DESIGN STUDIES IN THE DEVELOPMENT OF A BIOREACTOR FOR ARTIFICIAL LIVER SUPPORT

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by

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Declaration of Rights

List of Abbreviations

2CM	2 compartment model
3CM	3 compartment model
ADP	adenosine diphosphate
ALS	artificial liver support
ATP	adenosine triphosphate
BAL	bioartificial liver
BHI	brain heart infusion broth
BSA	bovine serum albumin
CCM	control culture medium
DMNA	dimethylnitrosamine
DMSO	dimethylsulphoxide
EEG	electroencephalogram
EROD	ethoxyresorufin O-dealkylation
FCS	foetal calf serum
FHF	fulminant hepatic failure
GABA	γ-aminobutyric acid
GSH	reduced glutathione
GSSG	oxidised glutathione
HBSS	Hank's buffer stock solution
HEPES	N - [2 - hydroxyethyl]piperazine - N' - [2 - ethanesulphonic acid]
KHBSS	Krebs-Henseleit buffer stock solution
LDH	lactate dehydrogenase activity
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCS	newborn calf serum

OUR	oxygen uptake rate
PAN	polyacrylonitrile
PB	pentobarbital
PBS	phosphate buffered saline
PEST	penicillin : streptomycin (5000 IU · ml^{-1} : 5000 $\mu g \cdot ml^{-1}$)
PID	proportional integral differential
PVF	polyvinyl formol
RLC	residual liver capacity
SEM	standard error of the mean
TCA	trichloroacetic acid
TSBA	total serum bile acids

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ABSTRACT

There has been much interest in recent years in the development of an hepatocyte bioreactor for artificial liver support, or bioartificial liver (BAL). Although considerable advances have been made in hepatocyte culture, the BAL environment is unlikely to be comparable to conventional culture conditions. For example:

- the culture medium is likely to be human serum or plasma, as opposed to a specifically prepared culture medium,
- very high density cultures will be required for the BAL, which may result in a rapid build-up of cell waste products (eg. bile),
- in contrast to conventional stagnant cultures, oxygen gradients may exist within a bioreactor.

Investigations were therefore undertaken to determine the effect of serum, bile and oxygen concentration on cell viability and function, as well as the effect of oxygen partial pressure (pO_2) and cell density on oxygen consumption. Hep G2 human hepatoma cells were frequently used as a model of hepatocyte behaviour.

When exposed to increasing concentrations of newborn calf serum (NCS) and human serum, cell growth and intracellular reduced glutathione (GSH) levels were well maintained, provided that the supply of amino acids was not limiting. Sheep bile was shown to be toxic to growing cultures of Hep G2 cells at concentrations as low as 0.1% (v/v). The evidence of toxicity was not as marked for confluent cultures of Hep G2 cells exposed to increasing concentrations of bile. Rat hepatocytes were shown to be extremely sensitive to oxygen concentration, with considerable differences in cytochrome P450 content, associated ethoxyresorufin O-dealkylase (EROD) activity, GSH content, ^{3}H-leucine incorporation into protein and cell morphology when cultured in the presence of 5, 12, 20, 28 or 35% O_2. By contrast, Hep G2 cells did not display this sensitivity. Hep G2 cells were, however, observed to respond to linear decreases in both

cell density and pO_2 with exponential increases in oxygen consumption. This information has been applied to the design considerations of the BAL, and the techniques and equipment implemented may be used in further investigations of cell behaviour within the bioreactor environment.

Chapter 1: Fulminant Hepatic Failure and Artificial Liver Support

1.1 Introduction

Acute or fulminant hepatic failure (FHF) is defined as rapid onset hepatic failure in patients with no pre-existing liver disease. It is a condition precipitated by severe widespread hepatic necrosis, generally originating in zone 3 of the acinar structure (Appendix A.1). Such necrosis commonly results from acute viral hepatitis, local ischaemia or exposure to hepatotoxins, as in paracetamol overdose. Progression is swift, leading to Stage III or IV hepatic encephalopathy (Table 1.1) within eight

Table 1.1: Stages of hepatic encephalopathy[1]

Stage I:

mood swings, mild confusion, slurred speech, sleep disturbances, slight tremor, no EEG changes

Stage II:

accentuation of Stage II, drowsiness, loss of sphincter control, tremor, abnormal EEG

Stage III:

stuporous but rousable from sleep, marked confusion, incoherent speech, abnormal EEG

Stage IV:

generally unresponsive to painful stimuli, tremor absent, abnormal EEG

[1] These categories are commonly used, but there is no standard classification (Kirsch et al, 1992).

weeks of onset. Although hepatic encephalopathy is often the first and most obvious sign of FHF, other symptoms include hypoglycaemia, cerebral and pulmonary oedema, reduced resistance to infection and gastrointestinal haemorrhage (Andreoli et al, 1986). 70 - 80% of patients die within two weeks of admittance to hospital (Kirsch, 1992). This high mortality is an index of the lack of suitable therapy and the current inability to replace the numerous metabolic/synthetic functions of the failing liver [Appendix A.2].

The development of a comprehensive system of artificial liver support has been impeded by incomplete understanding of the metabolic abnormalities characterising hepatic failure. In addition to changes in amino acid levels, abrupt cessation of hepatic function has marked physiological consequences. Table 1.2 outlines just a few of

Table 1.2: Metabolic changes in FHF patients

Increases in:

- mercaptans (blood, breath, urine)
- free fatty acids (blood)
- false neurotransmitters (brain, muscle, blood, urine)
- ammonia (blood, muscle, brain, spinal fluid)
- glutamine (muscle, brain, spinal fluid)
- respiratory quotient

Decreases in:

- glucose (blood)
- plasma proteins (including fibrinogen and immunoglobulins)
- ketone production
- neurotransmitters (brain, muscle)
- oxygen and glucose consumption (brain)
- oxygen affinity of haemoglobin

the changes that occur in the body during FHF (Berk 1977; Andrews, 1978). The aim of most detoxification systems is to normalise the altered biochemical parameters of the blood. The difficulty lies not only in assessing the relative importances of these abnormalities, but also in identifying synergistic relationships amongst toxins (Zieve et al, 1974) and the components of feedback relationships influencing hepatocellular regeneration or death. In a world-wide survey of FHF, it was found that a positive prognosis correlates most strongly with the efficacy of liver regeneration and the ability of the physician to maintain the patient until this regeneration can be realised (Trey, 1972). Studies have demonstrated full restoration of hepatic structure and function following up to 80% resection of the organ in rat, dog and guinea pig (Brunner, 1986). Even cirrhotic tissue demonstrates the ability to regenerate, with partial recovery of undamaged structure in restored tissue (Pack et al, 1962; Lin and Chen, 1965).

Although chronic liver diseases are more common, most studies have concentrated on support systems for FHF. The reasons for this are:

1. extensive damage inflicted by chronic dysfunction would require careful management over a long period to realise sufficient regeneration
2. there is currently no support regime adequate to replace the liver for extended lengths of time.

Patients surviving FHF are remarkable for their speed of hepatic restoration and rarely, if ever, develop chronic liver disease (Karvountzis et al, 1974). Even so, it is felt that an artificial support system may well have application in chronic liver disease. For example, it may be used post-operatively after resection of fatty or cirrhotic tissue to sustain the patient during liver regeneration, or pre-operatively as a bridge to transplantation. In the United States alone, there are over 3000 candidates for transplantation each year (Evans et al, 1993): those who do not receive a donor organ fare no better than FHF patients in terms of survival.

1.2 Conventional therapy and liver transplantation

1.2.1 Conventional therapy

Earlier therapies for hepatic failure identified diet as the key factor effecting recovery. During the 1940's, high protein, carbohydrate and fat intake were recommended, along with supplements of vitamins and Brewer's yeast (Chen and Chen, 1984). Modern conventional therapy (Table 1.3; ibid) has

Table 1.3: Conventional therapy for FHF

1. withdrawal of dietary protein
2. magnesium sulphate enema to empty bowel
3. administration of lactulose as a laxative
4. neomycin to prevent infection
5. infusions of dextrose as a nutritional support
6. supplements of vitamin K, thiamine and pyroxidine to encourage clotting factor synthesis
7. appropriate treatment of secondary ailments arising from hepatic insufficiency

eliminated the protein and fat from the diet, the metabolism of which taxes the injured liver. Conventional therapy clearly has not been very successful, with survival rates ranging from 6 - 20% (Trey, 1972). With glucagon-insulin therapy to stimulate liver regeneration, and infusions of specifically prepared amino acid solutions, survival rates as high as 31% have been recorded (Fischer, 1976; Kasai, 1986). Drug therapy has normally been limited to antibiotics and nutritional supplements, although there were claims of dramatic successes with cortisone

therapy in the early 1950's (Ducci and Katz, 1952). Later tests did not support this claim, and subsequent research implied that such therapy may actually increase liver damage (Davis and Williams, 1980).

1.2.2 Liver transplantation

Presently, the only treatment shown to significantly decrease mortality has been hepatic transplant. Early successes with dogs (Welsh, 1955; Moore, 1959) led to the first successful transplant in humans in 1963 (Starzl et al, 1963). Five year survival rates have been reported for 40 - 70% of patients (Brems et al, 1987; O'Grady et al, 1988) with 80% surviving their first year (O'Grady et al, 1988). Although transplantation is usually considered a more viable option in chronic irreversible liver disease, several examples of successful transplants for acute hepatic failure have been reported (Bismuth et al, 1987; Brems et al, 1987; Vickers et al, 1988). These transplant recipients were seven times more likely to be alive after one month than those given conventional treatment. After six months, these patients were still five times more likely to survive (Peleman et al, 1987). Nevertheless, it is far from the ideal solution. Lifelong immunosuppression is required and infection becomes the most prevalent complaint of transplant recipients. 50 - 70% of these patients experience repeated bacterial infection and 30 - 60% suffer viral infections. There are also risks of severe changes in the central nervous system, hepatic osteodystrophy, hepatic arterial occlusion, renal dysfunction and hypertension (Van Theil et al, 1990). Notwithstanding the hazards of major surgery and the threat of complications, expense is another important consideration. The cost of the transplantation procedure (excluding follow up care) can range from $30,000 - $1,500,000 (median ≈ $150,000; Evans et al, 1993). Transplantation has been estimated to be almost three times as expensive as other therapies (Evans, 1983).

1.3 Blood purification devices

1.3.1 Haemodialysis

Given the success of haemodialysis for kidney failure, attempts were naturally made to employ this technique for hepatic failure. Early attempts using cellulosic membranes were unsuccessful in producing even temporary amelioration of hepatic coma (Kiley et al, 1957; Kiley et al, 1958). Later trials (Wilson et al, 1972) showed excellent removal of unbound solutes (> 90%), but negligible reduction in protein bound substances such as bilirubin. The low percentage of unbound solutes in serum was identified as the rate limiting factor in haemodialysis for FHF. Thus, no advantage was found in using haemodialysis over conventional therapy (Keynes, 1968; Opolon et al, 1976). This deficiency led to the development of a haemodialyser using a polyacrylonitrile (PAN) membrane (Opolon et al, 1976; Opolon, 1979), which is approximately fifteen times more permeable than the conventional cellulosic Cuprophan® membrane. The Rhone-Poulenc haemodialysis system, using a PAN membrane, was effective in the removal of excess amino acids and middle molecular weight substances up to 5,000 Da, with apparently good biocompatibility (Henderson et al, 1973; Silk et al, 1976), but it did not improve the overall patient survival rate. Although this is in agreement with the bulk of studies on haemodialysis for FHF, Silk et al (1976) claimed a 33% rate of patient recovery. This was attributed not only to the efficient removal of middle molecular weight and water soluble compounds, but also to the rapid transmission of excess amino acids from tissue to plasma pools (Silk et al, 1977). However, because solute removal is dependent upon pore size, there is no discrimination between extractable toxins and beneficial solutes within the same molecular weight range. For example, large scale depletion of branched chain amino acids, which are effective in preventing hepatic coma

(Rossi-Fanelli et al, 1982), is a consequence of haemodialysis (Brunner and Loesgen, 1977). Diffusion of potentially beneficial molecules from the blood is easily prevented by supplementation of the dialysate with physiological concentrations of these substances, as in amino acid dialysis (Loesgen et al, 1981). Unfortunately, not all such compounds within the threatened molecular weight range have been identified.

1.3.2 Haemoperfusion

Perhaps in response to the inadequacy of haemodialysis, with its non-selective solute clearance, researchers began to investigate the use of sorbents in the treatment of FHF. Theoretically, since the pattern of solute removal is determined by the physical and chemical characteristics of the material, rather than by pore size, a sorbent may be selected to remove only specific compounds within a range of molecular weights. Although the lack of precise information about which substances should be removed makes this advantage difficult to exploit, there has been some success in the extraction of toxins using haemoperfusion over sorbents. Early tests involving uncoated charcoal showed great promise, with reversal of coma in 45% of subjects treated (Gazzard et al, 1974a). Charcoal has been demonstrated to remove methionine, tyrosine, phenylalanine (Gazzard et al, 1974a), mercaptans and phenols (Kazuika and Chang, 1979), as well as unspecified toxins with a molecular weight between 500 and 5000 Da (Chang, 1972) which have been implicated in the initiation of hepatic coma (Bessman and Bessman, 1955; Zieve et al, 1974). A further advantage of charcoal as a non-specific sorbent is its apparent removal of factors which inhibit hepatocyte regeneration (Hughes et al, 1975). Evidence exists that charcoal also adsorbs blood-borne entities associated with the suppression of leukocyte formation.

Most of the limitations of charcoal as an adsorbent are related to its poor biocompatibility. This is demonstrated by the large scale depletion of platelets and leukocytes from the blood, the release of thrombogenic microparticles from the charcoal and the retention of clotting factors, hormones and essential amino acids (Shi and Chang, 1984; Brunner, 1986). Aldosterone, T_3 and T_4, which are necessary for liver regeneration, are also quickly removed by charcoal haemoperfusion. Platelet losses are of great importance, given the prolonged clotting time already present in hepatic failure. Coating the charcoal may reduce the surface adhesion of platelets, but gains in biocompatibility are accompanied by even greater reductions in the efficiency of the adsorptive action (Gazzard et al, 1974b). In tests with 4% by weight acrylic hydrogel coating, a 20 - 60% reduction in platelet counts was noted after a 4 hour perfusion (Silk and Williams, 1978). Furthermore, of those lost, a disproportionate amount were the larger platelets, which are more efficient in clotting. The net effect was a longer clotting time than would be expected for the given platelet volume.

Charcoal is further limited by its poor adsorption of protein bound substances (Maini et al, 1984). Various resins have excellent capacity for the removal of protein bound toxins, but generally these are even less biocompatible than charcoal (Abouna et al, 1977). Affinity chromatography, for example by perfusion over albumin-conjugated agarose beads (Plotz et al, 1974; Scharschmidt et al, 1974), is another method by which protein bound substances may be eliminated. In theory, toxic compounds should have as great, or greater, affinity for the beads than for the plasma proteins to which they are bound. Although this method offers superior biocompatibility, and claims better removal of protein bound substances than charcoal, results of *in vitro* experiments on the removal of protein bound substances from blood are variable.

1.3.3 Plasma perfusion

The low biocompatibility of resins and the problems associated with charcoal haemoperfusion stimulated interest in plasma perfusion. In this scheme, plasma is first separated from the blood, perfused through beds of sorbents, and then reunited with bulk flow through a mesh to retain solvent particles (Weston et al, 1975; Solomon et al, 1978). This has several advantages over direct-contact perfusion:

- damage to formed elements of blood in the sorption phase are minimal, as are immune reactions
- release of particles into the blood stream is easily avoided
- the adsorptive capacity of the sorbent is unhampered by coatings
- toxins are more readily removed without the formed elements to dilute them (Yamazaki et al, 1977)
- sorbents which would otherwise damage the cellular elements of the blood may be used.

In addition, multiple sorbents may be used, and in quantities that would require a prohibitively large priming volume for direct contact haemoperfusion. Tests by Morimoto and co-workers (1989) suggest that the removal of aromatic amino acids from patients' blood is better in systems using serial perfusion of uncoated charcoal and anion-exchange resin than in either one alone.

Initially, plasma separation posed the greatest obstacle to the use of this technique. Centrifugal separation was explored, but centrifuge speeds high enough to remove sufficient plasma from the formed elements of blood caused considerable platelet damage. In tests with a continuous flow seal-less centrifuge, a 50% reduction in platelet count was reported within one hour of perfusion, rising to a maximum of 70% at the end of 12 hours (Ito et al, 1975).

Alternatively, a microporous membrane may be used to filter plasma from whole blood (Castino et al, 1978). Pore size is selected to allow passage of high molecular weight toxins and protein bound species into the reactor whilst denying access to blood corpuscles. Though no significant trauma to platelets or other formed elements has been demonstrated, filtrate flux has been shown to be reduced by as much as 48% during the process due to the deposition of platelets and proteins on the membrane (Ouchi et al,1978).

1.3.4 Multi-enzyme systems

A more direct route to toxin removal is by use of multi-enzyme systems (Freston et al, 1976; Shu et al, 1980). These employ chemical pathways of detoxification similar to those found in the body. One such detoxification device could be described as an enzymatic dialyser (Sofir et al, 1979). Plasma is separated from the whole blood and flows across a semi-permeable membrane. Toxins are driven across this membrane by a concentration gradient and are metabolised using appropriate enzyme systems. The non-toxic by-products then diffuse back into the plasma stream which is reunited with bulk flow before being returned to the patient. The enzymes in the dialysate are continuously regenerated to prolong the viability of the chemical reactions. Cousineau and Chang (1978) demonstrated that microencapsulated enzyme systems could be used to metabolise either urea or ammonia to the amino acid glutamate. The rate of removal of these chemicals from the system was estimated to be 2 $mg \cdot hr^{-1} \cdot ml^{-1}$ of microcapsules. Similarly, rapid reduction of free phenols (> 95% within 4 hours) was accomplished using microencapsulated UDP-glucuronyltransferase bound to sepharose beads (Brunner and Loesgen, 1977). Such systems are more selective and better tolerated physiologically then adsorbents, but have not been shown to alter the course of hepatic failure. Additionally, no single enzyme

system can deal with the multitude of toxic species present in the blood of the liver failure patient. Thus, several enzyme systems would be required, along with careful coordination of the regeneration or replacement of enzymes as necessary.

1.4 Therapies dependent upon biological systems

Although most of the support systems discussed in Section 1.3 claim some success, reports are variable and seem to depend strongly upon the condition of the patient at the beginning of therapy. The mean survival rate in the systems that have been tested clinically is only about 30%, and so these approaches are still inadequate. The common characteristic linking these therapies is that they centre on blood detoxification rather than normalisation of blood biochemistry. Many researchers believe that the synthetic functions of the liver must also be provided if an artificial support system is to significantly alter the course of hepatic failure.

1.4.1 Plasma exchange

Instead of removing toxic compounds from the patients' blood, the plasma itself may be removed and replaced with fresh plasma from a healthy donor. Clinical experiences with exchange transfusions have shown a fivefold increase in patient blood fibrinogen levels, while clotting factors II and V have been raised from 11% and 22% respectively before treatment, to 90% and 100% (normal) after treatment (Brunner, 1986). Plasma exchange has met with some success (Abouna, 1972; Redeker and Yamahiro, 1973), but the highest survival rates thus far, approximately 30%, (Kasai, 1986; de Groot et al, 1989) are no better than those obtained with the extra-corporeal detoxification systems of Section 1.3. There are also several important disadvantages:

- normal serum contains hepatocyte growth suppressing factors (Graw et al, 1970)
- there may be viral contamination of the donor serum (Buckner et al, 1973)
- significant amounts of microaggregates have been discovered in fresh frozen plasma, making careful filtration an additional

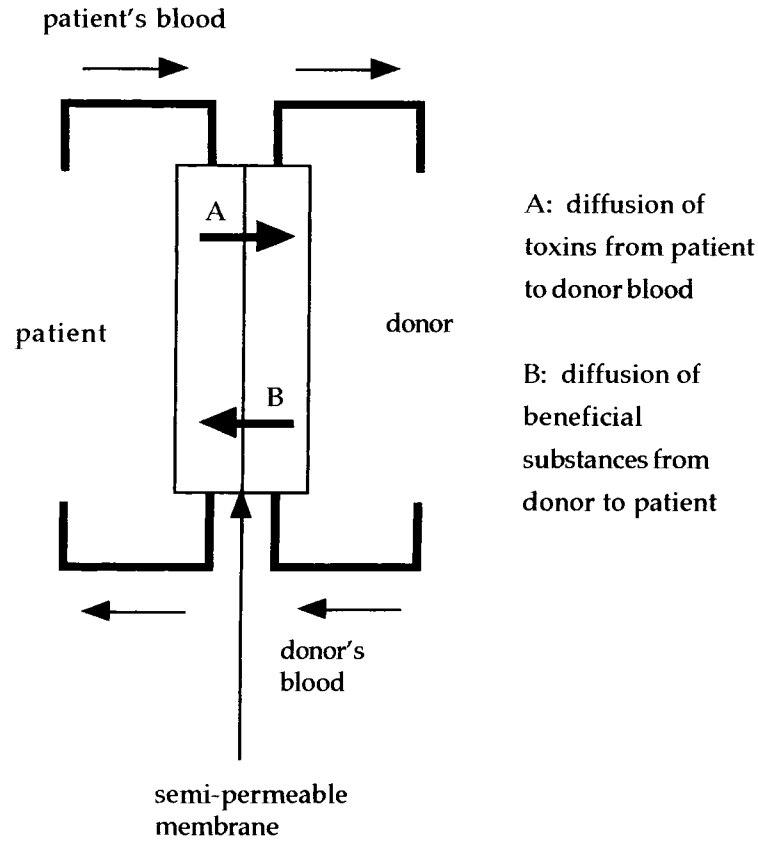
requirement to prevent thrombus formation (Akamatsu et al, 1990)

- plasma exchange is not always well tolerated, with reactions ranging from anaphylaxis to citrate toxicity with tetany, syncope or cardiac arrhythmias.
- a large volume of fresh plasma, which is expensive and difficult to obtain, is required (Freeman et al, 1986)
- daily plasma exchange may impair brain function and gas exchange in the lung (Brunner, 1986).

1.4.2 Cross circulation

Generally, cross circulation (Burnell et al, 1965; Swift et al, 1967; Roche-Sicot et al, 1974) can be described as a form of dialysis in which blood or plasma pumped continuously from a healthy individual is used as the dialysate for the hepatic failure patient (Figure 1.1, overleaf). Less commonly, direct blood flow between donor and recipient is maintained, by passing the patient's blood through either the systemic or hepatic circulation of the donor whilst donor blood is transferred to the patient's systemic circulation. In effect, the burden of blood normalisation is transferred from the damaged liver of the patient to a healthy donor liver. This method would seem to offer many of the advantages of plasma exchange: dialysis with plasma from a healthy patient would presumably remove toxins, add necessary metabolites and, unlike plasma exchange, would not require any extraneous plasma. However, the risk of anaphylactic reactions or viral transmission is paramount. Studies in rats have also demonstrated that this therapy is only effective in grade II coma and lower (Mohsini et al, 1980). This is potentially due to the low blood flows obtained with inter-systemic cross-circulation. It is estimated that only 2.7% of

Figure 1.1: Cross-circulation therapy for FHF



the blood from the patient that would normally be processed by the liver in a 24 hour period passes through the donor organ (de Groot et al, 1989).

1.4.3 Hepatocellular transplantation

Within the past fifteen years there has been much interest in the use of donor hepatocytes for transplantation (Mito et al, 1978; Demetriou et al, 1986a), either in free suspension (Demetriou et al, 1988; Makowka et al, 1980), microencapsulated (Dixit et al, 1990) or attached to microcarriers (Demetriou et al, 1986b; Fuller, 1988). In experiments with rats, these suspensions have primarily been injected intraperitoneally, although the portal vein, liver

parenchyma, spleen, kidney and abdominal cavity have also been examined as sites for transplantation (Kawai et al, 1987). Post experimental autopsy of rats has shown that transplanted cells proliferate and form functional groups, complete with vasculature and bile canaliculi (Jirtle et al, 1982; Jaffe et al, 1988). This is especially true with intrasplenic injection, where hepatocytes have been found to migrate and form a complex structural network within the pancreas (Jaffe et al, 1988). Early tests in dimethylnitrosamine (DMNA) treated rats indicated that the survival rate could be raised to 70% from a control (untreated) value of 6%, by intrasplenic transplantation of as little as 10% of the total normal rat liver cell mass (Mito et al, 1978). Later investigators, using animals with D-galactosamine induced hepatic failure (Kawai et al, 1987), hepatic ischaemia by ligation of the hepatic artery (Fuller, 1988) and up to 90% liver resection (Demetriou et al, 1988), have all resulted in extended survival times. Similarly, hepatocellular transplantation in Gunn rats (Demetriou et al, 1986a; Demetriou et al, 1986b; Dixit et, 1990), which are genetically incapable of bilirubin conjugation, and in Nagase analbuminaemic rats (Demetriou et al, 1986a; Demetriou et al, 1986b), at least partially restored the absent hepatic function.

There are conflicting reports on rejection rate of allo- and xeno- geneic implants (Fuller, 1988), but microencapsulation (Dixit et al, 1990) or irradiation (Kawai et al, 1987) of hepatocytes has been shown to obviate the need for immunosuppressants, without impairment of hepatocyte function. While microencapsulation provides a physical barrier to immunoglobulins, it is not clear how irradiation eliminates the immune reaction. It is felt that irradiation may lead to the destruction of functional groups on the hepatocyte cell membrane that would otherwise be recognised as foreign by the host.

While this system, at the present time, seems to have advantages over an

extracorporeal system in its relative simplicity and in the long-term preservation of hepatocyte function, there are a few serious drawbacks. Some studies indicate that the graft must have time to become established *prior* to hepatic failure to provide adequate support (Jaffe et al, 1988). There may also be an increased risk of local infarction, cardiac arrest and stroke related to hepatocellular aggregation within the blood vessels (Demetriou et al, 1988). Finally, the source of the grafts presents some difficulties. If human tissue is used, the same problems associated with whole liver transplantation are encountered. With the use of xenogeneic liver cells, there is inadequate information into the long-term effects of foreign tissue implantation. Nevertheless, these trials have demonstrated the ability of isolated hepatocytes to provide support for acute liver failure.

1.4.4 Whole liver perfusion

Extracorporeal perfusion of intact liver was introduced in 1965 (Eiseman, 1965). This therapy, using the whole livers of pigs (Watts et al, 1967; Parbhoo et al, 1971), baboons (Abouna et al, 1973; Gundermann and Lie, 1980) and human cadavers (Gundermann and Lie, 1980), produced excellent reduction of blood ammonia, nitrogen and bilirubin (Watts et al, 1967), as well as an increase in clotting factors (Abouna et al, 1973). In experiments using 300 - 500 g of baboon liver, 8 of 12 patients in "grade IV and V" coma regained consciousness, with 5 achieving full recovery (Gundermann and Lie, 1980). Similarly, trials by Abouna et al (1973) restored consciousness in 13 of 21 episodes of grades IV and V coma. However, because of contact between the blood and foreign tissue, immune reactions may develop after repeated perfusions, especially with porcine tissue (Bersohn et al, 1969). In addition, widespread application of this sort of perfusion is hampered by its extreme technical complexity, the difficulty in

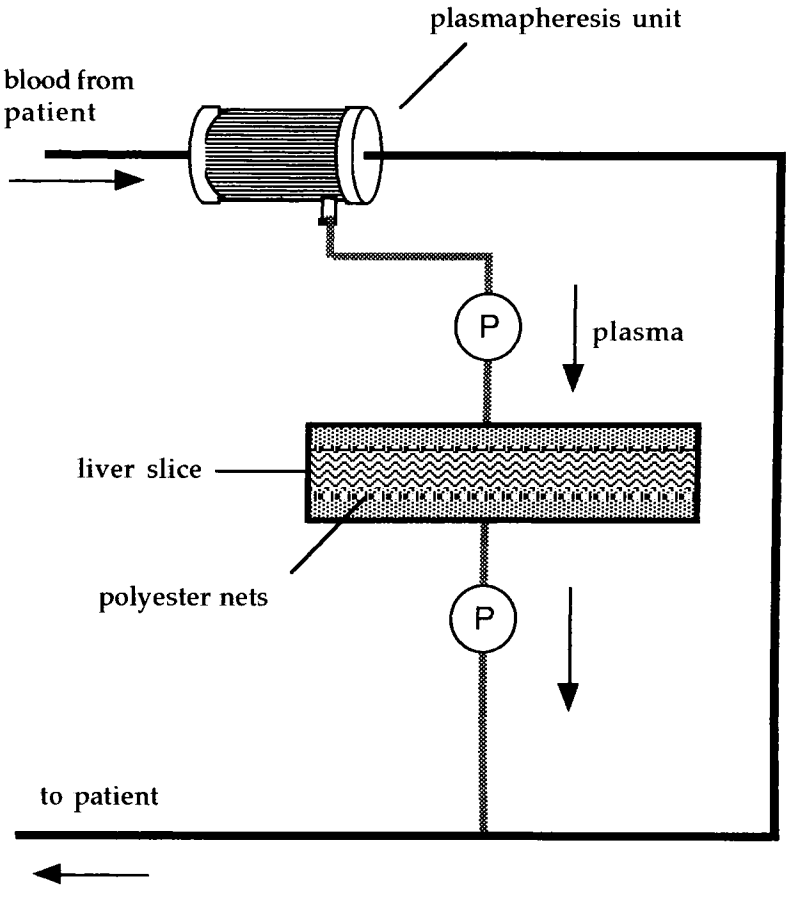
obtaining and maintaining primate donor livers and ethical arguments against the use of such livers.

1.4.5 Liver slice perfusion

Liver slice perfusion (Mikami and Nosé, 1959; Kimura et al, 1980) offers most of the advantages of whole liver perfusion, but it is technically simpler and easier to maintain. Early tests suggested that some essential metabolic activities were not provided by this technique: the average length of survival of hepatectomised pigs treated with their own tissue was just 30 hours (Soyer et al, 1973b). A later design (Nosé et al, 1977) employed a reactor chamber in which 1.5 mm thick tissue slices were sandwiched between porous (1 μm pore diameter) polyester nets. Plasma forced under pressure into the reactor chamber interacted with the liver tissue before being forced back into bulk flow (Figure 1.2, overleaf). Although these slices were found to be metabolically active, the rate of this activity was not sufficient for the needs of an averaged sized human. With an optimised design, it was estimated that only 250 g of tissue would be required to provide adequate support to an FHF patient (Soyer et al, 1973a).

The techniques described in this chapter were explored extensively during the 1970's and 1980's. Current trends are toward the development of a detoxification system utilising cultured hepatocytes or liver-derived cell lines as the primary unit of detoxification/metabolism in an extracorporeal liver assist device. These systems are discussed in great detail in the next chapter.

Figure 1.2: Schematic of liver slice perfusion by Nosé et al (1977)



Chapter 2: Bioreactors for Artificial Liver Support

2.1 Introduction

The shortcomings of the liver support systems discussed in Chapter 1 have led to increased interest in the use of isolated hepatocytes as the primary unit of detoxification in a bioartificial liver (BAL). It is envisaged that these cells would also provide essential synthetic and metabolic support. In its simplest form the BAL would consist of:

1. a biological reactor housing cultured hepatocytes. Several different reactor configurations are available commercially (eg. for hybridoma culture) and the choice of reactor would depend upon an evaluation of all system parameters.

2. a method of oxygenation, to maintain the proper balance of O_2 and CO_2 within the reactor. The gas balance may be controlled by:

    - circulation of an appropriate gas mixture through hollow fibres within the bioreactor, or
    - the perfusate may be oxygenated in a separate step prior to its entry into the bioreactor (this is feasible only for perfusates with a high oxygen solubility coefficient, eg. blood).

3. an immunological barrier between patient blood and the cultured hepatocytes. This may be accomplished by a plasmapheresis step, providing the pore size of the plasmapheresis membrane is small enough to exclude immunoglobulins. Alternatively, direct contact between patient blood or plasma and the cultured cells could be avoided entirely. If the reactor were composed of distinct blood and hepatocyte compartments (Figure 2.1, overleaf), an appropriate selection of

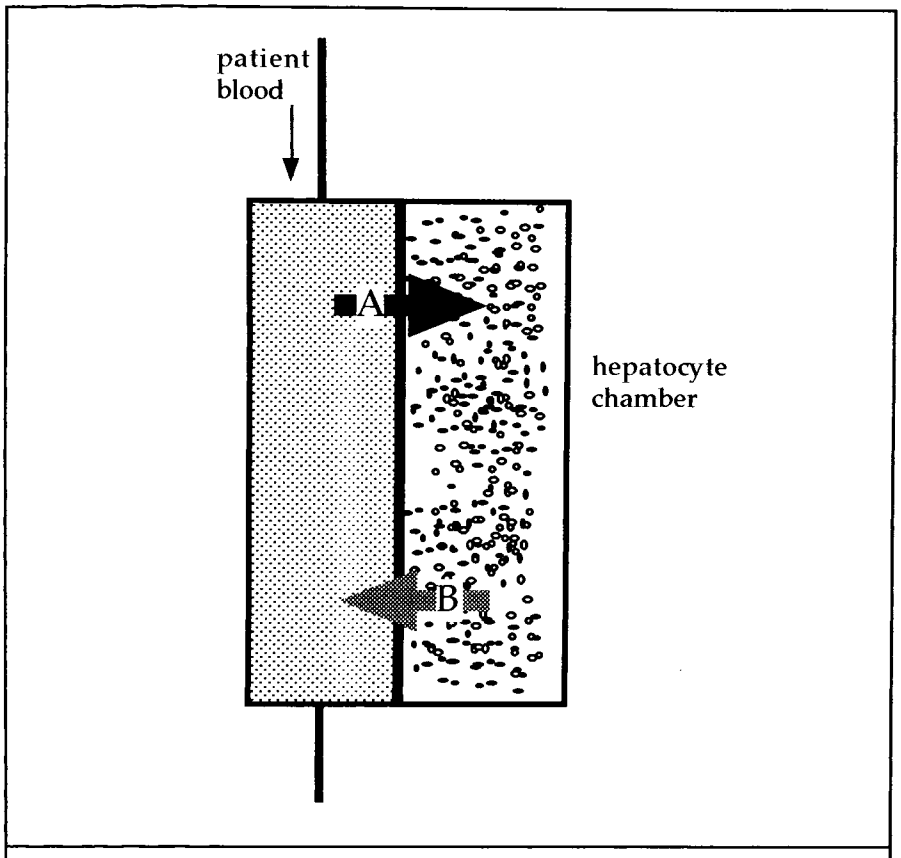


Figure 2.1: Schematic of an hepatocyte bioreactor with distinct blood and hepatocyte compartments. The hepatocytes are separated from the blood by a semipermeable membrane through which both toxins (A) and products formed by the hepatocytes (B) can diffuse.

separation membrane would allow diffusion of toxins and metabolites between compartments whilst preventing passage of immunoglobulins (molecular weight ≈ 150,000 Da; Smith et al, 1983). It would be essential for such a design to have a large membrane surface area to hepatocyte and blood compartment volume so that mass transfer resistance can be minimised. In each case, care should be taken not to restrict access of middle molecular weight toxins and protein bound substances to the hepatocytes. This would imply that the diffusion of

albumin, with a molecular weight of approximately 68,000 Da, should not be impeded. Nyberg et al (1992) have suggested that membranes with a nominal molecular weight cut-off between 67,000 - 100,000 Da are adequate for immunoprotection. It should therefore be possible to choose a membrane that excludes immunoglobulins but not protein bound toxins.

4. Some researchers (Takahama et al, 1983; Margulis et al, 1989; Rozga et al, 1993) believe that a sorbent stage should also be included, to supplement the detoxifying function of the cultured cells, and to remove potentially hepatotoxic substrates from the patients' blood so that hepatocyte function may be better preserved.

5. ancillary devices to regulate temperature, to detect and trap bubbles in the blood line and to detect haemolysis.

Implementation of 2 - 5 is fairly straightforward, and thus much effort has been directed toward improvements of the bioreactor module.

Design criteria for the BAL hepatocyte chamber (bioreactor) can be roughly divided into those which maximise the efficiency of the bioreactor, and those which seek to provide optimal conditions for hepatocyte function (Figure 2.2, overleaf). Unfortunately, these divisions are not always clear cut, and it is difficult to examine any one factor in isolation.

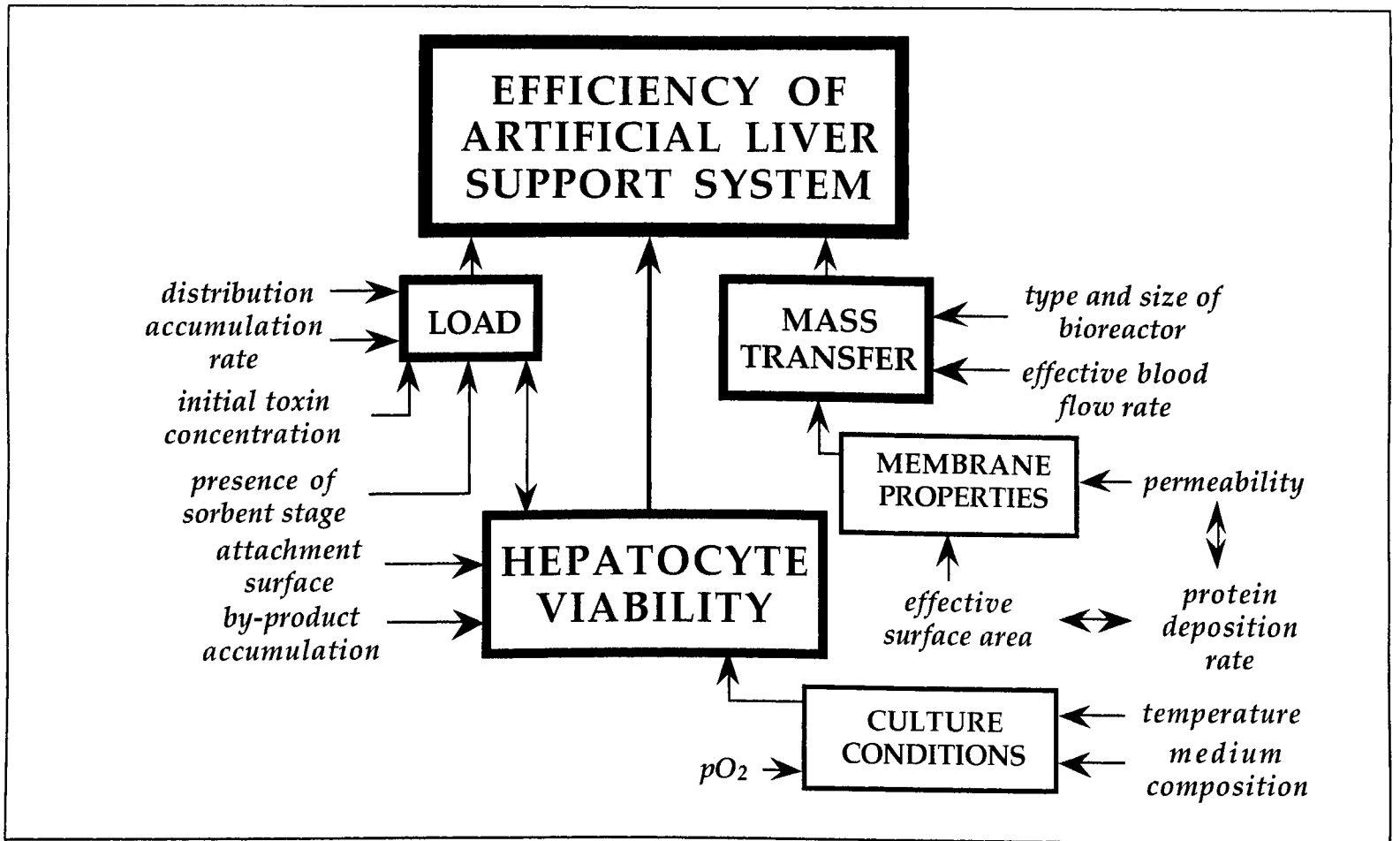


Figure 2.2: Relationships amongst some factors affecting the performance of the BAL

2.2 Model based bioreactor design

Bioreactor design as a part of the process industry is well researched and includes considerations such as (Venkatasubramanian et al, 1983):

- reducing the mass transfer resistance (eg. maximising the driving force, increasing surface area to volume ratio, etc.),
- elimination of stagnation regions (eg. through liquid residence time distribution analyses; Yarmush et al, 1992),
- provision of nutrients,
- elimination of unwanted by-products and
- regulation of hydraulic forces.

Much of this information can be applied to immobilised systems in general (Perry and Wang, 1989) and to the hepatocyte bioreactor in particular. Despite this fact, much research into the BAL has been empirical in nature. The two most notable exceptions (van Berlo et al, 1986; Ohshima et al, 1994) focus on mathematical models to predict the clearance of marker substances (bilirubin and ammonia respectively) from the blood of a patient supported by a BAL. It is understood that the levels of these marker substances are used only as an index of the biochemical normality of the patient's blood, and that their removal would not, in itself, be adequate for patient survival.

While the van Berlo and Ohshima models differ somewhat in their interpretation of the system (Figure 2.3, overleaf), both assume simple well-mixed compartments. A more detailed chemical engineering analysis, based on knowledge of the dynamics of a particular reactor type, might therefore be incorporated into these models to improve their predictive capacity. Nevertheless, even these simple models allow manipulation of some basic design parameters and predictions of bioreactor performance to be made (see Appendix B).

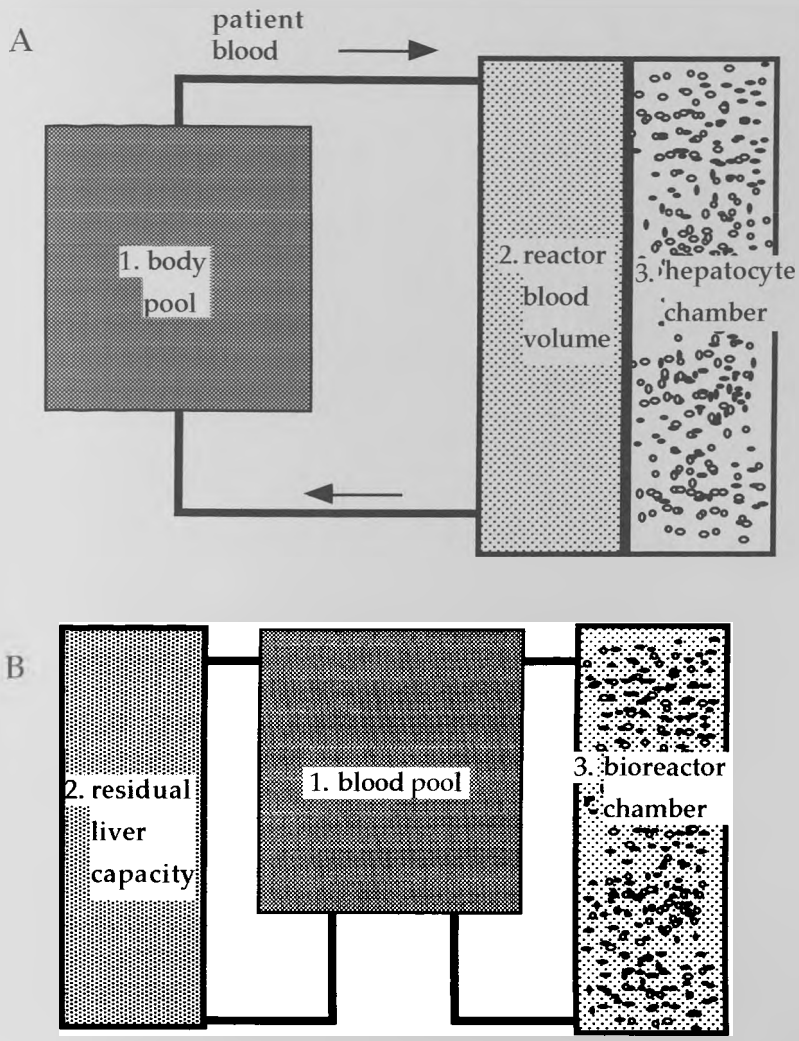


Figure 2.3: Organisation of compartments in Ohshima (A) and van Berlo (B) models of BAL detoxification capacity. For both models, each compartment has its own volume, flow rate and initial toxin concentration. Van Berlo's model, however, incorporates residual liver capacity as an additional enzyme reactor, whereas this function can only be represented in the Ohshima model by decreasing the coefficient for the generation rate of the marker toxin by the body. Furthermore, Ohshima's model assumes distinct hepatocyte and blood compartments whilst van Berlo's assumes even mixing of extracorporeal blood and hepatocytes.

Another model, employed by Rozga and coworkers (1993), is almost identical to the Ohshima model. The primary differences are:

- the Rozga model does not include the generation rate of the marker substance within the patient pool or the death rate of the hepatocytes within the bioreactor and
- the co-efficient describing mass transfer between the intra- and extra-capillary space is assumed to be variable in the Rozga model, to account for increased mass transfer due to transcompartmental fluid flow. *In vitro* testing of this model revealed substantial deviation (~ 100%, depending on overall flow rate) between theoretical and experimental mass transfer.

All of these models are notable in that they incorporate the system interaction with the patient, which is the greatest difference between the BAL and traditional bioreactor processes. Other aspects of the BAL reactor, such as pressure drop, transport processes and rates of clearance/product formation, are directly analogous to those encountered in the process industry and may be modelled as such.

2.3 Shear forces

Of the reactor variables which may be modelled, predictions of the drag force at the cell-fluid boundary are perhaps of special interest, as mammalian cells may be susceptible to shear damage (Born et al, 1992). Calculations by Perry and Wang (1989) demonstrate that the shear stress experienced by cells in one hollow fibre reactor is considerably less (3.6 dynes · cm^{-2} versus 8.8 dynes · cm^{-2}; 1 N · m^{-2} = 10 dynes · cm^{-2}) than that in a comparable microcarrier type packed bed reactor. While there does not appear to be any published data on the subject, it is possible that hepatocytes are similar in their shear resistance to endothelial cells, which exhibit metabolic but not morphologic changes in response to shear stresses of ~ 4 dynes · cm^{-2} (ibid).

High blood flow rates through the BAL may be necessary to treat a sufficient quantity of patient blood (de Groot et al, 1989). A healthy liver processes approximately 1500 ml · min^{-1} and works continuously, 24 hours a day. If, for example, only 20% of this daily flow were required and each perfusion session were 6 hours long, then a blood flow of approximately 1200 ml · min^{-1} through the reactor would be necessary. Depending on the reactor configuration, such flow rates could result in considerable shear stress.

The above argument assumes that biotransformation and/or toxin removal within the bioreactor are proportional to flow rate. This is not generally true in practice. For example, in haemodialysis it is well known that (see Figure 2.4, overleaf):

- clearance (the rate of toxin removal) does not increase linearly with increasing flow rates and
- clearance becomes increasingly flow independent as the molecular weight of the solute to be removed increases.

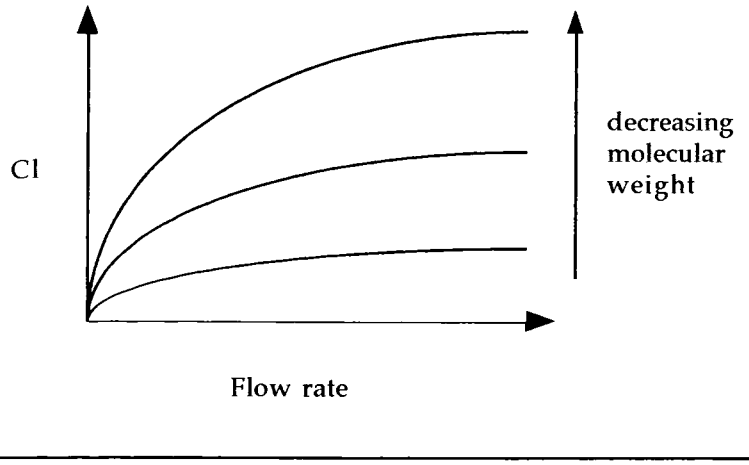


Figure 2.4: Dependence of clearance (Cl) on flow rate and solute molecular weight in haemodialysis

This effect is unlikely to be significant *in vivo*, due to the low local blood flow rates within the liver. Therefore, it is possible that a high daily throughput will need to be combined with low local flow rates (achieved through either extended lengths of perfusion or increased reactor volumes) within the bioreactor.

2.4 Hepatocyte numbers

An estimate of the number of cells required has been determined as follows (Matsumura et al, 1987):

> Surveys of liver disease patients have shown that health may be maintained in humans with a residual liver capacity (RLC) of only 30%. Acute liver failure results if approximately 10 - 30% of this critical mass is lost. It is therefore presumed that most patients have 20 - 27% RLC, and thus that only 3 - 10% of the total liver mass would have to be replaced in order to provide adequate support to the patient. Assuming that the average human liver is approximately 1.5 kg in mass, and that only 70% of this mass is accounted for by hepatocytes, then 30 - 100 g (~ 3×10^9 - 10^{10} individual cells) of isolated hepatocytes should be sufficient.

Although this calculation has been generally accepted (Takahashi et al, 1991), it assumes that hepatocyte function is not impaired during isolation and culture. Although isolated hepatocytes generally retain their *in vivo* metabolic functions, the capacity of these functions is often reduced and depends upon both culture conditions (eg. substratum for attachment) and culture medium composition (Uchino et al, 1988). An alternative estimate of the required cell mass might be obtained by comparing the minimum required removal rate of a putative toxin to the rate achieved experimentally in a BAL. For example, the minimum daily removal of ammonia in a

healthy person is estimated to be 345 mmol · day^{-1} (de Groot et al, 1989)[1]. One prototype bioreactor (Takahashi et al, 1992) was found to remove 1.28 ± 0.26 mg NH_3 · module^{-1} · h^{-1}. Each module contained 5 g hepatocytes, so this removal rate is approximately:

$$\frac{1.28 \times 10^{-3}\ \text{g NH}_3 \cdot \text{mole} \cdot \text{module} \cdot 24\ \text{h}}{\text{module} \cdot \text{h} \cdot 17\ \text{g NH}_3 \cdot 5\ \text{g cells} \cdot \text{day}} = \frac{0.36\ \text{mmol}}{\text{g cells} \cdot \text{day}}$$

and so therefore ~ 958 g hepatocytes would be needed. On the other hand, a bioreactor developed by Omokawa et al (1986) removed 6.96 x 10^{-4} mmol NH_3 · day^{-1} · g

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[1] de Groot uses this requirement to evaluate the efficiency of several non-biological liver support systems. However, he goes on to explain that his group have estimated the removal capacity required for survival to be 20% of this value, "since life can be sustained by about 20% of the total healthy liver". Unfortunately, there is a major flaw with this argument. The fact that 20% (or 30%, depending on the reference) of the total liver mass is sufficient to sustain life can not necessarily be extrapolated to say that only 20 - 30% of the required removal rate of toxins is sufficient. Presumably 20-30% of the total healthy liver mass is capable of removing 345 mmol · day^{-1} of ammonia (for example), and that is why that fraction is just sufficient for sustaining life. If these cells did not remove this amount, then ammonia (or whichever toxin was being studied) would begin to build up in the blood.

hepatocytes^{-1} which would imply that ~ 4.96 x 10^5 g hepatocytes would be needed.[2]

The lower of the two estimates, 958 g, is still a large requirement, ~ 9.6 x 10^{10} cells. If, however, the Matsumura analysis is combined with the de Groot information, and it is assumed that:

1. the patient has 20 - 27% RLC
2. 30% RLC is sufficient for the removal of 345 mmol of ammonia each day

then it can be predicted that the patient's RLC can metabolise 230 - 310 mmol of ammonia each day. The BAL would therefore have to remove only 35 - 115 mmol each day. Incorporating this result into the previous calculations would predict a required cell mass of 97.2 - 319 g for the Takahashi bioreactor or 50 - 165 kg for the Omokawa bioreactor. Improved cell performance could further reduce these requirements.

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[2] This result is not surprising as one would have expected superior performance from the Takahashi bioreactor (see section 2.10 for design description). Not only does this module have a greater mass transfer surface area to volume ratio (Takahashi: 100 cm^{-1}; Omokawa: 74.05 cm^{-1}) but the hepatocytes in the Takahashi bioreactor are provided with an attachment surface whilst those in the Omokawa bioreactor are in suspension culture. Hepatocytes are anchorage dependent cells and should thus exhibit poor performance in suspension culture in comparison to adhesion culture. Finally, the Takahashi bioreactor allows direct contact between the patient's plasma and cultured hepatocytes, whereas the Omokawa model has the additional mass transfer resistance imposed by the separation of the hepatocyte and plasma compartments. These examples illustrate not only the feasibility of a bioreactor as a means of artificial liver support (ALS), but also the potential importance of a combination of design parameters to the effectiveness of the BAL.

2.5 Cell densities

Many researchers feel that achievement of very high density cultures is of paramount importance in the development of BAL (Ohshima et al, 1986; Yanagi et al, 1990; Shatford et al, 1992). If, for example, it is assumed that 200 g of cells are to be used (~ 2×10^{10} cells) and that a system allowing direct contact[3] between patients' plasma and the cultured cells is employed, then even a relatively high density culture of 5×10^6 cells $\cdot$ ml^{-1} would require a priming volume of 4 litres. The average total plasma volume for an adult patient would be just 2.5 litres. This is not an insurmountable obstacle as the additional plasma could be supplied by donors. BAL therapy might then be viewed as an initial plasma exchange in which the normalised blood chemistry is perpetuated by subsequent hepatocyte haemoperfusion. However, it is worthwhile to point out that one of the primary motivations for the development of the BAL is the belief that synthetic functions of the liver cells, which cannot be provided by non-biological detoxification systems, are essential to survival. If a 4 litre priming volume were required, this extra plasma would result in a considerable dilution of those products manufactured by the cultured cells. As can be seen from Table 2.1 (overleaf) many BALs employ cell densities $\geq 10^7$ cells $\cdot$ ml^{-1}, ensuring more workable priming volumes.

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[3] The effect of cell density on priming volumes is also applicable to systems which employ distinct hepatocyte and blood compartments. Although the blood compartment need not necessarily increase in volume as the hepatocyte compartment increases for such a system, mass transfer resistance is generally minimised for a given diffusional surface area if both the volumes are equivalent.

Table 2.1: Comparison of culture methods and cell densities of some BALs in the literature. *Indicates methods which protect cells from fluid turbulence.

researchers	culture method	cells · ml^{-1}
Omokawa et al, 1986	suspension	10^7
Takahashi et al, 1992	monolayer	2×10^7
Margulis et al, 1989	external immobilisation on packed quartz granules	2×10^6
*Yanagi et al, 1990	immobilisation within a porous PVF resin	4.8×10^6
*Takabatake et al, 1991	encapsulated microaggregates	$1.5\text{-}2.3 \times 10^5$
*Nyberg et al, 1992	collagen gel-entrapped	10^7
*Ohshima et al, 1994	calcium alginate hydrogel-entrapped	1.2×10^7

Although high density culture techniques may permit improved BAL design, extremely high density cultures may obstruct fluid flows resulting in increased turbulence and shear stresses at the cell-liquid interface. In light of this, many of the methods used to support high density cultures (Table 2.1, above) provide a sort of external skeleton in which the cells are housed. Ohshima et al (1994) have reported that entrapment of hepatocytes within a calcium alginate hydrogel reduces the rate of cell death "significantly" in perfusion culture and that immobilisation of hepatocytes in a highly porous polyvinyl formol (PVF) resin results in a "practically negligible" death rate. There has been very little research to date on the effects of these types of "scaffolding" on transfer of substrates to and from the hepatocytes. Yanagi et al (1990)

found that perfusion cultures of rat hepatocytes in reticulated PVF exhibited stable levels of ammonia metabolism and urea production as cell density increased, whereas in conventional monolayer cultures there was a decline in these functions as cell density increased. This was attributed to a facilitated mass transfer of solute substances through the highly permeable PVF.

2.6 Provision of attachment surfaces

Another benefit of many of the high density culture techniques is that they provide a culture surface for the cells. Hepatocytes are highly polarised, anchorage dependent cells, whose function is modulated by interactions with neighbouring cells. The normal *in vivo* architecture is lost during cell isolation, and hepatocyte function declines thereafter. Additionally, damage to cell membranes during isolation reduces surface receptors, thus limiting substances which can be recognised and processed by the cell. Provision of an attachment surface enables at least partial reconstruction of the cell membrane and thus increases surface receptor numbers (Jauregui, 1991), slowing loss of function. Other culture methods, such as the hepatocyte sandwich configuration (Dunn et al, 1992), mimic the *in vivo* cell architecture, and thus help to reinstate cellular polarity. Researchers claim that this arrangement sustains function for up to 8 weeks, compared with conventional cultures on a single collagen layer, which last about 1-2 weeks (Yarmush et al, 1992). Even the collagen sandwich configuration, however, distorts normal hepatocyte polarity, as the two basolateral surfaces (those in contact with the collagen gel layers) cannot both participate in the uptake of oxygen and nutrients. *In vivo* cells receive nutrients and oxygen via diffusion through their basolateral membranes, whereas cultured cells are forced to obtain these through their apical surfaces. Alternative methodologies, such as culture on a permeable collagen coated membrane support (Cereijido et al, 1978), permit diffusion of nutrients through a single basolateral membrane and reinstate many polarised characteristics such as unidirectional transmembrane flow of fluids and ions and electrical potential differences, but it is not clear if this enhances cell function. Researchers have only attempted to maintain such hepatocyte cultures for up to 13 days (Sirica et al, 1978) and they exhibit unexplained morphological changes, such as a build-up of microfilaments beneath the apical surface. Further research is required to determine the most suitable and practicable culture configuration/attachment surface for the BAL.

2.7 Cell type

Many different types of cells have been used in prototype BALs, including primary cultures such as rat (Omokawa et al, 1986), dog (Uchino et al, 1986) and pig (Takahashi et al, 1992) hepatocytes as well as hepatocyte derived cell lines (Wolff et al, 1977; Sussman and Kelly, 1993). While human hepatocytes would appear to be the most appropriate in terms of reducing immunological reactions and in their similarity of metabolic response, sources of human cells are scarce. Furthermore, it is believed that immune reactions for all cell types could be eliminated by the appropriate selection of separation membrane (see section 2.1).

The greatest limitations to the use of primary hepatocytes are:

- fresh cells would have to be isolated for each reactor as hepatocytes have low mitotic activity in culture
- hepatocytes experience a rapid reduction in functional activity during culture.

Transformed cells might provide an alternative to the use of isolated hepatocytes. Requirements for such cells would be:

- strong maintenance of normal hepatocyte function
- no change in function as cells age/are passaged
- ability to proliferate in culture
- contact inhibition (so that BAL hepatocyte chambers are not overgrown)
- absence of viral sequences (to prevent transmission of oncogenes to the patient)

A cell line which is claimed to satisfy these requirements, C3A, has been developed by Sussman and Kelly (1993) and has had some success in the clinic where it was used in a

BAL to prolong the life of a 68 year old woman with acute liver failure. The patient showed dramatic improvement over 6 days of continuous treatment, but ultimately died of septic shock. More recently (Sussman et al, 1994) it has been reported that BAL cartridges using the C3A cell line have been used successfully as a bridge to transplantation in four cases of FHF (predicted mortality $\geq$ 90%) from July to August 1993.

2.8 Hepatocyte oxygen requirements

The dissolved oxygen concentration in the cell culture medium is another important variable. While provision of an adequate oxygen supply is often addressed in BAL designs, it is usually considered sufficient to deliver an excess of oxygen to the cells. Studies by Ohshima et al (1986, 1994) have determined that for pO_2 values > 200 mmHg, the metabolic reaction rate (eg. rate of metabolism of ammonia and n-caproic acid) remains stable whilst the cell death rate increases exponentially with increasing pO_2. Their results suggest that the optimal pO_2 for hepatocytes in culture lies between 100 and 200 mmHg. This differs from the normal *in vivo* environment, in which the range of pO_2 to which the cells are exposed lies between ~ 30 - 55 mmHg (Nauck et al, 1981). The endpoints of this internal gradient are a consequence of the supply and demand of oxygen within the organ: the liver is supplied with a mixture of venous and arterial blood (see Appendix A.1) and the liver cells steadily deplete oxygen from this stream as blood travels down the hepatic sinusoids.

It is probable that oxygen will be supplied directly to the cultured cells by diffusion through oxygen permeable hollow fibres within the bioreactor, rather than by a separate oxygenation step for the perfusate (see section 2.1). Although the latter might simplify design analysis, the flow rate necessary to deliver an adequate volume of oxygen by this method would probably result in excessive shear stresses at the fluid/cell interface. For example, assuming:

- plug flow
- uniform oxygen concentration at each cross section
- oxygen consumption is independent of oxygen concentration
- plasma as the perfusate (oxygen solubility similar to that of water; van Slycke et al, 1928)

then the volumetric flow rate (F_V) can be calculated from a macroscopic balance as follows:

$$F_V = \frac{R_C}{(C_i - C_o)}$$

where R_C is total oxygen consumed · bioreactor^{-1} · min^{-1}, and C_i and C_o are the concentrations of oxygen in the inlet and outlet streams, respectively. Given the following conditions:

- oxygen consumption of 24 nmol · 10^6 cells^{-1} · min^{-1} (Yarmush et al, 1992)
- 200 g hepatocytes (~ 2.0 x 10^{10} cells)
- inlet pO_2 = 200 mmHg (~ 280 nmol · l^{-1}; Noll et al, 1986)
- outlet pO_2 = 100 mmHg (~ 140 nmol · l^{-1})

then the volumetric flow rate would be ~ 3.4 x 10^3 l · min^{-1}. In reality, even this flow rate would be insufficient, as perfect mixing cannot be realised in practice.

Experimental values for oxygen consumption vary widely, and have increased by a factor of 20 from 1968 to 1992 (Table 2.2, overleaf). This is probably due to advances in hepatocyte culture; as greater cell function is retained, the cells become more active metabolically and thus require more oxygen. Oxygen consumption *in vivo* for rat liver has been determined to be as high as 16.88 x 10^{-12} ml O_2 · cell^{-1} · s^{-1} (McLimans et al, 1968; ≈ 2.7 μmol · 10^6 · cells^{-1} · h^{-1} if all cell types in the liver are assumed to have similar oxygen requirements, or ~ 3.9 μmol · 10^6 · cells^{-1} · h^{-1} if the parenchymal cells are assumed to be the predominant oxygen users), so this might be near the upper limit

Table 2.2: Oxygen consumption of cultured hepatocytes

researchers	reported value	in $\mu mol \cdot 10^6$ $cells^{-1} \cdot h^{-1}$
McLimans et al, 1968	0.46×10^{-12} ml $\cdot$ $cell^{-1} \cdot s^{-1}$	0.074
Jensen, 1977	0.11 $\mu mol \cdot 10^6$ $cells^{-1} \cdot h^{-1}$	0.11
Noll et al, 1986	23 nmol $\cdot$ 10^6 $cells^{-1} \cdot min^{-1}$	1.4
Yarmush et al, 1992	0.8 nmol $\cdot$ 2×10^6 $cells^{-1} \cdot s^{-1}$	1.44

of oxygen consumption for rat hepatocytes. However, oxygen consumption for whole liver varies depending on the species studied. For example, human liver consumes 2.89 x 10^{-12} ml O_2 $\cdot$ $cell^{-1}$ $\cdot$ s^{-1} (ibid; $\approx$ 0.46 - 0.66 $\mu mol \cdot 10^6$ $cells^{-1} \cdot h^{-1}$, following the argument used for rat hepatocytes). Information about the effect of pO_2 on the maintenance of cell function, as well as the effect of various culture conditions on oxygen consumption for a given cell type, would therefore help to improve the bioreactor environment by allowing optimal oxygenation; these effects are explored as part of this thesis.

2.9 Perfusate composition

In most biological reactors, the culture medium can be chosen to optimise cell function. Many BAL designs, however, require direct contact between the patients' blood or plasma and the cultured hepatocytes. While this minimises mass transfer resistance within the reactor chamber, patient blood/plasma may not satisfy the nutritional requirements of the cells. Much effort has been directed toward the development of serum-free chemically defined culture media; consequently, there has been little research into the metabolic competence of cultured cells in plasma. One of the few studies into this area (Cunningham and Hodgson, 1992) has shown encouraging results. Microcarrier attached Hep G2 cells cultured in human plasma for 24 hours demonstrated a significantly higher protein synthetic rate than those cultured in RPMI 1640 medium and maintained time linear glucose metabolism. No information is available on the effect of longer periods of culture in plasma or serum on either hepatocytes or liver-derived cell lines.

The effect of bile in the culture medium is also worth consideration. In addition to the loss of cellular polarity, another consequence of the disruption of the normal *in vivo* architecture of the liver during cell isolation is the loss of the normal bile circulation. In the liver, bile is channelled away from the hepatocytes into bile ducts toward the gall bladder, whereas in culture the bile must diffuse directly into the culture medium. In a high density BAL with no provision for bile removal, a build up of bile in the plasma and/or culture medium is possible, and may be counter-productive. In addition, serum bile acids are elevated in liver failure (Franco, 1991), and can be up to 800 times greater than in health (see section 6.2). At least one group has suggested that some components of bile in the patients blood may prematurely impair the function of the cells in the BAL (Takahama et al, 1983), and have thus recommended the use of sorbents specific to bilirubin and bile salts. Additional research to determine the effect of bile on cultured cells is required, and forms part of this thesis.

2.10 Prototype BALs

The following pages contain a synopsis of some of the BAL designs reported in the literature. Because the information provided by these reports appears to be selective and there is a lack of uniformity in the testing procedures employed, an effort has been made to present this data in a standardised form, and to particularly note details with reference to some of the parameters discussed in this chapter. All information given refers to the most recent publication from which the data could be obtained.

As precise design information is often lacking, it is difficult to make objective assessments of the merits or disadvantages of the individual BAL systems described. Some potential difficulties may, however, be addressed intuitively. For example, in the system described by Margulis et al (1989), it would seem that the extreme bioincompatibility of uncoated activated charcoal (as described in section 1.3.2) has not been addressed. This bioreactor configuration, which is essentially a slurry of patient blood, hepatocytes and activated charcoal, may therefore increase the likelihood of damage to or adsorption of the formed elements of the patients' blood and of toxic reactions with the hepatocyte suspension. In addition, no attachment surface has been provided for the hepatocytes, and no provision made to prevent an immune reaction between the patients' blood and the (porcine) hepatocytes. It would be expected, from the information provided, that the lifespan of the hepatocytes in this BAL would be significantly reduced in comparison to the other BALs presented. The success rate reported in the literature is surprising.

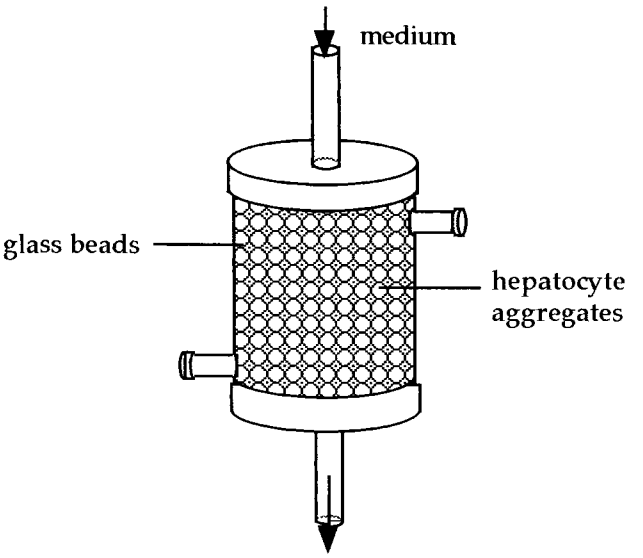
Other BAL configurations, while not so unfavourable for hepatocyte culture, would be expected to provide sub-optimal conditions. These include the BALs described by Matsumura et al (1987) and Omokawa et al (1986), which also employ suspension cultures and thus would not be expected to optimise hepatocyte lifespan. In addition, the BAL described by Yanagi et al (1989) does not appear to provide any form of immunoprotection for the (rat) hepatocytes.

The BAL reported by Takabatake et al (1991) has a potential drawback in the low hepatocyte culture density. It would therefore be expected that an unfeasibly large bioreactor volume would be required to effectively treat an FHF patient. In addition, oxygen is supplied to the cells via pre-oxygenated perfusate. If the cell density were increased, then the oxygen supply may not be adequate to maintain optimal cell function (see sections 2.1 and 2.8).

The other BALs, in the opinion of the author, are comparable in their strengths and weaknesses. The BAL described by Rozga et al (1993) would seem to present some obstacles to mass transfer, in that detoxification would rely upon diffusion of the chemical species present in the blood over a relatively large distance to the cultured hepatocytes, but this has been inferred solely from the drawings presented in the literature, rather than from specific design details. The BALs described by Nyberg et al (1993) and Sussman and Kelly (1993) have separate hepatocyte and blood/plasma compartments. However, losses in efficiency due to mass transfer limitations imposed by this design may be at least partially recovered by the fact that a large surface area for mass transfer has been provided. It is possible that the high density of the cultures and the irregularity of the flow boundary in the BALs described by Li et al (1993) and by Takahashi et al (1992) may induce significant turbulence and high shear rates at the cell-fluid boundary, and may also lead to a lack of flow uniformity. However, there is no evidence of these difficulties presented in the literature.

Overall, it appears that significant advances have been made in BAL design, and that the number and success of clinical results are improving. Nevertheless, significant scope exists for improvements in both hepatocyte culture technique and optimisation of the BAL design.

Li et al (1993)



brief description: The BAL is composed of multicellular hepatocyte aggregates entrapped within a cylinder by glass beads. This system was commercially developed as a method of long term hepatocyte culture, but the developers envisage its use as a potential BAL.

contact: direct (with plasma)

effective volume: ~ 96.2 ml

cell type: rat hepatocytes

culture method: packed bed of multicellular aggregates

cell number: 2×10^8

cell density: ~2.1×10^6 cells · ml^{-1}

perfusate flow rate: 40 ml · min^{-1}

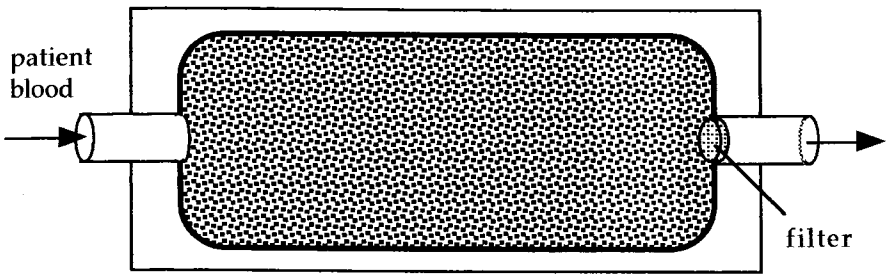
oxygenation: medium oxygenated prior to entry into bioreactor

immunoprotection: to be provided by plasmapheresis

testing procedure:

in vitro: With Waymouth 752 medium as the perfusate, cultures were able to maintain time linear synthesis of urea and albumin for up to 20 days.

Margulis et al (1989)



brief description: The BAL is composed of a polychlorovinyl capsule containing a mixture of hepatocytes and activated charcoal. The patient's blood is diverted through this suspension by an arteriovenous shunt and the non-blood components are retained against fluid flow by a nylon filter.

contact: direct (with blood)

volume: 20 ml

cell type: porcine hepatocytes

culture method: suspension

cell number: 4×10^7

cell density: 2×10^6 cells $\cdot$ ml^{-1}

blood flow rate: $\sim 90 \pm 10$ ml $\cdot$ min^{-1}

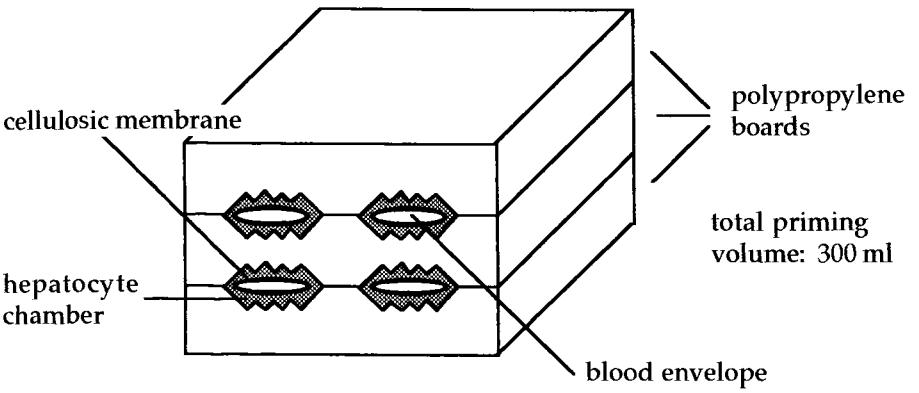
oxygenation: O_2 obtained from patient's blood

immunoprotection: none specified

testing procedure:

clinical: A group of 59 acute hepatic insufficiency patients treated with the BAL in addition to conventional therapy had a survival rate of ~ 63%, whereas a group of 67 patients given only conventional treatment had a survival rate of ~ 39%.

Matsumura et al (1987)



brief description: The BAL is a Kiil dialyser in which the dialysate compartment is filled with isolated hepatocytes. Blood flows through a cellulosic membrane envelope which is permeable to both toxins and products formed by the hepatocytes.

contact: separate blood and hepatocyte compartments

blood compartment volume: unspecified

hepatocyte compartment volume: ~ 300 ml

surface area for mass transfer: 1 m^2

cell type: rabbit hepatocytes

culture method: suspension

cell number: 10^{10}

cell density: ~ 3.3×10^7 cells · ml^{-1}

blood flow rate: 145 ml · min^{-1}

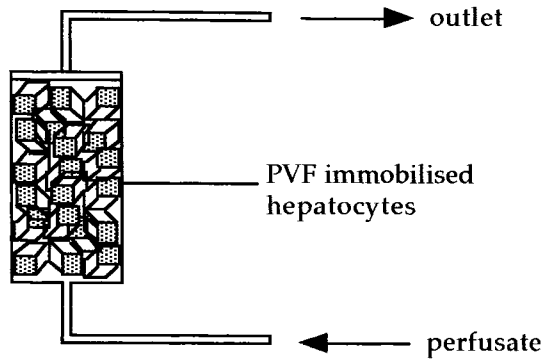
oxygenation: unspecified

immunoprotection: provided by cellulosic membrane barrier

testing procedure:

clinical case study: A 45 year old man with worsening hepatic failure due to advanced carcinoma of the bile duct was treated with 2 sessions of "hepadialysis" lasting 5 hours and 12 minutes each. Serum bilirubin was reduced from 25 mg · dl^{-1} to 8 mg · dl^{-1} over both sessions. He was discharged from hospital with little expectation of survival.

Miyoshi et al (1994)



brief description: The BAL is composed of packed bed filled with hepatocytes entrapped within 2 mm PVF resin cubes (500). Patient blood or plasma would be perfused through this bed.

contact: direct (with blood or plasma)

volume: 4 ml

cell type: rat hepatocytes

culture method: immobilised within a PVF resin

cell number: $1 - 2.4 \times 10^7$

cell density: $2.7 - 5.9 \times 10^6$ cells $\cdot$ ml^{-1}

perfusate flow rate: 13.2 - 17.4 ml $\cdot$ min^{-1}

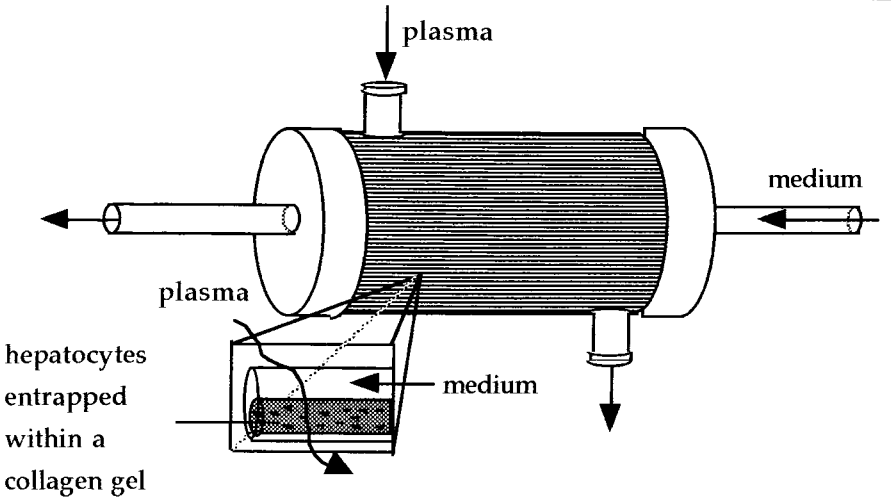
oxygenation: perfusate oxygenated prior to entry into bioreactor

immunoprotection: none specified

testing procedure:

in vitro: Ammonia metabolism, urea synthesis and albumin synthesis were measured over an eight day period. These functions were well preserved in comparison to monolayer cultures, but result varied according to the culture medium used.

Nyberg et al (1993a, 1993b, 1992); Shatford et al (1991, 1992)



brief description: Hepatocytes and collagen gel are injected into the intraluminal space of a hollow fibre bioreactor. The gel contracts, leaving a void space through which medium supplying nutrients can be perfused. The patient's plasma flows through the extracapillary space more or less diagonally across the hollow fibres.

contact: separate blood and hepatocyte compartments

plasma compartment volume: unspecified

hepatocyte compartment volume: ~ 10 ml

surface area for mass transfer: unspecified

cell type: rat hepatocytes

culture method: entrapped in a collagen matrix

cell number: $5 - 10 \times 10^7$

cell density: $5 - 10 \times 10^6$ cells $\cdot$ ml^{-1}

plasma flow rate: 30 ml $\cdot$ min^{-1}

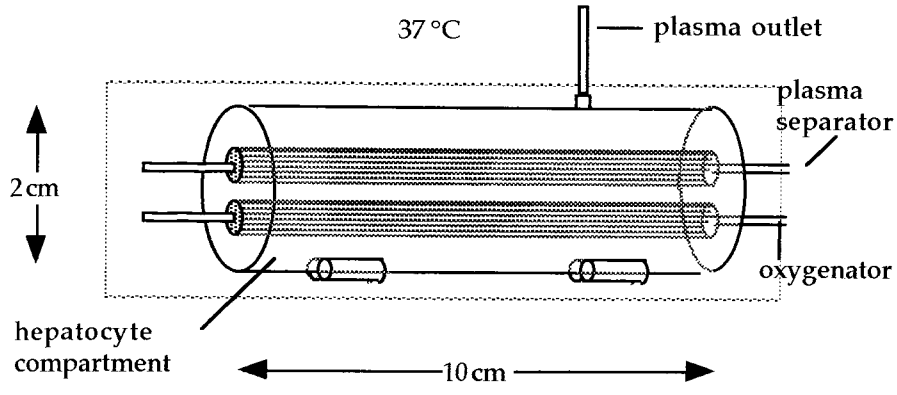
oxygenation: by extracapillary flow

immunoprotection: provided by hollow fibre barrier

testing procedure:

in vitro: Using Williams' E medium as the perfusate for both the intra- and extra-capillary spaces, albumin synthesis was maintained for 2 weeks. Arginine clearance and ornithine and urea production were maintained for 5 days.

Omokawa et al (1986)



brief description: The BAL is comprised of a hollow cylinder containing isolated hepatocytes, one hollow fibre plasmapheresis unit and one hollow fibre oxygenation unit. Whole blood flows through the plasma separator and the resulting plasma enters the hepatocyte compartment. Plasma is removed from the chamber and reunited with bulk flow. The entire unit is rolled back and forth to keep the hepatocytes in suspension.

contact: direct contact (with plasma)

effective volume: 30 ml

cell type: rat hepatocytes

culture method: suspension

cell number: 3×10^8

cell density: 1×10^7 cells $\cdot$ ml^{-1}

plasma flow rate: 1.0 - 1.2 ml $\cdot$ min^{-1}

oxygenation: O_2 is obtained from the hollow fibre oxygenation unit

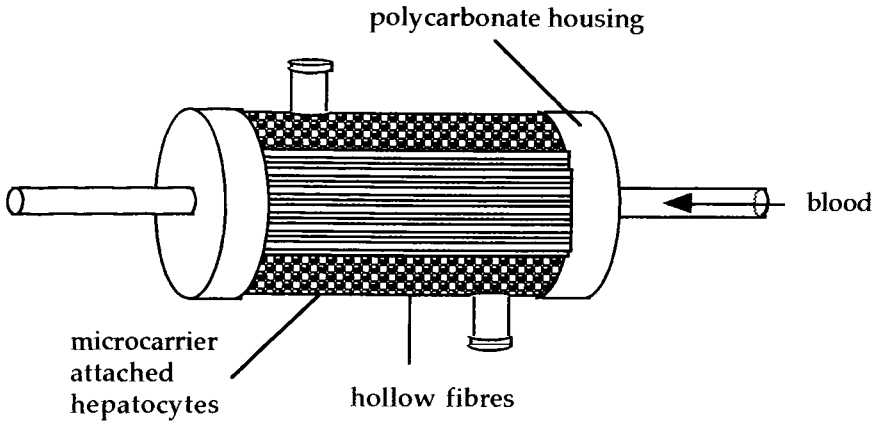
immunoprotection: provided by hollow fibre barrier

testing procedure:

in vitro: A three hour perfusion using Hanks' solution in place of plasma showed that ammonia was metabolised at a rate of approximately $2.9 \pm 0.5 \times 10^{-14}$ mol $\cdot$ cell$^{-1} \cdot$ h^{-1} by the BAL.

ex vivo: Survival time of anhepatic rabbits increased from 10.63 ± 1.6 hours in an untreated group to 15.3 ± 0.8 hours in a group treated with the BAL.

Rozga et al (1993a, 1993b, 1994); Neuzil et al (1993); Arnaout et al (1990)



brief description: The BAL is a hollow fibre module with microcarrier attached hepatocytes in the extracapillary space. Blood flows through the lumen of the hollow fibres, through which toxins in the blood and products formed by the hepatocytes diffuse. In some experiments, a column containing 300 g cellulose-coated activated charcoal was included in the perfusion stream.

contact: separate blood and hepatocyte compartments

blood compartment volume: unspecified

hepatocyte compartment volume: 200 ml

surface area for mass transfer: ~ 6000 cm^2

cell type: porcine hepatocytes

culture method: packed bed (microcarrier)

cell number: 4 - 6 x 10^9

cell density: 2 - 3 x 10^7 cells · ml^{-1}

blood flow rate: 220 - 400 ml · min^{-1}

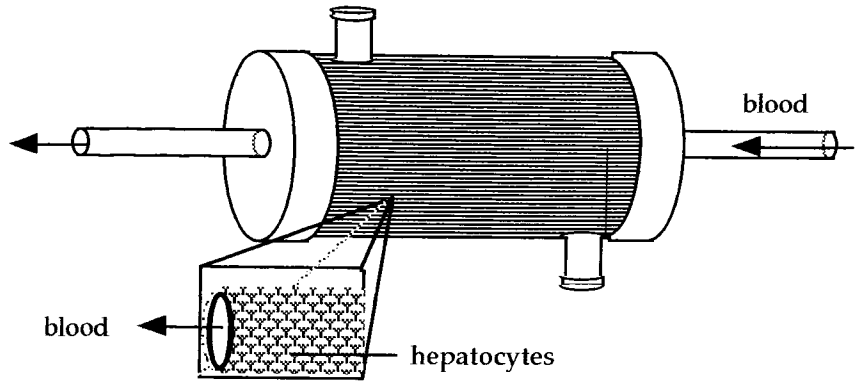
oxygenation: unspecified

immunoprotection: provided by hollow fibre barrier

testing procedure:

clinical: This BAL has been used successfully as a bridge to transplantation for 7 patients with FHF of varying etiology.

Sussman and Kelly (1993); Sussman et al (1994a, 1994b); Gislason et al (1994); Wood et al (1993)



brief description: Cells are cultured in the extracapillary space of a hollow fibre bioreactor, whilst patient blood flows within the hollow fibres. Toxins diffuse from the patient's blood through the hollow fibre membranes to the cell mass, and products formed by the cells diffuse into the patient's blood.

contact: separate blood and hepatocyte compartments

blood compartment volume: unspecified

hepatocyte compartment volume: unspecified

surface area for mass transfer: unspecified

cell type: a cell line, C3A

culture method: grown to fill bioreactor capacity

cell number: ~ 200 g

cell density: "high" (otherwise unspecified)

blood flow rate: ~ 150 ml · min^{-1}

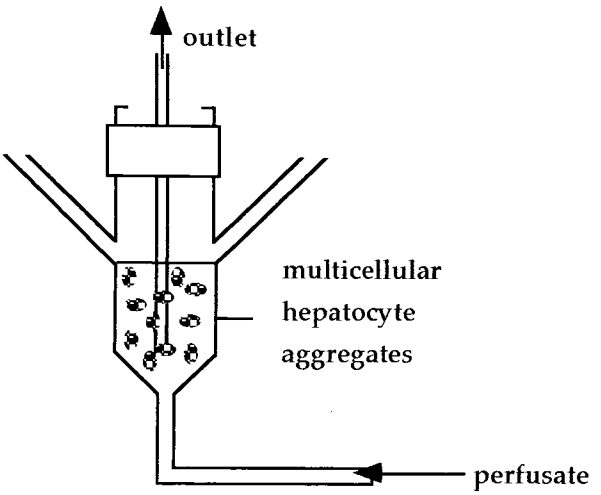
oxygenation: unspecified

immunoprotection: provided by hollow fibre barrier

testing procedure:

clinical: Of 11 FHF patients (varied etiology) treated with this BAL, 6 died (5 of cerebral oedema), 4 survived to liver transplantation and 1 made a full recovery.

Takabatake et al (1991)



brief description: The BAL is composed of multicellular hepatocyte aggregates in a spouted bed bioreactor. The aggregates are kept in suspension by the upward flow of the perfusate.

contact: direct (with blood or plasma)

volume: 70 ml

cell type: rat hepatocytes

culture method: multicellular aggregate suspension

cell number: 1.5 - 2.3 x 10^7

cell density: 2.1 - 3.3 x 10^5 cells · ml^{-1}

perfusate flow rate: 3 - 4 ml · min^{-1}

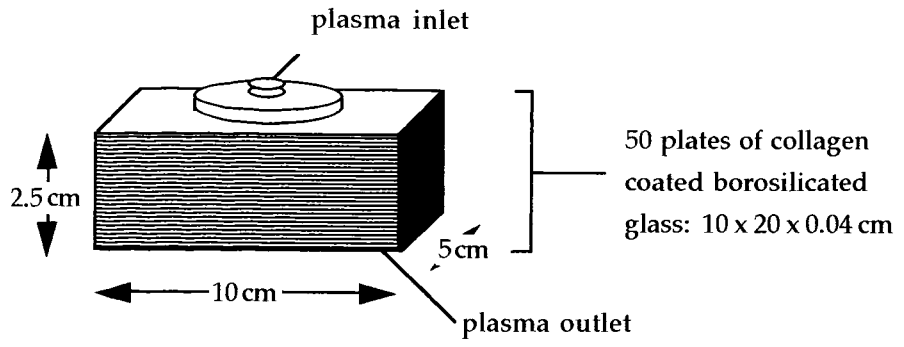
oxygenation: medium oxygenated prior to entry into bioreactor

immunoprotection: none specified

testing procedure:

in vitro: Albumin and urea synthesis were well preserved throughout a 66 hour perfusion with hormonally defined medium.

Takahashi et al (1992)



brief description: Hepatocytes are cultured on collagen coated borosilicated glass plates, which are then stacked within a transparent acrylic container. Plasma is perfused from top to bottom.

contact: direct (with plasma)

effective volume: 25 ml

culture surface area: 2500 cm^{-1}

cell type: porcine hepatocytes

culture method: monolayer

cell number: 5×10^8

cell density: 2×10^7 cells · ml^{-1}

blood flow rate: 40 ml · min^{-1}

oxygenation: plasma oxygenated prior to entry into bioreactor

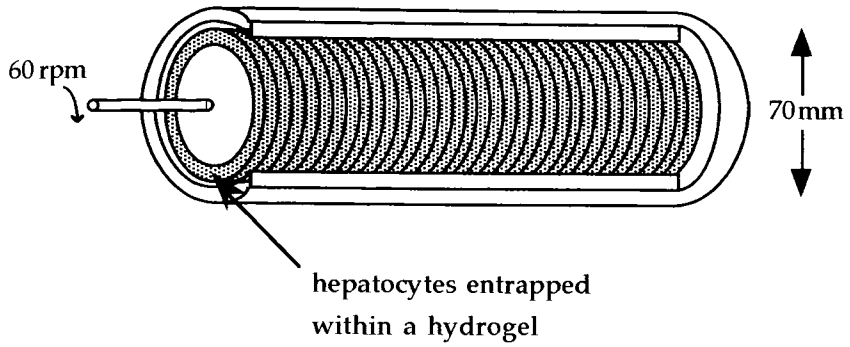
immunoprotection: provided by plasmapheresis

testing procedure:

in vitro: The bioreactor was perfused with Leibovitz L-15 Medium. After 24 hours, gluconeogenesis was measured to be 5.08 mg · $module^{-1}$ · h^{-1}, and urea synthesis was 0.7 mg · $module^{-1}$ · h^{-1}.

ex vivo: Anhepatic rabbits treated with this BAL had an average survival time of 24.5 h compared to 15.5 h for untreated anhepatic rabbits.

Yanagi et al (1989)



brief description: 40 disks containing hepatocytes entrapped within a calcium alginate hydrogel are mounted on a horizontally rotating axis and enclosed within a cylinder (material unspecified). Blood flows along the bottom of the cylinder and the total priming volume for the system is 80 ml.

contact: diffusion through thin layer of hydrogel

cell type: rat hepatocytes

culture method: entrapped within calcium alginate hydrogel

cell number: unspecified

cell density: 0.19 - 1.7 x 10^7 (within the hydrogel)

perfusate flow rate: 10 ml · min^{-1}

oxygenation: blood oxygenated prior to entry into bioreactor (pO_2 maintained > 300 mmHg)

immunoprotection: none specified

testing procedure:

in vitro: Williams' E supplemented with 10% FCS and 10 μM dexamethasone was used as the perfusate. Ammonia was measured over a 4 h period and was reduced from 10^{-4} M to 4 x 10^{-5} M.

ex vivo: Adult mongrel cats with acute hepatic insufficiency induced by end to end portocaval shunt and ligation of hepatic artery were used as a model of hepatic failure. Blood ammonia levels were reduced by 3.4 $\pm$ 3.3% h^{-1}.

2.11 Thesis objectives

Studies which supplement existing knowledge about the effects of perfusate composition and oxygen concentration on the function of cells[4] in the BAL are presented in this thesis. The purpose of these studies were to present:

I. a preliminary study on the long term effects of exposure to serum on the viability of cultured cells investigating:
   A. the effect of increasing concentrations of newborn calf serum (NCS) on the GSH content and growth rate of Hep G2 cells,
   B. the effect of NCS supplemented with amino acids on the GSH content and growth rate of Hep G2 cells and
   C. the effect of human serum and amino acid-supplemented human serum on the GSH content and growth rate of Hep G2 cells,

II. a supplementary study into the effect of exposure to serum on confluent Hep G2 cells measuring:
   A. the effect of exposure to increasing concentrations of NCS on the GSH and protein content of confluent Hep G2 cells,
   B. the timecourse of GSH depletion from confluent Hep G2 cells exposed to 100% NCS and

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[4] Many of these experiments were performed on Hep G2 cells, a human hepatoma derived cell line. Hep G2 are considered to be a good model system for predicting the metabolic and toxicological behaviour of human hepatocytes. The activity of the cytochrome P450 mixed function oxidase system, and several conjugation pathways have been compared in freshly isolated human hepatocytes and in Hep G2 cells. When Hep G2 cells are cultured in Williams' E medium their metabolic profile is similar to that of human hepatocytes (Doostdar et al, 1988; Grant et al, 1985).

C. the effect of supplementation of NCS with amino acids on the timecourse of GSH depletion,

III. a preliminary investigation into the effect of bile in the culture medium on Hep G2 cells measuring:

A. the effect of exposure to increasing bile concentrations on the growth, GSH content, and LDH activity of Hep G2 cells and

B. the effect of exposure to increasing bile concentrations on the protein content, GSH content and LDH activity of confluent cultures of Hep G2 cells,

IV. a study of the effect of environmental factors on the oxygen consumption of cultured cells investigating:

A. the effect of cell density on the oxygen consumption of Hep G2 cells and

B. the effect of dissolved pO_2 on the oxygen consumption of Hep G2 cells and

V. an examination of cell tolerance to variations in pO_2:

A. the effect of increasing oxygen concentration on ^{3}H-leucine incorporation, cytochrome P450 content, LDH and EROD activity, on cultured rat hepatocytes and

B. the effect of increasing oxygen concentration on ^{3}H-leucine incorporation, LDH and EROD activity, on Hep G2 cells.

Chapter 3: Tissue Culture Materials and Methods

3.1 Materials

All chemicals were obtained from Sigma, (Poole, England) except where otherwise indicated.

Ammonium chloride Cupric sulphate Mercuric chloride	BDH Chemicals Limited, Poole, England.
Penicillin : streptomycin (PEST) (5000 IU $\cdot$ ml^{-1} : 5000 μg $\cdot$ ml^{-1}) L-glutamine Fungizone	Flow Laboratories, Irvine, Scotland.
Trypsin (2.5% (w/v))	Northumbria Biologicals Limited, Cramlington, England.
Foetal Calf Serum (FCS)	Sera-Lab, Sussex, England.
Newborn Calf Serum (NCS) Williams' E Medium Modified Earle's Medium RPMI 1640 Modified Amino Acid Supplement x 100	Gibco, Poole, England.
Brain Heart Infusion Broth (BHI) Sabouraud's Medium	Oxoid Limited, Basingstoke, England.
L-[4,5] ^{3}H-leucine 120 - 190 Ci $\cdot$ mmol^{-1}	Amersham International PLC, Amersham, England.
75 cm^2 cell culture flasks 24 well tissue culture plates	Costar, Poole, England.
100 mm tissue culture dishes 0.22 μm bottle top filters	Falcon, Edinburgh, Scotland.

Sterivex-GS filter units (0.22 μm)	Millipore (UK) Limited, Middlesex, England.
Cytodex 3 microcarrier beads	Pharmacia Fine Chemicals, Uppsala, Sweden.
150 ml, 250 ml Techne stirrer flasks	Techne, Cambridge, England.

Sheep bile was generously supplied by Dr. V. Zammit of the Hannah Institute, Ayr, Scotland.

3.2 Culture media, buffers and stock solutions

3.2.1 Tissue culture media

(a) Media used for Hep G2 cell culture

Williams' E medium (450 ml supplemented with 2 mM L-glutamine, 5 ml PEST and 50 ml FCS) was the usual culture medium for Hep G2 cells. This mixture was further supplemented with 15 mM N - [2 - hydroxyethyl]piperazine - N' - [2 - ethanesulphonic acid] (HEPES) for use in microcarrier culture of Hep G2 cells.

Some experiments required the culture of Hep G2 in different concentrations of NCS, and in NCS supplemented with the amino acids L-cysteine, glycine and L-glutamic acid. These preparations are listed in Table 3.1

Table 3.1: Compositions of the media used

Medium composition	Abbreviation
Williams' E + PEST + L-glutamine + 10% (v/v) FCS	CCM
Williams' E + PEST + L-glutamine + 10% (v/v) NCS	10% NCS
Williams' E + PEST + L-glutamine + 25% (v/v) NCS	25% NCS
Williams' E + PEST + L-glutamine + 50% (v/v) NCS	50% NCS
Williams' E + PEST + L-glutamine + 75% (v/v) NCS	75% NCS
100% NCS	100% NCS
100% NCS + L-glutamic acid (50 mM), glycine (50 mM) and L-cysteine (50 mM).	100% NCS+

for clarity.

Human serum was also used as a culture medium and was prepared as follows: Blood was collected aseptically from human volunteers into sterile plastic bags without anti-coagulant. It was then centrifuged for 30 minutes at 800 g and the plasma expelled using a standard expresser. This plasma was centrifuged at 3000 g for 15 minutes. The supernatant was removed and allowed to clot at 4° C for 18 hours. The resulting serum was filtered with 125 μm sterile gauze mesh and stored at -20° C or used immediately. Human serum supplemented with 50 mM L-cysteine, glycine and L-glutamic acid was also used as a culture medium.

(b) Medium used for hepatocyte culture (KI medium)

KI medium was prepared from 450 ml Modified Earle's Medium supplemented with 50 ml RPMI modified amino acid solution, 25 ml FCS, 10 ml PEST and 5 ml fungizone. All experiments with rat hepatocytes used this medium.

All media were adjusted to pH 7.6 with sterile 1 M sodium hydroxide.

3.2.2 Buffers

(a) Sodium phosphate buffer

0.2 M Na_2HPO_4	87 ml
0.2 M NaH_2PO_4	13 ml

The pH was adjusted to 7.6 using the appropriate solution and the mixture diluted 1 : 1 with distilled water.

(a) P450 buffer

0.1 M sodium phosphate buffer, pH 7.6	80 ml
glycerol	20 ml
EGTA	37.5 mg
Dithiothreitol	15.45 mg
Nonidet P-40 10% (w/v)	200 μl

3.2.3 Stock solutions

(a) Trypsin

NaCl	4 g
Na_2HPO_4	0.05 g
glucose	0.5 g
Tris	1.5 g
KCl 19% (w/v)	1 ml
phenol red 1% (w/v)	0.75 ml

Dissolved in 500 ml distilled water. The pH was adjusted to 7.7 at room temperature with concentrated HCl. After sterilisation by autoclaving, 5 ml PEST and 50 ml sterile trypsin (2.5%, w/v) were added.

(b) Versene

NaCl	4 g
KCl	0.1 g
Na_2HPO_4	0.58 g
KH_2PO_4	0.1 g
EDTA	0.1 g
Phenol red 1% (w/v)	0.75 ml

Dissolved in 500 ml distilled water, and sterilised by autoclaving.

3.3 Maintenance and checks for sterility

Cell experiments and tissue culture manipulations were carried out under sterile conditions in a laminar flow hood. Glassware and heat stable solutions were sterilised by autoclaving for 20 minutes at 120° C. Solutions that could not be autoclaved were sterilised by membrane filtration using 0.22 μm Millipore filters. Culture media and other sterile solutions were checked routinely for bacterial contamination with BHI and fungal and yeast contamination with Sabouraud's Medium. These "checks" were incubated for at least three days at 37° C.

3.4 Culture of Hep G2 cells

3.4.1 Routine culture of Hep G2 cells

Hep G2 cells were routinely grown in 75 cm^2 tissue culture flasks in mono- or multi- layer culture in Williams' E Medium supplemented with 10% FCS. They were grown in a 95% air : 5% CO_2 environment at 37° C and subcultured every 7 days at a split ratio of 1 : 3 (seeding density 5 x 10^4 cells · cm^{-2}).

3.4.2 Harvesting Hep G2 cells

Hep G2 cells were harvested by treating confluent cultures with 5 ml of a 1 : 5 dilution of trypsin solution in versene. Suspensions were centrifuged at 500 g for 5 minutes to form a pellet. The cells were then resuspended in a small volume of culture medium and counted in 0.14% (w/v) Trypan blue solution in an improved Neubauer haemocytometer. Viability was noted: live cells were colourless, whereas dead cells appeared blue and granular.

3.4.3 Freezing Hep G2 cells

Excess Hep G2 cells were harvested, counted and resuspended in ice cold freezing medium (25 ml FCS, 20 ml Williams' E Medium, 5 ml dimethylsulphoxide (DMSO)) to yield a final concentration of 5 x 10^6 cells · ml^{-1}. 1 ml aliquots of cell suspensions were frozen at -70° C for 18 hours and then transferred to liquid nitrogen for long term storage. Microcarrier attached Hep G2 cells (section 3.4.4) were frozen directly on the microcarrier beads, using an altered freezing medium and methodology (section 3.4.4: Freezing of microcarrier attached Hep G2 cells).

3.4.4 Preparation of microcarrier attached Hep G2 cells

Siliconisation of glass culture vessels

All stirrer flasks were siliconised to prevent adherence of the microcarrier beads to the culture surfaces. A small volume of Sigmacote (~ 5 ml) was used to wet the interior of the culture vessels. Excess fluid was drained from the vessels which were then allowed to dry in a fume cupboard. They were then rinsed three times with distilled water and sterilised by autoclaving.

Preparation of microcarrier beads

Cytodex 3 microcarriers were hydrated in 100 ml phosphate buffered saline (PBS) · g Cytodex^{-1} at 37° C for at least three hours. After this time, the supernatant was removed and the microcarriers washed twice with PBS. The microcarriers were resuspended in 50 ml PBS · g Cytodex^{-1} and sterilised by autoclaving for 15 minutes at 120° C. Prior to use, the supernatant was removed and the microcarriers rinsed twice in 37° C culture medium.

Attachment of Hep G2 cells to microcarrier beads

All glassware was siliconised with Sigmacote prior to use. Using an inoculation density of 2.5 x 10^8 cell · g Cytodex^{-1}, Hep G2 cells in the exponential phase of growth were added to a final volume of 250 ml culture medium · g Cytodex^{-1} in Techne stirrer flasks. The cultures were immediately and continuously stirred at 60 rpm, and experiments carried out between days 2 and 3 of growth.

Determination of numbers of Hep G2 cells attached to microcarriers

1 ml of the microcarrier culture was removed into a plastic 5 ml Bijoux bottle. This sample was rinsed briefly in versene, the microcarriers allowed to settle and the supernatant removed. 1 ml of a 1 : 5 dilution of trypsin in versene was then added, and the cells left at 37° C for 15 minutes with occasional aspiration using a Pasteur pipette. Microcarriers did not interfere with cell counting using an improved Neubauer haemocytometer.

Freezing of microcarrier attached Hep G2 cells

Hep G2 cells were frozen directly on Cytodex 3 microcarrier beads as follows: The microcarrier-attached cells were allowed to settle and the supernatant removed. 50 ml of an ice-cold solution containing 25 ml Williams' E medium, 22.5 ml FCS and 2.5 ml DMSO was added for each gram of Cytodex (as measured when dry). The cells were then left in a -20° C freezer for 5 minutes, after which the supernatant was removed and replaced with a volume (equal to that of the supernatant removed) of the freezing mixture previously described for preserving Hep G2 cells (see section 3.4.3). The cells were left in a -20° C freezer for an additional 10 minutes with occasional gentle agitation. The supernatant was removed oncemore, and the microcarriers resuspended in freezing medium at a final concentration of 5×10^6 cells $\cdot$ ml^{-1}. 1 ml aliquots were cooled to -70° C overnight and then transferred to liquid nitrogen for long term storage.

Recovery of Hep G2 cells frozen on microcarriers

Each aliquot was thawed in a 37° C water bath and then rinsed in 10

ml Williams' E medium. The microcarriers were allowed to settle and the supernatant removed before the microcarriers were resuspended in a small volume (10 ml per 1 ml aliquot) of Williams' E medium. Typically, the percentage of cells remaining attached to the microcarriers was 40 - 50%. This value was determined by carefully rinsing random microcarrier cultures with fresh Williams' E medium to remove detached cells, counting the microcarrier attached cells as described previously and comparing this number with that obtained prior to freezing. Trypan blue could not be used to determine the viability of microcarrier attached Hep G2 cells because:

- the dye was quickly absorbed by the collagen coating on the surface of the microcarrier beads, making it difficult to distinguish dead cells from dyed collagen and
- the cells could not be counted accurately whilst attached to the microcarrier beads and harvesting the cells from the microcarriers inevitably resulted in some cell death.

The proportion of cells which remained attached to the microcarrier beads was therefore viewed as an index of viability. Culture of the revived Hep G2 cells was continued for 2 - 3 days after thawing, or until the cells had reached confluence.

3.5 Preparation of isolated hepatocytes

3.5.1 Preparation of rat tail collagen

Rat tails were soaked in ethanol until ready for use. Each tail was then broken into 2" pieces and the scaly skin removed. The tendons were pulled out from each tail fragment and then teased into finer filaments in a 100 mm diameter Petri dish containing sterile distilled water. Collagen was extracted from the rat tail tendons using 1% (w/v) tendon in 0.5 M acetic acid at 4° C for 48 hours. The extract was then passed through sterile gauze and dialysed against 5 l of 0.1x Eagles Medium, pH 7.4, for 2 x 24 hours at 4° C. The dialysed extract was sterilised by centrifugation at 10,000 g for 2 hours. The solution was quantified by evaporating a known volume to dryness and weighing the residue. This solution was stored at 4° C for up to 6 months.

3.5.2 Coating of culture dishes

Collagen solution was diluted to a final concentration of 0.5 mg protein·ml^{-1} in sterile distilled water for use in coating 100 mm Petri dishes. 3 ml collagen solution per dish was added and allowed to dry for 18 hours in a laminar flow hood. The plates were rinsed with PBS prior to addition of hepatocytè culture to remove any traces of acetic acid.

3.5.3 Solutions for preparation of isolated hepatocytes

Stock solutions

(a) Hank's Buffer Stock Solution (HBSS) x10 concentrate

NaCl	80 g
KCl	4 g
$MgSO_4 \cdot 7H_2O$	0.6 g
$Na_2HPO_4 \cdot 2H_2O$	0.6 g

Dissolved in 1 l distilled water and stored at 4° C.

(b) Krebs-Henseleit Buffer Stock Solution (KHBSS) x2 concentrate

distilled H_2O	785 ml
16.09% (w/v) NaCl	200 ml
1.1% (w/v) KCl	150 ml
0.22 M KH_2PO_4	25 ml
2.74% (w/v) $MgSO_4 \cdot 7H_2O$	50 ml
0.12 M $CaCl_2 \cdot 6H_4O$	100 ml

The solutions were added in the above order into a 2 l brown Winchester bottle and bubbled with 95% O_2 : 5% CO_2 for ten minutes. 9.71 g of $NaHCO_3$ was dissolved in 1 l distilled H_2O and bubbled in the same manner. The two solutions were combined and stored at 4° C.

Perfusion solutions

(a) Hank I

$NaHCO_3$	1.05 g
HEPES	15 g
BSA (bovine serum albumin)	3.33 g
EGTA	114 mg
distilled H_2O	450 ml
x10 HBSS	50 ml

(b) Hank II

$NaHCO_3$	1.05 g
HEPES	1.5 g
$CaCl_2 \cdot 2H_2O$	147 mg
distilled H_2O	450 ml
x10 HBSS	50 ml

(c) Krebs-Albumin

HEPES	1.5 g
BSA	5 g
distilled H_2O	250 ml
x2 KHBSS	250 ml

(d) Krebs-HEPES

HEPES	6 g
distilled H_2O	1 l
x2 KHBSS	1 l

The pH of all perfusion solutions was adjusted to 7.4 with 1 M NaOH. They were sterile filtered through a 0.22 μm filter and stored at 4° C.

3.5.4 Isolation of rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (200 - 250 g) by collagenase perfusion (Moldeus et al, 1978). The rats were anaesthetised with pentobarbitone (60 mg · kg^{-1}, i.p.) before dissection of the abdomen. 50 units of heparin were injected into the vena cava to prevent blood clotting. After cannulation of the portal vein using a metal cannula of external diameter 2.5 mm, the liver was dissected out and 150 ml of Hank I solution allowed to recirculate through the liver at a flow rate of 10 ml · min^{-1} for five minutes. Hank II (150 ml) containing 32 mg (equivalent to 14720 units of enzyme activity) collagenase (Type IV from Clostridium histolyticum specific for hepatocyte isolation, Sigma catalogue number C-5138) was then recirculated through the liver for 15 - 20 minutes until the cells inside the liver capsule were seen to be dissociated. The hepatocytes were dispersed into Krebs-Albumin solution, filtered through a sterile 125 μm gauze mesh and allowed to settle before being washed in Krebs-HEPES buffer. All solutions were maintained at 37° C and bubbled with 95% O_2 : 5% CO_2 throughout the perfusion.

3.6 Preparations of Cell Homogenates

Hep G2 and rat hepatocyte homogenates were prepared as follows: Cells were washed twice with PBS, pH 7.6, scraped off the culture dishes into the appropriate buffer using a "rubber policeman" and homogenised using 7 strokes of a motor driven Teflon-glass Potter-Elvehjem homogeniser. All solutions were kept on ice throughout the procedure. Both cell types were homogenised at a concentration of $5x10^6$ cells $\cdot$ ml^{-1} and stored at -70° C in 1 ml aliquots until analysed.

3.7 Assays

3.7.1 Protein determination

Total cell protein was measured by the method of Lowry et al (1951). Rat hepatocyte suspensions (5×10^6 cells $\cdot$ ml^{-1}) were diluted 25-fold to a final volume of 0.5 ml with 0.5 M NaOH. Hep G2 cells were diluted 20-50 fold, depending on initial cell concentration. Standards contained 0 - 200 $\mu g \cdot ml^{-1}$ of protein prepared from a stock solution of BSA (200 $\mu g \cdot ml^{-1}$ in 0.5 M NaOH, stored at -20° C). To each standard and sample were added 2.5 ml of a solution containing 98 parts sodium carbonate (2%, w/v), 1 part sodium potassium tartrate (2%, w/v), and 1 part copper sulphate (1%, w/v). After 10 minutes incubation, 0.25 ml of a 25% (v/v) solution of Folin-Ciocalteau's reagent in water was added, the samples mixed immediately and left for 30 minutes before the absorbance was measured at 725 nm (UV-2101PC spectrophotometer, Shimadzu Corporation).

3.7.2 Intracellular reduced glutathione (GSH) content

The concentration of GSH in Hep G2 cells was measured by the method of Saville (1958), using the following solutions:

Solution A:	10 parts 0.01 M sodium nitrite 1 part concentrated sulphuric acid 89 parts distilled water
Solution B:	0.5% (w/v) ammonium sulphamate in water
Solution C:	1 part 1% (w/v) mercuric chloride 4 parts 3.4% (w/v) sulfanilamide in 0.4 M HCl
Solution D:	0.2 % (w/v) N - 1 - naphthyl-ethylenediamine

Solution D: 0.2 % (w/v) N - 1 - naphthyl-ethylenediamine dihydrochloride

Medium was removed from cultured cells which were then rinsed twice with PBS. 100 $\mu l \cdot cm^{-2}$ trichloroacetic acid (TCA; 10%, v/v) was added and the samples allowed to stand for 10 minutes. The acidic fraction was then removed into Eppendorf tubes and stored at -20° C until ready for the assay, which was carried out as follows: Each sample was centrifuged at 6500 g for 5 minutes. To 150 μl of the supernatant, 150 μl of solution A and 30 μl of solution B were added, each addition being followed by a 5 minute incubation period at room temperature. 300 μl of solution C was then added, immediately followed by 120 μl of solution D. After 10 minutes, the absorbance of this mixture was read at 535 nm. GSH was quantified using standards (0 to 20 mM) prepared in 10% TCA.

3.7.3 Lactate dehydrogenase (LDH) activity

LDH leakage of rat hepatocytes and Hep G2 cells was measured by the method of Anuforo et al (1978). 100 μl of medium from the culture plates was added to 0.9 ml sodium phosphate buffer, pH 7.4, at room temperature. 40 μl of the same buffer containing 0.2 mM nicotinamide adenine dinucleotide (NADH) and 1.36 mM pyruvic acid was used to initiate the reaction. The change in absorbance due to NADH oxidation was recorded at 340 nm against a blank buffer. Results were calculated using the molar extinction coefficient for NADH: 6.22 $mM^{-1} \cdot cm^{-1}$.

3.7.4 Cytochrome P450 content

The concentration of cytochrome P450 in rat hepatocytes was determined as described by Omura and Sato (1964). Medium was removed from cultured cells which were then washed with PBS. The cells were then scraped into 1 ml P450

buffer, homogenised and stored at -70° C until ready for analysis. For the assay, the hepatocytes were diluted to a final concentration of 10^6 cells · ml^{-1} in P450 buffer, and saturated with carbon monoxide by bubbling for approximately 20 seconds. Each sample was equally divided between two 1 ml Quartz cuvettes with black sides and baseline recorded in the range of 500 - 390 nm. A few grains of sodium dithionite were then added to one cuvette, and the samples re-scanned to obtain the difference spectra. Cytochrome P450 content was determined by the difference in absorbance at 450 nm and 469 nm divided by the molar extinction coefficient 91 $mM^{-1} \cdot cm^{-1}$. Cytochrome P420 was also quantified by measuring the difference in absorbance between 420 nm and 469 nm, divided by the molar extinction coefficient, 110 $mM^{-1} \cdot cm^{-1}$.

3.7.5 Ethoxyresorufin O-dealkylation (EROD)

EROD reactions were measured by direct fluorimetric determination of the resorufin formed (Grant et al, 1988). Cultured cells were washed twice with PBS, then scraped into 1 ml of 0.1 M sodium phosphate buffer, pH 7.6, homogenised and stored at -70° C until analysed. EROD activity was measured using 0.2 ml cell homogenate (5×10^6 cells · ml^{-1}) with 0.3 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 5 μM ethoxyresorufin in a final volume of 0.5 ml 0.1 M sodium phosphate buffer in a 37° C water bath. Reactions were stopped after 30 minutes by the addition of 0.5 ml ice cold acetone and the samples were centrifuged at 13000 g for 5 minutes. The fluorescence of the supernatant was read at an emission wavelength of 600 nm and an excitation wavelength of 580 nm (RF-5001PC spectrofluorimeter, Shimadzu Corporation). The background fluorescence of the cell homogenate was also measured (by adding acetone at the beginning of

the reaction) and subtracted from the total fluorescence measured. These assays were carried out under yellow light. The assay was quantified using 10 μl of 0.1 M resorufin in 1 ml phosphate buffer.

3.7.6 ^{3}H-leucine incorporation

Radioactivity (0.1 μCi $\cdot$ ml^{-1} ^{3}H-leucine) was added to the culture medium (Williams' E and KI for Hep G2 cells and rat hepatocytes respectively) using a 10 μl glass Hamilton syringe. A sample of this medium was retained so that its radioactivity, and thus the total radioactivity added to the cultures, could be measured. After a 24 hour culture period the radioactive medium was removed from the cultured cells. The cells were then incubated in 0.5 mM leucine in a 0.9% saline solution for 5 minutes, followed by a 10 minute incubation in 10% (w/v) trichloraocetic acid. Each stage was followed by two rinses with PBS, pH 7.6, and all solutions were discarded. The cells were then digested in 1 ml 0.5 M NaOH (tightly sealed and left overnight at 37° C). 250 μl of the digested cells was taken and 4 ml scintillation fluid added before the radioactivity was determined by scintillation counting (1900-TR scintillation counter, Canberra Packard). Samples were counted for 10 minutes and automatic quench correction applied using the transformed spectral index of the external standard to compensate for changes in quench levels. ^{3}H leucine incorporation was determined as the percentage of the total radioactivity added which was incorporated into the cell protein.

Chapter 4: Experiments

4.1 The effect of serum concentration on the growth and viability of Hep G2 cells

The following aspects of exposure of Hep G2 cells to different serum concentrations were explored:

1. the effect of different serum concentrations on the growth and GSH content of Hep G2 cells:

   Hep G2 cells were cultured on 24 well tissue culture plates and exposed to 100% NCS or different concentrations (10, 25, 50 and 75%, v/v) of NCS in Williams' E medium supplemented with L-glutamine and PEST. Medium was changed on days 2 and 5 of culture and samples for protein and GSH were taken on days 1, 2, 3, 5, 6 and 7.

2. the effect of different serum concentrations on protein and GSH content of confluent Hep G2 cells:

   Hep G2 cells were grown to confluence (7 days post passage) on 75 cm^2 cell culture flasks. The culture medium was then removed and replaced with 100% NCS or with different concentrations (10, 25, 50 and 75%, v/v) of NCS in Williams' E medium supplemented with L-glutamine and PEST. Samples for protein and GSH were taken after 48 hours.

3. the timecourse of GSH depletion from confluent cultures of Hep G2 cells:

   Hep G2 cells were grown to confluence (7 days post passage) on 24 well plates. The culture medium was then removed and replaced with 100% NCS. Samples for protein and GSH were taken at t = 0, 1, 2, 4, 8, 22 and 48 hours.

4. the effect of supplementation of serum with amino acids on the growth and viability of Hep G2 cells:

   Hep G2 cells were cultured on 24 well tissue culture plates and exposed to 100% NCS or 100% NCS supplemented with 50 mM L-cysteine, glycine and L-glutamic acid. Medium was changed on day 3 of culture and samples for protein and GSH were taken on days 1, 2, 3, 4, 5 and 6.

5. the effect of supplementation of serum with amino acids on the timecourse of GSH depletion from Hep G2 cells:

   Hep G2 cells were grown to confluence (7 days post passage) on 24 well plates. The culture medium was then removed and replaced with 100% NCS or 100% NCS supplemented with 50 mM L-cysteine, glycine and L-glutamic acid. Samples for protein and GSH were taken at t = 0, 1, 2, 4, 8 and 22 hours.

6. the effect of human serum on the growth and GSH content of Hep G2 cells:

   Hep G2 cells were cultured on 24 well tissue culture plates and exposed to 100% human serum or human serum supplemented with 50 mM L-cysteine, glycine and L-glutamic acid. Medium was changed on day 3 of culture and samples for protein and GSH were taken on days 1, 2, 3, 4, 5 and 6.

Williams' E medium supplemented with 10% FCS was the control culture medium (CCM) for all of the above experiments. The schedule of medium replenishment was changed for experiments 4 and 6 (from that adopted for experiment 1 in this section and the experiment described in section 4.2) after it had become apparent

that there was significant resistance to mass transfer (i.e. of O_2 and CO_2 in the headspace) through the culture medium[1]. A reduction in the volume of medium added to each well (from 1 ml to 0.75 ml) reduced the thickness of medium through which the gases diffused (from 5 mm to 3.75 mm) and thus improved mass transfer between the gases in the headspace and the cultured cells. It was therefore only necessary to change the medium once, on day 3, as opposed to twice, on days 2 and 5.

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[1] by observation; the pH of the medium in the wells at the edges of the culture plate was maintained within normal limits for a longer period than that of the wells in the centre.

4.2 The effect of increasing bile concentrations on the growth and viability of Hep G2 cells

Hep G2 cells were cultured on 24 well tissue culture plates and exposed to 0.1 and 0.5% (v/v) sheep bile in Williams' E medium. The medium was changed on days 2 and 5 of culture and samples for protein, GSH and LDH were taken on days 1, 2, 3, 4, 5 and 6.

The effect of different concentrations of sheep bile on confluent cultures of Hep G2 was also investigated: Hep G2 cells were grown to confluence (7 days post passage) on 24 well plates. The culture medium was then removed and replaced with Williams' E medium containing different concentrations of sheep bile (0.1, 0.3, 0.5, 1 and 2%, v/v). The medium was changed on days 2 and 5 of culture (these experiments were performed prior to the discovery of the diffusional resistance imposed by the culture medium) and samples for protein, GSH and LDH were taken on days 1, 2, 3, 4, 5 and 6.

4.3 The effect of oxygen concentration on the viability of Hep G2 cells and rat hepatocytes

Hep G2 cells were grown on 100 mm diameter tissue culture treated Petri dishes (seeding density 5 x 10^4 cells · cm^{-2}) at 37° C in a 95% air : 5% CO_2 environment. At confluence (7 days post-passage) the medium was changed and the cells transferred to an enclosed polypropylene box in a shaking incubator (37° C, Figure 4.1, overleaf), where they were kept for 24 hours. The tray within the shaking incubator was rocked from +25° to -25° in a time span of 30 seconds, ensuring that the culture medium was well mixed. One of five gas mixtures (5, 12, 20, 28 and 35% O_2 with 5% CO_2 and the balance N_2; British Oxygen Company) was supplied to the cells at a constant flow rate of 200 ml · min^{-1}. This flow rate was decided upon after initial tests were conducted to determine the rate at which atmospheric air within the polypropylene box was replaced with the inlet gas. It was confirmed that 200 ml · min^{-1} was adequate not only to replace the air in the box in less than two hours, but also to maintain this new headspace gas composition throughout the 24 hour test period, without significantly elevating the pressure within the box (< 1 mmHg increase from barometric pressure). Gauge pressure (via manometer readings) and the O_2 fraction of the outlet gas (via a Servomex oxygen monitor) were monitored continuously. Moisture was eliminated from the sampled gas by allowing it to flow through a silica gel mini-column prior to its passage through the oxygen monitor. The oxygen monitor was calibrated following the manufacturer's guidelines using the pre-measured gas cylinders as standards. Typically, no drift was observed over the test period. At the end of the 24 hour test period, measurements were made of LDH and EROD activity, protein content and ^3H-leucine incorporation.

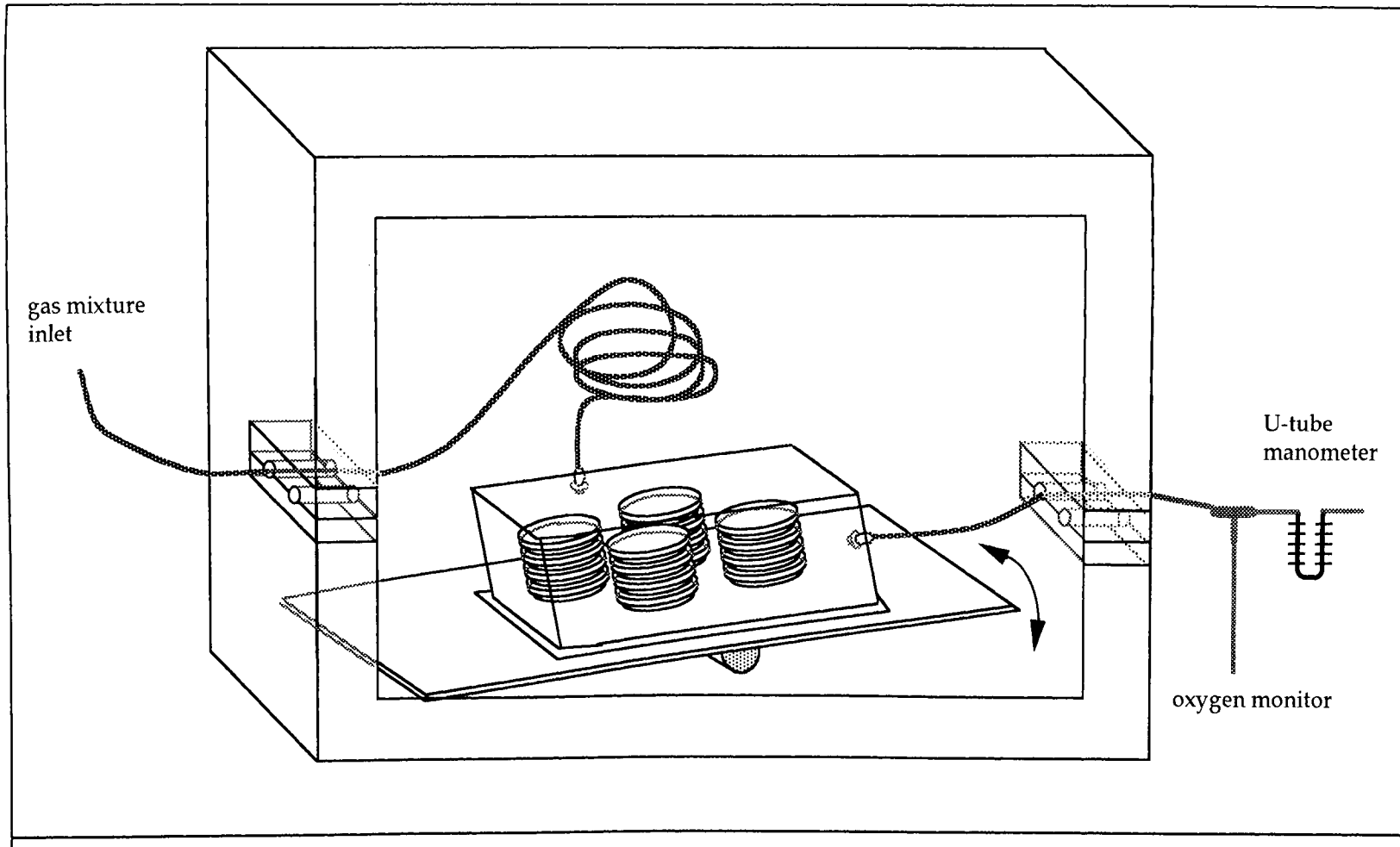


Figure 4.1: Experimental set up for studies on the effect of oxygen concentration on the viability of Hep G2 cells and rat hepatocytes

Attempts were made to determine the oxygen concentration of the culture medium as a function of time and headspace gas composition (experimentally using a 5 ml sample of water in a Petri dish placed in the shaking incubator and exposed to one of the five gas mixtures). These attempts were unsuccessful, as the process of obtaining samples of this water inevitably resulted in some mixing in the presence of room air, which made pO_2 measurements unreliable. However, McLimans et al (1968) demonstrated that the medium supplying oxygen to cells in monolayer rocker cultures under similar conditions (rocked from $+25^{\circ}$ to -25° in a time span of 40 seconds) could be considered to equilibrate with the headspace almost instantaneously, due to the frequent mixing of the culture medium and the periodic thinning of the fluid film over most of the culture surface to < 0.1 mm at the extremities of the rocking cycle. In the experiments to determine the effect of oxygen concentration on the viability of Hep G2 cells and rat hepatocytes, it was noted by direct observation that up to 75% of the culture surface was exposed to ambient air at each extremity of the rocking cycle. To determine if rocking (as opposed to stationary culture conditions) influenced cell viability and/or function, cultures of Hep G2 cells were kept in the non-rocking 95% air : 5% CO_2 incubator (i.e. ~ 19.8% O_2 in the headspace) and LDH and EROD activity, protein content and ^{3}H-leucine incorporation were measured at the end of the 24 hour test period. These values were compared with those obtained from Hep G2 cells exposed to 20% O_2 in the rocking incubator (no significant difference was noted). Hep G2 cells exposed to 20% O_2 in the shaking incubator served as the controls for these experiments.

Rat hepatocytes were cultured on collagen-coated 100 mm diameter Petri dishes (seeding density 5×10^6 cells $\cdot$ plate^{-1}) and allowed to attach for 24 hours. Rat hepatocytes were treated in the same manner as Hep G2 cells, with the rat hepatocytes

exposed to 20% O_2 in the shaking incubator serving as the controls for these experiments. At the end of the 24 hour test period, measurements were made of cytochrome P450 and P420 content, LDH and EROD activity, protein content and ^3H leucine incorporation, and differences in cell morphology noted. Pre-experimental values of cytochrome P450 and P420 content and EROD activity were obtained from samples of the freshly isolated hepatocytes which were ultimately cultured and exposed to the various oxygen concentrations.

4.4 Measurement of oxygen consumption in Hep G2 cells

The dependence of Hep G2 cell oxygen consumption on cell density and the pO_2 of the culture medium was examined.

Oxygen consumption was measured using a modification (described in section 4.4.5) of a system developed by Noll et al (1986), represented schematically in Figure 4.2 (overleaf). Briefly, Hep G2 cells in the test chamber deplete oxygen in the surrounding medium, which is well mixed; pO_2 is continuously monitored using a polarographic oxygen electrode (Type E5, 19 mm; Uniprobe Instruments Limited). As the pO_2 in the test cell falls below a certain level (the set point, specified by the user) an error signal generated by the control system drives a motor driven burette which supplies medium with a predetermined pO_2 (generally oxygen saturated) to the test cell. Oxygen consumption can thus be quantified from the volume of medium dispensed over time.

The entire system was kept in a 37° C cabinet, and the medium reservoir heated to 37° C, to prevent temperature fluctuations. Prior to each experiment, the culture medium was oxygenated and its pO_2 measured as described in section 4.4.3. Microcarrier attached Hep G2 cells were introduced into the test cell via the sampling port (see Figure 4.3, page 90). The oxygen electrode was connected to a chemical microsensor (model 1201; Transidyne General Corporation) which sent a constant analogue signal to the computer. A digital signal was obtained by an A/D converter within the computer and the resulting digital signal was used by the control system (UC Online© Version 2.3; University of California) to calculate the pO_2 within the test cell, following the relation detailed at the top of page 91 (specific to the Transidyne microsensor):

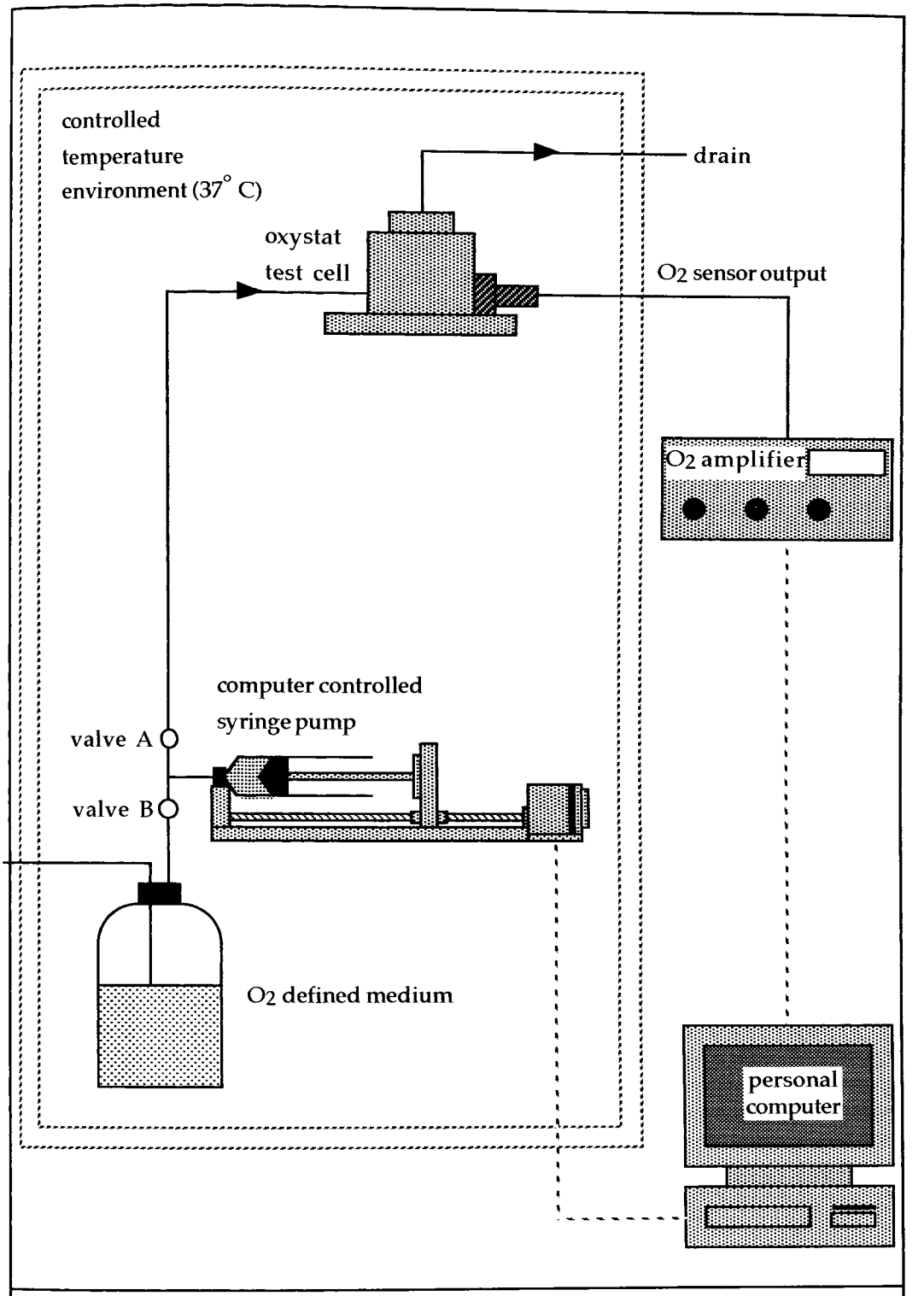


Figure 4.2: Apparatus used to measure the oxygen consumption of Hep G2 cells

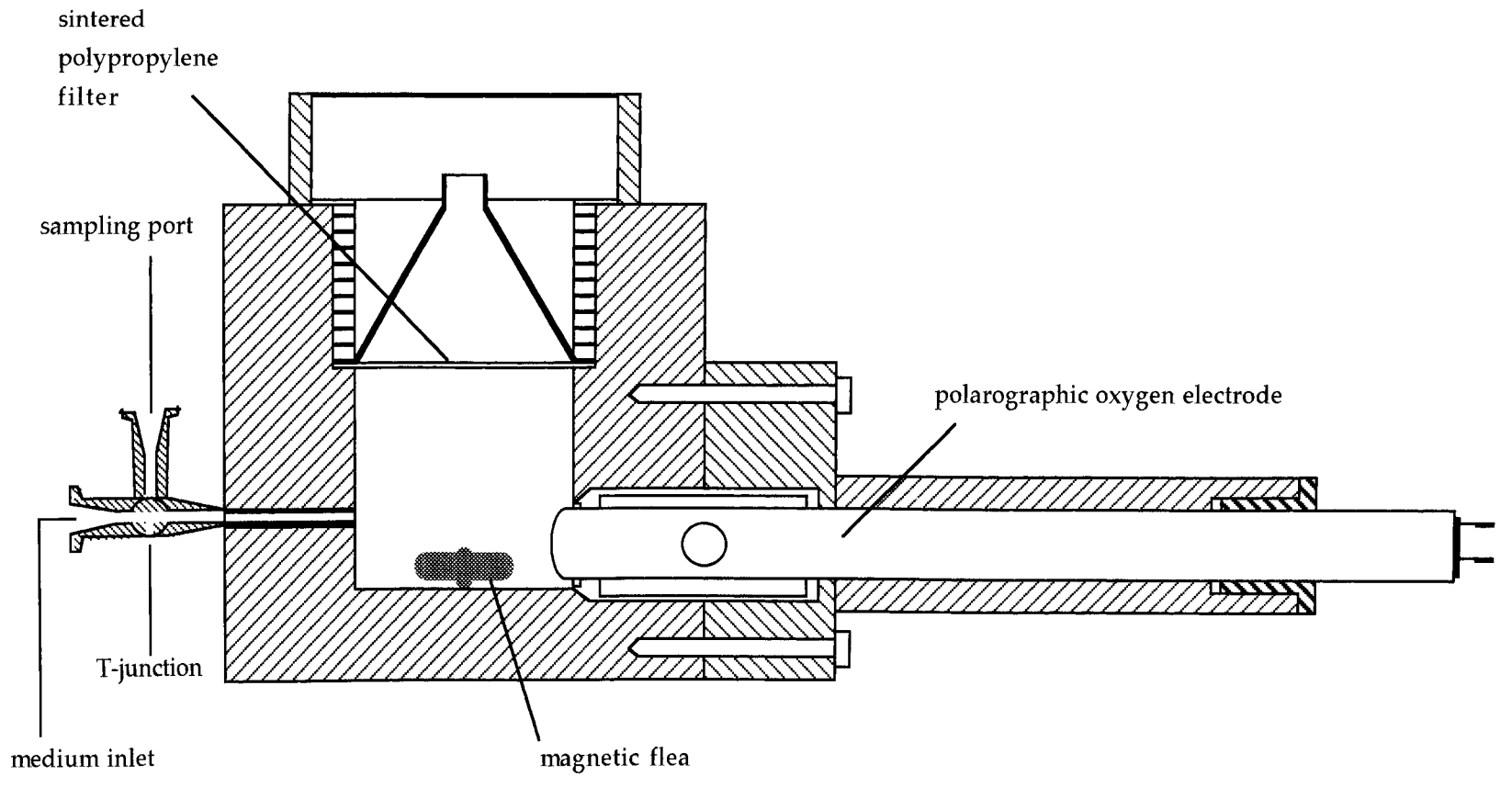


Figure 4.3: The oxystat test cell with the medium inlet open

$P_{tc} = -992.5\,R + 0.484$

where:

P_{tc} : pO_2 in the test cell (mmHg)

R : magnitude of the signal generated by the A/D converter

The control system then generated a signal proportional to the difference between the measured pO_2 within the test cell and the desired pO_2 (specified by the user, the set point). Although a standard PID controller was used, it was determined through trial and error that the best results in terms of stability and accuracy were obtained using only proportional control. The integral and derivative variables were therefore set to 0, whilst the proportional variable was generally set equal to 1.

The signal generated by the control system in response to a deviation between the measured pO_2 and the set point was used to drive a stepper motor (model number 440-442; RS Components Limited). The stepper motor was, in turn, used to depress the plunger on the glass syringe (via a threaded mechanism), so that an appropriate volume of oxygen saturated medium was dispensed into the test cell.

The volume of fluid dispensed by one step of the stepper motor was found gravimetrically: i.e. the syringe was filled with water and the quantity released after 1000 steps was weighed. The resulting value was divided by 1000. The average volume of liquid dispensed by one step of the stepper motor was 6.078 ± 0.0005 μl ($n = 4$). The pO_2 in the test cell, time elapsed and total number of steps were logged every 2.7 seconds by the control system.

Using only proportional control, the above system dispensed oxygen saturated medium with great accuracy (Figure 4.4 A, overleaf) and maintained pO_2 in the test chamber with an offset < 2 mmHg from the set point. Furthermore, once stability was achieved, pO_2 in the test cell never varied more than $\pm$ 0.5 mmHg from this offset value (Figure 4.4 B, overleaf).

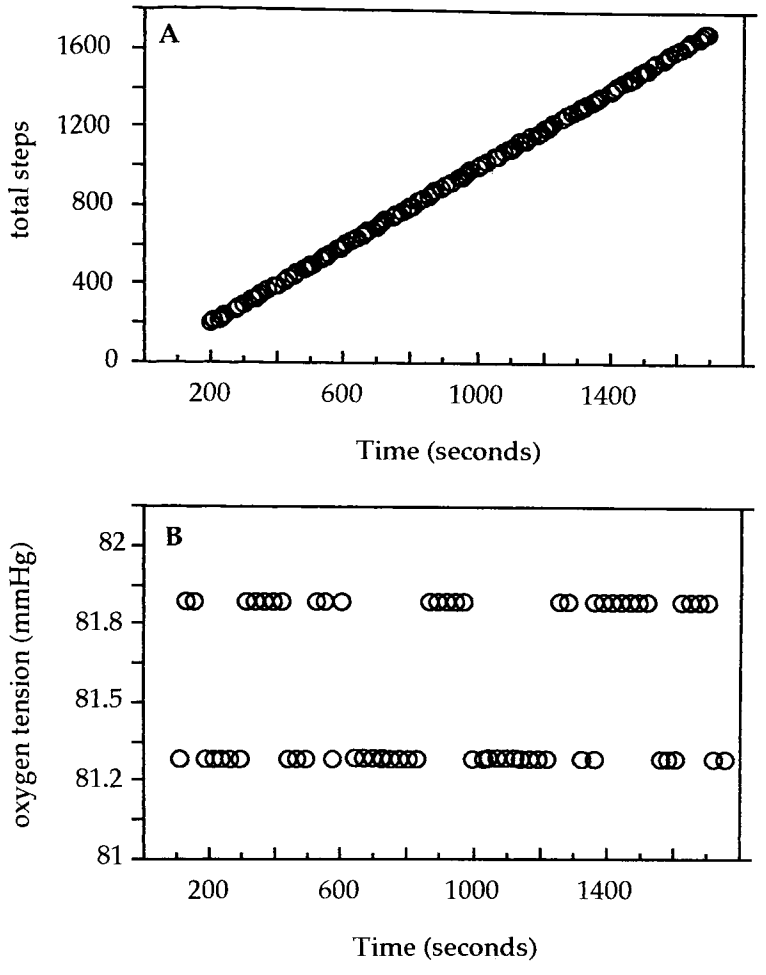


Figure 4.4: Accuracy of the control system.

(A) The total steps as prescribed by the control system demonstrated great linearity over time. This resulted in an even flow of oxygen saturated medium into the test cell which precisely matched the rate at which oxygen was depleted, without under- or overshoot.

(B) As a result of (A), dissolved oxygen concentration within the test cell medium was maintained with excellent precision. The set point for this experiment was 82 mmHg: therefore, the offset was < 0.5 mmHg and deviation from this offset value was ± 0.25 mmHg.

When not being used in the feedback control of the pO_2 in the test cell, UC Online© could also be used to control the emptying and filling of the glass syringe as necessary. This required the correct positioning of valves A and B (open or closed; Figure 4.2, page 89) and changes to the direction of the stepper motor as appropriate.

Oxygen consumption was calculated manually. The following equation was found by summing sources of O_2 into and out of the test cell:

$$V_{O2} = \frac{V_S \cdot N_S \cdot (C_i - C_t) + O_l}{N_c \cdot t}$$

where:

V_{O2} oxygen consumption ($nmol \cdot min^{-1} \cdot 10^6\ cells^{-1}$)

V_S volume of medium dispensed by 1 step of the stepper motor ($6.078 \times 10^{-3}\ ml \cdot step^{-1}$)

N_S total number of steps in time t

C_i concentration of oxygen in the inlet medium ($moles \cdot ml^{-1}$)

C_t concentration of oxygen in the test cell ($moles \cdot ml^{-1}$)

O_l O_2 leakage into the test cell over time t (nmol, see section 4.4.4)

N_c total number of cells ($\times 10^6$)

t time (min).

C_i and N_c were calculated as described in sections 4.4.3 and 4.4.4 respectively.

4.4.1 Sterility in the oxygen consumption test cell

Sterile syringes were used for the transfer of medium and microcarrier attached cells to the test chamber. Disinfectant tablets (Presept, 1/2 tablet for 5 litres) were added to the water bath. At the end of each experiment, the water bath was emptied, the medium reservoir, syringe, flow lines and test cell were rinsed and filled with 70% ethanol, and microcarrier attached cells examined for signs of visible contamination. Prior to each new experiment, the ethanol was drained from the system which was then rinsed twice with sterile distilled water. This was followed by two further rinses with culture medium. All surfaces were wiped with 70% ethanol before and after each experiment.

4.4.2 Calibration of the oxygen electrode

A two point calibration was made on the oxygen electrode at the beginning of each experiment:

(a) The test cell was filled with water through which 100% N_2 was bubbled at a rate of approximately 10 ml · min^{-1}. Once the water had been depleted of oxygen and a stable reading obtained, corrections to the zero were made as necessary. For the reasons discussed in the second paragraph of section 4.2, it was not possible to obtain reliable samples of this water for independent pO_2 measurement. Therefore, a stable reading over a 15 minute period was assumed to indicate that maximal depletion of oxygen from the test cell had been achieved.

(b) The test cell was opened and filled with fresh water. This water was stirred continuously (> 60 r.p.m.) and allowed to equilibrate with room air. The sensor was then adjusted so that

$pO_2 = 0.21\,(BP - 47)$ mmHg, where BP is the barometric pressure (at the time of the experiment) measured in mmHg and 47 mmHg is the saturated water vapour pressure at 37° C. A sample of this water was taken via a 2 ml glass syringe to a blood gas analyser (Corning 168) and the test cell value checked against that given by the blood gas analyser. Readjustment of the test cell value was seldom necessary.

4.4.3 Measurement of the inlet medium pO_2

Prior to each experiment, the reservoir was filled with Williams' E medium. 35% O_2 was bubbled through this medium for one hour at a rate of approximately 30 ml · min^{-1}. A silicone coated polyurethane mesh was placed in the reservoir to prevent foaming of the medium during this stage. Approximately 20 ml of this medium was removed from the reservoir and injected directly into the test cell via the sampling port. Extreme care was taken to ensure that no air remained trapped in the test cell. The remainder of the oxygenated medium was drawn into the glass syringe. After the reading given by the oxygen electrode had stabilised, the magnetic stirrer was turned off and approximately 15 ml of fresh medium was delivered to the test cell from the glass syringe. Stirring was then resumed while the reading on the oxygen electrode stabilised. This procedure was repeated until there was no difference between two successive readings.

4.4.4 Measurement of O_2 leakage into test cell

Great care was taken to minimise leakage of oxygen into and out of the test cell, supply lines and culture medium: steel tubing was used for all fluid transfer, a luer-locked gas impermeable glass syringe was used to dispense the culture

medium and "water tightness" was ensured for all inlet and outlet ports to the the test cell. Despite these precautions, O_2 leakage into the test cell (probably through the tubing leading to the drain) could not be completely eliminated and so was quantified as follows:

Sample water was depleted of oxygen by bubbling with 100% N_2 for approximately 20 minutes. This water was injected directly into the test cell, taking care to ensure that all air bubbles were removed. pO_2 was measured continuously for 18 hours. This procedure was repeated 4 times and a logarithmic best fit found for the resultant data (R^2 = 0.92, StatView, Abacus Concepts Incorporated). The derivative of this curve with respect to time provided $d(pO_2)/dt$, which, when re-plotted against pO_2 provided the following equation for $C_o' \geq C_i'$:

$$O_l = Q \cdot t \cdot (P_o' - P_i')^{2.3664}$$

where:

O_l O_2 leakage into the test cell over test period (nmol)

P_o' atmospheric oxygen concentration (mmHg)

P_i' oxygen concentration in the test cell (mmHg)

t time (min)

and $Q = 4.403 \times 10^{-6}$ incorporates a correction factor to change mmHg to nmol (1 mmHg = 1.4 nmol $\cdot$ l^{-1}; Noll et al, 1986)

The proportion of oxygen delivered to the cells by this leakage, as opposed to the control system, was dependent upon cell number and respiration rate, but was generally negligible for $pO_2 > 60$ mmHg.

4.4.5 Design modifications to prevent cell depletion

As fresh medium enters, an equal volume of medium is displaced from the test cell. In the original design, cells would leave the test chamber with this outflow and thus the decrease in cell number over time had to be incorporated into the computer algorithm that calculated oxygen consumption. By using microcarrier attached cells, it was possible to prevent this cell depletion by the insertion of a sintered polypropylene filter (5 μm pore diameter) inside the test cell just in front of the outlet port.

Efforts were also made to ensure that there was no cell loss through mechanical damage. Stirring in the test chamber followed microcarrier manufacturers' recommendations (60 rpm, no contact between surfaces of magnetic flea and test cell floor). Microcarrier cultures appeared to tolerate these conditions well and, after up to 8 hours in the test cell, did not show any measurable loss of cell numbers, counted as described in section 3.4.4 (Determination of cell number of microcarrier attached Hep G2 cells). Experiments on any one group of cells did not last longer than 4 hours.

Chapter 5: Results

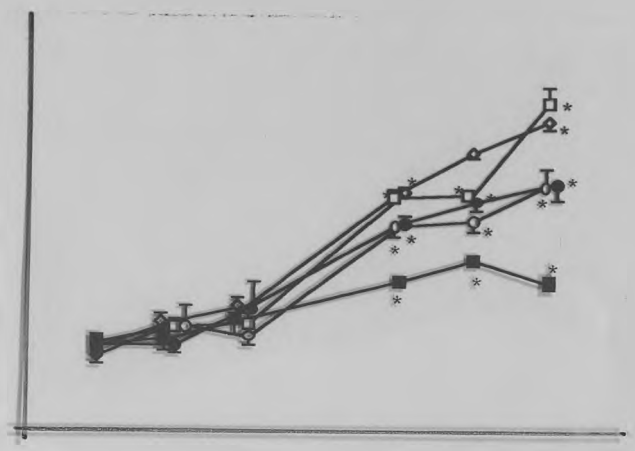
5.1 The effect of serum on the growth and viability of Hep G2 cells

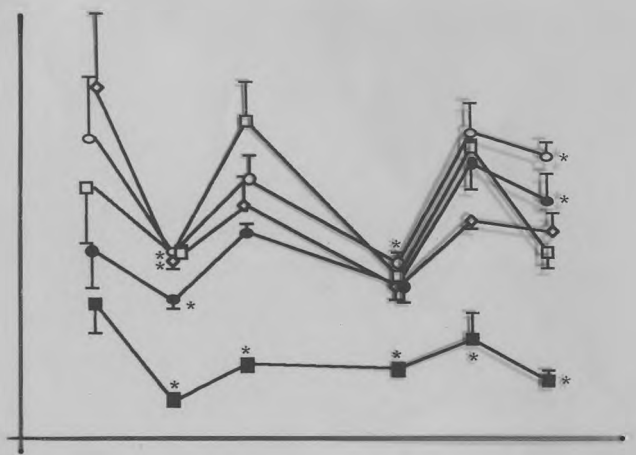
5.1.1 The effect of serum concentration on Hep G2 cell growth and GSH levels

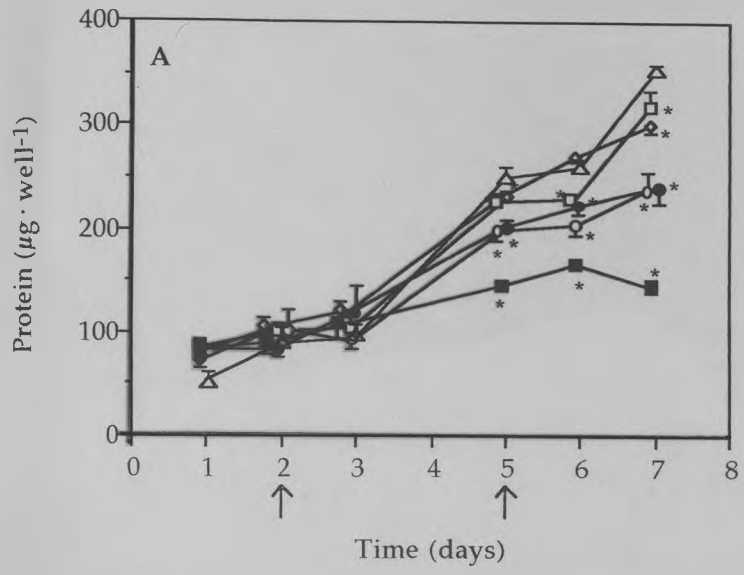
Figure 5.1 A (overleaf, with acetate) shows the effect of increasing serum concentrations on the growth rate of Hep G2 cells. With the acetate overlay removed, Figure 5.1 A also clearly shows the normal "log" and "lag" phase of growth experienced by cells cultured in CCM. All cultures experienced a lag phase, during which growth is minimal, for the first three days of growth following subculture. After this period, the control cultures were observed to enter a log phase of growth, in which cell numbers increase rapidly. Although cells cultured in $\leq$ 75% NCS were also observed to enter the log phase of growth by day 5, increases in cell numbers were slower in all cultures grown in the presence of NCS compared with those grown in CCM. No direct correlation was observed between growth inhibition and NCS concentration, but cell growth was severely inhibited in 100% NCS. Furthermore, NCS concentrations $\geq$ 25% were observed to affect cell morphology within the first 24 hours of culture, resulting in irregular cells with a granular appearance (Photos 5.1 a-f, page 101).

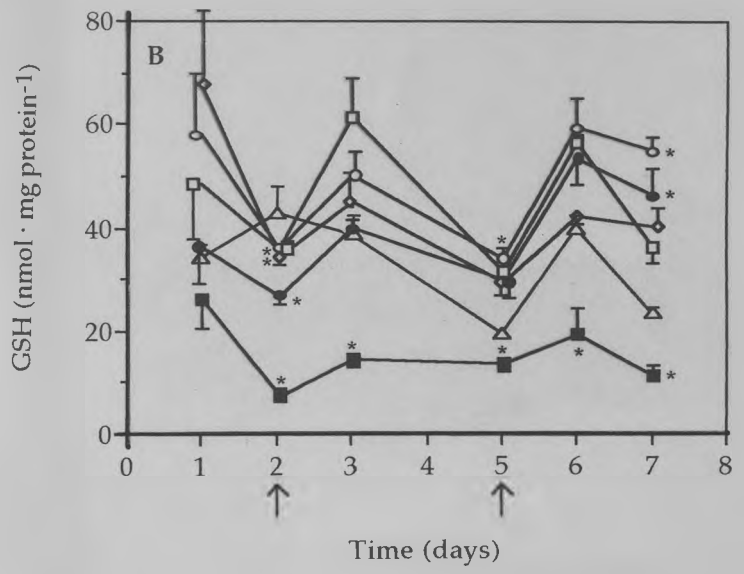
Compared with control, there was little difference in the GSH concentrations of cells grown in serum concentrations $\leq$ 75%, with GSH levels being neither consistently higher nor lower for cultures in NCS supplemented medium than for those in CCM (Figure 5.1 B, overleaf with acetate). All cultures, with the exception of those grown in CCM, experienced a drop in GSH levels on day 2. Nevertheless, these cultures were observed to respond to the medium change on day 2 with an increase in GSH content as measured on day 3. All cultures, with the exception of those grown in 100% NCS, experienced a sharp drop in GSH levels on day 5, coinciding with a burst of cell division between days 3 and 5. Furthermore, all cultures were observed to respond to the medium change on day

Figure 5.1: Growth (A) and GSH content (B) of Hep G2 cells grown in increasing concentrations of NCS. Results are the mean of 4 experiments and error bars represent the standard error of the mean (SEM). —▲— CCM; —○— 10% NCS; —□— 25% NCS; —◇— 50% NCS; —●— 75% NCS; —■— 100% NCS. * $P < 0.05$, by ANOVA followed by Dunnett's test. Significant values refer to differences between cells grown in CCM and in different concentrations of NCS. ↑ denotes change of medium.






A
Protein (μg · well-1)
400
300
200
100
0
0 1 2 3 4 5 6 7 8
Time (days)


B
GSH (nmol · mg protein-1)
80
60
40
20
0
0 1 2 3 4 5 6 7 8
Time (days)

Photos 5.1 a-f: Hep G2 cells (magnification: 257x), 24 hours after initiation of culture in:

a: CCM

b: 10% NCS

c: 25% NCS

d: 50% NCS

e: 75% NCS

f: 100% NCS

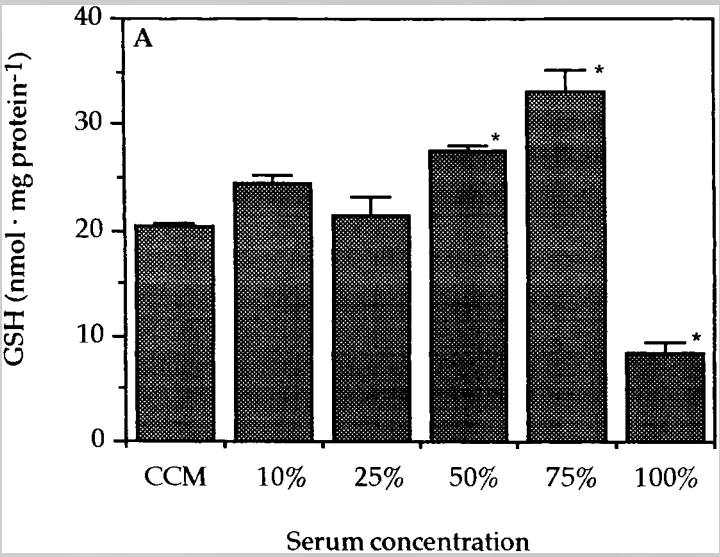

a
b
c
d
e
f

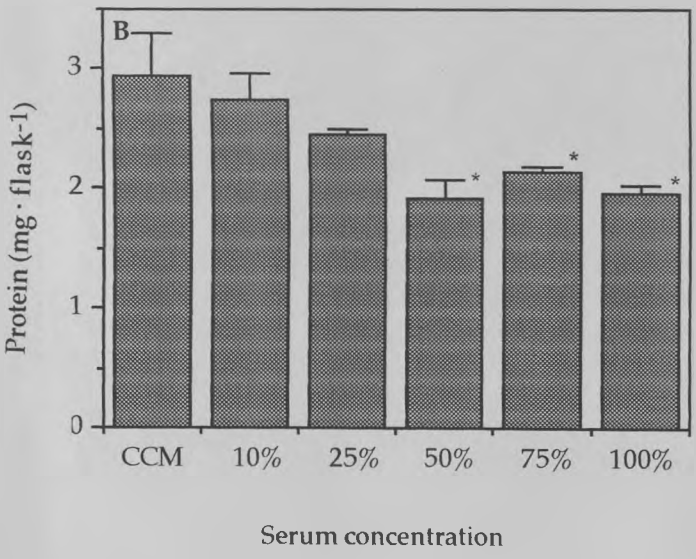
5, as evidenced by an increase in GSH content on day 6. However, the GSH levels of cells grown in 100% NCS were severely depleted, averaging less than 40% of control values throughout the growth period.

5.1.2 The effect of increasing serum concentrations on the GSH content of confluent Hep G2 cells

When confluent cultures of Hep G2 cells were exposed to increasing concentrations of NCS as a result of a medium change on day 7 of growth, these cultures maintained GSH levels somewhat higher than control, with the exception of those in 100% NCS, in which GSH was markedly depleted (Figure 5.2 A, overleaf). Morphologically, cultures appeared normal in concentrations of NCS $\leq$ 50%, whereas cells exposed to 75 and 100% NCS for 2 days were beginning to detach and float freely in the culture medium (Photos 5.2 a-c, page 105). It is due to this cell detachment and the subsequent rounding up of the cells in concentrations of NCS $\geq$ 75% that Photos 5.2 b & c could not be brought into focus. Evidence of cell detachment was also provided by measurements of cell protein, which revealed a significant decrease in the protein content of cultures exposed to concentrations of NCS > 25% (Figure 5.2 B, overleaf).

Figure 5.2: GSH (A) and protein (B) content of confluent Hep G2 cells maintained in increasing serum concentrations. Results are the mean of 4 experiments and error bars represent SEM. * $P < 0.05$, by ANOVA followed by Dunnett's test. Significant values refer to differences between cells grown in CCM and in different concentrations of NCS.


A
GSH (nmol · mg protein-1)
40
30
20
10
0
CCM
10%
25%
50%
75%
100%
Serum concentration

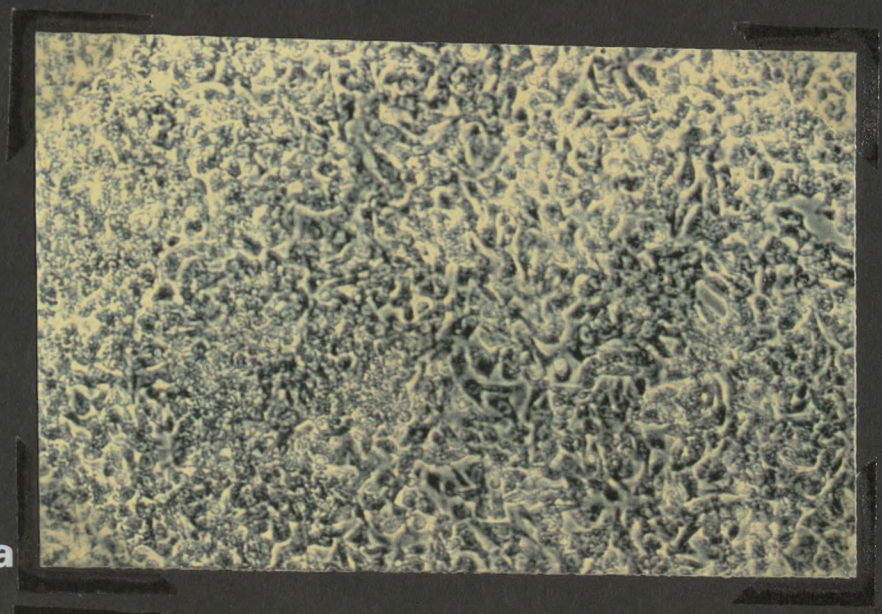

B
Protein (mg · flask-1)
3
2
1
0
CCM
10%
25%
50%
75%
100%
Serum concentration

Photos 5.2 a-c: Confluent Hep G2 cells (magnification: 128x), after 2 days exposure to:

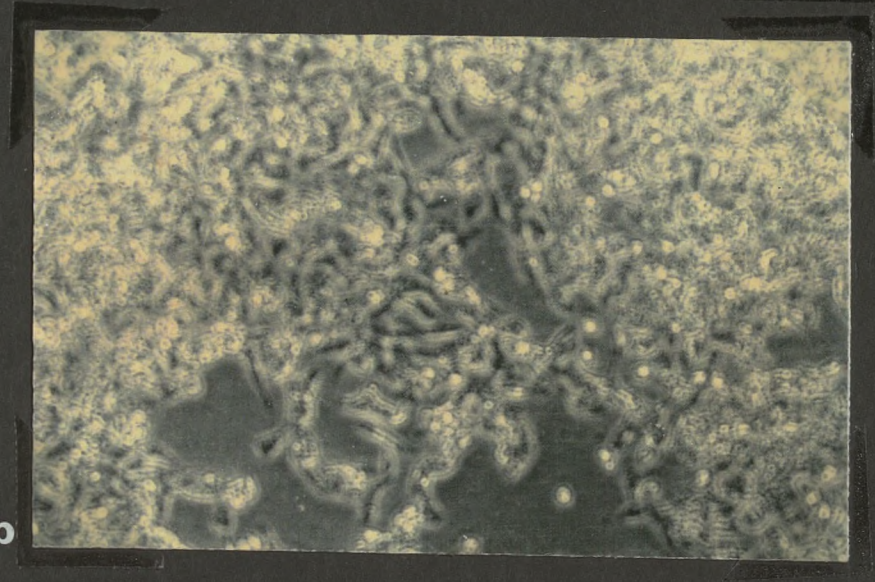
a: 50% NCS

b: 75% NCS

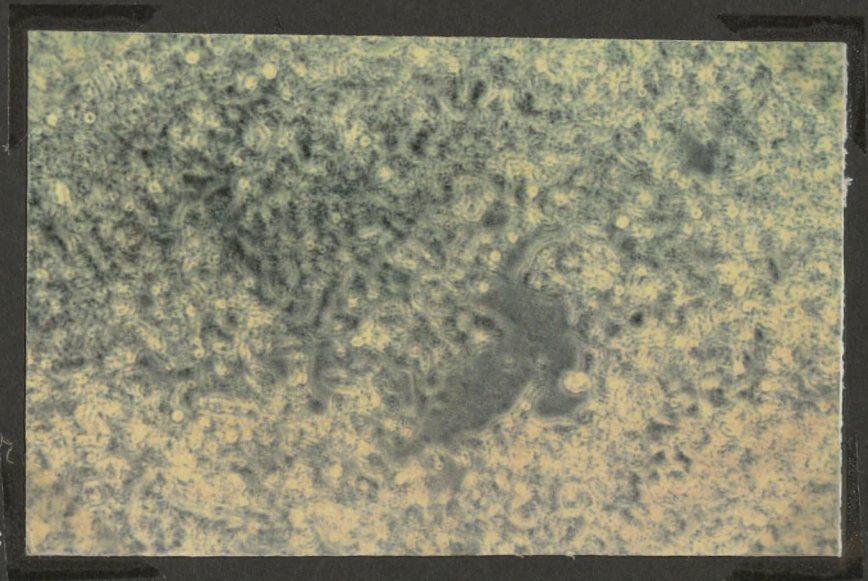
c: 100% NCS



a



b



c

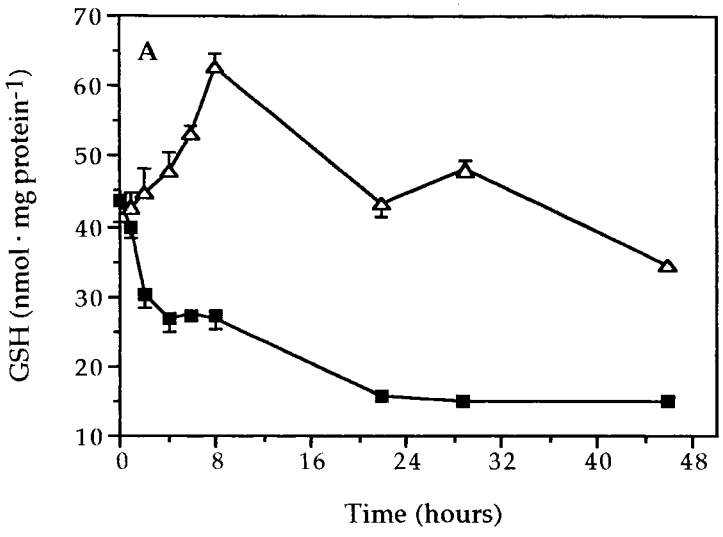
5.1.3 Timecourse of GSH depletion in confluent Hep G2 cells cultured in 100% NCS

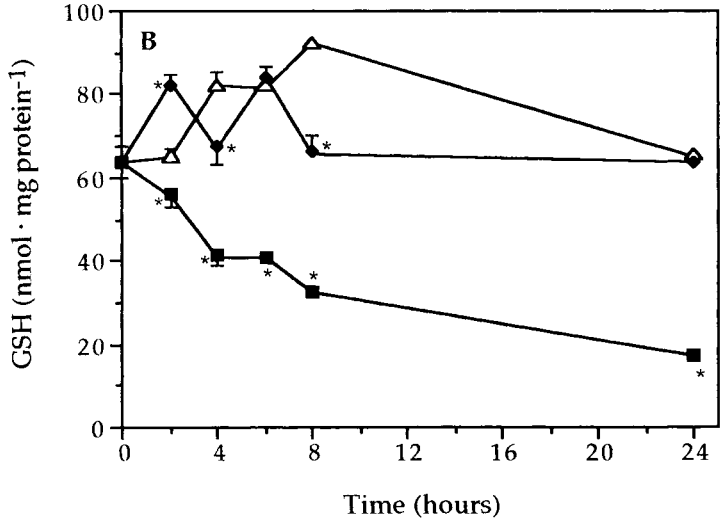
In contrast to the control cultures, in which GSH levels increased when the medium was renewed at time zero, cells which had received 100% NCS experienced a significant decrease in GSH content within 2 hours of this medium change (Figure 5.3 A, overleaf). Within 20 hours, GSH levels in these cultures had fallen to less than 30% of their initial value. Supplementation of NCS with amino acids prevented this decrease (Figure 5.3 B, overleaf); this result will be discussed in further detail in section 5.1.4.

Figure 5.3:

(A) GSH content of confluent Hep G2 cells maintained in CCM and 100% NCS for 46 hours. Medium was changed at time = 0. Results are the mean of 4 experiments and error bars represent SEM. —▲— CCM; —■— 100% NCS. All values measured for cells in 100% NCS at time ≥ 2 hours were statistically different ($P < 0.05$) from those measured for cells in CCM, by t-test.

(B) GSH content of confluent Hep G2 cells maintained for 24 hours in CCM, 100% NCS and 100% NCS supplemented with 50 mM L-cysteine, glycine and L-glutamic acid. Medium was changed at time = 0. Results are the mean of 4 experiments and error bars represent SEM. —▲— CCM; —■— 100% NCS; —◆— 100% NCS+. * $P < 0.05$, by ANOVA followed by Dunnett's test. Significant values refer to differences between cells grown in CCM and in supplemented or unsupplemented NCS.


A
GSH (nmol · mg protein-1)
70
60
50
40
30
20
10
0
8
16
24
32
40
48
Time (hours)

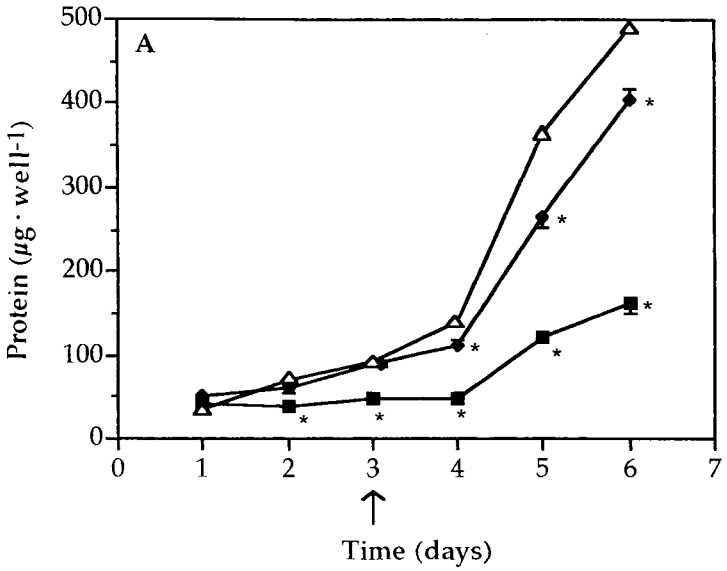

B
GSH (nmol · mg protein-1)
100
80
60
40
20
0
4
8
12
16
20
24
Time (hours)

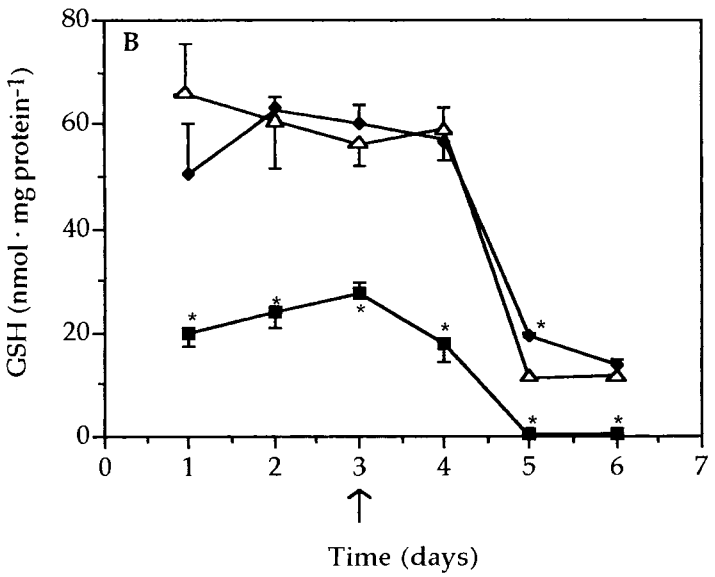
5.1.4 The effects of supplementation of NCS with amino acids

Addition of the amino acids (L-cysteine, glycine and L-glutamic acid) to NCS prevented the rapid depletion of GSH noted in section 5.1.3. The GSH content of confluent Hep G2 cells was well maintained in this medium, and, although some values were lower, the levels compared favourably with those of cells cultured in CCM (Figure 5.3 B, page 107). Figure 5.4 A (overleaf) shows that culture of Hep G2 cells in 100% NCS supplemented with the amino acids results in a much higher growth rate than that achieved in unsupplemented NCS, although the growth rate remains slower than for cells grown in CCM. Additionally, there was no consistent difference in GSH levels between cells grown in CCM and amino acid supplemented NCS (Figure 5.4 B, overleaf). All cultures experienced a sharp drop in GSH content between days 4 and 5. This corresponds to the rapid increase in cell division between days 4 and 5 for the cells grown in CCM and those in amino acid supplemented NCS, and also to the more moderate increase in cell numbers for those cells grown in unsupplemented NCS.

The difference in GSH profile between the control cell cultures in Figure 5.1 B and those in Figure 5.4 B may be attributed to the changes in the medium depth and in the schedule of medium replenishment as described in section 4.1. As explained in section 4.1, it was felt that the reduction in medium depth (to 3.75 mm for the cultures described in this section, from 5 mm for those described in section 5.1.1) allowed improved mass transfer between the gases in the headspace and the cultured cells, and thus improved the cell environment. This, in turn, resulted in a delay of the normal GSH depletion with time observed in Hep G2 cells in culture. It was therefore only necessary to change the medium once, on day 3, as opposed to twice, on days 2 and 5.

Figure 5.4: Growth (A) and GSH content (B) of Hep G2 cells grown in CCM, 100% NCS and 100% NCS supplemented with L-cysteine, glycine and L-glutamic acid (NCS+). Results are the mean of 4 experiments and error bars represent SEM. —▲— CCM; —■— 100% NCS; —◆— 100% NCS +. * $P < 0.05$, by ANOVA followed by Dunnett's test. Significant values refer to differences between cells grown in CCM and in supplemented or unsupplemented NCS. ↑ denotes change of medium.


A
Protein (μg · well-1)
500
400
300
200
100
0
0
1
2
3
4
5
6
7
Time (days)

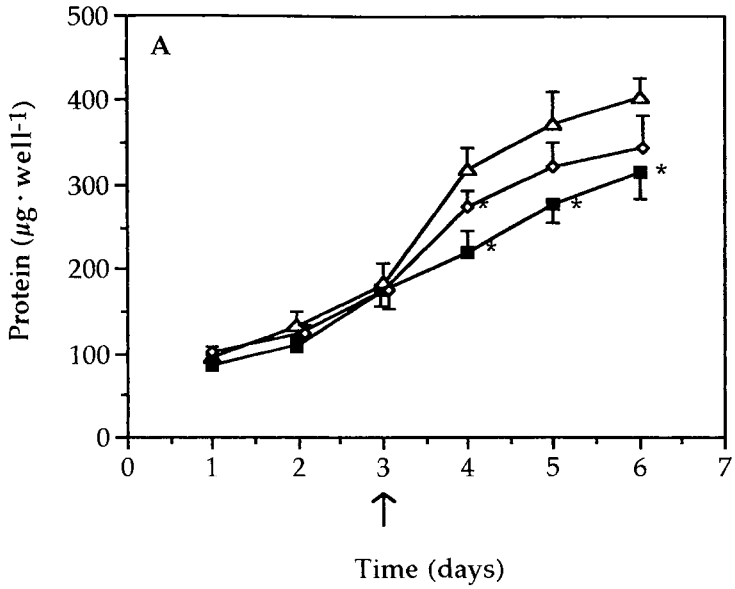

B
GSH (nmol · mg protein-1)
80
60
40
20
0
0
1
2
3
4
5
6
7
Time (days)

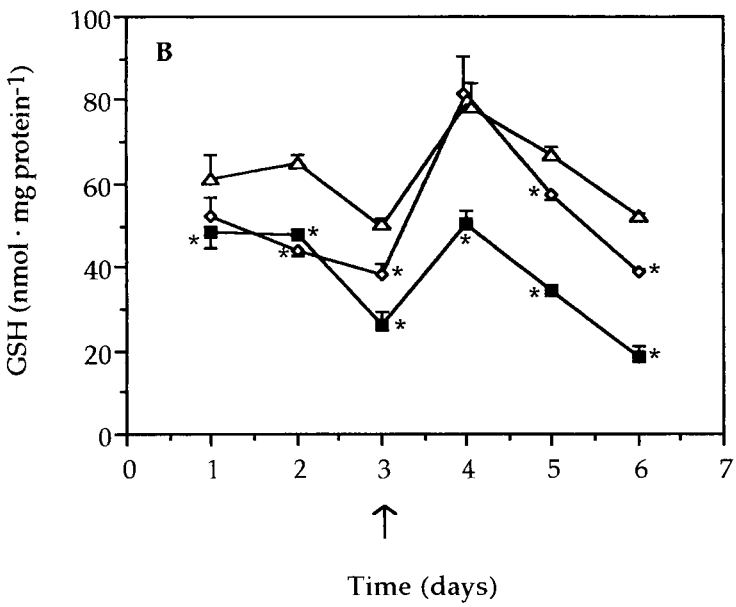
5.1.5 The effect of human serum on Hep G2 cell growth and GSH content

Human serum was well tolerated by Hep G2 cells. Even unsupplemented serum permitted cell growth at up to 80% of the control value (Figure 5.5 A, overleaf). Furthermore, this mild inhibition of growth in the cultures exposed to unsupplemented serum was not manifested until rapid cell growth in the log phase of growth occurred; the slight growth experienced during the lag phase was unaffected. Except on day 4, there was no significant difference between growth in amino acid supplemented serum and in CCM. In comparison with NCS and amino acid-supplemented NCS as a culture medium (section 5.1.4; Figure 5.4), both supplemented and unsupplemented human serum resulted in improved cell growth and maintenance of GSH levels.

GSH was generally depleted in cells grown in human serum (Figure 5.5 B, overleaf), especially in cultures grown in unsupplemented medium. All cultures were observed to respond to the medium change on day 3, as demonstrated by the increases in GSH content measured on day 4.

Figure 5.5: Growth (A) and GSH content (B) of Hep G2 cells grown in CCM, 100% human serum and 100% human serum supplemented with 50 mM L-cysteine, glycine and L-glutamic acid. Results are the mean of 4 experiments and error bars represent SEM. —△— CCM; —■— human serum; —◇— human serum supplemented with amino acids. * $P < 0.05$, by ANOVA followed by Dunnett's test. Significant values refer to differences between cells grown in CCM and in supplemented or unsupplemented human serum. ↑ denotes change of medium.


A
Protein (μg · well-1)
500
400
300
200
100
0
0 1 2 3 4 5 6 7
Time (days)


B
GSH (nmol · mg protein-1)
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Time (days)

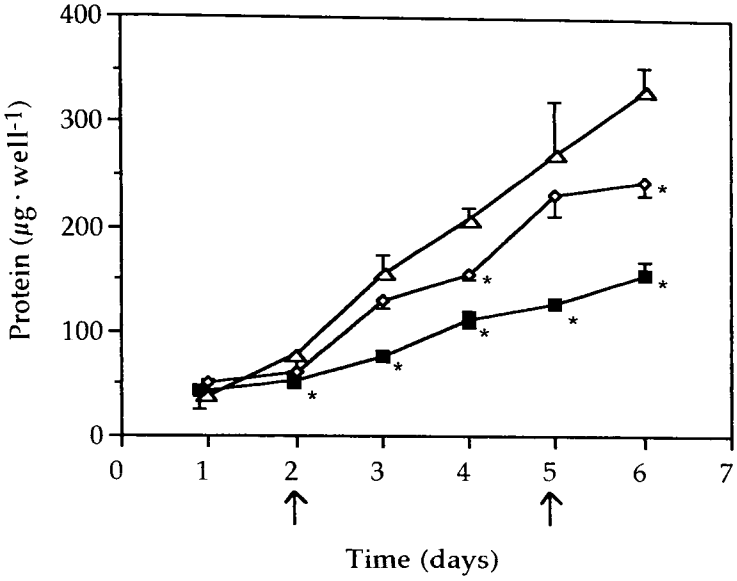
5.2 The effect of bile concentration on Hep G2 cell growth and viability and the viability of confluent Hep G2 cell cultures

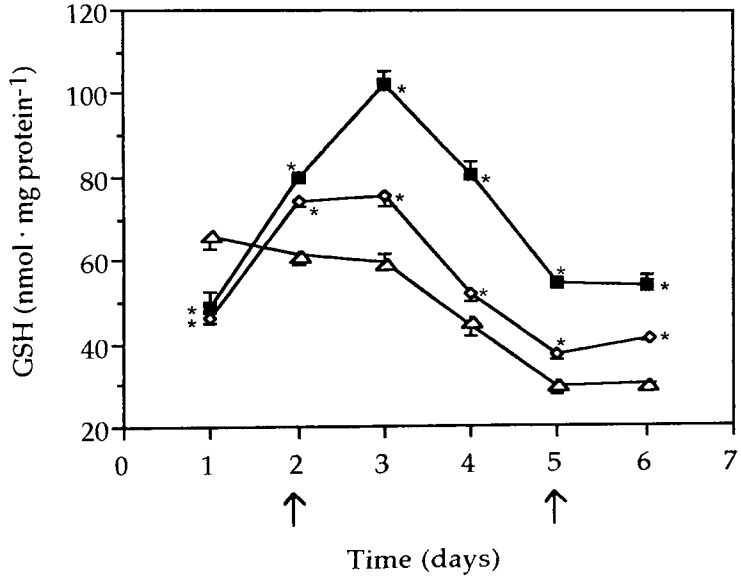
Figure 5.6 A (overleaf) shows the effect of 0.1% and 0.5% (v/v) bile in the culture medium on the growth rate of Hep G2 cells. The lag phase of growth was minimal (≤ 2 days) in all cultures. As this diminished lag phase (compared, for example, with those shown in Figures 5.1 A, 5.4 A and 5.5 A) was noted in all cultures, it may be attributed to some factor independent of the experimental variable, such as small batch to batch deviations in medium composition, serum quality or trypsin strength. Differences in the subjective aspects of the sub-culturing procedure (eg. the length of exposure to the trypsin solution or the extent of cell aspiration as required to encourage dispersion of cell "clumps") may also influence the degree of damage to the cell membranes and thus increase or decrease the length of the lag phase of growth.

Significant inhibition of growth was noted in the cultures grown in medium containing 0.1% bile on days 4 and 6 of culture. Although a decrease in total protein was also noted on day 5 of culture for these cells, this value was not statistically significant due to the large standard error of the measured protein value for the control cells on that day. In cultures grown in 0.5% bile the growth rate was markedly inhibited after day 2.

After 24 hours exposure to both 0.1 and 0.5% bile, cells experienced a 30% decrease in GSH content compared to control (Figure 5.6 B, overleaf). However, by day 2, the GSH content in these cultures had increased significantly with respect to control. These increases were greater for cells exposed to 0.5% bile than for those exposed to 0.1% bile. This elevated GSH content persisted throughout the growth period, but appeared to reach a peak on day 3, at which time cells exposed to 0.1% bile experienced a 25% increase in GSH content with respect to control while those exposed to 0.5% bile experienced a 75% increase with respect to control.

Figure 5.6: Growth (A) and GSH content (B) of Hep G2 cells grown in the presence of sheep bile. Results are the mean of 4 experiments and error bars represent SEM. —△— CCM; —◇— 0.1% bile; —■— 0.5% bile. * $P < 0.05$, by ANOVA followed by Dunnett's test. Significant values refer to differences between cells grown in CCM and in different concentrations of sheep bile. ↑ denotes change of medium.


Protein (μg · well-1)
400
300
200
100
0
0 1 2 3 4 5 6 7
Time (days)


GSH (nmol · mg protein-1)
120
100
80
60
40
20
0 1 2 3 4 5 6 7
Time (days)

Only the cells exposed to 0.5% bile demonstrated an increase in GSH content on day 3 in response to the medium change on day 2. As described in the first paragraph of this section, the lag phase of growth was reduced for all cultures in this experiment. Cell numbers increased more than two-fold between days 2 and 3 for those cells grown in CCM and in 0.1% bile. As was seen in Figures 5.1 and 5.4, a rapid rise in cell numbers is often associated with a corresponding drop in GSH content. It is therefore probable that the expected rise in GSH content was not observed in these cultures (those grown in CCM and in 0.1% bile) because the newly synthesised GSH was utilised during the cell division occurring between days 2 and 3.

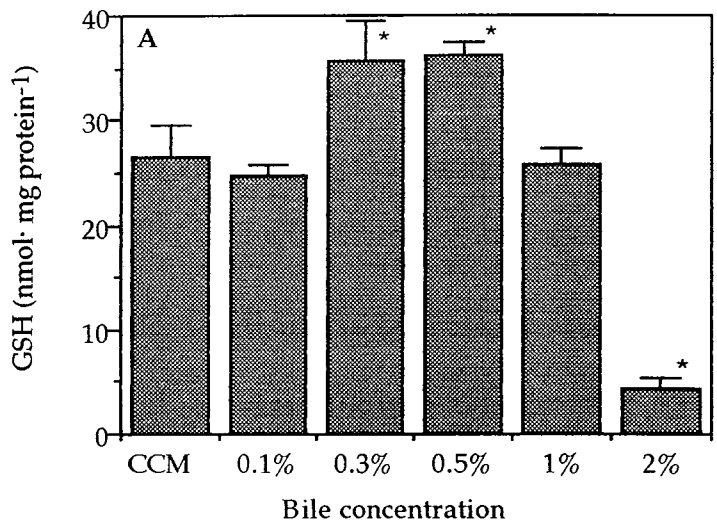
Only those cells cultured in 0.1% bile were observed to respond to the medium change on day 5 by a slight increase in GSH content on day 6. An approximately linear increase in cell numbers was associated with an approximately linear decrease in GSH for the cells grown in CCM between days 3 and 5. Although the linear growth pattern continued between days 5 and 6 for these cells, GSH levels did not continue to decline. This provides further support for the argument set out in the previous paragraph. Those cells cultured in 0.5% bile exhibited a similar, but less marked, pattern of growth and GSH fluctuations. A slight variation on this pattern can be seen in the results of the cultures grown in 0.1% bile. An approximately linear increase in cell numbers was associated with an approximately linear decrease in GSH between days 3 and 5 for these cells. Cell growth appeared to be reaching a plateau on day 6 and this flattening of the growth curve was accompanied by a modest increase in GSH content.

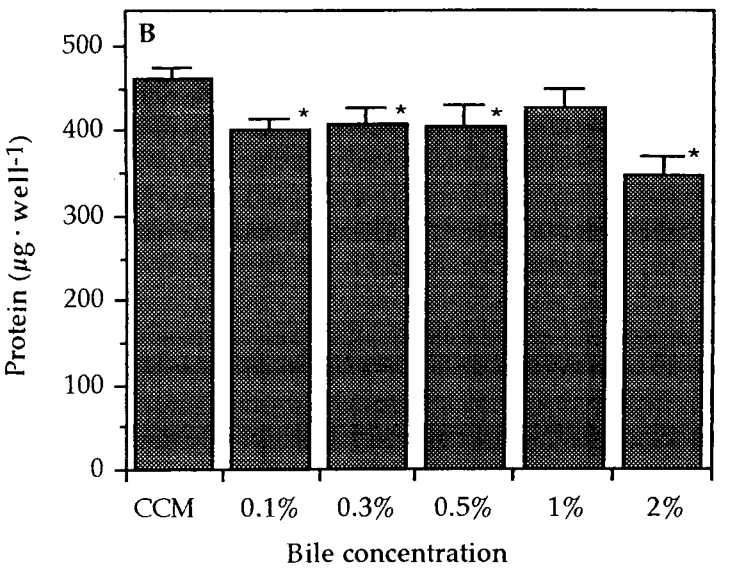
LDH activity, as measured on day 5, was seen to be higher in cultures grown in 0.5% bile (44.0 ± 2.9 nmol· min^{-1} · mg $protein^{-1}$; 3.7 ± 0.2% of total LDH), compared with 0.1% bile (27.4 ± 0.9 nmol · min^{-1} · mg $protein^{-1}$; 2.3 ± 0.08% of total LDH) and control (28.5 ± 0.9 nmol · min^{-1} · mg $protein^{-1}$; 2.4 ± 0.08% of total LDH).

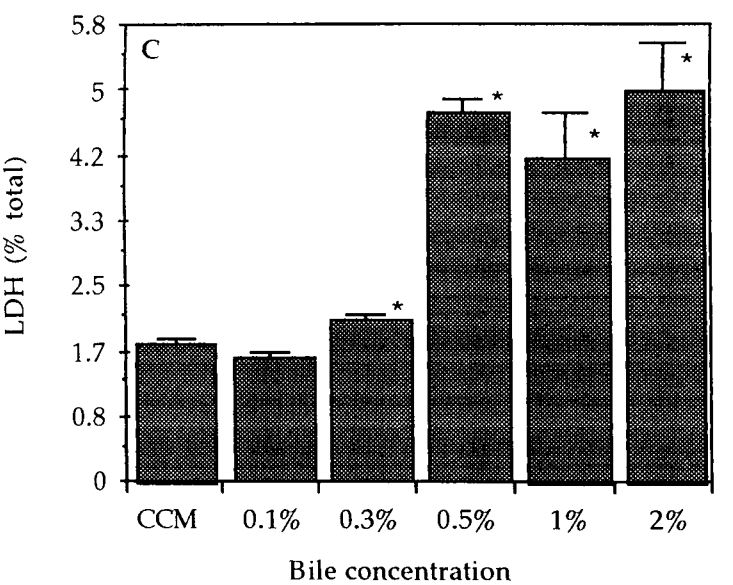
In confluent cultures, addition of sheep bile promoted an increase in GSH levels in

concentrations of 0.3% and 0.5%, but GSH declined at higher bile concentrations, and at 2% bile GSH was severely depleted (Figure 5.7 A, overleaf). Additionally, higher concentrations of bile resulted in detachment of cells from the culture flasks, as shown by a general decrease in the measured total protein (Figure 5.7 B, overleaf). In general, LDH values increased with increasing bile concentrations (Figure 5.7 C, overleaf).

Figure 5.7: GSH levels (A, below), protein (B) and LDH activity (C) of confluent Hep G2 cells maintained in increasing bile concentrations for 48 hours. Results are the mean of 4 experiments and error bars represent SEM. * $P < 0.05$, by ANOVA followed by Dunnett's test. Significant values refer to differences between cells grown in CCM and in different concentrations of sheep bile.




B
500
400
300
200
100
0
Protein (μg · well-1)
*
*
*
*
CCM
0.1%
0.3%
0.5%
1%
2%
Bile concentration


C
5.8
5
4.2
3.3
2.5
1.7
0.8
0
LDH (% total)
*
*
*
*
CCM
0.1%
0.3%
0.5%
1%
2%
Bile concentration

5.3 Tolerance of rat hepatocytes and Hep G2 cells to oxygen concentration

Rat hepatocytes were found to be sensitive to changes in the oxygen level of the culture medium gas phase, as the following results (Table 5.1, overleaf; Figures 5.8 & 5.9, pages 119 - 120; Photos 5.3 - 5.6, pages 121 - 124) demonstrate.

Cytochrome P450 was well preserved in hepatocytes exposed to 5% O_2, with levels approximately twice as great as that found in the control group (20% O_2), and nearly four times greater than that of the group exposed to 35% O_2, in which cytochrome P450 was significantly reduced (Figure 5.8 A). Cells exposed to 12 and 28% O_2 were similar to control in their P450 levels.

Compared to cells cultured in the presence of oxygen concentrations greater than 5%, hepatocytes exposed to 5% O_2 showed improved maintenance of EROD activity, again with values nearly twice that of control (Figure 5.8 B). No significant differences in EROD activity were observed amongst the remaining groups.

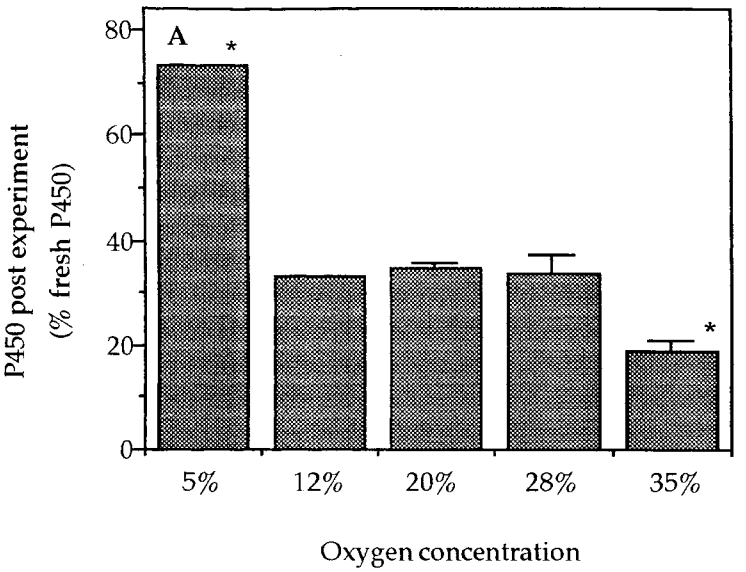
The post-experiment ratio of cytochrome P420 to P450 tended to increase with increasing oxygen concentration; only the group exposed to 5% O_2 maintained P420/P450 less than unity (Figure 5.9 A). There were no significant differences among measured values of ^{3}H-leucine incorporation except for the group exposed to 35% O_2, in which the value was doubled (Figure 5.9 B).

Although there were no significant differences in LDH activity between the experimental and control groups, cells exposed to higher oxygen concentrations appeared to age more rapidly in culture, with cells tending to flatten more quickly compared to cells exposed to lower concentrations of oxygen (Photos 5.3 - 5.6). Unfortunately, the film of the cells exposed to 20% oxygen was overdeveloped. The picture quality was therefore too poor to include in this thesis. Photo 5.6 a was also slightly overdeveloped and out of focus, but the initial rounded morphology of these cells can still be identified.

Table 5.1: Results of variations in oxygen concentration on rat hepatocyte function. Results are the mean of 4 experiments (except [a], where n = 3, [b], where n = 2, and [c], where n = 8) and error bars represent SEM. Statistical differences are shown in the graphs (Figures 5.8 & 5.9) obtained from this data.

Oxygen concentration (%)	P450: fresh cells (pmol · mg protein^{-1})	P450: post experiment (pmol · mg protein^{-1})	P420: fresh cells (pmol · mg protein^{-1})	P420: post experiment (pmol · mg protein^{-1})	EROD: fresh cells (pmol · min^{-1} · mg protein^{-1})	EROD: post experiment (fmol · min^{-1} · mg protein^{-1})	^{3}H-leucine incorporation (%)	LDH activity (nmol · min^{-1} · mg protein^{-1}/ % total)
5	140 ± 2.8[a]	103 ± 0.0	44 ± 2.3[a]	61 ± 3.5	22.8	196 ± 7.8	7.1 ± 1.1	17.4 ± 0.8/ 1.2 ± 0.05
12	219, 219[b]	72.5 ± 0.0	72, 91[b]	73 ± 7.5	35.6	123 ± 7.8	6.2 ± 1.5	21.8 ± 2.0/ 1.5 ± 0.14
20	253 ± 5.4[a]	96.2 ± 2.8	109 ± 3.2[a]	128 ± 3.5	42.4	139 ± 8.2	8.1 ± 1.7	23.2 ± 0.8/ 1.6 ± 0.16
28	155 ± 6.4[a]	52.2 ± 5.8	39 ± 3[a]	73 ± 4.1	48.0	187 ± 19	8.3 ± 0.3	16.7 ± 2.6/ 1.2 ± 0.18
35	297, 297[b]	55.6 ± 7.1	109, 100[b]	86 ± 5.0	4	166 ±14	15.9 ± 0.7[c]	14.8 ± 2.3 1.4 ± 0.16

Figure 5.8: P450 levels (A) and EROD activity (B) of cultured rat hepatocytes exposed to increasing oxygen concentrations for 24 hours. Results are the mean of 4 experiments and error bars represent SEM. * $P < 0.05$, by ANOVA followed by Dunnett's test. Significant values refer to differences between hepatocytes exposed to 20% O_2 and to other concentrations of oxygen.


A
*
*
80
60
40
20
0
P450 post experiment
(% fresh P450)
5%
12%
20%
28%
35%
Oxygen concentration

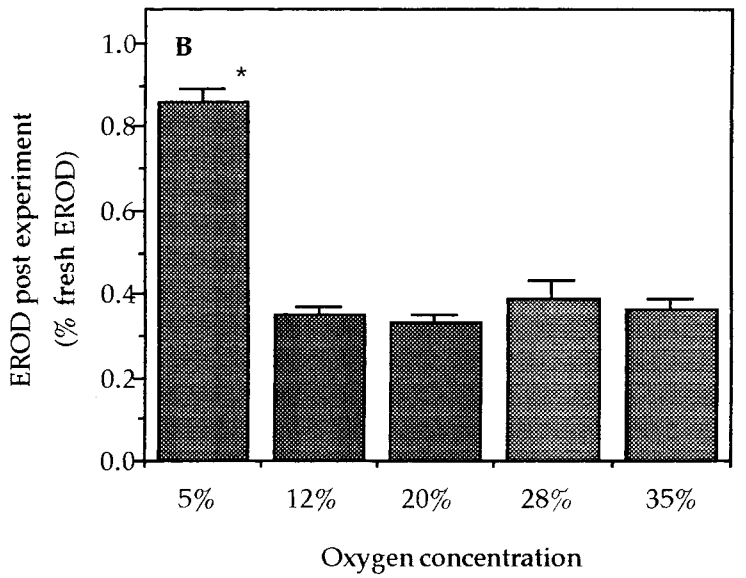
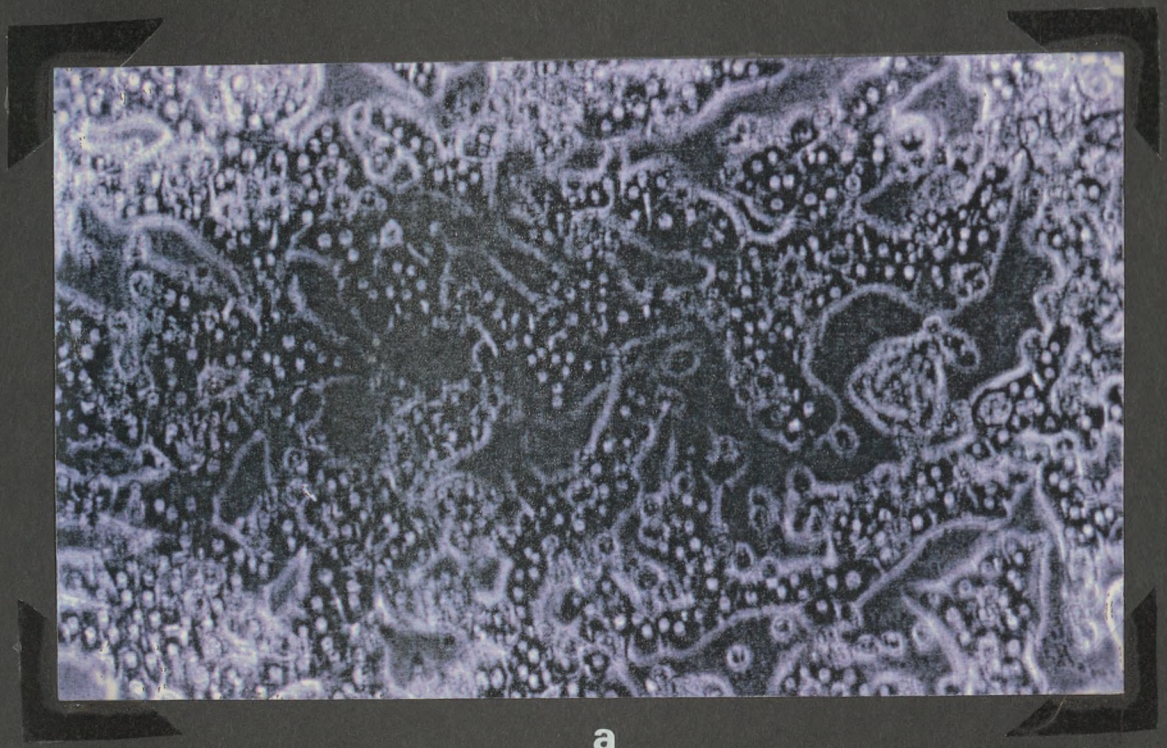
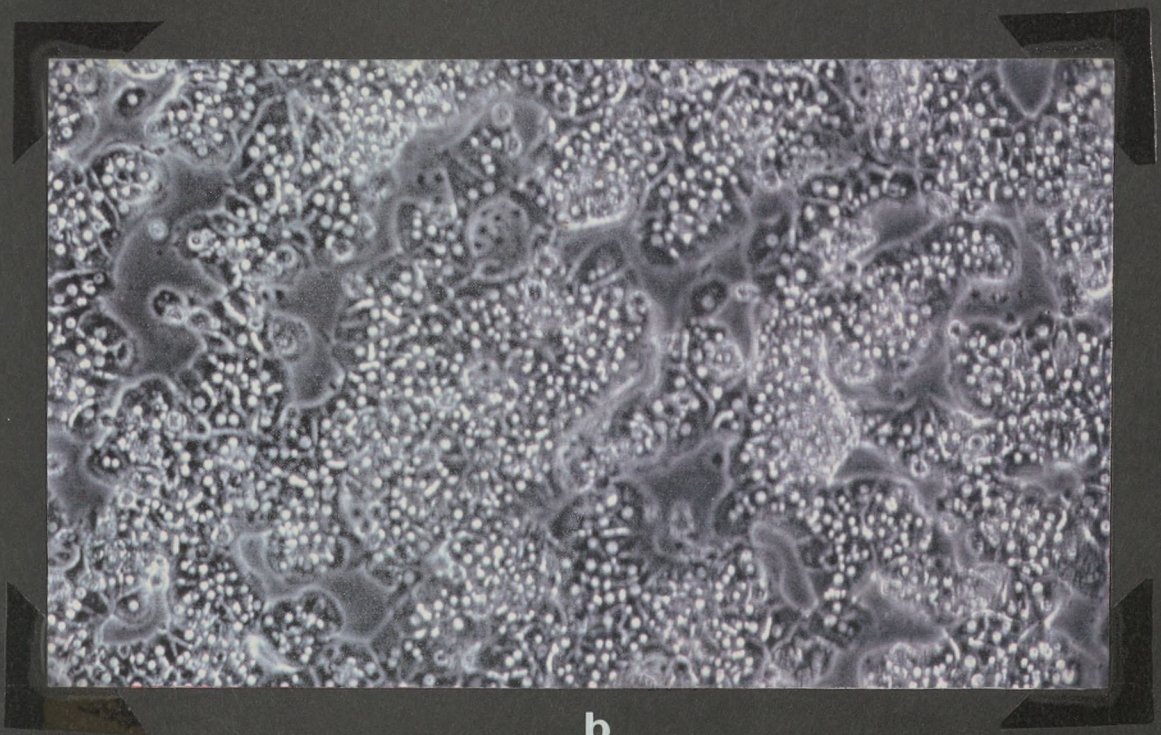

B
*
1.0
0.8
0.6
0.4
0.2
0.0
EROD post experiment
(% fresh EROD)
5%
12%
20%
28%
35%
Oxygen concentration

Photo 5.3: Cultured rat hepatocytes before (a) and after (b) 24 hours exposure to 5% O_2 (magnifications: 192x and 128x respectively).

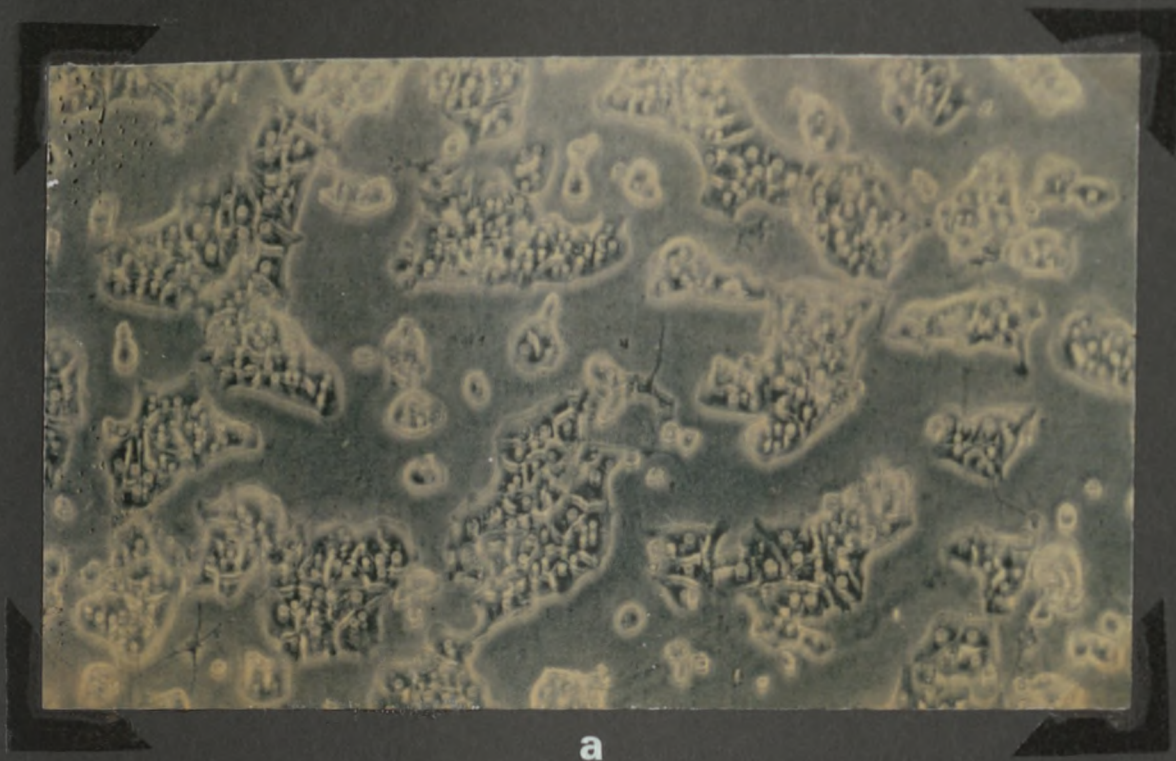


a

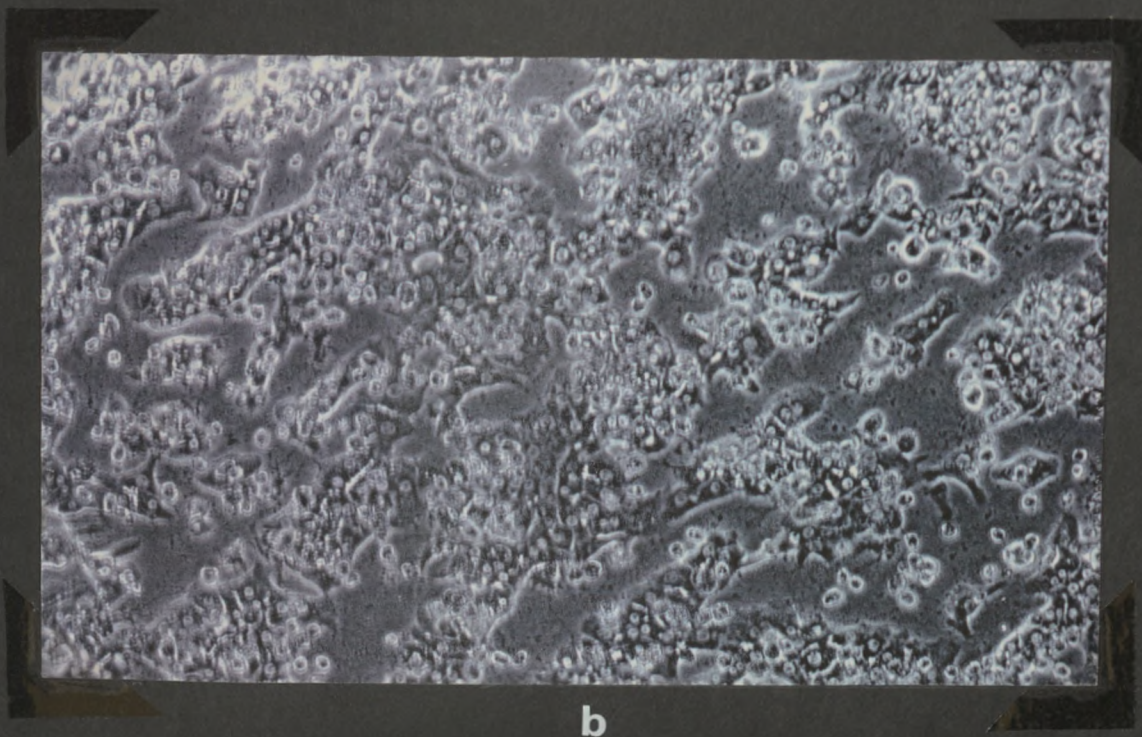


b

Photo 5.4: Cultured rat hepatocytes before (a) and after (b) 24 hours exposure to 12% O_2 (magnification: 128x).

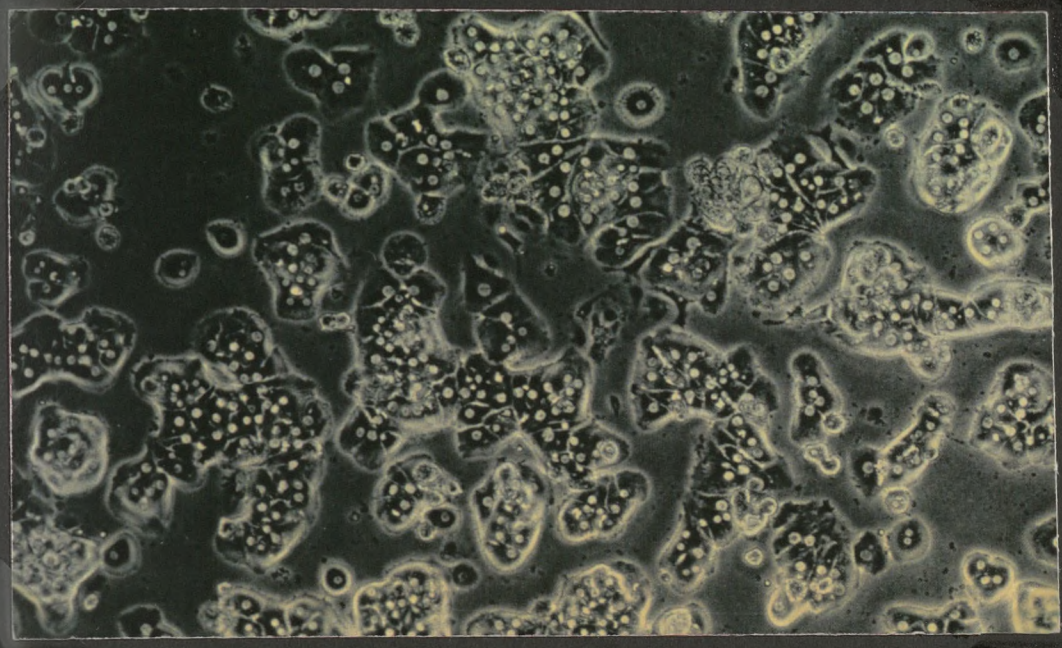


a

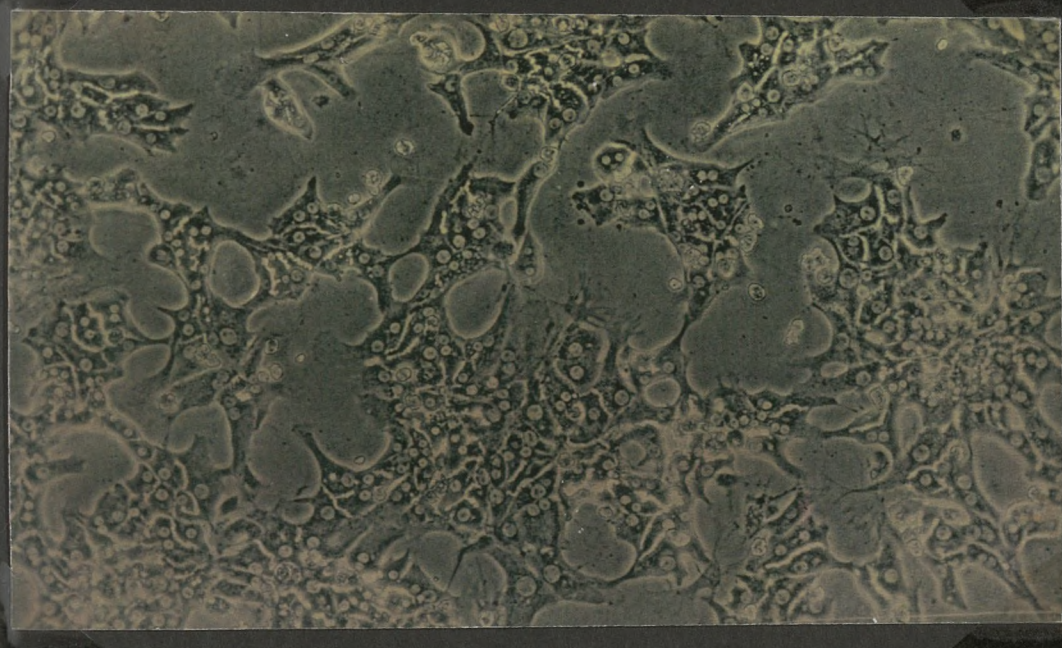


b

Photo 5.5: Cultured rat hepatocytes before (a) and after (b) 24 hours exposure to 28% O_2 (magnification: 128x).

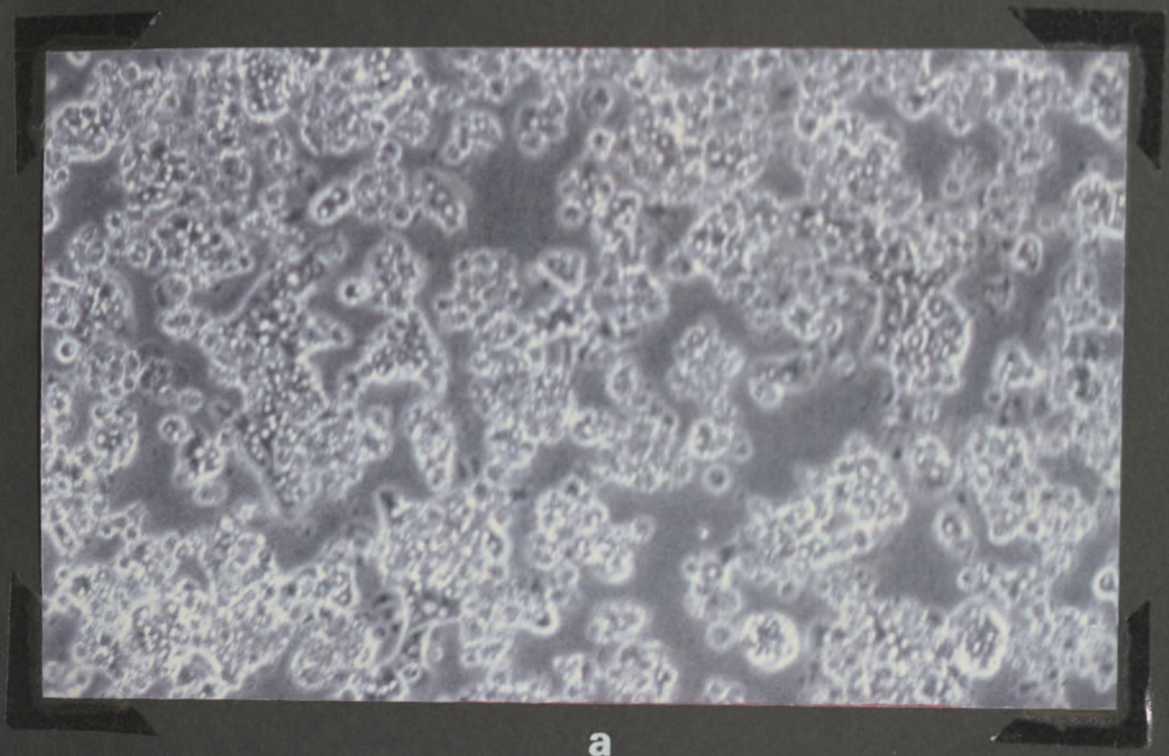


a

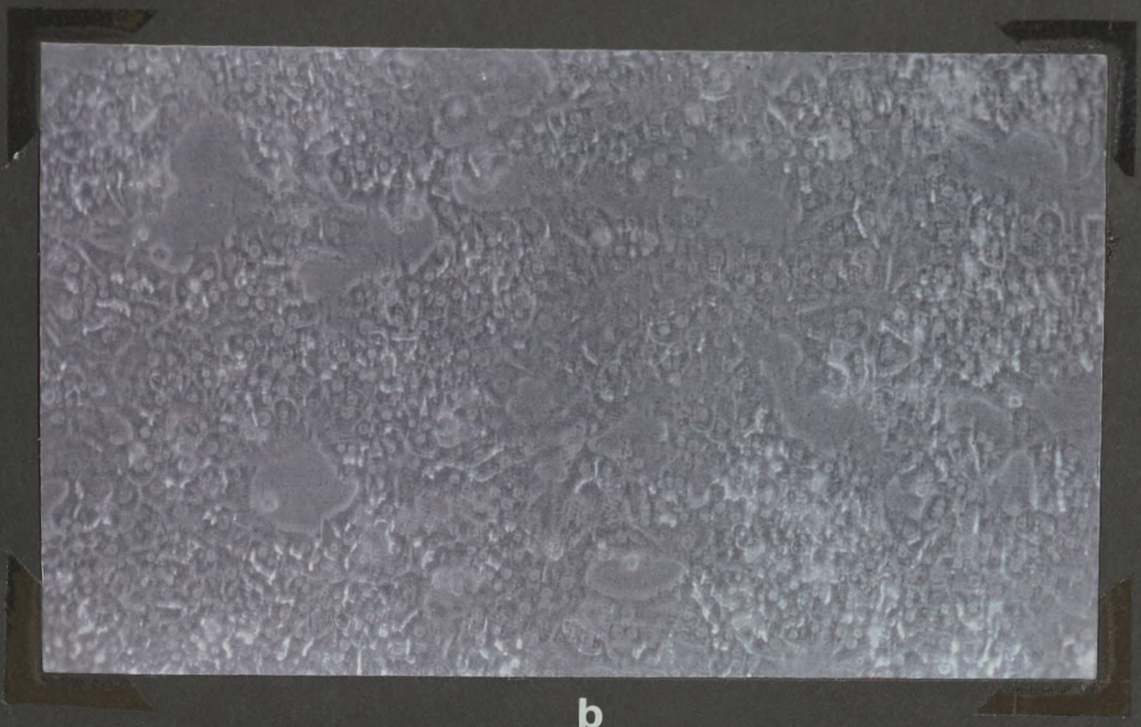


b

Photo 5.6: Cultured rat hepatocytes before (a) and after (b) 24 hours exposure to 35% O_2 (magnification: 128x).



a



b

By contrast, Hep G2 cells were unaffected by oxygen concentration. No significant differences could be found in EROD activity, LDH activity or ^{3}H-leucine incorporation amongst the various groups (Table 5.2, overleaf), and no morphological differences could be observed. Although LDH activity appears to be significantly lower for Hep G2 cells exposed to 20% O_2, it is likely that this is due to some factor independent of the experimental variable, as described in section 4.2. Cultures that were initiated at the same time as each of the experimental cultures but kept in the non-shaking 95% air: 5% CO_2 incubator were not significantly different in the measured LDH values from their experimental counterparts. In other words, while the standard error for an individual "batch" of Hep G2 cells cultured at the same time was very small, it was substantially higher when comparisons were made between batches (19.9 ± 4.3 nmol · min^{-1} · mg protein^{-1}; n = 20; exposed to 95% air: 5% CO_2 in the non-shaking incubator, measurements made on day 8 of culture). When a statistical analysis (ANOVA) was performed using all of these 95% air : 5% CO_2 "batches" as controls, it was determined that no significant difference existed between the experimental and control groups with respect to LDH activity.

Table 5.2: Results of variations in oxygen concentration on Hep G2 cell function. Results are the mean of 4 experiments and error bars represent SEM. No statistical differences were found between the experimental and control groups.

Oxygen concentration (%)	Protein (mg · plate^{-1})	^{3}H-leucine incorporation (%):	LDH activity: (nmol · min^{-1} · mg protein^{-1}/ % total)	EROD (fmol · min^{-1} · mg protein^{-1}):
5	4.5 ± 0.3	6.2 ± 0.4	19.9 ± 1.5/1.7 ± 0.13	52.3 ± 5.1
12	4.0 ± 0.2	6.3 ± 0.6	22.1 ± 1.8/1.8 ± 0.15	54.1 ± 6.1
20	4.6 ± 0.5	6.8 ± 0.7	14.7 ± 1.1/1.5 ± 0.11	59.4 ± 4.9
28	3.7 ± 0.1	6.4 ± 0.5	21.8 ± 2.7/1.8 ± 0.23	52.1 ± 3.8
35	4.6 ± 0.2	6.7 ± 0.4	20.0 ± 0.5/1.67 ± 0.04	53.4 ± 7.8

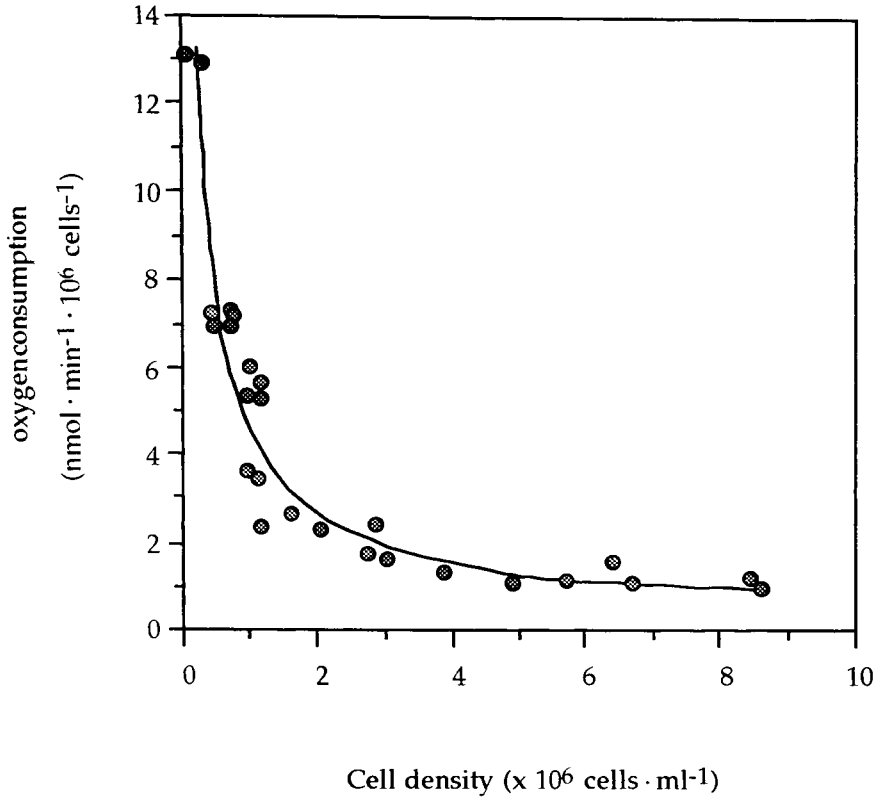
5.4 The effect of cell density and pO_2 on the oxygen consumption of Hep G2 cells

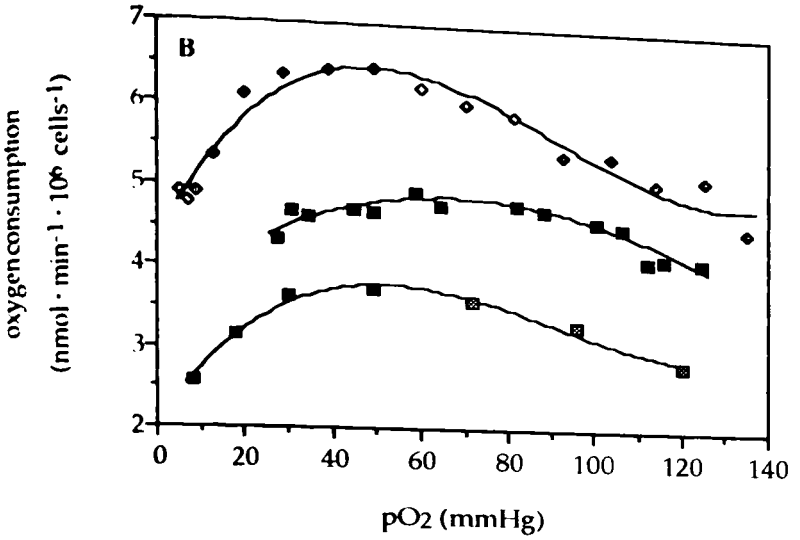
The rate of oxygen consumption of Hep G2 cells was found to be dependent on both cell density and the dissolved oxygen partial pressure in the culture medium. Oxygen consumption was found to increase exponentially with decreasing cell concentration (Figure 5.10, overleaf) when pO_2 was kept at a constant value between 140 and 150 mmHg. Oxygen consumption was also found to increase with decreasing pO_2, generally reaching a peak between 30 - 60 mm Hg O_2 before decreasing again (Figure 5.11 A - C, page 129; Table 5.3, below).

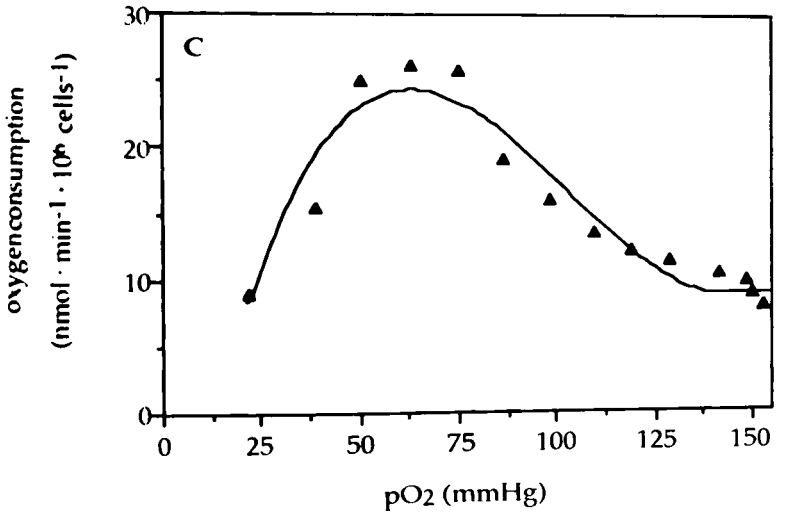
Table 5.3: Peak O_2 consumption data for Hep G2 cells at a range of cell densities.

Cell density (cells · ml^{-1})	O_2 consumption (nmol· min^{-1}· 10^6 $cells^{-1}$)	pO_2 for peak consumption (mmHg)
1.9×10^5	26	60
4.6×10^5	13.7	35
10^6	6.4	38
1.1×10^6	6.7	28
1.9×10^6	5.0	58
2.2×10^6	5.1	8
4×10^6	3.6	50

The scale of the individual graphs has been chosen so that this pattern may be seen more clearly. Only the cells tested at a density of 2.2×10^6 cells · ml^{-1} did not exhibit this pattern.


oxygenconsumption
(nmol · min-1 · 10^6 cells-1)
14
12
10
8
6
4
2
0
0
2
4
6
8
10
Cell density (x 10^6 cells · ml-1)


B
oxygenconsumption
(nmol · min-1 · 10⁶ cells-1)
7
6
5
4
3
2
0
20
40
60
80
100
120
140
pO2 (mmHg)


C
oxygenconsumption
(nmol · min-1 · 10⁶ cells-1)
30
20
10
0
0
25
50
75
100
125
150
pO2 (mmHg)

Chapter 6: Discussion

6.1 The effect of serum on Hep G2 cell growth and viability

The depletion of intracellular GSH and the inhibition of growth experienced by Hep G2 cells cultured in increasing concentrations of serum may be explained in conjunction with a brief discussion of some of the functions of GSH, and of the mechanisms by which intracellular GSH is depleted.

GSH is a ubiquitous tripeptide found in all living cells, and is essential for normal physiological cellular processes. Among its various functions (Meister, 1988; Bray and Taylor, 1994), GSH:

- detoxifies a diverse range of toxic electrophilic reactive metabolites,
- is essential for the maintenance of the cellular redox balance,
- is involved in deoxyribonucleotide synthesis,
- is involved in immune function and regulation and
- is necessary for cellular proliferation and attachment to substrata

It is therefore probable that the depletion of GSH in Hep G2 cells cultured in increasing concentrations of NCS is at least partly responsible for the inhibition of growth and the changes in cell morphology noted in these cultures. This argument is further supported by the observation that when GSH levels were maintained at normal levels in the amino acid supplemented NCS, cell growth also improved, and approached that of cells grown in the control Williams' E medium.

Intracellular GSH levels in Hep G2 cells normally decline as the cells approach confluence (Doostdar et al, 1990), and viable functional cells usually respond to the replenishment of the growth medium by an increase in GSH content. This effect was clearly seen in all cultures indicating at least some maintenance of cell viability, despite the severe reduction in GSH content for cells exposed to 100% NCS.

There are several methods by which intracellular GSH may be depleted (Meister, 1988; Seelig and Meister, 1985):

1. exposure to agents which either oxidise, or form conjugates with, GSH

(eg. in detoxication),

2. a reduction in the supply of the amino acids necessary for its synthesis (i.e. L-cysteine, glycine and L-glutamic acid),
3. inhibition of its biosynthesis (eg. inhibition of GSH-synthetase or γ-glutamylcysteinyl synthetase) and
4. increased efflux and degradation of GSH.

The ability of Hep G2 cells to maintain normal levels of intracellular GSH when cultured in NCS-supplemented with 50 mM L-cysteine, glycine and L-glutamic acid implies that an amino acid deficit is the most likely explanation for the GSH depletion observed in Hep G2 cells cultured in unsupplemented NCS. Generally, intracellular glycine and L-glutamic acid are present in substantial amounts, whereas L-cysteine is rapidly consumed, via oxidation to L-cystine or through cell metabolism. Supply of L-cysteine is therefore often the rate limiting factor in GSH synthesis (ibid). Although *de novo* GSH synthesis was not proven in these experiments, it is a probable explanation for the increases in GSH observed in response to:

- the replenishment of the growth medium (for growing cultures) and
- exposure to 50 and 75% NCS (for confluent cultures).

GSH metabolism in Hep G2 cells differs from that of rat hepatocytes in that L-cystine has been shown to help maintain intracellular levels of GSH by forming mixed disulphides from GSH released from the cells, thereby liberating L-cysteine for uptake (Lu and Huang, 1994). Furthermore, at physiologic concentrations of L-cysteine (10 μM), its uptake in Hep G2 cells is inhibited by the presence of certain other amino acids, such as L-threonine and L-alanine. Therefore, an imbalance of amino acids, or over-abundance of those which inhibit the uptake of L-cysteine, is a possible explanation for the depletion of GSH observed in the cultures exposed to 100% NCS (as opposed to a lack of L-cysteine due to its oxidation to L-cystine).

From these experiments, it appears that unsupplemented human serum does

contain sufficient quantities of L-cysteine and/or L-cystine to permit the maintenance of intracellular GSH levels at the same levels as seen in control cultures. However, there appears to be some inaccuracy in the measurement of the average values of L-cysteine and L-cysteine in human plasma or serum. For example, in a compilation of morphometric and biochemical data (Altman and Dittmer, 1974) there seems to be a contradiction: the concentration of L-cystine in human serum (1.47 mg · 100 ml^{-1} = 1.18 μM) was quoted as being considerably greater than the concentration of <u>both</u> L-cysteine and L-cystine in human serum (1.18 mg · 100 ml^{-1}; a molar concentration cannot be determined as the proportions of L-cysteine and L-cystine are not obvious from the information given). Another such compilation (Lentner, 1984) gave the concentrations of L-cysteine and L-cystine as 42 μM and 33 μM respectively. In addition, the introduction of a study by Gmünder et al (1990), reported that plasma concentrations of L-cysteine and L-cystine were in the range of 10 - 20 μM and 50 - 100 μM respectively. Even if the highest of these values is correct, it would seem that far less L-cysteine is required for GSH synthesis than is provided by Williams' E medium (52 mM). Regardless of the specific details, growth and maintenance of GSH were far superior in unsupplemented human serum than in unsupplemented NCS. Therefore, it is probable that NCS differs from human serum in some respect(s) that is (are) crucial for cell proliferation and maintenance of intracellular GSH.

Although no information could be found relating to the concentrations of amino acids in NCS, some variations have been observed in the composition of NCS compared to human serum in terms of total protein, ion concentration, pH and conductivity (Table <u>6.1</u>, overleaf). In addition, while there do not appear to be any published values for plasma or serum, whole bovine blood contains slightly higher concentrations of glutathione than whole human blood (Table <u>6.2</u>, overleaf). Finally, NCS has been found to have more than twice as many ß- and γ - globulins as human serum (MacCleod

and Drummond, 1980). These variations may extend to the amino acid balance of NCS, which might therefore be inadequate to allow sufficient L-cysteine transport into the cells for GSH synthesis. As GSH is known to be involved in immune function and regulation (Bray and Taylor, 1994), subsequent reductions in the levels of GSH may thus increase the susceptibility of the cells to immunological agents in the NCS.

Table 6.1: Differences between total protein, ion concentration, pH and conductivity between human serum and NCS (MacCleod and Drummond, 1980)

parameter	human serum	NCS
Total protein ($g \cdot l^{-1}$)	82.0	49.8 - 72.4
Sodium (mM)	130	110 - 147
Potassium (mM)	10	5 - 6
Chloride (mM)	103	80 - 109
Phosphate (mM)	1.3	2.16 - 2.46
pH	8.31	7.45 - 7.82
Conductivity (mmho)	10.9	8.15 - 10.8

Table 6.2: Concentrations of oxidised (GSSG) and reduced glutathione (GSH) in human and bovine blood (Altman and Dittmer, 1974)

parameter	human blood	bovine blood
GSH ($mg \cdot 100$ ml $blood^{-1}$)	26.7 - 31.9	40
GSSG ($mg \cdot 100$ ml $blood^{-1}$)	3.99 - 4.06	6

The improved growth and maintenance of intracellular GSH levels seen in Hep G2 cells cultured in both amino acid supplemented and unsupplemented human serum also supports the "amino acid variation" hypothesis. The composition of human serum is more likely to be appropriate for the nutritional requirements of Hep G2 cells, as they are a human hepatoma derived cell line. Immunological reactions are also likely to be reduced for human serum compared to NCS, due to the lower levels of immunoglobulins, and greater species similarity.

GSH regulates its own biosynthesis by feedback inhibition, and responds to GSH depletion by increased production of γ-glutamylcysteinyl synthetase (Seelig and Meister, 1985). The increases in GSH content seen for confluent cultures of Hep G2 cells exposed to 50 and 75% NCS for 48 hours may therefore be explained as follows: a decreased or restricted supply of amino acids in 50 and 75% NCS may result in an initial decrease in the GSH content of confluent cultures of Hep G2 cells at some time between t = 0 and t = 48 hours. This would then stimulate an over-production of γ-glutamylcysteinyl synthetase, ultimately resulting in the elevated levels of GSH observed in these cultures.

Hepatocyte phenotype is exceptionally sensitive to culture conditions, and there are marked interspecies differences in P450, GSH and protein content, and in urea synthesis, when these cells are cultured in media of varying compositions (Watts et al, 1994). This sensitivity must be addressed in the context of the design of the BAL, to prolong the functional lifespan of the cultured cells. These results imply that direct contact between cultured cells in the BAL and the patients' plasma may be a viable option. Hep G2 cells cultured on Cytodex 3 microcarrier beads have been shown to remain viable in human plasma for up to 24 hours (Cunningham and Hodgson, 1992), while rabbit hepatocytes survive and retain function in medium containing up to 90% rabbit serum (Jauregui et al, 1991). However, artificial liver support systems are likely to involve heterologous hepatocytes. Thus it is important to note that Uchino and co-

workers (1991) have reported that pig hepatocytes retained metabolic activities such as gluconeogenesis and ureogenesis for up to 3 days when cultured in normal human plasma, and in plasma from patients with hepatic failure. However, the same research group reported that dog hepatocytes did not survive in human plasma (Uchino et al, 1988), and that up to 6.8% of humans produce anti-porcine hepatocyte immunoglobulins (Takahashi et al, 1993). The indications are that the ability to survive in human plasma is species dependent, and may depend on immune interactions.

Immune recognition is mediated by white blood cells, antibodies and complement proteins. By designing the bioreactor device with direct contact between plasma and hepatocytes, the contribution of the white blood cells to any immune interactions is eliminated. However, immune interactions may still occur due to the antibodies and complement proteins, leading to a deterioration in cell function and ultimately to cell death. The use of hollow fibres with 100 kDa molecular weight cut off has been shown to exclude antibodies and complement proteins in a bioreactor based on rat hepatocytes, which was used to provide liver support for anhepatic rabbits (Nyberg et al, 1992). Although Takahashi and coworkers (1992) reported no immunological interactions when anhepatic rabbits were treated with an artificial liver support device involving the perfusion of plasma over immobilised pig hepatocytes, it is not clear at present whether this would be the case with human plasma and xenogenic hepatocytes. Ultimately, it may prove necessary to incorporate an immunoprotective membrane into the BAL.

There is broad scope for additional research into this area. A variety of cell types have been proposed for the BAL (see section 2.7). Future research may therefore include an effort to determine the effect of human plasma on several different cell types. It is anticipated that the Strathclyde BAL will use sheep hepatocytes. Studies at Harwell AEA Technology have shown that sheep hepatocytes cultured in heparinised sheep plasma exhibit significantly higher rates of urea synthesis than

those cultured in Williams' E medium (Iain Edwards, unpublished data). Future work is planned to test the effect of normal human plasma and plasma from FHF patients on the function and viability of sheep hepatocytes. A wider range of parameters (incorporating several tests of metabolic and synthetic competence) should be explored, to ensure that human serum can provide adequate nutrition to the cell type of choice.

Although current results appear promising, it may be that culture of heterologous hepatocytes with human serum will significantly impair one or more cell functions, or lead to a reduction in the lifespan of the cells. In addition, the membrane barrier required for immunoprotection may restrict the range of substrates that may enter the hepatocyte chamber for metabolism/detoxication. An examination may then need to be undertaken, in which these factors are weighed against the reductions in efficiency caused by the combined mass transfer limitations (eg. immunoprotection and diffusional resistance) that would be expected in a BAL with distinct hepatocyte and blood compartments.

6.2 The effect of bile on Hep G2 cell growth and viability

The increases in GSH content of Hep G2 cells exposed to 0.1 and 0.5% sheep bile in the culture medium appears to be part of a defensive response to a sub-lethal toxic insult. Many cell types respond to sub-lethal injury by an increase in the activity/concentration of cytoprotective intracellular systems (Brunton et al, 1990). In growing cultures of Hep G2 cells, this increase appears after an initial drop in GSH content, as observed on day 1 of culture. As described in section 6.1, GSH synthesis is controlled by feedback inhibition of the synthesis of γ-glutamylcysteinyl synthetase in the presence of GSH. Thus an initial depletion of GSH may occur due to conjugation with toxic substances present in the bile. This depletion would then stimulate production of the γ-glutamylcysteinyl synthetase which would ultimately promote the increases in GSH observed. The observed inhibition of cell growth in the presence of 0.1 and 0.5% bile may therefore be due to the utilisation of amino acids in the synthesis of GSH, rather than in cell proliferation.

A similar response can be observed in the confluent cultures exposed to increasing concentrations of bile, although the balance between the synthesis and depletion of GSH is perhaps more explicitly illustrated. For concentrations of 0.3 and 0.5% bile in the culture medium, intracellular concentration of GSH is elevated as the cells produce GSH in excess of that used to detoxify the bile. Ultimately, as the concentration of bile in the culture medium increases, the ratio of GSH utilised to GSH synthesised increases, with those cells exposed to 2% bile experiencing a sharp drop in GSH content.

The observed toxic response to bile is likely to be due to the presence of bile acids. Table 6.3 (overleaf) outlines the basic components of human gall bladder bile (information is unavailable for sheep gallbladder bile, but it is probable that the components of human gallbladder bile, if not the proportions, are representative of sheep gallbladder bile). It can be seen from this table that, with the exception of the bile acids, the major constituents of bile are unlikely sources of toxicity. Bile acids

Table 6.3: Chemical composition of human gallbladder bile	
General composition ($g \cdot l^{-1}$)	
Dry matter	180
Inorganic matter	5 - 11
Bile salts	115
Water	859
Electrolytes ($meq \cdot l^{-1}$)	
Bicarbonate	8 - 12
Chloride	15 - 30
Calcium	5 - 7
Iron (as Fe^{2+})	0.01 - 0.6
Organic substances (mg %)	
Protein	315 - 539
Bilirubin	1000
Urea	20 - 45
Amino acid nitrogen	6.0 - 21.6
Cholesterol	630
Fatty acids	970
Lecithin	3500

have been shown to be hepatotoxic by a number of researchers (Ohta et al, 1990; Delzenne et al, 1992; Sokol et al, 1993) and to induce lysis in red blood cell preparations (Child and Rafter, 1986). Although the mechanism by which hepatotoxicity is induced by bile acids is not fully understood, it is generally accepted that the cytotoxicity and hydrophobicity of bile acids are inversely related to the number of hydroxyl groups on their steroid moiety (Delzenne et al, 1992). Despite the correlation between hydrophobicity and bile acid toxicity, hepatocellular injury has been observed in isolated hepatocyte suspensions exposed to bile acid concentrations (100 - 500 μM) well below their detergent levels (Sokol et al, 1993). It is felt that free radicals generated by hepatocytes incubated in hydrophobic bile acids are the causative agent in hepatotoxicity. This corresponds well with the induction of GSH synthesis, as GSH is used in the detoxification of free radicals. Inclusion of antioxidants (eg. α-tocopherol) in the culture medium resulted in a dose related reduction in the cytotoxicity of bile acids (ibid).

Several arguments against the extrapolation of *in vitro* bile acid/salt cytotoxicity to their *in vivo* toxicity have been presented (Ostrow, 1994). These shall be considered in detail:

1. *The concentrations of bile salts/acids used in most* in vitro *studies are not relevant to the total bile salt/acid concentrations found in the plasma of patients with hepatobiliary diseases.*

   This argument is not relevant to the experiments described in this thesis. The total bile acid concentration of sheep hepatic bile is approximately 42.5 mM (Diem and Lentner, 1973). Gallbladder bile is more concentrated than hepatic bile; for example, in humans gallbladder bile is approximately 3 times more concentrated than hepatic bile (ibid), while rabbit gallbladder bile is 2-3 times more

concentrated than hepatic bile (Erlinger, 1988). If it is assumed that sheep gallbladder bile is approximately 3 times more concentrated than hepatic bile, then the upper level of the total bile acid concentration would be approximately 130 mM. This would correspond to total bile acid concentrations in the culture medium of 130 μM and 650 μM for 0.1 and 0.5% bile respectively. Even if sheep gallbladder bile were 5 times more concentrated than hepatic bile, the total bile acid concentrations in the culture medium would only be 210 μM and 1 mM for 0.1 and 0.5% bile respectively.

These values are well within the range of total serum bile acids (TSBA) that may occur in liver disease. Although the average TSBA for a healthy individual is approximately 1.6 μM (Makino et al, 1969), an analysis of a survey published by Osborn et al (1959) shows that the average TSBA in hepatic failure is 310 $\pm$ 37 μM. As Ostrow points out, even if it is assumed that hepatic extraction of bile salts is reduced due to severe cholestasis or cirrhosis, total bile salt concentration in portal venous blood may be up to twice as great as that measured in the peripheral blood. This would be of particular relevance if the BAL were connected to the portal (rather than systemic) circulation in an effort to increase total blood flow through the reactor.

2. *Only deoxycholates and lithocholates are significantly hepatotoxic, and these rarely constitute more than 40% of the TSBA.*

This is not true. The normal bile salt pool in humans comprises 40% chenodeoxycholic acid, 38% cholic acid, 19% deoxycholic acid and 3% lithocholic acid (Beher, 1976). The proportions of cholic, chenodeoxycholic and lithocholic acid in the blood of liver failure

patients varies according to the etiology and severity of the disease, but chenodeoxycholic acid is often the predominant primary bile acid, whilst lithocholate becomes the predominant secondary bile acid (ibid). Although deoxycholate appears to be absent in most liver disease (ibid), chenodeoxycholic and lithocholic acid are also highly toxic, and cholic acid demonstrates (to a lesser extent) significant cytotoxicity (Delzenne et al, 1992; Ohta et al, 1990). Each of these bile acids, when added to the culture medium individually at a concentration of 1 mM, resulted in lysis of 30% of hepatocytes after a brief (60 minute) incubation period (Delzenne et al, 1992). Furthermore, experiments by Latta and coworkers (1993) have shown that less than 45% of the colon cancer cell line T84 survived a 48 hour exposure to 50 μM lithocholic acid, and that none survived a 48 hour exposure to 100 μM lithocholic acid. In addition, the viability of T84 cells exposed to 100 μM chenodeoxycholic acid for 48 hours decreased to less than 20% (as determined by Trypan Blue exclusion). Similarly, lithocholic and chenodeoxycholic acid were shown to be extremely cytotoxic to the colon cancer cell lines HT29 and HCT116 in relatively low quantities (Table 6.4, overleaf).

3. In vitro *bile cytotoxicity tests generally examine the toxicity of individual bile acids, whereas* in vivo *tissues would be subjected to a mixture of bile acids, the less hydrophobic of which may reduce overall cytotoxicity.*

There does not appear to be any evidence to support the claim that any of the naturally occurring bile acids, either in health or disease, reduce the cytotoxicity of the more hydrophobic bile acids.

The fact that hepatotoxicity is reduced when a high ratio of trihydroxy to di- and mono- hydroxy bile acids is maintained (Paumgartner, 1975), is more likely to reflect the relative decrease in

Table 6.4: Viability (as determined by Trypan Blue exclusion) of the colon cancer cell lines HT29 and HCT116 when exposed to lithocholic and chenodeoxycholic acid for 24 hours (Latta et al, 1993).

Bile acid added to culture medium	HT29	HCT116
Lithocholic acid		
50 μM	< 75%	< 50%
100 μM	< 35%	< 25%
150 μM	< 25%	< 10%
200 μM	< 20%	0%
Chenodeoxycholic acid		
100 μM	< 60%	< 25%
150 μM	< 25%	< 10%
200 μM	< 20%	0%
250 μM	< 20%	0%
300 μM	< 20%	0%

the more cytotoxic bile acids as a proportion of the total bile acid pool, rather than a reduction in the individual toxicity of these acids. Furthermore, the least hydrophobic (and thus the least cytotoxic) of the commonly occurring bile acids, cholic acid, is often reduced in

hepatic disease (Beher, 1976).

It is just as possible that a combination of bile acids could act synergistically to increase overall cytotoxicity. Delzenne and coworkers (1992) have shown that oral administration of either cholic, deoxycholic or lithocholic acid in rats results in increased TSBA concentration, and is not restricted to an increase in the bile salt administered. Furthermore, these increases in TSBA (to between 200 - 660 μM) resulted in significant increases in liver necrosis as measured by the activity of alanine aminotransferase and aspartate aminotransferase in the serum.

Finally, even if this claim were legitimate, it is not relevant to the work presented in this thesis. The bile toxicity experiments described in this thesis were conducted using sheep gallbladder bile, from which cholic, deoxycholic and chenodeoxycholic acid have been isolated (Beher, 1976).

4. *The vast majority of the dihydroxy and monohydroxy bile salts in plasma are amidated or sulphated in both normal and cholestatic human beings, rendering them less hepatotoxic than their unconjugated moieties.*

Although the percentage of the total serum bile acids which are sulphated has been shown to increase in cholestatic patients, it can be significantly reduced (compared to levels seen in health) in other forms of hepatobiliary disease, and on average constitutes less than 10% of the serum bile acids (Makino et al, 1972). In addition, the ratio of conjugated serum bile acids to TSBA varies according to the origin of hepatic dysfunction. For example, Makino and coworkers (1969) have

also shown that, although TSBA are highly elevated in chronic active portal cirrhosis, a substantial percentage of this remains unconjugated (39.9 ± 24.9%, n=17).

It has been reported that > 88% of bile acids are conjugated with either glycine or taurine when TSBA concentrations exceed 50 μM (Neale et al, 1971), but it is debatable whether or not this conjugation reduces cytotoxicity. Experiments by Sokol et al (1993) have shown that less than 50% of rat hepatocytes remain viable (as determined by Trypan Blue exclusion) after a 4 hour exposure to 200 μM taurolithocholic acid. Although the hepatotoxicity of tauro-conjugated bile acids was somewhat diminished when rat hepatocytes were pre-treated with a 30 minute incubation in a reduced oxygen atmosphere (9% O_2, 5% CO_2, 86% N_2), cell viability was still reduced to less than 60% (as determined by Trypan Blue exclusion) after a 4 hour exposure to 500 μM taurolithocholic or taurochenodeoxycholic acid. Furthermore, Ohta et al (1990), in experiments with rat hepatocytes, have demonstrated that tauro- and glyco- conjugation increase the toxicity of chenodeoxycholate and ursodeoxycholate.

Finally, although it appears that Hep G2 cells have only limited ability to conjugate bile acids (Javitt, 1990; Everson and Polokoff, 1986), *in vitro* tests on rat hepatocytes indicate that primary cultures are similar to hepatocytes *in vivo* in their ability to conjugate bile acids (Hylemon et al, 1985). This ability to conjugate bile acids does not appear to have prevented the bile acid hepatotoxicity observed *in vitro* (Delzenne et al, 1992; Ohta et al, 1990; Miyazaki et al, 1984).

5. *More than 90% of conjugated or unconjugated dihydroxy and monohydroxy bile salts are bound to albumin* in vivo, *whilst the albumin concentration, and thus the binding capacity of the culture medium, is much lower* in vitro.

It is true that bile acids are bound extensively by serum albumin, as the following analysis will indicate:

Serum albumin in hepatic failure is generally lower than in health (468 ± 14 μM, n = 49, lowest value = 217 μM; data calculated from tables provided by Osborn et al, 1959) while serum bile acids are elevated (eg. chenodeoxycholic acid concentration = 18.5 ± 3.6 μM, n = 36, highest value = 89.2 μM, data calculated from tables provided by Neale et al, 1971). Therefore, even assuming moderately high patient serum levels of chenodeoxycholic acid (30 μM) in conjunction with relatively low levels of serum albumin (300 μM), it would be predicted that 99% of chenodeoxycholic acid in serum would be bound, using the relation given by Pico and Houssier (1989):

$$r = \sum_{i=1}^{x} \frac{n_i K_i c_f}{1 + K_i c_f}$$

where:

x = 2 provides sufficient accuracy

r: ratio of the concentration of bound bile acid to the concentration of serum albumin

c_f: the concentration of unbound (or free) bile acid

n_1 = 2.7

$n_2 = 2.4$

$K_1 = 70 \pm 4.6 \times 10^4$

$K_2 = 0.2 \times 10^4$

However, this analysis assumes that there is no competition for binding sites on the albumin by other bile acids and/or protein-binding species present in hepatic failure. Chitranukroh and Billing (1983) have shown that, in reality, albumin binding of serum bile salts is decreased in hepatic failure (Table 6.5). However, the albumin binding

Table 6.5: Degree of albumin binding of bile salts in hepatic failure (Chitranukroh and Billing, 1983). Values are given as % bound of total ± standard deviation, except where a range is indicated. GCDC, GCDCS and GC denote glycochenodeoxycholate, glycochenodeoxycholate-3-sulphate and glycocholate, respectively.

A: normal; n = 8

B: viral hepatitis; n = 10

C: chronic active hepatitis; n = 8

	A	B	C
GCDC	95.5 ± 0.9	91.3 ± 1.7	85.8 ± 1.9
GCDCS	92.9 ± 1.8	93.0 ± 2.7	88.3 ± 4.2
GC	79.9 ± 2.6	62 - 75	35 - 58

of the more toxic bile salts still appears to be greater than 85% in hepatic disease patients in general. By contrast, the concentration of albumin in FCS is ~ 660 μM (Altman and Dittmer, 1974; assuming albumin concentration is similar to that found in adult bovine serum), and so culture medium containing only 10% FCS would contain approximately 66 μM albumin. Although it is more difficult to estimate the proportions of free and bound bile acids in this culture medium due to the lack of information about the exact composition of sheep bile, a very rough calculation would indicate that over 90% of the bile acids would be unbound for 0.1 and 0.5% bile in the culture medium.

However, although some evidence exists which could be *interpreted* to imply that bound bile acids are less toxic in the short term than their unbound counterparts (Latta et al, 1993), there is none to suggest that high concentrations of protein bound serum bile acids are benign. *In vivo* determinations in the rat of bile acid toxicity have shown that increases in total hepatic bile acid concentration corresponding to 2.5 - 20 times higher than normal (~ 66 - 500 μM TSBA) are moderately to strongly hepatotoxic, resulting in changes in the architecture of the liver parenchyma including enlargement of portal spaces, excessive proliferation of bile canaliculi and periportal inflammatory infiltration (Delzenne et al, 1992). Neale et al (1971) have stated that TSBA in hepatic failure can be up to 100 times greater than in health, and independent calculations by the author from information presented in the literature show that this value can be up to 800 times greater than in health (see point 1. in this section). Thus, high levels of serum bile acids are reflected in increased hepatic bile

acid concentration, which may induce liver damage, or exacerbate existing damage.

As previously discussed in section 2.1, if an immunoprotection membrane is used in the BAL, it should be chosen so that the passage of protein bound toxins into the bioreactor chamber is not impeded. It is therefore possible that patient serum bile acids, whether bound or unbound, conjugated or unconjugated, may be significantly toxic to the cultured cells in the BAL, and thus reduce hepatocyte lifespan and function.

Many toxins are removed from the blood by conjugation with hepatic enzyme systems followed by active transport into the hepatic bile, which is ultimately disposed of in the faeces (Smith, 1973). The percentage of a substance that is excreted in bile (as opposed to urine) is positively correlated with both its molecular weight and degree of plasma binding, and often reaches 100% (ibid). Although some of these toxins are de-conjugated by colonic bacteria and diffuse through the intestinal wall back into the blood supply (i.e. enterohepatic circulation), in health there is a net efflux. However, there is no facility for bile removal in the BAL, and thus all metabolites removed in this way by the cultured cells would eventually be returned to the patient plasma. Protein bound serum bile acids therefore represent a pool of hepatotoxins that cannot be eliminated by the BAL, and can be only slowly depleted over time by sorbent systems such as activated charcoal (Dunlop et al, 1978). It may therefore be necessary to investigate an alternative method for the elimination of bile acids from the blood of FHF patients, potentially employing a combination of affinity chromatography and perfusion over activated charcoal (see section 1.3.2).

The possibility that the cytotoxicity of bile may be important during the initial attachment phase of the hepatocytes to the culture surfaces within the BAL has also been considered. As discussed in section 2.5, BALs generally employ extremely high density cultures of hepatocytes. Furthermore, evidence exists which suggests that, in

contrast to their *in vivo* counterparts, primary cultures of hepatocytes exhibit time linear bile acid synthesis that is not controlled by feedback inhibition (Kubaska et al, 1985). Therefore, bile acids may build up in the culture medium during this attachment phase. In addition, the culture medium will contain 10% FCS at most, so there is little question of albumin-binding reducing the toxicity of these bile acids. However, experiments by Hylemon et al (1985) suggest that bile acid production is minimal during the attachment phase. Extrapolation of the data provided by Hylemon et al would indicate that the rate of total bile acid synthesis in rat hepatocytes is less than 3.9×10^{-11} mol $\cdot 10^6$ cells$^{-1} \cdot$ h^{-1} during the first 24 hours, but increases to a rate of approximately 1.1×10^{-10} mol $\cdot 10^6$ cells$^{-1} \cdot$ h^{-1} after this period.

If the diffusivity of the bile acids is assumed to be sufficiently high, it may also be assumed that any bile acids secreted by the cultured cells in the BAL will be evenly distributed throughout the culture chamber at the end of the twenty four hour attachment period. Bile acid concentrations calculated in this way will be referred to as the "mean" concentration. However, if the bile acid diffusivity values are relatively low, then the "local" bile acid concentration (that accumulating within a short distance from the cell surface) may be substantially higher than this mean value. In the absence of bile acid diffusivity data, it is difficult to calculate local bile acid concentration at the end of a given time period. However, intuitively, one may suggest that the local bile acid concentration may be up to ten times as great as the mean value. In addition, as cell densities increase, a greater proportion of the culture volume is taken up by the cell, as opposed to the culture medium. For example, the average volume of a rat hepatocyte has been determined to be equal to 4.94×10^{-9} ml $\cdot$ cell^{-1} (Weibel et al, 1969). If the cells are cultured at a density of 2×10^7 cells $\cdot$ ml^{-1}, then approximately 10% of the volume is occupied by cells. If the cells are cultured at a

density of 5×10^7 cells $\cdot$ ml^{-1}, then this fraction increases to approximately 25%. If the cells were cultured at the maximum possible density, then 100% of the volume would be filled with cells. Therefore, as the cell density increases, the volume of medium available to dilute the bile produced by the cells decreases. However, although bile acids are actively transported out of the cell into the bile (Beher, 1976), it is not reasonable to assume that all of the bile acids will be concentrated in the culture medium. It may therefore be appropriate to weight the estimated bile acid concentration, so that (for example) it might be increased by 10% for cells cultured at a density of 2×10^7 cells $\cdot$ ml^{-1}, whereas it would be multiplied by a factor of 10 for cells cultured at the maximum density (approximately 2×10^8 cells $\cdot$ ml^{-1} for rat hepatocytes; ibid). These weighting factors are, however, purely speculative. In the following paragraph, mean bile acid concentrations are estimated, initially ignoring the effect of local bile acid build up and decreasing culture medium volume as cell density increases. Possible maximums for local values are also proposed, although these values cannot be credited with any degree of accuracy.

Currently, the maximum cell density achieved in hepatocyte cultures appears to be approximately 2×10^7 cells $\cdot$ ml^{-1} (Takahashi et al, 1992). It is not inconceivable that future advances may allow even higher density cultures of viable functional hepatocytes. If, for example, the cells in the BAL were cultured at a density of 5×10^7 cells $\cdot$ ml^{-1}, then the total concentration of bile acids in the culture medium at the end of a 24 hour attachment period would be approximately 47 nM. Even if it were assumed that the higher rate of bile acid synthesis (of the two calculated above) would be typical during the attachment phase, the total bile acid concentration in the culture medium at the end of a 24 hour attachment period would be just 0.132 μM. In fact, even if cell densities comparable to that found in intact rodent liver (~ 2×10^8 cell $\cdot$ ml^{-1};

Weibel et al, 1969) were achieved, the total bile acid concentration in the culture medium at the end of a 24 hour attachment period would be just 0.528 μM. Local bile acid concentrations might then be as high as 50 μM (incorporating both weighting factors and thus anticipating a hundred-fold increase in local bile acid concentration).

Although it is possible that bile acid concentrations of this magnitude would be toxic to the cultured cells in the BAL, culture conditions are unlikely to be this extreme in practice. However, "marked variations" in the rates of bile acid synthesis measured in rat hepatocytes have been observed between different cell isolations (Princen and Meijer, 1990). It is possible that hepatocytes of different species origin may exhibit much higher rates of bile acid synthesis, and that significant accumulation of bile acids may then be observed in the culture medium at the end of the attachment period. In addition, substantial variations in the sensitivity of different cell types to the cytotoxic effect of bile acids have been demonstrated (albeit indirectly) throughout this section. If the hepatocytes or cell lines considered for a BAL were determined to have a particularly high rate of bile acid synthesis, then the toxicity of this bile to that cell type would require further examination. It is, however, more probable that elevated bile acid concentrations in the patient's plasma would contribute to a bile acid related impairment of cell function.

6.3 The effect of oxygen concentration on rat hepatocyte and Hep G2 cell function and viability

Cytochrome P450 plays an important role in the detoxification function of hepatocytes. During the events of metabolism, the cytochrome P450 proteins activate oxygen in a two step process (Schenkman and Greim, 1993).

$$O_2 + P450\text{-}Fe^{2+} \longrightarrow P450\text{-}Fe^{2+}O_2 \longleftrightarrow P450\text{-}Fe^{3+}O_2^- \quad (1)$$

$$P450\text{-}Fe^{3+}O_2^- + e^- \longrightarrow P450\text{-}Fe^{2+}O_2^- \longleftrightarrow P450\text{-}Fe^{3+}O_2^{2-} \quad (2)$$

The one electron reduced haemoprotein seen at the end of reaction (1) and at the beginning of reaction (2) is seen in the cytochrome P450 spectrum as a peak at 418 nm rather than at 450 nm (ibid). Because of slight variations in the position of this peak, it has been referred to as cytochrome P420 in these experiments. As cells age in culture, the tendency is toward decreased levels of cytochrome P450 and its associated functions along with increased levels of cytochrome P420.

In comparison with control cells (those cultured in a 20% O_2 environment), rat hepatocytes cultured in a 5% O_2 environment retained higher levels of P450 and EROD activity, and demonstrated a decreased rate of degradation of P450 as measured by the post-experimental ratio of P420 : P450. By contrast, hepatocytes cultured in a 35% O_2 environment exhibited decreased levels of P450 and a higher ratio of P420 : P450 compared to control. Cells cultured in 12% O_2 were comparable to control cultures in every respect except for the reduction in post-experiment P420 : P450 ratio. This seems to indicate that low oxygen concentrations help to preserve hepatocyte function. Furthermore, the increased incorporation of ^{3}H-leucine (compared to control) observed in those cultures exposed to 35% O_2 may indicate increased protein synthesis to protect against free radical damage. Oxygen toxicity is a well established phenomenon and

oxygen reactive species are amongst the most abundant and toxic free radicals known: these oxygen free radicals are products of normal cellular oxidation-reduction processes, and their production increases markedly under conditions of hyperoxia (Frank and Massaro, 1980).

Hepatocytes *in vivo* are supplied with mixed venous : arterial blood (75% : 25%), and oxygen concentration in the hepatic sinusoids falls from 9 - 13% pericentrally to 4 - 5% perivenously (Wölfle et al, 1983). The lower oxygen concentrations examined in these experiments (5% and 12% O_2) are therefore closer to the physiologic norm, and should be well tolerated by cells in culture. Studies by Holzer and Maier (1987) have also suggested that the aging process is delayed and regenerating processes are better maintained in rat hepatocytes cultured in a low oxygen (4% O_2) environment for up to 7 days.

Alternatively, the increased retention of cytochrome P450 and EROD activity observed in response to lower oxygen concentrations may reflect a reduction in the rate of cell activity. For example, as reactions (1) and (2) imply, cytochrome P450 function is at least partially dependent upon the availability of oxygen. As cells have more oxygen available to them, certain functions may cease to be oxygen limited. As function increases, substrates within the cells and the surrounding medium will be depleted more quickly, increasing the rate at which cells age. If this is true, then the rise in protein synthesis indicated by the increased uptake of ^{3}H-leucine of cells exposed to 35% O_2 may be attributed to an acceleration of normal cellular metabolism rather than as a response to the replacement of proteins depleted during oxidative stress. Ohshima and coworkers (1986, 1994) have shown that, although the death rate of rat hepatocytes increases as the pO_2 is increased from 0 to 600 mmHg, this is accompanied by an increase in metabolic reaction rates (eg. the rate of conversion of ammonia to urea). They have estimated that the optimal pO_2 (that at which the death rate is

minimised and the reaction rate maximised) lies between 100 and 200 mmHg. Surprisingly, variations in oxygen concentration within the physiologic range have no effect on the ratio of ATP : ADP (Nauck et al, 1981; Hummerich et al, 1988).

It is also possible that neither low nor high oxygen concentrations are in themselves either advantageous or damaging to hepatocyte function, but that different functions are expressed in different environments. Considerable evidence exists, both *in vitro* and *in vivo*, which suggests that gene expression is modulated by oxygen concentration (Fanburg et al, 1992). Moreover, it is believed that the metabolic zonation of hepatocyte function *in vivo* is controlled by a combination of factors relating to nutrient supply, including oxygen tension (Wölfle et al, 1985). For example, *in vitro* simulation of periportal hepatic conditions encourages gluconeogenesis whilst perivenous conditions stimulate glycolysis (Wölfle et al; 1983, 1985). Furthermore, the induction of both phospho*enol*pyruvate carboxykinase and tyrosine aminotransferase by glucagon (0.1 μM) in rat hepatocytes were significantly increased when they were exposed to 13% O_2 as opposed to 4% O_2 (Nauck et al, 1981).

The positive correlation between enhanced cell spreading and increased oxygen concentrations (refer to Photos 5.3 - 5.6) seen in these experiments has also been observed by Rotem and co-workers (1994). These researchers found that the surface area of rat hepatocytes (24 hours after initiation of culture) decreased exponentially with linear decreases in oxygen concentration from 75% to 4% (Table 6.6, overleaf). In addition, the percentage of cells which had attached to the culture surfaces within one hour increased from 40% to 100% (approximately) as the oxygen concentration increased from 0.01% to 13%. Cells flattened most rapidly (as measured by relative increases in measured cell surface area) during the first 5 hours of culture. However, no evidence was given to suggest that increased cell spreading reflected increased hepatocyte function or metabolic competence.

In addition to total cytochrome P450, only one activity (EROD; dependent upon

Table 6.6: Effect of oxygen on hepatocyte spreading in culture. Values shown have been extrapolated from printed graphs (Rotem et al, 1994).

Oxygen concentration (%)	surface area ($\mu m^2 \cdot cell^{-1}$)
0	300
4	410
10	450
21	650
75	830

the cytochrome P450 subfamily CYP1A) of this family was measured in these experiments. Eight different subfamilies of the cytochrome P450 family of haemoproteins have been identified in rat liver, and nine subfamilies have been identified in humans (Schenkman and Greim, 1993). Members of these families, the individual isoenzymes of cytochrome P450, differ from each other in substrate specificity and catalytic activity. Therefore, changes in the distribution of these isoenzymes have major implications for the metabolism and toxicity of many compounds, and thus for the efficacy of the BAL. There do not appear to be any published observations on the relative importance of specific isoenzymes of cytochrome P450 for effective BAL therapy. However, a considerable body of evidence exists (Basile et al, 1991) which suggests that administration of γ-aminobutyric acid (GABA) receptor antagonists to hepatic failure patients results in an amelioration of the neurologic manifestations of hepatic encephalopathy (see section 1.1). Therefore, it may be especially important to preserve the cytochrome P450 isoenzymes responsible for the metabolism of GABA and its agonists (i.e. the benzodiazepienes).

Diazepam is a representative member of the benzodiazepienes, and thus the isoenzymes of cytochrome P450 necessary for its metabolism may also be relevant to the

metabolism of substances present in the blood of patients with hepatic encephalopathy. Unfortunately, although there has been much research into both *in vivo* and *in vitro* diazepam metabolism, very few studies have attempted to identify the individual isoenzymes involved (Neville et al, 1993). Recent investigations (ibid) have implicated CYP3A2, CYP2C11, CYP2B1 and CYP2C6 in various stages of diazepam metabolism in the rat. None of these isoenzymes were measured in the experiments undertaken in this thesis. However, results of a series of experiments to determine the effects of physiologic oxygen concentrations (4% and 13% O_2, in the presence of inducing agents) on the expression of several isoenzymes of cytochrome P450 have recently been published (Maier et al, 1994; Saad et al, 1994; Table 6.7, overleaf). At least one of the isoenzymes involved in diazepam metabolism (CYP2C6) exhibits increased activity (in the presence of 0.75 mM phenobarbital) in rat hepatocytes exposed to reduced oxygen environments.

The lack of effect of oxygen concentration on Hep G2 cells (for the parameters measured) may be due to the reduced metabolic function of these cells compared to that of isolated hepatocytes. This would imply a lower oxygen consumption of Hep G2 cells in comparison to that of isolated hepatocytes (see section 6.4) and thus a lower oxygen dependence for those functions that are retained. It is likely that some aspects of Hep G2 cell function not measured in this experiment would exhibit changes in response to variations in oxygen supply. For example, Hep G2 cells demonstrate an exponential increase in erythropoietin synthesis in response to a linear decrease in the oxygen concentration of the headspace from 40% to 1% (Ueno et al, 1989). This would seem to indicate a response to hypoxia, and thus perhaps might be accompanied by a decrease in other oxygen dependent functions of Hep G2 cells. This has not, however, been demonstrated by these experiments, and is contradicted by the results discussed in section 6.4.

These results demonstrate the extreme importance of oxygen supply to the function

Table 6.7: The effect of oxygen concentration (4 day exposure) on the induction of individual cytochrome P450 isoenzymes and cytochrome P450 dependent activities in rat hepatocytes. Values refer to increases with respect to cells cultured in a 95% air : 5% CO2 environment. The superscript[1] refers to data obtained from Maier et al (1994), and the superscript[2] to data obtained from Saad et al (1994). All cultures were exposed to 0.75 mM phenobarbital (PB) except for †, which were exposed to 3 mM PB, and *, which were exposed to 6.25 μM 3-methylcholanthrene. n/s signifies "not stated".

Isoenzyme or function measured	4% O_2	13% O_2
CYP1A1/2 [2†]	3-fold	n/s
CYP2B1/2 [1]	19-fold	27-fold
CYP2B1/2 [1*]	11-fold	8-fold
CYP2B1/2 [2]	20-25-fold	20-25-fold
CYP2C6 [1]	6-fold	6-fold
CYP2C6 [2]	6-fold	6-fold
CYP3A [2]	2-fold	n/s
CYP3A2 [1]	2-3-fold	not induced
EROD activity (CYP1A dependent) [1]	37-fold	30-fold
16 α/β hydroxylation of testosterone (CYP2B dependent)	42-fold	96-fold

of the cells within the bioreactor. Not only is it possible that a range of oxygen tensions may be required to maximise the metabolic potential of the cells, but this range must be carefully maintained to prevent either hypoxic or hyperoxic damage. Furthermore, the implications of oxygen concentration with respect to hepatocyte attachment and

spreading discussed in this chapter must be considered. It is possible that there may be an initial requirement for uniform high oxygen concentration (so that the attachment process may be facilitated) during the first 6 to 24 hours after the cells are introduced into the BAL chamber. Thus an initial high oxygen concentration may be followed by one of a range of oxygen concentrations, as previously proposed. The effect of these changes in oxygen concentration during the culture period should therefore be explored. In addition, different cell types display varying sensitivity to oxygen in culture. Any cell type proposed for use in the bioreactor should therefore be subjected to a detailed examination of the effect of oxygen concentration on a range of functions and on the lifespan of the cells in culture, so that bioreactor efficiency may be optimised.

6.4 The effect of cell density and pO_2 on the rate of oxygen consumption observed in Hep G2 cells

The exponential increase in oxygen consumption experienced by Hep G2 cells in response to linear decreases in cell density is identical[1] to that described for hybridoma cells (Wohlpart et al, 1990), mouse fibroblasts (Danes et al, 1963) and normal human lymphocytes in culture (Hedeskov and Esmann, 1966). Although further investigations implied that this effect was not due to local variations in oxygen tension, CO_2 concentration, or accumulation of lactic acid (Sand et al, 1977), there is some debate over the effect of oxygen gradients within the microenvironment of the cell culture vessel. Wohlpart et al (1990) suggested that increases in cell density may influence bulk mixing such that cells cultured at higher densities are surrounded by an extended boundary layer. Wohlpart et al further supported this argument by reference to the literature, which indicates that the boundary layer contributes approximately 80% of the mass transfer resistance to oxygen uptake in red blood cells. However, the apparent contradiction between this theory, and the independence of hybridoma cell oxygen consumption with respect to dissolved oxygen concentration was also acknowledged. That is, if mass transport is important in determining rates of oxygen consumption, then this consumption should increase with higher dissolved oxygen concentrations, in accordance with the higher driving force for transfer of oxygen into the cell. The density dependence of Hep G2 cell oxygen consumption is even less likely to be explained in this way, as oxygen consumption was observed to increase in response to decreases in dissolved oxygen concentration from 150 mmHg to approximately 40-60 mmHg.

It has also been suggested that cell-to-cell communication controlled by the

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[1] i.e. the shape of the curves are identical, although actual oxygen consumption differs

release of "humoral factors" (eg. cytokines) influences the density dependence of oxygen consumption. Therefore, cells could anticipate a "crowding" effect by a temporary suppression of oxidative metabolism. Wohlpart and coworkers (ibid) also investigated this possibility, by exposing cells initially cultured at a low density (10^5 cells $\cdot$ ml^{-1}) to the supernatant medium of cells cultured at a "high" density (8×10^5 cells $\cdot$ ml^{-1}). Although no changes to the pattern of density dependence on respiration rate was observed, the conclusion by Wohlpart et al that cell-to-cell communication is not responsible for the density dependence of oxygen consumption is somewhat premature. An almost immediate reduction in oxygen consumption was observed in response to increases in cell density. It is therefore possible that the chemicals (or "messengers") mediating intercellular communication are both synthesised and degraded rapidly, thereby reducing lag between communication and response within this context. This might also lead to a partial explanation of the observed phenomenon. For example, at higher cell densities, the distance and thus the travelling time between cells is decreased, and so a greater proportion of these messengers might reach neighbouring cells before degradation occurs. If the messengers in question exert an inhibitive effect on oxygen dependent cellular metabolism, then higher cell densities would result in decreased oxygen consumption, as observed in the experiments on Hep G2 cells.

This mechanism would also explain the exponential shape of the relationship seen between oxygen consumption and cell density. Although a large number of simplifying assumptions are required for this analysis, and the effect of mixing within the culture volume is ignored, it still demonstrates the observed effect with respect to cell density. Mixing may not be as important in an analysis of the microenvironment of microcarrier attached cells, as the position of the cells on individual microcarriers would remain fixed with respect to each other. Therefore, assuming:

1. the cell disburses the "messenger" at a constant rate b units $\cdot s^{-1}$. The total amount of messenger generated over a discrete time period would therefore be equal to $b \cdot dt$.

2. the rate of degradation of the messenger is proportional to the quantity of messenger present. Therefore, if k is the current quantity of "messenger", then $dk/dt = -f \cdot k$, where f is a constant. Then $k(t) = c_0 \cdot \exp(-f \cdot t)$ where c_0 is the initial amount before degradation $= b \cdot dt$. Thus the total amount of messenger within a given volume is $b \cdot \exp(-f \cdot t) \cdot dt$, and the concentration of messenger within a spherical shell a distance r from the centre of the cell would be:

$$b \cdot \exp(-f \cdot t) \cdot dt/(4 \cdot \pi \cdot r^2 \cdot dr) \qquad \text{eq. 6.1}$$

3. the messenger travels at a constant velocity (g) in a straight path directed radially outward from the cell surface, so that $dr = g \cdot dt$, and $t \approx r/g$.

Substituting the identities from 3. into equation 6.1 allows the concentration of the messenger substance to be calculated as a function of the distance from the cell as: $b \cdot \exp(-f \cdot r/g)/(4 \cdot \pi \cdot r^2 \cdot g)$. The quantities $b/(4 \cdot \pi \cdot g)$ and f/g are just constants, which can be designated c_1 and c_2 respectively, so that:

$$x = c_1 \cdot \exp(-c_2 \cdot r)/r^2 \qquad \text{eq. 6.2}$$

is the concentration of messenger at any given distance r from the cell. If it is assumed that oxygen consumption is inversely proportional to the concentration of messenger, and that the distance between cells is inversely proportional to cell density, then an

equation of the same form as 6.2 relating cell density to oxygen consumption may be obtained. This equation takes the same shape (Figure 6.1) as the oxygenconsumption

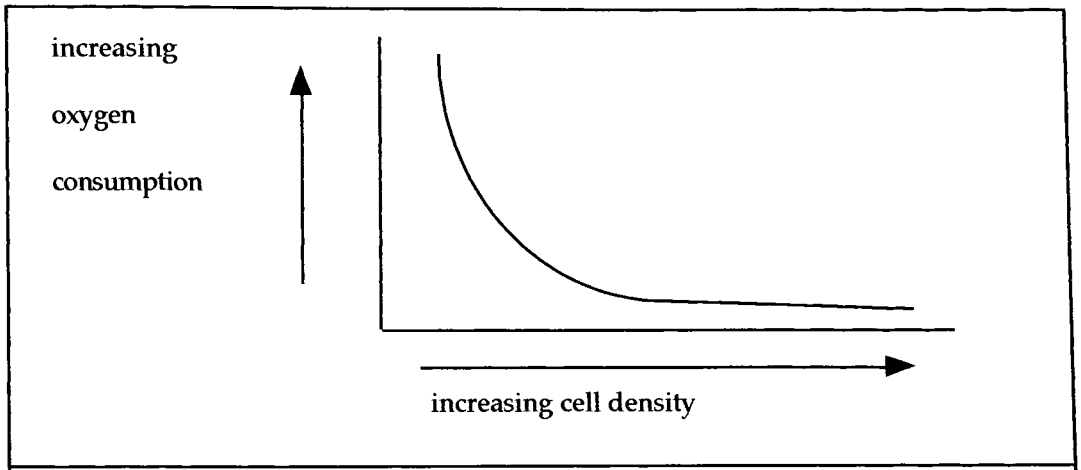

Figure 6.1: Predicted relationship between cell density and oxygen consumption

versus cell density curves obtained for both Hep G2 cells and the other cell types discussed at the beginning of this section.

Whatever the cause of the decreases in oxygen consumption observed for high cell densities, this effect could have important consequences for the BAL. If decreased oxygen consumption is accompanied by decreases in oxygen-dependent cell functions, then the high density cultures which are sought for the BAL may reduce cell viability. Further research should explore the effect of cell density on the oxygen consumption of primary cultures of hepatocytes, as well as its effect on cell function. If high densities are found to impair cell function, then further research into the cause of this phenomenon, coupled with improvements in culture conditions to overcome these limitations, would be required. The observed effect is unlikely to be due to cell density alone: hepatocytes co-exist within the liver at an average density of 2×10^8 cells $\cdot$ ml^{-1} (Weibel et al, 1969; Kasai et al, 1994), and *in vivo* oxygen consumption is higher than

that of isolated hepatocytes (see section 2.8). Given the emphasis placed upon achievement of high density hepatocyte cultures for use in a BAL (see section 2.5), these factors are of paramount importance.

Although the rate of oxygen consumption measured in Hep G2 cells was shown to be strongly dependent on both cell density and pO_2, it was generally much lower than the value of 24 $nmol \cdot min^{-1} \cdot 10^6 cells^{-1}$ reported by Noll et al (1986) for rat hepatocyte suspensions. This may be due to reduced metabolic activity and thus lower oxygen requirements for Hep G2 cells as compared to isolated hepatocytes. The maximum oxygen consumption for Hep G2 cells was usually observed between 40 and 60 mmHg. One possible explanation for this effect may be that the cells respond to a decrease in local pO_2 by an increase in consumption as part of a mechanism to maintain intracellular homeostasis. The increased synthesis of erythropoietin observed in Hep G2 cells cultured at low oxygen concentrations (Ueno et al, 1989) supports this hypothesis. The lack of effect of the headspace oxygen concentration on Hep G2 cell function described in section 6.3 might therefore be evidence of a successful control of cell functions under hypoxic conditions. This may be contrasted with observations on cultured rat hepatocytes, for which oxygen consumption is independent of pO_2 until oxygen supply becomes limiting, when pO_2 falls below 10 mmHg (Noll et al, 1986). The extreme sensitivity of rat hepatocyte function to oxygen concentration could therefore be attributed to the lack of the control system proposed for Hep G2 cells.

Alternatively, it is conceivable that Hep G2 cells prefer a low oxygen atmosphere, and that the increased oxygen consumption of Hep G2 cells observed between 40 and 60 mmHg reflects a direct increase in cellular metabolism and viability (in parameters not tested by the experiments discussed in section 6.3). Many tumour cells, such as those isolated from carcinoma, ovarian, colon and lung tissue, have demonstrated increased cloning efficiency in response to low oxygen environments (~ 40

mmHg; Sridhar et al, 1983). Research by Taylor et al (1978) has demonstrated that mammalian fibroblast cultures also have increased growth and plating efficiency at dissolved oxygen concentrations within the range of 40 - 60 mmHg. It is believed that tumour cells, which commonly experience *in vivo* pO_2 of approximately 40 mmHg, have a reduced capacity for protection against damage by oxygen free radicals, and thus higher oxygen concentrations are not as well tolerated by these cells. Hep G2 cells, in particular, do not exhibit GSH peroxidase activity. The ability of these cells to protect themselves from peroxides generated by high oxygen concentrations may therefore be reduced (Helen Grant, personal communication).

It is also possible that the oxygen consumption of hepatocytes and hepatocyte derived cell lines may change as they pass through the various stages of culture. In particular, hepatocyte oxygen extraction has been shown to increase during attachment to substrata (Yarmush et al, 1992; Rotem et al, 1992). Although the two studies referenced were conducted by the same research group, there appears to be some disagreement between the reported pattern of the changes in oxygen uptake rates (OUR) at various stages in culture (Figure 6.2, overleaf). This may be due to differences in culture configuration. The results reported with Yarmush as the first author (—◆—) are for cells cultured in a collagen sandwich configuration, while those from Rotem et al (—□—) are representative of the OUR of monolayer cultures. As —◆— appears to be a delayed (and exaggerated) version of —□—, the differences in these graphs could be due to the mass transfer resistance imposed by the collagen gel layer surrounding the collagen sandwich cultures. Nevertheless, the implications are that the oxygen requirements of these cultures vary with time, and may be particularly pronounced during cell attachment. Additional studies by this research group (Rotem et al, 1994) have been discussed in section 6.3, and suggest that oxygen supply may be critical to hepatocyte attachment and spreading in the initial stages of culture. Further oxygen uptake/consumption experiments are in progress at Strathclyde. These will evaluate:

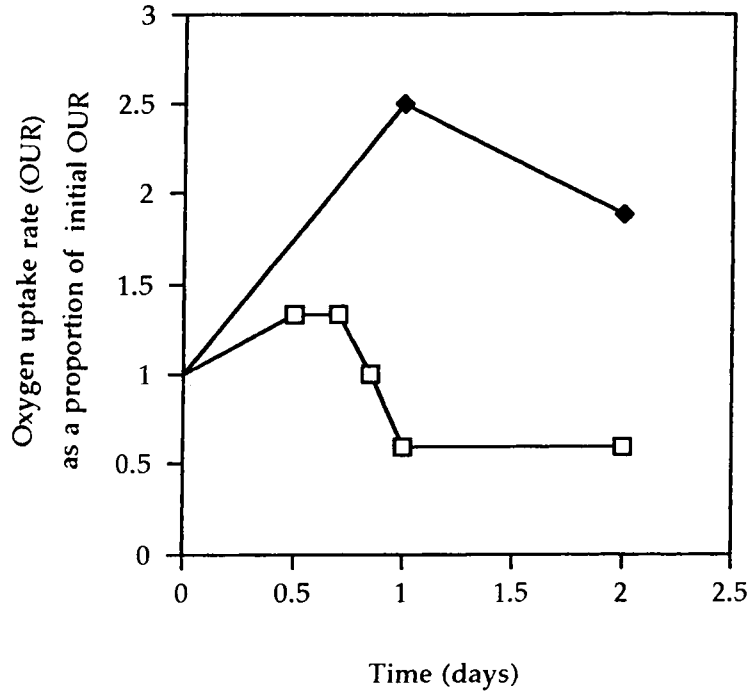

Figure 6.2: Pattern of oxygen uptake in cultured rat hepatocytes. Values are plotted as a fraction of the initial OUR and have been extrapolated from published data. —□— denotes monolayer culture (Rotem et al, 1992) and —◆— denotes collagen sandwich configuration (Yarmush et al, 1992).

- the oxygen consumption of a variety of cell types during attachment and
- the optimum oxygenation protocol.

The indications are that substantial interactions exist between pO_2, cell function, cell density and oxygen consumption. It is also evident that oxygen consumption varies according to cell species and origin. While low oxygen requirements throughout the culture period would simplify implementation of oxygen supply, a more detailed analysis of cell function and oxygen consumption as controlled by cell type, cell density, pO_2 and culture conditions is required.

6.5 Summary

The utility of a BAL in the treatment of hepatic failure is becoming increasingly apparent. At least one model is under commercial/clinical development (Sussman et al, 1994), and there is already evidence of clinical success (see section 2.10). However, there is still ample opportunity for research in this area, as the use and design of the BAL evolves. The first clinical applications of the artificial kidney were seen in the late 1940's, and yet improvements in dialyser materials, analysis and treatment prescription continue to the present day. Advances within the last 20 years have enabled a halving of average dialysis session duration (Ramirez et al, 1973; Shaldon and Koch, 1991). In the opinion of the author, it is probable that the BAL will follow a similar pattern of development.

Possible future work with respect to the experiments performed as a part of this thesis have been discussed in sections 6.1 to 6.4. Other areas for development have been discussed in Chapter 2. As BAL treatment for hepatic failure becomes more widespread, other factors relating to mass production and storage will have to be addressed. For example, although there has been recent success in the cryopreservation of rat hepatocytes (Watts and Grant, 1994), the size, density and heat transfer properties of the BAL may introduce difficulties into the application of a particular cryopreservation regime. While a uniform temperature distribution may be achieved relatively rapidly for a cryotube of approximately 5 mm diameter or a tissue slice of nominal thickness, this would not be the case for a full scale BAL (eg. hollow fibre) module. It is therefore possible that new strategies of cryopreservation may need to be developed. For example, if a hollow fibre BAL were employed, then cryopreservation might be achieved by simultaneous intra- and extra- luminal perfusion with supercooled cryopreservation fluid. If appropriate strategies cannot be devised, then it may be necessary for either:

- BALs to be manufactured in anticipation of need (i.e. no cryopreservation,

BALs to be used immediately upon construction), or

- a halfway stage between long term storage of the complete BAL, ready for use upon defrosting and manufacture as dictated by demand. This may involve separate cryopreservation of hepatocyte suspensions, which may then be thawed prior to their introduction into a mass produced BAL. Under the appropriate conditions, it may be possible to reduce the length of time required for hepatocyte attachment, and thus the waiting period between knowledge of patient need for treatment and subsequent treatment, to 6 hours (see section 6.3).

Problems relating to large scale manufacture should be much less difficult to overcome, especially if the hepatocytes are to be added to the BAL in a separate stage just prior to treatment. Although it is beyond the scope of this thesis to discuss possible manufacturing techniques, entire industries are devoted to the solution of such problems, and so these considerations should not present a substantial obstacle to widespread use of BAL treatment for hepatic failure. Still, it may be expected that there will be several intervening years between the present time and this ultimate goal of large scale use and production.

Finally, although the liver appears to be the most complex organ for which artificial replacement/support has been developed, the indications are that recent success in this area will be followed by sufficient advances to allow widespread use. Ultimately, the high mortality associated with hepatic failure may give way to effective BAL treatment and positive future prognoses.

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Appendix A: The liver structure and function

A.1 Liver Structure

The liver (Figure A1, overleaf) is both the largest visceral organ and the largest gland. Anatomically, it may be described as a large pyramidal body located directly beneath the diaphragm, in the upper right quadrant of the abdominal cavity. It is composed of two main lobes, the left and right, the latter being the largest and comprising 5/6 of the total liver mass. Additionally, two auxiliary lobes originate from the posterior surface of the right lobe, the caudate and quadrate. The outer surface of the organ is covered by a capsule, consisting of an outer peritoneal layer, and an inner layer, a fibrous sheath known as Glisson's capsule, which invaginates at the porta hepatis or hilum. This serves as the point of entry for the vessels conveying blood to the liver, the hepatic artery and the portal vein, as well as providing an outlet for the bile ducts.

Although variable, and increasing particularly during digestion, blood flow through the liver is on average 1500 ml $\cdot$ min^{-1}, amounting to 25% of the cardiac output. 80% of this flow (~ 1200 ml $\cdot$ min^{-1}) is furnished by the portal vein, which channels all blood leaving the stomach, small and large intestine, pancreas, gallbladder and spleen through the liver for processing prior to its return to the heart. The hepatic artery is responsible for the remaining 20%, and together they branch to form two intricate parallel vascular trees which ultimately merge to supply every cell in the organ with mixed arterial/venous blood. Blood begins its exit from the organ via the terminal hepatic venules, or central veins, which coalesce to form vessels of ever larger calibre leading to the left, central and right hepatic veins. These empty directly into the vena cava.

Blood occupies approximately 20% of the volume of the liver, with the largest part, 70%, belonging to the parenchymal cells, or hepatocytes. 7% is composed of sinusoidal cells, which form the hepatic "capillaries", or sinusoids. The majority of

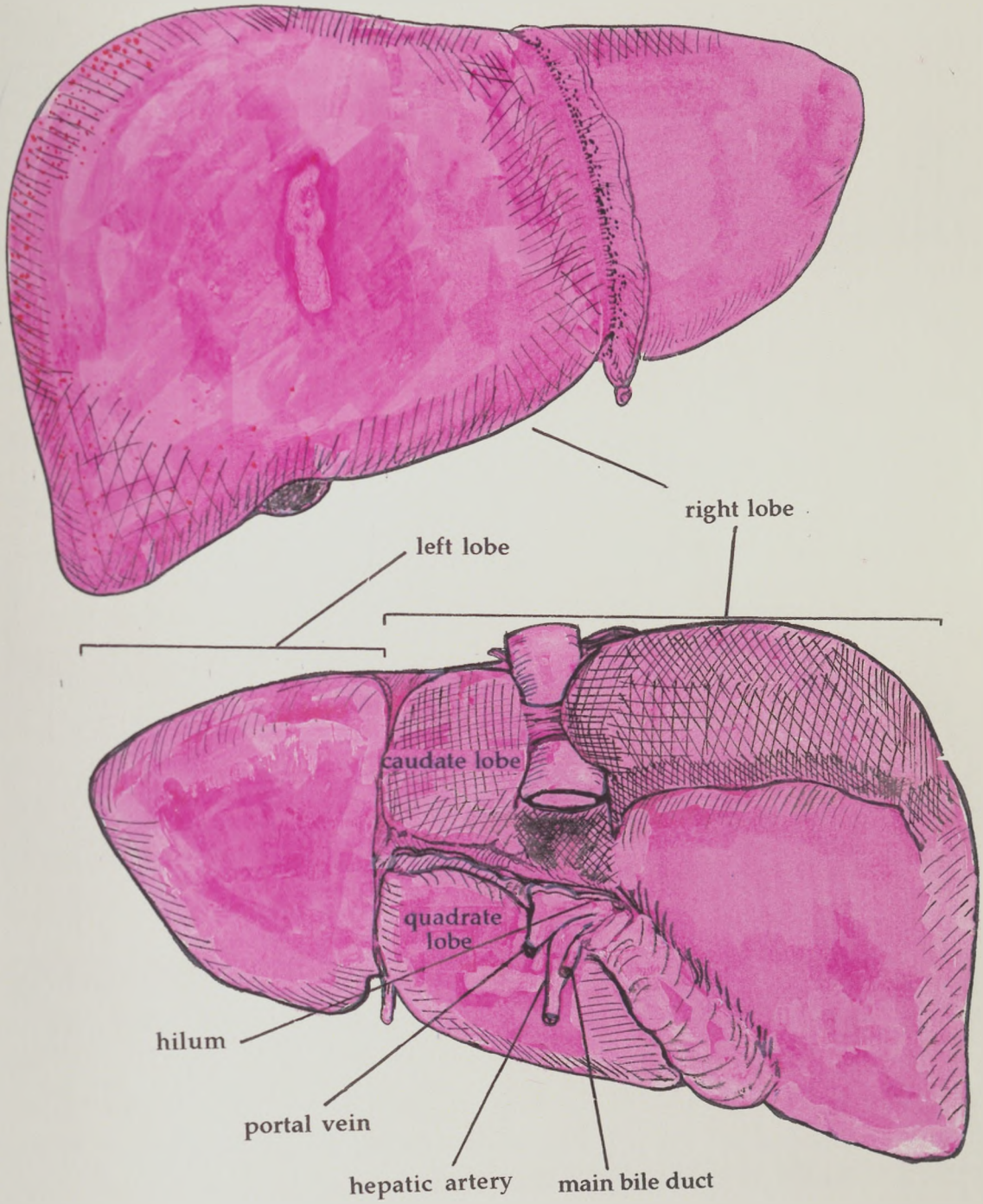


Figure A1: External features of the liver

the remaining 3% is attributed to the space of Disse.

The microscopic organisation of the liver is quite complex, and the apparent structural unit, the lobule, was originally believed to consist of chords of hepatocytes folded once over upon themselves to form sandwiches two cells thick, radiating outward from a central vein like spokes from a bicycle tyre (Figure A2, overleaf). Although this view is largely incorrect, it provides a useful reference from which the true three dimensional structure may be more easily visualised, and is in fact still referred to in some modern texts.

In this simplified model, bile canaliculi lie between the surfaces of the hepatocyte sandwiches, which are themselves separated by the liver sinusoids. Specialised endothelial cells form the sinusoidal wall, a discontinuous membrane with an average porosity of 9%. This barrier contains three other cell types: Kuppfer cells, perisinusoidal or fat-storing cells, and pit cells. It is separated from the hepatic parenchyma by the space of Disse (Figure A3, page 193), which is believed to increase communication between the hepatocytes and the blood supply. In addition, lymph is formed in the Space of Disse.

In reality, the hepatocytes have been found to be irregularly arranged as plates one cell thick (hepatic laminae), and constitute a continuous liver framework. Within each plate, individual bile canaliculi form a matrix which surrounds all of the cells. Adjacent laminae are connected by bridges of single cell thickness, and the sinusoids wind a tortuous path throughout this system (Figure A4, page 194). Although the portal vein empties more or less continuously into the sinusoids, it is believed that arterial blood flow is modulated by nervous input, so that the mixing of this high pressure influx (100 mmHg) with that of the portal supply (7 mmHg) does not become too turbulent.

A portal tract containing a terminal hepatic arteriole, a terminal portal venule, a bile ductule, nerves, and lymph vessels provides the vascular axis of the functional

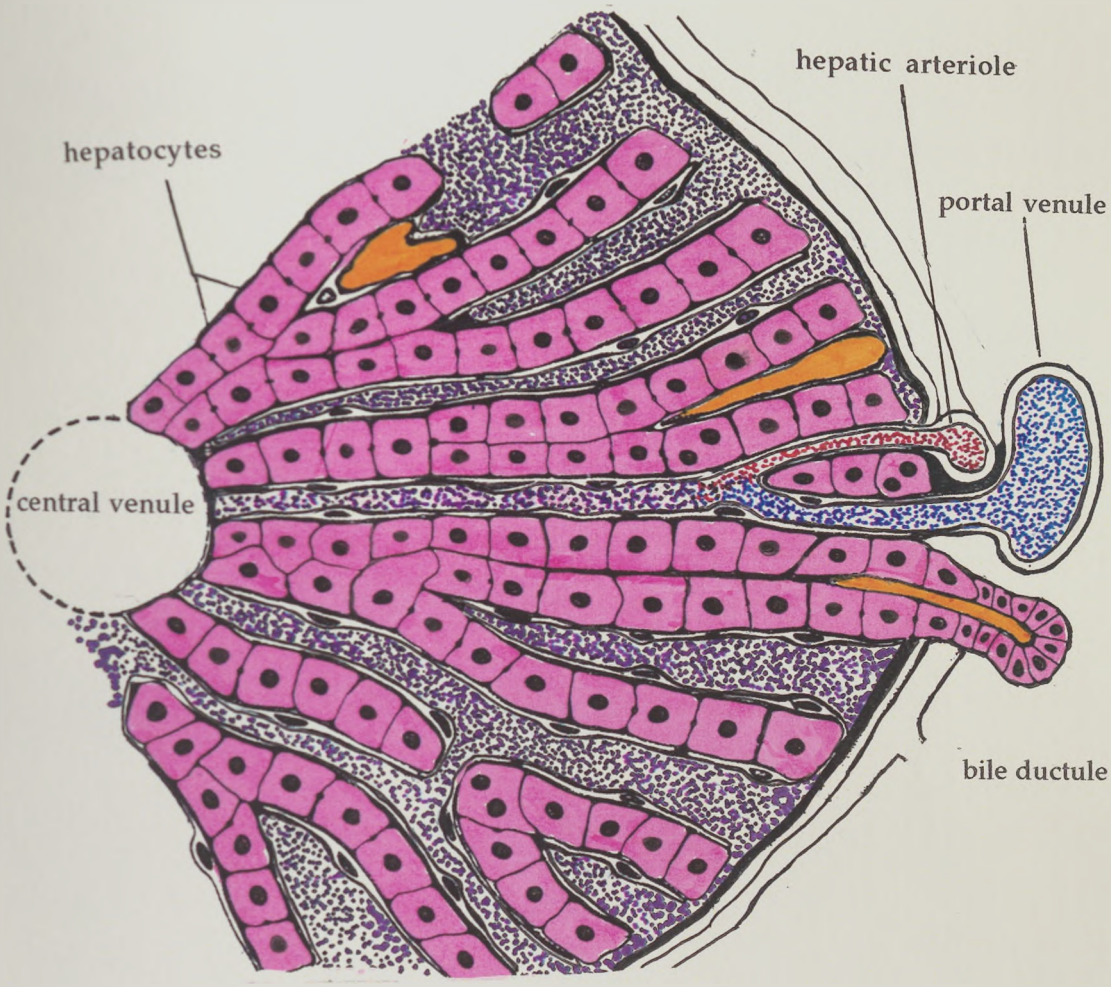


Figure A2: The liver lobule

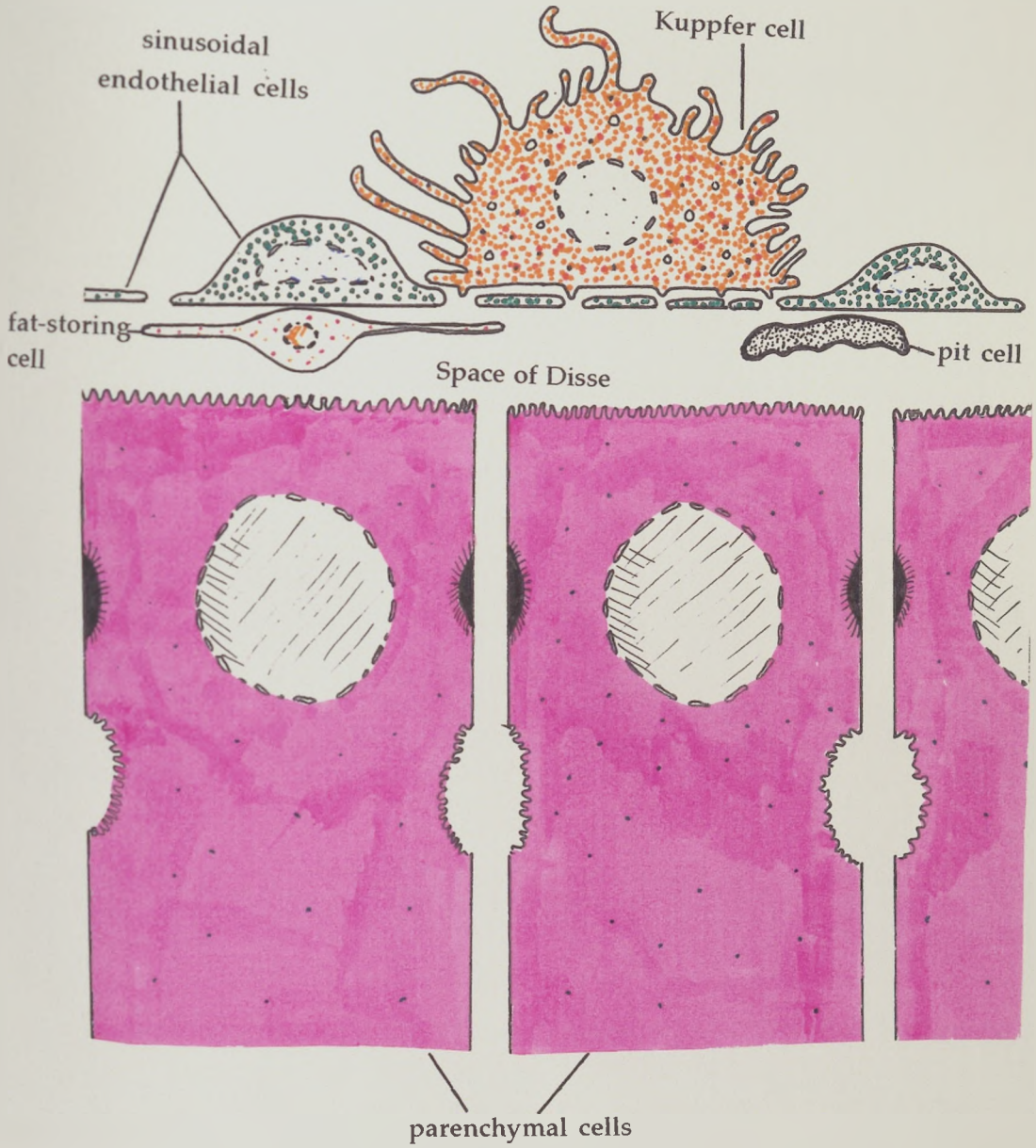


Figure A3: The hepatic sinusoidal barrier

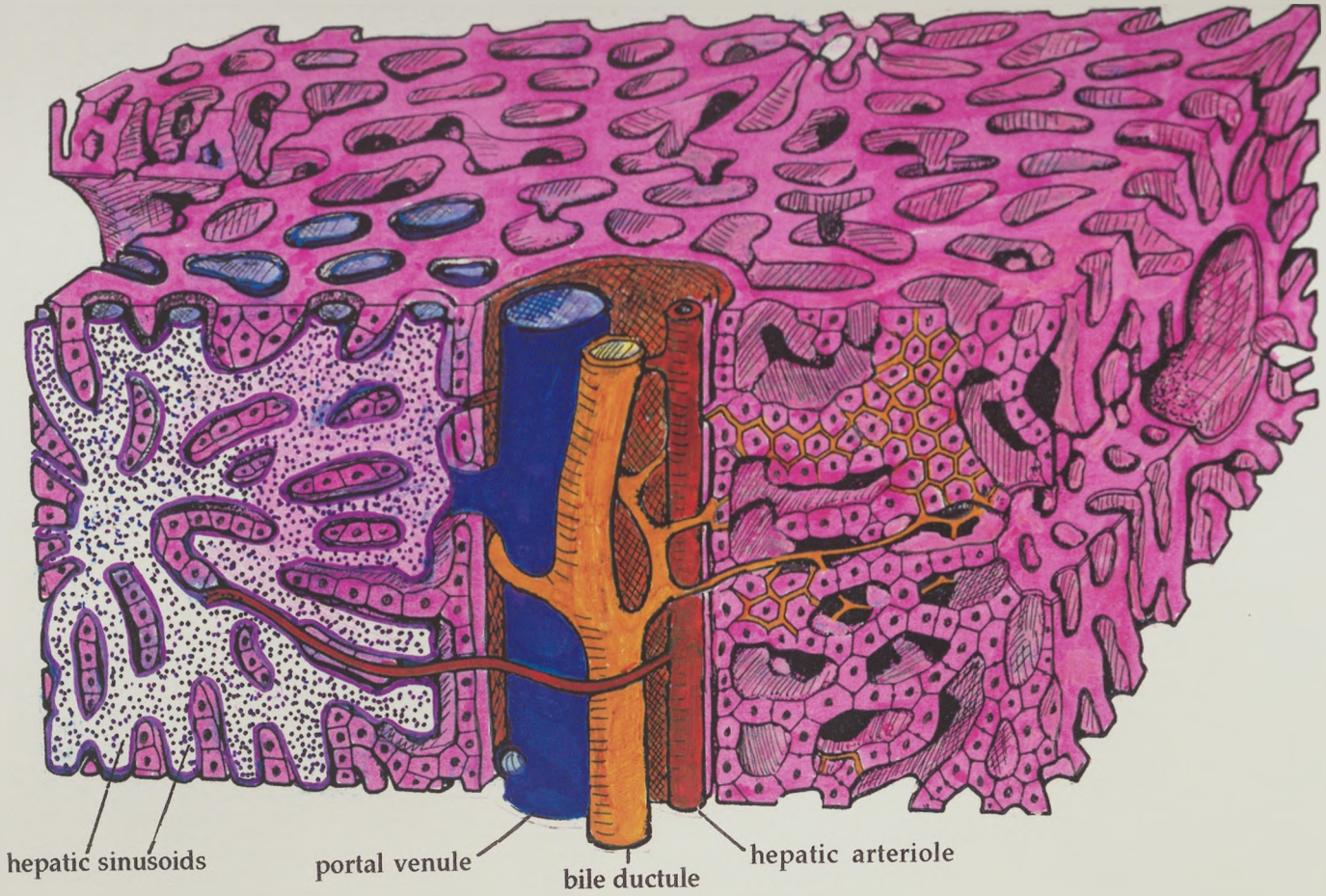


Figure A4: Three-dimensional hepatic structure

unit of this arrangement, the liver acinus (Figure A5, overleaf). The acinus is commonly divided into three regions: zone 1, the periportal, zone 2, the intermediary, and zone 3, the perivenous. The major differences between these zones may be summed up as follows:

1. Sinusoids become progressively wider and less contorted from zone 1 to zone 3. This results in a perivenous vascular space that is twice as large as that in the periportal region, creating a lower surface to volume ratio and lower cell density in the perivenous zone.
2. The porosity of the sinusoidal endothelia, as well as the surface area of the individual cells, increases from zone 1 to zone 3.
3. There is an uneven distribution of sinusoidal cells throughout these regions. For example, Kuppfer cells are situated predominately in the periportal area.
4. Concentration gradients exist within the sinusoids as substances are removed from or released into the blood supply. Perhaps the most important of these is oxygen, which is 250 mmHg in the periportal area, but decreases to 70 mmHg perivenously.

These differences affect not only the exchange of solutes within these regions, but are also believed to induce metabolic heterogeneity within the cells of a particular zone. The function of a particular cell is therefore likely to depend upon its position within the acinus.

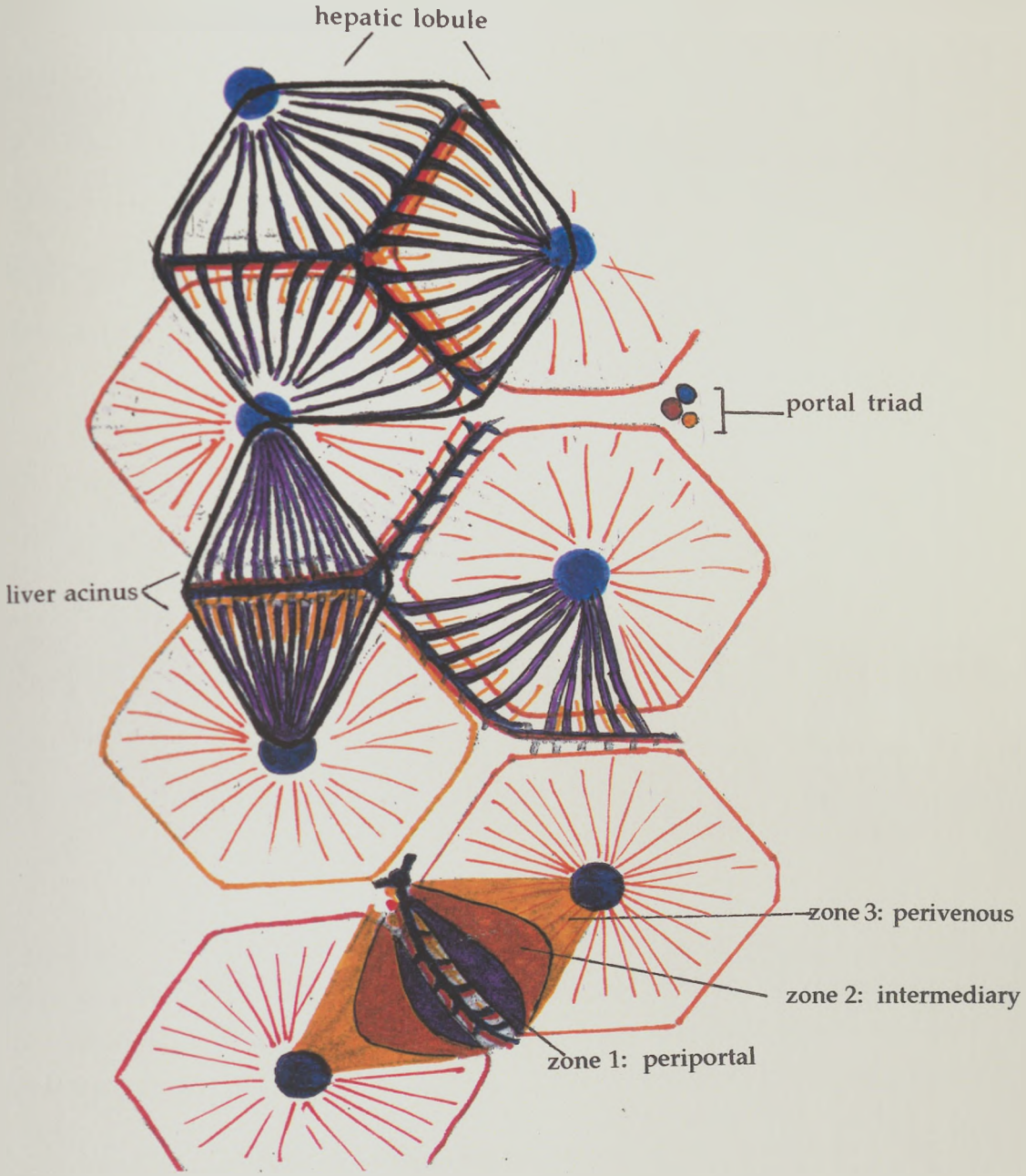


Figure A5: The hepatic acinus and the zonation of the liver

A.2 Liver function

As a result of its unique structure, origin of blood supply, combination of cell types, and their metabolic diversity, the liver has a wider variety of functions than any other organ in the body. Although not all of these have been fully elucidated, more than 500 have been catalogued. The liver is therefore essential for life; in cases of total hepatic failure or resection, survival cannot be expected beyond one to two days.

The following pages contain a brief synopsis of the functions of the liver. Although sometimes difficult to segregate, these may be roughly categorised as: detoxication, metabolism, synthesis, phagocytosis, and storage. All of these contribute to the maintenance of homeostasis within an organism.

Detoxication: Through ingestion, inhalation, or other routes of entrance to the body, we are exposed to an enormous number of exogenous compounds. In addition, the biological activity of endogenous substances, such as steroid hormones, must be carefully regulated. The major routes of excretion from the body are suitable only for the removal of hydrophilic substances. Therefore, in order to control the duration of activity of biologically active substances, and to facilitate the removal of potentially toxic compounds, the liver has evolved several enzyme systems with broad substrate specificity to increase the hydrophilicity, and thereby facilitate excretion, of these chemicals.

The process of biotransformation has been divided into two distinct phases. In phase I, the polarity of the parent compound is increased by the addition or activation of a specific functional group. This is generally accomplished by reactions involving either oxidation or reduction. In some cases, the products of these reactions are sufficiently polar for excretion, but usually further modification is required. Phase II, or conjugation reactions, result in the combination of the Phase I metabolite with specific endogenous substrates to form

a highly polar conjugate which may be readily excreted.

Intermediary metabolism: The liver plays a key role in the assimilation of nutrients, both for the maintenance of the integrity of the body tissues and the provision of substrates for their metabolic requirements. In this position, and in its location between the portal and peripheral circulation, it may be seen as a buffer between the dietary sources of amino acids, carbohydrates, and fats and the extrahepatic cells that utilise them. Although they are often dealt with separately, the metabolism of these three categories of foodstuffs are highly inter-related. For example, some amino acids can be converted to fats in the presence of excess glucose. Others participate, along with glycerol derived from fatty acids, in the *de novo* synthesis of glucose, or gluconeogenesis. Fatty acids, along with carbohydrates and amino acids, are necessary for the synthesis of proteins, which may themselves be metabolised to add to the free amino acid body pool. Similarly, there is considerable recycling amongst these metabolites. Fatty acids may be esterified in the liver to form neutral fats, which may then be degraded to produce free fatty acids and glycerol. Excess amino acids, whether obtained as part of the end products of digestion or from the breakdown of endogenous proteins, are deaminated in the liver, and the non-nitrogenous residues may be re-aminated to provide other necessary amino acids. In addition to the glucose already present in the portal blood supply after digestion and intestinal absorption, fructose and galactose reaching the liver are also converted to glucose. This may be stored in the liver or skeletal muscle as glycogen or completely oxidised in the citric acid cycle to form ATP. Glucose may be released from the glycogen stores as necessary (glycogenolysis) or reformed in the liver from lactic acid.

The fate of amino acids, fatty acids and monosaccharides in the liver are

illustrated in Figures A6 - A8 (pages 200 - 202).

Synthesis: One of the primary activities of the liver as a digestive organ is the production of bile and bile acids. These play an important part in the metabolism of fats and the excretion of bile serves as a route of excretion for some of the products of detoxication. In addition, the liver synthesises virtually all of the plasma proteins, with the exception of the immunoglobulins. These help to maintain plasma osmotic pressure, and allow the transport of various substances with low solubility via protein binding. They also serve as a blood buffer, as they will combine with both acids and bases. The liver also synthesises fibrinogen and several of the clotting factors.

Storage: The liver stores several important metabolites including glycogen, fatty acids, serum proteins, water soluble vitamins (eg. thiamine, riboflavin, vitamin B12 and folic acid), fat soluble vitamins (eg. vitamins A, D, E and K) and haemopoietic factors. The liver may also be said to store blood, in that 10 - 15% of the total blood volume resides in the organ at any given time. This provides an important blood reservoir, as up to half of this volume may be expelled by sympathetic nervous stimulation during acute haemorrhage.

Phagocytosis

The phagocytotic functions are performed mainly by the Kupffer cells. These provide the first line of defense against gut derived foreign material in the portal venous system, including endotoxin. These cells are also vital for the removal of substances from the systemic circulation, including erythrocytes, altered platelets, denatured albumin, thrombin, fibrin-fibrinogen complexes and circulating immune complexes.

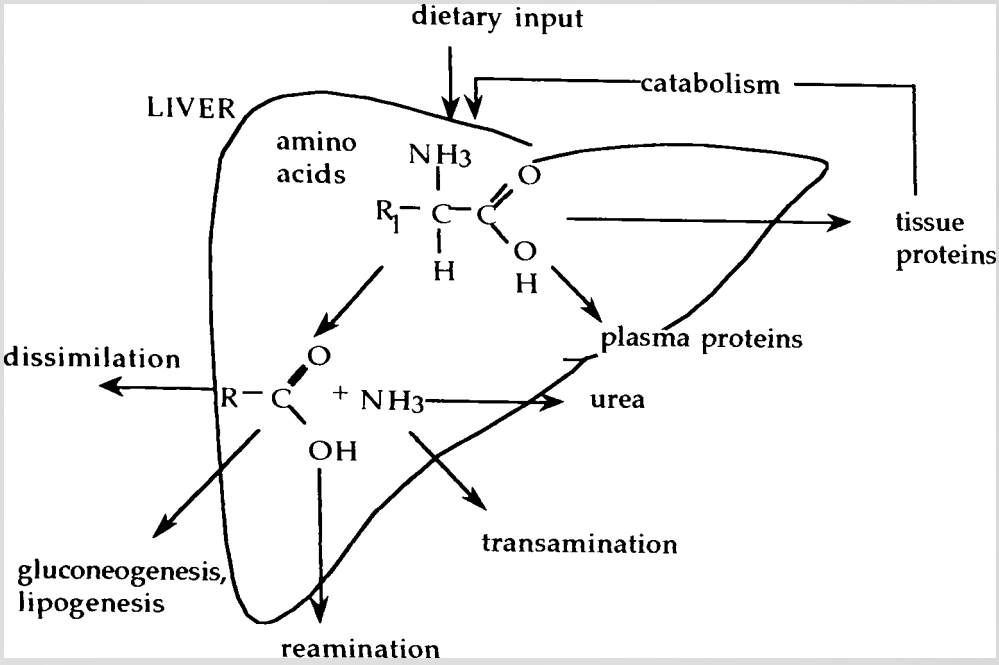


Figure A6: Amino acid metabolism

The liver has an important role in the maintenance of nitrogenous equilibrium in the body. Amino acids, whether obtained from the catabolism of endogenous tissues or from the digestion of dietary protein, are selectively removed from the liver. Excess amino acids are deaminated, and the non-nitrogenous residues may either undergo reamination or complete dissimilation, or participate in gluconeogenesis or lipogenesis. The resulting ammonia may be converted to urea for renal excretion, or used in the conversion of a keto-acid to an amino acid (transamination). Amino acids resulting from this reshuffling are used in the synthesis of plasma proteins or are incorporated into body tissues.

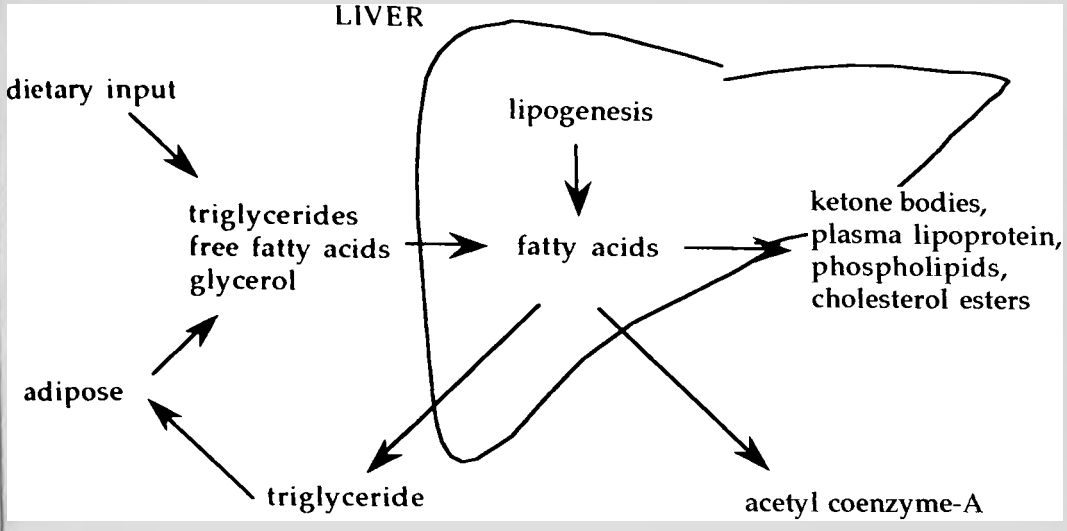


Figure A7: Fatty acid metabolism

From the digestion of foodstuffs and the breakdown of adipose tissue, the liver is presented with a mixture of triglycerides, free fatty acids (FFA) and glycerol. Triglycerides may be metabolised in the liver to form FFA (lipolysis), which may also be formed from excess amino acids and glucose (lipogenesis). FFA in the liver may be reconverted to triglycerides (esterification) for storage in adipose tissue, or oxidised to form acetyl coenzyme A, an important precursor for both lipogenesis and the citric acid cycle. FFA are also used in the synthesis of ketone bodies, plasma lipoproteins, phospholipids and cholesterol esters.

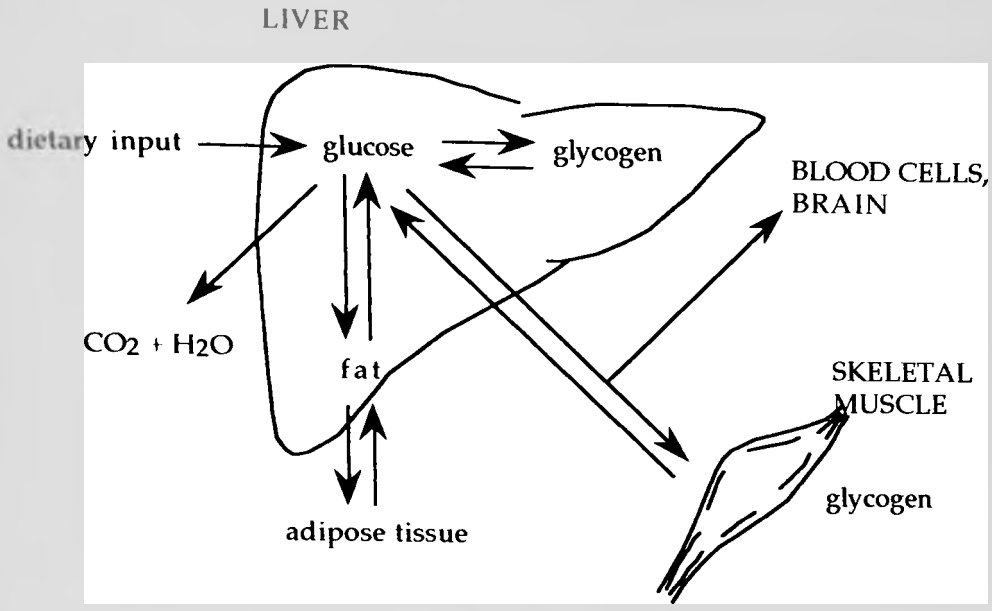


Figure A8: Glucose metabolism

Carbohydrate metabolism is best described by a comparison between the fed and fasted states. Although the dietary sources of carbohydrate are principally glucose, fructose and galactose, the latter two are converted to glucose in the liver, so that the metabolism of carbohydrate is essentially the metabolism of glucose. After a meal, some glucose passes directly into the peripheral circulation, for use by the brain and blood cells and to be stored as glycogen in skeletal muscle. The remainder may be converted to glycogen (glycogenolysis) for storage in the liver, converted to fats for storage in adipose tissue, or completely oxidised in the citric acid cycle. In the fasted state, glucose is steadily released into blood from the liver by glycogenolysis Free fatty acids and amino acids are also released respectively from adipose tissue and skeletal muscle, and used in gluconeogenesis. Glucose-6-phosphate may also be mobilised from skeletal muscle glycogen reserves, but its dephosphorylation to glucose occurs almost exclusively in the liver.

Appendix B: Compartmental mass transfer analyses of BAL toxin removal

CMTA (Compartmental Mass Transfer Analysis, listing provided on pages 210-220) is a program written in Turbo Pascal which evaluates the compartmental mass transfer analysis by Ohshima et al (1986, 1994; Figure B1, page 206) as well as a modification of this model to allow for direct contact between between the patient's blood and the cultured hepatocytes (Figure B2, page 207), where:

V: volume of compartment (l)

C: solute concentration ($mol \cdot l^{-1}$)

F: perfusion rate ($l \cdot h^{-1}$)

R_a: accumulation rate of solute in the body ($mol \cdot h^{-1}$)

K: overall mass transfer coefficient ($m \cdot h^{-1}$)

A: membrane surface area (m^2)

K_m: metabolic reaction rate constant ($l \cdot cell^{-1} \cdot h^{-1}$)

N_0: initial concentration of living hepatocytes ($cell \cdot l^{-1}$)

v: % hepatocyte viability = $\exp(-K_v t)$, where

K_v: death rate constant (h^{-1})

t: time (h)

A mass balance is performed on each compartment, and the resulting simultaneous equations solved using a fourth order Runge Kutta method with variable step size.

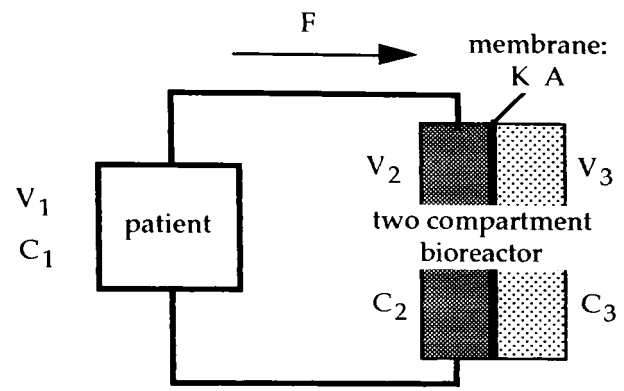
Although they were not always explicitly specified in the text, average values for the above parameters were obtained (or extrapolated from the information provided) from Ohshima et al (ibid; Table B1, page 208). In this case, ammonia was used as the "marker" substrate. Several runs were performed, for which only one parameter was varied at a time (Figure B3 a-g, page 209 and facing pages). It can be

seen in Figure B3 a that significant increases in the rate of ammonia removal would be expected with an increased reactor volume for the two compartment model (2CM). By contrast, the effect of such increases in reactor volume for the three compartment model (3CM; Figure B3 b) is much less dramatic, and no improvement in the rate of ammonia removal would be predicted as the volume increases from 2 to 50 l. The 2CM is also predicted to cope with increases in the rate of accumulation of a ammonia within the body (representing patient residual liver capacity and the rate of ammonia generation) much better than the 3CM (Figures B3 c & d). When $R_a = 5\ \mu mol \cdot h^{-1}$, the BAL modelled by the 2CM would still be able to produce a 40% reduction in ammonia concentration over an eight hour perfusion, whilst ammonia concentration *increases* ~ 50% for the same value of R_a in the 3CM. Similarly, when a relatively high death rate is specified in the model ($Kv = 1.0\ .\ h^{-1}$ instead of 0.06, as suggested by Ohshima and coworkers) the predicted reduction in the initial ammonia concentration is still > 25% at the end of an eight hour session, although it should be noted that the rate of ammonia generation would exceed that of ammonia removal by this time (Figure B3 e). The 3CM would not be able to sustain even a modest reduction in ammonia concentration under these conditions (Figure B3 f), and after eight hours there would be a net increase in ammonia concentration.

Unsurprisingly, the differences between BALs modelled by the 2CM and the 3CM are due to the mass transfer resistance of the membrane separating the hepatocyte compartment from the blood or plasma in the 3CM. When the overall mass transfer coefficient is increased, the 3CM behaviour begins to resemble that of the 2CM (Figure B3 g).

While such models may be an important tool in future BAL design, there are too many unknowns at present to assess these predictions. As discussed in Chapter 2 (section 2.4), it appears that bioreactor design strongly influences hepatocyte viability and

detoxifying capacity, and these effects are not addressed in this model. A more detailed analysis (eg. incorporating a wider range of factors influencing hepatocyte function, not assuming perfect mixing, etc.) may therefore be necessary to increase the usefulness of this model to the BAL design process.



1:

$$V_1 \frac{dC_1}{dt} = F\ (C_2 - C_1) + R_a$$

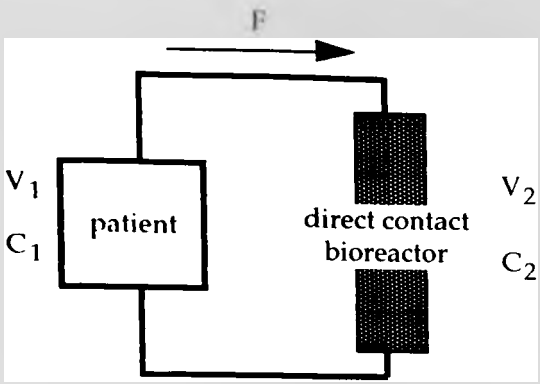
2:

$$V_2 \frac{dC_2}{dt} = F\ (C_1 - C_2) + K\ A\ (C_3 - C_2)$$

3:

$$V_3 \frac{dC_3}{dt} = K\ A\ (C_2 - C_3) - K_m N_0\ v\ C_3 V_3$$

Figure B1: Compartmental analysis of a two compartment BAL



1:

$$V_1 \frac{dC_1}{dt} = F\ (C_2 - C_1) + R_a$$

2:

$$V_2 \frac{dC_2}{dt} = F\ (C_1 - C_2) - K_m N_0\, v\, C_2 V_2$$

Figure B2: Compartmental analysis of a direct contact BAL

Table B1: Default values of the variables for the 2 compartment (A) and the 3 compartment mass transfer analysis (B). See main text for units.

	A	B
V:		
Reactor volume	2	2
Blood / plasma compartment volume	N/A	2
Patient blood volume	40	40
C:	0.0008	0.0008
F:	20	20
R_a:	0.0005	0.0005
K:	N/A	0.04
A:	N/A	0.04
K_m	1.8	1.8
N_0:	10^6	10^6
K_v:	0.06	0.06
t (total length of perfusion session):	8	8

Figure B3: Compilation of results from several test runs of Program CMTA. Curves labelled * indicate the ammonia concentration predicted in the hepatocyte compartment of the BAL, while • indicates the patient "compartment" ammonia levels.

a: 2CM; The effect of reactor volume on ammonia removal. The number at the end of each curve refers to the reactor volume in litres.

b: 3CM; The effect of reactor volume on ammonia removal. The number at the end of each curve refers to the reactor volume in litres.

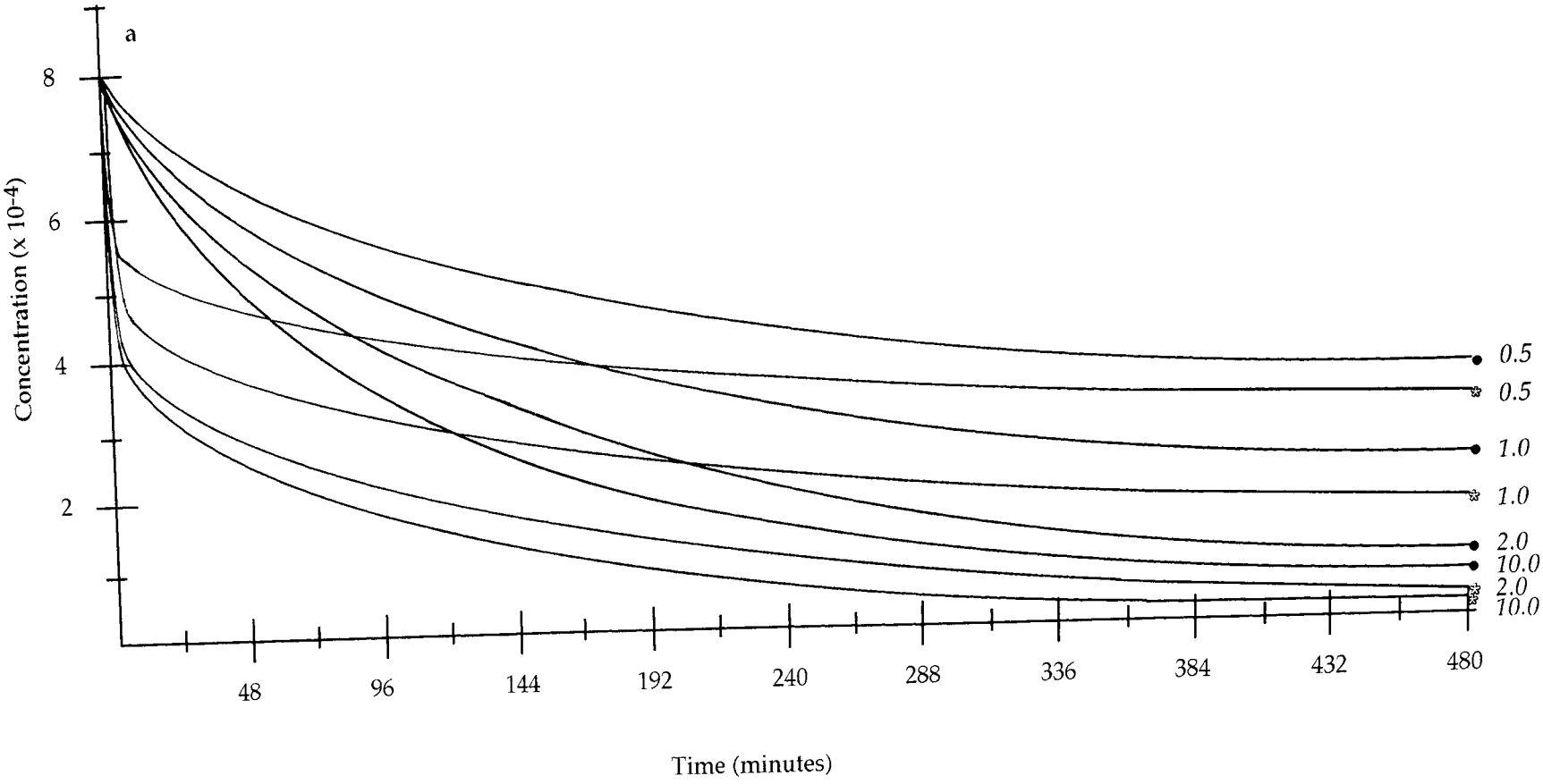
c: 2CM; The effect of the accumulation rate of ammonia on its removal. The number at the end of each curve refers to the accumulation rate in mol · h^{-1}.

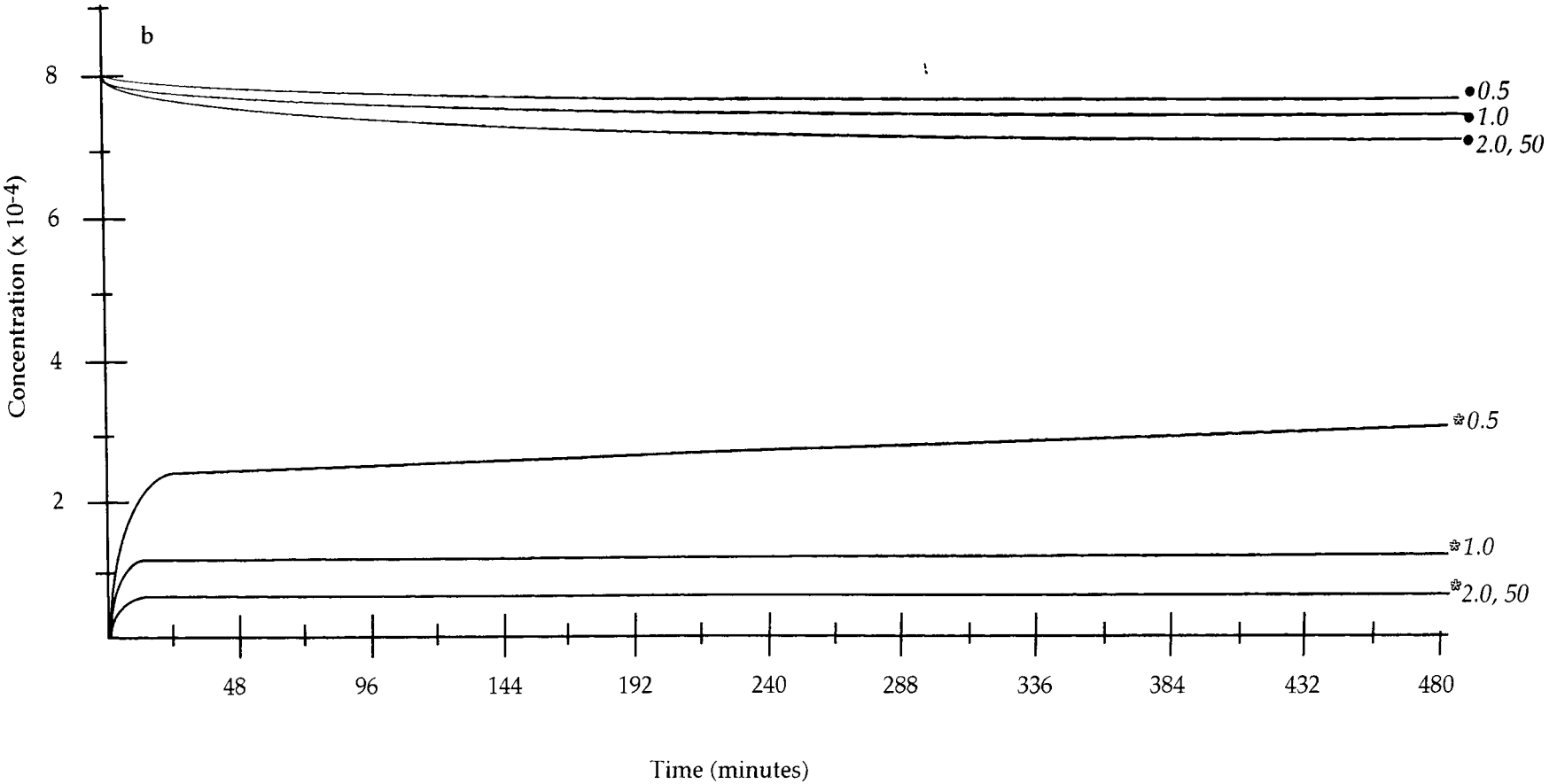
d: 3CM; The effect of the accumulation rate of ammonia on its removal. The number at the end of each curve refers to the accumulation rate in mol · h^{-1}.

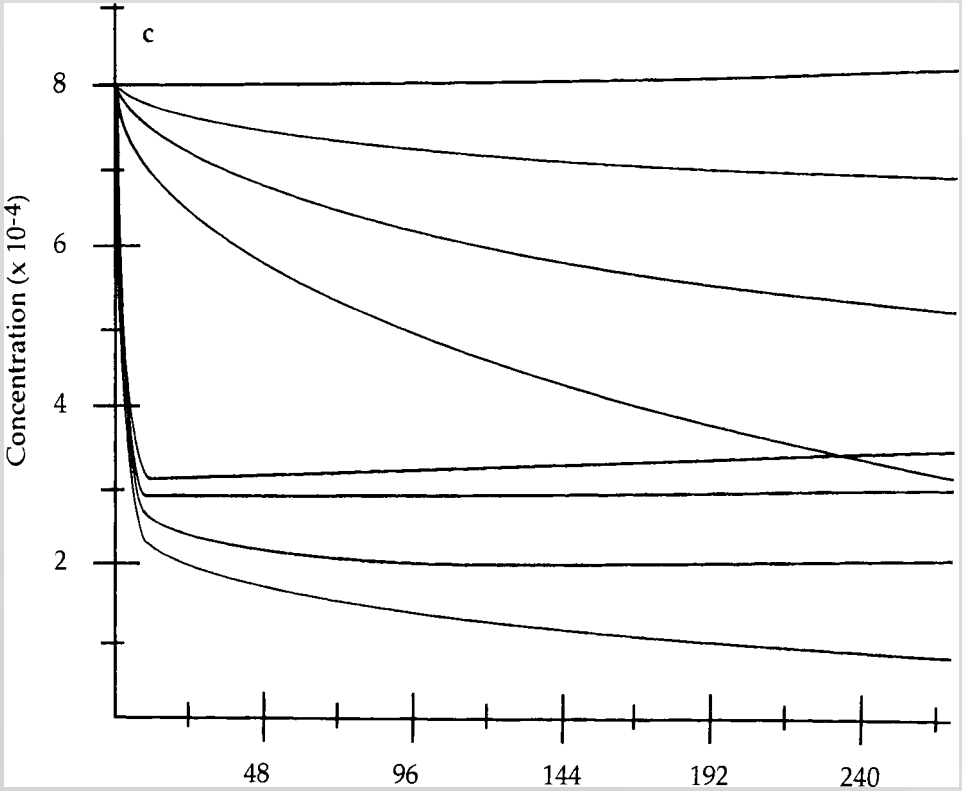
e: 2CM; The effect of the cell death rate on ammonia removal. The number at the end of each curve refers to the death rate constant in h^{-1}.

f: 3CM; The effect of the cell death rate on ammonia removal. The number at the end of each curve refers to the death rate constant in h^{-1}.

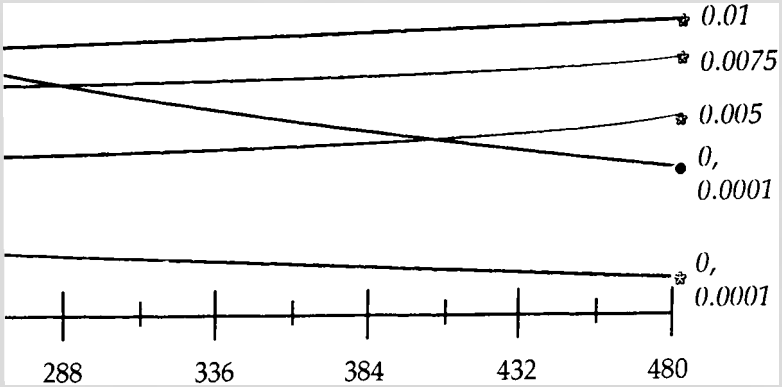
g: 3CM; Ammonia removal profile when the mass transfer/surface area coefficient is maximised.

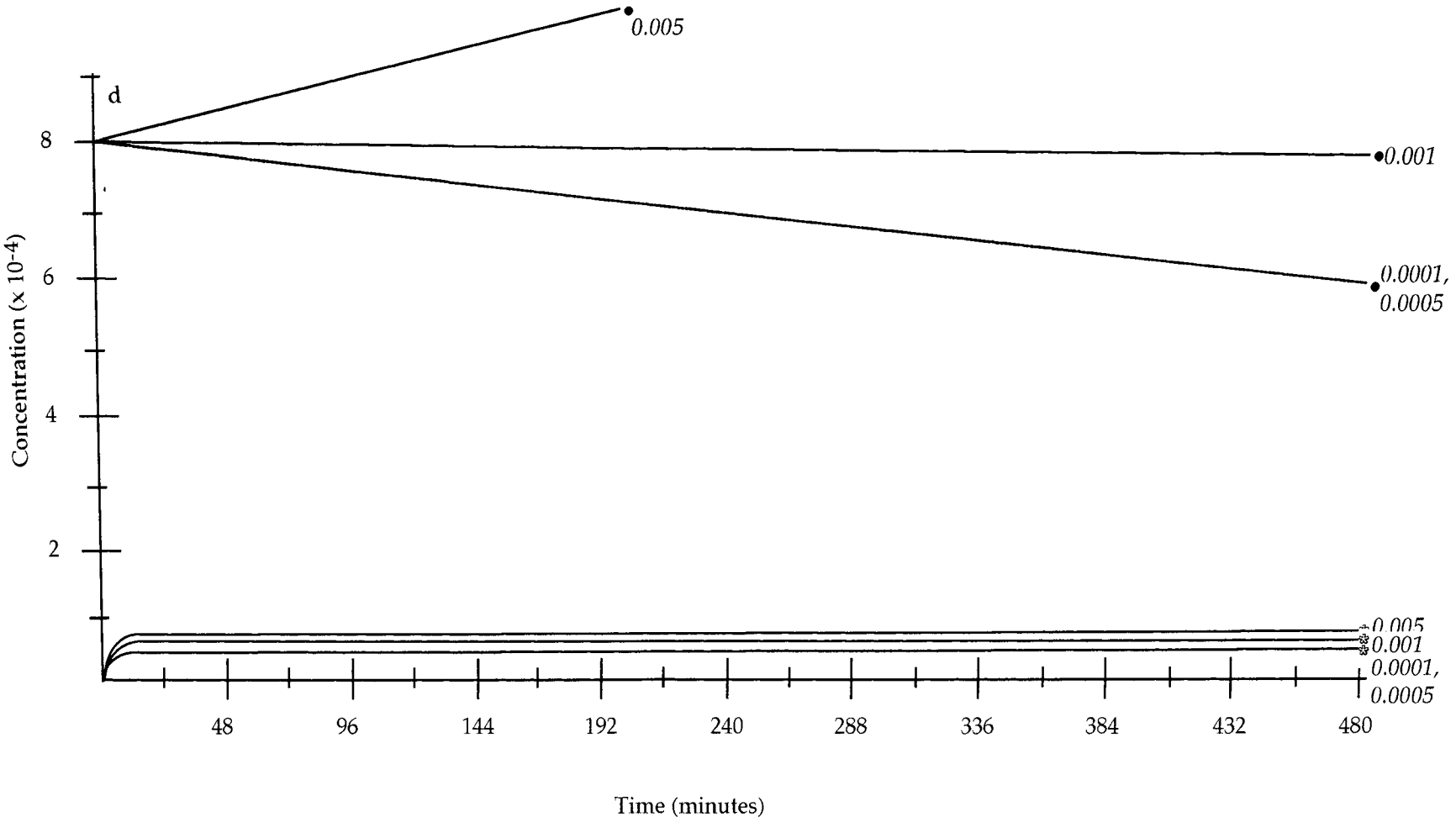

a
8
6
4
2
Concentration (x 10-4)
48
96
144
192
240
288
336
384
432
480
Time (minutes)
0.5
0.5
1.0
1.0
2.0
10.0
2.0
10.0

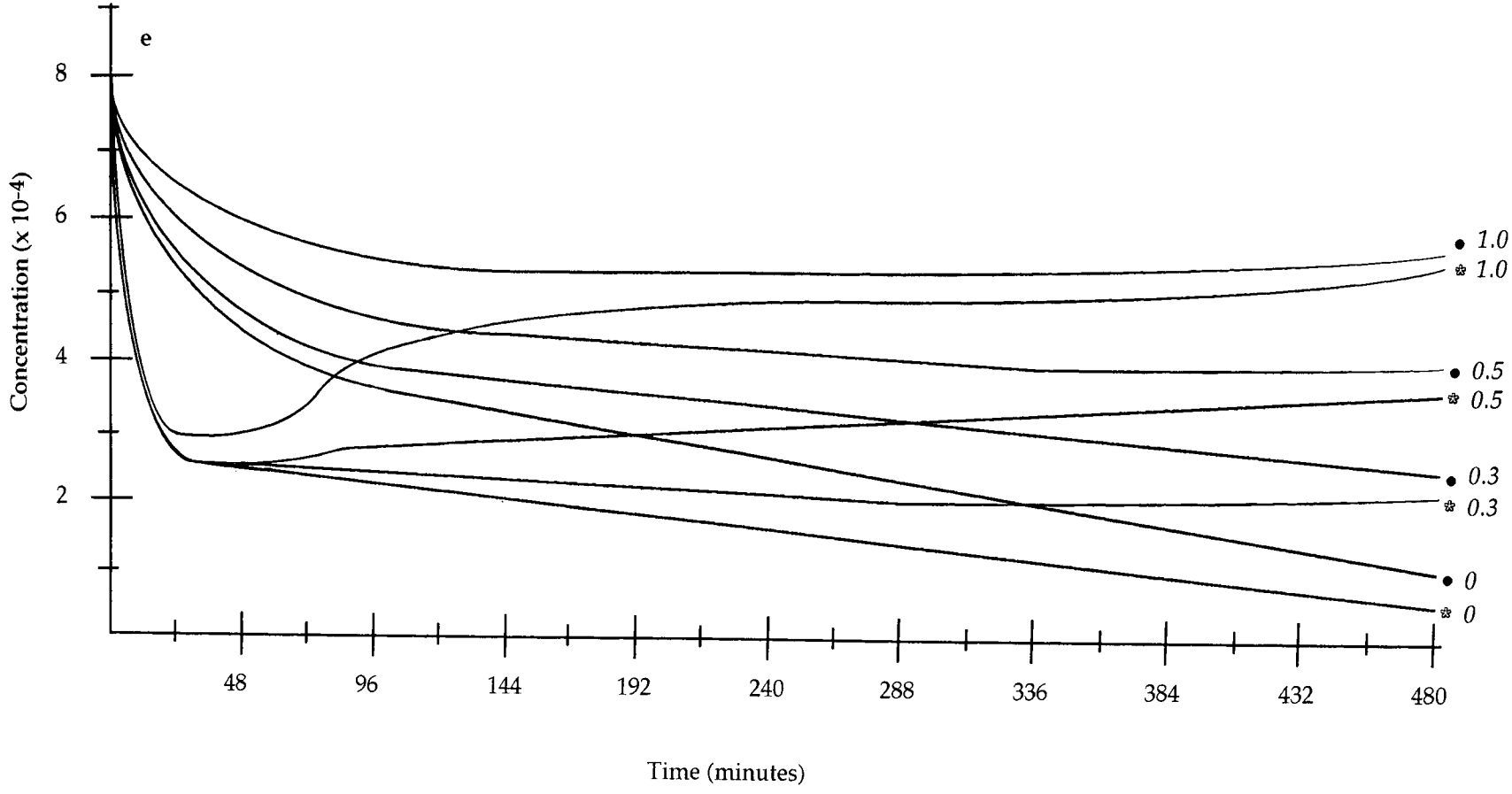

b
8
6
4
2
Concentration (x 10-4)
0.5
1.0
2.0, 50
0.5
1.0
2.0, 50
48
96
144
192
240
288
336
384
432
480
Time (minutes)

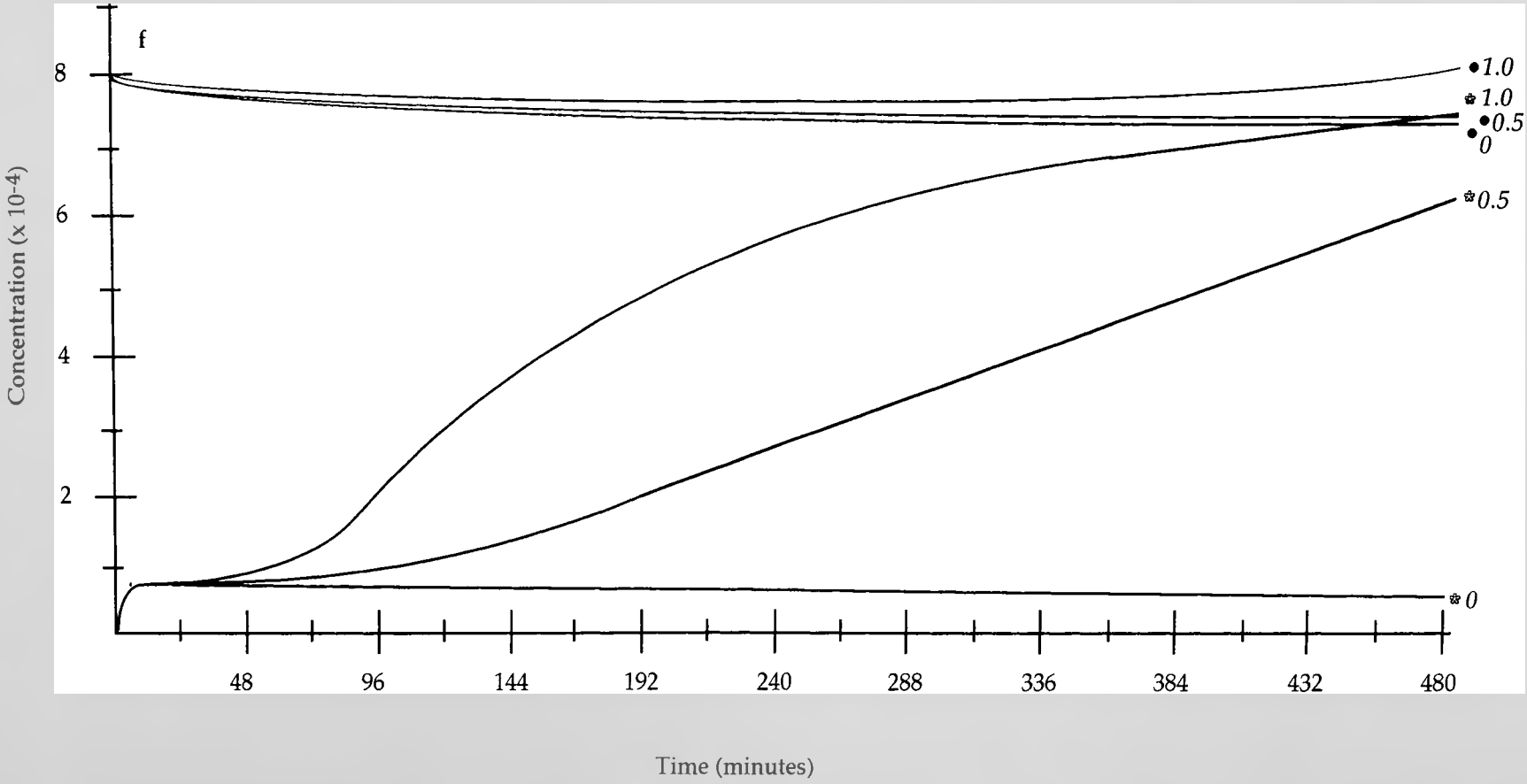

c
8
6
4
2
Concentration (x 10-4)
48
96
144
192
240
Time (minutes)

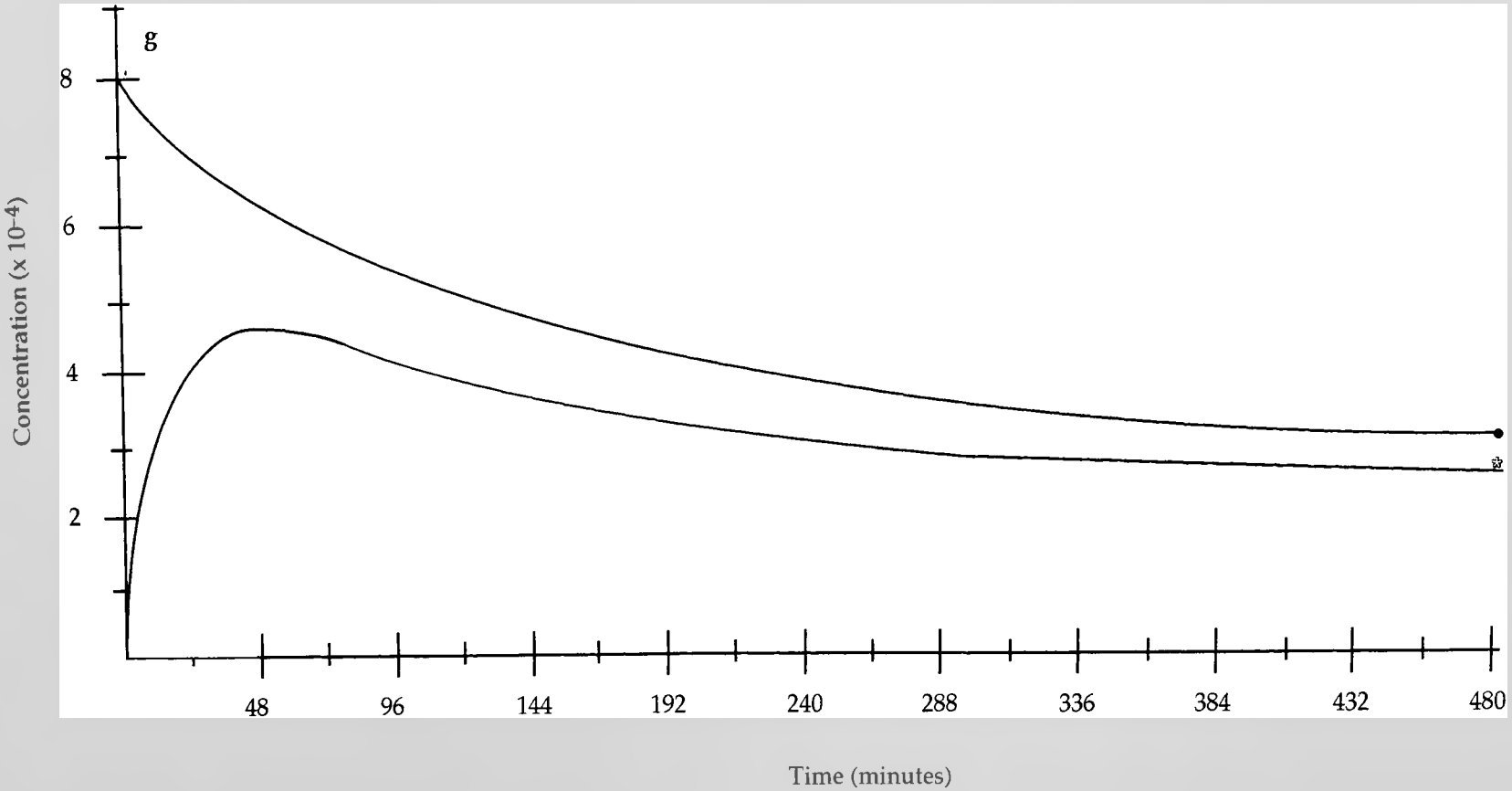

0.01
0.0075
0.005


0.01
0.0075
0.005
0,
0.0001
0,
0.0001
288
336
384
432
480


d
0.005
0.001
0.0001,
0.0005
8
6
4
2
Concentration (x 10-4)
0.005
0.001
0.0001,
0.0005
48
96
144
192
240
288
336
384
432
480
Time (minutes)


e
8
6
4
2
Concentration (x 10-4)
1.0
1.0
0.5
0.5
0.3
0.3
0
0
48
96
144
192
240
288
336
384
432
480
Time (minutes)


f
8
6
4
2
Concentration (x 10-4)
1.0
1.0
0.5
0
0.5
0
48
96
144
192
240
288
336
384
432
480
Time (minutes)


g
8
6
4
2
Concentration (x 10⁻⁴)
48
96
144
192
240
288
336
384
432
480
Time (minutes)

```
{   ************************************************************************   }

Program Compartmental_Mass_Transfer_Analysis;

{   ************************************************************************   }

USES
   CRT, DOS, GRAPH;

TYPE
   RealArray     =  array[1..500] of REAL;
   RealMatrix    =  array[1..3, 1..500] of REAL;
   IntegerArray  =  array[1..500] of INTEGER;
   IntegerMatrix =  array[1..3, 1..500] of INTEGER;

VAR
   t                                 :  RealArray;
   C                                 :  RealMatrix;
   NumVal, GraphDriver, GraphMode    :  INTEGER;
   NumXPix, NumYPix                  :  INTEGER;
   PathToDriver                      :  STRING;
   XorY                              :  BOOLEAN;
   Power, Q                          :  INTEGER;
   XMax, YMax, YMin, Time            :  REAL;
   XCoord                            :  IntegerArray;
   YCoord                            :  IntegerMatrix;
   Qc                                :  CHAR;

CONST
   XOffset    =  50;
   YOffset    =  20;

{   ************************************************************************   }

Procedure SetUpScreen;
{ Creates screen to prompt user for data }

   Begin
      ClrScr;
         GoToXY(8, 1);
            Write('COMPARTMENTAL MASS TRANSFER ANALYSIS FOR A
```

```
        HEPATOCYTE BIOREACTOR');
GoToXY(26, 2);
    Write('IN ARTIFICIAL LIVER SUPPORT');
GoToXY(1, 4);
    Write('Enter the required data:');
GoToXY(3, 6);
    Write('Bioreactor characteristics:');
GoToXY(43, 6);
    Write('Hepatocyte characteristics:');
If Q = 3 then
    Begin
        GoToXY(5, 7);
        Write('Vol. plasma pool (l)')
    End;
GoToXY(45, 7);
    Write('MRRC (mol/cl-h)');
GoToXY(5, 8);
    Write('Reactor vol. (l)');
GoToXY(45, 8);
    Write('Init cells (cell/l)');
If Q = 3 then
    Begin
        GoToXY(5, 9);
        Write('Contact area (m2)')
    End;
GoToXY(45, 9);
    Write('DRC (/hr)');
If Q = 3 then
    Begin
        GoToXY(5, 10);
        Write('MTC (m/h)')
    End;
GoToXY(3, 12);
    Write('Operating conditions:');
GoToXY(5, 13);
    Write('Perfusion rate (l/hr)');
GoToXY(5, 14);
    Write('Length of perfusion (h)');
If Q = 3 then
    Begin
        GoToXY(5, 15);
```

```
                Write('Init conc solute in bioreactor (mol/l)')
            End;
        GoToXY(5, 16);
            Write('Init conc solute in body (mol/l)');
        GoToXY(5, 17);
            Write('Accumulation rate, body (mol/h)');
        GoToXY(5, 18);
            Write('Volume, body pool (l)');
    End;        { procedure SetUpScreen }

{   ************************************************************************   }

Procedure GetData(   VAR      t       :   RealArray;
                     VAR      C       :   RealMatrix;
                     VAR   NumVal     :   INTEGER      );

{ gets information from the screen and performs calculations }

    VAR
        K1, K2, K3, K4, K5, K6 :   REAL;
        V2, V3, A, K, Km, N0   :   REAL;
        V0, Kv, F, r, V1       :   REAL;
        h, Ci                  :   REAL;
        i, j, m, Index         :   INTEGER;
        E                      :   RealArray;

    Function dC(Index : INTEGER; t : REAL; Ci : REAL) : REAL;
    { calculates the change in concentration for each compartment }

        Begin
            If Q = 3 then
                Case Index Of
                    1 : dC := F*(C[2,m-1]-Ci)/V1 + r/V1;
                    2 : dC := F*(C[1,m]-Ci)/V2 - 1000*K*A*(Ci-C[3,m-1])/V2;
                    3 :  dC :=  1000*K*A*(C[2,m]-Ci)/V3 - Km*N0*Ci*exp(-Kv*t)
                End  { case }
            else
                Case Index Of
                    1 : dC := F*(C[2,m-1]-Ci)/V1 + r/V1;
                    2 :  dC := F*(C[1,m]-Ci)/V2 - Km*N0*Ci*exp(-Kv*t)
                End  { case }
```

```
    End; { Function }

Begin
    If Q = 2 then
        GoToXY(30,8)
    else
        GoToXY(30,7);
    Read(V2);                { 2: bioreactor vol., 3: plasma compart }
    If Q = 3 then
        Begin
            GoToXY(30,8);
                Read(V3);    { volume of hepatocyte compartment }
            GoToXY(30,9);
                Read(A);     { contact area between V2 and V3 }
            GoToXY(30,10);
                Read(K);     { mass transfer coefficient }
        End;
    GoToXY(69,7);
        Read(Km);            { metabolic reaction rate constant }
    GoToXY(69,8);
        Read(N0);            { initial number of viable hepatocytes }
    GoToXY(69,9);
        Read(Kv);            { death rate constant }
    GoToXY(48,13);
        Read(F);             { rate of perfusion }
    GoToXY(48,14);
        Read(Time);          { length of perfusion }
    If Q = 3 then
        Begin
            GoToXY(48,15);
            Read(C[3,1])     { initial conc. of toxin in bioreactor }
        End;
    GoToXY(48,16);
        Read(C[1,1]);        { initial conc. in body }
    GoToXY(48,17);
        Read(r);             { accumulation rate of toxin }
    GoToXY(48,18);
        Read(V1);            { volume of body pool }

    ClrScr;
```

```
h := 0.005;
t[1] := 0;
m := 1;
C[2,1] := C[1,1];
If Q = 2 then
    E[3] := 0.001;

Repeat                          { loop for values to be plotted }
    m := m + 1;
    t[m] := t[m-1] + h;

    { determine concentration in each compartment using }
    { a fourth order Runge Kutta method                 }
    For i := 1 to Q Do
        Begin
            K1 := dC(i, t[m-1], C[i,m-1]);
            K2 := dC(i, t[m-1] + h/4, C[i,m-1] + h*K1/4);
            K3 := dC(i, t[m-1] + 0.375*h, C[i,m-1] + 0.09375*h*K1 +
                0.28125*h*K2);
            K4 := dC(i, t[m-1] + 12*h/13, C[i,m-1] + 1932*h*K1/2197 -
                7200*h*K2/2197 + 7296*h*K3/2197);
            K5 := dC(i, t[m-1] + h, C[i,m-1] + 439*h*K1/216 - 8*h*K2 +
                3680*h*K3/513 - 845*h*K4/410);
            K6 := dC(i, t[m-1] + h/2, C[i,m-1] - 8*h*K1/27 + 2*h*K2 -
                3544*h*K3/2565 + 1859*h*K4/4104 - 0.275*h*K5);
            C[i,m] := C[i,m-1] + (25*K1/216 +1408*K3/2565 + 2197*K4/4104 -
                K5/5)*h;

            { check error and vary stepsize accordingly }
            E[i] := (K1/360 -128*K3/4275 - 2197*K4/75240 + K5/50 +
                2*K6/55)*h;
            C[i,m] := C[i,m] + E[i];
            E[i] := Abs(100*E[i]/C[i,m]);
            If (i = Q) and (E[1] <= 0.01) and (E[2] <= 0.01) and (E[3] <= 0.01) then
                    If h< 0.04 then h := 2*h
                        else
                    h := 1.1*h;
            If E[i] >= 1 then
                Begin
                    i := Q;
                    h := 0.5*h;
```

```
                        m := m-2;
                        writeln('The error is > 1%')
                    End
                End;
            If Q = 3 then
                writeln('C1 = ', C[1,m]:7:6, '  C2 = ', C[2,m]:7:6, '  C3 = ', C[3,m]:7:6, '  t = ',
                    t[m]:5:4, '  h = ', h:9:8, '  E = ', E[3]:4:3)
            else
                writeln('C1 = ', C[1,m]:7:6, '  C2 = ', C[2,m]:7:6, '  t = ', t[m]:5:4, '  h = ',
                    h:6:5, '  E = ', E[2]:4:3, '  m = ', m:3);

        Until (t[m] >= Time) or KeyPressed or (m > 500);
        { end Repeat-Until }

        NumVal := m
End; { procedure GetData }

{   ************************************************************************   }

Procedure FindXCoord(    RealX     :   RealArray;
                         NumVal    :   INTEGER;
                     VAR XMax      :   REAL;
                     VAR NumXPix   :   INTEGER;
                     VAR XCoord    :   IntegerArray);

{ transforms real values of time into pixel values                          }

{ GetMaxX is a TurboPascal function which returns the number of pixel       }
{ columns for a given monitor                                               }

    VAR
        Scale  :   REAL;
        i      :   INTEGER;

    Begin
        XMax := Time;
        If Round(XMax) >= XMax
            then
                XMax := Round(XMax)
            else
                XMax := Round(XMax + 1);
```

```
        NumXPix := 50*Round((GetMaxX - XOffset)/50) - XOffset;
        Scale := NumXPix/XMax;

        For i:= 1 to NumVal Do
            XCoord[i] := Round(Scale*RealX[i] + XOffset)

    End;        { procedure FindXCoord }

{   ************************************************************************   }

Procedure FindYCoord(     RealY              :   RealMatrix;
                          NumVal             :   INTEGER;
                      VAR YMax, YMin         :   REAL;
                      VAR NumYPix, Power     :   INTEGER;
                      VAR YCoord             :   IntegerMatrix);

{ transforms real values of concentration into pixel values                 }

{ GetMaxY is a TurboPascal function which returns the number of pixel       }
{ rows for a given monitor                                                  }

    VAR
        Scale, Temp    :   REAL;
        i, j           :   INTEGER;

    Begin
        ClearDevice;

        { find maximum and minimum values to be plotted }
        YMax := RealY[1,1];
        YMin := YMax;
        For i := 1 to Q Do
            Begin
                For j := 1 to NumVal Do
                    Begin
                        If RealY[i,j] > YMax then
                            YMax := RealY[i,j];
                        If RealY[i,j] < YMin then
                            YMin := RealY[i,j]
                    End;  { for j statement }
            End;   { for i statement }
```

```
        { find power for scientific notation }
        power := 0;
        If YMax < 1.0 then
            Repeat
                YMax := YMax*10;
                power := power + 1
            Until YMax >= 1.0;

        YMin := YMin*exp(power*ln(10));
        If Round(YMin) < YMin
            then
                YMin := Round(YMin)
            else
                YMin := Round(YMin - 1)

        { round YMax to nearest integer }
        YMax := 1.1*YMax;
        If Round(YMax) < YMax
            then
                YMax := Round(YMax + 1)
            else
                YMax := Round(YMax);

        { find number of pixels available for plotting }
        NumYPix := GetMaxY - YOffset - 50;
        Scale := NumYPix/(YMax - YMin);

        { find coordinates }
        For i := 1 to Q Do
            For j := 1 to NumVal Do
                YCoord[i,j] := Round(NumXPix  - Scale * exp(power*ln(10)) * RealY[i,j]
                    - YMin) + YOffset)

    End;        { procedure FindYCoord }

{   ************************************************************************   }

Procedure GraphResults(   YCoord                  :   IntegerMatrix;
                          XCoord                  :   IntegerArray;
                          XMax, YMax, YMin        :   REAL;
```

```
                    NumXPix, NumYPix, Power    :    INTEGER);

CONST
    XLegend = 'Time (minutes)';
    GraphTitle = 'COMPARTMENTAL ANALYSIS FOR HEPATOCYTE
        BIOREACTOR';
    NumXMarks = 10;
    NumYMarks = 5;
VAR
    Place, MaxX, MaxY   :   INTEGER;
    X, Y, i, j, Temp    :   INTEGER;
    Ch                  :   CHAR;
    YLegend, iS, LbS    :   STRING;
    Lbl                 :   REAL;

Begin
    ClearDevice;
    MaxX := NumXPix + XOffset;
    MaxY := NumYPix + YOffset;

    { draw axes }
    SetLineStyle(0, 1, 3);
    Line(XOffset, YOffset, XOffset, MaxY);
    Line(XOffset, MaxY, MaxX, MaxY);

    { put legend on x axis and title on the graph }
    SetTextJustify(1, 2);
    OutTextXY(Round(GetMaxX/2), 5, GraphTitle);
    OutTextXY(Round(GetMaxX/2),MaxY + 20, XLegend);

    { put legend on y axis }
    Str(power, YLegend);
    YLegend := Concat('Conc x 10E-', YLegend);
    SetTextStyle(0, 1, 0);
    SetTextJustify(2, 1);
    OutTextXY(15, Round((MaxY + YOffset)/2), YLegend);

    { label values on y axis }
    SetLineStyle(0, 1, 1);
    SetTextStyle(0, 0, 0);
    SetTextJustify(2, 1);
```

```
  For i := 1 to NumYMarks Do
      Begin
          Place := YOffset + (i-1)*Round(NumYPix/NumYMarks);
          Lbl := YMax - (i-1)*(YMax-YMin)/NumYMarks;
          Line(XOffset + 5, Place, XOffset - 5, Place);
          Str(Lbl:2:1,LbS);
          OutTextXY(XOffset - 8, Place, LbS)
      End;  { for statement }

  { label values on x axis }
  SetTextJustify(1,0);
  For i := 1 to NumXMarks Do
      Begin
          Place := XOffset + (i)*Round(NumXPix/NumXMarks);
          Lbl := Round(60*i*XMax/NumXMarks);
          Line(Place, MaxY - 5, Place, MaxY + 5);
          Str(Lbl:3:0,LbS);
          OutTextXY(Place, MaxY + 15, LbS)
      End;  { for statement }

  { graph information }
  SetTextJustify(1, 1);
  SetLineStyle(0, 0, 1);
  Temp := 0;
  For i := 1 to Q Do
      Begin
          SetLineStyle(0, i, 1);
          For j := 2 to NumVal Do
              Line(XCoord[j-1], YCoord[i,j-1], XCoord[j], YCoord[i,j]);
          Str(i, LbS);
          OutTextXY( XCoord[j], YCoord[i,j], LbS)
      End;  { for statement }

  Repeat
      Delay(100)
  Until KeyPressed;

  While KeyPressed Do
      Ch := ReadKey

End;  { procedure GraphResults }
```

```
{    ************************************************************************    }

Begin            { main program }
    ClrScr;
    GoToXY(8,1);
    writeln('COMPARTMENTAL MASS TRANSFER ANALYSIS FOR A
        HEPATOCYTE BIOREACTOR');
    GoToXY(23,10);
    writeln('2 or 3 compartment analysis?');
    GoToXY(57,10);
    Read(Q);
    SetUpScreen;
    GetData(t, C, NumVal);
    DetectGraph(GraphDriver, GraphMode);
    PathToDriver := ' ';
    InitGraph(GraphDriver, GraphMode, PathToDriver);
    FindXCoord(t, NumVal, XMax, NumXPix, XCoord);
    FindYCoord(C, NumVal, YMax, YMin, NumYPix, Power, YCoord);
    GraphResults(YCoord, XCoord, XMax, YMax, YMin, NumXPix, NumYPix, Power)
End.             { main program }
```