A Hybrid Artificial Liver with Integral Membrane Oxygenation: Theory, developmental studies and prototype testing.

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Abstract

The purpose of this thesis is to identify, develop and evaluate a bioreactor design appropriate for use in a liver support system. The requirement for such systems is placed in context by discussing liver failure and options for therapy. The thesis objective is pursued by examining the natural organ and considering how the microarchitecture of the liver facilitates organ function. Thereafter, current bioartificial liver designs are studied with emphasis on analysis of; cell source, predicted mass transfer performance and mode of application. Reported clinical findings are also assessed. The experimental work investigates the influence of cell species, medium formulation and culture configuration on key liver function parameters. The oxygen consumption rate of hepatocytes under load and their response to fluid shear stress is investigated in order to develop design rules for the prototype bioreactor. The concluding work describes the development and testing of the bioreactor.

The microarchitecture of the liver proves to be adapted for efficient mass exchange and characterised by large capacity upstream processes and downstream fine tuning of solute concentrations facilitating organ function. The current designs analyzed are shown to have shortcomings especially with respect to oxygen transfer. These shortcomings seem more serious when the oxygen consumption experimental results are considered. The oxygen consumption rate recorded exceeds previously reported values and is significant since the experimental conditions are more akin to those anticipated in clinical application. These findings make a compelling argument for integral oxygenation in hepatocyte bioreactors. The hepatocytes responded favourably to shear stresses suggesting that from mechanical and functional perspectives, designs based on direct contact between hepatocytes and plasma are feasible. This configuration offers mass transfer advantages. The resultant prototype bioreactor offers efficient mass transfer and control of cell surface pO_2. *In vitro* testing demonstrates maintenance of urea synthetic activity for nine days under continuous ammonium load.

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Publications arising from this thesis

1. Edwards I.W., Watts P.L., Smith M.D., Grant M.H. & Brown V.A. "Comparison of hepatocyte function in different media." Mutagenesis 1993:8:482.

2. Watts P., Smith M.D., Zammit V., Edwards I., Brown V. & Grant M.H. "Comparison of the effect of medium composition on cytochrome P450 and glutathione content and urea synthesis on primary cultures of rat and sheep hepatocytes." British Journal of Clinical Pharmacology 1994:38(2):171-P.

3. Watts P., Smith M.D., Edwards I., Zammit V., Brown V. & Grant M.H. "The influence of medium composition on the maintenance of cytochrome P450, glutathione content and urea synthesis: A comparison of rat and sheep primary hepatocyte cultures." Journal of Hepatology 1995:23:605-612.

4. Smith M.D., Smirthwaite A.D., Cairns D.E., Cousins R.B. & Gaylor J.D.S. "Techniques for measurement of oxygen consumption rates of hepatocytes during attachment and post attachment." Int. J. Artif. Organs 1996:19(1):36-44

5. Smith M.D., Airdrie I., Cairns D. Cousins R.B., Ekevall E., Grant M.H. & Gaylor J.D.S. "A novel hybrid artificial liver with integral membrane oxygenation: Theory and initial in vitro evaluation." In Cell Biology and Toxicology 1996: Special Issue: International Congress on Hepatocytes: Applications in Cell Biology, Toxicology and Medicine: p56.

6. Ekevall E., Airdrie I., Cousins R.B., Smith M.D. & Gaylor J.D.S. "Effect of oxygen partial pressure within a bioartificial liver." Int. J. Artif. Organs 1996:19(9):545

7. Smith M.D., Cairns D.E., Veitch R., Cousins R.B. & Gaylor, J.D.S. "Analysis of oxygen transfer in hollow fibre hepatocyte bioreactors." Artificial Organs 1997:21(6):531

8. Smith M.D., Airdrie I., Cairns D., Courtney J.M., Cousins R.B., Ekevall E., Grant M.H. & Gaylor J.D.S. "Characterisation of a hybrid artificial liver bioreactor with integral membrane oxygenation." Artificial Organs 1997:21(6):531

9. Smith M.D., Ekevall E., Airdrie I., Grant M.H., Cousins R.B. & Gaylor J.D.S. "A modular hepatocyte bioreactor with integral membrane oxygenation: Design and development." Int. J. Artif. Organs 1997:20(9):509

10. Ekevall E., Smith M.D., Airdrie I., Grant M.H., Cousins R.B. & Gaylor J.D.S. "A modular hepatocyte bioreactor with integral membrane oxygenation: *In vitro* testing." Int. J. Artif. Organs 1997:20(9):529

11. Smith M.D., Airdrie I., Courtney J.M., Cousins R.B., Ekevall E., Grant M.H. & Gaylor J.DS. "Development and Characterization of a Hybrid Artificial Liver Bioreactor with Integral Membrane Oxygenator" In: Bioartificial Liver Support: the Critical Issues, Crepaldi G., Demetriou A.A. and Muraca M. Eds., International Up to Date - 208, C.I.C. Edizioni Internazionali (1997): pp. 27-35

Table of achronyms, abbreviations and symbols

ADP	adenosine di-phosphate
ALA	5-amino-laevulinic acid
ALT	alanine amino transferase
AST	aspartine amino transferase
ATP	adenosine tri-phosphate
BAL	bioartificial liver
BSDF	bile salt dependent fraction
BSIF	bile salt independent fraction
CHE	Chee's Medium
CLSM	confocal laser scanning microscopy
DNA	deoxyribose nucleic acid
ECM	extracellular matrix
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol-bis (beta-amino-ehtyl ether) N,N,N^1,N^1-tetra acetic acid
ELAD	extracorporeal liver assist device
FHF	fulminant hepatic failure
γGT	γ glutamyl transferase
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione sulfyl transferase
HA	hepatic artery
HIV	human immuno-deficiency virus
ICP	intracranial pressure
IgM	immunoglobulin class M
KI	A modification of Earle's medium
K_m	Michaelis constant
LOHF	late onset hepatic failure
M199	Medium 199

MHF	Mitsubishi hollow fibre
mmHg	millimetres of mercury
mRNA	messenger ribose nucleic acid
NADP	nicotinamide adenine dinucleotide phophate
NADPH	nicotinamide adenine dinucleotide phophate (reduced form)
NAPQI	N-acetyl-p-benzoquinone imine
NMWCO	nominal molecular weight cut-off
OCR	oxygen consumption rate
O.D.	outer diameter
ODC	oxygen dissociation curve
OLT	orthotopic liver transplantation
P450	cytochrome P450
PBS	phosphate buffered saline
pCO_2	carbon dioxide partial pressure
PES	micropolyethersulphone
PMMA	polymethyl methacrylate
PNF	primary non function
pO_2	oxygen partial pressure
PP	microporous polypropylene
PVC	poly(vinyl chloride)
RNA	ribose nucleic acid
SFHF	subfulminant hepatic failure
TCA	trichloroacetic acid
TCP	tissue culture polystyrene
THV	terminal hepatic venule
TPV	terminal portal venule
UK	United Kingdom
US	United States
WE	Williams' E medium

Chapter 1.

The Failing Liver: Current Clinical Situation and Prospective Therapies.

1.1. Introduction

At present, significant research efforts are being directed towards the development of novel therapies for liver failure. This thesis is concerned with particular aspects of one potential mode of treatment, namely hybrid artificial liver support. Currently, there is no consensus of opinion about several critical parameters of hybrid artificial liver development. The quantity of work being carried out in this field and the urgency with which it is being pursued by certain groups has resulted in diverse developments some of which have little scientific basis.

A critical analysis of current hybrid artificial liver developments is required and key parameters must be evaluated if optimised devices are to be realised. These requirements will be addressed in this thesis.

In order to place this thesis within the correct context, the various clinical syndromes and therapeutical approaches must first be understood. The review of the clinical situation and relevant recent and current research which follows will primarily consider the disease states, current trends in clinical therapy and the prospects of potential future developments.

1.2. Liver Failure

In general, liver failure follows an episode of severe liver damage which may have been sudden or protracted. The massive loss of functional liver tissue which results, deprives the sufferer of many of the detoxifying and synthesising functions of the body. As a consequence, the concentration of circulating toxins rises and some factors essential for homeostasis cease to be generated. From this point, the condition of the liver may decline further and other body systems may fail. The failure episode may be acute, from which mortality or a full recovery will usually result, or chronic, in which the condition of the patient can be intractible. The rate at which liver failure symptoms develop and progress is the principal characteristic used to define more accurately the disease state. This categorisation of syndromes is vital since speed of progression has a strong correlation with prognosis (Williams and Wendon 1990).

1.2.1. Fulminant Hepatic Failure

Fulminant hepatic failure (FHF) is the term used to describe the clinical condition of patients without previous liver disease, who have hepatic failure which progresses rapidly with encephalopathy developing within eight weeks of the appearence of initial symptoms (Trey and Davidson 1970). It has been suggested that this category of disease be sub-divided such that FHF should define onset of encephalopathy within two weeks of the start of symptoms, while the term subfulminant hepatic failure (SFHF) should be used to describe the appearence of encephalopathy between 2 and 8 weeks after initial symtoms develop (Bernuau, Rueff, and Benhamou 1986). A further definition; late-onset hepatic failure (LOHF) is applicable when the encephalopathy occurs after 8 weeks to 6 months have elapsed since symptoms began (Gimson et al. 1986).

1.2.2. Aetiology

The rate of progression of symptoms in liver failure often correlates with aetiology. The prevalence of causes varies between countries. In the UK, liver failure

Grade	Features
I	Slowing of Mentation
II	Confusion, euphoria, inappropriate behaviour or drowsiness
III	Permanent somnolence
IV	Coma

Staging of hepatic encephalopathy (Schalm and de Knegt 1990)

Table 1.1.

following paracetamol overdose is very common and is normally associated with a suicidal episode. One study reports that 55% of all FHF cases over a 16 year period at King's College Hospital, London were caused by paracetamol intoxication (O'Grady et al. 1989). In other countries this is a very rare cause of FHF. Globally, the most common cause of FHF is acute viral hepatitis (Bernuau, Rueff, and Benhamou 1986). The leading causative agent for virally induced FHF also differs geographically. In the 'developed world', hepatitis B virus is the commonest cause (O'Grady and Williams 1992), while enterically transmitted non-A non-B hepatitis virus is the principal causative agent in the African and Asiatic populations (Ramalingaswami and Purcell 1988). Other less common causes are; idiosyncratic reactions to drugs, notably halothane (Farrell, Prendergast, and Murray 1985), isoniazid (formerly used against tuberculosis) (Black 1975) and anti-depressants (Larrey, Berson, and Habersetzer 1989); poisoning by carbon tetrachloride (Ruprah, Mant, and Flanagan 1985) or mushrooms (Hanrahan and Gordon 1984); haemodynamic disturbances (Nouel, Henrion, and Bernuau 1980; Sandle et al. 1980) and heat stroke (Benhamou 1990).

1.2.3. Clinical Findings

The definitive clinical findings are jaundice and encephalopathy (Trey and Davidson 1970). In addition to the aetiology and the rate of progression to encephalopathy (see Table 1.1.), the appearance and severity of other symptoms carry clear prognostic features.

Coagulopathy is usually present. The increase in prothrombin time is one of the chief indicators of the extent of liver damage (Williams and Wendon 1990) and the reduced levels of antithrombin III which are also found (O'Grady et al. 1986) result in shortening the half life of any heparin administered for extracorporeal blood treatments. Other coagulopathic findings include thrombocytopenia and impaired platelet reactivity (Preston 1990).

The serum concentrations of bilirubin and ammonia are elevated. Total plasma amino acids are increased (Iber et al. 1957). In particular, aromatic amino acids (tryptophan, phenylalanine, tyrosine and methionine) are increased while branched

chain amino acids (valine, leucine and isoleucine) are decreased (Cascino et al. 1982; Morgan et al. 1982). Increased circulating levels of certain enzymes, notably alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase (γ-GT) can also be indicative of liver damage.

Renal failure occurs in approximately 30% of cases where grade IV encephalopathy develops, but this figure rises to 70% following paracetamol overdose (O'Grady et al. 1988). Hypoglycaemia and hypophosphataemia are also common (Bihari, Gimson, and Williams 1985). In patients with FHF resulting from paracetamol overdose, metabolic acidosis is frequently found, while in those with FHF arising from other causes, alkalosis, though rare in itself, is more common than acidosis (Bihari et al. 1985).

Haemodynamic instabilities such as diffuse vasodilation and secondary arterial hypotension often occur in FHF. Patients also display supply dependent oxygen consumption (Shoemaker, Appel, and Kram 1986) which implies an underlying tissue oxygen debt. One possible cause of this is the development of interstitial oedema giving an increased distance between capillaries and cells (Wendon, Alexander, and Williams 1990). The two organs most likely to suffer primary or secondary hypoxic damage are the liver (already damaged) and the brain (due to cerebral oedema, disussed later). The importance of avoiding hypoxic damage to the liver and achieving adequate oxygenation during liver support will become increasingly apparent as this thesis develops.

Increased succeptibility to bacterial and fungal infection is common in patients with FHF. One study of 35 patients with FHF revealed that 83% had bacterial infections while 40% had fungal infections (Rolando et al. 1990). These findings most probably relate to compromised immune function. Endotoxaemia is also prevalent.

The findings which have the greatest prognostic implications are the encephalopathy itself and other symptoms associated with it. In particular, the grade of encephalopathy which the patient ultimately attains correlates very strongly with outcome. Cerebral oedema develops in most patients who reach grade IV encephalopathy. It is associated with increased intracranial pressure and is a major

cause of death (Ware, D'Agostino, and Combes 1971).

1.2.4. Course of Disease

In cases of paracetamol overdose, the encephalopathy usually develops within 3 or 4 days from the time of drug ingestion and progresses rapidly through the various grades within 48 hours. Renal failure may appear before encephalopathy, probably as a result of direct renal toxicity.

In those patients with virally induced liver failure, the rate of progression varies but is usually fulminant (Benhamou 1990) and renal failure will normally only appear when encephalopathy reaches or exceeds grade III.

Cases of mushroom poisoning generally follow a fulminant course similar to that of paracetamol overdose while liver failure resulting from drug reactions, cardiovascular syndromes or metabolic disorders usually progresses more slowly.

1.2.5. Prognosis and Classification of Patients

A clear prognosis at the earliest opportunity is essential for the appropriate treatment and management of patients with hepatic failure. The primary prognosticator is the severity of encephalopathy. Prognosis is good if encephalopathy does not exceed grade I or II. (Williams and Wendon 1990). However, when encephalopathy progresses to grade III or IV additional parameters have to be considered. Major liver clinics have developed sets of prognostic criteria which are normally used to select appropriate treatments and in particular to indicate transplantation. Centres generally agree that, in addition to grade III or IV encephalopathy, severe coagulopathy and an age of > 40 years correlate with a poor prognosis (O'Grady et al. 1989; Tygstrup and Ranek 1986).

Quantitative liver function tests might also be used to predict outcome. Galactose elimination capacity and caffeine clearance determinations have been evaluated as prognostic aids. One group concluded that neither test was useful in predicting outcome from fulminant hepatic failure (Nagel et al. 1991) while other investigators found that serial determination of galactose elimination capacity was

useful as a prognostic indicator in patients with chronic liver disease (Reichen, Widmer, and Cotting 1991). Metabolism of drugs, such as lignocaine or midazolam, by the cytochrome P450 enzyme system has also been used to quantify liver function.

It is worth noting at this stage that there appears to be no clinical consensus on the value of such quantitative liver function tests. This is a cause of concern for those groups attempting to develop hybrid artificial liver support systems. The absence of clear clinical opinion about the significance of particular liver functions in terms of recovery from liver failure means that any *in-vitro* data from a given hybrid artificial liver system is, at best, an indication of what functions the cells within can perform rather than whether the system is likely to be effective as a clinical therapy.

As previously indicated, prognosis also correlates with aetiology and course. Survival rates are highest in paracetamol overdose or mushroom poisoning cases. FHF resulting from idiosyncratic drug reactions has a very poor prognosis. Broadly speaking, when grade IV encephalopathy develops survival rates are usually no more than 10% while in those patients with grade III encephalopathy survival ranges from as low as 10% (idiosyncratic drug reaction or indeterminate causes) to as high as 50% (hepatitis A virus or paracetamol) (Benhamou 1990; O'Grady et al. 1989). When hepatic failure is virally induced with hepatitis B or D, survival lies between these limits (at around 25%).

It would appear to an investigator who was examining survival statisitics that survival is most likely when symptoms progress rapidly providing encephalopathy does not exceed grade II, and complications, especially severe coagulopathy and renal failure, are absent. However, this sub-group of patients can only be identified in retrospect. Therefore, early classification of patients is imperative. Those patients with poor prognosis, and who meet any criteria for being placed on a transplant waiting list, must be identified as soon as possible since they will generally deteriorate and their condition at the time of operation will influence the outcome.

1.3. Intensive Care in Acute Hepatic Failure

Irrespective of prognosis or patient classification, aggressive liver-oriented

intensive care is essential since no general conservative therapy is available. For those patients awaiting transplantation, support is required to maintain other body systems until an organ is obtained. In cases where transplantation is not applicable, intensive care is neccesary to retain as much body function as possible for as long as possible in the hope that the damaged liver will regenerate. In principal, the clinician attempts to avoid the onset of life-threatening complications or to control them once recognised.

Lactulose can be administered in conjunction with a reduced protein diet as a prophylactic measure against encephalopathy (Conn 1977). Intracranial pressure (ICP) monitoring is effective in detecting cerebral oedema early. A subdural transducer can be applied for this purpose (Blei et al. 1993) where the only complication likely to occur is local wound bleeding (Keays, Alexander, and Williams 1990). If raised ICP is detected early then therapeutic intervention may prevent irreversible brain injury. Hyperventilation, infusions with hyperosmolar mannitol and administration of thiopentone can all be used to manage ICP (Ede and Williams 1986; Forbes et al. 1989). Haemodialysis may also be beneficial at this stage since renal failure is likely. When renal failure is secondary to liver failure, the kidney function will not improve until liver function improves.

In these patients, bleeding complications are common. Coagulopathy is normally treated with fresh frozen plasma and/or platelet concentrates. Clotting factors may also be administered.

The success rate of intensive care management is the yardstick by which other therapies are, and will continue to be judged. For a novel therapy to be deemed valuable it must be shown to be more effective than intensive care management alone in a controlled trial. To date, only liver transplantation has been universally accepted as being more beneficial than intensive care alone. As we have seen, survival rates depend on aetiology, rate of progression, age of patient and the presence and severity of complications. Generally speaking though, about 15-25% of patients with FHF can expect to survive if treated with intensive care medicine alone (O'Grady et al. 1989; Rueff and Benhamou 1973).

1.4. Liver Transplantation

For the time being, discussion will be limited to orthotopic liver transplantation (OLT). This is the process by which the patient's own liver is removed and subsequently replaced with a human donor organ. Several variations on this theme have been tried with varying degrees of success. These will be discussed later.

It is accepted that, for those FHF patients for whom major surgery could be considered, transplantation would ideally be the therapy of choice. The survival of FHF patients after OLT has been reported as ranging from 50% to 80% (Bismuth and Samuel 1989; Brems et al. 1987; Schafer and Shaw 1989; Vickers et al. 1988). However, there are many complicating factors.

The most apparent problem associated with OLT is the dire shortage of suitable donor organs. This shortcoming, and the monetary expense, means that unnecessary tranplants must be avoided. Therefore, stringent patient selection is essential. Patients are selected for transplantation if sufficient indicators of a poor prognosis are present and no absolute contraindications exist. The criteria used for this assessment vary to some extent from one clinic to another.

1.4.1. Indications for Liver Transplantation

At least two centres have reported placing all patients with grade IV encephalopathy, who lack contraindications, on transplant waiting lists (Lidofsky et al. 1992; Samuel and Bismuth 1990). In the U.K., where paracetamol induced liver failure is more frequently found, at least one group use different criteria for paracetamol and non-paracetamol cases (O'Grady, Tan, and Williams 1990). For paracetamol cases, the main prognosticator used is arterial pH. Transplantation is indicated when this falls below 7.3 or, when prothrombin time exceeds 100 seconds and serum creatinine is greater than 300 $\mu mol\ l^{-1}$ and encephalopathy of at least grade III is present. In other cases, transplantation is indicated when a prothrombin time of more than 100 seconds is recorded or any 3 out of 4 secondary criteria (jaundice for more than 7 days prior to encephalopathy, age <10 or >40 years, prothrombin time >50 seconds and

serum bilirubin >300 μmol l^{-1}) are met. In Berlin, transplantation is indicated when serum bilirubin exceeds 20mg dl^{-1} (342 μmol l^{-1}), prothrombin time is excessively prolonged (>100 seconds), clotting factors II, V and VII decline to 20% of normal values and acute renal failure or grade III or IV encephalopathy is present (Gerlach 1994 personal communication).

1.4.2. Contraindications for Liver Transplantation

The main medical contraindications are uncontrolled infection and severe brain stem dysfunction. Uncontrolled sepsis will progress rapidly once immunosuppression commences. Those patients with brain stem dysfunction (as a consequence of cerebral oedema) with prolonged fixation and dilation of the pupils will not usually recover sufficiently if attempts to alleviate raised ICP have been unsuccessful. A positive serological test for H.I.V.or active substance abuse at the time of admission may also preclude patients from being transplant listed (Lidofsky et al. 1992). Furthermore, any patient with significantly improving liver function will not normally be transplanted.

Despite these strict criteria, some FHF patients still die while waiting for a donor organ.

1.4.3. Emergency Liver Transplantation

Whilst it would be fair to say that all OLT operations are something of an emergency, this definition applies to those procedures in which a less than satisfactory donor organ is used to keep the patient alive in the short term, either because a suitable organ has not become available in time or because problems have arisen before, during or after surgery which have rendered the intended graft unusable or non-functional. The emergency grafts may be of an incompatible ABO blood group, a size mis-match or be generally of poor quality.

It is hoped that the incidence of these operations can be reduced. Some recent technical improvements should help to achieve this. Increased efficiency in donor organ preservation (Kalayoglu et al. 1988) and refined surgical methods (Neuhaus et

al. 1990; Neuhaus et al. 1994; Starzl, Iwatsuki, and Esquivel 1985) should mean that rates of organ matching can be improved and failures due to surgical problems can be reduced.

1.4.4. Primary Non Function

In some cases the grafted liver may fail to start functioning properly. When no technical or immunological reason exists, this is referred to as primary non function (PNF). This has occurred in between 2% and 23% of cases reported in recent literature (Clavien, Harvey, and Strasberg 1992; Strasberg et al. 1994). PNF is normally treated by early re-transplantation (Ringe, Neuhaus, and Lauchart 1989). This approach has been questioned (Agnes et al. 1992) since there is a possibility that these grafts might regenerate with appropriate treatment. Indeed, Grieg and co-workers reported survival in 8 out of 10 patients with PNF who were treated with prostaglandin E_1 (Greig et al. 1989).

1.4.5. Other Transplantation Techniques

There are a number of other surgical procedures used to graft new livers or parts of liver into, or to remove damaged liver tissue from patients with liver failure. Orthotopic living-related liver transplantation has become a proven therapy for children with chronic liver disease. In this procedure the child receives a lobe of liver from a living related donor. The likelihood of rejection is reduced, and the liver of the donor will usually regenerate, while the lobe transplanted into the child should grow normally. A series of such operations was reported in the national press (Fletcher 1994). There is no actual impediment to the use of this technique in FHF (Schafer 1993).

The placement of a cadaveric graft somewhere else in the body to preserve liver function while the native liver is left in place to recover is known as auxiliary liver transplantation. Very few cases have been reported as this is technically a very difficult procedure. It is hoped that the graft would be removed (or rejected) after a period. This is a potentially rewarding therapy as it leaves the native liver in place.

However, the presence of the graft might actually inhibit regeneration of the native organ. This dichotomy illustrates the attractiveness of temporary extracorporeal support measures.

Partial orthotopic cadaveric liver transplantation is another technically difficult procedure which has been successfully used to treat a number of children with FHF (Schafer 1993). In this technique, a portion of the left cadaveric lobe replaces the left lobe of the patient. Eventually immunosuppression therapy is stopped causing the graft to degenerate and hopefully leaving the patient with a functioning liver.

Partial orthotopic living related liver transplantation is an un-tested theoretical therapy by which a lobe from a living related donor could be grafted onto the failing liver.

The other significant area of transplantation is that of xenografts. Whole and partial xenografts have been conducted with chimpanzee, and more recently with baboon livers. Two areas of controversy exist in this field. Firstly there is the ethical problem of the use of animals for such purposes and secondly, primates can harbour viruses which can infect humans. Such infections are a major problem in immunosuppressed patients. The advent of the transgenic age means that purpose-bred, virus-free donor animals may be available in the future. Controversy surrounds this subject too. Recently, the Nuffield Committee on Ethics considered these topics. No absolute judgements were made but concerns were expressed about whether xenografts could be ethically used in humans whilst there is a possibility that infectious agents could be introduced at the same time.

Another controversial procedure used in the field of liver transplantation is total hepatectomy. The liver of the patient is removed with the aim of stemming deterioration. The rationale behind this is that the cirulating levels of necrotic factors (liberated by the decaying liver), which some investigators consider to contribute to accelerated liver degeneration and complications (Ringe and Pilchmayer 1988), are reduced. Advocates of this procedure claim that patients with severe acute liver failure have shown instant dramatic improvements when hepatectomised while awaiting transplantation (Ringe 1993; Rozga et al. 1993b), however the validity of

this process has been questioned recently (Lee 1994). In this article, Lee argues convincingly that it is bordering on unethical to remove the liver from FHF patients before a donor organ has become available since this absolutely removes any prospect of recovery which may have existed, however small, and ensures that the patient will die if a donor organ does not become available very quickly. His argument is strengthened further by the assertion that no positive evidence of benefit exists for this "heroic" therapy. In particular, the almost instantaneous, though very often fleeting, improvements reported after total hepatectomy suggest that any humoral factors liberated by the necrotic liver which contribute to patient decline must have an astonishingly short half-life in the body. Also, the contribution made by the hepatectomy cannot be isolated from the effects of any other therapies which are concurrently administered. Lee concludes that this procedure should be strictly limited to application in those patients with a certain prognosis of immediate mortality.

1.5. Requirement For Liver Support Therapies

The preceding sections have described the various disease states and accepted therapies for liver failure. It can be seen that several situations arise in which a novel liver support therapy could be applied.

In FHF, liver support therapy could in theory be used to keep patients awaiting transplants alive until a suitable organ became available. This would hopefully reduce the incidence of emergency OLT and may also improve patient condition at the time of surgery, thereby improving success rates. Indeed, if the therapy was sufficiently effective, some patients may improve to the extent that they no longer required a transplant. It could be argued, however, that if liver support devices simply kept patients alive while awaiting a transplant then the limited supply of donor organs would be stretched even further. The scenario of wards full of patients permanently attached to liver support devices might be a possible consequence.

In those FHF patients with grade I or II encephalopathy, liver support systems might be able to prevent the disease progressing further and hence aid regeneration. PNF might also be addressed with liver support therapies. A period of liver support

may allow the graft to regain function or may maintain the patient until another suitable organ is available thus avoiding early emergency re-transplantation.

Patients with chronic liver failure might also benefit from such supportive therapy. It may give some improvement in condition if applied periodically (rather like dialysis in kidney failure) or it might be used to treat those periods of acute decompensation which sometimes occur. However, since in the near future any effective liver support measures which emerge are likely to be extremely expensive, this particular type of application will probably be rare until costs can be reduced. (The immunological consequences of chronic therapy with heterologous cells in a hybrid artificial liver may also be serious.) On the other hand, in chronic liver failure patients awaiting a transplant, a support device could be administered for a short period prior to surgery with the aim of improving success.

Liver support devices could also be used as a surgical aid to bridge any anhepatic period or to allow more difficult transplantation procedures such as those mentioned above to be attempted with greater confidence.

In the resection of liver tumours, the amount of tissue which can be removed is limited by the requirement to leave behind sufficient liver mass to support the patient through the regenerative period. Effective liver support devices might therefore make surgical resection of liver tumours a safer procedure by allowing the surgeon to remove more tissue if neccesary.

Finally, neonates with impaired liver function, which may result in brain damage, might also be treated with such therapies.

1.6. Liver Support Therapies: Clinical Experience

Many approaches have been tested over the years. However, to this day no supportive measures outwith the realms of normal intensive care medicine have been universally accepted as being beneficial to the patient with FHF.

The various therapies applied can be divided into four categories:

I Ex-vivo liver perfusion techniques

II Extracorporeal filtration, separation or exchange of blood or plasma

III Use of adsorbents

IV Biological liver support

Each of these categories will now be discussed in turn. The discussion on hybrid artificial liver support in this chapter will be restricted to reported clinical usage since a detailed critical analysis of the various device configurations will be given in Chapter 3.

1.6.1. Ex-Vivo Liver Perfusion

Since orthotopic transplantation of primate livers can support human life, one might also expect perfusion of excised primate livers with human blood to work. However, Pohlein and co-workers have recently shown that immunological interactions take place when human blood is perfused through isolated rhesus monkey livers to a far greater extent than when isolated human livers are perfused with human blood (Pohlein et al. 1994).

Yet, in spite of earlier failures (Eiseman, Liem, and Raffucci 1973; Otto et al. 1958), a recent series of operations was undertaken by Chari and co-workers in which 4 patients underwent *ex-vivo* haemoperfusion with isolated pig livers (Chari et al. 1994). Of these 4 patients, 3 died, despite showing signs of improvement during perfusion sessions, and 1 survived after receiving a transplant. The authors attribute the failures to the limited time for which perfusions could be performed. The pig livers tended to fail (noted by a reduction in oxygen consumption and bile production) after some 2 to 4 hours of perfusion. This was considered to be due to immunological problems, manifested by massive invasion of the pig livers by human neutrophils. Lack of evidence of hyperacute rejection was reported. A recent experimental report described a system whereby hepatectomised pigs underwent continuous ex-vivo haemoperfusion with isolated pig livers for periods of up to 24 hours without significant loss of hepatic function or necrosis (Neuhaus and Blumhardt 1993). The authors of this paper, together with Schafer (Schafer 1993),

envisage that the best hope for effective clinical usage of this type of procedure lies with the use of isolated human livers. Only those human livers which are unsuitable for transplantation could ethically be used for this purpose. At this time it is unclear whether such organs would be useful in temporary ex-vivo perfusion systems. It is evident therefore that the future prospects of this type of therapy are difficult to gauge.

1.6.2. Extracorporeal filtration, separation and exchange

As previously discussed, haemodialysis and continuous arteriovenous haemofiltration are used to treat or prevent renal failure in FHF. However, the widely held belief that hepatic coma is caused by removable toxins led to many investigators attemting to treat FHF by dialysing, filtering or replacing the blood or plasma of their patients.

Elevated concentrations of abnormal 'middle molecules' are thought to promote cerebral oedema in FHF (Leber et al. 1981). However, the continuous removal of such 'middle molecules' by haemofiltration did not improve survival in one study of 16 patients with FHF (Matsubara et al. 1990). The authors suggest that this therapy might be useful when combined with plasma exchange as a means of supporting patients awaiting a transplant. Indeed, one uncontrolled series of experiments with this therapy has subsequently been reported (Yoshiba et al. 1993). Of the 27 patients given this therapy, 15 survived (after transplantation), while 4 out of 4 patients who only received intensive care and plasma exchange also survived. In another investigation, (Kondrup et al. 1992) 5 out of 11 patients with FHF survived when treated with high volume plasma exchange. All the survivors had paracetamol induced FHF. As we have discussed, survival after paracetamol induced FHF can be as high as 50% when treated with intensive care alone. It is very difficult to draw any meaningful conclusions from such studies. One thing is clear though; this type of therapy cannot itself reverse hepatic failure. The best hope for such measures is to sustain life during the wait for a transplant. Schafer concludes, in his review of therapies for FHF (Schafer 1993), that such measures will have to be combined with

other treatments to assist in the support of FHF patients.

One interesting development in this field merits further mention. Many of the toxins which accumulate in liver failure are protein-bound (unconjugated bilirubin, fatty acids and certain aromatic compounds) and these cannot be removed by conventional haemofiltration methods. Two techniques for removing these toxins have recently been reported. One describes the addition of human serum albumin to the dialysate in peritoneal dialysis. This was shown to enhance the clearance of bilirubin and phenols from rats with obstructive jaundice (Sarnatskaya et al. 1993). The second details the addition of albumin to dialysis fluid and the impregnation of the membranes of a hollow fibre dialyser with albumin to clear protein-bound toxins from liver failure patients during haemodialysis (Stange et al. 1993). In one case this was reported to result in 90% removal of toxic markers. This procedure was reviewed recently in New Scientist magazine (Phillips 1994). Here, it was again suggested that this technique should be employed in conjunction with other measures to produce maximum effect.

1.6.3. Adsorbents

Amongst the non-biological liver support therapies, perhaps the best hope of useful future therapy lies in the application of adsorbent materials. Chang was first to report the use of charcoal haemoperfusion in FHF (Chang 1973). Since then many studies have suggested that this therapy is beneficial (Asanuma et al. 1980; Gelfand, Winchester, and Knepshield 1978; Kennedy, Greaves, and Triger 1985). However, a large controlled trial demonstrated that this therapy did not offer an improvement over intensive care management alone (O'Grady et al. 1988). Despite these findings, many groups have continued to work in this field and the development of novel adsorbent materials has given fresh impetus to this effort. Improvements in sorbent technology mean that smaller particles can now be produced. A recently reported novel system for blood purification, with high recirculation rate treatment of plasma filtrate with such small particle adsorbents, markedly improves the kinetics of sorbent perfusion processes (Weber et al. 1994).

One of the reasons why early enthusiasm for sorbent application in FHF was later somewhat dampened might have been the dramatic improvements in intensive care management which were simultaneously appearing. These improvements might have masked the successes found in earlier efforts. Another reason for the lack of certainty about the actual contribution each therapy makes to any patient recoveries is the complexity of treatment each patient receives. It would not be ethical to deny patients components of therapy which are known to be beneficial in order to evaluate the efficacy of a novel unproven therapy. This problem makes the evaluation of novel therapies in animal models of liver failure a very attractive approach. However, this direction requires a representative and reproducible model of liver failure in a suitable animal and the development of specially modified apparatus for the therapy under evaluation. A notable contribution to this field was the development of 'Reverse Plasma Exchange' (Ryan, Aslam, and Courtney 1990). In this system, where plasma from rats with galactosamine induced liver failure is transferred to healthy donor animals, recipients of untreated plasma reproducibly develop the same symptoms as the donor animals when the 'coma plasma' is infused via the systemic circulation (bypassing the liver). When the 'coma plasma' is introduced to the portal vein, no symptoms are manifested. Using this model, the authors demonstrated that perfusion of 'coma plasma' across charcoal led to an improvement in the survival of rats receiving plasma from donors with grade II encephalopathy but no benefits were found when donors had grade III encephalopathy. A more complex sorbent system, which featured charcoal, a bilirubin and bile removing resin and sepharose beads with bound polymixin B sulphate (a material effective in removing endotoxin), did lead to increased survival when grade III encephalopthy donor plasma was treated prior to infusion into recipients, though the improvement was less marked than in grade II. This report suggests that sorbents might be most effective if administered at an early stage of disease but implies that they may be of limited use as an isolated therapy in advanced FHF.

1.6.4. Biological Liver Support

Most investigators consider the inability of the aforementioned support therapies to reproduce all of the de-toxifying and synthesising functions of the liver to be the chief reason for their overall lack of success. Because of this, more and more effort is being directed towards the development of devices and therapies that incorporate living parenchymal liver cells (hepatocytes). The potential advantages offered by this type of therapy are manifest. The native liver can be left in place, hopefully to completely regenerate, while 'liver function' is carried out by the extracorporeal device with minimal surgical intervention. However, the complexity of this approach has meant that devices and techniques have been slow to emerge and, as yet, not one has definitely proved to be beneficial. Some early work considered the possibility of transplanting living hepatocytes into the peritoneal cavity of patients with FHF (Demetriou, Rozga, and Moscioni 1990), however, this approach has largely been abandoned in favour of the development of extracorporeal bioreactors containing cultured hepatocytes. Several groups throughout the world are presently at an advanced stage of research in this area. Yet at this time, few groups currently working in the field have reported clinical applications of such devices.

One of the first such publications (Sussman et al. 1992) described the treatment of a 68 year old woman who had FHF of indeterminate cause with the Hepatix ELAD. The patient was reported to show signs of improvement during therapy despite going on to die from septic shock. The same group subsequently detailed the application of the same device in a case of syncytial giant-cell hepatitis where a complete recovery was obtained (Sussman et al. 1994a). This device went on to minor clinical trials at a small number of liver transplant centres. However, for reasons which have not been published, the development program of this system has come to a complete halt.

A second group initially described the treatment of 9 patients (Neuzil et al. 1993; Rozga et al. 1993b; Rozga et al. 1994b) with the Cedars-Sinai BAL (now known as the Circe Biomedical Hepatassist 2000). Of these 9 patients, 8 survived, though 7 of these 8 received a transplant within 24 hours of treatment with the device and

they each received normal intensive care treatment. These are impressive statistics but they do not permit any meaningful conclusions about the efficacy of the device to be drawn. In some cases a charcoal perfusion column was incorporated in the extracorporeal circuit. Due to the nature of this particular support system, these patients also underwent plasmapheresis. Once again it can be seen that the complexity of the FHF syndrome and the diversity of measures employed against it in each case, confound the scientist attempting to evaluate novel therapies.

Biological liver support systems, and in particular extracorporeal hybrid artificial liver devices, remain the best prospect for future treatment of FHF. However, much of the existing effort in this field has been based on methods which can, at best, be described as empirical.

With necessity being the mother of invention, pioneering artificial organ developments have usually resulted from the efforts of clinicians, the artificial kidney is a case in point. There has been a tendency for bioengineers to become involved at some later stage and (hopefully) they have helped to refine devices by employing established principles of science and engineering.

A methodical approach is now required if hybrid artificial liver devices are to fulfill their potential and become clinically useful and commercially viable. The rigorous analysis of existing devices which forms part of chapter 3 of this thesis will reveal why, along with others, the two devices mentioned above are, at best, sub-optimal in terms of design and mode of application.

1.7. General Thesis Objectives

The broad aims of this thesis are to establish critical design parameters for hybrid artificial liver support systems and to consider and evaluate prototype bioreactor configurations.

However, before identifying the design requirements of an optimal hybrid artificial liver, certain aspects of *in-vivo* liver physiology must first be discussed. This will be undertaken in the next chapter at the end of which the specific aims of this thesis will be detailed.

Chapter 2

Liver Structure and Function

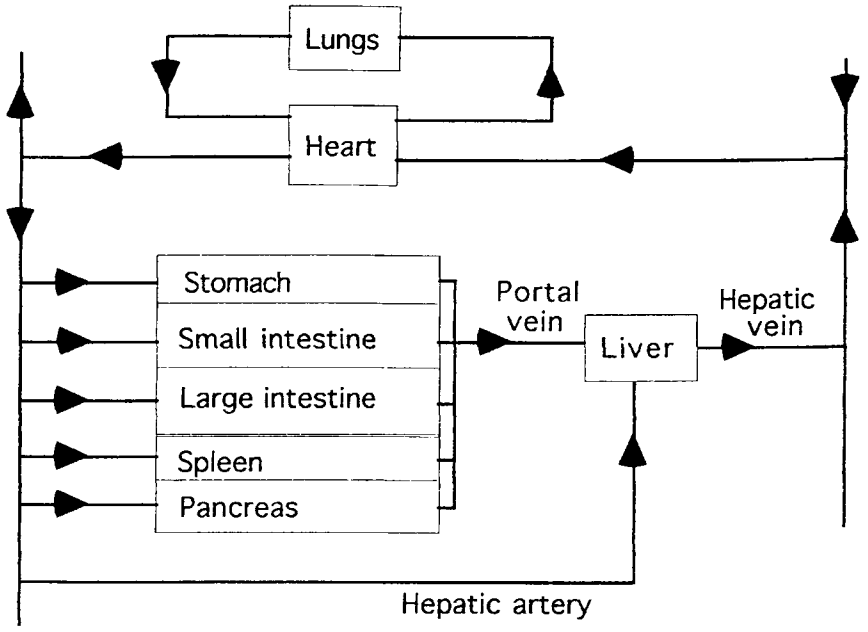
2.1. Introduction

This chapter will discuss those areas of liver biology and physiology which are immediately relevant to the development of hybrid artificial liver support devices. It is beyond the scope of this thesis to cover all aspects of liver function. A brief general description of the liver will be followed by a detailed assessment of the basic functional liver sub-unit (the hepatic acinus) and how its structure relates to liver function. Subsequently a discussion of key aspects of liver metabolism will provide the basis for an appraisal of the requirements of bioreactors for liver support.

2.2. The liver

The liver is the largest visceral organ in the body. It accounts for approximately 2% of body mass (1.5kg in a typical 70kg man).

Figure 2.1. indicates the position of the liver within the circulatory system. All of the blood leaving the absorptive digestive tract, the pancreas and the spleen passes through the liver via the portal vein prior to entering the systemic circulation.



The Location of the liver within the general circulatory system.

Figure 2.1.

The portal vein provides about three quarters of the liver's blood supply (Schenk et al. 1962) with the remainder being delivered via the hepatic artery. As a whole, the liver receives approximately 1.5 litres of blood flow per minute (Bradley et al. 1945), this is equivalent to 25% of total cardiac output. Total hepatic blood flow is increased by food ingestion (Orrego et al. 1965) and decreased during vigorous exercise (Wade et al. 1956). The liver is a highly vascularised organ containing approximately 30ml blood per 100g tissue (Lautt 1977). This represents some 10-15% of total body blood volume and makes the liver the largest blood reservoir in the body.

2.2.1. Overview of liver functions

The functions which the liver carries out can be divided broadly into three main categories. The first is metabolic regulation. The opportunity which the liver has to sample the entire blood flow from the absorptive intestinal tract before it reaches

the systemic circulation permits the regulation of the levels of a vast number of metabolites. Compounds at excessive concentrations are removed and stored (e.g. fat soluble vitamins) or toxins are modified to permit inactivation or excretion. Compounds which are depleted are replenished by mobilising reserves or by de-novo synthesis.

The second general function is haematological regulation. Dying or damaged red blood cells are removed by phagocytic cells in the liver. Many important plasma proteins are synthesised by the liver and these play roles in the osmotic regulation of blood, in the coagulation system and in the transport of nutrients.

Finally, the third general liver function is the synthesis and excretion of bile. Bile is an aqueous salt solution rich in lipids and bilirubin (the metabolic product of haeme units from phagocytosed red blood cells). The lipids in bile are mainly derived from cholesterol and are collectively known as bile salts. The liver secretes about 1 litre of bile each day. Between meals it is stored and concentrated in the gall bladder. Cholecystokinin stimulates the release of bile into the small intestine. This occurs when chyme enters the small intestine and bile secretion is increased when the dietary fat content is high. The bile salts help to emulsify this fat and promote interaction between lipids and lipid digesting enzymes from the pancreas.

The manner in which the architecture of the liver enables the organ to carry out this vast array of functions must be understood if critical design parameters for hybrid artificial liver support devices are to be identified.

2.3. The hepatic acinus

Histologically, the liver appears to consist of multiple three-dimensional sub-units known as lobules which are approximately hexagonal in section. These lobules are composed of several acini in which the parenchymal cells (hepatocytes) are arranged in mainly single cell thick plates. It is the acinus which is, in fact, the functional sub-unit of the liver. Within an acinus, each plate of hepatocytes is adjacent to a sinusoidal space through which blood flows. These sinusoids are grossly enlarged, specialised capillary lumens which connect the blood supply with blood

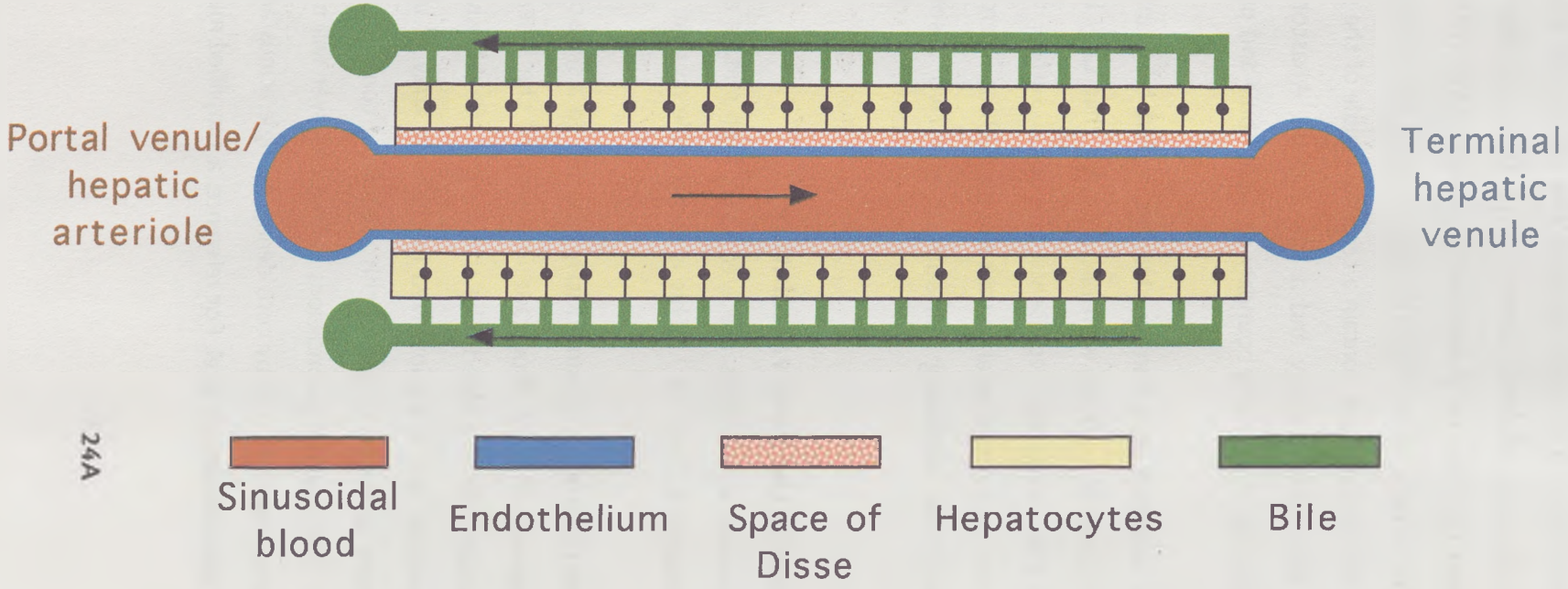
drainage. Covering each sinusoid is an irregular lining of endothelial cells, liver macrophages called Kupffer cells and occasional fat-storing Ito cells. Large fenestrations (up to 0.2μm) within the endothelium allow molecular species of up to 250,000 mol.wt. to pass freely back and forth between the circulation and the hepatocytes. A gap known as the Space of Disse, which is discontinuously filled with extracellular matrix, lies between the sinusoidal wall and the hepatocytes. The acini radiate outwards from a central venule of the hepatic vein towards portal triads at the six corners of each lobule. The portal triads contain a terminal venule of the portal vein, a terminal arteriole of the hepatic artery and a bile ductule. Anastomoses between these venules and arterioles create the boundary of each lobule and provide the blood for the sinusoids.

In each acinus, bile is secreted into narrow channels, called bile canaliculi, between adjacent cells. These canaliculi connect with bile ductules that carry bile to the nearest portal area. The bile canaliculus represents the apical surface of the hepatocytes and this surface has typical epithelial characteristics such as intercellular junctions. The canaliculi divide the remaining cell surface into two basolateral blood-contacting surfaces.

2.3.1. Operating conditions in the liver vasculature

The structure of the hepatic acinus gives the liver a very high surface area to blood volume ratio of approximately $0.27 m^2 ml^{-1}$. (This is comparable to that of the human lung.)

Within the acinus, every hepatocyte is immediately adjacent to a sinusoid and a bile canaliculus. This arrangement is very important in terms of the kinetics of exchange between the hepatocytes, bile and blood. The efficiency of the diffusive and carrier-mediated transport processes which are involved in the vast array of uptake, biotransformational and secretory activities is due to this specialised configuration in which all paths are very short. The operating conditions within the liver will now be discussed in terms of the macrocirculation and, by considering a single acinus and the separate fluxes within, at the microcirculatory level. For reference, a simplified model of



Idealised Model of the Hepatic Acinus

Figure 2.2.

the hepatic acinus is given in Figure 2.2.

2.3.2. Hepatic circulation

As indicated, the majority of the liver's blood supply arrives via the portal vein. Blood in this vessel has low kinetic energy so hepatic vascular resistance must be low for efficient perfusion. The primary venules of the portal vein are about 400μm in diameter narrowing to <280μm in the sinusoidal bed (Elias 1949). These vessels are valveless, enabling easy distribution of pressure changes to all the branches. The normal vascular pressure of the portal vein is about 6 to 10 mmHg above that of the inferior vena cava (Campra and Reynolds 1988).

The hepatic arterioles run adjacent to the branches of the portal vein and numerous anastomoses occur along each track. The pressure in the hepatic arterioles is approximately equivalent to that in the aorta. This merging of high pressure, high resistance arterial blood with low pressure, low resistance portal blood requires a mechanism of pressure equalisation. This is achieved through dilation or constriction of the range of arterial vessels by the action of smooth muscle cells. The larger arterioles (50-100μm O.D.) have two layers of smooth muscle while smaller arterioles (10-50μm O.D.) and arterial capillaries (<10μm O.D.) each have a single smooth muscle layer (Burkel 1970). These muscle cells are sensitive to flow and pressure changes (Johnson and Hanson 1966) and also respond to hormones, metabolites and bile salts (Rappaport and Schneiderman 1976).

These mechanisms achieve autoregulation of liver blood flow, that is, maintenance of local flow despite changes in pressure in the perfusing vessels.

The vascular pressure in the liver is a function of the contractile state of the arterial vessels and the intrahepatic resistance. When portal flow declines, the hepatic artery increases flow to the liver (Greenway and Oshiro 1972), although the converse does not occur (Ternberg and Butcher 1965). However, during episodes of portal hypertension, collateral flows may arise. Most significantly, portal collateral flow via anastomoses between the portal vein and the vena cava (these do not normally carry blood) can occur. This diverts intestinal blood around the liver and may expose the

brain to toxins (Campra and Reynolds 1988).

2.3.3. The microcirculatory unit

The sinusoids are 7 to 15 μm wide but may expand up to 180μm (Rappaport 1980). The sinusoidal pressure is 2 to 4 mmHg above that in the inferior vena cava. Arterial capillaries empty directly into the terminal portal venule or directly into the sinusoids. The terminal portal venule empties into a sinusoidal glomus feeding several acini. Each glomus of sinusoids is drained by at least two terminal hepatic venules, also known as collecting veins, with an outer diameter of about 45μm. Within the sinusoidal bed, there is total merging of portal and arterial blood.

The hepatic microcirculation represents an autoregulated system of low pressure flow in the sinusoidal bed which normally is effectively maintained despite changes in the flow, pressure or composition of blood in the perfusing vessels. This ensures the flux of solutes to the hepatocytes.

2.4. Hepatocyte heterogeneity and 'liver function'

Hybrid artificial liver devices must replace at least some functional aspects of the natural liver. An understanding of how the hepatic acinus produces 'liver function' would aid attempts to achieve such replacement. A consideration of the relationship between liver structure and function would enable design criteria to be established and would identify those unknowns which require to be resolved.

An important distinction must be made between the vast array of individual hepatic functional mechanisms and 'liver function' as a whole. This distinction is perhaps best understood by considering the question "What is the function of the liver?". Gumucio and Chianale have suggested that "the function of the liver is to regulate the concentration of solutes in the terminal hepatic venule and in bile" (Gumucio and Chianale 1988). This definition provides a good starting point from which to begin an analysis of how the structure of the hepatic acinus helps to bring about 'liver function' and how this might be reproduced *in vitro*.

The liver, as we have seen, is strategically located between the digestive

system and the systemic circulation. Therefore, the concentration of solutes in the terminal hepatic venule (THV) influences the concentration of solutes in the systemic circulation. The regulation of solute concentrations in the THV by uptake processes and synthetic and metabolic pathways contributes to the supply of nutrients for the brain, muscles and other organs. Regulation of the concentration of the components of bile acts as an excetory pathway and influences digestion.

Perfusion of hepatocytes within the acinus is unidirectional with blood from the terminal portal venule (TPV) being sequentially distributed to approximately 20 hepatocytes on each side of the acinar axis before emptying into the THV (Gumucio and Miller 1981). This distribution of blood flow is such that progressive removal of solutes gives rise to blood solute concentration gradients along the acinus, so different microenvironments exist around cells located near the inlet compared to cells near the outlet. Therefore, cells at the acinar inlet will contribute to solute transport differently to those at the acinar outlet. In other words, the contribution of any given hepatocyte to any given process depends upon its location within the acinus with respect to incoming sinusoidal blood. By extension, there will be a 'zonal' contribution to each liver function mechanism within the acinus. Furthermore, the composition of incoming blood will vary with time according to digestion and physical activity. Thus, while all hepatocytes in the acinus are potentially alike, they are not all doing the same thing at the same time and not all doing the same thing all the time.

This suggests that the function of the liver, as defined above, is the consequence of the overall expression of cellular mechanisms occuring with different intensities within different zones of the microcirculatory unit.

Several arbitrary classifications of hepatic acinar zones have been proposed. The initial observation that within a lobule, cells adjacent to the TPV or hepatic artery (HA) were morphologically different to those near the THV gave rise to the terms peri-portal and peri-venous which were used to describe these respective cell loci (Novikoff 1959). However, the structure and orientation of the acinus within the liver lobule means that while a cell may be geographically close to a TPV, it may in fact be some way along its own acinus towards its THV. Upon recognizing this,

Rappaport proposed that the acinus should be arbitrarily divided into 3 functional zones (Rappaport 1958).

In this classification, zone 1 defines those cells close to the acinar inlet, zone 3 identifies those cells adjacent to the acinar outlet and zone 2 represents a 'no man's land' where cells of an undefined nature exist. In reality there is obviously a gradual transition from 'zone 1' type cells to 'zone 3' type cells. Consequently, increases in the number of arbitrary zonal divisions along the acinar axis have been proposed (Jungermann 1995), but this does not seem particularly helpful. This hepatocyte heterogeneity requires to be placed within a functional context.

2.4.1. Solute transport in the hepatic acinus

The relative rates of uptake, metabolism and excretion of solutes at different points along the acinus will be influenced by modifiers; the uptake mechanism involved (diffusion or carrier mediated transport), the solute concentration in the TPV or HA, the affinity of the solute for binding proteins, the concentration of intracellular binding proteins and by sinusoidal structural determinants.

The structural determinants of solute uptake along the acinus are the sequential nature of perfusion and the changing geometrical configuration of the sinusoid.

The size and frequency of the fenestrations in the endothelium and the 'openness' of the Space of Disse mean that dissolved solutes of up to about 250,000 mol.wt. have free passage between sinusoidal blood and the basolateral hepatocyte membrane which is covered in microvilli to maximise its surface area. Therefore, any uptake resistances can be considered to occur at this membrane and/or from intracellular sequestration processes (Goresky and Schwab 1988). This means that the likelihood of collision of a solute with the hepatocyte membrane is a major factor determining its uptake rate.

Certain aspects of sinusoid structure differ along the acinar axis in a manner which would influence the frequency of such collisions. In zone 1, sinusoids are narrow and tortuous and have the greatest surface area to volume ratio (Miller, Zanolli, and

Gumucio 1979). Consequently, mixing and collisions are favoured. Two specific mechanisms occur as a consequence of the narrowness of sinusoids in this zone. 'Endothelial massage' is the process by which the rubbing of compliant red blood cells along the sinusoidal wall enhances transport and 'forced seiving' is the phenomenon resulting from compression of the sinusoidal wall by non-compliant white blood cells combined with the 'pumping' action of stellate cells (Wisse et al. 1985). In contrast, the sinusoids become wider and straighter in zone 3 and this configuration favours secretion by hepatocytes and fine adjustment of solute concentrations before the blood enters the THV (Gumucio and Chianale 1988).

Before considering the modifiers of solute uptake listed above, a short description of membrane carrier transport is required.

Membrane carrier transport is the process by which most solutes which are neither small enough nor sufficiently lipid soluble to permeate the membrane, enter the cell. There are three basic indications of whether transport of a solute is (predominantly) carrier mediated:

1. Saturation Kinetics:	As the concentration of solute increases, the rate of uptake increases until a plateau value is reached due to there being a finite quantity of carrier. The constant 'K_m' is quoted for a given process and this is equivalent to the solute concentration at which the uptake rate is half maximal.
2. Competitive Inhibition:	Introduction of a competitive solute with high affinity for a suspected carrier will inhibit transport.
3. Counter Transport:	Solutes can be transported against a concentration gradient when more free carrier exists on the low solute concentration side of the membrane.

The highest concentration of incoming solutes is found at the acinar inlet, so all other things being equal, those solutes which undergo purely diffusional transport

will have the greatest uptake rate in zone 1. Therefore, incoming solutes with low carrier binding affinity will be limited to a few hepatocytes near the acinar inlet and their concentration in the THV will be low. Carrier mediated solute transport will also initially occur at a high rate in this zone providing the relevant carriers are present. However, if there is an increased concentration of carriers further downstream, for which the solute in question has a high affinity, then the uptake rate will subsequently be greater in zone 2 or 3 once the process has become saturated in zone 1.

Having explained the physiological and transport phenomena which influence the fate of solutes in the hepatic acinus, the manner in which the acinus brings about 'liver function' can now be investigated. This will be undertaken by considering a set of key aspects of liver metabolism in sequence. In each instance, the objectives will be established and the specific mechanisms will be outlined before considering the whole process at the acinar level. The metabolic parameters to be considered were selected for their immediate relevance to liver support, their suitability for illustrating acinar function and their potential as indicators of 'liver function' *in-vitro*.

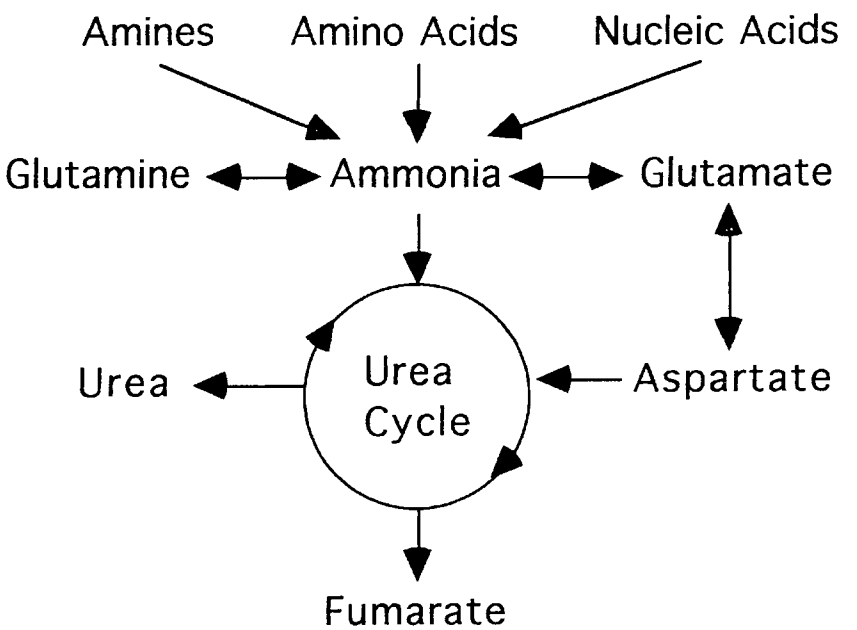
The synthesis of vital proteins such as coagulation factors is omitted from this stage of discussion. The reasons for this omission being that such factors can be directly administered to liver failure patients if necessary (so retention of these functions in a hybrid artificial liver is (arguably) relatively unimportant), little is known about the role of the hepatic acinus in the synthesis of these compounds and the measurement of the synthesis of such factors in extracorporeal systems is difficult.

2.5. Ammonia metabolism

In ammonia metabolism, the primary objective of the acinus is to regulate the concentration of ammonia in the THV. A secondary objective is to regulate the concentration of glutamine (a storage and transport form of ammonia) in the THV.

Ammonia arises mainly from protein and nucleic acid catabolism (much of this via glutamate). The liver is a major site of ammonia metabolism. The steady state concentration of ammonia and ammonium ions is at least 10 times greater in the

liver than in blood plasma. In the liver, ammonia is predominantly metabolised to urea via the urea cycle. About 90% of surplus nitrogen is disposed of in this way. This is essentially an irreversible reaction although, certain bacteria contain ureases which convert urea to free ammonia (Powers-Lee and Meister 1988). The other fates of ammonia in the liver are conversion to glutamine, catalysed by glutamine synthetase (reversible by glutaminase), quantitatively the second most important route, and formation of glutamate and fumarate, neither of which are quantitatively significant pathways (Bean and Atkinson 1984). A generalised schematic diagram of ammonia metabolism in the liver is given in Figure 2.3.



Pathways of ammonia metabolism

Figure 2.3.

2.5.1. Urea Synthesis

The liver is the only significant site of urea synthesis in the body. In the urea cycle, half of the nitrogen is directly sourced from free ammonia and half is derived from aspartate. The cycle is generally catalysed by cytosolic enzymes. However, two

urea cycle enzymes; carbamylphosphate synthetase and ornithine transcarbamylase, are restricted to the mitochondrial matrix. This requires that two compounds produced in the cycle, namely ornithine and citrulline enter and leave the mitochondrion respectively (Powers-Lee and Meister 1988). These processes require active transport. Overall, the cycle utilises four high energy phosphate cleavages, this together with transport phenomena make it an inefficient process from both an energetic and a mass transfer viewpoint.

The urea cycle is strictly regulated and maintains a steady state ammonia concentration in the liver of about 0.7mM with a resultant concentration in the circulation of approximately 30μM. This is necessary because, although highly toxic at excessive concentrations, ammonia is an essential precursor of many endogenous nitrogen compounds including amino acids.

2.5.2. Glutamine synthesis

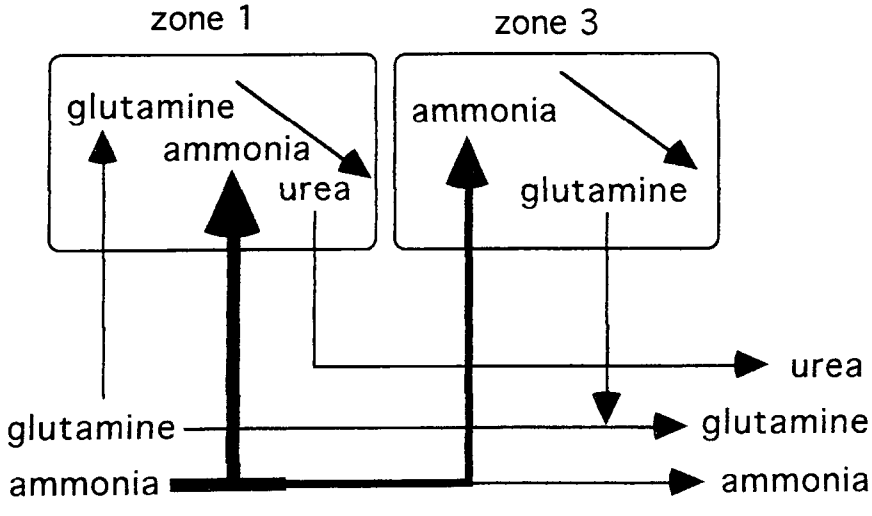
Glutamine synthesised in the liver is transported to the peripheral circulation. Upon delivery to other organs, local glutaminases catalyse the release of free ammonia as required for other synthetic reactions. This pathway can become quantitatively important in terms of bulk ammonia removal when urea synthesis is impaired (Haussinger and Gerok 1986).

2.5.3. Ammonia metabolism and the hepatic acinus

Studies have shown that the urea cycle enzymes occur at higher intracellular concentrations in zone 1, while glutamine synthetase occurs predominantly in zone 3 (Gaasbeek-Jansen et al. 1984; Gebhardt and Mecke 1983). These findings lead to the obvious hypothesis that ammonia in zone 1 is metabolised to urea while any remaining excess reaching the hepatocytes of zone 3 is scavenged and converted to glutamine. Support for this can be found from earlier studies on perfused rat livers (Haussinger 1983). This two-stage mechanism will clearly enable the two main objectives of ammonia metabolism cited above to be achieved. A heavy incoming load of ammonia and glutamine is processed to urea by a high capacity, low sensitivity

process. This in-turn neccesitates a downstream process with high sensitivity for fine adjustment of the concentration of ammonia and glutamine. The recycling of glutamine prevents it from accumulating in the circulation. A diagrammatic representation of this two-stage process is given in Figure 2.4.

This arrangement also enables the acinus to regulate the pH of sinusoidal blood and hence the blood pH in the systemic circulation. Regulation of blood pH requires the HCO_3^-/CO_2 ratio to be continually modulated. Hepatic urea synthesis provides a means of bicarbonate utilisation. (The kidney alone is unable to clear the quantity of bicarbonate generated by a meal of average protein content (Oliver et al. 1977)). Glutamine synthesis on the other hand does not consume bicarbonate. Studies have demonstrated that experimental acidosis and alkalosis change the relative rates of urea and glutamine synthesis (Haussinger 1983; Haussinger, Gerok, and Seis 1984). Therefore, it seems that fluctuations in sinusoidal blood pH affect the ratio of the rates of urea synthesis and glutaminase activity in zone 1 and the rate at which glutamine is synthesised in zone 3. This results in the regulation of blood pH as a consequence of changes in the relative quantity of hepatic ammonia which is removedby urea synthesis or by glutamine synthesis.



Ammonia metabolism in the hepatic acinus

Figure 2.4.

2.5.4. Ammonia metabolism and liver support

Evidently, efficient ammonia metabolism would be a requirement for any hybrid artificial liver device. As we have seen, build up of circulating toxins such as ammonia and loss of pH homeostasis are two major problems associated with hepatic failure. It is also evident that a bioreactor configuration which enables hepatocytes to function in a manner similar to the acinus should provide a regulated ammonia metabolism that is better suited to clinical application of such a device.

2.6. Biotransformation and bioactivation

The general objective of such metabolism in the liver is to regulate the concentration in the THV and in bile of compounds which are toxic or not required for other processes.

2.6.1. Detoxication

Humans are exposed to many exogenous and endogenous toxic compounds. In particular, lipophilic compounds which are readily absorbed must be modified to generate more polar compounds which can be excreted in urine or in bile. Enzyme systems exist which catalyse reactions to aid the elimination of toxic molecules. Some such enzymes have very broad substrate specificity and can therefore modify a vast range of compounds. Generally, these enzymes are concentrated in the liver. Amongst these low specificity enzymes are those which catalyse the oxidation or reduction of substrates. This group includes:

- Dehydrogenases, such as alcohol dehydrogenase, a cytosolic enzyme which catalyses the oxidation of alcohols to ketones or aldehydes.
- Reductases which catalyse reactions with a wide range of xenobiotics. These include NAD(P)H: quinone acceptor oxidoreductase, formerly known as DT-diaphorase, a cytosolic reducer of a wide range of quinone compounds and NADPH-cytochrome c reductase, a microsomal enzyme which catalyses reduction of many types of molecules including certain nitrogenous

compounds.

- The oxidases, aldehyde oxidase and monoamine oxidase, which catalyse transfer of electrons from a substrate to oxygen generating H_2O_2 via superoxide anions. These two enzymes have exceptionally broad substrate specificity.
- Monooxygenases which, as their name suggests, catalyse incorporation of one oxygen atom into substrates. Simultaneously, a reducing agent provides electrons for the remaining oxygen atom from one molecule of dissolved O_2 to produce a molecule of water. (This combination of oxidation and reduction has led to these enzymes being called mixed function oxidases.) The liver contains two types of monooxygenase both of which are microsomal. These are the flavin containing monooxygenases and the cytochrome P450 dependent monooxygenases.

The cytochrome P450 monooxygenase system consists of one flavoprotein (NADPH-cytochrome c reductase) and a family of distinct haemoproteins which are the genuine monooxygenases. Several isoenzymes of these haemoproteins exist in each species (Nelson et al. 1993). Some are constitutive while others are only detectable after induction by a foreign compound such as phenobarbitone. All P450 isoenzymes are membrane bound and each one catalyses the oxidation of a vast range of substrates. This enzyme family is probably the single most important detoxification system in the body.

In some instances, metabolic oxidation or reduction may produce a compound which is more toxic than the parent or which is still unsuitable for excretion. In these cases, a second phase of metabolism is required to render the compound non-toxic or readily excretable. The reactions involved in this secondary metabolism are conjugation and hydrolysis. Conjugation of substrates may also occur directly, that is, without prior oxidation or reduction.

Conjugation reactions involve participation of a second donor substrate. This donor, or a reactive group from it, is transferred to the metabolite in question. The main reactions are:

- Glucuronidation, in which UDP-glucuronosyl transferase catalyses the transfer of a glucuronyl group from UDP-α-glucuronic acid to a wide range of substrates.
- Sulfation, this entails compounds bearing hydroxyl groups receiving a sulfate group from phosphoadenylphophosulphate. These reactions are catalysed by a range of sulfotransferases.
- Glutathione conjugation, glutathione is a sufficiently important molecule to merit a brief discussion here. This tripeptide is ubiquitous in the body. It exists in a reduced (GSH) and an oxidised form (GSSG) largely controlling the cellular redox balance and is also involved in thiol transfer reactions and many other protective mechanisms. Glutathione also acts as a transport and storage form of its individual amino acid constituents, particularly cysteine. The role of GSH in protecting the cell from damage by bioactivated compounds will be discussed later in this chapter. In detoxication, GSH acts as a nucleophile by reacting with electrophilic carbon compounds to form thioethers. Thermodynamically, this is a spontaneous reaction, but in the liver it is enhanced by the catalytic action of glutathione S-transferases (GST). There are a large number of GST isoenzymes in humans (Jackoby 1978).

The remaining conjugation reactions are somewhat less important. These are; methylation, in which a group of methyltransferases catalyse the transfer of a methyl group from S-adenosyl-L-methionine to a range of substrates bearing phenol, amine or thiol groups; N-acetylation, here certain compounds undergo conjugation with an amino group donated by acetyl coenzyme A (catalysed by N-acetyltransferase) and amino acid conjugation by which some benzoate compounds are conjugated with glycine or glutamine.

The other form of reaction in this second phase of biotransformation is hydrolysis. Water acting as a nucleophile will hydrolyse certain bonds in organic molecules. Esterases, amidases and hydratases catalyse some such reactions while others occur spontaneously.

The message to carry forward from this section is that a relatively limited group of enzymes can catalyse reactions which modify a vast number of endogenous and exogenous substances in a variety of different ways which will normally render them more polar, and hence more excretable via the kidney or bile, and/or less toxic.

2.6.2. Hepatocellular toxicity and liver damage

The metabolism of some xenobiotics leads to damage to liver cells via the production of toxic metabolites. Some examples of the mechanisms which can lead to hepatocellular damage follow. In each case a brief description of the mode of action of a known agent (some identified as causes of liver failure) is given.

I. Bioactivation to stable but toxic metabolites

Stable but toxic metabolites are fortunately rare. An example is dichloromethane which is metabolised by the cytochrome P450 dependent monooxygenases and yields carbon monoxide.

II. Formation of reactive electrophilic metabolites

Reactive electrophilic metabolites are more commonly formed than stable toxic metabolites. Paracetamol can be metabolised by cytochrome P450 dependent monooxygenases (Hinson, Pohl, and Gillette 1979) to N-acetyl-p-benzoquinone imine (NAPQI) (Holme et al. 1984) which is toxic. This metabolite binds to proteins in the endoplasmic reticulum and cytosol (Streeter et al. 1984) leading to tissue necrosis. Reduced glutathione protects against paracetamol toxicity since it can react with NAPQI (Potter and Hinson 1986). Once glutathione is depleted, paracetamol toxicity progresses unhindered (Mitchell et al. 1973). N-acetylcysteine can be administered to prevent paracetamol toxicity. It has a two-fold effect, serving as a source of cysteine for glutathione synthesis and inhibiting the binding of NAPQI to proteins (Hazelton, Hjelle, and Klaassen 1986).

III. Free radical generation

The hepatotoxic effects of many drugs are a result of free radical formation.

Carbon tetrachloride metabolism liberates a trichloromethyl radical which binds to proteins leading to lipid peroxidation and membrane damage (Flander, Haas, and Kroner 1982).

IV. Liberation of reduced oxygen species

The formation of reduced oxygen species such as hydrogen peroxide is thought to produce cell damage by oxidative stress (Sies 1985). The liver is quite well protected against such damage by several antioxidative mechanisms such as the action of GSH, superoxide dismutases and catalases, so this is a less important hepatotoxic mechanism. One exception however, is the hepatotoxic quinone compound, menadione. This can undergo one electron reduction in the liver, predominantly by NADPH cytochrome c reductase, to yield a semiquinone and a superoxide radical. Redox cycling of the semiquinone can then give rise to more superoxide radicals and subsequent toxicity. Alternatively, DT-diaphorase may catalyse the two electron reduction of menadione to a relatively non-toxic hydroquinone (Kappus and Sies 1981).

All of the above mechanisms, in addition to the modes of toxicity described, appear to interfere with intracellular calcium homeostasis (Bellomo and Orrenius 1986; Nicotera et al. 1985; Recknagel 1983).

The hepatotoxicity of some drugs is far too complex to be explained in simple mechanistic terms. The effects of these compounds have been termed 'metabolic derangements' (Anders 1988). Galactosamine is an example of such a drug and no clear explanation for its mode of action exists.

2.6.3. Biotransformation, hepatotoxicity and the hepatic acinus.

The discovery that liver lesions resulting from drug induced liver damage were often restricted to hepatocytes of zone 3 prompted investigation into hepatotoxicity at the acinar level (Thurman, Kauffman, and Baron 1986). It appears that monooxygenation occurs predominantly in zone 3 while conjugation reactions, although occuring throughout the acinus, are more commonly by the sulfation route

in zone 1 and by the glucuronidation route in zone 3 (Jungermann 1995). Other enzymes occuring predominantly in zone 3 include; alcohol dehydrogenase (Salsano, Maly, and Sasse 1990) and NADPH-cytochrome c reductase (Sasse 1986). However, the concentration of glutathione decreases along the axis of blood flow in the acinus (Katz 1992). Thus, it seems as though the hepatocytes of zone 3 are at greater risk of toxic damage since there is a greater scope for bioactivation here, these cells are less well protected and oxygen may be close to a critical level in this zone (Sies 1977). Since the concentration of xenobiotics will be highest in zone 1, this enzyme distribution appears paradoxical. However, it should be noted that the zonal rate of drug metabolism depends on the rate of drug enzyme interaction to a greater extent than on the intracellular concentration of enzyme. Therefore, the interaction of drugs with the cells in zone 3 may, despite concentration gradients, be a consequence of selective uptake or binding of certain drugs.

However, the purpose of acinar heterogeneity in biotransfromation is not clear at this stage. Apart from an obvious 'division of labour' explanation, it is possible that biotransformational uptake processes saturate very quickly in zone 1 without bringing about a drastic reduction in substrate concentration (unlike zone 1 ammonium uptake in urea synthesis) and further high capacity downstream processing is required to eliminate these compounds.

2.6.4. Biotransformation and liver support

The importance of retaining as much of the biotransformational function of hepatocytes as possible in a liver support system cannot be overstated. In addition to the normal metabolic burden, the plasma of liver failure patients would deliver elevated levels of many toxins to a liver support device. These toxins would require oxidation, reduction or conjugation before they could be cleared. Furthermore, a device which delivered a high level of both phases of biotransformation in a single pass would be advantageous.

2.7. Oxygen transport and uptake

The objective of the liver is to extract sufficient oxygen from sinusoidal blood

to enable all oxygen dependent cell processes to operate in a non-oxygen limited manner. Adequate oxygen supply and uptake is therefore a fundamental requirement for liver function.

2.7.1. Hepatic oxygen consumption

The oxygen saturation in the hepatic artery is high and during fasting, portal blood has an oxygen saturation of about 85%, though this is substantially decreased after food ingestion. Estimates of the basal oxygen consumption rate in the cat liver place it at about 6 ml O_2 min^{-1} per 100g liver (Lutz, Henrich, and Bauereisen 1975), equivalent to approximately 70 mmol s^{-1} in man or about 25% of total body oxygen consumption (Myers 1947). This represents an extraction rate of about 40%. Experimental studies in rat, human and cat have demonstrated that the rate of oxygen utilisation in the liver is largely independent of liver blood flow and blood pO_2 (Lutz et al. 1978; Lutz, Henrich, and Bauereisen 1975). An interesting characteristic of hepatic oxygen consumption is that increased demand for oxygen by the liver is met by an increase in the extraction rate of oxygen from sinusoidal blood and not by vasodilation or increased flow (Lautt 1977). After all, the hydraulic pressure in the sinusoids is low and must remain so. In fact, it seems that the extraction rate can exceed 90% when demand is exceptional (Lutz, Henrich, and Bauereisen 1975), for example, when high ammonium chloride concentrations exist (Haussinger, Weiss, and Sies 1975; Trowell 1942). This suggests that the liver has a greater capacity for extracting oxygen from blood than most other tissues.

This increased efficiency of oxygen extraction reflects a fluctuating demand which reaches levels not found in other tissues. As discussed above, ammonia metabolism requires the energy released from the cleavage of high energy bonds. These are created by mitochondrial oxidative energy metabolism which has a high demand for oxygen. Other processes in the liver, including certain biotransformational reactions, have a high affinity for oxygen and consume large quantities directly.

2.7.2. Oxygen consumption and the hepatic acinus

The rate limiting oxygen transport steps in the liver are the diffusion of oxygen away from the sinusoid to the hepatocyte membrane and the transport of oxygen from the cell surface to the mitochondrion. An axial pO_2 gradient would be expected to exist in the hepatic acinus. This has been reported to have a magnitude of some 35-60mmHg, with the inlet pO_2 being around 55-65 mmHg and the pO_2 at the outlet being approximately 5-30 mmHg (deGroot, Littauer, and Noll 1987). This confirms that the pO_2 in zone 3 can be close to critical levels, since the potential for increased extraction could lead to a pO_2 in this region of around 1 mmHg. Oxygen consumption begins to decline below partial pressures of 10mmHg with a rapid decline occuring below 2 mmHg. The k_m value for this process in rat liver has been reported to be 0.5 to 0.7 mmHg (deGroot, Noll, and Sies 1985). The low pO_2 levels in zone 3 may limit transport of oxygen from the cell surface to the mitochondria. In cases where the sinusoidal pO_2 is less than 2mmHg, the magnitude of the intracellular pO_2 drop has been shown to be roughly equivalent to the cell surface pO_2 (deGroot, Littauer, and Noll 1987). In other words, the mitochondrial pO_2 approaches zero.

There are reportedly more numerous mitochondria in zone 3 than in zone 1 (Gumucio and Miller 1981). This may serve to shorten the intracellular diffusion distance. However, the mitochondria of zone 1 are larger (Loud 1968). This distribution reflects a large capacity upstream oxygen uptake system where oxygen is plentiful and a reduced capacity (but more efficient?) downstream uptake system where oxygen supply is close to limiting values. In this case, it appears that the hepatocytes within the acinar architecture have adapted to the consequences of axial oxygen perfusion with its resultant concentration gradient. Many of the more oxygen hungry processes, including oxidative energy metabolism and urea synthesis, occur predominantly in zone 1 where oxygen is plentiful while the cells in zone 3 have adapted to enable them to extract oxygen more efficiently from the oxygen deficient blood in this area.

2.7.3. Oxygen consumption and liver support

There is a clear indication from the information in the preceding section that oxygen supply is a critical design parameter for hybrid artificial liver support devices. Inadequate oxygen supply will lead to oxygen limited metabolism or possibly hypoxic damage and cell death.

Strategies for efficient oxygen transport must therefore be devised. In chapter 3, the current state of the art in hybrid artificial liver support will be critically analysed. One of the main themes developed in this chapter is the lack of consideration given to oxygen transport in current devices. In the analysis of designs in chapter 3, the hepatic oxygen consumption data described above will be used. However, one of the most important questions which must be answered is, what is the maximum specific cellular oxygen consumption rate? This value will evidently impose design restrictions. Failure to supply sufficient oxygen to meet this demand will result in oxygen-limited conditions within the device.

2.8. Bile secretion and flow

The objective of bile secretion and flow in the liver is to secrete products into the extracellular compartment of the intestinal lumen. A secondary objective is to remove certain solutes from the systemic circulation.

Some of the compounds secreted by hepatocytes into bile cannot be re-absorbed by the intestine while others, including bile salts, participate in digestion and are re-absorbed. These bile salts are then recirculated and taken up again by the liver.

2.8.1. Bile flow and the hepatic acinus

The recirculated bile salts and bile acids reach the hepatic acinus via the TPV and hence arrive in zone 1. Again axial concentration profiles exist (Jones, Hradek, and Renston 1980). Most of the bile salt transport occurs in zone 1. This has led to bile being classified as being formed by two fractions; the bile salt dependent fraction (BSDF) and the bile salt independent fraction (BSIF). The BSDF is secreted predominantly by cells in zone 1, while the BSIF is secreted by zone 3 cells (Gumucio

et al. 1978). However, if, as one would expect, the intracellular concentration of bile salts exerts a negative feedback on bile salt synthesis then most synthesis of bile salts would occur in zone 3.

Bile flow in the hepatic acinus is approximately countercurrent with respect to sinusoidal blood flow. Therefore, it is conceivable that the two fluid paths exert various forms of feedback on other cellular processes. There is no supportive evidence for this at present.

2.8.2. Bile flow and liver support

While it has been shown that total occlusion of the bile duct (and hence a cessation in bile transport) is not, in itself, life threatening over short periods and, therefore, that bile transport is not an essential component of a hybrid artificial liver support device, there are possible justifications for the incorporation of two (counter-current) fluid paths in such devices. It may be that provision of two discrete fluid paths on either side of a layer of cultured hepatocytes will enable the cells to function more effectively. This may enable the cells to develop the appropriate receptors on the correct surface, permit directional transport of certain solutes and might also allow regulation of cellular processes by feedback, if this exists in the liver.

2.9. Summary

The hepatic acinus produces liver function. The primary function of the liver is to regulate the concentration of solutes in the THV and in bile. The vast number of detoxifying and synthetic processes of the liver are achieved by a relatively small number of enzymes. Different zones in the hepatic acinus contribute to each process to differing degrees. This type of zonal heterogeneity is a consequence of the unidirectional mode of blood perfusion in the acinus.

All diffusional paths in the acinus are short. Despite this, cells in zone 3 are operating under conditions which are close to being oxygen limiting. The countercurrent flows of sinusoidal blood and bile may contribute to the regulation of liver metabolism by feedback between the two compartments.

2.10. Specific thesis objectives

The development of efficacious hybrid artificial livers requires a methodical approach. Certain variables must be considered and some unknowns must be resolved. With this in mind, the specific objectives of this thesis are:

I. To critically analyse current hybrid artificial liver support developments.

II. To evaluate the influence of the solute composition of culture media on the function of primary cultured hepatocytes of different species.

III. To evaluate the significance of different culture configurations with respect to the function of primary cultured hepatocytes.

IV. To determine whether the magnitudes of shear force anticipated in a hepatocyte bioreactor have adverse effects on primary cultured hepatocytes.

V. To quantify the specific cellular oxygen consumption rate of primary cultured hepatocytes under conditions of elevated metabolism.

VI. To develop and carry out initial testing on a prototype hybrid artificial liver device based upon the architecture of the hepatic acinus with input from data generated from the preceding thesis objectives.

Chapter 3

Analysis of hybrid artificial liver designs

3.1. Introduction

Several groups are currently reporting the development and testing of hybrid artificial liver devices. These include the two devices discussed briefly in Chapter 1 which have been used clinically in a number of cases. While researchers continue to advance ideas and designs, there remains uncertainty in three areas in the development of hybrid liver support.

The first area of debate is over the source of hepatocytes. Parameters to consider when selecting a cell source include; availability, nature and stability of phenotype, possible immunological problems, ability to proliferate in culture, costs and ethical issues. It is generally agreed that a stable, immortalised, non-tumorogenic human hepatocyte cell line which retains all of the functional characteristics of human hepatocytes *in vivo* would be ideal. However, at present, no cell line meets all of these criteria. Attempts to cryopreserve primary hepatocytes have met with limited success so far. These factors have compelled workers to compromise in their selection of cell source. In the Hepatix ELAD and Cedars-Sinai BAL devices, two radically different directions have been pursued.

The second uncertainty is the optimal reactor configuration. In chapter 2 the structure of the hepatic acinus was described and its role in hepatic function was elucidated. Clearly it would be enormously difficult, if not impossible, to recreate all aspects of acinar architecture in a hepatocyte bioreactor. This once again compels compromise in design. However, the best design path is less obvious due, in part, to

a paucity of data in a variety of areas. For example, little is known about the biochemical kinetics of liver failure at the acinar level. This means that the loads placed upon hepatocytes during liver failure cannot be accurately quantified. Consequently a number of fundamental questions pertaining to the design specification cannot be answered. These include:

- What is the required cell mass for support?
- At what rate must oxygen and nutrients be supplied?
- What are the maximum permissible diffusion distances?
- At what densities may the cells be cultured?
- Which are the critical biochemical pathways?

Each of these parameters is influenced by reactor configuration and they are obviously interrelated. Unfortunately, most reported devices lack quantitative design input and the development process has been largely empirical. This has inevitably produced a series of variations of commercial hollow fibre devices. In other words, an 'off the shelf' device, such as an ultrafiltration cartridge, has been used and in some cases modified. This direction has the advantages of low cost and ready availability

The third area of debate is the mode of application of the bioreactor and this itself consists of three uncertainties, namely: How will it be connected to a patient? What should be pumped through the device? and, how should it be operated? It is surprising that these questions still do not have unequivocal answers.

The matter of mode of connection may not appear critical but this too has ramifications for design since the site of blood access will influence permissible flow rates and the resultant inlet pO_2 in some devices.

The question of what should be pumped through the device includes the choice between blood or plasma and the possibility of including additional fluid paths in more complex devices for nutrient supply or other purposes. Commercial interests would prefer a blood perfused system for simplicity and cost reasons. This mode of operation is also favoured by some clinicians since it simplifies the perfusion apparatus

and negates the requirement for plasmapheresis which may further disturb haematological parameters in the patient. However, some form of immuno-isolation would have to be incorporated into a blood perfused bioreactor. This usually requires the interposition of a membrane between the patient's blood and the heterologous hepatocytes (as in the ultrafiltration cartridges previously mentioned). The membrane barrier would have to be absolutely reliable, the patient's white blood cells must not contact the hepatocytes and heterologous cells or cell fragments must not be returned to the circulation. This membrane would inevitably impose a diffusional resistance to solute transport between the blood phase and the hepatocytes. In light of these observations, a plasma perfused system appears a suitable alternative. However, direct hepatocyte plasma contact may also cause immunological problems (via immuno-competent proteins or if a small number of white blood cells or cell fragments were present in the plasma fraction) and arterial plasma has only approximately 2% of the total oxygen content of arterial blood.

Finally, the mode of operation requires to be considered. Would intermittent sessions of therapy be sufficient, as in haemodialysis? This may permit filters and, if necessary, bioreactors to be replaced between sessions. On the other hand one could argue in favour of long term (i.e. days or even weeks) continuous therapy analogous to the treatment of acute respiratory failure using blood oxygenators.

3.2. Critical analysis of current developments

In order to identify shortcomings in current developments and to resolve, where possible, optimal design parameters, case studies will be undertaken. Four widely reported hybrid artificial liver devices will be considered.

Each case study will consider the device in terms of the suitability of the cell source from performance, ethical and economic standpoints, the configuration in terms of hepatocyte function and oxygen transport and the envisaged mode of operation.

3.3. The Hepatix Extracorporeal Liver Assist Device (ELAD)

This device was developed in Houston, Texas. The principal investigators, N.L. Sussman and J.H. Kelly have reported its testing in animal models of liver failure (Kelly et al. 1992; Sussman et al. 1992) and some clinical case studies (Sussman et al. 1994a; Sussman and Kelly 1992). A US. patent relating to the development is pending. Most recently, use of this device has been described in the context of a review of the treatment of hepatic failure in which the authors look forward to FHF becoming a "simple management problem, analogous to the options available in the treatment of acute or chronic renal failure" (Sussman and Lake 1996).

The ELAD is based on a commercial membrane ultrafiltration cartridge. It contains a parallel bundle of approximately 10,000 cellulose acetate hollow fibres with a nominal molecular weight cut-off (NMWCO) of 70,000, a wall thickness of 30 μm and an outer diameter of 280 μm. The intraluminal (tube side) volume is 120 ml, the extraluminal (shell side) volume 125 ml and the fibre length is 32 cm. From this data, the mean inter-fibre distance can be calculated, on the basis of uniform radial distribution, to be approximately 50 μm.

The extraluminal space of this device is seeded with some 10 g (approximately $1x10^9$ cells) of C3A cells, a hepatoma cell line derived from the widely reported HepG2 line. These cells divide to fill the shell side space to give a final cell mass of 200 g ($2x10^{10}$ cells). During this culture period (which may last for up to 3 weeks), the cells are maintained by perfusing the intraluminal compartment with culture medium which is changed twice daily.

As reported in chapter 1, this device has been used to treat a number of patients and was in phase 1 trials at selected transplant centres. During therapy, the patient is connected to the device via a veno-venous double catheter. Blood is pumped through the intraluminal compartment at 100-250 ml min^{-1}.

3.3.1. The C3A cell line and liver support

This cell line was produced by sub-culture selection from the human hepatocarcinoma HepG2 cell line. It should be noted that this method does not

represent a true clonal selection process. The derived 'line' is reported to show elevated levels of liver specific synthetic functions such as albumin synthesis. By selecting an established cell line, the developers have avoided the costly and time consuming requirement for repeated isolations of primary hepatocytes from donor organs. Furthermore, the cells can be proliferated within the device. It is therefore relatively easy to ensure that devices are available upon demand.

However, there are a number of disadvantages associated with the use of this cell line in liver support devices. Firstly, it is of cancerous origin. While the risk of metastases arising in patients treated with this device is arguably small and may be contrasted with the life threatening condition of liver failure, the developers recognise that a definite risk exists and have had to incorporate safety features into the device to ensure that C3A cells are not returned to the patient. A positive pressure is reported to be maintained on the blood side of the device and the resultant ultrafiltrate is returned to the patient via a cell trapping filter (0.45 μm pore size). The importance of this safety feature is emphasised in a recent report comparing the performance of primary rat hepatocytes and the HepG2 cell line in a prototype hybrid liver support system (Nyberg et al. 1994). This study surprisingly reported that HepG2 cells were able to cross a polysulfone membrane (70,000 NMWCO) and enter the intraluminal compartment. A mechanism was not postulated for this phenomenon. The imposition of a positive hydraulic pressure on the blood side of the Hepatix device is intended to prevent transmembrane cell migration. However, postive blood side pressure would have to be maintained along the entire length of the cartridge requiring the axial pressure drop on the blood side (due to wall friction) to be taken into account.

The aforementioned paper also illustrated another disadvantage associated with the use of established cell lines for liver support. All reported hepatocyte cell lines have lost some critical aspects of the normal hepatocyte phenotype. HepG2 cells are known to be deficient in many metabolic pathways including ureogenesis and certain oxidation, glucuronidation and sulfation reactions (Everson and Polokoff 1986; Schuetz et al. 1993). These findings were confirmed by Nyberg and co-workers who

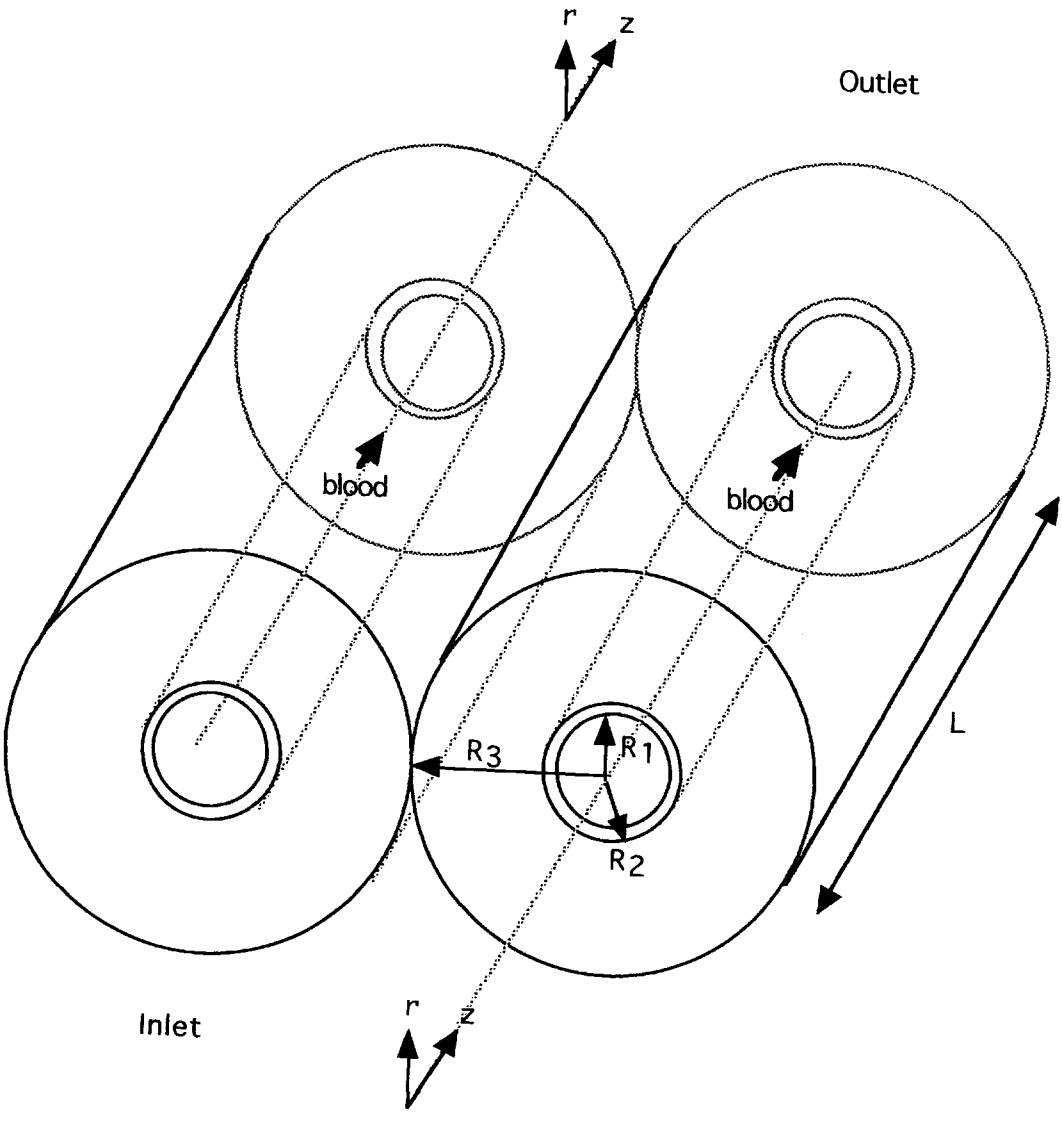
found that of several biotransformational and synthetic functions studied, only albumin synthesis was as high in HepG2 cells as in primary rat hepatocytes (Nyberg et al. 1994). Indeed, at longer culture times, albumin synthesis in HepG2 cells far exceeded that in primary rat hepatocytes at the same time. (This highlights one of the disadvantages of primary cells, namely the gradual loss of function with time which can be quite significant within 24-48 hours. This is normally only associated with increasing passage number in established cell lines.) It should be recognised that albumin synthesis could not realistically be considered as an important liver function in the context of FHF therapy.

Loss of expression of critical metabolic pathways in established hepatoma cell lines is also reflected by the low rate of oxygen consumption of HepG2 cells (Smirthwaite 1994).

The rationale for the selection of the C3A cell line would therefore appear to be that the convenience and availability of a cell line, allied to its consistency of performance outweighs the risk of tumour production in patients and the consequences of the reduced range of functions. It could be strongly argued that this is not a satisfactory justification for the incorporation of a functionally compromised and potentially dangerous cell source.

3.3.2. Analysis of Hepatix Configuration

The configuration can be modelled as a three compartment system with blood in the lumen of capillaries, a homogenous tissue mass filling the extraluminal space and a thin membrane layer in between. This is analogous to the *in vivo* situation in some tissues such as muscle. However, the synthetic membrane in the Hepatix device will have a much higher mass transfer resistance than the corresponding capillary membrane *in vivo*. While the device is operated with a transmembrane pressure difference producing an element of transmembrane convective flow (the magnitude of this flux has not been reported though it is likely to be small), the main mechanism of solute transport in this device will be diffusion. In these circumstances, the Krogh model, originally describing the diffusion of oxygen from capillaries to a surrounding



The Krogh-Erlang idealised tissue cylinder model
Figure 3.1.

tissue mass (Krogh 1919), is applicable, providing that diffusion across the capillary membrane is taken into account. A diagrammatic representation of this model is given in Figure 3.1.

In this model blood is considered to flow along along the inside of a narrow tube of radius R_1 and length L. At any given axial location z, blood is considered to be well mixed and pO_2 is not a function of r within the capillary. Surrounding this tube is a homogeneous cell annulus of radius R_3. The system is radially symmetric and since L is much greater than R_1, R_2 and R_3, diffusion of solutes in the axial direction can be ignored. Diffusion in the cell and tube wall compartments occurs in the radial direction r.

The transport of oxygen in the Hepatix system will now be considered on the basis of this model, with the governing equations for each compartment being described in turn:

The cell annulus phase

The O_2 diffusivity and solubility coefficients are presumed to be constant throughout the cell annulus (these being designated D_c and α_c respectively). The cell mass is considered to have a uniform rate of oxygen consumption Q (This is an approximation since previous work has suggested that oxygen consumption may not be a zero order kinetic reaction (deGroot, Noll, and Sies 1985)). Therefore, Fick's second law describing the unsteady-state diffusion of oxygen in the tissue annulus is:

$$\frac{\delta P_c}{\delta t} = \frac{1}{r}\frac{\delta}{\delta r}\left[D_c r \frac{\delta P_c}{\delta r}\right] - \frac{Q}{\alpha_c} \qquad \textit{(Equation 3.1)}$$

Where P_c is the pO_2 within the cell phase and t is time. Assuming steady state operating conditions for the bioreactor, then $\partial P_c/\partial t = 0$ and given the condition that there is no net diffusion at the tissue annulus boundary ($r = R_3$) then equation 3.1 can be integrated to give:

$$\frac{\delta P_c}{\delta r} = \frac{-Q}{2\alpha_c D_c}\left[\frac{R_3^2}{r} - r\right] \qquad \textit{(Equation 3.2)}$$

The membrane phase

Again, the values of D and α are presumed to be constant throughout the membrane layer (in this case D_m and α_m respectively). Hence, in the membrane, the steady-state diffusion of oxygen is given by:

$$\frac{1}{r}\frac{\delta}{\delta r}\left[D_m r \frac{\delta P_m}{\delta r}\right] = 0 \qquad \textit{(Equation 3.3)}$$

Integration of equation 3.3 and matching fluxes at the cell annulus/membrane interface ($r=R_2$) yields:

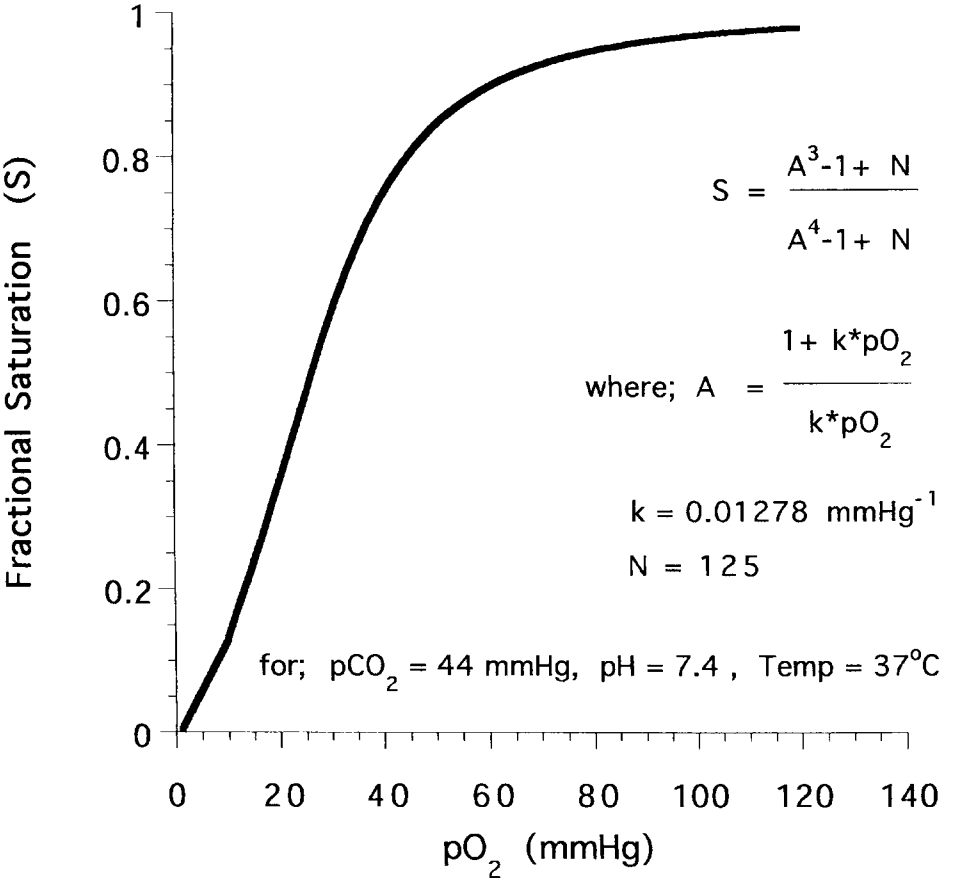
$$\frac{\delta P_m}{\delta r} = \frac{-Q}{2\alpha_m D_m}\left[R_3^2 - R_2^2\right]\frac{1}{r} \qquad \textit{(Equation 3.4)}$$

The blood phase

Here, the transfer of oxygen in dissolved and haemoglobin bound forms must be considered. The governing steady-state convective diffusion equation describing O_2 transfer in blood flowing in a cylindrical capillary is given by:

$$V_z\left[1 + m'(P_B)\right]\frac{\delta P_B}{\delta z} = \frac{1}{r}\frac{\delta}{\delta r}\left[D_B r \frac{\delta P_B}{\delta r}\right] \qquad \textit{(Equation 3.5)}$$

where V_z is the axial blood velocity (usually a function of radial position r), P_B is the O_2 partial pressure in the blood, D_B is the O_2 diffusivity in blood and $m'(P_B)$ is given by:



Margaria plot for human venous blood.

Figure 3.2.

$$m'(P_B) = \frac{B_{hbO_2} C_{hb}}{\alpha_B} \frac{dS}{dP_B} \qquad \textit{(Equation 3.6)}$$

$m'(P_B)$ represents the source/sink effect of haemoglobin bound oxygen and is proportional to the local slope (dS/dP_B) of the oxygen dissociation curve (ODC). B_{hbO2}, C_{hb} and α_B are respectively, the O_2 binding capacity of haemoglobin, the haemoglobin concentration and the O_2 solubility in blood.

The oxygen saturation S is a non-linear function of P_B as represented by the ODC and also depends on temperature, pH and pCO_2. The Margaria equation describes the ODC and is depicted in Figure 3.2 for normal human venous blood.

Because of the non-linearity introduced by $m'(P_B)$, equation 3.5 must be solved by numerical techniques. An analytical solution may be obtained with further simplifying assumptions:

(a) The blood is well-mixed and has a uniform velovity V

(b) $m'(P_B)$ is assigned a constant value M corresponding to the relevant P_B range.

Assumption (a) will yield an upper boundary for O_2 transfer since it implies no resistance to O_2 transfer in the blood phase. Assumption (b) is valid in that it corresponds to the linear segment of the ODC with venous blood as input to the device.

With these assumptions, a mass balance over an elemental length Δz of the blood compartment yields:

Rate of change of total O_2 convected in axial direction = rate of transfer of dissolved O_2 at membrane/blood interface over length Δz.

Hence:

$$V\pi R_1^2\alpha_B(1+M)\frac{\delta P_B}{\delta z} = 2\pi R_1\alpha_m D_m\frac{\delta P_m}{\delta r}\Big|r=R_1 \quad \textit{(Equation 3.7)}$$

from equation 3.4:

$$\frac{\delta P_m}{\delta r}\Big|r=R_1 = \frac{-Q\left[R_3^2-R_2^2\right]}{2\alpha_m D_m}\frac{1}{R_1} \quad \textit{(Equation 3.8)}$$

Hence substituting in equation 3.7 gives:

$$V\pi R_1^2\alpha_B(1+M)\frac{\delta P_B}{\delta z} = -\pi Q\left[R_3^2-R_2^2\right] \quad \textit{(Equation 3.9)}$$

therefore:

$$\frac{\delta P_B}{\delta z} = \frac{-Q}{V\alpha_B(1+M)}\frac{R_3^2-R_2^2}{R_2^2} \quad \textit{(Equation 3.10)}$$

Integration of equation 3.10 with $P_B = P_0$ at $z = 0$ yields:

$$P_B = \frac{-Qz}{V\alpha_B(1+M)}\frac{R_3^2-R_2^2}{R_1^2}+P_0 \quad \textit{(Equation 3.11)}$$

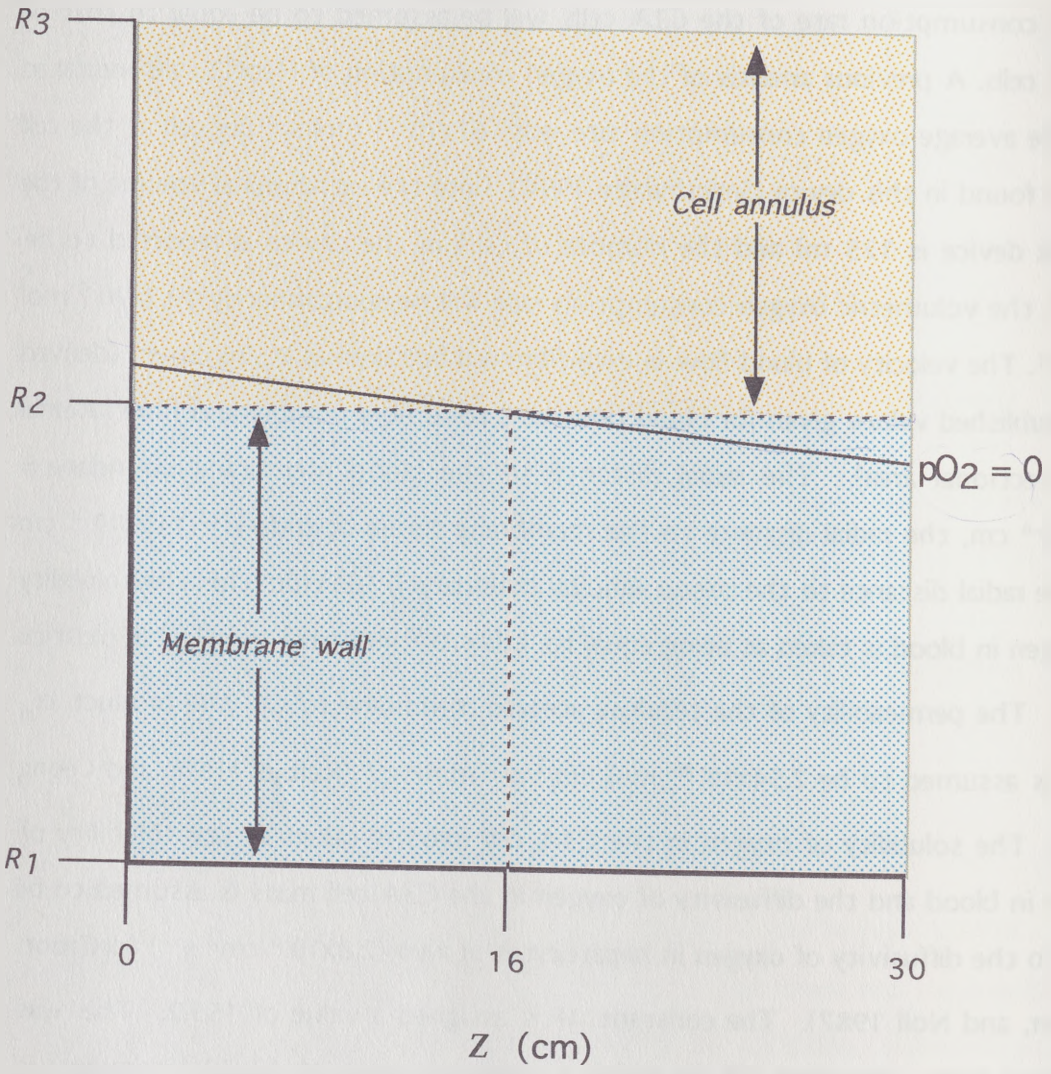
We can now obtain an expression for P (the pO_2 at any given point (r, z) in the cell annulus and membrane phases) by combining equations 3.2, 3.4 and 3.11 and applying boundary conditions to resolve the constants of integration. The resultant equation is:

$$P=P_0+Q\left[\frac{-z}{V\alpha_B(1+M)}\frac{R_3^2-R_2^2}{R_1^2}+\frac{\left(R_3^2-R_2^2\right)\ln\left(\frac{R_1}{R_2}\right)}{2\alpha_m D_m}+\frac{r^2-R_2^2+2R_3^2\ln\left(\frac{R_2}{r}\right)}{4\alpha_c D_c}\right]$$

(Equation 3.12)

As described, the Hepatix device is normally perfused with venous blood (Gislason et al. 1994). Therefore, the pO_2 at the device inlet will be approximately 40 mmHg. However, in the absence of relevant clinical data, the inlet pO_2 will be deemed to be equal to 40 mmHg for the purposes of this analysis. For convenience, the oxygen consumption rate of the C3A cells will be assumed to be equal to that of HepG2 cells. A previous analysis of the oxygen consumption of HepG2 cells indicated that the average oxygen consumption rate was 67×10^{-18} mol s^{-1} per cell at the cell density found in this device (Smirthwaite 1994). Since the extraluminal volume of the Hepatix device is 125 ml and the number of cells in the device is reported to be 2×10^{10}, the volumetric oxygen consumption rate will be approximately 10.7×10^{-9} mol s^{-1} cm^{-3}. The velocity of blood flow in each fibre will be taken as 0.526 cm s^{-1} (derived from published values given for blood flow rate, total fibre number and fibre internal cross sectional area). The radial distance to the blood membrane boundary = 110×10^{-4} cm, the radial distance to the membrane tissue boundary = 140×10^{-4} cm and the radial distance to the tissue annulus boundary = 165×10^{-4} cm. The solubility of oxygen in blood is taken as being equal to 1.07×10^{-9} mol cm^{-3} $mmHg^{-1}$ (Goldstick 1971). The permeability of the cellulose acetate membrane to O_2 (the product $\alpha_m .D_m$) is assumed to be 2.54×10^{-15} mol cm^{-1} s^{-1} $mmHg^{-1}$ (Refojo, Holly, and Leong 1977). The solubility of oxygen in the C3A cell mass is taken as the solubility of oxygen in blood and the diffusivity of oxygen in the C3A cell mass is assumed to be equal to the diffusivity of oxygen in hepatocytes *in vivo* (2.8×10^{-6} cm^2 s^{-1}) (deGroot, Littauer, and Noll 1987). The constant M is assigned a value of 153.2. This was calculated from equation 3.6. in which a constant value of 18×10^{-3} $mmHg^{-1}$ is assigned as the local slope of the Margaria plot and the haemoglobin oxygen binding capacity and blood concentration are taken as 60.714×10^{-6} mol g^{-1} and 0.15 g cm^{-3} respectively.

For convenience, a summary of the model parameters is given in Table 3.1.



2D plot of $pO_2 = 0$ as a function of r and z for the Hepatix ELAD

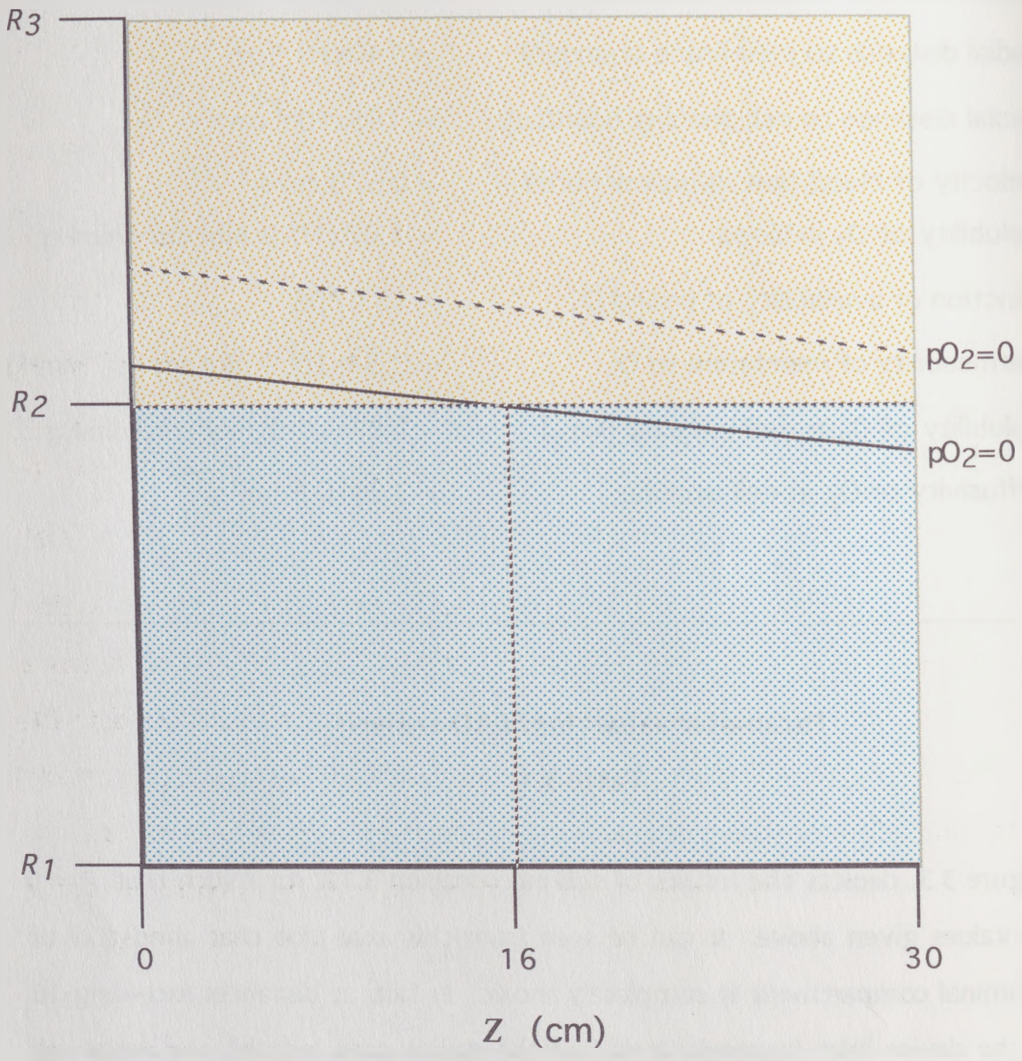
Figure 3.3.

P_O	pO_2 in blood at device inlet	= 40 mmHg
Q	O_2 consumption in cell mass	= 10.7×10^{-9} mol s^{-1} cm^{-3}
R_1	radial distance to blood boundary	= 110×10^{-4} cm
R_2	radial distance to membrane boundary	= 140×10^{-4} cm
R_3	radial distance to cell annulus boundary	= 165×10^{-4} cm
V	velocity of blood flow in hollow fibres	= 0.526 cm s^{-1}
α_B	solubility of O_2 in blood	= 1.07x10-9 mol cm^{-3} $mmHg^{-1}$
m'	function of availability of bound O_2	= 153.205
$\alpha_m D_m$	permeability of membrane to O_2	= 2.54×10^{-15} mol cm^{-1} s^{-1} $mmHg^{-1}$
α_c	solubility of O_2 in cell annulus ($\equiv \alpha_B$)	= 1.07x10-9 mol cm^{-3} $mmHg^{-1}$
D_c	diffusivity of O_2 in cell annulus	= 2.8×10^{-6} cm^2 s^{-1}

Parameter values for ELAD analysis

Table 3.1.

Figure 3.3. depicts the results of solving equation 3.12. for r such that $P = 0$ using the values given above. It can be seen from this axial plot that almost all of the extraluminal compartment is completely anoxic. In fact, at distances exceeding 16 cm from the device inlet (approximately half the device path length), the entire cell annulus is devoid of oxygen, while at shorter axial distances, only a very thin section of the cell annulus immediately adjacent to the membrane outer wall is supplied with oxygen. For reference, at position $z = 0$ and $r = R_2$, $P = 1.25$ mmHg. The tissue annulus could be considered to consist of stratified layers of cells. For an annular radius of 25μm there are likely to be 2 to 4 such layers (depending on the degree of cell flattening). In the defined conditions only the cell layer immediately adjacent to the hollow fibre wall would receive any oxygen and even this layer would only be



——— $\alpha_m.D_m$ = 2.54x10^{-15} mol cm^{-1} s^{-1} mmHg^{-1}

- - - - - $\alpha_m.D_m$ = 3.00x10^{-15} mol cm^{-1} s^{-1} mmHg^{-1}

(for $\alpha_m.D_m$ = 19.26x10^{-15} mol cm^{-1} s^{-1} mmHg^{-1} at z = 30 and $r = R3$, $pO_2 > 0$)

2D plot of $pO_2 = 0$ as a function of r and z for the Hepatix ELAD: Effect of changing membrane permeability to oxygen.

Figure 3.4.

oxygenated for half of the device length. In other words, somewhere between 75% and 87.5% of the cell mass is not supplied with oxygen.

(It has been reported that this device may be operated in series with a second device (Gislason et al. 1994). In these circumstances, the cell mass throughout the second cartridge would be exposed to entirely anoxic conditions. One would expect a device operating under such conditions to offer virtually nothing in terms of metabolic activity.)

Analysis of equation 3.12. reveals that the principal determinants of the resulting pO_2 in the cell mass are the device inlet pO_2 (P_0) and the permeability of the hollow fibre membrane to oxygen ($\alpha_m . D_m$). The device inlet pO_2 could, of course, be increased either by introducing arterial, rather than venous blood or by use of an oxygenator upstream of the ELAD in the extracorporeal circuit. In fact, only a relatively small increase is required since the oxygen demands of the cells may be satisfied (i.e. $P \rightarrow 0$ for z = 30 cm and $r = R_3$) if P_0 = 54 mmHg (for a single device). However, the requirement for such measures rather begs one to question whether electing to use whole blood perfusion is really advantageous in this instance. Arterial access to the patients blood stream would in most cases be less desirable than venous access even if the latter were combined with subsequent plasma separation, both from a haematological point of view and with regard to the necessary surgery. Also, one could argue from an engineering perpective that a requirement for additional oxygenation negates any potential design advantages inherent in blood perfused systems.

While we can have confidence in the value assigned to the permeability of the membrane to oxygen, it is worthwhile to investigate the effect of varying the magnitude of this resistance. The effect of changing the value is shown in Figure 3.4. This figure indicates that by assigning an increased membrane permeability value of 3.0×10^{-15} mol cm^{-1} s^{-1} $mmHg^{-1}$, which is equivalent to the permeability of oxygen in hepatocytes, to equation 3.12, the fraction of the cell annulus which is oxygenated is increased. However, as can be seen from the figure, even with this increased

membrane permeability, the majority of the cell annulus is still experiencing anoxic conditions. Only when the membrane permeability to oxygen is increased to values approaching that of blood (i.e. 19.26×10^{-15} mol cm^{-1} s^{-1} $mmHg^{-1}$) can the entire cell annulus be adequately oxygenated (i.e. for $z = 30$, at $r = R_3$, $P \geq 0$).

It should be noted that during the proliferation phase, the device is perfused with culture medium. There is likely to be increased oxygen debt during this culture phase since the oxygen carrying capacity of culture medium is far less than that of blood. In the culture phase, as in the blood perfusion phase, it is unlikely that the lack of oxygen could be effectively compensated for by increasing the perfusate flow rate.

3.3.3. Mode of Application of the Hepatix Device

The device is perfused with whole blood. One case report describes connection to the patient via the femoral artery (Sussman and Kelly 1992), although the normal access is via the subclavian vein (Sussman et al. 1994a). The inlet pO_2 and possibly blood flow rate would be affected by the arterial access mode. While perfusion with arterial blood will alleviate oxygen debt (as described above), some of the cell mass will inevitably be exposed to unusually high oxygen tensions. The response of the C3A cell line to elevated oxygen concentrations has not been reported.

The mode of therapy is continuous. However, cartridges are reported to require changing every 5 to 36 hours. In the two case reports identified in Chapter 1, one patient underwent 58 hours of continuous therapy while the other required 6 days of treatment. At best therefore, 2 cartridges might be sufficient for successful treatment of one patient. However, at worst it could be argued that some 29 cartridges may be required given a 5 hour replacement frequency over 6 days. This would almost certainly make such therapy prohibitively expensive.

The indications for cartridge replacement are not clearly explained by the authors. Blood clotting and loss of measured function in the device are likely to be contributing factors. It may also be the case that lack of improvement or relapse in the patient may lead to the device being replaced.

3.3.4. Conclusions from the Hepatix study

The selection of a cell line as opposed to primary cells has many practical advantages. Also, the decision to perfuse the device with whole blood potentially simplifies the design. However, the chosen cell line lacks several important metabolic pathways and is potentially tumorogenic. Furthermore, one of the potential advantages of blood perfusion namely a larger oxygen capacity in the perfusate, is offset by inefficient oxygen transport to the cells which is primarily the consequence in this case of the presence of an 'immuno-isolation' membrane between the perfusate and the cells. It is likely therefore, that the transport of other solutes will be insufficient to meet the demands of the cells. Analysis has shown that this configuration cannot supply sufficient oxygen for a cell line which is known to have a reduced oxygen consumption rate compared with primary hepatocytes. Therefore, increased oxygen debt would be expected if primary cells or a more highly differentiated cell line was used in this device.

It should be pointed out that this analysis is based on a constant oxygen consumption rate for the cells. If the model were to include Q as a function of P (for example, according to Michaelis-Menten kinetics), then the anoxic region may be reduced. However, this is not to say that device performance would be improved, since there would be no net increase in oxygen supply.

A continuous mode of therapy is envisaged for this device. This should be beneficial for the patient who may otherwise relapse between sessions. However, the device cannot be relied upon to remain functional for the duration of therapy required.

This group have published findings from their own initial clinical experience with the device (Sussman et al. 1994b). Of 11 patients reported, 6 died, 4 survived after receiving a transplant and 1 had a full recovery. The total amount of therapy administered was 579 hours and this required 36 cartridges. The average operational life span of the device is therefore 16 hours. Several patients were reported to show signs of improvement during therapy. These signs included improved mental status and increased galactose elimination capacity. However, improvements in these and other parameters were fleeting and did not generally correlate with outcome. In a

recent publication, it was stated that 23 patients have now been treated with this device in small trials at different centres (Sussman and Lake 1996). The authors claim that patients show signs of recovery while undergoing therapy but that the device soon gets "overwhelmed", after which, the condition of the patient declines again. They attribute this failure to inadequacy of cell mass and intend to use 2 cartridges in series in future. As we have seen, this is not likely to be a solution.

It has been shown that this device is sub-optimal in at least three critical areas. The selected cell line is functionally compromised. The transport of oxygen (and by extension, other low molecular weight solutes) is inadequate and the therapeutic life span of the cartridges is severely limited. These shortcomings are likely contributors to the lack of definitive evidence that this device is clinically efficacious.

3.4. The University of Minnesota Hepatocyte-Entrapment Bioreactor

The principal investigators in this group are F. B. Cerra and W-S. Hu. The group have reported the development and *in vitro* evaluation of their device (Nyberg et al. 1994; Nyberg et al. 1993b) and have published some results from preliminary animal model testing (Nyberg et al. 1993a; Nyberg et al. 1992).

This device is also based upon a commercially available hollow fibre cartridge. It contains polysulfone hollow fibres with a nominal molecular weight cut-off of 100,000 and an intraluminal diameter of 1,100 μm. The fibre length is 203 mm. The intraluminal volume of one fibre is therefore 0.193 ml. The device has a membrane surface area of 0.03 m^2. Neither the membrane wall thickness nor the number of fibres per cartridge is given. The total intraluminal volume can be calculated from reported cell seeding densities and resultant total cell numbers (Nyberg et al. 1993b). The calculated value is 10 ml. This means that there are some 52 fibres in the device.

The intraluminal compartment of the device is seeded with up to 10^8 primary rat hepatocytes suspended in a collagen solution. The collagen solution polymerises to form a gel which is reported to contract from an initial diameter of 1,100 μm to a final diameter of around 630 μm. The extraluminal space is perfused with whole blood

at 30 ml min^{-1}. The intraluminal space available as a result of gel contraction is reported to be perfused with a solution containing "growth factors and nutrients".

Evidently this device is at a prototype stage and most probably does not contain sufficient cells for human liver support. More recently, this group have reported techniques for the isolation and culture of primary pig hepatocytes (Lazar et al. 1995a; Lazar et al. 1995b; Sielaff et al. 1995) and have also published a paper describing the utilisation of porcine hepatocyte spheroids (discussed later in this chapter) in their device (Wu et al. 1996). It seems likely therefore that a scaled-up version will be reported in the future.

3.4.1. Primary Hepatocytes in Bioartificial Livers

The decision to utilise primary hepatocyte cultures in hybrid liver support devices is one which is taken with reluctance. It necessitates the preparation of fresh hepatocytes for each session of therapy since, to date, no reliable cryopreservation method has been described in the literature. The use of primary hepatocytes in a hybrid artificial liver device will therefore make preparation more labour intensive and will increase the overall cost of therapy.

However, there are no sufficiently well differentiated cell lines which could be considered suitable for use in a liver support device at present. This makes the decision a straightforward one. In effect one can elect to use an unsuitable cell line in the knowledge that the device is unlikely to work, or one may burden oneself with the inconvenience of having to perform repeated hepatocyte isolations while accepting the associated costs.

Since no hybrid liver support device has yet been proven to be efficacious in human trials and the precise requirements of hepatocytes in a hybrid liver support device have not been identified, the responsible approach must be to use a cell source which retains the highest degree of differentiated function and which has an acceptably low risk associated with its use in human therapy.

For most researchers this means working with primary non-human hepatocytes. (Human hepatocytes would of course be preferable however, few groups

have access to this resource and the supply is infrequent due to the demands of transplant centres.) The numbers of hepatocytes which are expected to be required for effective liver support means that large animals are likely to be the preferred source. Several devices are based on pig hepatocytes. Amongst these are the two remaining systems to be considered in this thesis. The salient points relating to the use of porcine hepatocytes in hybrid liver support devices will therefore be discussed in the two case studies later in this chapter.

As described, most of the published data on the University of Minnesota device is based on primary rat hepatocytes. This is only possible since the device is still at a prototype stage. One can expect to isolate no more than about $5x10^8$ hepatocytes from a rat liver. The use of primary rat hepatocytes in a clinically applicable device can therefore be discounted on purely numerical grounds since 40 or more animals would be required for each device. However, the relatively simple isolation method required and the reduced costs make rat hepatocytes an attractive model cell for use in small scale prototype devices. There are sufficiently few major metabolic differences between human and rat hepatocytes to make rat hepatocytes suitable for some in vitro metabolism studies. They can therefore be considered appropriate for evaluation of bioreactor designs for hybrid liver support devices.

3.4.2. Analysis of Minnesota Configuration

There are two major differences in the configuration of this device compared with the Hepatix device discussed previously. These are the location of the hepatocytes in the intraluminal compartment and the entrapment of the cells in a collagen gel. The principal reason for seeding the cells in the intraluminal compartment is a consequence of the decision to entrap the cells in collagen gel. Locating the collagen gel in the intraluminal compartment will give better control over cell distribution within the gel and also help to regulate gel diameter and gel contraction. It would be very difficult to achieve satisfactory control over gel contraction in the extraluminal space. There are no mass transfer advantages in having the blood in the extraluminal space in devices where the direction of blood flow is parallel to the fibres.

Indeed, if the interfibre distance is large (as it must be in this device) this is a disadvantageous configuration.

As mentioned previously, one of the problems associated with the culture of primary hepatocytes is the gradual loss of differentiated function with time. It has been reported that this phenomenon is a consequence of events triggered during the isolation process (Padgham and Paine 1993). In particular, the authors of this report describe alterations in the expression of mRNAs encoding cytochrome P-450 isoenzymes which occur during isolation however, no particular mechanism is postulated. Nevertheless, there are three parameters which, amongst others, may be considered as potential contributors to this effect. These are: the action of collagenase (the enzyme normally used to dissociate the liver into hepatocyte suspensions), the changes in intracellular pO_2 which occur during isolation and the effects of the loss of intercellular contacts and detachment from the extracellular matrix.

Workers have tried various approaches in order to combat the loss of function during culture. Non-enzymatic isolation methods have been evaluated (Gerlach et al. 1993a; Meredith 1988; Muller et al. 1972) but although some improvement in function has been observed with non-enzymatic techniques (Meredith 1988), cell yields have generally been very poor making this an inappropriate method for generating the large numbers of cells required for liver support devices.

The sparging of isolation solutions with various gas mixtures has been incorporated into some isolation methods. However, these experiments have been insufficiently controlled to determine the influence of liver pO_2 during isolation on hepatocyte function during the subsequent culture phase.

The most widely investigated aspect has been the role of extracellular matrix (ECM) in the maintenance of hepatocyte functions during culture. It has long been recognised that coating artificial materials with a layer of ECM material such as collagen promoted hepatocyte attachment and function. However more recent reports have suggested that particular culture configurations incorporating ECM can prolong the expression of certain hepatic functions in culture. One such report (Dunn et al. 1989) described a technique whereby hepatocytes could be cultured between

two layers of collagen gel. Hepatocytes cultured in this system were reported to exhibit normal morphology and albumin synthesis for 42 days. These workers attribute this marked improvement to the hepatocytes being able to recreate their in vivo structure and polarity. The presence of two layers of ECM will indeed support the development of two basolateral surfaces as in the hepatic acinus, however the benefits to the apical surface are less easy to see. Nevertheless, this group have demonstrated that the 'collagen sandwich technique' positively influences the transcription and translation of albumin RNA (Dunn, Tompkins, and Yarmush 1992) and improves cytoskeletal architecture (Ezzell et al. 1993) and membrane protein distribution (Yarmush et al. 1992).

Recently, this technique has been incorporated into a bioreactor design intended for application as an in vitro drug screening system (Bader et al. 1995; Bader et al. 1994). This device is based on a multi-plate oxygenator. However, it is of such complexity that scale-up to a clinically useful liver support device seems improbable.

It should be noted that there is an important distinction between the sandwich technique and the entrapment method reported for the Minnesota device under discussion. There is less evidence that the entrapment method, which cannot ensure the monolayer cell configuration produced by the sandwich technique, has such a positive effect on hepatocyte function. One recent paper has reported a similar prolongation of albumin synthesis for both gel entrapped and sandwich cultured hepatocytes (Koebe et al. 1994).

The presence of the collagen gel and the additional fluid phase between the membrane and the contracted gel in the lumen of the fibres are likely to impose significant transfer resistances in this device. Due to a lack of required data, a numerical analysis of oxygen transport in this device cannot be undertaken. However, since transfer resistances, diffusion distances and oxygen consumption rates will all be higher in this configuration than in the Hepatix system, we can reasonably expect there to be a large oxygen debt in this device even although the cartridge length is significantly shorter. This would explain the very low measured value of oxygen consumption reported for this device of around 28×10^{-18} mol s^{-1} per cell. In other

reports the oxygen consumption rate of primary rat hepatocytes has ranged from $328x10^{-18}$ mol s^{-1} per cell (McLimans, Blumenson, and Tunnah 1968) to $380x10^{-18}$ mol s^{-1} per cell (Foy et al. 1995). It would seem therefore, that the consumption rate in the Minnesota bioreactor is limited by poor oxygen supply.

Albumin synthesis is the most widely reported functional parameter for this device. This has also been the case for many culture configurations and bioreactor designs incorporating collagen gels. It may be the case that this parameter has tended to be reported since it gives the clearest demonstration of improvement. However, its relevance to liver support is questionable. The synthesis of albumin itself is not important for the treatment of liver failure although enhanced albumin synthesis may reflect improved synthesis of other proteins such as clotting factors which are of more therapeutic value. Even so, such factors can be directly administered if necessary. Most clinicians would agree that retention of a wide range of detoxification pathways would be of greater value for a prospective hybrid liver support system. One report has demonstrated that this device is capable of metabolising lidocaine via P-450 isoenzymes to give different metabolites (Nyberg et al. 1993a).

3.4.3. Mode of Application of the Minnesota Device

This device is intended to be perfused with whole blood. It will therefore be subject to the same advantages and disadvantages as the Hepatix device. One important distinction being an increase in the nominal molecular weight cut-off of the Minnesota device of some 30,000. This should give a small improvement in the transfer of low molecular weight proteins (such as albumin) although the albumin rejection ratio for such a membrane is likely to be high. This may well turn out to be a significant disadvantage since many species intended to reach the hepatocytes are protein bound and will not therefore undergo metabolism or transformation in the reactor.

3.4.4. Conclusions from the Minnesota Study

The utilisation of primary cells potentially offers improved function in the

short term. Entrapment of the cells within collagen gels may prolong the expression of some functions although the effect of this entrapment on critical functional parameters is unknown. The transport of oxygen in this system would seem to be inadequate and this is reflected by the very low cellular oxygen consumption rate reported for this device.

It is based on a commercially available hollow fibre cartridge and is intended for perfusion with whole blood so it is simple to construct, prepare and apply clinically. These factors make it an economically attractive proposition and it may therefore find industrial backing for scaled-up production.

However, there is no evidence which indicates that the device is likely to work in liver failure patients and this analysis would suggest that it will offer little in performance terms.

3.5. The Cedars-Sinai Bioartificial Liver (BAL)

This device has been developed at the Department of Surgery and the Liver Support Unit of Cedars-Sinai Medical Centre in Los Angeles with support from the Department of Chemical Engineering of the Vanderbilt University in Nashville. The principal investigators are A. A. Demetriou and J. Rozga. They have reported the development of their system (Rozga et al. 1993a), its performance in *in vitro* and animal studies (Giorgio et al. 1993; Rozga et al. 1993c) and have published numerous clinical papers (Chen et al. 1996; Demetriou et al. 1995; Neuzil et al. 1993; Rozga et al. 1994a; Rozga et al. 1993b; Rozga et al. 1994b). Recently, another group have reported the results of a small preclinical trial of a system based on the Cedars-Sinai device (Sheil et al. 1996).

Like the two previous devices, this system is based upon a commercially available hollow fibre module (Microgon Inc., California). It consists of 670 cellulose mixed ester fibres of 0.2 μm pore size, 635 μm internal diameter and a wall thickness of 62.5 μm. The fibres are housed in a polycarbonate cylinder giving a potted length of 445 mm and a shell side volume of 177 ml. The effective membrane surface area is 0.7 m^2. From this data, the mean interfibre distance can be calculated to be 240 μm.

The extraluminal space is seeded with up to $6x10^9$ primary porcine hepatocytes attached to microcarriers. Prior to therapy, the patient receives a double lumen catheter in the femoral vein. Blood from the patient passes through a plasma separator and in some instances the resultant plasma is pre-treated with a charcoal column before entering the device at a flow rate of approximately 80 ml min^{-1}. In more recent cases, a plasma transmission reservoir and a pump have been incorporated in the circuit to enable recirculation of plasma at high rates (220-400 ml min^{-1}) through the device.

Sessions of therapy are continuous and are reported to last 6 or 7 hours. Patients have tended to receive only 1 or 2 sessions of therapy although one case report describes one patient who had 3 sessions (Demetriou et al. 1995).

3.5.1. Porcine Hepatocytes in Hybrid Artificial Liver Devices

Primary porcine hepatocytes are currently the cell source of choice for several groups working on hybrid liver support. However, no precise reasons for this choice have been reported.

In the preceding sections, the current lack of an appropriate cell line was identified and the consequent (temporary?) reliance upon primary hepatocytes as a cell source for hybrid liver support systems was explained. Evidently, the large numbers of cells required for clinical scale devices could only realistically be harvested from a large animal. In this regard, the pig is an obvious source. Being physiologically similar to humans, pig tissues have been utilised in other areas of medicine and clinical familiarity with related practical and ethical matters exists.

However, the suitability of porcine cells in terms of metabolism and immunological parameters is less well understood.

There is a paucity of data on porcine liver metabolism. For example, the author has been unable to find published values for the oxygen consumption rate of cultured porcine hepatocytes. Furthermore there is insufficient data available for researchers to assess whether porcine hepatocytes are able to metabolise all potential human liver toxins. It is thought that pigs do not express many sulfation reaction enzymes (Grant

1995 personal communication). However, the significance of this is not clear since it is not reported whether the compounds which would undergo sulfation in humans are detoxified by another route in pigs.

One report has identified a potential immunological problem associated with the use of porcine hepatocytes (Takahashi et al. 1993). In a series of experiments in which porcine hepatocytes were cultured in human sera for metabolic studies, sera from 7 out of 103 donors proved to be toxic to the cells. The toxicity was dose dependent and was abrogated by heat treatment at 56°C but not by treating at 50°C. The authors concluded that the classical complement activation pathway was involved in the toxicity but was not the initiator of the toxic reactions. A naturally occurring anti-porcine hepatocyte IgM was detected more frequently on the surface of porcine hepatocytes incubated in the toxic sera than on those incubated in the non-toxic sera. The authors attributed the initiation of toxicity to the fact that the IgM in the toxic sera recognised different surface receptors on the porcine cells than the IgM in the non-toxic sera and that this in turn affected the extent of complement activation.

The sub-group of patients with toxic serum represents 6.8% of the test population. This is a small but significant population and indicates the requirement for further immunological experimentation on porcine and other candidate cell types.

Another report demonstrates that hepatocytes are in themselves non-immunogenic *in vitro* and that immunological phenomena observed in hepatocyte cultures are a consequence of the presence of non-parenchymal cells (especially Kupffer cells) in the culture or are due to activation by factors present in xenogenic sera (Karrer et al. 1995).

Investigators should proceed with caution if they intend to utilise primary hepatocytes in a hybrid artificial liver. Porcine hepatocytes represent a potentially useful cell source. However, there is no definitive evidence that they are metabolically sufficient and some potential immunological problems exist.

3.5.2. Analysis of Cedars-Sinai Configuration

From an analytical point of view the configuration is similar to the Hepatix device. The chief differences are that the intraluminal compartment is perfused with plasma rather than blood and that the cells in the extraluminal space are attached to microcarriers.

A significant transmembrane convective flux of both glucose and albumin has been reported to exist in this device (Giorgio et al. 1993). This flux is produced by reducing the rate of outflow from the intraluminal compartment by 20%. The measured magnitude of this convective flux was reported to depart slightly from theoretical values and this was attributed to the presence of a small diffusional component. However, for the purpose of this analysis, the 20% reduction in axial flow rate will be considered to produce an equal transmembrane convective flow rate, the oxygen concentration gradient in the membrane will be assumed to be zero and any contribution to oxygen transport by diffusion will be ignored.

Therefore, in this configuration, the maximum rate of delivery of oxygen to the extraluminal space will be the product of the transmembrane convective flow rate, the allowable oxygen partial pressure drop across the extraluminal space and the solubility of oxygen in the filtrate. This will be equivalent to the oxygen consumption rate of the cell mass. The maximum oxygen partial pressure drop occurs when the extraluminal outlet pO_2 is zero. In practice this is unlikely but this analysis will give the best possible case for oxygen transport in this device since it assumes zero membrane transfer resistance and 100% oxygen extraction by the cells.

This gives:

$$Q_F.\alpha.\Delta pO_2 = T_{O_2} \qquad \textit{(Equation 3.13)}$$

Where; Q_F is the convective flow rate (ml s^{-1}), α is the solubility of oxygen in plasma at 37°C (1.25×10^{-9} mol ml^{-1} $mmHg^{-1}$ (Battino 1981)), ΔpO_2 is the oxygen partial pressure drop across the entire extraluminal space (mmHg) and T_{O2} is the oxygen transfer rate (mol s^{-1}).

The reported maximum axial flow rate in this device is 400 ml min^{-1}. Therefore the maximum transmembrane convective flow rate will be 80 ml min^{-1} (1.33 ml s^{-1}). No information about the inlet pO_2 in this device has been published. The authors early clinical reports did not detail any form of plasma oxygenation although more recently, they have stated that they use a membrane oxygenator in the plasma recirculation loop. If we initially assume, despite potential oxygen toxicity, that the perfusate is equilibrated with pure oxygen as a result of such oxygenation (i.e. inlet pO_2 = 750 mmHg) then this will give a maximum theoretical rate of oxygen transfer in the device.

Inserting these values into equation 3.13. gives a maximum theoretical oxygen transfer rate of 1.25×10^{-6} mol s^{-1}. Since no reported oxygen consumption rates of pig liver *in vivo* or of isolated pig hepatocytes *in vitro* have been found, this theoretical value cannot be directly compared to actual cellular requirements. However, it may be compared to previously reported values for rat hepatocytes (328×10^{-18} mol s^{-1} per cell (McLimans, Blumenson, and Tunnah 1968) to 380×10^{-18} mol s^{-1} per cell (Foy et al. 1995)) and for human liver *in vivo* at basal metabolic activity (318×10^{-18} mol s^{-1} per cell (Myers 1947)). Based upon this data, the number of cells which could be supported, given 100% efficiency in O_2 exchange within the extraluminal space, lies between 3.2×10^9 and 3.9×10^9.

Conversely, the maximum rate of oxygen consumption which could, under ideal transfer conditions, be supported by this device is 208×10^{-18} mol s^{-1} per cell since the number of cells in this device is reported to be 6×10^9.

It can be seen that the maximum theoretical oxygen transfer rate in this device is significantly lower than that required to satisfy the oxygen consumption rate of rat hepatocytes or the human liver, according to previously reported values. The number of hepatocytes which could be supported by this device is equivalent to approximately 2% of the number typically present in an adult human liver. Therefore, one could only expect to achieve around 2% of basal human liver metabolic output from this device if it were populated with fully functional human hepatocytes and transfer conditions were ideal. This level of metabolism is highly unlikely to be

sufficient to support a patient in acute liver failure for any significant length of time.

In practice, the amount of oxygen available for consumption by hepatocytes will be far less than that assumed in this analysis. Channelling of filtrate flow is likely due to non-uniform distribution of microcarriers and fibres, the actual transmembrane convective plasma flow rate may well be less than that assumed since the redirection of 20% of axial flow across the membrane may not be possible at the highest axial flow rates and 100% oxygen extraction by hepatocytes from the filtrate is improbable since the filtrate flow is not well mixed.

Another aspect of the Cedars-Sinai configuration should also be considered. Although, in practice, this device could not support an increase in cell mass since it already appears to be oxygen limited, the volume occupied by the microcarriers in the extraluminal space would ultimately limit any increase in cell mass. In this context, the microcarriers appear quite literally to be 'a waste of space'. Their role in this device is unclear. Certainly in stirred tank systems, microcarriers offer the possibility of high surface area to volume ratio attachment dependent cultures and in the Cedars-Sinai device, the carriers will increase the overall available attachment area. However, this effect could also be achieved by increasing the number of hollow fibres in the device and reducing the internal fibre diameter and the interfibre distance.

It is more likely that the microcarriers are used to help overcome practical difficulties. The hepatocytes are attached to the carriers in a stirred culture flask prior to seeding in the reactor. This probably aids cell distribution within the device and may permit cryopreservation of attached cells which could be quickly thawed and seeded into a device upon demand.

As stated earlier, attempts to cryopreserve primary hepatocytes have so far met with limited success. It has been suggested that success can be improved by cryopreserving hepatocytes as an almost dry pre-attached monolayer (Watts 1995 unpublished data). Interestingly, the Cedars-Sinai group have reported that their porcine hepatocytes may be cryopreserved while attached to the microcarriers (Rozga et al. 1993c), but, as yet, such cryopreserved cells have not been explicitly stated to have been used in any clinical applications. The group have more recently reported a

slightly different method of seeding their device (Chen et al. 1996). This involves use of a reduced quantity of microcarriers. The total seeded cell number remains unchanged but the use of fewer microcarriers is reported to lead to formation of cell aggregates on and between the carriers. The utilisation of hepatocyte aggregates in hybrid liver support devices is discussed later in this chapter.

3.5.3. Mode of application of the Cedars-Sinai device

The device is perfused with plasma. The patients are normally connected to the system via a veno-venous double lumen catheter placed in the femoral vein. Blood is removed from the patient at approximately 80 ml min^{-1} and is pumped through a plasma separator. The plasma drawn from the separator is delivered to a secondary circuit in which it is recirculated at flow rates between 220 and 400 ml min^{-1}. In some cases, the recirculating plasma passes through a charcoal column before entering the device. A fraction of the treated plasma is re-united with the cell fraction and returned to the patient while the remainder is recirculated. Treatment sessions last for 6 to 7 hours during which perfusion through the device is continuous. At the end of this period, the patient is disconnected. Therapy is not usually repeated (of 12 clinical treatments reported so far, 6 patients received only 1 session of therapy, 4 received 2 sessions and the remaining 2 patients were treated 3 times) except in those cases where a suitable donor organ is not available immediately and the patient is a transplant candidate. It is not explained whether 6 to 7 hours represents the life span of one device or that this is the duration of therapy which the clinicians involved consider to be required. It is likely that some component of the extracorporeal circuitry, most probably the hybrid artificial liver device itself, the plasma separator or the charcoal column, requires to be changed after 6 or 7 hours and the decision to cease therapy at this point is a consequence of this requirement.

This is a complex system. The provision of plasma separation, charcoal perfusion and external membrane oxygenation is disadvantageous making the circuit complex, increasing priming volume and the number of pumps. The high recirculation rate has most probably been introduced for the purpose of oxygen transport since the

rate at which treated plasma is returned to the patient is not increased, although it might conceivably also serve to reduce the required duration of therapy. Some form of oxygenation is essential since the plasma from venous blood will have a pO_2 of approximately 40 mmHg and this combined with a relatively low solubility means the untreated perfusate will transport very little oxygen to the device. However, as demonstrated, delivery (via an external membrane oxygenator) of completely oxygen saturated plasma, which would probably be toxic to the hepatocytes in the entry region of the device will still not satisfy the oxygen demands of the cells so this mode of oxygenation is inappropriate.

The incorporation of a charcoal column upstream of the device is intended to 'protect' the hepatocytes from the potentially toxic effects of FHF plasma. This is a very important issue. It is reasonable to expect that plasma from patients with FHF will have some toxicity to cultured hepatocytes. Indeed this has been reported to be the case (Haas et al. 1981). Such serum has also been shown to inhibit DNA synthesis in hepatocytes from regenerating rat livers (Gove, Hughes, and Williams 1982). However, more recent findings suggest that serum from patients with FHF resulting from paracetamol overdose increases the total cytochrome P450 content and the rate of urea synthesis in cultured rat hepatocytes (Dillon et al. 1995).

In chapter 1 the role of adsorbents in liver support was discussed. It was concluded that in isolation they would be unlikely to be effective in treating patients with advanced liver failure (i.e. those with grade 3 or 4 encephalopathy). However, it was postulated that they may find application if combined with other supportive measures. It remains to be seen whether the combination of adsorbents and hybrid liver support is more beneficial than hybrid support alone. While an upstream sorbent column may protect the hepatocytes from toxicity, it may also remove compounds which ought to contact the cells such as nutrients or unknown factors which may enhance hepatocyte function. One definite conclusion which can be reached at this stage is that the presence of a charcoal column in the system will make it more difficult to attribute any apparent clinical successes directly to the hybrid artificial liver device.

3.5.4. Conclusions from the Cedars-Sinai study

Being based upon primary porcine hepatocytes, this device potentially offers good metabolic performance although there is a lack of data about which detoxification pathways are expressed by these cells and a small proportion of patients may have serum proteins which trigger immunological reactions upon binding to porcine cells. The reliance upon repeated harvesting of cells probably makes this device commercially unattractive until such time as a reliable cryopreservation method or a suitable cell line becomes available.

The cell mass ($6x10^9$ cells) is somewhat less than other groups have suggested might be required for support ($>2x10^{10}$). Although it should be stressed that such figures have been derived by indirect empirical methods and to date, one cannot reliably state how many cells will be necessary. Inevitably, the differentiated status and metabolic capacity of the cells will enter the equation. In this respect, the Cedars-Sinai device has advantages and disadvantages. Repeated isolations of fresh primary cells should provide a wide spectrum of functions in the short term. However, analysis has shown that this device is oxygen limited even under ideal transfer conditions and since the principal mechanism of solute transport in the device is convection, it can be stated with a fair degree of certainty that the transport of other solutes will also be limited in this system.

In papers detailing 22 clinical applications (Chen et al. 1996; Demetriou et al. 1995), 20 patients are reported to have survived although 19 of these received a transplant within 24 hours of treatment with the device. All but one of these treatments included a charcoal column upstream of the device.

The most striking effect seen in patients treated with the hybrid artificial liver was a marked reduction in intracranial pressure. Intracranial pressures measured immediately after treatment were significantly lower than those measured immediately prior to treatment. There was a concomitant increase in cerebral perfusion pressure. However, due to the complexity of the therapy it is not possible to attribute these effects to performance of the device itself.

The Cedars-Sinai BAL is part of a scaled-up, clinically applicable system which

has been used without serious incident in a number of patients. It lacks the controversy associated with the Hepatix cell line. While it is not yet clear whether primary porcine hepatocytes are a suitable cell source in terms of metabolism or immunological reactions, it could be argued that this device could accept a change in cell source with little or no requirement for re-design. In these respects it represents a major advance in the state of the art.

However, it suffers from design shortcomings. Oxygen transport is inadequate and the transfer of other solutes will also be limited. As in the Hepatix device, this is partly the consequence of the interposition of a membrane between the perfusate and the cells. In the Cedars-Sinai device this has a more deleterious effect due to the reduced oxygen carrying capacity of plasma in comparison to whole blood. (Having said this, it should be recognised that, in comparison to the Hepatix ELAD, the interposing membrane in the Cedars-Sinai BAL has an increased NMWCO so an improvement in the transfer of protein bound solutes might be expected.) The incorporation of a membrane oxygenator in the recirculating loop cannot be expected to alleviate the oxygen debt. The cell mass may also prove to be insufficient and the design will not permit the 4 or 5 fold increase in cell mass, which would take it to the level thought to be required for support, to be achieved within a single hollow fibre module. Attempts to operate multiple devices in series or in parallel would increase transfer limitations.

3.6. A novel multi-compartment bioreactor for liver support

A prototype device with a unique design concept has been developed in Berlin. The inventor, J. Gerlach has reported the development of his device and its subsequent testing *in vitro* and in an animal model of liver failure (Fuchs et al. 1994a; Fuchs et al. 1994b; Gerlach et al. 1994a; Gerlach et al. 1996a; Gerlach et al. 1993b; Gerlach et al. 1993c; Gerlach et al. 1994b; Gerlach et al. 1996b; Gerlach et al. 1994c).

Since this device is fabricated 'in house', some of the data which is available for the devices based upon commercially sourced modules is unavailable. It is based upon an array of woven hollow fibre mats. These mats are layered on top of each other to

create a three dimensional framework of capillaries between which the hepatocytes are cultured. By alternating mats, each composed of a different type of capillary membrane, a multi-compartment system is generated.

The device is intended to be perfused with plasma in the clinical setting. Plasma is delivered to the hepatocytes by one set of capillary mats and is collected from another set. The plasma carrying capillaries are composed of polyethersulphone or polyamide membranes. There is provision within the device for the incorporation of additional capillary systems which could, for example, provide oxygenation (via hydrophobic capillaries such as silicone rubber or polypropylene), heat exchange or co-culture.

Primary porcine hepatocytes are seeded into the extraluminal space where they are reported to organise themselves into structures resembling aggregates. The current prototype is reported to hold up to $2.5x10^{10}$ cells.

The associated circuitry includes a plasmapheresis system and pressure transducers which control the pumps. There is no plasma reservoir in the circuit so the rate of plasma filtration is equivalent to the rate at which the device is perfused with plasma. The envisaged mode of therapy is one of a series of repeated intermittent sessions. Each session is expected to last for 4 to 6 hours with a gap of similar duration during which plasma filters would be changed. The device itself is expected to survive several such sessions. If continuous therapy turns out to be necessary the system could be adapted to accommodate this.

3.6.1. Hepatocyte aggregates and liver support

A number of articles have described the formation of multicellular hepatocyte aggregates, sometimes referred to as spheroids, under different culture conditions. In this thesis, these structures will be referred to as aggregates. In 1988, Shinji and co-workers detailed the formation of aggregates of rat hepatocytes when the cells were cultured upon a surface coated with rat proteoglycans (Shinji, Koide, and Tsuji 1988). Subsequent papers have reported the formation of rat aggregates in the absence of extracellular matrix components (Koide et al. 1990), on the surface of novel polymeric

materials (Tobe et al. 1992a; Tobe et al. 1992b) and by chicken hepatocytes within collagen gels (Parsons-Wingerter and Saltzman 1993).

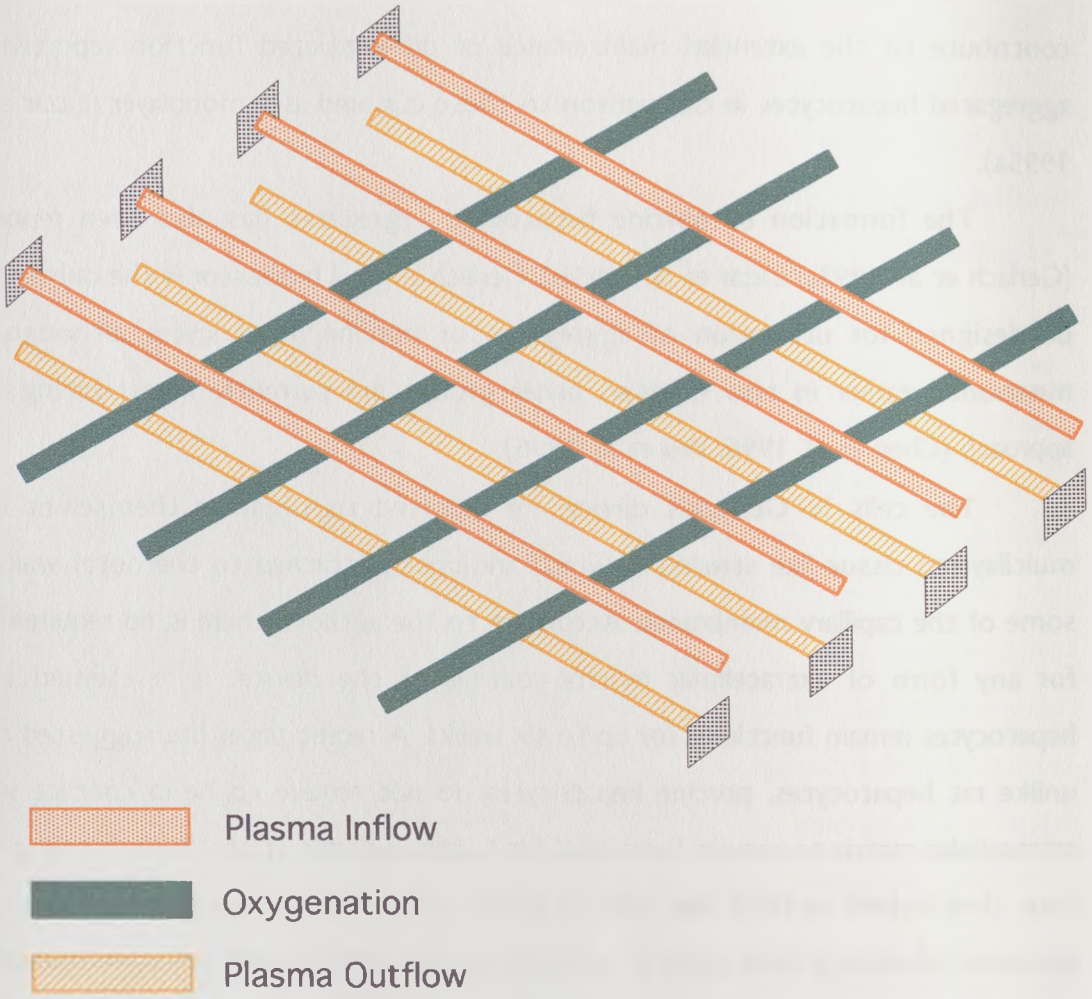
It has also been stressed that hormones (insulin and epidermal growth factor) are essential for the maintenance of rat aggregates and that aggregated hepatocytes remain in the G_0 phase of the cell cycle (Yuasa et al. 1993). This phenomenon may contribute to the extended maintenance of differentiated function reported for aggregated hepatocytes in comparison to those cultured as a monolayer (Lazar et al. 1995a).

The formation of porcine hepatocyte aggregates has also been reported (Gerlach et al. 1993c; Lazar et al. 1995b). Gerlach's novel bioreactor is the only one to be designed for utilisation of aggregates of porcine hepatocytes although, as mentioned earlier in this chapter, other groups are currently investigating this approach (Chen et al. 1996; Wu et al. 1996).

The cells in Gerlach's device are reported to organise themselves into multilayered tissue-like structures which are loosely attached to the outer walls of some of the capillary membranes. According to the authors, there is no requirement for any form of extracellular matrix coating of the device. It is claimed that hepatocytes remain functional for up to six weeks. A recent paper has suggested that unlike rat hepatocytes, porcine hepatocytes do not require to be in contact with extracellular matrix to remain functional for 5 days (teVelde et al. 1995). If this is the case, then hybrid artificial liver devices based upon porcine hepatocytes will have a particular advantage since there is no need to coat surfaces with collagen or another extracellular matrix extract, which is difficult and represents a contamination risk.

The novel multi-compartment device under discussion enjoys this particular advantage. A sterile suspension of porcine hepatocytes can be seeded directly into the sterile device immediately upon isolation. There is no requirement for attachment of the cells to microcarriers or for suspension of the cells in some collagen solution.

However, there is at least one potential disadvantage to the use of aggregated hepatocytes in a hybrid artificial liver device. These aggregates are reported to be quite large, having a diameter of approximately 100 μm in one report (Peshwa et al.



Idealised membrane configuration in the Berlin hybrid artificial liver

Figure 3.5.

1994) and to date a reliable means of controlling the size of aggregates has not been reported. Large aggregates may lead to mass transfer problems and this will be considered in the following section within the context of oxygen transport in Gerlach's bioreactor.

3.6.2. Analysis of Berlin configuration

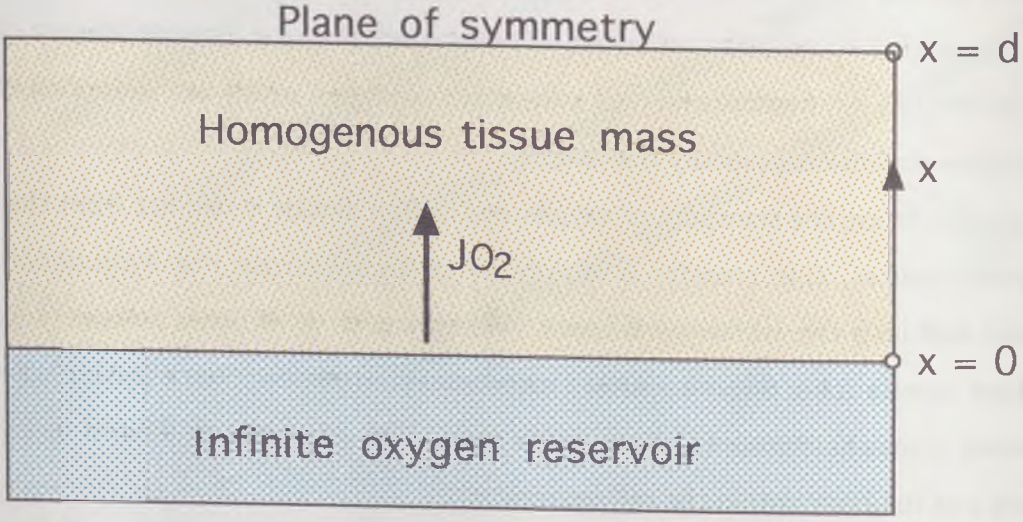
A diagrammatic representation of the membrane configuration in Gerlach's design is given in Figure 3.5.

The cells are seeded directly into the extraluminal space. They occupy most of this space, forming aggregated layers which fill gaps between the different membrane mats. The plasma inflow and outflow capillaries are both closed at one end which enables the plasma to be perfused across the extraluminal space. Integral delivery of oxygen is effected via hydrophobic gas permeable capillaries which are incorporated into the three-dimensional capillary framework.

In this device, the transport of oxygen and carbon dioxide is distinct from that of all other solutes. Gas transfer is mainly by diffusion across the hydrophobic membrane wall into the extraluminal space. The transport of all other solutes is by convection across the plasma inflow membranes. However, once inside the extraluminal space the transport of any given solute will ultimately be limited by its diffusivity and solubility within the cell aggregates.

There are two main areas of resistance to gas transfer in this device. The first consists of those resistances associated with the gas compartment membrane. However, by manipulating the conditions in the gas phase, axial and transmembrane concentration gradients can be adjusted to give the desired gas conditions at the outer wall of the hydrophobic membrane. Therefore, the second area of transfer resistance, i.e. those resistances encountered in the intracellular milieu, become the transfer limiting parameters.

If the extraluminal space is considered to be filled by a homogenous tissue mass having a permeability to oxygen equivalent to the intracellular environment of the hepatocytes and an oxygen consumption rate equal to that of the hepatocytes, a



Idealised oxygen diffusion model for Berlin device

Figure 3.6.

simple model of gas diffusion across a well defined liquid film from an interface with an infinite gas reservoir becomes applicable for this device. For the purposes of this analysis, it can be assumed that there is no axial pO_2 gradient at the outer wall of the gas compartment membrane provided that the gas flow rate is sufficiently high. The model system then becomes one dimensional. Models of this general form have been reported by McLimans *et.al.* (McLimans, Blumenson, and Tunnah 1968) and more recently by Yarmush *et.al.* (Yarmush et al. 1992) which describe the diffusion of oxygen from such a gas interface across a liquid film with zero oxygen consumption to a monolayer of cells with a known rate of oxygen consumption per unit area. The principle differences between these two models and the model system that is applicable to this device are that a homogenous cell mass replaces the liquid film and this cell mass is assigned a non-zero rate of oxygen consumption and secondly that a boundary condition stating that there is no net diffusion of oxygen at half the interfibre distance from the outer capillary wall is required. This boundary replaces the original cell monolayer boundary. A representation of this model system is given in Figure 3.6.

This approximation of radial diffusion to that of planar diffusion assumes that the diffusion distance is small with respect to the oxygenation fibre outer radius. As the diffusion distance becomes larger, this approximation becomes inappropriate. The diffusion distances considered in these analyses extend beyond the limits of applicability of the model. However, since the oxygenation membranes are arranged in flat woven mats with smaller interfibre distances in the plane of the mat than between adjacent mats, this approximation is probably more appropriate than a radial diffusion model. It follows that the data generated will give the best possible case for oxygen transport in this design.

The hydrophobic membrane is considered to be infinitely permeable to oxygen. This approximation holds for typical microporous oxygenation membranes. (Note: the analysis could be extended to homgeneous oxygenation membranes e.g. Silicone rubber by taking into account their permeability.) The tissue mass is considered to be homogenous with uniform oxygen diffusivity, solubility and consumption. At steady

state, Fick's first law of diffusion can be used to determine the oxygen partial pressure P at any distance x from the gas tissue boundary.

At steady state, the flux of oxygen from the membrane tissue mass boundary is entirely due to consumption by the cell mass. In these circumstances, equation 3.1. can be re-arranged and re-written as:

$$\frac{dpO_2}{dx} = \frac{-J_{O_2}}{D\alpha} \qquad \textit{(Equation 3.14)}$$

Where; JO_2 is the flux of oxygen through the cell mass and D and α are the diffusivity and solubility of oxygen in the cell mass respectively. The magnitude of the flux is equivalent to the rate Q at which oxygen is consumed by the cell mass.

This gives:

$$\frac{dpO_2}{dx} = \frac{-Q}{D\alpha} \qquad \textit{(Equation 3.15)}$$

Integration of equation 3.15. will produce a solution for the oxygen partial pressure at some distance x from the gas tissue interface.

Thus:

$$P_{(x)} = P_i - \frac{Qx}{D\alpha} \qquad \textit{(Equation 3.16)}$$

Where P_i is the oxygen partial pressure at the gas tissue interface. In order to solve this equation, we must assign values to Q, D and α which relate to the cell mass. For this purpose, the cell mass shall be considered to consist of that number of identical cells which occupy a unit volume. If each cell is considered to be a cuboid of equivalent volume to a sphere of diameter 25 μm (McLimans, Blumenson, and Tunnah 1968) and if $2x10^5$ such cells occupy a surface area of 1 cm^2 (Yarmush et al. 1992), then the dimensions of each cell element are 22.4 μm x 22.4 μm x 16.4 μm

Oxygen consumption rate	Cell type	Reference
12-25 nmol $min^{-1}/10^6$ cells	Primary rat hepatocytes	Noll et al 1986
15-23 " " "	Primary rat hepatocytes	Foy et al 1994
50 " " "	Theoretical value	n/a

Number of cell layers which can be oxygenated

pO_2 at cell mass membrane boundary (mmHg)

Oxygen consumption rate (nmol $min^{-1}/10^6$ cells)	50	100	200	400
10	2	4	7	14
25	1	2	3	6
50	1	1	2	3

Maximum permissible interfibre distance (μm)

pO_2 drop across cell mass (mmHg)

Oxygen consumption rate (nmol $min^{-1}/10^6$ cells)	49.5	99.5	199.5	399.5
10	65	131	229	459
25	32	65	98	196
50	32	32	65	98

Solution of oxygen diffusion model for Berlin configuration

_ Figure 3.7.

(length, breadth and height respectively). The diffusivity of oxygen in the cell mass shall be considered to be equal to the diffusivity of oxygen in the intracellular hepatocyte milieu, that is 2.8×10^{-6} cm^2 s^{-1} (deGroot, Littauer, and Noll 1987). Given that the cell area density is 2×10^5 cells cm^{-2}, this diffusivity is equivalent to 560×10^{-3} cells s^{-1}. The solubility of oxygen in the cell mass will be considered to be equivalent to that of oxygen in blood at 1.07×10^{-9} mol cm^{-3} $mmHg^{-1}$ (Goldstick 1971). If the volume of one cell is 8.23×10^{-9} cm^3, then this solubility can be expressed in cell terms as 8.81×10^{-18} mol $mmHg^{-1}$ per cell.

As discussed previously, there is no published data on the oxygen consumption rate of porcine hepatocytes. This analysis will therefore consider a set of oxygen consumption rates which encompass values previously reported for rat hepatocytes *in vitro* together with a significantly elevated value which is included for the purpose of illustrating the effect of increasing oxygen consumption rate on oxygen transport in this model system.

The basis used for the solution of equation 3.16 is that the minimum permissible pO_2 in the cell mass is 0.5 mmHg. This has previously been shown to be the K_m value for oxygen consumption by rat hepatocytes (deGroot, Noll, and Sies 1985; Yarmush et al. 1992), that is, the oxygen partial pressure at which oxygen consumption is half maximal.

On this basis, for given oxygen consumption rate and tissue membrane boundary pO_2, the number of cell layers which can be oxygenated and hence maximum permissible interfibre distance can be calculated.

In Figure 3.7., the oxygen consumption rates and their sources are given together with two sets of solutions for equation 3.16. These solutions are based upon the selected oxygen consumption rates and a set of values assigned for P_i.

It can be seen from this figure that if the aggregate membrane interface pO_2 is kept within the range normally associated with the liver *in vivo*, then no more than 4 cell layers may be adequately oxygenated provided that the oxygen consumption rate is at the lower end of the reported range. Any significant increase in oxygen consumption rate will reduce the number of layers which may be oxygenated to 1, or

at most 2. Significantly greater numbers of cell layers may be oxygenated if a very high aggregate membrane interface pO_2 is present. However, this would most probably cause oxygen toxicity in cells close to the oxygenation membranes. In design terms, these findings indicate that maximum permissible interfibre distances for this configuration are of the order of <100 µm.

Considering that this oxygen transport model lies within a 3-dimensional framework incorporating at least two further sets of capillary membranes, these requirements will be hard to satisfy. One possibility, which may improve oxygen transport to some extent if physical constraints mean that the maximum interfibre distance in the oxygenation system must be exceeded, would be to externally oxygenate the plasma which is perfused through the reactor. It has been shown in the Cedars-Sinai study, that this mode of oxygenation cannot alone meet the demands of the required cell mass. However, if combined with integral oxygenation in the Berlin configuration it might contribute sufficient oxygen transfer to meet a small oxygen debt if interfibre distances were slightly too large.

3.6.3. Mode of application of the Berlin device

This device is currently undergoing first clinical applications. As it is perfused with plasma, the mode of application is broadly similar to that of the Cedars-Sinai device. One major difference is that in the Berlin device, the entire plasma flow is directed across the extraluminal space whereas in the Cedars-Sinai device only a fraction of the plasma flow is diverted. This may lead to membrane fouling since proteins and other molecules from the plasma will be deposited upon the membrane surface. As a layer of deposition builds up on the membrane, the mass transfer performance of the membrane diminishes. After time the membrane may become completely occluded.

Besides the effect of reducing solute transport, this phenomenon has serious implications for the operation of the device. As membrane fouling proceeds, the pressure required to maintain flow increases. Hence the perfusion system used to operate this device must be primarily pressure regulated rather than flow controlled in

order to avoid dangerously high perfusion pressures. Fluctuations in pressure during operation are compensated for by adjusting flow rates and/or by having intermittent pump shut-downs. The effects of such alterations must be isolated from the patient in any clinical application. The necessary control hardware and modifications to the tubing system makes the perfusion apparatus more complex.

The membrane fouling problem may be addressed by periodic back flushing. This is an effective procedure but it does not negate the requirement for pressure regulated operation.

3.6.4. Conclusions from the Berlin study

This device, unlike any of those previously discussed, is purpose built as a hybrid artificial liver. The design is readily adaptable in terms of size, materials and configuration. It is probable therefore that it can be designed to accommodate any increases in cell mass which may prove to be necessary. On this basis, the Berlin device represents a landmark in hybrid artificial liver development.

It is the only reported scaled-up device with integral oxygenation. However, analysis has shown that few cell layers may be oxygenated in this manner without recourse to very high inlet oxygen partial pressures. This is not surprising when one considers the acinar architecture of the liver as described in chapter 2. It remains to be seen whether the required small interfibre distance in the oxygenation compartment can be achieved. If not, then at best only a proportion of the seeded cells will be metabolically active. Those cells at the centre of the cell mass will be under anoxic conditions.

The mode of perfusion of this device is disadvantageous since it courts pressure problems as a consequence of likely membrane fouling by plasma proteins.

3.6.5. Clinical application of the Berlin bioreactor

Dr. Gerlach has recently reported that his device has now been used clinically to treat three patients. Each patient had FHF resulting from drug toxicity and was treated for approximately 20-30 hours with the hybrid artificial liver system before

receiving a transplant. All three patients have survived to date and each showed improvements in general status and liver function during the treatment period (Gerlach 1997 personal communication). These results have not been published yet.

3.7. General conclusions from the case studies

There are a number of conclusions which may be drawn from the preceding studies. These centre upon the cell source, the perfusate, and the mode of oxygenation.

At present, there is no ideal cell line for application in a hybrid artificial liver. Therefore, those workers developing such devices should focus on primary cells in the meantime. For clinical application, the cells would have to be harvested from a large animal in order to obtain the necessary cell mass. The most obvious source animal is the pig. However, further work is required to elucidate the metabolic phenotype of pig hepatocytes and potential immunological problems have been identified. Therefore, alternative cell sources should be considered e.g. the sheep. In chapter 4, the function of primary sheep hepatocytes will be compared to primary rat hepatocytes. Different culture medium formulations will be tested for their suitability for maintaining these cells *in vitro* and for elucidating the sensitivity of the cell types to different fluid compositions.

The presence of an immuno-isolation membrane has a detrimental effect on mass transfer. However, these membranes are essential in blood perfused systems. If possible, direct contact between the hepatocytes and plasma would be the best configuration since this would offer optimal mass exchange. The immunological consequences of this configuration require to be elucidated.

The Cedars-Sinai study suggested that plasma cannot transport sufficient oxygen to satisfy the demands of the cell mass in a hybrid artificial liver. Integral oxygenation is therefore essential for a plasma perfused device (and would also be beneficial in a blood perfused system). The Berlin study indicated that even with integral oxygenation, at physiological oxygen partial pressures, only 1 or 2 cell layers may be supported if the anticipated rates of oxygen consumption are to be met.

A potentially suitable device configuration can now be discerned. This would consist of monolayers of hepatocytes (akin to the acinus) in direct contact with plasma and close to integral gas transfer membranes supplying oxygen at physiological partial pressures.

Exactly how and upon what the hepatocytes would be immobilised must also be decided. Some potential immobilisation regimes will be investigated in chapter 4.

It became apparent earlier in this chapter that there was a lack of data about the oxygen consumption rates of cultured hepatocytes. In particular, the rate at which monolayers of primary hepatocytes consume oxygen when they are perfused with solutions with a toxin load requires be elucidated. Such an investigation will form part of chapter 5.

One unique aspect of the envisaged configuration would be that the hepatocytes might be directly exposed to the shear stresses generated by the perfusate flow. The response of hepatocytes to such stresses has not previously been investigated. This will also be undertaken in chapter 5.

Finally, the conclusions drawn from the case studies in this chapter will be combined with the data generated in chapters 4 and 5 in order to design, build and test a prototype hybrid artificial liver device. This will be described in chapter 6.

Chapter 4

Comparison of primary rat and sheep hepatocyte function in different medium formulations and consideration of different culture configurations

4.1. Introduction

The aims of this chapter are; to investigate species differences in the function of cultured primary hepatocytes, to identify the most appropriate medium formulation for the maintenance of function of each cell type in bioreactors for liver support, to select appropriate functional tests and consider the relative benefits and suitability of different culture configurations for application in a prototype hybrid artificial liver.

4.1.1. Comparison of culture medium formulations

Many culture medium formulations have been used to culture hepatocytes. Several have proven to be successful for conventional culture methods (Gerlach et al. 1989; Grant et al. 1985; Paine and Hockin 1980; Waxman et al. 1990). The specific benefits of particular formulations have usually been unclear. However, two formulations will be considered in this comparison which offer two distinct advantages.

Chee's medium (Waxman et al. 1990) is a serum-free formulation. It must

therefore, be considered as a candidate medium for the culture of hepatocytes intended for medical application in a liver support system since at the present time, doubts are being expressed by regulatory bodies about whether cells which have been in contact with bovine serum will be permitted in medical devices.

A modification of Earle's Medium (Paine and Hockin 1980) has been reported to result in prolonged maintenance of intracellular cytochrome P-450 concentrations (Grant et al. 1985). This medium formulation is therefore also worthy of inclusion in this comparison.

These two media will be compared with Williams' E medium, the most widely used formulation for hepatocyte cultures and Medium 199, a formulation previously used in hepatocyte bioreactors (Gerlach et al. 1990).

4.1.2. Investigation of species differences

Much of the literature data about hepatocyte culture is based on the rat and since rat hepatocytes are relatively simple to isolate in numbers sufficient for in vitro investigations, they will form the basis of the experimental work in this thesis. However, as discussed in the previous chapter, it would require at least 40 rat livers to produce $2x10^{10}$ hepatocytes, the number considered by some investigators to be the minimum necessary cell mass required for liver support purposes. Evidently larger animals would be required to provide hepatocytes for a scaled-up hybrid artificial liver.

Sheep livers could provide the necessary cell numbers and since a source and a method of isolation (Emmison, Agius, and Zammit 1991) are available, sheep hepatocytes will be compared with those from the rat within the media comparison experiments. This will be performed in order to gain some insight into whether the medium of choice is likely to be species dependent and whether any major differences are evident between the two species.

4.1.3. Functional parameter and assay selection

There are a vast number of liver functions which could be investigated in

hepatocyte cultures. In chapter 2, aspects of liver metabolism of particular relevance to liver support were discussed. Ammonia metabolism, oxygen consumption and detoxification were identified as key parameters. Accurate investigation of oxygen consumption requires specialised culture methods so this will be undertaken separately. In the current chapter, ammonia metabolism will be investigated by quantifying the rate of urea synthesis in hepatocyte cultures. Two aspects of the hepatic detoxification phenotype will also be investigated by measuring the intracellular concentrations of reduced glutathione (GSH) and total cytochrome P-450.

In particular, a method for continuous monitoring of functional activity in hepatocyte bioreactors, possibly by means of reaction rate equations, and a sensitive assay for the assessment of the differentiated status of hepatocyte cultures are required. The suitability of the parameters and methods used in this chapter will be considered for these applications.

4.1.4. Culture configurations

In chapter 3, different culture configurations were described within the context of the case studies. Only those configurations based on hepatocyte monolayers (which therefore reproduce a single cell thick plate geometry similar to that found in the liver) were considered to be appropriate for inclusion in the prototype bioreactor which is described in this thesis. The initial evaluation of different configurations can therefore be carried out in conventional cell culture dishes since the appropriate configurations are all variants of the traditional monolayer technique. As indicated in the preceding chapter, previous work has shown that the maintenance of rat hepatocyte function can be prolonged if the cells are immobilised upon a layer of collagen or other extracellular matrix (ECM) component(s). The effect of changing the conformation of this ECM layer or by including a second covering layer will be investigated.

Traditionally, little attention has been paid to gas transport in conventional cell culture vessels. This rarely leads to problems since most cell lines such as

fibroblasts and hybridomas are relatively metabolically inactive when compared to hepatocytes, so oxygen consumption or CO_2 removal do not become limiting. (However, whether it is the case that insufficient oxygen supply to primary cell cultures contributes to de-differentiation or that reduced oxygen demand is a consequence of de-differentiation is unclear.) When culturing hepatocytes, one could run into oxygen limited conditions if the medium depth between the cells and the gas interface were too large. A theoretical consideration of this problem has been reported previously (McLimans, Blumenson, and Tunnah 1968). The simple diffusion model described in this paper is based on Fick's law. By applying experimentally derived oxygen consumption rates the authors predict that for hepatocytes cultured as a confluent monolayer, the limiting medium depth is 1.2 mm. The validity of this model will be investigated by determining the effect on hepatocyte metabolism, as measured by the conversion of ammonia to urea, of varying the depth of medium covering rat hepatocytes cultured as monolayers at different cell area densities (i.e. number of cells per unit attachment area).

Finally, since subsequent studies will require the hepatocytes to be cultured on synthetic membranes, a preliminary comparison of function between hepatocytes cultured on such materials with those cultured on tissue culture polystyrene will also be undertaken.

4.2. Materials and methods

4.2.1. Isolation of rat hepatocytes

Rat hepatocytes were isolated by a portal vein collagenase perfusion technique. This method is similar to that described by Moldeus et.al. (Moldeus, Hogberg, and Orrenius 1978). Male rats weighing 200-250 g were anaethsetised by an intraperitoneal injection of 6.0 mg Sagatal (sodium pentobarbital) per 100 g body mass. Heparin (0.1 ml of a solution of 1,000 units ml^{-1}) was introduced via the inferior vena cava above the renal branch. A small incision was then made in the

portal vein and a stainless steel cannula (3 mm OD) was inserted and held in place by means of a small vascular clamp. The animal was then euthanased and the liver was excised.

The perfusion protocol was as follows:

All four isolation solutions were sterile and briefly sparged with 95% O_2/5% CO_2. The perfusion solutions were recirculated by means of a peristaltic pump from a water bath at 37°C to a small raised reservoir which acted as a hydrostatic feed for the perfusate. The liver was perfused at a flow rate of 5 ml min^{-1}.

An initial perfusion with Hank's buffer containing 0.228 g/l ethylene glycol-bis(beta-amino-ethyl ether) N,N,N^1,N^1-tetra-acetic acid (EGTA), 2.1 g/l $NaHCO_3$, 3 g/l Hepes and 6.66 g/l bovine serum albumin (H1) for approximately 5 minutes was followed by perfusion with a second solution (H2) based on Hank's buffer. This contained 3 g/l Hepes, 2.1 g/l $NaHCO_3$, 0.294 g/l $CaCl_2$ and 0.21 g/l collagenase (type IV collagenase, Sigma Chemical Co., Poole, UK). This collagenase has a FALGPA activity of 0.5-2.0 units mg^{-1} (1 FALGPA unit hydrolyses 1 µmole of furylacryloyl-Leu-Gly-Pro-Ala per minute at 25°C, pH 7.5 in the presence of calcium ions). The collagenase perfusion step was continued for approximately 10-15 minutes or until the organ had completely blanched and softened. The liver capsule was then disrupted with an atraumatic instrument and the cells were gently combed into a sterile Petri dish containing Krebs Hensleit buffer with 10 g/l bovine serum albumin and 3.0 g/l Hepes (KA). The resultant 'cell soup' was then filtered through gauze to remove any remaining connective tissue and larger cell clumps. The filtrate was allowed to settle under gravity in a glass bottle containing KA. Once the cells had settled, the supernatant was removed and replaced with Krebs Hensleit buffer containing only 3 g/l Hepes (KH). The cell sediment was agitated and allowed to re-settle before another rinse with KH was performed. At the end of this phase, as much buffer as possible, without disturbing the cell sediment, was removed from the bottle. The yield and viability of the sedimented cells were then determined by light

microscopy using the Trypan blue test.

Finally, the cells were re-suspended in the desired culture medium at a known concentration and seeded into culture dishes as described in the following sections.

4.2.2. Isolation of sheep hepatocytes

Sheep hepatocytes were isolated by a caudate lobe collagenase perfusion technique, largely as previously described (Emmison, Agius, and Zammit 1991). 6 month old wethers underwent exsanguination. Immediately thereafter, an incision was made in the abdominal cavity and the liver was excised with a knife. The caudate lobe was separated and appropriate cannulae were inserted into those blood vessels which could be accessed. The lobe was initially perfused with Ca^{2+}-free buffer containing 140 mM NaCl, 6.7 mM KCl, 10 mM Hepes, 2.5 mM glucose and 0.5 mM ethylene diamine tetra-acetic acid (EDTA) at pH 7.4. This was followed by a recirculating perfusion with buffer containing 0.3 g/l collagenase (type 1 collagenase, Sigma Chemical Co., Poole, UK. This collagenase has a FALGPA activity of 0.25-1.0 units mg^{-1}), 0.025 g/l trypsin inhibitor (crude preparation, Sigma Chemical Co., Poole, UK.), 140 mM NaCl, 6.7 mM KCl, 30 mM Hepes, 2.5 mM glucose and 5 mM $CaCl_2$. The collagenase perfusion step took 30-70 minutes since the sheep livers were considerably more fibrous than those of the rat. The capsule was ruptured and the cells were combed into dishes as per rat hepatocytes. The resultant cell suspensions were centrifuged at 50 g for 2 to 3 minutes and the cell pellet was re-suspended in KH. After allowing the cells to sediment, excess buffer was removed as per rat hepatocytes and viability and yield were determined by Trypan blue exclusion. The cells were finally re-suspended in the desired culture medium at a known concentration and seeded into culture dishes as described under the subsequent headings.

4.2.3. Cell culture

For all experiments, except where indicated otherwise, $5x10^6$ viable cells in 10 ml of the desired medium were seeded into Petri dishes (nominal diameter 90 mm)

(Falcon, Becton Dickinson, UK) which had previously been coated with type 1 collagen, prepared from rat tail tendon as described elsewhere (Elsdale and Bard 1972), at a coverage of 25 μg cm^{-2}. This is achieved by allowing 3 ml of 0.5 mg ml^{-1} rat tail collagen to evaporate from each dish leaving a very thin dry layer of unpolymerised collagen on the dish. The Petri dish cultures were incubated at 37°C in a humidified air/5% CO_2 environment. The medium was changed after 4 hours and after every subsequent 24 hours.

All of the culture medium formulations were supplemented with penicillin (100 Units ml^{-1}), Streptomycin (100 μg ml^{-1}) and Fungizone (2.5 μg ml^{-1}). Williams' E medium (WE), Medium 199 (M199) and Modified Earle's Medium (KI) were also supplemented with 5% foetal bovine serum. Chee's medium (CHE) was used as a serum-free formulation. The serum was supplied by Seromed, UK. All other additives and the four medium formulations were supplied by Gibco BRL, Paisley, UK.

4.2.4. Assay methods

The rate of urea synthesis by hepatocytes exposed to 10 mM NH_4Cl was derived by processing the data obtained from a diagnostic kit (BUN 535-B, Sigma Chemical Co., Poole UK). Urea synthesis by freshly isolated cells was measured in gassed (95% O_2, 5% CO_2) rotary suspension cultures ($5x10^6$ cells in 10 ml medium supplemented with 10 mM NH_4Cl) in 50 ml round bottom flasks over the first 2 hours of culture by analysis of discrete samples (300 μl). Cells cultured in Petri dishes for 24 hours, 48 hours or longer periods, were exposed to 10 ml of the same medium formulation with additional 10 mM NH_4Cl for 4 hours and discarded thereafter. During the incubation period, discrete samples (300 μl) were removed for analysis after 0, 1, 2, 3 and 4 hours.

The intracellular concentration of reduced glutathione (GSH) was quantified as previously described (Hissin and Hilf 1976). $1x10^6$ freshly isolated cells were suspended in 0.5 ml 10% trichloroacetic acid (TCA) and stored at -70°C. Cells cultured in Petri

dishes were rinsed twice with phosphate buffered saline, pH 7.4 (PBS) to remove dead cells and debris. 2 ml 10% TCA was then added to each culture and the plates were allowed to stand at room temperature. After 15 minutes, the acidic extracts were removed and stored at -70°C. Prior to analysis, the cell suspension/TCA samples were centrifuged at 13,000 rpm for 1 minute to pellet any cellular debris.

Total cytochrome P450 concentrations were measured by a modification of the method of Omura and Sato (Omura and Sato 1964). $5x10^6$ freshly isolated cells were homogenised with 7 strokes of a motorised Teflon-glass homogeniser in 1 ml 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM EGTA, 20% glycerol, 0.02% Nonidet P-40 (Sigma Chemical Co., Poole, UK) and 1 mM dithiothreitol (P450 Buffer) and stored at -70°C. Cultures were rinsed twice with PBS and the cells were then detached into 2 ml P450 buffer by means of a cell scraper. The resulting slurries were then homogenised and stored at -70°C. Analyses were conducted within 72 hours of sample preparation.

4.2.5. Analytical methods

The urea concentration in the samples was obtained via a computerised standard curve of sample absorption at 535 nm versus concentration. Rates of urea synthesis were obtained from the slope of the line of urea concentration versus time plots.

GSH concentrations were determined from standard curves of concentration versus relative fluorescence intensity on a computer driven fluorimeter (RF 5001PC, Shimadzu, UK). 1.0 ml samples were measured in polymethyl methacrylate (PMMA) microcuvettes at excitation wavelength 350nm, emission wavelength 420 nm and 5 nm slit width.

The total intracellular cytochrome P450 content of the cultures was determined by an algorithm which compares the absorption spectra (390 to 500 nm wavelength) of carbon monoxide treated homogenates with that of carbon monoxide treated and sodium dithionite reduced homogenates. The difference in the peak

heights observed nearest to 450 nm between the two homogenates is proportional to the cytochrome P450 concentration. The samples were read in a computer controlled twin channel spectrophotometer (UV 2101PC, Shimadzu, UK). The concentration of P450 in the samples was calculated using an extinction coefficient of 91 mM^{-1} cm^{-1}.

Data were analysed by one-way analysis of variance. The post hoc test used was Fisher's pairwise least significant differences. The error quoted for mean values is the standard error of the mean in instances where cells from more than one isolation were used in an experiment. In cases where all cells were sourced from the same isolation, means ± standard deviations are given.

4.3. Experimentation

4.3.1. Species and Media comparisons

Rat hepatocytes were cultured in each of the test media for 48 hours or 6 days, thereafter the assays were performed as described. Sheep hepatocytes were cultured for 24 hours in each of the media prior to the determinations. (It proved difficult to avoid build up of bacterial contaminants in the sheep cultures for longer periods, perhaps due to the rather crude method of excision of the caudate lobe.) Urea synthesis, GSH concentration and total cytochrome P450 content were measured. For both rat and sheep hepatocytes, cells from 4 different isolations were used and within each experiment 4 replicates were included.

4.3.2. Influence of assay medium formulation on urea synthesis

Since the urea synthesis experiments were conducted over a period of 2 to 4 hours in each of the media in which the cells had previously been cultured, it was necessary to investigate whether differences in urea synthesis rates reflected changes in the differentiated status of the cells and/or differences in the capacity of each medium formulation to sustain the urea cycle during the assay period. This was

carried out in rat hepatocyte cultures by performing the urea synthesis assays in cultures in the same medium in which they had been maintained during the initial 48 hours and also, in replicate cultures, in a different medium. In these tests cells from 4 different isolations were used and 4 replicates were performed within each experiment.

4.3.3. Investigation of the influence of medium depth

In order to test the influence of hepatocyte area density and medium depth (and thus oxygen supply) on urea synthesis, rat hepatocytes were cultured for 48 hours at two area densities; 0.8×10^5 cells cm^{-2} and 2.4×10^5 cells cm^{-2}, and with two depths of covering medium; 1.6 mm and 0.8 mm. Chee's medium was used for these tests. Cells from 2 isolations were used, each with 4 replicates.

4.3.4. Effect of changing configuration

The influence of the configuration of ECM on hepatocytes was investigated with rat cells. In each experiment, half of the dishes were coated with a thin layer of collagen as described above and the other half received a collagen gel layer. In each Petri dish, this gel layer was prepared by allowing 6 ml of a 3 mg ml^{-1} solution of acid soluble rat tail collagen in culture medium at pH < 5, 4°C to gel by neutralising at 37°C with 5M NaOH. This process yields a partially polymerised highly hydrated gel layer with a thickness of approximately 1 mm.

All of the dishes were then seeded with 8×10^6 cells and cultured as before. After 20 hours, 50% of each of the two sets of cultures had the excess medium removed and a further collagen gel layer was placed on top of the cells as described.

This produced four different culture configurations as follows:

I.	Control	Hepatocytes on a thin collagen layer
II.	Gel	Hepatocytes on a layer of collagen gel
III.	Overlay	Hepatocytes on a thin collagen layer with a gel layer overlay
IV.	Sandwich	Hepatocytes between 2 collagen gel layers.

The cultures were then incubated for a further 2 or 6 days and urea synthesis was determined. Cells from 3 different isolations with 4 internal replicates were used for these experiments.

4.3.5. Investigation of synthetic membranes as substrata

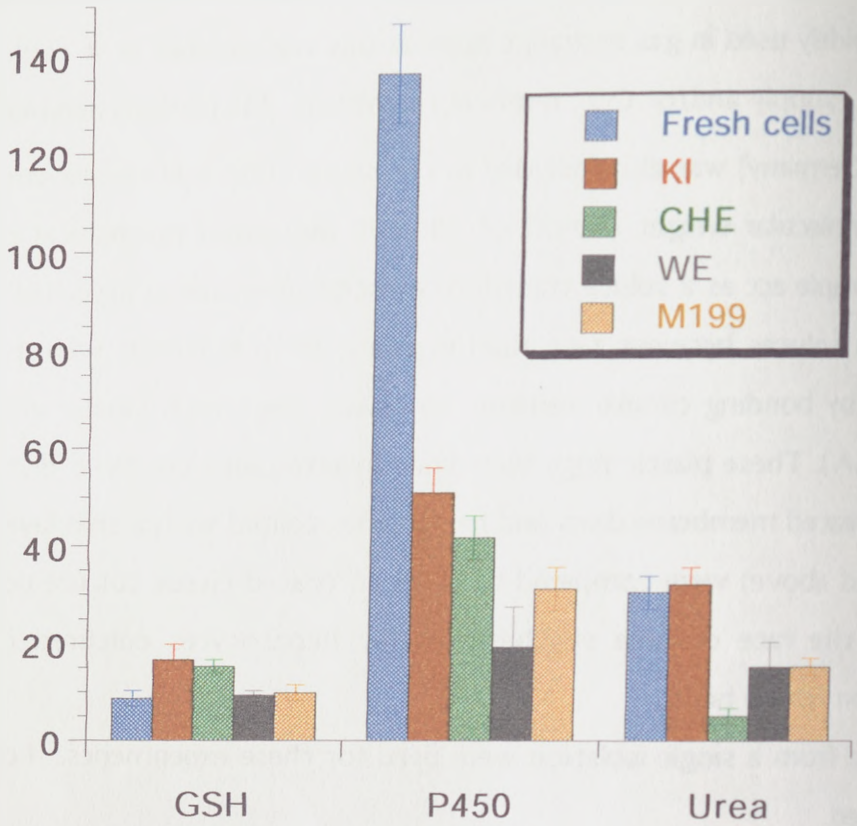
In a simple preliminary experiment, the suitability of two commercial synthetic membranes as support substrata for hepatocyte monolayers in a bioreactor was also investigated. Celgard 2400 (Hoescht Celanese, Germany), a microporous polypropylene membrane widely used in gas exchange applications was studied as it could in principle act as an O_2 supply and/or CO_2 removal membrane. Micropolyethersulphone (Akzo, Wuppertal, Germany) was also included in the study. This hydrophillic membrane has a nominal molecular weight cut-off of 100,000 and good mechanical properties. It could in principle act as a solute transport substratum enabling attached hepatocytes to partition solutes between two fluid streams. Both materials were tested in flat sheet form by bonding circular sections to plastic rings with Silastic adhesive (Dow Corning, USA). These plastic rings were then inserted into the Petri dishes described above. Untreated membrane discs and membranes coated with a thin layer of collagen (as described above) were compared to collagen coated tissue culture polystyrene by measuring the rate of urea synthesis of rat hepatocytes cultured in each dish configuration for 48 hours.

Cells from a single isolation were used for these experiments. Four replicates were included.

4.4. Results

4.4.1. Species and media comparison

The viability of isolated rat hepatocytes had a mean value of 89% (range 80-94%) and the average yield per rat liver was $330x10^6$ viable cells (range



Status of primary rat hepatocytes after isolation and after 48 hours of culture in four media formulations

(GSH, nmol/10^6 cells; P450, pmol/10^6 cells, Urea, pmol s^{-1}/10^6 cells. Bars represent means, error bars represent standard error of mean, n=4)

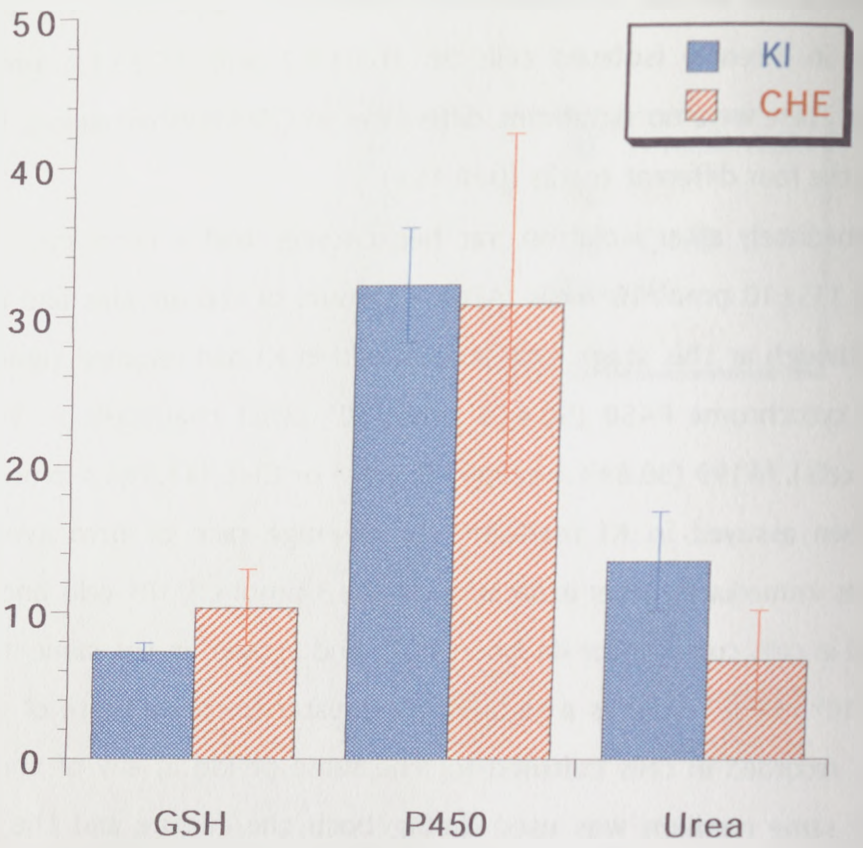
Figure 4.1.

190-400x10^6). Freshly isolated sheep hepatocytes had an average viability of 91% (range 87-98%). The mean yield per caudate lobe was 200x10^6 viable cells (range 130-450x10^6). The larger range and more skewed distribution of sheep hepatocyte yields reflects differences in the sizes of the lobes and in the efficacy of the perfusions. Rat hepatocytes had a mean intracellular GSH concentration of 8.7±1.6 nmol/10^6 cells immediately after isolation. After 48 hours, rat cells cultured in WE and M199 had similar GSH levels of 9.3±0.9 and 9.8±1.6 nmol/10^6 cells respectively. However, the GSH concentrations in cells cultured for 48 hours in KI and CHE was almost double that found in recently isolated cells at 16.6±3.2 and 15.2±1.4 nmol/10^6 cells respectively. There were no significant differences in GSH concentrations between cells cultured in the four different media ($p=0.153$).

Immediately after isolation, rat hepatocytes had a total cytochrome P450 content of 135±10 pmol/10^6 cells. After 48 hours of culture this had diminished in all media, though at this stage, cells maintained in KI had retained significantly more ($p<0.005$) cytochrome P450 (50.4±5 pmol/10^6 cells) than cells in WE (19.1±8.1 pmol/10^6 cells), M199 (30.8±4.4 pmol/10^6 cells) or CHE (41.3±4.4 pmol/10^6 cells).

When assayed in KI medium, the average rate of urea synthesis in rat hepatocytes immediately after isolation was 30±3 pmol s^{-1}/10^6 cells and this was not diminished in cells cultured for 48 hours in KI and assayed in the same medium (32±3 pmol s^{-1}/10^6 cells). This was a significantly greater ($p<0.001$) rate of urea synthesis than that recorded in cells cultured for the same period in any of the other media when the same medium was used during both the culture and the assay periods (15±5, 15±2 and 5±2 pmol s^{-1}/10^6 cells in WE, M199 and CHE respectively). The data for 48 hour rat hepatocyte cultures in the various media are depicted in Figure 4.1.

After having been cultured for 6 days in KI or CHE medium, rat hepatocyte intracellular GSH concentrations were similar to those found in cells immediately after isolation (7.3±0.6 and 10.3±2.6 nmol/10^6 cells in KI and CHE respectively). At the same time point, cytochrome P450 levels had diminished further in cells cultured in



Status of primary rat hepatocytes after 6 days of culture in two different medium formulations.

(GSH, nmol/10^6 cells, P450, pmol/10^6 cells, Urea, pmol s^{-1}/10^6 cells. Bars represent means, error bars represent standard error of mean, n=4)

Figure 4.2.

both KI (31.9±3.8 pmol/10^6 cells) and CHE (30.6±11.3 pmol/10^6 cells) but there was no longer a significant difference between the two. Interestingly, cells cultured for 6 days in either of these two media had retained as much P450 as cells cultured for just 48 hours in WE or M199.

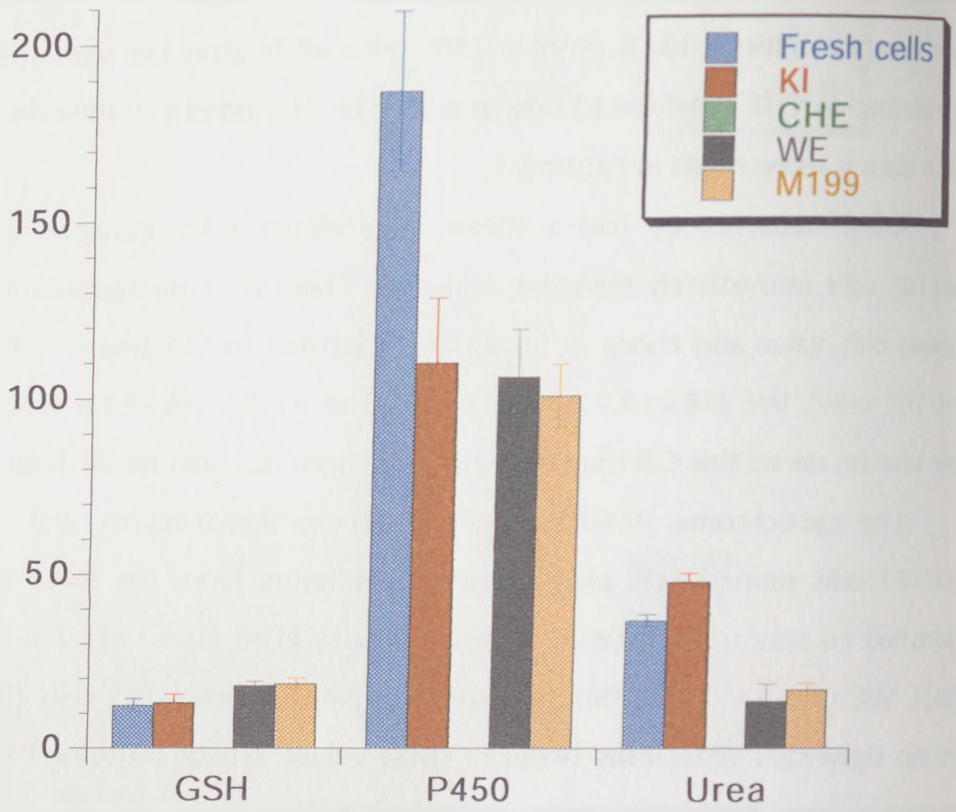
The pattern of diminishment of urea synthesis in cells cultured in KI and CHE was similar to the nature of P450 loss, in that the significant difference in the rate of urea synthesis between cells cultured in these two media which was recorded after 48 hours was not observed after 6 days. At this time, rat hepatocytes in KI medium had a urea synthesis rate of 13±3 pmol $s^{-1}/10^6$ cells, while after the same period, those cells cultured in CHE synthesised urea at a rate of 7±3 pmol $s^{-1}/10^6$ cells. This 6 day culture data is represented in Figure 4.2.

Sheep hepatocytes had a mean intracellular GSH content of 12.3±1.9 nmol/10^6 cells immediately following isolation. There were no significant differences between this value and those of sheep cells cultured for 24 hours in KI (13.1±2.3 nmol/10^6 cells), WE (18.0±1.2 nmol/10^6 cells) or M199 (18.5±1.6 nmol/10^6 cells). There was no detectable GSH in cells which had been cultured for 24 hours in CHE.

The cytochrome P450 content of sheep hepatocytes was 185.6±22.4 pmol/10^6 cells immediately after isolation. 24 hours later, the P450 content had diminished to approximately 50% of initial values in KI (109.3±18.2 nmol/10^6 cells {59%}), WE (105.2±13.5 {57%}) and M199 (99.8±8.8 nmol/10^6 cells {54%}). There were no significant differences between these values. No cytochrome P450 could be detected in sheep hepatocytes after 24 hours in CHE.

Immediately after isolation, sheep hepatocytes in KI synthesised urea at a rate of 35±2 pmol $s^{-1}/10^6$ cells). After 24 hours, those cells maintained in KI synthesised urea at a significantly ($p<0.05$) greater rate (47±2) than those in any of the other 3 media (WE, 13±5; M199, 18±2; CHE, 0 pmol $s^{-1}/10^6$ cells). Once again, the same medium formulation was used in both the culture and the assay periods.

It should be noted that Chee's medium did not support retention of cytochrome P450, maintenance of intracellular GSH or urea synthesis in sheep



Status of primary sheep hepatocytes after isolation and after 24 hours of culture in four different medium formulations

(GSH, nmol/10^6 cells; P450, pmol/10^6 cells; Urea, pmol s^{-1}/10^6 cells.
Bars represent means, error bars represent standard error of mean, n=4)

Figure 4.3.

hepatocytes. The findings for sheep hepatocyte cultures are shown in Figure 4.3.

4.4.2. Effect of assay medium on urea synthesis

The rate of urea synthesis of rat hepatocytes cultured for 48 hours in CHE was 7±2 pmol $s^{-1}/10^6$ cells when maintained during the assay in CHE and 15±3 pmol $s^{-1}/10^6$ cells when KI was the medium used during the assay period. Cells initially maintained in KI synthesised urea at a rate of 22±2 pmol $s^{-1}/10^6$ cells when KI was also the assay medium used and 5±2 pmol $s^{-1}/10^6$ cells when CHE was the assay medium.

When KI and CHE were compared, the culture medium in which rat hepatocytes were maintained during the period of urea synthesis measurement proved to be a more significant ($p<0.0001$) determinant than the medium in which the cells had been cultured for the preceding 48 hours ($p=0.0137$). This data is represented in Figure 4.4.

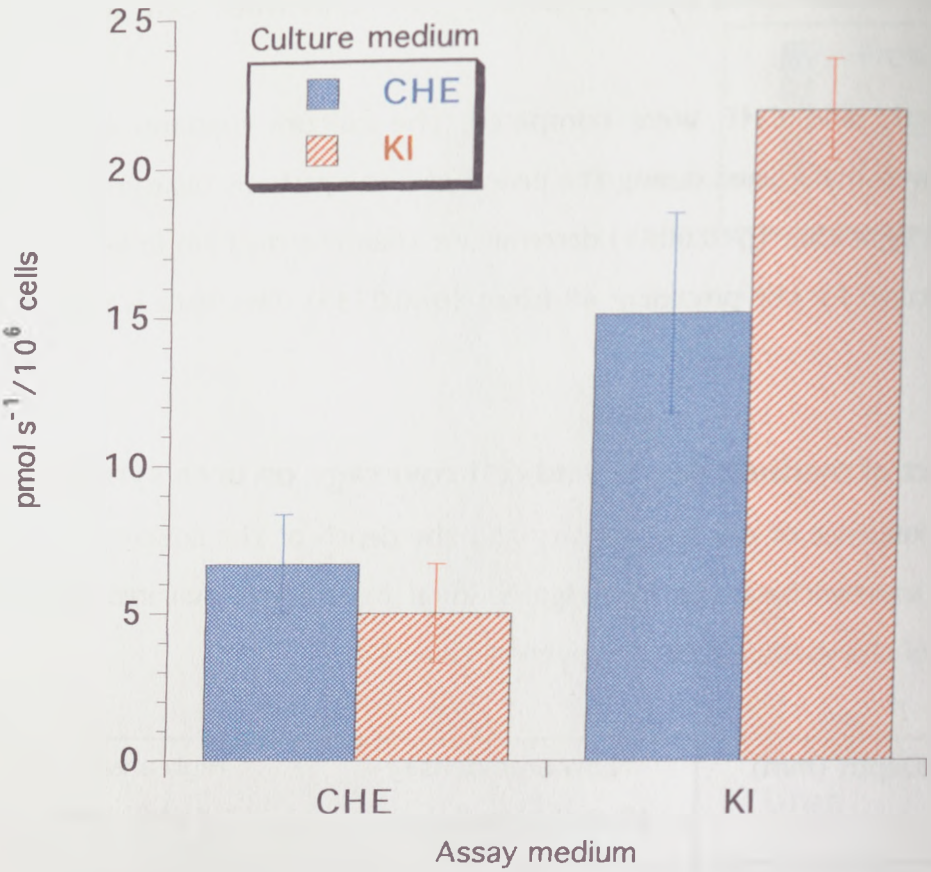
4.4.3. Effect of medium depth and cell coverage on urea synthesis

The influence of cell area density and the depth of the covering medium on the rates of urea synthesis was investigated in rat hepatocytes cultured for 48 hours. The results of this investigation are given in Table 4.1.

Medium Depth (mm)	Low area density ($0.8x10^5$ cells cm^{-2})	High area density ($2.4x10^5$ cells cm^{-2})
0.8	32±7	38±2
1.6	28±3	32±2

Effect of cell area density and medium depth on urea synthesis (pmol $s^{-1}/10^6$ cells) Values represent mean ± SEM (n=2)

Table 4.1.



Urea synthesis in 48 hour cultures of primary rat hepatocytes: Influence of culture medium and assay medium

(Bars represent means, error bars represent standard error of mean, n=4)

Figure 4.4.

Within the limits of this study, urea synthesis was not significantly influenced by cell area density itself. However, the rate of urea synthesis was significantly greater ($p<0.005$) in cells cultured at the higher density with the reduced depth of covering medium than in those cells which had been cultured with 1.6 mm of covering medium irrespective of the cell area density. When cells were cultured at the low area density, there was no significant difference in the rate of urea synthesis between cells which had 0.8 mm of covering medium and those which had 1.6 mm of covering medium.

4.4.4. Comparison of ECM configurations

4 different culture configurations were investigated. The average rates of urea synthesis of rat hepatocytes after 3 and 7 days culture in each configuration are given in Table 4.2.

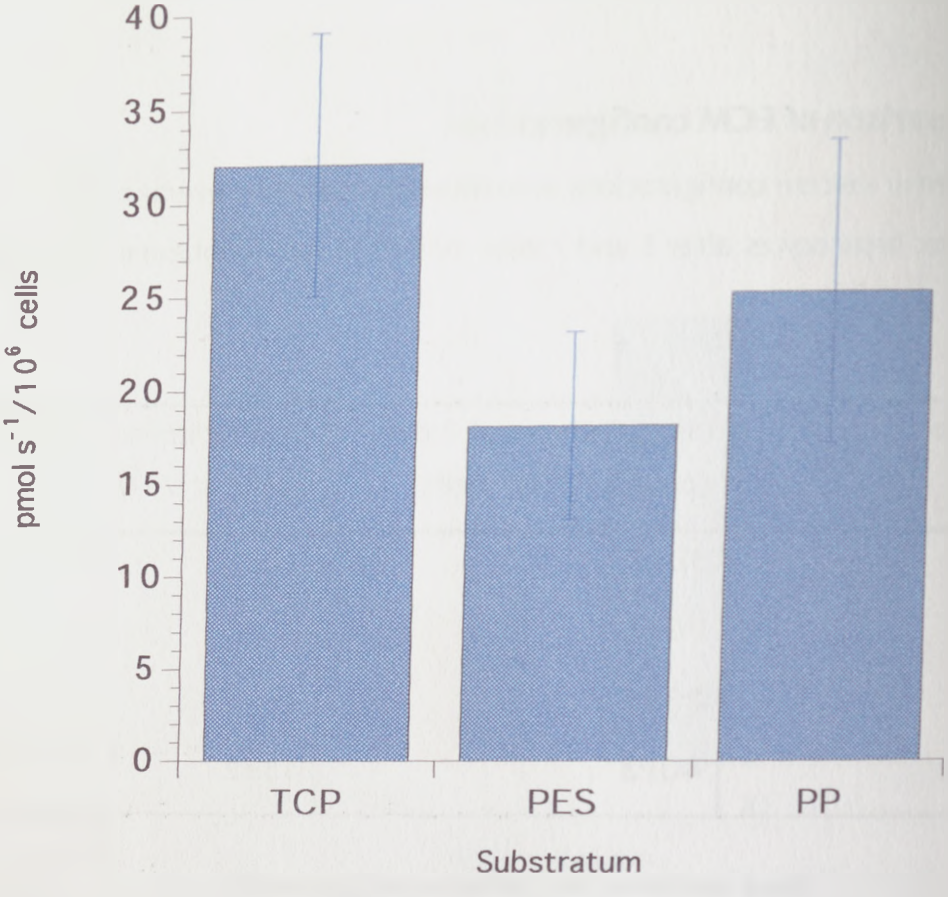
Configuration	Urea synthesis at 3 days (pmol $s^{-1}/10^6$ cells)	Urea synthesis at 7 days (pmol $s^{-1}/10^6$ cells)
FILM	32±2	27±2
GEL	28±2	22±2
OVERLAY	23±2	25±2
SANDWICH	40±3	45±2

Urea synthesis in 4 culture configurations

Values represent mean ± SEM (n=3)

Table 4.2.

A number of trends can be discerned from this table. After 3 days, the rate of urea synthesis of cells cultured in the sandwich configuration was significantly greater than those in the film ($p=0.029$), the gel ($p=0.011$) or the overlay ($p=0.001$)



Urea synthesis by primary rat hepatocytes cultured for 48 hours on 3 different collagen coated substrata.

(TCP = tissue culture polystyrene; PES = micropolyethersulphone; PP = microporous polypropylene. Bars represent means, error bars represent standard deviations, n=1)

Figure 4.5.

configurations. The same effect was recorded after 7 days ($p<0.0001$ in all three cases), although the duration of culture did not have a significant effect overall ($p=0.615$). Cells cultured for 3 days in the simple film configuration performed relatively well.

4.4.5 Suitability of synthetic membranes as substrata

The rate of urea synthesis of rat hepatocytes cultured for 48 hours on three different polymeric surfaces was investigated. No detectable quantities of urea were synthesised by cells which had been cultured on either of the membrane materials in the absence of a thin collagen layer. The rate of urea synthesis of cells cultured on collagen coated tissue culture plastic (TCP) was greatest (32 ± 7 pmol $s^{-1}/10^6$ cells). This was greater than that of cells cultured on collagen coated micropolyethersulphone (PES) in which the rate of urea synthesis was 18 ± 5 pmol $s^{-1}/10^6$ cells. Rat hepatocytes cultured on collagen coated microporous polypropylene (PP) synthesised urea at the rate of 25 ± 8 pmol $s^{-1}/10^6$ cells. The data is depicted in Figure 4.5.

4.5. Discussion

4.5.1. Cell isolation methods

The method for rat hepatocyte isolation proved simple and reliable in operation, producing good yields with an acceptable viability. It was possible to culture the rat cells for at least seven days without detectable contamination. The mean yield of $300x10^6$ viable cells (89% of total yield) is sufficient for >30 Petri dishes even if high cell area density cultures are used. Therefore, large controlled experiments (up to 6 effects and 1 negative control, each with 3 degrees of freedom) can be undertaken with each isolation. The number of viable cells isolated from one rat liver would be sufficient to seed an area of approximately $0.2m^2$ which is equivalent to 11 plates measuring 30 cm x 6 cm. This would be a satisfactory size for a 1st level

scale-up of a prototype bioreactor. Therefore, one can in theory avoid the requirement for isolating hepatocytes from larger animals until such time as a full size clinically applicable bioreactor is needed.

However, since species differences might conceivably influence bioreactor design, a simple method of isolating hepatocytes from any envisaged large donor animal, in numbers of a similar order of magnitude to that produced by the rat isolation method, would be advantageous. In this respect, the sheep hepatocyte isolation method proved to be unsatisfactory. While the initial viability of sheep hepatocytes was as high as that of rat hepatocytes, the yield per liver mass was lower in sheep and it proved difficult to maintain contamination free cultures. If sheep hepatocytes were to be used for further work, the isolation technique would have to be improved significantly. This would almost certainly require the use of aseptic operating theatre conditions and such measures could only be justified if such cell numbers as could only be obtained by whole liver isolations were required. In other words, within the context of hybrid artificial liver development, isolation of sheep hepatocytes, and by extension, those from other large animals (pigs for example) will only become economically and ethically acceptable when large scale bioreactors are in development.

4.5.2. Species and medium formulation study

The investigation of species differences and medium formulations produced some interesting data. The most striking species difference was that sheep hepatocytes cultured in CHE ruptured and died within 24 hours. Related work has shown that this effect is not due to the absence of serum in this formulation (Watts 1993 unpublished findings). There is no immediately apparent reason why this formulation should have such a lethal influence on sheep hepatocytes. Exhaustive research would be required to identify the cause of this effect.

Apart from this phenomenon, those aspects of the sheep hepatocyte phenotype which were investigated in this study were less sensitive to changes in

between rat hepatocytes maintained in KI and those cultured in CHE. The reason for this is unclear. One possibility is that the presence of serum may contribute to accelerated de-differentiation in older KI cultures. However, it should be noted that in rat hepatocytes, the P450 levels in 6 day old CHE and KI cultures were equivalent to those in 48 hour WE and M199 cultures. The rather poor performance of WE in this study is noteworthy since WE is one of the most widely used formulations for hepatocyte culture. Both WE and M199 contain L-cysteine and L-cystine. These two amino acids have been shown to increase degradation of P450 (Paine and Hockin 1980). However, it has been suggested that loss of P450 in rat hepatocyte cultures is a consequence of loss of mRNAs which code for the various P450 isoenzymes and that this may be triggered during the isolation process (Padgham and Paine 1993). If such irreversible mechanisms occur at such an early stage then any protective effect provided by L-cysteine free medium formulations would be less significant.

The rate of urea synthesis emerged as the most discriminatory parameter from the species and media comparison study. Both cell types had significantly differing rates of urea synthesis when cultured in different medium formulations and this was not the case for either GSH or P450 content. Such an outcome was not expected since previous work had suggested that quantification of urea synthetic activity is a less labile functional parameter than cytochrome P450 measurement (Dunn, Tompkins, and Yarmush 1991). However, it should be remembered that the synthesis of urea requires maintenance of activity of all of the urea cycle enzymes and a continuous energy supply so perhaps the fact that measurement of urea synthetic activity should appear to be a more sensitive parameter than quantification of total P450 content (as opposed to P450 activity) is less surprising. Further specific comment on the urea synthesis assay will be included in the following sections of this discussion.

In short term cultures of both rat and sheep hepatocytes (48 and 24 hours respectively), urea synthesis was greatest when the cells were maintained in KI. However, after 6 days of culture there was no longer a significant difference between

the rates of urea synthesis of rat hepatocytes maintained in KI and those cultured in CHE, although both sets of cultures had rates of urea synthesis which were lower than their respective 48 hour values.

The pattern of loss of function with time observed in rat hepatocyte KI and CHE cultures was similar for both P450 content and urea synthesis rate. In the short term (i.e. the first 48 hours of culture), both parameters diminished more rapidly in cells maintained in CHE, while in the subsequent 4 days, those cells cultured in KI must have undergone more rapid deterioration in KI since after 6 days of culture each parameter had declined to the same net level in both media. The mechanism responsible for this effect is unknown. It is tentatively postulated that KI might provide initial protection to cells in a wider spectrum of 'health' than does CHE, while some subpopulation of cells which can be supported by CHE receives better long term protection against de-differentiation. (Perhaps, as suggested above, this may be due in part to the absence of serum in CHE.) In other words, CHE may be exerting some selectivity upon the cell population. Additional work (which lies beyond the scope of this thesis) would of course be required to test this hypothesis.

It would be advantageous in terms of hybrid artificial liver bioreactor development if the hepatocytes could be maintained in a serum-free environment. As mentioned above, cells which have been in contact with bovine serum may be prohibited for use in medical devices by regulatory bodies. Furthermore, binding of substances to serum proteins may interfere with the results of biochemical assays during the an in vitro testing phase. However, the experience with sheep hepatocytes suggests that use of serum-free media must be individually evaluated for each species.

4.5.3. Urea synthesis and assay medium

The data from the investigation of the effect of the assay medium on the measurement of the rate at which urea is synthesised by rat hepatocytes suggest that the medium in which the cells were maintained for the initial 48 hours of culture is a less important determinant of the rate of urea synthesis measured than is the

medium in which the cells were cultured during the assay period itself. This suggests that the differences in the expression of urea cycle enzymes which may exist between rat hepatocytes cultured in CHE and those cultured in KI are exceeded by differences in the extent to which each formulation may support the activity of the urea cycle during the assay period. Why KI should support or produce a greater level of urea cycle activity is unclear. The principle rate limiting substrates in the urea cycle, and their respective experimentally derived limiting concentrations, are given in Table 4.3.

Compound	Rate limiting concentration (mM)	Potentially rate limiting in tests?
Ammonium	0.7	No
Ornithine	0.3	Possibly (all media)
Mg^{2+}	1.0	No
Arginine	0.05	No
Mg ATP	8.0	Not known
N-acetylglutamate	0.1	Not known

Rate limiting substances in the urea cycle.
{reproduced from (Powers-Lee and Meister 1988)}
Table 4.3.

There are no significant differences between the two media formulations in terms of the concentrations of those rate limiting substances listed above. Under normal circumstances, ammonium is likely to be the rate limiting substrate, since it has a relatively high limiting concentration. However, in the experiments under discussion, the ammonium concentration was 10 mM and from the quantities of urea generated it can be calculated that this concentration was not significantly depleted

during the assay periods. Since neither medium formulation contains ornithine and the concentration of this compound limits urea cycle activity below approximately 0.3 mM, it could be that the rate of urea synthesis is being limited in both cases by a lack of ornithine. N-acetylglutamate and Mg ATP might also be limiting the urea cycle in these experiments but this is more difficult to determine. In any case, differences in the formulations offer no apparent explanation for the results observed.

However, it would seem that rat hepatocytes maintained in CHE are capable of expressing the urea cycle enzymes and sustaining the urea cycle and that the rate of urea synthesis of these cells can be altered by changing the composition of the surrounding fluid. In this context, the effect on the rate of urea synthesis of a phase of culture in which the cells were maintained in normal plasma and plasma from liver failure patients would be of interest.

4.5.4. Influence of cell coverage and medium depth on urea synthesis

A previous model (McLimans, Blumenson, and Tunnah 1968) predicts that rat hepatocytes cultured as a confluent monolayer experience oxygen limited conditions when a depth of medium of 1.2 mm or greater separates the cells from a gas interface with 20% O_2. In order to determine whether the rat hepatocyte cultures in this series of experiments were suffering from oxygen debt, the influence of cell area density (i.e. the number of cells per cm^2) and the depth of covering medium on the rate of urea synthesis was investigated. The conversion of ammonia to urea via the urea cycle involves the cleavage of 4 high energy phosphate bonds (2 ATP to 2 ADP and 1 ATP to AMP) which in turn require large quantities of oxygen for their generation. Therefore, synthesis of urea is dependent upon a continuous supply of oxygen. If cells were experiencing oxygen debt, one would expect the rate of urea synthesis to diminish.

The rate of urea synthesis of cells cultured at the 'normal' area density ($0.8x10^5$ cells cm^{-2}) proved to be independent of the depth of covering medium when depths of 0.8 mm and 1.6 mm were tested. This is not necessarily at odds with the

model since $0.8x10^5$ cells cm^{-2} represents a sub-confluent area density. However, when cells were cultured at a super-confluent area density ($2.4x10^5$ cells cm^{-2}), the depth of covering medium proved to be important. Urea synthesis was significantly greater in cells which had only 0.8 mm of covering medium than in cells which were covered by 1.6 mm of medium. This effect is predicted by the model. However, an explanation for why the urea synthesis rate in cells cultured at the high area density with the thinner medium layer was greater than that in cells with the same depth of medium but a lower area density is less easy to find. Perhaps cells cultured at sub-confluent area densities are less metabolically active in general. Whether this might be a consequence of cytoskeletal alterations, lack of intercellular communications or some other phenomenon is unclear.

It is evident that the distance between the hepatocytes and the oxygen source might well be a critical design parameter for hybrid artificial liver bioreactors.

4.5.5. Effect of collagen configuration on urea synthesis

The rate of urea synthesis by rat hepatocytes was used as an indicator of cell function in order to determine which configurations of collagen (and by extension other extracellular matrix preparations) are best suited for inclusion in a prototype bioreactor. The results of these tests indicated that only the sandwich configuration produced better cell function (as quantified by the rate of urea synthesis) than the simplest collagen film configuration. This is an interesting outcome. It indicates that somehow, cell function is improved by sandwiching the cells between two deformable collagen layers. This in turn suggests that the most important function of the collagen might be to act as a structural component (rather like a scaffold). If the effect were purely chemical or was purely due to the presence of extracellular matrix contacts on both cell surfaces then one would have expected different results.

It has previously been suggested that the collagen sandwich configuration enables hepatocytes to preserve both their basolateral and apical surfaces (Yarmush et al. 1992). This may indeed be the case and associated improvements in cytoskeletal

architecture may lead to better preservation of cell organelles and consequently better function.

However, this culture configuration may not offer any net benefits in terms of solute transport in hepatocyte bioreactors. The theoretical advantages derived from the development of two basolateral surfaces in the plane of the collagen gels (and hence two potential blood or plasma exchange surfaces) cannot be harnessed. Current technology does not enable two fluid process streams to be maintained on either side of a hepatocyte monolayer in the sandwich configuration (without the further interposition of a synthetic membrane which will increase transfer resistance). In the first instance the collagen gels lack the mechanical strength required to withstand the fluid shear forces which would be present. Furthermore the surface areas required for support are large and such collagen layers could not be produced in thin enough sections to give adequate solute transport between the perfusate and the hepatocytes. Finally, this configuration does not provide for any bile or 'bile-like' transport since the apical surfaces of the hepatocytes must communicate with the same fluid stream(s) as the basolateral surfaces. It can be seen therefore that there may not be any net benefit obtained by incorporating the sandwich configuration into a prototype hepatocyte bioreactor since in the tests, the single collagen film layer performed reasonably well. This single film layer is also much easier to produce in a membrane based bioreactor.

The most efficacious design path may be to develop a system based initially on the simple collagen film configuration. This will be easier to build and prepare and would certainly be appropriate for collecting data from short to medium term (i.e. 24 hour to 7 day) cultures in bioreactors. A reactor design based upon a modular plate configuration could subsequently be adapted to include a more complex extracellular matrix arrangement after initial testing was completed.

4.5.6. Use of synthetic membranes as cell supports

The brief investigation into the suitability of two synthetic membranes to act

as cell support substrata suggests that both micropolyethersulphone and microporous polypropylene can be used to culture rat hepatocytes providing that they are coated with a thin film of collagen.

Rat hepatocytes cultured on these coated membranes had comparable rates of urea synthesis after 48 hours to those cultured on collagen coated tissue culture polystyrene for the same period of time. These encouraging initial findings suggest that both of these membranes could be used as cell support substrata in a prototype hepatocyte bioreactor. Microporous polypropylene could act as a gas exchange membrane. (Previous chapters having emphasised the requirement for integral oxygen supply). However, this material is widely used in membrane oxygenators for application in cardiopulmonary bypass and is known to suffer from pore wetting after extended exposure to blood. This in turn leads to leakage of plasma into the gas compartment causing oxygenator failure and loss of vital patient fluids. The partial blockage of the pores by plasma lipids rendering the pores more hydrophillic is thought to be the mechanism responsible for this phenomenon which may be exacerbated by a wide distribution of pore size within each sample. Microporous polypropylene may therefore ultimately prove to be an unsuitable gas transfer membrane for use in hepatocyte bioreactors since it is likely to be exposed to blood or plasma for extended periods during clinical application. It may find application in prototype devices used for in vitro studies. In such tests, cell culture media would be used and this would have a reduced pore wetting tendency in comparison to blood or plasma. Furthermore, the duration of such studies could be limited to the lifetime of the membrane. In chapter 5, the application of this membrane in a prototype hepatocyte perfusion chamber will be described.

Micropolyethersulphone (PES) could find application in a bioreactor for liver support as a fluid exchange membrane. If hepatocytes were cultured as a monolayer on this (coated) material, then two fluid streams could be separated by this membrane in order to produce an integrated dialysis effect, to supply nutrients to the cells or possibly to induce some form of bile transport (depending on the configuration of the

cells on the membrane). PES is an attractive membrane material since it has good mechanical strength and can be produced to give a tailored nominal molecular weight cut-off. It is therefore worthy of consideration for incorporation as a cell support material in a hepatocyte bioreactor.

It would be interesting to study the effect of the collagen coating process on the transport properties of the two membranes. However, direct measurement by transfer tests with specialised equipment would be required to do this. Such testing lies out with the scope of this thesis.

4.6. Conclusions

The rat hepatocyte isolation and culture methods are appropriate for further use and these will form the basis of the remainder of the experimental work in this thesis. At such time as larger numbers of cells are required, a specialised isolation technique, using a larger animal as the source of the cells, will require to be developed.

Chee's serum free medium formulation is suitable for culturing rat hepatocytes for periods of at least 6 days. The advantages offered by the absence of serum in this formulation make it a more appropriate choice than KI medium (which performed at least as well in the tests) for subsequent studies. However, the lethal effect of CHE on sheep hepatocytes makes it essential that use of this medium is reevaluated when hepatocytes from other species must be cultured.

Of the three functional parameters, urea synthesis was the simplest to quantify and had the unique advantage that no direct access to the cells was required for measurement. These two facts alone would make it the obvious choice as a primary indicator of function in a prototype bioreactor since large numbers of samples can be easily processed and these can be obtained from the perfusion lines, obviating the requirement for access to the reactor interior. However, urea synthesis also proved to be the most discriminatory parameter in the study. While this discriminatory effect was shown to be at least partly due to the different media supporting urea cycle activity during the period of measurement to differing extents, the complex cyclic

nature of the conversion of ammonia to urea ensures that quantification of the rate of urea synthesis will be a reliable indicator of hepatic function. A further benefit of this parameter is that reaction equations for reactor model systems can be developed in order to calculate reaction rates from the measured concentration of urea in discrete samples.

In confluent cultures, rat hepatocytes are sensitive to their distance from their oxygen source. Oxygen may become limiting when this distance exceeds approximately 1 mm. This was borne in mind when designing the perfusion chamber described in chapter 5 and the prototype reactor described in chapter 6.

The collagen gel sandwich configuration gave better maintenance of hepatocyte function (as quantified by urea synthesis) than the other culture configurations tested. However, the difficulties associated with producing this configuration within bioreactors whilst retaining appropriate geometry and adequate solute transport may outweigh its advantages in terms of function. Therefore, in the first instance, the prototype bioreactor will be based on the single collagen film configuration. At some future point, any benefits offered by switching to the sandwich configuration will be assessed.

Two synthetic membranes have been shown to be capable of acting as hepatocyte substrata when coated with collagen. These membranes will be used for subsequent studies.

In the next chapter, the development of a hepatocyte perfusion culture chamber is described. This device was designed to enable the measurement of the oxygen consumption rate of rat hepatocytes exposed to elevated toxin levels and the response of these cells to direct exposure to fluid shear stresses.

Chapter 5

Measurement of the oxygen consumption rate and the response to shear stress of primary rat hepatocytes cultured in a novel flow cell.

5.1. Introduction

The scarcity of published data pertaining to the oxygen requirements of cultured hepatocytes has been highlighted in previous chapters. Furthermore, there is no literature on the behaviour of cultured hepatocytes when exposed to fluid shear stresses. This chapter describes the design of a novel flow cell and its subsequent use in the evaluation of oxygen consumption rate (OCR) and shear response.

5.1.1. Methods for measurement of oxygen consumption rate (OCR)

There are various methods suitable for the determination of oxygen consumption. These can be divided into two groups distinguished by the behaviour of dissolved oxygen concentration with respect to time within the test environment.

- Dynamic systems

  The simplest arrangement involves recording the oxygen concentration of the test fluid in a simple vessel (e.g. a stirred tank) as it is allowed to fall (due to consumption) from an initial value. The rate of change of dissolved concentration being proportional to OCR. This dynamic technique produces

data which readily enables the relationship between OCR and pO_2 to be elucidated. However, the test fluid must be well mixed and any exchange of fluids during the experiment would complicate the analysis. The shear stresses produced by the agitator (which are difficult to quantify) and the limited time for which tests could be run (due to depletion of oxygen) make this an unsuitable technique for application in hepatocyte culture systems.

- Steady state systems

  It is possible to operate OCR tests in steady state systems with nutrient replenishment. In the oxystat technique, a constant pO_2 is maintained in a test chamber containing medium and cells. The pO_2 is maintained at the desired value by a control system which supplies fresh medium of known pO_2 upon demand. Fluid volume is constant since old medium is drained from the chamber at the same rate as fresh fluid is supplied. In this system, OCR is proportional to the rate of supply of fresh medium. Such apparatus has previously been used to quantify OCR of rat hepatocyte suspensions (Noll, deGroot, and Wisseman 1986) and more recently to measure OCR of microcarrier attached HepG2 cells (Smirthwaite 1994).

This is an attractive technique. However, Noll and co-workers were only able to measure OCR of hepatocytes in suspension culture. It is widely known that hepatocytes only remain viable in suspension for a few hours so this data may not reflect the oxygen requirements of hepatocytes cultured in a bioreactor. The work of Smirthwaite represents a distinct improvement since in this system, the cells were immobilised on microcarrier beads.

The immobilisation of primary hepatocytes upon such microcarriers is an unreliable technique. In a stirrer flask with dividing cells (such as HepG2), only a small fraction of the cell inoculum need attach to the beads since the progeny of attached

cells will normally remain attached. When working with primary hepatocytes (generally non-dividing), one must attempt to get a major fraction of the cell inoculum to attach to the beads. This is in itself difficult and even when successful, usually results in a highly uneven surface distribution of cells.

It would be desirable to have one test system which could be used for both OCR determinations and shear investigations. Therefore, it was decided that a novel test apparatus was required.

5.1.2. Hepatocytes and fluid shear stresses

In the *in vivo* situation, hepatocytes are not directly exposed to fluid shear forces. The flow of blood along the acinus produces shear. The resultant wall shear stresses are exerted on the sinusoidal lining. The fenestrations in the endothelium allow the development of small eddy currents in the parenchyma of the organ but the hepatocytes are largely protected from even these by the Space of Disse.

The direct contact between hepatocytes and flowing fluid which would occur in the proposed prototype reactor configuration outlined in the preceding chapters is a unique aspect of the design which does not occur *in vivo*.

The effects of exposing the hepatocytes to such shear forces cannot be predicted. It might be that any adverse (or beneficial?) effects resulting from such exposure would be manifested as observable morphological alterations or in the form of changes in measured function.

Digital photomicrography combined with computer based image processing could be used to assess any such morphological alterations. Quantification of the rate at which hepatocytes synthesise urea when exposed to different magnitudes of shear stress could be used to determine whether such exposure influences hepatic function.

5.1.3. Requirements of test apparatus

The test system must provide a means by which hepatocytes can be cultured

in a hydrodynamic environment. The shear stress in this environment should be quantifiable and near-uniform across the hepatocyte perfusate interface and a means of determining oxygen consumption rates would also have to be incorporated. A linear flow cell with a defined narrow slit geometry could form the basis of the system. More specifically, the flow cell would have to:

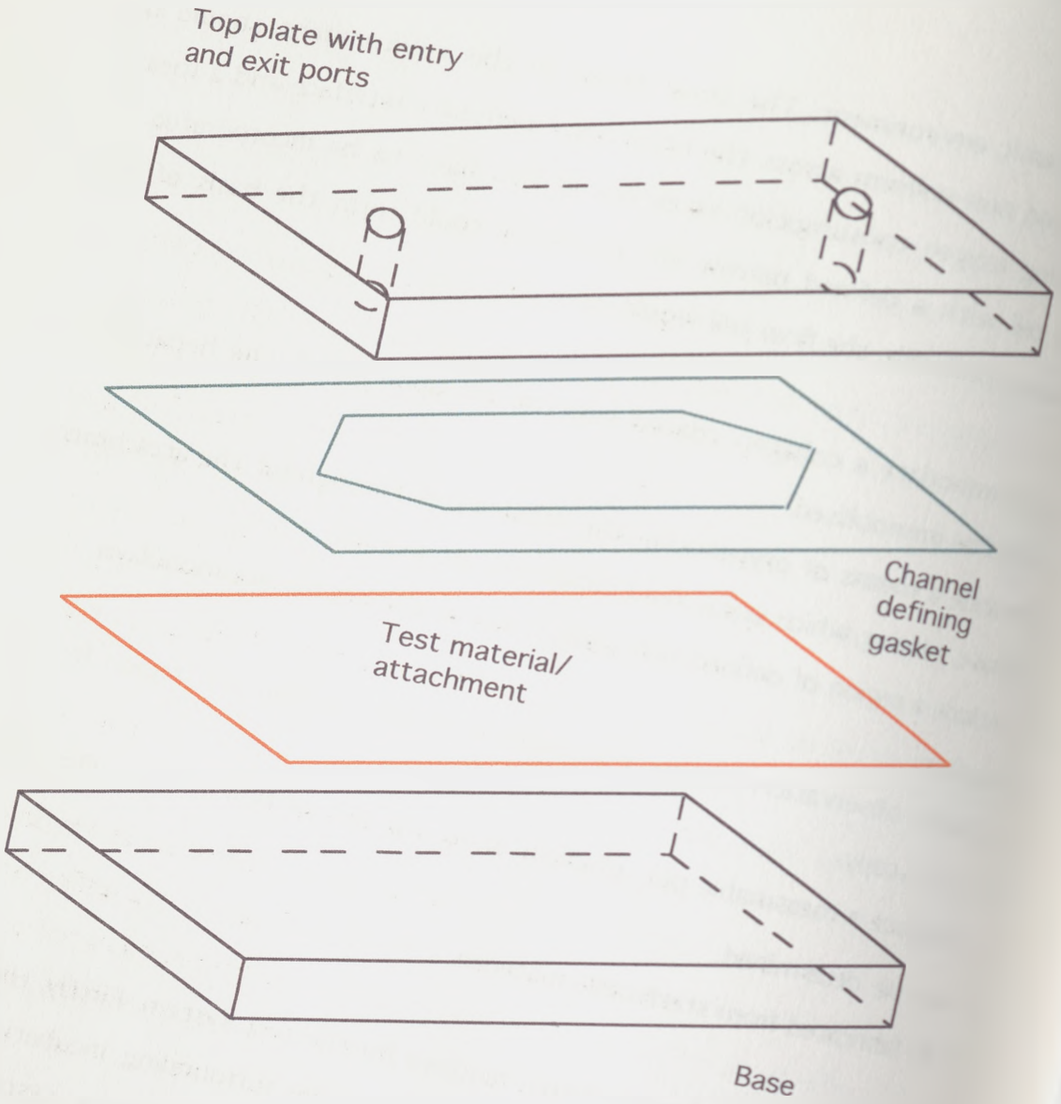
- Accommodate a collagen coated substratum upon which the hepatocytes could be immobilised.
- Provide a means of oxygenating the hepatocytes throughout the attachment phase, during which static fluid conditions are required.
- Include a region of defined wall shear stress at the hepatocyte monolayer surface.
- Enable observation of the hepatocyte monolayer within this region by light microscopy.
- Produce a measurable pO_2 gradient along the axis of flow in order that OCR can be determined
- Be fabricated from sterilisable materials.

There are two stages of design required for the test system. Firstly, the flow cell itself must be designed and built. Secondly, the surrounding incubation and perfusion apparatus, microscopical examination and image capturing system and dissolved oxygen monitoring equipment must be assembled. These two phases of development will be described in the following sections.

5.2. Apparatus

5.2.1. Hepatocyte flow cell design

Specialised flow cells for studying blood material interactions and cellular



Generalised parallel-plate flow cell

Figure 5.1.

adhesion have been described previously. Wilson and co-workers detailed a well designed narrow slit flow device for haemocompatibility investigations (Wilson et al. 1982). More recently, van Kooten and colleagues reported a similar parallel plate flow chamber for studying the behaviour of cultured fibroblasts in a hydrodynamic environment (van-Kooten et al. 1992). Wilson's device consisted of two plates between which a test membrane and a gasket assembly could be clamped in place by means of two additional external plates which were bolted together. The gasket assembly formed the liquid seal and defined a narrow slit flow channel. The general design of this device would be appropriate for use in the investigations outlined in the preceding sections. However, it would have to be modified to accommodate a means of oxygenating the hepatocytes during the static attachment phase. The device described by van Kooten does not offer any particular advantage over that of Wilson.

A generalised schematic diagram of a narrow-slit parallel plate flow cell of the type described by Wilson and van Kooten is given in Figure 5.1.

In order to have a region of defined, near-uniform shear stress in the flow cell, the geometry of the flow channel must be carefully designed. A gradually diverging entry region and a gradually converging exit region are required in order to have well developed laminar flow in a rectangular channel. According to the strictest criteria (Bowen 1985) the entry section should constitute no more than 2.5% of the channel length. Other workers have suggested a less exacting limiting ratio of the length of the flow channel to the length of the entry region of 10:1(van-Wagenen and Andrade 1980). It is also argued that the total internal angle of divergence in the entry region should not exceed 15° for the range of flow rates envisaged (<<100 ml min^{-1}) (Abramowitz 1949). Taken together, these represent criteria which are, for practical purposes, unattainable. For a flow channel width of as little as 2 cm, the entry section diverging from a 2 mm entry port would have to be some 7 cm in length. The overall device length, including the exit region (which can in theory converge more rapidly)

would therefore exceed 70 cm. Such a device would be unwieldy for use in the system of apparatus described. As we shall see, a wider channel will also prove to be necessary, making the entry section criteria even more difficult to attain.

The distribution of flow velocity in the y,z plane of such a rectangular duct of width w along the y-axis and height $\pm h$ along the z-axis is given by:

$$\frac{u_{(y,z)}}{u_0} = 1 - \left(\frac{z}{h}\right)^2 - \frac{4}{h^3}\sum_{n=0}^{\infty}(-1)^n \frac{\cos mz \cosh my}{m^3 \cosh\frac{mw}{2}} \qquad \textit{(Equation 5.1)}$$

where:

$$m = \left(n + \frac{1}{2}\right)\frac{\pi}{h}$$

u_0 = maximum fluid velocity and

$u_{(y,z)}$ = fluid velcocity at some point in the same plane of the channel (Berker 1963).

Since all the hepatocytes in the flow cell can be considered to lie at the same point along the z-axis, this equation can be simplified to consider only the y-axis flow distribution. Numerical solutions for Equation 5.1 demonstrated that only the first term in the summation series is significant. Ignoring all but the first term in the summation series, the following obtains:

$$\frac{u_{(y)}}{u_0} = 1 - \left\{\frac{32}{\pi^3}\left(\frac{\cosh\frac{\pi}{2}\frac{y}{h}}{\cosh\frac{\pi}{2}\frac{w}{h}} - \frac{\cosh\frac{3\pi}{2}\frac{y}{h}}{27\cosh\frac{3\pi}{2}\frac{w}{h}}\right)\right\} \qquad \textit{(Equation 5.2)}$$

This equation predicts that the velocity profile across the width of such a channel flattens, as w/h becomes larger. In other words, the uniformity of wall shear stress across the plane of the hepatocyte monolayer increases with the width to height ratio of the channel. A set of solutions for the distribution of flow across the width of rectangular channels according to Equation 5.2 is given in Table 5.1.

w/h	U at y = 0.9 *w*	U at y = 0.95 *w*	U at y = 0.99 *w*
10	0.785 U_o	0.533 U_o	0.142 U_o
20	0.955 U_o	0.786 U_o	0.261 U_o
40	0.998 U_o	0.955 U_o	0.455 U_o
60	0.999 U_o	0.990 U_o	0.600 U_o
80	0.999 U_o	0.998 U_o	0.707 U_o
100	$\cong U_o$	0.999 U_o	0.786 U_o

Fluid velocity distribution in a rectangular channel of width *w* and height *h*

(U_o = maximum fluid velocity)

Table 5.1.

The data in Table 5.1 indicate that there is less than 5% drop in fluid velocity across 95% of the channel width for those channels with a width to height ratio of 40 or more. In order to achieve good distribution of hepatocytes across the channel surface, a channel height of at least 1.0 mm would be advantageous. For a *w/h* ratio of 40, a channel of width 40 mm would be required, a manageable size.

For practical purposes, the overall length of the flow cell device should not

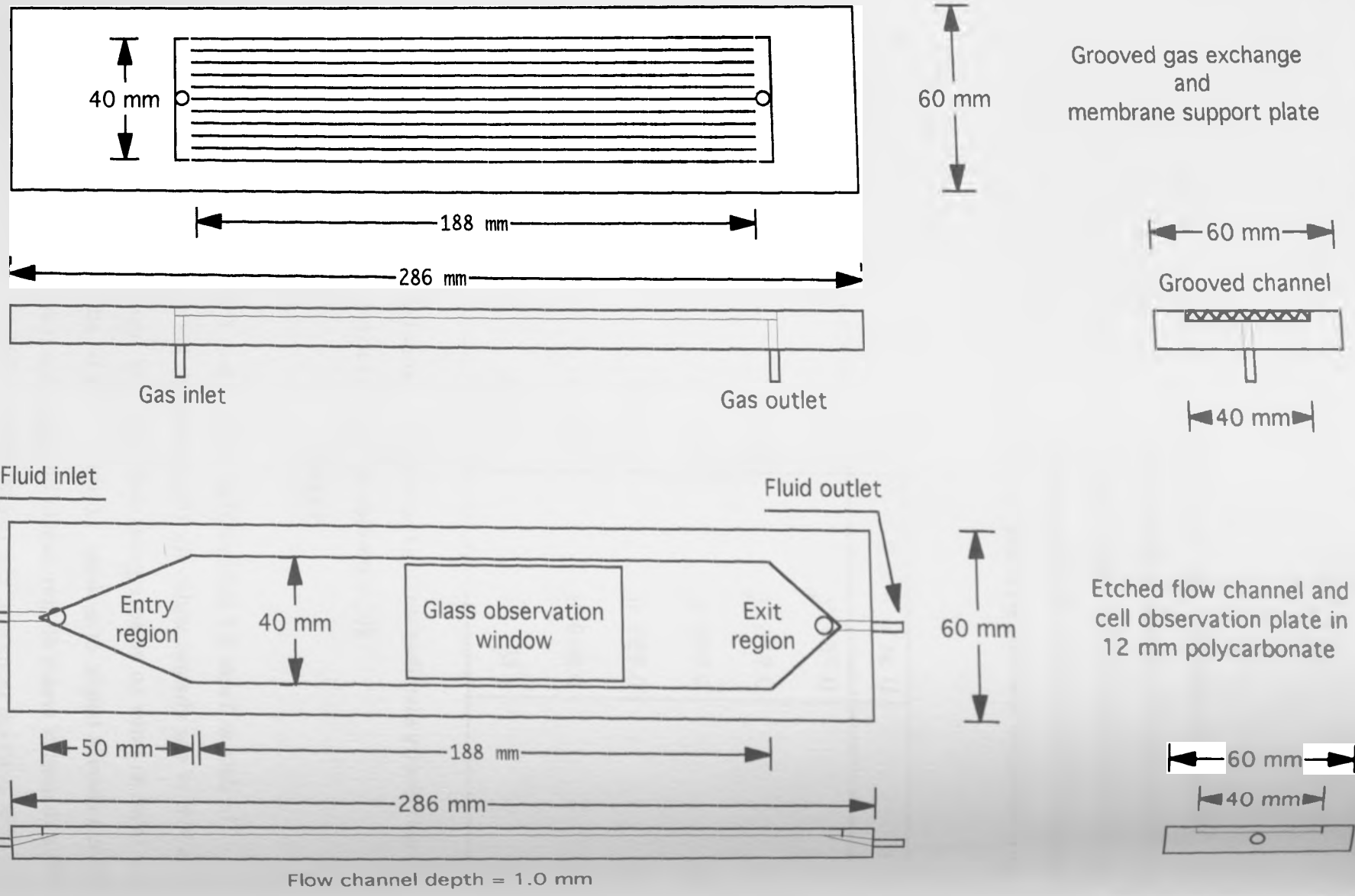


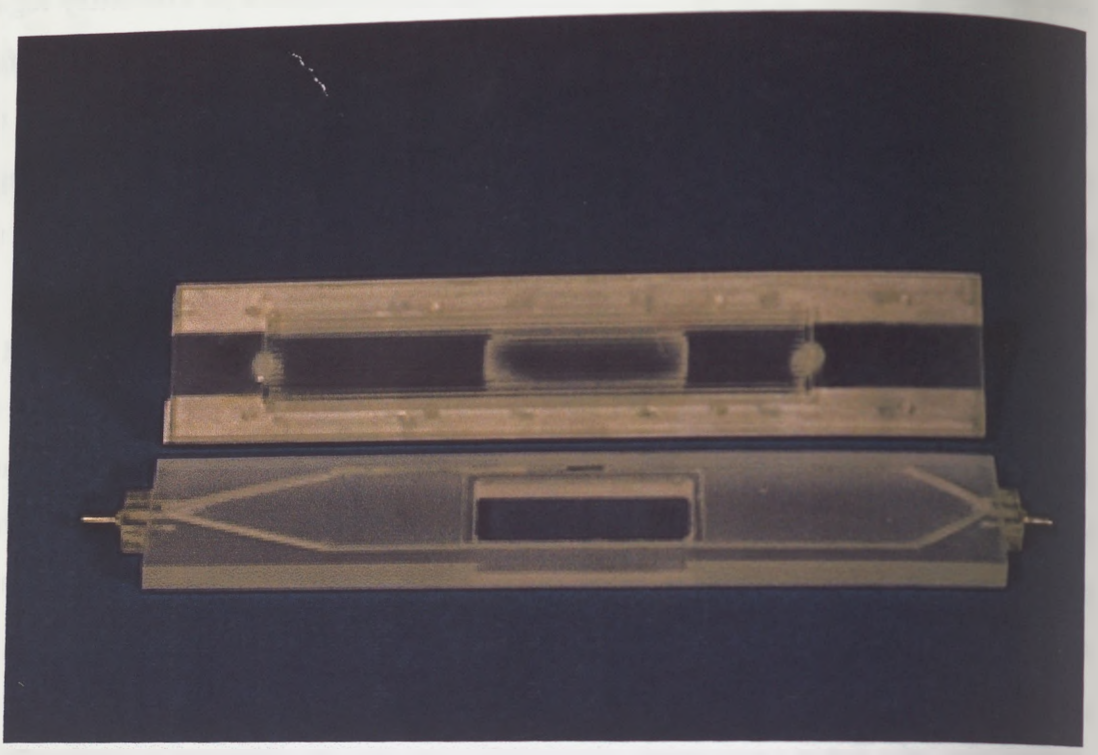
Figure 5.2.
119A

exceed 300 mm. A compromise between the angle of divergence of the entry region and the ratio of the total channel length to the entry region length is therefore required. For a channel of total length 260 mm, an angle of divergence of 22° in the entry region can be achieved by having an entry section of length 50 mm, giving a total channel length to entry region length ratio of 5.2 : 1. These dimensions offer the best compromise possible within the size constraints.

The general device design shown in Figure 5.1 requires to be modified to include a cell support substratum and some form of integral oxygenation during the attachment phase in which static fluid conditions are necessary. A plastic plate coated with collagen could act as the cell support substratum and a microporous polypropylene flat sheet membrane could provide oxygenation during the static phase. A device with this configuration would have two compartments separated by the flat sheet membrane. One containing the cells, immobilised upon the coated support, and the perfusate, the other containing the supply gas. In an alternative configuration, the oxygenation membrane might be coated with collagen and this could act as both the cell support and oxygenation surface. Both of these configurations could be provided by one flow-cell design. A plate with a grooved channel could provide both support for the flat sheet membrane and a gas supply compartment. A second plate could have the flow channel etched into its surface and a channel defining gasket included to act as a seal. The oxygenation membrane could be held between these 2 plates by means of external clamps. A drawing of the proposed flow-cell design is given in Figure 5.2.

5.2.2. Flow cell manufacture

The two plates were machined in 12 mm polycarbonate. The glass observation window (a microscope slide) was sealed into the channel defining plate with medical grade silicone adhesive (Silastic, Dow Corning, USA) ensuring a flush finish with the channel surface . A small groove was etched around the periphery of the flow channel



Hepatocyte Flow Cell

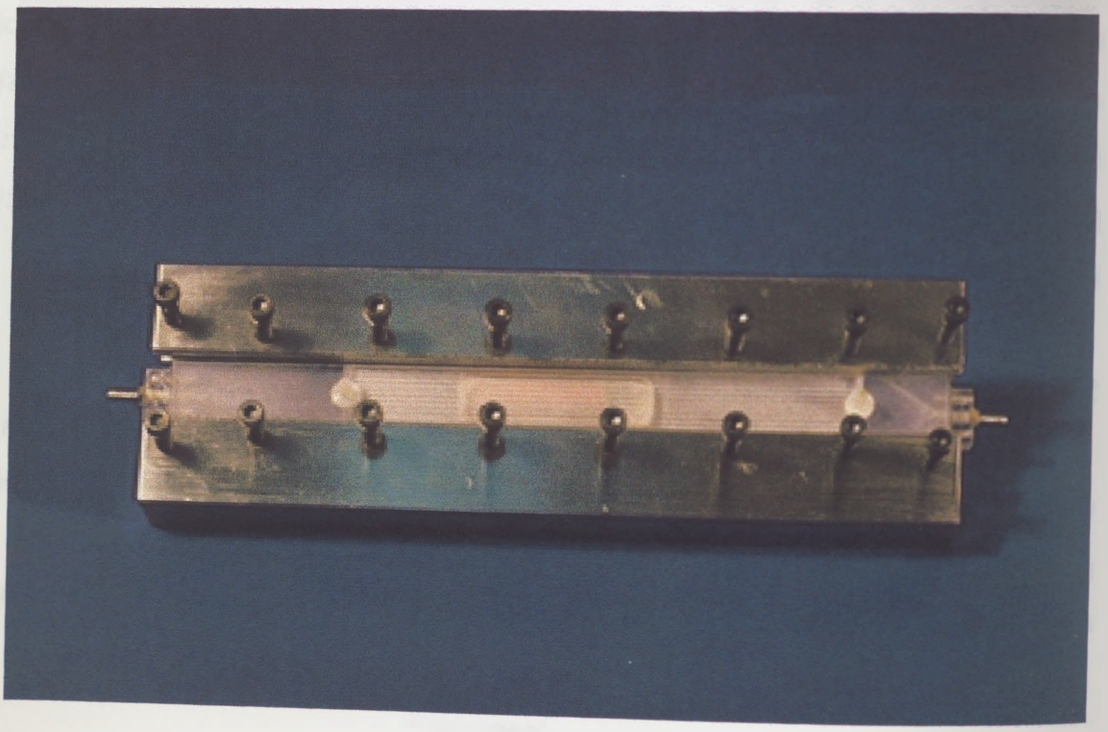


Figure 5.3

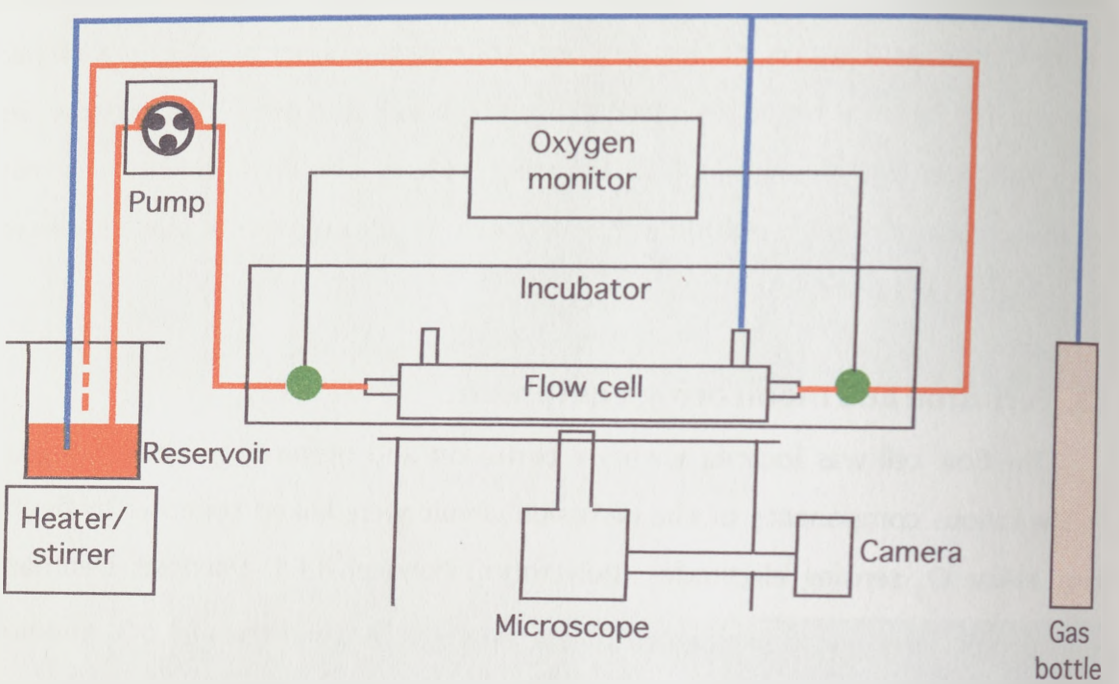
which accommodated a gasket of 0.4 mm bore, 0.8 mm wall silicone tubing (Altec Products Ltd., Alton, UK) to provide a liquid seal. Entry and exit ports were tapped (M4 thread) into which, threaded hose barb connectors (M4 x 3.2 mm barb) were fitted and sealed. A pair of stainless steel clamps were used to hold the two polycarbonate plates together and seal the flow channel by compressing the gasket via threaded bolts distributed along the length of the clamps.

The cell attachment surface which lies within the glass observation window can be considered as a region of near-uniform shear stress since all of the attached hepatocytes will lie on a single x-y plane along the z axis and since the window lies within a region of well developed flow covering 95% of the channel width, across which there is less than 5% variation in fluid velocity. A photograph of the completed flow cell is given in Figure 5.3.

5.2.3. Perfusion and monitoring equipment

The flow cell was located within a perfusion and monitoring system (Figure 5.4). The various components of the perfusion circuit were linked together by flexible tubing. In-line O_2 sensing electrodes (Polytrodes, Polystan Ltd., Vaerlose, Denmark) were sited upstream and downstream of the flow cell. A twin channel pO_2 monitor (Polystan Ltd., Denmark) was used to record data from the electrodes. Perfusate was recirculated by means of a peristaltic pump. A stirred and heated (37 °C) perfusate reservoir with a venting filter and a sampling port was also included in the circuit. The supply gas was connected directly to the gas channel of the flow cell and also, via a sparger, to the fluid reservoir. The flow cell and O_2 electrodes were housed within a 37°C incubator.

For microscopy, the flow cell was mounted on the stage of an inverted phase contrast microscope. A digital camera (Kodak DCS200, Eastman Kodak, UK) was connected to the microscope and used to record images from the flow cell.



Key:

Perfusate

Gas supply

Oxygen electrodes

Hepatocyte flow cell perfusion circuit

Figure 5.4.

5.3. Materials, methods and analysis

5.3.1. Hepatocyte isolation

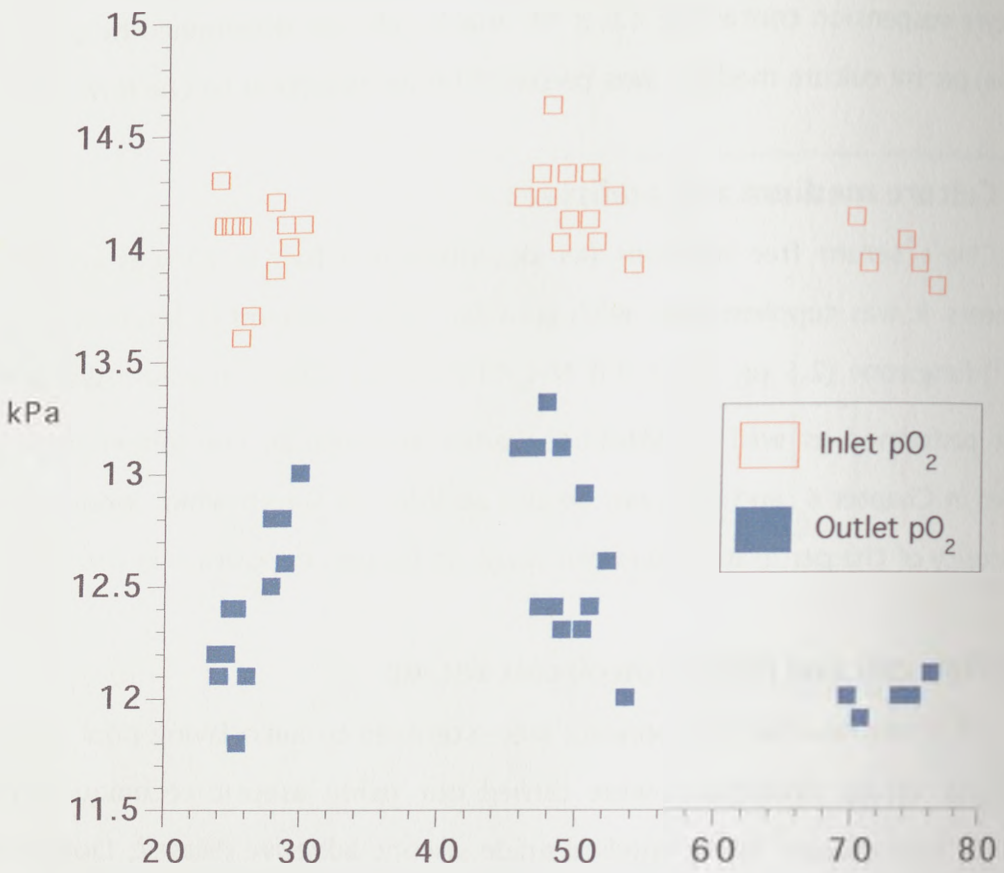
Primary rat hepatocytes were isolated as described in Chapter 4. A final hepatocyte suspension containing 1.0 x 10^6 viable cells (as determined by trypan blue exclusion) per ml culture medium was prepared for introduction to the flow cell.

5.3.2. Culture medium and additives

Chee's serum free medium (as described in Chapter 4) was used for all experiments. It was supplemented with penicillin (100 Units ml^{-1}), Streptomycin (100 µg ml^{-1}) fungizone (2.5 µg ml^{-1}) and NH_4Cl (2 mM). Chee's medium was selected since it performed as well as Modified Earle's medium in the longer term tests described in Chapter 4 and does not require addition of serum which would influence the viscosity of the perfusate and might result in protein deposition in the system.

5.3.3. Flow cell and perfusion circuit set-up

All of the flow cell components were sterilised by autoclaving prior to use. All subsequent set-up procedures were carried out using aseptic technique within a laminar air flow cabinet. Sterile medical grade silicone adhesive (Silastic, Dow Corning, USA) was diluted 1 in 10 with sterile hexane. A thin film of the resultant solution was painted around the periphery of the lower surface of the polycarbonate flow cell top plate. A pre-cut sheet of a microporous hydrophobic polypropylene membrane (Celgard 2400, Hoescht Celanese, Frankfurt, Germany) was then attached to this siliconised surface, producing a seal between the grooved gas supply channel and the membrane. The upper surface of the channel defining base plate was coated with rat tail collagen (as described in Chapter 4) at a density of 35µg cm^{-2} by allowing a thin film of collagen solution deposited upon the polycarbonate to evaporate. This process resulted in a thin layer (<25 µm) of unpolymerised collagen being retained on the



Sample raw pO_2 data from flow cell experiments

Figure 5.5.

surface. This coating formed the cell attachment surface of the channel defined by the sealing gasket in the base plate. The flow cell was then assembled and clamped.

The connecting tubing (3.2 mm bore, 1.6 mm wall Marprene tubing (Watson Marlow, Falmouth, UK)) and the fluid reservoir were also sterilised by autoclaving prior to use. An 0.2 µm hydrophilic liquid sampling filter and an 0.2 µm hydrophobic gas venting filter (both Minisart™, Sartorius Ltd., UK), supplied sterile, were attached to spare ports on the reservoir lid. The in line O_2 electrodes were also supplied sterile.

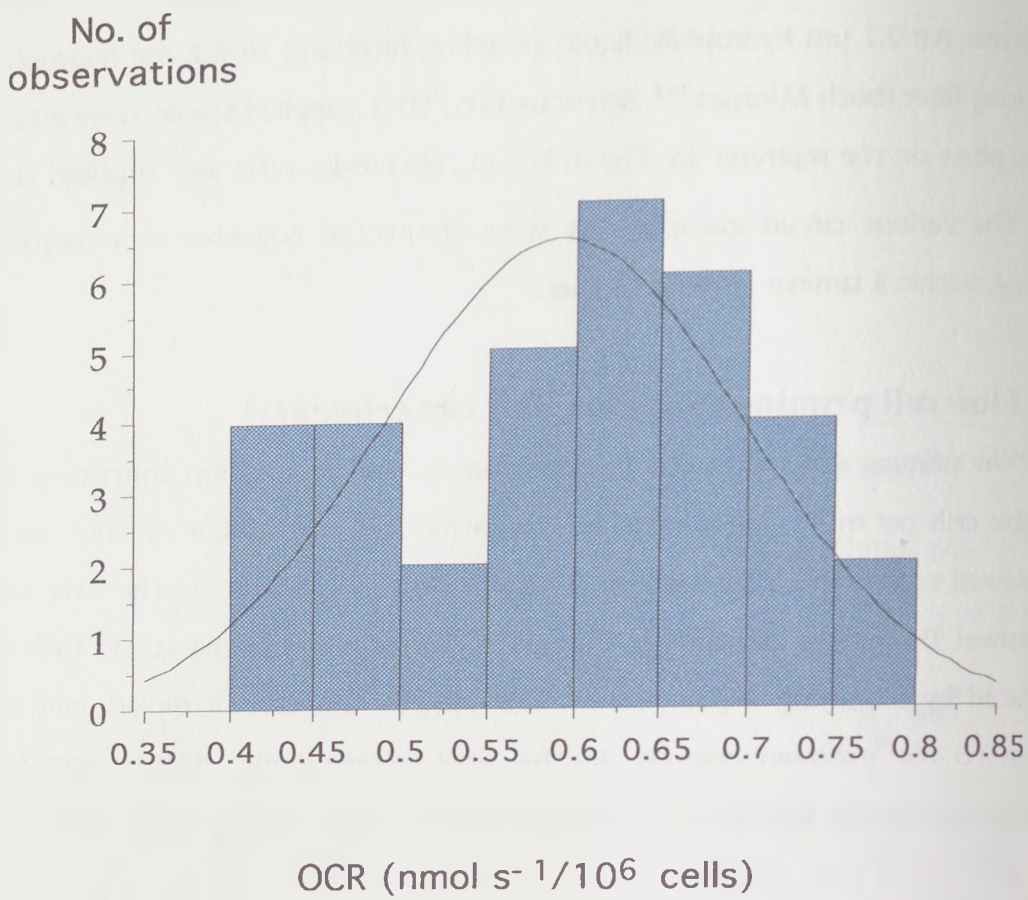
The various circuit components were connected together as indicated in Figure 5.4. within a laminar air flow cabinet.

5.3.4. Flow cell priming procedure *(all experiments)*

The priming volume of the flow cell was 9.7 ml. A solution containing 1.0 x 10^6 viable cells per ml in Chee's medium was introduced to the flow cell and the cells were allowed to attach to the collagen coated surface which formed the base of the flow channel. During the attachment phase, the fluid was completely static. Cells were oxygenated by supplying a gas mix of 12% oxygen, 5% carbon dioxide and 83% nitrogen to the grooved channel. In this way oxygen could diffuse across the membrane to the cells and the CO_2 balance in the medium could be maintained.

5.3.5. OCR experiments

After an initial static attachment phase of approximately 20 hours (the attachment process was generally completed after 6 hours but the static phase was maintained for 20 hours for logistical reasons), the gas supply to the grooved channel was discontinued and this channel was sealed from the external atmosphere. Any unattached cells were flushed from the flow channel. An excess of Chee's medium containing 2 mM NH_4Cl (to act as a metabolic load) was then recirculated through the flow cell at 20 ml min^{-1} via a peristaltic pump from the heated and stirred reservoir which was continuously sparged at 200 ml min^{-1} with the gas mix described



Oxygen consumption rate measurements

Figure 5.6.

above. The Clark type in-line oxygen electrodes which were sited upstream and downstream of the flow cell and the dual channel dissolved oxygen monitor were used to record pO_2 values at the flow cell inlet and outlet simultaneously.

5.3.6. Shear stress experiments

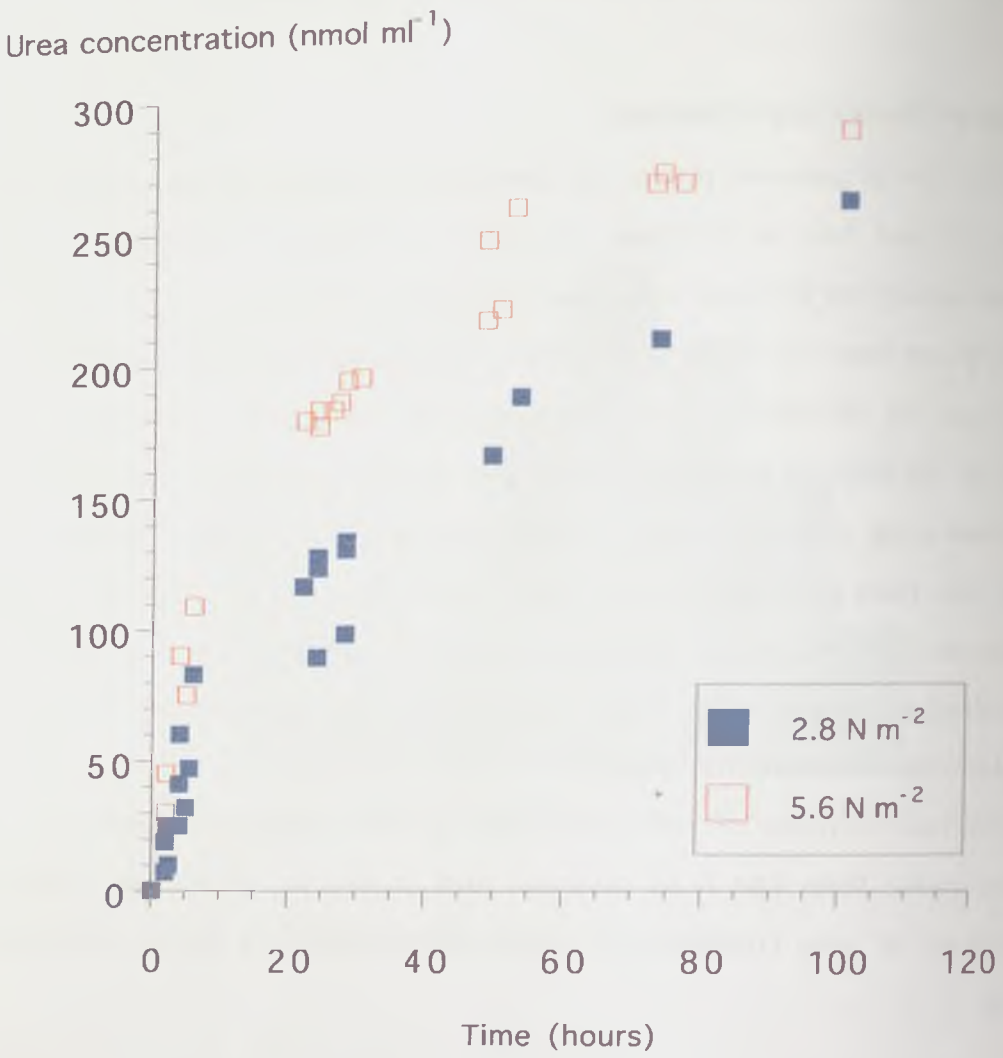
After the attachment phase, any unattached hepatocytes were flushed from the flow cell and 300 ml of Chee's medium containing 2 mM NH_4Cl (as an ammonium source for the urea cycle) was recirculated through the flow cell via the peristaltic pump from the reservoir which was continuously sparged at 200 ml min^{-1} with the gas mix described above. Two flow rates, 20 ml min^{-1} and 40 ml min^{-1} (selected on the basis of anticipated bioreactor operating conditions) producing two levels of wall shear stress were used for these experiments. The glass window in the flow cell base plate permitted morphological examination of the hepatocytes under phase contrast light microscopy. Photomicrographs of the hepatocytes in the flow cell were obtained as compressed digitised images via the digital camera and stored on an Apple Macintosh computer for analysis.

Discrete samples of perfusate (300 µl) were obtained throughout the perfusion phase from the fluid reservoir and stored at -20°C for subsequent determination of urea concentration which was carried out within 72 hours of sampling.

5.3.7. OCR Analysis

If the reservoir is well mixed and any drop in oxygen partial pressure across the flow cell is entirely due to consumption by hepatocytes, then the oxygen consumption rate of the hepatocytes (OCR_H) in this system is given by:

$$OCR_H = Q \alpha \Delta pO_2 \qquad \textit{(Equation 5.3)}$$



Urea synthesis in flow cell: Influence of shear stress

Figure 5.7.

where; Q is the medium flow rate, α is the solubility coefficient of oxygen in Chee's medium at 37°C, taken as being equivalent to 0.893 mmol l^{-1} at 1 atmosphere and ΔpO_2 is the drop in oxygen partial pressure across the flow cell.

5.3.8. Shear stress analysis

The wall shear stress (τ_w) at the hepatocyte perfusate interface was calculated from:

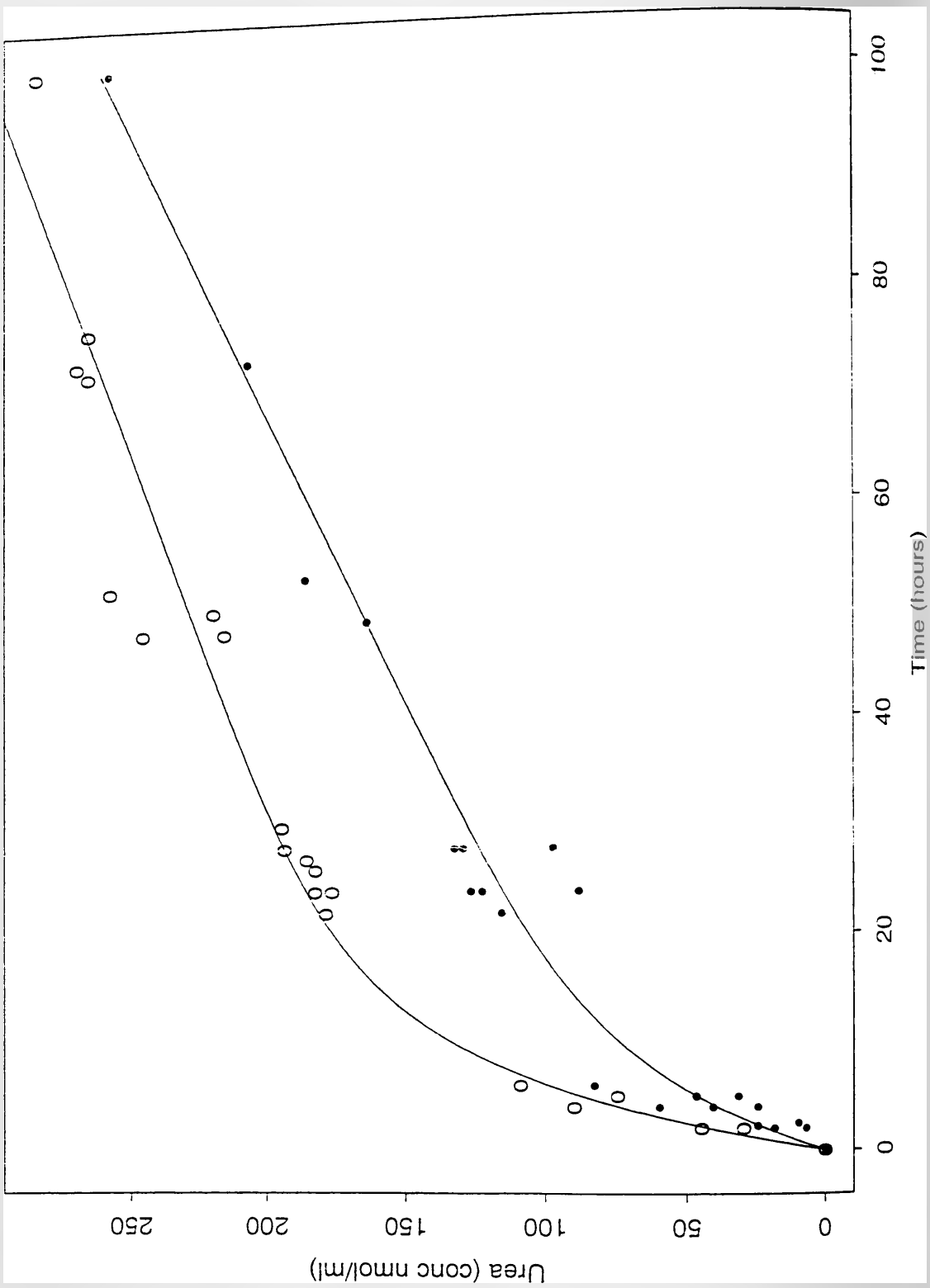
$$\tau_w = \mu \frac{6Q}{wh^2} \qquad \textit{(Equation 5.4)}$$

where: μ is the dynamic viscosity of the perfusate, taken as being equivalent to that of water at 37°C (6.913×10^{-3} N s m^{-1}), Q is the perfusate flow rate, w is the channel width and h is the channel height.

The two flow rates, 20 ml min^{-1} and 40 ml min^{-1} which were used for the shear response experiments were calculated to produce wall shear stresses at the hepatocyte perfusate interface of 2.8×10^{-3} N m^{-2} and 5.6×10^{-3} N m^{-2} respectively. The corresponding wall shear rates (from $\tau_w = \mu \varpi$ where ϖ = wall shear rate) were calculated to be 400 s^{-1} and 800 s^{-1}.

5.3.9. Urea analysis

The urea concentration in the reservoir during the perfusion phase was determined from the samples obtained using the diagnostic kit described in chapter 4 (BUN 535B, Sigma Chemical Co., Poole, UK). Instantaneous rates of urea synthesis were calculated by differentiating the equation of the best fit line of urea concentration against time plots. The influence of wall shear stress on urea synthesis was determined by statistical methods described below.



Fitted urea synthesis data, open symbols = 5.6×10^{-3} N m^{-2}, dots = 2.8×10^{-3} N m^{-2}

Figure 5.8.

5.3.10. Image analysis

The images from the digital camera were converted from proprietary compressed files to a standard image format (24 bit gray scale PICT files) using Photoshop software (Adobe Software, UK). Some preliminary image cropping and filtering was also conducted at this stage. The resultant images were then imported into Optilab software (Graftek, France). In this program, custom look-up table transformations and further filtering (Median and Sigma convolutions) were used to convert the file to a black and white image appropriate for morphological analysis. The following measurement parameters were then applied to the objects (assumed to be individual cells) in each image:

I. Basic descriptive statistics

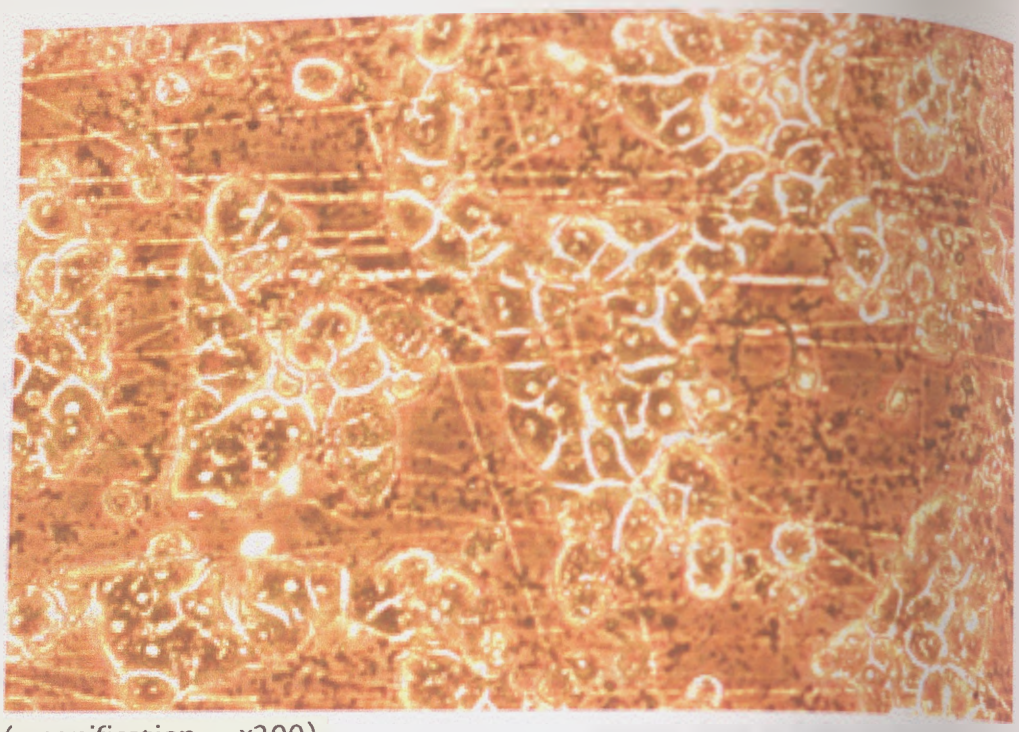
In order to determine whether significant image to image variations were present in terms of the total cell number, the extent of cell coverage within a field and the size range of the cells.

II. Compactness factor

This is defined as the area of an object divided by the product of its breadth and its width. The parameter belongs to the interval [0,1]. A perfect rectangle having a value of 1. Since hepatocytes in monolayer culture spread to form polygons during the attachment process, this parameter might highlight variations in the intimacy of hepatocyte to substratum contact.

III. Heywood circularity factor

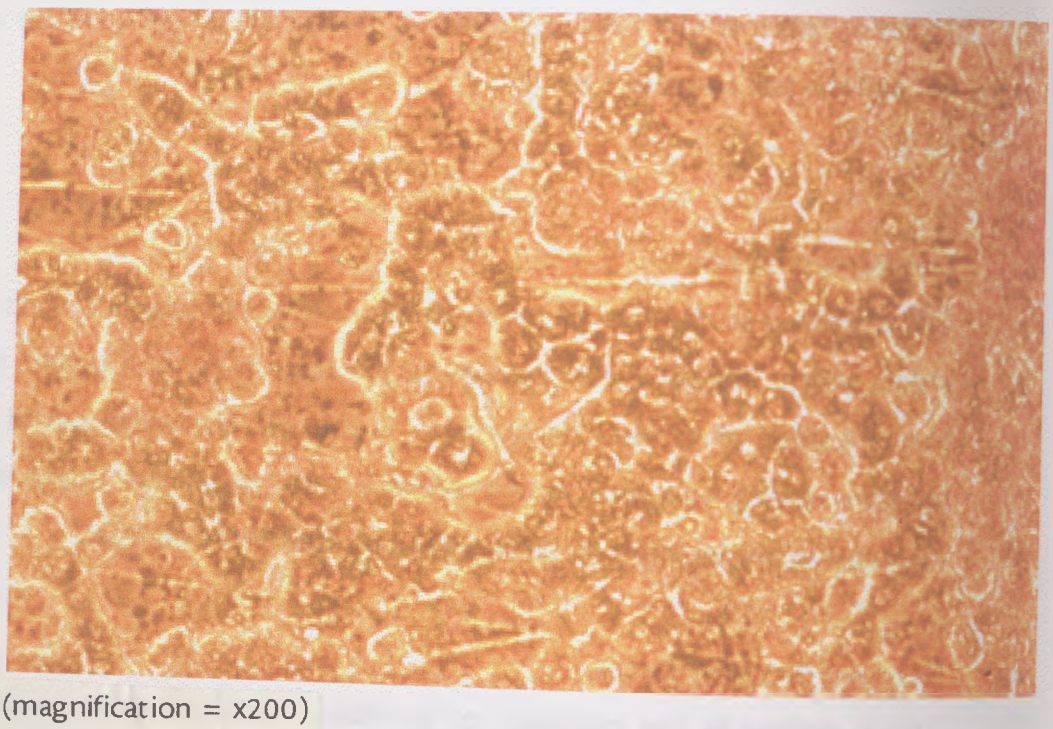
This is defined as the perimeter of an object divided by the perimeter of a circle with the same area as the object. The closer the circularity factor is to 1,



(magnification = x200)

Hepatocytes in flow cell (τ_w =2.8x10^{-3} N m^{-2})

Figure 5.9



(magnification = x200)

Hepatocytes in flow cell (τ_w =5.6x10^{-3} N m^{-2})

Figure 5.10

the closer to a perfect circle is the object. As cells begin to desquamate from their substratum, they tend to 'round up'. This parameter could therefore be used to determine the extent to which shear stresses were leading to hepatocyte detachment in the flow cell.

IV. Elongation factor

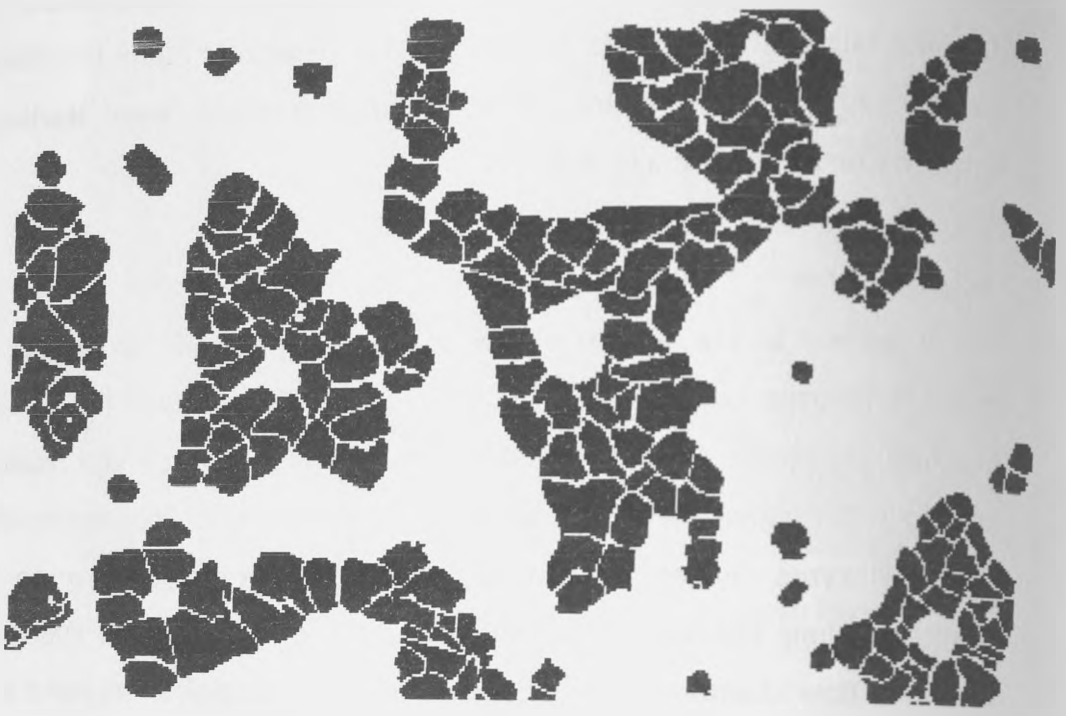
This is defined as the length of the longest segment of an object (the maximum intercept) divided by the mean perpendicular intercept (i.e. the mean length of the chords in an object which are perpendicular to the maximum intercept). The higher the elongation factor, the more elongated is the object. Some cell types (notably endothelial cells) have been shown to align themselves along the principal axis of shear (Levesque and Nerem 1985) when studied in flow chambers of this type. Whether hepatocytes respond in this way is unknown. The elongation factor could be used to determine the extent of this behaviour.

The Optilab software has algorithms enabling calculation of each of these parameters for every object (hepatocyte) in a suitable black and white image. Statistical methods (paired T tests) were used to determine whether these parameters were influenced by the level of shear stress to which the hepatocytes were exposed.

5.4. Results

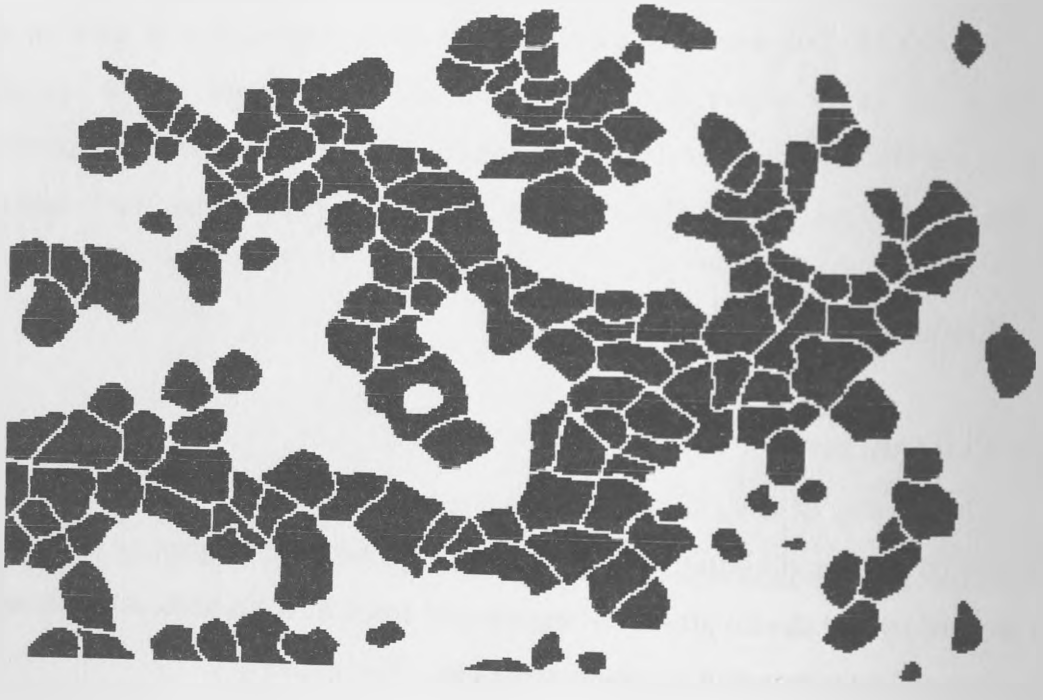
5.4.1. Cell Culture

The viability of the cell populations used for the flow cell experiments ranged from 81% to 93% as determined by trypan blue exclusion. The proportion of seeded cells retained by the device after flushing ranged from 86% to 95%. All retained cells were assumed to be attached to the membrane.



Processed image (Fig.5.9)

Figure 5.11.



Processed image (Fig.5.10)

Figure 5.12.

5.4.2. OCR Results

Rat hepatocytes from 4 different cell isolations were used for these experiments. Continuous recordings of the drop in oxygen partial pressure across the flow cell were made between 22 and 76 hours after seeding the device with hepatocytes. The mean values for each 4 hour period were calculated, producing a total of 34 time averaged ΔpO_2 values which were then used for the OCR calculations. The upstream pO_2 remained stable throughout the duration of the experiments (as shown in figure 5.5.) indicating that there was no significant oxygen debt and an absence of bacterial contamination. The maximum OCR recorded was 0.77×10^{-9} moles $s^{-1}/10^6$ cells and the minimum value was 0.40×10^{-9} moles $s^{-1}/10^6$ cells. The population of OCR measurements had a near normal distribution about a mean value of $0.59 \pm 0.10 \times 10^{-9}$ moles $s^{-1}/10^6$ cells. This is illustrated in figure 5.6. There was no significant difference between the mean OCR of different cell isolations (minimum: 0.52 ± 0.10, maximum: 0.66 ± 0.07) and there was no obvious correlation between OCR and time point. In a set of tests where no hepatocytes were present in the flow cell, the maximum oxygen partial pressure drop recorded was 0.1 kPa representing a possible error of approximately 2% in the measured pressure drop values in the OCR tests. This was included in the OCR calculations.

5.4.3. Shear stress results *(urea synthesis)*

Primary rat hepatocytes from 6 different isolations were used for the shear stress experiments. Urea synthesis was recorded throughout the duration of all tests. Figure 5.7 indicates that monotonic increases in urea concentration in the reservoir were measured over 5 day periods. At the longest time points, the NH_4Cl concentration in the perfusate was diminished by not more than 6%. The influence of shear stress level on urea synthesis was determined by curve fitting and statistical techniques. The data as seen in Figure 5.7, appear to consist of an initial non linear

Parameter	$2.8x10^{-3}$ N m^{-2} (Figure 5.11)	$5.6x10^{-3}$ N m^{-2} (Figure 5.12)
No. Objects detected	218	166
No. touching border	20	13
No. Objects analysed	208	153
Min. Object area (μm^2)	10.0	20.8
Max. object area (μm^2)	1339.8	1088.0
Mean object area (μm^2)	294.3±191.7	360.8±223.3
Total image area (cm^2)	0.192	0.192
Sum object area (cm^2)	0.064	0.060
% object coverage	33	31

Basic descriptive statistics for Figures 5.11 and 5.12.

Figure 5.13.

portion which is ultimately superceded by linear segments in both experimental conditions. Therefore, an exponential function, of the general form:

$$[Urea] = A\left(1 - e^{-Bt}\right) + Ct \qquad \textit{(Equation 5.5)}$$

where: *[Urea]* is the concentration of urea in the reservoir (nmol ml^{-1}), t is time (hours) and A, B and C are constants, was applied to the data.

This produced the following models:

$$[Urea]_{2.8} = 72.57\left(1 - e^{-0.143t}\right) + 1.89t \qquad \textit{(Equation 5.6)}$$

$$[Urea]_{5.6} = 155.92\left(1 - e^{-0.144t}\right) + 1.46t \qquad \textit{(Equation 5.7)}$$

where: $[Urea]_{2.8}$ is the concentration of urea in experiments where the wall shear stress was 2.8×10^{-3} N m^{-2} and $[Urea]_{5.6}$ is the concentration of urea at 5.6×10^{-3} N m^{-2}.

In both models, the constants A, B and C all proved to be significantly ($p<0.05$) different from zero. In other words, each constant has a significant effect on the fit. Upon closer analysis of the models, it emerged that there was a significant difference ($p<0.05$) between the 2 models in the values of both A and C. However, the value of B was not significantly different ($p>0.05$) in the two data sets. Therefore, the data was re-fitted using the same general function, but specifying that the time constant B must be the same in both models. This produced the following:

$$[Urea]_{2.8} = 72.47\left(1 - e^{-0.144t}\right) + 1.89t \qquad \textit{(Equation 5.8)}$$

$$[Urea]_{5.6} = 155.98\left(1 - e^{-0.144t}\right) + 1.45t \qquad \textit{(Equation 5.9)}$$

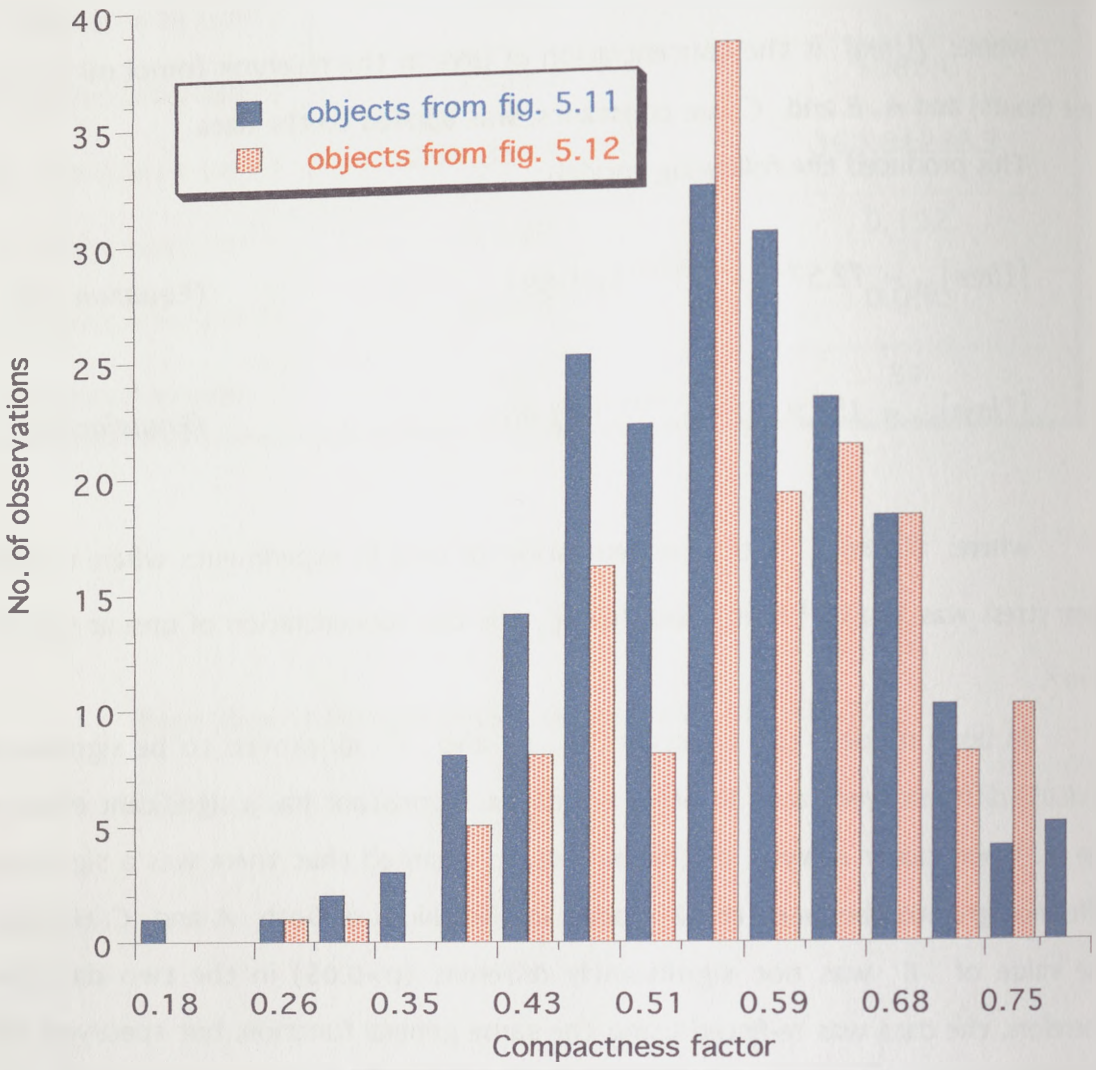


Figure 5.11, mean = 0.58 ± 0.11

Figure 5.12, mean = 0.61 ± 0.10

Histogram of compactness factor for objects from Figures 5.11 and 5.12

Figure 5.14

These two models are plotted along with raw data for both sets of experimental conditions in Figure 5.8. It can be seen that at short times the rate of urea synthesis was significantly greater ($p<0.05$) in cells exposed to $5.6x10^{-3}$ N m^{-2} than in cells cultured under $2.8x10^{-3}$ N m^{-2}. However, the ultimate rate of urea synthesis was greater ($p<0.05$) at $2.8x10^{-3}$ N m^{-2} than at $5.6x10^{-3}$ N m^{-2}.

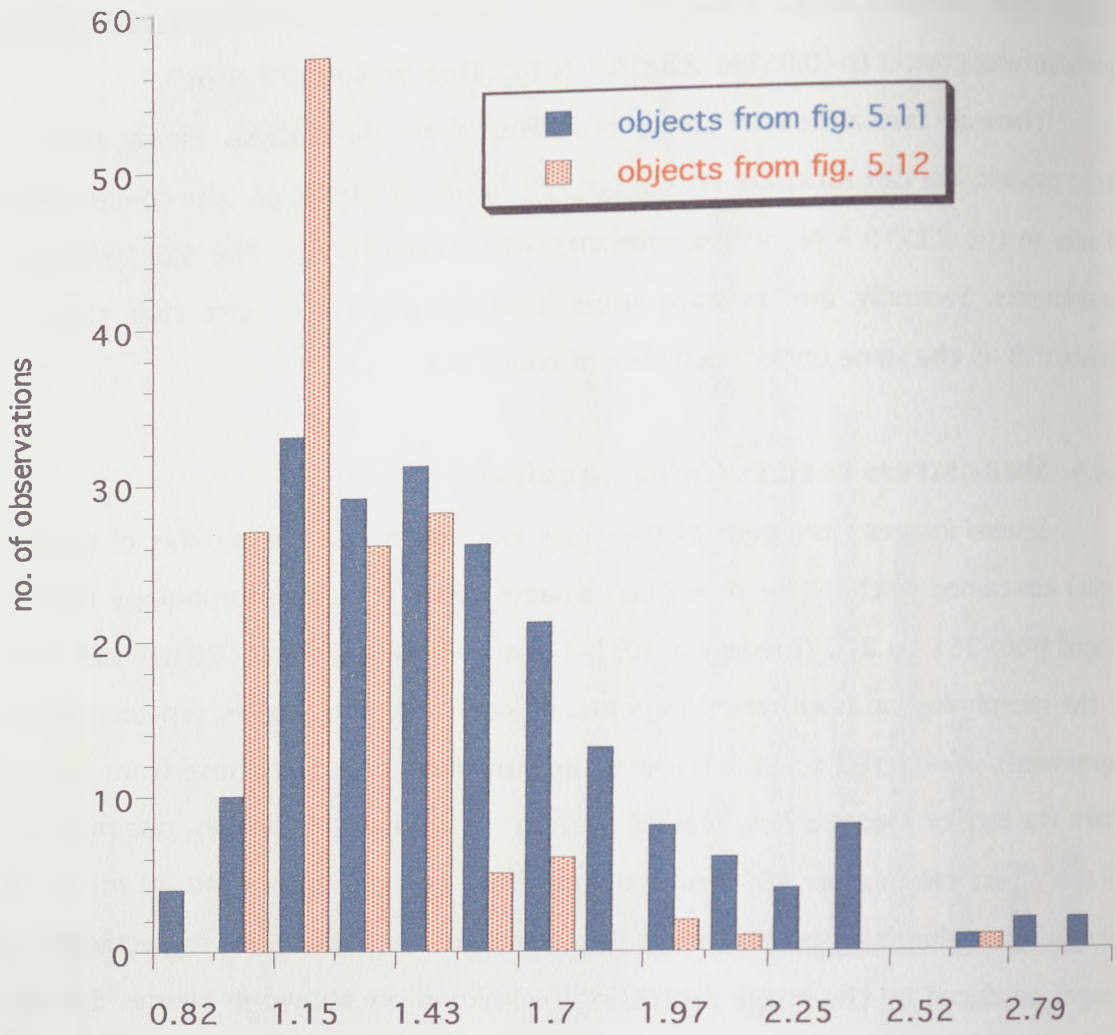
There are two additional points of interest from this analysis. Firstly, from the fitted models, we can estimate that after 192 hours of operation, the concentration of urea in the $2.8x10^{-3}$ N m^{-2} experiments will exceed that in the $5.6x10^{-3}$ N m^{-2} experiments. Secondly, and perhaps more interestingly, is the fact that the time constant B is the same under both sets of conditions.

5.4.4. Shear stress results *(image analysis)*

Several images from each of the tests were obtained. The number of particles (cells) contained within the processed images used for the morphology analyses ranged from 151 to 223 (median = 191). There were no significant differences in any of the morphological parameters between objects (cells) in images captured during experiments conducted at 20 ml min^{-1} medium flow rate and those from tests in which the rate of medium flow was 40 ml min^{-1}. Two example images, one from a 20 ml min^{-1} test taken after 100 hours of operation and one from a 40 ml min^{-1} test taken after 76 hours of operation are given in Figures 5.9 and 5.10 respectively. The images produced by the image processing techniques are shown in Figures 5.11 and 5.12. The results of the comparative morphological analyses for these 2 images are given in Figures 5.13 (basic statistics), 5.14 (compactness factor), 5.15 (Heywood circularity factor) and 5.16 (elongation factor).

5.5. Discussion

The primary rat hepatocytes attached to, and spread on, the coated



Histogram of Heywood circularity factor for objects from Figures 5.11 and 5.12

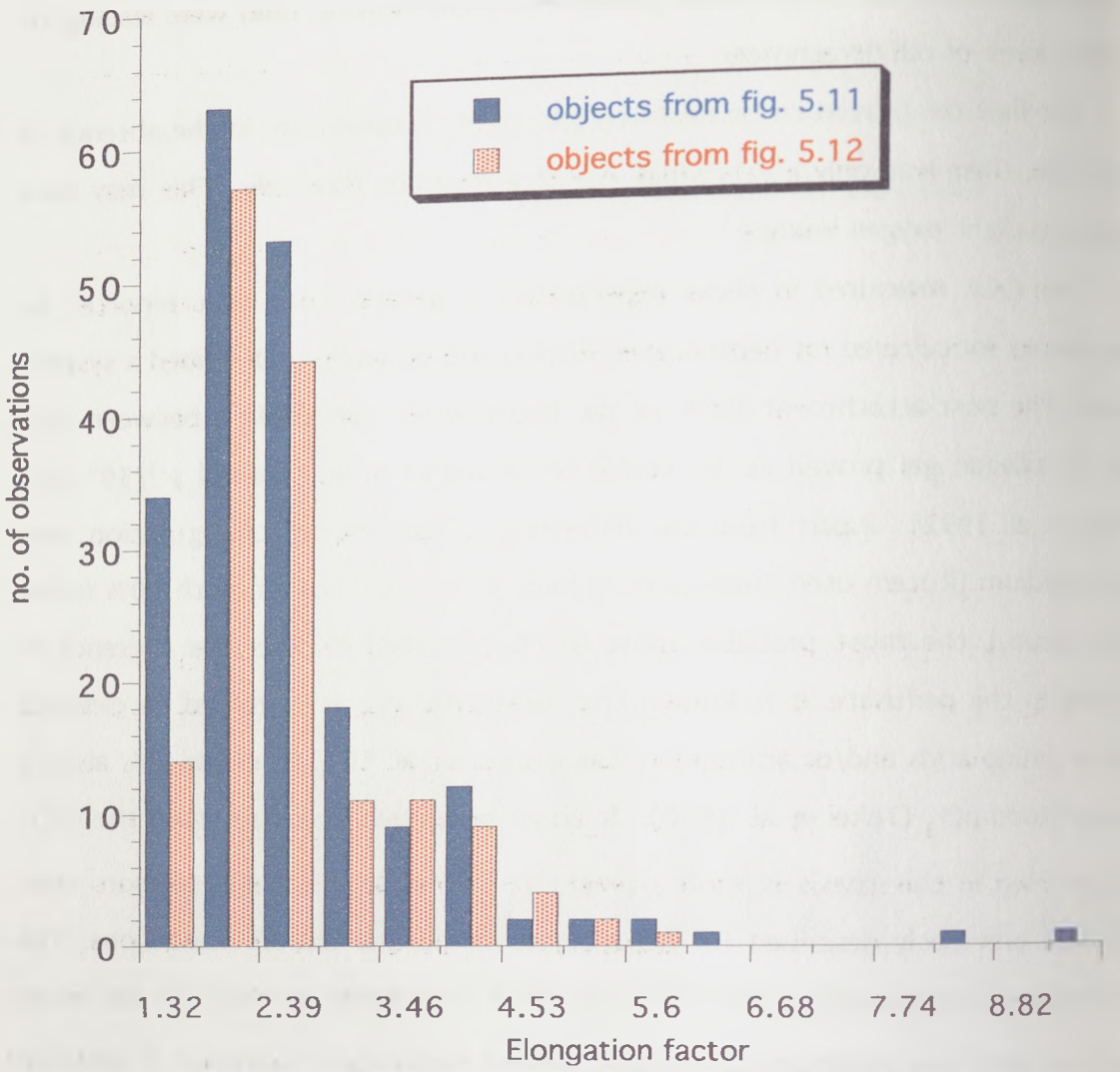
Figure 5.15

substratum and thrived under the perfused conditions in the flow cell. This is evidenced by the normal hepatocyte morphology seen in Figures 5.9 and 5.10 and by the continuous production of urea and consumption of oxygen recorded throughout the duration of all tests. The rates of attachment were consistently high and there was no evidence that the shear forces generated by the flowing fluid were leading to significant levels of cell detachment.

The flow cell proved to be relatively gas tight in operation. In the absence of hepatocytes, there was only a very small $\Delta pO2$ across the flow cell. This may have been due to slight oxygen leakage.

The OCR measured in these experiments is greater than that reported by other workers for cultured rat hepatocytes. Rotem and co-workers described a system in which the post-attachment OCR of rat hepatocytes sandwiched between two layers of collagen gel proved to be stable for 2 weeks at 0.27 nmol $s^{-1}/10^6$ cells (Rotem et al. 1992). Apart from the difference in substratum configuration, and culture medium (Rotem used Dulbecco's Minimal Essential Medium with 10% foetal bovine serum), the most probable cause of the elevated OCR is the presence of ammonia in the perfusate. It is known that urea synthesis is increased by elevated levels of amino acids and/or ammonium salts (Lutz et al. 1978) and possibly also by elevated blood pO_2 (Takei et al. 1990). It could be argued therefore, that the OCR data obtained in this thesis is more relevant to hybrid artificial liver support than OCR data previously described for hepatocytes in normal culture conditions. The hydrodynamic environment and the toxin load (simulated by NH_4Cl) are more consistent with the conditions anticipated when a hepatocyte bioreactor is perfused with blood or plasma from liver failure patients.

Because of the nature of operation of the flow cell, the OCR data were obtained for medium pO_2 ranging from 12-14 kPa. Noll and De Groot have reported that the O_2 consumption behaviour of rat hepatocytes in suspension culture approximates to Michaelis-Menten kinetics, with a plateau OCR (zero order kinetics)



Histogram of elongation factor for objects from Figures 5.11 and 5.12

Figure 5.16

occuring when the bulk pO_2 is greater than approximately 0.3-1.3 kPa.

The almost 10 fold difference in OCR between perfused primary rat hepatocyte monolayers, as reported here, and microcarrier attached HepG2 cells (Smirthwaite 1994) (0.59 and 0.07 nmol $s^{-1}/10^6$ cells respectively) further strengthens the argument that such transformed hepatocyte cell lines are not appropriate for use in hybrid artificial liver support systems where a large detoxification capacity is a prerequisite.

The analysis of the effect of shear stress on the rate of urea synthesis of rat hepatocytes cultured in the flow cell produced interesting results. The higher initial rate of urea synthesis recorded in experiments with the larger shear stress component might be explained in terms of fluid dynamics as a consequence of reduced boundary layer resistance. In other words, the supply of substrate (NH_4^+) to, and the removal of product (urea) from the hepatocytes has an increased convective element, and a decreased dependency upon diffusion, at higher shear rates. By extension, reactions can occur more frequently

However, the subsequent urea synthetic behaviour is more difficult to explain. One obvious explanation for the initial transitional phase in which the rate of urea synthesis declines with time would be that during this period a subpopulation of cells, which were sub-lethally damaged during or following isolation, dies off. At the end of this phase, a stable population remains and urea is synthesised at a constant rate. However, on the basis of the fitted models, such a remaining stable population would only constitute a tiny fraction of the initial population. The fact that those cells exposed to the lower shear stresses ultimately synthesise urea at a greater rate than the cells exposed to the higher level of shear might in principle be rationalised by attributing a time dependent cell damaging effect, which is proportional to stress magnitude, to the shear forces. On the other hand, one might argue that, while there were significant differences in the ultimate rates of urea synthesis between cells exposed to 2.8×10^{-3} N m^{-2} and those exposed to 5.6×10^{-3} N m^{-2}, that the differences

are in fact small and may be due to experimental artefacts. In the absence of a physical or physiological explanation for the initial transient urea synthetic behaviour, it is, unfortunately, not possible to discuss the interesting situation of both model fits having the same time constant.

It might be expected that any damaging effects caused by the shear stresses would result in observable changes in hepatocyte morphology. However, no such changes were seen in the image analysis study. In fact, hepatocytes cultured in the shear stress environment exhibited normal morphology after 100 hours with 2.8×10^{-3} N m^{-2} (Figure 5.9) and 76 hours with 5.6×10^{-3} N m^{-2} (Figure 5.10). Furthermore, there were no significant differences in any of the morphological parameters between hepatocytes exposed to 2.8×10^{-3} N m^{-2} and those cultured under a shear stress of 5.6×10^{-3} N m^{-2}. Whether observable morphological alterations would appear at longer times or when hepatocytes are exposed to greater levels of shear stress is open to debate. However, it would seem that for those levels of shear stress investigated, serious detrimental effects on the cultured hepatocytes are absent for several days.

The general design proposition of a stacked plate bioreactor based upon hepatocyte monolayers in direct contact with a plasma perfusate appears to be feasible. The assertion from chapter 3, that large scale hepatocyte bioreactors for hybrid liver support systems require an integral gas supply to satisfy the oxygen demands of the cells has been strengthened by the measurement of higher hepatocyte oxygen consumption rates than those previously published.

In the next chapter, the design, fabrication and initial in vitro testing of such a prototype hepatocyte bioreactor is discussed.

Chapter 6

Design, fabrication and initial *in vitro* testing of a hepatocyte bioreactor with integral membrane oxygenation

6.1. Introduction

A short review of some of the relevant findings from the preceding chapters is appropriate at this point. This will serve to introduce the concepts behind the bioreactor design.

In Chapter 1, it was argued that the specific requirements of a device for the treatment of acute liver failure have yet to be determined. For example, the cell mass and duration of therapy likely to be required are unknown. The uncertainty regarding these two parameters suggests that modularity and robustness should be two important design features. A modular design would enable the device to be scaled to accommodate the necessary cell mass. Robustness, both in terms of materials and reliability during operation should also be considered as a design priority since protracted sessions of therapy may be required. Chapter 1 also illustrated the multifactorial nature of liver failure and the associated uncertainty about which particular aspects of liver function need to be replaced for the reversal of advanced hepatic encephalopathy. This presents particular problems for those groups attempting to develop bioreactor designs for liver support systems. The relative importance of individual hepatic functional markers is unknown, so developers are

unsure what to measure and how to optimise the design. However, one could argue that the kinetics of liver failure suggest that a high capacity for removal of toxic compounds and metabolism of other molecules is likely to be of greater importance than synthetic function. Therefore, a design which performs well in clinically relevant hepatic clearance and metabolic tests should be sought.

The role of the architecture of the hepatic acinus in facilitating liver function was described in chapter 2. Here, the importance of certain physical parameters was emphasised. The relatively constant flow of blood along the sinusoids, the large oxygen carrying capacity of blood and the very short diffusion distances between sinusoidal blood and the hepatocytes ensures efficient mass transport and metabolism in the liver. This is enhanced by specialised transport phenomena such as cell seiving, endothelial massage and carrier mediated transport. It is obvious therefore, that efficient mass transfer will be of great importance in a hybrid artificial liver device. It is surprising that most groups have given little or no consideration to this aspect of design.

Direct contact between hepatocytes and the perfusate, ideally in a thin plate configuration akin to that in the acinus, will produce the most efficient exchange of solutes. The interposition of synthetic membranes between the perfusate and hepatocytes creates transfer resistances in diffusion based systems and leads to membrane fouling in designs where a large transmembrane convective perfusate flux is used.

In chapter 2, the case for well controlled dissolved oxygen conditions was also argued. Maintenance of physiological cell surface pO_2 may prove to be vital and the development of controlled oxygen gradients (as found in the acinus) might also be advantageous. This latter argument was recently advanced in a review of hepatocyte heterogeneity (Bhatia et al. 1996). A final suggestion in chapter 2 was that provision of two discrete fluid paths on opposing sides of hepatocyte monolayers, might improve 'liver function' (as defined in chapter 2) in bioreactor designs.

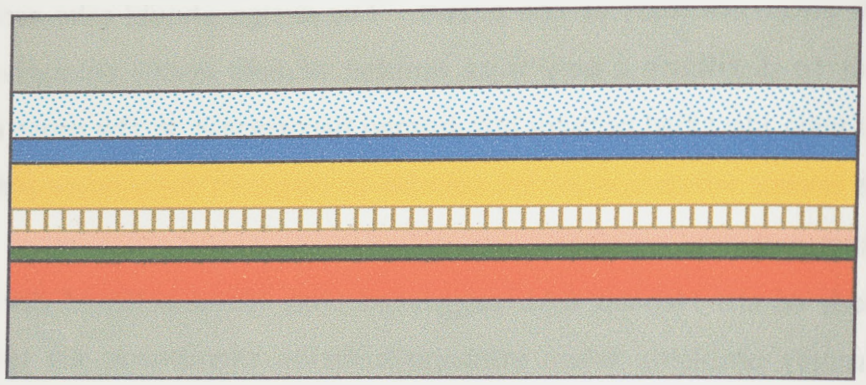
The design case studies undertaken in chapter 3 produced some valuable

information. At this time, it would seem that workers must rely upon primary hepatocyte cultures to provide 'liver function' in hybrid artificial liver devices since no appropriate cell lines are presently available. Consequently, devices would require to be seeded with the entire cell mass at the outset. The design should take account of this requirement to distribute a very large number of cells evenly throughout the device. While having identified primary non-human hepatocytes as the most practical cells, it remains unclear which species are suitable in terms of hepatocyte phenotype and potential immunological problems.

The study of the Hepatix BAL suggested that blood perfused devices can have oxygen supply problems when immuno-isolation membranes are used. The analysis of the Cedars-Sinai device proved that plasma perfused devices must have integral oxygenation since despite plasma equilibration with pure oxygen and ideal transfer conditions, massive oxygen debt occurs. Furthermore, study of Gerlach's novel device suggested that even with parallel oxygenation, careful design is necessary since gas diffusion distances must be kept short and multiple layers of hepatocytes must be avoided.

The investigation of hepatocyte species, culture medium formulations, functional parameters and culture configurations undertaken in chapter 4 indicated that:

- Different species of hepatocytes may favour different culture conditions.
- The composition of the culture medium can influence the results of function tests.
- Typical static culture configurations approach being oxygen limited for hepatocytes when medium depth exceeds 1 mm.
- The benefits of complex ECM configurations, such as collagen gel sandwiches, might not be significant in bioreactors for liver support where the improvement in some functional markers could be offset by reduced mass transfer efficiency.
- The physical nature of the hepatocyte support substratum may not be important



Block plastic

Gas phase

Hydrophobic gas transfer membrane

Perfusate (e.g. plasma)

Hepatocyte monolayer

Collagen coating layer

Cell support membrane

Second perfusate stream

Generalised schematic of proposed bioreactor design

Figure 6.1.

provided that it can be coated with collagen.

These findings help to identify a design development path which may be followed with some confidence. A design based upon hepatocyte monolayers with integral membrane oxygenation appears to be necessary. Complex ECM configuations might prove to be unnecessary but a design which could accommodate collagen gels or sandwiches at some later date should be considered. The reactor materials may be selected (providing they fulfill general biocompatibility requirements) for appropriate transfer characteristics, mechanical properties and sterilisation performance since they may be rendered suitable for hepatocyte attachment by collagen coating.

Finally, the experimental data generated in chapter 5 provided some information needed to finalise the general design of the prototype reactor. The analysis of oxygen consumption emphasised the need for integral oxygenation and the assessment of the effects of shear stress on hepatocytes indicated that, from a mechanical and functional point of view, a device based upon direct contact of hepatocyte monolayers with perfusate is feasible. Having said this, it should be borne in mind that an immuno-isolation membrane might, unfortunately, prove to be necessary. Therefore, the argument for a device with two perfusate paths is strengthened since, if the hepatocytes must be located on the other side of a synthetic membrane from the plasma of the patient, a perfusate compartment becomes essential, if only to provide a route for oxygen transfer in the cell compartment.

6.2. Proposed reactor design

A schematic of the generalised design envisaged for the reactor is given in Figure 6.1. It can be seen from this figure that the proposed design has certain facets in common with the geometry of the hepatic acinus as depicted in Figure 2.2. In principle, such a design is scalable by stacking identical elements and can be manufactured to give appropriate fluid channel geometries (as identified in Chapter 5)

and diffusion distances (as defined in Chapter 3). The cells are configured as monolayers giving the best possible mass transfer characteristics and while the preferred direct contact between hepatocytes and perfusate is offered, the provision of the second perfusate compartment allows the cell support membrane to be used for immunoisolation, without compromising gas exchange, by simply swapping the 2 perfusate streams.

6.3. Design and fabrication of prototype reactors

The design illustrated in Figure 6.1 could be produced from a similar plate and frame arrangement to that used for the flow cell described in Chapter 5. Three distinct compartments must be provided by incorporating two membrane systems. These requirements could be met by clamping the cell support membrane between two machined plates. The base plate would incorporate the second perfusate stream and a means of supporting the cell attachment membrane. The upper plate would incorporate the main perfusate channel and the gas exchange system.

6.3.1. Selection of materials

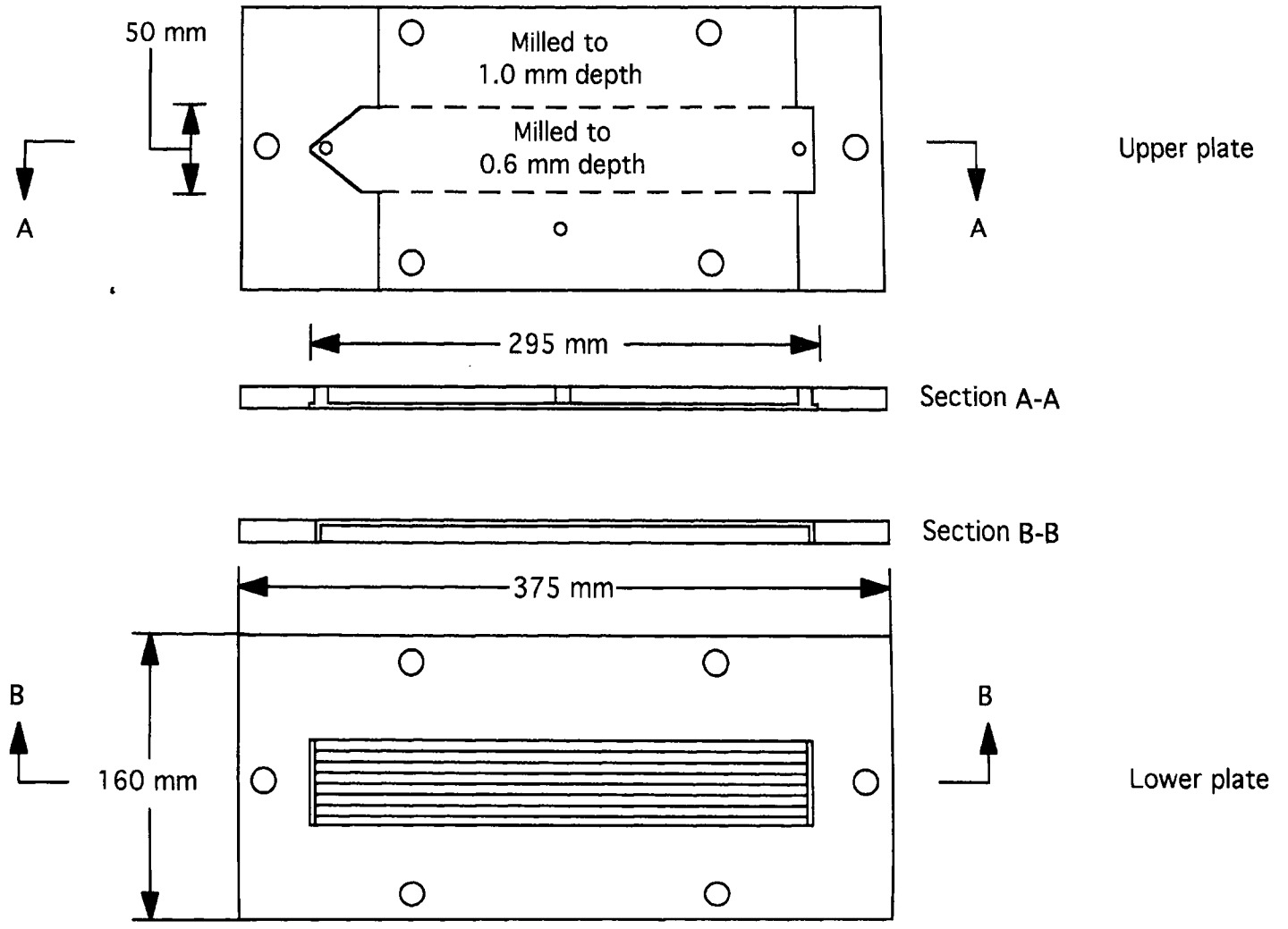
The material for the base and upper plates should have good machining properties and sterilisation performance, resist bending and distortion under mechanical and temperature stresses, be reasonably optically clear and should bond well to other materials where necessary. In these respects, polycarbonate would seem to be an ideal material. It is expensive though, so plates ought to be recyclable.

The possibilities for the cell support membrane are vast. It is beyond the scope of this thesis to investigate the suitability of different materials for inclusion in the reactor as a cell support or immunoisolation membrane. Such an investigation would be worthwhile. However, since it has been argued in this thesis that, at present, we are unsure about what transport properties such a membrane should possess and, furthermore, that for the initial *in vitro* evaluation of the reactor, immunoisolation will be unnecessary (since culture medium will normally be the

perfusate), the collagen coated hydrophobic polypropylene membrane (Celgard 2400) which was utilised as part of the work detailed in Chapter 5 (though not used for the experiments reported there) could be used as the cell support since it had already been established that primary rat hepatocytes attach to and remain functional on this material.

The selection of an appropriate gas transfer membrane requires that some rather more serious technical problems are considered. In the clinical setting, hydrophobic membranes are widely used in membrane blood oxygenators for cardiopulmonary by-pass and for acute respiratory failure. The most commonly used membrane materials in these devices are hydrophobic microporous membranes (typically polypropylene) or homogeneous silicone rubber membranes. Unfortunately, both of these materials suffer from limitations which would be likely to compromise the performance of the bioreactor.

Use of silicone rubber membranes would be likely to lead to oxygen transfer being limited due to its relatively low oxygen permeability. In any case, such membranes are not typically found in a size and form suitable for inclusion in the design. On the other hand, microporous hydrophobic polypropylene membranes have (in theory) virtually infinite oxygen permeability, are available in sheet and hollow fibre form with sufficiently small wall thicknesses to make them appropriate for use in the bioreactor from both a manufacturing and oxygen transfer perspective. However, the phenomenon of plasma breakthrough which can occur in prolonged clinical application of microporous membrane oxygenators must be considered. Plasma leakage rapidly compromises O_2 and CO_2 transfer leading to device replacement. It would be disastrous in a hepatocyte bioreactor for the therapy of liver failure. Plasma breakthrough is thought to occur as a result of the hydrophobic pores being rendered more hydrophilic or 'wetted' by the gradual deposition of plasma lipoproteins around the pores. In practise, this effect limits the application of microporous membrane oxygenators to hours rather than days. An alternative gas transfer membrane must therefore be sought for inclusion in the bioreactor.



Drawings of bioreactor plates
Figure 6.2.

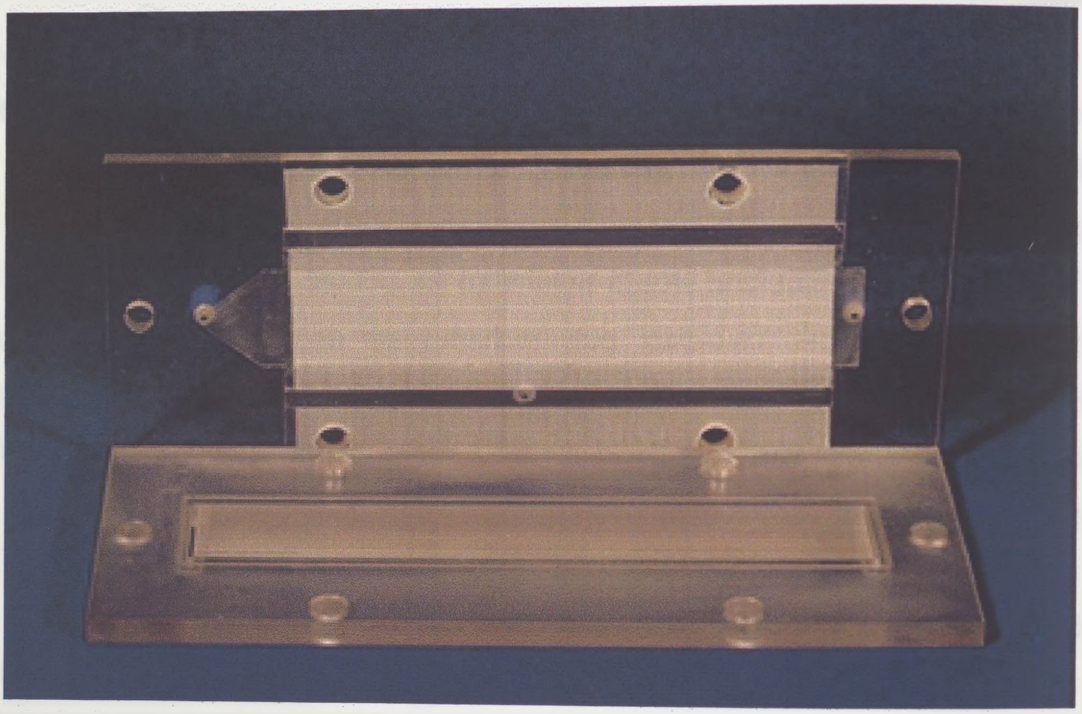
An exhaustive literature search and contacts with membrane manufacturers worldwide revealed that while several research groups and companies are attempting to fill the rather obvious gap in the market for a non-wetting, very thin-walled, highly gas permeable membrane, only one such membrane was available. Manufactured in Japan by Mitsubishi Rayon Co., Tokyo, the membrane (MHF) is a trilaminate hollow fibre (outer diameter 280 μm) with a wall thickness of approximately 20 μm. It consists of two layers of microporous polyethylene each 10μm thick, between which is sandwiched a contiguous polyurethane layer approximately 1 μm in thickness. It is this thin contiguous layer which renders the membrane resistant to pore wetting effects.

While the MHF membrane is, to the best knowledge of the author, currently clinically unproven, it potentially offers design advantages and is produced in hollow fibre woven mats of a suitable geometry. It was decided to incorporate it into the design.

The MHF membrane would require to be potted into the upper plate. In most medical devices, two-part polyurethanes are used as the potting agent. However, one can experience difficulties when trying to cure these materials in very thin sections. It was therefore decided that a room temperature vulcanising silicone compound would be used for the potting.

6.3.2. Manufacture of reactor plates

Figures 6.2, 6.3 and 6.4 illustrate respectively; drawings of the reactor plates, photographs of the finished reactor plates and a photograph of an assembled bioreactor. The plates were fabricated from 12 mm thick polycarbonate sheet. A grooved channel (1 mm deep), with inlet and outlet troughs (full plate thickness) at each end, was machined into the lower plate to act as both the second fluid channel and a support structure for the cell attachment membrane. A narrow groove was machined around the grooved channel to accommodate a gasket for sealing the main fluid channel in the upper plate. Narrow rectangular grooves were machined in the



Bioreactor Plates
Figure 6.3

lower surface of this plate around the periphery of the entry and exit troughs to accommodate a gasket for sealing against the stainless steel base plate (described below). The upper plate had a 600 µm channel with a gradually diverging entry region machined into the lower surface to act as the main perfusate compartment. This plate surface was also machined to accommodate the MHF membranes and associated potting. The MHF membranes were potted such as to lie against the upper surface of the main perfusate channel and configured such that the fibres were normal to the direction of perfusate flow. On the outlet side, the potting was cut to allow the MHF fibres to vent to the atmosphere along the side of the plate. This arrangement minimises pressure in the hollow fibres since the fibre lengths are short (~50 mm) and there is no convergence of gas flow to a small port at the outlet. This in turn should prevent rupturing of the membranes and hence avoid gas bubbles entering the perfusate channel. The cross-flow geometry might result in better mixing of dissolved gas in the perfusate (though at the low shear rates envisaged, this effect would be small) but was selected principally to simplify the arrangement of the ports for the three compartments.

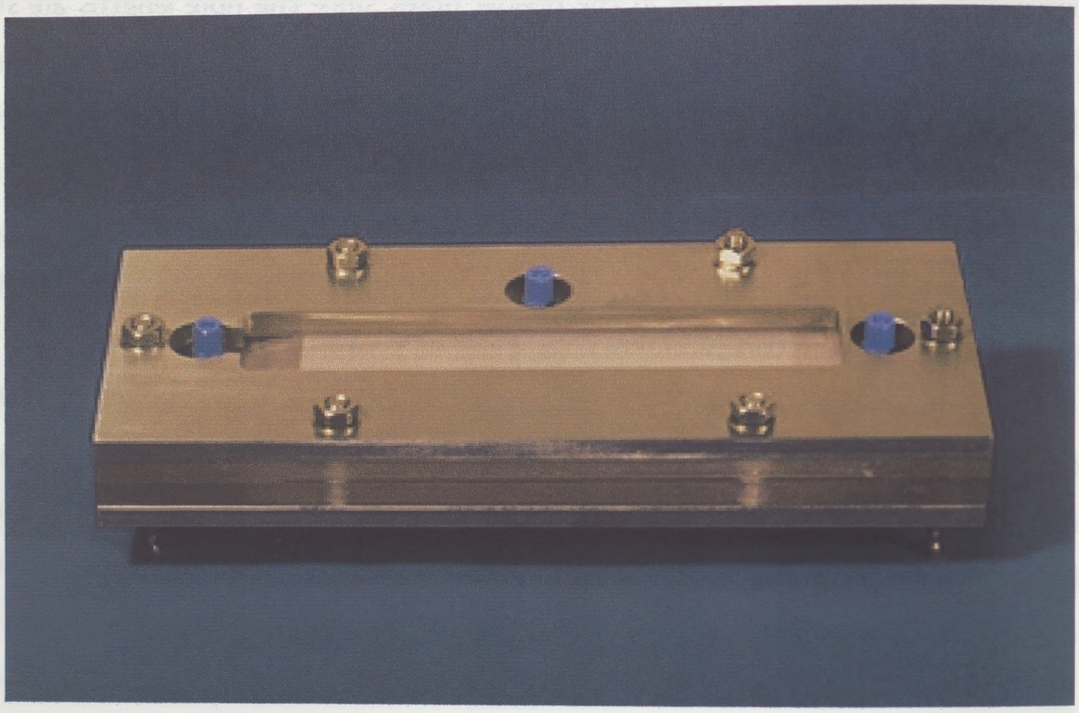
The ports were formed by machining tapped holes in the reactor plates (M10 thread). In the case of the second fluid channel, the ports were sited in the stainless steel base plate. Thread to hose barb polypropylene adapter fittings (Festo, UK) were inserted in the ports and short Marprene tube lengths were attached and finished with polypropylene Luer fittings (Altec, Alton, UK).

A stainless steel base plate with six threaded studs (M12 thread) distributed round the periphery was used to locate the reactor plates which were clamped together by means of a stainless steel top frame and tightening nuts.

6.4. Materials and methods

6.4.1. Bioreactor preparation

The bioreactor plates were washed and dried prior to assembly in order to



Assembled Bioreactor
Figure 6.4

remove any residues. The lower plate was located on the stainless steel base plate. A thin film of dilute silicone compound (1:10 dilution of Silicone Flowable Fluid (RS Components, UK) in hexane) was painted around the periphery of the grooved channel and allowed to partially dry. A pre-cut sheet of Celgard 2400 membrane (Hoescht Celanese, Germany) was then attached to the resultant tacky surface. This procedure was adopted as it gives a seal between the membrane and the grooved channel and it also enabled the membrane to be held in place while stretching out any bumps or wrinkles, resulting in an even surface. The membrane was then collagen coated according to the procedure described in Chapter 5, by means of a template which defined the membrane surface of the main perfusate channel. Thereafter, the upper plate was located and the whole assembly was clamped between the stainless steel plates. The torque on the tightening nuts was gradually increased and monitored until the main fluid compartment gave a complete seal against the external atmosphere when pressurised to 100 mmHg with a sphygmomanometer. At this point (generally at 4 to 8 Nm torque) the gas compartment was checked. (Since silicone potting is compressible, there was a possibility that excessive torque on the tightening nuts could lead to partial or complete occlusion of the MHF hollow fibres where the potting material abutted the channel sealing gasket of the base plate.) This was checked by closing the inlet and outlet ports of the fluid channels, connecting an air pump to the gas compartment inlet port and commencing air flow at low pressure (~100 mmHg) before submerging the whole reactor in a tank of water. Integrity of the gas system was confirmed by ensuring that bubbles were being emitted along the entire length of the gas outlet manifold. Once the reactor had passed these integrity tests, it was placed in a double layer of heat sealed sterilisation sheathing (Smith Brothers, Whitehaven, UK) and sterilised by an ethylene oxide process (4 hours at 60 °C followed by alternating pressure and vacuum air flushes). After sterilisation, reactors were usually stored for 48 to 72 hours prior to use. Residual ethylene oxide levels were expected to be sufficiently low at this stage.

6.4.2. Cell seeding of Bioreactor

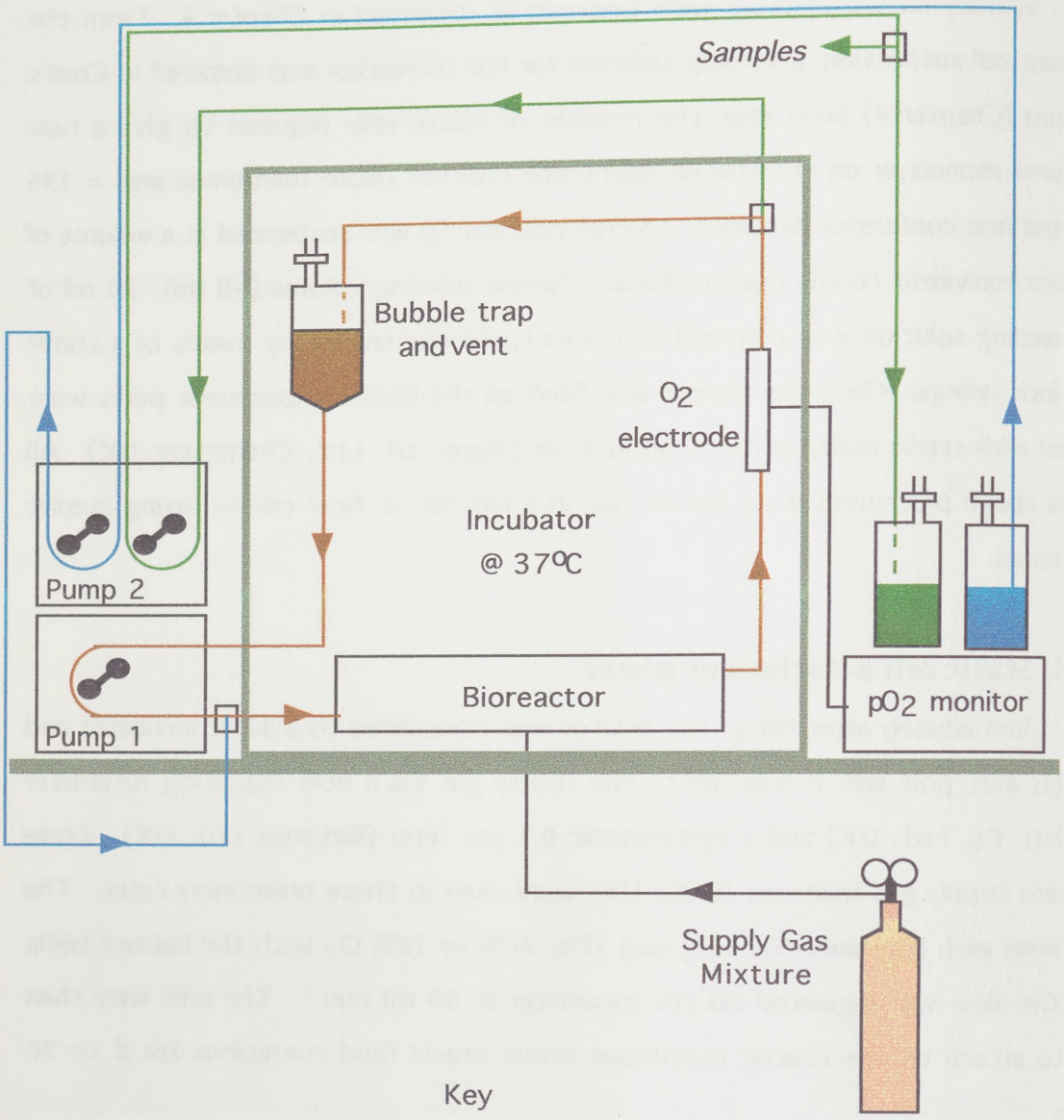
Primary rat hepatocytes were prepared as described in Chapter 4. From the resultant cell suspension, a seeding solution for the bioreactor was prepared in Chee's medium (Chapter 4) such that the number of viable cells required to give a near confluent monolayer on the coated membrane ($20x10^6$ (from membrane area = 135 cm^2 and near confluence density = $1.5x10^5$ cells cm^{-2})) was suspended in a volume of medium equivalent to the main perfusate channel priming volume (~9 ml). 20 ml of this seeding solution was prepared and used to fill the reactor by means of a sterile Luer lock syringe. Once the reactor was filled, all the fluid compartment ports were capped with sterile male Luer lock obdurators (Vygon UK Ltd., Cirencester, UK). All of the above procedures were carried out in a laminar air flow cabinet using aseptic techniques.

6.4.3. Static cell attachment phase

Immediately after filling, the reactor was transferred to a 37°C incubator and the gas inlet port was connected to the supply gas via a flow indicating rotameter (CP Inst. Co. Ltd., UK) and a hydrophobic 0.2 µm filter (Sartorius Ltd., UK). Three different supply gas mixtures (BOC, UK) were used in these preliminary tests. The gas mixes each contained 5% CO_2 and 12%, 20% or 28% O_2 with the balance being N_2. Gas flow was regulated via the rotameter at 60 ml min^{-1}. The cells were then left to attach to the coated membrane under static fluid conditions for 8 to 20 hours.

6.4.4. Reactor flushing and determination of cell retention

At the end of the static attachment phase, 40 ml of fresh Chee's medium was flushed through the reactor and the resultant wash out fluid was collected. The volume of wash out was determined and its cell concentration and viability were evaluated microscopically using the Trypan blue exclusion tests as described in Chapter 4. The extent of cell retention in the bioreactor was determined from the



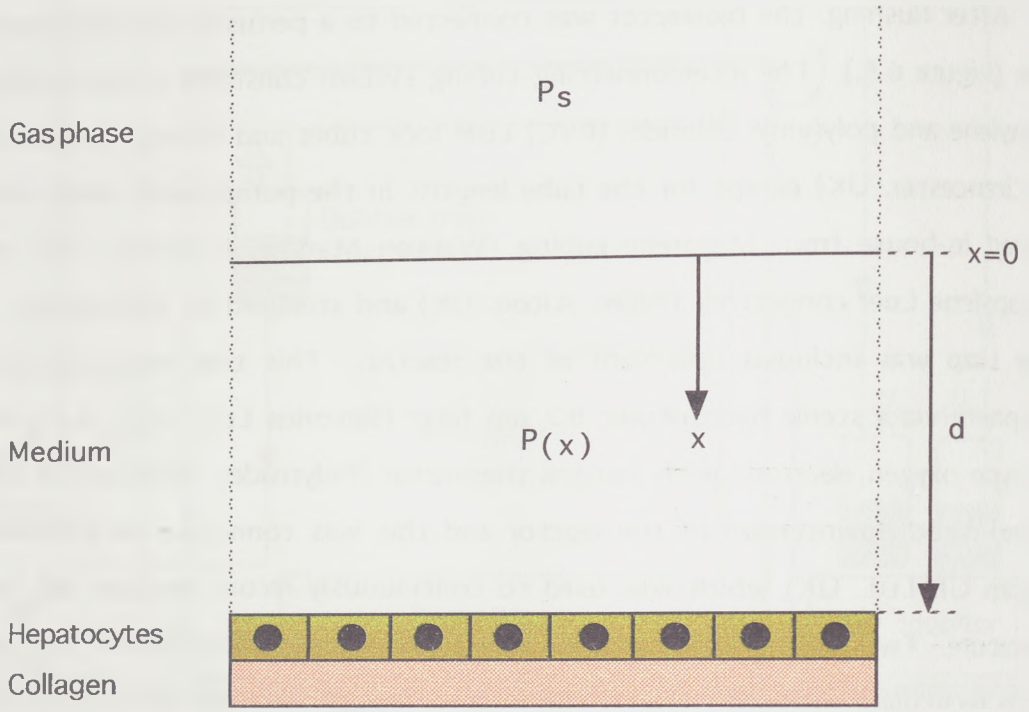
Bioreactor perfusion system

Figure 6.5.

total number of cells inoculated and the total number of cells flushed out.

6.4.5. Perfusion circuitry and monitoring equipment

After flushing, the bioreactor was connected to a perfusion and monitoring system (Figure 6.5.). The interconnecting tubing system consisted of pre-sterilised polyethylene and poly(vinyl chloride) (PVC) Luer lock tubes and fittings (Vygon UK Ltd., Cirencester, UK) except for the tube lengths in the pump heads which were produced in-house from Marprene tubing (Watson Marlow, Flamouth, UK) and polypropylene Luer connectors (Altec, Alton, UK) and sterilised by autoclaving. A bubble trap was included upstream of the reactor. This trap was vented to atmosphere via a sterile hydrophobic 0.2 µm filter (Sartorius Ltd., UK). An in-line Clark type oxygen electrode with integral thermistor (Polytrodes, Polystan GB Ltd., UK) was sited downstream of the reactor and this was connected to a monitor (Polystan GB Ltd., UK) which was used to continuously record medium pO_2 and temperature. Two peristaltic pumps were included in the perfusion system. One was used to recirculate medium through the reactor, oxygen electrode and bubble trap (subsequently referred to as the primary loop). The second was used to supply fresh medium (Chee's medium as described previously but containing 2mM NH_4Cl as an ammonium source for the urea cycle) to, and drain old medium from, the primary perfusion loop. The medium flow rate in the recirculation loop was 6 ml min^{-1}. The volume of the recirculation loop was 60 ml. The rate of fresh medium inflow and spent medium outflow was 2.5 ml $hour^{-1}$. Therefore, the volume of the primary recirculation loop was constant with a complete medium exchange being effected daily. The very large recirculation rate to medium exchange rate ratio meant that the medium in the primary recirculation loop could be considered to be well mixed. This is important for the calculation of reaction rates in the bioreactor. Sampling ports, capped with sterile hydrophilic 0.2 µm syringe filters (Sartotius Ltd., UK), were included in the primary recirculation loop (for removal of samples for blood gas analysis and contamination checks) and in the medium outflow line (for samples for liver



Predicts pO_2 at cell surface (x=d) as a function of:

- cell density
- gas phase pO_2
- medium depth

Attached cell fraction correlates with cell surface pO_2

Diffusion model for cell attachment (Yarmush *et al* 1992)

Figure 6.6.

liver function tests).

6.4.6. Prediction of cell surface pO_2 and correlation with cell attachment

A simple one-dimensional diffusion model for the calculation of cell surface pO_2 has been reported previously (Yarmush et al. 1992). This model describes a system where oxygen diffuses from an infinite gas reservoir of known pO_2 across a medium film of known thickness and oxygen permeability to a hepatocyte monolayer of known area density and rate of oxygen utilisation. It is therefore appropriate for application to the bioreactor design. The model is illustrated in Figure 6.6. Cell surface pO_2 (P_d) is calculated according to:

$$P_d = \frac{-\alpha + \sqrt{\alpha^2 + 4P_s k_m}}{2} \qquad \textit{(Equation 6.1)}$$

where:

$$\alpha = k_m + \frac{V_m \rho \delta}{D k} - P_s \qquad \textit{(Equation 6.2)}$$

and: P_s = gas phase pO_2, k_m = the medium pO_2 at which oxygen consumption is half maximal, V_m = maximal oxygen consumption rate, ρ = cell area density, δ = diffusion distance, D = diffusivity of oxygen in culture medium and k = solubility of oxygen in culture medium.

In our case, P_s is derived from the composition of the supply gas mixture in each experiment, k_m = 0.5 mmHg (De Groot Noll and Sies 1985), V_m = 0.63 nmol $s^{-1}/10^6$ cells (from Chapter 5 results), ρ is calculated from the data produced from the determination of cell retention, δ = 0.032 cm (from bioreactor geometry), D =

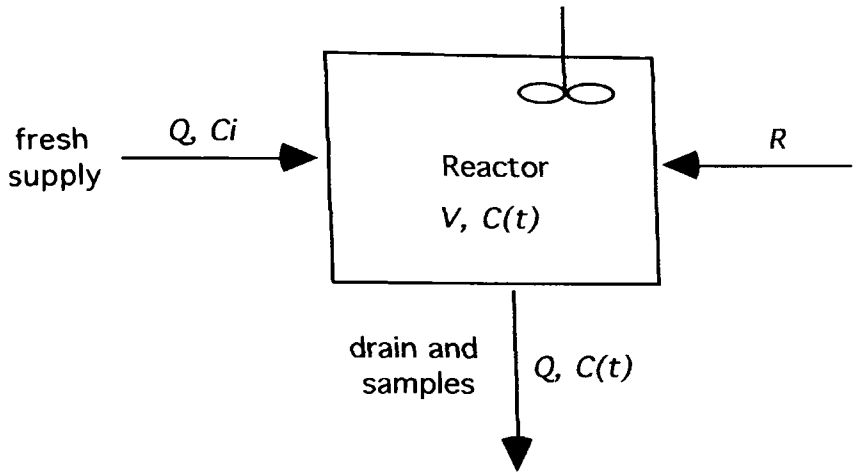
2.7×10^{-5} cm^2 s^{-1} (assumed to be equal to diffusivity of oxygen in water) and k = 1.25 nmol cm^{-3} $mmHg^{-1}$ (Battino 1981).

In a previous paper (Foy et al. 1993), this model was used to correlate the extent of attachment of rat hepatocytes to microcarriers with predicted cell surface pO_2.

The cell retention data from the bioreactor experiments were combined with the results from the prediction of cell surface pO_2 in order to investigate any relationship between hepatocyte surface oxygen tension and attachment of the cells to the coated membrane. For the purposes of this investigation, all of those cells retained by the bioreactor were assumed to be attached to, and evenly distributed on, the collagen coated Celgard membrane.

6.4.7. Determination of urea synthesis rates in bioreactor

The time averaged urea synthesis reaction rate (R) was derived by measuring the urea concentration in discrete samples obtained from the drain line. Quantification of the urea concentration in the samples was carried out using the same diagnostic kit (BUN-535, Sigma Chemical Co. Ltd., UK) and methods as described in Chapter 4. At any time (t) the concentration of urea (C) in the sample was assumed to be equal to that in the reactor. The reactor was assumed to be well mixed and the concentration of urea in the fresh medium supply was assumed to be zero. A diagramatic representation of this model system is given in Figure 6.7.



Model representation of urea synthesis in bioreactor

Figure 6.7.

From this model, a term for R between time points t_1 and t_2 can be obtained:

$$R = \frac{Q\,C_{t_2} - \beta\,Q\,C_{t_1}}{1-\beta} \qquad \textit{(Equation 6.3)}$$

where:

$$\beta = exp\frac{-Q\left(t_2 - t_1\right)}{V} \qquad \textit{(Equation 6.4)}$$

Cell batch	Initial viability (%)	No. cells retained	Retention (%)	Cell density cells cm^{-2}	Gas mix* (% O_2)	Predicted cell surface pO_2 (mmHg)	Duration (hours)
63	94	$24x10^6$	95	$1.8x10^5$	20	47.4	99
65	92	$22x10^6$	97	$1.6x10^5$	20	58.3	144
72	93	$20x10^6$	78	$1.5x10^5$	28	122.4	193
74	93	$21x10^6$	95	$1.6x10^5$	28	116.9	168
36	79	$27x10^6$	53	$2.0x10^5$	28	94.7	20
38	65	$31x10^6$	89	$2.3x10^5$	28	78.2	45
93	69	$30x10^6$	96	$2.2x10^5$	12	1.2	51

(* with 5% CO_2, balance N_2)

Summary of Reactor runs

Table 6.8.

and; Q = rate of supply of fresh medium (or removal of old medium), C_i = the concentration of urea in the fresh supply and V = the volume of the primary recirculation loop.

The data produced from this analysis were used to investigate the time course of urea synthesis throughout the bioreactor experiments as an index of the capacity of the bioreactor to maintain viable and functional hepatocytes over prolonged periods of operation.

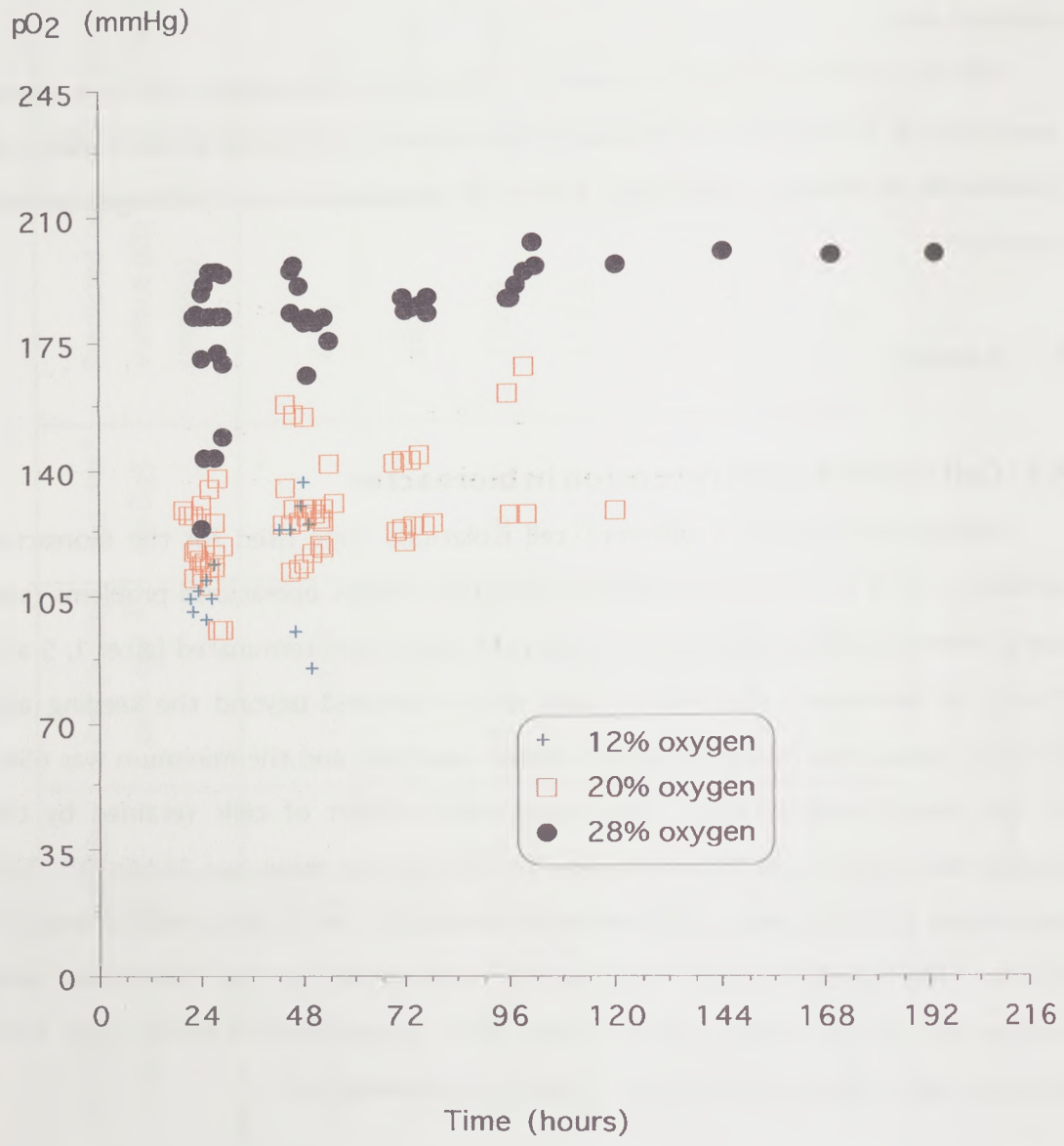
6.5. Results

6.5.1. Cell viability and retention in bioreactor

Hepatocytes from 15 different cell isolations were used for the bioreactor experiments. In 3 of the 15 experiments initiated, serious operational problems (seal failure or membrane damage) developed and the tests were terminated (after 1, 5 and 20 hours of operation). In the 12 runs which survived beyond the seeding and attachment phase, the maximum initial viability was 95% and the minimum was 65%, with the mean being 86±9%. The maximum number of cells retained by the bioreactor was $33x10^6$, the minimum was $12x10^6$ and the mean was $24±6x10^6$. This corresponded to a percentage cell retention range of 53% to 98% with a mean of 90±12%. The resultant area densities of hepatocytes on the membrane were therefore calculated to be $2.4x10^5$ cells cm^{-2} (maximum), $0.9x10^5$ cells cm^{-2} (minimum) and $1.8±0.5x10^5$ cells cm^{-2} (mean ± std. deviation).

6.5.2. Reactor operation and monitoring

In 5 of the remaining 12 runs, bacterial contamination of the culture medium was evident at the end of the experiment (this was evidenced by a significant reduction of perfusate pO_2 and by slight clouding of the culture medium) so the urea synthesis data from these tests were not used. The operational records of the 7 experiments from which urea synthesis data were obtained are shown in Table 6.8.



Perfusate pO_2 at bioreactor outlet

Figure 6.9

In these 7 runs, the perfusate pO_2 remained close to that of the supply gas mix throughout the duration of perfusion, indicating an absence of oxygen debt. Plots of perfusate pO_2 verus time for the runs are illustrated in Figure 6.9. Temperature was also stable at between 35.5 to 37.8 °C throughout each run and pH and pCO_2 were within the normal/expected range as determined by blood gas analyses (Coming 278) being 7.27 to 7.58 and 2.4 to 3.2 kPa respectively.

6.5.3. Correlation of cell attachment and cell surface pO_2

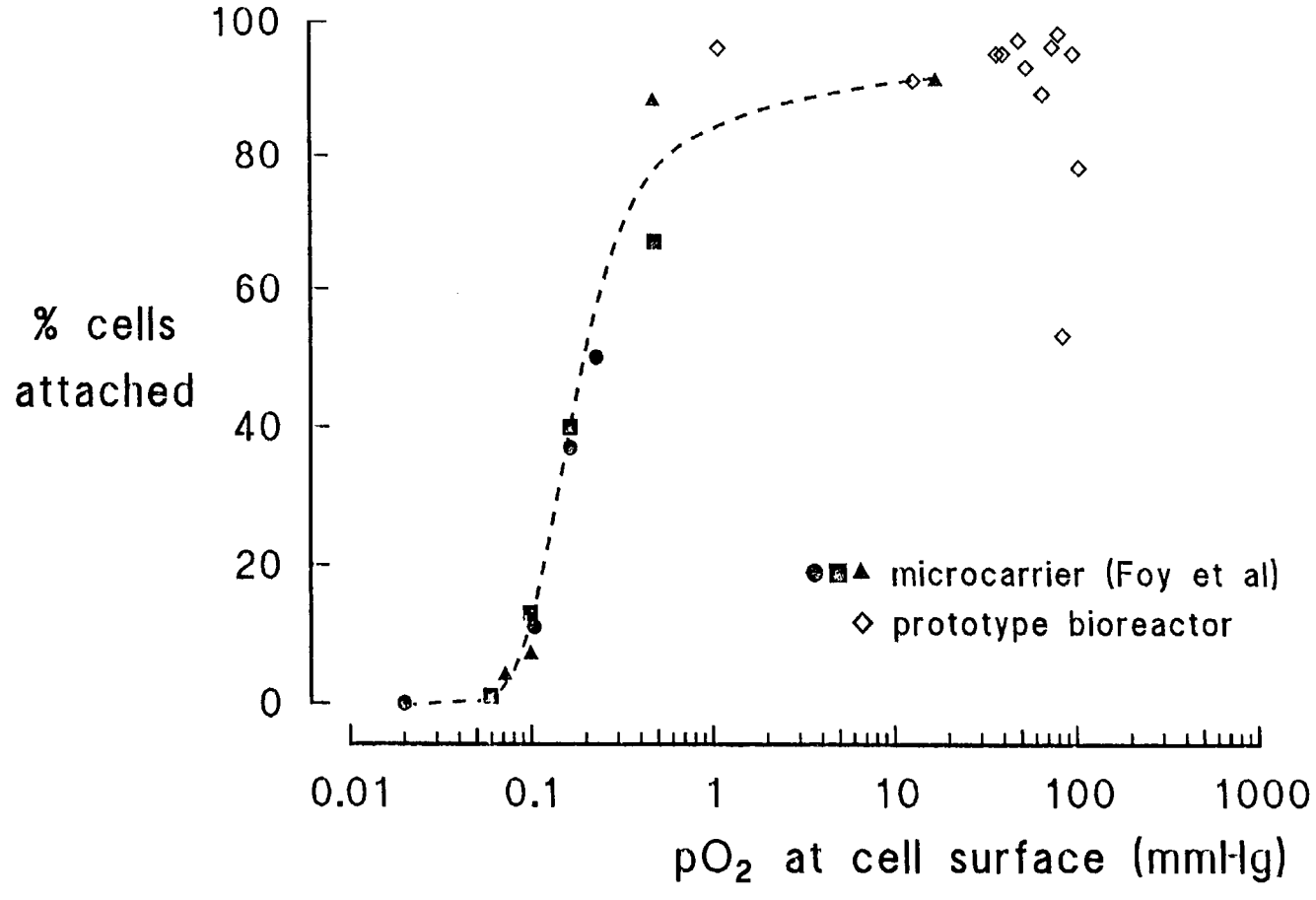
Cell surface pO_2 during the static attachment phase was predicted for each of the 12 runs identified in section 6.5.1. according to the supply gas pO_2 and cell area density for each experiment. These data were then plotted against the corresponding percentage cell attachment results (Figure 6.10.). For reference, data previously published (Foy et al. 1995) correlating the attachment of rat hepatocytes to microcarriers with predicted cell surface pO_2 are included.

6.5.4. Urea synthesis results

In each of the 7 experiments from which data were included, urea was continuously synthesised thoughout the duration of perfusion. The operating time (post static attachment phase) for these tests ranged from 20 to 193 hours with the median being 99 hours (mean = 103±62 hours). Of the seven tests included, 4 were run with a supply gas mixture containing 28% O_2, 2 with 20% O_2 and 1 with 12% O_2. Each experiment was characterised by an initially elevated urea synthesis rate which subsequently declined gradually. While no genuine stable base level of urea synthesis was reached, at longer times (i.e. greater than 100 hours post attachment) the rate of reduction of urea synthesis rate became very small. Data from 4 of these experiments (two 28% O_2 runs and both 20% O_2 runs) are depicted in Figure 6.11.

6.6. Discussion

A prototype hepatocyte bioreactor has been developed. It has advantageous



Effect of cell surface pO_2 on cell attachment

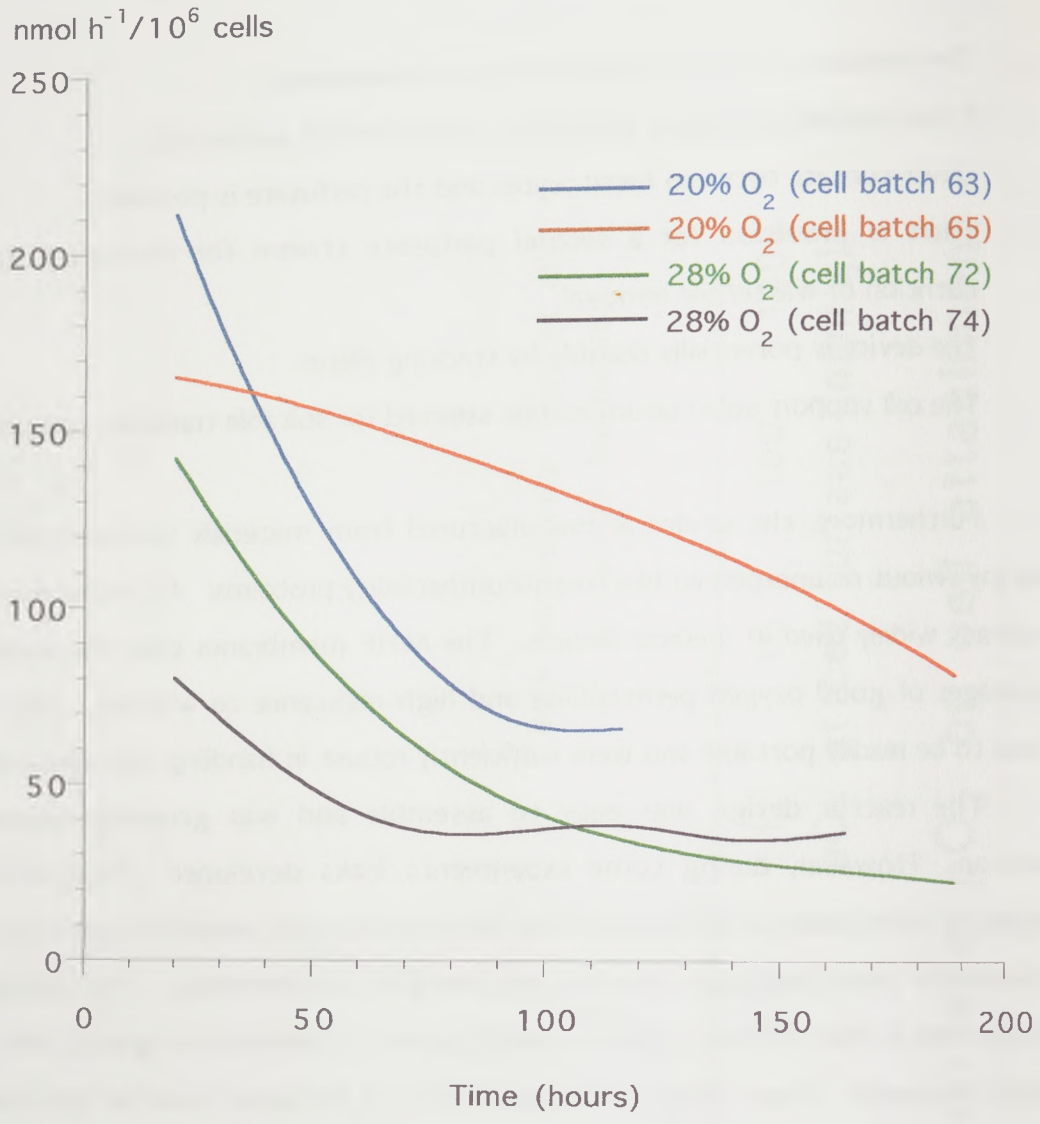
Figure 6.10.

design features, some of which are listed below:

- The bioreactor is based upon hepatocyte monolayers.
- It has integral gas supply permitting control of cell surface pO_2.
- Direct contact between hepatocytes and the perfusate is possible.
- There is provision for a second perfusate stream for immuno-isolation, nutrition or waste/bile removal.
- The device is potentially scalable by stacking plates.
- The cell support substratum can be selected for suitable transport properties.

Furthermore, the device is manufactured from materials which should not pose any serious or unexpected bio/haemocompatibility problems. All of the materials are already widely used in medical devices. The MHF membranes offer the particular advantages of good oxygen permeability and high resistance to wetting. They also proved to be readily pottable and were sufficiently robust in handling and operation.

The reactor design was easy to assemble and was generally reliable in operation. However, during some experiments leaks developed. Post mortem analyses of failed runs demonstrated that the problem was associated with the seal between the main perfusate channel and the gas compartment. The particular difficulty here is that the main channel sealing gasket is compressed against two very different materials. These being the polycarbonate of the upper plate at each end of the channel and the silicone rubber potting for the MHF membrane along each side of the channel. As the layer of silicone potting was cast by hand, it was difficult to guarantee that the surface was sufficiently even and flush with the polycarbonate surface at the ends of the channel. In some reactors this led to uneven compression of the main gasket and meant that the silicone potting was either over compressed, leading to distortion and possible leakage of medium or gas (depending on the pressure difference between the two compartments) between individual MHF fibres and the potting, or under compressed, giving rise to leakage of medium or gas



Urea synthesis during bioreactor runs

Figure 6.11

between the cell support membrane and the upper plate. Fortunately, in most cases, this defect was detected at the pre-sterilisation integrity testing stage. Plates which failed at this point could be quickly recycled by stripping out the MHF membranes and re-potting them. Only 3 reactors passed these tests and subsequently failed completely during operation. The reasons for these failures are unclear but it could be that pressure differentials larger than those applied during the integrity tests arose during operation.

Evidence for the adequacy of gas transfer in the bioreactor was provided in two ways. Firstly, the perfusate pO_2 downstream of the reactor was maintained at levels close to that of the supply gas throughout each experiment. Verification that increased oxygen demand could be detected in this manner was supplied in those runs which became contaminated with bacteria. These were characterised by exponentially decreasing perfusate pO_2. In fact, this parameter proved to be quite sensitive since small reductions in perfusate pO_2 could be reliably used to predict the appearance of detectable contamination. Secondly, the results of the cell attachment analyses provided confirmation that oxygen transfer was sufficient to meet the demands of the hepatocytes. Previous work has suggested that hepatocyte oxygen consumption is elevated during the attachment period (Foy et al. 1993). If the hepatocytes consumed oxygen during the static attachment phase at the rate measured in Chapter 5, they would completely deplete the perfusate in the reactor of dissolved O_2 in approximately 20 minutes. Therefore, integral oxygenation is obviously essential at this crucial stage. However, as the analyses in chapter 3 demonstrated, provision of parallel oxygenation does not, in itself, solve this problem. Rather, the gas transfer configuration must be carefully designed and in particular, large diffusion distances and multiple cell layers must be avoided. The oxygenation system in the prototype bioreactor was designed to overcome both of these difficulties. Oxygen is supplied via an array of hollow fibre membranes located approximately 320 μm from a single cell thick layer. In theory, this supply mode should be sufficient to satisfy oxygen demand in excess of that measured in Chapter 5 without recourse to supply gas

mixtures containing very high oxygen concentrations. It was encouraging to have this theory supported by the very high levels of cell attachment in the bioreactor. In fact, the level of cell retention proved to be independent of supply gas pO_2 in these experiments. This may be because for the three supply gas mixes used, the predicted cell surface pO_2 exceeded the k_m value in all experiments. The basis of this theory is that hepatocyte oxygen consumption is independent of cell surface pO_2 above a certain value. Previous reports have placed this value at between 0.7 and 10 mmHg (deGroot, Littauer, and Noll 1987; Lutz et al. 1978; Yarmush et al. 1992). It is important to note the assumptions made in the analyses correlating cell attachment with predicted cell surface pO_2. These were that all of those cells retained by the bioreactor after flushing are:

- viable
- attached to the collagen coated membrane
- evenly distributed over the attachment surface
- consuming oxygen at the same rate
- located at the same distance from the oxygen source

The assumption of viability can be considered to be fair on three counts. Firstly, the cell populations used were of a high initial viability. Second, most of those cells flushed out were dead or damaged suggesting that the net viability of those cells retained may have been enhanced and finally, the measured urea synthetic activity was consistent with such a viable cell population.

The second and third assumptions are more difficult to assess. Visual inspection of post-run disassembled reactors indicated an absence of cells everywhere other than the attachment surface and the MHF membranes (to which a small fraction of the cell mass appeared to be attached). At the macroscopic level, the distribution of the hepatocytes on the membrane appeared to be fairly even. However, one cannot determine anything from such an inspection about the

microscopic cell distribution. Microscopy analysis of the cell attachment surface would obviously be advantageous. In particular, a dual staining confocal laser scanning microscopy (CLSM) method would be appropriate. With such a technique, sections of the cell attachment membrane could be examined in an unfixed state with viable and non-viable hepatocytes being differentially stained. While this would be interesting and potentially useful for the assessment of the distribution and viability of hepatocytes on bioreactor membranes, the development of such a technique lies outwith the scope of this thesis. For the time being therefore, the validity of the second and third assumptions cannot be thoroughly tested.

The fourth and fifth assumptions are dependent on attainment of even cell distribution. If this is valid, we can have confidence in the assumptions of uniformity of oxygen consumption and diffusion distance. This is a consequence of the reactor design in which the channel dimensions and cross flow configuration minimise tangential and axial pO_2 gradients and the geometry of the MHF membrane array which minimises variations in the diffusion distance between different points on the cell attachment membrane.

It was also encouraging to note that, throughout the duration of perfusion in all experiments, there was no evidence of hepatocyte desquamation in the form of cells appearing in the perfusion loop where they could be expected to accumulate in the mesh filter in the bubble trap. This supported the data in Chapter 5 which suggested that hepatocytes were able to remain attached to a substratum and continue to function when exposed to fluid shear stresses. The levels of wall shear stress in the bioreactor were approximately $1x10^{-3}$ Nm^{-2} (i.e. less than, but of the same order of magnitude as, the stresses evaluated in Chapter 5).

The maintenance of urea synthetic function for up to 9 days of continuous operation and exposure to 2mM NH_4Cl was particularly significant. The author has been unable to find any previous reports of this hepatic function being maintained under such culture conditions for this length of time. In standard tissue culture (as described in Chapter 4) rat hepatocytes in Chee's medium demonstrate virtually no

urea synthetic activity at 7 days following a single unprecedented challenge with NH_4Cl. The continuous exposure of the hepatocytes to elevated levels of ammonium compounds is, arguably, more consistent with the conditions expected when a bioreactor is perfused with plasma from patients with liver failure. In this respect, these results represent a significant achievement. For perhaps the first time, a hepatocyte bioreactor may be expected to continue to function for prolonged periods under adverse conditions. Obviously further work requires to be carried out to determine whether other important functional markers are maintained. However, grounds for optimism exist. As described in Chapter 2, urea synthesis is a complex activity. A recent report which detailed an investigation of how the liver responds to different levels of anoxia contended that ammonia metabolism, particularly urea synthesis, was one of the functions which are "shut down" early in the liver in order that the limited oxygen resources are utilised for more vital reactions (Hochachka et al. 1996). If this were the case, it could be argued that maintenance of urea synthetic activity is an indication that the hepatocytes are in an "energy rich" state and are potentially capable of carrying out many other functions. Having said this, it is obvious that functions which are lost or irreversibly compromised are unlikely to be restored in any bioreactor system. Therefore, markers of other labile functions, most notably cytochrome P450 dependent metabolism, should be evaluated in the bioreactor. The findings from such investigations would help to identify the extent to which the bioreactor can preserve what remains of these fragile markers after cell isolation.

The initially elevated levels and the prolonged maintenance of urea synthesis and the apparent lack of serious toxicity arising from the ammonium load are probably a consequence of efficient mass transfer. As well as enhancing oxygen supply, the reactor geometry should reduce boundary layer resistances. This not only leads to increased reaction rates but also prevents the accumulation of solutes in the locality of the cells and should thus minimise any ammonium toxicity. The mass transfer efficiency derives from having a single cell thick layer of hepatocytes in direct contact

with the perfusate and the appreciable wall shear stresses which result from the perfusate flow.

The urea synthesis data also suggest a possible relationship between dissolved oxygen levels and urea synthesis rate at short times. However, further work would be required to test this theory.

In summary, the bioreactor developed in this thesis addresses some previously neglected design aspects and is a promising prototype for scale-up and further study. The overall conclusions from this thesis and some recommendations for further work are given in the following final chapter.

Chapter 7

Summary, suggestions for further work and opinion

7.1 The future for liver failure therapy

The requirement for a novel therapy for acute liver failure is undeniable. The future role of liver transplantation is unclear. At the same time as demand for transplants is increasing, the supply of donor organs is in decline (Hayes 1997 personal communication). It remains to be seen whether xenotransplantation of transgenic livers will become a routine therapy. Uncertainty exists as to the likelihood of cross-species transfer of so called 'infectious agents' occurring with this procedure. As long as such a potential hazard exists, this is unlikely to become a widespread therapy. Therefore, it is not surprising that the number of research groups working on novel methods of artificial liver support is increasing.

7.2 Parallel developments in artificial liver support

Since the work in this thesis began, several groups have entered the field. Improvements in sorbent technology have persuaded some workers to revitalize efforts towards development of wholly artificial support strategies. Some groups have endeavoured to combine adsorption processes and hepatocyte bioreactors in their systems. In the opinion of the author, it is yet to be demonstrated that any net benefit exists for such combined therapy when compared to purely hepatocyte based support regimes.

While this thesis has been in preparation, three groups have reported work on

hepatocyte based liver support systems and which merit particular mention here. Yarmush, Tompkins and co-workers in Boston, USA have published papers on the general topic of hepatic tissue engineering. One recent paper has considered in some detail, the role of local dissolved oxygen conditions in producing liver function as defined in Chapter 2 of this thesis (Bhatia et al. 1996). The findings in this paper support the argument developed in this thesis that the control of dissolved oxygen conditions at the cell surface is one of the major goals for the design of successful hepatocyte bioreactors.

Two further groups have identified this requirement and have set about designing their own devices. Chamuleau, Flendrig and colleagues in Amsterdam have reported a novel device based on a hollow fibre microporous membrane oxygenator (Flendrig 1997). Porcine hepatocytes are immobilized on a non-woven polyester mesh which is incorporated into the fibre winding. Bader and co-workers in Hannover, Germany have further developed their system which is based upon hepatocyte plates and incorporates integral oxygenation (Bader et al. 1997). Their device has a similar configuration to that proposed in this thesis.

The device described by Chamuleau and Flendrig is a simple and elegant design. However, it does not replicate the plate structure of the hepatic acinus and it may well prove to be prone to fail as a result of the pore wetting effect described in Chapter 6. Bader's device is more sophisticated in these respects since the acinar plate structure is preserved and the oxygenation membranes are coated with a collagen gel which may help to prevent plasma leakage.

It is to be hoped that these two developments together with that of Gerlach and the device reported in this thesis represent a movement away from the previously common practice of using inappropriate commercial filtration cartridges as hepatocyte bioreactors.

7.3 Theoretical and experimental findings

The discussion of liver structure and function in Chapter 2 of this thesis described the highly specialized geometry of the hepatic acinus and its axial heterogeneity in terms of structure, enzyme activity and local solute conditions. This 'design' could be considered to represent the optimal geometry for a hepatocyte bioreactor.

It was not too surprising therefore that the three commercial hepatocyte bioreactors which were analyzed in Chapter 3 were predicted to have serious mass transfer shortcomings. Not only are they likely to be inadequate in terms of oxygen transfer (and by extension, suboptimal in the transport of other low molecular weight solutes), but it is difficult to see how these devices can be expected to have any regulatory function at all.

While it seems unlikely that we will be able to reproduce the acinar architecture in an artificial environment, workers must endeavour to replicate the thin plate structure and attempt to provide appropriate oxygenation conditions and adequate cell nutrition. By satisfying these two requirements, one might expect a well designed device to regulate the concentration of some solutes. So far, this has not been demonstrated by any device. In the opinion of the author, this should be the manner in which devices are evaluated in the future. In other words, device performance should be quantified in terms of the magnitude and nature of load which can be input to the device whilst retaining desirable outlet conditions. In such circumstances, devices which have also been proven to be scalable could be tailored to suit predicted loading from the patient.

The device invented by Gerlach (also discussed in Chapter 3) is a novel attempt to replicate liver architecture. It is likely that there are regions in this device where hepatocytes benefit from appropriate dissolved oxygen conditions and solute gradients. However, the aggregated nature of the cell mass in this device will also result in areas which are potentially hypoxic and/or are poorly perfused. Until such

time as the interfibre distances in this device can be significantly reduced, it is likely to suffer from these drawbacks and consequently will be inefficient in terms of output per unit cell mass. Having said this, Gerlach's device is at present unique in terms of its operational lifespan, suggesting that it has significant clinical advantages over the commercial filtration cartridge based systems.

The experimental work described in Chapter 4 produced some interesting findings. Particular culture medium compositions may only be appropriate for the culture of hepatocytes from certain species. This may be significant in terms of comparing performance of different hepatocyte bioreactors. If the devices under consideration are seeded with cells from different sources, comparisons may be compromised by artefacts introduced by the culture medium formulation.

Chapter 4 also identified quantification of urea synthesis as a sensitive and convenient parameter for assessment of hepatocyte function. In many respects, it is an ideal parameter. Urea synthesis is a complex pathway which is at the core of liver nitrogen metabolism. Synthesis of urea demonstrates an intact urea cycle which comprises several enzyme systems located in different parts of the cell and therefore is also indicative of intact organelles (mitochondria) and intracellular transport systems. It is a convenient parameter to quantify since readily measurable concentrations of urea can be detected in the culture medium or perfusate. Furthermore, urea synthesis is a clinically significant function test since it can be correlated with ammonium clearance which is vital for the prevention of cerebral oedema.

The investigation of different culture configurations which concluded chapter 4 also gave food for thought. Workers should carefully consider the benefits of including complex extracellular matrices (ECM) in hepatocyte bioreactors. These will inevitably create transfer resistances and may give rise to manufacturing problems. When these drawbacks are considered, inclusion of, for example, collagen sandwiches (which barely increased measured function in this thesis) appears less advantageous.

In the opinion of the author, the restoration in bioreactors of the chiefly single

cell thick hepatocyte plate geometry found in the hepatic acinus will prove to be a more important criteria than the inclusion of extracellular matrices or what have come to be known (inappropriately?) as 3-D culture models.

In fact, some recent findings have suggested that the benefits of including ECM configurations in hepatocyte culture models may be species specific. Porcine hepatocytes have been reported to perform just as well in the absence of ECM when cultured in a prototype bioartificial liver (teVelde et al. 1995). Furthermore, scanning electron microscopy of porcine hepatocytes on different collagen coated and non-coated substrata demonstrated superior morphology in those cells cultured on uncoated materials (Ekevall 1997 unpublished data). The initial investigation of coated membranes as support substrata for rat hepatocytes in chapter 4 suggested that providing candidate materials fulfill general biocompatibility requirements, then they may be rendered suitable for rat hepatocyte culture by collagen coating.

Taken together, the findings described in the preceding paragraph suggest that researchers may be able to select membrane materials on the basis of their mechanical and transport properties, sterilization performance and suitability for reactor manufacturing processes.

Inspection of the literature reveals that there is, as yet, no consensus on critical parameters for hepatocyte bioreactor design. Several groups appear to be satisfied to take an inappropriate commercial product, fill it with an empirically derived number of cells, pump blood or plasma through it and expect it to function. Having said this, two particular aspects of design are beginning to be gain recognition as being very important. These are the provision of gas exchange (especially oxygen supply) and the mode of contact (or, indeed separation) of blood or plasma and the hepatocytes. These two parameters were investigated in chapter 5 of this thesis. Firstly, an investigation of the oxygen requirements of rat hepatocytes under load produced data which indicates that the rate at which rat hepatocytes consume oxygen when challenged with ammonia is higher than that previously published. This

finding strengthens the argument for parallel gas exchange provision within hepatocyte bioreactors.

The simplest of mathematical modelling identifies the requirement for parallel oxygen supply in plasma perfused bioreactors. Without this resource, cellular oxygen demand can only be met by imposing massive oxygen gradients on the device and/or by operating the system with ridiculously high plasma flow rates. To some extent, this is also true for blood perfused devices in which, most workers would agree, some form of immunoisolation is essential. In these circumstances, the oxygen transfer resistance imposed by the immunoisolation membrane may lead to hypoxic regions in the device when venous blood is supplied to the device as would normally be the case clinically. Furthermore, a study of liver physiology and architecture reveals the importance of controlled dissolved oxygen gradients within the hepatic acinus for the maintenance of liver function. It is quite alarming therefore, to find that several groups working in this field have either failed to identify oxygen supply as a critical design parameter or have chosen to disregard the shortcomings of their devices in this respect.

The work conducted in chapter 5 of this thesis also suggested that a bioreactor configuration based upon direct contact between patient plasma and hepatocytes may be possible from a mechanical and functional point of view. The author acknowledges that before this configuration can be accepted in a clinical liver support system, extensive immunological studies are necessary. However, the configuration should not be dismissed on the basis of what remain at present theoretical immunological problems. It offers significant mass transfer advantages and could potentially simplify reactor designs. Therefore, it was encouraging to discover that rat hepatocytes remained attached to a collagen coated substratum and continued to function when in direct contact with a culture medium perfusate. The shear stresses generated in these experiments did not adversely affect hepatocyte morphology or lead to cell desquamation. In fact, at short culture times, hepatic

function, measured by urea synthesis rate, was enhanced when the wall shear rate was increased. This is likely to be a result of reduced boundary layer resistances.

The findings from chapters 2 through 5 provided the basis for the prototype bioreactor design which was described and evaluated in chapter 6. This design proved to be reliable in manufacture and operation. Analysis of cell retention in the reactor and correlation with predicted cell surface pO_2, suggested that the design offers good cell attachment and distribution and efficient gas exchange. Furthermore, urea synthesis by rat hepatocytes in the bioreactor was maintained throughout prolonged periods of continuous ammonium loading. The rates of urea synthesis recorded were enhanced with respect to those obtained in the standard tissue culture experiments conducted in chapter 4 for comparable culture media and time points. The bioreactor design replicates the monolayer configuration of the hepatic acinus and the integrated oxygenator enables accurate control of cell surface pO_2. It is based upon direct contact between hepatocytes and perfusate. However, the design has provision for immunoisolation should it prove necessary.

In the opinion of the author, this device represents a significant development in hepatocyte bioreactor design. However, there is a great deal of work required to develop and scale-up the prototype and further studies are necessary for material selection, configuration optimization and operational refinement. Some suggestions for specific future research and development are given in the following section.

7.4 Future work

The most challenging goal for the prototype reactor is scale-up to a clinically applicable device. The most obvious route forward is to convert from the single plate ($20x10^6$ cells) prototype to a system of stacked plates. By increasing the usable surface area per plate, it is possible to envisage a 100 fold increase in cell mass whilst keeping the plates to a manageable number (approximately 20). However, $2x10^9$ cells might well prove to be insufficient for clinical application. Therefore, at some stage in

the future, the design may have to be re-thought. It is to be hoped that the core features of cell monolayers and integral oxygenation can be retained. The principal obstruction for scale-up of this prototype to a clinically useful size is likely to be the requirement to remove the bulk generated by the plastic plates in order to keep the device to a manageable size. Some emerging technologies such as microgrooved surfaces, membrane envelopes, ultrasonic welding and membrane potting might be able to be harnessed for this purpose.

In terms of general design development there is one obvious refinement which could be made. By switching the orientation of the gas hollow fibres to be parallel with, rather than normal to, the main perfusate channel, the potting arrangements could be simplified, there would be no need to compress the gas compartment potting to seal the main liquid channel and cell distribution might be improved since the cells would be distributed along the fibre axes rather than across the fibres. With this geometry, it might also be possible to allow axial pO_2 gradients to be developed along the direction of perfusate flow as found in the hepatic acinus.

Another area worthy of further study is the interaction between hepatocytes and synthetic membranes. The preliminary study undertaken in this thesis should be expanded to include further hydrophilic materials which would be appropriate for use in the bioreactor. These membranes might be required for immunoisolation, nutrient supply or waste removal. In particular, the interaction of such membranes both in collagen coated and uncoated forms with hepatocytes from a more clinically relevant species, such as the pig, would seem appropriate.

The effect of serum or plasma from liver failure patients on hepatocyte function is another topic which should be studied further. Critical functions such as biotransformation and ammonium metabolism should be evaluated and post-treatment cell viability could also be used as an index of the effects of toxic sera on hepatocytes.

Further information on hepatocyte oxygen requirements is needed. In

particular, specific oxygen consumption rates of hepatocytes during different phases of reactor operation should be elucidated. Oxygen requirements during the static seeding and attachment phases should be quantified for hepatocytes sourced from species considered suitable for clinical application and the effects of toxin load and contact with serum or plasma from liver failure patients on oxygen consumption also require to be evaluated.

Finally, and perhaps most significantly, a rather obvious gap in our understanding of liver failure and its therapy requires to be filled: we must identify exactly what a hepatocyte bioreactor is required to do in a clinical liver support system.

Bibliography

Abramowitz, M. 1949. On backflow of a viscous fluid in a diverging channel. J. Math. Phys. 28: 1.

Agnes, S., A.W. Avolio, S.C. Magalini, M. Focco, and M. Castagneto. 1992. Should retransplantation still be considered for primary non function after liver transplantation? Transplant. Int. : S170-S172.

Anders, M.W. 1988. Bioactivation mechanisms and hepatocellular damage. In The Liver: Biology and Pathobiology, Second Edition., ed. I.M. Arias, W.B. Jakoby, H. Popper, D. Schachter, and D.A. Shafritz: 389-400. New York: Raven Press Ltd.

Asanuma, Y., P. S. Malchesky, I. Zawicki, J. W. Smith, W. D. Carey, D. R. Ferguson, R. E. Herman, and Y. Nose. 1980. Clinical hepatic support by on-line plasma treatment with multiple sorbents - Evaluation of system performance. Transactions of the American Society for Artificial Internal Organs. 26: 400-405.

Bader, A., N. Fruhauf, G. Steinhoff, and A. Haverich. 1997. Development of a bioartificial liver by reconstructing 3-D cell plates and sinusoidal analogues. Artificial Organs 21 (6): 526.

Bader, A., E. Knop, K. Boker, N. Fruhauf, W. Schluttler, K. Oldhafer, R. Burkhard, R. Pilchmayer, and K-FR. Sewing. 1995. A novel bioreactor design for in vitro reconstruction of in vivo liver characteristics. Artificial Organs 19 (4): 368-374.

Bader, A., K. Zechs, O. Crome, U. Christians, B. Ringe, R. Pilchmayer, and K-FR. Sewing. 1994. Use of organotypical cultures of primary hepatocytes to analyse drug biotransformation in man and animals. Xenobiotica 24 (7): 623-633.

Battino, R., ed. 1981. Oxygen and Ozone. Solubility Data Series. Oxford: Pergamon Press.

Bean, E.S. and D.E. Atkinson. 1984. Regulation of the rate of urea synthesis in liver by extracellular pH; a major factor in pH homeostasis in mammals. J. Biol. Chem. 259: 1552-1559.

Bellomo, G. and S. Orrenius. 1986. Toxicological implications of perturbation of Ca2+ homeostasis in hepatocytes. In Calcium and Cell Function, ed. W.Y. Cheung, 6:186-208. Orlando: Academic Press.

Benhamou, J.P. 1990. Fulminant and subfulminant liver failure: Definitions and causes. In Acute Liver Failure Improved Understanding and Better Therapy, ed. R. Williams and R.D. Hughes:6-10. Dorset, U.K.: SmithKline Beecham Pharmaceuticals U.K.

Berker, R. 1963. Movement d'un fluid visquex incompressible. In Hanbuch der Physik, 8(2):70. Berlin: Springer Verlag.

Bernuau, J., B. Rueff, and J.P. Benhamou. 1986. Fulminant and subfulminant liver failure: Definitions and causes. Sem. Liv. Dis. 6: 97-106.

Bhatia, S.N., M. Toner, B.D. Foy, A. Rotem, K.M. O'Neil, R.G. Tompkins, and M.L. Yarmush. 1996. Zonal liver cell heterogeneity: effects of oxygen on metabolic functions of hepatocytes. Cellular Engineering 1 (3): 125-135.

Bihari, D., A.E. Gimson, J. Lindridge, and R. Williams. 1985. Lactic acidosis in fulminant hepatic failure. Some aspects of pathogenesis and prognosis. Journal of Hepatology 1: 405-416.

Bihari, D.J., A.E. Gimson, and R. Williams. 1985. Cardiovascular, pulmonary and renal complications of fulminant hepatic failure. Sem. Liv. Dis. 6: 119-128.

Bismuth, H. and D. Samuel. 1989. In Liver Transplantation in Fulminant and Subfulminant Hepatitis. Edited by P.J. Morris and N.L. Tilney. Transplantation Reviews. Philadelphia: Saunders.

Black, M. 1975. Isoniacid-associated hepatitis in 114 patients. Gastroenterology 69: 389-394.

Blei, A.T., S. Olafsson, S. Webster, and R. Levy. 1993. Complications of intracranial pressure monitoring in fulminant hepatic failure. Lancet 341 (8838) (Jan. 16): 157-158.

Bowen, B. D. 1985. Streaming potential in the hydrodynamic entrance region of cylindrical and rectangular capillaries. J. Colloid Interface Sci 106 (2): 367-376.

Bradley, S.E., F.J. Ingelfinger, G.P. Bradley, and J.J. Curry. 1945. The estimation of hepatic blood flow in man. J. Clin. Invest. 24 (890-897).

Brems, J.J., J.R. Hiatt, K.P. Ramming, W.J. Quinones-Baldrich, and R.W. Busutti. 1987. Fulminat hepatic failure: The role of liver transplantation as primary therapy. American Journal of Surgery 154: 137-141.

Burkel, W.E. 1970. The fine structure of the terminal branches of the hepatic arterial system in the rat. Anat. Rec. 167: 329-349.

Campra, J.L. and T.B. Reynolds. 1988. The hepatic circulation. In The Liver: Biology and Pathobiology, Second Edition., ed. I.M. Arias, W.B. Jakoby, H. Popper, D. Schachter, and D.A. Schafritz:911-930. New York: Raven Press Ltd.

Cascino, A., C. Cangiano, F. Fiaccadori, F. Ghinelli, M. Merli, G. Pelosi, O. Riggio, F.R. Fanelli, D. Sacchini and M. Stortoni. 1982. Plasma and cerebrospinal-fluid amino-acid patterns in hepatic-encephalopathy. Dig. Dis. Sci. 27(9): 828-832

Chang, T. M. S. 1973. Haemoperfusion over microencapsulated adsorbent in a patient with hepatic coma. The Lancet ii: 1371-1372.

Chari, R. S., B. H. Collins, J. C. Magee, J. M. DiMaio, A. D. Kirk, R. C. Harland, R. L. McCann, J. L. Platt, and W. C. Meyers. 1994. Treatment of hepatic failure with ex-vivo pig liver perfusion followed by liver transplantation. The New England Journal of Medicine 331 (4): 234-237.

Chen, S., S. Eguchi, F. Watanabe, W. Hewitt, E. Kahaku, J. Rozga, and A.A. Demetriou. 1996. Hepatic support strategies. Transplantation Proceedings 28 (4): 2036-2038.

Clavien, P.A., P.R.C. Harvey, and S.M. Strasberg. 1992. Preservation and reperfusion injuries in liver allografts. Transplantation 53: 957-978.

Conn, H.O. 1977. Comparison of lactulose and neomycin in the treatment of portal-systemic encephalopathy. Gastroenterology 72: 573.

deGroot, H., A. Littauer, and T. Noll. 1987. Metabolic and pathological aspects of hypoxia in liver cells. In Die Signalfunktion des sauerstoffes in biologischen systemen.:49-64.

deGroot, H., T. Noll, and H. Sies. 1985. Oxygen dependence and subcellular partitioning of hepatic menadione mediated oxygen uptake. Archives of Biochemistry and Biophysics 243 (2): 556-562.

Demetriou, A. A., J. Rozga, and A. D. Moscioni. 1990. Liver cell transplantation. In Acute Liver Failure. Improved Understanding and Better Therapy., ed. R. Williams and R. D. Hughes:66-69. Dorset, U.K.: SmithKline Beecham Pharmaceutical U.K.

Demetriou, A. A., J. Rozga, L. Podesta, E. LePage, E. Morsiani, A. D. Moscioni, A. Hoffman, M. McGrath, L. Kong, H. Rosen, F. Villamil, G. Woolf, J. Vierling, and L. Makowka. 1995. Early clinical experience with a hybrid bioartificial liver. Scandinavian Journal of Gastroenterology 30 (S208, S18): 111-117.

Dillon J., E. Ekevall, M.D. Smith, K. Anderson, M.H. Grant, J.D.S. Gaylor and P.C. Hayes. 1995. Serum from patients in fulminant hepatic failure enhances hepatocyte function in culture. Gut 37(Suppl. 2):T98.

Dunn, J. C. Y., R. G. Tompkins, and M. L. Yarmush. 1991. Long term in vitro function of adult hepatocytes in a collagen sandwich configuration. Biotechnology Progress 7: 237-245.

Dunn, J. C. Y., R. G. Tompkins, and M. L. Yarmush. 1992. Hepatocytes in collagen sandwich: Evidence for trancriptional and translational regulation. Journal of Cell Biology 116 (4): 1043-1053.

Dunn, J. C. Y., M. L. Yarmush, H. G. Koebe, and R. G. Tompkins. 1989. Hepatocyte function and extracellular matrix geometry: long-term culture in a snadwich configuration. Faseb Journal 3: 174-177.

Duthie, S. J., C. S. Coleman, and M. H. Grant. 1988. Status of reduced glutathione in the human hepatoma cell line, HepG2. Biochem. Pharmacol. 37: 3365-3368.

Ede, R.J. and R. Williams. 1986. Hepatic encephalopathy and cerebral edema. Sem. Liv. Dis. 6: 107-118.

Eiseman, B., D. S. Liem, and F. Raffucci. 1973. Heterologous liver perfusion in treatment of hepatic failure. Annals of Surgery 162: 329-345.

Elias, H. 1949. A re-examination of the structure of the mammalian liver; the hepatic lobule and its relation to the vascular and biliary systems. Am. J. Anat. 85: 379-456.

Elsdale, T. and J. Bard. 1972. Collagen substrata for studies on cell behaviour. J. Cell Biol. 54: 626-637.

Emmison, N., L. Agius, and V. A. Zammit. 1991. Regulation of fatty acid metabolism and gluconeogenesis by growth hormone and insulin in sheep hepatocyte culture: Effects of lactation and pregnancy. Biochemical Journal 274: 21.

Everson, G.T. and M.A. Polokoff. 1986. HepG2: a human hepatobalstoma cell line exhibiting defects in bile acid synthesis and conjugation. J. Biol. Chem. 261: 2197-2201.

Ezzell, R. M., M. Toner, K. Hendricks, J. C. Y. Dunn, R. G. Tompkins, and M. L. Yarmush. 1993. Effect of collagen gel configuration on the cytoskeleton in cultured rat hepatocytes. Experimental Cell Research 208: 442-452.

Farrell, G., D. Prendergast, and M. Murray. 1985. Halothane hepatitis. New England Journal of Medicine 313: 1310-1314.

Flander, U., W. Haas, and H. Kroner. 1982. The damage of the hepatic mixed function oxygenase system by CCl4: significance of incorporation of 14CCl4 metabolites in vivo. Exp. Mol. Pathol. 36: 34-43.

Flendrig, L.M. 1997. Semi-permeable hollow-fibre membranes in hepatocyte bioreactors: A pre-requisite for a successful bioartificial liver? Artificial Organs 21 (6).

Fletcher, D. 1994. Transplant breakthrough saves five liver children. The Daily Telegraph, Monday, July 18, 1994, 8.

Forbes, A., G.J.M. Alexande, J.G. O'Grady, R. Keays, R. Gullan, S. Dawling and R. Williams. 1989. Thiopental infusion in the treatment of intracranial pressure complicating fulminant hepatic failure. Hepatology 10(3): 306-311.

Foy, B.D., J. Lee, J. Morgan, M. Toner, R.G. Tompkins, and M.L. Yarmush. 1993. Optimisation of hepatocyte attachment to microcarriers: Importance of oxygen. Biotechnology and Bioengineering 42 (5): 579-588.

Foy, B. D., A. Rotem, M. Toner, R. G. Tompkins, and M. L. Yarmush. 1995. A device to measure the oxygen uptake rate of attached cells: Importance in bioartificial organ design. Cell Transplantation 3 (6): 515-527.

Fuchs, M., J. Gerlach, J. Encke, J. Unger, M.D. Smith, P. Neuhaus, and E. Riedel. 1994a. Amino acid metabolism by hepatocytes in a hybrid liver support bioreactor. Int. J. Artif. Organs 17 (12): 663-669.

Fuchs, M., J. Gerlach, J. Unger, J. Encke, M.D. Smith, P. Neuhaus, M. Nundel, and E. Riedel. 1994b. Alpha-keto acid metabolism by hepatocytes cultured in a hybrid liver support bioreactor. Int. J. Artif. Organs 17 (10): 554-558.

Gaasbeek-Jansen, J.W., W.H. Lamers, A.R. Moorman, A. Graaf, J. Los, and R. Charles. 1984. Immunohistochemical localisation of carbamoyl-phosphate synthetase amongst adult rat liver: evidence for a heterogeneous distribution. Cytochemistry 32: 557-564.

Gebhardt, R. and D. Mecke. 1983. Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cells in situ and in primary culture. EMBO Journal 2: 567-570.

Gelfand, M. C., J. F. Winchester, and J. H. Knepshield. 1978. Biochemical correlations of reversal of hepatic coma treated with charcoal hemoperfusion. Transactions of the American Society for Artificial Internal Organs 24: 239-242.

Gerlach, J., J. Brombacher, J. M. Courtney, and P. Neuhaus. 1993a. Nonenzymatic versus enzymatic hepatocyte isolation from pig livers for larger scale investigation of liver cell perfusion systems. Int. J. Artif. Organs 16: 677-681.

Gerlach, J. C., J. Encke, O. Hole, C. Muller, C. J. Ryan, and P. Neuhaus. 1994a. Bioreactor for a larger scale hepatocyte in vitro perfusion. Transplantation 58 (9): 1-5.

Gerlach, J.C., M. Fuchs, M.D. Smith, R. Bornemann, J. Encke, P. Neuhaus, and E. Riedel. 1996a. Is a clinical application of hybrid liver support systems limited by an initial disorder in cellular amino acid and a-keto acid metabolism, rather than by later gradual loss of primary hepatocyte function? Transplantation 62 (2): 224-228.

Gerlach, J., A. Jorres, O. Trost, O. Hole, J. Vienken, J. M. Courtney, G. M. Gahl, and P. Neuhaus. 1993b. Side effects of hybrid liver support therapy: TNF-alpha liberation in pigs, associated with extracorporeal bioreactors. Int. J. Artif. Organs 16 (8): 604-608.

Gerlach, J. C., K. Kloppel, C. Muller, N. Schnoy, M. D. Smith, and P. Neuhaus. 1993c. Hepatocyte aggregate culture technique for bioreactors in hybrid liver support systems. Int. J. Artif. Organs 16 (12): 843-846.

Gerlach, J., K. Kloppel, H. H. Schauwecker, R. Tauber, C. Muller, and E. S. Bucherl. 1989. Use of hepatocytes in adhesion and suspension cultures for liver support bioreactors. Int. J. Artif. Organs 12: 785-791.

Gerlach, J., K. Kloppel, P. Stoll, J. Vienken, and C. Muller. 1990. Gas supply across membranes in bioreactors for hepatocyte culture. Artificial Organs 14: 328-333.

Gerlach, J., N. Schnoy, M. D. Smith, and P. Neuhaus. 1994b. Hepatocyte culture between woven capillary networks: A microscopy study. Artificial Organs 18 (3): 226-230.

Gerlach, J.C., N. Schnoy, J. Vienken, M.D. Smith, and P. Neuhaus. 1996b. Comparison of hollow fibre membranes for hepatocyte immobilisation in bioreactors. Int. J. Artif. Organs 19 (10): 610-616.

Gerlach, J., T. Trost, C. J. Ryan, M. Meissler, O. Hole, and P. Neuhaus. 1994c. Hybrid liver support system in a short term application on hepatectomized pigs. Int. J. Artif. Organs 17 (10): 549-553.

Gimson, A.E., J. O'Grady, R.J. Ede, and et al. 1986. Late onset hepatic failure: Clinical, serological and histological features. Hepatology 6: 288-294.

Giorgio, T. D., A. D. Moscioni, J. Rozga, and A. A. Demetriou. 1993. Mass transfer in a hollow fiber device used as a bioartificial liver. ASAIO Journal 39: 886-892.

Gislason, G. T., D. D. Lobdell, J. H. Kelly, and N. L. Sussman. 1994. A treatment system for implementing an extracorporeal liver assist device. Artificial Organs 18 (5): 385-389.

Goldstick, T.K. 1971. Oxygen transport. In Engineering principles in physiology, ed. Brown and Gann:257-282.

Goresky, C.A. and A.J. Schwab. 1988. Flow, cell entry and metabolic disposal: Their interactions in hepatic uptake. In The Liver: Biology and Pathobiology. Second Edition., ed. I.M. Arias, W.B. Jakoby, H. Popper, D. Schachter, and D.A. Shafritz:807-831. New York: Raven Press Ltd.

Gove, C. D., R. D. Hughes, and R. Williams. 1982. Rapid inhibition of DNA synthesis in hepatocytes from regenerating rat livers by serum from patients with fulminant hepatic failure. British Journal of Experimental Pathology 63: 547-561.

Grant, M. H., M. A. L. Melvin, P. Shaw, W. T. Melvin, and M. D. Burke. 1985. Studies on the maintenance of cytochromes P-450 and monooxygenase and cytochrome reductases in primary cultures of rat hepatocytes. FEBS Letters 190: 99-103.

Greenway, C.V. and G. Oshiro. 1972. Intrahepatic distribution of portal and hepatic arterial blood flows in anaethsetised cats and dogs and the effects of portal occlusion, raised venous pressures and histamine. J. Physiol. 227: 473-485.

Greig, P.D., G.M. Woolf, M. Abecassis, J. Forster, B.R. Strasberg, S.M. Taylor, R.A. Superina, M.F. Glynn, M. Ettles, L.M. Blendis, and G.A. Levy. 1989. Prostaglandin E1 for primary non function following liver transplantation. Transplantation Proceedings 21 (2): 3360-3361.

Gumucio, J.J., C.P. Balabaud, D.L. Miller, L.J. Demason, H.D. Appelman, T.J. Stoecker and D.R. Franzbla. 1978. Bile secretion and liver cell heterogeneity in the rat. J. Lab. Clin. Med. 91(2): 350-362.

Gumucio, J.J. and J. Chianale. 1988. Liver cell heterogeneity and liver function. In The Liver: Biology and Pathobiology, Second Edition., ed. I.M. Arias, W.B. Jakoby, H. Popper, D. Schachter, and D.A. Shafritz:931-947. New York: Raven Press Ltd.

Gumucio, J.J. and D.L. Miller. 1981. Functional inplications of liver cell heterogeneity. Gastroenterology 80: 393-403.

Guzelian, P. S. and D. M. Bissell. 1976. Effects of cobalt on synthesis of heme and cytochrome P450 in the liver. J. Biol. Chem. 251: 4421-4427.

Haas, T. H., C. J. Holloway, V. Osterhuss, and I. Tranteshold. 1981. Hepatotoxic effects of sera from patients with fulminant hepatitis B on isolated rat hepatocytes in culture. J. Clin. Chem. Clin. Biochem. 19: 283-290.

Hanrahan, J.P. and M.A. Gordon. 1984. Mushroom poisoning. Case reports and a review of therapy. Journal of the American Medical Association 251: 1057-1061.

Haussinger, D. 1983. Hepatocyte heterogeneity in glutamine and ammonia metabolism and the role of an intracellular glutamine cycle during ureogenesis in perfused rat liver. Eur. J. Biochem. 133: 269-275.

Haussinger, D. and W. Gerok. 1986. Metabolism of amino acids and ammonia. In Regulation of hepatic metabolism. Intra- and intercellular compartmentation., ed. R.G. Thurman, F.C. Kauffman, and K. Jungermann: 253-291. New York: Plenum Press.

Haussinger, D., W. Gerok, and H. Seis. 1984. Hepatic role in pH regulation: role of the intercellular glutamine cycle. Trends. Biochem. Sci. 9: 300-302.

Haussinger, D., L. Weiss, and H. Sies. 1975. Activation of pyruvate dehydrogenase during metabolism of ammonium ions in hemoglobin-free perfused rat liver. Eur. J. Biochem. 52: 421-431.

Hazelton, G.A., J.J. Hjelle, and C.D. Klaassen. 1986. Effects of cysteine pro-drugs on acetaminophen-induced toxicity. J. Pharmacol. Exp. Ther. 237: 341-349.

Hinson, J.A., L.R. Pohl, and J.R. Gillette. 1979. N-Hydroxyacetaminophen: A microsomal metabolite of N-hydroxyphenacetin but apparently not acetaminophen. Life Sciences 24: 2133-2138.

Hissin, P. J. and R. A. Hilf. 1976. A fluorimetric method for the determination of oxidised and reduced glutathione in tissues. Analytical Biochemistry 74: 214-216.

Hochachka, F.W., L.T. Buck, C.J. Doll, and S.C. Land. 1996. Unifying theory of hypoxia tolerance: Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proc. Natl. Acad. Sci. USA 93: 9493-9498.

Holme, J.A., D.C. Dahlin, S.D. Nelson, and E. Dybing. 1984. Cytotoxic effects of N-acetyl-p-benzoquinono imine: a cytochrome P450 mediated oxidation product of acetaminophen. Proc. Natl. Acad. Sci. USA 81: 1327-1331.

Iber, F.L., H. Rosen, S.M. Levenson, and T.C. Chalmers. 1957. The plasma amino acids in patients with liver failure. J. Lab. Clin. Med. 50: 417-425.

Jackoby, W.B. 1978. The glutathione S-transferases: A group of mutifunctional detoxication proteins. Adv. Enzymol. 46: 384.

Johnson, P.C. and K.M. Hanson. 1966. Capillary filtration in the small intestine of the dog. Circ. Res. 19: 766-773.

Jones, A.L., G.T. Hradek, and R.H. Renston. 1980. Autoradiographic evidence for hepatic lobular concentration gradient of bile acid derivative. Am. J. Physiol. 238: G233-237.

Jungermann, K. 1995. Zonation of metabolism and gene expression in liver. Histochemistry 103: 81-91.

Kalayoglu, M., H.W. Sollinger, R.J. Stratta, R.M. Hoffmann, and F.O. Belzer. 1988. Extended preservation of the liver for clinical transplantation. Lancet (2): 617-619.

Kappus, H. and H. Sies. 1981. Toxic effects associated with oxygen metabolism: Redox cycling and lipid peroxidation. Experimentia 37: 1233-1241.

Karrer, F. M., R. Gill, A. Ostrowska, A. Bolwerk, B. Reitz, M. Stegall, G. Schroter, I. Kam, and K. J. Lafferty. 1995. Purified hepatic parenchymal cells are nonimmunogenic in vitro. Transplantation Proceedings 27 (1): 625-627.

Katz, N.R. 1992. Metabolic heterogeneity of hepatocytes across the liver acinus. J. Nutr. 122: 843-849.

Keays, R., G.J.M. Alexander, and R. Williams. 1990. Intracranial pressure monitoring and cerebral blood flow measurements in fulminant hepatic failure. In Acute Liver Failure. Improved Understanding and Better Therapy., ed. R. Williams and R.D. Hughes:45-47. Dorset, U.K.: SmithKline Beecham Pharmaceuticals U.K.

Kelly, J.H., T. Koussayer, D. He, M.G. Chong, T.A. Shang, H.H. Whissennand, and N.L. Sussman. 1992. Assessment of an extracorporeal liver assist device in anhepatic dogs. Artificial Organs 16 (4): 418-422.

Kennedy, H. J., M. Greaves, and D. R. Triger. 1985. Clinical experience with the use of charcoal hemoperfusion: Is prostacyclin required? Life Support Systems 3: 115-122.

Koebe, H. G., M. Wick, U. Cramer, V. Lange, and F. W. Schildberg. 1994. Collagen gel immobilisation provides a suitable cell matrix for long term human hepatocyte cultures in hybrid reactors. Int. J. Artif. Organs 17 (2): 95-106.

Koide, N., K. Sakaguchi, Y. Koide, K. Asano, M. Kawaguchi, H. Matsushima, T. Takenami, T. Shinji, M. Mori, and T. Tsuji. 1990. Formation of multicellular spheroids composed of adult rat hepatocytes in dishes with positively charged surfaces and other non-adherent environments. Experimental Cell Research 186: 227-235.

Kondrup, J., T. Almdal, H. Vilstrup, and N. Tygstrup. 1992. High volume plasma exchange in Fulminant Hepatic Failure. Int. J. Artif. Organs 15 (11): 669-676.

Krogh, A. 1919. The number and distribution of capillaries in muscles with calculations of the oxygen partial pressure head necessary for supplying the tissues. J. Physiol. 52: 409-415.

Larrey, D., F. Berson, and F. Habersetzer. 1989. Genetic predispostion to drug hepatotoxicity: the role in hepatitis caused by amineptine - a tricyclic anti-depressant. Hepatology 10: 168-173.

Lautt, W.W. 1977. Hepatic vasculature: A conceptual view. Gastroenterology 73: 1163-1169.

Lazar, A., H. J. Mann, R. P. Remmel, R. A. Shatford, F. B. Cerra, and W-S. Hu. 1995a. Extended liver-specific functions of porcine hepatocyte spheroids entrapped in collagen gel. In Vitro Cell. Dev. Biol. 31: 340-346.

Lazar, A., M. D. Peshwa, F. J. Wu, C-M. Chi, F. B. Cerra, and W-S. Hu. 1995b. Formation of porcine hepatocyte spheroids for use in a bioartificial liver. Cell Transplantation 4 (3): 259-268.

Leber, H. W., J. Klausmann, G. Goubeaud, and et al, eds. 1981. Middle Molecules in the Serum of Patients and Rats with Liver Failure: Influence of Sorbent Hemoperfusion. Edited by G. Brunner and F. W. Schmidt. Artificial Liver Support. New York: Springer-Verlag.

Lee, W. M. 1994. Total hepatectomy for acute liver failure: Don't take out my liver! Gastroenterology 107 (3): 894-897.

Levesque, M. J. and R. M. Nerem. 1985. The elongation and orientation of cultured endothelial cells in response to shear stress. J. Biomech. Eng. 107: 341-347.

Lidofsky, S.D., N.M. Bass, M.C. Prager, D.E. Washington, A.E. Read, T.L. Wright, N.L. Ascher, J.P. Roberts, B.F. Scharschmidt, and J.R. Lake. 1992. Intracranial pressure monitoring and liver transplantation for fulminant hepatic failure. Hepatology 16 (1): 1-7.

Loud, A.V. 1968. Quantitative description of the ultrastructure of normal rat liver parenchymal cells. J. Cell Biol. 37: 27-46.

Lutz, J., B. Decke, M. Bauml, and H-G. Schulze. 1978. High oxygen extraction combined with extensive oxygen consumption in the rat liver perfused with fluosol-DA a new perfluoro compound emulsion. Pflugers Archive 376: 1-6.

Lutz, J., H. Henrich, and E. Bauereisen. 1975. Oxygen supply and uptake in the liver and the intestine. Pflugers Archive 360: 7-15.

MacDonald, C., M. Vass, B. Willet, A. Scott, and M. H. Grant. 1994. Expression of liver functions in immortalised rat hepatocyte cell lines. Hum. Exp. Toxicol. 13: 439-444.

Matsubara, S., K. Okabe, K. Ouchi, Y. Miyakazi, Y. Yajima, H. Suzuki, M. Otsuki, and S. Matsuno. 1990. Continuous removal of middle molecules by hemofiltration in patients with acute liver failure. Crtical Care Medicine 18 (12): 1331-1338.

McLimans, W.F., L.E. Blumenson, and K.V. Tunnah. 1968. Kinetics of gas diffusion in mammalian cell culture systems. II. Theory. Biotechnology and Bioengineering 10: 741-763.

Meredith, M. J. 1988. Rat hepatocytes prepared without collagenase: Prolonged retention of differentiated characteristics in culture. Cell Biology and Toxicology 4 (4): 405-425.

Miller, D.L., C.S. Zanolli, and J.J. Gumucio. 1979. Quantitative morphology of the sinusoids of the hepatic acinus. Quantimet analysis of rat liver. Gastroenterology 76: 965-969.

Mitchell, J.R., D.J. Jollow, W.Z. Potter, J.R. Gillette, and B.B. Brodie. 1973. Acetaminophen induced hepatic necrosis. IV. Protective role of glutathione. J. Pharmacol. Exp. Ther. 187: 211-217.

Moldeus, P., J. Hogberg, and S. Orrenius. 1978. Isolation and use of liver cells. Methods in Enzymology 52: 60-71.

Morgan, M.Y., M.W. Marshall, J.P. Milson and S. Sherlock . 1982. Plasma amino acid patterns in liver disease. Gut 23(5): 362-370

Muller, M., M. Schrieber, J. Kartenbeck, and G. Schreiber. 1972. Preparation of single-cell suspensions from normal liver, regenerating liver and Morris hepatomas 9121 and 5123tc. Cancer Research 32: 2568-2576.

Myers, J.D. 1947. The hepatic blood flow and splanchnic oxygen consumption of man - Their estimation from urea production or bromosulpthalein during catheterisation of the hepatic veins. J. Clin. Invest. 26: 1130-1137.

Nagel, R.A., K.M. Hayllar, J.M. Tredger, and R. Williams. 1991. Caffeine clearance and galactose elimination capacity as prognostic indicators in fulminant hepatic failure. European Journal of Gastroenterology and Hepatology 3 (12): 907-913.

Nelson, D.R., T. Kamataki, D.J. Waxman, F.P. Guengerich, R.W. Estabrook, R. Feyereisen, F.J. Gonzalez, M.J. Coon, I.C. Gunsalus, O. Gotoh, K. Okuda, and D.W. Nebert. 1993. The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. DNA and Cell Biology 12 (1): 1-51.

Neuhaus, P. and G. Blumhardt. 1993. Extracorporeal liver perfusion: Applications of an improved model for experimental studies of the liver. Int. J. Artif. Organs. 16 (10): 729-739.

Neuhaus, P., G. Blumhardt, W.O. Bechstein, R. Steffen, and H. Keck. 1990. Side-to-side anastomosis of the common bile duct is the method of choice for biliary tract reconstruction after liver transplantation. Transplantation Proceedings 22: 1571.

Neuhaus, P., G. Blumhardt, W.O. Bechstein, R. Steffen, K-P. Platz, and H. Keck. 1994. Technique and results of biliary reconstruction using side-to-side choledochocholedochostomy in 300 orthotopic liver transplants. Annals of Surgery 219 (4): 426-434.

Neuzil, D. F., J. Rozga, A. D. Moscioni, M-S. Ro, R. Hakim, W. S. Arnaout, and A. A. Demetriou. 1993. Use of a novel bioartificial liver in a patient with acute liver insufficiency. Surgery 113 (3): 340-343.

Nicotera, P., M. Moore, F. Mirabelli, G. Bellomo, and S. Orrenius. 1985. Inhibition of hepatocyte plasma membrane Ca2+ATPase activity by menadione and its restoration by thiols. FEBS Letters 181: 149-153.

Noll, T., H. deGroot, and P. Wisseman. 1986. A computer-supported oxystat system maintaing steady-state O2 partial pressures and simultaneously monitoring O2 uptake in biological systems. Biochem. J. 236: 765-769.

Nouel, O., J. Henrion, and J. Bernuau. 1980. Fulminant hepatic failure due to transient circulatory failure in patients with chronic heart disease. Dig. Dis. Sci. 25: 49-52.

Novikoff, A.B. 1959. Cell heterogeneity within the hepatic lobule of the rate. J. Histochem. Cytochem. 7: 240-244.

Nyberg, S. L., W. D. Payne, B. Amiot, K. Shirabe, R. P. Remmel, W-S. Hu, and F. B. Cerra. 1993a. Demonstration of biochemical function by extracorporeal xenohepatocytes in an anhepatic animal model. Transplantation Proceedings 25 (2): 1944-1945.

Nyberg, S. L., J. L. Platt, K. Shirabe, W. D. Payne, W-S. Hu, and F. B. Cerra. 1992. Immunoprotection of xenocytes in a hollow fiber bioartificial liver. ASAIO Journal 38: M463-M467.

Nyberg, S.L., R.P. Remmel, H.J. Mann, M.V. Peshwa, W.S. Hu, and F.B. Cerra. 1994. Primary hepatocytes outperform HepG2 cells as the source of biotransformation functions in a bioartificial liver. Annals of Surgery 220 (1): 59-67.

Nyberg, S. L., R. A. Shatford, M. V. Peshwa, J. G. White, F. B. Cerra, and W. S. Hu. 1993b. Evaluation of a hepatocyte-entrapment hollow fiber bioreactor: A potential bioartificial liver. Biotechnology and Bioengineering 41: 194-203.

O'Grady, J., A.E.S. Gimson, C.J. O'Brien, A. Pucknell, R.D. Hughes, and R. Williams. 1988. Controlled trials of charcoal hemoperfusion and prognostic factors in fulminant hepatic failure. Gastroenterology 94: 1186-1192.

O'Grady, J.G., G.J.M. Alexander, K.M. Hayllar, and R. Williams. 1989. Early indicators of prognosis in fulminant hepatic failure. Gastroenterology 97: 439-445.

O'Grady, J.G., P.G. Langley, L.M. Isola, L.M. Aledort and R. Williams. 1986. Coagulopathy of fulminant hepatic failure. Sem. Liv. Dis. 6(2): 159-163.

O'Grady, J.G., K.C. Tan, and R. Williams. 1990. Selection criteria and results of orthotopic liver transplantation in the U.K. In Acute Liver Failure. Improved Understanding and Better Therapy., ed. R. Williams and R.D. Hughes:77-80. Dorset U.K.: SmithKline Beecham Pharmaceuticals U.K.

O'Grady, J.G. and R. Williams. 1992. Acute Hepatic Failure. Edited by Tinker and Zapol. Care of the Critically Ill Patient: Springer-Verlag.

Oliver, J., A.M. Koelz, J. Costello, and E. Bourke. 1977. Acid-base induced alterations in glutamine metabolism and ureogenesis in perfused muscle and liver of the rat. Eur. J. Clin. Invest. 7: 445-449.

Omura, T. and R. Sato. 1964. Carbon monoxide binding pigment of liver microsomes: evidence for its haemoprotein nature. J. Biol. Chem. 239: 2379-2385.

Orrego, J., I. Mena, E. Baraona, and R. Palma. 1965. Modifications in hepatic blood flow and portal pressure produced by different diets. Am. J. Dig. Dis. 10: 239-248.

Otto, J. J., J. C. Pender, J. H. Cleary, D. M. Sensenig, and C. S. Welch. 1958. The use of a donor liver in experimental animals with elevated blood ammonia. Surgery 43: 301-309.

Padgham, C. R. W. and A. J. Paine. 1993. Altered expression of cytochrome P-450 mRNAs, and potentially of other transcripts encoding key hepatic functions, are triggered during the isolation of rat hepatocytes. Biochemical Journal 289: 621-624.

Paine, A. J. and L. J. Hockin. 1980. Nutrient imbalance causes the loss of cytochrome P-450 in liver cell culture: formulation of culutre media which maintain cytochrome P-450 at in vivo concentrations. Biochemical Pharmacology 29: 3215-3218.

Parsons-Wingerter, P. A. and W. M. Saltzman. 1993. Growth versus function in the three-dimensional culture of single and aggregated hepatocytes within collagen gels. Biotechnology Progress 9: 600-607.

Peshwa, M. V., F. J. Wu, B. D. Follstad, F. B. Cerra, and W. S. Hu. 1994. Kinetics of hepatocyte spheroid formation. Biotechnology Progress 10: 460-466.

Phillips, M. 1994. Buying time for liver patients. New Scientist, 26 November 1994, 26.

Pohlein, Ch., P. Lemmens, M. R. Schon, P. Neuhaus, J. Thiery, and C. Hammer. 1994. Xenogeneic ex-vivo hemoperfusion of rhesus monkey livers with human blood. Transplantation Proceedings 26 (3): 1061-1062.

Potter, D.W. and J.A. Hinson. 1986. Reactions of N-acetyl-p-benzoquinone imine with reduced glutathione, acetaminophen and NADPH. Mol. Pharmacol. 30: 33-41.

Powers-Lee, S.G. and A. Meister. 1988. Urea synthesis and ammonia metabolism. In The Liver: Biology and Pathobiology, Second Edition., ed. I.M. Arias, W.B. Jakoby, H. Popper, D. Schachter, and D.A. Shafritz:317-329. New York: Raven Press, Ltd.

Preston, F.E. 1990. Haemorrhagic diathesis and control. In Acute Liver Failure. Improved Understanding and Better Therapy, ed. R. Williams and R.D. Hughes:36-39. Dorset, U.K.: SmithKline Beecham Pharmaceuticals U.K.

Ramalingaswami, V. and R.H. Purcell. 1988. Waterborne non-A non-B hepatitis. Lancet 1: 571-573.

Rappaport, A.M. 1958. The structural and functional unit in the human liver (liver acinus). Anat. Rec. 130: 673-686.

Rappaport, A.M. 1980. Hepatic blood flow: Morphologic aspects and physiologic regulation. Int. Rev. Physiol. 21: 1-63.

Rappaport, A.M. and J.H. Schneiderman. 1976. The function of the hepatic artery. Rev. Physiol. Biochem. Pharmacol. 76: 129-175.

Recknagel, R.O. 1983. A new direction in the study of carbon tetrachloride hepatotoxicity. Life Sciences 33: 401-408.

Reed, D. J. and P. W. Beatty. 1978. The role of the cystathione pathway in glutathione regulation in isolated hepatocytes. In Functions of glutathione in liver and kidney, ed. H. Sies and A. Wendel:13-21. Berlin: Springer-Verlag.

Refojo, M. F., F. J. Holly, and F-L. Leong. 1977. Permeability of dissolved oxygen through contact lenses. 1. Cellulose acetate butyrate. Contact Lens 3 (4): 27-33.

Reichen, J., T. Widmer, and J. Cotting. 1991. Accurate prediction of death by serial determination of galactose elimination capacity in primary biliary cirrhosis: A comparison with the Mayo model. Hepatology 14 (3): 504-510.

Ringe, B. 1993. Transplantation Proceedings 25: 1090-1091.

Ringe, B., P. Neuhaus, and W. Lauchart. 1989. Experience with hepatic retransplantation. Transplantation Proceedings 21 (1): 2407-2408.

Ringe, B. and Pilchmayer. 1988. Transplantation Proceedings 20 (Suppl. 1): 552-557.

Rolando, N., F. Harvey, J. Brahm, J. Philpott, G. Alexande, A. Gimson, M. Casewell, E. Fagan and R. Williams. 1990. Prospective study of bacterial infection in acute liver failure: an analysis of 50 patients. Hepatology 11(1): 49-53.

Rotem, A., M. Toner, R. G. Tompkins, and M. L. Yarmush. 1992. Oxygen uptake in cultured rat hepatocytes. Biotechnology and Bioengineering 40: 1286-1291.

Rozga, J., M. D. Holzman, M-S. Roo, D. W. Griffin, D. F. Neuzil, T. Giorgio, A. D. Moscioni, and A. A. Demetriou. 1993a. Development of a hybrid bioartificial liver. Annals of Surgery 217 (5): 502-511.

Rozga, J., E. Morsiani, E. LePage, A. D. Moscioni, T. Giorgio, and A. A. Demetriou. 1994a. Isolated hepatocytes in a bioartificial liver: A single group view and experience. Biotechnology and Bioengineering 43: 645-653.

Rozga, J., L. Podesta, E. LePage, A. Hoffman, E. Morsiani, L. Sher, G. M. Woolf, L. Makowka, and A. A. Demetriou. 1993b. Control of cerebral oedema by total hepatectomy and extracorporeal liver support in fulminant hepatic failure. The Lancet 342: 898-899.

Rozga, J., L. Podesta, E. LePage, E. Morsiani, A. D. Moscioni, A. Hoffman, L. Sher, F. Villamil, G. Woolf, M. McGrath, L. Kong, H. Rosen, T. Lanman, L. Makowka, and A. A. Demetriou. 1994b. A bioartificial liver to treat severe acute liver failure. Annals of Surgery 219 (5): 538-546.

Rozga, J., F. Williams, M-S. Roo, D. F. Neuzil, T. D. Giorgio, G. Backfisch, A. D. Moscioni, R. Hakim, and A. A. Demetriou. 1993c. Development of a bioartificial liver: Properties and function of a hollow-fiber module inoculated with liver cells. Hepatology 17 (2): 258-265.

Rueff, B. and J.P. Benhamou. 1973. Acute hepatic necrosis and fulminant hepatic failure. Gut 14: 805-815.

Ruprah, M., T.G. Mant, and R.J. Flanagan. 1985. Acute carbon tetrachloride poisoning in 19 patients: Implications to diagnosis and treatment. Lancet 1: 1027-1029.

Ryan, C. J., M. Aslam, and J. M. Courtney. 1990. Experimental procedures for the assessment of artificial liver support. In Acute Liver Failure. Improved Undestanding and Better Therapy., ed. R. Williams and R. D. Hughes:60-66. Dorset, U.K.: SmithKline Beecham Pharmaceuticals U.K.

Salsano, F., I.P. Maly, and D. Sasse. 1990. The circadian rythmn of intra-acinar profiles of alcohol dehydrogenase activity in rat liver: a microquantitative study. Histochem. J. 22: 395-400.

Samuel, D. and H. Bismuth. 1990. Selection criteria and results of orthotopic liver transplantation in fulminant and subfulminant hepatitis. In Acute Liver Failure. Improved Understanding and Better Therapy., ed. R. Williams and R.D. Hughes:73-77. Dorset, U.K.: SmithKline Beecham Pharmaceuticals U.K.

Sandle, G.I., M. Layton, C.O. Record, and W.K. Cowan. 1980. Fulminant hepatic failure due to Budd-Chiari syndrome. Lancet 1: 1199.

Sarnatskaya, V. V., V. G. Nikolaev, L. A. Osipova, A. I. Ivanov, V. N. Maslenny, A. A. Ivanyuk, and L. A. Yushko. 1993. Peritoneal dialysis for the removal of protein-bound markers of hepatic insufficiency. Artificial Organs 17 (10): 828-836.

Sasse, D. 1986. Liver structure and innervation. In Regulation of hepatic metabolism, ed. R.G. Thurman, F.C. Kauffman, and K. Jungermann:3-25. New York: Plenum Press.

Schafer, D. F. 1993. Desperate appliances: A short review of therapies for fulminant hepatic failure. Blood Purification 11: 158-162.

Schafer, D.F. and B.W. Shaw. 1989. Fulminant hepatic failure and orthotopic liver transplantation. Sem. Liv. Dis. 9: 189-194.

Schenk, W.G.Jr., J.C. McDonald, K. McDonald, and T. Drapanas. 1962. Direct measurment of hepatic blood flow in surgical patients: With related observations on hepatic flow dynamics in experimental animals. Annals of Surgery 156: 463-471.

Schalm, S.W. and R.J. de Knegt. 1990. Acute liver failure: Definitions and pathological variants. In Acute Liver Failure. Improved Understanding and Better Therapy., ed. R. Williams and R.D. Hughes: 11-13. Dorset, U.K.: SmithKline Beecham Pharmaceuticals U.K.

Schuetz, E.G., J.D. Schuetz, S.C. Strom, M.T. Thompson, R.A. Fisher, D.T. Molowa, D. Li and P.S. Guzelian. 1993. Regulation of human liver cytochromes P-450 in family 3A in primary and continuous culture of human hepatocytes. Hepatology 18(5): 1254-1262.

Sheil, A.G.R., J. Sun, D.C. Mears, M. Waring, K. Woodman, B. Johnston, M. Horvat, K.J. Watson, N. Koutalistras, and L-S. Wang. 1996. Preclinical trial of a bioartificial liver support system in a porcine fulminant hepatic failure model. Aust. N.Z. J. Surg. 66: 547-552.

Shinji, N., N. Koide, and T. Tsuji. 1988. Cell Struct. Funct. 13: 179-188.

Shoemaker, W.C., P.L. Appel, and H.B. Kram. 1986. Hemodynamic and oxygen transport effects of dobutamine in critically ill general surgical patients. Critical Care Medicine 14: 1032-1037.

Sielaff, T. D., M. Y. Hu, S. Rao, K. Groehlier, D. Olson, H. J. Mann, R. P. Remmel, R. A. Shatford, B. Amiot, W-S. Hu, and F. B. Cerra. 1995. A technique for porcine hepatocyte harvest and description of differentiated metabolic functions in static culture. Transplantation 59 (10): 1459-1463.

Sies, H. 1977. Oxygen gradients during hypoxic steady states in liver. Urate oxidase and cytochrome oxidase as intracellular O2 indicators. Hoppe-Seyler's Z. Physiol. Chem. 358: 1021-1032.

Sies, H. 1985. Oxidative Stress. New York: Academic Press.

Smirthwaite, A. 1994. Design studies in the development of a bioreactor for artificial liver support. PhD, University of Strathclyde.

Stange, J., S. Mitzner, W. Ramlow, and R. Schmidt. 1993. 'Liver bypass' on the basis of a new albumin-triggered dialsysis. In Eleventh Annual Meeting of the International Society of Blood Purification:Abstract 61.

Starzl, T.E., S.I. Iwatsuki, and C. Esquivel. 1985. Refinements in the surgical technique of liver transplantation. Sem. Liv. Dis. 5: 349

Strasberg, S.M., K.H. Todd, E.P. Molmenti, and M. Hertl. 1994. Selecting the donor liver: Risk factors for poor function after orthotopic liver transplantation. Hepatology 20: 829-838.

Streeter, A.J., D.C. Dahlin, S.D. Nelson, and T.A. Baillie. 1984. The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of acrylation in vitro. Chem. Biol. Interact. 48: 349-366.

Sussman, N.L., M.G. Chong, T. Koussayer, D. He, T.A. Shang, H.H. Whisennand, and J.H. Kelly. 1992. Reversal of fulminant hepatic failure using an extracorporeal liver assist device. Hepatology 16 (1): 60-65.

Sussman, N. L., M. J. Finegold, J. P. Barish, and J. H. Kelly. 1994a. A case of syncytial giant-cell hepatitis treated with an extracorporeal liver assist device. The American Journal of Gastroenterology 89 (7): 1077-1082.

Sussman, N. L., G. T. Gislason, C. A. Conlin, and J. H. Kelly. 1994b. The Hepatix extracorporeal liver assist device: Initial clinical experience. Artificial Organs 18 (5): 390-396.

Sussman, N. L. and J. H. Kelly. 1992. Improved liver function following treatment with an extracorporeal liver assist device. Artificial Organs 17 (1): 27-30.

Sussman, N.L. and J.R. Lake. 1996. Treatment of hepatic failure - 1996: Current concepts and progress towards liver dialysis. Scandanavian Journal of Kidney Diseases 27 (5): 605-621.

Takahashi, M., H. Ishikara, C. Takahashi, Y. Nakajima, M. Matsushita, H. Matsue, K. Sato, H. Noto, K. Taguchi, M. Koike, M. Nishikawa, H. Kamachi, H. Kon, J. Uchino, and T. Yoshiki. 1993. Immunologic considerations in the use of cultured porcine hepatocytes as a hybrid artificial liver. ASAIO Journal 39: M242-M246.

Takei, Y., F. C. Kauffman, U.K. Misra, H. Yamanaka and R.G. Thurman. 1990. Regulation of urea synthesis in regions of the liver lobule by oxygen. Biochim. Biophys. Acta 1036(3): 242-244.

Ternberg, J.L. and J.R. Jr. Butcher. 1965. Blood flow relation between hepatic artery and portal vein. Science 150: 1030-1031.

teVelde, A. A., N. C. J. J. Ladiges, L. M. Flendrig, and R. A. F. M. Chamuleau. 1995. Functional activity of isolated pig hepatocytes attached to different extracellular matrix substrates. Implication for application of pig hepatocytes in a bioartificial liver. Journal of Hepatology 23: 184-192.

Thurman, R.G., F.C. Kauffman, and J. Baron. 1986. Biotransformation and zonal toxicity. In Regulation of hepatic metabolism, intra and intercellular compartmentation., ed. R.G. Thurman, F.C. Kauffman, and K. Jungermann:321-382. New York, London: Plenum Press.

Tobe, S., Y. Takei, K. Kobayashi, and T. Akaike. 1992a. Receptor mediated formation of multilayer aggregates of primary cultured adult rat hepatocytes on lactose-substituted polystyrene. Biochemical and Biophysical Research Communications 184 (1): 225-230.

Tobe, S., Y. Takei, K. Kobayashi, and T. Akaike. 1992b. Tissue reconstruction in primary cultured rat hepatocytes on Asiaglycoprotein model polymer. Artificial Organs 16 (5): 526-532.

Trey, C. and C.S. Davidson. 1970. The management of fulminant hepatic failure. Progress in Liver Disease 3: 282-298.

Trowell, O.E. 1942. Urea formation in the isolated perfused liver of the rat. J. Physiol. 100: 432-458.

Tygstrup, N. and L. Ranek. 1986. Assessment of prognosis in fulminant hepatic failure. Sem. Liv. Dis. 6: 129-137.

van-Kooten, T. G., J. M. Schakenraad, H. C. Van der Mei, and H. J. Busscher. 1992. Development and use of a parallel-plate flow chamber for studying cellular adhesion to solid surfaces. J. Biomed. Mat. Res. 26: 725-738.

van-Wagenen, R. A. and J. D. Andrade. 1980. Flat plate streaming potential investigations: Hydrodynamics and electrokinetic equivelance. J. Colloid Interface Sci 76 (2): 305-314.

Vickers, C., J. Neuberger, J. Buckels, P. McMaster, and E. Elias. 1988. Tranplantation of the liver in adults and children with fulminant hepatic failure. Journal of Hepatology 7: 143-150.

Wade, O.L., B. Combes, A.W. Childs, H.O. Wheeler, A. Courmand, and S.E. Bradley. 1956. The effect of excercise on the splachnic blood flow and splanchnic volume in normal man. Clin. Sci. 15: 457-463.

Ware, A.J., A.N. D'Agostino, and B. Combes. 1971. Cerebral edema: A major complication of massive hepatic necrosis. Gastroenterology 61: 877-884.

Waxman, D. J., J. J. Morrissey, S. Naik, and H. O. Jauregui. 1990. Phenobarbital induction of cytochromes P450. Biochemical Journal 271: 113-119.

Weber, C., F. Loth, H. Schima, S. Mitzner, and D. Falkenhagen. 1994. A new technology of sequential filtration and adsorption based on a high speed circulation system of micro- and nanospheres for extracorporeal blood purification. Int. J. Artif. Organs 17 (8): 440.

Wendon, J., G.J.M. Alexander, and R. Williams. 1990. Cardiovascular monitoring and local blood flow. In Acute Liver Failure. Improved Understanding and Better Therapy., ed. R. Williams and R.D. Hughes:39-42. Dorset, U.K.: SmithKline Beecham Pharmaceuticals U.K.

Williams, R. and J. Wendon. 1990. Clinical syndrome and aetiology of fulminant hepatic failure. In Acute Liver Failure, Improved Understanding and Better Therapy, ed. R. Williams and R.D. Hughes:1-5. Dorset, U.K.: SmithKline Beecham Pharmaceuticals U.K.

Wilson, R. S., M. G. Goode, M. D. Lelah, and S. L. Cooper. 1982. An ex vivo narrow-slit flow device for evaluation of blood-material interactions. Transactions of the American Society for Artificial Internal Organs 28: 420-425.

Wisse, E., R.B. De Zanger, K. Charels, Van Der Smissen, and R.S. McCluskey. 1985. The liver sieve: considerations concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of Disse. Hepatology 5: 683-692.

Wu, F.J., J.R. Friend, A. Lazar, H.J. Mann, R.P. Remmel, F.B. Cerra, and W-S Hu. 1996. Hollow fiber bioartificial liver utilizing collagen-entrapped porcine hepatocyte spheroids. Biotechnology and Bioengineering 52: 34-44.

Yarmush, M. L., M. Toner, J. C. Y. Dunn, A. Rotem, A. Hubel, and R. G. Tompkins. 1992. Hepatic tissue engineering. Development of critical technologies. Annals of the New York academy of Sciences 665: 238-252.

Yoshiba, M., K. Sekiyama, Y. Iwamura, and F. Sugata. 1993. Development of reliable artificial liver support (ALS) - Plasma exchange in combination with hemodiafiltration using high performance membranes. Dig. Dis. Sci. 38 (3): 469-476.

Yuasa, C., Y. Tomita, M. Shono, K. Ishimura, and A. Ichihara. 1993. Importance of aggregation for expression of liver functions and regeneration demonstrated with primary cultured hepatocytes. Journal of Cellular Physiology 156: 522-530.

Appendix 1

Authors Publications

Papers in Refereed Journals

1. Smith M.D., Barbenel J.C., Courtney J.M. & Grant M.H. "Novel quantitative methods for the determination of biomaterial cytotoxicity." International Journal of Artificial Organs 1992:15(3):191-194.

2. Smith M.D., Shearer M.G., Srivastava S., Scott R. & Courtney J.M. "Quantitative evaluation of the growth of established cell lines on the surface of collagen, collagen composite and reconstituted basement membrane." Urological Research 1992:20:285-288.

3. Shearer M.G., Smith M.D., Courtney J.M. & Scott R. "Investigation of bladder carcinoma: consideration of in vitro assessment of the invasive potential of tumour cells." STP Pharma Sciences 1993:3(1):97-103.

4. Gerlach J.C., Kloppel K., Muller C., Schnoy N. Smith M.D. & Neuhaus P. "Hepatocyte aggregate culture technique for bioreactors in hybrid liver support systems." International Journal of Artificial Organs 1993:16(12):843-846.

5. Gerlach J.C., Schnoy N., Smith M.D. & Neuhaus P. "Hepatocyte culture between woven capillary networks: A microscopy study." Artificial Organs 1994:18(3):226-230.

6. Fuchs M., Gerlach J., Unger J., Encke J., Smith M.D., Neuhaus P., Mundel M. & Riedel E. "alpha-keto acid metabolism by hepatocytes cultured in a hybrid liver support bioreactor." International Journal of Artificial Organs 1994:17(10):554-558.

7. Fuchs M., Gerlach J., Encke J., Unger J., Smith M.D., Neuhaus P. & Riedel E. "Amino acid metabolism by hepatocytes cultured in a hybrid liver support bioreactor." International Journal of Artificial Organs 1994:17(12):663-669.

8. Smith M.D., Grant M.H., Blass C.R., Courtney J.M. & Barbenel J.C. "Poly(vinyl chloride) formulations: Acute toxicity to cultured human cell lines." Journal of Biomaterials Science. Polymer Edition 1995:7(5):453-459.

9. Watts P., Smith M.D., Edwards I., Zammit V., Brown V. & Grant M.H. "The influence of medium composition on the maintenance of cytochrome P450, glutathione content and urea synthesis: A comparison of rat and sheep primary hepatocyte cultures." Journal of Hepatology 1995:23:605-612.

10. Gerlach J.C., Schnoy N., Enke J., Smith M.D., Muller C. & Neuhaus P. "Improved hepatocyte *in vitro* maintenance in a culture model with woven multicompartment capillary systems: Electron microscopy studies." Hepatology 1995:22(2):546-562.

11. Smith M.D., Smirthwaite A.D., Cairns D.E., Cousins R.B. & Gaylor J.D.S. "Techniques for measurement of oxygen consumption rates of hepatocytes during attachment and post-attachment." International Journal of Artificial Organs 1996:19(1):36-44

12. Gerlach J., Brombacher J. Smith M.D. & Neuhaus P. "High yield hepatocyte isolation from pig livers for investigation of hybrid liver support systems: Influence of collagenase concentration and body weight." Journal of Surgical Research 1996:62:85-89.

13. Gerlach J., Jorres A., Berger A., Schon M., Smith M.D., Nohr R. & Neuhaus P. "Systemic liberation of interleukin-1ß and interleukin-1 receptor antagonist in the perioperative phase of liver transplantation." Transplant International 1996:9:408-414.

14. Gerlach J.C., Fuchs M., Smith M.D., Bornemann R., Encke J., Neuhaus P. & Riedel E. "Is a clinical application of hybrid liver support systems limited by an initial disorder in cellular amino acid and a-keto acid metabolism, rather than by later gradual loss of primary hepatocyte function?" Transplantation 1996:62(2):224-228.

15. Gerlach J.C., Schnoy N., Vienken J., Smith M.D. & Neuhaus P. "Comparison of hollow fibre membranes for hepatocyte immobilisation in bioreactors." International Journal of Artificial Organs 1996:19(10) 610-616.

16. Bornemann R., Smith M.D. & Gerlach J.C. "Consideration of potential immunological problems in the application of xenogenic hybrid liver support." International Journal of Artificial Organs 1996:19(11) 655-663.

17. Gerlach J., Jorres A., Schon M., Berger A., Spatkowski G., Smith M.D. & Neuhaus P. "Systemic liberation of interleukin-8 in the perioperative phase of liver transplantation." Transplant International 1997:10(5):401-404.

18. Gerlach J.C., Hentschel F., Spatkowski G., Zeilinger K., Smith M.D. & Neuhaus P. "Cell detachment during sinusoidal reperfusion after liver preservation." Transplantation 1997:64(6):

Published Abstracts

1. Barbenel J.C., Smith M.D., Courtney J.M. and Grant M.H. "Quantitative cytotoxicity assessment by cell culture." Medical and Biological Engineering and Computing 1991:29(World Congress Supplement):539.

2. Edwards I.W., Watts P.L., Smith M.D., Grant M.H. and Brown V.A. "Comparison of hepatocyte function in different media." Mutagenesis 1993:8:482.

3. Smith M.D., Shearer M.G., James A.S., Rajnoch C.E.A., Courtney J.M. and Scott R. "Utilisation of an artificial basement membrane in the assessment of the invasive potential of tumour cells." International Journal of Artificial Organs 1993:16(6):471.

4. Watts P., Smith M.D., Zammit V., Edwards I., Brown V. and Grant M.H. "Comparison of the effect of medium composition on cytochrome P450 and glutathione content and urea synthesis on primary cultures of rat and sheep hepatocytes." British Journal of Clinical Pharmacology 1994:38(2):171-P.

5. Dillon J., Ekevall E., Smith M.D., Anderson K., Grant M.H., Gaylor J.D.S. and Hayes P.C. "Serum from patients in fulminant hepatic failure enhances hepatocyte function in culture." Gut 1995:37(Suppl. 2):T98.

6. Ekevall E., Airdrie I., Cousins R.B., Smith M.D. and Gaylor J.D.S. "Effect of oxygen partial pressure within a bioartificial liver." International Journal of Artificial Organs 1996:19(9):545

7. Cairns D.E., Airdrie I., Smith M.D., Grant M.H., Gaylor J.D.S. & Cousins R.B. "Oxygen consumption and urea synthesis of isolated hepatocytes during attachment to a substratum." Artificial Organs 1997:21(6):526

8. Ekevall E., Plevris J., Smith M.D., Anderson K., Nelson L., Hayes P.C., Cousins R.B., Grant M.H. & Gaylor J.D.S. "Response of rat hepatocytes in culture to serum from fulminant hepatic failure patients." Artificial Organs 1997:21(6):527.

9. Smith M.D., Cairns D.E., Veitch R., Cousins R.B. & Gaylor, J.D.S. "Analysis of oxygen transfer in hollow fibre hepatocyte bioreactors." Artificial Organs 1997:21(6):531

10. Smith M.D., Airdrie I., Cairns D., Courtney J.M., Cousins R.B., Ekevall E., Grant M.H. & Gaylor J.D.S. "Characterisation of a hybrid artificial liver bioreactor with integral membrane oxygenation." Artificial Organs 1997:21(6):531

11. Ishida Y., Okamoto M., Keogh A., Sadamoto T., Auth M., Smith M.D., Wallace L., Gerlach J., Kelly D., McMaster P. And Strain A. "Investigation of an experimental bio-artificial liver support system using primary human hepatocytes." International Journal of Artificial Organs 1997:20(9):500

12. Smith M.D., Ekevall E., Airdrie I., Grant M.H., Cousins R.B. And Gaylor J.D.S. "A modular hepatocyte bioreactor with integral membrane oxygenation: Design and development." International Journal of Artificial Organs 1997:20(9):509

13. Ekevall E., Smith M.D., Airdrie I., Grant M.H., Cousins R.B. And Gaylor J.D.S. "A modular hepatocyte bioreactor with integral membrane oxygenation: *In vitro* testing." International Journal of Artificial Organs 1997:20(9):529

Published Conference Proceedings

1. Reed I., Brown V., Edwards I., Cousins R., Gaylor J. & Smith M.D. "Development of membrane bioreactor for liver support." In: Proc. Euromembrane '95 (eds W.R. Bowen, R.W. Field & J.A. Howell) 1995, University of Bath, Bath, UK. 315-320.

2. Cairns D.E., Cousins R.B., Smith M.D., Grant M.H. and Gaylor J.D.S. "Novel static culture oxystat system for hepatocyte oxygen consumption measurements: Applicatioj to high cell density hybrid artificial organs." In: Cell Biology and Toxicology - 1996: Special Issue: International Congress on Hepatocytes: Applications in Cell Biology, Toxicology and Medicine: p53.

3. Smith M.D., Airdrie I., Cairns D. Cousins R.B., Ekevall E., Grant M.H. and Gaylor J.D.S. "A novel hybrid artificial liver with integral membrane oxygenation: Theory and initial in vitro evaluation." In: Cell Biology and Toxicology 1996: Special Issue: International Congress on Hepatocytes: Applications in Cell Biology, Toxicology and Medicine: p56.

4. Smith M.D., Airdrie I., Courtney J.M., Cousins R.B., Ekevall E., Grant M.H. and Gaylor J.DS. "Development and Characterization of a Hybrid Artificial Liver Bioreactor with Integral Membrane Oxygenator" In: Bioartificial Liver Support: the Critical Issues, Crepaldi G., Demetriou A.A. and Muraca M. Eds., International Up to Date - 208, C.I.C. Edizioni Internazionali (1997): pp. 27-35

Unpublished Presentations/Posters

1. "The influence of medium composition on cytochrome P450, glutathione content and urea production on primary cultures of rat hepatocytes." 3rd Annual meeting of the Hepatocyte Users Group, Wentworth College, University of York, UK. 8th-9th September 1993.

2. "Hepatocyte Aggregate Culture for Liver Cell Bioreactors" 1st International Conference on Cellular Engineering, Keele University, UK. 12th-15th September 1993.

3. "Hepatocyte oxygenation in bioreactors." Invited lecture in special symposium on liver support systems at XXI Congress of the European Society for Artificial Organs Congress, Barcelona, Spain. 20th-22nd October 1994.

4. "Oxygen requirements of hepatocytes: Consideration of bioreactor configurations." Invited lecture in special symposium on hybrid liver support systems at XXII Congress of the European Society for Artificial Organs, Berlin, Germany. 19th-21st October 1995.

5. "Bioartificial liver devices must provide adequate oxygenation." Xth World Congress of the International Society for Artificial Organs, Taipei, Taiwan. 14th-18th Novemeber 1995.

6. "Membrane oxygenation in hybrid liver support systems." Invited lecture in special symposium on artificial liver systems at XXIV Congress of the European Society for Artificial Organs, Budapest, Hungary. 16th-18th October 1997.

Manuscripts in preparation

1. "Review of Bioartificial Liver Developments: A Bioengineering Perspective." Invited chapter in the forthcoming second edition of The Handbook of Hepatocyte Culture edited by Professor M.N. Berry.

2. "Hepatocyte Oxygen Consumption: Measurement Techniques and Importance in Bioreactor Design." Invited chapter in the forthcoming second edition of The Handbook of Hepatocyte Culture edited by Professor M.N. Berry.