MECHANISMS OF PORTAL-SYSTEMIC ENCEPHALOPATHY: A STUDY USING ISOLATED LIVER AND BRAIN PERFUSION

by

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DEDICATION

To my loving family

Peta, Zoe and Ross,

for their patience, understanding and constant encouragement. This thesis is also dedicated to the memory of my father whose untimely death prevented him witnessing its completion.

CONTENTS

TABLE OF CONTENTS

Page No.

A B S T R A C T

ABSTRACT

The pathogenesis of hepatic encephalopathy is obscure. This study examines some of the underlying mechanisms thought to be associated with portal systemic encephalopathy (PSE) by investigations of the interrelationship between the liver and brain. These mechanisms were studied experimentally in two preparations. The first involved the development and application of novel in vitro perfusion techniques, in order to examine the effect of liver perfusion upon the isolated perfused rat brain. The second made use of portacaval anastomosis (PCS) and portacaval transposition (PCT) in the rat as an in vivo model of PSE.

Detailed investigations of the liver/brain interrelationship necessitated the development of an isolated organ perfusion system which incorporated a new miniaturised membrane oxygenator. The oxygen permeability was accurately measured by a chemical technique developed during these investigations and optimum conditions for perfusion of the isolated rat liver were defined.

For the isolated perfused rat brain preparation new electrodes were constructed to improve the quality of electroencephalogram (EEG) signals recorded with conventional EEG electrodes. The authenticity of the EEG signal was established by administration of incremental doses of sodium pentobarbitone and by demonstrating a gradual attenuation in EEG on cessation of brain perfusion which recovered upon re-establishment of flow. These studies then established a survival time for the isolated perfused rat brain under optimal perfusion conditions. Further investigations demonstrated that the introduction of an isolated perfused rat liver into the perfusion circuit prolonged survival of the isolated perfused rat brain from a median of 35

minutes (range 22-53 minutes) to 326 minutes (range 172-480).

Brain amine measurements carried out for up to 75 weeks in PCS and PCT rats showed that changes in brain amines were probably largely due to portal diversion (as reflected in the PCT rat) rather than liver dysfunction (in the PCS rat). The only sustained increases above those measured in PCT rats were in 5 hydroxyindole acetic acid suggesting a reduced breakdown or uptake of the indole amine in PCS rats.

These results were partially confirmed through measurement of brain amine concentrations in isolated perfused brains of PCS and control rats. Elevated PCS brain amine concentrations declined to control levels during perfusion without a corresponding increase in survival time. This suggested that other fundamental mechanisms may be involved in the prolongation of survival time and may be implicated in the precipitation of PSE. It is concluded that the pathogenesis of PSE is not only due to changes in brain amine concentrations and that a hitherto unexplained liver-brain homeostatic interrelationship may also be involved in this complex, multifactorial syndrome.

LIST OF FIGURES

Page No.

Page No.

Page No.

Page No.

Page No.

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AAA	Aromatic amino acids
ACD	Acid citrate dextrose (bags)
ADP	Adenosine 5'-diphosphate
ADR	Adrenaline (epinephrine)
ALK	Alkaline phosphatase
AST	Aspartate-serine transaminase
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCAA	Branched chain amino acids
BSP	Bromosulphthalein
CPB	Cardiopulmonary bypass
CSF	Cerebrospinal fluid
DOP	Dopamine
ECoG	Electroencephalocorticogram
EEG	Electroencephalogram
FHF	Fulminant hepatic failure
Hb	Haemoglobin
HCT	Haematocrit
HE	Hepatic encephalopathy
HF	High frequency
5HIAA	5 hydroxyindole acetic acid
HOU	Hepatic oxygen uptake
HPLC	High performance liquid chromatography
5HT	5 hydroxytryptamine
5HTP	5 hydroxytryptophan

HV	High voltage
IPRB	Isolated perfused rat brain
IPRL	Isolated perfused rat liver
IVC	Inferior vena cava
KRBA	Krebs Henseleit buffer
mmHg	Millimetres of mercury (pressure)
NOR	Noradrenaline (norepinephrine)
OTC	Oxygen transfer (transport) capacity
PFH	Perfusate (plasma) free haemoglobin
PCS	Portacaval shunt (anastomosis)
PCT	Portacaval transposition
PCV	Packed cell volume (haematocrit)
PDS	Polydimethylsiloxane
PHE	Phenylalanine
PSE	Portal systemic encephalopathy
PV	Portal vein
TPT	Oxygen transport

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LIST OF CONTENTS

Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

C H A P T E R 1

OVERVIEW OF THESIS

γηρασκω δ' αιει πολλα διδασκομενος.

"I grow old ever learning many things"

Solon c.630 - c.555 B.C.

OVERVIEW OF THESIS

Portal systemic encephalopathy (PSE) is the term applied to a disorder of brain function which is seen in patients with severe acute and chronic liver disease. The manifestation of mental disturbances is believed to be related to the inability of the liver to process, metabolise and detoxify portal blood efficiently. The pathogenesis of the resultant cerebral dysfunction remains obscure and the aim of this study was to investigate some of the underlying mechanisms that are thought to be involved in the precipitation of PSE. These are discussed in Chapter 2.

The dependence of the brain upon a healthy functional liver was first demonstrated experimentally by Geiger and Magnes in 1947. Isolated perfused cat brain experiments demonstrated that electrical activity was preserved longer when a liver was included in the perfusion circuit. In addition, small quantities of the nucleotides cytidine and uridine added to the perfusate could prolong electrical activity of the brain in the absence of a liver (Geiger, 1958). This led Geiger to hypothesise that the liver may produce a factor or factors essential for normal cerebral function. Surprisingly, additional evidence in support of this hypothesis has not been reported and this early work has never been repeated. The current series of investigations was conducted to confirm these findings and further elucidate the liver/brain interrelationship (chapter 8).

In the studies reported in this thesis the rat was chosen as the experimental model because of its easy availability and the wealth of literature which exists upon the physiology and anatomy of the rat, especially with reference to PSE.

Existing systems for the perfusion of either rat liver (Hems et al 1966) or rat brain (Andjus et al 1967) were found inappropriate due to the complexity and inefficiency of these systems particularly if two organs were to be perfused concomitantly. This is discussed further in Chapter 3.

Chapter 4 describes the design, construction and in vitro evaluation of a new miniaturised membrane oxygenator suitable for isolated organ perfusion. The in vitro gas transfer characteristics of the membrane used were accurately measured by a new chemical technique which was superior to conventional measuring systems. In addition, the influence of haematocrit, membrane tubing curvature, flow rate and coating thickness upon the oxygen transport of the membrane and the percentage saturation difference were also investigated to provide data regarding optimum lengths and curvature of membrane.

This new oxygenator was incorporated into a simple design of isolated organ perfusion circuit with a small prime volume. Chapter 5 demonstrated the applicability of the new perfusion circuit to isolated perfused rat free skin flaps, rabbit hearts and in particular isolated perfused rat livers. Extensive investigations were undertaken to optimise the system for perfusion of an isolated rat liver (Chapter 6).

Reliable and reproducible EEG tracings from an isolated perfused rat brain preparation were then established (Chapter 7). Initially investigations were undertaken to verify the authenticity of the signals recorded in vivo. When these were completed, studies were then initiated with the isolated perfused rat brain preparation, which was a modification of a technique originally described by Thompson et al

(1968), to establish a baseline survival time.

These studies were extended in chapter 8 which describes the results from combined isolated perfused rat liver and brain preparations using a combination of the optimised circuits described in chapters 6 and 7 and confirms the important role of the liver in the regulation of normal brain function.

Elevated brain concentrations of 5HT (serotonin) and 5HIAA (5-hydroxyindole acetic acid) have been demonstrated in rats with experimentally induced portacaval anastomosis (PCS) (James et al 1979, Siemert et al 1978). Chapter 9 investigates the turnover of brain catechol and indole amines in vivo following portacaval diversion to determine their degree of involvement in the pathogenesis of PSE. In addition, the effects of liver dysfunction were differentiated from those of portal systemic diversion and related to previous experimental models of PSE (the PCS rat) where this was not possible. Finally brain amine results were compared to values obtained in perfused PCS and control brains in order that their relevance in the pathogenesis of PSE could be evaluated.

The aims of this study were therefore to 1) substantiate and elaborate the experiments of Geiger, this time using the rat rather than the cat as the experimental model, 2) investigate the influence of the liver upon brain amine turnover in PCS and PCT rats, and 3) relate these observations to those in vitro by use of the isolated perfused rat brain and thus attempt to elucidate some of the underlying mechanisms involved in the precipitation of PSE.

The conclusions of this study and future proposals are described in Chapter 10.

CHAPTER 2

REVIEW OF HEPATIC ENCEPHALOPATHY

"In a sense, much of the investigation governing hepatic coma has been like looking at the grounds of a grand mansion and trying to imagine the furnishing inside from a description of the formal gardens"

Josef E. Fischer, 1982.

REVIEW OF HEPATIC ENCEPHALOPATHY

2.1 WHAT IS HEPATIC ENCEPHALOPATHY?

Hepatic encephalopathy (HE) is the term applied to a disorder of consciousness which occurs in patients with severe liver disease (Zieve 1966). This mental derangement is thought to be due to the inability of the liver in acute or chronic liver failure to metabolise, process and detoxify portal blood.

Acute liver failure develops as a result of extensive necrosis of liver cells or follows any other sudden and severe impairment of hepatic function in a patient in whom there has been no previous evidence of liver disease (Trey and Davidson 1970). It is characterised by acute onset of mental changes which rapidly develop into stupor or coma. Jaundice appears later and the whole illness from the first symptom to death may be less than a week. Chronic liver failure may develop over a longer time period, as a result of cirrhosis of the liver for example, and may present similar neuropsychiatric disturbances which often precede the onset of coma.

Portal systemic encephalopathy (PSE) and HE are the terms used to describe these neuropsychiatric disturbances although these are of a very non-specific nature. However there are characteristic neuromuscular disorders which are nearly always associated with PSE. The familiar asterixis or "flapping tremor" is almost a diagnostic feature of PSE although it may be seen with other encephalopathies. The mental disturbances, being non-specific, are also found during hepatic encephalopathy in general, where portal-systemic shunting is absent, and range from mild changes in mood or character to major behavioural derangements and finally to coma.

For the purposes of this study, however, the two terms may be considered interchangable. Indeed history may prove that a difference may not exist between the pathogenesis of the two encephalopathies particularly since the nature of the cerebral intoxicants is still obscure.

2.2. HISTORICAL PERSPECTIVE

The dependence of the brain upon a healthy, functional, liver has been recorded since the earliest times. The Babylonians (circa 2000 BC) and the Chinese (circa 1000 BC) believed that examination of the appearance of the liver reflected information regarding the personality and character of the patient. Consequently the conclusion was drawn that the liver was responsible for expression of the 'soul' directly, since diseased livers were often discovered at autopsy in patients who had previously exhibited abnormal yet characteristic behavioural changes. Hippocrates (460-370 BC) described a patient with hepatitis who barked like a dog, could not be held and said things that could not be comprehended.

Other experimental physicians of the era such as Galen deduced from Hippocrates' and their own findings that the syndrome now known as encephalopathy was a direct result of liver disease. Glisson in the 17th century described one of the earliest functional attributes of the liver and provided one of the first documented accounts of the perfusion of an isolated liver. A cannula, which consisted of a modified swan quill, was introduced and secured into the portal vein of a human cadaver. Through this he squeezed an ox bladder full of milk which subsequently appeared in the vena cava. This experiment led him to conclude that one of the functions of the liver was to

strain, from the portal blood, noxious substances which originated from the gastrointestinal tract. Later Budd (1845) also stated that "the role of the liver is to remove noxious substances from the blood stream".

It was in 1877 that the history of experimental portal-systemic shunting was initiated by Nickolai Eck. Eck was a 29 year old Russian military surgeon serving in the Crimea who was investigating surgical treatment of mechanical ascites through diversion of the portal blood supply into the general circulation. He constructed a side to side anastomosis of the portal vein to the vena cava experimentally in dogs and concluded:

"The main reason to doubt that such an operation can be carried out on human beings has been removed because it was established that the blood of the portal vein, without any danger to the body, could be diverted directly into the general circulation and this by means of a perfectly safe operation".

Eck performed the anastomosis in 8 dogs of which 7 died through peritonitis or strangulation of the intestines and omentum. The remaining survivor recovered completely and lived in the laboratory for 2.5 months but escaped through "lack of attention".

Sixteen years later Pavlov and his associates (Hahn et al 1893) performed Eck fistulae in 20 dogs and reported weight and hair loss post-operatively. In addition when the dogs were fed upon meat, they developed the syndrome which became known as 'meat intoxication', which consisted of irritability and staggering followed by convulsions and death if meat feeding continued. At autopsy, liver atrophy and fatty infiltration were noted. Two important observations which Pavlov recorded during these experiments were that 1) the neurological abnormalities of meat intoxication were reversed if meat was withdrawn from the diet and 2) both the neurological abnormalities and liver

atrophy accompanied by fatty infiltration were reduced amongst surviving dogs where there was evidence of shunt thrombosis or stenosis at post-mortem.

The historical credit for the first portacaval shunt to be performed in man rests with the French surgeon Vidal in 1903. He fulfilled Eck's earlier ambition and successfully treated a case of ascites by the construction of a portacaval shunt. Unfortunately the patient died after six weeks from sepsis after suffering from recurrent haematemesis. Vidal recorded however that "all absorption of albuminoides provoked a severe intoxication". Drawing from the experimental observations of Eck and Pavlov and from his own records he concluded that

> "ammoniacal substances which are toxic for the organism when the liver is no longer there to guard against this danger and transform them into urea and other waste products ..... Even in limited amounts (albuminoides) produce sweats, muscular trembling, intense anxiety, cardiac arrhythmias - briefly a presentation characteristic of ammonia intoxication".

Medical science was now able to predict a direct relationship between deterioration in brain function with deteriorating liver function. Physiologists such as Von Coulaert in Strasbourg (1932) and Kirk in Denmark (1936) demonstrated that administration of ammonium chloride or urea to cirrhotic patients caused a marked mental disturbance similar to the observations of Vidal during administration of "albuminoides" to his patient. These also bore striking similarities to the effects of "meat intoxication" previously described by Eck and Pavlov.

It was not until 1954 that Professor Sheila Sherlock coined the term "portal systemic encephalopathy" and acknowledged the crucial relationship between PSE and impaired hepatocellular function. It was also during this time period that reports of PSE in non-cirrhotic

patients were beginning to appear in the literature: McDermott and Adams (1954) and Hubbard (1958) reported PSE in patients with carcinoma of the head of the pancreas whose livers appeared essentially normal except for biliary obstruction. End-to-side portacaval anastomosis had been performed as a part of a pancreaticoduodenectomy and PSE was reported in all four patients within a few weeks of operation. Further reports of PSE began to appear when portal diversion had become established as a means of surgical management of bleeding varices in portal hypertension (Blakemore and Lord 1945; Blakemore 1952).

Sherlock et al (1954) acknowledged and in fact rediscovered that PSE was a phenomenon which was not confined to patients with liver damage but that man with a normal liver is also susceptible to the effects of PSE due to portal diversion through much the same mechanism as seen in Pavlov's dogs.

Adams and Foley (1953) graded the encephalopathy according to the various grades of coma exhibited by the patients. This led to the construction of the table shown below (see Sherlock 1975).

Table 2.1

Grade	Observation
1	Confused, slurred speech
2	Drowsy. Inappropriate behaviour.
3	Stuporose but speaking and obeying simple commands. Inarticulate speech. Marked confusion.
4	Coma
5	Deep coma with no response to painful stimuli and no spontaneous movements.

Grading of hepatic encephalopathy.

The clinical features of HE also include disturbed consciousness, personality changes, intellectual deterioration, slow or slurred speech, "flapping" tremor and a slowing of the electroencephalographic alpha wave to 4Hz from the normal range of 13.2 to 32Hz.

Eleven years later Sherlock and her colleagues (Thompson et al 1964) reported encephalopathy in 15% of non-cirrhotic patients who underwent surgical portal diversion for portal vein thrombosis. This was one of the earliest examples of PSE associated with the formation of portal-systemic shunts in patients with near normal livers.

2.3 EXPERIMENTAL MODELS FOR THE STUDY OF PSE

It has been discussed how PSE may be precipitated by portal-systemic shunts in man and experimental models without pre-existing hepatocellular damage. The management of patients remains difficult and presents many complex problems which have yet to be totally reproduced in an experimental model. Nonetheless detailed investigations of experimental models which exhibit some of the manifestations of PSE, such as animal models with Eck fistulae, could contribute towards a better understanding of the various changes involved. The requirements of an animal model of fulminant hepatic failure were previously described by Terblanche et al (1975), but may also be applied to models of PSE and are listed below:

1. Reversibility : to respond and recover with suitable treatment.
2. Reproducibility
3. Fatality : as a direct result of liver failure. This is necessary to allow assessment of methods of treatment.
4. Applicability : should be applicable to larger animals so that the treatment can be readily extrapolated to man.
5. Safety : should be of minimal danger to personnel.

Criterion 3 is inapplicable to chronic PSE models, which should not be fatal but should exhibit a stable neurological defect that is not progressive.

Mullen and McCullough (1989) have recently outlined a more specific list of features which should be reproduced during experimental HE:

1. Occurrence of chronic liver disease.
2. Full range of encephalopathy ranging from subclinical to deep coma.

3. Reversibility.
4. Absence of significant extrahepatic complications.
5. Precipitation by gastrointestinal nitrogenous load.
6. Improvement by lactulose, neomycin treatment or protein withdrawl.
7. Associated with abnormal nitrogen metabolism.
8. Alzheimer Type 2 changes in brain astrocytes.

They qualify the list by stating that at present there is no animal model which can totally fulfil all of these criteria. They suggested that the presently available models of carbon tetrachloride induced cirrhosis and portacaval shunt held the greatest promise if produced in a standardised fashion.

2.3.1 Acute Models

One of the earliest acute models of hepatic failure was described by Mann and Magath (1922) who performed total hepatectomy in dogs and demonstrated that the impending coma could be subdivided into a reversible hypoglycaemic stage and a secondary irreversible stage which was glucose independent. Acute liver failure may also be surgically induced through a two stage devascularisation such as the technique reported by Rappaport et al (1953): they performed Eck fistulae in dogs and demonstrated that ligation of the hepatic artery produced a secondary irreversible stage of coma, associated with extensive hepatic necrosis.

A combination of the two techniques was reported by Professor Benhamou's group in France and incorporated a three stage hepatectomy in rats (Degos et al 1974) in which the inferior vena cava was ligated initially and a portacaval shunt performed two weeks later. Two days after the portacaval shunt the liver was removed from

some rats or the liver, spleen, stomach, intestine and pancreas were all removed from other "eviscerated" rats. They concluded from measurements of EEG and survival times that ammonia and other substances released from the intestine did not play a major role in the mechanisms of HE. However, acute surgical models of liver failure are generally irreversible and the various grades of coma may not be easily distinguishable. Anhepatic models of course do not produce damaged liver cells, and thus differ fundamentally from human fulminant hepatic failure.

Acute hepatic failure may also be induced chemically. Terblanche's group in Cape Town (Terblanche et al 1975) conducted extensive studies in pigs using carbon tetrachloride in one group and paracetamol intoxication in another. Characteristic hepatic histopathological changes such as centrilobular necrosis were identified but they concluded that it was difficult to assess the ensuing coma in either of the groups. Other chemicals such as dimethylnitrosamine (Fischer 1977) and galactosamine (Dekker and Keppler 1972) have also been used to induce acute hepatic failure but chemical models may suffer from individual differences in sensitivity (Mullen and McCullough 1989, Terblanche et al 1975). Cost may also be prohibitive, particularly in larger animals.

2.3.2 Chronic models

Chronic PSE may also be chemically or surgically induced. Chemically induced models of chronic PSE include some of the compounds discussed, such as paracetamol and dimethylnitrosamine, but used at a lower dosage and perhaps administered through a different route. Surgical models of PSE are more reproducible and are more easily

applied to larger animals.

The most familiar surgical model of PSE is the Eck fistula, whose history has already been described. More recently, the technique was modified by Lee and Fischer (1961) for application to the laboratory rat. The portacavally shunted (PCS) rat has subsequently been widely used for the experimental production of PSE in rats (Herz et al 1972, Lee et al 1974, Fischer 1982). The portal-systemic diversion results in liver atrophy accompanied by impaired liver function, and characteristic alterations in the brain which are often reported in man with PSE (Benjamin et al 1976, Doyle et al 1978, Kyu and Cavanagh 1970).

Unfortunately the PCS rat fails to distinguish between the two sub-components present in this model of hepatic insufficiency: portal-systemic diversion of blood and liver failure. Both of these sub-components are believed to be involved in the precipitation of the neuropsychiatric syndrome. Although the PCS rat reflects many aspects of PSE seen in man the extent of the individual contributions of these two principal components which characterise the model are still largely ill defined.

The technique of portacaval transposition (PCT) in the rat was a modification of a technique described by Child et al (1953) in the dog. The preparation consists of an end-to-end anastomosis between the distal end of the divided vena cava to the hepatic end of the portal vein and another end-to-end anastomosis between the proximal end of the ascending vena cava and the visceral end of the portal vein. The value of this model is that:

1. Portal diversion is achieved with minimal reduction in liver function (Benjamin et al 1976) or blood flow (Ryan et al 1978, Kreuzer

and Schenk 1971).

2. The effects of portal-systemic diversion may be distinguished from those of abnormal or reduced liver function (Benjamin et al 1976, Ryan et al 1974, Vaerman et al 1981).

3. The Alzheimer type II changes in astrocytes in the CNS have been found to be reduced when compared to PCS rats although an equivalent degree of portal-systemic diversion is maintained (Doyle et al 1978).

The contribution of these two surgical models towards the understanding of underlying mechanisms of PSE are discussed in detail in the following section.

2.4 EXPERIMENTAL EVALUATION OF PSE

A major problem with all models of PSE is the difficulty in objective assessment of encephalopathy in laboratory animals. This section discusses the evaluation of PSE from behavioural, electrophysiological, morphological and biochemical standpoints.

2.4.1 Behavioural modifications

The disturbed consciousness and hypersomnia characteristic of the early stages of PSE in patients (Sherlock 1975) is not readily detectable in PCS rats (Herz et al 1972) although PCS rats have been noted to be stuporose, inactive and to possess attenuated startle responses (Denis et al 1983). In addition, the PCS rat has been reported to develop an alteration of circadian rhythms with insomnia and nocturnal hyperactivity (Campbell et al 1979). Results from behavioural studies of this nature do present problems in interpretation. For example, PCS rats housed individually or in groups present differences in behaviour and activity (Tricklebank et al 1978). Careful quantification of such behavioural changes could help in the measurement of the degree of severity of chronic PSE. Such studies could confirm if the changes produced in PCS rat brains are of a stable neurological nature or not. Initial studies in PCS rats demonstrated a reduction in spontaneous locomotion and exploration following surgery which was sustained for up to 6 weeks post-operatively (Bengtsson et al 1985). Similar behavioural changes seen in PCS rats were attributed to alterations in brain indole amine metabolism and specifically to an increase in synthesis of 5HT (Bengtsson et al 1988a, Green 1981, Warbritton et al 1979). These

studies contrast with those of Martin and Baettig (1980) who reported few differences between the behavioural patterns of PCS and control rats.

2.4.2 Electrophysiological observations

Characteristic, but non-specific, EEG changes are seen in man with overt PSE or FHF. Moreover these EEG changes often precede the onset and detection of the clinical symptoms. Similarly, EEG changes have been reported to precede the onset of altered biochemistry and behavioural patterns in rats in FHF (Degos et al 1974) and in PCS rats with chronic PSE (Herz et al 1972, Martin and Baettig 1980).

A more recent application of electrophysiological measurements in the diagnosis of experimental PSE has been the use of the visually evoked potential (VEP). These are cortical potentials generated by a visual stimulus whose transit time is recorded between two electrodes set at a fixed distance. Alterations in VEPs have also been observed to mark a pre-clinical encephalopathic state (Levy et al 1987). In addition alterations in median somatosensory evoked potentials have been detected in patients with liver cirrhosis consequent on chronic viral hepatitis (Chu and Yang 1988). Galactosamine induced hepatic encephalopathy in rabbits resulted in VEPs that could also be reproduced by GABA (gamma-amino butyric acid) injections or GABA agonists such as diazepam and muscimol hydrate (Schafer et al 1984).

2.4.3 Morphological changes

The most prominent and well defined gross morphological change which occurs in the brain in man during HE is cerebral oedema (Sherlock 1975). The pathogenesis of this is still poorly understood

although it is a common characteristic of PSE which is also reproduced in the PCS rat in the form of cerebellar oedema (Zamora et al 1973). It may be related to alterations in the permeability of the blood-brain barrier (BBB) (Laursen and Westergaard 1977). This conceptual barrier between blood capillaries and brain neurones is believed to be controlled by a population of non-excitable brain cells, the astrocytes, so-called for their characteristic star shape. These non-excitable cells, or neuroglia (from the Greek 'nerve glue'), fill the spaces between neurones and blood capillaries, which would be filled by extracellular fluid in other organs. They thus serve an adhesive role amongst many of their other functions which will be discussed (Bowsher 1988). Neuroglia occupy at least 50% of the volume of the brain: neuroglia may be sub-divided into oligodendrocytes and astrocytes. Oligodendrocytes are associated with the myelination of axons located in the white matter of the brain. Astrocytes, which make up the largest proportion of neuroglia, are sub-divided into 2 main categories; fibrous astrocytes which are found in the white matter of the brain and protoplasmic astrocytes which are associated with the deep grey matter in the brain. The precise functions of these morphologically different types of astrocytes are not yet fully understood. However, it is highly likely that they could regulate the exit and entry of numerous substances across the brain (Kimelberg and Norenberg 1989). It is therefore not surprising that any alterations to the astrocyte population could have radical influences upon brain biochemistry, morphology and pathophysiology.

The major histological changes seen in the brain during PSE in man are a generalised increase in the number and size of astrocytes in the deep grey matter of the central nervous system. The most frequent changes reported during PSE are the so called "Alzheimer Type II"

changes whereby the astrocytic nucleus becomes greatly enlarged and watery and contains abnormal nucleoli which are also enlarged and occasionally possess indented outlines (Von Hosslin and Alzheimer 1912). These changes can be reproduced experimentally in the PCS rat (Cavanagh and Kyu 1971). "Alzheimer Type I" changes are also known to occur but much less frequently. This phenomenon is characterised by the presence of enlarged, deformed and often multilobular astrocytic nuclei which contain approximately twice the normal complement of DNA (Sherlock 1975).

The precise function of astrocytes has long been a subject of debate (Peters and Palay 1965). However, it is currently believed that they play a far more complex regulatory and supportive role in the maintenance of brain neurones than previously thought (Kimelberg and Norenberg 1989). The Alzheimer Type I and II changes may be related to the increase in permeability of the BBB during PSE (Sarna et al 1977, Laursen and Westergaard 1977) and this could possibly be responsible for the reported increases in transport of aromatic amino acids (AAA) across the BBB during PSE in rats (Bengtsson et al 1988b, Cardelli-Cangiano et al 1981). This increased transport of AAAs is thought to favour an increase in the formation of false neurotransmitters (see section 2.4.4.). Morphological changes have been reported in the brains of PCT rats, a model which retains liver blood flow and function in the presence of portal-systemic diversion (Doyle et al 1978). These were less prominent than those observed in PCS rat brains (Doyle et al 1978) where portal-systemic diversion was accompanied by reduced hepatocellular function (Ryan et al 1978a). These changes may therefore be more closely related to reduced hepatocellular function than to systemic diversion of portal blood. In addition, since the altered architecture of astrocytic cells may be

related to alterations in the permeability of the BBB, it may be that normal hepatocellular function is vital to the maintenance of the BBB.

2.4.4 Biochemical Alterations

Extrahepatic (portal-systemic) shunts are often formed during chronic forms of liver disease, such as cirrhosis, where portal blood bypasses the liver through large collateral vessels. Intrahepatic shunts, however, may also occur in cirrhosis by the formation of spontaneous portosystemic vascular shunts around the hepatic nodules. Portal blood, which may contain a variety of toxins originating from the gastrointestinal tract, will then enter the systemic circulation essentially unaltered and produce the profound effects described earlier: these are illustrated in figure 2.1. Consequently a variety of biochemical alterations have been reported and linked to altered behavioural patterns. These are discussed in the following sections.

Altered ammonia metabolism

It is hardly surprising that many of the earliest theories implicated gut ammonia as the main agent responsible for the precipitation of HE (Walker and Schenker 1970). Acute ammonia intoxication produces a mild encephalopathic preconvulsive state (Pappas et al 1982) similar to that observed during PSE in man (Sherlock et al 1954) or the 'meat intoxication' syndrome reported by Eck and Pavlov during their dog experiments. Dietary protein restriction has been shown to relieve the symptoms of chronic PSE in man and this has been attributed to a reduction in previously elevated concentrations of blood and CSF concentrations of ammonia and

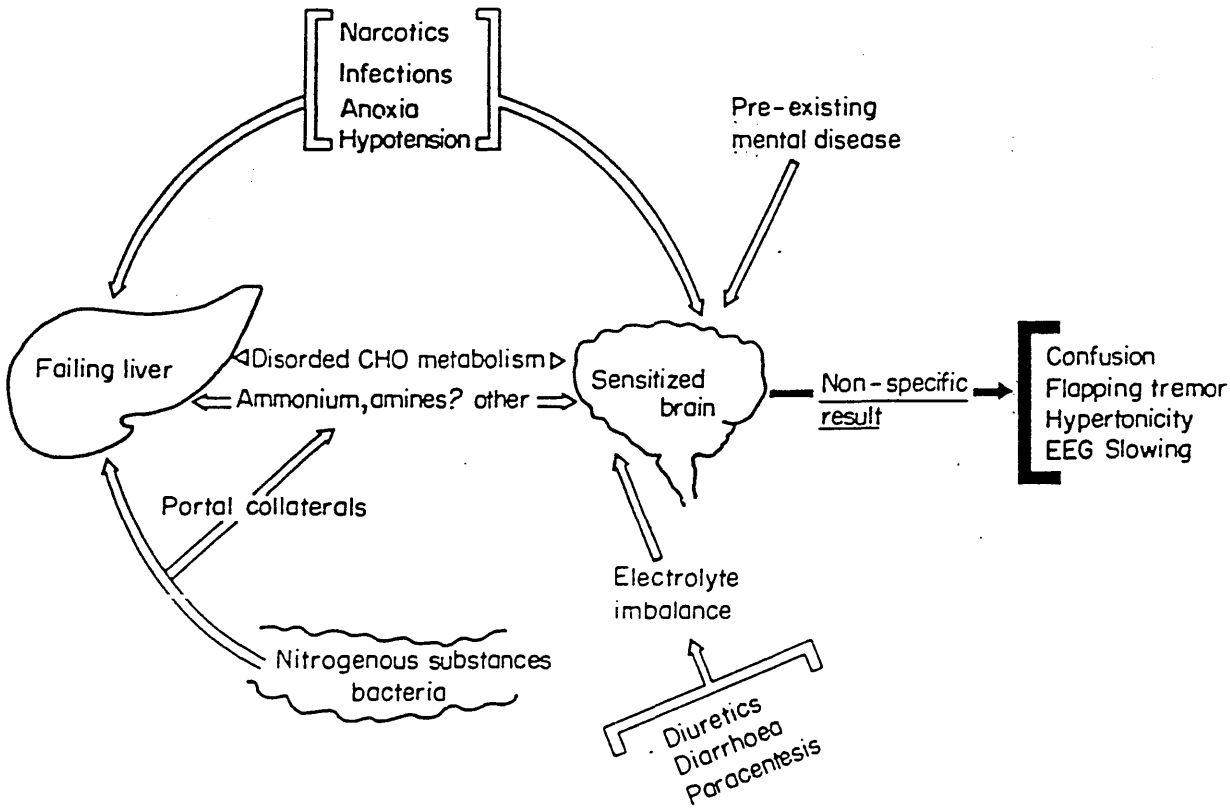


Figure 2.1. The pathogenesis of hepatic coma. Many factors affecting hepatic or cerebral function, or both, can lead to the picture of hepatic pre - coma in a patient with liver disease. The brain in such subjects may be particularly sensitive to these factors (from Sherlock 1975).

glutamine (Caesar 1962).

The primary source of ammonia production was originally thought to have been gut bacteria (Sherlock 1975) since oral broad spectrum antibiotics such as neomycin were also found to ameliorate the symptoms of HE in man (Dawson et al 1954). However PSE could still be produced in germ-free dogs with Eck fistulae (Nance et al 1971) and increased ammonia concentrations could be detected after a blood meal in both control and germ-free dogs (Sherlock 1975) and in germ-free PCS rats (Jeppsson et al 1979). In addition, the degree of coma in man with PSE or in PCS rats did not correlate with the concentrations of ammonia measured in CSF or blood (Fischer 1982). Also although ammonia produces a mild pre-convulsive state, the EEG patterns recorded are very different from those produced during chronic PSE in rabbits (Pappas et al 1982).

Blood ammonia levels reach an elevated steady state some two weeks after PCS in rats (Herz et al 1972, Kyu and Cavanagh 1970, Lee and Fischer 1961). This delayed hyperammonaemia has been attributed to reduced nutrition but a correlation between reduced food intake and delayed hyperammonaemia has not been demonstrated (Herz et al 1972). However, significant decreases in liver transamination enzymes have been shown to occur at similar postoperative time periods to the hyperammonaemia plateau and the hepatic parenchymal weight loss plateau (Colombo et al 1973). This implied that the delayed hyperammonaemia may be due to the active removal of circulating ammonia by the liver in the initial postoperative phase via the hepatic arterial supply before portal deprivation reduced the functional capacity of the liver (Lauterberg et al 1976).

Ammonia detoxification may also be achieved using other biochemical pathways. The hyperammonaemia observed in PCS rats has been shown to stimulate activity of the pyrimidine synthetic pathway (in other organs as well as the liver) due to elevated levels of carbamylphosphate, as a direct result of increased activity of aspartate transcarbamylase (Colombo et al 1977a). This hyperammonaemic plateau could have been due to stimulated activity of ammonia detoxication enzymes in organs other than the liver since these have been shown to be elevated following PCS in rats (Colombo et al 1977b).

Although these experiments question the direct involvement of intestinal bacterial ammonia in the precipitation of PSE, the role of ammonia may be indirect. Elevated ammonia from non-colonic sources was shown to enhance the transfer of phenylalanine, tyrosine and tryptophan across the BBB. Ammonia was also shown to increase the levels of brain indole amines in pigs following hepatic devascularisation (Denis et al 1983). Finally, Degos et al (1974) demonstrated few differences in EEG or survival times between experimental anhepatic and anhepatic eviscerated rats and concluded that intestinal compounds played only a minor role in the pathogenesis of PSE.

Amino acid imbalance

A consistent observation in patients and various experimental animals with chronic PSE has been the alterations in plasma and CSF concentrations of amino acids (Cascino et al 1978, Rosen et al 1977, Zanchin et al 1979). It has been postulated that an amino acid imbalance leads to increased entry of aromatic amino acids (AAA) into

the central nervous system (CNS) with a resultant accumulation of various false neurotransmitters (figure 2.2).

Much interest has centred upon the AAA precursors of the catechol and indole amines phenylalanine, tyrosine, methionine and tryptophan (Crossley et al 1983). These have all been shown to be elevated during chronic PSE in man but have also been accompanied by a corresponding decrease in the catechol amines, dopamine and noradrenaline (James et al 1982). These changes in indole amines and catechol amines have been demonstrated in PCS rats (Siemert et al 1978). In addition, a generalised increase in permeability of the blood brain barrier (BBB) to a range of molecules has been shown in PCS rats (Laursen and Westergaard 1977) although others failed to confirm this (Sarna et al 1977). This reported increase in BBB permeability could lead to an increased concentration of AAAs in the brain. However it is unclear if these elevations in aromatic amino acid concentrations in the brain are epiphenomena which occur after portal diversion or if they contribute to the precipitation of PSE. The raised concentrations of phenylalanine and tyrosine are thought to promote the synthesis of false neurotransmitters such as phenylethanolamine and octopamine (James et al 1979) (see fig 2.2). Injection of octopamine into the ventricles of rats produced characteristic decreases in brain dopamine and noradrenaline, associated with PSE, but failed to produce any disturbances in consciousness (Zieve and Olsen 1977). However, increased concentrations of the false neurotransmitter octopamine were reported in the plasma of patients with chronic PSE, although these did not necesssarily correlate with the severity of the symptoms (Bucci and Chiaverelli 1980).

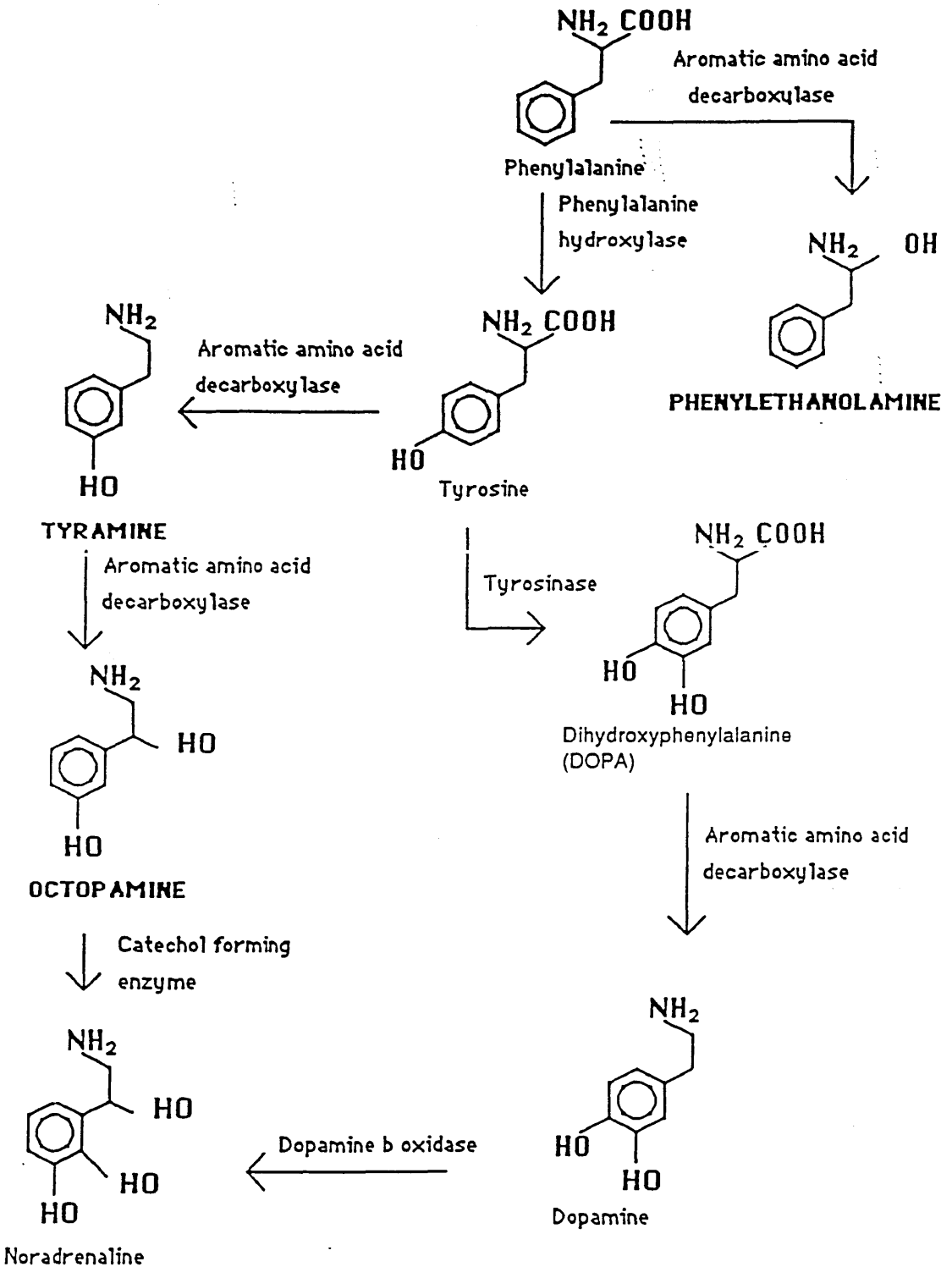


Figure 2.2. Catecholamine synthesis illustrating possible pathways for the generation of false neurotransmitters.

The so-called "unified hypothesis" proposed by Professor Fischer's group in Ohio (Fischer 1980, James et al 1979) states that the persistent hyperammonaemia observed during chronic PSE stimulates secretion of glucagon which promotes gluconeogenesis from amino acids and therefore proteolysis resulting in further ammonia production. The resultant hyperglycaemia stimulates insulin secretion, which promotes catabolism of BCAAs in skeletal muscle and fat, decreasing their concentration in the plasma and thereby inceases the AAA : BCAA ratio (see fig 2.3). The second effect of the hyperammonaemia is to increase the rate of entry of ammonia into the brain where it is rapidly detoxified to glutamine. Glutamine has a low but shared affinity for the AAA transport system across the BBB and it readily exchanges with tryptophan, tyrosine and methionine in the plasma, which favours entry of these amino acids into the brain and thus promotes a faster exit of glutamine (Fischer 1982).

There is little evidence to suggest that elevations in AAA may produce symptoms of PSE directly, but carotid artery infusions of L-tryptophan and phenylalanine have been shown to produce irreversible, coma-like states in dogs which could be prevented by simultaneous infusions of BCAAs (Rossi-Fanelli et al 1982). L-tryptophan has also been shown to modify sleep and pituitary prolactin secretion both of which are known to occur in Reye's syndrome, a paediatric illness characterised by hyperammonaemia, mitochondrial dysfunction, hepatic failure and hepatic encephalopathy (Payne 1982). The lack of overt behavioural disturbances associated with such high brain and plasma levels of L-tryptophan in PCS rats is difficult to explain (Zieve and Olsen 1977). It has been proposed that a synergism may exist between L-tryptophan and other AAAs which are present in raised concentration. High concentrations of

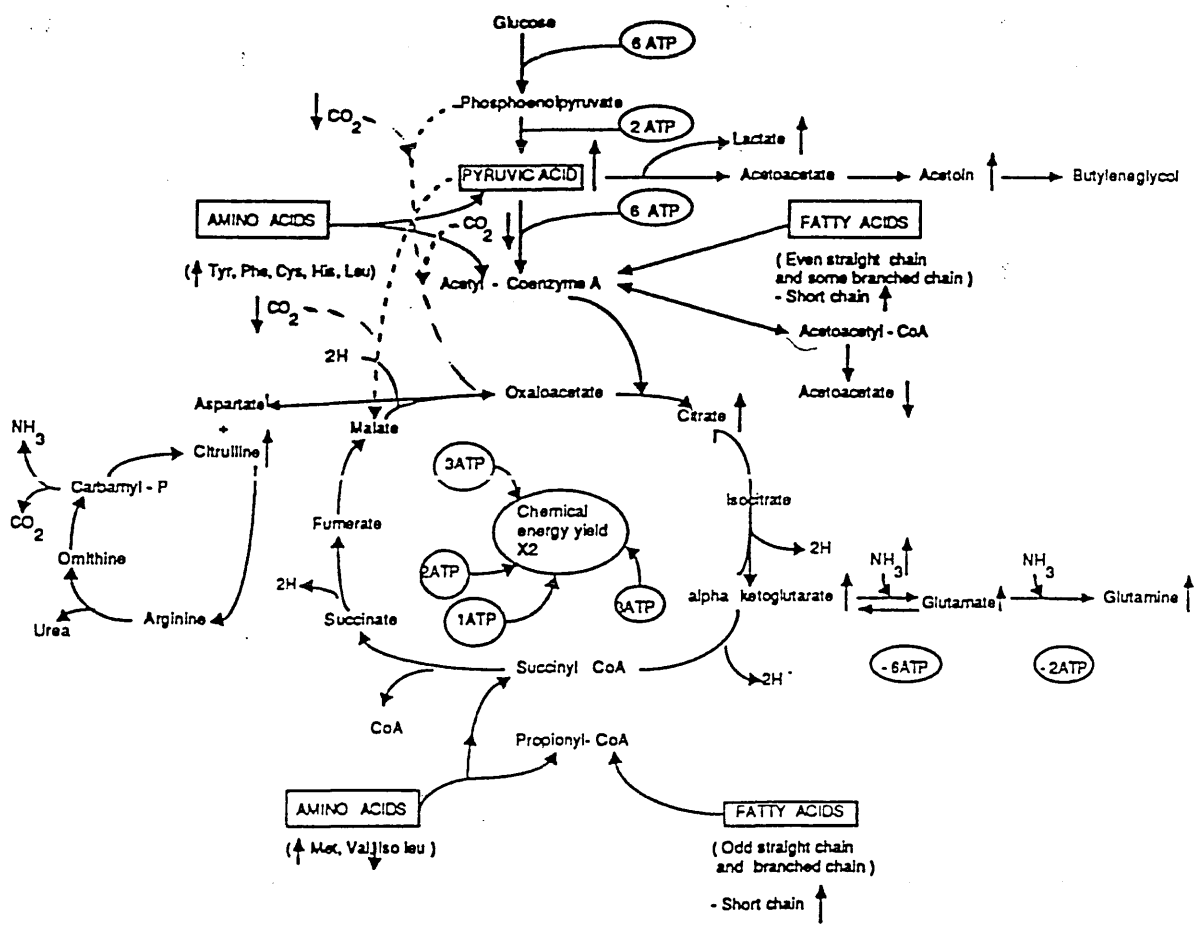


Figure 2.3. Zieve's original map of metabolic abnormalities in hepatic coma. The vertical arrows indicate abnormalities that have been detected in blood (from Sherlock 1975).

L-tryptophan may increase the rate of transport and entry of AAAs into the brain which, in turn, would promote synthesis of the false neurotransmitters octopamine and phenylethanolamine described earlier (Fischer 1982). The precise role of phenylethanolamine and octopamine in the precipitation of PSE, however, remains obscure (Crossley et al 1983, Payne 1982).

Deliberate attempts to manipulate the BCAA : AAA ratio in patients suffering from chronic PSE by infusion of BCAAs resulted in a regression of the symptoms of PSE in some studies (Fischer 1982), but this effect was not observed in other multicentre studies (Wahren et al 1983). Chronic alcoholics have elevated serum levels of BCAAs but this does not prevent patients from relapsing into deep coma. Decreases in BCAA and increases in AAA are not observed until patients have been abstinent for more than five days (Payne 1982). Recent studies, however, have shown that a BCAA enriched diet fed to PCS rats normalises CNS serotonin metabolism although behavioural abnormalities could not be totally reversed (Bengtsson et al 1988b). It is therefore difficult to ascribe the symptoms of PSE to elevated plasma concentrations of AAAs alone and the precise role of BCAAs in bringing relief from the syndrome of PSE is open to question. Differences between acute and chronic liver failure have been reported in the plasma amino acid profile. BCAAs are decreased during chronic liver failure leaving plasma concentrations of AAAs to predominate (Fischer 1982). During acute liver failure however, alterations in BCAAs are not observed which leaves the BCAA : AAA ratio largely unaffected and may suggest a different aetiology (Rosen et al 1977).

The use of L-dopa has been shown to alleviate the symptoms of PSE (Aor et al 1981, Loiuidice et al 1979). The rationale behind the use

of L-dopa has predominantly been the observed decreases in brain and CSF concentrations of noradrenaline and dopamine in experimental models and chronic PSE in man. It is thought that these decreases may occur as a consequence of the increase in concentrations of false neurotransmitters discussed earlier (Aor et al 1981, Fischer 1982). L-dopa had little effect upon increases in AAAs in PCS rat brains (Zanchin et al 1981) but effectively prevented elevations in 5-hydroxytryptamine (5HT) previously reported following PCS in rats. These increases in 5HT have been measured during clinical and experimental PSE, probably as a consequence of elevated plasma free tryptophan (Cummings et al 1976, Siemert et al 1978).

One criticism of the unified hypothesis is that the plasma BCAA : AAA ratio may be of little significance in the regulation of brain metabolism (Fischer 1982) since the brain is protected by a highly selective blood brain barrier (BBB) (Oldendorff 1977). However, non-specific increases in permeability of the BBB have been reported in PCS rats (Laursen and Westergaard 1977) and this could render the brain vulnerable to the entry of toxic molecules.

Role of gamma-aminobutyric acid (GABA)

GABA is a BCAA and is formed by the decarboxylation of glutamic acid. It is found in isolated regions in the nervous system and is believed to function as an inhibitory neurotransmitter. Attention has focussed upon the role of GABA as a causative agent for some of the manifestations of PSE. Coma-like states have been induced by minute (<1 μmole) quantities of GABA when injected microiontophoretically into the hippocampal region of the brain of rabbits (Smialowski 1978). This coma-like state was very similar to that produced in experimental models with fulminant hepatic failure and exhibited

inhibitory post-synaptic potentials often associated with HE and PSE (Schafer and Jones 1982a). In addition, a role for GABA was further suggested by increased sensitivity of patients with fulminant HE to benzodiazepines and barbiturates (which are known GABA agonists), possibly due to increases in the number of binding sites for these drugs and GABA during HE.

GABA has been shown to be produced by colonic bacteria and levels in portal blood are almost twice those in aortic blood. GABA from colonic sources is normally catabolised by GABA transaminase which has a high activity in the normal liver. Whether the increased plasma levels of GABA in hepatic failure are due to defective delivery of GABA to the liver or to impairment of hepatic catabolism of GABA remains to be answered (Schafer and Jones 1982b). However it is possible that GABA from sources other than the colon may precipitate PSE. Plasma GABA concentrations remained unaltered during oral administration of broad spectrum antibiotics to decrease gut flora (Schafer and Jones 1982a).

It is now believed that an endogenous CNS benzodiazepine ligand with properties of GABA-ergic transmission is present in encephalopathic animals and patients but may possibly be absent in normal animals (Mullen et al 1988). The amelioration of encephalopathy by benzodiazepine blockade using agents such as Flumazenil is thought to be mediated by antagonism of GABA receptors (Bansky et al 1989). In other studies where liver failure in rats was induced by galactosamine intoxication, no increases could be found in the permeability of the BBB nor was there any evidence to suggest an increased transport of GABA into the brain (Knudsen et al 1988). In addition, patients suffering from cirrhosis and diagnosed as having

PSE did not have elevated concentrations of plasma or cerebrospinal fluid GABA (Moroni et al 1987).

Clearly the precise role of GABA in the aetiology of PSE is still controversial. Experiments which revascularised the portal vein of PCS rats by microsurgery did not reduce plasma or brain GABA concentrations although the symptoms of PSE were reversed (Rigotti et al 1982). In addition, the release of GABA may be dependent upon or be modulated by other neurotransmitters. Waterhouse et al (1982) showed that the action of GABA in rat Purkinje neurons was enhanced by the specific beta adrenergic agonist isoproterenol. Also assessment of the coma that is produced by GABA injected microiontophoretically in rabbit ventricles has shown differences from the coma produced during PSE. GABA is a known inhibitory neurotransmitter and double inhibitory pathways in the CNS are extensive and propagate excitatory stimuli which are unlike those seen during hepatic coma (Roabe 1982). Finally elevated cerebral concentrations of ammonia reported during FHF have been shown to diminish the postsynaptic inhibition effected by GABA at the cellular level in the cat motor cortex (Roabe 1982).

Middle molecular weight compounds

The improvement in patients with fulminant hepatic failure after haemodialysis with polyacrylonitrile membranes has tended to suggest that substrates in the middle molecular weight range (500-5000) may be involved in the precipitation of HE and PSE (Crossley et al 1983, Denis et al 1983). Interestingly these same molecules appear in the plasma during liver failure regardless of the mechanism by which the liver has been damaged and are a common feature of liver failure in general (Leber et al 1980). It is believed that these molecules

inhibit the leucocyte Na+/K+ ATPase pump in cell membranes and perhaps could permit the entry of potential toxins into the brain that would have otherwise been removed by white blood cells (Leber et al 1980).

Glutamic acid depletion

Glutamic acid is found in higher concentrations in the brain than any other amino acid and is very important for the detoxification of ammonia (Waelsch 1951). Glutamic acid reacts with ammonia which it detoxifies to glutamine via the alpha-ketoglutarate-glutamate -glutamine transamination reaction through the tricarboxylic (Krebs) cycle (fig 2.3). Glutamic acid is a hyperpolarising excitatory amino acid whose levels have been shown to decrease in hepatectomised, comatose rats (Fischer and Baldessarini 1975) so that the predominantly inhibitory aspects of HE may be expressed through a decrease in excitatory neurotransmitter compounds. Furthermore increased concentrations of glutamine have been measured during chronic PSE in PCS rats (Williams et al 1972) and in the CSF of patients suffering from liver failure (Hourani et al 1971). The precise role played by glutamic acid during PSE is still unclear but recent evidence suggests that it is stored within astrocytes in the brain and it is believed that these cells are responsible for the detoxification of ammonia (Kimelberg and Norenberg 1989). Therefore any derangements in astrocytic function in PSE could readily increase the concentrations of ammonia in the brain.

2.5 SUMMARY

The precipitation of hepatic encephalopathy is difficult to ascribe to one single factor and the wealth of literature on the subject reflects the probable multifactorial nature of the disorder. Maintenance of homeostasis by the liver is partially dependent upon the removal of potential CNS toxins from portal blood possibly produced by gut bacteria. Experiments to remove these putative gut toxins were conducted using germ free Eck-fistula dogs (Nance et al 1971), PCS rats (Jeppsson et al 1979) and eviscerated rats (Degos et al 1974). The toxins produced by gut flora were virtually eliminated but the symptoms of PSE remained. These results therefore suggested that the liver may provide functions other than detoxification of portal blood to maintain homeostasis between the liver and the brain.

It remains difficult to identify these other functions in vivo due to interactions from other organs present in the body. Therefore this thesis adopted two approaches to unravel the problem: the first approach used a combined in vitro isolated rat liver and brain perfusion system in order to identify and isolate these functions, and the other related these results to an in vivo model of PSE, the PCS rat. The in vitro studies enabled the liver-brain interrelationship to be investigated in detail by the development of a new isolated organ perfusion system. The following chapter reviews isolated organ perfusion systems and discusses their relevance and merits.

CHAPTER 3

REVIEW OF ISOLATED ORGAN PERFUSION SYSTEMS

"The method of examining the physiological action of an organ or tissue by perfusing it by blood after its removal from the body has already proved of great value in many instances, and under proper conditions can solve many physiological problems"

T. G. Brodie, 1903

3.1 HISTORY OF ISOLATED ORGAN PERFUSION

One of the earliest attempts to investigate the metabolic properties of an organ by ex vivo perfusion was Loebell's perfusion of the isolated pig kidney (1849). However, this report provided few technical details but was probably responsible for the initiation of many future experimental perfusions. A wealth of literature now exists upon isolated organ perfusion preparations and this review will present those major contributions which have led to the establishment of reliable and reproducible preparations in use today. Most of this review will concentrate upon the isolated perfused rat liver (IPRL) and brain (IPRB) since these are most relevant to this study. However, many organs other than the liver and brain have been successfully perfused inlcluding the kidney, heart, stomach, duodenum, biliary tree, small bowel with ileo-caecal junction, colon, adrenal gland, bone, uterus and ovary. These are discussed in detail by Ritchie and Hardcastle (1973).

3.1.1 Tentative beginnings

Nineteen years after Loebell had demonstrated perfusion of the isolated kidney a technique for the perfusion of the skeletal muscle of the dog was reported. The muscle retained activity through the artificial circulation of fresh oxygenated blood (Ludwig and Schmidt, 1868) and these studies suggested that analysis of chemical changes in the blood, tissue and oxygenation mixture could yield significant information about the metabolic status of the isolated perfused organ. The observation that constant gassing of the perfusate could contribute significantly to the survival of the perfused organ was extended further by Asp (1873). He described one of the earliest

preparations for the isolated perfusion of the rabbit liver with dog blood, in which an extra circuit, in parallel, was added for the perfusion of an animal lung in order to improve oxygenation.

Skutul (1908) published a review which delineated the major contributions to the development of isolated organ techniques up to that time. The significance of temperature control, pulsatile and non-pulsatile flow, methods of oxygenation (to avoid direct mixing of gas with the perfusion medium) and measurement of perfusion pressure were appreciated but technological limitations restricted further development. Brodie (1903) described an apparatus (figure 3.1) which attempted to optimise many of the features thought necessary for the successful perfusion of isolated organs at that time. Unfortunately, the blood underwent considerable trauma upon direct contact with foreign materials such as wood, leather and metal. However, Brodie commented upon the greater degree of successful perfusion if autologous blood, rather than heterologous, was used as the perfusate.

3.1.2 Establishment of basic principles

The main objectives of isolated organ perfusion have changed little since those originally proposed by Baglioni (1910). He suggested that the three main objectives were

1) to isolate individual organs from the whole animal so that the organ might be studied in isolation, uninfluenced by others.

2) to enable the tissue or organ to survive the operative and perfusion procedure and exhibit some measureable parameter of survival; and

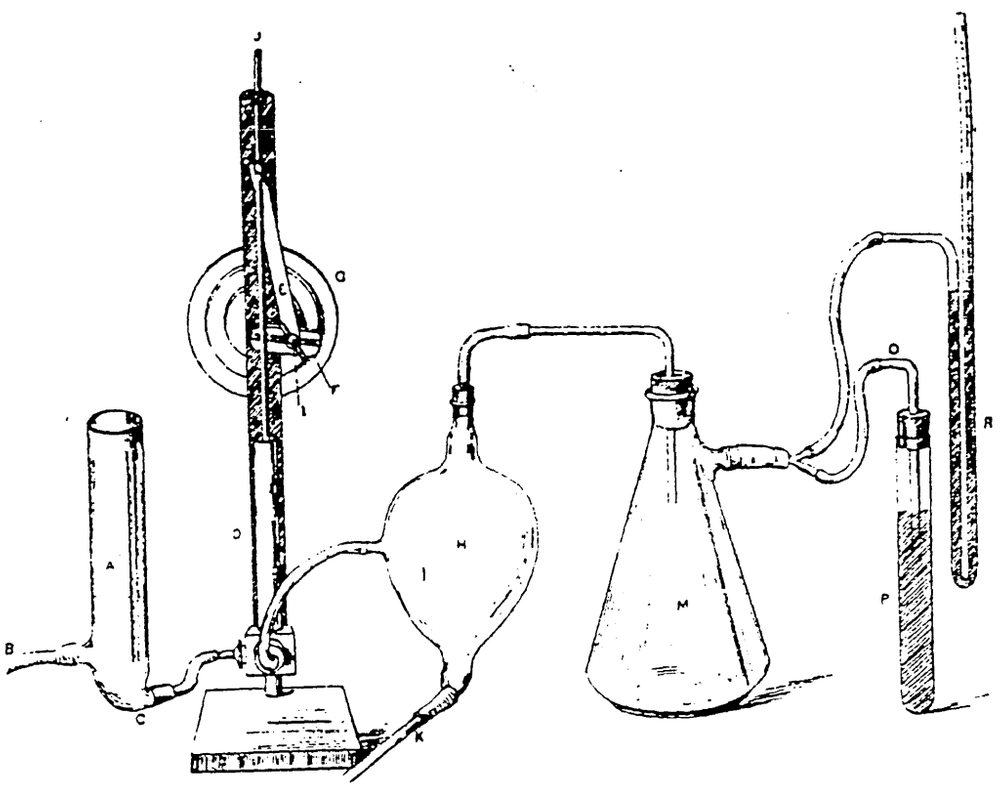


Figure 3.1. Illustration of Brodie's original equipment for the in vitro perfusion of isolated organs. Note the use of rubber tubing at points 'K' and 'B' for the transmission of perfusate to the cannulated organ and the inclusion of a manometer at point 'R' (Brodie 1903).

3) to attempt to imitate the natural circulation and maintain physiological conditions in the perfusion medium in order to prevent trauma and thereby enable the organ to survive for as long as possible in vitro.

In addition, Baglioni postulated that the composition of the perfusion medium would vary as perfusion progressed and a study of the perfusate could reflect the metabolic status of the perfused organ. Baglioni also suggested that the changes the organ underwent may have been subtle and that they could require amplification by the application of electrical, chemical (including noxious stimuli) or heat stimuli. It was also important that the organ be perfused via the arteries, or portal vein in the case of the liver, in a manner comparable to the in vivo situation. Occasionally, however, retrograde perfusion has been carried out, for example in isolated rat liver (Trowell 1942), rat diaphram (Brownlee and Straughan 1957) and rat adrenal gland (Hechter et al 1953), but with limited success.

Selection of cannulation sites was primarily determined by the anatomy of the afferent vasculature. In addition, the purpose of each investigation may influence selection of a particular perfusion technique. For example, two preparations were reported for the perfusion of the isolated rat brain, originally described by Andjus et al (1967). The simpler and speedier technique of Thompson et al (1968) provided a convenient method for the isolation of the rat head, in which survival was assessed by the continuous electroencephalogram (EEG). This preparation provided easier arterial cannulation, was less traumatic and produced more physiological flow distribution than other brain perfusion preparations, but specific metabolic uptake studies upon the brain could not be carried out because of the

inclusion of perfused tissues outside the central nervous system.

Nowadays the isolated rat liver preparation is almost exclusively perfused via the portal vein. Ligation of the hepatic artery has been shown not to exert any serious deleterious effects upon hepatic function (Ross 1972).

3.2 APPLICATIONS AND OBJECTIVES OF ISOLATED ORGAN PERFUSION CIRCUITS

The primary application for isolation and perfusion of organs in vitro was to increase knowledge of the physiology or pathophysiology of an organ (Ritchie and Hardcastle, 1973). However organs have also been isolated and perfused for transplantation in conjunction with cryopreservation techniques (Kamada et al 1980, Rijkmans et al 1984, Skibba et al 1983). Organs have also been preserved for toxicity testing of food additives, fertilisers and pesticides (Garattini et al 1973). In addition, techniques of isolated liver perfusion have been used for the harvest of viable, isolated hepatocytes for in vitro investigations such as hepatic drug metabolism experiments (Berry and Friend 1969, Seglen 1976). More recently the IPRL has been used as an investigative tool for mechanisms which control vascular tone. The presence and characterisation of adenosine A_2 purinoceptors in the hepatic vasculature was demonstrated (Buxton 1988, Buxton et al 1987).

3.2.1 Advantages of isolated organ perfusion

The technique of isolated organ perfusion offers the biochemist, physiologist and pharmacologist an opportunity to study the effects of different substrate concentrations upon metabolic rates. Another major advantage of isolated organ perfusion is the opportunity to study highly organ-specific phenomena. For example, the transport of glucose into the brain could perhaps be facilitated by insulin. Clearly this would be difficult to demonstrate in the intact animal. However, the isolated perfused rat brain perfusions of Sloviter and

Kamimoto (1967), in which an artificial perfusate was used, demonstrated that insulin had no effect upon the uptake of glucose by the brain.

The other major advantage of isolated organ perfusion procedures is that individual manipulations of blood flow, gaseous exchange (by alteration of blood gases) and temperature are all possible. This enables accurate quantification to be carried out under various reproducible, controlled conditions, some of which may mimic pathological conditions such as ischaemic heart disease (Opie 1965). In this particular example, the effects of reduced blood flow to the heart are readily separable from those of anoxia and acidosis, conditions difficult to achieve in the intact animal. Finally, the influences of neural control are also removed in the in vitro preparation.

All these investigations, however, can only contribute valid information if the morphology and function of the perfused organ has not been adversely affected by the isolation and perfusion procedure. One particularly rigorous method of examining the phenomenon is the ability of the perfused organ to function following transplantation. This was demonstrated successfully with kidney transplantations in dogs following a storage period of 3 days with extracorporeal perfusion (Belzer et al 1982, Rijkmans et al 1984). This was also demonstrated by autotransplantation of the liver in dogs following extracorporeal perfusion for one hour (Skibba et al 1983). Finally, Kamada et al (1980) in Cambridge successfully transplanted livers in rats after 25 hours continuous perfusion with a flourocarbon emulsion.

3.2.2 Isolated liver perfusion

The technique of isolated liver perfusion has been used for experimental purposes for over 300 years. One of the earliest reports of an isolated liver perfusion was provided by Glisson (1665) who perfused cadaveric livers to examine the blood flow patterns and physiological function of the liver. The first true isolated liver perfusion was attributed to Claude Bernard (1855) who investigated the conversion of glycogen to glucose in an isolated rabbit liver perfused with defibrinated dog blood. Later, Embden and Baldes (1913) reported a preparation for the investigation of amino acid, keto acid and lactate metabolism using an isolated dog liver perfused with defibrinated cow's blood.

The 1930's saw the dawn of the modern era of biochemistry with the establishment of enzymes as molecular protein entities. Intensive studies were initiated into carbohydrate metabolism and the isolated perfused cat or dog liver proved particularly useful in this context (Lundsgaard et al 1936). Between 1930 and 1950 the continued use of isolated liver perfusion preparations for this purpose lessened primarily due to the widespread acceptance of Warburg tissue slice and homogenate techniques (Miller 1973).

Isolated perfusion of the liver of the rat is thought to have originated with the English physiologist Dr O Trowell in 1942 who reported a technique for retrograde perfusion via the vena cava. The perfusate was composed of Krebs-Henseleit buffered saline and was used to demonstrate the synthesis of urea from ammonia, amino acids and carbohydrate intermediates of the Krebs cycle. The preparation was thought to be superior to liver slices since it remained functional for a number of hours.

A reliable and reproducible isolated perfused liver preparation upon which many are based today was reported by Miller et al (1951). These workers investigated the role of the liver in plasma protein synthesis by incorporation of ^{14}C labelled lysine. Concurrently, Brauer et al (1951) also used an isolated perfused rat liver preparation for detailed studies of bile production, blood flow and oxygen consumption and were able to demonstrate that hepatic artery perfusion was not essential since the liver perfused via the portal vein remained functional for many hours and produced bile throughout the perfusion period. Further refinements were made to the technique of IRLP and later Brauer et al (1953) established pressure-flow relationships and reported the presence of a 'hepatic vasoconstrictor' substance in fresh heparinised blood which reduced the activity of the IRLP preparation. He also demonstrated that perfusate flow through the isolated liver was almost 5 times greater than in vivo, probably due to a pronounced vasodilatation. Investigations of this nature established the rat as a popular species for isolated liver perfusion since it offered many advantages over larger animals:

1) Rats were more economical to use than larger animals.

2) The operative technique in general was less complex.

3) A larger amount of biochemical data was available.

4) Inbred strains were readily available and reduced genetic variations in anatomy and physiology.

5) The rat liver was divided into discrete lobes which could, if required, be individually ligated for excision of tissue during an experiment without excessive blood loss.

6) Use of small volumes of plasma reduced the volume and cost of isotopically labelled compounds.

The 1960's saw the widespread application of IRLP preparations. Brauer et al (1953), as mentioned earlier, highlighted the significance of surgical trauma, perfusate pH, perfusate oxygen tension and haematocrit upon the vasomotor activity of the IRLP. He reported later that the hepatic oxygen uptake (HOU) of the IRLP was reduced if low perfusate haematocrits were used (Brauer 1963). However other IRLP studies established that whole blood was not necessary for the support of an organ in vitro for periods of up to 4 hours (Hems et al 1966). Dilution of erythrocytes with physiological saline was found very satisfactory. Moreover, the source of erythrocytes was also thought to be of little consequence provided the cells were thoroughly washed prior to use (Ross 1972). This led to the almost universal use of washed erythrocytes from a wide variety of sources in perfusates (see chapter 6).

The significance of perfusate haematocrit has been investigated and shown to influence bile volume production in addition to HOU (Keiding et al 1980). An optimum haematocrit of 20% has been proposed, since this would provide the required oxygen carrying capacity but also avoid the problems of increased perfusion pressures and reduced flow rates due to increased viscosity (Hems et al 1966). Reidel pointed out that while oxygen uptake is increased at higher perfusate haematocrits in the IPRL, the exponential decrease in flow rate (or increase in perfusion pressure in a fixed flow preparation) is detrimental to the viability of the liver and thus a compromise has to be achieved (Riedel et al 1983).

Schmucker et al (1975) published a technique for perfusion of the isolated rat liver using a haemoglobin-free perfusate and stated that various liver functions, such as bile production, lasted for perfusion periods of up to 5 hours. However, very high flow rates were necessary to avoid tissue hypoxia and this may have disrupted the hepatocellular architecture. In addition, reduced volumes of bile were produced when compared to erythrocyte-containing perfusates, and the quality of the bile produced (in terms of bilirubin concentration) was not discussed.

Since the completion of this work, an excellent review by Gores et al (1986) has confirmed that measurements of bile volume production, HOU and pH provided an adequate global assessment of liver viability. More recently Reichen (1988) reported a dual-perfused model of the IPRL, perfused through the hepatic artery and portal vein. His studies suggested that biliary uptake of a indicator dye injected into the portal vein was faster than when injected into the hepatic artery: this suggested that dual-perfusion promoted a faster rate of bile volume production. Unfortunately his model had a failure rate of at least 40% predominantly due to variations in the hepatic arterial vasculature.

At the onset of these studies no systematic evaluations had been carried out on either the influence of the different perfusate haematocrits or of different sources of erythrocytes. These two fundamental aspects of IRLP were therefore investigated during this study (chapter 6).

3.2.3 Isolated brain perfusion

Initial investigations which explored the feasibility of brain perfusion were thwarted through problems of isolation of the venous drainage of the blood. This meant that metabolic investigations such as oxygen uptake studies could not be carried out because of the inclusion of perfused tissues outside the CNS. In addition, blood entering the CNS could be diverted away from the carotid vasculature and retrogradely down the vertebral arterial system with a subsequent loss of perfusion to the brain. Also venous blood from the brain, normally entering the jugular circulation, may enter the sinusii columnae vertebralis (Chute and Smyth 1939), which could deliver the entire venous return from the brain (Hill 1896). These routes therefore had to be isolated and occluded in order to ensure that the entire supply of perfusate could reach the brain and that CNS venous blood could be collected without contamination from other tissues.

Chute and Smyth (1939) were probably the first to measure the oxygen consumption of the cat brain in vivo, by means of a successful perfusion technique. They suggested that the lower values obtained in vitro from tissue slices or chopped brain segments were probably a result of trauma from the extraction procedure and that the in vivo values which they measured were closer to the true value. Geiger and Magnes (1947) were first to isolate the cerebral circulation of the cat and were able to carry out the first accurate brain metabolism studies. They used defibrinated ox blood as the perfusate and were able to maintain brain activity for up to 2 hours as measured by various physiological assessments such as corneal reflex. Geiger demonstrated a decreased brain oxygen consumption in the presence of

barbiturate and showed that activity was prolonged if the perfusate was first passed through a liver to remove various 'vasoconstrictor substances'. The technique was refined further by the incorporation of a heparinised perfusate containing well washed bovine erythrocytes suspended in Krebs-Henseleit buffer (Geiger et al 1954). Geiger demonstrated that the rate of glucose entry into the brain was dependent upon the presence of a liver in the circuit and that electrocortical activity, as measured by the electrocorticogram (ECoG), brain glucose utilisation and corneal reflexes were stable for up to 2 hours. Later he concluded that the addition of cytidine or uridine in the perfusate could mimic these actions and might be responsible for the prolongation of brain activity (Geiger and Yamasaki 1956).

Gilboe et al (1964) were probably first to report a true isolated brain perfusion technique. This was carried out in dogs and the brain remained viable for up to 90 minutes as verified by ECoG activity. They demonstrated that ECoG activity was the most sensitive index of brain function, decaying more rapidly than other physiological parameters used for brain viability assessments such as corneal reflex.

One of the major contributions towards simplification of isolated brain perfusion was described by Andjus et al (1967). He described a technique for total isolation of the rat brain and reported perfusion survival times in excess of 2 hours as measured by EEG activity.

Zivin and Snarr (1972) also reported a stable preparation for the isolated perfusion of the rat brain. In these studies the rats were anaesthetised by hypothermia since this was believed to prolong activity and produce survival times of up to 5 hours as verified by

ECoG activity. They demonstrated that a complex relationship existed between glucose uptake and perfusion flow rate through the brain.

Isolated brain perfusions have also been used for investigations of drugs believed to act upon cerebral metabolism (Krieglstein et al 1980). Krieglstein investigated the action of the tranquilliser chlorpromazine and its active metabolite 7-hydroxychlorpromazine upon EEG activity in the isolated perfused rat brain. He showed that 7-hydroxychlorpromazine had less effect upon EEG activity than its parent compound although both drugs elevated striatal concentrations of homovanillic acid to similar levels.

For the purposes of this investigation, a less complex and time consuming preparation was required if dual organ perfusion were to be carried out as described earlier. The simpler and faster isolated perfused rat head preparation reported by Thompson et al (1968) appeared to be a useful model for these investigations: figure 3.2 illustrates the cannulation procedure. This model was developed further during this study and the results are presented in chapters 7 and 8.

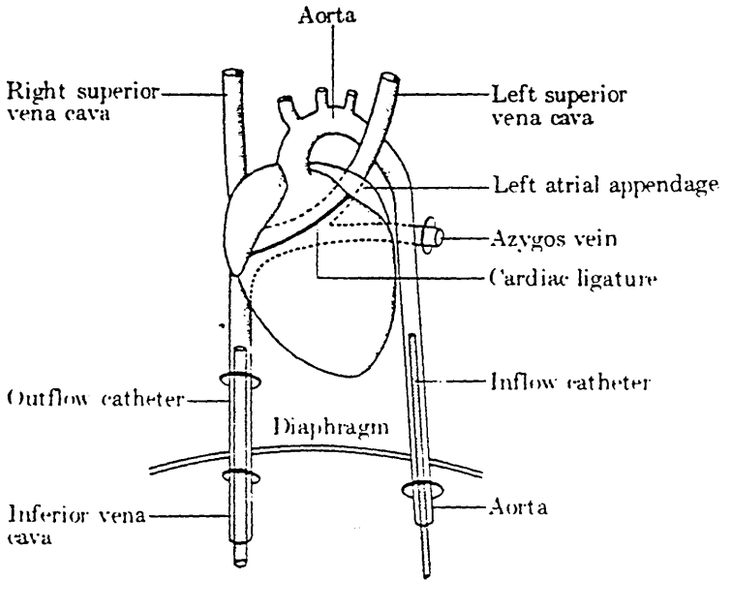


Figure 3.2. Diagrammatic representation of the cannulation sites used for the perfusion of the isolated rat brain prepared according to the technique of Thompson et al (1968).

3.3 COMPONENTS OF AN ISOLATED ORGAN PERFUSION CIRCUIT

The fundamental working principles and designs of many recent perfusion circuits have altered little from those described originally by Brodie (1903). His system was designed for the perfusion of a wide variety of organs including the heart, liver, kidney, spleen and lungs. The majority of this section will be focussed upon systems which have been designed for application to small animals, particularly the rat.

3.3.1 Features of an ideal system

The ideal perfusion system should permit accurate control of blood flow, gaseous exchange and perfusate temperature to the perfused organ. This will allow individual manipulation of many parameters and the influence of controlled blood flow, perfusion pressure, anoxia, hypothermia, changes in blood pH, effect of nerve stimulation or drug action can be accurately assessed (Ritchie and Hardcastle 1973).

3.3.2 Circuit design

A variety of technological improvements after Loebell (1849) published a report on perfusion of the isolated pig kidney enabled Langendorff to produce and publish in detail his celebrated paper in 1895 upon the isolated perfusion of the heart (Langendorff 1895). The circuit was sophisticated for its time and included electromagnetically controlled perfusate delivery to the heart (from a perfusate reservoir) and accurate temperature control of the organ and perfusion media by means of a heated water bath (figure 3.3). Many isolated heart preparations utilised today are still based upon the

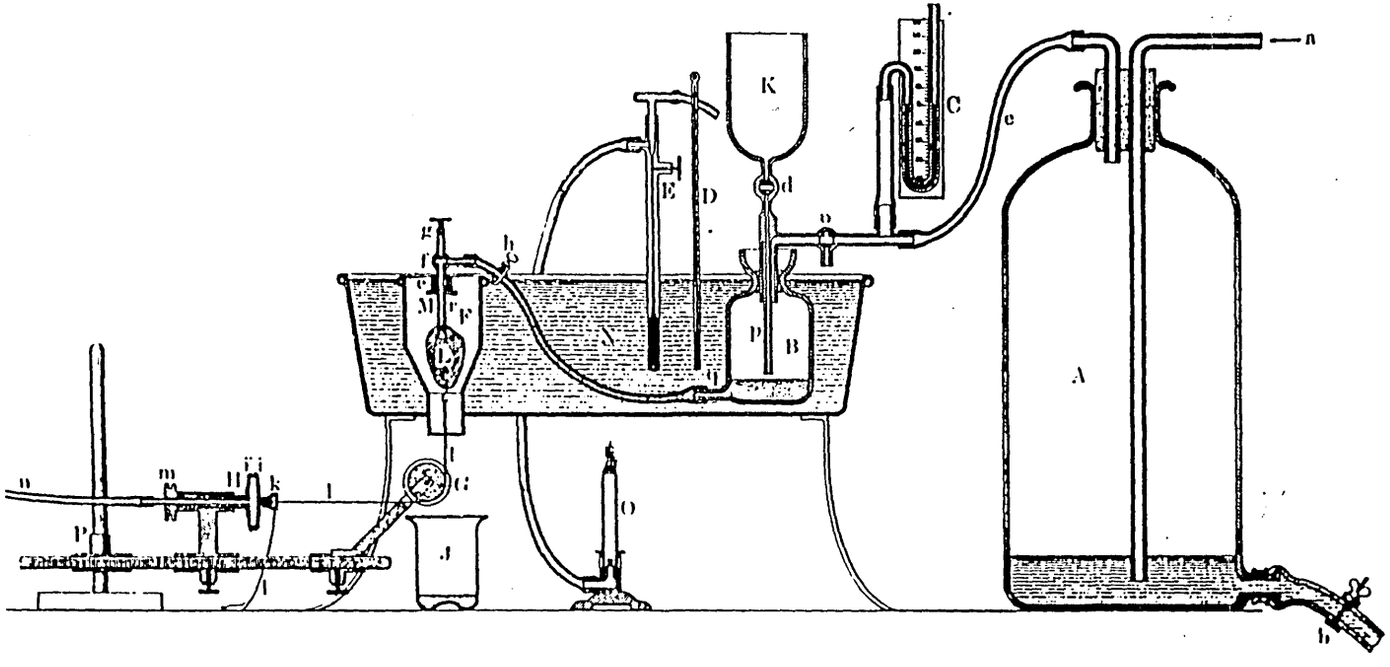


Figure 3.3. A reproduction from an engraving of the original apparatus used by Langendorff for in vitro perfusion of the isolated heart circa. 1895.

original Langendorff technique. The circuits described by Brodie (1903), Langendorff (1895) and Loebell (1849) established many of the fundamental principles upon which the modern foundations of isolated organ perfusion are based.

All isolated organ perfusion systems are essentially either single pass, flow-through designs or allow recycling of fluid. Figure 3.4 is an illustration of these two modes of perfusion as applied to the Langendorff isolated heart perfusion technique. In general, open circuit configurations are principally employed for drug or specific metabolic studies. Further discussion in this section refers to the closed circuit systems more commonly used for pathophysiological investigations. Figure 3.5 is a diagram of such a circuit as used by Miller et al (1951) for perfusion of the isolated rat liver. Perfusate flow to the organ is delivered by gravitational feed from a reservoir, and perfusion pressure is regulated by altering the height of the reservoir. This simple circuit was adapted for the perfusion of the isolated rat heart (Neeley et al 1967, Chain et al 1969) and rat kidney (Bauman et al 1963).

Miller's apparatus presents problems if dual organ perfusions are to be carried out because of the large priming volume required. The temperature-regulated cabinet restricted excessive heat losses but also hindered ready access to the system for collection of samples or for minor adjustments to the circuit. The complicated valve pumping system could also be avoided with the use of modern roller pumps and thus avoid problems with cleaning the apparatus. The problems of the falling film oxygenator used are considered in the next section. The circuit used by Morgan et al (1961) for the Langendorff preparation (figure 3.4) avoided some of these problems by use of a simplified

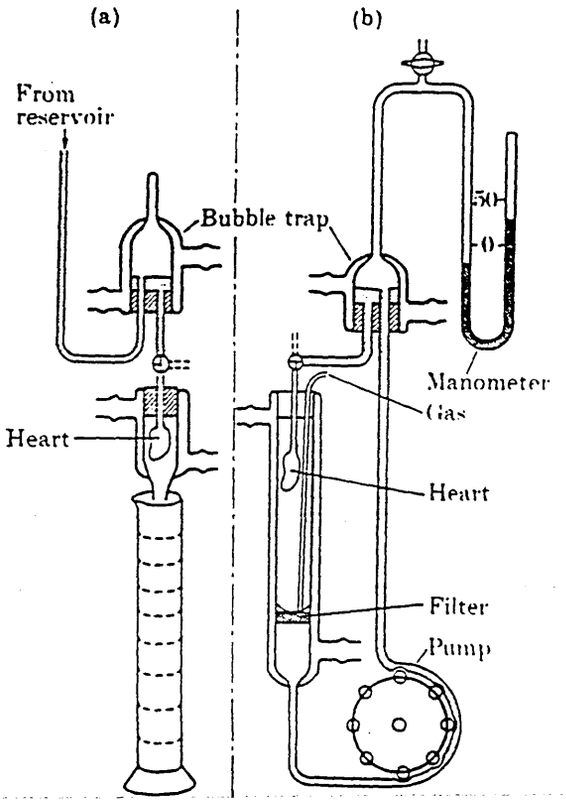


Figure 3.4. Apparatus for "Langendorff " perfusion of the heart according to the method of Morgan et al (1961). (a) Once through perfusion. Medium enters the aorta after passing from a reservoir circa 100 cm above a bubble trap. Outflowing medium is collected in a measuring cylinder. (b) Recirculation perfusion. In a similar circuit, a pressure of 150mmHg is provided by a roller pump. Pressure is recorded by a side - arm manometer and medium recirculates from a reservoir below the heart (from Ross 1972).

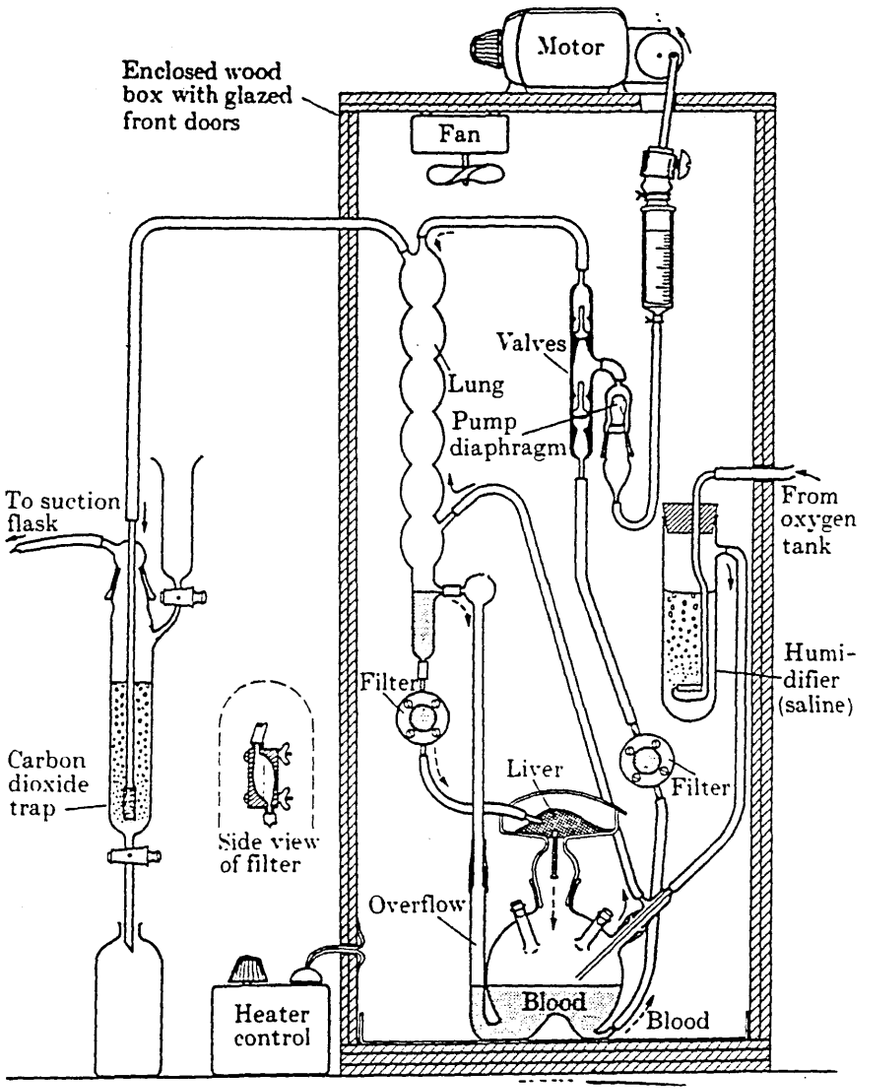


Figure 3.5. Liver perfusion apparatus developed by Miller et al (1951).

circuit and roller pump.

Andjus' circuit for the isolated perfusion of the rat brain (figure 3.6) was probably the closest to a true miniaturised circuit for the isolated brain perfusion. But according to his paper, a provision was not made to restrict heat loss nor was there any description of a heat source. In addition, disposable blood administration drip sets as oxygenators, although economical and convenient, are subject to the trauma of bubble oxygenation (see section 3.4). However, the fairly simple design of the circuit, coupled with the comparatively small priming volume, did make dual organ perfusion feasible provided that the oxygenator and heat loss characteristics could be improved.

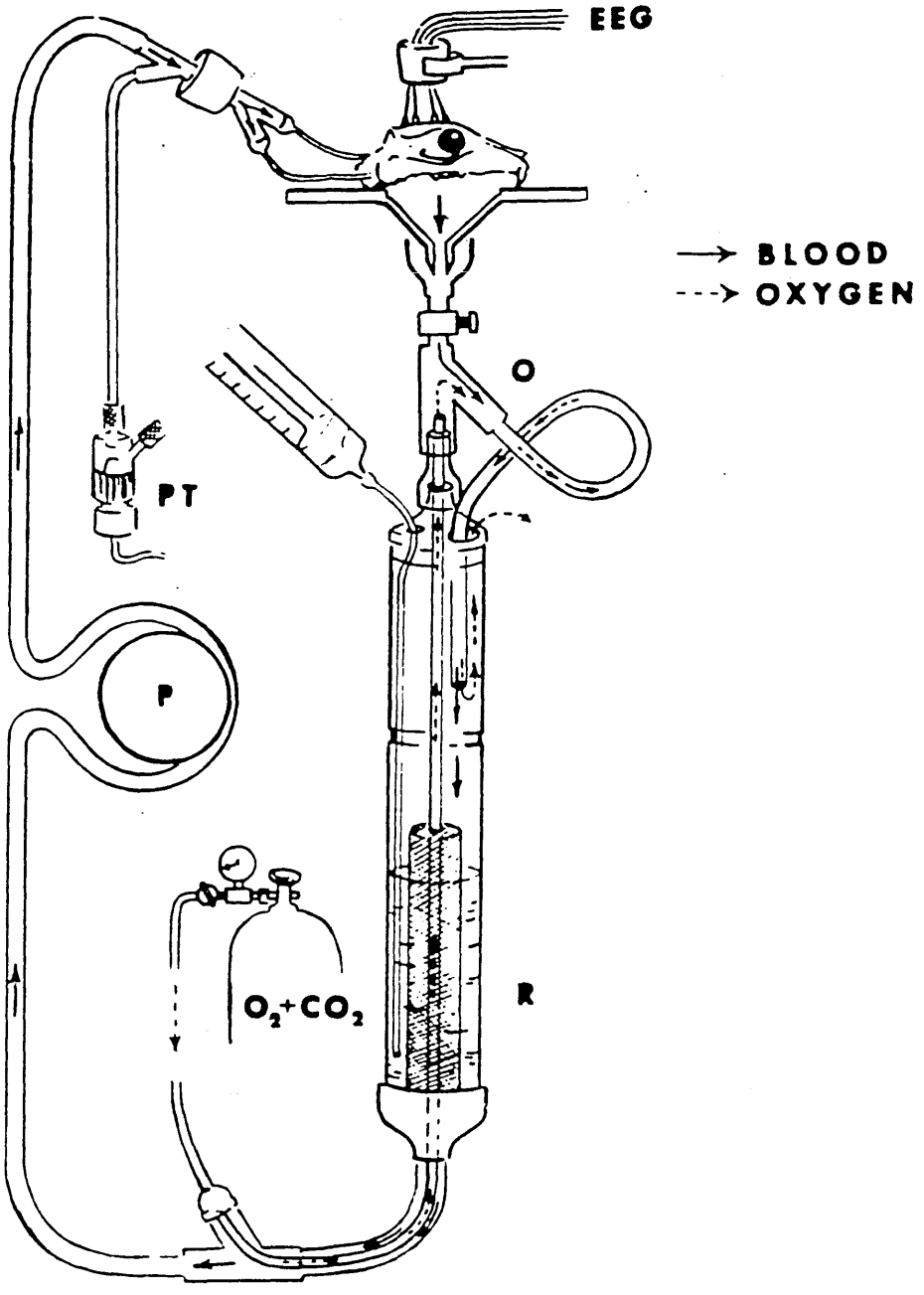


Figure 3.6. Schematic representation of the miniaturised circuit developed by Andjus et al (1967) for the perfusion of the isolated rat brain. Venous blood from the brain enters the perfusion circuit via a funnel, through a side branch of the bubble oxygenator (O) made from a modified blood administration set. The blood then enters a reservoir (R) where it is picked up by the perfusion pump (P) and delivered to the brain at a fixed perfusion pressure, monitored by a transducer at point (PT).

3.4 OXYGENATORS USED IN ISOLATED ORGAN PERFUSION CIRCUITS

The maintenance of a viable isolated, perfused organ is dependent upon efficient delivery and exchange of blood gases with the tissues. Adequate control of perfusate gas tensions is an essential feature of all isolated organ studies and is achieved by incorporation of artificial oxygenators into the circuit.

3.4.1 Properties of an ideal oxygenator

The ideal oxygenator is one which mimics the action of the natural lung. This ideal oxygenator should be atraumatic to the constituents of the perfusion medium, have a small prime volume (and therefore demand a highly efficient gas exchange rate) and be simple in design to facilitate cleaning. At present there are two basic categories of oxygenators: "open" system and "closed" system oxygenators. Open system oxygenators include bubble , falling column and rotating chamber oxygenators. Closed system oxygenators encompass the complete range of membrane oxygenators.

3.4.2 Open system oxygenators

The simplest type of open system oxygenator was the bubble oxygenator, which involved the bubbling of gas through a reservoir of perfusate. This presented complications due to foaming and haemolysis if blood was used.

Miniaturised bubble oxygenators constructed for cardiopulmonary bypass (CPB) in small laboratory animals, as well as for isolated organ perfusion (Proctor and Fernando 1973), were traumatic and caused

damage to erythrocytes (Galletti 1971, Siderys 1975), low density lipoproteins (Belzer et al 1968, Zapol et al 1969) and reticuloendothelial function as manifested by a reduced Kupffer cell phagocytic activity in rats and reduced blood clearance of cholesterol (Neveu et al 1956, Subramanian et al 1965).

Multibulb glass oxygenators, introduced by Miller in 1951, remain one of the most widely used designs of oxygenator for isolated organ perfusion. Two disadvantages of this system were the large prime volume demanded and the flow rate restrictions imposed by the rate of oxygenation.

The rotating chamber oxygenator or roller oxygenator was introduced for small organ perfusion by Mortimore et al (1959) and was used for the perfusion of cat spleen, liver and large intestine (Blakeley and Brown 1963, Powis 1970). The large prime volume and inclusion of an extra mechanical component restricted its widespread use.

3.4.3 Closed system oxygenators

These are membrane oxygenators in which the liquid phase is separated from the gaseous phase by a highly permeable membrane. Early investigations demonstrated that materials such as polyethylene were relatively impermeable to carbon dioxide and therefore unsuitable for this role (Melrose et al 1955). However, the high gas transfer characteristics and biocompatibility of other synthetic membranes such as polydimethylsiloxane (PDS) and teflon microporous membranes has led to their incorporation in many membrane oxygenators that are used today (Bartlett et al 1974, Hamilton et al 1974, Tanishita et al 1975).

3.4.4 Advantages of membrane oxygenators

The development of membrane oxygenators was stimulated by the considerable trauma which blood underwent on transit through a bubble oxygenator, and the adoption of membrane oxygenators has allowed extended periods of extracorporeal perfusion in man (Carlson et al 1972, Heimbecker 1977, Liddicoat et al 1975, Pierce 1974). Comparative investigations of bubble versus membrane oxygenators have not disclosed any definitive advantages of either design. However a reduction in haemolysis has been attributed to the use of membrane oxygenators during prolonged CPB in sheep (Fleming 1977). Initial investigations showed that plasma haemoglobin concentrations were lower during perfusion with membrane oxygenators (Fleming et al 1979). It was demonstrated that membrane oxygenators retained higher platelet numbers and function during prolonged CPB in man (Van den Dungen et al 1982, Boers et al 1983).

3.4.5 Membrane oxygenators used in isolated organ perfusion circuits

Mainly flat sheet or tubing membrane is used in oxygenators for isolated organ perfusion. One of the earliest designs of membrane tubing oxygenators specifically adapted for the guinea-pig and rat heart was described by Roskenbleck et al (1967). A flat sheet of cuprophane originally designed as a medium for exchange dialysis was used, but the device was limited by the complexity of the design and the large prime volume. Another design of flat sheet membrane oxygenator constructed from PDS (Windmueller and Spaeth 1972) was again complex and had a large surface area of $250cm^2$.

A spiral coiled flat sheet membrane oxygenator was utilised for isolated perfusion of the rat liver but this had a large surface area of 200cm^2 and probably a large prime volume (Collins and Skibba 1980). Spiral coiled flat sheet oxygenators have also been used for isolated perfusion of the canine kidney and liver where the magnitude of the prime volume and cost of the devices was not important (Skibba et al 1983, Rijkmans et al 1984). Flat sheet membrane oxygenators have therefore been largely abandoned in favour of membrane tubing oxygenators for purposes of isolated organ perfusion.

The biocompatability of PDS has been assessed previously (Alexander et al 1978b, Alexander and Al Ani 1983, Braley 1964, Fleming 1977) and was demonstrated to be less thrombogenic than polyvinylchloride (PVC) during prolonged extracorporeal membrane oxygenation in lambs (Fleming 1977, Melrose and Singh 1974) and rats (Alexander and Al Ani 1983). These investigations also suggested that the use of PDS oxygenators resulted in much fewer complications such as wound bleeding, probably due to the resultant thrombocytopaenia, often associated with the use of bubble oxygenators (Melrose and Singh 1974, Fleming 1977). Haematological changes were attributed to the extracorporeal circuit alone, since these were also observed in extracorporeal circuits without oxygenators (Alexander and Al Ani 1983). Teflon (GoreTex) (R) has been successfully used for membrane tubing oxygenators (Tanishita et al 1978) but, since the material appeared to be less pliable than PDS, miniaturisation would have been difficult.

One of the major disadvantages of membrane tubing oxygenators was the development of saturated erythrocyte interfaces along the inside edges of the walls (see chapter 4) which reduced the efficiency of the

devices. Disruption of these interfaces could be achieved by a mechanical oscillator (Melrose et al 1972, Alexander and Al Ani 1983) or by the construction of coiled tubes, in order to generate secondary blood flow profiles through centrifugal force (see chapter 4). One disadvantage of narrow coiled membrane tubes was the resistance to flow and the tendency of the membrane to collapse after use, especially with the early prototypes which were some 20 feet in length (Folkman et al 1966). Later designs were able to use shorter lengths of tubing (16 feet) for the isolated perfusion of organs derived from smaller animals but these were also limited since they could not be re-used (Finseth et al 1972) and were mainly for low flow rates (Hamilton et al 1974).

3.5 PERFUSION MEDIA USED FOR ISOLATED ORGAN PERFUSION

The earliest isolated organ perfusion investigations recommended the use, where possible, of homologous blood from the donor animal (Brodie 1903). Clearly, there were many instances where this was not possible and therefore the use of semi-synthetic or totally synthetic media (such as flourocarbon media -see later) was desirable. Ideally, such a perfusate was to retain a high oxygen binding capacity, a physiological pH (usually 7.4 with adequate buffering capacity), a high colloid osmotic pressure and an ionic composition comparable to the intact animal (Ritchie and Hardcastle 1973).

The majority of isolated organ preparations use perfusates which consist of haemodiluted, washed erythrocytes usually obtained from human or bovine sources (Hems et al 1966, Lee and Holland 1979). Occasionally, the perfusates used are pooled homologous blood and are diluted in a priming solution to produce a perfusate similar to blood in electrolyte and protein content but at a reduced haematocrit (Brauer et al 1951). Consequently haematocrit values may be selected (for example during oxygen uptake studies, Keiding et al 1980), whilst retaining physiological electrolyte and protein concentrations (Ritchie and Hardcastle 1973). The composition of these electrolytic primers is therefore crucial and is discussed together with a review of other perfusion media utilised for isolated organ perfusion.

3.5.1 Basic perfusion (primer) media

The primer medium most often used for haemodilution consisted of isotonically balanced saline solution (0.9% NaCl) which was usually made to one of the established formulations according to Locke, Tyrode or Ringer (Ross 1972). The earliest published report upon the

isolated perfused rat liver preparation which utilised an oxygenated saline solution according to the formulation of Krebs and Henseleit (1932) was that of Trowell (1942). The preparation of Miller et al (1951) used pooled rat blood, haemodiluted with Ringer's solution to a haematocrit which ranged from 25 to 40 per cent. This preparation formed the basis of many systems used today.

Some physiological solutions such as Locke's, which were suitable for isolated perfusion of the heart, could be modified and optimised for the perfusion of other organs. Consequently a range of Ringer solutions were formulated for the perfusion of the frog heart (frog Ringer's), mammalian heart (Locke's), rabbit intestine (Tyrode) and amphibian Ringer (Ross 1972). The composition of the modified perfusion medium favoured by Hems et al (1966) was based upon the original formulation devised by Krebs and Henseleit (1932).

Schimassek (1963) proposed the inclusion of 2.5g% albumin in Tyrode solution, and albumin was also included by Hems et al (1966) in their modification of the original Krebs formulation. The concentration of albumin in these perfusates was approximately equal to that measured in normal plasma but this did not account for the oncotic pressure exerted by other plasma proteins such as the globulins and fibrinogen. Consequently, concentrations of albumin were increased to 6g% (Andjus et al 1967).

3.5.2 Erythrocyte supplemented media

Isolated organ perfusion investigations have incorporated erythrocytes from homologous or heterologous blood in order to increase the oxygen carrying capacity of the perfusate. The necessity for the inclusion of an oxygen carrier was best illustrated by Trowell

(1942) where an unphysiological increase in flow rate was necessary to compensate for the much reduced solubility of oxygen in water compared to haemoglobin (Gores et al 1986). This problem was overcome by the inclusion of a hyperbaric oxygenator in the perfusion system, which avoided the necessity for the inclusion of an oxygen carrier in the perfusate (Schmucker et al 1975).

3.5.3 Haemoglobin as an oxygen carrier

Dissolved haemoglobin in perfusates has been used for perfusion of the thyroid (Williams 1966) and the isolated perfused rat liver (D'Silva and Neil 1954), but the possible deleterious consequences of haemolysates upon isolated perfused organs, such as reduced reticuloendothelial function (Fischer et al 1981) and organs in vivo (Miller and McDonald 1951) has prevented extensive use of this oxygen carrier (Ritchie and Hardcastle 1973).

3.5.4 Fluorocarbon supplemented media

Fluorocarbon emulsions, in particular FX80 (3M Company, St Paul, Minnesota), were extensively investigated during the late 1960's and early 1970's. FX80 was composed predominantly of perfluorobutyltetrahydrofuran and its isomers. When totally saturated with oxygen, it could carry 0.63ml of oxygen per ml of solution, in contrast to human erythrocytes, which carry 0.46ml oxygen per ml (Sloviter and Kamimoto 1967). However, isolated rat liver perfusions demonstrated that FX80 diminished the volumes of bile produced and increased perfusion pressures, when compared to diluted and pooled homologous rat blood. In addition histological evidence of vascular occlusions were also reported (Brown and Hardison 1972).

3.6 SUMMARY

The disadvantages of existing circuits for isolated organ perfusion preparations have been discussed. These necessitated the development of a miniaturised system for liver and brain perfusion. Moreover, fundamental studies on the basic features of liver perfusion such as choice of perfusate, erythrocyte source and influence of haematocrit upon liver viability requires investigation. These studies are the subject of chapters 4, 5 and 6.

CHAPTER 4

DEVELOPMENT AND EVALUATION OF A MINIATURISED MEMBRANE OXYGENATOR FOR ISOLATED ORGAN PERFUSION

4.1 INTRODUCTION

A circuit offering the facility of dual organ perfusion was necessary in order that the liver/brain interelationship could be examined. An efficient membrane oxygenator which is compact and with a small prime volume was needed to control blood gas tensions accurately. This chapter describes the construction and in vitro evaluation of a new miniaturised membrane oxygenator for isolated organ perfusion. The device consists of a length of coiled PDS membrane tubing, coated with a layer of elastomer to increase compactness and durability.

One of the commonest materials utilised for the construction of membrane oxygenators is PDS, which has the advantages of chemical inertness, pliability and high rates of oxygen and carbon dioxide exchange (Alexander et al 1978a, Galletti et al 1966, Robb 1968). Membrane tubing configurations utilising PDS have high oxygen transfer capacities (Cohen et al 1974, Melrose et al 1972) and good biocompatibility characteristics (Alexander and Al Ani 1983, Alexander et al 1978b, Fleming 1977).

Measurement of the rate at which oxygen crosses synthetic membranes (the oxygen transfer capacity, OTC) by a satisfactory chemical technique which can provide a continuously high oxygen gradient across the membrane had not been previously reported in the literature. Previous techniques require the use of specialised equipment and this limits measurements to a few laboratories (Brown and Tuwiner 1962, Robb 1968, Waak et al 1955). Furthermore, the oxygen permeability of membranes has generally been measured with gas or liquid on both sides of the membrane which could introduce inaccuracies through membrane distortion from physical pressure (Brown

and Tuwiner 1962). Occasionally blood has been used to maintain the oxygen gradient in test cells but under these conditions the diffusion coefficient of gases through blood may become a limiting factor (Heimbecker 1977, Huxley and Kutchai 1981, Lee et al 1961, Waak et al 1955). This problem is compounded when PDS membrane tubing is used since oxygen molecules have to diffuse through a layer of fully saturated erythrocytes lining the walls of the tubing before oxygenating unsaturated red cells. The rate of oxygen uptake by the blood will be influenced by the dimensions of this saturated layer (Marx et al 1962). This chapter describes measurement of the oxygen permeability of the membrane using a new chemical technique. Sodium hydrosulphite was used as the reducing agent to eliminate the possibility of saturated erythrocyte interfaces reducing the effective oxygen gradient with blood (Huxley and Kutchai 1981).

4.2 A NEW CHEMICAL METHOD FOR MEASUREMENT OF THE OXYGEN PERMEABILITY OF PDS MEMBRANE TUBING

4.2.1 Chemical technique for measurement of OTC

The circuit used for the testing of oxygenators is shown in figure 4.1. The circuit tubing was composed of 3mm internal diameter, 1mm wall thickness Teflon tubing held together with glass connectors. A rotary roller pump (Watson-Marlow, Falmouth, Cornwall) was used to recirculate the solution and the gas syringe was an inverted 20ml blood syringe without the plunger. A cylindrical glass tube 34cm long, 3cm diameter was constructed to accomodate 30cm lengths of straight membrane. Each end of the glass tube was sealed with a rubber bung with a hole drilled through the middle to accomodate the circuit tubing. In addition, the glass cylinder contained two side arms for connection to the gas portion of the circuit (see figure 4.1). The surface area of the membrane was calculated from the length and average diameter of the membrane measured in several places with a calibrated graticule in a jeweller's eyepiece. Solutions of sodium hydrosulphite from 0.05 to 2.0 Molar were made up fresh for each experiment and protected from atmospheric oxygen by a layer of liquid paraffin. The test cell was flushed with nitrogen before the circuit was primed with reducing agent and 20ml of liquid paraffin poured into the reservoir. The reservoir which held the reducing agent was filled to the 50ml level to result in a total priming volume of 65ml and the solution was recirculated.

Five ml of 1% methylene blue was introduced into the 65ml total prime volume of reducing agent in order to indicate full oxygen saturation of the reducing agent by turning from colourless to blue.

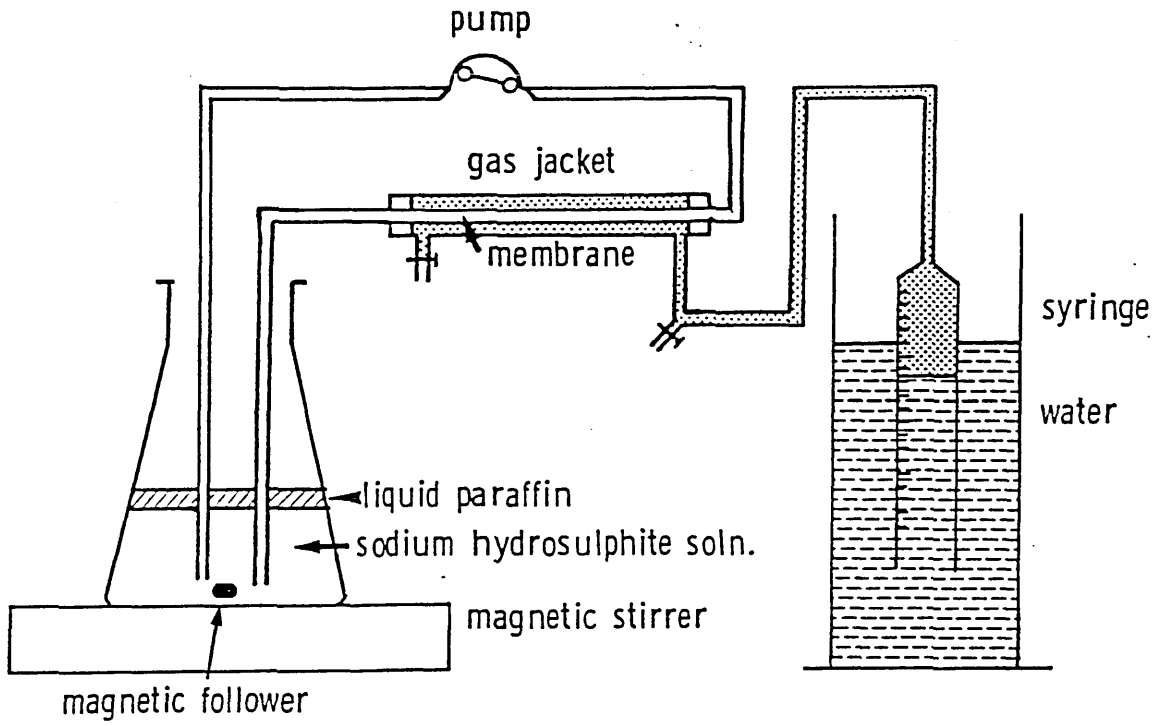


Figure 4.1 Circuit for chemical measurement of oxygen transport.

It was later observed that termination of the reaction could also be detected by measurement of progressively lower oxygen buffer rates. The water level in the syringe was carefully aligned with that in the cylinder whilst readings were taken to avoid pressure gradient effects altering OTC values (Galletti et al 1966). The test was commenced by substituting oxygen for nitrogen in the gaseous phase of the circuit via the inlet tap and the water level in the gas syringe was adjusted to that of the water vessel. The oxygen present in the syringe was gradually displaced with water sucked up by negative pressure as the gas passed through the membrane into the reducing agent. The volume of gas displaced by the water was measured directly from the inverted syringe. The water level in the syringe could be readjusted to the original level by reintroduction of more oxygen into the gaseous phase of the circuit. Readings from the gas syringe were noted every minute from the introduction of oxygen into the gas section, and enabled average values per minute to be calculated over 5 minute periods.

Finally the passage of oxygen across the membrane (OTC) was expressed in $ml^{-1}min^{-1}m^{-2}$ membrane surface area (Alexander et al 1978a, Melrose and Singh 1974, Robb 1968). The maximum value of the OTC that could be obtained was termed the permeability and expressed in ml $min^{-1}mmHg^{-1}\mu^{-1}$wall thickness.

4.2.2 Factors influencing the chemical OTC

An investigation was carried out to optimise all the factors likely to influence measurement of the OTC with sodium hydrosulphite in order that a maximum value could be finally obtained. Figure 4.2 demonstrates the influence of different concentrations of sodium hydrosulphite upon the OTC when a flow rate of 25ml min^{-1} was used.

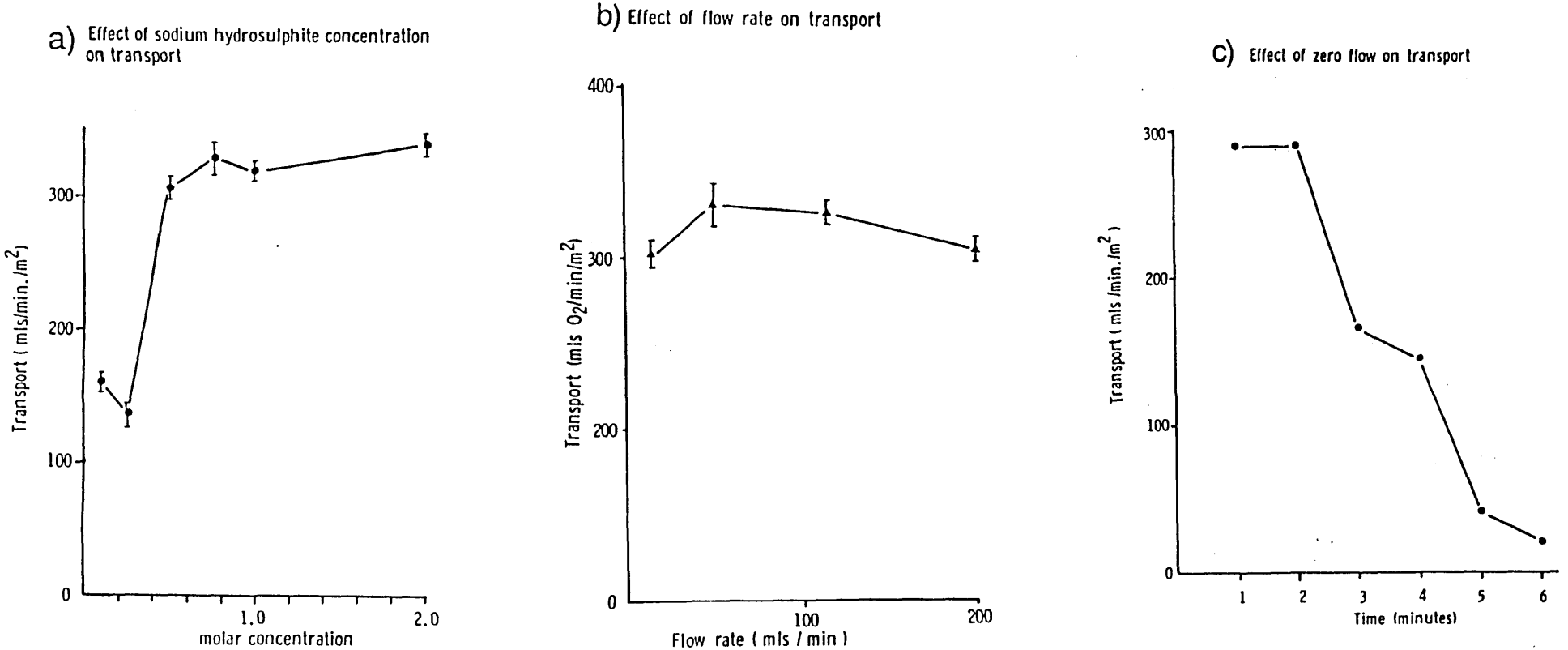


Figure 4.2 Effect of a) sodium hydrosulphite concentration, b) flow rate and c) zero flow upon oxygen transport. Points shown are mean ± standard error (s.e.), n = 6. See appendices A1, A2 & A3.

Since it was demonstrated that the OTC remained constant for all concentrations of hydrosulphite above 0.5 molar all further OTC measurements were conducted at a concentration of 0.75 molar to eliminate concentration effects.

It was important to assess the effect of progressive oxygen saturation of sodium hydrosulphite by measurement of OTC at one minute intervals from the start of the reaction. The OTC did not decrease for the first 20 minutes of the reaction at a flow rate of 50ml min^{-1} if a membrane of $7.5x10^{-4}m^{-2}$ surface area was tested. Therefore all OTC measurements were carried out within this time period.

Figure 4.2b shows that flow rates above 25ml min^{-1} did not alter the OTC. Another experiment demonstrated stable values at this flow rate for up to 30 minutes after the introduction of oxygen into the circuit. Zero flow (fig 4.2c) resulted in a decline in the OTC after 2 minutes and the volume of hydrosulphite that had remained in the membrane tubing became saturated after 6 minutes to result in very low values (fig 4.2c).

4.2.3 Optimum chemical conditions for measurement of oxygen permeability in PDS membrane

The results demonstrated that the lowest concentration of sodium hydrosulphite that could be used without creating an alteration in the OTC was 0.75 molar and a flow rate of 50ml min^{-1} was required to allow sufficient time for the maximum OTC to be measured with accuracy. The maximum OTC value measured was 340 ± 8ml $O_2min^{-1}m^{-2}$ which resulted in a value for the membrane permeability of $(2.55\pm0.06)x10^4 \pm 600$ ml $min^{-1}m^{-2}u/mmHg/\mu$.

4.3 THE DESIGN, CONSTRUCTION AND EVALUATION OF A NEW MINIATURISED PDS MEMBRANE OXYGENATOR

The generation of secondary blood flow vortices in PDS tubing oxygenators contributes greatly to an increased oxygenation efficiency and therefore a decreased priming volume. These secondary blood flow vortices are not necessarily required in conventional flat sheet oxygenators, or hollow fibre oxygenators, due to the large surface area of membrane to which a comparatively small volume of blood is exposed. Membrane tubing oxygenators, however, inherently possess the complication of a central 'core' or 'cylinder' of blood that rarely achieves total saturation unless 1) a comparatively low flow rate of blood is used (which would permit saturation by passive diffusion of oxygen through the plasma) or 2) secondary mixing is induced. Unfortunately, such low flow rates required a considerable surface area of tubing to be utilised and was reflected in the first miniaturised prototype developed in our laboratory which comprised 18 silastic membrane tubes, 1.5mm bore, 30cm in length with a prime volume of 12ml (Cohen et al 1974). The prototype was used for relatively low flow rates and therefore could function quite effectively, but at a much reduced efficiency. Other laboratories avoided the induction of secondary blood flow vortices by using large individual lengths of membrane ranging from 12 to 20 feet (Finseth et al 1972, Folkman et al 1966, Hamilton et al 1974) which imposed high input pressures. Secondary mixing was evidently required to reduce excessive lengths and number of tubes as a consequence of the decreased efficiency.

It seemed feasible, therefore, that if these saturated erythrocyte boundary layers might be continually disrupted, or

displaced by unsaturated erythrocytes from the centre of the tubing, the working oxygen gradient across the wall could be increased and maintained. The formation of these secondary vortices could be induced by two different methods.

The first method originally described by Melrose et al (1972) was by the use of a mechanical oscillator. The second method induced vortices passively by means of centrifugal forces generated by the formation of tubes into tight coils (Weissman and Mokros 1968). This principle was originally described by Dorson et al (1969) and others applied it to isolated organ perfusion systems (Folkman et al 1966, Nevasaari 1976, Schmucker et al 1975). The demonstration that fourfold increases in oxygenation efficiency could be achieved by the construction of much narrower coiled configurations (Alexander et al 1978a) permitted the development of the current model.

4.3.1 Construction of basic oxygenator

The membrane tubing was supplied as a 150 metre length of 1.5mm bore and 75μ wall thickness (Siltech Ltd, South Glamorgan). A knot was tied at one end of the tubing and a blunt ended 16 French gauge needle was fastened to the other end and attached to a pressure head of distilled water, to maintain inflation of the membrane during the remainder of the procedure. Approximately 23 metres of membrane, sufficient for the manufacture of 10 to 15 oxygenators, were inflated under the pressure head of water. The inflated membrane was wound on to aluminium rod formers to result in a ratio of rod diameter to membrane diameter of 4:1. This 4:1 ratio had been previously shown to be the optimum configuration with this membrane (Alexander et al 1978a).

A winding machine was designed and constructed to assemble the membrane upon aluminium rod formers 6.0mm internal diameter, and 53cm in length, at an even pressure and with a constant pitch between each turn (fig 4.3). The machine also prevented twisting of the membrane during the procedure. One and a half metres of membrane were wound upon each aluminium rod former, fastened and detached from the remainder of the unwound membrane. Another 16 French gauge needle was attached to the other end of the membrane and the assembly was then placed into a gas jacket (the glass cylinder used earlier) and the assembly connected to the testing circuit (fig 4.4).

4.3.2 Production of coated PDS oxygenators for isolated organ perfusion

Earlier studies had shown that the membrane was liable to rupture after extensive use and that it was difficult to store after use, due to the fragility of the material and also because the membrane tended to collapse. These problems were overcome by dip-coating the coiled membranes (on the aluminium rods) into a diluted silicone elastomer to result in a strengthened self-supporting unit which was resistant to collapse.

The membrane was inflated and wound upon the aluminium rod formers in the manner described previously (section 4.3.1). A knot was placed at each end of the coiled oxygenator and secured into position. A dip-coating solution, consisting of a partially cross-linked silicone elastomer, Silcoset 105 (ICI Ltd, Stevenson, Ayrshire, Scotland), was diluted in toluene at a concentration of 10% w/v and this solution was poured into a V-shaped tray. The rods were totally immersed in the dip-coating solution so that all portions of

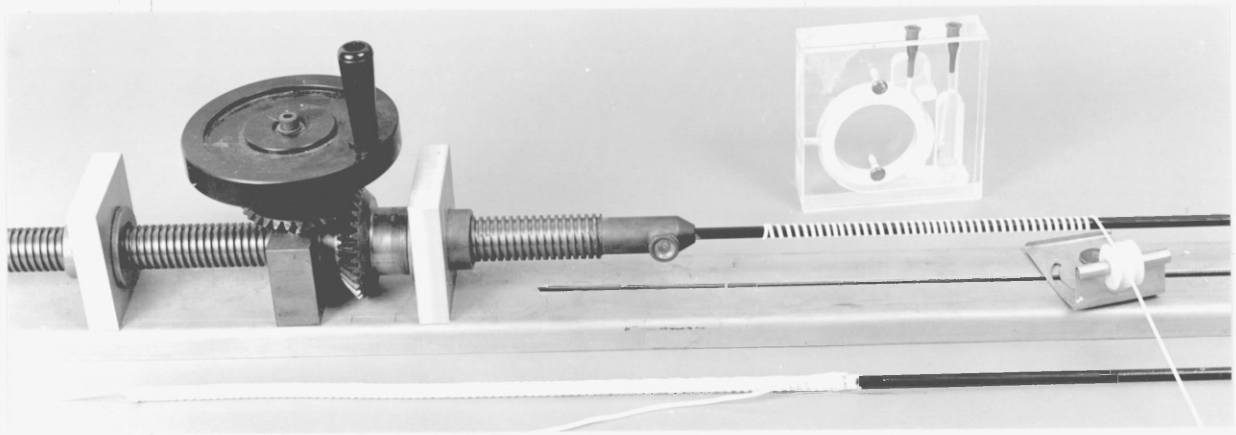


Figure 4.3 Various stages in the production of the oxygenator. Photograph of winding machine in action, a coated oxygenator after dipcoating and a finished miniaturised oxygenator.

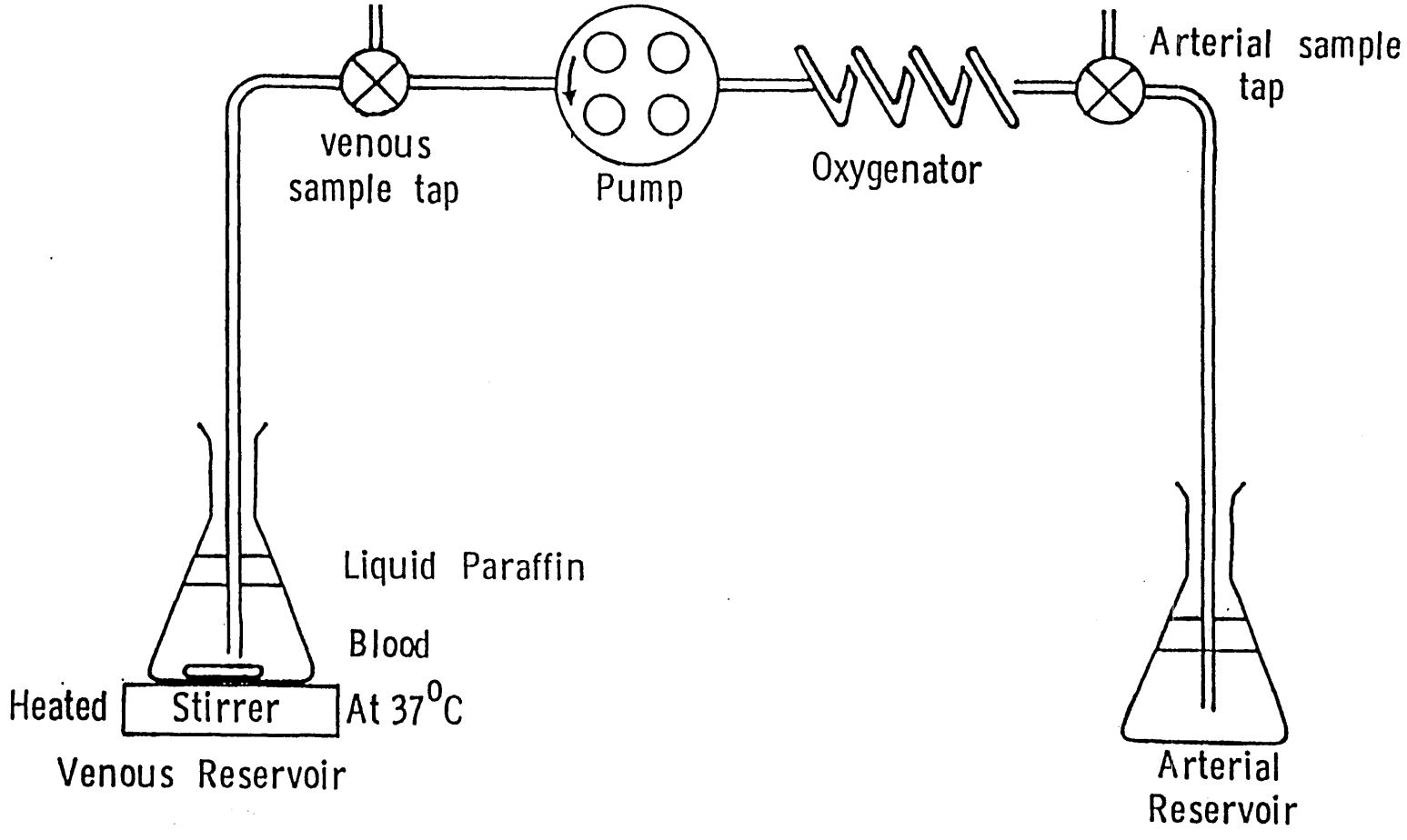


Figure 4.4 In vitro testing circuit for use with blood.

the rod and oxygenator received an even coating of the solution. The wet, coated oxygenator rod assemblies were then permitted to cure at room temperature for 5 hours to result in an even coating over the membrane approximately 75 to 120uMin thickness. The knotted ends of the oxygenator were detached and the water inside the membrane expelled to allow removal of the rods (see fig 4.3). Two 3cm lengths of 5FG cannulae (Portex Ltd, Hythe, Kent), were attached to each end of the now flexible coated oxygenator and placed into an acrylic gas jacket (fig 4.5).

4.3.3 Method for OTC measurement with blood

Tests were carried out upon measured lengths of uncoated coiled membranes supported upon cylindrical rod formers and also upon elastomer coated coiled membranes. The testing circuit was assembled as illustrated in figure 4.4 and incorporated an extra reservoir to enable "arterial" (post-oxygenator) and "venous" (pre-oxygenator) samples to be withdrawn simultaneously for gas analysis.

Outdated human banked blood, stored at $4^{o}C$, was prewarmed to $37^{o}C$ prior to each test and corrected with sodium bicarbonate to a physiological pH (7.28-7.42). In addition, the blood was diluted with saline to result in a haematocrit of 32-37% and a whole blood haemoglobin of 10-13g%. Blood was kept in the supplied ACD bags during preparation to protect it from atmospheric oxygen contamination. When the blood had been corrected for pH and haematocrit, 150ml was added to the venous reservoir and stored under liquid paraffin (fig 4.4). The pre-oxygenator reservoir was agitated by a magnetic stirrer to prevent plasma separation and a heater maintained the temperature at $37^{o}C$. The priming volume of the

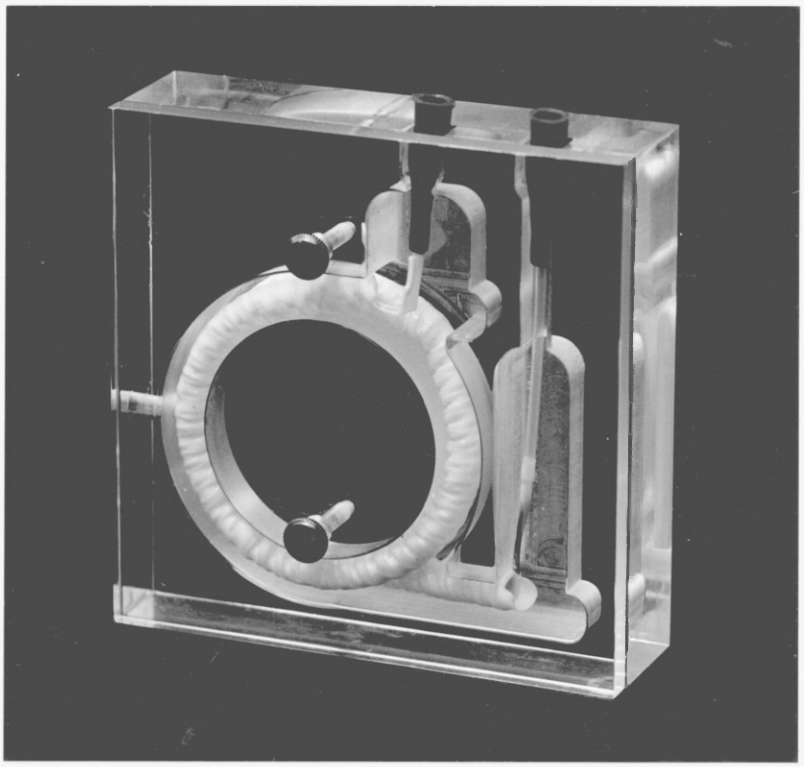


Figure 4.5 Photograph of the new miniaturised membrane tubing oxygenator housed in the specially designed gas jacket which was constructed from acrylic.

circuit excluding the reservoirs was 12ml and the circuit tubing and connectors were identical in dimensions to those used in the chemical tests.

Before commencement of each test a membrane (in a test cell) was connected to the circuit and the flow rate was selected (see section 4.3.4). Oxygen was then added to the gas phase of the system and the perfusion pump switched on. Samples were withdrawn from the pre- and post-oxygenator sample points at one minute intervals for 3 minutes from the start of perfusion at each flow rate. The flow rate was then altered, the pre-oxygenator reservoir refilled, if necessary, and the procedure repeated. Blood gases were measured upon a Radiometer ABL 1 blood gas analyser (Radiometer, Copenhagen), oxygen content upon a Lex O_2 Con (Lexington Inc, Massachusetts), haemoglobin using the cyanomethaemoglobin technique upon an EEL Haemoglobinometer (Corning EEL Ltd) and haematocrit upon a Hawksley microhaematocrit centrifuge (Hawksley Ltd, England).

4.3.4 Experimental design

Previous investigations had shown that a 1.5 metre length of membrane was the optimal length to use (Alexander et al 1978a). Therefore a comparative study was carried out between coated oxygenators and uncoated 1.5 metre lengths of basic coiled PDS membrane tubing. The study was divided into two sections to assess differences in oxygenation characteristics between the uncoated and coated membranes.

The first series of tests were designed to measure the OTC in uncoated and coated membranes and reflected the efficiency of the devices. However, this did not disclose any information regarding the

effectiveness of oxygenation in terms of the degree of oxygen saturation of haemoglobin. Therefore a second series of experiments investigated the total volume of oxygen in post oxygenator samples, rather than the volume entering per unit area of membrane, by measurement of the degree of oxygen saturation of haemoglobin in oxygenator output samples. Both series of tests were conducted at flow rates of 5, 12.5, 15, 17.5, 20 and 25ml per minute in coated and uncoated membranes. Flow rates above these values were not tested since 25ml per minute was previously shown to be the optimal flow rate for 1.5 metres of uncoated membrane (Alexander et al 1978a).

CALCULATIONS

1) OTC

Let:

Input oxygen content = x ml O_2 per 100 ml blood
Output oxygen content = y ml O_2 per 100 ml blood

Input - output oxygen content difference = y - x ml O_2 / 100 ml blood

Let flow rate = z ml per minute.

Then the total volume of oxygen entering the blood per minute

$$= \frac{y - x}{z} \text{ ml } O_2 \text{ per minute.}$$

Now if the surface area of membrane to be used = Ω sq M

Then the OTC = $\frac{y - x}{z\,\Omega}$ ml O_2 / min / sq M.

2) Percentage saturation difference

Let:

Input O_2 content = x ml O_2 per 100ml blood
Output O_2 content = y ml O_2 per 100ml blood

Assuming that 1g of haemoglobin, 100 % saturated, will bind 1.34 ml of oxygen and that the whole blood haemoglobin of the sample under analysis = " A " g Hb per 100ml of blood.

Then 100ml of blood, 100% saturated, will hold 1.34 A ml O_2

And,

$$\text{Input sample} = \frac{x}{1.34\,A} \times 100\ \%\ \text{saturated}$$

$$\text{Output sample} = \frac{y}{1.34\,A} \times 100\ \%\ \text{saturated}$$

so that the percentage saturation difference between input and output samples will be =

$$\frac{100y}{1.34A} - \frac{100x}{1.34A}\ \%.$$

4.3.5 OTC results with blood in uncoated and coated PDS membrane oxygenators

The maximum OTC value measured in uncoated membranes by the new chemical technique was 278ml $O_2/min/M^2$ compared to 348ml/min/M^2 in uncoated membranes, a decrease of 20%. However, this OTC value was obtained with the shortest length of membrane at a high flow rate which kept the oxygen gradient at a maximum.

Figure 4.6 (a) shows a series of single run tests to illustrate the difference in OTC between uncoated and elastomer coated membranes. The data shown were obtained from single run tests on the same batch of blood to ensure constant haematocrit and input pO_2. It has previously been shown that OTC and percent saturation difference vary in a parabolic relationship with flow rate (Galletti et al 1966, Robb 1968). Two variable parabolic regression analysis was therefore applied to fit the curves according to the standard equation for parabolic curves:

$$y = a_o x + a_1 x + a_2 x^2$$

This was carried out using a desktop Hewlett Packard Programmable Calculator (model no.9810A). A list of the values calculated for the coefficients for each of the 4 curves is provided in the table on the following page. The curves demonstrated a reduction in OTC values at all flow rates in the coated oxygenators.

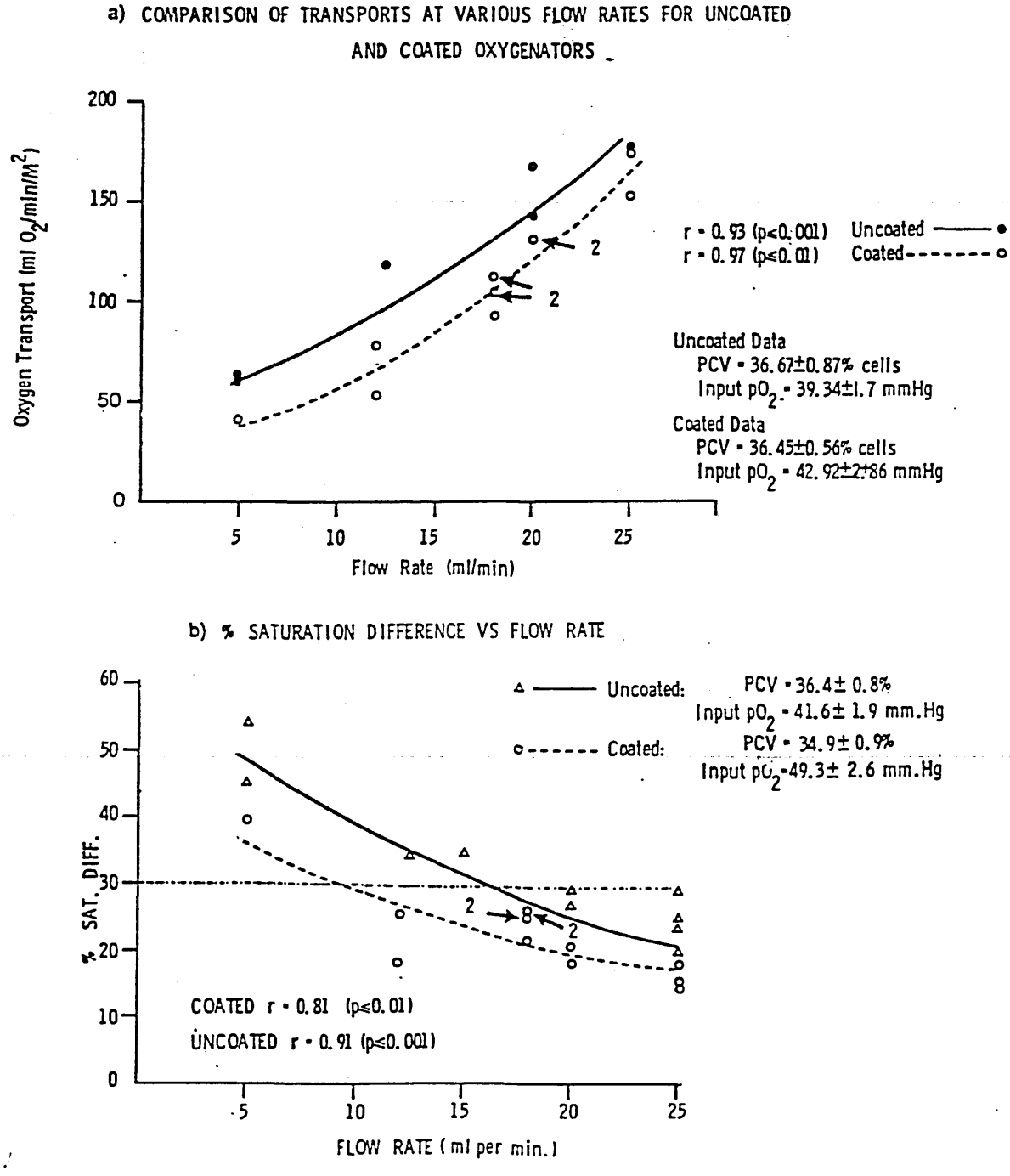


Figure 4.6 Effect of flow rate upon a) OTC and b) percentage saturation difference. See appendices A4 & A5.

Table 4.1

Coefficient	a_o	a_1	a_2	
Coated	28.884	1.666	0.172	Transport
Uncoated	41.706	4.104	0.058	ml/min/O_2
Coated	43.354	-1.593	0.022	% saturation
Uncoated	59.317	-2.150	0.025	difference

List of coefficients calculated for parabolic regression analysis for the curves shown in figure 4.6.

Figure 4.6 (b) demonstrates the difference in percent saturation between uncoated and elastomer coated membranes. The results were obtained from several samples of blood in order that several runs per membrane could be carried out and therefore permit statistics and parabolic regression analysis to be used to fit the two curves. Galletti (1971) proposed that the minimal satisfactory difference between input and output oxygenator samples should be 30%. This value was attained at a flow rate of 16ml per minute and 9ml per minute in uncoated and elastomer coated membranes respectively.

4.4 DISCUSSION

Previous measurements of the gas transfer rates of PDS and other membranes presented discrepancies due to gradual accumulation of oxygen on each side of the membrane, which reduced the overall effective oxygen gradient and consequently decreased OTC values (Brown and Tuwiner 1962, Robb 1968, Waak et al 1955). The technique described here overcame these problems by maintenance of a very high oxygen gradient, using sodium hydrosulphite, which delayed the onset of equilibrium while measurements were being taken. The remaining limiting factors influencing OTC were the reaction rate of oxygen with the hydrosulphite, assumed to be too high to influence results significantly and the structural characteristics of the membrane itself. Some laboratories have used blood inside the test cells to maintain the oxygen gradient (Heimbecker 1977, Waak et al 1955) but under these conditions, the diffusion coefficient of blood may become a limiting factor (Lee et al 1961, Waak et al 1955). The carbon dioxide transfer rate across the membrane was not calculated during these experiments since earlier investigations demonstrated that this was already maximal with less efficient configurations (Melrose et al 1955, Robb 1968).

The construction of a new type of miniaturised, silastic (PDS), membrane tubing oxygenator, 1.5 metres long, 1.5mm internal diameter, 75u wall thickness, incorporated in one self-supporting unit is described. This unit was designed to be applicable to isolated organ perfusion and small animal investigations by virtue of its simplicity, durability and prime volume of less than 3ml. The performance of the oxygenator in vitro, with outdated human banked blood demonstrated that the unit could effectively oxygenate blood of 30% haematocrit at

flow rates over 10ml per minute. Higher flow rates could be used through the oxygenator if blood or perfusates of lower haematocrit were utilised.

The 10% dilution of Silcoset (R) resulted in a dipcoat of reproducible thickness and strength combined with a pronounced resistance to membrane collapse. Attempts to dilute the elastomer further resulted in very fragile coatings which often fractured during construction. The effective wall thickness of the membrane was increased from 75 to 150μ with the 10% dilution of the elastomer but this did not reduce the oxygenation efficiency by a similar proportion.

In summary, it was concluded that the overall efficiency of the new oxygenator enabled it to be incorporated into the new isolated organ perfusion system for futher evaluation. The following chapter describes the incorporation of the oxygenator into a new isolated organ perfusion system designed for liver and brain support.

CHAPTER 5

A NEW ISOLATED ORGAN PERFUSION CIRCUIT (FOR LIVER AND BRAIN SUPPORT)

5.1 INTRODUCTION

This chapter is a detailed description of the development and performance of the perfusion circuit described in the previous chapter. It has been tested in three organ systems, the isolated perfused rabbit heart, the isolated perfused rat free skin flap and the isolated perfused rat liver (IPRL). This chapter describes the work carried out upon the IPRL in order to establish that the new liver perfusion system remained stable during six hours of perfusion. Measurements that have been previously cited as indices of liver function of the IPRL were assessed both immediately and retrospectively. The results of the isolated perfused rat brain (IPRB) preparation will be presented in chapter 7. The results obtained from the isolated perfused rat free skin flaps and isolated perfused rabbit heart are included in appendix B3 at the end of this thesis.

5.2 CONSTRUCTION AND PRINCIPLES OF OPERATION OF THE PERFUSION SYSTEM

5.2.1 Circuit description (Fig 5.1)

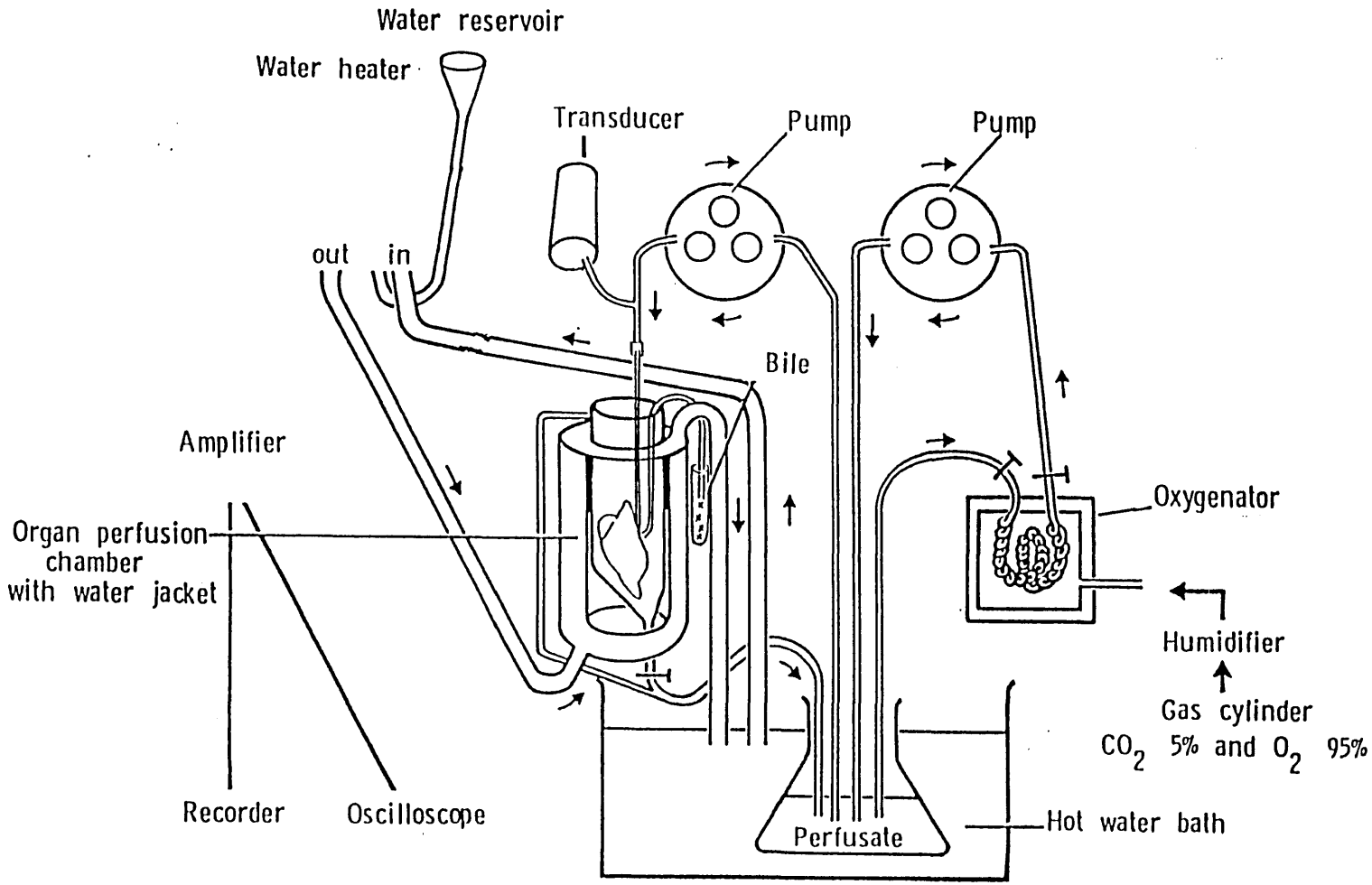
The systems used for the three isolated organ perfusion experiments are all minor adaptations of the basic system shown in figure 5.1. It comprises two circuits connected together in parallel to a common reservoir which contains a mixed arterio-venous perfusate. One circuit is connected to the miniaturised membrane oxygenator and the other supplies the organ with oxygenated perfusate. The major advantage of this design is that blood gases in the reservoir are controlled independently from the flow rate through the isolated organ.

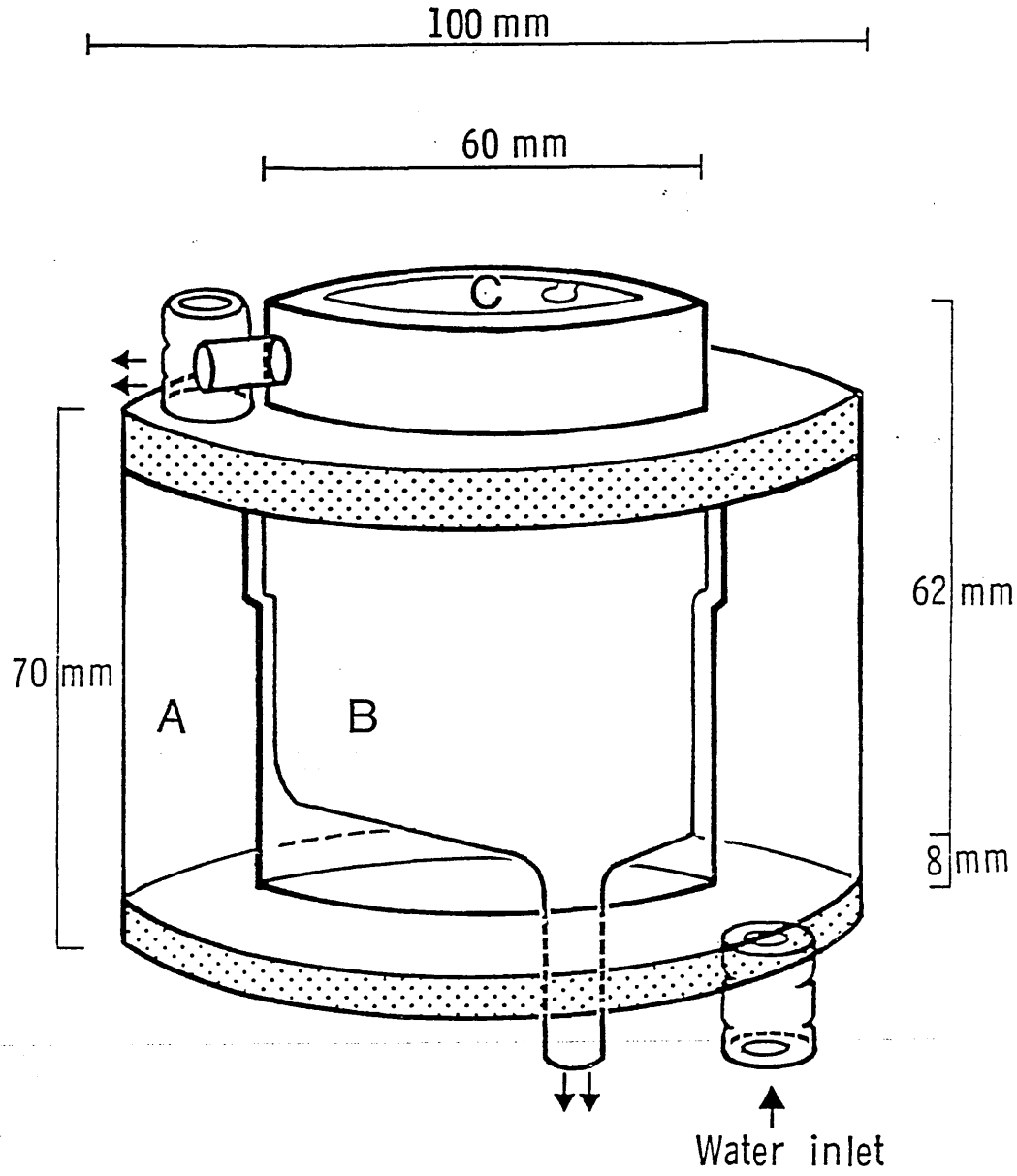
5.2.2 Design of organ chamber (Fig 5.2)

The organ bath has an angled base to facilitate drainage of perfusate via a drainage tube into the reservoir. The flow rate of liver effluent into the reservoir was controlled by a gate clamp on the drainage tube to ensure that a constant level of perfusate was retained in the bath. This retains organ buoyancy and is believed to facilitate successful perfusion of the periphery of the liver (Schmucker et al 1975). Careful adjustment of the gate clamp also prevents entry of air into the drainage tube which could result in bubbling and foaming in the reservoir.

The circular organ bath is constructed from acrylic and is placed inside an acrylic warm water jacket. The temperature in the organ bath is maintained at 37^{o} with the heated water jacket which is connected in series to a water bath in which the perfusate reservoir is immersed. The water bath is connected to the heat exchanger

Figure 5.1 Isolated rat liver perfusion circuit.





↑ Water inlet and outlet

A Water jacket

B Removable perfusate and organ container

C Lid

↑↑ Perfusate to reservoir

Figure 5.2 Organ perfusion chamber (bath).

(Churchill Instruments, England) which delivers warm water to the base of the organ bath water jacket.

5.2.3 Perfusion pressures and flow rates

Flow rates were governed by input perfusion pressure which was maintained at the appropriate physiological value after correction for the previously measured pressure gradient across the cannula for each flow rate and haematocrit.

5.2.4 Sterilisation and cleaning protocols

The cleaning protocols adopted were those described by Alexander and Al Ani (1983), used for prolonged CPB in rats. A first wash with sterile water was followed by a 2% (w/v) solution of sodium hydroxide. This solution was left in the circuit for one hour to deproteinise the circuit. The circuit was then flushed through with sterile distilled water for ten minutes and then perfused with a solution of 2% (w/v) hydrochloric acid. This ensured that any residual sodium hydroxide would be neutralised before the circuit was given a final wash through with one litre of sterile distilled water 15 minutes later. The circuit was often sterilised the day before use and was kept aseptic overnight with a solution of 10% formal-saline. This was thoroughly washed out of the circuit with sterile water prior to use and then washed through with a litre of sterile saline before the perfusate was introduced into the circuit.

5.3 SURGICAL PROCEDURES

Sprague Dawley rats weighing 250-300g were anaesthetised with ether. The abdomen was entered through a midline incision and the viscera were placed in a warm, saline-moistened swab to the left hand side of the animal. The median and left lateral lobes of the liver were mobilised by division of the suspensory ligaments and the entire liver was everted upon the thoracic cavity and placed into a warm, saline-moistened swab. This procedure readily exposed the portal vein and the bile duct to facilitate cannulation procedures. The bile duct was carefully dissected, mobilised and tied distally with a 5/0 silk to allow dilatation and facilitate cannulation of the duct. The duct was cannulated with a 2 French gauge cannula constructed from a 10cm length of Portex PP3 tubing (Portex Ltd, Hythe, Kent).

The left gastric vein, hepatic artery and splenic vein were mobilised and ligated together with single 5/0 silk ligature in order that the portal vein could be mobilised. The liver was returned to the abdominal cavity and bile collected into a sample tube for 15 to 30 minutes during the remainder of the operative procedure and the volume of in vivo bile production and biochemical composition measured. The liver was again everted upon the thoracic cavity and two 3/0 silk ligatures were placed under the portal vein for cannulation. A small incision was made just below the entry of the splenic vein and the portal vein was cannulated with a 5 French gauge cannula (Portex Ltd, Hythe, Kent), filled with heparinised KRBA (2 units/ml). Care was taken to ensure that portal vein branches to individual lobes of the liver were not occluded.

The liver was perfused with 20ml of cold heparinised KRBA and its colour changed to a uniform pale beige. The diaphragm and inferior

vena cava were incised around the hepatic veins and the inferior vena cava severed below the liver. The liver was removed and placed into a petri dish and the portal vein flushed with a further 20ml of cold KRBA. Incomplete colour change of the liver was indicative of poor perfusion, and such preparations were abandoned. The organ was weighed prior to connection to the perfusion circuit and the maximum ischaemic time permitted was 2.5 minutes. The liver was placed into the organ bath and perfusion commenced at a fixed perfusion pressure between 6 and 8mmHg. The first 10ml of effluent from the perfusion was discarded because of its content of toxic metabolites (Hems et al 1966).

5.4 MATERIALS AND METHODS

5.4.1 Preparation of perfusates

The perfusates for the preparations were based upon heparinised Krebs-Henseleit buffer (KRBA). The KRBA was prepared in the following manner according to the method of Krebs and Henseleit (1932).

100ml 0.9% NaCl : 1ml 0.15 M KH_2PO_4

4ml 0.154 M KCl : 1ml 0.154 M $MgSO_4$

3ml 0.11M $CaCl_2$: 21ml 0.154 M $NaHCO_3$

This mixture was gassed with 5% carbogen to clear the solution and to each 100ml of perfusate was added 6.0% bovine serum albumin (Sigma fraction 5), 200mg glucose, 4 units/ml insulin and 2 units/ml heparin. The solution was finally filtered through 45μ interstices filters (Millipore Ltd) and stored at $4^{o}C$ in sterile containers. Penicillin (Glaxo Laboratories Ltd) and streptomycin (Evans Medical Ltd) were added to the perfusate immediately prior to use at 2000 units/ml and 500ug/ml respectively.

The perfusate utilised for the IPRL preparation was based upon that described by Brauer et al (1951). Pooled rat blood was collected from Sprague Dawley rats by cannulation of the aorta under ether anaesthesia. Approximately 10-15ml of blood could be collected from a 200-250g rat and this was haemodiluted with oncotically balanced KRBA (Hems et al, 1966) to result in a haematocrit of 10-12% cells (whole blood haemoglobin 3-4g%). It was found that haemodiluted blood from three rats was sufficient to prime the perfusion circuit, which required 130-150ml of perfusate.

5.4.2 Physiological and dynamic measurements

Portal perfusion pressure was measured by a transducer (type 4-422-0001, Bell and Howell Ltd, England) connected to an SEM medical four channel recorder (SE Laboratories Ltd, Middlesex, England). Hepatic function was assessed by immediate and retrospective measurements. Immediate measurements of hepatic function included flow rate, perfusion pressure, blood gases, hepatic oxygen uptake, perfusate haematocrit, bile volume production and in one series of perfusions, bromosulphthalein (B.S.P.) removal. Blood gases were measured upon a Radiometer ABL 1 blood gas analyser (Radiometer, Copenhagen), oxygen content upon a Lex O_2 Con (Lexington Inc., Massachusetts) and haematocrit upon a Hawksley microhaematocrit centrifuge (Hawksley Ltd, England). Flow rate through the isolated liver was measured by timed diversion of a fixed volume of perfusate into a calibrated measuring cylinder.

5.4.3 Biochemical and histological evaluations

Plasma haemoglobin measurements were carried out retrospectively once all the samples from one complete series of experiments had been collected and stored at $-4^{o}C$. Plasma haemoglobin estimations were carried out by the direct ultra violet absorbance technique according to the method of Harboe (1959).

Histological examinations were carried out upon paraffin fixed, haematoxylin and eosin stained livers perfused initially with sterile saline at the end of perfusion. Formal buffered saline was substituted for the saline after 5 minutes of perfusion, once the liver exudate was visually clear of erythrocytes. This procedure

ensured that the organ was fixed efficiently and minimised autolysis. Representative sections were cut from each lobe, mounted and prepared for staining with haematoxylin and eosin.

5.5 RESULTS

A small but significant decline in the mean wet weight of the livers from 16.3 ± 0.75g to 14.46 ± 1.18g (mean ± standard error) was detected (Student's paired t-test, $P<0.05$). Perfusate flow rate was maintained at 1.91 ± 0.05ml/min/g liver after a 5 minute equilibration period. Appendix B1 summarises the blood gas data from these perfusions where the flow rate through the oxygenator was maintained at 30ml/min. In summary, the blood gases remained relatively constant at physiological concentrations throughout 6 hours of perfusion.

Figure 5.3a illustrates alterations in haematocrit during the 6 hours of perfusion. Haematocrit appeared to decline by 6 hours of perfusion although this was not statistically significant ($P>0.05$, Student's paired t-test). A significant increase in plasma free haemoglobin occurred from the first hour of perfusion (Student's paired t-test, $P<0.05$) and continued to increase in a linear fashion reaching 700mg/100 ml plasma by 6 hours.

The oxygenator readily corrected liver (tissue) effluent blood gases to retain physiological arterial values in the mixed arterio-venous reservoir (table 5.1). Occasionally it was necessary to correct metabolic acidosis of the liver by the addition of 0.1 ml sodium bicarbonate (8.4%) to the reservoir. HOU remained steady and no significant changes were measured in HOU during the perfusions ($P>0.05$, Student's paired t-test). Bile volume production increased after 2 hours of perfusion when compared to pre-perfusion (in vivo) values ($P<0.05$, Student's paired t-test: see figure 5.4b). However decreases in bile volume production were measured after 4 hours of perfusion and became significant after 5 and 6 hours ($P<0.05$, paired

t-test).

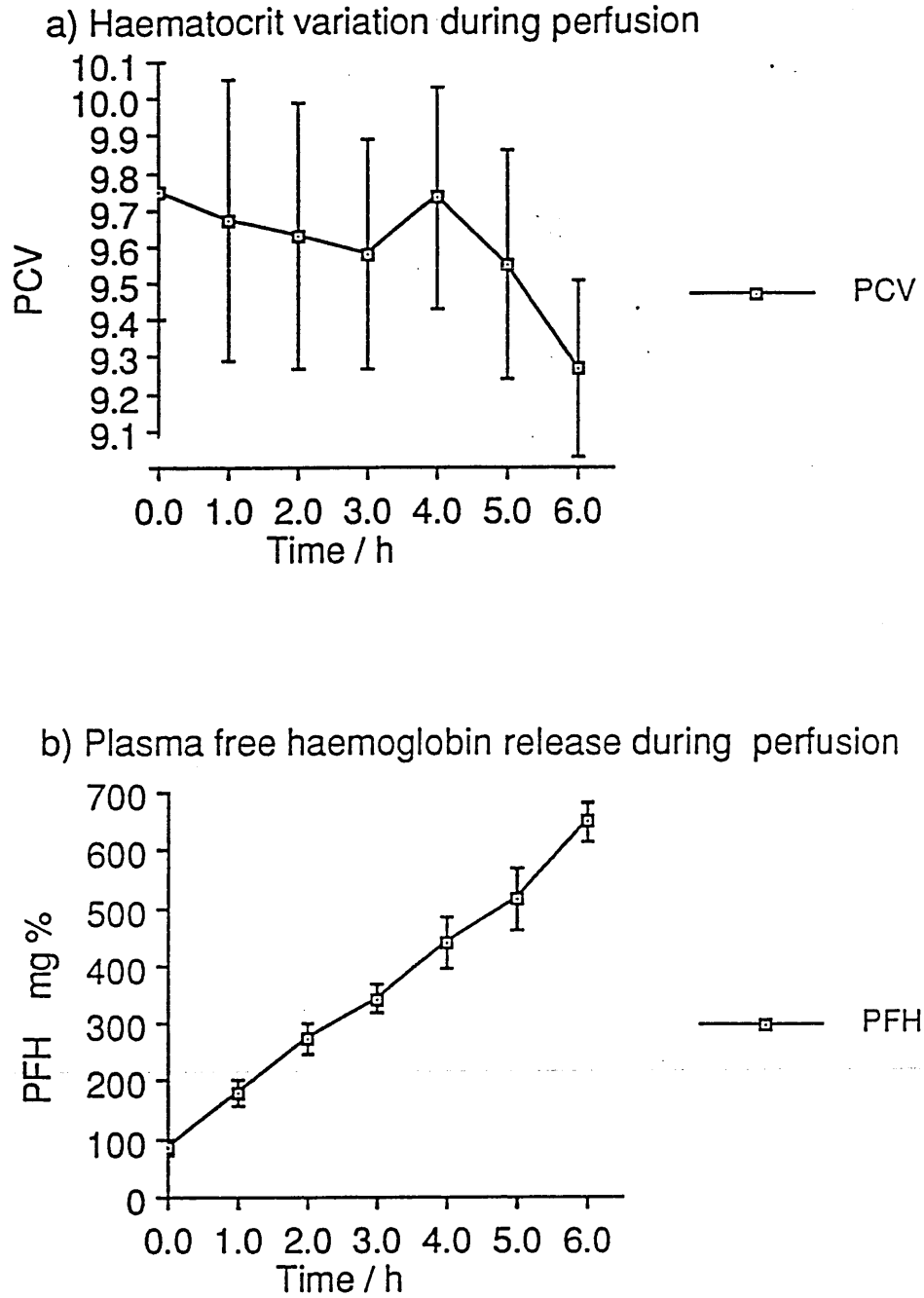


Figure 5.3 Measurements of a) haematocrit and b) plasma free haemoglobin during 6 isolated rat liver perfusions. Results showr are mean ± SE (see appendix B1).

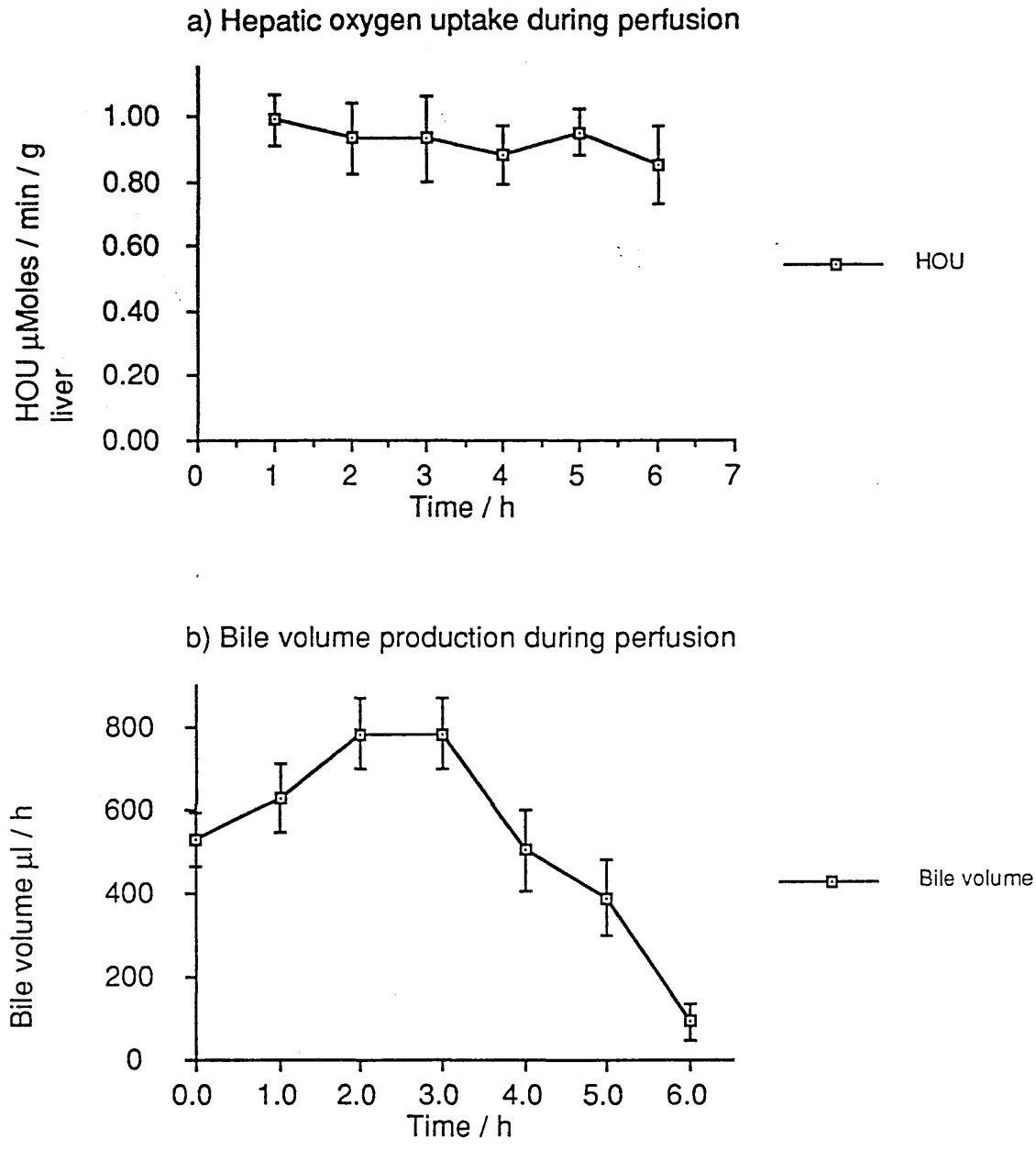


Figure 5.4 a) hepatic oxygen uptake (HOU) and b) bile volume production during 8 isolated rat liver perfusions.Results shown are mean ± SE (see appendix B1). * $P< 0.05$, ** $P< 0.01$ (Student's unpaired t-test, compared to time = 0).

TABLE 5.1

Sample	pH	PO_2 (mmHg)	pCO_2 (mmHg)
Post-oxygenator	7.44±0.05	227.2±9.2	32.9±1.8
Post-tissue	7.31±0.09	53.9±2.2	29.8±1.9
Reservoir	7.41±0.02	141.8±9.5	29.7±1.3

Summary of blood gas results obtained from 23 isolated rat liver perfusions PCV = 29.3±1.1 (mean±SE).

BSP removal

In 2 perfusions, 2 bolus injections of 2mg of BSP were made into the reservoir (figure 5.5). Decreases in plasma (and bile) BSP concentrations were measured by the 3 hour perfusion interval and these increased following an additional 2mg bolus of BSP into the perfusate. These experiments demonstrated that the viability of the liver as estimated by removal of plasma BSP was preserved for at least 4 hours of the perfusion period. After a second bolus injection plasma levels remained high, possibly due to a decrease in hepatic

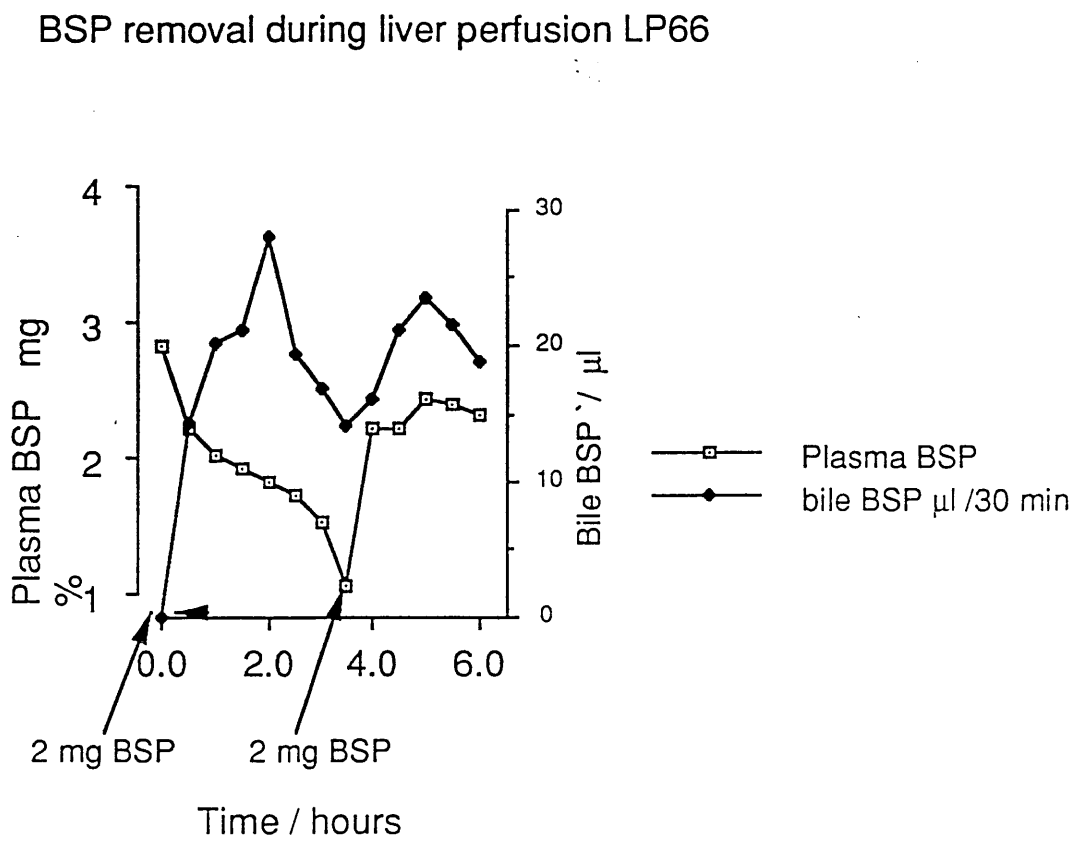


Figure 5.5 Demonstration of bromosulphthalein (BSP) removal during isolated rat liver perfusion LP66 (see appendix B2).

function and/or increases in haemolysis which could interfere with measurements of BSP.

Histological investigations revealed few changes in liver sections perfused for 6 hours (fig 5.6). The major changes detected were slight distension of the liver sinusoids which could have been attributed to the fixation procedure of the perfused livers.

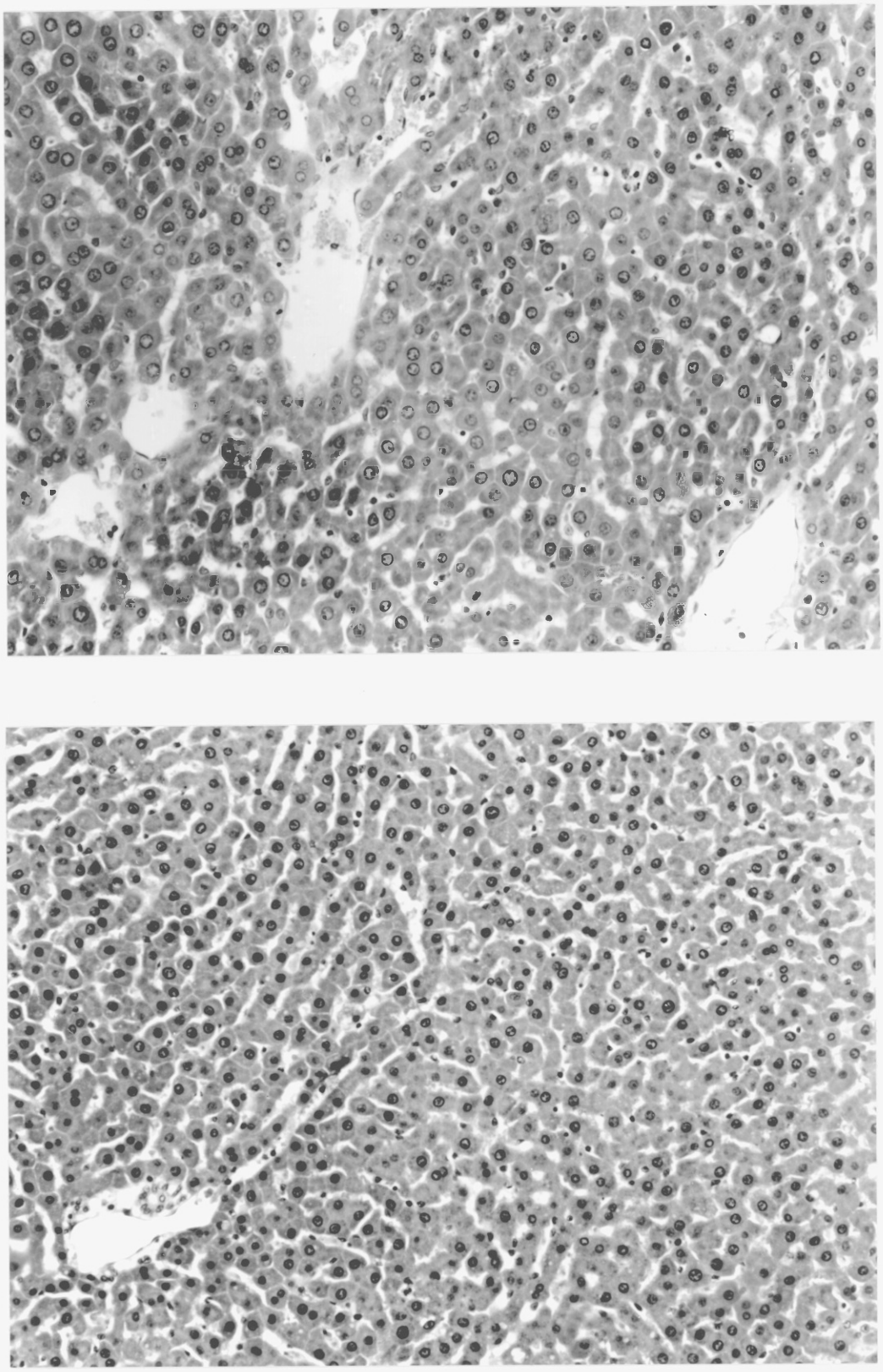


Figure 5.6 Centrilobular to midzonal areas of the left lateral lobe taken from a rat liver perfused for 6 hours with haemodiluted rat blood (X 200).

5.6 DISCUSSION

Conventional techniques and perfusates were used during the isolated rat liver perfusions in order to assess the new isolated organ perfusion system. The major limitation of the circuit was that the level of perfusate in the organ bath had to be closely monitored but it was felt that this disadvantage was outweighed by the advantages offered in a buoyant preparation which was reported to preserve hepatic architecture due to a more complete perfusion of the periphery of the organ (Ryoo and Tarver 1968, Schmucker et al 1975). Unfortunately, another disadvantage of this was that the prime volume was increased considerably.

Accurate control of perfusate blood gases was achieved by the separation of the oxygenation circuit from the perfusion circuit. In addition, the perfusion pressure and flow rate through the liver could be adjusted independently from the remainder of the perfusion circuit. It was demonstrated that a single oxygenator was sufficient for the flow rates and haematocrits chosen for this series of experiments.

These pilot studies demonstrated that the system was technically capable of maintaining the liver in a stable condition throughout the desired period of perfusion. In addition, the circuit caused little haemolysis as reflected in haematocrit or plasma haemoglobin measurements after six hours of perfusion.

Hepatic oxygen uptake (HOU) has often been used as an index of liver viability during isolated liver perfusion (Collins and Skibba 1980, Hems et al 1966, Mitzkat and Meyer 1973, Schmucker et al 1975, Schimassek 1962). This measure of hepatic function has been shown to

increase if the perfusate haematocrit or flow rate are increased in the IPRL (Keiding et al 1980, Riedel et al 1983). In addition, fluctuations in flow rate have been shown to alter the extraction of BSP (Brauer 1963) and therefore conditions should be standardised during comparative evaluations. Fluctuations in HOU may occur for several other reasons. A decline in the HOU of non-starved rats from 2.5μM/min/g to 1.1μM/min/g in 48 hour starved rats has been reported (Forasander et al 1965). A reduction in in HOU appears to be proportional to the duration of starvation (Gores et al 1986). Collins and Skibba (1980), reported an HOU of 3.0μM/min/g when washed, aged, human erythrocytes were used as the oxygen carrier at a haematocrit of 20%, with a preceding starvation period of 24 hours. Hems et al (1966), reported a value of 2.2μM/min/g, the reduction being probably due to a reduced haematocrit and decreased perfusion temperature. Schimassek (1962) reported a value of 2.0μM/min/g with a haematocrit of 30% at 37^{o}C, and later Powis (1970) reported a value of 2.7μM/min/g under identical conditions. These values are greater than those measured in vivo of 1.4μM/min/g (Mitzkat and Meyer 1973). However, those reported by Schmucker et al (1975) of 1.1μM/min/g and Stollman et al (1983) of 1.48μM/min/g are closer to in vivo values. It is possible, however, that the decreased value reported by Schmucker et al (1975) was due to the absence of an oxygen carrier in the perfusate (Keiding et al 1980, Lee and Walker 1977).

The value obtained in the present studies was 0.99μM/min/g after one hour of perfusion and 0.85μM/min/g after six hours of perfusion. It is probable that these low values were due to the low haematocrit of the perfusate and also due to the starvation period of 24 hours prior to the experiment. Furthermore, the source of erythrocytes may also influence the HOU: this is investigated in Chapter 6.

Bile volume production has probably been the most widely used index of hepatic function during isolated liver perfusion (Brauer et al 1951, Gores et al 1986, Hems et al 1966, Schmucker et al 1975). The rate of bile production is subject to many variables, including the presence of choleretic agents such as taurocholate in the perfusion medium (Boyer and Klatskin 1970, Gores et al 1986, Percy-Robb and Boyd 1970). It would be expected therefore that preparations based upon diluted rat blood would result in a higher rate of bile production compared to those composed of washed erythrocytes suspended in KRBA without the addition of any choleretic agents.

The rate of bile production has been shown to be sensitive to changes in the reticuloendothelial system (RES) (Brauer et al 1951). Injection of a colloid suspension (Indian ink) into the perfusate of an isolated liver preparation to confirm adequate reticuloendothelial function also reduced bile volume production upon uptake of the particles by Kupffer cells (Brauer et al 1951). In addition hypoxia and the type of anaesthetic used, for example ether or pentobarbitone, may alter the rate of bile production (Ashford and Burdette 1965). Dobbs et al (1979) reported that bile production was reduced when ether was used as an anaesthetic and suggested that halothane was preferable.

The in vivo rate of bile production was examined in each rat prior to cannulation of the portal vein. The rate of bile production measured in this series of experiments was 528±65 µl per hour. This is similar to the in vivo value reported by Schmucker and Curtis (1974). The rate of bile production increased above the value measured in vivo after 2 and 3 hours of perfusion and then declined

after 4 hours of perfusion. It is probable that this reduction was indicative of declining liver function. The decreased rates of bile production after 5 and 6 hours of perfusion (391±92 and 180±45 µl per hour) were similar to those reported in the literature (Boyer and Klatskin 1970, Brauer et al 1951, Hems et al 1966, Kvetina and Guaitani 1969).

The reduction in the wet weights of the livers following 6 hours of perfusion was not considered to be indicative of any injury to the liver. A weight gain would have caused greater concern as this would have been indicative of oedema which would have affected liver function.

The clearance of BSP by the isolated perfused rat liver has been used in the past as an index of hepatic function (Brauer et al 1951) and as a tool for the elucidation of mechanisms of bile secretion (Brauer et al 1953). It was only used in two studies during this series of experiments as further confirmation of transport, uptake and excretory functions during perfusion and as an additional marker of hepatocellular function. This was carried out purely to demonstrate that the perfused liver was capable of excreting exogenous substances during perfusion.

5.7 SUMMARY

The preliminary results of the isolated perfused rat liver preparations indicated that the system was capable of maintaining a liver under stable conditions for up to 4 hours. The viability of the liver was assessed by measurement of perfusion pressure, bile volume production, blood gases and hepatic oxygen uptake. The degree of trauma caused by the system was demonstrated by measurement of plasma free haemoglobin and perfusate haematocrit. Significant alterations in haematocrit were not apparent and although significant increases in plasma free haemoglobin were detected, these were considered as acceptable (by comparison to other circuits which used conventional bubble oxygenators for blood-gas exchange) and did not warrant any further refinements to the circuit. The following chapter describes a series of investigations which studied the influence of various perfusates upon the function of the isolated perfused rat liver since previous investigations had demonstrated that diluted rat blood was unsuitable for isolated perfused rat brain preparations (Andjus et al 1967).

CHAPTER 6

OPTIMUM CONDITIONS FOR PERFUSION OF THE ISOLATED RAT LIVER

6.1 INTRODUCTION

The previous chapter described the establishment of an isolated organ perfusion system which incorporated a new miniaturised membrane oxygenator. The perfusion conditions during that preliminary series of experiments were essentially those described by Brauer et al (1951). However, the present series of experiments was designed to investigate the influence of perfusate haematocrit and of different erythrocyte sources upon liver viability.

Diluted rat blood was not considered to be the ideal medium for the planned experiments using combined liver and brain perfusions because of the large prime volume required. Little attention has focussed upon the influence of the source of erythrocytes upon the maintenance of the IPRL (Gores et al 1986, Lee and Clarke 1977). Therefore rat erythrocytes (Brauer et al 1951, Miller et al 1951), human erythrocytes (Hems et al 1966) and washed canine erythrocytes (Reidel et al 1983) were tested seperately in the perfusion medium. In addition, a series of 'control' perfusions were conducted with Krebs-Henseleit buffer to assess the viability of the liver in the absence of an oxygen carrier (Ashford and Burdette 1965, Lee and Clarke 1977). The influence of the perfusate haematocrit upon the hepatic oxygen uptake (HOU) has been reported during IPRLs (Brauer 1963, Keiding et al 1980, Reidel et al 1983) but its influence upon other tests of liver function (as described in chapter 5) has not been fully assessed and was also investigated.

6.2 MATERIALS AND METHODS

6.2.1 Surgical procedures

These have been described in detail in chapter 5.3.3.

6.2.2 Circuit description

This has been described in chapter 5.2.2.

6.2.3 Preparation of perfusates

a) The preparation of the rat erythrocyte derived perfusates (REDP) has been described in detail in chapter 5.4.1.

b) Fresh canine erythrocytes were obtained from healthy greyhounds by withdrawal of blood from the jugular vein into a sterile, heparinised 50ml syringe. The blood was mixed thoroughly after withdrawal and transferred into two 30ml sterile universal containers (Sterilin Ltd) which were centrifuged at 2.5g for 10 minutes. The plasma was removed, discarded and the red cells resuspended in cold, heparinised, Krebs-Henseleit buffer (KRBA). The two solutions were further divided into 2 universal containers and all 4 containers filled to 25ml with cold KRBA. The containers were centrifuged for another 10 minutes and the supernatant discarded. This 'washing' procedure was repeated 3 times and the cells were then resuspended in a solution of oncotically balanced KRBA at room temperature. The final volume of KRBA added to the washed erythrocytes was carefully titrated to result in the haematocrit selected for each perfusion.

Perfusates which contained washed human erythrocytes were

prepared from outdated human banked blood obtained from the National Transfusion Service in A.C.D. bags. Fifty ml of blood was withdrawn and centrifuged at 2.5g for 10 minutes and the supernatant discarded. The remainder of the protocol was identical to that described for the canine erythrocyte derived perfusate.

6.2.4 Physiological measurements

The liver weights were measured both before and after perfusion for 6 hours. Bile volume, oxygen uptake, haematocrit, perfusion pressure, flow rate and blood gases were all measured at hourly intervals during perfusion (as described in chapter 5).

6.2.5 Biochemical measurements

Samples of bile and perfusate were taken every hour and measurements of potassium, urea, bilirubin, glucose and perfusate haemoglobin were carried out upon perfusate supernatants. All the measurements were carried out upon an automated Centrificam using 0.5ml sample volumes. Perfusate free haemoglobin was measured by the direct near ultraviolet absorption technique according to the method of Harboe (1959).

6.3 EXPERIMENTAL DESIGN

The majority of IRLP and IRBP experiments reported have used perfusate haematocrits which range from 10% to 20% and these 2 values were therefore tested in all 3 groups of erythrocytes under investigation. A total of 6 perfusions each lasting for 6 hours were conducted and in addition a series of 6 perfusions were carried out with the omission of erythrocytes from the perfusate (KRBA series). Table 6.1 is a summary of the experimental design.

TABLE 6.1

	Source of erythrocytes		
PCV	Dog	Human	Rat
10%	6	6	6
20%	6	6	6

Summary of experimental design of isolated rat liver perfusions to investigate the influence of haematocrit (PCV) and erythrocyte source upon liver function. A further series of 6 perfusions was carried out with Krebs-Henseleit buffer alone (KRBA).

6.4 RESULTS

Collectively, all the livers at the start of perfusion weighed 15.88 ± 2.55g and following 6 hours of perfusion weighed 14.90 ± 3.515g (mean ± s.d.) There were no significant differences in liver weights between any of the groups nor in individual groups before and after perfusion. Measured values dependent upon liver weight were therefore expressed in absolute terms since the weights of the livers did not alter significantly before and after perfusion or between groups. General comparison of the data was carried out using single factor analysis of variance (anovar) and specific sub-groups were compared using Student's unpaired t-test. The results of all the measurements are includued in Appendices C1 to C6.

6.4.1 Physiological measurements

Figure 6.1 demonstrates the influence of haematocrit upon bile volume in all 3 erythrocyte groups. It can be seen that bile volume production was constant from 1 to 3 hours of perfusion and declined thereafter. The dog erythrocytes produced the lowest volumes of bile and no significant differences ($p \geq 0.05$, anovar) were noted in this group amongst the 3 haematocrits (graph 6.1b). During the first 3 hours of perfusion the dog erythrocyte group produced smaller volumes of bile than rat ($p<0.05$, Student's unpaired t-test) and human ($p<0.05$, Student's unpaired t-test). Also during the first 3 hours in the human and rat erythrocyte groups, but not the dog, the 20% haematocrit group produced higher volumes of bile than lower haematocrit ranges ($p<0.05$, anovar). The human and rat erythrocytes produced higher volumes of bile than the dog erythrocytes after 2 and 3 hours of perfusion ($p<0.05$, anovar).

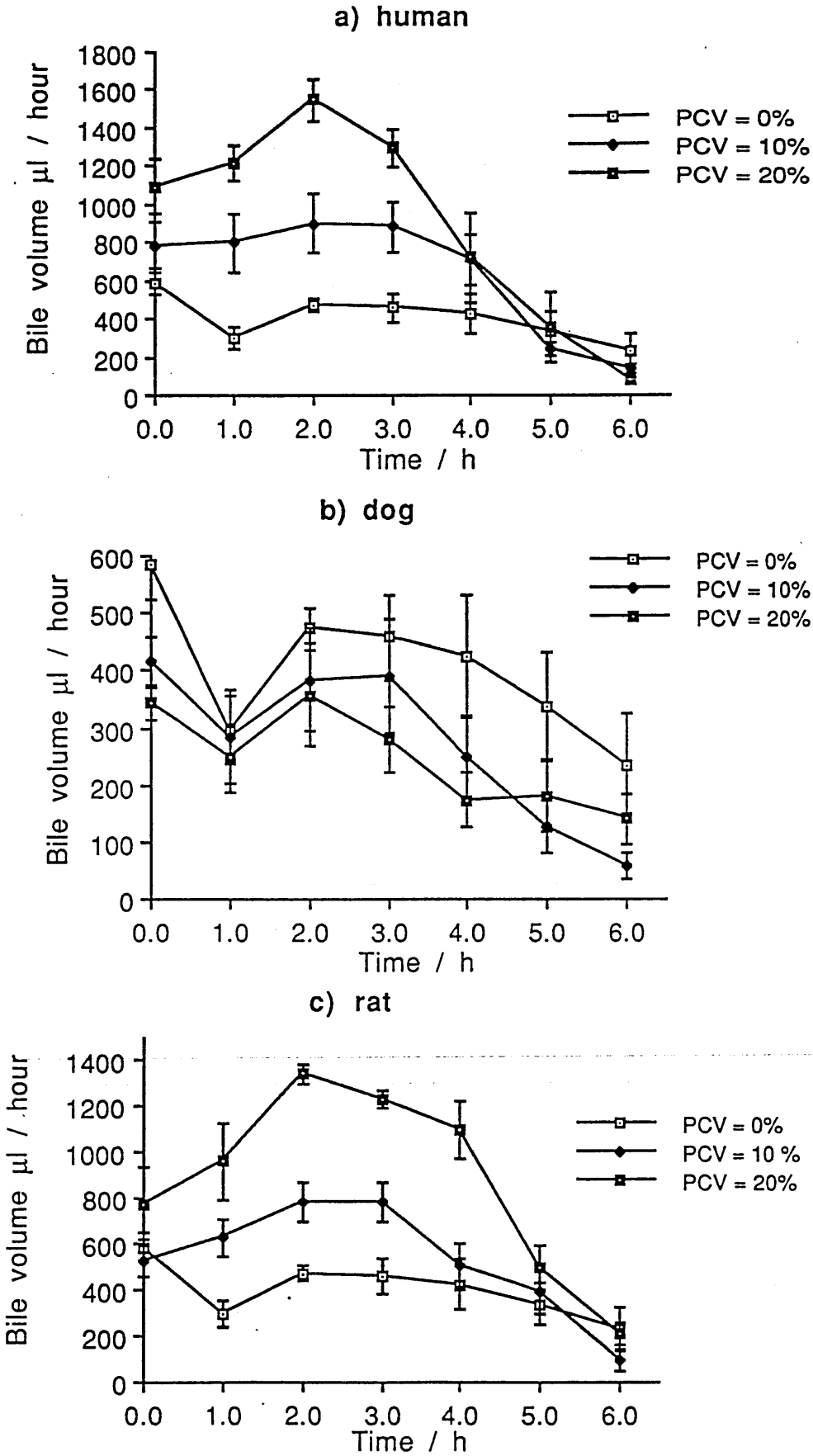


Figure 6.1 Bile volume production during isolated rat liver perfusions. Values shown are means ± standard error (n = 6 per group). See appendices B1 and C1 - C6.

Figure 6.2 demonstrates the influence of haematocrit upon oxygen uptake. The KRBA (PCV = 0%) perfusate group produced significantly lower HOU values than all the other groups. The first 3 hours of perfusion showed no differences in HOU between any of the different erythrocyte species and no differences between the 10% and 20% haematocrit ranges in any group ($p \geq 0.05$, anovar). Between the 4th and 6th hours of perfusion however, the dog HOUs declined progressively. The rat erythrocyte HOU was maintained throughout the perfusions at both haematocrit ranges. However, the human HOU at the 10% haematocrit range declined from 3 hours onwards and was significantly lower than the 20% haematocrit at 6 hours of perfusion ($P<0.05$). Also the 20% rat erythrocytes had higher HOU values than the 20% dog erythrocytes ($p<0.05$) after 6 hours of perfusion.

There were no significant changes during perfusion in perfusate haematocrit, liver weights, perfusion pressure or flow rate and therefore these results were not plotted but are included in the appendices.

6.4.2 Perfusate biochemistry

Figure 6.3 shows a gradual rise in perfusate free haemoglobin (PFH). The highest levels of PFH were measured in the dog erythrocyte group and during perfusion all the higher haematocrit ranges had the highest concentrations of PFH. The dog erythrocytes had the highest levels of haemolysis and the highest concentrations of PFH after 4 hours of perfusion ($p<0.001$, anovar).

Figure 6.4 shows the perfusate urea concentrations during perfusion in all 3 groups. Pre-perfusion concentrations of perfusate urea were higher in the rat group because haemodiluted rat blood was

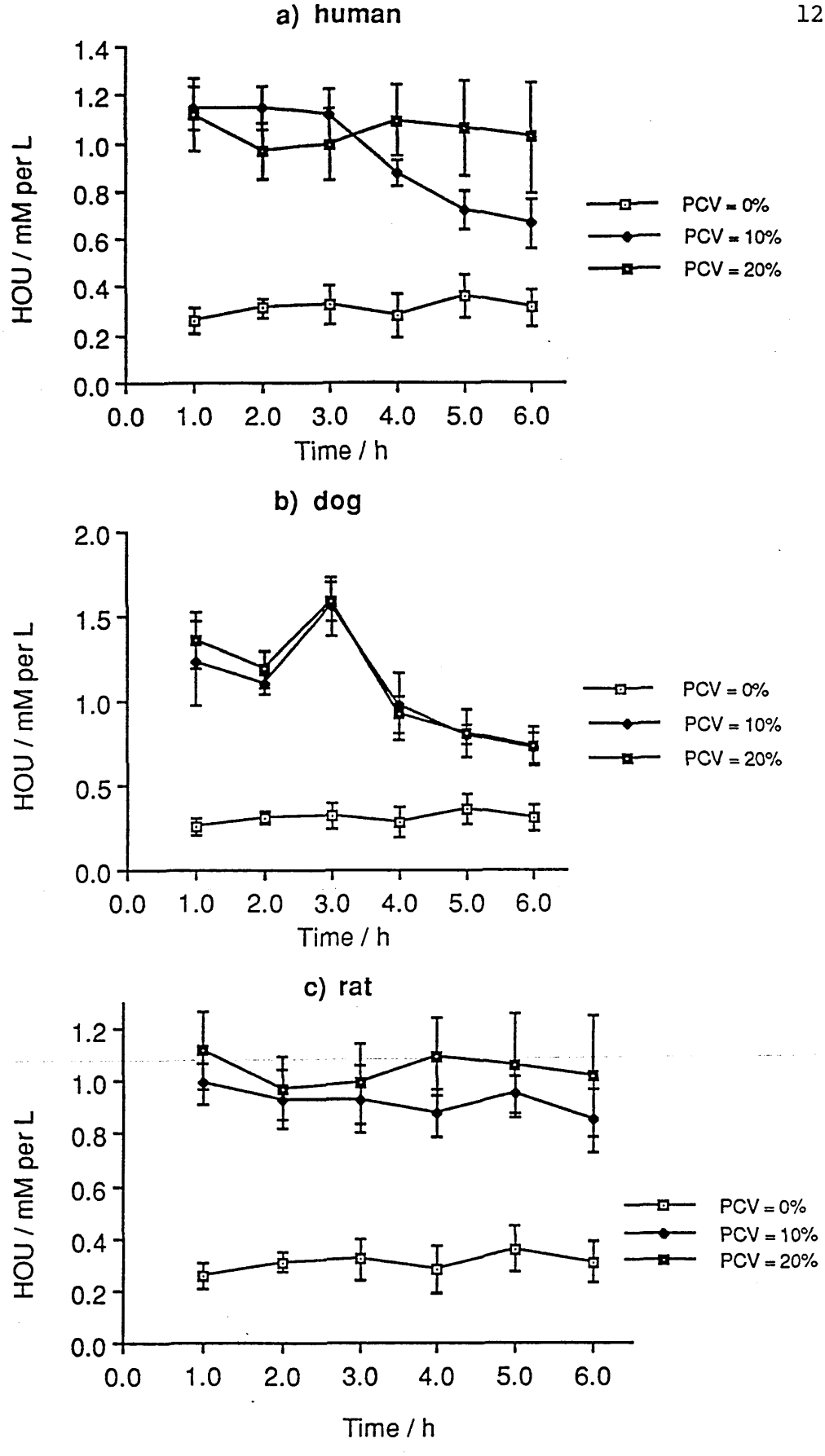


Figure 6.2 Hepatic oxygen uptake (HOU) during isolated rat liver perfusions. Values shown are means ± standard error (n = 6 per group). See appendices B1 and C1 - C6.

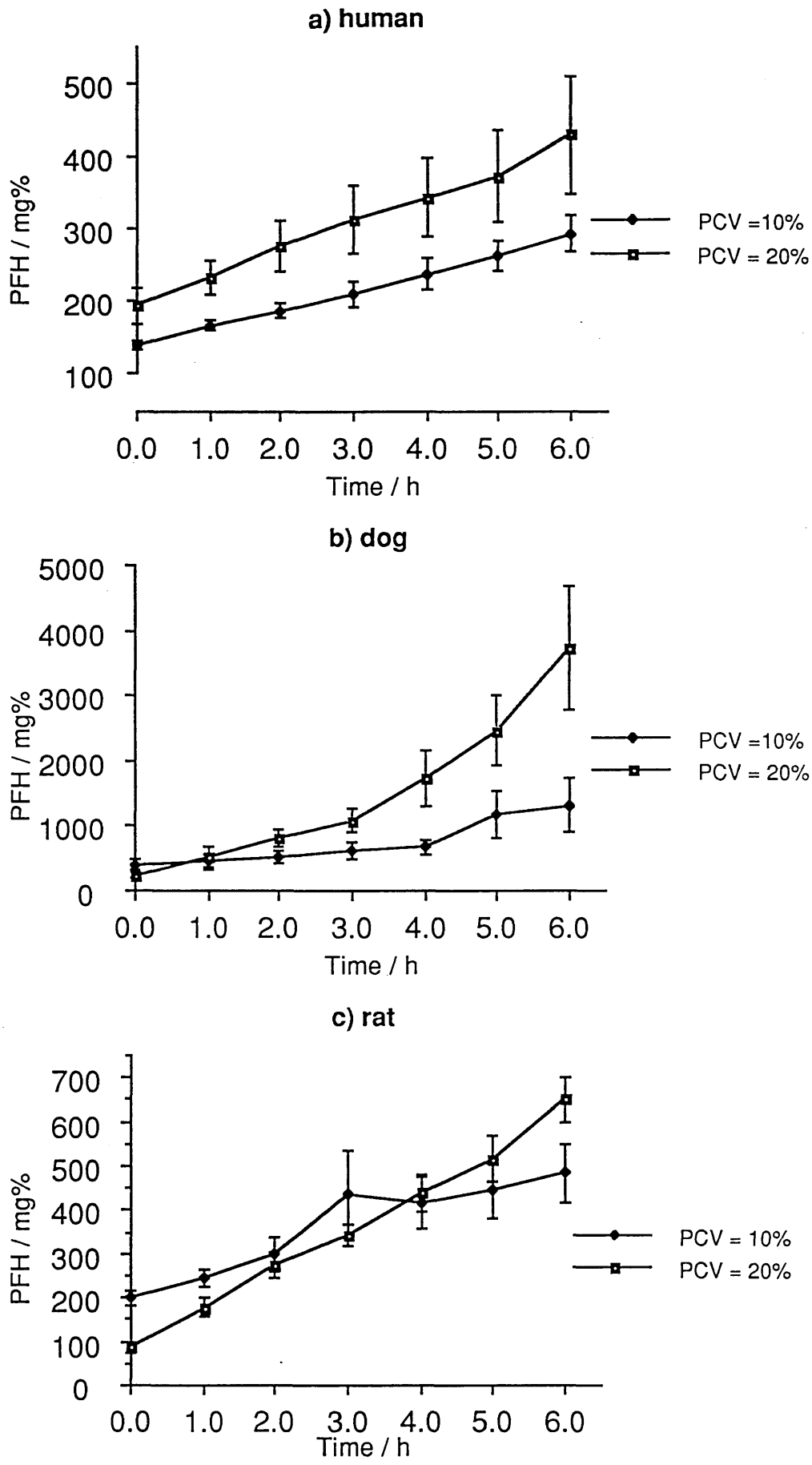


Figure 6.3 Perfusate free haemoglobin (PFH) during isolated rat liver perfusions. Values shown are means ± standard error (n = 6 per group). See appendices B1 and C1 - C6

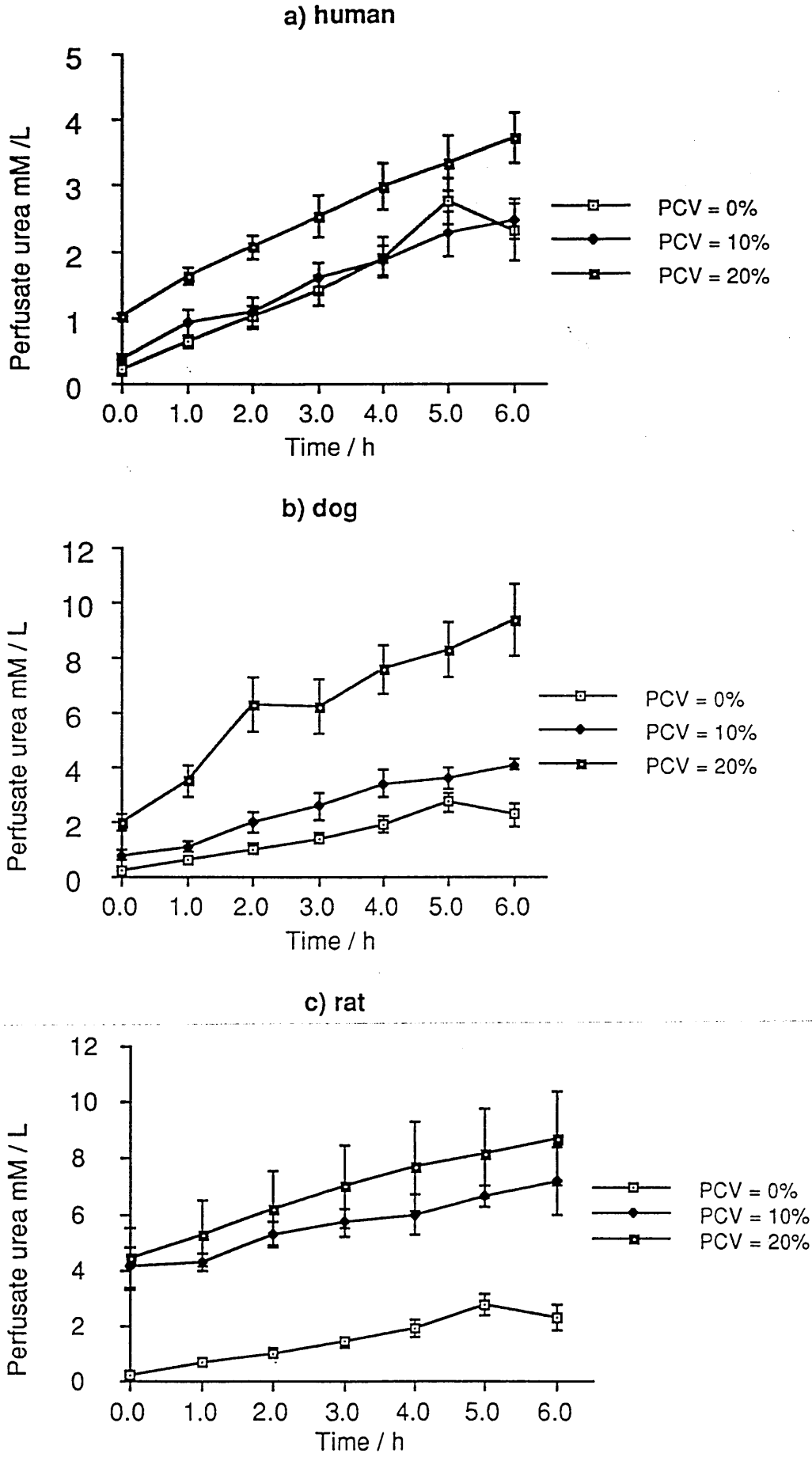


Figure 6.4 Perfusate urea concentrations during isolated rat liver perfusions. Values shown are means ± standard error (n = 6 per group). See appendices B1 and C1 - C6.

used while the other perfusates were erythrocytes suspended in KRBA. The highest concentrations were measured in the 20% dog and rat groups after 6 hours of perfusion ($p<0.05$, anovar) but there were no significant differences between the 2 groups after this time interval.

Figure 6.5 demonstrates that the 20% haematocrit range of the dog group had the highest concentrations of perfusate bilirubin of all the other groups ($p<0.001$, anovar). The KRBA group again had the lowest concentrations of bilirubin and did not increase during perfusion.

The efflux of potassium into the plasma has often been used as an indicator of hepatocyte damage during perfusion (D'Silva and Neil 1954, Bartosek et al 1973). No significant differences in plasma potassium were seen between any of the groups ($p>0.05$, anovar).

6.4.3 Bile biochemistry

The 20% haematocrit range of all the groups gave the highest concentrations of bile bilirubin after 6 hours of perfusion as shown in figure 6.6. ($p<0.001$, anovar). The dog series had the highest concentrations ($p<0.001$, anovar), but there were no significant differences between human and rat ($p\geq0.05$, anovar). The KRBA perfusion series consistently had the lowest concentrations of bilirubin although similar volumes of bile were produced. This measurement, often used as an indicator of bile quality, may be artificially elevated due to the presence of plasma free haemoglobin which has a similar absorbance spectrum.

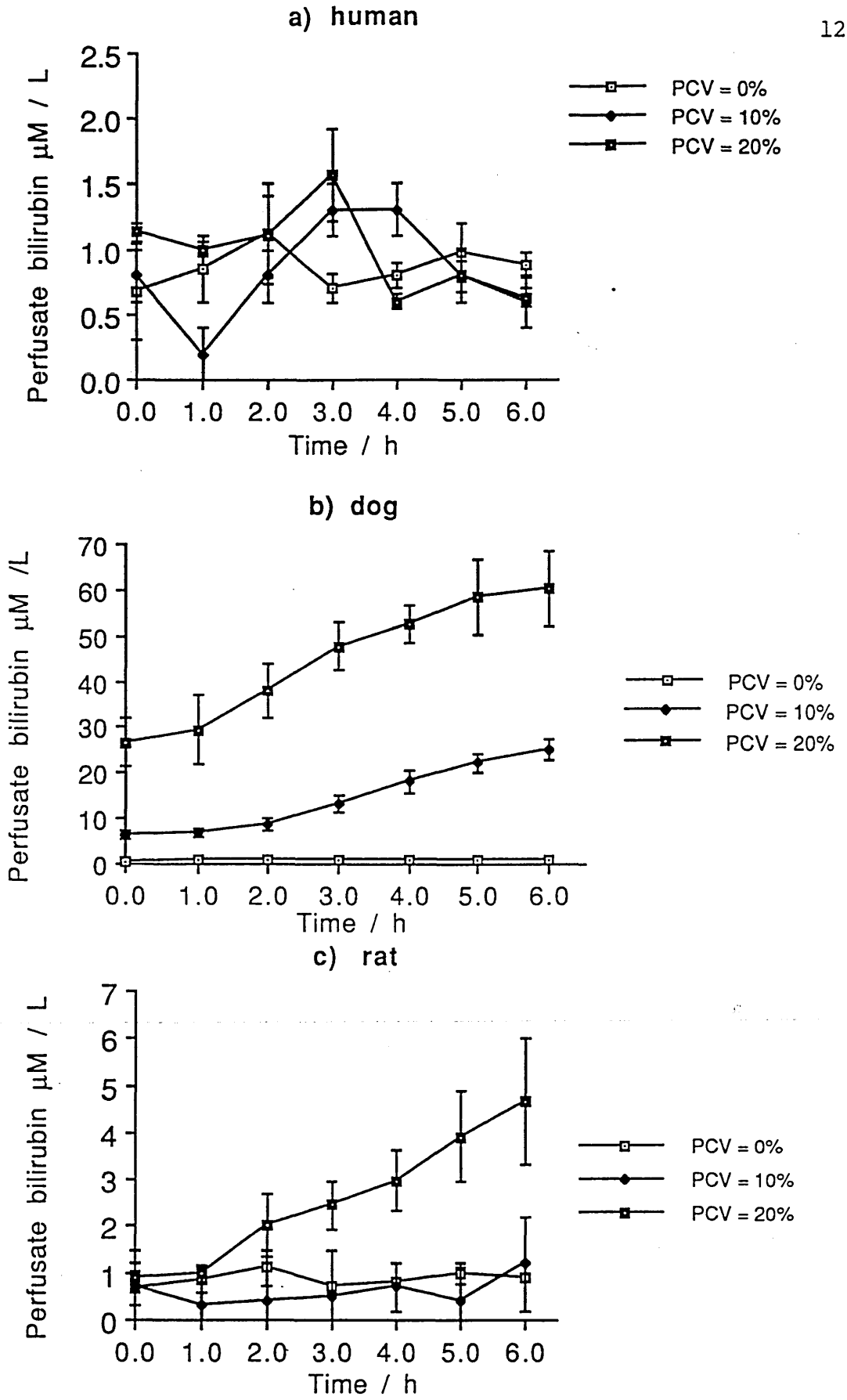


Figure 6.5 Perfusate bilirubin concentrations during isolated rat liver perfusions. Values shown are means ± standard error (n = 6 per group). See appendices B1 and C1 - C6.

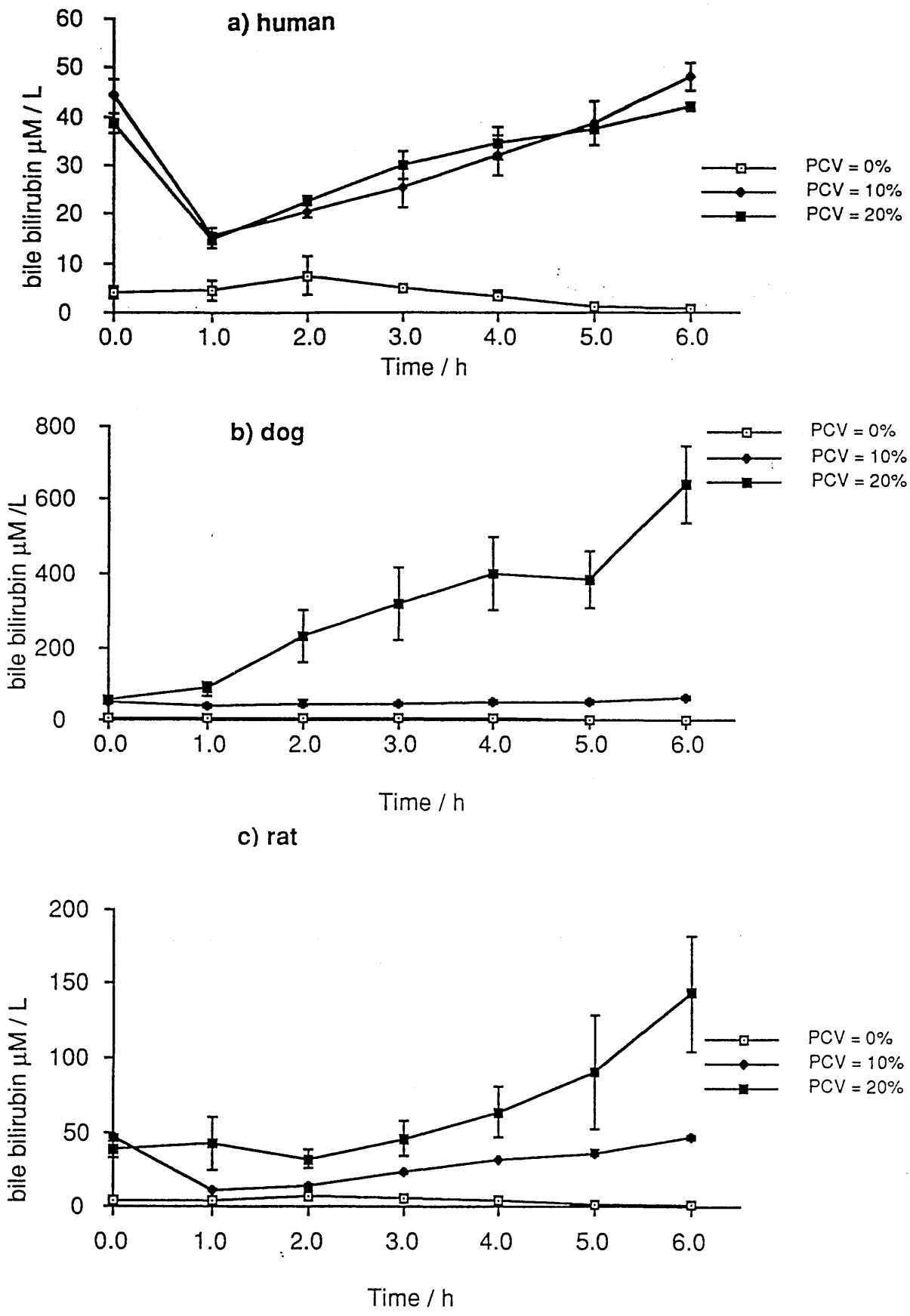


Figure 6.6 Bile bilirubin concentrations during isolated rat liver perfusions . Values shown are means ± standard error (n = 6 per group). See appendices B1 and C1 - C6.

6.5 DISCUSSION

The influence of the perfusate erythrocyte source upon the function of the IPRL has not been previously investigated. A wide variety of donor species including human, ox, horse, sheep, cat, dog and rat have been used in the past to perfuse isolated rat livers and selection was largely dependent upon availability and cost. The earliest perfusion experiments stated that the best results were obtained using homologous blood where possible (Brodie 1903) but this was restricted to larger animals. This investigation was confined to the commonly used rat and human erythrocytes (Brauer 1951, Collins and Skibba 1980, Hems et al 1966) and canine erythrocytes.

Dog erythrocytes were proposed for future use because while haemodiluted rat blood was reported unsuitable for the isolated perfused rat brain preparation (Andjus et al 1967), a spontaneous EEG could be maintained with this perfusate (Andjus et al 1967, Zivin and Snarr 1972). Later reports proved this was also suitable for isolated rat liver perfusions (Reidel et al 1983). Furthermore, canine erythrocytes were readily available in our laboratories and this enabled fresh blood to be collected just prior to an experiment. The use of fresh blood has been shown to be important during isolated organ perfusion, since aged erythrocytes exhibit reduced oxygen delivery (Rennie and Hollozosky 1977, Ruderman et al 1980).

The rate of bile production has been considered a convenient index of liver viability (Brauer et al 1951, Dobbs et al 1979, Gores et al 1986, Lee and Clarke 1977 and Schmucker et al 1975). The rates of bile production generally quoted in the literature ranged from 200µl to 490µl per hour (Boyer and Klatskin 1970, Lee and Holland 1979, Percy-Robb and Boyd 1970). The volumes of bile produced during

this series of investigations in the HEDP and REDP groups were generally higher than the measured in vivo pre-perfusion values, and were maintained for at least four hours of perfusion. Dog cells produced the lowest volumes of bile in the present series and indeed showed a decline from pre-perfusion values. This was similar to the findings of Brauer et al (1951) and Kvetina and Guaitani (1969) who reported a decrease of 60% in bile production on perfusion using haemodiluted rat erythrocytes. Brauer et al (1951) reported a value of 17.3 µl of bile per minute (1038 µl per hour) in vivo, compared to 6.3 µl per minute (378 µl per hour) on perfusion. This latter value of 384µl per hour is in contrast to peak values measured during this series of experiments of 1343µl per hour with the REDP series. Brauer's in vitro value was similar to the peak values obtained with the 20% haematocrit range of the CEDP series of 359µl per hour.

The peak values of more than 1000µl per hour obtained with the HEDP and REDP series were similar to the exceptional values reported by Schmucker and Curtis (1974) of 900-1000µl per hour. Schmucker and Curtis reported bile volumes of less than 200µl per hour during anoxic perfusion of the liver and an average of 70µl per hour after a prolonged pre-perfusion ischaemic time was reported by Lee and Clarke (1977); these lower volumes were also encountered in the present experiments after 4 hours of perfusion with the 20% haematocrit group of the CEDP series, indicating a reduction in hepatic function. It has already been noted that bile salts were present only in the REDP preparations, and the choleretic effect of these may have contributed to elevated bile volume production (Storer et al 1980). In addition the presence of bile acids may exert a synergistic effect upon HOU via the action of insulin and glucagon (Ostergaard et al 1983). However, this does not explain the unusually high bile volume production in the

HEDP series.

When these studies began, there was little information regarding the significance of perfusate haematocrit upon maintenance of the IPRL. Several reports have now shown that alterations in perfusate haematocrit affect HOU and liver function (Collins and Skibba 1980, Keiding et al 1980, Riedel et al 1983, Thompsen and Larsen 1983). Keiding et al (1980) suggested that lower perfusate haematocrits may cause a reduction in liver function and possibly oxygen uptake which cannot be compensated by physiological increases in flow rate. Our data showed that the highest HOU values were measured in the 10% haematocrit series of the CEDP group (1.59+_0.12uM/min/g liver) but these were not significantly different from the 20% haematocrit group of the same series after 4 hours of perfusion. In summary our results did not show a reduction in HOU with the 2 different haematocrits used. This may have been due to the adoption of a fixed perfusion pressure technique as described by Riedel et al (1983) who suggested that increases in perfusate haematocrit may in fact reduce HOU. This was because these values lay on the flow dependent portion of the oxygen consumption vs blood flow curve and that a substantial reduction in flow rate was necessary to maintain physiological perfusion pressure at these high haematocrits. Riedel also suggested that the higher HOU values recorded with higher haematocrits described by Brauer (1963), Keiding et al (1980) and Storer et al (1980) lay on the flow independent part of the curve and were thus calculated from preparations using excessive, non-physiological perfusion pressures to maintain the considerably higher flow rates of 90-100 ml/min/100g liver. Riedel concluded that an optimal haematocrit of 20% combined acceptable blood flow, perfusion pressure and oxygen carrying capacity. A constant flow preparation was adopted for these studies

rather than a constant pressure/passive flow preparation so that the influence of haematocrit could be investigated at identical flow rates, since flow rate may also influence hepatic viability (Brauer 1963, Gores et al 1986). Our studies confirmed Riedel's observations and suggested that a haematocrit of 20% did not appear to alter HOU values significantly but that haematocrits lower than 10% may reduce HOU values to approach those of the KRBA, hypoxic group.

The plasma urea and bilirubin concentrations showed similar trends to the plasma free haemoglobin estimations in the CEDP group (figure 6.3) and were possibly indicative of ruptured erythrocyte membranes (Bartosek et al 1972). In general the higher haematocrit ranges of all the perfusion groups gave the highest concentrations of PFH after 6 hours of perfusion (Student's unpaired t-test $P \leq 0.01$). The highest concentrations of PFH were measured in the dog group after 6 hours of perfusion. Significant differences were seen for each haematocrit range between the 3 species of erythrocytes. The rank order of PFH concentrations from highest to lowest was dog > rat > human for both haematocrit ranges. It was concluded that this order of haemolysis may have been a reflection of the fragility of each erythrocyte type particularly since the fragility of canine erythrocytes is well known (Miller and McDonald 1951, Ritchie and Hardcastle 1973). The changes in plasma potassium and urea concentrations were similar to those reported by Dobbs et al (1979) who used diluted rat blood as the perfusate. However, these authors did not report PFH estimations and the changes described may not have been due to haemolysis. Many reports have cited bile volume production as a reliable index of hepatic function during IRLP (Gores et al 1986). This series of investigations was conducted to determine the quality of bile produced in terms of electrolyte, bilirubin and

urea concentrations since previous reports have quoted similar rates of bile volume production under diverse perfusion conditions, including hypothermic perfusion, hyperbaric perfusion and with different perfusate haematocrits (Keiding et al 1980, Lee and Walker 1977, Schmucker et al 1975). The quality of the bile produced under these conditions however has not been measured and such changes may be a more valid index of hepatic function than bile volume production.

All the data were considered in order that a suitable perfusate could be selected for future perfusions of the liver and brain. Andjus et al (1967) showed rat erythrocytes to be unsuitable for the maintenance of the spontaneous EEG in the isolated perfused rat brain but found dog erythrocytes to be a suitable alternative. Although the canine erythrocytes produced the lowest bile volumes at 20% haematocrit, bile production was well maintained throughout the liver perfusions. These factors along with the easy availability of dog erythrocytes permitted their inclusion in the perfusate for future investigations.

6.6 SUMMARY

Having established the limitations of using washed canine erythrocytes in the perfusate for IPRLs it was now necessary to confirm that this was a suitable perfusate for an isolated perfused rat brain preparation. The following chapter describes the evaluation of a new isolated perfused rat brain preparation which enabled an accurate measurement of the survival time in vitro to be made before a perfused liver was included in the circuit.

CHAPTER 7

EVALUATION OF A NEW ISOLATED PERFUSED RAT BRAIN PREPARATION

7.1 INTRODUCTION

This chapter describes an isolated rat brain perfusion technique based upon a modification of the technique described by Thompson et al (1968). New designs of EEG electrodes were used to reduce extraneous electrical noise together with a new perfusion circuit modelled upon a minor modification of the circuit described in chapter 6. Various parameters, which were anticipated to influence the survival time of the brain during perfusion, were investigated and optimised to result in a reproducible model. Various factors such as the influence of the type of anaesthesia used (Krieglstein et al 1972), the source of perfusate erythrocytes (Rennie and Holloszy 1977) and the effects of total cervical dislocation upon the quality of the recorded EEG signal (Andjus et al 1967) were assessed and a standard survival time of an in situ isolated perfused rat brain was established.

7.2 MATERIALS AND METHODS

The perfusate used for all the isolated brain perfusion experiments was a 20% haematocrit perfusate, containing canine erythrocytes and is described in detail in chapter 6 (section 6.2.3) of this thesis. Briefly, 50 ml of blood was withdrawn from healthy greyhounds and heparinised. This was centrifuged and the plasma discarded. The remaining red cells were resuspended in cold, heparinised, KRBA and recentrifuged. The procedure was repeated twice more after which the red cells were resuspended in an oncotically balanced solution of KRBA containing 6g per 100ml bovine serum albumin.

Perfusate glucose concentrations were measured during the perfusions using 'Dextrostix' (Ames Co. Ltd., U.K.). through visual assessment and confirmed retrospectively by the glucose oxidase/peroxidase principle (Randox Laboratories Ltd, N.Ireland) upon a R.A. 1000 autoanalyser system (Technicon Ltd, U.K.). EEG measurements were carried out using a Siemens 'Myngograph' EEG monitor (Siemens Ltd, U.K.). Blood gases and perfusion pressures were measured as described in chapters 4 and 5 of this thesis.

7.3 IN VIVO EVALUATION OF AN EEG SIGNAL

7.3.1 EEG signals from conventional designs of surface and sub cortical electrodes

Rat EEGs are usually recorded from platinum or silver wire electrodes which rest upon the surface of the cranium or are implanted sub-cortically at the level of the dura mater. Table 7.1 is a summary of the normal range of EEG measurements which should be readily obtained from these types of electrodes. In order to obtain a clear trace however, the electrodes should fulfil the following criteria:

1) Produce a high signal to noise ratio.

2) Enable brain death to be easily recognised.

3) Remain secured in position.

4) Provide easy recognition of each waveform type.

Initial trials were carried out with various designs of conventional EEG electrodes constructed from platinum or silver wire, according to the technique of Sayers and Stille (1969), implanted sub-cortically with the aid of a stereotactic stand made in the workshops of the Royal Postgraduate Medical School (figure 7.1). The neurological authenticity of the recorded EEG was evaluated by investigation of the effect of incremental doses of pentobarbitone, leading up to a lethal dose. However recordings from conventional EEG electrodes implanted sub-cortically were severely contaminated by extraneous electrical

waveform	frequency range	
α	13.2	32.0 HZ
β	7.6	13.2 HZ
θ	4.0	7.6 HZ
δ	0.98	4.0 HZ

Table 7.1 Spectrum of EEG activity.

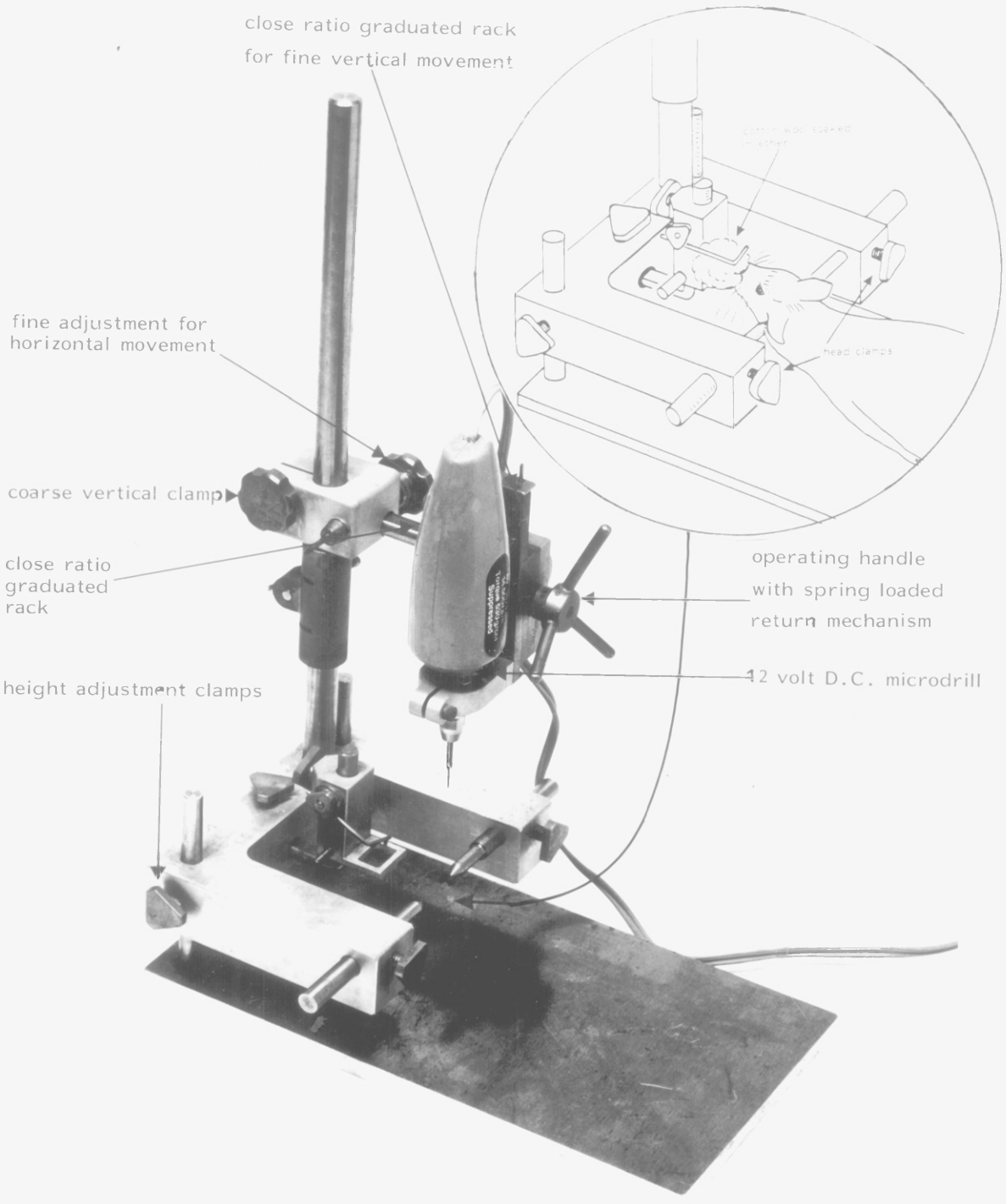


Figure 7.1. Stereotactic frame

noise from mains and other non-specific interference (figure 7.2a). Improvements to the earthing electrodes did not improve the quality. Andjus et al (1967) reported satisfactory signals using surface cortical electrodes. Our results using this technique are illustrated in figure 7.2b and were considered unsuitable due to the low signal to noise ratio. Attempts to isolate the noise contamination by the use of various screening and earthing techniques proved unsuccessful and a new design of sub-cortical electrode was therefore constructed and tested.

Figure 7.3(a) is an illustration of the final configuration of electrode assembly adopted which was tested in a series of 10 anaesthetised rats (section 7.2.4). It produced the highest signal to noise ratio and fulfilled all the above criteria. The electrodes were constructed from 30 gauge hypodermic syringe needles and were embedded into an acrylic template, 3.5mm apart and 3.5mm long. The purpose of the acrylic template was to secure the electrodes firmly into position and to prevent accidental movement of the electrode during the experiments and also to prevent the transmission of muscular activity artefact to the electrodes. The following sections of the chapter describe the surgical preparations for electrode insertion and the testing of the new electrode assembly in vivo and in vitro during isolated rat brain perfusions.

7.3.2 Surgical preparation for electrode insertion

250-300g male Sprague-Dawley rats were anaesthetised with ether, and for some studies, maintained upon iv sodium pentobarbitone. The new electrode was inserted subcortically since this produced a stronger and less contaminated signal. Once anaesthetised, a ventral

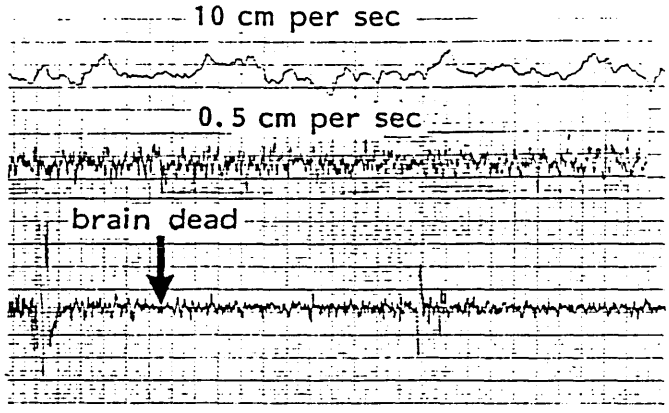


Figure 7.2(a) EEG trace with conventional electrodes inserted subcortically.

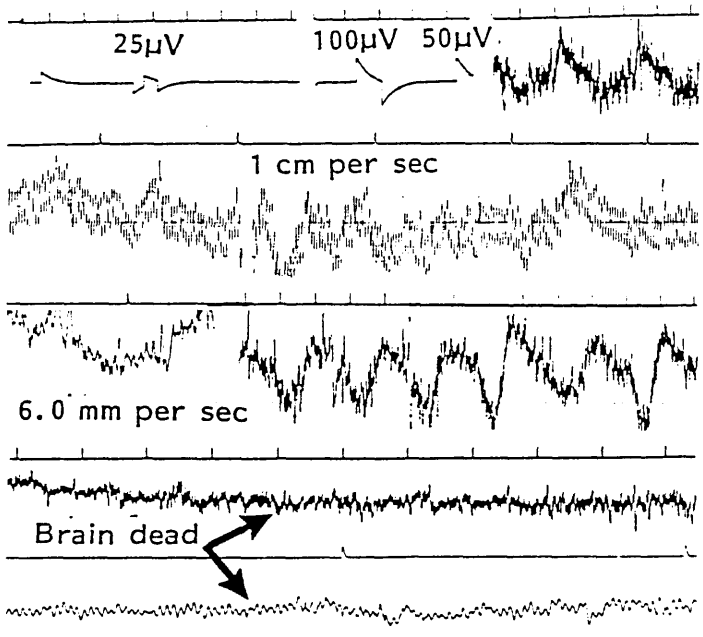


Figure 7.2(b) EEG trace with conventional electrodes showing surface cortical activity.

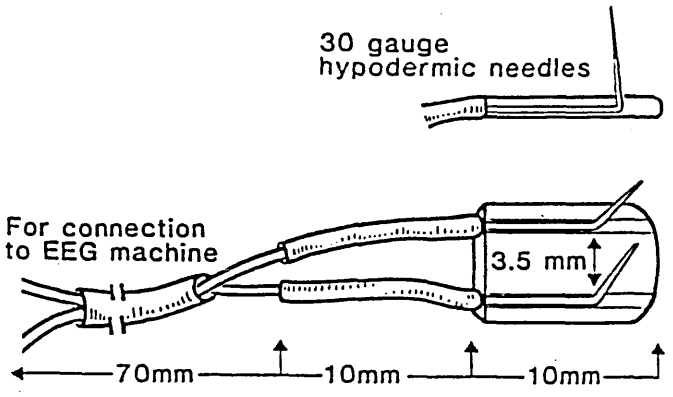


Figure 7.3(a) Design of the new EEG electrode assembly.

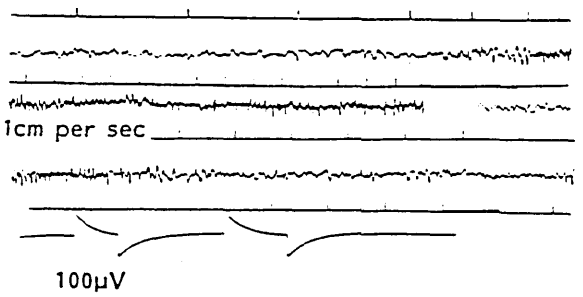


Figure 7.3(b) EEG trace with new design of electrode assembly.

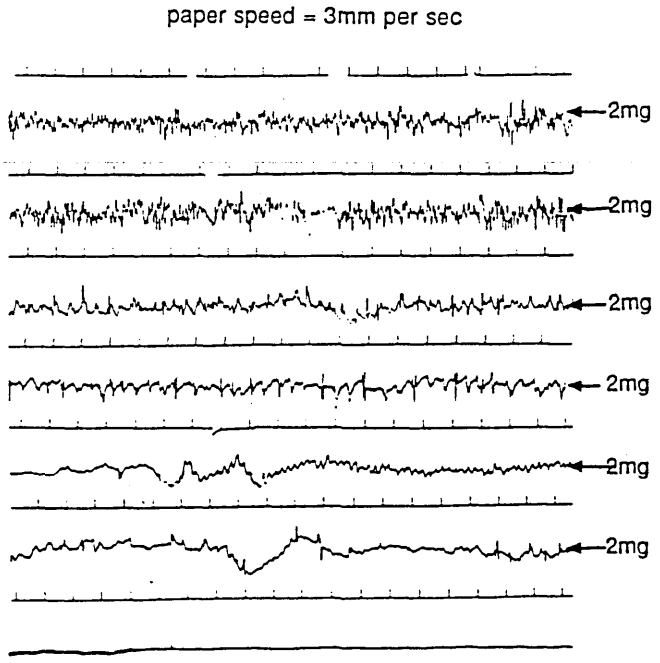


Figure 7.3(c) Influence of incremental doses of sodium pentobarbitone.

midline incision was made along the trachea, the skin retracted and the carotid arteries exposed for cannulation at a later stage if the brain was to be perfused. Some rats had the right jugular vein exposed and cannulated for barbiturate anaesthesia (sections 7.2.1 and 7.2.4). The rat was now turned on its abdomen and placed into the stereotactic stand so that the electrode assembly could be inserted into the cranium. The stand was modified to secure a microdrill (Radio Spares Ltd) which could be raised and lowered accurately upon a calibrated vertical rack.

A sagittal, dorsal incision was made along the length of the head, down through the periosteum. The skin was retracted with artery forceps, and the cranium exposed. Two holes were carefully drilled into the skull, 3.5mm apart, with a 600μM diameter drill, to seat the electrode assembly. During the drilling procedure great care was taken to prevent rupture of the dura by the use of the calibrated marks on the vertical rack and also through feeling less resistance to drilling once through the skull. This procedure ensured a close fit of the electrodes into the holes and thus prevented unnecessary movement. An electrode was positioned centrally, front-to-back, upon each cerebral hemisphere.

The new electrode assembly was placed into position over the skull and pushed into the drilled orifices. The length of the electrodes and the distance they projected into the skull was calculated from previous measurements of thickness of several skulls from dead rats. Care was taken not to rupture the dura during insertion of the template into position (figure 7.3.a). The skin retractors were removed from the cranial surface and the electrode assembly was secured into position with 3/0 sutures through the skin.

7.3.3 Results with a new design of electrode

Figure 7.3(b) is a typical example of the signal obtained with the new electrode configuration. The 100μvolt calibration signal on the trace confirmed that the range of signals recorded had amplitudes in the appropriate range of 10 to 100μ volts (Dirks et al 1980). The noise and contamination levels were reduced to values below 5μ volts, best illustrated by the faster paper speed setting of 15mm per second (top trace figure 7.3.b).

7.3.4 Effect of anaesthetic upon EEG trace

An investigation was now conducted to assess EEG changes which occur during narcosis with sodium pentobarbitone. This was carried out to determine the contribution of background noise to the trace and also to assess the sensitivity and response of the EEG trace to gradual increases in the depth of anaesthesia.

Figure 7.3(c) illustrates the EEG response to incremental doses of pentobarbitone using the new design of electrode pad. The signals varied in amplitude from 48.5±11 to 195±46μV (mean ±s.d.) with frequencies ranging from 0.5 to above 12Hz as assessed visually. Two milligram doses of the anaesthetic were given intravenously through the jugular vein for approximately one hour until the lethal dose was reached after which the EEG trace deteriorated to an amplitude of 3u volts.

7.4 MEASUREMENT OF THE SURVIVAL TIME OF AN ISOLATED PERFUSED RAT BRAIN

The previous series of experiments demonstrated the various changes in EEG which are encountered during anaesthesia with sodium pentobarbitone. The depth of anaesthesia was light immediately prior to commencement of perfusion in order that a stable EEG pattern could be maintained.

Isolated brain perfusion is achieved by cannulation of the carotid arteries and collection of effluent from the jugular veins. This may be accompanied by transection of the spinal cord and all soft tissue of the neck and complete separation of the head, or these steps may be omitted and 'in situ' isolated perfusion carried out.

Initially the former procedure was adopted as described by Gilboe et al (1964). In a series of 5 such totally isolated brain perfusion experiments we found that the majority of activity from 60 to 120 minutes of perfusion was confined to theta and delta waves. However, interruption of perfusate flow did not show any significant effect upon the trace until 16 minutes after the pump was switched off. Other workers have shown that an interruption of perfusate flow for time periods longer than 2 minutes was sufficient to depress EEG activity permanently (Andjus et al 1967, Krieglstein et al 1972). The validity of the recorded EEG signal in this preparation was therefore questionable. This problem was not encountered with the 'in situ' isolated perfusion technique and this method was used for the remainder of the studies. The technique is described in detail below.

7.4.1 Surgical isolation of the cerebral vascular supply and its influence upon the EEG

An investigation was conducted to determine whether any EEG changes could be attributed to the cannulation procedure. 250-300g male Sprague Dawley rats were anaesthetised with ether and maintained upon low doses of pentobarbitone. The electrode template was inserted as described in section 7.2.2 and the rats were removed from the stereotactic stand and then placed upon the perfusion table (see figure 7.4(a)). The table was composed of two flat sheets of acrylic held together by a universal joint to facilitate tilting of the table to any desired angle. In addition, channels were machined into the upper surface of the table to permit drainage of the perfusate into the reservoir.

The midline vertical incision, mentioned earlier for exposure of the carotid arteries and cannulation of the right jugular vein, was re-entered and the skin retracted to expose the trachea. The sternohyoid muscle was also retracted on both sides of the trachea to expose the left and right carotid arteries which lay alongside the thyrohyoid muscles.

The carotid arteries were carefully dissected out, exposed and two 5/0 silk ligatures placed loosely under each vessel ready for cannulation. Each carotid artery was cannulated with a 3 F.G. cannula (pink) (Portex, Hythe, Kent). Perfusion was commenced immediately after each vessel had been cannulated (to avoid occlusion of the carotid vasculature and risk deterioration of the EEG trace). Following cannulation of both vessels, perfusion was temporarily halted so that the two cannulae were connected together in parallel

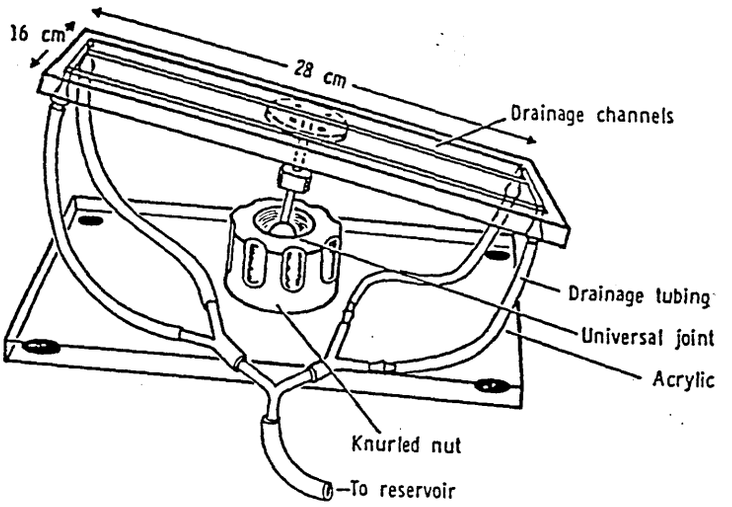


Figure 7.4(a) Diagram of brain perfusion table.

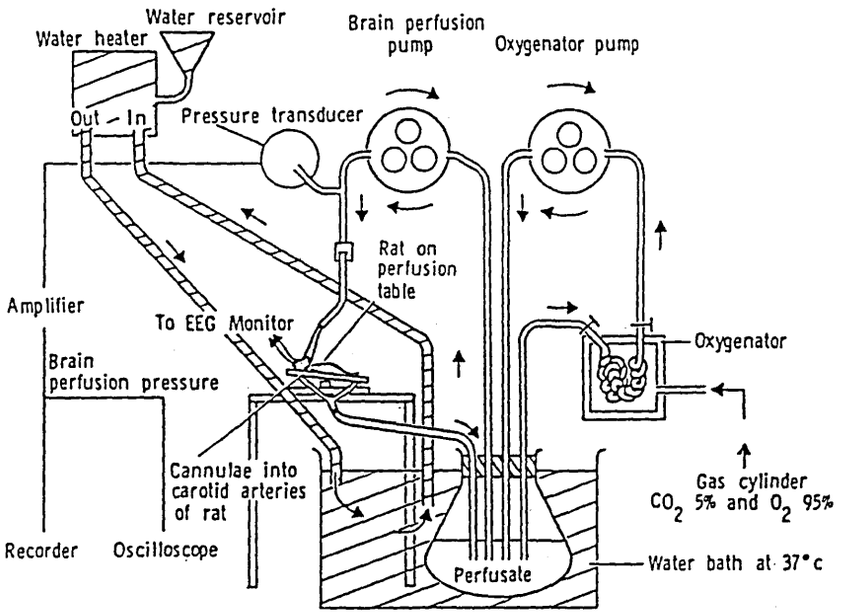


Figure 7.4(b) Diagram of isolated rat brain perfusion circuit.

to a luer 'Y' connector which was attached to the perfusion circuit. Perfusion was carried out at a fixed arterial perfusion pressure measured by a transducer in the arterial line (type 4-422-0001 Bell and Howell Ltd, England) which was connected to an SEM four channel medical recorder (SE Laboratories Ltd, Middlesex, England). The flow rate was determined previously by the perfusion pressure measured at various pump flow settings with the perfusion cannulae attached. The perfusate flow rate was increased until a perfusion pressure of 100-120mmHg was attained. This approximated to a flow rate of 2.5-3.0ml per minute. A lateral incision was made on each side of the head to transect the external jugular veins in order to exsanguinate the rat and permit drainage of the venous blood from the brain. Finally, a stout ligature was tied around the aorta and the inferior vena cava and the heart excised.

The perfusate used was similar to that described by Andjus et al (1967) and Zivin and Snarr (1972) and was described previously (section 7.2). The final haematocrit of perfusate was 20-25% since lower haematocrit perfusates were reported and demonstrated to be detrimental to the survival of the isolated perfused rat brain (Thompson et al 1968). In addition, chapter 6 also demonstrated that this was the optimum haematocrit for the maintenance of IPRL preparations.

The perfusion circuit used (figure 7.4(b)) included two separate circuits in parallel sharing a common perfusate reservoir, which was immersed in a water bath at $37^{o}C$. Perfusion pressure was monitored by a pressure transducer in the perfusate delivery line.

Figure 7.5 shows that few changes could be detected in the pattern of the EEG during the cannulation procedure. Cannulation of

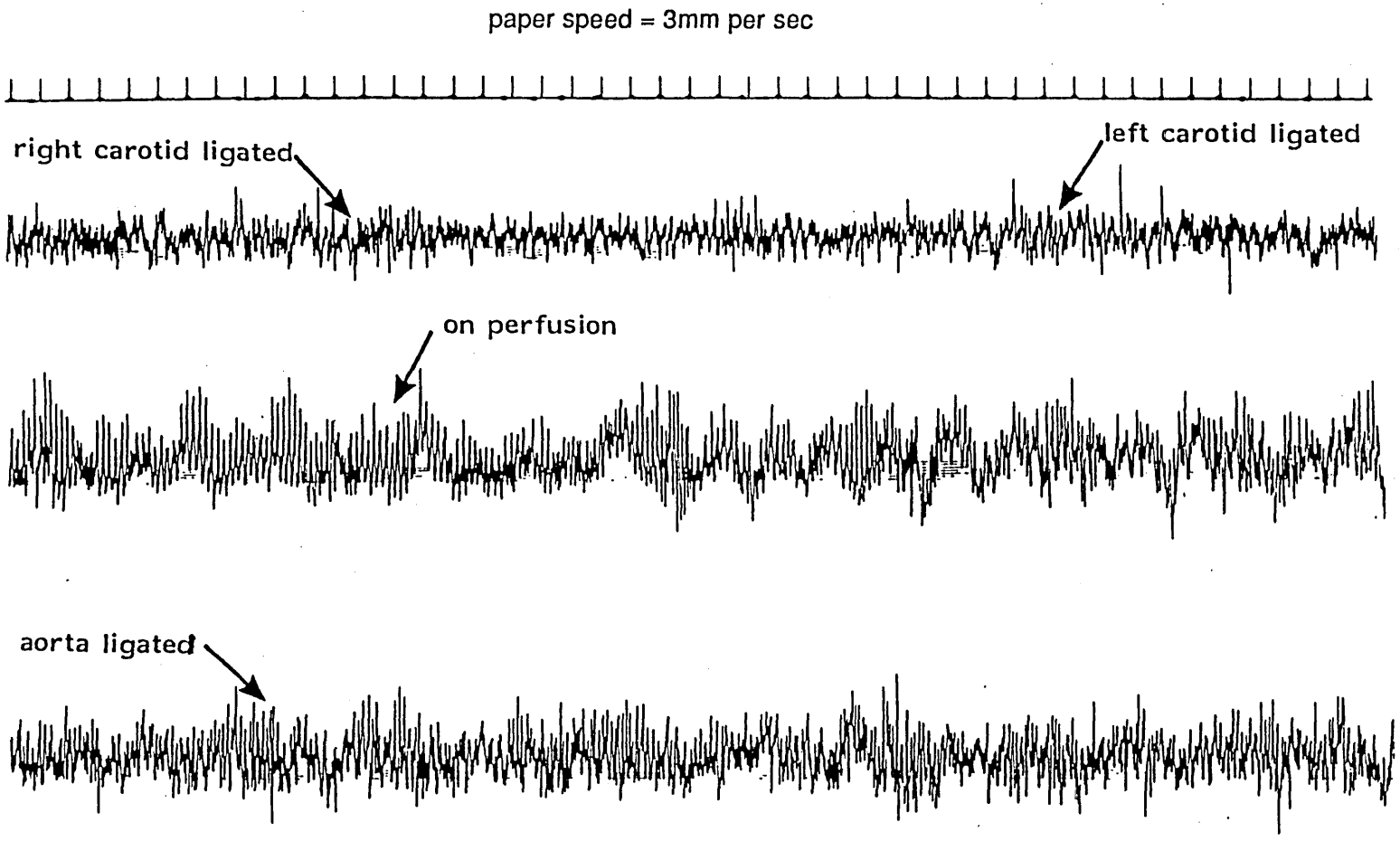


Figure 7.5 EEG trace recorded during the surgical preparation of the rat for perfusion.

the left and right carotid arteries produced little change and indeed other tests demonstrated that the EEG did not alter until at least fifteen minutes had elapsed after cannulation without perfusion.

7.4.2 Establishment of basic survival time of signal

To assess the quality and sensitivity of EEG signals recorded during perfusion of the isolated brain. the following standard criteria were adopted:

1) The recorded signal should include the range of frequencies and wave velocities quoted in figure 7.2(c).

2) A progressive and not abrupt reduction in the recorded signal should be observed upon termination of perfusate flow.

3) A gradual increase in the signal strength up to the original amplitude should be observed upon resumption of flow.

Six perfusions were conducted to demonstrate signal authenticity and also to establish the range of waveforms and frequencies to be expected during perfusion. It was found that if the perfusate flow was stopped for periods of more than one minute although the EEG returned on restoration of flow, the quality of the signal deteriorated progressively on each occasion. Such checks were therefore conducted infrequently. Figure 7.6 demonstrates the 4 frequency ranges and types of waveforms encountered during perfusion. The top trace was expanded to a faster paper speed of 5 cm per second in order to illustrate the faster frequency range of alpha waves.

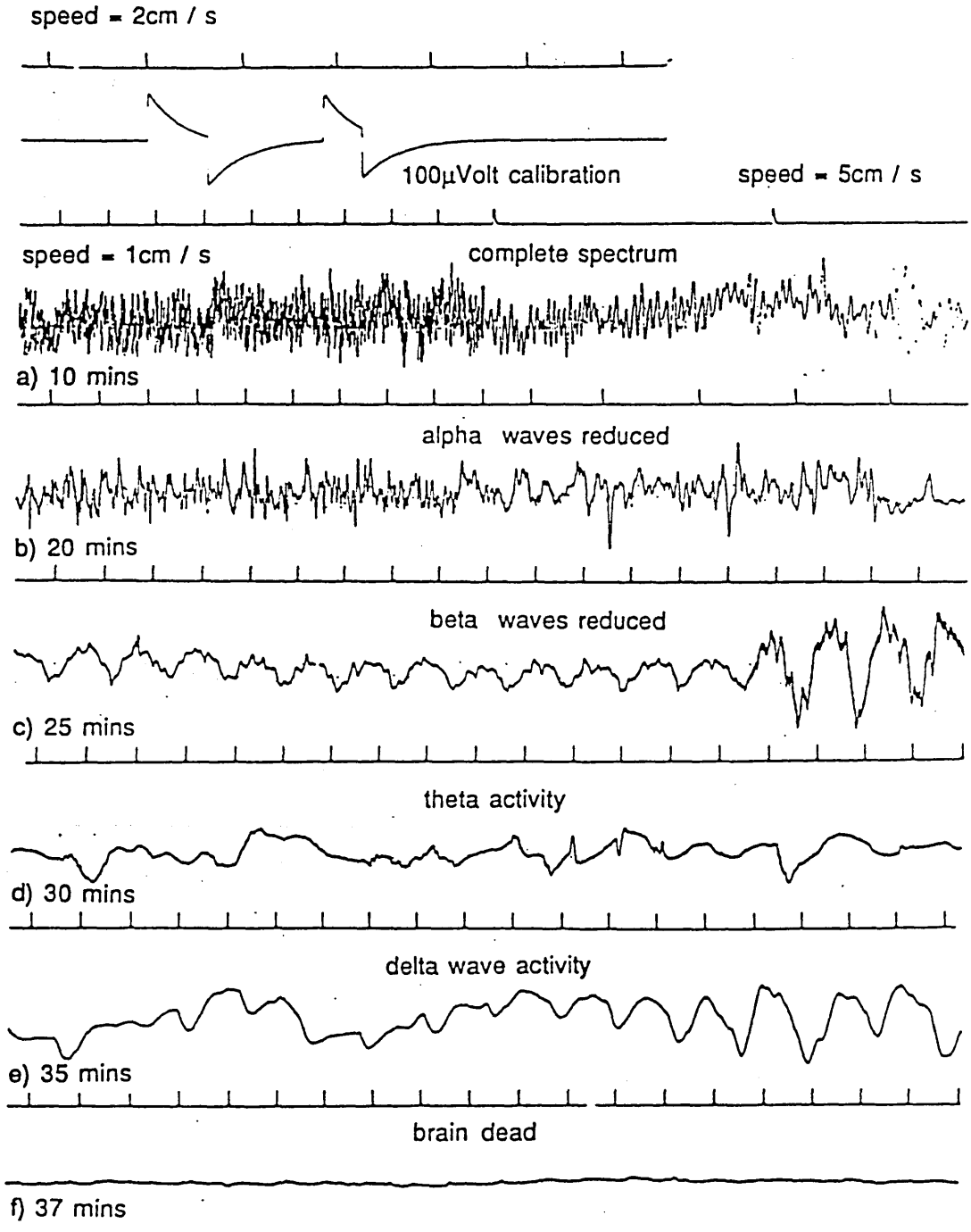


Figure 7.6 Range of wave amplitudes and frequencies seen during a typical isolated rat brain perfusion experiment.

Figure 7.7 shows an EEG trace recorded during perfusion and illustrates how the standard criteria described earlier were fulfilled. Initially the waveform was characterised by the presence of the full range of signals as described in figure 7.6 with waves ranging in amplitude from 12.5 to 155 μvolts. The EEG signal declined gradually on termination of perfusate flow, and then required a further 10 seconds to recover to the original amplitude after perfusion was re-established. Brain death was defined as the progressive deterioration of the EEG trace to a horizontal line or random background pattern of less than 5μvolts in amplitude which did not alter upon interruption of perfusate flow (Andjus et al 1967). The median survival time of the brain without interruption of flow was 35 minutes (range 22-53 minutes, n = 12): these results are presented in table 7.2 overleaf.

Table 7.2.

Perfusion No.	Survival time / minutes
015	33
023	30
025	37
029	42
031	35
032	38
034	43
036	34
049	35
065	53
072	22
073	31

Survival times of the 12 isolated rat brain perfusions using sodium pentobarbitone anaesthetic. Median survival time was 35 minutes (range 22-53 minutes).

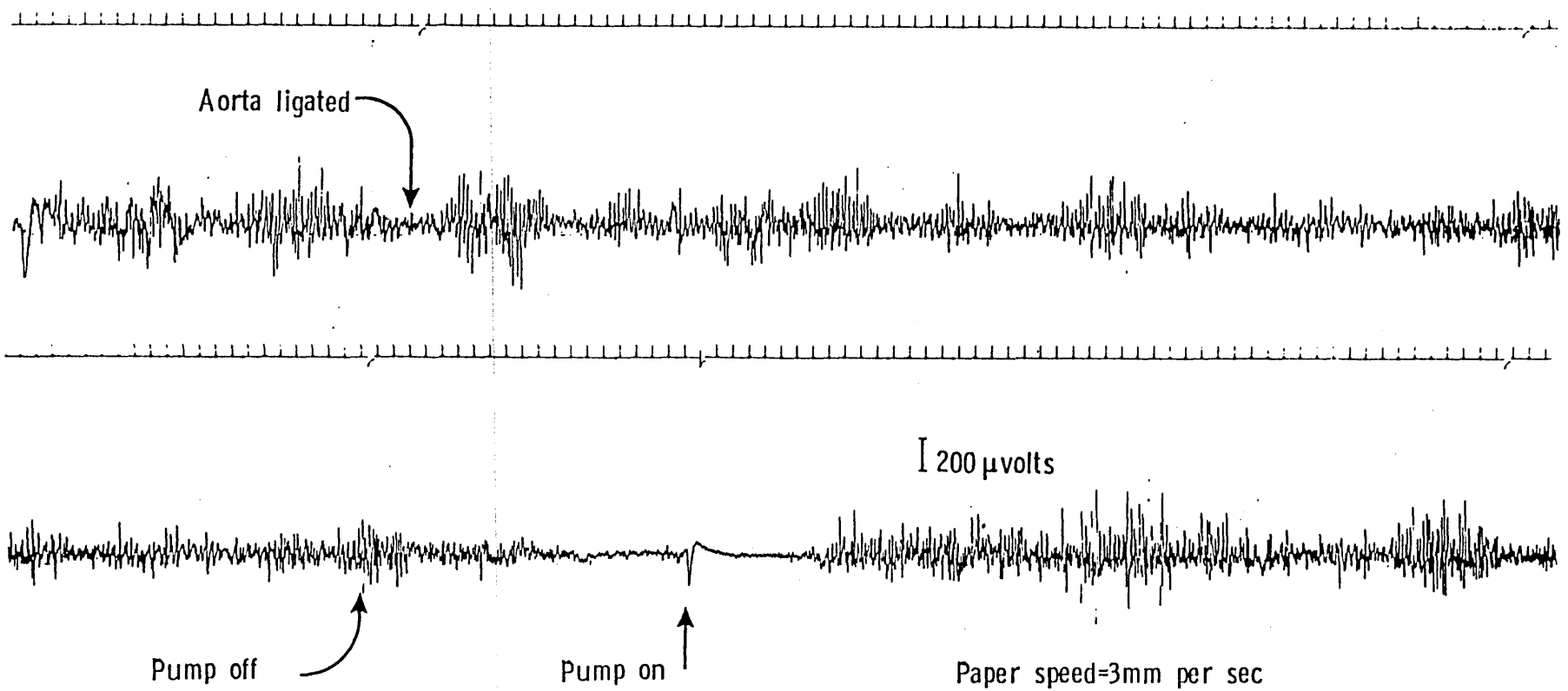


Figure 7.7 Confirmation of EEG authenticity during in situ isolated rat brain perfusion.

7.5 CONDITIONS DETERMINING THE SURVIVAL TIME OF THE BRAIN IN VITRO

A systematic evaluation of the preparation was now carried out to determine if the survival time of the isolated perfused rat brain could be extended by optimisation of the technique and thus permit extended studies upon combined liver and brain perfusions to be carried out.

7.5.1 Influence of choice of the anaesthetic

The type of anaesthetic used has been reported to influence the metabolism of the isolated perfused rat brain (Krieglstein et al 1972, Krieglstein et al 1980). Krieglstein et al (1972) reported an increase in brain phosphocreatine and glucose concentrations but a decrease in pyruvate, lactate and alpha glycerophosphate when sodium pentobarbitone was used as the anaesthetic. When urethane was used, cerebral glucose, adenosine triphosphate (ATP) and adenosine diphosphate (ADP) concentrations increased. Since such changes may influence the survival time of the brain in vitro a small series of experiments was conducted using rats which were anaesthetised with ether and compared to rats anaesthetised with intraperitoneal sodium pentobarbitone (60mg/kg). The brains of ether anaesthetised rats had a median survival time of 35.5 minutes (range 21-48 minutes), see table 7.3 overleaf, compared to a median of 35 minutes (range 22-53 minutes) for sodium pentobarbitone. Non parametric analysis, using the Mann Whitney U-test, showed no significant differences between the two groups ($P>0.05$).

Table 7.3

Perfusion No.	062	064	066	071	075	076
Survival time /minutes	33	21	42	33	38	48

Survival times of 6 isolated perfused rat brains using ether anaesthetic. Median survival time was 35.5 minutes (range 21-48 minutes).

7.5.2 The necessity for an oxygen carrier

A series of 6 in situ control perfusions were carried out with KRBA without an oxygen carrier. Figure 7.8.(a) shows a typical sequence of events during one of these investigations. Few changes were observed during the cannulation procedure since perfusion did not commence until both arteries were cannulated. The EEG trace declined rapidly and irreversibly on commencement of perfusion to a flat horizontal line on ligation of the aorta.

All 6 of these experiments produced similar results and confirmed that an oxygen carrier in the perfusate was obligatory for the survival of the isolated perfused rat brain. These experiments also confirmed that some of the unusual waveforms seen during perfusion were not simply due to the physical action of pumping perfusate through the brain.

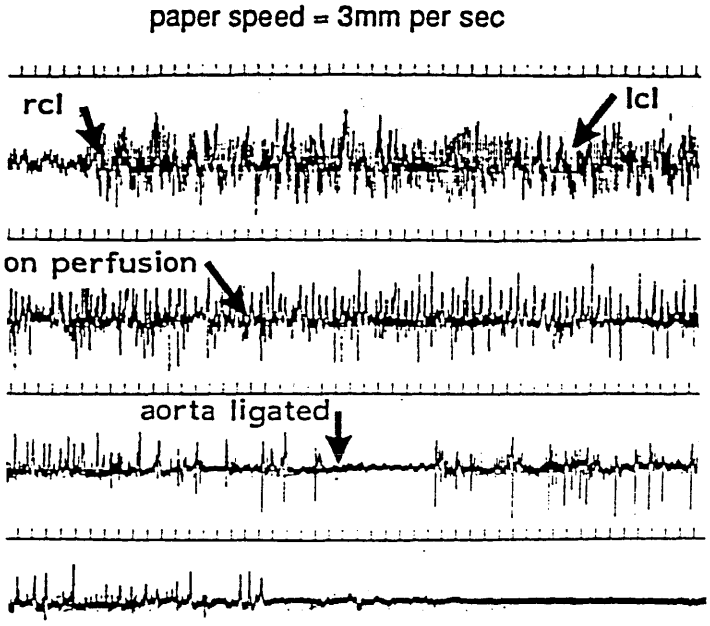


Figure 7.8(a) EEG trace during perfusion with the oxygen carrier omitted from the perfusate (lcl = left carotid ligated, rcl = right carotid ligated).

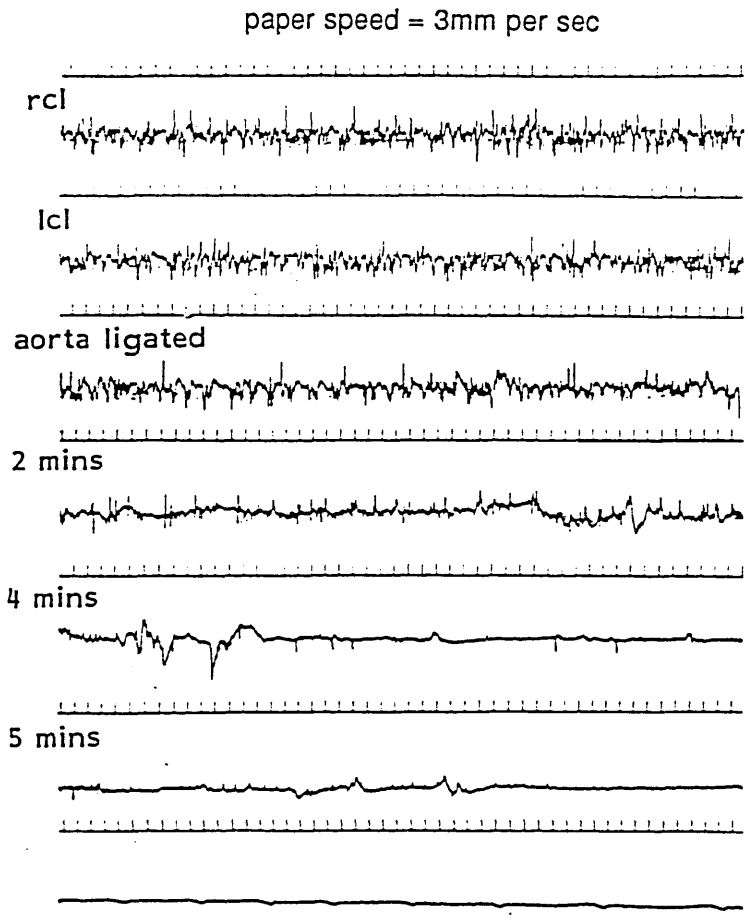


Figure 7.8(b) Isolated in situ rat brain perfusion with human erythrocytes as the oxygen carrier.

7.5.3 Selection of erythrocyte source

The controversy over the most suitable source of oxygen carrier for isolated brain perfusion has still to be resolved. Some investigators have used totally synthetic oxygen carriers such as fluorocarbon emulsions (Sloviter and Kamimoto 1967). A variety of sources of erythrocytes have been used in the past including human (Woods et al 1976), bovine (Krieglstein et al 1980), rat (Thompson et al 1968) and dog red cells (Andjus et al 1967, Zivin and Snarr 1972). An investigation was therefore carried out to assess the relative merits of rat, canine and human erythrocyte source.

Six perfusions were carried out with washed human erythrocytes, prepared as described in chapter 6. Figure 7.8.(b) is an example of the EEG obtained during one of these perfusions. A normal EEG trace was recorded before commencement of perfusion during every experiment and assessed visually. The trace declined as soon as perfusion had commenced through the first cannula and then declined further when perfusion through the second cannula commenced. The alpha wave portion of the EEG trace diminished after one minute of perfusion. At 2 minutes there was a reduction and eventual absence of beta activity which was also accompanied by an increase in perfusion pressure after 3 minutes. The absence of theta waves (4-7.6Hz) was most noticeable after 4 minutes and ligation of the aorta and transection of the jugular veins resulted in the absence of delta waves (0.98-4.0Hz). It was later discovered that this sequence of events was typical of all the perfusions carried out with human erythrocyte perfusion media. Attempts to increase the number of washes or include additional filtration techniques to the protocol did not influence the survival time of the brain.

Similar events were observed during perfusions with diluted rat blood to a haematocrit of 20%, prepared in a similar manner to that described by Thompson et al (1968). Here, however, the EEG trace persisted for 10 minutes to result in a total perfusion time ranging from 12 to 15 minutes in a series of 6 perfusions.

It was therefore concluded that the perfusates which contained the washed canine erythrocytes were best suited for the preparation (range 22-53 minutes) in agreement with Andjus et al (1967) and Zivin and Snarr (1972).

7.5.4 Influence of erythrocyte age and haematocrit upon the brain survival time

Fresh canine erythrocytes were usually obtained on the day of perfusion in order that the standard survival time could be established. However, if this was difficult blood was collected and stored at $4^{o}C$ in ACD bags until the day of use. However, blood stored for 48 hours or more caused significant decreases in the survival time of the brain on perfusion. A series of 6 perfusions carried out with canine erythrocytes that had been stored for 48 hours reduced the survival time of the brain to a range between 10 and 12 minutes. It was therefore decided the maximum permissable storage time of the erythrocytes was 24 hours.

The influence of the perfusate haematocrit was also assessed with canine erythrocytes during this series of experiments. Four perfusions were carried out with perfusate haematocrits of 13-16% all obtained and prepared within 24 hours of the perfusion. These lower haematocrit perfusates shortened the survival of the brain to a median of 15 minutes (range 9-18 minutes) and all further perfusions were

therefore conducted using higher haematocrit perfusates of 25-30%.

7.6 DISCUSSION

The background noise level with conventional electrodes was in excess of 50uVolts and prevented clear identification of a recognisable EEG trace. The new design of electrode assembly enabled EEG waveforms below 25u volts to be analysed and the characteristic high voltage EEG waveforms often associated with barbiturate anaesthesia (Krieglstein et al 1972) were readily observed.

The advantages of the isolated perfused rat brain over tissue slice experiments and other in vivo preparations has already been discussed (chapter 3). The complex vasculature of the brain precluded its widespread use for isolated perfusion experiments until the detailed report by Geiger and Magnes (1947). They identified the afferent and efferent vasculature of the cat brain as described by Hill (1896) and carried out successful perfusions by refinement and adaptation of a technique originally described by Chute and Smyth (1939).

Geiger and Magnes (1947) measured the survival time of the isolated cat brain by measurement of oxygen uptake, reflex reactions, blood flow and pressure, glucose consumption and cortical response to applied chemical stimuli, such as strychnine or metrazol. The main criteria for the interpretation of adequate cerebral function were the maintenance of near consciousness, spontaneous movements, natural respiration and very active ocular, respiratory and vasomotor reflexes.

Later, analysis of the EEG from the isolated cat brain demonstrated that cortical activity was present for 30 to 40 minutes (Geiger et al 1954). The EEG signal during this time period was

characterised by wave frequencies between 8 and 12Hz, and a high level of oxygen consumption, accompanied by spontaneous movements of the eyes and face. The present series of experiments established a rat brain survival time of 35 minutes (range 22-53 minutes). The quality of the recorded EEG waveform was similar to that described by Geiger et al (1954) and Andjus et al (1967), the absence of alpha and beta waves being noted towards the terminal stages of perfusion.

The major advantage of the technique described by Andjus et al (1967) was its miniaturisation for use in rats. The longest survival time reported with this system was 5 hours but this was achieved with a single pass system and it appeared the majority of residual activity was confined to the theta and delta wave frequencies. Few rats achieved survival times greater than 2 hours if the perfusate was recycled, presumably due to accumulation of toxic metabolites. Blood gas control in a recycling configuration may also have been a problem with the design of oxygenator described by Andjus et al (1967). Extensive haemolysis of the perfusate might also have occurred after one hour of perfusion (Proctor 1977).

One of the earliest reports of a totally isolated brain perfusion system was that of Gilboe et al (1964) in the dog. Electrocortical activity was maintained for up to 4 hours from the onset of perfusion although the other reflexes described earlier were still present after the EEG had ceased. This suggested that the EEG was the most sensitive index of brain death, in agreement with Krieglstein et al (1972). It was of interest to note that the alpha and beta wave activity mainly subsided during the first 60 minutes of perfusion and that the remainder of the EEG activity was confined to theta and delta wave activity in Gilboe's preparation.

In contrast to the totally isolated brain perfusion experiments, the results obtained from in situ isolated brain perfusion investigations were easier to interpret. The pilot studies conducted during these studies demonstrated that the quality of the EEG signal deteriorated abruptly on transection of the spinal column in the totally isolated preparation. The quality of the in situ EEG trace was also superior to the totally isolated brain perfusion traces. The authenticity of the EEG signal was readily demonstrable at any time throughout the in situ perfusions with alpha and beta activity detectable until 2 to 3 minutes prior to brain death.

Inadequate filtration of the perfusate may reduce the survival time of the isolated perfused rat brain (Zivin and Snarr 1972). The perfusate used in this series of experiments was prepared in two fractions. The Krebs-Henseleit buffer was filtered through 45μ interstices Millipore filters prior to addition to the washed erythrocytes. The erythrocytes were washed 3 to 4 times in phosphate buffered saline before combination with Krebs-Henseleit buffer. This mixture was again filtered through a sterile stainless steel mesh prior to incubation in the reservoir container and upon every subsequent pass into the reservoir.

The perfusate which drained from the perfusion table was also filtered before return to the reservoir to prevent the entry of clots, aggregates and other debris. The major difference with the perfusate prepared during this series of experiments and those described by Krieglstein et al (1972) or Andjus et al (1967) was in the preparation of albumin used. This was not pre-washed or passed over Amberleit (MB3) resin since the grade of albumin used was already of a purified grade (Sigma fraction V no. A-2153 Sigma Chemical Company).

Experiments were not conducted to investigate the influence of this factor upon the survival of the brain.

It was further demonstrated during this series of experiments that the source of erythrocytes influenced the survival time of the isolated brain. This, in common with isolated liver perfusion, had not previously been investigated in a systematic manner. Various sources of erythrocytes had been utilised in the past as oxygen carriers in artificial perfusates, the choice of which was largely determined by the species of animal under investigation. Geiger and Magnes (1947) used homologous blood for the isolated perfused cat brain preparation and, similarly, Gilboe et al (1964) used dog blood for his isolated dog brain perfusions. However, isolated perfused rat brains perfused with pooled rat blood were reported to produce poor results (Andjus et al 1967, Krieglstein et al 1972). The reasons for this were largely speculative although Ross (1972) suggested that 'certain vasoconstrictor substances' could be released during blood collection and perhaps this was the reason for poor results. Thompson et al (1968) however, reported that the spontaneous EEG of the rat brain could be recorded during perfusion with haemodiluted rat blood of haematocrit 12-16%. The results obtained during this series of investigations (see section 7.4.3) were similar to the findings of Andjus et al (1967) who concluded that pooled rat blood was unsatisfactory for the maintenance of the spontaneous EEG.

The poor results obtained with human erythrocytes were attributed to their age since Krieglstein et al (1972) commented that blood no older than 24 hours should be used. Woods et al (1976) incorporated washed human erythrocytes in the perfusate but did not report upon the quality of the resultant EEG trace. The spontaneous EEG measured

during similar investigations described here were of a poor quality and of short duration (less than 4 minutes).

Zivin and Snarr (1972), reported that it was possible to use washed canine erythrocytes for up to 6 days after storage. Canine blood was collected and stored in an identical manner during this investigation but it was found that blood stored for more than 48 hours reduced the survival of the isolated perfused rat brain. This result was in accordance with other observations of inadequate oxygen delivery characteristics of aged red cells and could have been responsible for the reduced brain survival times both in the aged dog erythrocytes and human erythrocytes (Rennie and Holloszy 1977).

7.6 SUMMARY

A series of investigations were conducted in order to establish an isolated perfused rat brain preparation. An EEG trace was authenticated in vivo by visual assessment during administration of incremental doses of sodium pentobarbitone and standard criteria for a normal trace were defined. Various parameters which influenced the brain survival time were investigated and optimised. It was found that the best signal to noise ratio was obtained by in situ isolated rat brain perfusion without transection of the spinal cord.

A closely reproducible survival time was observed (median 35 minutes, range 22-53 minutes). The longest survival times were recorded when fresh washed canine erythrocytes were used in the perfusate. The following chapter describes the use of this preparation to determine the influence of a concomitantly perfused rat liver upon an isolated perfused rat brain.

CHAPTER 8

CONCOMITANT PERFUSION OF THE ISOLATED PERFUSED RAT BRAIN WITH THE ISOLATED PERFUSED RAT LIVER

8.1 INTRODUCTION

The work described in chapter 6 established that after an equilibration period of one hour following excision, adequate function of the isolated perfused liver could be sustained for up to 4 hours. In chapter 7 it was established that the mean survival time of an isolated, perfused rat brain preparation was 35 minutes (range 22-53 minutes)

This chapter describes the combination of the two circuits in parallel in order to examine the interrelationship of the liver and the brain. The qualitative changes in the EEG waveforms produced during the combined perfusions were investigated in detail and compared with those during the separate perfusions of the brain.

8.2 MATERIALS AND METHODS

8.2.1 Perfusion medium

The investigations described in chapters 6 and 7 indicated that the perfusate best suited for the combined liver and brain perfusions was a washed canine erythrocyte medium with a haematocrit of not less than 20%. This perfusate was prepared as described in chapter 7 and used throughout the studies described in this chapter.

8.2.2 Perfusion circuit

The circuit adopted was an amalgamation of the 2 individual circuits described earlier (chapters 5 and 7) using a single oxygenator and a common reservoir in order to compact the system and reduce the prime volume (figure 8.1). The liver and brain were both perfused with a mixed arteriovenous perfusate. The combined flow rates of the liver and the brain were less than half the maximum blood flow calculated for the oxygenator to provide blood gas values within the normal physiological range.

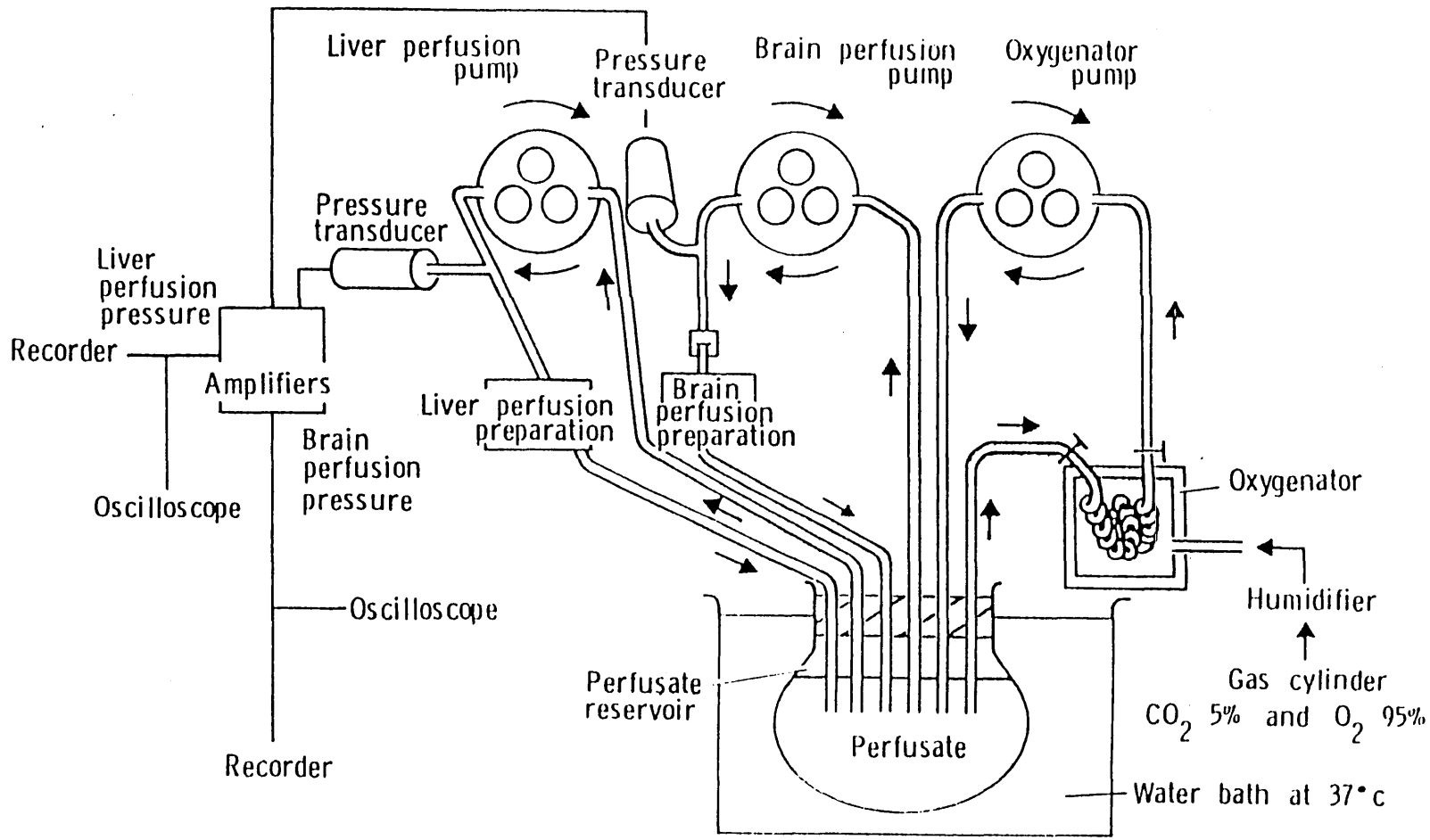
8.2.3 Surgical procedures

The rat liver was prepared for excision and cannulated for bile collection (as in chapter 6) during recirculation of perfusate medium. Once the liver was on perfusion a second rat was prepared for isolated perfusion of the brain as described in chapter 7 of this study.

8.2.4 Perfusion protocol

Twelve combined perfusions were carried out. The livers were perfused for 60 minutes prior to insertion of the brain upon the

Figure 8.1. Combined liver and brain perfusion apparatus.



perfusion apparatus. This allowed equilibration of the liver and permitted sufficient time to prepare the brain for perfusion.

EEG measurements were carried out using a Siemens 'Myngograph' EEG monitor (Siemens Ltd, U.K.). Perfusion pressures and flow rates were maintained at physiological levels for each organ throughout the perfusions and were measured as described in chapters 4 and 5 of this thesis. In addition, the blood gases of the reservoir were monitored every 30 minutes of perfusion and regulated either by alteration of flow through the oxygenator, alteration of gas mixture or addition of sodium bicarbonate to the reservoir. Outflow blood gases from the liver and the brain were also monitored.

Glucose concentrations were measured at regular intervals with Dextrostix (Ames Co. Ltd, U.K.) and measurements were confirmed retrospectively upon a R.A. 1000 autoanalyser system (Technicon Ltd, U.K.) using the glucose oxidase/peroxidase principle (Randox Laboratories, N.Ireland).

8.3 RESULTS

The nature of the traces recorded during these experiments was more complex than that observed during perfusion of the isolated brain alone. A gradual deterioration of the signal was observed, in contrast to previous experiments in which the decline was abrupt. Definition of the onset of "brain death" was therefore critical to the interpretation of these results.

Figure 8.2 is a typical EEG trace (type A, see table 8.1) from a combined perfusion experiment. The principle features during this perfusion are described in outline:

Traces a) and b): complete spectrum of EEG activity shown during the cannulation and initial perfusion phases.

c): no deterioration of signal after 30 minutes of perfusion.

d): 60 minutes perfusion - reduction in alpha waves recorded.

e): 90 minutes - further decrease in alpha and beta wave activity although occasional alpha activity in in excess of 100u volts still seen as a high voltage (HV) waveform.

f): 106 minutes - decline in theta and delta wave activity detected.

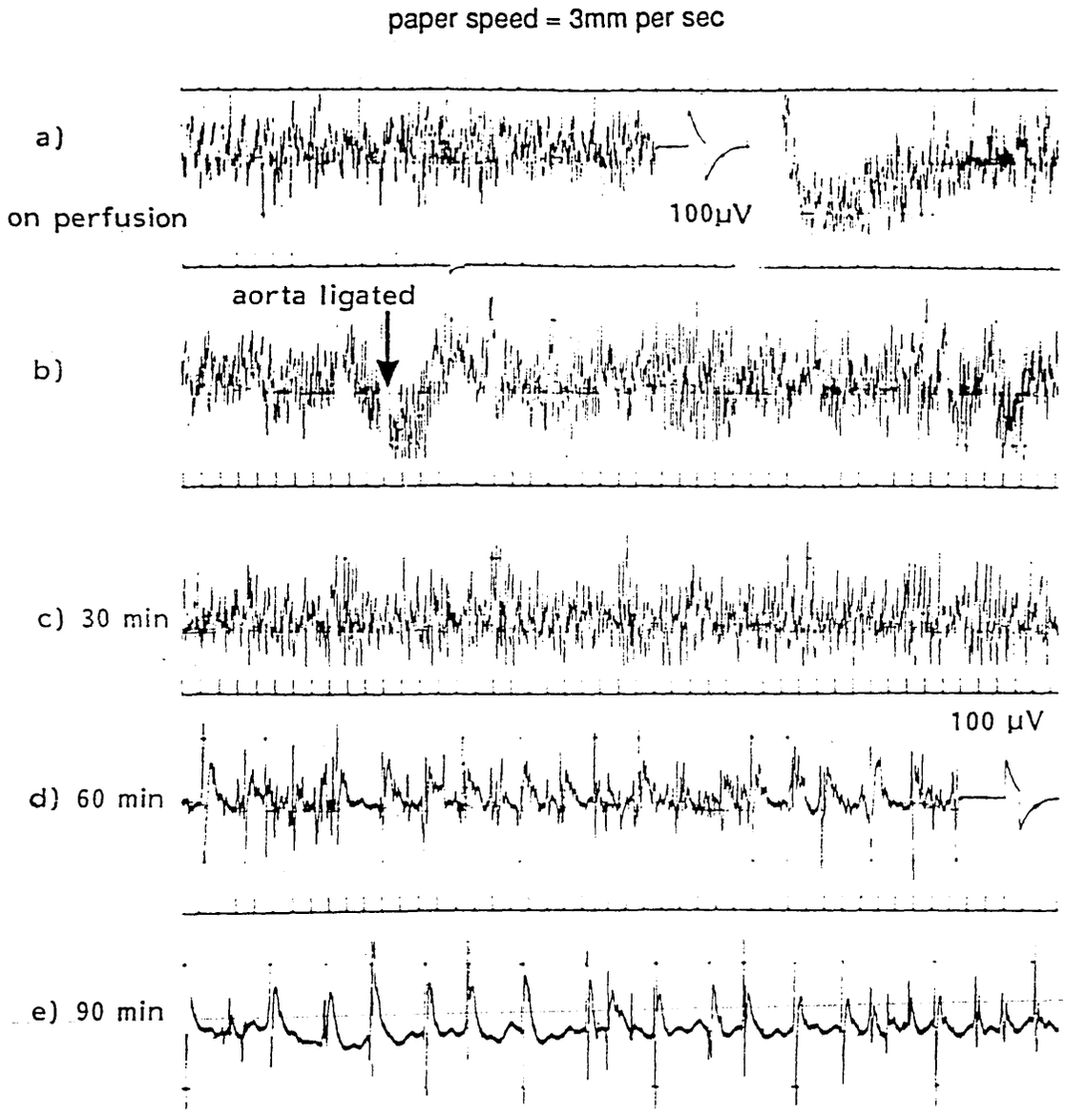


Figure 8.2 Typical EEG trace (type A) recorded during the concomitant perfusion of the isolated rat brain with the isolated rat liver (c.f. figure 8.3).

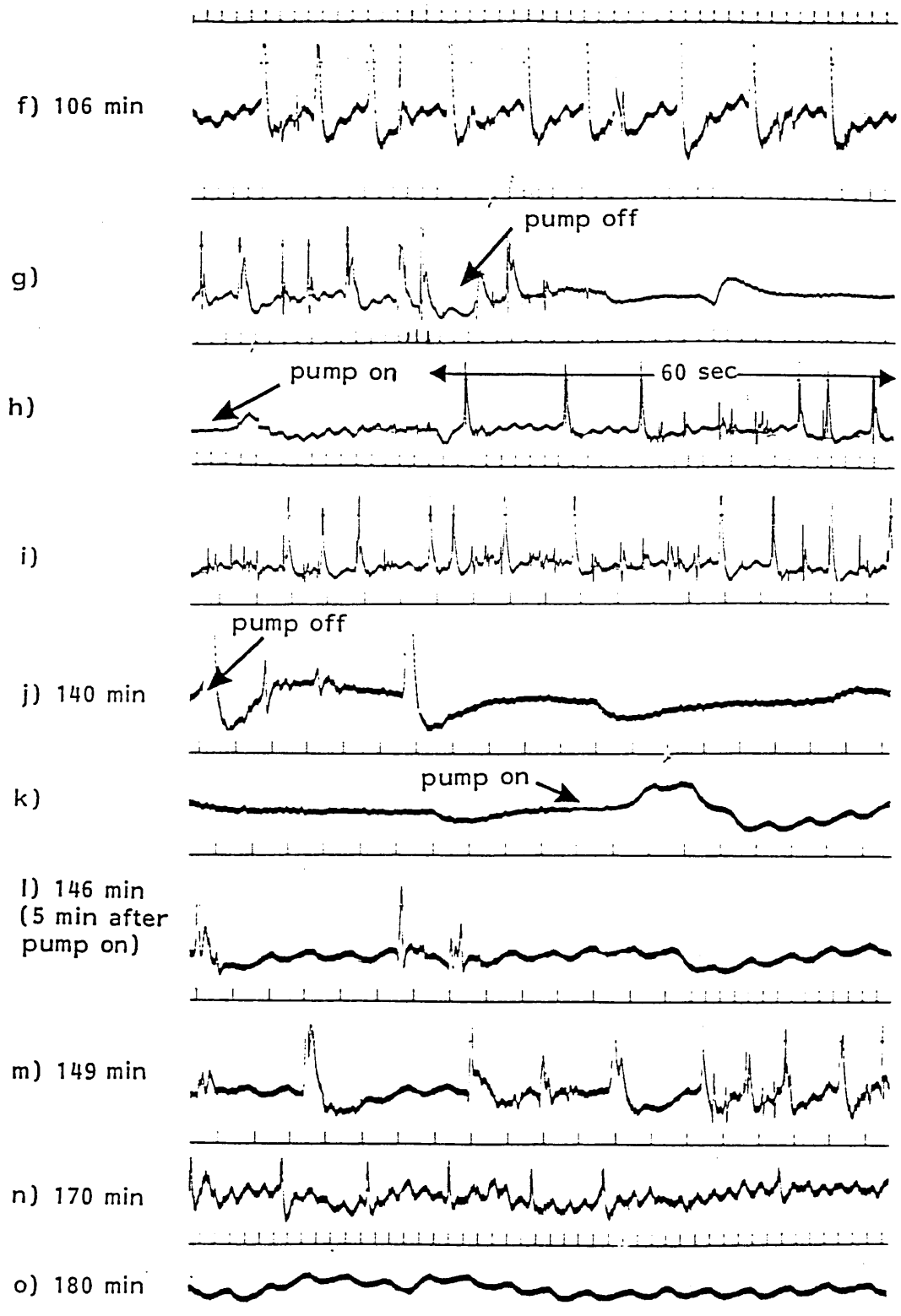


Figure 8.2. (contd).

g): authenticity of recording verified by switching pump off as described in chapter 7. Gradual decline in signal strength recorded.

h): re-establishment of perfusate flow resulted in return of EEG, predominantly as delta wave activity after approximately 60 seconds of perfusion.

i): 35 seconds of perfusion later, the original spectrum of EEG activity returned as in g).

j): 140 minutes - further reduction in signal strength noted, flow interrupted temporarily.

k): recognisable signal could not be identified.

l): return of isolated waveforms.

m): 149 minutes - EEG similar to trace f).

n): 170 minutes - decline in signal amplitude

o): 180 minutes - EEG deteriorated to flat line.

Figure 8.3 is a record of another type of trace (type B) recorded during combined perfusions.

In Summary:

Traces a) & b): pre-perfusion traces - similar to previous example.

c): 45 minutes perfusion - increasing incidence of high voltage (HV) waves approximating to theta wave frequency.

d): 60 minutes - theta activity more pronounced, reduction in alpha and beta activity.

e): 90 minutes - the rhythm recorded here could suggest theta activity or extraneous noise contamination.

f): However, temporary interruption of perfusate flow for 20 seconds results in loss of activity returning to normal after resumption of flow confirming signal authenticity of theta rhythm.

g): 120 minutes - delta activity prominent, theta activity reduced.

h): 125 minutes - resumption of regular theta rhythm.

i): 140 minutes - decline in theta activity, residual delta wave present.

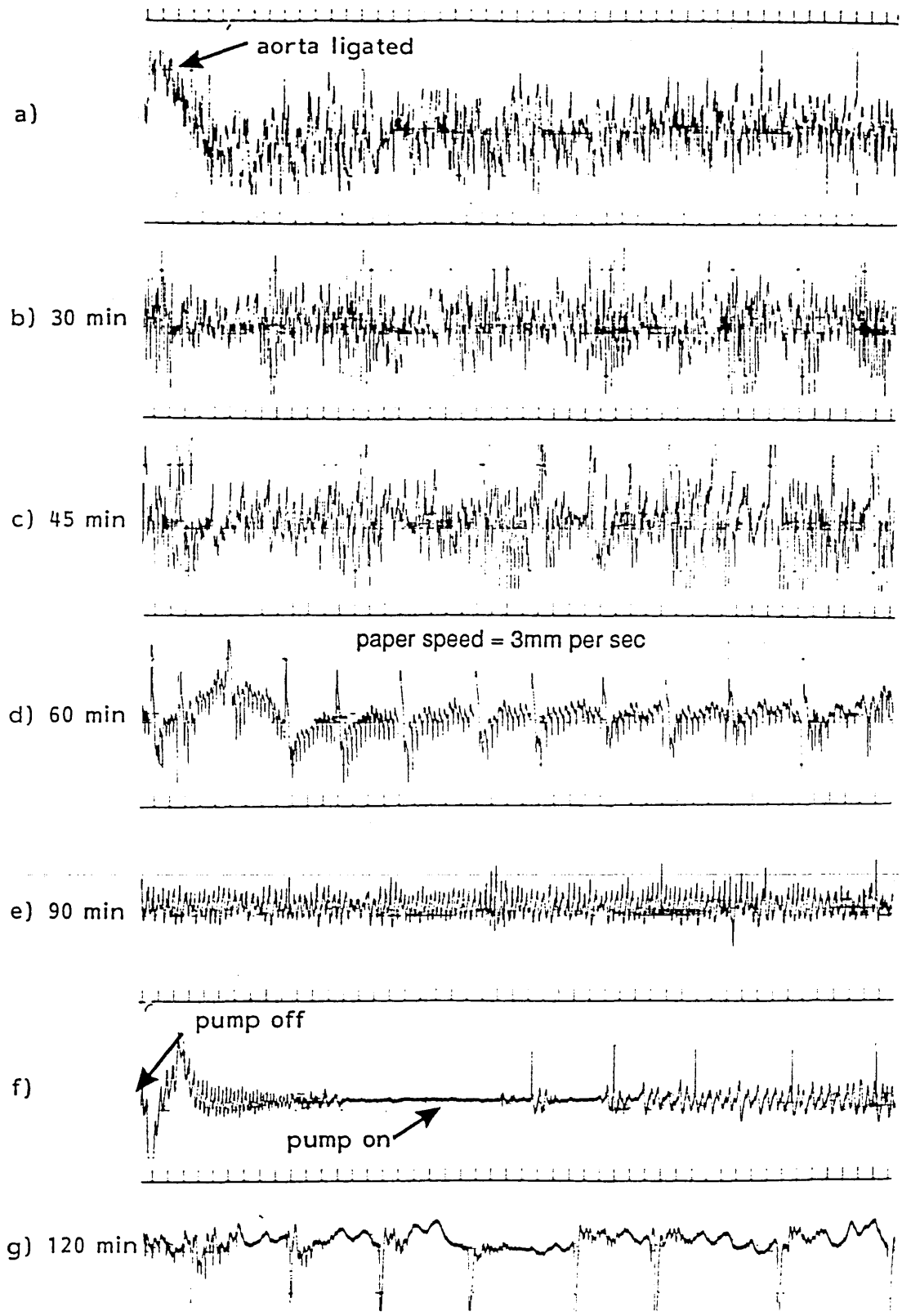


Figure 8.3 EEG waveform (type B) recorded during combined liver and brain perfusion experiments (c.f. figure 8.2).

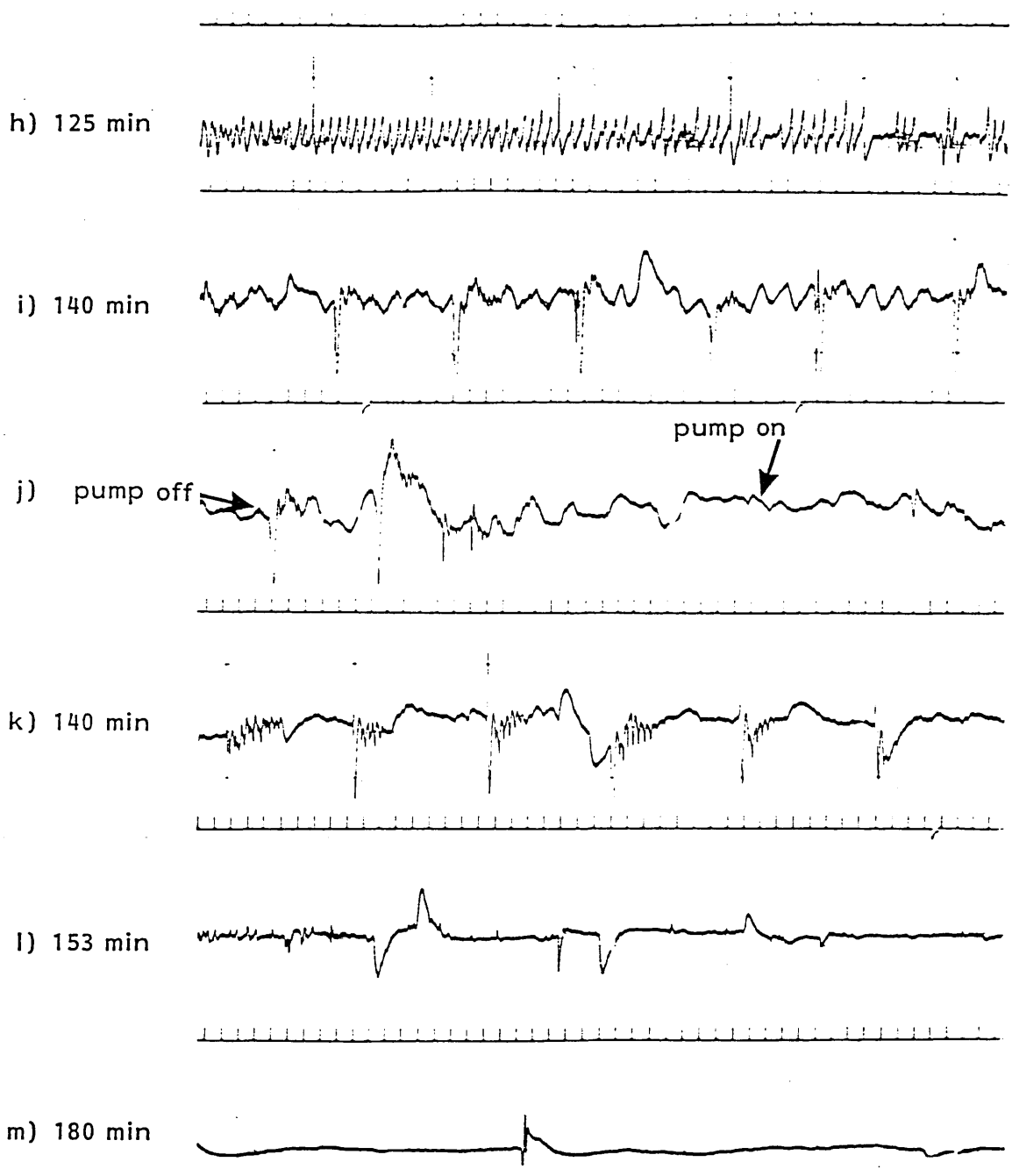


Figure 8.3. (contd).

j): temporary flow interruption demonstrating .....

k): .....signal authenticity by gradual resumption of signal 13 seconds later.

l): 153 minutes - generalised reduction in signal activity.

m): 180 minutes - brain dead (flat trace).

Figure 8.4 is a record of one perfusion which had a duration of over 7 hours.

In summary:

Trace a): full spectrum of EEG during initial stages of perfusion and during ligation of aorta.

b) & c): 30 & 90 minutes perfusion - no change in EEG pattern.

d): 440 minutes - unusual theta and delta rhythm recorded, some alpha and beta activity.

e): perfusate flow temporarily halted to check EEG.

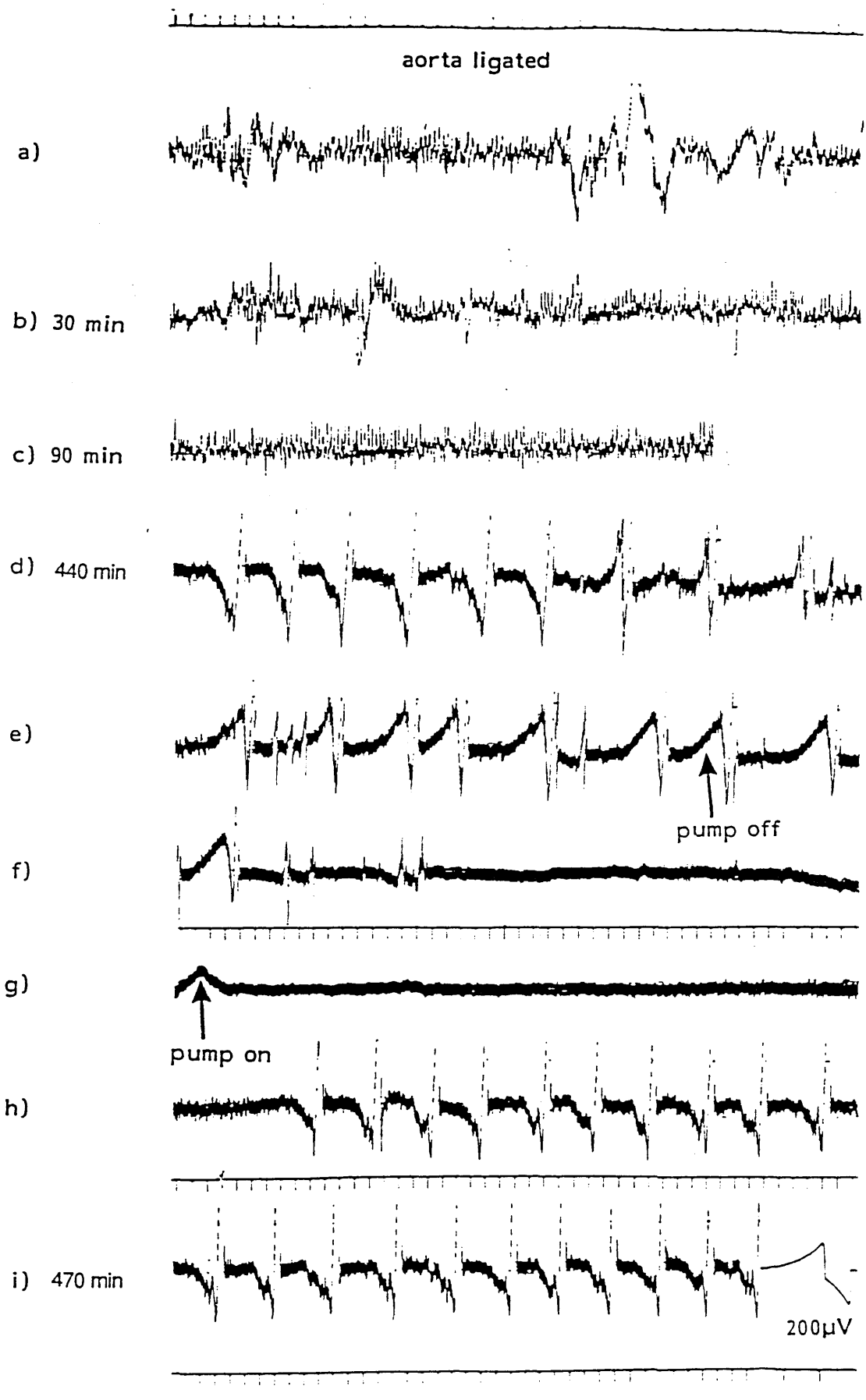


Figure 8.4. EEG waveform recorded from one of several prolonged perfusions which had a duration in excess of seven hours.

f): gradual decline in signal strength over 22 seconds noted.

g) & h): 30 seconds later - perfusate flow resumed demonstrating gradual recovery of signal.

i): 470 minutes - continued activity of EEG recorded.

Figure 8.5 is included to demonstrate that occasionally an apparently flat EEG trace was capable of a response to an external auditory stimulus.

Summarising:

Trace a): 135 minutes perfusion - EEG of 'dead' brain, auditory input applied, i.e. bell rung, at 'ai'.

b): restoration of a physiological EEG which lasted for over 30 seconds.

c): second stimulus applied - large fluctuations re-elicited accompanied by stronger EEG signal.

d): 3 minutes later - gradual decline in signal strength to flat line.

e): further burst of activity recorded following application of 3rd stimulus.

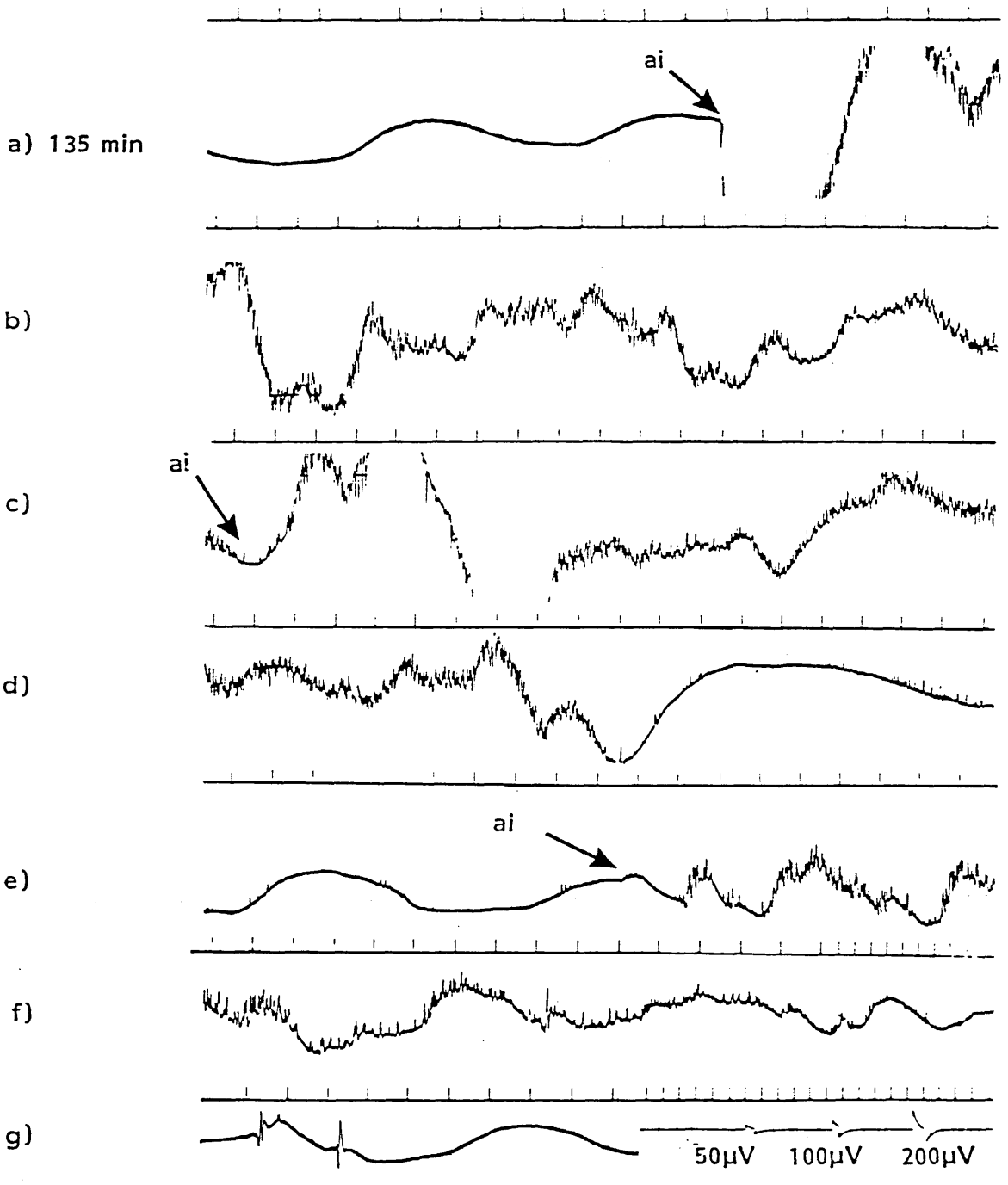


Figure 8.5. Restoration of the spontaneous EEG waveform from a flat trace previously assumed to derived from a non viable preparation.

ai = auditory stimulus applied

f): gradual reduction in signal strength over 2 minutes.

g): deterioration of signal to flat horizontal line. In this case there was no further response to an auditory stimulus.

This technique was often employed to confirm brain death during these prolonged perfusions.

Perfusate glucose concentrations declined to below 70mg per 100ml during each combined perfusion beyond 65 minutes. Glucose was added 2 or 3 times to the perfusate to restore a concentration of 200mg per 100ml perfusate.

This series of 9 perfusions showed that the survival of the isolated perfused rat brain could be extended to a median of 210 minutes (range 113-480 minutes) when perfused concomitantly with an isolated rat liver. The results from this series of experiments are presented in table 8.1 overleaf.

Table 8.1

Perfusion No.	Survival time /minutes	Trace type
LB001	172	A
LB002	210	A
LB003	180	B
LB004	253	A
LB005	350	B
LB006	113	A
LB008	435	B
LB010	480	B
LB011	184	A

Survival times of 9 combined isolated perfused rat liver and brain preparations. The median survival time was 210 minutes (range 113-480 minutes) and was shown to be significantly different ($P<0.001$) than brains perfused alone (see text).

Non parametric analysis using the Mann Whitney U-test showed a highly significant difference ($P<0.001$) in survival times from brains perfused without a liver where the median was 35 minutes (range 22-53 minutes).

8.4 DISCUSSION

Early investigations upon the interrelationship between the liver and the brain used isolated perfused cat brain preparations (Geiger and Magnes 1947). The viability of the brain was assessed by measurement of glucose uptake, oxygen uptake and blood flow rate; physiological activity was measured by corneal reflex reactions, respiration rates, vasomotor responses, spontaneous movements of face and eyes and also by the electrocorticogram (Geiger et al 1954).

These early experiments suggested that the presence of a liver in the perfusion circuit was essential for the regulation of glucose uptake and oxidative metabolism in the brain. A transient recovery of a 'dormant' perfused brain by the addition of cytidine and uridine to a 'non-liverised' perfusate led to the hypothesis that these substrates were responsible for the regulation of glucose oxidative metabolism (Geiger and Yamasaki 1956). In contrast Gilboe et al (1964) was able to demonstrate glucose uptake in the isolated perfused dog brain and Zivin and Snarr (1972) in the rat brain without the presence of a liver or 'liverised' blood. Later investigations extended the survival time of the IPRB to several hours without the inclusion of 'liverised' blood in the perfusion circuit or the inclusion of cytidine or uridine in the perfusate (Andjus et al 1967, Krieglstein et al 1972, Zivin and Snarr 1972).

The major difference between these later investigations and the early work of Geiger was that Geiger's preparation in the cat was not totally isolated since the spinal column was not transected during the operative procedure. Investigations which had used the rat in preparations directly comparable to Geiger's, i.e. perfusion of the head rather than the brain alone, produced very similar results with

an average survival time of 46 minutes when perfused with 'unliverised' blood (Thompson et al 1968). However the EEG traces which were provided began to decline after a period of 19 minutes of perfusion (Thompson et al 1968), which was in agreement with the findings of Andjus et al (1967) who reported poor maintenance of an EEG signal with the use of pooled rat blood.

The simplicity and speed of preparation of Thompson's model (1968) was very attractive (Krieglstein and Stock 1974). In addition, problems due to the large individual variation in carotid vasculature were avoided with this preparation since all the vessels were perfused and separate isolation of the internal and external carotid arteries was unnecessary (Thompson et al 1968). A major advantage of the preparation was that it required less time to prepare the brain for perfusion while the liver was being perfused and equilibrating in the perfusion circuit. The present investigations adapted Thompson's model by the substitution of washed canine erythrocytes for rat erythrocytes as the oxygen carrier, prepared according to the method of Zivin and Snarr (1972).

Perfusion traces recorded from individually perfused brains had deteriorated after 30 minutes of perfusion (see chapter 7) and many declined to a flat trace after 35 minutes. In contrast the majority of the combined liver/brain perfusions showed little or no change in the EEG trace after 30 minutes of perfusion when compared to pre-perfusion appearances (see figure 8.2, point (c) and figure 8.3, point (b)).

While the isolated brain perfusions showed clear evidence of brain death in under 1 hour, brain death during the combined

perfusions became increasingly difficult to determine due to the unusual nature of the EEG waveforms which developed.

The flat trace recorded after 135 minutes of perfusion in figure 8.5 was indicative of brain death. Application of a sound stimulus produced a short-lived signal which gradually deteriorated to a flat trace again. The signals induced by a sound stimulus which were published by Andjus et al (1967) were mainly composed of isolated HV waves. The complete spectrum of EEG activity was induced during this series of perfusions and this may have been due to the presence of a liver in the circuit (see figure 8.5).

The median brain survival time of 210 minutes (range 113-480 minutes) was similar to the time for which the isolated perfused rat liver (alone) remained in optimal functional condition (see chapter 6). In these studies the liver was perfused for 1 hour prior to insertion of the brain in the perfusion circuit and this therefore should be added to the brain survival time to calculate the liver perfusion time. It is therefore possible that the deterioration in brain function was due to a deterioration in liver function and that the liver plays a fundamental role in the regulation of brain metabolism. Possible evidence of liver failure could be drawn from a closer examination of the nature of the EEG traces at 90 minutes of perfusion and thereafter. Figure 8.2 shows characteristic slow wave, high amplitude, high voltage (HV) waves after 60 minutes of perfusion (point d) with an increased frequency after 90 minutes (point e). These were characterised by the early appearance of HV waves (point d) which subsided in frequency and disappeared after 90 minutes of perfusion (point e). These eventually reappeared at a much reduced frequency after 125 minutes of perfusion (point k).

The onset of HV waves has previously been reported to be associated with liver dysfunction and was recorded in rats which had undergone end-to-side portacaval anastomosis (PCS) (Grange et al 1974). Others however, failed to report an increase in the incidence of slow wave spindles after PCS (Bucci et al 1980). Derangements in the level of wakefulness have also been associated with hepatic insufficiency in rats (Beaubernard et al 1977) accompanied by a measured alteration in response to visual evoked potentials in rats with galactosamine induced fulminant hepatic failure (FHF) (Zeneroli et al 1981). Measurements of motor nerve conduction velocity in these rats demonstrated little or no changes between PCS and control rats but these were measured in the rat tail and possibly were not a true reflection of the central changes occurring in the brain (Holmin and Hindfelt 1980).

Holmin and Hindfelt suggested that the PCS rat model was perhaps too subtle a model of hepatic insufficiency to reflect the gross changes observed during hepatic encephalopathy and that this may have been the reason for the variable results obtained with visual evoked potentials in galactosamine induced FHF rats mentioned earlier (Schafer et al 1984, Zeneroli et al 1981). Several reports have appeared in the literature upon the incidence of slow HV waves during hepatic encephalopathy in rats (Bucci and Chiaverelli 1980, Yaar et al 1981) and therefore it seemed reasonable that a high degree of liver damage must occur before these spindles can be detected (Bucci et al 1980). The incidence of slow HV waves during the combined liver/brain perfusion experiments may therefore suggest that their presence is indicative of a failing liver after 4 hours of hepatic perfusion and 2 to 3 hours of brain perfusion.

Geiger and Yamasaki (1956) were able to prolong the activity of the isolated perfused rat brain by the addition of cytidine and uridine to the perfusate. It was proposed that these nucleosides regulated the oxidation of carbohydrates, thereby reducing lactic acid concentrations in the brain produced from glycolysis. The energy for glycolysis was probably derived from beta oxidation of lipids. Geiger and Yamasaki proposed that the addition of cytidine and uridine to the perfusate prevented a depletion of brain phospholipids and lipid galactose (probably through regulation of protein synthesis) thus stabilising carbohydrate (glucose) oxidation. The precise mechanisms by which cytidine and uridine may regulate these processes remains speculative. However, deficiencies in these nucleosides have not been reported during hepatic failure.

Perfusate concentrations of glucose were found to decrease during combined liver and brain perfusion and had to be replenished regularly. This is not thought to be due to hepatic glucose consumption since this had not been seen during the extensive series of IPRLs conducted in chapter 6. Alternatively, head muscle, which was also perfused in the IPRB "non-liverised" preparation, may have consumed glucose. This was also considered unlikely since glucose concentrations remained unaltered after 30 minutes of brain perfusion alone. Although it is accepted that the majority of perfusions did not exceed 45 minutes, reductions in glucose concentrations had been noted by this time during the combined perfusions, and furthermore, a small series of extended perfusions (beyond one hour) also did not show reduced glucose concentrations. It was therefore possible that the reduced glucose concentrations were due to the increased entry of glucose into the brain which was aided by the presence of 'liverised' blood or by the inclusion of a liver in the extracorporeal circuit

(Geiger and Magnes 1947, Geiger et al 1954). Also the presence of a liver in the circuit may have provided a factor or factors which increased the rate of entry of glucose into skeletal muscle. In order to compare glucose concentrations between the single and combined perfusions, results could only be assessed at time periods up to 45 minutes since the majority of individually perfused rat brains did not survive beyond this time point.

Finally the possibility remains that the perfused liver may have extended the survival of the brain by the detoxification of compounds released into the perfusate either by the brain or head muscles. The measurement of such putative toxic compounds was not attempted so that this interesting hypothesis remains outside the scope of the current study.

8.5 SUMMARY

The results from this series of isolated perfused rat brain investigations demonstrated that the survival of the brain could be extended from median 35 (range 22 - 53) minutes to 210 minutes (range 113 - 480) when perfused concomitantly with an isolated rat liver. The model studied during these investigations reflected some of the electrophysiological changes observed in vivo during experimental hepatic encephalopathy (Bucci and Chiaverelli 1980, Yaar et al 1981, Schafer et al 1984). In addition, this series of experiments demonstrated the physiological dependence of the brain upon the liver for the normal maintenance of cerebral glucose metabolism and electrophysiological activity in vitro. The precise nature of this interdependence is unknown.

Reports in PCS rats have suggested that altered brain amine metabolism could be responsible for the characteristic changes seen during chronic PSE (Siemert et al 1978) and that it is here that the interdependence between the liver and brain may lie. In order to investigate this further, measurements in brain amine metabolism were conducted in the following chapter in PCS and PCT rat brains and results were then related to measurements in perfused rat brains.

CHAPTER 9

THE ROLE OF BRAIN AMINES IN PSE

9.1 INTRODUCTION

The previous chapter demonstrated an important relationship between the isolated perfused rat brain and an isolated perfused rat liver, as measured by prolongation of the spontaneous EEG. This final series of experiments investigates some of the biochemical changes reported in vivo in PCS rats and relates them to observations with the in vitro IPRB model.

Previous studies have suggested that the ratio of plasma aromatic:branched chain amino acids (AAA:BCAA) is important in the pathogenesis of PSE both in man and in experimental models following PCS in rats and dogs (Fischer 1982). Hyperammonaemia and alterations in plasma and cerebrospinal fluid (CSF) concentrations of amino acids are a constant observation in patients with chronic PSE (Rosen et al 1977, Fischer 1982). This led Fischer to propose a "unified hypothesis" to link these clinical and experimental observations. Briefly, Fischer's hypothesis suggests that an increase in AAAs and a reduction in the concentration of BCAAs could promote an increased transport of AAAs across the blood brain barrier (BBB). This in turn could lead to an increased generation of false neurotransmitters (such as phenylethanolamine and octopamine) and also an increased production of indole amines such as 5HT, 5HTP and 5HIAA. Increased levels of phenylalanine, tyrosine, methionine and plasma free tryptophan, the AAA precursors of indole amines and the previously mentioned false neurotransmitters, have been observed during chronic PSE in man (Crossley et al 1983). In addition, increased levels of octopamine were measured in the plasma of patients with chronic PSE (Bucci and Chiaverelli 1980).

It is of course impossible to demonstrate cerebral amine changes in man during life. However, increases in the concentrations of the brain indole amines 5HT, 5HTP and 5HIAA (see fig 9.1) and the amino acid phenylalanine (PHE) following PCS in rats have been reported (Siemert et al 1978). These increases in indole amine concentrations have previously been attributed to elevations in indole amine metabolism. Furthermore the behaviour exhibited by rats microiontophoretically injected with 5HT into the brain ventricles is consistent with that seen following PCS in rats and with elevations in indole amine metabolism (Siemert et al 1978). Elevations of PHE may also account for the syndrome of PSE by the synthesis of the false neurotransmitters phenylethanolamine, octopamine and tyramine (fig 9.2).

The mechanism of increased transport of AAAs into the brain is still largely unknown. However, it has been suggested that the hyperammonaemia of PSE passively increases ammonia entry into the brain where it is rapidly detoxified to glutamine; since glutamine shares a common carrier across the BBB with tryptophan, tyrosine and methionine, increased exchanged transport of these AAAs into the brain leads to formation of indole amines and false neurotransmitters (Fischer 1982). Another hypothesis suggests that the increased entry of AAAs into the brain may occur through a generalised, non-specific increase in permeability of the BBB. This has been demonstrated with horseradish peroxidase in rats following PCS (Sarna et al 1977).

It has already been noted that the PCS rat model is characterised by two components, reduced hepatic function and portal-systemic diversion. It is essential to differentiate these two components in order to investigate those changes in brain amine metabolism that may

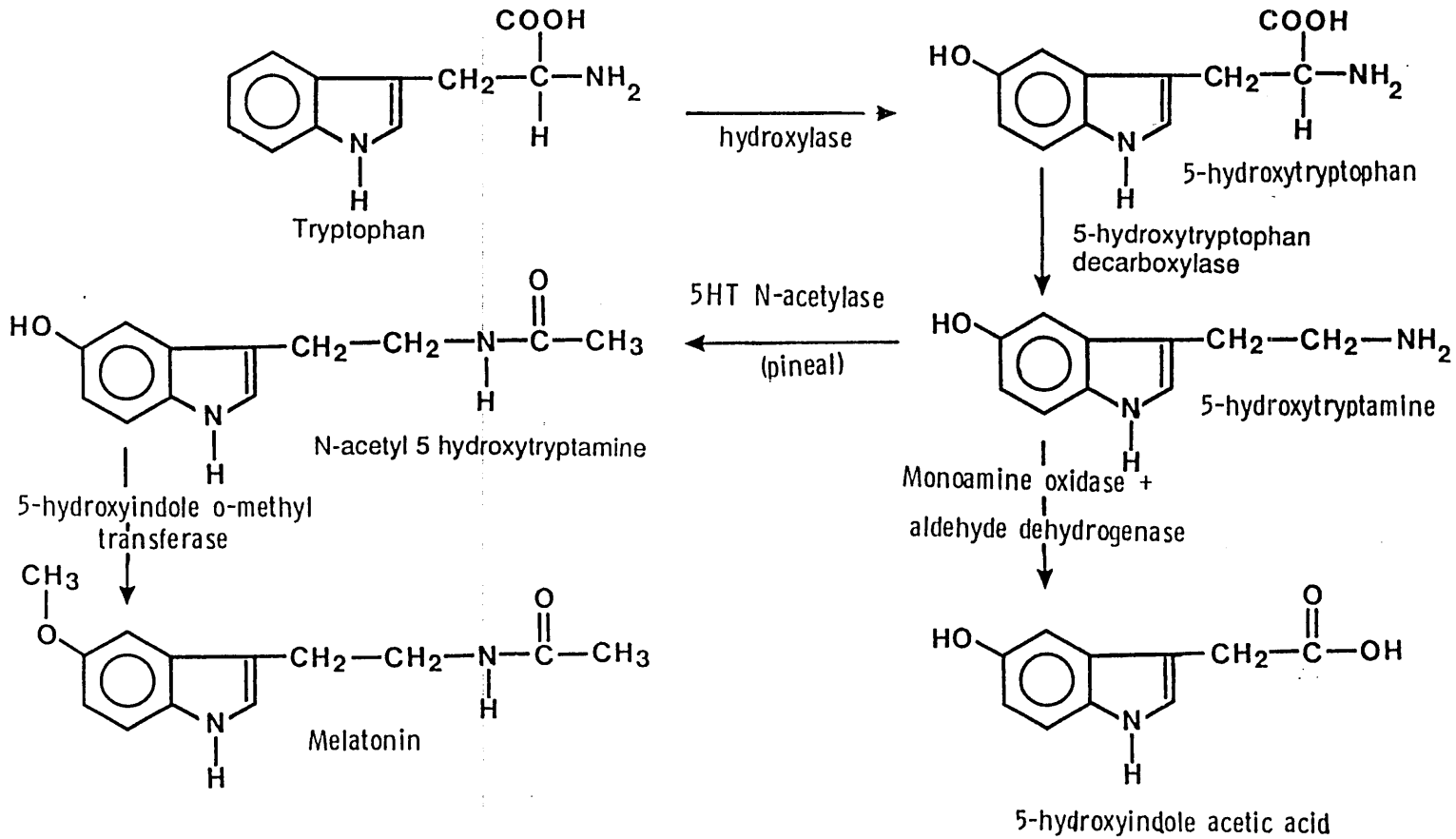


Figure 9.1 Metabolic pathways for the synthesis and catabolism of 5 hydroxytryptamine (5HT).

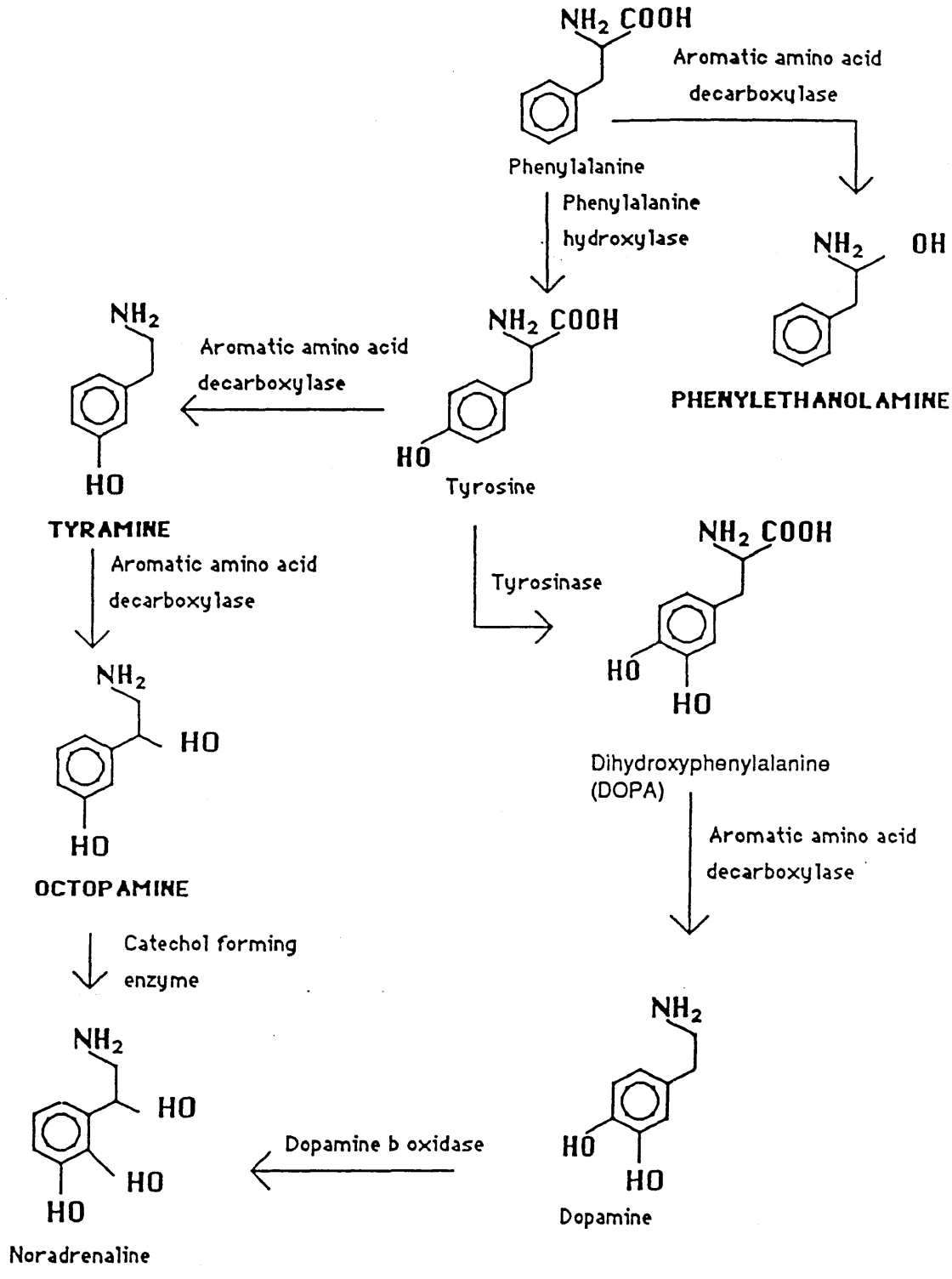


Figure 9.2 Generation of false neurotransmitters from catecholamine synthesis.

be directly attributable to hepatic insufficiency. The portacaval transposed (PCT) rat model achieves this by establishing total portal-systemic diversion with preservation of total hepatic blood flow and hepatic function (Ryan et al 1978b, Benjamin et al 1984).

In this study therefore, the concentrations of catechol and indole amines were measured in the brains of PCS and PCT rats at intervals up to 75 weeks to determine whether the changes described previously could be attributed to portal-systemic diversion or to reduced liver function. In addition the following studies were performed to determine:

1) The effect of perfusion on brain amines in PCS and control brains.

Although biochemical changes were observed in vivo in PCS rat brains it was uncertain if they were due to intrinsic fixed alterations of metabolic pathways in the CNS or to metabolic changes which are determined by the presence of portal-systemic diversion and hepatic dysfunction.

2) The difference in survival times between perfused brains of PCS and control rats.

PCS and control brains were perfused in order to compare the survival times and to determine whether any biochemical changes could be observed.

3) The optimum postoperative time to perfuse PCS rat brains.

The results of the long term PCS rats (described above) were used to determine the optimum postoperative time for perfusion experiments

in PCS rats.

9.2 MATERIALS AND METHODS

The catechol amines noradrenaline, adrenaline and dopamine and the indole amines 5HT (5 hydroxytryptamine), 5HTP (5 hydroxytryptophan) and 5HIAA (5 hydroxyindole acetic acid) were measured in the brains of PCS, PCT and control, sham operated, rats at 1, 3, 9, 15 and 75 weeks postoperatively. These amines were also measured in brains perfused according to the technique used in chapter 7. The main inactivation pathways of 5HT are through 5HT N-acetylase and monoamine oxidase (figure 9.1) and by reuptake into nerve vesicles. Therefore, in order to assess the rate of synthesis of indole amines the monoamine oxidase inhibitor NSD 10/15 (m-hydroxy benzylhydrazine, Sigma Corporation) was injected (100mg/kg IP) 30 minutes prior to sacrifice in PCS, PCT and control rats or added to the perfusion circuit at 1.0mMol/l 5 minutes after commencement of perfusion, according to the technique of Woods et al (1976). Measurement of 5 HTP or 5HT after blockage of breakdown at the monoamine oxidase level would therefore provide an estimation of the rate of formation of these indole amines. 5HT N-acetylase is believed to be localised in the pineal gland and this was therefore excluded from the tissues used in the assay (Cooper et al 1978).

9.2.1 Surgical techniques

Eighty male Sprague Dawley rats (250-300g) underwent surgery for the construction of end-to-side portacaval anastomosis or portacaval transposition. Portacaval anastomosis was carried out by exposure and mobilisation of the portal vein, which included the ligation and division of the gastroduodenal vein. Following mobilisation, the portal vein was carefully translocated to the ascending vena cava and the vessel secured by 2 stay sutures for the construction of an

end-to-side anastomosis, using a continuous suture of 7/0 silk (figure 9.3(a)). In general, the procedure did not take longer than 15 minutes.

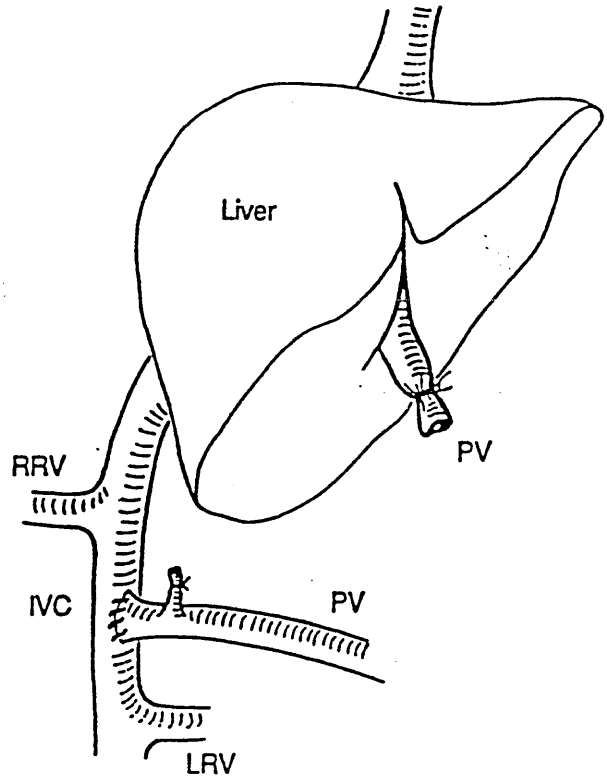
The technique of portacaval transposition was that originally reported by Ryan et al (1974) and involved the formation of 2 end-to-end anastomoses between the portal vein and the ascending vena cava. Briefly, an end-to-end anastomosis was constructed initially between the splanchnic end of the divided portal vein and the cephalic end of the divided vena cava. The second anastomosis was constructed between the caudal end of the vena cava and hepatic end of the portal vein (figure 9.3(b)). A continuous suture technique was adopted using 7/0 silk sutures as for the PCS. The construction of each anastomosis took approximately 15 minutes.

Sham operated controls were prepared by an identical procedure to PCT rats except that the ascending vena cava and portal vein were clamped for 15 minutes but not divided. Groups of rats (6 PCS, 6 PCT, 6 shams) were sacrificed at 1,3,9,15 and 75 weeks post operatively. The anastomoses were examined for patency at post mortem. The method of sacrifice is described in section 9.2.2.

9.2.2 Collection, extraction and storage of brain tissue

Rats were sacrificed by stunning and decapitation 30 minutes after intraperitoneal injection of the monoamine oxidase inhibitor. Skulls were then opened with a stout pair of forceps to expose the anterior facets of the cerebral hemispheres. Obstructing cranial bone was carefully removed with forceps to facilitate excision of the brain from the base of the skull speedily and with minimal trauma to the

Portacaval anastomosis (PCS)



Portacaval transposition (PCT)

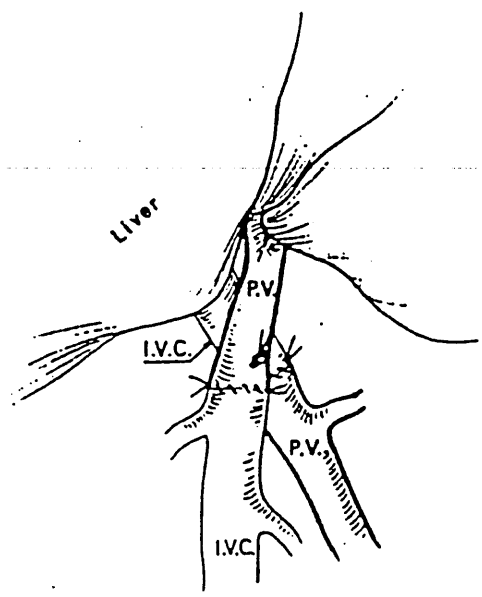


Figure 9.3 Anatomical details of a) portacaval anastomosis / shunt (PCS) and b) portacaval transposition (PCT). RRV = right renal vein, PV = portal vein, IVC = inferior vena cava, LRV = left renal vein.

underlying tissue. The pineal gland was discarded and the cerebellum removed to expose the midbrain which was excised and immediately stored in liquid nitrogen. The cerebral hemispheres were divided and each placed in a labelled container for storage into liquid nitrogen. The brains of both groups were then assayed for brain biogenic amines as described in section 9.2.3.

9.2.3 Chemical analyses

These were carried out as part of a collaborative study with the Department of Surgery, University of Lund, Sweden. Dopamine, noradrenaline, 5 hydroxytryptophan, 5 hydroxytryptamine and 5 hydroxyindole acetic acid were measured in the mid brain and the cerebral hemispheres by high performance liquid chromatography (HPLC) using an electrochemical detection system according to the methods described by Bengtsson et al (1985). Briefly, the tissue specimens were homogenised in 5ml 0.4M perchloric acid and then centrifuged at 34000g for 10 minutes. The supernatant from each sample was removed and filtered and the pellet discarded. The filtrate was then divided into a 2ml aliquot for indole amine measurements and a 1ml aliquot for catechol amine measurements.

Catechol amines

0.05µg of alpha methyl dopamine was used as an internal standard and 50µl of reduced glutathione (0.05mmol/l), 50µl EDTA (0.3mol/l, pH 7.0) and 20mg alumina were added to each sample. 1.0ml Tris buffer (1mol/l, pH 8.6) was then added to each sample, mixed and placed in a rotary mixer for 15 minutes. The supernatant was removed and the particles were washed 3 times in 3nmol/l EDTA (pH 7.0) and on the third wash each sample was centrifuged and the supernatant discarded.

The catechol amines were eluted from the alumina particles by mixing with 150µl perchloric acid (0.2mol/l). The sample was finally centrifuged and the supernatant frozen and stored in the dark. 50µl aliquots were thawed just prior to injection into the chromatographic column.

Catechol amine analytical equipment

A Varian 8500 high-pressure pump in combination with Rheodyne Model 7125 sampling value injector with a 100µl loop and a thin layer amperometric detector (Bioanalytical Systems Inc. model LC-2A) was used for the measurement of catechol amines. The functional electrode potential was maintained at 0.8 volts against a Ag/AgCl (3M NaCl) reference electrode using a graphite working electrode. The column was 250mm long, 5.0mm i.d. and packed with 5um Nucleoril 5SA. The mobile phase of the column contained 2.4g NaOH, 8.2g Na acetate (H_2O-free) and 8.4g citric acid per litre H_2O, pH 5.25. The flow rate was 1.0ml per minute and all the analyses were conducted at ambient temperature.

Indole amines

5HTP

The pH of each sample was adjusted to 4.0 with sodium hydroxide prior to adsorbance upon a Dowex 50wx4 column (5x10.0mm). 0.1µg alpha methyl 5HTP was added at this stage as an internal standard. Elution from the column commenced with the addition of 3ml 0.1M potassium phosphate buffer pH 7.0 and the eluate assayed for 5HTP by HPLC.

5HT

0.1µg alpha methyl 5HT was added to each sample as an internal standard. The pH of the sample was then adjusted to 6.0 with sodium hydroxide and passed through an Amberlite (IRP-64 column 5x12mm) in the Na+ form. The column was washed twice with 2ml 0.05M potassium phosphate buffer containing 0.2% EDTA, pH 7.0 and elution commenced with 2.0ml 1.2M hydrochloric acid, the concentration of 5HT being determined by HPLC.

5HIAA

5HIAA was prepared for analysis in a similar manner to 5HT except that 10µl of L-cysteine were added to the initial filtrate as an anti-oxidant.

Indole amine analytical equipment

A Waters M-600 high-pressure pump was used together with a Rheodyne Model 7125 sampling valve detector with a 100µl loop and a thin-layer amperometric detector (Bioanalytical Systems Inc, model L C-3). The working electrode potential was maintained at 0.85V against a Ag/Ag Cl (3M NaCl) reference electrode. The working electrode was a Glassy Carbon Electrode Model TL-5A.

9.2.4 Perfusion technique

The perfusion technique utilised for the isolated rat brain was identical to that described in chapter 7. A 5 minute equilibration period was permitted before NSD 10/15 was added to the reservoir of the perfusion circuit at 1.0mMol/l (Woods et al 1976). Thirty minutes after injection of the drug the brains were removed from perfusion and

stored in liquid nitrogen.

9.3 EXPERIMENTAL DESIGN

9.3.1 Effects of two different tissue collection techniques

It was necessary to establish whether the chosen method of sacrifice of the animals would affect the concentrations of brain amines. The two techniques which were compared were stunning and ether overdose and a total of 6 normal rats per group were analysed. The first of these involved the delivery of a sharp blow to the back of the head; the rat was then decapitated and the brain rapidly excised, dissected and placed into liquid nitrogen. Ether intoxication was achieved by placing the rat into a closed glass container which contained some cotton wool soaked in ether. After the animal became intoxicated through ether inhalation, a loss of righting reflexes was followed by a gradual reduction of respiratory movements which eventually led to total respiratory arrest. Once respiration had ceased the animals were decapitated and the brain rapidly excised, dissected and placed into liquid nitrogen. These results are presented in section 9.4.1.

9.3.2 Comparison between PCS and PCT rats

Portacaval anastomoses and portacaval transpositions were constructed in 2 groups of male Sprague Dawley rats and 6 rats from each group were sacrificed at 1,3,9,15 and 75 weeks post operatively. The brains were extracted and then assayed for the catechol amines noradrenaline, adrenaline and dopamine and indole amines 5 hydroxytryptamine (5HT), 5 hydroxytryptophan (5HTP) and 5 hydroxyindole acetic acid (5HIAA). These results are presented in sections 9.4.2 and 9.4.3.

9.3.3 Biogenic amines in perfused rat brains

Initial pilot experiments showed that elevations in amine metabolism were already established by 7 days post-operatively in PCS rats. This has subsequently been confirmed by other workers (Bengtsson et al 1988a). Brain amine concentrations were therefore measured in isolated perfused brains from PCS rats at this time point. In addition, biogenic amines were also measured in control perfused and control unperfused rat brains for direct comparison. This investigation was divided into 3 groups, a control non-perfused group, a control perfused group and a perfused PCS group, perfused one week post operatively. These results are presented in section 9.4.4.

9.4 RESULTS

9.4.1 Effects of two different tissue collection techniques

This study consisted of 12 normal rats of which 6 were killed by ether overdose and the remaining 6 by stunning. Figure 9.4 demonstrates that neither brainstem nor cerebral concentrations of the biogenic amines measured differed significantly between the two methods of killing (Student's unpaired t-test, $P>0.05$). This enabled the faster technique of stunning and decapitation to be used for collection of brain samples during future experiments. This also permitted comparisons to be drawn between ether anaesthetised rats used for perfusions and decapitated groups used for in vivo investigation.

9.4.2 Biogenic amine concentrations in PCS rats

Figure 9.5 shows the changes in catechol amines at various post-operative time periods extending to 75 weeks in rats following PCS. The major changes in cerebral catechol amines (figure 9.5.(b)) were in dopamine concentrations and were significant at one week post-operatively ($p<0.05$ Student's unpaired t-test). Similarly brainstem concentrations of dopamine increased at 1 and 3 weeks post-operatively ($p<0.001$ Student's unpaired t-test) but this decreased to pre-operative levels thereafter (figure 9.5(a)). The elevations in brainstem dopamine concentrations were much greater than those measured in the cerebrum. No significant trends in noradrenaline or adrenaline were observed.

Significant elevations ($p<0.05$ Student's unpaired t-test) were measured in cerebral 5HT, 5HTP and 5HIAA at one week and three weeks following PCS (figure 9.6(a)). Cerebral 5HT however returned to

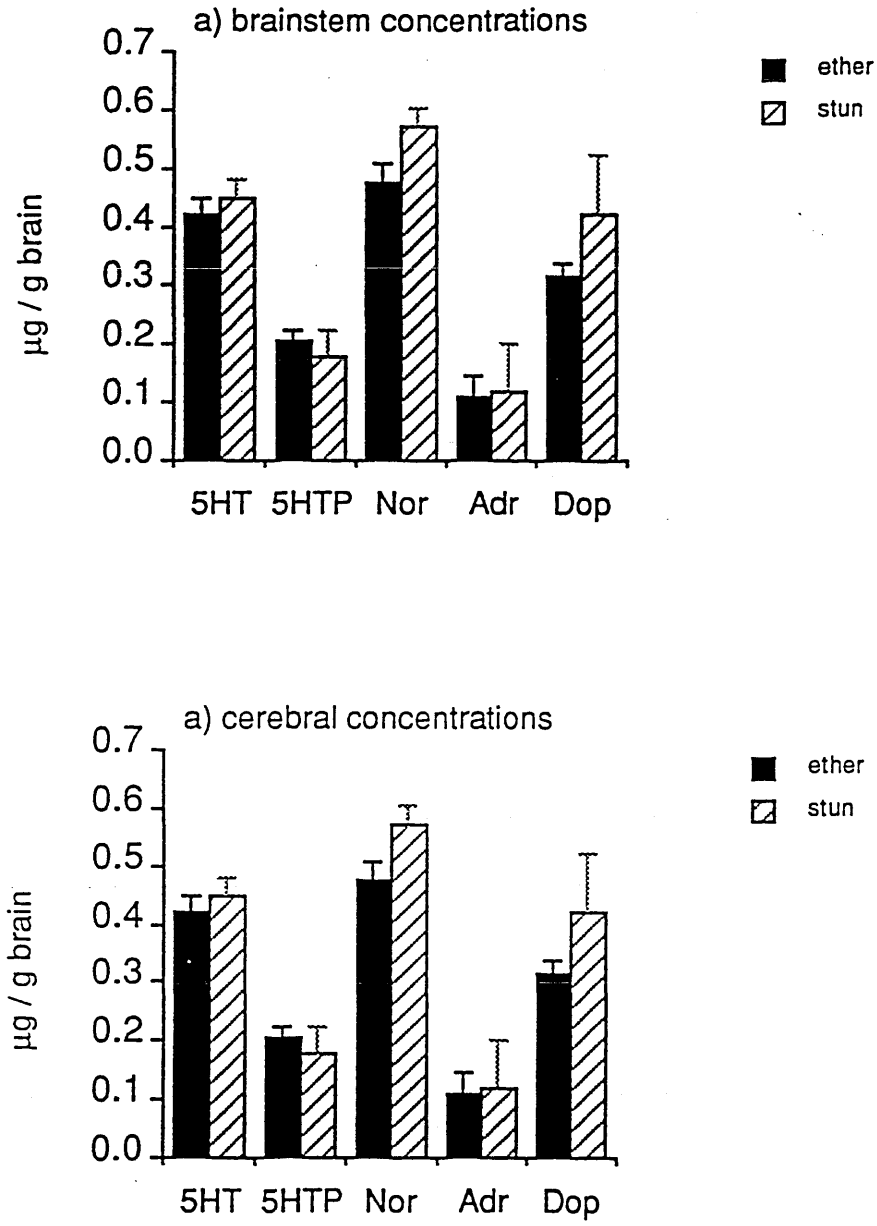


Figure 9.4 a) brainstem and b) cerebral concentrations of brain amines measured using ether overdose or stunning (stun) as methods of sacrifice (see appendix D1). Results shown are mean ± standard error, n = 6 per group.

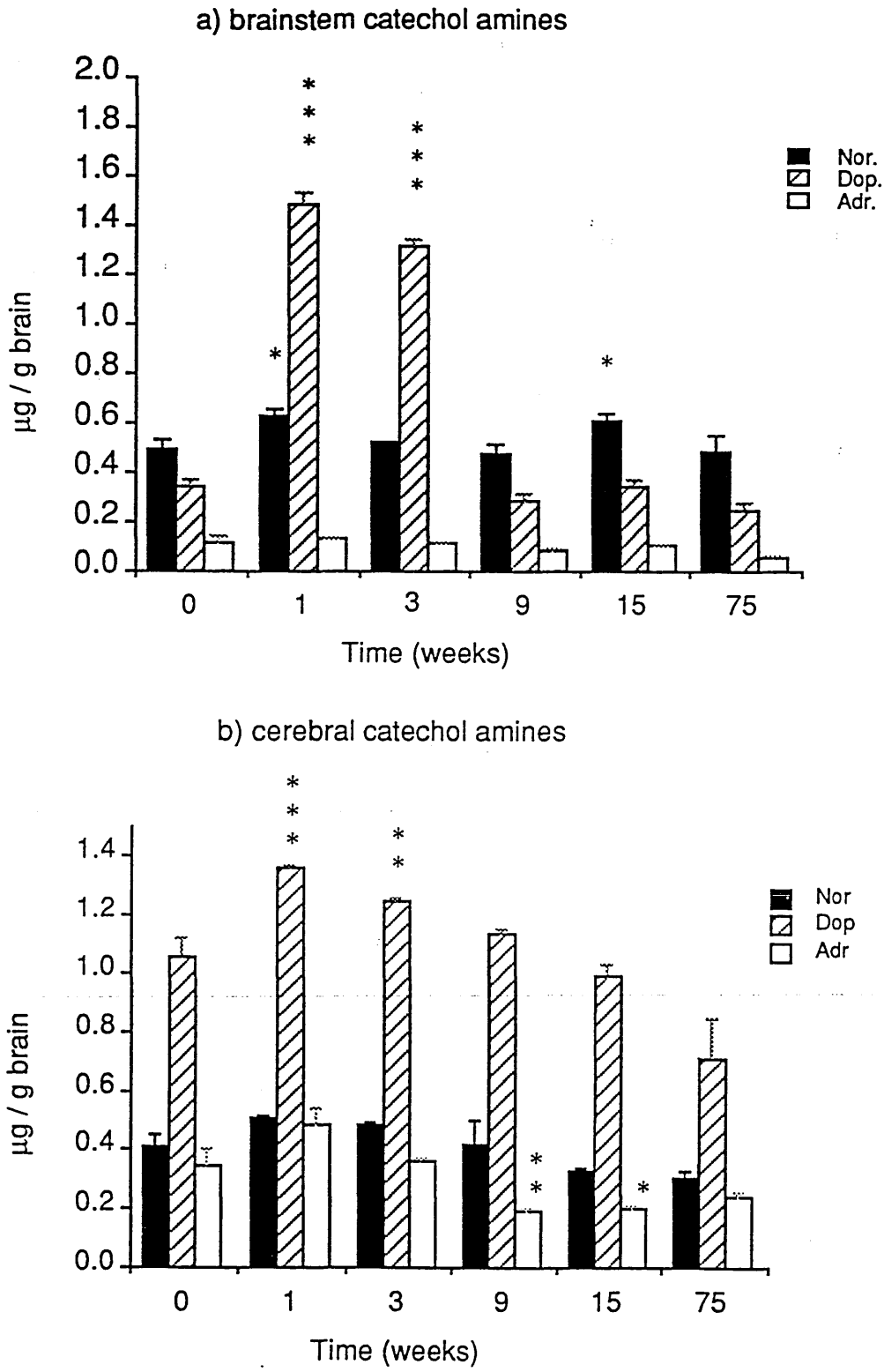


Figure 9.5 a) brainstem and b) cerebral catecholamines following PCS in rats. Results shown are mean ± standard error, n = 6 per group (see appendix D2). *P<0.05, **P<0.01, ***P<0.001 (Student's unpaired t-test, compared to time = 0 weeks).

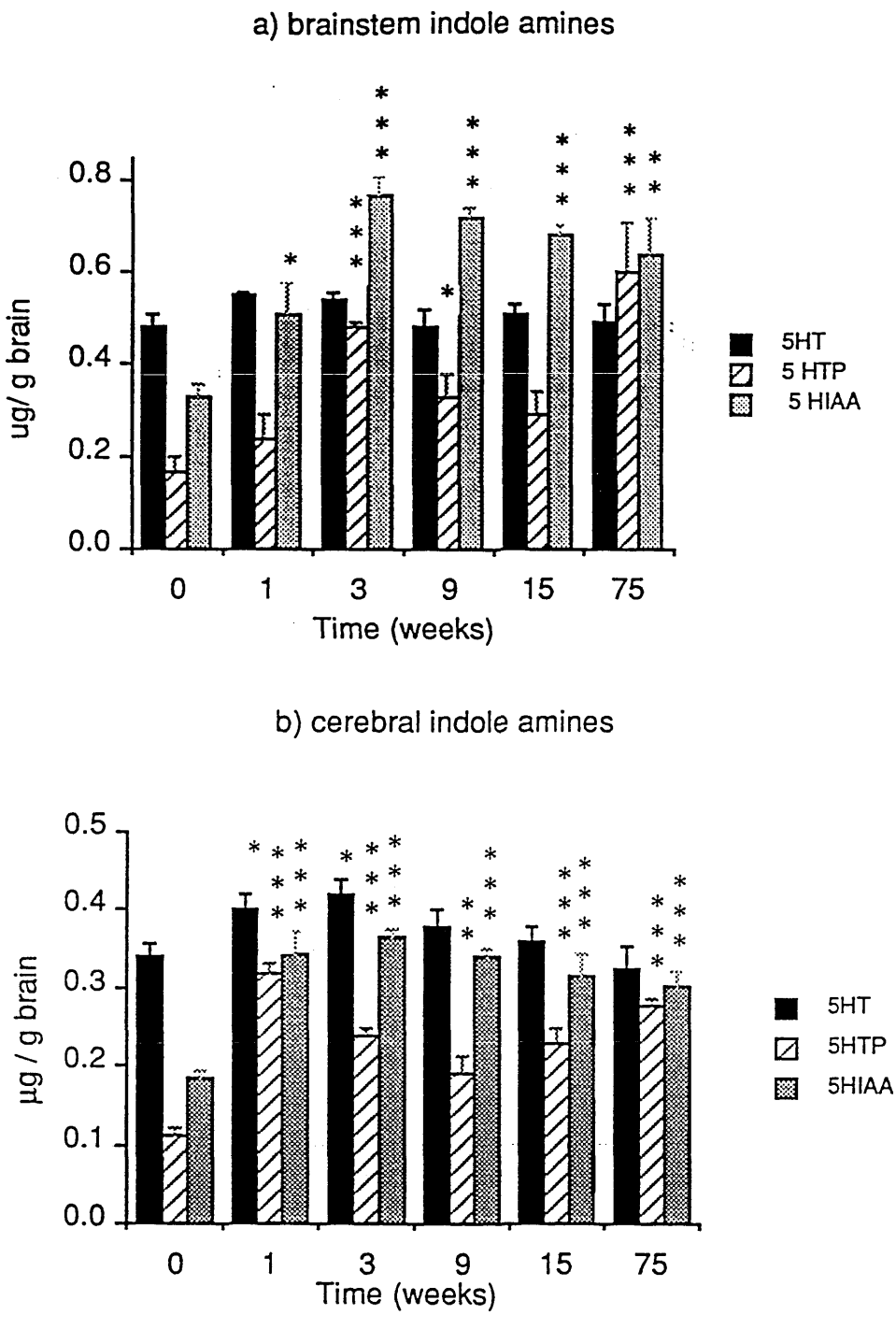


Figure 9.6 a) brainstem and b) cerebral indole amines following PCS in rats. Results shown are mean ± standard error, n = 6 per group (see appendix D3). *P< 0.05, **P< 0.01, ***P< 0.001 (Student's unpaired t-test, compared to time = 0 weeks).

preoperative values at 9 weeks (figure 9.6(b)). Both cerebral and brainstem 5HIAA and 5HTP concentrations remained elevated throughout most of the investigation. These results are in keeping with previous reports that brain biogenic amines are elevated significantly after portacaval anastomosis in rats (Bengtsson et al 1985, Siemert et al 1978).

9.4.3 Comparison of brain biogenic amine concentrations in PCS and PCT rats

This series of experiments assessed the degree to which portal diversion alone contributed to the elevations in brain biogenic amines previously measured in PCS rats. (The earlier series of experiments confirmed that the major changes in catechol amines were in dopamine, and therefore noradrenaline and adrenaline were not included in the next series of results.) Figure 9.7 illustrates the changes in brainstem and cerebral levels of dopamine in PCS and PCT rats. As in the previous series of experiments (figure 9.6), the largest elevations in dopamine concentrations were found in the brainstem of PCS rats at 1 and 3 weeks post operatively. Similar changes were measured in PCT rat brainstem concentrations figure 9.7(a), suggesting that these elevations were probably due to portal diversion rather than hepatic insufficiency. Cerebral dopamine concentrations (figure 9.7(b)) did not rise consistently above control values and were not significantly different between the PCS and PCT groups.

Figure 9.8 shows the changes in cerebral concentrations of indole amines in PCT and PCS rats at post-operative time periods up to 15 weeks. Figure 9.8(a) demonstrates that the concentrations of 5HT did not alter significantly in any of the 3 groups. In contrast, concentrations of 5HTP (figure 9.8(b)) and 5HIAA (figure 9.8(c)) were

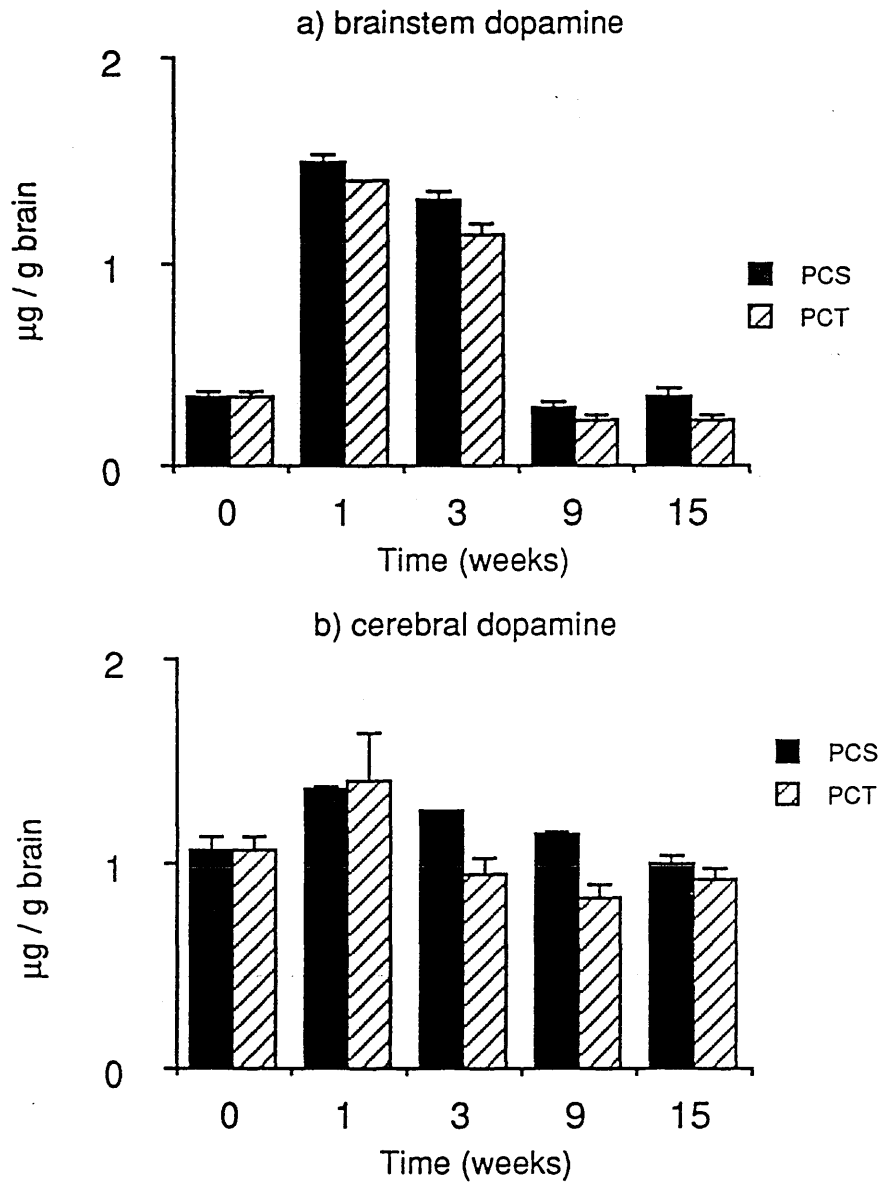


Figure 9.7 a) brainstem and b) cerebral concentrations of dopamine following PCS and PCT in rats. Results shown are mean ± standard error, n = 6 per group (see a appendix D5). No significant differences were found between the two groups at any time point (Student's unpaired t-test).

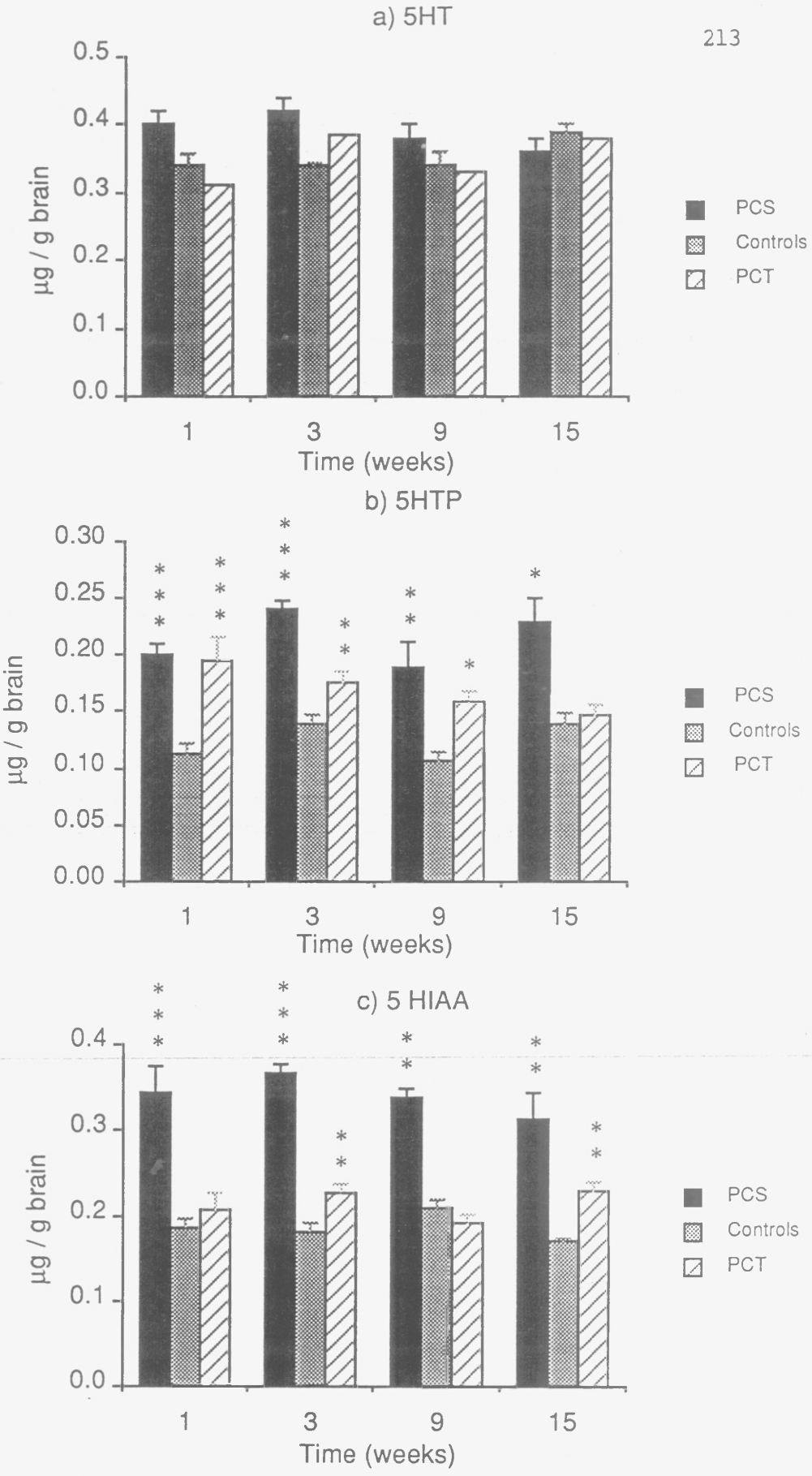


Figure 9.8 Cerebral concentrations of a) 5HT,b) 5HTP and c) 5HIAA following PCS and PCT in rats. Results shown are mean ± standard error, n = 6 per group (see appendix D6). * P< 0.05, **P< 0.01,***P<0.001 Student's unpaired t-test, compared to control rats at each time interval.

higher in PCS and PCT than control rats at most time points. PCS rats showed higher levels of 5HIAA than PCT rats at all time points, but higher levels of 5HTP at only 3 and 15 weeks.

Figure 9.9(a) shows that there were no differences in brainstem concentrations of 5HT between PCS, PCT and control rats except at 15 weeks post operatively. Figure 9.9(b) demonstrates that the PCS rat brainstem concentrations of 5HTP were elevated significantly above control values from 3 weeks onwards. The PCT rats also showed elevated levels of 5HTP up to 9 weeks, which declined to normal at 15 weeks. A comparable pattern was found in brainstem 5HIAA (figure 9.9(c)).

9.4.4 Biogenic amine concentrations in perfused rat brains

Previous work has suggested that alterations in biogenic amines could be a result of hepatic insufficiency and consequently one of the causative agents of hepatic encephalopathy (Fischer 1982). The next series of experiments was designed to assess the changes in biogenic amines in isolated perfused rat brains of PCS and control rats, in order to clarify whether the changes observed in PCS rats are due to an intrinsic CNS defect or to the presence of an abnormal liver and portal-systemic shunting in the intact animal.

In this experiment a series of perfused normal brains had a comparable survival time to the previous series of perfusions (median 17 minutes, range 15 - 26 minutes, n = 6). Perfused brains from PCS rats at 7 days after the operation had a median survival time of 18 minutes (range 16 - 27 minutes, n = 6) and were not significantly different from control brains.

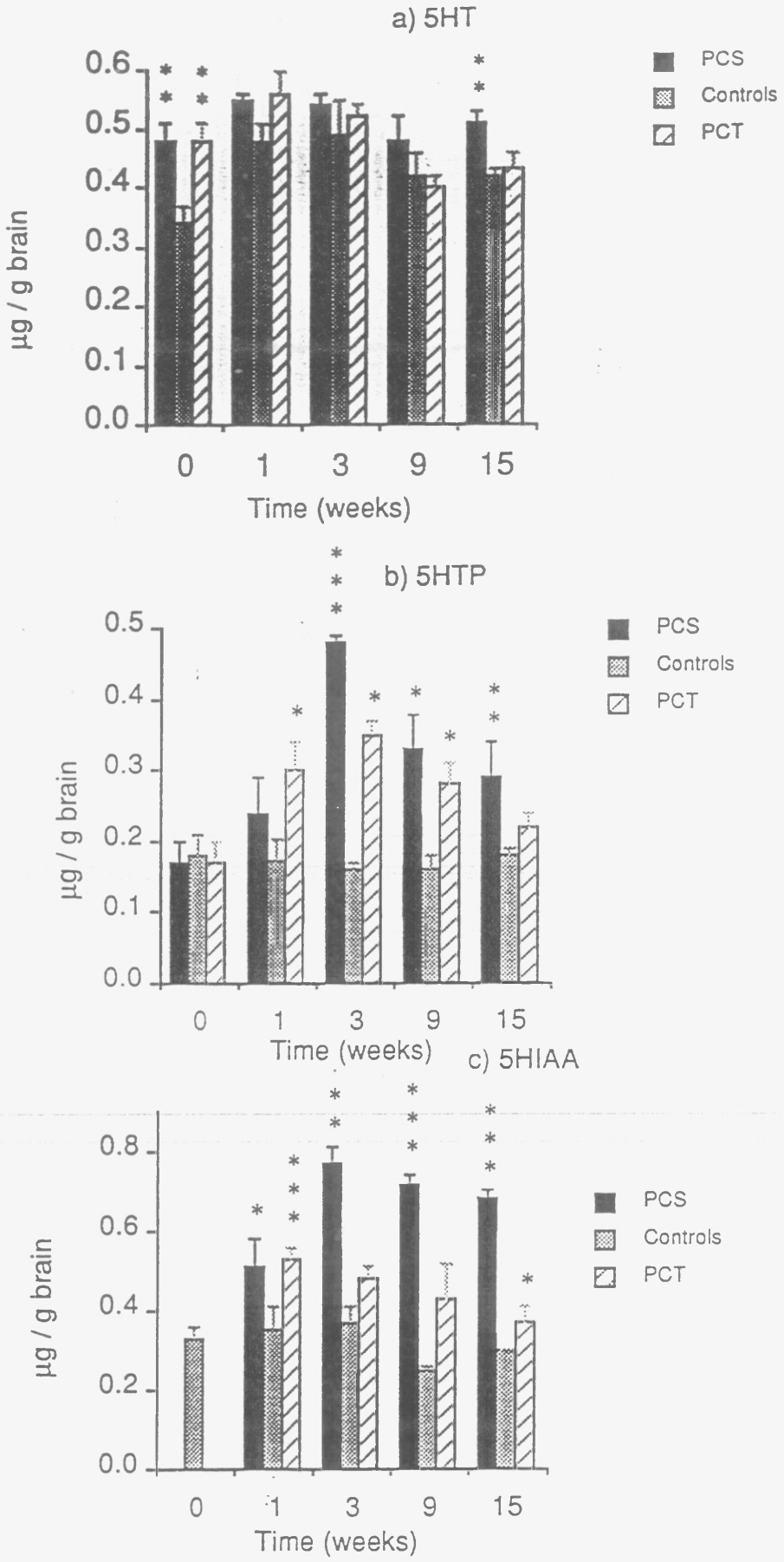
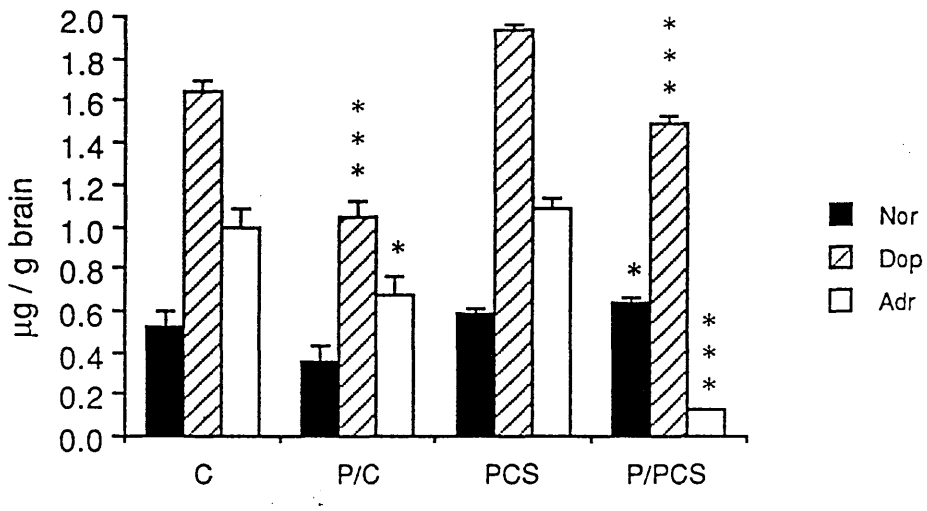


Figure 9.9 Brainstem concentrations of a) 5HT, b) 5HTP and c) 5HIAA following PCS and PCT in rats. Results shown are mean ± standard error, n = 6 per group (see appendix D7). *P< 0.05, **P< 0.01, ***P<0.001, Student's unpaired t-test, compared to control rats at each time interval.

The PCS brain amine investigations indicated that the major catechol and indole amine alterations were established by one week after operation (figure 9.5 and 9.6) and therefore this post-operative time interval was selected for perfusion of the brains of PCS rats. Cerebral catechol amines were not measured since the concentrations had been shown to be unaltered in PCS rats. Figure 9.10 shows brainstem catechol amine concentrations in a series of 6 perfused control and 6 perfused PCS brains one week post-operatively, and in 6 unperfused control and 6 unperfused PCS brains. Brainstem concentrations of dopamine and adrenaline measured in PCS and control brains after perfusion were lower than those in corresponding unperfused brains.

However, perfusion significantly decreased brainstem concentrations of 5HTP in both control and PCS groups (figure 9.11(a)). Figure 9.11(a) shows no significant differences in 5HT between any of the groups. These trends were also seen in the cerebral concentrations of 5HT and 5HTP (figure 9.11(b)). In general, PCS brains retained higher concentrations of 5HTP than controls following perfusion although, this procedure appeared to decrease brain amine concentrations.



C = unperfused controls, P/C = perfused controls

PCS = unperfused shunts, P/PCS = perfused shunts

Figure 9.10 Brainstem catechol amines in the isolated perfused and unperfused rat brain from control and PCS rats (1 week post-operatively). Results shown are mean ± standard error, n = 6 per group (see appendix D4). *P< 0.05, **P< 0.01, ***P<0.001, Student's unpaired t-test, perfused vs. unperfused rats.

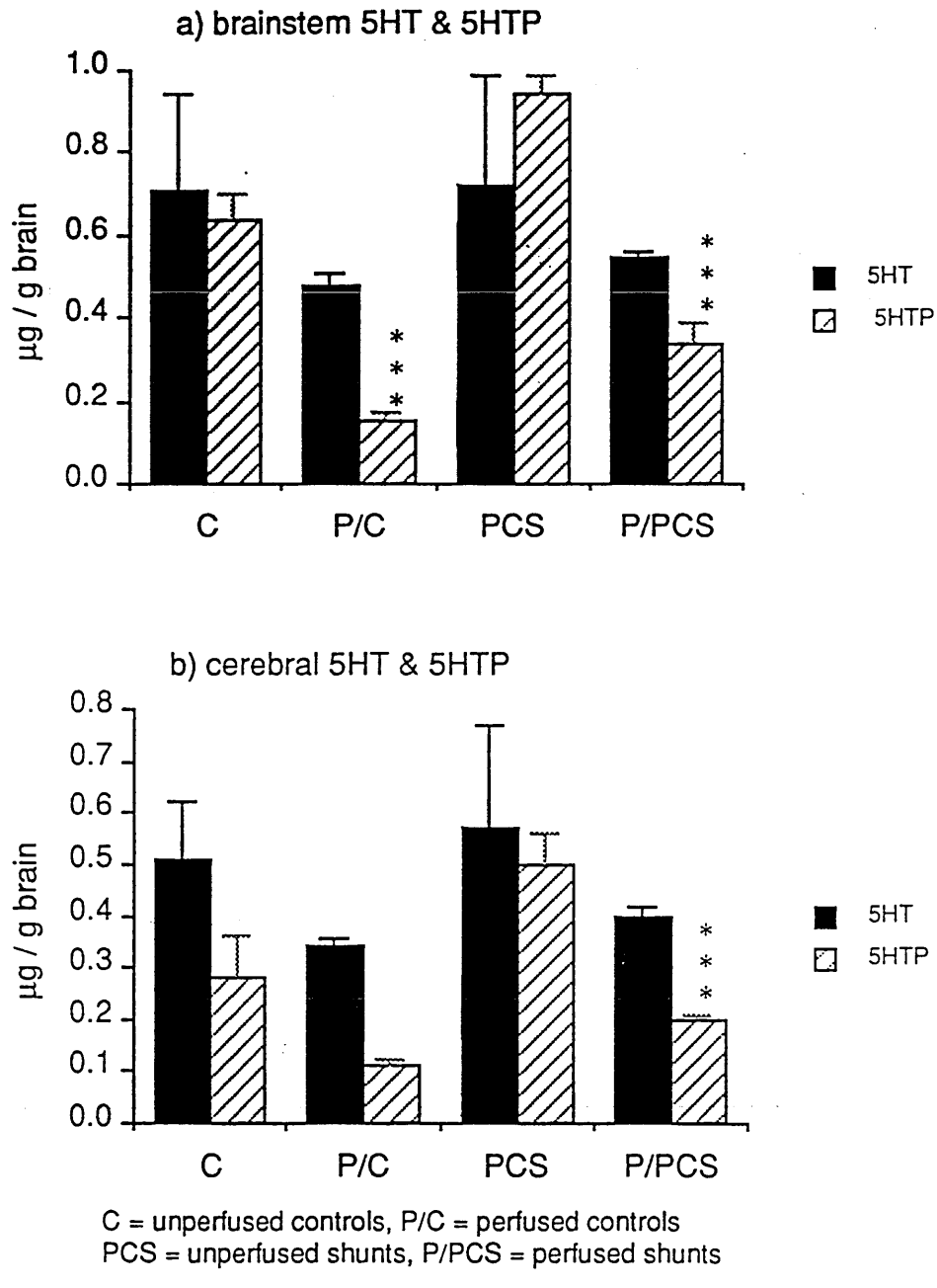


Figure 9.11 a) brainstem and b) cerebral concentrations of 5HT and 5HTP in the perfused and unperfused rat brain from control and PCS rats (1 week post-operatively). Results shown are mean ± standard error, n = 6 per group (see appendix D4). *P< 0.05, **P< 0.01, ***P<0.001, Student's unpaired t-test, perfused rats vs. unperfused.

9.5 DISCUSSION

Previous investigators have used the technique of stunning and decapitation as a rapid method for the excision of rat brains (Bucci et al 1980, Cummings et al 1976, Fischer and Baldessarini 1975, Siemert et al 1978, Zanchin et al 1981). The instability of catechol amine concentrations during stress and possibly during ether anaesthesia, could readily influence the concentration of biogenic amines in the rat brain. This study established that there were no differences between the 2 procedures studied.

Associations between increased brain indole amine turnover (Baldessarini and Fischer 1973, Curzon et al 1975, Tyce and Owen 1978, Bengtsson et al 1985), decreased catechol amine (dopamine) breakdown (Fischer 1982, Fischer et al 1976, Zieve and Olsen 1977) and hepatic encephalopathy remain speculative. Perhaps this is because the phenomenon of PSE is complicated by the existence of two components, portal-systemic diversion and hepatic insufficiency, both of which are seen in the PCS rat. The unique feature of the PCT rat, compared with the PCS rat, is that it provides an opportunity to segregate the shunting component from that of hepatic insufficiency. This series of investigations attempted to clarify whether previously observed changes in PCS rats were due to hepatic insufficiency or portal-systemic diversion by examining the various changes which occurred in the brain of PCS and PCT rats for up to 75 weeks post-operatively and also by assessing these changes during perfusion. The perfused, in vitro, investigations were of interest since the influence of other organs in the body was removed and therefore the status of PCS rat brains could be assessed directly. Furthermore, this model avoided influence of ammonia either from

dietary sources (Jeppsson et al 1979, Sherlock 1975) or from gut flora (Nance et al 1971). Finally this model permitted investigation of whether the changes observed in vivo persisted in vitro during perfusion.

9.5.1 IN VIVO STUDIES

Noradrenaline and adrenaline

Changes in catecholamine concentrations have produced varied results in previous studies. Our studies confirmed significant reductions in cerebral adrenaline but not noradrenaline, at 9 and 15 weeks. However after 75 weeks, no significant increases were found in cerebral noradrenaline or adrenaline in PCS or PCT rats. Reduced cerebral noradrenaline and dopamine concentrations were also measured in PCS dogs (Faraj et al 1981, Fischer 1982), but these studies only measured the changes for up to 4 weeks post operatively. It is therefore possible that some form of adaptive change may occur in the metabolism of noradrenaline and adrenalin between 4 and 75 weeks following the operation which tends to normalise brain concentrations of these catechol amines. It is further likely that these transient changes are due to portal systemic diversion rather than hepatic dysfunction.

Dopamine

The observed transient increases in dopamine concentrations reported in our studies confirm the findings of Siemert et al (1978) who found little change by 6 weeks after PCS. In addition, Tyce and Owen (1978) did not observe any significant changes in cerebral dopamine concentrations in hepatectomised rats. However in contrast

to the results of Siemert et al (1978) and Tyce and Owen (1978) increases were measured at 1 and 3 weeks following the operation in PCS rats and were accompanied by corresponding increases in PCT rats (figure 9.7). This observation suggests that these transient increases are due to portal-systemic diversion rather than hepatic dysfunction.

These results support the hypothesis that if alternative catecholamine pathways can be stimulated due to the increases in dopamine concentrations these could lead to the formation of other biogenic amines which act as false neurotransmitters (Fischer 1982) (see figure 9.2). Figure 9.2 shows how an increase in the formation of dopamine could be a result of increased tyrosine or phenylalanine levels. Increased concentrations of these two substrates could increase the formation of phenylethanolamine, tyramine and octopamine, all of which are false neurotransmitters as described in chapter 2 (Faraj et al 1981, Fischer 1982, Payne 1982, Tyce and Owen 1978). Aromatic amino acid decarboxylase does not show a high degree of specificity and acts, at least in vitro, on a variety of substrates oxidizing virtually any phenylethylamine to its corresponding phenylethanolamine (for example phenylalanine to phenylethanolamine and tyrosine to tyramine). Consequently a number of structurally analogous metabolites such as tyramine may replace noradrenaline at the nerve endings and function as a false neurotransmitter by the generation of atypical post-synaptic potentials (Cooper et al 1978).

In addition, these observations may also explain the discrepancies in the literature regarding the changes in catecholamine metabolism following PCS. For example Bucci et al (1980) reported no change in brain dopamine 7 weeks following PCS in rats. This suggests

that he missed the early peak in concentrations found in these studies. Siemert et al (1978) also did not look at the concentrations until 6 weeks post-operatively and again may have missed an earlier peak. Faraj et al (1981) showed increased plasma dopamine levels and decreased brain concentratons of dopamine following PCS in dogs. In contrast, Curzon et al (1975) reported no change in brain dopamine concentrations 3 weeks after PCS in rats.

5HT

The results showed that cerebral 5HT levels did not alter significantly during the time course of the experiment (figures 9.6a and 9.8a). Figure 9.9(a) shows lower values of brainstem 5HT in control animals (unoperated) than in PCS or PCT animals sacrificed immediately after the procedures. There is no obvious explaination for this difference.

5HTP and 5HIAA

Sustained increases in cerebral 5HTP and 5HIAA concentrations were observed throughout the experiment in PCS rats (figure 9.6a). However, only 5HIAA concentrations remained elevated in brainstem regions of PCS rats when compared to control values (figure 9.6b). The increased levels of 5HIAA suggest an increased breakdown of 5HT, particularly since the concentration of 5HT remained essentially unchanged. The increased cerebral levels of 5HTP suggest an increase in synthesis of 5HT, at least up to 9 weeks. This implies that 5HT is either being metabolised at a faster rate by monoamine oxidase and aldehyde dehydrogenase (reflected in the increased levels of 5HIAA, see figure 9.1), or that reuptake into nerve vesicles has been

accelerated.

Elevations in the cerebral (figure 9.8b) and brainstem (figure 9.9b) concentrations of 5HTP were seen in PCT rats up to 9 weeks. This suggested that previously reported increases in 5HT and tryptophan concentrations in PCS rats (Baldessarini and Fischer 1973) could be attributed to portal-systemic diversion up to this time. These levels were not significantly higher than controls at 15 weeks. These results imply that the reduction in 5HTP concentrations (the precursor of 5HT) in PCT rats after 15 weeks (figures 9.8b and 9.9b) might be due to the increased catabolism of 5HT thus reducing the concentrations of its precursor. It is therefore possible that previously reported increases in tryptophan and 5HT levels in PCS rats (Baldessarini and Fischer 1973) could have been due to portal-systemic diversion rather than liver dysfunction since increased levels of 5HTP were found in our PCT results up to 9 weeks post-operatively. However, significant elevations of cerebral and brainstem 5HIAA in PCS rats above PCT and control rats were measured throughout the study. These results suggest an increased catabolism of 5HT as a direct consequence of hepatic insufficiency and not due to portal-systemic diversion. Alternatively a reduction in the excretion rate of 5HIAA may have occurred as a consequence of hepatic insufficiency. This seems to be less likely because increases in 5HTP concentrations were found with no corresponding increase in its metabolite 5HT. This suggests an increase in the breakdown of 5HT through increased monoamine oxidase and aldehyde dehydrogenase activity or increased 5HT acetylase activity to reduce 5HT concentrations (figure 9.1). Thus while it is agreed that portacaval anastomosis increases 5HT metabolism in general (Bucci et al 1980, Baldessarini and Fischer 1973), the results from this study further suggest that liver

dysfunction per se may increase the activity of monoamine oxidase and aldehyde dehydrogenase, thus increasing the catabolism of 5HT.

9.5.2 PERFUSED SERIES

In general, perfusion of PCS and control rat brains decreased the concentrations of all brainstem catechol amines (figure 9.10). The observation that in vitro perfusion of 1 week PCS rat brains reduced the increased levels of dopamine and 5HTP measured in vivo was probably indicative of the transient nature of these changes at least during the early post-operative period. It is possible that these differences are due to oedema in the brain caused by perfusion. Future studies could confirm this by measurement of the wet weight of the brains in control and perfused brains. However, perfused PCS brains had significantly higher levels of dopamine but lower levels of adrenaline than perfused control brains. The differences in dopamine and adrenaline concentrations in each perfused group did not appear to influence survival time, which did not differ between the two groups.

A significant decrease in brainstem 5HTP was found between all perfused and corresponding unperfused rat brains (figure 9.11a). In addition a significant decrease in cerebral 5HTP was seen in PCS perfused brains. This contrasts with the results of Woods et al (1976) who reported few alterations during perfusion. The pre-perfusion values measured during these studies were higher than those reported by Woods et al (1976). However, these authors did not differentiate between cerebral and brainstem concentrations of each amine. Nor is it clear whether these authors excluded other sources of brain amines, especially indole amines from the pineal gland, the normal site of activity of 5HT-n-acetylase (figure 9.1). There were

also differences in the techniques used to measure the amines: Woods used flourometric analyses and this study used HPLC.

The data also showed increased dopamine concentrations in PCS brainstems compared with control brains following perfusion. This was accompanied by a reduction in adrenaline concentrations when compared to control values. However, these results do not preclude the possibility that a reversal of the PCS amine changes during perfusion might have influenced the survival time.

It is also possible that portacaval anastomosis may not be sufficient to precipitate the gross derangements usually associated with PSE. It has been suggested that PCS produces insufficient impairment in hepatic function to act as a reliable model of PSE (Bucci et al 1980). Investigation of the changes in brain biogenic amine concentrations in PCT rats during this study showed that much of the observed changes could be accounted for by systemic diversion of portal blood in the PCS rat. Therefore the hepatic dysfunction component of the PCS rat may be insufficient to present all the characteristics normally associated with the syndrome of PSE.

9.6 SUMMARY

Preliminary investigations upon the influence of the method of sacrifice on the concentrations of brain amines showed that there were no significant differences between the 2 methods studied.

The transient increase in dopamine concentrations seen in PCS rats at 1 and 3 weeks may have promoted the formation of the false neurotransmitters phenylethanolamine, tyramine and octopamine at these time points. These changes were also seen in PCT rats and this suggested that the changes were due to portal-systemic diversion rather than hepatic insufficiency. Moreover, the increases in 5HTP, and particularly 5HIAA, which were unique to PCS rats, suggest that the changes were due to hepatic dysfunction rather than portal-systemic diversion. However, the increases in 5HTP were transient and only 5HIAA concentrations remained elevated throughout the study. These data therefore suggest that breakdown or possibly re-uptake of 5HIAA are reduced in hepatic dysfunction.

In vitro perfusion of 1 week PCS rat brains reduced the increased in vivo levels of dopamine and 5HTP, possibly reflecting the transient nature of the alterations during the early post operative period. In addition, it is concluded that the nature of the changes seen in PCS rats are not due to intrinsic fixed alterations of metabolic pathways in the brain.

CHAPTER 10

CONCLUSIONS AND FUTURE PROPOSALS

CONCLUSIONS AND FUTURE PROPOSALS

It is difficult to ascribe the pathogenesis of hepatic encephalopathy to a single factor. The wealth of literature on the subject reflects the probable multifactorial nature of the process. In addition the considerable degree of pathological deterioration that the liver may undergo before any deleterious effect is reflected in the brain further complicates the picture. Results obtained from experiments with germ-free PCS dogs (Nance et al 1971) and rats (Jeppsson et al 1979), eviscerated rats (Degos et al 1974) and patients undergoing treatment with antibiotics (Dawson et al 1954) demonstrate that the liver may provide functions other than detoxification of portal blood to maintain liver/brain equilibrium. Original information in support of this theory was provided by the in vitro studies of Geiger and Magnes (1947) who demonstrated that the inclusion of an isolated perfused cat liver in an isolated perfused cat brain preparation prolonged the brain survival time and provided a regulatory role in the control of brain oxidative metabolism. This thesis has confirmed the observations of Geiger, using the rat as the experimental model, and related the results to the in vivo neuropharmacological changes associated with PSE in PCS and PCT rat preparations.

The inadequacy of many existing models of isolated organ perfusion preparations necessitated the development of a new circuit. The preliminary results of the isolated perfused rat liver preparations in chapter 5 indicated that the new system was capable of maintaining a functioning liver for up to four hours after which optimum conditions could not be maintained (chapter 6). The viability of the liver was readily assessed by measurement of perfusion

pressure, bile volume production and hepatic oxygen uptake. Bile volume production and hepatic oxygen uptake are probably the best indices of viability of the isolated perfused rat liver (Brauer et al 1951, Ashford and Burdette 1965, and Keiding et al 1980). Assessment of trauma to the perfusate in the system by measurement of plasma free haemoglobin and haematocrit showed that the circuit was adequately trauma-free for the time period necessary for these investigations.

A series of isolated rat liver perfusions (chapter 6) established that the choice of perfusate, erythrocyte source and haematocrit could influence liver viability. Washed canine red cells did not induce gross metabolic derangements although variations in bile volume production were observed. Optimum conditions for survival of the isolated perfused rat liver were defined and utilised for the series of concomitant liver/brain perfusions described in chapter 8. The use of haemodiluted rat blood as the perfusate could not maintain acceptable EEG signals from the isolated perfused rat brain, a finding in agreement with that of Andjus et al (1967).

A detailed series of investigations established the isolated perfused rat brain preparation (chapter 7). The EEG traces recorded were assessed in vivo and in vitro by administration of incremental doses of sodium pentobarbitone up to a lethal dose. Parameters such as flow rate, perfusion pressure and the PO_2 of the perfusate were investigated under constant experimental conditions. It was found that the best results were obtained by in situ isolated rat brain perfusions, which exhibited minimal extraneous electrical noise. These investigations established an average survival time of the in situ isolated perfused rat brain and served as a baseline for investigations upon the influence of concomitant perfusion with an

isolated rat liver (chapter 8).

The combined perfusion studies clearly demonstrated the physiological dependence of the brain upon the liver for the normal maintenance of the spontaneous EEG and oxidative glucose metabolism. This repeated the previously unconfirmed findings of Geiger and Magnes (1947). Future investigations with this model might concentrate upon more precise measurements of glucose concentrations to determine if decreases in glucose uptake correspond with a failing liver after 2 to 3 hours of perfusion.

The biochemical features of the liver/brain interrelationship were investigated further in chapter 9 by a series of experiments conducted using PCS and PCT rats. Chapter 9 examined the relevance of the PCS rat model to clinical hepatic encephalopathy: changes observed in the PCS rat were compared to the PCT rat, including the measurement of brain biogenic amines at post-operative time intervals of up to 75 weeks. These results were then compared with those from the in vitro isolated perfused brain described during chapter 7. These results implied that elevations in brain amines were not solely responsible for the reduction in the survival time of the perfused brains and that some other factors may be responsible, including the regulation of glucose entry into the brain. Previous investigations have associated variations in the concentration of brain amines of PCS dogs and rats with the precipitation of PSE (Bucci et al 1980, Fischer et al 1975, Siemert et al 1978) but the data from this thesis suggest that these changes may only be one aspect of the problem.

Other studies have measured visual evoked EEG potentials in galactosamine induced hepatic failure rats. These results were found different from those recorded in rats in which ammonia and AAA

concentrations were artificially elevated to simulate HE (Zeneroli et al 1981). These results also implied that elevated plasma ammonia and AAA concentrations are not the major factors involved in the precipitation of PSE.

The assessment of visual evoked potentials in PCS and PCT rats has not been reported in the literature to date. However behavioural and spontaneous EEG measurements in PCS rats up to 50 days post-operatively disclosed few changes, although increases in brain indole amine metabolism and ammonia concentrations were detected (Bucci et al 1980). Woods et al (1976) also reported an increase in brain concentrations of 5HT in PCS rats which was probably attributable to an increase in the formation of 5HT. However, 5HIAA concentrations were not measured and the increase could have been due to a decrease in 5HT catabolism and this remains to be clarified. Significant elevations in cerebral and brainstem concentrations of indole amines were also measured in PCS rats during this study with a decreased concentration in brainstem dopamine. The changes in catechol amine metabolism were also measured in PCT rats which implied that these were due to portal diversion rather than hepatic dysfunction. It therefore remained a possibility that the elevations in 5HT and 5HIAA which were exclusive to PCS rats may have a relevance in the precipitation of PSE.

In order to confirm the relevance of these agents to the syndrome of PSE, it would be of interest to measure them in acute forms of experimental hepatic failure induced either by hepatectomy or drugs such as carbon tetrachloride or galactosamine. These results could be related to the degree of severity of HE in these rats by means of EEG and behavioural measurements. This series of investigations could

help answer the criticism that the PCS rat model may lack the necessary degree of severity to reproduce all the features of PSE (Bucci et al 1980). These changes could then be compared to measurements conducted in totally hepatectomised rats or rats with drug induced hepatic failure in order that the degree of injury could be readily assessed. This could help establish a correlation between the severity of PSE (as measured by EEG and behavioural studies), the degree of hepatic dysfunction and the concentrations of putative neurotransmitters.

The influence of portal diversion alone on the electrophysiological status of PCS rat brain has yet to be reported. This component of the PCS rat model may be of equal importance to hepatic dysfunction during PSE and this could be readily investigated in the PCT rat. In addition, measurement of the blood brain barrier (BBB) permeability by the horseradish peroxidase technique (Laursen and Westergaard 1977) in PCT rats could establish the relevance of portal diversion alone upon the observed increases in BBB permeability in PCS rats.

Investigations into the numerous mechanisms that have been associated with PSE have been in progress for over 20 years. Recent studies, including the present one, have brought sophisticated techniques such as microvascular surgery and isolated organ perfusion into use to address some of the fundamental questions. Although these investigations have yielded only a limited amount of information, I feel that some valuable observations have been recorded which will contribute to our understanding of experimental PSE and PSE in man. In particular during this study, the concept of combined liver and brain perfusion was re-introduced, miniaturised using modern

technology and successfully applied to the rat. Consequently the results obtained here bear a greater relevance to the majority of current experimental data upon PSE which have been obtained in this animal. I hope that this work will stimulate others to adopt these new techniques as an investigative tool to help unravel the complex syndrome of portal systemic encephalopathy.

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APPENDICES

Appendix A1 Effect of sodium hydrosulphite concentrations on oxygen transport (TPT).

Conc/Moles	0.1	0.25	0.5	0.75	1.0	2.0
TPT ml/min/m^2	160±8	136±9	306±8	327±12	319±10	340±8

Appendix A2 Effect of flow rate on oxygen transport

Flow ml/min	25	50	100	200
TPT ml/min/m^2	302±10	332±15	329±10	305±8

Appendix A3 Effect of zero flow rate on oxygen transport

Time/mins	1	2	3	4	5	6
TPT/ml/min/m^2	291	291	166	146	42	21

Results shown are mean ± S.E n= 6

Appendix A4 OTC vs flow rate in uncoated and coated oxygenators

			UNCOATED MEMBRANE			
Flow ml/min	5	12.5	15	18	20	25
TPT ml/min/M2	61.5,63	118	118	112,112	142,167	114,114,127
					167	144,121,178
			COATED MEMBRANE			
Flow ml/min	5	12	15	18	20	25
TPT ml/min/M2	41	78,53	64	104,104	91,78	98,93
				92,112		78

Appendix A5 % Saturation difference vs flow rate in uncoated and coated oxygenators

Flow ml/min	5	12.5	15.0	17.5	20	25
Coated	39	25,18	-	22,28,26,26	18,21	19,17.5,17.0
Uncoated	45,55	35	37	28	29.5,28.5	29.5,27,26.2

Appendix B1 Table of parameters measured during the 6 isolated rat liver perfusions described in Chapter 5. Results shown are mean ± S.E. n=6

MEASUREMENT	HOURS OF PERFUSION 0	1	2	3	4	5	6
Oxygen uptake (μmol $min^{-1}g^{-1}$)	-	0.99 ± 0.08	0.93 ± 0.11	0.93 ± 0.13	0.88 ± 0.09	0.95 ± 0.07	0.85 ± 0.12
Bile Volume (μl)	527 ± 65	630 ± 81	784 ± 84	694 ± 88	505 ± 97	391 ± 92	180 ± 46
Haematocrit (% cells)	9.75 ± 0.35	9.67 ± 0.38	9.63 ± 0.36	9.58 ± 0.31	9.73 ± 0.30	9.55 ± 0.31	9.27 ± 0.24
Liver Wt (g)	16.34 ± 0.75	-	-	-	-	-	10.46 ± 0.18
Perfusion Pressure (mmHg)	18.0 ± 4.1	11.4 ± 1.6	11.5 ± 2.3	10.5 ± 1.9	11.6 ± 2.4	9.9 ± 2.1	8.7 ± 1.7
Flow rate (ml $min^{-1}g^{-1}$)	1.92 ± 0.06	1.92 ± 0.06	1.92 ± 0.06	1.92 ± 0.06	1.92 ± 0.06	1.92 ± 0.06	1.92 ± 0.06
K+ (μmol l^{-1})	5.56 ± 0.6	5.25 ± 0.36	5.9 ± 0.60	6.20 ± 0.40	6.70 ± 0.63	6.64 ± 0.38	7.30 ± 0.4
Urea (μmole l^{-1})	4.1 ± 0.7	4.3 ± 0.3	5.3 ± 0.4	5.7 ± 0.5	6.0 ± 0.7	6.0 ± 0.4	7.73 ± 1.2
Bilirubin (μmol l^{-1})	0.9 ± 0.31	0.97 ± 0.31	2.01 ± 0.66	2.45 ± 0.51	2.48 ± 0.65	3.9 ± 0.45	4.68 ± 1.34
Glucose mmol l^{-1})	17.83 ± 1.62	27.54 ± 1.52	21.94 ± 1.31	22.07 ± 1.36	20.54 ± 1.23	20.62 ± 3.16	19.52 ± 1.52
Plasma haemoglobin PFH (mg%)	85.8 ± 12.3	178 ± 22.1	274 ± 28.0	343.1 ± 25.9	439.7 ± 431	516.1 ± 52.8	650.1 ± 53.3
AST (IU l^{-1})	18.75 ± 1.25	34.67 ± 9.3	42.00 ±15.50	57.67 ± 17.89	85.67 ± 6.06	10267 ± 15.14	65.67 ± 23.59
ALK (IU l^{-1})	26.4 ± 3.4	26.2 ± 5.1	29.25 ± 9.1	41.0 ± 16.0	50.00 ± 18.1	57.7 ± 225	75.3 ± 30.8
Bilirubin (μmol l^{-1})	47.25 ± 12.06	11.50 ± 0.25	13.50 ± 0.90	23.00 ± 0.61	31.25 ± 0.41	36.00 ± 2.30	46.25 ± 2.13

Appendix B2 BSP removal during LP66. Results shown are BSP concentrations.

	HOURS OF PERFUSION												
MEASUREMENT	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
Plasma/mg%	3.0	2.4	2.2	2.1	2.0	1.9	1.7	1.23	2.4	2.4	2.6	2.5	62.5
Bile mg%	-	8.75	5.8	4.88	6.25	5.625	6.72	4.68	6.4	11.72	14.68	14.37	17.19

APPENDIX B3

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LABORATORY INVESTIGATION

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Methods in Laboratory Investigation

Practical Miniaturized Membrane Oxygenator for Isolated Organ Perfusion

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Silastic membrane tubing oxygenators possess valuable atraumatic properties, but no satisfactory miniature system for isolated organ perfusion has been achieved. We describe the construction of a new type of miniaturized Silastic membrane tubing oxygenator, 1.5 m long, 1.5-mm diameter, 75-μm wall thickness, incorporated in a single self-supporting unit readily applicable to isolated organ perfusion because of its simplicity, durability, and small prime volume of less than 3 ml. *In vitro* experiments using out-dated human banked blood demonstrated that the unit can effectively oxygenate blood of hematocrit 35% at flow rates of at least 10 ml/minute and that higher flow rates may be used to oxygenate blood or perfusates of a lower hematocrit. The results were verified in three different practical experimental applications: isolated rat liver, rabbit heart, and rat free skin flap perfusion.

Additional key words: Perfused liver, Perfused heart, Perfused free skin flap, Coiled tubing oxygenator, Small animal oxygenator.

Maintenance of a viable isolated, perfused organ is dependent upon an efficient delivery to, and exchange of, (blood) gases, with the tissues. Oxygenators used in the past for this purpose often required large prime volumes and were complicated by foaming due to direct contact of the perfusate with the gas phase (6, 22, 28, 30). The latter problem could be reduced by use of rotating spherical flasks (23), but these still required a large prime volume and introduced the added complication of a mechanical component. Bubble oxygenators used for cardiopulmonary bypass in small laboratory animals (29) still produce damage to erythrocytes (13, 32), low density lipoproteins (5, 39), and reticuloendothelial function (27, 33).

The advent of membrane oxygenators reduced many of these traumatic effects, and although smaller membrane oxygenators were produced for small animals and isolated organ studies, they still had comparatively large prime volumes, and economy restricted their widespread use (38).

Membrane oxygenators have been successfully produced using hollow Silastic membrane tubing (dimethylpolysiloxane), and their efficiency has been assessed (2). The atraumatic properties of this material have already been established (1, 11, 15, 21), and a miniaturized prototype was developed in our laboratory comprising 18 tubes, 1.5-mm bore, 30 cm in length, that reduced the prime volume to 12 ml (7). The header problem was decreased by the use of larger diameter membranes than those utilized, for example, in hollow fiber dialyz minimizing the dead space area exposed to blood. H ever, this was used for relatively low flow rates, anc compensate for this, other workers used lengths of terial ranging from 12 to 20 feet (10, 12, 15) impo high input pressures. Secondary mixing was require reduce the excessive length and number of tubes, this was accomplished either with the use of a mechar oscillator (1, 20) or, more simply, by coiling the t around a cylindrical former (37). Initial experiments tubes coiled around a cylindrical former (12, 26, 31) a comparatively large K value (ratio of former diam to tubing diameter) (8, 9). Use of a smaller K valu the present model produced more efficient oxygena (3), which is reflected in the small prime volume of than 3 ml.

Fluorocarbon emulsions are more efficiently oxy ated (19) due to the absence of a boundary layer (17 that shorter lengths of tubing have been used very e tively (18). The greatest problem of coiling Silastic tu into small coils is kinking, and this may be avoide the use of more permeable Teflon microporous m brane tubing (Gore-Tex) (34, 35). Effective oxygena was established with this material, but the manufac of these devices is complicated, and the cost ma prohibitive.

We have previously reported a novel technique coiling Silastic membranes and have assessed their formance (3). One disadvantage of these early protot

was the fragility of the 75-μm membrane thickness, which suffered irreversible collapse after use. Furthermore, if high hematocrit blood or high flow rates are used, excessive oxygenator input pressures (up to 250 mm Hg) tend to distend and burst the tubing. The present report describes the construction of a coiled Silastic tubing oxygenator that is compact, robust, and reusable, with a prime volume of less than 3 ml. It is composed of a 1.5-m length of 1.5-mm bore membrane tubing coiled in a $K^{\cdot} = 4$ formation with a 75- to 120-μm coating of silicone rubber, which obviates the cumbersome cylindrical former and increases durability and compactness. Header problems do not exist, since the oxygenator is composed of a single coiled tube connected to a Luer fitting on the transmission circuit. The performance of these units is assessed during outdated human banked blood and in three experimental perfusion applications: the isolated rat liver, rabbit heart, and rat free skin flap.

MATERIALS AND METHODS

The membrane was supplied on a reel as a 150-m length of 1.5-mm bore, 75-μm wall thickness hollow tubing (Siltech Ltd., South Glamorgan). A blunt-ended 16-French gauge (FG) needle was attached to one end of the tubing by immersing the tip of the membrane in cyclohexane, which allowed it to swell, producing a shrink fit. This needle was attached to a pressure head of distilled water to maintain inflation of the membrane during the remainder of the procedure. A winding machine was designed to wind the membrane with even pressure without twists and at a constant pitch between turns (Fig. 1). A 1.5-m length was wound onto the aluminum former (6.0-mm diameter, 53 cm long) and a 16-FG needle or a 3-cm length of 5 FG Portex cannula (Portex Ltd., Hythe, Kent, England) attached to the other end. The needles or cannulas were secured to the membrane with R.T.V. silicon adhesive (Dow Corning Corporation) to withstand high input pressures. The complete assembly was finally placed into a glass gas chamber and connected to a testing circuit (2).

All oxygenators were prepared in an identical manner up to the point of detachment of the 1.5-m length of membrane following the winding procedure. Two forms of oxygenator were investigated, uncoated and coated. For preparation of the coated oxygenators the metal rod formers were coated with the liquid detergent Teepol (B.P. Ltd., London, England) and allowed to dry prior to assembly to facilitate removal of the oxygenator. Knots were tied at each end of the wound oxygenator to maintain the head of pressure inside the tubing and this was detached from the remainder of the membrane; small crocodile clips were then attached to the loose ends to prevent uncoiling during the dipcoating procedure.

A partially cross-linked silicon elastomer, Silcoset 105 (ICI Ltd., Stevenson, Ayrshire, England) diluted in toluene at 10% w/v was used as the dipcoat solution. This solution was poured into a v-shaped tray, and the oxygenator rods were briefly immersed and then allowed to cure at room temperature for 5 hours to result in a coating 75 to 120 μm in thickness over the membrane. The knotted ends of the oxygenator were then detached, and the water inside the membrane was expelled to allow the metal rod formers to be removed. Figure 2 illustrates the shape and thickness of the coating. Finally, 3-cm lengths of 5-FG Portex cannulas were attached to each end, and the flexible coated oxygenator was placed into a Perspex housing, which also acted as a gas jacket (Fig. 3). The complete assembly may now be obtained commercially (Sci Med Products Ltd., East Molesey, Surrey, England).

In Vitro Testing with Outdated Human Bank Blood

The testing circuit used has been previously described (2). Silastic tubing (2 mm in diameter, 1-mm wall thickness) was used throughout the testing circuit, since this material minimizes hemolysis and any complications due to washout products such as the plasticizers often associated with polyvinyl chloride materials. The 1-mm wall thickness of the transmission tubing was sufficient to prevent any measurable entry of atmospheric oxygen into the circuit during the testing time. A total volume of 50 ml of blood was recycled repeatedly until saturation

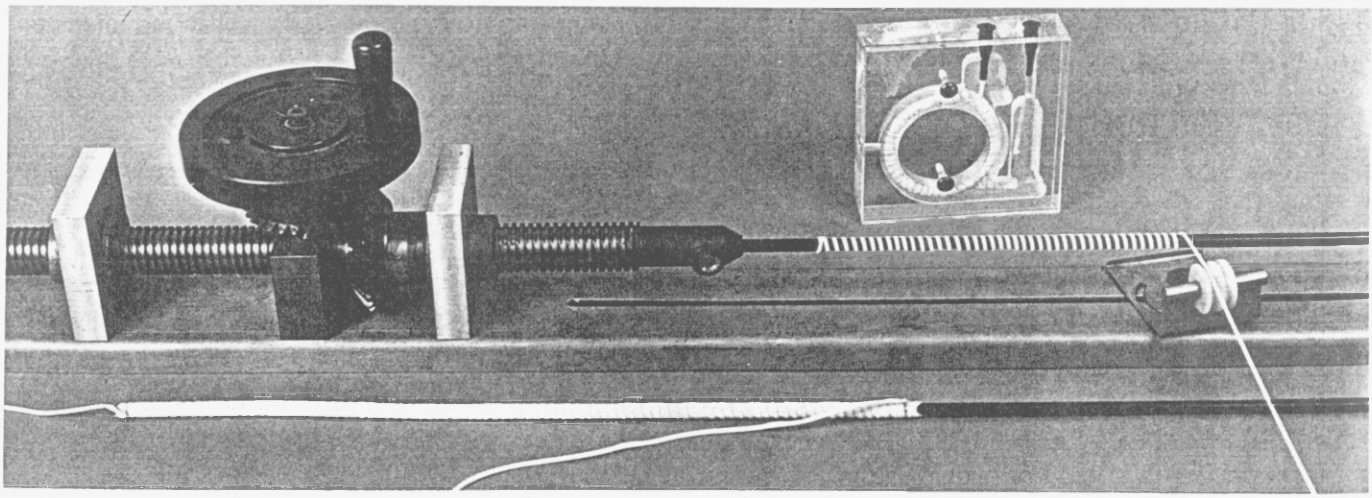

FIG. 1. Photograph illustrating the winding machine designed to produce evenly wound oxygenators, a coated oxygenator partially removed from its metal rod former (*foreground*), and a finished oxygenator seated in its perspex housing (*background*).

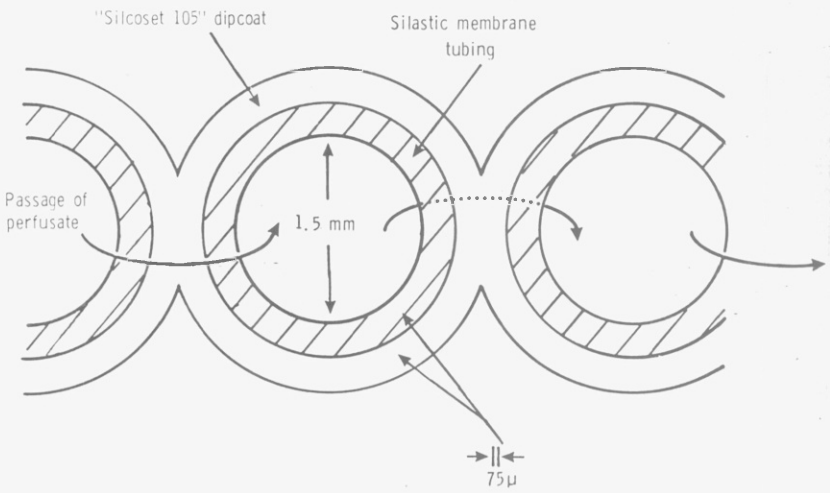

FIG. 2. Cross-section through oxygenator to illustrate shape of coating.

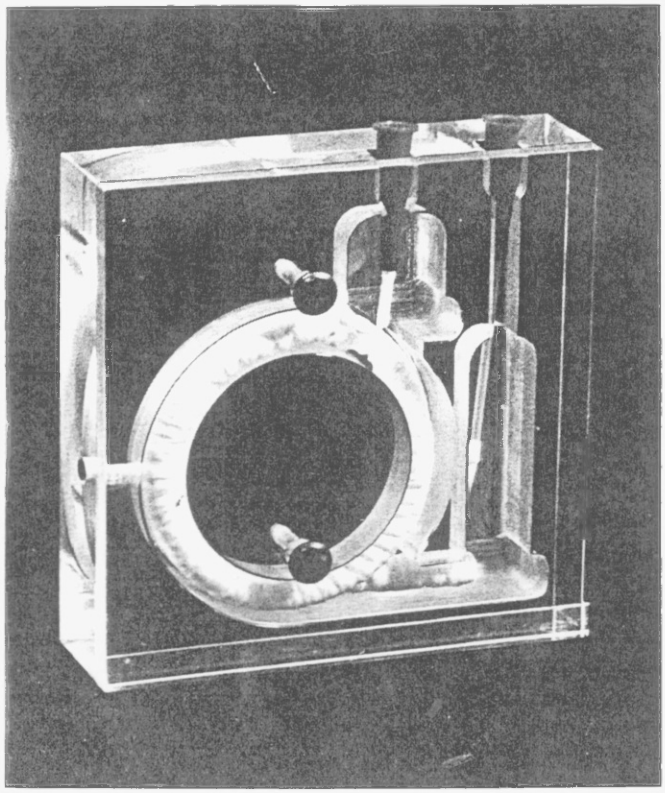

FIG. 3. Photograph of the new compact, spiral coiled Silastic membrane tubing oxygenator (dimensions: housing 82 mm^2, 24 mm deep; oxygenator tubing, 1.5-mm bore, 1.5 m long, 75-μm wall thickness, excluding coating).

was obtained. The pH of the stored blood was corrected to 7.4 by the addition of sodium bicarbonate, and the reservoir was protected from atmospheric oxygen by a layer of liquid paraffin. The hematocrit was adjusted by dilution with normal saline.

Isolated Liver Perfusions

Male Sprague-Dawley rats (250 to 300 gm) were anesthetized with ether and intraperitoneal sodium pentobarbital (Sagatal, May and Baker Ltd.) at 30 mg/kg. The excision of the liver and preparation of the human erythrocyte-derived, heparinized, perfusate with a fina matocrit of 36% was carried out according to the m of Hems *et al.* (16). Pressure in the portal venous ca was monitored by a transducer connected to a medical four-channel recorder (SE Laboratories, M sex, England). Pressures were maintained in the of 15 to 20 mm Hg by adjustment of the pump flow Blood gases were measured at the start of each perf and thereafter every hour from the outflow of the genator, the reservoir, and the venous effluent fro liver.

Isolated Rabbit Heart Perfusion

New Zealand white rabbits weighing 3.4 to 3.9 k anesthetized by intramuscular injection of fentan anisone (Hypnorm, Janssen R & D, Inc.), 0.5 ml/k diazepam (Valium, Roche Laboratories), 70 mg/k heparin, 1000 units/kg, was administered intraver Hearts were rapidly excised and placed into ic Krebs-Henseleit buffer. The maximum period of mia was 2 minutes before perfusion was begu heparinized Krebs-Henseleit buffer at 37° C as stant flow Langendorff preparation. This perfusa oxygenated with two spiral coiled oxygenators in p at a flow rate of 50 ml/minute. Perfusion pressu 50 to 90 mm Hg; left ventricular pressure was me with a fluid-filled latex balloon introduced via t atrium. Flow rates were recorded with an electrom flow meter (SE Laboratories) with a Skalar-Flow Flow, perfusion pressure, and left ventricular p were recorded on a Hewlett-Packard eight-chan corder.

Isolated Free Skin Flap Perfusion

Male SA Wistar rats (200 to 250 gm) were anest with ether and intraperitoneal sodium pentobarbi gatal, May and Baker), 30 mg/kg. The free skin based on the superficial epigastric artery and ve femoral artery and vein were ligated distally and lated with a 2-FG cannula (Portex Ltd.). The f immediately irrigated with heparinized perfusate of Haemaccel (Hoechst U.K. Ltd.), 11.5 ml c sodium bicarbonate, glucose (200 mg/100 ml), a

arin (2 units/ml)); the maximum period of ischemia was 5 minutes.

The flap was perfused inside a sterile organ bath. Arterial pressure was monitored by a transducer connected to an SEM medical four-channel recorder (SE Laboratories) and maintained in the range of 110 to 120 mm Hg by adjustment of the pump flow rate which rarely exceeded 400 μl/minute. Once flow rates and pressures had stabilized, samples for blood gas analysis (pre- and postoxygenator and posttissue) were taken at hourly intervals for the first 6 hours. A series of 12 perfusions were carried out, ranging from 24 to 48 hours duration.

Blood Analysis

Blood gases were analyzed on an ABL 1 Acid Base Laboratory (Radiometer, Copenhagen, Denmark) and a 166 micro pH/blood gas analyzer (Corning Medical, Essex, England), oxygen contents on a Lex O_2 Con (Lexington Instruments Inc., Massachusetts), hemoglobin estimations on an Eel hemoglobinometer, using the cyanomethemoglobin method, and hematocrit on a Hawksley microhematocrit centrifuge (Hawksley Ltd., London, England).

Statistical Methods

Results were analyzed by computerized regression analysis and by multivariate regression analysis using the Generalized Linear Interactive Modelling (GLIM) program of the Numerical Algorithms Group of the Royal Statistical Society.

RESULTS

In Vitro Experiments on Outdated Banked Blood

There was a significant parabolic relationship between percentage saturation difference and flow rate for both coated and uncoated membranes, but values for coated membranes were significantly lower than those for uncoated membranes ($p < 0.01$) (Fig. 4). The minimal satisfactory difference in oxygen saturation of 30% (14) between the input and output of the oxygenator blood was attained at approximately 16 ml/minute using the uncoated membrane and 9 ml/minute using the coated membrane.

Figure 5 demonstrates the influence of flow rate on the oxygen transport across the membrane at two different hematocrits. The two curves illustrating the oxygen transport in the uncoated membranes demonstrate an increase at the higher hematocrit (40%), although this becomes less prominent at lower flow rates. The coated membranes exhibited an approximate decrease of 30% in oxygen transport compared with uncoated membranes, although again this was less prominent at lower flow rates.

A linear relationship could be demonstrated, using regression analysis, between input and output pO_2 in the range of 35 to 200 mm Hg input pO_2, for both coated and uncoated membranes. A simplified graph (Fig. 6) has been provided to illustrate the operative working efficiency of the device. No significant difference was observed in this study between coated and uncoated membranes. To eliminate a possible effect of hematocrit on transport efficiency, these studies were repeated using perfusates with hematocrits of 33 to 35%, and similar results were obtained.

Isolated Liver Perfusion

Slight variations in input pO_2 to the oxygenator (due to the metabolism of the liver) influenced the output pO_2, but this was always maintained above 150 mm Hg (mean 227.16 ± 1.8 SE, $n = 42$) using a 5% carbogen mixture (Table 1). The low "posttissue" pCO_2 did not appear to be detrimental to the liver, in agreement with the findings of other workers using this method (15, 18). Fluctuations beyond the normal physiologic range could be compensated for by increasing or decreasing the flow rate through the oxygenator.

Isolated Rabbit Heart

Pre- and postoxygenator blood gases were compared with results obtained using conventional bubble and film oxygenators (4, 24). Initially, a single oxygenator was used, but the high input pressures at high flow rates in excess of 50 ml/minute necessitated the use of two oxygenators in parallel. Table 1 illustrates the blood gas values obtained during perfusions using two oxygenators in parallel at flow rates of 50 ml/minute. Little variation was detected between preparations, and the results

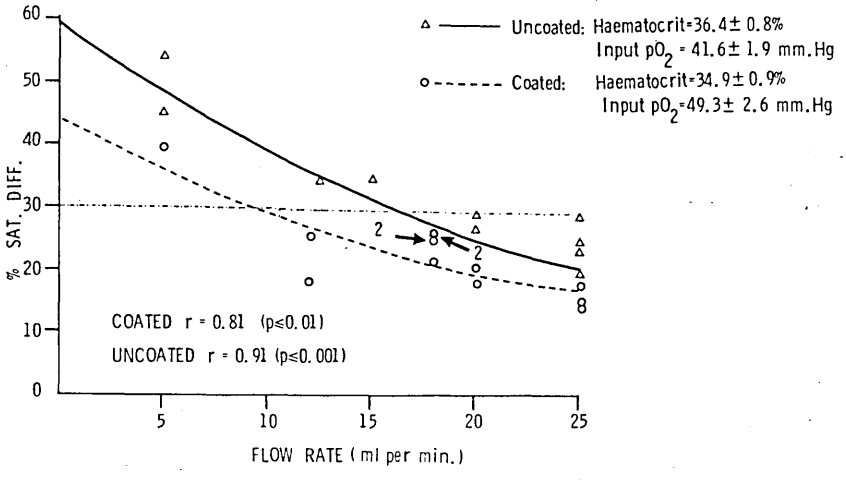

FIG. 4. Variations in the percentage of oxygen saturation difference at various flow rates in coated and uncoated membranes. *Dotted line* corresponds to the minimal satisfactory difference in oxygen saturation of 30% (14).

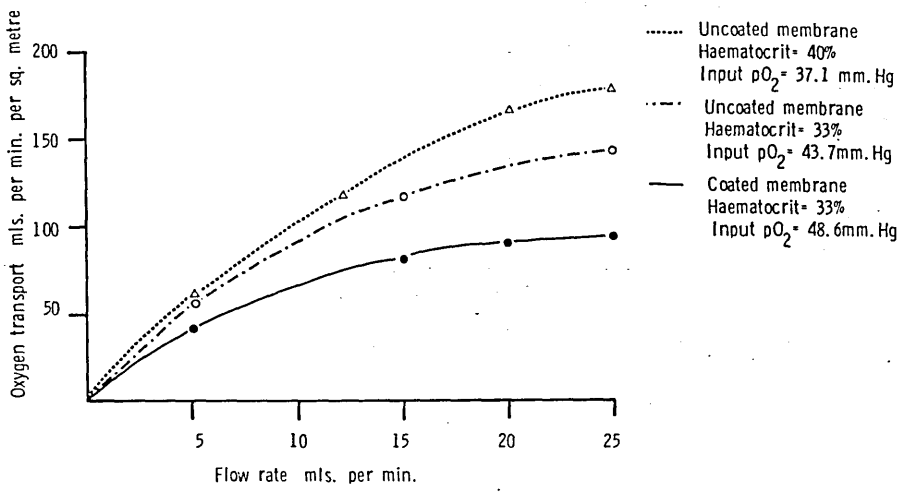

FIG. 5. Influence of dipcoating and flow rate on the oxygen transport of the oxygenator when perfusates of different hematocrits (33% and 40%) are used.

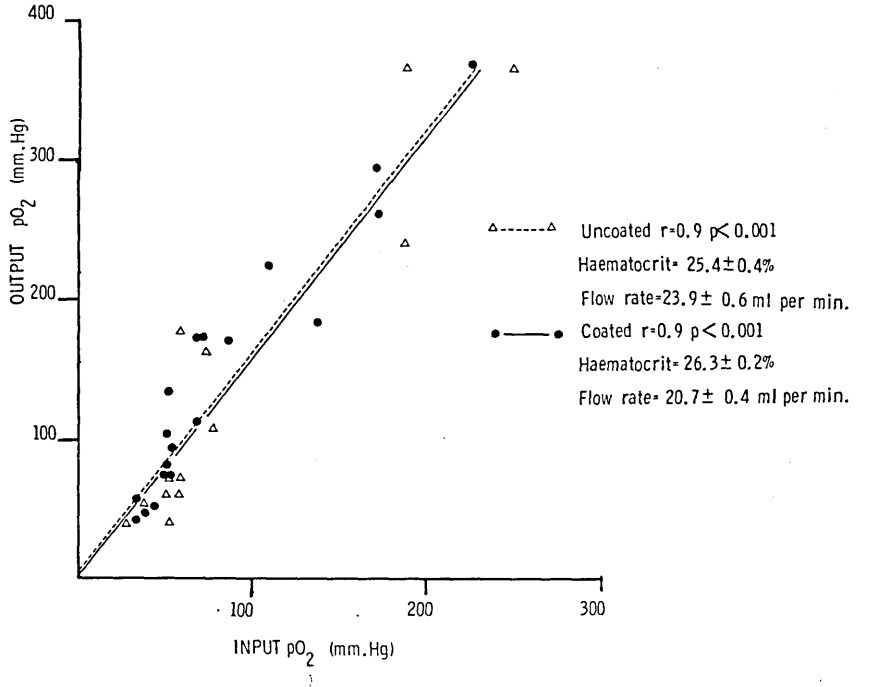

FIG. 6. Simplified graph to demonstrate the operative working efficiency of the oxygenator. Results were obtained using several different samples of outdated human banked blood diluted to the appropriate hematocrit with saline. pH of the blood was adjusted to physiologic values with sodium bicarbonate.

shown are representative of 33 perfusions each of 3 hours duration.

Typical postoxygenator blood gases using a bubble/film-type oxygenator are also shown in Table 1. Generally, the pO_2 values obtained were higher than those obtained with the new oxygenator, but either system was capable of producing pO_2 values greater than 400 mm Hg. This was high enough to maintain a stable preparation with constant perfusion pressure and myocardial contractility throughout a 3-hour perfusion. Preoxygenator pCO_2 levels in excess of 48 mm Hg were occasionally encountered due to pregassing the Krebs-Henseleit buffer with carbon dioxide to aid dissolution of the calcium chloride. This was readily compensated for by substituting 100% oxygen for the 5% carbogen mixture in the oxygenator.

Isolated Free Skin Flap Perfusions

Arterial perfusion pressure was monitored and maintained at 125 mm Hg, using a flow rate in the region of 300 μl/minute. Oxygen saturation was readily achiev due to both low flow rates and the use of hemoglobi free perfusates. The results of 12 perfusions were pr sented in Table 1.

After perfusion for more than 3 hours, the flaps becar edematous with a weight increase of more than 300 After perfusion the flap was replaced in the same anim by reanastomosis of the vessels to the femoral artery a vein on the contralateral side. Immediately after t clamps were released, the flaps became pink, and veno blood flow could be seen. Three days postoperatively, of the flaps became ischemic, and only one flap surviv for 5 days after perfusion. Dissection of the vessels a and 5 days postoperatively revealed intravascular thro bosis.

DISCUSSION

The 10% dilution of the partially cross-linked silic elastomer resulted in a dipcoat of reproducibly unifo thickness, strength, and resistance to collapse. Attem

TABLE 1. SUMMARY OF BLOOD GAS RESULTS FROM THREE DIFFERENT PERFUSION PREPARATIONS[a]

Experiment	Sample	pH	pO_2	pCO_2
Isolated liver perfusion (hematocrit = 29.3 ± 1.1); n = 23	Postoxygenator	7.44 ± 0.05	227.2 ± 9.2	32.9 ± 1.8
	Posttissue	7.31 ± 0.09	53.9 ± 2.2	29.8 ± 1.9
	Reservoir	7.41 ± 0.02	141.8 ± 9.5	29.7 ± 1.3
Isolated rabbit heart	Preoxygenator	7.54 ± 0.09	172.6 ± 3.2	29.3 ± 1.8
	Postoxygenator	7.39 ± 0.05	419.1 ± 10.0	38.8 ± 2.1
	Typical results post-bubble oxygenator	7.37	480.1	38.0
Isolated free skin flap	Preoxygenator	7.35 ± 0.05	207.6 ± 12.3	34.6 ± 3.7
	Postoxygenator	7.32 ± 0.0	387.3 ± 28.4	34.9 ± 3.8
	Posttissue	7.6 ± 0.06	179.8 ± 24.3	17.0 ± 2.4

[a] Results shown are means ± standard error. The isolated rabbit heart and free skin flap perfusions were of "open-circuit" design, and the oxygenator could be used to add both oxygen and carbon dioxide to the perfusate. The perfusates used in these two experimental situations were colloid-electrolytes (see text), and only the isolated liver perfusion used an erythrocyte-containing perfusate.

to further dilute the elastomer resulted in very fragile coats that often fractured during extraction of the oxygenator from the rod formers. The 10% dilution doubled the effective wall thickness of the membrane from 75 to 150 to 200 μm, but this did not produce a proportionate decrease in efficiency of the membrane.

Results obtained with outdated human banked blood were used to give a broad indication of the optimum lengths and configurations of tubing. Blood was used to test the oxygenator because initially it was proposed to use the device with an erythrocyte-containing perfusate in the isolated liver perfusions and for rats undergoing cardiopulmonary bypass (1). It should be noted that the increased oxygen-carrying capacity of the perfusate due to the presence of hemoglobin, the resultant boundary layer effect (17), and the shape of the oxygen dissociation curve might all influence the performance of the device.

The increase in efficiency of oxygenators by coiling Silastic tubing has already been reported (3). Therefore, these experiments were carried out using 1.5-mm diameter tubing, 75-μm wall thickness, in a coil configuration of $K = 4$ (ratio of coil diameter to tubing diameter) since smaller coils resulted in kinking. Efficiency of oxygenation was reduced by 25% in coated membranes (3), initially calculated from using a single unit of outdated human banked blood; a reduction of 37% in efficiency was later obtained from a range of different units of blood with hematocrits ranging from 28 to 36% with a wider range of input pO_2 values.

The linear output *versus* input pO_2 curve (Fig. 6) gives an approximate index of the operative capability of the oxygenator under experimental conditions. The output-input pO_2 difference appears to increase somewhat as the input pO_2 increases. This is because the majority of hemoglobin at the higher pO_2 values has become saturated with oxygen, and oxygen is dissolved in the plasma. Since the solubility of oxygen in plasma is much lower than that in a hemoglobin-containing solution, a very much smaller volume of oxygen was required to increase the total pO_2. Thus, although it might appear that the oxygenator is more efficient at higher pO_2 values, this is not the case, since a smaller volume of oxygen delivery is required to increase the pO_2 at this working range.

For liver perfusions a hematocrit of 30% or less and a flow rate of less than 26 ml/minute was used. Under these conditions, a single oxygenator was sufficient to maintain the mixed arteriovenous reservoir at physiologic arterial pO_2 values. Previous oxygenators of a similar design (31) required larger surface areas and, therefore, increased prime volumes.

For the isolated rabbit heart perfusion in which much higher flow rates were used, two oxygenators in parallel were necessary to maintain an adequate pO_2. Nevertheless, the system required a smaller prime volume and was more compact than bubble oxygenators used previously (24). Although the older oxygenators were able to achieve tissue input pO_2 of 600 mm Hg (4, 25), the present system maintained a tissue input pO_2 of 400 mm Hg and an output pO_2 in the region of 100 mm Hg, and the heart retained its contractility throughout the perfusion.

To our knowledge this is the first report of an isolated free skin flap perfusion in the rat. The perfusions were carried out to assess the effects of prolonged storage and possible improvement of functional viability of the dissected tissue at room temperature rather than at 4° C (36). Pronounced edema of the flap occurred after 3 hours of perfusion despite the use of Haemaccel or albumin as an osmotic agent in the perfusate and was not prevented by the addition of mannitol. This edema is not unique to the perfused flap but has been noted following reanastomosis of flaps that have not been perfused but have been stored aseptically at 4° C for 24 hours and may be a result of endothelial damage (36). Perfusion techniques have been used in other organ transplant procedures (18) and may be valuable for isolated skin flap preparations. Furthermore, this preparation may form the basis of a very compact model for the study of capillary transport phenomena (10).

Thrombogenicity, leukocyte, or platelet function tests have not been completed in this particular configuration of oxygenator. However, previous studies using an identical membrane assembled in a different configuration have shown satisfactory results (1, 11, 21), and it is probable that these properties will apply to the present configuration. This new compact membrane oxygenator may, therefore, prove useful in experiments involving cardiopulmonary bypass in rats or other small animals, and preliminary investigations in this area have been encouraging (1).

In summary, a compact, durable, and reusable oxygen-

Appendix C1. Table of parameters measured during IRLPs using KRBA with the omission of an oxygen carrier. Results shown are mean ± S.E n=6

MEASUREMENT	HOURS OF PERFUSION 0	1	2	3	4	5	6
Oxygen uptake (μmol $min^{-1}g^{-1}$)	-	0.26 ± 0.05	0.31 ± 0.04	0.32 ± 0.08	0.28 ± 0.09	0.36 ± 0.09	0.31 ± 0.08
Bile volume (μl)	585 ± 62	295 ± 59	472 ± 37	458 ± 75	425 ± 108	338 ± 94	232 ± 93
Haematocrit (% cells)	-	-	-	-	-	-	-
Liver wt (g)	15.7 ± 0.99	-	-	-	-	-	14.04 ± 1.39
Perfusion pressure (mmHg)	15.67 ± 5.8	18.3 ± 4.1	15.8 ± 4.4	15.5 ± 4.6	15.0 ± 4.4	17.4 ± 5.47	18.17 ± 5.09
Flow rate (ml $min^{-1}g^{-1}$)	2.35 ± 0.33	2.61 ± 0.22	2.60 ± 0.24	2.64 ± 0.20	2.45 ± 0.20	2.56 ± 0.19	2.73 ± 0.28
K+ (mmol l^{-1})	6.37 ± 0.66	6.31 ± 0.58	6.54 ± 0.62	6.87 ± 0.75	7.48 ± 0.97	8.38 ± 1.32	7.71 ± 0.71
Urea ($mmol^{-1}l^{-1}$)	0.22 ± 0.09	0.65 ± 0.09	1.02 ± 0.18	1.42 ± 0.22	1.90 ± 0.31	2.25 ± 0.35	2.30 ± 0.43
Bilirubin ($\mu mol^{-1}l^{-1}$)	0.67 ± 0.37	0.85 ± 0.25	1.12 ± 0.38	0.70 ± 0.11	0.8 ± 0.09	0.98 ± 0.22	0.88 ± 0.09
Glucose (mmol l^{-1})	11.43 ± 1.87	16.4 ± 0.82	16.82 ± 0.82	17.82 ± 0.94	17.85 ± 1.33	17.4 ± 1.28	17.06 ± 1.67
Plasma haemoglobin (mg%)	-	-	-	-	-	- -	
AST (IU l^{-1})	9.75 ± 3.14	13.25 ± 3.27	35.00 ± 10.86	55.5 ± 31.27	90.5 ± 32.11	34.75 ± 21.72	54.5 ± 20.07
ALK (IU l^{-1})	6.0 ± 2.04	6.25 ± 3.32	6.75 ± 2.95	3.75 ± 3.12	5.75 ± 2.84	6.25 ± 3.17	11.25 ± 3.06
Bilirubin (μmol l^{-1})	4.1 ± 1.28	4.63 ± 2.14	7.53 ± 3.84	4.9 ± 0.43	3.48 ± 1.90	1.22 ± 0.55	0.95 ± 0.46

Appendix C2. Table of parameters measured during IRLPs with pcv=20% haemodiluted rat blood. Results shown are mean ± S.E. n=6

	HOURS OF PERFUSION						
MEASUREMENT	0	1	2	3	4	5	6
Oxygen uptake (μmol $min^{-1}g^{-1}$)	-	1.12 ± 0.15	0.97 ± 0.12	0.99 ± 0.15	1.09 ± 0.15	1.06 ± 0.70	1.02 ± 0.23
Bile volume (μl)	778 ± 161	962 ± 169	1343 ± 40	1235 ± 38	1103 ± 124	495 ± 95	210 ± 45
Haematrocrit (% cells)	22.9 ± 1.9	32.2 ± 1.8	23.2 ± 1.8	23.0 ± 1.8	22.7 ± 1.4	22.2 ± 1.3	21.7 ± 1.3
Liver wt (g)	16.2 ± 1.3						15.3 ± 1.3
Perfusion pressure (mmHg)	23.4 ± 3.4	22.6 ± 2.7	21.7 ± 4.9	22.7 ± 3.3	22.3 ± 3.9	23.3 ± 4.0	22.2 ± 3.9
Flow rate (ml $min^{-1}g^{-1}$)	1.76 ± 0.18	1.97 ± 0.09	2.07 ± 0.07	2.10 ± 0.05	2.15 ± 0.11	2.10 ± 0.04	2.07 ± 0.04
K+ (mmol l^{-1})	5.7 ± 0.6	5.5 ± 0.7	6.1 ± 0.6	6.3 ± 0.4	6.8 ± 0.4	7.4 ± 0.3	8.1 ± 0.5
Urea (mmol l^{-1})	4.4 ± 1.1	5.3 ± 1.2	6.2 ± 1.4	7.0 ± 1.5	7.7 ± 1.6	8.2 ± 1.6	8.7 ± 1.7
Bilirubin (μmol l^{-1})	0.9 ± 0.31	0.97 ± 0.2	2.01 ± 0.66	2.45 ± 0.51	2.98 ± 0.65	3.92 ± 0.9 5	4.68 ± 1.34
Glucose (mmol l^{-1})	18.1 ± 1.9	22.3 ± 2.7	21.6 ± 1.9	20.9 ± 1.6	20.9 ± 2.1	21.1 ± 2.4	22.6 ± 2.3
Plasma haemoglobin (mg%)	85.8 ± 12.3	178.8 ± 22.1	274.7 ± 28.0	343.1 ± 25.9	439.7 ± 43.1	516.1 ± 52.6	650.1 ± 53.3
AST (IU l^{-1})	8.0 ± 1.6	14.2 ± 2.9	19.0 ± 3.6	25.0 ± 4.3	32.7 ± 5.5	52.2 ± 9.5	73.5 ± 14.1
ALK (IU l^{-1})	3.7 ± 0.3	5.0 ± 0.6	6.2 ± 0.4	10.4 ± 0.6	13.9 ± 1.4	17.3 ± 2.4	23.4 ± 4.1
Bilirubin (μmol l^{-1})	38.2 ± 5.1	42.7 ± 18.1	31.7 ± 6.3	45.8 ± 11.9	63.2 ± 17.1	90.4 ± 37.7	143.3 ± 39.3

Appendix C2 **Blood gas data from perfusions**

RED CELL SOURCE: RAT PCV: 20%

	HOURS OF PERFUSION						
MEASUREMENT	0	1	2	3	4	5	6
pH	7.913 ± 0.026	7.302 ± 0.044	7.379 ± 0.021	7.411 ± 0.017	7.451 ± 0.052	7.473 ± 0.045	7.407 ± 0.041
pO_2	290.2 ± 98.4	220.6 ± 18.3	235.0 ± 19.5	244.4 ± 19.5	229.3 ± 15.2	240.8 ± 10.2	232.2 ± 19.0
pCO_2	46.9 ± 6.7	42.4 ± 3.0	40.1 ± 2.5	35.6 ± 4.1	32.6 ± 4.1	34.8 ± 5.1	34.7 ± 3.7
HCO_3	20.1 ± 2.8	19.5 ± 2.4	23.9 ± 1.7	23.7 ± 1.3	23.2 ± 1.6	25.4 ± 4.1	23.0 ± 2.8
pH	7.277 ± 0.061	7.314 ± 0.044	7.386 ± 0.034	7.431 ± 0.018	7.380 ± 0.060	7.424 ± 0.045	7.389 ± 0.03
pO_2	211.5 ± 28.4	201.7 ± 21.0	117.1 ± 16.6	218.0 ± 23.3	171.1 ± 21.8	165.0 ± 26.3	153.3 ± 23.6
pCO_2	43.0 ± 4.9	43.5 ± 3.4	44.8 ± 3.5	41.1 ± 3.4	38.1 ± 5.2	37.9 ± 5.2	40.5 ± 4.3
HCO_3	19.9 ± 3.0	21.1 ± 2.9	23.5 ± 1.3	22.9 ± 0.8	21.0 ± 0.9	24.1 ± 3.3	23.2 ± 1.8
pH	7.248 ± 0.040	7.280 ± 0.044	7.352 ± 0.026	7.403 ± 0.027	7.377 ± 0.051	7.405 ± 0.048	7.382 ± 0.03
pO_2	72.5 ± 6.4	70.6 ± 7.1	65.6 ± 4.1	71.7 ± 6.4	66.1 ± 8.0	64.4 ± 8.6	63.4 ± 9.1
pCO_2	43.7 ± 7.0	45.2 ± 4.6	44.2 ± 4.2	42.0 ± 4.4	39.9 ± 5.5	39.0 ± 5.5	42.2 ± 4.8
HCO_3	21.3 ± 2.1	19.8 ± 2.1	23.7 ± 1.4	23.4 ± 1.0	22.1 ± 1.1	23.8 ± 3.1	23.3 ± 1.8

Appendix C3 Table of parameters measured during IRLPs with PCV=10% haemodiluted dog blood. Results shown are mean ± S.E. n=6.

RED CELL SOURCE: DOG PCV=20%

MEASUREMENT	HOURS OF PERFUSION 0	1	2	3	4	5	6
Oxygen uptake (μmol $min^{-1}g^{-1}$)	-	1.23 ±0.25	1.10 ±0.06	1.56 ±0.17	0.97 ±0.20	0.80 ±0.0	60.72 ±0.09
Bile volume (μl)	416 ±44	284 ±83	384 ±89	390 ±99	248 ±72	128 ±46	56 ±23
Haematocrit (% cells)	9.1 ±1.1	8.4 ±0.9	8.0 ±0.8	7.8 ±0.8	7.5 ±0.8	6.9 ±0.9	6.8 ±0.9
Liver wt (g)	17.48 ±0.7						18.78 ±2.08
Perfusion pressure (mmHg)	15.2 ±0.9	13.6 ±2.7	14.5 ±1.50	15.1 ±1.0	14.8 ±0.7	14.6 ±1.8	15.9 ±1.0
Flow rate (ml $min^{-1}g^{-1}$)	1.91 ±0.20	1.97 ±0.13	2.01 ±0.10	2.00 ±0.11	2.05 ±0.11	2.05 ±0.12	2.01 ±0.11
K+(mmol l^{-1})	8.9 ±1.1	8.2 ±0.3	8.4 ±0.3	8.6 ±0.4	9.3 ±0.8	9.4 ±0.8	9.4 ±0.9
Urea (mmol l^{-1})	0.8 ±0.2	1.1 ±0.2	2.0 ±0.4	2.6 ±0.5	3.4 ±0.5	3.6 ±0.4	4.1 ±0.2
Bilirubin (μmol l^{-1})	6.4 ±0.9	7.0 ±0.9	8.8 ±1.4	13.4 ±1.75	18.2 ±2.6	22.2 ±2.1	25.2 ±2.3
Glucose (mmol l^{-1})	20.0 ±0.7	24.1 ±0.9	23.9 ±1.7	27.0 ±1.9	27.9 ±1.9	28.8 ±2.4	31.8 ±1.4
Plasma haemoglobin (mg%)	407 ±73	448 ±93	521 ±106	621 ±119	676 ±123	1163 ±360	1322 ±412
Bilirubin (μmol l^{-1})	51.4 ±2.2	38.6 ±7.2	44.6 ±7.5	45.4 ±6.3	48.4 ±6.3	50.8 ±6.2	58.4 ±6.0

Appendix C3 Blood gas data from perfusions

RED CELL SOURCE: DOG PCV: 10%

	HOURS OF PERFUSION						
MEASUREMENT	0	1	2	3	4	5	6
pH	7.246 ± 0.080	7.438 ± 0.090	7.398 ± 0.047	7.358 ± 0.040	7.339 ± 0.070	7.430 ± 0.023	7.480 ± 0.059
pO_2	103.8 ± 49.2	216.6 ± 21.2	204.2 ± 25.0	234.1 ± 7.6	237.2 ± 14.1	229.2 ± 20.3	234.9 ± 28.5
pCO_2	46.5 ± 5.5	27.2 ± 3.1	38.4 ± 5.9	39.7 ± 4.2	32.3 ± 3.6	31.6 ± 2.8	29.2 ± 3.4
HCO_3	24.3 ± 1.9	17.8 ± 1.6	22.1 ± 2.4	21.2 ± 0.5	18.1 ± 3.0	20.72 ± 1.8	19.3 ± 2.0
pH	7.249 ± 0.075	7.440 ± 0.088	7.371 ± 0.051	7.375 ± 0.038	7.340 ± 0.071	7.426 ± 0.021	7.423 ± 0.025
pO_2	102.2 ± 47.8	138.4 ± 21.6	118.7 ± 14.5	120.0 ± 17.8	123.2 ± 19.6	106.5 ± 47.1	141.6 ± 20.7
pCO_2	46.7 ± 5.4	27.8 ± 3.3	37.4 ± 5.3	39.1 ± 4.6	32.9 ± 4.0	31.1 ± 3.1	30.0 ± 2.7
HCO_3	21.8 ± 4.1	18.3 ± 1.4	21.8 ± 2.2	21.0 ± 0.5	18.3 ± 3.1	20.3 ± 1.9	19.7 ± 1.6
pH	7.257 ± 0.081	7.439 ± 0.084	7.384 ± 0.041	7.352 ± 0.043	7.367 ± 0.088	7.439 ± 0.039	7.456 ± 0.050
pO_2	108.9 ± 46.9	56.8 ± 9.7	53.3 ± 2.6	51.6 ± 4.8	60.6 ± 9.8	44.8 ± 1.6	50.9 ± 9.0
pCO_2	39.25 ± 4.2	26.9 ± 2.6	39.3 ± 6.1	41.1 ± 4.6	31.1 ± 3.5	31.5 ± 3.3	30.4 ± 3.5
HCO_3	21.9 ± 4.1	17.7 ± 1.6	21.9 ± 2.4	21.5 ± 0.6	17.9 ± 3.0	20.6 ± 2.0	19.9 ± 1.5

Appendix C4. Table of parameters measured during IRLPs with PCV=20% washed dog erythrocytes. Results shown are mean ± S.E n=6

RED CELL SOURCE : DOG PCV: 20%

MEASUREMENT	HOURS OF PERFUSION 0	1	2	3	4	5	6
Oxygen uptake(μmol $min^{-1}g^{-1}$)	-	1.36 ±0.17	1.19 ±0.11	1.59 ±0.12	0.92 ±0.11	0.81 ±0.14	0.73 ±0.11
Bile volume (μl)	343 ±30	248 ±59	357 ±90	280 ±57	173 ±47	180 ±60	140 ±43
Haematocrit(%cells)	30.2 ±2.3	26.0 ±1.8	24.7 ±2.4	35.2 ±2.3	22.8 ±2.5	22.5 ±3.4	21.3 ±3.9
Liver wt (g)	15.7 ±0.8						15.5 ±0.9
Perfusion Pressure (mmHg)	17.7 ±1.8	20.4 ±1.8	21.3 ±2.0	18.8 ±2.0	21.8 ±2.5	18.8 ±1.8	19.8 ±2.2
Flow rate (ml $min^{-1}g^{-1}$)	1.94 ±0.05	2.05 ±0.06	2.09 ±0.08	2.07 ±0.08	2.07 ±0.04	2.03 ±0.05	2.08 ±0.04
K+ (mmol l^{-1})	5.1 ±0.5	5.5 ±0.5	5.7 ±0.5	6.0 ±0.5	6.3 ±0.5	6.6 ±0.4	7.0 ±0.5
Urea (mmol l^{-1})	2.0 ±0.3	3.5 ±0.6	6.3 ±1.0	6.2 ±1.0	7.6 ±0.9	8.3 ±1.0	9.4 ±1.3
Bilirubin (μmol l^{-1})	26.7 ±5.1	29.5 ±7.7	38.0 ±6.0	47.7 ±5.3	52.7 ±4.1	58.5 ±8.2	60.3 ±8.2
Glucose (mmol l^{-1})	20.9 ±2.6	24.4 ±2.4	18.6 ±1.7	17.4 ±2.4	16.8 ±1.7	14.8 ±1.7	13.0 ±2.4
Plasma Haemoglobin (mg%)	245 ±52	507 ±165	813 ±126	1080 ±181	1743 ±428	2460 ±541	3727 ±953
Bilirubin (μmol l^{-1})	52.8 ±9.6	86.2 ±19.5	231.8 ±71.5	316.8 ±100.0	399.5 ±96.8	382.3 ±77.1	639.7 ±102.8

Appendix C4 **Blood gas data from perfusions**

RED SOURCE CELL : DOG PCV: 20%

PERFUSION TIME/HOURS

	MEASUREMENT	0	1	2	3	4	5	6
OXYGENATOR	pH	7.315 ± 0.028	7.352 ± 0.038	7.399 ± 0.081	7.438 ± 0.050	7.466 ± 0.049	7.403 ± 0.038	7.401 ± 0.039
	pO_2	155.0 ± 12.8	212.6 ± 22.8	233.7 ± 22.8	245.3 ± 17.7	244.9 ± 19.1	238.3 ± 14.9	232.0 ± 26.5
	pCO_2	40.8 ± 8.1	29.8 ± 3.5	33.0 ± 4.9	29.5 ± 4.2	27.1 ± 2.3	30.8 ± 1.5	31.8 ± 1.5
	HCO_3	19.2 ± 2.9	16.0 ± 1.7	20.0 ± 2.0	18.7 ± 1.6	19.0 ± 0.8	18.9 ± 1.4	19.9 ± 1.5
EXCHANGER	pH	-	7.385 ± 0.036	7.463 ± 0.052	7.480 ± 0.041	7.467 ± 0.045	7.432 ± 0.048	7.426 ± 0.032
	pO_2	-	120.7 ± 21.6	97.0 ± 13.0	137.2 ± 30.7	137.3 ± 22.5	136.4 ± 23.4	112.8 ± 24.0
	pCO_2	-	29.2 ± 2.9	29.7 ± 4.1	28.9 ± 3.7	27.9 ± 2.1	29.7 ± 1.7	29.8 ± 1.6
	HCO_3	-	16.8 ± 1.2	20.4 ± 1.2	19.0 ± 1.5	19.6 ± 0.9	19.8 ± 2.1	19.7 ± 1.5
ORGAN OUTFLOW	pH	-	7.349 ± 0.038	7.413 ± 0.78	7.454 ± 0.045	7.460 ± 0.041	7.507 ± 0.094	7.426 ± 0.037
	pO_2	-	54.4 ± 5.0	67.0 ± 7.2	70.4 ± 13.5	65.1 ± 7.4	59.0 ± 6.0	60.3 ± 10.0
	pCO_2	-	31.2 ± 3.4	32.7 ± 4.8	28.4 ± 3.7	28.1 ± 1.9	30.3 ± 1.6	28.3 ± 1.8
	HCO_3	-	16.5 ± 1.5	19.5 ± 2.1	18.9 ± 1.6	19.5 ± 0.7	19.3 ± 1.5	19.3 ± 1.2

ator with a prime volume of less than 3 ml has been described, which may be used for a variety of perfusion experiments. It has a coil configuration of $K = 4$, a length of 1.5 m, a 1.5-mm external diameter, and 75- to 120-μm, wall thickness.

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Appendix C5 Table of parameters measured during IRLPS with PCV=10% using washed human erythrocytes. Results shown are mean ± S.E. n=6.

RED CELL SOURCE : HUMAN PCV: 10%

	HOURS OF PERFUSION						
MEASUREMENT	0	1	2	3	4	5	6
Oxygen Uptake (μmol $min^{-1}g^{-1}$)	-	1.15 ±0.09	1.15 ±0.09	1.11 ±0.11	0.87 ±0.05	0.72 ±0.08	0.66 ±0.10
Bile Volume (μl)	784 ±124	800 ±156	896 ±154	878 ±135	708 ±134	240 ±36	140 ±25
Haematocrit (% cells)	9.8 ±0.3	9.8 ±0.3	9.8 ±0.3	9.5 ±0.4	9.3 ±0.6	9.5 ±0.4	9.5 ±0.4
Liver weight (g)	13.5 ±0.1						12.1 ±0.8
Perfusion Pressure (mmHg)	15.4 ±2.5	14.4 ±1.7	15.6 ±2.7	15.6 ±2.6	13.4 ±1.0	13.6 ±1.08	13.8 ±1.21
Flow rate (ml min^{-1})	1.89 ±0.05	1.95 ±0.07	2.16 ±0.08	2.18 ±0.08	2.12 ±0.09	2.15 ±0.08	2.15 ±0.08
K+ (mmol l^{-1})	6.60 ±0.2	6.8 ±0.3	6.9 ±0.3	7.1 ±0.3	8.2 ±0.7	8.3 ±0.5	8.6 ±0.4
Urea (mmol l^{-1})	0.40 ±0.04	0.94 ±0.19	1.10 ±0.22	1.60 ±0.22	1.86 ±0.23	2.26 ±0.33	2.48 ±0.31
Bilirubin (μmol l^{-1})	0.8 ±0.2	0.2 ±0.2	0.8 ±0.2	1.3 ±0.2	1.3 ±0.2	0.8 ±0.2	0.6 ±0.2
Glucose (mmol l^{-1})	13.8 ±0.8	15.7 ±1.0	19.0 ±1.5	19.5 ±2.1	19.8 ±1.8	22.4 ±3.2	21.9 ±2.9
Plasma Haemoglobin (mg%)	138 ±5	166 ±7	186 ±11	208 ±18	236 ±22	262 ±21	292 ±25
Bilirubin (μmol l^{-1})	44.2 ±3.6	15.8 ±1.3	20.6 ±1.3	25.6 ±4.1	32.0 ±4.1	38.6 ±4.6	48.0 ±2.0

Appendix C5 **Blood gas data from perfusions**

RED CELL SOURCE : HUMAN PCV: 10%

HOURS OF PERFUSION

	MEASUREMENT	0	1	2	3	4	5	6
OXYGENATOR	pH	-	7.666 ±0.040	7.363 ±0.050	7.369 ±0.041	7.366 ±0.035	7.360 ±0.025	7.367 ±0.032
	pO_2	-	268.3 ±5.6	283.2 ±5.6	257.7 ±6.6	253.3 ±14.0	275.0 ±10.6	269.8 ±10.7
	pCO_2	-	41.7 ±2.3	41.7 ±3.3	42.2 ±3.1	40.1 ±3.3	38.9 ±2.7	39.8± 2.8
	HCO_3	-	20.5 ±1.3	22.3 ±1.1	22.3 ±1.3	22.0 ±2.8	21.4 ±1.1	21.7 ±0.6
RESERVOIR	pH	7.455 ±0.035	7.333 ±0.033	7.326 ±0.094	7.299 ±0.096	7.331 ±0.052	7.326 ±0.033	7.331 ±0.030
	pO_2	171.5 ±7.8	146.4 ±15.9	141.3 ±17.1	161.8 ±20.7	124.7 ±12.2	135.3 ±18.7	148.7 ±21.2
	pCO_2	34.1 ±2.2	41.9 ±2.6	42.3 ±3.5	44.5 ±3.4	42.8 ±3.3	40.9 ±3.5	41.9 ±3.5
	HCO_3	19.9 ±1.4	19.9 ±1.3	21.5 ±1.0	22.2 ±1.3	20.9 ±1.2	20.7 ±0.9	21.6 ±1.0
ORGAN OUTFLOW	pH	-	7.297 +0.024	7.314 ±0.043	7.277 ±0.035	7.297 ±0.019	7.303 ±0.035	7.292 ±0.034
	$P0_2$	-	51.7 ±4.7	59.5 ±10.9	63.8 ±12.0	63.4 ±9.9	58.6 ±9.2	65.0 ±11.6
	PCO_2	-	44.3 ±2.7	47.3 ±4.6	46.8 ±3.5	44.4 ±3.1	42.6 ±3.0	40.3 ±3.2
	HCO_3	-	19.7 ±1.2	20.9 ±1.1	22.0 ±1.4	21.6 ±1.2	20.9 ±0.6	20.4 ±0.8

Appendix C6 Table of parameters measured during IRLPs with PCV=20% using washed human erythrocytes. Results shown are mean ± S.E. n=6.

	HOURS OF PERFUSION						
MEASUREMENT	0	1	2	3	4	5	6
Oxygen Uptake (μmol $min^{-1}g^{-1}$)	-	1.36 ± 0.16	1.26 ± 0.12	1.06 ± 0.11	1.11 ± 0.29	1.04 ± 0.40	1.00 ± 0.10
Bile Volume (μl)	1093 ± 143	1216 ± 89	1543 ± 105	1290 ± 100	720 ± 236	357 ± 182	77 ± 19
Haematocrit (% cells)	24.7 ± 0.3	22.3 ± 1.0	22.7 ± 1.1	22.7 ± 1.5	22.7 ± 1.5	22.3 ± 1.4	21.7 ± 1.1
Liver weights (g)	15.3 ± 1.6						14.5 ± 0.9
Perfusion pressure (mmHg)	14.7 ± 3.4	17.7 ± 3.5	17.3 ± 3.2	17.0 ± 3.4	16.7 ± 3.6	17.7 ± 3.5	19.0 ± 3.2
Flow rate (ml min^{-1})	2.03 ± 0.03	2.10 ± 0.05	2.18 ± 0.06	2.21 ± 0.03	2.07 ± 0.04	2.09 ± 0.05	2.09 ± 0.05
K+ (mmol l^{-1})	7.83 ± 0.33	7.33 ± 0.19	7.10 ± 0.14	6.83 ± 0.32	7.33 ± 0.40	8.03 ± 0.24	10.17 ± 0.46
Urea (mmol l^{-1})	1.01 ± 0.05	1.63 ± 0.12	2.07 ± 0.18	2.53 ± 0.31	2.97 ± 0.35	3.34 ± 0.41	3.71 ± 0.38
Bilirubin (μmol l^{-1})	1.13 ± 0.07	1.00 ± 0.05	1.10 ± 0.31	1.57 ± 0.35	0.60 ± 0.05	0.80 ± 0.12	0.63 ± 0.07
Glucose (mmol l^{-1})	18.7 ± 2.6	20.9 ± 4.2	21.2 ± 3.4	21.3 ± 2.1	21.8 ± 1.3	24.2 ± 1.6	26.2 ± 0.7
Plasma Haemoglobin (mg%)	193 ± 24	233 ± 24	276 ± 35	312 ± 46	342 ± 55	372 ± 62	428 ± 82
Bilirubin (μmol l^{-1})	38.7 ± 2.1	14.7 ± 1.6	22.7 ± 1.1	30.0 ± 2.8	34.7 ± 3.3	37.3 ± 0.5	42.0 ± 0.9

Appendix C6 Blood gas data from perfusions.

RED CELL SOURCE = HUMAN PCV:20%

	HOURS OF PERFUSION						
MEASUREMENT	0	1	2	3	4	5	6
pH	7.293 ±0.066	7.364 ±0.024	7.428 ±0.030	7.368 ±0.024	7.368 ±0.024	7.448 ±0.044	7.388 ±0.019
pO_2	410 ±46.13	80.2 ±99.7	436.6 ±139.2	227.1 ±48.4	216.5 ±37.0	246.3 ±9.9	209.2 ±22.2
pC0	237.2 ±0.63	8.7 ±0.8	36.4 ±2.1	34.2 ±3.1	43.5 ±4.3	40.5 ±3.2	41.8 ±4.0
$HC0_3$	18.9 ±2.31	8.6 ±2.5	21.9 ±2.1	24.6 ±1.8	24.7 ±2.0	24.1 ±0.5	24.0 ±1.1
pH	-	7.314 ±0.069	7.362 ±0.049	7.3777 ±0.032	7.310 ±0.050	7.457 ±0.026	7.397 ±0.250
pO_2	-	208.0 ±23.3	151.4 ±19.2	238.6 ±23.4	159.3 ±28.9	152.0 ±17.7	140.6 ±7.9
$pC0_2$	-	44.8 ±3.7	44.1 ±1.7	40.8 ±3.1	42.9 ±5.5	42.4 ±3.4	44.5 ±2.9
$HC0_3$	-	19.1 ±2.2	22.2 ±1.9	21.6 ±0.6	21.5 ±2.1	23.3 ±0.8	23.5 ±0.2
pH	-	7.262 ±0.063	7.314 ±0.009	7.388 ±0.036	7.313 ±0.037	7.401 ±0.016	7.400 ±0.035
pO_2	-	54.0 ±1.4	75.8 ±11.9	63.8 ±2.9	42.3 ±10.2	49.2 ±4.7	44.5 ±4.1
$pC0_2$	-	47.5 ±7.8	46.6 ±5.0	44.6 ±5.5	45.4 ±5.4	45.0 ±3.7	48.9 ±1.4
$HC0_3$	-	19.5 ±2.1	22.1 ±2.4	22.7 ±0.7	23.0 ±2.0	23.7 ±0.8	26.6 ±2.3

Appendix D1 Effect of tissue collection technique upon brain biogenic amines. Results shown are mean± S.E. (n=6 per group). Concentrations shown are expressed as μg / g brain.

	Rat Weight	5HT	5HTP	NOR	ADR	DOP
			CEREBRAL CONCENTRATIONS			
Ether	313±10.0	0.423± 0.0025	0.205±0.020	0.476±0.03	0.112 ± 0.036	0.315±0.025
Stunned	316±6.0	0.448± 0.031	0.178±0.044	0.572±0.03	0.12 ± 0.080	0.423±0.09
			BRAINSTEM CONCENTRATIONS			
Ether	313±10.0	0.324± 0.017	0.126±0.017	0.300±0.020	0.380 ± 0.011	0.884±0.035
Stunned	316±6.0	0.311± 0.013	0.119±0.020	0.416±0.042	0.350 ± 0.060	1.05±0.072

Appendix D2 Changes in cerebral and brainstem concentrations of catechol amines following portacaval shunting (PCS). Results shown are mean ± S.E. (n=6 per group). Concentrations shown are expressed as μg / g brain.

TIME/WKS	NOR	ADR	DOP
		CEREBRAL CONCENTRATIONS	
0	0.41±0.04	0.35±0.05	1.06±0.06
1	0.51±0.01	0.45±0.06	1.36±0.01
3	0.48±0.01	0.36±0.01	1.25±0.01
9	0.42±0.08	0.19±0.01	1.14±0.01
15	0.33±0.005	0.20±0.01	0.99±0.04
75	0.31±0.02	0.24±0.02	0.71±0.14
		BRAINSTEM CONCENTRATIONS	
0	0.4998 ±0.03	0.114 ±0.025	0.34 ±0.031
1	0.633 ±0.026	0.13 ±0.003	1.49 ±0.04
3	0.52 ±0.005	0.113 ±0.003	1.31 ±0.03
9	0.48 ±0.03	0.09 ±0.006	0.286 ±0.03
15	0.612 ±0.03	0.102 ±0.003	0.3455 ±0.03
75	0.489 ±0.06	0.06 ±0.011	0.297 ±0.02

Appendix D3. Cerebral and brainstem concentrations of indole amines following PCS in rats. Results shown are mean ± S.E. (n = 6 per group). Concentrations shown are expressed as μg / g brain.

Time/Wks	5HT	5HTP	5HIAA
		CEREBRAL CONCENTRATIONS	
0	0.34 ±0.017	0.112± 0.01	0.186 ±0.009
1	0.397 ±0.02	0.298± 0.01	0.336 ±0.03
3	0.424 ±0.02	0.236± 0.008	0.363 ±0.01
9	0.382 ±0.02	0.192± 0.02	0.337 ±0.01
15	0.365 ±0.02	0.23± 0.02	0.314 ±0.03
75	0.32 ±0.03	0.277± 0.01	0.302 ±0.02
		BRAINSTEM CONCENTRATIONS	
0	0.48 ±0.03	0.17± 0.03	0.33 ±0.03
1	0.55 ±0.01	0.24± 0.05	0.51 ±0.07
3	0.54 ±0.02	0.48± 0.01	0.77 ±0.04
9	0.48 ±0.04	0.33± 0.05	0.72 ±0.02
15	0.51 ±0.02	0.29± 0.05	0.68 ±0.13
75	0.49 ±0.04	0.60± 0.11	0.64 ±0.08

Appendix D4 Changes in cerebral and brainstem concentrations of brain biogenic amines in perfused and non-perfused PCS and control brains. Results shown are mean ± SE, n = 6 per group. Concentrations shown are expressed as μg / g brain.

		INDOLE AMINES				BRAINSTEM CATECHOLAMINES		
	Rat weights/g	5HT		5HTP		Nor	Adr	Dop
		Cerebrum	Brainstem	Cerebrum	Brainstem			
Controls (C) Unperfused	286 ± 11.0	0.51 ± 0.11	0.71 ± 0.23	0.28 ± 0.08	0.64 ± 0.06	0.52 ± 0.08	0.99 ± 0.09	1.64 ± 0.05
Unperfused (PCS) Shunted brains (7 days)	261 ± 17.5	0.57 ± 0.20	0.72 ± 0.27	0.50 ± 0.06	0.94 ± 0.05	0.58 ± 0.02	1.08 ± 0.06	1.93 ± 0.03
Control Perfused C/P	275 ± 29.6	0.34 ± 0.017	0.48 ± 0.03	0.112 ± 0.01	0.153 ± 0.03	0.36 ± 0.07	0.67 ± 0.09	1.01 ± 0.08
Shunted (PCS/P) Perfused	253 ± 33	0.397 ± 0.02	0.55 ± 0.01	0.198 ± 0.01	0.34 ± 0.05	0.633 ± 0.026	0.13 ± 0.003	1.49 ± 0.04

Appendix D5 Cerebral and brainstem concentrations of catechol amines following PCS and PCT in rats. Results shown are mean ± S.E., (n=6 group). Concentrations shown are expressed as μg / g brain.

A) CEREBRAL CONCENTRATIONS

1. PCS

Time Wks	Nor	Adn	Dop
Control 0	0.41 ±0.04	0.35± 0.05	1.06 ±0.06
1	0.51 ±0.01	0.48± 0.06	1.36 ±0.01
3	0.48 ±0.01	0.36± 0.01	1.25 ±0.01
9	0.42 ±0.08	0.19± 0.01	1.14 ±0.01
15	0.33 ±0.005	0.20± 0.01	0.99 ±0.04
75	0.31 ±0.02	0.24± 0.02	0.71 ±0.14

2. PCT

1	0.543 ±0.05	0.386± 0.03	1.40 ±0.23
3	0.423 ±0.02	0.231± 0.02	0.943 ±0.08
9	0.307 ±0.02	0.177± 0.002	0.825 ±0.05
15	0.310 ±0.003	0.165± 0.002	0.921 ±0.06

3. Controls

0.41±0.04	0.35± 0.05	1.06 ±0.06

Appendix D5

B) BRAINSTEM CONCENTRATIONS

1. PCS

	Nor	Adn	Dop
0	0.4998 ± 0.03	0.114 ± 0.025	0.34 ± 0.031
1	0.633 ± 0.026	0.13 ± 0.003	1.49 ± 0.04
3	0.52 ± 0.005	0.113 ± 0.003	1.31 ± 0.03
9	0.48 ± 0.03	0.09 ± 0.006	0.386 ± 0.03
15	0.612 ± 0.03	0.102 ± 0.003	0.3455 ± 0.03
75	0.489 ± 0.06	0.06 ± 0.011	0.207 ± 0.02

2. PCT

0	0.4998 ± 0.03	0.114 ± 0.025	0.38 ± 0.031
1	0.586 ± 0.007	0.123 ± 0.003	1.14 ± 0.48
3	0.579 ± 0.008	0.105 ± 0.002	0.228 ± 0.02
9	0.42 ± 0.02	0.070 ± 0.002	0.223 ± 0.02
15	0.54 ± 0.02	0.07 ± 0.002	0.226 ± 0.020

Appendix D6 Cerebral concentrations of indole amines following PCS and PCT in rats. Results shown are mean ± S.E., (n=6 per group). Concentrations shown are expressed as μg / g brain.

Time (weeks)	1	3	9	15
		5HIAA		
PCS	0.34±0.03	0.36±0.01	0.34±0.01	0.31±0.03
PCT	0.21±0.02	0.23±0.01	0.19±0.01	0.23±0.01
Control	0.18±0.01	0.18±0.01	0.21±0.01	0.17±0.01
		5HTP		
PCS	0.20±0.01	0.24±0.01	0.19±0.02	0.23±0.02
PCT	0.19±0.02	0.17±0.01	0.16±0.01	0.15±0.01
Control	0.11±0.01	0.14±0.01	0.12±0.01	0.14±0.01
		5HT		
PCS	0.40±0.02	0.42±0.02	0.38±0.02	0.36±0.02
PCT	0.31±0.02	0.38±0.01	0.33±0.02	0.38±0.01
Control	0.34±0.02	0.34±0.01	0.34±0.02	0.34±0.01

Appendix D7 Brainstem concentrations of indole amines following PCS and PCT in rats. Results shown are mean ± S.E., (n=6 per group). Concentrations shown are expressed as μg / g brain.

Time (weeks)	0	1	3	9	15
			5HIAA		
PCS		0.51±0.07	0.77±0.04	0.72±0.02	0.68±0.01
PCT		0.53±0.03	0.48±0.03	0.43±0.09	0.37±0.04
Control	0.34±0.03	0.33±0.03	0.35±0.06	0.37±0.04	0.25±0.01
			5HTP		
PCS	0.17±0.03	0.24±0.05	0.48±0.01	0.33±0.05	0.29±0.05
PCT	0.17±0.03	0.30±0.04	0.35±0.02	0.28±0.03	0.22±0.02
Control	0.18±0.03	0.15±0.03	0.16±0.03	0.16±0.01	0.18±0.02
			5HT		
PCS	0.48±0.03	0.55±0.01	0.54±0.02	0.48±0.04	0.51±0.02
PCT	0.48±0.03	0.56±0.04	0.52±0.02	0.40±0.02	0.43±0.03
Control	0.34±0.03	0.48±0.03	0.49±0.03	0.42±0.02	0.42±0.03