UNIVERSITY COLLEGE LONDON

THE EFFECTS OF ISOLATION TECHNIQUE, SUBSTRATA AND PYRAZOLE ON CYTOCHROME P450 ENZYMES IN CULTURED MALE RAT HEPATOCYTES

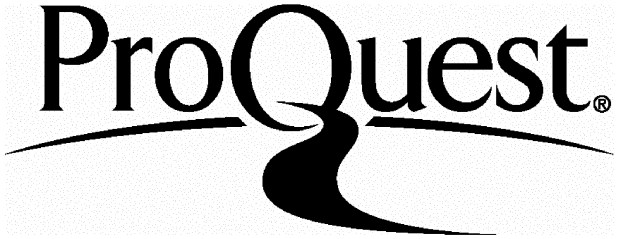
A DISSERTATION SUBMITTED TO THE FACULTY OF LIFE SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

DEBORAH HERRIOTT

LONDON, U.K.

JANUARY 2000



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ABSTRACT

The cytochrome P450 enzymes are of particular interest in the study of drug metabolism since they play a key role in the transformation of xenobiotics. The recent explosion of *in vitro* techniques has facilitated detailed examination of drug metabolism in isolated systems such as primary hepatocyte culture. However, the expression of cytochrome P450 enzymes declines under culture conditions, limiting the use of this method.

These studies examined the effects of combinations of isolation technique and substrata on the activity and amount of immunodetectable protein of several cytochrome P450 enzymes in male rat hepatocytes cultured for up to 96 hours. Two techniques, collagenase and EDTA, were used to isolate hepatocytes subsequently cultured on Matrigel®, Vitrogen®, fibronectin or uncoated plastic. Collagenase digestion was a significantly superior method of isolating hepatocytes compared to EDTA, in terms of cell yield and viability. The low cell yield and viability following EDTA isolation suggest that this technique is unsuitable for routine use.

The data generated show that both substrata and, to a greater degree, isolation technique significantly affected the activity and immunodetectable amounts of several cytochrome P450 enzymes in rat hepatocytes throughout the culture period examined. The rates of decline of these enzymes, and the effects of isolation technique and substrata, were variable. No combination of isolation technique and substratum was found to halt the decline in cytochrome P450 enzymes and it is likely that a number of factors involved in their expression are lacking in the culture system

The expression of CYP2E1 in cultured rat hepatocytes was also examined following exposure to the CYP2E1 inducing agent pyrazole. Increases in both CYP2E1 activity and amount of immunodetectable protein show that the hepatocytes have retained the ability to respond to pyrazole, demonstrating that the decline in cytochrome P450 expression is not irreversible.

ACKNOWLEDGEMENTS

The production of this dissertation has involved a long, and at times, difficult journey – definitely a marathon rather than a sprint! There are a number of people who have encouraged, nudged and consoled me along the way and I would like to give them my thanks. These include my parents who, in addition to being responsible for my existence, have always encouraged and believed in me, even when things weren't going so well. I know how delighted they will be that I managed to hang in there and complete these studies. Big thanks also to my husband who has supported and listened to me over the years and who realised how important this project was to me. He has also tried to keep the combined noise of our children, 3-year old Corrinne and 2-year old Callum, down to a mild roar while I write which probably deserves some sort of medal!

Thanks go to Liz Shephard and Mike Tarbit for being my supervisors and for inspiring me to carry out this work. Liz, probably without realising it, gave me a couple of nudges in the last few years when I was close to giving up and Mike has imparted much advice on 'life, the universe and everything'. Thanks also to Steve Hood for his help and patience with the 'molecular bits' – (and for teaching me the importance of adding nucleotides to a PCR reaction!) - and to Bob Kempson who gave invaluable help with the statistics to someone who last formally studied statistics quite a number of years ago and whose brain had to be gently coaxed into remembering the joys of variability and significance testing!

Finally I would like to thank Lynn and Louise, my sisters, for making me laugh and keeping things in perspecive and the friends and colleagues who have shown me both friendship and support through the years.

FIGURES AND TABLES *Page*

ABBREVIATIONS

AD	Androstenedione
ADME	Absorption, Distribution, Metabolism and Excretion
BSA	Bovine Serum Albumin
CAMP	Cyclic Adenine Monophosphate
CYP450	Cytochrome P450
DEPC	Diethyl pyrocarbamate
DNA	Deoxyribonucleic Acid
EBSS	Earles' Balanced Salt Solution
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethyleneglycol bis-(β-aminoethyl ether) N', N', N', N'-tetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
ERE	Estrogen Response Element
FAD	Flavin Adenine Dinucleotide
FDA	Food and Drug Administration
FMN	Flavin Mononucleotide
FMO	Flavin Monooxygenase
GRE	Glucocorticoid Response Element
HNF	Hepatocyte Nuclear Factor
HPLC	High Performance Liquid Chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
mRNA	Messenger Ribonucleic acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCE	New Chemical Entity
OHT	Hydroxy testosterone
PAH	Polycyclic Aromatic Hydrocarbons
PPAR	Peroxisome Proliferator Activated Receptor
RAR	Retinoic Acid Receptor
SD	Standard Deviation
TAE	Tris Acetate

TBS	Tris Buffered Saline
TLC	Thin Layer Chromatography
TTBS	Tris Buffered Saline containing Tween 20
TE	Tris-EDTA
TEMED	N,N,N',N',-tetramethyl-ethylenediamine
X-gal	5-bromo-4-chloro-indoyl-β-D-galactoside

CHAPTER 1 INTRODUCTION

1.1 THE PHARMACEUTICAL INDUSTRY

Mankind has sought, for thousands of years, medicines for the treatment of illness and disease. Documents such as the 3500 year-old Ebers Papyrus from Egypt, describing an early classification of medicine, diseases and treatments have been found. Today a more structured approach is made towards the treatment of disease, with chemical design an important aspect of the production of new medicines. GlaxoWellcome is a research based company whose purpose, as one of the largest pharmaceutical companies in the world, is the discovery, development, manufacture and marketing of safe, effective medicines.

The design and development of novel medicines is far from facile however. The complex biochemistry of the body can be disrupted by the administration of foreign molecules, which may lead to undesirable side effects. Further, no drug can be considered entirely free from toxic side effects if given in large enough doses. Therefore, when developing a new medicine the nature and severity of these side effects must be carefully considered and balanced against the nature and severity of the disease the drug is targeted towards. The risk-benefit balance will be different for a life threatening disease such as cancer than for a less serious disease such as migraine. The benefit of every new drug in terms of life-saving capability, reduction in patient suffering, improvement in patient quality of life and cost needs to be assessed.

The time-consuming, complex, and highly regulated process of developing a new drug is considered below and summarised in Figure 1.1 (Davies et al., 1986 for review). Each drug has an individual development program, since the problems and issues identified for each drug are unique. The process can also be extremely expensive with development costs as high as 300 million pounds for each compound (Halliday et al., 1997). The new drug is often protected for a number of years by a patent, although the long development time that is typically 5 to 10 years, is included in the patent life.

It is desirable, therefore, to bring new products to market in the minimum possible time while ensuring the safety and efficacy of the drug.

1.1.1 Drug Discovery

There are several identifiable stages involved in the production of a new medicine which can be arbitrarily broken down into research, pre-clinical and clinical development. The initial stage of the drug development process, research, is the identification of chemical entities that may have a therapeutic use. To minimise the potential for undesirable side-effects, the target of early research is to identify compounds that act selectively and are potent enough to be administered at a low dose. During this early stage the potency, selectivity and physicochemical properties of the new chemical entities (NCEs) are determined in a number of screens which often have a high-throughput capacity (reviewed by Tarbit and Berman, 1998).

The screening strategy usually involves simple *in vitro* tests or uses an animal model of the disease, which contains the proposed therapeutic target, such as an enzyme or receptor. Obviously an understanding of the cause of the disease-state is desirable in order to target the activity of the NCEs and to design the appropriate screens. Since inter-species differences are known to exist, it is preferable to use the human enzyme or receptor in the screen since this is the ultimate target of the drug. Determination of physicochemical properties, such as pKa and lipophilicity can also be an important component of the screening strategy. Knowledge of these properties help in understanding the relationships between structure and activity and aid in ensuring molecules chosen for further evaluation have optimum properties associated with kinetic parameters such as oral absorption.

Many thousands of compounds are synthesised for each therapeutic area under investigation. High-throughput primary screens of vast compound libraries are used to identify those compounds with high selectivity and potency as well as metabolic stability and good absorption. Secondary 'screens' are then used to identify those compounds that have activity *in vivo*. There are many reasons why a compound identified in the primary screen may be unsuitable for further development including high clearance of the drug due to extensive metabolism, high renal excretion or high

biliary excretion, low oral bioavailability and unacceptable toxicity. Some of these factors may be measured using pharmacokinetic parameters, and compounds with unfavourable pharmacokinetics may be eliminated during both the primary and secondary screening phases. This serves to ensure that compounds forwarded for further development are more likely to have the appropriate properties to progress as therapeutic agents. The majority of new chemical entities are discarded during the screening process, however, because of the difficulty in achieving this balance.

1.1.2 Drug Development

Once a drug has been identified as being suitable for further progression two main stages of drug development follow. These are often referred to as exploratory or pre-clinical development, and full or clinical development. During exploratory development extensive biological and pharmacological screening and pre-clinical trials using both *in vivo* and *in vitro* models are carried out. These tests provide information on the suitability of the compound to be a therapeutic agent in man. Both the safety of the compound and the efficacy of the candidate compound are established during the development process, prior to first administration to man.

During exploratory development important safety studies are performed which are a requirement of regulatory authorities throughout the world. Two species must be used in these toxicology studies: a rodent and a non-rodent. Often the rat and the dog are the species of choice due to the large amount of background information on these animals, although occasionally it may be necessary to use other species such as the marmoset or the guinea pig.

The compound is also evaluated in Genetic Toxicology for its potential to interfere with the translation of the genetic message and by Reproductive Toxicology for the potential to interfere with the reproductive process and to examine the effect, if any, on the progeny of animals receiving the compound. Finally, carcinogenicity studies are carried out to determine the effect of chronic dosing over several years. Drug metabolism studies are also performed at this time, to measure absorption, distribution, metabolism and excretion of compounds using pharmacokinetic

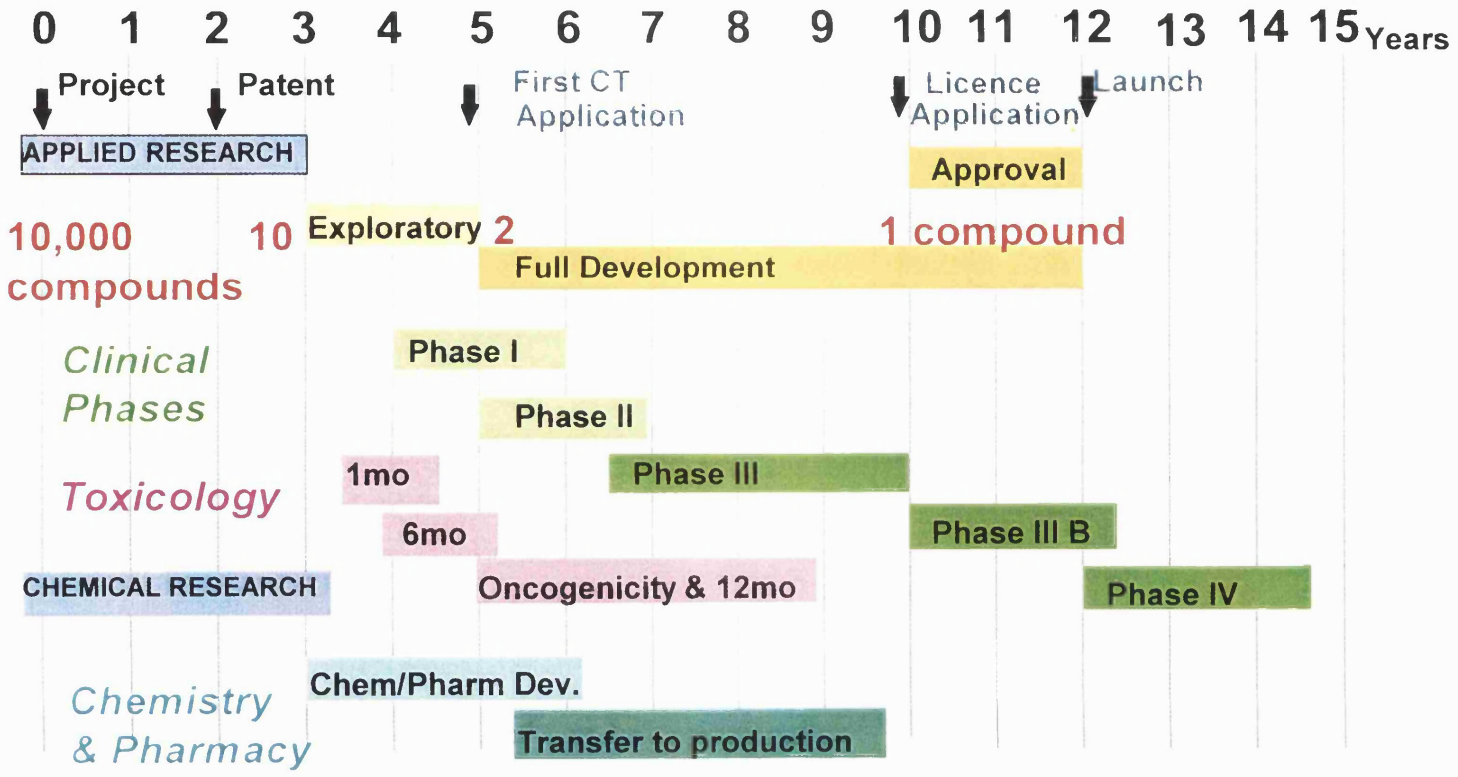
parameters and to support toxicology studies by measuring exposure of test animals to the compound.

It must be appreciated that these studies determine the efficacy and safety of the compound in animal species since it would not be ethical to carry out early studies in man. It is necessary therefore, despite the limitations, to extrapolate safety and efficacy data to man. However, the best model for man is man himself. It is therefore desirable to obtain information on the behaviour of drugs in man as early as possible and recent developments in the use of human tissues *in vitro* provide a useful tool to aid this process.

Once short-term animal safety studies have been successfully completed the compound may progress into Phase 1 clinical development. These studies involve the first administration of the compound to man and are performed in a limited number of healthy volunteers, usually males. The main aim of these studies is to establish the safety and the tolerability of the drug. Following successful completion of Phase I studies a compound can move into full development involving extensive clinical trials in human volunteers and patients under the strict supervision of clinicians. The approval of the appropriate regulatory authority is required before Phase 2 studies, in which the drug is administered to patients, are performed. The main aim of these studies is to establish safety and efficacy in the intended patient population. Phase 3 studies are then conducted in a larger number of patients in order to obtain information on efficacy and long term safety of the new drug. At this stage comparator studies with other medicines may also be performed.

Once these stages of development are complete all the data from each stage of development are submitted to regulatory authorities throughout the world, such as the Food and Drug Administration (FDA) in the USA and the European Medicine Evaluation Agency (EMEA) in Europe, as an application to market the new medicine. The regulatory authorities review the data and may grant a marketing licence for the drug that can then be launched as a new medicine. Pharmaceutical companies continue to monitor their drugs after they have been launched via pharmacovigilance groups which collate adverse event information from the medical community.

Figure 1.1 The process of drug discovery and development



1.2 The Role of Drug Metabolism

Regulatory authorities both in the UK and overseas, have made safety and efficacy studies in animals and man a legal requirement. An integral part of these requirements is proof of exposure and comparative data on the fate of the molecules. Thus the absorption, distribution, metabolism and excretion studies (ADME) are determined in samples obtained from animals and, ultimately, man.

1.2.1 ADME Studies

ADME studies are carried out to establish a number of parameters:

- The oral bioavailability of the drug is determined to ensure that a drug given by this route is absorbed and can reach the site of action.

- The exposure of animals to the drug and related metabolites is measured to validate toxicology studies and demonstrate dose-related exposure of animals to drug and any metabolites formed.

- The metabolic fate of the drug, the identification of metabolites and enzymes involved in metabolism and the route(s) of elimination of both drug and metabolites are determined for comparison between animals and man.

- The distribution of the drug in the body is assessed to identify any areas where the drug is present in high concentration or where the drug remains bound for long periods of time.

Drugs are often lipophilic in nature, as targets are often located in the cell membranes. Thus prior to removal from the body, most compounds will be subjected to a degree of metabolism via a large range of chemical reactions possible through the bodies xenobiotic clearance mechanisms. Drug metabolism occurs throughout the body particularly in ports of entry, and reactions have been documented in the skin

(Pendlington et al., 1994), gastrointestinal tract, (Watkins et al., 1992), lung, (Gram, 1993), blood, brain, (Warner and Gustaffson, 1993), nasal epithelium (Longo et al., 1991) and kidney as well as the liver (Baron et al., 1986). The purpose of metabolism is to increase the polarity of the compound, thereby lowering the partition of the drug into the lipid compartment of cell membranes and facilitating elimination of the drug from the body in urine and bile.

These metabolic reactions have traditionally been separated into two phases, known as Phase I and Phase II. Reactions considered to be Phase I involve 'functionalisation' of the compound such as oxidation, reduction, hydrolysis, hydration and isomerisation. The majority of Phase I metabolism occurs in the endoplasmic reticulum and is carried out by enzymes including cytochrome P450s (CYP450) and flavin monooxygenases (FMO). In contrast, Phase II reactions involve conjugation of the compound, or a Phase I metabolite of the compound, to a variety of very polar moieties including glucuronic acid, sulphate, glutathione and certain amino acids. Phase II metabolism occurs in both the endoplasmic reticulum and the cytoplasm.

1.2.2 In Vitro Drug Metabolism

As the process of developing new drugs becomes increasingly complex, new models for investigating the various facets of drug development have been sought. The recent explosion of *in vitro* technology within the pharmaceutical industry has not only reduced the numbers of experimental animals used but has enabled scientists to obtain data which would be difficult to extract from *in vivo* experiments due to the complexity of this system. The potential reduction of animals used is in accordance with the 3R concept proposed by Russell and Burch (1959) recommending the refinement, reduction and replacement of whole animal testing.

In vitro techniques have been used in all stages of development involving Drug Metabolism (Wrighton et al., 1995, review). This ranges from providing metabolic and absorption screens for research projects to the use of human hepatic microsomes to help predict drug metabolism and interactions in man at an early stage of drug development. The possibility of *in vitro* models using human material is of particular

interest since it avoids some of the problems of extrapolation of data obtained using other animal species to man. *In vitro* tests are also free from many of the ethical and legal constraints encountered with *in vivo* experiments, although obtaining human tissue can present its own ethical dilemmas. *In vitro* testing is often more manageable, quicker and cheaper than *in vivo* studies. Further, certain European laws require the use of these alternative methods if possible. Directive 86/609/EEC states that an animal procedure "*shall not be performed, if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practically available*".

In vitro technology can be used to answer many questions within drug metabolism. This includes the identification of drug metabolites and the enzyme(s) responsible in both man and in the toxicology species, allowing compounds that are metabolised by enzymes subject to genetic polymorphism to be identified. *In vitro* methodology can also be used to determine whether the compound is an inhibitor or an inducer/repressor of drug metabolising enzymes. This information can then provide a valuable insight into which drug interactions in man are more likely.

Generally, studies have centred on the liver since this is considered to be the major site of drug metabolism. It is important to note however, that many other tissues in the body are capable of metabolising xenobiotics and that metabolism in the liver may not be representative of whole body metabolism. A plethora of *in vitro* tools are now available including sub-cellular fractions such as microsomes, hepatocyte suspensions, primary hepatocyte cultures, immortalised cell lines, precision-cut liver and kidney slices, recombinant enzymes and specific inhibitory chemicals and antibodies. Using these tools either singly or in concert, many questions related to drug metabolism may be answered. To address questions relating to metabolism in man, human tissue, although scarce, can be obtained although the tissue must be fresh or suitable preserved (Skett et al., 1995). Recombinant human enzymes can also be obtained commercially. The tools and models available, and their respective merits, are considered in more detail below.

1.2.2.1 Heterologously Expressed Enzymes

Heterologously expressed enzymes are now commercially available, in which the enzyme of interest is expressed in isolation from other related enzymes (Lee et al., 1995). Microsomes prepared from cells expressing a single drug metabolising enzyme such as an isoform of cytochrome P450 or FMO can be used as a tool to determine whether the enzyme has the potential to metabolise the candidate drug. These preparations often have high activity and can be used in simple high throughput screens for metabolic stability. Although initially research has concentrated on achieving expression of active cytochrome P450 enzymes, FMO is also available and other enzymes will inevitably follow. The expression of drug metabolising enzymes using a variety of expression vectors is the subject of a review article (Guengerich and Parikh, 1997).

Certain problems can be encountered when trying to express drug metabolising enzymes in bacteria and yeast, such as incorrect folding of the protein, absence of a suitable membrane for insertion within the cell, lack of NADPH-dependent cytochrome P450 reducatse (P450 reductase) activity, insufficient incorporation of haem and over-expression of inactive protein. Despite these problems good results have been obtained for the cytochrome P450 isoforms using the baculovirus expression system. This method can be used to obtain a membrane-bound enzyme that has good activity, particularly if co-expressed with P450-reductase. Under optimised conditions it is possible to obtain active enzyme with similar characteristics to the native enzyme. However, in some cases the enzyme may be present at much higher levels than that found *in vivo* and, coupled with an absence of other enzymes to compete for the test compound and P450-reductase, misleading results may be produced.

1.2.2.2 Microsomes

Despite advances in recombinant technology one of the most commonly used preparations remains the microsomal sub-cellular fraction. This is obtained by centrifugation of homogenised tissue, usually liver, or more rarely other tissue such as

lung or gut, to obtain the endoplasmic reticulum. This preparation contains the majority of the cytochrome P450 enzymes, FMO and the Phase II enzyme glutathione transferase. Microsomes are easy to prepare in large amounts and are convenient to use. They may be stored at -80°C for long periods of time with minimal loss of activity during storage occurring, even with freeze/thawing (Pearce et al., 1996). Due to the absence of the cell wall cellular uptake of the drug is not involved, enabling metabolism to be investigated in isolation. However, co-factors such as NADPH must be added to incubations and the preparation lacks some of the Phase II enzymes which are present in the cytosol. The S9 fraction may be used when it is felt necessary to make available both membrane bound and cytosolic enzymes, although we have found S9 to be poor in Phase II activity.

1.2.2.3 Isolated Hepatocyte Suspensions

Suspensions of intact hepatocytes are another popular tool and models utilising whole cells have been reviewed (Guillouzo, 1998; Skett, 1994; Berry et al., 1992). Intact hepatocytes, unlike microsomes, can be used to investigate drug metabolism without the need for added co-factors such as NADPH and UDP-glucuronic acid, although a source of sulphate may be required. This preparation also contains the full complement of metabolising enzymes that are not lost during the culture period, including the Phase II enzymes, in their usual cellular environment. Finally, enzyme instability due to disruption of the tissue is minimised.

However, isolated hepatocytes are a more complex system than the microsomal preparation since the cell wall is present and transport of the drug into the cell is required. Hepatocyte suspensions are also more technically difficult to prepare and have a limited life span of approximately 3 to 4 hours because of cell damage caused during incubation (Berry et al., 1991). Hepatocytes must be prepared as required, since attempts to cryopreserve hepatocytes have met with varying degrees of success. Cryopreserved hepatocytes, both as suspensions and attached to alginate beads (Hammond et al., 1999), are available commercially although freshly isolated cells remain higher in drug metabolising activity and would be the preferred choice at this time. This will undoubtedly change as more sophisticated techniques for

cryopreserving cells are found, and some promising studies have been reported (Swales and Utesch, 1998).

1.2.2.4 Hepatocyte Culture

Hepatocyte cultures, either in monolayer culture, co-culture or as spheroids, have many of the same benefits as hepatocyte suspensions with the added advantage that cultured cells may be maintained for much longer periods of time. They are useful for studies of gene expression, growth control and toxicology. This model also offers an extended incubation time, which is useful for detecting metabolites that are produced slowly.

One of the major limitations is that hepatocytes maintained in this way rapidly lose liver-specific functions including drug metabolising enzymes. Despite this primary hepatocyte cultures may still be useful in determining enzyme induction by test compounds (Wortelboer et al., 1991). Human cells retain their differentiated status for longer than certain other species such as rat but the reason for this is not known (Grant et al., 1987). Alternatives to simple monolayer culture have been sought therefore and co-cultures, whereby hepatocytes are cultured on a layer of epithelial cells, have been reported to improve maintenance of liver-specific functions (Akrawi et al., 1993; Begue et al., 1984; Fraslin et al., 1985). However co-culture is technically more complicated and the epithelial cells have metabolic enzymes themselves including CYP2E1 (Lerche et al., 1996). Hepatocyte spheroids are another alternative to monolayer culture that has shown some promise in the maintenance of cytochrome P450 (Niwa et al., 1996, Hammamoto et al., 1998). Immortalised hepatoma cell-lines, such as HepG2, may also be useful if extended experimentation is required, but in general these cells have few of the characteristics of normal adult liver including the expression of cytochrome P450 enzymes (Silva et al., 1998). Finally, recent improvements in artificial livers using hepatocytes, and knowledge gained from such systems, may eventually lead to a system that can be used in drug metabolism (Kan et al., 1998; Yanagi et al., 1998).

1.2.2.5 Liver Slices

Liver slices are an increasingly popular tool since they are much easier to prepare than hepatocytes and retain the architecture of the liver (Ekins, 1996, review). The commercial availability of the Krumdiek and the Brendel-Vitron slicers have enabled slices of a uniform thickness to be achieved on a regular basis leading to a resurgence of interest in the use of this technique. Liver slices enable both cell-cell and cell-matrix interactions to be maintained, so that the three-dimensional architecture containing all cell types is not disrupted. In this system the gap junctions between cells which play a role in cell signalling are not destroyed, therefore intracellular communication between the various cell types can still occur (Green and Jones, 1996). Tissue slicing also avoids the damage caused by proteolytic enzymes used in the preparation of isolated hepatocytes.

Unfortunately, slices cannot be maintained for as long as cultures, possibly due to the low availability of oxygen and nutrients to the centre of the slice where a band of necrotic cells can often be observed. There is also growing evidence that the decrease in levels of drug metabolising enzymes with time observed with cultured hepatocytes also occurs in slices and that slices may be even less stable than hepatocytes (Vandenbranden et al., 1998). Finally, sequestration of the drug and metabolites into the slice can make quantification of metabolism technically difficult in this system.

1.3 THE CYTOCHROME P450 FAMILY OF ENZYMES

The cytochrome P450 enzymes (EC1.14.14.1, non-specific monooxygenase) are a large family of haemoproteins. Strictly speaking the enzymes are not cytochromes but heme-thiolate proteins. However the original name for these enzymes, derived from the discovery that the reduced pigment after binding to carbon monoxide had an absorption band with a λ_{max} at 450nm, (Omura and Sato, 1964) has been retained. Cytochrome P450s are involved in the Phase I metabolism of a large number of structurally diverse xenobiotics, acting as the terminal electron oxidase of an electron transport system (Chang and Kam, 1999). This family of enzymes is also involved in

the metabolism of endogenous substrates such as steroids, bile acids, fatty acids and prostaglandins (Coon et al., 1998). As such these enzymes are of particular interest in Drug Metabolism and are the subject of these studies. Many other enzymes, such as flavin monooxygenase and the conjugating enzymes, are involved in drug metabolism and, although not within the scope of this review, have been reviewed elsewhere (Beedham, 1997).

The large substrate specificity of this enzyme family is due both to the multiplicity of distinct enzymes and to the broad and often overlapping substrate selectivities of individual enzymes. Indeed, the widespread specificty of these enzymes make them ideally suited to respond to the many endogenous and xenobiotic challenges. Some of the cytochrome P450 enzymes metabolise both xenobiotics and endogenous compounds and it has been suggested that the true function of these enzymes was originally one of endogenous metabolism. The greater affinity for the endogenous substrates in some cases, such as for certain steroids, lends support to this theory. The metabolism of xenobiotics may therefore have been purely fortuitous.

Although not identified until 1958 (Klingenberg; Garfinkle), the cytochrome P450 superfamily is extremely old, with the ancestral gene thought to have been in existence more than 3.5 billion years ago. Cytochrome P450 has been identified in every class of biota, including mammalia, birds, fish, reptiles, amphibia, insects, plants, bacteria and fungi. (Andersson and Forlin, 1992; Feyereisen, 1993; Cohen and Feyereisen, 1995; Bozak et al., 1990; Nelson et al., 1996). Indeed, in the mammal cytochrome P450 has been detected in every type of cell except erythrocytes and skeletal muscle (Guengerich, 1993a and 1993b).

It has been speculated that the original role of cytochrome P450 was in the detoxication of tissue and atmospheric dioxygen to water, since the gene predates the existence of drugs and plant/animal interactions (Nebert and Feyereisen, 1994). Eukaryotes and ultimately, plants and animals subsequently retained the enzyme and gene duplication and mutation has resulted in the many enzymes found today. The proposed evolution of the cytochrome P450 superfamily provides an interesting

parallel with major events in the development of biological forms and this particular aspect of cytochrome P450 has been reviewed (Lewis et al., 1998).

The exact number of cytochrome P450 enzymes is not yet known although to date 481 P450 genes and 22 pseudogenes have been characterised (Nelson et al., 1996). This number will undoubtedly increase and up-to-date information can be found on the WorldWideWeb (<http://www/icgeb.trieste.it/p450). The enzymes have been classified into 74 families, which are then further divided into subfamilies on the basis of amino acid similarity. Members of the same family must have at least 40% sequence identity, while members of the same subfamily have at least 70% sequence similarity. This system of classification was adopted in 1987 to avoid the confusion that had arisen from different laboratories isolating the same protein but designating the enzyme with different names. Although aiding in the clarification of this area this system of classification does not facilitate understanding the biology of the enzymes and excludes similar enzymes such as cyclo-oxygenase and nitric oxide synthase.

1.3.1 Structure and Function

Most of the cytochrome P450 enzymes are integral membrane proteins of about fifty thousand Daltons. They consist of a single polypeptide chain approximately 400 to 500 amino acids in length, and contain a single, noncovalently bound haem group ligated to a fixed cysteinyl residue and a replaceable water ligand. They are present in the highest concentration in the endoplasmic reticulum but are also present in the inner mitochondrial membrane.

Binding to a membrane is thought to be essential for the function of most cytochrome P450 enzymes (Sakaguchi et al., 1994) and these enzymes are synthesised by the membrane bound ribosomes of the rough endoplasmic reticulum and co-translationally integrated into the membrane. Complexes of rough endoplasmic reticulum and mitochondria have been observed which may facilitate the integration of haem, which is synthesised in mitochondria, with the P450 apoprotein (Meier et al., 1978). The phospholipids in the immediate vicinity of P450 in liver microsomes have been reported to be highly organised (Ahn et al., 1998) and the interaction between the

P450 enzyme and the phospholipid may be necessary for active protein conformation and efficient transfer of electrons from P450 reductase.

The exact conformation of the enzyme has yet to be fully elucidated in most cases, although the soluble, non-membrane bound camphor 5-exo-monooxygenase from *Pseudomonas putida* has been crystallised (Poulos et al., 1987) as have other soluble bacterial P450s (P450BM-P, P450terp and P450eryF). The membrane topology of microsomal P450s is thought to contain a large cytoplasmic domain anchored to the membrane by either one or two amino-terminal transmembrane segments (Black, 1992). Information regarding the topology of cytochrome P450 from such sources as NMR, and knowledge of the structure of substrates, have enabled some models of the active site of a number of P450s to be proposed (Poliscaife et al., 1997; Smith et al., 1997 for review). This can then be used to gain information as to whether a compound is likely to be a substrate for this enzyme. The structural and functional aspects of cytochrome P450 enzymes have also been reviewed (Graham-Lorence and Peterson, 1996).

The proposed catalytic cycle of cytochrome P450 is shown in Figure 1.2 (Estabrook et al., 1999 for review). The cycle shown refers to the cytochrome P450 found in the endoplasmic reticulum system and not to the mitochondrial system which differs in the use of an iron-sulphur protein, adrenodoxin, that shuttles between the FAD-dependent NADPH-adrenodoxin reductase. Some speculation and controversy still exists as to the mechanism of cytochrome P450 reactions, particularly with respect to whether the active oxygenating species is an oxenoid or peroxide (White, 1994, Coon et al., 1998). Possibly both mechanisms are used depending on the catalytic conditions and substrate. The overall reaction can be represented simply by the equation:

$$\mathbf{RH + O_2 + NADPH / H^+ \Rightarrow ROH + H_20 + NADP^+}$$

The chemical mechanisms of cytochrome P-450 catalysis involve the incorporation of an oxygen atom from molecular oxygen into the substrate, with the second atom

P450 enzyme and the phospholipid may be necessary for active protein conformation and efficient transfer of electrons from P450 reductase.

The exact conformation of the enzyme has yet to be fully elucidated in most cases, although the soluble, non-membrane bound camphor 5-exo-monooxygenase from *Pseudomonas putida* has been crystallised (Poulos et al., 1987) as have other soluble bacterial P450s (P450BM-P, P450terp and P450eryF). The membrane topology of microsomal P450s is thought to contain a large cytoplasmic domain anchored to the membrane by either one or two amino-terminal transmembrane segments (Black, 1992). Information regarding the topology of cytochrome P450 from such sources as NMR, and knowledge of the structure of substrates, have enabled some models of the active site of a number of P450s to be proposed (Poliscaife et al., 1997; Smith et al., 1997 for review). This can then be used to gain information as to whether a compound is likely to be a substrate for this enzyme. The structural and functional aspects of cytochrome P450 enzymes have also been reviewed (Graham-Lorence and Peterson, 1996).

The proposed catalytic cycle of cytochrome P450 is shown in Figure 1.2 (Estabrook et al., 1999 for review). The cycle shown refers to the cytochrome P450 found in the endoplasmic reticulum system and not to the mitochondrial system which differs in the use of an iron-sulphur protein, adrenodoxin, that shuttles between the FAD-dependent NADPH-adrenodoxin reductase. Some speculation and controversy still exists as to the mechanism of cytochrome P450 reactions, particularly with respect to whether the active oxygenating species is an oxenoid or peroxide (White, 1994, Coon et al., 1998). Possibly both mechanisms are used depending on the catalytic conditions and substrate. The overall reaction can be represented simply by the equation:

$$\mathbf{RH + O_2 + NADPH / H^+ \Rightarrow ROH + H_20 + NADP^+}$$

The chemical mechanisms of cytochrome P-450 catalysis involve the incorporation of an oxygen atom from molecular oxygen into the substrate, with the second atom

incorporated into water (Guengerich, 1990, review). Electrons are fed into the cytochrome P450 catalytic cycle by P450 reductase, a flavin-containing enzyme that consists of both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). This enzyme is thought to act as a transducer of reducing equivalents, by accepting electrons from $NADPH/H^+$, a two-electron donor and transferring them to cytochrome P450, a one-electron acceptor. Although the complex redox biochemistry of this system has yet to be fully elucidated it is thought that FAD accepts electrons from $NADPH/H^+$ and FMN donates the electron to cytochrome P450 (Shen et al., 1999).

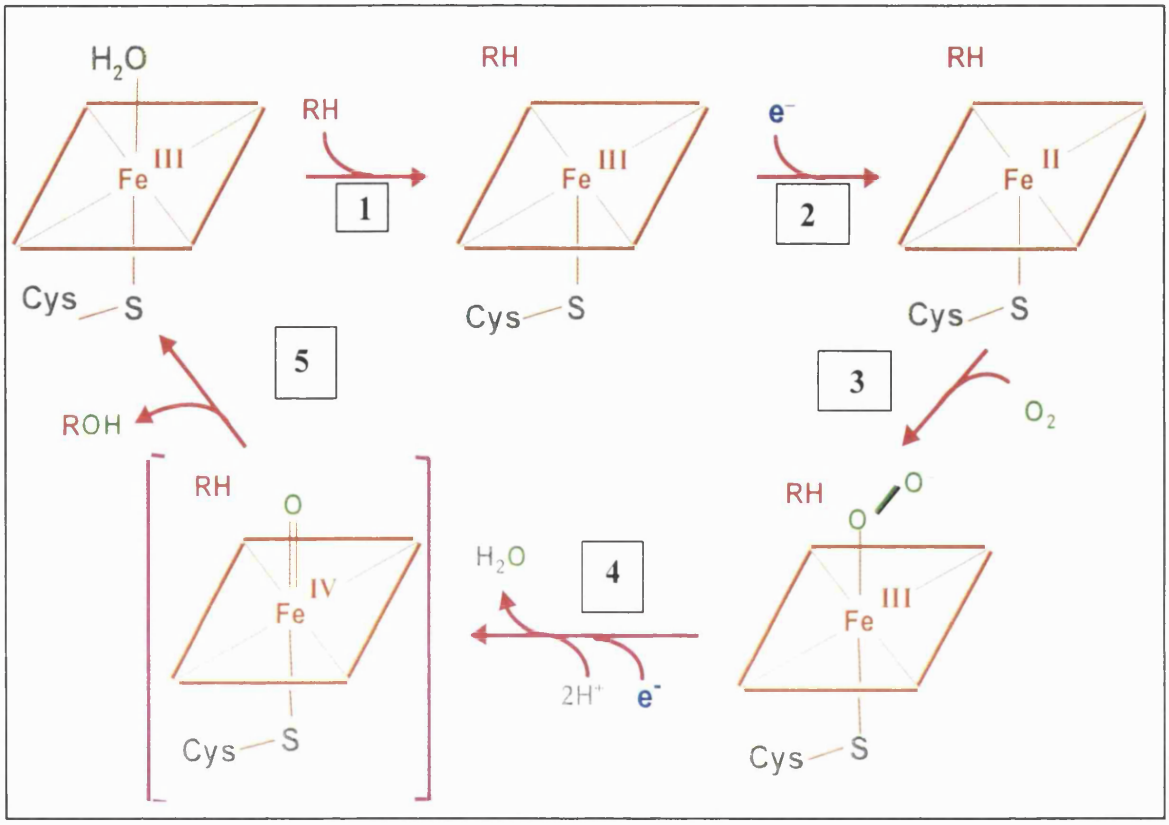
The first step in the cytochrome P450 cycle involves the binding of the substrate, such as a drug, to the oxidised form of cytochrome P450. Most P450 enzymes exist in a low-spin configuration of the electrons of the ferric iron (Fe^{3+}). Binding of drug to the protein portion of cytochrome P450 causes a conformational change and loss of a water molecule from the active site. This is accompanied by a shift in spin-state from low-spin to high-spin. This in turn causes an increase in the redox potential to a more positive value resulting in a greater electromotive force for the subsequent transfer of electrons to the cytochrome. Binding of the drug directly to the haem iron, particularly by compounds containing a nitrogenous base, results in a 6-co-ordinated low-spin haemoprotein with loss of enzyme activity.

The next step (2) involves the reduction of the substrate-bound cytochrome P450 to the ferrous form by a single electron. This electron is provided by P450 reductase. The spin-state of the cytochrome P450 is thought to be important for this first reduction. This is followed by the third step whereby molecular oxygen, which is usually freely available, binds to the cytochrome P450-substrate adduct. The input of a second electron in step four, either from P450-reductase or cytochrome b_5 /NADPH-cytochrome b_5 reductase, results in an iron-peroxide complex. Oxygen is inserted into the substrate resulting in the formation of the product, although the mechanism is not completely understood. The arguments for, and evidence of, the oxene or peroxide intermediate have been reviewed (Lewis, 1998, Coon et al., 1998). The most likely mechanism by which this occurs is hydrogen abstraction with the generation of a carbon radical (White, 1994; Guengerich and Macdonald, 1990). The product is then released from the complex (step 5).

P450 reductase donates electrons to cytochrome P450, although there may be competition for the P450 reductase enzyme due to the order of magnitude greater concentration of total cytochrome P450 in the membrane environment (Guengerich, 1984). The P450 enzymes are also known to vary in their affinity for P450 reductase (Guengerich, 1984). The role of cytochrome b_5 and NADPH-cytochrome b_5 reductase in the catalytic cycle of cytochrome P450 is not yet clear and is dependent both on the particular isoform of P450 involved and on the substrate (Yamazaki, 1996). Electrons can be transferred to cytochrome b_5 from NADH by cytochrome b_5 reductase or from

P450 reductase. For some metabolic reactions, including certain fatty acid oxidations (Estabrook, 1978) and a number of CYP2E1 mediated reactions (Yamazaki et al., 1996b) the presence of cytochrome b_5 is essential. The mechanism by which cytochrome b_5 stimulates metabolism is not fully understood, but may involve acceleration of the flow of the first electron from the NADPH-P450 reductase complex to the P450-substrate complex (Yamazaki et al., 1996c). Thus the catalytic cycle of cytochrome P450 is highly complex and despite years of intensive research is not yet fully understood.

Figure 1.2 The proposed catalytic cycle of cytochrome P450



Step 1	Substrate binding with shift from low spin to high spin Fe^{3+}.
Step 2	First electron transfer: Fe^{3+} is reduced to Fe^{2+}
Step 3	In the presence of oxygen the ternary P450-substrate-oxy-complex is formed
Step 4	The P450-substrate-oxy complex is reduced to an iron-peroxide complex.
Step 5	Release of product

1.3.2 Overview of Major Forms Involved in Drug Metabolism

The ability to differentially regulate the expression of specific genes is a fundamental characteristic of both prokaryotic and eukaryotic organisms. This includes the expression of cytochrome P450 enzymes, the amounts of which are in a state of constant flux. In man, for example, expression is regulated by a large number of factors, including age, hormonal status, dietary and environmental components as well as certain genetic differences (Bock et al., 1994). To this may be added the varied induction and inhibition of various P450 enzymes by exposure to pharmaceuticals (for reviews see Barry and Feely, 1990: Lin and Lu, 1998). This adaptive response is entirely appropriate given the intermittent and sometimes unpredictable nature of exposure to the xenobiotics and endogenous compounds which these enzymes metabolise.

Due to the large number of P450s, it is not surprising that the control of expression of the genes encoding these proteins is complex, with different P450 genes regulated by different mechanisms. In addition the vast array of xenobiotics to which the organism may be exposed and the intermittent nature of this exposure can differentially induce or suppress the levels of these enzymes (Guengerich, 1987). The levels at which gene expression control mechanisms are exerted, however, are no different to those operating in the control of other mammalian gene products (Goldfarb, 1990). Briefly, these include the rate of gene activation and transcription, pre-mRNA splicing, translocation of mRNA, mRNA stability, post-translational modifications and the rate of degradation of individual proteins (Figures 1.3 and 1.4).

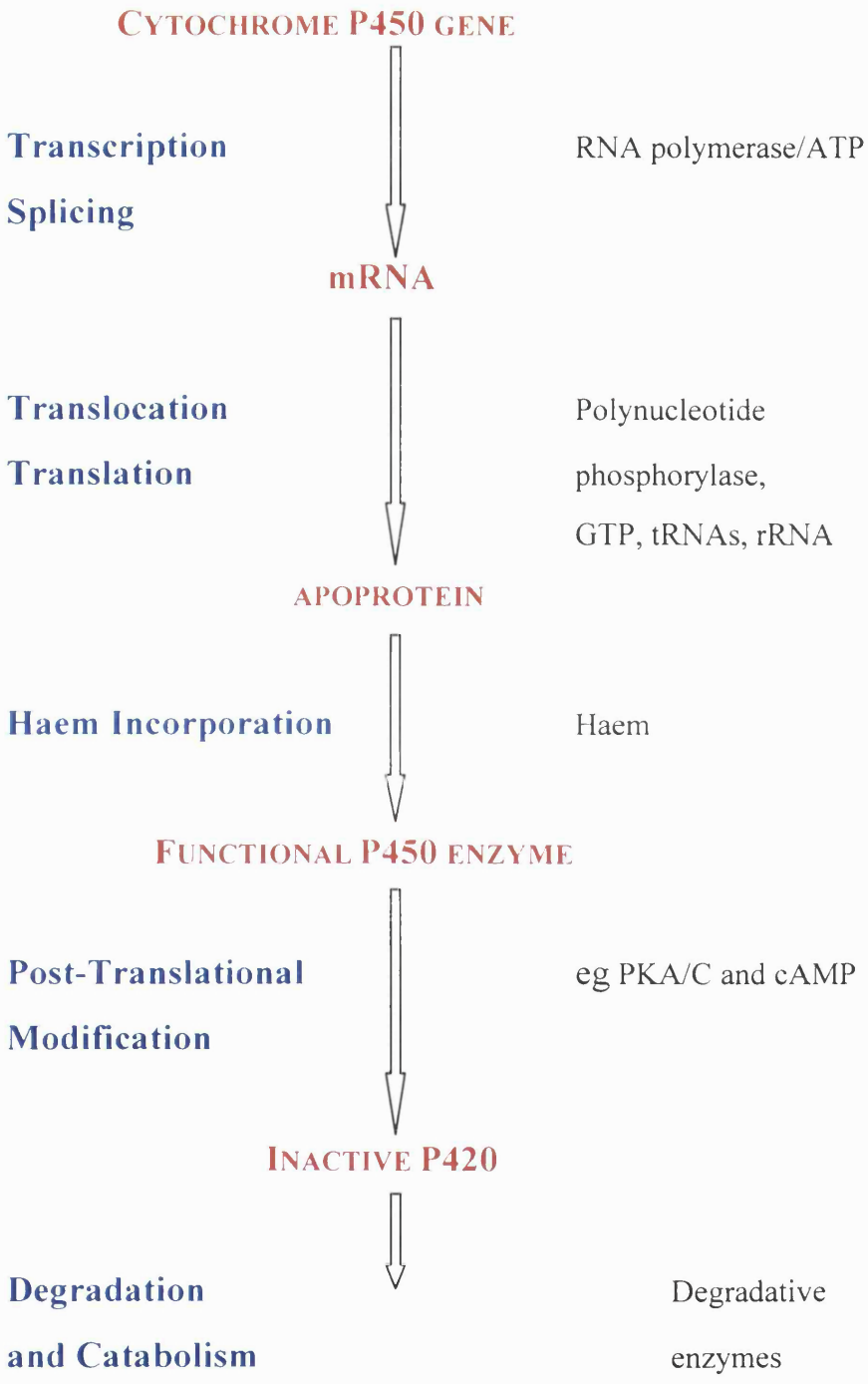


Figure 1.3 Control points in cytochrome P450 expression (adapted from Lewis, 1996)

Figure 1.4 Simplified Structure of a P450 gene (modified from Lewis, 1996)

≈/> -1000bp	-100 to –100bp	-40bp	-25bp	+1
Enhancer elements XREs etc	Tissue/development specific REs, GREs, factors etc	BTE	TATA Box	Transcription Initiation Site
	Regulatory Region			**Coding Region**

XRE = Xenobiotic Response Element

BTE = Basal Transcription Element

TATA = TATA box motif

Regulation of gene transcription is controlled by the presence of *cis*-acting sequences of DNA adjacent to or within the gene (see Dogra et al., 1998 and Ramana and Kohli, 1998 for review articles). In the case of cytochrome P450 genes these are referred to as drug responsive elements (DREs) or xenobiotic responsive elements (XREs) (Churchill and Travers, 1989). DNA binding proteins, or *trans*-acting factors, bind with a specific target regulatory region and facilitate the entry of RNA polymerase molecules into the RNA promoter site of the gene. The RNA polymerase is then able to transcribe the gene and synthesise a pre-mRNA. The control of CYP1A expression is unusual in that a cytosolic receptor rather than a nuclear receptor for inducers, the Ah receptor, has been shown to be involved. Demethylation events have also been shown to cause gene activation by the unwinding of condensed, inactive chromatin. This has been demonstrated for the CYP2E1 gene (Viera et al., 1996).

The nuclear hormone receptors are a family of DNA-binding proteins which control gene transcription when bound to responsive elements (Green, 1993; Waxman, 1999). Of these some are well characterised and the endogenous ligand identified, for example the oestrogen receptor (ER), the retinoid receptors (the retinoic acid receptor or RAR, and the retinoid X receptor or RXR) and the peroxisome proliferator-activated receptor (PPAR). For others such as PXR, a so-called orphan receptor, the

endogenous ligand has yet to be identified. Members of the RXR subfamily are able to dimerize both homologously, with themselves, or heterologously with many other nuclear transcription factors (Vecchini et al., 1994). The retinoid receptors are particularly interesting in the control of cytochrome P450 expression since ligands for RAR decrease hepatic cytochrome P450 levels whilst ligands for RXR increase P450 levels (Howell et al., 1998).

Nuclear hormone receptors are thought to act by the binding of a ligand inducing a conformational change. This in turn allows the formation of homodimers or heterodimers (often with RXR) which bind to the specific recognition sequences upstream of the target gene, thus triggering transcription. Drugs that induce gene transcription bind in the same way as the endogenous ligand and thereby initiate transcription. However, the CAR β-receptor (constitutive androstane receptor) which forms a heterodimer with RXR is thought to act differently in that the endogenous ligand bound form is inactive (Forman et al., 1998). Drugs such as phenobarbital displace the androstanes and allow binding to DNA and subsequent activation of transcription.

A second point of control is differential splicing, whereby an mRNA can give rise to more than one product. This is not a common phenomenon in the control of cytochrome P450 expression. It has, however, been demonstrated for CYP4A1 where an extra 75bp sequence in the 3' non-coding region of mRNA could be retained (Brawerman, 1989). Aberrant splicing has been observed in the 2C and 2D families, but this is due mutation within the splice site and is not a true alternative splicing (Gonzalez et al., 1988).

When the mRNA is translocated into the cytoplasm it can be degraded by ribonucleases. Therefore, the susceptibility of each individual cytochrome P450 mRNA will determine the amount of mRNA available for translation to protein. Inhibition of mRNA degradation is the cause for the induction of CYP3A by troleandomycin (Watkins et al., 1986).

A further point of control is the availability of the haem prosthetic group (Padmanban et al., 1989). It has been estimated that approximately 65% of the total haem content of the liver is used as the prosthetic group in cytochrome P450 enzymes. Haem itself can act as an intracellular regulator of a variety of metabolic pathways for systems that utilise oxygen. Haem has been shown to have a positive effect on drug-mediated activation of cytochrome P450 genes in rat liver and is required for the stability of newly synthesised apo-cytochrome P450 (Dwarki et al., 1987). Interestingly, the existence of a phenobarbital-induced transcription factor that is modulated by haem has been demonstrated (Rangarajan and Padmanban, 1989). The exact mechanisms by which haem controls the level of cytochrome P450 enzymes has yet to be fully elucidated.

Other post-translational mechanisms of control are known, including protein phosphorylation by cAMP dependent protein kinases. Protein phosphorylation appears to lead to inactivation or reduction in activity of several forms including CYP2B1 and CYP2C2 (Oesch-Bartlomowicz and Oesch, 1990: Bartlomowicz et al., 1989a). This mechanism of control allows for a very rapid response. To add to the complexity, the activity of kinases is regulated by the level of cAMP, which in turn is under hormonal control (Bartlomowicz et al., 1989a). It has been observed that although the initial effect is a decrease in activity, a long-term increase in cAMP causes an increase in P450 via protein synthesis (Berry and Skett, 1988).

The stability of the cytochrome protein is the final point at which control of activity has been demonstrated. The binding of certain substrates has been found to increase the half-life of the protein by blocking degradation in a process termed ligand stabilisation. CYP2E1 activity is partially affected in this way (Eliasson et al., 1988).

The cytochrome P450 isoforms responsible for the metabolism of xenobiotics belong to families 1,2,3 and 4 with many of the enzymes in the remaining families involved in steroidogenesis and lipid processing. A selection of typical substrates, inducers and inhibitors of the major human forms of cytochrome P450 involved in drug metabolism is shown in Table 1.1. These enzymes are involved in the oxidative metabolism classified as Phase I reactions whose purpose is to increase the aqueous solubility of

the foreign compound, facilitating elimination. This process usually produces metabolites which are less toxic than the parent compound, although there are many documented examples of activation of procarcinogens and production of toxic metabolites (Guengerich, 1988). In particular, enzymes in the CYP1A family carry out many activation reactions, including those involving polycyclic hydrocarbons. Activation of procarcinogens and production of toxic metabolites by other forms including CYP2E1 and CYP3A has also been documented (Guengerich and Shimada, 1991). The four families involved in drug metabolism, including a brief review of control of expression, are considered in more detail below.

1.3.2.1 The CYP1 Family

The CYP1A subfamily consists of two closely related isoforms, CYP1A1 and CYP1A2, which have distinct but overlapping substrate specificity (Nedelcheva and Gut, 1994). The high degree of conservation of these proteins across a range of species suggests that they have an important endogenous function, although this has not yet been determined. These enzymes have been the subjects of intense study, partly due to their involvement in chemical toxicity and of carcinogenicity in particular (Gonzalez and Gelboin, 1994). Differences are apparent in the constitutive expression of the enzymes, since although CYP1A1 does not appear to be expressed in human liver (Shimada et al., 1992), CYP1A2 has been detected in the livers of many species including man, comprising about 9 per cent of human hepatic P450 (Shimada et al., 1994). It has been suggested that the constitutive expression of CYP1A2 is under the control of hepatocyte-enriched transcription factors. The enzymes have many substrates including benzo[a]pyrene, acetaminophen and ethoxyresorufin.

Table 1.1 Substrates, inhibitors and inducers of human cytochrome P450 isoforms (Chang and Kam, 1999)

P450	SUBSTRATES	INHIBITORS	INDUCERS
CYP1A1	ETHOXYRESORUFIN, BENZO[A]PYRENE	α-NAPHTHOFLAVONE	POLYAROMATIC HYDROCARBONS, FLAVONES, INDOLOCARBAZOLES
CYP1A2	PHENACETIN, CAFFEINE, CLOZAPINE	FURAFYLLINE FLUVOXAMINE	POLYAROMATIC AMINES, ISOSAFROLE, OMEPRAZOLE
CYP2A6	COUMARIN		
CYP2B6	PHENOBARBITAL, PENTOXYRESORUFIN		
CYP2C9	TOLBUTAMIDE, DICLOFENAC	SULPHAPHENAZOLE	SECOBARBITAL, RIFAMPICIN
CYP2C18	WARFARIN		
CYP2C19	MEPHENYTOIN, DIAZEPAM, OMEPRAZOLE, PROGUANIL	FLUOXETINE, OMEPRAZOLE	PHENOBARBITAL, RIFAMPICIN
CYP2D6	DEBRISOQUINE, PROPANOLOL, HALOPERIDOL, CODEINE, IMIPRAMINE,	QUINIDINE, PAROXETINE METHADONE	
CYP2E1	CHLORZOXAZONE, ETHANOL, BENZENE, CHLOROFORM	DISULFIRAM	ETHANOL, PYRAZOLE
CYP3A4	MIDAZOLAM, LIDOCAINE, QUINIDINE, ERYTHROMYCIN, NIFEDIPINE, VERAPAMIL, CYCLOSPORIN, TERFENADINE	KETOCONAZOLE, GESTODENE, ERYTHROMYCIN	DEXAMETHASONE, RIFAMPICIN, ERYTHROMYCIN, PREGNENOLONE-16α-CARBONITRILE
CYP4A	LAURIC ACID		CLOFIBRATE, CIPROFIBRATE

The enzymes are highly induced by polycyclic aromatic hydrocarbons (PAHs), for example benz(a)pyrene results in a 100-fold increase in CYP1A levels (Ioannides and Parke, 1993). Analyses of the induction have revealed a novel transcriptional regulatory mechanism (Whitlock et al., 1996; Dogra et al., 1998, review). The first stage of this complex pathway involves binding of the drug to the ligand-binding domain of the Ah receptor causing a conformational change that exposes a nuclear localisation signal. Ligands for the Ah receptor include 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methylcholanthrene. The ligand-bound Ah receptor subsequently translocates to the nucleus where dimerization with the Ah receptor nuclear translocator (Arnt) occurs and in the process the heat shock protein 90 (HSP90) is displaced (Pongratz, 1992), although it is not yet clear when the HSP90 protein dissociates. The heterodimer product subsequently interacts with an upstream xenobiotic-responsive element, the aromatic hydrocarbon response element. This results in transmission of a signal to the *CYP1A* promoter and ultimately in changes in chromatin structure (Whitlock et al., 1996). The inductive effect is therefore due to an increase in transcription, an effect that can be seen rapidly, within minutes of exposure to the inducing agent.

The 5' upstream region of human *CYP1A1* also contains a negative regulatory element allowing further control of expression (Boucher et al., 1995). The regulation of *CYP1A1* is also at least partially controlled by a nuclear factor 1 site located in the proximal promoter (Morel and Barouki, 1998).

More recently a second subfamily, CYP1B, purified and characterised from mouse embryo fibroblasts (Pottenger et al., 1991), has also been identified in man (Sutter et al., 1994). This enzyme is constitutively expressed in steroidogenic tissues such as mammary, ovary and testis as well as embryonic cells leading to the suggestion that this enzyme has an endogenous role (Savas et al., 1997). The CYP1B members are induced by β-naphthoflavone and have a limited number of substrates that are polycyclic aromatic hydrocarbons typified by dimethylbenzanthracene (Savas et al., 1997).

1.3.2.2 The CYP2 Family

The CYP2 family is one of the most complex, consisting of seven subfamilies in mammals. For this reason they are considered separately below.

CYP2A: The CYP2A subfamily has been characterised in many species with the isoforms 2A1, 2A2 and 2A3 detected in the rat, 2A4 and 2A5 in the mouse and 2A6 and 2A7 in man. Substrates include testosterone, coumarin and aflatoxin B1. Differences in expression between male and female rats have been observed, with CYP2A1 female-dominant and CYP2A2 male-specific. Of the two human forms CYP2A6 and CYP2A7, which comprise about 4 per cent of hepatic P450 (Shimada et al., 1994) one, the latter appears to lack activity due to its inability to incorporate haem (Ding et al., 1995).

Induction of these enzymes is variable: *CYP2A1* and *CYP2A5* are weakly inducible by phenobarbital, *CYP2A2* and *CYP2A4* appear to be non-inducible and *CYP2A3* is weakly inducible by 3-methylcholanthrene. In man it is thought that *CYP2A6* is subject to genetic polymorphism (Gonzalez and Gelboin, 1994) and there is wide variability in activity. However, due to the very small number of drugs that are substrates for these enzymes this is not thought to be of great significance clinically.

CYP2B: The CYP2B subfamily has also been characterised in many species with isoforms CYP2B1, CYP2B2 and CYP2B3 detected in the rat, CYP2B4 and CYP2B5 in the rabbit and CYP2B6 and CYP2B7 in man. Substrates for these enzymes include phenobarbital and other barbiturates and a variety of organochloride pesticides including DDT. In the rat CYP2B2 but not CYP2B1 is constitutively expressed in the liver in low amounts although in other tissues such as the lung and testes, this situation is reversed (Soucek and Gut, 1992). The third rat form is constitutive but not inducible. In man expression is very low or non-detectable, and tissue-specific expression is evident with CYP2B6 found in the liver and CYP2B7 in the lung (Gonzalez, 1992).

The *CYP2B1* and *CYP2B2* genes are both inducible by phenobarbital, although *CYP2B1* is more highly induced. In contrast to induction of the CYP1A enzymes no cytosolic phenobarbital-binding receptor has been identified, and control of *CYP2B* genes is complex (Honkakoski and Negishi, 1998a, review). Basal transcription of the *CYP2B* genes is governed by proximal DNA elements such as the C/EBP binding site which are quite active (Luc et al., 1996). A bacterial barbiturate-responsive regulatory sequence, (the 'Barbie box') in which derepression results in increased transcription, has also been identified (Shaw and Fulco, 1993). Despite similar sequences in mammalian *CYP2B* genes which show increased binding of nuclear proteins in the presence of phenobarbital (He and Fulco, 1991) the majority of evidence indicates that the barbie box is not involved in the phenobarbital induction of mammalian cytochrome P450 genes (Park et al., 1996; Honkakoski et al., 1996).

Induction of mammalian *CYP2B* transcription by agents including phenobarbital is thought to be under the control of a distal element located upstream from the transcription start site. This has been designated either the phenobarbital-responsive element (PBRE) in the rat (Trottler et al., 1995) or the phenobarbital-responsive enhancer module (PBRM) in the mouse (Honkakoski and Negishi, 1997). Furthermore, a CAR-RXR heterodimer has been identified as a trans-acting factor for phenobarbital induction of the mouse *Cyb2b10* gene (Honkakoski et al., 1998b). Phenobarbital induction of *CYP2B* is further complicated a phosphorylation mechanism which acts as a switch to increase affinity of transcription factors for the negative rather than the positive regulatory element within *CYP2B1/2*. The complex regulation of the *CYP2B* genes is still a long way from being understood.

CYP2C: The CYP2C subfamily is very large with 26 isoforms identified to date, with four constituting approximately 16 per cent of hepatic P450 in man (Shimada et al., 1994). These include CYP2C6, CYP2C7, CYP2C11, CYP2C12, CYP2C13, CYP2C22, CYP2C23 and CYP 2C24 in the rat and CYP2C8, CYP2C9, CYP2C18 and CYP2C19 in man. A large number of identified substrates include retinol (CYP2C8), tolbutamide (CYP2C9), warfarin (CYP2C18) and mephenytoin (CYP2C19), with marked differences in substrate and stereo-selectivity existing between the isoforms.

Gender differences have been observed in the rat, with the female-specific CYP2C12 being under the control of growth hormone, an effect mediated by the transcription factor HNF-6 (Lahuna et al., 1997). Sex-related differences have not been observed in man although the 4-hydroxylation of S-mephenytoin by CYP2C19 is subject to genetic polymorphism (Wrighton et al., 1993) with approximately 3-5 per cent of Caucasian and 20 per cent of Oriental populations having poor activity. The CYP2C subfamily is weakly inducible by phenobarbital.

CYP2D: The CYP2D subfamily has been the subject of much research, since it has been estimated that up to 30 per cent of drugs are metabolised by the human CYP2D6 orthologue, and because this isoform is genetically polymorphic (Eichelbaum and Gross, 1990). Although constitutive, expression is surprisingly low, comprising about 2 per cent of total hepatic P450 (Shimada et al., 1994). The enzyme does not appear to be inducible by therapeutic agents although it is induced during pregnancy (Wadelius et al., 1997). Substrates include debrisoquine, bufuralol and tropisetron. The rat forms of CYP2D are CYP2D1, CYP2D2, CYP2D3, CYP2D4 and CYP2D5.

CYP2E: The CYP2E subfamily is small, with only two members: CYP2E1 and CYP2E2, which is a rabbit specific form. The CYP2E1 enzyme is constitutively expressed in many tissues in man, although primarily the liver where it comprises approximately 6 per cent of human hepatic P450. Substrates include chlorzoxazone and paracetamol.

The *CYP2E1* gene is inducible by many chemicals including pyrazole, pyridine and ethanol as well as by physiological status such as fasting and diabetes. It is of particular interest since there is growing evidence for the involvement of this enzyme in carcinogen activation, and in particular disease states possibly due to generation of free radicals and active oxygen species. As such it is the subject of an induction model in these studies, and this enzyme has been considered in detail in Section 1.6.

CYP2F, CYP2G, CYP2H, CYP2J and CYP2K: Little is known about these subfamilies of CYP2. Two isoforms of CYP2F, CYP2F1 and CYP2F2 have been

identified in human and mouse lung and CYP2F1 may be involved in skatole activation. The CYP2G subfamily consists of only one protein, CYP2G1, to date that has been located in rat and rabbit olfactory tissue. The two proteins in the CYP2H subfamily, CYP2H1 and CYP2H2 are found in avian liver and are phenobarbital inducible. Even less is known of the enzymes in the CYP2J and CYP2K subfamilies found in rabbit intestine and trout respectively.

1.3.2.3 The CYP3 Family

The CYP3A subfamily of enzymes are of particular interest in drug metabolism, comprising up to 63 per cent of human hepatic P450 (Gonzalez and Gelboin, 1994), and being responsible for the metabolism of a large number of currently used drugs (Wrighton and Stevens, 1992). In the rat four enzymes, CYP3A2, CYP3A9, CYP3A18 and CYP3A23 (previously CYP3A1) although constitutive and inducible, have less impact on drug metabolism than their human counterparts. These enzymes are expressed mainly in the male, with CYP3A23 male-dominant and CYP3A2 male specific. Of the four known human forms, CYP3A3 and CYP3A4 are almost identical, CYP3A5 is polymorphic being expressed in about 20 percent of Caucasians (Wrighton and Stevens, 1992) and CYP3A7 is a foetal form (Kitada et al., 1987) which is however, detectable in some adults. The many diverse substrates include nifedipine, midazolam, erythomycin and diazepam (Li et al., 1995). The large active site of CYP3A can even accommodate the macrolide antibiotic cyclosporin, and may even be able to bind two separate substrates at the same time (Shou et al., 1994).

The *CYP3A* genes are inducible by endogenous glucocorticoids and hormones such as growth hormone, by synthetic steroids such as pregnenolone-16α-carbonitrile and dexamethasone and by macrolide antibiotics such as erythromycin. The exact induction mechanism has yet to be fully elucidated, and evidence suggests that it is likely that more than one mechanism exists depending on the class of inducing agent (Okey, 1990). Steroid receptor response elements GRE (glucocrticoid respone element) and ERE (estrogen response element) are present in the 5'-flanking region of CYP3 genes and may mediate induction of these enzymes (Kemper, 1993) although evidence exists suggesting a role for a microsomal protein (Wright and Paine, 1994).

However, involvement of the GRE is not essential and some non-steroidal inducers such as metyrapone induce transcription for CYP3A but are not ligands for the GRE (Quattrochi et al., 1995).

More recently a new hormone receptor was identified, the pregnenolone X receptor (PXR), which mediates glucocorticoid agonist and antagonist activation of a hormone response element within the promoter region of the rat CYP3A23 gene (Kliewer et al., 1998). A number of xenobiotics including organochlorine pesticides, polychlorinated biphenyls and a number of antihormones such as spironolactone, induce CYP3A23 by a mechanism involving activation of the PXR (Schuetz et al., 1998). Indeed, it has been proposed that the purpose of PXR mediated induction of CYP3A23 by steroids and xenobiotics is to metabolise the very chemical which is causing the induction (Schuetz et al., 1998).

CYP3A is also negatively regulated and iodothyronines have been shown to decrease CYP3A-mediated metabolism (Liddle et al., 1998).

Due to the large number of drugs that are metabolised by CYP3A drug interactions involving this family of enzymes are frequently reported. A recent review of interactions involving human CYP3A *in vitro* and *in vivo* has been published (Thummel and Wilkinson, 1998).

1.3.2.4 The CYP4 Family

The CYP4A subfamily consists of three subfamilies: CYP4A, CYP4B, and CYP4F. The rat isoforms include CYP4A1, CYP4A2, CYP4A3, CYP4A8, CYP4B1 and CYP4F1. The human orthologues are CYP4A9, CYP4A11, CYP4B1, CYP4F2 and CYP4F3. Some tissue specificity has been observed with CYP4A11 found in the kidney and CYP4B1 found in the lung. This family of enzymes is involved in the metabolism of fatty acids and arachidonic acid products such as leukotrienes (Bains et al., 1985). The ω-hydroxylation of lauric acid is often used as a probe substrate for the CYP4A enzymes, which are expressed constitutively in liver and kidney. CYP4A gene expression is hormonally regulated and is induced in pregnant and lactating rats.

CYP4A proteins are inducible by a class of drugs which includes the hypolipidemic drug clofibrate, that cause peroxisome proliferation and induction of peroxisomal enzymes (Kimura et al., 1989). Induction of CYP4A by clofibrate is due to transcriptional activation, which may be mediated by a peroxisome proliferator activated receptor (PPAR), a nuclear hormone receptor for which the endogenous ligand is thought to be leukotriene B4 (Devchand et al., 1996). A binary complex of peroxisome proliferator activated receptor and retinoid X receptor acts as a transcription factor, increasing CYP4A expression by binding to the peroxisome proliferator responsive element. It is also possible that inducers displace an endogenous ligand from the PPAR rather than binding to it themselves (Lake, 1995).

1.4 THE LIVER

The liver is one of the major organs involved in metabolism of xenobiotics and has been the focus of a large amount of research aimed at developing *in vitro* models for toxicology and metabolism studies. The liver is unique among organs in that it has a dual blood supply, from both the hepatic artery and the hepatic portal vein. The portal vein conveys blood from the gastrointestinal tract, spleen, pancreas, gall bladder and stomach to the liver and then on to the systemic circulation. The liver is thus the first organ encountered by substances, including toxins and drugs, absorbed from the gastrointestinal tract and it is therefore the ideal location for enzymes involved in detoxification. The liver is also involved in metabolism and detoxification of endogenous substances and for certain toxins, such as ammonia, is the only site of detoxication. The liver therefore contains a high concentration of metabolic enzymes, including those involved in both Phase I and Phase II metabolism.

1.4.1 Liver Structure

As well as parenchymal cells the liver contains a stroma of connective tissue, blood vessels, nerves and lymphatics, sinusoids, a surrounding capsule and a serous cover on the capsule to prevent adherence to the abdominal wall. As far as is known the

commitment to being a certain cell type is irreversible since no adult liver cell has ever been seen to switch phenotype to that of another cell type under any physiological or pathological state or experimental condition.

The classical structural and functional unit of the liver, referred to as a lobule, was described some time ago (Kiernan, 1833). The lobule is shaped like a polyhedral prism and is comprised of a central venule from which radiate plates of hepatocytes one cell thick. Around the periphery of the lobule are the six portal tracts, or portal triads, which contain the terminal branches of the hepatic arterioles, the terminal branches of the portal venules and the bile ductules. Stem cells, or oval cells, that are found in the vicinity of these triads give rise to the plates of hepatocytes. Fewer cell divisions and a terminal differentiation process take place as hepatocytes progress towards the central vein.

The spaces between the plates of liver cells are referred to as sinusoids and these are lined with endothelial cells. These specialised capillaries provide the exchange of substances between the blood and the parenchyma. Blood derived from both the hepatic arterioles and the portal venules perfuses through the sinusoids, interacts with parenchymal tissue, and leaves the lobule via the hepatic vein from which venous blood is returned to the vena cava.

More recently an alternative model to describe the liver acinus (Rappaport, 1973) has been developed, with the portal tract in the middle instead of the central venule. The parenchyma is then divided into zone 1, the periportal zone, zone 2, the midacinar zone and zone 3, the peripheral or perivenous zone. This model therefore takes into account the existence of gradients between the zones. The peripheral tissue of several adjacent acini forms a star shaped area around the terminal hepatic venules with a simple acinus containing at least two terminal branches of the hepatic vein at its periphery.

Parenchymal cells are not directly accessible to molecules in the sinusoidal blood, being separated by the sinusoidal endothelial cells, which lie between the sinusoidal lumen and the space of Disse. The space of Disse contains several types of connective

tissue, including collagens, fibronectin, heparan sulphate proteoglycan and laminin. Hepatic sinusoidal endothelium is unique in the numerous fenestrae that are organised into sieve plates that act to filter particulate material during passage of blood through the sinusoids. Hepatocytes also produce bile that is drained into the bile cannaliculi that form a network of communicating channels between hepatocytes. From here the bile is discharged into bile ductules which eventually open into the larger bile ducts. Biliary excretion is a well-documented route of excretion of xenobiotics and metabolites with a certain minimum molecular weight.

The liver parenchyma is comprised of a number of different cell types, the most abundant of which is the parenchymal cell which accounts for 60% of the total cell number and 80% of liver volume (Geerts et al., 1987). The remainder of the liver is composed of Kupffer cells, which are macrophages, Ito cells, which act as fat storage depots, pit cells, which have natural killer activity and endothelial lining cells (Wisse and Knook, 1977). Some of these cells may also possess the ability to metabolise xenobiotics and this has been reported for the Kupffer cells, stellate cells and epithelial cells (Wickramasinghe, 1998; Parola et al., 1997; Lerche et al., 1996 respectively). Kupffer cells have also been reported to down-regulate cytochrome P450 mediated metabolism when co-cultured with rat hepatocytes (Milosevic et al., 1999).

Hepatocytes are polyhedral in shape and many are polyploid, containing double or more than the normal amount of DNA. Binucleated cells occur in the middle of the lineage of the plate of hepatocytes previously described and multinucleated cells are found near the central vein. Hepatocytes themselves demonstrate a marked heterogeneity with respect to ultrastructure and enzyme activity, resulting in different cellular functions within different zones of the liver lobuli (Gebhardt, 1992). The spatial organisation of the various metabolic pathways and functions forms the basis for efficient adaptation of the liver to the different nutritional and energetic requirements of the animal (Jungermann and Sasse, 1978). This concept of metabolic zonation is known to be applicable to other aspects of liver function. Functions preferentially located within hepatocytes present in zone I, surrounding the hepatic terminal portal vein, include oxidative energy metabolism, gluconeogenesis, amino

acid catabolism and β-oxidation. Glycolysis and lipogenesis, however, are functions more likely in hepatocytes located in zone III, surrounding the hepatic vein. This zonation extends to the cytochrome P450 enzymes, which are generally more prevalent in the perivenous region (Gebhardt, 1992).

1.4.2 Extracellular Matrix

The thin extracellular matrix, underlying hepatocytes is a complex mixture including collagens (mainly Type I with a lesser amount of Types III,IV,V and VI), to which are bound the adhesion proteins fibronectin and laminin (Martinez-Hernandez and Amenta, 1993). Adhesion proteins are attached to proteoglycans that are bound to the plasma membrane surface. Two types of proteoglycans (PG) are known to exist: heparan sulphate (HS) which has glycosaminoglycan chains that are polymers of glucuronic acid and glucosamine, and chondroitin sulphate (CS) which has chains of glucuronic acid and galactosamine. The glycoproteins elastin and entactin are also found in the extracellular matrix.

These components are all secreted by the cells and organised locally into a matrix on the basal surfaces of cells, providing structural connections between epithelia and mesenchymal cells. In fact, the appropriate proteins do not only have to be present at the basal surface but organised into the correct complex (Bissell et al., 1987). Certain patterns are apparent in the association of the different proteins forming the extracellular matrix. Fibronectins, for example, are affiliated with the fibrillar collagens (Types I and II) whereas the laminins are associated with Type IV collagen.

Evidence has emerged suggesting that as well as hormonal, nutritional and oxygen tension gradients an extracellular matrix gradient also exists which may effect metabolic zonation (Reid et al., 1992). This gradient is illustrated by the classical basal lamina found in the periportal region which changes to a more fibrillar extracellular matrix such as produced by mesenchymal cells, in the perivenous region. Therefore the matrix surrounding cells depends on the stem cell lineage and the state of differentiation and is summarised in Table 1.2.

Table 1.2 Extracellular matrix associated with hepatocytes (Reproduced from Reid, 1990)

LINEAGE POSITION	COLLAGEN TYPE	ASSOCIATED ADHESION PROTEIN	CELL SURFACE RECEPTOR	PROTEOGLYCAN
PRECURSORS				
OVAL CELLS	IV	Laminin	Laminin	HS-PG
INTERMEDIATE HEPATOCYTES				
: QUIESCENT	III and VI	Fibronectin	Integrin	HS-PG
: REGENERATING	IV	Laminin	Laminin	HS-PG
: CIRRHOTIC	I	Fibronectin	Integrin	CS-PG + DS-PG
TERMINALLY DIFFERENTIATING	I + III	Fibronectin	Integrin	Mixture

The components of the basement membrane play diverse roles in stabilising cells in specific configurations that optimise subsequent regulation by soluble signals such as hormones. It is even thought that the components themselves may exert some biological control of phenotypic expression due to the interaction of the receptor sites on the hepatocyte surface (Lin and Bissell, 1993, review). Possibly the cell maintains its polarity under these conditions, which may be vital to gene expression (Musat et al., 1993). The extracellular matrix has also been shown to play a critical role in the induction of certain cytochrome P450 enzymes, for example the induction of CYP2B1/2 by phenobarbital (Brown et al., 1995a).

There is a large amount of evidence showing that the composition of the matrix can also modulate the shape of cells in culture (Watt, 1986). Fibronectin, for example, promotes the spreading of many cell types during culture. A direct relationship between cell shape and gene expression has been documented (Ben-Ze'ev et al., 1988)

and maintenance of the *in vivo* configuration of the hepatocyte may be of importance in maintaining differentiated characteristics in cultured hepatocytes.

1.4.3 Expression of Cytochrome P450 in the Liver

The expression of cytochrome P450 enzymes in the liver exhibits zonal distribution, with total cytochrome P450 content showing a slightly increasing periportal to perivenous gradient (Kanai et al., 1990; Moody et al., 1983; Lindros, 1997 for review). This may be due to the differential perfusion of cells with gradients of oxygen, nutrients and hormones or that hepatocytes acquire different phenotypes as they mature. Some cytochrome P450-dependant enzyme activities have been shown to be localised, such as the predominantly perivenous metabolism of ethoxycoumarin, ethoxyresorufin, aniline p-hydroxylase, benzphetamine N-demethylase and ethanol oxidation (Vaananen, 1986; Bengtsson et al., 1987; Seibert et al., 1989; Gascon-Barre et al., 1989). Histochemical and *in situ* hybridisation studies support the enzyme activity data, suggesting that most of the cytochrome P450 enzymes are expressed predominantly in the perivenous hepatocytes.

This regional expression is sometimes preserved following induction (Bars et al., 1992) but different isoforms can show a striking individual variation in the extent of zonal distribution following induction by various chemicals and hormones (Oinonen et al., 1996). There is even some evidence suggesting that perivenous and periportal hepatocytes are somehow imprinted and retain their inducibility following isolation (Bars et al., 1992), although other authors have suggested that the microenvironment in the liver lobule is a more important determinant of gene expression (Gupta et al., 1999).

1.5 HEPATOCYTE CULTURE

Cell culture may be defined as the mechanical or enzymatic dispersal of cells in a tissue into a cell suspension, which may then be cultured as an adherent monolayer on a solid substrata. Tissue culture was first devised at the beginning of the 20th century

as a method for studying the behaviour of animal cells free of systemic variations. More recently, isolated hepatocytes in primary monolayer culture have become a useful tool for studying hepatic drug metabolism, liver regeneration, cytotoxicity and liver gene expression and regulation under defined conditions *in vitro* because of the ease in which the conditions can be varied. The isolated hepatocyte represents a unique opportunity to study liver function in isolation from other organs, whilst offering a more physiological model than widely used cell fractions such as microsomes.

The adult liver is normally a non-proliferating tissue with a cellular life span of several hundred days (Grisham, 1973). Within 24 hours of isolation hepatocytes assemble to form trabecular aggregates and monolayer sheets, with the development of bile cannaliculi between cells. (Chapman et al., 1973). Cultured hepatocytes can be overgrown by other more proliferative cells such as fibroblasts and this can be minimised by optimising the disaggregation technique used to isolate the hepatocytes and the culture conditions. The absence of a vascular network for the removal of metabolic products is also a problem encountered when using monolayer culture. This allows the accumulation of substances such as bile salts, that are detergents known to denature cytochrome P450. The build up of waste products may be harmful to the hepatocytes and may affect gene expression including that of the cytochrome P450 enzymes (Smirthwaite et al., 1998).

Optimal conditions for the long-term culture of hepatocytes with a maintenance of liver functions have not yet been defined and within 1 to 2 weeks most of the cells show progressive structural and functional deterioration. As a result, it has been found that differentiated hepatocytes lose their specialised properties *in vitro*, with regression of a number of liver specific functions (Potter, 1972). It is perhaps not surprising that cells removed from their normal environment in terms of both the cells in which they are in contact, and the enormous number of chemicals, hormones and xenobiotics found in the bloodstream, cease to maintain their functions. It is unclear, however, whether undifferentiated cells overgrow differentiated cells due to reduced proliferative capacity or whether the absence of the appropriate inducers such as hormones and cell/matrix interaction causes a change in previously differentiated

cells. The term de-differentiation is often used, although this implies that the specialised properties of the cell are lost irreversibly. Adaptation is probably a better description, since the presence of the certain hormones, matrix and chemicals in the media can restore certain differentiated functions including that of some cytochrome P450 isoforms.

1.5.1 Expression of Cytochrome P450 in Cultured Hepatocytes

One of the most dramatic changes in cultured hepatocytes, and of particular interest in the study of drug metabolism, is the loss of cytochrome P450 enzymes. The amounts of cytochrome P450 isoforms can show a decrease of up to 80% within the first 24 hours of hepatocyte culture in cells from both rats and man (Bissell and Guzelian, 1979; Goodwin et al., 1996; George et al., 1997). The decline and instability of levels of cytochrome P450 isoforms imposes some limitations on the use of this technique (Steward et al., 1985). Gene expression is not always affected and transcription of non-liver specific mRNA, such as that for tRNA and rRNA, are unchanged (Clayton and Darnell, 1983).

The decrease in cytochrome P450 enzymes may be triggered by the isolation procedure and the first 4 hours in culture (Padgham et al., 1994). The rapid effect is probably due to the short half-life of these enzymes, which is typically 10 to 20 hours (Correia, 1991). Hepatocyte survival and maintenance of specific functions are dependent on the status of the isolated cell, the contents of the media and on the matrix to which cell attachment occurs. Attempts have been made to increase the functional life of cultured hepatocytes, with the main areas of research being media design and supplementation, and the substratum for attachment including the use of co-cultures with other cells. To these may be added attempts to change the isolation procedure in light of the observation that this also affects hepatocyte function (Padgham and Paine, 1993). Finally, speculation has arisen recently that the loss of cytochrome P450 is due to the increase in nitric oxide levels which accompanies the decrease in cytochrome P450 and attempts are being made to inhibit the activity of nitric oxide synthase (Lopez-Garcia, 1998). The exact role of nitric oxide remains to

be elucidated, however, with different effects on different cytochrome P450 enzymes (Morgan et al., 1998).

1.5.2 Isolation Techniques

The development of methods to isolate hepatocytes has been recently reviewed in an interesting article considering the history, rationale and problems associated with these techniques (Berry et al., 1997). It has been known for some time that the processes used in the isolation of liver parenchymal cells affects the expression of several factors. These include carnitine (Christiansen and Bremer, 1976), glutathione (Hogberg and Kristoferson, 1977), albumin secretion (Miyazaki et al., 1985) and drug metabolising enzymes, including cytochromes P450 (Croci and Williams, 1985). Indeed it has been suggested that the greatest factor influencing the status of hepatocytes is the isolation procedure (Bader et al., 1999).

A variety of enzymes have been utilised to prepare isolated hepatocytes including trypsin, pronase and lysozyme, although the most popular technique for the isolation of hepatocytes remains the use of the enzyme collagenase. Use of collagenase allows isolation of large numbers of intact cells. However, collagenase may be responsible for the loss of surface factors (Kleitzein et al., 1976) including those that may play an important role in the expression of cytochrome P450 enzymes. Furthermore, cell absorption of proteases (Guzelian and Diegelmann, 1979) and their progressive releases into the culture medium have been shown to be involved in subsequent proteolytic events (Capuzzi et al., 1979).

For these reasons there was a resurgence of interest in isolation methods in the late 1980's concerning methods using only a chelating, agent such as EDTA, without enzymatic digestion (Wang et al., 1985; Meredith, 1988, and Bayad et al., 1991) and a resulting stabilisation in phenotype reported. This technique requires extended perfusion for up to one hour with EDTA, however, and the resulting calcium depletion within the hepatocytes may adversely effect yield and viability.

1.5.3 Substrata

The substratum has been found to be the primary determinant of attachment and survival of cells (Gatmaitan et al., 1983). Although isolated hepatocytes can adhere to tissue culture plastic, many workers prefer to provide an attachment factor for cells to adhere to. Cultured hepatocytes are not functionally indifferent to the physical surface onto which they adhere, and the extracellular matrix and cell-cell interaction between hepatocytes in monolayer culture is an important factor in maintaining morphology, gene expression and polarity (Awata et al., 1998; Clayton et al., 1985; Moghe et al., 1996). Therefore, the substratum has a more important role than simply aiding in attachment of hepatocytes to the culture dish and the choice of substratum is part of the optimisation of the culture system (Silva et al., 1998).

Several different types of simple substratum are in common use. These include 'natural' substrata such as collagens, fibronectins, laminins and proteoglycans which are found in the extracellular matrix *in vivo*, as well as the more artificial substrata such as specially treated plastics. To these may be added complex biological basement membrane such as Matrigel®, extracted from the Engelbreth-Holm-Swarm mouse tumour, which has been the subject of favourable reports (Schuetz et al., 1988; Guzelian et al., 1989). Different formulations of the same protein, for example simple collagen coating and collagen gels, are also used. It has been shown that the physical state of the matrix can also effect gene expression (Bucher et al., 1990) and can influence the basal lamina secreted by cultured cells (Cook and Buskirk, 1995). The use of gels in hepatocyte culture has been increasingly popular due to reports of maintenance of function, including that of the cytochrome P450 enzymes (Evans, 1995).

The interaction between the substratum and hepatocytes, and between neighbouring hepatocytes, is known to affect the cell shape and, in turn, gene expression (Ben-Ze'ev et al., 1988; Rana et al., 1994). It has been proposed that the composition of the matrix affects the number or distribution of receptors for matrix proteins in the plasma membrane. This influences the interaction of polymerised actin with the membrane which results in a change in cell shape, and hence the synthesis, assembly and

organisation of other cytoskeletal proteins. This could then effect gene expression either via transcription or translation. The effect of cell shape may, therefore, be a factor in the loss of differentiated function of cultured hepatocytes. The effect of substrata extends to cytochrome P450 expression, for example floating collagen membranes and gelatin gels maintain higher levels of cytochrome P450 than that in hepatocytes grown in ordinary monolayer culture maintained in the same culture medium (Evans, 1995; Michalopoulos and Pitot, 1975).

The density at which cells are seeded onto the substrata can also have an effect on gene expression. Many workers have traditionally used confluent monolayers, although there is now some evidence that for certain functions sub-confluent monolayers are better (Greuet et al., 1997). This study showed that loss of cell contact resulted in decreased expression of P450.

1.5.4 Media Composition

Two of the major advantages of tissue culture are the potential to control the physicochemical environment, such as pH, temperature and oxygen concentration, as well as the physiological conditions. One of the most basic requirements of cell culture is the media in which the cultured cells are bathed. The media composition can obviously have a major impact on gene expression, including that of the cytochrome P450 enzymes (Turner and Pitot, 1988). A review of tissue culture media components and requirements has been published (Bjare, 1992).

Most media contain a complex mixture of amino acids, inorganic salts, vitamins, co-factors, carbohydrates, proteins and salts. Several chemically defined media are now available, the choice of which will depend on individual requirements. Many basic media are commercially available such as Williams' media E, Dulbecco's Modified Eagle's media, Waymouth, L-15 and McCoy's media. Williams and Gunn (1974) developed Williams' media E during studies on the long-term culture of adult liver. This media has been consistently shown to perform at least as well as, and often better than, many other media with respect to hepatocyte function (Grant et al., 1986a;

Miyazaki et al., 1990). Chee's medium has also been reported to have a positive effect on the maintenance of cytochrome P450 function (Skett et al., 1992).

Many culture systems are based on a serum-free media because serum is an undefined and complex medium and may be subject to batch to batch variation. The presence of serum complicates design and interpretation of experiments aimed at the interaction of growth hormones and other factors on cultured cells, introducing an ambiguous factor into the system. Many workers have therefore eliminated this factor from their culture system since the use of a completely defined system makes experimental conclusions less speculative. However, this has introduced the need to have richer media, such as Ham's F12, or to supplement simple media with a range of additives. This is turn can introduce unwanted contaminants which may have effects even at low concentration, since the purity of chemicals cannot be guaranteed. For the purposes of these studies a serum-free system was adopted since, in addition to the problems stated, the presence of serum has been reported to decrease cytochrome P450 activity (Hammond and Fry, 1992).

Deficiency in the nutrients contained in culture medium has been extensively investigated and wide ranges of diverse supplements have been reported to maintain cell differentiation. However the requirements of cultured cells are complex and may vary under the influence of a number of factors. For example, the specific demands of certain cell-lines have been shown to vary depending on the cell density (Matsuya and Yamane, 1986). Supplements routinely included in culture media include vitamins, hormones, growth factors, antibiotics and lipids.

Vitamins and lipids tend to be included in commercially available media and for most cells this is sufficient. Occasionally enrichment of a particular vitamin or lipid is required, such as B_{12} when culturing at low cell density (Matsuya and Yamane, 1986).

Hormones and growth factors are frequently added to cell cultures to stimulate specific cell functions and promote growth. Insulin is the most common supplement to culture media either on its own or in combination with other hormones although it has recently been shown to suppress induction of CYP2E1 (Woodcroft and Novak,

1997). Inclusion of hormones into the media may, therefore, have varied results on different enzymes. Other hormones and growth factors that have been used include hydrocortisone, dexamethasone, triiodothyronine, transforming growth factor and certain prostaglandins to name but a few. The effects of hormones are, however, complex and can vary depending on the presence of other hormones in the media (Skett, 1990).

Antibiotics, such as penicillin and streptomycin are an almost compulsory addition to prevent overgrowth by unwelcome micro-organisms. Other agents such as fungizone, to prevent the growth of fungi, are also used. Use of these agents should be minimised however, and good aseptic technique coupled with a minimum of antibiotics should keep problems of contamination under control.

For many cells transferrin is an obligatory additive of defined media. The main function of this serum protein is the transfer of iron into cells. Transferrin appears to have other functions, however, and has been shown to counteract the effect of low-density lipoproteins (LDLs) in their action on specific mitogen stimulation of lymphocytes (Scupham et al., 1987).

Another important supplement to the media used in studies involving hepatocytes and cytochrome P450 in particular, is δ-amino levulinic acid. Inclusion of this haem precursor causes a small but significant increase in total cytochrome P450 (Engelman et al., 1985; Paine, 1990) in rat hepatocytes at 24 hours post-isolation. Since cytochrome P450 contains an essential haem group it has been proposed that the decline in cytochrome P450 levels is due to the paralleled rise in the activity of the microsomal enzyme system haem oxygenase (Bissell et al., 1974). However the observation that cytochrome P450 could be maintained in the presence of high levels of haem oxygenase indicates that this factor alone is not responsible for the decline in cytochrome P450 (Paine and Legg, 1978).

The oxygenation status of the media bathing hepatocytes is also important. The kinetics of gas diffusion through a typical culture medium have been calculated (McLimans et al., 1968a and 1968b) and compared with that of the liver *in vivo*. It

was found that the initial equilibrated concentration of oxygen would be exhausted in only 35 minutes. However, neither culture under conditions of a 100% oxygen atmosphere, nor the use of floating filters that are thought to facilitate gaseous exchange, were shown halt the decline in cytochrome P450. It is unlikely therefore, that lack of oxygen is the major factor in the loss of differentiated function (Paine et al., 1978) although it can affect cytochrome P450 induction (Kietzmann et al., 1999).

The addition of chemicals, such as metyrapone, to the media has been a popular approach to maintenance of cytochrome P450 (Paine, 1990). Unfortunately, total cytochrome P450 levels cannot be assumed to result from maintenance of the relative abundance of isoforms expressed *in vivo*. In fact this is unlikely to be the case since many inducers and chemicals have specific effects on a limited number of isoforms (Goodwin et al., 1996).

Other factors known to affect expression of cytochrome P450 are temperature (Blankson et al., 1991; Evans, 1995) and the presence of solvents such as dimethyl sulphoxide (Zangar and Novak, 1998: Villa et al. 1991 for mechanistic studies). The effect of solvents is particularly important since many drugs are added to the media in formulations containing solvents.

Although the majority of work has centred on the addition of supplements to the media, it has also been shown that certain constituents of the media may be detrimental to cytochrome P450 levels. For example, it is thought that the amino acid composition of these media is an important factor in control of protein degradation with cystine thought to enhance cytochrome P450 degradation (Seglen et al., 1985). However, the use of cytsine/cysteine free media does not allow the maintenance of cytochrome P450 levels and other factors must be involved in the loss of these enzymes.

To summarise, in the liver the control of gene expression is mainly by transcriptional regulatory mechanisms. This involves a very complex interaction between cell-cell contact, extracellular matrix and endogenous chemicals such as hormones. This environment is inevitably lost by the processes of cell isolation and culture, is difficult

to reproduce, and may never be regained during the culture period. Hepatocytes that have been isolated and cultured show rapid dedifferentiation within hours of isolation and the optimum conditions of isolation, substrata, media and supplements required to regain normal gene expression have yet to be defined.

1.6 CYP2E1: STRUCTURE, FUNCTION, EXPRESSION AND INDUCTION

The expression of the CYP2E1 enzyme is the subject of the latter part of these studies and a more detailed consideration of CYP2E1, including expression and induction, is appropriate. The biochemistry, molecular biology and clinical relevance of CYP2E1 have been reviewed (Song, 1996) as have its pathological and physiological role (Lieber, 1997) and its role in the oxidation of low molecular weight carcinogens (Guengerich et al., 1991).

1.6.1 Structure, Zonal Expression and Metabolic Activity of CYP2E1

CYP2E1 is the only gene expressed in this subfamily in most species including rat and man. Rabbits are an exception with two genes (*CYP2E1* and *CYP2E2*) being expressed. Although the sequence is only 78% homologous, CYP2E1 is 493 amino acids long in both man and rat. At least six polymorphisms have been reported in human *CYP2E1*, including the RsaI restriction length polymorphism in the 5'-flanking region, the DraI polymorphism in intron 6 and the PstI polymorphism in the upstream flanking region (Wu et al., 1998) or Taq I in intron 7 (Carriere et al., 1996). All mutations identified so far occur in the promoter and intron regions with structural regions conserved.

Transcription of the *CYP2E1* gene is activated immediately after birth in the rat and is maximally expressed within 1-week post-partum (Song et al., 1986; Hong et al., 1987a). The expression of CYP2E1 mRNA in rat liver at 6 hours post-parturition is detectable at a low level, followed by a dramatic rise within 24 hours of birth with continuing elevation to day 6 (Umeno et al., 1988). In man, CYP2E1 is non-

detectable in foetal liver, but protein and activity rise in the first few hours after birth independently of whether the neonate is full-term or premature. Since mRNA remains low it is possible that stabilisation of the low levels of protein is the reason for the rise in CYP2E1. The first intron/exon region in genomic DNA from foetal liver contains several hypermethylated spots, and it has been proposed that cytosine demethylation facilitates gene transcription in the neonate (Vieira et al., 1996).

CYP2E1 is expressed in the liver and other tissues such as the brain (McFayden et al., 1998) and is involved in the metabolism of many xenobiotics including acetaminophen and chloroform (Table 1.3). It is of particular interest to toxicologists due to its capacity to metabolise drugs, solvents and environmental procarcinogens compounds, such as carbon tetrachloride (Johansson and Ingelman-Sundberg, 1985), to cytotoxic compounds and carcinogenic metabolites (Koop, 1992). In fact, in a recent review, 85 compounds were listed as being activated by CYP2E1 (Lieber, 1997). It may also be of importance in the aetiology of certain diseases such as alcohol-associated cancers of the head and neck (Uematsu et al., 1992). CYP2E1, as well as being constitutively expressed in the lung, is induced dramatically by cigarette smoke (Villard et al., 1998) and it has been suggested therefore that CYP2E1 may be involved in pulmonary carcinogenesis in human smokers, though this has yet to be proved.

In common with other cytochrome P450 isoforms CYP2E enzymes contain a noncovalently bound haem group which is associated with a cysteine containing peptide near the carboxy terminus of the enzyme. The complete gene sequence for rat and human CYP2E1 were determined in 1988 (Umeno et al.) and a TATA box identified just upstream of the start site although there was no evidence for a CCAAT box. The enzyme contains a noncleaved signal sequence and is intrinsically bound to the endoplasmic reticulum membranes.

In general, cytochrome P450 enzymes predominate in the pericentral cells in the liver. The zonal expression of CYP2E1 in the control, well-fed rat, has been examined by several groups of researchers with differing results. The majority of studies are in agreement with immunohistochemistry, immunoanalysis and western blot data

showing that in untreated rats the CYP2E1 was almost exclusively expressed in perivenous cells surrounding the terminal hepatic venules (Ingelman-Sundberg et al., 1988). Previous observations by the same team (Haddad et al., 1985) showed that the zonal specific ρ-hydroxylation of aniline could be switched to periportal hepatocytes in chronically hypocalcemic rats, indicating that the phenotype is not fixed. Further studies involving human and rat samples also indicated that CYP2E1 expression was normally localised in the perivenous (zone 3) region, even following induction by ethanol (Tsutsumi et al., 1989; Cohen et al., 1997). However not all studies are in agreement with this and CYP2E1 has been shown to be higher in periportal hepatocytes, although the difference was not significant (Gascon-Barre et al., 1989). CYP2E1 has also been shown to exhibit zonal expression in the lung, with higher activity in the distal bronchioles and parenchyma than in the proximal airways (Lee et al., 1998), and the kidney (Hotchkiss et al., 1995).

In contrast with other forms, CYP2E1 enzyme is constitutively present in its high-spin form (Ryan et al., 1985) but is converted to its low spin form upon substrate binding (Morgan et al., 1982). It is possible that the substrate binds only after the activated oxygen complex has been formed, in a similar mechanism to the flavin-containing monooxygenase enzyme. The existence of this enzyme in the high spin form in the endoplasmic reticulum means that it may be readily reduced by P450 reductase in the absence of substrate and generation of active oxygen such as hydrogen peroxide is known to occur. It is for this reason that CYP2E1 may cause oxidative damage and attempts have been made to link CYP2E1 with development of diseases such as Parkinson's (Jenner, 1998, review) and alcoholic liver disease (Dupont et al., 1998).

In contrast to some other cytochrome P450 enzymes, cytochrome b_5 appears to be necessary for optimal activity of CYP2E1 towards several substrates including 7-ethoxycoumarin, chlorzoxazone, aniline and N-nitrosodimethylamine (Yamazaki et al., 1996b). The effect of cytochrome b_5 has also been studied using insect cell expressed CYP2E1. An increase in both K_m and K_{cat} for CYP2E1 mediated reactions was observed (Patten and Koch, 1995). Unlike certain reactions mediated by other cytochrome P450 isoforms in baculovirus insect cell expression systems, stimulation of CYP2E1 activity by b_5 could not be replaced by apo-b_5 (Yamazaki et al., 1997).

Other data obtained using baculovirus insect cell expressed CYP2E1 and b_5 have suggested that b_5 enhances the coupling between CYP2E1 and NADPH-P450 oxidoreductase (Wang et al., 1996).

A number of exogenous substrates for CYP2E1 have been identified and a limited selection is shown in Table 1.3. Most of the substrates are relatively small molecular weight compounds, which has led to the suggestion that CYP2E1 has a small active site. Molecular modelling also indicates that the active site of CYP2E1 is small, with mainly hydrophobic residues (Tan et al., 1997). CYP2E1 is also involved in endogenous metabolism including that of arachidonic acid, and is the major enzyme responsible for the omega-1 metabolism of the physiological monounsaturated fatty acid oleic acid in the liver (Adas et al., 1998).

Table 1.3. Substrates for CYP2E1

CYP2E1 SUBSTRATE	REFERENCE
N-NITROSODIMETHYLAMINE	YANG ET AL., 1990
TRIMETHADIONE	KURATA ET AL., 1998
ETHANOL	KOOP ET AL. 1986
ACETONE	KOOP ET AL., 1986
ANILINE	KOOP ET AL., 1986
HALOTHANE	SPRACKLIN ET AL., 1997
ENFLURANE	HOFFMAN ET AL., 1989
CHLOROFORM	BRADY ET AL., 1989
BENZENE	JOHANSSON ET AL., 1988a
TRIMETHADIONE	NISHIMURA ET AL., 1996
ETHERS	BRADY ETAL., 1987
ACETALDEHYDE	KUNITOH ET AL., 1997
ACETAMINOPHEN	RAUCY ET AL., 1987
CARBON TETRACHLORIDE	JOHANSSON ET AL., 1985
CHLORZOXAZONE	PETER ET AL., 1990

1.6.2 Control Of CYP2E1 Expression

Induction of drug metabolising enzymes can lead to serious, clinically significant drug interactions (Tanaka, 1998, review). Clearance of co-administered drugs that are metabolised by the induced pathway will be increased and the drug concentration may fall to sub-therapeutic levels. The interaction between rifampacin, a CYP3A inducer, and cyclosporin, an immunosuppressive agent metabolised by CYP3A, is an example of such an interaction (Koselj et al., 1994).

The pharmacokinetic consequences of CYP2E1 induction has been studied using isoniazid as the inducer on the formation of NAPQI (N-acetyl-benzo-quinoneimine) from acetaminophen (Raucy et al., 1989) and chlorzoxazone (Sarich et al., 1997) and this area is the subject of a recent review (Klotz and Ammon, 1998). The effect of isoniazid was, in both studies, an initial decrease in metabolism of drug, followed by an increase in drug metabolism when the inducer was removed. Persistent inhibition of activity was observed whilst the inducer was present. It is thought that an enzyme-inducer complex forms that is inactive. However, the complex is protected from degradation and the level of the enzyme is therefore increased. After the inducer is eliminated an increase in activity can then be observed. The time of reversal from inhibition to enhanced metabolism depends on the ability of the substrate to compete for the active site as the inducer is eliminated.

CYP2E1 is of particular interest in the study of gene expression since, in contrast to certain other cytochrome P450 enzymes, it has been shown to be under both transcriptional and post-transcriptional control. Stabilisation of protein and mRNA, and phosphorylation of the protein are all post-transcriptional methods by which CYP2E1 activity is modulated.

Initiation of transcription in eukaryotic cells requires both general transcription factors to guide RNA-polymerase II to the correct initiation site and specific nuclear factors to increase the formation of the transcription complex. Two factors that are thought to influence the expression of CYP2E1 *in vivo* have recently been the subject of a great deal of research. They are hepatocyte nuclear factors 1 and 4 (HNF-1 and HNF-4),

which are proteins found in high concentration, although not exclusively, in the liver. Using HepG2 cells transfected with the 5' upstream region of *CYP2E1*, Gonzalez (1988) was able to demonstrate that HNF-1 stimulates transcription of the *CYP2E1* gene. Further studies using a reporter gene assay have also showed that HNF-1 binds to and activates the *CYP2E1* promoter (Liu and Gonzalez, 1995). HNF-1 is itself under the control of HNF-4, which may indicate that a transcriptional hierarchy exists with respect to CYP2E1 expression (Kuo et al., 1992).

Stabilisation of CYP2E1 protein by the presence of small ligands has been demonstrated (Eliasson et al., 1988). The level of CYP2E1 protein was maintained in rat hepatocytes cultured in the presence of a number of ligands even when mRNA rapidly disappeared. Ligand-dependent stabilisation is thought to be due to ligand binding to the active site and protecting the enzyme from degradation, possibly by protecting the enzyme from cAMP-dependent phosphorylation and subsequent degradation (Eliasson et al., 1990; Chien et al., 1997).

Control of degradation of CYP2E1 protein is a second mechanism by which activity of CYP2E1 is modified. A number of degradative routes have been proposed: an ATP/ubiquitin-mediated proteolytic degradation (Roberts et al., 1995a), and a rapid phase Mg/ATP-dependent phosphorylation with a slower phase autophagosomal/autolysosomal process (Eliasson et al., 1992). Whilst the exact pathway has yet to be elucidated there is consensus that the degradation is biphasic.

Various models have been proposed to account for the biphasic degradation of CYP2E1. The simplest of these considers there to be a single enzyme pool with an endogenous ligand that slows the otherwise rapid degradation of CYP2E1. However, the ligand would have to have a very slow dissociation constant so that the half-life of dissociation would be several hours, with the dissociation of the ligand as the rate-limiting step. A more likely model would have two kinetically separate pools of enzyme, which can be either chemically or physically distinct.

In order to be chemically distinct the two forms of the enzyme would have to be produced by a post-translational modification that does not compromise the activity of

the altered enzyme. Although this would result in appropriate biphasic degradation kinetics there is no evidence that such a chemically altered enzyme exists which is still active. However, physically distinct pools of catalytically active CYP2E1 may exist.

Catalytically active CYP2E1 occurs in both the endoplasmic reticulum and the Golgi apparatus, although the Golgi compartment contains relatively little enzyme (Neve et al., 1996). CYP2E1 in the Golgi apparatus probably arises from the smooth endoplasmic reticulum, while newly synthesised CYP2E1 appears firstly in the rough endoplasmic reticulum. It is possible that the rapid degradative process occurs at only one site, while the slow process can operate in both. A model in which the enzyme in the rapidly turned over pool in the rough endoplasmic reticulum can be stabilised by ligand and therefore protected from rapid degradation whilst the slow process remains unaffected would result in the observed biphasic degradation kinetics. Further, more complicated models considering translocation between compartments have been proposed although it is not yet known which of these models is correct.

CYP2E1 shares a feature in common with members of the CYP2C subfamily, in possessing a consensus site for cAMP-dependant protein kinase A (PKA) stimulated phosphorylation (Koch and Waxman, 1989). Phosphorylation of CYP2E1 causes a decrease in activity and an increased rate of degradation (Eliasson et al., 1990). Phosphorylation of CYP2E1 therefore antagonises induction of this enzyme and compounds which activate PKC such as phorbol myristate acetate have been shown to inhibit induction of CYP2E1 by acetone (Menez et al., 1990).

The expression of CYP2E1 is affected by a number of factors including physiological status and a wide range of chemical entities. The control of CYP2E1 expression *in vivo* and *in vitro*, and the proposed mechanisms of induction are considered in detail below and summarised in Table 1.4.

1.6.3.1 Physiological Status

During fasting or starvation, changes in a number of biotransformation processes have been observed, including an increase in CYP2E1 in man (Mandl et al., 1995 for review) although at least one study has reported that prolonged fasting in man results in a decrease in CYP2E1 activity (O'Shea et al., 1994). Numerous studies examining the effect of fasting in rats showed an increase in CYP2E1 enzyme activity in hepatic microsomes (Hong et al., 1987b; Tu and Yang, 1983; Imaoka et al., 1990) with induction occurring after as little as 8 hours of fasting (Brown et al., 1995). CYP2E1 induction by fasting appears to involve an increase in both mRNA and protein and increased transcription is thought to be responsible for the rise in CYP2E1 mRNA (Johansson et al., 1990). The induction of CYP2E1 mRNA during fasting has been localised to the perivenous region in the rat, and is an example of zonal induction (Hu et al., 1995). The induction of CYP2E1 caused by fasting in the rat is accompanied by an increase in DNA methylation (Sohn and Fiala, 1995).

The body is known to produce acetone during fasting and it is possible that at least some of CYP2E1 induction seen during fasting is due to the presence of this established CYP2E1 inducer. Acetone is also a substrate for CYP2E1 suggesting that this enzyme is involved in gluconeogenesis through the utilisation of ketone bodies (Koop and Casazza, 1985). Briefly, CYP2E1 can catalyse the oxidation of acetone to acetal and then to methylglyoxal and this can lead to the synthesis of glucose. This may be an emergency gluconeogenesis pathway used during fasting, and the rationale behind an increase in CYP2E1 during this physiological state. However, the amount of acetone produced during fasting is probably insufficient to account for the total rise in CYP2E1 and fasting also increases CYP2E1 activity in acetone treated rats in a synergistic manner. Further, the mechanism of the increase in CYP2E1 during fasting is different to that caused by acetone induction.

Diabetes, both chemically induced and spontaneous, is also accompanied by an elevation of hepatic microsomal CYP2E1 activity in the rat (Past and Cook, 1982). Early studies further revealed that the induction of CYP2E1 in diabetic rats could be reversed by the administration of insulin (Dong et al., 1988). Induction of CYP2E1

activity is accompanied by a large elevation of CYP2E1 mRNA (Song et al., 1987), although nuclear run-on transcriptional analysis has failed to demonstrate an increased rate of transcription in the liver nuclei of diabetic rats. This suggests that mRNA stabilisation might be the mechanism of induction in the diabetic state (Hong et al., 1987a) and later studies in the rabbit have confirmed that CYP2E1 mRNA half-life is decreased by the presence of insulin (Peng and Coon, 1998).

It is not clear whether diabetes-induced increases in CYP2E1 is a direct effect of lack of insulin, or to metabolic consequences including decreases in growth hormone, glucagon and thyroid hormone and increased serum glucose and ketones. The change in the level of CYP2E1 following insulin treatment to the diabetic animal was believed to be due to the elimination of ketosis as opposed to a direct effect of insulin (Dong et al., 1988). However, recently *in vitro* studies have suggested that insulin present in culture medium directly down-regulates CYP2E1 in primary rat hepatocytes (Woocroft and Novak, 1997). It is likely that insulin results in a combination of direct and indirect effects. The inverse relationship between insulin and CYP2E1 is not surprising in light of the fact that when glucose and therefore insulin levels are low, fat will become a major source of energy and CYP2E1 is involved in the metabolism of fatty acids (Adas et al., 1998). In man the situation is less clear with some evidence of CYP2E1 induction in diabetic patients in certain studies (Song et al., 1990) but not in others (Berthou et al., 1997).

Obesity is a prevalent disease in some countries and the obese overfed rat has been used as a model for this condition. The cytochrome P450 content of hepatic microsomes prepared from obese rats was increased over control values, accompanied by a significant increase in ethanol metabolism (Salazar et al., 1988). The mechanism by which induction occured in these obese rats was not investigated. CYP2E1 induction in the obese overfed rat, and in rats fed a high fat diet, has been confirmed by later studies (Raucy et al., 1991; Chen et al., 1997). Induction of CYP2E1 activity in obese humans has also been reported (O'Shea et al., 1994). An interesting observation linking the induction of CYP2E1 in the diabetic, obese and fasted state is that systemic exposure to ketone bodies is high in all three states (Raucy et al., 1990)

Although some hormonal control of CYP2E1 has been observed, no significant sex related differences in CYP2E1 expression in liver have been observed in the rat. This suggests that androgens and oestrogens do not regulate this enzyme. However, an increase in the level of CYP2E1 in female mice to that found in the male mouse as a result of testosterone treatment has been demonstrated (Hong et al., 1989) suggesting some inter-species differences in control. Hypophysectomised rats have a 5-10 fold increase in hepatic CYP2E1 immunodetectable protein although only a slight increase in CYP2E1 activity (Williams and Simonet, 1988). Treatment of hypophysectomised rats with growth hormone in this study resulted in a decrease in CYP2E1 activity, suggesting that this hormone acts to repress CYP2E1 levels.

Inflammation and infection also tend to have a suppressive on intermediary metabolism and drug metabolism activity (Andus et al., 1991, Iber et al., 1999, review) with a corresponding decrease in P450 content and activity (Renton and Knickle, 1990). Nitric oxide released during the inflammatory response has been implicated in the decrease in cytochrome P450, and although nitric oxide synthase inhibitors do not halt the decrease in cytochrome P450 (Sewer and Morgan, 1998) they can protect cytochrome P450 apoprotein levels from suppression by cytokines (Khatsenko and Kikkawa, 1997).

A differential response to a number of cytokines for individual cytochrome P450 mRNAs and proteins has been observed (Morgan et al., 1994). Although the general effect of cytokines is to down-regulate the expression of cytochrome P450 isoforms including CYP1A1/2, CYP3A, CYP2C and CYP2E1, it has been reported that the cytokine interleukin-4 increased the mRNA for CYP2E1 up to five fold over untreated human hepatocytes in primary culture (Abdel-Razzak et al., 1993). An increase in levels of immunodetectable protein was also observed although since CYP2E1 activity was not determined it is not yet known whether an increase in CYP2E1-specific enzyme activity accompanies the rise in mRNA and apoprotein. Other studies have also reported CYP2E1 induction by cytokines including IL-1 and inflammation (Tindberg et al., 1996, Sewer et al., 1997).

Finally, one further factor found to influence CYP2E1 levels is oxygenation status, although there is little data published in this area. Continuous exposure of rats to a 95% oxygen atmosphere has been shown to cause an increase in the level of immunodetectable CYP2E1 with no concurrent increase in mRNA (Tindberg and Ingelman-Sundberg, 1989).

1.6.3.2 Chemical Inducing Agents

Pyrazole has been well documented both *in vivo* and *in vitro* to be an inducer of CYP2E1 via protein stabilisation (Tu et al., 1981; Ingelman-Sundberg and Jornvall, 1984; Hunt et al., 1991). CYP2E1 activity in periportal and pericentral hepatocytes isolated using digitonin-collagenase perfusion of liver from pyrazole treated rats has also suggested that induction is zone specific (Dicker et al., 1991; Kukielka and Cederbaum, 1995), with the activity of CYP2E1 preferentially localised in the pericentral zone of the liver acinus. Immunochemical staining in the same study confirmed an increase in CYP2E1 in pericentral hepatocytes compared to periportal hepatocytes.

It is also well documented that ethanol ingestion induces CYP2E1 even at low doses. Induction of CYP2E1 by ethanol is zonal with a preferential increase in the pericentral compared to the periportal hepatocytes (Ingelman-Sundberg et al., 1988: Hu et al., 1995). A number of *in vivo* studies have been carried out, with some conflicting results in the apparent mechanism of induction. Enzyme activity and immunodetectable CYP2E1 have been found to increase without a rise in mRNA following ethanol administration to rats, suggesting a protein stabilisation mechanism (Roberts et al., 1995b: Johansson et al., 1988). The presence of ethanol appeared to decrease the rapid phase of CYP2E1 degradation, leading to a longer half-life for immunodetectable CYP2E1 (Roberts et al., 1995b). The induction of CYP2E1 by ethanol in rats was rapidly reversed on withdrawal of treatment (Roberts et al., 1995b). In contrast, chronic administration of ethanol has been shown to occur via increased transcription (Badger et al., 1993). This has been supported by examination of the *in vitro* induction of CYP2E1 by ethanol using western blotting and mRNA analysis which revealed that ethanol treatment caused a selective induction of

CYP2E1 mRNA as well as immunodetectable protein in the rat (Ingelman-Sundberg et al., 1988).

Induction of CYP2E1 by ethanol *in vitro* has also been demonstrated using the rat hepatoma FGC-4 cell, with maximal induction (5 fold) observed within 8 hours of treatment (McGhehee et al., 1994). Extending either the treatment time or the concentration of ethanol did not increase the level of CYP2E1 induction, with CYP2E1 mRNA levels similar to untreated controls. It is interesting to note that increasing the level of ethanol in these studies did not result in the increased transcription observed *in vivo*. The mechanism of induction observed *in vitro* therefore, will not always be an indication of that occurring *in vivo*.

To rationalise differences between studies it has been proposed that ethanol induction of CYP2E1 may occur by a concentration-dependant 2-step mechanism (Ronis et al., 1993). The first step is associated with low blood alcohol levels and appears to be post-transcriptional. The second step, associated with high blood alcohol levels (above 300mg/dL) appears to be associated with increased *CYP2E1* gene transcription (Badger et al., 1993). The time period over which ethanol is administered may also effect the mechanism of induction (Hu et al., 1995). Differences in the mechanism of induction observed between studies are probably due to the concentration of ethanol used and to the length of treatment. A further complicating factor has been uncovered since it has been found that rats chronically administered ethanol at a high level have a poor nutritional status and it is known that fasting and starvation also induce CYP2E1 (Hu et al., 1995).

Recent data has suggested that inter-species variation in the CYP2E1 induction mechanism by ethanol may exist. Takahashi et al., 1993, found that induction of CYP2E1 in the human liver by ethanol is caused by an increase in mRNA. This is similar to the hamster (Kubota et al., 1988) but in contrast to the rat and rabbit (Johansson et al., 1988 and Porter et al., 1989). This process was found to occur primarily in perivenous hepatocytes. However, the authors could not rule out post-translational mechanisms

The fact that induction of CYP2E1 by acetone *in vivo* may be due to protein stabilisation or to increased protein synthesis was reported many years ago (Tu et al., 1981) and this has also been shown *in vitro* (Kraner et al., 1993). Treatment of rats with acetone increased CYP2E1 activity in hepatic microsomes, and this effect was abolished by co-administration of the protein synthesis blocker cycloheximide (Tu et al., 1983). Other studies have confirmed this (Song et al., 1989 and Johansson et al., 1988) with the level of mRNA for CYP2E1 found to be the same in rats treated with acetone as that in untreated animals. Labelling of the protein *in vivo* showed that administration of acetone abolished the rapid component of protein degradation, with only the longer half-life remaining. Some evidence has also been published suggesting that the very early response to acetone may involve increased translational efficiency (Kim et al., 1990). Inter-species differences in induction mechanism have been observed however, with acetone administration to isolated rabbit hepatocytes causing a rise in both protein and mRNA synthesis (Kraner et al., 1993).

Induction of CYP2E1 by chemicals is not always post-transcriptional however, since acetylsalicylic acid also induces CYP2E1 and is unusual in that this is thought to occur via increased transcription (Damme et al., 1996). Although rarer than induction, levels of cytochrome P450 can also be decreased by certain moieties and we have observed a decrease in CYP2E1 expression in rats treated with phenobarbital and β-napthoflavone (D.Herriott, unpublished data). This has been demonstrated for chloroform which reduces CYP2E1 post-translationally, probably by a decrease in protein synthesis (Sohn et al., 1991) and the mitogen human hepatocyte growth factor, which causes a decrease in the activity of several cytochrome P450s including CYP2E1 in human hepatocytes (Donato et al., 1998). Disulfiram also decreases CYP2E1 via both pre-translational and post-translational mechanisms (Martini et al., 1997).

Table 1.4. The mechanism of action of chemical and physiological inducers of CYP2E1 *In Vivo* and *In Vitro*

COMPOUND	*IN VIVO* / *IN VITRO*	MECHANISM OF INDUCTION	REFERENCE
ACETYLSALICYLIC ACID	*IN VIVO*	TRANSCRIPTION	DAMME ET AL., 1996
ACETONE	*IN VIVO* *IN VITRO*	TRANSLATIONAL EFFICIENCY/ PROTEIN STABILISATION	KIM ET AL., 1990 TU ET AL., 1981 KRANER ET AL., 1993
DMSO	*IN VITRO*	PROTEIN STABILISATION	ELIASSON ET AL., 1988
ETHANOL	*IN VIVO* *IN VITRO*	TRANSCRIPTION PROTEIN / MRNA STABILISATION	BADGER ET AL., 1993 ROBERTS ET AL., 1995 INGELMAN-SUNDBERG ET AL., 1988
PYRAZOLE	*IN VIVO* *IN VITRO*	PROTEIN STABILISATION	TU ET AL., 1981 HUNT ET AL., 1991
FASTING	*IN VIVO*	TRANSCRIPTION	JOHANSSON ET AL., 1990
OBESITY	*IN VIVO*	ND	SALAZAR ET AL., 1988
DIABETES	*IN VIVO*	TRANSCRIPTION/ MRNA STABILITY	SONG ET AL., 1987

ND = not determined

1.7 *THE AIM OF THESE STUDIES*

The loss of cytochrome P450 *in vitro* cannot be solely attributed to isolation technique, loss of cell-cell contact and substrata (Wright and Paine, 1992), and despite the many and varied media supplements the search for a system capable of maintaining *in vivo* levels goes on.

The aim of these studies was initially to establish two different techniques for the isolation of hepatocytes. Enzymatic digestion utilising collagenase, and non-enzymatic digestion utilising EDTA, were selected because of the differing method by which cells are liberated from liver. Subsequent to isolation the next aim was to establish hepatocytes in monolayer culture using four substrata for attachment: Matrigel®, a laminin-rich reconstituted basement membrane, Vitrogen®, a type I collagen, fibronectin and untreated plastic, with cells to be seeded to confluence. The media chosen was Williams' media E with a limited number of supplements including δ-aminolevulinic acid, insulin, L-glutamine, transferrin and penicillin-streptomycin.

Combinations of these isolation techniques and substrata were subsequently used to study the effect of these factors on the status of cytochrome P450 isoforms in male rat hepatocytes. For this purpose an assay using the substrate probe testosterone was to be used to measure the catalytic activity, and western blotting analysis was to be established to determine apoprotein levels of a number of cytochrome P450 isoforms.

Finally, using the optimal isolation technique and substratum defined in these studies it was intended to determine whether rat hepatocytes maintained under these culture conditions retained the ability to respond to the known CYP2E1 inducer pyrazole. It was intended to establish an assay using the CYP2E1-specific substrate probe chlorzoxazone to determine CYP2E1 catalytic activity and western blotting analysis to measure immunodetectable CYP2E1. To further extend the information available a further aim was to synthesise a riboprobe and to develop a Northern ELISA assay in order to determine CYP2E1 mRNA levels.

CHAPTER 2
MATERIALS AND METHODS

2.1 ANIMALS AND CHEMICALS

The animals and chemicals used throughout these studies are described in the following sections.

2.1.1 Animals

Adult male albino (AHA) rats (approximately 200g) were bred and supplied by the Animal Services Department (Glaxo Group Research Limited, Ware, UK). Rats were maintained on Rat and Mouse No 1. Modified SQC diet (Special Diets Services Limited, Witham, UK) and food and water were available *ad libitum*. Rat holding rooms were thermostatically maintained within accepted parameters and illuminated artificially on a 12-hour light dark cycle.

2.1.2 Chemicals

Earle's balanced salt solution (EBSS, calcium and magnesium free), sodium bicarbonate solution (7.5%), L-glutamine (200mM) and Glassmax^{TM} kits for DNA isolation were supplied by Gibco Limited (Paisley, UK).

Ethyleneglycol bis- (β-aminoethyl ether) N', N', N', N'-tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), calcium chloride, potassium chloride, magnesium sulphate, 4-(2-hydroxyethyl) piperazine-2-ethanesulphonic acid (HEPES), bovine serum albumin (BSA, fraction V), sodium pyruvate, foetal calf serum, deoxyribonuclease 5'-oliguonucleotidohydrolase (DNase I; EC 3.2.21.1) from bovine pancreas, trypsin inhibitor (from soyabean, Type II-S), zinc sulphate, iron saturated transferrin, insulin, δ-amino-levulinic acid, fibronectin, penicillin streptomycin, testosterone, carbodiimide (cyanamide), chlorzoxazone, TEMED (N,N,N',N',-tetramethyl-ethylenediamine), ammonium persulphate, phosphate-buffered saline

tablets, DL-isocitrate and isocitrate dehydrogenase were all supplied by the Sigma Chemical Co. (Poole, UK).

Collagenase (EC 3.4.24.3) from *Clostridium histolyticum*, β-glucuronidase, aryl-sulphatase, the DIG RNA (SP6/T7) labelling kit, PCR DIG probe synthesis kits, DIG nucleic acid detection kits, Northern ELISA detection kits, Hind III cloned from *Haemophilus influenzae* and Sac I cloned from *Sachromyces achromogenes* were supplied by Boehringer Mannheim (Lewes, UK). Trypan blue (4,4'-bis (8-amino-3, 6-disulpho-1-hydroxy-2-naphthylazo) 3,3'-dimethylbiphenyl tetrasodium salt) formulated as a 0.5% w/v solution in 0.85% saline and Williams' Media E (WME) without phenol red or L-glutamine, were obtained from Flow Laboratories (Rickmansworth, UK).

Matrigel® was supplied by Universal Biologicals, and Imperial Laboratories, UK, supplied Vitrogen®.

^{14}C-radiolabelled testosterone and ^{14}C-radiolabelled chlorzoxazone were obtained from Amersham (Amersham, UK) and Salford Ultrafine Chemicals (Manchester, UK) supplied 6-hydroxy chlorzoxazone.

Oxygene (Dallas, USA) supplied rabbit anti-rat cytochrome P450 antibodies to CYP1A1 and rabbit anti-rat reductase antibodies. Rabbit anti-rat antibodies to CYP1A2, CYP2B, CYP3A, CYP2E1, CYP2C6 and CYP2C11 were obtained from Dr. C.R.Wolf (I.C.R.F., Edinburgh, UK) Dr. G.G. Gibson (University of Surrey, Guildford, UK) supplied sheep anti-rat CYP4A antibodies. Nitro-cellulose sheets (0.45μM) were supplied by Hoefer Scientific Instruments (Newcastle, UK) and the pre-weighed acrylamide and immunodetection kit were supplied by Bio-Rad Laboratories (Hemel Hempstead, UK).

QIAGEN INC. (California, USA) supplied the QIAGEN plasmid midi kit, and Stratagene (Cambridge, UK) supplied the Bluescript II KS plasmid. The Wizard midi

prep DNA purification system and the PolyATtract®System 1000 for mRNA isolation were supplied by Promega (Southampton, UK).

All other chemicals and solvents were of Analar grade or equivalent, or molecular biology grade where appropriate.

2.2 *HEPATOCYTE ISOLATION AND CULTURE*

The methods used in the isolation and subsequent culture of male rat hepatocytes are described below.

2.2.1 Solutions for Collagenase Isolation

Perfusion Buffer Perfusion buffer consisted of calcium and magnesium free EBSS supplemented with sodium bicarbonate (final concentration 26mM). The pH of the perfusion buffer was adjusted to pH 7.4 using hydrochloric acid (1M).

Chelating Solution EGTA (25mM) was prepared in sodium hydroxide (0.1M) and the pH adjusted to pH 7.4 with hydrochloric acid (1M). An aliquot of EGTA solution (5mL) was added to perfusion buffer (500mL) to give a final EGTA concentration of 0.25mM.

Enzyme Solution Collagenase (0.12 Wunsch Units/mL) was dissolved in perfusion buffer (100mL). Collagenase activity as determined by the method of Wunsch and Heidrich (1963) was supplied by the manufacturer. Trypsin inhibitor (10mg) and calcium chloride (final concentration 2mM) were also added to the buffer.

Dispersal Buffer The dispersal buffer consisted of sodium chloride (0.14M), potassium chloride (5mM), Hepes (0.01M), magnesium sulphate (0.7mM) calcium chloride (0.01M) and BSA (1.5%w/v). The pH was adjusted to pH 7.4 with sodium hydroxide (1M).

2.2.2 Solutions for EDTA Isolation

Purification Buffer Calcium and magnesium free EBSS was supplemented with sodium bicarbonate (final concentration 26mM) and sodium pyruvate (6μM). The pH of the purification buffer was adjusted to pH 7.45 with hydrochloric acid (1M).

Dissociation Buffer Dissociation buffer was prepared from purification buffer with the addition of EDTA (final concentration 2mM) and bovine serum albumin (BSA, 1% w/v). The pH was adjusted to pH7.45 with hydrochloric acid (1M).

Hepatocyte Dispersal Buffer The hepatocyte dispersal buffer used was the same as that previously described in Section 2.1.3.

2.2.3 Culture Medium

Culture medium was composed of Williams' Medium E supplemented with L-glutamine (2mM), insulin (1μM), δ-aminolevulinic acid (100μM), bovine transferrin (5μg/mL) and penicillin-streptomycin (100U penicillin, 0.1mg streptomycin/mL). The composition of Williams' Medium E is provided in the appendix. For initial attachment of hepatocytes the culture medium was also supplemented with foetal calf serum (10%v/v).

2.2.4 Perfusion Apparatus

The equipment used for the perfusion of rat liver is shown in Figure 2.1. The flow of buffer from up to three reservoirs, linked by a series of three-way taps, was maintained by a Watson-Marlow 5025 peristaltic pump (Watson-Marlow Ltd, Falmouth, UK). The temperature of perfusion buffers was maintained by passage through a heat exchanger surrounded by a water jacket, such that on exit from the cannulae to enter the liver lobes the buffer was 37°C. The temperature of water in the water jacket was maintained by a thermoregulator (Harvard Apparatus Ltd., Edenbridge, UK). All perfusion buffers were continually gassed with carbogen (95% oxygen:5% carbon dioxide) to maintain the pH and to oxygenate.

Perfusion with buffers containing either collagenase or EDTA were used to investigate the effect of the method of isolation on the maintenance of cytochrome P450 enzymes in cultured male rat hepatocytes over a 96 hour period.

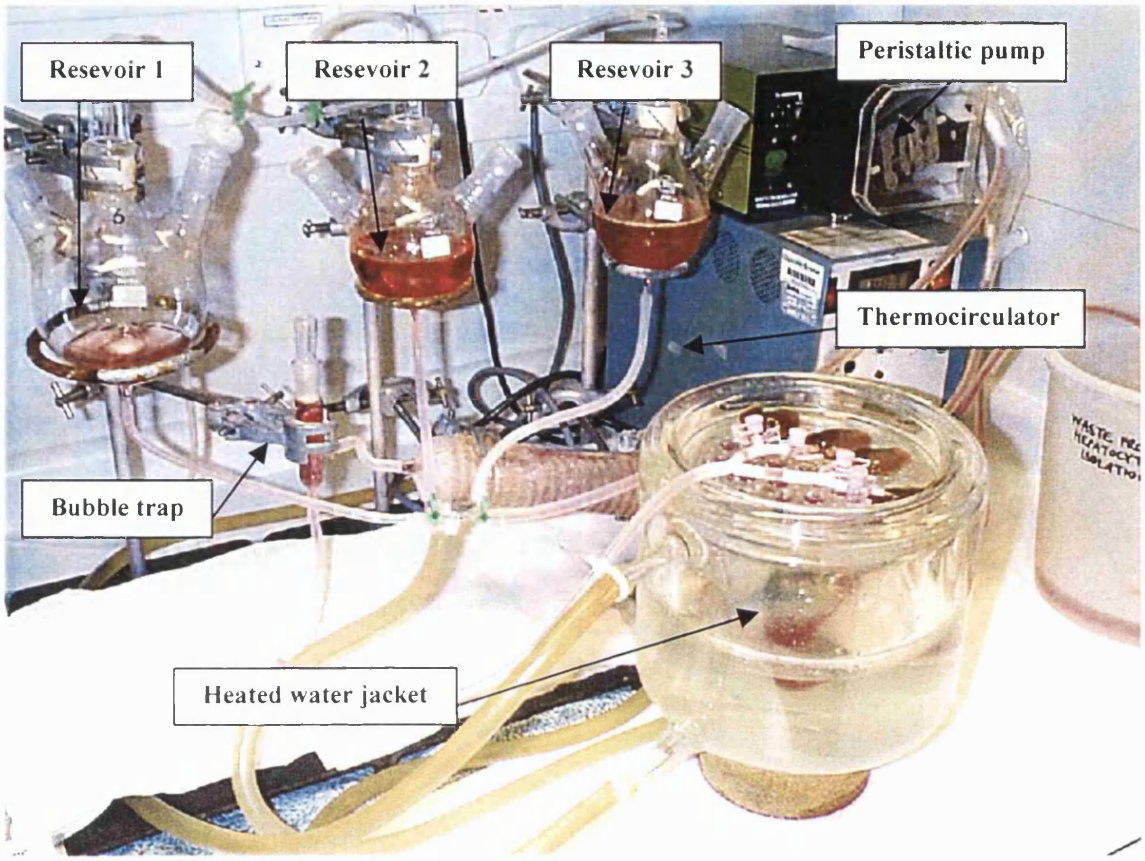


Figure 2.1 Perfusion apparatus used for hepatocyte isolation

2.2.5 *Preparation of Liver*

Male rats (approximately 200g) were sacrificed by cervical dislocation and the liver removed into either perfusion buffer or purification buffer for collagenase and EDTA isolation respectively. The left lateral, right lateral and median lobes were immediately separated by dissection and cut with a sterile scalpel to expose the blood vessels. The major vessel in each lobe was cannulated with an 18-gauge polyethylene catheter (Becton Dickinson, Cowley, UK).

2.2.6 *Isolation of Male Rat Hepatocytes by Collagenase Perfusion*

Perfusion of liver lobes was started immediately at a flow rate of 6 mL/minute/cannula. The cannulae were manipulated slightly until perfusion of the maximum area was achieved. Liver lobes were initially perfused with chelating solution containing EGTA (Reservoir 1) which also cleared the majority of blood from the lobes. After five minutes the perfusate was switched to perfusion buffer (Reservoir 2) to wash out the chelating solution. Following another five minutes the perfusate was switched to enzyme solution containing collagenase (Reservoir 3). The enzyme solution was recirculated for approximately 20 minutes when the appearance of the liver was seen to change as the liver softened.

The liver lobes were removed into ice-cold dispersal buffer (100mL) containing DNase I (4mg/mL). The Glisson capsule surrounding the liver was ruptured and metal forceps were used to comb the hepatocytes out. The resulting suspension of hepatocytes was filtered through pre-wetted Bolting cloth with a pore size of 64 microns. The suspension was centrifuged gently (50g, 5 minutes, 4°) to sediment viable hepatocytes. The majority of Kupffer cells and non-viable cells, which are lighter than parenchymal cells, were expected to remain in the supernatant that was removed by aspiration.

Hepatocytes remaining in the pellet were washed by gently resuspending them in dispersal buffer containing Dnase I. The centrifugation and wash steps were repeated once more in the presence of Dnase I and once without Dnase I. Hepatocytes in the

final pellet were resuspended in culture medium containing foetal calf serum (10% w/v).

2.2.7 Isolation of Male Rat Hepatocytes by EDTA Perfusion

Perfusion of liver lobes began immediately at a flow rate of 6 ml/minute/cannula. The cannulae were manipulated slightly until the perfusion of the maximum area was achieved. Liver lobes were initially perfused with purification buffer (Reservoir 1) which facilitated the elimination of red blood cells from the lobes. After two minutes the perfusate was switched to dissociation buffer containing EDTA (Reservoir 2) which was perfused through the lobes for approximately forty minutes.

Liver lobes were removed into ice-cold dispersal buffer and hepatocytes liberated by use of a metal comb as described before although a much more vigorous approach was required. The preparation of hepatocytes was then exactly as described following isolation by collagenase.

2.2.8 Preparation of Culture Dishes

Falcon 6-well culture dishes (35mm diameter) and culture dishes (10cm diameter, Becton Dickinson, Cowley, UK) were coated either with Matrigel®, fibronectin or Vitrogen® (collagen Type I) or left untreated as a control. The small 6-well culture dishes were used for determining testosterone metabolism in intact hepatocytes and for obtaining samples for Western blotting analysis for the untreated substrata control. The large dishes were used to obtain samples for Western blotting analysis for all substrata except control, and to determine metabolism of chlorzoxazone in intact hepatocytes. The preparation of the coated plates is described below. All culture dishes were washed twice with sterile phosphate buffered saline prior to use. Culture dishes not used immediately were stored at 4°C.

Matrigel® was thawed to 4°C and diluted 1/10 in ice-cold sterile water. Diluted Matrigel® was applied to culture dishes using a cooled sterile pipette to give coverage

of 0.1mL/cm^2. The dilute coating of Matrigel® was dried overnight in a laminar flow cabinet.

Fibronectin solution was diluted in sterile water to give a protein concentration of 0.1mg/mL. Diluted fibronectin was applied to culture dishes to give coverage of 10µg protein/cm^2. The fibronectin coating was dried overnight in a laminar flow cabinet.

Vitrogen® (10mg protein) and carbodiimide (0.1% w/v, 1.3ml) were diluted to 100 mL with sterile water. Culture dishes were coated with this solution to give a final coverage of 20µg protein/cm^2. Dishes coated with Vitrogen® were left overnight in an incubator maintained at 37°C. Water-soluble carbodiimides activate carboxy groups on the protein surface and catalyse covalent cross-linking of the charge-pairing complexes.

2.2.9 Plasma Membrane Integrity

Plasma membrane integrity was assessed by the trypan blue dye-exclusion method, a commonly performed criterion of cellular integrity. Cells with an intact plasma membrane exclude dyes such as trypan blue and eosin (Paul, 1970) whereas structurally damaged cells become stained, particularly in the nucleus.

Trypan blue solution (50µL) was mixed with rat hepatocytes suspended in culture medium (250µL) and left for 2 minutes at room temperature. Hepatocytes were examined using an improved Neubauer counting chamber (Weber Scientific International Ltd., Lancing, UK). The counting chamber was viewed using a Standard Laboratory 16 binocular microscope at 400-fold magnification (Carl Zeiss Ltd., Welwyn, UK). Cells were counted as being viable if they excluded trypan blue from the nucleus and had a shiny refractive surface. The cell number and viability were determined using the following equations:

Cell number/mL = Total number of cells in grid x 10 000 x 1.2

% Viability = (Number unstained hepatocytes ÷ Total number of hepatocytes) x 100

Each suspension of hepatocytes was counted four times and the mean result used. Hepatocyte suspensions following collagenase isolation were used if the viability exceeded 70%. However, hepatocytes isolated using EDTA had a much lower viability and preparations with a viability figure in excess of 40% were accepted.

The cell yield was calculated by dividing the total number of viable cells isolated by the amount of liver digested. The amount of liver digested was determined by weighing the liver prior to isolation and weighing the remaining liver and cell debris after isolation.

2.2.10 Attachment of Hepatocytes

Hepatocytes isolated either by collagenase or EDTA perfusion were suspended in culture medium containing foetal calf serum (10%v/v) to a concentration of 0.5 x 10^6 viable cells/mL. Culture dishes coated either with Matrigel®, fibronectin, Vitrogen®, or uncoated plastic, were seeded with hepatocytes at $1x10^5$ viable cells/cm^2 ($1x10^6$/35mm well and $8x10^6$/10cm dish).

Hepatocytes were left to attach to culture dishes for approximately 2 hours. The medium containing any unattached cells was then removed by aspiration and the hepatocyte monolayer gently washed twice with sterile phosphate-buffered saline. Hepatocyte monolayers were covered in serum-free culture medium (0.2mL/cm^2) and the medium was thereafter changed every 24 hours. Culture dishes were placed in an incubator maintained at 37°C with a humidified atmosphere of 5% carbon dioxide: 95% air.

2.3 *TESTOSTERONE METABOLISM IN MALE RAT HEPATOCYTES*

The metabolism of testosterone was determined by incubating cultured male rat hepatocytes with radiolabelled testosterone. Subsequent analysis was by high performance liquid chromatography (HPLC) following a solid phase extraction. The analysis of samples from preliminary experiments was by thin layer chromatography, autoradiography and radiometric scanning. However, the resolution of radioactive peaks was not sufficient to obtain good quality data and the HPLC method was developed as a superior technique.

2.3.1 Testosterone Incubation Procedure

A stock solution of ^{14}C-testosterone (4mM) was prepared as follows: radiolabelled testosterone (50μL of 50μCi/mL) was evaporated to dryness under nitrogen gas. The residue was dissolved in a methanolic solution of non-radiolabelled testosterone (0.5mL of 4mM) to give a final testosterone solution with a concentration of 4mM (5μCi/mL).

The reactions were initiated by the addition of the ^{14}C-testosterone solution (25μL) to fresh culture medium (1mL) overlaying the hepatocyte monolayers (35mm well) such that the final concentration of testosterone was 100μM. Culture dishes were placed in an incubator maintained at 37°C with a humidified atmosphere of 5% carbon dioxide and 95% air for 1 hour.

The reactions were terminated by the addition of ice-cold methanol (1mL) to the culture medium. The hepatocytes were released from the culture dishes using a cell scraper and, together with the culture medium, placed immediately on ice. The resulting samples were diluted with double distilled water (2mL), frozen and stored at -20°C until further analysis.

2.3.2 Solid Phase Extraction

Samples obtained by incubating testosterone with cultured rat hepatocytes were thawed, sonicated for two minutes and centrifuged (13000g, 5 minutes, 4°) prior to extraction. Testosterone and metabolites were isolated by solid phase extraction using the ASPEC automated extraction system (Anachem, Luton, UK). Bond Elute solid phase extraction cartridges (1cc, C18, Jones Chromatography Glamorgan, UK), were conditioned with methanol (2mL) and distilled water (5mL). The samples were applied and the cartridges washed with 25% methanol (5mL). Testosterone and metabolites were eluted from the cartridges with methanol (2mL). Methanolic eluates were evaporated to dryness under nitrogen gas and the residue resuspended in methanol (100%, 50μL) for analysis by thin layer chromatography (TLC) and methanol (35%, 500μL) for analysis by high performance liquid chromatography (HPLC).

Recovery of testosterone and metabolites by this solid phase extraction technique was determined. ^{14}C-testosterone was incubated with hepatocytes in monolayer culture as previously described in Section 2.3.1. Aliquots of incubate (100μL) and methanolic eluate (100μL) were added to Picofluor 30 scintillant (8mL) in triplicate and were counted in a Tracor Analytic Mark III liquid scintillation counter (Tracor Analytic, Illinois, USA). Small aliquots of eluate were also analysed by thin layer chromatography and autoradiography as described in Section 2.3.3. to demonstrate that metabolism of testosterone and recovery of metabolites had taken place.

2.3.3. Thin Layer Chromatography, Scanning and Autoradiography

Testosterone and metabolites were detected by TLC following application to G60 F254 silica gel prelayered plates (E.Merck, Darmstadt, Germany). TLC plates were developed under saturated conditions in two solvent systems as described by Ballard et al., 1988.

System 1. Dichloromethane : ethanol (20:1 v/v), plates developed 3 times

System 2. Dichloromethane : acetone (70:30 v/v), plates developed twice

Quantitation of radiolabelled testosterone and metabolites was carried out using a linear plate analyser (Isomess 3000, Lablogic, Sheffield, UK) linked to an Apple IIe data system. Metabolites were identified by co-chromatography with authentic standards supplied by the Steroid Reference Collection (London) visualised under UV light.

Autoradiographs were prepared by placing TLC plates in contact with X-ray film (Osray M3, AgfaGevaert) in light-tight envelopes. Films were exposed for up to 3 days and developed in G150 developer (AgfaGevaert) for 5 minutes, fixed in G334 fixer (AgfaGevaert), washed for 30 minutes in running tap water and dried.

2.3.4 High Performance Liquid Chromatography (HPLC)

Testosterone and metabolites were detected by HPLC, using a gradient system (Table 2.1). The HPLC column was a μ-Bondapak C18 stainless steel column (300x3.9mm, 10μm particle size, Waters, Watford, UK). The HPLC pump was an HP1050 set at a flow rate of 1 mL/minute and the UV detector was an HP1050 (Hewlett Packard) with a detection wavelength set at 240nm. For detection of radioactive entities a Berthold radio-detector (St. Albans, UK) was used and the scintillant was Picofluor 30 (Canberra Packard, UK), set at a flow rate of 2mL/minute. Metabolites were identified by co-chromatography with authentic standards supplied by the Steroid Reference Collection (London, UK).

Results were captured and analysed using the Multichrom data capture and analysis system.

Table 2.1 The gradient HPLC system used for the analysis of testosterone metabolism

	0 mins	12 mins	23 mins	30 mins	40 mins	50 mins
Solvent A	55%	55%	19%	20%	55%	55%
Solvent B	42.5%	42.5%	76%	78%	42.5%	42.5%
Solvent C	2.5%	2.5%	5%	2%	2.5%	2.5%

Solvent A = methanol : water 35:65 v/v

Solvent B = methanol : water 65:35 v/v

Solvent C = acetonitrile

2.3.5 Deconjugation of Testosterone and Metabolites

Samples (0.5mL) obtained from incubating cultured rat hepatocytes with testosterone and isolated using solid phase extraction, were incubated in the presence of β-glucuronidase (1/200 v/v), aryl-sulphatase (1/50 v/v) or without deconjugating enzymes to act as a control. Reactions were halted by the addition of acetonitrile (50μL). Chemical controls were carried out using phenolpthalein glucuronide (4mg/mL in sodium acetate 0.05M, pH6.0) acid and phenolpthalein disulphide (4mg/mL in sodium acetate 0.05M, pH6.0) to ensure that the two deconjugating enzymes were functional. Sodium hydroxide (1M, 3 drops) was added to chemical controls and the presence of deconjugated phenolpthalein indicated by a pink-coloured product. The samples containing testosterone were analysed by TLC and autoradiography as described previously, and the levels of metabolites measured.

2.3.6 Statistical Analysis

An analysis of variance was carried out to determine whether there was any difference between isolation technique, substrata, or an interaction between isolation and substrata with respect to testosterone metabolism for data obtained from samples taken at 0, 24, 48, 72 and 96 hours. Comparisons were made using a t-test with a

Sidak adjustment made to take account of multiple comparisons. This method of analysis makes the assumption that the variable under investigation is normally distributed with constant variance. These analyses were kindly carried out by Statistical Services (Glaxo Wellcome Research and Development)

2.3.7 Microsomal Preparation, Protein Measurement and Cytochrome P450 Determination

Microsomes were prepared for determination of testosterone metabolism in the absence of requirement for cell penetration by the substrate and for Western blotting analysis. Microsomes were prepared from hepatocytes at 0, 24, 48, 72 and 96 hours post-attachment.

Hepatocytes cultured for the purpose of making microsomes were cultured on large dishes (10cm diameter) to maximise the number of cells obtained. Smaller dishes (35mm diameter) were used as the untreated control, due to poor attachment of hepatocytes to the large dishes. The culture medium overlaying the hepatocytes was aspirated and replaced with phosphate buffer (0.1M, pH7.4) and hepatocytes were scraped from the dishes with a cell scraper. Hepatocytes obtained in this way were immediately placed on ice before being frozen and stored at -80°C. Immediately prior to the preparation of microsomes, hepatocytes were thawed and placed on ice. Hepatocytes from six experiments were pooled to enable a sufficient quantity of microsomes to be prepared.

Hepatocytes were homogenised using a mini glass hand-held homogeniser and the homogenate centrifuged in a Centrikon T-1180 ultracentrifuge (9000g, 20 minutes, 4°C). The resulting pellet was discarded and the supernatant centrifuged (100000g, 1hour, 4°C). The microsomal pellet obtained was resuspended in a small volume of phosphate buffer (0.1M, pH7.4) using a mini glass hand-held homogeniser. Microsomal preparations were snap frozen in liquid nitrogen and stored in small aliquots at -80°C until analysis.

The protein concentration of microsomal preparations was determined using a Coomassie blue kit (Pierce, UK) with a standard curve prepared from bovine serum albumin at known concentrations. The absorbance at 595nm was obtained using a Carey spectrophotometer and microsomal protein calculated from the standard curve.

Cytochrome P450 was measured following the addition of Renex buffer (see appendix for composition) to hepatocytes seeded onto culture dishes. A carbon-monoxide absorption spectra was performed using a Carey spectrophotometer and the difference in absorption at the wavelengths 450nm and 480nm and the molar extinction coefficient were used to calculate the concentration of cytochrome P450.

2.3.8 Microsomal Metabolism of Testosterone

Microsomal testosterone metabolism was determined by incubating microsomes (100µg protein) for up to 3 hours with ^{14}C-testosterone (100µM) and NADPH (2mM) in phosphate buffer (0.1M, pH7.4). An isocitrate-NADPH regenerating system consisting of isocitrate (5mM) and isocitrate dehydrogenase (5%v/v) was also included to ensure that a supply of NADPH was present throughout the extended incubation period. Incubations were carried out in a shaking water bath maintained at 37°C. Extraction of metabolites and HPLC analysis was as described in Section 2.3.2 and 2.3.4

2.4 WESTERN BLOTTING ANALYSIS

Western blotting analysis is a technique by which the amount of a particular protein in a sample may be determined using specific antibodies. Although the technique can be developed as a quantitative method, in the following experiments the technique is only semi-quantitative.

2.4.1 Sample Preparation

Hepatic microsomes were prepared from cultured rat hepatocytes as previously described (Section 2.3.6). Microsomes were initially diluted (1.2mg protein/mL) in a denaturing buffer (SDS, 1%w/v; sodium phosphate buffer, 10mM, pH 7.0; 2-mercaptoethanol, 1% v/v; and bromophenol blue, 0.01% w/v). Samples were then further diluted (1mg protein/mL) in loading buffer (EDTA, 0.5M, 2.5mL; sodium phosphate buffer, 1M, pH7.9, 1.25mL; bromophenol blue, 0.0125g; glycerol, 18.75mL; and 2-mercaptoethanol, 1.25mL). Samples prepared in this way were stored at -20°C. Immediately prior to electrophoresis samples were placed in a boiling water bath for approximately 3 minutes.

To aid in the identification of the cytochrome P450 band a sample of rat hepatic microsomes from rats treated with a variety of recognised inducing agents were supplied with the antibodies. These microsomes were diluted in denaturing and loading buffer in the same way as test microsomes. The relevant standard was then run alongside test samples.

2.4.2 Electrophoresis

The electrophoresis system used was based on the discontinuous system for resolving proteins denatured with SDS described by Laemmli (1970). Gels were cast in a SE250 multiple gel casting stand (Hoefer Scientific Instruments, Newcastle, UK) between a glass slide and a notched alumina plate separated by 0.75mm spacers.

The separating gel was prepared from pre-weighed acrylamide/bis-acrylamide (37.5:1) diluted in double distilled water to give a 30% solution. The composition of the 10% separating gel was acrylamide solution (10mL of a 30% solution), double distilled water (11mL), TEMED (23µL), ammonium persulphate (1.5mL of a 1.5% solution) and Tris-HCl buffer (7.5mL, 1.5M, pH8.8). The components of the gel were swirled gently to mix and the gel poured as quickly as possible. Gels were immediately overlaid with ethanol (95% v/v) and left to polymerise. Gels not used immediately were wrapped in Saran wrap and stored in a humidified box at 4°C until use.

The ethanol overlaying the separating gel was removed prior to pouring the stacking gel and the surface of the gel washed with distilled water. The stacking gel consisted of acrylamide solution (1mL of a 30% solution), Tris-HCl buffer (2.5mL, 0.5M, pH6.8), double distilled water (6mL), ammonium persulphate (0.5mL of a 1.5% solution) and TEMED (10μL). The solution was mixed gently, poured on top of the separating gel and a 10-well comb inserted. The stacking gel was left to set for approximately 30 minutes.

Aliquots (2.5 to 10μL) of each denatured microsomal sample were loaded onto the stacking gel. Samples were initially electrophoresed at a constant current of 8mA per gel until they were stacked into a narrow band, before the current was increased to 16mA per gel. The electrophoresis buffer (25mM Tris, 192mM glycine, 0.1% SDS), was cooled throughout this procedure by passage of water maintained at approximately 10°C by a temperature controlled recirculating water bath through the electrophoresis apparatus.

2.4.3 Electrotransfer

Protein transfer from the polyacrylamide gel to a nitrocellulose filter (0.45μm) was performed overnight at a constant current of 100mA per gel followed by 150mA per gel for 1 hour using a TE22 Transfer unit (Hoefer Scientific Instruments, Newcastle, UK). The transfer buffer (25mM Tris, 192mM glycine, 20% methanol) was cooled throughout this procedure by passage of water maintained at approximately 10°C by a recirculating water bath through the transfer apparatus.

2.4.4 Immunodetection

Detection of specific cytochrome P450 enzymes was achieved using a dual antibody technique. The second, alkaline phosphate-labelled antibody, and the buffers were supplied as a kit (BioRad) and the method used was as supplied by the manufacturer. Briefly, blocking solution was prepared by the addition of gelatin (3%) to TTBS (Tris-buffered saline containing Tween 20, 0.5%) with subsequent warming to approximately 56°C to dissolve the gelatin. Nitrocellulose membranes were

incubated with blocking solution for approximately 2 hours to block unoccupied protein binding sites on the membrane.

Membranes were washed in TTBS for 5 minutes before incubation with primary antibody specific for either CYP1A1, P450 reductase (Oxygene, diluted 1/100), CYP1A2, CYP2B1/2, CYP2C6, CYP2E1, CYP2C11, CYP3A4 (Dr CR Wolf, diluted 1/500) or CYP4A1 (gift, Dr G.G. Gibson, diluted 1/5000) for approximately 2 hours. Primary antibodies were all raised in the rabbit except for the CYP4A1 antibody that was raised in the sheep. Membranes were washed twice in TTBS for 5 minutes and then incubated with an alkaline phosphatase conjugated goat anti-rabbit or rabbit anti-sheep antibody, depending on which species the primary antibody was raised in.

Membranes were washed twice in TTBS for 5 minutes and once in TBS for 5 minutes before the enzyme labelled antibody was detected by incubating membranes in buffer containing 5-bromo-4-chloro-indolyl phosphate (BCIP) with nitroblue tetrazolium (NBT). Alkaline phosphatase cleaves the phosphate group of the BCIP and reduces NBT to an insoluble purple precipitate under the alkaline conditions used. The presence of the specific cytochrome P450 enzyme of interest was indicated by the appearance of a purple precipitate, the intensity of which was related to the amount of isoform present in the sample.

2.4.5 Densitometry

The colour density of the purple bands was measured using a Shimadzu CS9000 (Shimadzu, Japan) flying spot densitometer set in the zigzag-scanning mode using an absorbance wavelength of 555nm. The zigzag method of analysis allowed the absorbance of the whole band to be measured, thereby reducing inaccuracy due to non-uniformity of colour production across the band.

2.5 *THE EFFECT OF PYRAZOLE ON THE EXPRESSION OF CYP2E1*

The ability of male rat hepatocytes isolated using collagenase and cultured on Matrigel® to respond to the CYP2E1 inducing agent pyrazole was determined. The techniques used to measure the levels of CYP2E1 activity, protein and mRNA were metabolism of the substrate probe chlorzoxazone, Western blotting and Northern ELISA respectively.

2.5.1 Culture Conditions

Hepatocytes were isolated by collagenase perfusion and seeded onto Matrigel® coated dishes as previously described (Section 2.2.8). Low levels of chlorzoxazone 6-hydroxylation necessitated the use of large culture dishes (10cm diameter) to maximise the number of hepatocytes present. Hepatocytes were left for 24 hours prior to treatment by the addition of pyrazole (25, 50, 75 and 100μM in WME) for 48 or 72 hours. Untreated male rat hepatocytes were also cultured for up to 96 hours to act as a control.

2.5.2 Chlorzoxazone Metabolism in Rat Hepatocytes and Hepatic Microsomes

The metabolism of the CYP2E1 substrate probe chlorzoxazone was determined in male rat hepatocytes in monolayer culture and in microsomes prepared from a pool of male rat hepatocytes that had been maintained in culture.

2.5.2.1 Incubation Conditions

To measure chlorzoxazone metabolism in untreated and pyrazole-treated hepatocytes in monolayer culture the media overlaying the monolayers was first changed to a phosphate buffer (0.1M, pH7.4) prior to addition of the substrate probe. This avoided interference problems experienced with HPLC analysis when Williams' Media E was used as the incubation buffer. The reaction was initiated by the addition of

chlorzoxazone (50µL of a 50mM stock prepared in methanol) to the buffer overlaying the hepatocyte monolayers (5mL/ dish of 8 x 10^6 cells). Culture dishes were placed in an incubator maintained at 37°C with a humidified atmosphere of 5% carbon dioxide and 95% air.

Incubation times of up to three hours were used to establish linearity of the production of 6-hydroxy chlorzoxazone with respect to time, and thereafter an incubation time of one hour was used. The reaction was terminated by the addition of ice-cold PCA (0.5mL, 70%) to the culture medium. The hepatocytes were released from the culture dish using a cell scraper and, together with the culture medium, placed immediately on ice.

Chlorzoxazone metabolism was also measured in hepatic microsomes prepared from untreated and pyrazole-treated hepatocytes maintained in monolayer culture as described in Section 2.3.6. Hepatic microsomes (50µg protein) were incubated with NADPH (2mM) in phosphate buffer (0.1M, pH7.4, 0.1M $MgCl_2$) for up to 3 hours. An isocitrate-NADPH regenerating system consisting of isocitrate (5mM) and isocitrate dehydrogenase (5%v/v) was also included to ensure a supply of NADPH throughout the incubation period. Incubations were carried out in a shaking water bath maintained at 37°C. The reaction was initiated by the addition of chlorzoxazone (10µL of a 50mM stock solution, prepared in methanol/mL) and was terminated by the addition of ice-cold PCA (70%, 100µL/mL incubation mixture). The linearity of production of the 6-hydroxy chlorzoxazone metabolite with respect to time was determined and subsequently an incubation time of 3 hours was used.

To quantify chlorzoxazone metabolism a standard curve of the 6-hydroxy metabolite was prepared, extracted and analysed by HPLC alongside test samples. The retention time of these standards was also used to identify the metabolite peak in samples.

2.5.2.2 Solid Phase Extraction

Samples obtained by incubating chlorzoxazone with cultured male rat hepatocytes or microsomes were frozen and stored at -20°C until analysis. The chlorzoxazone and the 6-hydroxy metabolite were isolated by solid phase extraction using the ASPEC automated extraction system (Anachem, Luton, UK). Solid phase extraction cartridges (1cc, C8 Bond Elute) were conditioned with methanol (1mL) and distilled water (1mL). The samples were then applied to the cartridges which were then washed with distilled water (1mL). Chlorzoxazone and the 6-hydroxy metabolite were then eluted from the cartridges with methanol (1mL).

Recovery of chlorzoxazone by this solid phase extraction technique was determined by the use of ^{14}C-chlorzoxazone (500μM, 5μCi/mL). Radiolabelled chlorzoxazone was incubated with hepatocytes in monolayer culture as previously described in Section 2.5.2.1. Aliquots of incubate, water wash and methanolic eluate were added to Picofluor 30 scintillant (8mL) and were counted in a Tracor Analytic Mark III liquid scintillation counter (Tracor Analytic, Illinois, USA).

2.5.2.3 HPLC Analysis

Methanolic eluates were evaporated to dryness under nitrogen gas and the residue resuspended in mobile phase (500μL of 70% 0.02M sodium perchlorate : 30% acetonitrile, 0.0217% PCA). Samples were analysed by HPLC using a gradient system (Table 2.2). The HPLC column was a Spherisorb ODS2 (150 x 4.6mm, Waters, Watford, UK). The flow rate was 1 mL/minute and the detection wavelength was 287nm. The HPLC pump was a HP1050 and the UV detector was an HP1050. The amount of 6-hydroxy chlorzoxazone was determined by co-chromatography with authentic 6-hydroxy standards. Results were analysed using the Multichrom data capture and analysis system.

Table 2.2 The gradient HPLC system used for the analysis of chlorzoxazone metabolism

TIME (MINS)	% A	% B
0	100	0
30	75	25
32	100	0
38	100	0

A = 0.02M sodium perchlorate containing 0.0217% PCA

B = acetonitrile containing 0.0217% PCA

2.5.2.4 Deconjugation

Samples obtained from incubating rat hepatocytes with chlorzoxazone were treated as described in Section 2.3.5. The samples were analysed by HPLC as described previously, and the level of 6-hydroxy chlorzoxazone measured.

2.5.3 Western Blotting Analysis of Immunodetectable CYP2E1

Immunodetectable levels of CYP2E1 were determined as described in Section 2.4.

2.5.4 Statistical Analysis

CYP2E1 activity and relative levels of immunodetectable enzyme indicated by Western blotting analysis were analysed by a one-way analysis of variance. This was kindly carried out by Statistical Services (GlaxoWellcome, UK).

2.6 *PROBE SYNTHESIS AND DEVELOPMENT OF A NORTHERN ELISA TO MEASURE CYP2E1 MRNA*

A digoxigenin-labelled DNA probe to measure CYP2E1 mRNA was synthesised for use in an RNase protection assay or a Northern ELISA assay.

Synthesis of the probe and development of the Northern ELISA are considered fully in the Results and Discussion Section. Briefly, a probe was derived from a rat liver cDNA library using primers designed to bind around the target sequence of the rat *CYP2E1* gene. The probe was subsequently cut and cloned into a Bluescript plasmid. Bacterial cells transformed with the recombinant plasmid were used to synthesise the plasmid. A digoxigenin (DIG) labelled probe was produced from the recombinant plasmid by a polymerase chain reaction (PCR). The DIG-labelled probe was used to detect CYP2E1 mRNA.

2.6.1 Isolation of Plasmid DNA

MC1061 cells (10μL of a glycerol stock) containing a rat liver cDNA library (MC1061/P3) cloned into plasmid PCDM8 were inoculated into LB (Luria-Bertaini) medium (100mL) containing the antibiotics ampicillin (100μg/μL) and tetracycline (15μg/mL) to which the presence of the PCDM8 plasmid confers resistance. The culture was grown overnight at 37°C with vigorous shaking.

A single colony of JM109 cells containing the Bluescript KS plasmid (Figure 6, Stratagene) was inoculated into LB medium containing ampicillin (100μg/μL) to which the Bluescript plasmid confers resistance. The culture was grown overnight at 37°C with vigorous shaking.

The plasmids contained within the MC1061 and JM109 cells were isolated using the QIAGEN plasmid midi kit (QIAGEN Inc. California, USA). The Wizard midi prep system (Promega) was adopted in later experiments due to speed and elution of DNA

in buffer that does not have a high salt concentration. Brief descriptions of these two methods are given below. The composition of buffers is given in the appendix.

The Qiagen plasmid midi kit was used essentially as described in the protocol provided by the manufacturer with slight deviation in the final steps to try to remove salt. The bacterial pellet was resuspended in buffer P1 (4mL) and buffer P2 (4mL), mixed and incubated for 5 minutes at room temperature to lyse the cells. Buffer P3 (4mL, chilled) was added and incubated for 15 minutes on ice with intermittent shaking. Samples were centrifuged (30000g, 30 minutes, 4°C) and the supernatant immediately removed. A QIAGEN-tip 100 was equilibrated with buffer QBT (4mL) and the supernatant applied to the tip. The tip was washed twice with buffer QC (10mL) and the DNA eluted with buffer QF (5mL). DNA was precipitated with isopropanol (3.5mL) and centrifugation (15000g, 30 minutes, 4°C). The pellet was resuspended in water (0.5mL) before ammonium acetate (50µL, 3M) and ethanol (1mL, -20°C) were added. Samples were placed at -20°C for 30 minutes and then centrifuged (15000g, 30 minutes, 4°C). Supernatant was removed and the pellet air was dried then resuspended in TE buffer (100µL, 10mM).

The Wizard™ Miniprep was also used as described in the protocol provided by the manufacturer (Promega). The bacterial pellet was resuspended in cell resuspension solution (200µL), cell lysis solution added (200µL) and mixed by gentle inversion. Neutralising solution (200µL) was added and gently mixed, and the solution centrifuged (13000g, 5 minutes). The supernatant was removed and purification resin added (1mL) and mixed. The DNA/resin mix was applied to a Wizard™ minicolumn and a vacuum applied. The column was washed with wash solution (2mL) and the resin dried using a vacuum. The column was centrifuged (13000g, 2 minutes) and water (50µL) added to the column. After 1 minute the column was centrifuged (13000g, 20 seconds) to elute DNA.

2.6.2 Preparation of Bluescript Plasmid

A check digestion of the Bluescript plasmid was performed. A small aliquot of Bluescript plasmid (5μL) isolated as described was digested with the restriction endonucleases Hind III (2.5μL) and Sac I (2.5μL) in X10 concentration buffer A (2μL) diluted with sterile water (8μl). A sample of the cDNA plasmid library was also digested in this way. The restriction enzymes were added to the incubation mixture last, and the digestion carried out for 1 hour at 37°C.

RNA and DNA migrate towards the positive pole of an electric field due to their net negative charge and this property was used to separate and size fragments of DNA and RNA. Samples of cut plasmid, uncut plasmid, cut library, uncut library and a λ DNA Hind III digest ladder (5μL), were diluted with water (15μL) and bromophenol blue/xylene running dye (5μL) and loaded onto a 1% agarose gel (1g in 100mL TAE buffer). Samples were electrophoresed through the gel (approximately 1 hour, 100mA) and DNA visualised by staining the gel for about 20 minutes with the intercalating dye ethidium bromide (0.5μg/mL), washing the gel with water and viewing the gel under UV light (300nm). Ethidium bromide fluoresces at 590nm (visible) when excited with UV light (300nm) and photographs of transilluminated gels were taken to act as permanent records. In later experiments the image was captured using the AlphaImage system.

The plasmid was identified under UV light using the molecular weight ladder for guidance and was cut from the gel using a scalpel. The plasmid was isolated using the GLASSMAX™ isolation procedure essentially as described in the protocol provided (Gibco, UK). Binding solution (sodium iodide, 6M, 4.5mL) was added to the gel containing the plasmid (1g) and heated to 50°C until the agarose dissolved. Aliquots (500μL) of this solution were added to a GLASSMAX™ spin cartridge and centrifuged (13000g, 20 seconds). Wash buffer (0.4mL) was applied to the cartridge followed by centrifugation (13000g, 20 seconds) and this procedure was repeated a further two times. An extra wash step of 70% ethanol was also included. DNA was eluted with TE buffer (10mM Tris-HCl, 1mM EDTA, 40μL) preheated to 65°C

2.6.3 Generation of a CYP2E1 Probe by the Polymerase Chain Reaction (PCR)

Two primers were designed to bind around the region of the *CYP2E1* gene to be used as the probe but with a minor alteration in each case to include restriction sites for Hind III and Sac I in the sequence (Figure 2.3). The primers were synthesised using an ABI oligonucleotide synthesiser and were kindly supplied by the Molecular Pathology Group (Glaxo Group Research Limited). Primers were quantified using a Genequant (Pharmacia). The probe generated had an expected length of 249 bp.

The probe was generated by a PCR reaction, composed of Primer 1 (20μL, 100ng/mL), Primer 2 (20μL, 100ng/mL), dNTP (100μL, 2mM), buffer (100μL), PFU enzyme (10μL) and water (150μL). An aliquot of this reaction mixture (40μL) was added to a number of dilutions of plasmid DNA isolated from the rat cDNA library (10μL: 10pg/μL to 10ng/μL). The PCR reaction was carried out in a Perkin Elmer GeneAmp PCR system 9600. The temperature to break the double strands of DNA was set at 95°C for 5 minutes. The subsequent PCR cycle was composed of 30 seconds at 95°C to dissociate double strands, 30 seconds at 55°C to bind the primers and 90 seconds at 72°C for elongation. This cycle was repeated 35 times. The process was completed with a 5-minute incubation at 72°C before the samples were cooled to 4°C.

The products of the PCR reaction (50μL) were mixed with running dye (bromophenol blue/xylene cyanol; 5μL) and electrophoresed (100mA, approximately 90 minutes) through an agarose gel (2%) to separate the probe from other DNA.

The product of the PCR reaction with the size of 249bp was cut from the agarose gel with a scalpel and the expected DNA recovered using the Glassmax isolation procedure as described in the protocol provided. The sequence of the PCR product was kindly determined by the Molecular Pathology Group (Glaxo Group Research Limited) using the dideoxy chain termination sequencing technique with dye-labelled terminators on an automated ABI 373A sequencer.

Primer 1 (5'; 36bp)

Hind III

5' ACCTACCTGG**AAGCTT**TAGAAAAATCATGAAAAATG 3'

Primer 2 (34 bp)

Sac I

5' GCAGGAACTGAGACCAC**GAGCTC**AACTCTGAGAT 3'

Figure 2.3 Primer sequences with sites for restriction enzymes shown

To measure the amount of DNA or RNA in samples the absorbance at λ=260nm and λ=280nm were measured in a Carey spectrophotometer. The amount of RNA or DNA was determined using the molar extinction coefficients which are 40 and 50 respectively. The purity of the DNA or RNA was also determined by calculating the ratio of absorbance at 260nm:280nm.

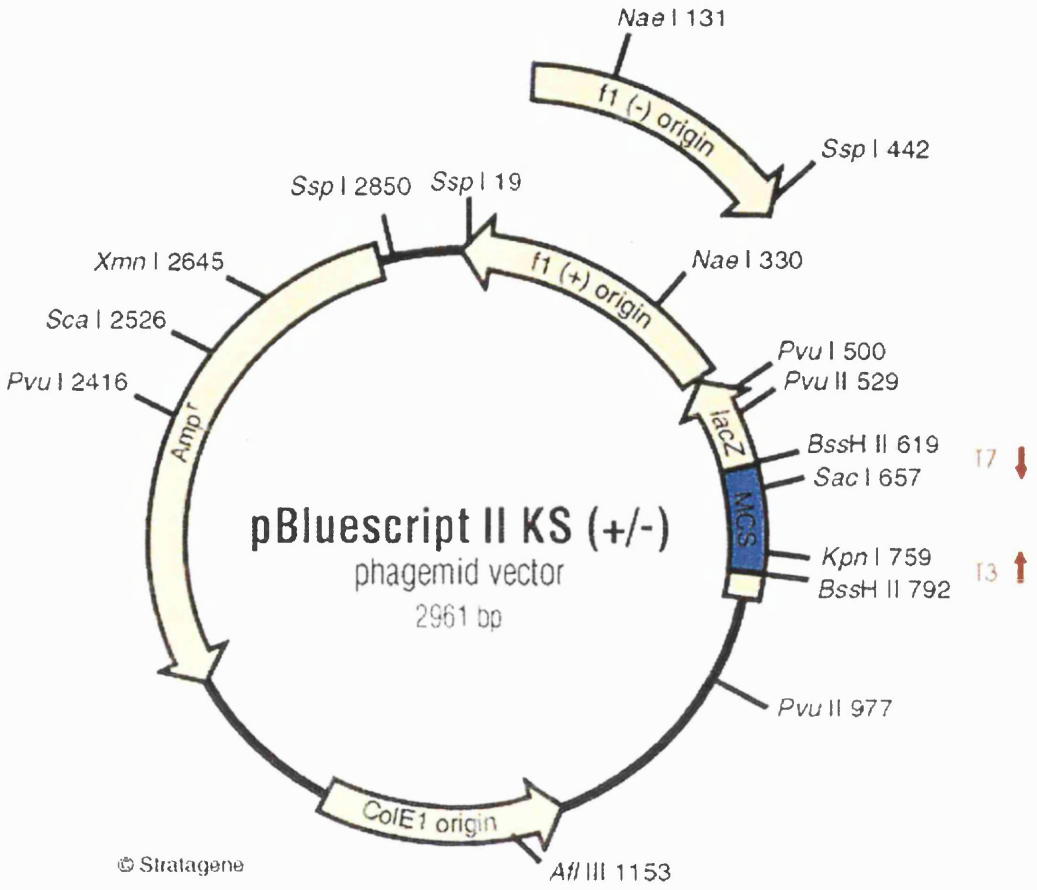


Figure 2.2 Map of the pBluescript II KS plasmid vector

2.6.4 Cloning the CYP2E1 mRNA Probe into the Bluescript Plasmid

The Bluescript KS plasmid (40μL) and the CYP2E1 PCR product (20μL) isolated as described were each digested using Hind III and Sac I restriction enzymes (1 U each) in buffer A. Multiple digests were performed (n=4) since this technique had not been found to respond well to scaling up of volumes used previously to check the activity of the restriction enzymes. The digestion was carried out at 37°C for 2 hours.

Following digestion the Bluescript plasmid and the PCR product were diluted with running dye and electrophoresed through an agarose gel (1%). The gel was stained with ethidium bromide and the plasmid and probe cut from the gel using a scalpel. The cut plasmid and PCR product were isolated using the Glassmax™ procedure.

A final check of the two DNA samples was made by electrophoresing (100mA, approximately 1 hour) an aliquot (5μL) of the resulting cut and purified DNA diluted with water (15μL) and running dye (5μL) through an agarose gel (1%). The gel was stained with ethidium bromide and photographed under UV transillumination.

Digested and purified PCR product and plasmid DNA were ligated with T4 DNA ligase (10U) overnight at 16°C. A range of ratios of plasmid to insert were used (1:2.5, 1:5 and 1:7.5). The digested plasmid was expected to have non-complementary protruding termini, and was not expected to self-ligate. The ligation of insert DNA and plasmid DNA resulted in a recombinant DNA molecule containing the DNA encoding *CYP2E1*.

2.6.5 Cell transformation

JM109 cells were inoculated into LB media (25 mL) and grown overnight at 37°C in an orbital shaker. An aliquot (0.5mL) was diluted with LB media (50mL) and the culture grown at 37°C in an orbital shaker until the culture achieved an optical density of approximately 0.4 (λ=600nm). At this stage the cells are considered to be 'competent', a physiological state that facilitates DNA uptake. Cells were sedimented

by gentle centrifugation (3000g, 10 minutes). The supernatant was removed and the cells resuspended in calcium chloride (40ml, 100mM) and placed on ice for 1 hour. The cells were gently centrifuged again (3000rpm, 10 minutes) and then resuspended in calcium chloride (2mL, 100mM). The final preparation of cells was approximately 10^6 cells/mL.

Recombinant plasmid DNA was added to aliquots of cells (5ng/μL) and cells placed on ice for 30 minutes. The introduction of DNA into competent bacteria in this way is referred to as transformation. The cells were heat shocked (42°C, 60 seconds) and recovered on ice for 5 minutes. LB media (1mL) was added and the cells incubated for 1 hour at 37°C in an orbital shaker. The cells were plated out on ampicillin selection plates and grown overnight at 37°C.

2.6.6 Screening for Bacteria carrying Recombinant DNA

Two techniques were used to screen for bacteria that had been transformed with the recombinant plasmid. These were blue/white screening for active β-galactosidase activity and PCR.

2.6.6.1 Blue/white screening for β-galactosidase

The Bluescript plasmid contains a sequence coding for the α-peptide of β-galactosidase that is interrupted by the multiple cloning site. Non-recombinant plasmids produce a functional α-peptide that complements the defective product of the host cell lacZM15 gene resulting in functional β-galactosidase activity. However, when the α-peptide is interrupted by cloning a fragment into the multiple cloning site, complementation does not take place and there is no β-galactosidase activity. Colonies of bacteria containing recombinant plasmid are white, while colonies of bacteria containing non-recombinant plasmid are blue when grown on selection plates containing the substrate X-gal (5-bromo-4-chloro-indoyl-β-D-galactoside). The ability of bacterial colonies to hydrolyse this chromogenic substrate is the basis for the blue/white screening process.

Plates were prepared from L-broth (10g tryptone, 5g yeast extract, 10g NaCl and 15g agar in 1L water) which had been autoclaved and cooled before ampicillin (50μg/mL), IPTG (isopropyl β-D-thiogalactopyranoside, 0.5mM) and X-gal (40μg/mL) were added. Bacteria obtained from the transformation process were inoculated onto the plates by spreading over the surface of the agar medium. Colonies were grown overnight at 37°C on inverted plates.

2.6.6.2 PCR

A number of discrete colonies obtained from the blue/white screening process were picked into LB media (100μL) containing ampicillin (100μg/mL) and tested for the presence of recombinant Bluescript plasmid using the PCR technique described in Section 2.6.3 to generate the CYP2E1 mRNA probe. However, taq polymerase was used instead of pfu polymerase since 100% fidelity was not required in this particular test. The products of the PCR reaction were electrophoresed (150mA, 45 minutes) through an agarose gel (3%). The presence of a product approximately 249bp in length indicated that the bacterial cells had been transformed with the recombinant plasmid. The gel was stained with ethidium bromide and those colonies found to contain the plasmid were grown overnight and stored as glycerol stocks. The recombinant plasmid was isolated from bacteria using the Glassmax™ technique.

2.6.7 Digoxigenin (DIG) Labelling of Probe.

Two methods were used to produce a DIG-labelled probe. The first method was the use of linearised plasmid to create DIG-labelled RNA 'run-off' transcripts. This method was not successful and a second technique using a DIG-PCR kit was successfully developed instead.

2.6.7.1 'Run-Off' Transcription

To create linearised DNA for the synthesis of 'run-off' transcripts the isolated recombinant Bluescript plasmid was linearised with the restriction enzyme Hind III.

Plasmid DNA (1μg) was diluted with water (16μL), enzyme buffer B (2μL) and Hind III (2μL). The Hind III enzyme was added last and the digest well mixed using a pipette. The digests were incubated for 1 hour at 37°C. An aliquot (2μL) of the digest was diluted with water (6μL) and running dye (1μL) and loaded onto an agarose gel (1%) gel alongside uncut Bluescript plasmid and a 100 bp ladder to determine whether the plasmid had linearised. The gel was electrophoresed (100mA, 1 hour), stained with ethidium bromide and a photograph of the UV illuminated gel taken. The linearisation digest was shown to have gone to completion by the presence of only one band on the check gel, which was retarded in comparison to the uncut plasmid.

To remove the Hind III from the recombinant plasmid digest, a phenol/chloroform extraction was carried out. The plasmid digest (18μL) was diluted with water (82μL) and phenol/chloroform (100μL, 1:1v/v) added. The sample was vortex mixed and centrifuged (13 000g, 5 minutes). The upper phase was removed and an equivalent volume (80μL) of phenol/chloroform (1:1 v/v) was added. The sample was vortex mixed and then centrifuged (13000g, 5 minutes). The supernatant was removed and sodium acetate (8μL, 3M) and ethanol (250μL, 100%, -20°C) added to the precipitate. The sample was left overnight at -20°C.

The linearised DNA was used as a template by RNA polymerase to create 'run-off' transcripts with a DIG label using a DIG RNA (SP6/T7) labelling kit (Boehringer Mannheim) as described by the manufacturer. The nucleotides ATP, CTP, GTP and UTP were supplemented with DIG-UTP to label the transcript so that approximately every 20-25th nucleotide of the newly synthesised RNA was a DIG-UTP

The sample was centrifuged (13000g, 15 minutes, 4°C) to sediment precipitated DNA. The supernatant was removed and the pellets resuspended in ethanol (70%, 200μL, -20°C) and centrifuged (13000g, 15 minutes, 4°C). The supernatant was removed and the pellet al.lowed to air dry to remove the ethanol. The DNA pellet was resuspended in DEPC-treated (diethyl pyrocarbamate, 0.1%) water.

To transcribe the antisense *CYP2E1* mRNA probe, linearised plasmid (20μL), NTP labelling mixture (3μL), RNase inhibitor (1μL) and T7 polymerase (3μL) were added to transcription buffer (X10, 3μL). The transcription reactions were performed for 2 hours at 37°C. Transcripts were briefly centrifuged and DNase I (2μL) added to each tube. Samples were incubated (15 minutes, 37°C) to digest the template DNA. The magnesium-dependant transcription reaction was terminated by the addition of EDTA to each tube (1μL, 0.5M, pH8). The transcribed RNA in all samples was precipitated by the addition of LiCl (5μL, 4M) and ethanol (150μL, 100%, -20°C). Samples were mixed well and left overnight at -20°C. Samples were centrifuged (13000g, 15 minutes, 4°C) to precipitate the expected DIG-labelled mRNA probe. The resulting pellet was washed with ethanol (50μL, 75%, -20°C) and centrifuged (13000g, 10 minutes, 4°C). The ethanolic supernatant was removed, the samples air-dried and resuspended in DEPC-treated water (50μL).

2.6.7.2 PCR DIG Probe Synthesis

The second technique used to synthesise a DIG-labelled CYP2E1 mRNA probe was the DIG PCR probe synthesis kit (Boehringer Mannheim). This kit is designed to enable the generation of DNA probes labelled with highly-sensitive digoxigenin via the PCR reaction.

PCR buffer (X10, 5μL), primer T3 (1μL, 25pmol), primer T7 (1μL, 25pmol), PCR DIG mix (5μL, 200uM dNTP), High Fidelity enzyme (0.75μL, 2.6U), template DNA (10μL, 100pg) and sterile water (27.5μL) were mixed. The PCR reaction was carried out in a Perkin Elmer GeneAmp PCR system 9600 using the procedure described in Section 2.6.3. The yield of DIG-labelled product was found to be too low and dilution of the PCR DIG mix with an equal volume of dNTP stock (200μM) was found to result in high yield of DIG-labelled PCR product.

2.6.8 Immunodetection of DIG-Labelled Probe

The DIG nucleic acid detection kit (Boehringer Mannheim) was used to detect the DIG-labelled probe as described in the manufacturers instructions. Initially DIG-

labelled probe was spotted directly onto nylon membrane to establish that the immunodetection was functional. During further assay development to check hybridisation, plasmid DNA was boiled for 5 minutes to separate the two strands and then placed on ice. A range of concentrations of plasmid (1 to 300pg) was spotted onto a nylon membrane (Boehringer Mannheim) alongside a range of concentrations of the DIG-labelled probe (1 to 300pg) and cross-linked in a UV transilluminator for 3 minutes.

The membrane was incubated in hybridisation buffer (20mL/100cm^2) at 68°C for at least one hour in a hybridisation oven (Techne). For detection of DNA, hybridisation buffer was composed of SSC (5X, sodium chloride 3M, sodium citrate, 0.3M), blocking reagent (1%), N-lauroylsarcosine (0.1% w/v) and SDS (0.02% w/v). For detection of RNA, hybridisation buffer was composed of formamide (50%), SSC (5X), sodium phosphate (50mM, pH7.2), blocking reagent (2%w/v), SDS (7% w/v) and N-lauroylsarcosine (0.1%). Membranes were incubated overnight at 68°C in hybridisation buffer (2.5mL/100cm^2) containing probe DNA (100ng/mL) which had been denatured by boiling for 5 minutes. The membranes were washed twice (2XSSC, 0.1% SDS, 50mL/100cm^2) for 5 minutes at room temperature and twice (0.1XSSC, 0.1% SDS) for 15 minutes at 68°C.

Membranes were washed for 1 minute in buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5, 0.3% w/v Tween 20). The digoxigenin was detected immunologically by incubating the membranes in blocking solution (1%, 100mL/100cm^2) for 1 hour and then in blocking solution containing alkaline-phosphatase conjugated anti-DIG antibody (1:5000, 20mL/100cm^2)) for 30 minutes. The membranes were washed twice (15 minutes) and the alkaline phosphatase detected using 5-bromo-4-chloro-indolyl phosphate with nitroblue tetrazolium. The presence of the DIG-labelled probe was indicated by the presence of a purple spot.

2.6.9 Isolation of sample RNA

RNA was isolated from rat hepatocytes using two techniques: RNazol in early experiments and the PolyATtract®System 1000 (Promega) in later experiments.

2.6.9.1 Isolation of RNA Using RNazol

RNA was extracted from two plates (10cm diameter containing $8x10^6$ cells) of rat hepatocytes using RNazol (2.5mL/plate). The resulting sample was placed on ice and chloroform (500μL) added. After 5 minutes samples were centrifuged (13000g, 15 minutes, 4°C).

The upper, aqueous layer was removed into a clean tube and the lower phase discarded. To each sample an equal volume of propan-2-ol (approximately 750μL) was added and the samples placed on ice for 15 minutes before and centrifugation (13000g, 4°C, 15 minutes) to precipitate RNA. The RNA was visible as a white precipitated pellet. The supernatant was removed and the pellet resuspended in ethanol (75%, -20°C) by vortex mixing. Samples were centrifuged again to precipitate RNA (7500g, 8 minutes, 4°C). The ethanol supernatant was removed and the pellet resuspended in sterile water (200μL).

A small aliquot (5μL) of each sample prepared above, along with the plasmid (2μL), were diluted with Milli-Q water (395μL) and the absorbance at λ=260nm and λ=280nm determined.

2.6.9.2 Isolation of mRNA Using the PolyATtract®System 1000

The PolyATtract®System 1000 (Promega), a faster technique with a higher yield, was used in later experiments. The procedure used was as described by the manufacturer for the isolation of mRNA from cultured cells.

Briefly, hepatocytes were lysed in extraction buffer containing guanidium thiocyanate and β-mercaptoethanol (1mL). The cells were lysed using a sterile pasteur pipette rather than a homogeniser as described in the protocol. Dilution buffer preheated to 70°C (2mL), β-mercaptoethanol (41μL) and the biotinylated oligo(dt) probe (3μL) were added, and the resulting sample was mixed and incubated at 70°C for 5 minutes. The lysate was centrifuged (12000g, 10 minutes, room temperature). Streptavadin Magnesphere® Paramagnetic particles (SA-PMPs) in suspension (2.5mL) were

decanted into tubes which were then placed on the magnetic stand provided. The storage buffer was removed and the SA-PMPs resuspended in SSC (0.5X solution, 1mL) and washed three times.

The supernatant from the lysate was added to the suspension of SA-PMPs and mixed by inversion. Following incubation (2 minutes, room temperature) the SA-PMPs were captured using the magnetic stand and the supernatant removed. Particles were resuspended in SSC (0.5X, 1mL) and washed three times. The mRNA was eluted in nuclease free water (200µL).

2.6.10 Development of an RNase Protection Assay to Measure CYP2E1 mRNA

The RNase protection assay was intended to be the analytical technique used for the measurement of CYP2E1 mRNA. The RNase protection assay may be divided into three stages; hybridisation between the DIG-labelled PCR probe and target mRNA, polyacrylamide gel electrophoresis and detection of digoxigenin-labelled nucleic acids by enzyme immunoassay (DIG Nucleic Acid Detection Kit, Boehringer Mannheim). These are described briefly below, while details of the buffers are given in the appendix.

The DIG-labelled CYP2E1 mRNA probe was diluted 1/300 in hybridisation buffer (0.02ng/µL). Sample RNA (5µg) was added to hybridisation buffer containing the CYP2E1 probe (30µL). Samples were denatured by heating (5 minutes, 95°C), after which the samples were transferred to a water bath maintained at 45°C for overnight incubation.

The hybridisation mixture was cooled to room temperature, diluted with RNase digestion buffer (350µL) and RNase T1 added (2.5µL). RNase A was omitted due to reports that this enzyme may denature double stranded RNA due to the presence of groups such as biotin (Turnbrow and Garner, 1993). Digestion of single stranded RNA was carried out (30 minutes, 30°C). Samples were treated with SDS (20%, 10µ L) and proteinase K (2.5µL), and incubated (15 minutes, 37°C). Yeast tRNA (1µL)

and ice-cold ethanol (1mL) were added and samples incubated at -20°C for 30 minutes. Samples were centrifuged (13000g, 15 minutes, 4°C) after which the ethanol was removed and the pellet stored at room temperature until the ethanol had completely evaporated.

The precipitate was resuspended in loading buffer and heated (5 minutes, 95°C). Samples were immediately transferred to a water bath. The digestion products were electrophoresed (180V, 50 minutes) through a polyacrylamide gel (6% containing 6M urea). RNA in the gel was transferred to a nylon membrane by capillary action and the transferred RNA crosslinked with UV light (3 minutes).

DIG labelled RNA hybrids were detected using enzyme immunoassay with colorimetric detection. All steps were carried out at room temperature using an orbital shaker, except for the colour development. Membranes were washed briefly (1 minute) with Buffer 1 containing Tween 20 (0.3%v/v) and incubated with blocking solution for 30 minutes. Membranes were incubated with anti-digoxigenin-AP conjugate (1:2000) for 30 minutes and unbound antibody subsequently removed by washing with buffer 1. Membranes were equilibrated with Buffer 3 for 2 minutes, before addition of the freshly prepared colour substrate solution 5-bromo-4-chloro-indolyl phosphate with nitroblue tetrazolium. The intensity of the colour precipitate was measured using a densitometer (λ=555nm)

2.6.11 Development of a Northern ELISA to Measure CYP2E1 mRNA

The Northern ELISA assay is a photometric enzyme immunoassay used for the detection and quantification of specific transcripts in isolated mRNA. In this instance it was established to measure CYP2E1 mRNA. The techniques used are essentially as described in the manufacturers protocol for the DIG nucleic acid detection kit and the Northern ELISA kit (Boehringer Mannheim). Details of buffers are given in the appendix.

Assays were initially developed using dot blots. Isolated recombinant plasmid was boiled for 5 minutes and placed on ice prior to being applied to a nylon membrane(1,

3, 10, 30, 100 and 300pg). To ensure that the immunodetection system was functional, DIG-labelled probe was also spotted directly onto the nylon (1, 3, 10, 30, 100 and 300pg). Samples were UV crosslinked to the nylon membrane for 3 minutes and the membrane incubated with hybridisation buffer (20ml/100cm^2) maintained at 68°C in a hybridisation oven (Techne) for 1 hour. DIG-labelled probe was boiled for 5 minutes, placed on ice and an aliquot (5µL, 1µg) was added to hybridisation buffer (10mL). Membranes were incubated in hybridisation buffer containing probe at 68°C overnight in a hybridisation oven. Membranes were washed twice for 5 minutes at room temperature (2X SSC, SDS 0.1% w/v) and twice for 15 minutes at 68°C.

Membranes were developed using the DIG-nucleic acid immunodetection method described in Section 2.6.8.

CHAPTER 3

RESULTS AND DISCUSSION

3. 1 ISOLATION PROCEDURES

Isolation of intact hepatocytes is the essential first step in primary hepatocyte culture. Two isolation techniques, based on knowledge of the components and mechanisms of cellular adhesion, were established for these studies. An enzyme-based method, collagenase, and a non-enzymatic method, EDTA, were selected due to the different mechanisms in which cells are liberated by these techniques. The effect of these different isolation techniques on cytochrome P450 expression in hepatocytes maintained in culture for up to 96 hours was subsequently determined.

The isolation methods used have evolved from early attempts of several workers that had limited success, with the simple mechanical procedures resulting in damaged cells despite occasionally good yields (Schreiber and Schreiber, 1973, review). One of the most successful early methods, developed by Anderson (1953), involved hepatic perfusion with an isotonic solution containing EDTA, followed by mincing the liver into small pieces, homogenisation with a loose fitting pestle, filtration and centrifugation. Certain aspects of this technique have been adopted in the EDTA isolation method used in these studies. Later, enzymatic isolation techniques were developed following elucidation of the fine structure of cell junctions by electron microscopy (Fawcett, 1961) coupled with studies on biochemistry, cell biology, microbiology and histology.

The connective skeleton within the liver lobule is comprised of a fine network of reticular and collagenous fibres embedded in the extracellular matrix. Each hepatocyte is also directly attached to neighbouring cells by means of several types of junctional complexes. The two isolation techniques selected represent different approaches to the breakdown of the connective skeleton and the cell junctions, which form the basis of hepatocyte isolation. It has been proposed, however, that neither

collagenase nor EDTA isolation results in the full cleavage of tight or gap junctions, and that after the desmosomes break the cells are separated by the tearing of the plasma membrane (Seglen, 1973). The gap and tight junctions are subsequently retained by one of the cells and the damage to the other cell is probably repaired (Berry et al., 1997).

The removal of a calcium-dependant cell-cell adhesion factor in the central plaque of the desmosomes by a chelating agent is utilised by both techniques selected (Green and Jones, 1996). Removal of the adhesion factor from within the central plaque material of the desmosomes is thought to cause the hemidesmosomes to part (Amsterdam and Jamieson, 1974). The removal of the adhesion factor in this way is irreversible and it is thought that the adhesion factor is detached and washed out (Modjanova and Malenkov, 1973). This is probably the reason that calcium free media, with or without a chelating agent, is an essential feature of the successful isolation of intact hepatocytes.

The use of enzymatic methods to isolate hepatocytes, in addition to that of simple chelation, developed later following the successful use of collagenase and hyaluronidase to isolate fat cells from adipose (Rodbell, 1964; Howard et al., 1967). The use of these enzymes as well as trypsin, pronase and lysozyme, greatly facilitated the preparation of intact cells although the resulting yield and viability were low. Hyaluronidase was later shown to be unnecessary (Ingebretsen and Wagle, 1972) and to enhance glycogen degradation (Wagle and Ingebretsen, 1974) and is no longer extensively used. Later, the introduction of physiological liver perfusion making tissue uniformly accessible to collagenase in oxygenated digestion media, was a major advance resulting in high yields and viability (Berry and Friend, 1969).

The collagenase isolation technique was further optimised into a two-step procedure by Seglen (1972). The first step involves chelation of calcium ions to break calcium dependent cell-cell interaction and the second step involves collagenase digestion in media supplemented with calcium, a co-factor required by collagenase. This technique, and variations of this technique, results in the production of large numbers of intact cells and is still widely used by many laboratories.

The two isolation techniques used in these studies are described below. A strong carbon-dioxide/bicarbonate buffering system was utilised for both isolation techniques, with the perfusate being gassed with 95% O_2 and 5% CO_2. This has the advantage of combining oxygenation with buffering capacity. This is important since the absence of oxygen supplementation may result in a higher proportion of damaged cells accompanied by blebbing of the plasma membrane (Berry et al., 1991) and in the absence of a buffering system perfused livers rapidly acidify the medium (Seglen, 1976).

The post-isolation purification method used was also the same. This was based on the separation of the parenchymal cells from non-parenchymal cells due to the 10-fold difference in the size of these cells (Blomhoff and Berg, 1990). It is important to eliminate as many non-parenchymal cells as possible since these cells also have metabolic capacity and contamination may effect the results (Wickramasinghe, 1998). However, this results in the isolation of a population of liver cells which are less representative of those present *in vivo*.

3.1.1 3-Step Collagenase Perfusion

The collagenase hepatocyte isolation technique adopted was a three-step perfusion technique based on the method of Oldham et al. (1985). The first step, perfusion with EGTA in calcium and magnesium free media, chelates the calcium and breaks down the desmosomes. EGTA was used in preference to EDTA as this has been reported to prevent loss of reduced glutathione (Vina et al., 1978) and magnesium was excluded because it has been shown to inhibit dispersion of hepatocytes (Seglen, 1973).

The second step, perfusion with media to remove EGTA, prevents the chelation of calcium which is added during the collagenase digestion where it acts as a co-factor (Berg and Boman, 1973). Perfusion with collagenase is the third and final stage, with enzymatic digestion of the connective skeleton. The collagenase solution was recirculated due to the expense of this enzyme despite reports that cells and cell debris may detach into the recirculating solution and subsequently block vessels (Wang et al., 1985)

Different preparations of coilagenase are commercially available and collagenase H was use in preference to collagenase C since it was found to be easier to dissolve in perfusion medium. Calcium was added during collagenase digestion to enhance collagenase activity (Berg and Boman, 1973) and to help prevent damage due to prolonged calcium deprivation. Trypsin inhibitor was also included to decrease the activity of any trypsin contaminating the collagenase preparation since this enzyme is thought to be have a deleterious effect on receptors present on the cell surface (Kletzien et al., 1976). The liver was seen to swell during the final perfusion stage and this has been shown to be due to expansion of the extracellular space (Seglen, 1973).

The optimum time to halt digestion of the lobe was based upon observation. The digestion was terminated when the structure of the liver under the outer capsule was seen to soften and deteriorate, with the appearance of the white, reticular network. This generally required approximately 20 minutes of perfusion with collagenase, with differences in : the exact area perfused, collagenase activity and the level of contaminants the most likely cause of variability. Contamination of commercial preparations by other proteolytic enzymes including trypsin, clostripain and caseinase is, however, thought to enhance cell dissociation and for this reason the collagenase obtained from commercial sources was not further purified (Berry et al., 1991).

Hepatocytes were released by rupturing the outer capsule, agitating the tissue and gently combing out the cells. The release of cells from the liver was relatively easy following collagenase digestion with minimal mechanical force required for cell dispersal.

3.1.2 2-Step EDTA Perfusion

A resurgence of interest occurred in the late 1980's in methods using only a chelating agent without enzymatic digestion and a resulting stabilisation in phenotype was reported using this approach (Wang et al., 1985; Meredith, 1988; Bayad et al., 1991). These methods avoid the presence of collagenase and non-specific proteases present in collagenase which are thought to attach to hepatocytes and hydrolyse proteins

(Capuzzi et al., 1979) and damage plasma membrane proteins (Tarentino and Galivan, 1980; Kato et al., 1979). A two-step technique based on the whole liver *in situ* method of Meredith (1988) was adopted in this study to isolate hepatocytes in a non-enzymatic method using the chelating agent EDTA.

The first step, perfusion with a simple purification buffer, eliminates red blood cells from the perfused area of the lobes. The second step involves perfusion with the chelating agent EDTA for approximately 40 minutes. The optimum perfusal time for each preparation was more difficult to determine using this technique since no disaggregation of the liver below the surface of the capsule was observed, even with prolonged perfusion. Perfusion buffers contained sodium pyruvate, a potential energy source which results in higher ATP levels and it is also effective in stimulating protein synthesis in hepatocytes (Tomita et al., 1995; Schwarze et al., 1982).

Cells were released by rupture of the outer capsule followed by combing out of the cells. The gentle combing and agitation for release of cells following collagenase isolation was inadequate to release cells following EDTA isolation, and a more forceful procedure was required. This has been observed by others when attempting this isolation technique (Seglen, 1976). The cell suspension obtained following EDTA isolation, therefore, contained more cell debris and larger clumps of cells than that following collagenase isolation.

3.1.3 Hepatocyte Viability

Assessment of hepatocyte viability was performed routinely so that each hepatocyte preparation satisfied a minimum criterion before use. The use of hepatocytes with a high proportion of non-viable cells was avoided as the undesirable release of enzymes into the culture medium by non-viable cells is unavoidable (Evarts et al., 1984). For collagenase-isolated rat hepatocytes a minimum viability value in excess of 80 to 90% is generally accepted (Berry et al., 1991) and was exceeded by all hepatocyte preparations isolated during these studies. However, due to much lower viability for EDTA-isolated hepatocytes a minimum viability value of 40% was adopted for these

studies since hepatocyte preparations with a viability value of less than 40% were found to attach poorly to culture plates.

The viability of hepatocyte preparations can be assessed by a variety of tests, the most commonly performed criterion of cellular integrity being the trypan blue exclusion test. This is based on the fact that cells with an intact plasma membrane exclude this dye (Paul, 1972) and can be identified by their pale yellow colour and well defined outline, whereas structurally damaged cells become stained, particularly in the nucleus. Trypan blue is negatively charged and it has been proposed that it is excluded by an energy-dependant maintenance of the plasma membrane potential which is negative inside the cell (Berry et al., 1988). This test is simple, reliable and gives an almost instant result. However, a cell may have internal metabolic lesions or surface alterations not revealed by this test, but from which cell death may result at a later time. The trypan blue test is, therefore, a measure of gross structural integrity rather than viability.

Other so-called viability tests include measurement of metabolic capacity such as glycogen synthesising ability (Seglen, 1973) but these can show great variability. Respiratory rates have also been used to assess the quality of hepatocyte preparations but are of limited value because both isolated mitochondria and those in dead cells can still respire. Other metabolic tests also suffer from this problem, whereby the process selected can take place in subcellular structures and can therefore also occur in dead or damaged cells. Alternative tests, such as measurement of cellular potassium, are unsuitable due to the reversible nature of the loss and uptake process (Tolbert and Fain, 1974). The leakage of soluble enzymes such as lactate dehydrogenase is also a useful determinant of structural integrity (Berg et al., 1972) but takes longer to perform than trypan blue.

For reasons of speed, reliability and simplicity, and in the absence of a test offering any major advantages, the trypan blue test was used to assess viability. Following isolation of cells from the liver, purification of parenchymal cells from the non-parenchymal cells, damaged cells, cell clumps, pieces of connective and vascular tissue and subcellular debris was necessary. This was achieved through a combination

of filtration to remove any aggregates and tissue fragments and differential centrifugation to remove damaged cells, cell fragments and non-parenchymal cells. Purification procedures were performed at approximately 4°C to minimise aggregation, to make the cells metabolically dormant and to minimise cellular damage (Berry et al., 1991). The purification process alters both the yield and viability of the cell preparation obtained, so that the values obtained are a reflection of both the isolation and purification procedure. The filtration and centrifugation steps were the same for both isolation methods however, and would not therefore be expected to contribute to any differences in viability.

The percentage viability, cell yield, amount of liver digested, liver weight and rat weight data for hepatocytes isolated by collagenase and EDTA are shown in Table 3.1 and a summary of this data with a comparison with published data is shown in Table 3.2. A larger number of isolations by collagenase are reported since all the preliminary work was performed in collagenase-isolated hepatocytes. All of the hepatocyte preparations isolated using collagenase met the minimum viability criterion and were used, however some EDTA-isolated hepatocyte preparations were not used due either to low viability or to an insufficient yield of cells for the purpose of a particular experiment. For this reason the summary of data for EDTA-isolated hepatocytes contains values both for total preparations including those which were not used and a second set of values for those cell suspensions which were used.

Hepatocyte viability was greatly affected by the isolation technique used to obtain the cells. The mean initial viability ± standard deviation (SD), as assessed by trypan blue exclusion, were 93% ± 3% (n=25), 52% ± 20% (n=19) and 66% ± 15% (n=13) for collagenase-, EDTA- and utilised EDTA-isolated hepatocyte preparations respectively (Table 3.2). The data was analysed using the Students' *t* test and the results indicated that the collagenase isolation technique was a significantly superior method of isolating viable hepatocytes than EDTA ($P< 0.001$).

A consistently high viability was obtained following collagenase perfusion, despite the fact that, although source of enzyme was consistent throughout these experiments, the activity and purity of the enzyme inevitably varied between batches. The source

and purity of the collagenase used for isolation have been shown to be critical (Queral et al., 1984). This study has frequently been cited to demonstrate the variability associated with the use of collagenase, however the extremely wide range of results obtained in this study (0% to 97%) were not observed throughout the studies presented here where, on the contrary, viability was consistently high. The difficulties experienced in the Queral study may be due to the number of sources of collagenase used and to the mechanical damage introduced by mincing the liver before incubation with the collagenase solution.

The viability both for collagenase- and EDTA-isolated hepatocytes obtained in these studies compare well with data published from a number of other laboratories. The low viability of EDTA-isolated hepatocytes preparations was a common feature of published work and for this reason a Percoll centrifugation step has been used in some laboratories to improve viability (Utesch et al., 1991; Wang et al., 1985; Meredith, 1988). This method, whereby damaged cells have been found to float while intact hepatocytes pellet during gentle centrifugation in an isotonic Percoll solution is one way in which cell viability following EDTA isolation may be improved. However, this may also result in the undesirable isolation of a sub-population of cells of a particular size which does not reflect the characteristics of hepatocytes from the whole liver (Wang et al., 1985; Kreamer et al., 1986). The high variability in the viability of hepatocytes isolated using EDTA has also been observed in other laboratories (Skett, 1994)

Of these studies only Bayad et al. (1991) obtained rat hepatocyte viability following EDTA perfusion to be comparable with that following collagenase perfusion. This was attributed to the inclusion of bovine serum albumin (BSA), protecting the hepatocytes from stress (Berg and Morland, 1975). However, viability was low throughout the current studies despite the inclusion of BSA in the wash buffer. Some differences exist between the Bayad study and the current study, including *in situ* perfusion under anaesthetic, perfusal buffers and culture medium and viability may have been effected by these factors. The EDTA isolation technique has also been reported to result in the isolation of hepatocytes with both high yield and viability in

other species, such as the catfish (Seddon and Prosser, 1999). It would appear that success in the use of EDTA isolation varies widely between laboratories and species.

These data indicate that collagenase isolation yields a population of cells with fewer dead and damaged cells than those obtained using the EDTA isolation technique. The most likely reason for the improved viability associated with the use of collagenase is the minimisation of mechanical damage. Disruption of the liver capsule and release of cells occurred with only a small amount of agitation and very little mechanical force, minimising damage to cells following collagenase perfusion. Conversely, it was observed that the use of EDTA resulted in clumps of poorly separated hepatocytes, and that a much greater degree of mechanical force was required to disperse these cells. It would appear that hepatocytes isolated by EDTA sustain more cellular damage than those isolated by collagenase. Mechanical damage to cells by the EDTA procedure is particularly crucial since parenchymal cells are thought to be very sensitive to shearing stress leading to rupture of the plasma membrane (Berry et al., 1991).

A suspension of hepatocytes containing a high proportion of damaged cells may have increased leakage of enzymes into the culture media. The EDTA-isolated hepatocyte preparations with a low viability and a high proportion of dead and damaged cells may suffer increased exposure to enzymes such as lysosomal proteases during the initial attachment phase. This may have a subsequent impact on the performance of those hepatocytes during culture (Evarts et al., 1984). Attempts to improve viability for EDTA isolation by including pyruvate in the perfusion medium and perfusing for longer time periods to enhance disaggregation were not successful.

Table 3.1 Data for hepatocytes isolated by collagenase and EDTA perfusion.

TECHNIQUE	% VIABILITY	CELL YIELD ($x10^6$/g liver)	LIVER DIGESTED (g)	LIVER WEIGHT (g)	RAT WEIGHT (g)
COLLAGENASE	97	30.5	3.3	ND	ND
COLLAGENASE	99	52.3	2.3	15.7	ND
COLLAGENASE	91	35.9	2.7	17.0	ND
COLLAGENASE	93	35.6	2.7	16.4	ND
COLLAGENASE	95	73.0	1.6	8.9	170
COLLAGENASE	95	56.1	4.6	9.0	169
COLLAGENASE	96	40.8	3.5	13.1	264
COLLAGENASE	98	53.7	4.1	14.5	268
COLLAGENASE	96	81.3	1.6	12.4	226
COLLAGENASE	87	60.0	3.4	10.6	200
COLLAGENASE	90	52.6	2.6	16.2	ND
COLLAGENASE	87	ND	ND	ND	ND
COLLAGENASE	88	59.4	2.0	11.1	227
COLLAGENASE	94	30.2	3.2	12.5	237
COLLAGENASE	92	36.5	3.7	12.3	215
COLLAGENASE	95	72.0	2.5	9.2	184
COLLAGENASE	99	82.9	2.1	13.9	263
COLLAGENASE	92	126.7	1.7	11.2	202
COLLAGENASE	93	115.8	3.7	11.7	223
COLLAGENASE	92	50.0	1.1	11.0	232
COLLAGENASE	93	45.4	1.4	10.2	235
COLLAGENASE	92	105.6	1.0	10.1	218
COLLAGENASE	94	37.3	1.8	10.8	220
COLLAGENASE	90	ND	ND	13.3	253
COLLAGENASE	95	ND	ND	16.5	340
EDTA	41	37.3	2.2	10.4	193
EDTA	91	51.5	1.2	9.9	204
EDTA	91	41.8	2.1	11.1	225
EDTA	57	32.2	1.5	11.2	221
EDTA	73	22.7	1.8	11.5	256
EDTA	59	20.1	1.7	10.1	216
EDTA	57	22.6	1.5	8.9	187
EDTA	68	43.2	1.0	9.8	191
EDTA*	32	15.5	1.5	12.8	295
EDTA*	34	8.9	2.5	15.6	299
EDTA*	18	ND	ND	8.9	197
EDTA	64	ND	ND	12.8	279
EDTA*	38	ND	ND	9.5	211
EDTA	47	ND	ND	9.8	216
EDTA	66	11.0	2.0	12.4	239
EDTA*	24	12.9	1.6	13.4	239
EDTA*	35	9.3	2.7	11.6	240
EDTA**	42	12.0	1.8	11.2	246
EDTA	48	18.5	1.3	11.8	250

* Not used due to low viability

** Not used due to low yield

ND Not Determined

Table 3.2 Summary of data obtained following hepatocyte isolation by collagenase and EDTA perfusion and comparison with published data.

ISOLATION TECHNIQUE	% VIABILITY MEAN±SD	CELL YIELD ($x10^6$/g liver) MEAN±SD	LIVER DIGESTED (g) MEAN±SD	LIVER WEIGHT (g) MEAN±SD	RAT WEIGHT (g) MEAN±SD
COLLAGENASE	93 ± 3	60.6 ± 27.4	2.6 ± 1.0	12.5 ± 2.5	229 ± 39
EDTA (TOTAL)	52 ± 20	24.0 ± 13.4	1.8 ± 0.5	11.2 ± 1.7	232 ± 33
EDTA (USED)	66 ± 15	29.3 ± 13.6	1.6 ± 0.4	11.5 ± 1.1	221 ± 23
COLLAGENASE (Meredith, 1988)	96	31			
COLLAGENASE (Bayad et al., 1991)	92	42.4			
COLLAGENASE (Drochmans et al., 1975)	70				
COLLAGENASE (Gustavsson and Mørland, 1980)	70				
COLLAGENASE (Wright and Paine, 1992)	85				
COLLAGENASE (Brendler-Schwaab, 1994)	86	70-90			
EDTA (Bayad et al., 1991)	92	37.1			
EDTA (Berry et al., 1983)	50				
EDTA (Meredith, 1988)	72 100*	28 20			
EDTA (Wright and Paine, 1992)	50-70				
EDTA (Wang et al., 1985)	50 (95*)	17.6*			

* Following Percoll separation

3.1.4 Hepatocyte Yield

Accurate determination of the number of hepatocytes isolated enables standardisation of the number of viable cells seeded onto culture dishes in different experiments. This is important since the density of seeding has been shown to affect gene expression, with more densely seeded cells retaining their differentiated status better than less densely seeded cells (Greuet et al., 1997). The number of cells was determined throughout these studies using a haemocytometer cell counting chamber.

The technique used during these studies was to mount the cover slip first and use capillary force to draw cells into the chamber. The alternative, placing a drop of cell suspension on the chamber and then placing the cover slip over the suspension, has been reported to selectively squeeze damaged cells to the periphery of the chamber resulting in inaccurate assessment of viability (Seglen, 1976). Complete mixing of the hepatocyte suspension and trypan blue, followed by rapid counting of cells, was found to be essential throughout these studies since hepatocytes were found to sediment very rapidly, resulting in inaccurate counting. Cell aggregation was also found to be an important factor when establishing an accurate cell count, since this made counting more difficult. This was a problem encountered with EDTA-isolated rather than collagenase-isolated cells. Two chambers were counted for each test and at least two tests performed to ensure that the cell count was as accurate as possible.

Alternative counting methods are available, for example electronic counting using the Coulter counter. This offers a rapid, more accurate measurement but cannot distinguish between viable and non-viable cells and is expensive. Other methods such as cell weight are considered to be inaccurate due to the variable amount of intracellular liquid. DNA determination may also be used but takes longer to determine and due to the polyploid nature of some hepatocytes may not be accurate. The determination of cell number using a haemocytometer was chosen to assess cell number because it was rapid, cheap, simple and, when used consistently, accurate.

The mean viable cell yield (± SD) was 60.6 (± 27.4), 24.0 (± 13.4) and 29.3 (± 13.6) $x10^6$ viable cells/g liver for collagenase-, EDTA- and utilised EDTA-isolated cells

respectively. The Students' *t* test was used to analyse these data. This indicated that the collagenase isolation technique resulted in a statistically significantly greater number of viable cells for each gram of liver digested than the EDTA method for both total EDTA preparations ($P<0.001$) and for those which were used in incubations ($P<0.02$). This could be due, in part, to the much larger amount of cell debris produced by EDTA isolation of hepatocytes. This was minimised by retaining as much of the cell debris as possible so that the amount of liver digested by EDTA was not overestimated. It is more likely that the greater mechanical force used to liberate hepatocytes when using EDTA caused a greater degree of plasma membrane shearing with the resulting loss of the hepatocyte throughout the centrifugation steps that is the cause of the difference in cell yield.

The amount of liver digested by each technique was also analysed using the Students' *t* test. This suggested that the amount of tissue digested with collagenase was significantly higher than that digested with EDTA for both total preparations and for those which were subsequently used ($P<0.02$). This was not unexpected since the use of collagenase resulted in the perfusion of a greater area of liver and almost complete liberation of hepatocytes with very little cell debris. In contrast, a smaller area of liver was perfused using EDTA, and liberation of hepatocytes was difficult with the production of large amounts of cell debris and clumps of cells and tissue.

A Students *t* test confirmed that there were no significant differences between isolation techniques in either the weight of rat used or in the weight of the liver obtained.

3.2 HEPATOCYTE CULTURE

A number of diverse substrata were selected to provide a surface for hepatocyte attachment during the culture period. The substrata selected were Matrigel®, fibronectin, Vitrogen® and untreated plastic.

Matrigel® is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins. The components of Matrigel® include laminin, collagen IV, heparan sulphate proteoglycans, enactin and nidogen (Kleinman et al., 1986). The major component of Matrigel® is laminin, which is specifically found in the periportal area of the liver lobule (Saad et al., 1993). Matrigel® also contains fibroblast growth factor β, tissue plasminogen activator and other growth factors including those that occur naturally in the EHS tumour (Dirami et al., 1995). Matrigel® thus contains a complex of the components of the extracellular matrix, in contrast with other popular attachment factors which are composed of a single factor. The advantage of this substratum, therefore, lies in its multi-component complexity. However, Matrigel® is not a completely defined and controlled medium, and derives from a tumour making the chemistry potentially both abnormal and unstable. To combat this, commercially available Matrigel® is routinely tested to ensure that the concentration of various components lies within set limits.

The fibronectins are high molecular weight multifunctional glycoproteins composed of large sub-units linked by disulphide bonds which have been used as substrata in cell culture (Marceau et al, 1982; Rubin et al., 1981). They are found on cell surfaces, in body fluids such as plasma, in soft connective tissue matrices in most basement membranes and throughout the liver lobule (Saad et al., 1993). Fibronectins apparently function as adhesive molecules although the full range of their biological functions is still under investigation (Martinez-Hernandez, 1993).

Collagen is one of the most popular substrata used for cell culture and Vitrogen®100, a commercially available collagen type I, was used in these studies. Since collagen can detach from plastic a technique to covalently link the collagen using the cross-linking reagent carbodiimide was utilised (Macklis et al., 1985). Collagen type I is found in the perivenous area of the hepatic lobule (Saad et al., 1993)

Polystyrene dishes have been used successfully in hepatocyte culture and provide the simplest substrata for cell attachment. The surface of the dish is flat, allowing a

uniform and reproducible culture to form. The polystyrene surface is usually hydrophobic which is unsuitable for cell growth, and so it is treated either with gamma radiation, chemicals, or an electric arc, to provide a charged surface that is then wettable. Dishes manufactured from other plastics such as polycarbonate are also available commercially. Permeable substrata such as the cellophane sandwich have been used and hepatocytes shown to have improved survival, possibly due to the higher oxygen tension (Sandstrom, 1965). However, the polystyrene surface has none of the proteins normally found in the extracellular matrix surrounding the hepatocyte *in vivo*, and as such must be regarded as a very synthetic surface for adhesion.

During preliminary work poly-D-lysine (0.1mg/mL) was also examined, but hepatocytes showed poor attachment to this particular substrata and it was not subsequently used.

The effect of these substrata on cytochrome P450 expression in hepatocytes isolated using collagenase and EDTA, and subsequently maintained in culture for up to 96 hours, was determined.

3.2.1 Cell Attachment

Hepatocytes attached successfully to all substrata in both small and large culture dishes, with the exception of the large (10cm) untreated plastic dishes where attachment was poor. The reason for the poor attachment is not known, but was not limited to a particular batch of dishes. Therefore, hepatocytes cultured for the preparation of microsomes on untreated plastic had to be cultured on small (35mm) dishes.

Hepatocytes were seen to attach to all substrata within one to two hours. Confluent monolayers formed with the typical pavement appearance associated with hepatocytes in culture (Rogiers and Vercruysse, 1993) and were maintained throughout the 96 hour culture period on fibronectin, Vitrogen® and untreated plastic. The appearance of hepatocytes maintained on Matrigel® was unusual in that the cells retained a rounded shape and were seen to form aggregates, or cords, which were visible to the

naked eye after a 96 hour culture period. However, despite the apparent gaps between the hepatocytes, attempts to seed greater numbers of hepatocytes on this substrata were not successful. The unusual appearance of cells maintained on Matrigel® has also been described by other groups using both Matrigel® and other gels (Bissell et al., 1987; Awata et al., 1998; Ben-Ze'ev et al., 1988; Brown et al., 1995; Moghe et al., 1996).

The effect of Matrigel® on hepatocyte shape is interesting since cell shape has been associated with differential gene expression. The composition of the extracellular matrix has been proposed to effect gene expression via the number or distribution of receptors for matrix proteins in the plasma membrane (Bucher et al., 1990). This is believed to influence the interaction of polymerised actin with the membrane, resulting in a change in cell shape, and hence the synthesis, assembly and organisation of other cytoskeletal proteins. This, in turn, may effect gene expression either via transcription or translation. The effect of isolation and substrata on hepatocyte shape may, therefore, be a factor in the change in differentiated function of cultured hepatocytes. Previous studies have shown that hepatocytes cultured on Matrigel® differentially regulate the expression of major cytoskeletal genes including actin and tubulin and other liver specific genes such as albumin (Ben-Ze'ev, 1988).

The attachment of viable hepatocytes to the substrata was measured by allowing hepatocytes to attach for two hours before the culture media was removed and the monolayers washed with phosphate buffered saline. The numbers of viable and non-viable cells that had not attached were determined by analysis of the combined media and wash. The results are shown in Table 3.3. These data were obtained from collagenase-isolated hepatocytes, since the large amount of cell fragments and non-viable cells made this procedure unacceptably inaccurate for EDTA-isolated hepatocyte suspensions.

The very low number of cells present in the wash indicated that attachment of hepatocytes was very good to all substrata but made the accurate estimation of unattached cells difficult. The mean attachment of viable hepatocytes was 94%

(n=16) with a similar level of attachment to all substrata. The calculation of viable cell attachment was based on the assumption that all viable cells not present in the wash had attached. The mean attachment of hepatocytes, both viable and non-viable, was 80% (n=16) of total cells, again with a similar level of attachment for all substrata. This data was based on the assumption that all cells not in the wash had attached rather than having lysed and the appearance of confluent monolayers would support this assumption. The lower attachment value for total cells is probably due to the lack of attachment of non-viable cells. This plating efficiency is comparable to that obtained elsewhere (te Velde et al., 1995; Rojkind et al., 1980)

Table 3.3 Attachment of hepatocytes to culture dishes.

SUBSTRATA	% CELLS ATTACHED (N=1)	% CELLS ATTACHED (N=2)	% CELLS ATTACHED (N=3)	% CELLS ATTACHED (N=4)	MEAN ± SD
MATRIGEL®					
Viable Cells	94	98	94	95	94.5 ± 0.6
Total Cells	70	85	81	82	79.5 ± 6.6
FIBRONECTIN					
Viable Cells	97	95	100	95	97 ± 2
Total Cells	70	83	98	74	81 ± 12
VITROGEN®					
Viable Cells	97	90	96	90	93 ± 4
Total Cells	54	79	96	82	78 ± 18
PLASTIC					
Viable Cells	94	95	89	90	93 ± 4
Total Cells	73	83	72	65	73 ± 7.
ALL SUBSTRATA					
Viable Cells	96	95	95	93	94 ± 3
Total Cells	67	83	87	76	80 ± 11

3.2.2 Cytochrome P450 in Cultured Hepatocytes

Total cytochrome P450 concentrations in cultured hepatocytes attached to Matrigel®, fibronectin and untreated plastic were determined following solubilisation of hepatocytes in Renex buffer. A typical carbon-monoxide absorption spectra is shown in Figure 3.1

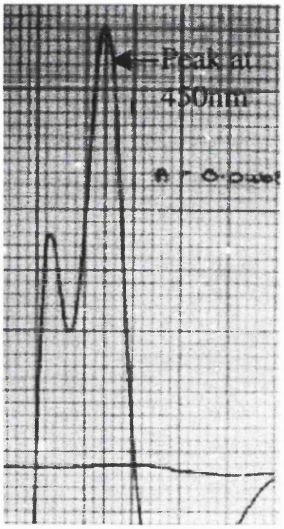


Figure 3.1 Cytochrome P450 carbon-monoxide binding spectrum from cultured hepatocytes.

The cytochrome P450 levels present in collagenase-isolated hepatocytes after the two-hour attachment period was determined (Table 3.4). The mean amount of cytochrome P450 present was 288pmol/ml (n=15) with the mean value for each substrata being similar to this value. This is in keeping with published data (Begue et al., 1984; Paine, 1990). Cytochrome P450 is generally expressed in the units pmol/mg microsomal protein, however, the cytochrome P450 was determined following solubilisation of hepatocytes in Renex buffer and, therefore, protein could not be determined in these samples. No data was obtained for Vitrogen® since this

substratum had not been selected at the time these experiments were carried out. The appearance of a peak at 420nm in the spectrum signifies the presence of partially degraded cytochrome P450 which is inactive (Taniguchi et al., 1985; Grant et al., 1985) and of cytochrome b_5 (McLean and Day, 1973). Although this data indicated the presence of cytochrome P450 in the rat hepatocytes the measurement of cytochrome P450 using the carbon-monoxide binding spectrum is not particularly sensitive and does not necessarily reflect active enzyme (Turner and Pitot, 1989).

Table 3.4 Cytochrome P450 levels in collagenase isolated hepatocytes post-attachment.

SUBSTRATA	P450 PMOL/ML N=1	P450 PMOL/ML N=2	P450 PMOL/ML N=3	P450 PMOL/ML N=4	P450 PMOL/ML N=5	MEAN PMOL/ ML
MATRIGEL®	133	280	499	176	492	316
FIBRONECTIN	122	236	365	162	479	273
PLASTIC	178	272	366	157	396	274
COMBINED						288

3.2.3 Culture Conditions

Hepatocytes isolated using both collagenase and EDTA were subsequently cultured for up to 96 hours on Matrigel®, fibronectin, Vitrogen® and untreated plastic as described in Chapter 2. The selection of optimal media and media supplements was difficult due to the enormous variation in results between different laboratories that have published their work, and the often poorly characterised effects of media additives (Skett, 1994). Williams' Media E was chosen because it has been shown to perform at least as well as, and often better than, many other media with respect to hepatocyte function (Grant et al., 1986; Miyazaki et al., 1990), although Chees media has also been reported to perform well (Waxman et al., 1990; Jauregui et al., 1986). For the purposes of these studies a serum-free system was adopted since, in addition to being undefined, complex, and subject to batch to batch variation (Bjare, 1992) the

presence of serum has been reported to decrease cytochrome P450 activity (Hammond and Fry, 1992).

The choice of supplements was also difficult since, despite claims that many additives have positive effects on hepatocyte function, results obtained by different laboratories have varied widely. It was decided to keep the system as simple as possible while retaining supplements extensively used in hepatocyte culture, or for which positive effects had been well documented. Therefore insulin (Dich et al., 1988), transferrin (Laskey et al., 1988), zinc sulphate (Gatmaitan et al., 1983) and the haem precursor δ-aminolaevulininc acid were included in the media. The antibiotics penicillin and streptomycin were also included to minimise contamination by bacteria and viruses which may trigger the release of cytokines which reduce cytochrome P450 activity (Calleja et al., 1998; Morgan et al., 1994). Many workers also add dexamethasone or other glucocorticoids. However, glucocorticoids were not used throughout these studies due to the reported inductive/suppressive effects on certain cytochrome P450 enzymes including CYP3A and 2C11 (Schuetz et al., 1984; Iber et al., 1997; Silva et al., 1998; Madan et al., 1999; Edwards et al., 1992).

3.3 TESTOSTERONE METABOLISM

The substrate probe testosterone (Figure 3.2) has proved to be a useful indicator of the activity of a number of cytochrome P450 isoforms (Wood et al., 1983) and has been used to assess the activity of these enzymes in cultured rat hepatocytes (Vind et al., 1989). The regio- and stereo- specific hydroxylation of testosterone makes it an ideal probe substrate for studying the activity of several cytochrome P450 isoforms simultaneously (Waxman et al., 1983; Sonderfan et al., 1987). The specificity of product formation by different P450 enzymes, determined using purified enzymes and specific chemical and immunological inhibitors, is shown in Table 3.5. The differences in catalytic specificity are thought to be due to variation in the primary sequence of the enzymes influencing the juxtaposition of the substrate (Guengerich and Macdonald, 1990) and to the ease with which various groups on a molecule undergo oxidation (Smith and Jones, 1992).

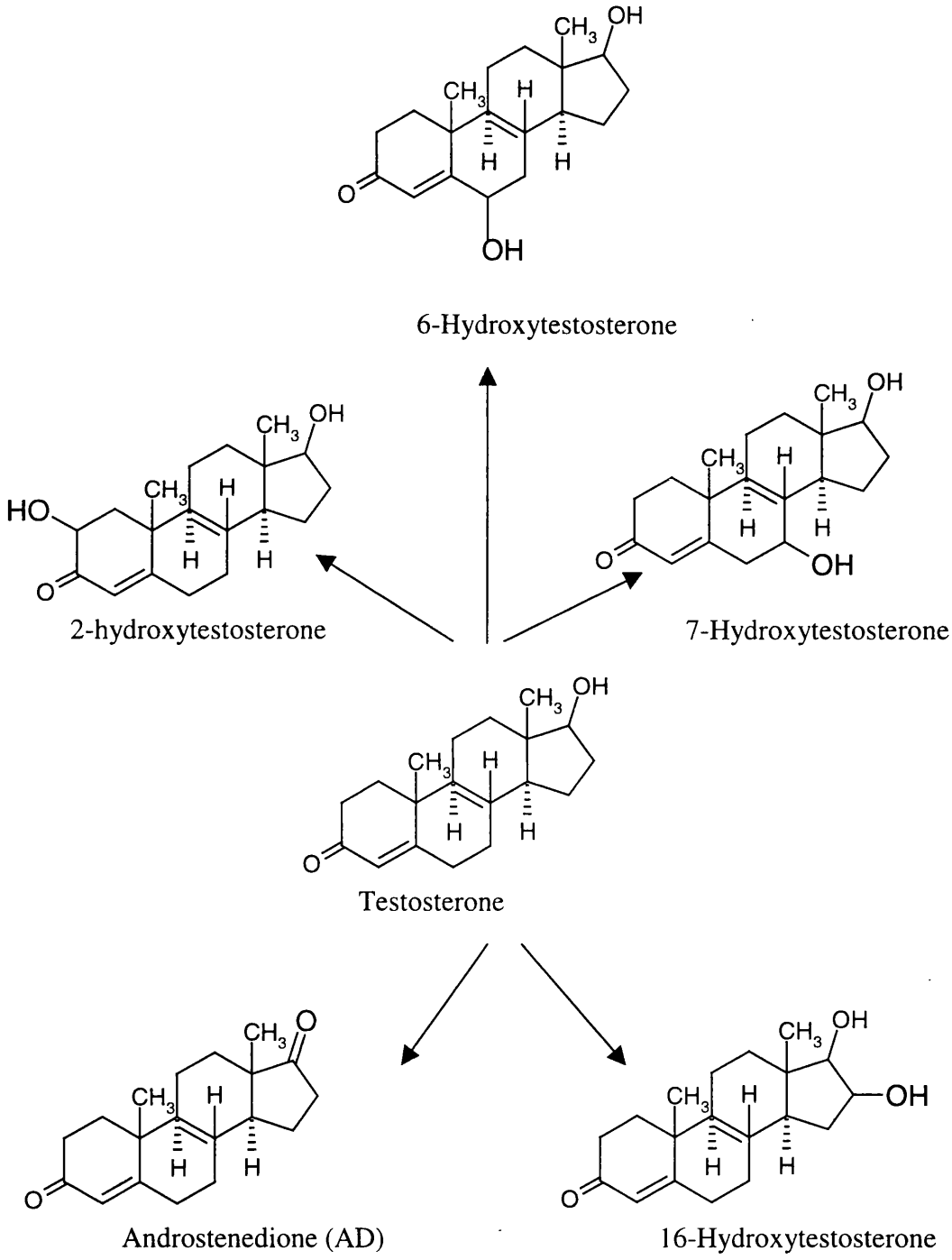


Figure 3.2 The structures of the substrate probe testosterone and major metabolites.

Table 3.5. Cytochrome P450 specific metabolism of testosterone.

TESTOSTERONE METABOLITE	CYTOCHROME P450	REFERENCE
2α	**2C11**	Waxman et al.,1984
6β	**3A1/2**, 2C13, 1A1, 1A2	Waxman et al., 1985 Wood et al., 1983
7α	**2A1**	Levin et al., 1987 Wood et al., 1983
16α	**2C11**, **2B1**,2B2, 2C7	Waxman et al., 1984
16β	**2B1**, 2B2	Reik et al., 1985 Wood et al., 1983
AD*	**2B1**, 2C11, 2B2	Sonderfan et al., 1987

(Major cytochrome P450 enzyme in bold type)

*AD is also produced by 17β-hydroxysteroid dehydrogenase (Waxman, 1988; Biswas and Russell, 1997; Bartlomowicz et al., 1989b) an enzyme which can also catalyse the reverse reaction (Martel et al., 1992).

The metabolism of ^{14}C-testosterone by hepatocytes isolated using collagenase and EDTA, and maintained on Matrigel®, fibronectin, Vitrogen® and untreated plastic, was determined following incubation for one hour as described by Vind et al., (1989). Following termination of the reactions testosterone and metabolites were isolated using solid phase extraction, with analysis by TLC and HPLC.

3.3.1 Solid Phase Extraction

The solid phase extraction method used was as described by Darby et al., 1986. The recovery of testosterone and metabolites by this method was determined in samples obtained following incubation with hepatocytes maintained for up to 7 days on Matrigel®, fibronectin, and untreated plastic, with testosterone for one hour. A small aliquot of each sample was also analysed using TLC and autoradiography to ensure

that metabolism of testosterone had occurred. The results are shown in Table 3.6. The multiple radioactive entities detected by autoradiography indicated that metabolism had taken place and that the subsequent determination of extraction related to both parent compound and metabolites.

The recovery of both testosterone and metabolites appears to be almost complete in most cases. The overall mean recovery ± SD was 105% ± 17 which is comparable with published data of 93% ± 4% (Ballard et al., 1988) and there were no apparent differences between hepatocytes maintained on different substrata and harvested at different times. In one sample, from hepatocytes maintained on Matrigel® immediately post-attachment, recovery was poor (31%). The value for all three aliquots of the eluate was similar discounting a problem with the scintillation counter or a pipette error. The duplicate sample had a recovery of 103% and it is likely therefore, that the low value obtained for the first sample was due to a fault in the extraction cartridge.

In some cases a recovery exceeding 100% was obtained, which may have been due to some evaporation of the methanol and consequent concentration of the eluate sample. Samples were analysed as rapidly as possible following extraction to minimise this effect. Overall these results indicate that the extraction procedure results in recovery of most of the radiolabelled material. It is possible, however, that some metabolites formed in small amounts were not successfully extracted.

Table 3.6 Recovery of radioactive testosterone and metabolites by solid phase extraction.

SUBSTRATA	TIME IN CULTURE (HOURS)	DPM (PRE-EXTRACTION)	DPM (POST-EXTRACTION)	% RECOVERY
MATRIGEL®	0	4568	1413	31
MATRIGEL®	0	4459	4612	103
MATRIGEL®	24	7441	7213	97
MATRIGEL®	24	6423	7411	115
MATRIGEL®	48	4823	4879	101
MATRIGEL®	48	4297	3980	93
MATRIGEL®	72	4843	4839	100
MATRIGEL®	72	4515	4173	92
MATRIGEL®	96	4892	5220	107
MATRIGEL®	96	4839	4832	100
MATRIGEL®	120	4471	4324	97
MATRIGEL®	120	4242	3339	79
MATRIGEL®	144	4164	4864	117
MATRIGEL®	144	3553	4183	118
FIBRONECTIN	0	5106	4644	91
FIBRONECTIN	0	6431	6032	94
FIBRONECTIN	24	5814	6828	117
FIBRONECTIN	24	6606	6460	98
FIBRONECTIN	48	4291	5632	131
FIBRONECTIN	48	4751	5289	111
FIBRONECTIN	72	3565	3823	107
FIBRONECTIN	72	3241	4061	125
FIBRONECTIN	96	4379	4460	102
FIBRONECTIN	96	3109	3851	124
FIBRONECTIN	120	13149	13059	99
FIBRONECTIN	120	3564	4452	125
FIBRONECTIN	144	4094	5500	134
FIBRONECTIN	144	3650	4684	128
PLASTIC	0	768	803	105
PLASTIC	0	462	519	112
PLASTIC	24	3484	2843	82
PLASTIC	24	4446	4695	106
PLASTIC	48	3102	2936	95
PLASTIC	48	2886	2901	101
PLASTIC	72	2561	2836	111
PLASTIC	72	2489	2621	105
PLASTIC	96	3699	3844	104
PLASTIC	96	2793	3229	116
PLASTIC	120	3463	3848	111
PLASTIC	120	2386	2875	120
PLASTIC	144	3214	3697	115
PLASTIC	144	3541	3733	105

3.3.2 Thin Layer Chromatography

During preliminary investigations the testosterone and metabolites present within hepatocytes and in the media overlying the hepatocytes, was extracted and analysed by TLC and autoradiography. An example of an autoradiograph obtained from these samples is shown in Figure 3.3. Radioactive testosterone and metabolites were present in both the intracellular and extracellular matrices, with the majority of radioactive moieties present in the culture media. This suggests that metabolites were secreted into the overlying media by the hepatocytes and in subsequent experiments both hepatocytes and media were collected for analysis and pooled. This data compares well with published data (Imanidis et al., 1996) which also shows that testosterone and metabolites did not accumulate but were effluxed back out of the cell.

A number of radioactive metabolites were identified by co-chromatography with authentic standards. However, due to the limited number of testosterone metabolite standards available at the time this work was carried out it was not possible to identify all metabolites. Other potential metabolites include 6α-, 7β-, 11α-, 11β-, 14α-, 15α-, 18α- and 19α- hydroxy testosterone (Darby et al., 1986). The levels of metabolites were found to decline throughout the 96 hour culture period, although one metabolite (M1) not detectable in samples obtained from hepatocytes immediately post-attachment was found in samples from later timepoints, and also as a slight impurity in the testosterone standard (lane 8). The identity of this metabolite is unknown.

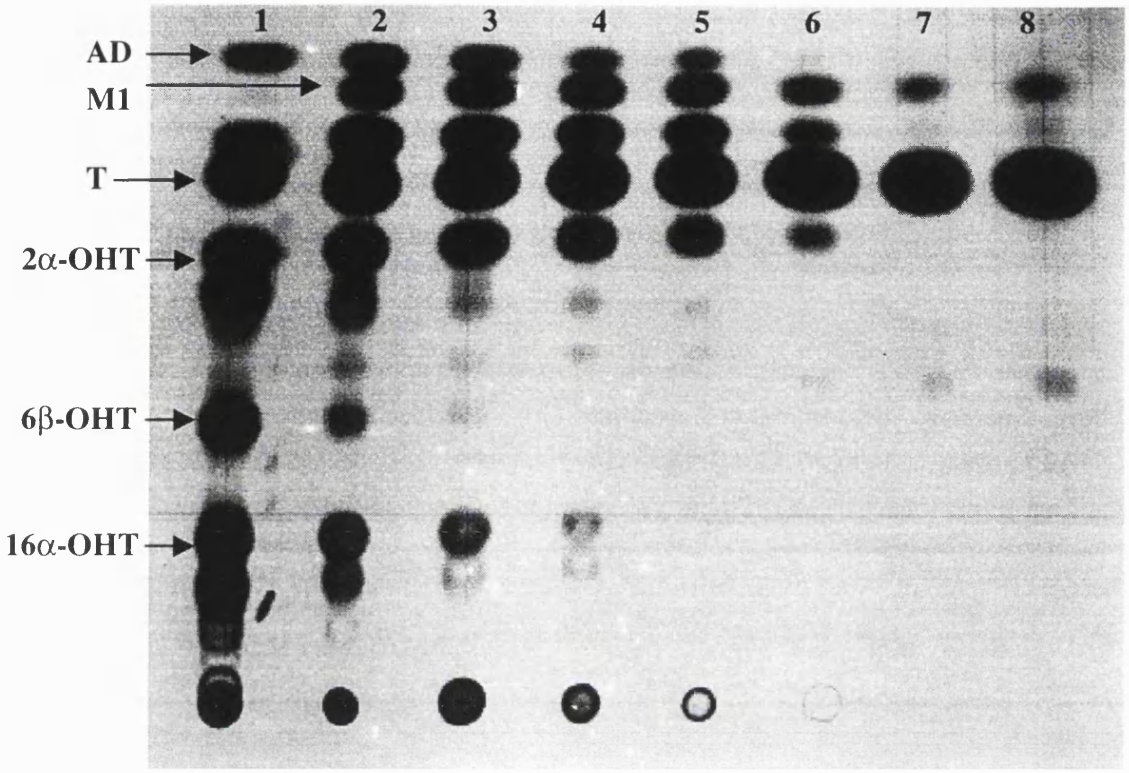


Figure 3.3 Autoradiograph showing separation of testosterone, hydroxy testosterone (OHT) metabolites and androstenedione. Samples were produced by hepatocytes cultured on fibronectin for 0, 24, 48, 72, 96, 120 and 144 hours (lanes 1 to 7 respectively). The testosterone solution added to the culture media is shown in lane 8.

3.3.3 Deconjugation

To determine whether any conjugates of testosterone metabolites had been formed a number of samples were divided into two and half treated with the deconjugating enzymes sulphatase and glucuronidase. Typical autoradiographs of control and deconjugated samples are shown in Figure 3.4. These indicated that there were no obvious differences between the metabolites in samples incubated with glucuronidase and sulphatase and those untreated samples. Conjugated metabolites would be expected to have a different retention time to the primary metabolite due to increased polarity.

It is unlikely that sulphate and glucuronide conjugation of testosterone metabolites has occurred in detectable amounts and treatment with deconjugating enzymes was not carried out in subsequent experiments. It should be noted however, that the deconjugating enzymes used in these studies do not cover all possible conjugates. The detection of testosterone-related conjugates has not been reported by other *in vitro* studies (Utesch et al., 1991; Vind et al., 1989; Wortleboer et al., 1990) although at least one report of glucuronides has been made (Guillemette et al., 1996) and in other species such as the monkey various hydroxy testosterone molecules are substrates for glucuronosyltransferase (Bélanger et al., 1999). Conjugates have also been observed when using human liver slices however this may be attributed due to the extensive incubation time used (Baumann et al., 1999).

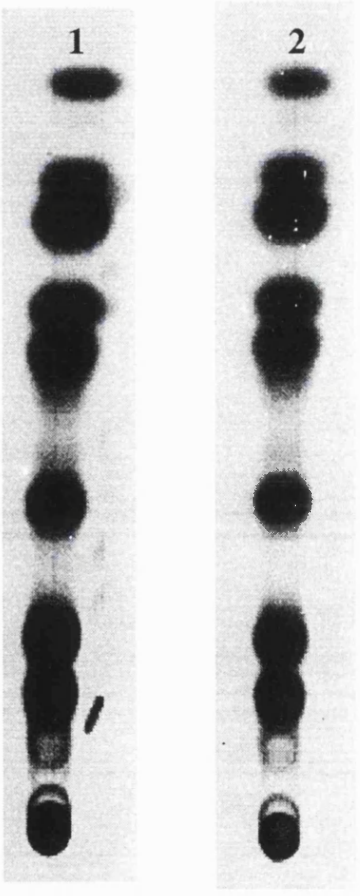


Figure 3.4 Autoradiographs of untreated (lane 1) and deconjugated (lane 2) samples from hepatocytes incubated with ^{14}C-testosterone immediately post-attachment.

3.3.4 High Performance Liquid Chromatography

In preliminary experiments reverse phase thin layer chromatography was used to separate metabolites spatially, with detection of radioactive moieties by radiometric scanning and autoradiography. Although good separation of several metabolites was observed on the autoradiographs, it was not possible to get clear enough resolution of peaks using the Isomess 300 radio-TLC scanner to allow accurate peak analysis (Figure 3.5). Therefore an HPLC (high performance liquid chromatography) system was developed to analyse samples.

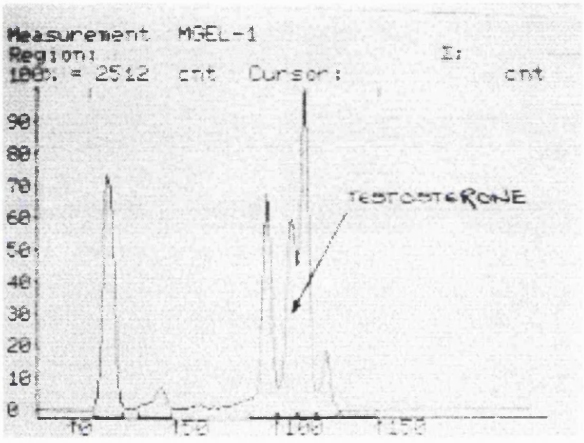


Figure 3.5 Typical scan produced by the Isomess 300 radio-TLC scanner.

The separation of testosterone and metabolites by the gradient HPLC system developed is shown in Figure 3.6. Although the exact retention times varied slightly within a run, and between runs, the approximate retention times were 13.5, 15.2, 17.4, 20.7, 23.0, 28.1 and 32.4 minutes for 7α-, 6β-, 16α-, 16β-, 2α-hydroxy testosterone, androstenedione and testosterone respectively. The metabolite (M1) identified by TLC that was found to increase between 0 and 24 hours was not detected using the HPLC system. It is possible that this metabolite was retained on the column and eluted after data collection for each injection was halted. Although the recovery of radiolabelled material from the HPLC column was determined to be greater than 95%,

it is possible that the small amount of radiolabel that was not accounted for was due to retained material rather than experimental error. The peak was also identified as a small impurity in the radiolabelled testosterone supplied, but was not seen in HPLC chromatographs of the standard, indicating that the entity was not co-chromatographing with other metabolites or eluting very early, but may have co-chromatographed with testosterone.

The 2α– and 2β– hydroxy metabolites could not be resolved by the HPLC system used. However, since the amount of 2α–hydroxy metabolite produced is approximately 10-fold that of 2β–hydroxy in the mature male rat (Wood et al., 1983; Sonderfan et al., 1987; Utesch et al., 1992) the peak may be considered to be a reflection of the amount of 2α–hydroxy metabolite. This was also supported by data from TLC analysis, where the 2α–hydroxy and the 2β–hydroxy metabolites do separate and a radioactive metabolite co-chromatographing with the 2α–hydroxy but not the 2β–hydroxy testosterone metabolite was detected. Therefore, no further HPLC method development to resolve these two metabolites was carried out.

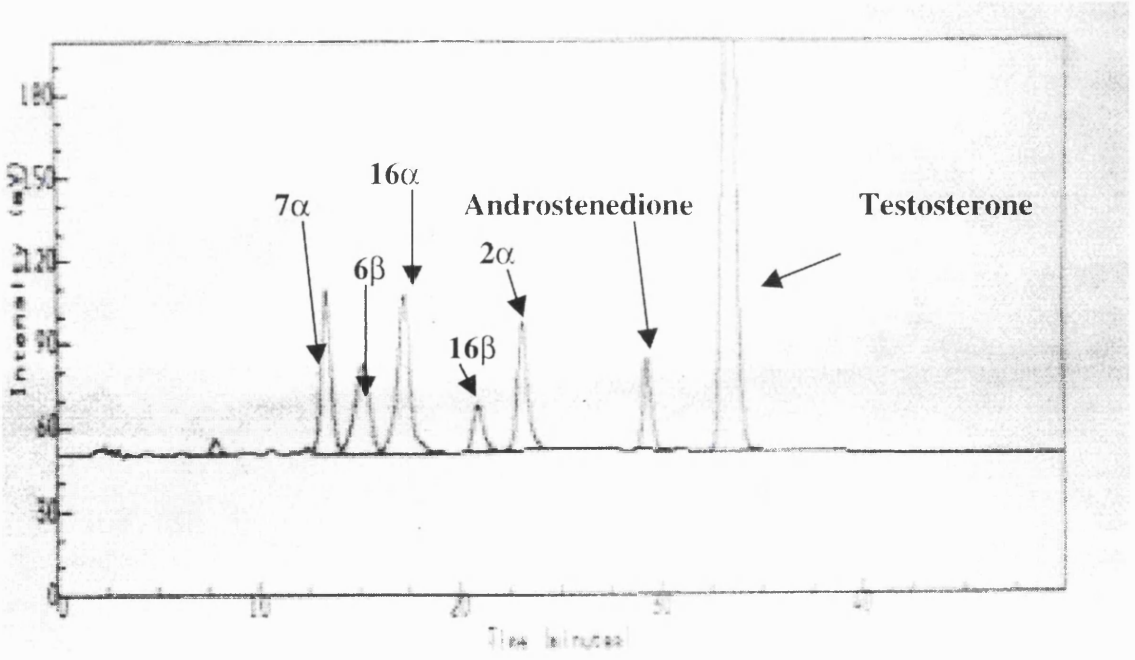


Figure 3.6 An HPLC chromatograph showing separation of testosterone and metabolites.

The metabolism of testosterone to specific metabolites by hepatocytes maintained in culture for 0, 24, 48, 72 and 96 hours was used to measure the activity of a number of cytochrome P450 enzymes. Six separate preparations of rat hepatocytes were isolated using both collagenase and EDTA based techniques, with hepatocytes subsequently cultured on the four substrata selected. Testosterone metabolites were identified by co-chromatography with authentic standards. The five major metabolites detected by HPLC, 2α–hydroxy, 6β–hydroxy, 7α–hydroxy, 16α– hydroxy, and androstenedione, are in agreement with published data (Sonderfan et al., 1987, Gemzik et al., 1990, van der Hoeven, 1981 and Wortelboer et al., 1991).

The relative abundance of metabolites in samples obtained immediately post-attachment was, in descending order, 16α-OHT, androstenedione, 6β-OHT, 2α-OHT, 7α-OHT and 16β-OHT in accordance with previous work (van der Hoeven, 1981). However, both isolation technique and substrata effected the relative amount of each metabolite produced so that in EDTA-isolated hepatocytes the 2α-OHT metabolite was more abundant than the 6β-OHT metabolite which has also been previously reported (Utesch et al., 1991).

The relative abundance of each metabolite varies between different laboratories and although no major rat strain-dependant effects on the regio- and stereo-selectivity of testosterone hydroxylation have been reported, the rate of hydroxylation does vary between strains (Koster et al., 1989; Sonderfan et al., 1987). The incubation conditions chosen may also be of importance since it has been demonstrated that cytochrome P450 activity is differentially effected by the choice, ionic strength and pH of buffer (Gemzik et al., 1990), by the choice of solvent in which the substrate is added to the incubation medium (Busby et al., 1999; Chauret et al., 1998), and also by the concentration of substrate used. Methanol had the least effect on cytochrome P450 activity, and this was the solvent used throughout these studies. Certainly interlaboratory comparison of cytochrome P450 activity has shown that even with the same microsomes results can vary enormously depending on the conditions used (Rutten et al., 1992).

The age of the rat used has also been shown to effect testosterone metabolism (Imaoka et al., 1991). The minor variations in metabolite abundance between different laboratories is likely, therefore, to be a result of differences in rat strain and age, incubation conditions used and analytical technique.

Conjugates of testosterone and metabolites were not apparent and there was no evidence of secondary metabolism of primary metabolites. The absence of secondary metabolites found in these studies is similar to other *in vitro* studies, although androstenedione is itself a substrate for the cytochrome P450 enzymes (Waxman et al., 1983) and monohydroxylated metabolites of testosterone can be further hydroxylated (Lim et al., 1992). It is possible that low levels of secondary metabolites may have been produced which are below the limit of detection, although the relative excess concentration of testosterone compared to that of primary metabolites would minimise this. It is also possible that the solid phase extraction does not retain these metabolites due to increased polarity. However, this would be expected to result in a decrease in recovery of radioactivity from early samples where production of primary metabolites was high, and this was not observed.

The results of this study, considered in detail below, show that a marked heterogeneity in the rates at which individual P450 isoforms are lost from cultured hepatocytes exists. This has been found in other laboratories where the conditions of hepatocyte culture have been shown to have a quite dramatic effect of cytochrome P450 levels (Steward et al., 1985; Blankson et al., 1991). However, all cytochrome P450 activity monitored was found to decrease, probably due to a combination of enhanced degradation and reduced synthesis (Paine and Villa, 1980) and a marked decline in mRNA which has been reported to occur within the initial 24 hours of culture (Padgham et al., 1994). The decline in each metabolite is considered below.

3.3.5 2α-Hydroxy Testosterone

The testosterone 2α-hydroxylase activity in hepatocytes isolated using collagenase and EDTA and cultured on Matrigel®, fibronectin, Vitrogen® and untreated plastic is shown in Figure 3.7. Data combined on the basis of substrata or isolation technique are shown in Figures 3.8 and 3.9. The data are summarised in Table 3.7. Data from individual experiments (n=6) are given in the appendix.

The testosterone 2α-hydroxylase activity in all cultured hepatocytes was seen to drop dramatically throughout the 96-hour culture period, to between 0 and 18% of initial activity. The largest decrease in testosterone 2α-hydroxylase activity, ranging from 37 to 56%, occurred during the first 24 hours for collagenase-isolated cells cultured on all substrata. In contrast, for EDTA-isolated cells the decline in activity was slower, with the decline in activity during the first 24 hours ranging from 6 to 29%. Activity was particularly well maintained in EDTA-isolated cells cultured on all substrata except plastic at 24 and 48 hours post-attachment, although was similar to collagenase-isolated cells at 72 and 96 hours post-attachment. In a small number of samples a slight increase was observed in the 2α-hydroxylase activity on consecutive days at the later timepoints.

Activity was very low in all samples and below the level of detection in many hepatocyte cultures, following a culture period of 96 hours. The low 2α-hydroxylase activity indicates that the level of active CYP2C11, the major enzyme responsible for 2α-hydroxylase activity, has greatly declined during the 96-hour culture period.

The analysis of variance indicates that both the isolation technique and the substrata used have resulted in significant differences in testosterone 2α-hydroxylase activity. Significant differences were observed due to the substrata used at 96 hours post-attachment ($P=0.0425$) while at 48 hours post-attachment the test statistic was close to borderline ($P=0.065$). The 2α-hydroxylase activity was lower in hepatocytes maintained on untreated plastic than those maintained on other substrata at these timepoints. No significant differences were apparent in hepatocytes immediately

post-attachment, at 24 hours, or 72 hours post-attachment with respect to the substrata used.

Significant differences in testosterone 2α-hydroxylase activity were also observed due to the isolation technique used after 24 and 48 hours of culture (P=0.003 and P=0.002 respectively). The 2α-hydroxylase activity was higher in hepatocytes isolated using EDTA compared to those that had been isolated using collagenase at these times. No significant differences were apparent due to isolation technique immediately following attachment, at 72 hours, or at 96 hours post-attachment. There was no significant interaction between isolation and substrata at any of the timepoints examined.

Table 3.7 Summary of testosterone 2α-hydroxylase activity in cultured hepatocytes.

SUBSTRATA / ISOLATION METHOD	0 HOURS	24 HOURS	48 HOURS	72 HOURS	96 HOURS
MATRIGEL®/COLLAGENASE (N=6)	6.4 ± 2.1 (100)	4.0 ± 1.2 (63)	1.4 ± 1.2 (22)	1.2 ± 0.7 (19)	0.6 ± 0.5 (9)
MATRIGEL®/EDTA (N=6)	7.4 ± 2.1 (100)	6.4 ± 2.5 (86)	3.5 ± 1.3 (47)	2.1 ± 0.8 (28)	1.3 ± 1.1 (18)
FIBRONECTIN/COLLAGENASE (N=6)	8.3 ± 1.1 (100)	4.0 ± 1.4 (48)	1.6 ± 1.4 (19)	1.5 ± 1.0 (18)	0.8 ± 0.5 (10)
FIBRONECTIN/EDTA (N=6)	8.0 ± 2.6 (100)	6.4 ± 2.3 (80)	3.7 ± 1.3 (46)	1.6 ± 0.8 (20)	0.2 ± 0.3 (3)
VITROGEN®/COLLAGENASE (N=6)	7.8 ± 1.4 (100)	4.1 ± 1.5 (53)	1.7 ± 1.5 (22)	1.3 ± 1.1 (17)	0.6 ± 0.5 (8)
VITROGEN®/EDTA (N=6)	6.4 ± 3.2 (100)	6.0 ± 2.4 (94)	3.6 ± 1.9 (56)	1.5 ± 1.3 (23)	0.1 ± 0.1 (2)
PLASTIC/COLLAGENASE (N=6)	7.5 ± 3.6 (100)	3.3 ± 1.0 (44)	1.2 ± 1.3 (16)	1.1 ± 0.9 (15)	0.4 ± 0.4 (5)
PLASTIC /EDTA (N=6)	5.6 ± 2.7 (100)	4.0 ± 2.4 (71)	2.2 ± 1.7 (39)	0.3 ± 0.5 (5)	0.0 ± 0.1 (0)
COMBINED DATA					
MATRIGEL® (n=12)	6.9 ± 2.1 (100)	5.2 ± 2.3 (75)	2.4 ± 1.6 (35)	1.7 ± 0.9 (25)	1.0 ± 0.9 (14)
FIBRONECTIN (n=12)	8.1 ± 1.9 (100)	5.2 ± 2.2 (64)	2.7 ± 1.7 (33)	1.6 ± 0.9 (20)	0.5 ± 0.5 (6)
VITROGEN® (n=12)	7.1 ± 2.5 (100)	5.1 ± 2.2 (72)	2.6 ± 1.9 (37)	1.4 ± 1.2 (20)	0.3 ± 0.4 (4)
PLASTIC (n=12)	6.6 ± 3.2 (100)	3.7 ± 1.8 (56)	1.7 ± 1.5 (26)	0.7 ± 0.8 (11)	0.2 ± 0.3 (3)
COLLAGENASE (n=24)	7.5 ± 2.2 (100)	3.8 ± 1.2 (51)	1.5 ± 1.3 (20)	1.3 ± 0.9 (17)	0.6 ± 0.5 (8)
EDTA (n=24)	6.8 ± 2.7 (100)	5.7 ± 2.5 (84)	3.2 ± 1.6 (47)	1.4 ± 1.1 (21)	0.4 ± 0.8 (6)

Data are expressed as mean ± SD percentage 2α-hydroxy metabolites represent of total radioactive entities in the sample. Figures in brackets are activity relative to t=0.

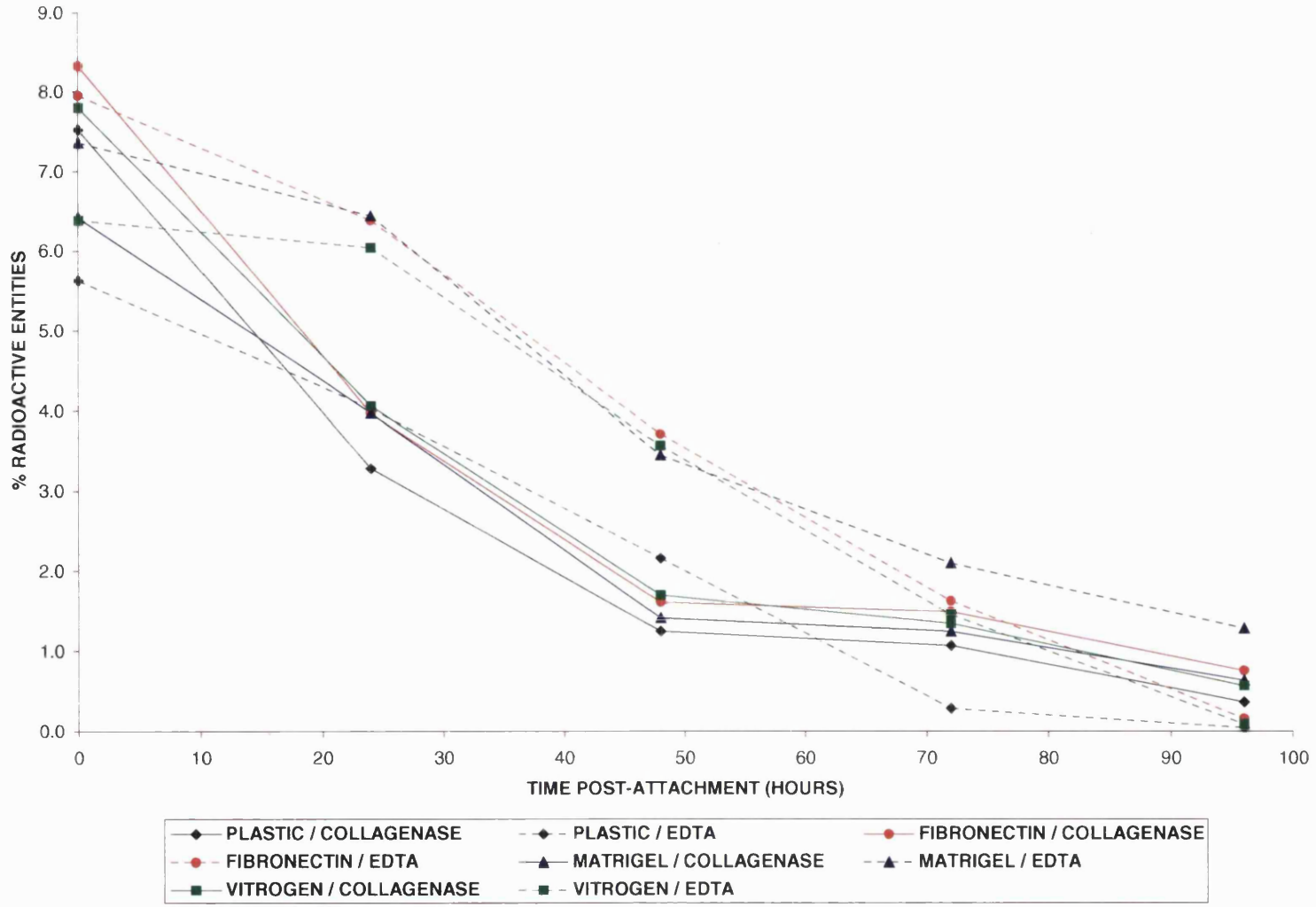


Figure 3.7 Testosterone 2α-hydroxylase activity in cultured rat hepatocytes at 0, 24, 48, 72 and 96 hours post-attachment

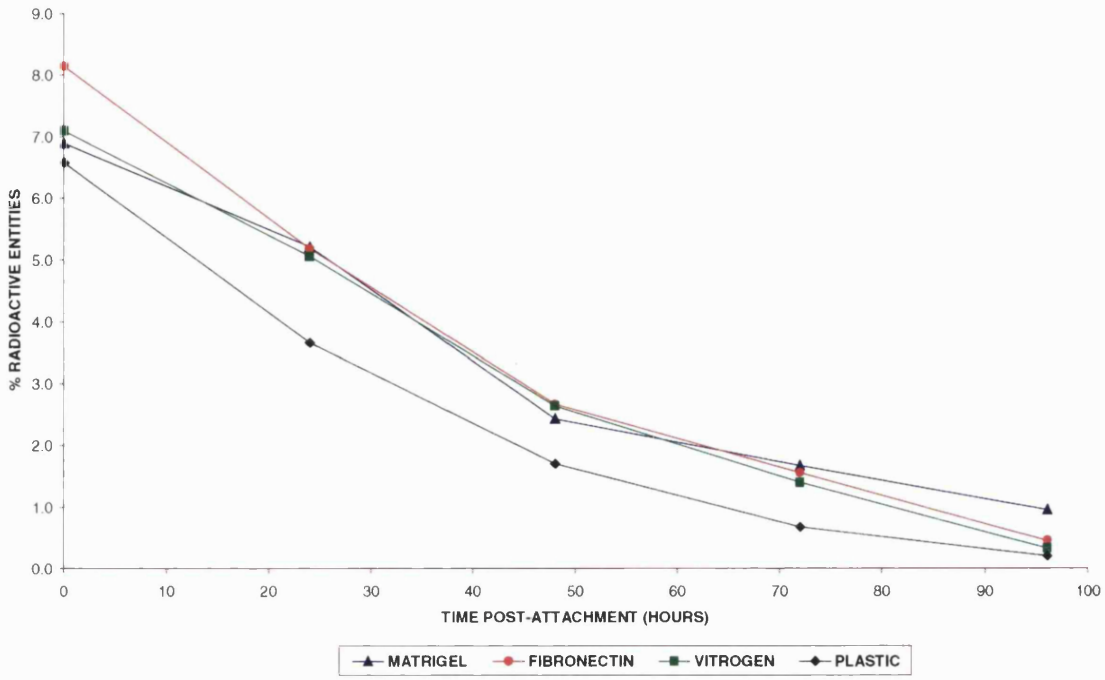


Figure 3.8 Summary of 2α-hydroxylase activity data combined on the basis of substrata

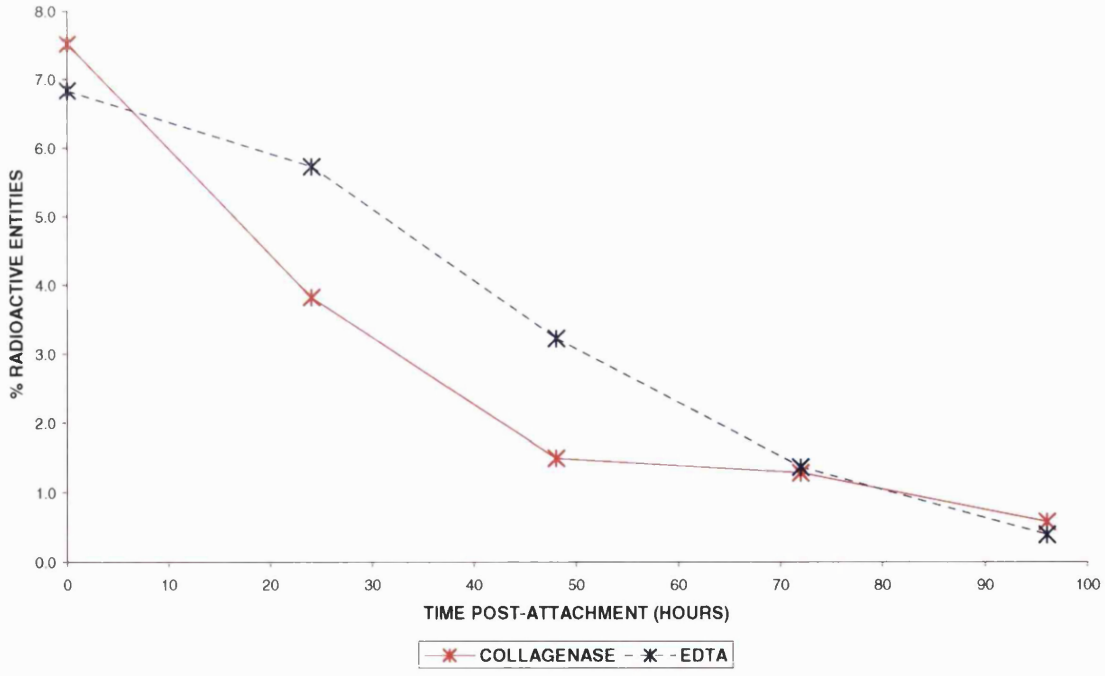


Figure 3.9 Summary of 2α-hydroxylase activity data combined on the basis of isolation technique

3.3.6 6β-Hydroxy Testosterone

The testosterone 6β-hydroxylase activity in hepatocytes isolated using collagenase and EDTA and attached to Matrigel®, fibronectin, Vitrogen® and untreated plastic is shown in Figure 3.10 and data combined on the basis of substrata or isolation technique are shown in Figures 3.11 and 3.12. The data are summarised in Table 3.8. Data from individual experiments (n=6) are given in the appendix.

The testosterone 6β-hydroxylase activity in all cultured hepatocytes was seen to decrease throughout the 96-hour culture period, to between 0 and 14% of initial activity. The largest decrease in testosterone 6β-hydroxylase activity, ranging from 47 to 72%, occurred during the first 24 hours for all combinations of isolation and substrata. A particularly large decrease (66%) was observed for collagenase-isolated cells during the first 24 hours. The decline in activity during the first 24 hours was less marked for EDTA-isolated cells, where initial activity was very low compared to that in collagenase-isolated cells. The rate of loss of activity for all combinations of isolation and substrata slowed down after the initial 24 hour culture period.

Activity was very low in all samples, although still detectable in the majority of samples, following a culture period of 96 hours. The loss of 6β-hydroxylase activity suggests that the levels of active CYP3A1/2, the major enzymes responsible for 6β-hydroxylase activity, have greatly declined during the 96-hour culture period.

The analysis of variance showed that there was no significant effect of substrata on testosterone 6β-hydroxylase activity. However, the test statistic was close to borderline at 24 hours post-attachment ($P=0.057$), with hepatocytes maintained on untreated plastic having lower 6β-hydroxylase activity than those maintained on other substrata.

The analysis of variance also indicates that there was a significant difference in testosterone 6β-hydroxylase activity due to the isolation technique used immediately after seeding ($P<0.0001$), at 48 hours post-attachment (P,0.01), at 72 hours post-

attachment ($P<0.001$) and at 96 hours post attachment ($p<0.0001$). The test statistic was close to borderline ($P=0.057$) at 24 hours post-attachment. Testosterone 6β-hydroxylase activity was higher in hepatocytes isolated by collagenase perfusion than those isolated by EDTA at these times with a particularly large difference apparent immediately post-attachment. There was no significant interaction between isolation and substrata at any of the timepoints measured.

Table 3.8 Summary of testosterone 6β-hydroxylase activity in cultured hepatocytes.

SUBSTRATA / ISOLATION METHOD	0 HOURS	24 HOURS	48 HOURS	72 HOURS	96 HOURS
MATRIGEL®/COLLAGENASE (N=6)	8.6 ± 3.5 (100)	3.8 ± 2.7 (44)	1.8 ± 1.0 (21)	1.4 ± 0.8 (16)	0.8 ± 0.7 (9)
MATRIGEL®/EDTA (N=6)	5.8 ± 3.9 (100)	3.1 ± 1.0 (53)	1.1 ± 0.6 (19)	1.5 ± 1.7 (26)	0.6 ± 0.7 (10)
FIBRONECTIN/COLLAGENASE (N=6)	10.8 ± 2.4 (100)	3.8 ± 3.1 (35)	1.3 ± 0.7 (12)	1.5 ± 0.8 (14)	1.1 ± 0.7 (10)
FIBRONECTIN/EDTA (N=6)	5.8 ± 3.4 (100)	2.8 ± 1.3 (48)	1.5 ± 1.3 (26)	1.4 ± 0.8 (24)	0.8 ± 0.7 (14)
VITROGEN®/COLLAGENASE (N=6)	11.5 ± 3.2 (100)	3.5 ± 2.4 (30)	1.3 ± 0.5 (11)	1.2 ± 0.8 (10)	1.1 ± 0.9 (10)
VITROGEN®/EDTA (N=6)	5.3 ± 5.3 (100)	2.6 ± 1.7 (49)	0.9 ± 1.0 (17)	0.4 ± 0.5 (8)	0.1 ± 0.1 (2)
PLASTIC /COLLAGENASE (N=6)	8.8 ± 3.8 (100)	2.5 ± 1.0 (28)	1.3 ± 0.4 (15)	1.7 ± 0.9 (19)	0.8 ± 0.3 (9)
PLASTIC /EDTA (N=6)	4.5 ± 5.1 (100)	1.5 ± 1.6 (33)	0.6 ± 0.7 (13)	0.2 ± 0.3 (4)	0.0 ± 0.1 (0)
COMBINED DATA					
MATRIGEL® (n=12)	7.2 ± 3.8 (100)	3.4 ± 2.0 (47)	1.5 ± 0.9 (21)	1.4 ± 1.3 (19)	0.7 ± 0.6 (10)
FIBRONECTIN (n=12)	8.3 ± 3.8 (100)	3.3 ± 2.3 (40)	1.4 ± 1.0 (17)	1.5 ± 0.8 (18)	1.0 ± 0.7 (12)
VITROGEN® (n=12)	8.4 ± 5.3 (100)	3.1 ± 2.0 (37)	1.1 ± 0.8 (13)	0.8 ± 0.6 (10)	0.6 ± 0.8 (7)
PLASTIC (n=12)	6.7 ± 4.8 (100)	2.0 ± 1.3 (30)	0.9 ± 0.7 (13)	0.9 ± 1.0 (13)	0.4 ± 0.4 (6)
COLLAGENASE (n=24)	9.9 ± 3.3 (100)	3.4 ± 2.3 (34)	1.4 ± 0.7 (14)	1.4 ± 0.8 (14)	1.0 ± 0.7 (10)
EDTA (n=24)	5.4 ± 4.2 (100)	2.5 ± 1.5 (46)	1.0 ± 0.9 (19)	0.9 ± 1.1 (17)	0.4 ± 0.6 (7)

Data are expressed as mean ± SD percentage 6β-hydroxy metabolites represent of total radioactive entities in the sample. Figures in brackets are activity relative to t=0.

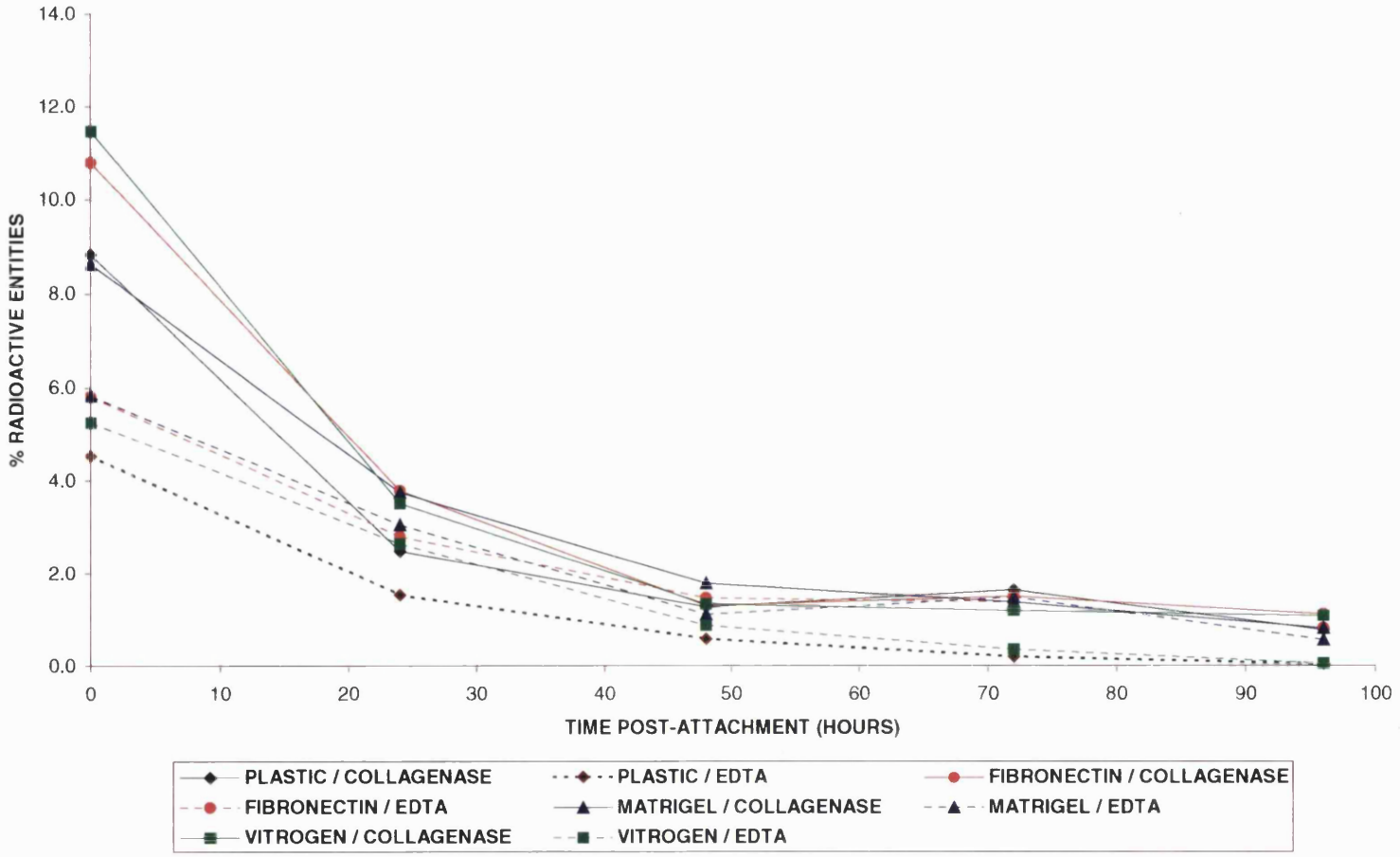


Figure 3.10 Testosterone 6β-hydroxylase activity in cultured rat hepatocytes at 0, 24, 48, 72 and 96 hours post-attachment

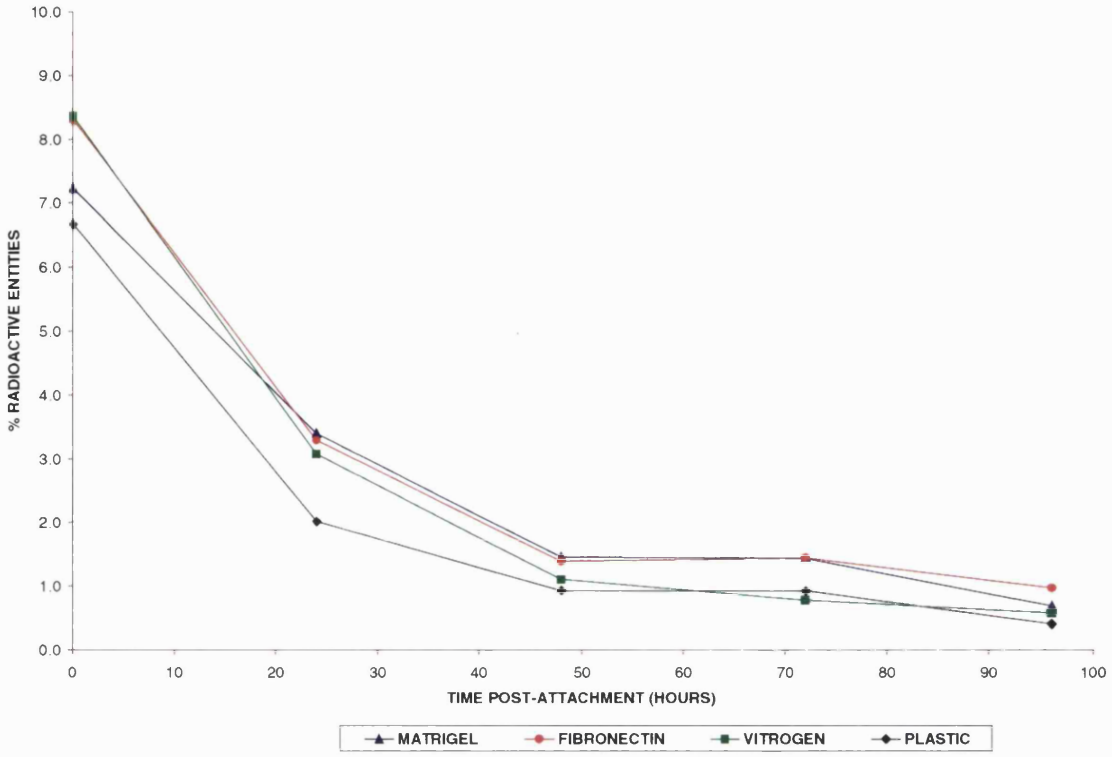


Figure 3.11 Summary of 6β-hydroxylase activity data combined on the basis of substrata

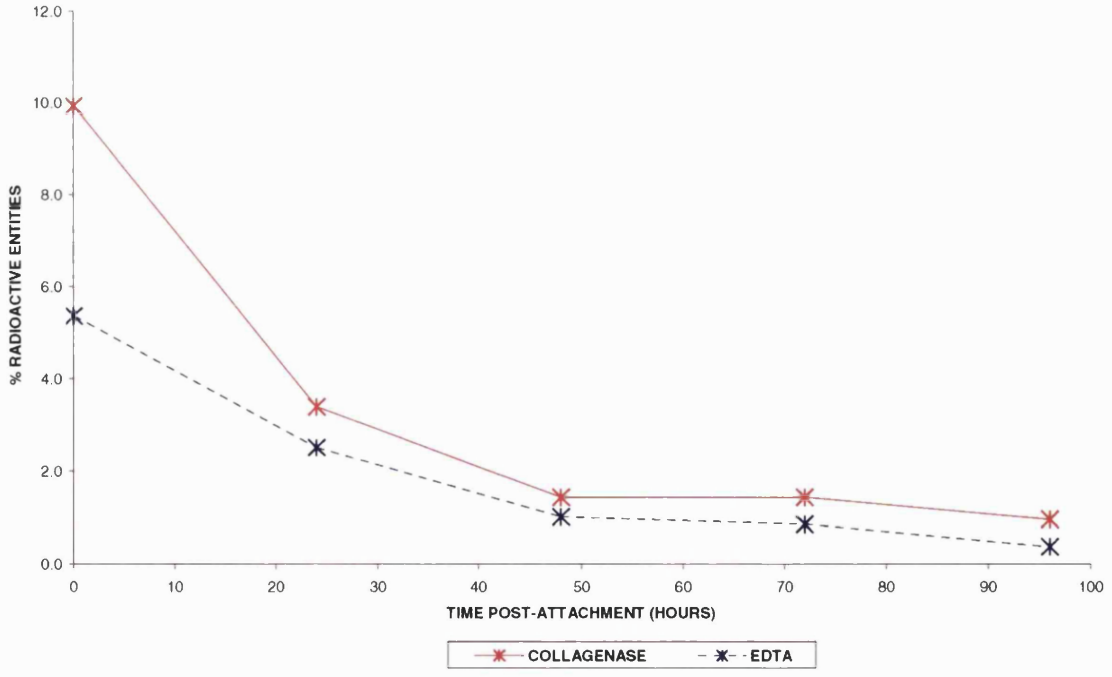


Figure 3.12 Summary of 6β-hydroxylase activity data combined on the basis of isolation technique

3.3.7 7α-Hydroxy Testosterone

The testosterone 7α-hydroxylase activity in hepatocytes isolated using collagenase and EDTA and attached to Matrigel®, fibronectin, Vitrogen® and untreated plastic is shown in Figure 3.13 and data combined on the basis of substrata or isolation technique are shown in Figures 3.14 and 3.15. The data are summarised in Table 3.9. Data from individual experiments (n=6) are given in the appendix.

The testosterone 7α-hydroxylase activity in cultured hepatocytes was seen to decrease throughout the 96-hour culture period, to between 0 to 55% of initial activity. A sharp drop in activity, ranging from 44 to 67%, was observed during the first 24 hours for collagenase-isolated hepatocytes. Following the initial 24 hour period the loss of activity in collagenase-isolated cells slowed down and was similar to that seen in EDTA-isolated cells. Despite the slower rate of decline in testosterone 7α-hydroxylase activity in EDTA-isolated hepatocytes, the activity immediately post-attachment was very low compared to that in collagenase-isolated cells.

Testosterone 7α-hydroxylase activity was very low in all cultures and below the level of detection in some samples following a culture period of 96 hours. The apparently high percentage activity remaining in EDTA-isolated cells cultured on Matrigel® (55%) is a reflection of the low initial activity in these hepatocytes. The loss of testosterone 7α-hydroxylase activity suggests that the level of active CYP2A1, the major enzyme responsible for 7α-hydroxylase activity, has greatly declined during the 96-hour culture period.

The analysis of variance suggests that there were no significant differences in testosterone 7α-hydroxylase activity due to substrata, although at 48, 72 and 96 hours post-attachment activity was highest in hepatocytes maintained on Matrigel®.

The analysis of variance indicates that there was a significant difference, however, in testosterone 7α-hydroxylase activity due to isolation technique immediately post-attachment ($P<0.0001$) and at 48 hours post-attachment ($P<0.05$), with higher 7α-

hydroxylase activity observed in the hepatocytes isolated by collagenase perfusion. There were no significant differences due to the isolation technique used at any other timepoints, and no significant interactions between isolation technique and substrata at any of the timepoints measured.

Table 3.9 Summary of testosterone 7α-hydroxylase activity in cultured hepatocytes

SUBSTRATA / ISOLATION METHOD	0 HOURS	24 HOURS	48 HOURS	72 HOURS	96 HOURS
MATRIGEL®/COLLAGENASE (N=6)	2.7 ± 1.5 (100)	1.5 ± 2.0 (56)	1.0 ± 2.2 (37)	1.3 ± 2.1 (48)	0.4 ± 0.5 (15)
MATRIGEL®/EDTA (N=6)	1.1 ± 0.5 (100)	0.9 ± 0.4 (82)	1.0 ± 0.8 (91)	0.9 ± 1.1 (32)	0.6 ± 0.8 (55)
FIBRONECTIN/COLLAGENASE (N=6)	3.8 ± 2.0 (100)	1.6 ± 2.2 (42)	0.9 ± 1.8 (24	0.8 ± 1.6 (21)	0.3 ± 0.5 (8)
FIBRONECTIN/EDTA (N=6)	1.2 ± 0.6 (100)	0.9 ± 0.5 (75)	0.7 ± 0.7 (58)	0.1 ± 0.1 (8)	0.0 ± 0.0 (0)
VITROGEN®/COLLAGENASE (N=6)	3.9 ± 2.4 (100)	1.6 ± 2.2 (41)	1.2 ± 2.7 (31)	0.7 ± 1.5 (18)	0.3 ± 0.4 (8)
VITROGEN®/EDTA (N=6)	0.6 ± 0.8 (100)	0.8 ± 0.6 (133)	0.4 ± 0.5 (67)	0.2 ± 0.3 (33)	0.0 ± 0.0 (0)
PLASTIC /COLLAGENASE (N=6)	2.4 ± 2.0 (100)	0.8 ± 1.2 (33)	1.4 ± 3.14 (58)	1.2 ± 2.5 (50)	0.3 ± 0.7 (13)
PLASTIC /EDTA (N=6)	0.6 ± 0.7 (100)	0.5 ± 0.6 (83)	0.3 ± 0.4 (50)	0.1 ± 0.3 (17)	0.0 ± 0.0 (0)
COMBINED DATA					
MATRIGEL® (n=12)	1.9 ± 1.4 (100)	1.2 ± 1.4 (63)	1.0 ± 1.6 (53)	1.1 ± 1.6 (58)	0.5 ± 0.7 (26)
FIBRONECTIN (n=12)	2.5 ± 2.0 (100)	1.2 ± 1.6 (48)	0.8 ± 1.3 (32)	0.5 ± 1.1 (20)	0.1 ± 0.3 (4)
VITROGEN® (n=12)	2.3 ± 2.4 (100)	1.2 ± 1.6 (52)	0.8 ± 1.9 (35)	0.5 ± 1.1 (22)	0.2 ± 0.3 (9)
PLASTIC (n=12)	1.5 ± 1.7 (100)	0.6 ± 0.9 (40)	0.8 ± 2.2 (53)	0.7 ± 1.8 (47)	0.2 ± 0.5 (13)
COLLAGENASE (n=24)	3.2 ± 2.0 (100)	1.4 ± 1.9 (44)	1.1 ± 2.3 (34)	1.0 ± 1.9 (31)	0.3 ± 0.5 (9)
EDTA (n=24)	0.9 ± 0.7 (100)	0.8 ± 0.5 (89)	0.6 ± 0.6 (67)	0.3 ± 0.6 (33)	0.2 ± 0.4 (22)

Data are expressed as mean ± SD percentage 7α-hydroxy metabolites represent of total radioactive entities in the sample. Figures in brackets are activity relative to t=0.

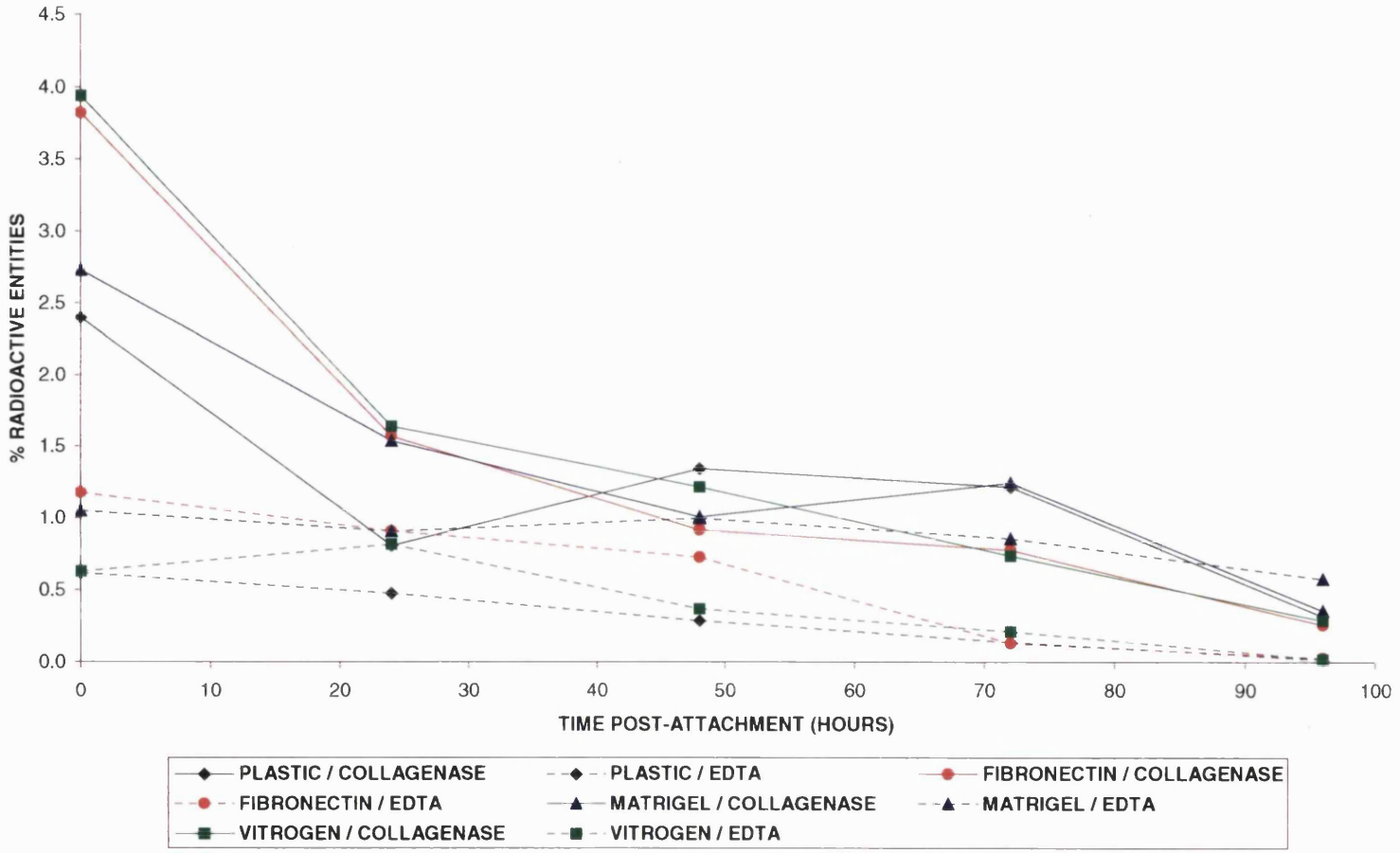


Figure 3.13 Testosterone 7α-hydroxylase activity in cultured rat hepatocytes at 0, 24, 48, 72 and 96 hours post-attachment

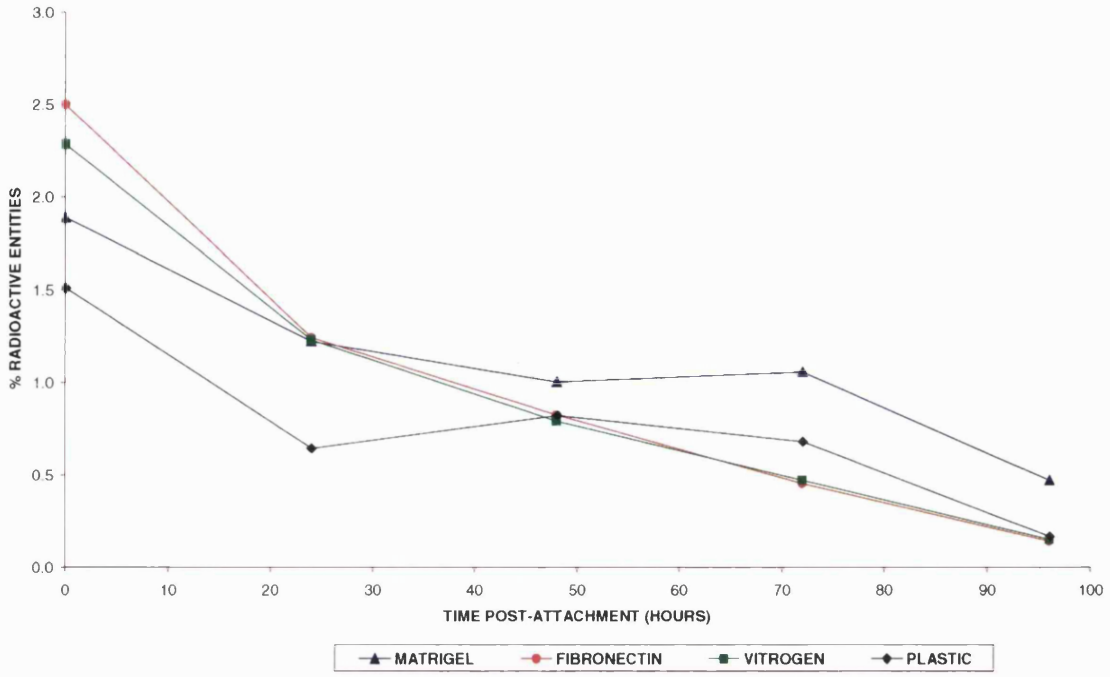


Figure 3.14 Summary of 7α-hydroxylase activity data combined on the basis of substrata

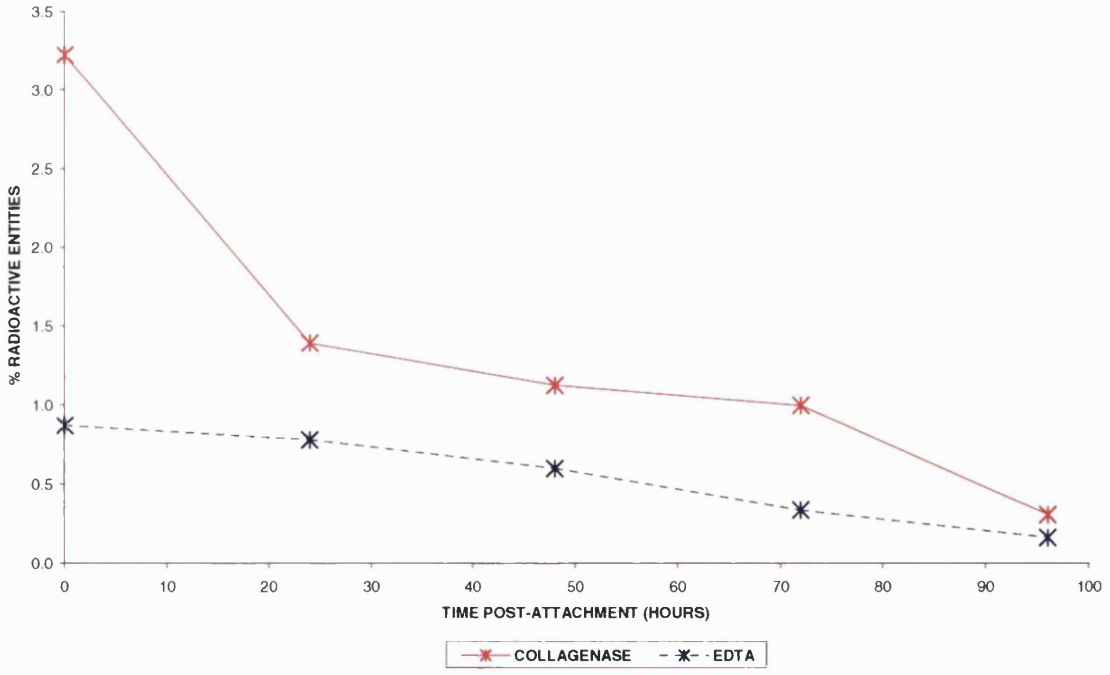


Figure 3.15 Summary of 7α-hydroxylase activity data combined on the basis of isolation technique

3.3.8 16α-Hydroxy Testosterone

The testosterone 16α-hydroxylase activity in hepatocytes isolated using collagenase and EDTA and attached to Matrigel®, fibronectin, Vitrogen® and untreated plastic is shown in Figure 3.16 and data combined on the basis of substrata or isolation technique are shown in Figures 3.17 and 3.18. The data is summarised in Table 3.10. Data from individual experiments (n=6) are given in the appendix.

The testosterone 16α-hydroxylase activity in all cultured hepatocytes was seen to decrease during the 96 hour culture period, to between 1 to 21% of initial activity. The greatest decline in activity, ranging from 39 to 60%, was observed during the first 24 hours for hepatocytes isolated using collagenase. For EDTA-isolated cells the decline in activity was less pronounced during the first 24 hours, with the loss ranging from 6 to 21%.

Following 96 hours in culture 16α-hydroxylase activity was very low in all cultures, although activity was still detectable in the majority of samples. This suggests that the levels of active CYP2C11 and CYP2B1/2, the major enzymes responsible for 16α-hydroxylase activity, have greatly declined over the 96-hour culture period.

The results from the analysis of variance show that significant differences in testosterone 16α-hydroxylase activity exist due to both the isolation technique and the substrata used. Significant differences in testosterone 16α-hydroxylase activity between hepatocytes maintained on different substrata were observed at 24 hours ($P<0.05$) and at 96 hours ($P<0.05$) post-attachment with 16α-hydroxylase activity higher in hepatocytes maintained on Matrigel®, fibronectin and Vitrogen® compared to untreated plastic. No significant differences were apparent due to the substrata used at 0 hours, 48 hours and 72 hours post-attachment.

Significant differences were also observed due to isolation technique at 24 hours ($P=0.001$) and 48 hours ($P<0.001$) post attachment, with EDTA-isolated cells having higher activity than those isolated using collagenase. There did not appear to be a

significant difference between isolation techniques immediately after seeding, at 72 hours, or at 96 hours post-attachment. There was no significant interaction between isolation technique and substrata used at any of the timepoints examined.

The effects of isolation technique and substrata on testosterone 16α-hydroxylation were the same as that seen for 2α-hydroxylation. This supports the fact that the same enzyme has been reported to catalyse both reactions.

Table 3.10 Summary of testosterone 16α-hydroxylase activity in cultured hepatocytes

SUBSTRATA / ISOLATION METHOD	0 HOURS	24 HOURS	48 HOURS	72 HOURS	96 HOURS
MATRIGEL®/COLLAGENASE (N=6)	13.5 ± 4.5 (100)	8.2 ± 6.8 (61)	2.7 ± 1.6 (20)	2.4 ± 1.2 (18)	1.3 ± 0.8 (10)
MATRIGEL®/EDTA (N=6)	16.0 ± 4.4 (100)	13.4 ± 3.4 (84)	7.7 ± 3.0 (48)	6.2 ± 3.7 (39)	3.3 ± 3.7 (21)
FIBRONECTIN/COLLAGENASE (N=6)	15.9 ± 6.4 (100)	7.9 ± 5.8 (50)	2.8 ± 1.6 (18)	3.1 ± 2.1 (19)	1.6 ± 0.9 (10)
FIBRONECTIN/EDTA (N=6)	17.3 ± 4.8 (100)	13.6 ± 4.4 (79)	7.6 ± 2.7 (44)	3.3 ± 2.3 (19)	0.3 ± 0.5 (2)
VITROGEN®/COLLAGENASE (N=6)	15.2 ± 5.6 (100)	7.8 ± 4.9 (51)	3.0 ± 1.7 (20)	2.5 ± 1.8 (16)	1.2 ± 0.9 (8)
VITROGEN®/EDTA (N=6)	12.7 ± 7.3 (100)	11.9 ± 3.8 (94)	7.0 ± 3.9 (55)	3.0 ± 3.3 (24)	0.2 ± 0.3 (2)
PLASTIC/COLLAGENASE (N=6)	14.0 ± 7.2 (100)	5.6 ± 1.9 (40)	2.5 ± 1.54 (18)	2.3 ± 1.6 (16)	0.8 ± 0.4 (6)
PLASTIC/EDTA (N=6)	10.4 ± 6.0 (100)	7.5 ± 4.9 (72)	4.4 ± 3.9 (42)	0.6 ± 1.1 (4)	0.1 ± 0.2 (1)
COMBINED DATA					
MATRIGEL® (n=12)	14.7 ± 4.4 (100)	10.8 ± 5.8 (73)	5.2 ± 3.5 (35)	4.3 ± 3.3 (29)	2.3 ± 2.7 (16)
FIBRONECTIN (n=12)	16.6 ± 5.4 (100)	10.7 ± 5.8 (64)	5.2 ± 3.3 (31)	3.2 ± 2.1 (19)	0.9 ± 1.0 (5)
VITROGEN® (n=12)	14.0 ± 6.3 (100)	9.9 ± 4.7 (71)	5.0 ± 3.6 (36)	2.7 ± 2.5 (19)	0.7 ± 0.8 (5)
PLASTIC (n=12)	12.2 ± 6.6 (100)	6.6 ± 3 7 (54)	3.4 ± 2.9 (28)	1.5 ± 1.6 (12)	0.4 ± 0.5 (3)
COLLAGENASE (n=24)	14.7 ± 5.7 (100)	7.4 ± 4.9 (50)	2.7 ± 1.5 (18)	2.6 ± 1.6 (18)	1.2 ± 0.8 (8)
EDTA (n=24)	14.1 ± 6.0 (100)	11.6 ± 4.6 (82)	6.7 ± 3.5 (48)	3.3 ± 3.3 (23)	1.0 ± 2.2 (7)

Data are expressed as mean ± SD percentage 16α-hydroxy metabolites represent of total radioactive entities in the sample. Figures in brackets are activity relative to t=0.

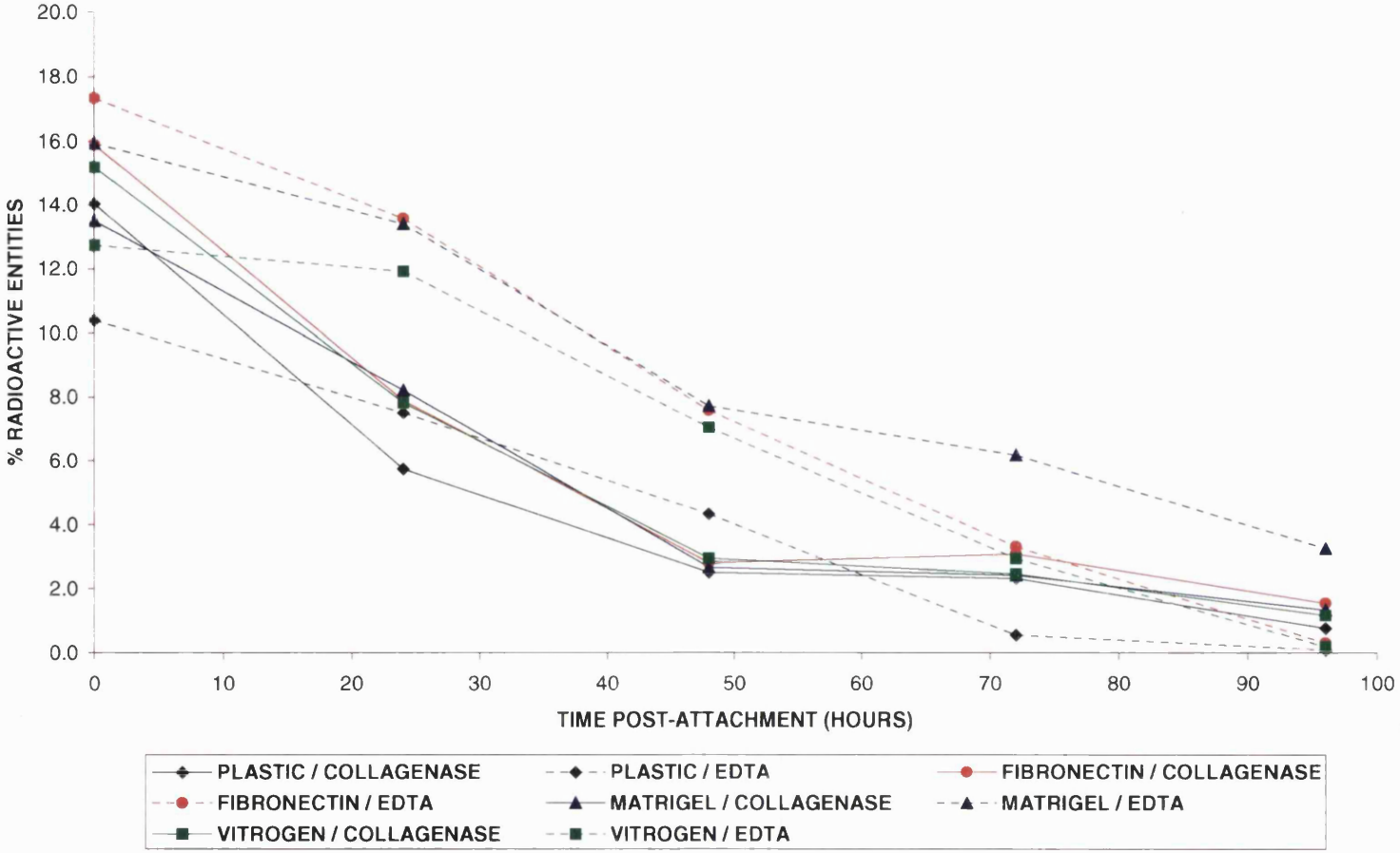


Figure 3.16 Testosterone 16α-hydroxylase activity in cultured rat hepatocytes at 0, 24, 48, 72 and 96 hours post-attachment

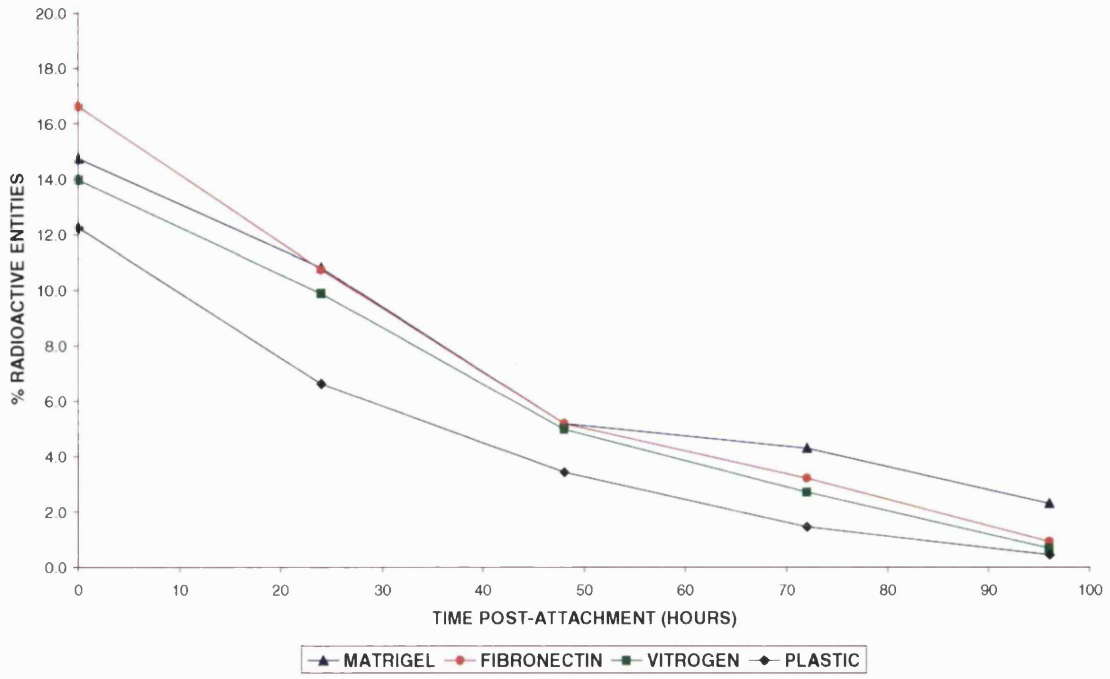


Figure 3.17 Summary of testosterone 16α-hydroxylase activity data combined on the basis of substrata

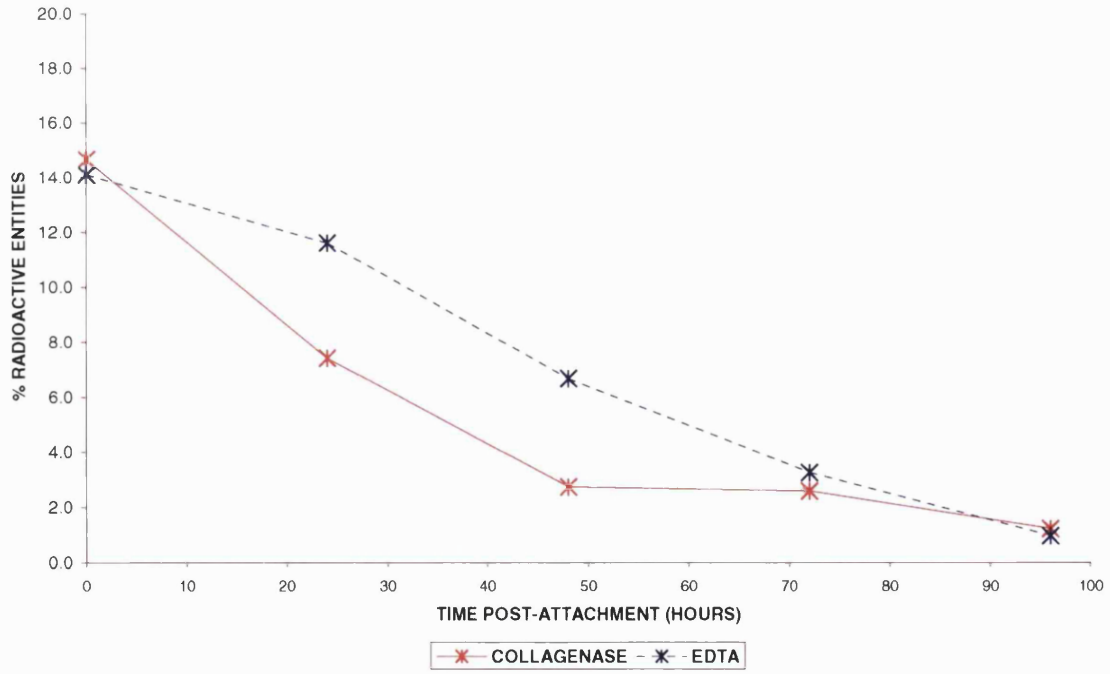


Figure 3.18 Summary of testosterone 16α-hydroxylase activity data combined on the basis of isolation

3.3.9 16β-Hydroxy Testosterone

The testosterone 16β-hydroxylase activity in hepatocytes isolated using collagenase and EDTA and attached to Matrigel®, fibronectin, Vitrogen® and untreated plastic is shown in Figure 3.19. 16β-hydroxy testosterone is a minor metabolite and the very low levels produced by cultured hepatocytes were below the limit of detection in the majority of samples. The low levels of activity towards 16β-hydroxylation has been observed previously where activity could not always been measured (Utesch et al., 1991). For this reason statistical data analysis could not be reasonably carried out. However, 16β-hydroxylase activity did decline from low levels of activity immediately post-attachment to undetectable activity by 96 hours post-isolation in most samples indicating that the level of active CYP2B1/2, the major enzymes involved in this reaction, have declined during the culture period.

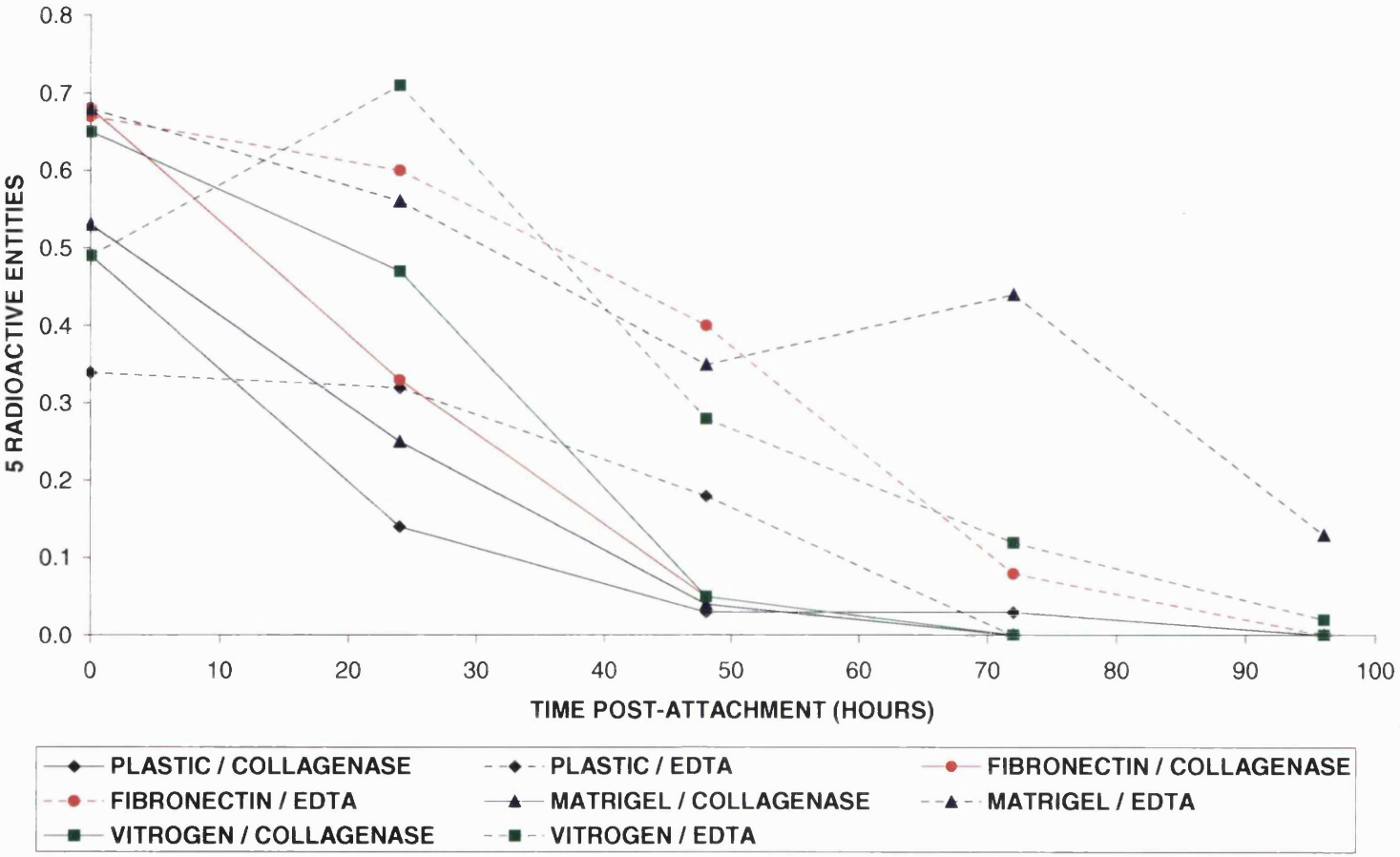


Figure 3.19 Testosterone 16β-hydroxylase activity in cultured rat hepatocytes at 0, 24, 48, 72 and 96 hours post-attachment

3.3.10 Androstenedione

The testosterone 17α-oxidase activity in hepatocytes isolated using collagenase and EDTA and attached to Matrigel®, fibronectin, Vitrogen® and untreated plastic is shown in Figure 3.20 and data combined on the basis of substrata or isolation technique are shown in Figures 3.21 and 3.22. The data is summarised in Table 3.11. Data from individual experiments (n=6) are given in the appendix.

The decrease in testosterone 17-oxidase activity during the 96-hour culture period was different to that observed for other metabolites. During the first 24 hours the testosterone 17-oxidase activity was either maintained or increased, ranging from 95 to 136% of initial activity. Activity then declined over the next 72 hours although the rate of decline varied greatly. The slowest rate of decrease in activity was associated with EDTA-isolated cells maintained on Matrigel® and the fastest with EDTA-isolated hepatocytes maintained on plastic.

Activity was still detectable in almost all hepatocyte cultures at 96 hours post-isolation, although the remaining activity varied greatly ranging from 4 to 80% of initial activity. These data suggest that some, or all, of the enzymes responsible for androstendione production, CYP2B, CYP2C11 and 17β-hydroxysteroid dehydrogenase, have declined during culture but are still present. Activity was particularly well maintained in hepatocytes cultured on Matrigel®, and in hepatocytes isolated by collagenase.

Results from the analysis of variance indicate that significant differences in testosterone 17α-oxidase activity exist due to both isolation technique and to the substrata used. Significant differences were observed due to the substrata used after 72 hours ($P=0.007$) and 96 hours ($p<0.0001$) post-attachment of cells with hepatocytes maintained on Matrigel® having a higher level of activity than hepatocytes maintained on other substrata. No significant differences were apparent due to substrata used at 0 hours, at 24 hours or 48 hours after cell attachment.

Results from the analysis of variance also suggest that significant differences exist due to isolation technique after 72 hours ($P=0.041$) and 96 hours ($P<0.0001$) post-attachment with activity with higher activity observed in collagenase-isolated hepatocytes. No significant differences were apparent due to isolation technique immediately following attachment, at 24 hours or 48 hours after cell attachment.

There was also evidence of significant interaction between isolation technique and substrata used at 96 hours ($P<0.05$) post-attachment and the test statistic was borderline at 72 hours ($P=0.071$) with cells isolated by EDTA and maintained on Matrigel® having the highest activity. This was the only example of an interaction between the isolation technique and the substrata used found in these studies. There was no evidence of significant interaction between these two factors at other timepoints.

Table 3.11 Summary of testosterone 17α-oxidase activity in cultured hepatocytes

SUBSTRATA / ISOLATION METHOD	0 HOURS	24 HOURS	48 HOURS	72 HOURS	96 HOURS
MATRIGEL®/COLLAGENASE (N=6)	11.9 ± 5.3 (100)	12.9 ± 5.4 (108)	10.1 ± 7.8 (85)	10.8 ± 4.5 (91)	8.0 ± 4.4 (67)
MATRIGEL®/EDTA (N=6)	11.9 ± 5.5 (100)	14.6 ± 7.1 (123)	13.9 ± 7.6 (117)	14.6 ± 10.6 (123)	9.5 ± 6.6 (80)
FIBRONECTIN/COLLAGENASE (N=6)	12.5 ± 3.7 (100)	12.3 ± 4.8 (98)	7.3 ± 4.5 (58)	9.9 ± 7.9 (79)	6.3 ± 4.4 (50)
FIBRONECTIN/EDTA (N=6)	11.7 ± 5.3 (100)	13.6 ± 7.1 (116)	13.2 ± 7.3 (113)	6.9 ± 3.6 (59)	0.9 ± 0.7 (8)
VITROGEN®/COLLAGENASE (N=6)	13.2 ± 4.1 (100)	14.0 ± 8.5 (106)	7.6 ± 4.8 (58)	6.8 ± 4.2 (52)	4.9 ± 3.5 (37)
VITROGEN®/EDTA (N=6)	9.4 ± 4.2 (100)	12.4 ± 4.8 (132)	10.4 ± 6.2 (111)	5.7 ± 7.4 (61)	1.1 ± 0.8 (12)
PLASTIC /COLLAGENASE (N=6)	10.8 ± 3.3 (100)	14.7 ± 9.3 (136)	6.7 ± 3.9 (62)	8.9 ± 8.3 (82)	4.8 ± 4.2 (44)
PLASTIC /EDTA (N=6)	9.4 ± 5.0 (100)	8.9 ± 5.7 (95)	6.7 ± 4.9 (71)	1.3 ± 1.2 (14)	0.4 ± 0.3 (4)
COMBINED DATA					
MATRIGEL® (n=12)	11.9 ± 5.2 (100)	13.8 ± 6.0 (116)	12.0 ± 7.6 (101)	12.7 ± 8.1 (107)	8.8 ± 5.4 (74)
FIBRONECTIN (n=12)	12.1 ± 4.4 (100)	12.9 ± 5.8 (107)	10.2 ± 6.5 (84)	8.4 ± 6.1 (69)	3.6 ± 4.1 (30)
VITROGEN® (n=12)	11.3 ± 4.4 (100)	13.2 ± 6.6 (117)	9.0 ± 5.5 (80)	6.2 ± 5.8 (55)	3.0 ± 3.1 (27)
PLASTIC (n=12)	10.1 ± 4.1 (100)	11.8 ± 8.0 (117)	6.8 ± 4.2 (67)	5.1 ± 6.9 (50)	2.6 ± 3.6 (26)
COLLAGENASE (n=24)	12.1 ± 4.0 (100)	13.5 ± 6.8 (112)	7.9 ± 5.3 (65)	9.1 ± 6.3 (75)	6.0 ± 4.1 (50)
EDTA (n=24)	10.6 ± 4.8 (100)	12.4 ± 6.2 (117)	11.1 ± 6.8 (105)	7.2 ± 8.0 (68)	3.0 ± 5.0 (28)

Data are expressed as mean ± SD percentage 17α-oxidase metabolites represent of total radioactive entities in the sample. Figures in brackets are activity relative to t=0.

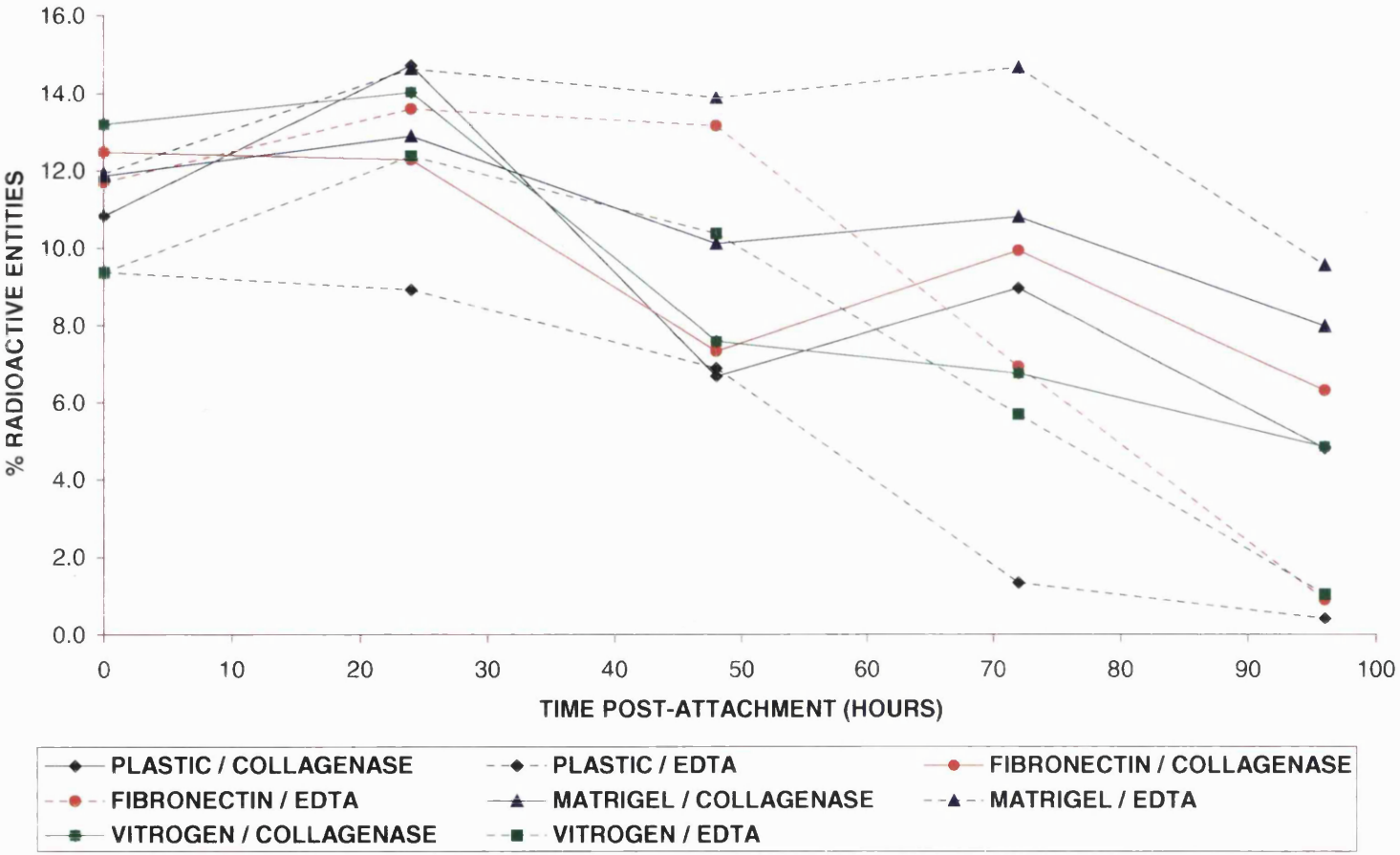


Figure 3.20 Testosterone 17-oxidase activity in cultured rat hepatocytes at 0, 24, 48, 72 and 96 hours post-attachment

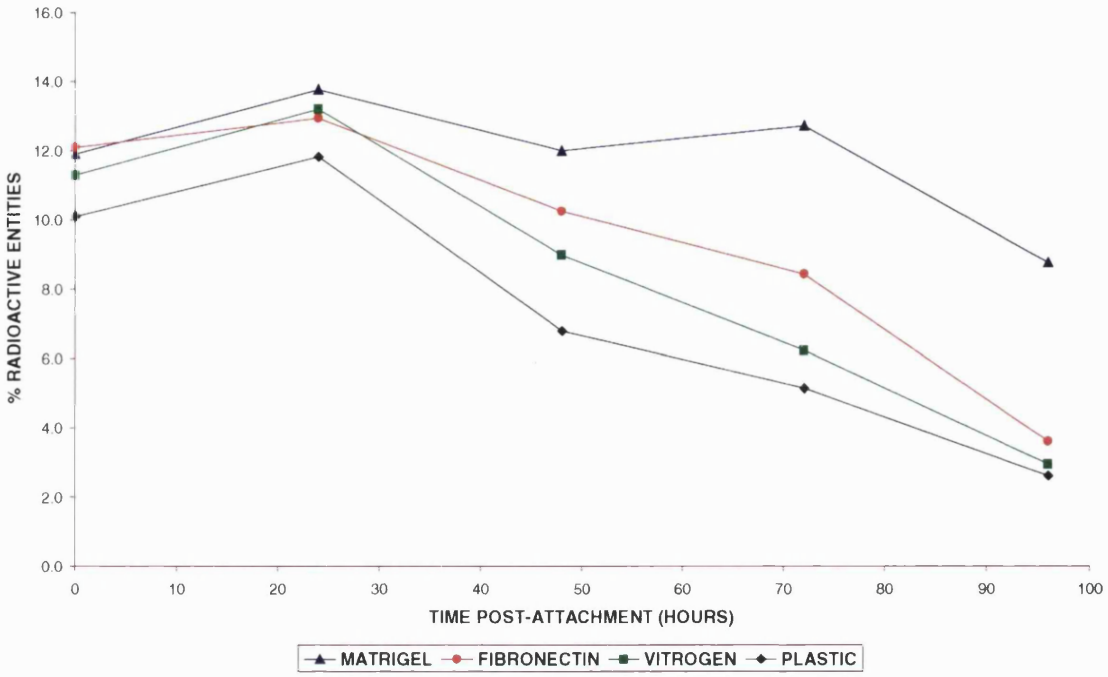


Figure 3.21 Summary of testosterone 17α-oxidase activity data combined on the basis of substrata

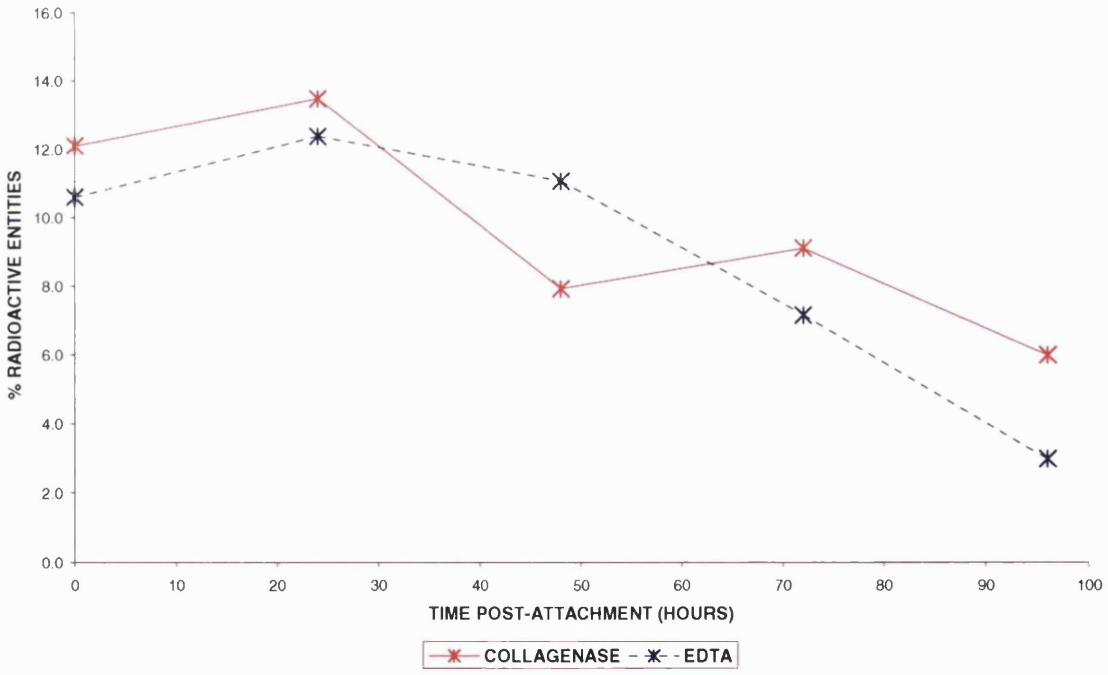


Figure 3.22 Summary of testosterone 17α-oxidase activity data combined on the basis of isolation technique

A summary of the statistically significant effects of isolation technique and substrata on the metabolism of testosterone is given in Table 3.12.

Table 3.12 Summary of significant effects on testosterone metabolism due to isolation technique, substrata or to an interaction between these factors.

METABOLITE	0 HOURS	24 HOURS	48 HOURS	72 HOURS	96 HOURS
2α-HYDROXY		ISOLATION SUBSTRATA*	ISOLATION		SUBSTRATA
6β-HYDROXY	ISOLATION	ISOLATION* SUBSTRATA*	ISOLATION	ISOLATION	ISOLATION
7α-HYDROXY	ISOLATION		ISOLATION		
16α-HYDROXY		ISOLATION SUBSTRATA	ISOLATION		SUBSTRATA
16β-HYDROXY					
AD				ISOLATION SUBSTRATA INTERACTION*	ISOLATION SUBSTRATA INTERACTION

* Borderline test statistic

Throughout these studies variation between experiments was found to be high, possibly due to the wedge biopsy technique employed for both methods of isolation. High variability has also been reported in other publications (Silva et al., 1998; Turner and Pitot, 1989; Kocarek et al, 1992) including that of testosterone metabolism in cultured rat hepatocytes (Wortelboer et al., 1990). Tissue samples are invariably heterogeneous and hepatocytes isolated from different livers, or even replicates from the same tissue, may vary in the constituent cell types. This is particularly important for cytochrome P450 since it is known that the different regions of the liver lobule vary in their expression of P450 enzymes (Gebhardt, 1992). This has probably resulted in the isolation of different populations of cells despite attempts to minimise this by standardising the lobe perfused and the vessel cannulated.

In addition, variation in the numbers of non-parenchymal cells such as Kupffer cells contaminating the isolation may result in the release of chemicals which effect cytochrome P450 activity (Iber et al., 1999) and which themselves have some metabolic capacity (Wickramasinghe, 1998; Lafranconi et al., 1986). It is likely, therefore, that the use of the wedge biopsy perfusion results in the isolation of a different population of cells on each occasion.

Whole liver perfusion carried out *in situ* is an alternative method of isolating hepatocytes which may help reduce this source of variability, although high variability can still occur (Skett et al., 1999; Daujat et al., 1987). However this technique requires the administration of an anaesthetic such as pentobarbitone which has been reported to cause induction of mRNA for cytochrome P450 enzymes (Padgham and Paine, 1993). The resulting altered expression of these enzymes would be an undesirable effect for the purposes of these studies. Moreover, for other species routinely used in metabolism studies such as dog and pig, the wedge biopsy is the most practical technique to use due to the size of the liver and for human samples, supplied as ends of lobe, the wedge biopsy is the only possible technique to use. To standardise methodology, the wedge biopsy technique has continued to be the method used within our laboratory despite limitations resulting from this choice.

A further cause of high variability between experiments may be due to the age and nutritional status of the rat used. Age-related and nutritional-related changes in the levels of cytochrome P450 enzymes have been described (Imaoka et al., 1991; Robinson et al., 1990; Friedman et al., 1989; Imaoka et al., 1990; Ioannides, 1999). To minimise the effect of rat age, manifested by a general decline in the metabolism of testosterone by hepatocytes, a target weight was selected (200g) and, as far as possible, rats were used which were near to this weight. It was not always possible to obtain rats close to the target weight, however, as shown in Table 3.1. The corresponding age range of the rats used was approximately 60 to 90 days.

Throughout these experiments rats were allowed free access to food and water, however comparison with other studies can be complicated by the effect of nutritional status on cytochrome P450 (Yang and Yoo, 1988; Brown et al., 1995; Ioannides, 1999) particularly since some researchers prefer to starve rats prior to use (Vind et al., 1989).

Accurate determination of the number of hepatocytes in suspension may be a further source of variability. A reciprocal relationship between growth and differentiation has been demonstrated (Nakamura et al., 1983) and cell density has been shown to effect the induction of cytochrome P450 enzymes that with sub-confluent cultures was substantially decreased (Greuet et al., 1997). Uneven spreading of the hepatocyte suspension under the cover slip, cell clumping and rapid settling of cell suspensions can all result in inaccurate counting of the hepatocytes. The high level of cell debris in hepatocytes isolated using EDTA made this a particular problem with this method.

3.3.11 Microsomal Metabolism of Testosterone

The decline in cytochrome P450-mediated testosterone metabolism may be due to other factors in addition to the loss of cytochrome P450 enzymes. These include transport of probe substrate into the hepatocytes and the availability of the co-factor NADPH. The partition coefficient of testosterone in octanol/water (2042) has been determined and is considered to be high (Imanidis et al., 1996). This suggests that the most likely entry of testosterone into the cell would be passive absorption which would not be effected by changes in the expression of proteins involved in transport of chemicals into the cell throughout the culture period. The supply of the co-factor NADPH by the pentose phosphate pathway or certain mitochondrial reactions may, however, be perturbed under culture conditions, limiting the rate of metabolic reactions (Berry et al., 1992). Therefore, the effect of the presence of the cell wall and NADPH on the metabolism of testosterone was investigated using microsomes prepared from hepatocytes isolated by collagenase and maintained on Matrigel®. In the microsomal system the cell wall is no longer present and an excess of NADPH can be added to the incubation media.

The linearity of testosterone metabolism by microsomes prepared from rat hepatocytes cultured for up to 96 hours was determined over a three hour incubation period. Production of all metabolites was linear over an incubation period of two hours and this was chosen for subsequent experiments. The metabolism of testosterone by microsomes prepared from cultured rat hepatocytes (pool of n=6 separate isolations) maintained for up to 96 hours is shown in Figure 3.23, and comparisons of the decline in activity between hepatocytes and microsomes are shown in figures 3.24 to 3.28.

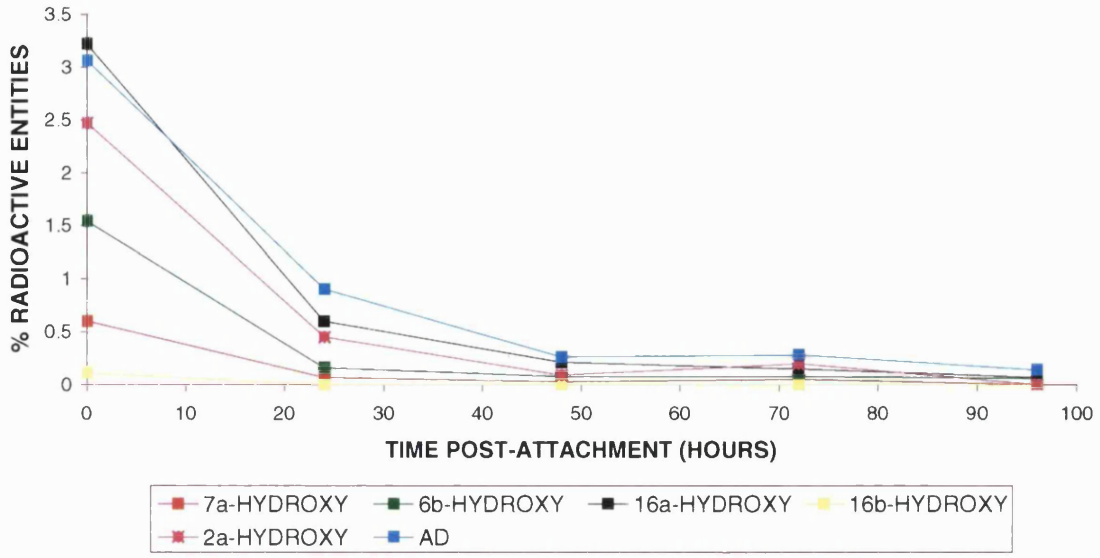


Figure 3.23 The metabolism of testosterone by microsomes prepared from cultured rat hepatocytes.

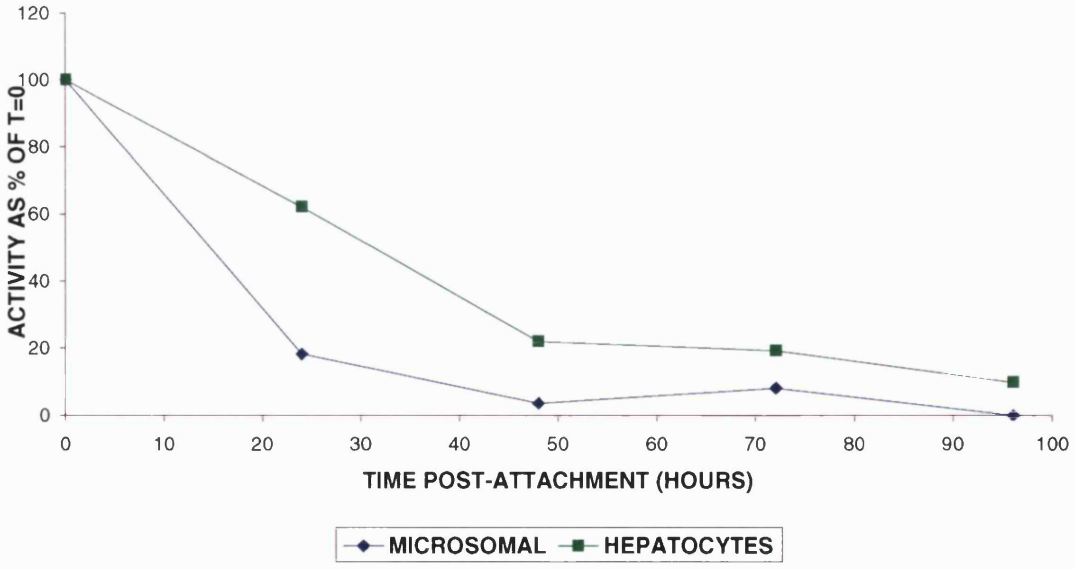


Figure 3.24 Comparison of hepatocyte and microsomal testosterone 2α-hydroxylase activity.

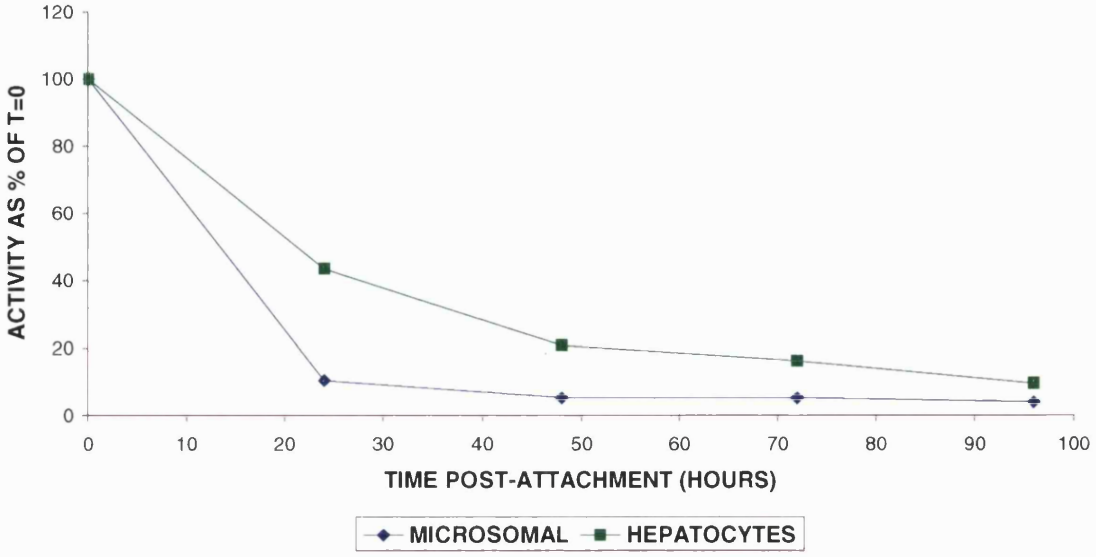


Figure 3.25 Comparison of hepatocyte and microsomal testosterone 6β-hydroxylase activity.

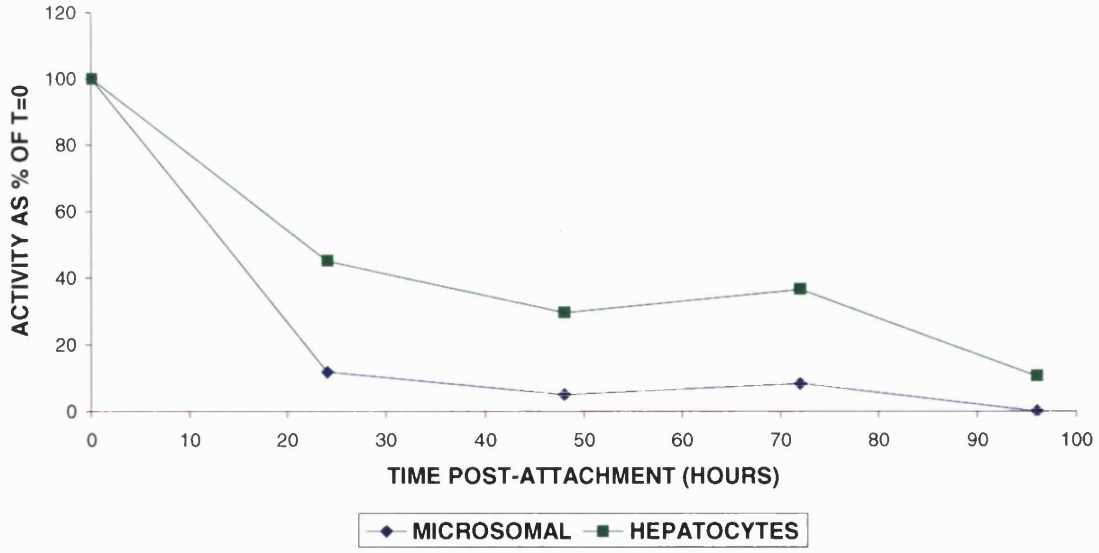


Figure 3.26 Comparison of hepatocyte and microsomal testosterone 7α-hydroxylase activity.

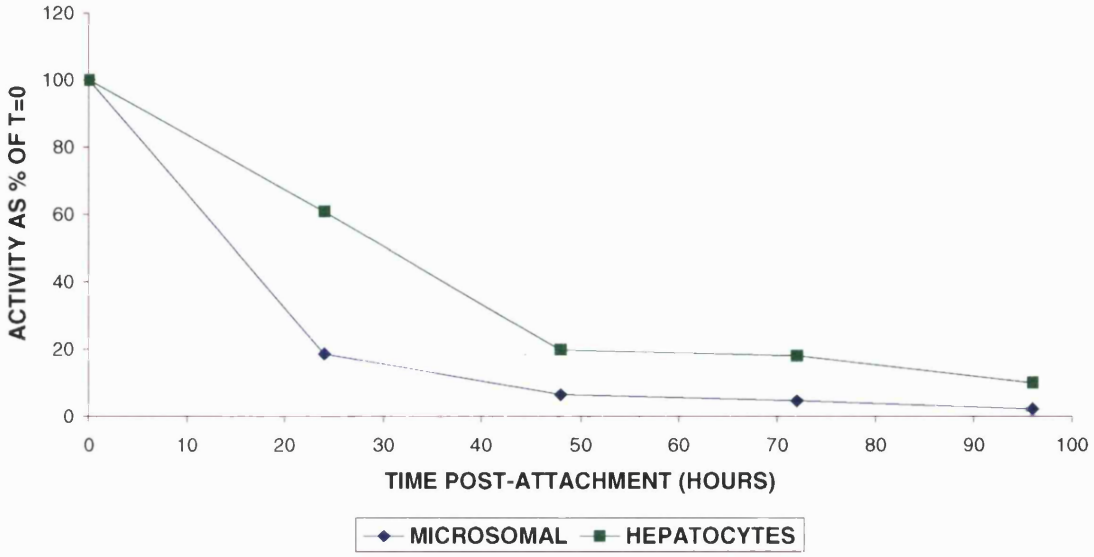


Figure 3.27 Comparison of hepatocyte and microsomal 16α-hydroxylase activity.

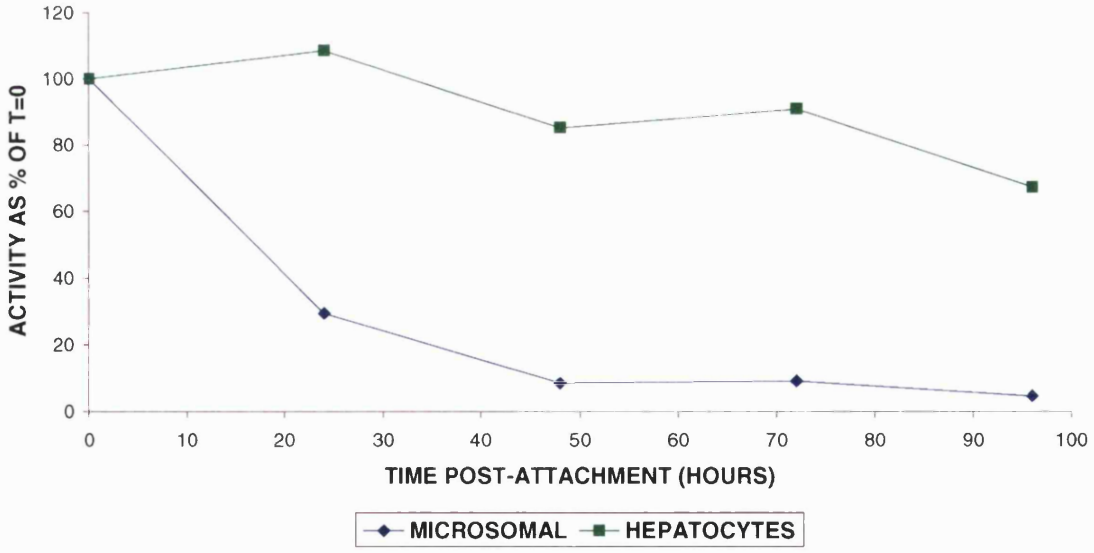


Figure 3.28 Comparison of hepatocyte and microsomal androstenedione production.

Microsomal metabolism of testosterone produced the same metabolites as those seen following incubation of testosterone with intact hepatocytes. However, a slight difference was noted in the relative abundance of metabolites. The 6β-hydroxy metabolite was more abundant than the 2α-hydroxy metabolite in intact hepatocytes isolated by collagenase and maintained on Matrigel®, while this was reversed in the microsomal system. This may be an artefact of the microsomal system, since as previously described, the ionic strength and pH of the buffer can influence the production of metabolites (Gemzik et al., 1990), although some studies in intact hepatocytes also report the greater production of 2α -hydroxy testosterone compared to 6β-hydroxy testosterone (Utesch et al., 1991).

Testosterone metabolism was slightly higher in intact hepatocytes compared to microsomes and this is in agreement with previous observations (Wortelboer et al., 1990). This may also be an artefact of the microsomal system relating to the incubation conditions, or may be due to a change in the lipid environment of the microsomes in which the cytochrome P450 enzymes are embedded since this has been shown to effect activity (Skett, 1988; Guengerich, 1984). The relative activity of the different cytochrome P450 enzymes may also be effected by other factors such as protein binding or to competition for the NADPH cytochrome P450 reductase enzyme (Li et al, 1997).

The results show that the decrease in testosterone metabolism in cultured hepatocytes is also seen in microsomes prepared from cultured hepatocytes. One notable difference, however, was that the well-maintained androstenedione production observed in intact hepatocytes is not seen in microsomes, an observation consistent with previous reports (Utesch et al., 1991; Wortelboer et al., 1990). These data may be due to the fact that not all components of the hepatocyte are retained in the microsomal preparation with loss of cytosolic and other sub-cellular fractions. The 17β-hydroxysteroid dehydrogenase enzyme has been reported to be present in the cytosol (Utesch et al., 1992), which is not retained in the microsomal preparation. The decline in activity observed in microsomes, therefore, is probably due to the

decrease in CYP2B1/2 and CYP2C11 which is consistent with the decline in 2α-, 16α- and 16β-hydroxy testosterone metabolites.

These data suggest that transport of probe substrate into the hepatocyte, and availability of the co-factor NADPH, are not responsible for the decline in testosterone metabolism by rat hepatocytes maintained in monolayer culture.

Overall, these results show a large decrease in testosterone metabolism mediated by the cytochrome P450 enzymes CYP2C11, CYP3A1/2, CYP2A1 and CYP2B1/2. This is in accordance with previous studies where extensive cytochrome P450 losses of up to 70 to 80 % within 24 hours have been reported (Utesch et al., 1991; Niwa et al., 1996; Evans et al., 1995; Dich et al., 1988; Evarts et al., 1984; Engelmann et al., 1985; Blankson et al., 1991; Nieman et al., 1991) in addition to the loss of many cytochrome P450 mRNAs (Padgham et al., 1994). The first 24 hours of culture was the period where the greatest decline in activity was generally observed in the studies reported here and in published studies (Goodwin et al., 1996), although the rate of decline in activity varied with the enzyme, the isolation technique and the substrata.

The activity of most enzymes measured was very low following 96 hours in culture for all combinations of isolation technique and substrata which is in agreement with published data (Utesch et al., 1991). Androstenedione was the only metabolite whose production was well maintained by hepatocytes isolated by collagenase and by hepatocytes isolated by EDTA and maintained on Matrigel®. The reason for this was not further investigated, but may be due to the continued presence of 17β-hydroxysteroid dehydrogenase which also catalyses this reaction.

The reason for the loss in cytochrome P450 gene expression has yet to be defined but may include loss of cell-cell contacts, the isolation technique, the substrata, the loss of regulatory factors such as glucocorticoids, growth hormones and other liver specific differentiation factors, or that a defect has occurred in either haem synthesis or in the utilisation of haem (Paine 1990). These factors have been investigated in large numbers of studies (Rogiers and Vercruysse, 1993, review). For example the loss of cell-cell contacts has been addressed using liver slices where the architecture of the

liver is maintained, revised isolation techniques and different substrata have been proposed, and the effect of many media additives determined (Gulati and Skett, 1989; Kleinman et al., 1985; Dich et al., 1988). The loss of cytochrome P450 may also be attributable to the decline in liver transcription factors such as HNF1, although the reason for the loss of these factors is also unknown (Padgham et al., 1994).

The issue of haem synthesis and utilisation has also been addressed, and the rate-limiting step in the synthesis of haem, the production of δ-aminolaevulinic acid, is avoided in these studies by the addition of this component to the culture media. The utilisation of haem, or the transport of haem to the cytochrome P450, may however be factors in the loss of cytochrome P450 activity in cultured hepatocytes since other haemoproteins such as tryptophan 2,3-dioxygenase that utilise large amounts of haem also show a rapid decrease in cultured hepatocytes while other non-haem enzymes such as lactate dehydrogenase are maintained (Paine, 1990).

3.3.12 Summary of the effect of isolation technique on cytochrome P450 activity

The effect of both isolation technique and substrata were examined in these studies and found to have statistically significant effects on subsequent testosterone metabolism by cultured rat hepatocytes. The rate of decline in activity varied with the enzyme, and this has been seen previously in both the rat and other species (Daujat et al., 1987).

The data obtained in these studies suggest that of these two factors, for the enzymes monitored by testosterone metabolism, the isolation technique has a greater influence on subsequent metabolism than the substrata. The isolation procedure has been described as the greatest stress factor involved in hepatocyte culture, and it is not surprising therefore that different isolation procedures effect the hepatocytes differentially (Bader et al., 1999).

The EDTA method had previously been reported (Wang et al., 1985) as a means of producing primary hepatocytes in culture which retained differentiated functions such as albumin and triglyceride synthesis and secretion. Meredith (1988) was able to

demonstrate no loss of cytochrome P450 during culture following EDTA isolation, but the complement of enzymes making up the total was not determined and it is possible that the total P450 measured was not made up of the enzymes expressed constitutively. Work carried out by Bayad et al. (1991) showed that the levels of phase I and II drug metabolising enzymes were more stable in EDTA-isolated cells, with P450 levels in collagenase-isolated cells only 35% of those in EDTA isolated cells after 4 days in culture. This percentage dropped further, to only 20% by day 8. This finding could not be reproduced in the work presented here or in other studies (Wright and Paine, 1992; Utesch et al., 1991).

Despite reports of enhanced cytochrome P450 expression, the use of EDTA does not appear to have been extensively adopted as an isolation technique and publications involving the use of EDTA-isolated hepatocytes are relatively rare. Therefore very little data has been published regarding specific cytochrome P450 activity in EDTA-isolated cultured hepatocytes for comparison. In the Meredith (1988) study only total cytochrome P450 measurements were made and the Bayad (1991) study followed only ethoxyresorufin O-deethylase (CYP1A) and pentoxyresorufin O-deethylase (CYP2B) activity. The results presented here suggest that EDTA isolation resulted in significantly higher activity for CYP2C11 catalysed reactions (2α-, and 16α-hydroxylation) at certain timepoints, supporting the claims that EDTA isolation can result in improved maintenance of certain cytochrome P450 enzymes. However, these studies also indicate that collagenase was the preferential method for the maintenance of 6β-hydroxylation (CYP3A1/2), 7α-hydroxylation (CYP2A1) and androstenedione production. The collagenase isolation technique was also the method of preference with respect to both hepatocyte yield and viability. Since CYP3A1/2 and CYP2A activity were not measured in previous studies the effect of EDTA isolation with respect to these enzymes cannot be compared.

The lower cell yield, cell viability and cytochrome P450-related activity in EDTA-isolated hepatocytes observed in these studies is probably multi-factorial in origin. Isolation of hepatocytes using the EDTA method was a longer process resulting in prolonged exposure of hepatocytes to hypocalcaemic conditions which may have resulted in calcium depletion within the hepatocytes. Cells prepared without calcium

have a lower K^+ uptake capacity and a lower membrane potential than cells prepared in the presence of calcium (Barnabei et al., 1974; Baur et al., 1975) so that a period of calcium depletion may have affected the hepatocytes isolated using this method. This method also required the use of greater mechanical force to liberate hepatocytes which may have caused a greater degree of plasma membrane shearing and damage when compared to collagenase-isolated cells. In addition to the reduced yield and viability, hepatocytes isolated using this technique would have been exposed to a greater level of enzymes released from the lysed cells during the initial attachment phase. This may have had a detrimental effect on the performance of the hepatocytes remaining in culture.

As discussed, collagenase isolation of hepatocytes offers certain advantages over EDTA isolation in terms of maintenance of most cytochrome P450-related activity measured, cell viability, and cell yield. However, there was still a rapid decline in activity of the enzymes measured in hepatocytes isolated by collagenase. This confirms other studies showing that within 24 hours of isolation using collagenase all cytochrome P450 mRNA had declined except that of CYP1A1 which is not normally expressed constitutively in the liver (Padgham and Paine, 1993). It has been concluded that the isolation procedure alters gene expression and this is supported by the data generated in the current work. Neither technique adopted enabled isolation of hepatocytes which maintained testosterone metabolism at initial levels.

3.3.13 Summary of the effect of substrata on cytochrome P450 activity

The effect of substrata on testosterone metabolism was found to be less pronounced than that of isolation technique. Metabolism of testosterone was generally lower in hepatocytes maintained on plastic, and androstenedione production was preferentially maintained on Matrigel®. However, apart from these effects the nature of the substrata did not appear to have had a major impact on cytochrome P450-mediated metabolism. This is in agreement with data showing the minimal effect of substrata on testosterone metabolism in rat hepatocytes cultured on collagen and plastic (Utesch et al., 1991), of collagen and fibronectin in biotransformation (van't Klooster et al.,1992), of Vitrogen® and Matrigel® on certain cytochrome P450 enzymes (Kocarek

et al, 1992) of collagen and Matrigel® on CYP3A1/2 and CYP2B1/2 (Silva et al., 1998) and of collagen, laminin and fibronectin on several cytochrome P450 enzymes (Saad et al., 1993).

In contrast to these there have been many reports on the effect of the composition and organisation of the substrata, and of Matrigel® as a substrata or overlay in particular, on cell differentiation (Sidhu et al., 1994; Nagaki et al., 1995; Schuetz et al., 1988; Bissell et al., 1987; Silva et al., 1998; Bissell et al., 1990). The proposed mechanism by which cell substratum interactions mediate regulation of tissue specific gene expression is via glycoproteins located on the cell surface and in the extracellular matrix (Corlu et al., 1991; Edelman et al., 1990). These extracellular matrix components work in co-operation with hormones and growth factors to regulate liver differentiation. The matrix can also bind certain hormones and growth factors and form complexes with them, affecting both stability and ability to bind to their respective receptors (Reid, 1990). In addition the extracellular matrix dictates the profile of hormone receptors, ion channels, intracellular pH, and the cytoskeletal arrangements and patterns that determine the cell shape (Watt, 1986) as well as influencing expression of liver-specific transcription factors such as hepatocyte nuclear factors 1 and 4 (Nagaki et al., 1995). It has also been shown that cell-extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes (Rana et al., 1994).

The matrix may, therefore, have reasonably been expected to have an effect on the expression of cytochrome P450 in cultured hepatocytes and the lack of effect of substrata in these studies was therefore disappointing. One reason for this may have been the absence of dexamethasone from the culture medium since it has been suggested that this is required in order to retain good basal expression of CYP3A1/2 (Silva et al., 1998).

The unfortunate fact remains that despite extensive work in this area there has been limited progress in the maintenance of cytochrome P450 enzymes in cultured hepatocytes. The studies reported here clearly show that the isolation technique and the substrata to which hepatocytes are attached can influence the cytochrome P450 enzymes but do not maintain activity at the level seen immediately post-attachment.

3.4 WESTERN BLOTTING ANALYSIS

Western blotting analysis is a technique enabling the detection and quantification of proteins, which has been used to quantify the cytochrome P450 enzymes (Shean and Paine, 1990). The technique involves sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer and immunodetection using enzyme-specific antibodies. This technique has the advantage over other methods, such as dot blots and ELISA assays, in that the proteins are separated spatially on a gel and therefore any non-specific binding has a minimal interference. Dot blots and ELISA have a greater reliance, therefore, on the availability of very specific antibodies which do not cross react with other proteins. However, although separation of the proteins by electrophoresis helps to minimise the effect of cross-reactivity this can still be problematic if the particular protein of interest is not separated from cross-reacting proteins.

The use of this technique expanded the number of cytochrome P450 enzymes examined compared to those detected by testosterone metabolism due to the availability of antibodies for a large number of cytochrome P450 enzymes. For each enzyme a standard curve was constructed to determine the range of protein over which a linear colorimetric response was obtained in microsomes prepared from freshly isolated hepatocytes. The time taken for blots to develop sufficient colour to be measured increased with the length of time the hepatocytes had been cultured, indicating that there was less enzyme in later samples. For hepatocytes obtained at these later timepoints sensitivity was more of a problem than linearity of response.

A single standard was included on each blot to aid in the identification of the enzyme of interest in samples. It was not possible to run a standard curve with the samples due to the limited number of sample wells available and consequently the technique used is only semi-quantitative under these conditions. It is also important to recognise that the peak areas from different blots cannot be directly compared since the exact conditions of development, for example the time taken for colour to develop and the number of times the antibody has been used, will not be constant from day to day.

The expression of several cytochrome P450 enzymes and NADPH-cytochrome P450 reductase in cultured rat hepatocytes was examined over a 96 hours culture period and the results shown below. The colorimetric density of the bands for CYP1A1, CYP1A2, CYP2B1/2, CYP2C6, CYP2C11, CYP2E1, CYP3A1/2, CYP4A and NADPH-cytochrome P450 reductase at 0, 24, 48, 72 and 96 hours post-attachment are shown in Tables 3.13 to 3.17.

3.4.1 The CYP1 Family

The primary antibody used for CYP1A1 has good specificity, showing only a single band in liver microsomes from BNF treated rats. In fact since CYP1A1 and CYP1A2 were spatially separated in the system used during these studies the specificity of the antibody was not crucial. A typical blot showing the detection of CYP1A1 is shown in Figure 3.29.

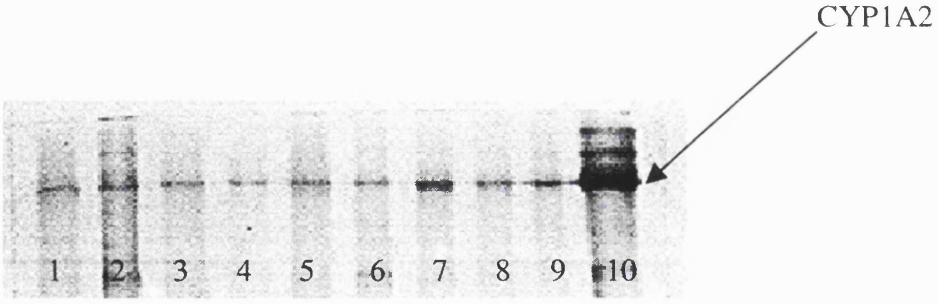
1 2 3 4 5 6 7 8 9 10

Lane	1	Plastic / collagenase (10µg)
	2	Matrigel® / collagenase (10µg)
	3	Fibronectin / collagenase (10µg)
	4	Vitrogen® / collagenase (10µg)
	5	Plastic / collagenase (10µg)
	6	Matrigel® / EDTA (10µg)
	7	Fibronectin / EDTA (10µg)
	8	Vitrogen® / EDTA (10µg)
	9	Plastic / EDTA (10µg)
	10	BNF induced microsomes (1µg)

Figure 3.29 Western blot of CYP1A1 obtained from microsomes prepared from cultured rat hepatocytes at 48 hours post-attachment.

The amount of immunodetectable CYP1A1 enzyme was below the limit of detection in all samples at 0 and 24 hours post-attachment. However, the enzyme was detected in all hepatocytes collected at 48, 72 and 96 hours post-attachment. The highest levels of immunodetectable CYP1A1 were in collagenase-isolated cells at 48, 72 and 96 hours post-attachment, particularly those maintained on Matrigel® and fibronectin. Following 96 hours in culture, the amount of CYP1A1 was highest in collagenase-isolated cells maintained on Matrigel® and lowest in EDTA-isolated cells maintained on fibronectin.

The antibody used to detect CYP1A2 was not specific for this enzyme and cross-reacted with CYP1A1. However, since in the early samples CYP1A1 was not expressed and the two enzymes were spatially separated, the lack of specificity did not interfere with analysis. Freshly isolated hepatocytes were found to give a linear response at up to 25ug protein loaded onto the gel. CYP1A2 was detectable in all samples obtained from hepatocytes immediately post-attachment, and a typical blot showing the detection of CYP1A2 is shown in Figure 3.30.



Lane 1 Plastic / collagenase (25µg)
2 Matrigel® / collagenase (25µg)
3 Fibronectin / collagenase (25µg)
4 Vitrogen® / collagenase (25µg)
5 Plastic / collagenase (25µg)
6 Matrigel® / EDTA (25µg)
7 Fibronectin / EDTA (25µg)
8 Vitrogen® / EDTA (25µg)
9 Plastic / EDTA (25µg)
10 BNF induced microsomes (1µg)

Figure 3.30 Western blot of CYP1A2 in cultured rat hepatocytes at 24 hours post-attachment.

The CYP1A2 enzyme was detected in the majority of samples including those obtained from hepatocytes cultured for 96 hours. The amounts of immunodetectable CYP1A2 were broadly similar in collagenase and EDTA-isolated hepatocytes at 0, 24 and 48 hours post-attachment but were generally higher in collagenase-isolated hepatocytes at 72 and 96 hours post-attachment. However, this was not exclusively the case, for example the highest amount of immunodetectable CYP1A2 at 72 hours was in EDTA-isolated cells cultured on Vitrogen®. Following 96 hours in culture the amount of CYP1A2 was highest in collagenase-isolated cells maintained on Matrigel® and lowest in EDTA-isolated cells maintained on fibronectin.

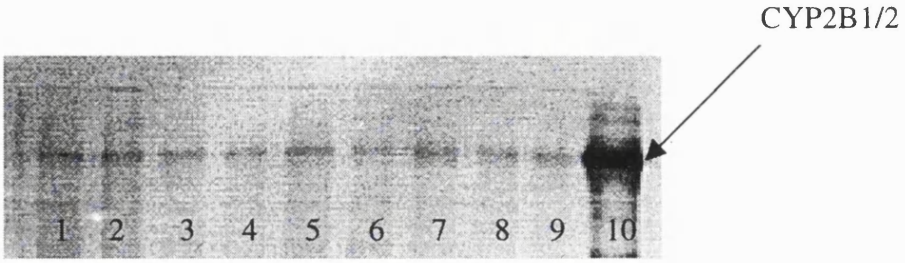
The results show that amounts of CYP1A2 were particularly low in hepatocytes isolated by EDTA and cultured on Matrigel® immediately post-attachment. This particular sample gave consistently low results in all subsequent blots for other enzymes. However, results from hepatocytes isolated by EDTA and cultured on Matrigel® for 24, 48, 72 and 96 hours were not unusually low. Furthermore, the immunodetectable levels of cytochrome P450 enzymes in hepatocytes isolated by EDTA and maintained on other substrata at 0 hours post-attachment were not unusually low. The microsomes were prepared at the same time as other samples and the protein content of this sample was much lower than that of the other samples. It is likely, therefore, that a problem has arisen during the preparation of the microsomes from these hepatocytes obtained immediately post-attachment and for this reason this data was omitted from the statistical analysis.

3.4.2 The CYP2 Family

Western Blotting analysis of a number of cytochrome P450 enzymes belonging to the CYP2 family were carried out, including CYP2B1/2, CYP2C6, CYP2C11 and CYP2E1.

The primary CYP2B antibody used cross-reacts with CYP2B1 and CYP2B2. Since only CYP2B2 is usually expressed in the liver the band detected by the antibody was most likely to be CYP2B2 (Soucek and Gut, 1992). However, since the antibody cross-reacts with both enzymes, and it is not known whether these enzymes would be

separated spatially on the gel, results have been expressed as CYP2B1/2. Freshly isolated hepatocytes were found to give a linear response at up to 10ug protein loaded onto the gel and a typical blot showing the detection of CYP2B1/2 is shown in Figure 3.31.

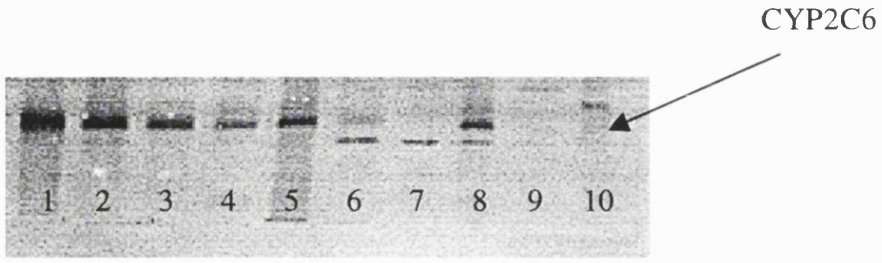


Lane 1 Plastic / collagenase (10µg)
2 Matrigel® / collagenase (10µg)
3 Fibronectin / collagenase (10µg)
4 Vitrogen® / collagenase (10µg)
5 Plastic / collagenase (10µg)
6 Matrigel® / EDTA (10µg)
7 Fibronectin / EDTA (10µg)
8 Vitrogen® / EDTA (10µg)
9 Plastic / EDTA (10µg)
10 PB induced microsomes (1µg)

Figure 3.31 Western blot of CYP2B1/2 in cultured rat hepatocytes at 24 hours post-attachment.

The CYP2B1/2 enzyme(s) was detected in the majority of samples including those obtained from hepatocytes cultured for 96 hours. The bands, however, had become very faint in samples obtained at 72 and 96 hours post-attachment suggesting that the amounts of this enzyme were low. The amount of immunodetectable CYP2B1/2 were broadly similar in collagenase and EDTA-isolated hepatocytes at 24, 48 and 96 hours post-attachment, but were generally higher in collagenase-isolated hepatocytes at 0 and 72 hours post-attachment. Following 96 hours in culture the amount of CYP2B1/2 was highest in collagenase-isolated cells maintained on Matrigel® and lowest in EDTA-isolated cells maintained on fibronectin.

The primary CYP2C6 antibody used was found to cross-react with one other protein in untreated rat microsomes. In samples obtained from hepatocytes at later timepoints a band was observed running faster than the CYP2C6 band. This indicates the presence of either a cross-reacting protein or possibly a partially degraded CYP2C6 which has retained the ability to interact with the antibody but which is smaller in size. Freshly isolated hepatocytes were found to give a linear response at up to 25ug protein loaded onto the gel. A typical blot showing the detection of CYP2C6 is shown in Figure 3.32.

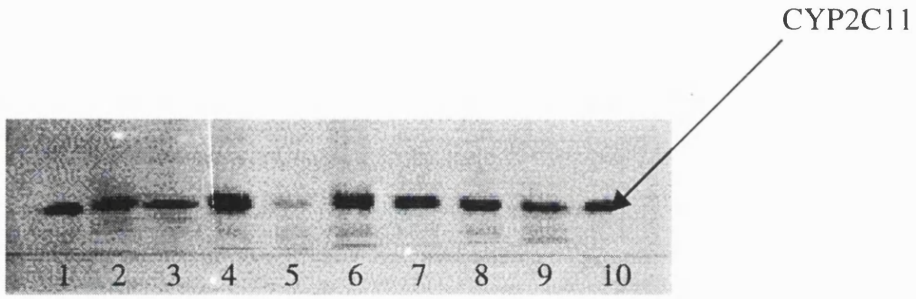


Lane 1 Matrigel® / collagenase (25µg)
2 Fibronectin / collagenase (25µg)
3 Vitrogen® / collagenase (25µg)
4 Plastic / collagenase (25µg)
5 Matrigel® / EDTA (25µg)
6 Fibronectin / EDTA (25µg)
7 Vitrogen® / EDTA (25µg)
8 Plastic / EDTA (25µg)
9 Empty (25µg)
10 Untreated rat microsomes (5µg)

Figure 3.32 Western blot of CYP2C6 in cultured rat hepatocytes at 24 hours post-attachment.

The CYP2C6 enzyme was detected in the majority of samples including those obtained from hepatocytes cultured for 96 hours. The amounts of immunodetectable CYP2C6 were similar in collagenase and EDTA-isolated hepatocytes at 0 and 24 hours post-attachment, but were generally higher in collagenase-isolated hepatocytes at 48, 72 and 96 hours post-attachment. Following 96 hours in culture the amount CYP2C6 was highest in collagenase-isolated cells maintained on Matrigel® and lowest in EDTA-isolated cells maintained on plastic.

The primary CYP2C11 antibody used produced a single band in samples from both hepatocytes maintained in culture and in hepatic microsomes from untreated rats. Freshly isolated hepatocytes were found to give a linear response at up to 7.5ug protein loaded onto the gel. A typical blot showing the detection of CYP2C11 is shown in Figure 3.33.

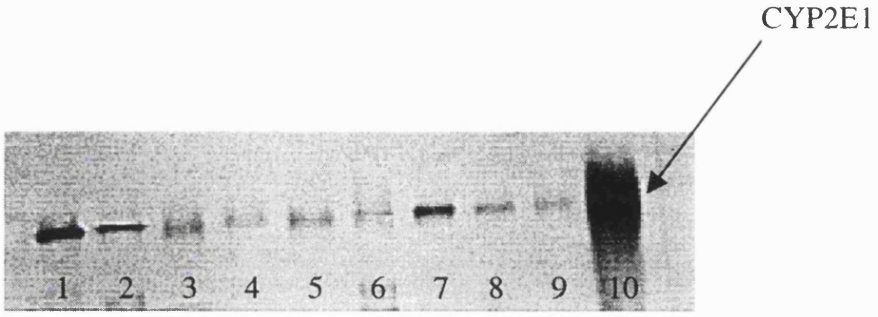


Lane 1 Matrigel® / collagenase (7.5µg)
2 Fibronectin / collagenase (7.5µg)
3 Vitrogen® / collagenase (7.5µg)
4 Plastic / collagenase (7.5µg)
5 Matrigel® / EDTA (7.5µg)
6 Fibronectin / EDTA (7.5µg)
7 Vitrogen® / EDTA (7.5µg)
8 Plastic / EDTA (7.5µg)
9 Fibronectin / collagenase (7.5µg)
10 Untreated rat microsomes (5µg)

Figure 3.33 Western blot of CYP2C11 in cultured rat hepatocytes at 24 hours post-attachment.

The CYP2C11 enzyme was detected in the majority of samples including those obtained from hepatocytes cultured for 96 hours. The amounts of immunodetectable CYP2C11 were broadly similar in collagenase and EDTA-isolated hepatocytes at 0 and 24 hours post-attachment, but were generally higher in collagenase-isolated hepatocytes at 48, 72 and 96 hours post-attachment. Following 96 hours in culture the amount of CYP2C11 was highest in collagenase-isolated cells maintained on Matrigel® and lowest in EDTA-isolated cells maintained on plastic.

The primary CYP2E1 antibody used was shown to recognise a single band. Freshly isolated hepatocytes were found to give a linear response at up to 5µg protein loaded onto the gel. A typical blot showing the detection of CYP2E1 is shown in Figure 3.34.



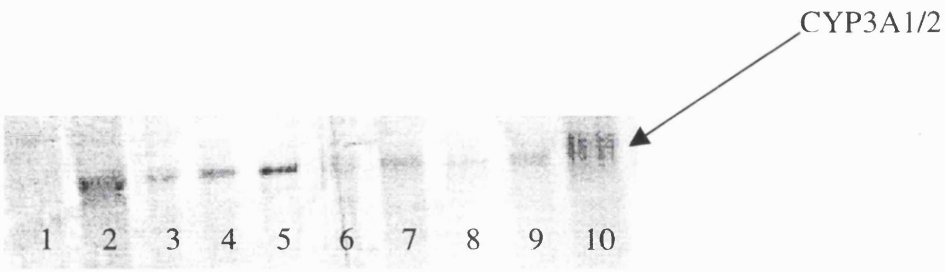
Lane
1 Matrigel® / collagenase (5µg)
2 Matrigel® / collagenase (5µg)
3 Fibronectin / collagenase (5µg)
4 Vitrogen® / collagenase (5µg)
5 Plastic / collagenase (5µg)
6 Matrigel® / EDTA (5µg)
7 Fibronectin / EDTA (5µg)
8 Vitrogen® / EDTA (5µg)
9 Plastic / EDTA (5µg)
10 Pyrazole induced rat microsomes (5µg)

Figure 3.34 Western blot of CYP2E1 in cultured rat hepatocytes at 24 hours post-attachment.

The CYP2E1 enzyme was detected in the majority of samples including those obtained from hepatocytes cultured for 96 hours. The amounts of immunodetectable CYP2E1 were broadly similar in collagenase and EDTA-isolated hepatocytes at 0, 24, 72 and 96 hours post-attachment, but was generally higher in collagenase-isolated hepatocytes at 48 hours post-attachment. Following 96 hours in culture the amount of CYP2E1 was highest in EDTA-isolated cells maintained on Matrigel® and lowest in EDTA-isolated cells maintained on fibronectin and plastic.

3.4.3 The CYP3 Family

The primary antibody used has been shown to react with two proteins in rat liver, probably CYP3A1 and CYP3A2. Since only one band was detected on blots it is possible that these two proteins have not separated on the gel and results have therefore been expressed as CYP3A1/2. Freshly isolated hepatocytes were found to give a linear response at up to 25µg protein loaded onto the gel. A typical blot showing the detection of CYP3A1/2 is shown in Figure 3.35.



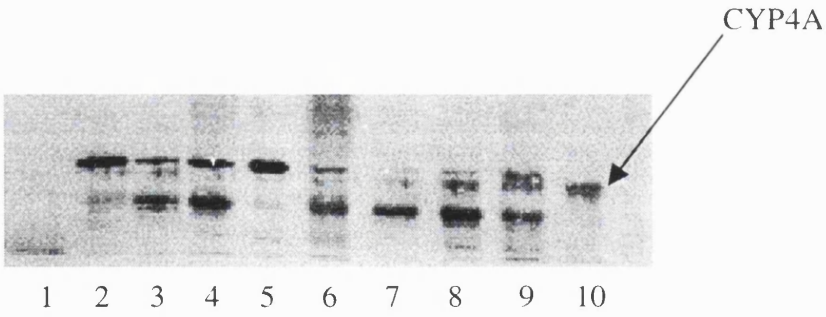
Lane 1 Empty
2 Matrigel® / collagenase (25µg)
3 Vitrogen® / collagenase (25µg)
4 Plastic / collagenase (25µg)
5 Fibronectin / collagenase (25µg)
6 Matrigel® / EDTA (25µg)
7 Fibronectin / EDTA (25µg)
8 Vitrogen® / EDTA (25µg)
9 Plastic / EDTA (25µg)
10 PCN induced rat microsomes (5µg)

Figure 3.35. Western blot of CYP3A1/2 in cultured rat hepatocytes at 0 hours post-attachment

The CYP3A1/2 enzyme was detected in the majority of samples including those obtained from hepatocytes cultured for 96 hours. The amounts of immunodetectable CYP3A1/2 were broadly similar in collagenase and EDTA-isolated hepatocytes at 24 and 72 hours post-attachment, but were generally higher in collagenase-isolated hepatocytes at 0, 48 and 96 hours post-attachment. Following 96 hours in culture the amount of CYP3A1/2 was highest in collagenase-isolated cells maintained on Matrigel® and lowest in EDTA-isolated cells maintained on fibronectin and plastic.

3.4.4 The CYP4 Family

The primary antibody produced one band in clofibrate induced rat microsomes. Extra bands were observed in hepatocytes which had been cultured for 24 hours or longer, and unfortunately no data was available with respect to the cross-reactivity of the antibody used. The extra bands may indicate the presence of either cross-reacting proteins or possibly partially degraded CYP4A enzymes, which have retained the ability to interact with the antibody, but which are smaller in size. There are at least four members of the CYP4A subfamily in the rat and the extra bands may indicate that the spectrum of CYP4A enzymes expressed in the hepatocytes have altered. Freshly isolated hepatocytes were found to give a linear response at up to 15µg protein loaded onto the gel. A typical blot showing the detection of CYP4A is shown in Figure 3.36.



Lane 1 Empty
2 Fibronectin / collagenase (15µg)
3 Plastic / collagenase (15µg)
4 Vitrogen® / collagenase (15µg)
5 Matrigel® / collagenase (15µg)
6 Matrigel® / EDTA (15µg)
7 Plastic / EDTA (15µg)
8 Vitrogen® / EDTA (15µg)
9 Fibronectin / EDTA (15µg)
10 Clofibrate induced rat microsomes (2.5µg)

Figure 3.36. Western blot of CYP4A in cultured rat hepatocytes at 24 hours post-attachment

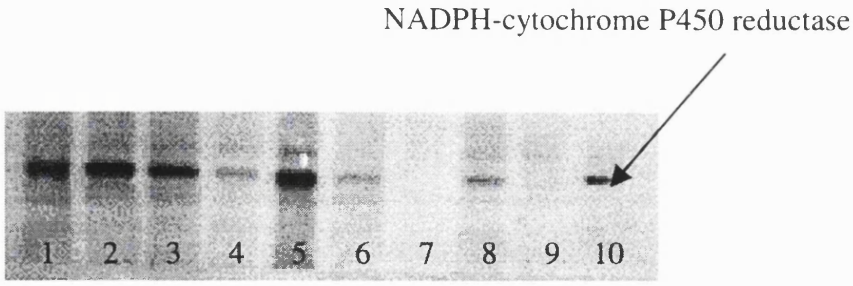
The CYP4A enzyme was detected in the majority of samples including those obtained from hepatocytes cultured for 96 hours. The amounts of immunodetectable CYP4A

were broadly similar in collagenase and EDTA-isolated hepatocytes at 48 hours post-attachment, but were generally higher in collagenase-isolated hepatocytes at 0, 24, 72 and 96 hours post-attachment. At 24 hours post-attachment, amounts of CYP4A were particularly high in hepatocytes maintained on fibronectin. Following 96 hours in culture the amount of CYP4A was highest in collagenase-isolated cells maintained on fibronectin and lowest in EDTA-isolated cells maintained on fibronectin and plastic.

3.4.5 NADPH-Cytochrome P450 Reductase

NADPH-cytochrome P450 reductase was analysed by Western blotting in addition to the cytochrome P450 enzymes to give some information about this part of the cytochrome P450 system. The primary antibody was shown to react with a single protein in rat liver microsomes. Freshly isolated hepatocytes were found to give a linear response at up to 20ug protein loaded onto the gel. A typical blot showing the detection of NADPH-cytochrome P450 reductase is shown in Figure 3.37.

The NADPH-cytochrome P450 reductase enzyme was detected in the majority of samples including those obtained from hepatocytes cultured for 96 hours. The amounts of immunodetectable NADPH-cytochrome P450 reductase were broadly similar in collagenase and EDTA-isolated hepatocytes at 0 and 48 hours post-attachment, but were generally higher in collagenase-isolated hepatocytes at 24, 72 and 96 hours post-attachment. At 0, 24 and 48 hours post-attachment amounts of NADPH-cytochrome P450 reductase were particularly high in hepatocytes maintained on fibronectin. Following 96 hours in culture the amount of NADPH-cytochrome P450 reductase was highest in collagenase-isolated cells maintained on Matrigel® and lowest in EDTA-isolated cells maintained on fibronectin.



Lane 1 Matrigel® / collagenase (20µg)
2 Matrigel® / collagenase (20µg)
3 Fibronectin / collagenase (20µg)
4 Vitrogen® / collagenase (20µg)
5 Plastic / collagenase (20µg)
6 Matrigel® / EDTA (20µg)
7 Fibronectin / EDTA (20µg)
8 Vitrogen® / EDTA (20µg)
9 Plastic / EDTA (20µg)
10 Untreated rat microsomes (5µg)

Figure 3.37. Western blot of NADPH-cytochrome P450 reductase in cultured rat hepatocytes at 96 hours post-attachment.

The increase in time taken for bands to develop with the increase in culture time for the cytochrome P450 enzymes was not observed for the NADPH-cytochrome P450 reductase enzyme, where bands developed quickly for samples from all timepoints. This suggests that the amounts of this enzyme had not decreased in the same way as those of the cytochrome P450 enzymes.

Table 3.13 The density of immunodetected CYP1A1, CYP1A2, CYP2B1/2, CYP2C6, CYP2C11, CYP2E1, CYP3A1/2, CYP4A and NADPH-P450 reductase bands in microsomes prepared from cultured hepatocytes at 0 hours post-attachment

SUBSTRATA	ISOLATION	CYP1A1	CYP1A2	CYP2B1/2	CYP2C6	CYP2C11	CYP2E1	CYP3A1/2	CYP4A	REDUCTASE
MATRIGEL™	COLLAGENASE	ND	14735	12452	19044	39911	**35374**	**25099**	**55489**	49560
FIBRONECTIN	COLLAGENASE	ND	12377	**14796**	**35520**	48139	25391	24118	32348	**52256**
VITROGEN™	COLLAGENASE	ND	**4894**	**1593**	19557	**24377**	**4567**	**4699**	31378	**10930**
PLASTIC	COLLAGENASE	ND	13284	1689	**15270**	37272	13658	12017	46046	19664
MATRIGEL™	EDTA	ND	ND	ND	1308	3064	1765	1255	23470	2720
FIBRONECTIN	EDTA	ND	**24832**	7652	31218	**53868**	15480	11406	25590	46900
VITROGEN™	EDTA	ND	9781	7382	25304	25061	14874	9606	**19811**	21654
PLASTIC	EDTA	ND	9331	3960	25638	25169	14606	11741	40108	27954

The highest value is denoted in bold black, the lowest value in bold red.

Table 3.14 The density of immunodetected CYP1A1, CYP1A2, CYP2B1/2, CYP2C6, CYP2C11, CYP2E1, CYP3A1/2, CYP4A and NADPH-P450 reductase bands in microsomes prepared from cultured hepatocytes at 24 hours post-attachment

SUBSTRATA	ISOLATION	CYP1A1	CYP1A2	CYP2B1/2	CYP2C6	CYP2C11	CYP2E1	CYP3A1/2	CYP4A	REDUCTASE
MATRIGEL™	COLLAGENASE	ND	20990	9918	37708	58634	36825	50933	47698	91047
FIBRONECTIN	COLLAGENASE	ND	12377	9486	23910	38370	29105	39665	**74964**	98423
VITROGEN™	COLLAGENASE	ND	9299	8719	55065	45588	12075	23823	49811	78244
PLASTIC	COLLAGENASE	ND	15331	15499	23988	**130235**	32766	33826	40301	113729
MATRIGEL™	EDTA	ND	**5602**	**5504**	30539	**10791**	17411	36169	7283	**37067**
FIBRONECTIN	EDTA	ND	**35293**	**15586**	**8588**	84577	**41647**	**55215**	66487	**171163**
VITROGEN™	EDTA	ND	13510	11353	21620	66150	17935	30225	21611	60625
PLASTIC	EDTA	ND	12824	5640	**56159**	66722	**10544**	**15924**	**5833**	50798

The highest value is denoted in bold black, the lowest value in bold red.

Table 3.15 The density of immunodetected CYP1A1, CYP1A2, CYP2B1/2, CYP2C6, CYP2C11, CYP2E1, CYP3A1/2, CYP4A and NADPH-P450 reductase bands in microsomes prepared from cultured hepatocytes at 48 hours post-attachment

SUBSTRATA	ISOLATION	CYP1A1	CYP1A2	CYP2B1/2	CYP2C6	CYP2C11	CYP2E1	CYP3A1/2	CYP4A	REDUCTASE
MATRIGEL™	COLLAGENASE	**26192**	13315	14861	54311	43074	**23947**	**31366**	**22180**	39306
FIBRONECTIN	COLLAGENASE	17029	12468	15660	51362	52506	18072	13344	18613	45510
VITROGEN™	COLLAGENASE	7805	**4085**	**4524**	17115	33550	9561	23877	**4593**	36433
PLASTIC	COLLAGENASE	15691	**15275**	12354	**58342**	**145322**	21824	10966	16705	30051
MATRIGEL™	EDTA	3354	5924	11525	30859	32802	10349	9199	8365	**17386**
FIBRONECTIN	EDTA	6628	10813	18548	18840	19420	18945	**4935**	7010	**78288**
VITROGEN™	EDTA	**2545**	6323	**28470**	41948	34770	16720	6873	15400	37768
PLASTIC	EDTA	6060	8775	17985	**8128**	ND	ND	6393	8278	39020

The highest value is denoted in bold black, the lowest value in bold red.

Table 3.16 The density of immunodetected CYP1A1, CYP1A2, CYP2B1/2, CYP2C6, CYP2C11, CYP2E1, CYP3A1/2, CYP4A and NADPH-P450 reductase bands in microsomes prepared from cultured hepatocytes at 72 hours post-attachment

SUBSTRATA	ISOLATION	CYP1A1	CYP1A2	CYP2B1/2	CYP2C6	CYP2C11	CYP2E1	CYP3A1/2	CYP4A	REDUCTASE
MATRIGEL™	COLLAGENASE	61354	30744	**15126**	**121465**	29173	**32342**	8740	30191	**72487**
FIBRONECTIN	COLLAGENASE	**62769**	33728	6035	105143	**30662**	18138	4943	**38396**	68078
VITROGEN™	COLLAGENASE	22744	15948	2158	57094	20718	5959	**2205**	21149	38045
PLASTIC	COLLAGENASE	25864	15366	6286	65900	9949	13986	2800	35991	45922
MATRIGEL™	EDTA	13366	2930	6234	11328	14936	27994	2278	11212	24908
FIBRONECTIN	EDTA	**ND**	**ND**	**ND**	**ND**	**ND**	20463	**23275**	**2813**	4175
VITROGEN™	EDTA	5415	**34945**	3865	5718	29968	29698	6335	30725	21980
PLASTIC	EDTA	**ND**	10160	**ND**	5613	7870	**3555**	6548	5188	**3398**

The highest value is denoted in bold black, the lowest value in bold red.

Table 3.17 The density of immunodetected CYP1A1, CYP1A2, CYP2B1/2, CYP2C6, CYP2C11, CYP2E1, CYP3A1/2, CYP4A and NADPH-P450 reductase bands in microsomes prepared from cultured hepatocytes at 96 hours post-attachment

SUBSTRATA	ISOLATION	CYP1A1	CYP1A2	CYP2B1/2	CYP2C6	CYP2C11	CYP2E1	CYP3A1/2	CYP4A	REDUCTASE
MATRIGEL™	COLLAGENASE	**82392**	**27582**	**9567**	**48093**	**60094**	58210	**15901**	30743	**59160**
FIBRONECTIN	COLLAGENASE	44340	16779	5390	34042	49219	10787	4837	**33634**	43195
VITROGEN™	COLLAGENASE	12193	10106	5912	14128	32301	11198	1034	13936	8530
PLASTIC	COLLAGENASE	17644	12244	6842	21240	48016	12004	11436	33410	54949
MATRIGEL™	EDTA	6424	9250	6689	9496	12533	**73685**	2041	5000	8604
FIBRONECTIN	EDTA	**1416**	ND	**1061**	1623	1370	ND	ND	ND	**1392**
VITROGEN™	EDTA	4052	1453	5601	12463	22995	16089	1005	19862	15730
PLASTIC	EDTA	2791	1116	2042	ND	ND	ND	ND	ND	2367

The highest value is denoted in bold black, the lowest value in bold red.

3.4.6 Statistical Analysis

Dr. R. Kempson (Statistical Services, Glaxo Wellcome) kindly performed the complicated statistical analysis of variance of this data. Significant differences in the levels of immunodetectable cytochrome P450 enzymes were observed due to isolation technique, substrata and to interactions between these two factors. As indicated previously the data obtained from EDTA-isolated hepatocytes maintained on Matrigel® immediately post-attachment were excluded and therefore, no analysis was possible for this substrata at this timepoint.

In samples obtained from hepatocytes immediately post-attachment significant differences ($P<0.05$) due to substrata were identified with higher immunodetectable levels of all enzymes except CYP4A detected in hepatocytes maintained on fibronectin. No significant differences were observed due to isolation technique or to an interaction between isolation technique and substrata.

In samples obtained from hepatocytes at 24 hours post-attachment significant differences ($P<0.05$) due to substrata, isolation technique and to an interaction between substrata and isolation technique were indicated. In particular, hepatocytes maintained on fibronectin had higher immunodetectable levels of enzymes, due to the particularly high results from hepatocytes isolated by EDTA, and this substrata-isolation technique interaction was statistically significant ($P<0.05$). For all other substrata the levels of immunodetectable enzymes were higher in collagenase-isolated hepatocytes although this was not quite significant.

In samples obtained from hepatocytes at 48 hours post-attachment significant differences ($P<0.05$) due to isolation technique and to an interaction between substrata and isolation technique were indicated. For all substrata except Vitrogen® collagenase isolation resulted in significantly higher levels of immunodetectable enzymes compared to EDTA isolation.

In samples obtained from hepatocytes at 72 hours post-attachment significant differences ($P<0.05$) due to both isolation technique and substrata were detected. The

collagenase isolation technique resulted in hepatocytes with higher levels of immunodetectable enzymes compared to EDTA-isolated cells for all enzymes except CYP2E1 and CYP3A1/2, with a particularly large difference in the levels of CYP2C6. Hepatocytes maintained on Vitrogen® were the only cells where results for collagenase-isolated cells were similar to EDTA-isolated cells. Overall the use of Matrigel® and fibronectin substrata resulted in collagenase-isolated hepatocytes with higher levels of immunodetectable enzymes than those maintained on Vitrogen® or plastic.

In samples obtained from hepatocytes at 96 hours post-attachment significant differences ($P<0.05$) due to isolation technique, substrata and to an interaction between the two were detected. Immunodetectable levels of all enzymes except CYP2E1 were much higher in collagenase-isolated compared to EDTA-isolated hepatocytes. Levels were also found to be highest in hepatocytes maintained on Matrigel® compared to other substrata. In contrast levels were particularly low in hepatocytes isolated by EDTA and maintained on fibronectin and plastic. The combination of collagenase isolation and Matrigel® substrata was particularly successful.

In conclusion these data indicate that the profile of the immunodetectable cytochrome P450 enzymes in hepatocytes varies with the isolation technique, the substrata and the length of time that the hepatocytes have been maintained under culture conditions. The pattern of effects is extremely complicated so that although generalisations can be made exceptions to these invariably exist. As with the previous data relating to testosterone metabolism, the isolation technique appeared to have more of an effect than the substrata on the cytochrome P450 enzymes. This also appears to be the case for enzymes not measured by testosterone metabolism, such as CYP1A2, CYP2C6 and NADPH-cytochrome P450 reductase.

The differences between the results for enzyme activity and immunodetectable levels of enzymes are interesting. This was particularly apparent immediately post-attachment where no effect of isolation technique were observed in the blotting data

but many effects were detected in the activity data. Furthermore the positive effect of EDTA isolation on CYP2C11 activity was not reflected in higher levels of immunodetectable CYP2C11 at 24 and 48 hours post-attachment. These, and many other differences between results obtained for enzyme activity and immunodetectable enzyme levels, were apparent. This suggests that immunodetectable levels of enzymes do not always give an accurate representation of whether the protein detected is catalytically active and this phenomenon has been reported in other studies (Johansson et al., 1988; Utesch et al., 1991) and in observations within our laboratory with other studies.

It has been known for some time that spectrally detectable cytochrome P450 is lost more rapidly than immunochemically detected cytochrome P450, which has been attributed to the presence of apo-P450 (Guengerich, 1984; Steward et al., 1985). The half-life for the protein and haem components of cytochrome P450 have been reported to be approximately 40 hours and 22 hours respectively, so that the apo-P450 will be present but not active (Omura, 1980). An apparent lack of cytochrome P450 activity, despite improved levels of immunodetectable cytochrome P450, suggests therefore that the immunodetectable protein is the apo-form of the enzyme, lacking the haem group that is essential for activity. A further possibility is that the enzyme detected immunochemically is partially degraded, whilst still of similar size and thus able to co-electrophorese with undegraded enzyme. It is also possible that the cytochrome P450 enzymes are not fully functional due to the oxygen-deficient culture conditions used, which are hypoxic compared to those *in vivo* (Bader et al., 1999; Toutain et al., 1998).

It is also possible that a defect has occurred in either haem synthesis or in the utilisation of haem (Paine 1990). The rate-limiting enzyme in the production of haem is δ-aminolaevulinic acid synthase and δ-aminolaevulinic acid was supplemented to the media throughout the current studies to help reduce the influence of haem synthesis on cytochrome P450 activity. The utilisation of haem may, however, remain a problem in the loss of cytochrome P450 activity in cultured hepatocytes. It is interesting to note that other haemoproteins such as tryptophan 2,3-dioxygenase that utilise large amounts of haem also show a rapid decrease in cultured hepatocytes

while other non-haem enzymes such as lactate dehydrogenase are maintained (Paine, 1990).

The appearance of CYP1A1, an enzyme not expressed constitutively in the liver, occurred in all hepatocytes by 48 hours post-attachment. This has been observed previously (Kocarek et al., 1993) and was attributed to the presence of tryptophan in the media. Other studies have also showed an increase in ethoxyresorufin O-deethylase activity, a reaction catalysed by CYP1A1, at 48 hours post-isolation (Grant et al., 1986; Niemann et al., 1991; Turner and Pitot, 1989) so that results obtained in the studies reported here are in accordance with those from other laboratories.

The length of time taken for the bands of colour to develop increased as the time in culture increased for all of the cytochrome P450 enzymes, although this was less so for the NADPH-cytochrome P450 reductase. This suggests that there were lower levels of cytochrome P450 enzymes in these samples, which is in agreement with the cytochrome P450 activity data. There were differences, however, in NADPH-cytochrome P450 reductase levels indicated by the western blots. This is interesting since this enzyme is responsible for feeding electrons into the cytochrome P450 enzymes. The most noticeable difference appears in EDTA-isolated hepatocytes 96 hours post-attachment where levels are lower than those in collagenase-isolated cells. This may be a further reason for the decrease in cytochrome P450 activity in those samples where NADPH-cytochrome P450 reductase levels appear to be lower.

The results indicate that there is improved maintenance of immunodetectable cytochrome P450 levels with hepatocytes isolated by collagenase. The effect of substrata was less unilateral, although immunodetectable cytochrome P450 levels in hepatocytes maintained on Matrigel® were often higher than cells cultured on other substrata, particularly at the 96 hour post-attachment timepoint. Despite this, little improvement in metabolic activity was observed.

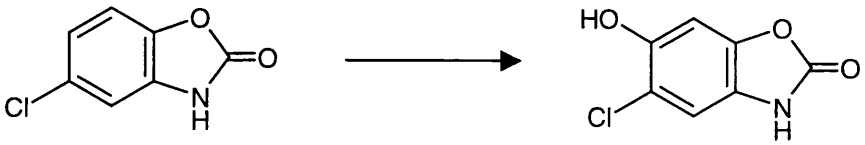
3.5 CYP2E1 EXPRESSION IN RAT HEPATOCYTES CULTURED IN THE PRESENCE OF PYRAZOLE

Although the decline of cytochrome P450 enzymes in rat hepatocyte culture has been clearly demonstrated in these studies the cells may have retained the ability for increased expression in response to chemicals known to induce these enzymes. The possibility of using cultured hepatocytes to study cytochrome P450 induction by new chemical entities would be of interest in industrial Drug Metabolism departments to study this phenomenon in candidate drugs.

It was decided to examine the expression of one enzyme, CYP2E1, in more detail and to determine whether levels of this enzyme could be increased in hepatocytes exposed to pyrazole, reported to be an inducing agent of this enzyme (Tu et al., 1981; Craft, 1985; Sinclair et al., 1986). Therefore, the levels of CYP2E1 activity and immunodetectable CYP2E1 protein were measured in rat hepatocytes maintained in monolayer culture in the presence of the CYP2E1 inducer pyrazole (25, 50, 75 and 100μM) and in control, untreated hepatocytes.

3.5.1 CYP2E1 Activity

Chlorzoxazone (5-chloro-2-hydroxybenzoxazole) is a compound used therapeutically as a centrally acting muscle relaxant. Chlorzoxazone was selected as a substrate probe for this enzyme because it has been documented that the main oxidative metabolite, 6-hydroxy chlorzoxazone, is a CYP2E1-specific product of metabolism (Peter et al., 1990) and because it has been extensively used as a CYP2E1 probe (Gebhardt et al., 1997; Schmalix et al., 1995; Court et al., 1996). The structure of chlorzoxazone and the 6-hydroxy metabolite are shown in Figure 3.38.



Chlorzoxazone 6-Hydroxy Chlorzoxazone

Figure 3.38 The structure of chlorzoxazone and 6-hydroxychlorzoxazone

It has been suggested, however, that this reaction is not completely specific for CYP2E1 with possible CYP1A and to a lesser extent, CYP3A1/2 involvement (Carriere et al., 1993 Gorski et al., 1997). CYP1A1 is not normally expressed in hepatocytes so the contribution of CYP1A1 to hepatic chlorzoxazone hydroxylation would normally be negligible. However, it was observed earlier in these studies that hepatocytes express CYP1A1 after a few days in culture and it is possible that at later times CYP1A1 has some contribution towards the metabolism of chlorzoxazone.

Other CYP2E1 substrates such as P-nitrophenol are available, however these may also be less than completely specific (Zerilli et al., 1997; Raucy et al., 1991). Chlorzoxazone was chosen as a metabolic probe for CYP2E1 activity because of increased sensitivity over nitrosamine substrates which also have the disadvantage of being established carcinogens (Koop, 1992). The issue of specificity was not felt to preclude chlorzoxazone since although other isoforms may also contribute to chlorzoxazone metabolism, the K_m for CYP2E1 has been shown to be 23 times lower than that for CYP1A1, with little involvement of other enzymes indicating that the majority of activity can be attributed to this form (Yamazaki et al., 1995). The suitability of chlorzoxazone as a CYP2E1 metabolic probe was also supported by an in-depth study by Hickman et al, 1998.

3.5.1.1 Solid Phase Extraction

Chlorzoxazone and 6-hydroxy chlorzoxazone were analysed using HPLC following solid phase extraction of samples. Samples obtained following incubation of rat hepatocytes maintained on Matrigel® for up to 96 hours post-attachment, with ^{14}C-chlorzoxazone, were used to determine recovery of radiolabelled chlorzoxazone-related material following solid phase extraction. The mean recovery of both chlorzoxazone and metabolite combined was 65%, and a non-radiolabelled 6-hydroxy chlorzoxazone standard curved was extracted which gave a linear response following HPLC detection (20 to 5000ng on column).

3.5.1.2 High Performance Liquid Chromatography

The HPLC system used was able to separate chlorzoxazone and the 6-hydroxy metabolite, with retention times of 5.4 and 14.1 minutes for metabolite and chlorzoxazone respectively. A typical chromatograph is shown in Figure 3.39.

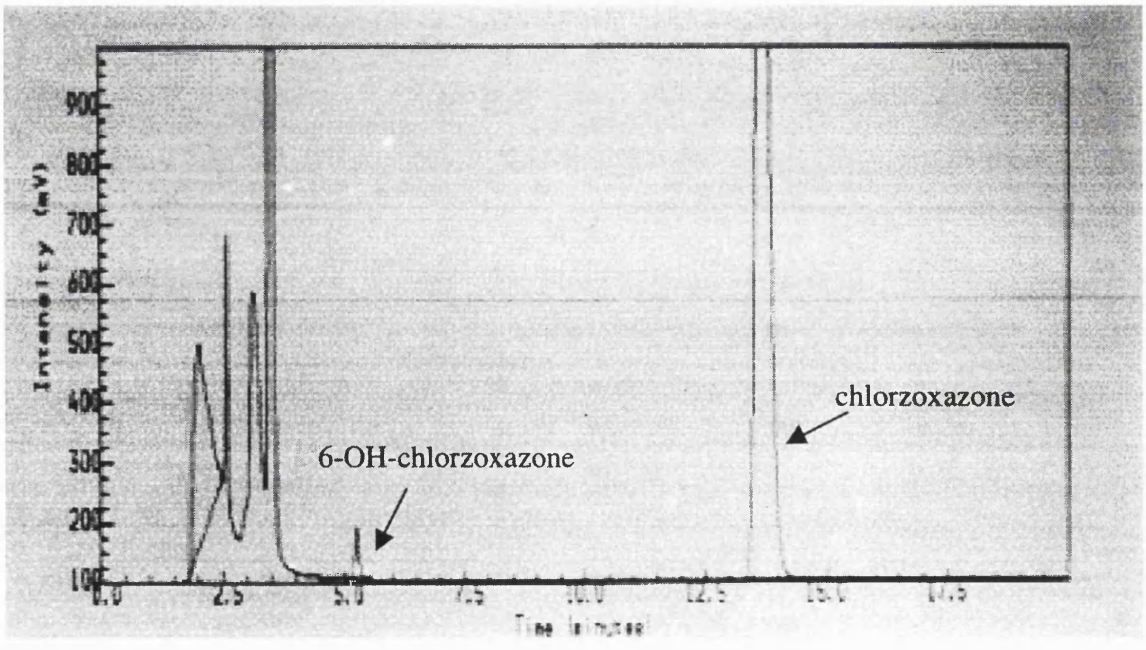


Figure 3.39 HPLC chromatograph showing separation of chlorzoxazone and 6-hydroxy chlorzoxazone.

3.5.1.3 Deconjugation

To determine whether any conjugates of the 6-hydroxy metabolite had been formed, samples were obtained by incubating 14C-chlorzoxazone with cultured rat hepatocytes for up to 4 hours. Samples were subsequently treated with glucuronidase, sulphatase, glucuronidase and sulphatase together, or left untreated. The levels of 6-hydroxy chlorzoxazone in control and deconjugated samples was determined by HPLC analysis, with any conjugated metabolites expected to have an earlier retention time due to increased polarity. Samples were not extracted by solid phase extraction since it was not known whether potential conjugates would be retained and for this reason, samples were injected directly onto the HPLC column. The use of radiolabelled chlorzoxazone avoided the problem of interference by endogenous peaks.

Results indicated that there were no obvious differences in samples incubated with glucuronidase and sulphatase and those untreated samples (Figure 3.40). The presence of conjugates of 6-hydroxy chlorzoxazone would have been reflected in lower levels of 6-hydroxy chlorzoxazone in untreated samples compared to those treated with deconjugating enzymes, since the 6-hydroxy metabolite and any conjugates would not be expected to co-chromatograph. The results indicate that sulphate and glucuronide conjugation of 6-hydroxy chlorzoxazone had not occurred in detectable amounts and that treatment with deconjugating enzymes would not be necessary in subsequent experiments. Very few publications concerning the use of chlorzoxazone refer to conjugates, although glucuronides were detected in an *in vivo* human study (Lucas and Berthou, 1993).

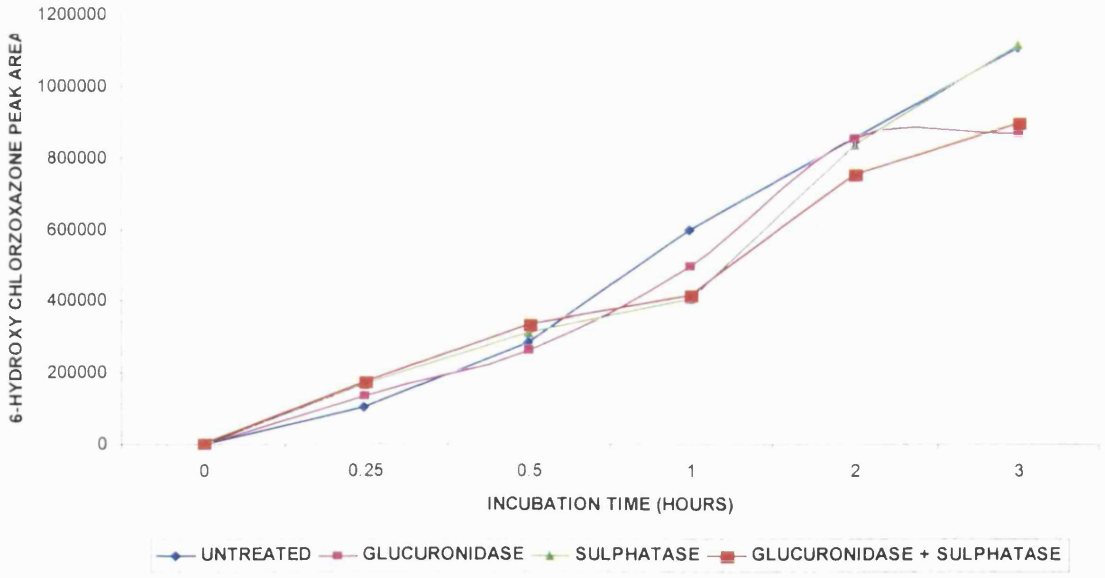


Figure 3.40 Effect of deconjugation on 6-hydroxy chlorzoxazone levels in samples obtained from cultured rat hepatocytes.

3.5.1.4 Chlorzoxazone activity in cultured hepatocytes and microsomes following treatment with pyrazole

To establish the assay conditions to be used, the linearity of chlorzoxazone 6-hydroxylation with respect to time and, for microsomal incubations, protein was measured. The production of 6-hydroxy chlorzoxazone by cultured rat hepatocytes was found to be linear up to 1 hour.

Due to the lower turnover of chlorzoxazone at later timepoints the sensitivity of the assay was limited by the specific activity of the radiolabelled chlorzoxazone. For this reason non-labelled substrate was used. Preliminary experiments showed that endogenous peaks interfered with the detection of non-radiolabelled 6-hydroxy chlorzoxazone. The use of phosphate buffer instead of Williams' media E as the incubation buffer reduced this.

Rat hepatocytes isolated using collagenase digestion and maintained on Matrigel® were incubated with chlorzoxazone (500μM) for a period of 1 hour at 0, 24, 48, 72

and 96 hours post-attachment (n=6). However, due to continuing problems with interference by endogenous peaks it was decided to repeat this work using microsomes prepared from cultured hepatocytes.

The metabolism of chlorzoxazone to the 6-hydroxy metabolite was therefore investigated in microsomes prepared from rat hepatocytes cultured for up to 96 hours. The reaction was found to be linear for the whole 3-hour incubation period investigated and this time was used in subsequent experiments. Microsomes prepared from rat hepatocytes cultured for up to 96 hours were incubated with chlorzoxazone for 3 hours in the presence of NADPH and an isocitrate dehydrogenase NADPH regenerating system (n=3, each prepared from a pool of 6 experiments). The regenerating system was added to provide a renewable source of NADPH throughout the prolonged incubation period.

CYP2E1 activity, as indicated by chlorzoxazone metabolism, decreased dramatically during the 96 hour culture period with a particularly high decline noted during the first 24 hours of culture where a 49% loss was observed (Figure 3.41). A small amount of activity was still detectable in microsomes from the hepatocytes cultured for 96 hours, representing 24% of activity immediately post-attachment. An analysis of variance showed that the activity was significantly lower at 24, 48, 72 and 96, hours post-attachment compared to activity immediately post-attachment ($P<0.01$).

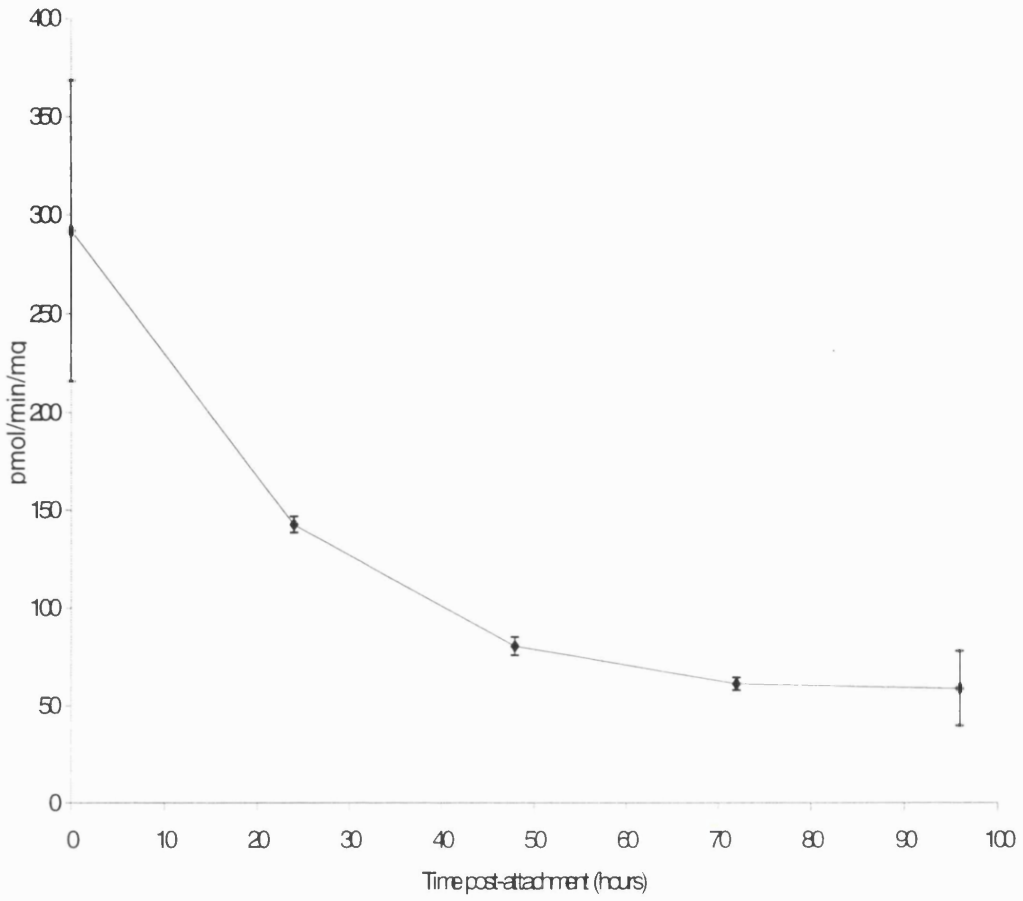


Figure 3.41 The decline in CYP2E1 activity in microsomes prepared from hepatocytes maintained in culture for up to 96 hours.

Hepatocytes were also cultured in the presence of the CYP2E1 inducing agent pyrazole (0, 25, 50, 75 and 100μM) for 48 or 72 hours (n=3, each from a pool of 6 experiments) following an initial 48 hour period in the absence of pyrazole. This initial culture period in the absence of compound is commonly used to allow the hepatocytes to adapt to the culture conditions prior to the attempted induction (Silva et al., 1998) and because hepatocytes have been shown to be non-responsive to inducers at this time (Schuetz et al., 1984). The activity of CYP2E1 as assessed using chlorzoxazone 6-hydroxylation is shown in Table 3.18 and Figure 3.42.

Table 3.18 The chlorzoxazone 6-hydroxylase activity in hepatocytes treated with pyrazole (0, 25, 50 and 100μM) for up to 72 hours.

PYRAZOLE CONC. (μM)	TREATMENT TIME (HOURS)	N=1 pmol/min/mg	N=2 pmol/min/mg	N=3 pmol/min/mg	MEAN±SD pmol/min/mg
0	48	64	57	62	61 ± 4
25	48	74	116	127	106 ± 28
50	48	79	113	121	104 ± 22
75	48	81	115	128	108 ± 24
100	48	62	119	87	89 ± 29
0	72	78	40	58	59 ± 19
25	72	61	91	122	91 ± 31
50	72	82	112	106	100 ± 16
75	72	92	72	77	80 ± 10
100	72	74	122	72	89 ± 28

The data expressed are chlorzoxazone 6-hydroxylase activity in pmol/min/mg protein.

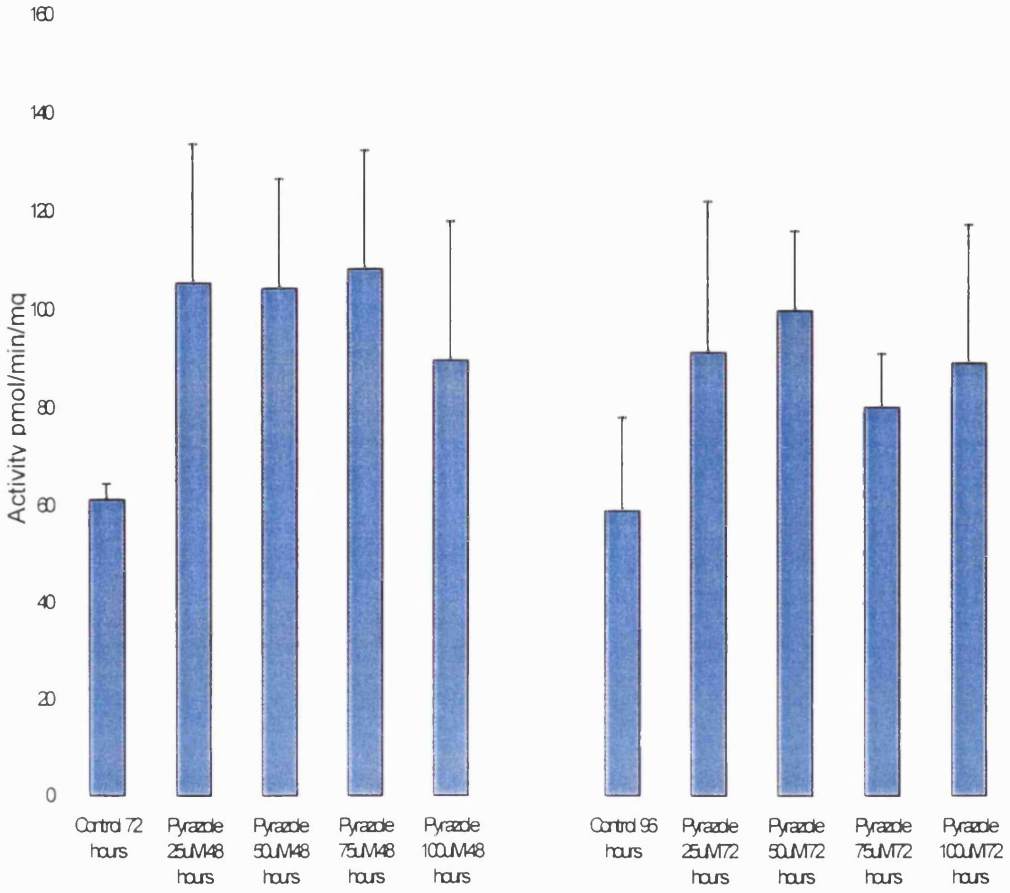


Figure 3.42 Summary of CYP2E1 activity in microsomes prepared from hepatocytes treated with pyrazole. Data shown are mean ± SD

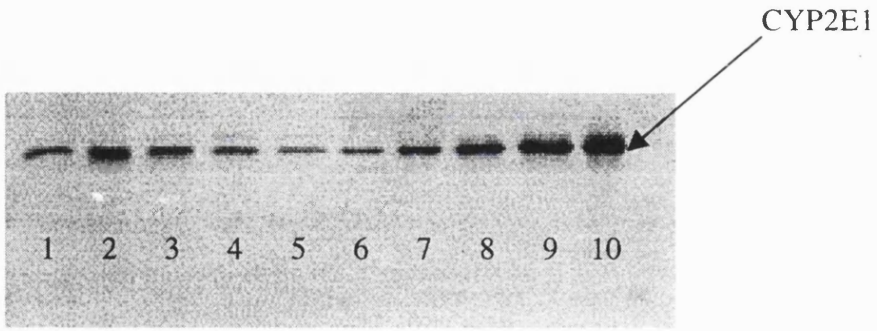
An analysis of variance was used to analyse the data, and this was kindly performed by Dr. R. Kempson (Statistical Services, GlaxoWellcome). This indicated that there were significant differences in the CYP2E1 activity in hepatocytes following a 48 hour exposure period to pyrazole at concentrations of 25μM, 50μM and 75μM ($P<0.05$). The difference in activity following hepatocyte exposure for 48 hours to pyrazole at the highest concentration used (100μM) was not significant ($P=0.166$). The data obtained following hepatocyte exposure to pyrazole for 72 hours showed that activity was slightly lower for all samples compared to those treated for 48 hours, and that only hepatocyte exposure to pyrazole at a concentration of 50μM produced a significant increase in activity ($P<0.05$).

3.5.2 Immunodetectable CYP2E1

Western blotting analysis was carried out to establish whether there was a decline in immunodetectable CYP2E1 in hepatocytes cultured for up to 96 hours. This method was also used to analyse microsomes from hepatocytes which had been cultured in the presence of pyrazole, to determine whether there had been an increase in immunodetectable CYP2E1 protein.

3.5.2.1 Immunodetectable CYP2E1 in cultured hepatocytes

The amount of immunodetectable CYP2E1 in rat hepatocytes cultured for up to 96 hours was determined using Western blotting analysis. CYP2E1 was identified by co-chromatography with a number of standards and the area of these CYP2E1 bands were determined by densitometry. An example of a CYP2E1 blot is shown in Figure 3.43. Unfortunately I did not have access to pure CYP2E1 and the standards used were microsomes from rats treated with pyrazole *in vivo*. The amount of CYP2E1 in each sample relative to that in the standards was determined from the standard curve. Results were expressed in this way so that data from different blots could be directly compared (Figure 3.44).



Lane	1	Pyrazole-induced microsomes (0.1µg)
	2	0 hours post-attachment (1µg)
	3	24 hours post-attachment (1µg)
	4	48 hours post-attachment (1µg)
	5	72 hours post-attachment (1µg)
	6	96 hours post-attachment (1µg)
	7	Pyrazole induced microsomes (0.25µg)
	8	Pyrazole induced microsomes (0.5µg)
	9	Pyrazole induced microsomes (0.75µg)
	10	Pyrazole induced microsomes (1µg)

Figure 3.43 Western blot of immunodetectable CYP2E1 in hepatocytes maintained in culture for up to 96 hours

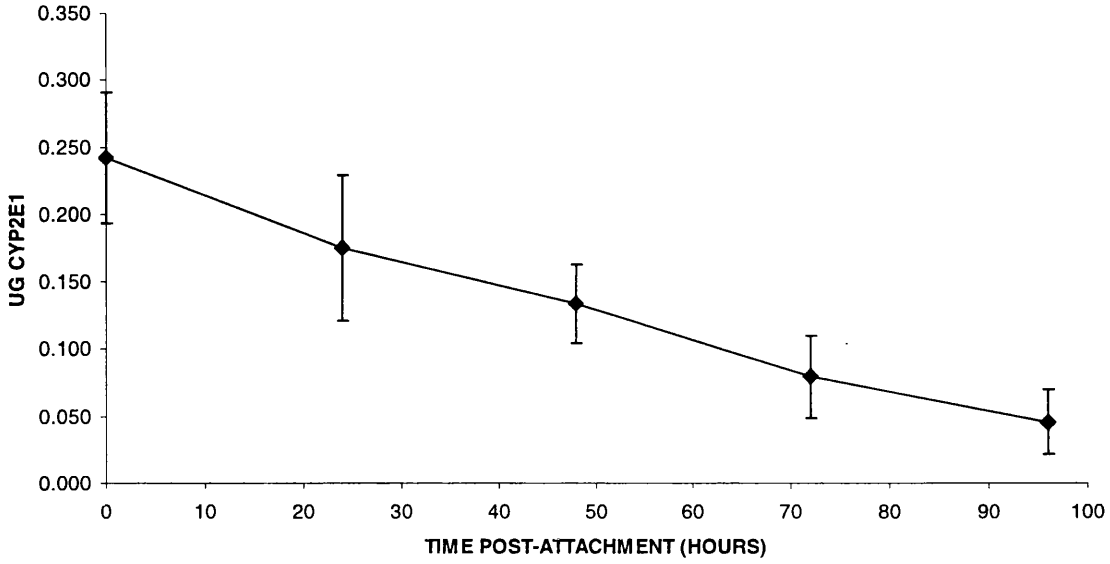
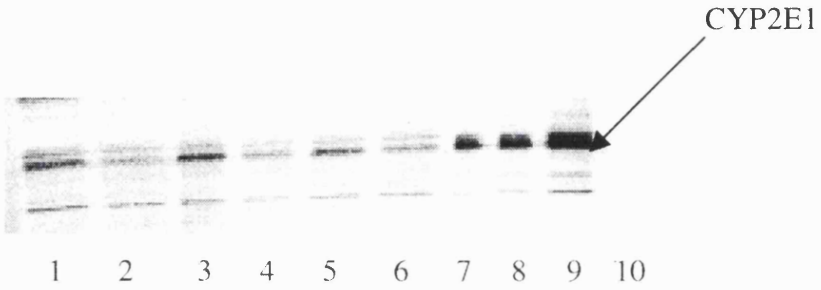


Figure 3.44 Decline in immunodetectable CYP2E1 levels in hepatocytes cultured on Matrigel® for up to 96 hours (n=6).

A decline in the level of immunodetectable CYP2E1 protein was observed, although immunodetectable protein was still detectable in hepatocytes cultured for 96 hours. This is in agreement with the CYP2E1 activity data, where chlorzoxazone 6-hydroxylation was also still detectable in microsomes prepared from hepatocytes cultured for 96 hours.

3.5.2. Immunodetectable CYP2E1 in hepatocytes cultured in the presence of pyrazole

Hepatocytes were cultured in the presence of the CYP2E1 inducing agent pyrazole (0, 25, 50, 75 and 100μM) for 48 or 72 hours (n=3, each from a pool of 6 experiments) and immunodetectable CYP2E1 measured using Western blotting analysis. A typical blot is shown in Figure 3.45. The amount of immunodetectable CYP2E1 is shown in Table 3.19. More than one band was detected in microsomes prepared from hepatocytes, with a cross-reacting protein running slightly later than CYP2E1. The identity of this protein is not known.



Lane 1 Pyrazole (100μM) 48hours (5μg)
2 Untreated control (5μg)
3 Pyrazole (100μM) 48hours (5μg)
4 Untreated control (5μg)
5 Pyrazole (50μM) 48hours (5μg)
6 Pyrazole (25μM) 48hours (5μg)
7 Pyrazole induced microsomes standard (0.25μg)
8 Pyrazole induced microsomes standard (0.5μg)
9 Pyrazole induced microsomes standard (0.75μg)
10 Empty

Figure 3.45 Immunodetectable CYP2E1 in hepatocytes treated with pyrazole

Table 3.19 Levels of immunodetectable CYP2E1 in hepatocytes treated with pyrazole (0, 25, 50, 75 and 100μM) for 48 or 72 hours.

PYRAZOLE CONC. (μM)	TREATMENT TIME (HOURS)	N=1 PEAK AREA	N=2 PEAK AREA	N=3 PEAK AREA
0	48	12352	10827	13949
0	48	14220	10241	12309
25	48	10894	22079	9501
50	48	27344	37089	12257
75	48	39438	45639	27770
100	48	27432	31475	52015
0	72	48323	48716	7528
0	72	48779	44958	9279
25	72	34165	40737	9572
50	72	56277	49256	12927
75	72	66691	47346	19855
100	72	127158	125683	22494

An analysis of variance carried out by Dr. R. Kempson (Statistical Services, GlaxoWellcome) indicated that there was a significant increase in immunodetectable CYP2E1 following 48 hours of exposure to pyrazole at concentrations of 50μM, 75μM and 100μM ($P<0.05$) but not at 25μM. Following 72 hours exposure to pyrazole, however, only pyrazole at the highest concentration had a significant effect ($P<0.05$).

Overall these data show a decline in CYP2E1 activity and immunodetectable enzyme in untreated hepatocytes during the 96 hour culture period. The greatest decline in CYP2E1 activity was during the first 24 hours of culture where a 49% decrease was observed. The sharp rate of decline of CYP2E1 activity in the first 24 hours was not accompanied by a sharp decline in immunodetectable CYP2E1, with the level of immunodetectable CYP2E1 decreasing by only 28% compared to initial values by 24 hours. This discrepancy is similar to that seen for other enzymes in the earlier part of these studies, and has also been demonstrated for CYP2E1 by others (Johansson et al., 1988). Both activity and CYP2E1 protein were still detectable in hepatocytes following 96 hours in culture, although in both instances at much lower levels than those immediately post-attachment.

The decrease in CYP2E1 activity is less here than that reported by Hunt et al., 1991, where a 60% decrease during the first 24 hours was reported, and by Padgham et al., 1994, where an 80% loss of CYP2E1 within 4 hours was observed. The decline in levels of immunoreactive CYP2E1 determined in these studies compare well with those found in one earlier study (Eliasson et al., 1988), but are improved over those measured in another (Hunt et al., 1991).

The data show that, despite some inconsistency, the activity and immunodetectable levels of CYP2E1 have been significantly induced by treatment with pyrazole at some concentrations and treatment times. This indicates that the hepatocytes have retained the ability to respond to a known inducer of CYP2E1. Although the scale of induction in these studies is small, low levels of induction have also been found *in vivo* (Goadstuff et al., 1996; Dicker at al., 1991). Other *in vitro* studies using rat hepatocytes have also reported lack of, or very limited, CYP2E1 induction (Wu et al., 1990; Eliasson et al, 1988; Schuetz et al., 1988; Hunt et al, 1991). A modest 3-fold increase in CYP2E1 *in vitro* has been demonstrated using pyrazole (Zangar et al., 1995) and limited CYP2E1 induction by pyrazole has been shown to occur preferentially in pericentral hepatocytes both *in vivo* and *in vitro* where Chee's medium was used as the culture medium (Dicker et al., 1991).

The reason for the modest level of CYP2E1 induction is not known, but recent data has indicated that inclusion of insulin in the culture media may repress the levels and induction of CYP2E1 (Woodcroft and Novak, 1997, Woodcroft and Novak, 1999). This effect was specific to CYP2E1, and the expression and induction of other cytochrome P450 enzymes including CYP2B, CYP3A, CYP4A and CYP2C11, was enhanced by the presence of insulin (Morgan et al., 1998; Woodcroft and Novak, 1999). However, other studies dispute this, with insulin suppression of CYP2B1/2 induction by phenobarbital reported (Yoshida et al., 1996). These observations highlight the difficulty in attempting to maintain several enzymes which have different regulatory controls.

3.6 METHOD DEVELOPMENT TO MEASURE CYP2E1 mRNA

The level of CYP2E1 mRNA has been reported to decline in cultured rat hepatocytes (Hunt et al., 1991) and it was felt that the ability to determine the effect of new chemical entities on CYP2E1 mRNA expression would be useful. A technique enabling the measurement of this mRNA was required and it was decided initially that an RNase protection assay should be developed.

The ribonuclease protection assay was initially selected to measure CYP2E1 mRNA because it is an extremely sensitive technique for quantifying specific RNAs in solution which has previously been used to detect cytochrome P450 mRNA (Friedberg et al., 1990). The RNase protection assay has several advantages over Northern blots which are often used to measure mRNA, which may suffer from inefficient RNA transfer and binding to the membrane and, once bound, lack of accessibility for some mRNA molecules for hybridisation. Perhaps of most importance is the greater sensitivity offered by the RNase protection assay.

During the course of these studies another method, the Northern ELISA, became available. Since the ribonuclease protection assay was still under development it was decided to halt further work on this technique and change to Northern ELISA.

3.6.1 Production of Probe and Insertion into the Bluescript Plasmid

The term 'probe' is used throughout this text to describe a DNA or an RNA molecule produced for the detection of the complementary mRNA sequence by molecular hybridisation. In this case the probe was designed to specifically detect rat CYP2E1 mRNA molecules, and the steps involved in production are summarised in Figure 3.46. For the ribonuclease protection assay, the probe was an RNA molecule whilst for the Northern ELISA the probe was a DNA molecule. The first stage in the production of the probe was the design of primers used to initiate transcription of the probe from the template DNA in the cDNA library.

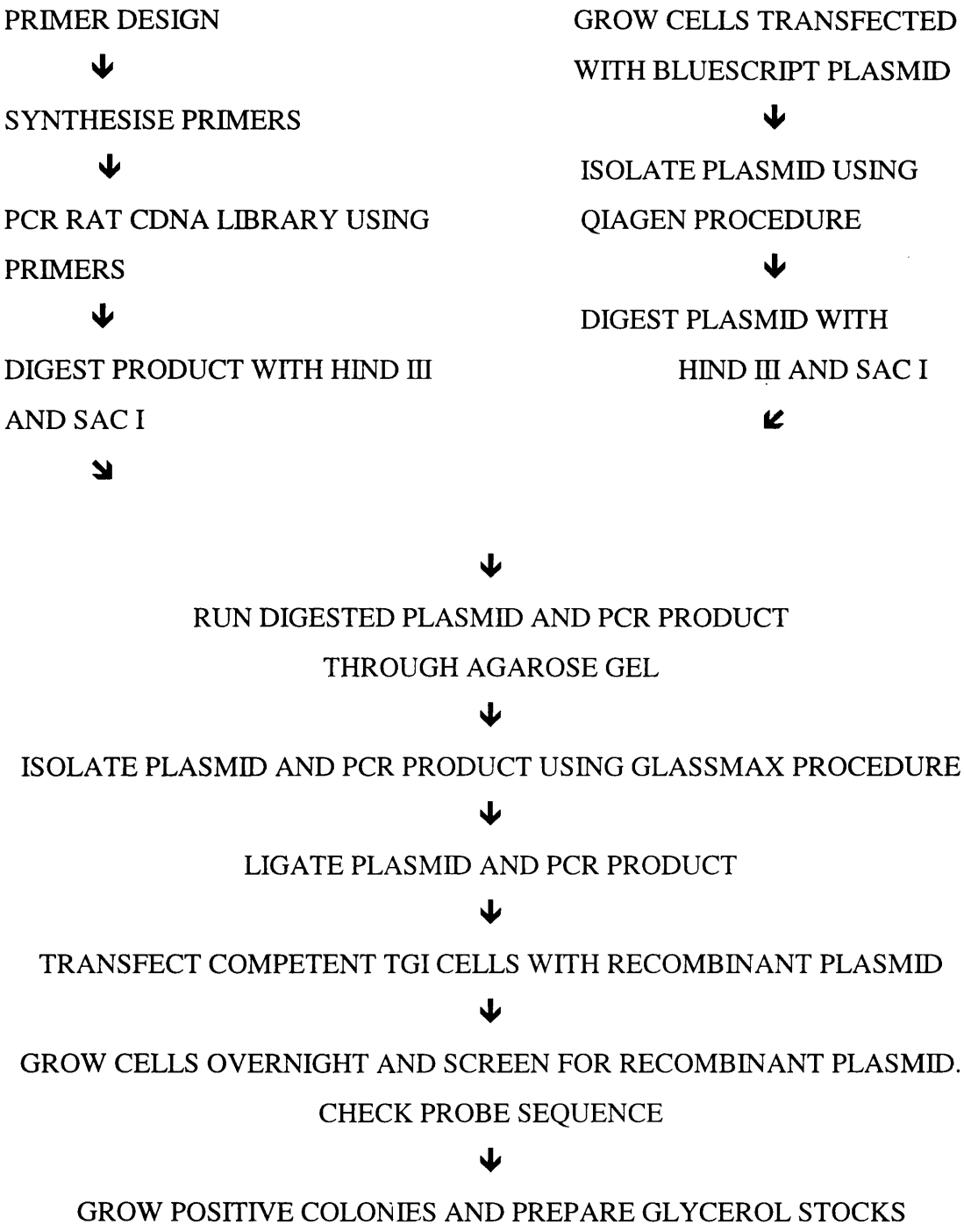


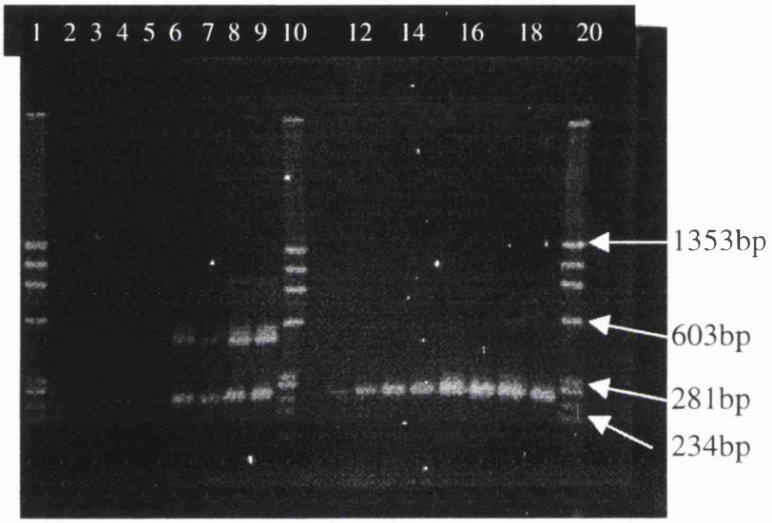
Figure 3.46 Steps involved in the production of a probe for CYP2E1 mRNA

The region of the CYP2E1 mRNA proposed as the template for the probe was checked for restriction enzyme sites and enzymes found to cut the sequence within the region of the probe were not suitable and were rejected. No naturally occurring sites for transcription enzymes were present at the 3' or 5' ends of the target sequence. Therefore, it was decided to generate primers which would be able to hybridise to each of the 3' and 5' ends but which contained a restriction site introduced into the primer sequence (see Section 2.6.3, Figure 2.2.3). The generation of sites for Hind III and Sac I required few base changes and restriction sites for these enzymes were included in the primer sequence. These sites were also present in the multiple cloning site of the cloning vector, the Bluescript plasmid.

Restriction enzymes, which cut DNA reproducibly and predictably at precise sequences, used in this study were Hind III and Sac I both of which cut asymmetrically leaving protruding single stranded sequences known as 'sticky ends'. This can be utilised in solving one of the major difficulties in cloning in plasmid vectors which is distinguishing between plasmids which contain inserted foreign DNA and those which have recircularised without the insert. The easiest fragments to clone carry non-complementary protruding termini generated by digestion with two different restriction enzymes such as Hind III and Sac I. Plasmids cut with these enzymes are not expected to be able to recircularise and since Hind III and Sac I generate different protruding termini or 'sticky ends' foreign DNA can be inserted in only one possible orientation, known as directional cloning.

The primers were used to initiate transcription by hybridising to the 5' and 3' regions at either end of the DNA used as a template for the probe. The DNA used as a template in these experiments was a rat cDNA library derived from rat liver. Briefly, preparation of a cDNA library involves isolation of mRNA from the tissue in which the target mRNA is known to be expressed followed by transcription by RNA polymerase into complementary DNA (cDNA). The cDNAs produced in this way are fragments of genes and these can then be cloned into plasmids. Bacteria transformed with these plasmids can then be used to replicate the plasmids.

The probe to be inserted into the cut Bluescript plasmid was obtained by PCR (polymerase chain reaction) using the designed primers with the rat cDNA library. The products of PCR were electrophoresed through an agarose gel which was subsequently stained with ethidium bromide (Fig 3.47). A band corresponding to the expected size of the probe (249bp) was observed indicating that the PCR reaction had been successful.



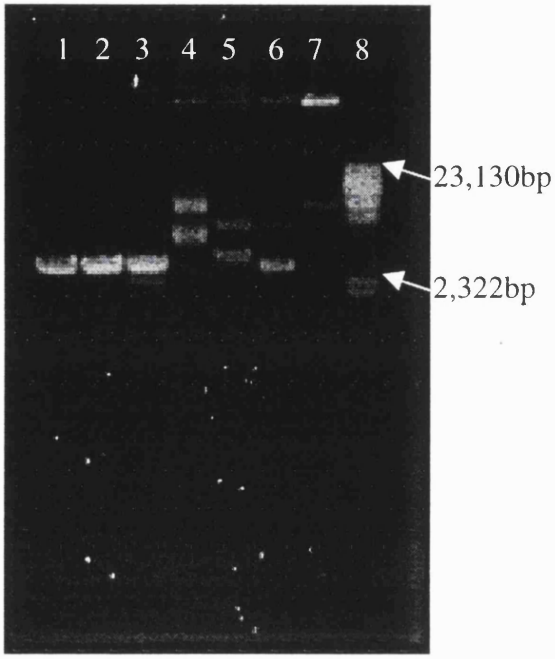
Lane 1.	φX ladder
Lanes 2 to 9 are not related to this study	
Lane 10.	φX ladder
Lane 11	Empty
Lanes 12 and 13	0.1ng rat cDNA library
Lanes 14 and 15.	1ng rat cDNA library
Lanes 16 and 17.	10ng rat cDNA library
Lanes 18 and 19.	100ng rat cDNA library
Lane 20.	φX ladder

Fig 3.47 Photograph of PCR products of rat cDNA library.

The PCR product was excised and isolated from the gel using the Glassmax procedure and was subsequently cut with the restriction enzymes Hind III and Sac I. The products of digestion were electrophoresed through an agarose gel and the probe band identified by comparison with a molecular weight ladder following staining with ethidium bromide. The probe was isolated by the Glassmax procedure and was then ready for insertion into a plasmid vector. Subsequent sequencing indicated that the PCR product had the same sequence of the expected probe.

Bacterial plasmids are double-stranded closed circular DNA molecules, ranging in size from 1 kbases (kb) to more than 200 kb. They are inherited independently, replicate autonomously of the bacterial chromosome but rely on enzymes and proteins encoded by the host bacterial cell for their replication and transcription. In these studies the Bluescript plasmid was used as a vector to carry the probe DNA allowing this material to be cloned.

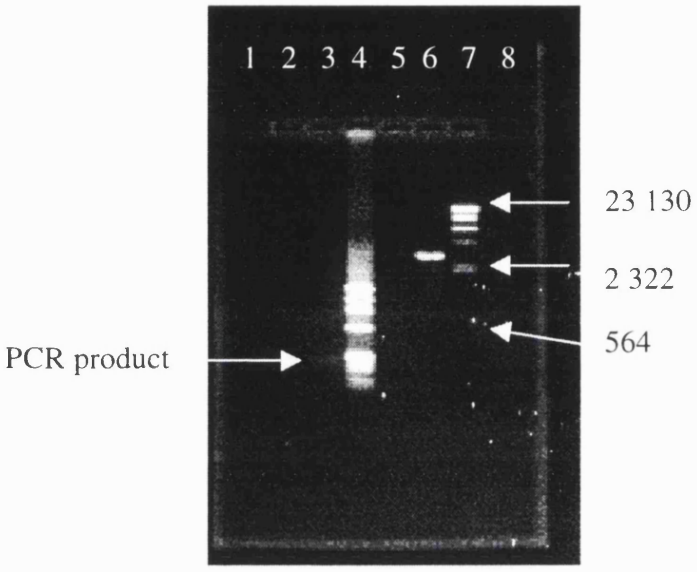
Bacterial cells (JM109) containing the Bluescript plasmid were grown overnight and the plasmid isolated using the Qiagen plasmid midi kit. The plasmid was subsequently cut with the restriction enzymes Hind III and Sac I. Cut and uncut samples were electrophoresed through an agarose gel and plasmid, of approximately 3kb, identified using a molecular weight ladder following staining with ethidium bromide (Fig 3.48). Cut and uncut plasmid electrophorese differently due to differences in both a molecular weight difference and shape. The cut plasmid was excised from the gel and isolated using the Qiagen plasmid midi kit



Lane 1.Bluescript plasmid cut with Hind III and Sac I
Lane 2.Bluescript plasmid cut with Hind III
Lane 3.Bluescript plasmid cut with Sac I
Lane 4. Uncut Bluescript
Lanes 5 and 6 are not related to these studies
Lane 7.Uncut DNA library
Lane 8.λ/Hind III ladder

Fig 3.48 Photograph showing isolated cut and uncut Bluescript plasmid.

Samples of the PCR product and Bluescript plasmid were electrophoresed through an agarose gel which was subsequently stained with ethidium bromide to ensure that the cutting and isolation procedures had been successful (Figure 3.49). The probe and plasmid were then ligated overnight.



Lane 3 Rat PCR product / tentative CYP2E1 probe(cut and purified)
Lane 4 φX ladder
Lane 5 Empty
Lane 6 Bluescript (cut and purified)
Lane 7 λ / Hind III ladder

Figure 3.49 Photograph of cut and isolated Bluescript plasmid and PCR product.

The recombinant plasmid was then introduced into competent JM109 cells, a process known as transformation. The first technique used to screen for bacteria that had been transfected with the recombinant plasmid was blue/white screening for active β-galactosidase activity on X-gal plates. All colonies screened, however, were blue indicating that transformation may not have been successful. However, when the template DNA is small and the reading frame is not disrupted the blue white screening can fail and it was decided to screen a number of colonies by a second method.

A number of colonies were picked, plasmid contained within the cells isolated and PCR used to generate the probe to determine if the recombinant plasmid was present. Of 25 colonies screened, 8 appeared to contain recombinant plasmid as determined by the presence of a PCR product of the expected size of the mRNA probe. Colonies which had been successfully transfected were retained and glycerol stocks prepared.

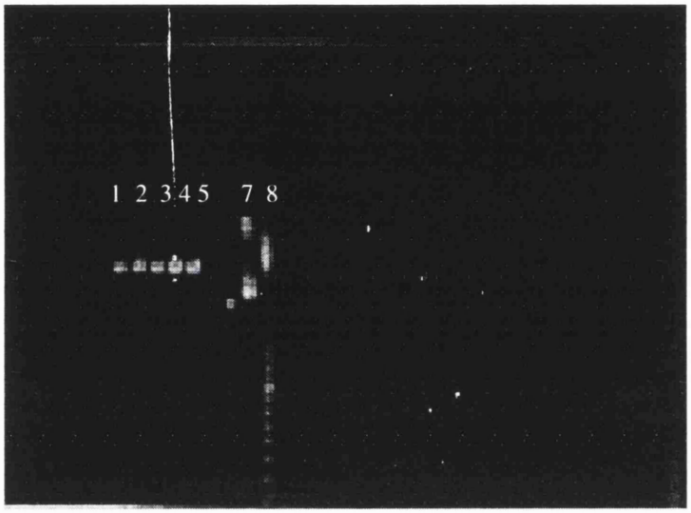
3.6.2 DIG-Labelling of the PCR Product

The next stage of method development was transcription of the probe sequence in the presence of digoxigenin labelled nucleotide followed by isolation of the DIG-labelled probe. The digoxigenin (DIG) label was selected since probes labelled in this way are stable for up to a year in contrast to radio labelled probes which should be used within approximately 24 hours of preparation with the nucleotides used to prepare them disintegrating within about seven days. Use of the DIG label eliminates the risk of radioactive contamination or the requirement for special disposal procedures.

3.6.2.1 *'Run-Off' Transcription*

'Run-off' transcription was used to prepare a DIG-labelled probe for the RNase protection assay. Linearised DNA is required for the synthesis of 'run-off' transcripts and isolated recombinant Bluescript plasmid was linearised with the restriction enzyme Hind III. Linearised plasmid and native plasmid were electrophoresed and, following staining of the gel, the presence of a single, retarded band in the linearised sample which did not co-electrophorese with uncut plasmid indicated that the digestion was both successful and complete (Figure 3.50). A phenol/chloroform

extraction was carried out to remove the Hind III from the recombinant plasmid digest. The linearised DNA was then used as a template by RNA polymerase to create 'run-off' transcripts with a DIG label using a DIG RNA (SP6/T7) labelling kit.

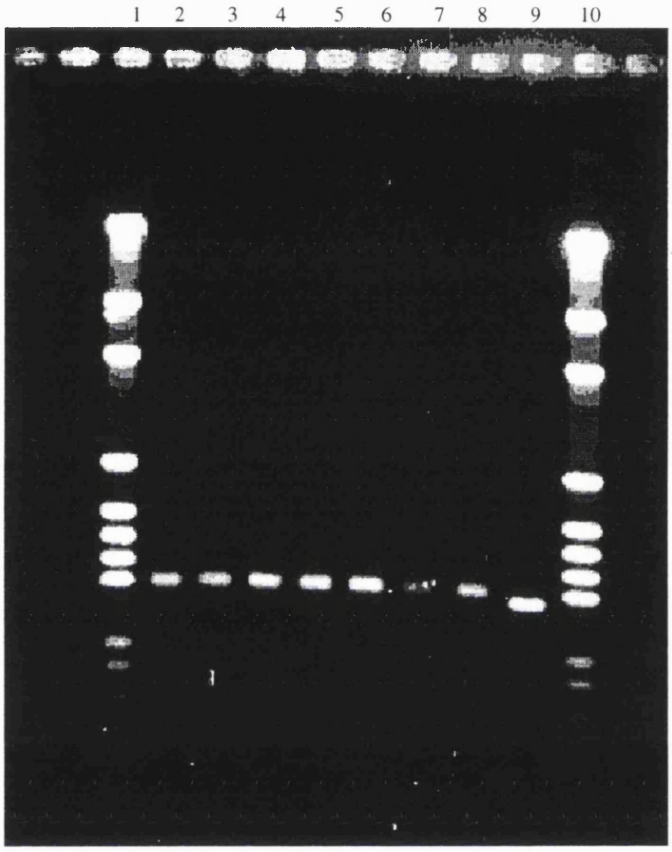


Lane 1 Hind III cut plasmid
2 Hind III cut plasmid
3 Hind III cut plasmid
4 Hind III cut plasmid
5 Hind III cut plasmid
7 Uncut Bluescript
8 100Kb ladder

Figure 3.50 Photograph of gel showing linearisation of plasmid.

3.6.2.2PCR DIG Probe Synthesis

The second technique used to synthesise a DIG-labelled CYP2E1 mRNA probe, for the Northern ELISA, was the DIG PCR probe synthesis kit. This kit is designed to enable the generation of DNA probes labelled with highly sensitive digoxigenin via the PCR reaction. The double-stranded DNA probe produced is more stable than the single stranded RNA probe produced by run-off transcription. The conditions were determined to enable successful labelling of the probe as shown by the increased size of the probe due to the incorporation of the DIG label (Figure 3.51).



Lane 1 φX ladder
2-8 DIG-labelled probe
9 Non-labelled probe
10 φX ladder

Figure 3.51 Photograph of a gel showing labelled and unlabelled probe.

3.6.3 Immunodetection of of DIG-Labelled Probe

The DIG nucleic acid detection kit (Boehringer Mannheim) was used to detect the DIG-labelled probe. Isolated recombinant plasmid was used to demonstrate that the hybridisation and immunodetection using the DIG-labelled probe was successful. Initial blots, with DIG-labelled probe directly spotted onto the membrane indicated that the immunodetection was working satisfactorily by the appearance of purple spots. Later, hybridisation between plasmid DNA which had been made single stranded by heat treatment, and DIG-labelled probe was also shown to have been successful by the appearance of purple spots. A typical dot blot obtained showing successful detection of the CYP2E1 sequence is shown in Figure 3.52.

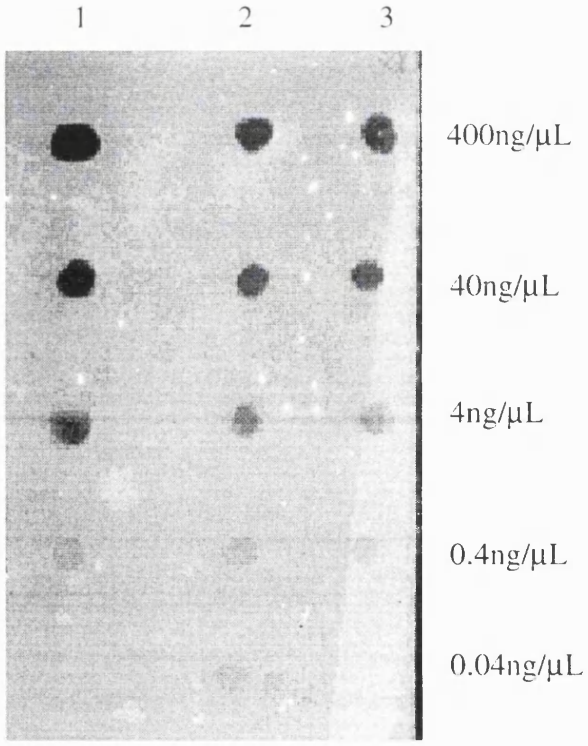


Figure 3.52 Dot blot showing immunodetection of DIG-labelled probe. Lane 1 is DIG-labelled probe hybridised with plasmid and lanes 2 and 3 are DIG-labelled probe.

The next stage of assay development required mRNA derived from hepatocytes to show that CYP2E1 present in the hepatocytes could be detected. It was intended to carry out initial work using dot blots and then to transfer the method to a 96-well plate format suitable for an ELISA assay

3.6.4 Isolation of RNA

Two techniques were used in these studies to isolate RNA from cultured rat hepatocytes. During development of the RNase protection assay RNazol was successfully used to isolate total RNA while during the development of the Northern ELISA the PolyATtract®System 1000 was used to directly isolate mRNA.

3.6.4.1 Isolation of RNA Using RNazol

The purity and integrity of isolated RNA is critical for the RNase protection assay. There are four important features of RNA isolation, which result in intact RNA. These are effective disruption of cells, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease activity and purification of RNA away from contaminating DNA and protein. Possibly the most important factor is the immediate inactivation of endogenous ribonucleases and the RNazol used to isolate RNA from hepatocytes in these study contains the RNase inhibitor guanidium thiocyanate (Chirgwin et al., 1979). In addition most procedures were carried out on ice, which significantly slows the rate of RNA degradation (Han et al., 1987), gloves were worn at all times and sterile techniques used to minimise contamination by airborne dust.

Isolation of RNA from hepatocytes using RNazol was successsful. The purity and concentration of RNA was determined spectrophotometrically and an aliquot from each sample was electrophoresed through an agarose gel. Following staining of the gel the appearance of three bands (28s, 18s and 5.8s rRNA) indicated that RNA isolation had been successful.

3.6.4.2 Isolation of mRNA using the PolyATtract®System 1000

During the development of the Northern ELISA assay the PolyATtract®System 1000 had become available, which had the advantage that mRNA could be isolated directly from cells in contrast to the RNazol method which isolates total RNA. The PolyATract method was used to isolated mRNA from hepatocytes which had been stored at -80°C for a number of years. The method was not successful although it was not established whether the technique or degradation of the mRNA in the cells was the cause of this.

3.6.4 RNase Protection Assay

The ribonuclease protection assay is an extremely sensitive technique used for the quantification of specific RNAs. A probe, labelled in this case with digoxigenin, and complementary to the RNA of interest, is used in solution hybridisations with sample RNA. Digestions with nuclease removed unhybridised material. The protected fragment is analysed by denaturing gel electrophoresis followed transfer to nitrocellulose and immunodetection.

Development of this assay reached the point at which RNA had been isolated using RNazol but hybridisation with the DIG-labelled probe had not be achieved. Due to family illness work was halted for some time and on re-commencement the Northern ELISA technique had become available and no further development of the RNase protection assay was carried out

3.6.5 Northern ELISA

The Northern ELISA technique has certain advantages over the RNase protection assay. Reliable quantification where results can be read by a plate reader is possible in contrast to the rough estimation possible with X-ray films. The results can be obtained rapidly, with the complete procedure from labelling isolated mRNA to detection in less than one day. The sensitivity is high and the format allows large

numbers of samples to assayed simultaneously. In addition, the use of radioactivity is avoided.

Early work was very promising indicating that the DIG-labelled probe coupled with the DIG immunodetection system was functional. However due to the lack of success with the mRNA extraction it was not possible to measure the amount of CYP2E1 mRNA in samples obtained from hepatocytes which had been cultured in the presence of pyrazole. The RNA isolated previously using RNazol had deteriorated during storage and it was not possible therefore to use this material. Unfortunately due to constraints of time and resource it was not possible to repeat the culture work to obtain more samples for RNA extraction or to continue development of the Northern ELISA.

Current developments in the detection of specific mRNAs include DNA chip technology. Briefly a robotic machine, the arrayer, spots cDNAs or expressed sequence tags (ESTs) onto a matrix such as a glass slide and the well position of each sequence is recorded. Complementary DNA is then produced from the mRNA from the tissue or sample of interest. The cDNA produced has to be detectable following hybridisation with the defined cDNA or EST, and transcription is therefore in the presence of a tagged nucleotide such as fluorescence entity. Following hybridisation a laser is used to read the chip and the simultaneous analysis of the expression thousands of genes is possible (Kurian et al., 1999). Other techniques are also emerging including a high throughput ribonuclease assay carried out *in situ* in real time without the need for RNA isolation (Surry et al., 1999). Due to the emergence of these techniques the Northern ELISA has not been further developed.

3.7 CONCLUSIONS

A number of conclusions may be drawn from these studies.

- The data generated in these studies clearly show that both isolation technique and substrata have affected the activity and immunodetectable amounts of several cytochrome P450 enzymes in male rat hepatocytes cultured for up to 96 hours.
- The rates of decline of cytochrome P450 enzymes, and the effects of isolation technique and substrata on these enzymes, were variable.
- The statistically significant effects of isolation technique on the expression of cytochrome P450 enzymes were greater than those of substrata under the culture conditions used.
- For future work, collagenase would be recommended as the isolation method of preference. This is due to the higher yield, viability and reliability of this method, in addition to the higher activity of the CYP3A1/2, CYP2A and 17α-oxidase enzymes in hepatocytes following collagenase isolation. Matrigel® would be recommended as the substratum for further studies, since some improvement in immunodetectable amounts of cytochrome P450 enzymes and in activity were observed.
- The hepatocytes had retained the ability to respond to the CYP2E1 inducing agent pyrazole, with increases in CYP2E1 activity and amount of immunodetectable protein demonstrated. This suggests that the decline in cytochrome P450 enzymes is not irreversible.

Unfortunately there have been no dramatic improvements in the maintenance of cytochrome P450 activity as a result of the many studies in this area including those reported here. This may indicate that the loss of cytochrome P450 enzymes from the hepatocytes is, in fact, a reasonable response to the simple components of the culture system in which the hepatocytes are placed relative to those *in vivo*. The expression of cytochrome P450 enzymes is an adaptive system and hepatocytes may simply cease to produce cytochrome P450 enzymes under conditions where these enzymes are not required or where the transcription factors affecting cytochrome P450 expression to which hepatocytes are exposed *in vivo* are absent.

The ability of the hepatocytes to respond to the CYP2E1 inducing agent pyrazole, although inconsistent and lower than that seen *in vivo,* were demonstrated in these studies. This suggests that the loss of these enzymes is not necessarily irreversible and improvements in the expression of cytochrome P450 enzymes in cultured hepatocytes may be possible. Indeed, the current development of artificial livers in which hepatocytes are exposed to transcription factors present in the systemic circulation may offer the opportunity for culturing hepatocytes in an environment where cytochrome P450 expression is better maintained.

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APPENDIX

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on plastic at 0 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.62	1.78	4.35	ND	5.15	2.51
6β	4.71	12.01	13.53	4.32	9.74	8.58
16α	21.2	14.67	8.91	4.33	12.09	23.06
16β	0.52	0.68	ND	0.19	0.56	1
2α	7.32	10.89	6.39	1.19	10.35	9
AD	14.97	6.37	8.85	13.95	11.28	9.48
T	42.85	40.89	50.17	74.38	35.74	28.95

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on plastic at 24 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.28	0.11	3.3	0.39	0.42	0.38
6β	4.14	1.57	3.03	1.98	1.75	2.49
16α	6.91	4.11	5.86	4.24	4.51	8.85
16β	ND	0.16	ND	0.2	0.13	0.37
2α	2.66	2.17	4.62	2.84	2.99	4.4
AD	32.17	6.11	8.55	15.17	15.7	10.54
T	51.61	81.29	73.71	73.27	73.25	70.13

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on plastic at 48 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.18	7.57	ND	0.05	0.3
6β	1.00	0.78	2.01	1.41	1.34	1.11
16α	3.64	2.46	4.59	1.59	0.43	2.44
16β	ND	ND	ND	ND	ND	0.17
2α	1.82	1.23	3.38	ND	ND	1.02
AD	12.55	4.1	7.39	7.84	0.96	7.26
T	80.33	87.18	73.28	89.15	96.3	87.2

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on plastic at 72 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.42	0.1	6.32	ND	ND	0.5
6β	3.15	0.54	1.23	1.7	1.45	1.82
16α	3.97	1	3.6	1.4	0.41	3.68
16β	ND	ND	ND	ND	ND	0.2
2α	1.53	0.62	2.36	0.51	ND	1.37
AD	24.3	1.8	8.02	7.42	1.57	10.55
T	64.64	93.65	78.48	88.5	95.45	81.69

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on plastic at 96 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.11	1.7	ND	0.01	0.1
6β	1.27	0.69	0.59	0.79	0.89	0.37
16α	1.21	0.67	1.27	0.92	0.27	0.32
16β	ND	ND	ND	ND	ND	ND
2α	0.66	0.4	0.94	ND	ND	0.17
AD	11.52	1.66	4.81	7.86	0.72	2.28
T	83.9	92.81	90.03	90.43	97.39	96.42

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on plastic at 0 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.91	0.87	0.3	0.12	0.4	0.11
6β	9.21	12.52	1.4	0.93	2.42	0.68
16α	18.59	16.33	6.91	5.43	10.96	4.25
16β	1.07	0.79	ND	ND	0.2	ND
2α	5.33	8.98	4.47	3.27	8.84	2.88
AD	18.57	7.4	5.14	7.68	6.24	11.2
T	28.21	47.43	75.57	76.65	65.84	76.18

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on plastic at 24 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.58	0.68	0.22	0.1	0.27	ND
6β	3.87	3.14	0.57	0.28	1.02	0.33
16α	13	14.1	4.23	3.32	7.15	3.31
16β	1.11	0.61	ND	ND	0.18	ND
2α	4.6	7.89	2.78	1.84	5.36	1.74
AD	20.03	8.6	3.88	7.32	6.03	7.66
T	43.42	60.75	82.61	85.52	77.04	82.14

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on plastic at 48 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.1	0.47	ND	0.04	0.15	ND
6β	1.77	1.17	0.09	0.11	0.35	ND
16α	8.59	9.68	1.03	1.53	3.96	1.35
16β	0.6	0.46	ND	ND	ND	ND
2α	2.84	5.14	0.75	0.98	2.33	0.9
AD	15.43	9.46	1.51	4.78	5.45	4.68
T	64.55	68.95	90.76	92.61	90.99	85.84

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on plastic at 72 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.11	0.68	ND	ND	0.04	ND
6β	0.15	0.42	ND	ND	0.6	ND
16α	0.5	2.67	ND	ND	0.22	ND
16β	ND	ND	ND	ND	ND	ND
2α	0.21	1.33	ND	ND	0.15	ND
AD	2.07	3.34	0.1	1.18	0.57	0.79
T	93.06	80.93	96.95	97.34	97.4	95.38

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on plastic at 96 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.05	0.04	ND	ND	ND	ND
6β	0.13	0.1	ND	ND	ND	ND
16α	0.26	0.33	ND	ND	ND	ND
16β	ND	ND	ND	ND	ND	ND
2α	ND	0.11	0.11	ND	ND	ND
AD	0.69	0.48	0.83	0.25	0.07	0.19
T	96.25	96.28	97.19	98.7	98.7	96.28

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on fibronectin at 0 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.24	2.14	5.12	6.25	5.39	2.77
6β	7.88	14.26	10.09	12.97	9.05	10.52
16α	22.56	16.32	7.46	20.56	8.68	19.74
16β	0.62	0.74	ND	1.08	0.38	1.27
2α	9.04	9.65	8.34	6.5	8.01	8.4
AD	17.45	7.66	9.27	14.62	14.25	11.61
T	33.1	40.35	56	30.55	40.86	33.97

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on fibronectin at 24 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.15	2.84	5.55	0.67	0.23
6β	4.91	1.44	2.71	9.59	2.24	1.86
16α	8.07	4.06	5.11	19.42	4.79	5.89
16β	ND	0.14	ND	1.24	0.4	0.22
2α	3.67	2.53	4.62	6.5	3.34	3.17
AD	16.69	6.26	7.79	13.08	18.49	11.36
T	58.83	82.33	76.93	24.71	67.12	74.97

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on fibronectin at 48 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.1	4.51	ND	ND	0.89
6β	0.44	0.78	1.64	2.32	1.05	1.63
16α	2.34	2.95	4.97	2.88	0.18	3.57
16β	ND	ND	ND	ND	ND	0.27
2α	1.28	1.68	4.18	1.14	ND	1.4
AD	6.28	4.57	7.25	12.03	1.1	12.78
T	89.5	86.48	77.46	81.08	96.89	77.8

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on fibronectin at 72 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.15	0.11	4.03	ND	ND	0.36
6β	1.93	0.66	1.03	2.82	1.06	1.53
16α	6.14	1.73	3.87	3.78	0.1	2.96
16β	ND	ND	ND	ND	ND	ND
2α	2.47	0.88	2.78	1.59	ND	1.22
AD	22.17	2.42	7.92	13.78	0.76	12.46
T	66.32	91.63	79.39	77.34	97.29	79.49

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on fibronectin at 96 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.03	1.17	ND	0.06	0.27
6β	0.89	0.46	0.66	2.21	1.87	0.67
16α	1.65	0.95	2.34	2.77	0.32	1.31
16β	ND	ND	ND	ND	ND	ND
2α	0.77	0.55	1.53	1	ND	0.65
AD	9.19	2.13	6.16	13.02	1.13	6.29
T	86.57	93.17	87	81.01	95.71	90.53

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on fibronectin at 0 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	2.23	0.94	0.63	1.24	0.89	1.14
6β	9.17	10.8	2.24	5.33	3.65	3.69
16α	24.79	14.7	11.1	19.88	15.2	18.28
16β	1.16	0.78	0.22	0.84	0.33	0.66
2α	5.16	7.93	6.53	7.9	12.81	7.41
AD	19.77	7.01	7.37	14.59	7.18	14.28
T	31.31	51.47	64.75	36.79	53.69	35.69

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on fibronectin at 24 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.66	0.98	0.25	1	0.64	0.93
6β	4.57	3.78	0.7	3.02	2.22	2.48
16α	14.46	15.91	5.63	16.87	11.43	17.19
16β	1.16	0.77	ND	0.57	0.37	0.74
2α	4.93	8.52	3.62	5.87	9.81	5.56
AD	19.8	8.69	4.74	20.57	8.33	19.41
T	40.25	56.35	77.87	42.62	62.18	40.54

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on fibronectin at 48 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	2.08	0.89	0.14	0.53	0.32	0.43
6β	3.6	2.42	0.18	1.14	0.83	0.64
16α	9.95	10.29	2.67	7.89	7.02	7.67
16β	0.83	0.7	ND	0.29	0.21	0.36
2α	3.81	5.11	1.7	3.48	4.95	3.26
AD	22.07	9.93	3.39	17.86	7.3	18.32
T	52.2	63.85	88.22	64.86	76.35	60.96

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on fibronectin at 72 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.19	0.37	0.07	0.1	0.06	ND
6β	0.57	1.05	ND	0.16	0.07	0.08
16α	3.01	7.75	1.18	2.99	2.05	3.02
16β	0.2	0.28	ND	ND	ND	ND
2α	1.51	3.08	0.82	1.53	1.38	1.43
AD	9.46	7.09	2.04	9.66	2.94	10.43
T	80.34	75.55	92.91	83.79	91.48	81.15

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on fibronectin at 96 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.04	0.09	ND	ND	0.04	ND
6β	0.13	0.37	ND	ND	ND	ND
16α	0.28	1.27	ND	ND	0.18	ND
16β	ND	ND	ND	ND	ND	ND
2α	0.11	0.69	ND	ND	0.13	ND
AD	1.5	1.89	0.46	ND	0.73	0.84
T	95.7	94.35	95.44	98.63	97.85	95.57

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Matrigel® at 0 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.97	1.8	3.19	4.71	4.06	1.64
6β	4.68	12.56	13.2	6.62	8.67	6.05
16α	16.85	14.69	7.89	19.16	8.6	13.89
16β	0.66	0.6	ND	0.43	0.63	0.87
2α	4.8	9.7	4.75	5.31	8.36	5.57
AD	9.79	7.63	9.23	21.9	13.58	9.09
T	40.86	45.99	61.75	29.95	41.41	27.8

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Matrigel® at 24 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.26	0.1	3.19	4.92	0.44	0.31
6β	3.7	1.2	3.52	9	2.53	2.57
16α	14.4	3.17	4.4	18.99	4.68	3.59
16β	ND	0.14	ND	0.81	0.16	0.37
2α	4.47	2.31	4.77	5.57	3.03	3.72
AD	20.57	5.7	8.29	13.43	16.17	13.16
T	50.47	82.82	74.22	31.24	71.17	70.36

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Matrigel® at 48 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.12	5.5	ND	ND	0.45
6β	2.21	0.66	1.71	3.5	0.98	1.69
16α	1.89	2.44	4.8	3.94	0.16	2.81
16β	ND	ND	ND	ND	ND	0.24
2α	0.89	1.47	3.74	1.18	0.13	1.06
AD	23.23	4.57	7.71	12.23	0.8	12.09
T	69.85	88.59	76.56	78.49	97.39	79.56

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Matrigel® at 72 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.34	0.12	5.39	ND	0.18	1.48
6β	1.85	0.54	1.36	2.23	0.31	2.03
16α	3.18	1.32	2.9	4.13	1.12	1.91
16β	ND	ND	ND	ND	ND	ND
2α	1.57	0.83	2.08	1.83	0.36	0.77
AD	17.28	4.17	7.39	12.08	11.68	12.12
T	74.65	89.11	79.96	79.05	85.16	81.18

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Matrigel® at 96 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.06	1.21	ND	0.06	0.83
6β	0.33	0.71	0.62	1.79	0.06	1.36
16α	0.76	0.87	1.7	2.83	0.92	0.98
16β	ND	ND	ND	ND	ND	ND
2α	0.51	0.44	1.26	1.16	ND	0.4
AD	4.83	2.72	5.28	11.28	14.17	9.6
T	93.26	89.48	89.95	82.94	84.03	86.49

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Matrigel® at 0 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.99	0.93	0.53	1.04	0.68	1.12
6β	8.69	12.39	2.46	4.55	3.07	3.82
16α	20.33	15.59	10.92	22.06	13.15	13.61
16β	1.12	0.84	0.22	0.91	0.28	0.68
2α	4.82	8.76	6.53	6.84	10.73	6.5
AD	18.72	7.06	6.88	16.02	7.06	15.83
T	33.5	47.81	64.73	43.24	58.97	42.98

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Matrigel® at 24 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.65	1.08	0.43	0.86	0.71	0.71
6β	3.65	4.32	1.45	2.91	2.32	3.64
16α	12.92	18.44	8.64	13.87	11.21	15.45
16β	0.85	0.79	0.19	0.62	0.37	0.54
2α	4.73	9.67	5.18	4.91	9.68	4.47
AD	19.62	9.52	6.52	22.32	8.84	20.98
T	42.95	49.54	71.78	44.74	60.25	44.38

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Matrigel® at 48 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.41	2.27	0.08	1	0.42	0.81
6β	1.78	1.2	0.16	1.43	0.86	1.28
16α	8.96	11.06	2.2	8.7	7.23	8.14
16β	0.65	0.72	0	0	0.21	0.51
2α	3.62	4.74	1.37	3.04	4.97	2.98
AD	16.1	11.94	3.08	22.58	8.2	21.32
T	61.6	62.11	82.84	57.31	74.55	55.35

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Matrigel® at 72 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	10.11	5.5	0.05	0.76	0.16	0.06
6β	4.54	2.44	ND	0.98	0.37	0.58
16α	9.71	11.05	0.93	5.58	4.14	5.64
16β	1.67	0.68	ND	ND	ND	0.27
2α	1.78	2.96	0.64	2.33	2.71	2.2
AD	30.58	14.06	1.49	19.11	4.36	18.26
T	24.38	44.68	92.31	66.99	86.91	67.2

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Matrigel® at 96 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.92	1.17	ND	.28	.06	.07
6β	1.18	1.59	0.09	0.21	0.06	0.24
16α	5.25	9.9	0.28	1.41	0.63	2.08
16β	0.37	0.3	ND	ND	ND	0.08
2α	2.11	3.18	0.21	0.89	0.43	0.84
AD	17.66	10.22	1.37	13.1	1.75	13.06
T	67.07	71.68	93.97	82.25	95.64	78.59

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Vitrogen® at 0 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.12	1.5	5.75	6.28	6.2	2.78
6β	8.13	13.36	17	9.8	10.43	10.01
16α	20.96	16.72	7.46	20.91	9.94	15.14
16β	0.6	0.67	ND	0.93	0.54	1.17
2α	8.27	9.56	6.84	5.53	8.51	8.1
AD	18.49	7.81	9.48	15.64	15.43	12.36
T	35.08	41.35	43.55	28.61	33.78	33.63

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Vitrogen® at 24 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.12	3.16	5.5	0.67	0.39
6β	3.34	1.52	3.39	8.24	2.13	2.44
16α	6.86	4	6.18	17.55	4.95	7.41
16β	ND	0.15	ND	2.18	0.18	0.31
2α	2.84	2.34	5.24	6.42	3.6	3.96
AD	29.35	5	8.39	14.37	15.78	11.27
T	55.81	81.35	73.66	27.54	71.02	72.05

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Vitrogen® at 48 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.13	6.72	ND	0.06	0.4
6β	0.74	1.08	2.06	1.3	1.28	1.55
16α	2.42	2.87	5.58	3.27	0.34	3.27
16β	ND	0.09	ND	ND	ND	0.21
2α	1.23	1.54	4.6	1.49	ND	1.34
AD	8.12	3.99	7.3	14.86	1.07	10.23
T	87.28	82.82	73.75	79.09	96.42	82.16

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Vitrogen® at 72 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.14	3.85	ND	ND	0.42
6β	0.59	0.6	1.19	2.45	0.64	1.71
16α	1.04	1.8	4.56	4.17	0.09	3.16
16β	ND	ND	ND	ND	ND	ND
2α	0.58	0.89	3.31	1.87	0.1	1.29
AD	5.37	3.13	8.47	12.09	1.4	10.08
T	91.98	89.82	78.22	77.77	96.98	82.73

Metabolism of testosterone in hepatocytes isolated by collagenase and maintained on Vitrogen® at 96 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	ND	0.04	1.06	ND	0.28	0.34
6β	0.57	0.23	0.57	2.7	1.76	0.73
16α	0.29	0.74	2.07	2.35	0.41	1.15
16β	ND	ND	ND	ND	ND	ND
2α	0.13	0.52	1.36	0.82	ND	0.56
AD	3	1.82	5.68	10.67	1.6	6.41
T	95.74	94.67	86.6	83.47	95.07	89.76

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Vitrogen® at 0 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	2.13	1	0.58	0.29	0.59	0.2
6β	10.21	13.64	2.04	1.4	3.23	1.05
16α	24.66	17.15	10.13	5.16	12.81	6.5
16β	1.36	0.86	0.24	0.08	0.27	0.12
2α	4.65	9.03	6.57	3.5	11.24	3.32
AD	17.82	7.48	7.15	8.46	6.73	8.6
T	26.8	44.08	63.8	76.6	59.51	75.77

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Vitrogen® at 24 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.81	1.12	0.45	0.45	0.68	0.42
6β	4.76	4.73	1.09	1.62	2.24	1.42
16α	14.51	18.47	8.24	9.47	10.59	10.32
16β	1.31	1.76	ND	0.47	0.37	0.35
2α	4.89	9.1	4.75	4.31	9.15	4.06
AD	19.77	9.05	6.88	15.05	9.13	14.31
T	39.06	50.97	71.19	61.6	61.27	59.7

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Vitrogen® at 48 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	1.24	0.49	0.05	0.08	0.26	0.08
6β	2.55	1.36	0.17	0.27	0.76	0.1
16α	11.21	12.03	2.39	4.55	7.24	4.79
16β	0.96	0.51	ND	ND	0.12	0.09
2α	4.52	6.4	1.61	2.23	4.53	2.15
AD	21.22	10.26	2.86	10.54	6.36	10.94
T	50.08	63.58	89.29	80.99	79	77.51

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Vitrogen® at 72 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.49	0.66	ND	ND	0.04	0.06
6β	1.4	0.37	0.08	ND	0.06	0.2
16α	9.08	3.73	1.02	0.19	0.76	2.91
16β	0.71	ND	ND	ND	ND	ND
2α	3.66	1.93	0.72	0.18	0.52	1.71
AD	20.64	5.01	1.74	2.18	1.29	3.33
T	54.45	75.5	93.65	95.82	95.78	87.81

Metabolism of testosterone in hepatocytes isolated by EDTA and maintained on Vitrogen® at 96 hours post-attachment.

METABOLITE	N=1	N=2	N=3	N=4	N=5	N=6
7α	0.04	0.03	ND	ND	ND	0.04
6β	0.19	0.11	ND	ND	0.01	ND
16α	0.5	0.58	ND	ND	0.01	ND
16β	0	0	ND	ND	0.01	ND
2α	0.22	0.32	ND	ND	ND	ND
AD	2.23	0.82	0.45	ND	1.84	0.93
T	94.61	96.95	95.75	98.43	96.82	96.42

The composition of Williams' Media E.

INGREDIENT	MG/LITRE	INGREDIENT	MG/LITRE
L-Alanine	90.00	Biotin	0.50
L-Arginine HCL	60.50	Calciferol	1.00
L-Asparagine H_2O	20.00	D-Calcium pantothenate	1.00
L-Aspartic acid	30.00	Choline chloride	1.50
L-Cysteine HCL	52.05	Folic acid	1.00
L-Cystine, disodium salt H_2O	25.20	i-Inositol	2.00
L-Glutamic acid	50.00	Menaphthone sodium salt $3H_2O$	0.01
Glutathione	0.05	Nicotinamide	1.00
Glycine	50.00	Pyridoxal HCl	1.00
L-Histidine HCL H_2O	20.30	Riboflavin	0.10
L-Isoleucine	50.00	Thiamin HCl	1.00
L-Leucine	75.00	DL-α tocopherol phosphate disodium salt	0.01
L-Lysine HCl	87.50	Vitamin A acetate	0.10
L-Methionine	15.00	Vitamin B12	0.20
L-Phenylalanine	25.00	$CaCl_2.2H_2O$	264.9
L-Proline	30.00	$CuSO_4.5H_2O$	0.00009
L-Serine	10.00	$Fe(NO_3)_3.9H_2O$	0.0001
L-Threonine	40.00	KCl	400.0
L-Tryptophan	10.00	$MgSO_4.7H_2O$	200.0
L-Tyrosine disodium salt	43.49	$MnCl_2.4H_2O$	0.0001
L-Valine	50.00	NaCl	6800
L-Ascorbic acid	2.00	NaHCO3	2200
Methyl linoleate	0.03	$NaH_2PO_4.2H_2O$	158.3
Sodium pyruvate	25.00	$ZnSO_4.7H_2O$	0.0002
		D-Glucose	2000

Components of the QIAGEN Plasmid Midi Kit.

P1 (resuspension buffer): 100µg/ml Rnase A, 50mM Tris/HCl, 10mM EDTA, pH 8.0
P2 (lysis buffer): 200mM NaOH, 1% SDS
P3 (neutralisation buffer): 3M Kac, pH 5.5
QBT (equilibration buffer): 750mM NaCl, 50mMMOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100
QC (wash buffer): 1M NaCl, 50mM MOPS, 15% ethanol, pH 7 .0
QF (elution buffer): 1.25M Tris/HCl, 50mM EDTA, 15% ethanol, pH 8.5
TE: 10mM Tris/HCl, 1mM EDTA, pH 8.0

Components of the Wizard™ Plasmid Midi Kit

Cell resuspension solution: 50mM Tris/HCl, pH 7.5, 10mM EDTA, 100µg/ml Rnase A
Cell lysis solution: 0.2M NaOH, 1% SDS
Neutralisation solution: 1.32M potassium acetate, pH 7.5
TE buffer: 10mM Tris/HCl, pH 7.5, 1mM EDTA
Column wash solution: 200mM NaCl, 20mM Tris/HCl, pH 7.5, 5mM EDTA

Components of the Rnase Protection Assay

RNase digestion buffer :
Buffer 1: Maleic acid 100mM, NaCl 150mM, pH7.5 with NaOH
Blocking solution: 10%w/v stock diluted 1/10 in buffer 1
Buffer 3: Tris-HCl 100mM, NaCl 100mM, $MgCl_2$ 50mM, and pH9.5

Components of the DIG Nucleic Acid Detection Kit

Buffer 1: Maleic acid 100mM, NaCl 150mM, pH7.5 with NaOH, (autoclaved)
Blocking Stock Solution: Blocking reagent (50g) was diluted in buffer 1 (500mL) and autoclaved to give a 10% w/v solution.
Washing buffer: Buffer 1 with Tween-20, 0.3% (w/v).
Buffer 2: Blocking solution diluted 1/10 in buffer 1.
Buffer 3. Tris-HCl 100mM, NaCl 100mM, $MgCl_2$ 50mM, pH9.5
Buffer 4: Tris-HCl 10mM, EDTA 1mM, pH 8.0
Hybridisation buffer: 5X SSC, blocking reagent 1% (w/v), N-lauroylsarcosine 0.1% (w/v), SDS 0.02% (w/v).
Hybridisation buffer for mRNA samples: Formamide 50%, 5X SSC, $NaPO_4$ 50mM, pH7.2, blocking reagent 2% (w/v), SDS 7% (w/v), N-lauroylsarcosine 0.1% (w/v)