DRUG METABOLISM IN HUMAN LIVER

Being a Thesis Presented for the Award of a Degree of Doctor of Philosophy in the University of Surrey.

by

Jeffrey R. Fry, B.Sc. (Surrey)

August 1974

Dept. of Biochemistry
University of Surrey
Guildford
SUMMARY

The phenomenon of species differences in drug metabolism and toxicity is in many instances dependent on the ability of the liver to handle the drug in question. This problem is particularly important when attempting to extrapolate metabolism and toxicity data from laboratory animal species to man. In an effort to establish test systems that would enable this extrapolation to be predicted more reliably and accurately two different but inter-linked approaches were adopted.

Firstly, isolated fractions of adult human liver were investigated. The obtention of small amounts of fresh biopsy tissue and of larger samples of post-mortem material necessitated a thorough assessment of the techniques suitable for speedy, small-scale analysis of microsomal drug metabolizing system components together with a study into the storage and post-mortem stabilities of this system. By use of these techniques large species differences in the ability of microsomes to bind drugs were noted which appeared to be related to differences in metabolism.

The second approach used was that of tissue culture of adult rat hepatocytes. Once again a thorough examination of the available methods of liver culture had to be made and based on these findings a suitable culture system was devised. The cells present in culture were subjected to a battery of tests designed to show liver-specific functions. These tests proved that the epitheliocytes present in monolayer culture were in fact functional adult rat hepatocytes, a situation which appears to have been rarely, if ever, achieved previously.
ACKNOWLEDGEMENTS

Throughout this work I have been the grateful recipient of the valued guidance, interest, patience and encouragement of Dr. J.W. Bridges to whom I express a very honest and sincere gratitude. My thanks are also due to the other members of staff and postgraduates of the department for their several rewarding discussions, especially Mr. F. McPherson, my collaborator in some of the early storage experiments, and Mrs. Kathy Burke, Mr. Ahmed Shabaan and Mr. P. Redmond for valued assistance in the latter stages of the cell culture work. My particular appreciation is accorded to Dr. A. Munro (Pfizer Ltd.) and Prof. K.J. Netter (University of Mainz) for their valuable contributions during this study. The skilled assistance of Mr. G. Dufou in processing the photomicrographs is gladly acknowledged, as is the typing of Mrs. L. Mitchell by whose efforts this work has been transposed into a legible form.

The unceasing and selfless enthusiasm of my wife and parents has been a constant source of encouragement throughout this study which itself would not have been initiated were it not for the generous financial support of the Thomas and Elizabeth Williams Scholarship Fund and of Pfizer Ltd.
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summary</td>
<td>1</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>2</td>
</tr>
<tr>
<td>Glossary of Terms</td>
<td>4</td>
</tr>
<tr>
<td><strong>Chapter 1. General Introduction</strong></td>
<td>6</td>
</tr>
<tr>
<td><strong>Drug Metabolism Studies</strong></td>
<td>9</td>
</tr>
<tr>
<td>- Chapter 2. Introduction</td>
<td>10</td>
</tr>
<tr>
<td>- Chapter 3. Materials and Methods</td>
<td>46</td>
</tr>
<tr>
<td>- Chapter 4. Results</td>
<td>63</td>
</tr>
<tr>
<td>- Chapter 5. Discussion</td>
<td>129</td>
</tr>
<tr>
<td><strong>Tissue Culture Studies</strong></td>
<td>146</td>
</tr>
<tr>
<td>- Chapter 6. Introduction</td>
<td>147</td>
</tr>
<tr>
<td>- Chapter 7. Materials and Methods</td>
<td>177</td>
</tr>
<tr>
<td>- Chapter 8. Results</td>
<td>189</td>
</tr>
<tr>
<td>- Chapter 9. Discussion</td>
<td>224</td>
</tr>
<tr>
<td><strong>Appendix: Suppliers of Chemicals, Media and Media Supplements</strong></td>
<td>234</td>
</tr>
<tr>
<td><strong>References</strong></td>
<td>236</td>
</tr>
</tbody>
</table>
**GLOSSARY OF TERMS**

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Acid' Microsomes</td>
<td>Those produced by isoelectric precipitation</td>
</tr>
<tr>
<td>$b_5$</td>
<td>Cytochrome $b_5$</td>
</tr>
<tr>
<td>B.S.S.</td>
<td>Balanced salt solution</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(£(aminoethylether)$N,N$-tetraacetic acid</td>
</tr>
<tr>
<td>e.r.</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>G-6-P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>IP</td>
<td>Isoelectric precipitation</td>
</tr>
<tr>
<td>$K_S$</td>
<td>Binding constant for spectrally-apparent microsome-substrate interactions</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MFO</td>
<td>Mixed function oxidase</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>Oxidised nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>'Normal' Microsomes</td>
<td>Those produced by conventional centrifugation</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical density</td>
</tr>
<tr>
<td>P$_{450}$</td>
<td>Cytochrome P$_{450}$</td>
</tr>
<tr>
<td>P.A.S.</td>
<td>Periodic-acid schiff</td>
</tr>
<tr>
<td>PBS'A'</td>
<td>Dulbecco’s phosphate buffered saline lacking calcium and magnesium salts</td>
</tr>
<tr>
<td>P.M.</td>
<td>Post Mortem</td>
</tr>
<tr>
<td>P.M.S.</td>
<td>Post mitochondrial supernatant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TPB</td>
<td>Tetrphenylboron</td>
</tr>
</tbody>
</table>

Other abbreviations are those recommended by the 'Biochemical Journal'.

Chemical formulae are those in general use.
CHAPTER 1. GENERAL INTRODUCTION AND RATIONALE

From ancient civilizations on it has been common practice to use animals on which to test for possible toxic effects the food and drink meant for human consumption. Comparatively recently, this use of experimental animals has been applied, with some sophistications, to the routine pharmacology and toxicity testing of therapeutic agents designed for human use. This approach is based on the belief that all animals, including man, show essentially similar responses on exposure to a particular agent and that the effect observed in experimental animals can be extrapolated to the human situation. Whilst this is true in many instances, particularly in the early studies on the role of chemotherapeutic agents in combating bacterial infection in rats and mice, it is almost equally true to say that species differences in the responses to administered pharmacodynamic and other agents are so great that any extrapolation to man is at best highly tenuous. Furthermore, these species differences are highly unpredictable in that the pattern of species variation in response to one drug does not necessarily match that for any other drug.

It is now widely recognized that in many instances these species differences in both pharmacological and possible toxic responses are related to the rates and patterns of metabolism of the drugs themselves (see Chapter 2). It is for this reason that metabolism studies play an important role in the testing programme of a new drug, being involved in virtually every phase of the programme (Drug Research Board, 1969). Even after extensive metabolism studies in experimental animals there still exists the one major critical stage in the complete testing schedule, that of initial pre-clinical trials in man himself. This step is, in many
respects, very much a step into the unknown but the uncertainty inherent in this transition to man could be greatly minimized if an appropriate in vitro system be devised which would accurately predict metabolism and metabolism-related hepatotoxicity of the drug in man.

The liver is the major organ responsible for the metabolism of drugs and other nutrients within the mammalian body (see Chapter 2). Because of this, and also due to the fact that the liver is the first major internal organ to receive a drug after administration, the liver often shows adverse reactions to the drug prior to the more obvious grosser clinical irregularities. It is for these reasons that human liver preparations could prove the most appropriate in vitro system with which to monitor the probable in vivo metabolism of nutrients in man. This in turn would lead to a safer and more rational approach to the problem of pre-clinical and clinical trials in man.

It was with this rationale in mind that the present study was initiated. Early on it became apparent that two approaches might prove the most worthwhile to investigate further. These approaches were based on the use of isolated adult human liver fractions and of tissue culture techniques.

As the isolated fractions would be obtained from either small amounts of surgical or needle biopsy samples or from much larger amounts of post-mortem material it was considered necessary

a) to assess the existing assay procedures for enzyme analysis with a view to scaling them down for the small samples, and

b) to investigate the feasibility of storing, and rapidly isolating, the fractions from the much larger samples.

Tissue culture of adult human liver was an attractive alternative in that hepatotoxicity studies as well as metabolism studies could be
meaningfully carried out using such a technique. However, a survey of the current literature on the tissue culture of adult mammalian liver revealed adversity of methods in use which in itself implied a high degree of dissatisfaction as to the applicability and usefulness of each technique (see Chapter 6). This necessitated a survey of some of the more useful techniques in order to ascertain the most applicable to the present study, during which rat liver was employed as a model for human liver.
DRUG METABOLISM STUDIES
## CHAPTER 2. CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Characteristics of the Hepatic Mixed Function Oxidase System</td>
<td>11</td>
</tr>
<tr>
<td>2. <em>In Vitro</em> Metabolism of Xenobiotics by the Human Hepatic MFO System</td>
<td>17</td>
</tr>
<tr>
<td>3. Factors Influencing Activity of the Hepatic MFO System</td>
<td>23</td>
</tr>
<tr>
<td>A. Genetic Factors: Species and Strain Variations</td>
<td>23</td>
</tr>
<tr>
<td>B. Physiological Factors</td>
<td>28</td>
</tr>
<tr>
<td>C. Environmental Factors</td>
<td>29</td>
</tr>
<tr>
<td>4. Isolation of Hepatic Endoplasmic Reticulum Fragments for use in <em>In Vitro</em> Drug Metabolism Studies</td>
<td>33</td>
</tr>
<tr>
<td>5. Stability of the Hepatic MFO System - Storage and Post Mortem Characteristics</td>
<td>36</td>
</tr>
<tr>
<td>6. Metabolism of Xenobiotics in Isolated Cells and Tissue Culture Systems</td>
<td>42</td>
</tr>
</tbody>
</table>
CHAPTER 2. INTRODUCTION

1. Characteristics of the Hepatic Mixed Function Oxidase System

There exists within the mammalian liver an enzyme system which has a major responsibility for the oxidative metabolism of various drugs, nutrients, environmental pollutants etc., collectively termed xenobiotics. At the subcellular level, this oxidative metabolism of xenobiotics is localized in the endoplasmic reticulum (Mueller and Miller, 1959) and has an absolute requirement for both NADPH and oxygen. This dual requirement for both a reducing agent and an oxidizing agent has led to this system being termed a "mixed function oxidase" (MFO) (Mason, 1957).

Several excellent reviews on the hepatic MFO system have been written (Gillette, 1966, 1971; Estabrook et al., 1971; Mannering, 1971; Ramnar, 1972) and only a brief description of the system will be described here. The essential components of this system, and their interrelationships, are shown in Fig. 2.1. Although metabolism by the MFO system is manifested by a number of seemingly different reactions - aromatic and aliphatic hydroxylation, N-, O- and S-dealkylation, S-oxidation and deamination (Gillette, 1966) - it has been shown (Brodie et al., 1955) that all these reactions can be viewed as hydroxylation processes.

At the heart of the MFO system is the terminal oxidase cytochrome P450, so-called because of the wavelength maximum at 450 nm of the reduced form in the presence of carbon monoxide (Omura and Sato, 1964). It has been calculated (Estabrook et al., 1971)
that cytochrome P₄₅₀ is the major cytochrome species existing within the liver, and is also present, in lesser amounts, in the adrenals (Lewis and Bryan, 1971), placenta (Juchau et al., 1973), lung (Gram, 1973), kidney (Jakobsson and Cinti, 1973), testis (Menard and Purvis, 1973), small intestine (Lehrmann et al., 1973) and skin (Alvares et al., 1973).

The first stage in the cycle (Fig. 2.1) is the interaction of the lipid-soluble substrate (S•H) with the ferric form of P₄₅₀ (Mannering, 1971).
Fig. 2.1. The Hepatic Mixed Function Oxidase System (from Remmer, 1972)

$S'\text{H}$

$P_{450}^{Fe^{++}}$

$P_{450}^{Fe^{+++}}$

$e \leftarrow "X" \rightarrow Fp^c \rightarrow NADPH$

$e \leftarrow b_5 \rightarrow Fp^d \rightarrow NADH$

$P_{450}^{Fe^{++}O_2}$

$H_2O \leftarrow 2H^+$

$S'OH$

$S\text{OH}$

$P_{450}^{Fe^{+++}}$

$P_{450}^{Fe^{+++}}$

$P_{450}^{Fe^{++}}$

$P_{450}^{Fe^{++}}$

$P_{450}^{Fe^{++}}$

$P_{450}^{Fe^{++}}$

$CO$

$S'\text{H}$ — Substrate to be oxidized

$P_{450}$ — Cytochrome $P_{450}$

$S'\text{OH}$ — Hydroxylated product

$b_5$ — Cytochrome $b_5$

$Fp^c$ — NADPH-cytochrome $c$ reductase

$Fp^d$ — NADH-cytochrome $b_5$ reductase

"$X$" — Non-haem iron intermediate

$CO$ — Carbon monoxide
These interactions are of two major general types, type I and type II (Remmer et al, 1966; Imai and Sato, 1966), although a third, minor, type, designated reverse type I, has also been described (Schenkman et al, 1972). These type I and type II interactions appear as alterations in the absolute spectrum of oxidized $P_{450}$ (see Remmer, 1972) which when observed as difference spectra yield plots characteristic of the two classes (Schenkman et al, 1967) (Fig. 2.2).

Type I compounds give a difference spectrum with $\lambda_{\text{max}}$ at 385 - 390 nm. and $\lambda_{\text{min}}$ at 418 - 427 nm.; the $\lambda_{\text{max}}$ and $\lambda_{\text{min}}$ given by type II compounds are 425 - 435 nm. and 390 - 405 nm. respectively. Type I spectra are produced by the majority of substrates of the MFO system and are caused by changes in $P_{450}$ due to the formation of the enzyme-substrate complex, probably involving an association with components of the e.r. membrane (see Mannering, 1971). Type II spectra, on the other hand, are produced by various basic nitrogen-containing compounds (such as aniline) which bind to the sixth ligand of the haem iron (Mannering, 1971). The reverse type I spectrum has been shown to be due to the removal of endogenous bound material (Schenkman et al, 1972). An important observation that arose from studies involving difference spectra was that in many instances the spectral dissociation constants ($K_S$) derived from the changes in optical density at different concentrations of certain drugs bore a direct relationship to the Michaelis constants obtained for the metabolism of the same drugs (Remmer et al, 1966; Imai and Sato, 1966; Mannering, 1971). Indeed, it has been postulated that the spectral changes are in fact visible representations of the Michaelis complexes (Gillette, 1969).
Once formed the ferric-substrate complex can accept an electron to give the ferrous-substrate complex which in turn can interact with carbon monoxide to give the characteristic absorbance band at 450 nm. of the reduced $P_{450} - CO$ compound; reduction by dithionite is necessary for a full expression of the reducible $P_{450}$ \textit{in vitro}. This electron donation can only come from NADPH (and not from NADH) via NADPH-cytochrome $c$ reductase (Estabrook \textit{et al.}, 1971), a term synonymous with NADPH-cytochrome $P_{450}$ reductase (Masters \textit{et al.}, 1973). This single electron donation is presumed to require the presence of an unknown non-haem iron intermediate ("X").

The ferrous-substrate complex can then interact with oxygen (Baron \textit{et al.}, 1973) and pick up an electron donated by either NADH or NADPH, giving rise to an "active oxygen" intermediate. This electron transfer from either NADH or NADPH is believed to flow via cytochrome $b_5$, the other cytochrome present in the e.r. and which is quantitatively the second most prominent cytochrome in the liver (Estabrook \textit{et al.}, 1971; Baron \textit{et al.}, 1973; Sesame \textit{et al.}, 1973).

Subsequent to the formation of the "active oxygen" intermediate the bound substrate is hydroxylated and then released, returning the $P_{450}$ to its original ferric state.
Fig 2.2. Difference Spectra of Substrate Interactions with Cytochrome P₄₅₀ (from Schenkman et al, 1967).

Protein Concentration: 1mg/ml

- 5 mM Hexobarbital
- 18 mM Aniline

Wavelength (nm)
2. **In Vitro Metabolism of Xenobiotics by the Human Hepatic MFO System**

There are now a large number of reports concerning the presence in human liver, foetal and adult, of various components of the microsomal MFO system. Thus, cytochrome b₅ and P₄₅₀, NADPH-cytochrome c (P₄₅₀) reductase activity and metabolism of type I and type II compounds have all been shown to be present in adult human liver, whether the specimens be biopsy or post-mortem material. The present (December 1973) state of the literature on this subject is briefly summarized in Table 2.1.

The ethical problems associated with the obtention of surgical or needle biopsies from normal undiseased human adults are quite clearly immense and because of this a large number of the studies mentioned in Table 2.1 have had to be performed using post-mortem material. Consequently, caution should be exercised when attempting to extrapolate results from the *in vitro* post-mortem state to the state appertaining *in vivo*. Comparison of the levels of P₄₅₀ in liver samples from fresh biopsy specimens (Ackermann, 1970, 1972; Alvares et al., 1969; Black et al., 1973; Darby et al., 1973; Pelkonen et al., 1973; Thorgeirsson and Davies, 1971) and from post-mortem specimens (Darby et al., 1970; Kamataki et al., 1973; Nelson et al., 1971) reveals a markedly lower level in the latter specimens, no doubt reflecting the autolytic changes that occur post-mortem. Results obtained from needle biopsy material can also be questioned due to the large amounts of contaminating capsular tissue which is invariably found in such specimens, as well as in surgical
biopsies although to a lesser extent (Schmidt and Schmidt, 1970).
The pattern that emerges from the studies of the MFO system in
human hepatic biopsy tissue (see Table 2.1) is that the levels
of all the components of this system are lower than those found
in rat hepatic tissue. This is true for cytochromes b5 and
P450 and for type I and type II metabolism. Furthermore,
marked individual differences in the levels of P450 and type I
and type II metabolism in human liver biopsies have also been
noted (Pelkonen et al., 1973). Whether these individual variations
were genetically-linked or were caused by prior medication was not
established.

The MFO system of adult human liver is similar in many
respects to that of rat liver. 1) It has an absolute
requirement for an NADPH-generating system (Ackermann, 1970;
2) Antibody to porcine NADPH-cytochrome c reductase has been
shown to inhibit human hepatic NADPH-cytochrome c reductase,
NADPH-cytochrome P450 reductase and aminopyrine N-demethylation
(Masters et al., 1971; Nelson et al., 1971) thus verifying that a
common flavoprotein is involved in the MFO system of human and
animal livers. 3) Induction of the human hepatic MFO system
by both barbiturates (Black et al., 1973; Gold and Ziegler, 1973)
and by ethanol (Rubin et al., 1970) has been reported. One point
of difference between the adult human and rat hepatic MFO system
lies in their differing sensitivity to the inhibitor SKF 525-A
(Ackermann, 1970). SKF 525-A inhibited aminopyrine N-demethylation
in man only at a concentration eight times greater than in rat
<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Component</th>
<th>Source of Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ackermann (1970)</td>
<td>Aminopyrine N-demethylase, Codeine O-demethylase, NADPH-cytochrome c reductase, Cytochrome b$<em>5$ and P$</em>{450}$</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Ackermann (1972)</td>
<td>Ethylmorphine N-demethylase, Aniline 4-hydroxylase, Cytochrome P$_{450}$</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Ackermann and Heinrich (1970)</td>
<td>N-monomethyl-p-nitroaniline, N-demethylase, 4-nitroanisole O-demethylase, NADPH-cytochrome c reductase</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Alvares et al (1969)</td>
<td>Cytochromes b$<em>5$ and P$</em>{450}$, Benzpyrene hydroxylase</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Behr et al (1971)</td>
<td>Nortriptyline hydroxylase, Desmethyliemipramine hydroxylase</td>
<td>P.M.</td>
</tr>
<tr>
<td>Black et al (1973)</td>
<td>Cytochrome P$_{450}$</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Coccia and Westerfeld (1967)</td>
<td>Various pathways of chlorpromazine metabolism</td>
<td>P.M.</td>
</tr>
<tr>
<td>Creaven and Williams (1963)</td>
<td>Biphenyl hydroxylase, Coumarin hydroxylase</td>
<td>P.M.</td>
</tr>
<tr>
<td>Darby et al (1970)</td>
<td>Codeine O-demethylase, Hexobarbital hydroxylase, Aniline 4-hydroxylase, NADPH-cytochrome c reductase, Cytochrome P$_{450}$</td>
<td>P.M.</td>
</tr>
<tr>
<td>Darby et al (1972)</td>
<td>Tolbutamide hydroxylase</td>
<td>Biopsy and P.M.</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Component</td>
<td>Source of Material</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Darby and Grundy (1973)</td>
<td>Tolbutamide hydroxylase</td>
<td>Biopsy</td>
</tr>
<tr>
<td></td>
<td>NADPH-cytochrome c reductase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P₄₅₀</td>
<td></td>
</tr>
<tr>
<td>Deckert and Remmer (1972)</td>
<td>Aminopyrine N-demethylase</td>
<td>Biopsy</td>
</tr>
<tr>
<td></td>
<td>Aniline 4-hydroxylase</td>
<td></td>
</tr>
<tr>
<td>Doshi et al (1972)</td>
<td>Pentobarbital hydroxylase</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Enomoto and Sato (1967)</td>
<td>2-Acetylaminofluorene N-hydroxylase</td>
<td>P.M.</td>
</tr>
<tr>
<td></td>
<td>2-Acetylaminofluorene 7-hydroxylase</td>
<td></td>
</tr>
<tr>
<td>Gold and Ziegler (1973)</td>
<td>Aminopyrine N-demethylase</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Kamataki et al (1973)</td>
<td>Aminopyrine N-demethylase</td>
<td>P.M.</td>
</tr>
<tr>
<td></td>
<td>Hexobarbital hydroxylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aniline 4-hydroxylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADPH-cytochrome c reductase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P₄₅₀</td>
<td></td>
</tr>
<tr>
<td>Kitagawa and Kamataki (1971)</td>
<td>Aminopyrine N-demethylase</td>
<td>P.M.</td>
</tr>
<tr>
<td></td>
<td>Aniline 4-hydroxylase</td>
<td></td>
</tr>
<tr>
<td>Korten and Van Dyke (1971)</td>
<td>Aminopyrine N-demethylase</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Aniline 4-hydroxylase</td>
<td></td>
</tr>
<tr>
<td>Kuntzman et al (1966)</td>
<td>Benzpyrene hydroxylase</td>
<td>Biopsy</td>
</tr>
<tr>
<td></td>
<td>Pentobarbital hydroxylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetophenetidin O-dealkylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-methyl-4-monomethylaminoazobenzene N-demethylase</td>
<td></td>
</tr>
<tr>
<td>Masters et al (1971)</td>
<td>Aminopyrine N-demethylase</td>
<td>P.M.</td>
</tr>
<tr>
<td>Nelson et al (1971)</td>
<td>Aminopyrine N-demethylase</td>
<td>P.M.</td>
</tr>
<tr>
<td></td>
<td>NADPH-cytochrome c reductase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P₄₅₀</td>
<td></td>
</tr>
<tr>
<td>Ozols (1972)</td>
<td>Cytochrome b₅</td>
<td>P.M.</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Component</td>
<td>Source of Material</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Pelkonen (1973)</td>
<td>Aminopyrine N-demethylase</td>
<td>Biopsy</td>
</tr>
<tr>
<td>Pelkonen et al 1973</td>
<td>Aniline 4-hydroxylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Benzpyrene hydroxylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADPH-cytochrome c reductase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P$_{450}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hexobarbital oxidase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Benzpyrene hydroxylase</td>
<td></td>
</tr>
<tr>
<td>Saggars et al (1970)</td>
<td>Quinine 2-hydroxylase</td>
<td>P.M.</td>
</tr>
<tr>
<td></td>
<td>Aniline 4-hydroxylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P$_{450}$</td>
<td></td>
</tr>
<tr>
<td>Schoene et al (1972)</td>
<td>Aminopyrine N-demethylase</td>
<td>Biopsy</td>
</tr>
<tr>
<td></td>
<td>4-nitroanisole O-demethylase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADPH-cytochrome c reductase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P$_{450}$</td>
<td></td>
</tr>
<tr>
<td>Strother (1970)</td>
<td>Various pathways of methylcarbamate metabolism</td>
<td>Biopsy</td>
</tr>
<tr>
<td>(1972)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thorgeirsson and Davies (1971)</td>
<td>Ethylmorphine N-demethylase</td>
<td>Biopsy</td>
</tr>
<tr>
<td></td>
<td>NADPH-cytochrome c reductase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P$_{450}$</td>
<td></td>
</tr>
<tr>
<td>Vieu et al (1972)</td>
<td>Prazepam N-demethylase</td>
<td>P.M.</td>
</tr>
</tbody>
</table>
liver to obtain a comparable degree of inhibition. Furthermore, codeine O-demethylation in human microsomes was not inhibited up to a concentration of SKF 525-A of 0.8mM. Similar results have been shown for the inhibition by warfarin on aniline hydroxylase (Deckert and Remmer, 1972) there being no species difference with its effect on aminopyrine N-demethylation.

Activity of the human hepatic MFO system does not appear to be preferentially influenced by a specific disease process (Gold and Ziegler, 1973) but is markedly decreased upon severe liver damage (Doshi et al, 1972; Gold and Ziegler, 1973; Schoene et al, 1972). Interestingly, activity of NADPH-cytochrome c reductase does not decrease during severe liver damage whereas the levels of P_450 and of N- and O-demethylation do (Schoene et al, 1972).

The one component of the adult human hepatic MFO system that has received little attention is that of the spectrally-apparent substrate-P_450 interactions. Whilst aniline has been shown to be a type II compound with human and rat hepatic microsomes (Pelkonen, 1973; Kamatskki et al, 1971, 1973; Ackermann, 1972), hexobarbital and aminopyrine, both type I in the rat, have been described as being either type I (Pelkonen, 1973) or type II (Kamatskki et al, 1971, 1973) in man. Desmethylinipramine has been stated to be a weak type I compound in man (Rane et al, 1971) whilst SKF 525-A has been described as being type II (Kamatskki et al, 1971); both these substrates are type I in the rat. Warfarin produces a modified type II spectrum in both rat and human liver microsomes (Deckert and Remmer, 1972).
Various components of the MFO system have also been found in foetal human liver microsomes (Ackermann et al., 1972; Pelkonen, 1973a; Yaffe et al., 1970). It is extremely interesting to note that foetuses from other animal species do not possess an active hepatic microsomal MFO system (see Pelkonen 1973a). Activity of the MFO system was lower in human foetal microsomes when compared to adult levels (Pelkonen et al., 1973) but could be induced by treatment of the mothers with phenobarbitone (Pelkonen et al., 1973a).

3. Factors Influencing Activity of the Hepatic MFO System

A number of factors are known which can modify the functional ability of the liver to metabolize xenobiotics and these factors can be broadly classified into three groups — genetic, physiological and environmental. Within the context of the present work the most important factor is that of species variation. Gillette (1971) has pointed out that variations and alterations in the hepatic MFO system brought about by these factors cannot be universally related to any specific component of the system but may arise from alterations in any of the components.

A. Genetic Factors: Species and Strain Variations

It is well known that the duration of therapeutic action and toxicological response of many drugs and other xenobiotics is species dependent. For example, there is a marked species
difference in the hexobarbital-induced sleeping time for mouse, rabbit, rat and dog (Quinn et al, 1958). A similar species variation has been found for the carcinogenicity of 2-acetylaminofluorene (see Parke, 1968), the guinea pig and lemming being refractory whilst the mouse, rabbit, hamster and dog are susceptible. Furthermore, there is great unpredictability in the patterns of species differences when comparing one compound with another.

In the testing of new drugs and other selectively-toxic chemicals for use in man, studies of the toxicity and pharmacological activity are usually carried out in two or three mammalian species, and from these limited data predictions are made for man. As illustrated above the extent of species variation is such that these extrapolations to man cannot be made with any degree of certainty. As yet no species has been found which shows toxicological and pharmacological responses to all compounds similar to those in man and even the use of non-human primates has not been found to be satisfactory (Weiner et al, 1968).

In the past fifteen years it has become increasingly apparent that these species variations in the effects of drugs and carcinogens are frequently due to differences in the rate of metabolism of the compounds themselves. Thus the species variation in sleeping time has been shown to be due to differences in the rate of metabolism of hexobarbital to its inactive products (Quinn et al, 1958), and the species variation in the carcinogenicity of 2-acetylaminofluorene is related to the
proportion of the two metabolites, N-hydroxy and 7-hydroxy-2-acetylaminofluorene, produced in the liver (see Parke, 1968). The N-hydroxy derivative is carcinogenic whereas the other is not.

By virtue of their relatively complex chemical structure the vast majority of xenobiotics have the potential for metabolism by apparently independent different routes. It is thus not surprising that species differences in the pattern of metabolite formation have been frequently noted. An excellent example is the metabolism of amphetamine (Dring et al., 1966) (Fig. 2.3). Aromatic hydroxylation is the major route of metabolism in rat, whereas in man, dog and rabbit the major route is oxidative deamination. A further example is that of phenacetin. In man phenacetin is metabolized by O-dealkylation to yield \( p \)-acetamidophenol, itself an analgesic, but in dog it is metabolized by deacetylation to give \( p \)-phenitidine and subsequently, by O-dealkylation, \( p \)-aminophenol, both of which are toxic and cause methaemoglobinaemia (Nery, 1971) (Fig. 2.4).

Although most of the studies mentioned above were carried out by analysis of various body fluids following in vivo xenobiotic administration, a great number of studies have been made under in vitro conditions using microsomal fractions. Thus, species variations in the activity of the hepatic MFO system have been noted with hexobarbitone (Quinn et al., 1958), 2-acetylaminofluorene, coumarin, biphenyl (Parke, 1968), pentobarbital, benzpyrene, phenacetin (Kuntzman et al., 1966), codeine (Ackermann, 1970), methylcarbamates (Strother, 1972) and tolbutamide (Derby et al., 1972).
Work by Davies et al (1969) and Flynn et al (1972) has shown that these species differences in the rates of metabolism by the hepatic MFO system cannot be attributed to variations in the level of NADPH-cytochrome c reductase and are not entirely due to differences in the magnitude of the substrate-induced binding spectrum. Flynn et al (1972) have observed that prolonged storage of microsomes with aminopyrine or aniline resulted in atypical type I or type II spectra which had maximum absorption at different wavelengths in various species. This observation implies that subtle differences in the state of cytochrome $P_{450}$ may exist in various species. The role that differences in cytochrome $P_{450}$ content may play as determinants in species variation has not been resolved. Davies et al (1969) state that it is not a determinant whilst Flynn et al (1972) state that it is, even though they could show no great difference in the $P_{450}$ level in the various species. The one parameter that does appear to be a determinant is the NADPH-cytochrome $P_{450}$ reductase level. Although they suggested that the quantity of this enzyme is in general the most important factor involved in determining overall drug oxidation activity, they concluded that it could not account for all the observed species variation (Flynn et al, 1972).

Strain variations in the hepatic metabolism of drugs have been reported for rats (Quinn et al, 1958; Siegert et al, 1964), rabbits (Cram et al, 1965) and mice (Vesell, 1968), and the strain variations in the extent of metabolism of 2-naphthylamine in mice has been correlated with the incidence of chemical carcinogenesis (Dewhurst, 1963a).
Fig 2.3. Pathways for the Metabolism of Amphetamine

![Diagram of pathways for the metabolism of amphetamine]

- Amphetamine
- Oxidative deamination
- Ring hydroxylation
- p-Hydroxyamphetamine
- Oxidation
- Benzoic acid
- Benzylmethylketone
- Reduction
- 1-Phenylpropan-2-ol

Fig 2.4. Pathways for the Metabolism of Phenacetin

![Diagram of pathways for the metabolism of phenacetin]

- Phenacetin (p-Ethoxyacetanilide)
- Deacetylation
- O-Deethylation
- p-Acetamidophenol
- p-Phenetidine
- p-Aminophenol
8. **Physiological Factors**

This class encompasses age, sex, diurnal, hormonal (including pregnancy), nutritional and disease factors.

The levels of all the components of the hepatic MFO system so far studied change with age. For a short period following birth they are very low in the mouse, guinea pig, rabbit, rat, pig and man (Jondorf *et al.*, 1958; Fouts and Devereux, 1972; Macleod *et al.*, 1972; Basu *et al.*, 1971; Short and Stith, 1973; Pelkonen, 1973). After birth there is a rapid rise in the capability of the newborn to metabolize drugs during which the levels of all the parameters of the hepatic MFO system also rapidly increase.

A large number of hormones influence activity of the hepatic MFO system. Thus, thyroid hormone, adrenal hormones, insulin, ACTH and growth hormones have all been shown to exert such an influence (Parke, 1968; Kato and Takanashi, 1968; Pfeiffer *et al.*, 1972; Wilson, 1973). The sex differences in microsomal drug metabolism that are observed in rats are also hormonal-linked (Quinn *et al.*, 1958; Macleod *et al.*, 1972), as are the effects of diurnal variation (Radzialowski and Bousquet, 1968; Jori *et al.*, 1971; Burke *et al.*, 1972) and pregnancy (Parke, 1968).

The nutritional state of an animal can influence its capabilities to metabolize drugs by the hepatic MFO system. Fasting, starvation, protein deficiency, low dietary levels of Fe and Ca and ascorbic acid deficiency in guinea pigs have all been shown to have an influence in this respect (Bock *et al.*, 1973; Cram *et al.*, 1970; McLean and McLean, 1966; Becking, 1972; Dingell *et al.*, 1966; Chedwick *et al.*, 1973). Also, various components of the diet, whether
natural or synthetic, may also influence the hepatic \textit{MFO} system. These include Cedrene (Hashimoto \textit{et al}, 1972), caffeine (Lombrozo and Rizoma, 1970), and flavones (Cutroneo \textit{et al}, 1972), as well as various environmental contaminants such as DDT and polychlorinated biphenyls (Fouts, 1970; Litterst \textit{et al}, 1972).

Damage to the liver leads to an impairment in its ability to metabolize drugs (Green \textit{et al}, 1973). \textit{In vitro} experiments in animals have shown that cholestasis leads to an impairment of the \textit{MFO} system (Schaffner \textit{et al}, 1971) and it is thought that accumulation of bile acids which block the active site of the appropriate enzyme may explain this (Denk, 1973). Infection of mice with murine hepatitis virus also reduces activity of the \textit{MFO} system (Kato \textit{et al}, 1963). Similar \textit{in vitro} results have been reported in man (Doshi \textit{et al}, 1972; Gold and Ziegler, 1973; Schoene \textit{et al}, 1972).

Rats with abdominal carcinosarcomas, intramuscular transplants and breast tumours show impaired activity of the hepatic \textit{MFO} system (Kato \textit{et al}, 1963; Khandekar \textit{et al}, 1972; Brown \textit{et al}, 1971) whilst this activity is severely impaired or even absent in hepatomas (Brown \textit{et al}, 1971). Liver regeneration following partial hepatectomy is accompanied by a fall in activity of the \textit{MFO} system which returns to its original value once the rapid cellular proliferation is complete (Henderson and Kersten, 1970).

C. \textbf{Environmental Factors}

Stress conditions such as cold and noise lead to a rapid rise in activity of the hepatic \textit{MFO} system (Inscoe and Axelrod, 1960; Dawhurst, 1963; Fuller \textit{et al}, 1972). This rise is probably due to a rapid induction of the system and is under the control of the pituitary-adrenal axis (Drievo and Bousquet, 1965).
A characteristic property of the hepatic MFO system is its ready \textit{in vivo} susceptibility by a wide range of foreign compounds to which man maybe potentially exposed, including drugs, pesticides and polycyclic hydrocarbons (Remmer, 1973). The most widely studied inducers are phenobarbitone and the polycyclic hydrocarbons such as 3-methylcholanthrene (3-MC). These two compounds appear to exert their inducing effects by different mechanisms. For example, phenobarbitone induction enhances almost all of the reactions catalysed by $P_450$ whereas 3-MC induces relatively few pathways. These differential inductive effects are also apparent on the components of the hepatic MFO system including $P_450$, NADPH-cytochrome $c$ ($P_450$) reductase and type I and type II spectral changes. The selective induction by 3-MC is accompanied by the appearance of a different form of $P_450$ termed $P_{448}$, the formation of which probably accounts for the relatively selective induction of drug metabolizing enzymes by 3-MC. Whether the $P_{448}$ represents the formation of a new haemoprotein or is merely a different allosteric form of the existing $P_450$ has not been fully resolved. For a more comprehensive review on enzyme induction see Gillette (1971), Remmer (1973), Conney \textit{et al}, 1973).

In addition to their role as inducers of the hepatic MFO system phenobarbitone and 3-MC have also been shown, amongst other things, to induce the level of serum hemopexin, the haem-binding $\beta$-glycoprotein (Smibert \textit{et al}, 1972), to inhibit the activity of ribosomal and microsomal ribonucleases (Lechner and Pousada, 1971; Louis-Ferdinand and Fuller, 1972; Pousada and Lechner, 1972; Smith \textit{et al}, 1972), and to increase bile flow and bile salt excretion (Paumgartner \textit{et al}, 1971; Redinger and Small, 1973).
Moreover, inducers of the hepatic MFO system possess the following additional characteristics:

a) They increase the microsomal conjugation of bilirubin with uridine diphosphoglucuronic acid and hence decrease the serum level of free bilirubin (Catz and Yaffe, 1968).

b) They increase the excretion of glutathione-conjugated bromsulphophthalein (BSP) (Fujimoto et al., 1965; Klaassen and Plea, 1968).

c) They increase the microsomal 6β-hydroxylation of cortisol and subsequent excretion of this product (Conney et al., 1965).

d) They increase the excretion of D-glucaric acid in ascorbic acid-requiring animals (Hunter et al., 1973).

Evidence that phenobarbitone and other inducers of the hepatic MFO system behave similarly in man emerges from several sources:

1) Analysis of the plasma half-lives of various drugs has revealed that phenobarbitone, tricyclic antidepressants, diphenylhydantoin and ethanol, all known inducers in the rat, also induce drug metabolism in man (Levi et al., 1968; O'Malley et al., 1973; Hausen et al., 1971; Vesell et al., 1971).

2) Phenobarbitone treatment decreases the free bilirubin levels in the serum of jaundiced infants (see Cao et al., 1973).

3) The excretion of BSP is more rapid in patients receiving phenobarbitone (Gogl, 1971).
4) Diphenylhydantoin, tricyclic antidepressants and phenobarbitone all increase the excretion of \( \alpha \)-hydroxycortisol in humans (Conney et al., 1965; O'Malley et al., 1973; Choi et al., 1973).

5) Anticonvulsant drugs and phenobarbitone increase the urinary excretion of D-glucaric acid (Hunter et al., 1971).

6) A few authors have also shown induction of the human hepatic MFO system in liver samples obtained from patients on alcohol and tricyclic antidepressants (Rubin et al., 1970; Mezey and Tobon, 1971; Black et al., 1973; Gold and Ziegler, 1973).

Inhibition of the hepatic MFO system by competition of substrates and by other means can also occur and although the inhibition patterns are complex it does appear that SKF 525-A and metyrapone, the two most commonly used inhibitors, act by competing with the substrate for the active site of the enzyme (Anders and Mannering, 1966; Roots and Hildebrandt, 1973). Moreover it also appears that, at least in some cases, metabolism of the inhibitor by the MFO system is a prerequisite for its action (Schenkman et al., 1972; Parli et al., 1973). The administration of steroids, antibiotics, \( \text{CCl}_4 \), lead, methylmercury and other hepatotoxins have all been shown to inhibit hepatic microsomal drug metabolism (Juchau and Fouts, 1966; Dixon and Fouts, 1962; Peters and Fouts, 1969; Archakov and Karuzina, 1973; De Matteis, 1973; Sweeney et al., 1973; Alvaros et al., 1972). Similar inhibition by oral contraceptives and antibiotics has been noted in humans (O'Malley et al., 1972; Christensen and Skovsted, 1969).

In the light of the numerous, previously described, genetic and environmental factors capable of altering hepatic microsomal
drug metabolizing activity in experimental animals it comes as no surprise that humans display large, if not extreme, individual differences due to a complex interrelationship of these genetic and environmental factors (Vesell, 1972).

4. Isolation of Hepatic Endoplasmic Reticulum Fragments for In Vitro Drug Metabolism Studies

When the liver is homogenized the endoplasmic reticulum fragments, the fragments collectively being termed the microsomal fraction of the resulting homogenate. Classically this microsomal fraction is harvested by the method of Schneider and Hogeboom (1950) involving a scheme of differential centrifugation. Using this approach the homogenate is first centrifuged at 10,000 g for 10 - 20 minutes to remove intact cells, erythrocytes, nuclei and mitochondria. The supernatant resulting from this preliminary centrifugation is then subjected to a spin at 105,000 g for 60 minutes. The supernatant (cytosol) is discarded, the pellet being the microsomal fraction. For many studies of in vitro drug metabolism this fraction is too impure and has to be purified by a further centrifugation at 105,000 g for 60 minutes.

From this brief description two major disadvantages to the use of differential centrifugation as a means of isolating microsomes are apparent. Firstly, it can take up to 4 hours from the time of obtaining the liver to the stage where a microsomal suspension is ready for use; this is particularly disadvantageous when the liver specimen itself has been left for some time before processing, as may occur with human biopsy and autopsy samples. Secondly, this technique involves the use of costly high-speed centrifugation equipment.
Unfortunately, until recently no alternative technique has been available which overcomes these two disadvantages. In the last six years, however, three different techniques have been described for the rapid isolation of hepatic microsomes obviating the use of high-speed centrifugation. All of these techniques use as a starting point the 10,000 g post-mitochondrial supernatant described above.

The first such technique is the use of gel filtration involving Sepharose 2B (Staron and Kaniuga, 1971; Tangen et al, 1973). Comparable levels of NADPH-cytochrome c reductase and aminopyrine N-demethylase activity have been found in microsomal fractions prepared by conventional centrifugation and gel filtration (Tangen et al, 1973). However, certain disadvantages do exist with this technique.

1) The yield of microsomal protein and cytochrome P450 is lower in microsomes prepared by gel filtration (Tangen et al, 1973).

2) It takes approx. 1 hour to obtain a usable microsomal fraction when timed from the commencement of the gel filtration. This preparation time is obviously greatly increased when more than one sample is being processed.

3) When more than one sample is processed the high cost of precision columns, fraction collectors and monitors becomes prohibitive.

Another recently described technique for the rapid isolation of hepatic microsomes is that of Ca^{++}-induced aggregation (Kamath et al, 1971). Later reports by Kamath and co-workers (Kamath and Narayan, 1971; Kamath and Rubin, 1972) have shown that Ca^{++}-aggregated microsomes contain levels of in vitro drug metabolizing activity, when measured as type I and type II metabolism and
cytochrome content, comparable to those in microsomes prepared conventionally, a finding confirmed by Kupfer and Levin (1972), Schenkmann and Cinti (1972), Cinti et al (1972) and Baker et al (1973). It has also been shown that Ca**+-induced aggregation leads to microsomes typical with regard to their inducibility by phenobarbital (Kupfer and Levin, 1972) and their ability to bind substrates of the MFO system (Cinti et al, 1972; Baker et al, 1973). Once again, this technique has its own disadvantages:-

1) If the original method of Kamath et al (1971) is used a large dilution of the PMS has to be made, hence leading to unwieldy volumes for the subsequent centrifugation.

2) If, on the other hand, the modified method of Cinti et al (1972) is considered, a spin of 27,000g for 15 minutes has to be used, which together with the run-up, run-down and washing times, constitutes a lengthy high-speed centrifugation step.

3) One report (Schenkmann and Cinti, 1972) has observed that Ca**+-aggregated microsomes lose their ribosomes, although it should be noted that other reports (Kamath et al, 1972; Baker et al, 1973) have refuted this claim.

The other recent technique for the rapid isolation of microsomal fractions is that of isoelectric precipitation, by which the pH of PMS is lowered to 5.0-5.4 and the aggregated microsomes harvested by brief centrifugation (Karler and Turkanis, 1968; Mitchard, 1969). As early as 1946 Claude was able to show that aggregation occurred at acid pH, and suggested that this isoelectric
precipitation represented a membrane phenomenon common to all membranous intracellular components. This pH-dependent aggregation of hepatic microsomes was later confirmed by Leone and Redstone (1962). Karler and Turkanis (1968) and Ritcherd (1969) have recently shown that microsomes prepared by isoelectric precipitation have levels of hexobarbital oxidase and benzphetamine N-demethylation activity comparable to those present in conventionally-prepared microsomes. Unfortunately, no further work on the use of acid-precipitated microsomes in studying in vitro MFO activity was, or has since been, mentioned.

In light of the apparent speed and simplicity of operation it was felt that this technique could prove a valuable alternative to conventional centrifugation in studies of hepatic MFO activity in vitro. Accordingly, microsomes prepared by isoelectric precipitation were thoroughly studied for their ability to exhibit characteristics of the MFO system in vitro, both in rat liver and, as far as possible, in human liver.

5. Stability of the Hepatic MFO System — Storage and Post Mortem Characteristics

A number of workers have commented on the storage characteristics of the hepatic MFO system as a factor influencing their results. Thus Clark (1967) has stated that freezing of 10,000 g supernatant of rat liver homogenate does not inactivate pethidine N-demethylation over "several days" whilst Pederson and Aust (1970) have stated that storage at -15°C under N₂ and in the presence of 50% glycerol has no effect on aminopyrine demethylation in rat liver microsomal suspensions, although no time limit for the storage was specified.
Similarly, storage of rat or pig hepatic microsomes, either as pellets or the intact liver, at -18°C for 1 - 4 days has no effect on the in vitro metabolism of imipramine (Gigon and Bickel, 1971). Maintenance of a microsomal suspension in buffer at -5°C for 7 days largely destroys the type I spectral interaction but not that of type II (Shoeman et al, 1969), whilst storage at 4°C for 24 hours brings about a reduction in the magnitude of the type I interaction without greatly changing the $K_g$ (Kutt et al, 1970). Storage of the microsomal suspensions at -15°C for 18 days brings about no loss of activity with respect to aniline hydroxylation, aminopyrine demethylation and binding affinity (Deckert and Rammer, 1972). Generally similar findings have been observed in respect to the storage of human biopsy liver samples (Black et al, 1973; Gold and Ziegler, 1973) although contamination of microsomes by haemoglobin after overnight storage of frozen solid human liver samples has been reported (Alvares et al, 1969).

A short-term study into the aging in vitro (90 hours at 1°C under $N_2$) of hepatic microsomal suspensions has been reported by Hewick and Fouts (1970). The decreases in the aniline type II difference spectra paralleled the loss of total haem and of cytochrome P450 content whilst the type I spectra were less stable to aging in vitro than the previously mentioned components. The decreases in type I and type II metabolism were loosely correlated with the decreases in the magnitude of the binding spectra of the appropriate substrate.

Few authors have, however, investigated either the optimal storage conditions or the causes for the decay changes that occur on storage. Early work by Leadbeater and Davies (1964) showed that
storage for 30 days at $-40^\circ C$ was most successful with 10,000 g supernatant, freeze-dried 10,000 g supernatant or microsomal suspension being less successful. Storage at $0^\circ C$ was not recommended as all enzyme activity was lost within 7 days. Furthermore, the stability varied according to the enzyme parameters being studied, a finding echoed by Levin et al (1969) who also observed that where storage of microsomes for more than one day is absolutely necessary they should be stored as pellets overlaid with buffer at $-15^\circ C$ rather than as a suspension or freeze-dried powder. Similar findings with regard to cytochrome P$_{450}$ content have recently been reported by Wade et al (1972) and Burke (1973).

Gram et al (1966), Jacobson et al (1973) and Archakov and Karuzina (1973) have postulated that the inactivation of the microsomal MFO system that occurs during incubation at $37^\circ C$ in the presence of an NADPH-generating system is due to peroxidative destruction of microsomal lipids which are known to be essential for the full realization of this MFO system. Studies by Burke (1973) have revealed that the degradative changes that occur on storage of hamster hepatic microsomes are also accompanied by increases in lipid peroxidation, as measured by malonaldehyde production. Thus, it can be postulated that it is the peroxidative damage to the microsomal membrane lipids that is intimately involved in the storage decay in activity of the MFO system.

Lipid peroxidation is the reaction of oxidative deterioration of polyunsaturated lipids. Microsomal membranes contain relatively large amounts of polyunsaturated fatty acids in their phospholipids and some of the most powerful catalysts that initiate lipid peroxidation, namely haemoproteins, are in close molecular proximity to these polyunsaturated lipids (Tappel, 1973).
Indeed, it does appear that the system responsible for lipid peroxidation in microsomal membranes is, or is intimately allied with, the NFO system itself (see Fig. 2.5). Both NADPH-cytochrome c reductase and NADPH-cytochrome b\textsubscript{5} reductase have been shown to be involved in the initial propagation of lipid peroxidation in microsomal membranes (Aust et al., 1972; Pederson et al., 1973; Bidlack et al., 1973) by converting molecular oxygen to an active intermediate, probably a superoxide, (a), (Pederson and Aust, 1972). The superoxide then reacts with the polyunsaturated membrane lipids so producing semistable lipid peroxides, (b), which then commence a chain reaction of lipid peroxidation on adjacent lipids, (c), (Tappel, 1973). Furthermore, these two enzymes also possess a peroxidative activity, (d), by which the lipid peroxides are converted to other radicals which are themselves capable of propagating lipid peroxidation within the cyclic system, (e), (Hrycay and O'Brien, 1973; Bidlack et al., 1973). The cumulative effects of this lipid peroxidation cycle include the loss of polyunsaturated lipids and protein from the membrane, loss of cytochrome P\textsubscript{450} which is paralleled by decreased activity of the NFO system and, finally, ultrastructural changes to the membrane itself (Bidlack and Tappel, 1973; Hogberg et al., 1973).

In addition, inactivation of cytochrome P\textsubscript{450} by both carbon tetrachloride and allylisopropylacetamide may be associated with their ability to stimulate microsomal lipid peroxidation (Archakov and Karuzina, 1973; Schacter et al., 1973). Interestingly, there is little loss of cytochrome b\textsubscript{5} on storage of microsomal fractions (Wade et al., 1972; Hewick and Fouts, 1970) and neither is there any
Fig 2.5. Tentative Pathway of Lipid Peroxidation in Hepatic Microsomes

Superoxide \([O_2^-]\) → NADP → NADPH

\(\text{P}_{450}^{b_5}\) → Fe++ → (a) → 0₂

LO• → OH

Lipid (L) → LOOH

Destruction of Membrane Lipids

Destruction of Membrane Integrity

Disruption of MFO System
appreciable loss of cytochrome \( b_5 \) on stimulation of lipid peroxidation \textit{in vitro} (Schacter \textit{et al}, 1973).

The post-mortem stability of the hepatic MFO system has been little studied. An early report by Creaven and Williams (1963) indicated that leaving rat carcasses at room temperature for up to 2 hours reduced the biphenyl 4-hydroxylase level to 50% that of the fresh sample. Most of this fall-off occurred in the first hour and if the liver samples obtained after 2 hours were then placed in the deep freeze for 24 hours the enzyme activity remained at the 50% level. Similar experiments with rabbit livers showed that the loss in enzyme activity was no more than 10% in the first hour and only about 15 - 20% in three hours, this latter value being maintained if the liver was then stored for 24 hours in the deep freeze. These latter findings were later confirmed by Leadbeater and Davies (1964) who further showed that only 15 - 40% of the original level of enzyme activity remained after 24 hours \textit{in situ} storage post-mortem but that after this time the rate of inactivation was very much lower.

Recent work by Macleod \textit{et al} (1973) has shown that \textit{in situ} storage post-mortem of rabbit livers under conditions approximating to those at human autopsy led to decreases in the levels of type I and type II metabolism, NADPH-cytochrome \( c (P_{450}) \) reductase and cytochrome \( P_{450} \) in the order of 50 - 80%. In livers removed immediately after death and kept in ice for the same period of time (4.5 hours) the levels of these components remained stable. These findings are in agreement with those of Jondorf and Donahue (1970). Macleod \textit{et al} (1973) came to the conclusion that slow cooling of the liver with
coincidental autolysis was the major cause for this post-mortem decay. The possibility exists that these autolytic changes may well be lipid peroxidative in nature.

For obvious reasons no direct study has been made of the post-mortem characteristics of the human hepatic MFO system but some useful facts on this topic may be gleaned from one or two relevant papers. Thus, Kitagawa and Kamataki (1971) have measured type I and type II metabolism in human livers obtained post-mortem and then placed at 24°C for various periods of time. By extrapolating back from these results they endeavoured to assess the levels of metabolism in the living state. This approach did not prove very successful due to the widely differing individual decay rates. Darby et al. (1973) have reported that the Vmax, and Km values for [ureyl-14C]-tolbutamide metabolism were the same in human biopsy and post-mortem material obtained less than 4 hours after death. This finding implies that no significant autolytic alteration in the human hepatic microsomal MFO system is present up to 4 hours subsequent to death.

6. Metabolism of Xenobiotics in Perfused Liver, Isolated Cells and Tissue Culture Systems

The systems of liver perfusion, isolated cells and tissue cultures are potentially a much closer approximation to the state in vivo than is the use of isolated liver fractions, large because the cell architecture is, in these systems, essentially unchanged. However, for various technical reasons much less work has been done on the application of these systems in studies of MFO activity.
Metabolism of drugs in perfused liver systems has been reported for a number of compounds including benzpyrene (Juchau et al., 1965), chlorpromazine (Cordelli et al., 1969), imipramine (Cordelli et al., 1969; Bickel and Rinder, 1970), phenylbutazone, antipyrine and nortriptyline (von Behr et al., 1970). Unfortunately, certain problems of technique do exist which are serious drawbacks when considering the validity of the liver perfusion system. These drawbacks include the possible interference from hormones and vasoactive factors in the circulating blood (particularly if the blood is heterologous), the problem of adequate gas exchange, the short time (less than 8 hours) during which each preparation is viable, and the difficulty in setting up more than one perfusion at a time (see Bartosek et al., 1973). These problems do not arise when using cell culture systems and consequently a system of cultured adult normal mammalian liver could potentially be the most appropriate in vitro method with which to study hepatic MFO activity.

As far as the author is aware no work has been reported on the metabolism of xenobiotics in cultures of normal adult mammalian liver. Nonetheless, a large number of such studies have been performed with other cell systems. Thus Henderson and Dewaide (1969), Holtzman et al. (1972) and Centrell and Bresnick (1972) have all reported drug metabolism in freshly isolated hepatocytes; Centrell and Bresnick (1972) also finding inducible drug metabolism in non-parenchymal cells. Cultures of human embryonic lung have been shown to metabolize carbaryl (Baron and Locke, 1970) and dimethoate (North and Menzer, 1972), the metabolite pattern of the former being similar to that found in the intact animal (Dorough, 1970). The metabolic profile of dimethoate varied according to
the cell-line studied (North and Menzer, 1972). A cloned line of hepatoma cells conjugates both p-aminophenol and p-nitrophenol with glucuronic acid (Dybing, 1972) while primary cultures of chick embryo liver demethylate both aminopyrine (Poland and Kappas, 1971) and chlorcyclizine (Poland and Kappas, 1973). Organ cultures of chick and rat embryonic liver are capable of conjugating p-aminophenol (Dutton, 1973).

A lot of work has been carried out into the metabolism of polycyclic hydrocarbons by cells in culture especially with regard to their carcinogenic potential (Gelboin and Weibel, 1971). A large number of different culture systems have been used including human lymphocytes (Kellerman et al., 1973), mouse embryo (Sims, 1970), monkey kidney, human foetal lung (Diamond, 1971) and many others (Diamond, 1971; Huberman et al., 1971; Huberman and Sachs, 1973). By using such a wide spectrum of culture systems it has been found that in general the susceptibility of cells in culture to the cytotoxic effects of the hydrocarbon is related to the amount of water-soluble metabolites produced from the hydrocarbon by the cells themselves (Huberman et al., 1971; Diamond, 1971; Gelboin and Weibel, 1971).

The enzyme largely responsible for the oxidative metabolism of the polycyclic hydrocarbons, aryl hydrocarbon hydroxylase, has been studied in great detail in foetal rodent cell and organ culture. Thus, aryl hydrocarbon hydroxylase activity in foetal rat liver explants has been shown to be inducible by 3-MC (Burki et al., 1971), flavones (Cutroneo et al., 1972) and phenobarbital (Cutroneo and Bresnick, 1973), similar findings having also been described for foetal rodent liver cells in short-term culture (Gielen and Nebert,
1971 a, b, c, 1972). Indeed much of the present knowledge regarding the early responses of cells to microsomal enzyme inducers and the fate of the inducer has been derived from this work of Nebert and co-workers (Nebert and Bausserman, 1970 a, b; Gielen and Nebert, 1971 a, b, 1972).

Nebert (1973) has suggested that foetal cell cultures, not necessarily from foetal liver, may prove useful in predicting toxic damage due to drugs in the intact foetus in utero, although the importance of metabolic transformation of the drug by the mother must also be studied. As Holtzman et al (1972) have pointed out the embryonic liver cells grow rapidly and are relatively undifferentiated, having little endoplasmic reticulum. For this reason they may be a poor model with which to study drug metabolism in the relatively stable adult hepatocyte. Consequently, adult mammalian liver cells in culture offer a much better model with which to study the mechanisms and patterns of drug metabolism and hepatotoxicity in the intact adult animal even though the procedures for obtaining such cultures are more difficult and unreliable.
# CHAPTER 3. CONTENTS

## MATERIALS

1. Animals and Phenobarbitone Treatment 47
2. Chemicals and Instruments 47

## METHODS

A. Preparation of Liver Fractions

1. Preparation of Liver Homogenate 48
2. Preparation of PMS 49
3. Preparation of 'Normal' Microsomes 49
4. Preparation of 'Acid' Microsomes 50

B. Assay Methods

1. Measurement of Protein 50
2. Extraction and Assay of RNA 51
3. Measurement of Total Haem Levels 52
4. Measurement of Malonaldehyde 52
5. Measurement of Cytochromes $b_5$ and $P_{450}$ 53
6. Assay for NADPH-cytochrome c Reductase Activity 54
7. Measurement of Ethylmorphine, Aminopyrine and Imipramine N-demethylase Activity 55
8. Measurement of Aniline 4-hydroxylase Activity 57
9. Measurement of 4-chloro-N-methylaniline N-demethylase Activity 59
10. Inhibition of Hepatic Microsomal Mixed Function Oxidase Activity in vitro 60
11. Observation and Quantitation of Spectrally Apparent Substrate-Microsome Interactions 60
12. Analysis of Results 61
CHAPTER 3. MATERIALS AND METHODS

MATERIALS

1. Animals and Phenobarbitone Pretreatment

Male rats of the Wistar/Albino strain were used throughout this work; their weights varied from 150 - 350 g, and they were bred and maintained in the University Animal House. All animals were allowed free access to food and water, the feed being Spiller's No. 1 Laboratory Diet. 'Sterolit' (Engelhard, New Jersey) was used as bedding to eliminate any possible interference from enzyme induction caused by wood shavings (Fujii et al., 1968).

Pretreatment with phenobarbitone was administered for 12 days prior to sacrifice, in the form of a 0.1% (W/V) solution of phenobarbitone sodium in the drinking water.

2. Chemicals and Instruments

The suppliers of certain chemicals mentioned in this work are listed in the Appendix. All other chemicals and solvents used were of the highest grade obtainable. Before use, biphenyl was purified by recrystallization (twice) from 96% ethanol. SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate HCl) was a gift from the Smith, Kline and French Laboratories Ltd. (Welwyn Garden City) and imipramine-HCl was a gift from Biorex Ltd.

pH measurements were made using an EIL pH meter, model 7039. For low speed centrifugation an MSE Minor bench centrifuge and Mistral 6L were used, whereas for high speed centrifugation an MSE High Speed 18 and Superspeed 50 were employed; all centrifugations were carried
Spectrophotometers used comprised the Pye-Unicam SP 1800 and Perkin Elmer 356 for high resolution measurements and the Pye Unicam SP 500 and 600 and Cecil CE 272 for routine measurement of colour production. When small scale enzyme assays were used, optical measurements were made using the micro-cell carriage and optical system modification on the Pye-Unicam SP 500.

Enzyme assays were carried out at 37°C in a shaking water bath (Mickle Engineering Co., Gomshall, Surrey) the tubes being shaken at a speed of 75 oscillations/minute. Sample mixing was achieved by use of a 'Whirlimixer' and extraction procedures involved the use of a rotary shaker built in the department.

METHODS,

A. Preparation of Liver Fractions

1. Preparation of Liver Homogenate

Rats were killed between 9.00 a.m. and 11.00 a.m. so as to minimize any differences caused by diurnal variation in drug metabolism (Burke et al., 1972). The animals were killed by cervical dislocation and the livers rapidly removed into cold (4°C) 1.15% (w/v) KCl. After removal of excess blood and mesentery, the liver was weighed in 10 ml KCl solution and this was followed by rough scissors-mincing. The contents of the beaker were then transferred to a cold Potter-Elvejhem homogenizer and homogenized by 3 up-and-down strokes of the Teflon plunger operated by an electric drill at max. speed. The homogenate was diluted with KCl solution such that 1 ml of homogenate contained the equivalent of 250 mg liver. (From the mincing stage onwards all operations were carried out at 4°C).
Human liver specimens, due to their more fibrous nature, were homogenized in a different manner. After transfer to the Potter-Elvejhem homogenizer the mince was first subjected to three 15 second bursts at max. speed with a 'Polytron' homogenizer (Northern Media Supply Ltd.) followed by homogenization and dilution as outlined above.

2. Preparation of Post Mitochondrial Supernatant (PMS)

The 25% (w/v) homogenate, obtained as outlined above, was transferred to a 50 ml. polypropylene centrifuge tube and centrifuged at 10,000 g av. for 20 minutes (11,000 r.p.m., 6 x 50 ml rotor, High Speed 18 centrifuge). As recommended by Bjorntorp et al (1965), human liver homogenates were centrifuged at 12,000 g av. for 20 minutes (14,000 r.p.m., 10 x 10 ml rotor, Superspeed 50 centrifuge) so as to minimize lysosomal contamination in the ensuing microsomal fraction. The supernatant derived from these spins is designated the 'post mitochondrial supernatant' (PMS).

3. Preparation of 'Normal' Microsomes

The resulting PMS was transferred to a 10 ml. polypropylene centrifuge tube and centrifuged at 105,000 g av. for 60 minutes (40,000 r.p.m., 10 x 10 ml. rotor, Superspeed 50 centrifuge). After this time the supernatant was discarded and the pellet washed by resuspension in 1.15% (w/v) KCl using a single complete stroke of the glass-Teflon homogenizer and further centrifugation at 105,000 g av. for 60 minutes. The pellet was finally resuspended in 0.1M phosphate buffer pH 7.4: glycerol (4:1 v/v), to a final concentration of 250 mg liver equivalent per ml. of microsomal suspension.
4. **Preparation of Acidic Microsomes**

To an aliquot of the PMS was added sufficient 0.2M acetate buffer pH 4.0 to lower the pH to 5.4. In early experiments a pH meter was used to monitor the pH changes; however, in later experiments it was found that 0.075 ml of the acetate buffer per ml of PMS was sufficient to lower the pH to 5.4. In all the experiments (approx. 100 PMS samples) the pH of the PMS, using unbuffered 1.15% (w/v) KCl as the homogenizing medium, lay in the range 6.70 - 6.85. This same pH range was found in the limited number of human liver PMS tested.

The aggregated microsomes were collected by centrifugation (see Chap. 4) and the pellet was washed by resuspension in 1.15% (w/v) KCl: glycerol (4:1 v/v), readjustment to pH 5.4 if necessary and recentrifugation. The final pellet was resuspended in 0.1M phosphate buffer pH 7.4: glycerol (4:1 v/v), to a final concentration of 250 mg liver equivalent per ml of microsomal suspension.

In some experiments 0.2M citrate buffer pH 4.0 or 0.1N HCl was used in place of the acetate buffer (see Chap. 4).

5. **Assay Methods**

1. **Measurement of Protein (Goodwin and Choi, 1970)**

This method, originally described for serum proteins, is based on the reaction of lysine residues in the protein with trinitrobenzene-sulphonic acid (TNBS), which at pH 10.5 and in the presence of sulphite results in the formation of a highly coloured complex with absorption maximum at 420 nm. This method was chosen in preference
to that of Lowry et al. (1959) due to its ease of operation and obeyance of Beer's Law up to at least 250\(\mu\)g protein per ml. incubation mixture.

To 0.5 ml diluted sample (dilutions: homogenate 1/500, PMS 1/200, microsomes 1/50, all in 0.1M sodium tetraborate) was added 3ml of a freshly-prepared mixture of equal volumes of 0.05\%(w/v) TNBS, 0.1M sodium tetraborate and 0.05M sodium sulphite (the TNBS was stored in the dark at 4°C and the sodium sulphite was freshly prepared each day). The contents of each tube were mixed and the tubes placed at 70°C for 15 minutes. After cooling, the samples were read at 420 nm against a standard of bovine serum albumin (500\(\mu\)g/ml in 0.1M sodium tetraborate) which had been similarly treated.

2. Extraction and Assay of RNA (Hatcher and Goldstein, 1969)

In this procedure the nucleic acids are precipitated with cadmium ions, followed by washing and hydrolysis, the hydrolysate then being assayed for RNA by a modified orcinol reaction.

To 1 ml of the microsomal suspension was added an equal volume of 1M cadmium chloride. After mixing, the tubes were placed at 37°C for 10 minutes. The precipitate was collected by centrifugation and the supernatant discarded. The pellet was washed twice in 0.1M cadmium chloride, again followed by standing at 37°C for 10 minutes and centrifuging.

3 ml of 0.1N HCl was then added to the pellet and the tube was placed in a boiling-water bath for 20 minutes to achieve complete hydrolysis. After cooling, 1 ml of hydrolysate was transferred to
another tube, followed by 1 ml. of 4% (w/v) orcinol in ethanol; 
1-butanol (7:3 v/v) and finally 3 ml. of 0.5% (w/v) ferric chloride 
in conc. HCl was added. The tubes were returned to the boiling-water 
bath for a further 30 minutes and after cooling the contents of each 
tube were read at 660 nm. against a similarly-treated standard 
containing 100 µg. hydrolysed RNA (Torula yeast) per ml. of 
0.1N HCl.

3. **Measurement of Total Haem Levels (Gilbert, 1972)**

Two glass cuvettes were set up each containing 1 ml. of 
0.1M NaOH, 1 ml. of 20% (v/v) eq. pyridine and 1 ml. microsomal 
suspension and, after mixing, a base line was recorded between 
490 - 600 nm. 0.03 ml. of 3mM potassium ferricyanide was added 
to the reference cuvette and an equal volume of water and approx. 
1 mg. sodium dithionite was added to the test cuvette. The spectrum 
was again recorded after mixing and the total haem level determined 
using the extinction coefficient of 32.4 cm⁻¹ mM⁻¹ for the O.D. 
difference between 557 and 575 nm. (Omura and Sato, 1964).

4. **Measurement of Malonaldehyde (Slater and Sawyer, 1972)**

To 2 ml. microsomal suspension was added 2 ml. 10% (w/v) TCA and 
after mixing the tubes were left at 4°C for 30 minutes. The tubes 
were centrifuged to remove the denatured protein and the malonaldehyde 
assayed by adding 2 ml. of 0.67% (w/v) thiobarbituric acid to 2 ml. 
of the deproteinized supernatant and placing in a boiling-water 
bath for 10 minutes. After cooling, the colours produced were 
read at 535 nm. against a similarly-treated malonaldehyde standard
of 2.5 n mole/ml. in 5% (w/v) TCA. The standard was prepared by diluting 1, 1, 3, 3-tetraethoxypropane in 5% (w/v) TCA; this resulted in the quantitative production of malonaldehyde as the bis-diethyl acetal.

5. Measurement of Cytochrome b₅ and P₄₅₀

Cytochrome b₅ levels were measured according to Omura and Sato (1964). Briefly, 2 cuvettes were set up each containing 2 ml. 0.1M phosphate buffer pH 7.4 and 1 ml microsomal suspension. A few grains of NADH (disodium salt) were added to the test cuvette and the spectrum recorded between 390 - 500 nm. Cytochrome b₅ was quantitated as the O.D. difference between 426 nm (peak) and 410 nm (trough). In some experiments this O.D. difference was converted to concentration units using the extinction coefficient of 161 cm⁻¹ mm⁻¹ (Omura and Sato, 1964).

The measurement of cytochrome P₄₅₀ was carried out using one of 2 methods, the method chosen depending on the particular subcellular fraction being studied. When rat liver microsomes were under investigation the method was essentially that of Omura and Sato (1964) and was performed in the same cuvettes as used for the cytochrome b₅ estimation. A few grains of sodium dithionite were added to each cuvette and carbon monoxide was bubbled through the contents of the test cuvette for 30 seconds. The spectrum was again recorded from 390 - 500 nm.

The cytochrome P₄₅₀ levels of human liver microsomes, rat liver homogenate and PMS were measured by the method of Greim as described by Schoene et al (1972). In this assay the contents of both cuvettes
were bubbled with carbon monoxide for 30 seconds and sodium dithionite was added to the test cuvette only; the spectrum between 390 - 500 nm. was then recorded. In this way the marked interference by haemoglobin in such fractions could be eliminated. It has been reported (Schoene et al, 1972) that both methods for cytochrome P<sub>450</sub> estimation give identical results.

The cytochrome P<sub>450</sub> level was quantitated as the O.D. difference between 450 - 490 nm. and in some instances the extinction coefficient of 91 cm<sup>-1</sup> mM<sup>-1</sup> was also used (Omura and Sato, 1964).

6. Assay for NADPH-Cytochrome c Reductase Activity
   (Williams and Kamin, 1962)

The rate of reduction of cytochrome c was measured in a double-beam spectrophotometer using an appropriate blank.

Into each of two cuvettes was added 1 ml. of 3 mM potassium ferricyanide, 1 ml. of cytochrome c solution (1.85 mg/ml), 0.6 ml. 0.1M phosphate buffer pH 7.4 (0.8 ml. in reference cuvette), 0.1 ml. of glucose-6-phosphate dehydrogenase solution (10 IU/ml) and 0.1 ml. microsomal suspension. The reaction was started by the forceful addition of 0.2 ml. of an NADPH-generating system (2 μmole NADP, 10 μmole G-6-P, 10 μmole MgCl<sub>2</sub>) into the test cuvette. The increase in absorbance at 550 nm. was then recorded and the enzyme activity determined from the initial velocity using an extinction coefficient for reduced cytochrome c of 27.7 cm<sup>-1</sup> mM<sup>-1</sup> (Williams and Kamin, 1962).

This assay procedure was adapted for use in a single cell dual-wavelength spectrophotometer (Perkin Elmer 356) thus eliminating the
need for a reference cuvette. A preliminary experiment involving repeat wavelength scans showed 650 nm to be a suitable reference wavelength for this assay (Fig. 3.1). Both procedures gave identical results (Fig. 3.2).

7. Measurement of Ethylmorphine, Aminopyrine and Imipramine N-demethylase Activity

Ethylmorphine, aminopyrine and imipramine are metabolized in vitro to the N-demethylated derivatives, the methyl group being lost as formaldehyde which, after trapping with semicarbazide, can be measured by the Nash reaction (Nash, 1953).

The incubation system used is outlined below:

<table>
<thead>
<tr>
<th>Control</th>
<th>Test</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal suspension</td>
<td>← 0.3 ml →</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>0.1M phosphate buffer pH 7.4</td>
<td>0.9 ml</td>
<td>0.9 ml</td>
</tr>
<tr>
<td>2% Semicarbazide buffered to pH 7.4</td>
<td>← 0.2 ml →</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase (10 I.U./ml)</td>
<td>0.2 ml</td>
<td></td>
</tr>
<tr>
<td>NADP (10 μmole/ml)</td>
<td>0.2 ml</td>
<td></td>
</tr>
<tr>
<td>G-6-P (50 μmole/ml)</td>
<td>0.2 ml</td>
<td></td>
</tr>
<tr>
<td>MgCl₂ (50 μmole/ml)</td>
<td>0.2 ml</td>
<td></td>
</tr>
<tr>
<td>Pre-equilibration (2 min, at 37°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate (75 μmole/ml)</td>
<td>0.2 ml*</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Formaldehyde standard (1 μmole/ml)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* denotes added after incubation
Fig. 3.1. NADPH-Cytochrome c Reductase Activity - Wavelength Scan at Various Incubation Times

![Wavelength Scan Graph]

Incubation Times:
a) 3 min. Incubation
b) 4 min.
c) 5 min.
d) 6 min.

Fig. 3.2. NADPH-Cytochrome c Reductase Activity - Comparison of Methods

![Comparison of Methods Graph]

Incubation Time (min.) vs. O.D. Change 550-650 nm.

Comparison Methods:
- Single Beam Assay
- Double Beam Assay
The incubation time was 10 min. at 37°C after which time the reaction was stopped by the addition of 1 ml. 15% (w/v) ZnSO₄. Substrate and standard were added to appropriate tubes followed by 1 ml. sat. Ba(OH)₂.

The tubes were centrifuged at 2,000 r.p.m. for 15 minutes and to 2 ml. of the supernatant was added 2 ml. of double-strength Nash reagent (4M aq. ammonium acetate containing 0.4% (v/v) acetyl-acetone). The tubes were incubated for 40 min. at 37°C and, after cooling, the contents of each tube were read at 412 nm.

6. Measurement of Aniline 4-Hydroxylase Activity

The product of this enzymic reaction, 4-aminophenol, can be measured by its reaction with phenol in alkaline solution to yield a measurable blue colour. The presence of non-microsomal fractions in the incubation system in some way interferes with this colour reaction and an extraction procedure is required to eliminate this problem (Chhabra et al., 1972).

The incubation system used for this assay is outlined below:—
Control  Test  Standard

Microsomal suspension  ←  0.5 ml  →

0.1M phosphate buffer pH 7.4  0.6 ml  0.6 ml  0.4 ml

Glucose-6-phosphate dehydrogenase  ←  0.2 ml  →

(10 I.U./ml)

NADP (10μ mole/ml)

G-6-P (50μ mole/ml)

MgCl₂ (50μ mole/ml)

Pre-equilibration (2 min. at 37°C)

Aniline (40μ mole/ml)  0.5 ml*  0.5 ml  0.5 ml*

4-Aminophenol standard

(0.5μ mole/ml)  -  -  0.2 ml*

* denotes added after incubation

Incubation time was 20 min. at 37°C. When microsomes were used as the enzyme source the reaction was terminated with 2 ml. 15% (w/v) TCA, substrate and standard added as appropriate, and, after mixing, the tubes were centrifuged at 2,000 r.p.m. for 15 min. To 2 ml. of the supernatant was added 2 ml. 10% sodium carbonate and 2 ml. 0.25 N NaOH containing 5% (w/v) phenol. After mixing, the tubes were left for 1 hour prior to reading at 620 nm.

When this enzyme was assayed in the presence of non-microsomal material the following procedure was adopted. The enzyme reaction was terminated by plunging the tubes into ice-water. Approx 1g. solid NaCl was added to each tube and substrate and standard added
as appropriate. 15 ml. of 1.5% (v/v) iso-amyl alcohol in diethyl ether was added and the contents of the incubation mixture extracted for 30 minutes. The tubes were centrifuged to separate the layers and 10 ml. of the other phase was back-extracted with 4 ml. of 1% (w/v) phenol in 0.5M potassium triphosphate for 30 minutes. The phases were again separated and 1 hour after commencing the final extraction the colours in the aqueous layers were read at 620 nm.

9. **Measurement of 4-Chloro N-Methylaniline N-Demethylase Activity**

In this assay the product, 4-chloraniline, is measured by its reaction with 4-dimethleminobenzaldehyde in acid solution (Kupfer and Bruggeman, 1969).

The incubation system used is outlined below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>Test</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal suspension</td>
<td>⊢ 0.3 ml(\rightarrow)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1M phosphate buffer pH 7.4</td>
<td>1.2 ml</td>
<td>1.2 ml</td>
<td>1.1 ml</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>⊢ 0.2 ml(\rightarrow)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10 \text{ I.U./ml})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADP (10 (\mu) mole/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-6-P (50 (\mu) mole/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl(_2) (50 (\mu) mole/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-equilibration (2 min. at 37(^{\circ})C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Chloro N-methylaniline (30 (\mu) mole/ml)</td>
<td>0.1 ml*</td>
<td>0.1 ml</td>
<td>0.1 ml*</td>
</tr>
<tr>
<td>4-Chloraniline (1 (\mu) mole/ml)</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

* denotes added after incubation
Incubation time was 20 min. at 37°C, after which time the reaction was terminated by plunging the tubes into a boiling-water bath for 1 min. Substrate and standard were added as appropriate followed by 3 ml. 2% (w/v) 4-dimethylaminobenzaldehyde in 1N H₂SO₄. The tubes were then centrifuged at 2,000 r.p.m. for 15 minutes and left overnight prior to reading at 445 nm.

10. Inhibition of Hepatic MFO Activity In Vitro

Two inhibitors of microsomal MFO activity were used in this work: SKF 525-A and carbon monoxide. SKF 525-A was used at a final concentration of 10⁻⁴M in the incubation mixture. Carbon monoxide was administered to the incubation mixture by gentle bubbling from a cylinder for 10 minutes prior to the incubation; during the incubation itself the gassed tube was kept tightly stoppered.

11. Observation and Quantitation of Spectrally Apparent Substrate - Microsome Interactions

The microsomal suspension was diluted in 0.1M phosphate buffer pH 7.4: glycerol (4:1 v/v) to a final concentration of 2 mg protein/ml. 2.5 ml. of this diluted suspension was placed into each of 2 cuvettes which were then stoppered with 'Suba Seals' (Gallenkamp). These cuvettes were placed in a double-beam spectrophotometer and a base-line recorded between 350 - 500 nm. The substrate, dissolved either in distilled water or absolute ethanol, was added, from a microsyringe, through the 'Suba Seal' into the microsomal suspension; an equivalent volume of solvent was added to the
reference cuvette. The spectrum was again recorded. For each concentration of substrate present in the cuvette the maximal O.D. difference (compensated for base-line) between trough and peak was calculated. A double reciprocal plot of O.D. difference against substrate concentration was then drawn and the binding constant \( K_s \) calculated from the intercept on the abscissa (Fig. 3). As can be seen this graph is analogous to a Lineweaver-Burk plot for enzyme-catalysed reactions. Similarly the definition of \( K_s \) is analogous to that of the Michaelis constant, \( K_m \), namely: \( K_s \) is defined as that concentration of substrate which produces half the maximal O.D. difference for that particular substrate-microsome interaction.

12. Analysis of Results

Whenever 3 or more samples were being assessed the results are given as mean ± SEM. Statistical analysis was carried out using Student’s t-test. When comparing microsomes prepared by normal centrifugation and isoelectric precipitation, a paired-sample t-test was adopted (Campbell, 1967).
Fig. 3.3. Double Reciprocal Plot to Determine Binding Constant $(K_s)$ for Aniline

$K_s = 3.28 \times 10^{-4} \text{M}$
## CHAPTER 4. CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scale-Down of Assays for the Measurement of Hepatic MFO Activity</td>
<td>65</td>
</tr>
<tr>
<td>The Isolation of Hepatic Microsomes — A Comparison of Isoelectric Precipitation with the Conventional Centrifugation Procedure</td>
<td></td>
</tr>
<tr>
<td>1. Effect of Isoelectric Precipitation on Microsomal Yield</td>
<td>66</td>
</tr>
<tr>
<td>2. Centrifugation Requirements of Acid-Precipitated Microsomes</td>
<td>67</td>
</tr>
<tr>
<td>3. MFO Capabilities of Hepatic 'Acid' Microsomes</td>
<td>69</td>
</tr>
<tr>
<td>4. Observation and Quantitation of Spectrally-Apparent Substrate-Microsome Interactions</td>
<td>70</td>
</tr>
<tr>
<td>5. Low-Speed Sedimentation of Microsomes by Means of Isoelectric Precipitation of the Post-Nuclear Supernatant of Rat Liver Homogenate</td>
<td>71</td>
</tr>
<tr>
<td>Storage Characteristics of the Hepatic MFO System</td>
<td>72</td>
</tr>
<tr>
<td>1. Storage of Hepatic Microsomal Fractions</td>
<td>72</td>
</tr>
<tr>
<td>2. Storage of Solid Liver</td>
<td>75</td>
</tr>
<tr>
<td>3. Post-Mortem Characteristics of the Microsomal MFO System of Liver</td>
<td>77</td>
</tr>
<tr>
<td>Studies on the Microsomal MFO System of Human Liver</td>
<td>78</td>
</tr>
<tr>
<td>1. Details of Human Liver Samples Received</td>
<td>78</td>
</tr>
<tr>
<td>2. Applicability of Isoelectric Precipitation to the Isolation of Human Liver Microsomes</td>
<td>79</td>
</tr>
</tbody>
</table>
3. Nature of the Human Hepatic MFO System \textit{In Vitro} \hspace{1cm} 80

(i) Time Dependence of Enzymic Reactions \hspace{1cm} 80
(ii) Cofactor Dependence of Enzymic Reactions \hspace{1cm} 80
(iii) Effects of Inhibitors of Enzymic Reactions \hspace{1cm} 80

4. The Microsomal MFO System of Human Liver - Levels of Certain Parameters \hspace{1cm} 81

(i) Cytochromes $b_5$ and $P_{450}$ \hspace{1cm} 81
(ii) Metabolism of Type I and Type II Compounds \hspace{1cm} 83

5. Microsome-Substrate Interactions in Human Liver \textit{In Vitro} \hspace{1cm} 84
CHAPTER 4. RESULTS

A. Scale-Down of Assays for the Measurement of Hepatic Microsomal \textit{MFO} Activity

In the initial period of this investigation it was quickly realized that small-scale enzyme assays were essential when dealing with small samples of human liver biopsy material and samples of cultured liver tissue.

Consequently, various experiments were performed in attempts to improve method sensitivity using rat liver and the existing spectrophotometric methods of product measurement. The results from these various experiments (Table 4.1) showed that decreasing the level of microsomal-containing fraction from 0.3 - 0.5 ml (approx. 1.5 - 2.5 mg protein) to 0.1 ml (approx. 0.5 mg protein) and halving the volumes of the other incubation components in a total volume of 1 ml, with subsequent halving of volumes for the ensuing extractions and colour measurements gave results totally comparable to those obtained using the conventional assay systems as described in Chapter 3. The overall improvement in sensitivity produced in this way was in the order of 3 - 5 fold.

Furthermore, scale-down in the NADPH-cytochrome c reductase assay could be achieved by means of the single-cell dual-wavelength modification as described in Chapter 3.

It provided impossible, however, to scale-down the cytochrome P$_{450}$ estimation without loss of accuracy and sensitivity.
B. The Isolation of Hepatic Microsomes - A Comparison of Isoelectric Precipitation with the Conventional Centrifugation Procedure

1. Effect of Isoelectric Precipitation on Microsomal Protein Yield

From preliminary studies it soon became apparent that a major difference between IP and conventional centrifugation techniques lay in the increased yield of protein found in 'acid' microsomes. Accordingly, this problem was studied initially using various acid precipitants; the results are shown in Table 4.2. 50 - 60% extra protein is found in the 'acid' preparations which is not accompanied by any increase in the 'true' microsome content as measured by the recovery of $P_{450}$. It appears that this increased protein yield is independent of the nature of the acid precipitant in that citrate buffer or dilute HCl can be used in place of the acetate buffer (Table 4.2), but is related to the lowering of the pH to 5.4.

These findings imply that the increased protein content found in 'acid' microsomes is non-microsomal and is probably derived from proteins normally located in what is termed, in conventional centrifugation parlance, the cytosol i.e. the 105,000 g. supernatant. This extra protein is non-ribosomal as judged from a comparison of the RNA levels in 'acid' and 'normal' microsomes (Table 4.3). This extra protein is also not derived from haemoglobin as shown by a lack of any appreciable peak at 420 nm. in the spectrum for $P_{450}$ (Fig. 4.1). Also, the protein content of 'acid' microsomes approximates to that found in unwashed microsomes prepared by normal means (Table 4.4).

2. Centrifugation Requirements of Acid-Precipitated Microsomes

During the preliminary studies the 'acid' microsomes were harvested by the use of a bench centrifuge maintained at 4°C operated
at max. speed (approx. 2,000 g av.). The effects of varying the time of centrifugation at this speed on the resulting pellet composition are shown in Table 4.5.

Two facts emerge from these results. Firstly, quantitative harvesting of 'acid' microsomes is complete after 5 minutes centrifugation and, secondly, the 'acid' microsomes remain stable (as judged by $b_5$ and $P_{450}$ levels) during the centrifugation procedure. During this centrifugation procedure there was no variation in the protein content of the microsomal pellets.

After the completion of this work it was felt desirable to obtain a tighter-packed microsomal pellet so as to simplify removal of the overlying supernatant, and this was achieved by centrifuging the 'acid' microsomal suspensions at 10,000 g av. for 10 minutes. All results hence refer to 'acid' microsomes harvested in such a manner.

All comparative results between 'acid' and 'normal' microsomes are on a paired basis i.e. both 'acid' and 'normal' preparations are derived from the same liver.

From these studies a standardized isolation scheme for microsomes using IP was adopted and this is outlined below:-
P.H.S.
(from liver homogenate (25% w/v) in 1.15% (w/v) KCl)

pH lowered to 5.4 with acetate buffer (0.2M, pH 4.0)

Centrifuge 10,000 g av. (10 minutes)

Discard—Supernatant

Pellet

Resuspend in 1.15% (w/v) KCl: glycerol (4:1 v/v)

Re-centrifuge (10,000 g av., 10 min.)

Discard←Supernatant

Pellet

Resuspend in 0.1M phosphate buffer pH 7.4: glycerol (4:1 v/v)
Table 4.6 summarizes the results relevant to this study. 'Acid' microsomes are identical to 'normal' microsomes in regard to their capability to metabolize both type I (aminopyrine) and type II (aniline) drug substrates. Furthermore, the levels of $P_{450}$ and $b_5$ are similar in both microsomal preparations. The spectrophotometric appearance of both cytochromes is unaltered by acid treatment (Fig. 4.1).

NADPH-cytochrome c reductase activity in vitro is believed to reflect NADPH-cytochrome $P_{450}$ reductase activity although the absolute levels for these enzymes are greatly different (see Chap. 2). This being so, it is interesting to note that 'acid' and 'normal' microsomes contain equivalent levels of NADPH-cytochrome c reductase activity.

Furthermore, pre-treatment of the rats with phenobarbitone raises the hepatic levels of cytochrome $b_5$ and $P_{450}$, type I and type II metabolic activity to the same extent in 'acid' microsomes as in 'normal' microsomes (Table 4.7). This is more clearly illustrated in Table 4.8 in which the percentage increases in the haemoproteins and Type I and type II metabolic activity after induction are recorded. All 4 parameters studied show similar increases in both 'normal' and 'acid' microsomes. The extent of the increases in type I and type II metabolism follows that of the cytochrome $P_{450}$ more closely than that of cytochrome $b_5$.

It is interesting to note that the extra protein present in 'acid' microsomes from control rat liver mentioned earlier is absent in similar preparations derived from phenobarbitone-induced rat liver (Table 4.7). The reasons for this are not known although it may be
that phenobarbitone induction of rat liver leads to an absolute decrease of cytoplasmic protein in addition to its obvious relative decrease compared to microsomal protein. Alternatively it may be that excess of acid buffer may be present in control 'acid' preparations which accounts for the aggregation of cytoplasmic protein. Obviously, in induced 'acid' preparations this excess of acid buffer is utilized in aggregating the extra microsomal protein that is present, so leading to a negligible cytoplasmic protein contamination.

4. Observation and Quantitation of Spectrally-Apparent Substrate-Microsome Interactions

A representative substrate for each type of binding class was used, namely biphenyl (type I) and aniline (type II).

The type of binding for each substrate was unaltered when the microsomes were prepared by IP from both induced and control rat livers, biphenyl remaining type I and aniline remaining type II (Fig. 4.2 and 4.3). The magnitude of the O.D. change at any concentration of substrate is slightly less in 'acid' microsomes prepared from control rat liver than in corresponding 'normal' microsomes due to their differing protein content. However, when the difference spectrum is redrawn on a g. liver basis the two preparations yield identical results (Fig. 4.4).

Table 4.9 shows that the affinity of binding of microsomes for the substrate (both type I and type II) as measured by the $K_d$ is unaltered when the microsomes are prepared by IP. If anything, the affinity for aniline is greater in 'acid' microsomes as compared to 'normal' microsomes from control and induced rat liver.
5. **Low-Speed Sedimentation of Microsomes by Means of IP of the Post-Nuclear Supernatant of Rat Liver Homogenate**

The success of IP on the PNS of rat liver prompted the question: Can microsomes be sedimented at low speed using IP on rat liver homogenate? If this were possible it could prove valuable as an initial step in a scheme for the clinical analysis of liver function in human biopsy material using MFO activity as the organ-specific parameter. It was decided to assess this possibility using rat liver homogenate which had been 'cleaned up' of nuclei by low-speed (600 g, av.) centrifugation for 5 minutes at 4°C in a bench centrifuge. Acid precipitation was then attempted on this post-nuclear homogenate ('acid homogenate').

From preliminary studies certain problems regarding assay conditions were countered. Firstly, $b_5$ levels could not be measured in crude homogenate or their low-speed-derived fractions due to the presence of a large excess of mitochondrial cytochromes thereby completely masking the cytochrome $b_5$ difference spectrum.

Also due to this mitochondrial contamination was the greatly increased cofactor levels required for optimal activity of both aminopyrine N-demethylase and aniline hydroxylase when assayed in homogenate, as shown in Fig. 4.5.

Table 4.10 gives the levels of protein, $P_{450}$ and type I and type II metabolic activity in various rat hepatic fractions — homogenate, 'acid homogenate', PNS and 'acid' microsomes. These results are also shown, in percentage form, in Tables 4.11 and 4.12.

These results clearly show that IP of liver homogenate yields a microsomal fraction comparable to other more commonly prepared fractions.
with regard to both its $P_{450}$ content and its drug-metabolizing capability, thereby making this method of low-speed aggregation of hepatic microsomes a suitable alternative in the absence of high-speed centrifugation facilities. Indeed, where a crude microsomal fraction is adequate for a particular need, the 'acid homogenate' fraction, due to its probable higher $P_{450}$ content and speed of preparation, would appear to be a better proposition than the use of the PMS.

C. Storage Characteristics of the Hepatic MFO System

The storage characteristics of the rat hepatic microsomal MFO system were compared in 3 systems, viz-

1. Storage of microsomal fractions
2. Storage of solid liver
3. Storage of the liver in situ subsequent to death

The aims of this study were two-fold: firstly, to establish the optimal storage conditions for valuable material such as human biopsy and p.m. samples and to study the effect of in situ p.m. autolysis. The second aim was to utilize these storage characteristics as a probe in understanding the complex structural relationships of this membrane-bound MFO system.

1. Storage of Hepatic Microsomal Fractions

In this experiment microsomal fractions were prepared by conventional centrifugation and IP and stored as washed pellets and suspensions both at 4°C and 0°C. To ensure a sufficient quantity
of material livers from 24 rats were homogenized and the homogenate pooled; thus each point on the following graphs represents a single measurement. The samples stored at -20°C were slow-thawed by placing in ice.

At various time intervals the activities of aminopyrine N-demethylase, biphenyl 4-hydroxylase, aniline 4-hydroxylase and NADPH-cytochrome c reductase were measured together with the corresponding levels of P$_{450}$ and malonaldehyde in each fraction. The level of malonaldehyde was taken to represent the level of lipid peroxidation and hence the state of membrane integrity at any chosen time.

The decay curves for the activities of aminopyrine N-demethylase, biphenyl 4-hydroxylase, aniline 4-hydroxylase and NADPH-cytochrome c reductase and the level of P$_{450}$ are shown in Fig. 4.6 - 4.10 respectively, whilst the increase in malonaldehyde levels is shown in Fig. 4.11. Whilst it is readily accepted that each point, being a single measurement, may contain some error, it is nonetheless felt that these graphs do enable valid comparisons of decay characteristics of the various fractions to be made.

The first observation that can be made from these results is that drug-metabolizing activity of stored microsomes is best maintained by storage at -20°C irrespective of the method of preparation of, or storage state of the microsomes. In general, storage of microsomes at -20°C in the form of pellets leads to a more stable preparation than microsomes stored as a suspension. This storage state-dependent activity is more marked in the case of aminopyrine N-demethylase as compared to the other enzymes and P$_{450}$. 
Concomitant with this decay of drug-metabolizing activity on storage is an increase in the malonaldehyde levels (Fig. 4.11); for each microsomal preparation increase in the malonaldehyde content roughly parallels the decrease in enzyme activity and P^450 content. This inverse relationship of enzyme activity and P^450 content to the level of malonaldehyde is illustrated in Fig. 4.12 - 4.15 using each fraction of the 'normal microsomes' series; where the enzyme activity after 162 hours storage is between 0 - 20% that of the fresh sample (Fig. 4.12 and 4.14) the malonaldehyde level is between 700 - 1600% that of the starting value; where the enzyme activity after a similar time is between 40 - 90% that of the fresh sample (Fig. 4.13 and 4.15) the malonaldehyde level is correspondingly low (120 - 140% that of the starting value). These results indicate that membrane integrity plays an important role in determining the in vitro efficiency of the hepatic microsomal MFO system.

Another fact to emerge from Fig. 4.6 - 4.11 is that 'acid' microsomes are more labile than 'normal' microsomes on storage. The initial (20 hours) decay in enzyme activity and P^450 content is more rapid in 'acid' microsomes and, once again, this more rapid fall-off is associated with elevated malonaldehyde levels which are higher than those in 'normal' microsomes. After these initial activity reductions the resulting rates of decay of both 'normal and 'acid' microsomes are similar for all the parameters studied but, due to the more rapid initial fall-off, the final (162 hours) levels of enzyme activity and P^450 content are lower in 'acid' microsomes. It would appear that although 'acid' microsomes are initially comparable to 'normal' microsomes with regard to their drug metabolizing potential the acid treatment itself activates their decay on storage;
this is substantiated by the approx. 100% extra malonaldehyde present in fresh 'acid' microsomes when compared to 'normal' microsomes (Table 4.13).

Another fact to emerge from Fig. 4.12 - 4.15 is that the decay in metabolism of biphenyl, a type I substrate, follows that of aniline (a type II compound) more closely than that of aminopyrine (another type I compound). Furthermore, in the initial stages decay in NADPH-cytochrome c reductase follows that of aminopyrine N-demethylase more closely than that of aniline 4-hydroxylase whilst in the later stages this is reversed, the reductase activity more closely following the aniline 4-hydroxylase activity.

2. Storage of Solid Liver

Short term (approx. 1 week) storage of solid liver placed at -40°C immediately after excision from the animal maintained the activity of the microsomal MFO system at virtually 100% that of fresh liver; this is illustrated in Fig. 4.16. Both the type I and type II metabolism and the P450 content were unaffected by this storage and the malonaldehyde content also did not appear to alter to any great extent.

This short term stability of the microsomal MFO system of stored solid liver was confirmed by the results of a much longer term experiment (Fig. 4.17). In this experiment, various parameters of the MFO system were measured in microsomes isolated from livers maintained solid at -40°C for up to 43 days. This was devised as a paired experiment in that one half of the liver was measured fresh and served as a control for the other half. The results are illustrated
Under these storage conditions the type I metabolism was decidedly the most labile parameter studied, the decay in enzyme activity beginning as soon as storage started such that by day 43 only 11% of the original activity remained. Type II metabolic capability was more stable on storage, enzyme activity falling off only after 16 days' storage and the activity remaining after 43 days' storage being 50% the original value. The cytochromes b5 and P450 were both more stable than either type I or type II metabolism. Fall off in cytochrome levels occurred after 16 days' storage and by 43 days' storage these levels had fallen to only 87% and 74% the original value for b5 and P450 respectively. Furthermore, once fall-off in the levels commenced the rate of decay for each parameter varied considerably.

During the storage period it became increasingly difficult to obtain a clean separation of the PMS from its sediment, leading to a somewhat contaminated PMS; the cause of this inability to form a tightly-packed pellet was not resolved. Also occurring during this storage period was the emergence of a peak at 420 nm, which was observed when running the P450 spectrum (Fig. 4.18). This was not the degraded form of cytochrome P450, cytochrome P420, as no loss in P450 content could be discovered (this was prior to the cytochrome P450 decay i.e. less than 16 days' storage). This peak, therefore, probably represented contamination of the microsomes with haemoglobin and made it impossible to decide if the loss of P450 after 16 days' storage could be accounted for by its conversion to P420. It is probable that the haemoglobin contamination was related to the previously mentioned contamination of the PMS.
3. **Post-Mortem Characteristics of the Microsomal MFO System of Liver**

Macleod *et al.* (1973) have recently shown with rabbit liver that maintaining the liver in situ after death for 4.5 hours resulted in a decrease in the type I and type II metabolism, NADPH-cytochrome c reductase and NADPH-cytochrome \( P_{450} \) reductase activities and \( P_{450} \) content of microsomes derived therefrom. The enzyme levels decayed to approx. 25% those of fresh tissue and the \( P_{450} \) content fell to approx. 50%. However, the effect of p.m. storage in situ on the binding affinity of hepatic microsomes for substrates of the MFO system was not studied. For this reason, in addition to determination of any possible species differences in p.m. in situ decay, the experiment detailed below was performed.

Six rats (80 - 100 g) were killed and 3 were maintained intact for 1 hour at room temp. followed by 2 hours at 4°C; these conditions approximated to those existing prior to human autopsy. The remaining 3 rats served as controls in which the livers were immediately removed and kept at 4°C during the 3 hours. Both sets of livers were then processed identically using IP for preparation of the microsomes.

In agreement with the findings of Macleod *et al.* (1973) was the significant fall in \( P_{450} \) content on in situ storage together with a, previously unreported, similar fall in \( b_5 \) content (Table 4.14). There was also a less marked fall in microsomal protein content, the extent of this decrease being approx. one-half that found for the cytochromes.

However, during this period of p.m. decay in the microsomal cytochrome and protein content there was no effect on the binding
affinity of the microsomes for both type I and type II compounds, as shown in Table 4.15.

Using microsomes from such young rats (80 - 100 g) revealed the presence of two binding sites for biphenyl as described by Burke (1973) for the hamster; there was no evidence for more than one binding site for aniline in these microsomes. In previous experiments using microsomes from older animals (250 g) and from phenobarbitone-induced animals there was no evidence for this dual-binding phenomenon with biphenyl. In situ storage p.m. did not significantly alter the \( K_s \) for either biphenyl-binding site and the ratio \( K_s \) low : \( K_s \) high was also unaffected.

D. Studies on the Microsomal MFO System of Human Liver

1. Details of Human Liver Samples Received

<table>
<thead>
<tr>
<th>Code</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Nature of Sample/ Cause of Death</th>
<th>Storage Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL1</td>
<td>F</td>
<td>56</td>
<td>Surgical Biopsy</td>
<td>Received Fresh</td>
</tr>
<tr>
<td>HL2</td>
<td>F</td>
<td>53</td>
<td>Surgical Biopsy</td>
<td>Received Fresh</td>
</tr>
<tr>
<td>HL3*</td>
<td>F</td>
<td>mid 30s</td>
<td>P.M./Salicylate Poisoning</td>
<td>14 hours at 4°C, 4 hours at -70°C, Thereafter at -40°C</td>
</tr>
<tr>
<td>HL4*</td>
<td>M</td>
<td>22</td>
<td>P.M./Road Accident</td>
<td>Samples were placed at -40°C within 1 hour of removal from body. Thereafter at -40°C</td>
</tr>
</tbody>
</table>

* Unfortunately the time that elapsed from death to autopsy was not recorded in samples HL3 and HL4.
Analyses for the presence of salicylate and 4-aminosalicylate in samples of HL3 homogenate proved negative (methods used as described by Varley, 1969). Binding studies performed with rat liver microsomes showed 4-aminosalicylate to be a very weak type II substrate. It is felt that these two observations taken together strongly indicate that 4-aminosalicylate, even if present in sample HL3, would not interfere with the studies as described below.

2. Applicability of Isoelectric Precipitation to the Isolation of Human Liver Microsomes

A comparison of protein and cytochrome levels and of 4-chloro N-methylaniline N-demethylase activity in human hepatic microsomes isolated by both conventional centrifugation and IP is shown in Table 4.15. IP of human hepatic microsomes yielded a fraction comparable to that isolated by conventional centrifugation with regard to its MFO activity.

These results are in accord with those presented earlier for rat liver. A point of interest is that the protein content of both microsomal fractions is very similar, a point substantiated by the results shown in Table 4.17.

This similarity in the protein content of both washed 'normal' and 'acid' human hepatic microsomes differs somewhat from the situation existing in rat liver (Table 4.4) and may reflect interspecies differences in cytosol protein structure and/or content.

Furthermore, both 'normal' and 'acid' human hepatic microsomes contain 'non-P450b5' haem which is regarded as representing the haemoglobin content of such fractions (Table 4.18). It should be
noted that the concentrations of the various haem moieties had to be calculated using extinction coefficients derived from non-human species.

The haemoglobin content of 'acid' microsomes roughly corresponds to that found in washed 'normal' microsomes, both of which represent one-third to one-quarter that found in unwashed 'normal' microsomes. Whilst a small part of the decrease in total haem content on washing 'normal' microsomes is due to a loss in the levels of cytochrome b\textsubscript{5} and P\textsubscript{450} by far the major bulk is due to a loss in haemoglobin content.

3. Nature of the Human Hepatic Microsomal M\textsubscript{FO} System In Vitro

(i) Time Dependence of Enzymic Reactions

The time dependence of 4-chloro-N-methylaniline N-demethylase and aniline 4-hydroxylase activities are shown in Fig. 4.19. The activity of the former enzyme is linear for at least 20 minutes and that of the latter for at least 30 minutes.

(ii) Cofactor Dependence of Enzymic Reactions

The dependence of 4-chloro-N-methylaniline N-demethylase activity on the concentration of NADP and G-6-P in the incubation system is shown in Fig. 4.20. In the absence of exogenously-added NADP or G-6-P there was no detectable enzyme activity.

(iii) Effect of Inhibitors of Enzyme Reactions

Carbon monoxide and SKF 525-A, known inhibitors of the microsomal M\textsubscript{FO} system in the rat, were tested for their ability to
inhibit 4-chloro-N-methylanilino N-demethylase and aniline 4-hydroxylase activities in human hepatic microsomes. The results of this study are shown in Table 4.19.

The extent of inhibition by CO was similar for both enzymes studied and was probably not greater than 65% due to endogenous oxygen and/or substrate remaining bound on the cytochrome P_{450}. SKF 525-A on the other hand produced no inhibition whatsoever. Further work carried out in conjunction with Prof. K.J. Netter showed that 4-nitroanisole O-demethylase activity in human liver microsomes was totally resistant to any inhibitory effect of both SKF 525-A (10^{-4} - 10^{-3}M) and metyrapone (10^{-5}M).

4. The Microsomal MFO Activity of Human Liver - Levels of Certain Parameters

(i) Cytochromes b_5 and P_{450}

The presence of haemoglobin in the human hepatic microsomal suspensions resulted in a peak at 420 nm, when attempting to measure cytochrome P_{450} by the usual method of Omura and Sato (1964); this is illustrated in Fig. 4.21. This 420 nm peak distorted the peak of cytochrome P_{450} so moving the peak to 454 - 457 nm. This interference could be eliminated by using the modified method as described in Chapter 3, as seen in Fig. 4.21.

The presence of cytochrome b_5 in human hepatic microsomes could readily be detected using the normal method of assay (Fig. 4.22). The positions of the trough and peak in the
cytochrome $b_5$ spectrum (410 and 426 nm, respectively) were identical in both rat and human hepatic microsomes.

Furthermore, the addition of an oxidizing agent, potassium ferricyanide at a final concentration of $10^{-5} M$, to the control cuvette resulted in an apparent increase in the $P_{450}$ concentration (Fig. 4.23). This apparent increase was of the order of 20% and indicated that there was endogenous reduced $P_{450}$ prior to in vivo reduction with dithionite, this endogenous reduced $P_{450}$ possibly representing an in vivo state. No such effect on addition of oxidizing agent was observed for cytochrome $b_5$.

The levels of cytochromes $b_5$ and $P_{450}$ found in the 2 samples of human liver studied are listed in Table 4.20. Whilst admitting that these 2 results are hardly statistical, it should be noted that the levels of cytochrome $b_5$ in both samples were extremely similar whilst there was a two-fold difference in the corresponding levels of $P_{450}$, the lower level of cytochrome $P_{450}$ being found in the sample with the poorer storage conditions. These results are broadly in keeping with those observed during the various experiments on storage phenomena in rat liver, namely that $b_5$ has greater stability than $P_{450}$ and that preservation of $P_{450}$ content is dependent on the storage conditions.

Furthermore, the level of cytochrome $b_5$ in human liver microsomes lies in the lower region of the normal range for male rat liver microsomes (0.30 - 0.85 $O.D./g. liver$) whilst the level of cytochrome $P_{450}$ in the better preserved sample also lies in the lower region of the normal range for male rat liver microsomes (0.30 - 0.95 $O.D./g. liver$).
(ii) Metabolism of Type I and Type II Compounds

a) Type I Compounds

The results for the metabolism of three type I compounds - ethylmorphine, aminopyrine and imipramine - by human liver microsomes is shown in Table 4.21. Two of the liver samples were fresh i.e. unstored and one was a stored sample; following 6 weeks' storage there was no detectable metabolism of these type I compounds by HL3, although there was metabolism of type II compounds and detectable cytochromes b_5 and P_450.

The level of type I metabolism in fresh human liver microsomes is approx. 50% that found in adult male rat liver microsomes, on a g. liver basis. Human liver microsomes metabolized ethylmorphine and aminopyrine via N-demethylation at an identical rate whereas the N-demethylation of imipramine was only 20% of this rate; this difference in rate of N-demethylation is probably related to the known multiplicity of metabolic routes available for the microsomal metabolism of imipramine (Bickel, 1971). Furthermore, aging of the microsomes by in situ storage p.m. led to a fall in the type I metabolizing potential, which, in the case of HL4, was of the order of 75 - 80%.

b) Type II Compounds

Metabolism of type II compounds (aniline and 4-chloro-N-methylaniline) was demonstrable in all the
samples tested (Table 4.22). This type II activity was more stable than type I metabolism and in one instance was demonstrated 42 days subsequent to storage of the sample at −40°C, by which time all type I metabolism had been lost.

These results indicate that the level of aniline 4-hydroxylase activity in fresh human liver microsomes is similar to that found in adult male rat liver microsomes. Aging of human liver by in situ storage p.m. decreased the aniline 4-hydroxylase activity by approx. 60%. The level of 4-chloro-N-methylaniline N-demethylase activity in stored human hepatic microsomes was approx. 10% that found in adult male rat liver microsomes.

5. Spectrally-Apparent Interactions of Substrates with Human Liver Microsomes In Vitro

A number of substrates were tested for their ability to elicit any spectrally-apparent response on addition to human liver microsomes; where one did occur the interaction was defined (i.e., type I or type II) and, if possible, quantified using the binding-affinity constant $K_b$.

Biphenyl, SKF 525-A, naphthalene, 1-naphthol and safrole are all type I compounds in the rat. On addition of these substrates to human liver microsomes the spectrally-apparent responses were those associated with type I compounds in that at wavelengths below 410 nm, the O.D. change was positive with respect to the base-line, and at wavelengths above 410 nm, the O.D. change was negative. Unfortunately, due to excessive absorption at both ends of the spectrum (not found when using rat liver microsomes) the typical horizontal S-shape was not
observed. This is illustrated in Fig. 4.23 using 1-naphthol as an example. The other substrates elicited similarly-shaped responses.

These binding spectra when analysed, using standard wavelengths of 390 nm and 430 nm, gave workable double reciprocal plots from which the $K_d$ could be assessed; this is exemplified in Fig. 4.25. The $K_d$ values for the various substrates are given in Table 4.23, together, where possible, with those corresponding for adult male rat liver.

Human liver microsomes did not bind type I substrates as avidly as did rat liver microsomes. In fact human liver microsomes were approx. 5 times weaker in their binding with biphenyl and naphthalene and at least 25 times weaker with SKF 525-A. It was of interest to notice that SKF 525-A showed evidence of 2 binding sites with markedly differing affinities (approx. 20-fold difference).

Human liver microsomes also bound substrates known to be type II in rat, the type of binding remaining unaltered. The substrates tested comprised aniline, 4,4'-bipyridyl and metyrapone. In this instance the binding spectra were identical in shape to those accepted as being archetypic for this class, as shown in Fig. 4.26 for 4,4'-bipyridyl.

The double reciprocal plot for all 3 substrates showed evidence for the existence of 2 binding sites of differing affinity. This is shown in Fig. 4.27 using metyrapone as an example. The binding affinity constants for the substrates are shown in Table 4.24 together with the corresponding values for male rat liver.

The presence of 2 binding sites makes the comparison with rat liver difficult. Taking into account the low affinity for aniline
shows that human liver microsomes bind the substrate as readily as
do rat liver microsomes but that human liver microsomes show greater
affinity at the high site. The same situation holds true for
4,4'-bipyridyl but not for metyrapone where there exists a vast
difference between the binding affinities for this compound in human
and rat liver microsomes even allowing for the fact that the figure
quoted for rat liver was derived from pre-induced livers.

Furthermore, human liver microsomes also elicited a spectrally-
apparent response with butan-1-ol which constituted what has been
termed a "Reverse Type I" spectrum (Schenkman et al., 1972). This
reverse type I spectrum is shown in Fig. 4.28 and could be distinguished
from a type II spectrum by its different wavelength maxima and minima.*

In conclusion, these results show that human liver microsomes can
produce spectrally-apparent interactions with various compounds typical
of the three classes of binding - type I, type II and reverse type II.

*(Table 4.25)
### Table 4.1. Comparison of Conventional and Scaled-Down Assays for the Measurement of Hepatic Microsomal MFO Activity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Conventional Assay</th>
<th>Scaled-Down Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>19.5 ± 7.1</td>
<td>19.3 ± 4.7</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>1.14 ± 0.16</td>
<td>1.16 ± 0.13</td>
</tr>
<tr>
<td>4-Chloro-N-methyleniline</td>
<td>7.5 ± 0.9</td>
<td>7.7 ± 0.6</td>
</tr>
<tr>
<td>N-demethylase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. Figures in brackets indicate number of experiments

a μ moles formaldehyde/g.liver/hr.
b μ moles 4-aminophenol/g.liver/hr.
c μ moles 4-chloraniline/g.liver/hr.

### Table 4.2. Effect of Isoelectric Precipitation on the Yield of Microsomal Protein and Cytochrome P<sub>450</sub>

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Protein Content&lt;sup&gt;(a)&lt;/sup&gt;</th>
<th>P&lt;sub&gt;450&lt;/sub&gt; Content&lt;sup&gt;(b)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>25.5 ± 2.2</td>
<td>0.89 ± 0.08</td>
</tr>
<tr>
<td>0.1N HCl</td>
<td>37.7 ± 2.4</td>
<td>0.93 ± 0.10</td>
</tr>
<tr>
<td>Acid</td>
<td>40.0 ± 3.0</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.2N Citrate Buffer pH 4.0</td>
<td>38.4 ± 2.8</td>
<td>0.95 ± 0.14</td>
</tr>
<tr>
<td>0.2N Acetate Buffer pH 4.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean of 3 rats ± S.E.M. N.D. = not determined

<sup>(a)</sup> mg/g.liver  <sup>(b)</sup> O.D./g.liver
### Table 4.3. RNA Levels in 'Acid' and 'Normal' Microsomal Fractions

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>RNA Levels (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Normal'</td>
<td>3.47 ± 0.29</td>
</tr>
<tr>
<td>'Acid'</td>
<td>3.50 ± 0.32</td>
</tr>
</tbody>
</table>

Values are mean of 9 rats ± S.E.M. (a) mg/g liver

### Table 4.4. Protein Content of Unwashed 'Normal', Washed 'Normal' and 'Acid' Microsomal Fractions

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Protein Content (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwashed 'Normal'</td>
<td>27.0</td>
</tr>
<tr>
<td>Washed 'Normal'</td>
<td>17.7</td>
</tr>
<tr>
<td>'Acid'</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Values are mean of 2 rats. (a) mg/g liver

### Table 4.5. Influence of Centrifugation Time on the Composition of 'Acid' Microsomes

<table>
<thead>
<tr>
<th>Time of Centrifugation (minutes)</th>
<th>Cytochrome b_65 (a)</th>
<th>Cytochrome P_450 (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.34</td>
<td>0.32</td>
</tr>
<tr>
<td>10</td>
<td>0.35</td>
<td>0.30</td>
</tr>
<tr>
<td>20</td>
<td>0.32</td>
<td>0.33</td>
</tr>
<tr>
<td>(Control - 'Normal' Microsomes)</td>
<td>0.38</td>
<td>0.30</td>
</tr>
</tbody>
</table>

(a) mg/g liver
### Table 4.6. Parameters of the MFO System in Hepatic Microsomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>'Normal' Microsomes</th>
<th>'Acid' Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P&lt;sub&gt;450&lt;/sub&gt; (a)</td>
<td>0.89 ± 0.08</td>
<td>0.95 ± 0.14</td>
</tr>
<tr>
<td>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (a)</td>
<td>0.83 ± 0.08</td>
<td>0.81 ± 0.16</td>
</tr>
<tr>
<td>Aminopyrine N-Demethylase (b)</td>
<td>16.7 ± 1.7</td>
<td>16.5 ± 2.0</td>
</tr>
<tr>
<td>Aniline 4-Hydroxylase (c)</td>
<td>0.80 ± 0.04</td>
<td>0.83 ± 0.04</td>
</tr>
<tr>
<td>NADPH-Cytochrome c Reductase (d)</td>
<td>111.9 ± 27.3</td>
<td>109.7 ± 30.2</td>
</tr>
</tbody>
</table>

Results are mean of 3 rats ± S.E.M.

(a) O.D./g.liver
(b) µ mole formaldehyde/g.liver/hr.
(c) µ mole 4-aminophenol/g.liver/hr.
(d) µ mole cytochrome c reduced/g.liver/hr.

### Table 4.7. Parameters of the MFO System in Hepatic Microsomes from Phenobarbitone-Induced and Control Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Induced</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (a)</td>
<td>41.9 ± 1.9</td>
<td>42.4 ± 3.2</td>
</tr>
<tr>
<td>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (b)</td>
<td>1.34 ± 0.09</td>
<td>1.39 ± 0.05</td>
</tr>
<tr>
<td>Cytochrome P&lt;sub&gt;450&lt;/sub&gt; (b)</td>
<td>2.44 ± 0.34</td>
<td>2.48 ± 0.24</td>
</tr>
<tr>
<td>Aminopyrine N-Demethylase (c)</td>
<td>55.5 ± 4.5</td>
<td>50.0 ± 5.5</td>
</tr>
<tr>
<td>Aniline 4-Hydroxylase (d)</td>
<td>2.92 ± 0.18</td>
<td>2.83 ± 0.15</td>
</tr>
</tbody>
</table>

Values are mean of 4 rats ± S.E.M. The differences in the values between control and induced for each fraction were significant (P < 0.01)

(a) mg/g.liver  (b) O.D./g.liver  
(c) µ mole formaldehyde/g.liver/hr.  (d) µ moles 4-aminophenol/g.liver/hr.
Table 4.8. **Percentage Increases in Haemoprotein Content and Type I and Type II Metabolic Activity and Hepatic Microsomes After Phenobarbitone Induction**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cytochrome b&lt;sub&gt;5&lt;/sub&gt;</th>
<th>Cytochrome P&lt;sub&gt;450&lt;/sub&gt;</th>
<th>Aminopyrine N-Demethylase</th>
<th>Aniline 4-Hydroxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Increases after Induction</td>
<td>Normal* Microsomes</td>
<td>Acid* Microsomes</td>
<td>Normal* Microsomes</td>
<td>Acid* Microsomes</td>
</tr>
<tr>
<td></td>
<td>184</td>
<td>282</td>
<td>288</td>
<td>278</td>
</tr>
<tr>
<td></td>
<td>174</td>
<td>288</td>
<td>321</td>
<td>270</td>
</tr>
</tbody>
</table>

Table 4.9. **Binding Constants for the Spectrally-Apparent Substrate-Induced Hepatic Microsome Interactions**

<table>
<thead>
<tr>
<th>Phenobarbitone Induction</th>
<th>Microsomes</th>
<th>K&lt;sub&gt;s&lt;/sub&gt; (μM): Biphenyl</th>
<th>Aniline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'Acid'</td>
<td>1.25 ± 0.09 (3)</td>
<td>5.19 ± 0.53 (3)</td>
</tr>
<tr>
<td></td>
<td>'Normal'</td>
<td>1.21 ± 0.01 (3)</td>
<td>5.96 ± 0.71 (3)</td>
</tr>
<tr>
<td>+</td>
<td>'Acid'</td>
<td>0.17 ± 0.07 (4)</td>
<td>5.03 ± 0.20 (4)</td>
</tr>
<tr>
<td>+</td>
<td>'Normal'</td>
<td>0.17 ± 0.01 (3)</td>
<td>5.35 ± 0.30 (4)</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. Figures in brackets indicate the number of rats used.
### Table 4.10. Levels of Protein, Cytochrome P<sub>450</sub> and Type I and Type II Metabolic Capacity in Various Rat Hepatic Microsome-Containing Fractions

<table>
<thead>
<tr>
<th>Microsome-Containing Fraction</th>
<th>Protein (mg/g liver)</th>
<th>Cytochrome P&lt;sub&gt;450&lt;/sub&gt; (O.D./g liver)</th>
<th>Aminopyrine N-Demethylation (u mole 4-aminophenol/g liver/hr.)</th>
<th>Aniline 4-Hydroxylation (u mole formaldehyde/g liver/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>254 ± 12.7</td>
<td>1.46 ± 0.07</td>
<td>N.D. (e)</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>'Acid' Homogenate</td>
<td>117 ± 3.1</td>
<td>1.17 ± 0.02</td>
<td>17.1 ± 0.5</td>
<td>0.89 ± 0.08</td>
</tr>
<tr>
<td>PMS</td>
<td>170 ± 3.7</td>
<td>N.D.</td>
<td>19.2 ± 0.5</td>
<td>0.93 ± 0.02</td>
</tr>
<tr>
<td>'Acid' Microsomes</td>
<td>51 ± 3.1</td>
<td>0.88 ± 0.03</td>
<td>13.0 ± 0.7</td>
<td>0.65 ± 0.01</td>
</tr>
</tbody>
</table>

Values are mean of 4 rats ± S.E.M.

(a) mg/g liver  
(b) O.D./g liver  
(c) μ mole formaldehyde/g liver/hr.  
(d) μ mole 4-aminophenol/g liver/hr.  
(e) not determined

### Table 4.11. Levels of Protein and Cytochrome P<sub>450</sub> in Various Fractions of Rat Liver Homogenate, Expressed as a Percentage of the Homogenate Value

<table>
<thead>
<tr>
<th>Source of Microsomes</th>
<th>Protein</th>
<th>Cytochrome P&lt;sub&gt;450&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Acid Homogenate'</td>
<td>46</td>
<td>80</td>
</tr>
<tr>
<td>PMS</td>
<td>68</td>
<td>-</td>
</tr>
<tr>
<td>'Acid' Microsomes</td>
<td>20</td>
<td>60</td>
</tr>
</tbody>
</table>
### Table 4.12. Type I and Type II Metabolic Activities in Various Fractions of Rat Liver Homogenate, Expressed as a Percentage of the PMS Value

<table>
<thead>
<tr>
<th>Source of Microsomes</th>
<th>Aminopyrine N-Demethylase</th>
<th>Aniline 4-Hydroxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Acid Homogenate'</td>
<td>89</td>
<td>96</td>
</tr>
<tr>
<td>'Acid' Microsomes</td>
<td>60</td>
<td>70</td>
</tr>
</tbody>
</table>

### Table 4.13. Enzyme Activity, Cytochrome P<sub>450</sub> and Malonaldehyde Content of Fresh 'Acid' and 'Normal' Microsomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>'Normal' Microsomes</th>
<th>'Acid' Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopyrine N-Demethylase&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td>11.6</td>
<td>13.1</td>
</tr>
<tr>
<td>Biphenyl 4-Hydroxylase&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>2.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Aniline 4-Hydroxylase&lt;sup&gt;(c)&lt;/sup&gt;</td>
<td>0.50</td>
<td>0.62</td>
</tr>
<tr>
<td>NADPH-Cytochrome c Reductase&lt;sup&gt;(d)&lt;/sup&gt;</td>
<td>70.1</td>
<td>74.6</td>
</tr>
<tr>
<td>Cytochrome P&lt;sub&gt;450&lt;/sub&gt;&lt;sup&gt;(e)&lt;/sup&gt;</td>
<td>22</td>
<td>31</td>
</tr>
<tr>
<td>Malonaldehyde&lt;sup&gt;(e)&lt;/sup&gt;</td>
<td>8.1</td>
<td>15.1</td>
</tr>
</tbody>
</table>

(a) μ mole formaldehyde/g.liver/hr.
(b) μ mole 4-hydroxybiphenyl/g.liver/hr.
(c) μ mole 4-aminophenol/g.liver/hr.
(d) μ mole cytochrome c reduced/g.liver/hr.
(e) nmole/g.liver
### Table 4.14. Post-Mortem Changes in Hepatic Microsomal Cytochrome and Protein Levels

<table>
<thead>
<tr>
<th>Source of Microsomes</th>
<th>b$_5$ (a)</th>
<th>P$_{450}$ (a)</th>
<th>Protein (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Liver</td>
<td>0.61 ± 0.07</td>
<td>0.50 ± 0.06</td>
<td>35.3 ± 3.5</td>
</tr>
<tr>
<td>Aged Liver</td>
<td>0.37 ± 0.10*</td>
<td>0.33 ± 0.04*</td>
<td>29.3 ± 3.6*</td>
</tr>
<tr>
<td>% Decrease</td>
<td>39</td>
<td>34</td>
<td>17</td>
</tr>
</tbody>
</table>

Values are mean of 3 rats ± S.E.M.

(a) O.D./g.liver (b) mg/g.liver

* P < 0.025  + P < 0.01

### Table 4.15. Kinetics of the Spectrally-Apparent Drug-Microsome Interactions: Effects of In Situ Storage Post-Mortem

**Binding Constants, K$_B$(X10$^4$M)$^{-1}$:**

<table>
<thead>
<tr>
<th>Source of Microsomes</th>
<th>Aniline Low Affinity</th>
<th>Biphenyl High Affinity</th>
<th>Low/High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Liver</td>
<td>3.55 ± 0.19</td>
<td>5.11 ± 0.24</td>
<td>1.36 ± 0.07</td>
</tr>
<tr>
<td>Aged Liver</td>
<td>3.55 ± 0.16(NS)</td>
<td>4.60 ± 1.06(NS)</td>
<td>1.26 ± 0.06(NS)</td>
</tr>
</tbody>
</table>

Values are mean of 3 rats ± S.E.M.

N.S. not significant
### Table 4.16. A Comparison of MFO Activity in 'Acid' and 'Normal' Human Hepatic Microsomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>'Normal' Microsomes</th>
<th>'Acid' Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (a)</td>
<td>21.1 ± 1.09 (4)</td>
<td>20.7 ± 0.79</td>
</tr>
<tr>
<td>Cytochrome P&lt;sub&gt;450&lt;/sub&gt; (b)</td>
<td>0.18 ± 0.00 (3)</td>
<td>0.20 ± 0.08</td>
</tr>
<tr>
<td>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (b)</td>
<td>0.32 ± 0.09 (3)</td>
<td>0.33 ± 0.11</td>
</tr>
<tr>
<td>4-Chloro-N-Methylaniline N-Demethylase (c)</td>
<td>0.31 ± 0.02 (2)</td>
<td>0.30 ± 0.06</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. Figures in brackets indicate number of estimations.

(a) mg/g liver, (b) O.D./g liver, (c) 4-chloraniline/g liver/hr.

### Table 4.17. Protein Content of Human Hepatic Microsomes: Effect of Isolation Procedure

<table>
<thead>
<tr>
<th>Source of Microsomes</th>
<th>Protein Content (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwashed 'Normal'</td>
<td>30.9 ± 2.5</td>
</tr>
<tr>
<td>Washed 'Normal'</td>
<td>20.9 ± 4.1</td>
</tr>
<tr>
<td>'Acid'</td>
<td>23.0 ± 0.3</td>
</tr>
</tbody>
</table>

Values are mean of 3 determinations ± S.E.M. (a) mg/g liver
Table 4.18. Analysis of Haem Content in Human Hepatic Microsomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unwashed</th>
<th>Washed</th>
<th>'Acid'</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{450}$</td>
<td>2.64</td>
<td>1.66</td>
<td>1.95</td>
</tr>
<tr>
<td>$b_5$</td>
<td>3.26</td>
<td>2.52</td>
<td>2.97</td>
</tr>
<tr>
<td>$P_{450} + b_5$</td>
<td>5.90</td>
<td>4.18</td>
<td>4.92</td>
</tr>
<tr>
<td>Total Haem</td>
<td>11.10</td>
<td>5.50</td>
<td>6.65</td>
</tr>
<tr>
<td>Total Haem - ($P_{450} + b_5$)</td>
<td>5.20</td>
<td>1.32</td>
<td>1.73</td>
</tr>
</tbody>
</table>

All results are in units of nmole/g liver

Table 4.19. Effect of Carbon Monoxide and SKF 525-A on the Microsomal MFO Activity of Human Liver

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline 4-Hydroxylase</td>
<td>62</td>
</tr>
<tr>
<td>4-Chloro-N-Methylaniline</td>
<td>65</td>
</tr>
<tr>
<td>N-Demethylase</td>
<td></td>
</tr>
</tbody>
</table>

CO  
SKF 525-A ($10^{-4}$N)  
0  
Not Tested
Table 4.20.  Microsomal Cytochrome Content of Human Liver

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Cytochrome b$_5$ (a)</th>
<th>Cytochrome P$_{450}$ (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL3</td>
<td>0.478</td>
<td>0.177</td>
</tr>
<tr>
<td>HL4</td>
<td>0.461</td>
<td>0.345</td>
</tr>
</tbody>
</table>

(a) OD/g.liver

Table 4.21.  Microsomal Metabolism of Type I Compounds by Human Liver

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Substrate</th>
<th>N-Demethylating Activity (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL1</td>
<td>Ethylmorphine</td>
<td>9.80</td>
</tr>
<tr>
<td>HL2</td>
<td>Ethylmorphine</td>
<td>6.50</td>
</tr>
<tr>
<td>HL4</td>
<td>Ethylmorphine</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>Aminopyrine</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td>Imipramine</td>
<td>0.36</td>
</tr>
</tbody>
</table>

(a) µ mole formaldehyde/g.liver/hr.
### Table 4.22. Microsomal Metabolism of Type II Compounds by Human Liver

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Aniline 4-Hydroxylase (a)</th>
<th>4-Chloro-N-Methylaniline N-Demethylase (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL2</td>
<td>1.090</td>
<td>Not Determined</td>
</tr>
<tr>
<td>HL3</td>
<td>0.430</td>
<td>0.360</td>
</tr>
<tr>
<td>HL4</td>
<td>0.345</td>
<td>0.265</td>
</tr>
</tbody>
</table>

(a) μ mole 4-aminophenol/g liver/hr
(b) μ mole 4-chloraniline/g liver/hr.

### Table 4.23. Binding Affinity Constants for the Interaction of Type I Substrates with Human Liver Microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_s$ (Human) ($\times 10^4 \text{M}$)</th>
<th>$K_s$ (Rat) ($\times 10^4 \text{M}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biphenyl</td>
<td>6.67 (HL3) (a)</td>
<td>1.25 ± 0.19 (b)</td>
</tr>
<tr>
<td></td>
<td>7.14 (HL4)</td>
<td></td>
</tr>
<tr>
<td>Naphthalene</td>
<td>11.0 (HL4)</td>
<td>2.60 ± 0.19 (b)</td>
</tr>
<tr>
<td>SKF 525-A</td>
<td>33.3; 1.59 (HL3)</td>
<td>0.067 (c)</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>4.88 (HL4)</td>
<td>-</td>
</tr>
</tbody>
</table>

(a) Figures in brackets indicate sample number
(b) Values are mean of 3 rats ± S.E.M.
(c) Mannering (1971).
Table 4.24. Binding Affinity Constants for the Interaction of Type II Compounds with Human Liver Microsomes

\[ K_g \times 10^4 M = \]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>High Affinity</th>
<th>Low Affinity</th>
<th>Rat Low/High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline</td>
<td>1.00</td>
<td>5.00 (HL4)(a)</td>
<td>5.0</td>
</tr>
<tr>
<td>4,4*-Bipyridyl</td>
<td>0.20</td>
<td>1.54 (HL4)</td>
<td>7.0</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>0.86</td>
<td>5.00 (HL3)</td>
<td>5.8</td>
</tr>
</tbody>
</table>

(a) Figures in brackets indicate sample number.
(b) Values are mean of 3 rats ± S.E.M.
(c) Phenobarbitone-induced liver, Liebman et al (1967).

Table 4.25. Wavelength Maxima and Minima for Type II and Reverse Type I Spectra Induced in Human Liver Microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Spectrum</th>
<th>Minima (nm.)</th>
<th>Maxima (nm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,4*-Bipyridyl</td>
<td>Type II</td>
<td>393 - 395</td>
<td>420 - 425</td>
</tr>
<tr>
<td>Aniline</td>
<td>Type II</td>
<td>390 - 395</td>
<td>422 - 427</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>Type II</td>
<td>392 - 395</td>
<td>423 - 426</td>
</tr>
<tr>
<td>Butan-1-ol</td>
<td>Reverse</td>
<td>385 - 390</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>Type I</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The reduced:oxidised spectrum of NADH-reducible cytochrome *b*₅ is illustrated on the left whilst the CO-induced difference spectrum of dithionite-reduced cytochrome *P*-450 is illustrated on the right.

The amount of microsomal fraction used corresponded to 250 mg wet weight liver equivalent.
Fig. 4.2. Spectrally Apparent Interactions of Biphenyl with 'Acid' and 'Normal' Microsomes from Control and Phenobarbitone-Induced Rat Liver.

Biphenyl Conc: 0.20 mM.
Fig. 4.3. Spectrally Apparent Interactions of Aniline with 'Acid' and 'Normal' Microsomes from Control and Phenobarbitone-Induced Rat Livers

Aniline Conc: 0.80 mM.
Fig. 4.4. Spectrally-Apparent Interactions of Biphenyl and Aniline with 'Acid' and 'Normal' Microsomes from Control Rat Liver (Redrawn on a g: liver basis)

Each graph represents the binding spectrum for microsomes equivalent to 1 g wet weight of liver.
Fig. 4.5. Cofactor Dependence of Aminopyrine N-Demethylase and Aniline 4-Hydroxylase Activities as Assayed in Rat Liver Homogenate

a) Aminopyrine N-Demethylase

Optimal Levels for Microsomal Fractions

b) Aniline 4-Hydroxylase

\[
\begin{align*}
\text{O.D. at} & \quad 412 \text{ nm.} \\
0.30 & \quad 1 \quad 0.20 \\
0.10 & \\
\end{align*}
\]

\[
\begin{align*}
\text{O.D. at} & \quad 620 \text{ nm.} \\
0.7 & \quad 0.5 \\
0.3 & \\
\end{align*}
\]

NADP (μmole/tube)

G-6-P at 25 μmole/tube

NADP at 10 μmole/tube
Fig. 4.6.- 4.10. Storage of Hepatic Microsomal Fractions: Time-Dependent Decay Curves for Various Parameters of the MFO System

Fig. 4.11. Storage of Hepatic Microsomal Fractions: Time-Dependent Increase in Malonaldehyde Levels

Key

- **4°C.**, Pellet
- **-20°C.**, Pellet
- **4°C.**, Suspension
- **-20°C.**, Suspension

--- 'Normal' Microsomes

--- 'Acid' Microsomes

Ordinate: Level as % that of Fresh Sample

Abscissa: Hours of Storage
Fig. 4.6. Aminopyrine N-Demethylase
Fig. 4.7. Biphenyl 4-Hydroxylase
Fig. 4.8. Aniline 4-Hydroxylase
Fig. 4.9. NADPH-Cytochrome c Reductase
Fig. 4.10. Cytochrome P450
Fig. 4.11. Malonaldehyde
Fig. 4.12 - 4.15. Effect of Different Forms of Storage on the MFO and Malonaldehyde Content of Hepatic Microsomal Fractions

Key

A  Aminopyrine N-Demethylase
A  Biphenyl 4— Hydroxylase
□  Aniline 4-Hydroxylase
□  NADPH-Cytochrome c Reductase
○  Cytochrome P 450

— Malonaldehyde

Left-Hand Ordinate: Levels of Certain Components of the MFO System as % that of Fresh Sample

Right-Hand Ordinate: Malonaldehyde level as % that of Fresh Sample

Abscissa: Hours of Storage
Fig. 4.12. Pellets at 4°C.
Fig. 4.13. Pellets at -20°C
Fig. 4.14. Suspensions at 4°C.
Fig. 4.16. Microsomal MFO Activity and Malonaldehyde Content in Rat Liver Maintained Solid at -40°C.

a. Aminopyrine N-Demethylase  
b. Aniline 4-Hydroxylase  
c. Cytochrome P_{450}  
d. Malonaldehyde

1. Fresh Sample  
2. 18 hours storage  
3. 66 hours storage  
4. 162 hours storage

Ordinates:  
a. \( \mu \text{ mole formaldehyde/g.liver/hr.} \)  
b. \( \mu \text{ mole 4-aminophenol/g.liver/hr.} \)  
c. nmole/g.liver  
d. nmole/g.liver
Fig. 4.17. Levels of Certain Components of the MFO System in Microsomes Isolated From Livers Maintained Solid for Varying Periods at \(-40^\circ\text{C}\).

% Original Level

Days of Storage

- Δ Cytochrome b$_5$
- Δ Cytochrome P$_{450}$
- ○ Aniline 4-Hydroxylase
- ○ Aminopyrine N-Demethylase
Fig. 4.18. Emergence of 420 nm. Peak After Storage of Solid Liver at $-40^\circ$C for 16 Days.

The two preparations were paired i.e. derived from the same liver and in both cases the above traces were obtained from microsomes equivalent to 250 mg, wet weight liver.
Fig. 4.19. Time Dependence of Enzymic Reactions in Human Hepatic Microsomes

Aniline 4-Hydroxylase

4-Chloro-N-Methylaniline N-Demethylase
Fig. 4.20. Cofactor Dependence of 4-Chloro-N-Methylaniline N-Demethylase Activity in Human Liver Microsomes

a. NADP

- M mole product/mg. protein/hr.
- G-6-P = 8mM
- NADP (mM)

b. G-6-P

- M mole product/mg. protein/hr.
- Optimal Level for Rat Liver Microsomes
- G-6-P (mM)
Fig. 4.21. Cytochrome $P_{450}$ Spectrum of Human Hepatic Microsomes
Fig. 4.22. Presence of Cytochrome $b_5$ in Human Hepatic Microsomes

![Graph showing absorbance vs wavelength (nm).]
Fig. 4.23. Apparent Increase in Cytochrome P<sub>450</sub> Content of Human Liver Microsomes After Addition of Oxidizing Agent to Control Cuvette.
Fig. 4.24. Spectrally-Apparent Interaction of Human Liver Microsomes with 1-Naphthol

1-Naphthol Concentration
1. 0.08 mM
2. 0.16 mM
3. 0.24 mM
4. 0.40 mM
5. 0.79 mM

Wavelength (nm)
Fig. 4.25. Double Reciprocal Plot to Calculate $K_s$ for the Interaction of Human Liver Microsomes with 1-Naphthol.
Fig. 4.26. Spectral-Apparent Interaction of Human Liver Microsomes with 4,4'-Bipyridyl

4,4'-Bipyridyl Conc\(^\circ\):
1. 0.04mM
2. 0.08mM
3. 0.12mM
4. 0.20mM
5. 0.30mM
6. 0.40mM

Wavelength (nm)
Fig. 4.27. Double Reciprocal Plot to Calculate the $K_s$ for the Interaction of Human Liver Microsomes with Metyrapone
Fig. 4.28. Spectrally-Apparent Interaction of Human Liver Microsomes with Butan-1-ol.

Vol. of Butan-1-ol Added: 1. 10μl
2. 20μl
3. 30μl

Wavelength (nm.)
## CHAPTER 5. CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Scale-Down of Assays for Components of the Hepatic Microsomal MFO System</td>
<td>130</td>
</tr>
<tr>
<td>2. Isolation of Hepatic Microsomes by Isoelectric Precipitation</td>
<td>133</td>
</tr>
<tr>
<td>3. Storage Characteristics of the Hepatic Microsomal MFO System</td>
<td>136</td>
</tr>
<tr>
<td>4. Post-Mortem Storage Characteristics of the Hepatic Microsomal MFO System</td>
<td>139</td>
</tr>
<tr>
<td>5. The MFO System in Human Liver Microsomes</td>
<td>141</td>
</tr>
</tbody>
</table>
CHAPTER 5. DISCUSSION

1. Scale-Down of Assays for Components of the Hepatic Microsomal MFO System

As mentioned earlier (see Chap. 2) the liver has a major responsibility for the metabolism of the various xenobiotics to which the mammalian body may be exposed. Such a specialized ability could in principle prove extremely valuable as a laboratory tool in the clinical diagnosis of liver function in man. Indeed, a few workers have demonstrated a relationship between the degree of severity of liver damage and the level of hepatic MFO activity (see Chap. 2). One could, in principle, use in vivo metabolism of a drug as a method of assessing liver function but difficulties exist due to the complexity of the in vivo system - the drug or its metabolite might undergo non-hepatic metabolism, etc. - and consequently the use of liver samples appears to be a more valid proposition. Unfortunately, a major problem in the use of MFO activity as a measure of liver function in man has been the obvious difficulty in obtaining appropriate samples. It is ethically unreasonable to submit a patient to surgery simply for the purpose of obtaining a liver specimen for subsequent analysis. This has meant that needle biopsy samples of human liver have had to be considered for this purpose. Needle biopsy specimens are approx. 30 - 50 mg. in weight, part of which has invariably to be used for histological analysis. Since 75 - 150 mg. of liver is required for a conventional study of MFO activity, small scale assay procedures have to be adopted when attempting to measure MFO activity in the remaining 15 - 25 mg. of sample.
A number of methods exist for the micro-scale assay of hepatic MFO activity, these being based largely on radioactivity techniques (Kuntzman et al., 1967; Gang et al., 1972; Hayakawa and Udenfriend, 1973; Poland and Nebert, 1973; Kupfer and Rosenfeld, 1973) although micro-scale spectrophotometry techniques have also been used (Schoene et al., 1972). Enzymes measured by these techniques include pentobarbital and hexobarbital hydroxylase (Kuntzman et al., 1967; Kupfer and Rosenfeld, 1973), aniline hydroxylase (Gang et al., 1972), benzpyrene hydroxylase (Hayakawa and Udenfriend, 1973) and aminopyrine and \( p \)-nitroanisole dealkylation (Poland and Nebert, 1973; Schoene et al., 1972). Whilst these techniques do use very small amounts of tissue (1 - 25 mg. liver wet weight) they do suffer from the major disadvantages that there is an absolute need for costly ready-made or specially-manufactured equipment, coupled with the cost and difficulty in obtaining radioactively-labelled substrates.

We have found that reduction of the total volume of assay medium together with a concomitant reduction in the volume of microsomal fraction, and the use of a spectrophotometer capable of accepting micro cells (as outlined in Chap. 4) has proved successful for scaling-down the assays for aminopyrine or ethylmorphine \( N \)-demethylase, aniline hydroxylase and \( 4 \)-chloro-\( N \)-methylaniline \( N \)-demethylase. By these means the equivalent of 25 mg. wet weight of liver can be used for each enzyme assay as opposed to the 75 - 150 mg. normally used (Table 4.1). Although these assays were not assessed on needle biopsies of human liver they were used on surgical biopsy samples (HL1 and HL2). The results obtained for these samples (Tables 4.21 and 4.22) agree very well with the values quoted by other
workers using similar samples and 'normal' assays (Ackermann, 1972; Deckert and Rammer, 1972), thus implying the applicability of these micro-scale assays to the human situation. It is probable that less than 25 mg. liver equivalent could be used in these assays if the incubation time is increased accordingly and a protein stabilizing agent, such as serum albumin (Jacobson et al., 1972), is added to the assay medium.

The applicability of these assays to homogenates of rat or human liver was not tested although problems to this approach are apparent. Firstly, there is an increased requirement for the cofactors G-6-P and NADP within the assay medium (Fig. 4.5 and 4.6) which probably reflects involvement of the contaminating mitochondria (Bachmann et al., 1970) and of hydrolytic enzymes. Secondly, the presence of large amounts of protein in the homogenate makes it impossible to accurately measure formaldehyde production as assayed by the conventional Nash reaction due to turbidity of the coloured solution. This could possibly be greatly minimized by either high-speed centrifugation (Ziegler O.N., personal communication) or, more simply, by extraction of the coloured complex into an organic solvent (Hasegawa, 1963).

No such difficulty exists in the aniline hydroxylase assay although it does appear that an inhibitor of this enzyme is present in rat liver homogenate (Table 4.10).

Scale-down of the cytochrome P₄₅₀ assay system either by diluting the microsomal suspension or by using micro-cuvettes did not prove successful. As the microsomal suspension was diluted so the apparent concentration of P₄₅₀ per g. of liver decreased, - finding also observed by Wade et al. (1972) who considered this to be related to some form of membrane-aggregation effect.
2. Isolation of Hepatic Microsomes by Isoelectric Precipitation

In certain situations, e.g., where precise localization of metabolism is required or in substrate binding studies, isolated microsomal systems have to be used in place of either homogenates or PMS. We have shown that isolation of hepatic e.r. fragments by isoelectric precipitation yielded a microsomal fraction comparable, on a g. liver basis, in activity of the MFO system to that obtained by conventional high-speed centrifugation techniques, whether these fractions be obtained from normal or induced rat liver (Tables 4.6 and 4.7). Similar findings with regard to type I metabolism only have been reported by Karler and Turkenis (1968) and Mitchard (1969). The precipitate formed after addition of acid buffer to a PMS could be harvested by low-speed centrifugation for short periods, maximum sedimentation occurring after 5 minutes centrifugation at approx. 2,000 g av. (Table 4.5).

The major difference between 'acid' and 'normal' microsomes lay in their differing protein content, there being 50 - 60% extra protein in the 'acid' preparations (Table 4.2). This difference has also been noted by Karler and Turkenis (1968) as well as by Takesu and Omura (1970). It is believed that this additional protein is derived from what is termed the 'cytosol' fraction of the liver homogenate. These cytosol proteins show minimum solubility at pH 4-5 (Lione and Redstone, 1962) and would be expected to co-sediment with the microsomes prepared by isoelectric precipitation. Further evidence for this belief comes from a number of results:

1) The additional protein can be precipitated when the acetate buffer is replaced by either dilute HCl or by citrate buffer.
(Table 4.2), implying that it is the lowering of the pH and not the effect of the acetate per se which is responsible;

2) The extra protein is non-ribosomal in origin as judged by the yields of RNA (Table 4.3);

3) It is not derived from haemoglobin contamination as assessed by a lack of any appreciable peak at 420 nm. in the spectrum of P\textsubscript{450} (Fig. 4.1);

4) The protein content of 'acid' microsomes is similar to that of unwashed 'normal' microsomes, washing of which results in the removal of trapped soluble cytosol proteins (Table 4.4).

This difference in protein content between 'acid' and 'normal' microsomes is not observed when using livers obtained from phenobarbitone-induced animals (Table 4.7). The reason for this is obscure although it may reflect a relative or absolute decrease in the level of cytosol protein on phenobarbitone induction.

It is clear from these studies that both acid pH and the presence of acetate ions exert no short-term deterious effect on the functional state of the MFO system in hepatic microsomes. Karler and Turkanis (1968) were able to show that hexobarbital metabolism stayed at a normal level even if the pH went as low as 4.6 during the IP stage although it has been reported (Betz et al., 1973) that exposure of hepatic microsomes to pH 5.0 for 40 minutes leads to a fall in the levels of b\textsubscript{5}, P\textsubscript{450} and NADPH-cytochrome c reductase activity. The final concentration of acetate present when the pH is lowered to 5.4 is of the order of 0.02M. Incubation of hepatic microsomes with sodium acetate at pH 8.0 for 10 minutes produces no loss of P\textsubscript{450}, even if the acetate concentration is 2M or greater (Imai and Sato, 1967).
As indicated earlier, the overall rate of drug metabolism (as measured by the levels of type I and type II metabolism), the rate of $P_{450}$ reduction (as assessed by the level of NADPH-cytochrome c reductase) and the level of $P_{450}$ itself are exactly comparable in 'acid' and 'normal' microsomes. In addition, the affinity of 'acid' microsomes for substrates of the MFO system is strikingly similar to that for 'normal' microsomes (Table 4.9). This is true for control and induced livers. If anything, the affinity for aniline is greater in the 'acid' microsomes although this is not reflected as differences in the level of aniline hydroxylase activity. Furthermore, the type of binding is unaltered by the acid treatment (Fig. 4.2 and 4.3).

Thus at all stages of the MFO system so far studied - substrate binding, cytochrome $P_{450}$ reduction and overall rate of metabolism - hepatic microsomes prepared by IP are at total parity with those prepared by conventional techniques. This technique of isoelectric precipitation offers distinct advantages in respect to its speed and its obviation for the need of a costly high-speed centrifuge.

These results concerning IP, which were obtained using rat liver microsomes, have been further confirmed in two samples of human p.m. liver. Once again, the levels of $P_{450}$, $b_5$ and enzyme activity were comparable in 'normal' and 'acid' microsomes whilst the protein levels were, surprisingly, also similar (Table 4.16). In this instance, the protein level of 'acid' microsomes more closely approached that of washed 'normal' microsomes as opposed to unwashed 'normal' microsomes (Table 4.17). The extra protein present in unwashed 'normal' microsomes could be attributed, at least in part, to the haemoglobin contaminating the samples. This study has conclusively
shown that isoelectric precipitation is indeed a highly feasible and attractive alternative to high-speed centrifugation when studying human liver microsomes for their drug-metabolizing potential.

IP can also be successfully employed to achieve a microsome-rich fraction from rat liver homogenate cleared of nuclei and cellular debris which is distinct from a PMS (Table 4.10). The preparation of a 'pure' microsomal fraction via a PMS results in the loss of 40% of the P450 present in the homogenate (Table 4.11 and Schoene et al., 1972), this value being reduced to only 20% in the 'acid' homogenate. The level of type I and type II metabolism in 'acid' homogenates is approx. 90% and 95% respectively that of PMS fractions derived from the same livers; the corresponding figure for microsomes derived from these PMS fractions is 70% (Table 4.12). By use of this scheme of IP on liver homogenate it is possible to achieve a microsome-rich fraction requiring the use of a common bench centrifuge only. These microsome-rich fractions appear a suitable tool with which to assess the functional ability of the liver to metabolize various drugs without the disadvantages (discussed earlier) of using the homogenate proper.

3. Storage Characteristics of the Hepatic Microsomal MFO System

Whilst it is advisable to measure MFO activity as soon as possible after preparing microsomal fractions situations do arise where this is not possible. In these circumstances it would be obviously advantageous to know the best method of storing microsomes, either as the solid unprocessed tissue or as isolated fractions. This has been studied in the present work.
Although the short-term stability of hepatic microsomes prepared by IP appears to be very high they are more susceptible to the loss in MFO activity associated with storage, this being reflected in the high level of malonaldehyde present even in fresh samples (Table 4.13). In general, activity of the hepatic MFO system in isolated fractions is best maintained if the samples are stored at -20°C in the form of microsomal pellets (Fig. 4.7 - 4.11). These findings are broadly in agreement with those of Leadbeater and Davies (1964), Levin et al. (1969) and Wade et al. (1972). Also, the 'normal' microsomes remain more stable at -20°C than do their 'acid' counterparts (Fig. 4.7 - 4.11); with storage of both microsome preparations at 4°C the decay is more rapid and so meaningful comparisons between the various preparations cannot be made. The storage decay curves with time for two enzymes responsible for the metabolism of type I substrates, aminopyrine N-demethylase and biphenyl 4-hydroxylase, in 'normal' microsomes at -20°C show discernible differences (Fig. 4.13 and 4.15) which may possibly indicate the presence of more than one terminal oxidase within the MFO system (Levin et al., 1969).

Concomitant with the time- and storage-dependent decay in the levels of various parameters of the MFO system is a time- and storage-dependent increase in the malonaldehyde content (Fig. 4.12 - 4.15). The level of malonaldehyde is generally regarded as a sensitive index for lipid peroxidation (Tappel, 1973) and so one can deduce that the storage dependent decay in activity of the MFO system is associated with peroxidation of the lipid present in the microsomal membrane. This provides further evidence for the vital role played...
by microsomal membrane lipid in maintaining the correct structural
and lipophilic features of the MFO system (Estabrook et al, 1971).
Thus it is probably not specific degradation of the MFO system per se
within the microsomal membrane which leads to its decay on storage
but rather it is degradation of the microsomal membrane which leads
to inactivation of the MFO system.

This poses the problem as to what initiates this membrane
degradation. This is probably the MFO system itself via its role
as a peroxide-generating system as outlined in Chap. 2. The finding
of Deckert and Remmer (1972) that rat liver microsomal suspensions
could be maintained for 20 days at -20°C with no loss in activity
(which is in disagreement to the findings presented above and the
work of Wade et al, 1972) can be ascribed to the presence within the
suspending system of EDTA, a known inhibitor of lipid peroxidation
(Bidlack et al, 1973). This use of EDTA has its limitations in that
it might interfere in the subsequent assays performed on the
suspensions.

Our work has shown that if storage of a valuable sample is
necessary it is best achieved by storing the solid liver specimen
itself and not by storing the isolated microsomal fraction. An
experiment carried out in parallel with that on storage of isolated
microsomes showed there to be no decrease in the levels of aminopyrine
N-demethylase, aniline 4-hydroxylase activity and P_{450} content, and
no increase in the level of malonaldehyde if liver was stored solid
at -40°C for up to one week (Fig. 4.16). This was confirmed in a
further experiment which also showed that during 6 weeks' storage fall
off in activity did occur although the rate of decay varied according
to the parameter under study, type I metabolism being the most labile and b<sub>5</sub> the most stable (Fig. 4.18). Once again, the loss in MFO activity in these samples is probably linked to a membrane lipid peroxidation effect, the greater short-term stability of these solid-stored samples (cf. isolated microsomes) reflecting the protective effect of intracellular compartmentalization and integrity of the endoplasmic reticulum. It is also possible that lipid peroxidative damage of lysosomal membranes could lead to the release of hydrolases (Tappel, 1973) which in turn could directly inactivate the microsomal MFO system (Betz et al., 1973).

4. Post-Mortem Storage Characteristics of the Hepatic Microsomal MFO System

In studies of the human hepatic microsomal MFO system it is usually very difficult to obtain fresh normal samples and recourse has often been made to the use of p.m. material. However, the autolytic changes that occur p.m. are a major drawback to this approach and it is well known that p.m. storage of liver in situ results in a decrease in the levels of P<sub>450</sub> type I and type II metabolism, P<sub>450</sub> reduction and of protein content (Jondorf and Donahue, 1970; Macleod et al., 1973). These results have been confirmed with regard to P<sub>450</sub> and protein content, together with a previously unreported fall in b<sub>5</sub> content (Table 4.14). However, during this period of p.m. decrease in cytochrome and protein content, there is no corresponding decrease in the binding affinity of the microsomes (on a mg. protein basis) either for aniline or biphenyl (Table 4.15). It is feasible to assume that the overall metabolism
of type I and type II compounds, being dependent on the levels and/or activities of various components in the MFO system, would be expected to show greater susceptibility to p.m. changes compared with the comparatively simple reaction of microsome-substrate binding. Based on these findings it is probable that analysis of substrate interactions with microsomes derived from post-mortem and/or stored liver samples could provide a more rational extrapolation to the functional in vivo compared to, say, the measurement of metabolism or cytochrome content.

It is apparent from these proceeding studies that extrapolation from MFO activity in stored and/or p.m. liver to that existing in vivo is, with the possible exception of analysis of microsome-substrate interactions, of somewhat doubtful value. Yet for obvious reasons this extrapolatory approach has had to be employed in many instances when investigating the MFO system in human liver (see Table 2.1). Whilst the use of p.m. human liver may not prove valid for quantitative studies on microsomal drug metabolism it is, nevertheless, a perfectly acceptable tool with which to study qualitative patterns of microsomal drug metabolism, and has been so used to assess pathways of chlorpromazine metabolism (Coccia and Westerfeld, 1967) and the relative proportion of N- and 7- hydroxylation of 2-acetylaminofluorene (Enomoto and Sato, 1967). It is most likely that this qualitative attitude to the use of p.m. human liver in drug metabolism studies could prove to be of great value in the pre-clinical testing of potential drugs in that, although absolute values for metabolism could not be given, the relative proportions of metabolites produced could be assessed and compared with the patterns obtained
from laboratory animal species in vivo and in vitro. This system would obviously entail an awareness as to the possible storage-dependent differential decay in activities of the enzymes responsible for the various pathways of metabolism and could involve the use of a marker of storage decay such as malonaldehyde production.

5. The MFO System in Human Liver Microsomes

Only four samples of human liver were available for our study and these ranged from fresh biopsy samples to p.m. specimens subjected to long periods of storage. It is important that the history of each sample be known with regard to the method of its obtention, the storage conditions and the medical state of the donor; these were obtained as complete as was possible. Due to this paucity in the number of samples only preliminary findings can be reported.

In agreement with other workers is the finding that human liver microsomes do contain cytochromes P₄₅₀ and b₅ (Table 4.20; Alvares et al., 1969; Ackerman, 1970; Black et al., 1973; Nelson et al., 1971; Schoene et al., 1972). The presence of large amounts of haemoglobin necessitate the use of a modified assay for P₄₅₀ as described by Schoene et al. (1972) (Fig. 4.21). It has been reported that storage of solid human liver in a frozen state overnight leads to haemoglobin contamination of the ensuing fraction (Alvares et al., 1969). One interesting observation is the possibility that up to 20% of P₄₅₀ may be in the reduced state within the human liver in vivo (Fig. 4.23).
The levels of $P_{450}$ in our study are much lower in the p.m. liver samples (1.9 – 3.8 nmole/g.liver) when compared to those reported for fresh biopsy samples (approx. 12 nmole/g.liver) (Black et al., 1973; Schoene et al., 1972; Thorgeirsson and Davies, 1971) whilst the levels of $b_5$ in the fresh and p.m. samples are comparable (approx. 3 nmole/g.liver) (Ackermann, 1970). This no doubt reflects the greater storage stability of $b_5$ as observed in our previous studies for rat liver.

Human liver microsomes are capable of metabolizing a number of compounds known to be either type I or type II in the rat (Tables 4.21 and 4.22). Metabolism of type I compounds in fresh biopsy samples of human liver is at a level (6.5 – 10$\mu$ moles product/g.liver/hr.) one third to one half that present in male rat liver microsomes; similar relationships have been reported by Ackermann (1970), Darby et al. (1972) and Pelkonen et al. (1973). Metabolism of aniline on the other hand is at a level equal to that found in male rat liver microsomes (1$\mu$ mole product/g.liver/hr.). Comparison of enzyme activities for both type I and type II compounds in fresh biopsy and stored p.m. human liver samples reveals the decrease brought about by the post-mortem and storage conditions, (Tables 4.21 and 4.22).

A few studies have shown that metabolism of various substrates by human liver microsomes is mediated by a typical MFO system (Ackerman and Heinrich, 1970; Kuntzman et al., 1966; Rauers et al., 1971). This has been confirmed in these studies with regard to time and cofactor dependency for the demethylation of 4-chloro-N-methyl-aniline (Fig. 4.19 and 4.20) and the susceptibility of aniline hydroxylase and 4-chloro-N-methylaniline N-demethylase to inhibition.
One striking difference, however, between human and rat liver microsomes lies in the resistance of human liver microsomes to any inhibition by either SKF 525-A or metyrapone, even when the concentration of SKF 525-A is increased by a factor of 10 over the level effective in the rat. This difference in sensitivity to inhibition by SKF 525-A has also been noted by Ackermann (1970). This aspect will be discussed further.

The present study has shown that human liver microsomes will bind substrates of the MFO system in a manner similar to that observed for rat liver microsomes. Thus, biphenyl, naphthalene, SKF 525-A and 1-naphthol are type I substrates in human and rat liver microsomes (Table 4.23; Fig. 4.24; Mannering, 1971; von Bahr et al., 1971) whilst aniline, metyrapone and 4,4'-bipyridyl are type II in both species (Table 4.24; Fig. 4.25). In addition, butan-1-ol has been shown to give a typical reverse type I spectrum on addition to human liver microsomes (Fig. 4.20; Table 4.25). Kamatsaki et al. (1971; 1973) have reported that addition of high concentrations of hexobarbital, aminopyrine and SKF 525-A to suspensions of adult human liver microsomes yielded characteristic type II spectra whilst Pelkonen (1973) has shown that these compounds elicit type I spectra. This discrepancy between the results of Kamatsaki et al. (1971; 1973) on the one hand and those of the present study and of Pelkonen (1973) on the other can possibly be allied with the known alteration in the class of binding which occurs with certain compounds (hexobarbital, aminopyrine, imipramine, dexamethylimipramine and (+)-amphetamine) as their concentration in the sample cuvette is increased (Kamatsaki et al., 1973b; Anders 1972; Hoffstrom and Orrenius, 1973; von Bahr and Orrenius, 1971). However, no evidence for this transition from
As discussed earlier, the binding affinity of hepatic microsomes is much less affected by p.m., and probably storage, changes than are the levels of P₄₅₀ or overall drug metabolizing capability. Based on this, it is reasonable to assume that the K₈ values obtained for the p.m. human liver microsomes would bear more relation to those obtained for fresh samples than would the levels of overall drug metabolizing activity. Consequently, analysis of the K₈ values obtained from these p.m. human liver microsomes could prove extremely valuable in elucidating the differences in MFO activity between human liver microsomes and those derived from the various laboratory animal species. It has been argued that K₈ values are linked to the Michaelis constants for various substrates (see Chap. 2). The present findings support this contention. Thus, the low rate of metabolism of type I compounds in man appears to be related to the lower affinity of human liver microsomes for the substrate (see biphenyl and naphthalene in Table 4.23) as well as to the lower P₄₅₀ content, whilst the similar level of aniline hydroxylase activity in human and rat hepatic microsomes is paralleled by near parity in their K₈ values for aniline (Table 4.24). In this context, the level of aniline used for the enzyme assays (10 mM) lies within the range for which the low affinity K₈ value applies. Evidence for two binding sites was obtained for all three type II substrates and the similarities in the values for the low K₈:high K₈ ratio strongly suggest that these two binding sites are common to all three substrates. There was no evidence for two binding sites for these compounds in male rat liver microsomes. Furthermore, the previously-noted resistance of the MFO system in human liver microsomes to inhibition by either
SKF 525-A or metyrapone is palpably associated with a very much lowered affinity of human liver microsomes for these inhibitors (Table 4.23 and 4.24). The reasons for these species differences in substrate-microsome affinities are at present unknown although differences in the structure of P450, which lead to alterations in the charge density and/or lipophilicity of the relevant molecule, are probably involved.

These studies have revealed three major conclusions. Firstly, techniques exist by which small samples of human liver can be processed and assayed for their drug metabolizing potential, analysis of which could prove a useful tool in the diagnosis of liver-specific disease situations. Secondly, stored p.m. human liver cannot be used for quantitative studies on drug metabolism. They could, however, with reservation be profitably used for qualitative studies into the patterns of drug metabolism and these patterns compared with those found when using fresh animal liver microsomes. Finally, analysis of drug-microsome interactions using p.m. human liver and fresh animal liver could prove valuable in assessing the relative rates of hepatic drug metabolism in man and laboratory animal species. This is of further importance due to the difficulty in obtaining a regular supply of fresh human liver suitable for direct drug metabolism studies.
TISSUE CULTURE STUDIES
CHAPTER 6. CONTENTS

1. Rationale 148

2. Brief Description of the Anatomy and Histology of Mammalian Liver 149
   a. Blood Supply 149
   b. Liver Lobules 150
   c. Hepatic Sinusoids 150
   d. Hepatocytes 152
   e. Bile Canaliculi 153
   f. Connective Tissue 153

3. Some Characteristics of Mammalian Liver of Possible Importance to its Successful Culture 154
   a. Hepatic Connective Tissue 154
   b. The Cell Types of Mammalian Liver 157
   c. The Hepatic Cell Cycle and its Regulation 159

4. The Isolation of Mammalian Liver Cells 162

5. Liver Culture: Techniques and Results 165
   i) Culture of Foetal Mammalian Liver 166
      a. Non-Human Species 166
      b. Man 169
   ii) Culture of Adult Mammalian Liver 170
      a. Non-Human Species 171
      b. Man 174
1. **Rationale**

The mammalian liver is a complex and heterogeneous organ, both morphologically and functionally. It is this functional heterogeneity that makes mammalian liver the ideal organ with which to study various metabolic processes, their control and their interrelationships. These metabolic processes, including those concerned with the transformation of xenobiotics, can be studied at four levels of tissue organization viz. whole animal, isolated liver, isolated liver cells and isolated subcellular fractions. In the field of drug metabolism it is only the two extremes of this organizational spectrum that have been used to any great degree. The reasons for this are not hard to find; both systems are relatively easy to use and, in the case of the whole animal, gives a true picture of the overall state of metabolism. The use of isolated liver preparations either as the perfused organ or the isolated cells offers distinct advantages when examining in detail the role of the liver in drug metabolism and obviously represent a truer approximation to the *in vivo* state than do the subcellular fractions. The major limitation to the use of perfused liver in such metabolic investigations is the short length of time (less than 10 hours) during which the preparation remains viable; there is potentially no such limitation in the case of cultured cells. Thus, this system of cultured liver cells would appear ideal for studies into the metabolism of xenobiotics and also, possibly more important, into drug-induced hepatotoxicity and the role of metabolism therein.
Furthermore, obvious ethical difficulties exist to using human volunteers and perfused post-mortem human liver as systems for studying metabolism and possible hepatotoxicity of novel compounds developed by the pharmaceutical industries. Once again, the most feasible system for these preliminary clinical trials is prospectively that of cultured human liver.

In spite of these considerations there are no published reports as to the use of cultured adult mammalian liver in examining the metabolism of xenobiotics (although foetal cultures and freshly-isolated cell systems have been used for this purpose—see Chap. 2). The reasons for this lay in the recognized difficulty in obtaining cultures suitable for such a purpose. Foetal liver can be cultured quite successfully (see later) although these cells are unsuitable for most drug metabolism studies due to their known state of immaturity with regard to the microsomal MFO system (see Chap. 2). Many culture systems whilst being successful in a number of respects are unsuitable for metabolism and toxicity studies due to their difficulty and scale of operation. This is discussed elsewhere. Firstly, however it is appropriate to give a brief description of the mammalian liver and also of the factors which may possibly affect its successful culture.

2. Brief Description of the Anatomy and Histology of Mammalian Liver

a. Blood Supply

In the adult the liver is supplied by blood from two sources. The principal afferent blood vessel, supplying 90% of the blood, is the portal vein. It collects the blood from the viscera of the
digestive tract and enters the liver at the porta together with the hepatic artery. As the portal vein enters the liver it branches up into a series of vessels which supply a capillary bed in the liver tissue, from which the blood drains into the hepatic veins which discharge into the inferior vena cava. The minor source of blood supply (10%) is by way of the hepatic artery a relatively small vessel which delivers oxygenated blood from the dorsal aorta and supplies the interlobular connective tissue and its contained structures and helps to nourish the parenchyma of the gland.

b. Liver Lobules

The mammalian liver is comprised of small polygonal areas each of which represents an architectural unit or lobule, 0.7 mm. to 2 mm. in diameter. The lobule is a polygonal prism which in cross section has 5–7 sides. Running through the centre of the lobule, in its long axis, is the central vein, while at the periphery are the branches of the portal (interlobular) vein, the interlobular bile ducts, branches of the hepatic artery and the lymphatics which form a network about the portal vein and its branches (Fig. 6.1).

c. Hepatic Sinusoids

The cords of liver cells are separated one from another by the hepatic sinusoids. These are irregular blood spaces which pursue a radial course in the lobule and connect the interlobular portal veins with the intralobular central veins. They also receive blood from the branches of the hepatic artery. The lining of the sinusoids is
Fig. 6.1. Idealized Transverse Section of Mammalian liver to Show Lobule Structure

Each portal canal transmits a branch of the hepatic portal vein, hepatic artery, bile duct and lymphatic vessels.

Fig. 6.2. Intralobular Structure of Mammalian Liver
composed of an irregular alternation of 2 kinds of cells. These are the undifferentiated lining cells and the fixed macrophages — the stellate cells of von Kupffer — both types being part of the reticuloendothelial system (Fig. 6.2).

d. Hepatic Cells

The liver cells (hepatocytes) are arranged regularly in cords which form columns extending radially from the central vein to the periphery of the lobule. The cords may branch slightly and anastomose with nearby cords, but in spite of this their general direction is perpendicular to that of the central vein. Between those cords are the sinusoids (Fig. 6.2).

The liver cells are polygonal in shape and have 6 or more surfaces. Most hepatocytes have one large, round nucleus, although binucleated cells are quite common; a recent study has estimated that binucleated cells comprise about 30% of hepatocytes in adult rats and that large numbers appear shortly after weaning (Wheatley, 1972). The nucleus is quite vesicular; it has a smooth membrane and one or more very prominent nucleoli and a few small chromatin dots.

The cytoplasm of the hepatocyte presents an extremely variable appearance which reflects to some degree the functional state of the cell. Both glycogen and fat inclusions can be readily demonstrated. The cytoplasm also contains mitochondria of variable appearance, a Golgi net and vacuoles.

In spite of the multitude of functions which hepatocytes perform, there is a marked similarity in all of them. This is at variance with what is seen in other organs, in which highly specialized functions
are carried on by cells which morphologically are highly differentiated. It seems that all the hepatocytes are equally endowed with the same functional properties, but that their active participation in these processes under normal conditions depends on the location of the cell in the lobule.

e. Bile Canaliculi

In adult man the liver cell cords in cross section consist of two adjacent cells between which runs a thin bile canaliculus which is a condensation of the membrane of the hepatocytes. These bile canaliculi, after pursuing a tortuous route, eventually join up with the interlobular bile ducts.

f. Connective Tissue

The liver lobules are partially separated by very thin strands of dense connective tissue termed periportal connective tissue. This is a part of Glisson's capsule, the dense connective tissue sheathing the intrahepatic portions of the portal vein, bile duct and hepatic artery, and is also continuous with the thin layer of connective tissue covering the liver. In man the outlines of the lobules are usually indistinct because the connective tissue partitions between them are poorly developed. In the pig, polar bear and raccoon, on the other hand, each lobule is completely surrounded by a layer of connective tissue and the lobulation is obvious. The periportal connective tissue continues directly into the dense network of intralobular reticular fibres which surrounds the sinusoids and supports the liver parenchyma.
Some Characteristics of Mammalian Liver of Possible Importance to its Successful Culture

a. Hepatic Connective Tissue

Connective tissue is found wherever there is a need for support of other tissues and is composed of two phases, the cellular and the extracellular. The proportion of these two phases governs the type of connective tissue.

Dense connective tissue occurs where strength is more important than looseness (e.g., as organ capsules, support for skin, muscle fascias and tendons) and has an abundance of the extracellular phase with a few cells. The predominant cells are fibroblasts which synthesize and secrete the collagen fibres which, together with the ground substance of mucopolysaccharides, constitute the extracellular phase.

Loose connective tissue is found where exchange of metabolites and fluids between an epithelial layer and the vascular system is desirable. Although the major cell type is again the fibroblast various other cell types are also observed including macrophages, the lymphoid cells, eosinophils, mast cells and plasma cells. The extracellular phase consists of three types of fibres viz., collagenous, elastic and reticular. The reticular fibres are collagenous in form but with smaller diameter than the mature collagen fibre. This reticular connective tissue is found in the interior of most organs.

Basement membranes form another extracellular material of rather amorphous structure and are considered to be a supporting structure,
mostly for epithelia and vascular channels. They are composed of reticular fibres and a ground substance.

As mentioned earlier, the periportal connective tissue and Glisson's capsule account for the bulk of the dense connective tissue found in the liver. Basement membranes surround the blood vessels and lymphatic channels found in the portal tracts and the epithelium of the bile ducts and ductules also rests on a basement membrane. Within the liver lobules the only connective tissues present are the reticular fibres which surround the sinusoids, although the space between the endothelial cells and the hepatocytes, the space of Disse, contains protein-bound mucopolysaccharides (Rubin and Hutterer, 1967).

In man, the infant liver contains less than one-half of the collagen content of the adult liver. This has been taken to indicate that in normal growth the parenchymal cells proliferate much more rapidly than do the stromal cells (Rubin and Hutterer, 1967). A similar situation has been found in rat liver where the hepatic collagen content was related to both age and diet (Deyl et al, 1971).

The ground substance of hepatic connective tissue has been shown to be largely a mixture of chondroitin sulphates A and C together with some hyaluronic acid (Rubin and Hutterer, 1967).

This brief discussion on connective tissue and its presence in mammalian liver highlights some points extremely relevant to the successful isolation of, and hence culture of, hepatocytes. These points are summarized below.

1) Connective tissue is the only tissue which supports the liver whether this connective tissue be surrounding the blood vessels or the liver itself, or whether it be the fine reticular fibres which
support the hepatocytes.

ii) The vast bulk of the hepatic connective tissue is extralobular with only the reticular fibres being intralobular.

iii) In both the dense and reticular connective tissue the outstandingly predominant extracellular fibre is collagenous whilst the ground substance is virtually all chondroitin sulphates A and C.

Two conclusions can be drawn from these points. Firstly, successful isolation of liver cells should be possible by use of a system which breaks down the collagen/chondroitin sulphate phase of the connective tissue. Secondly, it should be theoretically easier to isolate and culture foetal or newborn liver cells than adult hepatocytes.

The Ca\(^{++}\)-free collagenase/hyaluronidase system which breaks down collagenous structures has been proposed by Howard and Pesch (1968). This couples the specific action of Clostridial collagenase with that of testicular hyaluronidase, an enzyme effective against hyaluronic acid and chondroitin sulphates A and C. The dissociating medium is free of Ca\(^{++}\), presumably to minimize the possibility of Ca\(^{++}\)-induced re-aggregation of the dissociated cells (Moscona et al., 1965). The Clostridial enzyme progressively removes small fragments from the terminal carboxy end (Evanson, 1971); for activity the enzyme requires a P-R-R\(^{1}\) structure where P represents proline or hydroxyproline, the actual break occurring between R and R\(^{1}\) (Banga, 1966). Testicular hyaluronidase by itself has no action on collagen (Banga, 1966). The use of this enzyme system will be discussed elsewhere.
The mammalian pancreas contains an enzyme, termed a collagen mucoproteinase, which is able to exert a collagenase effect provided the substrate is adequately disintegrated (Banga, 1966), although Evanson (1971) has stated that this mammalian collagenase is strongly inhibited by a potent serum factor. Other more general proteases, such as trypsin, appear to be essentially inactive against collagen. Banga (1966) has given evidence that only associated accessory substances are removed during tryptic digestion, whereas the collagen protein is not attacked.

b. The Cell Types of Mammalian Liver

Mammalian liver contains a large number of cell types in addition to the hepatocytes; these cell types are listed in Table 6.1.

It should not be imagined that these non-parenchymal cell types represent an insignificant figure in the total cell population of the liver. Gates et al. (1961) computed that approx. 16% of the cells in human liver were non-parenchymal while in rats this figure approached 40% (Daoust, 1958). A breakdown of these results is shown in Table 6.2.

This multitypic conglomeration of cells in mammalian liver leads to one of the most serious and continual problems in liver culture, namely, the appearance of either more than one cell-type or of a single undesired cell-type, leading in both cases to a suppression in the growth of the desired cell-type, the hepatocyte.
### Table 6.1. The Cell Types of Mammalian Liver

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell Classes</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenchyma</td>
<td>Hepatocytes</td>
<td>Intralobular</td>
</tr>
<tr>
<td>Reticulo-Endothelial</td>
<td>Littoral (Kupffer cells and indifferntiated lining cells)</td>
<td>Intralobular</td>
</tr>
<tr>
<td>Bile Duct</td>
<td>Bile Duct Epithelium</td>
<td>Interlobular</td>
</tr>
<tr>
<td>Connective Tissue</td>
<td>Fibroblasts</td>
<td>Interlobular</td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>Erythrocytes</td>
<td>Inter- and Intra-lobular</td>
</tr>
<tr>
<td></td>
<td>Leucocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasma Cells</td>
<td></td>
</tr>
<tr>
<td>Blood Vessel Walls</td>
<td>Fibroblasts</td>
<td>Inter- and Intra-lobular</td>
</tr>
<tr>
<td></td>
<td>Pavement Epithelium</td>
<td></td>
</tr>
</tbody>
</table>

### Table 6.2. Distribution of Cell Types in Mammalian Liver

(from Daoust, 1958; Gates et al, 1961)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Rats (%)</th>
<th>Man (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenchymal</td>
<td>60.6</td>
<td>84.2</td>
</tr>
<tr>
<td>Littoral</td>
<td>33.4</td>
<td>14.7</td>
</tr>
<tr>
<td>Bile Duct</td>
<td>2.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Connective Tissue</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Blood Vessel Walls</td>
<td>1.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>
The Hepatic Cell Cycle and its Regulation

The cell cycle of hepatocytes is identical to that of other cells and has four phases, the pre-DNA-synthesis stage ($G_1$), the DNA synthesis stage ($S$), post-DNA-synthesis stage ($G_2$) and a mitotic stage ($M$) (Lehmiller, 1971). This cycle is outlined in Fig. 6.3.

**Fig. 6.3.** The Hepatic Cell Cycle

![Cell Cycle Diagram](image)

**G$_2$**

$S$  $M$

**G$_1$**

**G$_0$**

Proliferation Kinetics in normal adult hepatocytes are unusual in that few cells continuously cycle through $S$ and $M$ phases although cells can be readily stimulated to rapidly cycle through these phases. To account for these characteristics, a $G_0$ phase has been postulated (Baserga, 1968). While in this phase the cells are considered to be outside the replicative cycle in a type of "siding" from which they can be recalled by appropriate stimuli.

The growth fraction (the proportion of hepatocytes that are involved in the replicative cycle) declines markedly as rats age.
From a level of about 36% on the first day after birth it declines to 4% - 6% in rats 6 - 8 weeks old (see Grisham, 1973); the corresponding proportion of mitoses is 3.8% and 0.6% respectively (Buetow, 1971). This discrepancy between the proportion of cells in the replicative cycle and the proportion actually undergoing mitoses can partially be explained by the gradual appearance of tetraploid and octaploid nuclei over this time (Wiest, 1973). Furthermore, the duration of the cell cycle appears to lengthen shortly after birth, reaching constant values at 3 weeks (see Grisham, 1973). This is illustrated in Table 6.3.

Table 6.3. Phases of the Hepatic Cell Cycle in Intact Livers of Maturing Rats (from Grisham, 1973).

<table>
<thead>
<tr>
<th>Age</th>
<th>Phase</th>
<th>Time (hours)</th>
<th>Total Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>S</td>
<td>G&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>1 Day</td>
<td>5.0</td>
<td>7.0</td>
<td>1.5</td>
</tr>
<tr>
<td>3 Weeks</td>
<td>9.0</td>
<td>9.0</td>
<td>1.8</td>
</tr>
<tr>
<td>8 Weeks and older</td>
<td>9.0</td>
<td>9.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Once formed, rat hepatocytes have a "life-span" or turnover time of 400 - 450 days, the mouse hepatocytes having a turnover time of 480 - 620 days whilst the littoral cells of mouse have a turnover time of 160 days; these figures should be compared to the turnover times for mouse duodenal epithelium and erythrocytes which are 3 and 1 - 2 days respectively (Cameron, 1971).
The regulation of hepatic cell proliferation is poorly understood although a number of hypotheses have been proposed. Bullough and Laurence (1967) have proposed the chalone concept to explain the regulation of hepatic cell proliferation, a chalone being a tissue-specific mitotic inhibitor produced by the cell type on which it acts. A liver-specific chalone has been isolated by Bullough and Laurence (1967). On the other hand, various investigators have suggested the existence of humoral regulator factors of hepatic cell proliferation. Glinos (1967) has postulated such a humoral regulator whose site of synthesis is the liver itself thereby enabling a feedback control system to be established. One of these factors appears to be associated with the plasma proteins although this is not the only factor involved (Glinos, 1967). In agreement with this proposal is the finding by Onda and Yoshikawa (1973) of an hepatocyte-specific mitotic inhibitor in normal rat plasma which may be present in the γ-globulin fraction. A very similar hypothesis has been put forward by Grisham (1973).

It is conceivable that all those above-mentioned points have a bearing on the successful cultivation of adult mammalian liver tissue. Thus, in explant culture of liver, in which the liver architecture is largely unaltered, the proliferation and outgrowth of hepatocytes may be very poor due to the maintenance of the normal inhibitor level within the explant despite the fact that the hepatocytes may remain functionally intact within the explant until their turnover time expires. In cell cultures, on the other hand, where the cells have firstly been separated, the mitotic inhibitor present whether it be intrahepatic or humoral is likely to be washed out leaving the cells ready for mitotic
growth by altering the hepatocyte state from the $G_0$ phase to the $G_1$ phase of the replicative cycle.

3. The Isolation of Mammalian Hepatocytes

The isolation of adult mammalian liver cells whether for immediate use or for culturing has been attempted by a large number of investigators (for review see Muller et al., 1972), although many of these studies have paid insufficient attention to the viability of the cell suspensions. The most commonly used viability test is exclusion of the dye trypan blue, which occurs only with viable cells. This is generally regarded as a sensitive index of membrane integrity, on which many hepatic functions are dependent. Each method of cell isolation falls into one of three overlapping categories, namely, mechanical, chemical and enzymic, although this classification is somewhat misleading in that every method contains an element of mechanical dissociation e.g., the use of a stirring or mixing apparatus.

The employment of a wholly mechanical system for the isolation of liver cells has occasionally been reported. Thus, Kattenbach (1952), Le Page (1953) and Ontko (1967) have described the use of a tissue press whilst Bucher et al. (1951) dissociated liver cells by shaking liver slices with glass beads. However, it has been much more common to use some form of in situ perfusion of the liver in conjunction with these mechanical techniques; the perfusing fluid commonly contains a metal chelator. An early example of this technique is that of Anderson (1953) who perfused a solution of
sodium citrate through the liver in situ prior to dissociation of the liver by means of a modified Potter-Evejhem homogeniser. This technique was later modified by Jacob and Bhargava (1962), who reported high cell yields, and by Rutter and Brosemer (1961) who used a sucrose/sodium citrate mixture for the perfusion. Suzungar and Dickson (1970) have recently examined this system in detail and assessed the various perfusion media.

Two interesting variations on this perfusion-mechanical dissociation procedure have been reported. Branister and Morton (1957) and Dajani and Orten (1959) have described the use of polyvinylpyrrolidone as the perfusing medium whilst McLimans (1969) has reported the use of a hypertonic, hyperosmolar salt solution as perfusing medium.

Whilst most of these perfusion-mechanical dissociation techniques produce excellent cell yields (Suzungar and Dickson, 1970), the harsh treatment of the homogenization step invariably leads to damaged cells and cell membranes. This damage is manifested in a number of ways: lack of aerobic glycolysis and of endogenous respiration, impairment of protein and RNA synthesis, leakage of enzymes, potassium ions and NAD, and, finally, staining with trypan blue (for references see Muller et al., 1972).

The one example of chemical dissociation of liver is the use of tetraphenylboron (TPB) as a potassium-chelator, originally suggested by Rapaport and Howze (1966). Casanello and Gerschenson (1970) have described this use of TPB and have shown that these dissociated cells are capable of growth in suspension culture. The viability of cells dissociated by this method has been questioned by a number of authors.
on the basis of trypan blue exclusion tests and leakage of enzymes (Gallai-Hatchard and Gray, 1971; Rutzky et al, 1971), lack of uptake of acetate into cellular lipid (Lipson et al, 1972), lack of endogenous oxygen consumption (Suzangar and Dickson, 1970) and low potassium content (Murthy and Petering, 1969).

In 1968 Howard and Pesch described the first successful enzymic digestion of adult mammalian liver into appreciable numbers of largely intact cells. This procedure of Howard and Pesch (1968) basically involved brief perfusion of the liver in situ with a mixture of collagenase and hyaluronidase in Ca⁴⁺-free solution, slicing the liver and incubating the slices with the Ca⁴⁺-free enzyme solution. Approx. 5 x 10⁶ cells per g. liver could be obtained with viability score (as judged by trypan blue exclusion of between 75 and 95%; the level of endogenous respiration was higher than that found in mechanically-isolated cells (Howard and Pesch, 1968). Since then the method has been continually modified, largely with the aim of increasing cell yield (Berry and Friend, 1969; Capuzzi et al, 1971; Ingebretsen and Wagle, 1972). All these modified techniques involve the use of a continuous perfusion of the liver in vitro in the presence of circulating collagenase/hyaluronidase. Various studies have shown that these enzymically-prepared adult hepatocytes are superior in morphology and functional activity to both mechanically- and chemically-prepared cells (Lipson et al, 1972; Gallai-Hatchard and Gray, 1971; Murthy and Petering, 1969; Muller et al, 1973) whether the cells are isolated from normal liver, regenerating liver or hepatoma (Muller et al, 1973). These enzymically-dissociated hepatocytes have been reported to be able to synthesize and secrete albumin.
(Weigand et al, 1971; East et al, 1973), the ratio of albumin synthesis to total protein synthesis being the same as in intact liver in vivo (Weigand et al, 1971). Furthermore, such cells can synthesize glycogen (Seglen, 1973), are responsive to glucagon and epinephrine in stimulating glycogenolysis (Wagle and Ingebretsen, 1973; Garrison and Haynes, 1973) and are susceptible to the inductive effects of glucocorticoid hormone on tryptophan oxygenase (Berg et al, 1972). More recent work (Seglen, 1972; Howard et al, 1973) has shown that the presence of Ca$^{++}$ in the dissociation medium stimulated the enzymic dissociation and also increased the potassium content of the dissociated cells (Howard et al, 1973); it should be noted that previously Ca$^{++}$ was excluded from the dissociation medium (Howard and Pesch, 1968). This Ca$^{++}$-stimulated enzymic dissociation is dependent either on a preliminary removal of tissue Ca$^{++}$ with a chelator (Seglen, 1972) or on the time of addition of the Ca$^{++}$ (Howard et al, 1973).

In spite of the obviously superior morphological and functional qualities of hepatocytes prepared by collagenase/hyaluronidase digestion very few authors have commented on the possibility of culturing these hepatocytes (see later).

4. Liver Culture: Techniques and Results

The culture of animal tissues can be achieved using a variety of techniques which fall into 3 categories viz., organ culture, explant culture and cell culture (Moscona et al, 1965). In organ culture the organ or a piece of the organ is maintained such that the cellular architecture of the organ is unaltered and that peripheral outgrowth
is discouraged. This is usually achieved by placing the specimen on a solid support such as a sponge, grid or raft and allowing sufficient medium to just come into contact with the specimen-support interface. Explant culture is very similar to organ culture in that small pieces of tissue are used but differs from it in that the peripheral outgrowth is encouraged and the outgrowing cells, not the explant, are examined. The third system for culturing animal tissue is cell culture in which cells isolated from the desired tissue are maintained as independent units either by attachment to a support or in suspension culture. The behaviour of animal cells and tissues in vitro depends very largely on the size of the units which are cultured and, according to the size selected, the conditions for their culture must be varied.

All three types of culture system have been applied to mammalian liver both foetal and adult, non-human and human in origin.

1) **Culture of Foetal Mammalian Liver**
   
   a. **Non-Human Species**

   Culture of dissociated foetal mammalian hepatocytes has been described by a number of workers (Schapira et al, 1971; Plas et al, 1973; Lambiotte et al, 1973; Leffert and Paul, 1972; Gielen and Nebert, 1971). It appears to be much easier than culture of adult hepatocytes, which is predicted on theoretical grounds. The most widely-used dissociating agent is trypsin (Schapira et al, 1971; Plas et al, 1973; Lambiotte et al, 1973) although collagenase (Leffert and Paul, 1972) and viokase, an enzyme mixture (Gielen and Nebert, 1971) have also been used.
The number of isolated cells actually attaching to the culture surface never exceeds 10% that of the initial seeding level (Leffert and Paul, 1972; Plas et al., 1973) even when collagen, a substratum known to be beneficial to cultured cells (Elsdale and Bard, 1972), is used in place of plastic (Plas et al., 1973). Two types of cell, fibroblastic and epithelial, were distinguishable in these cultures (Plas et al., 1973) although after 4 days in culture the extent of fibroblast involvement never exceeded 15% (Plas et al., 1973).

Lambiotte et al. (1973) were able to selectively remove the fibroblasts (2-3 days after plating) using a collagenase subculturing stage, during which the hepatocytes were almost completely selectively replated. Leffert and Paul (1972) suppressed growth of non-parenchymal cells by use of a medium deficient in arginine although it should be noted that the hepatocytes in culture were either non- or at best slow-growing. This non-growth of cultured hepatocytes in arginine-deficient medium may be related to the lack of extracellular arginine; this deficiency of arginine has been shown to lead to a decrease in the level of ornithine-δaminotransferase, an important enzyme in the urea cycle, over a 24-hour incubation period (Strecker and Hammer, 1971). It is possible that such a build-up of toxic nitrogenous waste produced as occurs in blockage of the urea cycle could inhibit cellular growth.

These cultured hepatocytes have been shown to retain liver-specific urea-cycle functions together with synthesis and secretion of albumin (Leffert and Paul, 1972) as well as benzpyrene hydroxylase activity, both endogenous and inducible
(Gielen and Nebert, 1971). However, the continual presence of cortisol in the medium was required for cultured foetal hepatocytes to exhibit glycogen storage function (Plas et al., 1973) and formation of bile canaliculi (Lambiotte et al., 1973). Furthermore, Schapira et al. (1971) have shown that these cells lose a liver-specific aldolase on culturing. It is very possible that these differences in the ability of foetal hepatocytes in culture to retain specific functional activity may be a reflection in some way of the use of the different dissociating enzymes for their isolation (see above).

The only reports regarding explant culture of mammalian foetal liver from non-human species appear to be those of Sandstrom (1964, 1965, 1966). Four cell types could be distinguished in the explant outgrowth, only one type of which resembled true hepatocyte morphology (Sandstrom, 1965). Moreover, the predominance of any cell type in the culture depended largely on the means of culture. The only system of culture in which hepatocytes predominated was one wherein the liver explant was held firmly compressed between 2 sheets of cellophane just moist enough to adhere to each other by capillary force (Sandstrom, 1964, 1965).

Wicks (1968) has described a system, involving a metal grid as a support, for the organ culture of foetal rat liver. Tourian (1973) has shown that such a system maintains the tissue in a differentiated state over a 48-hour period, at least with regard to its high baseline tyrosine aminotransferase activity and the hydrocortisone inducibility of this activity. This
culture system has been used to study the phenylalanine hydroxylase activity (Tourian, 1973) and the inducibility of tyrosine aminotransferase (Wicks, 1968) and aryl hydrocarbon hydroxylase (Burki et al., 1971) in foetal liver. A system of organ culture, similar to that of Wicks (1968) has been described by Klein and Weller (1970). Using this system Eisen et al. (1973) have demonstrated the role of insulin in regulating hepatic glycogen synthesis. A slightly modified culture system, employing a lens paper and metal grid support, has been used by Raiha et al. (1971) to confirm the finding of Wicks (1968) regarding the inducibility of tyrosine aminotransferase.

b. Man

The first report of culture of human foetal liver was that of Hillis and Bang (1959) using an explant culture system in collagen-coated roller tubes; collagen was considered essential for growth. Cell outgrowth was good but by the tenth day of culture cells of mesenchymal origin began to overrun the hepatocytes. Further work (Hillis and Bang, 1962) showed that more selective growth of hepatocytes could be obtained by infrequent medium changes, and that the presence of arginine provided a more abundant healthier spread of hepatocyte bands and cords. Feldenstein (1963) maintained human foetal hepatic cells in culture by allowing cell suspensions (prepared by trypsinization) to settle by gravity onto collagen-coated polythene discs. Zuckerman et al. (1967) using a system involving
pressure implantation of trypsin-softened human foetal liver onto polythene discs, reported fibroblast interference in cultures after approx. 10 days culture. Mechanical disaggregation and the use of pronase as dissociating agent proved unsuitable for the initial preparation of cell clumps. Lie (1972) in a recent paper has demonstrated the effect of portal serum in enhancing hepatocyte outgrowth from human foetal liver explants. This effect was specific to portal serum, fibroblastic outgrowths being repeatedly observed when using peripheral serum.

A recent report regarding the culture of trypsin-dissociated human foetal liver is that of Bissell and Tilles (1971). The presence of 10% calf serum or 1% human serum in the growth medium accelerated the proliferation of the liver cells with subsequent rapid loss of characteristic morphology and specific functional activity. In the absence of serum the cultured liver cells retained their morphology and their function for at least 4 weeks as evidenced by secretion of serum albumin and storage of glycogen and iron although the division rate was very low.

ii) Culture of Adult Mammalian Liver

For the sake of simplicity the term 'adult' in this context refers to any period after birth. This distinction is purely arbitrary and, as discussed earlier, the liver does not attain its adult morphology and functions for some time after birth (3 weeks in the case of the rat).
a. Non-Human Species

Literature reports regarding culture of adult mammalian liver place much less emphasis on the role of organ culture techniques and much more emphasis on the cell culture techniques. The only reports concerning organ culture of adult liver appear to be those of Laufs and Walker (1970) and Campbell and Hales (1971), the former employing marmoset liver and the latter rat liver. These reports place great stress on the gas atmosphere required for optimal survival of the tissues, both agreeing on the desirability of an oxygen-enriched atmosphere. Campbell and Hales (1971) noted that during the first 6 days of culturing the tissue pieces showed a gradual loss in glycogen content, the extent of decrease being dependent on the composition of the gas overlay.

The first report regarding explant culture of adult liver was that of Hillis and Bang (1959). Using a culture system identical to that used for foetal human liver, they were able to observe that adult monkey liver followed the same pattern of growth to that of foetal human liver except that the outgrowth was more irregular and began somewhat later and at a much slower rate. Watanabe (1966), using a system very similar to that of Hillis and Bang (1959), reported that outgrowth from explants of newborn (24 hours old) rat liver was essentially identical to that described by Hillis and Bang (1959). More recently, Alexander and Grisham (1970) using a system very similar to that of Sandstrom (1965) but incorporating a collagen substratum, have reported on the light and electron microscopic appearance of cells
which grew out from explants derived from livers of rats 1–5 days old. Several types of cells were observed, the most prominent being hepatocytes. On the other hand, Rutzky et al (1971) obtained a fibroblastic cell-line from explants of trypsin-treated neonatal rat liver. Rabee et al (1972) using a simple culture system (no collagen, large fluid volume) could not demonstrate any outgrowth from rat liver explants (rats older than three weeks) in culture; only when the rats had been pretreated with diethylnitrosamine for 107 days did they observe cell proliferation and then only from explants derived from the induced 'preneoplastic' enzyme-deficient nodules. Douglas et al (1966) attempted to culture explants derived from the livers of nine chimpanzees. The outgrowth was exclusively fibroblastic and only from the liver of one animal could a cell-line be established which eventually transformed itself to an epitheloid line.

Because of the difficulties inherent in using explant and organ cultures of adult liver a number of investigators have concentrated on the use of cell cultures for such tissue. Unfortunately, after many years of research there still is no widely accepted system for the culture of adult mammalian hepatocytes. Consequently, hepatocytes prepared by all three isolation methods (mechanical, chemical and enzymic) have been claimed to be able to survive in culture although the morphological and functional state of some of these cultures must remain in question.

McLimans (1969) has reported that mouse hepatocytes isolated
by perfusion/mechanical dissociation can grow in culture provided that a collagen substratum and a thin film of medium overlay is used. However, work by Gallai-Hatchard and Jones (1971) has shown that hepatocytes prepared by this means are not viable on the basis of dye-uptake tests. Dickson (1971) has also described the culture of mechanically-disaggregated hepatocytes; during the 10-day culture period there was no storage of glycogen and the histological appearance of the cultured cells showed marked irregularities especially of the nuclei. These results are not unexpected in light of previous work, mentioned earlier, which conclusively showed that the hepatocytes isolated by mechanical disaggregation were damaged even before the culture proper was initiated.

Hepatocytes dissociated by treatment with TPB have also been maintained in culture (Casanello and Gerschenson, 1970) although, once again, this method of dissociation is not recognized as one which yields viable cells; it is probable that cultures of this type are derived from clonal growth. Moreover, evidence obtained from chromosomal analysis and carcinogenic potential (Oshiro et al., 1972) and from enzymic analyses (Walker et al., 1972) strongly indicates that the cells of this established line do not resemble normal functional hepatocytes.

The establishment of cultures from trypsin-dissociated liver has been reported (Umeda and Saito, 1969; Armato et al., 1972a, b; Williams et al., 1971) although the rats used were either newborn (Umeda and Saito, 1969; Armato et al., 1972a) or 10-day-old (Williams et al., 1971). Up to seven cell types have been
described in such cultures, the predominant types being morphologically hepatocyte-like and fibroblastic (Armato et al., 1972b). Williams et al. (1973) later demonstrated that treatment of these cultures with carcinogens yielded cells which on injection into rats produced carcinomas not found after injecting untreated cells.

Borek (1972) has described the establishment of a clonal line of epithelial-like cells obtained from rat liver by means of a trypsin/collagenase dissociating mixture. Conditions of nutritional stress led to neoplastic transformation into differentiated hepatoma-like cells. Iype (1971) using the method of Howard and Posch (1968) for the isolation of hepatocytes has reported the establishment of cell lines which appear normal with regard to karyology, morphology and growth pattern; unfortunately no tests for liver-specific function were tested for. Iype (1971) also reported that an oxygen-rich atmosphere was harmful to the growth of these cells. In a subsequent paper, Iype et al. (1972) demonstrated organ-specific antigens on the surface of cells freshly isolated from normal rat liver and from the liver cell lines.

b. Man

Attempts to obtain cell cultures of adult human liver have so far not been too successful. Chang (1954) described a cell line consisting of epithelial cells derived from normal human liver. These cells developed the characteristics of undifferentiated
neoplastic tissue and it was not suggested that these cells represented differentiated hepatic cells. A cell morphology similar to that of the Chang cells was observed in a cell-line developed by Syvertson and McLaren (1957) from a liver biopsy of an infant. More recently, Sullman et al (1960) published a brief report on the applicability of the method of Zuckerman et al (1967) to the culture of adult human liver biopsies; no follow-up work has been reported. Two cell-types could be distinguished in these cultures, namely hepatocytes and fibroblast-like cells.

Explant culture of adult human liver has met with some success. Thus, Sandstrom (1973) using a method previously described (Sandstrom, 1964) for rat liver was able to show outgrowth of hepatocytes from explants of human liver biopsies, there being histochemical evidence for the presence of bile canaliculi. A thorough investigation into explant culture of adult human liver has been carried out by Bourel and co-workers (Bourel et al, 1968; Lenoir et al, 1968; Le Guilly et al, 1969, 1971, 1973; Guillouzo et al, 1972). After a lag of 4-5 days cell outgrowth commenced, and fibroblasts and macrophages became apparent. In the days following, cells resembling hepatocytes became noticeable (Bourel et al, 1968). Incorporation of a proton-acceptor, e.g. dipyridamole, into the culture medium greatly facilitated this growth (Lenoir et al, 1968). Later work (Le Guilly et al, 1971; Guillouzo et al, 1972) showed that five cell types could be observed and that the type of serum protein produced by any culture was determined by the predominating
cell type. By day 20 of culture all cells had simpler ultrastructures (Guillouzo et al, 1972); dedifferentiation was claimed to account for this phenomenon. On the other hand, Damoise et al (1971) proposed on electron microscopy analysis that the epithelial cells which outgrew from explants of adult human liver biopsy material were mesenchymal rather than parenchymal in origin.
CHAPTER 7. CONTENTS

Materials

1. Animals
2. Materials, Media and Chemicals

Methods

1. Explant Culture
2. Cell Culture

A. Cell Dissociation Procedures
   1) Method of Jacob and Bhargava
   2) Method of McLimans
   3) Use of Tetraphenylboron
   4) Use of Trypsin, Pangestin and Other General Proteases
   5) Use of Collagenase/Hyaluronidase

B. Cell Count and Viability Assessment - Trypan Blue Exclusion Test

C. Subculturing Procedures

3. Staining Methods
   1) Haematoxylin and Eosin
   2) PAS Stain for Glycogen
   3) Oil Red O Stain for Lipid

4. Scanning Electron Microscopy Studies
5. Tests of Functional Differentiation of Cultured Hepatocytes

i) Storage of Glycogen

ii) Urea Production

iii) Albumin Production

iv) Histochemical Demonstration of Glucose-6-Phosphatase Activity

v) Effect of Insulin and Glucagon on Glucose Metabolism

vi) Metabolism of $^{14}$C-Impiramine

6. Determination of Mitotic Index
CHAPTER 7. MATERIALS AND METHODS

Materials

1. Animals

All animals used in this study were male Wistar/Albino rats, weight range 60 - 150 g, which were bred and maintained in the University Animal House. The animals were allowed free access to food and water, the feed being Spiller's No. 1 Laboratory Diet.

2. Materials, Media and Chemicals

Culture vessels used were 'Falcon' treated-polystyrene flasks of area 25 sq.cm, and in some experiments 'Falcon' microtitre plates were employed; both were supplied by Scientific Supplies, London. The Bolting nylon cloth was obtained from P.A. Simon Ltd., Cheadle Heath, Stockport.

The suppliers of the various media, media supplements and chemicals are listed in the Appendix. Sheep portal serum was a generous gift from Pfizer Ltd., Sandwich.

All solutions prepared in the laboratory were sterilized by either autoclaving at 15 lb pressure for 15 minutes in the case of salt solutions, or by filtration through a Millipore filter pore size 0.22μ. All media contained gentamycin (70μg/ml) whilst those media based on MEM also contained non-essential amino acids.

Methods

1. Explant Culture

Rats were killed by cervical dislocation and the abdomens
swabbed with 70% (v/v) aq. ethanol prior to removal of the livers, which were subsequently placed in PBS 'A' containing gentamycin at a level of 70 μg/ml (PBS 'A'/gentamycin). The livers were then roughly scissor-minced and the mince washed 3 times in PBS 'A'/gentamycin. Pieces of liver were transferred to a Petri dish containing a small volume of PBS 'A'/gentamycin and finely minced, using a scalpel, to cubes of approx. 1 mm³ dimensions. 8-12 of these finely-minced pieces in a minimum of fluid were transferred to the culture flask and allowed to adhere to the culture surface by partial drying at 37°C for one hour. After this time 5 ml. of the appropriate medium was added to the flask which was then incubated at 37°C. The medium was changed 24 hr. after the initiation of the culture and thereafter at 2 day intervals. From the scissor-mince stage onwards all operations were carried out under sterile conditions.

2. Cell Culture

A. Cell Dissociation Procedures

The rats were killed and the livers removed as described above. In some methods the liver was perfused prior to removal from the body, the perfusion fluid being introduced via the inferior vena cava. Each liver was finely minced and then subjected to one of a variety of cell dissociation procedures described below.

i) Method of Jacob and Bhargava (1962)

The liver was perfused with 50 ml. of 0.027M sodium citrate in Ca³⁺-free Locke's B.S.S. until the blanched liver was fully
distended. The liver was removed and finely minced, and this mince was transferred to a glass boiling-tube. 10–12 complete strokes of a loose-fitting rubber bung on a handle served to completely dissociate the cells. The resulting cell suspension was filtered through one layer of Bolting cloth to remove strands of connective tissue and clumps of cells. The filtered suspension was centrifuged at 100 g av for 2 min. to remove blood cells and washed twice in PBS 1gentamycin. The cells were finally resuspended in culture medium and, after counting and viability assessment (see later), were diluted to $5 \times 10^5$ cells/ml. Two ml aliquots of this cell suspension were then placed into culture flasks and examined microscopically at various intervals to check for growth.

ii) Method of McLimans (1969)

In this method the liver was perfused with 50 ml. of an alkaline, hyper-osmolar salt solution as described by McLimans (1969). The liver was removed and minced, the mince being placed in this salt solution for 30 minutes at 37°C. This mince solution was then subjected to 2 complete strokes in the homogenizer described above. The filtration, washing and initiation of culture were then carried out as outlined above.

iii) Use of Tetraphenylboron (TPB)

The minced liver was subjected to four 30-minute cycles of dissociation in 3mM TPB in isotonic saline (10 ml), the temperature being maintained at 37°C. After each cycle the supernatants were pooled. The centrifugation, washing and initiation of culture was carried out as described above.
iv) **Use of Trypsin, Pangestin and Other General Proteases**

The trypsin, pangestin and protease (from *Strep. griseus*; pronase) were dissolved in PBS 'A' at various concentrations (0.05% - 0.50% (w/v)). The minced livers were subjected to enzymic digestion at varying temperatures (room temp. and 37°C), varying concentrations of enzyme and various times of digestion (30 - 120 min.), including overnight at 4°C followed by 30 minutes at 37°C. A magnetic stirring bar was used in all experiments to aid mixing and dissociation.

v) **Use of Collagenase/Hyaluronidase**

The minced liver was subjected to enzymic digestion in collagenase/hyaluronidase (0.05%/0.10% (w/v)). A variety of conditions for the digestion procedure were investigated and these included the effect of temperature, overnight digestion in the cold and the effect of initial Ca²⁺-removal and subsequent replacement in the enzyme solution.

After much investigation (see Chap. 8) the following procedure was adopted. Following removal from the body the liver was cut into slices approx. 0.5 - 1 cm. in thickness by means of a sharp blade and these slices transferred to a 250 ml. conical flask. The contents of the flask were washed twice in PBS 'A'/gentamycin at 37°C. in a shaking water bath for 10 minutes followed by two 10 minute cycles at 37°C in 0.5mM EGTA in PBS 'A'. After this, the liver pieces were digested in 10 ml. of enzyme solution. The incubation time was 60 minutes at 37°C.
in a shaking water bath (approx. 90 oscillations/min.); the
enzyme solution consisted of collagenase/hyaluronidase
(0.05%/0.10% (w/v)) in Hank’s B.S.5, containing 5mM Ca++. 
At intervals during the incubation the pH of the medium was
checked and if necessary adjusted to approx. 7.5.

The filtration, washing and initiation of culture were
carried out as described in i) above.

B. Cell Count and Viability Assessment — Trypan Blue
Exclusion Test

An aliquot of the cells suspended in culture medium was mixed
with trypan blue to give a final concentration of 0.14% trypan blue.
This stained suspension was placed in an improved Neubauer counting
chamber (Hawksley, England) and examined under the microscope.
Cells considered to be non-viable were those which took up the
stain into their cytoplasm and nucleus (Howard et al, 1973). Thus,
a total cell count and a viable cell count could be performed and
from these a value for the %-viability could be calculated.

C. Subculturing Procedures

In a few experiments subculturing was attempted and this was
carried out in one of two ways:-

i) After washing the cell sheet in PBS 'A', 0.25%/w/v) trypsin in
PBS 'A' was added and left in contact with the cells for 30 seconds
at room temperature. The bulk of the enzyme solution was decanted
and the flask placed at 37°C. The cell layer was examined under a
microscope at various intervals until virtually all the cells had
become detached. Fresh medium was then added and, after dispersing the cells, the suspension was inoculated into fresh flasks.

ii) After washing, the cells were subjected to a 5 minute treatment with collagenase/hyaluronidase (0.05%/0.10% (w/v)) in PBS 'A' at 37°C. After this time the cell-containing enzyme solution was removed and centrifuged at 100 g av. for 2 minutes. The supernatant was discarded and the cell pellet suspended in fresh medium which was then placed in fresh flasks.

3. Staining Methods

All the staining was performed with the cells fixed in situ on the culture surface. Photomicrography was carried out using an Olympus PMG camera and EM VI exposure meter, the camera being mounted on an Olympus inverted microscope.

1) Haematoxylin - Eosin Stain

The cells were fixed in acetic alcohol:formalin (5:85:10) for 5 minutes following a preliminary rinse in PBS 'A'. After fixation, the monolayer was rinsed twice with tap-water and stained with Harris' haematoxylin for 3 minutes. The stain was decanted and replaced by 0.5% (w/v) aq. lithium carbonate for 5 minutes. After removal of this solution the cells were stained with 0.5% (w/v) alcoholic eosin for 1 minute, after which time the stain was discarded and the cell-sheet rinsed twice with absolute alcohol. In order to make the preparations semi-permanent each flask received a small volume of iso-amyl alcohol (approx. 0.3 ml) which covered the monolayer.
ii) **Periodic Acid - Schiff (PAS) Stain for Glycogen**

Following fixation in methanol (10 minutes) the cells were subjected to a 10 minute treatment with 1% (v/v) periodic acid. After this time the periodic acid was removed and the cell sheet was thoroughly rinsed in 70% (v/v) ethanol. The cells were then stained for 20 minutes with the Schiff stain as described by Horobin and Kevill-Davies (1971). After this staining the cells were thoroughly rinsed in absolute alcohol prior to microscopic examination.

Intracellular PAS positive material was taken as presumptive evidence for the presence of glycogen.

iii) **Oil Red O Stain for Lipid**

The cells were fixed in buffered neutral formalin and stained with oil red O for 30 minutes, the flask being kept closed during this time. The stain was prepared by diluting 6 ml of a stock solution of oil red O, saturated solution in iso-propyl alcohol, with 4 ml distilled water, leaving for 10 minutes and filtering; the filtrate was the staining solution. After staining, the monolayer was rinsed with 60% (v/v) aq. ethanol and examined microscopically.

4. **Scanning Electron Microscopy Studies**

For these studies the cells were cultured on small (12 mm, diameter), sterile cover-slips maintained in sterile plastic Petri dishes. At
various intervals some of these cover-slips were processed for
scanning electron microscopy.

Following rinsing of the cover-slips, the cells were fixed
in situ in cold glutaraldehyde (1% in 0.1M phosphate buffer pH 7.4)
for 1 hour. The cover-slips were washed twice in buffer and dehydrated
sequentially in 25, 50, 75 and 100% acetone, 30 minutes each. After
air-drying, the cover-slips were vacuum-coated with palladium and
then examined in a Cambridge Mark II Stereoscan scanning electron
microscope.

5. Tests of Functional Differentiation in Cultured
Hepatocytes

A variety of tests were performed on hepatocytes isolated by
collagenase/hyaluronidase digestion and maintained in culture for
3 days in order to assess their state of functional differentiation.
These tests included the following.

1) Storage of Glycogen

This was qualitatively assessed by the presence of
intracellular PAS positive material, as determined by the
PAS stain (see above).

ii) Urea Production

3 days after initiating the culture the cells received
fresh medium which 24 hr. later was collected and assayed for
urea by the diacetylmonoxime method as described by Ellis (1971).
Medium not in contact with cells was used as a control.
iii) **Albumin Production**

Following 3 days in culture the monolayer was washed three times with PBS 'A' and received fresh medium which was free of serum so as to prevent cross-reaction with the albumin antiserum. Twenty-four hours later the medium was collected and assayed for the presence of albumin by Ouchterlony gel diffusion using goat anti-rat albumin antiserum.

Visualization of the line of precipitation was carried out by staining with amidoschwarz 10B as recommended by Varley (1969).

iv) **Histochemical Demonstration of Glucose-6-Phosphatase Activity**

Glucose-6-phosphatase activity was assessed in unfixed cells using the method of Wachstein and Meisel (see Culling, 1963). The cells were post-fixed in 10% formalin. Owing to the hypotonicity of the incubation medium the cells assumed a rounded appearance and consequently the intracellular localization of the enzyme could not be ascertained.

v) **Effect of Insulin and Glucagon on Glucose Metabolism**

24 hr. after establishing the culture, the cells received fresh medium (MEM) containing $0.25 \mu$Ci $^{14}$C-glucose per ml; 2 ml. of this radioactive medium was used per flask. In some flasks the radioactive medium also contained insulin at a final concentration of 200 or 400 $\mu$units/ml. Twenty-four hours later the medium was collected and counted for radioactivity.
These flasks then received non-radioactive medium containing 200 ng glucagon per ml. and twenty-four hours later this medium was also collected and counted.

For counting, 20 μl of medium was pipetted into scintillation vials and 10 ml scintillator added. The scintillator used was toluene-based and contained 0.4% PPO and 0.01% POPOP.

vi) Metabolism of $^{14C}$-imipramine

Twenty-four hours after commencing the culture the cells received 2 ml of fresh medium containing $0.2 μCi$ $^{14C}$-imipramine at an imipramine concentration of $0.1 m$M. The cells were cultured for a further 1–2 days after which time the medium was assayed for imipramine and its metabolites using the extraction technique and chromatography system of Bickel and Weder (1969). Following the chromatography the silica gel was scraped off in bands 0.5 cm width and placed in scintillation vials containing 1 ml methanol. The vials were shaken to elute any material off the silica and 10 ml scintillator added. Scintillator used was that outlined above.

Radioactivity counting was performed using a Packard Tricarb. Counting efficiency was assessed by internal standardization.

6. Determination of Mitotic Index

Mitotic index was calculated by incubating the cells in the presence of colcemid, $0.05 μg/ml$, for 5 hours. After this time the cells were stained with haematoxylin and eosin, and the percentage of cells in mitosis was ascertained.
CHAPTER 8. CONTENTS

1. Explant Culture of Mammalian Liver 190
2. Isolation of Adult Rat Hepatocytes 192
3. Culture of Freshly Isolated Rat Hepatocytes 195
4. The Functional State of Cultured Hepatocytes 199
CHAPTER 8. RESULTS

Two tissue culture systems were chosen with which to investigate the feasibility of culturing adult mammalian liver. These systems were explant culture and cell culture, the latter technique also involving an assessment of the methods available for the isolation of hepatic cells. The available literature (see Chapter 6) indicated that these two systems appeared to be the most applicable for the study of culture of adult mammalian liver.

1. Explant Culture of Mammalian Liver

The technique of explant culture is outlined in Chapter 7. The standard medium used throughout these studies was based on MEM with Hank's B.S.S., antibiotics and HEPES as buffer; serum was added at a final concentration of 20%. For each experiment 40 - 80 explants were cultured, 8 - 12 explants per flask. When using foetal bovine or calf serum cell outgrowth was usually observed from approx. 75% of the explants although the extent of outgrowth varied quite considerably from explant to explant.

Outgrowth of cells was invariably apparent 3 days after commencing the culture and took the form of isolated cells which were stellate in shape (Plate 8.1). As the growth progressed these cells began to assume a typically fibroblastic form with numerous bundles of cells (Plate 8.2). The cells were consistently mononucleate, the cytoplasm was lightly staining and numerous cytoplasmic projections could be observed (Plate 8.2). Throughout this period these cells were consistently negative for the PAS stain. The outgrowth appeared to have a limiting size above which no further outgrowth occurred although "stacked" layers of fibroblasts could be seen (Plate 8.3).
This stage was followed by one during which the fibroblast-like cells nearest the explant contracted together to produce an arc effect of spindles around the explant (Plate 8.3). In many instances sheets of fibroblastic cells could be observed round explants and mitotic figures were frequently observed (Plate 8.3). If at this stage the explant was removed and resettled on another area of growth surface this sequence of outgrowth could be repeated, even if this transplanting was carried out in the same flask in which the original culture was initiated. In some instances a few bizarre cells could be seen after the fibroblast outgrowth was complete. These took the form of giant multinucleate cells containing a large number of vacuoles which stained positively for lipid. At no time was any cell-type observed which could be identified as epithelial or epithelial-like.

The nature of the substratum had a dramatic effect on this cell outgrowth. Treated polystyrene (Falcon' flasks) was the best substratum studied and the foregoing discussion of the cell types was based on cultures growing on these flasks. On the other hand, cell outgrowth was extremely poor on glass and the deposition of a gelatin layer (Moscona et al, 1965) on this glass did not improve this situation.

A number of media other than MEM (i.e. L-15, medium 199 and McCoy's 5A) were tested for their ability to induce outgrowth of epithelial cell types, but of these only L-15 was as successful. Furthermore, a number of different sera were also tested (foetal bovine, calf and horse serum and sheep portal serum) but no major differences in the pattern of cell outgrowth could be detected other than for a greater initial cell outgrowth when using the portal serum. Other
biological media such as chick embryo extract, tryptose phosphate broth and liver digest ultrafiltrate used at concentrations ranging from 10⁻⁶ to 30% produced no beneficial effect on the pattern of cell outgrowth, fibroblasts remaining the only cell type observed. Putrescine used at 0.3 - 2.0 x 10⁻⁶M increased the outgrowth of fibroblastic cells, in agreement with the work of Pohjanpelto and Raina (1972) using skin fibroblasts, but did not induce the appearance of any epithelial-like cells.

Subculture of the cell outgrowth with trypsin yielded cell-lines which were characteristically fibroblastic in appearance.

2. Isolation of Adult Rat Hepatocytes

A number of systems which have been reported for the isolation of rat hepatocytes were investigated with regard to the yield and viability of the isolated cells. These techniques were based on perfusion (Jacob and Bhargava, 1962; McLimans, 1969), dispersion by chemicals (i.e. TPB) and dispersion by enzymes (trypsin, pangeatin, pronase, subtilisin and collagenase/hyaluronidase). The results of this study are shown in Table 8.1.

Perfusion of the liver in situ followed by dispersion of the cells in a loose-fitting homogenizer resulted in the release of a large number of hepatocytes, particularly if the perfusion medium contained a Ca⁺⁺-chelator (Jacob and Bhargava method) or was hyperosmolar and hypertoncic (McLimans method). The cell suspension produced by the latter method was significantly 'cleaner' than that produced by the Jacob and Bhargava method there being much less contamination by nuclei and other cell debris and red blood cells.
However, the viability of these isolated cells, as measured by dye uptake, was extremely poor (see Table 8.1) virtually all of the cells taking up the dye. In an attempt to improve the viability of cells prepared by these methods dispersion of the liver was carried out either in glycerol-containing solution (25 - 100% v/v glycerol) or in 2M sucrose, both being membrane-protective agents. None of these modifications, however, proved effective in increasing the cell viability.

Incubation of liver pieces with the K⁺-chelator tetraphenylboron also produced large numbers of cells the vast majority of which were again non-viable (Table 8.1). These cell preparations were contaminated with large numbers of isolated nuclei implying that the dissociation step had progressed beyond the cell isolation stage and the TPB had started to destroy the newly-isolated cells.

A number of nonspecific proteases were also tested as possible dissociating agents for rat hepatocytes. These proteases included trypsin, pangestin (a pancreatic extract) and pronase, and a number of different incubation conditions were tried:— varying the level of enzyme (0.05% - 0.25% w/v), varying the dissociation temperature (room temp. 37°C and overnight at 4°C) and sequential treatment with two enzymes (trypsin and pangestin). Treatment with these enzymes proved singularly unsuccessful, very few cells being released, the cells that were being virtually 100% non-viable (Table 8.1).

The only system to yield reasonable numbers of viable hepatocytes was that of dissociation by collagenase/hyaluronidase as adapted from the technique of Howard and Pesch (1968) but omitting the initial perfusion stage (Table 8.1). By this technique approx. 1 x 10⁶ viable cells could be obtained from 1 g. liver. It was noticed that this
yield was decreased somewhat when larger rats were used and was increased if the dissociation was carried out at 37°C rather than at 25°C. Prolonged exposure of the liver tissue to enzymes at 25°C in an attempt to improve the viable cell yield should be avoided as this would lead to excessive destruction of the fragile newly-isolated cells. It was also apparent that slicing of the liver was superior to chopping into cubes with regard to the final yield and viability of cells. These dissociated cells were very susceptible to mechanically-induced damage, the cell yield being lower if a magnetic stirrer was used in place of a shaking water bath to maintain the liver slices in an agitated state. Once again, neither treatment with collagenase/hyaluronidase overnight at 4°C, nor the use of a secondary sequential enzyme dissociation step (e.g. with trypsin or pangeostin) raised the viability score or the viable cell yield.

By incorporating a preliminary Ca++-removal stage with EGTA and by adding Ca++ to the enzyme dissociating mixture, as outlined in Chapter 7, into the collagenase/hyaluronidase dissociation scheme it was possible to increase both the viability score and total cell yield (Table 8.1: Modified Collagenase/Hyaluronidase method). The average total cell yield was 5.5 x 10^6 hepatocytes per g. liver of which 78% were viable. This represents a more than 2-fold enhancement of the total cell yield coupled with a higher viability score, when compared to the method of Howard and Pesch (1968). The cells isolated by this modified technique were largely resistant to uptake of the dye trypan blue and possessed highly retractile cell membranes (Plate 8.4). Red blood cells were also present in these preparations together with another cell-type, smaller in size than the hepatocytes, which was presumed to be of fibroblastic origin.
5. Culture of Freshly Isolated Rat Hepatocytes

The only cell suspensions to show consistent reproducible growth in vitro were those which contained a high percentage of viable cells. Thus, although isolated areas of living cells could be seen using various isolation methods, the culture of cells obtained by methods other than collagenase dissociation was on the whole unsuccessful. Indeed, it appeared that once attachment of the viable cells to the substratum was complete (approx. 4 hours after initiating the culture) the continued presence of non-viable cells markedly decreased the number of living cells present 20 hours later (Table 8.2), implying a direct toxic effect of the dead cells or their contents on the living cells.

The pattern of in vitro growth was the same whether the cells were obtained by the original Howard and Pesch (1968) method, omitting the initial perfusion stage, or its present modification employing a preliminary Ca\(^{++}\)-removal and its subsequent replacement, but as the latter approach gave higher yields of viable cells this was used in all subsequent studies.

The plating efficiency, i.e. the percentage of inoculated viable cells that remained viable and attached to the surface 24 hours after inoculation, varied considerably between preparations but lay in the range 10 - 20%. Similar values were obtained if the cells were cultured in microtitre plates. Once again, the nature of the substratum markedly influenced the appearance of the culture. Thus, cell attachment and growth was almost non-existent on ordinary glass culture bottles (i.e. 2 oz. medical flat bottles) although growth on glass coverslips was possible albeit on a limited scale
compared with the treated-polystyrene 'Falcon' flasks.

Three distinct cell-types could be seen 24 hours after initiating the culture (Plate 8.5), together with large numbers of dead cells which lay over the epithelial colonies (Plate 8.6). The most abundant type (60%) was an epitheliocyte, usually highly granular, present singly or, more commonly, in groups ranging from 4 - 20 cells in number (Plates 8.7 and 8.8). The epithelial cells in the groups showed a marked polygonality strongly reminiscent of true hepatocytes. The cell nuclei were round, large and very apparent and contained one or two nucleoli centrally placed; chromatin dots were also apparent. A number of these epitheliocytes (approx. 20 - 30%) were binucleate and use of a metaphase-arresting agent (colcemid, 0.05 μg/ml) revealed that the epithelial cells had a mitotic index of approx. 2 - 3%. Distinct intercellular gaps could often be seen in the cell clusters. The cell cytoplasm was highly granular and occasionally showed a small number of vacuoles which stained positively for lipid. The number of cells containing lipid droplets was greater in both medium 199 and MEM when compared to L-15 which strongly indicated that lipid build-up was related to the composition of the medium. The cytoplasm stained positively for glycogen although the intensity of staining varied from cell to cell. On the basis of their microscopical appearance and their functional abilities (to be discussed later) these epitheliocytes were designated as hepatocytes in culture.

The second most common cell-type apparent after 1 day in culture was a rather bizarre 'foamy' cell (Plate 8.9). These cells were always found singly, had a lightly-staining nucleus and cytoplasm, and the cytoplasm contained large vacuoles which did not stain for lipid.
The one remaining cell-type present was fibroblastic in origin (Plate 8.10). These cells were small in size and number with spindle and stellate forms apparent. The nuclei were elongated and the cytoplasm was clear and consistently PAS negative.

After 2 days in culture the areas of epitheliocyt formation had increased in size and, especially at the periphery of the culture flask, started to assume a sheet-like appearance (Plate 8.11). Although the proportion of fibroblasts increased during this time (Table 8.3) their actual numbers as seen on the culture surface appeared to change little.

By day 4 the culture began to take on a different appearance in that the fibroblasts began to dominate (Plate 8.12) even though the actual cell number decreased (Table 8.3). This led to the eventual encircling of the hepatocyte colonies by the fibroblasts (Plate 8.13) which by day 7 was complete and a virtually complete cell monolayer composed almost entirely of fibroblasts was obtained (Plate 8.14). One or two areas of epithelial presence could occasionally be seen at this time and it was considered that the sudden increase in the rate of fibroblast division utilized the available culture surface so rapidly that the hepatocytes with their much lower mitotic rate were unable to keep abreast of this sudden fibroblast growth and so became, as it were, strangled by the fibroblasts. It is also possible that the restraints imposed on the hepatocytes by the fibroblasts surrounding them compelled the hepatocytes to assume a fibroblastic shape and so lose their observable characteristic epithelial morphology.

Substantially similar patterns of growth were observed when the isolated cells were grown on thin round glass coverslips and examined by the scanning electron microscopy. Thus, 3 distinct cell-types could
again be seen which correspond to those already mentioned. Fibroblasts could be readily distinguished by their cylindrical appearance (Plate 8.15) and the hepatocytes possessed a characteristic polygonal appearance (Plate 8.16). The hepatocytes appeared to attach to the culture surface by one of two mechanisms. Firstly, the cytoplasm may 'fuse' with the culture surface and this is exemplified in Plate 8.16; alternatively, where the cytoplasm is raised off the surface, attachment could be achieved by means of cytoplasmic processes extending down from the mass of cytoplasm (Plate 8.17). The 'foamy' cells could also be observed by the scanning electron microscopy and the vacuoles observed in light microscopy showed some evidence for the presence of structure within or under these vacuoles (Plate 8.17).

The fibroblast overgrowth of the hepatocyte colonies was obviously undesirable and preliminary experiments showed that the incorporation of either dexamethasone (final concentration $2 \times 10^{-6}M$) or collagenase (final concentration of $0.05\% \text{ w/v}$) into the medium or the use of arginine-free medium were successful in keeping down the overgrowth although the fibroblast presence could not be completely eliminated. Each method of fibroblast suppression was initiated on or before the third day of culture so as to minimize the initial fibroblast involvement. By these means it proved possible to maintain the hepatocyte dominance in the cultures for up to two weeks or, in the case of dexamethasone incorporation, three weeks. Throughout these periods the hepatocyte morphology appeared to be essentially unchanged although further work on this matter was not carried out. No tests on the functional abilities of these long-term cultured hepatocytes was carried out.

Subculture of the hepatocytes proved completely unsuccessful.
Trypsin (0.25% w/v) and collagenase (0.05% w/v) were tested for their ability to release cells from the culture surface and whilst the fibroblasts could be readily subcultured, the hepatocytes could not. This refractory nature of the hepatocytes to the action of proteases is well exemplified by the previously noted observation that the hepatocytes could be successfully grown in collagenase-containing medium.

4. The Functional State of Cultured Hepatocytes

As the granular epithelial cell-type predominated for only the first 3 days in culture it was decided to test these cells for hepatocyte-specific functions within this time-scale. The following functions were detectable in the 3 day culture.

a) Glycogen storage could be detected histochemically in the cultured hepatocytes although the extent of storage varied from cell to cell. The other two cell-types did not store glycogen.

b) The hepatocytes could be successfully maintained on glucose-free medium, the glucose being replaced by galactose. This implied that the cells possessed a gluconeogenic function.

c) Experiments (carried out in collaboration with Mr. A. Shabaan) showed that glucose uptake by the epitheliocytos was influenced by insulin and glucagon, there being a dose-dependent response with insulin (dose-dependency with glucagon was not assessed).

Table 8.4 shows that the glucose uptake into the epitheliocytos was markedly enhanced in the presence of insulin, this enhancement
increasing as the insulin concentration was increased.

Furthermore, the subsequent release of glucose into non-radioactive medium in the absence of exogenously-added hormones was greater if the cells had been exposed to insulin at the same time as the $[^{14}C]$-glucose (Table 8.5). This insulin-enhanced release of glucose was further enhanced in the presence of glucagon which strongly indicated a glycogenolytic function for glucagon on the epitheliocytes.

\[ \text{[4C]}\text{-glucose (Table 8.5). This insulin-enhanced release of glucose was further enhanced in the presence of glucagon which strongly indicated a glycogenolytic function for glucagon on the epitheliocytes.} \]

d) An experiment (performed in conjunction with Mr. M. Bird) showed there to be good histochemically-detectable activity of glucose-6-phosphatase, a liver-specific enzyme, in the epithelial cells, although by the nature of the method this activity could not be quantitated. The other two cell-types did not possess histochemically-detectable glucose-6-phosphatase activity.

e) Analysis of the spent medium showed that the epitheliocytes produced urea at a rate of approx. 0.2 mg. per flask per day.

f) The cells could be successfully maintained on arginine-free medium.

These last two points strongly indicated a functional urea cycle in these presumptive hepatocytes.

g) Secretion of albumin into the medium could be readily detected by Ouchterlony gel diffusion, there being lines of identity between rat albumin standard and the albumin detected in the culture medium. (Plate 8.18)
h) Finally, extensive metabolism of $^{14}$C-imipramine by the epitheliocytes could be detected (see Fig. 8.1). When medium containing $^{14}$C-imipramine was incubated for 44 hours in the absence of cells, no metabolism of the imipramine could be demonstrated (Fig. 8.1a). When the same medium was incubated for 20 hours in the presence of rat liver epitheliocytes, two metabolites could be readily detected (Fig. 8.1b). The use of a reference marker of desmethylimipramine and comparison of the obtained $R_f$ values with those quoted by Bickel and Weder (1969) strongly indicated that the major metabolite produced (28% of the total radioactivity on the thin layer plate) was the N-oxide ($R_f$ 0.1-0.2), whilst the minor metabolite (7%) was the desmethyl derivative ($R_f$ 0.3-0.4) (Fig. 8.1b). After a further 24 hours incubation the amount of N-oxide produced had risen to 39% and that of the desmethyl derivative to 15% (Fig. 8.1c). No analysis for possible glucuronide/sulphate conjugates was carried out. During the incubation period of 44 hours the imipramine appeared to have no toxic effect on the epitheliocytes.

Imipramine is known to undergo complex metabolic transformations in the liver in vivo which leads to the production of large numbers of metabolites (Bickel and Weder, 1969). That only two metabolites could be detected upon incubation of imipramine with the presumptive cultured rat hepatocytes may possibly be due to one or two factors. Firstly, each flask received only a low level of radioactivity (200nCi per flask) and possibly the level of radioactivity attributable to each minor metabolite was too low to be detected. Secondly, the possibility exists that many of the minor metabolites as well as some of the desmethylimipramine would be conjugated within the liver cells and so would not be detectable as the free compounds in the organic extract of the medium.
<table>
<thead>
<tr>
<th>Method of Isolation *</th>
<th>Number of Experiments</th>
<th>Total Cell Yield (x10^6/g liver)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jacob and Dhargava</td>
<td>6</td>
<td>21.4(9.1-31.2)⁺</td>
<td>0</td>
</tr>
<tr>
<td>McLimans</td>
<td>8</td>
<td>22.8(14.3-32.3)</td>
<td>0</td>
</tr>
<tr>
<td>Tetraphenylboron</td>
<td>3</td>
<td>11.8(9.3-13.4)</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>15</td>
<td>&lt; 1</td>
<td>0</td>
</tr>
<tr>
<td>Pangestin</td>
<td>5</td>
<td>&lt; 1</td>
<td>0</td>
</tr>
<tr>
<td>Pronase</td>
<td>2</td>
<td>&lt; 1</td>
<td>0</td>
</tr>
<tr>
<td>Collagenase/Hyaluronidase</td>
<td>11</td>
<td>2.6(0.3-9.9)</td>
<td>70(50-89)⁺</td>
</tr>
<tr>
<td>Modified</td>
<td>20</td>
<td>5.5(2.9-14.0)</td>
<td>84(70-96)</td>
</tr>
</tbody>
</table>

* Methods used were those outlined in Chapter 7

Collagenase/Hyaluronidase - method of Howard and Pesch (1968) but omitting the initial perfusion

Modified Collagenase/Hyaluronidase - method as detailed in Chapter 7 i.e. initial Ca²⁺-removal and subsequent replacement in enzyme mixture

⁺ Results are mean with range in brackets
Table 8.2.  Effect of Dead Cells on the Numbers of Viable Cells

Existing 24 Hours After Initiating Culture

<table>
<thead>
<tr>
<th>Medium</th>
<th>Viable Hepatocyte Count ((\times 10^4/\text{flask})^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium undisturbed for 24 hours</td>
<td>7.2</td>
</tr>
<tr>
<td>Medium changed 4 hours after initiating culture</td>
<td>13.9</td>
</tr>
</tbody>
</table>

* Cells were prepared by the modified collagenase/hyaluronidase method. Both flasks received \(2 \times 10^6\) viable hepatocytes per flask. Viability of initial suspension was 71%.

Table 8.3. Monolayer Culture of Rat Liver – Proportion of Cell Types at Various Times of Culture

<table>
<thead>
<tr>
<th>Culture</th>
<th>Granular Epithelial-</th>
<th>Fibroblasts</th>
<th>'Foamy'</th>
<th>Total Cell Number ((\times 10^6/\text{flask}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period (days)</td>
<td>iocytes (%)</td>
<td>Fibroblasts</td>
<td>Cells (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>62.4</td>
<td>7.3</td>
<td>30.3</td>
<td>1.02</td>
</tr>
<tr>
<td>2</td>
<td>61.0</td>
<td>17.1</td>
<td>21.0</td>
<td>0.76</td>
</tr>
<tr>
<td>4</td>
<td>9.0</td>
<td>83.0</td>
<td>7.2</td>
<td>0.62</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>98.0</td>
<td>2.0</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Cells were prepared by the modified collagenase/hyaluronidase method. All flasks were seeded initially at \(2 \times 10^6\) viable hepatocytes.
Table 8.4. Glucose Uptake by Cultured Rat Hepatocytes — Influence of Insulin

<table>
<thead>
<tr>
<th>Level of Insulin (μunits/ml, medium)</th>
<th>% [^{14}C]-Glucose Remaining in Medium$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>97.0 ± 0.5</td>
</tr>
<tr>
<td>200</td>
<td>93.0 ± 0.5$^*$</td>
</tr>
<tr>
<td>400</td>
<td>91.5 ± 0.5$^*$</td>
</tr>
</tbody>
</table>

$^+$ Results are mean of 3 experiments ± S.E.M.

$^*$ Significantly different from control (P<0.01)

Table 8.5. Glucose Release from Cultured Rat Hepatocytes — Influence of Glucagon

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>% [^{14}C]-Glucose Released (Control as 100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin$^+$ Glucagon$^*$</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>+</td>
<td>117</td>
</tr>
<tr>
<td>+</td>
<td>138</td>
</tr>
</tbody>
</table>

$^+$ Insulin concentration 200 μunits/ml, medium

$^*$ Glucagon concentration 200 ng/ml, medium
Plate 8.1. Explant Culture of Adult Rat Liver. Morphological Appearance After 3 Days Culture

a) x 100

The dark masses at the bottom of the plates are the explanted pieces of liver.

b) x 200
Plate 8.2. Explant Culture of Adult Rat Liver. Morphological Appearance After 5 Days Culture

a) $\times 100$

b) $\times 200$
Plate 8.3. Explant Culture of Adult Rat Liver. Morphological Appearance After 8 Days Culture

a) x 100

b) x 200

Mitotic figures are indicated by arrows
Plate 8.4. Morphological Appearance of Freshly-Isolated Adult Rat Hepatocytes

The cells are suspended in trypan blue solution. The darkly-staining cells (indicated by arrows) are regarded as being non-viable.
Plate 8.5. Monolayer Culture of Adult Rat Liver - The Three Cell-Types Present

x 100

Hepatocytes are indicated by ★

A fibroblast is shown in the centre (indicated by arrow)

'Foamy' cells surround this fibroblast
The amorphous material overlying the hepatocyte groups are dead cells. Twentyfour hours in culture.
Plate 8.7. Monolayer Culture of Adult Rat Liver

x 200

Hepatocyte group after twentyfour hours culture
Hepatocyte group. Binucleation is apparent as are the intercellular spaces.
Plate 8.9. Monolayer Culture of Adult Rat Liver

'Foamy' cell. Large vacuoles can be observed around the cell nucleus.
Plate 8.10. Monolayer Culture of Adult Rat Liver

x 400

Fibroblasts. Shown are the typical stellate and spindle forms.
Plate 8.11. Monolayer Culture of Adult Rat Liver.

x 200

Two-day culture. Sheet-like appearance of hepatocytes.
Plate 8.12. Monolayer Culture of Adult Rat Liver

x 200

Four day culture. This plate illustrates the emergence of fibroblast clusters (in centre).
Plate 8.13. Monolayer Culture of Adult Rat Liver

Five day culture. Overgrowth of hepatocyte groups by fibroblasts.
Plate 8.14. Monolayer Culture of Adult Rat Liver

x 100

Seven day culture. Overgrowth by fibroblasts is now complete.
The polygonal nature of the cells is very apparent.
Plate 8.17. Scanning Electron Microscopy of Adult Rat Liver Cells in Culture - 'Foamy' Cell

The 'foamy' cell is at the bottom left-hand of the photograph.
Plate 8.18. Ouchterlony Gel Diffusion Precipitation Patterns of Albumin in Containing Medium

1. Goat anti-rat albumin antiserum
2. Rat albumin standard
3. Unincubated medium
4. Medium incubated with cells

For method see Chapter 7.
Fig. 8.1. Radioactivity Profile of Thin Layer Chromatograms of Organic Extracts Obtained from Media Containing $^{14}C$-Imipramine Incubated with Rat Liver Epitheliocytes.

a) Medium alone; 44 hr.

b) Medium + Cells; 20 hr.

c) Medium + Cells; 44 hr.

Abscissa: Distance along chromatogram (in cm)

Ordinate: d.p.m. ($\times 10^3$)
CHAPTER 9. CONTENTS

1. Explant Culture 224
2. Isolation of Adult Rat Hepatocytes 226
3. Culture of Cells Liberated by Collagenase/Hyaluronidase 228
   Digestion of Rat Liver
CHAPTER 9. DISCUSSION

1. **Explant Culture**

   The basis of explant culture is the attachment of small tissue pieces to a suitable substratum and observation of the cells that grow out from these explants (Moscona *et al.*, 1965). However, when adult rat liver was cultured using the most simple explant technique viz. explants attached directly to glass, the results were most disappointing in that virtually no outgrowth was observed from any of the explants (see Chapter 8). Gelatin and other proteins have often been reported to be suitable substrata for cell outgrowth studies (see Moscona *et al.*, 1965), largely because these are thought to represent a more physiological approach to the problem of cell attachment and spreading. In the case of liver explants the deposition of a gelatin layer onto a glass surface did not induce any cell outgrowth whatsoever.

   Successful reproducible outgrowth of cells from liver explants could only be achieved if the explants were attached to polystyrene specially treated for tissue culture purposes (i.e. 'Falcon' flasks). The cells present in these outgrowths were, however, of typical fibroblastic appearance (see Chapter 8) and did not stain for glycogen at any stage in the development of the culture. Using a similar technique (i.e. treated polystyrene as substratum) essentially identical results have been reported for the explant culture of foetal human liver (Lie, 1972), adult human liver (Domoise *et al.*, 1971) and adult monkey liver (Douglas *et al.*, 1966).

   The overwhelming predominance of fibroblasts in the cell outgrowths when using plastic flasks was not influenced by the source of the serum...
present in the medium. Thus, the presence of foetal bovine serum, calf serum or horse serum produced no differences in the cell outgrowth pattern (see Chapter 8). Lie (1972) has reported that incorporation of portal serum into the culture medium led to cell outgrowth of a largely hepatocyte nature from explanted human foetal liver, but this could not be confirmed using explanted adult rat liver (Chapter 8). This discrepancy between the foetal and adult situations is perhaps related to the much greater repression of cell division by tissue-specific mitotic inhibitors which are present to a considerably higher extent in the adult animal (see Grisham, 1973).

It would appear from a survey of the literature that successful outgrowths of hepatocytes from explant cultures of adult mammalian liver can only be achieved using highly complex culture conditions. Thus, Alexander and Grisham (1970) employed a collagen-coated glass coverslip covered with a piece of perforated cellophane and encased in a Sykes-Moore chamber as a culture vessel and cellulose sponge as a medium reservoir. With this system they were able to observe the outgrowth of several cell types, including hepatocytes from livers of rats 1 - 5 days old. A similar system has been employed by Sandstrom (1965), the coverslip being replaced by a second piece of cellophane which is unperforated. By this technique foetal rat hepatocytes (Sandstrom, 1965) and more recently, adult human hepatocytes (Sandstrom, 1973) have been successfully cultured.

Whilst these cultures are of undoubted value in observing morphological changes of the cells in response to any given stimulus they are of doubtful value in metabolism studies, largely because of the difficulty of producing exactly parallel replicate cultures.
For these reasons the explant culture approach was discarded and the cell culture aspect was further studied. Before this, however, it was necessary to examine what is, perhaps, the most critical step in cell culture, namely the initial cell isolation.

2. Isolation of Adult Rat Hepatocytes

A number of workers have shown that collagenase/hyaluronidase digestion of rat liver yields a cell suspension superior in viability to those produced by mechanical, chemical or other enzymic means (Gallai-Hatchard and Gray, 1971; Jezyk and Liberti, 1969; Lipson et al, 1972; Muller et al, 1972; Murthy and Petering, 1969). This conclusion has been amply confirmed in the present study in that collagenase/hyaluronidase treatment was the only system to produce cell suspensions of a largely viable nature as judged by trypan blue exclusion. Furthermore, maintenance of hepatocytes in culture as morphologically viable entities was also only possible when the cells were produced by collagenase/hyaluronidase digestion (see Chapter 8). It has been argued by Dickson (Dickson, 1970; Suzangar and Dickson, 1970) that viable hepatocytes normally take up trypan blue, mainly in the nucleus, and that non-viable hepatocytes be considered as those which stain uniformly within the cell. If this were true all the cells produced by the perfusion techniques and by TPB treatment were in fact viable, which should have been reflected by reproducible maintenance and growth of these cells in vitro. That this was not in practice the case strongly argues against this contention of Dickson.
The viability test most commonly used in studies of isolated liver cells is that of dye exclusion which is based on the ability of the dye to enter only non-viable cells via a damaged plasma membrane. This test is purely one of plasma membrane integrity and it might be thought that cells with damaged plasma membranes might still be viable with regard to various metabolic capabilities. However, all the available information on the metabolic capabilities of mechanically- and chemically-isolated liver cells (as outlined in Chapter 6) points to the view that non-viability as measured by dye uptake is only one of a number of abnormal aspects of cell morphology and function, although whether these arise as a result of the plasma membrane damage is as yet unknown. Conversely, maintenance of membrane integrity is accompanied by near normality in various metabolic parameters (see Chapter 6). Thus, if isolated liver cells are required for short term use only (less than, say, 6 hours) the question of cell viability is still of extreme importance, especially if a comparison to the in vivo state is contemplated.

A major drawback to the method of hepatocyte isolation by collagenase/hyaluronidase as reported by Howard and Pesch (1958) and its subsequent modifications (Berry and Friend, 1959; Capuzzi et al, 1971; Ingebretsen and Wagle, 1972; Seglen, 1972) has been the necessity to use a preliminary perfusion of the liver with the enzyme mixture, either in situ or in vitro. This initial perfusion step is obviously impractical when using foetal liver or human biopsy material. It was found in this present study that reasonable numbers of viable cells could be obtained if this perfusion step was omitted (see Chapter 8) the yield of viable cells (0.2 - 6.6 x 10^6/g liver) being
comparable to that reported by Howard and Pesch (1968) (approx. 4 x 10⁶/g liver). Introduction of a Ca⁺⁺-removal stage prior to the enzymic digestion and the presence of Ca⁺⁺ in the digestion stage itself as suggested by Seglen (1972) increased the yield of viable cells still further (range 2.2 - 11.0 x 10⁶/g liver).

It is generally recognized that Ca⁺⁺ plays an important role in the maintenance of intercellular contacts in intact tissues (Moscona et al., 1965) and it appears to act via a Ca⁺⁺-dependent adhesion factor (Modjanova and Malenkov, 1973). It is probable that initial washing of the liver pieces with Ca⁺⁺-free buffer containing EGTA removes both the Ca⁺⁺ and the adhesion factor (Seglen, 1973b), and the cellular adherence is reduced sufficiently to facilitate subsequent access for the matrix-dissolving enzymes (Moscona et al., 1965). Collagenase itself requires the presence of Ca⁺⁺ for maximal activity (Seglen, 1972; Howard et al., 1973).

3. Culture of Cells Liberated by Collagenase/Hyaluronidase Digestion of Rat Liver

Culture of collagenase/hyaluronidase-digested adult rat liver cells yields 3 morphologically distinct cell-types, the relative proportions of which alter as the culture progresses (see Chapter 8). Twenty-four hours after initiating the culture the major cell-type present is a granular epitheliocyte (62%) together with fibroblasts (7%) and 'foamy' cells (31%), these relative proportions being strongly reminiscent of the values quoted for rat liver in vivo i.e. parenchymal cells representing 60% of the total cell population (Dacouet, 1959). During the following days in culture there is a fall in the total cell
number (arising largely from the loss of the 'foamy' cells) which is accompanied by an absolute, as well as relative, increase in the fibroblast population. The rate of division of the epitheliocytes is much slower than that of the fibroblasts so that by 5 - 7 days the fibroblasts completely dominate the culture (see Chapter 6).

Morphologically, the epitheliocytes are strikingly similar to normal hepatocytes in vivo and resemble the granular epitheliocytes observed by Alexander and Grisham (1970), Sandstrom (1965) and Watanabe (1966). The fibroblasts are believed to be of connective tissue origin. Introduction of collagenase into the culture medium for 3 days markedly decreased the number of fibroblasts remaining on the culture surface, which possibly suggests that these fibroblasts are functional in that they secrete, and attach to, collagen fibres. The origin of the 'foamy' cells could not be established. The morphological appearance of the three cell-types present in culture could be confirmed by the scanning electron microscopy (see Chapter 8) and it is possible that use of this technique might prove useful in investigating the short-term effects of various carcinogens on the surface morphology of cultured cells.

A large number of differing liver-specific functions were tested for in the three-day cultures of epitheliocytes (see Chapter 8). Firstly, the cells appear to have a normal carbohydrate metabolism. They store glycogen, they can grow in medium in which glucose is replaced by galactose, and glucose uptake and release by the cells is strongly influenced by insulin and glucagon respectively. Those last results indicate that the epitheliocytes possess functional hormone cell receptor sites and from this it appears that this culture system is potentially a very useful tool in the study of liver-hormone
interactions. The advantages of this technique are that the concentration of hormone and the period in which it is in contact with the cells can be critically monitored, and also that the hormone does not act on organs other than the liver and so lead to misleading results. Two situations can be readily imagined in which this culture system could prove a valuable experimental tool: a) studies into hormone receptor sites, and b) studies into the role of adenosine 3' : 5'-cyclic monophosphate as a secondary messenger in mediating many effects of hormones acting on the liver.

Secondly, evidence suggests that these epitheliocytes possess a functional urea cycle which although not truly liver-specific is found in mammals only in liver, brain and kidney (Conn and Stumpf, 1972). The epitheliocytes can be maintained in culture for at least a week in medium which is essentially free of arginine and they produce urea at a rate of approx. 0.2 mg. per flask per day.

Furthermore, these cells secrete immunochemically-detectable serum albumin into the medium, and they also show intense staining for glucose-6-phosphatase, a liver-specific enzyme.

Finally, metabolism of $[^{14}C]^{-}$imipramine by these cells has been detected (see Chapter 8). The major routes of imipramine metabolism by the liver in vivo are N-demethylation and N-oxidation (Gigon and Bickel, 1971). These routes of metabolism have also been detected in this present in vitro study. Imipramine administration in vivo leads to the production of numerous minor metabolites, none of which could be detected in this study; various technical limitations (see Chapter 8) probably account for this discrepancy.

All this information when taken together with the morphological appearance of the cells very strongly suggests that these epitheliocytes
can, with some confidence, be designated "cultured hepatocytes". Whilst certain of these parameters have been used previously as markers for adult hepatocyte cultures (Alexander and Grisham, 1970; Le Guilly et al, 1973) this present study is, as far as is known, the only one to combine so many parameters of hepatocyte-specific function as a tool in assessing the nature and origin of cultured liver cells. The various reports of adult mammalian hepatocyte monolayer cultures that have appeared in the literature base their justification for designating the cells "hepatocytes" purely on morphological appearance (Suliman et al, 1968; Armato et al, 1972a, b; Iype, 1971) and not on any functional criteria whatsoever. In the cell culture situation the use of morphological appearance may not, by itself, help in elucidating cell derivation (see Moscona et al, 1965), mainly due to difficulties in translating the 3-dimensional structure in vivo to an essentially 2-dimensional structure in vitro, and it is for this reason that functional criteria have to be used both to elucidate cell derivation and the differentiated state of the cells. This has been done in the present study and the wide range of hepatocyte-specific functions that have been detected in these cultures most strongly argue for the existence, in culture, of differentiated hepatocytes.

Preliminary experiments have shown that the fibroblast overgrowth which is complete by 7 days in culture may be successfully minimized. In agreement with Leffert and Paul (1972) was the finding that the use of an arginine-free medium eliminates the fibroblast presence to a large extent without morphologically affecting the hepatocytes. The incorporation of dexamethasone (2 x 10^{-6}M) or collagenase (0.05%)
into the culture medium produced similar findings. This effect of collagenase in minimizing fibroblast growth has been utilized by Lasfargues (1973) to enable mammary epithelial cells to be maintained in culture in the presence of fibroblasts. It should be pointed out that as yet these results are based solely on morphological patterns and that biochemical tests on the cells surviving these treatments has not been attempted.

It proved impossible by the various techniques employed to subculture the hepatocytes although the fibroblasts could be readily subcultured. It is of interest to note that in keeping with the results obtained from the cell isolation experiments, trypsin was not successful in detaching the hepatocytes from the substratum in a viable state. The lack of effect of collagenase as a subculturing agent for the hepatocytes indicates that the attachment and spreading of the hepatocytes in culture is by a different mechanism to that operating in vivo.

Three different cultures have been developed to satisfy the requirements of any particular experiment. The cultures can be grown in flasks, these being ideal for analysis of medium contents. The cells can be grown on coverslips to enable detailed examination of the cells to be made, and, finally, the cells can be grown in microtitre plates suitable for micro-scale work. In all three culture situations the morphological appearance of the hepatocytes is identical.

In conclusion, the system described for the culture of differentiated rat hepatocytes is potentially a very useful tool with which to study a wide range of problems concerned with hepatocytes and their involvement
in metabolism and toxicity. Examples in which this application of hepatocyte cultures could prove useful include the role of covalent binding of drugs to macromolecules in toxic hepatic damage, and the influence of hormones on hepatic drug metabolism. In both situations the effects on the hepatocytes can be studied without the problem of possible interference by other organs of the body. Another example is the study of the short-term effects of hepatocarcinogens and other hepatotoxic agents on the morphology and function of hepatocytes with a view to the potential use of hepatocyte cultures as a test in determining the possible hepatotoxicity of novel compounds. Obviously, this culture system would be totally unsuitable where the toxic effect on the liver is directed not to the hepatocytes but to the other cellular elements e.g. the bile duct epithelium. In such instances the experimental approach would have to be based on an organ culture system in which the cellular architecture is maintained in vitro.

Furthermore, if this method could be adapted for use with human liver, a paired system of rat and human hepatocyte cultures should prove valuable in elucidating species differences in drug metabolism and toxicity and should, in turn, lead to a more rational transition of drug studies from animals to man. It is perhaps worthwhile at this stage to speculate on the feasibility of culturing differentiated human hepatocytes. The probability exists that, owing to the greater fibrous content of adult human liver, the viable cell yields would be lower from human specimens, but the microtitre system which has been developed using rat hepatocytes could prove very useful where the viable cell yields from human liver are low, due either to poor dissociation or to the size of sample received. This culture system now appears to be at a stage where its applicability to human liver could profitably be investigated.
APPENDIX

SUPPLIERS OF MEDIA, MEDIA SUPPLEMENTS AND CHEMICALS

- Albumin, Bovine Serum: Koch-Light
- Albumin, Rat: Sigma
- Aminopyrine: Ralph N. Emmanuel
- Aniline: BDH
- Antiserum, Goat Anti-Rat Albumin: Calbiochem
- 4,4'-Bipyridyl: Ralph N. Emmanuel
- Calf Serum: Biocult
- Chicken Embryo Extract: Biocult
- 4-Chloroaniline: Calbiochem
- 4-Chloro-N-methylaniline: Calbiochem
- Colcemid: Calbiochem
- Collagenase (Types I and II): Sigma
- Cytochrome c (Type II, Horse Heart): Sigma
- Dexamethasone: Roussel
- Ethylmorphine Hydrochloride: May and Baker
- Foetal Bovine Serum: Biocult
- Gentamycin: Biocult
- Glucagon: Sigma
- \( ^{14}\text{C} \)-Glucose: Radiochemical Centre, Amersham
- Glucose-6-phosphate, Disodium Salt: P-L Biochemicals
- Glucose-6-phosphate Dehydrogenase (Type XV, Baker's Yeast): Sigma
- Horse Serum: Biocult
- Hyaluronidase (Type II, Bovine Testis): Sigma
[14C]-Imipramine
Insulin Hydrochloride
Liver Digest Ultrafiltrate
McCoy's 5A Medium
MEM with Earle's B.S.S. without Arginine
MEM with Hank's B.S.S. and HEPES Buffer
NADH, Disodium Salt
NADP
Naphthalene
1-Naphthol
Non-Essential Amino Acids
Phenobarbitone Sodium
Protease (Type V, Streptomyces griseus)
Putrescino Dihydrochloride
Ribonucleic Acid (Torula Yeast)
1,1,3,3-Tetraethoxypropane
Tetraphenylboron Sodium
Trinitrobenzenesulphonic Acid
Trypan Blue
Tryptose Phosphate Broth
Trypsin (2.5%, 1:250)

Radiochemical Centre, Amersham
BDH
Wellcome
Biocult
Biocult
Biocult
Koch-Light
P-L Biochemicals
Ralph N. Emmanuel
Ralph N. Emmanuel
Biocult
BDH
Sigma
Sigma
Sigma
Koch-Light
Ralph N. Emmanuel
BDH
Biocult
Biocult
Biocult
REFERENCES

(1972) - Biochem. Pharmacol., 21, 2169.


(1963b) - Experentia, 19, 646.


Dutton, G. J. (1973) - Enzyme, 15, 304.


(1971c) - Science, 172, 167.


Hansen, J.M., Nielson, K.S., Kristensen, R., Skovsted, L.,


Kupfer, D., Rosenfeld, J. (1973) - Drug Metab. Dispos., 1, 760.

De Matteis, F. (1973) - Drug Metab. Dispos., 1, 267.

(1972b) - Molec. Pharmacol., 6, 304.

Onda, H., Yoshikawa, J. (1973) - Gann, 64, 139.


Perli, C.J., Lee, N.W., McFehon, R.E. (1973) - Drug Metab. Dispos., 1, 628.


Redzialowski, F. M., Bousquet, W. F. (1968) - J. Pharmacol. Exp. Ther.,
163, 229.
5, 70.
Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J. R.,
22, 569.
Smith, D. E., Williams and Wilkins.
Sasame, H. A., Mitchell, J. R., Thorgeirsson, S., Gillette, J. R. (1973) -
Drug Metabol. Dispos., 1, 150.
1, 286.
Schaffner, F., Bacchin, P.G., Hutterer, F., Scharnbeck, H.H., Sarkozi, L.L.,
Schapira, F., Delain, D., Lacroix, Y. (1971) - Enzyme, 12, 545.
Schenkman, J.B., Cinti, D.L., Orrenius, S., Moldeus, P., Krasznitz, R.
(1972) - Biochemistry, 11, 4243.
3, 113.
21, 2373.
Schoene, B., Fleischmann, R.A., Remmer, H., Oldershausen, H.F. (1972) -
Shoeman, D.W., Chaplin, M.D., Mannering, G.J. (1969) - Molec. Pharmacol.,
5, 412.
Siegert, M., Alsleben, B., Liebenschultze, U., Remmer, H. (1964) -
Pharmacol., 21, 1753.
Pharmacol., 8, 69.


Vessell, E.S. (1968) - Pharmacol., 1, 81.
(1972) - in "Liver and Drugs" ed. Orlandi, F., Jerzequel. A.M.

Academic Press.


Walker, P.R., Bonney, R.J., Becker, J.E., Potter, V.R. (1972) –
In Vitro, 8, 107.


Life Sci., Part II, 9, 1189.