NEW ASPECTS OF THE CELLULAR EFFECTS OF PARACETAMOL AND RELATED ANTIOXIDANTS

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ABSTRACT

In order to further understand the cellular effects of paracetamol and other related antioxidants, two different aspects have been studied:

(i) Inhibition of DNA synthesis by paracetamol and related antioxidants.

Paracetamol is known to have antioxidant and radical scavenging properties and it has been recently observed that paracetamol inhibits DNA synthesis through inhibition of ribonucleotide reductase. This property is similar to that of hydroxyurea, a well known DNA synthesis inhibitor which also inhibits ribonucleotide reductase activity by destroying the tyrosyl free radical. An objective of the present study is to determine whether paracetamol concentrations found in human therapeutic use or overdose are likely to inhibit DNA synthesis in humans. Other antioxidants were also included to broaden the scope of the investigations since it seems likely that any antioxidant which has the right shape and size to inhibit ribonucleotide reductase will inhibit DNA synthesis.

Tissues (testis, spleen and liver) from male Wistar rats were used in an in vitro tissue slice system to study the concentration-response relationships for inhibition of DNA synthesis by different compounds. Inhibition of protein synthesis was used as a control to assess non-specific cell damage.

Paracetamol and hydroxyurea were found to inhibit DNA synthesis in a dose-dependent manner in vitro, with little effect on protein synthesis. Considerable variation in the sensitivity of the different tissues was also observed with an order of most sensitive to least sensitive tissue of spleen > testis > liver. Some other phenolic antioxidants have also been found to inhibit DNA synthesis specifically but this was not a property shared by all phenolic antioxidants.

(ii) Role of apoptosis pathways in paracetamol-induced liver cell injury.

Previous studies have suggested that paracetamol can cause apoptotic changes. An objective of the present study is to determine whether apoptotic pathways are involved in
the hepatotoxic effects of paracetamol.

A dose-response and a time course study were conducted with groups of male C57Bl/6 mice and necrotic and apoptotic cell injury assessed. Necrotic cell injury was assessed at the microscopic level (H&E) and the degree of damage was correlated with plasma alanine aminotransferase (ALT) levels. Apoptotic changes were investigated using the TUNEL method in liver sections, Western blot analysis for poly (ADP-ribose) polymerase (PARP) cleavage in liver protein lysates and immunohistochemical staining for Bax protein expression in liver sections. Essentially no evidence was found for the involvement of apoptotic pathways in liver cell injury due to paracetamol overdose.
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CHAPTER 1: INTRODUCTION

1.0 Aims

The main aim of this project is to study new aspects of the cellular effects of paracetamol and related antioxidants. Two different aspects have been studied: (i) the inhibition of DNA synthesis by paracetamol and related antioxidants, and (ii) the role of apoptosis pathways in paracetamol-induced liver injury. The individual aims are listed below:

- **Inhibition of DNA Synthesis by Paracetamol and Related Antioxidants**
  * To investigate inhibition of DNA synthesis by paracetamol using an *in vitro* test system.
  * To assess whether tissue and plasma concentrations of paracetamol found in human therapeutic use or overdose are likely to inhibit DNA synthesis in humans.
  * To determine if differential inhibition of DNA synthesis occurs in different tissues.
  * To investigate inhibition of DNA synthesis by antioxidants related in structure to paracetamol.

- **Role of Apoptosis Pathways in Paracetamol-induced Liver Cell Injury**
  * To determine whether or not detectable apoptosis occurs in paracetamol-induced liver cell injury.
  * To determine the kinetics of any apoptotic changes relative to necrosis.
  * To investigate the expression of specific proteins known to play a role in apoptotic pathways in order to provide some insight as to the mechanism of apoptosis in
paracetamol-dosed livers.

By determining whether paracetamol-induced liver injury has an apoptotic component, other strategies could be developed for preventing injury after overdose.
Chapter 1A - Introduction to Paracetamol

1.1 Paracetamol

Paracetamol (acetaminophen) is an effective analgesic and antipyretic with weak anti-inflammatory activity, which is available without prescription and is present in many over-the-counter products. Paracetamol is moderately soluble in hot water. After an oral dose it is rapidly absorbed from the gastrointestinal tract as it readily crosses cell membranes and rapidly and relatively uniformly distributes to the different tissues. Gwilt et al (1963) demonstrated using a spectrophotometric method that the tissue:plasma ratio of paracetamol in the dog, 2 hours after an oral dose of 300 mg/Kg paracetamol, was approximately unity in the tissues studied with the exception of fat (Table 1.1). The plasma half-life of paracetamol is 2.0 ± 0.1 hours as Prescott (1971) demonstrated after measuring plasma paracetamol levels in 17 healthy adults using a gas-liquid chromatography method (1.5 - 1.8 g dose). Therapeutic doses of paracetamol lead to plasma levels of around 5 - 15 mg/L (≈0.1 mM).

Side effects at therapeutic oral doses of 0.5 - 1 g (3 to 4 times daily) are rare, however, in overdose, paracetamol can cause centrilobular liver necrosis and occasionally renal tubular necrosis. Unfortunately it has also become an increasingly popular choice for deliberate self-poisoning. Generally it is found that there is a high risk of illness or death from liver failure when the blood level is more than 200 mg/L of blood (≈1.3 mM) at 4 hours after ingestion.

There is a delay of several days between ingestion of an overdose and presentation of clinical symptoms. Although the mechanism of paracetamol-induced cell injury has been extensively researched over the years, the exact cell injury pathways involved in the late
stages of injury are still somewhat a mystery. Currently it is known that the early initiation stage involves oxidation of paracetamol to a toxic intermediate (N-acetyl-p-benzoquinone imine, NAPQI), and conjugation of this metabolite with glutathione leading to eventual depletion of cellular glutathione. For the late stages of injury many mechanisms involving the NAPQI metabolite have been studied but none of these completely accounts for the events leading up to liver cell injury (see section 1.7). More recently there has been interest in the role of apoptosis pathways in paracetamol-induced hepatotoxicity (see section 1.15). Hallmarks of apoptotic change were observed in the mouse in vivo and in isolated cells after exposure to cytotoxic doses of paracetamol (Shen et al, 1991; Ray et al, 1996; Wiger et al, 1997). It is also possible that other cellular effects of paracetamol and its metabolites contribute to the cell injury observed. For example, it has been shown that paracetamol can inhibit DNA synthesis both in vitro and in vivo (Hongslo et al, 1989; Lister and McLean, 1997).
Table 1.1. Paracetamol levels in different tissues after oral dosing

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>PARACETAMOL TISSUE LEVELS (mg/Kg TISSUE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>140 ± 12</td>
</tr>
<tr>
<td>Liver</td>
<td>144 ± 18</td>
</tr>
<tr>
<td>Kidney</td>
<td>149 ± 22</td>
</tr>
<tr>
<td>Heart</td>
<td>135 ± 19</td>
</tr>
<tr>
<td>Spleen</td>
<td>117 ± 16</td>
</tr>
<tr>
<td>Lung</td>
<td>125 ± 15</td>
</tr>
<tr>
<td>Brain</td>
<td>124 ± 17</td>
</tr>
<tr>
<td>Muscle</td>
<td>132 ± 16</td>
</tr>
<tr>
<td>Fat</td>
<td>18 ± 2</td>
</tr>
</tbody>
</table>

Tissue levels of paracetamol in 5 dogs, 2 hours after an oral dose of 300 mg/Kg. Adapted from Gwilt et al, 1963. Results are expressed as mean ± SEM.
1.2 Pharmacology of Paracetamol

Much of our early understanding of the antipyretic and analgesic properties of paracetamol comes from studies using phenacetin and acetanilide. Brodie and Axelrod (1948; 1949) later showed that paracetamol is the active metabolite of acetanilide and phenacetin in man.

1.2.1 Antipyretic activity

Flower and Vane (1972) were first to demonstrate that the antipyretic activity of paracetamol could be explained by central inhibition of prostaglandin synthesis. Paracetamol was shown to inhibit prostaglandin synthetase in rabbit and dog brain homogenates with an ID$_{50}$ of 14 μg/ml and 12.5 μg/ml, respectively, i.e. at therapeutic plasma concentrations. This was compared to an ID$_{50}$ of 100 μg/ml found in dog spleen, which suggests that at therapeutic plasma concentrations, paracetamol does not inhibit prostaglandin synthesis in the periphery.

1.2.2 Analgesic activity

The mechanism of action of the analgesic activity is far more complex and cannot be fully explained by prostaglandin synthesis inhibition. There is a lot of contrasting data in the literature that indicates that under different conditions, paracetamol may inhibit, stimulate, or have no effect on prostaglandin synthesis (reviewed by Prescott, 1996). Increasing evidence is emerging that the analgesic activity of paracetamol is mediated through central pathways. Piletta et al (1991) found that paracetamol increased the pain threshold in 10 human subjects after electrical sural nerve stimulation which evokes C-fibre reflex activity. But because this model only stimulates central pain pathways and not peripheral pathways no conclusions can be drawn regarding any peripheral action of paracetamol.

The specific central pathways that are involved in the analgesic effect are not known. There is some evidence that activation of spinal serotonergic pathways is involved. Tjolsen et al (1991) demonstrated in rats that lesioning of the serotonergic pathways abolished the reduction in pain-related behaviour induced by paracetamol after hind paw injection of formalin. Further evidence was demonstrated by Pelissier et al (1995) who
showed that tropisetron, a 5HT$_3$ receptor antagonist, reversed the effects of paracetamol on C-fibre evoked reflex activity after electrical nerve stimulation. Another pathway thought to be involved is the inhibition of L-arginine-nitric oxide pathways. Bjorkman et al (1994) demonstrated that the nociceptive behaviour of rats after intrathecal dosing with NMDA and substance P was antagonised by paracetamol and that this antagonism was reversed by administration of L-arginine, a natural substrate for nitric oxide synthase.

1.3 Normal Metabolism of Paracetamol

When paracetamol is ingested it is rapidly absorbed from the GI tract (mainly in the small intestine). Not all of the ingested paracetamol enters the circulation as a certain amount undergoes first pass metabolism in the liver and GI tract.

Paracetamol is mainly metabolised in the liver by conjugation with sulphate and glucuronide and excreted in the urine. A minor pathway of metabolism involves oxidation followed by conjugation with glutathione that breaks down to cysteine and mercapturic acid conjugates (Figure 1.2). Brodie and Axelrod (1949) showed that within the first 24 hours after administration of phenacetin (1 - 2 g) to human subjects, about 3.6 % of the drug was recovered in the urine as paracetamol, and about 75 % of the drug was recovered in the urine as sulphate and glucuronide conjugated paracetamol. In 3 normal volunteer subjects given doses of 0.4 - 4 g of paracetamol, Davis et al (1976) showed that 85 - 90 % of the drug was recovered in the urine as the parent compound and the various metabolites within the first 24 hours. The glucuronide metabolite made up 45 - 55 % of the total, the sulphate metabolite made up 20 - 30 %, the unmetabolised drug made up 0 - 2 % and the cysteine and mercapturate conjugates made up 15 - 25 %.

Oxidation of paracetamol is catalysed by the cytochrome P450 mixed-function oxidase system in the liver. This produces a short-lived reactive toxic intermediate, N-acetyl-p-benzoquinone imine (NAPQI), which is normally rapidly conjugated with glutathione and further broken down to mercapturic acid and cysteine conjugates. The P450 enzymes involved in paracetamol metabolism in man are 1A2, 2E1, and 3A4 (Raucy et al, 1989, Thummel et al, 1993).
1.4 Paracetamol Metabolism in Overdose

During overdose, increasing amounts of NAPQI are produced in the liver and these rapidly deplete cellular glutathione levels. When glutathione is sufficiently depleted the intermediate is no longer removed and can go on to cause hepatotoxicity and centrilobular necrosis. There is a long delay between presentation of clinical symptoms and ingestion of overdose.

Liver injury following oxidation and formation of a reactive metabolite through a P450-dependent pathway, was first demonstrated by Mitchell et al (1973a). They found that pre-treatment of rats and mice with inducers of cytochrome P-450 enzymes potentiated paracetamol-induced liver injury whereas inhibitors of cytochrome P-450 enzymes reduced the degree of injury observed.

There are different views as to why there is an increase in oxidation of paracetamol during overdose. One commonly held view is that glucuronidation and sulphation pathways become saturated and a larger proportion of paracetamol goes through the oxidation pathway. This was demonstrated by Davis et al (1976) in overdose patients and probably holds true for the sulphation pathway. However, Prescott (1983) has demonstrated that the glucuronidation pathway does not become saturated after overdose in man indicating that the proportion of paracetamol going through the glucuronidation and oxidation pathways remains constant but more oxidation occurs because of the presence of more substrate. The reported apparent Km and Vmax values for the sulphation pathway are 7.14 mmoles/person (~0.12 mM) and 0.65 mmoles/hour/person, respectively. And for the glucuronidation pathway, the apparent Km and Vmax values are 13.4 mmoles/person (~0.22 mM) and 2.8 mmoles/hour/person, respectively (Slattery and Levy, 1979). It has more recently been reported that the glucuronidation pathway has Km and Vmax values of 7.37 ± 0.99 mM and 4.76 ± 1.35 μmol/min/g protein, respectively (mean ± SD) (Miners et al, 1990).
Figure 1.2. Paracetamol metabolism pathways

Adapted from Jollow et al (1974).
1.5 Glutathione

Glutathione conjugation of the oxidised metabolite is an important mechanism for protection against paracetamol-induced injury. It is also important in the detoxification of many other reactive molecules and a co-factor for many enzyme systems, and is regarded as an essential cellular component without which the cell will die. Glutathione is a non-protein thiol present in all cells at concentrations ranging from 0.1 to 10 mM. In freshly isolated rat hepatocytes cellular levels of $3.8 \pm 0.55$ mM (mean $\pm$ SEM) have been reported (Leszczynska-Bisswanger and Pfaff, 1985). It is a tripeptide with the sequence $\gamma$-glutamyl-cysteine-glycine with is normally abbreviated to GSH. Oxidation of the thiol group of the cysteine residue to form a disulphide is usually abbreviated to GSSG. GSH can act as a nucleophile, forming conjugates with electrophilic compounds or as a reductant forming GSSG with free radicals or oxidising species.

![Figure 1.3. Structural formula of glutathione.](image)

Glutathione is synthesised by a 2-step reaction as follows:

$$
\text{\textit{\(\gamma\)-glutamylcysteine}} \text{ synthetase} \\
\text{L-cysteine + L-glutamate + ATP} \rightarrow \text{\(\gamma\)-glutamylcysteine + ADP + Pi} \quad (1)
$$

$$
\text{glutathione} \text{ synthetase} \\
\text{\(\gamma\)-glutamyl-cysteine + L-glycine + ATP} \rightarrow \text{GSH + ADP + Pi} \quad (2)
$$
Highly reactive radicals such as NAPQI are able to conjugate with GSH and are then converted in separate stages to the mecapturic acid derivatives. Firstly by removal of the γ-glutamyl moiety then removal of the glycine moiety to form the cysteine conjugate and then thirdly by N-acetylation of the cysteine conjugate to form the mercapturic acid derivative. GSH can also be oxidised to form GSSG, although this does not appear to be a major route in paracetamol detoxification (Vermeulen et al, 1992).

1.6 Extrahepatic Injury

Whilst the primary site of injury after paracetamol overdose is the liver, other organs have also been found to be affected. The kidney is another well-known site of injury. Newton et al (1983; 1985) have shown that paracetamol can cause nephrotoxicity in rats both in vivo and in vitro. Again this injury is associated with overproduction of NAPQI by cytochrome P450 metabolism. A further study in mice by Placke et al (1987) showed that 600 mg/Kg paracetamol induced injury in liver, kidney, lung, testes, and lymphoid tissues. Histological signs of injury in liver and kidney occurred as early as 2 hours after dosing. Signs of injury in the lung and testes began at 4 and 6 hours, respectively, and in lymphoid tissues injury did not occur until 24 hours after dosing. The mechanism of this late injury is unlikely to be due to NAPQI. Other cellular effects of paracetamol and its metabolites such as inhibition of DNA synthesis may be the mechanism.

1.7 Cellular Mechanism of Injury

Metabolism of paracetamol to its reactive metabolite takes place in the first few hours after ingestion but signs of cell injury appear some hours later. The initial delay is taken up by the time required to deplete glutathione in the liver. But after this point the exact mechanism of injury is not known. Various factors thought to be involved include covalent binding to cellular macromolecules, oxidative stress, disruption of calcium homeostasis, and disruption of mitochondrial function (for reviews see Nelson, 1990, and Vermeulen et al, 1992). Figure 1.4 illustrates some of the pathways thought to be involved in paracetamol-induced injury.
1.7.1 Covalent binding to cellular macromolecules

One of the earliest mechanisms discovered for paracetamol-induced liver injury involved the covalent binding of the reactive metabolite to cellular proteins. Studies have found that hepatotoxic doses of paracetamol covalently bind to mouse liver protein both in vivo and in vitro in a dose-dependent manner after a 70 % or more depletion in glutathione levels (Jollow et al, 1973; Mitchell et al, 1973b; Potter et al, 1973). It must be noted that because total glutathione levels in the liver were measured, it is not known if glutathione is lost uniformly from all hepatocytes. It is possible that certain hepatocyte populations within the liver, e.g. in the centrilobular region, could be more depleted than other regions making them more susceptible to injury.

More recent studies have demonstrated that injury can be inhibited with no significant change in the degree of covalent binding. In vivo studies in mice have shown that some compounds that protect from liver injury do so without reducing the degree of covalent binding (Labadarios et al, 1977). In vitro studies with rat liver slices and isolated hepatocytes have also shown protection from injury without reduction in covalent binding (Devalia et al, 1982; Devalia and McLean, 1983; Beales et al, 1985). Overall it has been found that covalent binding to cellular macromolecules occurs during paracetamol-induced liver injury but it may be a side effect of injury rather than a cause of injury.

1.7.2 Disruption of calcium homeostasis

The intracellular calcium (Ca$^{2+}$) concentration in normal cells is far lower (10$^{-7}$ M) than the extracellular concentration (10$^{-3}$ M). The low level of Ca$^{2+}$ in the cell is maintained by sequestration into the endoplasmic reticulum and mitochondria, binding to protein, and active transport of calcium out of the cell. Prolonged elevation in intracellular Ca$^{2+}$ levels can activate cell injury pathways associated with depletion of energy stores, dysfunction of microfilaments and activation of degradative enzymes. There is much experimental evidence linking disruption of Ca$^{2+}$ homeostasis with paracetamol-induced injury (reviewed by Vermeulen et al, 1992). The mechanisms involved include depletion of glutathione levels and inhibition of the ability of mitochondria to sequester Ca$^{2+}$, all leading to an elevation in cytosolic Ca$^{2+}$. All this evidence points to dysregulation of Ca$^{2+}$ homeostasis as an important effector mechanism.
1.7.3 Oxidative stress and lipid peroxidation

The NAPQI metabolite of paracetamol is a strong oxidant. Many studies have attempted to elucidate whether oxidative stress underlies the mechanism of toxicity of paracetamol. Studies using rat liver slices and isolated hepatocytes have supported the idea of a pro-oxidant pathway by showing protection by certain free radical scavengers and antioxidants, although not all antioxidants were found to be effective (McLean and Nuttall, 1978; Devalia et al, 1982). Lipid peroxidation is one of the major mechanisms which can result from oxidative stress and was observed after paracetamol exposure in mice in vivo and in mice and rats in vitro (Wendel et al, 1979; Albano et al, 1983). However, more recent studies have found that inhibiting lipid peroxidation did not affect mouse liver injury in vivo (Younes and Siegers, 1985) and this has also been observed in isolated rat hepatocytes in vitro (Beales et al, 1985). This evidence suggests that lipid peroxidation is more likely to be a result rather that a cause of liver necrosis (reviewed by Vermeulen et al, 1992).

1.7.4 Mitochondrial function and ATP synthesis

Most of a liver cell's requirement for ATP is met by oxidative phosphorylation in mitochondria. Any impairment of respiratory activity can lead to loss of the mitochondrial membrane potential and a reduction in ATP synthesis. Lowering of intracellular ATP levels in the cell can contribute to cell death, although in the case of paracetamol-induced injury, a 70 % depletion in ATP levels alone is not a sufficient cause (Martin and McLean, 1995). Studies in isolated mouse hepatocytes and rat liver slices have found that mitochondrial function is impaired by paracetamol exposure before cell injury develops (Burcham and Harman, 1990; Nazareth et al, 1991).

A more recent study by Beales and McLean (1996) has found that the combination of fructose, cyclosporin A and trifluoperazine can protect rats from the late stages of paracetamol-induced liver cell injury in vivo and also in an in vivo/in vitro model. Fructose provides a substrate for glycolytic ATP production, cyclosporin A is able to block the non-specific permeability transition pore in the inner mitochondrial membrane and prevent the loss of the mitochondrial membrane potential, and trifluoperazine is
thought to protect the mitochondria by inhibiting phospholipase A₂ activity. Each of these compounds interacts closely with mitochondrial function indicating a key role of mitochondrial injury in paracetamol-induced hepatotoxicity.
Figure 1.4. Proposed pathways leading to cytotoxicity of paracetamol

Adapted from Nelson (1990).
1.8 Clinical Treatment of Overdose

1.8.1 Assessment

A nomograph of plasma paracetamol levels against time (see Figure 1.5) is used by clinicians to assess whether a patient requires treatment. If blood plasma paracetamol levels lie above the ‘treatment line’ at the corresponding time after ingestion then there is risk of liver injury and treatment is usually required. If levels are below the line, then treatment is not usually administered. The treatment line which is most widely used is the ‘200 line’ (Prescott et al, 1974; Rumack and Matthew, 1975). This has recently been modified in the US to the ‘150 line’ (Smilkstein et al, 1991).

Other factors that increase the toxic effects of paracetamol include a low protein diet, enzyme inducing drugs, and chronic alcohol abuse. Chronic alcohol abusers have lower glutathione levels in their livers and are therefore at higher risk from lower doses of paracetamol. For assessment of these patients the ‘100 line’ has been recommended (Vale and Proudfoot, 1995).

1.8.2 Treatment

The first line of therapy (within 6 hours of overdose) is to reduce absorption of paracetamol by giving activated charcoal or inducing emesis. Patients are then treated with either oral or i.v. N-acetyl cysteine (GSH precursor). If N-acetyl cysteine is given within 15 hours of overdose, little hepatotoxicity results. After this timepoint and up to 24 hours its effectiveness at preventing injury varies. After 24 hours giving N-acetyl cysteine has been found not to be very effective at preventing liver failure. However it has been reported that when administered between 10 - 36 hours after overdose, mortality in patients not receiving the antidote was 58 % compared to 37 % in those that did receive the antidote (Harrison et al, 1990).
Figure 1.5. Nomograph used to assess whether a person requires treatment after taking a paracetamol overdose

1.8.3 Paracetamol-methionine tablet

Another antidote, which can be used clinically to treat paracetamol poisoning, is oral methionine (also a precursor of GSH). This antidote is not used clinically very often because it is only effective if administered within 10 hours of overdose. It was first suggested that methionine should be included in paracetamol tablets by McLean (1974) and there is an ongoing debate about whether or not to include methionine in paracetamol tablets.

Arguments “for” include the prevention of impulsive overdosing amongst a group of ‘at risk’ people and reduction of the risk of unintentional therapeutic overdose. It has also been shown in controlled trials that the combination tablet is efficacious.

Arguments “against” include the lack of information regarding the side effects and long-term risks of methionine and the ethical issue of whether people who take paracetamol responsibly should be forced to take methionine also. Whilst the exact side effects and long term risks of methionine are not known, it is known that high doses of homocysteine, a metabolite of methionine, causes vascular toxicity. It is also not known how much methionine should be included in the tablet. Currently only one formulation called Paradote (500 mg paracetamol, 100 mg methionine) is on the market in Britain.

1.9 Other Therapeutic Properties of Paracetamol

Paracetamol has been reported to have therapeutic properties in addition to its antipyretic and analgesic action. In a case-control study of 300 cataract patients and 609 controls in Oxford (UK), van Heyningen and Harding (1986) reported that a larger percentage of control groups took analgesics compared to cataract patients and that paracetamol and other aspirin-like drugs had significant anticataract properties. Paracetamol was reported to reduce the relative risk of cataract to 0.42. In another case-control study of 563 epithelial ovarian cancer patients and 523 controls in eastern Massachusetts and New Hampshire (USA), Cramer et al (1998) reported that there was a reduced risk of ovarian cancer in women who took paracetamol on a daily basis, had been taking paracetamol for more than 10 years, or took more than 20 tablet years (tablets per day x years of use). The overall odds ratio for risk of ovarian cancer for paracetamol was 0.52 with a 95 % confidence interval of 0.31 - 0.86. The mechanism of these protective effects is not known.
Chapter 1B - Mechanisms of Cell Death

1.10 Cell Death

There are two major forms of cell death - necrosis and apoptosis (reviewed by Wyllie and Duvall, 1992). Although there is growing evidence that these two forms of cell death have overlapping characteristics they are generally differentiated by their morphology (summarised in Table 1.2). During necrosis, the cell becomes swollen due to intake of water whereas during apoptosis, the cell shrinks, as water is lost. Cell shrinkage leads to loss of cell-cell contacts and cell surface microvilli, and cytoplasmic condensation. The pattern of disruption of the organelles also differs. During necrosis, the mitochondria and smooth endoplasmic reticulum become swollen and very early on the rough endoplasmic reticulum lose their ribosomes; the nucleus remains relatively intact. During apoptosis, chromatin condenses, DNA is cleaved into multiples of 180 - 200 base pairs and nuclear fragmentation occurs, but all other organelles are relatively intact at first. DNA fragmentation also occurs during necrosis but unlike apoptosis the DNA fragments are not of any particular size.

Blebbing from the plasma membrane occurs at the end of both necrosis and apoptosis with differing results. At the end of necrosis, the internal and plasma membranes rupture, which leads to leakage of cellular contents and causes an inflammatory response and scarring. At the end of apoptosis, the plasma membrane involutes and forms membrane-bound vesicles called apoptotic bodies. These are rapidly engulfed by neighbouring cells or phagocytes and broken down in lysosomes. An inflammatory response is avoided as cellular contents are contained within the apoptotic bodies.

1.11 Necrosis

Necrosis occurs after a severe alteration of the cellular environment that can lead to an inflammatory response. Injury can be caused by virus infections, hypoxia, toxins (e.g. paracetamol), attack by complement, or extremes of temperature (Wyllie and Duvall, 1992). Necrosis often involves a large volume of cells and early changes such as mild dilatation of organelles, dissociation of ribosomes from the rough endoplasmic reticulum
and slight ‘blebbing’ of the cell membrane are reversible. Late changes such as massive swelling of the mitochondria and organelles and membrane rupture are irreversible and cell death is inevitable. The changes in the cell that occur during necrosis are illustrated in Figure 1.6.

1.12 Apoptosis

Apoptosis, also known as programmed cell death, occurs in many normal physiological processes, e.g. during development of organs and tissues, in tissue homeostasis, and in the immune defence system. This form of cell death is regulated by gene expression and allows individual cells to die without causing a widespread inflammatory response so unwanted cells are removed with minimum disruption to surrounding healthy cells (Wyllie and Duvall, 1992). The morphological changes in apoptosis are illustrated in Figure 1.7.

It must be noted that whilst apoptosis works well for the removal of small volumes of cells or individual cells, if a large volume of adjacent cells are apoptosing and there are not enough healthy cells to remove the dying cells, secondary necrosis results (Ogasawara et al., 1993). Apoptosis can also contribute to or cause pathological processes. This can be due to inappropriate activation or inactivation of apoptosis.

Apoptosis can be divided into different phases. The first phase is the internal or external stimulus signal that causes apoptosis. The next phase is the detection of the stimulus signal and transduction of the signal to the effector phase. The effector phase includes the activation of the caspases, a family of proteolytic enzymes and their positive and negative regulators. The Bcl-2 family of proteins either inhibit or promote the proteolytic activity of the caspases. At this point the cell is not yet committed to dying. Only when the promotory signal is stronger or the inhibitory signal is absent will the cell progress to the next step. The activated caspases cleave their substrates and activate or inactivate different proteins, which eventually lead to the death of the cell. The final phase is the removal of the dead cell.

Figure 1.8 shows a model of some of the apoptosis control pathways. This model does not include all known control pathways, for example, the role of cytochrome c and apoptosis inducing factor (AIF) in the mechanism of apoptotic cell death are not shown.
Table 1.2. Comparison of morphology of apoptosis and necrosis

<table>
<thead>
<tr>
<th></th>
<th>APOPTOSIS</th>
<th>NECROSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histology</strong></td>
<td>Single cells affected within living tissues</td>
<td>Sheets of cells die together, disrupting tissue structure</td>
</tr>
<tr>
<td><strong>Cytology</strong></td>
<td>Pyknotic nuclei, condensed cytoplasm, apoptotic bodies (rounded cell fragments)</td>
<td>Cellular oedema</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nuclei intact but stain faintly</td>
</tr>
<tr>
<td><strong>Dye exclusion tests</strong></td>
<td>Dyes initially excluded</td>
<td>Dyes enter</td>
</tr>
<tr>
<td><strong>Ultrastructure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Compacted, intact organelles</td>
<td>Mitochondria show high amplitude swelling and matrix densities</td>
</tr>
<tr>
<td></td>
<td>Dilated endoplasmic reticulum</td>
<td>Dilated organelle profiles</td>
</tr>
<tr>
<td></td>
<td>Plasma membrane intact</td>
<td>Ruptured plasma and internal membranes</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Chromatin condensed in caps and toroids</td>
<td>Coarse chromatin patterns which retain normal distribution</td>
</tr>
<tr>
<td></td>
<td>Nucleolar disintegration</td>
<td></td>
</tr>
<tr>
<td><strong>Circumstances</strong></td>
<td>Often in ‘programmed cell death’</td>
<td>Never physiological</td>
</tr>
<tr>
<td></td>
<td>Atrophy</td>
<td>Complement</td>
</tr>
<tr>
<td></td>
<td>Cell-mediated immune killing</td>
<td>Hypoxia</td>
</tr>
<tr>
<td></td>
<td>Toxins (low dose)</td>
<td>Toxins (high dose)</td>
</tr>
<tr>
<td><strong>Tissue effects</strong></td>
<td>No inflammation</td>
<td>Acute inflammation</td>
</tr>
<tr>
<td></td>
<td>Phagocytosis by adjacent cells</td>
<td>Scarring later</td>
</tr>
<tr>
<td></td>
<td>Rapid involution without collapse of overall tissue</td>
<td></td>
</tr>
<tr>
<td></td>
<td>structure</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Wyllie and Duvall (1992).
Figure 1.6. Representation of the major morphological events in necrosis

Adapted from Wyllie and Duvall (1992).
Figure 1.7. Representation of the major morphological events in apoptosis

1. Loss of surface contact
2. Shrinkage
3. Organelles intact
4. Nuclear change
5. Phagocytosis

Adapted from Wyllie and Duvall (1992).
Figure 1.8. A model of some of the apoptosis signal transduction pathways in mammalian cells

Arrows do not necessarily indicate direct interactions. Adapted from Vaux and Strasser (1996).
1.13 Mitochondrial Effects

Mitochondrial damage is a common step in response to both necrotic and apoptotic injury. During necrosis, the mitochondria appear denser, the inner membrane shrinks away from the outer membrane and there is impairment of oxidative phosphorylation and energy production. This leads to failure of the ionic pumps, followed by swelling and an accumulation of calcium and related dense bodies in the mitochondrial matrix (Wyllie and Duvall, 1992). Morphologically, the mitochondria appear to be intact during apoptosis but it has been found that the mitochondria play a central role in the control of apoptotic pathways (reviewed by Mignotte and Vayssiere, 1998).

An event that occurs during mitochondrial damage is an abrupt increase in the permeability of the inner membrane to solutes of molecular mass < 1500 Da, leading to the dissipation of the mitochondrial membrane potential. This is known as the mitochondrial permeability transition and occurs in both necrosis and apoptosis. Crompton et al (1988) and Szabo and Zoratti (1991) have demonstrated that there is a non-specific pore in the inner mitochondrial membrane. Opening of this pore leads to the mitochondrial permeability transition, and this opening can be blocked by cyclosporin A. The open state of this pore is promoted by calcium ions, inorganic phosphate, membrane depolarisation and oxidation of thiols in the pore complex. The closed state of this pore is promoted by magnesium ions, ADP, acidic matrix pH and high membrane potential (reviewed by Lemasters et al, 1998).

1.14 Model of Apoptosis - *Caenorhabditis elegans*

*C. elegans* is a nematode worm whose embryonic development is reproducible and precisely mapped. 131 of the 1090 somatic cells in the adult undergo apoptosis and the cell death abnormal genes (*ced*) that are responsible for apoptosis have been identified. Because of the similarities between programmed cell death in the nematode worm and mammalian cells, the nematode worm has become a very powerful model system for the analysis of the cell and molecular biology of apoptosis in mammalian cells (reviewed by Hale et al, 1996). Three genes have been found to control the death of all 131 cells, these are *ced*-9, *ced*-3 and *ced*-4. *ced*-9 is a suppressor of apoptosis and has been found to have sequence and structural similarities with a mammalian proto-oncogene, Bcl-2 (Hengartner and Horvitz, 1994). Both *ced*-3 and *ced*-4 are promoters of apoptosis. *ced*-3
has been found to be a homologue of the mammalian interleukin-1β-converting enzyme (ICE) (Yuan et al, 1993) and *ced-4* has been found to be a homologue of apoptotic protease-activating factor 1 (Apaf-1) (Zou et al, 1997).

### 1.14.1 Bcl-2 Family Proteins

The Bcl-2 family of proteins act upstream of the caspases. Bcl-2 was the first protein of this family to be isolated and sequenced and is considered to be the main anti-apoptotic protein. Members of this family of proteins have been found to be anti-apoptotic (Bcl-2, Bcl-X<sub>L</sub>, Bcl-w, Bfl-1, Brag-1, Mcl-1 and A-1) and pro-apoptotic (Bax, Bak, Bcl-X<sub>S</sub>, Bad, Bid, Bik and Hrk) (reviewed by Kroemer, 1997). In fact, under different experimental circumstances the same protein can function to prevent apoptosis or accelerate it (Kiefer et al, 1995). All Bcl-2 proteins contain at least one of four Bcl-2 homology (BH) domains. Most contain hydrophobic regions in their structure that localises them to the mitochondrial, nuclear, and sometimes endoplasmic reticulum membrane.

Bcl-2 family proteins can form heterodimers and homodimers. Different models have been proposed as to how the interaction of the Bcl-2 proteins determines the cell’s fate. One suggested model is that formation of a homodimer of Bax leads to a death signal and that heterodimerisation (with Bcl-2 or Bcl-X<sub>L</sub>) gives rise to cell survival. Another suggested model is that Bcl-2 and Bcl-X<sub>L</sub> actively repress apoptosis and that heterodimerisation to Bax and Bad blocks this active repression.

The localisation of many Bcl-2 proteins to the mitochondria has lead to much research into the interaction between the Bcl-2 proteins and mitochondria. The release of cytochrome c and apoptosis inducing factor (AIF) from the mitochondria have both been shown to trigger apoptosis by activating caspases (Susin *et al*, 1996; Zou *et al*, 1997). Bcl-2 has been shown to inhibit the release of cytochrome c whereas AIF and Bax promote the release of cytochrome c (Susin *et al*, 1996; Kluck *et al*, 1997; Rosse *et al*, 1998) demonstrating the importance of the Bcl-2 proteins in the mitochondrial control of apoptosis.

### 1.14.2 Caspases

The caspases are also known as the interleukin-1β-converting enzyme (ICE) -like
proteases because ICE was the first homologue of *ced-3* identified. This family of cysteine proteases is involved in different signalling pathways and has been implicated in many of the underlying effector mechanisms in apoptosis. The caspases are proteolytic enzymes that cleave the peptide bond immediately after aspartate residues in highly specific 4 amino acid cleavage sites. Table 1.3 shows some substrates of caspases, their cleavage sites and effects of cleavage.

Caspases are normally present in the cell as pro-enzymes and require cleavage to become activated. Cleavage of caspases leads to formation of a large (17-20 kDa) and small subunit (9-12 kDa) that dimerise to form the active protease. Although there is some evidence that caspases can be homo-activators, i.e. able to activate themselves, activation by proteases (including other caspases) is viewed as the classic way of activating caspases (reviewed by Stennicke *et al*, 1998).

### 1.14.3 Apaf-1

Whilst mammalian homologues for *ced-9* and *ced-3* were found early in the 1990's the mammalian homologue for *ced-4* was elusive until Zou *et al* (1997) identified a protein named Apaf-1 that showed 22 % identity and 48 % similarity to *ced-4*. Zou *et al* (1997) further demonstrated that Apaf-1 then activates caspase 3 by binding to cytochrome c.
Table 1.3. Some substrates of caspases, their cleavage sites and effects of cleavage

<table>
<thead>
<tr>
<th>CASPASE</th>
<th>SUBSTRATE</th>
<th>CLEAVAGE SITE</th>
<th>EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 1</td>
<td>ProIL-1β</td>
<td>YVHD/A</td>
<td>Activates IL-1β</td>
</tr>
<tr>
<td></td>
<td>ProIGIF (IL-18)</td>
<td>LESD/N</td>
<td>Activates IGIF</td>
</tr>
<tr>
<td>Caspases 3 and 7</td>
<td>DFF</td>
<td>DETD/S</td>
<td>Initiates DNA fragmentation</td>
</tr>
<tr>
<td></td>
<td>PARP</td>
<td>DEVD/G</td>
<td>Inactivates DNA repair</td>
</tr>
<tr>
<td></td>
<td>PKCδ</td>
<td>DMQD/N</td>
<td>Activates kinase</td>
</tr>
<tr>
<td></td>
<td>MEKK</td>
<td>DTVD/G</td>
<td>Activates kinase</td>
</tr>
<tr>
<td></td>
<td>U1-70kD</td>
<td>DGPD/G</td>
<td>Inactivates mRNA splicing</td>
</tr>
<tr>
<td>Caspase 6</td>
<td>Lamin A</td>
<td>VEID/N</td>
<td>Nuclear collapse</td>
</tr>
<tr>
<td></td>
<td>Keratin 18</td>
<td>VEVD/A</td>
<td>Cytoskeletal collapse</td>
</tr>
</tbody>
</table>

1.15 The Role of Apoptosis Pathways in Paracetamol-induced Liver Cell Injury

Whilst liver necrosis induced by paracetamol overdose has been extensively studied over the years it has only recently begun to be reported that apoptosis may be involved in paracetamol-induced liver injury. Ray et al (1996) found that after i.p. dosing of mice with hepatotoxic doses of paracetamol, the characteristic hallmarks of apoptotic change, i.e. DNA fragmentation and nuclear condensation, occurred before the onset of necrosis. Shen et al (1991) found that DNA fragmentation occurred in isolated mouse hepatocytes after exposure to paracetamol before the onset of necrotic injury. Another study in HL-60 cells also found nuclear condensation and fragmentation after exposure to 2 - 3 mM paracetamol (Wiger et al, 1997).

Reviews of the literature have focused on the killing of hepatocytes as well as the possible genotoxic and carcinogenic effects of paracetamol. These have overall concluded that any damaging effects occurred at doses above certain threshold levels which are not reached at therapeutic doses (reviewed by Bergman et al, 1996). The role of apoptosis in paracetamol-induced liver injury has not been thoroughly investigated and it is not known if apoptosis is occurring in the livers of people taking therapeutic doses or overdoses. There may be an underlying increase in apoptosis in the liver which has not been detected before. The present study is attempting to fill in the gap in our knowledge.
Chapter 1C - Inhibition of DNA Synthesis by Paracetamol

1.16 Inhibition of DNA Synthesis by Paracetamol

As well as its analgesic and antipyretic therapeutic properties, paracetamol has been shown to specifically inhibit replicative DNA synthesis in V79 cells at close to the therapeutic plasma level of 0.1 mM (Hongslo et al, 1989). This inhibition was demonstrated to be due to quenching of the tyrosyl free radical present in the active site of ribonucleotide reductase (Hongslo et al, 1990). This property is similar to that of hydroxyurea, a well-known DNA synthesis inhibitor which also inhibits ribonucleotide reductase activity by destroying the tyrosyl free radical (Krakoff et al, 1968; Thelander et al, 1985). The present study is attempting to determine whether the concentrations found in therapeutic use or overdose are likely to inhibit DNA synthesis in humans. The question remains whether inhibition of DNA synthesis contributes to, or actually protects from cell injury.

Paracetamol is known to have antioxidant and radical scavenging properties (Binkova et al, 1990; Woollard et al, 1990) and it has also been observed that paracetamol inhibits DNA synthesis both in vitro and in vivo (Hongslo et al, 1989; Lister and McLean, 1997). The question remains whether it is the antioxidant or anti-DNA replication property of paracetamol, which is mediating the other long-term therapeutic properties (non-analgesic and non-antipyretic) mentioned in section 1.9.

1.17 Ribonucleotide Reductase

Ribonucleotide reductase or ribonucleoside diphosphate reductase (EC 1.17.4.1) is an enzyme that is involved in the de novo synthesis of all four deoxyribonucleotides by catalysing the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates. This is a rate-limiting step in DNA synthesis and inhibition of this enzyme stops synthesis of deoxyribonucleotides which in turn leads to disruption of the deoxyribonucleotide pools and inhibition of DNA synthesis (reviewed by Thelander and Reichard, 1979). The mammalian ribonucleotide reductase is a class I ribonucleotide
reductase and is made up of two different types of subunit, a large subunit called R1 (or M1) and a small subunit called R2 (or M2). Both subunits are homodimers and they weakly bind together to form the active enzyme. The R1 subunit contains allosteric and substrate binding sites and thiol groups which donate hydrogen to the substrate during catalysis and the R2 subunit contains a binuclear iron centre and a stable tyrosyl free radical which is essential to catalytic activity (Uhlin and Eklund, 1994).

Figure 1.9 illustrates the mechanism by which ribonucleotide reductase catalyses the reduction of ribonucleoside diphosphate. The enzyme utilises the stable free radical in the active site to catalyse the replacement of the hydroxyl group at position 2 of the ribose sugar with a hydrogen atom. The donation of a hydrogen atom results in the formation of oxidised thioredoxin, which is re-reduced at the expense of NADPH. In *E.coli* and some animal tissues, glutaredoxin is utilised instead of thioredoxin.

Paracetamol has been found to specifically inhibit DNA synthesis by inhibiting ribonucleotide reductase activity. It has been demonstrated by Hongslo *et al* (1990) that a hydroxyurea-resistant mouse mammary tumour cell line (TA3H2), which is shown to overproduce the small subunit of ribonucleotide reductase, is more resistant to DNA synthesis inhibition by paracetamol than wild type cells (TA3H). Moreover, it was shown that paracetamol inhibits DNA synthesis by quenching the tyrosyl free radical in the active site of ribonucleotide reductase. The present study is attempting to determine whether the concentrations found in therapeutic use or overdose are likely to inhibit DNA synthesis in humans.

Iron chelators are also known to inhibit ribonucleotide reductase activity by depleting cellular iron and preventing regeneration of the iron centre of the M2 subunit of ribonucleotide reductase which is essential for enzyme activity (Hoyes *et al*, 1992; Cooper *et al*, 1996).
The thioredoxin system operates in all cells containing ribonucleoside diphosphate reductase activity. Glutaredoxin can also supply reducing power, but is generally thought to be less important in vivo. Adapted from Halliwell and Gutteridge (1999).
1.18 Examples of Compounds that Inhibit DNA Synthesis

There are many compounds that are DNA synthesis inhibitors that can act at different points in the DNA synthesis pathway.

1.18.1 Hydroxyurea

Hydroxyurea is a DNA synthesis inhibitor which specifically inhibits ribonucleotide reductase by destroying the tyrosyl free radical present in the active site of this enzyme (Krakoff et al, 1968; Thelander et al, 1985). It is used clinically as an anti-neoplastic and anti-sickle cell disease drug and is readily absorbed in the GI tract. Hydroxyurea has a plasma half-life of approximately 2 hours. Approximately 80% of the drug is recovered in the urine within 12 hours after either oral or i.v. administration (Calabresi and Chabner, 1990). In clinical use as an anti-neoplastic drug, hydroxyurea is administered either by (i) continuous daily oral doses ranging from 20 - 40 mg/Kg/day or (ii) intermittent oral doses of 80 mg/Kg every third day. An oral dose of 40 - 80 mg/Kg hydroxyurea, leads to peak plasma levels of 0.5 - 2 mM within 1 - 2 hours of administration (Donehower, 1992). Hydroxyurea is an example of a drug with anticancer properties that is based on its ability to inhibit DNA synthesis via a free radical mechanism. It is possible that paracetamol has similar therapeutic activity.

1.18.2 Aphidicolin

Aphidicolin is a tetracyclic diterpenoid antibiotic that inhibits DNA synthesis by specific inhibition of DNA polymerase α with no effects on the DNA polymerases β or γ (Ikegami et al, 1978; Ohashi et al, 1978). Aphidicolin has antiviral and anti-mitotic activity and acts by competing with deoxycytidine-5'-triphosphate (dCTP) binding to DNA polymerase α (Pedrali-Noy and Sparadi, 1980). Experiments using murine and human neoplastic cells have shown that aphidicolin inhibits cell growth (measured by \(^3\)H-thymidine incorporation) by 50% at doses of 0.04 - 0.09 µg/ml. Furthermore, RNA
and protein synthesis were not affected at 100-fold higher doses (Pedrali-Noy et al., 1982).

1.18.3 Cytosine arabinoside

Cytosine arabinoside also known as 1-β-D-arabinofuranosylcytosine (ara-C) is an example of a DNA synthesis inhibitor that is a nucleotide analogue. Ara-C must first be converted to cytosine arabinoside triphosphate (ara-CTP) by a series of kinases in the cell before it can inhibit DNA synthesis. Ara-CTP is thought to block DNA synthesis by being incorporated into DNA and preventing further elongation of the DNA chain and thereby causing chain termination. Major et al (1982) have shown in L1210 cells that inhibition of $^3$H-thymidine incorporation increased with increasing levels of ara-C incorporation into DNA. Ara-C is used clinically as an anti-neoplastic drug and is most effective against human acute myelogenous leukaemia (Kufe et al, 1985).
Chapter 1D - Introduction to Antioxidants

1.19 Historical Perspective of Antioxidants

It is a well-known fact that rancidity occurs in fats on storage. This is due to spontaneous oxidation on exposure to oxygen. Cellular lipids are also subjected to attack by oxygen and its derivatives, and the cell has evolved antioxidant defence mechanisms to protect itself from this attack. Vitamin E is one of the earliest lipid-soluble antioxidants studied and probably one of the most important inhibitors of the chain reaction of lipid peroxidation. Its importance in the diet is illustrated by the fact that diets low in vitamin E induce sterility in female laboratory animals (Evans and Burr, 1927). Administering diets that contain high vitamin E levels reverses this effect. It was also reported by Evans and Burr (1927) that an increase in dietary saturated fats lead to a higher requirement for vitamin E; probably due to increased levels of lipid peroxidation.

Subsequently, the use of antioxidants was mostly restricted to industrial purposes and it wasn’t until the 1970's when studies showed that antioxidants could protect experimental animals from the carcinogenic effects of different compounds that interest in the potential therapeutic effects of antioxidants arose. Wattenburg (1972) was one of the first to report that phenolic antioxidants could inhibit the carcinogenic effects of benzo(α)pyrene and 7,12-dimethylbenz(α)anthracene in rodents. In the present day, research is centred on investigating the nutritional value and therapeutic properties of antioxidants.

1.20 Reactive Oxygen Species and Antioxidants

Oxygen is a highly reactive substance that can form even more damaging reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide anion radical (O₂⁻) and hydroxyl radical (OH⁰). Some of these are free radicals (any species capable of independent existence that contains one or more unpaired electrons) and some of these are non-radicals. ROS can react with cellular components such as DNA, protein, lipids, and carbohydrate leading to dysfunction and oxidant damage. The cell employs a range of primary antioxidant defences to protect against this oxidant damage.
A general definition of an antioxidant is ‘any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate’ (Halliwell, 1995). An oxidisable substrate can be any biological component of the cell.

Antioxidants can be broadly divided into two different groups, non-enzymatic and enzymatic. Some of the most common non-enzymatic antioxidants include ascorbates, tocopherols, carotenoids, uric acid, glutathione and flavonoids. These are either ingested in the diet or produced endogenously. Another dietary source of antioxidants is the synthetic compounds that are used as preservatives in the food industry. These include the gallates, ethoxyquin, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Enzymatic antioxidants include superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-POD).

1.21 Mechanism of Action of Non-Enzymatic Antioxidants

Various mechanisms can be employed by non-enzymatic antioxidants to prevent cell injury by ROS or their products. An antioxidant can employ a combination of any of the following mechanisms to either prevent the formation of ROS or remove the species rapidly after formation.

1.21.1 Free radical scavengers

Many ROS are free radicals. Free radicals can react with other molecules by donating its unpaired electron, abstracting an electron (or hydrogen atom), or joining onto another molecule. When a free radical reacts with a non-radical molecule, the non-radical becomes a free radical. This free radical may then go on to react with another non-radical and form a further free radical. If left unchecked, this continues in a chain reaction until the free radical reacts with another free radical (termination reaction). Free radical scavengers stop this amplifying chain reaction by reacting directly with free radicals. When this occurs the free radical scavenger itself becomes a free radical. The oxidant properties and stability of the products formed determines whether it can initiate further radical production or stop it. \( \alpha \)-tocopherol (vitamin E) is an example of a lipid-soluble free radical scavenger and will be discussed further in section 1.27.
1.21.2 Reducing agent

Some antioxidants are readily oxidisable compounds that protect cellular components from oxidative damage by being preferentially oxidised by reactive oxygen species. When the antioxidant is oxidised it may also become a free radical. As a rule, antioxidants can become 'pro-oxidants' under certain circumstances, e.g. high concentrations. Again the stability of the radical formed determines how effective the antioxidant is and whether it will have pro-oxidant properties. Ascorbate (vitamin C) is an example of a water-soluble cytosolic antioxidant that is a powerful reducing agent.

1.21.3 Hydrogen donor

A hydrogen atom contains a single electron. When a hydrogen atom is abstracted from an organic compound it leaves an unpaired electron, i.e. the organic compound becomes a free radical. Hydrogen donors can remove this free radical by donating an electron. When a hydrogen atom reacts with a free radical a termination reaction occurs and stops further free radical production. Again the stability of the hydrogen donor which becomes a radical after losing a hydrogen atom determines how effective the antioxidant is.

1.21.4 Transition metal ion chelator

Transition metals can promote free radical generation by different routes. One route involves a spontaneous reaction of the reduced forms of the metal ions (Me^{(n-1)+}), e.g. Fe (II) and Cu (I), with oxygen (O_2) to produce O_2^-\textsuperscript{−}. The oxidised metal ions (Me^{n+}) can then react with O_2^-\textsuperscript{−} to regenerate the reduced transition metal that can again react with oxygen and so on. This continuous oxidation and reduction leading to formation of ROS is known as redox cycling.

\[
\text{Me}^{(n-1)+} + \text{O}_2 \rightarrow \text{Me}^{n+} + \text{O}_2^-\textsuperscript{−} \\
\text{Me}^{n+} + 2\text{O}_2^-\textsuperscript{−} + 2\text{H}^+ \rightarrow \text{Me}^{(n-1)+} + \text{H}_2\text{O}_2 + \text{O}_2
\]

Another route of free radical generation involves the reduction of H_2O_2 to OH\textsuperscript{−}.
radical, one of the most reactive free radical species. One of the most studied transition metals is iron. The reaction of Fe (II) with $H_2O_2$ is called the Fenton reaction and involves the formation of intermediate complexes.

$$Fe(II) + H_2O_2 \rightarrow FeOH^{3+} (or \ FeO^{2+}) \rightarrow Fe(III) + OH^- + OH^- \ \text{Fenton reaction}$$

Fe (III) can then be reduced back to Fe (II) by reaction with $O_2^-$. The net result of these reactions (omitting intermediate complexes) is known as the ‘iron-catalysed Haber-Weiss reaction’.

$$Fe(III) + O_2^- \leftrightarrow Fe(II) + O_2$$
$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH^-$$

Net: $O_2^- + H_2O_2 \rightarrow O_2 + HO^- + OH^- \ \text{‘Iron-catalysed Haber-Weiss reaction’}$

The generation of ROS by transition metals means that intracellular concentrations must be kept very low. This is usually the case because metal ions are normally protein bound in the cell. By removing transition metals from the cell, chelators can prevent the formation of the highly reactive $OH^-$ radical making them very effective antioxidants. Calcium EDTA is one of most commonly used metal chelators in both experimental and therapeutic use.

1.22 Enzymatic Antioxidants

Three of the major enzymatic antioxidants present in the cell are superoxide dismutase (SOD), glutathione peroxidase (GSH-POD) and catalase. SOD catalyses dismutation of superoxide by the following reaction:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The $H_2O_2$ produced by SOD is then catalytically broken down by catalase and GSH-POD to produce water and oxygen. GSH-POD requires glutathione (GSH) to breakdown $H_2O_2$. The oxidised glutathione (GSSG) produced by this reaction is reduced back to
GSH by glutathione reductase.

Catalase : \( \text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \)

Glutathione peroxidase : \( \text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \)

Glutathione reductase : \( \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \)

In addition, GSH-POD can also reduce lipid hydroperoxides, an intermediate produced during lipid peroxidation.

### 1.23 Lipid Peroxidation

Lipid peroxidation is one of the major mechanisms of cellular injury caused by ROS. ROS can initiate lipid peroxidation by abstracting a H atom from polyunsaturated fatty acids (PUFAs) leading to formation of a conjugated diene that on rearrangement will readily combine with oxygen to form a lipid peroxyl radical. This radical in turn abstracts a H atom from an adjacent PUFA (propagation) and forms a lipid hydroperoxide and a new lipid free radical that can go on and repeat the whole process in a chain reaction. The lipid hydroperoxide is then converted to lipid alkoxyl radical which then breaks down to other reactive species such as aldehydes, alkanes, alcohols and lipid epoxides (Figure 1.11). If the chain reaction is not terminated by chain-breaking antioxidants and enzymatic removal of peroxides, accumulation of peroxidised lipids in the membrane will affect fluidity, permeability and in extreme conditions, membrane integrity. This can lead to organelle dysfunction, release of lysosomal enzymes and ultimately cell death. As well as injury to the cell membrane, reactive products of lipid peroxidation can diffuse to other cellular components and cause injury (Gregus and Klaassen, 1996).
Figure 1.11. Mechanism of lipid peroxidation

1.24 Antioxidants in the Diet

There are many naturally occurring and synthetic antioxidants present in the diet. Ascorbates, tocopherols, and flavonoids are the most common naturally occurring antioxidants. Synthetic antioxidants include the gallates, ethoxyquin, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). These are added to foods and packaging and a certain amount are ingested in the diet. As well as ingesting naturally occurring antioxidants in their food many people take large quantities of antioxidant supplements in the hope that they can prevent or ameliorate the symptoms of diseases that have been associated with oxidative damage.

1.25 Levels of Synthetic Antioxidants Allowable in Foods

Legislation regarding the levels allowable in foods and ADI (allowable daily intake) have been set for the main types of synthetic antioxidants used (see Table 1.4). Ascorbates and tocopherols do not have ADI’s, and levels in foods are not restricted by legislation as normal daily intake from other sources is high compared to intake as a food additive. The estimated average daily intake of tocopherols in the USA is 5 - 20 mg per person (Tomassi and Silano, 1986).
Table 1.4. Allowable levels and ADI’s of selected synthetic antioxidants in foods

<table>
<thead>
<tr>
<th>ANTIOXIDANT</th>
<th>MAXIMUM ALLOWABLE LEVEL*</th>
<th>FOOD TYPE*</th>
<th>ADI mg/kg body weight/day**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallates (propyl, octyl &amp; dodecyl)</td>
<td>100 mg/kg total mixture or any one of, 1000 mg/kg total mixture or any one of,</td>
<td>Dairy products, edible fats and oils, vitamin oils, emulsifiers and stabilisers Essential oils</td>
<td>0 - 0.5 (sum of gallates)</td>
</tr>
<tr>
<td>BHA / BHT</td>
<td>200 mg/kg total mixture or any one of, 1000 mg/kg total mixture or any one of, 25 mg/kg total mixture or any one of, 140 mg/kg total mixture of, 140 mg/kg total BHT only</td>
<td>Dairy products, edible fats and oils, vitamin oils, emulsifiers and stabilisers Essential oils Potato powder, flakes and granules Walnuts (shelled) Chewing gum</td>
<td>Temporary values 0 - 0.5 (BHA) 0 - 0.05 (BHT)</td>
</tr>
<tr>
<td>Ethoxyquin</td>
<td>3 mg/kg total</td>
<td>Apples and pears</td>
<td>-----</td>
</tr>
</tbody>
</table>

1.26 Oxidative Damage and Disease

Oxidative damage by free radicals and ROS has been associated with the pathophysiology of many different diseases such as cancers, cataract, and atherosclerosis. Non-enzymatic antioxidants have in recent years become the focus of much attention due to their possible therapeutic potential in certain diseases, especially in prevention of atherosclerosis and many cancers. Several dietary antioxidants that are thought to be important in the prevention of atherosclerosis and cancers will now be discussed in detail.

1.27 Vitamin E

Vitamin E is the major lipid-soluble chain-breaking antioxidant present in the cell involved in protection of PUFAs from lipid peroxidation. There are eight naturally occurring forms of vitamin E including α-, β-, γ, and δ-tocopherols and tocotrienol. Vitamin E acts as a free radical scavenger and reacts with the lipid radicals produced during lipid peroxidation, thereby causing a termination of the chain reaction. Recent in vitro studies suggest that the tocopheryl radical that is produced, is reduced back to vitamin E by ascorbate (Niki, 1991). However this is yet to be confirmed in vivo (reviewed by Halliwell et al., 1992).

High intake of vitamin E in the diet has been associated with protection from cardiovascular disease and a lower risk of some cancers. Epidemiological studies conducted in the USA have found that high intake of vitamin E supplements reduced the risk of coronary heart disease in both men and women (Rimm et al., 1993; Stampfer et al., 1993). The Cambridge Heart Antioxidant Study (CHAOS), a double-blind, placebo-controlled study in patients with coronary atherosclerosis, showed that patients receiving α-tocopherol treatment had a significantly lower risk of cardiovascular death and non-fatal myocardial infarction, though the number of ‘cardiac events’ was unaltered (Stephens et al., 1996). Vitamin E has been reported to inhibit low density lipoprotein (LDL) oxidation which is thought to be a key process in atherosclerosis (Sato et al., 1990).

Studies of the effects of vitamin E on the risk of some cancers have produced mixed results. A Finnish study with 453 males showed a 0.7 adjusted relative risk of cancer in
the two highest quintiles of blood vitamin E levels (Salonen et al, 1985). A UK study reported that serum vitamin E levels were significantly lower in patients subsequently diagnosed with cancer within one year after blood collection but not in other cancer patients (Wald et al, 1987). However, a study in the USA showed no relationship between blood antioxidant levels and subsequent risk of cancer (Willett et al, 1984).

1.28 Phenolic Compounds As Antioxidants

Phenolic compounds are often found to have antioxidant properties due to the hydrogen donating ability of the hydroxyl group. Paracetamol is one such compound. Phenolic acids such as the hydroxybenzoic, hydroxycinnamic, and hydroxyphenylacetic acids are well known antioxidants of plant origin. As a general rule, the higher the number of hydroxyl groups in the molecule the better its antioxidant activity (Rice-Evans et al, 1996).

1.29 Flavonoids

Flavonoids are a large group of naturally-occurring polyphenolic compounds found in high concentrations in tea, wine, fresh fruit, and vegetables. Figure 1.12 shows the structural formulae of the different subclasses of flavonoids. The antioxidant activity of flavonoids is thought to be due to a combination of free radical scavenging and iron chelating properties. Many reports have shown flavonoids to be scavengers of peroxyl radicals, superoxide radicals and hydroxyl radicals (Torel et al, 1986; Husain et al, 1987; Robak and Gryglewski, 1988) and various studies have shown iron chelating activity (Afanas’ev et al, 1989; Morel et al, 1993; van Acker et al, 1996). Flavonoids can act in both the aqueous phase and the lipid phase.

The Trolox equivalent antioxidant activity (TEAC) or total antioxidant activity (TAA) is a measure of the antioxidant activity of a putative antioxidant in the aqueous phase. The interaction of metmyoglobin with 2,2’-azinobis-(3-ethyl benzthiazoline-6-sulphonic acid) (ABTS) in the presence of hydrogen peroxide leads to the formation of the ABTS⁺⁺ radical cation that absorbs strongly at 734 nm. The TEAC measures the ability of hydrogen-donating antioxidants to scavenge the ABTS⁺⁺ radical cation and compares this with Trolox, a water-soluble vitamin E analogue. The TEAC is defined as the concentration of Trolox solution with equivalent antioxidant potential to a 1 mM
concentration of the compound under investigation (reviewed by Rice-Evans, 1996). Table 1.5 shows the TEAC values of some flavonoids. As the values indicate a large number of flavonoids are much stronger antioxidants than Trolox in this assay, quercetin in particular has a very high mean TEAC value of 4.7 mM.

Estimates of the daily flavonoid intake in the Western diet range from 23 mg to 1 g per person (Kuhnau, 1976; Hertog et al., 1993). Limited information is available on the absorption and metabolism of flavonoids in humans. Many flavonoids occur in the glycosylated form in foods and it was thought that these glycosylated forms did not pass through the gut wall. However, Hollman et al (1995) has demonstrated that 52% of the quercetin glycosides in onions and 17% of pure quercetin rutinoside was absorbed when orally administered to ileostomy patients. Furthermore, when quercetin aglycone (nonglycosylated) was administered 24% was absorbed. Metabolism of flavonoids in animal studies has shown that flavonoids can be broken down by many different mechanisms. Conjugation of hydroxyl groups with glucuronic acid or sulphate in the liver is the most common step of metabolism in mammals. These conjugates are excreted in the bile. The intestinal microflora plays a large part in the metabolism of flavonoids. They can hydrolyse glycosylated flavonoids, thereby releasing the aglycone to be absorbed in the intestine but at the same time they also degrade the flavonoid structure by ring scission. The phenolic compounds produced can then be reduced, oxidised, dehydroxylated, demethylated, or decarboxylated. The phenolic acids are absorbed and excreted in the urine (reviewed by Hackett, 1986).

The bioavailability of the ingested flavonoids is important if it is to have an in vivo effect. Hollman et al (1997) measured the plasma levels of quercetin over 36 hours after ingestion of one of the following: onions containing 225 ± 43 μmol (mean ± SD) of quercetin, apples containing 325 ± 7 μmol of quercetin, or a capsule containing 331 μmol of quercetin-3-O-β-rutinoside. The peak plasma concentration of quercetin was 224 ± 44 ng/ml (± 0.74 ± 0.15 μM) at 0.7 hours after ingestion of onions. For apples and rutinoside, the levels were 92 ± 19 ng/ml after 2.5 hours and 90 ± 93 ng/ml after 9 hours, respectively. The elimination half-life of flavonoids after consuming onions or apples was approximately 25 hours. These data suggest that significant levels of quercetin would be accumulated in the plasma after repeated ingestion of quercetin-containing foods.

A high dietary intake of flavonoids has been associated with a reduced risk of
cardiovascular disease. An epidemiological study in the Netherlands has found that a high intake of flavonoids in the diet is associated with a lower risk of death from coronary heart disease (Hertog et al, 1993). Another epidemiological study showed that the Southern French have a very low incidence of coronary heart disease despite having a high fat diet and smoking tendencies, the so-called ‘French Paradox’ (Renaud & De Lorgeril, 1992). The precise mechanism of action for this protective effect is not known. However, it is thought that it is in part due to the high consumption of red wine in the diet, which contains several flavonoids.

In order to examine the mechanism of the possible anti-cancer effects of flavonoids, studies on the growth of cancer cell lines have been conducted. Kaur et al (1992) showed that a novel flavone L86-8275, quercetin and genistein inhibited growth of a breast carcinoma cell line (MDA468). L86-8275 was further shown to inhibit the growth of several other breast carcinoma and lung carcinoma cell lines. Other studies have shown that inhibition of the proliferation of various tumour cells in culture is due to cell cycle arrest or by induction of apoptosis (Matsukawa et al, 1993; Wei et al, 1994). Activation of p53 may also play a role. It has recently been reported that apigenin (45 - 180 µM), luteolin (53 - 110 µM) and quercetin (60 - 120 µM) induce p53 accumulation in C3H10T1/2CL8 cells (Plaumann et al, 1996).

Quercetin makes up a large percentage of the flavonoids that are ingested in the diet. It has been found to be a highly effective antioxidant and has been studied extensively. Yoshida et al (1990) showed that quercetin dose-dependently inhibited the growth of four human gastric cancer cell lines, suppressed DNA synthesis and blocked cell cycle progression from G1 to S phase. Quercetin has also been found to inhibit the cell growth of Ehrlich ascites tumour cells, and leukaemic T-cells (Suolinna et al, 1975; Yoshida et al, 1992).

Quercetin has also been reported to have mutagenic and genotoxic effects in various in vitro models (Bjeldanes and Chang, 1977; Brown and Dietrich, 1979; Rueff et al, 1986). Laughton et al (1989) have demonstrated that quercetin can mediate the production of oxygen radicals under certain conditions and these may go on to cause cell injury. However, the actual mechanism of the genotoxic effect is yet to be elucidated (Gaspar et al, 1994).
Figure 1.12. Structural formulae of different subclasses of the flavonoid family

Flavanone

Flavan-3-ol

Flavon-3-ol

Flavone

Chalcone

Adapted from van Acker et al (1996).
Table 1.5. Examples of each subclass of naturally occurring flavonoids and their respective TEAC values (mM)

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Compound</th>
<th>OH ARRANGEMENT</th>
<th>TEAC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavanol</td>
<td>Catechin</td>
<td>3,5,7,3',4'</td>
<td>2.40 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Epicatechin</td>
<td>3,5,7,3',4'</td>
<td>2.50 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Epigallocatechin</td>
<td>3,5,7,3',4',5'</td>
<td>3.80 ± 0.06</td>
</tr>
<tr>
<td>Flavanone</td>
<td>Naringenin</td>
<td>5,7,4'</td>
<td>1.53 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Taxifolin</td>
<td>3,5,7,3',4'</td>
<td>1.90 ± 0.03</td>
</tr>
<tr>
<td>Flavonol</td>
<td>Kaempferol</td>
<td>3,5,7,4'</td>
<td>1.34 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>3,5,7,3',4'</td>
<td>4.70 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Myricetin</td>
<td>3,5,7,3',4',5'</td>
<td>3.10 ± 0.30</td>
</tr>
<tr>
<td>Flavone</td>
<td>Chrysin</td>
<td>5,7</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td>5,7,4'</td>
<td>1.45 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>5,7,3',4'</td>
<td>2.10 ± 0.05</td>
</tr>
<tr>
<td>Anthocyanidin</td>
<td>Cyanidin</td>
<td>3,5,7,3',4'</td>
<td>4.40 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>Delphinidin</td>
<td>3,5,7,3',4',5'</td>
<td>4.44 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Apigenidin</td>
<td>5,7,4'</td>
<td>2.35 ± 0.20</td>
</tr>
</tbody>
</table>

Adapted from Rice-Evans et al (1996).
Chapter 1E - Methods Used to Study Inhibition of DNA synthesis and Cell Injury

1.30 In vitro Tissue Slice System

An in vitro tissue slice model was chosen as it allows concentration-response relationships to be determined in different tissues of a single animal under uniform experimental conditions. The main tissues investigated were testis, spleen and liver. Testis was chosen as a tissue containing dividing cells, spleen as a tissue containing many lymphoid cells, and liver as the known target for paracetamol injury. Spleen and liver slices rather than cell cultures were studied because tissue organisation, cell types and cell-cell interactions were maintained with this method. The effects of paracetamol and other compounds on DNA synthesis were studied. Protein synthesis was used as a control to assess non-specific cell injury.

1.31 Functional Measurements Used in in vitro Tissue Slice Experiments

1.31.1 DNA synthesis

DNA synthesis was measured by $^3$H-thymidine incorporation. Whilst thymidine is not the endogenous precursor for thymine nucleotides, it can be phosphorylated by thymidine kinase to produce thymidine monophosphate, which can be further phosphorylated and incorporated into DNA (Cleaver, 1967). Any inhibition of DNA synthesis, at any point, leads to inhibition of thymidine incorporation.

1.31.2 Protein synthesis

Inhibition of protein synthesis was chosen as an indicator of cell viability in these experiments because protein synthesis requires ATP. The cell’s supply of ATP is produced by the mitochondria. As mentioned previously in chapter 1B, during cell injury the mitochondria are damaged and can therefore no longer produce ATP. Inhibition of
both DNA and protein synthesis would suggest that non-specific injury is occurring within the cell as mitochondrial function is disrupted. Inhibition of DNA synthesis and not protein synthesis would imply a specific effect on DNA.

\(^{14}\text{C}-\text{leucine} was chosen as the marker of protein synthesis in these experiments because it is an essential amino acid that is not broken down in the liver (Peters, 1986). A \(^{14}\text{C} labelled amino acid was used so that thymidine and leucine incorporation could be measured simultaneously.

### 1.32 Cycloheximide

![Figure 1.13. Structural formula of cycloheximide. MW 281.3.](attachment:image)

Cycloheximide is an antibiotic which is a known specific inhibitor of protein synthesis. It inhibits protein synthesis by inhibiting the peptidyl transferase activity of the 60S ribosomal subunit (Stryer, 1988).

### 1.33 Indicators of Cell Injury Used in *in vivo* Experiments on Cell Injury by Paracetamol

A range of methods can be used to assess necrotic injury in the liver. These include biochemical measurement of alanine aminotransferase (ALT) and histological methods. For the detection of apoptosis, immunohistochemical staining for specific proteins in tissue sections, the TUNEL method, and Western blot analysis are employed.

#### 1.33.1 Biochemical measurement of plasma ALT levels

Biochemical measurement of enzymes released from the damaged liver into the blood is a widely used method for assessing liver injury. ALT in particular is an enzyme that is widely distributed in animal tissues, with the highest levels found in liver and
kidney. An elevated level of this protein in plasma or serum samples is considered to be a common indicator of liver cell injury (McGee, 1992).

1.33.2 Histological methods

Qualitative assessment of tissue sections that have been stained with specific staining techniques enables identification of the type of lesion present. Image analysis enables quantitation of the degree of injury.

1.33.3 Immunohistochemical staining

Immunohistochemical staining using specific antibodies enables detection of the expression of specific proteins in tissue sections. Immunohistochemical staining can also identify where within the cell a protein is expressed, and also whether it is localised to a specific cell type.

1.33.4 TUNEL method

The terminal deoxynucleotidyl transferase–mediated dUTP-digoxigenin nick end labelling (TUNEL) method is a commonly used method for in situ labelling of cells with new DNA ends generated during apoptosis. Developed by Gavrieli et al (1992), TUNEL is a much more sensitive method for detection of DNA fragmentation compared to agarose gel electrophoresis as apoptosis in single cells can be detected. Another advantage of this method is that the location of apoptotic cells and the cellular morphology can be assessed.

1.33.5 Western blot analysis

Western blot analysis allows the detection of specific proteins in cell lysates. Both qualitative and quantitative data can be obtained from Western blot analyses. In the present study, Western blot analysis is being utilised to detect specific cleavage of poly (ADP-ribose) polymerase (PARP) protein.
2.0 Materials

All chemicals were of the highest grade available from Merck Ltd. (Lutterworth, Leics, UK), Sigma (Poole, Dorset, UK), or Aldrich Chemical Company (Poole, Dorset, UK) unless otherwise stated.

\[6^-H\] thymidine (22 - 26 Ci /mmol, 98.5 - 99.4 % purity) and L-[1^-14C] leucine (54 mCi /mmol, 98.5 - 99.7 % purity) were purchased from Amersham (Little Chalfont, UK). Liquid scintillation fluid was purchased from National Diagnostics (Hessle, Hull, UK). Bicinchoninic acid (BCA) protein assay reagents and bovine serum albumin (BSA) protein standard were obtained from Pierce Inc. (Illinois, USA).

Proteinase K and ApopTag Plus in situ apoptosis detection kit (peroxidase) were obtained from Appligene Oncor (Strasbourg, Germany). 10 or 15 well ready-made 4 - 20 % gradient Tris-glycine sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) were obtained from Novex (Frankfurt, Germany). Goat anti-mouse PARP (A-20) polyclonal antibody, goat anti-human PARP (D-20) polyclonal antibody, rabbit anti-mouse Bax (P-19) polyclonal antibody, biotin-conjugated goat anti-rabbit antibody, avidin-biotin peroxidase reagent and diaminobenzidine (DAB) solution was obtained from Santa Cruz Biotech (Santa Cruz, California, USA). Nickel-enhanced DAB solution was obtained from Vector Labs (Burlingame, USA). Goat anti-mouse PARP polyclonal antibody was obtained from R&D systems (Abingdon, UK). Rabbit anti-human Bax (Ab-1) polyclonal antibody was obtained from Oncogene Research Products (Cambridge, USA). Biotin-conjugated rabbit anti-goat antibody was obtained from Sigma Chemical Co. (Poole, UK). Streptavidin AuroprobeBLplus and IntenSE BL silver enhancement reagents were obtained from Amersham (Little Chalfont, UK).
2.1 *In vitro* Experiments on Inhibition of DNA Synthesis by Paracetamol and Related Antioxidants

2.1.1 Animals and diet

Male Wistar rats (Harlan Olac, Bicester, UK) of final weight 90 - 215 g were fed stock pellets (SDS no. 1 diet containing 69 mg/Kg of α-tocopherol, and 11 mg/Kg of vitamin C, SDS Ltd, Witham, UK) and given unlimited access to drinking water. Rats were killed by cervical dislocation after inducing deep anaesthesia by giving fentanyl citrate (0.02 mg /100g body weight) (Hypnorm, Janssen, Wantage, UK) and loprazolam (0.08 mg /100g body weight) (Roussel Ltd, Uxbridge, UK) as a single i.m. injection. Animals were immediately exsanguinated and liver, spleen, and testes were rapidly removed and placed into ice-cold 0.15 M NaCl solution.

Rats were not pre-induced with phenobarbitone to minimise the metabolism of paracetamol by P450 enzymes in the liver so that the effects observed would be representative of the effects of the parent compound and not its oxidative metabolite. Previous work in our laboratory showed P450 levels of 31.6 ± 15.2 nmol/g (mean ± SD, n = 4) in control rats compared to 98.8 ± 21.8 nmol/g (n = 6) in phenobarbitone-treated rats (Beales and McLean, 1995).

2.1.2 Tissue preparation

Liver slices of 0.35 ± 0.036 mm (mean ± SD, n = 10) thickness and 60 - 100 mg wet weight, and spleen slices of 0.34 ± 0.026 mm (n = 12) thickness and 10 - 25 mg wet weight were cut by hand using a Stadie-Riggs tissue slicer (A. H. Thomas Co. Philadelphia, USA) (illustrated in Figure 2.1). Slice thickness was estimated by measuring the weight of the slice and dividing this by the surface area of the slice. Surface area was measured by placing the slice on graph paper and counting the number of square millimetres.

Tissue slices have been used successfully to study xenobiotic metabolism in liver, spleen, kidney, heart, lung, adrenal and intestine (reviewed by Bach *et al*, 1996). Previous work using liver slices has demonstrated that this system can give a good indicator of likely *in vivo* response to toxic injury as slices can metabolise xenobiotics,
maintain good adenosine-5'-triphosphate (ATP) levels, glutathione and ion balance (McLean and Nuttall, 1978; Miller et al, 1993; Martin and McLean, 1995). Testis slices have also been successfully used to study glucose-stimulated protein synthesis in the rat (Dills et al, 1981) and human spleen slices have been used to study cytokine and immunoglobulin production (Skibinski and James, 1997).

Tissue slices were gently blotted, weighed, and placed into individual 25 ml Erlenmeyer flasks containing 5 ml Ringer solution at room temperature. After decapsulation, samples of testis tubules were gently pulled into approximately 50 - 90 mg samples with forceps, weighed, placed into individual 25 ml Erlenmeyer flasks containing 5 ml Ringer solution at room temperature and gently teased apart with a pair of mounted needles.

The Ringer solution had the following composition:

- NaCl 125 mM
- KCl 6 mM
- MgSO₄ 1.2 mM
- NaH₂PO₄ 1 mM
- CaCl₂ 1 mM
- Glucose 10 mM
- Bovine serum albumin (BSA) 5 mg/ml
- Gentamicin 50 μg/ml
- Bicarbonate buffer 15 mM, pH 7.4
- 20 amino acids each at 0.1 mM
  - Glycine
  - Isoleucine
  - Serine
  - Threonine
  - Valine
  - Phenylalanine
  - Tyrosine
  - Tryptophan
  - Aspartic acid
  - Asparagine
  - Glutamine
  - Proline
  - Arginine
  - Histidine
  - Lysine
  - Methionine
  - Cystine
  - Cysteine
  - Hydroxyproline
  - Alanine

(Peters, 1983). The Ringer solution was bubbled through with O₂/CO₂ (95%/5%) before use to stabilise the pH.
Water-soluble drugs were dissolved in distilled water and made up as concentrated stock solutions and added to the Ringer solution in the Erlenmeyer flasks to give the final concentration. Trolox was directly dissolved in Ringer. Nordihydroguaiaretic acid (NDGA), quercetin, and naringenin were first dissolved in dimethylsulphoxide (DMSO) and added to Ringer solution to give a final DMSO concentration of 0.1% (v/v). This concentration of DMSO has been found not to interfere with incorporation of radiolabel.

**Caution:** *Latex gloves and protective clothing must be worn when handling all compounds. In particular, p-aminophenol, m-aminophenol and p-cresol are highly toxic compounds and in addition to the use of latex gloves and protective clothing these compounds must be weighed in a fume cupboard to avoid inhalation of powder.*

Tissues were incubated in the presence or absence of test drug at 37°C under O₂/CO₂ (95%/5%) in a magnetic-coupled shaking water bath (80 strokes/minute, 6 cm/ stroke). After 30 minutes, a dual label of [6-³H] thymidine (5 μCi) and L-[1-¹⁴C] leucine (0.25 μCi) was added to each flask, with carrier thymidine (25 nmol) and carrier L-leucine (0.5 μmol). Incubation was continued for a further 30 minutes.

After incubation the flasks were taken out of the water bath and spleen and liver slices removed with forceps, rinsed in ice-cold 0.15 M NaCl solution and precipitated in 5 ml 10% trichloroacetic acid containing 10 mM L-leucine (TCA/leu). For the testes samples, the flask contents were spun down at 3,000 rpm for 5 minutes, and the supernatant poured off. The pellet was then re-suspended and washed with 2 ml of ice-cold 0.15 M NaCl solution, spun down, the supernatant discarded and the resultant pellet precipitated in 5 ml 10% TCA/leu. Each sample was homogenised using an Ultra-Turrax homogeniser (10 mm diameter) for 8 - 15 seconds at full speed.

To check the amount of radioactivity added to each flask, 50 μl of the flask contents was added to scintillation fluid and counted using a Packard Tricarb liquid scintillation counter.
Figure 2.1. Illustration of a Stadies-Rigg stage with a long blade used to obtain liver and spleen slices

Adapted from Peters (1983). Slices were cut with a side-to-side sawing motion towards the body as opposed to away from the body as illustrated by above figure.
2.1.3 Measurement of thymidine and leucine incorporation into tissues

The tissue homogenate was spun down (3,000 rpm for 5 minutes) and the precipitated pellet resuspended and washed at least a further two times with 5 ml of fresh 10 % TCA/leu. 400 μl of the supernatant from the last wash was taken from a few samples, added to scintillation fluid and counted using a Packard scintillation counter. If there was still a significant amount of radioactivity present, then the pellets were washed with another 5 ml of 10 % TCA/leu, spun down and 400 μl of the supernatant counted. When the radioactivity in the supernatant was sufficiently low (i.e. less than 20 dpm /400 μl), the pellets were digested with 1 ml of 1 M NaOH at 37°C overnight.

Incorporation of $^3$H-thymidine and $^{14}$C-leucine into DNA and protein, respectively, were measured by adding 200 μl of digested sample to scintillation fluid containing 200 μl of 1 M hydrochloric acid (to neutralise the NaOH). This was counted in a Packard Tricarb liquid scintillation counter. Results are expressed as dpm/mg protein.

Non-radiolabelled carrier thymidine and leucine were included in the incubation media as this reduces variability in radiolabel incorporation between samples by providing a steady-state concentration of thymidine and leucine.

Using a dual label programme, counts were converted to disintegrations per minute (dpm) using automatic external standardisation calibrated with quenched $^3$H and $^{14}$C samples of similar counting geometry to the tissue samples.

Duplicate or triplicate tissue samples from the same animal were taken for each incubating condition. Controls were samples taken from the same animal and incubated in the absence of drug.

2.1.4 Measurement of thymidine and leucine uptake into tissues

Whilst thymidine and leucine uptake into tissue was not directly measured, the amount of radiolabel in the TCA-soluble fraction (or acid fraction) of the tissue homogenate was measured in a number of experiments. The acid fraction represents the amount of unincorporated radiolabel in the cytoplasmic and remaining extracellular fluid and is an indicator of the amount of thymidine and leucine taken into the tissue samples. The amount of radiolabel in the acid fraction was measured by adding 200 μl of the
supernatent of the first spun down tissue homogenate to scintillation fluid and counting using a Packard Tricarb liquid scintillation counter. Results are expressed as dpm/mg tissue.

2.1.5 Measurement of protein secretion from liver slices

It is well known that proteins are secreted by the liver and both in vivo methods and liver slices have been used to study this secretion (Peters, 1983). In order to determine whether the liver slices used in the present study are secreting radiolabelled proteins, the level of $^{14}$C in the protein of the incubating medium was measured. 2 ml of Ringer solution was taken from flasks that were used to incubate liver slices and each sample was precipitated in 5 ml 10 % TCA/leu. This was spun down at 3,000 rpm for 4 minutes and the resultant pellet washed repeatedly by pouring off the supernatent, resuspending the pellet in fresh 10 % TCA/leu and spinning down the suspension. This was repeated until the level of radioactivity in 400 µl of supernatent was less than 20 dpm. The pellet was then digested in 1 ml 1M NaOH and 200 µl of digest was added to scintillation fluid containing 200 µl 1M HCl and counted. Results are expressed as dpm/mg tissue.

2.1.6 Lowry protein assay - protein determination

The protein content of the digested samples was determined by the method of Lowry et al (1951) using the following protocol:

1. Digested tissue samples were diluted with 0.15 M NaCl solution, e.g. 1/20 for testis, 1/5 for spleen, 1/50 for liver.
2. 100 µl of each diluted sample was pipetted into tubes containing 400 µl 0.15 M NaCl solution, in duplicate.
3. 500 µl standards (0, 10, 20, 50, 75, 100 µg BSA in 0.15 M NaCl solution) were then prepared fresh from a 2 mg/ml BSA standard solution and pipetted into tubes, in duplicate.
4. Folin C was prepared fresh by mixing Folin A (2 % anhydrous Na$_2$CO$_3$ in 0.1 M NaOH), with Folin B (0.5 % CuSO$_4$ .5H$_2$O in 1 % Na / K tartrate) in the proportions 50:1, respectively. Folin D was prepared by mixing Folin & Ciocalteu’s phenol reagent and distilled water in the proportions 1:1.8, respectively.

NB. pH can greatly affect the results of this assay, therefore 0.1M NaOH was included in Folin A to buffer the pH.
5. 2.5 ml of Folin C was added to each 500 μl standard and sample, vortexed immediately, and left for 10 minutes at room temperature.

6. 0.25 ml of Folin D was then added to each sample, vortexed immediately, and left for 30 minutes at room temperature.

7. Absorbance was then measured at 710 nm using a Ciba-Corning 257 colorimeter blanked with distilled water.

8. A standard curve was plotted and the protein content of each sample determined from this curve.

Figure 2.2A shows a typical protein standard curve obtained using the Lowry assay.

2.1.7 BCA protein assay - protein determination

The reaction of protein with Cu$^{2+}$ under alkaline conditions to produce Cu$^{1+}$ is well known and called the Biuret reaction. Protein determination by the Pierce BCA protein assay is based on the highly sensitive and specific interaction of the sodium salt of bicinchoninic acid (BCA) with Cu$^{1+}$. Two molecules of BCA interacts with one Cu$^{1+}$ ion to produce a water-soluble purple product that shows a strong absorbance at 562 nm that increases proportionally with increasing protein concentrations (Smith et al, 1985).

The protein content of the digested samples was determined by the BCA protein assay using the following protocol:

1. As with the Lowry protein assay, the digested samples were diluted down.
2. 100 μl of each diluted sample was pipetted in duplicate into tubes.
3. 100 μl standards (0, 5, 10, 20, 30, 40, 50 μg) were prepared fresh from a 2 mg/ml BSA standard solution and pipetted into duplicate tubes.
4. BCA reagent was then prepared by mixing reagent A with reagent B in the proportion 50:1, respectively.
5. 2 ml of the BCA reagent was added to each sample and standard, mixed, and incubated at 37°C for 30 minutes.
6. The absorbance was then measured at 562 nm using a Pye Unicam SP8-100 UV/Vis spectrophotometer blanked with distilled water.

Figure 2.2 B shows a typical protein standard curve obtained using the BCA assay.
Figure 2.2. Typical standard curves for the Lowry protein assay (A) and BCA protein assay (B)

(A) Abs at 710 nm vs. protein (µg)
(B) Abs at 562 nm vs. protein (µg)
2.1.8 Statistical analysis

For measurement of the inhibition of thymidine and leucine incorporation, the mean incorporation of thymidine and leucine for duplicate or triplicate flasks (expressed as dpm/mg protein) was considered as a single data point for analysis of results from at least three separate experiments. % inhibition of synthesis was calculated by comparing the dpm from dosed tissues with control tissues incubated in the absence of test drug. All data are presented as mean % inhibition ± SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) with comparisons to a control group (Bonferroni correction). The level of significance was established at $P < 0.05$.

The concentration of drug that gave 50 % inhibition of synthesis (IC$_{50}$) was determined using a sigmoidal curve fitting programme in GraphPad Prism 2.01 (GraphPad Software, Inc., San Diego, CA, USA). All concentration-response relationships that showed >40 % inhibition were plotted with fitted curves, otherwise graphs were plotted with straight lines between data points.

Coefficient of variation (COV) was determined by the following equation:

$$\text{COV} (\%) = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

For the measurement of thymidine and leucine uptake, the mean of the $^3$H-thymidine and $^{14}$C-leucine levels in the acid fraction (expressed as dpm/mg tissue or Ringer solution) from duplicate flasks was used as a single data point for analysis of results. Statistical significance was determined using one-way analysis of variance (ANOVA) with multiple comparisons (Bonferroni correction). The level of significance was established at $P < 0.05$.

For the measurement of protein secretion from liver slices, the mean of the $^{14}$C counts in the TCA-insoluble fraction of Ringer solution from 4 - 6 flasks was used as a single data point for analysis of results.
2.2 *In vivo* Experiments on Cell Injury by Paracetamol

2.2.1 Animals and diet

Male C57Bl/6 mice (Harlan Olac, Bicester, UK) of final weight 17 - 22 g, were fed CRM(E) diet from Special Diet Services Ltd (SDS Ltd) (Witham, UK) and given drinking water *ad libitum*. Mice were killed by cervical dislocation after inducing deep anaesthesia by i.p. injection of a mixture of fentanyl citrate (0.008 mg /10 g body weight) (Hypnorm, Janssen, Wantage, UK) and midazolam (0.05 mg /10 g body weight) (Hypnovel, Roche, Welwyn Garden City, UK). Blood samples were collected by removing blood from the aorta with a syringe and injecting into a 1.5 ml Eppendorf tube containing heparin, or cutting the carotid artery and bleeding directly into an Eppendorf tube containing heparin. Liver sections (~3 mm) from the left lobe were cut using a scalpel and fixed in 10 % formalin. The rest of the liver was cut into pieces and snap frozen in isopentane cooled on dry ice.

2.2.2 Assessment of injury

Various measures of necrosis and apoptosis were used to assess cell injury. Irreversible liver cell injury was assessed by measurement of alanine transaminase (EC 2.6.1.2) (ALT) leakage into blood and by histological analysis of haematoxylin and eosin (H&E) stained liver sections. Some liver sections were also stained using the periodic acid Schiff (PAS) method to assist with histological scoring of cell injury. Apoptotic changes were investigated using the terminal deoxynucleotidyl transferase (TUNEL) method in liver sections and by Western blot analysis of poly (ADP-ribose) polymerase (PARP) in liver protein lysates. Expression of the pro-apoptotic protein Bax was also studied in liver sections by immunohistochemical staining.

2.2.3 Measurement of plasma ALT levels

Alanine transaminase (EC 2.6.1.2) (ALT) is an enzyme that specifically catalyses the transfer of the amino group of alanine to 2-oxoglutarate. ALT is widely distributed in animal tissues, with the highest levels found in liver and kidney and is normally present at low levels in the blood. An elevated level of this protein in plasma or serum samples is
ALT measurement is based on a coupled reaction based on the rate of NADH oxidation by lactate dehydrogenase (LDH) as follows:

\[
L\text{-Alanine} + 2\text{-Oxoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + L\text{-Glutamate} \tag{1}
\]

\[
\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} L\text{-Lactate} + \text{NAD}^+ + H_2O \tag{2}
\]

Heparinised blood samples were spun down at 10,000 rpm for 5 minutes. The plasma was removed and assayed for ALT activity using the Sigma ALT 20 reagent. 20 μl of plasma sample was added to 0.98 ml of ALT reagent in a 1.5 ml cuvette, inverted to mix and placed in a Kontron Instruments UVIKON 930 spectrophotometer blanked with distilled water and set at 340 nm. Change in absorbance (Δ Abs) was recorded for up to 4 minutes. ALT activity was calculated from the following equation:

\[
\frac{\Delta \text{Abs}_{340\text{nm}} / \text{minute}}{6.22} \times 1000 \times \text{dilution factor}
\]

\[
\Delta \text{Abs}_{340\text{nm}} / \text{minute} = \text{Initial Abs} - \text{Final Abs over 1 minute}
\]

1000 = conversion of μmol/ml to nmol/ml

dilution factor = 50 for a 20 μl sample

6.22 = mM extinction coefficient of NADH at 340 nm (with 1 cm path length)

Results are expressed as nmoles/min/ml. The limit of sensitivity for this method is 5 nmoles/min/ml.

2.2.4 Preparation of livers for histological analysis

~3 mm liver sections taken from the left lobe were fixed in 10 % formalin for at least one week. Sections were then dehydrated by graded alcohols (70 %, 90 %, 100 %), cleared to remove the alcohol and embedded in paraffin wax blocks. 5 μm sections were cut with a MSE rotary microtome and mounted onto microscope slides (coated and uncoated). These were air-dried overnight and stored in slide boxes ready for use.
2.2.5 Haematoxylin and eosin staining of tissue sections

5 μm mouse liver sections mounted onto uncoated microscope slides were dewaxed, rehydrated and stained with H&E by the histology lab at SmithKline Beecham Pharmaceuticals, Welwyn, UK. Stained sections were dehydrated with graded alcohols, mounted, coverslipped and observed under an Olympus BH-1 microscope. Slides from each animal were scored for injury using the key in Table 2.1.

2.2.6 Periodic acid Schiff staining of tissue sections

The liver stores a large amount of glucose in the form of glycogen. The PAS method allows the visualisation of glycogen in cells. Because of the abundance of glycogen in liver cells, loss of glycogen is used as an early indicator of liver cell injury and often occurs before any obvious morphological changes. Only a small number of slides were stained using the PAS method to assist in the histological analysis of H&E stained slides that were difficult to score for injury.

5 μm mouse liver sections mounted onto uncoated microscope slides were dewaxed, rehydrated and stained using the PAS method by the histology lab at SmithKline Beecham Pharmaceuticals, Welwyn, UK. Stained sections were dehydrated with graded alcohols, mounted, coverslipped and observed under an Olympus BH-1 microscope.
Table 2.1. Histology scoring key for injury in H&E stained mouse liver sections.

<table>
<thead>
<tr>
<th>SCORE</th>
<th>DESCRIPTION OF INJURY</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>Occasional, small foci of a few necrotic cells</td>
</tr>
<tr>
<td>2</td>
<td>Centrilobular degeneration with small areas of necrosis affecting $&gt;10 &lt; 40%$ of the section</td>
</tr>
<tr>
<td>3</td>
<td>Centrilobular degeneration and large areas of necrosis affecting $&gt;40 &lt; 80%$ of the section; often with haemorrhage and affected areas merging together</td>
</tr>
<tr>
<td>4</td>
<td>As 3 but affected area $&gt;80%$ of the section</td>
</tr>
</tbody>
</table>
2.2.7 TUNEL method

Fragmentation of DNA into multiples of 180 - 200 base pairs is a hallmark of apoptosis. Agarose gel electrophoresis is often employed to assess DNA fragmentation, producing a characteristic ladder pattern if apoptosis is occurring. However, this method is not very sensitive, requiring a large number of cells, and does not provide any additional information. In order to detect apoptosis in tissue sections, an in situ method for detecting the new DNA ends generated by DNA fragmentation was developed and called TUNEL (terminal deoxynucleotidyl transferase (dUTP)-digoxigenin nick mediated nick end labelling) (Gavrieli et al., 1992). TUNEL is a much more sensitive method for detection of DNA fragmentation compared to agarose gel electrophoresis as apoptosis in single cells can be detected. Another advantage of this method is that the location of apoptotic cells and the cellular morphology can be assessed.

5 μm mouse tissue sections mounted onto coated slides were dewaxed, rehydrated and incubated for 15 minutes with 20 μg/ml proteinase K in phosphate-buffered saline (PBS), washed in distilled water, quenched for 15 minutes with 2 % hydrogen peroxide in PBS, washed in PBS, and TUNEL stained using the ApopTag Plus in situ apoptosis peroxidase kit according to the manufacturers instructions (mechanism illustrated in Figure 2.3).

Briefly, terminal deoxynucleotidyl transferase (TdT) enzyme was used to catalyse a template independent addition of deoxyribonucleotide triphosphate to the 3'-OH ends of DNA fragments. The incorporated nucleotides form a random oligomer of digoxigenin-11-deoxyuridine triphosphate (dUTP) and deoxyadenosine triphosphate (dATP), in a ratio that has been optimised for anti-digoxigenin antibody binding. An anti-digoxigenin antibody conjugated with peroxidase was then bound. Peroxidase was then visualised by incubating with diaminobenzidine (DAB) substrate to produce localised permanent brown staining. Sections were counterstained with haematoxylin, dehydrated, mounted and coverslipped.

Caution: DAB solution is carcinogenic. When handling this solution latex gloves and protective clothing must be worn. All spillages and leftover DAB solution must be mixed with an equal volume of 10 % potassium permanganate solution and collected in a brown glass container with screwcap lid and disposed of by incineration when the container is full.
In addition to the experimental samples, positive and negative control sections were stained simultaneously to confirm staining. Mouse testis sections were used as a positive control tissue and for negative controls, TdT enzyme was omitted from the reaction buffer.

Stained cells were examined by light microscopy at x 40 magnification. Using the stained cells as a guide, the number of apoptotic bodies was counted. Each stained cell was counted as a single apoptotic body if the cell showed shrinkage in size and loss of contact with adjacent cells, or the cell did not display any morphological hallmarks of necrosis i.e. enlarged nucleus, swelling, infiltration of inflammatory cells. Small clusters of stained fragments in adjacent cells were counted as a single apoptotic body as these were likely to have originated from the same cell.

The total number of apoptotic bodies in each liver section was counted twice. If the two numbers varied greatly then the count was repeated until a consistent value was obtained. The section surface area was estimated by electronically drawing around the section using computer visualisation software (OPTIMAS 5.2) linked to a microscope set at low magnification. Results are expressed as mean number of apoptotic bodies observed per cm$^2$ of section area in one liver section from each animal in the dose-response study and two separate liver sections from the same animal in the time course study.
Figure 2.3. Mechanism of TUNEL staining

Modified from instruction leaflet provided with Appligene Oncor ApopTag Plus kit.
2.2.8 Western blot analysis for poly (ADP-ribose) polymerase cleavage

Poly (ADP-ribose) polymerase (PARP, EC 2.4.2.30) is a ubiquitous nuclear enzyme that catalyses the polymerisation of the nucleic acid homopolymer poly (ADP-ribose). PARP is thought to be involved in DNA repair as it binds to damaged DNA, becomes activated and transfers ADP-ribose onto various nuclear proteins (including PARP itself). The native protein is 116 kDa, and is cleaved, mostly by caspase 3, to 85 and 23 kDa signature fragments during apoptosis (Kaufmann et al, 1993; Tewari et al, 1995). Detection of the cleaved signature fragments is believed to be a reliable early marker of apoptosis.

Frozen liver pieces were thawed in 5 ml of lysis buffer containing 0.9 % Triton X-100, 0.9 % sodium deoxycholate, 0.09 % sodium dodecyl sulphate (SDS), 0.082 % EDTA (disodium, dihydrate), 0.2 % bestatin, 0.2 % leupeptin, 0.2 % aprotinin in Tris-buffered saline (pH 7.4) (Sambrook et al, 1989), homogenised with an Ultra-Turrax homogeniser for 10 seconds at medium speed, and spun down. The supernatant was removed and protein content was determined and adjusted to 1 mg/ml with lysis buffer. Equal volumes of lysates from individual samples in each group were pooled together for analysis.

The pooled lysate samples were mixed with SDS reducing loading buffer and boiled for 5 minutes prior to loading onto a 4 - 20 % gradient Tris-glycine SDS-PAGE gel. Proteins were separated by electrophoresis (180 V constant voltage for 80 minutes) and transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer system (Bio-Rad, Hemel Hempstead, UK). Membranes were blocked overnight with blocking buffer (1 % BSA, 0.02 % Ficoll 400, 0.02 % polyvinylpyrrolidone-40 in PBS) at 4°C.

Blocking buffer was discarded and immunodetection of membrane-bound proteins was carried out by incubating the membrane with a goat anti-PARP polyclonal antibody (at a suitable dilution) for one hour at room temperature, with agitation. The antibody solution was then decanted, the membrane washed, and then incubated with a biotin-conjugated rabbit anti-goat antibody (1:10,000) for one hour at room temperature, with agitation. After washing, signal was detected by incubating with streptavidin AuroprobeBLplus solution for 1.5 to 2 hours, followed by washing in PBS and a 30-60
second rinse in deionised water and incubating in intense BL silver enhancement reagent (made up fresh) until the desired level of staining was obtained. Membranes were rinsed in deionised water and allowed to dry.

All antibody solutions were made up in 1 % BSA, 0.5 % Tween 80, 0.01 % sodium azide in PBS. Membranes were washed three times with 0.5 % Tween 80 in PBS between incubation steps.

2.2.9 Immunohistochemical detection of Bax protein in tissue sections

Immunohistochemistry was used to visualise the expression of Bax protein in tissue sections. Bax is a member of the Bcl-2 family of proteins and is pro-apoptotic. The expression of Bax protein in normal mouse liver has previously been demonstrated by Krajewski et al (1994). Bax protein is normally located in the cytosol, however on induction of apoptosis, Bax appears to localise to the mitochondria (Wolter et al, 1997). Any changes in the pattern of Bax protein expression in paracetamol-treated livers would give an indication that apoptosis is occurring.

Figure 2.4 illustrates the mechanism of staining. All incubation steps were performed in a humidified chamber at room temperature unless otherwise stated, and all washing steps were performed with three PBS washes.

5 μm mouse liver sections mounted onto coated slides were dewaxed, rehydrated and washed. Endogenous peroxidase activity was quenched in 0.6 % (v/v) H₂O₂ in PBS for 30 minutes. After washing, sections were immersed in near boiling 10 mM citrate buffer, pH 6.0, and exposed to microwaves. Sections were then washed in PBS, the surrounding area blotted with a tissue, and then circled with a paraffin pen to contain the incubation solutions.

If a pre-blocking step was included, sections were first incubated with 1 % BSA, 0.3 % Triton X-100, 0.01 % sodium azide, 2 % normal goat serum in PBS, for 1 hour. Slides were gently tapped to remove excess blocking solution before the next step. If a pre-blocking step was not included then the first incubation was with primary antibody.

Sections were incubated with primary antibody made up in 0.3 % Triton X-100, 0.01 % sodium azide, 2 % normal goat serum in PBS, at a suitable dilution (1:200; 1:500; 1:1000), overnight at 4°C. Sections were then washed and gently tapped to remove
excess PBS and incubated with a biotin-conjugated goat anti-rabbit antibody made up in 0.3 % Triton X-100 in PBS (at a suitable dilution) for 30 minutes. 15 minutes after incubation began, avidin-biotin peroxidase reagent was made up in 0.3 % Triton X-100 in PBS (must be prepared fresh 30 minutes before use). Sections were washed, tapped gently to remove excess PBS and then incubated with avidin-biotin peroxidase reagent for 30 minutes.

Avidin-biotin peroxidase reagent was removed by washing in PBS and then rinsed with distilled water. Peroxidase was visualised by incubating with DAB substrate for 10 seconds - 5 minutes until brown staining was visible. As well as normal DAB substrate which gives brown staining, nickel-enhanced DAB substrate was also used in some staining experiments. Incubating with nickel-enhanced DAB substrate gives blue/black staining. DAB solution was removed by washing in PBS. Sections were then either counterstained with haematoxylin, or not counterstained, before being dehydrated, mounted and coverslipped. Sections were observed under an Olympus BH-1 microscope for staining.

Again DAB solution was handled with caution and disposed of as previously described in methods section 2.2.7.
Figure 2.4. Mechanism of immunohistochemical staining in tissue sections

Step 1. Label antigen with primary antibody

Step 2. Bind biotin-conjugated secondary antibody

Step 3. Bind horse radish peroxidase-conjugated avidin-biotin reagent

Step 4. Stain with DAB substrate

Key: + Horse radish peroxidase ☐ Avidin ☐ Biotin ← Antibody

2.2.10 Protein determination

The principle behind this assay is described previously in chapter 2.1.7. However, the protocol used in this set of experiments differs. Protein content was determined by the following protocol:

1. 10 μl samples and standards (0, 0.4, 0.8, 1.2, 1.6, 2 mg/ml BSA in lysis buffer) were pipetted in duplicate into separate wells of a 96 well plate.
2. BCA reagent was made up fresh by mixing reagent A with reagent B (50:1 respectively).
3. 200 μl of BCA reagent was added to each well, the plate was agitated gently to mix, covered, and incubated at 37°C for 30 minutes.
4. Absorbance at 595 nm was read using a 96 well plate reader. A standard curve was plotted and the protein content of samples determined from this curve.

Figure 2.5 shows a typical protein standard curve obtained using this method.

2.2.11 Statistical analysis

For the plasma ALT results, data were log transformed and statistical significance was determined using the Mann Whitney U test. For the H & E histology scores in the dose-response study, statistical significance was determined using one-way analysis of variance (ANOVA) with multiple comparisons (Bonferroni correction). For the H & E histology scores in the time course study, statistical significance was determined using Student’s t-test (unpaired). For the TUNEL results, the limit of detection was arbitrarily set at 1. All 0 values were converted to 1 before data were log transformed. Statistical significance was determined using the Mann Whitney U test. The level of significance was established at $P < 0.05$ for all statistical analyses.
Figure 2.5. Typical standard curve for BCA protein assay
CHAPTER 3: RESULTS

Inhibition of DNA Synthesis Using An *in vitro* Tissue Slice System

3.0 Introduction

As mentioned in chapter 1C, paracetamol has been shown to specifically inhibit replicative DNA synthesis at close to the therapeutic plasma level of ~0.1 mM (Hongslo *et al*, 1989; Hongslo *et al*, 1990; Wiger *et al*, 1997). However, these studies were conducted in cell lines and are hard to relate to the possible *in vivo* effects. An *in vivo* study in our laboratory has also shown inhibition of DNA synthesis in rat tissues by paracetamol (Lister and McLean, 1997). However, the lack of concentration-response data did not enable determination of the degree of inhibition that may or may not occur at different concentrations, in particular at therapeutic concentrations. Another observation from this *in vivo* study was that there seemed to be a difference in the sensitivity of different tissues to the inhibition of DNA synthesis, though this could be due to pharmacokinetic factors.

The present study looks at the inhibition of DNA synthesis by paracetamol and other compounds in an *in vitro* tissue slice system. DNA synthesis was measured by $^3$H-thymidine incorporation and protein synthesis was measured by $^{14}$C-leucine incorporation. Inhibition of protein synthesis was included as an indicator of non-specific cell injury. This *in vitro* experimental model can show when a compound causes specific inhibition of DNA synthesis without interference with protein synthesis, and by inference, without block to ATP synthesis, or cytotoxic effects.

An *in vitro* tissue slice system was used instead of an *in vivo* system because many samples can be produced from a single animal and the tissue is exposed to a uniform environment. Whilst there are other *in vitro* systems, the tissue slice system was chosen because it maintains cell-cell interactions, tissue organisation and cell types similar to the
intact organ. However, the cell types involved are unknown as they are not visualised by autoradiography and the degree of penetration of the test drugs is not known. This method also provides no mechanistic information.

Concentration-response relationships in rat testis tubules, spleen slices, and liver slices were obtained for paracetamol, hydroxyurea, cycloheximide, n-propyl gallate and (+)-catechin.

Paracetamol is a phenolic antioxidant (Woollard et al, 1990) and it is this antioxidant property which is thought to be the mechanism by which paracetamol inhibits ribonucleotide reductase by quenching the tyrosyl free radical (Hongslo et al, 1990). Inhibition of DNA synthesis by paracetamol raises the question about whether there are other substances in the environment that inhibit DNA synthesis, and also whether some tissues are more sensitive than others to this type of inhibition.

Hydroxyurea is a DNA synthesis inhibitor that also inhibits ribonucleotide reductase activity by destroying the tyrosyl free radical (Krakoff et al, 1968; Thelander et al, 1985). It was included as a positive control compound to which paracetamol could be compared.

Cycloheximide is an antibiotic that is a specific inhibitor of protein synthesis. It inhibits protein synthesis by inhibiting the peptidyl transferase activity of the 60S ribosomal subunit (Stryer, 1988) and was used as a positive control for protein synthesis inhibition.

n-propyl gallate is a synthetic phenolic antioxidant used in the food industry as a preservative. (+)-catechin is a member of the flavonoid family of natural antioxidants. Both of these compounds were selected to study the inhibition of DNA synthesis in different tissues.
3.1 Summary of *in vitro* experiments with paracetamol, hydroxyurea, cycloheximide, (+)-catechin and *n*-propyl gallate

These experiments demonstrate that:

- Paracetamol specifically inhibits DNA synthesis in testis tubules, spleen and liver slices in a dose-dependent manner. IC$_{50}$ values for DNA synthesis inhibition are 0.22, 0.48, 1.33 mM for spleen, testis and liver samples, respectively.
- Hydroxyurea specifically inhibits DNA synthesis in testis tubules and spleen slices, but in liver slices the inhibition only occurs at concentrations which also inhibit protein synthesis. IC$_{50}$ values for DNA synthesis inhibition are 0.04, 0.1 mM for spleen and testis samples, respectively.
- Cycloheximide specifically inhibits protein synthesis in liver slices, but in testis tubules and spleen slices inhibition of protein synthesis is accompanied by significant DNA synthesis inhibition at micromolar concentrations.
- Paracetamol differentially inhibits DNA synthesis in different tissues. This difference in sensitivity between tissues was also observed with hydroxyurea, and to a lesser extent *n*-propyl gallate and (+)-catechin, with spleen being most sensitive, followed by testis and then liver.
- (+)-catechin specifically inhibits DNA synthesis in testis tubules and spleen slices, and preferentially inhibits DNA synthesis in liver slices.
- *n*-propyl gallate specifically inhibits DNA synthesis in rat spleen slices, preferentially inhibits DNA synthesis in testis tubules, but does not inhibit specifically in liver slices.
3.2 Methods

3.2.1 In vitro tissue preparation

In addition to liver, spleen and testes, in preliminary studies, kidney and thymus samples were also used. Tissues were rapidly removed from rats and placed in ice-cold 0.15 M NaCl solution (see chapter 2.1). Liver and spleen slices were cut by hand as described in methods section 2.1.2. Kidney cortex slices weighing 10 - 35 mg of approximately 0.35 mm thickness were also prepared in a similar manner. Tissue slices were blotted gently, weighed and placed into 25 ml Erlenmeyer flasks at room temperature, containing 5 ml Ringer solution in the presence or absence of test drug. Testis samples were prepared as described in methods section 2.1.2. Thymus samples were cut into small pieces weighing 20 - 50 mg with a sharp scalpel, weighed, placed into individual 25 ml Erlenmeyer flasks containing 5 ml Ringer solution at room temperature and gently teased apart with a pair of mounted needles.

3.2.2 Measurement of thymidine and leucine incorporation into tissues

Measurement of thymidine and leucine incorporation into kidney slices was carried out according to the method described for spleen and liver slices in methods section 2.1.3. Measurement of thymidine and leucine incorporation in thymus samples was carried out according to the method described for testis.

3.2.3 Protein determination

The Lowry protein assay was used throughout these experiments but when protein values appeared to be exceptionally high in testis and thymus samples, the BCA protein assay was also used so results from both assays could be compared. Very little difference was observed between the two assays. The problem was due to an error in the method where BSA added to the Ringer solution was found to have contaminated samples. The method was modified to include a washing step (shown in methods chapter 2.1) and this error no longer occurs.
3.2.4 Scintillation counting

A dual label programme was set up on the Packard Tricarb scintillation counter to convert counts per minute to disintegrations per minute (dpm) using automatic external standardisation calibrated with quenched $^3$H and $^{14}$C samples of similar counting geometry to the tissue samples. Problems were encountered when blank controls gave high counts and a series of investigations were conducted to find and eliminate the cause. It was found that high counts were due to static electricity between the sample holder and the vial holder, and the scintillation fluid being exposed to too much light that gave rise to chemiluminescence. The sample holders and vial holders were treated with anti-static solution and exposure of sample vials to light was kept to a minimum before samples were read.
3.3 Validation of *in vitro* Slice Technique

Before presenting results of studies using the *in vitro* slice technique, results of experiments on the validation of this technique will first be shown.

### 3.3.1 Uptake of thymidine and leucine into tissue samples

The level of $^3$H-thymidine and $^{14}$C-leucine uptake into cells can be indirectly determined by measuring the level of unincorporated radiolabel in the cytoplasmic and remaining extracellular fluid of the tissue (acid fraction) and comparing it with the level in the incubation medium (Ringer solution). In addition to comparing the acid fraction and Ringer solution radiolabel levels of control tissue samples, the acid fraction and Ringer solution levels were measured in drug-exposed (dosed) tissue samples to determine if drug treatment had a major effect on uptake. Dosed tissue samples were those incubated with either hydroxyurea, paracetamol, cycloheximide or n-propyl gallate at concentrations that showed a high level of DNA and/or protein synthesis inhibition.

Changes in uptake might cause apparent inhibition of protein or DNA synthesis, or changes in the ratio of $^{14}$C to $^3$H incorporation. Some control slice samples showed very low $^{14}$C counts in the acid-soluble fraction, together with high levels of $^{14}$C incorporation. This could be due to very rapid incorporation of label, or perhaps to errors in the dual label counting system at very high ratios of $^3$H to $^{14}$C. Figures 3.1 to 3.3 show that there were no major changes in uptake and in particular in none of these cases did the ‘inhibited’ tissues show low levels of isotope in the acid fractions.

Table 3.1 and 3.2 show the mean, SD, SEM and coefficient of variation in radiolabel levels in the acid fraction and surrounding medium of control, and drug-exposed tissue samples. As expected the variation in radiolabel levels in the incubating Ringer solution was reasonably low with COV values ranging from 4.7 to 21.9 %. The COV values for thymidine levels in the acid fraction of control and drug-exposed tissues ranged from 16.4 to 28.7 % whereas the COV values for leucine levels in the acid fraction of control and drug-exposed tissues ranged from 26.7 to 92.8 %.

#### 3.3.1.1 Testis samples

Figure 3.1 shows the levels of $^3$H-thymidine and $^{14}$C-leucine in the acid fraction of control and dosed testis sample homogenates and in the incubating Ringer solution. In
control testis samples, the level of $^3$H-thymidine per mg of tissue found in the acid fraction was not significantly different from that in the incubating Ringer solution. However, the $^3$H-thymidine level per mg of tissue found in the acid fraction of dosed testis samples was significantly higher compared to the surrounding medium. The $^{14}$C-leucine levels in the acid fraction of dosed tissues was not significantly different from the surrounding medium but the level of $^{14}$C-leucine in the acid fraction of control samples was significantly lower compared to the surrounding medium (Table 3.1 and 3.2).

3.3.1.2 Spleen slices

Figure 3.2 shows the levels of $^3$H-thymidine and $^{14}$C-leucine in the acid fraction of control and dosed spleen slice homogenates and in the incubating Ringer solution. The level of $^3$H-thymidine in the acid fractions and Ringer solution of both control and dosed spleen slices were not significantly different from each other. $^{14}$C-leucine levels in the acid fraction of both control and dosed spleen slices were less than half the level in the surrounding incubation medium. The levels in the Ringer solution were not significantly different from each other (Table 3.1 and 3.2).

3.3.1.3 Liver slices

Figure 3.3 shows the levels of $^3$H-thymidine and $^{14}$C-leucine in the acid fraction of control and dosed liver slice homogenates and in the incubating Ringer solution. The levels of $^3$H-thymidine and $^{14}$C-leucine in the Ringer solution of both control and dosed liver slices were not significantly different. However, in the acid fraction of control and dosed liver slices the $^3$H-thymidine levels were approximately double those in the surrounding medium whereas the $^{14}$C-leucine levels were less than half those in the surrounding medium (Table 3.1 and 3.2).
Figure 3.1. Testis - comparison of the levels of $^3$H-thymidine and $^{14}$C-leucine in the acid fraction and Ringer solution of control and dosed samples

$^3$H-thymidine (black symbols) and $^{14}$C-leucine (red symbols) levels were measured in the acid fraction of rat testis tubules and compared to levels in Ringer solution as described in methods section 2.1.4. Results are expressed as dpm/mg tissue or dpm/mg Ringer solution. Each data point represents the mean of duplicate samples from n separate animals. *significantly different from all other groups, $P < 0.05$ (one-way ANOVA with Bonferroni correction). Horizontal line represents mean of group.
Figure 3.2. Spleen - comparison of the levels of $^3$H-thymidine and $^{14}$C-leucine in the acid fraction and Ringer solution of control and dosed samples

$^3$H-thymidine (black symbols) and $^{14}$C-leucine (red symbols) levels were measured in the acid fraction of rat spleen slices and compared to levels in Ringer solution as described in methods section 2.1.4. Results are expressed as dpm/mg tissue or dpm/mg Ringer solution. Each data point represents the mean of duplicate samples from n separate animals. *significantly different from all other groups, **significantly different from acid fraction levels, $P < 0.05$ (one-way ANOVA with Bonferonni correction). Horizontal line represents mean of group.
Figure 3.3. Liver - comparison of the levels of $^3$H-thymidine and $^{14}$C-leucine in the acid fraction and Ringer solution of control and dosed samples

$^3$H-thymidine (black symbols) and $^{14}$C-leucine (red symbols) levels were measured in the acid fraction of rat liver slices and compared to levels in Ringer solution as described in methods section 2.1.4. Results are expressed as dpm/mg tissue or dpm/mg Ringer solution. Each data point represents the mean of duplicate samples from n separate animals. *significantly different from all other groups, **significantly different from acid fraction levels, +significantly different from Ringer solution levels, $P < 0.05$ (one-way ANOVA with Bonferonni correction). Horizontal line represents mean of group.
Table 3.1. Control tissues - mean levels of $^3$H-thymidine and $^{14}$C-leucine in the acid fraction of testis, spleen and liver samples, and in the Ringer solution.

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>Label</th>
<th>n</th>
<th>Acid fraction radiolabel levels of control tissues (dpm/mg tissue)</th>
<th>Ringer solution radiolabel levels of control tissues (dpm/mg Ringer solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Testis</td>
<td>Thymidine</td>
<td>55</td>
<td>2323.8</td>
<td>585.5</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>55</td>
<td>60.6*</td>
<td>21.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>Thymidine</td>
<td>38</td>
<td>2216.1</td>
<td>416.6</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>38</td>
<td>31.7*</td>
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</tr>
<tr>
<td>Liver</td>
<td>Thymidine</td>
<td>39</td>
<td>5553.5*</td>
<td>1452.5</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>39</td>
<td>27.5*</td>
<td>20.6</td>
</tr>
</tbody>
</table>

$^3$H-thymidine and $^{14}$C-leucine levels were measured in the acid fraction of control testis, spleen and liver samples and compared to levels in Ringer solution as described in methods section 2.1.4. *significantly different from corresponding Ringer solution levels, $P < 0.05$ (one-way ANOVA with Bonferonni correction).
Table 3.2. Drug-exposed tissues - mean levels of $^3$H-thymidine and $^{14}$C-leucine in the acid fraction of testis, spleen and liver samples, and in the Ringer solution

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>Label</th>
<th>n</th>
<th>Acid fraction radiolabel levels of dosed tissues (dpm/mg tissue)</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>COV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis</td>
<td>Thymidine</td>
<td>18</td>
<td></td>
<td>3010.8*</td>
<td>493.1</td>
<td>116.2</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>18</td>
<td></td>
<td>92.0</td>
<td>24.6</td>
<td>5.8</td>
<td>26.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>Thymidine</td>
<td>18</td>
<td></td>
<td>2110.8</td>
<td>469.1</td>
<td>110.6</td>
<td>22.2</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>18</td>
<td></td>
<td>48.5*</td>
<td>15.7</td>
<td>3.7</td>
<td>32.4</td>
</tr>
<tr>
<td>Liver</td>
<td>Thymidine</td>
<td>20</td>
<td></td>
<td>4643.7*</td>
<td>1331.6</td>
<td>297.8</td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>20</td>
<td></td>
<td>40.8*</td>
<td>37.9</td>
<td>8.5</td>
<td>92.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>Label</th>
<th>n</th>
<th>Ringer solution radiolabel levels of dosed tissues (dpm/mg Ringer solution)</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>COV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis</td>
<td>Thymidine</td>
<td>18</td>
<td></td>
<td>2232.5</td>
<td>104.5</td>
<td>24.6</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>18</td>
<td></td>
<td>79.2</td>
<td>13.9</td>
<td>3.3</td>
<td>17.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>Thymidine</td>
<td>18</td>
<td></td>
<td>2263.1</td>
<td>122.4</td>
<td>28.9</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>18</td>
<td></td>
<td>91.3</td>
<td>19.3</td>
<td>4.6</td>
<td>21.1</td>
</tr>
<tr>
<td>Liver</td>
<td>Thymidine</td>
<td>20</td>
<td></td>
<td>2139.1</td>
<td>121.4</td>
<td>27.2</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>20</td>
<td></td>
<td>86.2</td>
<td>16.3</td>
<td>3.7</td>
<td>18.9</td>
</tr>
</tbody>
</table>

$^3$H-thymidine and $^{14}$C-leucine levels were measured in the acid fraction of dosed testis, spleen and liver samples and compared to levels in Ringer solution as described in methods section 2.1.4. *significantly different from corresponding Ringer solution levels, $P < 0.05$ (one-way ANOVA with Bonferonni correction).
3.3.2 Time course of radiolabel incorporation

Figure 3.4 shows a time course of radiolabel incorporation into testis, spleen and liver samples. Incorporation is approximately linear over the time course studied in all three tissues.

3.3.3 Quantity of DNA and protein synthesis as estimated from radiolabel incorporation

Table 3.3 shows the number of pmoles of thymidine and leucine incorporated per mg protein per hour as estimated from isotope incorporation. This table illustrates the large differences in the quantity of DNA synthesis between the different tissue samples and also illustrates the high rate of protein synthesis compared to DNA synthesis. This is especially true for liver slices. Although the radioactive counts incorporated into DNA of liver slices may be roughly equivalent to those incorporated into protein, the actual quantity of DNA synthesis is much lower compared to protein synthesis.

3.3.4 Effect of pre-incubation period on incorporation of radiolabel

Figure 3.5 shows the effect of pre-incubation time on radiolabel incorporation into testis, spleen and liver samples. It was found that the period of pre-incubation did not have a major effect on subsequent radiolabel incorporation.
Time course of radiolabel incorporation into DNA (●) and protein (△) was measured in control tissues after pre-incubation for 30 minutes in Ringer solution followed by addition of radiolabel and further incubation for the indicated times. Data points represent the mean of duplicate tissue samples from one animal.
Table 3.3 Quantity of DNA and protein synthesis as estimated from radiolabel incorporation

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>Thymidine</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/mg protein/hr</td>
<td>pmol/mg protein/hr</td>
</tr>
<tr>
<td>Testis</td>
<td>13855</td>
<td>30.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>70533</td>
<td>153.3</td>
</tr>
<tr>
<td>Liver</td>
<td>1303</td>
<td>2.8</td>
</tr>
</tbody>
</table>

The quantity of DNA and protein synthesis (pmol/mg protein/hr) in control tissues was estimated from the number of counts incorporated into DNA and protein (dpm/mg protein/hr) and the number of counts in the incubation medium (dpm/mg protein/hr). Carrier leucine and thymidine (0.1 mM and 5 μM) were added to the incubation medium. The number of counts per pmole of thymidine and leucine in the incubation medium were approximately 460 dpm/pmol thymidine and 1 dpm/pmol leucine. dpm/mg protein/hr values were taken from the results of the time course study (Figure 3.4).
Figure 3.5. Effect of pre-incubation period on radiolabel incorporation

Radiolabel incorporation into DNA (●) and protein (△) in rat testis, spleen and liver slices was measured in control tissues after different periods of pre-incubation in Ringer solution followed by addition of radiolabel and incubation for a further 30 minutes. Data points represent the mean of triplicate tissue samples from two experiments.
3.3.5 Effect of leaving ‘on ice’ in isotonic saline on incorporation of radiolabel

Immediately after dissection samples are placed in ice-cold 0.15 M NaCl solution. Table 3.4 shows that leaving the testes in isotonic saline on ice for 8 - 12 minutes after dissection did not affect subsequent radiolabel incorporation when compared to testes used immediately after dissection with minimal time in ice-cold saline.

3.3.6 Secretion of protein from liver slices

The level of labelled protein secretion from liver slices has been found to be very low at 30 minutes after addition of radiolabel. $^{14}\text{C}$ count in the TCA-insoluble fraction of the incubation medium (Ringer solution) taken from flasks that were incubated with liver slices ranged from 0 to 11.8 dpm/mg of tissue. The mean ± SD for 32 experiments is 4.0 ± 2.4 dpm per mg of tissue slice. This is negligible when compared with the several hundred dpm per mg of tissue incorporated into the tissue slice itself.
Table 3.4. Effect of leaving testes ‘on ice’ in 0.15 M saline after dissection on incorporation rates of radiolabel

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>Incorporation</th>
<th>dpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt; 1 min*</td>
</tr>
<tr>
<td>Testis</td>
<td>Thymidine</td>
<td>22819</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25372</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>3332</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3166</td>
</tr>
</tbody>
</table>

*Tissue kept on ice in 0.15 M NaCl solution for time indicated after dissection.

$^3$H-thymidine and $^{14}$C-leucine incorporation was measured in control tissues after 30 minutes of pre-incubation in Ringer solution, followed by addition of radiolabel and incubation for a further 30 minutes.

Data represent mean incorporation values for triplicate tissue samples from two animals.
3.3.7 Variation in radiolabel incorporation

The variation in thymidine and leucine incorporation was measured in different control tissues after 30 minutes of pre-incubation in Ringer solution, followed by addition of radiolabel and a further 30 minutes of incubation.

3.3.7.1 Between replicate samples

Table 3.5 shows the variation in thymidine and leucine incorporation between triplicate testis, spleen and liver samples from the same animal. The coefficient of variation ranges from 1.1 to 13.1%, indicating that there is only a small variation between triplicate samples.

3.3.7.2 Between different tissues

Figure 3.6 and Table 3.6 illustrate the large variation in thymidine and leucine incorporation rates between different control tissues. Table 3.6 shows the mean, SD and range of incorporation levels encountered in this study. Spleen and thymus were found to have the highest rates of thymidine incorporation, testis and kidney have intermediate rates, and liver, the lowest rate of incorporation. These results are in line with the expected rates of cell turnover and hence DNA synthesis.

3.3.7.3 Between animals

Figure 3.6 and Table 3.7 demonstrate the range and variation in thymidine and leucine incorporation between different animals in various tissues. These results show that there is a large variation between different animals of the same species. The coefficient of variation ranges from 21.4 to 77.8%.

3.3.7.4 Between different sized animals

Table 3.8 and 3.9 show the variation in thymidine and leucine incorporation between different sized animals. The older animals (> 180 g) tended to have lower rates of DNA and protein synthesis compared to the younger animals. The rate of DNA and protein synthesis in liver slices was much higher in the youngest animals (90 - 120 g) compared to the older animals. Most experiments were carried out on animals between 120 - 180 g as these animals provided a large number of samples and the rates of DNA and protein
synthesis were still relatively high, even in the liver.
Table 3.5. Variation in radiolabel incorporation between triplicate

testis, spleen and liver samples from the same animal

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>COV (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thymidine incorporation</td>
<td>Leucine incorporation</td>
<td></td>
</tr>
<tr>
<td>Testis</td>
<td>7.9</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.8</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>5.2</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.1</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.7</td>
<td>6.0</td>
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<tr>
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<td>8.1</td>
<td>1.2</td>
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</tr>
<tr>
<td></td>
<td>4.8</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>5.0</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.9</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.4</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>4.0</td>
<td></td>
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<td></td>
<td>3.7</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>8.6</td>
<td></td>
</tr>
</tbody>
</table>

\(^3\text{H}-\text{thymidine and } ^{14}\text{C}-\text{leucine incorporation was measured in control tissues after 30 minutes of pre-incubation in Ringer solution, addition of radiolabel, followed by a further 30 minutes of incubation. The coefficient of variation (COV) was calculated from the dpm/mg protein values of triplicate tissue samples. Each row represents the results from an individual animal.}

95
Figure 3.6. Variation between animals of incorporation of radiolabel into DNA and protein in control tissues

Results are expressed as dpm/mg protein. Each data point represents the mean dpm/mg protein values of duplicate or triplicate tissue samples from one animal. Horizontal line represents the mean of the group.
Table 3.6. $^3$H-thymidine and $^{14}$C-leucine incorporation levels in control tissues

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>n</th>
<th>Thymidine incorporation (dpm/mg protein)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Lowest value</td>
</tr>
<tr>
<td>Testis</td>
<td>75</td>
<td>9,446</td>
<td>5,143</td>
<td>1,811</td>
<td>20,938</td>
</tr>
<tr>
<td>Spleen</td>
<td>60</td>
<td>52,900</td>
<td>32,800</td>
<td>8,605</td>
<td>152,505</td>
</tr>
<tr>
<td>Liver</td>
<td>60</td>
<td>1,996</td>
<td>1,552</td>
<td>342</td>
<td>6,029</td>
</tr>
<tr>
<td>Thymus</td>
<td>11</td>
<td>54,770</td>
<td>12,870</td>
<td>35,856</td>
<td>74,702</td>
</tr>
<tr>
<td>Kidney</td>
<td>4</td>
<td>2,190</td>
<td>830</td>
<td>1,499</td>
<td>3,319</td>
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</table>

<table>
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<tr>
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<th>n</th>
<th>Leucine incorporation (dpm/mg protein)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Lowest value</td>
</tr>
<tr>
<td>Testis</td>
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<td>2,821</td>
<td>837</td>
<td>396</td>
<td>5,684</td>
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<td>794</td>
<td>2,053</td>
<td>5,370</td>
</tr>
<tr>
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<td>508</td>
<td>515</td>
<td>2,620</td>
</tr>
<tr>
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<td>11</td>
<td>3,778</td>
<td>871</td>
<td>2,600</td>
<td>5,218</td>
</tr>
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<td>4</td>
<td>1,520</td>
<td>370</td>
<td>1,243</td>
<td>2,034</td>
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</table>

$^3$H-thymidine and $^{14}$C-leucine incorporation was measured in control tissues after 30 minutes of pre-incubation in Ringer solution, addition of radiolabel, followed by a further 30 minutes of incubation. Mean dpm/mg protein values of duplicate or triplicate tissue samples were taken as a single data point. n = number of animals. The amount of labelled marker and carrier was the same in all experiments.
Table 3.7. Variation in radiolabel incorporation between animals

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>n</th>
<th>COV (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thymidine incorporation</td>
<td>Leucine incorporation</td>
<td></td>
</tr>
<tr>
<td>Testis</td>
<td>75</td>
<td>54.4</td>
<td>29.7</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>60</td>
<td>62.0</td>
<td>21.4</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>60</td>
<td>77.8</td>
<td>38.2</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
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<td>23.5</td>
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<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>4</td>
<td>37.9</td>
<td>24.4</td>
<td></td>
</tr>
</tbody>
</table>

\(^3\text{H}\)-thymidine and \(^{14}\text{C}\)-leucine incorporation was measured in control tissues after 30 minutes of pre-incubation in Ringer solution, addition of radiolabel, followed by a further 30 minutes of incubation. Mean dpm/mg protein values of duplicate or triplicate samples were taken as a single data point and used to calculate the coefficient of variation (COV) between animals. n = number of animals.
Table 3.8. Variation in $^3$H-thymidine incorporation between different sized animals

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>Rat weight range (g)</th>
<th>n</th>
<th>Thymidine incorporation dpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Testis</td>
<td>90 - 120</td>
<td>9</td>
<td>7611</td>
</tr>
<tr>
<td></td>
<td>121 - 150</td>
<td>27</td>
<td>11506‡</td>
</tr>
<tr>
<td></td>
<td>151 - 180</td>
<td>34</td>
<td>9213</td>
</tr>
<tr>
<td></td>
<td>181 - 220</td>
<td>5</td>
<td>3219</td>
</tr>
<tr>
<td>Spleen</td>
<td>90 - 120</td>
<td>9</td>
<td>36300</td>
</tr>
<tr>
<td></td>
<td>121 - 150</td>
<td>27</td>
<td>64438</td>
</tr>
<tr>
<td></td>
<td>151 - 180</td>
<td>20</td>
<td>49577</td>
</tr>
<tr>
<td></td>
<td>181 - 220</td>
<td>4</td>
<td>28959</td>
</tr>
<tr>
<td>Liver</td>
<td>90 - 120</td>
<td>9</td>
<td>3956†</td>
</tr>
<tr>
<td></td>
<td>121 - 150</td>
<td>26</td>
<td>1827*</td>
</tr>
<tr>
<td></td>
<td>151 - 180</td>
<td>18</td>
<td>1537*</td>
</tr>
<tr>
<td></td>
<td>181 - 220</td>
<td>7</td>
<td>1287*</td>
</tr>
</tbody>
</table>

$^3$H-thymidine incorporation was measured in control tissues after 30 minutes of pre-incubation in Ringer solution, addition of radiolabel, and further incubation for 30 minutes. Mean dpm/mg protein values of duplicate or triplicate tissue samples were taken as a single data point and used to calculate the above values. n = number of animals.

*significantly different from 90 - 120 g group, $P < 0.05$ (one-way ANOVA with Bonferronni correction). ‡significantly different from 181 - 220 g group, $P < 0.05$ (one-way ANOVA with Bonferronni correction).
Table 3.9. Variation in $^{14}$C-leucine incorporation between different sized animals

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>Rat weight range</th>
<th>n</th>
<th>Leucine incorporation dpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g)</td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Testis</td>
<td>90 – 120</td>
<td>9</td>
<td>3581†</td>
</tr>
<tr>
<td></td>
<td>121 – 150</td>
<td>27</td>
<td>2790*†</td>
</tr>
<tr>
<td></td>
<td>151 – 180</td>
<td>34</td>
<td>2826*†</td>
</tr>
<tr>
<td></td>
<td>181 – 220</td>
<td>5</td>
<td>1585*</td>
</tr>
<tr>
<td>Spleen</td>
<td>90 – 120</td>
<td>9</td>
<td>4272</td>
</tr>
<tr>
<td></td>
<td>121 – 150</td>
<td>27</td>
<td>3797</td>
</tr>
<tr>
<td></td>
<td>151 – 180</td>
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<td>3425*</td>
</tr>
<tr>
<td></td>
<td>181 – 220</td>
<td>4</td>
<td>3361</td>
</tr>
<tr>
<td>Liver</td>
<td>90 – 120</td>
<td>9</td>
<td>1974†</td>
</tr>
<tr>
<td></td>
<td>121 – 150</td>
<td>26</td>
<td>1312*</td>
</tr>
<tr>
<td></td>
<td>151 – 180</td>
<td>18</td>
<td>1109*</td>
</tr>
<tr>
<td></td>
<td>181 – 220</td>
<td>7</td>
<td>1149*</td>
</tr>
</tbody>
</table>

$^{14}$C-leucine incorporation was measured in control tissues after 30 minutes of pre-incubation in Ringer solution, addition of radiolabel, and further incubation for 30 minutes. Mean dpm/mg protein values of duplicate or triplicate tissue samples were taken as a single data point and used to calculate the above values. n = number of animals.

*significantly different from 90 - 120 g group, $P < 0.05$ (one-way ANOVA with Bonferroni correction). †significantly different from 181 - 220 g group, $P < 0.05$ (one-way ANOVA with Bonferroni correction).
3.3.8 Choice of tissues for study

Preliminary experiments were performed to determine which tissues would be most suitable for study. The candidate tissues must give reproducible results, a small variation between replicate samples and also produce a sufficient number of samples in one experiment to allow study of at least 3 different compounds or doses. The tissues tested were liver, spleen, testis, thymus, and kidney, and the antioxidant n-propyl gallate was used as the test compound in this series of experiments.

The results for kidney slices were highly variable, presumably because of the non-uniformity of the slices in cell composition. The incorporation of radiolabel could sometimes vary greatly between triplicate control samples (Table 3.10) and the tissue response to n-propyl gallate was very variable (shown in Table 3.11). Further experiments were not performed on this tissue.

For thymus samples, the variation between triplicate samples could be quite high and the tissue response to n-propyl gallate varied greatly between experiments (Table 3.10 and Table 3.12). Further experiments were not performed on this tissue.

Testis, spleen and liver samples were selected to test further compounds since they gave much lower COV between samples. These tissues were also chosen for the following reasons:

- Liver - a much studied target tissue of paracetamol with large cells, slow metabolic turnover and a large quantity of tissue available that is easily accessible.
- Spleen - a lymphoid tissue with small cells and a fast metabolic turnover, that is easily accessible.
- Testis - a reproductive tissue with dividing cells, complex cell-cell interactions and a large quantity of tissue available that is easily accessible.

Duplicate samples instead of triplicate samples were taken in each experiment to maximise the number of doses that could be studied in each experiment.
Table 3.10. Comparison of variation in radiolabel incorporation between control triplicate kidney slices and thymus samples from the same animal

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>Thymidine incorporation</th>
<th>Thymine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>7.4</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>12.3</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>24.5</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Thymus</td>
<td>6.8</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>11.9</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>18.8</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>6.1</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>10.2</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>26.2</td>
</tr>
<tr>
<td></td>
<td>24.2</td>
<td>16.9</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

$^3$H-thymidine and $^{14}$C-leucine incorporation was measured in control tissues after 30 minutes of pre-incubation in Ringer solution, addition of radiolabel, followed by a further 30 minutes incubation. Coefficient of variation (COV) was calculated for triplicate tissue samples. Each row represents the results from separate animals.
Table 3.11. Inhibition of DNA and protein synthesis by n-propyl gallate in rat kidney slices

<table>
<thead>
<tr>
<th>[n-propyl gallate]</th>
<th>% inhibition of synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
</tr>
<tr>
<td>(mM)</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>26.1</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>0.3</td>
<td>17.1</td>
</tr>
<tr>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>0.3</td>
<td>42.8</td>
</tr>
</tbody>
</table>

Inhibition of DNA and protein synthesis was measured in rat kidney slices incubated in Ringer solution ± n-propyl gallate as described in methods chapter 2.1. Mean dpm/mg protein values of triplicate tissue samples were taken as a single data point and used to calculate % inhibition compared to control samples. Each row represents the results from separate animals.
Table 3.12. Inhibition of DNA and protein synthesis by \( n \)-propyl gallate in rat thymus samples

<table>
<thead>
<tr>
<th>[( n )-propyl gallate]</th>
<th>% inhibition of synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mM)</td>
<td>DNA</td>
</tr>
<tr>
<td>0.001</td>
<td>25.0</td>
</tr>
<tr>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td>0.03</td>
<td>11.7</td>
</tr>
<tr>
<td>0.03</td>
<td>16.4</td>
</tr>
<tr>
<td>0.03</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>18.9</td>
</tr>
<tr>
<td>0.1</td>
<td>60.9</td>
</tr>
<tr>
<td>0.1</td>
<td>29.0</td>
</tr>
</tbody>
</table>

Inhibition of DNA and protein synthesis was measured in rat thymus samples incubated in Ringer solution ± \( n \)-propyl gallate as described in methods chapter 2.1. Mean dpm/mg protein values of triplicate samples were taken as a single data point and used to calculate % inhibition compared to control samples. Each row represents the results from separate animals.
3.4 Results of *in vitro* experiments with paracetamol, hydroxyurea, cycloheximide, (+)-catechin and *n*-propyl gallate

3.4.1 Effect of paracetamol on DNA and protein synthesis *in vitro*

Figure 3.7 shows the concentration-response of rat testis, spleen and liver samples to paracetamol. Inhibition of DNA and protein synthesis by paracetamol in tissue samples was measured by comparing $^3$H-thymidine and $^{14}$C-leucine incorporation into tissue samples incubated in the presence of paracetamol, with samples incubated in the absence of paracetamol. Tissue samples were pre-incubated for 30 minutes, radiolabel was then added and incubation continued for a further 30 minutes. Paracetamol was found to be a highly specific dose-dependent inhibitor of DNA synthesis in this experimental system. Inhibition of DNA synthesis in testis and spleen samples reached ~90% or more, whereas in liver slices inhibition of DNA synthesis plateaued at ~70%. Protein synthesis was essentially unaffected at the concentrations tested. There was also a difference in sensitivity between the three tissues to the inhibitory effects of paracetamol. DNA synthesis in spleen slices was found to be much more sensitive to inhibition than testis samples, and testis samples were more sensitive than liver slices. The IC$_{50}$ values for inhibition of DNA synthesis are 0.22, 0.48, 1.33 mM for spleen, testis and liver samples, respectively.

3.4.2 Effect of hydroxyurea on DNA and protein synthesis *in vitro*

Hydroxyurea was used in this study as a control drug that is a known DNA synthesis inhibitor (Calabresi and Chabner, 1990). Figure 3.8 shows the concentration-response of rat testis, spleen and liver samples to hydroxyurea. Tissue samples were pre-incubated for 30 minutes, $^3$H-thymidine and $^{14}$C-leucine were then added and incubation was continued for a further 30 minutes. In spleen and testis samples, hydroxyurea was found to specifically inhibit DNA synthesis in a dose-dependent manner with very little effect on protein synthesis. Liver slices were highly resistant to inhibition of DNA synthesis with 15.6 ± 5.7% inhibition observed at 0.3 mM. At higher doses (1 - 10 mM) inhibition of
DNA synthesis was accompanied by an almost equivalent level of protein synthesis inhibition. Again a difference in sensitivity of the tissues to inhibition of DNA synthesis was observed with the same order of sensitivity demonstrated with paracetamol, i.e. spleen > testis > liver. The IC$_{50}$ values for DNA synthesis inhibition are 0.04 and 0.1 mM for spleen and testis samples, respectively. Inhibition of DNA and protein synthesis in liver slices did not increase above 50 % at 10 mM so no IC$_{50}$ values could be determined.

3.4.3 Effect of cycloheximide on DNA and protein synthesis \textit{in vitro}

Cycloheximide was used in this study as a control drug that is a known protein synthesis inhibitor (Vazquez, 1975). Figure 3.9 shows the concentration-response of rat testis, spleen and liver samples to cycloheximide. Tissue samples were pre-incubated for 30 minutes, $^3$H-thymidine and $^{14}$C-leucine were then added and incubation was continued for a further 30 minutes. Cycloheximide was found to specifically inhibit liver slice protein synthesis in a dose-dependent manner with an IC$_{50}$ value of 0.22 µM. Little effect on DNA synthesis was observed in liver slices. In spleen and testis samples, cycloheximide displayed a certain degree of preferential inhibition of protein synthesis. However, this was also accompanied by significant DNA synthesis inhibition. The IC$_{50}$ values for inhibition of DNA and protein synthesis in spleen slices are 0.60 and 0.31 µM, respectively. The IC$_{50}$ values for inhibition of DNA and protein synthesis in testis samples are 0.43 and 0.28 µM, respectively.
Figure 3.7. Specific inhibition of DNA synthesis by paracetamol in rat testis, spleen, and liver in vitro

Inhibition of DNA (closed symbols) and protein synthesis (open symbols) was measured in rat testis tubules (■), spleen slices (▲) and liver slices (○) incubated in Ringer solution ± paracetamol as described in methods chapter 2.1. Data points represent mean ± SEM of triplicate or duplicate samples from 4 separate animals. *significantly different from control group incubated in the absence of drug, \( P < 0.05 \) (one-way ANOVA with Bonferonni correction).
Figure 3.8. Inhibition of DNA and protein synthesis by hydroxyurea in rat testis, spleen, and liver *in vitro*

Inhibition of DNA (closed symbols) and protein synthesis (open symbols) was measured in rat testis tubules (■), spleen slices (▲) and liver slices (●) incubated in Ringer solution ± hydroxyurea as described in methods chapter 2.1. Data points represent mean ± SEM of triplicate or duplicate samples from 4 - 8 separate animals. *DNA synthesis inhibition significantly different from control group incubated in the absence of drug, P < 0.05 (one-way ANOVA with Bonferonni correction). **DNA and protein synthesis inhibition significantly different from control group incubated in the absence of drug, P < 0.05 (one-way ANOVA with Bonferonni correction).
Figure 3.9. Inhibition of DNA and protein synthesis by cycloheximide in rat testis, spleen, and liver *in vitro*

Inhibition of DNA (●) and protein (▲) synthesis was measured in rat testis tubules, spleen slices, and liver slices incubated in Ringer solution ± cycloheximide as described in methods chapter 2.1. Data points represent mean ± SEM of triplicate or duplicate samples from 3 - 5 separate animals. *significantly different from control group incubated in the absence of drug, \( P < 0.05 \) (one-way ANOVA with Bonferroni correction).
3.4.4 Effect of (+)-catechin on DNA and protein synthesis \textit{in vitro}

Because paracetamol has antioxidant properties, I wanted to look at other antioxidants using the same method to see if they would also specifically inhibit DNA synthesis and to see if the difference in sensitivity to both paracetamol and hydroxyurea between rat testis, spleen and liver samples could be reproduced with other compounds. (+)-catechin is a naturally-occurring flavonoid antioxidant present in abundance in tea. Figure 3.10 shows the concentration-response of rat testis, spleen and liver samples to (+)-catechin. Tissue samples were pre-incubated for 30 minutes, $^3$H-thymidine and $^{14}$C-leucine were then added and incubation was continued for a further 30 minutes. In spleen and testis samples, DNA synthesis was specifically inhibited in a dose-dependent manner with little effect on protein synthesis. IC$_{50}$ values are 1.02 and 0.81 mM for testis and spleen, respectively. In liver slices, DNA synthesis was inhibited at 0.5 mM by 12.2 ± 2.0 % with little effect on protein synthesis. However, at 1 and 2 mM, inhibition of protein synthesis was also seen, but to a lesser extent than DNA synthesis inhibition. At 5 mM, both DNA and protein synthesis were equally inhibited. The IC$_{50}$ values for DNA and protein synthesis were 1.88 and 2.83 mM, respectively. The differences observed in the sensitivity of the various tissues to the inhibitory effects of (+)-catechin on DNA synthesis were not as great as for paracetamol and hydroxyurea.
Figure 3.10. Inhibition of DNA and protein synthesis by (+)-catechin in rat testis, spleen, and liver in vitro

Inhibition of DNA (●) and protein (▲) synthesis was measured in rat testis tubules, spleen slices and liver slices incubated in Ringer solution ± (+)-catechin as described in methods chapter 2.1. Data points represent mean ± SEM of triplicate or duplicate samples from 4 separate animals except in spleen slices at 0.02 mM where n=3 animals and at 0.5 mM where n=5 animals. *significantly different from control group incubated in the absence of drug, P < 0.05 (one-way ANOVA with Bonferonni correction).
3.4.5 Effect of \textit{n}-propyl gallate on DNA and protein synthesis \textit{in vitro}

\textit{n}-propyl gallate is a synthetic phenolic antioxidant used as a preservative in the food industry. Figure 3.11 shows the concentration-response of rat testis, spleen and liver samples to \textit{n}-propyl gallate. Tissue samples were pre-incubated for 30 minutes, \textsuperscript{3}H-thymidine and \textsuperscript{14}C-leucine were then added and incubation was continued for a further 30 minutes. In testis samples, DNA synthesis was preferentially inhibited but this was accompanied by significant protein synthesis inhibition. At 3 mM, both DNA and protein synthesis were almost maximally inhibited. IC\textsubscript{50} values for inhibition of DNA and protein synthesis in testis samples are 0.32 and 0.82 mM, respectively. In spleen slices, a dose-dependent specific inhibition of DNA synthesis was observed with an IC\textsubscript{50} of 0.13 mM. In liver slices, DNA synthesis did not appear to be specifically inhibited at any dose. IC\textsubscript{50} values for inhibition of DNA and protein synthesis in liver slices are 1.94 and 2.65 mM, respectively. Liver slices were found to be more resistant to DNA synthesis inhibition compared to testis and spleen samples.
Figure 3.11. Inhibition of DNA and protein synthesis by \( n \)-propyl gallate in rat testis, spleen, and liver \textit{in vitro}

Inhibition of DNA (○) and protein (▲) synthesis was measured in rat testis tubules, spleen slices and liver slices incubated in Ringer solution ± \( n \)-propyl gallate as described in methods chapter 2.1. Data points represent mean ± SEM of triplicate or duplicate samples from 4 - 5 separate animals. *significantly different from control group incubated in the absence of drug, \( P < 0.05 \) (one-way ANOVA with Bonferonni correction).
3.5 Discussion

These experiments show that:

• The present *in vitro* experimental model can show when a compound causes specific inhibition of DNA synthesis without interference with protein synthesis.

• Paracetamol specifically inhibits DNA synthesis in testis tubules, spleen and liver slices in a dose-dependent manner.

• Hydroxyurea specifically inhibits DNA synthesis in testis tubules and spleen slices, but in liver slices the inhibition only occurs at concentrations that also inhibit protein synthesis.

• Cycloheximide specifically inhibits protein synthesis in liver slices, but in testis tubules and spleen slices inhibition of protein synthesis is accompanied by significant DNA synthesis inhibition.

• (+)-catechin specifically inhibits DNA synthesis in testis tubules and spleen slices, and preferentially inhibits DNA synthesis in liver slices.

• *n*-propyl gallate specifically inhibits DNA synthesis in rat spleen slices, preferentially inhibits DNA synthesis in testis tubules, but does not inhibit specifically in liver slices.

• Paracetamol differentially inhibits DNA synthesis in different tissues. This difference in sensitivity between tissues is also observed with hydroxyurea, and to a lesser extent *n*-propyl gallate and (+)-catechin, with spleen being most sensitive, followed by testis and then liver.

3.5.1 Experiments on validation of methods

The present *in vitro* experimental model can show when a compound causes specific inhibition of DNA synthesis without interference with protein synthesis. The results for paracetamol are a typical example of this. Inhibition of protein synthesis was chosen as an indicator of cell viability in these experiments because protein synthesis requires ATP, which is produced by the mitochondria. During both necrotic and apoptotic cell injury, mitochondria are damaged and can no longer produce ATP (Wyllie and Duvall, 1992; Mignotte and Vayssiere, 1998). A fall in intracellular ATP levels leads to inhibition of protein synthesis. Paracetamol is thought to inhibit DNA synthesis by inhibition of a single enzyme, ribonucleotide reductase, and does so
without inhibition of protein synthesis. Simultaneous inhibition of DNA and protein synthesis in the present *in vitro* model suggests effects more widespread in the metabolic pathways or cytotoxic effects.

Although the *in vitro* slice system used in the present study can show whether a compound causes specific inhibition of DNA synthesis, a lack of DNA synthesis inhibition does not necessarily mean that this compound cannot inhibit DNA synthesis under other circumstances. Failure of the compound being tested to penetrate cells or rapid metabolism could be the reason. A further limitation of this method is that no information about the specific sites of the DNA or protein synthesis pathways that are affected can be elucidated. While the inhibition of DNA synthesis by paracetamol is mainly attributable to inhibition of ribonucleotide reductase through the radical quenching property of the parent compound, it is not known if other properties affecting signalling pathways might play a role in the inhibition of DNA synthesis. This is also true for the other compounds, especially the flavonoids. Despite its limitations, this method can be very useful as a broad screen for DNA synthesis inhibitors.

A one hour incubation period with test drug was chosen as previous studies have shown inhibition of DNA synthesis at one hour after paracetamol dosing both *in vivo* and within a few minutes *in vitro* (Hongslo *et al.*, 1989; Lister and McLean, 1997). A longer time period was not chosen as other studies showed that if the time of exposure was extended cytotoxic effects became observable.

No control experiments were performed to determine if any non-specific binding of radiolabel to the tissue samples occurs, but it is expected to be quite low as inhibition of radiolabel incorporation into DNA and protein by specific inhibitors was found to reach levels of > 98 % in all three tissues tested.

In the present experiments, the uptake, utilisation and secretion of the radiolabel can affect availability of radiolabel to the tissue sample, which can in turn affect the incorporation rate. Apparent inhibition of synthesis by a test compound may be observed if radiolabel uptake were sufficiently reduced to be rate-limiting.

### 3.5.2 Uptake of thymidine and leucine into tissue samples

After a number of experiments were conducted, the question of uptake of radiolabel into the tissue was raised. In an attempt to determine the level of uptake of
radiolabel, retrospective analysis of the counts in the acid-soluble fraction of the homogenised tissue was used as an indirect measure. Although this measurement does indeed give an approximation of the level of uptake it must be borne in mind that the rate of utilisation and secretion can also affect the levels measured after 30 minutes of exposure. The level of uptake was compared to the concentrations in the incubation medium (Ringer solution). The radioactivity in the incubation medium was checked to make sure that isotope had been properly added. Because the Ringer solution was about 100-fold greater in volume than the slices (5000 µl Ringer solution compared to ~50 mg slice), uptake was in fact negligible in comparison with the large volume of incubation medium. Variations in radioactive counts in the Ringer sample are due to pipetting and counter variation.

3.5.2.1 Testis samples

Experiments on the uptake of radiolabel into testis tubules showed that the level of 3H-thymidine in the acid fraction of control samples was not significantly different from the concentration in the surrounding Ringer solution. However, the level of 3H-thymidine in the acid fraction of some drug-exposed testis samples was significantly higher compared to the concentration in the Ringer solution. Similarly, the 14C-leucine levels in the acid fraction of the drug-exposed testis samples were not significantly different from the concentration in the Ringer solution. But, in control samples the 14C-leucine levels in the acid fraction was significantly lower compared to the Ringer solution concentrations (Figure 3.1). Overall, it was found that radiolabel was present in amounts that were unlikely to cause errors in the estimation of inhibition of DNA or protein synthesis. In general, inhibition of synthesis was accompanied by increased isotope in the acid fraction, as would be expected from loss of a utilisation pathway.

3.5.2.2 Spleen slices

Experiments on the uptake of radiolabel into spleen slices showed that the levels of 3H-thymidine in the acid fraction and incubation medium of control and drug-exposed tissue slices were not significantly different from each other (Figure 3.2). However, 14C-leucine levels in the acid fraction of both control and drug-exposed spleen slices were less than half the level in the surrounding medium (Table 3.1 and 3.2). The lack of
difference between $^3$H levels in the acid fraction of control and drug-exposed slices and the Ringer solution suggest that the low $^{14}$C levels in the acid fraction are not due to inhibition of uptake by the test drug. The low level of $^{14}$C in the acid fraction is probably due to rapid incorporation of $^{14}$C-leucine into protein in spleen slices.

3.5.2.3 Liver slices

Experiments on the uptake of radiolabel into liver slices showed that as expected the levels of $^3$H-thymidine and $^{14}$C-leucine in the incubation medium (Ringer solution) of control and dosed liver slices were not significantly different (Figure 3.3). However, in the acid fraction of control and dosed liver slices $^3$H-thymidine levels were approximately double those in Ringer solution whereas $^{14}$C-leucine levels were less than half those in Ringer solution (Table 3.1 and 3.2). The high level of $^3$H-thymidine in the acid fraction indicates active uptake of thymidine, perhaps with metabolism to a differing form, together with low levels of incorporation into DNA. Figure 3.6 shows that the thymidine incorporation rate of liver slices is much lower than spleen slices and testis samples. The low level of $^{14}$C-leucine in the acid fraction is probably due to rapid incorporation into protein in liver slices.

Figure 3.3 also shows that in the acid fraction of drug-exposed liver slices there are 5 data points, which are much higher, compared to the rest of the group. These values all belong to slices that had been exposed to 6 or 10 mM $n$-propyl gallate. At these concentrations both DNA and protein synthesis were maximally inhibited. It is likely that the high $^{14}$C-leucine levels found in the acid fraction of these liver slices are due to inhibition of incorporation. However, no similar increase in the $^{14}$C levels in the acid fraction of liver slices exposed to 10 μM cycloheximide was seen.

3.5.3 Utilisation of thymidine and leucine in tissue samples

The utilisation of radiolabel is illustrated by the time course of incorporation shown in Figure 3.4. Radiolabel incorporation increases in an approximately linear manner up to 60 minutes. From this time course, 30 minutes of radiolabel exposure was chosen because this gave a reasonable level of thymidine and leucine incorporation. In addition, the time course indicates that at 30 minutes there is still plenty of radiolabel available for incorporation. Any inhibition of incorporation is therefore attributable to
the test drug and not because incorporation is slowing down because radiolabel levels are low. It was also found that neither the pre-incubation period nor the time spent in ice-cooled isotonic saline after dissection affected subsequent radiolabel incorporation (Figure 3.5 and Table 3.1).

Table 3.3 shows the approximate quantity of DNA and protein synthesis in the different tissue samples as estimated from isotope incorporation and radiolabel concentration in the incubation medium. There are large differences in the quantity of DNA synthesis between the different tissues as expected and a much higher rate of protein synthesis compared to DNA synthesis in all three tissues. Liver slices especially demonstrate that although the radioactive counts incorporated into DNA and protein are approximately equivalent, the actual quantity of DNA synthesis is much lower than protein synthesis. In the present study, in order to allow for this difference in DNA and protein synthesis, the concentration of $^3$H-thymidine added to the incubation medium was 20 times that of $^{14}$C-leucine, to give countable incorporation.

Experiments on the variation in thymidine and leucine incorporation into testis, spleen and liver samples show a low level of variation between replicate samples (Table 3.5) but a large variation between animals (Figure 3.6, Table 3.6 and 3.7). The variation in thymidine incorporation into control testis, spleen and liver samples from different animals is very large compared to the variation in thymidine levels in the acid fraction (COV range is 54.4 - 77.8 % vs. 18.8 - 26.2 %). This indicates that although incorporation of thymidine into DNA may vary greatly between animals, the uptake of thymidine into the tissue is reasonably constant.

It is generally found that thymidine and leucine levels vary together, i.e. low thymidine incorporation levels are associated with low leucine incorporation levels. This is probably due to poor health of the slice leading to reduced thymidine and leucine incorporation.

3.5.4 Secretion of leucine from liver slices

It is well known that proteins, especially albumin, are secreted by the liver and both in vivo methods and liver slices have been used to study this secretion (Peters, 1983). Experiments on the secretion of $^{14}$C-labelled proteins from liver slices showed very little secretion during the time period that slices were incubated, with levels of $4.0 \pm 2.4$
dpm/mg tissue (mean ± SD, n = 32). This represents on average approximately 2 - 3 % of the ¹⁴C levels incorporated into control liver slices and is sufficiently small to not affect the concentration of radiolabel within the cell.

3.5.5 Effect of different compounds on DNA and protein synthesis in vitro

3.5.5.1 Paracetamol

Paracetamol is shown to specifically inhibit DNA synthesis in a dose-dependent manner with little effect on protein synthesis in the rat in vitro (Figure 3.7). Concentration-response curves obtained in liver, spleen and testis samples demonstrate that as well as a specific inhibition of DNA synthesis there is a variation in the sensitivity of the different tissues. The order of most sensitive to least sensitive tissue is spleen > testis > liver with IC₅₀ values of 0.22, 0.48, 1.33 mM, respectively.

It must be emphasised at this point that the inhibition of DNA synthesis by paracetamol is not due to cell injury caused by the parent compound or its metabolites as liver slices from normal rats, not induced with phenobarbitone, are resistant to cell injury for many hours, even at 2 or 10 mM paracetamol (Miller et al, 1993; Martin and McLean, 1995). The lack of inhibition of protein synthesis in the present study is also an indicator of the good health of the cells.

The inhibition of DNA synthesis by paracetamol has been proposed as one of the mechanisms by which paracetamol might possibly cause genotoxicity (Bergman et al, 1996). In order for this to be relevant to the in vivo situation, therapeutic concentrations must be shown to inhibit DNA synthesis. It has been demonstrated by Hongslo and co-workers (1989; 1990) that paracetamol specifically inhibits replicative DNA synthesis in cell lines at close to the therapeutic level of 0.1 mM. The present work demonstrates that at 0.1 mM paracetamol, little or no inhibition of DNA synthesis would be expected in the liver or testes in vivo. But in the spleen, approximately 20 % inhibition of DNA synthesis was demonstrated in slices, suggesting that a low level of inhibition of DNA synthesis may be occurring in vivo. In overdose where plasma paracetamol concentrations are often > 1 mM, inhibition of DNA synthesis is highly likely to be occurring in some tissues in vivo.

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3.5.5.2 Hydroxyurea

Hydroxyurea is shown to specifically inhibit DNA synthesis in a dose-dependent manner in rat testis tubules and spleen slices but not in liver slices (Figure 3.8). The difference in sensitivity between tissues for inhibition of DNA synthesis is also observed with hydroxyurea with a similar order of sensitivity to inhibition, i.e. spleen > testis > liver. IC$_{50}$ values of 0.04, 0.1 mM, were obtained for spleen and testis, respectively. In clinical use as an anti-neoplastic drug, hydroxyurea is administered either by continuous daily oral doses ranging from 20 - 40 mg/Kg/day or intermittent oral doses of 80 mg/Kg every third day. An oral dose of 40 - 80 mg/Kg hydroxyurea, leads to peak plasma levels of 0.5 - 2 mM within 1 - 2 hours of administration (Donehower, 1992). The results of the present study demonstrate that DNA synthesis in both testis and spleen would most probably be maximally inhibited at therapeutic doses in vivo, with very little DNA or protein synthesis inhibition expected in the normal liver. However, with a plasma half-life of only 2 hours this inhibition would be short-lived.

3.5.5.3 Cycloheximide

Cycloheximide is shown to specifically inhibit liver slice protein synthesis in a dose-dependent manner with an IC$_{50}$ value of 0.22 μM (Figure 3.9). In spleen and testis samples, cycloheximide displays a certain degree of preferential inhibition of protein synthesis. However, this is also accompanied by significant DNA synthesis inhibition. The IC$_{50}$ values for inhibition of DNA and protein synthesis in spleen slices are 0.6 and 0.31 μM, respectively. The IC$_{50}$ values for inhibition of DNA and protein synthesis in testis samples are 0.43 and 0.28 μM, respectively. The high level of DNA synthesis inhibition in testis and spleen samples indicate either a non-specific cellular effect of cycloheximide on protein and DNA synthesis or a requirement for protein synthesis for DNA synthesis. Both of these explanations are equally likely and they may both contribute to the inhibition of DNA and protein synthesis. It has been previously shown by Venkatesan (1977) that cycloheximide inhibits DNA synthesis as well as protein synthesis in Balb/3T3 cells. Weiss (1969) has also demonstrated that the rate of DNA synthesis in HeLa S3 cells is highly dependent on protein synthesis during S-phase.
Inhibition of protein synthesis has been used in the present study as a marker of cell viability. However, as the results for cycloheximide in liver slices show, inhibition of protein synthesis does not always indicate that a test drug has cytotoxic effects.

3.5.6 Differential inhibition of DNA synthesis in different tissues

It is not known why there is a differential inhibition of DNA synthesis in different tissues and in particular why the liver slices were so much more insensitive to inhibition compared to testis and spleen samples. Several explanations are possible:

(i) Differences in the pool size of the DNA precursors, deoxyribonucleotides (dNTP).
(ii) Differences in the ribonucleotide reductase levels.
(iii) Differences in the activity of ribonucleotide reductase.
(iv) Alternative dNTP synthesis pathways are available.
(v) Metabolism pathways in the liver. (vi) Slower cell turnover.

The rate of DNA synthesis is highly dependent on the size of the dNTP pools within the cell. The reduction of all four ribonucleoside diphosphates to deoxyribonucleoside diphosphates by ribonucleotide reductase is an important rate-limiting step in the DNA synthesis pathway. Inhibition of ribonucleotide reductase activity leads to depletion of DNA precursors and inhibition of DNA synthesis. Table 3.5 and Figure 3.6 show the control rates of thymidine incorporation between different tissues. Spleen slices have the highest incorporation rate, testis samples have an intermediate rate whilst liver slices have the lowest rate. In spleen and testis samples, DNA synthesis is occurring at a fast rate because a large number of cells in the tissue are in S-phase. This would rapidly deplete the dNTP pools in the tissue which are normally replenished by various synthesis pathways. If ribonucleotide reductase activity were inhibited then de novo dNTP synthesis would stop, the pool of dNTP’s would be used up and inhibition of DNA synthesis would occur. In liver, a low level of DNA synthesis is occurring presumably because less than 0.01 % of cells is likely to be in mitosis (Short et al, 1973). Even if ribonucleotide reductase activity were inhibited, the large pool of dNTP’s in the liver slice could support further DNA synthesis, particularly if those cells not in S-phase can “feed” other liver cells with dNTP’s via gap junctions. Furthermore, it has been found that the acute cytotoxic effects of hydroxyurea is cell
cycle-dependent and restricted to cells in S-phase as only tissues with high rates of cellular proliferation are affected (Philips et al, 1967). Because only a small proportion of liver cells are in S-phase, even if ribonucleotide reductase activity were maximally inhibited in these cells, other liver cells would again be able to supply these cells with dNTP's from the pre-existing dNTP pool, via gap junctions.

Variation in ribonucleotide reductase levels in different tissues may also explain the difference in sensitivity to DNA synthesis inhibition between tissues. Hongslo et al (1990) has demonstrated that hydroxyurea-resistant cell lines with elevated levels of the small subunit of ribonucleotide reductase are more resistant to inhibition of cell growth by paracetamol than wild type cells. The amounts of ribonucleotide reductase in the different tissues are not known and the difference in sensitivity of the tissues could in part be due to a variation in ribonucleotide reductase levels. Ribonucleotide reductase levels are under cell cycle control and can increase 3 to 7-fold prior to and during S-phase (Eriksson et al, 1984; Engstrom et al, 1985). As discussed previously the different tissues have different rates of DNA synthesis that would in turn affect the cellular levels of ribonucleotide reductase.

The activity of ribonucleotide reductase has been found to affect the sensitivity of the enzyme to quenching of the tyrosyl free radical by hydroxyurea. A recent study using purified and reconstituted vaccinia virus ribonucleotide reductase has found that inhibition by hydroxyurea is dependent on ribonucleotide reductase enzyme activation produced by addition of substrate and cofactors (Hendricks and Mathews, 1998). The higher the activity of the enzyme, the more rapidly the tyrosyl free radical was lost after hydroxyurea addition. The authors proposed that activation of the enzyme exposes the tyrosyl free radical and makes it more susceptible to being quenched by hydroxyurea. Tissues with a high number of cells in S-phase would be expected to have a high level of ribonucleotide reductase activity and would therefore be more sensitive to inhibition. Likewise, tissues with a low number of cells in S-phase would be expected to have a low level of ribonucleotide reductase activity and would therefore be less sensitive to inhibition.

It was found that the level of inhibition of DNA synthesis in liver slices by paracetamol plateaued at ~70 % and a similar plateau effect occurred with hydroxyurea at ~50 %. This effect is not due to a lack of radiolabel in the flasks. As previously discussed the volume of the incubation medium was about 100-fold greater than the
liver slices and uptake of radiolabel was negligible in comparison to the large amount of radiolabel present in the incubation medium. It is also unlikely to be due to a delayed uptake of the drug because tissues are pre-incubated with drug for 30 minutes prior to isotope addition. Some of the previous explanations for the insensitivity of liver slices to DNA synthesis inhibitors deal with the effects of ribonucleotide reductase activity on dNTP synthesis. There are however other dNTP synthesis pathways present in the cell. This plateau effect occurs at > 2 mM, concentrations which would probably inhibit most or all ribonucleotide reductase activity in the liver slices. The fact that some DNA synthesis is still occurring in these liver slices suggests that other synthesis pathways not involving ribonucleotide reductase are being utilised.

In both the testis and the spleen samples, hydroxyurea was found to be approximately 5-fold more potent than paracetamol at inhibiting DNA synthesis. However, liver slices exposed to hydroxyurea were found to be highly insensitive to inhibition of DNA synthesis and inhibition did not occur until concentrations that also inhibited protein synthesis were reached. In contrast, paracetamol was specific even at doses up to 10 mM with no change in viability after one hour of incubation. A possible explanation for the insensitivity of the liver to the inhibitory effects of hydroxyurea lies with the highly active metabolic pathways within the liver. It has been previously shown that hydroxyurea is metabolised to urea in the mouse liver by an enzymatic reduction reaction (Adamson et al, 1965; Colvin and Bono, Jr, 1970). The intracellular concentrations of hydroxyurea in the liver slices would therefore be much lower compared to the concentration in the incubation medium leading to a lower level of inhibition. The ability of the liver to metabolise many foreign compounds could also partly explain why the liver slices were so much more insensitive to inhibition of DNA synthesis by paracetamol and other compounds.

3.5.7 Effect of other phenolic antioxidants on DNA and protein synthesis in vitro

Because paracetamol has antioxidant properties, other antioxidants were studied using the same method to see if they would also specifically inhibit DNA synthesis and to see if the difference in sensitivity between rat testis, spleen and liver samples could be reproduced with other compounds.
3.5.7.1 (+)-catechin

(+)-catechin, a flavonoid antioxidant, was found to specifically inhibit DNA synthesis in spleen slices and testis samples with little effect on protein synthesis (Figure 3.10). IC$_{50}$ values for DNA synthesis are 0.81 and 1.02 mM for spleen and testis samples, respectively. In liver slices, DNA synthesis was preferentially inhibited between 0.5 - 2 mM with significant inhibition of protein synthesis at 1 and 2 mM. At 5 mM, both DNA and protein synthesis were equally inhibited indicating a possible cytotoxic effect at this concentration. The IC$_{50}$ values for DNA and protein synthesis are 1.88 and 2.83 mM, respectively. Although a similar order of tissue sensitivity to DNA synthesis inhibition of spleen > testis > liver was observed with (+)-catechin, the differences between tissues were not as great as for paracetamol and hydroxyurea. It is not known why (+)-catechin specifically inhibited DNA synthesis in testis and spleen samples and not in liver slices but it is possible that (+)-catechin is metabolised to a toxic metabolite in the liver.

3.5.7.2 n-propyl gallate

n-propyl gallate, a synthetic phenolic antioxidant used as a preservative in the food industry, was also found to specifically inhibit DNA synthesis in spleen slices with an IC$_{50}$ of 0.13 mM (Figure 3.11). However, this specificity did not occur in testis and liver samples. In testis samples, DNA synthesis was preferentially inhibited at 0.3 - 1 mM but this was accompanied by significant protein synthesis inhibition. At 3 mM n-propyl gallate was probably cytotoxic as both DNA and protein synthesis were almost maximally inhibited. IC$_{50}$ values for inhibition of DNA and protein synthesis in testis samples are 0.32 and 0.82 mM, respectively. In liver slices, DNA synthesis did not appear to be specifically inhibited at any dose. IC$_{50}$ values for inhibition of DNA and protein synthesis in liver slices are 1.94 and 2.65 mM, respectively. Again a similar order of tissue sensitivity to DNA synthesis inhibition of spleen > testis > liver was observed with n-propyl gallate.

It is difficult to explain why n-propyl gallate specifically inhibits DNA synthesis in spleen slices but not in testis or liver samples. Whilst, n-propyl gallate has been shown to cause dose-dependent cytotoxicity in isolated rat hepatocytes at 0.5 - 2 mM
(Nakagawa et al, 1995), little is known about the possible toxic effects of \textit{n}-propyl gallate in testes and spleen. A long term feeding study in mice showed no significant toxicological effects after 90 weeks at dietary levels of 750 or 1500 mg \textit{n}-propyl gallate /Kg/day (Dacre, 1974).

The likelihood that \textit{n}-propyl gallate would inhibit DNA synthesis \textit{in vivo} is very low. In 1973, the average daily consumption of propyl gallate in the US was estimated to be 1.4 - 3.9 mg/Kg per person (reviewed by Kahl, 1984). This approximates to an exposure of about 6 - 18 μM. These concentrations are at least tenfold lower than those required for inhibition of DNA synthesis in the present study.

The results for (+)-catechin and \textit{n}-propyl gallate show that the difference in sensitivity to inhibition of DNA synthesis between tissues also occurs with other inhibitors, not just paracetamol and hydroxyurea. These results also show that other phenolic antioxidants can inhibit DNA synthesis specifically, possibly through inhibition of ribonucleotide reductase. However, it must be borne in mind that although the compounds tested were selected on the basis of their antioxidant activity, they also have other properties that may contribute to the inhibition of DNA synthesis. The lack of specific effects on DNA synthesis in some tissues also illustrates that these compounds have other cellular effects.
CHAPTER 4: RESULTS

Inhibition of DNA Synthesis by Phenolic Antioxidants and Flavonoids

4.0 Introduction

Paracetamol is a phenolic antioxidant that can act both as a transition metal chelator and radical scavenger (Woollard et al, 1990) and it is this radical quenching antioxidant property which is thought to be the mechanism by which paracetamol inhibits ribonucleotide reductase. It has been shown in chapter 3 that paracetamol can specifically inhibit DNA synthesis. Inhibition of DNA synthesis by paracetamol raises the question about whether there are other substances in the environment which inhibit DNA synthesis.

Another group of antioxidant drugs with both metal chelating and radical scavenging properties is the dietary flavonoids. Flavonoids are a large group of naturally-occurring polyphenolic antioxidants found at high concentrations in tea, wine, fresh fruit, and vegetables. Quercetin in particular is found in high abundance in many vegetables and has been found to be a highly effective antioxidant. It has been shown in chapter 3 that (+)-catechin, a flavonoid, specifically inhibits DNA synthesis in testis and spleen samples with very little protein synthesis inhibition. n-propyl gallate, a synthetic phenolic antioxidant was also shown in chapter 3 to inhibit DNA synthesis specifically in spleen slices.

It is not known if inhibition of DNA synthesis is a common property of the phenolic structure, or if all drugs with radical quenching activity can inhibit DNA synthesis, or even if other structural requirements determine whether DNA synthesis is inhibited.

Simple phenolic compounds have also been shown to inhibit DNA synthesis in another in vitro system. A study by Richard et al (1991) showed that a series of structurally similar phenolic paracetamol analogues were potent inhibitors of replicative
DNA synthesis in V79 Chinese hamster cells. Non-phenolic analogues were generally found to have lower potencies or were inactive.

The tyrosyl free radical and nonhaem iron centre of the M2 subunits of ribonucleotide reductase are essential for enzyme activity. Hydroxyurea inhibits enzyme activity by quenching the tyrosyl radical whilst leaving the iron centre intact (Yarbro, 1992). Iron chelators are also known to inhibit ribonucleotide reductase activity by removing the iron centre and causing simultaneous loss of the tyrosyl free radical and loss of activity (Hoyes et al, 1992; Cooper et al, 1996). It seems likely that small molecule antioxidants that are iron chelators and/or radical quenchers with both water and lipid solubility would penetrate ribonucleotide reductase and inhibit the enzyme.

The present study is attempting to determine whether inhibition of DNA synthesis is a common property of phenolic antioxidants or of antioxidants in general. From the results shown in chapter 3, testis tissue was chosen to screen candidate phenolic and non-phenolic antioxidants for specific inhibition of DNA synthesis. Different compounds were studied. These were:

- flavonoids - quercetin, naringenin.
- phenolic acids - caffeic acid, nordihydroguaiaretic acid (NDGA).
- phenolic paracetamol analogues - para-aminophenol (p-aminophenol), (meta-aminophenol (m-aminophenol), para-cresol (p-cresol).
- Trolox, a vitamin E analogue and highly effective antioxidant which acts mainly on lipids.
- calcium EDTA, a heavy metal chelator.

Figure 4.1 shows the structural formulae of paracetamol and the other compounds studied in this chapter.

The effect of hydrogen peroxide on DNA and protein synthesis was also studied in testis samples as a control for cytotoxicity.
Figure 4.1. Structural formulae of compounds studied

paracetamol

$m$-aminophenol

$p$-aminophenol

$p$-cresol

caffeic acid

nordihydroguaiaretic acid (NDGA)

quercetin

naringenin

Trolox

calcium EDTA
4.1 Summary of \textit{in vitro} experiments with various phenolic and non-phenolic antioxidants

These experiments demonstrate that:

- Simple phenolic compounds i.e. \textit{p}-aminophenol, \textit{m}-aminophenol, \textit{p}-cresol specifically inhibit DNA synthesis in rat testis \textit{in vitro}. IC$_{50}$ values for DNA synthesis inhibition are 0.02, 0.28, 0.17, respectively.
- NDGA preferentially inhibits DNA synthesis in rat testis samples, but at doses of 0.3 mM and above, displayed increasing protein synthesis inhibition.
- Quercetin preferentially inhibits DNA synthesis in rat testis samples but significant inhibition of protein synthesis also occurs.
- Caffeic acid and naringenin do not inhibit DNA synthesis specifically as protein synthesis is equally inhibited at the doses tested.
- Trolox and CaEDTA have very little effect on DNA and protein synthesis.
4.2 Methods

4.2.1 In vitro tissue preparation

Testis samples were prepared by decapsulating the testes and gently pulling into approximately 50 - 90 mg samples with forceps. Samples were weighed, placed into individual 25 ml Erlenmeyer flasks containing 5 ml Ringer solution at room temperature and gently teased apart with a pair of mounted needles as described previously in methods section 2.1.2.

4.2.2 Measurement of thymidine and leucine incorporation into tissues

Measurement of thymidine and leucine incorporation into testis samples was carried out as described previously in methods section 2.1.3.

4.2.3 Protein determination

The Lowry protein assay was used to determine protein content as previously described in methods section 2.1.6.
4.3 Results

4.3.1 Effect of hydrogen peroxide on DNA and protein synthesis in rat testis in vitro

Table 4.1 shows the concentration-response of rat testis samples to hydrogen peroxide. Inhibition of DNA and protein synthesis by hydrogen peroxide in rat testis samples was measured by comparing $^3$H-thymidine and $^{14}$C-leucine incorporation into samples incubated in the presence of hydrogen peroxide, with samples incubated in the absence of hydrogen peroxide. Testis samples were pre-incubated for 30 minutes, radiolabel was then added and incubation continued for a further 30 minutes. Concentrations of 1, 5 and 10 mM showed a dose-dependent non-specific inhibition of DNA synthesis as protein synthesis was also inhibited to a similar degree.

4.3.2 Effect of $p$-aminophenol on DNA and protein synthesis in rat testis in vitro

Because paracetamol has been found to inhibit DNA synthesis specifically, paracetamol analogues were selected to see if they would also specifically inhibit DNA synthesis. Figure 4.2 shows the concentration-response of rat testis samples to $p$-aminophenol. Inhibition of DNA and protein synthesis was measured in testis samples after pre-incubation for 30 minutes, addition of $^3$H-thymidine and $^{14}$C-leucine, followed by a further 30 minutes incubation. $p$-aminophenol has been found to specifically inhibit DNA synthesis with very little inhibition of protein synthesis. The IC$_{50}$ value for inhibition of DNA synthesis is 0.02 mM.

4.3.3 Effect of $m$-aminophenol on DNA and protein synthesis in rat testis in vitro

Figure 4.3 shows the concentration-response of rat testis samples to $m$-aminophenol. Inhibition of DNA and protein synthesis was measured in testis samples after pre-incubation for 30 minutes, addition of $^3$H-thymidine and $^{14}$C-leucine, followed by a further 30 minutes incubation. $m$-aminophenol has been found to specifically inhibit
DNA synthesis with very little inhibition of protein synthesis. The \( IC_{50} \) value for inhibition of DNA synthesis is 0.28 mM.

4.3.4 Effect of \( p \)-cresol on DNA and protein synthesis in rat testis

\textit{in vitro}

Figure 4.4 shows the concentration-response of rat testis samples to \( p \)-cresol. Inhibition of DNA and protein synthesis was measured in testis samples after pre-incubation for 30 minutes, addition of \(^3\text{H}\)-thymidine and \(^{14}\text{C}\)-leucine, followed by a further 30 minutes incubation. \( p \)-cresol has been found to specifically inhibit DNA synthesis with very little inhibition of protein synthesis. The \( IC_{50} \) value for inhibition of DNA synthesis is 0.17 mM.
Table 4.1. Effect of hydrogen peroxide on DNA and protein synthesis in rat testis \textit{in vitro}

<table>
<thead>
<tr>
<th>[Hydrogen peroxide] (mM)</th>
<th>% inhibition of synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
</tr>
<tr>
<td>1</td>
<td>73.4</td>
</tr>
<tr>
<td>5</td>
<td>91.6</td>
</tr>
<tr>
<td>10</td>
<td>99.6</td>
</tr>
</tbody>
</table>

Inhibition of DNA and protein synthesis was measured in rat testis tubules, incubated in Ringer ± hydrogen peroxide as described in methods chapter 2.1. Each value represents the mean of duplicate tissue samples from one animal. *significantly different from control Ringer group, $P < 0.05$ (one-way ANOVA with Bonferroni correction).
Figure 4.2. Specific inhibition of DNA synthesis by p-aminophenol in rat testis *in vitro*

Inhibition of DNA (○) and protein (△) synthesis was measured in rat testis tubules incubated in Ringer ± p-aminophenol as described in methods chapter 2.1. Data points represent mean ± SEM of duplicate samples from 4 separate animals. *significantly different from control Ringer group, *P* < 0.05 (one-way ANOVA with Bonferroni correction).
Figure 4.3. Specific inhibition of DNA synthesis by $m$-aminophenol in rat testis *in vitro*

Inhibition of DNA (●) and protein (▲) synthesis was measured in rat testis tubules incubated in Ringer ± $m$-aminophenol as described in methods chapter 2.1. Data points represent mean ± SEM of duplicate samples from 4 separate animals. *significantly different from control Ringer group, $P < 0.05$ (one-way ANOVA with Bonferroni correction).
Figure 4.4. Specific inhibition of DNA synthesis by p-cresol in rat testis in vitro

Inhibition of DNA (●) and protein (▲) synthesis was measured in rat testis tubules incubated in Ringer ± p-cresol as described in methods chapter 2.1. Data points represent mean ± SEM of duplicate samples from 4 separate animals. *significantly different from control Ringer group, $P < 0.05$ (one-way ANOVA with Bonferonni correction).
4.3.5 Effect of quercetin on DNA and protein synthesis in rat testis 

*in vitro*

Figure 4.5 shows the concentration-response of rat testis samples to quercetin, a flavonoid antioxidant. Inhibition of DNA and protein synthesis was measured in testis samples after pre-incubation for 30 minutes, addition of $^3$H-thymidine and $^{14}$C-leucine, followed by a further 30 minutes incubation. Quercetin has been found to preferentially inhibit DNA synthesis but this inhibition is accompanied by significant inhibition of protein synthesis. The IC$_{50}$ values are 0.48 and 0.93 mM for DNA and protein synthesis inhibition, respectively.

4.3.6 Effect of NDGA on DNA and protein synthesis in rat testis 

*in vitro*

Figure 4.6 shows the concentration-response of rat testis samples to NDGA, a phenolic antioxidant. Inhibition of DNA and protein synthesis was measured in testis samples after pre-incubation for 30 minutes, addition of $^3$H-thymidine and $^{14}$C-leucine, followed by a further 30 minutes incubation. NDGA has been found to preferentially inhibit DNA synthesis at low concentrations but at 0.3 mM and above significant inhibition of protein synthesis was also seen. The IC$_{50}$ values are 0.07 and 0.48 mM for DNA and protein synthesis inhibition, respectively.
Figure 4.5. Preferential inhibition of DNA synthesis by quercetin in rat testis *in vitro*

Inhibition of DNA (●) and protein (▲) synthesis was measured in rat testis tubules incubated in Ringer ± quercetin as described in methods chapter 2.1. Data points represent mean ± SEM of duplicate samples from 4 separate animals. *significantly different from control Ringer group, \( P < 0.05 \) (one-way ANOVA with Bonferroni correction).
Figure 4.6. Preferential inhibition of DNA synthesis by NDGA in rat testis \textit{in vitro}

Inhibition of DNA (●) and protein (▲) synthesis was measured in rat testis tubules incubated in Ringer ± NDGA as described in methods chapter 2.1. Data points represent mean ± SEM of duplicate tissue samples from 4 separate animals. *significantly different from control Ringer group, \( P < 0.05 \) (one-way ANOVA with Bonferonni correction).
4.3.7 Effect of naringenin on DNA and protein synthesis in rat testis \textit{in vitro}

Table 4.2 shows the concentration-response of rat testis samples to naringenin, a flavonoid antioxidant. Inhibition of DNA and protein synthesis was measured in testis samples after pre-incubation for 30 minutes, addition of \textsuperscript{3}H-thymidine and \textsuperscript{14}C-leucine, followed by a further 30 minutes incubation. Concentrations of 0.3 and 1 mM showed non-specific inhibition of DNA synthesis as protein synthesis was also inhibited to a similar degree.

4.3.8 Effect of caffeic acid on DNA and protein synthesis in rat testis \textit{in vitro}

Table 4.2 shows the concentration-response of rat testis samples to caffeic acid, a phenolic antioxidant. Inhibition of DNA and protein synthesis was measured in testis samples after pre-incubation for 30 minutes, addition of \textsuperscript{3}H-thymidine and \textsuperscript{14}C-leucine, followed by a further 30 minutes incubation. Concentrations of 0.3 and 1 mM showed non-specific inhibition of DNA synthesis as protein synthesis was almost equally inhibited at both doses.
Table 4.2. Non-specific inhibition of DNA synthesis in rat testes in vitro after exposure to different compounds

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibition</th>
<th>% inhibition of synthesis</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.3 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>DNA</td>
<td>2.7 ± 1.6</td>
<td>25.3 ± 6.5*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>0.3 ± 0.3</td>
<td>13.7 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Naringenin</td>
<td>DNA</td>
<td>5.9 ± 2.9</td>
<td>55.7 ± 6.3*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>10.7 ± 2.8</td>
<td>54.8 ± 7.2*</td>
<td></td>
</tr>
</tbody>
</table>

Inhibition of DNA and protein synthesis was measured in rat testis tubules, incubated in Ringer ± test drug as described in methods chapter 2.1. Data points represent mean ± SEM of duplicate tissue samples from 4 separate animals. *significantly different from control Ringer group, $P < 0.05$ (one-way ANOVA with Bonferonni correction).
4.3.9 Effect of Trolox on DNA and protein synthesis in rat testis *in vitro*

The inhibition of DNA and protein synthesis by Trolox in rat testis samples has been examined for 1 and 2 mM. Inhibition of DNA and protein synthesis was measured in testis samples after pre-incubation for 30 minutes, addition of $^3$H-thymidine and $^{14}$C-leucine, followed by a further 30 minutes incubation. Trolox has been found to have very little effect on DNA and protein synthesis even at the high doses tested (Table 4.3).

4.3.10 Effect of calcium EDTA on DNA and protein synthesis in rat testis *in vitro*

The inhibition of DNA and protein synthesis by CaEDTA in rat testis samples has been examined for 0.3 and 1 mM. Inhibition of DNA and protein synthesis was measured in testis samples after pre-incubation for 30 minutes, addition of $^3$H-thymidine and $^{14}$C-leucine, followed by a further 30 minutes incubation. CaEDTA has been found to have very little effect on DNA and protein synthesis even at the high doses tested (Table 4.3).
Table 4.3. Compounds that did not inhibit DNA and protein synthesis in rat testes *in vitro*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibition</th>
<th>% inhibition of synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>of</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>Trolox</td>
<td>DNA</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>ND</td>
</tr>
<tr>
<td>CaEDTA</td>
<td>DNA</td>
<td>6.0 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>4.0 ± 2.3</td>
</tr>
</tbody>
</table>

Inhibition of DNA and protein synthesis was measured in rat testis tubules, incubated in Ringer ± test drug as described in methods chapter 2.1. Data points represent mean ± SEM of duplicate tissue samples from 4 separate animals. *significantly different from control Ringer group, P < 0.05 (one-way ANOVA with Bonferonni correction). ND, not determined.
4.4 Discussion

These experiments show that:

- Hydrogen peroxide inhibits both DNA and protein synthesis to a similar degree in the testis tubules in a dose-dependent manner.
- *p*-aminophenol, *m*-aminophenol and *p*-cresol all specifically inhibit DNA synthesis in testis tubules in a dose-dependent manner.
- Quercetin preferentially inhibits DNA synthesis in testis tubules but this inhibition is accompanied by significant inhibition of protein synthesis.
- NDGA preferentially inhibits DNA synthesis in testis tubules but this inhibition is accompanied by significant inhibition of protein synthesis. Inhibition of DNA synthesis becomes non-specific at 1 mM.
- Caffeic acid and naringenin do not specifically inhibit DNA synthesis in testis tubules.
- Trolox and CaEDTA have very little effect on DNA and protein synthesis in testis tubules.

4.4.1 Non-specific effect of hydrogen peroxide on DNA and protein synthesis in rat testis *in vitro*

The results for paracetamol and hydroxyurea in chapter 3 demonstrate that the *in vitro* experimental model used in this study is a useful method for studying potential inhibitors of DNA synthesis. The effect of hydrogen peroxide on DNA and protein synthesis was studied in testis samples as a control for cytotoxicity. It was found that both DNA and protein synthesis were inhibited to similar degrees with ~70 % inhibition at 1 mM, 91.6 % at 5 mM and 99.6 % at 10 mM. These results further demonstrate that inhibition of DNA and protein synthesis in the present *in vitro* model suggests a non-specific effect of the test drug on the tissue.

4.4.2 Effect of different phenolic antioxidants on DNA and protein synthesis in rat testis *in vitro*

The results for paracetamol, (+)-catechin and *n*-propyl gallate (chapter 3) also showed that some phenolic antioxidants can specifically inhibit DNA synthesis. Further experiments utilising rat testis tubules were performed to determine if inhibition of DNA
synthesis was a common property of phenolic antioxidants.

4.4.2.1 p-aminophenol, m-aminophenol and p-cresol

p-aminophenol, m-aminophenol and p-cresol all displayed a highly specific inhibition of DNA synthesis with surprisingly little inhibition of protein synthesis. The IC\textsubscript{50} values for inhibition of DNA synthesis are 0.02, 0.17, 0.28 mM for p-aminophenol, p-cresol, and m-aminophenol, respectively. These IC\textsubscript{50} values for inhibition of DNA synthesis are similar to those previously demonstrated by Richard \textit{et al.} (1991) in V79 Chinese hamster cells. Richard \textit{et al.} (1991) also demonstrated that aminophenols were more potent inhibitors than their acetylated forms and that inhibition activity decreased in order of para, ortho and meta substitution. This order of potency was also demonstrated in the present study as p-aminophenol is more potent than p-cresol and p-aminophenol is also more potent than m-aminophenol.

4.4.2.2 Quercetin, NDGA, caffeic acid and naringenin

Quercetin has been found to preferentially inhibit DNA synthesis but this inhibition was accompanied by a significant inhibition of protein synthesis (Figure 4.4). The IC\textsubscript{50} values were 0.48 and 0.93 mM for DNA and protein synthesis inhibition, respectively. NDGA also displayed preferential inhibition of DNA synthesis but the cytotoxic effects of this antioxidant at higher doses overtook this effect (Figure 4.5). The IC\textsubscript{50} values are 0.07 and 0.48 mM for DNA and protein synthesis inhibition, respectively.

As the results in Table 4.1 show, not all phenolic antioxidants were effective inhibitors of DNA synthesis. Caffeic acid and naringenin did not display any specific inhibition of DNA synthesis. This may be because these two compounds cannot penetrate the ribonucleotide reductase enzyme and quench the tyrosyl free radical. As Figure 4.1 shows caffeic acid has a large side group, which may hinder penetration into the active site of ribonucleotide reductase. Naringenin is a large molecule and may also have difficulty penetrating the enzyme.

Another possible explanation of the non-specificity of action by the various phenolic antioxidants is that many antioxidants can also display pro-oxidant properties and so can cause cell injury under different circumstances (Stadler \textit{et al.,} 1995; Cao \textit{et al.,} 1997). It is often found that at low doses these drugs have antioxidant properties, but at high doses
they have pro-oxidant properties.

The mixed results obtained for quercetin were unexpected as quercetin is considered to be a highly effective antioxidant. However, it has been demonstrated that quercetin has pro-oxidant as well as antioxidant properties (Laughton et al., 1989). This is thought to be the cause of mutagenic and genotoxic effects in various in vitro models of genotoxicity and mutagenicity (Bjeldanes and Chang, 1977; Brown and Dietrich, 1979; Rueff et al., 1989) and could be the cause of the non-specific effects shown in the present model.

The results for p-aminophenol, m-aminophenol, p-cresol and NDGA show that certain phenolic antioxidants can inhibit DNA synthesis specifically. The mechanism of this inhibition may be through inhibition of ribonucleotide reductase by quenching the tyrosyl free radical. However, although the drugs tested were selected on the basis of their antioxidant activity, they also have other properties that may contribute to the inhibition of DNA synthesis. For example, p-aminophenol is known to be nephrotoxic in rats (Newton et al., 1982; Burnett et al., 1989) and p-cresol has been shown to be highly toxic in rat liver slices (Thompson et al., 1994). Flavonoids have also been reported to have many other biological effects as well as antioxidant properties. These include: vasodilatory, anti-carcinogenic, anti-allergic and anti-inflammatory activities (reviewed by Rice-Evans et al., 1996).

4.4.3 Effect of Trolox and calcium EDTA on DNA and protein synthesis in rat testis in vitro

In an attempt to determine if all drugs with antioxidant properties could inhibit DNA synthesis, Trolox, a vitamin E analogue, was tested. Trolox is a highly effective antioxidant that mainly acts by protecting cellular lipids from lipid peroxidation. In the present study, Trolox had little effect on DNA or protein synthesis, even at the high doses tested (1 - 2 mM) (Table 4.2).

It has been demonstrated by Hue et al. (1986) that rat liver slices are impermeable to CaEDTA, a highly effective metal chelator. The absence of any substantial effect of CaEDTA on DNA and protein synthesis in rat testis tubules in the present study further shows that this in vitro model reflects the in vivo situation, and that permeability barriers were intact.
The existence of permeability barriers in the *in vitro* slice system means that antioxidants that are not inhibitors of DNA synthesis in the tissue slices might still be inhibitors of ribonucleotide reductase in an isolated enzyme system. However, the slice system brings results closer to the *in vivo* situation and allows assessment of whether drugs are likely to interfere with DNA synthesis *in vivo*. 
CHAPTER 5: RESULTS

In Vivo Experiments on Cell Injury by Paracetamol

5.0 Introduction

As mentioned in chapter 1A, there is a long delay between ingestion of an overdose of paracetamol and signs of liver cell injury. The initial delay is due to the time required to metabolise paracetamol to its reactive metabolite and subsequent depletion of glutathione in the liver. But after this point the exact mechanism of injury is not known. Various factors thought to be involved include: covalent binding to cellular macromolecules, oxidative stress, disruption of calcium homeostasis, and disruption of mitochondrial function. However, none of these factors fully explain the mechanism of cell injury (reviewed by Nelson, 1990, and Vermeulen et al, 1992).

The objective of the present study is to determine if apoptotic pathways are involved in paracetamol-induced liver cell injury. A few studies have reported that apoptotic cell death may be involved in paracetamol-induced liver injury. An in vivo study by Ray et al (1996) demonstrated DNA fragmentation and nuclear condensation prior to onset of necrosis in mice dosed with hepatotoxic doses of paracetamol. Shen et al (1991) also demonstrated DNA fragmentation in isolated mouse hepatocytes before the onset of necrotic injury after exposure to paracetamol. Another study in HL-60 cells (which do not metabolise paracetamol) also found nuclear condensation and fragmentation after exposure to paracetamol (Wiger et al, 1997).

Reviews of the literature have focused on the killing of hepatocytes as well as the possible genotoxic and carcinogenic effects of paracetamol and these have overall concluded that any damaging effects occurred at doses above certain threshold levels which are not reached at therapeutic doses (reviewed by Bergman et al, 1996). The role of apoptosis in paracetamol-induced liver cell injury has not been thoroughly investigated and it is not known if increased apoptosis is occurring in the livers of people taking therapeutic doses or overdoses.
Male C57Bl/6 mice were used in these experiments as it is an inbred mouse line with reduced genetic variability between animals. Liver necrosis was assessed by measuring plasma ALT levels and by histological analysis of haematoxylin and eosin (H & E) -stained liver sections. A semi-quantitative scoring system was devised to assess the level of injury in the stained liver sections. The periodic acid Schiff (PAS) method was also used to visualise loss of glycogen in the injured liver. Apoptosis was assessed using the following methods:

1. terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labelling (TUNEL) method,
2. detection of poly (ADP-ribose) polymerase (PARP) cleavage by Western blot analysis in total liver protein lysates,
3. immunohistochemical staining for Bax protein expression in tissue sections.

In order to detect apoptosis in tissue sections, the classic TUNEL method was used to detect the new DNA ends generated by DNA fragmentation. This method allows the detection of apoptosis in single cells and also allows the assessment of the location of apoptotic cells and cellular morphology. Mouse testis sections were run concurrently with the experimental liver sections as a positive control tissue to make sure that lack of staining was not due to poor reagents. Mouse testes were used because spermatogonia undergo spontaneous apoptosis during normal spermatogenesis (Allan et al, 1992).

PARP is a ubiquitous nuclear enzyme that catalyses the polymerisation of poly (ADP-ribose). PARP is thought to be involved in DNA repair as it binds to damaged DNA, becomes activated and transfers ADP-ribose onto various nuclear proteins (including PARP itself). The native protein is 116 kDa, and is cleaved, mostly by caspase 3, to 85 and 23 kDa signature fragments during apoptosis (Kaufmann et al, 1993; Tewari et al, 1995). Detection of the cleaved signature fragments is believed to be a reliable early marker of apoptosis. Western blot analysis was used to detect the presence of PARP protein and the appearance of any cleavage products in total protein liver lysates.

Bax protein is a member of the Bcl-2 family of proteins that promotes apoptosis (Oltvai et al, 1993). It has been previously demonstrated that in normal mouse liver, Bax protein is located in the cytosol (Krajewski et al, 1994). Cytosolic distribution was also demonstrated by Wolter et al (1997) in normal L929 murine fibrosarcoma cells and Cos-
7 green monkey renal epithelial cells. Wolter et al (1997) further demonstrated that after induction of apoptosis with staurosporine, Bax appeared to localise to the mitochondria in both cell lines. Immunohistochemistry was used to visualise the expression of Bax protein in tissue sections in the present study to determine if any changes in the pattern of Bax protein expression in paracetamol-treated livers occurs.

From the results of the dose-response study on the effects of increasing doses of paracetamol on the different measures of necrosis and apoptosis (section 5.4), a dose of 450 mg/Kg paracetamol was chosen for a time course study. This dose was chosen because it gave a clear-cut necrotic end-point at 24 hours after paracetamol dosing without causing lethal injury.
5.1 Summary of Dose-response Study on Cell Injury by Paracetamol

These experiments demonstrate that:

- Control mice dosed with 0.075 M NaCl solution and those dosed with 130 mg/Kg paracetamol, showed no signs of necrotic cell injury at 24 hours post dose.
- Necrotic cell injury is not apparent until a dose of 450 mg/Kg paracetamol is administered at 24 hours post dose.
- Loss of PAS staining in mouse liver sections occurs in the groups of animals dosed with 200 - 450 mg/Kg paracetamol.
- Western blot analysis in control and paracetamol-treated mouse livers show no cleavage of PARP protein.
- Overall there is no significant difference between control and paracetamol-dosed animals in the number of apoptotic bodies per cm$^2$ in TUNEL-stained mouse liver sections. There is however, a large variation between the number of apoptotic bodies per cm$^2$ between animals in the same dosing group.
5.2 Summary of Time Course Study on Cell Injury by Paracetamol

These experiments demonstrate that:

- Control mice dosed with 0.075 M NaCl solution show no signs of necrotic cell injury at any time point.
- After dosing with 450 mg/Kg paracetamol, plasma ALT levels are significantly elevated above control levels in the groups of mice sampled at 4.5 - 6 hours and then appear to dip to control levels in the groups of mice sampled at 8.5 and 10.5 hours. In the groups of animals sampled at 16 and 24 hours, plasma ALT levels are again significantly elevated above control levels.
- H & E histology scores in 450 mg/Kg paracetamol-dosed liver sections show cell injury from 2.75 hours onwards.
- Overall there does not appear to be a significant difference between groups of control and paracetamol-dosed animals in the number of apoptotic bodies per cm² in mouse liver sections. An exception occurs in the group sampled at 16 hours post dose where the number of apoptotic bodies per cm² in control liver sections are significantly higher than the paracetamol-dosed mice. Again there is a large variation between the number of apoptotic bodies per cm² between animals in the same dosing group.
- Western blot analysis in control and paracetamol-treated mouse livers showed no cleavage of PARP protein at any time point.
- Immunohistochemical staining for Bax protein in liver sections showed no change in the pattern of staining between paracetamol-dosed and control mice at any time point.
5.3 Methods

5.3.1 Dose-response study

Groups of male C57Bl/6 mice (17 - 22 g) received a single dose of paracetamol (130, 200, 300, 450 mg/Kg, i.p.) dissolved in warm 0.075 M NaCl solution in a final volume of 200 μl /10g body weight. Controls received 200 μl /10g body weight of warm 0.075 M NaCl solution. Animals were kept in a warm cage at 26 - 28°C after dosing until sacrifice. Blood samples and liver samples were harvested at 24 hours post dose. Some testis samples were also taken for use as a positive control tissue for apoptosis. 675 mg/Kg paracetamol (n=2 animals) was also administered in preliminary studies but the mice died within 12 hours of dosing so this concentration was not used again. Feed and drinking water was available ad libitum throughout the experiment.

5.3.2 Assessment of injury

Necrotic cell injury was assessed by measuring plasma ALT levels, histological analysis of H & E and PAS-stained liver sections. Apoptotic cell injury was assessed by histological analysis of TUNEL-stained liver sections, and Western blot analysis for PARP cleavage in liver lysates. Assessment of injury was carried out as described in methods chapter 2.2.

5.3.3 Time course study

Groups of male C57Bl/6 mice (17 - 22 g) received a single dose of paracetamol (450 mg/Kg, i.p.) as a 22.5 mg/ml solution in a final volume of 200 μl /10 g body weight, dissolved in warm 0.075 M NaCl solution. Controls received 200 μl /10 g body weight of warm 0.075 M NaCl solution. Animals were kept in a warm cage at 26 - 28°C after dosing until sacrifice. Blood samples and livers from a dosed group and a control group were harvested at the following time points 2.75, 4.5, 6, 8.5, 10.5, 16 and 24 hours post dose. Some testis samples were also taken for use as a positive control tissue for apoptosis. Feed and drinking water were available ad libitum throughout the experiment.
5.3.4 Assessment of injury

Necrotic cell injury was assessed by measuring plasma ALT levels and histological analysis of H & E-stained liver sections. Apoptotic cell injury was assessed by histological analysis of TUNEL-stained liver sections, Western blot analysis for PARP cleavage in liver lysates and immunohistochemical staining for Bax protein in liver sections. Assessment of injury was carried out as described in methods chapter 2.2.
5.4 Validation of TUNEL Method

5.4.1 TUNEL staining in positive control tissue sections

Several different types of tissue sections were used as positive controls for TUNEL staining using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor, Strasbourg, France). Mouse testis sections were used because spermatogonia undergo spontaneous apoptosis during normal spermatogenesis (Allan et al, 1992). To show staining in mouse liver sections, livers from mice that have been treated with anti-Fas antibody were used because apoptotic cell death has been previously demonstrated to occur in the liver after i.p injection of an anti-Fas antibody (Ogasawara et al, 1993). In addition, rat liver sections known to contain apoptotic bodies were used to demonstrate staining in liver.

5.4.1.1 TUNEL staining in mouse testis sections

Testes were obtained from control mice in the present study and fixed in 10% formalin, dehydrated and embedded in paraffin wax. 5 μm sections were cut, mounted onto microscope slides and TUNEL-stained using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor) as described in section 2.2.7. Figure 5.1 shows a TUNEL-stained apoptotic cell in a testis section taken from a control mouse.

5.4.1.2 TUNEL staining in anti-Fas antibody-treated mouse liver sections

Anti-Fas antibody-treated liver sections embedded in paraffin wax were kindly supplied by Professor Gerald Cohen (MRC Toxicology Unit, Leicester, UK) and Dr Heath Thomas (Toxicology & Safety Assessment, SmithKline Beecham (SB) Pharmaceuticals, King of Prussia, USA). Sections were TUNEL-stained using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor) as described in section 2.2.7.

Figure 5.2 shows a TUNEL-stained liver section from a mouse that has been treated with anti-Fas antibody at 4 hours post dose. Figure 5.3 shows a section from the same sample that has been stained with H & E for comparison. The TUNEL-stained section shows very widespread brown staining that is both nuclear and cytoplasmic. The H & E
section shows very severe hepatocyte damage with infiltration of red blood cells and inflammatory cells. Staining in sections that have been exposed to longer periods of anti-Fas antibody show increasingly widespread brown staining in the cytoplasm.

5.4.1.3 TUNEL staining in rat liver section after partial hepatectomy

Paraffin-embedded rat liver sections known to contain apoptotic bodies were kindly supplied by the Histology lab at SB Pharmaceuticals (Welwyn, UK) and TUNEL-stained using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor) as described in section 2.2.7. This rat liver sample was obtained from one animal in a group of undosed Sprague-Dawley rats (~290 g) that underwent a 70% partial hepatectomy and were subsequently sacrificed 7 - 10 days later. Previous work in the Histology lab showed that this liver sample contains many apoptotic bodies, however, liver samples from other animals of the same treatment group did not show a similar response.

Figure 5.4 shows a liver section from this rat that has been TUNEL-stained. Many apoptotic cells can be observed and staining appears to be highly specific to apoptosing cells with a very low level of background staining.

5.4.2 Variation between sections from the same sample

Consecutive sections and sections cut more than 50 μm apart in the tissue block were TUNEL-stained in one experiment using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor) and the number of apoptotic bodies per cm² counted to determine the level of variation between sections from the same sample. Only stained cells that fulfilled the criteria set out in methods section 2.2.7 were counted as apoptotic bodies. Sections from one representative control and one representative paracetamol-dosed animal were examined at 4.5, 10.5 and 24 hours post dose. All control sections have H & E scores of 0. For the paracetamol-treated sections, the section at 4.5 hours has a H & E score of 2, the section at 10.5 hours has a score of 0/1 and the section at 24 hours has a score of 2/3. Figure 5.5 shows that there is a considerable variation in the number of apoptotic bodies per cm² between consecutive sections from the same liver. Similar results are shown for sections cut more than 50 μm apart.
Figure 5.1. TUNEL-stained testis section from a control mouse

Objective magnification x 40. Arrow indicates an apoptotic body.
Figure 5.2. TUNEL-stained liver section from anti-Fas antibody treated mouse at 4 hours post dose

Objective magnification x 20. Arrows indicate several cells with nuclear and/or cytoplasmic TUNEL staining.

Objective magnification x 40. Arrows indicate several cells with nuclear and/or cytoplasmic TUNEL staining.
Figure 5.3. H & E-stained liver section from anti-Fas antibody treated mouse at 4 hours post dose

Objective magnification x 20.

Objective magnification x 40.
Figure 5.4. TUNEL-stained liver section taken from a rat several days after a partial hepatectomy

Objective magnification x 20. Arrows indicate several cells with nuclear and/or cytoplasmic TUNEL staining.

Objective magnification x 40. Arrows indicate several cells with nuclear and/or cytoplasmic TUNEL staining.
Figure 5.5. Comparison of number of apoptotic bodies per cm$^2$ in consecutive sections and sections cut >50 μm apart in the tissue block

Sections from one representative control and one representative 450 mg/Kg paracetamol-treated animal were examined at the indicated time points. Two liver slices from each animal were fixed in 10% formalin, dehydrated, embedded in paraffin wax, cut and mounted onto each slide. Sections were dewaxed and TUNEL-stained using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor) as described in methods section 2.2.7. All 0 values were converted to 1 before data was log transformed and statistically analysed. Results are mean ± SD of number of apoptotic bodies per cm$^2$. Solid bars represent consecutive sections (n = 6 sections). Hatched bars represent sections cut more than 50 μm apart (n = 3 sections).
5.5 Method Development and Validation of Western Blot Analysis for PARP Cleavage

The concentration of BSA in blocking buffer was initially 0.1 %. However, Western blots blocked with this buffer showed a lot of background staining, even in the absence of primary and secondary antibody incubations. The concentration of BSA was therefore increased to 1 % and this led to a significant reduction in background staining.

The dilution of each anti-PARP antibody was optimised by incubating strips of PVDF membrane loaded with the same samples in different dilutions of antibody solution and selecting the dilution with a strong signal and low background. The dilution of the secondary antibody, a biotin-conjugated rabbit anti-goat antibody (Sigma) was 1:10,000 throughout.

5.5.1 Comparison of different anti-PARP antibodies

Initial results with anti-PARP (A-20) antibody showed a band at 116 kDa with little other staining. In order to determine whether lack of staining for any cleavage products was due to low affinity of the antibody, different goat anti-PARP polyclonal antibodies were used. These antibodies were reported to detect the epitopes corresponding to the following:

1. amino acids 1-20 of murine PARP (A-20) - detects full length protein and 23 kDa fragment (Santa Cruz Biotech, Santa Cruz, California, USA).
2. amino acids 980-999 of human PARP (D-20) - cross reacts with mouse and detects full length protein and 89 kDa fragment (D-20, Santa Cruz Biotech).
3. amino acids 71-329 of murine PARP - detects full length protein and 23 kDa fragment (R&D systems, Abingdon, UK).

Figure 5.6 shows the Western blots obtained using these different antibodies for liver protein lysate samples. There is very little difference in the pattern of staining between the different antibodies.
5.5.2 Positive controls for PARP cleavage

Two different positive controls were used to demonstrate that the antibodies used to detect PARP protein would also detect cleavage products. Incubation of human neuroblastoma cells (SH-SY5Y) with staurosporine is known to cause apoptotic cell death and PARP cleavage (Posmantur et al, 1997). Untreated and staurosporine-treated SH-SY5Y cells were kindly provided by Dr John Davis (Neurosciences, SB Pharmaceuticals, Harlow, UK) and Western blotted using the anti-PARP antibody obtained from R&D systems. Testis samples were also used as a positive control for apoptosis because as mentioned previously spermatogonia undergo spontaneous apoptosis during normal spermatogenesis (Allan et al, 1992).

Figure 5.7 shows Western blots obtained for untreated and staurosporine-treated SH-SY5Y cells. There is a clear loss of staining at 116 kDa in the staurosporine-treated samples compared to the untreated samples and an appearance of several cleavage products. The mouse testis sample also showed a very faint band at 116 kDa with several cleavage products.

5.5.3 Negative controls for Western blotting

Negative control Western blots for each antibody were obtained by either omitting the primary and secondary antibody incubation steps or performing the incubation steps with antibody diluent in the absence of antibody. It was found that some faint bands could be observed in the negative control blots but the intensity of the bands was always much lower compared to blots incubated with anti-PARP antibody.

An additional negative control Western blot was obtained for the anti-PARP (A-20) antibody from Santa Cruz Biotech. A blocking peptide that was specific to this antibody was obtained from Santa Cruz Biotech, added to the antibody solution and allowed to pre-absorb the anti-PARP (A-20) antibody overnight. This solution was used in place of the primary antibody solution for Western blotting. The blot obtained with this solution also showed some faint bands but again these were much lower in intensity compared to those incubated in anti-PARP antibody.
Figure 5.6. Comparison of different anti-PARP antibodies

A. anti-PARP (A-20) antibody (1:100,000 Santa Cruz Biotech).
B. anti-PARP (D-20) antibody (1:100,000 Santa Cruz Biotech).
C. anti-PARP antibody (1:5,000 R&D systems).

Samples analysed were liver samples from mice dosed with 450 mg/Kg paracetamol (1), and 0.075 M NaCl solution (controls) (2) at 24 hours post dose.

~9 µg of protein was loaded onto each lane of gel A and B and ~6.7 µg of protein was loaded onto each lane of gel C. Proteins were separated by SDS-PAGE and transferred to PVDF membrane (as described in methods section 2.2.8). Immunodetection of membrane-bound proteins was carried out using the above goat anti-PARP polyclonal antibodies and biotinylated anti-goat antibody (1:10,000). Signal was detected using streptavidin AuroprobeBL and silver enhancement reagents.
Figure 5.7. Western blot analysis for PARP cleavage in positive controls for apoptosis

Samples analysed were untreated SH-SY5Y cells (A), mouse testis (B), SH-SY5Y cells pre-treated with 0.3 μM staurosporine for 6.75 hours (C), and molecular weight markers (MW).

~10 μg of protein was loaded onto lane B. Protein concentration in the SH-SY5Y cell samples was not known as samples were provided ready-mixed with SDS-loading buffer.

Proteins were separated by SDS-PAGE and transferred to PVDF membrane (as described in methods section 2.2.8). Immunodetection of membrane-bound proteins was carried out using a goat anti-PARP polyclonal antibody (1:2,5000 R&D systems) and biotinylated anti-goat antibody (1:10,000). Signal was detected using streptavidin AuroprobeBL and silver enhancement reagents.
5.6 Method Development and Validation of Immunohistochemical Staining for Bax Protein

Immunohistochemical staining for Bax protein was performed using two different rabbit anti-Bax polyclonal antibodies. The first antibody (P-19) was obtained from Santa Cruz Biotech, and detects an epitope corresponding to amino acids 43-61 of murine Bax. The second antibody (Ab-1) was obtained from Oncogene Research Products (Cambridge, USA) and detects an epitope corresponding to amino acids 150-165 of human Bax. The secondary antibody used was a biotin-conjugated goat anti-rabbit antibody obtained from Santa Cruz Biotech.

5.6.1 Optimisation of primary and secondary antibody dilutions

Tissue sections were stained with different primary antibody dilutions whilst keeping the secondary antibody dilution constant. The dilution that gave the best level of staining with low background was chosen for further immunohistochemical staining experiments. The secondary antibody dilution was also optimised for anti-Bax (P-19) antibody by staining tissue sections with different secondary antibody dilutions whilst keeping the primary antibody dilution constant.

For the anti-Bax (P-19) antibody, a 1:500 dilution showed the best level of staining with relatively low background staining in tissue sections. Staining with a 1:200 dilution was too dark with lots of background staining and staining intensity was lost with a 1:1,000 dilution. Background staining was also much cleaner when the secondary antibody dilution was changed from 1:200 to 1:500.

For the anti-Bax (Ab-1) antibody, a 1:50 dilution showed the best level of staining in tissue sections. Dilutions of 1:100 or more reduced staining significantly. A secondary antibody dilution of 1:200 was used throughout.

5.6.2 Positive control tissues for Bax staining

To aid optimisation of the method and show positive staining with these antibodies, sections from mouse thymus and human tonsil were used. Mouse thymus has been shown previously to stain positively for Bax in the thymic medulla at low magnification. At high magnification, however, it was shown that much of this Bax staining was associated with
thymic epithelial cells (Krajewski et al, 1994). Another lymphoid tissue, human tonsil was also used because a high level of Bax expression has been shown in germinal centre B cells and memory B cells by immunoblot analysis (Ohta et al, 1995).

Paraffin-embedded mouse thymus and human tonsil sections were kindly provided by Dr Suzanne Newman (Analytical Sciences, SB Pharmaceuticals, Harlow, UK).

Figure 5.8 shows a mouse thymus section that has been immunohistochemically stained using anti-Bax (Ab-1) antibody. Some specific staining is seen in filamentous cells throughout the tissue section. Staining was confirmed by incubating sections with pre-absorbed antibody which showed very little staining and incubating sections with rabbit IgG which showed dark staining in the whole section.

Figure 5.9 shows a human tonsil section that has been immunohistochemically stained using anti-Bax (P-19) antibody. DAB staining occurs in the germinal centres, but it is very light and the haematoxylin counterstain tends to cover up the staining. When a human tonsil section was immunohistochemically stained in the absence of antigen unmasking by microwaving in citrate buffer, no DAB staining was seen.

5.6.3 Optimisation of microwave heating time in citrate buffer

In the present study, antigen unmasking in tissue sections was performed by exposure to microwaves in citrate buffer (pH 6) at temperatures up to 100°C. Paraffin sections mounted on microscope slides were dewaxed, rehydrated, incubated in 0.6 % H₂O₂ to quench endogenous peroxidase activity and washed with PBS before being subjected to heating in near boiling citrate buffer in a microwave oven. ~500 ml citrate buffer was placed in a plastic container, loosely covered and pre-heated in a microwave oven at 1000W power for 5 minutes until bubbling. The sections were placed in a plastic rack, then submerged in the citrate buffer and heated in a microwave oven at 700W power for the time periods indicated below.

In order to optimise antigen unmasking, consecutive sections from the same paraffin-embedded tissue block were subjected to different periods of microwaving in citrate buffer. For the anti-Bax (P-19) antibody, time periods ranging from 0 to 20 minutes of microwaving were performed with human tonsil sections. It was found that 0 minutes of microwaving produced no staining and 20 minutes of microwaving caused the section to detach from the slide. 10 minutes of microwaving showed the best level of
staining with low background. For the anti-Bax (Ab-1) antibody, time periods ranging from 5 to 15 minutes of microwaving were performed with mouse thymus sections. It was found that 15 minutes of microwaving showed the best level of staining. Sections microwaved for shorter time periods showed only faint staining.

5.6.4 Controls for immunohistochemical staining

To show that Bax staining is specific, several negative controls were used. Tissue sections were either incubated with anti-Bax antibody that had been pre-absorbed with specific peptides, or, the primary and secondary antibody incubation solutions were replaced with antibody diluent, or the primary, secondary and tertiary antibody incubation solutions were replaced with antibody diluent.

Normal rabbit serum or rabbit IgG were also used in place of primary antibody as a control for immunohistochemical staining. Because of the non-specificity of normal rabbit serum and rabbit IgG, the pattern of staining in tissue sections should be different to that of sections incubated with anti-Bax antibody.

Figure 5.10 shows immunohistochemical staining for Bax protein in mouse liver sections with anti-Bax (Ab-1) antibody. For comparison, immunohistochemical staining with pre-absorbed anti-Bax (Ab-1) antibody and rabbit IgG is also shown. The section immunohistochemically stained with pre-absorbed antibody showed very light DAB staining compared to the section immunohistochemically stained with anti-Bax antibody. The section immunohistochemically stained with anti-Bax antibody showed cytosolic staining whereas the section immunohistochemically stained with rabbit IgG showed darker generalised DAB staining which occurred in both the nucleus and cytosol. Similar results were also obtained for anti-Bax (P-19) antibody.
Figure 5.8. Immunohistochemical staining for Bax protein in a mouse thymus section

Paraffin-embedded mouse thymus sections were dewaxed, rehydrated and immunohistochemically stained with anti-Bax (Ab-1) antibody (1:50) and biotin-conjugated goat anti-rabbit antibody (1:200) as described in methods section 2.2.9. Signal was detected using peroxidase-conjugated avidin-biotin reagent and nickel-enhanced DAB substrate. Sections were not counterstained with haematoxylin.
Figure 5.9. Immunohistochemical staining for Bax in human tonsil sections

Paraffin-embedded human tonsil sections were dewaxed, rehydrated and immunohistochemically stained with anti-Bax (P-19) antibody (1:500) and biotin-conjugated goat anti-rabbit antibody (1:500) as described in methods section 2.2.9. Signal was detected using peroxidase-conjugated avidin-biotin reagent and nickel-enhanced DAB substrate. Sections were counterstained with haematoxylin.
Objective magnification x 40. Paraffin-embedded mouse liver sections were dewaxed, rehydrated and immunohistochemically stained with anti-Bax (Ab-1) antibody 1:50 (A), pre-absorbed anti-Bax (Ab-1) antibody (B) or rabbit IgG 1:50 (C) as described in methods section 2.2.9. A biotin-conjugated goat anti-rabbit antibody (1:200) was used as the secondary antibody. Signal was detected using peroxidase-conjugated avidin-biotin reagent and nickel-enhanced DAB substrate. Sections were not counterstained with haematoxylin.
5.7 Results of Dose-response Study on Cell Injury by Paracetamol

5.7.1 Effect of increasing paracetamol dose on plasma ALT levels at 24 hours post dose

Figure 5.11 shows that plasma ALT levels at 24 hours post dose in the group of control mice is around 55 nmoles/min/ml. The group dosed with 130 mg/Kg paracetamol had plasma ALT levels that were not significantly different from the control group. Plasma ALT levels for the groups of mice dosed with 200 and 300 mg/Kg paracetamol were more widespread, however, the majority had levels of below 100 nmoles/min/ml and as a whole these were not significantly different from the control group. The group dosed with 450 mg/Kg paracetamol had significantly elevated plasma ALT levels compared to the control group; all but one animal in this group had plasma levels of over 100 nmoles/min/ml.

5.7.2 Histological analysis of H & E-stained liver sections

At 24 hours after dosing, livers from groups of control and paracetamol-dosed animals were removed and ~3 mm sections fixed in 10 % formalin. These livers were embedded in paraffin wax and 5 μm sections were cut, mounted onto microscope slides and stained with H & E. Slides were scored for injury using the histology key shown in Table 2.1. Scores range from 0 for normal livers to 4 where very severe necrosis is evident and very few normal cells are present.

Table 5.1 shows the histology scores obtained for control and paracetamol-dosed liver sections. Control animals and those dosed with 130 mg/Kg paracetamol showed normal livers. The majority of the animals dosed with 200 and 300 mg/Kg paracetamol showed normal livers with a few showing minor injury with histology scores of 1. Animals dosed with 450 mg/Kg paracetamol showed a range of histology scores, the majority had scores of 2 or 3.
5.7.3 H & E-stained liver sections from control and paracetamol-dosed mice at 24 hours post dose

Figure 5.12 shows a liver section from a control mouse at 24 hours post dose. Healthy hepatocytes with normal nuclei and granular staining of cytoplasm can be seen. Figure 5.13 shows a liver section from a mouse dosed with 450 mg/Kg paracetamol at 24 hours post dose. A large area of hepatocytes surrounding the central vein show loss of nuclei, paler cytoplasmic staining, swelling and infiltration of inflammatory cells, all indicators of necrotic injury.

5.7.4 Correlation between different measures of liver injury in control and paracetamol-dosed mice at 24 hours post dose

Table 5.2 shows that plasma ALT levels and H & E histology scores in individual mice roughly correlate with each other. Plasma ALT levels of more than 100 nmoles/min/ml were generally found to be associated with histology scores of 1 or above and levels of less than 100 nmoles/min/ml were generally found to be associated with histology scores of 0.

5.7.5 PAS-stained mouse liver sections from control and paracetamol-dosed mice at 24 hours post dose

Figure 5.14 shows a control liver section that has been stained using PAS. Hepatocytes show high levels of staining for glycogen. Figure 5.15A and 5.15B shows PAS-stained liver sections from mice dosed with 200 mg/Kg and 450 mg/Kg paracetamol, respectively. Hepatocytes surrounding the central veins show a pronounced loss of staining for glycogen, an early indicator of cell injury. The number of cells affected increases with increasing paracetamol dose.
Figure 5.11. Effect of increasing paracetamol dose on plasma ALT levels at 24 hours post dose

Groups of mice were i.p. dosed with 0.075 M NaCl solution (controls) or increasing concentrations of paracetamol. Plasma samples were assayed for ALT activity at 24 hours. Results are expressed as nmoles/min/ml. Each data point represents the results from one animal. n = number of animals in group. Data was log transformed before statistical analysis. *significantly different from control group, $P < 0.05$ (Mann Whitney U Test).
Table 5.1. Histological analysis of H & E-stained liver sections from control and paracetamol-dosed mice at 24 hours post dose

<table>
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<tr>
<th>H &amp; E Histology Score</th>
<th>Control</th>
<th>130 mg/Kg paracetamol</th>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Groups of mice were dosed with 0.075 M NaCl solution (controls) or increasing concentrations of paracetamol. At 24 hours post dose, liver sections were taken, fixed in 10% formalin, embedded in paraffin wax, cut and stained with H & E. Histology scores were assigned to stained sections using the key shown in Table 2.1.
Figure 5.12. H & E-stained liver section from control mouse at 24 hours post dose

Objective magnification x 20. CV, central vein

Objective magnification x 40.
Figure 5.13. H & E-stained liver section from 450 mg/Kg paracetamol-dosed mouse at 24 hours post dose

Objective magnification x 20. Necrotic cells around the central vein are surrounded by normal cells.

Objective magnification x 40. Necrotic cells around the central vein are surrounded by normal cells.
Table 5.2. Correlation of plasma ALT levels and H & E histology scores at 24 hours post dose in individual mice.

<table>
<thead>
<tr>
<th>Control</th>
<th>130 mg/Kg paracetamol</th>
<th>200 mg/Kg paracetamol</th>
<th>300 mg/Kg paracetamol</th>
<th>450 mg/Kg paracetamol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ALT levels</td>
<td>Histology score</td>
<td>Plasma ALT levels</td>
<td>Histology score</td>
<td>Plasma ALT levels</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>17</td>
<td>ND</td>
<td>0 / 1</td>
</tr>
<tr>
<td>31</td>
<td>0</td>
<td>32</td>
<td>239</td>
<td>1</td>
</tr>
<tr>
<td>51</td>
<td>0</td>
<td>24</td>
<td>88</td>
<td>0</td>
</tr>
<tr>
<td>67</td>
<td>0</td>
<td>54</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>88</td>
<td>0</td>
<td>64</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Groups of mice were dosed with 0.075 M NaCl solution (controls) or increasing concentrations of paracetamol. At 24 hours, blood samples and liver sections were taken. Plasma samples were assayed for ALT activity. Results are expressed as nmoles/min/ml. Liver sections were fixed in 10% formalin, embedded in paraffin wax, cut and stained with H & E. Histology scores were assigned to stained sections using the key shown in Table 2.1. ND, not determined.
Figure 5.14. PAS-stained liver sections from control mouse at 24 hours post dose

Objective magnification x 20. CV, central vein.
Figure 5.15. PAS-stained liver sections from 200 mg/Kg (A) and 450 mg/Kg (B) paracetamol-dosed mice at 24 hours post dose.

Objective magnification x 40. CV, central vein.
5.7.6 Histological analysis of TUNEL-stained liver sections from control and paracetamol-dosed mice at 24 hours post dose

Table 5.3 shows the number of apoptotic bodies per cm\(^2\) in liver sections from control and paracetamol-dosed mice at 24 hours post dose (one section from each mouse, 5 animals per group). Table 5.4 shows the mean ± 1 SD range of each treated group. The limit of detection was arbitrarily set at 1 so that all 0 values were converted to 1 before data was log transformed and statistically analysed. There is a large variation between animals in the same dose group and with such a high variation it is difficult to determine whether paracetamol dosing has any effects on the number of apoptotic bodies per cm\(^2\). However none of the means of the paracetamol-dosed groups were significantly different from the control group. Many livers showed apoptotic bodies by this staining technique.

5.7.7 TUNEL-stained mouse liver sections

Figure 5.16 shows a typical TUNEL-stained apoptotic body in a liver section taken from a control mouse at 24 hours post dose.
Table 5.3. Histological analysis of TUNEL-stained liver sections from control and paracetamol-dosed mice at 24 hours post dose

<table>
<thead>
<tr>
<th>Number of apoptotic bodies per cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>10.4</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>25.6</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>5.9</td>
</tr>
</tbody>
</table>

Groups of mice were dosed with 0.075 M NaCl solution (controls) or increasing concentrations of paracetamol. At 24 hours post dose, liver sections were taken, fixed in 10% formalin, embedded in paraffin wax, cut and TUNEL-stained using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor) as described in methods section 2.2.7. All sections were stained in one experiment. Results are expressed as number of apoptotic bodies per cm². Data represents the results from one section from each animal.
Table 5.4. Summary of histological analysis of TUNEL-stained liver sections from control and paracetamol-dosed mice at 24 hours post dose

<table>
<thead>
<tr>
<th>Paracetamol dose (mg/Kg)</th>
<th>Number of apoptotic bodies per cm$^2$</th>
<th>Mean</th>
<th>± 1 SD range</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.4</td>
<td>1.0-18.4</td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>4.0</td>
<td>1.5-11.2</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>1.2</td>
<td>0.8-1.8</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>4.5</td>
<td>1.5-12.9</td>
<td></td>
</tr>
<tr>
<td>450</td>
<td>2.2</td>
<td>0.4-11.9</td>
<td></td>
</tr>
</tbody>
</table>

Groups of mice were dosed with 0.075 M NaCl solution (controls) or increasing concentrations of paracetamol. At 24 hours post dose, liver sections were taken, fixed in 10% formalin, embedded in paraffin wax, cut and TUNEL-stained using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor) as described in methods section 2.2.7. All sections were stained in one experiment. Results are expressed as number of apoptotic bodies per cm$^2$. n = 5 animals per group. All 0 values were converted to 1 before data was log transformed and statistically analysed.
Figure 5.16. TUNEL-stained liver section from a control (A) and 450 mg/Kg paracetamol-dosed (B) mouse at 24 hours post dose.

Objective magnification x 40. Arrow indicates an apoptotic body.
5.7.8 Comparison of plasma ALT levels, H & E scores and level of apoptosis in TUNEL-stained liver sections

Figure 5.17 shows the mean ± SD for plasma ALT levels, histology scores in H & E-stained liver sections and number of apoptotic bodies per cm² in TUNEL-stained liver sections. Comparison of plasma ALT levels and H & E histology scores again shows a dose-dependent correlation. However, there does not appear to be a dose-dependent correlation with the number of apoptotic bodies per cm² in mouse liver.

5.7.9 Western blot analysis for PARP cleavage in liver lysates from control and paracetamol-dosed mice at 24 hours post dose

Figure 5.18 shows the Western blot obtained for pooled liver lysates from control and paracetamol-dosed mice. A band at 116 kDa was observed but no cleavage products of PARP protein was detected in control or paracetamol-dosed livers. A mouse testis sample analysed at the same time shows loss of the 116 kDa band.
Figure 5.17. Comparison of plasma ALT levels, H & E scores and number of apoptotic bodies per cm$^2$ in TUNEL-stained liver sections at 24 hours post dose

Groups of mice were dosed with 0.075 M NaCl solution (controls) or increasing concentrations of paracetamol. At 24 hours, blood and liver samples were taken. Plasma samples were assayed for ALT activity and formalin-fixed paraffin-embedded liver sections were cut and stained with H & E or TUNEL as described in chapter 2.2. Histology scores were assigned to H & E-stained sections using the key shown in Table 2.1. Number of apoptotic bodies per cm$^2$ were determined for TUNEL-stained sections. Results are mean ± SD of n animals. *significantly different from control group, $P < 0.05$ (Mann Whitney U Test). **significantly different from control group, $P < 0.05$ (one-way ANOVA with Bonferroni correction).
Figure 5.18. Detection of PARP protein cleavage by Western blot analysis of liver lysates from control mice and paracetamol-dosed mice at 24 hours post dose

Samples analysed were pooled liver samples from mice dosed with 0.075 M NaCl solution (A), 130 mg/Kg paracetamol (B), 200 mg/Kg paracetamol (C), 300 mg/Kg paracetamol (D), 450 mg/Kg paracetamol (E), mouse testis (F) and molecular weight markers (MW).

Groups of mice were dosed with 0.075 M NaCl solution (controls) or increasing concentrations of paracetamol. At 24 hours, liver samples were taken and protein lysates prepared (A - E) as described in methods section 2.2.8. ~6 µg of protein was loaded onto each lane and proteins were separated by SDS-PAGE and transferred to PVDF membrane. Immunodetection of membrane-bound proteins was carried out using a goat anti-PARP polyclonal antibody (1:5,000 R&D systems) and biotinylated anti-goat antibody (1:10,000). Signal was detected using streptavidin AuroprobeBL and silver enhancement reagents.
5.8 Results for Time Course Study on Cell Injury by Paracetamol

5.8.1 Time course of plasma ALT levels after dosing with 450 mg/Kg paracetamol

Figure 5.19 shows a time course of plasma ALT levels in control animals and paracetamol-dosed animals. All control mice had plasma ALT levels below 100 nmoles/min/ml. In the 2.75 hour groups, plasma ALT levels in paracetamol-dosed and control animals were not significantly different. In the 4.5 and 6 hour groups, plasma ALT levels in paracetamol-dosed animals rose above 100 nmoles/min/ml and were significantly higher than control animals at the same time point. In the 8.5 and 10.5 hour groups, the level of cell injury detected in the paracetamol-dosed animals was not significantly different from control animals at the same time point. In the 16 and 24 hour groups, plasma ALT levels in paracetamol-dosed animals again elevated above those of control animals at the same time point.

5.8.2 Histological analysis of H & E stained liver sections

At different times after dosing, livers from control and paracetamol-dosed animals were removed and ~3 mm sections were fixed in 10 % formalin. These livers were embedded in paraffin wax and 5 μm sections were cut, mounted onto microscope slides and stained with H & E. Slides were scored for injury using the histology key in Table 2.1. Scores range from 0 for normal livers to 4 where very severe necrosis is evident and very few normal cells are present.

Table 5.5 shows the histology scores obtained for control and paracetamol-dosed liver sections. Control animals at all time points had normal livers.

5.8.3 H & E-stained liver sections from control and paracetamol-dosed mice

Figure 5.20A shows a liver section from a control mouse at 16 hours post dose. Healthy hepatocytes with normal nuclei and granular staining of cytoplasm can be seen.
Figure 5.20B shows a liver section from a mouse dosed with 450 mg/Kg paracetamol at 16 hours post dose. A large area of hepatocytes surrounding the central vein shows a loss of nuclei, paler cytoplasmic staining, swelling and infiltration of inflammatory cells.

5.8.4 Correlation of plasma ALT levels and H & E histology scores

Table 5.6 and 5.7 show the plasma ALT levels and liver H & E histology scores for individual control mice and those dosed with 450 mg/Kg paracetamol. Again the plasma ALT levels and H & E histology scores roughly correlate with each other. Plasma ALT levels of more than 100 nmoles/min/ml were often found to be associated with histology scores of 1 or above and plasma ALT levels of less than 100 nmoles/min/ml were often found to be associated with histology scores of 0.

The time of dosing and time of sacrifice were included to illustrate the staggered dosing regimen used in the present study.
Figure 5.19. Time course of plasma ALT levels from control and paracetamol-dosed mice

Groups of mice were dosed with 0.075 M NaCl solution (controls) (▲) or 450 mg/Kg paracetamol (●). Plasma samples were assayed for ALT activity at different time points. Results are expressed as nmoles/min/ml.

n = 4 animals except at 24 hours after dosing with paracetamol where n = 8 animals. Data was log transformed before statistical analysis. *significantly different from controls at the same time point, P < 0.05 (Mann Whitney U Test).
Figure 5.20. H & E-stained liver sections from control (A) and paracetamol-dosed (B) mice at 16 hours post dose.
Table 5.5. Time course of liver injury in control and paracetamol-dosed mice assessed by histological analysis of H & E-stained sections

<table>
<thead>
<tr>
<th>Time post Dose (hr)</th>
<th>Histology Score Control</th>
<th>450 mg/Kg paracetamol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 0 0 0 0</td>
<td>0 1 0/1 1</td>
</tr>
<tr>
<td>2.75</td>
<td>0 0 0 0 0</td>
<td>2 2 1/2 1/2</td>
</tr>
<tr>
<td>4.5</td>
<td>0 0 0 0 0</td>
<td>2 2 2 1/2</td>
</tr>
<tr>
<td>6</td>
<td>0 0 0 0 0</td>
<td>1/2 1/2 1/2</td>
</tr>
<tr>
<td>8.5</td>
<td>0 0 0 0 0</td>
<td>1/2 1/2 1/2</td>
</tr>
<tr>
<td>10.5</td>
<td>0 0 0 0 0</td>
<td>0/1 1 1/2 0/1</td>
</tr>
<tr>
<td>16</td>
<td>0 0 0 0 0</td>
<td>2/3 3 2/3 2/3</td>
</tr>
<tr>
<td>24</td>
<td>0 0 0 0 0</td>
<td>0/1 2 3 2/3</td>
</tr>
</tbody>
</table>

Groups of mice were dosed with 0.075 M NaCl solution (controls) or 450 mg/Kg paracetamol. Liver sections were taken at different time points, fixed in 10% formalin, embedded in paraffin wax, cut and stained with H & E. Histology scores were assigned to stained sections using the key shown in Table 2.1.
Table 5.6. Control mice - correlation between plasma ALT levels and liver H & E histology scores in individual mice at different times post dose

<table>
<thead>
<tr>
<th>Time of dosing</th>
<th>Time of sacrifice</th>
<th>Time post dose (hr)</th>
<th>Plasma ALT levels</th>
<th>Histology score</th>
<th>Plasma ALT levels</th>
<th>Histology score</th>
<th>Plasma ALT levels</th>
<th>Histology score</th>
<th>Plasma ALT levels</th>
<th>Histology score</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.25 am</td>
<td>2.10 pm</td>
<td>2.75</td>
<td>34</td>
<td>0</td>
<td>93</td>
<td>0</td>
<td>37</td>
<td>0</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>11.00 am</td>
<td>3.30 pm</td>
<td>4.5</td>
<td>18</td>
<td>0</td>
<td>19</td>
<td>0</td>
<td>35</td>
<td>0</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>10.30 am</td>
<td>4.30 pm</td>
<td>6</td>
<td>19</td>
<td>0</td>
<td>48</td>
<td>0</td>
<td>19</td>
<td>0</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>8.50 am</td>
<td>5.20 pm</td>
<td>8.5</td>
<td>24</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>8.30 am</td>
<td>7.00 pm</td>
<td>10.5</td>
<td>19</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>6.30 pm</td>
<td>10.30 am</td>
<td>16</td>
<td>19</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>16</td>
<td>0</td>
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<tr>
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<td>9.30 am</td>
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<td>0</td>
<td>26</td>
<td>0</td>
<td>23</td>
<td>0</td>
</tr>
</tbody>
</table>

Groups of mice were dosed with 0.075 M NaCl solution. At the different time points indicated, blood samples and liver sections were taken. Plasma samples were assayed for ALT activity. Results are expressed as nmoles/min/ml. Liver sections were fixed in 10 % formalin, embedded in paraffin wax, cut and stained with H & E. Histology scores were assigned to stained sections using the key shown in Table 2.1.
Table 5.7. Paracetamol-dosed mice - correlation between plasma ALT levels and liver H & E histology scores in individual mice at different times post dose

<table>
<thead>
<tr>
<th>Time of dosing</th>
<th>Time of sacrifice</th>
<th>Time post dose (hr)</th>
<th>Plasma ALT levels</th>
<th>Histology score</th>
<th>Plasma ALT levels</th>
<th>Histology score</th>
<th>Plasma ALT levels</th>
<th>Histology score</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.55 am</td>
<td>1.40 pm</td>
<td>2.75</td>
<td>32</td>
<td>0</td>
<td>193</td>
<td>1</td>
<td>64</td>
<td>0 / 1</td>
</tr>
<tr>
<td>10.30 am</td>
<td>3.00 pm</td>
<td>4.5</td>
<td>341</td>
<td>2</td>
<td>322</td>
<td>2</td>
<td>42</td>
<td>1 / 2</td>
</tr>
<tr>
<td>10.00 am</td>
<td>4.00 pm</td>
<td>6</td>
<td>407</td>
<td>2</td>
<td>1608</td>
<td>2</td>
<td>75</td>
<td>2</td>
</tr>
<tr>
<td>8.20 am</td>
<td>4.50 pm</td>
<td>8.5</td>
<td>35</td>
<td>1 / 2</td>
<td>26</td>
<td>1 / 2</td>
<td>23</td>
<td>1 / 2</td>
</tr>
<tr>
<td>8.00 am</td>
<td>6.30 pm</td>
<td>10.5</td>
<td>19</td>
<td>0 / 1</td>
<td>42</td>
<td>1</td>
<td>990</td>
<td>1</td>
</tr>
<tr>
<td>6.00 pm</td>
<td>10.00 am</td>
<td>16</td>
<td>3834</td>
<td>2 / 3</td>
<td>1646</td>
<td>3</td>
<td>3518</td>
<td>2 / 3</td>
</tr>
<tr>
<td>9.00 am</td>
<td>9.00 am</td>
<td>24</td>
<td>43</td>
<td>0 / 1</td>
<td>605</td>
<td>2</td>
<td>1830</td>
<td>3</td>
</tr>
</tbody>
</table>

Groups of mice were dosed with 450 mg/Kg paracetamol. At the different time points indicated, blood samples and liver sections were taken. Plasma samples were assayed for ALT activity. Results are expressed as nmoles/min/ml. Liver sections were fixed in 10% formalin, embedded in paraffin wax, cut and stained with H & E. Histology scores were assigned to stained sections using the key shown in Table 2.1. ND, not determined.
5.8.5 Histological analysis of TUNEL-stained liver sections from control and paracetamol-dosed mice at different time points post dose

Table 5.8 shows the number of apoptotic bodies per cm$^2$ in TUNEL-stained liver sections from control and paracetamol-dosed mice at different time points post dose (two sections from each mouse, 4 animals per group). Table 5.9 shows the mean ± 1 SD range of each group. The limit of detection was arbitrarily set at 1 so that all 0 values were converted to 1 before data was log transformed and statistically analysed. There is a large variation between animals in the same dose group and with such a high variation it is difficult to determine whether dosing with paracetamol has any effects on the level of apoptosis compared to control animals and whether there is any difference between the different time points. The overall trend suggests that there is little difference in the number of apoptotic bodies per cm$^2$ between groups of paracetamol-dosed and control animals at any time point.

5.8.6 TUNEL-stained mouse liver section from a paracetamol-dosed mouse at 16 hours post dose

Figure 5.21 shows a typical TUNEL-stained apoptotic body in a paracetamol-dosed mouse liver section at 16 hours post dose.

5.8.7 Comparison of plasma ALT levels, H & E scores and level of apoptosis in TUNEL stained liver sections at different time points post dose

Figure 5.22 shows the mean ± SD for plasma ALT levels, histology scores in H & E-stained liver sections and number of apoptotic bodies per cm$^2$ in TUNEL-stained liver sections. Comparison of plasma ALT levels and H & E histology scores, show a correlation but it is difficult to determine whether the level of apoptosis is affected by paracetamol-dosing or the time after dosing.
Table 5.8. Histological analysis of TUNEL-stained liver sections from control and paracetamol-dosed mice at different time points post dose

<table>
<thead>
<tr>
<th>Time post dose (hr)</th>
<th>Number of apoptotic bodies per cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>0.075 M NaCl solution (controls)</td>
</tr>
<tr>
<td>2.75</td>
<td>1.4 1.3 0 0</td>
</tr>
<tr>
<td>4.5</td>
<td>1.6 0 2.3 2.5</td>
</tr>
<tr>
<td>6</td>
<td>2.2 0 0 2.3</td>
</tr>
<tr>
<td>8.5</td>
<td>22.2 5.1 5.0 6.1</td>
</tr>
<tr>
<td>10.5</td>
<td>16.0 13.8 8.4 12.4</td>
</tr>
<tr>
<td>16</td>
<td>2.3 11.8 11.2 3.1</td>
</tr>
<tr>
<td>24</td>
<td>1.9 6.3 0 1.5</td>
</tr>
</tbody>
</table>

Groups of mice were dosed with 0.075 M NaCl solution (controls) or 450 mg/Kg paracetamol. At different time points, liver sections were taken, fixed in 10% formalin, embedded in paraffin wax, cut and TUNEL-stained using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor) as described in methods section 2.2.7. Results are expressed as number of apoptotic bodies per cm².
Table 5.9. Summary of histological analysis of TUNEL stained liver sections from control and paracetamol-dosed mice at different time points post dose

<table>
<thead>
<tr>
<th>Time post dose (hours)</th>
<th>Number of apoptotic bodies per cm$^2$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>450 mg/Kg paracetamol</td>
</tr>
<tr>
<td></td>
<td>Mean ± 1 SD range</td>
<td>Mean ± 1 SD range</td>
</tr>
<tr>
<td>2.75</td>
<td>1.2 ± 1.0 - 1.4</td>
<td>1.6 ± 0.8 - 3.0</td>
</tr>
<tr>
<td>4.5</td>
<td>1.7 ± 1.2 - 2.6</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>6</td>
<td>1.5 ± 0.9 - 2.4</td>
<td>3.6 ± 1.3 - 10.6</td>
</tr>
<tr>
<td>8.5</td>
<td>7.7 ± 3.8 - 15.7</td>
<td>4.2 ± 1.5 - 11.5</td>
</tr>
<tr>
<td>10.5</td>
<td>12.3 ± 9.4 - 16.2</td>
<td>1.2* ± 0.8 - 1.8</td>
</tr>
<tr>
<td>16</td>
<td>5.6 ± 2.4 - 13.0</td>
<td>4.4 ± 0.9 - 21.1</td>
</tr>
<tr>
<td>24</td>
<td>2.1 ± 1.0 - 4.5</td>
<td>4.4 ± 1.0 - 20.1</td>
</tr>
</tbody>
</table>

Groups of mice were dosed with 0.075 M NaCl solution (controls) or 450 mg/Kg paracetamol. At different time points, liver sections were taken, fixed in 10% formalin, embedded in paraffin wax, cut and TUNEL-stained using the ApopTag plus in situ apoptosis peroxidase kit (Appligene Oncor) as described in methods section 2.2.7. Sections were stained over two experiments. Results are expressed as number of apoptotic bodies per cm$^2$. All 0 values were converted to 1 before data was log transformed and statistically analysed. *significantly different from controls at the same time point, $P < 0.05$ (Mann Whitney U Test).
Figure 5.21. TUNEL-stained liver section from a mouse dosed with 450 mg/Kg paracetamol at 16 hours post dose

Objective magnification x 40. CV, central vein. Arrow indicates a cell with the classic morphology of an apoptotic cell, where the cell has shrunk and become detached from its neighbours.
Figure 5.22. Comparison of plasma ALT levels, H & E scores and level of apoptosis in TUNEL stained liver sections at different time points post dose

Groups of mice were dosed with 0.075 M NaCl solution (controls, ▲) or 450 mg/Kg paracetamol (●). At different time points, plasma samples were assayed for ALT activity and formalin-fixed paraffin-embedded liver sections were cut and stained with H & E or TUNEL as described in chapter 2.2. Histology scores were assigned to H & E-stained sections using the key shown in Table 2.1. Number of apoptotic bodies per cm² were determined for TUNEL stained sections.

Results are mean ± SD of n = 4 animals except for plasma ALT levels after 450 mg/Kg paracetamol at 24 hours where n = 8 animals. *significantly different from controls at the same time point, $P < 0.05$ (Mann Whitney U Test). **significantly different from controls at the same time point, $P < 0.05$ (Student’s t-test).
5.8.8 Western blot analysis for PARP cleavage in liver lysates from control and paracetamol-dosed mice at different time points post dose

Figure 5.23 shows the Western blot obtained for pooled liver lysates from control mice and paracetamol-dosed mice at different time points. There does not appear to be any cleavage of PARP protein in any of the samples analysed, and no apparent difference in the pattern of staining between paracetamol-dosed and control animals.

5.8.9 Immunohistochemical staining for Bax protein in liver sections from control and paracetamol-dosed mice

Figure 5.24 shows the pattern of immunohistochemical staining observed with the anti-Bax (P-19) antibody in control and paracetamol-dosed mouse liver sections at 24 hours post dose. The pattern of staining shown is representative of the immunohistochemical staining obtained in control and paracetamol-treated sections from different time points. The general pattern of staining showed no apparent differences between control and paracetamol-dosed animals at any time point.
Figure 5.23. Detection of PARP protein by Western blot analysis of liver lysates from control mice and paracetamol-dosed mice at different time points

Groups of mice were dosed with 0.075 M NaCl solution (controls) or 450 mg/Kg paracetamol. At different times post dose, liver samples were taken and total protein lysates prepared as described in methods section 2.2.8. Samples from each group were pooled and ~6 μg of protein was loaded onto each lane and proteins were separated by SDS-PAGE and transferred to PVDF membrane as described in methods section 2.2.8. Immunodetection of membrane-bound proteins was carried out using a goat anti-PARP polyclonal antibody (1:5,000 R&D systems) and biotinylated anti-goat antibody (1:10,000). Signal was detected using streptavidin AuroprobeBL and silver enhancement reagents.

Samples blotted were liver samples from mice dosed with paracetamol at 6 hours (A), 0.075 M NaCl at 6 hours (B), paracetamol at 4.5 hours (C), 0.075 M NaCl at 4.5 hours (D), paracetamol at 2.75 hours (E), 0.075 M NaCl at 2.75 hours (F), paracetamol at 10.5 hours (G), 0.075 M NaCl at 10.5 hours (H), paracetamol at 8.5 hours (I), 0.075 M NaCl at 8.5 hours (J), paracetamol at 24 hours (K), 0.075 M NaCl at 24 hours (L), paracetamol at 16 hours (M), 0.075 M NaCl at 16 hours (N) and molecular weight markers (MW).
Figure 5.24. Immunohistochemical staining for Bax protein in control (A) and paracetamol-treated (B) liver sections at 24 hours post dose

Paraffin-embedded mouse liver sections were dewaxed, rehydrated and immunohistochemically stained with anti-Bax (P-19) antibody (1:500) and biotin-conjugated goat anti-rabbit antibody (1:500). Signal was detected using peroxidase-conjugated avidin-biotin reagent and DAB substrate as described in methods section 2.2.9. Sections were counterstained with haematoxylin.
5.9 Discussion

One of the questions that the present study has set out to investigate is whether the mechanism of paracetamol-induced liver injury involves activation of apoptotic pathways. A dose-response study was first conducted to determine a suitable dose for a time course study. The dose-response study showed that:

- Significant necrotic cell injury at 24 hours post dose was only apparent when a dose of 450 mg/Kg paracetamol was administered to this strain of mice.
- Overall there was no significant difference between the number of apoptotic bodies per cm$^2$ in TUNEL-stained mouse liver sections from control and paracetamol-dosed animals.
- Western blot analysis in control and paracetamol-treated mouse livers showed no cleavage of PARP protein.

From the results of the dose-response study, 450 mg/Kg paracetamol was chosen to dose animals in a time course study. This dose was chosen because it gave a clear-cut necrotic cell injury end-point at 24 hours without causing lethal injury. The time course study showed that:

- After dosing with 450 mg/Kg paracetamol, plasma ALT levels were significantly elevated above control levels in the groups sampled at 4.5 - 6 hours and then appeared to dip to control levels in the groups sampled at 8.5 and 10.5 hours. At 16 and 24 hours, plasma ALT levels in the paracetamol-dosed animals were again significantly elevated compared to control levels at the same time point.
- H & E histology scores in 450 mg/Kg paracetamol-dosed liver sections showed cell injury at all time points, i.e. from 2.75 hours onwards.
- Overall there did not appear to be a significant difference in the number of apoptotic bodies per cm$^2$ in mouse liver sections between control and paracetamol-dosed animals. An exception occurred in the groups sampled at 16 hours post dose where the number of apoptotic bodies per cm$^2$ in control liver sections were significantly higher than the paracetamol-dosed liver sections.
- Western blot analysis in control and paracetamol-treated mouse livers showed no cleavage of PARP protein at any time point.
- Immunohistochemical staining for Bax protein in liver sections showed no apparent
change in the pattern of staining between paracetamol-dosed and control mice at any time point.

5.9.1 Necrotic cell injury

Plasma ALT levels and H & E histology scores in liver sections were used to assess necrotic cell injury in the liver. In both the dose-response study and time course study the H & E histology scores in liver sections approximately correlated with plasma ALT levels (Tables 5.2, 5.6 and 5.7). It was found that all control mice had plasma ALT levels of less than 100 nmoles/min/ml and H & E histology scores of 0. The results for paracetamol-dosed mice were more variable but it was mostly found that animals with plasma ALT levels of less than 100 nmoles/min/ml showed liver histology scores of 0 and animals with histology scores of ≥ 1 had plasma ALT levels of more than 100 nmoles/min/ml.

The lack of injury seen in the time course study at 8.5 to 10.5 hours post dose in the groups of paracetamol-treated mice was an unexpected result. Plasma ALT levels which were at the “necrotic” level in the groups of mice sampled at 4.5 - 6 hours, dipped in the groups sampled at 8.5 and 10.5 hours to “normal” levels and then increased again in the groups sampled at 16 to 24 hours. Histological analysis of liver sections from these groups of animals showed less injury in the livers sampled at 8.5 and 10.5 hours when compared to those taken at 4.5, 6, 16 and 24 hours, although injury was not completely absent. Because it is unlikely that the injured cells have undergone rapid repair, this may be an experimental artefact due to random variation in the response of individual animals to paracetamol; these animals could have been more resistant to cell injury induced by paracetamol. Or it may simply be that the animals were not dosed properly.

Another possible explanation for the lack of injury seen in the time course study at 8.5 to 10.5 hours post dose in the groups of paracetamol-treated mice are related to the sleep-eating cycle. Animals that have been fasted prior to paracetamol dosing are more susceptible to paracetamol-induced liver injury because liver glutathione levels are reduced. The animals used in the present study were not fasted prior to dosing, however, they were dosed using a staggered dosing regimen. For example, paracetamol-dosed animals that were sacrificed at 4.5 hours were dosed at 10:30 am and sacrificed at 3 pm, and animals that were sacrificed at 16 hours were dosed at 6 pm and sacrificed at 10 am.
the next day (see Table 5.6 and 5.7). If an animal were dosed immediately after feeding
then the hepatotoxic effects of paracetamol would be much less severe compared to an
animal that had not fed for several hours. Alternatively, the influence of diurnal rhythms
on protein expression and/or enzyme activity within the liver could also play a role in the
apparent lack of injury in paracetamol-dosed mice sampled at 8.5 to 10.5 hours.

5.9.2 Apoptotic cell injury

The present study has found little evidence to support the view that apoptosis is
involved in paracetamol-induced hepatotoxicity in this model. Western blot analysis of
pooled liver protein lysates showed no visible cleavage of the full length 116 kDa PARP
protein and no apparent differences in the pattern of staining between control and
paracetamol-dosed animals. Immunohistochemical staining for Bax protein in liver
sections showed no apparent changes in the pattern of staining between control and
paracetamol-dosed animals. The number of apoptotic bodies per cm$^2$ in TUNEL-stained
liver sections in paracetamol-treated mice were not significantly different compared to
control mice except at 10.5 hours, where the control mice appeared to have a higher level
of apoptosis occurring in their livers compared to the paracetamol-dosed mice. The
number of apoptotic bodies per cm$^2$ observed in the control mice at 10.5 hours were also
significantly elevated compared to other control animals at different time points,
indicating that this result may be due to random variation between animals.

The results of the present study are in direct contrast to the results reported by Ray et
al (1996), which demonstrated that after a 500 mg/Kg dose (i.p.), DNA fragmentation in
the form of a ladder occurred in paracetamol-treated mice from 2 - 24 hours and
histological analysis showed nuclear condensation at 2 - 6 hours after dosing. Necrotic
cell injury as assessed by increasing serum ALT levels occurred from at 4 - 24 hours.
This contrast in results may simply be due to variation in the response of different strains
of mice to the hepatotoxic effects of paracetamol, as Ray et al (1996) used ICR (CD-1)
mice and the present study used C57Bl/6 mice.

Another possible explanation for the contrasting results between the present study
and the Ray et al (1996) study is the differences in the sensitivity of the methods used. If
apoptosis were occurring at only a slightly elevated level in paracetamol-treated mice
compared to controls, then the TUNEL method may not be sensitive enough to detect

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this. Quantification of the level of apoptosis in mouse livers by determining the number of apoptotic bodies per cm² in TUNEL-stained sections has been shown to be highly variable, even when consecutive sections were stained (Figure 5.5). The duration of the histologically visible stages of apoptosis is relatively short at ~3 hours (Bursch et al, 1990). This could result in some apoptotic bodies not being detected even if apoptotic cell death is occurring at a high rate. There is also the problem of specificity of the TUNEL method. It has only recently been reported that this method does not discriminate between apoptosis, necrosis and autolytic cell death and that DNA fragmentation is common to different types of cell death (Grasl-Kräupp et al, 1995). To avoid this problem of non-specificity, morphology at the light microscopic level was taken into account when counting the number of apoptotic bodies in each section.

Another consideration that must be taken into account is the degree to which necrosis and apoptosis share common pathways. Whilst the final cell morphology of apoptosis and necrosis are quite distinct, there are certain overlapping pathways and mechanisms which are found in both modes of cell death, such as DNA fragmentation, disruption of intracellular calcium homeostasis, and disruption of mitochondrial membrane potential. For example, Tsujimoto et al (1997) has demonstrated that overexpression of Bcl-2 and Bcl-XL (anti-apoptotic proteins) can block hypoxia-induced apoptosis and necrosis in a rat hepatoma cell line, 7316A, and a rat pheochromocytoma cell line, PC12. Furthermore, overexpression of these anti-apoptotic proteins partially blocked KCN-induced necrosis in these cell lines. Interestingly, previous work has demonstrated that Bcl-2, a protein which prolongs cell survival by blocking apoptosis, is not expressed in normal or pathological hepatocytes (Charlotte et al, 1994).

Cell conditions can also influence whether a cell dies by apoptosis or necrosis. Cell death in human leukaemic cell lines induced by alkylating agents was studied by Fernandes and Cotter (1994) and it was found that depleting glutathione levels in these cells changed the mode of cell death from apoptosis to necrosis.
6.0 Project Achievements

6.0.1 In vitro experiments on inhibition of DNA synthesis by paracetamol and related antioxidants

The experimental results presented in chapter 3 and 4 show that the in vitro experimental rat model used can be utilised to investigate specific inhibition of DNA synthesis by paracetamol and other compounds. Simultaneous measurement of inhibition of protein synthesis was used as an indicator of non-specific cell injury and correlated well with these data. Experiments with paracetamol show a dose-dependent specific inhibition of DNA synthesis with little inhibition of protein synthesis in rat testis, spleen and liver samples.

The experimental results also show that there is a difference in the sensitivity of testis, spleen and liver samples to inhibition of DNA synthesis by inhibitory compounds, with spleen being most sensitive, followed by testis and then liver.

A range of phenolic compounds including paracetamol were found to specifically inhibit DNA synthesis with little inhibition of protein synthesis. However, not all phenolic compounds tested were specific inhibitors of DNA synthesis as these also caused significant inhibition of protein synthesis.

The concentration-response curves obtained for paracetamol show that little or no inhibition of DNA synthesis would be expected in the liver and testes in vivo at therapeutic doses with peak plasma levels of ~ 0.1 mM. However, approximately 20 % inhibition of DNA synthesis was demonstrated in spleen slices, suggesting that a low level of inhibition of DNA synthesis may possibly be occurring in the spleen in vivo. In overdose where plasma paracetamol concentrations are often > 1 mM and may go up to 5 mM for several hours, inhibition of DNA synthesis is likely to be occurring in some tissues in vivo.
6.0.2 *In vivo* experiments on cell injury by paracetamol

The experimental results presented in chapter 5 show that apoptotic cell death is unlikely to be involved in paracetamol-induced hepatotoxicity. Paracetamol-dosed mice did not show a dose-dependent or time-dependent elevation in the level of apoptosis compared to control mice as assessed by quantitative measurement of the number of apoptotic bodies per cm$^2$ in TUNEL-stained liver sections or qualitative measurement of PARP protein cleavage in liver lysates by Western blot analysis. Immunohistochemical staining for Bax protein in liver sections also showed little difference in the pattern of staining between control and paracetamol-dosed mice at different time points after dosing.
6.1 Methods Used to Study Inhibition of DNA synthesis and Cell Injury and their Limitations

6.1.1 *In vitro* experiments on inhibition of DNA synthesis by paracetamol and related antioxidants

Tissues taken from male Wistar rats were utilised in an *in vitro* experimental model to measure the effects of different compounds on DNA synthesis. $^3$H-thymidine and $^{14}$C-leucine incorporation were used to measure DNA and protein synthesis, respectively.

A limitation of this *in vitro* experimental model is that as with all *in vitro* experiments, extrapolation of results to the *in vivo* situation is sometimes difficult. However, the use of tissue slices instead of isolated cells brings results closer to the *in vivo* situation as cell-cell interactions, cell types and tissue organisation are relatively similar to the intact organ and permeability barriers are maintained.

Another limitation of this *in vitro* experimental model is that although specific inhibition of DNA synthesis can be demonstrated, a lack of DNA synthesis inhibition does not necessarily mean that this compound cannot inhibit DNA synthesis. Failure of the compound being tested to penetrate cells or rapid metabolism could be the reason. Similarly, inhibition of DNA synthesis *in vitro* may not indicate that the same effect will occur *in vivo*.

A further limitation of this method is that no information about the specific sites of the DNA or protein synthesis pathways that are affected can be elucidated. While the inhibition of DNA synthesis by paracetamol is mainly attributable to inhibition of ribonucleotide reductase through the radical quenching property of the parent compound, it is not known if other properties affecting signalling pathways might play a role in the inhibition of DNA synthesis. This is also true for the other compounds, especially the flavonoids. Despite its limitations, this method can be very useful as a broad screen for DNA synthesis inhibitors.

The results for cycloheximide in liver slices show that inhibition of protein synthesis is not always a good marker of cell injury. Inhibition of protein synthesis by cycloheximide in liver slices is not accompanied by inhibition of DNA synthesis.
6.1.2 In vivo experiments on cell injury by paracetamol

Male C57Bl/6 mice were dosed i.p. with paracetamol or 0.075 M NaCl solution (controls) and assessed for necrotic and apoptotic cell injury in the liver. Liver necrosis was assessed by measuring plasma ALT levels and by histological analysis of H & E-stained liver sections. A semi-quantitative scoring system was devised to assess the level of injury in the stained liver sections. The periodic acid Schiff (PAS) method was also used to visualise loss of glycogen in the injured liver. Apoptosis was assessed using the following methods:

1. terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labelling (TUNEL) method,
2. detection of poly (ADP-ribose) polymerase (PARP) cleavage by Western blot analysis in liver protein lysates,
3. immunohistochemical staining for Bax protein expression in tissue sections.

One of the main limitations of these experiments is the variation in the response of individual animals to paracetamol dosing. This is illustrated by the variation in the plasma ALT levels between mice dosed with 450 mg/Kg paracetamol at 24 hours post dose. Very little can be done to reduce this variation, one can only increase the numbers of animals used and use statistical analyses to determine whether paracetamol-dosed animals are significantly different from control animals.

A limitation of using the TUNEL method to stain apoptotic cells in situ is that this method does not only stain cells that are apoptosing. Grasl-Kraupp et al (1995) has found that the TUNEL method stained positive in rat livers after a cytotoxic oral dose of carbon tetrachloride or N-nitrosomorpholine. These are well-defined models of necrotic cell death. Furthermore, in insufficiently fixed livers where autolysis has taken place, the TUNEL method also stained positive. In the present study, to minimise the number of false positive counts, the morphology of each stained cell was observed at high power under a light microscope. Those cells that displayed signs of necrotic cell death such as swelling, an enlarged nucleus, or infiltration of inflammatory cells, were not counted.

Another limitation of the TUNEL method is that the duration of the histologically visible stages of apoptosis is relatively short at ~3 hours (Bursch et al, 1990). This could result in a small number of apoptotic bodies not being detected even when many cells are
dying.

There is a large variation in the number of apoptotic bodies per cm\(^2\) between TUNEL-stained sections cut from the same sample (Figure 5.5). The number of sections examined was probably not enough to take into account this variation between sections and give a true approximation of the level of apoptosis in the liver. In the dose-response study only one section from each animal was examined. In the time course study, two sections from each animal were examined. Examination of at least 6 sections from each animal (3 non-consecutive sections from 2 liver samples) would give a better estimate of the level of apoptosis in the treated livers. Overall it was found that the TUNEL method is not sensitive enough to quantify the level of apoptosis in the liver sections and other methods should be utilised.

PARP cleavage is considered to be a good early marker of apoptosis that occurs before histological signs of injury (Kaufmann et al, 1993). Western blot analysis for PARP cleavage should provide an indicator as to whether apoptosis is occurring in the liver or not. The lack of cleavage seen in the paracetamol-treated mice and lack of difference between the pattern of staining when compared to controls indicates that apoptosis is not significantly contributing to paracetamol-induced liver injury. However, it has recently been reported that PARP cleavage is not involved in Fas-mediated hepatocyte apoptosis (Jones et al, 1998). It is not known if this is relevant to the current work. Whilst anti-Fas antibody treatment of livers is a good model of apoptotic cell death, if there are not enough healthy hepatocytes to phagocytose the dying cells, the mode of cell death becomes necrotic (Ogasawara, 1993).

Detection of changes in the pattern of Bax protein expression in liver sections by immunohistochemistry proved to be very difficult to interpret. Although it was concluded that the pattern of staining did not differ between paracetamol-treated and control mice, changes in a few cells could easily have escaped detection.
6.2 Other Markers

6.2.1 *In vitro* experiments on inhibition of DNA synthesis by paracetamol and related antioxidants

Other than protein synthesis, measurement of other markers of cell viability could have been used. For example, ATP levels and potassium levels in the tissue, or leakage of enzyme markers such as LDH into the incubation medium. Markers of mitochondrial function could have also been used. One such marker is the cationic probe triphenyl phosphonium (TPMP⁺) which accumulates mostly in the mitochondrial matrix because of its high negative potential relative to the cytoplasm. Loss of the mitochondrial membrane potential releases TPMP⁺ from the mitochondria (Hoek *et al*, 1980).

6.2.2 *In vivo* experiments on cell injury by paracetamol

Electron microscopy remains one of the most reliable methods for detection of apoptosis. However, with the number of samples involved in the present study and the difficulty in obtaining quantitative data, this method would not have been useful in determining the level of apoptosis in paracetamol-treated mouse livers.

Agarose gel electrophoresis could be used to detect DNA laddering in tissue samples. Although recent studies show that apoptosis can occur in the absence of a cell nucleus and DNA fragmentation (Cohen *et al*, 1992; Schulze-Osthoff *et al*, 1994), this method is still considered to be one of the key methods for detecting apoptotic cell death.

The use of formalin-fixed paraffin sections to study the role of apoptosis in paracetamol-induced hepatotoxicity greatly restricts the number of methods that could be used. For example, detection of externalisation of phosphatidylserine on the plasma membrane (an early event that occurs during apoptosis) by specific high affinity binding with labelled annexin V can only be utilised with living cells (reviewed by van Engeland *et al*, 1998).
6.3 General Discussion

The main aim of this project was to study new aspects of the cellular effects of paracetamol and related antioxidants. Inhibition of DNA synthesis by paracetamol and related antioxidants and the role of apoptosis pathways in paracetamol-induced liver cell injury were studied. Different aspects of the cellular effects of paracetamol were looked at to allow for a more informed risk assessment and also to provide an insight to the mechanism of its non-antipyretic and non-analgesic properties. The inhibition of DNA synthesis by paracetamol via an antioxidant mechanism also lead to the study of other antioxidants to determine if this property also extends to these other compounds.

Paracetamol has been reported to have therapeutic properties in addition to its antipyretic and analgesic action. It was reported by van Heyningen and Harding (1986) in a case-control study that a larger percentage of control groups took analgesics compared to cataract patients and that paracetamol consumption reduced the relative risk of cataract. Cramer et al (1998) also reported in another case-control study that there was a reduced risk of ovarian cancer in women who took paracetamol on a daily basis, had been taking paracetamol for more than 10 years, or took more than 20 tablet years (tablets per day x years of use). The mechanism of these protective effects is not known, however the antioxidant properties of paracetamol may possibly contribute.

6.3.1 Antioxidant properties of paracetamol

Much research has centred around the therapeutic properties of antioxidants, in particular the protective effects of antioxidants against oxidative damage. Oxidative damage by free radicals and ROS has been associated with the pathophysiology of many different diseases such as cancers, cataract, and atherosclerosis. It is possible that paracetamol may be exerting its protective effects by inhibiting oxidative damage of cells. Woollard et al (1990) have shown that concentrations of paracetamol ranging from 62.5 to 100 μM can reduce the 1,1-diphenyl-2-picrylhydrazyl free radical to 1,1-diphenyl-2-picrylhydrazine, protect erythrocytes from butyl peroxide-induced lysis, and chelate ferrous ions. These concentrations are well within the range of the plasma concentrations found in man after ingesting a therapeutic dose.
6.3.2 Inhibition of DNA synthesis by paracetamol and hydroxyurea

Whilst protection against oxidative damage is likely to be one of the mechanisms by which antioxidants exert their therapeutic effect, it is known that these compounds also have other cellular effects. One such cellular effect is the inhibition of DNA synthesis. Hydroxyurea is an example of a drug with anti-neoplastic properties, which is based on its ability to inhibit DNA synthesis by quenching the tyrosyl free radical present in the active site of ribonucleotide reductase. Hydroxyurea is an effective anti-neoplastic drug because it only targets cells that are in S-phase, i.e. only those cells that are in S-phase are sensitive to its inhibitory effects. This may partly explain the difference in the sensitivity of the different tissues in the present study to the inhibitory effects of hydroxyurea. Control spleen slices had a high $^3$H-thymidine incorporation rate, control testis samples had an intermediate rate and control liver slices had a low $^3$H-thymidine incorporation rate. Indeed it was found that the most sensitive tissue to inhibition of DNA synthesis by hydroxyurea was spleen followed by testis and then liver. Paracetamol can also inhibit DNA synthesis in a similar fashion and this explanation also applies to the differential inhibition of DNA synthesis observed between tissues in the present study.

It is possible that paracetamol has similar therapeutic activity to hydroxyurea. In order for this to be feasible, inhibition of DNA synthesis must be demonstrated at therapeutic paracetamol concentrations. It has been previously shown in cell lines that paracetamol specifically inhibits replicative DNA synthesis at close to the therapeutic level of 0.1 mM (Hongslo et al, 1989; Hongslo et al, 1990). However, the concentration-response curves obtained for paracetamol in the present study show that little or no inhibition of DNA synthesis would be expected in the liver and testes in vivo at therapeutic doses, and in the spleen a low level of inhibition of DNA synthesis may be occurring. These results suggest that at therapeutic doses, paracetamol probably does not have similar therapeutic activity to hydroxyurea.
6.3.3 Differential inhibition of DNA synthesis in different tissues and its implications

As previously mentioned the experimental results show that there is a difference in the sensitivity of testis, spleen and liver samples to inhibition of DNA synthesis by inhibitory compounds, with spleen being most sensitive, followed by testis and then liver. Possible explanations for the differential inhibition of DNA synthesis in different tissues have been thoroughly discussed in results chapter 3.5.6. Differences in the pool size of the DNA precursors (dNTPs), differences in the levels of enzyme and activity of ribonucleotide reductase in the different tissues, availability of alternative dNTP synthesis pathways and the high metabolic activity in the liver have all been considered as possible reasons for this differential inhibition. What has yet to be discussed is the possible implications of this differential inhibition. Many studies on the cellular effects of paracetamol are conducted in experimental systems using a single tissue or cell type. The differential inhibition of DNA synthesis in different tissues demonstrates the need for caution when extrapolating results from studies that only look at one tissue or cell type. Lymphoid tissues and cell lines in particular, appear to be much more sensitive to inhibition of DNA synthesis by paracetamol and results of studies using these may be biased. In order to prevent this bias, several cell types should be studied to allow for a proper assessment.

The effects of therapeutic doses of paracetamol has not been investigated thoroughly in the spleen and other lymphoid tissues. It is not known if a low level of DNA synthesis inhibition is occurring in the spleen and lymphoid tissues of people taking regular therapeutic doses and it is also not known what the possible effects of this inhibition are.

6.3.4 Inhibition of DNA synthesis by phenolic antioxidants

The results of the present study show that some phenolic antioxidants can also inhibit DNA synthesis in a specific manner with little inhibition of protein synthesis. Although the doses used in the present study are rather high and a person is not likely to be exposed to such doses, these results nonetheless add to our knowledge of the cellular effects of these antioxidants.
6.3.5 Role of apoptotic pathways in paracetamol-induced liver cell injury

The events that occur after metabolism of an overdose of paracetamol and depletion of cellular glutathione, and before presentation of clinical symptoms are still not completely understood. Although many factors such as covalent binding to cellular macromolecules, disruption of calcium homeostasis, oxidative stress, and disruption of mitochondrial function have all been investigated, none of these fully explain the mechanism of cell injury and some appear to occur subsequent to injury. Recent reports that apoptotic cell death may be involved in paracetamol-induced liver injury have provided a new area of research which has not been looked at before.

One particular study showed that nuclear condensation and fragmentation occurred in HL-60 cells after exposure to 2 - 3 mM paracetamol (Wiger et al, 1997). HL-60 cells do not metabolise paracetamol so the mechanism of this apoptotic cell death is not via NAPQI. It is possible that this apoptotic cell death is a consequence of inhibition of ribonucleotide reductase activity in these cells. Although the primary site of injury after paracetamol overdose is the liver, Placke et al (1987) has demonstrated that 600 mg/Kg paracetamol induces injury in the liver, kidney, lung, testes, and lymphoid tissues of the mouse. Histological signs of injury in liver and kidney occurred as early as 2 hours after dosing. Signs of injury in the lung and testes began at 4 and 6 hours, respectively, and in lymphoid tissues, injury did not occur until 24 hours after dosing. The lung, testes and lymphoid tissues have a much lower capacity for metabolising paracetamol to NAPQI so injury is less likely to be through this pathway. Again inhibition of ribonucleotide reductase in dividing cells by unmetabolised paracetamol may trigger cell injury; possibly by activation of apoptotic cell death.

The results of the present study found little evidence for apoptotic change in the livers of mice dosed with 450 mg/Kg paracetamol compared to control animals. This result contrasts with the study conducted by Ray et al (1996) which found DNA fragmentation in the form of a ladder and nuclear condensation in histological sections before the onset of necrosis after dosing mice with 500 mg/Kg paracetamol. Various reasons for the contrasting results have been discussed previously in chapter 5, and include: differences in the mouse strains used, differences in the sensitivity of the methods used to detect apoptosis and the degree to which necrosis and apoptosis share
common pathways. Another consideration which has not been discussed and must not be discounted is the cellular effects of unmetabolised paracetamol on the liver after an overdose. As the results of the present study have shown, paracetamol at doses of more than 1 mM significantly inhibit DNA synthesis in rat liver slices. Plasma concentrations of more than 1 mM often occur after overdose so it is likely that DNA synthesis inhibition occurs in vivo at these concentrations. If indeed inhibition of ribonucleotide reductase does trigger apoptosis as Wiger et al (1997) suggest, then the apoptosis detected by Ray et al (1996) may in part be due to this cellular effect of paracetamol rather than NAPQI-induced injury. The lack of apoptotic injury observed in the present study may simply be due to a difference in the dose administered. The study by Ray et al (1996) used a higher dose of paracetamol so the amount of unmetabolised paracetamol remaining in the liver would probably be higher.

6.3.6 Implications of apoptotic cell death in paracetamol-induced cell injury

Reviews of the literature have focused on the killing of hepatocytes as well as the possible genotoxic and carcinogenic effects of paracetamol. These have overall concluded that there are certain threshold levels which must be exceeded before any damaging effects occur and that these levels are not reached at therapeutic doses (reviewed by Bergman et al, 1996). Because apoptotic cell injury has not been investigated before, it is not known if there really is a threshold before injury occurs. Above normal levels of apoptosis may be occurring in the livers of people taking regular therapeutic doses. Although a large increase in apoptotic cell death in the livers of people taking regular therapeutic doses is highly unlikely, as such an effect would have been reported by now, a small increase may go unnoticed.

Clearly, even if this study had found evidence for apoptotic changes preceding necrosis in paracetamol-dosed livers, the interpretation of such a finding would have been difficult i.e., would this be a desirable trait or a pathological feature contributing to hepatotoxicity? If apoptosis were occurring at a greatly elevated level then this would be a damaging effect. A large volume of cells would be affected and secondary necrosis would result. If however, apoptosis were occurring at a slightly elevated level, then this may be beneficial as a mechanism for deleting damaged cells. During overdose, it may
also delay necrotic cell death and allow the liver to remove its damaged cells. At this point, the liver cells are still not committed to necrotic cell death. Only when too many cells are apoptosing and there are not enough healthy hepatocytes to phagocytose the apoptotic cells would the mode of cell injury become necrotic.
6.4 Future Developments

The present in vitro experimental model provides a useful method for screening compounds for DNA synthesis inhibitory activity. Only a small number of compounds were examined in the present study. It would be interesting to see if other phenolic antioxidants, in particular other flavonoids are also specific inhibitors of DNA synthesis.

Although inhibition of DNA synthesis by paracetamol and hydroxyurea are known to be mediated by inhibition of ribonucleotide reductase, the mechanism of DNA synthesis inhibition by the other compounds studied are unknown. A ribonucleotide reductase assay could be used to determine if these compounds are also inhibitors of this enzyme. Two methods can be used to measure the activity of ribonucleotide reductase, (i) direct measurement of the tyrosyl free radical which is essential for enzyme activity and present in the active site of the enzyme, (ii) indirect measurement of the products of enzyme activity, i.e. deoxyribonucleotides.

The reason for the differential inhibition of DNA synthesis in different tissues is still unclear. Differences in the ribonucleotide reductase levels and differences in the activity of ribonucleotide reductase between the tissues have been suggested. It would be interesting to see if there are indeed these differences.

The present study has shown that very little inhibition of DNA synthesis in the liver and testes would be expected in vivo at therapeutic doses (0.1 mM) with a possible low level of inhibition of DNA synthesis in the spleen. And at doses encountered in overdose (1 - 5 mM), it is likely that inhibition of DNA synthesis is occurring in some tissues in vivo. But, it is not known what the consequences are of this inhibition of DNA synthesis. The inhibition of DNA synthesis by paracetamol has been proposed as one of the mechanisms by which paracetamol might possibly cause genotoxic effects (Bergman et al, 1996). It would be interesting to see if this were true and at what doses these effects occurred.

There have been suggestions that there are common mediators in the apoptotic and necrotic signalling pathways. Overexpression of Bcl-2 and Bcl-XL (proteins that prolong cell survival by blocking apoptosis) has been shown to protect cells from hypoxia-induced apoptosis as well as necrosis (Shimizu et al, 1996; Tsujimoto et al, 1997). Interestingly, previous work has demonstrated that Bcl-2 is not expressed in normal or pathological hepatocytes (Charlotte et al, 1994; Skopelitou et al, 1996). Recent studies in
transgenic mice expressing the Bcl-2 gene in their livers have demonstrated protection from anti-Fas antibody-induced liver failure (Lacronique et al., 1996; Rodriguez et al., 1996) and another study has shown that transfection of the human Bcl-2 gene in vivo prevents hypoxic liver cell necrosis (Yamabe et al., 1998). Preliminary work in our laboratory by Dr D Beales showed that rat liver slices could be transfected with an inactivated herpes simplex virus-1 vector (unpublished results). It would be interesting to see if a viral vector containing the Bcl-2 gene could induce expression in rat liver slices and afford protection against paracetamol exposure.
6.5 Summary

It has been shown in the present study that the *in vitro* experimental rat model used in chapter 3 and 4 can show when a compound causes specific inhibition of DNA synthesis without interference with protein synthesis. This method can be used as a broad screen for potential specific inhibitors of DNA synthesis. Compounds which have shown dose-dependent inhibition of DNA synthesis with little protein synthesis inhibition are paracetamol, hydroxyurea, (+)-catechin, *n*-propyl gallate, *p*-aminophenol, *m*-aminophenol and *p*-cresol.

The present study has also shown that paracetamol and other inhibitors differentially inhibit DNA synthesis in different tissues. This difference in sensitivity between tissues was also observed with hydroxyurea, and to a lesser extent *n*-propyl gallate and (+)-catechin. The order of most sensitive to least sensitive tissue is spleen > testis > liver. This differential inhibition of DNA synthesis in different tissues also demonstrates the need for caution when extrapolating results from studies that only look at one tissue or cell type.

Other compounds which were screened using this method gave more mixed results and demonstrated that not all phenolic antioxidants are specific inhibitors of DNA synthesis. Whilst NDGA and quercetin displayed preferential inhibition of DNA synthesis with a certain degree of protein synthesis inhibition, caffeic acid and naringenin did not inhibit DNA synthesis specifically as protein synthesis was equally inhibited at the doses tested.

It has also been shown in the present study that apoptotic pathways are unlikely to be involved in the mechanism of paracetamol-induced liver cell injury in C57Bl/6 mice. Apoptotic cell death was assessed by quantification of the number of apoptotic bodies per cm² in TUNEL-stained liver sections, Western blot analysis for PARP protein cleavage in liver lysates and detection of changes in the distribution of Bax protein in liver sections using an immunohistochemical staining technique. The present study showed no dose-dependent or time-dependent increase in the level of apoptosis in the livers of paracetamol-dosed animals compared to controls.
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van Engeland, M., Nieland, L.J., Ramaekers, F.C., Schutte, B. and Reutelingsperger, C.P.


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### APPENDIX: ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs</td>
<td>absorbance</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azinobis-(3-ethyl benzthiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>ADI</td>
<td>allowable daily intake</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine transaminase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease-activating factor 1</td>
</tr>
<tr>
<td>ara-C</td>
<td>1-β-D-arabinofuranosylcytosine</td>
</tr>
<tr>
<td>ara-CTP</td>
<td>cytosine arabinoside triphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
</tr>
<tr>
<td>BHA</td>
<td>butylated hydroxyanisole</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Ca EDTA</td>
<td>calcium ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>ced</td>
<td>cell death abnormal genes</td>
</tr>
<tr>
<td>CHAOS</td>
<td>Cambridge Heart Antioxidant Study</td>
</tr>
<tr>
<td>cm²</td>
<td>square centimetre(s)</td>
</tr>
<tr>
<td>COV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>CV</td>
<td>central vein</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>GSH-POD</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidised glutathione</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>hr</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HU</td>
<td>hydroxyurea</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>ICE</td>
<td>interleukin-1β converting enzyme</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>Kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LOO⁻</td>
<td>lipid peroxy radical</td>
</tr>
<tr>
<td>LOOH</td>
<td>lipid hydroperoxide</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>m⁻</td>
<td>meta-</td>
</tr>
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</tr>
<tr>
<td>min</td>
<td>minute</td>
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<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>MW</td>
<td>molecular weight markers</td>
</tr>
<tr>
<td>μCi</td>
<td>microcurie</td>
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<tr>
<td>μg</td>
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<tr>
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<td>micrometre(s)</td>
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<td>micromolar</td>
</tr>
<tr>
<td>μmol</td>
<td>micromole(s)</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>NAD(P)</td>
<td>nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>reduced nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NAPQI</td>
<td>N-acetyl-p-benzoquinone imine</td>
</tr>
<tr>
<td>ND</td>
<td>not determined</td>
</tr>
<tr>
<td>NDGA</td>
<td>nordihydroguaiaretic acid</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nmol</td>
<td>nanomoles</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>superoxide anion radical</td>
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<tr>
<td>OH$^-$</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>p-</td>
<td>para-</td>
</tr>
<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid Schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>programmed cell death</td>
</tr>
<tr>
<td>PG</td>
<td>propyl gallate</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>pmol</td>
<td>picomoles</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SB</td>
<td>SmithKline Beecham</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel</td>
</tr>
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</table>
SEM: standard error of the mean
SOD: superoxide dismutase
TCA: trichloroacetic acid
TCA/leu: trichloroacetic acid containing 10 mM L-leucine
TdT: terminal deoxynucleotidyl transferase
TEAC: Trolox equivalent antioxidant activity
TPMP*: triphenyl phosphonium
TUNEL: TdT-mediated dUTP-digoxigenin nick end labelling
Effects of phenolic antioxidants and flavonoids on DNA synthesis in rat liver, spleen, and testis in vitro

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Abstract

Paracetamol (acetaminophen) and hydroxyurea were found to inhibit DNA synthesis in a dose-dependent manner in tissue slices in vitro, with little effect on protein synthesis. Considerable variation in the sensitivity of the different tissues was also observed with an order of least sensitive to most sensitive tissue of liver < testis < spleen. The phenolic antioxidant properties of paracetamol are thought to be the mechanism by which paracetamol inhibits DNA synthesis, which led us to study other phenolic antioxidant molecules and flavonoids for specific inhibition of DNA synthesis. (+)-catechin, m-aminophenol, p-aminophenol and p-cresol all displayed a highly specific inhibition of DNA synthesis. Quercetin displayed a preferential inhibition of DNA synthesis but a significant level of inhibition of protein synthesis was also seen. Nordihydroguaiaretic acid (NDGA) and -propyl gallate showed preferential inhibition of DNA synthesis at the lower doses tested, but at higher doses showed significant inhibition of protein synthesis, presumably because of cytotoxicity. Caffeic acid and naringenin did not display any specific inhibition of DNA synthesis as protein synthesis was equally inhibited at all doses tested. This study demonstrates that certain phenolic antioxidants can inhibit DNA synthesis specifically but this is not a property shared by all phenolic antioxidants; and that these inhibitors show considerable variation in effectiveness between different tissues. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Paracetamol; Hydroxyurea; Phenolic antioxidants; Flavonoids; DNA synthesis; Protein synthesis; Testis; Spleen; Liver

1. Introduction

As well as its analgesic and antipyretic therapeu- tic properties, paracetamol has been shown to specifically inhibit replicative DNA synthesis in V79 cells at close to the therapeutic plasma level of 0.1 mM (Hongslo et al., 1989). This inhibition was demonstrated to be due to quenching of the tyrosyl free radical present in the active site of ribonucleotide reductase (Hongslo et al., 1990).
This property is similar to that of hydroxyurea, a well known DNA synthesis inhibitor which also inhibits ribonucleotide reductase activity by destroying the tyrosyl free radical (Krakoff et al., 1968; Thelander et al., 1985).

Paracetamol is a phenolic antioxidant that can act both as a transition metal chelator and radical scavenger (Woollard et al., 1990) and it is this radical quenching antioxidant property which is thought to be the mechanism by which paracetamol inhibits ribonucleotide reductase. Inhibition of DNA synthesis by paracetamol raises the question about whether there are other substances in the environment which inhibit DNA synthesis, and also whether some tissues are more sensitive than others to this type of inhibition.

Another group of antioxidant compounds with both metal chelating and radical scavenging properties is the dietary flavonoids. Flavonoids are a large group of naturally-occurring antioxidants found at high concentrations in tea, wine, fresh fruit, and vegetables. Estimates of the daily flavonoid intake in the Western diet range from 12 mg to 1 g per person and a high intake of flavonoids in the diet has been associated with a lower risk of death from coronary heart disease (Pierpoint, 1986; Hertog et al., 1993).

There have been reports that flavonoids can inhibit growth of cancer cell lines (Yoshida et al., 1990; Kaur et al., 1992). Hirano et al. (1989) also demonstrated that flavonoids could inhibit mitogen-induced proliferation in human lymphocytes. It is not clear whether inhibition of cell growth is specifically due to inhibition of DNA synthesis or interference with some basic function of cells leading to blocking of all cell function. Moreover, it is not known if inhibition of DNA synthesis is a common property of the flavonoid structure, or if all compounds with radical quenching activity can inhibit DNA synthesis, or even if other structural requirements determine whether DNA synthesis is inhibited.

The tyrosyl free radical and nonhaem iron centre of the M2 subunit of ribonucleotide reductase are essential for enzyme activity. Hydroxyurea inhibits enzyme activity by quenching the tyrosyl radical whilst leaving the iron centre intact. However, iron chelators may also remove the iron centre and cause simultaneous loss of the tyrosyl free radical and loss of activity (Yarbro, 1992). It seems likely that small molecule antioxidants that are iron chelators and/or radical quenchers with both water and lipid solubility would penetrate ribonucleotide reductase and inhibit the enzyme.

In the present study, the effect of paracetamol and different phenolic compounds (structures shown in Fig. 1) on DNA synthesis was investigated using an in vitro tissue synthesis in liver, spleen and testes of male Wistar rats to investigate concentration-response relationships of candidate compounds.

2. Materials and methods

2.1. Animals and diet

Male Wistar rats (Harlan Olac, Bicester, UK) of final weight 120–180 g were fed stock pellets (SDS Ltd, Witham, UK) and given unlimited access to drinking water.

2.2. Materials

[6-3H] thymidine (22–25 Ci/mmol) and L-[1-14C] leucine (54 mCi/mmol) were purchased from Amersham (Amersham, Bucks, UK). Liquid scintillation fluid was purchased from National Diagnostics (Hessle, Hull, UK). All other chemicals were of the highest grade available from Merck Ltd. (Lutterworth, Leics, UK), Sigma (Poole, Dorset, UK), or Aldrich Chemical Co. (Poole, Dorset, UK).

2.3. In vitro experiments

Rats were killed by cervical dislocation after inducing deep anaesthesia by giving fentanyl citrate (Hynpom, Janssen, Wantage, UK, 0.02 mg/100g b.w.) and loprazolam (Roussel Ltd, Uxbridge, UK, 0.08 mg/100g b.w.) as a single i.m. injection. Liver, spleen and testes were rapidly removed and placed into ice-cold 0.15 M saline.

Liver slices of ≈0.3 mm thickness and 60–100 mg wet weight, and spleen slices of ≈0.3 mm thickness and 10–25 mg wet weight were cut by hand using a Stadie-Riggs tissue slicer (A. H.
Thomas Co. Philadelphia, USA). This technique has been shown to give good preservation of potassium ion, glutathione and ATP levels in liver slices, by previous work in this laboratory (McLean and Nuttall, 1978; Martin and McLean, 1995). Tissue slices were gently blotted, weighed, and placed into 25 ml Erlenmeyer flasks containing 5 ml Ringer solution at room temperature. After decapsulation, samples of testis tubules were gently pulled into approximately 60–90 mg samples with forceps, weighed, placed into Ringer and gently teased apart with a pair of mounted needles. The Ringer solution had the following composition: NaCl 125 mM; KCl 6 mM; MgSO₄ 1.2 mM; NaH₂PO₄ 1 mM; CaCl₂ 1 mM; glucose 10 mM; 20 amino acids (no leucine) each at 0.1 mM; bovine serum albumin 5 mg/ml; gentamicin 50 μg/ml; and 15 mM bicarbonate buffer, pH 7.4 (Peters, 1983).

Tissues were incubated in the presence or absence of test compound at 37°C under 95% O₂/5% CO₂ in a shaking water bath (80 strokes/minute, 6 cm/stroke). After 30 min a dual label of [6-³H] thymidine (5 μCi) and L-[1-¹⁴C] leucine (0.25 μCi) was added to each flask, with carrier thymidine (25 nmol) and carrier L-leucine (0.45 μmol). Incubation was continued for a further 30 min. The flasks were then removed from the water bath and spleen and liver slices removed with forceps, rinsed in ice-cold 0.15 M saline and homogenised in 5 ml 10% trichloroacetic acid containing 10 mM L-leucine (TCA/leu). For the testes samples,
the flask contents were spun down at 3000 rpm for 5 min, and the supernatant poured off. The pellet was then resuspended and washed with 2 ml of ice-cold 0.15 M saline, spun down, the supernatant discarded and the resultant pellet homogenised in 5 ml 10% TCA/leu.

Water soluble drugs were dissolved in distilled water and made up as concentrated stock solutions and added to Ringer solution to give the final concentration. Trolox was directly dissolved in Ringer. Nordihydroguaiaretic acid (NDGA), quercetin, and naringenin were first dissolved in dimethylsulphoxide (DMSO) and added to Ringer solution to give a final DMSO concentration of 0.1% (v/v). This concentration of DMSO has been found not to interfere with incorporation of radiolabel.

2.4. Measurement of thymidine and leucine incorporation into tissue

The homogenate was spun down (3000 rpm for 5 min) and the precipitated pellet resuspended and washed at least a further two times with 5 ml of fresh 10% TCA/leu until the counts in the supernatant were negligible. Pellets were digested with 1 ml of 1 M sodium hydroxide at 37°C overnight.

Incorporation of $^3$H-thymidine and $^{14}$C-leucine into DNA and protein, respectively, were measured by adding an aliquot of digested sample to scintillation fluid containing an equal volume of 1M hydrochloric acid (to neutralise the sodium hydroxide). This was counted in a Packard Tricarb liquid scintillation counter. Using a dual label programme, counts were converted to disintegrations per minute (dpm) using automatic external standardisation calibrated with quenched $^3$H and $^{14}$C samples of similar chemical composition. Duplicate samples were taken for each incubating condition. Controls were samples taken from the same animal and incubated in the absence of drug.

Non-radiolabelled carrier thymidine and leucine were included in the incubation media as this reduces variability in radiolabel incorporation between samples by providing a steady-state concentration of thymidine and leucine.

2.5. Protein determination

Protein content of the digested tissue was determined by the method of Lowry et al. (1951) using BSA standards.

2.6. Statistical analysis

The mean incorporation of thymidine and leucine for duplicate flasks (expressed as dpm/mg protein) was considered as a single data point for analysis of results from at least three separate experiments. % inhibition was calculated by comparing the dpm from dosed tissues with control tissues. All data is presented as mean% inhibition ± SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) with comparisons to a control group (Bonferroni correction). The level of significance was established at $P < 0.05$. The concentration of drug that gave 50% inhibition of synthesis (IC$_{50}$) was determined using a sigmoidal curve fitting programme in GraphPad Prism 2.01 (GraphPad Software, Inc., San Diego, CA, USA). All concentration-response relationships that showed > 40% inhibition were plotted with fitted curves, otherwise data points were plotted with straight lines.

3. Results

Thymidine and leucine incorporation into the slices was linear with time for over 1 h (data not shown). Typical $^3$H-thymidine incorporation levels in control tissue samples after 60 min of incubation with radiolabel exposure from $T_{30}$ to $T_{60}$, ranged from 4800–10 000 dpm/mg protein for testes ($n = 14$), 13 500–77 400 dpm/mg protein for spleen ($n = 11$), and 800–4000 dpm/mg protein for liver ($n = 11$). Typical $^{14}$C-leucine incorporation levels in control tissue samples after 60 min of incubation and radiolabel exposure from $T_{30}$ to $T_{60}$ ranged from 2600–3600 dpm/mg protein for testes ($n = 14$), 2600–4800 dpm/mg protein for spleen ($n = 11$), and 800–1900 dpm/mg protein for liver ($n = 11$). As these values indicate there can be a large variation of radiolabel incorporation in control tissues between ani-
Fig. 2. Effect of paracetamol (A) and hydroxyurea (B) on DNA synthesis (solid symbols) and protein synthesis (open symbols) in various tissues of the male rat in vitro. Testis tubules (■), spleen slices (▲) and liver slices (●) were incubated in 5 ml Ringer at 37°C under 95% O₂/5% CO₂ in a shaking water bath. After 30 min, DNA and protein synthesis was measured by simultaneously incubating tissues with [³H]-thymidine (5 μCi) and [¹⁴C]-leucine (0.25 μCi), respectively, for a further 30 min. Tissues were homogenised in TCA (containing carrier leucine), washed, dissolved in 1M sodium hydroxide and radioactivity counted. Data points represent mean ± SEM of duplicate samples from 4-8 separate animals. Controls were duplicate samples taken from the same animal and incubated in Ringer in the absence of drug. *significantly different from control, P < 0.05 (one-way ANOVA with Bonferroni correction).

mals, however, variations between duplicate samples were relatively small. Percentage inhibition of DNA and protein synthesis by the compound studied, did not vary with the variation in radiolabel incorporation between animals in control slices.

Paracetamol has been found to be a highly specific dose-dependent inhibitor of DNA synthesis in this experimental system (Fig. 2A). There is also a difference in sensitivity of the three tissues to the inhibitory effects of paracetamol. The IC₅₀ values as determined by a sigmoidal curve fitting programme (GraphPad Prism 2.01) are 1.39, 0.49, 0.23 mM for liver, testis and spleen, respectively. DNA synthesis in spleen slices was found to be much more sensitive to inhibition than testis or liver samples, and testis was more sensitive than liver. Protein synthesis was essentially unaffected at these concentrations and times of exposure.

Hydroxyurea was used in this study as a control drug that is thought to inhibit DNA synthesis specifically (Calabresi and Chabner, 1990). In this study it has been found to inhibit DNA synthesis with very little effect on protein synthesis, in testis and spleen samples (Fig. 2B). Again a difference in sensitivity of the tissues to inhibition of DNA synthesis was observed. Spleen slices were more sensitive than testis samples with IC₅₀ values of 0.04 and 0.1 mM, respectively. Liver slices were highly insensitive to inhibition of DNA synthesis with 15.6 ± 5.7% inhibition observed at 0.3 mM. Higher doses (1–20 mM) no longer specifically inhibited DNA synthesis (protein synthesis was equally inhibited) suggesting that cytotoxic effects are coming into play at these doses (data not shown).

In both the testis and the spleen samples, hydroxyurea is approximately fivefold more potent than paracetamol at inhibiting DNA synthesis. In liver slices, hydroxyurea does not specifically inhibit DNA synthesis, whereas paracetamol is specific even at doses up to 10 mM with no change in viability after one hour of incubation.

Table 1 shows the dose-related effects of (+)-catechin, a naturally-occurring flavonoid, on DNA and protein synthesis in the three tissues studied. In spleen and testis samples, DNA synthesis was specifically inhibited in a dose-dependent manner. In liver slices, DNA synthesis was inhibited at 0.5 mM by 12.2 ± 2.0% with little effect on protein synthesis. However, at 1 and 2 mM, inhibition of protein synthesis was also seen, but to a lesser extent than DNA synthesis inhibition. The differences observed in the sensitivity of the various tissues to the inhibitory effects of (+)-catechin on DNA synthesis were not as great as for paracetamol and hydroxyurea.
Table 2 shows the dose-related effects of n-propyl gallate, a synthetic antioxidant, on DNA and protein synthesis in the three tissues studied. In testis tubules, DNA synthesis was inhibited at 0.1 mM, but from 0.3 to 3 mM this inhibition became increasingly non-specific as protein synthesis was increasingly inhibited as well. At 3 mM, n-propyl gallate was probably cytotoxic as both DNA and protein synthesis were almost maximally inhibited. In spleen slices, a dose-dependent specific inhibition of DNA synthesis was observed. In liver slices, DNA synthesis did not appear to be specifically inhibited and was much more resistant to inhibition.

Following these results, testis was chosen to screen candidate phenolic compounds and flavonoids for preferential inhibition of DNA synthesis (Fig. 3Table 3). For comparison, phenolic paracetamol analogues which have also been found to inhibit replicative DNA synthesis in V79 cells (Richard et al., 1991) were included in this study. p-aminophenol, m-aminophenol and p-cresol showed a highly specific inhibition of DNA synthesis (Fig. 3A–C) with very little protein synthesis inhibition. p-aminophenol was an order of magnitude more potent than m-aminophenol and p-cresol with approximate IC$_{50}$ values of 0.02, 0.29, 0.19 mM, respectively.

At low concentrations (0.03–0.1 mM), NDGA inhibited DNA synthesis with very little effect on protein synthesis. However, at doses of >0.1 mM, there was an increasing level of protein

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% inhibition of synthesis</th>
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<tbody>
<tr>
<td></td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Testis</td>
<td>DNA</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Spleen</td>
<td>DNA</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Liver</td>
<td>DNA</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
</tr>
</tbody>
</table>

$^a$ Values represent mean ± SEM of duplicate samples from n separate animals. Incubation conditions are the same as in Fig. 2. Controls were duplicate samples taken from the same animal and incubated in Ringer in the absence of drug.

$^b$ Significantly different from control, P<0.05 (one-way ANOVA with Bonferroni correction).

Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% inhibition of synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.03 mM</td>
</tr>
<tr>
<td>Testis</td>
<td>DNA</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Spleen</td>
<td>DNA</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Liver</td>
<td>DNA</td>
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<tr>
<td></td>
<td>Protein</td>
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</tbody>
</table>

$^a$ Values represent mean ± SEM of duplicate samples from n separate animals. Incubation conditions are the same as in Fig. 2. Controls were duplicate samples taken from the same animal and incubated in Ringer in the absence of drug.

$^b$ Significantly different from control, P<0.05 (one-way ANOVA with Bonferroni correction).

$^c$ ND, not determined.
synthesis inhibition. Inhibition of DNA synthesis became non-specific at 1 mM (Fig. 3D).

The results for quercetin are less clear. Quercetin shows a small degree of preferential inhibition of DNA synthesis but this was accompanied by significant protein synthesis inhibition (Fig. 3E).

Table 3 shows the results for drugs that did not inhibit DNA synthesis specifically. Caffeic acid did not display any specific inhibition of DNA synthesis and at 1 mM caused only a small cytotoxic effect. Naringenin also did not display any specific inhibition of DNA synthesis and caused significant cytotoxicity at 1 mM. Calcium ethylenediaminetetraacetic acid (CaEDTA) had very little effect on DNA and protein synthesis. Trolox (a water-soluble vitamin E analogue) also had little effect on DNA or protein synthesis, even at high doses (1–2 mM).

Cycloheximide inhibited liver slice protein synthesis without effects on DNA synthesis. In spleen and testes samples, cycloheximide displayed a certain degree of preferential inhibition of protein synthesis. However, this was also accompanied by significant DNA synthesis inhibition (Table 4).
4. Discussion

The present in vitro experimental model can show specific inhibition of DNA synthesis without interference with protein synthesis, and by inference, without block to ATP synthesis, or cytotoxic effects. The results for paracetamol are a typical example of this. However, if DNA synthesis is not inhibited, this may be due to failure of the compound being tested to penetrate cells, while simultaneous inhibition of DNA and protein synthesis only suggests cytotoxic effects and gives no information about the specific DNA synthesis pathways that are affected.

A limitation of this method is that the underlying mechanism of DNA or protein synthesis inhibition cannot be elucidated. While the inhibition of DNA synthesis by paracetamol is mainly attributable to inhibition of ribonucleotide reductase through the radical quenching property of the parent compound, it is not known if other properties affecting signalling pathways might play a role in the inhibition of DNA synthesis. This is also true for the other compounds, especially the flavonoids.

We have demonstrated that paracetamol specifically inhibits DNA synthesis in a dose-dependent manner in vitro. Full concentration-response

<table>
<thead>
<tr>
<th>Drug</th>
<th>% inhibition of synthesis</th>
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<tr>
<td>0.3 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>2 mM</td>
<td>n</td>
</tr>
<tr>
<td>Caffeic acid DNA</td>
<td>2.7 ± 1.6 4</td>
</tr>
<tr>
<td>Protein     0.3 ± 0.3 4</td>
<td>13.7 ± 4.6 4</td>
</tr>
<tr>
<td>Naringenin DNA</td>
<td>5.9 ± 2.9 4</td>
</tr>
<tr>
<td>Protein     10.7 ± 2.8 4</td>
<td>54.8 ± 7.2&lt;sup&gt;b&lt;/sup&gt; 4</td>
</tr>
<tr>
<td>CaEDTA DNA</td>
<td>6.0 ± 2.5 4</td>
</tr>
<tr>
<td>Protein     4.0 ± 2.3 4</td>
<td>5.6 ± 2.8 4</td>
</tr>
<tr>
<td>Trolox DNA ND&lt;sup&gt;c&lt;/sup&gt; 3.4 ± 1.7 4</td>
<td>6.6 ± 3.8 4</td>
</tr>
<tr>
<td>Protein     ND 5.3 ± 3.1 4</td>
<td>12.1 ± 2.3 4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent mean ± SEM of duplicate samples from <i>n</i> separate animals. Incubation conditions are the same as in Fig. 2. Controls were duplicate samples taken from the same animal and incubated in Ringer in the absence of drug.

<sup>b</sup> Significantly different from control, <i>P</i> <0.05 (one-way ANOVA with Bonferroni correction).

<sup>c</sup> ND, not determined.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% inhibition of synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 μM</td>
<td>0.1 μM</td>
</tr>
<tr>
<td>0.2 μM</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>1 μM</td>
<td>n</td>
</tr>
<tr>
<td>Testis DNA 10.8 ± 6.1 4</td>
<td>12.6 ± 6.0 5</td>
</tr>
<tr>
<td>Protein 18.4 ± 7.1 4</td>
<td>23.4 ± 7.7&lt;sup&gt;b&lt;/sup&gt; 5</td>
</tr>
<tr>
<td>Spleen DNA 1.1 ± 1.1 4</td>
<td>2.0 ± 1.4 4</td>
</tr>
<tr>
<td>Protein 18.4 ± 7.1 4</td>
<td>20.2 ± 8.1 4</td>
</tr>
<tr>
<td>Liver DNA ND&lt;sup&gt;c&lt;/sup&gt; 0.8 ± 0.8 4</td>
<td>0.1 ± 0.1 5</td>
</tr>
<tr>
<td>Protein ND 26.3 ± 9.2&lt;sup&gt;b&lt;/sup&gt; 4</td>
<td>47.5 ± 5.0&lt;sup&gt;b&lt;/sup&gt; 5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent mean ± SEM of duplicate samples from <i>n</i> separate animals. Incubation conditions are the same as in Fig. 2. Controls were duplicate samples taken from the same animal and incubated in Ringer in the absence of drug.

<sup>b</sup> Significantly different from control, <i>P</i> <0.05 (one-way ANOVA with Bonferroni correction).

<sup>c</sup> ND, not determined.
curves have been obtained in rat liver, spleen and testis samples demonstrating that as well as a specific inhibition of DNA synthesis there is also a variation in the sensitivity of the different tissues. The order of least sensitive to most sensitive tissue is liver < testis < spleen with IC_{50} values of 1.4, 0.49, 0.23 mM, respectively. The difference in sensitivity between tissues was also observed with hydroxyurea with a similar order of sensitivity to inhibition, that is, liver < testis < spleen.

The inhibition of DNA synthesis by paracetamol has been proposed as one of the mechanisms by which paracetamol might cause genotoxicity. It has been demonstrated by Hongslo et al. (1989, 1990) that paracetamol specifically inhibits replicative DNA synthesis at close to the therapeutic level of 0.1 mM. However, these studies were conducted in cell lines and are difficult to relate to the possible in vivo effects. A previous in vivo study in our laboratory has also shown differential inhibition of DNA synthesis in rat tissues (Lister and McLean, 1997). However, the lack of concentration-response data did not enable us to determine to what degree DNA synthesis might be inhibited at different concentrations. The present study has demonstrated that very little inhibition of DNA synthesis would be expected in vivo at therapeutic doses. However, in overdose, inhibition of DNA synthesis may play a significant role in the mechanism of injury.

It is not known why there is a differential inhibition of DNA synthesis between tissues. One possible explanation is that there are variations in the size of the deoxyribonucleotide (dNTP) pools. The rate of DNA synthesis is highly dependent on the levels of dNTP's within the cell and studies have shown that different tissues have varying levels of dNTP's (Schneider, 1955).

The control rates of thymidine incorporation varied greatly between tissues. Spleen slices had the highest incorporation rate, testis samples had an intermediate rate whilst liver slices had the lowest rate. In spleen and testes samples, overall DNA synthesis is occurring at a faster rate because a large number of cells in the tissue are in S-phase. It appears that in liver slices, a low level of DNA synthesis is occurring presumably because less than 1 in 1000 cells is likely to be in cycle. In liver slices, even if ribonucleotide reductase activity was completely inhibited, the large pool of dNTP's in the liver slice could support further DNA synthesis if those cells not in S-phase can 'feed' other liver cells with dNTP's via gap junctions.

A major reason for the resistance of liver slices to inhibition of DNA synthesis by all the compounds studied is the liver's ability to metabolise many foreign compounds. The intracellular concentrations of these agents may be much lower in the liver slices than in the incubation medium, leading to a reduced level of inhibition. In particular, flavonoids are rapidly metabolised in vivo, by the sulphation and glucuronidation pathways found predominantly in the liver (Hackett, 1986).

It must be emphasised that the inhibition of DNA synthesis by paracetamol is not due to cell injury caused by the parent compound or its metabolites as liver slices from normal rats, not induced with phenobarbitone, are resistant to cell injury for many hours even at 2 or 10 mM concentrations of paracetamol (Miller et al., 1993; Martin and McLean, 1995). The lack of inhibition of protein synthesis is also an indicator of the good health of the cells. Protein synthesis requires normal ATP and GTP synthesis and this can only occur if mitochondrial function is normal. Inhibition of protein synthesis as well as DNA synthesis would indicate that the compound is causing a general cytotoxic effect rather than a specific effect on DNA.

The results for paracetamol and hydroxyurea demonstrated that the in vitro experimental model used in this study is a useful method for studying potential inhibitors of DNA synthesis. Utilising the same model we have also demonstrated that a range of phenolic compounds can inhibit DNA synthesis specifically. (+)-catechin, m-aminophenol, p-aminophenol, p-cresol all displayed a highly specific inhibition of DNA synthesis with surprisingly little inhibition of protein synthesis. NDGA displayed specific inhibition of DNA synthesis to a certain degree but this effect was overtaken by the cytotoxic effects of this antioxidant at higher doses. n-propyl gallate displayed specific inhibition of DNA synthesis in spleen slices and to a certain extent in testis samples.
Again, liver slices were highly resistant to inhibition. Possible explanations for this difference in sensitivity have been discussed previously. These results lead us to conclude that certain phenolic antioxidants can inhibit DNA synthesis specifically; possibly through inhibition of ribonucleotide reductase.

Although the compounds tested were selected on the basis of their antioxidant activity, they also have other properties which may contribute to the inhibition of DNA synthesis. Flavonoids in particular are known to have multiple biological and pharmacological activities (Rice-Evans et al., 1996).

As the results in Table 3 show, not all phenolic antioxidants were effective inhibitors of DNA synthesis. Caffeic acid and naringenin did not display any specific inhibition of DNA synthesis. In an attempt to determine if any drug with antioxidant properties could inhibit DNA synthesis, Trolox, a vitamin E analogue, was tested. Trolox is a highly effective antioxidant that mainly acts by protecting cellular lipids from lipid peroxidation. In this study, Trolox had little effect on DNA or protein synthesis, even at the high doses tested (1–2 mM).

A possible explanation of the non-specificity of action by the various phenolic antioxidants is that many antioxidants can also display pro-oxidant properties and so can cause cell injury under different circumstances (Stadler et al., 1995; Cao et al., 1997). It is often found that at low doses these drugs have antioxidant properties, but at high doses they also have pro-oxidant properties.

The mixed results obtained for quercetin were unexpected as quercetin is considered to be a highly effective antioxidant. However, it has been demonstrated that quercetin has pro-oxidant as well as antioxidant properties (Laughton et al., 1989). This is thought to be the cause of mutagenic and genotoxic effects in various in vitro models of genotoxicity and mutagenicity and could be the cause of the non-specific effects shown in this model (IARC, 1983).

It has been demonstrated by Hue et al. (1986) that rat liver slices are impermeable to CaEDTA, a highly effective metal chelator. The absence of any substantial effect of CaEDTA on DNA and protein synthesis in this study (Table 3) further shows that this in vitro model reflects the in vivo situation, and that permeability barriers were intact.

The existence of permeability barriers in the slice system means that antioxidants that are not inhibitors of DNA synthesis in the slice might still be inhibitors of ribonucleotide reductase in an isolated enzyme system. However, the slice system brings results closer to the in vivo situation and the assessment of whether compounds are likely to interfere with DNA synthesis in vivo.

Acknowledgements

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References


