CHARACTERISATION OF TRI-IODOTHYRONINE AS A PRIMARY MITOGEN FOR THE LIVER

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ABSTRACT

Hepatocyte proliferation can be induced in one of two distinct processes, COMPENSATORY REGENERATION and DIRECT HYPERPLASIA. In compensatory regeneration, the initial event is hepatic injury (chemical/viral) or surgical resection with a reduction in liver mass. These events are followed by proliferation, initially of hepatocytes and then other cell populations of the liver, to rapidly restore the normal precisely regulated liver mass. In contrast, in direct hyperplasia, hepatocytes are stimulated to proliferate by primary mitogens in the absence of liver injury or resection, resulting in an increase in liver mass to above the normal value.

In this thesis we have characterised the effects of thyroid hormone as a primary mitogen for the rat liver, with particular reference to its potential exploitation within the fields of hepatic and transplant surgery. We have demonstrated that a single injection of tri-iodothyronine, initiates proliferation in hepatocytes, and 96h later over 30% of the hepatocytes (predominantly midzonal) have gone through S-phase and cell division. The less well-recognised property of T₃ was the potential of the intact liver to grow in vivo to a size and more importantly to a functional capacity greater than normal. We have shown that the simultaneous co-administration of tri-iodothyronine with 70% partial hepatectomy increases the proliferative response of hepatocytes following surgery, and thus enhances regeneration during one of the strongest known stimuli to hepatocyte proliferation.

In the final chapters we have attempted to identify a mechanism for this intriguing phenomenon and tried to develop an in vitro model of T₃ function. The overall aim of this project was to determine whether regimes administering thyroid hormone could provide a useful enhancement of liver function in man.
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Publications

Articles


Abstracts


2001  Malik R, Tootle R, Selden C and Hodgson HJ. Does the increases in hepatic mass induced by the mitogen tri-iodothyronine confer a useful increase in liver function. Hepatology 2001; 34/4 page 392A

ABBREVIATIONS

AST  Aspartate Aminotransferase
ALT  Alanine Aminotransferase
BrdU Bromodeoxyuridine
CHD-1 Chromodomainhelicase-DNA binding protein
DNA  Deoxy-ribonucleic acid
DENA Diethylnitrosamine
EDTA Ethylenediamine-tetraacetic acid
EGF  Epidermal Growth Factor
ELISA Enzyme Linked Immunosorbant Assay
FAH  Fumarylacetoacetate Hydrolase
FCS  Foetal Calf Serum
GEC  Galactose Elimination Capacity
HGF  Hepatocyte Growth Factor
I.P  Intra-peritoneal
I.V  Intra-venous
Kip1p Kinesin-like protein 1
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
RNA  Ribonucleic acid
SC   Subcutaneous
SD   Standard Deviation
T₃   Tri-iodothyronine
T₄   Tetra - iodothyronine
TSH  Thyroid Stimulating Hormone
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Synopsis

This project has developed from the observation that tri-iodothyroine administered to rats at high doses induces hepatocyte proliferation in the intact liver. My PhD thesis gives a systematic account of the effects of tri-iodothyroine as a hepatic mitogen, and shows how this property affects the structure and function of the liver.

The first chapter is a general introduction that can be broadly divided into two distinct topics, the first topic giving an account of the effects of the thyroid hormones with particular reference to their interactions with the liver. This review takes a logical scheme that includes a) Thyroid hormone nomenclature and secretion, b) Thyroid hormone metabolism, c) Intracellular signalling, d) Physiological interactions between the thyroid and the liver, e) Pathological interactions between the thyroid gland and the liver including dual pathology. The second topic describes the distinct mechanisms by which hepatocyte proliferation and liver growth can occur. It begins by describing how liver cell proliferation, which results in hepatic growth can occur in one of two distinct patterns, compensatory regeneration and direct hyperplasia. In compensatory regeneration, the initial event is hepatic injury (chemical/viral) or surgical resection with a reduction in liver mass. These events are followed by proliferation, initially of hepatocytes and then other cell populations of the liver, to rapidly restore the normal precisely regulated liver mass. In contrast, in direct hyperplasia, hepatocytes are stimulated to proliferate by primary mitogens in the absence of liver injury or resection, resulting in an increase in liver mass to above the normal value. The liver mass subsequently returns to its normal value following the removal of the mitogenic stimulus. This description is followed by a detailed review of the molecular mechanisms that regulate the process of compensatory regeneration, which is best exemplified by the well-characterised paradigm of the 70% partial hepatectomy in the
rat. The thesis then introduces thyroid hormone as a primary mitogen for the liver, emphasising how the mechanism of action of this (and other) primary mitogens are yet to be elucidated. The chapter ends by discussing how the mitogenic properties of T₃ have been utilised experimentally to correct inborn errors of hepatic metabolism, either by replacing the defective hepatocytes or by introducing the stable gene into the host cell. The first chapter discusses both the thyroid hormones and hepatic growth, both of which are prerequisites to an overall understanding of the thesis.

The second chapter characterises the effects of a single dose of T₃ on the rat liver in vivo. A peak increase in cell proliferation was seen 24 hours after the administration of T₃; being followed by a gradual decline. There were 7 ± 1.1% of hepatocytes in S phase at 24 hours, as compared to <1% in controls and these BrdU labelled hepatocytes were predominantly in the midzonal region of the hepatic lobule. The growth stimulation seen with T₃ resulted in an increase in the cellular mass of the liver, with the peak increase (15%) occurring at 10 days. We used the TUNEL assay to show that the restoration of the liver mass to pre-experimental values after 10 days occurs through the apoptotic deletion of excess cells. We investigated the toxic and systemic endocrine effects of a mitogenic dose of T₃ showing that the single subcutaneous injection of T₃ at 4mg/kg was well tolerated by recipient rats, with the rats maintaining an adequate appetite and bodyweight, with no unexpected mortality over the time period.

The third chapter assesses if the increase in liver mass induced by T₃ confers an increase in hepatic metabolism/function. Initially, we used a variety of strategies that included the assessment of serial plasma albumin levels following hepatic stress and survival following 85% partial hepatectomy. These initial strategies showed no differences between rats given T₃ ten days prior to assessment and control rats that had
received vehicle, however our final strategy of using galactose elimination capacity, showed a 15-20% increase in *in vivo* galactose metabolism in the T\textsubscript{3} group as compared to controls.

The fourth chapter studies the effects of combining the two distinct pathways of liver growth, direct hyperplasia and compensatory regeneration. The first part shows that pre-treatment (10 days) with a single injection of T\textsubscript{3} prior to a 70% partial hepatectomy was associated with a larger liver mass 24 hours after surgery, with the remnant liver retaining its regenerative capacity. It subsequently shows that the simultaneous co-administration of tri-iodothyronine with 70% partial hepatectomy increases the proliferative response of hepatocytes following surgery, and thus enhances regeneration during one of the strongest known stimuli to hepatocyte proliferation. The final part of this chapter shows the administration of T\textsubscript{3} following thioacetamide-induced liver injury enhances cell proliferation in the liver.

In chapter 5 we attempted to identify a possible mechanism by which this intriguing phenomenon was occurring. In both direct hyperplasia and compensatory regeneration, hepatocytes emerge from the G\textsubscript{0} (resting) stage of the cell cycle, enter G\textsubscript{1}, and then S-phase. It is apparent that the cell signals converge at the time of transition through G\textsubscript{1} (G\textsubscript{1} restriction point), so that both lead for example to Cyclin D activation and subsequent steps. However, the molecular processes in the early stages of direct hyperplasia appear to differ strikingly from those involved in compensatory growth. To identify the early molecular signals involved in T\textsubscript{3} induced cell proliferation we analysed the data available from a DNA microarray of the thyroid hormone regulation of hepatic genes *in vivo* in mice. It identified a candidate gene called Bcl-3 that is regulated by T\textsubscript{3} and involved in cell proliferation. We showed an early increase in Bcl-
3 mRNA (real time PCR) and protein (western analysis) in the liver of rats stimulated with T₃.

In chapter 6 we characterised the effects of thyroid hormone on primary rat hepatocyte cultures, to assess if T₃ could be shown to have similar mitogenic effects *in vitro* as it does *in vivo*. This work could then be applied to human liver cell culture systems to determine whether T₃ may constitute an effective primary mitogen in man. Unfortunately, we were unable to model the effects of T₃ *in vitro*, and so the final chapter (chapter 7) is a general discussion with a critique.
Chapter 1

Introduction
The thyroid gland secretes two iodine containing amine hormones derived from the amino acid tyrosine, L-thyroxine (T₄) and 3,5,3'-L-triiodothyronine (T₃).

1.1 Thyroid hormone metabolism

In normal subjects the gland secretes 110 nmol of thyroxine and 10 nmol of triiodothyronine each day (Larsen 1975). Tri-iodothyronine has a ten times greater affinity and efficacy than thyroxine for the nuclear receptor, therefore even though thyroxine is quantitatively secreted at much higher levels it should be regarded as a prohormone that requires deiodination and conversion to T₃ to become biologically active (Hassi et al. 2001). There are three groups of enzymes that regulate thyroid hormone metabolism forming part of the iodothyronine seleno-deiodinase enzyme system (type 1 = D1, type 2 = D2 and type 3 = D3). They are responsible for the activation of T₄ to T₃, inactivation of T₄ to rT₃ and the conversion of rT₃ and T₃ to T₂ (figure 1.1).

![Figure 1.1 - Structures and interactions between the principle iodothyronines.](image-url)
The conversion of T₄ to T₃ in extrathyroidal tissue occurs through a rapidly equilibrating pool via the D1 enzyme system and a slowly equilibrating pool via the D2 system. The type 1 deiodinase is mainly found in the liver and kidney (Sanders et al. 1997) and accounts for approximately 30-40% of extrathyroidal production of T₃ (12nmol). The type 2 deiodinase is found in pituitary, CNS, skeletal muscle and contributes 60-70% of the extrathyroidal production of T₃ (30nmol) (Leonard et al. 2000). Although this enzyme system performs similar actions to the D1 group of enzymes, its enzyme kinetics, regulation and susceptibility to propylthiouracil differ (Bianco et al. 2002). Although both the D1 and D2 system can inactivate T₄ and T₃, the major inactivator is the type 3 deiodinase system, which primarily exhibits inner ring deiodination (unlike the other systems) (Tu et al. 1999). It is found in the liver, skin and CNS where it catalyses the conversion T₄ to rT₃, T₃ to T₂ both inactive metabolites and also rT₃ to rT₂ (Bianco et al 2002). This enzyme system is present in placenta where it provides a protective role for the foetus from maternal thyroid hormones (Darras, Hume, & Visser 1999).

1.2 Thyroid receptor signalling and mechanism of action

Free T₃ and T₄ enter all cells through the plasma membrane and bind to a nuclear T₃ receptor. The thyroid receptor is part of the nuclear superfamily group of receptors (retinoic acid, retinoid X, vitamin D and peroxisome proliferator receptor) (Evans 1988). The thyroid receptor is encoded by two separate genes TRα (chromosome 17) and TRβ (chromosome 3) in humans. Alternate splicing from each gene generates multiple isoforms, with the TRα1, TRα2 isoform from the TRα gene and TRβ1, TRβ2 isoform from the TRβ gene, all having been cloned in humans (Lazar 1993;Williams 2000).
The thyroid receptor can be divided into six parts with three functional regions (Ribeiro, Kushner, & Baxter 1995) (figure 1.2):

<table>
<thead>
<tr>
<th>A/B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF1</td>
<td>DBD</td>
<td>LBD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activation</td>
<td>DNA binding</td>
<td>ligand binding, dimerization, AF2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.2 - Modular structure of the thyroid hormone receptor. Nuclear receptors, including TR, can be divided into six regions with three functional domains.

AF1, activation function 1 (A and B regions); DBD, DNA- binding region (C region); LBD, ligand binding domain (D, E and F region).

The TRα1 isoform is a ligand binding receptor whereas the TRα2 isoform does not interact with hormone and functions to suppress expression of genes containing thyroid response elements (Apriletti et al. 1998). The TRβ1 and TRβ2 are both ligand-binding receptors.

The main function of the thyroid receptor is as a ligand activated transcription factor that regulates target gene expression directly through DNA response elements (thyroid response elements - TREs) (Glass 1994). However, an important property of these receptors is their ability to bind thyroid response elements constitutively, independent of ligand occupancy (Mangelsdorf et al. 1995). The consensus sequence recognised by nuclear receptors often contains a hexamer AGGTCA known as the half site, of which two half site sequences with a specific orientation are required for functional and efficient binding (Umesono et al. 1991). In the central DNA binding region of the thyroid receptor are two zinc-containing modules that mediate the specific recognition sites (and spacing specificity) for the receptor to bind genomic DNA.
(Ribeiro et al. 1998). A DNA recognition helix (P box) in the carboxy terminus of the first zinc finger mediates the half site sequence recognition by directly contacting the major groove nucleotides (Apriletti et al. 1998). Although thyroid receptors can bind to TREs as monomers, the majority bind in the form of a heterodimer with the retinoid X receptor (RXR). Heterodimer formation is thought to enhance DNA binding affinity as well as provide target gene specificity. The retinoic acid receptor (RAR), vitamin D receptor (VDR) and peroxisome proliferator receptor (PPR) also form heterodimers with the retinoid X receptor (Kliewer et al. 1992) however they activate through different response elements.

Two groups of co-factors are essential to mediate activation (co-activators) and repression (co-repressors) of TR transcriptional activity. Unliganded thyroid receptors are bound to co-repressor molecules that have histone deacetylase activity. Deacetylation results in a closed chromatin configuration on genomic DNA that represses basal transcription of the target gene (Nagy et al. 1997). In the presence of T3 a conformational change occurs within the thyroid receptor that allows release of the co-repressor molecule and recruitment of a co-activator complex (Ribeiro et al. 1998). The SRC family and CBP proteins are thought to form part of this co-activator complex and these proteins have histone acetyltransferase (HAT) activity (Ribeiro et al 1998).

Enzymatic acetylation of the nucleosome allows for an open chromatin configuration of the TRE on genomic DNA. This open structure is thought to facilitate the assembly of the basal transcription machinery and enhances the rate of mRNA transcription of thyroid responsive genes (Torchia et al. 1997). This alters the expression TRE related peptides within cells, which mediate the metabolic effects of the thyroid hormones (Barettino, Vivanco-Ruiz, & Stunnenberg 1994) (figure 1.3).
Figure 1.3 - Model for activation and repression of the thyroid hormone receptor. In the absence of T₃, T₃/RXR recruits a repressor complex that has histone deacetylase (HDAC) activity. Enzymatic deacetylation results in a closed chromatin modification of the nucleosome that leads to transcriptional repression. In the presence of T₃, TR/RXR releases the repressor molecule and recruits an activator complex that has histone acetyltransferase activity. Enzymatic acetylation results in an open chromatin structure that initiates the enhancer region of the TRE and leads to promoter activation.
1.3 Physiological interactions between the thyroid and the liver

In addition to the central role in deiodination to activate and deactivate thyroid hormones, the liver performs specific functions relating to thyroid hormone transport and metabolism. The liver extracts 5 to 10 per cent of plasma T\(_4\) during a single passage as is shown by studies employing \([^{131}\text{I}] T_4\). This value is much higher than can be accounted for by the amount of free T\(_4\) delivered to the liver, indicating that a substantial amount of protein bound T\(_4\) is available for uptake (Mendel, Cavalieri, & Weisiger 1988). An active stereospecific transport mechanism has been identified for transporting T\(_4\) and T\(_3\) across the hepatocyte membrane. The intracellular concentrations of the free hormone have been found to be higher than the plasma levels and the process is energy dependent (Hennemann et al. 2001). The liver synthesises a number of plasma proteins that bind the lipophilic thyroid hormones and thereby provide a large, rapidly exchangeable pool of circulating hormone. The thyroid hormones are over 99% bound to thyroxine binding globulin, thyroxine binding prealbumin and albumin in plasma. The free hormone component within plasma is in equilibrium with the protein bound hormone and it is this free fraction which accounts for the hormone’s biological activities. The plasma concentrations of free T\(_4\) and T\(_3\) are at a steady concentration so that the tissues are exposed to the same concentrations of the free hormone. However, the free hormone concentrations in different tissues vary according to the transport and deiodinase activity within specific tissues (Bianco et al. 2002). The liver is the major site for cholesterol and triglyceride metabolism and the thyroid hormones play an integral part in hepatic lipid homeostasis. Thyroid hormones increase the expression of LDL receptors on the hepatocytes (Ness et al. 1998) and increase the activity of lipid lowering liver enzymes, resulting in a reduction in low density lipoprotein levels (Ness & Lopez 1995). Thyroid hormones also increase the
expression of apolipoprotein A1, a major component of high-density lipoprotein (Taylor et al. 1997). Clearly, the above effects of the thyroid hormones could be beneficial in reducing the onset of atherosclerosis if they were elicited without the deleterious effects, most particularly the cardiac effects such as the atrial arrhythmias (Dillmann 1990; Woeber 1992). A series of 3, 5 - dideoxy - 3 - aryl substituted - thyronines have been developed, which showed a potent cholesterol reducing effect in hypercholesterolaemic rats without the undesired tacycardia. The tissue selectivity of these agents was attributed to selective uptake by the liver rather than TR subtype selectivity (Leeson et al. 1989). Subsequently, a series of novel thyronine type derivatives (dimethyl-isopropyl-benzylphenoxy-acetic acid) (GC-1) have been shown to reduce serum cholesterol in rats without tacycardia through the selective activation of the TRβ isoform (Baxter et al. 2001). This is compatible with the predominant distribution of the TRβ isoform in the liver and the TRα isoform in the cardiovascular system.

Thus tissue thyroid status depends not only on thyroxine secretion but also on normal thyroid hormone metabolism, delivery of T₃ to nuclear receptors and on receptor distribution and function. Normal thyroid function, which is essential for normal growth, development and the regulation of energy metabolism within cells, is dependent on a normally functioning thyroid and liver axis.

1.4 Pathological interactions between the thyroid and the liver

1.4.i Thyroid metabolism in chronic illness

In most chronic illness defects arise in thyroid hormone metabolism, resulting in the sick euthyroid syndrome. This is characterised by a normal total T₄, normal/high free T₄, low total T₃, low free T₃ and an elevated rT₃. These changes reflect a reduction in D1 activity, an increase in D3 activity (Bianco et al. 2002) and changes in the plasma
concentration of thyroid binding proteins and free fatty acids (which displace thyroid hormones from binding proteins). There are also non-thyroidal influences on the hypothalamic-pituitary-thyroid axis, eg cortisol inhibiting TSH secretion (Camacho & Dwarkanathan 1999).

It has been suggested that this syndrome may confer a survival advantage, which adapts an organism to chronic illness by reducing the basal metabolic rate within cells and thereby reducing caloric requirements.

1.4. ii Thyroid abnormalities in liver disease

In different types of liver disease similar processes may occur to those seen in the sick euthyroid syndrome but in addition a number of changes specific to the type or stage of liver disease are also found.

Cirrhosis

A prospective study in 118 patients with cirrhosis demonstrated a 17% increase in thyroid glandular volume, assessed by ultrasonography, as compared to controls (Bianchi et al. 1991). The most consistent thyroid hormone profile in patients with cirrhosis is a low total and free T3 (L'age et al. 1980) and an elevated rT3 (Faber et al. 1981), similar changes to the sick euthyroid syndrome, probably reflecting a reduced deiodinase type 1 activity resulting in reduced conversion of T4 to T3. This results in an increase in conversion of T4 to rT3 by the deiodinase type 3 system and an increase in the rT3 to T3 ratio. The plasma T3 to rT3 ratio has a negative correlation with the severity of cirrhosis when assessed in non-alcoholic cirrhotics (Guven, Kelestimur, & Yucesoy 1993). Since T3 and rT3 bind to the same plasma proteins, the T3/rT3 ratio provides a parameter of liver function that is largely independent of protein binding. Both the T3/rT3 ratio and free T3 levels in plasma thus provide a correlate of liver function in
cirrhosis and have been shown to be of prognostic value though seldom used in clinical practice (Van Thiel et al. 1985).

The low total and free T3 levels may be regarded as an adaptive hypothyroid state that serves to reduce the basal metabolic rate within hepatocytes and preserve liver function and total body protein stores. Indeed, a recent study in cirrhotic patients showed that the onset of hypothyroidism from intrinsic thyroid disease of various aetiologies during cirrhosis resulted in a biochemical improvement in liver function (eg coagulation profiles) as compared to cirrhotic controls (Oren et al. 2000).

Hypothyroidism has also been associated with lesser degrees of decompensation in cirrhosis (Oren et al. 1998). Controlled induction of hypothyroidism might therefore be beneficial in cirrhotic patients, but further studies are required to test this hypothesis.

Acute hepatitis and acute liver failure

In acute hepatitis of mild or moderate severity, patients have elevated serum levels of total T4 due to increased thyroid binding globulin, which is synthesised as an acute phase reactant, but normal levels of free T4. In more severe cases with impending liver failure the data is variable and low total T4 levels may reflect reduced hepatocellular synthesis of thyroid binding globulin (Kano et al. 1987). Serum T3 levels are extremely variable, but the free T3 to T4 ratio correlates negatively with the severity of the liver disease and has been shown to have prognostic value (Kano et al. 1987). Again this probably reflects diminished type 1 deiodinase activity resulting in a reduced conversion of T4 to T3, in general however these patients are clinically euthyroid. Some series have described patients with acute hepatic failure (esp. viral hepatitis) having goitres that resolve with improvement in liver function (Hegedus 1986).
Specific forms of chronic liver disease

In patients with chronic hepatitis associated with primary biliary cirrhosis (PBC) or chronic autoimmune hepatitis there is an increased prevalence of autoimmune thyroid disease (Crowe et al. 1980; Krawitt 1996). Thus abnormalities may arise reflecting both the thyroid gland dysfunction or as a consequence of the liver disease. Autoimmune hypothyroidism is a prominent feature in PBC occurring in 10 to 25% of patients (Sherlock & Scheuer 1973). There is often an increase in total T\textsubscript{4} in PBC due to an increase in thyroid binding globulin levels and this may mask hypothyroidism emphasising the need to perform a free T\textsubscript{4} and TSH assay. Anti-thyroid microsomal antibodies are common in PBC (34%) as are anti-thyroglobulin antibodies (20%) (Elta et al. 1983). Thyroid dysfunction may precede or follow the diagnosis of PBC. In autoimmune hepatitis both Grave’s disease (6%) and autoimmune hypothyroidism (12%) are relatively common (Krawitt 1996). Primary sclerosing cholangitis is associated with an increased incidence of Hashimoto’s thyroiditis, Graves’s disease and Riedel’s thyroiditis (Saarinen, Olerup, & Broome 2000).

In patients with chronic hepatitis who do not have co-existing autoimmune liver and thyroid disease, total T\textsubscript{4}, total T\textsubscript{3}, thyroxine binding globulin levels are often increased but TSH and free T\textsubscript{4} levels are usually normal and the patients are clinically euthyroid (Borzio et al. 1983).

Currently the treatment of viral hepatitis with alpha interferon has added another dimension to the abnormalities of thyroid function seen in chronic liver diseases. In different studies assessing patients treated with alpha interferon for hepatitis C 2.5% - 10% developed thyroid dysfunction (Benelhadj et al. 1997; Shimizu, Joho, & Watanabe 1994), with both thyrotoxicosis due to acute thyroiditis and hypothyroidism being observed. Although the reason is not altogether clear, the induction of an autoimmune
reaction has been postulated, which results in the development of anti-thyroid and anti-thyrotrophin receptor antibodies (Fonseca, Thomas, & Dusheiko 1991). However, a distinct effect on intrathyroidal organification of iodine has also been suggested (Roti et al. 1996). The risk factors for developing thyroid dysfunction with alpha interferon (which may persist after discontinuation of the drug) are female sex, underlying malignancy, high doses of long duration, combination immunotherapy (especially II-2) and the presence of anti-thyroid peroxidase antibodies prior to commencing treatment (Koh, Greenspan, & Yeo 1997; Lisker-Melman et al. 1992). It should be noted that interferon therapy causes weakness and muscle aching and in this setting the myopathy of hypothyroidism may be missed. It is therefore recommended that thyroid function tests (inc. thyroid antibodies) are performed prior to therapy and subsequently monitored at 3-6 month intervals during interferon therapy (Deutsch et al. 1997).

In chronic hepatitis B, predominantly a disease of males, the frequency of pretreatment thyroid antibodies and the induction of thyroid antibodies and thyroid dysfunction during interferon therapy, are all lower than in chronic hepatitis C (Melman 1994).

Overall, the majority of patients with liver disease are clinically euthyroid and this can be confirmed with a normal high sensitivity TSH test and a normal free T4. The latter test is routinely performed and obviates the need to take into account the variation in thyroid binding globulin levels seen in patients with liver disease.

1.4.iii Liver abnormalities in thyroid disease

Hypothyroidism

Hypothyroidism may have features that mimic liver disease (pseudo-liver disease), examples include myalgias, fatigue and muscle cramps in the presence of an elevated aspartate aminotransferase from a myopathy (Laycock & Pascuzzi 1991), coma
associated with hyperammonaemia in myxoedema coma (Thobe, Pilger, & Jones 2000) and myxoedema ascites (Klein & Levey 1984). Myxoedema ascites, generally a high protein content ascites suggestive of an exudate has been varyingly interpreted as an intrinsic liver defect or a phenomenon mimicking liver disease. It had been proposed that the ascites was a consequence of chronic right sided heart failure resulting in central scarring of the liver (Kinney, Wright, & Caldwell 1989). The liver biopsy findings of central congestive fibrosis in a number of patients would support this (Klein et al 1984). However another study reported normal right heart pressures and proposed that severe hypothyroidism caused enhanced permeability of vascular endothelium resulting in ascites and serous effusions throughout the body (Hemmann et al. 1996). Following initiation of thyroid replacement therapy, myxoedema ascites resolves over a few months.

There is also evidence that hypothyroidism may directly affect the liver structure or function. Hypothyroidism has been associated, in a few case reports with cholestatic jaundice attributed to reduced bilirubin and bile excretion. In experimental hypothyroidism the activity of bilirubin UDP-glucuronyltransferase is decreased resulting in a reduction in bilirubin excretion (Van Steenbergen et al. 1989). The reduction in bile flow may be in part due to an increase in membrane cholesterol-phospholipid ratio and diminished membrane fluidity (Van Steenbergen et al 1989), which may affect a number of canalicular membrane transporters and enzymes, including the Na\(^+\), K\(^+\) - ATPase. The triad of reduced bilirubin excretion, hypercholesterolaemia and hypotonia of the gall bladder seen in hypothyroidism increase the incidence of gallstones (Inkinen, Sand, & Nordback 2000). Recent studies have shown that the hepatic abnormalities associated with hypothyroidism can be
reversible over a matter of weeks with thyroxine replacement, with no residual liver
damage (Gaitan & Cooper 1997; Huang & Liaw 1995).

Experimentally, hypothyroidism may protect against acetaminophen toxicity and
diminish thioacetamide toxicity, but there is no evidence for this in man (Bruck et al.
1999) (Bruck et al. 1998).

Hyperthyroidism

The clinical features of hyperthyroidism are diverse involving nearly every
system in the body. The liver injury caused by thyrotoxicosis is relatively common and
can be conveniently divided into a hepatitic or cholestatic picture.

Hepatitic Injury

An increase in the aspartate aminotransferase (AST) and alanine
aminotransferase (ALT) was reported in 27% and 37% of patients respectively
(Thompson et al. 1978), although the majority of those patients showed no other clinical
or biochemical features of liver impairment. The mechanism of injury appears to be
relative hypoxia in the perivenular regions due to an increase in hepatic oxygen demand
without an appropriate increase in hepatic blood flow. In mild cases, liver histology
shows non-specific changes, which on light microscopy consist of a mild lobular
inflammatory infiltrate consisting of polymorph neutrophils, eosinophils and
lymphocytes, associated with nuclear changes and Kupffer cell hyperplasia. Electron
microscopy may reveal hyperplasia of the smooth endoplasmic reticulum, a paucity of
cytoplasmic glycogen and increased number and size of mitochondria, which may
contain more cristae (Huang & Liaw 1995). A small proportion of patients have a
progressive liver injury which histologically consists of centrizonal necrosis and
perivenular fibrosis, affecting the areas in which hypoxia may be most prevalent. The
severity of centrizonal necrosis can be assessed using plasma isocitrate dehydrogenase
levels in plasma, offering a convenient method for grading the hepatic injury (Chung et al. 2001). The clinical presentation of this type of injury is usually that of a self-limiting hepatitis, however there are a few case reports of thyrotoxic patients presenting with fulminant hepatic failure. The precipitation of the clinical presentation is generally attributable to the onset of cardiac failure, often precipitated by arrhythmias (Choudhary & Roberts 1999).

**Cholestatic injury**

An elevated serum alkaline phosphatase is seen in 64% of patients with thyrotoxicosis (Doran 1978). However this is not necessarily liver specific as it can originate from bone and/or liver. It is therefore important to look at elevations in γ glutamyl transpeptidase (17%) and bilirubin (5%) as an indicator of cholestasis (Doran 1978). In patients with the cholestatic injury the histological features are similar to the non-specific changes seen in hepatic injury. However in addition there appears to be centri-lobular intrahepatocytic cholestasis (Sola et al. 1991).

Jaundice is uncommon but when it occurs complications of thyrotoxicosis (cardiac failure/sepsis) or intrinsic liver disease need to be excluded.

It is difficult to establish which features seen in thyrotoxic liver injury are from tissue thyroid status alone or are in combination with complications such as cardiac failure, malnutrition and sepsis. It is probably impractical to try and separate the causes out, as awareness of the presentation, complications and treatment are of greater importance. The early reports of patients developing a spectrum of pathological changes from focal necrosis with fatty change to cirrhosis can be attributed to untreated hyperthyroidism (Sola et al 1991). Modern therapies have made chronic liver disease a very rare complication of hyperthyroidism. In the vast majority of cases the hepatic
abnormalities associated with hyperthyroidism are reversible following the early recognition and treatment of the disorder (Fong, McHutchison, & Reynolds 1992).

However therapy may itself cause hepatic complications. Increased serum levels of aspartate aminotransferase and alanine aminotransferase occur in about 30 per cent of patients treated with propylthiouracil (Williams et al. 1997). The rise in AST appears to be dose related, so that AST and ALT levels are highest during the first few weeks of treatment, falling rapidly with a dose reduction (Kim et al. 2001). In the majority of patients serum aminotransferases return to normal with clinical improvement following withdrawal of treatment. A persistent hepatitis rarely occurs with clinical, biochemical (elevated bilirubin, AST and ALT) and histological features of hepatocellular necrosis (Levy 1993). This is an idiosyncratic reaction that can develop at any time but usually occurs within the first 2 to 3 months of treatment in about 1% of patients, usually occurring in women below 30. It is considered to be an allergic host response, which generally resolves over a protracted period of time (Gurlek, Cobankara, & Bayraktar 1997). A small proportion of patients develop fulminant hepatic failure, and in the presence of severe acidosis or a combination of grade 111/IV encephalopathy, renal failure and coagulopathy, may require orthotopic liver transplantation (Levy 1993;O'Grady, Schalm, & Williams 1993). Abnormalities of liver function are much less common with carbimazole and methimazole. These agents induce cholestasis, as an idiosyncratic reaction to the drug (Blom et al. 1985). An elevation of the bilirubin, alkaline phosphatase, and γ glutamyl transpeptidase levels are the predominant abnormalities. Such liver dysfunction usually presents within 2 to 3 weeks of initiation of treatment and can persist for several months despite discontinuation of the offending drug (Ayensa, Diaz, & Cia 1986). The predominant feature on liver biopsy is intrahepatic cholestasis.
It can be seen that hepatic injury can develop over the first few months in relatively few patients starting anti-thyroid therapy. Predicting the incidence in individual patients is difficult and it is therefore recommended that liver function tests are performed in all patients within three months of commencing therapy (Kim et al 2001).

1.4.iv  Dual pathology

A number of disease processes can affect both the liver and the thyroid gland simultaneously. The autoimmune diseases, which may occasionally occur in the setting of a multisystem autoimmune disorder have been discussed (Inoue et al. 1999).

A less common setting in which generalised pathology occurs is that of organ infiltration such as malignancy, amyloid or in secondary haemachromatosis when iron is diffusely deposited. Of the infiltrating malignancies, non-hodgkins lymphoma is the commonest cause and the presentation is dominated by a goitre (± lymphadenopathy), jaundice and a paraneoplastic illness (Wirtzfeld et al. 2001). Occasionally, other forms of hepatic impairment (eg coagulopathy) and hypothyroidism can occur as part of the presentation (Thieblemont et al. 2002). Secondary amyloidosis due to systemic inflammatory diseases (eg Crohns, TB and Familial Mediterranean Fever) is the commonest cause of amyloid deposition into the liver and thyroid gland (D'Antonio et al. 2000); being characterised by the deposition of the serum amyloid A (AA) protein (Lovat et al. 1998). The synthetic function of each organ is usually well maintained, therefore amyloid organ function is better followed by serial measurement of serum amyloid A protein and amyloid P scintigraphy (Hawkins et al. 1998). Aggressive treatment of the underlying inflammatory disorder to maintain serum amyloid A values within the reference range (<10 mg/l) is associated with a 50% reduction in mortality at 10 years (Gillmore et al. 2001). Transfusion related iron deposition (secondary
haemochromatosis) can rarely cause multiple endocrine abnormalities (including hypothyroidism) and cirrhosis from iron deposition into the respective organ (Phillips et al. 1992; Shirota et al. 1992). The toxicity of the iron deposition into the thyroid (and thus degree of hypothyroidism) is potentiated by hypoxia and anaemia making these patients difficult to treat (Magro et al. 1990).

Amiodarone is the most notable drug that effects both the liver (fibrosis) and the thyroid gland (hypo/hyperthyroidism) (Sanoski et al. 1998), the effects of which may remain even following withdrawal of the drug. The antimalarial drug Mefloquine has been shown to cause a self limiting hepatitis and thyrotoxicosis from acute thyroiditis, however the symptoms appear to resolve when the drug is withdrawn (Croft & Herxheimer 2002). Another major drug class affecting both organs are the anti-epileptics, of which carbamazepine can cause hepatic impairment and sub-clinical hypothyroidism from abnormal thyroid hormone metabolism (Isojarvi et al. 2001). Finally it is worth highlighting that the treatment of malignant disease using radical radiotherapy regimes including those containing $^{131}$I MIBG and modern chemotherapy schedules have been associated with a greater degree of toxicity effecting both organs (van Santen et al. 2002).
1.5 Hepatic growth

It has been shown that liver growth can be induced in one of two distinct patterns (Figure 1.4):

Direct Hyperplasia

Mitogen → Cell Proliferation → Apoptosis

Liver Mass (Precisely regulated)

Compensatory Regeneration

Surgery/injury → Cell loss → Cell Proliferation

Figure 1.4 – The two distinct patterns by which liver cell proliferation can occur.

1. Compensatory Regeneration is a process in which liver cell proliferation appears to be a secondary event in response to injury (chemical/viral) or surgical resection with a restoration of the normal liver mass (Fausto 2000). In modern times, the most recognised experimental model of compensatory regeneration is the 70% partial hepatectomy in the rat, introduced by Higgins and Anderson as a simple operation in which approximately two-thirds of the liver of a rat are removed (Higgins and Anderson 1931). Cell proliferation is an early response and follows a highly ordered sequence in all the mature cellular populations of the liver, with a peak in DNA synthesis in hepatocytes at 24 hours, cholangiocytes at 48 hours, kupffer cells at 72 hours and
sinusoidal endothelial cells at 96 hours (Alison 1986). This well regulated proliferative response is integrated into the histological expansion of the liver lobule to restore the normal liver mass over a period of 7 – 10 days (Kitamura, Watanabe, & Sato 1998).

2. In contrast, in direct hyperplasia, hepatocytes are stimulated to proliferate by primary mitogens in the absence of liver injury or resection, resulting in an increase in liver mass to above the normal value (Schulte-Hermann, R 1971). The liver mass subsequently returns to its normal value following removal of the mitogenic stimulus through the apoptotic deletion of excess cells (Larsen 1975; Leonard et al 2000). A variety of agents can act as primary mitogens including lead nitrate, barbiturates and ligands of the superfamily of nuclear receptors: retinoic acid, peroxisome proliferators and tri-iodothyronine (Columbano & Shinozuka 1996).

Like all mammalian cells, in both direct hyperplasia and compensatory regeneration, hepatocytes emerge from the G₀ (resting) stage of the cell cycle, enter G₁, and then S-phase (Fig 1.5)

![Figure 1.5 - The cell cycle](image-url)
The molecular processes in the early stages of direct hyperplasia (although unknown) appear to differ strikingly from those involved in compensatory growth (Ledda-Columbano et al. 1998; Ledda-Columbano et al. 2000). However, it is apparent that cell signals converge at the time of transition through G_1 (G_1 restriction point), so that both lead for example to Cyclin D1 activation and subsequent steps. The processes of compensatory hyperplasia take approximately 16h to reach this stage, contrasting with direct hyperplasia, in which Cyclin D1 activation occurs within 8 hours. The activation of Cyclin D1 corresponds to a G_1 state within the hepatocyte, which is characterised by a greater macromolecule synthetic rate, a higher enzyme activity and transmembrane transport rate within the cell (Nasmyth 1996). The expression of Cyclin D1 is accompanied by the sequential activation of the corresponding cyclin dependent kinase (CDK 4 and 6). The CDK 4 and 6 phosphorylate the retinoblastoma protein complex (HDAC-hSWI/SNF-Rb), which releases histone deacetylase (HDAC) from its constitutive protein complex (Zhang et al. 2000). The hepatocyte then initiates DNA synthesis following the enzymatic acetylation of E2F target genes on genomic DNA by the hSWI/SNF-Rb complex (Krude et al. 1997). The S phase is closely followed by G_2 and mitotic division.

1.6 Molecular events in compensatory regeneration

A large number of genes are either newly expressed or increase their expression after 70% partial hepatectomy in the rat (Haber et al. 1993). The enormous complexity of gene expression in the regenerating liver makes it essential to develop a logical and systematic approach to assigning a mechanism to this multistep process. The patterns of gene expression can be conveniently divided into a sequence of immediate early genes, delayed genes, cell cycle genes resulting in DNA replication and mitosis (Figure 1.6).
Immediate Early Delayed Cell Cycle DNA replication & Mitosis
Genes Genes Genes
(c-fos, c-jun, c-my) (bclx) (p53, cyclin D1) (cyclin E, A)

0 4h 8h 20h 48h

\[ \text{G}_0 \rightarrow \text{G}_1 \rightarrow S \text{ phase, G}_2 \text{ and Mitosis} \]

**Figure 1.6 - The sequence of gene activation in the liver during compensatory regeneration, following 70% partial heptectomy in the rat.**

The initial event in compensatory regeneration after partial heptectomy appears to be an increase in TNF-\(\alpha\) levels in both plasma and liver. TNF-\(\alpha\) primes liver cells to be transformed from a quiescent \(\text{G}_0\) state to an activated \(\text{G}_1\) state. The proliferative effects of TNF - \(\alpha\) on the liver occur through the TNFR-1 receptor, identified through studies on knockout mice. In TNFR-2 knockout mice there was completely normal DNA replication and an appropriate increase in liver mass following partial heptectomy (Fausto 1999). In contrast, lack of signalling through TNFR-1 greatly inhibited DNA synthesis, liver regeneration and increased mortality 24-40 hours after the operation (Fausto 2000). The activation of the TNFR-1 receptor by TNF - \(\alpha\) results in an increase in hepatic levels of the transcription factor NF\(\kappa\)\(\beta\) at 1-4 hours, which occurs in both hepatocytes and non-parenchymal cells (Cressman et al. 1994). The activation of NF\(\kappa\)\(\beta\) occurs through the removal of the inhibitor I\(\kappa\)\(\beta\) from the NF\(\kappa\)\(\beta\) heterodimer (formed by the p65 (rel A) and p50 subunits). Following NF\(\kappa\)\(\beta\) activation there is a subsequent increase in the levels of Interleukin - 6 occurring at 2-6 hours.
(Shiratori et al. 1996). IL-6 is a cytokine produced by non-parenchymal cells and it is likely that intracellular signalling through the TNFR-1 receptor in activated kupffer cells induces secretion of this cytokine. Interestingly, a single injection of IL-6 to TNFR-1 knockout mice corrects the defects in DNA synthesis (and liver regeneration), thereby establishing a pivotal role for the non-parenchymal cell secreting IL-6 in the regenerative process (Figure 1.7)(Fausto 1999).

**Figure 1.7 - The interactions between hepatocyte and kupffer cell, showing the early molecular events in compensatory regeneration.**

IL-6 ligands the IL-6 receptor complex present on the hepatocyte membrane. This receptor complex consists of an 80kD IL-6 binding glycoprotein termed IL-6R (gp
80) and the signal transducer gp130 (Taga & Kishimoto 1997). IL-6 bound to gp 80 activates its associated gp 130 molecule, which induces intracellular homodimerisation of two gp 130 molecules, activating the receptor (Hirano 1998). Dimerisation of the intracellular domains of two gp130 molecules brings the receptor associated Janus Kinases (JAK 1, JAK 2 and TYK) into close proximity. The intracellular part of the gp 130 molecule contains a series of distinct tyrosine residues that become phosphorylated through these JAK kinases. The resulting phosphotyrosines interact with SH 2 domains on several proteins leading to the activation of the STAT 3 signal pathway (Hemmann et al 1996) and an immediate early gene response.

The immediate early gene response is followed by a delayed gene response, which is blocked by protease inhibitors, indicating that the gene activation is a secondary event. There is also a concurrent increase in hepatocyte growth factor (and other growth factor) activity leading to the occupation of their corresponding tyrosine kinase receptors on hepatocytes. This delayed gene response in combination with increases in hepatocyte growth factor activity induces the expression of the cell cycle protein cyclin D1 and the subsequent cell cycle events leading to cell division.

Following the peak in hepatocyte proliferation at 24 hours, there are subsequent peaks in DNA synthesis and cell division in cholangiocytes, kupffer cells and sinusoidal cells at 24- hour intervals. The molecular mechanisms that regulate cell division in these non-parenchymal cells are yet to be elucidated.

1.7 Regulation of liver regeneration

During liver regeneration after 70% partial hepatectomy, approximately 90% of hepatocytes in the liver divide once or twice and return to quiescence. Recent experiments on the repopulation of damaged livers and serial transplantation studies demonstrated that hepatocytes have a very high replicative capacity (Overturf et al.
Overturf developed an animal model for liver repopulation using tyrosinaemic mice, which are deficient of the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (FAH). The damaged livers of these mice can be repopulated by transplantation of normal hepatocytes from syngeneic animals. The investigators transplanted normal syngeneic male hepatocytes into the liver of FAH knockout female mice and used a Y chromosome marker as a method of hepatocyte identification. The transplanted hepatocytes repopulated 90% of the damaged livers and provided a pool of hepatocytes for further transplantation. The process of serial transplantation was performed eight times with equal success, without a decrease in replicative capacity. This set of serial transplantations involved a minimum of 69 doublings, an astonishing proliferative capacity that resembles haemopoietic stem cells (Overturf et al 1997). In this light, the surprising fact is no longer that hepatocytes can proliferate following partial hepatectomy but that the proliferative capacity is so tightly regulated as to allow liver mass to be restored precisely. The mechanisms that regulate hepatocyte proliferation to precisely regulate liver mass during liver regeneration are yet to be elucidated.

1.8 Thyroid hormone regulation of liver regeneration.

There are a number of complex changes affecting the thyroid hormones following 70% partial hepatectomy in the rat. There is a reduction in both the thyroxine and tri-iodothyronine content of peripheral blood; the nadir is proportional to the amount of liver excised (Leffert & Alexander 1976). Metabolic studies suggest that the decreased levels are due to increased turnover and utilisation in the liver remnant, diminished hormone secretion and a slightly reduced concentration of carrier proteins. Thyroid stimulating hormone (TSH), after an initial fall, increases by 60% to 70%. Dillman et al observed a 50% reduction in nuclear $T_3$ binding sites within 12 hours of
partial hepatectomy followed by a 50% reduction in the rate of liver regeneration in thyroidectomized rats. They showed that even though the rate of regeneration was slower the overall time course was longer and therefore the regenerative process was complete (Oppenheimer et al. 1973). This work shows that the physiological effects of thyroid hormones are critical for the normal regenerative response following partial hepatectomy.

1.9 Thyroid hormone as a primary mitogen

This project has developed from the observation that T3 is a primary mitogen for the liver in rodents when administered at high doses (X20 - X40 physiological). The mitogenic properties of the thyroid hormones have been utilised experimentally to correct inborn errors of hepatic metabolism, either by replacing the defective hepatocytes or by introducing the stable gene into the host cell (Gupta et al. 1999; Gupta & Chowdhury 2002).

An experimental model inhibiting hepatocyte proliferation in vivo in rats has been developed utilising the alkaloid retrosine (Oren et al. 1999). The investigators transplanted syngenic hepatocytes into mice pre-treated with retrosine and showed that treatment with tri-iodothyronine resulted in complete parenchymal (>80%) replacement by the transplanted hepatocytes (Oren et al 1999).

A different approach is to develop a gene delivery system to replace the defective gene and allow its stable integration into the host cell genome (Forbes et al. 1998). Retroviral based vectors are the most extensively studied vectors in animal models and clinical trials (Forbes et al. 2000). The liver is mitotically quiescent and to date transduction with retroviral vectors has occurred when hepatocytes have been stimulated into mitoses. Our laboratory utilised the mitogenic properties of tri-iodothyroine to allow the transduction of retroviral vectors into the rat liver in vivo,
offering the potential for hepatic gene therapy, especially when one considers that the corrected cells could potentially repopulate the liver (through a survival advantage) (Forbes et al. 2000).

The repopulation of the liver with transplanted hepatocytes has also been used experimentally in the treatment of acute hepatic failure (Malhi et al. 2002) and radiation induced hepatitis (Malhi & Gupta 2001), with the rationale that in the clinical arena it may alleviate widespread donor organ shortage.

1.10 Aims of the Thesis

The overall aim of this project was to determine whether regimes administering T3 could enhance liver mass and improve hepatic function.

In order to achieve this, we have followed a logical scheme:

a) To characterise the effects of tri-iodothyroine (as a primary mitogen) on the rat liver.
   • The second chapter characterises the effects of T3 on the intact rat liver in vivo.
   • The third chapter assesses whether the increase in liver mass associated with T3 confers a useful increase in hepatic function.
   • The fourth chapter studies the effects of administering T3 with partial hepatectomy/drug induced liver injury, thus combining the two distinct pathways of liver growth, direct hyperplasia and compensatory regeneration.

b) To investigate the mechanism of action of T3 as a primary mitogen in rats.
   • The fifth chapter attempts to identify a mechanism by which this intriguing phenomenon may be occurring.

c) To develop an in vitro model of T3 action.
   • The sixth chapter characterises the effects of thyroid hormone on primary rat hepatocyte cultures, to assess if T3 can be shown to have similar effects in vitro as it
does *in vivo*. This work could then be applied to human liver cell culture systems to determine whether T₃ may constitute an effective primary mitogen in man.
Chapter 2

Characterising the effects of thyroid hormone on the intact rat liver
2.1 Background

2.1.1 Intact rat liver

The distinct cell subpopulations of the liver are arranged in a highly organised architectural form with individual cells in intimate relationships with each other and with extracellular structures. Hepatocytes constitute the largest cell number and by far the largest proportion of liver mass (>80% cellular mass). The sinusoidal structures that flank the hepatocyte cords are lined with endothelial cells and the hepatic tissue macrophages (kupffer cells). The biliary canaliculi formed between the hepatocytes drain via fine cholangiolar cells into the biliary epithelial cells. Stellate cells of mesenchymal origin lie scattered between the hepatocytes and the sinusoidal lining cells, and sundry lymphocytes reside in and traffic through the hepatic parenchyma (Malik, Selden, & Hodgson 2002).

In the adult, the liver is in a state of mitotic quiescence, its energies being channelled into an array of differentiated functions. These functions include protein synthesis, vitamin/mineral storage, carbohydrate and fat metabolism and are performed by the major cell type within the liver, the hepatocyte. This cell type is however unique in retaining an intrinsic ability to proliferate in response to various stimuli and hence regulate liver mass (Kountouras, Boura, & Lygidakis 2001).

In recent times a cell type with its own distinct proliferative capacity (hepatic stem cell) has been identified lining the biliary ductules (Canal of Hering) of adult animals and humans. These ductular cells form a reserve compartment capable of generating mature hepatocytes and biliary cells after toxic injury, massive necrosis or when hepatocyte proliferation has been inhibited (Forbes et al. 2002). I will not be discussing the hepatic stem cell in any detail during this thesis.
2.1.ii Endogenous markers of DNA synthesis

Endogenously expressed antigens of cell proliferation provide an opportunity for monoclonal detection of cells that are progressing through the cell cycle and undergoing cell division. The most commonly used proliferation markers are the Proliferative Cell Nuclear Antigen (PCNA) and Ki-67, both of which can be detected immunohistochemically using specific commercially available antibodies.

The PCNA is a 36 kDA auxiliary protein for DNA polymerase delta that is essential for cell division. The expression of PCNA varies with the cell cycle; rising through G1 to peak in S phase, followed by a decrease in G2 and M phase (Prelich et al. 1987).

Ki-67 is an IgG1 mouse monoclonal antibody that recognises a nuclear non-histone protein. Ki-67 antigen first appears in mid G1 and rises through the cell cycle to peak in G2 and M phase (Sasaki et al. 1987).

The advantage of both these markers is that they are expressed endogenously and therefore may be detected in archival specimens. The disadvantage is that non-proliferating cells may be weakly stained (high false positive rate) and the staining intensity may be influenced by the method of fixation (Gerdes et al. 1991).

2.1.iii Exogenous marker of DNA synthesis

Bromodeoxyuridine (BrdU) is a synthetic thymidine analogue incorporated into DNA during S phase. Once incorporated into the nucleus, BrdU forms a stable compound that can be reliably recognised by a monoclonal antibody within tissue sections. The main advantage of the BrdU method when compared to the detection of endogenous antigens is its reproducibility and greater specificity (Ryser et al. 1999).

A radiolabelled thymidine incorporation method can also be used to detect DNA synthesis within tissues. Due to the difficulty of in vivo radioactivity handling this
technique is rarely used, however we adopted the technique for the *in vitro* detection of DNA synthesis within primary rat hepatocyte culture (chapter 6).

2.1.iv  **Liver mass indices**

The liver has the unique capacity to regulate its mass in humans and animals. Deficits are bought about by surgical resection or by cellular necrosis caused by chemicals or viruses. In the latter case there is occasionally a functional deficit without a reduction in mass. Functional capacity is however, a relative rather than an absolute parameter in regulating hepatic growth (Fausto 2000). The absolute parameter for growth regulation is the ratio between the liver mass and the body mass (Fausto 2000). The optimisation of the ratio indicates that the liver reaches a state in which it performs the amount of metabolic work to meet the functional requirements of the body (Michalopoulos & DeFrances 1997). The total protein content of the liver accounts for 10%-15% of the total liver mass, with the total DNA content only accounting for 0.01%-0.001% of the total mass (Sapan, Lundblad, & Price 1999). If there are changes in liver mass associated with hyperplasia there are corresponding changes in total protein and total DNA (ie increase in liver mass associated with a corresponding increase in protein and DNA), reflecting the increase in the cellular content of the organ (Krawetz, States, & Dixon 1986).

2.1.v  **Hepatocyte apoptosis**

Primary mitogens can enhance liver mass and increase the liver/body mass ratio to above normal levels through the process of direct hyperplasia. The liver mass in turn returns to its normal level through a non-inflammatory process known as apoptosis (Columbano *et al.* 1985). Multicellular animals have a dedicated molecular programme for cell death known as apoptosis to tightly control cell numbers and tissue size. The morphological features of apoptosis are distinct from necrotic cell death, classic features
including cytoplasmic condensation, chromatin margination/capping, DNA fragmentation and the formation of apoptotic bodies (Afford & Randhawa 2000). These morphological changes occur through the effects of a set of cysteine proteases known as “Caspases”. The caspases are the terminal executioners of the apoptotic pathway within cells, however it should be recognised that a variety of distinct molecular signals can converge to activate these enzymes. All known caspases possess an active cysteine site that cleaves the covalent bonds adjacent to aspartate residues (Hengartner 2000).

There are a variety of methods available to identify apoptotic cells. The classical morphological changes are best viewed by transmission electronic microscopy, however this is a labour intensive procedure. The routine H&E stained slide is adequate for measuring apoptosis, however recently investigators have sought more rapid and reproducible methods of quantifying the process. The vast majority of these are based on the well-known fact that genomic DNA in cells undergoing apoptosis is cleaved into histone-associated DNA fragments, oligonucleosomal lengths. There are many commercially available kits to study DNA ladders on agarose gels, however these are only relevant to cell culture systems. DNA degradation can also be detected by enzymatic labelling of the 3’ - hydroxy end using modified nucleotides (eg flourescein labelled or biotinylated dUTP). There are two different enzymatic labelling methods, in situ nick translation and in situ end labelling (TdT mediated d-UTP Nick End Labelling – TUNEL). Nick translation relies on the specific binding of the large (Klenow) fragment of DNA polymerase 1 to the nicked DNA and catalyses the template dependent addition of nucleotides. TUNEL relies on the binding of terminal deoxynucleotidyl transferase (TdT) to free 3’ - hydroxy ends of single or double stranded DNA breaks, catalysing the incorporation of labelled and unlabelled nucleotides. Both these methods are applicable to paraffin embedded sections, however
it should be noted that DNA fragmentation is a characteristic feature of both apoptotic and necrotic cell death (Alison 1999).

Recently commercial kits detecting caspase activity, apoptotic cytokeratins and a variety of molecular activators of apoptosis have become available but their efficacy needs assessment.

2.2 Aim

Our aim was to characterise the effects of a single mitogenic dose of thyroid hormone on the intact rat liver. Tri-iodothyronine is of particular interest as a primary mitogen in view of its potential exploitation as a pharmaceutically available hormone that has been critically appraised in humans.

2.3 Methods

2.3.1 Induction of hepatocyte proliferation

Tri-iodothyronine was obtained from Sigma (Poole, England), dissolved in 0.01M NaOH at a concentration of 5mg/ml. It was administered to rats routinely at 9am by subcutaneous injection at a dose of 4mg/kg bodyweight (see protocol 1 below). Previous investigators have used a dose/response curve to show that 4mg/kg bodyweight is the optimum mitogenic dose of T₃ in rodents (Forbes et al 1998). In the portal injection protocol (see protocol 2 below) T₃ was administered directly into the portal vein at laparotomy.

2.3.iii Animal protocols

Male Sprague Dawley (SD) rats weighing 200-250g were used. They were housed in a temperature and light controlled room (12 hour light/dark cycle). The rats had access to food and water ad libitum. All animals were sacrificed on the same day to equilibrate body weights within groups. Animal care and all procedures were compatible with the Animals (Scientific Procedures) Act 1986, British Home Office.
Protocol 1

The rats (n=6 per group) were injected with a single dose of tri-iodothyronine and sacrificed at intervals of 6 hours, 12 hours, 1 day, 2 days, 4 days, 7 days, 10 days and 14 days.

Protocol 2

Rats (n=3 per group) were injected with a single dose of tri-iodothyronine into the hepatic portal vein and sacrificed 24 hours later. The rats were anaesthetised, secured prone and the abdomen swabbed with ethanol. A midline laparotomy incision was made to expose the liver and the liver delivered through the incision with gentle abdominal pressure. The liver was mobilised by cutting the falciform and gastrohepatic ligaments and the T3 injected by directly cannulating the portal vein with a 22 gauge paediatric cannula.

Serum analysis

Blood was taken from rats receiving subcutaneous T3 at 0, 8, 24, 48 and 96 hours for serum analysis and compared to control samples taken from rats receiving vehicle only. Standard laboratory liver tests were performed and a radioimmunoassay (Harbor-UCLA, USA) used to determine serum TSH levels.

Sacrifice

The animals were sacrificed by exsanguination under isoflurane anaesthesia. The livers were removed, washed in normal saline, allowed to dry and weighed. Both the liver and body mass were measured to allow the calculation of the liver/body mass ratio, the most sensitive index of liver mass. Sections were cut for biochemical and histological analysis, with histological sections being placed in methacarn and formalin.
Identification of hepatocyte proliferation

Cell proliferation was assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation into nuclei and immunohistochemical recognition. The BrdU was administered intraperitoneally at a dose of 50mg/kg one-hour prior to sacrifice to estimate the proportion of cells in DNA synthesis at sacrifice – Flash labelling. Upon sacrifice the liver was fixed in methacarn (60% methanol, 20% chloroform and 10% acetic acid) and processed with wax embedding in the histopathology department.

For immunohistochemical analysis 4µm – thick sections were used. The sections were deparaffinized in xylene and then placed in graded ethanol solutions (100% - 70%) prior to rehydration. Non specific peroxidase activity was blocked by incubation in a 0.3% solution of hydrogen peroxide for twenty minutes. The sections were then immersed in 1M hydrochloric acid heated to 60°C for five minutes (this disassociates the histones and partially denatures the DNA) and washed in distilled water. The slides were then incubated for ten minutes with 10% normal rabbit serum. The rabbit serum was gently tapped off and the primary antibody (mouse anti BrdU, DAKO, UK) immediately pipetted onto the section at the optimal dilution of 1:100. The sections were left in a humid incubator for one hour at room temperature. The sections were then washed in X1 PBS for ten minutes and then incubated for 45 minutes with the biotinylated rabbit anti mouse immunoglobulin (DAKO, UK) diluted 1:200 in PBS. The sections were then washed in X1 PBS for ten minutes prior to the addition of the ABC complex (streptavadin/biotinylated horseradish peroxidase mixture-DAKO, UK) for 30 minutes. The sections were then washed well in X1 PBS prior to development with a 1% DAB solution (150mls PBS, 1.5mls DAB and 0.5mls H₂O₂). The sections were then counterstained with haematoxylin, dehydrated and mounted ready for viewing under the microscope.
2.3.iv Identification of apoptosis

Sections of paraffin embedded liver was stained for DNA fragmentation characteristic of apoptosis by the terminal UDP-nick end labelling (TUNEL) reaction using the R&D systems TUNEL reagents (kit no – TA 4625, London, UK). For immunohistochemical analysis 4μm – thick sections were used. The sections were deparaffinized in xylene and then placed in graded ethanol solutions (100% - 70%) prior to rehydration. A 1% proteinase K solution was placed on to the sections for 5 minutes to enhance antigen retrieval. Non-specific peroxidase activity was blocked by incubation in a 0.3% solution of hydrogen peroxide for twenty minutes. The slides were immersed in a in TdT labelling buffer (5mls of X10 TdT buffer with 45mls distilled water) for five minutes and then incubated with the apoptosis reaction mixture (1μl biotinylated dNTP, 1μl Mn stock, 1μl TdT enzyme and 47μl X1 TdT labelling buffer) for one hour. The sections were then immersed in TdT stop buffer for five minutes (5mls of X10 TdT stop buffer with 45mls distilled water) prior to washing with X1 PBS. The sections were then incubated with the ABC complex (streptavidin/ biotinylated horseradish peroxidase mixture) for 30 minutes prior to development with a 1% DAB solution (150mls PBS, 1.5mls DAB and 0.5mls H₂O₂). The sections were then counterstained with methyl green, dehydrated and mounted ready for viewing under the microscope.

2.3.v Measurement of hepatocyte proliferation and apoptosis

The BrdU and apoptotic labelling indices were determined by counting 4000 hepatocyte nuclei per section from consecutive light microscopy fields. The immunolabelled sections were evaluated blindly to avoid bias with the number of positively stained nuclei being expressed as a percentage.
2.3.vi DNA estimation

To quantify levels of DNA in the liver a DNA tissue kit (Quiagen, UK; DNeasy kit [no 69504]) was used. A 15-25mg liver section was weighed and then lysed using protease enzymes and lysis buffers. The lysate was then loaded onto a DNA column and following repeated centrifugation, DNA was eluted using an elution buffer. The DNA content was determined by measuring absorbance at 260nm in a UV spectrophotometer.

Quantification Procedures - Calculations were performed to obtain a DNA concentration per gram of liver tissue. The DNA concentration was then used to obtain a total liver DNA using the total liver weight

2.3.vii Protein estimation

A 200-300mg section of rat liver was weighed and suspended in 5mls of 1% Phosphate Buffer Solution (PBS). The tissue was homogenised for 60 seconds at full speed in Potter Eljveham homogeniser with PTFE pestle. A 5ml volume of 0.6M perchloric acid was added to the mixture prior to centrifugation at 3400 rpm for 20 mins. The supernatant was discarded and the pellet resuspended in 5ml 0.2M perchloric acid, using 5 strokes of the homogenizer. The sample was again centrifuged at 3400 rpm for 20 mins and the supernatant discarded. A 5ml solution of 0.3M KOH was added to the pellet and homogenised using 10 strokes of the homogenizer. The solution was then transferred to a thin walled glass tube and placed on a shaking platform at 37 degrees Celsius for 90 minutes. A 50 µl aliquot of the sample was used for protein estimation using a micro-modification of the Lowry method. Thus, a 50 µl sample was added to 50 µl of copper sulphate solution and following the addition of 200 µl of F/C reagent absorbance was measured @ 620nm in a microplate reader to obtain a protein content.
Quantification Procedures - Calculations were performed to obtain a protein concentration per gram of liver tissue. The protein concentration was then used to obtain a total liver protein using the total liver weight.

2.3.viii Statistical analysis

Results are displayed as the mean ± standard deviation of sample ($\mu \pm \text{SD}_{n-1}$). Statistical differences were determined using the two tailed t-test and reported if $p<0.05$ as compared to controls.
2.4 Results

2.4.1 Induction of hepatocyte proliferation

We first characterised the effects of a single subcutaneous injection of T₃ on hepatocyte proliferation in the intact rat liver. A peak in cell proliferation was seen 24 hours after the administration of T₃, the peak being followed by a gradual decline (fig 2.1). There were 7 ± 1.1% of hepatocytes in S phase at 24 hours, as compared to <1% in controls (p<0.001).

Fig 2.1 - The flash BrdU labelling index in vivo in adult rat liver (n = 6) after a single dose of T₃.

* p<0.001 as compared to vehicle alone. ** p<0.05 as compared to vehicle alone
These BrdU labelled hepatocytes were predominantly in the midzonal region of the hepatic lobule (fig 2.2 and fig 2.3).

*Immunolabelled section of the liver for BrdU following vehicle only (Fig 2.2-above) showing no effect and following a single injection of T3 (Fig 2.3 below) showing midzonal distribution of staining reflecting midzonal cell proliferation.*
2.4.ii Effect of T₃ on hepatic mass, total DNA and protein levels.

The growth stimulation seen with T₃ resulted in an increase in the cellular mass of the liver. The maximum effect was seen on day 10 with a 15% larger liver mass than in control rats (p<0.01 – Fig 2.4 & Fig 2.5).

The effect of T₃ administration on liver mass (Fig 2.4 - above) & liver/body mass ratio (Fig 2.5 - below), showing a peak increase in liver mass at 10 days.

* p<0.01 as compared to vehicle alone.
The increase in liver mass was matched with corresponding increases in total DNA (p<0.01 – (Fig 2.6) and total protein levels (p<0.01 – (Fig 2.7) confirming the increase in liver mass was cellular and not the result of intra-extra cellular fluid retention in the liver.

*The effect of T3 administration on total DNA (Fig 2.6 – above) and total protein (Fig 2.7 – below) content of the liver. * p<0.01 as compared to vehicle alone.
Interestingly, there was a regression in liver mass between day 10 and day 14, which we investigated to delineate whether an apoptotic or necrogenic mode of liver cell death was occurring to restore the liver mass to its normal precisely regulated value.

**2.4.iii Effect of T₃ on hepatocyte apoptosis**

We characterised the effects of T₃ on hepatocyte apoptosis using the TUNEL assay. The TUNEL index peaked at day 14 with 2.2% of cells labelled as compared to 0.58% in controls (p<0.01 – fig. 2.8).

![Fig. 2.8 - TUNEL labelling index in vivo in adult rat liver after a single dose of T₃.](image)

* p<0.01 as compared to vehicle alone.
These positively labelled cells were randomly distributed throughout the hepatic lobule (Fig. 2.9 and Fig. 2.10).

*Immunolabelled section of the liver for apoptosis following vehicle only (Fig. 2.9 - above) showing no effect and 14 days after a single injection of $T_3$ showing enhanced TUNEL staining (Fig. 2.10 - below).*
The higher rate of hepatocyte apoptosis at day 14 (as compared to control liver) corresponded to the time point at which a regression in liver mass was occurring. These results in combination with the absence of necro-inflammatory changes in sections from day 14 confirm that the restoration of the liver mass to its normal value was through the apoptotic deletion of excess cells.

2.4.iv Investigating the toxic and systemic endocrine effects of T₃.

We investigated the effects of T₃ on serum levels of bilirubin, alanine aminotransferase (ALT) and inspected histological sections of the liver. There were no differences in serum bilirubin and ALT levels following T₃ stimulation at 8, 24, 48 and 96 hours as compared to the vehicle only group (table 2.1).

<table>
<thead>
<tr>
<th></th>
<th>Vehicle only</th>
<th>T₃ group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8 hours</td>
</tr>
<tr>
<td>body weight (g)</td>
<td>277 ± 13</td>
<td>N/A</td>
</tr>
<tr>
<td>bilirubin (µmol/L)</td>
<td>1.4 ± 0.9</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>ALT (units/l)</td>
<td>83 ± 9.1</td>
<td>85 ± 6.2</td>
</tr>
</tbody>
</table>

Table 2.1 - Effect of T₃ on body weight, serum bilirubin and alanine aminotransferase (ALT) levels.

Histological examination of the livers of rats receiving T₃ showed no evidence of a necrogenic liver injury. There was no centrifugal necrosis, a characteristic feature of thyrotoxic liver injury in humans (Sola et al 1991), probably reflecting the temporal difference between a single high dose of T₃ in a rat and the sustained elevated levels of
T₃ in hyperthyroidism in humans (Salata et al 1985). This confirms that the mitogenic doses of T₃ used induced cell proliferation in the absence of liver injury.

The single subcutaneous injection of T₃ at 4mg/kg was well tolerated by recipient rats, with the maintenance of an adequate appetite and bodyweight (table 2.1), and no unexpected mortality over the time period. As anticipated the mitogenic doses of T₃ resulted in hyperthyroidism in recipient rats, shown by the suppression of TSH secretion, with the trough level at 48 hours being below 1 ng/ml and the levels returning to near normal at 96 hours at 2.6 ng/ml (figure 2.11).

![Figure 2.11](image.png)

**Figure 2.11 - Serum TSH levels following the administration of a single dose (4mg/kg) of tri-iodothyronine to rats. *p<0.01 as compared to 0 hours.**

The final experiment in this chapter was aimed at performing a pilot study to identify if portal vein injection of T₃ could utilise its high rate of first pass metabolism to maintain its proliferative effects, while reducing the systemic endocrine effects. A dose response curve was generated after T₃ was injected into the portal vein and flash BrdU labelling performed at 24 hours, which allowed us to compare the results with the effect of a subcutaneous injection at 24 hours (7%). The intra-portal administration of
T₃ showed a lower trend in the rate of cell proliferation at 24 hours when compared to subcutaneous injection (fig 2.12).

![Graph showing the flash BrdU index of hepatocytes at 24 hours for different dose levels of T₃ administered intra-portal.]

**Fig 2.12** shows the effects of intra-portal administration of T₃ (n=3) with the flash BrdU labelling index of hepatocytes at 24 hours.
2.5 Discussion

We have confirmed that tri-iodothyronine is a primary mitogen for the liver using the BrdU labelling method. Bromodeoxyuridine is a thymidine analogue that is incorporated into the DNA of cells undergoing DNA synthesis and is thereby incorporated into the nuclei of actively replicating cells. Immunohistochemical recognition can be used to accurately identify the number of BrdU labelled cells on histological sections and thereby provide a proliferation index. There are a small number of false negatives using this technique; representing cells that are in G1, G2 or M phase of the cell cycle that are replicating but are not actively in DNA synthesis. There is also a small false positive group that represent hepatocytes undergoing DNA repair at the time of sacrifice. However, we have demonstrated in chapter 4 that the predominant amount of BrdU incorporation following T3 is a direct result of DNA synthesis within the context of cell proliferation, this confirmation being attained through the frequent demonstration of labelled twin daughter cells with continuous BrdU labelling. In spite of minor inaccuracies the flash BrdU labelling method is a reliable technique for quantifying the number of actively proliferating cells following T3 injection (Miyagawa et al. 1997). The hepatocyte is a quiescent cell within the intact liver as seen in control animals with less than 1% of cells proliferating at any one-time (Masuhara et al. 1996;Matsumoto & Nakamura 1992). We have confirmed that a single dose of subcutaneous tri-iodothyronine is a non-damaging way of inducing hepatocyte proliferation. Thyroid hormone induces a proliferative response which peaks at 24 hours with a 7% BrdU labelling index, being followed by a gradual decline. The hepatocytes entering S-phase are predominantly in the midzonal region of the hepatic lobule. This is an intriguing observation when one considers that the highest concentration of O2, nutrients, growth factors and hormones is in the periportal region.
of the liver. The mitogenic effects of T₃ occur without any biochemical or histological evidence of hepatic damage and the high doses used are tolerated by the recipient rats. Tri-iodothyronine is a prime candidate to manipulate liver cell proliferation in vivo to help design gene therapy (Forbes et al. 1998) and cell transplantation (Gupta, Gorla, & Irani 1999) approaches to liver disease.

The growth stimulation with T₃ results in a larger liver than in control animals. The increase in liver mass follows a normal distribution over the protracted time period with the peak increase occurring at ten days with a 15% larger liver than in control animals. The peak increase in liver mass was matched by corresponding increases in total DNA and total protein levels. The DNA assay is a sensitive assay that allows quantification of DNA using UV spectrophotometry. There are however a number of difficulties in obtaining accurate reading. These include:

1. Loss of tissue during preparation.
2. Contamination of tissue during preparation.
3. Variations in tissue solubility when using lysis buffers.

The liver protein assay can be broadly categorised into a preparation/purification stage and a quantification stage. The major inaccuracies arise during the preparation/purification stage, which is when the tissue is homogenised, centrifuged and buffered with perchloric acid repeatedly. However these inaccuracies appeared to be eliminated by the sample sizes (n=6), which provided the adequate power to produce significant results in both the total DNA and total protein calculations. The DNA and protein results confirm the increase in liver mass with T₃ is associated with an increase in cell number and not from extra/intra cellular fluid retention within the liver.

The decrease in liver mass between day 10 and day 14 occurs through the apoptotic deletion of excess cells. We characterised the effects of T₃ on hepatocyte
apoptosis using the TUNEL assay. The higher rate of hepatocyte apoptosis at day 14 (as compared to controls) corresponded to the time point at which a regression in liver mass was occurring. These results in combination with the absence of necroinflammatory changes in sections from day 14 confirm that the restoration of the liver mass towards its normal value was through the apoptotic deletion of excess cells.

The single subcutaneous injection of T\textsubscript{3} at 4mg/kg was well tolerated by recipient rats, with the maintenance of an adequate appetite and bodyweight, and no unexpected mortality over the four-day period. As anticipated the mitogenic doses of T\textsubscript{3} resulted in hyperthyroidism in recipient rats, shown by the suppression of TSH secretion, with the trough level at 48 hours being below 1 ng/ml and the levels returning to near normal at 96 hours at 2.6 ng/ml. The intra-portal administration of T\textsubscript{3} showed a lower trend in the rate of cell proliferation at 24 hours when compared to subcutaneous injection, for which there could be a number of reasons. The greater technical difficulty with the intraportal injection may have reduced the amount of T\textsubscript{3} being administered to the rat, an alternative for which could have been oral administration in food however again the immediate and complete delivery of the drug into the system would be difficult to assess. Another reason could have been that the high rate of first pass metabolism of T\textsubscript{3} could have reduced the systemic delivery of the hormone, which may have reduced the mitogenic effects if the effect was occurring indirectly (discussed in chapter 5 and 6). It is clear that larger sample sizes with the administration of radiolabelled T\textsubscript{3} (to accurately assess dose administration, uptake and excretion) could have allowed us to answer some of these questions. However, in view of the technical difficulties with the procedure we felt that the remaining experiments in the thesis should be performed using the standard subcutaneous dose, which has been well documented (Ribeiro et al, 1998).
Chapter 3

Does the increase in hepatic mass induced by $T_3$ confer an increase in hepatic function
3.1 Assessment of liver function

In the previous chapter we demonstrated that hepatocytes can be stimulated to proliferate by a primary mitogen ($T_3$) resulting in an increase in liver mass. It is astonishing that hepatocytes not only possess this intrinsic ability to proliferate in response to various stimuli but also perform over 4,000 metabolic functions (Burke 2002). The next logical step in this thesis is to establish if the increase in liver mass associated with $T_3$ confers a useful increase in hepatic function.

In man strategies to assess liver function have developed in relation to the syndrome of fulminant hepatic failure or in chronic liver disease. Fulminant hepatic failure occurs when there is an insufficient number of hepatocytes or severe loss of hepatocyte function preventing normal hepatic metabolism. A variety of causes including hepatotoxic drugs, viral agents, major surgical resection and metabolic conditions have been cited (Riordan & Williams 1999). The syndrome of liver failure is a heterogeneous disorder with a number of definitions reflecting a variety of presentations both in terms of the clinical characteristics and the temporal course of the illness. The clinical assessment determines if a patient has features of encephalopathy, jaundice, coagulopathy, renal failure or sepsis. The biochemical features include:

1. Prolonged prothrombin time/INR
3. Elevated serum urea or creatinine.
4. Elevated bilirubin.

These features represent only a minor proportion of the metabolic functions carried out by the hepatocyte but have been shown to be useful parameters to assess the need for orthotopic liver transplantation (O'Grady, Schalm, & Williams 1993).
The difficulty in developing a strategy for T₃ appears to be from:

   a) The clinical and biochemical parameters that assess liver function have been appropriately developed over time to determine the difference between a normally functioning liver and a failing organ. We require a strategy to determine the functional difference between a normal organ and a liver that has had its mass enhanced by a primary mitogen.

   b) Even within the parameters developed to assess hepatic function in liver failure, it is notoriously difficult to predict the outcome of patients with liver failure. This suggests that the effect of a causal agent on a specific metabolic function is idiopathically variable (Riordan & Williams 2000). Therefore finding a single metabolic parameter that reflects the overall function of the liver appears to be a quest for “The Holy Grail” of hepatology.

In developing different strategies to assess functional hepatic mass we have performed experiments where the animals were assigned to either T₃ or vehicle only, ten days prior to assessment, to assess function when the increase in liver mass was maximal. The following strategies were adopted:
3.2 Effect of T₃ induced increases in liver mass on the synthetic capacity of the liver following a single hepatic stress of a 3 ml venesection.

3.2.i Background

An assessment of serial plasma albumin levels following hepatic stress would be used to see if rats in the T₃ group with larger livers could support the plasma albumin to a greater extent than the control group. In this context we aim to use the plasma albumin as a surrogate marker of the synthetic capacity of the liver following hepatic stress. The plasma albumin has partly been used as a measure of synthetic function because of the availability of the assay in the biochemistry department. The prothrombin time, retinal binding protein, and pseudocholinesterase assays are not available and measurements of bilirubin (excretory function) and other liver tests (ALT, AST) were thought of to be less satisfactory (Burke 2002).

The first hepatic stress we have used is the 3 ml venesection in the rat (1/4 of the total blood volume) removing a significant amount of plasma albumin, which will result in an increased rate of albumin synthesis in the liver.

3.2.ii Aim

To assess if the increase in liver mass induced by the primary mitogen tri-iodothyronine confers an increase in hepatic function using serial measurements of the plasma albumin following a 3ml venesection.

3.2.iii Method

SD rats (n=5) were injected with a single dose of tri-iodothyronine 10 days and 1 day prior to venesection (this cohort was included to determine the effects on plasma albumin of a large number of proliferating cells in the liver). A control group received vehicle only 10 days prior to venesection. In order to perform the venesection the rats
were anaesthesised, secured prone and the chest swabbed with ethanol. A small incision
was made in the right supraclavicular fossa and the layers of fascia carefully dissected
to expose the internal jugular vein. The vein was cannulated with a 22 gauge paediatric
cannula and a 3ml venesection performed. Haemostasis was achieved and the incision
stitched up. Blood taken at the time of venesection was sent for biochemical analysis
and two further tail vein venesections were performed on day 1 and 3. A standard
laboratory plasma albumin assay was carried out by the department of clinical
chemistry, Royal Free Hospital.

Statistical analysis

Results are displayed as the mean ± standard deviation of the sample (μ ± SDn⁻¹).
A 2-way ANOVA was performed with the experimental group and the sampling day as
independent variables and reported if p<0.05 as compared to controls.

3.2.iv Results

The animals were assigned to either T₃ or vehicle only ten days prior to the
assessment of liver function, with this time point ensuring that the T₃ induced increase
in liver mass was at a maximum. There was no significant drop in the plasma albumin
concentrations from surgery to day 3 in both control rats and in rats given a single dose
of T₃ ten days prior to venesection. There was however, a significant difference in
plasma albumin between the group given a single dose of T₃ one day prior to
venesection and the control group on day 1 and day 3 (p<0.05 cf controls) following
venesection (fig 3.1).
Fig 3.1 – Effect of pre-treatment (1 and 10 days) with T3 on plasma albumin levels after a hepatic stress of a 3 ml venesection.

* - p<0.05 as compared to the pre-venesection sample in the T3 pre-treated day 1 group

3.2. v Discussion

A 3ml venesection does not produce a drop in the plasma albumin over a 3 day period in control rats. This suggests that the intact liver has a large reserve capacity to increase the rate of albumin synthesis and the 3 ml venesection is not an adequate hepatic stress to drop plasma albumin levels. It should be noted that there is a large pool of albumin available for redistribution from the extravascular space (40% total body albumin), which may also contribute to the homeostatic mechanisms that maintain the plasma albumin concentration. The ability of the liver to compensate for minor stresses
is supported by evidence that total protein and albumin concentrations can be maintained in humans after sub-clinical toxic injury (Redaell et al). The assessment of serial plasma albumin concentration following a hepatic stress of a 3 ml venesection cannot be used to assess a difference in liver function in 10 day pre-treated and control rats as the stress does not induce a drop in albumin levels in controls.

However, it is interesting to note that there was a significant drop in serum albumin following venesection, when T₃ was administered 24 hours prior to venesection. This probably reflects the large number of undifferentiated proliferating hepatocytes (from the mitogenic effects of T₃) present in the liver at the time of venesection. These hepatocytes are committed to proliferation rather than differentiated synthetic function and are thereby unable to increase the liver's rate of albumin synthesis to compensate for the loss at venesection. This finding provides the rationale for the addition of an additional stress to the 3 ml venesection to test differences between the groups.

We have chosen the 70% partial hepatectomy as an additional stress to venesection as it will:

a) Removing a significant proportion of functioning hepatocytes.

b) Induce a proliferative response in a significant proportion of the remaining hepatocytes (these will be in an undifferentiated state).
3.3 Effect of T<sub>3</sub> induced increases in liver mass on the synthetic capacity of the liver following a dual hepatic stress of a 3ml venesection and partial hepatectomy.

3.3.1 Aim

To assess if the increase in liver mass induced by the primary mitogen tri-iodothyronine confers a useful increase in hepatic function by serial measurements of the plasma albumin following a dual stress of 3ml venesection and 70% partial hepatectomy.

3.3.2 Method

SD rats (n=5) were injected with a single dose of tri-iodothyronine/vehicle ten days prior to operation. The rats were anaesthetised, secured prone and the abdomen swabbed with ethanol. A midline laparotomy incision was made to expose the liver and the liver delivered through the incision with gentle abdominal pressure. The liver was mobilised by cutting the falciform and gastrohepatic ligaments. A silk suture was placed around the vascular pedicle of the median and left lateral lobes of the liver and the lobes removed following ligation. A 3ml venesection was performed at the time of 70% partial hepatectomy by directly cannulating the portal vein with a 22 gauge paediatric cannula. The animals recovered in a warm quiet place and were observed regularly. Blood was taken for biochemical analysis at surgery and on days 1 and 3 following surgery. A standard laboratory plasma albumin assay was carried out by the department of clinical chemistry, Royal Free Hospital.

Statistical analysis

Results are displayed as the mean ± standard deviation of the sample (μ ± SD<sub>n-1</sub>). A 2-way ANOVA was performed with the experimental group and the sampling day as independent variables and reported if p<0.05 as compared to controls.
3.3.iii Results

There was a significant drop in plasma albumin concentration from surgery to day 3 in both the T3 and control groups (fig 3.2) (p<0.05 for both groups). This confirmed that this dual stress has a significant effect on the synthetic capacity of the liver. However, the T3 group was not supporting the plasma albumin to a greater extent than the control group and there were no differences between groups at any of the time points.

Fig 3.2 – Effect of a single dose of tri-iodothyronine administered ten days prior to PH on plasma albumin levels following a dual hepatic stress of PH and venesection.

* - p<0.05 as compared to pre-op T3 group

$ - p<0.05 as compared to pre-op control group
3.3.iv Discussion

A large stress on the synthesising capacity of the liver following venesection and 70% partial hepatectomy results in a significant drop in the plasma albumin in both the T3 and control groups. This major hepatic resection results in a marked reduction in functional hepatocyte mass and with the additional removal of 25% of the circulating blood volume the liver is unable to maintain albumin levels. It is not surprising that this dual stress results in a drop in serum albumin since recent work has shown that the 70% partial hepatectomy alone in the rat causes a 30-50% drop in total protein and albumin levels at day 5 and this takes 10 – 14 days to become normal (Panis et al).

However, comparative analysis demonstrates that the T3 group was not supporting the plasma albumin to a greater extent than controls and this experiment cannot be used to determine if the increase in liver mass induced by T3 confers an increase in hepatic function. It should be noted that there is a large pool of albumin available for redistribution from the extravascular space (40% total body albumin) and the half life of the plasma albumin is relatively long (35 days). This is likely to make the serum albumin a poor surrogate marker of hepatic synthetic capacity. The possibility of assessing synthetic capacity using radiolabelled albumin with gas chromatography mass spectroscopy as a more sensitive test exists. However this is a laborious procedure requiring specialised equipment and we have therefore pursued other strategies to test our hypothesis.
3.4 Effect of T₃ induced increases in liver mass on mortality following 85% partial hepatectomy.

3.4.1 Background

We have utilised a model of the 85% partial hepatectomy in the rat. This major surgical resection in the rodent has a mortality rate of 80% at 5 days (Panis et al 1997). There is a 50% mortality within the first 24 hours of surgery as a result of the complications of the major resection. The remaining 30% of mortality occurs in the next 4 days as a direct result of fulminant hepatic failure. In animals treated with T₃ ten days prior to surgery the remnant liver segment following 85% partial hepatectomy is larger than in control animals. Therefore residual liver function in the T₃ group may be preserved when compared to the control group resulting in an increased survival at 5 days.

3.4.2 Aim

To assess the effects of T₃ administered ten days prior to an 85% partial hepatectomy on mortality following surgery.

3.4.3 Method

Two groups of rats (n=12) were assigned to either T₃ administration or control (vehicle only) ten days prior to surgery.

The rats were anaesthetised, secured prone and the abdomen swabbed with ethanol. A midline laparotomy incision was made, the abdominal contents and peritoneum reflected to the left and the liver completely exposed. The liver was mobilised by cutting the falciform and gastrohepatic ligaments. A silk suture was placed around the vascular pedicle of the left lateral, median, caudate anterior and right inferior lobe of the liver and the lobes removed following ligation. The abdomen was closed in
two layers using silk and ethilon sutures. The animals received 15 mls sc dextrose saline and buprenorphine analgesia and recovered in a warm quiet place and were observed regularly.

Statistical analysis

Statistical differences were determined using a two sided log-rank test. Power calculations were performed in collaboration with Professor Senn (department of statistics – UCL) to identify the animal numbers required to achieve statistical significance between groups.

3.4.iv Results

There were 6 deaths (50% mortality) in both groups in the first 24 hours after surgery. The remaining animals developed features of hepatic encephalopathy after 24 hours. These features included slowness of movement, a reduction in reflex response, drowsiness followed by coma in a number of cases. There were 5 deaths in the control group between 24 and 72 hours and 4 deaths in the T3 group over the same time frame. These results confirm that the 85% partial hepatectomy is the most severe animal model of surgical resection of the liver. Only a single rat in the control group survived at the end of the experiment at five days, with two rats surviving in the T3 group (Table 3.1 and fig 3.3).
<table>
<thead>
<tr>
<th>T3 group</th>
<th>1-24hrs</th>
<th>24-48hrs</th>
<th>48-72hrs</th>
<th>72-96hrs</th>
<th>96-120hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 death</td>
<td>5 deaths</td>
<td>3 deaths</td>
<td>1 death</td>
<td>0 deaths</td>
<td>0 deaths</td>
</tr>
<tr>
<td>controls</td>
<td>2 deaths</td>
<td>4 deaths</td>
<td>4 deaths</td>
<td>1 death</td>
<td>0 deaths</td>
</tr>
</tbody>
</table>

Table 3.1 (above) and Figure 3.3 (below) showing the effect of T3 administered ten days prior to an 85% partial hepatectomy on mortality following surgery.
Liver regeneration occurs rapidly after the 70% partial hepatectomy in the rat, restoring the liver mass to its original value after 14 days, resulting in a complete recovery. Incremental increases in resection size are associated with marked increases in mortality rates: a) 80% resection associated with an 80% survival at 5 days, b) 85% resection associated with a 20% survival at 5 days, c) 90% resection associated with a 0% survival at 5 days. The size of the residual lobe is critical to survival for two reasons, with the most obvious one being that a larger lobe is associated with a greater functional hepatocyte mass. The second relates to the fact that after hepatectomy the entire stream of portal blood flow is directed through the small remnant liver, leading to a comparative increase in portal flow per unit liver tissue. The capacity for portal flow is adequately compensated after 70% partial hepatectomy, however decompensation occurs with larger resections. The inadequately sized residual lobe is vulnerable to a flow injury, which activates kupffer cells to release inflammatory cytokines. Serum levels of TNF-α, IL-6 and arginase correlate with the severity of the liver injury and correspond temporally to massive rises in ALT, AST and bilirubin (peak at 24 hours). Furthermore, PGE₂ levels are inversely proportional to levels of TNF-α suggesting a protective role for the prostaglandin.

In this experiment the induction of liver failure in rats after 24 hours and the mortality figures equate closely to those published by Panis et al. We had hypothesised that pre-enhancement of liver mass with T₃ could result in a survival advantage following major hepatic resection. The difference in survival in T₃ and control animals (one rat) was not statistically significant. The following power calculation was used to assess how many rats would be required to achieve statistical significance:

A two sided log-rank test using the survival curve in both groups shows:
Group 1 – control ($\pi_1$) = 0.085 (survival probability at five days)

Group 2 – $T_3$ group ($\pi_2$) = 0.170 (survival probability at five days)

Test level = 0.05.

When $n=169$ then the test has an 80% power of accepting the alternative hypothesis. A sample size of 169 was determined to be too high to continue the experiments for ethical and financial reasons.

The lack of difference between groups is in part more likely to represent an inadequacy of the surgical model. Panis et al demonstrated that survival after >70% partial hepatectomy is dependent on the haemodynamic complications of the surgical procedure, the effects of the flow injury induced by the redistribution of blood flow into the residual liver and the functional hepatocyte mass of the residual lobes. Thus factors more than just functional hepatic mass influence survival after 85% partial hepatectomy.
3.5 Effect of T\textsubscript{3} induced increases in liver mass on the synthetic capacity of the liver using the galactose elimination capacity as a dynamic liver function test.

3.5.i Background

The principle of a dynamic liver function test is to measure in plasma the activity of an enzyme secreted by the liver (eg lecithin-cholesterol acyltransferase – LCAT) or measure the rate of clearance of a substance preferentially metabolised by the liver (Jalan & Hayes 1995). Clearance assays are available for a variety of metabolic processes:

a) Cytochrome P450 metabolites - Caffeine clearance and \textsuperscript{14}C aminopyrine breath test.

b) Excretory function – bromosulphthalein and idiocyamine green clearance.

c) Galactose metabolism – galactose elimination capacity.

The animals would be assigned to either T\textsubscript{3} or vehicle only ten days prior to the assessment of liver function, with this time point ensuring that the T\textsubscript{3} induced increase in liver mass was at a maximum. The galactose elimination capacity was chosen as the method of choice as this was one of the first dynamic liver tests designed, it is easily reproducible and there is a large database available for comparison in the rat.

3.5.ii Aim

To assess if the increase in liver mass associated with T\textsubscript{3} confers an increase in hepatic function using the galactose elimination capacity.

3.5.iii Method

Two groups of rats (n=5) were assigned to either T\textsubscript{3} or control (vehicle only) ten days prior to assessing the galactose elimination capacity (GEC). The rats were anaesthetised, secured prone and the chest swabbed with ethanol. A small incision was
made in the right supraclavicular fossa and the layers of fascia carefully dissected to expose the internal jugular vein. The vein was cannulated with a 22 gauge paediatric cannula and 0.5 mls of 50% galactose administered via the internal jugular vein approach. A 0.5ml venesection was performed every ten minutes between 20 and 50 minutes. A bladder puncture was performed at the end of the experiment to collect urine.

The galactose elimination capacity was calculated as the ratio of the injected amount of galactose (with correction for urinary excretion) and the extrapolated time to zero concentration. All animals were approximately 250g at the start of the experiment to eliminate variations in GEC due to bodyweight. No correction factor for uneven distribution of galactose within body compartments was applied.

Statistical analysis

Results are displayed as the mean ± standard deviation of the sample (μ ± SDn-1). Statistical differences were determined using the two tailed t-test and reported if p<0.05 as compared to controls.

3.5.iii Results

The animals in both groups tolerated the procedures well with no unexpected mortality in the groups. The animals were assigned to either T₃ or vehicle only, ten days prior to the assessment of the galactose elimination capacity, to assess function when the increase in liver mass was maximal. The rate of elimination of galactose from plasma (Fig 3.4) and the calculated galactose elimination capacity was greater in the T₃ group as compared to control rats (9.3 ± 0.6 μmol/min cf 7.9 ± 0.4 μmol/min - p<0.05, Table 3.2).
Fig 3.4 (above) shows the mean galactose concentrations in both groups and gives an indication of the rate of galactose elimination and table 3.2 (below) compares the galactose elimination capacity in both groups. * - p < 0.05 as compared to animals receiving vehicle only ten days prior to assessment.

<table>
<thead>
<tr>
<th></th>
<th>Body mass (g)</th>
<th>Liver mass (g)</th>
<th>Total GEC (micmol/min)</th>
<th>GEC/100g BW (micmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td>224 ± 11</td>
<td>11.3 ± 0.7</td>
<td>7.9 ± 0.4</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td><strong>T3 group</strong></td>
<td>235 ± 12</td>
<td>13.0 ± 0.8</td>
<td>9.3 ± 0.6 *</td>
<td>4.2 ± 0.35 *</td>
</tr>
</tbody>
</table>
3.5.iv Discussion

We used a single dynamic liver function test to examine the difference between a normal liver and a mitogen enhanced liver. The principle of a dynamic liver function test is to measure in plasma the activity of an enzyme/metabolite secreted by the liver (e.g., lecithin-cholesterol acyltransferase -LCAT) or measure the rate of clearance of a substance preferentially metabolised by the liver (Jalan & Hayes 1995). Clearance assays are available for a variety of metabolic processes including cytochrome P450 metabolites, excretory products (bromosulphthalein and idiocyanine green clearance) and galactose metabolism. The recent advances in techniques using MR spectroscopy to dynamically assess liver function have added another dimension to the choices available. The galactose elimination capacity was chosen as the method of choice as this was the first dynamic liver test designed and even though technically demanding, there is a large database available for comparison in the rat.

There have been different tests utilising galactose elimination as an estimate of liver function for more than 60 years. Keidling (1973) defined the conditions for the estimation of the galactose elimination capacity during saturated and unsaturated kinetics in the rat (Keiding 1973). The following points appear to be critical:

1. A low concentration of galactose in serum (unsaturated galactose elimination) follows first order kinetics. Therefore the rate of elimination is dependent on the concentration in serum and independent of hepatic metabolism.

2. A high level of galactose in serum (saturated galactose elimination) follows zero order kinetics. The rate of elimination is dependent on hepatic (90%) and renal (10%) metabolism.
3. A plasma galactose concentration of above 2.5 mmol/l throughout the experiment equates to 85% - 95% of the \( V_{\text{max}} \). Any concentrations above this figure follows saturated kinetics and represents hepatic and renal clearance.

4. The galactose elimination rate is more representative of hepatic metabolism if a correction is made for renal excretion.

Tygstrup followed up this work by showing that a single intravenous injection of galactose was as good as a continuous infusion to determine galactose elimination (Tygstrup 1966). A continuous infusion had previously been used to minimize the influences of variations in volume of distribution on the GEC. The following calculation was developed to determine galactose elimination:

\[
\text{GEC} = \frac{\text{Total amount of galactose injected (\( \mu \text{mol} \))} - \text{Urinary excretion (\( \mu \text{mol} \))}}{\text{Time to zero galactose concentration (mins)}}
\]

Within our experiment the animal weights were kept within 50g of each other to eliminate variations in galactose elimination for different bodyweights. In addition, calculations were made to estimate the GEC per 100g bodyweight to improve the accuracy of results. The plasma galactose concentration in all our experiments was above 2.5 mmol/l (equates to 85% - 95% of the \( V_{\text{max}} \)), which allowed an assumption to be made that the elimination of galactose was independent of the galactose concentration in plasma and represented hepatic metabolism (Keiding 1973). There was a 15-20% increase in \textit{in vivo} galactose metabolism in the T3 group as compared to the control group in both the total GEC and the GEC/100g body weight. In this model of assessing galactose metabolism by the liver, the increase in hepatic mass induced by triiodothyronine confers an increase in hepatic metabolism. The increase in galactose metabolism is likely to represent an increase in functional hepatocyte mass induced by
T₃ rather than changes in galactose metabolism from changes in the basic metabolic rate induced by the thyroid hormones, as we have previously shown that hyperthyroidism persists for only 4 days after the T₃ injection.
Chapter 4

Combining the two distinct pathways of liver growth, direct hyperplasia and compensatory regeneration
4.1.1 Background

In the previous chapters we have demonstrated that tri-iodothyroinine is a primary mitogen for the liver inducing liver cell proliferation through the process of direct hyperplasia, resulting in an increase in liver mass to above normal levels. This type of liver growth is distinct from compensatory regeneration, in which liver cell proliferation appears to be a secondary event in response to injury (chemical/viral) or surgical resection, to restore the liver mass to its original value.

In modern times, the most recognised experimental model of compensatory regeneration is the 70% partial hepatectomy in the rat, introduced by Higgins and Anderson as a simple operation in which two-thirds of the rat liver is removed (Higgins et al. 1931). Cell proliferation is an early response and follows a highly ordered sequence in all the mature cellular populations of the liver, with a peak in DNA synthesis in hepatocytes at 24 hours, cholangiocytes at 48 hours, kupffer cells at 72 hours and sinusoidal endothelial cells at 96 hours (Alison 1986). This well regulated proliferative response is integrated into the histological expansion of the liver lobule to restore the normal liver mass over a period of 7 – 10 days (Kitamura et al 1998).

The study of animal models of acute hepatic failure following hepatic injury can also provide insight into the mechanisms of compensatory regeneration. These models can be broadly classified into two categories, surgical and pharmacological (Rahman & Hodgson 2000). The surgical models applicable to the rat include:

1. A sub-total hepatectomy (>70%) to induce functional impairment.
2. Partial/complete devascularisation of the liver.

A variety of pharmacological hepatotoxins have also been used extensively to develop models of acute hepatic failure. These toxins include D-galactosamine, acetaminophen (paracetamol), carbon tetrachloride, concanavalin A and thioacetamide.
These toxins induce hepatocyte damage which results in liver failure through a variety of distinct mechanisms.

In this chapter we characterise the effects of combining the two distinct types of liver growth – direct hyperplasia and compensatory regeneration, by administering the primary mitogen T₃ with established models of compensatory regeneration. The first model of compensatory regeneration we used was the 70% partial hepatectomy in the rat, which is a well-established non-inflammatory model of liver cell proliferation and secondary hepatic growth. The second model was of thioacetamide induced liver injury in the rat, which is an inflammatory model of compensatory hyperplasia during acute liver failure.
4.2 Effect of the primary mitogen, tri-iodothyroine on the 70% partial hepatectomy in the rat.

4.2.1 Background

We broadly categorised the dual combination of T₃ and partial hepatectomy into two distinct groups that represented different approaches available for hepatic surgery:

a) Staggered approach – The hypothesis in this experiment was that pre-treatment with T₃ could enhance liver mass, resulting in a larger remnant liver segment following surgery. The assessment of the regenerative capacity of the remnant liver would also be required as this would be critical for any potential clinical exploitation. During this protocol T₃ would be administered 10 days prior to partial hepatectomy, when T₃ induced increases in liver mass were maximal. This would be followed by an assessment of the liver mass indices 24 hours after surgery, with an additional assessment of the regenerative capacity of the liver at this time point.

b) Simultaneous administration – The hypothesis in this experiment was that the T₃ administered at the time of partial hepatectomy could enhance the proliferative response of hepatocytes following 70% partial hepatectomy, and thus increase the rate of liver regeneration.

It is clear that both these scenarios could have widespread clinical implications if applicable to man. A larger liver mass or enhanced regeneration following hepatic resection (with a commensurate increase in functional capacity) could make larger liver resections possible and indeed the principal may be applied to living related liver transplantation.
4.2.2 **Aim**

There were two major aims:

1. To characterise the effects of administering T₃ ten days prior to PH on the liver mass indices and the regenerative response of the liver 24 hours after surgery.
2. To characterise the effects of the simultaneous co-administration of T₃ and PH on the regenerative response of the liver at 24 hours and 4 days after surgery.

4.2.3 **Method**

4.2.3.1 **Induction of hepatocyte proliferation**

Tri-iodothyronine was obtained from Sigma (Poole, England), dissolved in 0.01M NaOH and administered to rats routinely at 9am at a dose of 4mg/kg.

4.2.3.2 **Animal protocols**

Male Sprague Dawley rats weighing 200-250g were used. Animal care and procedures were compatible with the Animals (Scientific Procedures) Act.

a) Rats were injected with a single dose of T₃ or vehicle only 10 days prior to a 70% partial hepatectomy (procedure described in chapter 3.3.ii). All animals were sacrificed 24 hours after surgery.

b) Rats were injected with a single dose of T₃ 24 hours prior or at the time of partial hepatectomy and sacrificed 24, or 96 hours after PH. Partial hepatectomy controls received vehicle only at partial hepatectomy and were sacrificed 24 or 96 hours after PH. Two further control groups included rats that received vehicle only, T₃ only and which were sacrificed at 96 hours.

4.2.3.3 **Tissue Harvest**

Animals were sacrificed by exsanguination under isoflurane anaesthesia and both the liver and body mass measured to allow a calculation of the liver/body mass ratio, the most sensitive index of liver mass.
4.2.3.iv Measurement of Hepatocyte Proliferation

Cell proliferation was assessed by 5-bromo-2-deoxyuridine incorporation into nuclei and immunohistochemical recognition (see reference with chapter 2.3.). There were two BrdU labelling methods used:

1) *Flash labelling* – In animals sacrificed at the 24 hour time point, BrdU was administered intraperitoneally at a dose of 50mg/kg one-hour prior to sacrifice to estimate the proportion of cells in DNA synthesis at sacrifice.

2) *Continuous labelling* – In animals sacrificed at 4 days BrdU was injected I.P. at a dose of 50mg/kg every 6 hours between 15 and 72 hours following surgery or injection. The pharmacokinetics of repeated injections of BrdU are such that the compound will remain in the body for up to 24 hours after the final injection (Miyagawa et al 1997). Thus with the S-phase duration being approximately 8 hours this technique ensures that all cells that have gone into DNA synthesis from 15 hours to sacrifice at 4 days will be labelled.

4.2.3.v Immunohistochemistry

For immunohistochemical analysis 4μm - thick sections were used. Incorporation of BrdU into hepatic nuclei was detected using a mouse monoclonal antibody (Dako Ltd, Cambridge) followed by a biotinylated rabbit anti-mouse secondary antibody (Dako Ltd). Visualization was achieved by the indirect peroxidase technique using 3,3' diaminobenzidine (Sigma, UK) as a substrate (see reference with chapter 2.3.).

Quantification Procedures

The BrdU labelling index was determined by counting 4000 hepatocyte nuclei per section from consecutive light microscopy fields. The immunolabelled sections were evaluated blindly to avoid bias with the number of positively stained nuclei being
expressed as a percentage. Specificity of staining was confirmed by examining serial sections.

4.2.3. VI Protein Estimation

A 200-300mg segment of rat liver was weighed, suspended in 5mls of 1% phosphate buffered saline (PBS) and homogenised in 5ml 0.6M perchloric acid. The homogenate was centrifuged at 2200XG for 20 mins and the supernatant removed. The pellet was dissolved in 5mls of 0.3M NaOH/1% SDS. A 50 μl sample was assayed using a micromodification of the Lowry method (see reference with chapter 2.3.).

4.2.3. VII DNA Estimation

DNA was measured using a quantification DNA analysis kit (DNeasy kit [no 69504], Qiagen, UK). A 15-25mg liver portion was weighed, dissolved in solubilisation buffer and loaded onto a DNA column. The DNA content was determined by measuring absorbance at 260nm in a UV spectrophotometer (see reference with chapter 2.3.).

4.2.3. VIII Statistical analysis

Results are displayed as the mean ± standard deviation of the sample (μ ± SDn-1). Statistical differences were determined using the two tailed t-test and reported if p<0.05 as compared to controls.
4.2.4 Results

A single injection of tri-iodothyronine administered 10 days prior to a 70% partial hepatectomy resulted in an increased (remnant) liver mass 24 hours after surgery as compared to partial hepatectomy controls (Fig 4.1). There was also an increase in the liver/body mass ratio in animals pre-treated with T3 (Fig 4.2).

Effect of T3 administered 10 days prior to PH on residual liver mass (Fig 4.1 - above) and liver/body mass ratio (Fig 4.2--below) when animals were sacrificed 24 hours after surgery - *p<0.05 compared to vehicle 10 days pre-PH.
As expected, the increase in liver mass in rats treated with T3 was matched by corresponding increases in total DNA (Fig 4.3) and total protein levels (Fig 4.4) confirming the increase in liver mass was cellular.

Effect of T3 administered 10 days prior to 70% PH on total DNA (Fig 4.3- above) and total protein levels (Fig 4.4- below) when animals were sacrificed 24 hours after surgery - * p<0.05 compared to vehicle given 10 days prior to 70% PH.
However, the regenerative response to surgery (both in terms of the proportion of proliferating cells and the distribution of the proliferative response) was the same in both groups at this 24 hour time point. The flash BrdU labelling index in rats pre-treated with T₃ being 24.6 ± 4.5% cf. 26.0 ± 3.8% in the partial hepatectomy controls. The distribution of labelled cells was also similar in both groups being predominantly confined to the periportal regions of the hepatic lobule. These results confirm that pre-treatment with T₃ could enhance liver mass following hepatic resection while retaining the regenerative capacity of the liver.

The aim of the next set of experiments was to determine if the simultaneous co-administration of T₃ with partial hepatectomy could enhance the regenerative response of the liver following surgery.

*Effect of co-administration of T₃ and partial hepatectomy on hepatocyte proliferation.*

*Flash labelling assessment* - We quantified the flash BrdU labelling index 24 hours after surgery *in vivo* in adult rat liver comparing 70% partial hepatectomy alone, T₃ administered 24 hours prior to 70% partial hepatectomy and T₃ administered at 70% partial hepatectomy. The flash labelling index 24 hours after partial hepatectomy alone showed 26.5 ± 2.8% hepatocytes were in S phase (Fig 4.5), confirming the major effect of 70% partial hepatectomy on hepatocyte proliferation. When T₃ was administered 24 hours prior to partial hepatectomy, the flash labelling index 24 hours after hepatectomy was 34.2 ± 4.1% (Fig 4.5) representing a combination of the 24 hour effects of partial hepatectomy and the residual effects 48 hours after T₃. When T₃ was administered at the time of partial hepatectomy the flash labelling index reached a maximum at 39.5 ± 5.0% (Fig 4.5) representing the combined 24 hour effects of partial hepatectomy and T₃. Intriguingly, when partial hepatectomy was combined with T₃ (either 24 hours prior to or at the time of PH) and flash labelling performed 24 hours after surgery, the two
stimuli appear to be synergistic. Thus the flash labelling index 24 hours after surgery was greater than the sum of the effects of 70% partial hepatectomy alone (26.5%) and T₃ alone (7%).

Fig 4.5 The flash BrdU labelling index 24 hours after surgery in vivo in adult rat liver comparing 70% partial hepatectomy alone, T₃ administered 24 hours prior to 70% partial hepatectomy (combination of the 24 hour effects of PH and the residual effects 48 hours after T₃) and T₃ administered at 70% partial hepatectomy (combination of the 24 hour effects of PH and T₃).

* p<0.05 compared to PH  # p<0.01 compared to PH  (n=9)

Continuous labelling assessment - To further investigate the effects of T₃ on 70% partial hepatectomy, continuous labelling experiments were performed to determine whether a greater proportion of cells went through S-phase with the combined stimulation. In the intact liver the continuous BrdU labelling index following vehicle alone was less than 1% reflecting the quiescent nature of the hepatocyte (Fig
The continuous labelling index following T₃ alone was 31.0 ± 3.2% (Fig 4.6), confirming that a significant proportion of cells had undergone a semi-synchronous proliferative response over the four-day period. The continuous labelling index following partial hepatectomy alone was 71.0 ± 4% (Fig 4.6). When the two stimuli were combined, the index was 78.0 ± 5.2% when T₃ was administered 24 hours prior to partial hepatectomy (Fig 4.6 - p<0.05 cf PH alone), and 84.0 ± 4.0% when T₃ was administered at the time of the surgery (Fig 4.6 - p<0.01 cf partial hepatectomy). Thus, combining T₃ with 70% partial hepatectomy increased the number of cells undergoing DNA synthesis above PH alone, though not in a simple summative manner.

**Fig 4.6 - The continuous BrdU labelling index in vivo in adult rat liver comparing vehicle only, T₃ alone, 70% partial hepatectomy, T₃ given 24 hours prior to PH and T₃ given at PH. * p<0.001 as compared to vehicle alone (n=6). # p<0.05 as compared to 70% partial hepatectomy (n=9). ~ p<0.05 as compared to T₃ administered 24 hours prior to partial hepatectomy (n=9).**
The synergistic effects of T₃ and partial hepatectomy on cells in S phase at 24 hours (flash labelling), as compared to the enhancement of effect at 4 days (continuous labelling) prompted us to analyse the histological distribution of cells entering S-phase.

**Distribution of BrdU labelling**

**Flash labelling**

The cells proliferating at 24 hours after T₃ alone were predominantly in the midzonal region of the hepatic lobule (Fig 4.7 – see reference with chapter 2.4.i), while those after partial hepatectomy alone were predominantly in the periportal region of the liver (Fig 4.8). When T₃ was administered at 70% partial hepatectomy the labelled cells occupy both the midzonal and periportal regions of the liver (Fig 4.9). Thus within the first 24 hours of T₃ administration or partial hepatectomy, cells in different parts of the hepatic lobule (midzonal - T₃, periportal - PH) are recruited into the cell cycle to induce proliferation.

**Continuous labelling**

The continuous labelling pattern over 4 days after T₃ again showed labelled cells predominantly in the midzonal region of the hepatic lobule (Fig 4.10). The distribution with partial hepatectomy alone showed labelling of cells in both the periportal and midzonal regions of the liver (Fig 4.11). The pattern when T₃ was administered at partial hepatectomy showed labelling in both the periportal and midzonal regions of the liver, however the labelling was more complete in the midzonal region than with hepatectomy alone (Fig 4.12). In all cases, the pericentral population of hepatocytes had less intense BrdU staining when compared to the other regions of the liver.
Fig 4.7 - $T_3$ alone: this flash labelled section demonstrates midzonal staining.

Fig 4.8 - PH alone: this flash labelled section demonstrates periportal staining.

Fig 4.9 - $T_3$ administered at PH: this section demonstrates a combination of periportal and midzonal staining.
Fig 4.10 - T3 alone: this continuous labelled section shows the midzonal staining and the twin daughter cell (tw) appearance of true hyperplasia.

Fig 4.11 - PH alone: continuous labelling showing midzonal and periportal staining.

Fig 4.12 - T3 administered at the time of 70% partial hepatectomy: this continuous labelled section shows a combination of midzonal (more complete than in (E)) and periportal staining.
Additionally confirmation that the BrdU incorporation was a direct result of DNA synthesis within the context of cell proliferation, rather than DNA synthesis as part of a DNA repair mechanism within the hepatocyte was attained through the continuous labelling experiments with the frequent demonstration of labelled twin daughter cells (Fig 4.10). These were BrdU labelled cells occurring in pairs, separated by a visible plasma membrane.

*Effect of T3 and partial hepatectomy on hepatic mass at 24 hours and 4 days.*

We investigated whether the increase in the proliferative response with co-stimulation resulted in an increase in liver mass, and total DNA and protein levels. There was no significant difference in liver mass, total DNA or liver protein levels between the T3 at partial hepatectomy group and partial hepatectomy alone group when animals were sacrificed 24 hours after surgery. However, there was a significant difference between the groups 4 days after surgery, where the liver mass was increased in the group receiving T3 at partial hepatectomy as compared to the partial hepatectomy alone group (p<0.05 cf PH alone – Fig 4.13 and Fig 4.14). There were corresponding increases in total DNA (p<0.05 cf PH alone - Fig 4.15) and total protein levels (p<0.05 cf PH alone–Fig 4.16) confirming the increase in liver mass was cellular and not a result of intra/extra cellular fluid retention in the liver.
The effect of T3 administration one day prior to and at the time of 70% partial hepatectomy, on liver mass, liver/body mass ratio, total DNA and total protein 4 days after surgery.

Fig 4.13 and Fig 4.14 - T3 administration at the time of 70% partial hepatectomy resulted in a larger liver mass and liver/body mass index at 4 days when compared to PH alone. Fig 4.15 and Fig 4.16 - T3 administration at the time of 70% partial hepatectomy resulted in a corresponding increase in total DNA and protein levels at 4 days when compared to partial hepatectomy alone.

*p<0.05 as compared to 70% partial hepatectomy alone (n=9).
4.2.5 Discussion

It was not surprising that the post-operative liver segment twenty-four hours after partial hepatectomy was greater in rats receiving T$_3$ ten days prior to PH than in hepatectomy controls. We have previously demonstrated that tri-iodothyronine increases liver mass, therefore a proportionately larger liver segment 24 hours after 70% partial hepatectomy would be expected in rats pre-treated with T$_3$. It is intriguing that the regenerative response to surgery (both in terms of the total number of proliferating cells and the distribution of the proliferative response -periportal) was the same in both groups at this 24-hour time point. There is other evidence to suggest that hepatocytes can be stimulated to proliferate beyond physiological thresholds when adequately conditioned. In our experiments it has allowed mitogen enhanced liver mass to achieve regenerative competence following surgery. In another setting, the administration of the alkaloid, retrorsine inhibits hepatocyte proliferation in vivo in mice. If this is followed by hepatocyte transplantation with repeated administration of T$_3$ it induces complete replacement of endogenous hepatocytes with those transplanted (Oren et al 1999). The FAH knockout mouse model of tyrosinaemia that is described in the introduction is probably the most impressive model of the proliferative capacity of hepatocytes. Syngeneic transplantation of healthy hepatocytes into FAH knockout mice results in the damaged livers being repopulated by normal hepatocytes through a survival advantage. The process of serial transplantation was performed eight times with equal success, without a decrease in replicative capacity. This set of serial transplantations involved a minimum of 69 doublings, an astonishing proliferative capacity that resembles haemopoietic stem cells (Overturf et al 1997). One can speculate that even though the livers of rats receiving T$_3$ had been pre-conditioned to
enhance liver mass, the overall stimulus following partial hepatectomy was so intense that regenerative competence was restored.

The second part of this work shows that the simultaneous co-administration of tri-iodothyronine with 70% partial hepatectomy increases the proliferative response of hepatocytes following surgery, and thus enhances regeneration during one of the strongest known stimuli to hepatocyte proliferation (Alison 1986). The cells proliferating 24 hours after partial hepatectomy were predominantly in the periportal area of the liver (Michalopoulos et al 1997), while those proliferating 24 hours after T3 were predominantly in the midzonal area. The histological pattern at 24 hours with combined stimulation shows a combination of both periportal and midzonal staining. However, recruitment of cells from different regions of the hepatic lobule by each stimuli appears to be only a partial explanation for the flash labelling result, as the effect of combined stimulation is greater than the sum of the individual stimuli at 24 hours. This is likely to represent the recruitment of a population of cells at this time point within the midzonal or periportal regions of the liver (the predominant areas in which labelling is occurring), that are not recruited by each of the individual stimuli.

This explanation appears plausible as each stimulus initiates DNA synthesis in hepatocytes via different pathways that ultimately converge. The initial event in compensatory regeneration appears to be an increase in TNF-alpha levels (Diehl 2000) leading to several sequelae including:

- the priming of liver cells through the activation of the TNFalpha-1 receptor to generate NF-kappa B (Cressman et al 1994) followed by increases in IL 6 levels (Streetz et al. 2000) to activate the transcription factor STAT 3.
- activation of immediate early genes such as: c-fos, c-jun, c-myc (AT-1 complex) (Haber et al 1993).
• The release and activation of Hepatocyte Growth Factor (and other factors) in serum, leading to the occupation of their corresponding receptors (tyrosine kinases) on liver cells (Matsumoto et al. 1992).

• A combination of growth factor stimulation (Burr et al. 1998) and a delayed gene expression results in cyclin D1 expression within liver cells (Fausto 2000), which allows the hepatocyte to pass beyond the G1 restriction point, initiate DNA synthesis and become irreversibly committed to cell division.

In contrast, the mitogenic effects of T3 on the liver are independent of activation of the transcription factors NF-kappa B and STAT 3 (Pibiri et al. 2001). Pibiri et al identified an earlier onset of expression of cyclin D1 when compared to partial hepatectomy suggesting that there were different mechanisms regulating T3 induced proliferation to those seen after partial hepatectomy (Pibiri et al 2001). Thus the synergistic effect at 24 hours is likely to represent the activation of different pathways within the same cell. We hypothesise this simultaneous activation increase the chances for individual cells to have made the critical G1 to S transition through the cell cycle and initiate DNA synthesis at the 24h time point. This is supported by our previous observations that exogenous administration of T3 and HGF (Forbes et al 2000), a major component of the compensatory hyperplasia pathway after partial hepatectomy, are synergistic to hepatocyte proliferation in vivo.

After 4 days, the continuous labelling data showed that proliferation after T3 administration remained confined to the midzonal region, whilst after partial hepatectomy cells were recruited from both the periportal and midzonal areas. With the addition of T3 at partial hepatectomy, there was an enhancement of the number of midzonal cells recruited when compared to hepatectomy alone. The enhancement is less striking than that seen in the flash labelling experiments at 24h, without the
synergy noted in those experiments but simple enhancement. We suggest that this is because there is an overlap of the two stimuli for many cells in the midzone. It is noteworthy that cells in the pericentral area of the lobule remain predominantly unrecruited into DNA synthesis at 4 days, even by the combination of the two stimuli. This centrilobular population of hepatocytes receives the lowest concentrations of oxygen, nutrients, hormones and growth factors from the circulation, which may in part explain their lack of recruitment into the cell cycle.

The increase in the proliferative response with co-stimulation resulted in a larger postoperative liver mass at 4 days in rats receiving T3 at partial hepatectomy as compared to partial hepatectomy alone. The differences in liver mass were matched with corresponding increases in both total DNA and total protein confirming that the increase in liver mass was cellular. The frequent appearance of twin labelled daughter cells histologically indicated that the BrdU incorporation was a direct result of DNA synthesis within the context of cell proliferation, confirming cell division had occurred within the previous 4 days and that the increase in liver mass included true hyperplasia. However as T3 is known to induce hypertrophic changes within the hepatocyte as well (Sola et al 1991), hypertrophy may also be contributing to this mass effect.

These experiments could have widespread clinical implications and be therapeutically valuable if applicable to man. The first set of experiments offers the potential to increase liver mass following liver resection and enhance hepatic reserve. The second set of experiments offers the ability to enhance proliferation during compensatory hyperplasia, enhancing liver regeneration. In transplant surgery, small-for-patient liver grafts could be encouraged to grow more rapidly to the appropriate mass and thereby prevent the onset of portal hypertension in the graft (Ranson et al.
1972), and indeed the principle may be applied prior to donation in living related transplantation.
4.3 Effect of the primary mitogen, tri-iodothyronine on a model of thioacetamide induced liver injury.

4.3.1 Background

The above experiments have characterised the effects of T₃ on a non-inflammatory model of compensatory regeneration, namely the 70% partial hepatectomy in the rat. The other mode by which liver cell proliferation can occur within the context of compensatory regeneration is following a necrogenic liver injury. A variety of viruses, chemicals and other agents can induce liver injury resulting in widespread hepatocyte loss and a reduction in hepatic mass/functional hepatic mass. This inflammatory stimulus to compensatory growth induces proliferation in hepatocytes and other cell types in an attempt to restore normal liver mass and function (Rolando et al. 2000). It is however clear that in relation to the syndrome of fulminant hepatic failure in man, a substantial proportion of people are unable to homeostatically compensate for widespread hepatocyte necrosis and their regenerative response is inadequate (Rahman & Hodgson 2001). The ability to enhance compensatory regeneration with a primary mitogen following necrogenic liver injury offers a potential therapeutic tool to enhance the regenerative response and prevent decompensation in some cases, providing of course the hepatotoxic environment allows the cells to divide and survive. In developing experiments to assess if T₃ could be applicable in this setting of liver injury, there are two major requirements:

1. To utilise a model of acute hepatic failure in rats.
2. To integrate the primary mitogen T₃ into this model and characterise its effects on the regenerative response of the liver.

In developing a model of acute hepatic failure in rats there were a variety of pharmaceutical agents available to us. D-galactosamine is an amino acid sugar...
metabolised in hepatocytes, resulting in the depletion of uridine nucleotides and causing hepatic transcriptional blockade. The resulting reduction in protein synthesis sensitises hepatocytes to endotoxin mediated liver damage that results in varying degrees of hepatic failure (Takahashi et al. 1990). Concanavalin A is a plant lectin that binds avidly to mannopyranosyl residues present on the surface of membrane glycoproteins. The binding of this lectin to the hepatocyte membrane is thought to initiate a T lymphocyte response with the activation of numerous cytokines including TNF α and interferon γ (Leist & Wendel 1996). Concanavalin A and D-galactosamine are considered to be models of T-cell activated hepatitis, which are relevant to autoimmune liver disease and chronic active hepatitis. The fact that D-galactosamine is hepatotoxic \textit{in vitro} makes it especially attractive to model these diseases in cell culture.

Acetaminophen is the most common cause of acute liver failure in humans in the United Kingdom (Bernal et al. 1998). Its metabolism occurs in the liver and in normal circumstances the drug undergoes biotransformation by a combination of glucuronidation and sulphation. In excess these pathways are saturated and the drug is metabolised by the cytochrome P450 enzyme system to a toxic metabolite N-acetyl-p-benzoquinonamine, which results in severe hepatocyte necrosis and liver failure (Mohandas et al. 1981). There is a marked variation in species and age related susceptibility with acetaminophen, making it a difficult agent to use in animal models (Hirayama et al. 1983).

The hepatotoxic effects of carbon tetrachloride have been known for some time. The mechanism of action involves the cytochrome P450 enzyme system, which produces an active metabolite that results in the production of free radicals and widespread necrotic and apoptotic liver cell death (Mukai et al. 2002). Again however,
carbon tetrachloride shows widespread species variation and poor reproducibility within species.

Thioacetamide has been shown to cause hepatic failure in rats using protocols of varying dose and timing. Thioacetamide is metabolised to an active metabolite by the cytochrome P450 enzyme system. The accumulation of the toxic metabolite results in free radical formation leading to lipid peroxidation and hepatocyte necrosis.

We have chosen to exploit thioacetamide induced liver injury as an animal model of acute liver failure in rats. This model has been extensively characterised in the laboratory by Dr Tony Rahman. It is easily reproducible and has similar histological characteristics to paracetamol induced liver injury in humans. A dose range between 400-600mg/kg bodyweight has been shown to induce widespread centrilobular necrosis and acute liver failure (Chu et al. 2001).
4.3.2 **Aim**

The hypothesis was that the administration of the primary mitogen T3 to rats with thioacetamide induced liver failure would enhance cell proliferation in the liver.

4.3.3 **Methods**

4.3.3.1 **Animal protocols**

Male Sprague Dawley rats (250-300g) were housed in a temperature and light controlled room (12-hour light/dark cycle) with free access to food and water. Animal care and all procedures were compatible with the Animals (scientific procedures) Act 1986, UK Home Office.

The following protocols were used:

*Protocol 1*

Rats (n=50) were injected with two intra-peritoneal injections of thioacetamide at a dose of 500mg/kg body weight (dissolved in normal saline) separated by 8 hours. The clinical, biochemical and histological pattern of liver failure was documented over 96 hours.

*Protocol 2*

Rats (n=7) were injected with two intra-peritoneal injections of thioacetamide at a dose of 500mg/kg body weight (dissolved in normal saline) separated by 8 hours. The animals received T3/vehicle 12 hours after the second thioacetamide injection. The animals were sacrificed 24 hours after the T3/vehicle with flash BrdU labelling performed one hour prior to sacrifice.

*Protocol 3*

Rats (n=12) were injected with two IP injections of thioacetamide at a dose of 500mg/kg body weight (dissolved in normal saline) separated by 8 hours. The animals
received \( T_3 \)/vehicle 12 hours after the second thioacetamide injection. The clinical, biochemical and histological pattern of liver failure was documented over 96 hours.

### 4.3.3.ii Clinical parameters

**Encephalopathy Scoring** - Encephalopathy was assessed twice daily using an adaptation of the system of Zimmerman (Zimmermann et al. 1989), assessing, flexion, grasping, righting, corneal, pupillary and auditory reflexes, and also examining placing, equilibrium tests, toe spreading, and head shaking. The cumulative score from 10 tests reflects the encephalopathy grade (Grade I-IV) of the animal as demonstrated in table 4.1:

<table>
<thead>
<tr>
<th>Stage of HE</th>
<th>Prominent neurobehavioral change</th>
<th>Total Reflex Score (Maximum score=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Lethargy</td>
<td>20-25</td>
</tr>
<tr>
<td>II</td>
<td>Lethargy and Ataxia</td>
<td>15-20</td>
</tr>
<tr>
<td>III</td>
<td>Gradual loss of reflexes</td>
<td>10-15</td>
</tr>
<tr>
<td>IV</td>
<td>Coma</td>
<td>0-10</td>
</tr>
</tbody>
</table>

*Table 4.1- Hepatic encephalopathy scoring system uses basic reflexes that can be tested in the rats. Each reflex is scored 0-3 and converted to an encephalopathy grade.*

### 4.3.3.iii Biochemical parameters

Blood was taken from rats for serum analysis over the stated time period. Standard laboratory liver tests were performed and a serum ammonia assay performed by the Department of Clinical Biochemistry, Royal Free Hospital.
4.3.3.iv Histological assessment

*Flash labelling* – BrdU was administered intraperitoneally at a dose of 50mg/kg one-hour prior to sacrifice to estimate the proportion of cells in DNA synthesis at sacrifice. Sections of rat liver were fixed in formalin post-mortem and processed in the Department of Histopathology, University College London.

For immunohistochemical analysis 4μm thick sections were used and stained with Haematoxylin and Eosin or for Bromodeoxyuridine (see reference with chapter 2.3.3). Incorporation of BrdU into hepatic nuclei was detected using a mouse monoclonal antibody (Dako Ltd, Cambridge) followed by a biotinylated rabbit anti-mouse secondary antibody (Dako Ltd). Visualization was achieved by the indirect peroxidase technique using 3,3' diaminobenzidine (Sigma, UK) as a substrate (see reference with chapter 2.3.).

Quantification Procedures

The BrdU labelling index was determined by counting 4000 hepatocyte nuclei per section from consecutive light microscopy fields. The immunolabelled sections were evaluated blindly to avoid bias with the number of positively stained nuclei being expressed as a percentage. Specificity of staining was confirmed by examining serial sections.

4.3.3.v Statistical differences

Results displayed as the mean ± standard deviation of the sample (μ ± SD_{n-1}). Statistical differences were determined using the two tailed t-test and reported if p<0.05 as compared to controls.

Power calculations were performed by Professor Senn (department of statistics – UCL) using a two-sided log-rank test to identify the animal numbers required to achieve statistical significance.
4.3.4 Results

We found that two injections of thioacteamide at 500mg per kg body weight given eight hours apart produced a model of acute liver failure, with similar clinical, biochemical and histological manifestations, and a reproducible mortality of 60-70% at 96 hours.

The animals became lethargic and developed grade I encephalopathy within the first 24 hours of the second dose of thioacetamide. The animals then progressed to grade II encephalopathy and by 48 hours, all survivors had developed grade III/IV encephalopathy. Overall the mortality over 96 hours was 60%.

The biochemical profile showed a progressive increase in serum ALT indicating severe hepatocellular necrosis (Fig 4.17). The serum ammonia reflected the clinical course of encephalopathy with a peak at 48 hours corresponding to the time point at which the animals were in grade III-IV encephalopathy (Fig 4.18).
Time course following the two thioacetamide injections showing serum ALT levels (Fig 4.17 – above) and serum ammonia levels (Fig 4.18 – below)
The histological appearance of sections of the liver in animals that survived 96 hours showed the classical centrizonal necrosis, a characteristic feature of thioacetamide induced liver injury (Fig. 4.19).

Fig 4.19 shows the classical centrizonal appearance of thioacetamide induced liver injury.
The next set of experiments introduced T₃ as a primary mitogen into the model of acute liver failure. The overall time course for the experiment is outlined in figure 4.20.

Figure 4.20 – Time course for the T₃/Thioacetamide experiment. Thyroid hormone or vehicle was administered to rats (n=7) 12 hours after the second thioacetamide injection and the animals sacrificed 24 hours later with flash BrdU labelling. NB – the time for sacrifice corresponded to the time point between which the animals were in grade 1/11 and grade 111/1V encephalopathy.
Both groups of animals followed a similar clinical course during the experiment developing encephalopathy appropriately. One rat from each group died prior to sacrifice leaving a total of six rats per group at sacrifice. There were no differences in serum ALT or ammonia between groups at sacrifice. However there was a difference between groups in the cell proliferation index, with a greater rate of cell proliferation in the T₃ group as compared to the vehicle group (table 4.2).

<table>
<thead>
<tr>
<th></th>
<th>Flash labelling</th>
<th>FlashBrdU labelling with centrizonal subtraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle alone</td>
<td>20 ± 2.5%</td>
<td>9 ± 3.5%</td>
</tr>
<tr>
<td>T₃ group</td>
<td>27 ± 3.5% *</td>
<td>15 ± 3% *</td>
</tr>
</tbody>
</table>

Table 4.2 – The cell proliferation index during acute liver failure when T₃ or vehicle had been administered 12 hours after the second TAA injection.

* - p<0.05 compared to vehicle only.

Two indices are included for the BrdU index (flash labelling and flash labelling with centrizonal subtraction) because the BrdU incorporation in the necrotic centrizonal regions is also due to white cell proliferation with a more speckled appearance (figure 4.21 and figure 4.22). Thus in this situation, the true index of hepatocyte proliferation is the index with centrizonal subtraction.

Histological inspection revealed that the increase in the rate of cell proliferation in the T₃ group was a result of increased BrdU labelling in the midzonal region of the hepatic lobule (figure 4.21 and figure 4.22).
Histological inspection of the rat liver at sacrifice showed an increase in the rate of cell proliferation in the T3 group was a result of increased BrdU labelling in the midzonal region of the hepatic lobule (figure 4.21 – above) as compared to controls where the labelling is predominantly confined to the periportal region (figure 4.22 - below).
Finally, we wanted to assess if the increase in hepatocyte proliferation in the T$_3$ group could enhance regeneration to show a survival difference between the groups. A pilot study was performed simulating the previous experiment however instead of the animals being sacrificed at the 36 hours time point they were monitored over 96 hours to look for differences in survival. The experiment showed no difference in survival as outlined in figure 4.23.

![Survival Curve](image)

*Figure 4.23 shows the survival curve comparing rats that received T$_3$ and those that received vehicle only.*
4.3.5 Discussion

Terblanche and Hickman suggested the following criteria are critical for animal models of acute hepatic failure (Terblanche & Hickman 1991):

1. Reversibility – The ability of the liver failure to resolve or respond to treatment is essential to the success of the model.

2. Reproducibility – Reproducible endpoints are required to standardize the animal models.

3. Death from liver failure – A key feature of the events seen in liver failure in humans.

4. Therapeutic window – Time should be available between insult and death to investigate potential treatments.

In this study we have characterised the effects of thioacetamide induced liver injury in a large number of rats to produce a model of acute liver failure that fulfils the criteria of reproducibility, reversibility and substantial severity that provides a therapeutic window to allow treatments to be tested.

Two intra-peritoneal injections of thioacetamide were administered at an 8-hour interval. Thioacetamide is oxidized to thioacetamide (di)sulfoxide in the liver, probably by the cytochrome P450 enzyme system (CYP2E1). The (di)sulfoxide compound is a reactive intermediate that induces oxidative stress, which results in a depletion of hepatic glutathione levels and an increase malondialdehyde concentration (markers of oxidative stress). The free radical and oxidative damage to intracellular membranes and organelles causes widespread hepatocyte necrosis. The characteristic lesion with thioacetamide begins centrizonally reflecting the sensitivity of this population of hepatocytes to oxidative injury (Bruck et al. 1998; Chu et al. 2001).
The rats showed a widespread sensitivity to this dose of thioacetamide and we characterised the clinical development of hepatic encephalopathy with an adapted reflex score. The fact that the peak in the serum ammonia corresponded temporally to when the rats were in grade III/IV encephalopathy was reassuring to our clinical assessment and confirmed that the animals had indeed developed liver failure. As expected there was a progressive increase in the serum ALT reflecting severe hepatocellular necrosis but it should be recognised that this is no indication of the severity of acute liver failure or of liver function. There was however a substantial mortality at the end of the experiment (60-70%) confirming that a severe liver injury had occurred. Histological assessment showed a limited centrilobular injury in rats that survived, whereas rats that died from liver failure in the first 96 hours had a more severe centrilobular/pan-zonal necrosis. Thus animals that survived had intact midzonal and periportal hepatocytes and a greater residual functional capacity.

A major advantage of this model was the fact that the rats did not develop encephalopathy for a protracted period of time after the second TAA injection. This allowed us to administer T$_3$ twelve hours after the second thioacetamide injection and characterise its effects on the regenerative process during acute liver failure. Previous studies have administered primary mitogens at the onset of the necrogenic liver injury and have therefore not provided appropriate models for clinical scenarios when there will be a time lapse between liver injury and presentation.

One rat per group died prior to the scheduled sacrifice 36 hours after the second thioacetamide injection as one would expect. The cell proliferation index was greater in the T$_3$ group as compared to controls in both the total count and after a correction to exclude centrilobular BrdU staining. The centrilobular BrdU staining produced false positives as a result of BrdU incorporation from white cell proliferation giving a
characteristic speckled appearance. The increase in the cell proliferation index with T₃ was the result of enhanced staining in the midzonal region of the hepatic lobule, which is an intriguing observation when one considers that T₃ has a predilection for this area of the liver lobule. However, it should be noted that intact midzonal hepaticocytes were required for T₃ to have its effect.

There were no differences in clinical, biochemical or survival outcomes in the T₃ group when compared to controls. One reason why there was no difference in survival may be that the differences in cell proliferation are small, making it difficult to find a difference in a notoriously difficult end point such as mortality. A two-sided log rank test using the survival curves in both groups allowed us to perform a power calculation:

Group 1 – vehicle = 0.33 survival probability at 96 hours
Group 2 – T₃ = 0.4 survival probability at 96 hours

A power calculation with a test level of 0.05 shows that when the sample number was greater than 105 per group (ethically and financially inappropriate) there is 80% power of accepting the alternative hypothesis of a survival difference.

The other reason for not finding a survival difference may be that there is no difference in survival between groups because enhanced cell proliferation does not improve mortality. The reasons for this may be that even though there was a greater rate of cell proliferation in the T₃ group there may not have been an improvement in liver function, ie the new cells were immature non-functioning hepatocytes. The systemic toxicity from the liver failure may itself suppress hepatocyte function making the small increase in cell proliferation insignificant on outcome. I think the use of T₃ in this model appears less attractive than combining it with surgery, however it may potentially have a role as a valuable adjunct with other forms of therapy in the future.
Chapter 5

Identifying a possible mechanism for the mitogenic effects of tri-iodothyronine
5.1 Background

In the previous chapters we have characterised the effects of tri-iodothyronine as a primary mitogen for the liver \textit{in vivo} in adult rats, with particular reference to its applications in hepatic surgery and liver injury. Whether \(T_3\) could be effective in man is unknown – the pharmacological doses used, though survivable, could not be tested clinically. It is therefore important to identify the mechanism of action, as the manipulation of cell signal pathways may allow for a significant dose reduction in the future or alternatively allow different agents to be introduced. In this chapter we try to identify a mechanism by which \(T_3\) may be acting as a primary mitogen for the liver.

In investigating the mode of action of primary mitogens comparison has been made with compensatory hyperplasia. In both direct and compensatory hyperplasia, hepatocytes emerge from the \(G_0\) (resting) stage of the cell cycle, enter \(G_1\), and then \(S\)-phase prior to mitotic division. It is apparent that the cell signal pathways in both direct hyperplasia and compensatory regeneration converge at the time of transition through \(G_1\) (\(G_1\) restriction point), so that both lead for example to cyclin D activation and subsequent events that result in DNA replication and cell division. However, the molecular processes in the early stages of direct hyperplasia appear to differ strikingly from those involved in compensatory growth.

Critical events in compensatory hyperplasia after partial hepatectomy include:

(a) priming resting hepatocytes to proliferate by an early event involving TNF-alpha, the TNF\(\alpha\)-1 receptor (TNFR-1) and activation of NF-kappa B and STAT 3 (Cressman \textit{et al} 1994; Streetz \textit{et al} 2000),

(b) activation of Hepatocyte Growth Factor and TGF-alpha to act on their corresponding tyrosine kinase receptor(s) (Matsumoto \textit{et al} 1992),

(c) eventual activation of cyclin D and initiation of DNA synthesis (Fausto 2000).
The mitogenic effects of T₃ are independent of the activation of the transcription factors NF-kappa B/ STAT 3 and not dependent critically on TNF-alpha/ IL 6 as shown by an unchanged effectiveness in TNFR-1 and IL 6 knockout mice. Pibiri et al identified an earlier onset of expression of cyclin D1 in the liver with T₃ as compared to partial hepatectomy suggesting that there were different mechanisms regulating T₃ induced cell proliferation to those seen after PH (Pibiri et al 2001).

Feng (Feng et al 2000) used a quantitative fluorescent cDNA microarray to identify hepatic genes regulated by thyroid hormone. Fluorescent-labeled cDNA was prepared from the hepatic RNA of T₃-treated (hypothyroid) mice and hypothyroid mice. This was then hybridized to a cDNA microarray, representing 2225 different mouse genes, followed by computer analysis to compare relative changes in gene expression. Fifty five genes, 45 not previously known to be thyroid hormone-responsive genes, were found to be regulated by thyroid hormone. Among them, fourteen were positively regulated by thyroid hormone, and unexpectedly forty-one were negatively regulated. Thyroid hormone affected gene expression for a diverse range of cellular pathways and functions, including gluconeogenesis, lipogenesis, insulin signaling, adenylate cyclase signaling, cell proliferation, and apoptosis (Feng et al. 2000).

The genes regulated by T₃ affecting cell proliferation included kinesin-like protein (KipIp), chromodomain helicase-DNA binding protein (CHD-1) and Bcl-3. The first two proteins are mitotic spindle regulators, which intuitively are unlikely to be initiators of cell proliferation. Bcl-3 belongs to a family of IκB proteins that also include IκBα, IκBβ, IκBγ, p105, and p100. The Bcl-3 protein contains a proline-rich amino terminus, a series of seven tandem ankyrin repeats, and a proline- and serine-rich carboxyl terminus (figure 5.1 and figure 5.2).
Structure of Bcl-3:

Figure 5.1(A) Domain organization of Bcl-3 and IκBα. The basic nuclear localization sequence (NLS) of Bcl-3 is indicated in blue, while the leucine-rich NLS and NES of IκBα are in green.

Figure 5.2(B) Ribbon diagram of the Bcl-3 ankyrin repeat domain. The molecule curves towards the α1 helices, which together with the β-hairpins forms the presumed binding surface for p50 and p52 homodimers.

This figure is courtesy of bbscript and Raster3D.
The binding of Bcl-3 to p50 and p52 NF-κB homodimers in the nucleus activates transcription through the transactivation domain of Bcl-3. This enhances NF-κB activity resulting in the expression of a variety of cellular genes, including those involved in immune and stress responses, apoptosis and cellular proliferation (Dechend et al. 1999). Bcl-3 mutations in humans resulting in an over expression of this IκB protein have resulted in uncontrolled cell proliferation and been implicated in the pathogenesis of B-cell chronic lymphatic leukaemia (McKeithan et al. 1997). We found it intriguing that Bcl-3 up-regulation was shown to induce cell proliferation in lymphocytes (be it in the context of malignant proliferation) and hypothesised that T3 may be inducing its proliferative effects in a similar way.

To support this claim we analysed the Bcl-3 promoter to identify if any of the well-characterised thyroid hormone response elements were present. Thyroid hormone response elements are DNA sequences that bind thyroid hormone receptors and cause nearby genes to respond transcriptionally to thyroid hormone. Thyroid hormone response elements are built from one or more copies of a sequence that matches a consensus motif: 5' AGGTCA 3'. The Bcl-3 gene has been cloned in both mice and humans. The mouse Bcl-3-coding region is 1746 base pair long and exhibits 80% identity with human Bcl-3 at both the nucleotide and amino acid level. The Bcl-3 locus maps to the proximal end of mouse chromosome 7, which is syntenic to human chromosome 19. We analysed the nucleotide sequence 3-5 kilo bases upstream from the coding sequence in mice and humans and identified the promoter from a TATA repeat box. We identified a consensus sequence (AGGTCA) for thyroid hormone upstream from the promoter (fig 5.3).
Fig 5.3 – The promoter region for the Bcl-3 gene was identified by a TATA box repeat (7402364-7402501) 4-5 Kilo bases upstream from the coding sequence. There is a hexameric thyroid hormone consensus motif AGGTCA (7401955-7401960) upstream from the promoter.
However it is important to recognise that Feng et al were investigating the hepatic gene expression in hypothyroid mice treated with a high dose of T₃. In this chapter we investigate the expression of Bcl-3 in our experimental setting, which is when euthyroid mice are given a high dose of T₃.
5.2 **Aim**

To assess the mRNA and protein expression of Bcl-3 in liver in vivo in euthyroid adult rats, following mitogenic stimulation with tri-iodothyroinine.

5.3 **Method**

5.3.1 **Materials**

i) Promega total RNA isolation kit (Cat. No – Z5110) consisting of:
   - Guanidine thiocyanate/phenol mono-phase solution.
   - Chloroform and Isopropanol

ii) RNAse free water

iii) Agarose (Sigma, UK)

iv) MOPS solution (Sigma, UK)

v) 37% formaldehyde (Sigma, UK)

vi) Deionized formamide (Sigma, UK)

vii) 10% SDS

viii) Ethidium Bromide (10 mg/ml stock)

ix) DNA, RNA and protein molecular markers (Sigma, UK)

x) Reverse transcriptase and RT buffers (Invitrogen, UK)

xi) Random hexamers (Invitrogen, UK)

xii) PCR kit (Qiagen, UK) consisting of:
   - PCR buffer
   - Mg(Cl)_{2} – 50 mmol/l
   - dNTP – 10 mmol/l
   - Q solution
   - TAQ DNA polymerase

xiii) DNA gel extraction kit (Qiagen, UK)
xiv) Internal non-competitive standard PCR mix (Oswell, Southampton, UK)

xv) 10% Tris-glycine resolving gel consisting of H₂O, 30% acrylamide, 1.5M tris, 10% SDS, 10% ammonium persulphate and 0.01% TEMED.

xvi) 5% stacking gel consisting of H₂O, 30% acrylamide, 1.0M tris, 10% SDS, 10% ammonium persulphate and 0.01% TEMED

xvii) Running buffer – (100 ml tris/gly/SDS (TGS) reagent and 900ml water)

xviii) Transfer buffer – (50ml TGS reagent, 200 ml methanol and 750 ml water)

xix) Milk blocking agent (10% milk powder dissolved in 0.5% PBS TWEEN)

xx) Rabbit anti-BCL-3 antibody (Santa Cruz antibody, USA)

xxi) HRP labelled goat anti-rabbit antibody (Santa Cruz antibody, USA)

xxii) ECL Western analysis reagent (Santa Cruz antibody, USA)

xxii) SDS gel-loading buffer consisting of 50mM Tris, 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol.

5.3.2 Animal protocols

Male sprague dawley rats weighing 200-250g were used. Animal care and all procedures were compatible with the Animals (Scientific Procedures) Act 1986.

The rats (n=4) were injected with a single dose of tri-iodothyronine and sacrificed at 1 hr, 4 hr, 8 hr, 24 hr and 48 hr. The liver was sectioned after sacrifice, snap frozen in liquid nitrogen and stored at –80 °C until use.

5.3.2 Isolation of RNA

RNA was prepared from liver tissue by a modification of the single step method reported by Chomczynski and Sacchi 1987.

- The samples were thawed on ice and approximately 100mg of tissue was added to 1 ml of a commercial guanidine thiocyanate/phenol mono-phase solution and homogenised 3 times for 10 seconds.
• Samples were allowed to stand at room temperature for 5 minutes to allow dissociation of nucleoprotein complexes and 200µl of chloroform added. The mixture was shaken for 15 seconds and allowed to stand for 15 minutes.

• Samples were then centrifuged for 15 minutes at 4°C and a speed of 12000XG separating the mix into an aqueous phase containing RNA, and interphase containing DNA and an organic phase containing protein.

• The aqueous phase was removed and 500µl of isopropanol added prior to mixing. The sample was allowed to stand for 15 minutes at room temperature and then centrifuged for 10 minutes at 4°C and a speed of 12000XG to precipitate and pellet the RNA.

• The supernatant was removed and the pellet washed by the addition of 1ml of 75% ethanol, mixing and a further 5 minutes of centrifugation at 7500XG.

• Finally the supernatant was removed and the pellet allowed to air-dry before being re-suspended in 30-50µl of RNAse free water.

5.3.3 Determination of RNA yield

The yield of total RNA was determined spectrophotometrically at 260nm, where 1 absorbance unit (A_{260}) equals 40 µg of single stranded RNA/ml. The purity was also estimated by the relative absorbances at 260 and 280 nm (A_{260}/A_{280}). The ratio between the readings at 260 and 280 nm provides an estimate of purity of the nucleic acid, with the ratio varying between 1.8 and 2.0 for pure samples. We were able to obtain a ratio above 1.8 for all samples.

5.3.4 Checking RNA integrity

We checked the integrity of the total RNA by running an aliquot of the RNA sample on a denaturing agarose gel stained with ethidium bromide.
We used the following agarose gel preparation (1%):

- 1.5 g agarose
- 26 ml 37% formaldehyde
- 15 ml 10X MOPS
- 109 ml RNAse free water.

A 3 µl aliquot of ethidium bromide (10mg/ml) was added to the total gel volume of 150ml. The RNA samples were heated to 65°C for 5 minutes in loading buffer and then loaded onto the gel and agarose gel electrophoresis (60 volts) was run for approximately 2 hours. The RNA was examined under UV light to check the integrity of the RNA (fig 5.4). Intact RNA has a sharp 28S and 18S rRNA bands with a 2:1 ratio (28S:18S).

![Image of gel showing RNA bands](image)

Fig 5.4 – A gel showing intact RNA from liver samples stimulated with T₃:

(a) Intact RNA, (b) control liver, (c) 1 hour, (d) 4 hour, (e) 8 hours, (f) 24 hours and (g) 48 hours. The RNA samples were run on a (1%) agarose gel and stained with ethidium bromide. The RNA is intact in all the samples as shown by the sharp 28S (top) and 18S (bottom) bands and the 2:1 ratio of 28S:18S, although it is clear the loading is unequal.
Developing an RT-PCR reaction to assess Bcl-3 gene expression.

The development of the polymerase chain reaction (PCR) technique in the 1980s has allowed rapid advances in the field of molecular biology. Thermostable DNA polymerase is utilised to extend a DNA sequence from synthesised oligonucleotide primer pairs, which are designed to flank an area of interest.

We designed a set of primers to amplify a sequence of DNA from the Bcl-3 coding region using a primer selection site from the UK genome mapping project (http://alces.med.umn.edu/rawprimer.html). Good primer design is essential for an effective PCR reaction and we applied the following rules:

- Cross mapped the Bcl-3 gene in both humans and mice (the rat gene has not been sequenced) to identify areas of complete homology. The rationale for designing primers flanking these areas was that areas of complete homology in the mouse and human gene are likely to coincide with homologous sequences in the rat.
- The Bcl-3 coding sequence is 1741 (human)/1746 (mouse) bases long, from which we designed primers to produce a product of less than 150 base pairs in areas of complete homology. The small PCR product is essential for developing a real-time PCR protocol to determine the levels of mRNA expression within our liver samples.
- We designed primers with a 50% ratio of GC/AT base pairs. However, to reduce the probability of secondary structures forming in the DNA we designed primers that did not have prolonged (consecutive) G/C sequences.

The sequences of the primers we designed and the predicted PCR product from the mouse Bcl-3 database are given in figure 5.5:
forward primer - 5' GTGACAGCTGGTGCCAGC 3'
reverse primer - 5' GTTGCGCTGTCCAGCAGGGC 3'

PCR product (112 bases) -
5'GTGACAGCTGGTGCCAGCCCCATGGCCCTGGATCGTCACGGCCAGACTGC
AATTCACTGGCATGCGAGCACCACCCAGCTGCCTGCAGGCCCTGCT
GGCAGCGCAAC 3'

Figure 5.5 – The forward primer (position 600-618) and reverse primer (position 692-712) sequences for the Bcl-3 gene. The predicted PCR product from the mouse database is 112 bases.

The addition of a reverse transcriptase step prior to the PCR has provided the opportunity to quantify the amount of mRNA within any given sample. Reverse transcriptase is an enzyme utilized by RNA viruses to convert their genomic material (ie RNA) into transcribable DNA within the host cell. If added to RNA isolated from the tissue, along with suitable RT-primers to initiate reverse transcription, it will produce cDNA that can then be used in a subsequent PCR. RT-primers can be either random (which will produce cDNA of all the mRNA in the sample) or sequence specific which produces cDNA only from mRNA containing the sequence of interest.

We incubated a 20μl RT mixture with the RNA template (5μl) from the 4 control liver samples at 37°C for one hour to produce a cDNA profile of the total RNA. The RT mixture (invitrogen, UK – kit no. 10966-026) consisted of:

- RT buffer – 5.0μl
- 0.2μmol/L dNTP – 2.0μl
- Random hexamers – 1.0μl
- MMLV reverse transcriptase – 1.0μl
- RNAse free water – 11μl
We incubated 5μl of the cDNA template from the samples with 20μl of an optimized PCR reaction mixture (Qiagen, UK – kit no. 201203) consisting of:

- X10 PCR buffer – 2.0μl
- 5 mmol/L Mg(Cl)₂ – 3.0μl
- 0.2μmol/L dNTP – 2.0μl
- 0.5 units TAQ DNA polymerase – 0.5μl
- Q solution – 5.0μl
- Bcl-3 forward and reverse primers (25 picomolar/μl) – 1.0μl per primer
- RNAse free water – 5.5μl

A PCR control was also run which consisted of the 20μl PCR reaction mix and 5μl of water. The PCR samples were placed in a thermal cycler and heated to 90°C to separate the double stranded DNA, followed by a cooling to a temperature of 60°C to allow the primers to anneal to a DNA strand. A further raise in temperature to 72°C allowed the extension to take place from the 5’ end of each primer. The reaction was then re-heated to 90°C and the cycle was repeated for 30 cycles.

**Gel electrophoresis**

Once the reaction had completed (1%) agarose gel electrophoresis was undertaken on the PCR products to identify the appropriate band(s) of DNA. The agarose gel preparation consisted of:

- 1.5g of agarose was dissolved in 150 mls X1 TBE Buffer and heated to 90°C.
- Once cooled to 50°C 1 μl of ethidium bromide solution (0.5 μg/ml) was added and the gel poured into a perspex mould with a gel comb in situ and left to set.
- 20 μl of PCR reactant was added to 5 μl of 5X DNA Loading Buffer
- Once set, the agarose gel was placed in an electrophoresis tank with X1 TBE.
• 25 μl of sample (20 μl of PCR reactant + 5 μl of DNA loading buffer) was loaded into each well and gel electrophoresis performed at a constant voltage of 80v for 90 minutes. Once completed the gel was visualised by ultra-violet trans-illumination, digital photography and specialised software.

The PCR product from the control liver samples was 112 bases (as expected for the Bcl-3 gene product), and produced a single DNA band (figure 5.6).

![Figure 5.6](image)

Figure 5.6– (1%) agarose gel electrophoresis of the PCR products using (a) DNA ladder (b) PCR control (c) control liver sample A (d) control liver sample B (e) control liver sample C (f) control liver sample D. A single band of 112 nucleotides (in size) was seen in the control liver samples consistent with the size of the expected PCR product for Bcl-3. This band was not present in the PCR control sample confirming it was not a result of DNA contamination.
5.3.6 Gel extraction and sequencing of the PCR product.

As previously mentioned we obtained a single band of appropriate size (112 bases) for the Bcl-3 gene from the control liver samples. The absence of this band in the PCR control confirmed it was not a result of DNA contamination. This result suggested that the PCR product was indeed the coding region of the Bcl-3 gene we were investigating. However as the rat sequence had not been identified previously, we wanted to gel extract and sequence the PCR product for definitive confirmation.

We used a Qiagen (kit – 28106) protocol for the gel extraction, in which we:

- Excised the DNA fragment from the agarose gel with a scalpel and placed it in an eppendorf tube.
- Added 300µl of lysis buffer to the tube to dissolve the agarose and incubated at 50°C for 10 minutes.
- 300µl of isopropanol was then mixed in with the sample.
- The sample was placed into a DNA column and centrifuged at 1200XG for 1 min.
- 0.5 ml of buffer QG was loaded onto the column and centrifuged at 1200XG for 1 min.
- The DNA was then eluted in 50µl of elution buffer.

The DNA sample was then sent for automated sequencing to Qiagen, Hamburg, Germany to confirm the PCR product was indeed from the Bcl-3 gene (figure 5.7).
Figure 5.7- (A) shows the chromatogram for the PCR product followed by the base sequence. The automated sequencing program was able to read 81 bases of the total PCR product sent to Hamburg.

(B) - shows the predicted sequence of the PCR product from the mouse Bcl-3 gene (previously shown in figure 5.3.5.A).

The automated sequence begins at position 34 (red) of the predicted PCR product. A BLAST search using the Genebank database shows >95% homology with the mouse Bcl-3 gene. This confirms the PCR product is a coding sequence for the Bcl-3 gene in rats.
5.3.7 Developing a real time PCR protocol to quantify the expression of Bcl-3 mRNA in the liver.

In the previous two sections we have developed an RT-PCR protocol to amplify up a coding region of the Bcl-3 gene in rats. Thus, in this protocol during reverse transcription, random primers convert all the m-RNA in the total RNA (from the liver tissue) to complementary DNA. The PCR reaction then amplifies the specific Bcl-3 cDNA into an exponential number of copies from synthesised oligonucleotide primer pairs. The next logical step was to integrate this protocol into a reproducible method of quantifying the amount of Bcl-3 m-RNA expressed in our liver samples.

The application of fluorescence techniques to the RT-PCR, together with suitable instrumentation capable of combining amplification, detection and quantification, has led to the development of kinetic RT-PCR methodologies that are revolutionizing the quantification of messenger RNA.

We used a quantitative RT-PCR protocol using biogene (Australia) real time PCR instrumentation. We used the following protocol:

1. We incubated 20μl of the RT mixture (invitrogen, UK – kit no. 10966-026 – as per section 5.3.5) with 5μl of the RNA template (this volume was corrected at the end according to the amount of 18S RNA to ensure an equal amount of total RNA was being loaded) from each of our liver samples at 37°C for one hour to produce a cDNA profile of the total RNA.

2. We incubated 5μl of the cDNA template from the liver samples/standard Bcl-3 DNA with 20μl of a PCR reaction mixture (Qiagen, UK – kit no. 201203) consisting of:
   - X10 PCR buffer – 2.0μl
   - 5 mmol/L Mg(Cl)$_2$ – 3.0μl
   - 0.2μmol/L dNTP – 2.0μl
0.5 units TAQ DNA polymerase – 0.5μl
Q solution – 5.0μl
Bcl-3 forward and reverse primers (25 picomolar/μl) – 1.0μl per primer
SYBR green (1:3000) - 1μl
RNAse free water – 4.5μl

The contents of this PCR mix is similar to the optimized PCR mix in section 5.3.5, apart from the addition of a fluorescent DNA binding dye (SYBR green).

3. We also incubated 5μl of the cDNA template from the liver samples/standard 18S with an internal non-competitive standard PCR mix consisting of:

- X10 PCR buffer – 2.0μl
- 5 mmol/L Mg(Cl)\(_2\) – 3.0μl
- 0.2μmol/L dNTP – 2.0μl
- 0.5 units TAQ DNA polymerase – 0.5μl
- Q solution – 5.0μl
- 18S standard forward and reverse primers (25 picomolar/μl) – 1.0μl each
- SYBR green (1:3000) - 1μl
- RNAse free water – 4.5μl

This non-competitive mix contained 18S standard primers for rats provided by Oswell laboratory, Southampton, UK. These internal standards acted as a loading control, allowing us to standardize the amount of RNA being loaded into each PCR reaction.

4. The PCR samples were placed into the biogene real time PCR machine with the appropriate PCR control samples for both the Bcl-3 and 18S reactions. The light cycler uses small volume capillary tubes, contained within a rotor-like carousel, which is heated and cooled in an air stream. We used the same thermogenic PCR conditions used for the Bcl-3 RT-PCR protocol, with a denaturing temperature of 90\(^\circ\)C to separate
the double stranded DNA, followed by a cooling temperature of $60^\circ\text{C}$ to allow the
primers to anneal to a DNA strand and a further raise in temperature to $72^\circ\text{C}$ to allow
the extension of DNA to occur. The SYBR green protocol detects the binding of a
fluorescent dye to DNA. The unbound dye exhibits little fluorescence in solution, but
during the extension phase increasing amounts of DNA bind to the nascent double
stranded DNA molecules. When monitored in real-time, this results in an increase in
the fluorescence signal during the polymerisation step. Consequently, fluorescence
measurements at the end of the elongation step of every PCR cycle are performed to
monitor the amount of amplified DNA.

The concept of the threshold cycle (Ct) is at the heart of accurate and
reproducible quantification using fluorescence-based RT-PCR. Fluorescence values are
recorded during every cycle and represent the amount of product amplified to that point
in the amplification reaction. The more template present at the beginning of the
reaction, the fewer number of cycles are required to reach a point in which the
fluorescent signal is first recorded above background. This point is defined as the Ct
and will always occur during the exponential phase of amplification. The computer
software then generates a standard curve from the appropriate standard Ct values and
gives a reading for all the samples within a large dynamic range. We initially set out to
verify the accuracy of real time PCR in measuring Bcl-3 DNA levels. We ran duplicate
samples on serial dilutions of standard Bcl-3 DNA to establish the reproducibility
(figure 5.8) and accuracy (figure 5.9) of the assay. The assay for the standard 18s
internal controls had previously been verified by Oswell, Southampton, UK. We tested
standard concentrations of 18S RNA in a number of PCR reactions provided by the
manufacturer to verify the accuracy of the 18S internal controls prior to testing with our
liver samples.
Figure 5.8 shows the fluorescence profile for standard concentrations of Bcl-3 DNA in duplicate using real time PCR. The fluorescence profile follows the classical sigmoidal shape, with each duplicate in close proximity (reproducibility) and a serial drop in the threshold Ct value representing each dilution ($\log_{10}$):

- a - Bcl-3 DNA = $5 \times 10^8$ copies/mcl
- b - Bcl-3 DNA = $5 \times 10^7$ copies/mcl
- c - Bcl-3 DNA = $5 \times 10^6$ copies/mcl
- d - Bcl-3 DNA = $5 \times 10^5$ copies/mcl
- e - Bcl-3 DNA = $5 \times 10^3$ copies/mcl
- f - PCR control = water
To establish how accurate this real time PCR protocol was in quantifying Bcl-3 DNA, we used the biogene software to generate a standard curve for serial dilutions.

Figure 5.9 shows the standard curve that was generated for the serial dilutions of Bcl-3 DNA. A regression coefficient >0.99 confirms the real time PCR protocol is accurate at quantifying levels of Bcl-3 DNA.
5.3.8 Developing a western analysis protocol to quantify the expression of the Bcl-3 protein in the liver.

The preparation of protein lysates from liver tissue for analytical electrophoresis of proteins has been facilitated by the use of commercial lysate buffers containing protease inhibitors (Sigma, UK). The tissue was homogenised in 0.5ml of lysis buffer and centrifuged at 1200XG for 10 mins. The supernatant containing the protein lysate is then ready for SDS-PAGE electrophoresis followed by western blotting to determine the presence of the protein, which in this case is the 63KDalton protein Bcl-3.

*SDS-polyacrylamide gel electrophoresis*

Almost all analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits with minimal aggregation. The following protocol was used:

- We assembled the glass plates to make a gel mould according to the manufacturers instruction (Biorad, UK) and poured the constituents of the 10% tris-glycine resolving gel into the mould. A gap was left at the top into which we poured the 5% stacking gel and inserted a gel comb. The gel was left vertically to set.
- A protein estimation on the lysate samples was performed using a micro-modification of the lowry method (see reference chapter 2.3.vii) to allow us to aliquot 300mcg of total protein per sample.
- Each sample was heated at 100°C for 3 minutes in SDS gel-loading buffer.
- The gel was mounted into the electrophoresis apparatus and the running buffer added. Each sample was added in a predetermined order with the molecular weight marker added first.
- The electrophoresis apparatus was connected to the electrical power supply and a constant voltage of 80v applied.
After the samples have run to the bottom of the gel the apparatus is dissembled and the glass plates pulled apart carefully. The gel is then ready for a transfer of the polypeptides in the gel to a nitrocellulose filter by western blotting.

Western blotting

We used a nitrocellulose filter containing polyvinylidene – difluoride (Hybond P-Amersham, UK) for western blotting using the following protocol:

- We cut two pieces of Whatman 3mm paper and one nitrocellulose filter (transfer membrane) and soaked them in transfer buffer for five minutes.
- We then set up the western blotting tray as shown in figure 5.10:

![Western Blotting Tray Diagram](image)

*Figure 5.10 shows the configuration of the western blotting tray*

- The western blotting tray containing transfer buffer was then connected to an electricity supply.
- A constant voltage of 80v was applied across the tray for two hours to facilitate the transfer of the polypeptides from the gel onto the membrane.
Staining the nitrocellulose filter

We used an HRP immunological detection system to stain the nitrocellulose filter for the Bcl-3 polypeptide using the following protocol:

- We incubated the nitrocellulose filter in a blocking solution containing 10% non-fat dried milk and 0.5% TWEEN in X1 PBS for 12 hours at 4°C.
- The filter was then incubated with the primary antibody (rabbit anti-rat Bcl-3 antibody - 1:200 in milk blocking solution) for 2 hours.
- The filter was washed (X3) for 20 mins in 0.5% TWEEN in X1 PBS.
- The nitrocellulose filter was then incubated with the secondary antibody (HRP labelled goat anti-rabbit antibody - 1:5000 dissolved in X1 PBS for 1 hours.
- The filter was washed (X3) for 20 mins in 0.5% TWEEN in X1 PBS.
- The nitrocellulose filter was then incubated with ECL substrate for 1 minute and exposed onto a photographic film for the result.

5.3.9 Statistical analysis

Results are displayed as the mean ± standard deviation of sample (μ ± SDn-1).

Statistical differences were determined using the two tailed t-test and reported if p<0.05 as compared to controls.
5.4 Results

5.4.1 Quantifying the level of expression of Bcl-3 mRNA in vivo in rat liver following mitogenic stimulation with T₃

We used real time PCR to quantify levels of Bcl-3 mRNA per unit 18S ribosomal RNA in our liver samples. There was increased expression of Bcl-3 mRNA in the livers of rats receiving T₃ (fig 5.11). There was a progressive increase in the level of Bcl-3 mRNA expression over the 48 hour period, beginning at 4 hours and peaking at 24 hours.

![Graph showing BCL-3:18S ratio over time](image)

Fig 5.11 shows the level of expression of Bcl-3 mRNA in vivo in rat liver following mitogenic stimulation with T₃. We used real time RT-PCR to quantify levels of Bcl-3 mRNA/18S RNA and have expressed the results as the ratio of Bcl-3:18S.

* - p<0.05 and ** - p<0.01 as compared to vehicle alone (n=4).
5.4.2 Quantifying the level of Bcl-3 protein expression in vivo in rat liver following mitogenic stimulation with $T_3$

We used SDS-PAGE electrophoresis followed by western blotting to quantify levels of the 63KDalton protein Bcl-3 in our liver samples. There were corresponding increases in Bcl-3 protein expression in the livers of rats receiving $T_3$ (fig 5.12).

![Image of western blot showing Bcl-3 protein expression](image)

63 KDaltons

Fig 5.12 shows the level of Bcl-3 protein expression in vivo in rat liver following mitogenic stimulation with $T_3$. A 300 µg aliquot of total protein lysate was loaded into each well prior to SDS-PAGE electrophoresis and western blotting:

- a = control liver
- b = 1 hour
- c = 4 hours
- d = 8 hours
- e = 24 hours
- f = 48 hours post $T_3$
5.6 Discussion

In this chapter we attempt to identify a possible mechanism by which T₃ may be acting as a primary mitogen for the liver. We have used the data available from a quantitative cDNA microarray in mice to identify hepatic genes regulated by thyroid hormone to generate a hypothesis that T₃ induced cell proliferation was occurring through the activation of the Bcl-3 gene (Pibiri et al 2001).

We set out to test this hypothesis by developing an RT-PCR protocol to quantify levels of expression of Bcl-3 mRNA in the liver samples of normal rats stimulated with T₃. The conditions required for the generation of a specific, essentially unique product (single strong band on the gel) from liver tissue required optimization. There were a number of PCR optimising strategies that we used to alter the specificity characteristics of the PCR reaction:

1. Primer design – As well as the specific considerations relevant to the Bcl-3 primer design given in the methods, there are some general considerations that are relevant. The two primers are generally 20-30 bp long, contain a relatively balanced GC vs AT content (eg 45-55% GC) with no long stretches of one base (prevents secondary structures). The primer pairs should not have more than 2 adjacent complementary base pairs (especially at the 3’ end) to avoid primer dimer formation.

2. Magnesium concentration – The specificity of the PCR reaction can be increased by decreasing the Mg²⁺ concentration in the reaction mix. The magnesium concentration must therefore be optimised for an efficient PCR reaction to occur, as a concentration too low may result in no product forming and a concentration too high may result in a variety of unwanted products.

3. Annealing characteristics- The annealing temperature is particularly important in determining the specificity of the PCR reaction: at lower temperatures the primers
may anneal to similar but irrelevant sequences elsewhere in the genome and amplify these, resulting in the formation of multiple products. We were able to optimise the annealing temperature to obtain a single band on the gel, a critical requirement for an accurate quantification during the PCR. Additionally, we were able to use the melt point analysis of the PCR products on the real time PCR machine during quantification to confirm that only a single PCR product was being formed.

4. Template considerations – The efficiency of the Bcl-3 PCR was also critically dependent on the presence of solution Q (Sigma, UK) in the PCR reaction mix. This is a solution that prevents secondary structures forming within the primers and maintains normal stoichiometric interactions.

The goal of most quantitative RT-PCR methods is to use PCR product yield as a measure of relative differences in mRNA template abundance. Because the efficiency of the RT reaction has been assumed to be constant, the quantitative capacity of the PCR has been the prime focus of debate. Early on, the feasibility of the quantitative PCR was questioned because of two theoretical constraints: (1) Given the exponential nature of the process, small tube to tube variations in efficiency would grossly affect the final yield of the PCR products (2) PCR product yield could only provide a valid measure of template input during the exponential phase of amplification. The development of competitive PCR methods allowed investigators to address such theoretical concerns experimentally (Giulietti et al. 2001). To date the pre-requisites to allow quantitative RT-PCR (Bustin 2000) appear to be:

1. PCR product yield is determined during the exponential phase of amplification.

2. A standard curve demonstrates the range over which PCR product yield provides a measure of mRNA input.
3. The number of samples tested allow for a statistical analysis of differences in
PCR product yield.

The real time PCR protocol with appropriate sample number (n=4) and
computer software allowed us to fulfil the required criteria and show that Bcl-3 mRNA
is up regulated in the livers of rats treated with a single mitogenic dose of T3.

There has been recent work in the study of genes regulating liver regeneration
that shows that certain genes appear to be activated at the mRNA level (increased
mRNA expression) however are not expressed at the protein level, through post
transcriptional alterations in mRNA stability (Fausto 2000). We felt it was therefore
critical to measure the corresponding levels of protein expression in our liver samples.

We used SDS-PAGE electrophoresis followed by western blotting to quantify
levels of the 63KDalton protein Bcl-3 in our liver samples. Almost all analytical
electrophoresis of proteins is carried out with prior heating to ensure dissociation of the
proteins into their individual polypeptide subunits with minimal aggregation. In our
protocol we added the strongly anionic detergent SDS during the heating stage to binds
to the denatured polypeptides and make them negatively charged. The SDS binds
proportionately to the molecular weight of the peptide, independently of sequence
configuration. The SDS-polypeptide complexes then migrate across the electrical
current through the polyacrylamide gel during electrophoresis according to the size of
the polypeptide. The electrophoretically separated components are transferred from a
gel to a solid support/nitrocellulose filter and probed indirectly with antibodies (western
blotting). The critical difference between southern (DNA) and western blotting (protein)
lies in the nature of the probe. Whereas nucleic acid probes hybridize with a specificity
and rate that can be predicted by simple equations, antibodies behave in a much more
idiosyncratic way and require greater optimisation. Virtually all western blots are
probed in two stages after which they are exposed on photographic film. In the first step an unlabelled antibody specific to the target protein is incubated with the nitrocellulose filter in the presence of blocking solution. The blocking reagent prevents non-specific binding of the antibody. The second step requires incubating an anti-immunoglobulin antibody that can be radiolabelled or coupled to an enzyme to allow detection.

These experiments have shown there is an up-regulation of Bcl-3 mRNA and protein in the livers of rats receiving T₃. However there are still many unresolved questions regarding the exact mechanism of action by which T₃ may be inducing its effects on cell proliferation. The gene array data and this work have shown an up-regulation of Bcl-3 in the livers of rodents receiving T₃. It is important to determine whether these changes in Bcl-3, express themselves functionally and affect the rate of cell proliferation in the liver. The simplest way to achieve this would be to perform experiments injecting T₃ to Bcl-3 knockout mice and their parent strain to determine if there is a lack of responsiveness in the knockout animals. There are two different colonies of Bcl-3 knockout mice worldwide, both in the USA. These colonies have been developed to identify the mechanisms involved in lymphocyte proliferation, within the context of studying lymphoproliferative and allergic disorders (Schwarz et al. 1997). The laboratory is in the process of setting up a collaboration with Professor U Siebenlist, Centre for Allergy, National Institute of Health, Bethesda, USA to utilise BCL-3 knockout mice.
Chapter 6

Characterising the effects of thyroid hormone on primary rat hepatocytes
6.1 Background

In this chapter we investigate the effects of tri-iodothyroine on primary rat hepatocyte cultures. This cell culture work has two important implications relating to both the clinical application of the work and the mechanism of action of T₃. Whether T₃ could be an effective primary mitogen in man is unknown – the pharmacological doses used, though survivable, could not be tested clinically, thus making it essential to develop an *in vitro* model of its effects on primary rat cells. Subsequently, if T₃ could be demonstrated to be mitogenic in rat cells, the cell culture system could be applied to human liver cell cultures to determine whether T₃ may constitute an effective primary mitogen in man. The cell culture work may also provide some insight into the mechanism of action, as a major question arising in the past few years has been whether the actions of T₃ are occurring directly (on liver cells) or indirectly from its effects on other organs. If we assume the effect is occurring through the up-regulation of Bcl-3, then the fact that there is a thyroid response element in the Bcl-3 gene and Bcl-3 levels increase in the liver may suggest that it is a direct effect. Additionally, thyroid/retinoid X heterodimer activation has been shown to activate Bcl-3 directly in yeast two-hybrid tests using glutathione S-transferase pull-down assays (Na et al. 1998). However, the fact that T₃ has previously not been shown to have a mitogenic effect on primary rat hepatocyte cultures may suggest the effect is indirect.

In order to quantify the mitogenic properties of T₃ in cell culture an exogenous marker of DNA synthesis was used. We chose to use the radiolabelled thymidine method to detect DNA synthesis within cells. This relies on the uptake of radiolabelled substrate (thymidine) by cell undergoing DNA synthesis followed by radioactive quantification of a specified number of cells within the system. There are a number of
problems and limitations with the radiolabelled thymidine method, which are discussed in the conclusion of this chapter.

Our aim was to follow a logical scheme for the cell culture work, which began with a simple dose response experiment studying the effects of T₃ on a monolayer of primary rat hepatocytes. These results were compared to the effects of epidermal growth factor (a known stimulator of hepatocyte DNA synthesis *in vitro*) on primary rat hepatocytes. The aim of this experiment was to confirm that thyroid hormone is not a primary mitogen *in vitro* and to provide the basis for developing further cell culture experiments. Subsequently, we performed cell culture experiments to determine whether the *in vivo* mitogenic properties of T₃ may be occurring indirectly, i.e. independently of the interactions between tri-iodothyronine and the hepatocyte. The most obvious candidates would be TSH and TRH, from changes due to feedback on the hypothalamic-pituitary- T₃ axis, however these factors do not regulate cell proliferation. The thyroid hormones have recently been shown to have an effect on a variety of other endogenous systems, most notably the growth hormone/IGF-1 system that regulates growth (Ramos *et al.* 2002). With this in mind we characterised the effects of a combination of T₃ and growth hormone and also T₃ and IGF-1 (the peptide through which the majority of growth hormones actions are mediated) on primary rat hepatocytes (Ramos *et al.* 2001). We also wanted to determine if T₃ could be having its effects through an unknown plasma factor, whose secretion/activation is regulated by T₃. Thus, we characterised the effects of plasma taken from rats stimulated with T₃ on DNA synthesis in primary cells.

Having performed experiments to identify whether T₃ may be working indirectly through a plasma factor, we moved on to experiments aimed at conditioning the liver in order to demonstrate its effects. We performed priming experiments on hepatocytes to
assess if they become sensitised to T₃. Hepatocytes are primed in vivo (following 70% partial hepatectomy) by a cytokine cascade involving the activation of the TNFalpha-1 receptor to generate NF-kappa B followed by increases in IL 6 levels to activate the transcription factor STAT 3 (Fausto 2000). Interleukin – 6 is a critical cytokine that sensitises hepatocytes to growth factors, required to initiate DNA synthesis. We performed experiments with various combinations of T₃ and interleukin - 6 on primary cells to determine if this cytokine may be required to prime hepatocytes prior to T₃ having its effect (Runge et al. 1999). In the final experiment of the chapter we characterised the effects of combination treatment with T₃ and retinoic acid on primary cells. The rationale for this experiment came from the observation that T₃ forms a heterodimer with the retinoid X receptor in vivo, leading to the hypothesis that dual receptor activation may be required to initiate the biological effect (Kliewer et al 1992).

6.2 Aim

Characterising the effect of tri-iodothyronine on primary rat hepatocyte cultures.

6.3 Methods

6.3.1 Materials

i) Collagenase enzyme (Worthington biochemical corp., New Jersey)

ii) Perfusion buffer (pH 7.4) –

- HEPES 10mM
- KCl 3mM
- NaCl 30mM
- NaH₂PO₄ 1mM
- Glucose 1mM
- Phenol red 1mM
iii) Calcium chloride supplemented perfusion buffer – components as above with the addition of 1.9mM CaCl₂.

iv) EDTA supplemented perfusion buffer – components as above with the addition of 5mM EDTA.

v) Williams E medium (Gibco, UK).

vi) Calcium-free HBSS medium (Gibco, UK).

vii) Trypan blue solution (Sigma, UK).

viii) Heparin (1000u/ml – Leo laboratories, UK).

ix) Midazolam (5mg/ml – Roche, UK).

x) Dexamethasone (Calbiochem, UK).

xi) Fetal calf serum (Royal Free Hospital, Department of Medicine).

xii) Penicillin (Royal Free Hospital, Department of Medicine).

xiii) Streptomycin (Royal Free Hospital, Department of Medicine).

xiv) Insulin (100 iu/ml – Novo nordisc pharmaceuticals, UK).

xv) Epidermal growth factor (Calbiochem, UK).

xvi) Growth hormone (Calbiochem, UK).

xvii) Insulin-like growth factor 1 (Calbiochem, UK).

xviii) Interleukin 6 (R & D systems, UK).

xix) Cis-retinoic acid (R & D systems, UK).

xx) Paediatric cannulas (Abbocath, UK).

xxi) Acetic acid (Sigma, UK).

xxii) Perfusion apparatus consisting of water bath, buffer reservoirs, peristaltic pump and a bubble trap.

xxiii) Standard tissue culture plates and materials.
6.3.2 Isolation of primary rat hepatocytes

The isolation of primary rat hepatocytes was undertaken in collaboration with Dr Neil Mellor. Rats were anaesthetised with an intramuscular injection midazolam at 0.2ml/kg bodyweight. The abdomen was swabbed with ethanol and a laparotomy incision made to expose the abdominal contents. The bowel was reflected to reveal the liver and portal vein. A silk suture was placed around the fat bed at the base of the portal vein and the vein cannulated with a heparin-primed paediatric cannula. The cannula was secured using a silk suture and another suture was placed around the inferior vena cava distal to the renal vein. The perfusion apparatus pumping EDTA supplemented buffer at 5 ml/min was connected to the portal vein cannula and the abdominal aorta was ligated. The liver typically became pale as the blood was cleared being replaced by buffer. Non-supplemented perfusion buffer was substituted after 3 minutes. After 5 minutes the non-supplemented perfusion buffer was replaced with calcium-supplemented buffer that had 25 mg of collagenase dissolved in it. The perfusion was allowed to continue for another 20 minutes, exhausting the buffer resevoir. During the final moments of the perfusion the liver swelled, became flaccid, from which point the liver was excised and placed in a petri dish. Cells were released by gently teasing the tissue and allowing the separation of the cells from the biliary remnants.

Thus in this protocol, the liver was sequentially perfused with buffers and enzymes to degrade the structural stroma of the liver to release the cells. The initial EDTA-buffer cleared the blood and disrupted calcium dependent gap junctions between cell. The calcium dependent buffer acted as a co-factor for collagenase to allow stromal digestion and the release of cells.
6.3.3 Hepatocyte separation and assessment of cell viability

The cell suspension was filtered twice through a coarse and thin (56 μm) mesh to remove cell debris. Hepatocytes were separated from non-parenchymal cells by centrifugation at 50 XG for 90 seconds. Hepatocytes were washed twice with calcium-free HBSS and resuspended in Williams E medium supplemented with antibiotics at a concentration of 5X10⁶ cells/ml.

The cell viability was estimated by assessing the integrity of the cell membrane. In this method injured/dead cells have impaired membrane integrity and are unable to exude vital dyes. A 20μl aliquot of trypan blue was mixed with 20μl of cell suspension and placed in 160μl of HBSS. After 3 minutes the cells were assessed using a haemocytometer, with dead cells appearing blue and live ones excluding the dye. This allowed an estimation of viability to be made and in most instances was greater than 80%.

6.3.4 Preparation of collagen coated plates

Collagen type I was prepared from rat tails, by sequentially fracturing the tail and removing the inner central tendons. This was followed by dissolution in acetic acid and purification to yield a solution that may be applied to tissue culture plates to promote hepatocyte monolayer culture.

Collagen coating of a 96 well tissue culture plate

- Add 200ul of collagen per well, leave for 5 minutes at room temperature.
- Shake plate vigorously into waste container (to avoid troughs in collagen coat )
- Wash twice with 200ul of HBSS (Ca + Mg free) to remove acetic acid
- Replace with 100ul of sterile saline (0.9%w/v)
- Irradiate for 15 mins, long wave UV light
- Store at 4° C until use
6.3.5 Culture of hepatocytes

Primary hepatocytes were plated at a density of 50,000 cells/cm² on collagen coated tissue culture plates, and maintained with Williams E medium supplemented with fetal calf serum (10%), penicillin (200U/ml), streptomycin (200U/ml), dexamethasone (10⁻⁸M) and insulin (10⁻⁷M). The culture plates were maintained in a humidified incubator with an atmosphere of 95% oxygen and 5% carbon dioxide. The culture plates were washed with HBSS two hours after the cell were plated to remove non-viable cells and the culture medium replaced daily during the course of the experiment.

6.3.6 Experimental protocol for determining the effects of T₃ in vitro.

- After plating the primary hepatocytes and replacing the medium the cells were incubated in new supplemented Williams E medium overnight.
- The following morning the factors being investigated were dissolved in new supplemented medium (dextrose and insulin supplementation) and added to the cells in the tissue culture plate. Combinations of dexamethasone, insulin and epidermal growth factor (10ng/ml) were also added in each experiment as controls.
- After 18 hours 2 μCi of ³H-methyl-thymidine (in serum free medium) was added per well.
- After a further 12 hours (total 30 hrs after the addition of factors), we stopped labelling by emptying contents of plate and washing X3 times with 200ul of distilled water.
- The cell monolayer was dissolved by incubating it with 200ul of 1 M KOH for 1 hour.
- A 50μl aliquot of sample was removed and mixed with 1 ml of scintillation fluid.
- Obtain the radioactivity count (DPM).
6.4 Results

The first experiment was a simple dose response study on the effects of T$_3$ on hepatocyte DNA synthesis on a monolayer of primary rat hepatocytes. As expected in the control groups with dexamethasone alone and dexamethasone with insulin there was little effect on hepatocyte DNA synthesis (fig 6.1) The control group containing epidermal growth factor had a profound effect on hepatocyte DNA synthesis confirming that EGF is a potent mitogen on primary rat hepatocytes (fig 6.1). However, T$_3$ appeared to have no effect on hepatocyte DNA synthesis at a dose between 0.1-100ng/ml (fig 6.1). We had calculated that the equivalent mitogenic dose in vitro was approximately 1-5ng/ml, and had thus studied a broad range.

![Graph showing the effects of different doses of T$_3$ on hepatocyte DNA synthesis on primary rat hepatocytes in vitro.](image)

Fig 6.1 – showing the effects of different doses of T$_3$ on hepatocyte DNA synthesis on primary rat hepatocytes in vitro. The dose of T$_3$ in ng/ml is given as a prefix in the figure. The effects of dexamethasone (Dx) alone, dexamethasone and insulin (I), and dexamethasone, insulin and epidermal growth factor (E) are also given. Each cohort was performed in quadruplicate (n=4) and results expressed as mean ± S.D.
In the next set of experiments we characterised the effects of a combination of T₃ and growth hormone on primary rat hepatocytes. In this experiment we assessed if growth hormone alone at 4 different doses between 0.1ng/ml and 100 ng/ml induces DNA synthesis in rat hepatocytes and also determined if different combinations of T₃ (0.1 –10ng/ml) and growth hormone (0.1 and 100 ng/ml) had an effect. We found that growth hormone alone and also combinations of growth hormone and T₃ had no effect on hepatocyte DNA synthesis in primary rat hepatocytes (Fig 6.2).

Fig 6.2 – showing the effects of growth hormone (G) alone and different combinations of growth hormone and tri-iodothyronine(T) on hepatocyte DNA synthesis on primary rat hepatocytes in vitro. The doses in ng/ml of both hormones are given as a prefix. The effects of dexamethasone (Dx) alone, dexamethasone and insulin (I), and dexamethasone, insulin and epidermal growth factor (E) are also given. Each cohort was performed in quadruplicate (n=4) and results expressed as mean ± S.D.
Having characterised the effects of T3 and growth hormone on primary rat hepatocytes, we wanted to complete this story by characterising the effects of T3 and IGF-1 on primary cells. In this experiment we assessed if IGF-1 alone at 3 doses between 10ng/ml and 75 ng/ml induces DNA synthesis in rat hepatocytes and also determined if different combinations of T3 (0.1–10ng/ml) and IGF-1 (10-75ng/ml) had an effect. Again we showed that IGF-1 alone and also combinations of IGF-1 and T3 had no effect on hepatocyte DNA synthesis (Fig 6.3).

**Fig 6.3 – showing the effects of IGF-1 (Ig) alone and different combinations of IGF-1 and tri-iodothyronine (T) on hepatocyte DNA synthesis on primary rat hepatocytes in vitro. The doses in ng/ml of both hormones are given as a prefix. The effects of dexamethasone (Dx) alone, dexamethasone and insulin (I), and dexamethasone, insulin and epidermal growth factor (E) are also given. Each cohort was performed in quadruplicate (n=4) and results expressed as mean ± S.D.**
We investigated if the mitogenic effects of T₃ may be occurring through an unknown plasma factor, whose secretion/activation is regulated by T₃. We obtained plasma samples from rats stimulated with T₃ at 1, 4 and 8 hours, performing serial 1:2 and 1:4 dilutions on the samples with supplemented conditioning medium. We also obtained plasma from control rats receiving vehicle only.

There was no effect on hepatocyte DNA synthesis from the serum of rats stimulated with T₃ or controls at any of the time points or with serial dilutions (Fig 6.4).

![Graph showing the effects of serum from rats stimulated with T₃ at various time points with serial dilutions on hepatocyte DNA synthesis on primary rat hepatocytes in vitro. The time points and serial dilutions (%) are given. The effects of dexamethasone (Dx) alone, dexamethasone and insulin (I), and dexamethasone, insulin and epidermal growth factor (E) are also given. Each cohort was done in quadruplicate (n=4) and results expressed as mean ± S.D.](image)

Fig 6.4 – showing the effects of serum from rats stimulated with T₃ at various time points with serial dilutions on hepatocyte DNA synthesis on primary rat hepatocytes in vitro. The time points and serial dilutions (%) are given. The effects of dexamethasone (Dx) alone, dexamethasone and insulin (I), and dexamethasone, insulin and epidermal growth factor (E) are also given. Each cohort was done in quadruplicate (n=4) and results expressed as mean ± S.D.
Having not been able to show an effect for a variety of plasma factors that may be regulated by T₃, we moved on to experiments aimed at conditioning the liver in order to demonstrate an effect. We initially determined if IL-6 alone at 4 doses between 0.1ng/ml and 50 ng/ml induces DNA synthesis in rat hepatocytes and also determined if different combinations of T₃ (0.1–10ng/ml) and IL-6 (0.1-50ng/ml) may be able to induce DNA synthesis in the hepatocytes. In this experiment we showed that IL-6 alone or in combination with T₃ had no effect on hepatocyte DNA synthesis (Fig 6.5).

Fig 6.5 – showing the effects of IL-6 (IL) alone and different combinations of IL-6 and tri-iodothyronine(T) on hepatocyte DNA synthesis in vitro. The doses in ng/ml of both IL-6 and T₃ are given as a prefix. The effects of dexamethasone (Dx) alone, dexamethasone and insulin (I), and dexamethasone, insulin and epidermal growth factor (E) are also given. Each cohort was performed in quadruplicate (n=4) and results expressed as mean ± S.D.
The final experiment in this chapter was to determine if combinations of retinoic acid and tri-iodothyronine were mitogenic to primary rat hepatocytes. In this experiment we assessed if cis-retinoic acid alone (retinoid X receptor agonist) at 3 doses between 0.1ng/ml and 10 ng/ml induced DNA synthesis in rat hepatocytes and also investigated the effects of combinations of T₃ (0.1–10ng/ml) and cis-retinoic acid (0.1-10 ng/ml). Again we found that retinoic acid alone or in combination with T₃ had no effect on hepatocyte DNA synthesis (Fig 6.6).

Fig 6.6 – showing the effects of cis-retinoic acid (R) alone and different combinations of retinoic acid and tri-iodothyronine(T) on hepatocyte DNA synthesis in vitro. The doses in ng/ml of both retinoic acid and T₃ are given as a prefix. The effects of dexamethasone (Dx) alone, dexamethasone and insulin (I), and dexamethasone, insulin and epidermal growth factor (E) are also given. Each cohort was performed in quadruplicate (n=4) and results expressed as mean ± S.D.
6.5 Discussion

In the previous chapters we have shown that tri-iodothyronine is a potent primary mitogen *in vivo* in adult rats. Whether T₃ could be effective in man is unknown – the pharmacological doses used, though survivable, could not be tested clinically, thus making it essential to develop an *in vitro* model of its effects. One of the aims of this chapter was to characterise the effects of T₃ on primary rat hepatocyte cultures. Subsequently, if T₃ could be demonstrated to be mitogenic in rat cells, the primary culture system could be applied to human liver cell cultures to determine whether T₃ may constitute an effective primary mitogen in man.

The rat liver perfusion was a technically challenging procedure that took a protracted period of time to develop. However, once the technique was developed, it was easily reproducible and provided a large supply of viable primary hepatocytes. In order to quantify the magnitude of cell proliferation in these primary rat hepatocytes an exogenous marker of DNA synthesis was used. We therefore used the radiolabelled thymidine method to detect DNA synthesis within cells, which relies on the uptake of radiolabelled substrate (thymidine) into the nucleus of cells undergoing DNA synthesis followed by radioactive quantification of a specified number of cells within the system. The problems with the thymidine incorporation method include:

1. The radiolabel is integrated into cells in DNA synthesis, both in the context of DNA replication in S-phase and also during DNA repair.

2. There may be non-specific labelling of RNA molecules by the ^3H.

3. The integration of exogenous thymidine is dependent on both the available concentration and the competition with endogenous levels of thymidine.

We aimed to reduce these inaccuracies by saturating the system with the radiolabelled substrate and by adding the thymidine at 18 hours instead of 8-12 hours to
yield increased counts (approx 2 fold-giving a better signal to noise ratio, which reflects
a reduced degradation of the tritiated molecule in culture). A confirmation that we had
both a significant proportion of viable hepatocytes and a sensitive \textit{in vitro} method of
detecting DNA synthesis came from the observations seen with epidermal growth
factor. This peptide known to be a potent hepatic mitogen on primary rat hepatocytes
caused a 3-6 fold increase in the radioactivity count in all experiments (compared to
supplemented medium). We were looking for at least a 2-3 fold increase in our samples
to show a significant result (Ohira \textit{et al.} 1996). If we had obtained any positive results
with the radiolabelled thymidine method it would have been important to follow this up
with morphological studies to confirm that cell proliferation was indeed occurring.

The first experiment was a simple dose response study investigating the effects
of \textit{T}_3 on a monolayer of primary rat hepatocytes. We had calculated that an equivalent
mitogenic dose \textit{in vitro} was approximately 1-5ng/ml, thus had studied a broad range
beyond this. We showed that \textit{T}_3 had no effect on hepatocyte DNA synthesis at a dose
between 0.1-100ng/ml. This result confirmed similar finding by other investigators
leading to the question as to why \textit{T}_3 was having such a profound effect on hepatocyte
DNA synthesis \textit{in vivo} but not \textit{in vitro}. The following appeared to be a reasonable list
of possibilities:

1. The mitogenic effects of \textit{T}_3 \textit{in vivo} may not be occurring by the direct action of
tri-iodothyronine on hepatocytes, but indirectly as a result of a different plasma factor,
whose secretion/activation is regulated by \textit{T}_3.

2. Thyroid hormone itself is not sufficient to be mitogenic to hepatocytes. \textit{T}_3 and
an endogenous factor present \textit{in vivo} are required to induce DNA synthesis in
hepatocytes.
3. The monolayer of primary rat hepatocytes does not provide the 3D architectural integrity of the *in vivo* liver, and additionally the interaction between hepatocyte and non-parenchymal cells is lost. The combination of these and other factors (eg changes in hepatocyte integrity following perfusion) may result in the loss of responsiveness of hepatocytes to T3.

The first set of experiments were aimed at investigating if T3 may be inducing its mitogenic effects indirectly as a result of a different plasma factor, whose secretion/activation is regulated by T3. The most obvious candidates would be TSH and TRH, from feedback on the hypothalamic-pituitary- T3 axis, however these factors are unrelated to cell proliferation. The thyroid hormones have recently been shown to have an effect on a variety of other endogenous systems, most notably the growth hormone/insulin type growth factor 1 system (Ramos *et al* 2002). Thyroid hormone response elements have been shown to be present in the gene responsible for growth hormone secretion in the pituitary gland. Moreover, thyroid hormone has independent actions increasing IGF-1 activity in the absence of its effects on growth hormone. Clinically, hypothyroid patients show low levels of IGF-1 activity where as hyperthyroid patients show increased levels of IGF-1 activity (Ramos *et al* 2001). Considering IGF-1 is one of the most potent mitogens known to man we felt it necessary to characterise the effects of growth hormone and IGF-1 alone and in combination with T3 on hepatocytes. Any significant results in these experiments would allow us to investigate GH/IGF-1 levels *in vivo* following T3 stimulation. We were however unable to show an effect on hepatocyte DNA synthesis for GH/IGF-1 individually or in combination with T3.

With no other obvious peptide/growth factor regulated by T3 we decided to investigate the effects of T3 stimulated plasma on primary rat hepatocytes. The
rationale for this would be that even unidentified plasma factors stimulated by T3 would be available for characterisation as a possible candidate. We obtained plasma samples from rats stimulated with T3 at 1, 4 and 8 hours and compared these with control samples from rats receiving vehicle only. Again, there was no effect on hepatocyte DNA synthesis from the serum of rats stimulated with T3 at any time point or with serial dilution.

The next set of experiments were aimed at conditioning the liver to assess if an endogenous factor present in vivo in combination with T3 was required to induce DNA synthesis in hepatocytes. The idea of liver cell priming came from the well-characterised paradigm of the 70% partial hepatectomy in the rat. Hepatocytes are primed in vivo (following 70% partial hepatectomy) by a cytokine cascade involving the activation of the TNFalpha-1 receptor to generate NF-kappa B followed by increases in IL6 levels to activate the transcription factor STAT3 (Fausto 2000). Interleukin – 6 is the final critical cytokine that sensitises hepatocytes to growth factors to initiate DNA synthesis. We therefore performed priming experiments with a combination of T3 and interleukin-6 to assess if this cytokine may be required to prime hepatocytes prior to T3 having an effect (Runge et al 1999). We were unable to show an effect on DNA synthesis with IL-6 alone or in combination with T3. Retrospectively, this was not a surprising result as the mitogenic effects of T3 in vivo have been shown to be independent of NF-kappa B and STAT3, thus suggesting that IL-6 is also unlikely to be involved in the underlying mechanism.

The final experiment in this chapter investigated the effects of T3 and retinoic acid on primary rat hepatocytes. We hypothesised that stellate cells, a major non-parenchymal cell population that stores retinoids, may play a role in modulating T3 responsiveness. In the presence of both T3 and retinoids, dual receptor activation (of the
T₃/retinoid X receptor heterodimer) in vivo may be critical for the biological effect (Kliewer et al. 1992). Unfortunately, again we were unable to show any significant effect during this experiment. It is clear that we had difficulties in modelling the effects of T₃ in vitro. However one of the limitations of the methodology was in measuring Thymidine uptake in KOH digests, where cytosolic (unpolymerised) uptake had not been removed by washing in acid. This may have given a high background masking any small affect T₃ may have had.

It is likely that the monolayer of primary rat hepatocytes do not provide the appropriate 3D architectural integrity to that of the liver in vivo. Previous workers have shown that RNA synthesis, RNA polymerase activity, and the expression of a variety of enzymes (e.g. malic acid) in rodent cells in response to T₃ are all down-regulated in monolayer culture when compared to 3D culture (Menjo et al. 1999). Parallel work in our laboratory aimed at culturing HepG2 cell into 3D spheroids within alginate beads (showing enhanced function) may provide further experimental opportunities for the 3D culture of primary rat hepatocytes in the future. The interaction between hepatocytes and non-parenchymal cells are also lost in primary cell culture. In the past the laboratory has shown that interactions between different cell populations in vitro can provide powerful new insights into the induction of hepatocyte proliferation. We used this technique to identify a role for IL-1 from non-parenchymal cells in regulating hepatocyte proliferation during compensatory hyperplasia (Boulton et al. 1998). The evidence that some primary mitogens initially interact with Kupffer cells to induce hepatocyte proliferation also provides another reason why interactions between hepatocyte and non-parenchymal cells may be required (Boulton et al. 1997). It is clear that we need to improve cell separation techniques to allow us to perform co-culture experiments and to generate 3D hepatocyte colonies to appropriately model the liver.
These techniques would allow us to recreate the architectural integrity of the liver, thus possibly allowing us to identify an underlying mechanism of action and to determine whether \( T_3 \) could play this role in man.
Chapter 7

General Discussion
In this thesis we have shown that tri-iodothyronine has a profound effect as a hepatic mitogen *in vivo* in adult rats. We have demonstrated that a single injection of T$_3$, initiates proliferation in hepatocytes, and 96h later over 30% of the hepatocytes (predominantly midzonal) have gone through S-phase and cell division. The mitogenic properties of the thyroid hormones have been utilised experimentally to correct inborn errors of hepatic metabolism, either by replacing the defective hepatocytes or by introducing the stable gene into the host cell. An experimental model inhibiting hepatocyte proliferation *in vivo* in rats has been developed utilising the alkaloid retrosine. The investigators transplanted syngenic hepatocytes and showed that treatment with tri-iodothyronine resulted in complete parenchymal (>80%) replacement by the transplanted hepatocytes (Oren *et al* 1999). A different approach is to develop a gene delivery system to replace the defective gene and allow its stable integration into the host cell genome. Retroviral based vectors are the most extensively studied vectors in animal models and clinical trials. Our laboratory utilised the mitogenic properties of tri-iodothyroine to allow the transduction of retroviral vectors into the rat liver *in vivo*, offering the potential for hepatic gene therapy, especially when one considers that the corrected cells could potentially repopulate the liver (through a survival advantage) (Forbes, *et al* 2000).

Tri-iodothyronine is of particular interest as a primary mitogen in view of its potential exploitation as a pharmaceutically available hormone that has been critically appraised in humans. The single subcutaneous injection of T$_3$ at 4mg/kg was well tolerated by recipient rats, with the maintenance of an adequate appetite and bodyweight, and not associated with any features of hepatocellular damage (no change in circulating hepatic enzyme levels or features of hepatocellular necrosis on sections) or unexpected mortality. As anticipated the mitogenic doses of T$_3$ resulted in
hyperthyroidism in recipient rats, shown by the suppression of TSH secretion, with the trough level at 48 hours returning to near normal at 96 hours.

The less well-recognised property of T₃ was the potential of the intact liver to grow in vivo to a size and more importantly to a functional capacity greater than normal. Previously, most analyses of the relationship between liver mass, function and functional reserve have investigated livers of normal or decreased size. Experimentally, acute reduction in liver mass by 70% partial hepatectomy is immediately and proportionately paralleled by a loss of metabolic function, with a reduction in urea-N synthesis, galactose elimination capacity and antipyrine clearance (Adel & Enghusen 1986). This translated clinically into residual liver mass after resectional surgery being a recognised parameter used to assess the feasibility of tumour resection (Redaelli et al. 2002). We hypothesised that the ability to increase liver mass and functional reserve with a primary mitogen could provide a therapeutic option to allow larger liver resections to be performed. A single injection of tri-iodothyronine administered 10 days prior to a 70% partial hepatectomy produced a larger remnant liver mass 24 hours after surgery when compared to hepatectomy controls. However, it was intriguing that the regenerative response to surgery (both in terms of the proportion of proliferating cells and the distribution of the proliferative response -periportal) was the same in both groups at this 24-hour time point. There is some other evidence to suggest that hepatocytes can be stimulated to proliferate beyond physiological thresholds when adequately conditioned. In our experiments it allowed a mitogen to enhance liver mass, yet retain regenerative competence following surgery. The FAH knockout mouse model of tyrosinaemia is probably the most impressive model of physiological conditioning of the proliferative capacity of hepatocytes. Syngeneic transplantation of healthy hepatocytes into FAH knockout mice results in the damaged livers being
repopulated by normal hepatocytes through a survival advantage. The process of serial transplantation was performed eight times with equal success, without a decrease in replicative capacity (Overturf et al 1997). One can therefore speculate that though the livers of rats receiving T₃ had been pre-conditioned to enhance liver mass, the overall stimulus following partial hepatectomy was so intense that regenerative competence was restored. The second part of this work shows that the simultaneous co-administration of tri-iodothyronine with 70% partial hepatectomy increases the proliferative response of hepatocytes following surgery, and thus enhances regeneration during one of the strongest known stimuli to hepatocyte proliferation (Alison 1986). The mitogenic properties of T₃ inducing proliferation in midzonal hepatocytes, not recruited by hepatectomy alone is central to the explanation. The ability T₃ has to enhance proliferation above a previously regarded threshold is also supported by our previous observations that exogenous administration of T₃ and HGF (Forbes et al 2000), a major component of the compensatory hyperplasia pathway after partial hepatectomy, are synergistic to hepatocyte proliferation in vivo.

This work could be therapeutically valuable if applicable to man. Increased liver mass prior to and following surgery could enhance hepatic reserve and prevent decompensation. In transplant surgery, small-for-patient liver grafts could be encouraged to grow more rapidly to the appropriate mass and thereby prevent the onset of portal hypertension in the graft. Indeed the principal could be applied to living related liver transplants, thus ensuring provision of an appropriate mass of liver to provide immediate function in the recipient, whilst maintaining the appropriate mass and function in the donor.

It is important to determine whether the enhanced cell proliferation with a primary mitogen increases the likelihood of malignant transformation. The long-
standing debate regarding enhanced cell proliferation and susceptibility to malignancy was addressed during the development of a mouse model of preneoplastic hepatic lesions using diethylnitrosamine (DENA) (Ledda-Columbano et al. 2000). The administration of DENA to mice produced glutathione – S – transferase positive, pre-malignant foci in the liver over a few weeks. It was shown that the administration of mitogenic doses of T\textsubscript{3} following DENA therapy reduced the number of GSTP-positive foci in the liver, with the authors citing the differentiating properties of T\textsubscript{3} to account for this effect (Ledda-Columbano et al 2000). However, this study involved relatively small numbers of animals and larger studies are required to investigate this apparently paradoxical effect to confirm that primary mitogens are safe for testing in humans.

In chapter 5 we attempted to identify a possible mechanism by which T\textsubscript{3} may be acting as a primary mitogen for the liver. We have used the data available from a quantitative cDNA microarray in mice to identify hepatic genes regulated by thyroid hormone (Feng et al 2000). The genes regulated by T\textsubscript{3} affecting cell proliferation included kinesin-like protein (Kip1p), chromodomain helicase-DNA binding protein (CHD-1) and Bcl-3. We found it intriguing that Bcl-3 up-regulation was shown to induce cell proliferation in lymphocytes (be it in the context of malignant proliferation in B cell leukaemia) and hypothesised that T\textsubscript{3} may be inducing its proliferative effects in a similar way. This hypothesis was supported by the fact that there is a thyroid response element in the Bcl-3 promoter and that the thyroid/retinod X receptor heterodimer have been shown to activate Bcl-3 in yeast two-hybrid tests using glutathione S-transferase pull-down assays (Na et al 1998). Our experiments have shown an up-regulation of Bcl-3 mRNA and protein in the livers of rats stimulated with T\textsubscript{3}. It is important to determine whether these changes in Bcl-3, express themselves functionally and affect the rate of cell proliferation in the liver. The simplest way to
achieve this would be to perform experiments injecting T₃ to Bcl-3 knockout mice and their parent strain to determine if there is a lack of responsiveness in the knockout animals. The laboratory is in the process of setting up a collaboration with Professor U Siebenlist, Centre for Allergy, NIH, Bethesda, USA to utilise Bcl-3 knockout mice. If Bcl-3 were implicated in T₃ induced cell proliferation its interesting to speculate how this mechanism would integrate into the findings of other investigators in the field of liver cell proliferation. Pibiri et al demonstrated that T₃ induced cell proliferation did not result from an increase in NFκB levels (electro mobility shift assays), a finding that would be consistent with our work, as Bcl-3 is an adaptor molecule for NFκB that enhances its activity but does not change the overall levels of the transcription factor. In compensatory growth increases in NFκB activity result in a cascade of IL-6/STAT-3 activation prior to cell proliferation (Fausto 2000). Pibiri et al demonstrated there were no changes in STAT 3 levels in T₃ induced cell proliferation, inviting the question (of our hypothesis) as to why enhanced NFκB activity with the Bcl-3 adaptor molecule would not produce a similar effect. The reason is unclear, however NFκB is a ubiquitous intracellular molecule that can be activated by a variety of cytokines/hormones. It is conceivable that the NFκB response is critically dependent on the signal driving it. This is supported by the fact that NFκB regulates a variety of cellular responses including immune and stress responses, apoptosis and cellular proliferation (Dechend et al 1999). It is therefore conceivable that NFκB activation with T₃ is occurring independently of IL-6/STAT-3 and that cell proliferation is occurring through a distinct mechanism to that seen in compensatory regeneration. It is unfortunate that the intracellular signalling pathway in chronic lymphatic leukaemia with the Bcl-3 mutation has not been identified to date as this may have provided further insight (McKeithan et al 1997).
The final chapter attempted to develop an *in vitro* model of T₃ action, to assess if T₃ could be shown to have similar effects *in vitro* as it does *in vivo*. This cell culture work has two important implications relating to both the clinical application of the work and to the mechanism of action of T₃. Whether T₃ could be an effective primary mitogen in man is unknown – the pharmacological doses used, though survivable, could not be tested clinically, thus making it essential to develop an *in vitro* model of its effects on primary rat cells. Subsequently, if T₃ could be demonstrated to be mitogenic in rat cells, the cell culture system could be applied to human liver cell cultures to determine whether T₃ may constitute an effective primary mitogen in man. The cell culture work may also provide some insight into the mechanism of action, as a major question arising in the past few years has been whether the actions of T₃ are occurring directly (on liver cells) or indirectly from its effects on other organs.

Unfortunately we were unsuccessful in our attempts to model the effects of T₃ *in vitro*. Additionally, further insight into the mitogenic effects of T₃ on the human liver cannot be drawn from acute thyrotoxicosis in humans. The single mitogenic dose in the rat is a different entity to acute thyrotoxicosis in humans, in which there are sustained elevated levels of thyroid hormones for a number of weeks prior to presentation.

However following a logical scheme in the future could provide further insight and allow us to use this hormone in the clinical arena (the ultimate aim of this work). It appears critical to determine if T₃ is inducing its proliferative effects through the up-regulation of Bcl-3 or whether this is a passive effect. If shown to be critical, then quantifying levels of Bcl-3 expression and DNA synthesis can be used *in vitro* to dissect out the pathways of the T₃ effect. This in combination with the development of cell separation techniques to allow us to perform co-culture experiments and to generate 3 D hepatocyte colonies to appropriately model the liver may provide insight.
Finally, even in the absence of any success in the cell culture experiments, options are available to determine whether T₃ may constitute an effective primary mitogen in man. An organ culture bath system encapsulating rat liver biopsy samples, which could then be applied to human liver biopsy samples could be used to determine if T₃ is mitogenic to human hepatocytes. Indeed, primate studies could be performed to establish the missing link between rodent and human, prior to testing in man. These strategies are important to determine whether T₃ may constitute an effective primary mitogen in man as there is very little data available on the effects of primary mitogens in humans.
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