 300 kDa nuclear protein whilst its E1B protein binds to p53, and the human papillomavirus E6 protein binds to p53 whilst its E7 protein binds to Rb (Levine, 1993 and refs therein). When Rb.E2F is exposed to E1A or E7, there is increased transcription at the E2F-responsive element (Raychaudhuri, *et al.*, 1990).

There is considerable evidence that PP2A at least is both used by the virus to enable its replication and also targeted by it to circumvent certain cellular regulatory blocks (Reviewed in Mumby, 1995). SV40 DNA replication *in vitro* is activated by the dephosphorylation of the large T antigen by PP2A (Virshup, *et al.*, 1989). The phosphates at Ser-120 and Ser-123 of SV40 large T antigen, which are known to effect its binding to the origin of SV40 DNA, are removed *in vitro* by PP2A (Scheidtmann, *et al.*, 1991). Furthermore, the effect is cell cycle specific since S-phase,

but not $G_1$- or M-phase cell extracts, are able to dephosphorylate [$^{32}$P]-labelled large T (Ludlow, 1992). Also, SV40 small t antigen and polyomavirus middle and small t antigens can each bind to PP2A (Pallas, *et al.*, 1990). SV40 small t can associate with both the A subunit and with the AC form, but not with the catalytic subunit alone or with the ABC holoenzyme. Therefore, the primary interaction appears to be with the A subunit. SV40 small t seemed to mimic the B subunit in that it caused inhibition of dephosphorylation of all substrates assayed except histone H1, with which there was a slight stimulation of activity (Yang, *et al.*, 1991). In human diploid cells with a deletion on the short arm of chromosome 11, SV40 small t was not required for viral transformation to occur. This was correlated with overproduction of a 55 kDa "small t-like" factor which is thought to be the PP2A B subunit (Smits, *et al.*, 1992). Upon binding of SV40 small t antigen, PP2A is unable to inactivate the mitogen-activated protein kinase ERK1 and the mitogen-activated protein kinase kinase MEK1 (Sontag, *et al.*, 1993). Okadaic acid treatment mimicked the effects of small t in normal cells to induce transformation (Smits, *et al.*, 1992). Therefore, it appears that this virus can control the activity of PP2A, leading to the promotion of viral DNA replication (Cegielska, *et al.*, 1994). From the foregoing, it would seem that certain viruses have targeted PP2A as part of the scheme to circumvent mammalian cell cycle controls. This may be an indication that PP2A has a central role in the normal regulation of these controls. Disruption of this system by indiscriminate inhibition of this and other enzymes may be a more clumsy way of achieving similar ends.

## 1.5 **Summary**

Due to their active transport into hepatocytes by a bile acid-type transporter, the microcystins are generally regarded as liver specific toxins. In the whole animal they cause death due to hypovolaemic shock secondary to haemorrhage into the liver. This haemorrhage is caused by

destruction of hepatic structure as the cytoskeleton within liver cells depolymerises with concomitant loss of intercellular junctions. Cytoskeleton depolymerisation is associated with a hyperphosphorylation of cytokeratin subunits, a finding linked to specific inhibition of the activity of the protein phosphatases PP1 and PP2A by the microcystins and certain other natural toxins.

These microcystin-group toxins have also been shown to be tumour promoters in a number of animal models. This effect is probably due to the intimate involvement of PP1 and PP2A in the regulation and coordination of cellular metabolism and the cell cycle. By use of these toxins, the role of the protein phosphatases in the regulation of the cell division cycle kinases, is being elucidated, as is their involvement with the tumour suppressor proteins p53 and Rb. The fact that PP2A in particular is targeted by the transforming viruses is another strong indication of their involvement in regulation of the replicative cycle. However, there has also been evidence presented of transformed cell-lines reverting to a normal phenotype or entering apoptosis upon exposure to the toxins. Therefore, it is likely that the effect of protein phosphatase inhibition will be modulated by "local conditions" such as tissue type, stage of differentiation, which if any oncogenes are activated, and whether the cell is infected with a virus (eg, hepatitis virus B). Hence, although there is good evidence that these toxins *may* act as tumour promoters, it is not established they *will* do so. In particular, the link is tenuous between the oral consumption of microcystin-contaminated drinking water and the promotion of tumours *in vivo*. Also, there is very little knowledge of the effects of the microcystins on the cell cycle at the cellular level, of the effective concentrations at which these effects occur, and of how these effects compare with those induced by okadaic acid (with which most *in vitro* studies in this area have been carried out).

## 1.6 Aims

### 1.6.1 Rationale.

The primary aim of these studies was to elucidate the mechanisms by which microcystins might act as tumour promoters. Factors influencing the potential for tumour promotion by these toxins include route of exposure, tissue specificity, and cellular and sub-cellular responses. Therefore, a two-pronged approach seemed appropriate.

Oral consumption of contaminated drinking water is the most likely route of exposure, as well as the most likely to lead to the promotion of tumour growth in the intact animal. Therefore, long term *in vivo* dosing studies are necessary in order to gauge the potential for microcystin-induced tumour promotion in a semi-realistic setting. They can give a better idea of the diversity of sites at which the toxin can act, allow for the metabolic conversion of the toxin to potentially more widely acting or potent forms, and permit tissues to interact in their responses to the toxin. Although microcystin toxicity is essentially liver-specific, this does not mean that tumour promotion must also be limited to this organ. Furthermore, any metabolism of the toxin may be modulated by other cyanobacterial compounds in the drinking water and these would not supplied if pure microcystin was used to dose the animals. Hence, *in vivo* studies can provide a framework based in the multicellular organism with which to assess findings from more defined experimental models. Therefore, a chronic *in vivo* study was undertaken which had the potential to detect microcystin-induced tumour promotion at multiple sites in the animal and permitted the toxin/tissue and tissue/tissue interactions just mentioned to occur.

However, the use of relatively defined and controlled models is also essential for the precise assessment of potential mechanisms. Immortalised cell-lines were not considered ideal for this purpose since they are by definition abnormal, and many are largely undefined in terms of the mutations which make them immortal. This did not seem like a good basis upon which to assess a toxin's effects "in the real world". Furthermore, toxin uptake was thought likely to be problematic in such systems. Therefore, given the liver specificity of microcystin-LR and the greater difficulty in using any other perhaps more hydrophobic microcystins, studies were undertaken using primary hepatocyte cultures.

### 1.6.2 Specific aims

(1) To carry out a chronic dosing study in which mutagen-treated mice could be exposed via the oral route to microcystin-containing *Microcystis* extract, the mutagen and the microcystin content of the extract being chosen with the aim of maximising the number of tissues in which tumour promotion might be induced. This study is the subject of Chapter 3.

(2) To develop an *in vitro* cellular model system with which to study the influences of microcystin-LR on the cell cycle. Such a system would require the use of proliferation-capable, microcystin-sensitive, cells in culture. The development of this model system is described in Chapters 4 and 5.

(3) To develop methods for the quantification of the microcystin-induced effects on the cellular model system, and then to quantify those effects. These studies are presented in Chapters 4-9.

(4) To apply the results of these experiments to a consideration of how such effects could promote tumour growth *in vivo*, having regard to normal exposure levels versus those found effective *in vitro*. This discussion is the subject of Chapter 10.

# CHAPTER 2

# GENERAL METHODS

## 2.1 Animals

### 2.1.1 Sources

C57BL/6J and Swiss Albino mice were bred and supplied by the University of Adelaide Animal Services Facility. C3H/HeJ mice were obtained from the Animal Resource Centre, Western Australia.

### 2.1.2 Housing

Animals were housed in the clean or carcinogen handling rooms of the University of Adelaide Medical School Animal House, as appropriate. Except for the *in vivo* tumour promotion study (see Chapter 3), mice were kept in groups of up to 6 animals per cage, and were fed Laboratory ration L 103 (Ridley Agriproducts, Pty Ltd) and water *ad libitum*. They were maintained on a 12-12 hr light/dark cycle at 19-23°C.

### 2.1.3 General procedures and handling

All animals were given at least one week to acclimatise before an experiment began. Approval was obtained from the University of Adelaide Animal Ethics Committee for all studies prior to their commencement.

## 2.2 Statistical analyses

Data analysis was carried out using the GraphPad Prism or Microsoft Excel computer packages. Bartlett's test was used to show homogeneity of variance. Analysis of variance (ANOVA) followed by Tukey's or Dunnett's *post hoc* tests were generally used to compare treatments (Sokal and Rohlf, 1973). Kaplan-Meier survival analysis was used to analyse some of the *in vivo* tumour promotion experiment data (Collett, 1994). Student's t-test, linear regression, correlations, and the $\chi^2$ test were used as necessary. In all studies, $p<0.05$ was taken to be significant. Values and points on graphs are shown as the means ± SEM. Levels of significance of differences from control at $p<0.05$, $p<0.01$, and $p<0.001$ are indicated by *, **, and *** respectively. Where comparisons are made with other treatments different symbols are used, and these are indicated on the graph as needed.

# CHAPTER 3

# IN VIVO TUMOUR PROMOTION BY TOXIC MICROCYSTIS EXTRACT

## 3.1 Introduction

Tumour promotion following exposure of animals to microcystins or *Microcystis aeruginosa* extracts has been demonstrated in a number of animal models. In the mouse skin model, tumours initiated by topical application of dimethylbenzanthracene (DMBA) were larger and more numerous on animals given *Microcystis* extract in their drinking water when compared to those given a non-toxic *Anabaena* extract or just water (Falconer and Buckley, 1989). This is despite the supposed liver-specificity of the microcystins. In F-344 rats that had been 2/3 hepatectomised and treated with diethylnitrosamine (DEN) prior to ip administration of microcystin-LR, altered enzyme foci expressing the placental form of γ-glutamyl transferase were noted in the livers of animals given microcystin-LR (Nishiwaki-Matsushima, *et al.*, 1992). Similar foci were seen in the livers of 2/3 hepatectomised, aflatoxin-$B_1$ initiated F-344 rats when given concentrated Chinese pond waters to drink for 8 months (Zhou and Yu, 1990; Zhang, *et al.*, 1993; cited in Yu, 1995). These pond waters are subject to cyanobacterial blooms and are known to contain significant quantities of microcystins (Ueno, *et al.*, 1996). As noted in the General Introduction, there was a significant correlation between the consumption of these pond and ditch waters and the incidence of hepatocellular carcinoma in the human population in certain regions of China (Yu, 1995).

Other factors contributing to the high rates of hepatocellular carcinoma in China are endemic Hepatitis B virus infection and consumption of foodstuffs contaminated with aflatoxins (Yu, 1995). These factors do not occur significantly in Australia, and the incidence of liver cancer here

is low. However, if some microcystins, for example the more hydrophobic varieties such as microcystin-YM (tyrosine, methionine), are not liver-specific, then interactions with other forms of cancer, such as skin or intestinal neoplasias, may be of greater concern here. As noted above, an interaction between the consumption of *Microcystis*-contaminated drinking water containing microcystin-YM and the development of skin papillomas in mice has been demonstrated (Falconer and Buckley, 1989), as has the likelihood that contaminated drinking water can reach exposed human populations to elicit a toxic response (Falconer, *et al.*, 1983). For microcystin to be hepatotoxic via the oral route, it must pass into the hepatic blood supply via the gut. In fact isolated chicken enterocytes have been to shown to be susceptible to deformation by low concentrations of microcystin (Falconer, *et al.*, 1992), implying that they do take it up and are effected in an analogous manner to hepatocytes. It is assumed that they would actively pass the toxin into the hepatic circulation *in vivo*, although leakage through the gut wall secondary to disruption of this structure by the toxin has not been experimentally disproven.

In order to investigate further the possibility that oral exposure to microcystins might lead to the promotion of non-liver neoplasias, a dosing study was devised in which a *Microcystis* extract containing a broad range of microcystins was provided in drinking water to C57BL/6J male mice, which are known to have relative resistance to induction of liver tumours (Drinkwater and Ginsler, 1986). The mice were exposed by oral gavage to the non-tissue specific tumour initiator N-nitroso N-methylurea (NMU) so that the production of alimentary tract tumours might be maximised. Weight and appearance of the animals were monitored throughout, and those showing a marked decline in health were culled. Therefore, survival time was used as the primary indication of the rate of tumour growth, with the histological determination of tumour size and number being used to refine this assessment.

Falconer and Buckley (1989) used a 1:1 dilution of a *Microcystis* extract equivalent in toxicity to 80 mg microcystin-LR per litre as drinking water supplied to their test group to produce significant promotion of the growth of skin papillomas. In a one year dosing study an extract of 56.6 mg/L equivalent in drinking water produced significant rises in serum alanine aminotransferase levels after 5 weeks in both sexes, whilst a 1/2 dilution caused such a rise in males only after 9 weeks (Falconer, *et al.*, 1988). Therefore, an extract with a microcystin content equivalent to 40 mg/L microcystin-LR was chosen as the high dose, with 10 mg/L as the low dose, to be supplied in the drinking water.

## 3.2 **Materials and methods**

### 3.2.1 Characterisation of bloom material

Bloom material had been collected from Malpas Dam, NSW, and Lake Mokoan, NSW, on 8 March, 1981, and 14 March, 1991, respectively, by Professor I.R Falconer. They were stored at -20°C until being oven dried in January/February, 1992. Previous assay by HPLC had shown that the major toxic constituent of the Malpas sample was microcystin-YM (Botes, *et al.*, 1985; Falconer, *et al.*, 1986; Runnegar, *et al.*, 1986), whereas the Mokoan sample contained a number of unknown microcystins, the major peak being tentatively identified as the more hydrophilic microcystin-YR (tyrosine, arginine) (Falconer, *et al.*, 1994).

### 3.2.2 Extraction protocol

100 g of 1:1 Malpas/Mokoan dried *Microcystis* was placed in a blender (Moulinex Power Blender Model W36) with 500 ml absolute ethanol, chopped for 2 min before a further 285 ml of ethanol was added and the suspension blended for 10 min more. The suspension was left to stand for a short time and the supernatant poured off. This was centrifuged (Sorvall) at 8,000 rpm (10,000 g) for 10 min. The supernatant from this centrifugation was then rotary evaporated (Büchi, Switzerland) at 50°C to 1/10 volume and frozen. This constituted the ethanolic extract.

The pellets from the centrifugation plus the settled material from the blender were resuspended in water to a volume of 3 L and sonicated in a sonication bath for 30 min before being frozen overnight at -80°C. Next day the frozen material was thawed in a 50°C water bath, mixed, and then centrifuged as above. The supernatant was collected and stored at 4°C as the first aqueous extract. The pellet was resuspended in tap water to the original volume, sonicated as above and refrozen overnight. In the morning it was thawed, mixed, and recentrifuged, and the supernatant collected as the second aqueous extract.

Seven 100 g lots were processed in this way to produce the total extract. The volumes collected were: ethanoic extract (after evaporative concentration), 500 ml; first aqueous extract, 13.1 L; second aqueous extract, 17.2 L. Samples of each were taken for HPLC, then the extracts were mixed thoroughly, with water being added to make the volume to 35 L, and the pH raised from 6 to pH 7.5-8.0. The combined extracts were bottled in 500 ml aliquots and frozen at -20°C until used.

### 3.2.3 Determination of extract toxicity

#### 3.2.3.1 Mouse bioassay

The toxicity of the final extract was determined by mouse bioassay using intraperitoneal injection into male C57BL/6J mice. This was found to be 38.7 μg microcystin-LR equivalent/ml extract by assuming an average microcystin $LD_{100}$ of 100 μg/kg.

#### 3.2.3.2 HPLC

The microcystin content of ethanolic extract, first and second aqueous extracts, and the final combined mixture were kindly determined by Joanna Rositano, Australian Water Quality Centre, using reverse phase HPLC with photo-diode array detection. Twenty-seven percent acetonitrile in 0.1M $KH_2PO_4$ was used as mobile phase at a flow rate of 1 ml/min. Up to 9 microcystins of widely differing hydrophobicity where present, as determined by their characteristic absorption spectra, although these could not be identified due to lack of suitable standards. Microcystin-LR was not detected. The major toxin in the final extract (71% of area of all microcystin-like peaks) was more hydrophilic than microcystin-LR and so may have been microcystin-YR (Falconer, *et al.*, 1994).

### 3.2.4 Choice of mouse strain

C57BL/6J mice were chosen for this experiment because of their relative resistance to initiating carcinogens such as DEN (Drinkwater and Ginsler, 1986) and their relative susceptibility to the induction of intestinal tumours by direct-acting mutagens (Deschner, *et al.*, 1984) when compared to other available strains.

### 3.2.5 Choice of tumour initiator

N-Nitroso-N-methylurea (NMU; IARC, 1978) was chosen as the mutagen because it is a direct-acting genotoxin not requiring prior activation in the liver (Pitot and Dragan, 1994). Thus by administering it via oral gavage, tumours could be initiated in the alimentary tract without prior hepatic bioactivation and exposure of the liver. It was noted that lymphoma was initiated in many of the animals treated (Ito, *et al.*, 1989), but this is another common cancer in western countries and therefore this was seen as an added advantage. Ito *et al,* (1989) used 8 x 20 mg/kg ip doses of NMU over 4 weeks as a tissue non-specific tumour initiator in male F344 rats. Over a series of experiments they found that this level of NMU dosage allowed the detection of significant tumour promotion by 6 different promoters in up to 9 different tissues. Similar results were reported by Uwagawa *et al*, (1991). To minimise the chance of undue weight loss caused by exposing the animals via the gastric route, a lower total dose was used over a shorter period. Therefore, 2 x 40 mg/kg treatments were given 4 days apart.

### 3.2.6 Mouse dosing protocol

One hundred and fifteen male C57BL/6J mice were obtained at 5 weeks of age. They were randomly divided into treatment groups of roughly equal average weight, up to 10 animals per cage, and then left for 2 weeks to acclimatise to normal food and tap water (filtered and UV-sterilised on site) ad libitum. To initiate tumours, mice were given 2 doses of NMU (Sigma) in water/acetic acid (pH 4) at 40 mg/kg by gastric intubation on two occasions 4 days apart. Two days after the second NMU administration, *Microcystis* extract diluted in tap water was started as the only source of drinking water. Three doses were supplied equivalent in toxicity to: 0.0, 10, and 40 mg microcystin-LR per litre to treatment groups comprising 46, 39, and 30 mice, respectively. Ethanol was added to low dose and control water supplies so as to match the high dose ethanol content of 1.4%. Water was changed every 2 days.

### 3.2.7 Data collection

Water consumption was noted at each change and mouse weights were recorded weekly. Mice showing signs of distress were put down by either $CO_2$ asphyxiation or, if blood samples were to be taken, by ip injection of 240 mg/kg phenobarbitone (Nembutal; Bomac Laboratories Pty. Ltd.). The weight was recorded and a detailed post-mortem(PM) examination performed. Livers, spleens, and duodenae plus any other tissues appearing abnormal by eye were preserved in buffered formalin for histological examination. Blood smears were also prepared. The carcass was also preserved for later reappraisal if necessary. Tissue sections were routinely stained with Haematoxylin and Eosin (H+E), and blood smears with Giemsa.

Livers were always sectioned through the midpoint of the left lobe. The area of the section, and the proportion of this area composed of infiltrating lymphocytes, were determined using image analysis (Video Pro, Leading Edge Pty. Ltd.). The infiltrating cells were also typed on morphological grounds, as were those infiltrating any other tissues collected at PM, along with the circulating lymphocytes seen in the blood smears.

Image analysis was also used to determine the number and area of duodenal tumours per unit duodenal length. Cell or tumour typing was not attempted in these sections since a great range of cell types was seen within each tumour, although all tumours (both within and between animals) appeared to be generally composed of the same range of cell types.

Sorbitol dehydrogenase and alanine aminotransferase activities were determined in the serum of a number of animals using standard spectrophotometric assays (Henry, *et al.*, 1974; Rose and Henderson, 1975; Pakuts, *et al.*, 1988).

### 3.2.8 Data analysis

Time-to-death was analysed by determination of the Kaplan-Meier estimate of the survivor function for each dose group, and these were then compared using the log-rank test and the log-rank test for trend (Collett, 1994). Serum liver enzyme activities, area of liver section, proportion of the liver infiltrated by lymphocytes, and area and number of duodenal tumours per unit length were analysed by one way ANOVA, with or without prior Ln-transformation as appropriate, followed by Tukey's Multiple Comparison Test (Sokal and Rohlf, 1973). Distribution of type of infiltrating lymphocyte was compared across dose groups by $\chi^2$ test.

## 3.3 Results

### 3.3.1 Water and microcystin consumption

The water and calculated microcystin-equivalent consumption averages are given in Table 3.1.

| Water: | (ml/mouse/day) |
|---|---|
| Control: | 3.05 |
| Low: | 2.98 |
| High: | 2.31 |
| **Microcystin:** | **(µg/mouse/day)** |
| Control: | 0.00 |
| Low: | 28.87 |
| High: | 89.44 |

**Table 3.1** - Water and calculated microcystin consumptions. Microcystin quantified as microcystin-LR equivalents, based on the acute $LD_{100}$ of the *Microcystis* extract.

The high dose consumption of microcystin was calculated to be approximately half of the reported oral $LD_{50}$ (Falconer, 1991). This was not confirmed by a separate oral $LD_{50}$ determination of this extract. On this basis, the low dose animals were exposed to approximately 16% of the oral $LD_{50}$.

### 3.3.2 Animal weight

The average weight per mouse in each dose group is shown in Figure 3.1 for the period of the experiment. Note that as sick, and therefore light-weight, mice were removed the average weight periodically recovered.

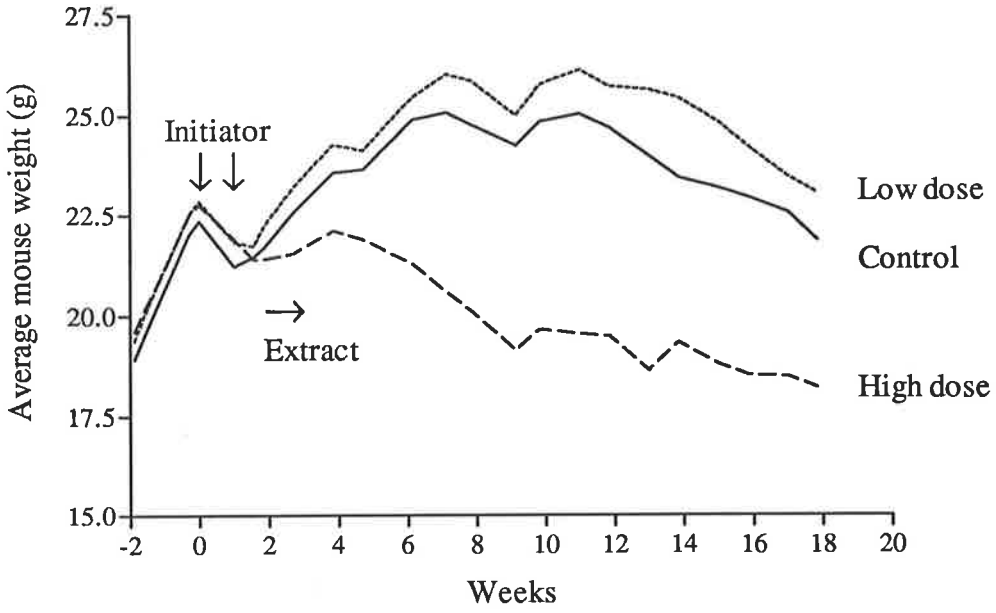



**Figure 3.1** - Average mouse weight in each treatment group. Tumours were initiated in mice with two doses of 40 mg/kg N-nitroso-N-methylurea by gavage four days apart. *Microcystis* extract (0, 10, or 40 mg/L equivalent of microcystin-LR) was given as sole source of drinking water two days later. Mouse weights were monitored weekly.

After an initial decline in weight in all groups due to administration of the initiator, mice in the control and low dose groups quickly recovered and resumed normal growth. However, the mice in the high dose group never recovered and instead exhibited a slow decline in body weight, notwithstanding that the lightest mice were, from week 8, continually removed. This decline amounted to an approximately 16% loss of growth potential, as compared to the control group, and is somewhat higher than the 10% loss of growth potential which is used as a benchmark when

setting the Maximum Tolerated Dose (MTD; Weisburger and Williams, 1984). Control and low dose mice started to lose weight again from about 7 weeks post-initiation due to tumour growth.

### 3.3.3 Time to death

Figure 3.2 shows the proportion of each dose group surviving at a give time.

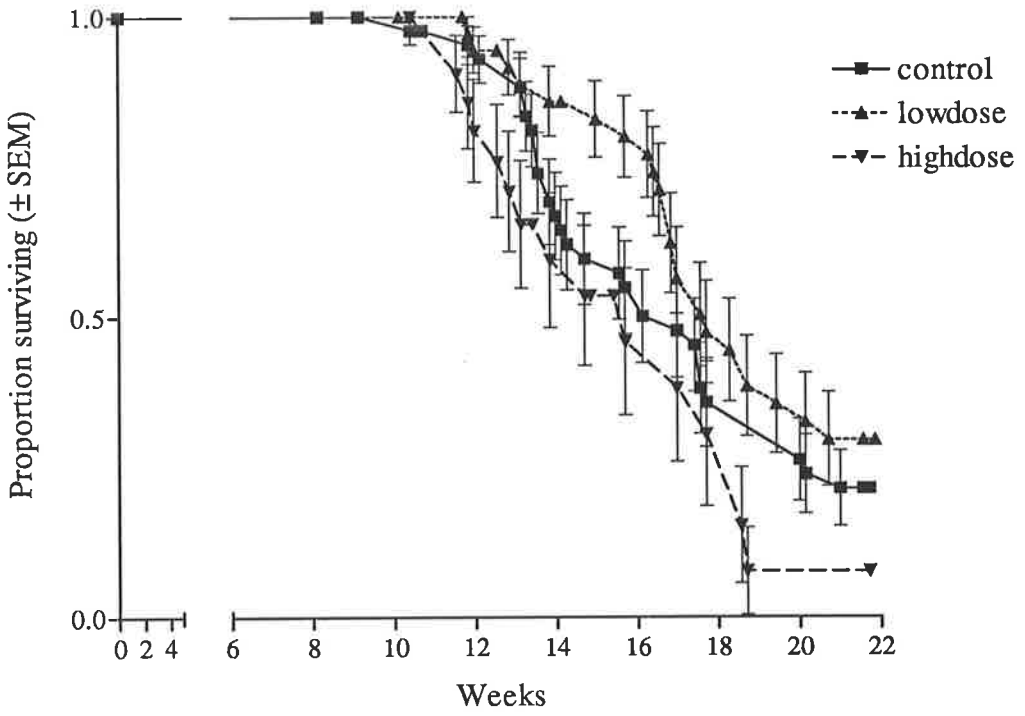



**Figure 3.2** - Survival curves for mice in each treatment. The Kaplan-Meier estimate of the survivor function was calculated for each treatment group. Error bars showing the SEM are superimposed on uncensored results (censored results being, for example, animals surviving until the end of the experiment, for whom death was not attributable to one of the disease processes under study).

The Logrank Test for difference between the curves yielded a p-value = 0.0670. There was not, therefore, a significant difference in survival between dose groups at the $\alpha$=0.05 level. As can be seen, it appeared for a while that there might be an increased survival in the low dose group, but this was not sustained until the end of the experiment. Survival was not dependant upon dose of extract.

When animals were grouped as to post mortem result (enlarged thymus, enlarged spleen, change in liver size, or presence of duodenal plaques or tumours), and these groups subjected to survival analysis, it was found that death with enlarged thymus best corresponded with the survival curves shown above (data not shown). This suggests that enlarged thymus was the most common reason for an animal dying or being culled.

### 3.3.4 Post mortem findings

The most conspicuous and frequent post mortem findings were enlarged spleen, enlarged thymus, and hepatomegaly, with abnormal renal colouration and size also being common. The most common finding in the duodenum were small in-foldings or small rounded depressions ("plaques") in the intestinal wall. These, when examined histologically, were found to correspond to duodenal adenomas and adenocarcinomas (see Section 3.3.5.3). Larger tumours were less common. Apart from the plaques there were also a few cases in which the intestine was intussuscepted (shortened and greatly thickened, apparently being folded longitudinally within itself). Quite often animals exhibited a large elongated lymphoid body, or series of bodies, amongst the folds of the intestine. Mice in all groups lacked any significant amount of abdominal fat (loss of weight being one of the main determinants of time to cull). Some animals showed signs of subcutaneous and/or intra-abdominal oedema.

Apart from the direct effects of the toxin on the liver in the high dose group (described later in Section 3.3.5.1), the two main disease processes seen were lymphoma/lymphocytic leukaemia and intestinal (predominantly duodenal) tumours. It was therefore these two processes which were examined for evidence of tumour growth promotion.

### 3.3.5 Histological findings

The histological examination of tissues concentrated on three main disease processes: (1) the toxic effects of the extract especially in the liver (where these could be distinguished from effects secondary to other processes); (2) lymphoma/lymphocytic leukaemia, with examinations of blood smears and infiltrated tissues such as the liver, spleen, lymph nodes, kidneys and lungs; (3) and duodenal tumours.

#### 3.3.5.1 Toxic effects

##### 3.3.5.1.1 Microscopic examination

Upon microscopic examination the major differences between the livers of control and low dose mice were that the sinusoidal spaces of the latter were often markedly wider throughout the lobule than those of the former. Differences in cytoplasmic granularity of hepatocytes (cells being more granular closer to the portal triad) and differences in staining intensity (centrilobular cells appearing more eosinophilic) were also noted but these traits did not appear to be especially confined to a particular dose group. Almost universally, livers from high dose group mice exhibited marked disruption of normal hepatic architecture due to an extensive infiltration by cells of a number of types, associated with amorphous lightly eosinophilic material. These cells and associated material contributed especially to a thickening of the walls of the major blood vessels, but also extended interlobularly. These findings are consistent with an active chronic liver injury showing early signs of post-necrotic cirrhosis (Robbins, 1974; Falconer, *et al.*, 1988). Intrahepatic haemorrhage and/or necrotic foci were also sometimes observed. Areas of infiltration by more homogeneous populations of cells, probably lymphomatous, were also seen in livers from high

dose mice but they rarely dominated the picture as was the case in sections from mice of other dose groups. Therefore, although the degree of white cell infiltration of high dose livers was markedly higher than that seen in the other two groups, as found by image analysis (see below), the patterns of infiltration were also markedly different. This made any sort of quantitative comparison of the livers of the high dose group with those of other groups very difficult.

The villi in the duodenae of high dose group mice were markedly stunted compared to those of the controls and low dose treated animals. In previous chronic dosing studies (Falconer, *et al.*, 1988; Falconer, *et al.*, 1994; Fawell, *et al.*, 1994), GI tract injury was not observed. Therefore, it is likely that the marked effects seen here were due to the combination of microcystin toxicity and tumour growth on the cells lining the gut. The reduced absorption of nutrients likely to result from such a loss of functional surface area was likely to be one of the main contributors to the weight loss seen in this group.

#### 3.3.5.1.2 Image Analysis

Since the microcystins have such marked acute effects on liver size it was of interest to see what chronic treatment might cause in this respect. The area of a section of the left lobe of the liver taken transversely through the mid-point (widest part) was determined by image analysis. The mean area of this liver section from high dose mice was significantly smaller than the mean area of the corresponding section from both control and low dose mice ($p<0.01$). There was no significant difference between control and low dose liver sections. However, when liver section areas were normalised for the animal's weight at death, the differences were no longer significant. Thus, the reduction in size of the liver in the high dose group may have been due to the general weight loss in these animals rather than a tissue specific effect of the toxin.

3.3.5.1.3 Liver Enzymes

Determinations were made of the serum levels of activity of sorbitol dehydrogenase (SDH) and alanine amino transferase (ALT) in animals from the latter part of the study. Means ± standard deviations are shown in Table 3.2a and the p-values for the statistical comparison of the treatments are shown in Table 3.2b.

| | SDH | ALT |
|---|---|---|
| Control | 50.2 ± 33.3 | 34.3 ± 32.4 |
| Low dose | 97.1 ± 96.0 | 217.7 ± 301.8 |
| High dose | 125.5 ± 100.5 | 190.2 ± 169.1 |

**Table 3.2a** - Liver enzymes in the serum of mice chronically treated with *Microcystis* extract and suffering concurrent lymphomatous hepatic infiltration. Values are means ± standard deviations. (SDH, n=47; ALT, n=25)

| | SDH | ALT |
|---|---|---|
| Control-High | $p<0.05$ | $p<0.01$ |
| Control-Low | $p>0.05$ | $p<0.01$ |
| Low-High | $p>0.05$ | $p>0.05$ |

**Table 3.2b** - Statistical comparison of the effects on the serum levels of liver enzymes of the consumption *Microcystis* extract and concurrent lymphomatous hepatic infiltration. One way ANOVA was performed on the Ln-transformed data followed by Tukey's Multiple Comparison Test for comparisons of the treatments.

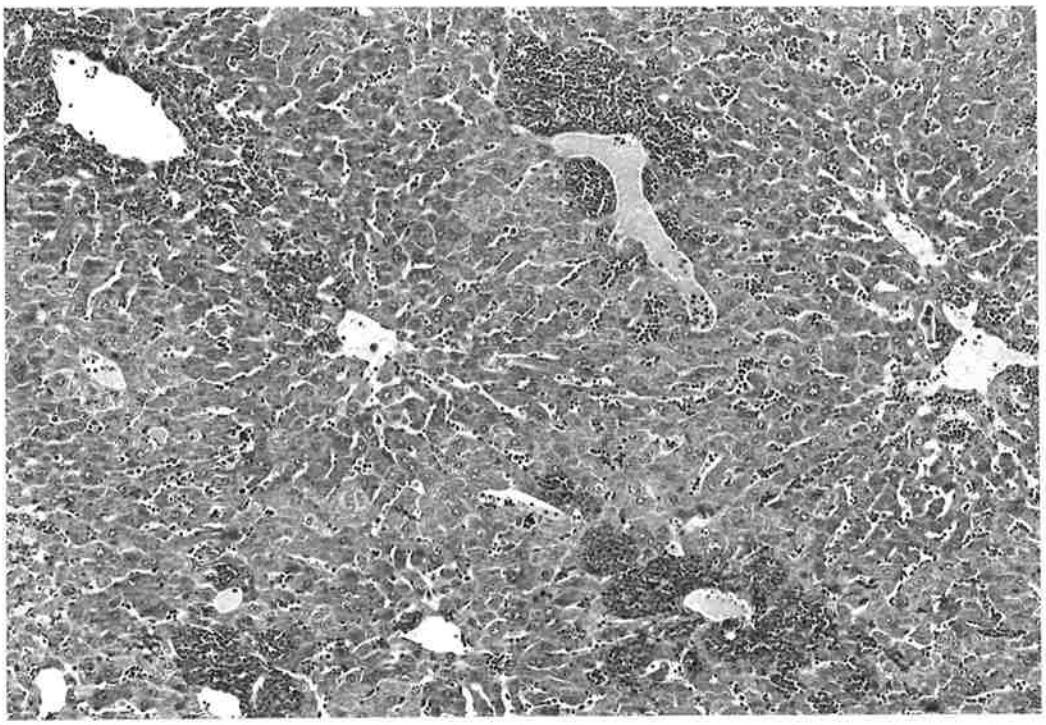
This shows that at least the high dose, and probably both *Microcystis* extract doses, caused significant elevations in the serum levels of these enzymes, indicative of liver damage.

3.3.5.2 Lymphoma/lymphocytic leukaemia

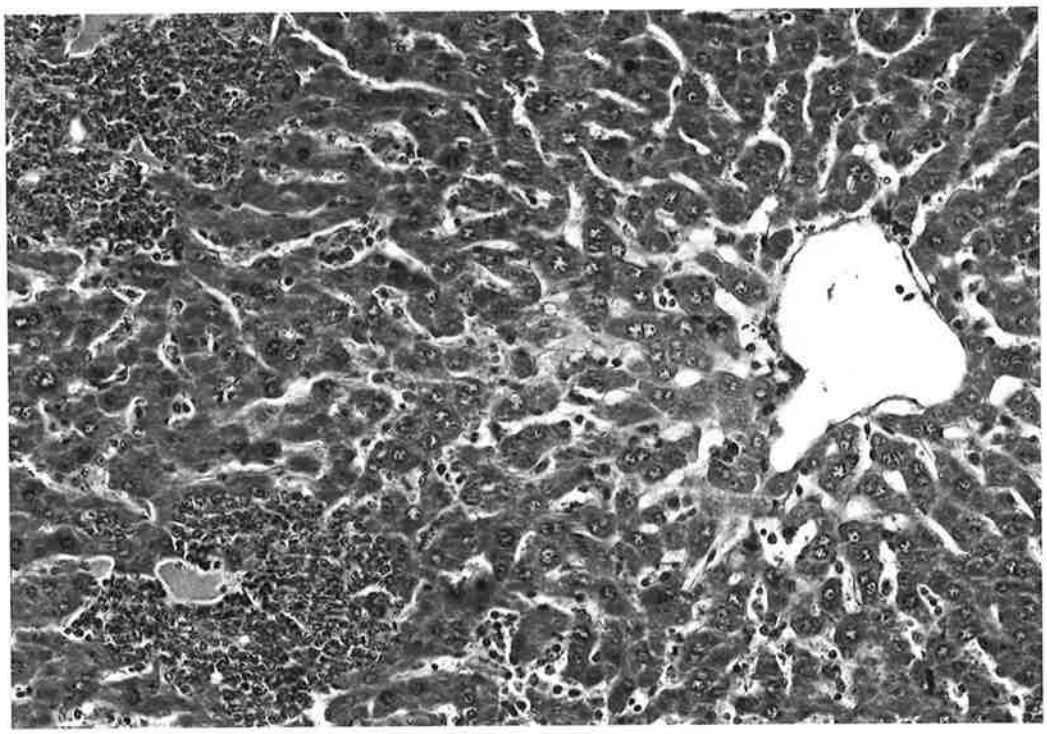
Lymphoma and/or lymphocytic leukaemia were found in almost 100% of mice of all dose groups. Gross findings were enlarged (infiltrated) thymus, spleen, liver, lymph nodes and/or kidneys, and usually large numbers of abnormal lymphocytes in the peripheral blood. A number of cell types were seen in different mice, indicating that multiple types of lymphoma were produced. The predominant types of lymphoma appeared to be lymphoblastic leukaemia, and follicular centre cell lymphomas of the large, small, or mixed types (Pattengale and Taylor, 1983; Pattengale, 1994).

There was no significant difference between degree of infiltration of control and low dose livers ($p=0.788$). As noted above, the pattern of infiltration in high dose livers could not be attributed solely to lymphomatous cells, and so a valid comparison of this group with the others could not be made. There was no significant correlation between degree of liver infiltration and time to death, probably due to the range of lymphomas found, each with a different rate of progression. An example of lymphocyte infiltration is presented in Figure 3.3.

**Figure 3.3** - Lymphomatous infiltration of the liver of a C57BL/6J mouse which had been treated with the tumour initiator NMU at 7 weeks of age and given *Microcystis* extract (equivalent to approx. 10 mg/L microcystin-LR) as sole source of drinking water. The mouse was killed and its tissues sampled at 16 weeks post-initiation.



(a) General view showing clearly defined islands of lymphocytes mainly clustered around the hepatic blood vessels. The area of these islands as a proportion of the total section area was determined by image analysis. (Magnification 100x).



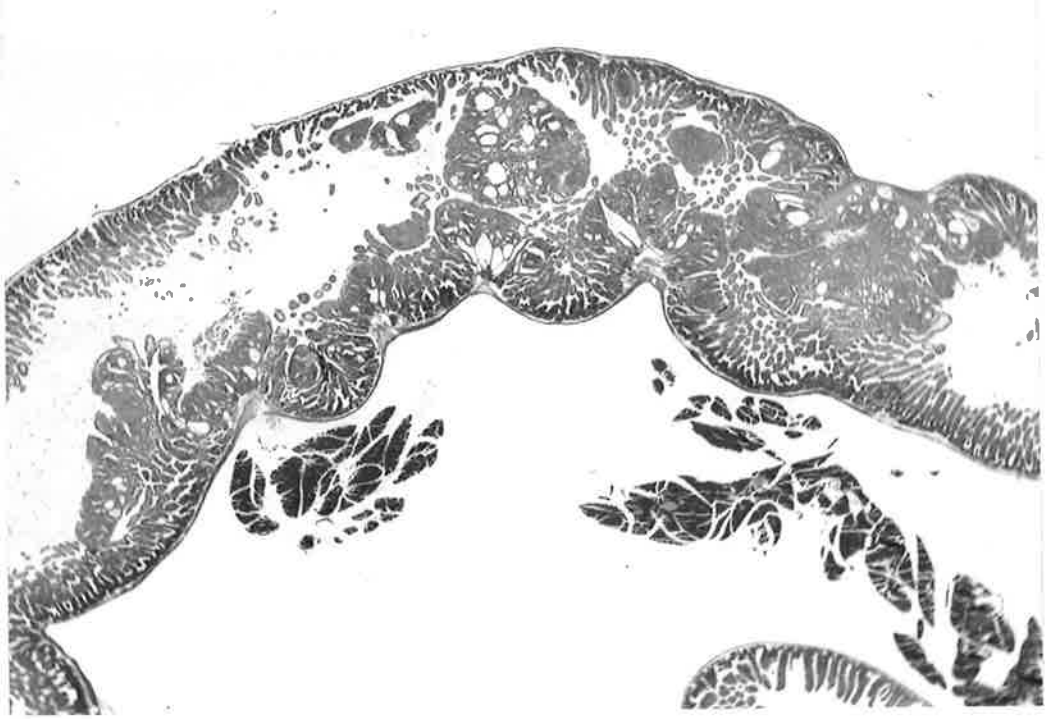
(b) Closer view showing circumscribed islands of lymphocytes, but also relatively large numbers within abnormally dilated sinusoids. Hepatocyte nuclei appear normal, but the cytoplasm is dense and inter-cellular boundaries are poorly defined. (Magnification 400x).

To examine the possibility that there might be differences in distribution of type of lymphoma between control and low dose groups, lymphocytes in the peripheral blood, and those infiltrating the liver and the spleen were independently grouped on morphological grounds. Because of the toxic effects on the liver, the high dose group had to be excluded from this examination. Therefore, 45 animals of the control and low dose groups for which all three tissues (blood, liver and spleen) had been histologically prepared were examined. Each mouse was assigned to three independent groups: one for blood cell type, one for the cell type infiltrating the liver, and one for the cell type infiltrating the spleen. When correspondences between cell type groups were analysed it was found that they fell into three clusters: one of which is probably lymphoblastic leukaemia, the other two probably being follicular centre cell lymphomas of the large and small cell types (although a few of these may have been of the mixed type) (Pattengale and Taylor, 1983; Pattengale, 1994). Only 4 animals could not be assigned to one of these three clusters. The distribution of animals from the two dose groups amongst the three clusters was then analysed by the $\chi^2$ test. No significant difference was found.

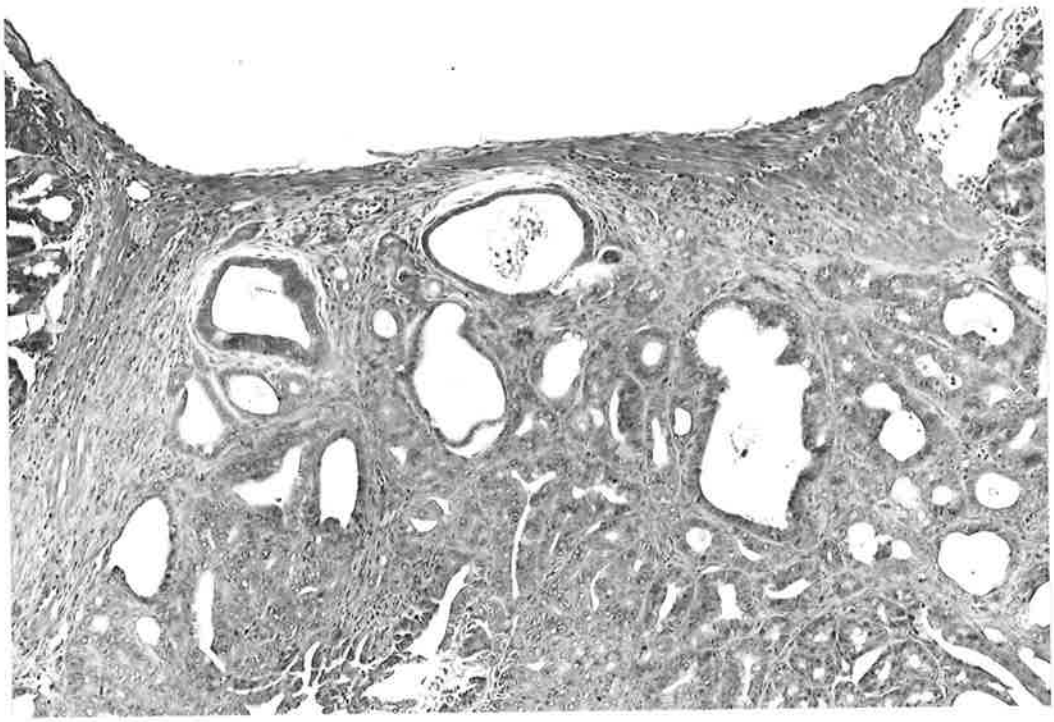
#### 3.3.5.3 Duodenal tumours

The top few centimetres of the duodenum were excised from each animal at post mortem and prepared for histological examination (see Figure 3.4). Image analysis was used to determine both the number and size (in $mm^2$) of the tumours present, and to precisely determine the length of the piece of duodenum examined. Thus, number of tumours/mm, tumour area ($mm^2$)/mm, and average tumour area ($mm^2$) were tabulated and analysed for dose dependent differences by one way ANOVA followed byTukeys MCT (see Tables 3.3a and 3.3b below).

**Figure 3.4** - Multiple adenomas and adenocarcinomas within the duodenal lumen of a C57BL/6J mouse treated with the tumour initiator NMU at 7 weeks of age and given *Microcystis* extract (equivalent to approx. 10 mg/L microcystin-LR) as sole source of drinking water. The mouse was killed and its tissues sampled at 16 weeks post-initiation.



(a) General view showing the extent of tumour growth and the almost complete occlusion of the duodenal lumen. The area of each tumour and the total length of duodenum sampled were determined using image analysis. (Magnification 10x).



(b) Higher magnification view of an invasive adenocarcinoma growing within the wall of the duodenum. (Magnification 100x).

| | Control | Low | High |
|---|---|---|---|
| Tumour area ($mm^2$/mm) | 0.36 ± 0.24 | 0.33 ± 0.26 | 0.12 ± 0.16 |
| Tumours/mm duodenal length | 0.28 ± 0.17 | 0.24 ± 0.17 | 0.16 ± 0.10 |
| Average Area of tumours ($mm^2$) | 1.29 ± 0.60 | 1.39 ± 0.79 | 0.84 ± 1.01 |

**Table 3.3a** - Effect of the consumption of *Microcystis* extract on the size and number of duodenal tumours in treated animals. Values are means ± standard deviation.

| | Control/Low | Control/High | Low/High |
|---|---|---|---|
| Tumour area ($mm^2$/mm) | $p>0.05$ | $p<0.001$ | $p<0.05$ |
| Tumours/mm duodenal length | $p>0.05$ | $p<0.05$ | $p>0.05$ |
| Average Area of tumours ($mm^2$) | $p>0.05$ | $p>0.05$ | $p<0.05$ |

**Table 3.3b** - Statistical comparison of the effects of the consumption of *Microcystis* extract on duodenal tumour growth. Data in Table 3.3a were analysed for dose dependent differences by one way ANOVA followed by Tukey's Multiple Comparison Test.

All of these parameters were markedly lower in the high dose group compared to the others, the difference attaining statistical significance in most cases. There was no significant difference between control and the low dose group for any parameter. Therefore, it appears that tumour growth in the high dose group was retarded by the high levels of toxin present.

Distribution of tumour type amongst the dose groups was not examined quantitatively since it appeared from an informal examination that the majority of tumours were of the tubular adenocarcinoma type, most infiltrating the submucosa but only a minority invading through the muscularis propria (Fenoglio-Preiser, *et al.*, 1990).

## 3.4 Discussion

Due to the low prevalence of primary liver cancer in Western populations not exposed significantly to hepatitis B virus and aflatoxins, the present experiments were carried out to determine whether microcystins may interact with other, more common cancers, to promote their growth. Mice were exposed to a non-tissue specific mutagen by gavage to initiate gastro-intestinal tumours. They were then fed one of three dose levels of a cyanobacterial extract containing a number of microcystins of widely varying hydrophobicity thereby hopefully maximising the penetration of toxins into a range of cell-types.

Mice receiving the high dose developed clear signs of toxicity in terms of weight loss, serum enzymes and histological effects in the liver and duodenum. These effects were as expected since this dose level was intended to approximate a Maximum Tolerated Dose (MTD; Weisburger and Williams, 1984). In fact, the high dose mice lost somewhat more weight than is usually produced by the MTD, probably due to the combined effects of toxicity and tumour growth, particularly in the gut. Nevertheless, their survival did not differ to a significant degree from that of those mice exposed to the other doses. In mice exposed to a 1/4 dilution of the *Microcystis* extract, the only sign of toxicity was an elevation of the serum levels of one (ALT) of the two liver enzymes assayed.

The most consistent and conspicuous findings upon post mortem were signs of lymphoid hyperplasia (grossly enlarged thymus and spleen, and intestinal lymphoid bodies), and duodenal infoldings and depressions (plaques). Thus despite the "topical" application of NMU to the gastrointestinal tract, lymphoma was the dominant finding at post mortem. The presence of an enlarged thymus at post mortem was, along with substantial weight loss, the major determinant of

an animal being culled. This is as might be expected, since the acute distress to an animal caused by an enlarged thymus causing pressure on the heart and lungs, is more likely to lead to the animal's being culled than are the less obvious symptoms due to hepatic, splenic or intestinal disease, notwithstanding that these were very often also present. Such complications would seem to suggest that time-to-death analyses are not particularly useful interpretive tools in this experiment. However, if it had been decided instead to end the experiment at some arbitrary intermediate time point, eg 15 weeks (see Figure 3.2), a completely different and possibly illusory result might have been found.

Histological analysis confirmed the presence of these major processes, that is, liver and duodenal toxicity in the high dose group, and lymphoma/lymphocytic leukaemia and duodenal adenocarcinoma in all groups. The effects of microcystin toxicity were very evident at the histological level in the tissues of high dose mice, complicating the picture and leading to this group's exclusion from the analysis. Despite elevated ALT levels in their serum, there was no histological evidence of toxicity in the low dose animals. Therefore ALT levels were a more sensitive indicator of microcystin toxicity than histological examination in this study. Nevertheless, the presence of microcystins in the drinking water of the low dose group did not effect the rate or degree of development of these neoplastic processes in these animals either. Therefore, there was no evidence in this study for microcystins being promoters of murine lymphocytic or duodenal neoplasias.

To investigate the tumour promotional effects of the microcystins in a more controlled system, it was considered important that *in vitro* techniques be developed. These studies are the subject of the remaining Chapters of this thesis.

# CHAPTER 4

# STUDIES ON IN VITRO TUMOUR PROMOTION BY MICROCYSTIN-LR. GENERAL METHODS

## 4.1 Introduction

To maximise growth in culture, hepatocytes are often cultured in a medium containing foetal calf serum (FCS; Berry, *et al.*, 1991). However, this makes the interpretation of effects on cell control difficult since the FCS is not fully defined in terms of growth factors. It was considered preferable for the present study to use a chemically defined medium in which factors influencing the growth of cells could be controlled individually.

Rodent hepatocytes in primary culture in chemically defined medium can be stimulated to synthesise DNA and undergo cytokinesis by addition of Epidermal Growth Factor (EGF). However, the peak of DNA synthesis occurs at 48-72 hours and generally stops with only 2-3 rounds of synthesis and cytokinesis having been completed (Michaelopoulos, 1990). Sawada *et al*, (1988) reported far better growth stimulation by EGF in hepatocytes from young (5-6 week old) C3H/N mice than in cells from similarly aged Sprague-Dawley rats. This stimulation also lasted longer, with the peak of increase in cell numbers occurring after 7 days in culture. The cells were still capable of albumin production at this time.

Sawada *et al*, (1988) also reported that a small number of the cells from the C3H/N mice were capable of growth without EGF. Furthermore, Lee *et al*, (1989) found that some cells derived from C3H/HeNJcl mice and kept in normal culture for up to 3 months developed into

immortalised colonies, whereas this was extremely rare in cells derived from C57BL/6NJcl mice. In the case of the C3H-derived cells only, this effect could be enhanced markedly by the addition of phenobarbital to the growth medium, a known tumour promoter in rodents. C3H mice have a higher incidence of spontaneous hepatocellular carcinoma than do C57BL mice and are also 20- to 50-fold more sensitive to tumour initiation by chemicals such as DEN (Drinkwater and Ginsler, 1986). This is linked to possession of hepatocarcinogen sensitivity (Hcs) loci by C3H mice, which appear to promote the proliferation of both normal and initiated cells from these animals (Hanigan, *et al.*, 1988). Therefore, it appears that hepatocytes from C3H mice carry a mutation which allows, given the right circumstances, unregulated growth, and so they might be considered to be constitutively "pre-initiated". Use of such cells was considered to be an advantage in studies using a putative promoter since it might obviate the need to chemically initiate mutations in cells prior to exposure of the cells to the test chemical (microcystin-LR). The slow background proliferation of non-EGF stimulated cells might also be useful, allowing examination of the influence of microcystin-LR on their growth without the potentially confounding presence of EGF.

## 4.2 **Materials and methods**

### 4.2.1 Sources of chemicals

Nodularin, okadaic acid, culture medium (DMEM/F12), and HEPES were purchased from ICN. Microcystin-LR, dexamethasone, Insulin-Transferrin-Selenium (ITS), penicillin, streptomycin sulphate, and insulin were from Sigma. Recombinant Epidermal Growth Factor, comprising the human EGF amino acid sequence plus a 53 amino acid N-terminal extension peptide, was purchased from GroPep Pty Ltd, Adelaide, Australia. Heparin was from

Commonwealth Serum Laboratories, Australia, and Nembutal (60 mg/ml pentobarbital) was from Boehringer-Ingelheim. All other chemicals were of the highest quality available.

### 4.2.2 Age and weight of mice

To minimise the possible influence of the sex, age and weight of donor animals on experimental outcomes, male C3H/HeJ mice were acquired at 4 weeks of age with weights of 17-19 g. They were kept on normal animal house chow for one week and then used at 5 weeks (± 1 day) when their weight was 20.86 g ± 1.92 (SD). Generally two mice were used per experiment, the isolated hepatocytes from each animal being mixed prior to the Percoll centrifugation stage of the cell isolation procedure (see below). The hepatocyte isolation procedure was performed in the morning between 10:00 am and 12:00 pm.

### 4.2.3 Cell isolation protocol

The method of cell isolation used was a modification of the two-step collagenase perfusion procedure introduced by Seglen (1976). Perfusion was retrograde, via the abdominal portion of the inferior vena cava (Martin, *et al.*, 1990).

All solutions, except those supplied sterile, were sterilised by passage through 0.22 μm filters. Recipes for media are given in Appendix 1. Perfusion tubing was sterilised by flushing with 70% ethanol followed by filter-sterilised MQ.$H_2O$ before use. Perfusion media were pre-equilibrated in a 37°C water bath and constantly bubbled with carbogen (5% $CO_2$, 95% $O_2$). The perfusion procedure was carried out in the open laboratory. However, the area used for the surgical

procedure was thoroughly sprayed with 70% ethanol, as were all surgical instruments such as scissors, forceps, etc. After removal of the perfused liver, all subsequent steps were carried out in a laminar flow cabinet.

Five week old C3H/HeJ male 21 g mice, Mammary Tumour Virus-free, (Animal Resource Centre, Perth, Western Australia) were given 50 U (50 µl) heparin and 100-150 µl of 60 mg/ml Nembutal (ip) in a single injection. Upon anaesthesia, the belly and chest were doused in 70% ethanol and the skin was cut away from these areas. Instruments were resterilised in 70% ethanol before opening the abdominal wall (Berry, *et al.*, 1991). The abdominal inferior vena cava was freed from the underlying connective tissue, cannulated and then ligated distally. The thoracic vena cava was occluded and the portal vein severed. The calcium-free perfusion buffer was begun at 4 ml/min and continued for 5 minutes, after which the collagenase buffer was commenced at the same flow rate. This was continued for between 7 and 10 minutes, until the liver became "buttery" and liquid leaked through the capsule.

When the collagenase perfusion was complete, the gall bladder was cut off, and the liver excised. It was washed in 20 ml of ice-cold wash buffer and then transferred to 20 ml of clean ice-cold wash buffer. The hepatic capsule was opened with scissors, and the cells were gently scraped into the medium with a spatula. The cell suspension was passed through a layer each of 200 µm and 100 µm nylon mesh into a 50 ml centrifuge tube. The mesh was washed with more buffer until the volume was 25 ml. Twenty ml of Percoll isodensity centrifugation buffer was added, mixed gently, and the resulting suspension was centrifuged at 50 x g for 10 min at 4°C (Kreamer, *et al.*, 1986). The supernatant was aspirated and the pellet resuspended in 45 ml ice-cold wash buffer and recentrifuged at 50 x g for 2 min at 4°C. The wash step was repeated with a sample

being taken for the determination of Trypan Blue exclusion and cell counts prior to the final centrifugation. After the final wash, the pellet was resuspended in an appropriate volume of DMEM/F12 culture medium which had been equilibrated in an incubator at 37°C in a 5% $CO_2$ atmosphere. Two ml, usually containing 5 x $10^5$ Trypan Blue-excluding cells, was added to each collagen-coated 36 mm culture plate. After 2 hours the medium was replaced, following a single wash with Hank's buffered salts solution (HBSS), with 2 ml of DMEM/F12 (with additions as below plus test chemicals).

In many experiments, 2 mice were perfused in parallel, the second being started once the first mouse was on the first perfusion buffer. The first liver was washed and the cells liberated into ice-cold wash medium, those from the second liver being added when ready. This generally entailed a delay of less than 10 minutes for the first cells. The mixed cell suspension was then processed as just described.

### 4.2.4 Collagen coating of plates

Forty µl of a preparation of rat-tail collagen (Berry, *et al.*, 1991) was applied to each 36 mm plate, and spread with a glass rod to produce an even coating. This volume contained 51.6 µg protein (± SEM of 3.1). In an effort to optimise conditions for cell attachment, quantities down to 10 µl of a stock solution of rat-tail collagen, as well as various more dilute solutions in 2% acetic acid, were also tried. Although the absolute number of cells which attached appeared to be independent of the amount of collagen applied to the plates, the variation between cell counts in different fields was the least with the 40 µl stock collagen preparation, suggesting that the collagen coating was not as uniform with lesser quantities or the various dilutions.

### 4.2.5 Cell culture protocol

The cell culture method used was essentially that of Sawada *et al*, (1986) with some modification. The basic culture medium was a 1:1 mix of Dulbecco's Modified Eagle'e Medium and Hank's F12 medium (DMEM/F12), supplemented with 60 mg/l penicillin, 100 mg/l streptomycin sulphate, 1 g/l BSA (fatty acid free), 15 mM HEPES, 1.2 g/l $NaHCO_3$ , $10^{-7}$ M dexamethasone, and ITS at 1/100 the recommended concentration (see Section 4.3.3) giving 50 ng insulin/ml, 50 ng transferrin/ml, and 50 pg selenium/ml (see Appendix 1). Dexamethasone and microcystin-LR were dissolved in ethanol. This vehicle was shown to have no effect on cell growth at levels up to 2%, which was much higher than the usual medium content. DMEM/F12 includes relatively high levels of amino acids, and has been shown to support good growth of hepatocytes (Sawada, *et al.*, 1986). EGF was used at 20 ng/ml. In most experiments, medium was changed every 24 hours.

### 4.2.6 Cell counts

During cell isolation, cell viability was determined by Trypan Blue exclusion (Berry, *et al.*, 1991). One tenth volume of 2% Trypan Blue was added to a sample of the preparation, and clear versus blue cells quantified using a haemocytometer after a 5 minute incubation. Percent of cells viable and number of viable cells/ml were then calculated.

On plates, five random fields were counted using the 10x objective lens and a grid graticule in the eye-piece. Calculations were based on the number of flat, healthy cells in a full grid area of 1 $mm^2$, a 36 mm plate having an area of 1017.9$mm^2$.

### 4.2.7 Protein determination

Protein determinations were performed using the Bicinchonic Acid (BCA) Protein Assay Kit supplied by Pierce Ltd. Some modification was necessary in order that protein, DNA, and $^{3}$H-thymidine uptake could all be determined on the same cell digest from a single plate. Thus, after cells had been counted as described above, the plates were washed twice in phosphate buffered saline, 4°C, and fixed in 2 changes of 5% trichloroacetic acid (TCA) at 4°C (Raines and Ross, 1985). The last traces of TCA were aspirated and then the cells were dissolved in 1 ml of 0.33 M NaOH at 65°C for 30 minutes (complete dissolution was checked under the microscope). This solution was then frozen at −80°C until assayed. All samples and standards were assayed for protein in a constant sample volume of 250 µl of 0.33 M NaOH in a total assay volume of 2.25 ml against a water blank. A standard curve of absorbance at 560 nm (± SEM) versus µg BSA is shown in Figure 4.1.

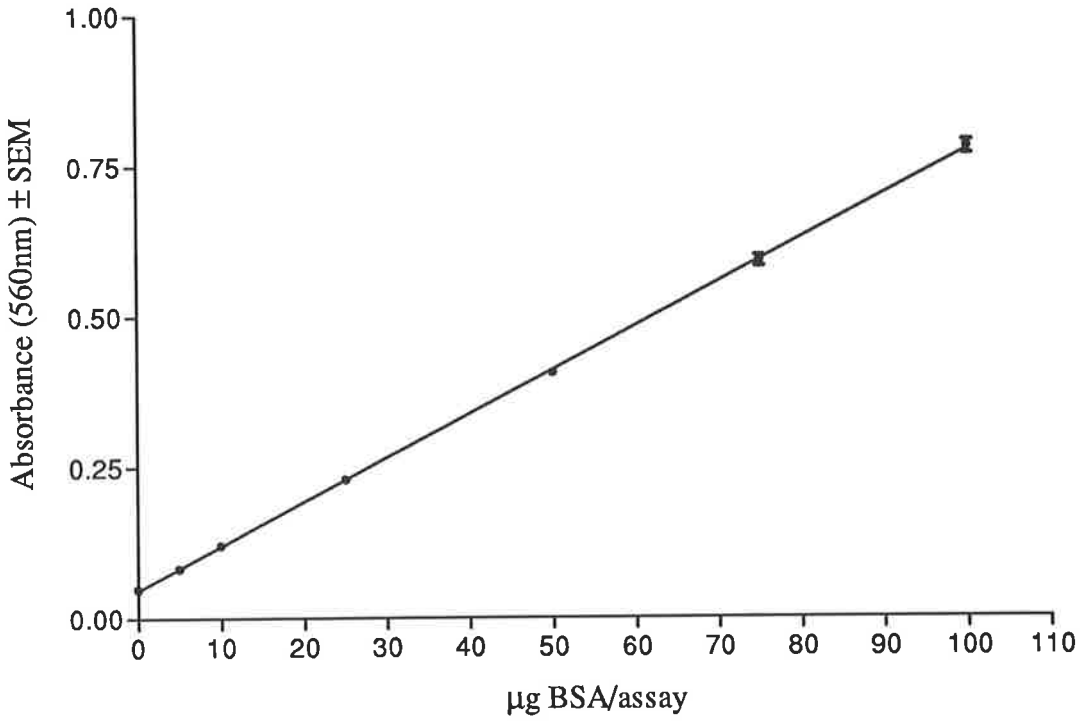



**Figure 4.1** - Protein assay standard curve. This curve was calculated from the duplicated standards from 5 separate runs, and has a slope of 0.007283 AU/µg BSA ± standard deviation of 0.00005955, Y-intercept of 0.04690 AU± 0.003092, and $r^2$ of 0.9955.

### 4.2.8 DNA determination

Determination of DNA was based on the method of Labarca and Paigen (1980), again with some modification. This method is sensitive to variations in both pH and ionic strength, and so the assay buffer (normally 0.05 M $NaPO_4$, 2.0 M NaCl, pH 7.4) was modified to accommodate the use of a sample volume of 250 µl of 0.33 M NaOH. Thus, 0.5 M $NaPO_4$, pH 7.2, was used in these studies, with 2.0 mM EDTA added as recommended by Labarca and Paigen (1980). This gave a solution of ionic strength $\mu$ = 2.63 M, rather than $\mu$ = 2.27 M for the original medium, which also allowed for increased dilution by the larger sample volume used here. Ten µl of a 200 µg/ml solution of Hoechst 33258 (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl-6-(1-methyl-4-

piperazyl)-benzimidazol•3HCl, dissolved in the original Labarca and Paigen assay buffer) was used as chromophore in a total assay volume of 2.26 ml. Figure 4.2 shows a standard curve of fluorescence (±SEM) versus µg DNA calculated from the duplicated standards from 4 separate runs, with calf thymus DNA dissolved in 0.33 M NaOH as standard.

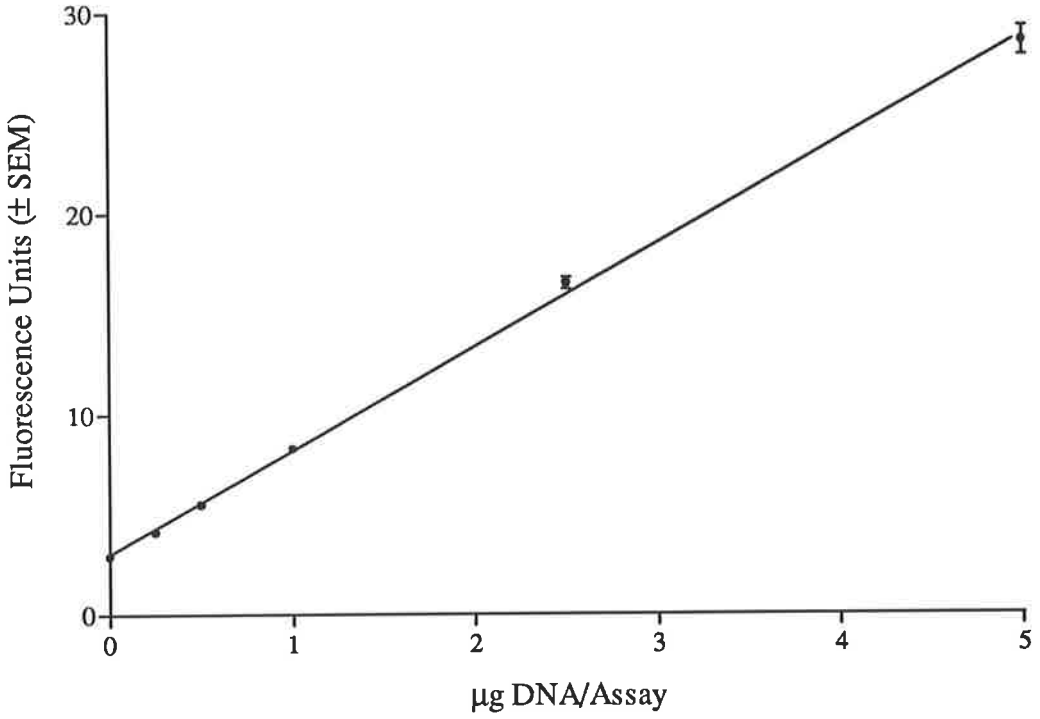



**Figure 4.2** - DNA assay standard curve. Fluorescence at 458 nm was monitored with excitation at 356 nm. Slope = 5.162 FU/µg DNA ± standard deviation of 0.07752, Y-intercept = 3.020 FU ± 0.1806, and $r^2 = 0.9897$.

### 4.2.9 Thymidine uptake

$^3$H-Thymidine uptake was usually determined using a sample volume 350 µl of the 0.33 M NaOH cell digest. This was added to 5 ml of "Ready Protein" scintillation fluid (Beckman). Samples were counted for 5 minutes per sample in a Beckman LS-3801 scintillation counter, and

dpm were calculated based upon a pre-defined quench curve determined using supplied quench standards. Dpm calculated for a series of vials containing a constant volume of $^3$H-thymidine but varying volumes of 0.33 M NaOH were constant. Vials containing 350 µl of 0.33 M NaOH plus scintillant had similar dpm to vials with scintillant alone.

### 4.2.10 Intra- and inter-experiment controls

For most experiments, a set of replicate plates were cell-counted at 2 hours' culture, immediately after the first medium change, and then placed on ice for a few minutes before $^3$H-thymidine was added as per the rest of the plates. This was done in order to estimate the non-specific binding of the $^3$H-thymidine and the efficiency of the wash process. After 5 minutes on ice, these plates were washed, fixed and dissolved in 0.33 M NaOH, as described above, as the "t=0" sample. These samples were used as the blanks for the thymidine uptake assay, and for inter-experiment standardisation for protein (where used), DNA, and cell counts.

Small changes in cellular protein were generally not measurable due to the larger variation introduced by the collagen added to plates, and so $^3$H-thymidine was generally standardised in terms of µg DNA or cell number.

Thymidine uptake, in terms of dpm/µg DNA, was standardised between experiments by normalisation to non-EGF stimulated, non-toxin treated controls.

In most experiments, toxins were added to non-EGF stimulated cells (see Section 7.4). To test for metabolic and proliferative competence of cell preparations, a set of replicates was always

included in which cells were stimulated by addition of 20 ng/ml EGF. These cells were morphologically different to unstimulated cells and incorporated 3-5 times as much $^{3}$H-thymidine over the course of an experiment (see Sections 7.3.1.1 and 7.4).

## 4.3 **Results**

### 4.3.1 Cell yield, viability and plating efficiency

Cell yield based on haemocytometer counts after Percoll centrifugation was generally about $10^{7}$ cells per mouse, of which (on average) 92% excluded Trypan Blue. Despite this level of "viability" however, only 30-50% of cells had stuck down and were starting to flatten after 2 hours, at the time of the "t=0" cell count. This suggests that Trypan Blue exclusion, while convenient, may not be a very accurate predictor of a cell's longer term survival in culture.

### 4.3.2 Growth of Swiss Albino and C3H/HeJ primary hepatocytes in culture

To examine the proliferative capabilities of cells derived from C3H/HeJ mice, they were compared with Swiss Albino-derived hepatocytes. Cells were isolated, and then cultured in the presence of ITS, as recommended by Sawada *et al*, (1986), to give final concentrations of 5 μg insulin/ml, 5 μg transferrin/ml and 5 ng selenium/ml. EGF was included at 20 ng/ml. Cells were counted in 3 fields on 5 replicate plates, and percent change in cell number was calculated (normalised to the number of cells at 24 hours). As can be seen in Figure 4.3, C3H/HeJ cell numbers increased 2-fold over the first 5 days before levelling off and finally beginning to decline

after 7.5 days. In contrast, Swiss Albino cell numbers declined rapidly over the first 2 days and then more slowly thereafter.

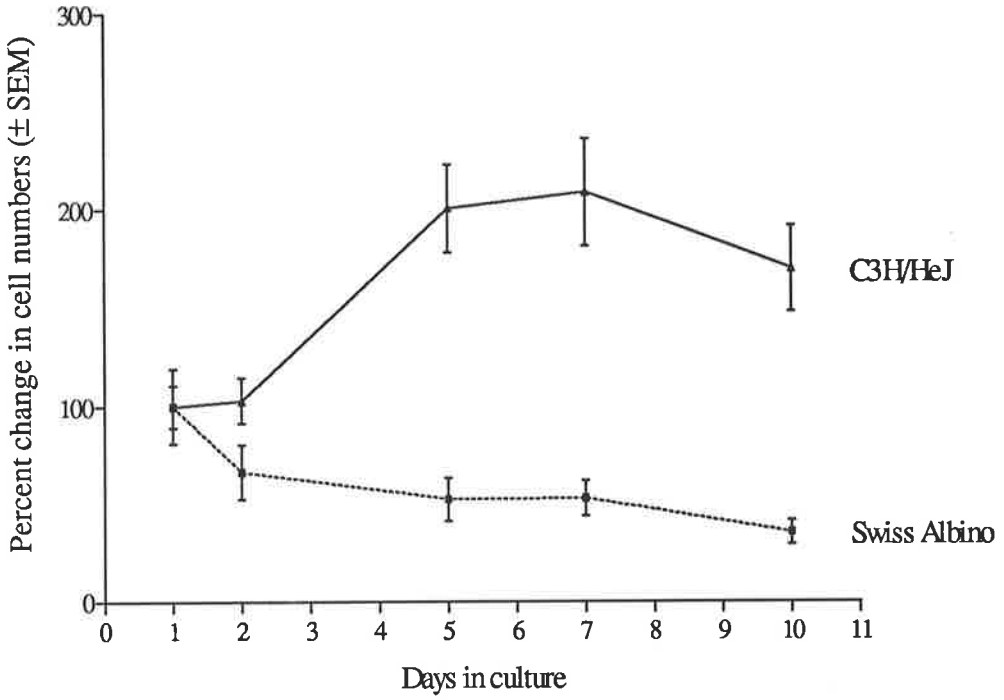



**Figure 4.3** - Growth of Swiss Albino and C3H/HeJ mouse hepatocytes in monolayer culture. Cells grown in DMEM/F12 medium containing EGF at 20 ng/ml, and ITS to give final concentrations of 5 µg insulin/ml, 5 µg transferrin/ml and 5 ng selenium/ml. Data points represent the average percent change in cell numbers in 3 fields on 5 replicate plates ± SEM normalised to numbers at day 1.

This 2-fold level of proliferation by C3H/HeJ-derived cells must be compared with greater than 5-fold reported by Sawada *et al*, (1986) when using C3H/N-derived cells. However, the animals used in this experiment were 6-7 weeks old whereas Sawada *et al.*, (1986) used mice of 5-6 weeks of age.

### 4.3.3 Effect of hormonal constituents of the culture medium on C3H/HeJ cell growth

To quantify the effect of the ITS medium supplement on hepatocyte growth, a preliminary experiment was carried out in which various concentrations of this additive were used. Cells isolated from a 5 week old male C3H/HeJ mouse were cultured in triplicate plates in DMEM/F12 medium plus dexamethasone ($10^{-7}$ M), with addition of ITS (at 4 concentrations equivalent to 0.0, 0.05, 0.5, and 5.0 µg insulin/ml), with and without EGF (20 ng/ml). Triplicate fields were counted on each plate, and percent change in cell number was calculated (normalised to the number of cells at 24 hours). The results are shown in Figures 4.4 (without EGF) and 4.5 (with EGF).

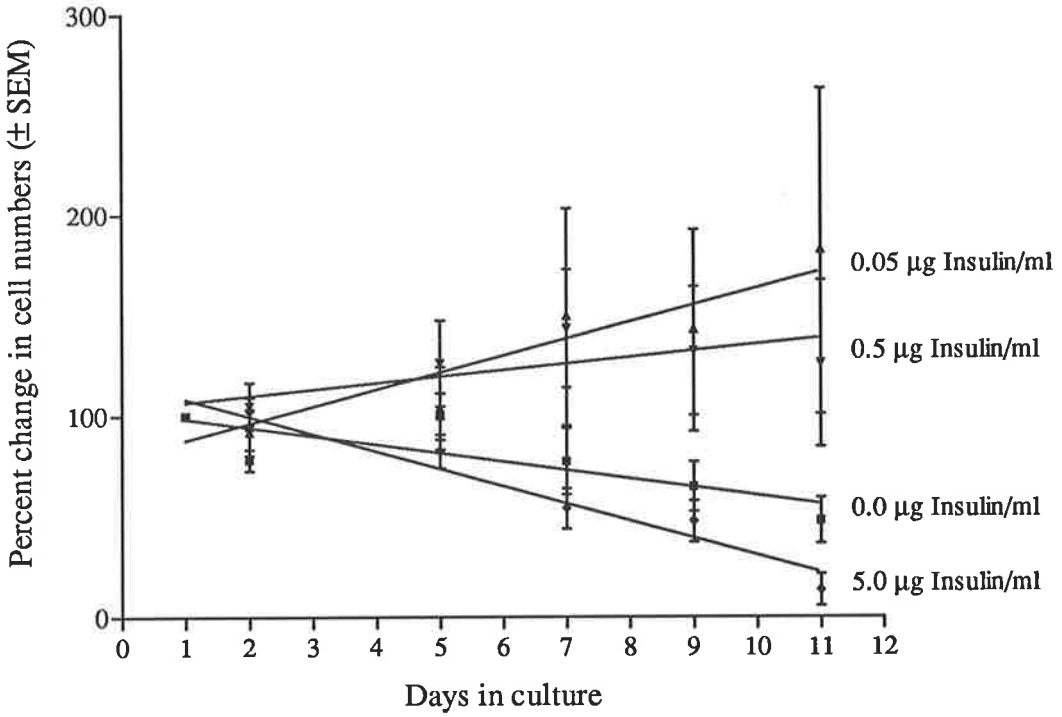



**Figure 4.4** - Effect of various dilutions of ITS on cell numbers without addition of EGF. ITS dilutions are shown as the effective insulin concentration, where ITS/1 gave final concentrations of 5 µg insulin/ml, 5 µg transferrin/ml and 5 ng selenium/ml. Differences in the rates of change of cell numbers between treatments was analysed by linear regression. None of the curves differed significantly from linearity by the Runs Test.

The percent change in cell number during culture in various concentrations of ITS was analysed by linear regression, as shown in Figure 4.4. None of the growth curves deviated significantly from linearity. The slopes of the 4 curves were −4.2 %/day ± standard deviation = 1.5, 8.4 ± 1.7, 3.2 ± 1.4, and −8.6 ± 1.0 for the 0.0, 0.05, 0.5, and 5.0 μg insulin/ml treatments respectively. Only the slope of the 0.5 μg insulin/ml curve was not significantly different from zero. These results suggest that the ITS supplement, at the concentration recommended by Sawada *et al*, (1986) and the manufacturers (Sigma), that is 5.0 μg insulin/ml, had a negative effect on cell proliferation, or survival, or both, in this system with non-EGF-stimulated cells. However, a concentration of 1% this level did not have this effect, cell numbers instead increasing on average by 8.4 %/day. Thus at this concentration, with cell death occurring at a rate of at least 4.2 %/day (cf. no ITS), proliferation must be occurring at an average rate of at least 12.6 %/day. The marked variability in the counts from the middle two ITS levels might suggest that not all cells are proliferating at an equal rate, that is, a sub-population of the total is actually responsible for this apparent (overall) growth rate.

To determine whether there is a synergistic effect between ITS and EGF on hepatocellular proliferation, the effect on cell numbers of the addition of EGF to hepatocytes cultured in medium containing various concentrations of ITS was quantified. A marked potentiation of the growth-promoting effect of ITS was seen in the presence of EGF, as shown in Figure 4.5.

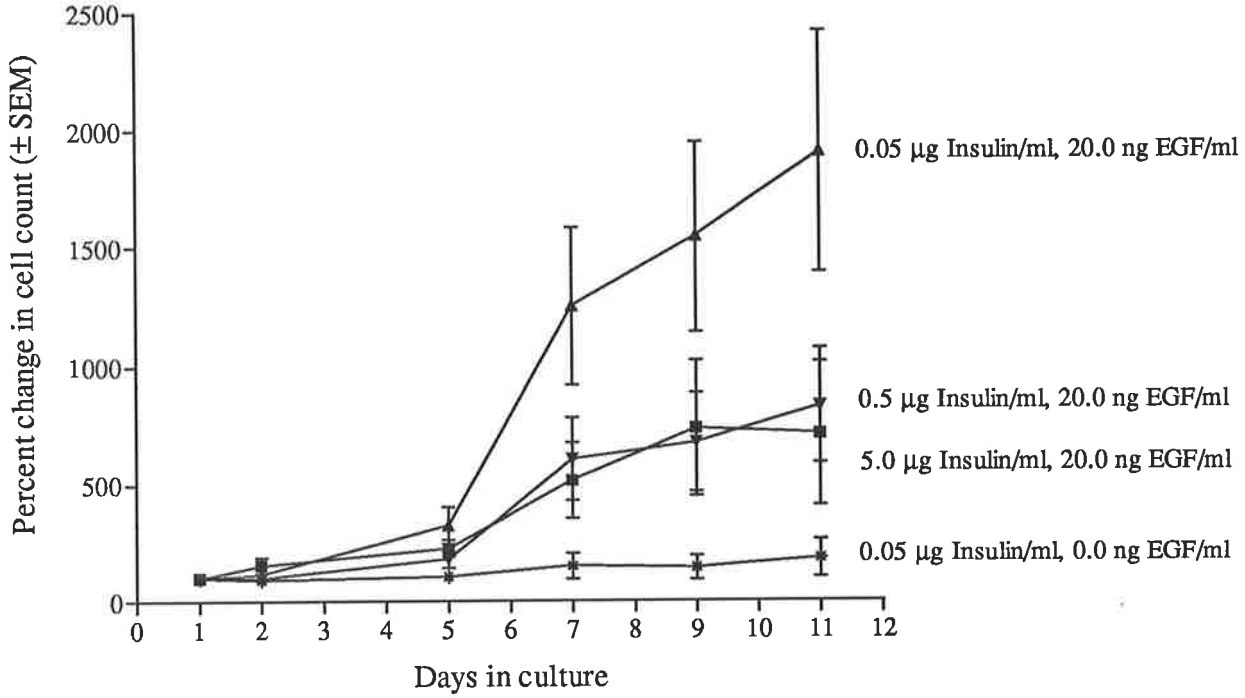



**Figure 4.5** - Effect of 20 ng/ml EGF and various ITS concentrations on cell numbers. The effective insulin concentrations are shown. ITS added at the recommended rate (ITS/1) would give final concentrations of 5 µg insulin/ml, 5 µg transferrin/ml and 5 ng selenium/ml. C3H/HeJ hepatocytes were grown in DMEM/F12 medium, the various ITS concentrations, and with or without EGF. Three fields on triplicate plates per treatment were counted at the times shown. Error bars are ± SEM.

Clearly the same effect is seen upon decreasing the ITS concentration to 1% its recommended concentration, and so in subsequent experiments this level was used, designated ITS/100 for simplicity. Furthermore, growth in the higher two ITS concentrations now compares well with the 5-fold increase by 7 days reported by Sawada *et al*, (1986). This indicates that cells from younger mice are more likely to proliferate, or that those which do, do so at a faster rate.

### 4.3.4 Effect of Epidermal Growth Factor (EGF) on C3H/HeJ cell growth

The addition of 20 ng/ml EGF to the culture medium clearly had a marked effect on cell numbers, as seen in Figure 4.5. To determine whether the effects of EGF on cell growth were

concentration dependent, the effect of various concentrations of EGF on the synthesis of DNA was examined. Hepatocytes from two 5 week old C3H/HeJ male mice were grown in quadruplicate plates containing DMEM/F12 plus dexamethasone, ITS/100, $^{3}$H-thymidine (0.5 μCi/ml), and EGF at concentrations of 0.0, 0.3, 0.6, 1.3, 2.5, 5.0, 10.0, or 20.0 ng/ml. Medium was changed every 24 hours. The cells were harvested at 65 hours by dissolution, after PBS wash and TCA fixation, in 0.33 M NaOH (65°C, 30 minutes), and the DNA content and radioactivity analysed as previously described. The relationship between EGF concentration and radioactivity incorporated, in terms of dpm/μg DNA, is shown in Figure 4.6.

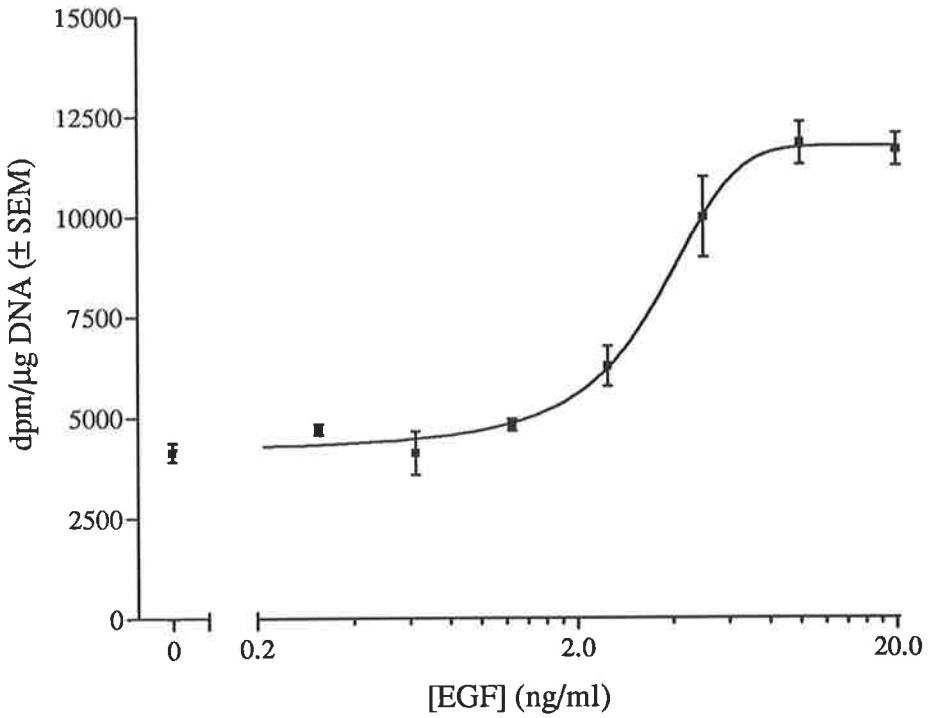



**Figure 4.6** - Dose response curve for 3H-thymidine incorporation/μg DNA with increasing concentrations of EGF between 0.2 and 20.0 ng EGF/ml. C3H/HeJ cells grown in DMEM/F12 medium containing ITS supplement at 1% the recommended level. Data points are the average of triplicate plates ± SEM.

EGF had little effect below 1 ng/ml, and its effect was maximal at 10 ng/ml. This corresponds well with the findings of others, for example Tomomura *et al*, (1987), who also found an upper plateau at 10 ng/ml when studying DNA synthesis in response to EGF stimulation in Sprague-Dawley rat hepatocytes.

### 4.4 Discussion

Based on the findings of the various experiments described in this Chapter, the culture conditions to be used in subsequent experiments were defined as follows. Hepatocytes from 5 week old male C3H/HeJ mice were used. These were isolated using retrograde collagenase perfusion and Percoll isodensity centrifugation. They were cultured on collagen coated plastic tissue culture plates in DMEM/F12 medium (pH 7.4) supplemented with 60 mg/l penicillin, 100 mg/l streptomycin sulphate, 1 g/l BSA (fatty acid free), 15 mM HEPES, 1.2 g/l NaHCO3 , $10^{-7}$ M dexamethasone, and ITS at 1/100 the recommended concentration giving 50 ng insulin/ml, 50 ng transferrin/ml, and 50 pg selenium/ml. When used, EGF was added at a concentration of 20 ng/ml. Culture plates were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$.

# CHAPTER 5

# UPTAKE OF MICROCYSTIN-LR BY PRIMARY HEPATOCYTES IN CULTURE

## 5.1 Introduction

The difficulty inherent in trying to monitor changes caused by microcystin on the proliferation rates of growing cultured hepatocytes, when those cells are rapidly losing the ability to take up the toxin, has lead to a paucity of such studies using this toxin. This is despite the obvious role which microcystin might play in elucidating the relative roles of the various protein phosphatases in cell cycle regulating events.

### 5.1.1 Previous studies

The following difficulties apply to the study of microcystin in proliferating cells: First, all of the readily available pure microcystins are hydrophilic and so require active uptake by a hepatocyte transporter related to those for bile acids (Falconer, *et al.*, 1981; Thompson, *et al.*, 1988). The lack of susceptibility to microcystin-LR of a number of other cell types, including mouse thymocytes (Falconer and Runnegar, 1987b), mammary alveolar cells, 3T3 fibroblasts (Falconer and Runnegar, 1987a), NIH-3T3 mouse fibroblasts and a human neuroblastoma cell line (Eriksson, *et al.*, 1990a) appears to confirm this. Second, none of the readily available hepatocyte-derived immortal cell-lines, for example Hep G2 (Eriksson, *et al.*, 1990a), express the bile acid type transporters required for microcystin uptake. And third, primary hepatocytes also apparently stop taking up bile acids after a few days in culture (Follmann, *et al.*, 1990). Intestinal enterocytes are susceptible to microcystin toxicity (Falconer, *et al.*, 1992), but perhaps because

culture conditions are less well defined for these cells than for hepatocytes, they have not been used in culture with microcystins.

Mellgren *et al*, (1993) appear to be the only group so far to have studied the effects of microcystin on DNA synthesis in growing hepatocytes. They found that when EGF-stimulated hepatocytes were treated with toxin from the 44$^{th}$ to the 55$^{th}$ hours in culture and then pulse-labelled with $^{3}$H-thymidine for a further hour, okadaic acid had inhibited DNA synthesis, whereas microcystin-LR had not, even when the cell was clearly morphologically effected by this toxin. They suggest that this difference is due to the selective activity of these compounds towards the different protein phosphatases, PP2A being more strongly inhibited by okadaic acid than PP1 (Cohen, 1991), whereas microcystin-LR is equipotent against both (MacKintosh, *et al.*, 1990). The PP1-specific inhibitors I-1 and I-2 did not inhibit DNA synthesis either, again suggesting that it is PP2A inhibition causing this observed inhibition of DNA synthesis by okadaic acid.

Mellgren *et al,* (1993) also report that uptake of microcystin-LR was very poor by 44 hours in culture, when they exposed their cells to the toxin. By directly injecting microcystin into the cells, they found that an internal concentration of about 1 μM was needed to have a 50% chance of causing visible morphological changes in a cell. However, the external concentration necessary in the medium to cause this degree of disruption in the cell population was 2.2 μM, which does not seem consistent with active transport, although the possibility of enhanced re-export of the toxin was not addressed by these authors. This latter possibility does not seem very likely in the light of evidence from various studies showing loss of bile acid transport in cultured hepatocytes.

Furthermore, Runnegar *et al*, (1991), in a study using radio-labelled microcystin-YM to follow uptake of the toxin by freshly isolated rat hepatocytes with time, found that the cells actively imported the microcystin to achieve, from a medium concentration of 17 nM, an internal concentration of 0.9 μM by 60 minutes exposure time, and, in fact, continued to do so despite blebbing of the plasma membrane. The coincidence of these morphologically active internal concentrations, despite the difference in culture-age of the cells, also suggests the loss of active uptake rather than gain of enhanced export in the older cells because otherwise the amount injected by Mellgren *et al* would have needed to be higher than the figure found by Runnegar *et al* in order to overcome the rate of export.

However, another possibility altogether was suggested by Follmann *et al*, (1990). These authors reported that bile acid (cholate, bumetanide and taucholate) uptake could be restored in monolayer cultured primary hepatocytes after they were resuspended by trypsinisation, and that if the cells were grown on Transwell® membranes, so that their basolateral surface was accessible to the medium, then bile acid uptake remained intact. These results suggest that bile acid transporter expression was not lost, but rather that the transporter is "hidden" from the medium on the cell's underside. Since hepatocytes are known to be polar in their interactions with their surroundings, this possibility cannot be discounted. To date however, the sensitivity to microcystins of cells grown on Transwell® membranes has not been investigated.

Dimethyl sulfoxide (DMSO) at a level of 2% in culture medium has been proposed as a means of maintaining the expression of differentiated characteristics by hepatocytes in primary culture (Isom, *et al.*, 1985). Levels of mRNA for albumin and other proteins characteristic of differentiated hepatocytes were found to still be significant after 40 days in culture (Isom, *et al.*, 1987), although this appeared to be due more to a stabilisation of mRNA than to maintenance of

transcription. Follmann *et al*, (1990) reported that DMSO (2%) did not maintain bile acid transport in rat hepatocytes cultured in their system. However, microcystin uptake appears to be facilitated by a number of different anion transporters, not all of them well characterised (Runnegar, *et al.*, 1995b), and so lack of uptake of some bile acids may not be indicative of a total lack of uptake of microcystin.

Microcystin-LR is a hydrophilic molecule which is obviously sparingly membrane permeant, as already discussed. The situation is less clear for some of the less hydrophilic variants, for example, microcystin-YM (tyrosine, methionine). In the *in vivo* experiment described in Chapter 3, in which non-hepatic sites were targeted, the *Microcystis* extract fed to mice included material from Malpas Dam, NSW, Australia, because it had previously been shown to contain microcystin-YM as a major component (Botes, *et al.*, 1985; Falconer, *et al.*, 1986). An extract of Malpas Dam bloom material was therefore used to challenge cells which had lost sensitivity to microcystin-LR.

The above considerations lead to the formulation of a preliminary series of experiments aimed at trying to maintain sensitivity to microcystin-LR or Malpas extract in C3H/HeJ hepatocytes in culture until the period of maximum cellular proliferation, that is, 5 to 7 days.

## 5.2 **Materials and methods**

### 5.2.1 Malpas Dam *Microcystis* extract

Oven dried Malpas Dam *Microcystis* material (50 mg) was suspended in Hank's Buffered Salts Solution (HBSS) at 10 mg/ml, sonicated at 80W for 2 minutes on ice with a probe sonicator and then frozen overnight. After thawing, the preparation was centrifuged at 3000 rpm in a bench-top

centrifuge and the supernatant applied to a pre-conditioned SEP-PAK $tC_{18}$ PLUS (Waters) SPE cartridge (400mg). This was washed with MQ.$H_2O$, followed by 20% methanol in MQ.$H_2O$, before the microcystins were eluted in 100% methanol. The methanol was evaporated under a stream of $N_2$ at 40°C and then the residue redissolved in 5 ml of HBSS. This preparation was added to the medium of overnight cultures of hepatocytes in the range 2.0 - 200 µl/ml medium and the cellular response observed. It was found that 2 µl/ml gave a response, in terms of rate and degree of rounding and loss of contact with the substrate, most similar to 1 µg/ml microcystin-LR added to parallel plates. Therefore, 2 and 20 µl/ml were used in the subsequent challenge experiments. Cells were cultured in standard DMEM/F12 culture medium plus dexamethasone ($10^{-7}$ M), ITS/100 and EGF (20 ng/ml), and treated with the extract at 5 days. Morphological effects were then noted over the next 24 hours.

### 5.2.2 Dimethyl sulfoxide

Dimethyl sulfoxide (DMSO, 2%) was added to the standard DMEM/F12 culture medium plus dexamethasone ($10^{-7}$ M), ITS/100 and EGF (20 ng/ml), either at 24 hours with the first medium change and maintained at subsequent medium changes, or it was added a few minutes before toxin challenge. Use of both these regimes was used to determine whether any effect seen was due to maintenance of transporter expression, or simply to membrane permeabilisation. Cultures were challenged at 5 days with 1 and 10 µg/ml microcystin-LR, and morphological effects noted for the following 24 hours.

### 5.2.3 Falcon® membranes

Microporous Falcon® membranes were used suspended in the wells provided (Becton Dickinson, USA). The 23 mm diameter membranes were described by the manufacturer as having 1.0 μm diameter pores at a density of $1.6 \times 10^6$ pores/cm$^2$. To check that the addition of collagen to the surface of these membranes would not hamper microcystin diffusion, 4.2 μg microcystin-LR (equivalent to 1 μg/ml in the total well plus membrane compartment volume) was added to the medium in the bottom well of the apparatus. After 7 hours incubation, 1.5 ml was removed from both above and below the membrane and added to separate 36 mm plates of cells which had been cultured for 24 hours. Both samples had a similar rate of onset and degree of toxicity as seen with 1 μg/ml microcystin-LR. Cells were seeded onto the collagen coated membranes at a cell density equivalent to that used on the usual 36 mm culture plates and cultured in standard DMEM/F12 culture medium plus dexamethasone ($10^{-7}$ M), ITS/100 and EGF (20 ng/ml). Toxin was added to the lower compartment after 5 days' culture, and then cells were observed for 24 hours.

### 5.2.4 Synthesis of tritiated microcystin

In order to monitor the uptake of low amounts of toxin, microcystin-LR was tritiated by reduction of the N-methyldehydroalanine moiety with $^3$H-$NaBH_4$ (Meriluoto, *et al.*, 1990; Williams, *et al.*, 1995). Specifically, 200 μg of microcystin-LR (Sigma) dissolved in 50 μl MQ.$H_2O$ was reacted with 114 μg $^3$H-$NaBH_4$ (Dupont NEN Ltd. Cat# NET 023 - 1000 mCi/mmol.) added in 200 μl propan-2-ol (molar ratio of $^3$H-$NaBH_4$ to microcystin-LR was 12.5:1) overnight at room temperature. The reaction was stopped by dropwise addition of 10%

acetic acid, pH 4, until bubbles no longer formed. This was diluted with 7 ml MQ.$H_2O$ and then applied to a conditioned SEP-PAK $tC_{18}$ PLUS (Waters) SPE cartridge (400mg). After washing with 10 ml MQ.$H_2O$ the product was eluted in 6 ml methanol. The methanol was dried off under a stream of $N_2$ at 40°C. The residue was taken up in 1 ml methanol and this solution was then purified further by the HPLC cleanup procedure described below.

Using a Waters μBondapak (10 μm particle size) 7.8 x 300 mm HPLC column with a mobile phase consisting of 65% methanol: 35% MQ.$H_2O$ containing 0.05% trifluoroacetic acid (TFA) and a flow rate of 2 ml/minute, reduction of microcystin-LR by the above method moved the major peak from an elution time of 13.8 minutes to 14.8 minutes. None of the 13.8 minute peak was detectable in the trace of the reduced product. This second peak corresponded to the peak of radioactivity in fractions collected across this region. Separation of the two enantiomers in the reduced product was not possible with this system but this was not considered critical to the outcome of the proposed experiment since they have very similar toxicity (Meriluoto *et al*, 1990). Twenty-five μl aliquots of the reaction product solution were sequentially processed, the 14.8 minute peaks being collected and pooled. The methanol was dried off under $N_2$, and then the water and TFA removed by lyophilisation. Two hundred and twenty-two μg of the reaction product was collected with a specific activity of 36.7μCi/μmole.

### 5.2.5 $^3$H-Microcystin-LR uptake assay

Hepatocytes were cultured in standard DMEM/F12 culture medium plus dexamethasone ($10^{-7}$ M), ITS/100 and EGF (20 ng/ml), with or without 2% DMSO for the duration of the culture period. Medium was changed at 2, 24 and 72 hours. Microcystin uptake after 2 and 5 days in

culture was assayed by addition of 1.1 μg/ml $^{3}$H-microcystin-LR to the culture medium in plates equilibrated at either 37°C or 0°C. After a 2 hour incubation the cells were washed (2x HBSS) and then detached from the plate by treatment with 0.2% collagenase in HBSS for 10 minutes at 37°C. The cell suspension was collected into an eppendorf tube and centrifuged at 8,000 rpm for 20 seconds. The cells, resuspended in 50 μl of buffer, were then centrifuged through a 100 μl layer of Silicon oil into 50 μl of 3M KOH (Barritt, *et al.*, 1981; Runnegar, *et al.*, 1991). The layers were then sequentially removed, and the radioactivity in the bottom layer was determined by liquid scintillation counting. For the day 2 sample, three replicate plates per treatment were processed, and for the day 5 sample, two plates were pooled for each of the three replicates per treatment. Triplicate average radioactivity in samples treated with $^{3}$H-microcystin-LR at 0°C was subtracted from that found in samples treated at 37°C to give an estimate of active uptake from each culture treatment group. Time- and culture treatment-matched plates were also assayed for DNA content, as previously described.

## 5.3 Results

### 5.3.1 Loss of hepatocyte sensitivity to microcystin-LR in culture

To estimate the rate of loss of microcystin uptake in cultured hepatocytes in this culture system, cells were exposed to 100 nM microcystin-LR from 0-18 hours, 18-42 hours, and 42-96 hours. Cells were counted (5 x 1 mm$^{2}$ fields per plate) before and after treatment and the percentage remaining calculated. From the studies of Runnegar *et al*, (1991), and Mellgren *et al*, (1993), an internal concentration of 1 μM appears to be required for morphological effects to be induced by microcystin. Therefore, a level of 100 nM microcystin-LR was used which would be

low enough that active uptake would be a prerequisite for cell death, but also high enough so that toxin availability would not be limiting if the rate of uptake was not constant between cells. The results are shown in Figure 5.1.

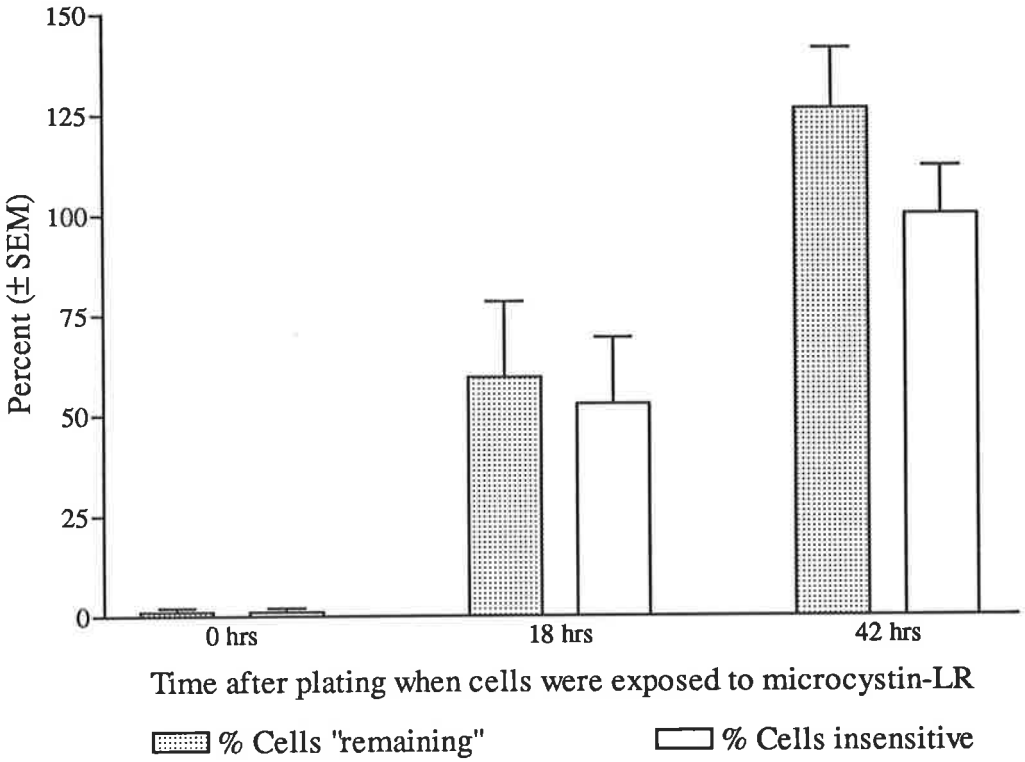



**Figure 5.1** - Loss of hepatocyte sensitivity to 100nM microcystin-LR as shown by cells surviving in culture in the presence of this concentration of toxin. Microcystin was added at 0, 18 or 42 hours culture time and the change in cell numbers was determined at 18, 42 and 96 hours respectively. Data are shown as cells remaining at the second time point as a percentage of those present before the addition of toxin, and also as a calculated estimate of the percentage of cells which were therefore insensitive to microcystin-LR at the time of addition allowing for subsequent proliferation in surviving cells.

At 18 hours, only 0.9% of cells remained, indicating greater than 99% of cells retained active microcystin transport during this period and hence were adversely affected (and also, incidentally, that the cell isolation procedure produced at least this level of purity of parenchymal hepatocytes). Furthermore, toxin concentration was high enough that it was not sequestered in dying cells to the extent that cells with slow uptake were spared a lethal dose. Of cells treated at 18 hours and

counted at 42 hours, 59% of cells remained. Of cells treated at 42 hours and counted at 96 hours, 126% remained. Since new cell production was estimated in Section 4.3.3 to be about 13% per day in these culture conditions, a correction equal to this must be applied to the above figures. Therefore, at 0 hours 98.9% of cells were killed (1.1% insensitive), 18 hours 48% of cells were killed (52% insensitive), and at 42 hours 0% of cells were killed (100% insensitive). This calculation assumes no inhibitory or stimulatory effect of the toxin on the remaining cells, and so can only be taken as an estimate.

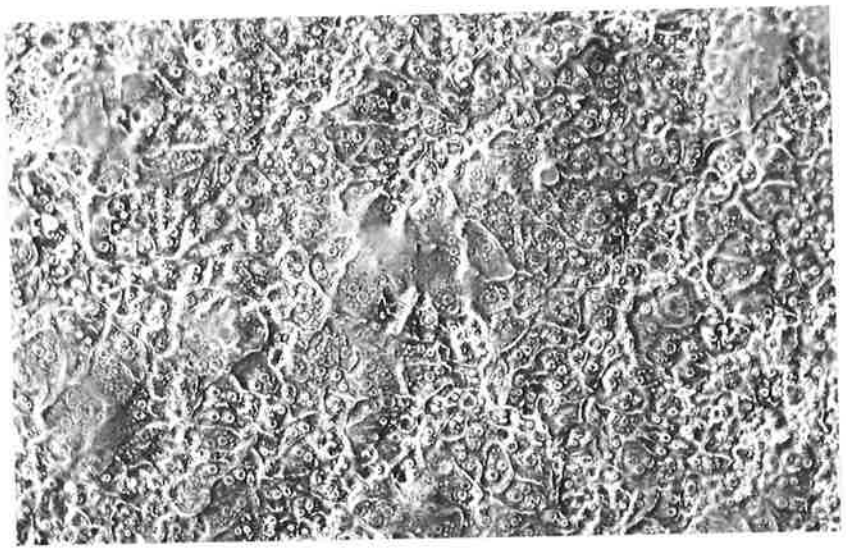
### 5.3.2 Attempts to retain hepatocyte sensitivity to microcystin-LR - DMSO, Falcon Membranes, and Malpas Dam *Microcystis* extract

Hepatocytes cultured for 5 days in the presence of 2% DMSO, but not those to which DMSO had been added just prior to microcystin-LR addition, were found to retain some sensitivity to 10 µg/ml microcystin-LR. Treatment with this concentration of toxin for up to 20 hours at this time resulted in most cells rounding up and many completely losing contact with the substrate (Figure 5.2c). Note that this concentration of toxin would be lethal to freshly isolated cells within 60 minutes (Figure 5.2a,b), so the rate of uptake is drastically reduced. However, in contrast, hepatocytes grown in standard culture medium for 5 days and then exposed to this concentration of toxin did not respond at all during a 24 hour observation period. In fact, one cell was observed to progress through mitosis with this level of microcystin present (see Figure 5.3), a process not likely to occur if a toxin known to disrupt the cytokeratin was present intracellularly. Exposure at 5 days to 40µl/ml Malpas Dam *Microcystis* extract induced a toxic response in 10-20% of cells after 20 hours. However, the morphology of the dead cells was different to that seen in microcystin-treated cells (Figure 5.2d), and so it was likely that cell death was due to some other

cytotoxin(s) normally masked by microcystin. Growth on Falcon® membranes had no effect on the sensitivity of hepatocytes when compared with controls, as shown by the retention of normal morphology when treated at 5 days culture time with 10 mg/ml microcystin-LR for 20 hours. Therefore, only continuous culture in 2% DMSO showed any likelihood of preserving hepatocyte sensitivity to microcystin-LR and this was explored further in uptake studies using tritiated microcystin-LR.

**Figure 5.2** - Effect of culture conditions on the morphological response of hepatocytes to exposure to 1.0 or 10 μM microcystin-LR

(a) C3H/HeJ mouse hepatocytes in culture after 24 hours. Culture medium (DMEM/F12) contained ITS/100 and 20 ng EGF/ml. Before addition of microcystin-LR. (Magnification 100x).



(b) Same cells as in (a) above after 2.5 hours exposure to 1.0 μg/ml microcystin-LR. Most cells are rounded and have lost contact with the substrate, although a few still appear to be unaffected. Note that the toxin-affected (rounded) cells are spherical, and in some cases smooth regions are developing in their membranes. (Magnification 200x).

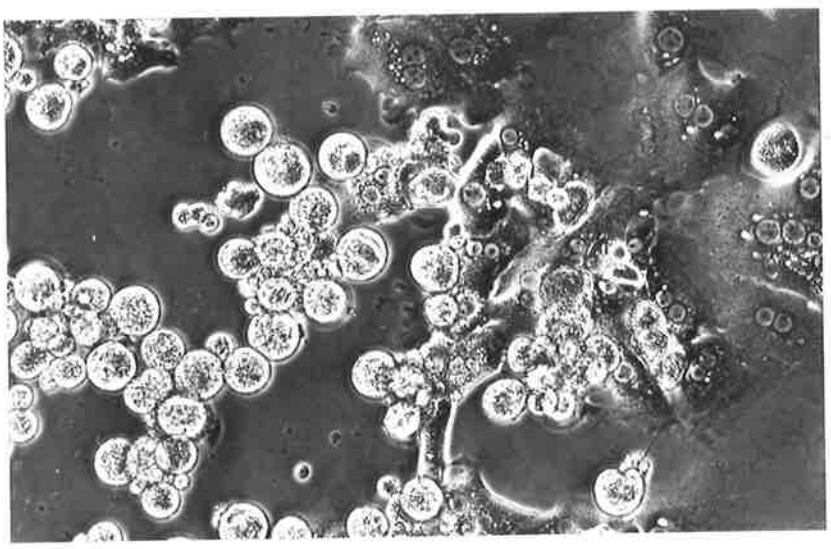
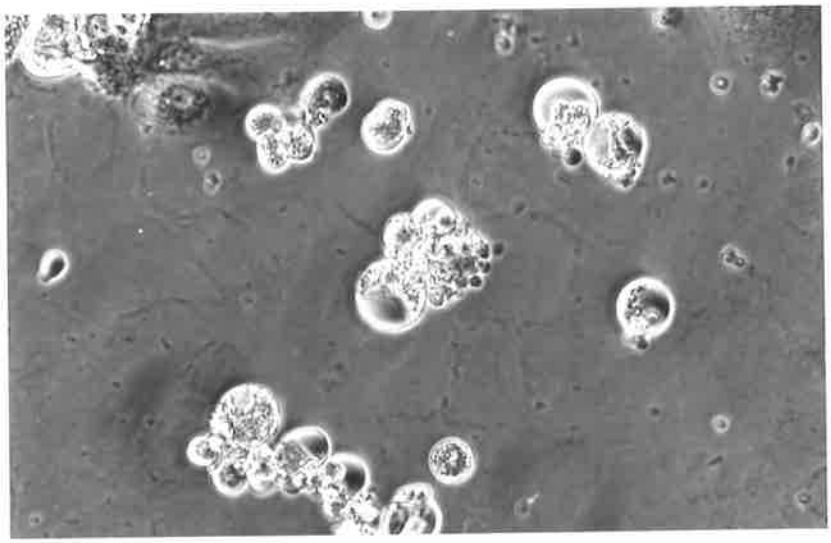


**Figure 5.2** continued.

(c) C3H/HeJ mouse hepatocytes grown for 5 days in standard culture medium supplemented with 2% DMSO, and then exposed to 10 µg/ml microcystin-LR for 20 hours. Cells are rounded and have lost contact with the substrate. Toxin-affected cells are spherical and have developed regions of smooth membrane. (Magnification 200x).



(d) Hepatocytes grown for 5 days in standard culture medium and then exposed to Malpas Dam Microcystis extract at a dilution (20 µl/ml) previously found to be toxic to 24 hour old cultures. Many cells have rounded up and lost contact with the substrate. However, the toxin-effected cells are often irregular in shape do not exhibit any smoothing of the membrane. It was concluded from these observations that the toxic agent acting on these cells was probably not a microcystin. (Magnification 200x)

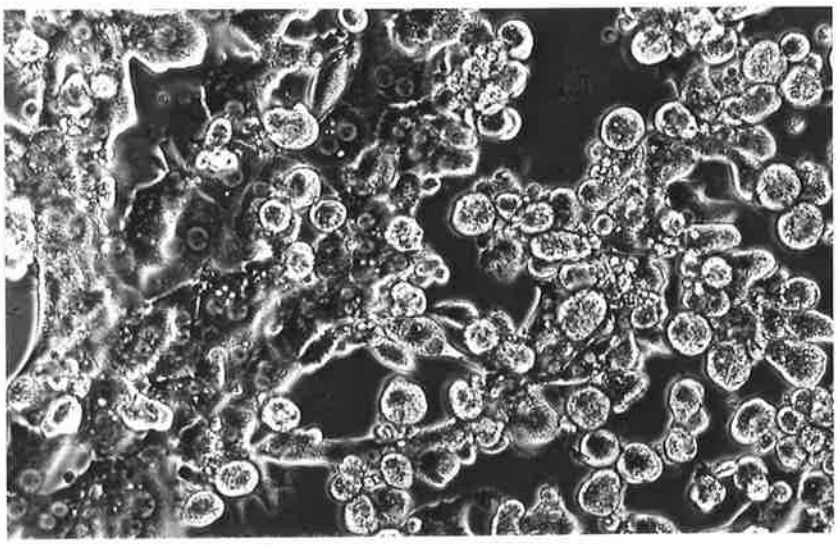
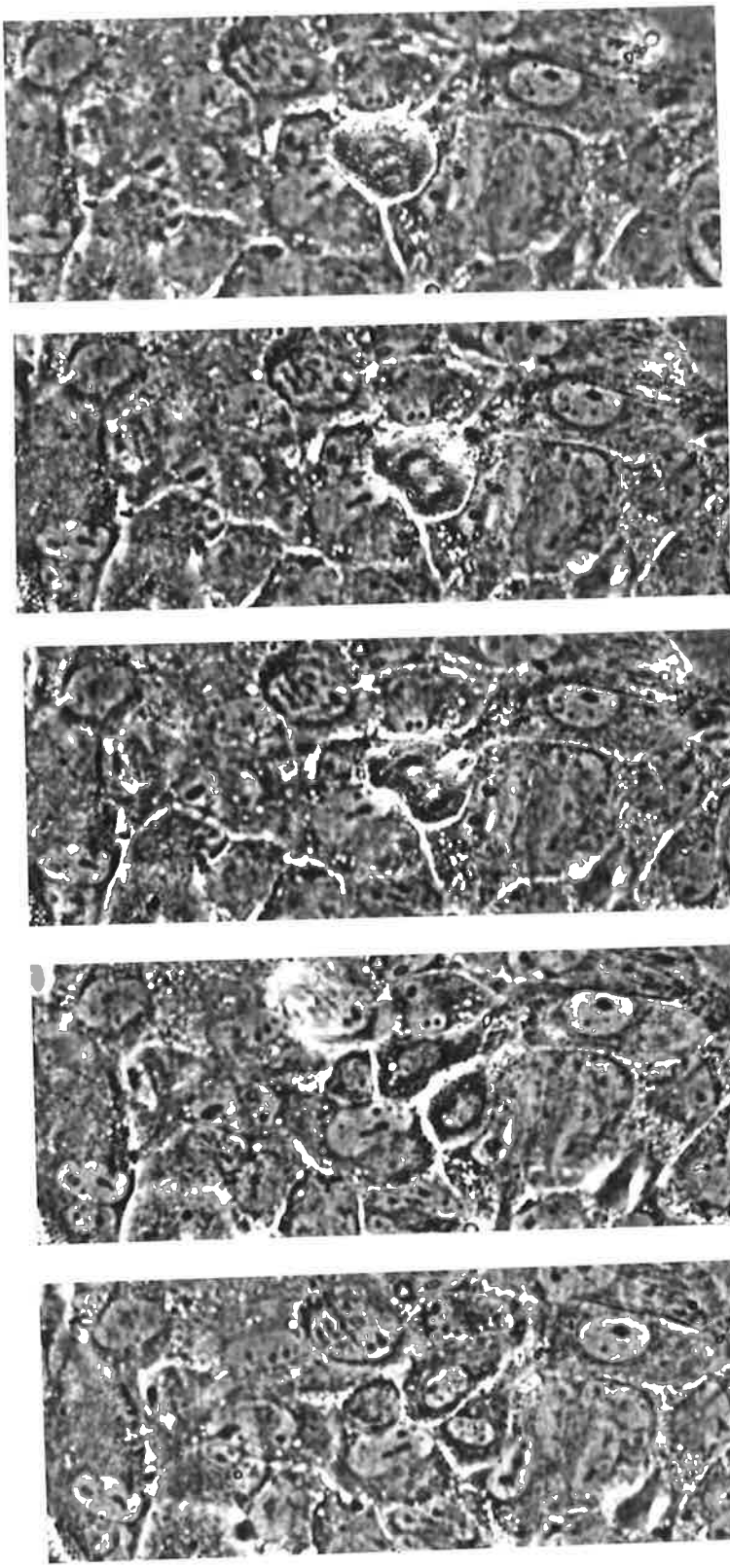


**Figure 5.3** - Insensitivity of a mitotic hepatocyte after five days in culture to the presence of 10 µM microcystin-LR as demonstrated by continued passage through mitosis. The time elapsed between the first (early anaphase) and last (division complete) photomicrographs was approximately 90 minutes which is consistent with observations made with time-lapse photography of rat hepatocytes in monoculture (Sattler, *et al.*, 1988). (Magnification 400x)



(a) Early anaphase.

(b) Late anaphase

(c) Telophase

(d) Late cytokinesis

(e) Cell division complete.

5.3.3 Comparison of $^{3}$H-microcystin-LR uptake in DMSO treated and untreated hepatocytes.

Mouse hepatocytes were cultured with or without 2% DMSO as described in the Materials and Methods section of this chapter. After 2 and 5 days in culture, triplicate plates were incubated with 1.1 μg/ml $^{3}$H-microcystin-LR at either 0°C or 37°C for 2 hours before the cells were harvested, as described. Morphological distortion was observed to be minimal in response to toxin treatment with very few cells rounding up or detaching from the plate in any of the treatments over this 2 hour period. Active uptake was calculated from the difference between the radioactivity found at the 2 incubation temperatures, and expressed in terms of both ng $^{3}$H-microcystin-LR /μg DNA and pmole $^{3}$H-microcystin-LR/ $10^{6}$ cells, the latter based on a value of 17 μg DNA/ $10^{6}$ cells (Berry *et al*, 1991). These results are shown in Table 5.1.

| | | DMEM | DMSO |
|---|---|---|---|
| μg DNA/plate, ± SD | 2 days | 5.7 ± 0.43 | 5.4 ± 0.24 |
| | 5 days | 24.7 ± 1.49 | 3.2 ± 0.19 |
| ng $^{3}$H-microcystin-LR /μg DNA, ± SD | 2 days | 0.56 ± 0.28 | 0.45 ± 0.25 |
| | 5 days | 0.016 ± 0.005 | 0.034 ± 0.015 |
| pmole $^{3}$H-microcystin-LR / $10^{6}$ cells, ± SD | 2 days | 9.4 ± 4.8 | 7.6 ± 4.2 |
| | 5 days | 0.26 ± 0.08 | 0.58 ± 0.25 |

**Table 5.1** - Changes with culture time of μg DNA per plate and $^{3}$H-microcystin-LR uptake in cells cultured in medium with or without 2% DMSO. Hepatocellular uptake of $^{3}$H-microcystin-LR at 2°C (average of 3 plates) was subtracted from uptake by triplicate plates at 37°C (incubation time 2 hours in each case). DNA was determined on a separate set of plates. Data are presented as means ± standard deviation.

As can be seen from the DNA estimates shown in Table 5.1, cells grown in DMEM/F12 plus EGF grew well over five days, whereas those grown in the same conditions but with the addition

of 2% DMSO failed to do so after day 2. This may be due to the "differentiating" effect of DMSO on hepatocytes (Isom *et al*, 1985, 1987) which might be expected to inhibit proliferation.

Neither culture medium enabled cells to retain significant ability to take up microcystin beyond the second day in culture. The 2-fold greater uptake by DMSO treated cells at five days still signified an approximately fifteen-fold loss from day two.

### 5.4 Discussion

Mouse hepatocytes in culture were found to lose sensitivity to microcystin-LR over a period of a few days. Although 99% of cells were sensitive after 2 hours in culture, this number was reduced to 48% by 18 hours and to 0% by 42 hours. However, it is not until between 5 and 7 days that cell proliferation is maximal (see Figure 4.5), by which time microcystin sensitivity would be essentially non-existent. Therefore, a number of methods were tried in an attempt to preserve the sensitivity of primary hepatocytes in culture to microcystin for longer than a few days. Only continuous culture in 2% DMSO showed any promise in preliminary experiments, and so this was explored further by measurement of uptake of tritiated microcystin after 2 and 5 days in culture.

The intracellular concentration of $^{3}$H-microcystin at 2 days, based on 9.4 pmole $^{3}$H-microcystin/$10^{6}$ cells and an intracellular volume of 11.2 µl/$10^{6}$ cells (derived from information in Berry *et al*, 1991), was about 0.8 µM. This seems unexpectedly high given that Mellgren *et al*, (1993) and Runnegar *et al*, (1991) both found that on average an intracellular microcystin concentration of 1 µM gave rise to morphological deformation characteristic of toxicity. Also,

Meriluoto *et al,* (1990) measured an uptake of 11.76 pmole/$10^6$ cells after a 5 minute exposure of fresh hepatocytes (which also corresponds to an approximately 1 μM internal concentration) and so an uptake of 9.4 pmol/$10^6$ cells at 48 hours without a corresponding morphological effect is puzzling. MacKintosh *et al* (1990) report that the intracellular concentrations of protein phosphatases 1 and 2A are about 1 μM each in fresh tissue, and it seems unlikely that the levels of these key enzymes would be reduced markedly during culture. However, the reduced derivatives have about half the toxicity, by mouse bioassay, of the original toxin (Meriluoto *et al,* 1990) probably due to loss of covalent binding.

Never-the-less, the overall results from the uptake experiment suggest that there is no substantial difference in the retention of the microcystin transport capacities of hepatocytes grown in the presence or absence of 2% DMSO. The fact that DMSO-treated cells did not proliferate between days 2 and 5, as indicated by a decrease in μg DNA per plate during this period, when DNA in the DMEM-treatment group increased more than 4-fold, could imply greater differentiation resulting in poorer proliferative capacity.

Because of the uncertain mechanism of the increased sensitivity of DMSO-treated cells to microcystin, and because such cells lost the ability to proliferate beyond 2 days in culture in any case, the use of DMSO was not continued.

# CHAPTER 6

# EFFECTS OF A SINGLE TREATMENT WITH MICROCYSTIN-LR ON DNA SYNTHESIS AND CELL GROWTH IN CULTURED HEPATOCYTES

## 6.1 Introduction

From the results described in the previous chapter it is apparent that microcystin-LR does not invoke a quantitative response in hepatocytes at times after 2 days in culture. The question then arises: Does this matter? Robinson *et al*, (1991), demonstrated that 70% of an ip injected sample of radiolabelled microcystin-LR remained within the hepatic cytosol from 1 hour after injection for at least 6 days. Of this, 83% was covalently bound at 1 hour and 42% at 6 days. It has been shown since that microcystins bind covalently to the active sites of PP1 and PP2A (Goldberg, *et al.*, 1995; Mackintosh, *et al.*, 1995; Runnegar, *et al.*, 1995c). Therefore, they may remain attached to these phosphatases for this length of time, the decrease in covalently bound toxin by 6 days representing normal protein, and protein phosphatase, turnover (Runnegar, *et al.*, 1995c). Thus, a cell might remain affected, at a sub-lethal level, by microcystin-LR for a period after uptake of the toxin has ceased.

The first wave of DNA synthesis in this culture system begins by 24 hours and peaks at about 50 hours (Sawada, *et al.*, 1986). Hepatocyte mitosis takes between 65 and 117 minutes (Sattler, *et al.*, 1988). Therefore, by about 3 days in culture, most proliferation-capable cells will have passed through at least one complete cell cycle. Since the cells are sensitive to additions of microcystin-LR early in the culture period, and are likely to remain affected to some extent for

some time after, it was decided to look for effects of the toxin on cell cycle parameters over a 3 day period.

## 6.2 **Methods and materials**

### 6.2.1 Experimental protocol

The general experimental protocol was outlined in Chapter 4, and so only a brief precis will be given here. Hepatocytes were isolated by parallel perfusion of two C3H/HeJ male mice with removal of dead cells and cell aggregates by Percoll isodensity centrifugation. Cells were plated onto collagen-coated 36 mm diameter tissue culture plates at a cell density of $4x10^5$ cells/plate. Non-adherent cells were washed off after 2 hours and the remaining cells cultured in DMEM/F12 medium containing dexamethasone ($10^{-7}$ M), ITS/100 and $^3$H-thymidine (0.3 μCi/ml), with or without EGF. Microcystin-LR at 1.0 nM, 3.0 nM, or 10.0 nM was also added to non-EGF treated plates at this time. No other medium changes were made for the duration of the experiment.

Triplicate "t=0" samples were taken at 2 hours, and then additional plates were sampled after 18, 42, and 66 hours in culture. Triplicate plates from each treatment group were cell-counted and then processed (see Section 4.2.7) for DNA and thymidine incorporation assays.

## 6.3 **Results**

## 6.3 Results

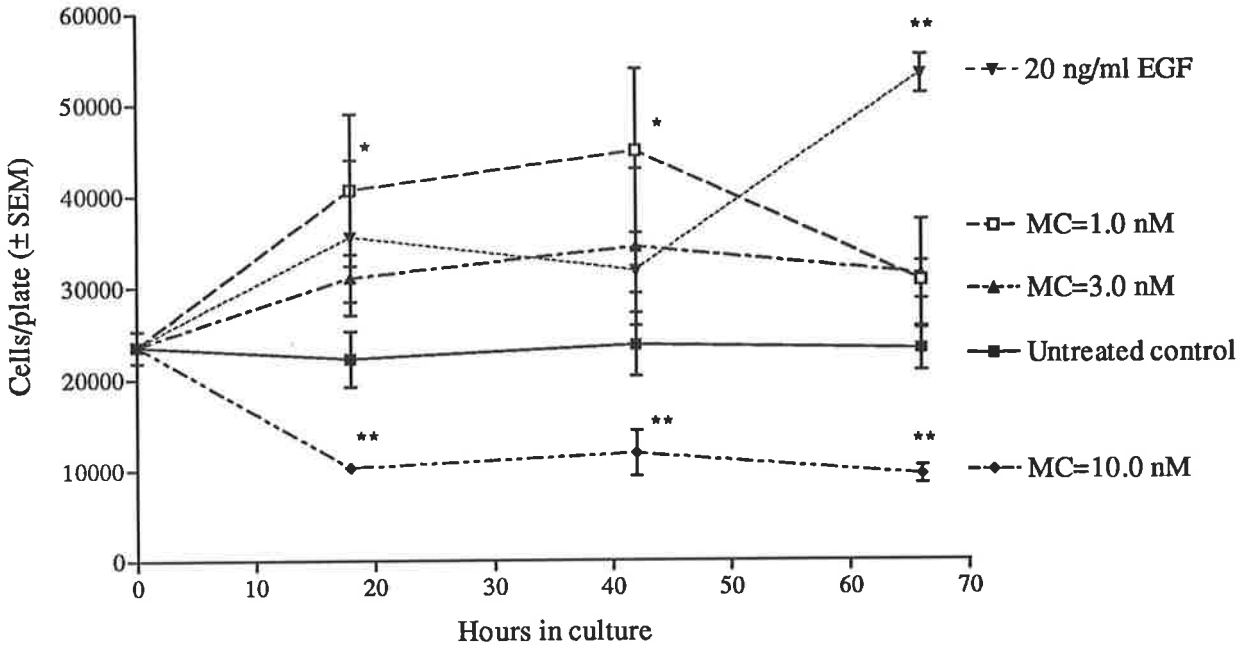



**Figure 6.1** - Effect of a single addition of microcystin-LR (MC) at 2 hours on hepatocyte cell numbers over time in culture. Cell numbers were counted in 5 random fields per plate on triplicate plates per treatment. Significant differences from cell counts in the untreated control at the same time point are indicated by * ($p<0.05$) and ** ($p<0.01$) as determined by one-way ANOVA followed by Dunnett's *post hoc* Test.

The data for cell numbers are shown in Figure 6.1. Interestingly, 1.0 nM microcystin-LR caused a significant ($p<0.05$) increase in cell numbers with respect to untreated control at the 18 and 42 hour time points. However, cell numbers had dropped again to about 1/3 their former peak above initial levels by the 66 hour time point. A similar but smaller effect was seen with 3.0 nM microcystin-LR. Ten nM microcystin-LR clearly had a negative impact on cell numbers by the first time point due to toxicity ($p<0.01$), although the remaining cells appeared to be insensitive to the acute effects of the toxin. Note that EGF stimulation induced a different pattern, starting to increase slowly at first but rising more quickly in the latter stage of the experiment to become

significantly different to the untreated control by 66 hours ($p<0.01$). This suggests that it is acting through a different cellular process to that of microcystin-LR.

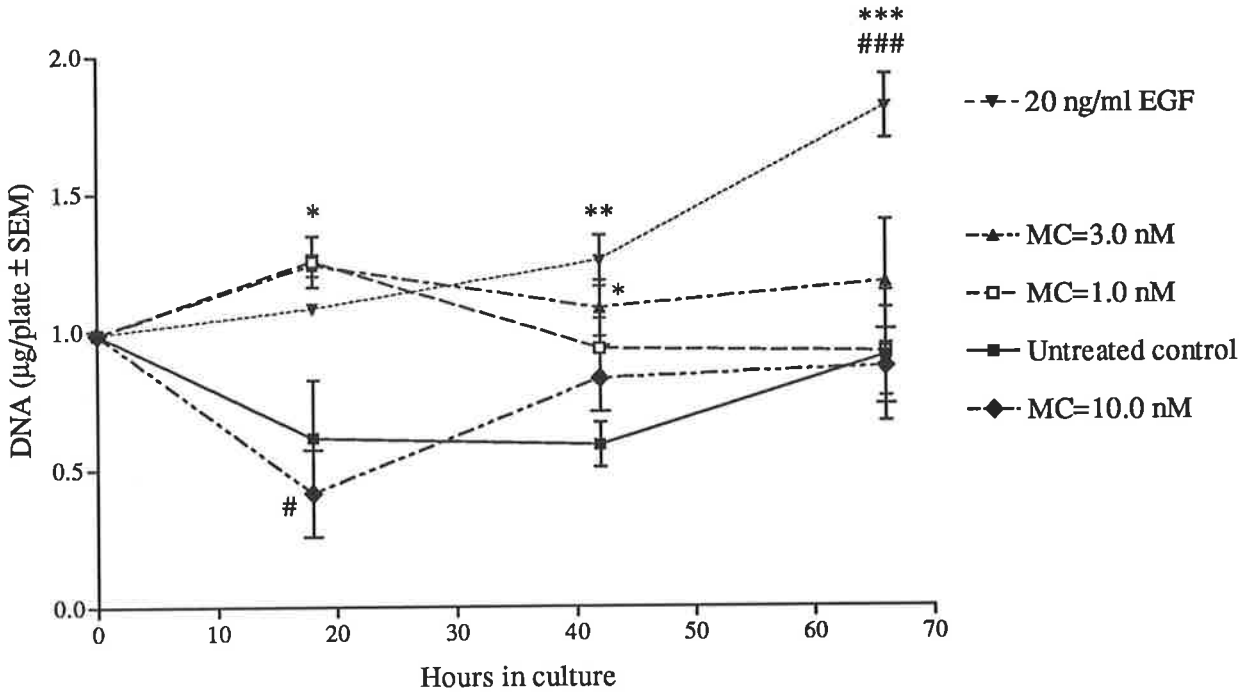



**Figure 6.2** - Effect of a single addition of microcystin-LR (MC) on DNA per plate over time in cultured hepatocytes. DNA was determined on triplicate plates per treatment. Significant differences from DNA levels in untreated control at the same time point are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) and significant changes from t=0 are indicated by # ($p<0.05$) and ### ($p<0.001$), as determined by one-way ANOVA followed by Dunnett's*post hoc* Test.

The effect of microcystin-LR on DNA per plate is presented in Figure 6.2. The levels of DNA on the untreated control plates appeared to fall by 40% at 18hrs, then remain at this level until 42 hours before recovering by 66 hours, although none of these changes were significantly different from t=0.

In contrast to cell numbers, DNA/plate in response to 1.0 and 3.0 nM microcystin-LR remained essentially constant throughout. However, they were significantly higher ($p<0.05$) than untreated control at 18 hours (both microcystin concentrations) and 42 hours (3.0 nM only).

The effect of 10 nM microcystin-LR at 18 hours was in line with the effect seen on cell numbers, that is, to cause a reduction of the DNA on the plate to a level significantly lower than t=0 ($p<0.05$) (and 1.0 and 3.0 nM microcystin-LR ($p<0.01$)). However, after this time point DNA recovered to nearly original levels whilst cell numbers remained low. Therefore, when the data were analysed as pg DNA/cell, as seen in Figure 6.3, the DNA content of cells surviving treatment with 10.0 nM microcystin-LR increased to be approximately double that of the original population ($p<0.01$).

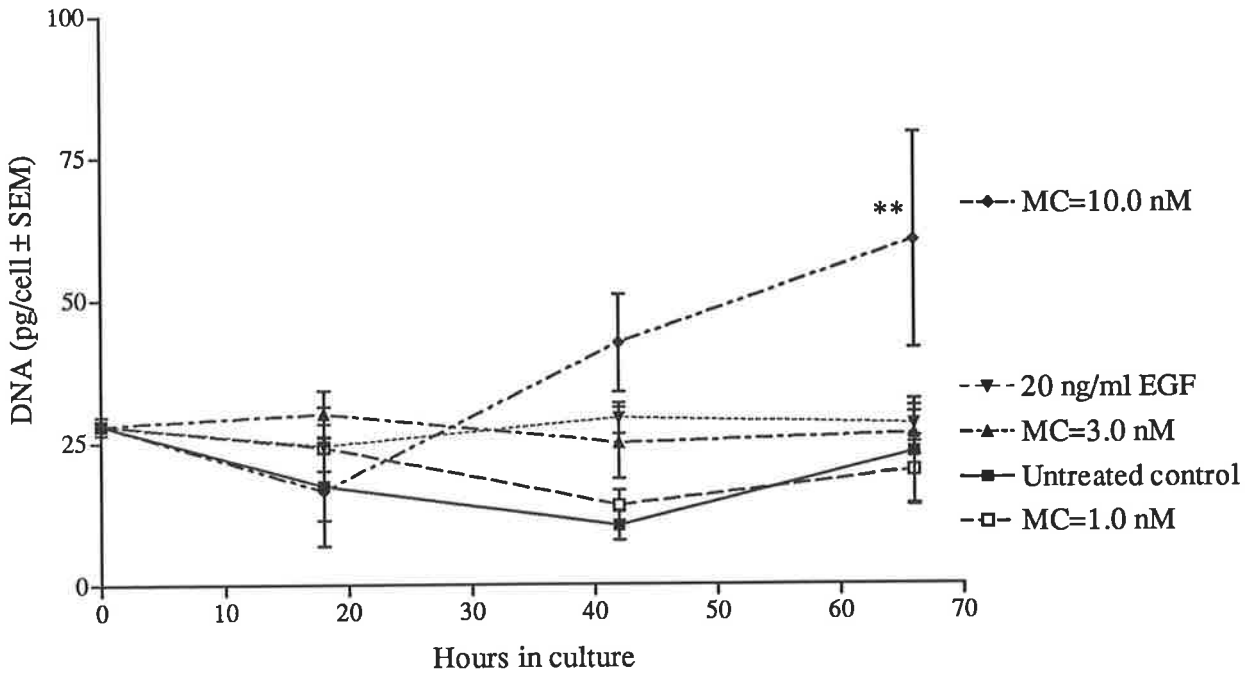



**Figure 6.3** - Effect of a single addition of microcystin-LR (MC) at 2 hours on DNA per cell over time in culture. Significant differences from DNA levels in untreated control at the same time point are indicated by ** ($p<0.01$), as determined by one-way ANOVA followed by Dunnett's *post hoc* Test.

The DNA content of untreated cells and those exposed to 1.0 nM microcystin-LR appeared to fall to approximately 50% of the original levels by 42 hours before recovering, but this was not statistically significant, largely due to greater variability at the 18 and 66 hour time points.

Thymidine incorporation per plate is shown in Figure 6.4. The incorporation by untreated control cells rose with time, indicating an inherent DNA synthetic capability in these cells. As none of these curves (with the exception of EGF stimulated) deviated significantly from linearity, toxin treatments were compared with untreated control using linear regression.

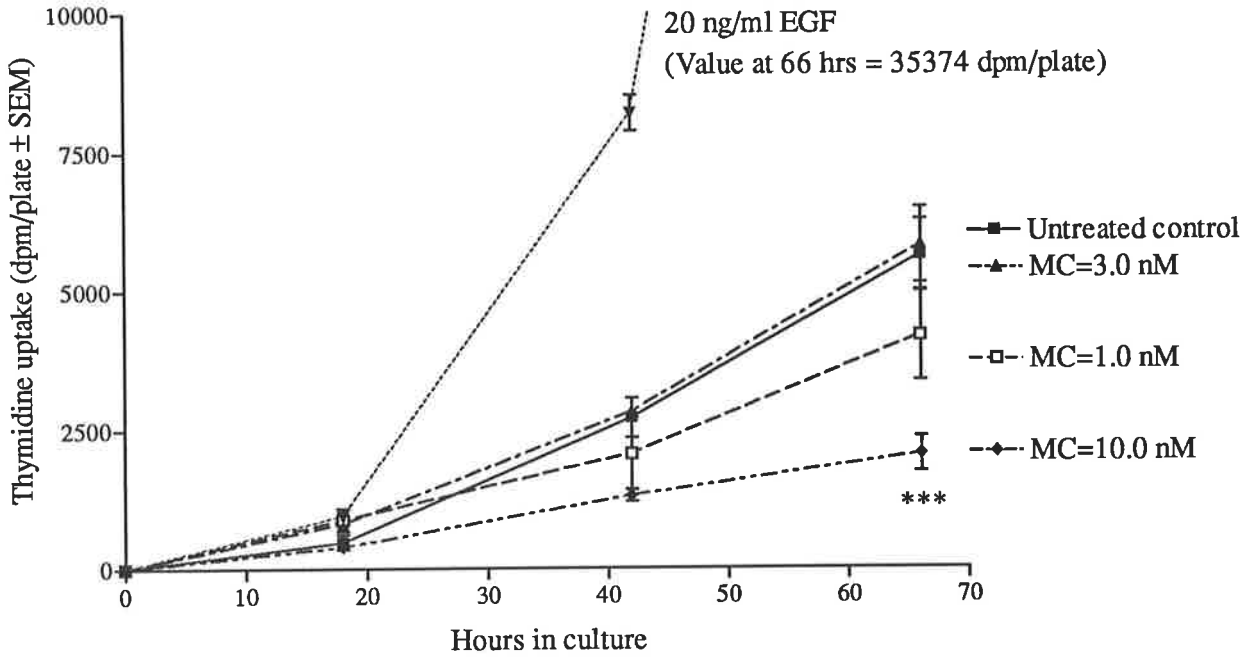



**Figure 6.4** - Effect over time in culture of a single addition of microcystin-LR (MC) at 2 hours on $^{3}$H-thymidine incorporation per plate. $^{3}$H-thymidine incorporation was determined on triplicate plates per treatment. Differences between rates of incorporation were analysed using linear regression (except the EGF treatment). A significant difference of the 10.0 nM microcystin-LR treatment slope from untreated control is indicated by *** ($p<0.001$).

There was a significant reduction of radiolabelled thymidine incorporation into DNA in cells that were treated with 10.0 nM microcystin-LR ($p<0.001$). However, this finding was not

surprising since there were fewer viable cells present to incorporate thymidine in the 10nM treatment as acute toxicity had reduced cell numbers.

When thymidine incorporation per cell was calculated it was found that, indeed, the 10 nM microcystin-LR treatment group was very similar to that of the untreated control in this parameter, suggesting that the remaining "microcystin-insensitive" population had a normal capacity for DNA synthesis. These results are presented in Figure 6.5.

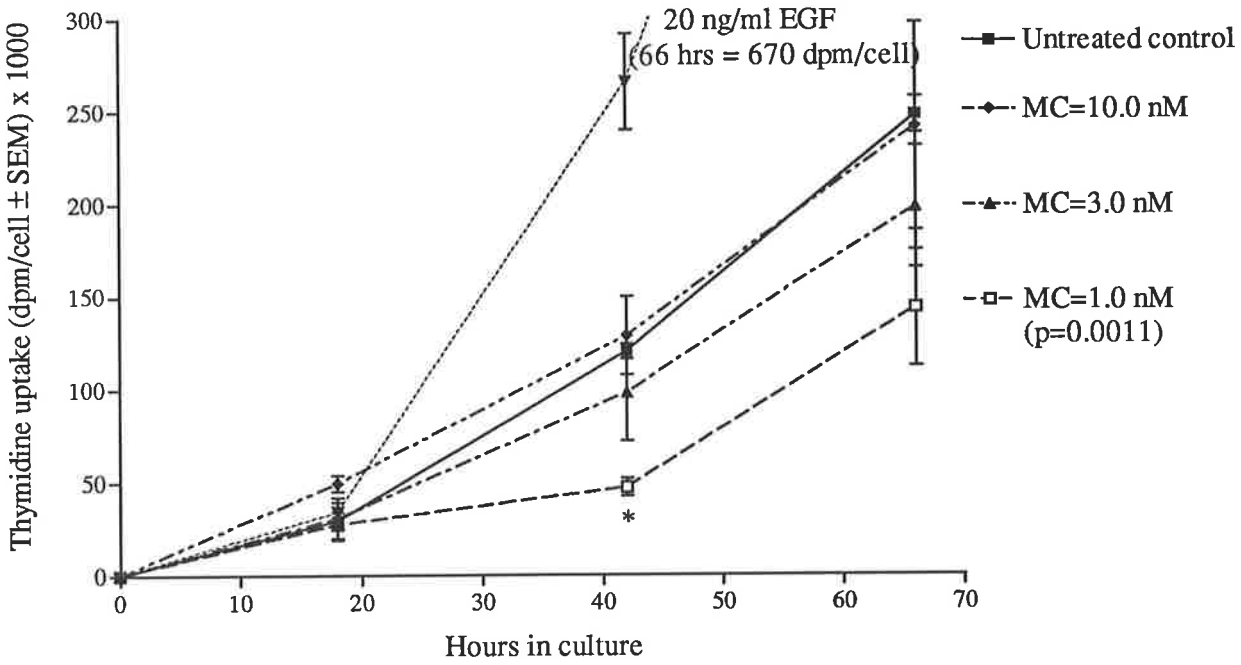



**Figure 6.5** - Effect over time in culture of a single addition of microcystin-LR (MC) at 2 hours on $^{3}$H-thymidine incorporation per cell. Differences between rates of incorporation per cell were analysed using linear regression (except the EGF treatment). P-values for the comparison of the treatment slopes with untreated control are shown. Significant differences from untreated control at the same time point are indicated by * ($p<0.05$), as determined by one-way ANOVA followed by Dunnett's *post hoc* Test.

In contrast to the effects produced by 10.0 nM microcystin-LR, toxin at 1.0 nM (p=0.001) caused an overall inhibition of the incorporation of thymidine into DNA when analysed on a cellular basis. This arises from a lowering of the rate of incorporation until 42 hours, after which

the rate of incorporation more closely approximated that of cells in the untreated control treatment. This suggests that the effect of 1.0 nM microcystin-LR was only temporary.

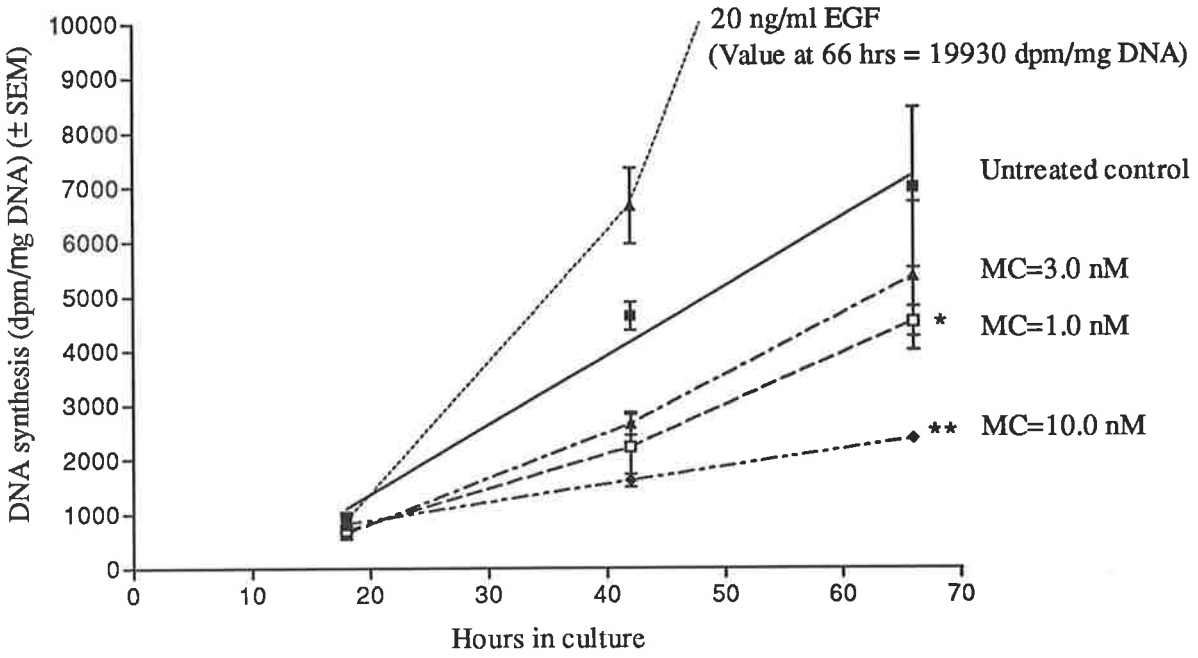



**Figure 6.6** - Effect over time in culture of a single addition of microcystin-LR (MC) at 2 hours on $^{3}$H-thymidine incorporation per µg DNA. Differences between rates of incorporation were analysed using linear regression (except the EGF treatment). Significant differences in slopes with respect to untreated control are indicated as * ($p<0.05$) and ** ($p<0.01$).

The ratio of thymidine labelled DNA to total DNA is presented in Figure 6.6. This ratio is reduced at all microcystin concentrations, most strikingly in the 1.0 ($p<0.05$) and 10.0 nM treatments ($p<0.001$). Note that this ratio is not simply a measure of the rate of DNA synthesis in this system, since changes in either the denominator or the numerator can occur to affect the outcome, and so these results must be interpreted in the light of other changes in the other parameters measured (see below).

## 6.4 Discussion

### 6.4.1 Controls

The results throughout for the EGF treatment group indicate that the cell population under study was capable of stimulation by a mitogen and able to complete all phases of the cell cycle. Thymidine incorporation per cell (Figure 6.5) or per μg DNA (Figure 6.6) was dramatically enhanced, and this resulted in increased DNA per plate over untreated control (Figure 6.2). DNA per cell (Figure 6.3) remained constant, however, indicating that both nuclear and cellular division occurred after DNA synthesis, and this in turn was translated into an increase in cell numbers by 66 hours (Figure 6.1). The fact that microcystin-LR induced effects differed markedly from those elicited by EGF indicated that it is unlikely that these compounds promoted cell growth by similar mechanisms, or at least that microcystin-LR acted on such a small proportion of the hepatocyte population that this effect was undetectable in this system.

The cellular and population processes occurring in the untreated control group act as the baseline for the effects of microcystin-LR and so these will now be examined in detail. Cell numbers in the untreated control remained constant throughout the study period (Figure 6.1), indicating that cell losses were balanced by cell proliferation. Thymidine incorporation per plate (Figure 6.4) showed an approximate doubling per sample period, as did the amount of $^{3}$H-thymidine per cell (Figure 6.5), indicating substantial DNA synthesis. DNA per plate (Figure 6.2), and per cell in particular (Figure 6.3), seemed to drop initially before recovering by 66 hours. These observations suggest that the hepatocellular population was changing over time such that initial cell losses were predominantly from the higher ploidy portion of the population, with their replacement by 66 hours occurring largely from proliferation within the remaining lower ploidy

portion. Therefore, two sub-populations can be identified: Non-proliferative cells which die early in the study period without undergoing DNA synthesis; and proliferative cells which do synthesise DNA and then divide, replacing the cells lost. An estimate of the size of these groups of cells can be provided by the following calculations.

In hepatocytes, whose DNA content is not fixed at the diploid level, there is a more complex relationship between cell number and DNA per plate than in other cell types. This is illustrated in Table 6.1.

| | Diploid | Tetraploid | Octaploid |
|---|---|---|---|
| Cellular proportions | 25% | 65% | 10% |
| DNA proportions | 13% | 66% | 21% |

**Table 6.1** - Relationship between the percentage of hepatocellular ploidy groups in a population and the percentage of the DNA they contribute to the total DNA pool, for a typical cellular ploidy distribution (After Severin, *et al.*, 1984b).

It appears from the results just discussed that it is higher ploidy cells which are the cells most likely to be lost in the first few hours of culture. If it is assumed for the purposes of the calculation that all octaploid cells are lost (approximately 20% of DNA) and 1/3 of tetraploid cells are lost (approximately 20% of DNA) then this could account for the approximately 40% of DNA lost by 42 hours (Figure 6.2). For this to be the case a number equivalent to at least 30% of the original cells plated must have died by this time and been replaced by cell proliferation. This must be an underestimate because cell death is unlikely to be so completely ploidy dependent, and also because this does not take into account DNA replacement via synthesis during this period. Nevertheless, this 30% cell loss by 42 hours (15%/day) corresponds well with the estimate of 13%/day net proliferation made in Section4.3.3.

However, there is yet another source of underestimation of this net proliferative rate. That is, it does not take into account those cells which die during or soon after DNA synthesis via apoptosis. These cells will not be detected in the assays unless the microcystin or EGF treatments affect their rate of loss, in which case they might be detected as part of any changes seen. This adds a third sub-population to the two already mentioned. The size of this sub-population can be estimated from data from an experiment to be reported in Chapter 8, in which apoptosis was inhibited with the peroxisome proliferator Nafenopin. It is necessary to discuss that data here in order to be able to estimate the contribution of this third group of cells to the outcomes found in this experiment. The reader is referred to the above mentioned section for further detail of experimental conditions, etc.

In the nafenopin experiment, suppression of apoptosis with this agent induced a two-fold increase in cell numbers by 65 hours in culture (Figure 8.1). However, thymidine incorporation per cell was unchanged from untreated control (Figure 8.5). This indicates firstly that the increase in cell numbers was not due to a stimulation of proliferation. But it also shows that the number of cells rescued from apoptosis was similar in both the non-proliferating and proliferating cohorts of cells. That is, thymidine incorporation per cell, which is measured as a population average, would be reduced by nafenopin treatment if only non-proliferative cells were rescued. Since this was not the case, the actual proliferation rate must be higher by an amount roughly equal to the rate of loss of non-proliferating cells, or about 15%/day. Therefore, the true proliferation rate in the untreated control cells is this undetected 15%/day plus the 15%/day which remains alive to balance the observed DNA loss. These estimates are not intended to be quantitative, but rather a qualitative illustration of the population dynamics of the system under study, upon which microcystin-LR might have an influence.

The above illustrates the complexity of the system under study. The following points can be made about the various parameters which were quantified or derived:

(1) Cell numbers as counted on the plate at a particular time point are the outcome of three dynamic processes: (a) Cell proliferation to increase cell numbers, counter-balanced by;

(b) Losses of cells during or soon after proliferation, and;

(c) Losses of cells with little or no proliferative ability which were present in the initial isolate.

(2) Total DNA on the plate broadly reflects the balance between DNA synthesis in proliferating cells and DNA losses in dying cells. The ploidy of the cells in question will determine the relationship between DNA per plate and cell numbers.

(3) DNA per cell is an estimate of the average ploidy of the cells on the plate. This will be determined by the rate of cell division versus the rates of formation of binucleate or higher ploidy mononucleate cells in the proliferative cohort (that is, the relative rates of karyokinesis and cytokinesis), as well as the ploidy of the cells lost from the plate. Thus if cell numbers rose but DNA per plate remained constant then DNA per cell (average ploidy) must have declined. However, the same outcome might also arise, perhaps with different implications, if cell numbers remained constant but DNA per plate fell.

(4) $^{3}$H-Thymidine incorporation per plate is a record of the rate at which DNA which has been synthesised and remained on the plate up to that point. Thus the increase between two time points is an estimate of the average incorporation rate of the cells still present at the second time point, minus any loss of labelled DNA which had been detected at the first time point.

(5) $^{3}$H-Thymidine incorporation per cell is an estimate of the average rate of DNA synthesis up to that time of the cells present at the time of determination. The ratio of proliferative versus non-proliferative cells present will determine this average, however, as with DNA per cell this will be modified by their average ploidy since higher ploidy cells will have needed to incorporate more

thymidine to replicate their DNA than lower ploidy cells. Similarly, the relative rates of karyokinesis and cytokinesis after DNA synthesis will have an effect.

(6) $^{3}$H-Thymidine incorporation per μg DNA represents the proportion of the total DNA present which has been newly synthesised and so labelled with radioactive thymidine. It is determined by the balance between the rate of DNA synthesis and the rate of DNA loss (labelled and unlabelled) up until the time of detection. Note that addition of newly labelled DNA, or loss of previously labelled DNA, will have less of an effect on the ratio than loss of unlabelled DNA since the former appear in both the denominator and numerator of the ratio whereas unlabelled DNA only appears in the denominator. Thus given equal rates of either DNA synthesis or loss of unlabelled DNA, the latter will have the more marked effect, perhaps giving the impression of a higher overall rate of DNA synthesis. Therefore, this ratio must be interpreted in the light of the cell number, DNA per plate, and thymidine incorporation per plate data.

### 6.4.2 Microcystin-LR treatments

The effects of microcystin-LR on this complex system will now be discussed. Exposure to 1.0 nM microcystin-LR caused an initial 1.6-fold rise in cell numbers by 18 hours (Figure 6.1) with a lesser increase in DNA per plate (which only increased by 25% from t=0; Figure 6.2). However, any change in DNA per cell was minimal at this time point (Figure 6.3). Radioactive thymidine incorporation into DNA, expressed on a per cell basis (Figure 6.5), was equal to that seen in untreated control although radioactivity per plate appeared to be approximately 2x higher (Figure 6.4). The DNA per plate of the control treatment was reduced to half that of the 1.0 nM microcystin-LR treatment at this time ($p<0.05$). A reduction of this loss of cells would have the effect of raising the baseline upon which proliferative gains could accrue. Since thymidine incorporation per cell did not drop in the microcystin-LR treatment, and thymidine incorporation

per plate may have risen, it is likely that a reduction in the rate of cell death has occurred in both the proliferative and non-proliferative cohorts. These "rescued" proliferative cells would therefore give rise to the increases in DNA per plate and cell numbers observed. The reduction of cell loss might be explained by the known inhibitory effects of phosphatase inhibition on the rate of autophagy in hepatocytes (Blankson *et al*, 1995) which may be linked to inhibition of microtubule-based vesicle movement (Hamm-Alvarez *et al*, 1996) and the general slow down of metabolism that this implies.

Between 18 and 42 hours cell numbers in the 1.0 nM microcystin-LR treatment increased further to be approximately double the starting numbers (Figure 6.1), but DNA per plate may have declined slightly (Figure 6.2). Therefore, DNA per cell was reduced to a level about half that of cells at t=0 (Figure 6.3). Thymidine incorporation per plate was lower than, and thymidine incorporation per cell only 1/3, that of untreated control (Figures 6.4 and 6.5, respectively). Since DNA per plate appears to have decreased, cells have been lost and this loss will be underestimated as thymidine incorporation per plate is still increasing slowly, indicative of some DNA synthesis. However, cell numbers have risen further from the already high level at 18 hours. This increase in cell numbers, in a background of some loss of DNA from the plate and reduced DNA synthesis, can only have come about by an increase in cytokinesis in binuclear hepatocytes, or via karyokinesis followed by cytokinesis in higher ploidy mononuclear cells, these events occurring without additional DNA synthesis. If cell numbers were increased in this way then thymidine incorporation per cell would be reduced, as was found (Figure 6.5).

Since only 50-60 % of C3H hepatocytes are binucleate (Severin, *et al.*, 1984b, and results presented in Chapter 9), this represents the maximum that cell numbers can increase due to an induction of cytokinesis only. However, since karyokinesis in cultured hepatocytes takes about 70

minutes in mononucleates and 80 minutes in binucleates, and cytokinesis only 20 - 30 minutes (Sattler *et al*, 1988), either scenario is possible within the time available. Whether induction of karyokinesis would automatically lead to cytokinesis is unknown in this case since the linkage of these two events appears to be dependent on the characteristics of the individual cell as well as on the factors to which it is exposed (see for example Saeter, *et al.*, 1988b; as well as results presented on the effect of EGF stimulation on the different cellular ploidy groups in Section 9.3.3). This issue is explored further in Chapter 9.

Between 42 and 65 hours in the 1.0 nM microcystin-LR treatment cell numbers decreased by 25% (Figure 6.1). DNA per plate was steady (Figure 6.2) and DNA per cell rose by 50% from the 42 hour level (Figure 6.3). Thymidine incorporation per plate rose slightly (Figure 6.4) and so the rate of thymidine incorporation per cell between 42 and 65 hours was only slightly lower than that in the untreated control (Figure 6.5). These data suggest that the earlier stimulation of cytokinesis had ceased and that the underlying loss of cells was now dominating the balance which determines cell numbers. Nevertheless, the rate of proliferation in the remaining population was fairly normal meaning that DNA lost in dying cells was being replaced by synthesis. Therefore, DNA per plate remained constant despite the fall in cell numbers. However, these proliferating cells had yet to divide since DNA per cell and thymidine incorporation per cell had increased.

It is unclear which cells are dying in this final period and which are proliferating. At 42 hours, the ratio of labelled to non-labelled DNA was markedly lower in the 1.0 nM microcystin-LR treatment than in the control (Figure 6.6) because of the initial retention on the plate of cells normally lost due to necrosis or apoptosis, and also perhaps because of a diversion of cells from DNA synthesis to cytokinesis. This ratio increased somewhat by the 65 hour time point but the rate of increase was still not as rapid as that of the control. However, if it was only the

microcystin-affected, and therefore comparatively less radioactively labelled cells which were being lost, then the rate of increase of this ratio would be much higher, resulting in a ratio similar to that of untreated control by the time that their cell numbers coincided. Thus a proportion of the cells affected by 1.0 nM microcystin-LR have become proliferative. Hence it seems likely that the long term effect of a single dose of 1 nM microcystin-LR could be to induce cytokinesis in a cohort of sensitive cells, and that at least some of these cells can survive to undergo further rounds of DNA synthesis.

When the microcystin-LR concentration was 10 nM, cell death occurred in about 50% of the population by 18 hours (Figure 6.1). Note that in Section 5.3.1, exposure to 100 nM microcystin-LR caused the loss of 99% of hepatocytes by this time. Therefore, practically all cells must have some uptake capacity. This suggests that a selective process underlies the acute response of cells to this toxin, probably in part determined by relative levels of expression of the transporter(s) needed for its active uptake. However, the exposure time in this experiment was relatively long which would allow even slow active uptake rates to achieve high internal concentrations of toxin. Nevertheless, there was a very sharp threshold between the acute toxicity seen at 10.0 nM microcystin-LR and the more sub-acute effects of the lower concentrations. Therefore, it may be that some other mechanism limits the acute effects of microcystin-LR until a sufficiently high concentration is achieved to overcome this threshold.

This halving of cell numbers in the 10 nM microcystin-LR treatment was corroborated by a similar drop in DNA per plate at 18 hours (Figure 6.2). The cells which remained after exposure to 10 nM microcystin-LR continued to synthesise DNA since thymidine incorporation per cell increased (Figure 6.5), and they accumulated this DNA until at least 66 hours as shown by the increase in DNA per cell (Figure 6.3). Thus, it may be that microcystin-LR at this concentration is

having an inhibitory effect on cytokinesis. That is, since DNA per cell rises constantly throughout the study period it seems likely that these cells either cannot undergo cytokinesis at all, or that this process is significantly delayed with respect to an untreated population. If, as an alternative, the microcystin-surviving sub-population had an inherently slower cell cycle, or the commencement of S-phase was delayed with respect to an untreated population, then the rate of thymidine incorporation per cell ought to be slower, or the onset of the rise in thymidine incorporation should be delayed, respectively, neither of which appeared to be the case. Therefore, it does seem more likely that microcystin-LR had a disruptive effect on cytokinesis in the microcystin-surviving hepatocyte population, leading to a substantial increase in DNA per cell. This seems consistent with its known effects on the cytoskeleton. Furthermore, the constant increase in DNA per cell to such high levels suggests that most cells were accumulating DNA. It is also conceivable that these cells represent a "synchronised" population and that at (say) 67 hours most of these cells could divide leading to a normalisation of DNA per cell and a sudden increase in cell numbers.

The effect of 3.0 nM microcystin-LR was to increase cell numbers by about 1/3 by 42 hours which thereafter remained steady for the rest of the study period (Figure 6.1). DNA per plate rose by 25% at 18 hours before decreasing slightly subsequently (Figure 6.2). However, it was still almost double that of the untreated control at 42 hours before the latter increased again by 65 hours. Cellular DNA content was fairly steady throughout (Figure 6.3) and furthermore, in contrast to untreated control, it did not fall initially, suggesting that fewer large cells were lost soon after being placed in culture in this treatment. Again, this would effectively raise the baseline upon which proliferating cells are added, and could thus account for the rises in DNA per plate and cells numbers as proliferation continued. Since DNA per cell remained constant, there was no evidence for the major effects on cytokinesis seen at the other two concentrations, unless they both occurred in separate sub-populations and cancelled each other out.

The most likely explanation for these observations is that the combined effects of microcystin-LR on five sub-populations of hepatocytes determined the overall outcome. These five sub-populations are: One in which normally fatal, possibly autophagocytic and/or apoptotic, processes are at least delayed by 1.0 nM microcystin-LR; A second which is stimulated to undergo at least cytokinesis, and possibly both karyokinesis and cytokinesis at 1.0 nM microcystin-LR, without promotion of DNA synthesis; A third in which DNA synthesis continues irrespective of 1.0 nM or 10.0 nM microcystin-LR, but in which cytokinesis is sensitive to microcystin-LR concentration; A fourth in which the cell dies due to acute toxicity at 10.0 nM microcystin-LR; And a fifth comprising cells which are completely unaffected except at much higher concentrations of toxin. The proportion of the hepatocyte population which appear in each of these groups will of course depend on the toxin concentration. However, it will also depend upon the relative ability of a cell to take up the toxin and respond (for example, its differentiation and/or ploidy status, and possibly its position in the cell cycle), as well as other influencing factors which include the time after cell isolation when the cells are exposed, and the overall health of the hepatocyte population (determined in part by the quality of preparation, the age, health, sex, and nutritional status of the donor animals, and time of day when the cell isolation procedure was performed). As already described, the experiments were standardised as much as possible to minimise these sources of variability.

Having characterised the processes which appear to be induced in this hepatocyte population by a single exposure to microcystin-LR, further experiments were carried out using a more constant exposure regime. This was thought to better reflect the type of exposure likely to occur due to the consumption of microcystin-contaminated drinking water. These experiments are described in the following Chapters.

# CHAPTER 7

# EFFECTS ON CULTURED HEPATOCYTES OF CONTINUOUS EXPOSURE TO MICROCYSTIN-LR, ITS INTERACTION WITH EPIDERMAL GROWTH FACTOR, AND COMPARISON WITH OKADAIC ACID

## 7.1 Introduction

Having characterised, in the experiment described in Chapter 6, the types of cellular processes which are likely to be induced in hepatocytes by microcystin-LR, it was considered important to pursue further studies under treatment conditions more likely to reflect a natural exposure regime. It seems very unlikely that an individual would receive a single large dose of the toxin in their drinking water. It is equally unlikely that such a dose would enhance tumour growth. Therefore, rather than a single addition of microcystin-LR, culture conditions were modified to allow a more continuous exposure of the cells to the toxin. Moreover, a greater range of concentrations incorporating toxin levels below those causing an acute toxic response were used. These experiments are generally referred to as "multi-addition" experiments to differentiate them from the "single-addition' experiment described in Chapter 6.

Two further experiments are described in this Chapter. The first is a comparison of the effects of microcystin-LR on cultured hepatocytes in the presence of EGF with those seen in its absence. The second is a comparison of the effects of microcystin-LR with okadaic acid. The impetus for these experiments was the contrast in the dose-response found in the multi-addition experiment (presented below) with those reported by Mellgren *et al*, (1993), whose data show effective concentrations approximately 100-fold higher than those found to apply in this system.

Furthermore, since okadaic acid has, to some extent, been presented as the archetypical protein phosphatase-inhibiting tumour promoter, with the unstated assumption that results obtained using this toxin can be extrapolated to explain the mechanism of action of the microcystins, it was also of interest to observe their relative effects in the same culture system.

## 7.2 Materials and Methods

The cell isolation and general culture protocols used in the experiments described in this Chapter were essentially the same as those used previously (Chapter 4), and so will not be described in detail again. Briefly, cells from two male 20 g 5 week old C3H/HeJ mice were used on triplicate plates for each treatment plus untreated and EGF-treated controls. EGF controls were used to check the metabolic and proliferative capability of each cell preparation. Rather than using a single addition of toxin to the culture medium soon after cell isolation, the medium in the present experiments was changed, with the addition of fresh toxin, every 24 hours in order to maintain a more constant level of exposure. Therefore, microcystin-LR or EGF was added, along with 0.3 μCi/ml $^{3}$H-thymidine, at 2 hours, and again when the medium was changed at 18 and 42 hours. Also, in order to examine the effects of microcystin-LR over a broader range of concentrations it was necessary to reduce the sampling to a single time point, at 65 hours culture time. Nevertheless, 3 separate experiments were necessary to cover the concentration range from 10 pM to 100 nM microcystin-LR, and so the results of these were normalised to untreated control levels in order to minimise inter-experiment variation. Where other additions or modifications were made to this general protocol they will be described in the introduction to the relevant Section.

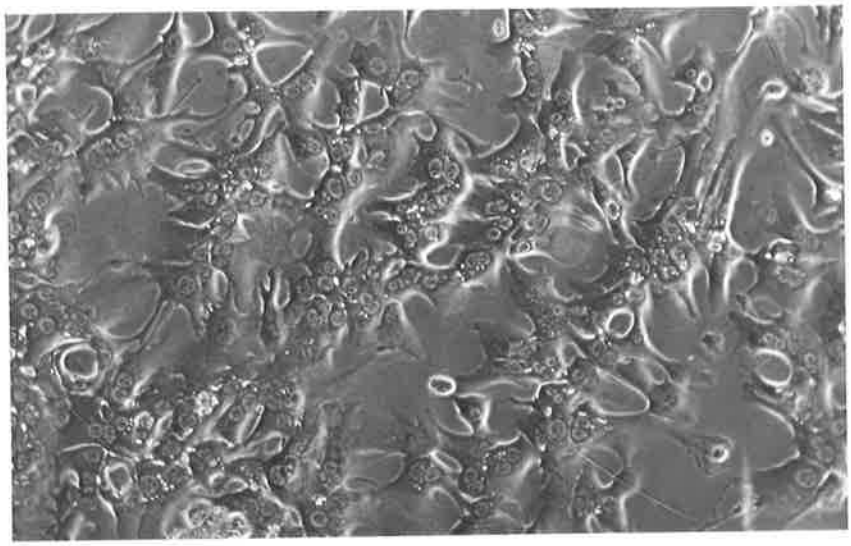
## 7.3 Effect of repeated additions of microcystin-LR to the culture medium on the hepatocyte cell cycle

### 7.3.1 Results

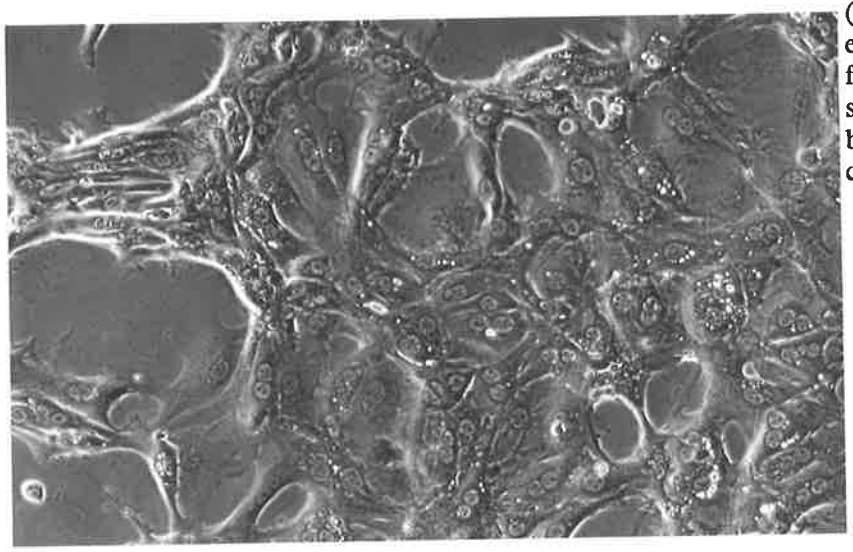
#### 7.3.1.1 Morphological effects

The effect of exposure to three changes of microcystin-LR containing medium over 65 hour of culture time on cellular morphology is shown in Figure 7.1, along with untreated and EGF treated cells for comparison. Untreated hepatocytes adhered to the substrate but did not form close cell-cell contacts when compared with EGF treated cells. Beginning at about 0.3 nM microcystin-LR, some patches of cells were observed which appeared to be very tightly packed, with smaller or less flattened nuclei. This effect was especially pronounced in the 1.0 nM photomicrograph, taken of an unusually large group of cells to show the effect. Some clumps of sloughed off dead cells are also seen in this region. At 10.0 nM microcystin-LR many cells had rounded up and lost their attachment to the substrate, although those that remained attached appeared to exhibit a more normal morphology than those highlighted in the previous micrograph. At 100 nM few cells survived.

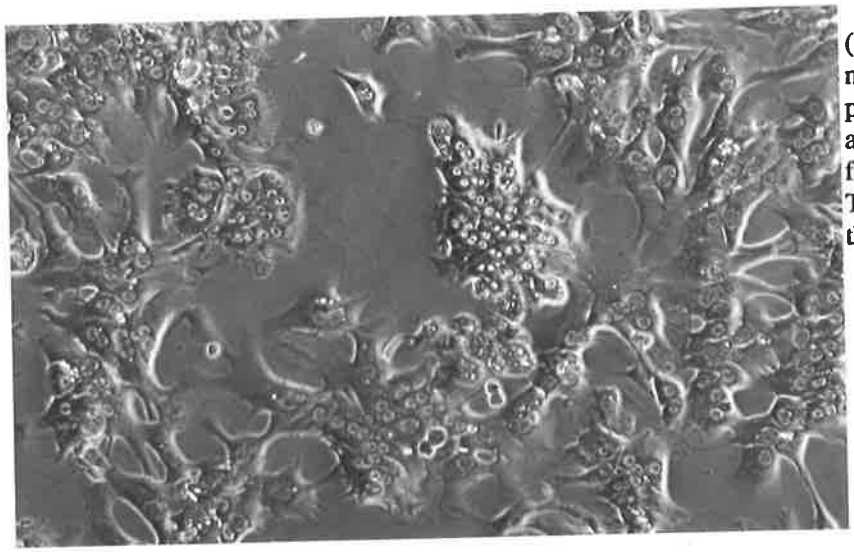
**Figure 7.1** - Effect of three changes of microcystin-LR containing medium over 65 hours of culture time on the morphology of C3H/HeJ hepatocytes. (Magnification 100x).



(a) Untreated control cells. Note minimal spreading and intercellular contacts when compared with the EGF-treated cells below.

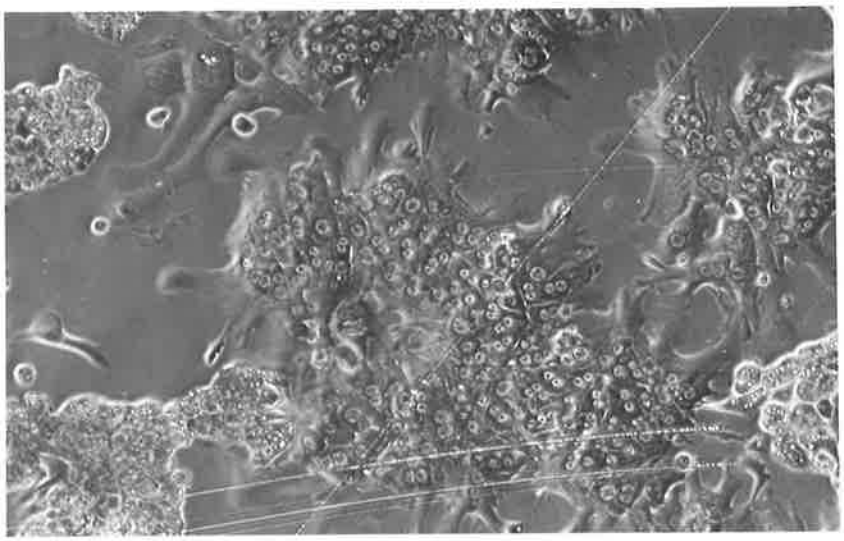


(b) EGF-treated cells. These cells formed extensive intercellular contacts and flattened out in contact with the substrate so that their nuclei appear larger and better defined than those in the untreated control.

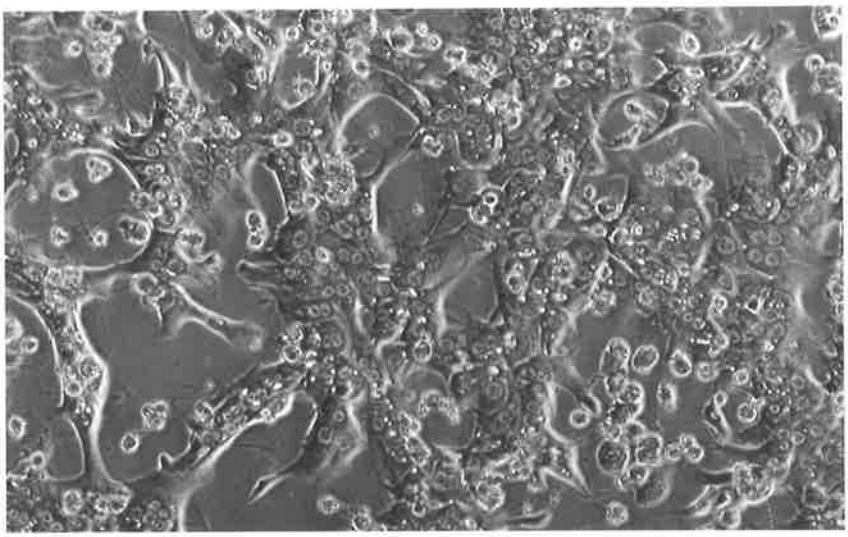


(c) Hepatocytes growing in 0.3 nM microcystin-LR. Some cells, most prominent in the group near the centre, appear more closely packed and are not as flattened as in the untreated control. Therefore, their nuclei appear smaller and the group has a prominent boundary.

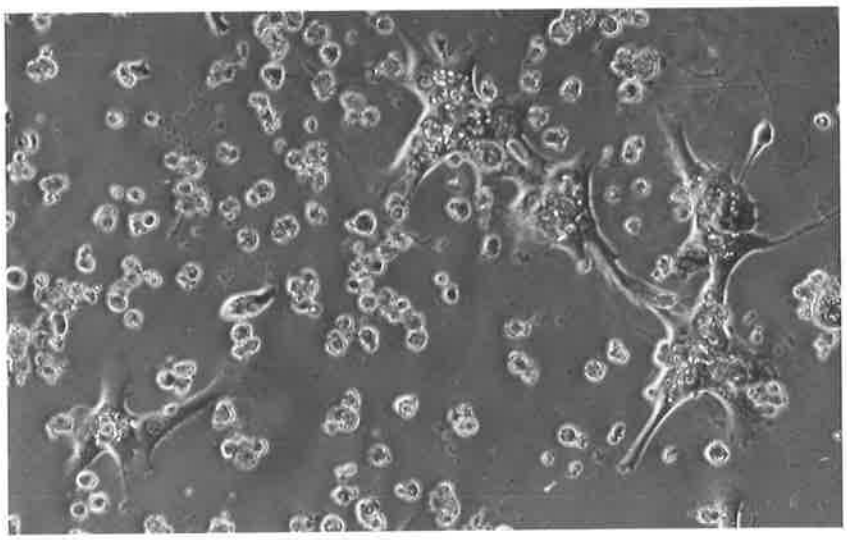
**Figure 7.1** continued.



(d) Hepatocytes growing in 1.0 nM microcystin-LR. An unusually large group of toxin-effected cells similar to those seen in (c) above. Also shown are clumps of dead cells, at the edges of the photograph, which are probably whole sheets of cells which have become detached from the collagen substrate.



(e) Hepatocytes growing in 10.0 nM microcystin-LR. There were fewer groups of cells with obviously abnormal morphology in this treatment, perhaps indicating that those cells were killed by the toxin at this concentration. Others remained alive and proliferated however. The small round, refractile cells have rounded up and become detached from the plate. They were presumed to be dead.



(f) 100.0 nM microcystin-LR. Most cells were killed by this concentration of toxin and are seen as the small round refractile cells which were not attached to the collagen substrate. Note that even at this toxin concentration, however, a few cells survived. Since many of these were binucleate, eg at the top centre of the photograph, it is probable that they were parenchymal hepatocytes.

7.3.1.2 Effects on cell cycle parameters

The data for hepatocyte numbers are shown in Figure 7.2. There appeared to be a rise in cell numbers up to a peak when the microcystin-LR concentration was 0.1 nM, although this did not reach statistical significance. In the experiment which is the subject of Chapter 8, this rise was significant. Furthermore, cell counts were always raised to some extent with respect to untreated control in this region (data not shown). After this peak there was a gradual decline in cell numbers until a microcystin-LR concentration of 10 nM, followed by a more rapid decline to almost 100% cell death at 100 nM. This latter finding matches earlier observations (see Section 5.3.1).

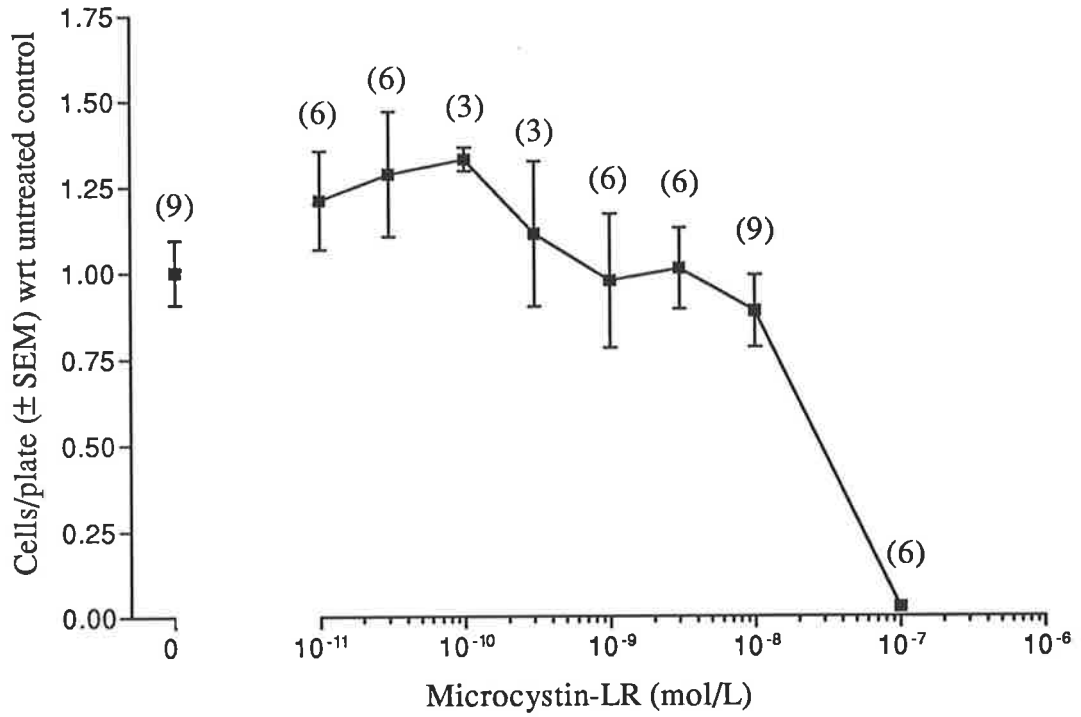



**Figure 7.2** - Effect on cell numbers of 3 additions of microcystin-LR, with change of medium, at 2, 24 and 48 hours. Cells were counted at 65 hours. Data points represent the average of 5 random fields on each of 3 plates ± SEM. A number of experiments were conducted to cover microcystin-LR concentrations from 0.01 - 100.0 nM. The numbers in brackets are the replicate determinations of that point. Data from each experiment were normalised to the untreated control for that experiment.

These findings are reminiscent of the data presented in the previous Chapter (Figure 6.1) in that cell numbers were elevated over untreated control at low microcystin-LR concentrations but then declined as the toxin concentration was increased further. The first addition of toxin in these experiments could be expected to produce the effects seen previously but the subsequent additions appear to modify these initial effects such that the dose-response curve is shifted to the left. Thus, the peak seen here at 0.1 nM microcystin-LR is approximately 1/3 higher than untreated control, and this ratio is of similar magnitude to that observed in the 1.0 nM treatment at 65 hours in the single-dosing experiment (Figure 6.1). In contrast, cell numbers appear to have been unaffected after 65 hours exposure to the three doses of 1.0 nM microcystin-LR in the present experiment. Also, cell numbers at 10 nM microcystin-LR were very similar to levels seen in untreated control, whereas in the previous study at this concentration, a decline in cell numbers was observed which did not recover by 66 hours.

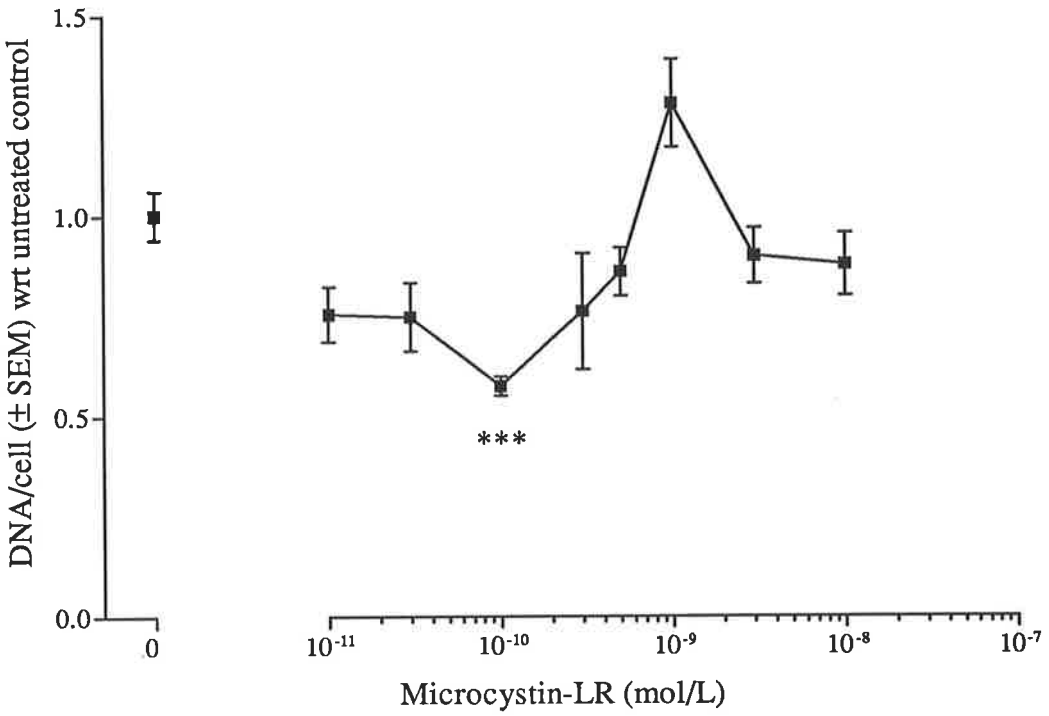



**Figure 7.3** - Effect on DNA per cell of 3 additions of microcystin-LR, with change of medium, at 2, 24 and 48 hours. Cells were harvested at 65 hours. Data points represent the average of between 3 and 9 plates ± SEM. Data were analysed by one-way ANOVA followed by Tukey's Multiple Comparison *post hoc* Test. Significant difference with respect to untreated control is indicated by *** ($p<0.001$). Differences between treatments are discussed in the text.

Hepatocyte DNA content, expressed on a per cell basis, is shown in Figure 7.3. At 0.1nM microcystin-LR, there was a significant ($p<0.001$) reduction of hepatocyte DNA content indicating that many of the exposed cells were stimulated to divide. This corroborates the indications found in the previous Chapter, namely that cell numbers increase in populations exposed to low doses of microcystin-LR because some hepatocytes are stimulated to undergo cytokinesis and/or karyokinesis followed by cytokinesis.

At 1.0 nM microcystin-LR, an apparent rise in cellular DNA content occurred. Although this rise was not statistically different from untreated control, it is significantly different from the effects of 0.1 nM and 0.5 nM toxin ($p<0.001$) and 10.0 nM toxin ($p<0.01$). It is a moot point as

to where the baseline for this comparison lies in a system where effects apparently opposite to those observed at one concentration, in this case 1.0 nM, were seen at lower concentrations. If it is assumed that the concept of sub-populations of cells proposed earlier is valid, then this represents a significant rise in the DNA content of these cells and is reminiscent of the effect elicited by a single dose of 10.0 nM microcystin-LR in the previous experiment.

The DNA content of cells exposed to 100 nM microcystin-LR was not shown on the above graph because of large errors generated by counting such small numbers of surviving cells.

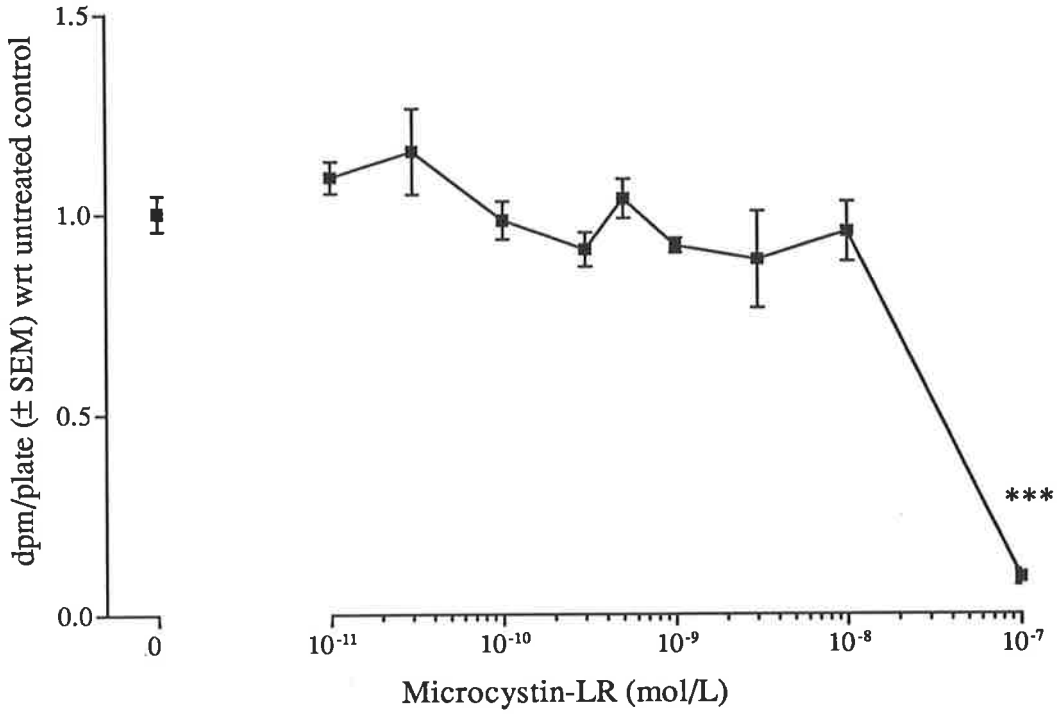



**Figure 7.4** - $^{3}$H-thymidine incorporation into DNA per plate following 3 additions of microcystin-LR, with change of medium, at 2, 24 and 48 hours. Cells were harvested at 65 hours. Data points represent the average of between 3 and 9 plates ± SEM. Data were analysed by one-way ANOVA followed by Dunnett's *post hoc* Test. Significant difference with respect to untreated control is indicated by *** ($p<0.001$).

The effect of microcystin-LR on thymidine incorporation per plate is shown in Figure 7.4. There was no statistically significant difference between any toxin dose below 100 nM, where cell numbers were dramatically reduced. Since cell numbers rise and then fall with increasing toxin concentration as previously described, this produces a thymidine incorporation per cell response curve very similar to that presented for DNA/cell above (Figure 7.3), and so this graph is not shown. It was considered of more interest to express thymidine incorporation in terms of μg DNA, giving an estimate of the ratio of newly synthesised DNA to original DNA at the end of culture period. This is shown in Figure 7.5.

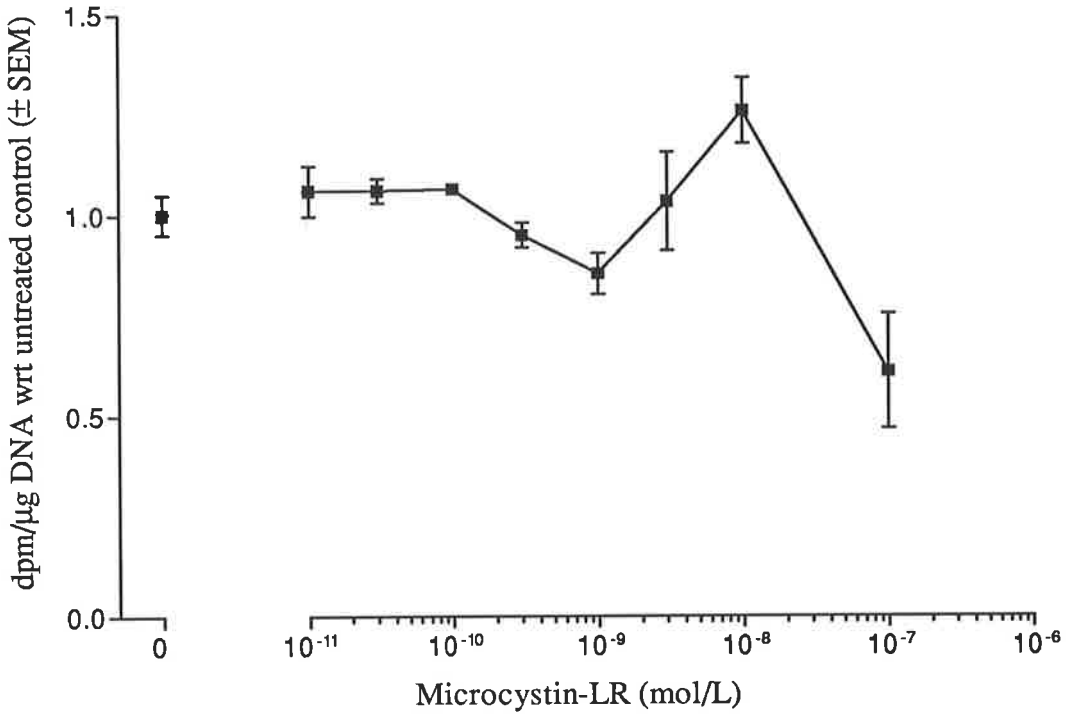



**Figure 7.5** - Effect on $^{3}$H-thymidine incorporation per µg DNA of 3 additions of microcystin-LR, with change of medium, at 2, 24 and 48 hours. Cells were harvested at 65 hours. Data points represent the average of between 3 and 9 plates ± SEM. Data were analysed by one-way ANOVA followed by Tukey's Multiple Comparison *post hoc* Test. There were no significant difference between any treatment and untreated control. Differences between treatments are discussed in the text.

As can be seen in Figure 7.5, microcystin had little effect on this ratio below 0.1 nM toxin after which there was a decreasing trend between 0.1 and 1.0 nM microcystin-LR although the measured $^{3}$H-thymidine incorporation/DNA at 1.0 nM was not significantly below that of the untreated control. Between 1.0 and 10.0 nM toxin, a reversal of this trend was seen. The peak of this apparent stimulation of DNA synthesis, at 10.0 nM, was not significantly different from untreated control, but was significantly different ($p<0.05$) from that at 1.0 nM, and that at 100.0 nM ($p<0.001$). The above argument as to the choice of baseline to use to prove significance applies here also.

### 7.3.2 Discussion of Section 7.3

The general outcomes from these experiments can be summarised as follows. Between 0.01 and 0.1 nM microcystin-LR an apparent increase in cell numbers was seen. The average cellular DNA content was low indicating a predominance of low ploidy cells (Figure 7.3). Thymidine incorporation per plate was comparable with control (Figure 7.4), so that thymidine incorporation per cell was reduced (not shown). This pattern is similar to that observed after a single 1.0 nM dose of microcystin-LR in the previous experiment, further corroborating the argument that the cells affected by microcystin-LR at low concentrations are being stimulated to divide without promotion of S-phase. More consistent protein phosphatase inhibition, a different timing of this inhibition or fewer cell losses due to acute toxicity may account for the fact the these effects are only apparent at pM toxin concentrations in these latter experiments.

Repeated dosing with 1.0 nM toxin had no apparent effect on cell numbers at 65 hours (Figure 7.2). In the previous single exposure experiment, a significant increase in cell numbers was observed at 18 and 42 hours, but this had declined by about 1/3 relative to its previous peak by 66 hours (Figure 6.1). This rise in cell numbers in the single dose experiment was attributed to a stimulation of cytokinesis in a sensitive population of cells, and the decline to subsequent loss of some of the cytokinesis-stimulated cells plus others rescued from cell death during the first 18 hours in culture. It might be expected therefore that the first dose of 1.0 nM microcystin-LR in these multi-addition experiments would cause a similar rise in cell numbers followed by a subsequent reduction. However, previously there was a marked drop in DNA per cell (Figure 6.3), concomitant with the stimulation of cytokinesis, followed by an increase again towards control levels as these cells began to synthesise DNA. In this experiment three doses of 1.0 nM

microcystin-LR appear to have caused a rise in average cellular DNA content compared with lower toxin concentrations (Figure 7.3), similar to the effect of 10 nM microcystin-LR in the single exposure experiment.

At 10.0 nM microcystin-LR, one addition was previously shown to kill approximately half of the cells within the first 18 hours (Figure 6.1). However, three additions of microcystin-LR did not cause a decrease in cell numbers at 65 hours (Figure 7.2). Since these cells were still actively incorporating thymidine, it is likely that the cells surviving the first exposure subsequently divided so that cell numbers returned to the original levels. This was accompanied by a reduction of cellular DNA to the original level (Figure 7.3), and by a peak in the ratio of labelled to unlabelled DNA (Figure 7.5). This peak does not necessarily imply a mitogenic stimulation of DNA synthesis, since this outcome could also arise if the "microcystin-insensitive" cells left to grow were, in general, inherently more proliferatively active than the general population, that is, a greater proportion of them were likely to be in the cell cycle at any given time. This is necessarily the case if cell numbers increased by 100% in approximately one complete cell cycle. It follows therefore that the cells killed initially were less likely than the general population to proliferate, which would be the case if they were more differentiated (and therefore expressed higher numbers of bile acid transporters). It may also be the case that further additions of toxin, perhaps taken up less avidly by older cells, had a stimulatory effect on cytokinesis, causing the cells to divide sooner and leading to a quicker replacement of cell numbers.

The results obtained in the experiments just described can be explained in terms of the cellular processes elucidated in the previous experiment. There were differences in the outcomes between the experiments at any given microcystin-LR concentration, indicating that a more or less constant level of exposure to a particular toxin concentration does not have the same effect as a

single dose of that concentration. Perhaps the most surprising finding in these multi-addition experiments was the fact that microcystin-LR concentrations in the pM range appeared to have an effect on a substantial proportion of the hepatocyte population, significantly reducing the average cellular DNA content by about 40% (Figure 7.3). Although low concentrations of okadaic acid (0.16 nM) have previously been shown to recruit SHE cells from G0 into G1 and to promote DNA synthesis (Afshari and Barrett, 1994; Afshari, *et al.*, 1994), an induction of mitosis was not reported. The effects seen on cell numbers (Figure 7.2) and thymidine incorporation (Figure 7.5) in the present experiments were induced by nM microcystin-LR concentrations, that is, about 100x less than those found necessary by Mellgren *et al*, (1993). Higher concentrations of microcystin-LR have generally been used in the past, partly because frank cytoskeletal disruption was being studied. However, because hepatocytes from most strains of mouse require mitogenic stimulation to grow for any length of time in culture (Berry, *et al.*, 1991), it may be that such stimulation confounds the interpretation of any effects seen in response to microcystin-LR. This possibility was examined in the experiment discussed in the following section.

## 7.4 Effect of microcystin-LR on EGF stimulated cells.

An experiment was conducted using a similar protocol to the previous experiment, except that EGF-treated cells were exposed to microcystin-LR (0.1, 1.0, and 10.0 nM) and harvested at 66 hours.

### 7.4.1 Results

The data for the ratio of labelled to unlabelled DNA are shown in Figure 7.6.

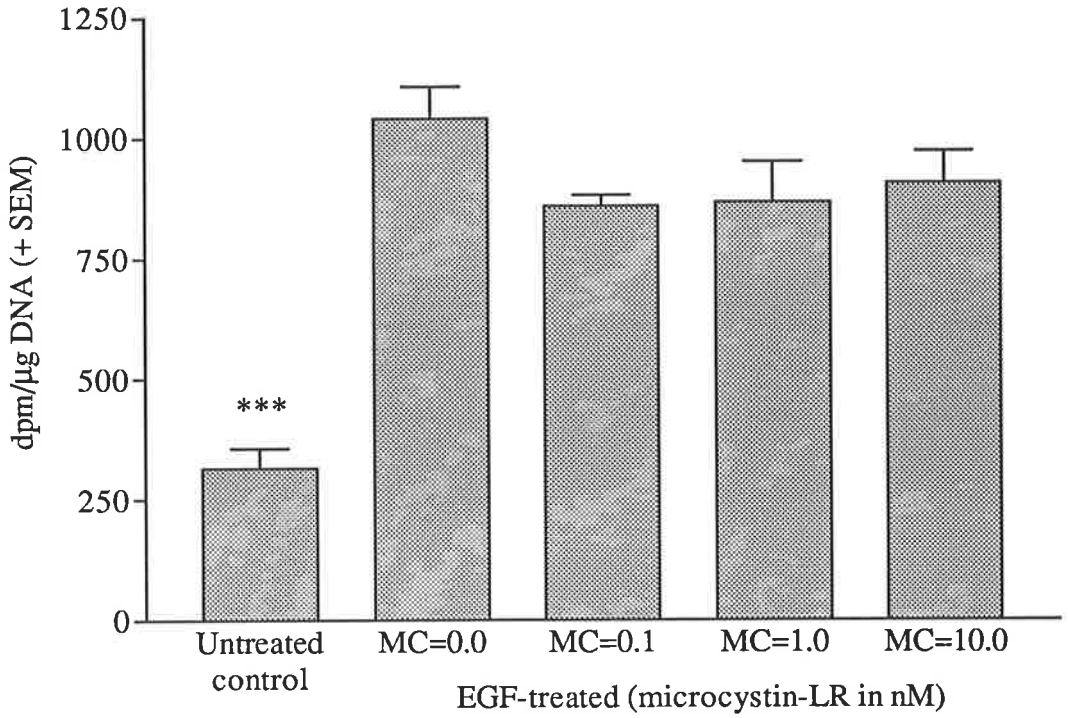



**Figure 7.6** - Effect of EGF on the response of hepatocytes to exposure to microcystin-LR. C3H/HeJ hepatocytes were grown in standard culture medium including 20 ng EGF/ml. Various concentrations of microcystin-LR (MC) were added at 2, 24 and 48 hours with medium changes. Cells were harvested at 65 hours and the $^{3}$H-thymidine incorporation per µg DNA was determined. Untreated control was significantly lower ($p<0.001$) than all other treatments. There were no significant difference between microcystin-treated groups (cf. Text accompanying Figure 7.5).

As seen in Figure 7.6, none of the EGF and toxin-treated groups had significantly different rates of new DNA synthesis compared to the EGF-alone treatment, although they all appear to be slightly decreased. More significantly, there were no differences between microcystin-LR treatment groups where in non-EGF treated cells they were readily apparent (Figure 7.5). The untreated control had a markedly slower rate of DNA synthesis than all other treatment groups ($p<0.001$).

### 7.4.2 Discussion of Section 7.4

These results indicate that non-EGF treated cells are more susceptible to microcystin-LR than are EGF treated cells. This is not surprising since a mitogen such as EGF is likely to have marked effects on hepatocyte survival and the level of differentiation of the cells. For example, the initial cell and DNA losses seen in untreated controls were not apparent in EGF treatments (Figures 6.1 and 6.2). Furthermore, the proportion of cells proliferating upon EGF treatment will be higher, their rate of divisional mitosis will be different, and the ratio of proliferation to cell loss will be altered. Since microcystin-LR appears to have effects on the rates of both cell death and cell cycle dynamics of the hepatocyte population, treatment with EGF would at least complicate the observation of these effects and possibly counter them. The hepatocyte population *in vivo* is not likely to be exposed to such high levels of mitogenic stimulation.

## 7.5 Comparison of microcystin-LR with okadaic acid

At concentrations less than 100 nM, microcystin-LR appeared to be influencing only part of the total hepatocyte population, a phenomenon possibly mediated by differential transporter expression and hence microcystin-LR uptake. It was therefore of interest to see what effect okadaic acid might have in the same concentration range. Okadaic acid does not require active uptake and so should not exhibit a threshold below which only some cells respond. However, nor does it bind covalently to the protein phosphatase catalytic subunits, and it causes about 100x greater inhibition of PP2A than PP1. These factors may lead to other variations between the effects of microcystin-LR and okadaic acid, but if all cells are responding to okadaic acid based solely on its concentration in the medium, then a simple dose-response curve should still result, in contrast to many of the effects induced by microcystin-LR. This has, in fact, been reported

previously by Mellgren *et al* (1993), but following on from the results of the preceding Sections, it was also of interest to determine the concentration range in which okadaic acid was active in this culture system.

The hepatocyte isolation and culture protocols were the same as for the other experiments described in this Chapter, with the exception that okadaic acid was added in place of microcystin-LR.

### 7.5.1 Results

The comparative effects of these toxins on cell numbers, hepatocyte DNA content, thymidine incorporation per plate, thymidine incorporation per cell and the ratio of thymidine labelled to unlabelled DNA are shown in the following figures.

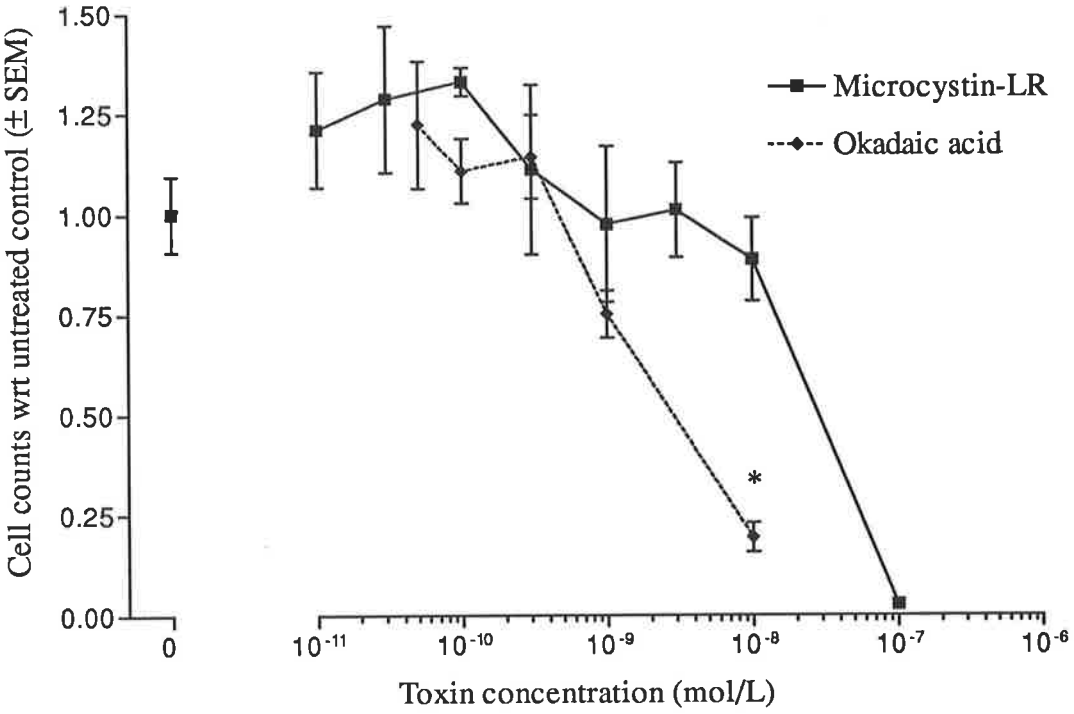



**Figure 7.7** - Comparison of the effects of microcystin-LR and okadaic acid on hepatocyte numbers. Three additions of toxin were made, with the change of medium, at 2, 24 and 48 hours. Cells were counted at 65 hours. Data were normalised to the untreated control. Significance of differences between the treatments at each concentration were determined with the t-test.
* ($p<0.05$)

Okadaic acid was non-toxic below 0.3 nM. However, above this concentration toxicity increased rapidly so that by 10 nM, okadaic acid appears to have killed approximately 5-fold more cells than microcystin-LR ($p=0.026$). Cell numbers at okadaic acid concentrations below 0.3 nM may be slightly raised with respect to untreated control, but not to the same degree as in the microcystin-LR treatments.

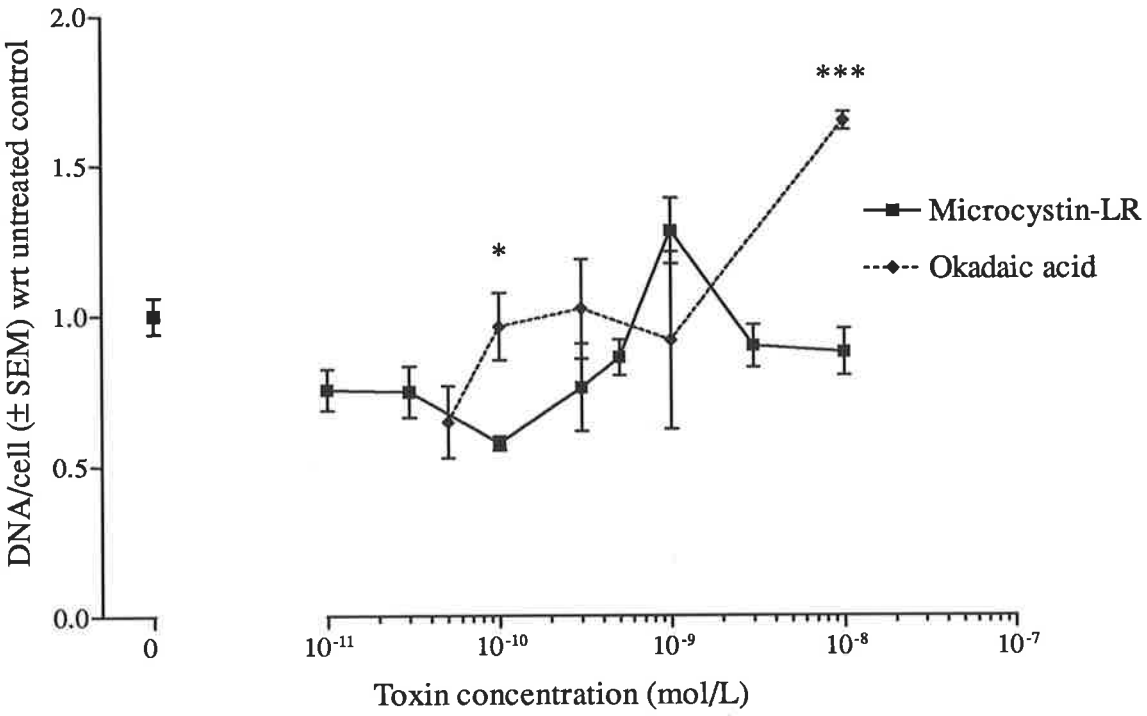



**Figure 7.8** - Comparison of the effects of microcystin-LR and okadaic acid on hepatocyte DNA content. Three additions of toxin were made, with the change of medium, at 2, 24 and 48 hours. Cells were harvested at 65 hours. Data were normalised to the untreated control. Significance of differences between the treatments at each concentration were determined with the t-test.
* ($p<0.05$), *** ($p<0.001$).

Consistent with the observation that cell numbers did not rise as much in response to low okadaic acid concentrations as to low levels of microcystin, the average DNA content of the hepatocytes was not lowered by okadaic acid. This suggests that cytokinesis has not been stimulated by okadaic acid in this concentration range. At the 10 nM toxin level, the DNA content of okadaic acid exposed cells was markedly higher than in microcystin-LR treated cells. This increase due to okadaic acid could result either from a block of cytokinesis in most of the 20% of the original cells remaining or from a selection against low ploidy cells.

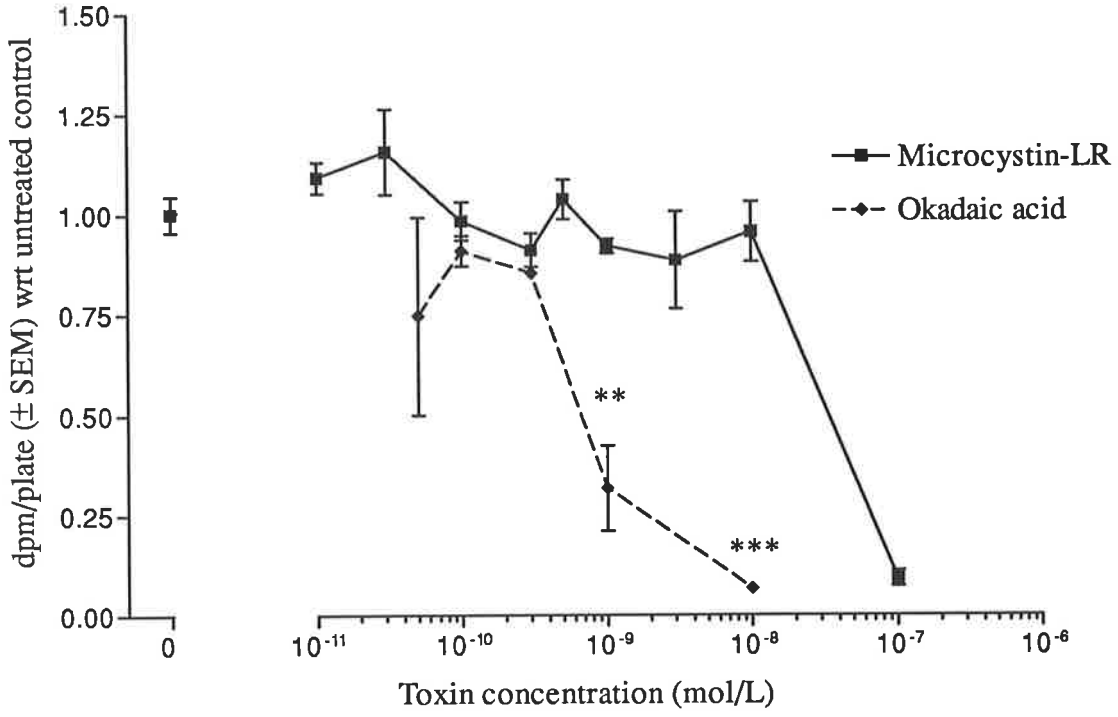



**Figure 7.9** - Comparison of the effects of microcystin-LR and okadaic acid on $^{3}$H-thymidine incorporation per plate. Three additions of toxin were made, with the change of medium, at 2, 24 and 48 hours. Cells were harvested at 65 hours. Data were normalised to the untreated control. Significance of differences between the treatments at each concentration were determined with the t-test. ** ($p<0.01$), *** ($p<0.001$).

Thymidine incorporation per plate was inhibited by okadaic acid at concentrations above 0.3 nM. This was even more marked than its effects on cell numbers, since at 1.0 nM okadaic acid thymidine incorporation was reduced to 30% of untreated control whereas cell numbers were still 75% of control. Similarly at 10 nM okadaic acid, thymidine incorporation was reduced to 10% of controls whilst cell numbers were 20%. Even at the lower concentrations, thymidine incorporation appeared to be reduced compared with control levels. These data therefore suggest that DNA synthesis inhibition occurs at concentrations substantially below those at which cells are killed. In contrast, microcystin had essentially no effect on thymidine incorporation per plate until the concentration was greater than 10 nM.

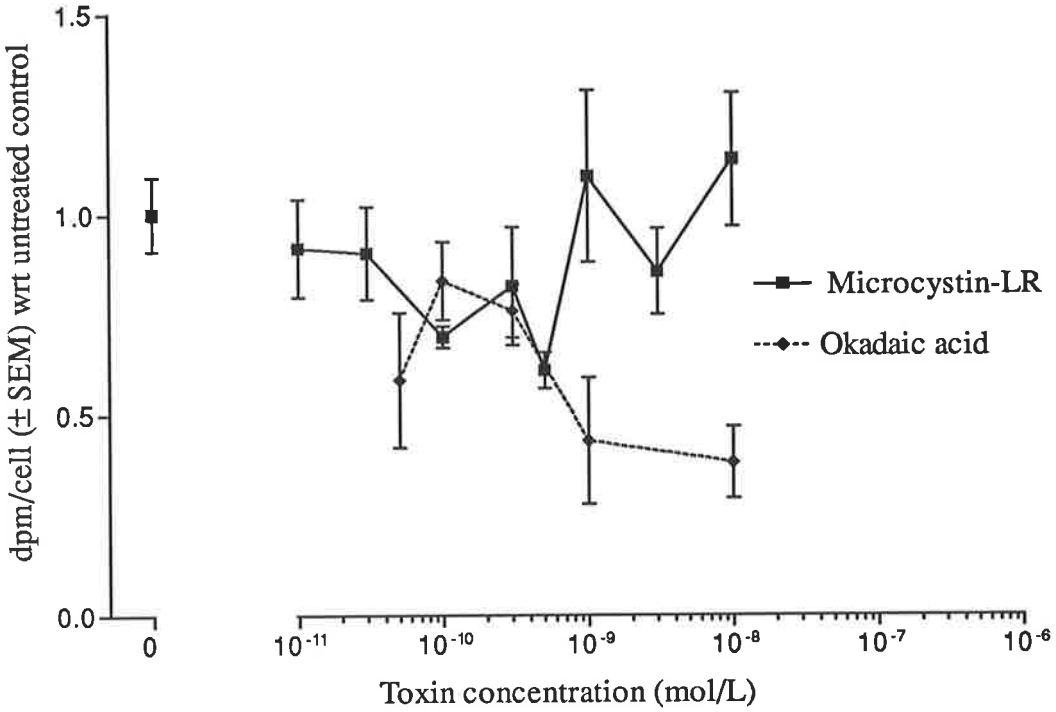



**Figure 7.10** - Comparison of the effects of microcystin-LR and okadaic acid on $^{3}$H-thymidine incorporation per cell. Three additions of toxin were made, with the change of medium, at 2, 24 and 48 hours. Cells were harvested at 65 hours. Data were normalised to the untreated control. There were no significance of differences between the treatments at any single concentration as determined by t-test. However, when the trend slopes for each toxin between 0.1 and 10.0 nM were compared using linear regression, it was found that they were significantly different ($p<0.05$).

When thymidine incorporation on a per cell basis was compared between these toxins, there was a significant difference in the response trends at concentrations between 0.1 nM and 10 nM ($p=0.023$). That is, the slopes of linear regression lines computed for each toxin between these concentrations were significantly different. This again indicates that the effects of these toxins on DNA synthesis are different, with okadaic acid becoming increasingly inhibitory with increasing concentration.

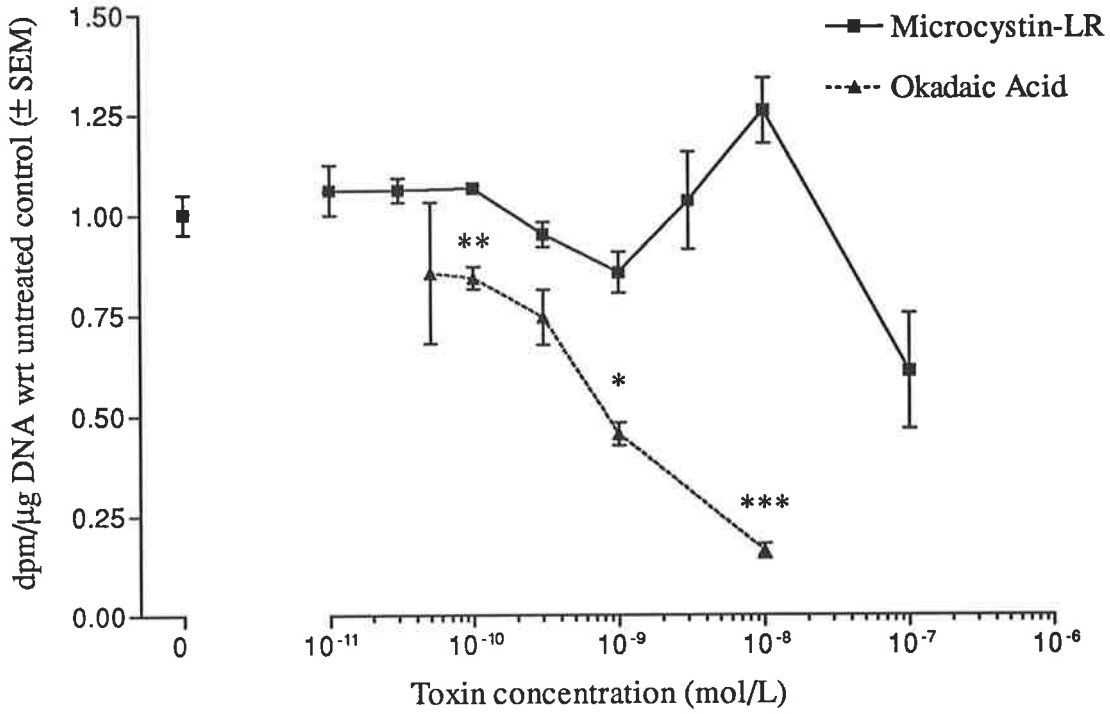



**Figure 7.11** - Comparison of the effects of microcystin-LR and okadaic acid on $^{3}$H-thymidine incorporation per µg DNA. Three additions of toxin were made, with the change of medium, at 2, 24 and 48 hours. Cells were harvested at 65 hours. Data were normalised to the untreated control. Significance of differences between the treatments at each toxin concentration were determined by t-test. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

When the ratio of radiolabelled to unlabelled DNA was plotted it is evident that there was a marked difference in the way that these toxins interact with the hepatocyte population. Okadaic acid caused a significant reduction in this ratio throughout the concentration range examined. The trends caused by the two toxins were similar at concentrations below 1.0 nM, but there was no increase again above 1.0 nM with okadaic acid, the trend instead continuing downward.

### 7.5.2 Discussion of Section 7.5

From the cell number data above (Figure 7.7) okadaic acid is approximately 10x more toxic than microcystin-LR in terms of cell killing. However, as became apparent in earlier experiments,

this is an over-simplistic view since these data represent not only cell killing but also subsequent proliferation. This point is especially important in the higher concentration range above 1.0 nM where toxicity dominates the picture and where the difference between the toxins is most marked. It was found previously that 10 nM microcystin-LR kills approximately 50% of cells soon after exposure (Figure 6.1). Therefore, since by 65 hours in culture cell numbers are not significantly reduced from normal (Figure 7.7), there must have been substantial regrowth and this is reflected in the continued thymidine incorporation (Figure 7.9), the high thymidine incorporation per cell (Figure 7.10) and in the high ratio of labelled to unlabelled DNA (Figure 7.11). In contrast, these values are all reduced in the okadaic acid treatment at this concentration, more or less in line with cell numbers. Therefore, unlike microcystin-LR toxicity, there do not appear to be any cells which are resistant to okadaic acid toxicity and which can therefore grow in its presence.

This lack of sensitivity to microcystin-LR at later culture times could be explained by loss of uptake. However, in the $^{3}$H-microcystin-LR uptake study (Section 5.3.3) there was still evidence of toxin uptake after 42 hours culture, when morphological signs of acute toxicity were not evident and after cell killing had apparently ceased, suggesting that these hepatocytes had only lost sensitivity to the acutely toxic effects of microcystin during culture, rather than the ability to transport it. However, others have shown a typical morphological response of cells micro-injected with microcystin-LR after 55 hours in culture (Mellgren, *et al.*, 1993). But microinjection into the cytosol may be functionally different from delivery via membrane transport to an intracellular transport system (Petzinger, 1994). If both the intra-cellular transport system and active bile acid transport are lost in culture, leaving only slow toxin uptake via a non-saturable mechanism (Frimmer and Ziegler, 1988), or an as yet uncharacterised transporter (Runnegar, *et al.*, 1995a), then an intracellular distribution of the toxin would occur which was markedly different from that arising from either microinjection of a bolus of toxin, or active uptake and concentrated delivery

to intracellular sites of action. Therefore, this loss of sensitivity to microcystin-LR in cultured cells could also occur as the result of slower uptake in combination with poor delivery of toxin to sensitive sites.

Resistance to the first dose of microcystin-LR could also be explained by differential expression in the freshly isolated cells of the transporters needed for its uptake. It is known for example that uptake of bromosulphthalein, a compound known to interfere with hepatocellular uptake of microcystins (Runnegar, *et al.*, 1981; Eriksson, *et al.*, 1990a), varies with differentiation state (Deschenes, *et al.*, 1981). The zonal distribution of biochemical functions, and of the pattern of acute toxic effects, in the intact liver, support this view. Microcystin-LR would therefore be selecting against more differentiated cells.

At lower concentrations the differences between these toxins were less marked. The apparent increase in cell numbers due to 0.1 nM microcystin-LR was more pronounced than the effect caused by this concentration of okadaic acid (Figure 7.7). This may be linked to the lack of a decrease in DNA per cell at this concentration (Figure 7.8), suggesting that okadaic acid did not stimulate cytokinesis. Thymidine incorporation per cell was the same with either toxin (Figure 7.10) but the ratio of labelled to unlabelled DNA was significantly lower with okadaic acid compared to microcystin-LR (Figure 7.11). Since a larger cell contains more DNA, requiring more thymidine incorporation for its replication, the larger cells in the okadaic acid treatment must have replaced their DNA less frequently than the smaller cells in the corresponding microcystin-LR treatment. This may indicate a greater inhibition of DNA synthesis by okadaic acid at pM concentrations, or it may simply reflect a slower rate of growth in these higher ploidy cells.

## 7.6 General Discussion of Chapter 7

The findings reported in Chapter 6 suggested that a number of processes were induced by various concentrations of microcystin, including inhibition of normal cell death, both stimulation and inhibition of cytokinesis, and death due to acute toxicity. Even at a single concentration of toxin there are likely to be a range of responses induced in different cells depending on their ability to actively take up the toxin and their ability to respond to its effects. This ability might depend upon cell cycle, ploidy, differentiation, and general metabolic status. Therefore, the experimental outcome seen will be an amalgam of these various cellular responses. This is the inevitable consequence of having to use such a heterogeneous model system.

In the present Chapter, further experiments covering a wider concentration range and triple additions of microcystin-LR over three days were reported, and similar processes to those described in Chapter 6 were identified. However, the concentration at which promotion of cytokinesis occurred was about 0.1 nM or one tenth of that inducing this effect in the single dose experiments, whereas inhibition of cytokinesis occurred at about 1.0 nM, again about one tenth of the effective concentration observed in the previous experiment. A number of reasons might be suggested for these discrepancies. At lower concentrations it is likely that there will be less cell loss due to acute toxic effects. Instead, sub-toxic effects might be more pronounced as more of the sensitive population remain to be observed. Also, with three doses of toxin a more constant inhibition of protein phosphatases might occur, counteracting the replacement of enzyme via *de novo* synthesis. Furthermore, for about the first 12 hours in culture, cellular metabolism will be devoted to re-establishing contact with other cells and with the collagen substrate, and perhaps entry into the cell cycle, a regeneration-like response having been initiated by the isolation procedure (Fausto and Webber, 1994). At later times, the sensitivity of the hepatocyte will change

as uptake decreases, exposing each cell to less toxin than would otherwise be the case earlier in the culture period. Therefore, a single exposure to toxin early in the culture period might be expected to have quantitatively different effects than lower doses given over a longer time.

At 10.0 nM microcystin-LR in the multi-addition experiments, cell numbers and cellular DNA content had returned to control levels by 65 hours, whereas in the single dose experiments cell numbers were still at half control levels and cellular DNA content at twice these. If the further doses of toxin had killed the cytokinesis-blocked cells to return cell DNA content to normal, then cell numbers should have been even lower than in the first experiment. Therefore, the initially surviving cytokinesis-blocked cells were still alive and had divided. The additional toxin doses did not appear to have any major influence on these surviving cells. For example, there was no evidence of a stimulation of cytokinesis in the previously undivided population, although this cannot be discounted. There was evidence that these surviving cells were able to undergo DNA synthesis, and that a high proportion of them were doing so because cell numbers returned to normal levels and they contained a relatively high proportion of radioactively labelled DNA. However, there was no evidence of an EGF-like stimulation of proliferation beyond the effect caused by preferentially killing cells less likely to proliferate.

The effects of microcystin-LR and okadaic acid were different in a number of ways despite their shared inhibition of protein phosphatases. At low doses (pM) there was no evidence that okadaic acid could promote cytokinesis since the average hepatocellular DNA content was not reduced. The average rate of DNA synthesis was lower in the okadaic acid exposed cells than in their microcystin-exposed counterparts, although whether this was due to inhibition by okadaic acid, or because this toxin did not change the ploidy and therefore proliferative profile of the population, cannot be determined. At higher (nM) concentrations, in contrast to microcystin-LR

treated cells, there was a marked reduction of cell numbers. DNA synthesis inhibition preceded cell death since the degree of inhibition of thymidine incorporation at a given concentration was greater than the corresponding reduction in cell numbers. However, the two processes occurred in parallel, indicating that they are linked in all cells present. Therefore, as toxin concentration increased, thymidine incorporation per cell tended to decrease in okadaic acid treated cells but increase in microcystin-LR treated cells. There was no evidence for a cell selective toxicity by okadaic acid, a major difference with microcystin at these concentrations.

These effects of nM microcystin-LR and okadaic acid can be compared with the qualitatively very similar findings of Mellgren *et al*, (1993). These authors also found, using different but analogous analytical endpoints to those used here, that decreases in the proportions of morphologically normal cells and those which were $^{3}$H-thymidine labelled were closely correlated when okadaic acid was used, but that for microcystin-LR, there was a critical concentration at which the proportion of normal cells began to fall but the proportion of thymidine labelled cells increased.

Mellgren *et al*, (1993) speculate that the reason for the different dose-responses of hepatocytes to microcystin-LR and okadaic acid is these toxins' different specificities towards the protein phosphatases, such that inhibition of PP2A alone by okadaic acid causes a reduction in the rate of DNA synthesis, whereas inhibition of both PP2A and PP1 by microcystin-LR does not. This may well be the case, however in the light of all of the foregoing, it seems likely that at least part of the difference between these toxins can be explained in terms of uptake. That is, microcystin-LR requires active uptake by cells expressing high copy numbers of transporters and/or with high specificity, whereas okadaic acid is passively acquired by the cells and hence affects all cells more or less equally.

Interestingly, the concentrations of each of these toxins required to produce a given degree of effect were about 100 times higher in the experiments of Mellgren *et al.* (1993) than here. Their primary rat hepatocytes were stimulated to proliferate with 9 nM EGF and were treated with toxins from 44 until 56 hours in culture. Okadaic acid at approximately 200 nM caused the loss of normal morphology in 50% of cells remaining on the plate after a 12 hour exposure which began at 44 hours in culture. In the present experiments, only 3 nM okadaic acid, when added at 2 hours culture time, was required to reduce cell numbers to 50% of initial levels (Figure 7.14). Similarly, in the Mellgren work, approximately 3 μM microcystin-LR caused deformation of 50% of cells, whereas in this system 10 nM microcystin-LR caused an initial loss of 50% of cells and 30 nM was enough to cause cell numbers to be at 50% of initial levels at 65 hours in culture. Since hepatocyte sensitivity to both toxins was higher in the present work than in that reported by Mellgren *et al.*, (1993) this precludes loss of uptake of microcystin-LR in culture as a reason for this difference. Similarly, both rat and mouse hepatocytes respond similarly to toxic concentrations of microcystin-LR, so it seems unlikely that there would be such a marked difference in their responses to lower concentrations, although this cannot be discounted. However, results presented in Figure 7.6 showed that DNA labelling variations due to microcystin-LR were much less marked in EGF-treated cells, thus it seems that cells so treated are less sensitive to microcystin-LR. This probably accounts for the difference in the results observed.

Some effects indicative of a delay of cell death were found in hepatocytes exposed to microcystin-LR. These might be explained by known effects of phosphatase inhibitors on processes likely to be involved in cell death. For example, okadaic acid at 30 nM during short incubations with rat hepatocytes has been shown to inhibit autophagy (Blankson, *et al.*, 1995). Also, microtubule-based vesicle transport is inhibited by similar concentrations of either okadaic

acid or microcystin-LR (Hamm-Alvarez, *et al.*, 1996). It is likely that one or both of these processes are involved in the breakdown of cellular components during cell death (Bursch, *et al.*, 1994), and therefore this latter process might also be susceptible to a cytoskeleton-disrupting toxin. The levels of toxin used here were far lower than those used in the above mentioned studies, but the exposure times were much longer, and more critically, microcystin-LR is concentrated by the cell, leading to much higher internal concentrations than exist in the culture medium. Therefore, it is possible that microcystin-LR, but less probably okadaic acid, could have an inhibitory effect on the rate of cell death, leading to the observed retention of non-proliferative DNA in the system. This issue is explored further in the next Chapter.

The production of binuclear cells normally occurs via acytokinetic mitosis in previously diploid cells. These accumulate as binucleate tetraploids, some of which subsequently undergo a second round of DNA synthesis before karyokinesis and cytokinesis, producing two tetraploid daughter cells. Similarly, a proportion of these go on to produce binuclear and mononuclear octaploids. A stimulation of karyokinesis and/or cytokinesis must occur in these polyploid cells since diploids are unlikely to able to be stimulated to enter karyokinesis or cytokinesis without prior DNA synthesis since they would end up with a haploid nuclear content, which would probably make them non-viable. There are too few octaploids to produce all of the cell increase seen, and so the tetraploid cells must also be involved. That protein phosphatase inhibition can promote a karyokinesis-like phenotype has been demonstrated previously. For example, okadaic acid was able to promote nuclear envelope breakdown and premature chromosome condensation (PCC) in mouse eggs (Dyban, *et al.*, 1993). Similarly, in a Golden hamster fibroblast cell line, okadaic acid caused a transient activation of cdc2 and a pre-mitotic phenotype (Yamashita, *et al.*, 1990). MPM-2 monoclonal antibody staining, indicative of mitosis-specific phosphorylations, was increased, nuclear lamins became dispersed in the cytoplasm, and mitotic asters, but not spindles,

formed, and PCC occurred. However, all of these changes were transitory even with continued incubation with okadaic acid. But there is critical difference between the cellular models just referred to and hepatocytes. The cells used by these groups were synchronised in the cell cycle so that effects could be observed. Since they were actively growing, the regulatory framework defining the sequence of events would likely be strictly enforced, with any perturbation causing an abortion of the cell cycle. In contrast, most of the hepatocytes used here were polyploid, "frozen" part-way through the cell cycle, and therefore these effects would not be "premature". Since they are polyploid, a promotion of nuclear and cellular division may not necessarily be fatal, as it is likely to be in a diploid cell. Conceivably, such an occurrence might not be as strongly prohibited. It might even be the case that polyploid hepatocytes are primed to receive such a stimulation, depending upon how such a block on mitosis comes about in the first place. Since cyclin B is synthesised during $G_1$ and the cdc2/cyclin B complex accumulates during S-phase, it may be that only the final activation step is withheld in mononuclear polyploid cells. This step is known to involve dephosphorylation of cdc2 by the cdc25 dual specificity phosphatase, which in turn is thought to be a substrate for PP1 or PP2A (Izumi, *et al.*, 1992; Lorca, *et al.*, 1992). The cdc2/cyclin B complex must also be phosphorylated on Thr-161 for activity, and so if an inactivating phosphatase was microcystin sensitive, this might provide an additional means for the activation of the mitotic kinase. There are a great many nuclear and cytoplasmic cellular targets of active cdc2/cyclin B, the phosphorylative activation of which are implicated in processes such as chromosome condensation, nuclear envelope breakdown, rearrangement of actin microfilaments, and activation of the centrisome and initiation of microtubule shortening for formation of spindles (Tiwari, *et al.*, 1996). PP2A has also been implicated as a countervailing phosphatase at some of these sites (Ferrigno, *et al.*, 1993), and so its inactivation might allow an increase in the rate of phosphorylation of these sites, leading to many of the experimental outcomes quoted above.

Initiation of cytokinesis, with or without microcystin-initiated karyokinesis, requires firstly, the deactivation of cdc2/cyclin B. This could arise via cdc2/cyclin B mediated activation of the cyclin proteolytic degradation system, either as the result of previously increased cdc2/cyclin B activity or decreased countervailing phosphatase activity, in response to microcystin-LR. A second requirement for initiation of cytokinesis, once the actin microfilament system has assembled at the contractile ring, is both phosphorylation and dephosphorylation of the myosin II regulatory light chain (Satterwhite, *et al.*, 1992; Holloway, *et al.*, 1993; Surana, *et al.*, 1993). Inhibitory phosphorylation is catalysed by cdc2/cyclin B, but the opposing phosphatase for this action is unknown (Satterwhite, *et al.*, 1992). Activating phosphorylations are catalysed by myosin light chain kinase (MLCK) and a Rho-associated kinase (Amano, *et al.*, 1996), and the opposing phosphatase to these activities appears to be a myosin-associated PP1 (Cohen, *et al.*, 1988; Cohen, 1989; Johnson, *et al.*, 1996) the activity of which, in smooth muscle, is reduced in the presence of microcystin-LR (Ikebe and Brozovich, 1996). From the foregoing, it seems credible that inhibition of protein phosphatase activity could lead to initiation of cytokinesis, with or without prior karyokinesis, but more detailed knowledge of the biochemical mechanisms involved in these complex processes is required to confirm this.

An alternative mechanism by which microcystin-LR could activate cytokinesis is via $Ca^{2+}$ mobilisation. Actomysin filament contraction is known to be stimulated by $Ca^{2+}$ treatment (Ikebe and Brozovich, 1996) since MLCK is calcium/calmodulin dependent (Satterwhite and Pollard, 1992), and microcystin-LR treatment (2.5 - 5.0 μM) has been shown to cause a rapid release of $Ca^{2+}$ from intracellular stores in isolated rat hepatocytes (Falconer and Runnegar, 1987a). Therefore, this $Ca^{2+}$ mobilisation could also be involved in a stimulation of cytokinesis, although again, too little is known of the specific role of microcystin in this process to evaluate the likelihood of this mechanism.

A cytokinetic inhibition can only be detected in a proliferating cohort because without continued DNA synthesis a block of cytokinesis could not cause an accumulation of cells with increased levels of DNA. An inhibition of cytokinesis by okadaic acid (20 μM) has been reported in *Schizosaccharomyces pombe* (Kinoshita, *et al.*, 1993). The uptake of this toxin was reported to be poor in yeast, perhaps explaining the high concentration used. In the present study, three additions of medium containing 10 nM okadaic acid appeared to cause a rise in DNA per cell (Figure 7.8), but since this is only one data point and only about 20% of the cells remained, this cannot be relied upon. A single addition of 10 nM microcystin-LR was also shown to cause a delay in cytokinesis (Figure 6.3), but this did not appear to be a permanent block, and prolonging the exposure by renewing the toxin-containing medium every 24 hours did not maintain the effect. Whether this effect is a block of cytokinesis specifically or some process earlier in the cell cycle, for example karyokinesis, or actin microfilament assembly at the contractile ring, cannot be determined from the data.

# CHAPTER 8

# INHIBITION OF CELL DEATH WITH NAFENOPIN

## 8.1 Introduction

Apoptotic cell death in response to μM concentrations of microcystin-LR has previously been postulated (Boe, *et al.*, 1991). Furthermore, effects on the ratio of proliferation to cell loss were implied in previous experiments described in this thesis. Nafenopin is a peroxisome proliferator which has been shown to inhibit apoptosis in cultured primary rat hepatocytes (Bayly, *et al.*, 1994). Fifty μM nafenopin was shown to maintain primary hepatocytes in culture for at least six weeks, whereupon its withdrawal allowed the recommencement of apoptotic cell death. Nafenopin is thought to change the balance between apoptosis and cell growth, allowing cells which would otherwise be "self-culled" to instead survive and proliferate (Gill, *et al.*, 1995).

Nafenopin was used in the following experiment to assess the potentially confounding effect of apoptosis on the results. In particular, between 0.01 and 0.1 nM microcystin-LR it is possible that a component of the observed increase in cell numbers was due to a reduction in losses due to cell death, that is, an anti-apoptotic effect was being induced. Therefore, it would be of interest to determine whether the effects of nafenopin and microcystin-LR at this concentration on cell numbers are additive. If so, then different processes probably mediate the cell preserving effects of these compounds. Whether or not a cytokinetic stimulation was induced in the larger nafenopin-rescued population would also be of interest. This should be distinguishable from cell preservation on the basis of measurements of cellular DNA content.

Also, at 10.0 nM microcystin-LR, it was suggested earlier that the observed peak in the ratio of labelled to unlabelled DNA (Figure 7.5) was most likely due to normal cell replacement by an inherently active sub-population of cells following the initial loss of a less active group due to acute toxicity. If this is the case then reduction of initial cell losses should reduce this effect, and it would also imply that the type of cell death induced by microcystin-LR was that which is inhibitable by nafenopin, that is apoptosis. However, if cell numbers were initially halved by the first addition of 10.0 nM microcystin-LR, as was seen in the single-addition experiment (Figure 6.1), then they must have doubled after the first exposure to the toxin in this multi-addition experiment in order to return to original levels by 65 hours. Since there was no evidence for a stimulation of cytokinesis to account for such a marked increase, the possibility remains of some stimulatory effect of microcystin-LR on the rate of entry of cells into the cell cycle at this concentration. If such cells were more likely to become apoptotic as a result of this, or if microcystin-LR could induce a similar effect in the nafenopin-rescued population, then the effects of the two compounds together on cell numbers and thymidine incorporation would be additive. Therefore, two concentrations of microcystin-LR in the low region (0.01 and 0.03 nM) and two in the high region (3.0 and 10.0 nM) were analysed for interactions with nafenopin.

## 8.2 Materials and Methods

Hepatocyte isolation and culture conditions were as previously described for the multi-addition experiments, except that 50 μM nafenopin (the kind gift of Dr. Paul Wright, Royal Melbourne Institute of Technology) was used in one set of plates, other set being nafenopin-free. Microcystin-LR was added to both sets of plates at concentrations of 0.01, 0.03, 3.0 or 10.0 nM. Medium was changed at 2, 24 and 48 hours, and the cells counted and harvested at 65 hours.

## 8.3 Results

### 8.3.1 Controls

The effect of nafenopin (50 μM), EGF (20 ng/ml), alone and in combination, on hepatocyte numbers, their rate of DNA synthesis, and their DNA content, is shown in Figure 8.1. Microcystin-LR was not added to any of these control treatments.

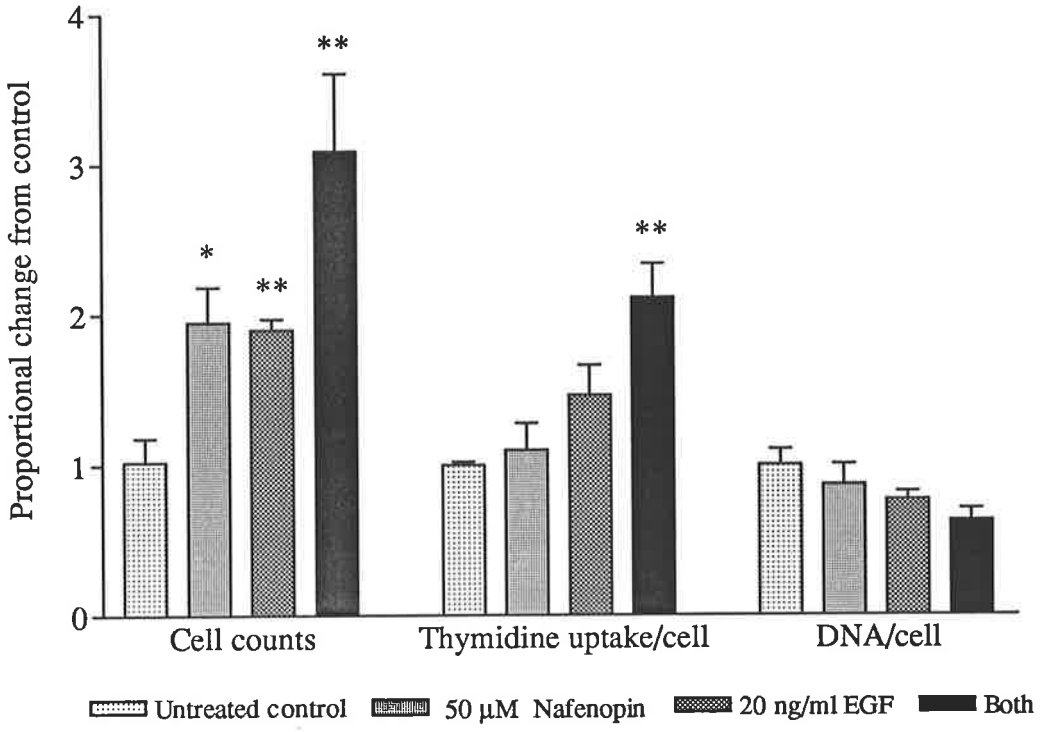



**Figure 8.1** - Effect of nafenopin (50 μM) and EGF (20 ng/ml) on hepatocyte numbers, $^{3}$H-thymidine incorporation per cell, and μg DNA per cell. C3H/HeJ cells were grown in standard medium with or without additions of nafenopin, EGF, or both. Medium was changed at 2, 24, and 48 hours and cells were harvested at 65 hours. Data are normalised to untreated control. Significant differences from untreated control are indicated as * ($p<0.05$) and ** ($p<0.01$).

Each of these mitogens caused a doubling in hepatocyte numbers (nafenopin alone, 1.95x, $p<0.05$; EGF alone, 1.9x, $p<0.01$). However, their means of producing these increases was different since their combined effect on hepatocyte numbers was additive (both, 3.1x, $p<0.01$).

Since EGF caused a two-fold increase in cell numbers but only a 1.5-fold increase in thymidine incorporation, this suggests that half as many cells again were synthesising DNA as in the untreated control. However, the vast majority of them then underwent cytokinesis after S-phase and karyokinesis, whereas in the untreated control many of the cells which synthesised new DNA subsequently remained as binucleate cells. In fact, this is precisely the effect that EGF was shown to have in later experiments (see Section 9.3.3), and is indicated by the decrease in DNA/cell due to EGF in Figure 8.1 (0.77x).

On the other hand, nafenopin alone had little effect on the rate of DNA synthesis, as measured by thymidine incorporation per cell (1.1-fold). Therefore increased cell proliferation cannot account for the observed doubling in cell numbers. Thus this compound must be acting, as expected, primarily by reducing the rate of cell loss from the population.

Some further effects might also be inferred from the data. Since the average cellular DNA content is slightly reduced (0.87x) this might indicate that the cells normally lost are mononuclear and/or low ploidy. The fact that the combined effect of nafenopin and EGF was again additive in this parameter (EGF=0.76x; both = 0.63x) suggests that the small reduction seen with nafenopin probably is real.

In fact, lower ploidy cells will contribute a less than average incremental rise to thymidine incorporation per cell since they synthesise less DNA per cell division than do higher ploidy cells. This implies that a high proportion of the rescued population must be synthesising DNA, even without EGF stimulation, since thymidine incorporation per cell remained constant. The presence of nafenopin caused an apparent 1.25-fold increase in thymidine incorporation per DNA when compared to untreated control (p=0.07; see Figure 8.6) which also implies that the rescued

population were more likely than the general population to synthesise DNA. These data therefore suggest that proliferating cells were more likely to enter apoptosis than non-proliferating cells.

However, these low ploidy cells have a lower tendency to divide than does the average untreated control cell, by the time of sampling, as otherwise thymidine incorporation per cell would decrease. Since EGF-stimulated cells can complete cell division within the experimental period it seems likely that the nafenopin-rescued proliferating cells tend not to grow for reasons of internal regulation rather than chance experimental selection. Also, since the combined effect of these two mitogens on thymidine per cell was greater than a simple addition of the two effects (2.1x), it may be that the cells rescued by nafenopin are more prone to EGF activation than the rest of the population. This synergism between EGF and nafenopin has been found previously in rodent hepatocytes (James and Roberts, 1995). These results are therefore in agreement with the view that nafenopin is allowing cells which would otherwise die to complete S-phase, but is not causing an EGF-like stimulation of cell proliferation (Gill, *et al.*, 1995), although many can be so stimulated in the presence of a mitogen.

### 8.3.2 Microcystin-LR treatments

The results of an experiment designed to examine the interaction of varying microcystin-LR concentration and nafenopin (50 μM) on a number of cellular parameters are described below. The data for cell numbers is presented first, in Figure 8.2.

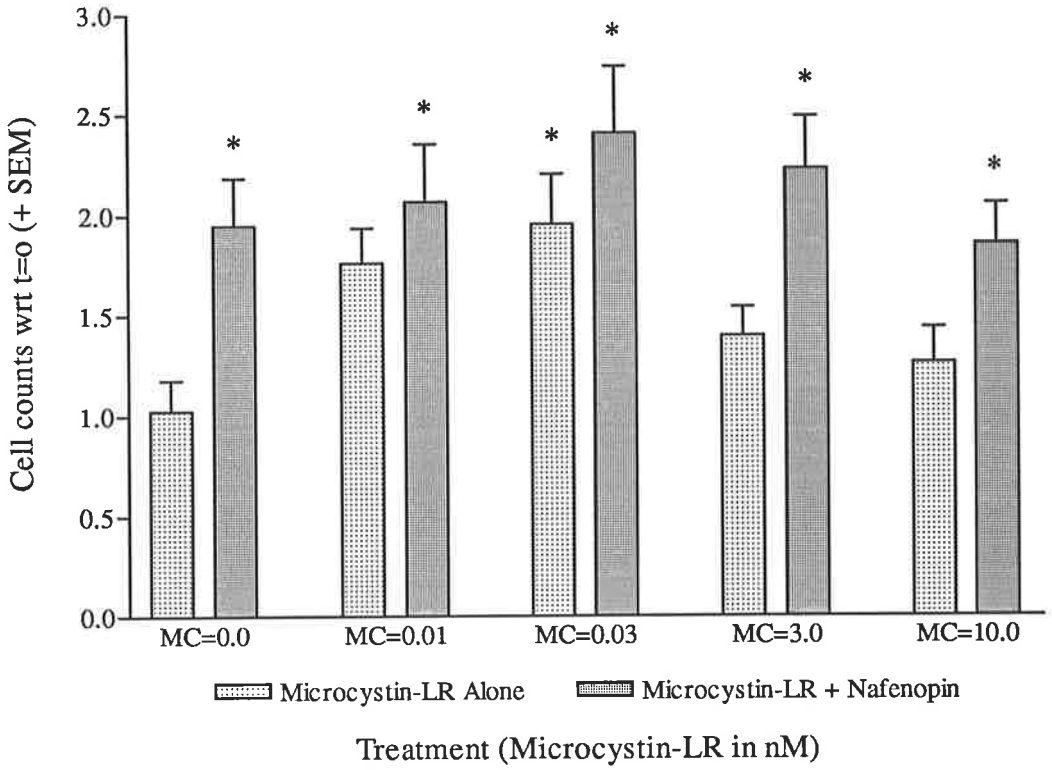



**Figure 8.2** - Effect on hepatocyte numbers of the interaction of nafenopin and microcystin-LR (MC). C3H/HeJ hepatocytes were grown in standard medium with or without 50 μM nafenopin, and with 0.0, 0.01, 0.03, 3.0, or 10.0 nM microcystin-LR. Medium was changed at 2, 24, and 48 hours, and cells counted at 65 hours. Microcystin-only data were analysed by one-way ANOVA followed by Dunnett's *post hoc* Test and comparisons of untreated control (no nafenopin or microcystin-LR) with nafenopin treatments were by t-test (2-sided). Significant differences from untreated control are shown as * ($p<0.05$).

The effect of low concentrations of microcystin-LR on cell numbers was again demonstrated in this experiment, with 0.03 nM microcystin-LR causing a significant ($p<0.05$) rise in cell numbers. Cell numbers also appeared to be increased at 0.01 nM microcystin-LR but this was non-significant. None of the nafenopin-plus-microcystin-LR treatments were significantly different from the nafenopin-only control, suggesting that nafenopin had reduced this effect of low concentrations of microcystin-LR. Therefore, when both microcystin-LR (0.01 nM - 0.03 nM) and nafenopin were present, the ratio of nafenopin to non-nafenopin-treated cell numbers was much lower than the 2-fold increase caused by either alone.

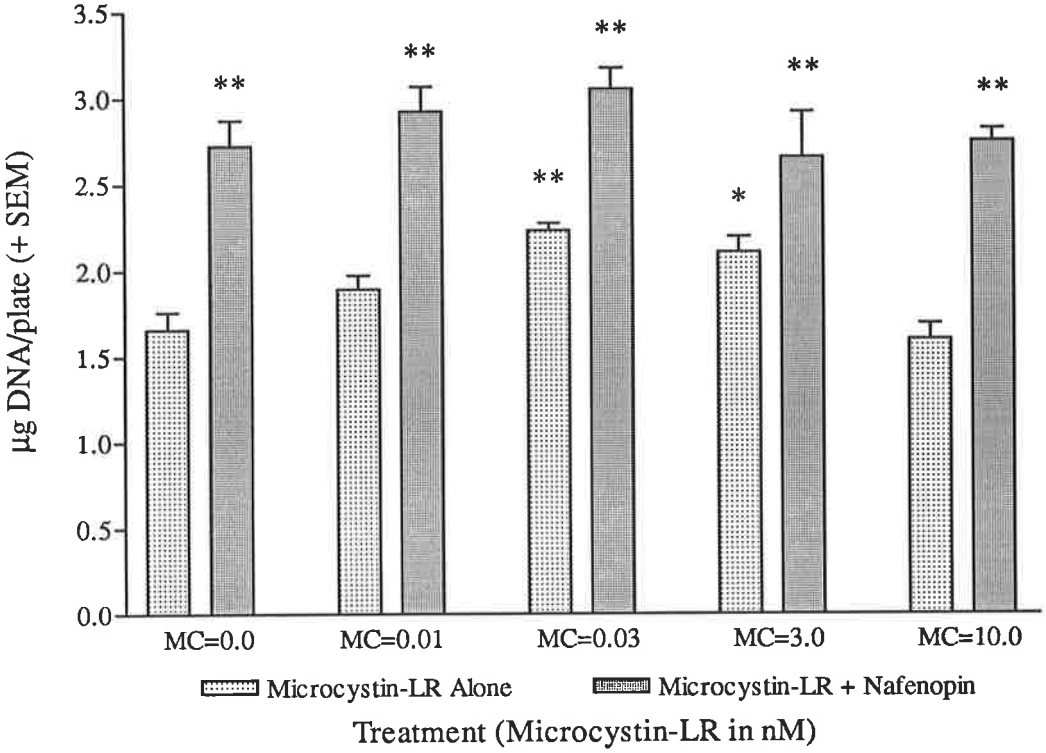



**Figure 8.3** - Effect on DNA per plate of the interaction of nafenopin and microcystin-LR (MC). C3H/HeJ hepatocytes were grown in standard medium with or without 50 µM nafenopin, and with 0.0, 0.01, 0.03, 3.0, or 10.0 nM microcystin-LR. Medium was changed at 2, 24, and 48 hours, and cells harvested at 65 hours. Microcystin-only data were analysed by one-way ANOVA followed by Dunnett's *post hoc* Test and comparisons of untreated control (no nafenopin or microcystin-LR) with nafenopin treatments were by t-test (2-sided). Significant differences from untreated control are indicated by * ($p<0.05$) and ** ($p<0.01$).

The DNA per plate data are shown in Figure 8.3. The pattern of effects across the treatments was essentially in agreement with cell numbers. There were significant rises in DNA per plate at 0.03 nM microcystin-LR ($p<0.01$) and at 3.0 nM microcystin-LR ($p<0.05$) on the non-nafenopin treated plates. As with cell numbers, none of the nafenopin plus microcystin treatments was significantly different from nafenopin alone. The rise in DNA per plate at 3.0 nM occurred in line with cell numbers (1.3x versus 1.4x) so that the small reduction in DNA/cell was not statistically significant, as shown below in Figure 8.4, and as found previously (Figure 7.3).

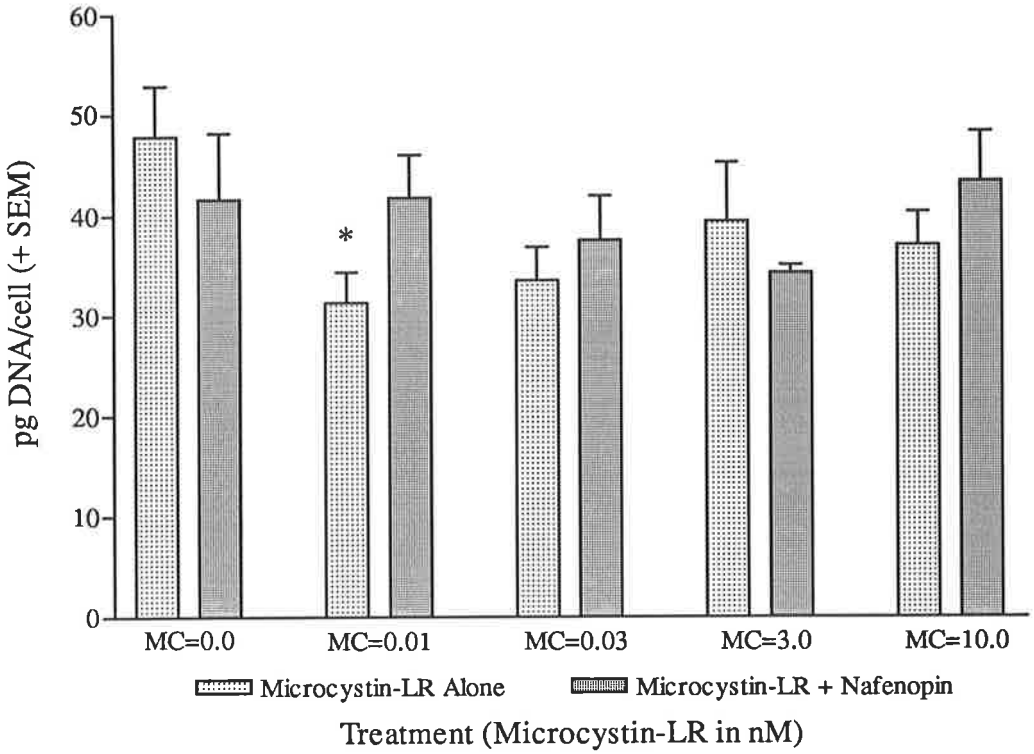



**Figure 8.4** - Effect on DNA per cell of the interaction of nafenopin and microcystin-LR (MC). C3H/HeJ hepatocytes were grown in standard medium with or without 50 μM nafenopin, and with 0.0, 0.01, 0.03, 3.0, or 10.0 nM microcystin-LR. Medium was changed at 2, 24, and 48 hours, and cells harvested at 65 hours. Microcystin-only data were analysed by one-way ANOVA followed by Dunnett's *post hoc* Test and comparisons of untreated control (no nafenopin or microcystin-LR) with nafenopin treatments were by t-test (2-sided). Significant differences from untreated control are indicated by * ($p<0.05$).

The rise in DNA per plate at 0.03 nM was not as great as that seen in cell counts at this concentration (1.3x versus 2x) and so the DNA/cell declined, also as found previously. Furthermore, there was also a marked decrease in cellular DNA content in the 0.01 nM microcystin-LR treatment ($p<0.05$). These reductions were negated in the presence of nafenopin.

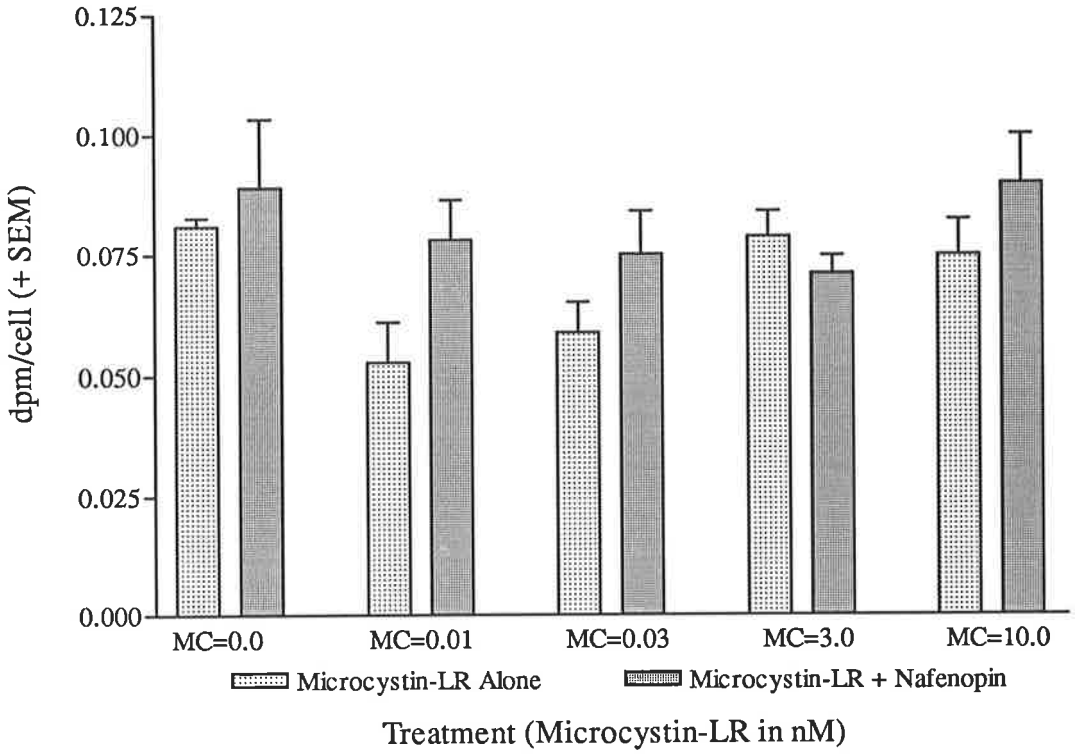



**Figure 8.5** - Effect of the interaction of nafenopin and microcystin-LR (MC) on $^{3}$H-thymidine incorporation per cell. C3H/HeJ hepatocytes were grown in standard medium with or without 50 μM nafenopin, and with 0.0, 0.01, 0.03, 3.0, or 10.0 nM microcystin-LR. Medium was changed at 2, 24, and 48 hours, and cells harvested at 65 hours. Microcystin-only data were analysed by one-way ANOVA followed by Dunnett's *post hoc* Test and comparisons of untreated control (no nafenopin or microcystin-LR) with nafenopin treatments were by t-test (2-sided). No single treatment was significantly different from untreated control although the one-way ANOVA for comparisons between microcystin-without-nafenopin treatments was found to be significant ($p<0.05$).

The effect of the interaction of microcystin and nafenopin on thymidine incorporation per cell is shown in Figure 8.5. There was a significant difference between microcystin-only treatments by ANOVA ($p=0.032$), although no one treatment was identified by *post hoc* analysis to be significantly different from the others. However, there was an apparent reduction of cellular thymidine incorporation by hepatocytes treated with 0.01 nM and 0.03 nM microcystin-LR. Again, there was no significant difference between nafenopin-treated groups, indicating that the effects induced by microcystin-LR in the non-apoptotic population did not occur in the nafenopin-rescued population.

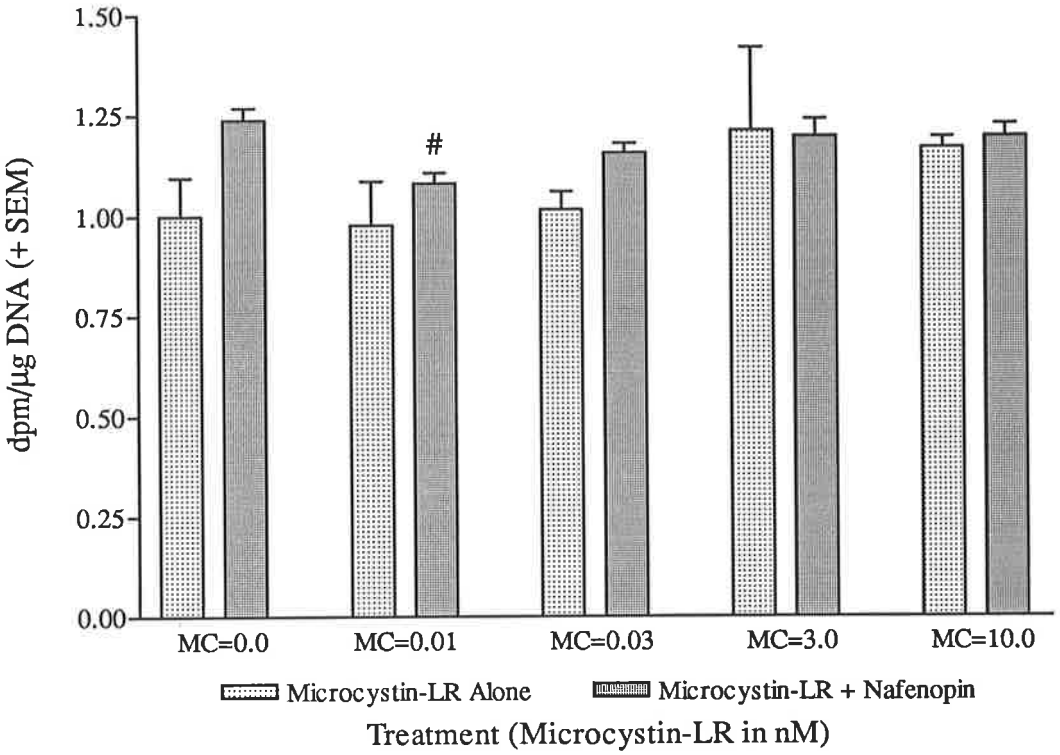



**Figure 8.6** - Effect of the interaction of nafenopin and microcystin-LR (MC) on $^{3}$H-thymidine incorporation per µg DNA. C3H/HeJ hepatocytes were grown in standard medium with or without 50 µM nafenopin, and with 0.0, 0.01, 0.03, 3.0, or 10.0 nM microcystin-LR. Medium was changed at 2, 24, and 48 hours, and cells harvested at 65 hours. Microcystin-only data were compared with untreated control (no nafenopin or microcystin-LR) by one-way ANOVA followed by Dunnett's *post hoc* Test, as were nafenopin treatments with nafenopin-only. Comparisons of nafenopin treatments with untreated control were by t-test (2-sided). A significant difference between nafenopin-plus-0.01nM microcystin-LR and nafenopin only is indicated by # ($p<0.05$). No other significant differences were found.

New DNA synthesis, as estimated by thymidine incorporation per µg DNA, is shown in Figure 8.6. There was a significant reduction ($p<0.05$) in new DNA synthesis with respect to nafenopin alone in nafenopin treated cells when concurrently exposed to 0.01 nM microcystin-LR. The possible significance of this will be discussed later in conjunction with other results. No treatments were significantly different to the untreated control.

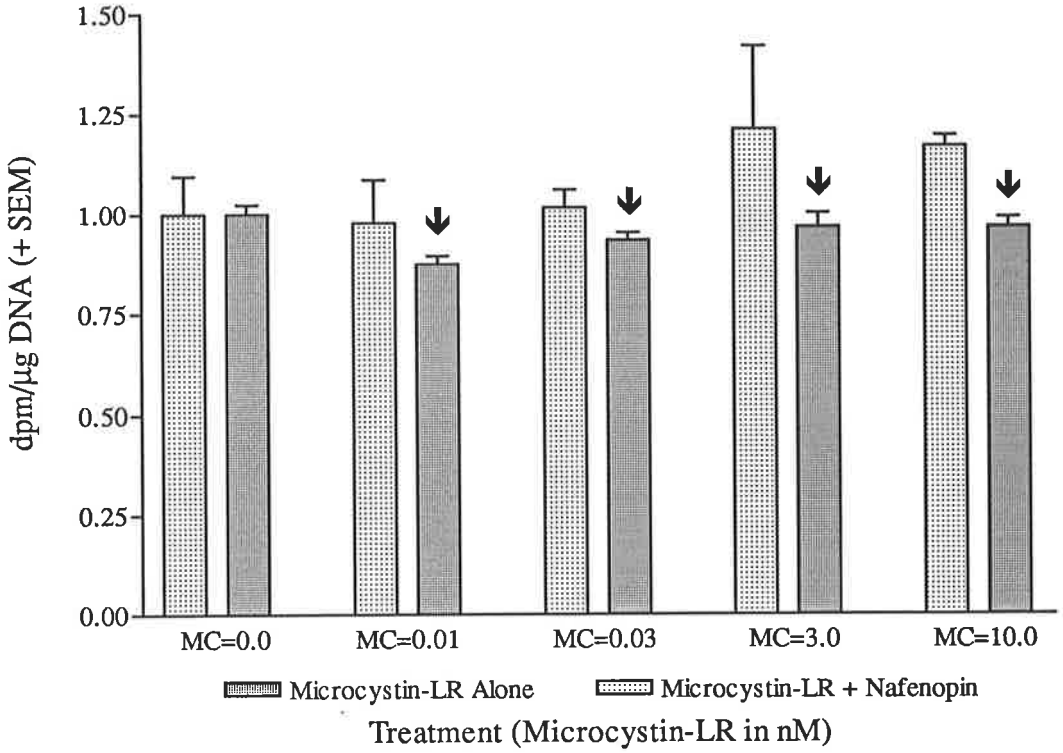



**Figure 8.7** - Effect of the interaction of nafenopin and microcystin-LR (MC) on $^{3}$H-thymidine incorporation per µg DNA Data for microcystin-alone and nafenopin treatments were normalised to untreated control and nafenopin-alone, respectively, in order to control for the effect of nafenopin. These data were then compared by two-way ANOVA. The combination of microcystin-LR plus nafenopin was found to have had a significant effect, with respect to microcystin-alone and controls, on the rate of incorporation of $^{3}$H-thymidine into DNA ($p<0.05$). This is indicated by the ↓ symbol on the graph.

When the plus- and minus-nafenopin treatment groups were normalised to their respective non-microcystin-treated controls and analysed by two-way ANOVA, it was found that the combination of nafenopin and microcystin-LR caused a small but significant reduction, with respect to the microcystin-only treatments, in the proportion of newly synthesised DNA ($p=0.026$; Figure 8.7).

All of these results are summarised in Table 8.2 below in terms of proportional change from the untreated control.

| | NAF Only | 0.01nM MCLR | | 0.03nM MCLR | | 3.0nM MCLR | | 10.0nM MCLR | |
|---|---|---|---|---|---|---|---|---|---|
| | | -NAF | +NAF | -NAF | +NAF | -NAF | +NAF | -NAF | +NAF |
| Cell Numbers | 1.9x* | 1.7x | 2.0x* | 1.9x* | 2.4x* | 1.4x | 2.2x* | 1.2x | 1.8x* |
| DNA/plate | 1.6x** | 1.1x | 1.8x** | 1.4x** | 1.8x** | 1.3x* | 1.6x** | 0.96x | 1.7x** |
| DNA/cell | 0.87x | 0.66x* | 0.87x | 0.70x | 0.79x | 0.8x | 0.7x | 0.77x | 0.91x |
| Thymidine incorp./cell | 1.1x | 0.65x* | 0.96x | 0.73x* | 0.93x | 0.98x | 0.88x | 0.93x | 1.1x |
| dpm/DNA | 1.2x | 0.98x | 1.1x | 1.0x | 1.2x | 1.2x | 1.2x | 1.2x | 1.2x |

**Table 8.2** - Summary of the interaction of nafenopin (NAF) and microcystin-LR (MCLR) on cultured hepatocytes. Results are expressed in terms of proportional change from untreated control, with statistically significant differences from this control indicated by * ($p<0.05$) or ** ($p<0.01$). See relevant graphs for details of statistical tests.

## 8.4 Discussion

The mechanism of action of nafenopin implied by these results was discussed in detail earlier. This can be summarised as follows: Nafenopin acts primarily by reducing the rate of cell death in the hepatocyte population. There is no evidence for an EGF-like stimulation of proliferation by this tumour promoter. However, it appears that the cells rescued by nafenopin are slightly more likely to be synthesising DNA at time of rescue and to continue to do so, or to synthesise DNA once rescued. They also appear to be of lower average ploidy than the general population.

Microcystin-LR at a concentration of 0.01 nM also raised cell numbers in a similar proportion to that produced by nafenopin. Microcystin-LR at this concentration also significantly lowered the

average cellular DNA content, indicating an induction of cytokinesis. However, when the two compounds were added together their actions on cell numbers and cellular DNA content were not additive (Figure 8.2). Therefore, microcystin-LR cannot be inducing cytokinesis to a significant degree in the larger nafenopin-protected population. Any cell preserving effects of microcystin were too small to cause a significant rise above that already induced by nafenopin. There was a small reduction ($p<0.05$) in the rate of new DNA synthesis in the nafenopin-sensitive population when 0.01 nM microcystin-LR was present (Figure 8.6). This might indicate some slight inhibitory effect of this level of microcystin-LR on the average rate of DNA synthesis in the nafenopin-rescued population, perhaps due to a diversion of cells into cytokinesis.

Microcystin-LR at 0.03 nM, unlike at 0.01 nM, caused a significant increase in DNA per plate (Figure 8.3), whilst all other measures were similar to those of the 0.01 nM microcystin-LR treatment. Increased DNA synthesis would imply increased thymidine incorporation per DNA, which did not occur (Figure 8.6). Therefore, cell preservation seems to be part of the mechanism of the toxin's action at this concentration. This effect was not as great as that induced by nafenopin, and the profile of the target population, in terms of ploidy and thymidine incorporation rate, was different. Whilst the rise in cell numbers induced by the two compounds together appeared to be greater than either alone, this was not as great as might be expected if they were acting on completely separate hepatocyte populations. The stimulation of cytokinesis caused by 0.01 nM microcystin-LR alone may also have occurred with 0.03 nM, as shown by the reduction in DNA per cell, although this was non-significant at this microcystin-LR dose (Figure 8.4).

In non-nafenopin treatments at 10.0 nM microcystin-LR, any effects, most especially on cell numbers and DNA per plate (Figures 8.2 and 8.3, respectively), must be analysed in the light of the initial cell losses caused by acute toxicity. It was previously shown that 10 nM microcystin-LR

caused an initial loss of 50% of cells (Figure 6.1) and that with multiple doses of toxin cell numbers returned to levels similar to those in the untreated control by 65 hours in culture (Figure 7.2). Therefore, the proportional rise in cell numbers, and hence DNA per plate, is likely to be greater than a comparison with untreated cells would suggest. In the present experiment cell numbers at 65 hours in these dose groups were slightly higher than untreated control, but not significantly so, and markedly lower than the low dose groups (Figure 8.2). However, if an initial kill of 50% is assumed, then cell numbers in the high dose group must have also increased by a little over 2-fold to reach this point. This suggests that a higher proportion of DNA should be thymidine labelled than in untreated control, which again appears to be the case since the ratio of labelled to unlabelled DNA was raised on a par with the nafenopin only treatment (Figure 8.6).

Cell numbers in the nafenopin plus 10 nM microcystin-LR treatment were significantly higher than in the untreated control (Figure 8.2). This suggests that nafenopin reduced the initial losses caused by 10 nM microcystin-LR. This is because if nafenopin was instead only acting on the microcystin-surviving population, the ratio of labelled to non-labelled DNA should be higher with both than with nafenopin alone since the microcystin-surviving population has a higher proportion of cells undergoing DNA synthesis, and so rescue from apoptosis in this group would mean that proportionally more DNA should be thymidine labelled. Since this was not the case (Figure 8.6), nafenopin must have prevented the initial cell loss induced by microcystin. Therefore, if nafenopin did act by preventing apoptosis, then an apoptotic process may have been involved in the microcystin induced cell deaths as well.

With 3.0 nM microcystin alone the proportion of DNA which was thymidine labelled was increased in similar proportion to that in 10 nM microcystin alone and in nafenopin alone (Figure 8.6). Since DNA per plate was also raised significantly (Figure 8.3) and DNA per cell reduced

somewhat (Figure 8.4), these findings might be explained as a gentler version of the 10 nM microcystin-LR response in which a slightly more proliferatively active cohort of cells were selected for, whilst the rate of toxic cell death was reduced giving rise to the observed higher DNA per plate (Figure 8.3). These effects were not seen in the presence of nafenopin.

To control for the effect of nafenopin on the ratio of labelled to unlabelled DNA, nafenopin plus microcystin treatments were normalised with respect to the nafenopin only treatment. It was found that there was a significantly lower proportion of thymidine labelled DNA than in their microcystin only counterparts (Figure 8.7). That is, once the effect of nafenopin alone had been excluded, then the combination of the two compounds was found to consistently lower this ratio when compared to microcystin-LR at the corresponding dose. At the two high doses of microcystin-LR, this could occur if nafenopin blocked the initial acute kill by microcystin, thus limiting the selective influence of this toxin, and so making the result equivalent to nafenopin only. This supports the argument above that nafenopin reduced microcystin-induced cell death. Similarly, if, in the nafenopin enlarged population, the two lower doses of microcystin-LR were promoting cytokinesis without prior S-phase, then the pool of DNA synthesising cells would be reduced. This would lower the average rate of DNA synthesis and so a reduction of the labelled to unlabelled DNA ratio to below nafenopin-only levels would be seen, as in Figure 8.7. Also, 0.01 nM microcystin-LR significantly reduced the proportion of radiolabelled DNA in the nafenopin rescued population (Figure 8.6). Therefore, although a promotion of cytokinesis was not evident at 0.01 nM microcystin-LR plus nafenopin in the DNA per cell data (Figure 8.4), the above arguments suggest that such a process might still be occurring in a reduced proportion of the larger nafenopin-protected population.

From the results of this experiment the following hypotheses can be proposed. First, the induction of cytokinesis seen at pM concentrations of microcystin-LR in non-nafenopin treated cells did not occur additively in the cells rescued by nafenopin. If it is true that the cells protected from apoptosis by nafenopin were more likely than the general population to be in the cell cycle, then it is quite conceivable that they would not be receptive to a signal to undergo cytokinesis since they were already committed to some other phase of the cell cycle. Second, the cell preserving effect of microcystin-LR alone, seen at 0.03 nM toxin in this experiment, did not appear to be additive with that of nafenopin. This suggests that both compounds acted on the same hepatocellular population and therefore that microcystin-LR might also have inhibited apoptosis. Third, the acute toxicity of 10.0 nM microcystin-LR was reduced by nafenopin, suggesting that at this concentration microcystin induced apoptosis. However, at these higher doses of microcystin-LR which caused an acute toxic response, there were likely to be multiple disruptive effects on many phosphorylation/dephosphorylation reactions, and so it was perhaps the loss of a coordinated intracellular regulation, rather than any single mechanism, which led to cell death. Whether a coordinated apoptotic response could occur in such an environment will require further work to clarify.

# CHAPTER 9

# EFFECTS OF MICROCYSTIN-LR ON HEPATOCYTE POPULATION DYNAMICS IN VITRO

## 9.1 Introduction

Murine hepatocytes are not an homogeneous population, but differ widely in their ploidy and differentiation, and thus in their ability to respond to cytotoxic challenges (Gumucio, *et al.*, 1994). Therefore, it is not surprising that a toxin like microcystin-LR, which requires active uptake by the cell, will act differentially in such an hepatocyte population. In order to analyse such selective effects, a method which can differentiate to some extent between the hepatocyte sub-populations is needed, and the use of flow cytometry was investigated with this end in mind.

### 9.1.1 Hepatocyte cell cycle and the development of polyploidy

As was described in Chapter 1, in mouse hepatocytes an acytokinetic mitosis can lead to the production of a single binuclear daughter cell. The cell can then undergo a subsequent round of DNA synthesis, followed by a cytokinetic mitosis, producing two mononuclear polyploid daughter cells. This process can continue through the series diploid (2N), binuclear tetraploid (2x2N), mononuclear tetraploid (4N), binuclear octaploid (2x4N), etc. The expression of differentiated characteristics is positively correlated with ploidy (Deschenes, *et al.*, 1981), and so this process continues as the animal ages until hepatic maturity (Brodsky and Uryvaeva, 1977). Cell size also increases with ploidy so that after about 1 month of age the number of hepatocytes

in a mouse liver is largely static even though its size and weight keep pace with increasing body weight (Brodsky and Uryvaeva, 1977; Fausto and Webber, 1994).

### 9.1.2 Effects of animal age, partial hepatectomy, and carcinogens on ploidy

As stated above, average hepatocyte ploidy increases with animal age until hepatic maturity. In C3H mice, diploid cells, which constitute the total hepatocyte population at birth are reduced to about 30% of the total by 1 month of age. At this time tetraploids (mainly binucleate, 2x2N) form about 50% of the total and octaploids 12%. Hexadecaploids are barely detectable. With continuing age, diploids continue to reduce in number, and tetraploid numbers also begin to fall, whereas octaploid and hexadecaploid numbers increase. By 24 months, octaploids constitute 50%, tetraploids 25%, diploids 20%, and hexadecaploids 5% of the population (Severin, *et al.*, 1984b). After hepatotoxicosis or partial hepatectomy, cytokinetic mitosis occurs in all mononucleate ploidy groups to replace lost cells (Saeter, *et al.*, 1988b). The diploid compartment increases at a slightly faster rate than those of higher ploidy. In chemically induced carcinogenesis in the rat liver after partial hepatectomy, a selective proliferation of diploid hepatocytes is induced (Saeter, *et al.*, 1988a; Saeter, *et al.*, 1988b). In general, the cells in hepatocellular carcinoma, in humans as well as animal models, are of low ploidy (mainly diploid) undergoing cytokinetic mitoses almost exclusively (Saeter, *et al.*, 1988c).

### 9.1.3 Flow cytometry

A flow cytometer consists of a laser aimed at a stream of single cells and an array of detectors arranged about the point of illumination (for a general introduction, see Shapiro, 1994). A cell suspension is forced into a narrow stream with a flow rate such that a line of single cells passes

the illumination point with a low probability of coincidence of cells. This stream is surrounded by an outer layer of sheath fluid to avoid lamina flow distortions of the stream. The detectors (photomultipliers) are arranged so that reflected light is gauged at angles of 10° and 90° to the incident light beam (forward and side, or orthogonal, scatter, respectively). The 90° signal passes through a number of dichroic mirrors and filters, being gauged after each, to detect fluorescent emissions in three wave-bands. Each cell is therefore detected as an "event" characterised by the intensity of its forward and side scatters, and by the intensity of fluorescent emission in whichever bands were chosen for recording. The signal intensities for all events are stored in the form of frequency distribution histograms, one for each parameter. The photomultiplier gain can be set for each parameter, to place the signals of interest within the range of the histogram (which usually consists of 1024 channels). Log or linear amplification is available depending on the breadth of the signal range of interest. The fluorescence filters do not have absolute wavelength cut-offs, and so cross-talk between wave-bands is possible, especially if the intensity in one band is much greater than in another band of interest. Therefore, circuitry is also provided to try to compensate for this.

## 9.2 **Methods and materials**

### 9.2.1 Flow cytometry

The flow cytometer used for the analysis of most experiments was a FACScan (Becton Dickinson Immunocytometry Systems). This was fitted with an air-cooled 15 mW argon-ion laser as the excitation source. Data analysis was performed using the Cell-Quest package supplied by Becton Dickinson.

Cell suspensions were analysed by flow cytometry at a flow rate of 60 µl/min in PBS with PBS as sheath fluid. Forward and orthogonal (90°) scatter were collected for all runs and used to gate out either cells or free nuclei as necessary, as well as cell debris. The 488 nm line of the Argon-ion laser was the excitation source. Propidium iodide (PI) staining was monitored in the 585 ± 21 nm band (channel 2), except when cells were also stained with 5-(6-)-carboxyfluorescein diacetate, succinimidyl ester (CFSE). Then CFSE was monitored in channel 1 (530 ± 15 nm) and PI in channel 3 (> 650 nm) so as to minimise cross-talk and simplify compensation. At least $10^4$ events were collected per run. Duck erythrocytes were used as internal standard, and to set voltage and gain prior to collecting data. These cells have 2.3 pg DNA/cell (in a single nucleus), whereas murine diploid hepatocytes have 6 pg DNA/cell (Brasch, 1980).

### 9.2.2 DNA staining with Propidium Iodide (PI)

Cells were permeabilised by incubation in 0.1% saponin in PBS for 10 min at room temperature. Nuclei were then stained for 30 min at 37°C in a PBS solution containing 50 µg/ml propidium iodide and 12.5 µg/ml RNase A.

### 9.2.3 Cytoplasmic staining with 5-(6-) Carboxyfluorescein diacetate, succinimidyl ester (CFSE)

In some experiments (Section 9.3.2) hepatocyte protein was stained with CFSE before plating out so that cell division could be monitored by the reduction in average cellular fluorescent intensity. To determine the optimum staining conditions, freshly isolated hepatocytes were incubated for 10, 20, or 40 minutes in 0, 10, 20, or 50 µM CFSE in BSA-free Krebs-Henseleit Wash Buffer (see Appendix 1), washed once in BSA-containing Krebs-Henseleit Wash Buffer,

and then plated at 2 x $10^5$ cells/plate in collagen-coated 36mm plates in DMEM/F12 medium (including dexamethasone and ITS/100) at 37°C in a 5% $CO_2$ atmosphere. Plating efficiency was determined 3 hours later by counting the proportion of adherent cells to total cells with >300 cells being counted per plate. Both 10 and 20 μM CFSE were found to not effect plating efficiency in 10 minute incubations. Longer incubations were detrimental, as was 50 μM CFSE, even at 10 minutes. Since 10 μM for 10 minutes was found to give adequate staining intensity both in the fluorescence microscope and the FACScan, this treatment regime was used in future experiments. CFSE was shown not to effect the rate of thymidine incorporation or cell number increase over 65 hours of culture in both EGF stimulated and unstimulated cells (results not shown).

### 9.2.4 Lymphocyte isolation

Mouse splenic lymphocytes were used during validation to check the staining procedures and to identify the channels collecting diploid (2N) and tetraploid (4N) cells (Section 9.3.1). These were collected by homogenising the spleen from a 5 week old C3H/HeJ male mouse in PBS, passing the suspension through a 50 μm nylon mesh, and washing once in PBS. They were then fixed in either ice-cold 70% ethanol, or in ice-cold buffered formalin. They were washed once in PBS before PI staining and analysis on the flow cytometer.

### 9.2.5 Hepatocyte ploidy group separation and characterisation

To further validate the FACScan identification of hepatocyte ploidy groups, CFSE plus PI stained mouse hepatocytes were sorted on a FACStar-PLUS flow cytometer by Alan Bishop at the Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Adelaide.

The sorted cells were then photographed at 50x magnification under the fluorescence microscope. These transparencies were then projected onto a screen, and the cell images were measured and nuclearity (mono- or binuclear) noted.

### 9.2.6 Harvesting of cultured cells from plates

A number of methods were examined for the preparation of single cell suspensions from culture plates. These included trypsinisation (0.5 - 1.0%) with EDTA (0.02%) or EGTA (0.02%), EGTA (0.02%) followed by 0.1% collagenase, mixtures of trypsin and collagenase with EDTA, all with and without scraping the cells off the plate with the obliquely cut end of a piece of Tygon tubing. None of these methods proved entirely satisfactory. Microcystin-treated hepatocytes proved especially difficult to disaggregate, with many cell clumps remaining and high numbers of free nuclei generated. The method settled upon involved washing a plate with ice-cold PBS, and then scraping the cells off into 0.02% EGTA/0.02% EDTA in PBS at 4°C. No enzymes were used as these increased the proportion of damaged cells and free nuclei in the preparation. This was incubated on ice for 5 minutes before washing the cells in PBS. They were then passed through a 21G needle and filtered through 50 μm nylon mesh. Cells were then preserved until flow cytometry as described below.

### 9.2.7 Preservation of cells prior to flow cytometry

Buffered formalin, glutaraldehyde, and 70% ethanol were examined as fixatives for hepatocytes prior to flow cytometry. Cells were thoroughly suspended, prior to addition of the fixative, in "saline-GM" (g/l: glucose 1.1; NaCl, 8.0; KCl, 0.4; $Na_2HPO_4.12H_2O$, 0.39; $KH_2PO_4$, 0.15; containing 0.5 mM EDTA; Crissman and Hirons, 1994). Fixative was added with vortexing.

All fixatives worked well for the preservation of murine lymphocytes, but caused clumping of hepatocytes. Therefore, the method of preservation settled upon was freezing at −80°C in citrate buffer (250 mM sucrose, 40 mM trisodium citrate.$2H_2O$, 5% DMSO, pH 7.6; Vindelov and Christensen, 1994). Cells could be stored in this form indefinitely without clumping or appreciable loss of morphology.

### 9.2.8 Determination of binuclearity

The proportion of binuclear cells in a cell suspension (its "binuclearity") was determined by counting PI-stained cells on a haemocytometer under the fluorescence microscope. Generally, >200 cells were counted per plate or preparation.

### 9.2.9 Isolation of nuclei

Preparation of free nuclei for flow cytometric determination of nuclear ploidy was performed by the method of Vindelov and Christensen, (1994). The cytoplasm is removed by treatment with trypsin (0.3%) in a citrate(3.4 mM)/tris (0.5 mM) buffer (pH 7.6) containing Nonidet-P40 (0.1%) and spermine tetrahydrochloride (1.5 mM) for 15 minutes at 37°C. This is followed by inhibition of the trypsin (soyabean trypsin inhibitor, 0.5 mg/ml) and removal of RNA (RNase A, 0.1 mg/ml), 15 minutes, 37°C. Finally the free nuclei are stained with PI (0.42 mg/ml) on ice. The DNA is stabilised throughout by the inclusion of spermine tetrahydrochloride. Duck erythrocytes were included in the procedure to act as internal standard. Preparations were checked under the fluorescence microscope to check for complete removal of the cytoplasm and even suspension of single nuclei.

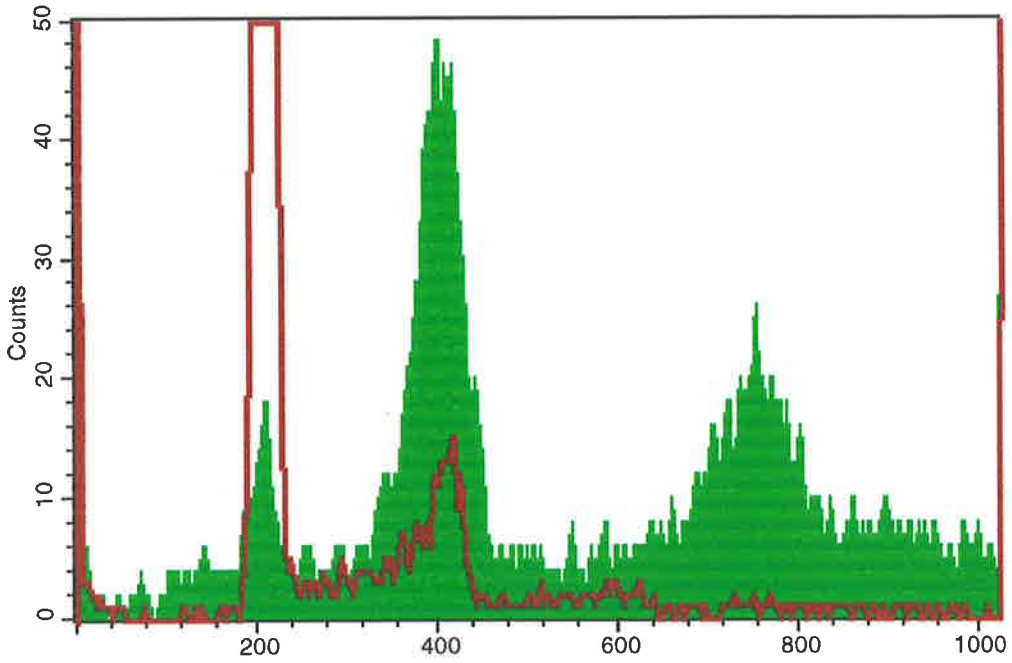
## 9.3 Results

### 9.3.1 Comparison of lymphocyte and hepatocyte ploidy groups

To help correlate different PI-stained peaks with hepatocyte ploidy, murine lymphocytes were prepared as described and used to set the fluorescence voltage and gain on the flow cytometer. Hepatocytes were then run through the machine using the same settings. The resulting histograms have been overlaid and are presented in Figure 9.1.

The large peak of diploid lymphocytes at channel 200 lines up with a small hepatocyte peak centred on this channel, whilst the small peak of M-phase lymphocytes at channel 400 (twice the DNA and hence twice the PI fluorescence intensity) corresponds to the larger hepatocyte peak. There is a third hepatocyte peak just below channel 800. Therefore, the hepatocyte peak at channel 200 is diploid cells (2N), the larger peak at channel 400 is tetraploid cells (4N and 2x2N), and the third peak is octaploid cells (8N and 2x4N). Note that these cell preparations were fixed in 70% ethanol, a procedure later abandoned for hepatocytes due to the excessive debris formation and cell clumping, which contribute to the peak widening and the high background "noise" seen between peaks in this preparation. Note also that in later analyses the diploid peak was placed at channel 100, the tetraploid at channel 200 and the octaploid at channel 400.

Figure 9.1 - Overlay of FACScan-detected DNA staining intensity distributions in murine splenocytes and hepatocytes used to identify the hepatocellular diploid, tetraploid, and octaploid peaks.



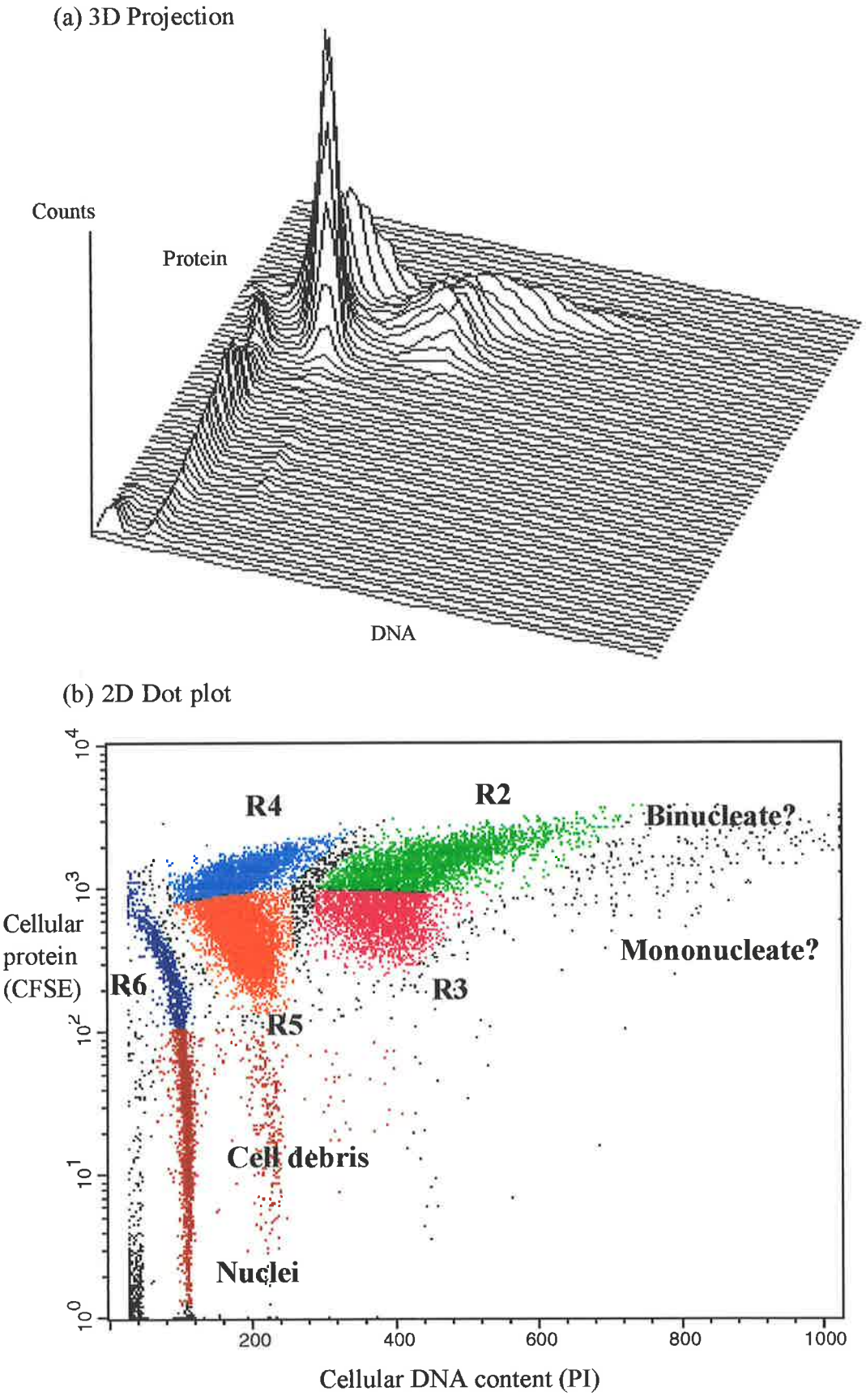

Splenic lymphocytes are diploid. The FACScan amplifier gain was set so that this group (large red peak) was counted with mean fluorescence intensity at channel 200. A small peak of mitotic cells is then seen at channel 400, with S-phase cells distributed between these peaks. A single-cell suspension of hepatocytes freshly isolated from a C3H/HeJ mouse was then analysed using the same settings to identify the peaks corresponding to the various ploidy groups. Therefore, the hepatocellular diploid peak (2N) is the small green peak at channel 200, the tetraploid peak (4N and 2x2N) is at channel 400, and the octaploid peak (8N and 2x4N) is at about channel 800. (This latter peak maximum is actually slightly lower than channel 800, most likely because of a non-linear amplification by the flow cytometer.)

### 9.3.2 Characterisation of hepatocytes separated on the basis of PI and CFSE staining intensity

To further confirm that the PI peaks seen in the FACScan were in fact the 2N, 4N, and 8N hepatocyte ploidy groups, and also to determine whether binucleate cells could be distinguished from mononucleates on the basis of CFSE stain intensity, five regions (denoted R2 - R6) were gated as shown in Figure 9.2 and sorted into separate test-tubes on the FACStar-PLUS flow sorter.

PI staining intensity increases along the horizontal axis, while CFSE staining intensity increases up the vertical axis. It can be seen that the 4N and 8N groups appear to be able to be further distinguishable into lower and higher intensity groups based on CFSE staining. It was thought possible that these regions might correspond to mononucleate and binucleate cells, respectively, since it is known that binucleate cells are slightly larger than mononucleate cells (Jack, *et al.*, 1990), and so should have a higher degree of CFSE staining. Events occurring below $10^2$ on the vertical axis are likely to be cell fragments and, lower down still, free nuclei.

Figure 9.2 - FACScan analysis of combined protein (CFSE) and DNA (PI) staining of murine hepatocytes. See text for details.



Sorted cells were counted under the fluorescence microscope to determine the binuclearity of each group and then photographed. These results are presented in Table 9.1 and Figure 9.3.

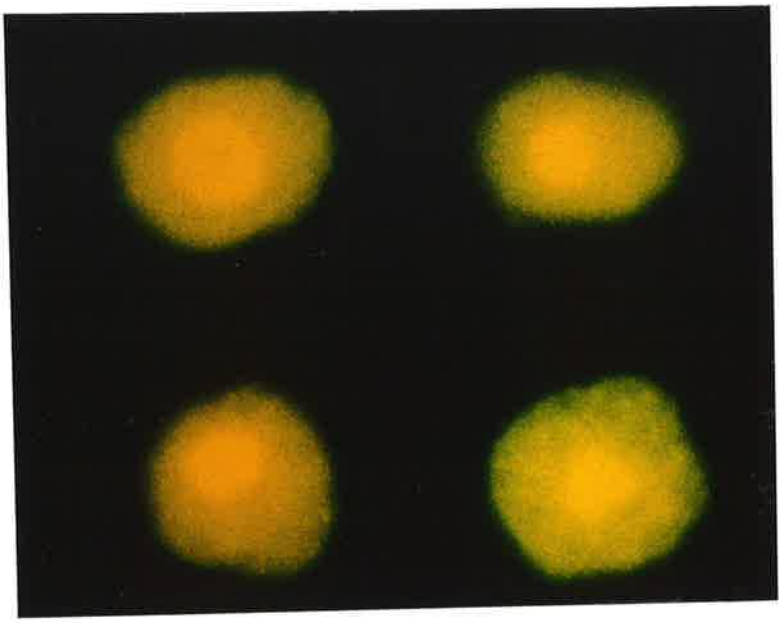
| Sorted region | Mononuclear (%) | Binuclear (%) | Total cells counted |
|---|---|---|---|
| R2 | 71 (13.6) | 451 (86.4) | 522 |
| R3 | 78 (14.4) | 465 (85.6) | 543 |
| R4 | 207 (38.4) | 332 (61.6) | 539 |
| R5 | 219 (42.1) | 301 (57.9) | 520 |
| R6 | 495 (94.6) | 28 (5.4) | 523 |

**Table 9.1** - Nuclearity of flow-sorted hepatocytes. Freshly isolated C3H/HeJ mouse hepatocytes were double stained with CFSE and PI, and then sorted using a FACStar flow cytometer. See Figure 7.2 and text for sorting conditions. Mononuclear and binuclear cells in each group were then counted under the fluorescence microscope and the proportions of each calculated for each group.

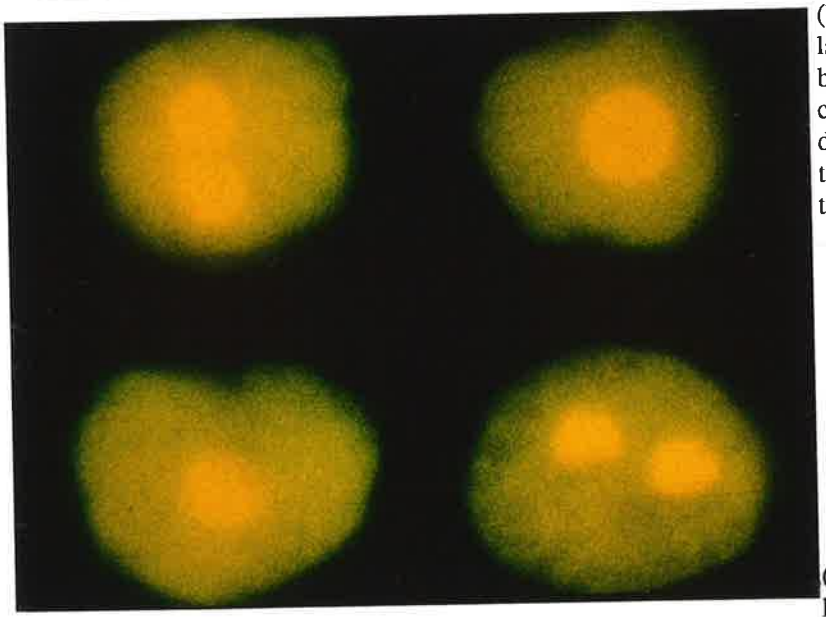
It can be seen that in each of the regions of the same ploidy, sorted on the basis of CFSE staining intensity, that is R2, R3 and R4, R5, the proportions of mononuclear cells are virtually identical. Therefore, CFSE intensity does not correlate with nuclearity of a cell. Note that there are 5.4% binucleate cells in the supposedly totally mononuclear diploid group, R6, suggesting that this is an estimate of the error rate inherent in the sorting procedure.

The size of the cell image, projected from a photographic transparency, was measured and the cell again classified as to its nuclearity. Representative cells from the different ploidy groups are shown in Figure 9.3, and their relative size distributions are shown in Table 9.2.

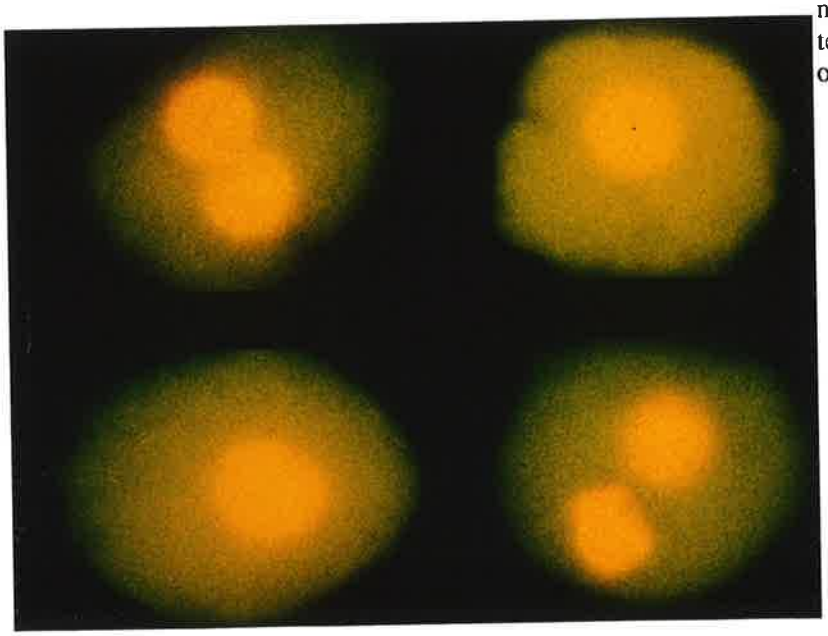
**Figure 9.3** - Hepatocytes separated on the basis of CFSE (cellular protein) and PI (DNA) fluorescence intensities on a FACStar flow sorter. (Magnification 2000x).



(a) Diploid hepatocytes. Note however that one cell appears to have a larger nucleus than the other three and may therefore actually be a higher ploidy cell which has been missorted because it has lost some cytoplasm.



(b) Tetraploid hepatocytes. These cells are larger than the diploid cells and many are binucleate. The nuclei of the binucleate cells are the same size as those seen in the diploid cells, whereas the single nuclei in the mononuclear cells are larger, having twice the DNA content.



(c) Octaploid hepatocytes. These cells are larger again, with the binucleates having nuclei the same size as mononuclear tetraploids and the mononuclear octaploids having still larger nuclei.

| | Diploid (R6) | Tetraploid (R4+R5) | | Octaploid (R2+R3) | |
|---|---|---|---|---|---|
| | Mononucleate | Mono | Binuc. | Mono | Binuc. |
| Mean (cm) | 6.5 | 8.1 | 8.3 | 9.5 | 9.9 |
| Standard deviation | 0.54 | 0.70 | 0.87 | 1.24 | 0.88 |
| Number sampled | 13 | 16 | 19 | 10 | 15 |

**Table 9.2** - Size distributions of the projected image of flow-sorted hepatocytes. Photomicrographs were taken of the sorted hepatocytes and transparencies made. These were projected onto a screen and the relative size distributions of the cells in the different groups determined. There were no significant differences between mononucleate and binucleate cells within a ploidy group (t-test, 2-sided) and so these were pooled for comparison of ploidy groups. A one-way ANOVA indicated that there was a significant difference in the size distributions of the 3 ploidy groups ($p<10^{-12}$).

It appears from these small samples that binucleates might be slightly larger than mononucleates, but the difference is too small to be significantly different with this data. However, when mononucleates and binucleates were grouped within a ploidy group, there was a significant difference between the three groups ($p=4.99\times10^{-13}$). Since cell size is known to increase with nuclear ploidy (Deschenes, *et al.*, 1981), this further confirms the identity of the PI peaks determined earlier.

### 9.3.3 Effect of EGF stimulation on hepatocellular and hepatonuclear ploidy, and binuclearity

To examine the effect of a growth stimulus on the distribution of ploidy within a population, C3H/HeJ hepatocytes were isolated, stained with CFSE (10 μM, 10 min), and grown in culture with or without 20 ng/ml EGF. They were harvested after 96 hours in culture, having had daily medium changes, and frozen at −80°C until use. Triplicate plates from each treatment were prepared for flow cytometric analysis of both cellular and nuclear ploidy. Binuclearity was determined on the samples prepared for cellular ploidy. CFSE staining intensity was also analysed

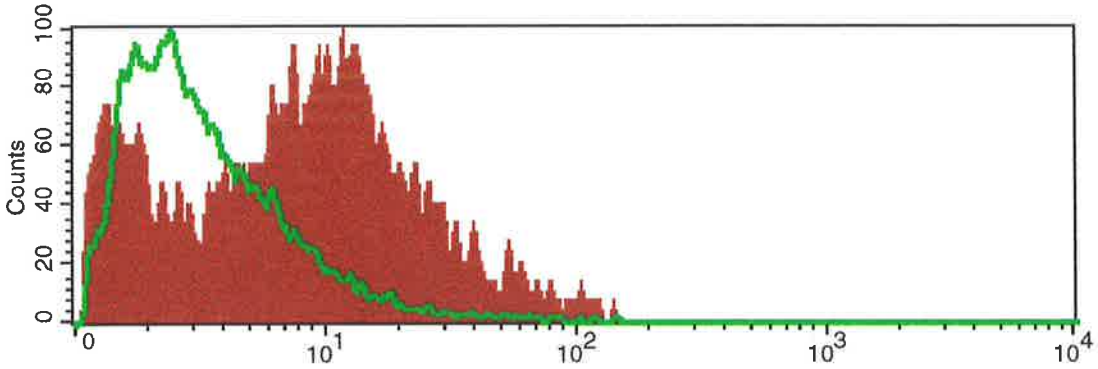
as an indication of cell division since the stain concentration is halved with each round of cytokinesis (Lyons and Parish, 1994). The results are shown in Figure 9.4.

A comparison of CFSE labelling is shown in Figure 9.4 (a). The EGF-stimulated cells have, in general, approximately 20-25% the CFSE intensity of non-stimulated cells, suggesting that two more rounds of replication have been completed by the stimulated cells. There is also a low intensity peak in the unstimulated cell histogram, which might suggest that there is a more active cohort of cells within the general population which proliferates independently of EGF. This pattern was seen in all three replicates.

In Figure 9.4 (b), the distribution of PI staining intensities is shown for EGF-stimulated and unstimulated cells. The general effect of EGF is to cause an increase in the average ploidy of the hepatocyte population when compared with unstimulated cells, that is, the proportion of the population which is diploid is lower, whereas the tetraploid and octaploid components are higher. This difference is even more marked when the nuclear ploidy is examined, Figure 9.4 (c).

Figure 9.4 - Effect of EGF stimulation on hepatocellular division rate (CFSE staining) and cellular and nuclear ploidies (PI staining).

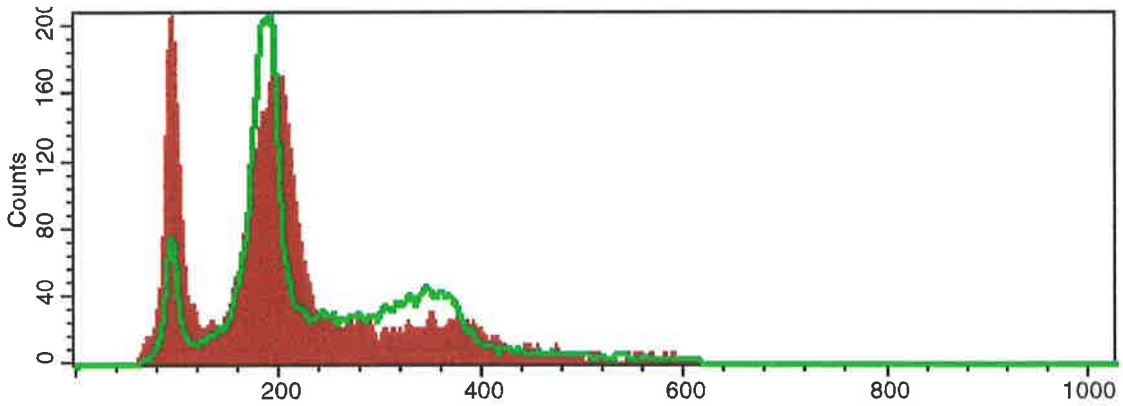
(a) Intensity of fluorescein-stained cellular protein




CFSE fluorescence intensity

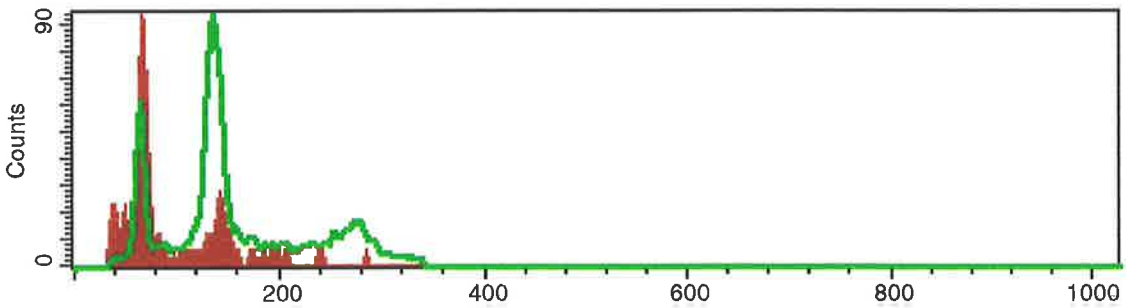
As fluorescein-stained cells divide the fluorescent label is divided between the daughter cells, reducing its cellular intensity. Here, EGF-treated cells (green) have a much lower fluorescence intensity than the unstimulated (brown) cells indicating a greater rate of cellular division during culture over 65 hours.

(b) DNA-staining: Cells




PI fluorescence intensity

(c) DNA-staining: Nuclei




PI fluorescence intensity

Hepatocyte ploidy groups (diploid, tetraploid, octaploid) are seen here with incrementally increasing staining intensity. In the above histograms (b and c), for example, it can be seen that EGF stimulated cells (upper histogram (b), green) have generally higher ploidy than unstimulated (brown) cells. Furthermore, the proportion of tetraploid nuclei (lower histogram (c), middle peak) has increased markedly, suggesting that most of the cells in this group are now mononucleate, whereas in the unstimulated cells most tetraploid cells are binucleate.

When the relative proportions of cells in each ploidy group are compared with the proportions of nuclei of that respective ploidy, an estimate of the binuclearity of each cellular ploidy group can be made. Thus, it can be seen, for example, that in the unstimulated hepatocyte population, a large proportion of the cellular tetraploid group must be binucleate since their nuclei are not found in the tetraploid nuclear peak. This is despite the fact that most of the octaploid cells must also have been binucleate (as there were very few free octaploid nuclei detected) and are thus contributing nuclei to the nuclear tetraploid peak. This effect is not nearly as marked in the EGF-stimulated distributions, suggesting that there is a higher proportion of mononucleate tetraploids in this population. When the binuclearity of the cellular preparations was determined by counting under the fluorescence microscope, it was found that the unstimulated population contained 53.5% ± 0.71 (SD) binucleates, whereas the EGF-stimulated population contained only 18.0% ± 3.0 (SD) binucleates. Note that binuclearity of the unstimulated treatment group here (53.5%) is slightly lower than that determined by Severin *et al*, (1984) who found that the binuclearity in C3H mice aged from 1 - 18 months was 61% (with a standard deviation of 10%). This may be due to the sub-population of actively cycling cells detected by CFSE staining (Figure 9.4 (a)).

The number of events (cells or nuclei) recorded in each of the PI-based ploidy groups above can be expressed as a proportion of the total cellular or nuclear events respectively. The number of cells were counted on the plates prior to harvest, and so the actual number of cells in each group can be calculated for each treatment. It was found that EGF-stimulation caused the diploid cohort to increase by 1.2-fold compared to unstimulated "background", the tetraploid cohort to increase by 3.7-fold, and the octaploid cohort to grow by 6.0-fold. Note that the unstimulated "background" is not static, but since its growth is much lower than that of the stimulated population, these estimates are likely to be close to true estimates of the effects of EGF on these

hepatocytes in culture. This increase in average ploidy and decrease in binuclearity is typical of hepatocyte population dynamics seen in the regenerating liver after partial hepatectomy (Jack, *et al.*, 1990; Fausto and Webber, 1994).

From the foregoing, it was clear that flow cytometry could be used to detect, via changes in ploidy and binuclearity, and in conjunction with other techniques already used, changes in the population dynamics of growing hepatocytes.

A further check on the overall protocol was made as follows. If it is assumed that the population binuclearity is a reasonable estimation of the binuclearity of the tetraploid cellular group and that that of the octaploid cellular group is 1.42x this (see Table 9.1), then based on the binuclearities determined under the fluorescence microscope, the numbers of mononuclear and binuclear cells can be calculated for each ploidy group. From this can be calculated an expected number of nuclei in each nuclear ploidy group, since mononucleate cells contribute a single nucleus to the nuclear group of the same ploidy, whereas binucleate cells contribute two nuclei to the next lower nuclear ploidy group. If these expected numbers of nuclei are then expressed as percentages, they ought to approximate those determined in the flow cytometer if the cell counting, binucleate counting, and flow cytometric determinations are relatively error free. The outcome of this process is presented in Table 9.3.

| | | Unstimulated | EGF stimulated |
|---|---|---|---|
| Calculated nuclear percentages: | Diploid | 62.7 | 28.9 |
| | Tetraploid | 34.2 | 58.3 |
| | Octaploid | 3.1 | 12.8 |
| Found nuclear percentages: | Diploid | 73.0 | 21.2 |
| | Tetraploid | 23.6 | 59.9 |
| | Octaploid | 3.4 | 18.8 |

**Table 9.3 -** Calculated percentages of nuclei, and those determined from the flow cytometric data. The expected proportions of nuclei of different sizes in the hepatocyte population were calculated based on the experimentally determined proportions of cellular ploidy groups, the ratio of mononuclear to binuclear cells, and the cell counts. These were then compared with the experimentally determined proportions of nuclei using a chi-squared test. The expected and observed were not found to be significantly different, confirming that the data were self-consistent.

It can be seen that the percentages of nuclei in each ploidy group and treatment are similar whether calculated from flow cytometric cellular data, cell counts and binuclearity counts, or determined directly from the flow cytometric nuclear data. The Chi-squared test p-values for the unstimulated and stimulated treatments are 0.2924 and 0.2954, respectively, indicating that there is no significant difference between the means of determination of these nuclear percentages. Therefore, despite the difficulty encountered in reliably harvesting microcystin-treated cells from plates as single cell suspensions, enough relevant information could be obtained from determinations of cell number on the plate, binuclearity of the cells, and flow cytometric determination of nuclear ploidy to be able to determine if changes in the cell population were occurring in response to microcystin-LR. Nuclear ploidy was expressed either as average population nuclear ploidy, calculated as

$$ANP = \frac{2Nn \times 2 + 4Nn \times 4 + 8Nn \times 8}{2Nn + 4Nn + 8Nn} \qquad \text{Eqn. 9.1}$$

where $2Nn$ = diploid nuclei,

$4Nn$ = tetraploid nuclei, and

$8Nn$ = octaploid nuclei.

or simply as the percentage of nuclei which were diploid if cell numbers were low due to toxin treatment, since octaploid nuclei were then so few as to make their quantification unreliable.

### 9.3.4 Effect of microcystin-LR on hepatonuclear ploidy and binuclearity after 18hrs exposure

Cells were exposed to a range of microcystin-LR concentrations at 2 hours culture time and triplicate plates from each treatment were harvested at 18 hours. Results from this sampling time were assumed to represent a non-proliferation-dependent response since DNA synthesis in the population is minimal before this time (see Figure 6.2). The results are shown in Figures 9.5 and 9.6.

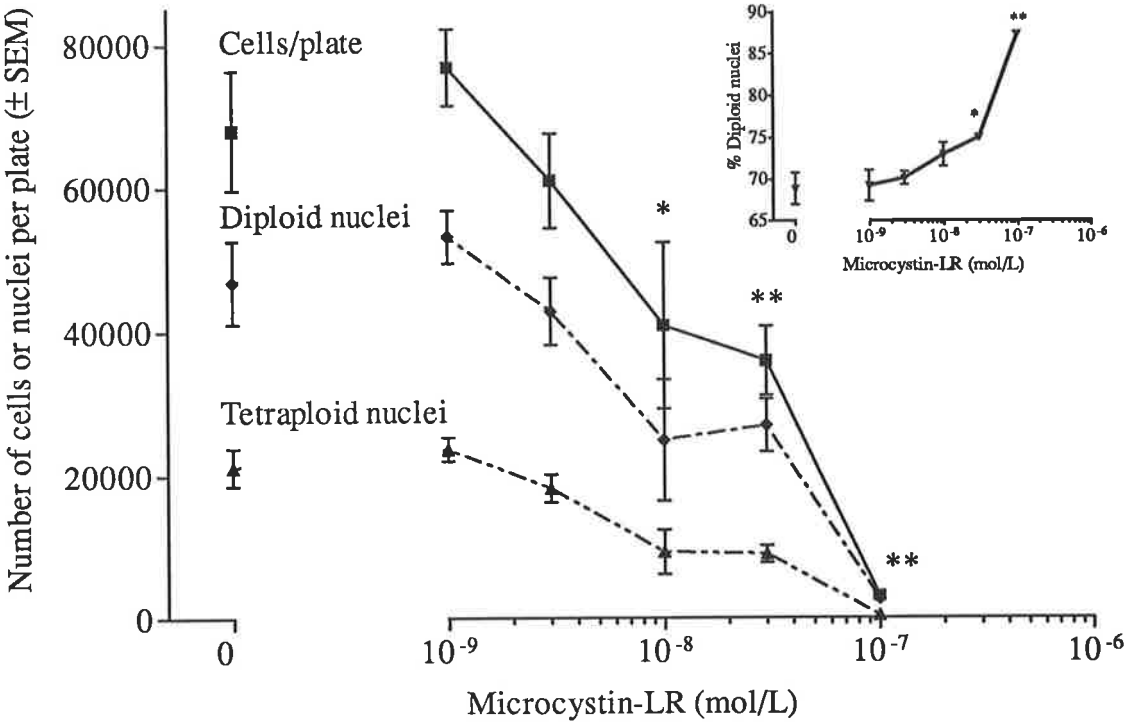



**Figure 9.5** - The effect of microcystin-LR on total cell numbers, and on the numbers of diploid and tetraploid nuclei at 18 hours in culture. Inset shows the percentage of diploid nuclei. Hepatocytes in culture were exposed to various concentrations of microcystin-LR at 2 hours, and the cells were counted and then harvested at 18 hours. Proportions of PI-stained nuclei in the diploid and tetraploid groups were quantified by flow cytometry. There were too few octaploid nuclei in some treatment groups to be accurately determined so these were not included. The numbers of nuclei per ploidy group were calculated based on cell numbers. Significant differences with respect to untreated control were analysed by one-way ANOVA followed by Dunnett's*post hoc* Test. * ($p<0.05$), ** ($p<0.01$).

From Figure 9.5 it can be seen that cell numbers decreased from a peak at 1.0 nM microcystin-LR to 100.0 nM toxin by which concentration almost all cells were killed. As was found previously (see Figure 6.1), 10.0 nM microcystin-LR killed approximately 50% of cells. In the present experiment, however, increases in cell numbers were not seen at 1.0 nM and 3.0 nM microcystin-LR. This issue is addressed in the discussion section of this Chapter. Proportions of diploid and tetraploid nuclei, determined by flow cytometry, are also shown, both as numbers per plate and, in the inset, as the percentage of nuclei which were diploid. There were too few octaploid nuclei to count reliably and so these were omitted. Both diploid and tetraploid nuclei decreased roughly in parallel with cell numbers, indicating that there was no absolute selectivity of

the toxin for higher ploidy cells. However, when the data for numbers of nuclei were analysed by two-way ANOVA, there was found to be a significant ($p=0.01$) interaction between toxin concentration and relative numbers of nuclei. This is illustrated in the inset, indicating that as the toxin concentration increased so did the proportion of diploid nuclei, becoming significantly higher than control ($p<0.05$) at 30.0 nM microcystin-LR. Therefore, although all cells were sensitive to microcystin-LR, higher ploidy cells tended to be more so at relatively high toxin concentrations.

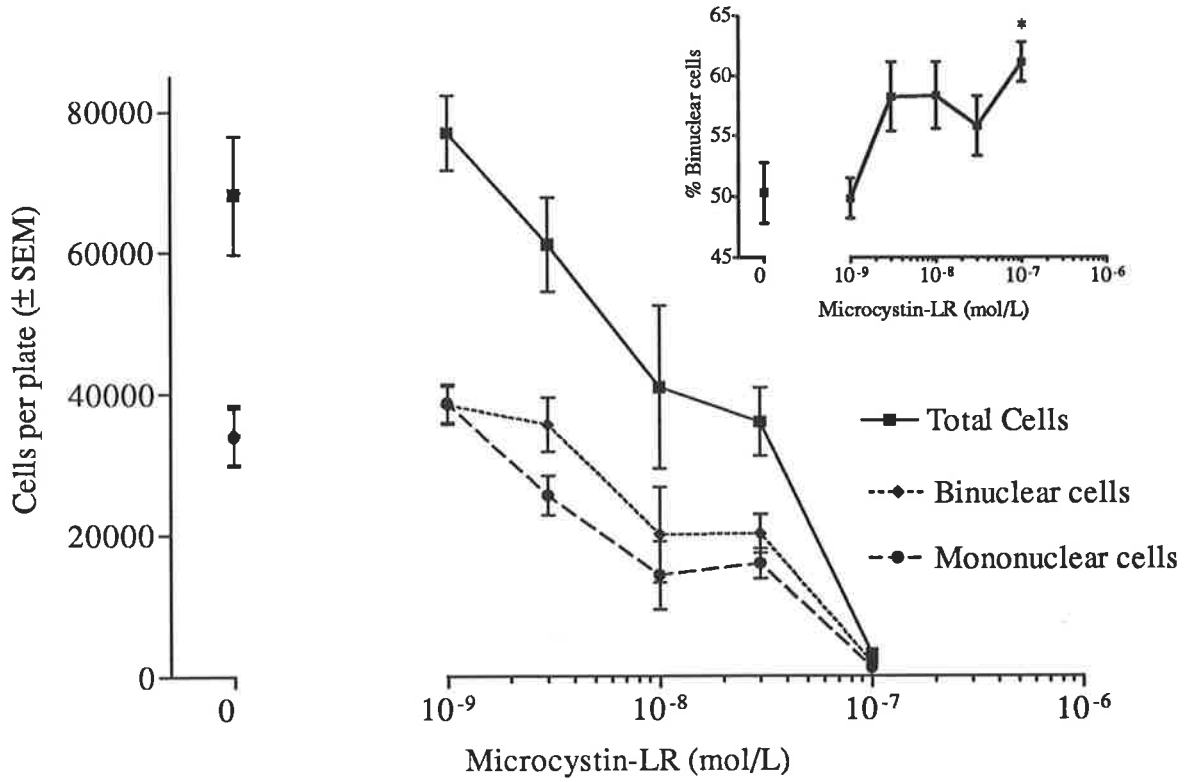



**Figure 9.6** - Effect of microcystin-LR on the numbers of mononuclear and binuclear cells per plate after 18 hours in culture. Inset shows percentage of cells which were binuclear. Hepatocytes in culture were exposed to various concentrations of microcystin-LR at 2 hours, and the cells were counted and then harvested at 18 hours. Proportions of PI-stained cells which were mononuclear or binuclear were quantified by fluorescence microscopy. The numbers of cells in each group were calculated based on total cell numbers. Significant differences with respect to untreated control were analysed by one-way ANOVA followed by Dunnett's *post hoc* Test. * ($p<0.05$).

The numbers of cells which were mononuclear or binuclear, derived from counts under the fluorescence microscope and counts of total cells on the plate, are shown in Figure 9.6, along with an inset depicting the percentage of cells which were binuclear. There was some evidence of toxin selectivity against mononuclear cells, but again this was not significant until high toxin concentrations when very few cells survived.

### 9.3.5 Effect of microcystin-LR on hepatonuclear ploidy and binuclearity after 65hrs exposure

Hepatocytes were exposed to a range of microcystin-LR concentrations from 2 hours until 65 hours culture time. Medium and toxin were renewed every 24 hours. Triplicate plates from each treatment were harvested as described in Section 9.2.6, and these cell suspensions were analysed using a haemocytometer under the fluorescence microscope for both total cell numbers and proportions of binuclear and mononuclear cells. Total mononuclear and binuclear cells, and the percentage of cells which was binuclear were then calculated. These results are presented in Figure 9.7.

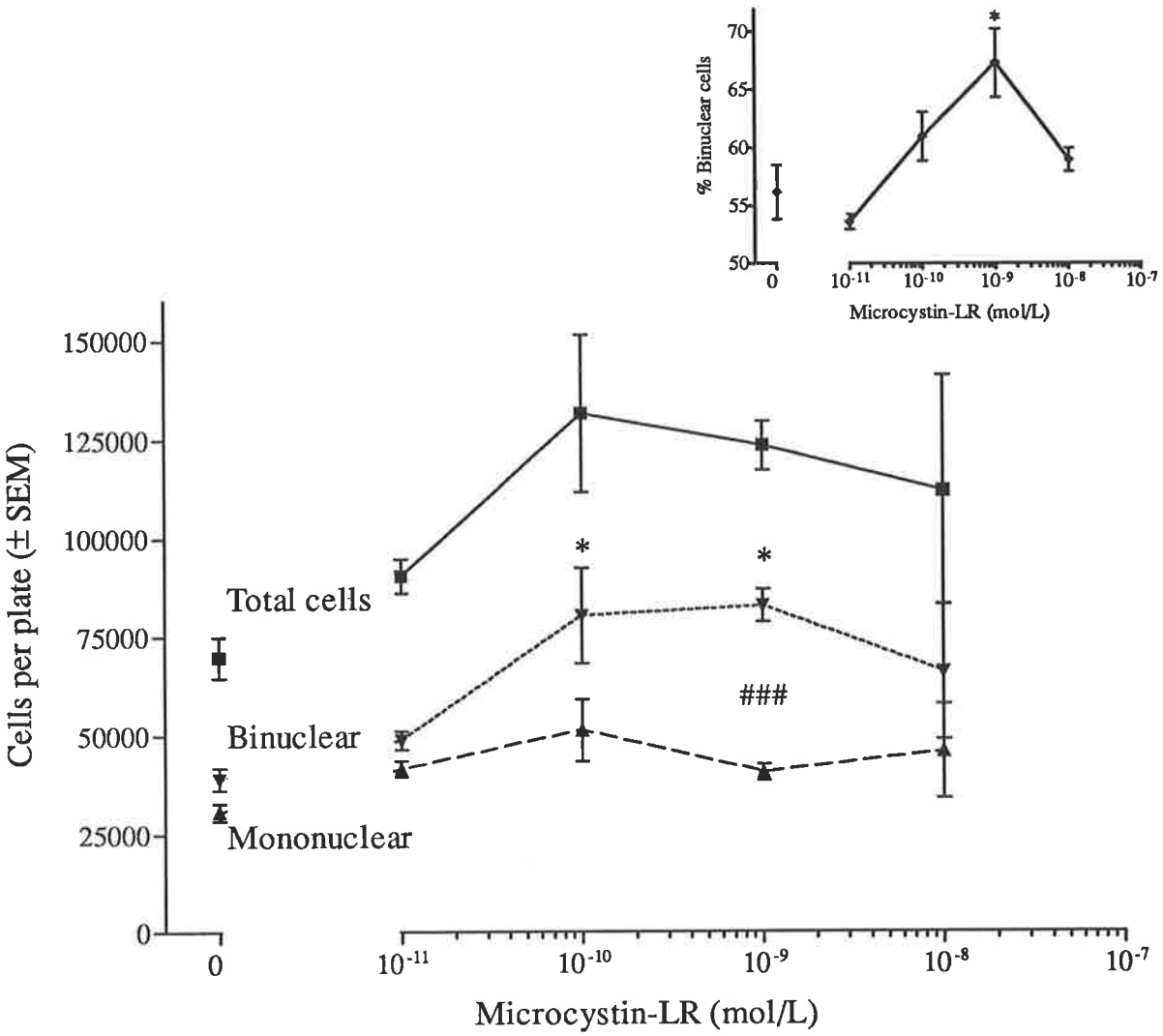



**Figure 9.7** - Effect of microcystin-LR on numbers of mononuclear and binuclear cells after 65 hours in culture. Inset shows change in the percentage of binuclear cells. Three additions of toxin were made at 2, 24 and 48 hours, with changes of medium. Cells were harvested at 65 hours. Cell numbers and the proportions of binuclear and mononuclear cells were determined by fluorescence microscopy. Significant differences between treatment groups with respect to the appropriate untreated control are marked by * ($p<0.05$), whereas a significant difference between the numbers of mononuclear and binuclear cells at 1.0 nM microcystin-LR is marked by ### ($P<0.001$).

The percentage of binuclear cells increased with increasing microcystin-LR concentration between 0.01 and 1.0 nM before falling again at 10.0 nM. It was significantly higher than in untreated control at 1.0 nM ($p<0.05$). It can be seen that this arises due to an increase in the number of binuclear cells in these treatment groups, rather than a fall in mononuclear cells. Binuclear cells were significantly higher in the 0.1 nM and 1.0 nM treatments than in untreated

control (one way ANOVA, Dunnett's post hoc test, $p<0.05$), and also significantly higher than mononuclear cell numbers in the 1.0 nM treatment (t-test, $p<0.001$).

Average nuclear ploidy was calculated from flow cytometric determinations of the proportions of nuclei in each ploidy group from parallel treatment groups to those counted above. This shown in Figure 9.8.

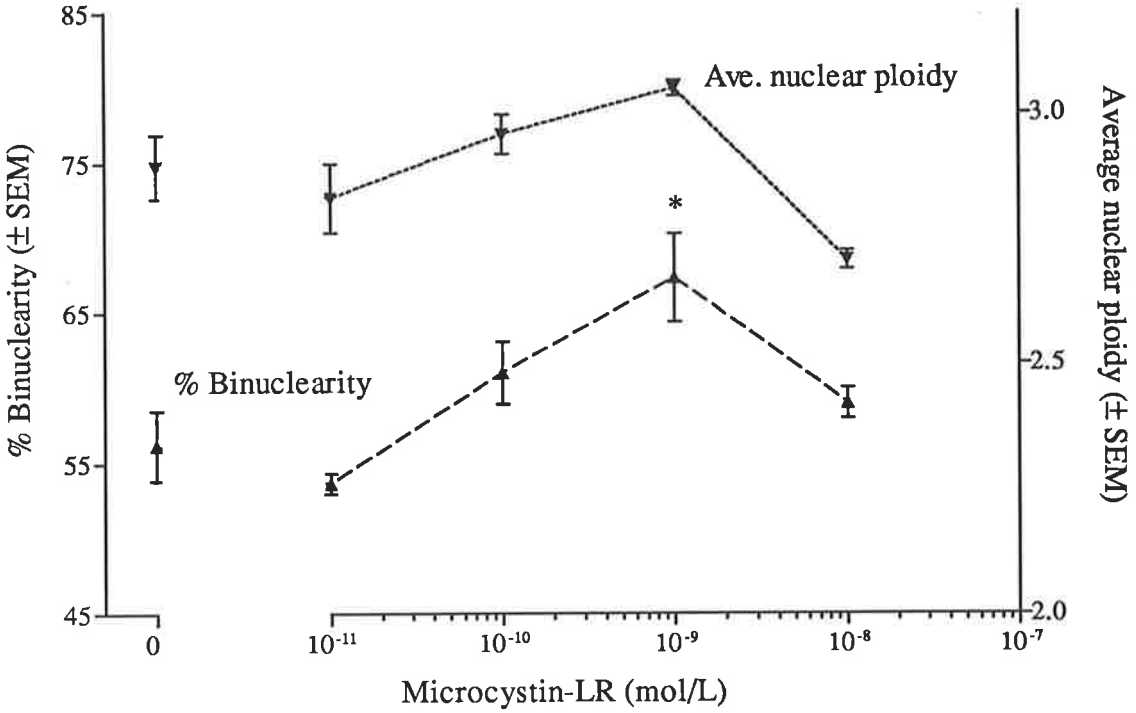



**Figure 9.8** - Effect of microcystin-LR on hepatonuclear ploidy and cellular binuclearity after 65 hours in culture. Three additions of toxin were made at 2, 24 and 48 hours, with changes of medium. Cells were harvested at 65 hours. Proportions of nuclei belonging to the different ploidy groups were quantified by flow cytometry, and the average nuclear ploidy was calculated using Eqn. 9.1. Percent binuclearity is shown again for comparison. Significance of differences between treatment groups was determined by one-way ANOVA followed by Tukey's Multiple Comparison *post hoc* Test. * ($p<0.05$) a significant difference from untreated control. For a description of the comparison of other treatment groups, see text.

Average nuclear ploidy followed a similar pattern to the binucleation rate, although due to some variability in the untreated control, none of the treatments were significantly different from

control. Nevertheless, 1.0 nM microcystin-LR produced a significantly higher average nuclear ploidy than 0.01 nM ($p<0.05$). Microcystin-LR at 10.0 nM caused a significant reduction with respect to both 0.1 nM ($p<0.05$) and 1.0 nM ($p<0.01$). Thus it does appear likely that changes are induced in average nuclear ploidy as well as in the binuclearity of the hepatocyte population by microcystin-LR at concentrations below those shown to induce a purely toxic response, that is below 10.0 nM microcystin-LR.

These results generally support the findings of the previous chapters. The occurrence of slight reductions in both percent binuclearity and average nuclear ploidy at 0.01 nM microcystin-LR could indicate a stimulation of cytokinesis, although since a DNA assay was not logistically possible in this experiment, this cannot be confirmed. An inhibition of cytokinesis was evident with increasing microcystin-LR concentration between 0.01 and 1.0 nM since percent binuclearity increased significantly through this range. Average nuclear ploidy also increased over the same concentration range suggesting that it was higher ploidy cells which were cytokinesis-inhibited. At 10.0 nM microcystin-LR, the toxin-surviving hepatocytes were characterised by essentially the same percent binuclearity and average nuclear ploidy as the untreated control. This again suggests that any effects that microcystin-LR has on this cohort of cells are not detectable from a single determination at this time point.

An attempt was made to elucidate the response patterns of each cellular ploidy group to microcystin-LR by way of a mathematical model, the rationale for which is detailed in Appendix 2. There are a number of uncertainties involved in using a model such as this to predict effects beyond the known data. Nevertheless, it was considered a useful exercise as an aid to understanding the underlying processes occurring when proliferating hepatocytes are exposed to

microcystin-LR. The above results were used to calculate the cell numbers in each ploidy group for each treatment. The result is presented in Figure 9.9.

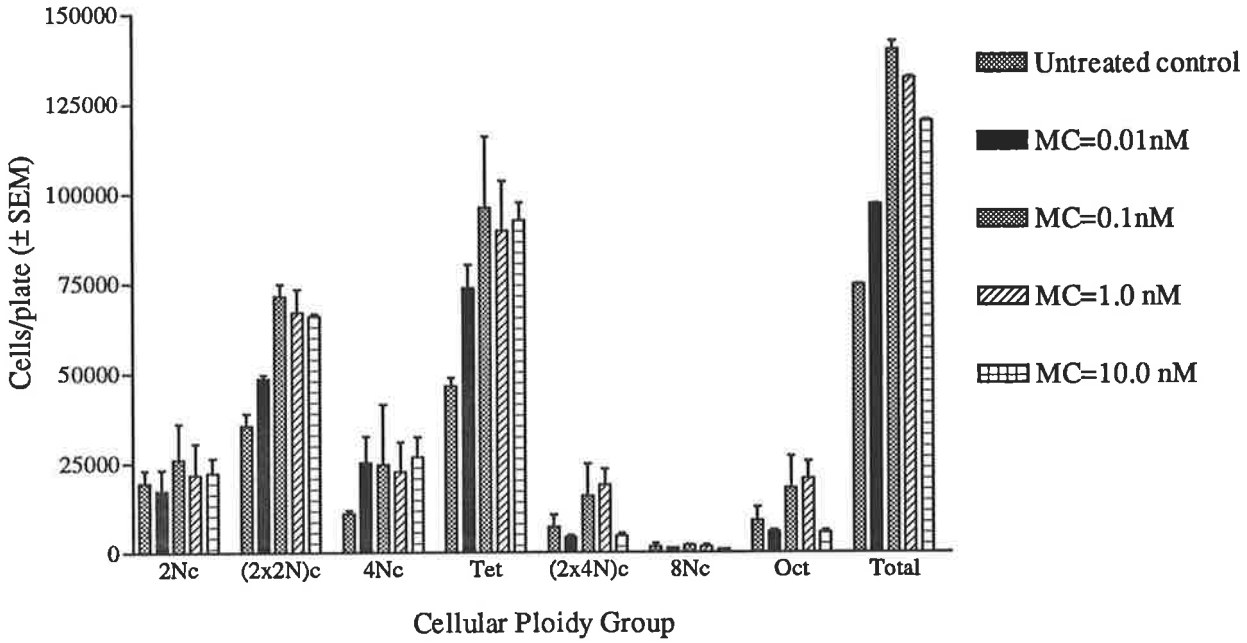



**Figure 9.9** - Change in calculated numbers per cellular ploidy group in relation to microcystin-LR concentration, as determined after 65 hours in culture. The calculation was based on the nuclear ploidy, binuclearity and cell number data presented in Figures 9.7 and 9.8. The calculation procedure is described in detail in Appendix 2.

From the above analysis it appears that binucleate cells in particular accumulate during toxin exposure in a dose-dependant manner up to 1nM microcystin-LR, and that at 10 nM toxin it is primarily the octaploid binucleate, (2x4N)c, group which is growth-inhibited and/or removed by the toxin. There is also a reduction of (2x4N)c cells with 0.01 nM microcystin-LR and a corresponding rise in the 4Nc group at this concentration, consistent with increased cytokinesis in these binuclear cells. The results of this exercise were therefore in agreement with those of previous experiments and help give some insight into the ploidy group dynamics of an hepatocyte population exposed to microcystin-LR.

## 9.4 Discussion

Flow cytometry was investigated as a means of determining the effects of microcystin-LR on various hepatocyte sub-populations. Validation experiments with untreated and EGF treated cells proved the efficacy of this technique for detecting cell division and changes in the proportions of ploidy groups in response to mitogenic stimulation. However, difficulties encountered in quantitatively releasing cells from culture plates as single-cell suspensions, especially those treated with microcystin-LR, threatened to limit its application to the elucidation of this toxin's effects. To overcome this problem, preparations of free nuclei were used, along with the determination of percent binuclearity, as a surrogate for quantification of effects at the cellular level. This was shown to be a valid alternative to cellular determinations, and to yield results confirming the findings of previous experiments using other techniques.

In cells exposed to microcystin-LR for 18 hours, when only immediate, that is non-proliferation-dependent, processes were likely to be observed, there appeared to be minimal ploidy based cell selectivity by the toxin. Only at relatively high concentrations of 30 - 100 nM microcystin-LR was there evidence of a selection process occurring. This was somewhat surprising since it is known, for example, that bromosulphthalein uptake rate is higher in larger, and hence high ploidy, hepatocytes (Deschenes, *et al.*, 1981). It was therefore expected that microcystin-LR uptake and toxicity might follow a similar trend. However, as previously noted in Section 5.4 and in relation to the results of Chapters 6-8, acute toxicity does not appear to correspond linearly to toxin uptake. Nor does toxin uptake appear to be predictable from bile acid uptake by known transporters (Runnegar, *et al.*, 1995b). Therefore, the relationship between the capacity for toxin uptake and the degree of hepatocyte differentiation is likewise unknown. Hence

it appears that the acute toxic effects of microcystin-LR are not markedly ploidy dependent. This is not true, however, for the sub-acute effects of this toxin.

In cells exposed to microcystin-LR for 65 hours there were marked effects on binuclearity and average nuclear ploidy at concentrations below 1 nM microcystin-LR. Both percent binuclearity and average nuclear ploidy rose with increasing concentration between 0.01 nM and 1.0 nM microcystin-LR. These results can be interpreted in a number of ways. For example, cytokinesis could have been inhibited. This would occur most simply in proliferating binuclear cells, and lead to the accumulation of binuclear cells with nuclei of twice the previous DNA content. However, the normally alternating pattern of acytokinetic and cytokinetic divisions is not obligatory, especially in the diploid cohort, because otherwise there would be no diploids left after one round of replication. Therefore, some mononucleate cells must normally complete a cytokinetic mitosis within a single cycle. If the relative rates of completion of single versus double cell cycles was effected by microcystin-LR, perhaps resulting from an inhibition of cytokinesis, such that acytokinetic mitoses were favoured in proliferating mononuclear cells, then this would also lead to the accumulation of binuclear cells, but with nuclei of the same DNA content as previously. Because average nuclear ploidy also increased with toxin concentration, this mechanism appears the less likely of the two. However, the increase in average nuclear ploidy with increasing toxin concentration could also be explained by an inhibition of karyokinesis in proliferating mononuclear cells. This would lead to an accumulation of mononuclear cells with twice the previous DNA content. In this case binuclear cells would accumulate due to independent effects in other cells, as discussed above. However, this gives two conflicting mechanisms being postulated to be occurring in mononuclear cells, although this could conceivably happen if the cells in question had different sensitivities to microcystin-LR. The modelling of the flow cytometric data did suggest that tetraploid mononuclear cells (4Nc), as well as binuclear cells,

were higher in the presence of microcystin-LR, but further experimental work would need to be carried out to verify this. Therefore, the simplest mechanism which can be postulated to account for the effects of 0.01 nM - 1.0 nM microcystin-LR on average nuclear ploidy and percent binuclearity is that cytokinesis was inhibited predominantly in proliferating binuclear cells, although effects on mononuclear cells cannot be discounted.

It should be noted that in these flow cytometry experiments there was no evidence of a change in ploidy to support the previous findings suggesting a stimulation of cytokinesis. This was particularly surprising in the experiment sampled at 18 hours because in a similar previous experiment (Chapter 6) a marked increase in cell numbers with a concomitant reduction in DNA per cell suggested that this had occurred. The results of the multi-addition experiments supported this interpretation. Furthermore, cell numbers did increase with microcystin-LR treatment in the flow cytometry experiment sampled at 65 hours, and there was some evidence of a cytokinetic stimulation from the modelling of this data in that tetraploid binuclear cells [(2x4N)c] were reduced and tetraploid mononuclear cells (4Nc) increased at 0.01 nM microcystin-LR. However, cell numbers were greater than expected at higher toxin concentrations in this experiment, based on previous findings. Since these cells were not counted on the plate as previously, but instead on a haemocytometer after removal from the plate and propidium iodide staining, this may account for the difference. Further experimentation is therefore required to clarify the conditions under which the cytokinetic stimulation occurs and also to monitor the yield of cells during harvesting for flow cytometry.

# CHAPTER 10

# DISCUSSION

The results of the research presented in this thesis were discussed in detail at the conclusion of the chapter in which they were presented. In this final chapter the findings from this research will be discussed in more general terms relating the possible mechanisms by which microcystins may promote tumour growth to the wider issues of exposure levels and public health.

There is mounting evidence that the microcystins can act as tumour promoters. This evidence comes from epidemiological surveys (Su, 1979; Yu, 1989; Yu, 1995) as well as *in vivo* studies (Falconer and Buckley, 1989; Zhou and Yu, 1990; Nishiwaki-Matsushima, *et al.*, 1992). However, little work has been done on the mechanism of action by which these toxins might have effects on the cell cycle (Mellgren, *et al.*, 1993), mainly because of the requirement for a model system capable of actively transporting the toxins into the cell (Eriksson, *et al.*, 1994). Therefore, most of our appreciation of possible mechanisms is by extrapolation from analogous studies carried out using other protein phosphatase inhibitors, such as okadaic acid (Fujiki and Suganuma, 1993; Fujiki and Suganuma, 1994). However, there are marked differences in the tissue and protein phosphatase specificities of these toxins and so such extrapolation is not strictly valid. For example, Mellgren *et al.* (1993) and this study have shown differences in the way in which primary hepatocytes in culture react to exposure to okadaic acid versus microcystin-LR. This suggests that for a proper assessment of the risk posed to human populations by the presence of microcystin in drinking waters, studies using this toxin rather than alternatives are vital.

The purpose of the research presented in this thesis was to address some of the outstanding questions relating to tumour promotion by microcystin. The *in vivo* study described in Chapter 3 was designed to assess the effects of these toxins at the whole animal level using a crude *Microcystis* extract as the source of a range of microcystins. Therefore, possible interactions between multiple influences could be permitted to play a part in the final outcome. For example, effects of constituents of the *Microcystis* extract on toxin uptake by the gut and metabolism in the liver, as well as effects of the toxin on various tissues, and the interactions of toxin-affected tissues with other tissues are all likely to be part of the "real life" response of an animal to exposure to these blue-green algal products in drinking water. Since hepatocellular carcinoma is rare in Western countries, mainly due to the lack of exposure to aflatoxins and hepatitis B virus, it was considered more appropriate to make the gut the primary focus of this investigation. Western rates of alimentary tract cancer are far higher than for liver cancer. Therefore, the directly acting carcinogen N-nitroso-N-methylurea was given by gavage, and *Microcystis* extract supplied as the only source of drinking water. By 20 weeks, the majority of the mice given the highest concentration of *Microcystis* extract had succumbed to the combined effects of the toxin and tumour growth, and the experiment was terminated. Duodenal adenomas and adenocarcinomas, and various types of lymphoma, were found in the animals. However, no significant differences were found in the incidence or progression of these neoplastic processes between the groups of animals exposed to different levels of toxic extract. Nor was there a difference in survival time. In mice drinking the undiluted extract a suppression of duodenal tumour growth was seen due to its acute toxicity, but the extract also had a deleterious effect on the general health of the mice. Therefore, there was no evidence from this experiment that the toxic *Microcystis* extract used here contained compounds which could significantly promote the growth of duodenal or lymphoid cancers. The high numbers and rates of growth of tumours in the control group reduced the

sensitivity of the study somewhat, and so further studies, with lower doses of initiator and extract exposures, will be required in the future.

*In vitro* studies were also carried out using cultured primary mouse hepatocytes. The advantage of using these cells is that they have, at least initially, the ability to actively take up microcystin. However, the cells lost sensitivity to the toxin, in terms of an acute morphological response, during culture. In Chapter 5, attempts to prolong the time that these cells retain expression of the microcystin transporter(s) were described. Since DMSO is known to cause retention of many differentiated characteristics in hepatocytes in culture (Isom, *et al.*, 1985; Isom, *et al.*, 1987), the effect of the addition of this compound to the culture medium was investigated. It was found that culturing hepatocytes in the presence of 2% DMSO significantly prolonged their sensitivity to the toxin but that, paradoxically, there appeared to be no difference from non-DMSO treated control cells in the time course for the loss of ability to take up $^{3}$H-microcystin-LR. This suggested that DMSO was promoting the retention of toxin sensitivity, rather than toxin uptake *per se*, and therefore it was this sensitivity which was primarily lost in non-DMSO treated cells during the first 2 days in culture. If the intracellular transport of microcystins plays a part in directing these toxins to specific responsive sites within the cell, it is conceivable that differences in culture conditions could alter the expression or functioning of such a mechanism. This could explain the observed differences between cells in untreated control and those cultured in the presence of DMSO.

Unfortunately, in order to study the effects of microcystin-LR on the hepatocyte cell cycle, proliferating cells were required and DMSO treatment significantly reduced the proliferation rate in comparison with untreated cells. Therefore, DMSO was not used. However, these results imply that the reduction in proliferation rate and the retention of toxin sensitivity were related in DMSO

treated cells, perhaps by a general retention of a more differentiated regulatory regime in the cell. This is supported by the finding (Section 7.4) that the highly proliferative EGF-treated cells were less sensitive to microcystin-LR than in untreated cells. If the same selectivity by the toxin applied *in vivo*, the more actively proliferating the (cancerous) cell and the less differentiated characteristics it expressed, the less sensitive to the acute toxic effects of microcystin-LR that cell would be. This could be seen as a growth advantage to that cell during microcystin-induced hepatotoxicosis.

Further *in vitro* studies, described in Chapters 6-9, were carried out in proliferating hepatocyte cultures in which cells were exposed to a range of sub-toxic microcystin-LR concentrations. The major disadvantage associated with using primary cultures is that they are not an homogeneous population and so various sub-populations can react in different ways to experimental stimuli. This complicates the interpretation of experimental outcomes. Nevertheless, evidence was found that microcystin-LR at different concentrations has effects on the rate of hepatocellular cytokinesis and also that toxicity is cell-selective. Thus at low concentrations a stimulation of cytokinesis was observed, whereas at higher concentrations either a delay of cytokinesis or synchronisation of cells, so that they all synthesised DNA and then passed through cytokinesis together, was seen. The actual concentrations of microcystin-LR required to produce these effects varied with the exposure regime. With continuous exposure, cytokinetic stimulation occurred in the 10 - 100 pM range, whereas cytokinetic delay or synchronisation, as well as cell-selective toxicity, occurred in the 1-10 nM range. If this stimulation of cytokinesis occurs *in vivo* it might be expected to lead to a lowering of the average ploidy of the hepatocyte population in the liver. Lower ploidy cells, lacking the genetic redundancy of their higher ploidy counterparts, may be more susceptible to mutagenic transformation (Saeter, *et al.*, 1988b). Whether the effects of 1-10 nM microcystin-LR on cytokinesis arose from a specific effect in the toxin-surviving population,

or simply a selection against all cells except those which had just entered the cell cycle at the time of exposure is unclear from the data. Flow cytometry could be usefully employed in this regard to detect changes in hepatocyte ploidy in exposed animals subjected to chronic microcystin treatment.

There was also evidence that microcystin-LR at sub-toxic levels could reduce cell losses from the culture system. The interaction of microcystin with nafenopin suggested that it was apoptotic cells which were rescued by microcystin-LR at low concentrations and also that it was a nafenopin-sensitive apoptotic process which was induced by microcystin at higher selectively toxic concentrations. Since apoptosis is a mechanism invoked by a cell which might otherwise enter S-phase prematurely, for example, if DNA excision repair is incomplete, a suppression of this could allow the continued proliferation of mutation-carrying cells, as is thought to occur in cells with defective p53 expression.

A comparison of microcystin-induced effects was made with those of okadaic acid over a range of toxin concentrations. As noted above, it was found that these two protein phosphatase inhibitors do not act in the same fashion. In particular, okadaic acid inhibited DNA synthesis in affected cells whereas microcystin-LR did not. Furthermore, no evidence was seen for an okadaic acid-induced stimulation of cytokinesis nor a selective induction of cell death. Further studies in a more homogeneous population of cells will be necessary to determine the relative roles of toxin uptake and inhibition of PP1 versus PP2A in the generation of these differences. Nevertheless, these results highlight the difficulty in extrapolating findings derived from experiments using okadaic acid to explain the effects of microcystin.

Flow cytometric methods were developed to allow the investigation of the effects of microcystin-LR on the hepatocyte population. It was shown, using EGF stimulation of hepatocytes in culture, that changes in cellular population dynamics could be detected using this technology. These changes were consistent with those reported by others in proliferating cells (Saeter, *et al.*, 1988b; Jack, *et al.*, 1990; Fausto and Webber, 1994). When these techniques were applied to microcystin-LR treated cells, a number of observations were made. During short-term exposure (18 hours) there was some effect of the toxin on the relative sizes of the different ploidy groups, in that binuclear tetraploid and possibly diploid cells were less likely to be killed by the toxin, but this only became apparent at relatively high (>10 nM) concentrations. The stimulation of cytokinesis by 1 nM microcystin-LR seen earlier was not confirmed in this experiment and so further work aimed at resolving this contradiction is required. However, with longer-term exposure (65 hours), during which time the cells were proliferating, there was evidence that microcystin-LR concentrations below 1 nM increasingly caused an accumulation of binuclear cells. Since this was accompanied by a parallel increase in the average nuclear ploidy, it seems that it was higher ploidy cells, and binucleate octaploid cells in particular, which were most affected by the toxin. This could be a combination of greater uptake in higher ploidy cells in general, and also an inhibition of cytokinesis in these cells, but as other processes can also be envisaged (as explored in the discussion of Chapter 9), further experiments will be necessary to clarify this. Above 1nM microcystin-LR this effect was not observed, and since it is in this range of toxin concentrations where selective toxicity was found to occur, this suggests that the cells most affected at lower concentrations were preferentially killed by microcystin-LR at these higher concentrations. Therefore, from these flow cytometric studies it can be concluded that microcystin-LR selectivity is, in part, based on the ploidy of the exposed cell.

This view is consistent with the known properties of the different hepatocyte ploidy groups. Higher ploidy cells are more differentiated than diploid hepatocytes, for example having greater rates of uptake of the bile acid analogue bromosulfophthalein (Deschenes, *et al.*, 1981), and so may accumulate higher internal concentrations of toxin. Furthermore, since they only have similar rates of RNA synthesis to diploids (Collins, 1978), they may be less able to respond quickly to loss of functional enzymes. Such a selective mechanism of action by microcystin may well lead to promotion of tumour growth. If it is true that the more differentiated higher ploidy cells have greater sensitivity to this toxin due their more avid uptake, then it is likely that in a more or less constantly exposed individual these cells will be constantly killed, leading to a functional deficit and hence a regenerative response by the organism (Fausto and Webber, 1994). Thus a "background" growth stimulus, higher than would normally be present, will be generated. Diploid hepatocytes, possibly including those which make up most hepatocellular tumours (Saeter, *et al.*, 1988a; Saeter, *et al.*, 1988c; Schwarze, *et al.*, 1991), have a faster rate of proliferation in such a regenerative environment (Saeter, *et al.*, 1988b) and so will have a growth advantage. This scenario could lead to an apparent promotion of tumour growth by microcystin. From this point of view, flow cytometric analysis of *in vivo* changes in hepatocellular ploidy following a relatively low chronic exposure to microcystin, with and without prior tumour initiation, might provide a means to assess the likelihood that this mechanism plays a role in tumour promotion by microcystin.

The selectivity exhibited by microcystin was not entirely ploidy-dependent, however, since not all cells appear to be affected solely on the basis of ploidy. Other modulating factors must also apply, examples of which might be whether a cell is quiescent or proliferative, or the balance of apoptotic versus proliferative regulatory signals pertaining, or once committed, its position in the cell cycle. The results presented in this thesis suggest that microcystin-LR may have effects on

many of these processes also, for example, on cytokinesis and apoptosis, and that it is therefore likely that tumour promotion by this toxin is the result of multiple interacting effects. In order to investigate these effects further, some means of making the hepatocyte population more homogeneous would be desirable. Attempts were made during this project to synchronise the isolated hepatocytes in the cell cycle using vinblastin or hydroxyurea (data not shown). This did not prove successful as these compounds caused high cell loses and because the time required for their effect to be useful would have prevented quantitative application of microcystin-LR. An alternative, although very expensive, approach could be to use a sorting flow cytometer to separate the cells into more homogeneous sub-populations prior to culture. However, sorting on the basis of ploidy would be difficult as the cells are not likely to remain viable if DNA-stained. Therefore, sorting on less specific characteristics such as their forward and side scatter would have to be employed, and so the resulting sub-populations could only be enriched in cells of a particular size and could not be considered to be at all homogeneous. Similar reservations apply to cells sorted by centrifugal elutriation. Nevertheless, these approaches could be used to correlate various markers of differentiation including expression of bile acid transporters with sensitivity to, or uptake of, microcystin. The transfection of immortalised hepatocyte cell lines with a microcystin transporter will most likely be a fortuitous by-product of such studies investigating the various bile acid transporters (Platte, *et al.*, 1996), particularly as a specific microcystin transporter has yet to be identified(Runnegar, *et al.*, 1995a).

There is a paucity of information on the hepatic availability of orally ingested microcystin at realistic exposure levels. Most animal studies tracing the fate of radiolabelled microcystin have used relatively high doses given intraperitoneally (Falconer, *et al.*, 1986; Runnegar, *et al.*, 1986; Brooks and Codd, 1987; Meriluoto, *et al.*, 1990; Robinson, *et al.*, 1991c). These studies confirm that the majority (70-90%) of the injected toxin is sequestered in the liver, reliant on its active

uptake by this organ (Eriksson, *et al.*, 1990a; Runnegar, *et al.*, 1991). However, when $^{3}$H-dihydromicrocystin-LR was given orally, only 0.68% of the total dose was found in the liver, whereas 70% of an ip dose was found in this organ (Nishiwaki, *et al.*, 1994). This liver uptake of orally given microcystin was comparable to that of non-liver specific okadaic acid and so it has been suggested that there may be other compounds in crude extracts which facilitate the liver specificity of the microcystins (Fujiki, *et al.*, 1996). However, Nishiwaki *et al.*, (1994) used 8 week old mice in their study, and it has since been suggested that young (5 week old) mice are markedly less sensitive than older (32 week old) mice to microcystins given orally (Ito, *et al.*, 1997). Therefore, the age of the test mice may have effected the magnitude of the difference observed by Nishiwaki *et al*, (1994). Nevertheless, the oral toxicity to mice of purified microcystin-LR was found to be between 30- and 100-fold lower than by the ip route (Fawell, *et al.*, 1994), which is reasonably consistent with the 100-fold difference in uptake suggested by Nishiwaki *et al.*, (1994) although the age of the mice used by Fawell *et al.*, (1994) was not reported. Using freeze-dried *Microcystis* bloom material having a 24 hour ip $LD_{50}$ of 28.5 mg dry weight/kg mouse, Falconer *et al.*, (1994) found that 2.6 g dry weight/kg mouse/day killed about 50% of orally treated mice over 21 days of exposure. This represents a ratio of approximately 90-fold, again in broad agreement with the results quoted above. Therefore, from these results it seems reasonable to assume that most of an ip dose is available to the liver, and the majority of this is sequestered by this organ, but that only perhaps one to two percent of a large, near fatal, oral dose is taken up by liver.

Since the liver can take up toxin if it is available in the blood, that is if given ip or iv, the limiting step in the uptake of orally administered toxin is that which takes the toxin from the gut and transfers it to the blood stream. It has been shown in a number of studies that the cells lining the small intestine are sensitive to microcystin toxicity (Falconer, *et al.*, 1992; Ito, *et al.*, 1997;

and results presented in Chapter 3 of this thesis) when the toxin is administered at a high enough concentration, and it is quite conceivable that toxin-induced damage to these cells would limit the active uptake and transfer of toxin to the blood. However, the transfer of toxin to the blood may not an active process but could rather be due to leakage through the gut wall once this barrier has been damaged by the toxin. But since this scenario still requires uptake of the toxin by enterocytes, it seems simpler to assume that the toxin is taken up by the cells via a mechanism analogous to that identified in hepatocytes, that is by an organic anion transporter, the purpose of which is to transfer bile acids and other compounds from the gut to the hepatic blood supply. If this is the case, then the efficiency of toxin uptake would be likely to be much higher in intact enterocytes than in damaged cells. Furthermore, since bile acids are not metabolised in the enterocytes but passed directly to the hepatic blood supply for processing in the liver, the intra-enterocyte bile acid transport system is likely to be functionally different to that of hepatocytes. That is, it is likely to be directed towards immediate re-export at the opposite pole of the cell, thereby delivering the bile acids and other compounds to the hepatic blood supply. If it is assumed that microcystins are delivered by the trans-membrane transporter, along with bile acids, to a specific intra-cellular transport system whose function is to transfer organic anions to the opposite cellular pole for re-export, then as long as the levels of toxin (plus other transported compounds) are low enough that they do not overload this system, toxicity to the enterocyte will be minimal and so the transfer of microcystin into the blood may be highly efficient. This would be enhanced further by any enterohepatic circulation of the toxin (Falconer, *et al.*, 1986). Therefore, a larger fraction of a low concentration oral dose might reach the liver than that estimated from findings based on the administration of a high concentration dose. Hence, the estimate of one to two percent made above should be seen as an lower limit of this fraction.

There are two possible processes which may reduce the efficiency of transfer of microcystins from the gut to the hepatic circulation, that is, metabolism of the toxin by microflora within the gastrointestinal tract, and by the cells of the gut lining. Both of these mechanisms are known to play a part in determining the efficiency of absorption of other xenobiotics, for example, many therapeutic drugs, from the gastrointestinal tract (Parkinson, 1996). They are only likely to be relevant during low exposure levels as they would be swamped by a large bolus dose of toxin. Neither of these possibilities appear to have been investigated in relation to their effects on microcystin uptake from low oral exposures, and so their relevance is unknown.

In the *in vitro* studies described in Chapters 6, 7and 8, effects on the hepatocyte cell cycle were seen at microcystin-LR concentrations in the range 10 - 100 pM, with cell selective toxicity at about 100 times these concentrations. This was despite the progressive loss of sensitivity of the hepatocytes to microcystin-LR in culture. If these concentrations in the culture medium are assumed to equate, in terms of availability to the hepatocyte, to similar concentrations in the blood, then no more than 100 times these concentrations would be required in the drinking water to produce similar effects *in vivo*. Thus a concentration of between 1 and 10 nM in drinking water could lead to these effects. This concentration corresponds to the recommended safe level of microcystin in drinking water of 1 μg/L or 1 nM (WHO, 1997). However, uncertainty will remain until the rates of intestinal uptake and hepatic sequestration of microcystin from realistic oral exposure levels can be determined.

# APPENDIX 1 MEDIA, BUFFERS AND SOLUTIONS

## A.1.1 Hepatocyte isolation buffers

### A.1.1.1 Hank's Buffered Salts Solution (HBSS)

10x Hank's Buffered Salts Solution

To make 1 L:

| | |
|---|---|
| NaCl | 80 g |
| KCl | 4.0 g |
| $KH_2PO_4$ | 0.6 g |
| $Na_2HPO_4$ | 0.48 g |
| Phenol Red | 100 mg |

Adjust to pH 7.3, make to 1 L, store at 4 °C.

For the working solution, take 100 ml of 10x stock HBSS, 100 ml of 10x Stock Magnesium Sulfate and 1 ml of 1000 mM $CaCl_2$, and make up to 1 L. Check pH, filter sterilise, store at 4°C.

### A.1.1.2 Krebs-Henseleit buffer

Stock 10x Modified Krebs-Henseleit Buffer

To make 1000 ml:

| | g/1000 ml | Conc. in 1/10 |
|---|---|---|
| NaCl | 67.3 g | 116 mM |
| KCl | 4.0 g | 5.4 mM |
| $KH_2PO_4$ | 0.54 g | 0.4 mM |
| $Na_2HPO_4$ | 0.43 g | 0.3 mM |
| $NaHCO_3$ | 21.8 g | 26 mM |
| HEPES | 47.6 g | 20 mM |
| BSA | 10.0 g | 0.1% |
| Glucose | 10.0 g | 5.6 mM |
| Phenol Red | 100 mg | 0.001% |

Adjust the pH to approx. 7.3. Store at 4 °C.

For the working buffer, dilute 100 ml 10x Modified Krebs-Henseleit Buffer, 100 ml 10x MgSO solution to almost 1000 ml with MQ.$H_2O$, set pH to 7.3 using HCl, top up to 1000 ml, filter sterilise, store at 4°C.

### A.1.1.3 Stock 10 x Magnesium Sulfate solution

Dissolve 2.0 g $MgSO_4.7H_2O$ in 1 L $MQ.H_2O$. Store at room temp.

### A.1.1.4 Stock 1000 x $CaCl_2$

Dissolve 14.7 g $CaCl_2$ in 100 ml $MQ.H_2O$. Autoclave. Store at room temp.

### A.1.1.5 Stock 100x EGTA solution

Dissolve 380 mg EGTA in 80 ml 1x Modified Krebs-Henseleit Buffer (keeping pH near 7 with NaOH to aid process). Adjust to approx. pH 7.3, make to 100 ml, autoclave, store at room temp.

### A.1.1.6 Stock 200x Insulin solution

Dissolve 3.5 mg Insulin in 35 ml saline, filter sterilise, aliquot into 20 x 1.5 ml lots (each enough for one perfusion), and store at -20°C.

### A.1.1.7 Stock 200x Antibiotic solution

Dissolve 420 mg Penicillin G and 700 mg Streptomycin in 35 ml saline, filter sterilise, aliquot into 20 x 1.5 ml lots (each enough for one perfusion), and store at -20°C.

### A.1.1.8 Stock 10x Collagenase solution

Dissolve 200 mg Collagenase (Sigma; Type IV) in 100 ml 1x Modified Krebs-Henseleit Buffer. Centrifuge at 15,000 x g for 30 min to remove particulate matter, filter sterilise, aliquot into 5 ml lots (each enough for one perfusion), and store at -20°C.

### A.1.1.9 First Perfusion Medium

To 1x Modified Krebs-Henseleit Buffer add:

0.6 ml 100x EGTA solution
0.3 ml of 200x Insulin solution
0.3 ml of 200x Antibiotic solution

Make up to 60 ml, bring to 37°C in water bath. Using a sterile Pasteur pipette bubble Carbogen slowly through (bubbling too fast causes it to foam).

### A.1.1.10 Second Perfusion Medium

To 1x Modified Krebs-Henseleit Buffer add:

0.05 ml 1000x $CaCl_2$ solution
0.25 ml of 200x Insulin solution
0.25 ml of 200x Antibiotic solution

Make up to 45 ml, bring to 37°C in water bath. Bubble Carbogen slowly through.

Just before beginning second perfusion, add 5 ml of 10x Collagenase solution to bubbling medium.

### A.1.1.11 Wash Medium

To 1x Modified Krebs-Henseleit Buffer add:

0.15 ml 1000x $CaCl_2$ solution
0.75 ml of 200x Insulin solution
0.75 ml of 200x Antibiotic solution

Make up to 150 ml, put on ice. Bubble Carbogen slowly through until ready to use.

### A.1.1.12 Percoll Isodensity Centrifugation Medium

To 18 ml Percoll, add:

2 ml 10x Modified Krebs-Henseleit Buffer
20 µl 1000x $CaCl_2$ solution
100 µl of 200x Insulin solution
100 µl of 200x Antibiotic solution
4 mg $MgSO_4.7H_2O$

Mix to dissolve $MgSO_4$, put on ice until ready to use.

### A.1.2 Hepatocyte culture media

#### A.1.2.1 Modified DMEM/F12 Culture Medium

In 900 ml MQ.$H_2O$ dissolve:

1 x 1L DMEM/F12 sachet ($NaHCO_3$/GLN-Free; ICN Chemicals)
60 mg Penicillin G
100 mg Streptomycin
1.0 g BSA
3.58 g HEPES (15 mM)
1.2 g $NaHCO_3$

Set pH to 7.45 and make up to 1 L. Centrifuge at 15,000 x g for 30 min to remove particulate matter which blocks the filter, then filter sterilise. Store at 4°C. Add L-Glutamine at 3.6 mg/10 ml, filter sterilised, just before use as it only lasts about a week in solution. Equilibrate medium in $CO_2$ incubator before use.

#### A.1.2.2 Stock Insulin-Transferrin-Selenium (ITS; Sigma) solution

This medium supplement contains Human transferrin and so should be treated as potentially infectious, as should all solutions containing it.

Add 50 ml MQ.$H_2O$ to supplied vial to dissolve lyophilised contents (which are under negative pressure). Divide as 1 ml aliquots into sterile Eppendorf tubes, store at -20°C.

#### A.1.2.2 Stock Dexamethasone solution

Dissolve 1.97 mg dexamethasone (Sigma) in 1 ml Ethanol. Store at -20°C.

#### A.1.2.3 Stock Long-Epidermal Growth Factor (Long-EGF; Gro-Pep) solution

Dissolve supplied lyophilised material at 1 mg/ml in 0.1 M HCl. Store at -80°C.

For working solution, add 10 µl Stock Long-EGF to 0.99 ml 0.1 M HCl containing 0.1 mg/ml BSA as carrier protein. Store at -80°C.

### A.1.3 Flow cytometry buffers

#### A.1.3.1 Preservation medium for cell freezing

"Saline-GM" (Crissman and Hirons, 1994)

| | g/L |
|---|---|
| Glucose | 1.1 |
| NaCl | 8.0 |
| KCl | 0.4 |
| $Na_2HPO_4.12H_2O$ | 0.39 |
| $KH_2PO_4$ | 0.15 |
| EDTA | 0.5 mM |

Solution pH set to 7.4. Stored at 4 °C.

#### A.1.3.2 Vindelov's nuclear isolation buffers

(Vindelov and Christensen, 1994)

##### A.1.3.2.1 Citrate buffer

| | |
|---|---|
| Sucrose | 85.5g |
| Trisodium citrate.$2H_2O$ | 11.76g |
| Dissolve in distilled water | approx 800ml |
| DMSO | 50ml |
| Distilled water to | 1000ml |

Adjust pH to 7.6

##### A.1.3.2.2 Stock solution

| | |
|---|---|
| Trisodium citrate.$2H_2O$ | 1.0g |
| Nonidet-P40 | 1.0ml |
| Spermine tetrahydrochloride | 522mg |
| Tris | 61mg |
| Distilled water to | 1000ml |

##### A.1.3.2.3 Solution A

| | |
|---|---|
| Stock solution | 1000ml |
| Trypsin | 30mg |

Adjust pH to 7.6

#### A.1.3.2.4 Solution B

| | |
|---|---|
| Stock solution | 1000ml |
| Trypsin inhibitor | 500mg |
| Ribonuclease A | 100mg |

Adjust pH to 7.6

#### A.1.3.2.5 Solution C

| | |
|---|---|
| Stock solution | 1000ml |
| Propidium iodide | 416mg |
| Spermine tetrahydrochloride | 1160mg |

Adjust pH to 7.6

## APPENDIX 2. CALCULATION OF CELLULAR PLOIDY GROUPS FROM NUCLEAR PLOIDY AND CELLULAR BINUCLEARITY

### Definition of terms

| | Diploid | Tetraploid | Octaploid |
|---|---|---|---|
| Cells | *2Nc* | *(2x2N)c* *4Nc* | *(2x4N)c* *8Nc* |
| Nuclei | *2Nn* | *4Nn* | *8Nn* |
| Nuclear aggregates | | *(2Nn+2Nn)* | *(4Nn+4Nn)* |

It is assumed that these are the only relevant groups. Since the *(2x8N)c* fraction is less than 1% of the total, the contribution of this fraction to *8Nn* is negligible (Severin, *et al.*, 1984a; Saeter, *et al.*, 1988b). Heterogeneous nuclear aggregates are dealt with below.

Therefore, total cells, $Tc = 2Nc + (2x2N)c + 4Nc + (2x4N)c + 8Nc$ (1)

Population binuclearity, $BIN = \frac{(2x2N)c + (2x4N)c}{Tc}$ (2)

Tetraploid binuclearity, $BINt = \frac{(2x2N)c}{4Nc + (2x2N)c}$ (3)

Octaploid binuclearity, $BINo = \frac{(2x4N)c}{8Nc + (2x4N)c}$ (4)

Nuclear fractions, $2Nn = 2Nc + 2(2x2N)c - 2(2Nc+2Nc)$ (5)

$4Nn = 4Nc + 2(2x4N)c + (2Nc+2Nc) - 2(4Nc+4Nc)$ (6)

$8Nn = 8Nc + (4Nc+4Nc)$ (7)

## Correction factors

Nuclear ploidy

Nuclear fractions are quantified by flow cytometry and expressed as percentages of the total number of nuclei detected within the three counting regions. These are in error to the extent that aggregates of two diploid or two tetraploid nuclei appear as single nuclei of the next higher ploidy group. Aggregates of unlike nuclei fall between the regions used for quantification and so can be ignored. These nuclei are being lost to counting in proportion to the size their group, and so the effect is more or less evenly spread amongst the ploidy groups. The proportion of flow cytometric events which arise from aggregates, *AGG*, can be estimated by microscopic counting of the PI-stained nuclear preparation, and assuming that nuclei aggregate randomly, this can be used as an estimate of *(2Nc+2Nc)* and *(4Nc+4Nc)*. Therefore, a correction of the nuclear fraction percentages can be applied as follows:

$$2Nn = 2Nc + 2(2x2N)c - 2(2Nc+2Nc) + 2(4Nn \times AGG)$$

$$= 2Nc + 2(2x2N)c \quad (8)$$

$$4Nn = 4Nc + 2(2x4N)c + (2Nc+2Nc) - 2(4Nc+4Nc) - 4Nn \times AGG + 2(8Nn \times AGG)$$

$$= 4Nc + 2(2x4N)c \quad (9)$$

$$8Nn = 8Nc + (4Nc+4Nc) - 8Nn \times AGG$$

$$= 8Nc \quad (10)$$

A second correction needs to be made to the nuclear fraction percentages in order to be able to calculate cellular fraction percentages since 100% of nuclei is greater than 100% of cells. Therefore, if cellular percentages were calculated directly, they would add up to less than 100%. Hence a correction is introduced as follows:

$$XNn_{corr} = (XNn \times BIN) + XNn \quad (11)$$

Note that these corrected estimates of the nuclear fractions are still in error since as *(2xXN)c* increases, *BIN* increases by $\frac{(2xXN)c}{XNc + (2xXN)c}$ from (3) and (4), but *XNn* increases by $XNc + 2(2xXN)c$ from (5) and (6). However, this too can be corrected by calculating the total of the cellular percentages, *TOTc*, after the first round of the calculation and then using this to iteratively correct $XNn_{corr}$, as follows:

$$XNn_{corr} = \frac{XNn_{corr} \times 100}{TOTc} \quad (12)$$

Binuclearity

Since the tetraploid cellular fraction is the largest, *BIN* is the best first estimate of *BINt*. The presence of *2Nc* in the denominator tends to make *BIN* < *BINt* but *BINo* has the opposite effect. This is because binucleation rate in the octaploid fraction is, in this age of mouse, higher than *BINt*. In fact *BINo* was found experimentally (see Section 9.3.3) to be 1.42-fold *BINt*. Therefore, the best initial estimates of *BINt* and *BINo* are

$$BINt = BIN, \quad \text{and} \tag{13}$$

$$BINo = BIN \times 1.42 \tag{14}$$

The accuracy of these assumptions can be gauged by calculating the binuclearity of the calculated cellular distribution, *NewBIN*, and comparing this with *BIN* (which at the end of the calculation is equal to the *BINt* of the calculated cellular distribution). In most cases these will differ by more than just a few percent, with *BINt* >> *NewBIN*, and hence *2Nc* will be overestimated and *(2x2N)c* will be underestimated. In this case, this can be corrected by incrementing *BIN* iteratively by 1% per round until *NewBIN* approximates the original *BIN*. During this process *BINo* is kept constant at its last calculated value as otherwise it will soon reach 100%. Therefore, as *BIN* is increased, the *2Nn* nuclei are redistributed between *2Nc* and *(2x2N)c* until the correct ratio is achieved, when *NewBIN* = *BIN*.

## Calculation of cellular ploidy groups

Calculation of *2Nc* and *(2x2N)c*

Let *BIN* be an estimate of *BINt*, and let *2Nc* be an initial estimate of *4Nc*

Then, substituting into (3)
$$BIN = \frac{(2x2N)c}{2Nc \times F + (2x2N)c} \tag{15}$$

where
$$F = \frac{4Nc}{2Nc} \tag{16}$$

therefore
$$(2x2N)c = BIN(2Nc \times F + (2x2N)c) \tag{17}$$

Also,
$$1 - BIN = \frac{2Nc \times F}{2Nc \times F + (2x2N)c} \tag{18}$$

therefore
$$2Nc = \frac{(1 - BIN)(2Nc \times F + (2x2N)c)}{F} \tag{19}$$

From (8),
$$2Nn = 2Nc + 2(2x2N)c$$

therefore $$2Nn = \frac{(1-BIN)(2Nc \times F + (2x2N)c}{F} + 2 \times BIN\,(2Nc \times F + (2x2N)c) \quad (20)$$

and so $$\frac{2Nn \times F}{2Nc \times F + (2x2N)c} = (1-BIN) + 2 \times BIN \times F \quad (21)$$

Rearranging (15), $$2Nc \times F + (2x2N)c = \frac{(2x2N)c}{BIN} \quad (22)$$

and substituting into (21) $$\frac{2Nn \times F}{(2x2N)c \,/\, BIN} = (1-BIN) + 2 \times BIN \times F \quad (23)$$

therefore $$\frac{2Nn \times F \times BIN}{(2x2N)c} = (1-BIN) + 2 \times BIN \times F \quad (24)$$

and so $$(2x2N)c = \frac{2Nn \times F \times BIN}{(1-BIN) + 2 \times BIN \times F} \quad (25)$$

Similarly, $$2Nc = \frac{2Nn \times (1-BIN)}{(1-BIN) + 2 \times BIN \times F} \quad (26)$$

Calculation of *4Nc* and *(2x4N)c*

From (4) $$BINo = \frac{(2x4N)c}{8Nc + (2x4N)c}$$

and substituting (10) and (14)

$$BIN \times 1.42 = \frac{(2x4N)c}{8Nn + (2x4N)c} \quad (27)$$

therefore $$\frac{1}{BIN \times 1.42} = \frac{8Nn}{(2x4N)c} + 1 \quad (28)$$

and so $$(2x4N)c = 8Nn / (\frac{1}{BIN \times 1.42} - 1) \quad (29)$$

From (9) $$4Nc = 4Nn - 2(2x4N)c \quad (30)$$

The mononuclear octaploid fraction is got, after correction of (7), from (10), as

$$8Nc = 8Nn$$

## Calculation of cellular ploidy group proportions

A spreadsheet was set up for the calculation of the cellular ploidy group proportions from nuclear ploidy fractions and binuclearity. In the first round of the calculation, *F* was set equal to 1, and $XNn_{corr}$ was set equal to (*XNn* x *BIN*) + *XNn*. For subsequent rounds, *F* was derived from the newly calculated *2Nc* and *4Nc* by (16), and $XNn_{corr}$ was corrected using *TOTc* by (12). After each subsequent round, these corrections were again implemented using the latest estimates of *2Nc, 4Nc,* and *TOTc*. This iterative process was continued until *TOTc* = 100%, at which time the estimates of the cellular ploidy groups had become stable. At this point, *BIN* incrementation by 1% per round was started in order to correct for the use of *BIN* as an approximation for *BINt*. This process ceased when the binuclearity of the newly calculated cellular ploidy distribution approximated the original *BIN*.

It should be noted that this calculation works well with good data. However, *8Nn* is the smallest and so least accurately determined parameter, but is central to the calculation (in Eqn. 29) of *(2x2N)c* and so *4Nc*, and via *F*, *2Nc* and *(2x2N)c*. Due to the unstable nature of the calculation, small errors in *8Nn* can lead very quickly to nonsense results (e.g. negative numbers) for *2Nc* and *4Nc* especially. This then "flags" that *8Nn* is probably in error, generally overestimated due to a high background in the flow cytometric determination. This can be checked on the histogram, and if this looks like being the case, *8Nn* can be reduced in the calculation until sensible (e.g. non-negative) results are obtained. This process will not of course give an "exact" result but the approximation will be close enough to be able to detect differences between treatments as long as these modifications are kept constant between treatment groups.

## Calculation of actual cell numbers

Actual cell numbers in each cellular ploidy group are got from cell counts multiplied by the above calculated cellular ploidy group proportions. These are then plotted to show how a treatment effects individual cellular ploidy groups.

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