THE USE OF ISOLATED HEPATOCYTES TO STUDY
THE TOXIC EFFECTS OF CHEMICALS

by

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A thesis submitted in accordance with the
requirements of the University of Surrey
for the degree of Doctor of Philosophy.

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November 1981.
To Jim and Dorothy
ACKNOWLEDGEMENTS

The completion of this thesis would not have been achieved without the dedicated supervision of Professor James W. Bridges. There are many others, too numerous to mention, who have contributed by their practical assistance, expert advice and encouragement.

I would like to thank particularly Dr. Jeffrey Fry, Dr. Carol Jones and Dr. Philip Wiebkin, with whom I worked so closely, for passing on their valuable experience in the use of isolated hepatocytes. I would also like to thank Dr. Tim Fennel, Dr. Richard Hinton and Dr. Diane Benford for their assistance in some of the specialised techniques used here.

I am also extremely grateful to my friends and colleagues at the D.H.S.S. and Smith Kline and French whose continued encouragement has helped me complete this manuscript. In particular I would like to mention Dr. Susan Sullman, Dr. Peter Johnson and Dr. Paul Sharpe.

Finally I am indebted to Mrs Jan Hamilton whom with extreme care and unending patience typed this manuscript and to the Medical Research Council for financial support.
ABSTRACT

Some characteristics of rat hepatocytes, isolated by a non-perfusion technique, have been examined in order to validate their use for the investigation of chemically induced toxicity.

Untreated hepatocytes synthesized protein, RNA and GSH and metabolised exogenous substrates. GSH was depleted during isolation but active resynthesis was evident on incubation in complete culture medium. The levels of GSH, ATP and cyclic AMP were similar to those reported in the literature for hepatocytes isolated by perfusion. Hence the hepatocytes were shown to possess a number of in vivo characteristics. Although there was an inherent variability between individual preparations characterised by the variability in, and lack of correlation between, the rate of protein synthesis, cell yield and viability, the effect of a number of xenobiotics were investigated.

Paracetamol induced a rapid fall in ATP, dose-dependent inhibition of protein, RNA synthesis, and the metabolism of 7-EC, depletion of GSH and covalent binding of drug related material to protein. Exposure of cultured hepatocytes to 40mM paracetamol for < 4 hrs produced reversible inhibition of protein synthesis, but cell death was the result of a longer exposure.

Safrole and phenobarbitone also induced a rapid fall in ATP levels followed by dose-dependent inhibition of protein and RNA synthesis. This was, therefore, a common sequence of events in response to a toxic insult but was unlikely to be the direct cause of cell death.

The activities of 7-EC O-deethylase, NADPH₂ diaphorase, SDH and G6PDH were all increased in cultures treated with 2mM phenobarbitone (a concentration which did not cause cell death). Paracetamol also caused increases in NADPH₂ diaphorase activity.

The effects of these xenobiotics in hepatocytes were compared and discussed. It was concluded that the cause of cell death was not identified and that it was unlikely to be due to one factor alone but to
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BSP</td>
<td>sulphobromophthalein</td>
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<td>DNA</td>
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<tr>
<td>NADPH</td>
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<tr>
<td>PAPS</td>
<td>3-phosphoadenosine 5'-phosphosulphate</td>
</tr>
<tr>
<td>PBS'A'</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PGK</td>
<td>3-phosphoglycerate kinase</td>
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<tr>
<td>PMS</td>
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CHAPTER 1

INTRODUCTION
INTRODUCTION

1.1 GENERAL BACKGROUND
The evolution of society to the sophistications of modern day life has been made possible by the numerous discoveries in the field of science since the dawn of mankind. Development of fuels and machinery has facilitated the foundation of an industrially based society in which life expectancy has been substantially increased. The population explosion has necessitated the processing of foods to reduce wastage and to enable more mouths to be fed. The invention of the steam and combustion engines has improved means of transportation and communication. In more recent years the advancement of medical science has provided many new drugs to combat disease in humans and animals.

Scientists have, therefore, provided much to improve the quality of life. However, they also realise that they have introduced hazards, many of which are still unrecognised. For example, the industrial revolution began the large scale pollution of the environment. Food processing requires the use of unnatural additives to stabilise, colour and flavour products to improve their quality, appearance and storage life. It is now recognised that many of these agents can cause damage to humans and animals especially if the duration of exposure covers a lifetime. In the race to provide new and more effective drugs mistakes have been made. The appalling effects of thalidomide, which caused congenital deformities in children born to mothers who had taken the drug during pregnancy, hastened the advancement of the science of toxicology in the United Kingdom.

1.2 INTRODUCTION TO TOXICOLOGY
The fundamental basis of toxicology was recognised many centuries ago by Paracelsus (1493-1541) who observed that "All things are poisons, for there is nothing without poisonous qualities. It is only dose which makes a thing a poison" (cited by Sigerist, 1958). Since the end of the nineteenth century there has been a vast increase in the number and availability of effective and potentially toxic drugs.
Advances in organic chemistry have enabled the synthesis of numerous new therapeutic agents, many by manipulation of existing structures. Increased usage of drugs has inevitably led to the observation of more and varied toxic reactions. These reactions can manifest themselves in a number of ways which may be categorised as follows: dose related, idiosyncratic, allergic, tolerated, physical dependency, carcinogenic, mutagenic and teratogenic.

Dose-related toxicity may be exemplified by the action of the coumarin anticoagulants which are used to prolong the clotting time of blood. Excessive dosing will lead to a tendency to bleed due to the prolonged clotting time.

Idiosyncracy, which is defined as a characteristic distinguishing an individual, may be demonstrated by the differing responses to isoniazid which is used in the chemotherapy of tuberculosis. The major route of isoniazid metabolism is via N-acetylation (Jenne, 1965) catalysed by an acetyl transferase enzyme. Epidemiological studies have found groups of people who are slow acetylators (La Du, 1972). A good example of this is found in Eskimos who therefore detoxify the drug less quickly so that it can accumulate to toxic levels within the body.

Drug allergy is an adverse reaction which is the result of previous sensitisation to the same drug, or a closely related one. The most dangerous of these types of adverse reactions is the onset of anaphylaxis which can lead to death in a very short time. One well known drug which will cause this type of reaction in some patients is penicillin (Schwartz and Vaughan, 1963).

Repeated dosing of drugs can cause two undesired side effects. The first of these is tolerance which is a decreased responsiveness to the action of a drug. This type of effect is demonstrated by sympathomimetic amines which, with repeated dosing, elicit smaller and smaller cardiovascular effects. A classic example of this is the experiments done with ephedrine in the dog (Chen and Meek, 1926).
The second undesired effect of repeated dosing is that of physical dependence where the presence of the drug becomes necessary for the normal functioning of the body. The withdrawal symptoms suffered by a person addicted to morphine are a striking example of physical dependence (Andrews and Himmelsbach, 1944).

Carcinogenesis is the development of cancer which is the malignant unrestrained proliferation of somatic cells. Chemical carcinogenesis was discovered in man long before it could be demonstrated in animals. In 1775 cancer of the scrotum was recognised as an occupational disease of chimney sweeps and it was the prolonged exposure to soot that was presumed to be the cause (Wolf, 1952). It has since been realised that the agents involved are the polycyclic hydrocarbons. Much research is now dedicated to elucidating the mechanisms by which cancer is produced.

Any stimulus which causes a permanent change in the genotype which could cause adverse effects in future generations is classified as a mutagen. One of the best examples of this is radiation. Although the serious implications of exposure to mutagens are understood the methods for assessing whether or not a chemical is mutagenic in vivo are still in their relative infancy.

The classic example of a teratogen is thalidomide which is known to cause abnormalities in foetal development (Williams, 1963). Teratogens may act in a number of ways, many of which may not be apparent due to the early abortion of the foetus. The discovery of the dreadful effects of the drug thalidomide shocked the world and has caused governments to lay down strict legislation concerning the production and marketing of new drugs. This has led to extensive testing of drugs in animals to determine any adverse effects to enable the safety of a drug in humans to be more accurately predicted.

This brief summary demonstrates the broad implications of the study of toxicology and suggests some of the many problems in predicting whether or not a drug or chemical exposed to humans or animals will be safe. A further realisation is that it is not only important to know if an agent is toxic, but also how it causes toxicity.
Approaches to the study of mechanisms of toxicity have been many and varied. One of the main areas is the investigation of effects of drugs on the liver. When a drug or chemical is exposed to the body, whether orally, injected, inhaled or applied topically once it enters the blood stream it very rapidly reaches the liver. One main function of the liver is the breakdown of exogenous compounds by metabolism to more water soluble conjugates which may then be excreted, mainly by the kidney. The liver is therefore subjected to potentially toxic chemicals, many of which manifest their symptoms within the liver, although others will be observed in other organs of the body. The liver as a metabolising organ may also produce toxic metabolites from relatively innocuous parent compounds. The importance of active metabolites in relation to toxicity will be discussed later.

The investigation of the mechanisms of toxicity of chemicals in the liver is therefore of great importance and will hopefully give a greater understanding of the overall toxicity of a compound. It may also help to provide information for the production of less hazardous chemicals and drugs in the future.

1.3 THE STRUCTURE OF THE LIVER

The structure of the liver has been classically described in terms of the hexagonal lobule (see Fig.1). This functional unit contains the terminal hepatic venule at its centre, with the portal space, hepatic arteriole and bile duct situated at the periphery of the lobule. According to this configuration pathological lesions of the liver have been classified as centrilobular, midzonal or periportal.

More recent evidence has indicated that this is not a true description of the functional unit of the liver (Rappaport, 1969). If coloured gelatin is injected into the portal vein or hepatic artery it becomes apparent that the terminal afferent vessels supply blood to only sections of the hepatic lobule. These sectors have been demonstrated to lie around the terminal portal branches and extend from the central vein of one hexagon to the central vein of an adjacent hexagon. These
Schematic representation of the classic hexagonal hepatic lobule (from Plaa, 1975).
PS is the portal space, consisting of a branch of the portal vein, a hepatic arteriole, and a bile duct; THV is the terminal hepatic venule (central vein).
Functional units have been defined by Rappaport and co-workers as liver acini (Rappaport, 1969) (see Fig. 2). The simple acinus consists of a small parenchymal mass which is irregular in size and shape, arranged around an axis consisting of a terminal portal venule, an hepatic arteriole, a bile ductule, lymph vessels and nerves. This acinus lies between two or more terminal hepatic venules (central veins) with which its vascular and biliary axis interdigitate. The hepatic cells of a simple acini are in cellular and sinusoidal contact with the cells of adjacent or overlapping acini. However, the cells of one particular acinus are preferentially supplied by their parent vessels. This concept has been developed to show that there are circulatory zones within each acinus which can be divided into three depending on their distance from the terminal vascular branch. It has been demonstrated that three or more simple acini can constitute a complex acinus. This unit consists of three simple units and a sleeve of parenchyma around the preterminal afferent vessels, the lymph vessels and the nerves that eventually give rise to the terminal axial vessels of the simple acini.

It has been demonstrated that there is a lack of uniformity of function of the liver cells (Chayen, 1974). The activity of the respiratory enzymes has been found to be highest in the zone closest to the terminal afferent vessel, whereas the most distant zone has a high level of microsomal enzymes. The classical descriptions of focal, midzonal, periportal and centrilobular lesions can be explained by Rappaport's theories. Centrilobular necrosis, a lesion caused by carbon tetrachloride (Recknagel, 1967), occurs in the region most distant from the terminal portal venule (zone 3). Midzonal necrosis, similar to that caused by beryllium (Witschi and Aldridge, 1967), would occur in the area corresponding to the acinar zone closest to the terminal afferent vessel (zone 1). A classic example of a compound producing periportal necrosis is allyl alcohol (Rouiller, 1964).

Morphologically chemically-induced liver injury can manifest itself in different ways. The acute effects can consist of accumulation of lipids (fatty liver) and the appearance of degenerative processes leading to cell death (necrosis). The necrotic process can affect
Schematic representation of a simple hepatic acinus (Rappaport, 1969)

PS and THV are as in Fig. 1. 1, 2 and 3 represent the various zones draining off the terminal afferent vessel.
small groups of isolated parenchymal cells (focal necrosis),
groups of cells located in zones (centrilobular, midzonal, or periportal
necrosis) or virtually all of the cells within a hepatic lobule (massive
necrosis).

Chemical-induced liver injury resulting from chronic exposure can
produce marked alterations of the entire liver structure giving rise
to the degenerative and proliferative changes observed in the
different forms of cirrhosis. Neoplastic changes may be another end
point of chemical liver injury.

1.4 MECHANISMS OF TOXIC LIVER INJURY
The study of pathology deals mainly with the structured response to
disease. The toxicity of any compound is, however, the sum total of
its interactions with cellular constituents to produce chemical
alterations and the response of the cell to those aberrations. It is
therefore important to determine the biochemical lesion which occurs
within the cell. The concept of the biochemical lesion was formulated
by Peters (1963), who together with others have laid the solid modern
foundation for biochemical pathology.

Two approaches have developed within the field of toxicology:
(a) the response of cells or organs to interference with
selected metabolic processes, i.e. the response to known
biochemical lesions - ATP deficiency, inhibition of
protein synthesis, RNA and DNA synthesis.
(b) the biochemical mechanisms underlying the induction of
discrete and selective biological responses to some toxic
agents such as selected experimental disease, e.g. carbon
tetrachloride, galactosamine and ethionine.

A wide variety of toxic chemicals, viruses and other hazards in the
cells environment such as anoxia, are known to induce hepatic cell death.
It has also become clear that persistent or repeated episodes of liver
cell necrosis seems to be the basis for the chronic progression of liver
disease to cirrhosis.
Magee (1966) made the following observations on the approach to toxic liver cell necrosis based on research carried out up to 1966. It would appear that the mechanisms of cell death are quite specific and studies have shown that one can interfere with most of the basic properties of the cell without inducing cell death. Cell death is not a degenerative phenomenon in which the essential lesion is some inhibiting effect on one of the essential metabolic processes in the cell. Neither is it simply the consequence of slowing down the cells metabolic activity as a result of the interference with one or more of its major metabolic functions. Hence any biochemical change in liver cells in response to agents that do not produce necrosis must be by itself insufficient to produce cell death and necrosis.

There have been three major hypotheses as to the primary target for the molecular mechanisms that produce cell death. Historically the first of these was the mitochondrial hypothesis of Christie and Judah (1954) based on experiments with carbon tetrachloride which was shown to cause severe mitochondrial damage. This involved the uncoupling of oxidative phosphorylation, loss of respiratory control and the activation of ATPase. It is now known, however, that this is not causative of cell death as the cells have already shown disruption of intracellular membranes, inhibition of protein and RNA synthesis, and an increase in cellular levels of triglyceride. The hepatotoxicity of carbon tetrachloride was extensively reviewed by Recknagel (1967) and Plaa and Witschi (1976). Mitochondrial injury is therefore simply a manifestation of the general metabolic deterioration of a dying cell.

The second of the major hypotheses was the lysosomal theory suggested by de Duve after his discovery of lysosomes in 1955. He implied that lysosomes could be potential suicide bags, knowing that they contain soluble hydrolases, with an acid pH optimum, surrounded by a lipoprotein membrane preventing their escape. De Duve (1963) suggested that the normal segregation of the lysosomal enzymes might have played an essential role in keeping the lytic processes of the cell localised and therefore protecting against autolysis. This theory raised a major question as to whether the release of lysosomal enzymes causes cell death, or only occurs when the cell is dead or
dying? Or one could ask whether cell death is simply autolysis? However, it has been shown that acute liver cell injury by carbon tetrachloride (Dianzani, 1963), thioacetamide (Slater et al., 1965), dimethylnitrosamine (Rees, 1962) and beryllium salts (Witschi and Aldridge, 1967) gave no evidence implicating lysosomal disruption as a factor in the cause of cell death. Lysosomes in fact appeared to be relatively stable particles during the development of cell death. The changes in the distribution of the lysosomal hydrolases between the particulate and soluble phases of the cell that occur late after the administration of hepatotoxins probably only reflects a scavenging role for the lysosomes.

The third theory indicates the plasma membrane as the primary target in the pathogenesis of cell death. A very early effect of carbon tetrachloride toxicity is a destructive attack on the membrane components of the cell. This is thought to involve homolytic cleavage of a carbon-chloride bond followed by free radical attack with peroxidative decomposition of the membrane structured lipids (Recknagel, 1967). Within the first hour of exposure changes within the endoplasmic reticulum are evident and the calcium content of the cell is increased within twenty four hours. Associated with the raised levels of calcium are other electrolyte disturbances, swelling of the liver cells, with intracellular enzymes appearing in the plasma. These changes suggest a severe breakdown of the selective permeability of the cell plasma membrane. Electron microscopic studies of liver cells poisoned with dimethylnitrosamine (Emmelot and Benedetti, 1960) and thioacetamide (Rees et al., 1961) also demonstrated alterations in the membranes of the endoplasmic reticulum. Plasma membranes were found to be abnormal as early as three hours after the administration of dimethylnitrosamine.

It has been known for many years that calcium accumulates in necrotic tissue. Judah et al (1964) suggested that the specific functional consequence of direct membrane injury is an increase in calcium ion concentration within the cell and that the calcium ion is the common agent mediating toxic liver cell death. This view is supported by results demonstrating the protective effect of promethazine and related compounds on the toxicity of carbon tetrachloride (Rees and Spector, 1961).
All of the above observations have the same problem to face. How does one decide if any one given specific disturbance is causally related to cell death, or simply a manifestation of the fact that the cell is irreversibly injured, dead or dying, as this implicates that all metabolism and cellular functions are going to be perturbed and eventually cease?

Research into the mechanisms of hepatic necrosis has centred around a few model compounds including carbon tetrachloride, galactosamine and ethionine. By understanding the mechanisms of toxicity of one compound in its minutest detail it is hoped that the mechanism of action of other compounds might be more easily understood.

The hepatotoxicity of ethionine has been briefly reviewed by Farber (1971). It is known to produce a variety of pathological changes in animals including fatty liver and liver cancer (Stekol, 1963 and Farber, 1967). This methionine analogue has at least four metabolic effects on the liver:

1. rapid induction of ATP depletion (Shull, 1962).
2. ethylation of RNA, of highly selective proteins, especially nuclear proteins, and of several other nuclear proteins.
3. inhibition of methylation of RNA, and other components utilizing methionine as donor.
4. incorporation into protein in place of methionine.

The evidence strongly suggests that most of the acute hepatic effects of ethionine are due predominantly or exclusively to the deficiency of ATP. These include fatty liver (Farber, 1967), inhibition of protein synthesis (Villa-Trevino et al, 1963), a number of ultrastructural alterations to cytoplasmic organelles of the liver cell and nucleolus and inhibition of RNA synthesis (Villa-Trevino et al, 1966). It has been suggested that the ratio of ATP to ADP to AMP is probably more important to cellular function than the absolute value of any single adenine nucleotide (Atkinson, 1966). The inhibition of protein synthesis and RNA synthesis closely parallel the decrease in ATP levels although the mechanism by which this occurs is not known. Ethionine also causes a striking loss of liver potassium with a corresponding increase in
sodium levels (Judah et al., 1966 and Christie and Judah, 1968) but no apparent effect on hepatic BSP uptake, conjugation with GSH, or excretion as free or conjugated BSP in the bile (Schenker and Combes, 1967).

The effects of galactosamine on the liver have been reviewed by Farber and El-Mofty (1975). Figure 3 shows the proposed pathway leading to cell death as caused by galactosamine. Its major initial effect is to deplete the uridine nucleotides by its metabolism to UDP hexosamine which traps the uridine nucleotides. This leads to reversible plasma membrane injury and increased intracellular calcium. At higher dose levels of galactosamine the plasma membrane becomes irreversibly damaged, followed by a marked increase in intracellular calcium and cell death.

The experiments described above with ethionine and galactosamine demonstrate that the depletion of ATP and UTP respectively do not cause cellular necrosis on their own, as these changes may be reversible.

Protein synthesis, and to a lesser extent, RNA synthesis are inhibited by a wide range of toxic agents. Alterations in the subcellular organisation and function of the rat liver parenchyma have been attributed to this inhibition (Trump and Ericsson, 1965). It has been difficult to correlate structural changes to a specific biochemical lesion, as the majority of compounds have many biochemical effects in addition to the inhibition of protein synthesis. It is also of interest that marked inhibition of protein synthesis by cycloheximide in rats for many hours leads to only a few cellular changes in selected organs and its effects are reversible (Harris et al., 1968).

The inhibition of protein synthesis also appears to be associated with several types of fatty liver, e.g. carbon tetrachloride, ethionine, and puromycin (Farber, 1967; and Lombardi, 1965 and 1966). The liver plays a major role in the conversion of fatty acids from the blood to triglycerides which are put back as plasma lipoproteins into the blood. If protein synthesis is inhibited, the supply of essential protein is halted causing an accumulation of triglyceride within the
Figure 3.

Tentative scheme for the sequence of events in the development of liver cell necrosis induced by galactosamine (Farber and El-Mofty, 1975)
liver. This, however, does not always occur, for cycloheximide (Verbin et al., 1969) and ethionine (Schlunk et al., 1968) both inhibit protein synthesis without an accompanying increase in triglyceride levels. The mechanisms must, therefore, be more complex perhaps involving changes in polysomes. Carbon tetrachloride causes disaggregation of polysomes to ribosomal monomers and subunits, whereas with cycloheximide and with ethionine (in the male rat) this does not occur. The bulk of triglyceride is synthesized in the endoplasmic reticulum. It may, therefore, follow that intact polysomes closely associated with the endoplasmic reticulum may allow a regulatory control of triglyceride synthesis. The main conclusion is that inhibition of protein synthesis alone is probably not the cause of cell death.

Attempts to associate cell death with inhibition of a specific reaction have also implicated RNA synthesis. Similar interpretation problems arise to those cited above. For example, α-amanitin, which induces severe lesions in several organs of experimental animals, inhibits RNA polymerase (Tata and Widnell, 1966). It causes cell death in the liver and kidney of the mouse, but its effects are reversible in rat liver.

1.5 PROTEIN BIOSYNTHESIS AND THE MODE OF ACTION OF SOME INHIBITORS
Proteins are synthesized by both eukaryotic and prokaryotic cells. Since this thesis is concerned with the effect of chemicals in eukaryotic organisms the emphasis of this brief summary is on eukaryotic protein synthesis although much of the work to elucidate the pathways has been carried out in prokaryotic organisms like E. coli. The pathways are, however, essentially similar in both types of cell.

The synthesis of proteins takes place on the surface of the ribosomes which in the eukaryotic cell occur either free in the cytoplasm or bound to the endoplasmic reticulum. Furthermore the mitochondria are rich in ribosomes. The types of protein synthesized at these different sites vary in their functions.
The first stage of protein synthesis requires the activation of amino acids, the subunits of proteins, to their corresponding aminoacyl-tRNAs. This stage requires ATP and Mg^{++} and the aminoacyl-tRNA synthetase specific for the amino acid undergoing activation. The aminoacyl-tRNA synthetases are highly specific for both the amino acid and its corresponding tRNA.

Errors can occur at this activation stage since certain amino acid analogues such as p-fluorophenylalanine, an analogue of phenylalanine, and ethionine and nor-leucine, analogues of methionine may be accepted by the aminoacyl-tRNA synthetase and hence be incorporated into proteins in the positions normally occupied by methionine or phenylalanine.

The second stage of protein synthesis involves the initiation of the polypeptide chain which occurs on the ribosomes. Ribosomes consist of RNA and proteins and can dissociate into two functional subunits. In extramitochondrial protein synthesis the initiating amino acid is methionine in the form of a specific methionyl-tRNA which responds to the initiating AUG codon in the mRNA. The formation of this initiation complex on the 40S subunit of the ribosome also requires initiation factors and GTP. The initiation complex then combines with the 60S subunit. The methionyl-tRNA is positioned at the initiation codon AUG so that the ribosomes start translation at the correct point on the mRNA.

The third stage of protein synthesis is the elongation of the polypeptide chain which occurs in three major steps. The first of these is the binding of the incoming aminoacyl-tRNA to the aminoacyl site on the 80S ribosome facilitated by the binding of an elongation factor and GTP to the aminoacyl-tRNA. This step can be inhibited by certain antibiotics, particularly the tetracyclines.

The second step in elongation concerns the formation of the peptide bond between the methionyl-tRNA and the aminoacyl-tRNA which is catalysed by peptidyl transferase and results in the displacement of tRNA from the peptidyl-tRNA at the peptidyl site.
The antibiotic puromycin can inhibit protein synthesis at this stage by becoming bound to the aminoacyl site resulting in the formation of a covalent peptidyl puromycin derivative. Since this derivative is not recognised by the mRNA elongation is halted and protein synthesis inhibited.

The third step in elongation of the polypeptide chain is translocation. The ribosome now moves to the next codon on the mRNA and shifts the newly formed peptidyl-tRNA from the aminoacyl to the peptidyl site on the ribosome leaving the aminoacyl site free to accept another aminoacyl-tRNA. This step requires a specific elongation factor as well as GTP and Mg++. In eukaryotic cells this elongation factor (EF2) can be inactivated by diphtheria toxin resulting in an inhibition of translocation.

The final stages of protein synthesis involve the termination of the polypeptide chain formation which is signalled by a special termination codon on the mRNA. Releasing factors then promote the release of the completed polypeptide chain from the ribosome. The completed polypeptide chain may then undergo covalent modification to yield its biologically active form.

Some other inhibitors of protein synthesis include chloramphenicol which blocks the peptidyl transfer reaction in mitochondrial protein synthesis. Cycloheximide blocks peptide bond formation in extramitochondrial protein synthesis. Both chloramphenicol and cycloheximide bind to the large ribosomal subunits of dissociated ribosomes.

Streptomycin on the other hand binds to the small ribosomal subunit of prokaryotic cells causing inhibition of protein synthesis and misreading of the genetic code on the mRNA. Other aminoglycoside antibiotics like the neomycins and kanomycins act in a similar fashion.

The alkaloid emetine inhibits the binding of aminoacyl-tRNAs. Abrin and ricin, which are toxic plant proteins, inhibit protein synthesis in eukaryotic cells by inactivating the 60S subunit of the ribosome and thus blocking elongation.
1.6 ACTIVE METABOLITES AND TOXICITY

One of the main functions of the liver is to metabolise drugs and foreign compounds to more polar compounds to facilitate their excretion. The main route of elimination is via the kidney, excretion via the biliary system, the intestine and the lungs being secondary to this route. During recent years research into drug metabolism has demonstrated that many foreign compounds are metabolised to potent alkylating, arylating or free radical intermediates which are in fact more toxic than their parent compounds. These reactive intermediates are very short lived and many exert their toxic action by binding irreversibly to tissue macromolecules. The lesions caused by this type of reaction are normally located in the part of the metabolising tissue which contains the enzyme pathways responsible for their metabolic activation. Many of the initial concepts of metabolic activation were developed during studies of chemical carcinogenesis (Miller and Miller, 1947, 1951 and 1966). It is now known that similar reactions occur with some drugs at high dosage levels (Gillette et al, 1974, Mitchell and Jollow, 1975). The reactive metabolites can lead to various toxicities including cellular necrosis, cancer, mutations, immunological reactions, blood dyscrasias and foetal damage. A few examples of these types of agents are given in Table 1, some will be discussed in more detail.

The hepatotoxicity of carbon tetrachloride has been extensively investigated (Recknagel, 1967). It was not until 1965-1966 that evidence that the metabolism of carbon tetrachloride was essential for its toxicity was published (Goshal and Recknagel, 1965; Slater, 1966; McLean and McLean, 1966). It was demonstrated in vitro that the carbon tetrachloride stimulation of lipid peroxidation in liver microsomes is dependent on a supply of NADPH, suggesting the involvement of the NADPH-cytochrome P450 complex. This was supported by the fact that phenobarbital induction of the complex greatly increased the toxicity of carbon tetrachloride (McLean and McLean, 1966). It has since been suggested that the most likely reactive product of metabolism is the trichloromethyl radical, CCl$_3^*$. Gordis (1969) showed that the CCl$_3^*$ group attaches covalently to lipids and proteins, the pattern produced by labelled CCl$_4$ in vivo being similar to that obtained after producing
### Table 1.

<table>
<thead>
<tr>
<th>Toxic Agent</th>
<th>References</th>
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<tbody>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Slater (1972)</td>
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<tr>
<td>CBrCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Koch &lt;em&gt;et al&lt;/em&gt; (1974)</td>
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<tr>
<td>Halothane</td>
<td>Burdino &lt;em&gt;et al&lt;/em&gt; (1973)</td>
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<td></td>
<td>Wood &lt;em&gt;et al&lt;/em&gt; (1976)</td>
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<tr>
<td>Dimethylnitrosamine</td>
<td>Magee &lt;em&gt;et al&lt;/em&gt; (1976)</td>
</tr>
<tr>
<td></td>
<td>Gravela &lt;em&gt;et al&lt;/em&gt; (1974)</td>
</tr>
<tr>
<td>Trichloroethylene</td>
<td>Van Duuren and Banerjee (1976)</td>
</tr>
<tr>
<td>Vinyl Chloride</td>
<td>Bolt &lt;em&gt;et al&lt;/em&gt; (1976)</td>
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<td></td>
<td>Kappus &lt;em&gt;et al&lt;/em&gt; (1976)</td>
</tr>
<tr>
<td>Carbon Disulphide</td>
<td>De Matteis and Seawright (1976)</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>Jollow &lt;em&gt;et al&lt;/em&gt; (1973)</td>
</tr>
<tr>
<td></td>
<td>Labadarios &lt;em&gt;et al&lt;/em&gt; (1977)</td>
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<tr>
<td>Aflatoxin</td>
<td>Patterson (1978)</td>
</tr>
<tr>
<td>N-acetylaminofluorene</td>
<td>Miller (1970)</td>
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<tr>
<td>Substituted azobenzenes</td>
<td>Kadlabar &lt;em&gt;et al&lt;/em&gt; (1976)</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Nelson &lt;em&gt;et al&lt;/em&gt; (1976)</td>
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<td>Nakatsugawa and Dahm (1967)</td>
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<tr>
<td>Parathion</td>
<td>Ptashne &lt;em&gt;et al&lt;/em&gt; (1971)</td>
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<td></td>
<td>Poore and Neale (1972)</td>
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<tr>
<td>Phenylthiourea</td>
<td>De Matteis and Seawright (1976)</td>
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<tr>
<td>α-naphthylisothiocyanate</td>
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Examples of toxic agents that require metabolic activation in the liver in order to exert their full potential as hepatotoxins (Slater, 1978).
the CCl$_3^*$ radical in vitro in the presence of liver microsomes. Studies by Uehleke et al (1973) and Recknagel et al (1976) have shown that covalent binding by CCl$_3^*$ occurs anaerobically as well as aerobically, but lipid peroxidation (which is a consequence of CCl$_3^*$ production) only occurs in the presence of oxygen. Associated with the lipid peroxidation is the liberation of biologically reactive breakdown products, some of which cause increases in capillary permeability and platelet aggregation (Ugazio et al., 1976). It can be seen from the above effects that the metabolic activation of carbon tetrachloride to the trichloromethyl radical is vital to the production of its hepatotoxic effects.

Further work by Slater et al (1980) has identified, using techniques including pulse radiolysis and electron spin resonance, a more reactive free radical, CCl$_3$O$_2^*$, derived from carbon tetrachloride after its interaction with active oxygen. The amount of O$_2$ present at the site of CCl$_4^*$-activation may affect the reactivity of the free radical products and may, therefore, have a qualitative and quantitative affect on the cellular lesion produced by CCl$_4^*$.

The halogenated hydrocarbon bromobenzene, which is known to be hepatotoxic has been shown to be converted to a chemically reactive metabolite, bromobenzene 3,4 epoxide, by a cytochrome P450 mediated metabolism (Brodie et al, 1971; Jollow et al, 1974a; and Zampaglione, 1973). About 70% of this epoxide is converted to a glutathione conjugate by the glutathione transferases in the liver. The glutathione conjugate is then converted to a mercapturic acid which is excreted via the kidney. It has been demonstrated that centrilobular necrosis of the liver does not occur until the dose of bromobenzene is high enough to deplete the level of glutathione below a threshold level. When the glutathione levels are reduced the epoxide metabolite covalently binds to tissue macromolecules causing tissue lesions. The relationship of glutathione to hepatotoxicity has been demonstrated for other hepatotoxins including paracetamol which will be discussed in greater detail in a later section.
1.7 RELATIONSHIP OF ENZYME INDUCTION AND INHIBITION TO TOXICITY

The previous section demonstrated the importance of the cytochrome P450 mediated enzyme system in the metabolism of drugs and foreign compounds by the liver. It is now known that the activity of this system can be induced in vivo by a number of agents such as phenobarbitone (Conney, 1967; Goldstein et al., 1974). Other known inducers include the carcinogenic hydrocarbon 3,4-benzpyrene, some of the methylenedioxyphenyl compounds, ethyl alcohol and the steroid hormones. If toxicity is metabolism mediated, induction of the drug metabolizing enzyme system will increase toxicity. This has been made use of in investigations of the toxicity of drugs and chemicals in experimental conditions. It is also of great importance in the clinical situation, especially if polypharmacy is being practised. The co-administration of barbiturates may enhance or diminish the toxicity of other drugs, or of foreign chemicals present in the body from other sources. It has also become widely recognised that there are inducers of drug metabolizing enzymes present in the environment and diet. These must be taken into consideration when designing experiments with animals, as any variation in conditions of the diet or environment may produce different results from those expected, especially when investigating effects related to metabolism.

Inhibition of drug metabolism is also of great importance. A toxic response will be reduced if toxicity is metabolite mediated, or increased if the parent molecule is the toxic agent. SKF 525A, or β-diethylaminoethyl diphenylpropyl acetate, is a potent inhibitor of cytochrome P450 mediated drug metabolism with no pharmacological action of its own but with wide uses in medical research. α-Naphthyl isocyanate and cobalt chloride are also used as experimental inhibitors.

Use of inhibitors of drug metabolism has been made in the clinical situation. Disulfiram produces very unpleasant side effects including flushing, dyspnoea, nausea, vomiting and hypotension, when it is ingested concomitantly with alcohol. It has, therefore, become a useful agent in the treatment of alcoholism. Its action is thought to be due to the accumulation of acetaldehyde (Hald et
caused by the inhibitory effect of disulfiram on aldehyde dehydrogenase, an enzyme which normally oxidises acetaldehyde to acetic acid.

Inhibitors of the monoamine oxidase enzyme system have been used in the treatment of severe depression, for example tranylcypromine and pargyline. A side effect of these drugs is caused by ingesting tyramine, a monoamine present in a number of food stuffs like cheese and marmite. Tyramine would normally be rapidly oxidised by the monoamine oxidases, but if the latter are inhibited by compounds like tranylcypromine the tyramine will accumulate and may cause serious lesions due to cardiovascular effects (Goldberg, 1964).

Another useful inhibitor of drug metabolism is allopurinol, an inhibitor of xanthine oxidase which catalyses the oxidation of xanthine to uric acid. In gout uric acid production is excessively high and allopurinol has proved to be of great value in alleviating the suffering caused by this disease.

1.8 PARACETAMOL HEPATOTOXICITY

Paracetamol was used as the main model hepatotoxin in the research carried out for this thesis. A fuller review of the literature relating to paracetamol hepatotoxicity will, therefore, be given here.

Paracetamol (acetaminophen, N-acetyl-para-aminophenol) is a commonly used analgesic with antipyretic properties. M.I.M.S. (the monthly index of medical specialities, 1979) lists forty-five preparations containing paracetamol which are in regular use within the United Kingdom. Over two hundred preparations are listed by Ameer (1977) as being used in the United States. This drug was synthesized at John Hopkins University in 1877, and it was first used in clinical medicine by von Mehring in 1893 (Spooner and Harvey, 1976). Its use in medicine did not become extensive until it was recognised as the principal active metabolite of acetanilide and phenacetin by Brodie and Axelrod in 1949 (1948, 1949). Paracetamol is now used regularly in the treatment of colds, coughs and mild pain, and its use in preference to aspirin, which is known to cause bleeding, is encouraged (Madan, 1976). In a double blind trial
carried out by Mielke et al (1976) aspirin was shown to have effects on bleeding time, platelet aggregation and platelet factor 3 release, whereas paracetamol did not demonstrate these effects.

Paracetamol is very safe when ingested at the normal therapeutic dose, usually one gram taken up to four times daily. Large overdoses, however, are now known to produce hepatic necrosis. This was first reported to occur in rats by Boyd and Bereczky (1966), followed by a report of its occurrence in man by Davidson and Eastham (1966). Fatal hepatic necrosis has been demonstrated in rats and mice (Mitchell et al, 1973a), hamsters (Davis et al, 1974), dogs (Gazzard et al, 1975) and man (Prescott et al, 1971). The evidence suggests that the minimum lethal dose in adult humans is about 25g (Proudfoot and Wright, 1970), although Prescott and Wright (1973) reported a fatal case after ingestion of only 18g. One of the main problems associated with paracetamol is its usage in self poisoning. In 1973 alone about 5000 cases were reported in Britain with about fifty deaths (McLean, 1974). The normal blood levels of paracetamol after a 500-600 mg dose are between 5 and 20 \mu g/ml in humans, toxic effects are not evident until it reaches 300 \mu g/ml (Madan, 1977).

Dixon et al (1975) carried out a detailed analysis of the cellular events leading to necrosis using light- and electron-microscopic, and histochemical studies in rats sacrificed at intervals of up to forty-eight hours following overdosage with paracetamol (3g per kg). They found glycogen depletion, loss of ribosomes and cytoplasmic matrix swelling commencing between three and six hours after administration of a dose which caused frank coagulative necrosis between twelve and twenty-four hours in centrilobular hepatocytes. Midzonal cells showed more prominent aqueous swelling, with vesiculation of the endoplasmic reticulum and some hydropic vacuolation. There was an early transient rise in succinate dehydrogenase activity followed by a progressive reduction between six and twelve hours which appeared to parallel swelling and disruption of mitochondria in degenerating cells. There appeared to be no early involvement of the lysosomes,
acid phosphatase activity being reduced only in cells already necrotic. Between twenty-four and forty-eight hours macrophages accumulated in the necrotic zones where the phagocytosis of cellular debris was accompanied by a prominent mitotic activity in surviving hepatocytes.

Buttar et al (1976) investigated the effects of sub-lethal, but toxic doses of paracetamol, in rats using biochemical parameters as indices of hepatotoxicity. They found dose-dependent elevations in activities of the serum enzymes glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and sorbitol dehydrogenase, together with an increase in hepatic triglyceride levels, following single doses of 0.5 and 1g per kilogram bodyweight. Maximal damage occurred within twelve to eighteen hours, hepatic function, in terms of serum enzymes and hepatic triglyceride returning to normal, between forty-eight and seventy-two hours. They also found an increase in hexobarbital induced sleeping time indicating that paracetamol inhibits the microsomal enzyme activity of the liver.

Thorgeirsson et al (1976) have investigated similar parameters in mice using doses of 400 mg/kg. They found decreases in microsomal protein concentration within three hours of paracetamol administration, reductions in cytochromes P450 and b5 occurring after nine hours, but no effect on triglyceride levels. There was also a 60% reduction in in vivo protein synthesis measured by [3H]-Leucine incorporation into total liver proteins.

The LD50 of paracetamol in rats was found to be 5.2g/kg (McLean and Day, 1975). This value was affected by diet with low protein and yeast diets reducing the LD50. In a chronic study where rats were given 200mg/kg paracetamol for 200 days no adverse effects were found (Thomas et al, 1977). It has also been demonstrated to have no teratogenic effects in rats (Lubway and Burriss Garrett, 1977), or mice (Lambert and Thorgeirsson, 1976).

The clinical significance of paracetamol toxicity in man has generated a lot of research into the mechanism by which paracetamol manifests its toxicity. Much of this work has been carried out at the National
Institute of Health (Bethesda, U.S.). Mitchell et al (1973a) demonstrated that pretreatment of rats and mice with phenobarbitone or 3-methyl cholangthrene, inducers of drug metabolism, increased the disappearance of paracetamol from the plasma and tissues of the animals and increased the severity of hepatic necrosis. Conversely pretreatment with inhibitors of drug metabolism, cobalt chloride and piperonyl butoxide, inhibited the metabolism and disappearance of paracetamol from the tissues, yet protected against necrosis. On the basis of these observations they postulated that paracetamol-induced hepatotoxicity is mediated by a toxic metabolite of paracetamol.

Covalent binding of radiolabelled paracetamol to mouse liver protein was found to be dose-dependent (Jollow et al, 1973), as was hepatic necrosis. Pretreatment with inducers or inhibitors of drug metabolism were found to have effects on the degree of covalent binding which correlated with effects on necrosis. It was, therefore, suggested that the hepatic necrosis may be caused by the covalent binding of a chemically reactive metabolite of paracetamol to vital hepatic macromolecules. Further studies by Potter et al (1973) on the binding of $^{3}$H-paracetamol to hepatic microsomes in vitro demonstrated that the binding was mediated by a cytochrome P450 dependent mixed function oxidase. The treatments which altered the toxicity of paracetamol in vivo similarly affected the extent of binding in vitro indicating that in vitro binding was a valid index of paracetamol hepatotoxicity.

Potter et al (1973) also investigated the binding of 2-acetylamino-fluorene demonstrating that its binding was also dependent on cytochrome P450. The toxicity of 2-acetylamino-fluorene is thought to result from its conversion to an N-hydroxy derivative (Grantham et al, 1965). The similarities between the two compounds suggested that the hepatotoxic metabolite of paracetamol might also be an N-hydroxy derivative.

The arylation by the hepatotoxic metabolite of paracetamol was found to be prevented in vitro by the addition of electrophilic sulphhydryl compounds like glutathione and cysteine (Potter et al, 1973). Mitchell et al (1973b) then demonstrated that glutathione also protected against hepatotoxicity in vivo in mice. Pretreatment of
mice with diethyl maleate, which depletes glutathione, potentiated paracetamol-induced necrosis. Paracetamol caused a dose dependent depletion of glutathione, with covalent binding of the toxic intermediate to the tissue macromolecules not occurring until the glutathione had been depleted by more than 70%.

Davis et al (1974) investigated the hepatotoxic effects of paracetamol in different animal species finding that the mouse and hamster were more susceptible to hepatotoxicity than the rat and guinea pig. The severity of damage was correlated to the rate of formation of the arylating metabolite by the hepatic microsomal enzymes, and to the rate of depletion of glutathione. The N-hydroxylation of 2-acetylaminofluorene, also an N-acetylarylamine, occurs to a greater extent in those species more susceptible to paracetamol-induced hepatotoxicity, further supporting the theory that the toxic metabolite of paracetamol results from N-hydroxylation.

It is known that drug-glutathione conjugates are further metabolised in vivo and appear in the urine as mercapturic acids (Boyland, 1971). Investigations by Jollow et al (1974b) demonstrated that the amount of paracetamol metabolised to a mercapturic acid was greater in the species more susceptible to toxicity. When the dose of paracetamol reached hepatotoxic levels the fraction of the dose excreted as the mercapturic acid decreased markedly, supporting the evidence that glutathione is depleted in paracetamol hepatotoxicity. They also suggested that the normal metabolic intermediate which conjugates with glutathione in the mercapturic acid pathway could also be the electrophilic metabolite, which in the absence of glutathione arylates hepatic macromolecules and causes cell death.

Further studies were carried out in hamsters, a species very susceptible to paracetamol toxicity, which are known to N-hydroxylate other N-acetylarylamines more rapidly than other species (Potter et al, 1974). 3-Methylcholanthrene pretreatment increased the severity of the damage whereas phenobarbitone did not. Further studies on the metabolism of other N-acetylarylamines demonstrated that 3-methylcholanthrene induced N-hydroxylation, whereas phenobarbitone in hamsters increased the glucuronidation of the parahydroxyl group of paracetamol which is
a detoxifying reaction resulting in a faster clearance of paracetamol from the tissues.

Figure 4 shows the postulated metabolic pathway for paracetamol (Mitchell et al., 1976a) which suggests that the toxic pathway is via an N-hydroxy derivative of paracetamol to an imidoquinone, which then irreversibly binds to the cellular macromolecules, leading to necrosis and death of the liver cells. The glutathione conjugate is excreted as a mercapturic acid which has been identified in the urine of animals and man (Mitchell et al., 1974). On the basis of this observation that metabolism of paracetamol in humans also involves glutathione much research is now being directed to finding an antidote to paracetamol overdosage. Mitchell et al. (1974) suggested cysteamine, a sulphydryl group containing compound which protected against the toxic effects of paracetamol in animals, supported by Gazzard et al. (1974). Boyland and Chasseaud (1969) give further support to the role played by glutathione in mercapturic acid biosynthesis. Similarly there is much evidence to support the role of the cytochrome P450 mixed function oxidases in the formation of active metabolites (Sims and Grover, 1974; Jerina and Daly, 1964; and Nebert et al., 1975).

In further studies by Davis et al. (1976) on species variation it was postulated that the different susceptibilities of animals to paracetamol toxicity could be due to differing capacities to synthesize glutathione, or to form glucuronide or sulphate conjugates. They found that the sulphate conjugation of paracetamol was a saturable process which could, therefore, lead to a greater formation of toxic metabolite. Slattery and Levy (1977) have suggested the use of inorganic sulphate as a protective agent against toxicity by its ability to increase the sulphate conjugating capacity, having demonstrated a reduction of toxicity in mice after treating with sodium sulphate.

Further work is still being carried out to determine the identity of the toxic metabolic intermediate, and to develop the ideal therapy in overdosage of paracetamol, as well as continuing studies to develop animal models of toxicity. Already some doubt has been cast, on the actual mechanisms already postulated, by Labadarios et al., (1977).
Postulated metabolic pathways of paracetamol (Mitchell et al, 1976a)
They demonstrated that a sulphydryl type compound, α-mercaptopropionylglycine, reduced necrosis but did not affect the degree of covalent binding in mice. They concluded that the binding of the reactive metabolite of paracetamol to hepatocyte macromolecules need not lead to hepatic necrosis provided that sulphydryl groups are present in the liver to prevent the deleterious effects of such binding.

More recently N-hydroxyparacetamol has been synthesized by Gemborys et al. (1978). This postulated metabolite of paracetamol, at a physiological pH in vitro, is far more stable than previously suggested with a half-life of slightly less than 20 minutes.

N-hydroxyparacetamol has been shown to deplete glutathione and cause hepatotoxicity (Healey et al, 1978). McMurtry et al. (1978) have demonstrated that the kidney toxicity which can also be produced by paracetamol is probably due to a metabolite produced in situ since pretreatment with 3-methylcholanthrene increased liver but not kidney toxicity.

It could be expected that if the N-hydroxy metabolite was responsible for toxicity production increased production in the liver would also cause increased kidney toxicity since the half-life is relatively long. This however is not the case. These results have therefore questioned the postulate that N-hydroxyparacetamol is the toxic metabolite (Gillette, 1981).

De Vries (1981) has suggested that the hepatotoxic action of paracetamol is dependent upon oxidation to a toxic metabolite via another pathway. He postulated that oxidation of paracetamol or transfer of electrons to a semiquinone, which is more reactive than the quinoneimine, is theoretically possible and can also explain the conjugation of glutathione:
The hepatotoxicity of paracetamol may therefore be due to inactivation of intracellular proteins via the reactive semiquinone or active oxygen species.

The toxic metabolite of paracetamol still remains unidentified. Furthermore the complete mechanistic pathway involved in the production of the toxic metabolite of paracetamol has not been completely elucidated.
CHAPTER 2

ISOLATED HEPATOCYTES
2.1 INTRODUCTION
The use of isolated cell systems has become widespread in recent years due to the development of reliable isolation techniques. This area of research has become important for two main reasons.

Firstly the increasing use of animals in the testing of drugs and chemicals has led to criticism from the general public who feel that the use of large numbers of animals is not only unnecessary, but also cruel. Recently a number of pressure groups have been formed to voice these feelings more forcefully. Research laboratories and pharmaceutical companies have become the targets of anti-vivisection groups who will do their utmost to stop any animals being used in experiments. Although researchers and industrialists are sympathetic towards these views they have to comply with the increasing demands of regulatory agencies to provide evidence for the safety of their products from animal tests. Many scientists, however, are becoming aware of the need to develop alternative methods for the safety screening of drugs and chemicals which will utilise less animals. Hence much research is now directed towards developing in vitro screening tests for predicting the safety of chemicals. One area in which some progress has been made is the testing of chemicals for mutagenic potential. A number of tests have been designed which utilise bacteria, yeasts, drosophila flies or cultured mammalian cells rather than whole animals. The use of these types of tests is becoming routine among chemical and pharmaceutical companies although many of the tests have given equivocal results in some cases which are difficult to interpret. There is therefore a need to develop and validate in vitro tests which will enable drugs and chemicals to be tested for potential toxicity. Isolated hepatocytes provide one possible alternative which uses small numbers of animals. If these types of tests can be developed the number of chronic toxicity tests which at the present time have to be carried out on animals may be reduced, together with the vast financial investments necessary to develop new and safer products. This last point is becoming a major factor in limiting original research aimed at finding new drugs.
Secondly the recent advances in biomedical research have demonstrated that cellular functions and the mechanisms of action of toxic agents are very complex. Isolated cells provide an experimental tool whereby mechanisms may be investigated in a system removed from the many influences involved in whole animal experimentation including pharmacokinetics, environmental and hormonal controls.

The liver is a major organ within the body involved in endogenous and exogenous metabolism and it is the principal target organ for the toxic action of many chemicals. It is therefore particularly relevant to use it in an isolated situation. The fact that it is easily removed from the body and its texture renders it suitable for the manipulations required for the isolation of separate cells is a great advantage.

The liver contains a number of different cell types which fall within two main classes, parenchymal and non-parenchymal. Parenchymal cells are now known to have different functions dependent on their location within the liver acinus. It is therefore important that the yield of cells isolated from a liver should be high enough to provide a sample containing representative numbers of the different cell types.
2.2 ISOLATION TECHNIQUES

The techniques initially employed for isolating liver cells used chemical and mechanical means. Although the cell yields were often high the viability was invariably very low, the majority of the cells having damaged plasma membranes. The many studies reported using these cells were, therefore, inadequate as they were not representative of in vivo functions. Many of these studies have been reviewed by Schreiber and Schreiber (1973).

In 1967 Howard et al introduced a technique involving digestion of the liver with a mixture of enzymes, collagenase and hyaluronidase. The liver was initially perfused with the enzymes in a Ca\(^{++}\)-free solution in the cold, this was followed by slicing the tissue and incubating the slices with the enzymes at 37\(^\circ\)C. This method produced cells with a more acceptable viability but the yield was still relatively low. The major breakthrough came when Berry and Friend (1969) first described a technique for the perfusion of the liver with collagenase under physiological conditions which resulted in high yields of intact cells. This technique is relatively difficult to carry out, and numerous modifications have been introduced by different research groups. This has resulted in a number of problems associated with the standardisation of the technique, and comparisons of results between different laboratories.

Fry et al (1976) introduced a further significant modification in the technique which removed the perfusion. Their technique involves the enzymic digestion of liver slices with collagenase and hyaluronidase. This is the method that was used for the research presented here and it will be described in full in the methods section. This method has a number of advantages over the other available techniques, the main one being that it does not require the sophisticated surgical operations necessary to set up a liver perfusion, which means that the operators do not require a Home Office animal licence. The method may also be applied to pieces of liver tissue including biopsy samples from humans and larger mammals, which may enable more direct comparisons to be made between animals and humans. The cell yield by this method is lower than that obtained by perfusion but the viability is high and the numbers are
adequate for a wide range of experiments. The technique employed preferentially isolates the parenchymal cells of the liver, which have been shown to have intact plasma membranes, and carry out a number of biochemical functions which require the presence of intact and functional hormone receptor sites and a full status of endogenous cofactors (Fry et al, 1976). Cells have been isolated from a number of different species by this technique, and a method has also been developed to culture the hepatocytes in primary monolayers.

2.3 BIOCHEMICAL AND MORPHOLOGICAL CHARACTERISTICS OF ISOLATED HEPATOCYTES

A large number of studies are documented concerning the functional and morphological characteristics of isolated hepatocytes using the many variations of the isolation methods. A short review of the more recent studies will be given here. There are a number of more comprehensive reviews available (Seglen, 1976c; Jeejeebhoy and Phillips, 1976; Fry and Bridges, 1979)

Prior to using isolated hepatocytes it must be demonstrated that the cells are intact and functionally viable. The most widely used method for determining viability is dependent on the ability of cells with an intact plasma membrane to exclude dyes like trypan blue, nigrosin and eosin (Paul, 1970). This technique is quick and simple and is considered by many to be the most reliable, however some workers have questioned its accuracy and suggested other methods. Expression of the cell's metabolic capacity, e.g. its ability to synthesize glycogen or respire, have been suggested as alternatives. These methods, however, are not completely reliable as there is an intrinsic variability in the cells capacity to carry out various metabolic functions. Damaged cells will continue to respire due to the presence of intact mitochondria, similarly other cellular functions controlled by subcellular organelles may continue to be active. Baur et al (1975) compared a number of different methods for evaluating cell viability, suggesting that the measurement of cellular potassium levels and membrane potential may be more suitable criteria. The loss and uptake of cellular potassium is, however, a reversible process and may not be a true indicator of cellular damage. The leakage of soluble enzymes has also been utilised and is probably equivalent to the trypan blue test in assessing irreversible membrane damage. If it is accepted that the latter test
is only a measure of gross structural integrity and not necessarily the cells true viability, it is probably the most reliable parameter for assessing the success of a cell isolation procedure. The survival of the cell after this initial trauma is dependent on many factors including the incubation media and conditions.

Isolated parenchymal liver cells are spherical in shape with the whole of their surface covered with microvilli (Seglen, 1976c). The internal structure is the same as that of the organised tissue (Howard et al., 1967; Berry and Friend, 1969; Moldeus et al., 1974). Specialised membrane regions like bile canaliculi do not appear to be present on the surface of the cell, although these may reappear when the cells are cultured.

Many facets of carbohydrate metabolism have been investigated in isolated rat hepatocytes, with results suggesting that they closely resemble the intact perfused liver. Cells isolated from fed rats retain glycogen, and synthesize glucose which is released into the surrounding medium, whereas cells isolated from starved rats contain no glycogen and can only synthesize glucose in the presence of added substrates like lactate and pyruvate (Garrison and Haynes, 1973; Geelen et al., 1977; Story et al., 1976; Mullhofer et al., 1977). Gluconeogenesis is stimulated by hormones and cyclic nucleotides (Garrison and Haynes, 1973; Pilkis et al., 1975). Further work on the hormonal control of gluconeogenesis has been reported by Byus et al. (1976), Riou et al. (1976), Arinze (1977) and Siess et al. (1977 and 1978). More specific studies on the levels of cyclic AMP in rat hepatocytes and its relationship to gluconeogenesis have also been reported (Moxley and Allen, 1975; Birnbaum and Fain, 1977; Fain and Shepherd, 1977; Chan and Exton, 1977). Hems et al. (1978) found that glycogen breakdown stimulated by hormones is dependent on the presence of extracellular Ca++. Dichloroacetate has also been shown to inhibit gluconeogenesis in isolated hepatocytes (Stacpoole, 1977; Demaugre et al., 1978). Walker (1977a) found that isolated hepatocytes are influenced by diurnal rhythms, their ability to synthesize glycogen being affected by the time of day at which they are prepared.
Isolated rat hepatocytes will synthesize fatty acids and glycerolipids. Soler-Argilaga et al (1978) have demonstrated that calcium plays a role in fatty acid synthesis, a depletion reducing the basal rate of triacylglycerol synthesis and diminishing the effects of glucagon and the cyclic nucleotides. Alterations in the dietary status of animals affect the synthesis of glycerolipids, these being reflected by isolated rat hepatocytes (Groener and van Golde, 1977). The same authors have also investigated the role played by exogenous and endogenously synthesized fatty acids in glycerolipid synthesis (Groener and van Golde, 1978). Akesson et al (1976) investigated the metabolism of specific monoacylphospholipids finding that the monoacylphospholipid acyltransferase reactions operating at positions one or two yield different saturated acyl chain profiles in phosphatidyethanolamine and phosphatidylcholine of a specific unsaturation. This is of importance because many membrane functions are related to the physical state of membrane lipids. Isolated rat hepatocytes will synthesize cholesterol (Gibbons and Pullinger, 1977). Fatty acid synthesis is significantly inhibited by very low density lipoproteins whereas cholesterol synthesis is not (Lakshmanan et al, 1977). Panek et al (1977) reported that both fatty acid and cholesterol synthesis were inhibited by 5-(tetradecyloxy)-2-furoic acid, a lipid lowering agent. Hormones also play an important role in fatty acid and glycerolipid synthesis in the liver. Geelen et al (1978) investigated the influence of glucagon and insulin on the synthesis of glycerolipids, and Ochs and Harris (1978) have studied the relationship between glycolysis, lipogenesis, gluconeogenesis and pyruvate kinase activity in isolated rat hepatocytes.

Urea is synthesized by the liver from aspartate and ammonia. Rognstad (1977) has shown that production of ammonia by the proline nucleotide cycle is not a major pathway because hadacidin which inhibits that pathway does not inhibit urea synthesis in rat liver cells. L-Norvaline and L-leucine however do inhibit urea synthesis with their main site of action at ornithine transcarbamylase. Hensgens et al (1978) suggest that the synthesis of urea from proline is primarily limited by the rate of formation of ammonia from glutamate via glutamate dehydrogenase. Further studies on ureogenesis in isolated rat hepatocytes have been reported by Meijer et al (1978), Siess et al (1977) and Triebwasser and Freedland (1977).
Isolated rat hepatocytes will synthesize and secrete proteins (Seglen, 1976a and b), synthesis being measured by the incorporation of radioactivity from a $[^{14}\text{C}]$-labelled amino acid mixture into the cellular proteins. The incorporation rate varies depending on the concentration of the cells in suspension and the extracellular amino acid concentration. Hepatic protein synthesis is subject to regulation by amino acids, exemplified by the effect of alanine which when added to the extracellular medium stimulates synthesis. The substrates lactate and pyruvate also stimulate synthesis, their effects being abolished by aminoacetate, an inhibitor of glutamic transaminases, indicating the presence of a positive control of protein synthesis by a transamination-dependent agent (Seglen and Solheim, 1978a). Seglen has also investigated the nature of the precursor pools for protein synthesis using $[^{14}\text{C}]$-Valine, an amino acid which is not metabolised to any significant extent by the liver. The results suggested that both the intracellular and extracellular valine pools can directly provide precursors for protein synthesis, the relative contribution from each pool being proportional to the relative valine concentration in that pool (Seglen and Solheim, 1978b). The effects of bioregulators and hormones on protein synthesis and amino acid uptake have been investigated by Donner et al (1978) and Jeejeebhoy et al (1977). Van Bezooijen (1977) found that the synthesis of total proteins was increased in older rats aged between 24 and 36 months. Ricca et al (1978) however reported an age related decrease in protein synthesis in rat hepatocytes. The extracellular media and amino acids used by these two groups differed, however, which could explain their differing results.

The normal process of protein degradation also occurs simultaneously with protein synthesis in isolated hepatocytes (Seglen, 1977a). Further studies into the control of protein degradation carried out by Hopgood et al (1977b) demonstrated that degradation of prelabelled protein was inhibited by insulin and certain amino acids, ammonia, proteinase inhibitors and by inhibitors of cell-energy production.

Bile acids are also synthesized by isolated rat hepatocytes (Yousef et al, 1978) at a rate, and with a composition, similar to that in vivo.
The uptake of the bile acid taurocholate by isolated hepatocytes is inhibited by cholestatic steroid hormones (Schwarz et al., 1977) and tauroolithocholate (Schwenk et al., 1977). The respiratory activities of isolated hepatocytes have been investigated, showing that there is no age-related decline in the functional integrity of the mitochondria (Brouwer et al., 1977). Further studies on cytosolic phosphorylation states have been carried out by van der Meer et al. (1978). Evidence also strongly suggests that energy dependent, carrier mediated, active transport systems are existent in isolated rat hepatocytes. This is supported by the work on α-aminoisobutyric acid (Chen and Lee, 1977), ouabain (Eaton and Klaassen, 1978), ATP (Akerboom et al., 1977) and amino acids (Le Cam and Freychet, 1977 a and b; Edmondson et al., 1977).

2.4 ISOLATED HEPATOCYTES IN CULTURE

The culturing of isolated rat hepatocytes involves a number of technical problems, with the result that relatively less studies have been carried out in primary monolayer cultures than with suspensions of freshly isolated cells. The method of cell isolation introduced by Fry et al. (1976) may be carried out under sterile conditions which has enabled the successful culturing of adult rat liver cells. One of the major problems associated with cell cultures is the loss of certain specialised cellular functions as the time in culture increases, with the result that the cells may eventually bear no relationship to the tissue from which they were originally derived. This is especially the case with cell lines which are composed of undifferentiated epithelial cells. A number of workers have, therefore, investigated the functional aspects of primary monolayer cultures of adult rat hepatocytes in some detail.

Functional studies carried out by Bissell et al. (1973) demonstrated that cells derived from regenerating adult liver synthesized proteins. They also produced glucose from lactate and pyruvate indicating that gluconeogenesis was operational together with an integrated interaction between the mitochondrial and cytoplasmic compartments of the cell. The response of the cells to insulin and glucagon verified the presence of intact receptors for those hormones as well as demonstrating the
integrity of the adenyl cyclase system. The structure of the smooth endoplasmic reticulum was retained and the levels of ATP returned to physiological levels within a few hours of incubation. Further studies were carried out on glucose metabolism (Bissell et al., 1978), showing that primary cultures utilised relatively small amounts of glucose like the intact liver, although on extended culture this pattern changed to that observed in cell lines where the utilisation of glucose is much greater.

Studies carried out by Tanaka et al. (1978), using adult cells from normal rat liver, demonstrated that some of the functions lost during the cell isolation were recovered during culturing. These included the induction of tyrosine transaminase by dexamethasone, reaggregated polysomes, and the stimulation of protein synthesis by insulin and dexamethasone which was inhibited by glucagon. They also demonstrated that the cells secreted proteins into the medium, albumin, transferrin, fibrinogen and lipoproteins being specifically identified.

Williams and co-workers (Laishes and Williams, 1976; Williams et al., 1977; Williams et al., 1978) have carried out a systematic examination of the conditions affecting the primary cell cultures of adult rat hepatocytes finding that a medium supplemented with dexamethasone increased the survival time in culture. Goldfarb et al. (1978) provided further support for the recovery of the ability to synthesize lipids and glycogen, and an improvement in morphology of isolated adult rat hepatocytes in primary monolayer culture.

Further studies on gluconeogenesis have been carried out by Oliver et al. (1978), Walker (1977b) and Kletzien et al. (1976). Pariza et al. (1977) found that the induction of amino acid transport by epinephrine is independent of cyclic AMP levels in primary cultures, and Hopgood et al. (1977b) found cells in primary culture were more sensitive to the effects of insulin and glycogen on protein degradation than hepatocytes in suspension.

Bellemann et al. using the isolation method of Fry et al. (1976) found that the primary monolayer cultures retain their metabolic and enzymic
characteristics for several days (Gebhardt et al, 1978). They also found that in cultures derived from normal and alloxan diabetic rats insulin stimulated glycogen synthesis in both, and protein synthesis more effectively in normal cells (Bellemann et al, 1977a).

2.5 **DRUG METABOLISM IN HEPATOCYTE SUSPENSIONS AND CULTURES**

The metabolism of xenobiotics in cell suspensions and cell cultures has been well reviewed (Bridges and Fry, 1976; Fry and Bridges, 1977b; Orrenius et al, 1977). The generally accepted method for the investigation of drug metabolism *in vitro* has been the use of microsomes. There are, however, a number of reasons why subcellular fractions are probably not representative of *in vivo* metabolism. Tissue homogenization may liberate lysosomal enzymes, and it removes the compartmentalisation of the enzymes and membrane components within the cell. Hence cellular metabolism which requires the integration of different cellular components will not be seen in cellular fractions. It is also necessary to add unnaturally high levels of co-factors to enable metabolism to proceed. Before metabolism commences the drug or chemical must enter the cell, the mechanisms controlling this may affect metabolism, and this will not be assessed in cell fractions.

The perfused liver and liver slices have also been employed in studies of drug metabolism but there are problems inherent in the use of these techniques. The perfused liver, although a good system, is difficult and expensive to set up, and the number of tests that can be run on one liver is limited. The main problems associated with liver slices are firstly the outer layers of the slices contain damaged cells as a result of the slicing procedure. Secondly the uptake of xenobiotics in slices is controlled by passive diffusion with the result that little if any will reach the centre of the slice, this also applies to the nutrients added to the medium. The cells in the centre of the slices will become necrotic quite rapidly resulting in a preparation that is not homogeneous.

Isolated hepatocytes in suspension and culture do not suffer these many drawbacks, and as techniques are now available for the preparation of viable and functional cells they have proved to be very useful in the study of drug metabolism. A number of drug oxidations dependent on
cytochrome P-450 have been investigated in isolated hepatocytes and a number of these are listed in Table 1.

Moldeus et al (1978a) used isolated hepatocytes to investigate the interaction between ethanol and the metabolism of foreign compounds. They specifically investigated the effects of ethanol on glucuronidation of a number of substrates including harmol, 2-naphthol, 4-methylumbelliferone and phenolphthalein finding that it was inhibited by up to 50% by low concentrations of ethanol (10mM). Further investigations were made into the effect of ethanol on the intracellular NADH/NAD\(^+\) ratio and the synthesis of UDP glucuronic acid, and the effect of 4-methylpyrazole an inhibitor of alcohol dehydrogenase dependent ethanol oxidation. This led the authors to suggest that the inhibitory effect of ethanol on glucuronidation is due to a decreased synthesis of UDP glucuronic acid caused by an increased NADH/NAD\(^+\) ratio resulting from the alcohol dehydrogenase dependent oxidation of ethanol.

The same group (Norling et al, 1978) investigated the uptake of glucuronides into isolated hepatocytes and their effects on the glucuronidation of model substrates.

The reaction sequence involved in the metabolism of paracetamol via cytochrome P-450 oxidation to the formation of the mercapturic acid derivative has been delineated in isolated rat liver and kidney cells by Moldeus et al (1978b). Oxidation and glutathione conjugation were catalysed primarily by the liver cells, while conversion of the glutathione conjugate to the mercapturic acid derivative was catalysed primarily by the kidney cells.

Moldeus (1978) performed a detailed analysis of the formation of the different metabolites from paracetamol in hepatocytes isolated from mouse and rat. The hepatocytes from both species catalysed the formation of glucuronide, sulphate, glutathione and cysteine conjugates of paracetamol. Sulphation had a higher affinity for paracetamol than glucuronidation in both the rat and mouse, whereas glucuronidation had a higher capacity. The maximal rate of glucuronidation was similar in hepatocytes from both species but that of sulphation was several-fold less in the mouse. The rate of glutathione conjugate formation was found to be directly correlated to the loss of intracellular glutathione, this
<table>
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<tr>
<th>Drug studied</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Alprenelol</td>
<td>Orrenius et al (1975)</td>
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<tr>
<td>Aminopyrene</td>
<td>Weigl et al (1977)</td>
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<tr>
<td>Antipyrene</td>
<td>Aarbakke et al (1977)</td>
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<td>Ethanol</td>
<td>Crow et al (1977)</td>
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<td>Harmol</td>
<td>Andersson et al (1978)</td>
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<td>Barbiturates</td>
<td>Yih and Rossum (1977)</td>
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<tr>
<td>Biphenyl</td>
<td>Wiebkin et al (1976 and 1978b)</td>
</tr>
<tr>
<td>Benzoic Acid</td>
<td>Kao et al (1978)</td>
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<td>Ethoxycoumarin</td>
<td>Fry et al (1978)</td>
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<td>4-Methyl Umbelliferone</td>
<td>Fry et al (1978)</td>
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<td>Benzo(a)pyrene</td>
<td>Jones et al (1978)</td>
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<tr>
<td>Diphenylhydantoin</td>
<td>Inaba et al (1975)</td>
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<td>4-androstene-3, 17-dione</td>
<td>Stenberg et al (1978)</td>
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<td>17α-ethinylestradiol</td>
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<td>Oestrone sulphate</td>
<td>Schwenk et al (1978)</td>
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<td>Sulphadimidine and sulphanilamide</td>
<td>Morland and Olsen (1977)</td>
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<td>Ethoxyresorufin</td>
<td>Burke and Orrenius (1978)</td>
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<td>Burke and Hallman (1978)</td>
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<td>Phenacetin and N-Hydroxyphenacetin</td>
<td>McLean (1978)</td>
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<td>Benzo(a)pyrene</td>
<td>Ashurst and Cohen (1980)</td>
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<td>Paracetamol</td>
<td>Moldeus et al (1980)</td>
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<tr>
<td>Pyridine and substituted pyridines</td>
<td>Blaauboer and Paine (1979)</td>
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<td>Carbon tetrachloride</td>
<td>Anundi et al (1979)</td>
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rate however was far less in the rat than the mouse. The mouse is a species more susceptible to the toxic effects of paracetamol. This was supported here by the progressive loss of plasma membrane integrity of the mouse hepatocytes after 90 minutes of incubation with 2mM paracetamol which did not occur in the rat hepatocytes incubated in the presence of 10mM paracetamol for five hours.

Further investigations by Moldeus et al (1980) have shown that chronic administration of ethanol to rats for 6-8 weeks caused an elevation in the concentration of cytochrome P-450 in isolated rat hepatocytes. These hepatocytes showed enhanced rates of oxidation and UDP-glucuronic acid conjugation of paracetamol, with the binding affinity of paracetamol to cytochrome P-450 being increased from 2.8mM⁻¹ in control hepatocytes to 0.75mM⁻¹ in ethanol-treated rat hepatocytes.

There have only been a limited number of studies in primary cultures of adult rat hepatocytes. This is mainly due to the inability of the earlier techniques to produce cultures in which the cells maintained physiological levels of cytochrome P-450.

2.6 MAINTENANCE OF CYTOCHROME P-450 IN PRIMARY MONOLAYER CULTURES OF RAT HEPATOCYTES

It has been well documented that during the first 24 hours of culture the concentration of cytochrome P-450 falls markedly in primary maintenance cultures of adult rat hepatocytes (Guzelian and Bissell, 1976; Guzelian et al, 1977). A further study by the same group (Guzelian and Barwick, 1979) presented supplementary evidence that this phenomenon occurred but was prevented by incubating cultures with inhibitors of protein synthesis such as cycloheximide, puromycin, actinomycin D, or azaserine. It was postulated since cycloheximide had no effect on the degradation of total protein and its effect appeared to be due to a decreased breakdown of haem, that the selective degradation of cytochrome P-450 in cell culture may require protein synthesis.

Earlier studies had suggested that the reduction in cytochrome P-450 could be causally related to an increase in haem oxygenase activity
(Maines and Kappas, 1977). This hypothesis was investigated by Paine and Legg (1978) using cultured adult rat hepatocytes. When cells were cultured in William's medium E supplemented with 5% foetal calf serum they obtained results similar to those of Guzelian and Barwick (1979). A loss in concentration of cytochrome P-450 was associated with a rise in haem oxygenase activity, although it was demonstrated by Guzelian and Barwick in their study that the rise in haem oxygenase occurred after the decrease in cytochrome P-450 became marked suggesting that there is not a direct link. Decad et al (1977) reported that levels of cytochrome P-450 could be maintained in cultured hepatocytes if the culture media was supplemented with pharmacological levels of hormones. Paine and Legg (1978) utilised media supplemented with hormones and measured cytochrome P-450 concentration and haem oxygenase activity after varying the isolation and culturing conditions. They found that if the liver perfusate was supplemented with hormones in addition to the culture media and the plastic petri dishes were coated with collagen the levels of cytochrome P-450 after 24 hours of culture of 121pmole/mg protein were similar to in vivo levels and the activity of haem oxygenase was high. It would appear therefore that high levels of cytochrome P-450 can be maintained even when high levels of haem oxygenase were present.

Paine et al (1979a) have also shown that the sole addition of high, unphysiological concentrations of nicotinamide (25mM) to culture media maintained cytochrome P-450 levels. These levels of cytochrome P-450 were further increased to those similar to whole liver values in vivo if 1mM nicotinamide was added to the perfusate for the isolation of hepatocytes. In a subsequent study (Paine et al., 1979b) hepatocytes cultured for 24 hours were shown to lose 60% of their NADH content as well as a marked reduction in cytochrome P-450 concentration. Addition of 2mM nicotinamide to the culture medium caused a marked increase in NADH levels after 24 hours of culture, as did 25mM nicotinamide. Since cytochrome P-450 concentration can only be maintained with the high level of nicotinamide it was suggested that there is not a causal relationship between the content of NADH and the maintenance of cytochrome P-450 in cultures. This was further confirmed by the finding that isonicotinamide was twice as efficient as nicotinamide at maintaining cytochrome P-450 but had no effect on NADH levels.
Paine et al. have also demonstrated that culture of hepatocytes in a simple balanced salt solution prevents the loss of cytochrome P-450 (Paine and Hockin, 1980) suggesting that a component(s) of the culture media is responsible for the loss of cytochrome P-450. Investigation of the effects of different culture media on cytochrome P-450 content of hepatocytes after 24 hours of culture indicated that cystine and cysteine were the components of media responsible for the decreased levels. They also found that incubation in media without cystine or cysteine, but with added 5-amino laevulinic acid maintained even higher levels of cytochrome P-450. It is of interest to note here that cysteine has been shown to be a cytotoxic constituent of culture media by Nishiuch et al. (1976). Added pyruvate had a protective effect probably due to complex formation with cysteine in the media since preincubation of media at 37°C was shown to have a dose-dependent effect on the stabilisation of cysteine reducing the concentration of oxidisable cysteine.

Leibovitz L-15 medium, used in the studies reported in the present work contains cysteine at a concentration of 120mg/l and pyruvate (550mg/l sodium salt) but no cystine. Paine and Hockin (1980) compared the effect of L15 with that of other media (Waymouth's MB752/1: cysteine-81mg/l, cystine 18mg/l; William's medium E: cysteine-52.05mg/l, cystine-23.70, pyruvate-25.0mg/l; RPMI 1640: cystine-59.15mg/l) and found that the level of cytochrome P-450 after 24 hours in culture fell to between 26 and 39% of the initial level with all four media.

Paine's group have investigated the structure-activity relationship for the substituted pyridines in relation to their ability to maintain cytochrome P-450 (Villa et al., 1980) together with their effect on protein synthesis inhibition compared to that of cycloheximide to test the hypothesis of Guzelian and Barwick (1979). They found that pyridine itself was able to maintain high levels of cytochrome P-450 as were the 3-acetyl- and 3-amino-pyridines. These results provided further support for their earlier findings for the effects of nicotinamide and isonicotinamide, although the amide group of nicotinamide was found to be important as thionicotinamide was not effective. It was also demonstrated that both nicotinamide and isonicotinamide inhibited protein
synthesis by 48 and 60% respectively after 24 hours in culture. Cycloheximide at a concentration of between 0.5 and 1.0 μM produced similar effects on inhibition of protein synthesis but the levels of cytochrome P-450 were lower. Pyridine, however, was found to maintain high levels of cytochrome P-450 with only minimal inhibition of protein synthesis. These results do not therefore support the hypothesis of Guzelian and Barwick (1979) that the inhibition of protein synthesis is the primary mechanism underlying the maintenance of cytochrome P-450 in hepatocyte culture.

Paine et al (1980) have further weakened this hypothesis by demonstrating that the ability of pyridines to maintain cytochrome P-450 in hepatocyte culture is highly correlated with their ability to bind to cytochrome P-450, suggesting that ligand formation prevents its accelerated turnover in liver cell culture. Furthermore metyrapone maintains cytochrome P-450 levels due partially to preventing degradation of the cytochrome (a property of ligand forming compounds) but mainly by its effect of increasing the synthesis (Paine and Villa, 1980). Increased synthesis of cytochrome P-450 was known to occur since the specific activity of labelled cytochrome P-450 in the cultures was reduced (animals were injected with labelled laevulinic acid prior to the isolation of hepatocytes). This increased synthesis by metyrapone was not completely prevented by concomitant incubation with 5 μM cycloheximide which inhibited protein synthesis by 95%.

It may therefore be concluded from the present evidence that the loss of cytochrome P-450 in cultures is due to impaired synthesis and enhanced degradation.

2.7 THE USE OF ISOLATED HEPATOCYTES IN TOXICOLOGY
Isolated hepatocytes in suspension and culture maintain many biochemical functions with activities similar to those in vivo. They, therefore, lend themselves to the study of the mechanisms of toxic action of drugs and chemicals. Within the last few years a number of studies have been carried out, some of which will be reported here. This review will be limited to isolated hepatocytes in suspension and primary culture, for
more general studies on in vitro toxicity evaluation the reviews of Tardiff (1978), Nardone (1977), Bridges (1976) and Worden (1974) are relevant.

The main endpoint of toxicity is the loss of cell viability. Zawydiwski and Duncan (1978) described a method for measuring spontaneous $[^{51}^{\text{Cr}}]$ release as an indicator of membrane damage. The leakage of cellular enzymes into the extracellular medium has been used by Zimmerman et al. (1974) to demonstrate the hepatotoxicity of erythromycin and chlorpromazine, glutamic oxaloacetic transaminase being used as the enzyme. Similar studies were carried out to investigate the toxicity of the tricyclic antidepressants (Abernathy et al., 1975) and the thioxanthine neuroleptics (Abernathy and Zimmerman, 1975).

Orrenius et al. (1976) investigated the effects of anoxia on isolated hepatocytes. It caused an increase in plasma membrane permeability demonstrated by a decrease in trypan blue exclusion frequency, leakage of cytosolic lactate dehydrogenase, and an increase in succinate-stimulated oxygen uptake. These changes appeared to be preceded by a marked decrease in cellular ATP concentration. Similar effects to those caused by anoxia were observed if the cells were incubated with an uncoupler of oxidative phosphorylation.

A number of studies have been carried out in isolated hepatocytes to investigate the effect of xenobiotics on the metabolic processes of the liver. Story and Freedland (1978) found that feeding DDT to starved rats inhibited gluconeogenesis from lactate at the step between pyruvate and phosphoenolpyruvate in the isolated hepatocytes. D-Galactosamine inhibits glycogenolysis by causing a decrease in the $P_{i}$ content of hepatocytes (Sternmann et al., 1978). Mapes (1977) concluded that the inhibition of lipogenesis by halothane is probably caused by inhibition of the oxidation of NADH. Gluud and Dich (1977) observed during studies on the role of cyclic AMP in liver protein metabolism that the xanthines aminophylline, caffeine and theophylline, inhibited the incorporation of $[^{14}C] -$Valine into the proteins of isolated hepatocytes and the secretion of albumin. Protein secretion is also inhibited by colchicine, ammonia and anoxia (Seglen and Reith, 1977).
A series of studies have been carried out on the toxin phalloidin which is obtained from *Amanita phalloides*. Phalloidin is toxic via its direct action on the plasma membrane of isolated hepatocytes (Frimmer, 1977), and at very low concentrations (20 μg/ml) it inhibits amino acid incorporation into proteins (Gravela and Poli, 1977). Trypsin delays or prevents the plasma membrane response of hepatocytes to phalloidin (Frimmer et al., 1977a) as do temperatures below 21°C, which suggests that the fluidity of the membrane lipids might control an early step of the phalloidin response (Frimmer and Rufeger, 1977). The uptake of phalloidin by hepatocytes is not by simple diffusion but probably via the pathway of physiological substrate. Systematic screening has shown that the bile acids inhibit the response of isolated hepatocytes to phalloidin in a dose dependent manner (Frimmer et al., 1977b). Further studies indicate that phalloidin induces an accumulation of triglycerides within the liver cell probably by impairing protein synthesis and secretion (Gravela et al., 1977).

The peroxidation of polyunsaturated lipids is associated with the hepatotoxicity of several chemicals, especially carbon tetrachloride. Hogberg and co-workers have utilised isolated hepatocytes for the investigation of factors which may contribute to the protection of live cells from lipid peroxidation (Hogberg et al., 1975a, b and c). These factors include the low permeation rate of iron into the cell, the inactivation of iron by its complexing to apoferritin, the metabolism of lipid peroxides by glutathione peroxidase, and the oxidation of malonaldehyde. Weddle et al. (1976) studied the effect of carbon tetrachloride on lipid peroxidation in isolated hepatocytes finding that it increased. Isolated hepatocytes from rats having consumed alcohol for several months, produce malondialdehyde - a measure of lipid peroxidation, in significantly higher amounts than cells from untreated animals, without the accumulation of triglycerides (Remmer et al., 1977).

Further studies on the effects of carbon tetrachloride in isolated hepatocytes have been carried out by Poli et al. (1978 and 1979). They also demonstrated that lipid peroxidation is stimulated in isolated rat hepatocytes which is dependent on the metabolism of carbon tetrachloride by cytochrome P-450. It was also shown to inhibit protein synthesis and protein and lipoprotein secretion and induce the
accumulation of fat within the cells. The free radical scavanger promethazine was found to inhibit malondialdehyde production due to carbon tetrachloride but not to influence its effects on reducing the cytochrome P-450 content of isolated hepatocytes.

Lindstrom et al (1978) demonstrated that isolated hepatocytes from phenobarbital-treated rats showed an increased rate of bioactivation of carbon tetrachloride producing a more marked effect on the release of lactate dehydrogenase from these cells than on cells from untreated rats. This effect was more prominent than the effect of phenobarbital pretreatment on malondialdehyde production. Depletion of the glutathione levels of hepatocytes by diethyl maleate was, however, associated with increased malondialdehyde formation.

Studies by Stacey and Priestly (1978a), using a number of agents shown to be hepatoprotective in other experimental models of carbon tetrachloride induced toxicity including SKF-525A, promethazine, glutathione and cysteine, demonstrated that they were ineffective in isolated rat hepatocytes in reducing the toxicity of carbon tetrachloride. Further studies (Stacey and Priestly, 1978b) suggested that lipid peroxidation is not a requisite step in carbon tetrachloride induced toxicity in isolated hepatocytes.

Bromotrichloromethane, another halogenated hydrocarbon, has been used by Koster-Albrecht et al (1978) to investigate lipid peroxidation and cell damage in isolated rat hepatocytes. It produces similar alterations in liver cells to carbon tetrachloride but within a lower concentration range.

Bromobenzene is another compound which is metabolised by the cytochrome P-450 system to produce an active metabolite which is toxic to the liver. The conjugation of the active metabolite with glutathione appears to be a major protective mechanism in preventing the hepatotoxicity of bromobenzene Zampaglione et al (1973). Thor et al (1978a, 1978b and 1979) have used isolated rat hepatocytes to investigate the metabolism and toxicity of bromobenzene. They found that the intracellular level of glutathione in the hepatocytes is of major importance in relation to the
cytotoxicity of bromobenzene and that hepatocytes incubated in a complete medium are protected against toxicity by their ability to resynthesize glutathione. Pretreatment of rats with diethyl maleate potentiated the hepatotoxic effect whereas the presence of cysteine, methionine and N-acetylcysteine in the incubation medium protected against toxicity.

Depletion of glutathione leads to covalent binding of reactive metabolites to tissue macromolecules. Dent and Sun (1980) have developed a model for studying the specificity of covalent binding in isolated hepatocytes. Using this model they investigated the effects of bromobenzene finding that binding occurs first with macromolecules in the 20-40,000 dalton range. The cell appears to be able to recognise these macromolecules and by some mechanism degrades them. When the rats are pretreated with phenobarbitone the rate at which the binding occurs is greater than with untreated hepatocytes resulting in an accumulation of the high molecular weight fraction since the rate of formation appears to exceed that of degredation. This method may prove to be useful in identifying the nature of the macromolecules with which covalent adducts have formed.

Glutathione plays a valuable role in the cell in protecting against the toxicity of endogenous chemicals. A number of studies have been carried out to investigate the level and turnover of this thiol in hepatocytes, and the effect of isolation techniques and incubation media. Reed and Orrenius (1977) demonstrated that hepatocytes freshly isolated from rats treated with diethyl maleate perform a net biosynthesis of glutathione at a rate similar to that observed in vivo of about 2 μmoles/hr/g wet wt. of liver. Vina et al (1978) however found that the level of glutathione in isolated hepatocytes fell during incubation. Hogberg and Kristoferson (1977 and 1978), who had also experienced loss of glutathione levels during incubation, found that by decreasing the time of isolation procedure and by supplementing the medium with amino acids and serum the levels of glutathione in freshly isolated hepatocytes were increased and a net rate of biosynthesis occurred thereafter.

The characteristics of primary hepatocyte cultures are less well defined than those of isolated hepatocytes in suspension with the result that only a few studies have been carried out so far. However,
the indications are that this system will be utilised far more in the future. Most studies to date have been carried out in cell lines, the reviews mentioned previously may be supplemented here by those of Desi et al (1977) and Grisham et al (1978). Michalapolous et al (1976) provide data within their review to support the use of primary hepatic cell cultures in the investigation of hepatocarcinogenesis by chemicals. Huberman and Jones (1980) have used liver cell cultures in mutagenesis studies. Two recent reviews provide more information on the use of primary liver cell cultures in assessing the toxicity of chemicals (Fry and Bridges, 1979; Grisham, 1979).

Williams (1977) described a system where ten carcinogens produced unscheduled DNA synthesis in primary cultures, suggesting it as a possible predictor of potential carcinogens. Fry and Bridges (1977a) describe another system for assessing the cytotoxicity of chemicals which are metabolism mediated. They took advantage of the fact that primary cultures of hepatocytes become overgrown with fibroblasts after three or four days, by using the hepatocytes as the metabolising component and the fibroblasts as the testing component. Using this system cyclophosphamide was shown to be far more toxic to the fibroblasts in the presence of hepatocytes, this being reduced if the hepatocytes are treated with SKF-525A, an inhibitor of drug metabolism. This fibroblast suppression test was used to test a wider range of chemicals with promising results by Wiebkin et al (1978a).

The leakage of cellular enzymes has again been used as a measure of toxicity in cultured cell lines to investigate the effects of carbon tetrachloride (Watanabe et al, 1977) and some general anaesthetics (Goto et al, 1976). This could also be applied to primary monolayer cultures of hepatocytes.

There are still many problems to be solved, and a greater standardisation of isolation and culturing techniques, for isolated hepatocytes, before batteries of short term tests can be introduced for the toxicity screening of new drugs and chemicals. A couple of problems may be highlighted by the following examples. Firstly by Seglen's study (1977b)
demonstrating that ammonia accumulating in the suspending culture medium inhibited protein degradation, and secondly the suggestion by Nishiuch et al (1976) that cysteine, a component of many tissue culture media, is highly toxic to cultured cells.

2.8 AIMS
The main aims of the work presented in this thesis were to validate the use of isolated hepatocytes, in suspension or culture, for the investigation of the toxicity of drugs and chemicals in the liver. Some model hepatotoxins, primarily paracetamol, were then investigated in these systems for their effects on the hepatocytes with the aim of providing further evidence for the mechanism of action of hepatotoxins.
CHAPTER 3

MATERIALS AND METHODS
3.1 MATERIALS

3.1.1 Animals
Male Wistar albino weanling rats were obtained from the University of Surrey breeding stock. They were housed in the University of Surrey animal unit in Makralon® cages on a bedding of wood shavings or Sterolet®. They had free access to water and a pelleted standard laboratory diet (Spillers No. 1, Spillers, Croyden) and were maintained under controlled environmental conditions.

Male Golden Syrian weanling hamsters were obtained from suitable suppliers, dependent on availability, and were housed and maintained in a similar manner to the rats.

3.1.2 Apparatus
The sonicators used were as follows: Soniprobe (Dave Instruments Ltd, London) and sonic bath (Kerry, England). Manufacturers for other apparatus used are as specified in the Methods Section.

3.1.3 Chemicals
Collagenase (type IV, or specific use re: C-2139), hyaluronidase (type II), β-glucuronidase (type H-1), sulphatase (type H-1), saccharo-1, 4-lactone, ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetra-acetic acid, paracetamol, NAD, 2,4-dinitrophenyl-hydrazine, N-methylphenazonium methosulphate, NADPH, N-1-naphthylethlenediamine dihydrochloride, reduced glutathione, GGPD, NADP, cycloheximide and neotetrazolium chloride were obtained from Sigma Ltd, London. Pure β-glucuronidase ('Ketodase') was obtained from William Warner and Co. Ltd, Hampshire.

7-Ethoxycoumarin was synthesized by the method of Ullrich and Weber (1972).

All tissue culture media and supplements were obtained from Gibco-Biocult Ltd, Scotland.

Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.
Phenobarbitone, amylobarbitone, quinalbarbitone and thiopentone (as the sodium salt) were all obtained from May and Baker Ltd, Dagenham, Essex.

L—\([U-^{14}C]\)-Leucine (specific activity 351mCi/mmol), \([U-^{14}C]\)-Uridine (specific activity 497mCi/mmol), \(p\)-Hydroxy \([\text{ring-3-5}^{14}C]\)-acetanilide (specific activity 14.6mCi/mmol) and the cyclic AMP assay kit (Code TRK.432) were all obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

ATP and GOT assay kits were obtained from Boehringer Mannheim Diagnostica.

All other chemicals used were of the best reagent grade available.
METHODS

3.2 HEPATOCYTE ISOLATION

3.2.1 The enzymic isolation of adult rat hepatocytes

Adult rat hepatocytes were isolated by a collagenase/hyaluronidase technique developed by Fry et al (1976) from the original method of Howard et al (1967).

Male Wistar Albino rats, weighing between 60 and 100g, were killed by cervical dislocation. The abdomen was swabbed with 70% alcohol prior to dissection to expose the liver. Each of the six lobes of the liver was removed separately and placed in Dulbecco's Ca\(^{++}\)- and Mg\(^{++}\)-free phosphate buffered saline (PBS'A')(Paul, 1970). Each lobe was carefully dried between two filter papers to remove any excess blood then placed on a clean filter paper supported by a square sheet of glass which acted as an anchor for the lobes and simplified the cutting. The lobes were cut with single strokes of a microtome blade (Swann-Morton PM40, Swann-Morton, Sheffield) to obtain slices of approximately 0.5mm thickness which were placed in PBS'A'. It is important to complete this process quickly so that the liver is not allowed to deteriorate. PBS'A'(10ml) containing about 3g of tissue was placed in a 250ml conical flask. The flask was shaken for 10 minutes in a shaking water bath (approximately 90 oscillations per minute) maintained at a temperature of 37\(^\circ\)C. The supernatant was then removed carefully and replaced with fresh PBS'A'. This washing procedure was repeated twice more. After three washes with PBS'A' the supernatant was removed and replaced by 10ml of PBS'A' containing 0.5mM ethylene glycol-bis-(\( \beta \)-amino ethylether) N,N'-tetracetic acid (EGTA). The slices were incubated for a further 10 minutes, followed by a second similar incubation. The supernatant was removed and replaced by 10ml of Hanks Mg\(^{++}\)-free balanced salt solution (BSS)(Paul, 1970) containing 5mM CaCl\(_2\) with collagenase/hyaluronidase (0.05%/0.10% W/V). The flask was incubated for approximately 45 minutes at 37\(^\circ\)C in the shaking water bath. The resulting supernatant was filtered through a layer of bolting cloth (150\( \mu \)m pore size; Henry Simon Ltd, Cheadle Heath, Stockport) to remove any large cell clumps and undigested material. The filtrate was centrifuged at 50g for one minute
in an MSE bench centrifuge, the supernatant was carefully removed and the pellet resuspended in PBS'A' (10ml) to wash the cells. After spinning the cells to a pellet this washing procedure was repeated again. The final cell pellet was resuspended in 10ml Liebovitz L15 medium containing 10% v/v foetal calf serum. The cell viability and yield were determined using the trypan blue exclusion method (see 3.3.1). All the solutions used in the isolation procedure contained phenol red as an indicator.

3.2.2 The enzymic isolation of adult hamster hepatocytes
The method followed was similar to that described in section 3.2.1. Hamster liver has only five lobes and contains a gall bladder, not present in rats, which must be carefully removed prior to slicing the lobes. The only modification to the above method (3.2.1) was that the slices were incubated for a shorter period of time (approx. 30 mins) with the collagenase/hyaluronidase solution. The reason for this was that the liver tissue of the hamster is less robust and dissociates more readily. The final cell pellet was similarly suspended in L15 plus 10% foetal calf serum and the cell viability and yield determined by the trypan blue exclusion method (3.3.1).
3.3 CRITERIA USED TO ASSESS CELL VIABILITY

3.3.1 Dye exclusion test
This test was used to assess the yield of viable cells directly after cell isolation, and also as a measure of changes in viability with time and due to toxicity.

The test is based on the fact that viable cells can exclude certain polar dyes whereas non-viable cells do not.

A cell suspension (0.25ml) was added to 0.1ml of trypan blue solution (0.4%) and mixed gently with a Pasteur pipette. A cover slip was placed over the grid of an improved Neubauer haemocytometer applying gentle pressure until Newtons rings were visible. The mixed cell suspension was allowed to pass from a Pasteur pipette under the cover slip. The viable and non-viable cells which settled within the central grid were then counted. The total cell yield and viability were calculated as follows:

\[
\text{Viability} = \frac{X}{X + Y} \times 100\%
\]

Total yield of viable cells = \(X \times \frac{7 \times 10^4}{5}\) cells/ml

where \(X = \) number of viable cells
\(Y = \) number of non-viable cells

After an initial cell isolation calculation of the viability enabled an assessment of the quality of the isolation procedure, calculation of the yield enabled the original cell suspension to be diluted to contain the number of cells required for experimentation.

Experiments to assess toxicity, using cell viability as the criterion of toxicity, were carried out using one ml samples of cell suspension containing \(10^6\) cells/ml and test chemical at the required concentration, in L15 plus 10% foetal calf serum. The incubations were carried out in 10ml conical flasks in a shaking incubator at \(37^\circ\text{C}\). Viability was assessed by dye exclusion as quickly as possible after the samples were removed from the incubator.
3.3.2 Lactate Dehydrogenase assay for viability

When hepatocytes are damaged intracellular enzymes are released into the extracellular medium, this fact was used to develop an assay whereby the amount of lactate dehydrogenase (LDH) released from isolated hepatocytes could be used as a measure of cell viability.

The method used was a modification of the colorimetric method of King (Varley, 1967) based on the formation of a coloured hydrazone, i.e. pyruvate-dinitrophenyl-hydrazone.

Reagents:
(a) Buffered substrate: 4g lithium lactate in 125ml 0.1M glycine buffer containing 75ml 0.1N NaOH.
(b) Solution of nicotinamide adenine dinucleotide; a solution of 10mg in 2ml distilled water was freshly prepared.
(c) 2,4-dinitrophenylhydrazine reagent; 200mg of the reagent was dissolved in hot N HCl and made up to a litre with this acid.
(d) Sodium hydroxide: 0.4N.

Procedure:
One ml samples of isolated hepatocytes containing $10^6$ cells/ml and the required concentrations of test chemical were incubated in 10ml conical flasks at 37°C. After the specified incubation time 0.5ml of the cell suspension was placed in an LP3 tube and the cells spun to a pellet by centrifuging at 50g for one minute. The supernatant (0.1ml) was added to 0.75ml of distilled water and used to determine the amount of LDH released from the cells into the extracellular medium. To the remaining supernatant and cell pellet was added 0.1ml 5% Triton X-100 (aqueous), mixing well to lyse all the cells; 0.1ml of the latter was added to 0.75ml of distilled water and used to determine the total amount of LDH present in the cells and the medium.
Assay:

0.5ml buffered substrate (a) +
50 μl sample
↓ mix and leave at 37°C for 2 mins
Add 0.1ml NAD solution (b)
↓ incubate at 37°C for 15 mins
Add 0.5ml colour reagent (c)
↓ incubate at 37°C for 15 mins
Add 5ml 0.4N NaOH (d)
↓ mix and read absorbance at 440nm

The standard curve was linear for the concentrations of LDH found at a cell concentration of 10^6/ml. Total LDH was represented by the absorbance at 440nm multiplied by a factor of 1.25 to allow for dilution. The LDH released was calculated by dividing the absorbance of the medium, containing released LDH, by the absorbance of the total LDH, expressed as a percentage.

A number of experiments were carried out to determine the effects of different chemicals on cell viability, some with a simultaneous assessment of the trypan blue exclusion conducted on the same cell sample.

3.3.3 Measurement of the rate of oxygen uptake in isolated hepatocytes

The rate of cellular respiration in isolated hepatocytes was determined using a Clark Oxygen Electrode (Rank Bros., Bottisham, Cambridge). This oxygen electrode consists of a platinum electrode covered by a Teflon® membrane which forms the base of a reaction chamber into which small volumes (approx. 3ml) of reaction medium may be placed. The reaction chamber is sealed to exclude air except for a small hole through which additions may be made to the reaction medium using a syringe. A small magnetic flea is placed in the reaction chamber so that the medium may be stirred at a constant rate. The platinum electrode is polarised at -0.6V with respect to a silver-silver chloride reference electrode. Under these conditions the oxygen, which diffuses through the Teflon® membrane, at the surface of the electrode produces a current. Any change in the
oxygen activity causes a corresponding change in current which may be monitored on a chart recorder (Heathkit).

Calibration of the oxygen electrode:
The electrode was calibrated by the method of Robinson and Cooper (1970), a chemical method using N-methylphenazonium methosulphate (PMS) as a catalyst instead of isolated mitochondria.

L15 plus 10% foetal calf serum (3ml) containing 800 units of catalase and 20 μg PMS was placed in the reaction chamber, which was surrounded by a water-jacket containing water at 37°C. The reaction medium was stirred for 20 minutes to allow temperature equilibration, and air saturation. The chamber was then sealed and 10 μl of NADH (0.1-0.3 μ mole) added to the stirred medium by syringe. When the current reached a steady state the addition of NADH was repeated. The mean scale deflection was calculated for a known concentration of NADH (calculated spectrophotometrically), from these values the oxygen concentration of the air saturated solution may be calculated, and hence the nmoles oxygen consumed per unit of deflection for one ml of reaction medium determined.

Procedure:
Isolated hepatocytes were incubated at 37°C in a shaking water bath. Twenty ml volumes containing between 0.5 and 2.0 x 10⁶ cells/ml and test chemical were used, incubating in 250ml conical flasks, to allow 3ml samples to be removed at time intervals for estimation of the cellular respiration rate. The reaction was monitored on the chart recorder until a straight line response was recorded that was adequate to determine the rate of oxygen consumption.

3.3.4 Measurement of 7-ethoxycoumarin 0-deethylase activity in cell suspensions
7-Ethoxycoumarin 0-deethylase activity was used as a measure of the metabolic capacity of the cells with the aim of finding out whether this could be used as a sensitive measure of cell viability.
Reagents:
(a) 7-Ethoxycoumarin (7-EC); 35mM in dimethyl formamide (DMF).
(b) 7-Hydroxycoumarin standard; 400 μg/ml in DMF.
(c) 0.3M acetate buffer; pH 4.5.
(d) β-glucuronidase/sulphatase; dissolve crude β-glucuronidase containing sulphatase activity (type H-1, Sigma) in acetate buffer (c) at 5mg/ml.
(e) Diethyl ether containing 1.5% isoamyl alcohol.
(f) 0.2M Glycine-NaOH buffer pH 10.4.
(g) Ketodase (pure β-glucuronidase in acetate buffer).
(h) Sulphatase; 100mg/6ml in acetate buffer.
(i) Saccharo-1, 4 β-lactone; 100mg in 2ml acetate buffer.

Procedure:
Samples of cell suspension (1ml) containing 10^6 cells/ml were placed in 10ml conical flasks containing 2 μl of 35mM 7-EC to give a final concentration of 70 μM. The incubation was continued for the required time, then the reaction was stopped by placing the samples on ice. A 0.5ml aliquot of the cell suspension was then added quickly to 4.5ml distilled water to lyse the cells and also dilute the fluorescence. Suitable blanks and standards were also run. A number of experiments were carried out to determine the effect of chemicals on the activity of 7-ethoxycoumarin O-deethylase, some were carried out in the presence or absence of added NADPH since it is known that NADPH only readily penetrates damaged plasma membranes.

Assay:
One ml samples (in triplicate) of the diluted incubate were placed in 10ml Soveril tubes to which was added 0.2ml of crude β-glucuronidase. The tubes were incubated overnight at 37°C then extracted with 5ml ether/alcohol for 10 minutes. The phases were separated by centrifuging at 2000g for 10 minutes. One ml samples of the ether phase were back-extracted into glycine/NaOH buffer for 10 minutes. The ether phase was aspirated and the fluorescence present in the alkali phase was determined in a fluorimeter set at λ_ex 370nm and λ_em 450nm. This gave the total metabolites produced by the cell suspension.
The products could be separated into free and conjugated metabolites by extracting with ether prior to treating with deconjugating enzymes. The free metabolites would be extracted into the ether phase and could be back-extracted into glycine buffer. The conjugated metabolites could be further separated into glucuronide and sulphate by treating samples separately with ketodase (pure β-glucuronidase) and sulphatase with added 1,4 saccharolactone.
3.4 PROTEIN SYNTHESIS IN ISOLATED HEPATOCYTES
The incorporation of $^{14}\text{C}$-Leucine into trichloracetic acid insoluble protein of isolated hepatocytes was used as a measure of protein synthesis. The method used was adopted from that of Seglen (1976a). Preliminary experiments were carried out to determine the ideal cell concentration and incubation times before the following procedure was adopted.

3.4.1 Measurement of $^{14}\text{C}$-Leucine uptake into isolated hepatocytes
One ml aliquots of a suspension of isolated hepatocytes containing $2 \times 10^6$ cells, 100 nCi L-$^{14}\text{C}$-Leucine (specific activity 351 mCi/mmol) and test chemical at the required concentration were incubated in 10ml conical flasks in a shaking water bath at 37°C. After the required incubation time the cell suspension was transferred to LP3 tubes and the cells spun gently to a pellet. The supernatant was removed and replaced by 1ml of 10% trichloroacetic acid (TCA) to precipitate the protein from the cell pellet. The precipitate was washed three times with 1% TCA to remove any residual labelled amino acids not incorporated into the proteins. The final protein pellet was solubilised with one ml of 0.5N NaOH overnight at 37°C. Protein content was estimated by the method of Lowry et al (1951) using 0.1ml of the solubilised sample. The remaining 0.9ml of sample was neutralised with 0.1ml 4.5N HCl. 250 $\mu$l aliquots of the neutralised sample were added to 4ml of a scintillation cocktail composed of: Toluene, 65% PPO, 1.5%; Metapol HC100, 32.5%) together with 250 $\mu$l of water.

The well mixed scintillation cocktail containing the sample was left overnight at 0°C in the dark to reduce quenching then counted for activity using a Hewlett Packard Tricarb scintillation counter.

A quench curve was determined for the above preparation using carbon tetrachloride as a quenching agent. The factors for the equation of this curve, obtained using a computer program, were programmed into the scintillation counter to enable decompositions per minute to be determined directly from the sample counts. The $^{14}\text{C}$-Leucine incorporated into the hepatocytes was expressed in terms of dpm/mg of cellular protein, or as a percentage of the control.
3.4.2 Preliminary investigations
One ml samples of isolated rat hepatocytes containing 0.5, 1 and 2 x 10^6 cells/ml were incubated for 30, 60 and 90 minutes. Samples were run in triplicate. After spinning the cells to a pellet the samples were treated as described in the method above (3.4.1).

3.4.3 The effect of xenobiotics on $^{14}$C-Leucine uptake by isolated hepatocytes
The effects of a number of xenobiotics, over a range of concentrations, on the uptake of $^{14}$C-Leucine by isolated rat hepatocytes were investigated. Hepatocytes at a concentration of 2 x 10^6 cells/ml were incubated for one hour, with triplicate samples for each test chemical concentration and control. Where the experiments were repeated on more than one occasion means and standard deviation were calculated. The test chemicals were dissolved directly in the medium if possible, or in DMF at concentrations which enabled 10 µl or less of DMF to be added to a one ml sample of suspended cells. Samples were treated as described in 3.4.1.

Experiments with hamster hepatocytes were carried out according to the same procedure.

3.4.4 The effect of paracetamol on $^{14}$C-Leucine uptake in hepatocytes isolated from rats pretreated with phenobarbitone
Rats were treated with i.p. injections of phenobarbitone (70mg/kg) daily for three days prior to sacrificing the animals and isolating hepatocytes. Untreated animals were maintained under the same conditions. After isolation of the cells cytochrome P450 was measured in both the phenobarbitone pretreated and the untreated cells spectrally by the method of Omura and Sato (1964) to check that induction of the metabolising enzymes had occurred. $^{14}$C-Leucine uptake was determined as previously described (3.4.1). This experiment was repeated on three occasions, each sample being run in triplicate. Means and standard deviations were calculated for each concentration of paracetamol.
3.4.5 Effect of paracetamol on protein degradation
After isolation hepatocytes were diluted to 10ml with L15 medium plus 10% foetal calf serum to contain a final concentration of 100 nCi $[^{14}C]$-Leucine per ml. This 10ml sample of cell suspension was incubated for one hour in a 100ml conical flask in a shaking water bath at 37°C. After about 50 minutes incubation a sample of cell suspension was taken to determine the cell viability and number. At one hour the cells were spun gently to a pellet and the supernatant removed. The cell pellet was washed twice carefully with medium to remove residual radioactivity and then made up to a cell concentration of $4 \times 10^6$ cells/ml with medium not containing $[^{14}C]$-Leucine. Aliquots of the cell suspension were then added to medium containing paracetamol to give a final cell concentration of $2 \times 10^6$ cells/ml and paracetamol concentrations of 0, 0.1, 5 and 40mM in one ml duplicate samples. Two samples, without paracetamol were processed immediately to determine the label present in the cellular protein after the one hour incubation. Control and treated samples were then incubated for a further hour and processed as described previously (3.4.1).

3.4.6 Effect of paracetamol on the uptake of $[^{14}C]$-Leucine into isolated hepatocytes (in relation to incubation time)
Isolated rat hepatocytes were incubated as one ml samples containing $2 \times 10^6$ cells at 37°C in a shaking water bath. At 5, 10, 15, 20, 25, 30, 45 and 60 minutes two samples were removed the cell suspension placed in LP3 tubes and spun gently to a pellet. The cell pellets were washed twice with unlabelled medium and then the protein was precipitated with 6.5% TCA and the protein precipitate spun to a pellet. The supernatant was removed carefully and a sample was counted to determine the TCA soluble radioactivity, the protein pellet was washed three times with 1% TCA and then solubilised with NaOH (0.5N) and treated as previously described (3.4.1).

This experiment was carried out with control and samples treated with 40mM paracetamol.
3.4.7 Effect of paracetamol on protein secretion
Hepatocytes were isolated from two rats and the cells were pooled and made up to 20ml with medium containing 100 nCi $[^{14}C]$-Leucine/ml. The cells were incubated for 30 minutes in a 250ml conical flask at 37°C in a shaking water bath then spun gently to a pellet and washed twice carefully with medium. The final pellet was made up to 10ml with medium and 2.5ml of the suspension dispensed to four 50ml conical flasks. Medium (2.5ml) was added to two samples of cells and 2.5ml of medium containing 80mM paracetamol was added to the remaining two 50ml conical flasks. The four samples were incubated for one hour the cells were then spun to a pellet and the supernatants removed and retained. TCA (5ml of 20%) was added to the supernatants to precipitate the protein. The precipitate was then spun to a pellet and treated as already described (3.4.1).

3.4.8 To determine whether the effect of paracetamol on protein synthesis is reversible
Isolated rat hepatocytes were incubated with or without 40mM paracetamol for one hour. The cells were then washed carefully and the media replaced with LI5 + 10% serum containing 100 nCi/ml $[^{14}C]$-Leucine. Control and paracetamol treated samples were removed at 5 minute intervals, for 30 minutes, the cells spun to a pellet then treated as previously described (3.4.1).

3.4.9 Effect of paracetamol on protein synthesis in vivo in the rat
Wistar Albino rats weighing approximately 70g were injected with 1000 mg/kg paracetamol in DMF containing 2μCi $[^{14}C]$-Leucine or DMF with label alone. The animals were observed for one hour and then killed by cervical dislocation. The livers were removed immediately and placed in 20ml 10% TCA, chopped finely and homogenised with three strokes of a Potter Eveljhem homogeniser. A 5ml sample of the homogenate was placed in a Soveril tube and the precipitate spun to a pellet and washed twice with 1% TCA then once with 70% ethanol, twice with 96% ethanol and finally twice with ether. The final protein pellet was solubilised in 5ml of 0.5N NaOH. Samples were taken to determine the protein concentration and the activity of $[^{14}C]$-Leucine present in the protein. The experiment was repeated on three occasions using three
test animals in each case. The animals were treated and killed by cervical dislocation in a staggered fashion at 10 minute intervals to enable each animal to have received the label for the same length of time prior to sacrifice.
3.5 TO DETERMINE WHETHER PARACETAMOL HAS A SPECIFIC EFFECT ON EXPORT PROTEINS

3.5.1 Preparation of cyanogen bromide activated Sepharose conjugates

Sepharose 4B (10ml) consisting of an equal volume of gel and water, was washed with 100ml of water through a sintered-glass funnel. The Sepharose was placed in a small beaker with 10ml water and 20ml 2M Na₂CO₃ and stirred in a fume cupboard. Cyanogen bromide (1ml) in acetonitrile was then added rapidly, the mixture was stirred vigorously for 2 minutes and then returned to the sintered-glass funnel and washed with 200ml of cold 0.1M NaHCO₃ pH 9.5 followed by 200ml of cold water and 200ml 0.1M NaHCO₃ pH 9.0. After the last wash the slurry was filtered to a moist compact cake under vacuum. (Three times this quantity was actually prepared, as one batch, to produce sufficient activated Sepharose.) Two thirds of the Sepharose was transferred to a bottle to which was added 17ml 0.1M NaHCO₃ pH 9.0 with 0.5M NaCl and 3ml of rat antisera (a), the remaining third of the Sepharose was transferred to another bottle to which was added 8.5ml of the same buffer and 1.5ml of non immune rabbit serum (b). Coupling of the activated Sepharose with conjugates was done overnight at 4°C on a shaker.

Conjugates:

(a) An antiserum prepared against the serum of laparotomised rats. The injection schedule was that used by Mullock et al (1974). The antiserum was shown by two dimensional crossed immune electrophoresis (Axelson et al, 1973), with a commercially available anti-(rat serum) incorporated into the upper gel, to react with all the major proteins of rat serum.

(b) Non immune rabbit serum.

In each case (a) and (b), a globulin fraction was prepared by precipitation with an equal volume of ammonium sulphate.

The next day the two samples of conjugated Sepharose were returned to separate sintered-glass funnels and drained quickly under vacuum. The following procedure is described for a 10ml quantity of conjugated Sepharose, the quantities were doubled for the rat antisera conjugate.
The slurry was washed with 70ml of coupling mix (0.1M NaHCO$_3$, 0.5M NaCl, pH 9.0) and allowed to stand in the cold for about 10 minutes while 1M ethanolamine pH 9.0 was prepared. The Sepharose was transferred to a glass beaker containing 40ml of the ethanolamine per 10ml of Sepharose, and left at room temperature for 2 hours. The mixture was then returned to the funnel and washed alternately with 60ml portions of coupling mix (pH 9.0) and 0.1M acetate buffer pH 4.0 containing 0.5M NaCl, starting and finishing with coupling mix, the last portion of each wash being drained gently under vacuum; there were 5 acid washes in all. The Sepharose was then placed in a graduated tube washing out the funnel with 0.15M NaCl (+ 15mM NaN$_3$). The Sepharose was allowed to settle. Each conjugated sample was adjusted so that it contained 50% Sepharose beads. The conjugated Sepharose was then incubated with labelled serum to determine whether or not it was active as follows:

Sample 1. 0.3ml normal rabbit conjugated Sepharose
       0.1ml $^{[14C]}$-fucose labelled rat serum
       0.6ml 0.15M NaCl

Sample 2. 0.3ml anti-rat conjugated Sepharose
       0.1ml $^{[14C]}$-fucose labelled rat serum
       0.6ml 0.15M NaCl

These two samples (1 and 2) were placed in LP3 tubes and shaken overnight at 4°C. The Sepharose was then spun to a pellet and washed twice with 0.15M NaCl. Sodium hydroxide (one ml of 0.5M) was then added to the pellet of Sepharose beads and heated at 80°C for 30 minutes. Aliquots were then added to scintillant (previously described in 3.4.1) and counted in a scintillation counter.

$^{[14C]}$-labelled rat serum:
A rat was injected intravenously with $^{[14C]}$-fucose 135 minutes prior to collection of serum. Analysis by crossed immunoelectrophoresis, followed by autoradiography demonstrated that the label was incorporated into all the major serum glycoproteins.
3.5.2 Preparation of $^{14}$C-labelled protein from isolated rat hepatocytes

Isolated rat hepatocytes were prepared as described previously (3.2.1). The final hepatocyte suspension was divided into two portions (a) and (b), each containing $20 \times 10^6$ cells in 10ml.

Sample (a) contained L15 + 10% foetal calf serum with $2 \mu$Ci $^{14}$C-Leucine
Sample (b) contained 40mM paracetamol in the medium with $2 \mu$Ci $^{14}$C-Leucine

Both samples were incubated at 37°C in a shaking water bath for 2 hours. The cells were then spun to a pellet in Soveril tubes. The incubation medium was removed carefully and stored frozen. Lubrol (5ml of 0.5%) was added to each cell pellet and mixed well to lyse the cells completely. This was checked microscopically. The Lubrol samples containing the cellular material were then centrifuged at 40,000g in an MSE Superspeed 50 for 1½ hours to remove all the membraneous material. The supernatant was removed and stored frozen until required, the pellets were also retained. All samples were stored at -20°C until required.

3.5.3 Affinity experiments with activated Sepharose conjugate

The Lubrol extracts, containing intracellular protein, and the incubation media, containing extracellular proteins, were thawed and 0.6ml aliquots of the samples were added in duplicate to both activated Sepharose conjugates (0.4ml) separately in LP3 tubes. The samples were incubated overnight at 4°C on rollers to continuously mix them. After incubation the samples were spun in a bench centrifuge (2000g) to pellet the Sepharose beads. The supernatant was carefully removed and retained. The pellets were washed three times with one ml 0.15M NaCl and the washings pooled with the supernatant. One ml of 0.5N NaOH was added to the final pellet of Sepharose which was then heated at 80°C for 30 minutes. Aliquots were taken for counting radioactivity. To the supernatant plus washings was added 3ml of 10% TCA to precipitate the protein. The precipitate was washed three times with 1% TCA then one ml of 0.5N NaOH was added to the final protein pellet and left overnight at 37°C to solubilise the protein. Samples were then taken for counting. The membrane pellet obtained after the preparation of the Lubrol supernatant...
Figure 1.

Flow chart for procedures used in experiments with activated Sepharose conjugates

- Cell suspension
  - incubate at 37°C for 2 hours
  - centrifuge at 200g for one min.

- Cells
  - add 5 ml Lubrol (mix well)
  - centrifuge 40,000rpm for 75 mins
  - supernatant
  - membranous pellet

- MEDIUM
  - incubate 0.6ml with 0.4ml of conjugated CNBr Sepharose

- Sepharose beads (bound)
  - heat at 90°C for 30 mins with 0.5N NaOH
  - count radioactivity

- Supernatant (unbound)
  - spin down Sepharose and wash x 3 with NaCl
  - acid ppt proteins (wash well)
  - protein precipitate
  - solubilise with 0.5N NaOH
  - count radioactivity
was also counted. The procedure is presented as a flow chart (Fig. 1) for clarity.

The dilution factors were calculated. Radioactivity was expressed as dpm per $10^6$ cells.
3.6 THE EFFECT OF XENOBIOTICS ON RNA SYNTHESIS

The incorporation of $[^{14}\text{C}]\text{-Uridine}$ into the ribonucleic acids of isolated rat hepatocytes was used as a measure of RNA synthesis.

3.6.1 Measurement of $[^{14}\text{C}]\text{-Uridine}$ uptake into isolated hepatocytes

The protein precipitate obtained from isolated hepatocytes after treatment with TCA also contains RNA. It was found that further treatment of the precipitate by heating at 90°C for 30 minutes followed by an extraction with 70% alcohol to remove lipids had no effect on the RNA content of the precipitate in terms of activity of $[^{14}\text{C}]\text{-Uridine}$. The method already described for measuring the uptake of $[^{14}\text{C}]\text{-Leucine}$ was, therefore, followed for $[^{14}\text{C}]\text{-Uridine}$.

A preliminary experiment was carried out to compare the effect of different cell concentrations on the rate of uptake. Isolated rat hepatocytes at concentrations of 0.5, 1.0, 1.5 and $2.0 \times 10^6$ cells/ml were incubated with 100 nCi $[^{14}\text{C}]\text{-Uridine}$ (specific activity 497 mCi/mmol) per ml for 30, 60 and 90 minutes. There was an initial time lag but after 30 minutes the uptake was approximately linear with time and cell concentration. It was, therefore, decided to maintain a concentration of $2 \times 10^6$ cells/ml for incubations as this gave a relatively high activity.

The effects of paracetamol, phenobarbitone and safrrole at different concentrations on the uptake of $[^{14}\text{C}]\text{-Uridine}$ were investigated.
3.7 COVALENT BINDING

3.7.1 Measurement of covalent binding of paracetamol metabolites to cellular protein

Isolated rat hepatocytes (2.3 x 10^6 cells/ml) were incubated in L15 + 10% foetal calf serum containing paracetamol at a concentration of 40 and 20mM with 0.422% p-hydroxy [ring-3, 5-[^14C]]-acetanilide (specific activity 14.6 mCi/mmol, 97μCi/mg). Twenty-four samples were prepared for incubation, the labelled paracetamol being added to the samples just prior to commencing incubation at 37°C. Samples, in duplicate were removed from incubation at 5, 10, 20, 40, and 60 minutes for both 20 and 40mM paracetamol. The cells were spun to a pellet, the supernatant removed and one ml of 1N perchloric acid added to precipitate the protein. The precipitate was washed five times with perchloric acid to remove all the unbound labelled paracetamol. The precipitate was solubilised in 0.5N NaOH and aliquots were counted to determine the amount of activity bound to the cellular protein. Protein was assayed by the method of Lowry et al (1951). The results are expressed in nmoles of total paracetamol bound per mg of protein, assuming that a similar percentage binding of unlabelled paracetamol (or metabolite) also occurred.
3.8 GLUTATHIONE IN ISOLATED HEPATOCYTES

The glutathione content of isolated hepatocytes was assayed by a modification of the method of Saville (1958).

Glutathione was extracted by adding 6.5% TCA (1ml) to the cell pellet (2 x 10^6 cells in one ml) to precipitate the protein. After mixing well the samples were spun at 1500g for 15 minutes and 0.1ml of the supernatant was used for the assay.

3.8.1 Glutathione assay
Reagents:
(a) 0.001M aqueous sodium nitrite.
(b) 0.5% aqueous ammonium sulphonate.
(c) Mix one volume of a 1% aqueous solution of mercuric chloride with 4 volumes of a 3.4% solution of sulphanilamide in 0.4N HCl.
(d) 0.1% solution of N-1-naphthylethlenediamine dihydrochloride in 0.4N HCl freshly prepared.

0.1ml of sample in 6.5% TCA was added to 0.5ml of solution (a) and left for 5 minutes. 0.1ml of (b) was added, mixed well and left for two minutes. One ml of solution (c) was then added followed by 0.8ml of solution (d). After 10 minutes the absorbance was read at 540nm.

Suitable controls and standards were always run. At concentrations of up to 100μg/ml of glutathione the standard curve was linear, passing through zero.
3.9 ASSAY OF ADENOSINE TRIPHOSPHATE IN ISOLATED HEPATOCYTES

Isolated hepatocytes (1ml) were incubated at a cell concentration of 0.5 x 10^6 cells/ml with 40mM paracetamol, 20mM phenobarbitone, 10mM safrrole with control samples for three minutes at 37°C. The reaction was stopped by the addition of 4ml cold IN perchloric acid. After 10 minutes the precipitated protein was spun at 1500g for 15 minutes to a pellet. The supernatant was carefully removed and neutralised with the required amount of 5M K_2CO_3 which precipitated the perchloric acid. After 30 minutes the perchloric acid precipitate was spun to a pellet (1500g for 15 minutes) and the supernatant was used as quickly as possible to assay for ATP.

ATP was assayed using a Boehringer test kit based on the method of Bucher (1947).

The following reagents were used:

(1) **Buffer/Glycerate-3-phosphate**
   - containing triethanolamine buffer pH 7.6 0.5mol/l
   - MgSO_4 4mmol/l
   - glycerate-3-phosphate 6mmol/l

(2) **NADH** 2.5mmol/l

(3) **GAPDH/PGK/GDH/TIM** ≥ 560u/mo1; ≥ 450u/ml; ≥ 800u/ml; ≥ 1000u/ml

Two ml of solution (1), 0.2mls of solution (2) and the deproteinised supernatant, were placed in a cuvette and mixed well. The absorbance was read at 340nm then 0.02ml of suspension (3) was added. After 10 minutes the absorbance was read again, readings being repeated until the reaction stopped.

Using the conversion factor provided, and allowing for appropriate dilutions, the concentration of ATP/10^6 cells was calculated for each sample.
3.10 ASSAY OF CYCLIC AMP IN ISOLATED HEPATOCYTES

Cyclic AMP was assayed using a kit supplied by the Radiochemicals Centre, Amersham (code TRK.432). The assay is based on the competition between unlabelled cyclic AMP and a fixed quantity of the tritium labelled compound for binding to a protein which has a high specificity and affinity for cyclic AMP. The amount of labelled protein:cyclic AMP complex formed is inversely related to the amount of unlabelled cyclic AMP present in the assay sample. Measurement of the protein bound radioactivity enables the amount of unlabelled cyclic AMP in the samples to be calculated.

Separation of the protein bound cyclic AMP from the unbound nucleotide is achieved by adsorption of the free nucleotide on to coated charcoal, followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabelled cyclic AMP in the sample is then determined from a linear standard curve.

Preparation of samples for the assay:

Isolated rat hepatocytes (2 x 10⁶ cells/ml) were incubated with control medium, 40mM paracetamol, 20mM phenobarbitone, and 10mM safrole at 37°C in a shaking water bath. At intervals of 5, 10, 30 and 60 minutes duplicate samples were removed from the incubator and placed on ice. The samples were transferred to LP3 tubes and the cells spun gently to a pellet followed by removal of the supernatant. One ml of cold 5% TCA was added to the pellet, mixed well and left for 15 minutes at 0°C. The samples were mixed again then the precipitate was separated by spinning the samples of 2000 rpm for 15 minutes. The supernatants were carefully removed and placed in Soveril tubes. HCl was added to bring the final concentration to 0.1N HCl (i.e. 0.1ml of 1.1N HCl to one ml of sample). TCA was extracted from the samples with 5ml water saturated diethyl ether. The 10 minute extractions were repeated five times. After the last extraction any excess ether was removed by heating the samples in a water bath at 70-90°C in a fume cupboard. The samples were then lyophylised and reconstituted in a small volume of acetate buffer (50mM, pH 6.2) and aliquots of this used in the cyclic AMP assay.
3.11 THE PREPARATION OF MICROSOMES FROM ISOLATED RAT HEPATOCYTES

A number of attempts were made to obtain a microsomal fraction from isolated hepatocytes with the aim of carrying out polyacrylamide gel electrophoresis on the fraction in order to give further information concerning the effects of xenobiotics on protein synthesis in hepatocytes. It was realised, however, that this was technically very difficult as the yields were far too small to enable further fractionation into samples containing smaller molecular weight ranges of proteins and distinction between the separate protein bands on the gels. Some of the techniques attempted are described below.

Isolated hepatocytes were suspended in 1.15% KCl pH 7.4 and homogenisation was attempted using the following techniques: Potter-Elvehjem, a polytron, a Dounce homogeniser, sonic bath and a sonic probe. Microscopy was used initially to determine whether or not the cells were disrupted. The homogenates were then centrifuged at 20,000g for 20 minutes. If it was felt that the homogenisation had been successful the supernatant was further centrifuged at 100,000g for one hour in an attempt to obtain a microsomal pellet which could be resuspended in a sucrose/tris/EDTA buffer. The protein concentration of the microsomes were assayed by the method of Lowry et al (1951).

The viability of some microsomal preparations was assessed by measuring the activity of NADH cytochrome C reductase or ethoxycoumarin O-deethylase activity.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was used for a couple of the microsomal preparations in an attempt to separate the proteins.

3.11.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was used as a means of separating microsomal proteins following the solubilisation of microsomes in buffer containing SDS. The method used was that of Laemmli (1970) which utilised a discontinuous buffer system.
Reagents:

Gel stock: 30% ($V_{\%}$) Acrylamide containing 0.8% ($W_{\%}$) NN'-methylene bisacrylamide (bis)

Buffer 1: 1.5M Tris-HCl (pH 8.8) containing 0.4% ($W_{\%}$) SDS

Buffer 2: 500mM Tris-HCl (pH 6.8) containing 0.4% ($W_{\%}$) SDS

Buffer 3: 25mM Tris-HCl (pH 8.3) containing 0.1% ($W_{\%}$) SDS and 192mM glycine

Buffer 4: 62.5mM Tris-HCl (pH 6.8) containing 2.3% ($W_{\%}$) SDS, 15% ($V_{\%}$) glycerol, 5% ($V_{\%}$) mercaptoethanol and 0.001% ($W_{\%}$) bromophenol blue

Propan-2-ol/acetic acid/water (25:10:65, by vol) containing 0.05% ($W_{\%}$) Coomassie Blue G-250

Propan-2-ol/acetic acid/water (10:10:80, by vol).

Procedure:

An air-cooled vertical slab gel apparatus was used for these studies and electrophoresis was always carried out in a cold-room at 4°C. A glass cuvette was used to contain the gel, which had final dimensions of 120 x 80mm x 1.5mm.

The glass cuvette was assembled by clamping perspex spacers between the glass plates to form the sides and bottom of the cuvette. The gaps between the perspex and glass were sealed by running a warm Agar solution (2% $W_{\%}$) along the edges. The glass cuvette was then clamped in a vertical position.

The lower (separating) gel was prepared by mixing Buffer 1 (10ml), gel stock (13.4ml), water (16.6ml), ammonium persulphate solution (freshly prepared, 10% ($W_{\%}$), 240 μl) and N,N,N',N'-tetramethylethylenediamine (TEMED, 20 μl). The solutions were mixed thoroughly and pipetted into the glass cuvette to a height of about 70mm, using a Pasteur pipette. A layer of water (about 5mm) was carefully introduced above the gel mixture to ensure a completely flat interface between the water and the acrylamide gel after it had polymerised. When polymerisation was complete, the water layer was removed and the upper (stacking) gel, consisting of a mixture of Buffer 2 (2.5ml), gel stock (1.0ml), water 6.5ml), ammonium persulphate solution (10% ($W_{\%}$), 6( μl) and TEMED (20 μl)
was added to the cuvette above the separating gel. A perspex comb was introduced into the top of the cuvette to form the sample wells within the stacking gel after polymerisation had occurred. When the stacking gel had set, the comb was carefully removed and a small volume of Buffer 3 introduced into the top of the cuvette to ensure separation of the sample wells. The perspex spacer was removed from the bottom of the cuvette before clamping the cuvette into the electrophoresis apparatus. The upper and lower buffer reservoirs were filled to suitable levels with electrode buffer (Buffer 3). Microsomal suspensions were diluted with sample buffer (Buffer 4) to a final concentration of 3mg microsomal protein/ml. The samples were placed in a boiling water bath (2-3 min) prior to application to the stacking gel. The amount of microsomal protein applied to the gel was 5μg. Electrophoresis was carried out using a constant current of 20mA in the stacking gel and this was increased to 40mA when the bromophenol blue tracking dye entered the separating gel. The electrophoresis was continued until the tracking dye was within 5mm of the end of the gel.

After the electrophoresis was complete, the cuvette was removed from the apparatus and the two halves separated. The stacking gel was cut away from the separating gel and the latter immersed overnight in a solution which stained for protein (propan-2-ol/acetic acid/water, 25:10:65, by vol, containing 0.05% (w/v) Coomassie Blue G-250). Destaining of the background gel was achieved by immersion of the gel in a mixture of propan-2-ol/acetic acid/water (10:10:80, by vol) for a suitable length of time. Gels were stored in 3% (w/v) glycerol at 4°C.

3.11.2 NADH - cytochrome C reductase assay
NADH - cytochrome C reductase activity was measured by monitoring the rate of reduction of exogenous cytochrome C at 550nm (E= 21,000M⁻¹cm⁻¹) in a dual beam spectrophotometer (Cary)(Prough et al, 1976). The reaction mixture consisted of 40μM cytochrome C, 200μM NADH, 100μM EDTA in 0.1M potassium phosphate buffer pH 7.7 contained in a 1ml cuvette (1cm light bath). KCN (500μM) was added to the incubation to inhibit the reoxidation of cytochrome C. The reaction temperature was 25°C.
Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Initial Conc.</th>
<th>Final Volume in Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C</td>
<td>400μM</td>
<td>0.1ml</td>
</tr>
<tr>
<td>NADH</td>
<td>2mM</td>
<td>0.1ml</td>
</tr>
<tr>
<td>KCN</td>
<td>5mM</td>
<td>0.1ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>1mM</td>
<td>0.1ml</td>
</tr>
<tr>
<td>buffer pH 7.7</td>
<td>0.1M</td>
<td>0.5ml</td>
</tr>
<tr>
<td>cells/supernatant</td>
<td>2 x 10^6 cells/ml</td>
<td>0.1ml</td>
</tr>
</tbody>
</table>

The addition of 10μl of 1mM Rotenone was used to determine its effect on the activity of NADH cytochrome reductase.

Protein content of the supernatant was assayed by the method of Lowry et al (1951).

3.11.3 Assay of 7-ethoxycoumarin 0-deethylation in microsomes

7-Ethoxycoumarin 0-deethylase activity was measured by determining the amount of 7-hydroxycoumarin produced from exogenously added 7-ethoxycoumarin during a 10 minute incubation with microsomes. The method was adapted from that of Aitio (1978).

Reaction mixture:

Buffer (0.1M Tris/HC1 pH 7.4) containing 100μM 7-EC

G6PD (10u/ml)  

Regenerating system:

NADP  10μmol/ml  0.05ml
G6P  50μmol/ml  0.05ml
MgCl₂  50μmol/ml

Incubate for 2 minutes

Microsome (10mg protein/ml) in tris/HCl buffer  0.05ml

Incubate for 10 minutes
The reaction was stopped by adding 0.5ml of 0.3M TCA. After thorough mixing the pH of the mixture was brought to about pH 10 by adding 4ml of 1.6M NaOH-glycine buffer (pH 10.3). The protein precipitate was removed by centrifugation and the amount of 7-hydroxycoumarin measured fluorimetrically ($\lambda_{ex}$ 370, $\lambda_{em}$ 450). Suitable blanks and standards were run simultaneously.
3.12 PRIMARY MONOLAYER CULTURES OF ADULT RAT HEPATOCYTES

Hepatocytes were isolated from adult rat liver according to the method previously described with the following modifications. All the procedures were carried out aseptically in a laminar air flow cabinet using sterile glassware, solutions and media. Sterile PBS'A' was supplemented with gentamicin (76 μg/ml) and the collagenase/hyaluronidase solution was filter sterilised prior to use. After isolation the final cell pellet was resuspended in sterile Liebovitz (L15) medium supplemented with 10% (v/v) foetal calf serum, 10% (v/v) tryptose phosphate broth and 1% (v/v) penicillin/streptomycin (complete medium). After determining the cell yield and viability the cells were diluted to 10^6 cells/ml.

Hepatocyte cultures were prepared in Falcon flasks (sometimes with the plastic substratum coated, then air dried with rat tail collagen), or Leighton tubes containing collagen-coated coverslips (10.5mm x 22mm). Suspension (2ml) was introduced into the former, one ml into the latter. The cultures were incubated at 37°C for 2 hours. The medium containing unattached cells was then carefully removed and replaced with fresh medium. About 20 hours later the medium was again removed and the cell monolayer was washed carefully with PBS'A' then fresh medium was introduced. After a further incubation of about 24 hours, during which the cultures were able to recover from the trauma of isolation yet contained only very small numbers of fibroblasts, the cultures were ready to use to study the effects of xenobiotics on a number of different parameters. Xenobiotics were added to the cultures as a solution, or a sonicated mixture in complete medium, or dissolved in DMF at a concentration which enabled less than 10 μl to be added per ml of incubation medium.

3.12.1 Hepatocyte architecture
Changes in hepatocyte architecture were investigated after staining cultures with Giemsa, or haematoxylin and eosin stains.

(a) Giemsa staining of cultured hepatocytes:
Reagents:
1. PBS'A'
2. Absolute methanol
3. Giemsa stain - Difco 'Bacto 71'
4. Buffers (a) $\frac{M}{15} \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$
   (b) $\frac{M}{15} \text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

5. Working buffer:- solution  
   (a) 50ml
   (b) 50ml
   Distilled water to 900ml.

Method:
Cultures were mixed with PBS'A' then flooded with methanol and left for one minute. They were drained and replaced with fresh methanol which was left for a further 10 minutes. The methanol was discarded and the culture flooded with the prepared Giemsa stain. The undiluted stain was allowed to remain in contact with the cells for one minute then working buffer was added to dilute the stain 1 in 5 and the preparation left for a further two minutes, followed by rapid rinsing in working buffer until free of excess stain. After air drying glass cover slips were either immersed in xylene and mounted in DPX, or for examination by microscopy and photography of the cultures in plastic flasks the working buffer was added or the flasks simply stored after air drying.

(b) Haematoxylin and eosin staining of cultured hepatocytes:
Cultures on cover slips from Leighton tubes were used in the following procedure: 
   1. Rinse with PBS'A'.
   2. Fix in Acetic Alcohol Formalin for 5 minutes.
   3. Rinse with tap water.
   4. Stain with 50% $\text{v/v}$ aqueous Haematoxylin (Harris) for 2 minutes.
   5. Rinse with tap water.
   6. Immerse in 0.5% $\text{HCl}$ for 15 seconds.
   7. Immerse in 0.05% lithium carbonate for 5 minutes.
   8. Rinse with 70% alcohol.
   9. Stain with Eosin for one minute.
   10. Rinse twice in absolute alcohol.
   11. Clear with xylene.
   12. Mount in DPX.
3.12.2 The effect of xenobiotics on cell attachment
This was determined by counting the nuclei of the attached cells.

Method:
The incubation was removed from the culture flask and replaced with 2ml of 0.033% crystal violet in 0.1M citric acid. The flasks were incubated at 37°C for one hour. The cells were removed from the culture surface by agitating them with a Pasteur pipette. An aliquot of the well mixed nuclei suspension was then counted to determine the number of nuclei using an improved Neubauer counting chamber.

3.12.3 Assay for glutamic oxaloacetic transaminase
Glutamic oxaloacetic transaminase (GOT) was assayed using a Boehringer Test Combination Kit (Cat. No. 124478). After the appropriate treatment the medium was removed from the culture flasks. The monolayer culture was washed twice with PBS'A', the washings being pooled with the medium. Water (1ml) was then added to the cultures and the cells were detached from the culture surface using a "rubber policeman". The cell suspension was transferred to LP3 tubes, total lysis of the cells was ensured by freezing and thawing three times. GOT activity was measured in both the medium and the cells. The amount of GOT leaked from the cells was calculated from the total present in both the cells and the medium.

3.12.4 Enzyme cytochemistry
The following enzymes were monitored in cultured rat hepatocytes using cytochemical methods based on those of Chayen et al (1973).

(a) Glucose-6-phosphate dehydrogenase:
Reaction medium: 0.05M glycyglycine buffer pH 7.9
0.3% neotetrazolium chloride .
5.0% polypeptide.
Just before use 2.5mg/ml NADP (disodium salt), 1.5mg/ml G6P (disodium salt) and 0.2mg/ml phenazine methosulphate were added, the final solution was bubbled with nitrogen and one ml introduced into Leighton tubes.
(b) NADPH₂ diaphorase:
To the reaction medium (a) was added 5mg/ml NADPH₂, this was then bubbled with nitrogen and one ml introduced into Leighton tubes.

(c) Succinate dehydrogenase:
Sodium succinate hexahydrate (68mg) was added to 5ml of the reaction medium and 0.2mg/ml phenazine methosulphate. The solution was bubbled with nitrogen and added to Leighton tubes as before.

The reaction time for all three enzymes was 45 minutes. The cover slips were then removed from the Leighton tubes, air-dried, and mounted in glycerin jelly. The colour reactions were quantified using a densitometer (Vickers, M85, Scanning and Integrating Microdensitometer).

These cytochemical techniques had been used previously in our laboratory. It was therefore considered that they had been suitably validated, further supported by the work of Chayen et al (1973). Prior to quantifying the colour reactions for any experiment the microdensitometer was adjusted to give the optimal wavelength and spot size. The machine settings were then unchanged for the assessment of all slides of a specified colour reaction within each experiment. The results are expressed in terms of arbitrary machine units.
3.12.5 Protein synthesis in primary cultures of adult rat hepatocytes
The method used was essentially the same as that already described for hepatocyte suspensions. Complete culture medium containing 100nCi [14C] -Leucine per ml together with the required amount of test compound was added to the cultures and incubated at 37°C for one hour. The medium was then removed and the cells washed with PBS'A' to remove any residual label not incorporated into the cells. PBS'A' (1ml) was then added to the cultures and the cells were detached from the culture surface with the aid of a "rubber policeman". The cell suspension was then removed from the Falcon flask and placed in LP3 tubes and treated as previously described (3.4.1).

3.12.6 7-Ethoxycoumarin O-deethylase activity in cultures
The method used was identical to that described in 3.3.4 for hepatocyte suspensions except that 10 μl of 140mM 7-ethoxycoumarin was added to 20ml of culture medium. The cultures were incubated with a 4ml volume of the substrate/medium solution. After deconjugation the samples were extracted with 5ml hexane prior to the ether alcohol extraction to remove any unreacted 7-ethoxycoumarin. Samples could be stored for up to one week at -20°C without loss of 7-hydroxycoumarin activity.
RESULTS

4.1 INTRODUCTION
Hepatocytes were isolated by the method of Fry et al (1976) from adult male Wistar Albino rats or Golden Syrian hamsters. It was assumed that if greater than 80% of the hepatocytes excluded trypan blue the preparation was viable for the purposes of the work described in this thesis. This assumption was based on the work carried out by Fry et al (1976) within the same laboratory. These workers demonstrated that isolated hepatocytes metabolise foreign compounds without the addition of exogenous cofactors, which together with the ability of the cells to exclude trypan blue, is indicative of a functional intact plasma membrane (Fry et al, 1976; Wiebkin et al, 1976). They also demonstrated that endogenous glycogenolysis was stimulated by glucagen providing evidence for the existence of functioning hormonal receptor sites within the isolated hepatocytes (Fry et al, 1976).

Figure 1 is a photograph of a typical sample taken from a preparation of isolated hepatocytes. Trypan blue was added and the sample viewed under a light microscope at x200 magnification. The cells coloured blue are the non-viable cells which have taken up the dye.

4.2 VIABILITY ASSESSMENT
The initial series of experiments to be reported investigated the effect of exogenously added chemicals on the viability of isolated hepatocytes. A number of different criteria of viability were compared.

4.2.1 Trypan blue exclusion
The first criterion investigated was the ability of hepatocytes to exclude the dye trypan blue. Tables 1a and 1b give the results from two experiments which are representative of a series carried out to determine the effect of a range of concentrations of paracetamol on the viability of isolated rat hepatocytes. Table 1a demonstrates that there was very little change in viability with time except at the 40mM level at which, after 90 minutes, the viability fell to 46%. Table 1b shows the results of a very similar experiment where there were no dramatic changes in the viability of the hepatocytes as a result of treatment with paracetamol. Viability was
reduced only slightly to 82% after 90 minutes treatment with 40mM paracetamol. In view of the variability experienced with the effect of paracetamol on isolated rat hepatocytes, supported by earlier investigations (Gwynn, 1976), the species was changed. The hamster has been shown to be more susceptible to the effects of paracetamol in vivo (Davis et al., 1974) and has also been used as an in vitro model for toxicity (Smith and Jollow, 1977).

The effect of paracetamol was then investigated in hamster hepatocytes (Figure 2) using a slightly different method. The isolated cells were incubated for two hours in medium containing 25mM paracetamol, the control was incubated under identical conditions but without paracetamol. After two hours the cells were spun to a pellet and the medium was replaced with control medium without paracetamol. The viability of the cells was assessed by trypan blue exclusion at two hours when the medium was changed and again at intervals for another three hours. The paracetamol was found to be more toxic to the hamster hepatocytes than it was in the previous experiments with rat hepatocytes.

In further experiments with hamster hepatocytes a range of concentrations of paracetamol was used. It is interesting to note (see Figure 3) that the lower concentrations were consistently more toxic to the hepatocytes in the early stages of the time course than the higher concentrations. Although this phenomenon was reproduced in five similar experiments some doubt was cast as to the validity of the trypan blue exclusion test as a criterion of viability assessment in hamster hepatocytes. This was due to the fact that with hamster hepatocytes the non-viable cells formed large aggregates during the incubations which meant that an accurate determination of the viability index was not possible. The cause of these aggregates was investigated. The addition of DNAase had no effect on the large clumps of cells indicating that DNA was not responsible (Bellemann et al., 1977b). The addition of protease, however, helped to disaggregate the clumps and it was, therefore, considered that protein was being secreted by the cells, or being released when a cell was damaged, causing the cells to clump together. These cell aggregates were far more apparent with the hamster hepatocytes, where they were present both after treatment with paracetamol at all concentrations and in control preparations after two to three hours of incubation, than with rat hepatocytes.
A typical suspension of isolated rat hepatocytes stained with trypan blue. The dark blue stained cells are non-viable. (Magnification x200)
Table 1.

Effect of paracetamol on the viability of isolated rat hepatocytes using trypan blue exclusion as the criterion of assessment.

(a)

<table>
<thead>
<tr>
<th>Time Mins.</th>
<th>Percentage viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>91</td>
</tr>
<tr>
<td>30</td>
<td>86</td>
</tr>
<tr>
<td>60</td>
<td>82</td>
</tr>
<tr>
<td>90</td>
<td>81</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Time Mins.</th>
<th>Percentage viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>30</td>
<td>92</td>
</tr>
<tr>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>90</td>
<td>88</td>
</tr>
</tbody>
</table>

These two tables give the results of two experiments (typical of 6) demonstrating the effects of a range of concentrations of paracetamol.
Figure 2: Effect of paracetamol on the viability of isolated hamster hepatocytes using trypan blue exclusion as the criterion of assessment.

Isolated hepatocytes ($10^6$/ml) were preincubated in the presence, ••••• or absence, ▼▼▼▼▼, of 25mM paracetamol for two hours, the medium was then changed for medium without paracetamol (indicated by arrow). Each point is the mean of three values and the experiment is typical of two carried out.
Figure 3: Effect of a range of concentrations of paracetamol on cell viability of hamster hepatocytes assessed by trypan blue exclusion.

Isolated hepatocytes (10^6/ml) were preincubated with paracetamol for two hours; ▼, control; ▼, 1mM; ■, 10mM; ○, 20mM. The medium was then changed for control medium (indicated by arrow). Each point is the mean of three estimations. This experiment is one typical of five.
4.2.2 Lactate dehydrogenase release

The second criterion investigated for viability assessment was leakage of the cytoplasmic enzyme lactate dehydrogenase from isolated hepatocytes. Lysis of the cells with Triton X100 enabled an estimation of the total intracellular lactate dehydrogenase. The percentage of the total cellular lactate dehydrogenase released into the extracellular medium was used as the index of viability. There was always some lactate dehydrogenase detectable in the extracellular medium which was due to either the presence of a small percentage of non-viable cells within the suspension, or to the foetal calf serum used in the medium which may have contained a small amount of the enzyme.

Table 2 shows the effect of a range of concentrations of paracetamol and acetanilide on the release of lactate dehydrogenase after one hour's incubation. There was a slight increase in the amount of lactate dehydrogenase detectable in the extracellular medium at concentrations greater than 10mM but the results were not very marked indicating that paracetamol and acetanilide were not very toxic to the plasma membranes of isolated rat hepatocytes within the conditions of the experiment.

Figure 4 demonstrates, however, that safrole had a much more marked effect on cell viability. At a concentration of 0.5mM there was little effect, with 1.0mM causing a slight increase in the extracellular lactate dehydrogenase. At higher concentrations of 5 and 10mM safrole caused a large release of lactate dehydrogenase demonstrating that safrole is relatively toxic to the rat hepatocytes at higher concentrations within a very short time of incubation.
Table 2: Effect of paracetamol and acetanilide on viability of isolated rat hepatocytes using lactate dehydrogenase release as the criterion of assessment

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>LDH released (% of total cellular LDH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paracetamol</td>
</tr>
<tr>
<td>0</td>
<td>23.5</td>
</tr>
<tr>
<td>2.5</td>
<td>25.0</td>
</tr>
<tr>
<td>5.0</td>
<td>23.5</td>
</tr>
<tr>
<td>7.5</td>
<td>-</td>
</tr>
<tr>
<td>10.0</td>
<td>25.0</td>
</tr>
<tr>
<td>15.0</td>
<td>-</td>
</tr>
<tr>
<td>20.0</td>
<td>28.5</td>
</tr>
<tr>
<td>40.0</td>
<td>32.0</td>
</tr>
</tbody>
</table>

Hepatocytes at 10^6 cells/ml were incubated for one hour with paracetamol or acetanilide at various concentrations. Samples of cells were taken at zero time to assess total cellular LDH. (One experiment typical of two.)
Figure 4: Effect of safrole on the viability of isolated rat hepatocytes using lactate dehydrogenase release as the criterion of assessment.

Hepatocytes were incubated at $10^6$ cells/ml as described in 'Methods'.

- Control; ▼——▼, 0.5mM; □——□, 1.0mM;

- ▼——▼, 5mM; □——□, 10mM safrole.

Each sample is the mean of two estimations. The results are from one experiment typical of two.
4.2.3 Comparison of trypan blue exclusion and lactate dehydrogenase release

Figures 5 to 8 give the results of a number of experiments where the effects on viability in terms of trypan blue exclusion and lactate dehydrogenase release were directly compared by measuring both parameters on the same incubation sample. Figure 5 demonstrates that paracetamol, on this occasion, exhibited a direct dose response effect in reducing the viability of isolated rat hepatocytes. The profile obtained for the effect of different concentrations of paracetamol on viability measured in terms of lactate dehydrogenase released from the cells was very similar to that for trypan blue exclusion.

Figure 6 gives the results of a comparison between the two criteria of plasma membrane integrity for hamster hepatocytes treated with paracetamol. The 1mM paracetamol dose was again more toxic than that at 10 or 20mM in terms of the trypan blue exclusion test and this was endorsed by the lactate dehydrogenase release measured on the same samples giving support to the results already discussed from Figure 3.

Phenobarbitone was demonstrated to reduce the viability of isolated rat hepatocytes by both criteria at a 20mM concentration (Figure 7).

Safrole (Figure 8) was also found to be very toxic at 10mM, by both criteria supporting the results from Figure 4. In this experiment the viability was zero according to the trypan blue exclusion method but only 75% according to lactate dehydrogenase released into the extracellular medium. This is probably due to some of the lactate dehydrogenase remaining within the non-viable cells which were removed from the medium by centrifugation prior to assaying the lactate dehydrogenase.

These results suggest that there was little difference between these two criteria of viability assessment. Both parameters provided a measure of irreversible plasma membrane damage. For a rapid assessment of viability trypan blue exclusion was the method preferred.
Figure 5: A comparison of the effect of paracetamol on trypan blue exclusion and the release of lactate dehydrogenase from isolated rat hepatocytes.

Hepatocytes ($10^6$/ml) were incubated as described in 'Methods'.

- Control; ■■■, 10mM; ○○○, 20mM

- , 40mM paracetamol.

Both assays were performed on the same incubation sample.
Figure 6: A comparison of the effect of paracetamol on trypan blue exclusion, and lactate dehydrogenase release from isolated hamster hepatocytes.

Hepatocytes ($10^6$/ml) were incubated as described in 'Methods'. Both assays were performed on the same sample.

Control; 1mM; 10mM; 20mM paracetamol.
Figure 7: A comparison of the effect of phenobarbitone on trypan blue exclusion and release of lactate dehydrogenase from isolated rat hepatocytes.

Hepatocytes (10⁶/ml) were incubated as described in 'Methods'. Both assays were performed on the same sample.

——, Control; ——, 1mM; ———, 10mM; ————, 20mM phenobarbitone.
Figure 8: A comparison of the effect of safrrole on trypan blue exclusion and the release of lactate dehydrogenase from isolated rat hepatocytes.

Hepatocytes (10^6/ml) were incubated as described in 'Methods'. Both assays were performed on the same sample.

- Control; ○-○, 0.1mM; □-□, 1mM; ●-●, 10mM safrrole.
4.2.4 Cell respiration

The rate of cellular respiration was next investigated as an index of viability. The amount of oxygen consumed per minute by a suspension of hepatocytes was determined using a calibrated Clark oxygen electrode. The effect of carbon tetrachloride was first examined as a positive control. Carbon tetrachloride is a well recognised hepatotoxin and when incubated with isolated rat hepatocytes at a concentration of 10,000 ppm (approx. 100mM) it has a dramatic effect on the viability of rat hepatocytes when assessed by trypan blue exclusion (see Figure 9). Figure 10 demonstrates that carbon tetrachloride at a concentration of about 3000 ppm (30mM) reduces the rate of respiration to about 25% of the control value within 15 minutes. Unfortunately there was only one Clark electrode available for these experiments hence all samples had to be run in rotation and no identical duplicates were possible.

Figure 11 demonstrates that paracetamol had a dose-dependent effect on cellular respiration of isolated rat hepatocytes. The 10mM and 40mM paracetamol reduced the rate of respiration to less than 50% of the initial control rate within twenty minutes of its addition to the cells.

The effects of paracetamol, phenobarbitone and safrole were then compared (see Figure 12). Safrole at a concentration of 1mM did not appear to reduce the rate of respiration whereas 20mM phenobarbitone and 40mM paracetamol had very similar marked effects on the rate of cellular respiration. The selection of the concentrations used in this comparative experiment was based on the similarity of effects of the three compounds on viability in terms of trypan blue exclusion.
Figure 9: Effect of carbon tetrachloride on the viability of isolated rat hepatocytes using trypan blue exclusion as the criterion of assessment.

Hepatocytes were incubated as described in 'Methods'.

\(\triangledown\triangledown\), Control; \(\circ\circ\), 10,000 ppm CCl\(_4\).
Isolated hepatocytes ($10^6$/ml) were incubated as described in 'Methods' in the presence of 3000ppm carbon tetrachloride (O—O), or absence (▼—▼). Oxygen consumption was determined using a Clark electrode.
Figure 11: Effect of paracetamol on the respiration of isolated rat hepatocytes

Hepatocytes 1.6 x 10^6 cells/ml were incubated as described in 'Methods' with: ▼▼, Control; ○○, 1mM paracetamol; □□, 10mM paracetamol; and ▼▼, 40mM paracetamol.
Oxygen consumption was determined using a Clark electrode.
Figure 12: Comparison of the effects of paracetamol, safrole and phenobarbitone on respiration of isolated rat hepatocytes.

Hepatocytes at $0.84 \times 10^6$ cells/ml were incubated as described in 'Methods'. ▼▼, Control; ○○, 40mM paracetamol; □□, 1mM safrole; ▼▼, 20mM phenobarbitone.

Oxygen consumption was determined using a Clark electrode.
4.2.5 7-Ethoxycoumarin O-deethylase activity

The xenobiotic metabolising capability of isolated hepatocytes was investigated as an index of viability using the activity of 7-ethoxycoumarin O-deethylase as the parameter.

The results shown in Figure 13 were from an experiment in which isolated rat hepatocytes were treated with different concentrations of paracetamol for one hour. The metabolites were then extracted and separated into the free and conjugated products of 7-hydroxycoumarin. The total activity of the O-deethylase metabolising enzymes is a composite of the free, sulphate and glucuronide metabolites. Paracetamol was observed to have a major influence on the total activity, a practically linear fall off in deethylolation with concentration occurred up to 20mM which then plateaued. Similar effects were seen with glucuronidation and sulphation.

In a subsequent experiment (Figure 14) isolated rat hepatocytes were incubated with different concentrations of paracetamol, and at time intervals 70nmole of 7-ethoxycoumarin was added to a sample and the incubation continued for 10 minutes. Only the total metabolites were measured however, instead of separating into glucuronide and sulphate conjugates. Again there was a nearly linear dose-dependent reduction in the activity of the 7-ethoxycoumarin O-deethylase and also a reduction with time. It would, therefore, appear that there is an effect, direct or indirect of paracetamol, on the viability of the metabolising enzyme system.

The effect of safrole was then examined at different concentrations (Figure 15). Safrole also has a direct dose-dependent effect on the activity of 7-ethoxycoumarin O-deethylase which did not appear to be very dependent on the time of exposure. Experiments were also carried out with paracetamol and safrole on the activity of 7-ethoxycoumarin O-deethylase activity in the presence or absence of exogenously added NADPH. A reduction in cellular NADPH is associated with loss of plasma membrane integrity and here addition of excess NADPH might have enabled an increase in the enzymic activity of 7-ethoxycoumarin O-deethylase. However, the addition of NADPH did not appear to have any effect on the production of metabolites by the hepatocytes.
Figure 13: Effect of paracetamol on 7-ethoxycoumarin O-deethylation activity in isolated rat hepatocytes

Hepatocytes were incubated as described in 'Methods' at $2 \times 10^6$ cells/ml for one hour with 7-ethoxycoumarin. The metabolites were then extracted to separate: ▼—▼, free; ○—○, sulphate conjugated and □—□, glucuronide conjugated metabolites.
Figure 14: Effect of paracetamol on 7-ethoxycoumarin O-deethylase activity in isolated rat hepatocytes

Hepatocytes were incubated at $2 \times 10^6$/ml. At 30 minute intervals 70nmole 7-ethoxycoumarin was added and the incubations continued for 10 minutes. The results are expressed as total metabolites and are the mean of duplicate determinations.
Hepatocytes were incubated at $2 \times 10^6$/ml. At 30 minute intervals 70nmole 7-ethoxycoumarin was added and the incubations continued for 10 minutes. The results are expressed as total metabolites and are the mean of duplicate determinations.
4.3 THE EFFECT OF EXOGENOUS CHEMICALS ON PROTEIN SYNTHESIS IN ISOLATED HEPATOCYTES

The amount of protein synthesis was assessed in isolated hepatocytes by measuring the incorporation of $[^{14}C]$ -Leucine into trichloroacetic acid insoluble proteins. The aim was to determine the effect of paracetamol and other xenobiotics on protein synthesis after a relatively short exposure time.

Preliminary experiments were carried out to determine the ideal cell concentration and incubation time necessary to obtain a measurable incorporation of radioactivity into cellular proteins and a linear response with time. Figure 16 demonstrates that $[^{14}C]$ -Leucine, added at a concentration of 100nCi to each incubation sample containing 0.5, 1 or $2 \times 10^6$ hepatocytes per ml, was incorporated into the cellular protein in a linear fashion for the first 60 minutes of incubation. Between 60 and 90 minutes there was a slight fall off in the incorporation with time. There was also a good correlation between cell number and the amount of radioactivity incorporated over the first 60 minutes of incubation. An incubation sample containing $2 \times 10^6$ cells in one ml of L15 medium plus 10% foetal calf serum and 100nCi $[^{14}C]$ -Leucine treated as described in the methods section after one hour's incubation gave values of incorporation into proteins in excess of 400dpm (per 250 μl sample). As the incorporation was linear up to one hour, and was well in excess of background levels of radioactivity, these conditions were adopted for the incubations in the majority of the following experiments.

In order to determine whether or not reduction in the incorporation of radioactivity was likely to be a valid indicator of protein synthesis inhibition, cycloheximide, a well documented inhibitor of protein synthesis, was added at a range of concentrations to isolated hepatocytes and the amount of $[^{14}C]$ -Leucine incorporated after one hour's incubation was then determined. Figure 17 demonstrates that concentrations of cycloheximide greater than $10^{-8}$M caused a marked decrease in the incorporation of labelled amino acid. Inhibition was greater than 90% at $10^{-5}$M. At very low concentrations ($<10^{-10}$M), however, there was a tendency for the rate of incorporation to be increased slightly above the control value.
The effect of a number of xenobiotics on protein synthesis in isolated hepatocytes was investigated.

Figure 18 demonstrates that 40mM paracetamol inhibited protein synthesis by greater than 90%, with 50% inhibition occurring when isolated rat hepatocytes were incubated with about 9mM paracetamol for one hour. The results given here for paracetamol give the means and standard deviations from three separate experiments where each sample was run in triplicate. In one of the experiments the concentrations of paracetamol used included dose levels down to 0.1mM. In this experiment there was an indication that 0.1mM caused an increase in the rate of protein synthesis. A couple of experiments using low levels of paracetamol ranging from $5 \times 10^{-4}$ to $5 \times 10^{-2}$mM were carried out to determine whether this was a reproducible effect. In the first of these protein synthesis was increased at all the concentrations with the greatest increase occurring at $2.5 \times 10^{-3}$mM. In the second experiment using the same range of concentrations of paracetamol there was no increase in the rate of protein synthesis but instead a slight dose-related decrease. It is of interest to note that the incorporation of labelled amino acid into the hepatocytes in the control incubations was nearly doubled in the latter of these experiments.

Phenacetin was a more effective inhibitor of protein synthesis in isolated rat hepatocytes with 50% inhibition ($ID_{50}$) occurring at a 2.5mM concentration (Figure 19). The $ID_{50}$ values for acetanilide, aniline hydrochloride, phenobarbitone, safrole and diethyl maleate were about 7.5, 11.0, 3.75, 1.4 and 2.0mM respectively (see Figures 20-24).

The effect of paracetamol on protein synthesis was greater in hamster hepatocytes (4mM) than in rat hepatocytes (Figure 25). There was also evidence that a concentration of 0.1mM increased the rate of protein synthesis.

In order to determine whether paracetamol or its metabolites were inhibiting protein synthesis rats were pretreated with phenobarbitone by intraperitoneal injection of 70mg/kg daily for three days prior to sacrificing the animals and isolating the hepatocytes. The aim of the phenobarbitone pretreatment was to induce the drug metabolising enzymes of the liver. After the cells were isolated the cytochrome P450 content
of the cells was estimated rapidly and found to be increased in the phenobarbitone pretreated cells. The ID$_{50}$ for the phenobarbitone treated cells was found to be slightly lower than the untreated controls, the mean values from three experiments being about 7 and 9mM respectively (Figure 26). There was a significant difference ($p<0.01$) between the percentage of inhibition at a 10mM concentration of paracetamol but not at any of the other concentrations investigated.

Further experiments were carried out with paracetamol to investigate in more detail its effects on protein synthesis, degradation and secretion.

To investigate the effect on protein degradation isolated hepatocytes were incubated with $[^{14}C]$-Leucine prior to treatment with paracetamol. The hepatocytes were incubated for one hour as previously described then separated from the medium and washed twice with unlabelled medium to remove any residual label not incorporated into the hepatocytes. Samples were processed at this stage to determine how much label had been incorporated into the hepatocytes. The hepatocytes were then incubated with a range of paracetamol concentrations for a further hour. After one hour the amount of label incorporated into the protein of control incubations was reduced to 67% of the initial value indicating that degradation of the proteins was occurring. In samples containing 40mM paracetamol, however, the amount of radioactivity was reduced to only 85% of the initial level after prelabelling. It would appear, therefore, that paracetamol also has an affect on protein degradation.

Depletion of reduced glutathione is known to increase the potential toxicity of paracetamol (see Chapter 1). Pretreatment of rats with diethyl maleate (0.05ml/kg) by intraperitoneal injection thirty minutes prior to sacrifice of rats and preparation of isolated hepatocytes had no effect on the rate of protein synthesis inhibition by paracetamol, compared to its effect on hepatocytes prepared from untreated rats. Experiments to be described later demonstrate that incubation of isolated hepatocytes with L15 plus 10% foetal calf serum increases the level of reduced glutathione within the cells. Isolated rat hepatocytes were, therefore, incubated with control medium for one hour prior to adding paracetamol. Again paracetamol produced no marked differences in effect on protein synthesis inhibition.
In order to investigate whether the effect of paracetamol on protein synthesis in isolated rat hepatocytes was reversible they were pre-incubated with 40mM paracetamol for one hour to maximally inhibit protein synthesis, in the absence of labelled amino acid. The cells were washed to remove the paracetamol, control cells were washed in a similar manner. The treated and control cells were then incubated with medium containing labelled amino acid and the rate of incorporation of label into the hepatocytes was monitored at 5 minute intervals for 30 minutes. The rate of protein synthesis in the paracetamol treated hepatocytes was less than that of the control cells (see Figure 27). At 30 minutes the rate of incorporation in the paracetamol pretreated hepatocytes was about 25% of the controls, a less marked inhibition than was seen in the presence of paracetamol, indicating that the effect of 40mM paracetamol is probably reversible if the cells are only pretreated for one hour.

An experiment was carried out to determine the rate of uptake of $[^{14}\text{C}]$-Leucine into isolated rat hepatocytes. Hepatocytes were incubated with labelled medium, samples were removed from the incubator at 5 minute intervals up to 30 minutes and at 45 and 60 minutes. The cells were immediately spun to a pellet and washed to remove any excess label not incorporated into the cells. The protein was then precipitated with 10% trichloroacetic acid. To determine the amount of labelled amino acid incorporated into the cell samples of the trichloroacetic acid supernatant were counted for radioactivity content. The amount of radioactivity present in protein was estimated as previously described. The level of amino acid present in the soluble fraction of the cells was very high ($>3000\text{dpm }[^{14}\text{C}]$-Leucine) within 5 minutes. This level of labelled amino acid remained stable thereafter for the 60 minute incubation period. Incorporation of labelled amino acid into the protein of the hepatocytes was linear and time dependent as described previously. This experiment was then repeated in the presence of paracetamol (40mM) to determine whether it had any effect on the uptake of amino acids into the hepatocytes. The results for the control untreated cells were very similar to those in the previous experiment. Similarly there was no difference in the rate of uptake of labelled amino acid into the soluble amino acid pool of the hepatocytes between the paracetamol treated and control hepatocytes. Paracetamol, however, at the concentration of 40mM used here inhibited
protein synthesis as shown previously but this inhibition was apparent from as early as 5 minutes.

The effect of paracetamol on protein secretion was investigated by preincubating isolated rats hepatocytes pooled from two rats (138 x 10^6 cells) in 20ml of medium with 2μCi [^{14}C] -Leucine for 30 minutes. The cells were then carefully washed, diluted and separated into two control and two paracetamol (40mM) treated samples as described in the methods. After one hour's incubation the cells were spun to a pellet and the medium removed carefully. The proteins in the medium were precipitated with trichloroacetic acid then processed in the normal way. The mean values were calculated for the amount of radioactivity present in the control and treated samples. The paracetamol treated samples contained only 39% of the radioactivity contained in the control indicating therefore, that paracetamol inhibits protein secretion.

To investigate further the early effect on protein synthesis inhibition isolated rat hepatocytes were incubated with levels of paracetamol, safrole and phenobarbitone which would inhibit protein synthesis by about 50% (10mM, 1mM and 5mM respectively). The incorporation of [^{14}C] -Leucine was monitored from 5 to 60 minutes at intervals and it was apparent that all three compounds inhibited protein synthesis within 10 minutes (see Figure 28).

Three experiments were conducted to determine whether the effects of paracetamol on isolated hepatocytes could be reproduced in vivo. For each experiment there were three rats designated controls and injected intraperitoneally with 2μCi/70g body weight of [^{14}C] -Leucine, and three rats injected intraperitoneally with 1000mg/kg paracetamol and 2μCi/70g body weight [^{14}C] -Leucine. The animals were sacrificed one hour after the injections, the livers were removed and homogenised as described in Chapter 3. Samples of the homogenate were used to determine the amount of labelled protein. The amount of label incorporated into the livers of the paracetamol treated rats was 80%, 83.5% and 93% of the control respectively for the three experiments. The standard deviations for each experiment were large and these values would not be statistically significant. There was, however, an obvious trend towards an inhibition of protein synthesis
by paracetamol. The dose of paracetamol was equivalent to about 50 times the human therapeutic dose and caused central nervous system depression in the treated rats which was observed to be partially reversible in some of the animals within the later period of time after injection.
Figure 16: Effect of cell concentration on the incorporation of $[14C]$-Leucine into protein of isolated rat hepatocytes

Hepatocytes were incubated as described in 'Methods' at the following concentrations: $0.5 \times 10^6$/ml, $\n$, $10^6$/ml, $2 \times 10^6$/ml. (Each point in the mean of three estimations.)
Hepatocytes were incubated as described in 'Methods' for one hour. 
$[^{14}C] -$Leucine incorporation was estimated from duplicate samples for each concentration.
Figure 18: Effect of various doses of paracetamol on $^{14}$C-Leucine incorporation into proteins of isolated rat hepatocytes after one hour of incubation.

Experimental conditions were as described in 'Methods'. $^{14}$C-Leucine incorporation was estimated in triplicate for each concentration investigated. Values are means ± S.D. for three experiments.
Figure 19: Effect of phenacetin on $^{14}$C-Leucine incorporation into proteins of isolated rat hepatocytes after one hour of incubation

Experimental conditions were as described in 'Methods'. $^{14}$C-Leucine incorporation was estimated in duplicate for each concentration. Values are means ± S.D. for two experiments.
Figure 20: Effect of acetonilide on the incorporation of $^{14}$C-Leucine into proteins of isolated rat hepatocytes after one hour of incubation.

Experimental conditions were as described in 'Methods'. $^{14}$C-Leucine incorporation was estimated in duplicate for each concentration. Values are means ± S.D. for two experiments.
Figure 21: Effect of aniline hydrochloride on the incorporation of $^{14}$C-Leucine into proteins of isolated rat hepatocytes after one hour of incubation.

Experimental conditions were as described in 'Methods'. $^{14}$C-Leucine incorporation was estimated in duplicate for each concentration. Values are means ± S.D. for two experiments.
Figure 22: Effect of phenobarbitone on the incorporation of $^{14}\text{C}$-Leucine into protein of isolated rat hepatocytes after one hour of incubation.

Experimental conditions were as described in 'Methods'. $^{14}\text{C}$-Leucine incorporation was estimated in triplicate for each concentration investigated. Values are means ± S.D. for two experiments.
Figure 23: Effect of safrole on the incorporation of $^{14}$C-Leucine into protein of isolated rat hepatocytes after one hour of incubation.

Experimental conditions were as described in 'Methods'. $^{14}$C-Leucine incorporation was estimated in triplicate for each concentration investigated. Values are means ± S.D. for two experiments.
Figure 24: Effect of diethyl maleate on the incorporation of \( ^{14} \text{C} \)-leucine into protein of isolated rat hepatocytes after one hour of incubation.

Experimental conditions were as described in 'Methods'. \( ^{14} \text{C} \)-leucine incorporation was estimated in triplicate for each concentration investigated.
Figure 25: Effect of paracetamol on the incorporation of $[^{14}\text{C}]$-Leucine into proteins of isolated hamster hepatocytes after one hour of incubation.

Experimental conditions were as described in 'Methods'. $[^{14}\text{C}]$-Leucine incorporation was estimated in duplicate for each concentration. Values are means ± S.D. for two experiments.
Figure 26: Effect of paracetamol on $^{14}\text{C}$-Leucine incorporation into hepatocytes isolated from rats treated with phenobarbitone in vivo.

Experimental conditions were as described in 'Methods'. $^{14}\text{C}$-Leucine incorporation was estimated in triplicate for each concentration investigated. Values are means ± S.D. for three experiments. ○○, Control; ••, phenobarbitone pretreated.
Figure 27: To determine whether the effect of 40mM paracetamol on $[^{14}C]$-Leucine incorporation into isolated rat hepatocytes is reversible.

Experimental conditions were as described in 'Methods'. $[^{14}C]$-Leucine incorporation was estimated in triplicate for each time point. ○○○, Control; - - - , paracetamol treated.
Experimental conditions were as described in 'Methods'. The concentration of chemicals chosen was that which gave approximately a 50% inhibition after one hour. \(\triangle\), Control; \(\circ\), 10mM paracetamol; \(\bullet\), 5mM phenobarbitone; \(\square\), 1mM safreole. Results are the mean of duplicate samples.
4.4 DETERMINATION OF THE SPECIFICITY OF THE ACTION OF PARACETAMOL ON PROTEIN SYNTHESIS

In order to determine whether paracetamol had an effect on specific proteins rather than a general inhibitory effect on protein synthesis attempts were made to isolate a microsomal fraction from isolated rat hepatocytes with an aim to carrying out SDS polyacrylamide gel electrophoresis.

4.4.1 The preparation of microsomes from isolated rat hepatocytes

A number of different techniques were investigated including homogenisation with a Potter-Elvehjem, a Dounce homogeniser, a Polytron, and two types of sonicator - a sonic probe and a sonic bath. It was found with both the Potter-Elvehjem and the Dounce homogeniser that even with a large number of strokes with the pestle the hepatocytes still remained largely intact when examined microscopically. The Polytron was found to be more successful in disrupting the hepatocytes therefore a number of experiments were conducted to determine the ideal time for homogenisation.

After the exposure of each hepatocyte suspension to the Polytron the activity of NADH cytochrome C reductase was measured in the homogenates. Table 3 gives the individual values for the activities obtained from two experiments. In the first experiment activities were measured after 10, 30 and 60 second exposures in the presence and absence of rotenone with the results expressed in nmole/min/10^6 cells. In the second experiment activities were also measured after a 5 second exposure, activities were expressed in nmole/min/mg protein. The total enzyme activity available within the cells was obtained by treating the cells with Triton X100 which totally disrupts the cells and the intracellular organelles. In the first experiment a 10 second exposure released the maximal activity from the cells with greater exposure times causing a decrease in activity. In the second experiment maximal activity was released within 5 seconds with a marked reduction in activity by 10 seconds. The decrease in activity was probably due to the severe shearing forces and heat produced by the Polytron causing denaturation of the enzyme. It was apparent from the results that a very short period of homogenisation time was required to disrupt the cells although the effective time was variable, as were the results within each time point for each separate
A larger sample of hepatocytes containing $74 \times 10^6$ cells in 5ml was homogenised for 10 seconds with the Polytron. The homogenate was then centrifuged at 10,000g for 20 minutes followed by 105,000g for 1 hour to isolate the 105,000g pellet. At the end of the second centrifugation the pellet was barely visible to the naked eye. The use of the Polytron was, therefore, abandoned as unsatisfactory.

Homogenisation with a sonic probe proved to be more effective in terms of disrupting the cells. The probe that was available, however, had inherent problems due to its large size and the necessity for a container for the suspension of hepatocytes to have a diameter only slightly greater than the probe itself. These restrictions meant that only very small volumes could be sonicated at one time, and there was also a percentage of material lost during sonication.

The most effective method was found to be the sonic bath. Samples of isolated hepatocytes containing $10^6$ cells/ml in 10ml of buffer were placed in a 10ml conical flask. A range of sonication times were tried and 60 seconds was found to be enough to disrupt the majority of the cells when observed microscopically. A 10ml suspension of rat hepatocytes was then homogenised for 60 seconds followed by centrifugation at 10,000g and 105,000g to produce a small pellet which was resuspended in tris/ETDA buffer to give a concentration of 10mg microsomal protein per ml. The activity of 7-ethoxycoumarin O-deethylase was measured in this microsomal pellet and found to be 10.25nmoles/10 min/0.05mg protein. This activity was similar to that found in a control microsomal preparation (12.05nmoles/10 min/0.05mg protein) assayed at the same time. The control microsomes had been prepared from whole rat liver by someone experienced in the standard method.

Samples of the microsomal fraction, derived from the cells treated in the sonic bath, were applied to an SDS polyacrylamide gel for electrophoresis to separate the proteins into bands according to their respective molecular weights. Even though a small amount of protein (5 g per application) was applied to the gel the resulting separation was complex and difficult to interpret. The fraction was not a pure microsomal pellet, and probably contained fragmented membranes derived from other organelles due to the non-selectivity of the sonication. Hence further
separation of the pellet would have been necessary to gain any more information. This, however, would have proved difficult as the original pellet obtained was so small.

An alternative technique was considered instead of the SDS polyacrylamide gel electrophoresis, which although less specific would distinguish between intracellular proteins produced for export and those produced for other purposes to be utilised intracellularly. This technique could potentially provide an answer to the question as to whether the effect of paracetamol was specific for either of the two major classes of protein or purely a non-specific action.

This alternative technique involved conjugating anti-rat serum to cyanogen bromide activated Sepharose. The Sepharose conjugate was incubated with samples derived from isolated hepatocytes which had been labelled with $^{14}$C-Leucine, with or without prior treatment with paracetamol. These samples were also incubated with activated sepharose conjugated with anti-rabbit serum which acted as a control by quantifying the amount of non-specific binding.

Table 4 gives the results obtained from this experiment. Lysis of the washed cell samples with lubrol after incubation with $^{14}$C-Leucine released all the cellular protein. Scintillation counting of the Sepharose beads after overnight incubation with the cell samples demonstrated that paracetamol inhibited the amount of labelled protein, and hence protein synthesis, which was available to bind to the anti-rat serum conjugate from 412.4 to 39.15dpm/10^6 cells. This represents the export proteins present within the cells. The labelled proteins in the incubation not bound to the anti-rat serum conjugate represent the non-export proteins which were also inhibited from 387.5 in the control sample to 21.25dpm/10^6 cells in the paracetamol treated samples. Hence it would appear that paracetamol inhibits the synthesis of both groups of proteins to a similar extent.

After the two hour incubation of isolated hepatocytes with or without paracetamol, the incubation medium was also retained. The protein present in the medium, which represents the protein exported during the two hour incubation was precipitated from the medium and solubilised in NaOH.
These samples were also incubated with the activated Sepharose conjugates. There was a greater amount of non-specific binding to the anti (rabbit serum) conjugate for these samples. There was also some labelled protein which did not bind to the anti (rat-serum) conjugate which was probably derived from cells damaged or dying during the two hour incubation period. Allowing for these factors it would still appear that isolated rat hepatocytes produced export proteins which they secreted, either actively or passively, and that the production of these export proteins was inhibited by paracetamol.
Table 3: Effect of homogenisation time with a Polytron on the activity of NADH cytochrome C reductase

<table>
<thead>
<tr>
<th>Homogenisation time (sec.)</th>
<th>NADH cytochrome C reductase</th>
<th></th>
<th></th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Rotenone Insensitive</td>
<td>nmole/min/10^6 cells</td>
<td>nmole/min/mg protein</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>51.1</td>
<td>51.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>73.1</td>
<td>73.1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>68.0</td>
<td>49.0</td>
<td>57.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>63.0</td>
<td>52.0</td>
<td>36.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>71.0</td>
<td>47.0</td>
<td>52.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>44.4</td>
<td>36.9</td>
<td>48.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>15.9</td>
<td>15.5</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>28.7</td>
<td>20.9</td>
<td>28.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TX 100</td>
<td>58.0</td>
<td>49.0</td>
<td>76.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated cells</td>
<td>60.0</td>
<td>50.0</td>
<td>77.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hepatocytes (2 x 10^6/ml) in sucrose/tris/EDTA buffer pH 7.4 were exposed to different times of homogenisation using a Polytron. NADH cyt.C reductase activity was measured in the resulting suspensions. The results from two experiments are given here. Treatment of the cells with TX 100 gave the total enzyme activity available within the hepatocytes.
Table 4: The effect of paracetamol (40mM) on the synthesis of intracellular and extracellular proteins in isolated rat hepatocytes

<table>
<thead>
<tr>
<th></th>
<th>$[^{14}C]$ -labelled protein (BOUND)</th>
<th>$[^{14}C]$ -labelled protein (NOT BOUND)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a) anti(rat serum) conjugate</td>
<td>(b) anti(rabbit serum) conjugate</td>
</tr>
<tr>
<td></td>
<td>DPM/10^6 cells</td>
<td>DPM/10^6 cells</td>
</tr>
<tr>
<td><strong>INTRACELLULAR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROTEINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>412.4 ± 19.1</td>
<td>84.15 ± 52.49</td>
</tr>
<tr>
<td>Paracetamol treated</td>
<td>39.15 ± 19.14</td>
<td>49.97 ± 2.36</td>
</tr>
<tr>
<td><strong>EXTRACELLULAR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROTEINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1838 ± 129</td>
<td>1446.65 ± 18.87</td>
</tr>
<tr>
<td>Paracetamol treated</td>
<td>1286 ± 33</td>
<td>1314.97 ± 143.79</td>
</tr>
</tbody>
</table>

Experimental conditions were as described in the 'Methods'. The proteins bound to the antisera conjugates are export proteins, those not bound constitute the remaining protein produced by the cell. The anti(rabbit serum) conjugate samples were used as a control to determine the level of non-specific binding. The results are the mean ± SD for duplicate determinations.
4.5 THE EFFECT OF PARACETAMOL AND OTHER XENOBIOTICS ON RNA SYNTHESIS

RNA synthesis was measured by monitoring the uptake of $^{14}C$-Uridine into the TCA insoluble fraction of isolated hepatocytes. Figure 29 demonstrates that the rate of uptake of $^{14}C$-Uridine increased with time up to 30 minutes and then became linear. In this experiment different cell concentrations were investigated to determine which would be the ideal concentration for further experiments. The concentrations used were 0.5, 1, 1.5 and $2 \times 10^6$ cells/ml. There was a direct relationship between the amount of label incorporated into the cells and the cell concentration. The levels achieved for the $1.5 \times 10^6$ cells did not quite fit the general pattern and this could be explained by a slight inaccuracy in the original cell dilution. The ratio of uptake in the 0.5 and $2.0 \times 10^6$ cells/ml samples was approximately four fold at 90 minutes. Since the uptake was still linear with the highest cell concentration this was utilised for further experiments as there would be less inaccuracy in the techniques due to the higher activity of the samples.

The effects of a range of concentrations of paracetamol, phenobarbitone and safrole on the incorporation of $^{14}C$-Uridine into isolated hepatocytes are shown in Figures 30-32. The concentrations which resulted in a 50% inhibition of RNA synthesis after one hour's incubation were about 5mM, 6mM and 1.2mM for paracetamol, phenobarbitone and safrole respectively. It is of interest to note in Figure 32 the lower concentrations of safrole caused an increase in RNA synthesis rather than an inhibition.
Figure 29: The effect of cell concentrations on the incorporation of $^{14}$C-Uridine into RNA of isolated rat hepatocytes.

Experimental conditions were as described in 'Methods'.

- $\downarrow$, $0.5 \times 10^6$ cells/ml;
- $\bigtriangleup$, $10^6$ cells/ml;
- $\blacklozenge$, $1.5 \times 10^6$ cells/ml;
- $\square$, $2 \times 10^6$ cells/ml.
Figure 30: Effect of paracetamol on the incorporation of \([^{14}\text{C}]\) -Uridine into the ribonucleic acids of isolated rat hepatocytes

Experimental conditions were as described in 'Methods'. \([^{14}\text{C}]\) -Uridine incorporation was estimated in triplicate for each concentration. Values are means \(\pm\) S.D. for two experiments.
Figure 31: Effect of phenobarbitone on the incorporation of $^{14}$C-Uridine into the ribonucleic acids of isolated rat hepatocytes

Experimental conditions were as described in 'Methods'. $^{14}$C-Uridine incorporation was estimated in triplicate for each concentration.
Figure 32: Effect of safrole on the incorporation of $[^{14}\text{C}]$-Uridine into the ribonucleic acids of isolated rat hepatocytes

Experimental conditions were as described in 'Methods'. $[^{14}\text{C}]$-Uridine incorporation was estimated in triplicate at each concentration.
4.6 COVALENT BINDING

Isolated hepatocytes were incubated with [14C]-paracetamol at the same percentage concentration in all samples. Samples of hepatocytes in duplicate were incubated for 5, 10, 20, 30, 40 and 60 minutes. On removal from the incubator the cells were spun to a pellet and perchloric acid was added to precipitate the protein. Repeated washing was carried out with the perchloric acid to ensure removal of all the unbound label. The final precipitate was then solubilised in NaOH and the samples counted in a scintillation counter. The results (Figure 33) indicate that the majority of the binding occurred within the first five minutes of incubation. There also appears to be a direct correlation between the concentration of paracetamol and the amount of covalent binding.
Figure 33: Effect of paracetamol concentration on the covalent binding of $[^{14}C]$-paracetamol to cellular perchloric acid-insoluble material of isolated rat hepatocytes.

Experimental conditions were as described in 'Methods'. Covalent binding was estimated in duplicate for each time point.
4.7 EFFECTS ON REDUCED GLUTATHIONE

Depletion of the level of glutathione within the liver is known to be closely linked with the toxicity of many xenobiotics, depletion often resulting in increased toxicity. The effects of various chemicals on the levels of glutathione were, therefore, investigated in isolated hepatocytes. The assay used was based on the method of Saville (1958) and is described fully in Chapter 3.

Initial experiments were carried out to determine the level of glutathione in isolated rat hepatocytes, to determine whether or not the levels remained stable during incubation, and to compare these findings with the levels present in the whole liver prior to its use in the isolation procedure. Two such experiments were carried out. In the first the level of glutathione in the freshly isolated hepatocytes was 9.0 μg/10^6 cells, which then increased over a 90 minute incubation period in L15 + 10% foetal calf serum to 14.5 μg/10^6 cells. If it is assumed that one gram of whole wet liver is equivalent to 120 x 10^6 hepatocytes these two values become 1.08mg/g liver at zero incubation time and 1.74mg/g liver after 90 minutes incubation time. The glutathione level in the whole wet liver was found to be 1.6mg/g. It would, therefore, appear that the isolation procedure depletes the level of glutathione which then is repleted during subsequent incubation with a full nutrient media.

In a second experiment following a similar procedure to above the levels in the isolated hepatocytes and whole liver of glutathione were both slightly lower (0.79mg/g and 1.19mg/g in hepatocytes and whole liver respectively) than previously. The pattern of depletion and repletion of the glutathione levels was, however, the same.

Figure 34 shows the effect of paracetamol on the glutathione level of isolated hepatocytes. There was a dose related response where all the concentrations effectively depleted the glutathione levels although only the 40mM concentration reduced the level below that of the initial value at zero time. The 40mM paracetamol also only had a marked effect between 30 and 45 minutes of incubation. The effects of 4 and 20mM phenobarbitone are presented in Figure 35. Both concentrations reduced the rate at which the levels of glutathione were increased in the control hepatocytes but
there was no evidence of a dose-related relationship in the effects of phenobarbitone. Neither concentration was as effective as the 40mM paracetamol at reducing the levels of glutathione.

Figure 36 demonstrates the effects of 1 and 10mM safrole. There was a consistent increase in glutathione levels in the control with the level nearly doubling during the 60 minute incubation time. The 1mM safrole treated cells were maintained at the initial level of glutathione whereas 10mM safrole caused a nearly linear reduction to about 50% of the initial value at zero time and 25% of the glutathione level in the control at 60 minutes.

Diethyl maleate is known to deplete glutathione and is used as an experimental tool in vivo for that purpose. Figure 37 shows the effects of diethyl maleate on the glutathione levels of isolated rat hepatocytes. Depletion of the glutathione level occurred at all three dose levels within the first fifteen minutes of incubation. Earlier time points which were not monitored might have indicated an even greater depletion. After 15 minutes, however, the levels increased again quite rapidly although they only increased above the initial level at zero time in the 10μM treated samples.
Figure 34: Effect of paracetamol concentration on reduced glutathione levels in isolated rat hepatocytes

Experimental conditions were as described in 'Methods'. The assay was carried out with duplicate determinations. ▲▲, Control; ○○, 10mM; ▲▲, 20mM; ●●, 40mM paracetamol.
Figure 35: Effect of phenobarbitone concentration on reduced glutathione levels in isolated rat hepatocytes

Experimental conditions were as described in 'Methods'. The results expressed here are from two experiments where each estimation was carried out in duplicate. ▼—▼, Control; ○—○, 4 mM; □—□, 20 mM phenobarbitone.
Figure 36: Effect of safrole on reduced glutathione levels in isolated rat hepatocytes

Experimental conditions were as described in 'Methods'. The results expressed here are from two experiments where each estimation was carried out in duplicate. ▼▼, Control; □□, 1mM; □□, 10mM safrole.
**Figure 37:** Effect of diethyl maleate concentration on the levels of reduced glutathione in isolated rat hepatocytes

Experimental conditions were as described in 'Methods'. Each value is the mean of duplicate determinations. ✷️, 10 μM; ○, 20 μM; ●, 30 μM; ■, 40 μM diethyl maleate.
4.8 EFFECTS ON ADENOSINE TRIPHOSPHATE (ATP)

There were a number of problems experienced with the assay of ATP levels in isolated rat hepatocytes. The fluorimetric method that was first attempted provided no useful results as it was too insensitive. Adaptation of the method to a UV spectrophotometric method which could be assayed on a very sensitive double beam spectrophotometer, however, enabled some preliminary experiments to be carried out. Due to the shortage of materials for the assay the experiments were limited to investigating the effects of one concentration for each of paracetamol, phenobarbitone and safrole. Table 5 gives the results from the two experiments carried out. The level of ATP is expressed in nmoles/10^6 cells for each treatment, and as a percentage of the control. Paracetamol (40mM) and phenobarbitone (20mM) both caused a very similar reduction in the ATP level of about 40% with safrole (10mM) having a more marked effect with a 17% reduction. These measurements were carried out on samples where the effect of the exogenous compounds were neutralised within two minutes by the addition of perchloric acid. The effects on the ATP levels were, therefore, very rapid.
Table 5: Comparison of the effect of paracetamol, phenobarbitone and safrole on ATP levels in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATP (nmol/10^6 cells) at 2 minutes</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.25 ± 1.42</td>
<td>100</td>
</tr>
<tr>
<td>Paracetamol (40mM)</td>
<td>6.28 ± 0.77</td>
<td>38.64</td>
</tr>
<tr>
<td>Phenobarbitone (20mM)</td>
<td>6.75 ± 1.21</td>
<td>41.53</td>
</tr>
<tr>
<td>Safrole (10mM)</td>
<td>2.79 ± 0.93</td>
<td>17.16</td>
</tr>
</tbody>
</table>

Experimental conditions were as described in the 'Methods'. Each sample was repeated in duplicate. The results are the means ± SD from two experiments.
4.9 EFFECTS ON CYCLIC ADENOSINE MONOPHOSPHATE (Cyclic AMP)

Cyclic AMP was assayed by radioimmunoassay using a kit supplied by the Radiochemical Centre (Amersham). The method for producing the standard curve was sensitive and very reproducible. Problems, however, were encountered with the preparation of the samples from the isolated hepatocytes for use in the assay. The values obtained in the preliminary experiments were all very high based on those quoted in the literature. The main reason for this was probably the complex nature of the medium used for incubating the cells and the acid used to precipitate the proteinacious material. It was found that if trichloroacetic acid fraction was adjusted to 0.1N HCl and then exhaustively extracted five times with water saturated ether the samples were cleaner and the freeze dried material much less in quantity. When this method had been perfected it was found that the results were more reproducible and reflected levels of cyclic AMP reported in the literature. An experiment was carried out to ensure that the level of cyclic AMP obtained was directly related to the number of cells in the original sample by diluting samples to determine whether the levels were the same or whether they decreased. If impurities had been present in the sample dilution this would have been expected to reduce their effect. This, however, was found not to be the case if the exhaustive extraction procedure was employed.

The results in Table 6 are from an experiment to determine the effects of the paracetamol, phenobarbitone and safrole, used at the same concentration as in the ATP experiments, on cyclic AMP levels. It is evident that paracetamol and phenobarbitone have minimal effects. Safrole appears to have a significant effect in reducing the level of cyclic AMP after a thirty minute incubation period.
Table 6: Comparison of the effects of paracetamol, phenobarbitone, and safrrole on cyclic AMP levels in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclic AMP pmol/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Control</td>
<td>2.3</td>
</tr>
<tr>
<td>40mM Paracetamol</td>
<td>1.75</td>
</tr>
<tr>
<td>20mM Phenobarbitone</td>
<td>1.33</td>
</tr>
<tr>
<td>10mM Safrrole</td>
<td>1.55</td>
</tr>
</tbody>
</table>

Cyclic AMP was determined by radioimmunoassay, details of the method are in Chapter 3. The results are the means of duplicate determinations.
4.10 INVESTIGATIONS WITH PRIMARY MONOLAYER CULTURES OF ADULT RAT HEPATOCYTES

Suspensions of isolated hepatocytes provide an ideal system for investigating those effects of xenobiotics on the liver in vitro which are evident in short time periods of up to three or four hours. If hepatocytes, however, are incubated for longer periods their viability and functional capacity will deteriorate and any similarity to the in vivo situation will become increasingly remote. The use of primary monolayer cultures enables longer periods of investigation of up to several days. Hepatocytes in monolayer culture attach to a culture surface in a manner which resembles the in vivo appearance of hepatocytes. The cells spread out, adopting "hexagonal" shapes, forming sheets of cells with evidence of structures similar to bile canaliculi. Figure 38 is an example of a monolayer culture of adult rat hepatocytes after two days in culture, stained with haematoxylin and eosin. A number of parameters have been investigated in culture the results of which will now be presented.
Figure 38: Primary monolayer culture of isolated rat hepatocytes after 48 hours in culture stained with haematoxylin and eosin (x400)
4.10.1 The effects of xenobiotics on protein synthesis in cultures

It has already been demonstrated that paracetamol inhibits the rate of protein synthesis in vitro in freshly isolated hepatocyte suspensions. Experiments were carried out in cultures to investigate the effects of paracetamol over a similar time period, of one hour, and range of concentrations. The results of these experiments are given in Figure 39. The concentration of paracetamol which caused a 50% inhibition in the rate of protein synthesis was about 10mM, which together with the slope of the curve for the dose response effect compares well with the effects found in the isolated hepatocyte suspensions (Figure 18). There is also evidence to suggest that concentrations of paracetamol less than 0.5mM caused an increase in the rate of protein synthesis.

Thus it was confirmed that hepatocytes in culture responded in a similar manner to hepatocytes in suspension with regard to effects on the rate of protein synthesis. The cultures were now used as a system for investigating the longer term effects of paracetamol. The unique property of monolayer cultures where the cells become attached to a plastic surface enables the incubation medium to be changed without damaging the cells. Hence facilitating the use of different exposure times to xenobiotics and the capacity to monitor the cultures for some time after the removal of the xenobiotic.

The concentration of paracetamol which causes near maximal inhibition of protein synthesis was found to be 40mM both in suspension and culture. This concentration was therefore used and added to cultures, after a two day period of incubation with control medium, for 2, 4, 6 and 8 hours. The rate of protein synthesis was monitored for one hour; directly after the addition of paracetamol, immediately after the completion of each exposure period, then again at further intervals of two and 24 and 48 hours after the start of the experiment. All estimations apart from the first were monitored in the absence of paracetamol.

The results of this rather complex experiment are expressed graphically in Figure 40 with the rate of protein synthesis being expressed as a percentage of the control at each time point. At zero time in the presence of paracetamol the rate of protein synthesis was reduced to about 7% of the control, a near maximal inhibition. After two hours of incubation, when
the paracetamol had been removed, protein synthesis was inhibited by about 80%. After a further two hours the rate of protein synthesis increased to 50% of the control. At 24 hours the rate was 70% of the control and by 48 hours the rate was nearly back to control levels. The profile for the cultures exposed for four hours to paracetamol was very similar to the two hour exposure although the rate of recovery over the first 24 hours was not quite as rapid. The results for the cultures exposed for 6 and 8 hours to 40mM paracetamol, however, were very different. The rate of protein synthesis was reduced to less than 10% of the control value directly after exposure and was further reduced in the subsequent determinations. In the cultures exposed for 8 hours inhibition was 100% within twelve hours.

A separate experiment was conducted using the same exposure times to investigate the toxicity of paracetamol to the cultures. Table 7 gives the results of the nuclei counts determined for the cultures at time points similar to those used in Figure 40 for the effects on protein synthesis. Exposure of the cultures to 40mM paracetamol for two hours had no marked toxic effect on the cultures. Exposure for four hours, however, did decrease the nuclei number after four hours with further reductions at 24 and 48 hours. Exposure for 6 and 8 hours caused marked toxicity with negligible numbers of nuclei being present at 24 hours and no evidence of any attached cells after 48 hours.

A further experiment was conducted to compare the effects of phenobarbitone and safrrole with paracetamol on protein synthesis in culture. The concentration of paracetamol utilised was the same as in the previous experiments (40mM) whereas the concentrations of phenobarbitone and safrrole chosen were those that would inhibit protein synthesis by about 50% (5mM and 1mM respectively) as it was considered that these compounds were likely to be potentially more toxic to the cultures. The experimental protocol followed was similar to that utilised in the previous experiments with paracetamol above.

The cultures prepared for this experiment were of inferior quality due to there being quantitatively less cells attached per culture to the monolayer. This could have affected the results of the experiment, especially towards the end when even less cells were attached and any small errors in determination could have been compounded.
The results of this experiment are given in Table 8. The initial determination of the rate of protein synthesis in the presence of the three compounds indicate that inhibition of protein synthesis was about 90% in the presence of 40mM paracetamol, 34% in the presence of 5mM phenobarbitone and 27% in the presence of 1mM safrole. The rate of protein synthesis in the presence of safrole was only just measurable due to the fact that the addition of safrole to the cultures caused the cells to detach from the culture surface. The detached cells did not take up trypan blue and were, therefore, assumed to be viable. Safrole must, therefore, effect the capacity of the cells to form a stable monolayer culture. The results of this experiment were difficult to interpret due to the variability in the rates of protein synthesis in the control cultures. It would appear, however, that the longer the exposure to 40mM paracetamol the greater the inhibition of protein synthesis. Treatment of the cultures for 8 hours with paracetamol did not, in this case, cause marked cell death nor did it appear to maintain a permanent reduction in the rate of protein synthesis. The effect of 5mM phenobarbitone on protein synthesis was not as great as that of 40mM paracetamol, and its duration of action was not as long because after each exposure time its effect on the rate of protein synthesis demonstrated quite a rapid recovery.
Figure 39: Effect of paracetamol on the incorporation of $[^{14}C]$-Leucine into protein of primary monolayer cultures of adult rat hepatocytes.

Experimental conditions were as described in 'Methods'. $[^{14}C]$-Leucine incorporation was estimated in triplicate for each concentration. Values are means $\pm$ S.D. for two experiments.
Primary monolayer hepatocytes were prepared as described in 'Methods'. On day 2 of the culture, flasks were treated with 40mM paracetamol for \(\text{△}\), 2 hrs; \(\text{○○○}\), 4 hrs; \(\bullet\)\(\bullet\), 6 hrs; and \(\text{△△△}\), 8 hrs. 

\([^{14}\text{C}]\)-Leucine incorporation, over one hour, was measured after paracetamol treatment, then again 2 hours later, and at 24 and 48 hours after the commencement of the experiment. \([^{14}\text{C}]\)-Leucine incorporation was 7% of the control value when measured in the presence of paracetamol at the commencement of the experiment.
Table 7: The toxicity of paracetamol to primary monolayer cultures of adult rat hepatocytes after different exposure times

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of culture (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>2.98</td>
</tr>
<tr>
<td>2hr paracetamol</td>
<td>-</td>
</tr>
<tr>
<td>4hr paracetamol</td>
<td>-</td>
</tr>
<tr>
<td>6hr paracetamol</td>
<td>-</td>
</tr>
<tr>
<td>8hr paracetamol</td>
<td>-</td>
</tr>
</tbody>
</table>

The results are expressed as nuclei counts (x 10^-6).

The experimental conditions for this experiment were the same as for the results described in Fig. 40. The cultures were exposed to 40mM paracetamol for 2, 4, 6 or 8 hours. The nuclei counts were determined at the end of the exposure period and at 24 and 48 hours after the start of the experiment. All counts were done in duplicate (two flasks).
Table 8: Effects of paracetamol, phenobarbitone and safrole on protein synthesis in monolayer cultures of adult rat hepatocytes

<table>
<thead>
<tr>
<th>Treatment (Time in hours)</th>
<th>Time in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>953</td>
</tr>
<tr>
<td>Paracetamol 2</td>
<td>92(9.6%)</td>
</tr>
<tr>
<td></td>
<td>287</td>
</tr>
<tr>
<td></td>
<td>272</td>
</tr>
<tr>
<td></td>
<td>618</td>
</tr>
<tr>
<td>Phenobarbitone 2</td>
<td>392(34%)</td>
</tr>
<tr>
<td></td>
<td>478</td>
</tr>
<tr>
<td></td>
<td>638</td>
</tr>
<tr>
<td></td>
<td>842</td>
</tr>
<tr>
<td>Safrole</td>
<td>258(27%)</td>
</tr>
</tbody>
</table>

Cultures were treated with 40mM paracetamol, 5mM phenobarbitone, or 1mM safrole for 2, 4, 6, or 8 hours. Protein synthesis was measured over one hour at zero time, two hours after removal of treatment and at 24 and 48 hours. Results are expressed as DPM/mg protein (means of triplicate estimations). Values in parenthesis are the percentage of the control value at zero time.
4.10.2 Comparison of the effects of paracetamol and carbon tetrachloride on cultures using cytochemical and other methods

The preparation of monolayer cultures of adult rat hepatocytes on cover slips in Leighton tubes, rather than Falcon flasks, provides a suitable system for investigating effects on intracellular enzymes by cytochemical methods. The cultures can be incubated with specific reagents which will react with enzymes causing a coloured chemical to be deposited at the point in the cell where the enzyme is present. The amount of enzyme may then be quantified using microdensitometry.

The effects of paracetamol and carbon tetrachloride on three intracellular enzymes, NADPH₂ diaphorase, succinate dehydrogenase and glucose-6-phosphate dehydrogenase were investigated in this way with the results being quantified with a Vickers Scanning and Integrating Microdensitometer. The results of these experiments are summarised in Figure 41. Carbon tetrachloride was used at two concentrations of 1000 and 10,000ppm being incubated with the cultures for up to six hours, with only one concentration of paracetamol (10mM) being used. The higher concentration of carbon tetrachloride caused a reduction in the levels of all three enzymes over the six hour incubation period, its effects being most marked on the glucose-6-phosphate dehydrogenase. Paracetamol (10mM) and carbon tetrachloride (1000ppm) both caused reductions, of a similar magnitude, in the levels of activity of succinate dehydrogenase and glucose-6-phosphate dehydrogenase, and in contrast to the effects of 10,000ppm carbon tetrachloride, they both caused increases in the activity of NADPH₂ diaphorase. Figure 42b is an example of a culture treated with a low level of carbon tetrachloride (0.01ppm) where the activity of NADPH₂ diaphorase, present as a purple formazan deposit, is markedly increased over that of a similar control culture (Figure 42a).

A further experiment was conducted with three concentrations of carbon tetrachloride with incubations of up to 24 hours to confirm the effects on NADPH₂ diaphorase. The results in Table 9, given as percentages of control enzyme activity, indicate that at levels of 1000 and 500ppm carbon tetrachloride causes a marked increase in the activity of NADPH₂.
diaphorase reaching nearly 300% of the control value after 8 hours treatment with 1000ppm. The 1000ppm carbon tetrachloride appeared to be the most active concentration in increasing the enzyme activity, whereas 5,000ppm was obviously toxic to the cultures.

A follow up experiment was attempted to determine whether the increase in enzyme activity for the NADPH$_2$ diaphorase was due to induction or activation. Cultures were, therefore, incubated with cycloheximide as well as carbon tetrachloride the rationale being that if the increase in enzyme levels was due to induction this should be masked by the effects of cycloheximide on inhibiting the synthesis of new enzyme. This experiment was not successful due to the poor nature of the culture and the effort which would have been involved in quantifying the results by microdensitometry could not be justified. One interesting fact did arise from the experiment, however, which was that carbon tetrachloride toxicity to the cultures appeared to be reduced in the presence of cycloheximide. This was just a visual observation and could not be quantified. There was insufficient time to confirm this observation.

Some results were also obtained for the effects of phenobarbitone, an inducer of liver microsomal enzyme activity, on the activity of NADPH$_2$ diaphorase, succinate dehydrogenase, and glucose-6-phosphate dehydrogenase. These results are detailed in Table 10. Enzyme activities expressed as a percentage of control activity are given after 2, 6, 26 and 30 hours of exposure to 2mM phenobarbitone. The results tended to be slightly inconsistent probably due to the variability of the values obtained for the controls in this experiment. NADPH$_2$ diaphorase activity appeared to be increased to a high level after six hours which was still apparent at 30 hours. Succinate dehydrogenase activity was also increased after two hours of incubation but this level of activity then returned to control levels. Glucose-6-phosphate dehydrogenase activity was also increased with a greater increase at 26 and 30 hours.

The effects of paracetamol (10mM) and carbon tetrachloride (1000 and 10,000ppm) on cell number, glutamic oxaloacetic transamines (GOT) levels and protein synthesis were then compared (Figure 43). This experiment confirms that 10,000ppm carbon tetrachloride was very toxic
to the cultured hepatocytes, the number of nuclei being reduced to less than 20% of the control within two hours of incubation. This is further supported by the low level of GOT present within the cells still attached to the surface of the culture and a marked effect on protein synthesis with nearly maximal inhibition apparent at two hours. Carbon tetrachloride at a concentration of 1000ppm had a slight effect on cell number at two hours but this was not more marked at six hours where the cell number was similar to that in the cultures treated with 10mM paracetamol. The effects of 1000ppm carbon tetrachloride and 10mM paracetamol on GOT levels and protein synthesis were very similar over the six hour incubation period.
Figure 41: Effect of paracetamol and carbon tetrachloride on enzyme levels in primary monolayer cultures of rat hepatocytes.

Paracetamol at 10mM (□) and carbon tetrachloride at 1000ppm (■) and 10,000ppm (▲) were added to cultures on day 2. Enzymes were measured after 2 and 6 hours as described in 'Methods'.
Figure 42:

Cytochemical illustrations of the activity of NADPH₂ diaphorase in primary monolayer cultures of adult rat hepatocytes.

(a) Untreated (magnification x200)

(b) Treated with 0.01ppm CC₁₄
Table 9: The effect of CCl$_4$ on the activity of NADPH$_2$ diaphorase in primary monolayer cultures of adult rat hepatocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time in culture after addition of treatment (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>500 ppm CCl$_4$</td>
<td>131</td>
</tr>
<tr>
<td>1000 ppm CCl$_4$</td>
<td>96</td>
</tr>
<tr>
<td>5000 ppm CCl$_4$</td>
<td>87</td>
</tr>
</tbody>
</table>

The results are expressed as a percentage of the control value at each time point.

Primary cultures were prepared on coverslips in Leighton tubes with duplicates for each treatment and time point. The cultures were exposed continuously to their individual treatment after the start of the experiment (time 0).
Table 10: The effect of phenobarbitone (2mM) on the activity of three intracellular enzymes in monolayer cultures of adult rat hepatocytes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time in culture (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>NADPH$_2$ Diaphorase</td>
<td>134</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>142</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>133</td>
</tr>
</tbody>
</table>

Cultures were incubated continuously with 2mM phenobarbitone.

Enzyme activity was measured by quantitative cytochemistry. The results are expressed as a percentage of control values and are the mean of 10 readings.
Figure 43: Effect of paracetamol and carbon tetrachloride on nuclei number, GOT leakage, and $[^{14}C]$-Leucine incorporation in primary monolayer cultures of adult rat hepatocytes

[Graph showing data]

Paracetamol at 10mM (●) and carbon tetrachloride at 1000ppm (■) and 10,000ppm (●) were added to cultures on day 2. Parameters were measured after 2 and 6 hours as described in 'Methods'.
4.10.3 Effect of cycloheximide on the metabolic activity of primary cultures of rat hepatocytes

The maintenance of physiological levels of cytochrome P-450 in cultures is a major problem associated with primary maintenance cultures (see 2.6). Guzelian and Barwick (1979) demonstrated that the marked loss in levels of cytochrome P-450 occurring during the first 12 hours of culture may be prevented by incubating cultures with inhibitors of protein synthesis. The purpose of the following two experiments was to investigate whether the addition of cycloheximide to the incubation medium would maintain the activity of the mixed function oxidase system and to verify the findings of Guzelian and Barwick.

On the basis of previous results (see Fig. 17) a concentration of $10^{-7}$M cycloheximide was chosen since it inhibited protein synthesis in suspensions of isolated hepatocytes by about 50%. A further concentration of $10^{-5}$M cycloheximide was also employed. The cycloheximide was added to the cultures on day 0 when the cells were first seeded into culture flasks and was present at the same concentrations throughout the three day experiment. Cell number, in terms of the number of nuclei, and the activity of 7-ethoxycoumarin O-deethylase (an indicator of the activity of the microsomal mono-oxygenase system) were monitored daily for three days. The rate of protein synthesis was estimated after 24 hours in culture.

Two experiments were carried out with the hepatocytes seeded into Falcon flasks coated with collagen in the first but not the second. Some cultures were stained with Giemsa stain to illustrate the cellular morphology. The morphology of the cultures from the first experiment is described in the following section. Figures 44-50 are illustrations of the cultures from the second experiment.

Cellular morphology:
After 24 hours in culture large numbers of cells were attached to the monolayer in the untreated cultures. The cells were predominantly epithelial in character with some reticuloendothelial cells and fibroblasts also present. They appeared largely as clusters with
occasional single cells. The epithelial cells were spread out with well-defined morphology and intercellular gaps with evidence of occasional duct-like structures. The cellular cytoplasm was granular with occasional vacuoles, the nuclei containing two to four nucleoli with binucleate cells predominating. After a further day in culture there were still large numbers of cells attached with the epithelial morphology largely preserved in the centre of the cell sheets but becoming less distinct at the periphery. The plasma membranes were becoming less distinct due to the spreading of the cytoplasm, resulting in less well-defined intercellular spaces, together with some misshapen nuclei. Fibroblasts and reticuloendothelial cells were still apparent with the latter demonstrating phagocytic activity. By day three the morphological degeneration was becoming more marked with some cells showing pseudopodia-like projections with mitotic figures present in some fibroblasts. By day four the fibroblasts were beginning to predominate in the culture with the epithelial morphology degenerating further with large numbers of vacuoles present in the cytoplasm of the epithelial cells.

After one day of culture in the presence of $10^{-7}$M cycloheximide the monolayer had similarities to the untreated cultures although there appeared to be less cells attached and the cell size tended to be smaller. There were a number of round cells with a large nucleus to cytoplasm ratio, the epithelial cells tended to contain more vacuoles, and there were also some necrotic cells apparent. By the second day of culture the vacuolation was more extensive with some cells containing large perinuclear vacuoles. Some of the epithelial cells maintained their epithelial morphology better than those of the controls and there were also fewer irregularly shaped nuclei. By the third day of culture about 50% of the epithelial cells were showing extensive vacuolation with the remaining cells showing bizarre nuclei and extensive spreading of the cytoplasm such that the epithelial morphology observed on day one had largely disappeared.

Cycloheximide at the higher concentration of $10^{-5}$M had a severe effect on the cultures. Although there were some cells attached on day one of the culture they were mostly round, densely stained and necrotic
with a high nucleus to cytoplasm ratio. The surface of the culture flask was littered with amorphous material which was probably cell debris. By the second day of culture there were even fewer cells attached and all appeared necrotic.
Figure 44: Primary monolayer culture of adult rat hepatocytes after 24 hours (day 1), stained with Giemsa stain (x200)

Figure 45: Primary monolayer culture of adult rat hepatocytes after 48 hours (day 2), stained with Giemsa stain (x200)
**Figure 46:** Primary monolayer culture of adult rat hepatocytes after 72 hours (day 3), stained with Giemsa stain (x200)

**Figure 47:** Primary monolayer culture of adult rat hepatocytes treated with $10^{-7}$M cycloheximide after 24 hours (day 1), stained with Giemsa stain (x200)
Figure 48: Primary monolayer culture of adult rat hepatocytes treated with $10^{-7}$M cycloheximide after 48 hours (day 2), stained with Giemsa stain (x200)

![Image of primary monolayer culture treated with cycloheximide for 48 hours]

Figure 49: Primary monolayer culture of adult rat hepatocytes treated with $10^{-7}$M cycloheximide after 72 hours (day 3), stained with Giemsa stain (x200)

![Image of primary monolayer culture treated with cycloheximide for 72 hours]
Figure 50: Primary monolayer culture of adult rat hepatocytes treated with $10^{-5}$M cycloheximide after 24 hours (day 1), stained with Giemsa stain (x200)
7-Ethoxycoumarin O-deethylase activity, protein synthesis and cell number after treatment with cycloheximide:

The above three parameters were also measured after treatment with cycloheximide at $10^{-5}$ M and $10^{-7}$ M. In both experiments, as previously described, $10^{-5}$ M cycloheximide was very toxic to the cells causing a large reduction in the cell numbers attached by day one of the culture. A marked decrease in the rate of protein synthesis was also evident being 1.4% and 3.0% of the control values for the two experiments respectively.

The results of the first experiment are detailed in Figure 51. The rate of protein synthesis was reduced to 50.8% of the control value by $10^{-7}$ M cycloheximide although the effect of this concentration on cell number did not differ from the controls over the three day period. 7-Ethoxycoumarin 0-deethylase activity was measured for four hours in the cultures days one to three and expressed in terms of the number of nuclei present in the flask. After one day in culture the 7-ethoxycoumarin 0-deethylase activity in the $10^{-7}$ M cycloheximide treated cultures was twice that of the control value. This was followed by a near linear reduction in activity on days two and three of the culture. In the control cultures the activity increased on the second day of culture to twice the level on day one. This was followed by a marked fall in activity by day three.

In the second experiment described in Figure 52, $10^{-7}$ M cycloheximide caused a reduction in protein synthesis to 44.5% of the control level. At this level the cycloheximide did not appear to be toxic to the cells compared to the controls over the three day period of incubation. The results in this second experiment for the activity of the 7-ethoxycoumarin 0-deethylase activity differed, however, from those of the previous experiment. In each case the activity in the controls was greater than that in the cycloheximide treated cultures but the activity increased in both from days one to two of culture followed by a fall off in activity in both treated and untreated by day three although the activity in the controls was more than double that in the cycloheximide treated cultures on the third day of culture.
Figure 51: The effect of cycloheximide on nuclei number, and 7-ethoxycoumarin 0-deethylase activity in primary monolayer culture of adult rat hepatocytes.

Cultures were incubated with medium containing 10^{-7}M cycloheximide (○○○), 10^{-5}M cycloheximide (□□□) or control medium (▼▼▼). Nuclei counts and 7-ethoxycoumarin 0-deethylase activity were measured at the time points indicated. All results were the mean of three samples. (The nuclei count for day 0 was estimated from the number of cells seeded into the flasks assuming a 30% binucleation of the cells.)
Figure 52: Second experiment to determine the effect of cycloheximide on nuclei number, protein synthesis and 7-ethoxycoumarin 0-deethylase activity in primary monolayer cultures of adult rat hepatocytes.

Culture flasks were incubated with medium containing $10^{-7}$M (○○○), $10^{-5}$M (□□□) cycloheximide with controls (▼▼▼). Nuclei counts and 7-ethoxycoumarin 0-deethylase activity were measured at the time points indicated. All results were the mean of three samples. (The nuclei count for Day 0 was estimated from the number of cells seeded into the flasks assuming a 30% binucleation of the cells.)
4.10.4 Comparison of the inducing capabilities of four barbiturates in primary monolayer cultures of adult rat hepatocytes

The experiments described in the previous section (4.10.4) demonstrated that the microsomal mono-oxygenase system was active in primary cultures and that the levels of activity may increase after the first day in culture even in control cultures. Cultures should therefore serve as a suitable model for investigating the effects of inducing agents and studying the mechanisms of induction. Furthermore by comparing different barbiturates it may be possible to identify structure activity relationships requisite for induction of the microsomal mono-oxygenase system.

In the following experiments the effect of four barbiturates were compared, each with a differing duration of activity in vivo, on the metabolic activity of adult rat liver cells in primary monolayer cultures using the activity of 7-ethoxycoumarin 0-deethylase as a measure of microsomal mono-oxygenase activity.

Preliminary experiments were conducted to assess the toxicity of the four barbiturates, phenobarbitone, amylobarbitone, quinalbarbitone and thiopentone (as their sodium salts) to the cultures. They were added to the cultures, after an initial 24 hour period of incubation with control media to allow the cells to recover, on day one and were incubated continuously until day four when the nuclei were counted as a measure of the number of cells attached to the monolayer cultures. The results in Table 11 demonstrate that all four of the barbiturates were extremely toxic at a concentration of 10mM with no cells remaining attached. At 1mM concentrations phenobarbitone was not toxic whereas the other three barbiturates were quite markedly toxic. There was no evidence of any of the barbiturates being toxic at 100μM or less.

Two experiments were then conducted to determine the concentration for each barbiturate which would cause the optimal increase in enzyme activity with minimal toxicity. The results of these two experiments are given in Table 12. 7-Ethoxycoumarin 0-deethylase activity was measured after the cultures had been exposed to the barbiturates for 48 hours (day three). The results were expressed as nmole of
7-ethoxycoumarin 0-deethylase activity per $10^6$ nuclei over a 4 hour incubation period. The optimal concentration of phenobarbitone was found to be 1mM after the second experiment. Amylobarbitone and quinalbarbitone were both found to induce maximal activity at 0.1mM and thiopentone at a concentration of 0.2mM.

7-Ethoxycoumarin 0-deethylase activity was then measured days one to four inclusively for amylobarbitone and thiopentone at their optimal concentrations and days one to four and day seven for phenobarbitones and quinalbarbitone. The results of these two experiments are given in Figures 53 and 54. In the untreated controls the activity on day two increased to at least double the initial activity on day one and then tended to fall off. Phenobarbitone caused a marked increase in activity which was approximately linear from days one to four and then fell dramatically by day seven although it was still higher than the control values. Amylobarbitone and quinalbarbitone had similar profiles of activity, increasing to day two and then tending to plateau or decrease slightly. Thiopentone, however, was delayed in its action. It caused a marked increase in activity by day four but the increases were small and incremental days two and three.

A further experiment was carried out using the same optimal concentrations for the four barbiturates over a seven day culture period to try and confirm the results of the previous experiments. The quality of the cultures prepared for this experiment was not as good as the previous ones and the control cultures gave some spurious results. The results for the barbiturates (Fig. 55) indicate that 1mM phenobarbitone was again the most active over the first four days of culture, with the activity increasing still further by day seven. The profiles for amylobarbitone and quinalbarbitone were again similar although the increase in enzyme activity was not as great on day one for amylobarbitone. Thiopentone again had a delayed effect in inducing the activity of the enzyme but by day seven there was a marked increase in activity. The nuclei counts, which were very low in many of the control cultures, were also very low in the amylobarbitone and quinalbarbitone cultures on day seven and this may have affected the day seven activity results for these two barbiturates in this experiment. Although this latter
experiment was not entirely successful it did tend to confirm the previous results for the effects of the four barbiturates on microsomal mono-oxygenase activity.
Table 11: Toxicity study with four barbiturates on primary monolayer cultures of adult rat hepatocytes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration of barbiturate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10mM</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>0</td>
</tr>
<tr>
<td>Thiopentone</td>
<td>0</td>
</tr>
<tr>
<td>Quinalbarbitone</td>
<td>0</td>
</tr>
<tr>
<td>Amylobarbitone</td>
<td>0</td>
</tr>
</tbody>
</table>

Toxicity was measured in terms of the number of nuclei present in each flask (each value is the mean of three flasks). The values were obtained on day 4 of culture. The control values on days 1 and 4 were 29.4 x 10^4 and 18.9 x 10^4 respectively.
Table 12: Determination of the optimal concentrations for four barbiturates for maximal induction of ethoxycoumarin 0-deethylase activity

<table>
<thead>
<tr>
<th>7-Ethoxycoumarin 0-deethylase activity (nmole/10^6 nuclei/4hr)</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (day 1)</td>
<td>30.8</td>
<td>108.2</td>
</tr>
<tr>
<td>Control (day 3)</td>
<td>44.7</td>
<td>46.8</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3mM</td>
<td>12.3</td>
<td>-</td>
</tr>
<tr>
<td>2mM</td>
<td>61.9</td>
<td>108.1</td>
</tr>
<tr>
<td>1mM</td>
<td>134.0</td>
<td>145.8 *</td>
</tr>
<tr>
<td>0.5mM</td>
<td>-</td>
<td>111.8</td>
</tr>
<tr>
<td>0.1mM</td>
<td>-</td>
<td>65.9</td>
</tr>
<tr>
<td>Amylobarbitone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6mM</td>
<td>3.5</td>
<td>-</td>
</tr>
<tr>
<td>0.3mM</td>
<td>91.2</td>
<td>-</td>
</tr>
<tr>
<td>0.2mM</td>
<td>89.5</td>
<td>200.9</td>
</tr>
<tr>
<td>0.1mM</td>
<td>120.4</td>
<td>126.1 *</td>
</tr>
<tr>
<td>0.05mM</td>
<td>-</td>
<td>118.1</td>
</tr>
<tr>
<td>0.01mM</td>
<td>-</td>
<td>128.6</td>
</tr>
<tr>
<td>Quinalbarbitone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3mM</td>
<td>2.1</td>
<td>-</td>
</tr>
<tr>
<td>0.2mM</td>
<td>75.2</td>
<td>85.2</td>
</tr>
<tr>
<td>0.1mM</td>
<td>118.2</td>
<td>72.8 *</td>
</tr>
<tr>
<td>0.05mM</td>
<td>-</td>
<td>70.1</td>
</tr>
<tr>
<td>0.01mM</td>
<td>-</td>
<td>62.3</td>
</tr>
<tr>
<td>Thiopentone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3mM</td>
<td>24.6</td>
<td>-</td>
</tr>
<tr>
<td>0.2mM</td>
<td>217.2 *</td>
<td>-</td>
</tr>
<tr>
<td>0.1mM</td>
<td>159.2</td>
<td>-</td>
</tr>
</tbody>
</table>

All values above are the means of three individual flasks. Wider concentration ranges were tested but the results are only given for those levels which were not very toxic to the cultures, i.e. the nuclei count was not too low.

* These concentrations were regarded as being optimal.
Figure 53: The effect of phenobarbitone and quinalbarbitone on 7-ethoxycoumarin 0-deethylase activity in primary monolayer cultures of adult rat hepatocytes.

Cultures were incubated with medium containing 1mM phenobarbitone (O---O), 0.1mM quinalbarbitone (□---□), or control medium (▼---▼). 7-Ethoxycoumarin 0-deethylase activity was measured at the time points indicated. Each point was the mean of duplicate samples.
Figure 54: The effect of amylobarbitone and thiopentone on the activity of 7-ethoxycoumarin 0-deethylase activity in primary monolayer cultures of adult rat hepatocytes.

Cultures were incubated with medium containing 0.1mM amylobarbitone (■—■), 0.2mM thiopentone (●—●), or control medium (▼—▼). 7-Ethoxycoumarin 0-deethylase activity was measured at the time points indicated. Each point was the mean of duplicate samples.
Figure 55: The effect of four barbiturates on 7-ethoxycoumarin O-deethylase activity in primary monolayer cultures of adult rat hepatocytes.

Cultures were incubated with medium containing 1mM phenobarbitone (○○○), 0.1mM quinalbarbitone (□□□), 0.1mM amylobarbitone (■■■), or 0.2mM thiopentone (●●●). 7-Ethoxycoumarin O-deethylase activity was measured at the time points indicated. Each point was the mean of duplicate samples.
CHAPTER 5

DISCUSSION
DISCUSSION

5.1 VIABILITY CHARACTERISTICS OF ISOLATED HEPATOCYTES

Throughout the studies reported here the method used to isolate hepatocytes was the non perfusion collagenase/hyaluronidase digestion of liver slices developed by Fry et al. (1976). An alternative method widely used is via perfusion of the liver which results in larger yields of hepatocytes with a shorter preparation time. The perfusion method, however, suffers from a number of disadvantages. It requires the use of live animals under anaesthesia and surgical skills are necessary to perform the operative procedures. The perfusate contains larger quantities of enzymes hence the technique is more expensive and there is a tendency for less reproducibility. The slicing technique also enables ready comparison of species differences (Jones, 1978) including the use of human liver biopsy samples.

Within the studies reported here a number of parameters were monitored directly following the completion of hepatocyte isolation including the yield of cells which excluded trypan blue. The rate of protein synthesis, respiration, metabolic activity, glutathione levels, ATP and cyclic AMP levels were all monitored in untreated preparations of isolated rat hepatocytes. It is therefore of interest to compare the properties of individual preparations of isolated hepatocytes and furthermore to compare the properties of hepatocytes isolated by the method utilised here with values in the literature where the method of perfusion predominates.

An approximate analysis has been made of the yield of viable cells from hepatocyte isolations prepared during the three years contributing to this research (Table 1). In the first month that this technique was employed, prior to the work reported here, the yield of viable cells was about \(50 \times 10^6\) with a viability of 79%. During the year 1976-1977 the yield was again \(50 \times 10^6\) but the viability had increased to about 86%. In the following two years, 1977-1978 and 1978-1979, the yield of hepatocytes had increased quite markedly from the previous year with a corresponding, but less marked, increase in
viability. These results suggest that the isolation of hepatocytes via the slicing method as with most approaches involving biological materials requires some degree of experience. It is clear however that reasonably high yields of cells with acceptable viability were obtained within a relatively short time.

Hamster hepatocytes proved easier to isolate since the liver tissue dissociated more readily. The results given in Table 1 indicate that this did not necessarily result in higher yields and viabilities. Experience with this species, however, was limited and the time the tissue was incubated with enzyme was not optimised.

A further analysis was made to determine whether or not there was a correlation between cell yield and viability. Figure 1 demonstrates that there is a very poor correlation since the results show a very wide scatter. It therefore follows that an increased yield of hepatocytes does not ensure increased viability. On the other hand a preparation with a low yield may still prove useful since the apparent viability in terms of trypan blue exclusion may be high. Once experience had been obtained with this technique visual observation of the suspension during preparation enabled preliminary judgement of its acceptability from the appearance, the relative ease with which it passed through the Boulting cloth filter, and the clarity of the supernatants during the final centrifugation procedure.

A number of experiments were carried out in which the rate of protein synthesis was assessed by measuring the incorporation of $^{14}C$ -Leucine into cellular protein over a time period of one hour. It was observed during these that although the between sample variation within one experiment was small there was a marked variation between experiments in the quantity of labelled amino acid incorporated into the cellular protein. This inter-experimental variation was unusually large compared to that experienced for other cellular parameters like glutathione levels and basal respiration. The drug metabolising activity of hepatocytes varied somewhat but this was probably related to induction of the microsomal monoxygenase
Table 1: An analysis of cell yield and viability of freshly isolated hepatocytes over a three year period

<table>
<thead>
<tr>
<th>Species</th>
<th>Period</th>
<th>Yield of viable cells (x 10^6)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>August 1976</td>
<td>50.3 ± 17.4 (n = 10)</td>
<td>79 ± 6 (n = 10)</td>
</tr>
<tr>
<td>Rat</td>
<td>1976-1977</td>
<td>50.3 ± 16.5 (n = 27)</td>
<td>86 ± 6 (n = 27)</td>
</tr>
<tr>
<td>Rat</td>
<td>1977-1978</td>
<td>68.5 ± 16.0 (n = 28)</td>
<td>89 ± 3 (n = 28)</td>
</tr>
<tr>
<td>Rat</td>
<td>1978-1979</td>
<td>67.0 ± 17.9 (n = 52)</td>
<td>90 ± 3 (n = 52)</td>
</tr>
<tr>
<td>Hamster</td>
<td>1976-1979</td>
<td>56.0 ± 17.5 (n = 16)</td>
<td>88 ± 3 (n = 16)</td>
</tr>
</tbody>
</table>
Figure 1: Scattergram comparing yield of viable cells and viability for a number of preparations of freshly prepared isolated rat hepatocytes.
enzymes by environmental factors related to the housing of the animals. The two scattergrams (Figs. 2 and 3) demonstrate that there was no correlation between the rate of protein synthesis (DPM/mg protein/hr), initial cell viability (Fig. 2) or the yield of viable cells (Fig. 3). This infers that the variability in the rate of protein synthesis is not a result of the isolation procedure but is related to the in vivo capacity of the animal from which the cells were derived.

In two experiments in which the rate of protein synthesis was measured in isolated hamster hepatocytes, under equivalent experimental conditions to the rat hepatocytes, the rate of incorporation of amino acid was about 225 DPM/mg protein/hr. This rate of incorporation was markedly less than any rate recorded for rat hepatocytes which may indicate a species difference for this parameter.

The rate of endogenous respiration, determined in freshly isolated rat hepatocytes, was about 10nmole O₂ consumed/min/10⁶ cells at 37°C. This is of the same order of magnitude as values reported by Baur et al. (1975) for viable preparations, prepared by the perfusion method, of between 5 and 6nmoles O₂ consumed/min/mg protein at 25°C. It should be noted that even cells with very poor viability may show some oxygen consumption. For example Baur et al. (1975) also reported that freshly isolated but fractured hepatocytes with a viability of about 5% according to trypan blue exclusion showed a rate of oxygen consumption which was about 55% of control values.

Freshly isolated hepatocytes maintain the ability to metabolise exogenous substrates without the addition of unnaturally high levels of exogenous cofactors. They differ in this respect from microsomal preparations. The demonstration in this report of active 0-deethylation of 7-ethoxycoumarin to 7-hydroxycoumarin followed by conjugation to the phenolic sulphate and glucuronide metabolites without the addition of cofactors confirms this metabolic property.
Figure 2: Scattergram comparing the viability of freshly prepared isolated rat hepatocytes and their capacity to synthesize protein.
Figure 3: Scattergram comparing the yield of freshly prepared rat hepatocytes and their capacity to synthesize protein.
The level of ATP in freshly isolated rat hepatocytes was found to be 16.25 ± 1.42nmoles/10^6 cells. This value is similar to those reported by Baur et al (1975) of between 15 and 16nmole/mg protein but substantially less than those reported by Hirata et al (1977) of greater than 40nmole/10^6 cells. Both these groups used Sprague Dawley rats of a similar age (200-300g) and isolated hepatocytes by the perfusion method. Wistar albino rats of 60-100g were used here. Baur et al, however, incubated cells in balanced salt solution after slow perfusion whereas Hirata et al used a culture medium supplemented with serum after a rapid perfusion which may have accounted for the differences reported since the cellular levels of ATP may be reduced very rapidly.

Basal cyclic AMP levels were of the order of 2.3pmole/10^6 cells. Little can be concluded from this result compared with the findings of other workers. Values reported in the literature include: 0.41 ± 0.05pmole/mg wet weight of liver (Garrison and Haynes, 1973), 0.45pmole/mg liver (Pilkis et al, 1975), 2.03pmole/mg protein (Moxley and Allen, 1975).

Rat hepatocytes isolated by the method used here also have the ability to synthesize RNA demonstrated by the incorporation of [14C] -Uridine. Unlike the incorporation of [14C] -Leucine which was completely linear over one hour the rate of incorporation of [14C] -Uridine was slightly less over the first 30 minutes of incubation but then increased and was linear for the following 60 minutes. Baur et al (1975) reported a linear incorporation of [14C] -Uridine with time although his results also suggest a slight lag phase within the first 10 to 15 minutes.

The glutathione levels in isolated rat hepatocytes were investigated in more detail since the level of this tripeptide in the liver is intimately associated with protection of hepatocytes from the potential toxicity of a number of xenobiotics.

In two experiments the concentration of glutathione in the whole liver was estimated at the same time as that in hepatocytes isolated from the same rat. In both of these experiments the levels within the freshly isolated hepatocytes of 1.08 and 0.78mg/g liver were
significantly lower than those in the original tissue, of 1.6 and 1.19mg/g liver respectively, from which they were derived (Table 2). However after incubation in complete medium the levels of glutathione rose to above those in the original tissue within one hour of incubation at 37°C hence demonstrating a 'rebound' effect.

Table 2: Comparison of the levels of glutathione in whole liver and isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Glutathione Levels</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mg/g liver</td>
<td>µmole/g liver</td>
<td>mg/g liver</td>
</tr>
<tr>
<td>Whole liver homogenate</td>
<td>1.6</td>
<td>5.3</td>
<td>1.19</td>
</tr>
<tr>
<td>Freshly isolated hepatocytes</td>
<td>1.08</td>
<td>3.3</td>
<td>0.78</td>
</tr>
<tr>
<td>Hepatocytes after 1 hrs incubation</td>
<td>1.74</td>
<td>5.8</td>
<td>1.35</td>
</tr>
</tbody>
</table>

Hogberg et al (1977) reported glutathione levels of 6.1µmole/g liver (1.84mg/g liver) and Vina et al (1978) 5.3µmole/g liver (1.6mg/g liver) in whole liver preparations.

The values obtained for the levels of glutathione in Table 2 again indicate some variability between animals. The level of glutathione is affected by the dietary status of animals (McLean and Day, 1975), it also shows a marked diurnal variation (Isaacs and Binkley, 1977) with the level at 6am being about twice that at 6pm.
The values obtained for glutathione in freshly isolated hepatocytes were 7.52 ± 1.12 μg/10^6 cells which compare well with those obtained by Thor et al. (1978) and Hogberg et al. (1977) of about 10.13 μg/10^6 cells. Hogberg et al. (1977) found that by using a more rapid perfusion technique the glutathione levels obtained in the freshly isolated hepatocytes were higher (13.5 μg/10^6 cells).

The results obtained for the preparations of rat hepatocytes used here indicate that incubation in complete medium facilitates the rapid resynthesis of glutathione. This is in agreement with the findings of the Karolinska group but at variance with the findings of Vina et al. (1978). These variations in the glutathione levels reported by different groups may be an indication of possible differences in viability characteristics of hepatocytes prepared by different methods but are more likely a reflection of the variations in the incubation media used. The L15 medium used throughout the studies reported here contains sufficient amounts of the amino acids, cysteine (120mg/l) and methionine (75mg/l), which are utilised by the cell to resynthesize glutathione. The Karolinska group also used culture medium containing amino acids whereas Vina et al. used Krebs-Heinslet buffer which had to be supplemented with methionine or homocysteine to just maintain the levels of glutathione during incubations. Krebs-Heinslet buffer therefore may not provide a suitable intracellular environment for the synthesis of glutathione.

The results discussed here suggest that the quality of the isolated hepatocytes prepared by the slicing method of Fry et al. (1976) does not differ markedly from that of hepatocytes prepared by perfusion of intact liver. They appear to carry out a number of functions which occur in vivo but without the addition of exogenous cofactors. There is however some variability between individual preparations of hepatocytes in terms of their yield and viability characteristics which may reflect the status of the animal from which they are derived and serves to emphasise that there is a considerable variation between different animals of the same species. This variation may be affected by age, diet, environmental conditions which may also cause stress effects and the time of day the animals are killed. For the
experiments reported here these variables were standardised as far as possible in that animals of similar ages were used throughout, they were fed a standard laboratory diet and were usually sacrificed between 9am and 11am in the morning. The time that the animals had to acclimatise to the environment within the housing facilities used however was variable and stress factors may also have varied. It should be possible to standardise most variables within one laboratory but between laboratory comparisons may be hindered however by the involvement of different variables.

5.2 ISOLATED RAT HEPATOCYTES IN PRIMARY MAINTENANCE CULTURE
5.2.1 The properties of cultures compared to suspensions of isolated hepatocytes
Suspensions of isolated rat hepatocytes can normally only be used for short term experiments of a few hours. After this time the viability of the hepatocytes is reduced to below levels at which the cells are likely to function as they would under normal physiological conditions in vivo.

The incubation method used here was to place one ml volumes of cell suspension into 10ml glass conical flasks in a shaking incubator. As the incubation time increased not only did the viability of untreated cells tend to decrease but the cells also tended to aggregate in clumps and attach to the sides of the flask, predominantly around the meniscus of the incubating medium. This localisation probably contributes to the reductions in viability since the hepatocytes would not be homogeneously dispersed within the medium hence the availability of essential nutrients from the medium would be hindered and oxygen supply no longer consistent with cells in the centre of clumps possibly being starved of oxygen. This became a serious problem for incubations of greater than three hours.

Alternative incubation techniques have been used by other research groups which may enable longer incubations. For example the Karolinska group (see Moldeus, 1978) incubate hepatocyte suspensions in round
bottomed flasks which are continually rotated. This factor may contribute to the fact that they claim to achieve longer incubation times. Other factors which may affect the viability of the hepatocytes over long periods may be the isolation procedure itself. If this is carried out under sterile conditions a suspension culture may be achieved. Subtle differences in the supplements added to culture media, whose role is as yet unidentified, may assist in maintaining viability of suspensions. Furthermore, differences in the apparatus such as the quality of the glass used may affect the characteristics of a hepatocyte suspension.

The characteristic property of reaggregating and attaching to surfaces like glass has been utilised in developing techniques for culturing hepatocytes. If hepatocytes, isolated under aseptic conditions, are inoculated into culture flasks, e.g. plastic flasks, or glass treated with collagen, they will attach to the surface of the flask. They will then tend to reaggregate as if to regain the configuration of the tissue from which they were derived. With time they flatten and spread to form a confluent monolayer. It takes about 24-48 hours for a culture to achieve a confluent monolayer if the non-viable detached cells are removed at intervals and the culture medium replaced thereby removing any toxic lytic enzymes and other matter. Epithelial hepatocytes will not divide to any significant extent in such primary cultures but non-epithelial fibroblasts will. After about 5 days in culture fibroblasts usually begin to predominate. It therefore follows that primary maintenance cultures of rat hepatocytes may be used to investigate the effects of chemicals for up to 3 to 5 days which is a marked improvement over hepatocyte suspensions.

This property of cultures was considered to be potentially useful for investigating the longer term effects of paracetamol on rat liver in vitro. It has been well reported (see Chapter 1) that an acute overdose of paracetamol causes liver damage in the rat in vivo. The onset of this liver damage however is not apparent for some hours after the administration of the toxic dose. It was therefore considered relevant to utilise cultures to investigate in particular the effects
of a large dose of paracetamol (40mM) on hepatocytes over a 48 hour period after exposure to paracetamol for between 2 and 8 hours. In the experiments carried out it became apparent that treatment with 40mM paracetamol for more than 4 hours could cause irreversible inhibition of protein synthesis cell damage and death, whereas a shorter exposure time led to reversible effects on protein synthesis. The effects of 2mM phenobarbitone on protein synthesis were also found to be reversible after up to 8 hours of exposure to cultured hepatocytes with no evidence of cell damage when compared to control cultures in terms of cell attachment.

5.2.2 Mixed function oxidase activity in primary maintenance cultures of adult rat hepatocytes
Suspensions of isolated rat hepatocytes have been demonstrated to have the ability to metabolise foreign compounds whose metabolism is mediated by the microsomal monooxygenase enzyme system. Primary monolayer cultures of rat hepatocytes have been reported to lose much of this activity however during the first twelve hours of culture (see Chapter 2). Guzelian and Barwick (1979) reported that supplementing culture medium with cycloheximide maintained the level of cytochrome P-450 in primary maintenance cultures. Two experiments were carried out here in order to test the findings of Guzelian and Barwick and furthermore to indicate whether the culture system used here would metabolise foreign compounds via the microsomal monooxygenase pathways. In our study cytochrome P-450 levels were not measured, instead the activity of the microsomal enzyme ethoxycoumarin O-deethylase was assessed.

In the first of these experiments cultures were maintained on a substraction of collagen whereas in the second no collagen was used. The presence of collagen did not appear to significantly affect the attachment of the cells to the plastic culture surface. The morphological appearance of the untreated control cultures, however, appeared to deteriorate more rapidly in the presence of collagen which is why it was omitted in the second experiment.
In both experiments treatment of cultures with $10^{-5}$ M cycloheximide which inhibited protein synthesis by $> 95\%$ was markedly toxic causing a rapid decrease in the number of cells attached to the monolayer over the first 24 hours. Treatment with cycloheximide ($10^{-7}$ M) which inhibited protein synthesis by about 50\% did not cause significantly different levels of cell attachment compared to control cultures. The numbers of cells attached fell by about 60\% during the three days of culture in both the control and cycloheximide ($10^{-7}$ M) treated cultures.

7-Ethoxycoumarin 0-deethylase activity was present in both experiments although there was a marked difference in the levels of activity with about a 6-fold greater activity in the second experiment. It is unlikely that the absence of collagen would have contributed to this difference in activity. It is more likely that the animals had their mixed function oxidase activity induced prior to the isolation of the hepatocytes for the second experiment. In both experiments the level of activity of 7-ethoxycoumarin 0-deethylase in the controls doubled between days one and two to about 20 and 120 nmoles/10$^6$ nuclei/4 hr which would not support the generally accepted finding that the levels of microsomal monooxygenase activity are reduced in cultures. By day 3 the levels of activity were reduced.

The findings from the two experiments for the effect of $10^{-7}$ M cycloheximide were not consistent. In the first experiment the level of activity of 7-ethoxycoumarin 0-deethylase activity was doubled by the presence of cycloheximide on day 1 followed by a linear fall off during days 2 and 3 in culture. In the second experiment, however, the activity on day 1 was 20\% less than in the control cultures. The activity increased on day 2 but not to the same extent as in the controls. By day 3 the activity had fallen again but was still less than that of controls.

Since the results of these two experiments were variable little may be concluded in relation to the experiments of Guzelian and Barwick (1979). It may be suggested however that the characteristics of the cultures used here were different to those of Guzelian and Barwick.
and other groups who have reported a marked loss of cytochrome P-450 when hepatocytes are cultured. The level of activity of the microsomal monooxygenase enzymes of the cultures used here was not very different to the levels seen in suspensions of hepatocytes. Furthermore the activity rose during culture which may be due to the presence of an inducing agent in the foetal calf serum. It therefore appears to be unnecessary to supplement the medium with an unnatural physiological additive like cycloheximide to maintain the levels. In fact the results obtained with cycloheximide here do not tend to support the results of Guzelian and Barwick. It may be that cycloheximide is only effective at maintaining levels of cytochrome P-450 in cells of poor quality.

5.2.3 Comparison of the inducing capabilities of some barbiturates
The experiments carried out with cycloheximide indicate that the primary maintenance cultures of rat hepatocytes used here contain an active microsomal monooxygenase system involving cytochrome P-450. In initial cultures the levels of mixed function oxidase activity were relatively low, although not very different to those of suspensions, but increased in controls during the first two days in culture. It has been demonstrated that phenobarbitone can induce the microsomal monooxygenase system in these cultures (Fry et al., 1980); benzothrinacin also induced microsomal monooxygenase activity and the effects of the two inducers were additive indicative of the fact that two types of haemoprotein were induced.

The barbiturate class of drugs contains a number of compounds with differing duration of action and physicochemical properties. The activity of the microsomal monooxygenase system, as reflected by the activity of 7-ethoxycoumarin O-deethylase, was used to compare the inducing capacities of four different barbiturates.

Phenobarbitone, the classic barbiturate inducer, is a lipid soluble long acting barbiturate with a relatively long in vivo half life. Amylobarbitone is an intermediate acting and quinalbarbitone a short acting barbiturate. Thiopentone is a highly lipid soluble, very short
acting barbiturate which is used as an anaesthetic. The structures for these barbiturates are given in Figure 4.

Prior to investigating the inducing capabilities of these four barbiturates their potential toxicity was investigated in cultures by monitoring the effects of various concentrations of each on the number of cell nuclei attached to the surface of cultures. Phenobarbitone was found to be the least toxic of the four barbiturates. Once the approximate toxic levels of the barbiturates had been identified concentrations of each were selected for the effects on the activity of 7-ethoxycoumarin 0-deethylase with the aim of identifying the levels which would achieve a maximal induction of activity with minimal toxicity over a two day culture period. These concentrations were identified as 1mM, 0.1mM, 0.1mM and 0.2mM for phenobarbitone, amylobarbitone, quinalbarbitone and thiopentone respectively. The profile of induction for these four barbiturates was then investigated over a seven day period measuring the activity of 7-ethoxycoumarin 0-deethylase on days 1, 2, 3 and 4, and in some cases day 7 (day 1 was 24 hours after the flasks were inoculated with hepatocytes).

Phenobarbitone was found to be the most effective inducer in this system. Of the four barbiturates studied phenobarbitone is the least lipid soluble and is metabolised and eliminated from the cell more slowly. It is of interest that Pelkonen and Karki (1973) found an inverse relationship in vivo between lipid solubility and enzyme induction for the barbiturates.

The profiles of induction for amylobarbitone and quinalbarbitone were similar in that there was a marked increase in the activity of 7-ethoxycoumarin during the first 24 hours of exposure which then remained constant or fell slightly. Both were far less effective than phenobarbitone which may be explained by the fact that they are both more lipid soluble and also more rapidly metabolised by the liver. There is however a marked difference between the structures of these two barbiturates. Quinalbarbitone contains an allyl side chain, not present in any of the other barbiturates studied here, which may potentially be metabolised to an active metabolite, possibly an allylic
Figure 4: Structures of barbiturates

Phenobarbitone

Amylobarbitone

Quinalbarbitone

Thiopentone
epoxide which could lead to the destruction of cytochrome P-450. The fact that the activity of 7-ethoxycoumarin O-deethylase was still relatively high after 6 days of treatment with quinalbarbitone may suggest that another haemoprotein is induced or that a limited amount of the allyl epoxide metabolite is formed resulting in less damage to cytochrome P-450.

Thiopentone, although highly lipid soluble, did cause an induction of 7-ethoxycoumarin O-deethylase activity but this effect was delayed. After three days the induction by thiopentone was quite marked. This delayed effect suggests that thiopentone itself was not responsible for the inducing activity since it is metabolised very rapidly by the liver. One of its metabolites however is pentobarbitone and this or another metabolite may be responsible for the effect of thiopentone on the delayed increase in activity of the microsomal monooxygenase system. The early lag may be related to the production of active sulphur (S') from thiopentone which would be toxic to haemoproteins.

It may be concluded from these preliminary investigations that the mixed function oxidase system may be induced in primary maintenance cultures of adult rat hepatocytes and this may prove to be a useful property for investigating the mechanisms of toxicity of other foreign compounds to the liver in vitro. This, however, may be dependent on the nature of haemoprotein induced (Fry et al, 1980) and whether this is similar to the in vivo response of the liver. Further investigations would be required to clarify this.

5.2.4 The application of quantitative cytochemistry to primary cultures
During the last few years much work has been dedicated to improving techniques for the histochemical and cytochemical analysis of enzymes in tissues. There are numerous advantages to being able to identify the cell types of a tissue where a particular enzyme is located and to quantify the amounts of enzyme in situ rather than use disrupted tissues and hence only being able to determine overall enzyme activities.
The identification of tissue stabilisers which are chemically inert (Altman, 1980) has enabled the measurement of enzymes within unfixed tissue sections without loss of enzyme from its active site in the tissue or loss in its activity.

Primary cultures of rat hepatocytes can be maintained on collagen coated glass coverslips in Leighton tubes. The resultant cell monolayer can be treated like an unfixed tissue section and therefore provides a suitable system for investigating cytochemistry in an \textit{in vitro} situation. Methods have been developed for the cytochemical identification of a number of dehydrogenase enzymes (Chayen \textit{et al}, 1973). The dehydrogenases oxidise their substrates by removing hydrogen and passing it to a suitable acceptor. Tetrazolium salts have been identified as suitable acceptors which when reduced yield a coloured precipitate of their corresponding formazan. This reaction can be speeded up by the addition of a powerful hydrogen acceptor phenazine methosulphate which rapidly takes hydrogen from the donor and passes it to the tetrazolium acceptor.

The effects of exogenous chemicals on the activity of three dehydrogenases were investigated: succinate dehydrogenase (SDH), glucose 6-phosphate dehydrogenase (G6PDH) and NADPH$_2$ diaphorase.

SDH is a mitochondrial marker enzyme which plays an essential role in the tricarboxylic acid cycle during the oxidation of succinate to fumarate. The hydrogen yielded from this reaction is normally utilised in the electron transport chain. The use of phenazine methosulphate will short circuit the electron transport chain as a strong hydrogen acceptor.

G6PDH is a cytoplasmic enzyme involved in the pentose phosphate pathway which is a major pathway involved in the oxidation of glucose. This particular pathway produces pentose sugars which may be used to produce ribose and deoxyribose for their corresponding nucleic acids. G6PDH requires NADP as a coenzyme and is therefore a major source of NADPH. This is of considerable importance in a number of essential metabolic reactions including the synthesis of steroids and fatty acids.
and in the hydroxylation of many lipid soluble compounds by cytochrome P-450.

NADPH$_2$ diaphorase is a marker enzyme for the endoplasmic reticulum, or more specifically for the microsomal respiratory system, which passes hydrogen from NADPH$_2$ to react with atmospheric oxygen by means of cytochrome P-450. Its true physiological role remains controversial. It may be equivalent to cytochrome P-450 reductase.

The activities of these three enzymes were quantified in cultured hepatocytes using a Vickers scanning and integrating microdensitometer to measure the absorbance of the precipitated formazan produced by the appropriate enzyme reactions in the presence of phenazine methosulphate, tissue stabiliser (PVA) and necessary cofactors.

Carbon tetrachloride a very widely used model hepatotoxin was found to cause dose dependent reductions in cell number attached to cultures, inhibition of protein synthesis and increases in GOT leakage. It also caused dose related decreases in the activity of G6PDH after two and six hours of incubation, 10,000ppm causing a rapid fall in activity within two hours such that it was not detectable at 2 or 6 hours. SDH activity was decreased by 1000ppm carbon tetrachloride by 20% within two hours remaining at this level after six hours. At the higher concentration of 10,000ppm however there was a continued fall in the activity of SDH between two and six hours to about 10% of the control activity. The activity of NADPH$_2$ diaphorase, however, showed a biphasic response to exposure to carbon tetrachloride. At high concentrations (5,000 and 10,000ppm) its activity was reduced. At lower concentrations (1000 and 500ppm) however the activity of NADPH$_2$ diaphorase was increased. This increase in activity over controls was maintained for 24 hours although the peak activity was seen at 8 hours. The reductions in enzyme activity could be due to inhibition or damage to the enzyme. G6PDH is a cytoplasmic enzyme which may explain why the effects of carbon tetrachloride were more marked on this enzyme since this enzyme would be more readily lost through damaged plasma membranes and also more accessible. An attempt
was made to determine whether the increase in NADPH\textsubscript{2} diaphorase activity was due to induction or activation by incubating in the presence of cycloheximide. This experiment was not however successful and further investigation would be needed to identify the mechanism by which this increase in activity occurs.

The results obtained for 10mM paracetamol were very similar to those obtained for 1000ppm carbon tetrachloride. After six hours of incubation there were slight reductions in cell number and slight increases in GOT leakage with a 40% inhibition of protein synthesis. Although the activities of SDH and G\textsubscript{6}PDH were reduced the activity of NADPH\textsubscript{2} diaphorase increased in a linear manner over 6 hours to about 200% of the control level.

Phenobarbitone (2mM) on the other hand produced increases in all three dehydrogenases. This effect was more marked and prolonged on the activities of NADPH\textsubscript{2} diaphorase and G\textsubscript{6}PDH with the effect tending to increase over a 30 hour period. For SDH the effect of phenobarbitone was more marked within the first two hours of exposure. The effect of phenobarbitone on the hepatocytes is therefore different to that of paracetamol.

5.3 THE EFFECTS OF XENOBIOTICS ON THE VIABILITY CHARACTERISTICS OF ISOLATED HEPATOCYTES

5.3.1 Paracetamol

The main compound whose toxic effects were investigated in the studies reported here was paracetamol. It is known to cause hepatotoxicity in animals and man when administered at high doses, vastly in excess of therapeutic levels. The susceptibility of different animal species to the hepatotoxic effects of paracetamol is however very variable (see Chapter 1).

Paracetamol was found to be relatively non-toxic to isolated rat hepatocytes in terms of its effect on cell viability as measured by trypan blue exclusion or lactate dehydrogenase release, parameters which are likely to be indicators of irreversible plasma membrane damage.
Incubations of rat hepatocytes with paracetamol at concentrations of up to 40mM for one hour caused little change in either parameter when compared to those of control incubations of a similar duration. There was however some evidence to suggest that 40mM paracetamol did produce plasma membrane damage when the exposure time was increased to 90 minutes although this was not a consistent finding.

From the above data it may therefore be assumed that, for the other effects investigated, the integrity of the cell membrane was preserved after one hour of incubation of hepatocytes with concentrations of up to 40mM paracetamol. It is therefore of interest that paracetamol was found to cause dose dependent reductions in both $[^{14}\text{C}]$-Leucine and $[^{14}\text{C}]$-Uridine incorporation. Protein synthesis was inhibited by $\geq 75\%$ within 5 minutes of exposure to high concentrations of paracetamol (40mM). This concentration caused a maximal inhibition throughout one hour of incubation since the amount of label incorporated into protein was not increased after 5 minutes. If paracetamol (40mM) was removed from the hepatocytes after 30 minutes of incubation the hepatocytes began to synthesize protein, albeit at a slower rate than controls, indicating that this effect on protein synthesis is reversible if the exposure time is relatively short.

The incorporation of $[^{14}\text{C}]$-Uridine was also maximally inhibited (95%) by 40mM paracetamol, with a concentration of about 5mM causing a 50% inhibition (compared to $\approx 9\text{mM}$ for 50% inhibition of protein synthesis). The effects of paracetamol at early time points and on reversibility were not investigated for this parameter.

As mentioned above in control hepatocytes glutathione was depleted during the isolation procedure but resynthesis began rapidly following incubation of hepatocytes in complete medium. The addition of 10 and 20mM paracetamol inhibited the rate of resynthesis by 77% and 67% respectively but only at a concentration of 40mM paracetamol was depletion of the levels of glutathione to below the initial control levels in rat hepatocytes observed. Evidence from preliminary experiments with paracetamol suggested that inhibition of the rate of resynthesis of glutathione occurred within 5 minutes of incubation.
It would therefore appear that protein synthesis and RNA synthesis are both inhibited by paracetamol with the effect of paracetamol on these two parameters being more marked than its effects on cellular glutathione levels. There also appears to be no direct correlation between cellular glutathione levels and the effect of paracetamol on the inhibition of protein synthesis. This is supported by the fact that if hepatocytes are preincubated for one hour prior to the addition of paracetamol, which allows the resynthesis of glutathione to physiological levels or above, there was no marked difference in the dose dependent inhibition of protein synthesis in rat hepatocytes.

It is of interest to note here that it has been suggested that glutathione depletion per se may lead to lipid peroxidation (Anundi et al., 1979). This hypothesis is further supported by the results of Younes and Siegers (1981). Glutathione, together with glutathione peroxidase, provides a defence mechanism within the cell by removing hydrogen peroxide and hydroperoxides formed during lipid peroxidation as well as electrophilic intermediates which may covalently bind to macromolecules or initiate lipid peroxidation. Glutathione depletion in isolated hepatocytes may lead to lipid peroxidation resulting in cell death prior to the alkylation of macromolecules expressing itself as an important cell destructive mechanism. In the experiments carried out by Anundi et al. (1979) paracetamol served as a protective agent against lipid peroxidative attack which may be due to its affinity for the microsomal monooxygenase system and cytochrome P-450 hence reducing lipid peroxide formation via the metabolic activation of other substrates. The in vivo significance of the protective action of paracetamol may only be slight since it also depletes glutathione and is hepatotoxic, although competition for cytochrome P-450 may lead to reductions in active metabolite production from both substrates.

More detailed investigations into protein metabolism indicated that paracetamol also caused inhibition of both protein degradation and protein secretion. Furthermore the inhibition of protein synthesis and protein secretion was not specific to one major class of proteins, e.g. intracellular or export, but rather was a general inhibition. The results from two experiments suggested that although synthesis was
inhibited at a concentration of 40mM paracetamol the uptake of amino acids into the intracellular amino acid pool was not. This latter finding must however be treated with some caution because subsequent investigation of the method used for these two experiments indicated that the hepatocytes may have been contaminated with labelled amino acid since the quantity of intracellular labelled amino acid counted for both the treated and controls was unexpectedly high (>3000 DPM/mg cellular protein) and any differences would not have been seen.

Paracetamol was also found to cause a dose- and time-dependent decrease in the 0-deethylation of 7-ethoxycoumarin. When the effect of different concentrations of paracetamol on the production of the individual and conjugated metabolites was investigated it was found to have a more marked effect on sulphate conjugation than on glucuronic acid conjugation. At 20mM paracetamol both conjugation reactions were inhibited markedly. At 40mM paracetamol there was minimal conjugation of the metabolites of 7-ethoxycoumarin whereas the level of free 7-hydroxycoumarin was increasing.

Paracetamol (40mM) caused a rapid decrease in cellular ATP levels within two minutes of exposure. It also inhibited endogenous respiration in a dose dependent manner. In contrast paracetamol had little or no effect on cyclic AMP levels.

Finally incubation of isolated rat hepatocytes with paracetamol at 20 and 40mM, spiked with \( ^{14}C \)-paracetamol, produced dose-dependent covalent binding of paracetamol (31.8 and 65.4nmole paracetamol/mg protein respectively) or its metabolites to cellular macromolecules within 5 minutes of incubation. The rate of covalent binding tended to plateau after the initial rapid phase.

Paracetamol has been shown to cause acute variations in a number of characteristics of rat hepatocytes which are not attributable to leakage of cofactors from the cell. One of the earliest responses of the cells to exposure with high levels of paracetamol appears to be a marked fall in ATP. Marked reductions in endogenous respiration and protein synthesis also occur quite rapidly. These changes occur prior to a marked depletion of glutathione and are probably reversible.
if exposure time is relatively short. Exposure for longer periods of time in primary culture however leads to irreversible plasma membrane damage and subsequent cell death.

A number of recent studies have been carried out using in vitro systems to investigate the metabolism and toxicity of paracetamol. Isolated hepatocytes have been used by a number of workers (Moldeus, 1978; Moldeus et al., 1980; Jollow, 1980; McLean, 1978) where hepatocytes from rat, mouse or hamster have been investigated. The results of Moldeus (1978) and McLean (1978) support our finding of the relatively low susceptibility of isolated rat hepatocytes to the hepatotoxic effects of paracetamol. Moldeus (1978) identified the formation of glucuronic acid, sulphate, glutathione and cysteine conjugates of paracetamol by both the rat and mouse. His results suggested that sulphation had a higher affinity for paracetamol than glucuronidation whereas glucuronidation had a higher capacity. The level of sulphate conjugate formed by mouse hepatocytes was lower than that seen in the rat whereas the formation of the glutathione conjugate was faster in the mouse. His results also suggested that the mouse was more susceptible to the hepatotoxic effects of paracetamol.

In relation to varying susceptibility of different animal species some results were obtained here for the hamster, a species which in vivo is more susceptible to the toxic effect of paracetamol. Protein synthesis was inhibited in a dose-dependent manner by paracetamol. The profile of this inhibition was however slightly different to that observed in rat hepatocytes. The effect of lower concentrations was more marked with the ID₅₀ being about 5mM paracetamol (9mM in rat) and 1mM causing about a 35% inhibition. Furthermore, an interesting phenomenon was observed with the effects of paracetamol on the integrity of the plasma membrane of hamster hepatocytes with low concentrations being consistently more toxic than higher concentrations. This phenomenon may be partially explained by the results of Jollow (1980) who found that increasing the concentration of paracetamol led to a greater production of glucuronic acid metabolites than glutathione conjugates. This may be regarded as
a protective mechanism since the affinity of paracetamol in the hamster is greater for the glutathione conjugation pathway and therefore at low concentrations more active metabolite is produced.

The results reported have demonstrated that rat hepatocytes can actively metabolise exogenous substrates. Furthermore paracetamol caused dose dependent reductions in glutathione levels and is covalently bound to protein. It may therefore be assumed that paracetamol was metabolised by the system and at high concentrations an active metabolite was produced which formed a glutathione conjugate.

As already discussed paracetamol caused dose dependent reductions in the activity of 7-ethoxycoumarin O-deethylase and at low concentrations it had a more marked effect on the sulphate conjugation of 7-hydroxycoumarin than on glucuronic acid conjugation. This could be due to the fact that there was competition for available active sulphate or that paracetamol has a higher affinity for the sulphotransferase than 7-hydroxycoumarin. To participate in sulphate conjugation cellular sulphate must first be activated to 3-phosphoadenosine 5′-phosphosulphate (PAPS) which requires two molecules of ATP. Sulphate conjugation is therefore relatively expensive in ATP and since paracetamol has a higher affinity for sulphation than glucuronidation (Moldeus, 1978) exposure of hepatocytes to high levels of paracetamol may result in a depletion of cellular ATP and sulphate. The results reported here would tend to support this.

The formation of glucuronic acid and glutathione conjugates also utilise ATP as an energy source, either directly or indirectly. As supplies of ATP become limiting the systems with the greater affinities for ATP will be spared at the expense of those systems with poorer affinities. The enzymes catalysing the synthesis of glutathione have a higher affinity for ATP than those required for the formation of active sulphate (PAPS) (Jones, 1981).
A therapeutic dose of 0.6-1g of paracetamol in man leads to a concentration of 1-2mM paracetamol in the portal plasma. The concentrations used in the experiments with isolated hepatocytes of up to 40mM paracetamol are therefore very high. Exposure of hepatocytes to paracetamol might be expected to lead to priority utilisation of available ATP to enable metabolic clearance of paracetamol via more water soluble metabolites which can be excreted from the cell. At high concentrations the sulphate and glucuronic acid pathways become saturated (Moldeus, 1978) hence the metabolism of paracetamol via the glutathione conjugation pathway mediated by the microsomal monooxygenase system will become more active leading to a depletion of cellular glutathione.

The first stage of protein synthesis also requires ATP to esterify individual amino acids to their corresponding tRNAs:

\[
\text{Amino acid} + \text{tRNA} + \text{ATP} \rightarrow \text{aminoacyl-tRNA} + \text{AMP} + \text{PP}_i
\]

A consequence of depletion of cellular ATP may well be an inhibition of protein synthesis at the activation stage of the translation process. A further consequence of this may be a feedback inhibition of RNA synthesis since less would be required for protein synthesis.

Paracetamol will cause gross toxicity to hepatocytes if the concentration is high enough and the exposure for long enough. Prior to the development of serious cell damage paracetamol causes reductions in protein synthesis, degradation and secretion, glutathione, ATP, and basal respiration. The effects of all xenobiotics however are not similar as has been seen with results for phenobarbitone and safrole reported here. For example Gluud et al. (1979) have shown that xanthine and some of its derivatives inhibited the synthesis of proteins in isolated rat hepatocytes at the translational level in isolated rat hepatocytes with no effects on cellular cyclic AMP, protein secretion, amino acid transport or degradation of proteins. Furthermore Mattei et al. (1979) have shown that diethyl- and dimethylnitrosamine inhibit protein synthesis in isolated rat hepatocytes with no effect on the energy status of the cells and that this is a reversible process. They suggest that the capacity for these nitrosamine derivatives to inhibit protein synthesis indicates that the hepatocytes
are capable of metabolising them to their active metabolites mediated by cytochrome P-450.

The N-hydroxylation of paracetamol is also mediated by cytochrome P-450 with a requirement for NADPH and oxygen. High concentrations of paracetamol will lead to saturation of the sulphate and glucuronic acid conjugation pathways resulting in more being metabolised by cytochrome P-450 dependent pathways to active metabolites. Paracetamol at a concentration of 10mM, which was shown to have negligible effects on the plasma membrane of isolated hepatocytes but inhibited protein synthesis by about 50%, was investigated cytochemically in cultures. It was found to cause slight reductions in G6PDH activities (30% within 6 hours) with more marked effects on SDH activities (reduced by about 60% within 6 hours). The fall in SDH activity is indicative of mitochondrial effects of paracetamol which would cause a reduction in the production of ATP and a reduced oxygen consumption by the hepatocyte. The effect of 10mM paracetamol on NADPH₂ diaphorase however was a marked increase in activity to about 200% of the control value within 6 hours.

This result may suggest that the response of the cell to a potentially toxic chemical is to activate the microsomal monooxygenase system to remove the insult more rapidly via its metabolism. This hypothesis is further supported by the evidence obtained which indicated that low levels of paracetamol enhanced the rate of protein synthesis which could provide the cell with an increased source of enzymes and haemoproteins necessary to accommodate the increased activity of the microcomal monooxygenase system or to repair damaged macromolecules. This redirection of the cells activity could result in a compensatory slowing of other anabolic and catabolic cellular processes. The reduction in ATP supplies, supported by the reduced mitochondrial activity of the cell, will in itself lead to a slowing of protein metabolism with reductions in protein degradation as well as protein synthesis.

The active metabolites produced by the microsomal monooxygenase system will be inactivated by conjugation with glutathione until the cellular
glutathione is reduced to a level where it cannot provide this protective action. This will result in covalent binding of active metabolite to cellular macromolecules which may include proteins, enzymes, RNA, DNA, lipids, and low molecular weight substances like ATP and UDPG (Gillette, 1981). Covalent binding per se probably does not lead to cell death since the cell is usually able to repair the damage and degrade the covalently bound products. A consequence of chronic exposure to high levels will however lead to damage from which the cell cannot recover with resultant cell death.

There may be other mechanisms directly or indirectly involved in the overall toxic response of the cell to paracetamol including effects on Ca\(^{++}\) flux and lipid peroxidation. It is clear however that once the level of chemical insult to the cell increases beyond a critical level (as yet unknown) the compensatory mechanisms of the cell which can protect it from toxicity may fail to operate. Furthermore the toxic action of paracetamol on the liver cell is not a single mechanism but a composite of a number of pathological changes which are still not fully understood.

5.3.2 Aniline analogues
A variety of structural analogues of aniline related to paracetamol, e.g. aniline, phenacetin and acetanilide were also investigated for their effects on protein synthesis in suspensions of isolated rat hepatocytes. They each caused a dose-dependent inhibition although the concentrations which caused a 50% inhibition (ID\(_{50}\)) varied (Gwynn et al., 1979).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>ID(_{50}) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol</td>
<td>9</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>3</td>
</tr>
<tr>
<td>Acetanilide</td>
<td>6.5</td>
</tr>
<tr>
<td>Aniline</td>
<td>13.5</td>
</tr>
</tbody>
</table>
The metabolism of these four compounds in vivo is interrelated (see Fig. 5). Phenacetin, acetylilide and paracetamol all have analgesic and antipyretic properties although the activities of phenacetin and acetylilide are probably via their metabolic conversion to paracetamol. The target organ for paracetamol toxicity in vivo is the liver whereas for phenacetin it is primarily the kidney or blood causing renal necrosis, or methaemaglobinaemia also caused by acetylilide. All four analogues, however, are hepatotoxic under the right experimental conditions. Investigations by Nelson et al. (1981) on the metabolism of phenacetin indicated that phenacetin can be metabolised to both reactive arylating and alkylating agents by hamsters. Furthermore the multiple pathways leading to reactive metabolite formation from phenacetin are different to those for paracetamol.

The outline in Figure 5 suggests that paracetamol would have the greatest capacity for active metabolite production in the liver since for the other three analogues more metabolic steps are required before active metabolite production becomes a major factor. Active metabolite formation from phenacetin in vivo is probably via the kidney rather than by the liver which may also be the case for acetylilide and aniline. It would therefore appear that there is no direct correlation between active metabolite formation and the effect of compounds on liver protein synthesis since phenacetin and acetylilide are slightly more effective inhibitors than paracetamol. The inhibition of protein synthesis may be related to other effects of the compounds on the mitochondria or indirect effects on cellular ATP levels.

5.3.3 Safrole
Safrole (4-allyl-1,2-methylenedioxybenzene) is a naturally occurring substance which has been used as an artificial flavouring agent in foods. It is hepatotoxic (Crampton et al., 1977) and has also been
Figure 5: The metabolic relationship of aniline and some analogues
found to be a weak hepatocarcinogen in rats and mice (Long et al, 1963). These effects are considered to be due to the formation of active metabolites. One of the metabolic fates of safrole is conversion in vivo to its 1-hydroxy analogue and excretion in the urine as the O-glucuronide (Borchert et al, 1973). The likely activation pathway is via the formation of 1-hydroxy safrole, mediated by cytochrome P-450, which is then esterified to form an electrophilic reactant.

A characteristic property of safrole is that it is capable of forming metabolic products which complex with cytochrome P-450. These tight complexes (product-adduct complexes) which are identifiable spectrophotometrically, effectively decrease the cellular content of functional cytochrome P-450, thereby inhibiting the metabolism of a number of exogenous (and endogenous) substrates (Elcombe et al, 1979; Werringloer and Estabrook, 1979).

Because it is likely that the hepatotoxicity of safrole, in common with paracetamol, is probably mediated via metabolic activation it was considered appropriate to use safrole as a model for the investigation of the early toxic changes which can occur in isolated hepatocytes.

Quantitatively safrole was more hepatotoxic to isolated hepatocytes than paracetamol. At 0.1 and 1.0 mM there were negligible effects on trypan blue exclusion and LDH leakage within two and a half hours of incubation but at 5 and 10 mM there was evidence within 10 minutes of a rapidly induced plasma membrane damage. Protein synthesis and RNA synthesis were also inhibited. Since marked inhibition did not occur until the concentration of safrole was greater than 1 mM the observed reductions in protein and RNA synthesis may be directly related to a reduction in cell viability. Experiments were not carried out to determine whether the effects of safrole on protein synthesis in isolated hepatocytes were reversible.

At a concentration of 1 mM safrole inhibited the resynthesis of glutathione. 10 mM caused a marked depletion of glutathione, as well as causing a marked decrease in ATP levels (to 17% of control values) within two minutes of exposure. The rate of endogenous respiration
was not affected however by 1mM safrole. Safrole was not investigated at higher concentrations for its effects on respiration since under such conditions it was seen to cause rapid plasma membrane damage. Unlike the situation with safrole the concentration of paracetamol which caused a 50% inhibition of protein synthesis (≈10mM) was found to cause decreases in endogenous respiration as well. This may indicate that there is not an obligatory correlation between endogenous respiration and protein synthesis in terms of the toxic response by the liver cell. Cyclic AMP levels were also reduced by 10mM safrole however this effect may simply reflect the plasma membrane damage caused by safrole.

The metabolism of 7-ethoxycoumarin was inhibited in a dose-dependent manner. The pattern of inhibition would suggest that once product-adduct formation with cytochrome P-450 was completed the level of inhibition remained at a steady state. At a 10mM concentration of safrole the metabolism of 7-ethoxycoumarin was completely inhibited which could be due to either product-adduct formation with all available cytochrome P-450 or cofactor leakage due to plasma membrane damage. This pattern of inhibition was different to that seen for paracetamol which does not form product-adduct complexes with cytochrome P-450. The inhibition of 7-ethoxycoumarin by paracetamol was both dose- and time-dependent. The major water soluble metabolite of safrole in vivo is the glucuronide therefore the metabolism of safrole has a low requirement for active sulphate (PAPS). The marked depletion of ATP by safrole (10mM) is unlikely to be due to the increased formation of PAPS but rather to plasma membrane damage.

The attempt to investigate the effects of safrole (1mM) on protein synthesis in cultured hepatocytes was largely unsuccessful. Safrole caused the rapid detachment of hepatocytes from the culture surface although it did not appear to impair the viability of these hepatocytes over one hour of incubation. The toxicity of safrole to isolated hepatocytes may be related to more specific effects on the plasma
membrane resulting in rapid changes in its integrity or to the affinity of safrole for plastic. Irreversible plasma membrane damage occurred rapidly at concentrations greater than 1mM safrole. The reductions in glutathione, an inhibited rate of synthesis seen at 1mM and marked depletion at 10mM, may be related to active metabolite production. Safrole is also an inducer of the microsomal monooxygenase enzyme system. Cytochrome P-450 mediates the production of an active metabolite of safrole, probably an epoxide and the reductions in glutathione may be related to the further metabolism of this epoxide via conjugation. Dihydrosafrole also depletes glutathione in hepatocytes (personal communication: Benford, 1981). This active metabolite is the undoubted cause of the toxicity of safrole in vivo.

5.3.4 Phenobarbitone
Phenobarbitone is a member of the barbiturate class of drugs which cause CNS depression in vivo and hence sedation. It has an additional important property in that it induces the activity of the microsomal monooxygenase system by increasing the amounts of cytochrome P-450 and a number of other microsomal enzymes. In vivo this results in liver enlargement, and both in vivo and in vitro in the increased rate of microsomal monooxygenase mediated metabolism of many xenobiotics.

This property of phenobarbitone has led to its extensive use as a tool both for in vivo and in vitro experiments on drug metabolism and toxicity. For example phenobarbitone pretreated preparations are commonly used to assess the contribution of active metabolites to cellular toxicity. It was therefore appropriate to determine what effects phenobarbitone has on the viability characteristics of isolated rat hepatocytes as it may influence the toxicity of xenobiotics in other ways than simply enhancing the rate of oxidative metabolism.

In experiments on trypan blue exclusion and LDH release there was no evidence of marked toxicity after one hours incubation with the concentrations of up to 20mM that were investigated. After three hours, however, 20mM caused a reduction of up to 70% in cell viability
although the effects of 1 and 10mM phenobarbitone were indistinguishable from the controls. Similar effects were seen with LDH release.

It was interesting to find that phenobarbitone caused a dose-dependent inhibition of total protein synthesis and total RNA synthesis, similar to that found for paracetamol, although the concentration of phenobarbitone which caused a 50% inhibition was lower, because it is well established that both in vivo and in vitro phenobarbitone causes an increase in liver microsomal protein. This result is however supported by the results of Tuma et al (1978) who also demonstrated that secretion of glycoproteins by rat liver slices was impaired by phenobarbitone. They suggested that the inhibition of secretory proteins may be an additional factor contributing to liver enlargement as a result of treatment with phenobarbitone.

Although phenobarbitone was found to be more toxic to cultures after chronic exposure for a few days, a concentration of 1-2mM allowed continued cell attachment. A concentration of 1mM phenobarbitone was found to cause marked increases in the activity of the microsomal monooxygenase system demonstrated by the increased activity of 7-ethoxycoumarin 0-deethylase. Furthermore 2mM phenobarbitone produced marked increases in the activity of NADPH$_2$ diaphorase, G6PDH and SDH activities which were demonstrated cytochemically.

Phenobarbitone at a concentration of 4mM caused about a 50% inhibition of protein synthesis and inhibited the resynthesis of glutathione within the hepatocyte. At a high concentration (20mM) which caused about a 90% inhibition of protein synthesis the effect on glutathione was similar to that of 4mM phenobarbitone. The levels of glutathione were not depleted below the original control levels by phenobarbitone. Direct dose-related depletion of glutathione by phenobarbitone therefore did not occur instead an inhibition of the resynthesis which is maximal at concentrations of phenobarbitone greater than 4mM. This may be related to the effects of phenobarbitone on the electron transport chain where it probably acts as an uncoupler causing a reduced production of ATP and reduced oxygen consumption, supported by the results obtained here. The large reduction in the level of
ATP may be responsible for the inhibition of the resynthesis of glutathione.

Phenobarbitone therefore causes induction and possibly activation of certain haemoproteins and enzymes in isolated hepatocytes. It may be that after acute exposure the cell switches to support the enhanced synthesis of these proteins due to the specific affinities of phenobarbitone for the microsomal monooxygenase system. As a result the synthesis of other proteins and RNAs not required may be inhibited resulting in an overall inhibition of total protein and RNA synthesis. Experiments with phenobarbitone in cultures suggested that the marked effect on protein synthesis over one hour in suspensions of hepatocytes was not maintained on longer term exposure. The results may even be interpreted to suggest that exposure to 5mM phenobarbitone for eight hours caused an increase in protein synthesis between 24 and 48 hours of culture although too much weight should not be placed on the results of this experiment since the results were very variable. A further implication from the results with phenobarbitone is that protein synthesis inhibition is not directly related to depletion of glutathione levels.

5.3.5 Carbon tetrachloride
Carbon tetrachloride is one of the classical model hepatotoxins and much work has been dedicated to elucidating the mechanism of its hepatotoxicity. Carbon tetrachloride is metabolised by the microsomal monooxygenase system to a free radical \( \text{CCl}_3^- \). It was thought that this active species was the cause of lipid peroxidative attack on cellular membranes resulting in cell death. Slater et al (1980) have demonstrated, with the use of pulse radiolysis, that \( \text{CCl}_3^- \) is produced by the liver cell and although relatively active and capable of covalently binding to cellular macromolecules it is not the peroxidative species. \( \text{CCl}_3^- \) is further metabolised by an oxygen dependent reaction to produce a highly reactive peroxyradical (\( \text{CCl}_3\text{O}_2^- \)). This latter species is more likely to be responsible for cell death due to lipid peroxidation and protein damage. The high reactivity of this species will lead to damage occurring at its site of formation in the endoplasmic
reticulum. The less reactive \( \text{CCl}_3^- \) and secondary products can diffuse from the site of activation and cause cell damage at other sites.

Kiezcka and Kappus (1980) provide further support for the oxygen dependence of the formation of lipid peroxides with the suggestion that carbon tetrachloride competes for the oxygen binding sites on cytochrome P-450. Low levels of oxygen will allow greater access of carbon tetrachloride for metabolism to active metabolites yet a low oxygen concentration will inhibit the formation of a peroxidative species.

The few results reported here for carbon tetrachloride indicate that it is markedly toxic to the plasma membrane of isolated rat hepatocytes and causes reductions in oxygen consumption and protein synthesis. Indications were also seen that low levels produced increases in NADPH\(_2\) diaphorase activity suggesting the initiation of self protective mechanisms within the liver cell. There was also a suggestion from cytochemical investigations, although not quantified, that cycloheximide had a mild protective action against the toxicity of carbon tetrachloride in cultures.

The protective effect of cycloheximide is supported by the results of Castro et al. (1977), Lindstrom and Anders (1977) and Popp et al. (1978). The concept that protein synthesis plays an active role in the production of cellular damage produced by carbon tetrachloride is probably however too simplistic. Carbon tetrachloride induced liver injury is probably a composite of more than one pathological change.

Endogenous protection against lipid peroxidation may well be mediated by glutathione via glutathione peroxidase. Lowering of glutathione levels by diethyl maleate has been associated with enhanced malondialdehyde formation by carbon tetrachloride (Lindstrom et al., 1978). Diethyl maleate alone however produces lipid peroxidation and glutathione depletion (Stacey and Priestly, 1978) whereas only high doses of carbon tetrachloride (20 µl/ml) produced glutathione depletion and cell damage without concomitant lipid peroxidation.
Poli et al (1979) have recently confirmed that carbon tetrachloride stimulates lipid peroxidation in isolated hepatocytes. It also inhibits both protein synthesis and protein and lipoprotein secretion and stimulates fat accumulation within liver cells, all similar findings to those seen after in vivo treatment with carbon tetrachloride. It was also confirmed that the increase in lipid peroxidation is mediated by its microsomal monooxygenase metabolism.

5.3.6 A summary of some postulated mechanisms of toxicity

Isolated hepatocytes in suspension or primary monolayer culture have been used here to investigate the effects of paracetamol, phenobarbitone and safrole on the liver. Chemically induced changes in a number of parameters have been identified which may or may not be related to toxicity.

Examination of the changes caused indicates that there is one sequence of events which is common to all three compounds. This is characterised by a rapid fall in ATP levels followed by inhibition of protein synthesis and RNA synthesis. Furthermore irreversible plasma membrane damage was observed in isolated hepatocytes after exposure to all three compounds:

\[ \text{ATP} \rightarrow \text{protein synthesis} \rightarrow \text{RNA synthesis} \rightarrow \text{cell death} \]

Safrole was found to have the most marked toxic effects in hepatocytes with irreversible plasma membrane damage occurring after a short exposure time to relatively low concentrations. Phenobarbitone was toxic to hepatocytes in suspension and culture although irreversible plasma membrane damage occurred later than with safrole. The effects of paracetamol in suspensions of rat hepatocytes were inconsistent. Marked toxicity was seen, however, in cultured hepatocytes after prolonged exposure to high concentrations.

Scale of toxicity:

- Safrole
- Phenobarbitone
- Paracetamol

Paracetamol caused marked reductions in ATP and protein synthesis at a concentration of 40mM. The inhibition of protein synthesis was found to be reversible, however, in cultures which were exposed to paracetamol
for short periods of time. It is therefore probable that the reductions in ATP are also reversible. Furthermore it is unlikely that reductions in protein synthesis and RNA synthesis, or ATP are direct causes of irreversible plasma membrane damage:

\[
\downarrow \text{ATP} \quad \not\rightarrow \text{cell death}
\]
\[
\downarrow \text{protein synthesis} \quad \not\rightarrow \text{cell death}
\]
\[
\downarrow \text{RNA synthesis} \quad \not\rightarrow \text{cell death}
\]

The microsomal monooxygenase (MMO) enzyme system is involved in the metabolism of paracetamol, safrole and phenobarbitone. The major metabolic pathways for paracetamol are via metabolism to the sulphate and glucuronic acid conjugates. At high concentrations, however, these pathways become saturated and metabolism via the microsomal monooxygenase system becomes a major pathway producing more toxic rather than less toxic metabolites in the form of active arylation or alkylating species. Safrole forms a product-adduct complex with cytochrome P-450 hence the MMO enzyme system plays a major role in the metabolism of safrole probably via formation of an epoxide. One minor metabolite of phenobarbitone may be a dihydrodiol which would require initial metabolism to an epoxide derivative. Active metabolite formation may also involve the production of reactive oxygen species. Reactive electrophiles, inactivated by conjugation with glutathione catalysed by glutathione transferase or peroxidase, will deplete intracellular glutathione:

\[
\text{GSH} \quad \not\rightarrow \text{GSSG}
\]

\[\text{GSSG} = \text{oxidised glutathione}\]
Paracetamol and safrole both caused dose-dependent depletion of glutathione. Phenobarbitone, however, reduced the rate of resynthesis of glutathione but did not actually deplete it in a dose-dependent manner. It has been postulated that depletion of glutathione alone may lead to cell death via lipid peroxidation. Prolonged treatment with paracetamol may therefore result in cell death due to lipid peroxidation.

Active arylating or alkylating metabolites may covalently bind to protein or other cellular material. High concentrations of paracetamol were shown here to cause rapid dose-dependent binding of drug-related material to proteins. Furthermore covalent binding of safrole and isosafrole is known to occur at levels below those which cause toxicity. Thus it is unlikely that covalent binding alone causes cell death.

Oxygen consumption was reduced by both paracetamol and phenobarbitone, but not by safrole, at the concentrations investigated here. Paracetamol was also found to inhibit succinate dehydrogenase activity. The mitochondrion may, therefore, be susceptible to the direct effects of paracetamol and phenobarbitone but is unlikely to be a direct target for the toxic effects of safrole.

The properties of these three compounds differ further in that phenobarbitone and safrole are both inducers of the microsomal monooxygenase enzyme system. Phenobarbitone induces cytochrome P-450 whereas safrole induces a novel form of cytochrome P-450. Phenobarbitone's role as an inducing agent was supported here by its effects on 7-ethoxycoumarin O-deethylase and NADPH$_2$ diaphorase activities in cultured hepatocytes. Phenobarbitone also caused increases in the activity of glucose-6-phosphate dehydrogenase and succinate dehydrogenase. Paracetamol caused increases in the activity of NADPH$_2$ diaphorase but decreases in glucose-6-phosphate dehydrogenase and succinate dehydrogenase. Substrates with an affinity for cytochrome P-450 may cause activation of the MMO enzyme system which could explain the effect of paracetamol on NADPH$_2$ diaphorase activity. The effects of safrole on these parameters were not investigated in cultured hepatocytes since safrole caused rapid detachment of cells from the culture surface. One would anticipate, however, that concentrations of safrole,
which did not cause rapid toxicity, would increase the activity of 7-ethoxycoumarin O-deethylase and NADPH$_2$ diaphorase in hepatocytes.

The results obtained here have not identified the direct cause of cell death although they would tend to suggest that it is not due to one factor alone but the combination of a number of factors. In other words chemically induced toxicity is related to the level of insult and the duration of exposure and a reduced capacity of the hepatocyte to repair or reverse chemically induced toxic changes.

A number of common mechanisms have been identified which may be related to the cell death caused by paracetamol, safrole and phenobarbitone:

\[
\begin{align*}
\uparrow & \text{ covalent binding} \\
\uparrow & \text{ lipid peroxidation} \\
\downarrow & \text{ protein synthesis} \\
\downarrow & \text{ RNA synthesis} \\
\downarrow & \text{ GSH} \\
\downarrow & \text{ ATP} \\
\end{align*}
\]

The rapid decrease in ATP levels was the earliest change identified in the studies carried out here. It is not known, however, whether this was a direct effect of the chemicals investigated or whether it was secondary to an earlier chemically induced change. Furthermore there might be other changes occurring which are causally related to cell death which have not been identified.

More detailed investigations into the effects on ATP may yield further information into mechanisms of toxicity. Other areas which might be looked at in hepatocytes are the relationship between glutathione levels and the onset of lipid peroxidation and the effect of chemicals on calcium flux or the transport of other essential electrolytes.
CONCLUSIONS

6.1 THE VALUE OF ISOLATED HEPATOCYTES FOR STUDYING THE TOXIC EFFECTS OF CHEMICALS

Isolated hepatocytes have a number of advantages over the use of whole animals for investigating the toxic effects of chemicals which may be summarised as follows:

1. Less animals required
2. Smaller tissue samples therefore a smaller requirement for chemicals
3. A large number of reproducible samples from one animal
4. The tissue is removed from the influences of hormonal control, and the environment
5. The nutrients may be carefully defined in the media used for incubations
6. The "diet" is easily manipulated via media variation
7. The overall cost of experiments is less.

These advantages are supplemented by the technique used here in that comparison between different species including humans is facilitated. Furthermore the historical data base for these preparations, giving support to the similarities of isolated hepatocytes to liver tissue in vivo, is increasing. There are however a number of disadvantages to using isolated hepatocytes some of which have been highlighted by the results reported here.

The liver is a complex tissue made up of a number of different cell types and areas. The toxic effects of chemicals are often restricted to one area of the liver, e.g. paracetamol causes centrilobular necrosis. Chemicals whose toxicity is caused by highly reactive metabolites produced by the microsomal monooxygenase system are more likely to produce centrilobular necrosis since cytochrome P-450 is more prevalent in the centrilobular hepatocytes (Gooding et al, 1978). Furthermore Smith et al (1979) demonstrated an uneven distribution of glutathione within the liver lobule by cytochemical means finding that the centrilobular cells contained less reduced glutathione than other regions in the liver lobule which may further support the susceptibility
of these cells to the toxic effects of metabolically activated chemicals.

A suspension of hepatocytes contains a homogeneous mixture of liver cell types from the different regions of the liver lobule. It is therefore impossible to identify histologically the site within the liver at which damage is occurring. It is also possible that hepatocytes may lose their individual specific functions in isolation. Furthermore if the effects of a chemical are slight in the acute phase, or even the chronic phase, these will be quantitatively diluted out by the presence of large numbers of unaffected cells. A toxic effect may therefore need to be quite marked before it is detected in a cell suspension. If the chemical is affecting all the cells to a similar extent then it is possible that the toxic effect in vitro differs from that in vivo. The isolation procedure may also introduce a further problem of relative resistance to damage by adaptation of hepatocytes to their new environment.

Some of these problems can be overcome by using cultured hepatocytes since a histological examination can be made and cytochemistry can be used to identify changes in particular cell types.

It is apparent from the results reported here that there is a marked variability in the preparations of hepatocyte suspensions and cultures. This variability is less of a problem in terms of the quantitation of functional parameters like cofactor levels and metabolic capacity than in the response to toxic effects of chemicals. To clarify this point it was apparent from these results that the hepatocytes derived from some animals were far more susceptible to the toxic effects of paracetamol than others. We are therefore unable to remove the individual variation between animals when using hepatocytes in vitro since they not only function as they would in vivo but also maintain the individual characteristics of the animal from which they were derived. This problem may be partially overcome by maintaining more rigorous standards for animal husbandry and animal selection.
The fact that hepatocytes in an isolated situation are removed from the in vivo hormonal controls and a blood supply is probably a major advantage if one is trying to characterise a well defined metabolic pathway or physiological function of the liver. It is clearly apparent however that mechanisms of toxicity are not easily defined and consist of many variables. Furthermore in vivo the pharmacokinetic distribution of a drug and its metabolites can play a major role in its toxicity, and the body has overall defense mechanisms which although undefined clearly aid in reversing toxicity.

The in vivo blood supply provides a continued source of nutrients and oxygen to the liver cell, and serves to remove rapidly components produced by the liver including proteins and drug metabolites. The bile and lymph also provide further pools into which the liver cell may excrete or secrete. The liver is therefore under continuous flux. The suspension of hepatocytes and cultures used here suffer from the disadvantage of not being under continuous flux thereby subjecting the liver cell to high concentrations of potentially toxic chemical for abnormally prolonged periods of time. Furthermore the incubation media may concentrate unwanted toxic by-products produced by the liver which may enhance the toxic response of the cell and influence the mechanistic pathway by which toxicity occurs.

Although the disadvantages discussed here may seem numerous and insurmountable the use of isolated hepatocytes has a wide potential. The research reported here serves to illustrate that the system in use needs to be well defined before subjecting it to toxic chemicals. Once the control hepatocyte in vitro is more fully understood interpretation of toxic changes should prove to be more easily accomplished. The use of hepatocytes as a cytotoxic screen is now being investigated. Tyson et al (1980) compared the effects of 23 chemicals on GOT release finding a good correlation between the effects seen in vitro, in hepatocytes prepared by the method used here, and those reported in vivo. This type of screening for chemicals is not, however, very informative and can give misleading results. It is hoped that it will be some time, if at all, before this type of routine
testing is adopted for screening the toxicity of chemicals. Continued investigations in hepatocytes however may lead to the provision of a more suitable model than the whole animal for elucidating the mechanisms by which chemicals are toxic, which is far more valuable than just knowing whether or not they are toxic.

Future investigations into mechanisms of toxicity will probably be more productive if greater attention is given to the use of isolated hepatocytes in primary monolayer culture rather than in suspension. Cultured hepatocytes allow greater flexibility with the applicability of a wider range of experimental techniques.

To achieve this end the quality of cultures must first be improved to enable reproducibility of preparations with similar properties, larger numbers of cells attached and a more predictable life span. Further investigations into the identity and activity of the MMO enzymes present in culture with the aim of obtaining a similar profile to that present in vivo would provide a more reliable system to work with.

Further improvements may be made by using animals of better quality or even germ free animals. Consideration of diet and environmental conditions prior to sacrifice may also help to produce preparations of a better quality.

There is a need to redefine the constituents of the media used during the isolation procedure and for incubation of the cultures. Careful manipulation of the constituents with a consideration of other supplements may define more suitable media. This is especially relevant to the levels of glutathione and cytochrome P-450 within the hepatocytes.

The availability of a well defined culture system will provide a more reliable in vitro model for the investigation of chemically induced toxicity. The wider range of techniques that may be used to aid these investigations include phase contrast electron microscopy and the application of quantitative cytochemical analyses, in addition to the enzyme marker approach used here. The use of hepatocytes isolated from genetically different strains of rats or the greater use of selective
metabolic inhibitors may provide further information. More detailed time sequencing of chemically induced changes may enable more positive conclusions to be made on the mechanisms of toxicity.

The results from well designed and carefully executed experiments may eventually lead to the elucidation of the mechanisms leading to chemically induced cell death. Furthermore the validation of isolated hepatocytes as a tool for investigating the toxic effects of chemicals may enable their use for predicting more accurately the effects of chemicals in man.
REFERENCES


Miller, E.C. (1951) Cancer Res. 11, 100.


Williams, G.M. (1977) Cancer Res. 37, 1845.


Williams, R.T. (1963) Lancet 1, 723.


