NEAR INFRARED SPECTROSCOPY IN THE
INVESTIGATION OF FETAL RESPONSES TO
STRESS

A thesis submitted for the degree of

Doctor of Philosophy

of the

University of London

by

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For my parents......
Intrauterine stressors are implicated in the development of a significant proportion of cases of perinatal brain injury, which may result in death of the infant or in deficits in motor and/or intellectual development. Whilst stressors such as reduction in oxygen delivery to the fetus or fetal infection are believed to have important roles in the development of perinatal brain injury, our understanding of the sequences of events leading from stressor to injury is incomplete.

My aim in performing the experiments presented in this thesis was therefore to use biophysical monitoring and near infrared spectroscopy to investigate fetal cerebral and peripheral cardiovascular and metabolic responses (A) to simulated fetal infection by intravenous administration of bacterial lipopolysaccharide to the fetus, and (B) to severe hypoxia-asphyxia induced in the mid- and late-gestation fetal sheep by either reduction in uterine artery flow or complete umbilical cord occlusion.

In these experiments chronically instrumented fetal sheep at either 0.6 or 0.8 of gestation were implanted with vascular catheters and a range of probes to permit chronic fetal monitoring without the confounding effects of anaesthesia. Fetal heart rate and mean arterial pressure were monitored by use of vascular catheters placed in brachial and/or femoral artery. These catheters allowed blood samples to be taken which could be analysed to provide data on blood pH, blood gas tensions, oxygen content and haemoglobin saturation. Coupled with measurements of flow from jugular and/or femoral vein, calculation of oxygen delivery to and consumption by both the brain and femoral skeletal muscle bed was possible. Additionally, use of a purpose-built continuous wavelength CCD near infrared spectrometer (NIR) provided continuous measurements of change in the absolute concentration of oxyhaemoglobin (HbO2), deoxyhaemoglobin (Hb) and oxidised cytochrome oxidase (CcO) in fetal brain and peripheral muscle during hypoxia and asphyxia.

My important findings are:

1. Fetal haemodynamic responses to acute severe reduction in uterine artery flow at 0.6 & 0.8 of gestation are qualitatively similar but differ in magnitude, indicating that the systems required for reflex adaptation to hypoxia – asphyxia are present and functional at 0.6 of gestation.

2. The ability of mid-gestation fetuses to maintain adaptations to severe hypoxia are attenuated compared to late-gestation animals and, despite receiving severe asphyxic challenges from uterine artery occlusion, there was no evidence of repeatable, severe brain injury at either 0.6 or 0.8 of gestation.

3. Cerebral CcO, at both 0.6 and 0.8 of gestation, oxidises during hypoxia. Simultaneously, femoral skeletal muscle CcO became reduced. Since O2 consumption fell in both brain and skeletal muscle, the CcO data indicates that subtle metabolic alterations can be made regionally, with perhaps more important tissues retaining more metabolic activity during hypoxic stress.

4. In late-gestation, severe umbilical cord occlusion results in temporary haemodynamic adaptation and CcO oxidation. When this insult is maintained, fetal cardiovascular collapse and CcO reduction begin. Histological evidence of hippocampal injury was present in all bar one of the fetuses exposed to this insult.

5. Exogenous infusion of adenosine, at a rate which does not dramatically alter haemodynamics, reduced fetal cerebral O2 consumption through an unknown mechanism and results in CcO oxidation similar to that seen during hypoxia.

6. A high nanogram dose of bacterial lipopolysaccharide administered iv to the fetal results in mild haemodynamic perturbation and severe brain injury.
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PERSONAL STATEMENT

Except for histological examinations, as noted in each appropriate chapter, the work in this thesis was performed solely by the candidate and is original.

James P. Newman

Certification by Supervisors:

Professor Mark A. Hanson  Dr Donald M. Peebles
% Percentage
\(\alpha\) Alpha, also Specific extinction coefficient
\(A_2\) Adenosine \(A_2\) receptor subtype
\(A_{2A}\) Adenosine \(A_{2A}\) receptor subtype
\(A_1\) Adenosine \(A_1\) receptor subtype
ACTH Adrenocorticotropic hormone
Ado Adenosine
AMPA \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANP Atrial natriuretic peptide
ATP Adenosine triphosphate
AVP Arginine vasopressin
\(\beta\) Beta
BBB Blood-Brain barrier
c Concentration of an absorbing compound when in solution
c.a. Circa
\(Ca^{2+}\) Calcium ion
\(C_aO_2\) Arterial oxygen content
CBF Carotid artery blood flow
CoO Cytochrome c oxidase, also called Cytochrome Oxidase (concentration when referring to NIRS data)
CMR\(O_2\) Cerebral metabolic rate for oxygen
CNS Central nervous system
CO Cardiac output
CoA Coenzyme A
\(CO_2\) Carbon dioxide
CP Cerebral palsy
CSF Cerebrospinal fluid
CRF Corticotrophin Releasing Factor
CuA Copper A centre of cytochrome oxidase
CuB Copper B centre of cytochrome oxidase
CVO (Fetal) Combined ventricular output
\(C_vO_2\) Venous oxygen content
cyt Cytochrome
\(\Delta\) Delta (Change in)
Abbreviations

δ  Delta (subunit of protein)
d  Thickness
DA  Ductus arteriosus
DO₂  Oxygen delivery
EAA  Excitatory amino acid
ECoG  Electrocorticogram
e⁻  Electron
e.g.  For example
et al.  And colleagues
FAD⁺  Flavin-adenine dinucleotide (oxidised)
FADH₂  Flavin-adenine dinucleotide (reduced)
FBF  Femoral artery blood flow
Fe²⁺  Iron ion (Valency 2)
Fᵢ  Inspired fraction of gas, e.g. FᵢO₂, expressed as fraction of whole
γ  Gamma
GMH  Germinal matrix haemorrhage
Hb  (Deoxy-) Haemoglobin (concentration when referring to NIRS data)
HbO₂  Oxyhaemoglobin (concentration when referring to NIRS data)
H₂O  Water
HIE  Hypoxic-ischaemic encephalopathy
H⁺  Proton
HPA  Hypothalamo-Pituitary axis
I₀  Intensity of incident light
I  Intensity of light transmitted through an absorbing compound
IPH  Intraparenchymal haemorrhage
IVH  Intraventricular haemorrhage
KA  Kainate
Kₘ  Michaelis constant (affinity)
ln  Natural logarithm (base e)
LV  Lateral ventricle
MAP  Mean arterial pressure
MPT  Mitochondrial permeability transition pore
MRI  Magnetic resonance imaging
MRS  Magnetic resonance spectroscopy
NADH  Nicotine-Adenine dinucleotide (reduced)
NAD⁺  Nicotine-Adenine dinucleotide (oxidised)
NIR  Near infrared
CHAPTER 1

INTRODUCTION

The materno-fetal relationship has evolved to provide the optimal conditions for fetal growth and development. Whilst in utero, these conditions may, either acutely or chronically, fall below the level at which optimal fetal condition can be maintained and fetal adaptation to sub-optimal conditions must occur. Globally, intrauterine stresses such as reduction in fetal oxygenation or nutrition and fetal infection may not be uncommon. Stressors such as these are implicated in the development of significant fetal morbidity and mortality, the incidence of which may be reduced by timely and appropriate intervention. Such intervention can only be attempted when there is a thorough understanding of the physiological responses of the fetus to these stressors throughout gestation, and especially at times in development when the fetus may be particularly susceptible to certain types of injury. Timely intervention also requires monitoring equipment and techniques which can accurately advise clinicians about appropriate indices of fetal wellbeing.

Hypoxia is probably the most well-studied intrauterine stressor and has been implicated in brain injury and other pathologies which can lead to the death of the infant. The rate of infant mortality ascribed to hypoxia-ischaemia or asphyxia at birth has been steadily falling over the last 50 years, probably as much as a result of technical improvements in care as to increases in the knowledge base about hypoxia. Increases in our understanding about the fetus and its responses to stressors has improved with the introduction of new techniques for fetal monitoring, including electronic fetal heart rate monitoring, ultrasound scanning, magnetic resonance imaging and spectroscopy, near infrared spectroscopy and with the development of new animal experimental models, especially the chronically-instrumented fetal sheep.

The use of the chronically instrumented fetus has allowed many aspects of fetal physiology to be investigated which would otherwise be impossible because of the ethical boundaries rightly placed upon studies of human infants. By studying fetal physiology and pathophysiology in the relatively undisturbed setting of the chronically instrumented animal, it is hoped that we can expand our knowledge of fetal responses to in utero stressors and identify their clinical outcomes. In this way, fetal physiologists working with clinicians and medical physicists may be able to develop new methods to confirm appropriate fetal development and new paradigms for the identification of fetal stress in utero.
1.1 Why do we monitor fetuses before and during labour?

In viviparous mammals, the developing fetus is buffered from external influences by both its placement within the uterus and by the function of the placenta. Whilst, in evolutionary terms, the internal location of the fetus provides the opportunity for development without the danger of predation associated with egg-laying species. For the clinician, it also prevents direct monitoring of fetal wellbeing which may impact on the management of the pregnancy. This has potential health consequences for both the mother and fetus. For these reasons, techniques have been developed for both the direct, invasive and indirect, non-invasive monitoring of the fetus.

Monitoring of fetal movements and heart rate has been performed for well over a century (John Burns, 1828) and, for many years, electronic intrapartum fetal heart rate monitoring has been one of the main methods of determining fetal health. Historically, the ability of the clinician and scientist to monitor the developing fetus has waited upon the development of sophisticated engineering and computing technologies. Development in the last thirty years of non-invasive imaging and spectroscopic techniques such as ultrasound scanning and magnetic resonance imaging (MRI) and spectroscopy (MRS) (Mattison et al., 1988; Wyatt et al., 1989) have given unique insights into the development of the fetus and provided new paradigms by which to measure fetal health, which had not previously been available in healthy infants before birth. Using such techniques, it is now possible to identify some of the fetuses in distress or who are developing abnormally and this may allow appropriate and timely intervention in some pregnancies.

Fetal asphyxia both antepartum and intrapartum is a problem that can lead to fetal death or brain injury, leaving the surviving infant with developmental delay and motor and/or intellectual deficits. Whilst the numbers of infants suffering asphyxia in the perinatal period is small, at ~2 per 1000 live births (Thomberg et al., 1995), it is a persistent proportion of births and is costly, both emotionally for the families and in terms of the money and resources needed for the care of these infants as they grow older. Whilst neonatal mortality rates have fallen substantially in the UK since the beginning of the century (Hull & Dodd, 1992; Smith, Wells & Dodd, 2000), further substantial reductions in neonatal mortality do not appear to be occurring with the introduction of new monitoring modalities and the subsequent increase in elective surgical interventions (Martin, 1998). The fall in mortality rate amongst preterm infants seen over the latter half of the century does not mean that the problem of brain injury has been solved for this group. The incidence of lesions in immature infants is proportional to the degree of prematurity so, whilst the number of infants born with or developing severe neurological deficits in the first days or months of life may be relatively stable in many centres, the number of infants developing subtle motor and cognitive deficits may, in fact, be increasing (Kuban & Leviton, 1994). In light of these data, investigation of the aetiology of hypoxic-ischaemic neuronal injury is both warranted and timely.
Considering the complexity of embryological development, it is unsurprising that current evidence suggests that the aetiology of brain injury is multifactorial, ie that hypoxic insults are not necessarily the primary cause in a majority of cases. Similarly in the preterm infant, evidence is accumulating that periventricular leukomalacia, previously reported as a lesion of cerebral hypoperfusion to which immature fetuses are particularly susceptible, may also be caused by infection. However, hypoxia is still the most well-investigated intrauterine stressor and is still believed to be an important cause of brain injury in a significant minority of cases of infants diagnosed with developmental difficulties as school age (Cooke & Abemethy, 1999). In the following sections, I will review the known fetal responses to acute hypoxia.

1.2 Defining hypoxia

Hypoxia, hypoxia-ischaemia and asphyxia are three terms used commonly to indicate low arterial oxygen tension (\(P_{a\text{O}_2}\)) and are, generally, used interchangeably by many authors. Since science relies upon precision (the precision of language as much as the precision of monitoring and measuring), it is important that we begin this discussion of hypoxia with definitions of the terms I will be using throughout this thesis. Some effort was made by Connett (Connett, 1990) to impose a language framework for the study of hypoxia, and this has been used as a starting point. Since this paper came from a cell physiology background and not from whole-body physiology, some of the terms cannot be used here with any degree of accuracy or certainty. The term dysoxia, for example, will not be used here in favour of more commonly used terminology. The terms I will use are as follows:

- **Hypoxaemia** is a term which will be used to indicate low arterial \(P_{a\text{O}_2}\) without accompanying acidaemia, hypercapnia or reduction in tissue perfusion; specifically, I will use this to mean a hypoxic insult initiated by lowering maternal inspired oxygen fraction.

- **Ischaemia** will be used to indicate a reduction in delivery of substrate such as glucose or \(O_2\) to a tissue due to a fall in tissue perfusion. This may be caused, for example, by a constriction of resistance vessels causing local stasis of blood flow.

- **Asphyxia** is a difficult term to use because is has an everyday meaning which is not the same as that used in scientific literature. Here it will mean reduced \(P_{a\text{O}_2}\) with hypercapnia.

Just from these simple examples, it is obvious that there can be a problem with the terminology. Hypoxia cannot, for example, exist without ischaemia if a reduced \(O_2\) delivery counts as ischaemia. Also, all but the mildest maternal inhalational hypoxic insults can cause some degree of increased arterial \(P_a\text{CO}_2\) or \([\text{H}^+]\). Owing to the progressive changes in tissue \(P_{a\text{O}_2}\) and organ blood flows during development, the normal \(P_{a\text{O}_2}\) for a given fetus will be continuously changing. This makes it difficult to say definitively whether a fetus is mildly hypoxic or within its own normal range of values. Taking these problems into account, I shall use the term hypoxia or hypoxia-ischaemia to signal a reduced \(P_{a\text{O}_2}\) for a given gestation and asphyxia for
INTRODUCTION

a hypoxia where there is definite acidaemia or hypercapnia. When referring to the literature, I shall
endeavour to use this same scheme or just “hypoxia” if it is not clear what the exact fetal conditions are
other than reduced PO$_2$.

1.3 Experimental hypoxia

Since interruption of oxygen delivery is believed to be one of the most common in utero insults, much
investigation of the fetal responses to intrauterine stressors has concentrated upon this challenge. A
number of methods of achieving hypoxia are in use, including reduction in maternal inspired O$_2$ fraction
(Cohn et al. 1974), umbilical cord occlusion (Bennet et al., 1999), restriction of uterine artery flow (Block et
al., 1990), placental embolisation (Bocking et al., 1988) and fetal haemorrhage (Matsuda et al., 1999).
Details of these methods are given below:

1.3.1 Maternal inhalation hypoxia

The most commonly used and easiest to apply of the experimental hypoxias, maternal inhalation hypoxia
involves reducing the fraction of inspired oxygen breathed by the mother and thus reducing maternal P$_a$O$_2$.
In this way the fetal P$_a$O$_2$ is also reduced from ca. 25 mmHg in late-gestation to 11-16 mmHg. The most
common method of applying this insult is by placing over the ewe’s head a clear polythene bag into which
the hose of the experimental gas supply is taped. The bag is secured around the head by means of a
drawstring. The ewe is then piped normally oxygenated air and allowed to acclimatise to the conditions
before control recording is begun. When it is time to begin the hypoxia, air and pure N$_2$ flows are altered
so as to maintain overall gas flow rate but to reduce O$_2$ fraction to the desired level. In this way, very mild
or quite severe O$_2$ reduction can be achieved. During hypoxia, chemoreceptors in the ewe initiate
hyperventilation in an attempt to increase arterial PO$_2$. This has the secondary effect of reducing maternal
P$_a$CO$_2$, causing respiratory alkalosis. By altering the partial pressure of CO$_2$ in the inspirate, respiratory
alkalosis, and its effects on the fetal response to hypoxia, can be minimised.

This method has the advantage of being very easy to apply, requiring no additional surgery in excess of
that normally used to monitor the fetus. However it is slow in onset and does expose the placenta to
hypoxia as well as the fetus, with possible confounding effects.

1.3.2 Umbilical cord compression

Another commonly used technique for reducing O$_2$ delivery to the fetus, an inflatable occluder around the
umbilicus (either as a whole, or around the umbilical veins alone) can produce a rapid reduction in blood
flow from the fetus to the placenta, and thus in O$_2$ transfer to the fetus. This method can produce graded
insults which can be applied singularly or repetitively. The volume of fluid injected into the occluder and the duration of the insult can be controlled either manually or by computer-linked infusion pump.

This method provides the benefit of a fast insult which may simulate umbilical cord problems in utero. However, in addition to restricting O\textsubscript{2} transfer to the fetus, this insult has the secondary effect of reducing CO\textsubscript{2} elimination, thus causing a fetal asphyxia where fetal PO\textsubscript{2} falls whilst PCO\textsubscript{2} rises and arterial pH drops. Placental oxygenation by the mother is not affected by cord compression, but compression mechanically alters fetal haemodynamics by interrupting a major path for blood flow. This has two immediate consequences which add to the fetal haemodynamic environment. Firstly there is a sudden increase in peripheral vascular resistance which will have baroreflex repercussions. Secondly, the fetal blood is directed away from the placenta and has to go elsewhere. This has implications for the study of the fetal redistribution of blood flow during hypoxia.

1.3.3 Reduction in uterine artery flow

This insult uses an occluder (inflatable or mechanical screw occluder) which, in sheep, is placed around the maternal common internal iliac artery. The maternal blood supply to the placenta can therefore be reduced in a graded or complete fashion as with umbilical occlusion. By reducing the flow of blood to the placenta, O\textsubscript{2} transfer to both the placenta and the fetus is reduced and elimination of CO\textsubscript{2} by the fetus is also impaired. Experimentally this is a difficult insult to apply owing to the difficulty of maintaining an occlusion of the common internal iliac arteries against the high maternal blood pressure, and there is often some degree of return of uterine flow as the challenge progresses.

Despite this reservation, this is an effective and potentially very severe insult which affects not only the fetus, but also the placenta. The asphyxia produced by this insult can usually be withstood by healthy late-gestation fetuses for at least 60 minutes.

1.3.4 Placental embolisation

Microspheres have been used for some time as a method of measuring blood flows to fetal tissues. The microspheres can be injected from either the maternal or fetal side of the placenta. When injected into a fetal vein (Gagnon et al., 1994), microspheres are transported around the fetal circulation with the blood until they lodge in capillaries which are too small for them to pass through. This technique has the unfortunate side effect of blocking capillaries, which limits the number of doses of microspheres which can be used in any given experiment. However, this blocking of vessels can be used advantageously in the experimental ovine fetal preparation to reduce the surface area of the placenta available for gaseous exchange. This method experimentally reproduces placental insufficiency and can be used to monitor the effects of chronic reduction of placental transfer of O\textsubscript{2} and substrate to the fetus.
1.3.5 Fetal haemorrhage

By reducing the fetal blood volume, we can manipulate the fetal blood's capacity to carry oxygen without affecting maternal or placental PO$_2$. As with the other insults detailed above, this can be achieved in a graded manner. The drawback to this method is that, unless the lost fetal blood is replaced with an equivalent volume of donor plasma, plasma proteins and electrolytes are also lost, along with their buffering, and other, functions.

1.4 Fetal responses to acute hypoxia

Many workers have investigated aspects of the response of the late gestation fetus to an acute hypoxic-ischaemic challenge, such as changes in organ blood flow, endocrine, neuronal or metabolic responses. The main thrust of this section will be to examine some of the cardiovascular and endocrine responses to an acute decrease in arterial PO$_2$ in an attempt to provide an appropriate and relevant background for the experimental work in this thesis. The effects of chronic hypoxaemia are beyond the scope of this thesis and will not be examined.

1.4.1 Cardiovascular response to acute hypoxia

Early work to elucidate the fetal cardiovascular responses to hypoxia was performed in anaesthetised, exteriorised fetuses (see Barcroft, 1973 for history). Although these data were complicated by the additional effects of the anaesthesia, many of the conclusions drawn from these experiments are still valid. It was this early work that established that the late gestation fetus can effect circulatory centralisation in order to protect vital organs and suggested the reflex nature of this response (Campbell et al., 1967; Barclay et al., 1944). The development of the chronically catheterised in utero fetal sheep preparation (Meschia et al., 1965) allowed for the first time the study of the cardiovascular effects of hypoxia without significant complications due to the methodology. Many of these early experiments using radiolabelled microspheres established the distribution of the fetal combined ventricular output to each organ or tissue during normoxia and under different hypoxic conditions. With the advent of real-time blood flow monitoring using electromagnetic and then transit-time ultrasonic flow probes, it became possible under various circumstances to continuously measure flow in selected arteries. In this way both immediate (Giussani et al., 1993) and slow onset (Giussani, Spencer & Hanson, 1994 for review) phases of the cardiovascular response to hypoxia were described. These fetal responses to hypoxia vary slightly with the methodologies employed to induce the insult but are variations on a common theme. These cardiovascular responses to hypoxia are explained below.
1.4.1.1 Fetal heart rate (FHR) and mean arterial pressure

In late-gestation the fetal cardiovascular response to hypoxia is typified by a rapid, transient bradycardia and increased heart rate variability (Dalton et al., 1977; Parer et al., 1980). The bradycardia reaches a nadir about two minutes after the onset of the hypoxic insult (Cohn et al., 1974; Westgate, Bennet & Gunn, 1999) and, presumably, economises on myocardial oxygen consumption. Fetal hypoxic bradycardia occurs at the onset of hypoxic hypoxia (Cohn et al., 1974), umbilical cord occlusion (Bennet et al., 1999) and uterine artery occlusion (Newman et al., 2000) – the three most common experimental hypoxic challenges.

The speed of onset of the bradycardia is suggestive of a neural reflex. The sensors for this reflex have been identified as the carotid chemoreceptors (Giussani et al., 1993; Bartelds et al., 1993), which send afferent fibres in the carotid sinus nerve to a central integrating area in the brainstem (Mifflin et al., 1992) and whose efferent arm consists of parasympathetic cholinergic fibres to the heart carried in the Vagus nerve (cranial nerve X), and α-adrenergic sympathetic fibres to resistance vessels in peripheral vascular beds (Giussani et al., 1994).

The depth of the bradycardia and its rapidity of onset increase with the severity and speed of onset of the insult (author’s observations, unpublished) and late-gestation fetal heart rate can briefly fall to 70 or fewer beats per minute with a severe challenge. Fetal mean arterial pressure responses to hypoxia are also variable. Cohn and colleagues reported in 1974 that there was no significant change in fetal mean arterial pressure with isocapnic hypoxaemia, but that mean pressure increased when the fetus became acidaemic during the insult. Subsequent authors have described a significant increase in mean arterial pressure with maternal inhalation hypoxia (Giussani et al., 1993), umbilical cord occlusion (Bennet et al., 1998 & 1999) and interruption of uterine artery flow (Field et al., 1990).
Figure 1.1 Acute fetal responses to hypoxia can be demonstrated to be chemoreflex in origin. Right hand graph shows carotid (upper) and femoral (lower) vascular resistance, left hand graph shows fetal heart rate (upper) and perfusion pressure (lower). With intact carotid sinus nerves (○), we can see the normal late-gestation fetal responses to hypoxia. When the carotid sinus nerve are cut (●), the initial bradycardia is not seen and there is no early increase in femoral vascular resistance. From Giussani et al., 1994.

HYPOXIA

\[ \text{CHEMORECEPTORS} \]

\[ \text{CAROTID} \]

\[ \text{HEART RATE} \]

\[ \text{PERIPHERAL RESISTANCE} \]

\[ \alpha\text{-adrenergic} \]

\[ \text{Cholinergic} \]

\[ \text{BRAINSTEM} \]

\[ \text{Carotid sinus nerve} \]

Figure 1.2 Diagrammatic representation of the ideas put forward by Giussani and colleagues (Giussani et al., 1993) which demonstrate the initial chemoreflex-driven responses to acute isocapnic hypoxaemia in the late-gestation fetal sheep in utero. Adapted from LR Green’s PhD thesis.
1.4.1.2 Altered distribution of combined ventricular output (CVO)

Experimental findings indicate that during hypoxia the fetal CVO is generally redistributed in favour of the placenta, heart, brain and adrenal glands at the expense of other tissues (Cohn et al., 1974). However, the precise pattern of this redistribution depends upon the type of hypoxic challenge used (Figure 1.4 and review by Jensen & Berger, 1991), and blood flow to the brain and placental circulation may not always be maintained as a percentage of CVO (Figure 1.4).

During hypoxia the redistribution of combined ventricular output is accompanied by altered distribution of umbilical venous flow. During normoxaemia, 55% of umbilical venous flow bypasses the liver through the ductus venosus (Rudolph & Heymann, 1970) and enters the inferior vena cava, from where it is preferentially streamed across the foramen ovale (Barclay, Franklin & Prichard, 1944) and sent to the head and upper limbs (Reuss & Rudolph, 1980). In this way the brain and heart always receive the most highly oxygenated blood. During hypoxaemia, these processes are augmented such that the percentage of oxygenated blood bypassing the liver increases from 55 to 65% and a greater proportion of umbilical venous blood passes through the foramen ovale to supply the brain and heart (Reuss & Rudolph, 1980).

The data given in Figure 1.4 demonstrates how the blood flow to adrenal and myocardial circulations, as a proportion of CVO, is increased during each of the hypoxic challenges shown. However, contrary to popular belief, cerebral flow as a percentage of CVO is increased during most but not all hypoxic challenges in late-gestation.

1.4.1.3 Flow to the myocardial circulation

Under normoxic conditions fetal myocardial blood flow is an inverse function of arterial $O_2$ content (Peeters et al., 1979), with flow increasing as $O_2$ content decreases in order to maintain $O_2$ delivery to the myocardium. The work of Cohn established that during hypoxaemia, myocardial blood flow is increased as a percentage of the CVO (Cohn et al., 1974), thus maintaining $O_2$ delivery and protecting myocardial function during hypoxaemia. This increase in myocardial flow as a percentage of CVO during hypoxia has been confirmed by other authors using a variety of hypoxic insults (Itskovitz et al., 1987; Block et al., 1990; Jensen et al., 1987; Yaffe et al., 1987; Ball et al., 1994a & b). Fisher and colleagues reported that the fetal and adult sheep myocardial $O_2$ consumption under in vitro conditions were similar (Fisher, Heymann & Rudolph, 1980), suggesting that the work output is similar at these different ages. Interestingly, Fisher noted that, although the $O_2$ consumption of the fetus and adult were similar, the substrate consumed was very different, with the fetal myocardium consuming lactate as much as, or perhaps more readily than it does glucose; this suggests myocardial adaptation to the fetal environment.
1.4.1.4 Flow to the cerebral circulation

Maintenance of an adequate blood supply to the fetal brain requires matching of flow to metabolic demand. Whilst in the adult this can be achieved through a fall in cerebrovascular resistance and an increase in cardiac output (CO), the fetus does not appear to be able to increase CO and must rely instead on altering the distribution of its combined ventricular output (Barclay et al., 1944; Rudolph & Heymann, 1970). Alteration of CVO is achieved through control of regional microvascular tone at the local level while plasma concentrations of substrates such as O₂ and glucose (and lactate in the fetus) are kept within tight limits.

Since the placenta performs oxygenation of the fetal blood and not the lung, fetal blood flow not required for lung development is shunted away from the lung tissue by the ductus arteriosus.

Without the need to perfuse the lung at postnatal levels, fetal cardiovascular flow is reorganised to given preferential supply of highly oxygenated blood to the upper body, including the heart and brain.
**Figure 1.4** Examples of alterations in distribution of fetal combined ventricular output during experimental hypoxic challenges. (A) maternal hypoxaemia, (B) complete umbilical cord occlusion, (C) reduction of uterine artery flow by 50%, and (D) complete arrest of uterine artery flow. From Jensen & Berger 1993 – adapted from various authors' work.

**Figure 1.5** Brain blood flow at different gestational ages in the sheep fetus as measured by Fick principle calculation. Adapted from Rudolph, 1970.
Fetal global and regional cerebral blood flow and metabolic rate increase with birth weight and gestational age (Figure 1.5; Szymonowicz et al., 1988; Northington et al., 1997) and although $O_2$ delivery to the fetal brain in late-gestation is about 170% of that in the adult, there is no significant difference in $O_2$ consumption per unit weight between the two ages (Jones et al., 1982).

As in the adult, the fetal cerebral vasculature is regulated by $P_{a}O_2$, $P_{a}CO_2$, by metabolic demand and by neural factors (Jones et al., 1978; Bissonette et al., 1984; Busija & Heistad, 1984; Rudolph, 1985; Helou et al., 1994). Of these factors, arterial $O_2$ content seems to be more important than $PCO_2$ (Jones et al., 1974) and has been shown to be sensitive to reduction in mean arterial pressure, especially in mid-gestation when fetal arterial pressure is near the lower end of the autoregulatory range (Helou et al., 1984). Fetal adenosine levels are also believed to be an important determinant of local vascular tone in the fetal brain, where adenosine concentrations are much higher than they are in the adult (Sawa et al., 1991). Fetal adenosine levels rise further in response to systemic hypoxia (Kubonoya & Power, 1997) and may mediate the hypoxic increase in cerebral blood volume (Cohn et al., 1974). Sympathetic innervation of some areas of the brain are particularly dense and may also be implicated in mediating decreases in cerebrovascular resistance during hypoxia in a regionally-selective fashion (Busija & Heistad, 1984).

In addition to the controls mentioned above, the fetal vasculature exhibits both myogenic responses and flow-mediated vasodilatation— a whole area of vascular regulation which is beyond the scope of this introduction, but which must be borne in mind when interpreting haemodynamic responses to intrauterine stresses. Some specific examples of regulation are given below.

1.4.1.5 Vascular resistance responses to hypoxia

Redistribution of CVO is achieved through alterations in resistance vessel tone exerted by local, endocrine and neural factors. The net result of these alterations are increases in vascular resistance in the gut, and femoral circulation and, presumably to the other peripheral vascular beds whilst resistance in the cerebral, adrenal and myocardial circulations is decreased (Bennet et al., 1998; Ball et al., 1994; Jensen, Hohmann & Künzel, 1987; Yaffe et al., 1987). The local conditions, reactivity to vasoactive hormones and autonomic innervation will therefore determine how each vascular bed reacts to hypoxia, with flow increasing to some whilst being maintained or falling in others.

There is evidence for a rapid rise in vascular resistance at the onset of hypoxia in femoral (Giussani et al., 1993) and superior mesenteric arteries (Bennet et al., 1999). Flow in the carotid arteries shows a variable response and is either increased (Giussani et al., 1994), maintained (Newman et al., 2000) or decreased
(Jensen et al., 1987b) depending upon the severity of the insult and its rapidity of onset. Nitric oxide synthase inhibition alters the cerebral blood flow response to hypoxaemia from an increase to a decrease in flow (Green et al., 1996) which suggests that nitric oxide maintains cerebral blood flow during hypoxaemia and that this vasorelaxation is either absent or overridden during the most severe hypoxic insults when CBF falls.

1.4.1.6 Nitric oxide (NO)

Nitrovasodilators such as nitroglycerine and nitroprusside are amongst the oldest cardiovascular drugs available for clinical use (Kelly & Smith, 1996). Until the early 1980s the mechanism by which they act to relax vascular smooth muscle was not apparent. It was first suggested by Furchgott (Furchgott & Zadowski, 1980) that these nitrovasodilators mimicked an endogenous paracrine or autocrine agent, whose action was dependent upon the presence of the vascular endothelium. This so-called endothelium-derived relaxing factor (EDRF) was believed to be nitric oxide or one of its congeners (i.e. nitrogen oxides of formula NO₂), which were known to be produced in many cells types and which subserved a number of physiological functions (for review see Nathan & Xie, 1994). EDRF was later confirmed to be nitric oxide (Palmer, Ferrige & Moncada, 1987; Moncada, Herman & Vanhoutte, 1987; Palmer, Ashton & Moncada, 1988), and a number of NO synthase enzymes were identified whose action are dependent upon NADPH, O₂ and often on calmodulin and, therefore, on Ca²⁺ levels (Palmer, Ashton & Moncada, 1988).

Confirmation that EDRF and NO are the same entity started a revolution in our understanding of physiology. Subsequent to this discovery, this diatomic free radical gas has been reported to be intimately involved in a number of physiological processes including neurotransmission, neuromodulation, intracellular Ca²⁺ homeostasis and gene expression.

1.4.1.6.1 Properties and Synthesis

Nitric oxide owes its ability to exert its specific biological effects to its chemical and physical properties. It is a gas, but is relatively hydrophobic and highly diffusible. For a free radical, it has low reactivity compared to other species such as superoxide or hydroxyl radicals, this increases the distance it can diffuse before reacting with other molecules. It does, however, react readily with a number of biological and non-biological target molecules, including superoxide radicals, lipids and molecules containing metal centres, such as haem and copper proteins (Figure 1.6).

Nitric oxide is synthesised by at least three isoforms of nitric oxide synthase (NOS). There are two classification systems in use, but these enzymes are commonly known as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Both nNOS and eNOS are constitutively expressed and Ca²⁺ dependent, whilst iNOS is inducible and Ca²⁺ independent. These isoforms can be present in
isolation on a cell, or can be co-expressed. Table 1.1 below gives a summary of each isoform. The mechanism by which NO is synthesised is shown in Figure 1.7 below. Briefly, L-arginine is catalysed to L-citrulline and NO, a process which uses five electrons and O₂.

The nitric oxide produced by eNOS targets soluble guanyl cyclase (McDonald & Murad, 1996) in endothelial smooth muscle which catalyses the production of cyclic guanidine monophosphate (cGMP) (Dawson & Dawson, 1995). Relaxation of smooth muscle could be achieved by interference with any number of steps in the process of vasoconstriction. However, the proposed mechanism for NO vasorelaxation is that cGMP interferes with the increase in intracellular Ca²⁺ required for the activation of myosin light chain (Murad, 1986) by activation of cGMP-activated protein kinases, which in turn phosphorylate and activate Ca²⁺-ATPases or other components associated with Ca²⁺ transport (Schmidt, Lohmann & Walter, 1993; Hirata & Murad, 1994).
**Figure 1.7** Biosynthesis of nitric oxide from L-arginine. NOS catalyses a 5-electron oxidation of an amide nitrogen of L-arginine, forming L-hydroxyarginine as an intermediate, tightly bound to the enzyme. The final products are L-citrulline and nitric oxide. As explained in the text, this process can be Ca\(^{2+}\) dependent or independent. The process also consumes oxygen.

<table>
<thead>
<tr>
<th>Type I: nNOS</th>
<th>Type II: iNOS</th>
<th>Type III: eNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity depends upon elevated Ca(^{2+})</td>
<td>Activity is independent of Ca(^{2+})</td>
<td>Activity depends upon elevated Ca(^{2+})</td>
</tr>
<tr>
<td>First identified in neurons</td>
<td>First identified in macrophages</td>
<td>First identified in endothelial cells</td>
</tr>
<tr>
<td>Constitutively expressed, but inducible under pathological conditions</td>
<td>Inducible under pathological conditions</td>
<td>Constitutively expressed, but inducible under pathological conditions</td>
</tr>
<tr>
<td>Plays a prominent role in the early stage of neuronal injury after cerebral ischaemia</td>
<td>Plays a role in the late stages of neuronal injury after cerebral ischaemia</td>
<td>Plays a protective role in cerebral ischaemia by maintaining cerebral blood flow</td>
</tr>
<tr>
<td>Protein and catalytic activity upregulated within 10 minutes and peak at 3 hours after cerebral ischaemia</td>
<td>Protein and catalytic activity upregulated within 12 hours and peak activity at 48 hours after cerebral ischaemia</td>
<td>Protein and catalytic activity upregulated within 1 hour and peak activity at 24 hours after cerebral ischaemia</td>
</tr>
</tbody>
</table>

**Table 1.1** NOS subtypes and summary of main differences.
1.4.1.6.2 NO and hypoxia

There is evidence that eNOS activity is increased during hypoxia (Nagafuji, Sugiyama & Matsui, 1994; Gajkowska & Mossakowski, 1997). Immunohistochemical localisation has shown eNOS to be present in the area of ischaemia in the rat middle cerebral artery occlusion model (Gajkowska & Mossakowski, 1997). Also, increased eNOS activity has been detected after a day of ischaemia and large increases after 3-7 days of ischaemia in the same model (Nagafuji, Sugiyama & Matsui, 1994). Acute hypoxia also stimulates eNOS activity in piglets (Beasley et al., 1998), specifically in the hippocampus where neuronal injury is very common after late gestation hypoxic insults.

Of particular interest to this thesis are nitric oxide’s activity as a vasodilator (Moncada, Herman & Vanhoutte, 1987), its actions at the mitochondrion which alter metabolic rate (Brown, 1995) and its role in neuronal injury and neuroprotection (Beckman, 1991). A recent investigation demonstrated that in the late-gestation sheep fetus, inhibition of basal release of nitric oxide by L-NAME infusion results in an increase in mean arterial pressure (MAP), presumably by removing the vasodilatory effects of NO in peripheral resistance vessels (Green et al., 1996). This study also indicated that the increased cerebral blood flow observed during hypoxaemia in the near term fetal sheep is mediated by an increase in NO synthesis. After an hypoxic episode ends, there may be two periods of increased cerebral vasodilation. The first occurs immediately after the insult and may last between 1 and 2 hours (Marks et al., 1996). The second begins approximately 12 hours after the end of hypoxia and is associated with fetal seizure activity and intracellular oedema (Williams, Gunn and Gluckman, 1991), decreased ATP/Pi ratio with phosphorus MRS (Lorek et al., 1994) and subsequent brain injury (Wyatt et al., 1989). Of these two reperfusion phases, NO seems to be involved in mediating the secondary phase of reperfusion, but not the early phase, which may be attributable to an increase in fetal MAP whilst autoregulation of fetal cerebral blood flow is impaired.

1.4.2 Endocrine responses to hypoxia

Whilst the initial bradycardia at the onset of hypoxia can be explained in terms of neural reflex pathways, during prolonged hypoxia this bradycardia is slowly abolished (Figure 1.1, 1.2 and Giussani et al., 1993) suggesting that slower-acting mechanisms may also be active during hypoxia. Carotid denervation may abolish the initial bradycardia and increase in peripheral resistance seen at the start of hypoxia (Giussani et al., 1994), but there is still a late-onset rise in peripheral resistance (Giussani et al., 1993). This suggests endocrine influences during hypoxia.

The obvious endocrine candidates to mediate slower cardiovascular responses to hypoxia are those with known vasoactive properties in the adult; catecholamines, cortisol, angiotensin II and AVP. From studies with α-adrenoreceptor blockade, hypertension during hypoxia is believed to originate from α -adrenergic
stimulation (Lewis et al., 1980; Reuss et al., 1982) with some contribution to increased peripheral resistance from increased AVP (Rurak, 1978; Akagi et al. 1990).

Figure 1.8 Hypoxia stimulates both carotid chemoreflex and hormonal responses. Catecholamines are released from the adrenal medulla in response to fetal hypoxia and act to increase femoral vascular resistance and to increase fetal heart rate.

The adrenal medulla is the source of most of the catecholamines circulating in the fetus (Jones et al., 1988) which act to initiate and co-ordinate the metabolic and cardiovascular responses discussed above. In late-gestation, this release of catecholamines from adrenal medulla (Figure 1.8) is primarily mediated by splanchnic cholinergic nerves (Jones et al., 1988). However, before the development of functional innervation the adrenal gland is still capable of secreting catecholamines in response to hypoxia and this suggests the presence of oxygen-sensitive cells in medullary tissue (Rychkov et al., 1998). Hypoxic stimulation acting via autonomic nerves causes the circulating levels of adrenaline and noradrenaline to rise between 50 to 100-fold during hypoxia (Jones & Robinson, 1975), an effect which is abolished by adrenalectomy or chemical sympathectomy (Jones et al., 1988).

Cortisol levels are lower in the fetus than in the mother (Shepherd et al., 1992), but increase over gestation and may progressively inhibit the CRH-elicited ACTH rise during hypoxia by acting at the level of the pituitary (Akagi, Berdusco and Challis, 1990). Some investigators have reported an increase in plasma cortisol during hypoxaemia (Akagi et al., 1990; Sug-Tang et al., 1992; Giussani, McGarrigle, Moore,
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Bennet, Spencer & Hanson, 1994) whilst others have not (Shepherd et al., 1992). These differences may be a function of the severity of the insult used. In addition to its function as a vasoconstrictor, cortisol may modulate the responsiveness of the cardiovascular system to other vasoactive hormones (Walker et al., 1992; Tangalakis et al., 1992) and alter gene expression to elicit more generalised adaptations to hypoxic stress.

Angiotensin II levels in the late gestation fetus are in the same range as those in the maternal circulation, although plasma renin activity in the fetus is much higher (Broughton-Pipkin et al., 1974). Hypoxia induces rises in both plasma renin activity and in angiotensin II levels in the fetal circulation, but only renin activity in the maternal circulation.

Experimental studies of the endocrine responses to hypoxaemia show that many of the hormone levels peak at least two hours after the onset of sustained hypoxia (Hooper et al., 1990; Challis et al., 1986) and may either remain high (PGE$_2$) or may return to pre-insult levels if the hypoxia is sustained (AVP, adrenaline). This emphasises that the functional significance of hormonal responses to hypoxia may lie in maintaining and augmenting the initial chemoreflex responses, but that indefinite cardiovascular redistribution may not be in the long-term interests of the fetus.

In addition to its role in altering gene expression (e.g. POMC), plasma arginine vasopressin (AVP) levels also increase in response to fetal hypoxia-ischaemia (Raff, Kane & Wood, 1991; Rurak, 1978) and AVP is a known vasoconstrictor when infused exogenously (Iwamoto et al., 1979; Pérez et al., 1989), indicating that this hormone is also an important mediator of the peripheral vasoconstriction which develops during hypoxia.
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Other vasoconstrictor systems stimulated by hypoxia-ischaemia include those for neuropeptide Y, prostaglandins and endothelin-1. The combined action of these systems maintains peripheral vasoconstriction to vascular beds including those to the lung, kidney, gut, liver and spleen. This is an extensive area of research and a detailed discussion is beyond the scope of this introduction.

1.4.3 Metabolic responses to hypoxia

Unlike that of the adult, fetal metabolism is geared towards tissue accretion and maturation in preparation for birth (Bell et al., 1986), remaining at a constant percentage of the increasing fetal O₂ consumption between mid and late gestation (Figure 1.10; Jones et al., 1982; Chesler & Himwich, 1944). However, like the adult, there is an absolute requirement for oxidative metabolism to achieve these ends. The fetus, especially in early to mid-gestation, has a much lower metabolic rate than its adult counterpart. Yet, the fetus is capable of withstanding prolonged periods of profound hypoxia and anoxia to which the adult is intolerant. I have already described how the fetal responses to hypoxic challenges result in cardiovascular redistribution in favour of tissues whose metabolism requires the most urgent protection – the heart, adrenal glands and brain.

The fetus also responds to hypoxia in a way which reduces tissue oxygen demand. This response makes teleological sense since the duration of the hypoxic episode cannot be predicted and the fetus does not have oxygen stores per se. Thus, if the fetal oxygen demand stays high and the hypoxia is extended,
tissue PO$_2$ will fall below the level at which metabolic rate can be sustained and cellular energy stores will become depleted; this is a circumstance which can only result in death of the most susceptible cells and is a disaster for a fetus and adult alike. Metabolic demand can be curtailed in a number of ways which will be examined below.

![Graph showing cerebral O$_2$ consumption across different stages of development](image)

**Figure 1.10** Development of ovine CMRO$_2$. Brain metabolism increases with gestation, reaching a peak after birth before slowly falling to adult levels. *Adapted from Jones et al., 1982 and Gleason et al., 1989.*

### 1.4.3.1 Behavioural adaptations

In late gestation, the ovine fetus experiences periods of active and quiet "sleep" associated with low and high voltage electrocortical states, respectively (Prechtl, 1974). Fetal breathing and body movements develop with gestation and, when electrocortical states become differentiated at ca. 120 days gestation, these activities are highest in the low voltage electrocortical state (Dawes, 1973; Walker *et al.*, 1984). During normoxia, these movements account for about 17% of fetal basal metabolic rate (Rurak & Gruber, 1983a & b; Wilkening & Boyle, 1990; Berger *et al.*, 1994) and thus can result in a substantial energetic saving to the fetus when they cease in high voltage state. In late-gestation, there is also a rapid decrease in both fetal breathing and body movements at the onset of hypoxia which result in a fall in oxygen consumption (Bissonnette, Hohimer & Willeke, 1989).

### 1.4.3.2 Cellular adaptations

Although it is generally stated that the primary purpose of fetal cardiovascular adaptive responses to hypoxia is to protect the function of essential fetal organs (the heart, brain and adrenal glands), this may be only partially true *in vivo*. In recent years it has been reported that, *in vitro* at least, some fetal tissues
are capable of actively reducing their metabolic rate in response to hypoxia (Braems & Jensen, 1991; Chandel et al., 1997; Budinger et al., 1998; Clementi et al., 1999). This experimental observation provides support for the theoretical argument that, during acute severe reduction in fetal oxygen delivery, cellular metabolism cannot be maintained at control levels and some reductions in metabolic rate must occur (Arai et al., 1991).

1.4.3.3 Myocardial metabolism

Data indicates that fetal sheep myocardium metabolises both glucose and lactate, with some small contribution from ketone bodies and free fatty acids (Bartelds et al., 1999). These substrates subserve a myocardial metabolic rate which is not significantly different to that of the adult (Fisher, Heymann & Rudolph, 1980) but which operates at a much lower \( P_aO_2 \) and arterial \( O_2 \) content, necessitating either a higher myocardial blood flow (Fisher et al., 1980) or an increase in myocardial efficiency.

In vitro work indicates that in the low-oxygen fetal environment, myocyte mitochondria are able to respond to their relatively low operating \( PO_2 \) with a reduction in contractile function initiated by mitochondrially-generated reactive oxygen species (Budinger et al., 1998). This metabolic suppression can be augmented during de facto hypoxia such that further reductions in energy requirements, ATP demand and contractile function can be achieved without any observable energy depletion or ischaemia and the increases in lactate production that that would entail.

Of course, these adaptations are only possible when reductions in myocardial oxygenation are moderate or short-term. Severe reductions in myocardial flow for longer than 20 minutes are reported to be associated with loss of contractile function leading to irrevocable loss of function (Heusch & Schulz, 1996) and myocardial necrosis. There is no histological evidence for irrevocable myocardial infarction following experimental hypoxic challenges.

1.4.3.4 Cerebral metabolic adaptations to experimental hypoxia

Whilst evidence for metabolic adaptation in cardiomyocytes comes from in vitro studies, data for cerebral hypoxic adaptation comes mainly from in vivo work (Parer, 1994). Electrocorticogram evidence from experiments using chronically instrumented fetal sheep indicates that the fetal brain undergoes a progressive reduction in neuronal activity during mild to severe hypoxia. During moderate hypoxia, the electrocorticogram of the late gestation-fetal lamb switches from low voltage, high frequency activity to high voltage, low frequency waves. High voltage; Gun et al., 1992 is associated with non-REM sleep in the fetal lamb and is indicative of a low oxygen consumption state (Richardson, Patrick and Abduljabbar, 1985). Further increases in the severity of hypoxia cause the ECoG to become isoelectric (Boddy et al., 1974; Ball et al., 1994; Mallard et al., 1992 & 1994; Bennet et al., 1998, 1999 & 2000), and this state can
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Persist for some time after the cessation of the original insult (Bennet et al., 1999). ECoG abnormalities may persist into neonatal life, providing post hoc markers for brain injury (Watanabe, Hayakawa & Okumura, 1999; Connell et al., 1987).

Metabolic data from fetal brain studies indicates that moderate hypoxia or asphyxia can be accommodated for with increases in cerebral blood flow and/or fractional oxygen extraction (Purves et al., 1969; Jones et al., 1977; Cohn, Heymann & Rudolph, 1974; Peeters et al., 1979; Johnson et al., 1979; Gleason, Hamm & Jones, 1989). Under these conditions, reductions in cerebral oxygen consumption are small compared to the reduction in arterial oxygen content (Figure 1.11 from Field, 1990). However, during severe or prolonged asphyxia, reductions in cerebral oxygen consumption are observed.

Acute severe reduction in oxygenation, such as that seen with complete arrest of uterine flow or complete umbilical cord occlusion, is not associated with a decrease in cerebrovascular resistance, as is seen in more mild insults. The rate at which the fetal arterial O2 content falls may be a determinant of the response elicited — a rapid onset of ECoG isoelectricity (Bennet et al., 1998), reduced regional cerebral blood flows (Jensen et al., 1989), a failure of cardiovascular adaptive responses (Block et al., 1990), an increase in cerebral lactate production (Ikeda et al., 1998) and a 50% reduction in cerebral metabolic rate (Jones et al., 1990). It is under such acute conditions as these that failure to maintain cerebral ATP levels is seen with nuclear magnetic resonance spectroscopy in neonates (Rutherford et al., 1996) and recently in the instrumented fetal sheep in utero (Sibony et al., 1998; Van Cappellen et al., 1998 & 1999). These conditions are believed to lead on to oxidative stress during reoxygenation and a subsequent secondary energy failure which progresses on to neuronal injury in susceptible cell populations.

By contrast, the late-gestation fetus can accommodate for a slowly developing hypoxia for several hours (Richardson et al., 1989). Metabolic rate reduction under these conditions may be influenced by increases in the rate of nitric oxide production. In vitro studies of cardiomyocytes, vascular smooth muscle cells and hepatocytes have shown that there are reversible reductions in metabolic rate in cells held at a PO2 of 40mmHg or less for 2 hours or more (Chandel et al., 1997; Clementi et al., 1998 & 1999). This reduction in O2 consumption may be the result of a functional change in cytochrome oxidase with prolonged hypoxia (Chandel et al., 1997). However, with severe reductions in O2 availability, increased NO production may begin an irreversible nitrosylation of thiol groups on mitochondrial complex I (see Section 1.4.4.1) which could result in permanent decreases in mitochondrial function, and thus in O2 consumption (Clementi et al., 1998).
1.4.4 Mechanisms of metabolic inhibition

1.4.4.1 Nitric oxide

As mentioned in Section 1.4.1.6.1, nitric oxide (NO) is a freely diffusible free radical gas that can be generated as needed from L-arginine by three different NO synthase (NOS) isoforms; nNOS, eNOS or iNOS. Whilst NO has a number of cellular targets (Figure 1.6), its interaction with mitochondrial electron transfer chain complexes suggests that NO is able to modulate metabolic rate in some fashion.

Several recent publications have reported NO’s ability to interact with mitochondrial complexes I, III and IV (cytochrome oxidase, CcO). The most rapid interaction appears to be that of NO with the O₂ binding site on mitochondrial complex IV during acute increase in NO levels. This competitive inhibition of CcO thus reduces mitochondrial O₂ consumption in a concentration dependent manner (Clementi et al., 1999). The mechanics and kinetics of the interaction of NO with the CcO binuclear centre responsible for the reduction of molecular O₂ to water has been described previously (see Torres, 1998 a & b and Brown, 1999 for review). For the purposes of this thesis it is relevant that moderate acute increases in NO levels results in competitive inhibition of CcO turnover, resulting in a reduction in the rate of transfer of electrons from the electron transfer chain to O₂. In effect, electrons are held at metal centres for an increased period of time, and the transfer chain becomes more reduced.

Figure 1.11 Shows how there is relatively little change in cerebral oxygen consumption over a wide range of fetal arterial oxygen contents (1.5 - 3.5 mM) in the late-gestation fetal sheep in utero. From Field et al., 1990.
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The question as to whether these effects of NO on mitochondrial respiration are functionally relevant is addressed in part by *in situ* hybridisation studies (Figure 1.12) in fetal and adult animals and humans (Ohyu & Takashima, 1998; Northington et al., 1997). From these studies, it appears that total NOS activity is low at 0.48 of gestation, but increases rapidly to 0.75 of gestation, when NOS activity is not different to adult levels in the frontal lobe and hippocampus. By 0.92 of gestation, all tested areas of the sheep brain have total NOS activity which is not different to that of the adult. Northington and colleagues do not provide statistical interpretation of their nNOS-immunoreactive cell density data, however, it appears that there is a biphasic expression of nNOS is the brain areas illustrated, with increases towards mid-gestation falling to adult levels over time (Figure 1.12, lower panel). So these two studies indicate that nNOS is present and that NOS activity is also present. The different developmental characteristics of these variables suggests that more than one NOS isoforms is present during development *in utero*.

However, in addition to the mere *presence* of NO in a cell, it is probably necessary for NOS to be localised *within* mitochondria to have full effectiveness in modulating metabolic rate, by creating microdomains of elevated NO which would not affect the other systems illustrated in Figure 1.6. One study using cultured cell lines has demonstrated such localisation (López-Figueroa et al., 2000). Using separate fluorescent markers for mitochondria (MitoTracker Red CM-H$_2$Xros; 10-100nM) and NO (10µM DAF-2/DA for 10 min at 37°C), this group demonstrated colocalisation of dye indicating the presence of NO within mitochondria, thus suggesting active physiological and pathophysiological roles for NO.
1.4.4.2 Adenosine

Adenosine is a breakdown product of adenine ribonucleotides (Figure 1.13). Present in the extracellular space and plasma at low concentration, adenosine has potent cardiovascular effects, effected through three ubiquitous classes of adenosine receptor; A1, A2 and A3. These receptors in turn couple to either stimulatory or inhibitory cGTP-binding components of adenylate cyclase, or to similar proteins modulating Ca\(^{2+}\) or K\(^+\) channels. The adenine nucleotides, including adenosine, are also intimately involved with energy metabolism. ATP regulates its own formation by negative feedback inhibition and is an activator of enzymes of purine degradation (AMP deaminase & 5' nucleotidase), whilst ADP and AMP are regulators of mitochondrial and substrate-level phosphorylation (Berne, Rall & Rubio, 1983). The presence of plasma membrane transporters for adenosine indicates that it, like cAMP, may be involved in intracellular
communication – perhaps as a signal of cellular energy status. Since adenosine is rapidly broken down in the extracellular space, it is likely that such signalling is autocrine and, perhaps, paracrine.

Although the functions of adenosine may seem at first to be disparate, they in fact form part of a complex system whose function appears to be the matching of energy supply to demand. These actions include:

(a) Regulation of organ blood flow. Adenosine increases flow to myocardial, cerebral and skeletal muscle vascular beds. It causes vasoconstriction in the renal vascular bed.

(b) Inhibition of cell activation. In addition to the inhibition of platelet activation, adenosine acts to hyperpolarize neurones, which reduces the likelihood of achieving the threshold necessary to initiate an action potential, reducing energy demand.

(c) Inhibition of those catabolic hormones which act to raise [cAMP]

(d) Promotion of insulin’s anabolic actions

(e) Modulation of fetal breathing.

The importance of adenosine to the fetus is not known. However, fetal plasma adenosine levels are higher than those of the mother (Koos & Doany, 1991) at approximately 1μM, whilst cisterna magna levels are reported to be 0.6μM (Winn, Rubio & Berne, 1981) and these levels increase even further during hypoxia, reaching 3-4 μM (Koos & Doany, 1991). The inverse relationship between P₂O₂ and adenosine concentrations has been demonstrated in the adult P₂O₂ range by Mentzer and colleagues (Mentzer et al. 1985) and in the fetal range by Koos and Doany (Koos & Doany, 1991), as shown in Figure 1.14.

Assuming that the fetal receptor and transduction systems for adenosine are intact, the relatively high fetal plasma concentration of adenosine would suggest higher tonic effects of adenosine during intrauterine life. This would, presumably, mean a chronic vasodilated state in many vascular beds and a tonic (although mild) inhibition of energy metabolism; effects which could be enhanced during hypoxic stimulation of adenosine levels.

In fetal neurones, these inhibitory actions of adenosine are, at least in part, effected through its ability to hyperpolarize neuronal plasma membranes (Dunwiddle & Fredholm, 1989) and to reduce excitatory neurotransmitter release (Dolphin & Archer, 1983; de Mendonca & Ribeiro, 1993), shifting the balance of neurotransmitter release in favour of the inhibitory transmitters. These effects on neurones may, either solely or in conjunction with direct effects on neurones, be responsible for the reduction in fetal breathing and body movements seen with hypoxia and during exogenous infusion of adenosine or adenosine analogues (Boddy et al., 1974; Walker, 1984; Runold, Lagercrantz & Fredholm, 1986; Runold et al., 1989; Koos & Doany, 1991; Yamamoto et al., 1994) and thus may contribute to brain temperature regulation (Anderson, Sheehan & Strong, 1994), although this point is not supported by all experimental evidence.
Figure 1.13 Pathways of adenosine formation. Reproduced from Newby et al., 1990.

Figure 1.14 Relationship between arterial O₂ content and plasma adenosine concentration in the near-term fetal sheep in utero. Under normal fetal oxygenation, [adenosine] remains constant at approximately 1 µM; [adenosine] rises with falling oxygenation, reaching 3-4 µM. Reproduced from Koos & Doany, 1991.
available to date (Suzuki & Power, 1999). A shift away from excitatory to inhibitory neurotransmitters may also be responsible for the decreased incidence of low-voltage electrocortical activity seen during exogenous adenosine infusion (Koos & Doany, 1991; see also Section 5.3). All of these effects of hypoxia and adenosine supplementation would act to reduce fetal energy demand.

1.5 Outcome of hypoxia-ischaemia in preterm infants

Even as long ago as the early 19th century, William Little had noted the link between prematurity and motor and intellectual deficits in infants surviving premature birth. Infants born prematurely are, by definition, not fully adapted for conditions outside the womb. This immaturity has meant that, historically, premature infants had poor prognoses and, as with the infants described by Little, many died soon after birth. One of the major problems with such premature fetuses was respiratory distress caused by lung immaturity. With the development of maternal glucocorticoid treatments (Pender, 1976), early lung maturation is possible in fetuses likely to be delivered early and this, along with advances in neonatal intensive care, have led to increases in survival rates for premature infants (Bernstein et al., 2000). However, these infants are also at risk of neuronal injury in the neonatal period.

The common lesions in the premature infant brain are periventricular haemorrhagic infarction (PVH) and periventricular leucomalacia (PVL) (Volpe, 1995). The efficacy of modern post partum assessment and monitoring techniques to adequately predict those premature infants at risk of long-term neurological sequelae arising from these lesions is poor. The scheme of Samat (Samat & Samat, 1976), for instance, uses observation of neurological indicators (including level of consciousness and the presence of seizures) to provide a score of hypoxic-ischaemic encephalopathy in the term infant and is not predictive of neurological outcome in the premature infant. The Apgar scoring technique (Apgar, 1953) assesses the health of the term infant immediately after birth but is not predictive of neurological outcome and, again, is inadequate for the preterm infant.

These measures are, of course, only available once the infant has been delivered and cannot be used as indicators of in utero fetal distress. Ultrasound scanning is the only commonly available method for real-time imaging of the fetus during labour and at the cot-side after labour. Unfortunately, this technique is currently only able to detect injuries once they are well established and even then the specificity of the technique is low (Hope et al., 1988). This can be seen in one such example of a prospective study aiming to predict brain injury from ultrasound scanning. A study performed at UCL in 1979-80, monitored very low birth weight (VLBW) infants (<1250g) plus those requiring mechanical ventilation (Stewart et al., 1983).
This study found that the ability of ultrasound scanning to successfully predict good outcome on the basis of a normal scan was good (positive prediction 85%), but the ability to predict poor outcome based upon an abnormal scan was low (59%). This may be due to numerous factors:

- The resolution of some ultrasound scanners may be inadequate to detect small abnormal echodensities or echolucencies.
- The appearance of injured tissue on ultrasound scan changes as the lesions develop and regular (daily) serial scans may be required for adequate diagnosis.
- Intra- and inter-observer variability in detecting lesions
- Some types of injury are diffuse, not focal, and are unlikely to be imaged by ultrasound scanners presently available.

Since it is believed that hypoxic-ischaemic damage may be caused antepartum in about 25-30% of cases of preterm delivery (de Vries et al., 1998; Murphy et al. 1996) and intrapartum in a significant minority of term labours where the infant goes on to develop motor and/or intellectual deficits (McCormick, 1989), there is an obvious need for a non-invasive technique which can adequately assess fetal well being whilst in utero and accurately indicate where a fetus is in relation to the point at which neurological damage is inevitable. In order to be able to do this we need (A) a better understanding of the susceptibility of the fetus at each stage of gestation to neuronal injury, and (B) accurate indicators of the progression towards brain injury which can be detected at a time when rescue therapies can still be applied. A summary of the relevant types of brain neuropathology is given below.

1.5.1 Intracranial haemorrhages

The common intracranial haemorrhages identifiable at post mortem which are associated with preterm birth are, in no particular order, germinal matrix-intraventricular haemorrhage and periventricular haemorrhage. These may be present singularly, or together.

1.5.1.1 Germinal matrix-intraventricular haemorrhage

1.5.1.1.1 Locus of origin

Intraventricular haemorrhage appears to be due to a primary bleed in the germinal matrix ventrolateral to the lateral ventricle (De Reuck, 1984), possibly due to rupture of the fragile vascular rete of the germinal matrix in early to mid gestation (Greisen, 1986; Meek et al., 1999). Towbin (Towbin et al., 1968) explained this rupture as the result of venous stasis resulting from systemic circulatory failure causing a thrombosis in the germinal matrix, leading to IVH. Others (e.g. Meek et al., 1999) have explained GMH-IVH in terms of fluctuations in cerebral blood flow or arterial blood pressure. De Reuk discussed these themes and
explained the consistent distribution of GMH-IVH in terms of the maturational susceptibility of primitive vascular beds in the germinal matrix and subependymal regions.

In early embryonic stages, the germinal matrix forms the entire wall of the cerebral vesicles. This entire area is fed by a dense primitive vascular rete (thin-walled vessels) connected to the surface of the cerebral vesicles by large channels. At around the third month of gestation, the germinal matrix and its associated rete begin to regress. This begins at the third ventricle, then at the posterior parts of the temporal horn of the lateral ventricle and, finally, from around the external angle of the anterior part of the anterior horn of the lateral ventricle (Figure 1.15). The final regression of primitive rete around the external angle of the lateral ventricles occurs just before term and thus the time scale of the regression may provide evidence why GMH-IVH remains common in preterm but not term infants.

One area especially vulnerable to haemorrhage is that slightly anterior to the head of the caudate nucleus (Figure 1.16), close to the foramen of Monro (Volpe, 1995). Bleeding into the foramen of Monro is a common effect of GMH.

**1.5.1.1.2 Predictive Value**

If, as scientists and clinicians, our ultimate aim is to prevent death and neuronal injury in infants, we must identify markers of morbidity. Many studies have been published providing models which can be used in the postnatal prediction of those infants at risk of developing brain lesions whilst our understanding of the events underlying neuronal injury remain incomplete. Part of the problem may be in the different descriptive systems in use for describing brain injury. For example, Papile's IVH terminology (Papile et al., 1978) divided IVH into four categories which were supposed to describe the "varied natural history of IVH". Other authors (Kuban et al., 1999) have used the term "white matter damage", an umbrella term covering a range of lesions.
Figure 1.15 3D schematic of the ventricles of the brain from anterolateral (upper panel) and from below (lower panel). The terminology for ventricular regions given in this diagram will be used throughout this thesis. The parts of the lateral horn shown are; body, anterior horn, atrium and temporal horn.

Figure 1.16 Diagram showing caudate nucleus (green) in close relation to the lateral ventricles (light blue). The foramen of Monro is medial to the head of the caudate nucleus and is a common site for blockage after germinal matrix – intraventricular haemorrhage.
There is a great deal of confusion in the literature as to how predictive germinal matrix-intraventricular haemorrhage is for subsequent neurological impairment. Older, pre-ultrasound publications were based on autopsy correlations or CT scanning and indicated that a strong positive correlation existed between IVH and PVL (Papile et al., 1978). Most later work with real-time ultrasound imaging has led to the current stance, which is that GMH-IVH without parenchymal involvement or ventricular enlargement is not predictive of later white matter damage. Any substantial degree of parenchymal involvement is, however, highly likely to cause neurological dysfunction, ranging from mild to severe spasticity with or without intellectual deficits and is currently fatal in around 50% of cases (Pidcock et al., 1990; Paneth et al., 1990; Roth et al., 1992; Pinto-Martin et al., 1995). However, there are still some publications which report increased risk of white matter damage following IVH (Kuban et al., 1999).

Some evidence has recently surfaced which raises the question as to which direction the aetiological arrow points, since WMD has been suggested to be present before IVH in a recent study (Inder et al., 1999), and demands a rethink of how WMD may be occurring. Without adequate, precise nomenclature based in knowledge of the antecedents of each type of damage, we are unlikely to progress in our knowledge of those lesions currently imaged by ultrasound. Some lesions are not imaged with ultrasound but which are often seen at post mortem. These lesions, such as diffuse white matter injury and infarcts in basal ganglia and cerebellum may be imaged with techniques like diffusion-weighted MRI (Cowan et al., 1994). Our knowledge of these lesions is poor indeed, and we have a long way to go before any progress can be made in preventing these particular lesions.

1.5.1.2 Periventricular haemorrhage

1.5.1.2.1 Location and terminology

Periventricular haemorrhage (PVH) is the most common neonatal intracranial haemorrhage and is usually associated with prematurity (de Vries et al., 1998). The lesion is usually highly asymmetrical and consists of haemorrhagic necrosis along the external angle of the lateral ventricles and, as such, is dorsal and lateral to the lateral ventricle. The area of necrosis has been described as fan-shaped; the area affected by this lesion is roughly outlined in Figure 1.17, below.
Figure 1.17 Brain from a human infant born at 24 weeks gestation who died shortly after birth. Note the highly asymmetrical intraventricular haemorrhage and ventricular dilation. The dashed line roughly traces the usual location of periventricular haemorrhagic infarction.

Figure 1.18 Schematic representation of the medullary veins draining the cerebral white matter. Compare with the location of PVL traced in Figure 1.16. PVH is mainly present at the external angle of the lateral ventricle (LV) where medullary veins converge. The terminal vein, germinal matrix and foramen of Monro are shown for information purposes.
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Papile and colleagues (Papile et al., 1978) provided terminology to describe PVH which is still in common use today, but which is problematic. Papile's scheme classifies this haemorrhage into four levels, where each level includes the signifiers in all the levels below it. This scheme is:

Grade I  Haemorrhage confined to the germinal layer or occupying less than half of one or both ventricles
Grade II Haemorrhage occupying more than half a lateral ventricle but not distending it
Grade III Haemorrhage distending any part of a lateral ventricle
Grade IV Haemorrhage extending from the region of the germinal layer into the brain tissue with or without IVH

Unfortunately, this scheme leaves no room for such entities as isolated ventricular enlargement or parenchymal haemorrhage without ventricular involvement; i.e. they are not part of Papile's "natural history of intraventricular haemorrhage". The terminology used in the literature to describe haemorrhage and white matter damage is not uniform and can be somewhat vague, reflecting, perhaps, the difficulties of using non-invasive techniques to diagnose these lesions (see, for example, Kuban et al., 1999) and the assumption which is not always clearly stated, that ultrasound abnormalities are being interpreted as particular neuropathological entities (Nwaesei et al., 1984).

1.5.1.2 Aetiology of periventricular haemorrhage

Microscopic studies, such as the one by Gould and co-workers (Gould et al., 1987) have shown that PVH is not necessarily a simple extension of existing germinal matrix or intraventricular haemorrhage into the parenchyma of the periventricular white matter, but instead that this is a primary venous haemorrhagic infarction. This idea is supported by the separate study of de Reuk (de Reuk, 1984), which emphasises the development of the angioarchitecture of the area. By comparing Figures 1.17 and 1.17, we can see that the area most affected by PVH is also the location of the confluence of the cerebral medullary veins draining the cerebral white matter. These veins converge to form a terminal vein which is prone to haemorrhaging near the foramen of Monro (Volpe, 1995). This site is particularly susceptible to haemorrhage into the ventricle. Primary haemorrhage in the periventricular region is distinguishable histologically from secondary bleeding into pre-existing periventricular leucomalacia (see later), but may not be distinguishable on cranial ultrasound (Hope, 1988).

However, the main contributory factor in many cases of PVH appears to be germinal matrix-IVH; it has been reported that some 80% of cases of periventricular haemorrhage also present with germinal matrix-IVH (Guzetta et al., 1986), although as with other types of injury in these infants, the two may merely share common antecedents. One theory of evolution of PVH is given below (Figure 1.19). Put simply, this suggests that germinal matrix-IVH leads to congestion in the terminal vein and ischaemia in the
periventricular white matter, with subsequent (haemorrhagic) infarction at the external angle of the lateral ventricle.

Figure 1.19 Theoretical scheme showing development of periventricular haemorrhagic infarction. Whilst not an “extension” of germinal matrix haemorrhage per se, PVH may often be a consequence of GMH-IVH.

Figure 1.20 Descending corticospinal tracts passing through or close to the location of periventricular haemorrhagic infarction determines the degree of motor and/or intellectual deficit in the patient.
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1.5.1.2.3 Clinical correlates

Unfortunately, these theories of the development of PVH are not substantiated by experimental technique or by indirect observations, such as ultrasound imaging or computed tomography. The clinical correlates of PVH are, however, well documented with many retrospective and prospective studies having been published describing the neurological associations of PVH. The main clinical manifestations of PVH are a spastic paresis affecting the lower extremities more than the upper (i.e. spastic quadriplegia or spastic hemiparesis) with or without intellectual deficits. Figure 1.20 shows the approximate locations of descending fibres from the primary motor cortex in relation to PVH distribution; this should be considered in conjunction with Figure 1.18, which shows the locations of medullary veins near the external angle of the lateral ventricles. These diagrams show that the descending motor fibres from the primary motor cortex pass through the areas most often damaged by PVH in the premature infant. The degree of extension of the lesion into the periventricular parenchyma determines the degree of motor and/or intellectual deficit, with greater involvement of the upper limbs and intellect with greater extension of the haemorrhage into the parenchyma (Volpe, 1995).

1.5.1.3 Periventricular leucomalacia

The other common lesion of immaturity is periventricular leucomalacia, a characteristic necrosis of white matter with a distribution similar to that of periventricular haemorrhagic infarction, i.e. dorsal and lateral to the external angles of the lateral ventricles. In recent years, in addition to this overt form of lesion which, in time, becomes cystic in character, a diffuse PVL injury with a wider distribution has also been described (Inder et al., 1999).

1.5.1.3.1 Diagnosis and evolution of injury

As with PVH, this is typically a lesion of the preterm infant and of those suffering respiratory and/or cardiovascular distress with an incidence which is inversely related to gestational age (Zupan et al., 1996). Diagnosis in surviving infants is performed with cranial ultrasound through the anterior fontanelle (Cooke, 1987 & 1989; Costello et al., 1988; de Vries et al., 1998; Eken et al., 1994; Fowlie et al., 1998; Hansen & Leviton, 1999; Hope et al., 1988; Kuban et al., 1999; Lai & Tsou, 1999; Nwaesei et al., 1984; Paneth et al., 1990; Pinto-Martin et al., 1995; Rodriguez et al., 1990; Trounce, Fagan & Levene, 1986), and this injury has a characteristic bilaterally symmetrical appearance. Initially, the lesion appears as a group of abnormal echodensities along the external angle of the lateral ventricle or, at post mortem, as focal coagulation necrotic lesions at the end zones of the long penetrating arteries. Over a period of days to weeks, these abnormal echodensities evolve into many individual echolucencies. These echolucencies are characteristic of focal PVL and have been confirmed by post mortem examination of non-surviving infants to be small cysts in the periventricular white matter (De Reuck, Chattha & Richardson, 1972).
The "diffuse component of PVL" has been identified using diffusion-weighted magnetic resonance imaging (MRI) (Inder et al., 1999) and appears as a widespread area of decreased apparent water diffusion coefficient lateral and posterior to the lateral ventricles (Figure 1.21). This lesion is poorly imaged by ultrasound imaging and appears before both focal WMD and signs of haemorrhage, suggesting that substantial abnormalities of white matter occur very early after birth and questions the direction of the causal arrow between IVH and WMD.

**Figure 1.21** Diffusion-weighted MRI image showing bilateral marked restriction of water diffusion in the periventricular white matter (arrows) at 7 days post partum. There were no abnormalities visible on cranial ultrasound taken on the same day. *From Inder et al., 1999*

### 1.5.1.3.2 Antecedents of PVL

The debate surrounding the factors contributing to PVL has been ongoing since the techniques of light microscopy first emerged into common practice in the mid 19th century. In the sections below I will look at the features believed by the majority of workers to be likely to contribute significantly to PVL in preterm infants.

### 1.5.1.3.3 Loss of cerebral pressure autoregulation and changes in cerebral blood flow

It has been known for some time that the distressed newborn infant does not have functional cerebral blood flow autoregulation (Lou et al., 1979a & b). Without autoregulation, such infants are susceptible to hyperperfusion during episodes of increased arterial pressure and to periods of ischaemia during
hypotensive episodes. These fluctuations in cerebral flow are believed to be one of the major causative agents in the aetiology of periventricular leukomalacia.

1.5.1.3.4 Antepartum infection

Periventricular leukomalacia is reported to be the single most common cause of cerebral palsy in infants surviving preterm birth (Kuban & Leviton, 1994). Since there has been no progress in producing positive correlations between the development of PVL and postnatal events (Bejar et al., 1988a & b), attention has recently turned to antepartum factors in the search for the antecedents of CP. Over the past ten years, there has been increasing interest in the role of antepartum infection in the development of cerebral palsy, fetal morbidity and mortality immediately post partum (Leviton, 1993 & 1999; Murphy et al., 1995; Grether & Nelson, 1997; Alexander et al., 1998; Morales, 1987; Hardt et al., 1985; Dexter et al., 1999; Bejar et al., 1988; Baud et al., 1999). There is an almost uniform agreement that maternal infection, premature rupture of membranes, chorioamnionitis, premature birth and subsequent death or appearance of motor and/or intellectual deficits in surviving infants are a linked cycle of events encompassing a progressive pathophysiology (Figure 1.22). Only in one recent study has the link between chorioamnionitis and short-term neonatal neurologic sequelae been refuted (Alexander, McIntyre & Leveno, 1999). However, the absence of histologic chorioamnionitis is not a guarantor of the absence of the inflammatory cascade which is likely to be the mediator of white matter injury in these preterm infants. Indeed, my own data presented in Chapter 7 shows that severe white matter injury can be produced without histologic evidence of chorioamnionitis.

Figure 1.22 Leviton's Scheme for the progression from maternal intrauterine infection to either preterm birth. PVL and cerebral palsy. Note the importance placed upon the action of TNF. From Leviton, 1993.
1.6 Hypoxic ischaemic encephalopathy in term infants

Unlike PVL and GMH-IVH, hypoxic-ischaemic encephalopathy is generally understood to be a lesion typical of the term infant subjected to an acute reduction in cerebral oxygen delivery leading, in the severest cases, to widespread cerebral metabolic disruption. This metabolic disruption and the reperfusion phase which may follow it are implicated in widespread apoptosis and necrosis of vulnerable cell populations (Volpe, 1995). Clinical studies indicate that this primary insult, where severe enough, causes widespread but not uniform cellular loss in the brain, with the exact pattern of injury depending on the maturity of the tissue and the severity of the insult.

Some studies indicate that advances in obstetric care over past years have not necessarily been accompanied by concomitant reductions in the rate of hypoxic-ischaemic encephalopathy (Westgate, Gunn & Gunn, 1999), although rates of neonatal encephalopathy have been falling in other centres (Hull &
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Dodd, 1992; Smith, Wells & Dodd, 2000). These studies are likely to have been affected by the infants available for study in tertiary centres, where high-risk infants are more likely to be delivered and thus we must regard these data with some caution. Despite this reservation, it has become apparent over the last ten years that whilst management of high risk pregnancies has resulted in falling incidence of injury in this group (Figure 1.23), many cases of HIE occur in apparently low risk patients (Westgate, Gun & Gunn, 1999). The prevention of HIE is thus hampered by the ability of the clinician to diagnose adequately those infants in which brain injury is likely to occur – whether through observer error, inadequate monitoring or poor indicators of those fetuses likely to develop HIE.

Hypoxia-ischaemia is now not believed to be the only route to encephalopathy in the neonate. For example, as already indicated, maternal fever & amniotic infection are predictive of subsequent neurodevelopmental deficits, including cerebral palsy (Hardt et al., 1985; Morales, 1987; Alexander, McIntire & Leveno, 1999; Baud et al., 1999; Dexter et al., 1999); there is also data indicating that both clotting and immune cascades are involved in the aetiology of cerebral palsy (Nelson et al., 1998) and that genetic factors may be involved in other injuries (Strater et al., 1999).

1.6.1 Early and delayed phases of injury

Nuclear magnetic resonance spectroscopy (MRS) studies of human patients and experimental animals have provided data on changes in certain markers of fetal metabolic status, namely, the ratio of phosphocreatine (PCr) to inorganic phosphate (Pi), as well as intracellular pH (pHi) and adenosine triphosphate (ATP). Like the microsphere method for blood flow, however, this system provides snapshots of the relative (not absolute) amounts of each substrate but cannot give data regarding substrate fluxes.

Sequential MRS scans have shown that at the onset of an hypoxic insult, there is a progressive decrease in [PCr]:[Pi] as PCr stores are used, presumably to buffer a maintained ATP demand. At some point, however, PCr will be exhausted and [ATP] will begin to fall. Simultaneous with these phosphagen changes, there is a fall in pHi and an increase in lactate concentration (Cady, 1990; Azzopardi et al., 1989). Cortical impedance measurements during late gestation asphyxia have demonstrated a developing cytotoxic (intracellular) oedema during these severe insults, adding to the stress on membrane functionality which can be irrecoverable.

With reoxygenation, there is a rapid but short lived hyperperfusion in some infants (see Chapter 3) and a rapid rise in cellular oxygenation; pHi and lactate levels return to normal over a few hours and phosphagen levels are quickly restored. In this way, shortly after a severe hypoxic-ischaemic insult, the infant can appear to be normal with this method. Despite this, some cells lose viability and do not recover from the insult. Indeed, a cascade of processes can have been started which begin to be manifested approximately
10 hours later, when there is a decline in [PCr]:[Pi] without any sign of impaired gas exchange, lactate levels increase and pH rises (Lorek et al., 1994). The magnitude of these changes is correlated with the severity of the primary insult and also with the ensuing brain injury (Blumberg et al., 1996). The mechanisms underlying these changes are not fully understood, although several pieces of information are available which provide a patchy picture of associated events. However, the very fact of such a long latency between the primary insult and secondary effects may well provide investigators with a therapeutic window in which treatment may ameliorate or prevent brain, and other organ, damage.

Brain damage at this gestation is not uniform, reflecting the maturity of the tissue and the method and severity of the insult. Damage has been reported in the hippocampus (Mallard et al., 1992), basal ganglia (Krageloh-Mann et al., 1995; Rutherford et al., 1996) and cortex (Reddy et al., 1998).

1.6.2 Cellular and molecular mechanisms of damage

As would be expected, the mechanism by which cerebral cell death occurs during and after hypoxia-ischaemia is multifactorial and, again, is dependent upon the maturation of the tissue and the severity of the insult. The pathological results of hypoxia-ischaemia which lead to injury in the neonate are likely to be a breakdown in plasma membrane ability to maintain transmembrane ionic gradients due to low [ATP], a rapid release of excitatory amino acids into the extracellular space and mitochondrial and Ca\textsuperscript{2+} homeostasis dysfunction. Elucidation of these events and their interactions have only begun, but are producing some interesting results.

1.6.2.1 Excitotoxins

Severe hypoxia in the late gestation fetal sheep stimulates the release of excitatory amino acids (EAA), of which the most common in the CNS are glutamate and aspartate. EAA levels rise rapidly to high concentrations in the cerebral extracellular spaces during hypoxia (McDonald, Silverstein & Johnson, 1988) and act at EAA receptors which are present on almost all CNS cells (Wong & Kemp, 1991 for NMDA receptors).

The most common of the excitatory amino acids, glutamate, binds to three classes of postsynaptic receptors which can be distinguished pharmacologically and are referred to as N-methyl-D-aspartate (NMDA), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate (KA). In addition, neurons express a class of glutamate receptor present both pre- and post-synaptically, and which are coupled to G-proteins. Activation of the first three types of receptor is associated with increases in intracellular Ca\textsuperscript{2+}. Because, compared to adult brain, that of the fetus has a relative excess of EAA receptors, it is particularly vulnerable to excess EAA which have toxic effects at high concentrations (Choi, 1994; Andiné et al., 1991; Riikonen et al., 1992), exerted both through increases in intracellular Ca\textsuperscript{2+} and
via activation of G-protein coupled receptors and subsequent alterations in cAMP and cGMP levels.

Experimental blockade of excitatory amino acid receptors performed both pre- and post-hypoxia has demonstrated reductions in infarct volumes (Clark, 1989; Andiné et al., 1988). Although the results of such studies show that blockade of single members of the four receptor types (NMDA, AMPA, KA & G-protein linked) does not provide the same degree of neuroprotection, that such neuroprotection is possible underlines the importance of these neurotransmitters in brain injury under certain circumstances.

1.6.2.2 Ca\(^{2+}\), mitochondria and cell death

Although transient depolarisations of the mitochondrial membrane potential (\(\Delta \psi_m\)) have been seen in excitable cells in response to excitation (Duchen, 1992), this is a normal result of mitochondrial Ca\(^{2+}\) uptake through its electrogenic uniporter. The depolarisation of \(\Delta \psi_m\) in response to the rise in intracellular Ca\(^{2+}\) caused by the release of excitotoxins during hypoxia can lead to mitochondrial dysfunction and cell death (Choi, 1994). It has been known for some time that prolonged mitochondrial Ca\(^{2+}\) uptake during oxidative stress may result in the opening of the mitochondrial megachannel (Lemasters et al., 1998) or mitochondrial permeability transition pore (MPT). The MPT is a disaster for the cell, since it represents certain cell death, through a number of mechanisms including energy depletion, release of cytochrome c and the initiation of pre-programmed cell death (apoptosis).

Focal apoptosis and necrosis are seen as histological outcomes of hypoxic injury at autopsy in experimental animals subjected to hypoxia-ischaemia (Shimizu et al., 1996; Yue et al., 1997). Apoptosis is a highly regulated mechanism for cell death which requires time, energy and gene transcription. Cells undergoing apoptosis have a distinct morphology associated with the manner of their demise; plasma membrane integrity is maintained, cytoplasmic volume is reduced whilst organelles are processed and packaged for phagocytosis by surrounding cells. Necrotic cell death is less organised with loss of plasma membrane integrity and extravasation of cellular contents into the extracellular space. Owing to the energy requirements of apoptosis, it is logical that the decision to begin this process occurs while the cell still has energy for completion of the task. It has recently been proposed that the difficulty in observing histologically classic apoptosis in neuronal injuries is a function of the types of insult received by the brain and that a subpopulation of the cells undergoing apoptosis simply run out of energy to complete the task and revert to necrosis, resulting in a disorganised mixture of apoptotic and necrotic cells (Roy & Sapolsky, 1999).

1.7 The basis of biological spectroscopy

Historically, physiological observations have required the application of invasive experimental procedures to one degree or another. Eisenberg's uncertainty principle tells us that such observations, by their nature,
may alter the variables we are attempting to investigate, thus invalidating our data. Application of non-invasive monitoring techniques may limit such confounding influences, resulting in data which more closely resemble the situation in the uninstrumented subject. In the experiments described in this thesis, non-invasive near infrared spectroscopy has been used to monitor changes in concentrations of three physiologically important chromophores (oxyhaemoglobin, deoxyhaemoglobin and oxidised cytochrome c oxidase). The following sections give a basic overview of the physics of tissue spectroscopy, and near infrared spectroscopy in particular.

Tissue spectroscopy has its origins in observations made in the 18th century which are today known as the Beer-Lambert law. This physical law states that consecutive layers of a light-absorbing material of thickness $d$ absorb a constant fraction $I_0/I$ of the light incident upon it. This loss of light (attenuation, $A$) is measured in optical densities (OD), where:

$$ A = \lg \left( \frac{I_0}{I} \right) = \alpha \cdot c \cdot d $$

1.1

where:

- $A$ = attenuation in OD
- $I_0$ = intensity of incident light
- $I$ = intensity of light transmitted through the absorbing compound
- $\alpha$ = specific extinction coefficient of the absorbing compound measured in $(\mu\text{mol}^{-1} \text{ cm}^{-1})$
- $c$ = the concentration of the absorbing compound (when in solution)
- $d$ = the thickness of the material or distance between light entry and exit points.

Since the attenuation is measured to logarithmic base 10, it gives a measure of the number of orders of magnitude by which light intensity falls on passing through the absorbing compound. Figure 1.24 below shows this absorbing system:

![Figure 1.24 Cuvette model of absorption](image_url)

Figure 1.24 Cuvette model of absorption, where incident light $I_0$ is not scattered and may be absorbed by compound of concentration $c$, attenuation is described in equation 1.1 above.
The simple cuvette model does not take into account the possibility that the compound (or chromophore) might scatter light as well as absorb it. This would result in some proportion of the incident light being lost, which would effectively increase the attenuation of light above that expected from the extinction coefficient alone. In this instance, the Beer-Lambert must be modified to take account of scattering. The mathematics used to describe the properties of individual molecules and their shape on scattering is beyond the scope of this work, suffice it to say that variations in refractive index in tissues (such as between the extracellular fluid and plasma membrane, or between the cytosol and intracellular structures) will cause light to be scattered. This is explained further below.

1.7.1 Scattering of light by biological tissue

As indicated, scattering in biological tissues occurs at boundaries of differing refractive index. In a complex structure containing many micro- and macroscopic particles, this may include the membranes of cells and their organelles, between intra- and extra-cellular fluid and at the boundaries of macromolecules, such as starch or proteins (Ross, 1967).

When considering multiple scattering in biological tissue, the Beer Lambert law must be modified to include terms which account for:

1. light loss due to scattering, and
2. increased photon pathlength due to scattering.

Loss due to scattering can be included by an additive term (G), whilst increased photon pathlength can be expressed by a scaling factor, called the differential pathlength factor (B). The Beer-Lambert law then becomes:

$$A = \log \left( \frac{I_0}{I} \right) = \alpha \cdot c \cdot d \cdot B + G \quad 1.2$$

Taking this into account, our cuvette model becomes:
This modification of the Beer-Lambert law results in a problem for the investigator. Since the term $G$ is unknown, we are unable to calculate changes in concentration ($c$) with this equation. In order to circumvent this problem, we can make the assumption that only the term $c$ changes in the equation to cause changes in attenuation. In that circumstance, we can calculate relative changes in concentration, which would have real units. This can be explained graphically as follows:

![Diagram](image)

Figure 1.25 Scattering model for compound in solution. Note that the actual photon pathlength is longer than the geometrical pathlength, $d$. The modified Beer-Lambert law described in equation 1.2 includes terms $B$ and $G$ to account for loss of light due to scattering.

$$A_1 = \log \left( \frac{I_0}{I} \right) = -c_1 \cdot d \cdot B + G$$

$$A_2 = \log \left( \frac{I_0}{I} \right) = -c_2 \cdot d \cdot B + G$$

Figure 1.26 Differential pathlength calculation assumes that all variables remain constant except for the concentration of chromophore. In this way, differential attenuation is proportional to change in concentration. By taking the differential attenuation ($\Delta A$, i.e., $A_1 - A_2$), the problem of pathlength correction is avoided since the additive factor $G$ drops out of the equation and relative changes can be calculated in absolute units.

All other things being equal, the change in attenuation ($\Delta A$) is proportional to the change in chromophore concentration ($\Delta c$) and has real units:

$$\text{Differential Attenuation} (\Delta A) = A_1 - A_2 = (c_1 - c_2) \cdot \alpha \cdot d \cdot B$$  \hspace{1cm} 1.3
The calculations above show that it is possible to use spectroscopy to quantify changes in chromophore concentrations in tissue and that, due to the uncertainties caused by multiple light scattering in biological tissue, these are quantified relative changes.

1.7.2 Absorbing compounds in biological tissue

Now that we can see that it is possible to measure changes in chromophore concentrations in biological tissue, we need to identify the chromophores present in living tissue and their relative contributions to total attenuation. The chromophores present in biological tissue may be in concentrations which are fixed or variable over the period of the experiment and can have absorption spectra which are oxygen dependent or independent. The main chromophores are detailed in the following sections.

1.7.2.1 Water

As the human is comprised mostly of water (80% in adult tissue), it follows that attenuation by water may make the greatest contribution to overall attenuation. Figure 1.27 shows absorption spectra for water between 200 and 2000 nm (a) and between 650 and 1000 nm (b). Attenuation below 600 nm is less than 0.001 cm\(^{-1}\), and can be considered to be negligible. Between 650 and 920 nm, attenuation is low, but measurable and increases markedly at ~960 nm. Above 1000 nm, attenuation is so large that very little tissue penetration occurs. As the fetal brain is believed to be ~90% water (Fillerup & Mead, 1967), the relative transparency between 600 and 900 nm goes some way to explain why NIR light penetrates deeply into biological tissue. This transparency window to near infrared wavelengths allows a large volume of brain to be interrogated and, in the case of the fetus, means that transillumination of the head is possible.
Figure 1.27 Shows absorption spectra for water between (a) 200 and 2000 nm and (b) 650 and 1000 nm. Note the different scales on the x axis and thus the relative size of the 970 nm peak (arrows) in (a) and (b).

1.7.2.2 Plasma and Cerebrospinal Fluid

Plasma is a yellowish solution in which red and white blood cells are suspended in the vascular system. Its colour derives from a low concentration of bilirubin, a breakdown product of haem metabolism. The absorption spectrum of plasma in the near infrared is dominated by bilirubin absorption centred around 500 nm (Figure 1.28) but, between 600 and 900 nm, the absorption is nearly linear. Plasma is treated as a colourless liquid for NIRS purposes.

Cerebrospinal fluid (CSF) is produced in the choroid plexus and fills the ventricles of the brain and bathes brain cells. It is a clear fluid containing little protein. No absorption features are present in the near infrared and it, too, is treated as a clear liquid for the purposes of NIRS work.
1.7.2.3 Bone and surface tissues

Any transillumination of the fetal head must first pass through the skin and bone as well as a layer of CSF to reach the brain tissue. In the adult, these layers represent a considerable barrier to light transit and may preclude NIRS work. In the fetus, however, these layers are relatively insubstantial. The main absorber in the different layers of the skin is melanin.

1.7.2.4 Lipids

All cell plasma membranes, organelles and myelin sheaths are made of lipid. The lipid concentration of different tissue types varies, but is unlikely to be greater than 40% of the mass of a given tissue and may be much less. Neonatal brain is poorly myelinated and contains perhaps 5% lipid, a concentration which increases as development progresses. The concentration of lipid in the fetal brain over the period of days which constitutes the upper limit for the experiments here will not change significantly and thus we can consider lipid to be a fixed absorber. The attenuation of pure lipid (taken from Conway et al., 1984) in the near infrared is given in Figure 1.29. Lipid attenuation in the near infrared is of similar magnitude to that of water, but lipid is present in a much smaller concentration. The overall effect of lipid on NIRS measurements in the fetus is unlikely to be significant.
1.7.2.5 Haemoglobin and oxyhaemoglobin

The main variable absorber in the fetal brain is haemoglobin. Haemoglobin, as its name suggests, is an iron-containing protein (Figure 1.30(a)). It is found exclusively in red blood cells in high concentration and has the function of carrying oxygen from the lung to the tissues and transporting carbon dioxide from the tissues to the lungs. Haemoglobin is comprised of four protein chains, each containing one haem group. Available chains are denoted α, β, δ and γ; (Figure 1.31) the exact combination of chains used determines the properties of the haemoglobin molecule. Adult haemoglobin is mainly of the α2β2 form, whilst fetal is of the α2γ2 form.

Haem is a porphyrin structure containing an iron atom in the Fe2+ configuration (Figure 1.32). Each haemoglobin molecule can, therefore, bind four oxygen molecules, which it does in a physical and not chemical manner. Oxygen binding is permissive, i.e. the $K_m$ for binding of the first oxygen is higher than that for the second, which is greater than binding for the third, etc. The plot of oxygen saturation with increasing $P_{O_2}$ is, therefore, a sigmoid curve, and is known as the oxygen dissociation curve for haemoglobin.

Haemoglobin attenuation is oxygenation dependent as can be clearly seen in Figure 1.30(a) between 400 and 1000 nm. Within the transparency window in the near infrared provided by water absorption characteristics, haemoglobin has a significantly different attenuation in its oxy- and deoxy- forms (Figure

![Figure 1.29 Absorption spectrum for lipid in the infrared spectrum. Note that the extinction coefficient begins to rise at around 875 nm.](image-url)
1.30(b)), with only a single wavelength (~800 nm) at which attenuation is the same for both compounds (an isobestic point). The clinical significance of this difference is the obvious colour change of blood when going from well oxygenated (bright red) to deoxygenated (dark purple, bordering on black).

Figure 1.30 Shows absorption spectrum for HbO₂ and Hb (a) between 400 and 100 nm and (b) in the transparency window provided by water's absorption characteristics.

Figure 1.31 Ribbon diagram of the four-subunit structure of haemoglobin. Note that each subunit has a haem group, shown here as stick diagrams. From http://www-bioc.rice.edu/~graham/CcO.html
1.7.2.6 Cytochrome oxidase

Mitochondrial complex IV (cytochrome oxidase) is the last electron acceptor in the mitochondrial electron transfer chain (Figure 1.33 & 1.34). The enzyme contains four redox active metal centres; one of which (the binuclear Cu₄-haem a₂ centre) is the site at which 90% of the oxygen used by the human body is reduced to water, whilst another (the Cu₃ centre) has a strong absorbance in the near infrared which is redox-state sensitive (Figure 1.33). Electrons from cytochrome c are passed to the cytochrome oxidase Cu₃ centre, thence to the haem a centre before being passed to the Cu₄-haem a₂ binuclear centre, where oxygen binds before being reduced to water (Malatesta et al., 1998; Regan et al., 1998).

Since the upstream elements of the electron transport chain are more reduced than the downstream elements (Roger Springett, personal communication), it follows that the Cu₄ centre which gives cytochrome oxidase its spectroscopic properties in the near infrared, will be slightly more reduced than the oxygen binding site. Despite this, it is believed that the NIRS behaviour of cytochrome oxidase provides a good reflection of the redox state of the cell. This belief is supported by nuclear magnetic resonance studies of the effects of hypoxia ischaemia (Kleinshmidt et al., 1996; Matsumoto et al., 1996).
Figure 1.33 Absorption spectrum for CcO between 650 – 1000 nm. Note the different scale to those for haemoglobin; cytochrome oxidase is present in concentrations one order of magnitude smaller than haemoglobin.

Figure 1.34 Schematic representation of the mitochondrial electron transfer chain. Electrons pass either from NADH to complex I or from FADH$_2$ to complex II, thence electrons pass to complexes III via ubiquinone (UQ) then to complex IV, where they are used to reduce oxygen to water.
1.7.3 Fetal responses to hypoxia-ischaemia as measured with NIRS

There are few studies of fetal haemodynamics or metabolism using NIRS which are directly applicable to this thesis. Owing to fetal inaccessibility, studies of human fetuses are confined to monitoring during labour (Peebles et al., 1992; Rolfe et al., 1992; Doyle et al., 1994). However, NIRS has been used to monitor chronically instrumented fetal sheep during periods of hypoxia-ischaemia or asphyxia, both by maternal inhalation hypoxaemia (Bennet et al., 1998) and umbilical cord occlusion (Bennet et al., 1999).

The above-mentioned fetal sheep studies both used the Hamamatsu NIRO500 NIR spectrometer, which uses 4 discreet NIR wavelengths to provide data on changes in the concentrations of HbO$_2$ and Hb, but not on changes in cytochrome oxidase oxidation. Thus the emphasis in these publications is on the cerebral haemodynamic consequences of hypoxia, data which is used in conjunction with other fetal variables (mean arterial pressure, heart rate, carotid flow) to provide a fuller picture of the fetal responses to hypoxia.

From these publications, we can see that there are rapid reciprocal changes in cerebral HbO$_2$ and Hb at the start of hypoxia, whilst cerebral tHb (an index of cerebral blood volume) increases (Figures 1.36 &
suggesting either cerebral vasodilatation or cerebral venous congestion. These findings are in agreement with the data presented in Chapters 3 & 5 of this thesis.

Figure 1.36 NIRS data showing significant changes in cerebral concentration of HbO₂, Hb and tHb during isocapnic hypoxaemia. Adapted from Bennet et al., 1998.

Figure 1.37 Cerebral NIRS data during umbilical cord occlusion. Showing reciprocal changes in HbO₂ and Hb, with little change in tHb during the challenge, but significant increase after release of the occlusion. Adapted from Bennet et al., 1998.
CHAPTER 2

GENERAL METHODS

2.1 Home Office Licensing

All work was conducted in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office Project Licence number PPL 70/03908 and Personal Licence number PIL 70/13466.

2.2 Sheep

All ewes were date-mated and tagged for identification. Ewes were confirmed pregnant by ultrasound scan at circa 0.4 gestation and parity was noted. Only ewes bearing singleton fetuses were considered for these studies.

- Late gestation studies:
  Mule ewes were used for the studies on late gestation uterine artery and umbilical cord occlusion, weighing approximately 50 – 60 kg, supplied by Mr Bob White.

- Early gestation studies:
  Romney marsh ewes were used for early gestation studies supplied by the Royal Veterinary College at Potter’s Bar. These ewes weighed between 60 – 70kg each.

It was found that the quality of the ewes (and, by implication, the fetuses) from the Royal Veterinary College was better than those supplied by Mr White. By this, it is meant that ewes tended to be heavier, had better condition scores and it was noted during surgery that they appeared to have a greater percentage of body fat; both subcutaneous and peri-aortic.

2.3 Transportation and housing

Each ewe was always accompanied by a companion whilst in the floor pens and when housed in metabolic carts. Upon delivery from the farm, ewes were housed indoors in floor pens at the Biological Services Rockefeller Unit and allowed unrestricted access to hay and water. Ewes were kept in floor pens for at least 24 hours before moving to metabolic carts, this gave the ewes some time to acclimatise to the new environment and was intended to minimise the stress caused to the animals.

At least 48 hours prior to surgery, ewes were transferred to metabolic carts. The carts were built in steel by the UCL physiology department workshop (Figure 2.1).
cart allowed the ewe to sit or stand comfortably but did not allow them to turn round. A water bucket and hay bags were provided at the front of each cart. Hay and water were changed each morning and afternoon. Sawdust-filled trays below the slatted floor of the cart caught urine and faeces; the trays were cleaned once per day.

Upon delivery, each ewe was inspected for any obvious signs of ill health. Subsequently, a daily record of by-eye observation and procedures performed (blood samples, IV antibiotics or fluids given etc) was kept. Daily record sheets were attached to the metabolic cart to provide up to date information to other research staff, Biological Services staff, the university vet and Home Office Inspector. Daily record sheets were filed with anaesthetic records and post mortem records after sacrifice of the ewe.

2.4 Starvation prior to surgery

Food, but not water, was removed from the carts prior to surgery to minimise the volume of rumen in the sheep stomachs. This was felt to be necessary because in the supine position during general anaesthesia, ruminal contents can compress the aorta and venae cavae leading to circulatory embarrassment (also
called supine hypotensive syndrome) and could restrict the free movement of the diaphragm giving rise to respiratory acidosis, reduced tidal volume and alveolar collapse. The duration of starvation required to significantly reduce rumen volume to prevent these problems is believed to be at least 4 hours, and starvation overnight was used as standard. Starvation typically started at 5 PM on the evening before surgery. Surgeries were generally started at 8.30 – 9.00 AM the next morning.

2.5 Equipment making

2.5.1 Catheters

Polyvinyl tubing cut to 1m was used to make all catheters. Each was fitted with a non-sharp hub and bagged with electrodes (see below) for gamma sterilisation.

2.5.2 Electrodes

Electrodes for ECoG, EMG and ECG were made from thin-walled polyvinyl tubing cut to 1.1m length. Either two or three wires were threaded through and the ends sealed with silicone sealant (RS Components). One end of each was bared before packing into bags with catheters for gamma sterilisation.

2.5.3 Autoclaving

On the evening before surgery, the wrapped surgical tray was autoclaved along with a double-wrapped laparotomy drape and double-wrapped gowns for the surgeons. The autoclave used was a Getinger Pulsmatic, with a 25 minute wet cycle.

2.5.4 Occluders

2.5.4.1 Inflatable occluders

Occluders were supplied by In Vivo Metric (Healdsburg, CA, USA) and were either of 12 or 14 mm diameter. Each occluder was sterilised in Novasapa before implantation and the free end kept clean by washing with chlorhexidine prior to occlusion. Inflation of the balloon was achieved by injection of a volume of sterile saline. Without exceeding the tolerance of the occluder, saline would be injected until the back pressure on the syringe prevented further injection. The tubing of the occluder would then be clamped with a surgical clamp.

Release of the occluder would be achieved by release of the clamp and withdrawal of the volume of saline injected.
2.5.4.2 Mechanical occluder

A mechanical screw occluder was donated by Dr L Bennet of Auckland University, New Zealand. The metal cable of this occluder was made to turn more easily by covering it with a black rubber cable sheath bought from a local ironmonger. In addition, a silastic sheath was made but cutting down a length of medical suction tubing and slid over the cable of the occluder. The silastic sheath would this prevent tracking of foreign material into the maternal abdomen from outside.

2.5.4.3 Placement of the occluder

Before each surgery, the occluder was sterilised with Novasapa cold sterilising solution and kept in aseptic conditions until needed. The occluder used was placed either at the point where the umbilicus leaves the abdomen or around the maternal common internal iliac artery.

Figure 2.2 shows how the external iliac arteries leave the aorta before it bifurcates to form the internal iliac arteries. Between the external and the separate internal iliac arteries is (usually) a short segment of common internal iliac artery where an occluder can be placed.

2.6 Anaesthesia

2.6.1 Induction

Ewes were anaesthetised with intrajugular injection of 0.8 – 1.0 g of sodium thiopentone (Intraval, Rhône Mérieux, Tallaght Dublin, Republic of Ireland). The ewe was rolled onto its back onto the operating table and intubated with a size 9 cuffed endotracheal tube (Portex) with the aid of a laryngoscope and
appropriate Rowson blade. Once intubated, halothane (Mallinckrodt Veterinary Ltd, Uxbridge, UK) was supplied at 3% in 100% O\textsubscript{2} (BOC Gases, Guildford, UK) using a closed-circuit anaesthetic machine (Ohmeda, Englewood, US).

2.6.2 Maintenance

Ewes were usually maintained on 2–2.5% halothane in 100% O\textsubscript{2} throughout the surgery. It was occasionally necessary to increase the halothane level higher, but was never below 2%, since this was the minimum level needed to keep the ewes unconscious.

Where possible, ewes were allowed to self-ventilate. Those ewes that were unable to self-ventilate were ventilated using a tidal volume of 200ml at a ventilation rate of 25–30 breaths per minute. In this way, arterial saturation remained greater than 90% and end-tidal PCO\textsubscript{2} less than 50 mmHg. There were no anaesthetic fatalities using this method.

2.6.3 Anaesthetic record keeping

An anaesthetic monitoring sheet was used to provide a discontinuous record of the wellbeing of the ewe during the surgery. The ewe was checked every 15 minutes and details noted on the sheet. Variables recorded were:

- % halothane given
- arterial saturation
- heart rate
- respiration rate
- corneal reflex
- pupillary dilation

2.6.4 Replacement fluids

During surgery each ewe was given 500ml Hartmann's solution IV (Baxter, UK) into the jugular vein using an 18 gauge Venflon (Ohmeda, as before) and set to infuse over about 3 hours.

Where it was thought to be necessary, 500ml to 1 litre of 0.9 % saline (Baxter, as before) was given to the amniotic cavity to replace fluids lost during surgery. This fluid was administered through the amniotic catheter placed during surgery and took place after surgery, whilst the ewe was recovering from the anaesthesia.
2.7 Fetal surgical procedure

In late gestation, instrumentation of both the fetal head and hind limb was undertaken to provide simultaneous cerebral and peripheral data; the leg data will be discussed Chapter 4. Instrumentation of the hind limb was not attempted with the mid gestation fetuses since, owing to their small size, it was felt catheterisation of femoral vessels would add extra surgery time and may have reduced the operative success rate. Finally, ECoG electrodes were not placed at mid gestation owing to the difficulty of burring holes in the thin skull at this age. It was felt to be a lesser evil to not have ECoG data than to damage the fetal brain in attempting to puncture the skull with the hand drill available at the time.

Apart from the above, the surgical procedure was identical for mid and late gestation fetuses, as follows. Under aseptic conditions, the ewe's abdomen was opened by low midline incision. In-dwelling instrumentation was fed into the abdominal cavity through a tunnel in the ewe's flank made with a trochar and scalpel. These instrumentation was tied to one side until needed.

After a change of gloves, the maternal posterior abdominal wall was palpated to locate the bifurcation of the aorta into external iliac arteries and the common internal iliac artery. Bowel and uterus were packed to one side with abdominal gauze packs (Johnson & Johnson, UK) and the common internal iliac artery dissected free of the posterior abdominal wall using long instruments. The ovarian arteries were next located, dissected free and ligated with 2/0 silk. Finally, the occluder was placed and secured as described above. The abdominal packs were removed and the cord of the occluder was placed out of the way within the abdomen and the rest of the surgery completed. Individual surgical details are given in relevant chapters.

2.7.1 Recovery of the ewe

Ewes were moved back into a clean cart after surgery and allowed to recover from the anaesthetic. The ET tube was left in place until the ewe was breathing well. Ewes were given food and water immediately after the ET tube was removed and began eating within 20-30 minutes. Ewes were normally standing from 30 minutes to 1 hour after the end of surgery.

2.8 NIR data collection and analysis

2.8.1 Collection

NIRS data were collected using a near-infrared spectrometer purpose-built by Dr Roger Springett at UCL Department of Medical Physics and Bioengineering. A filtered broadband white-light source (Oriel Instruments, Stratford, US) using a 100W quartz-halogen bulb provides incident infrared light between 700 - 1000 nm, which was transmitted to the fetal head and leg through fibre optic bundles custom made for these experiments (Schott, UK). Unabsorbed light falling on the receiving optodes was transmitted to the
spectrometer (Jobin Yvon Spex 270M, Groupe Instruments SA, Longjumeau, France) by a second set of optical fibres and a spectrum was collected.

Figure 2.4 shows the data acquisition screen of MOGINA, the NIRS program designed by Roger Springett (UCL Medical Physics Dept) to run the NIRS hardware. Camera exposure time was set to give a signal amplitude of approximately 100,000 photon counts using an entrance slit width of 200-300 μm. This represented a compromise whereby sufficient signal was received to maximise signal-to-noise ratio whilst preventing saturation with light if tissue absorption fell during the experiment. Exposure time was set at the beginning of each experiment and held constant for the duration of the experiment. Exposure times ranged from 2 to 30 seconds in the experiments performed in this study.

2.8.2 NIR data analysis

Spectra from head and leg were saved to disk for later analysis. In order to obtain absolute changes in chromophore concentration, a difference spectrum was first generated from each raw absorption spectrum, which is the arithmetic difference between a spectrum at time x and the reference spectrum taken at time 0 (the start of the experiment). Each difference spectrum was then fitted between 780 and 900nm to previously determined absorption spectra (one for each chromophore) using a least-squares multilinear regression algorithm. Residual changes in optical density, not accounted for in the fitting process, were analysed to look for large or systematic changes that might indicate the presence of another chromophore not included in the algorithm. Optical pathlength was obtained using second order differential analysis from the 840nm water absorption feature.

Data for Δ[HbO2] and Δ[Hb] are absolute changes in concentration (in μmol/l) from a zero control set at the start of the experiment. However, because the amount of cytochrome oxidase present is fixed over the time scale of the experiment, changes in CcO represent changes in the amount of the oxidised enzyme present relative to the start of the experiment and not to changes in enzyme levels. In order to get an impression of the magnitude of changes in CcO observed relative to control values, CcO was fully reduced just prior to culling in several experiments by infusion of sodium cyanide (NaCN) 10mg/ml/min. A value for changes in total haemoglobin concentration (Δ[tHb]) can be generated from the sum of Δ[HbO2] and Δ[Hb] at any time point. tHb is related to blood volume (BV) through the haematocrit.

2.9 Statistical analysis

2.9.1 Repeated measures ANOVA with post hoc Newman-Keuls test

Serial measurements in research present a problem for statistical analysis, since the tests available tend to be geared towards comparing groups; a with b and c, for example. There are a number of methods for the analysis of serial measurements, usually between a control group and one receiving a treatment or challenge,
where the consequence can be dissected into two or three simple parameters which adequately describe the response; time to peak, maximum value, etc (Altman, 1995). The haemodynamic and NIRS data presented pose a problem for statistical analysis, since they are not simple functions of time, are not linear and have complex underlying physiology where layers of control are exerted sequentially such that some simple summary measures would disguise the dynamic control underlying their responses to hypoxia.

This is illustrated in Figure 2.3. The shape of the NIRS curves are not simple and summary measures are insufficient to describe these events.

![Figure 2.3 Example NIRS data showing complexity of raw data. Simple analyses such as summary measures are insufficient to fully describe these fetal responses to hypoxia (grey box).](image)

In order to adequately analyse the data, I have chosen to use repeated measures ANOVA with post hoc Newman-Keuls tests. Repeated measures ANOVA in these circumstances is based upon a comparison of the observed variation between the groups' means (in this instance, between the control group and the data points taken after initiation of the occlusion) with that expected from the observed variability between subject animals. The test takes the form of an F test to compare the variances, assuming that each data group tested is Normally distributed population. This test thus provides us with an overall p value, indicating whether the test was significant, and the level of that significance.

However, ANOVA testing in this way has a cumulative chance of finding a significant difference where there is none (ie a high false positive rate). In order to account for this, we use a post hoc test which aims to control the
rate of Type I error to no more than 5%. There are a number of tests available, such as the Bonferroni method, Duncan, Scheffé and Newman-Keuls tests. The problem with these tests is that they are rather conservative; in other words, they err on the side of safety (non-significance), such that it is not inconceivable to have a significant F test but a non-significant post hoc test. Owing to this, I have chosen to use the Newman-Keuls test which provides a balanced (not too conservative but not the least stringent) result.

Using these tests, within age group comparisons a significance test result yields a p value of < 0.05. These data are denoted by the use of the letter a.

2.9.2 Testing between age groups

When testing between age groups is required, say between fetal responses at 0.6 and 0.8 gestation to the same insult, unpaired Student's t test has been used. As with other tests, significance is considered to be when p is < 0.05.

Figure 2.4 Screen capture from MOGINA program showing typical setup, with individual windows for attenuation spectrum, difference spectrum and fitting and another showing concentration change of the NIRS chromophores.
2.10 Histological examination

Histological examination was performed by Dr Rosemary Scott, who was blinded to the experimental protocol. At post mortem, the ewe and fetus were killed with an overdose of pentobarbitone sodium after which the fetus was removed from the uterus. The fetal carotid arteries were catheterised, with the tips of the catheters lying as near to the base of the brain as possible. First 500ml of isotonic saline (Baxter, UK), then 1 litre of 2% buffered formalin (Sigma Aldrich, UK) were infused through the brain. A hole was cut into the heart to allow drainage of fluid. After formalin infusion, the brain was allowed to stand for at least one week before cutting for histological purposes.

At autopsy, each brain was weighed, the brain stem was removed at the level of the anterior border of the pons and weighed separately. The right hemisphere and brain stem were marked with ink. Four millimetre coronal slices of the hemispheres were made, based on an initial cut at the level of the mamillary body. Three millimetre slices were made of the brain stem. Selected slices, (to include medulla, pons, midbrain, cerebellum, basal ganglia, hippocampus, and frontal and occipital cortex and white matter) were embedded in paraffin, and sectioned and stained with haemotoxylin and eosin (H&E). Additional stains were performed to delineate further lesions identified on H&E.

Evidence of injury was assessed by low and high power examination of the selected areas, recording the presence of cortical infarcts, white matter damage, and selective neuronal injury. The method of examination resembled the approach used in assessing brain injury in human clinical material. It was designed to detect substantial degrees of damage, rather than subtle levels of cell dropout, and numeric quantitation was not attempted.
CHAPTER 3

OVINE FETAL RESPONSES TO COMMON INTERNAL ILIAC ARTERY OCCLUSION IN MID- AND LATE-GESTATION

3.1 Introduction

As discussed in section 1.1, infants born both at term or preterm (before 37 weeks gestation) are at risk of neurological injury resulting in motor and/or intellectual deficits. Whilst the cause of the majority of severe disabilities in children of school age are congenital abnormalities or inherited illnesses, in a significant minority of infants these injuries are due to the effects of intrauterine hypoxia-ischaemia. Considering the increase in the number of infants born preterm who are surviving into childhood with neurodevelopmental impairments, it is unsurprising that scientists and clinicians are seeking methods of detecting those infants at risk of perinatal hypoxia-ischaemia and strategies to prevent or ameliorate neurological injury.

The clinical debate over the antecedents of neurodevelopmental disabilities, such as those grouped under the term “cerebral palsy”, has been ongoing for many years (Goodlin, 1995; Alberman, 1982). Experiments mainly using animal models have begun to dissect the question and, as might be expected, multiple factors appear to be important. At present, however, little effort has been made to provide a gestational map of the fetal responses to the various types of insult known to cause neuronal injury. Previously, this may have been due in part to methodological problems associated with keeping alive chronically prepared experimental fetal animals at early gestation, but this hurdle has been overcome to a great extent. However, there is still little systematic work of this kind in evidence. The late gestation fetal sheep data presented in this chapter has been published in a comparison of head and peripheral muscle (Newman et al., 2000) but is compared here with data from the brain of the mid gestation fetus. This may provide an appreciation of gestational changes in the cerebral haemodynamic and metabolic responses to common internal iliac artery occlusion, as detected by NIRS.

3.2 Methods

3.2.1 Fetal surgery

Theatre and ewe were prepared for surgery as described in Chapter 2. In late gestation, instrumentation of both the fetal head and hind limb was undertaken to provide simultaneous cerebral and peripheral data; the leg data will be discussed in a later chapter. ECoG electrodes were not placed at mid gestation owing to the difficulty of burring holes in the thin skull at this age. It was felt to be a lesser evil to not have ECoG data than to damage the fetal brain in attempting to puncture the skull with the hand drill available at the time.
Apart from the above, the surgical procedure was identical for mid and late gestation fetuses, as follows. The head and one upper limb were exteriorised and catheters placed in the carotid artery and jugular vein on one side such that their tips were close to the heart. A reference catheter was placed in the amniotic cavity. A Transonic flow probe (size 3R, Transonic Systems Inc, Ithaca, US) was placed around the carotid artery contralateral to the jugular catheter. Using a T-incision, the skin of the scalp was peeled back. Burr holes were made in the skull overlying the parasagittal cortex using a hand drill and electrodes were placed on the dura. The burr holes were sealed using rubber caps and cyanoacrylate. Infrared optodes were placed on the skull overlying the parasagittal cortex and held firmly in place by a custom made black rubber holder which was sutured to the edges of the skin incision. Apposition of optodes to scalp was improved by closure of the scalp incision over the holder and optodes. Two pairs of electrodes were sewn onto the chest wall, one for monitoring of fetal ECG and another to act as a common earth. The fetus was replaced and the uterus and maternal abdomen were closed in two layers with 2/0 silk (Pearsalls Sutures, UK). Finally, a catheter was placed in the maternal recurrent metatarsal vein for administration of antibiotics. Anaesthesia was discontinued and the ewe allowed to recover. Antibiotic regime was as described in Chapter 2.

3.2.2 Experimental protocol

Experiments were performed on day three after surgery. Cardiovascular and NIRS data were collected for at least 1 hour prior to, during and 1 hour after the insult. Fetal asphyxia was induced by maximally tightening the screw of the mechanical occluder for 1 hour for late gestation fetuses and until mean arterial pressure fell below 20 mmHg in the mid gestation fetuses.

Arterial, central venous and amniotic pressure lines were connected to pressure transducers (SensoNor 840, SensoNor a.s, Horten, Norway) and then into amplifiers (Digitimer Ltd, Welwyn Garden City, UK). Arterial and central venous pressures were corrected for amniotic pressure, using the signal from the reference catheter, and monitored by use of MacLab software (ADInstruments, UK). Other biophysical variables (FHR, CaBF in all fetuses and FBF in late gestation fetuses only) were monitored using MacLab software in a similar way and saved to optical disk for later analysis. As indicated in the general methods chapter, the fetal ECoG waveform was monitored to confirm normal cerebral function by the presence of sleep cycling prior to the onset of hypoxia.

Blood samples were collected at the following times relative to occlusion: -55, -30, -5, +5, +30, +55 (when the insult lasted 60 minutes) and then at +15, +35 and +55 minutes after release of the occluder. 0.5 ml samples were collected from carotid artery and jugular vein in all fetuses and from femoral artery and vein in late gestation fetuses only. Samples were immediately tested for gases and electrolytes (BGElectrolytes 14008-01 and CO-Oximeter 482, Instrumentation Laboratories, UK) and for glucose and lactate (YSI 2300 STAT Plus), values being corrected to the fetal temperature of 39.5°C.
Oxygen delivery (DO₂) values were calculated as the product of either carotid flow and carotid arterial oxygen content (Ca), corrected to 39.5°C, or from femoral flow and femoral arterial oxygen content where appropriate. Where possible, oxygen consumption (VO₂) values were calculated in a similar manner using, in addition, data for the appropriate venous oxygen content (Cv) and Fick’s law, i.e. \( VO_2 = Q(Ca - Cv) \), where \( Q \) is carotid or femoral blood flow (ml/min).

3.2.3 Collection of near-infrared spectra
Data acquisition was performed as described in detail in section 2.7.1, and saved for off-line analysis. As with all other experiments, chromophore changes were tracked in real time using MOGINA to assess the success of the occlusion (see Figure 2.Y for MOGINA screen), in addition to monitoring biophysical variables and blood gas results.

3.2.4 Data presentation and statistical methods
NIRS data were obtained simultaneously from the brain and hindlimb of ten late-gestation fetuses. Two late-gestation fetuses failed to recover from the insult and died between reversal of the insult and the end of the experiment, so for this group in the recovery period, \( n = 8 \) (head). Nine mid-gestation fetuses were used, all of whom were instrumented for cerebral monitoring only. All data are expressed as mean ± SEM. Time points taken for analysis are, except where specifically noted, 5 minutes prior to occlusion, 5 minutes into occlusion, 5 minutes before release of the occluder, 5 minutes after release of the occluder and 60 minutes after the end of occlusion. Within age group comparisons are made using repeated measures ANOVA and Newman-Keuls post hoc test where necessary and is signified in diagrams and tables with the superscript \( a \). Between age group analysis was performed using unpaired t-test and is represented with a superscript \( b \). Statistical significance is taken as \( p \leq 0.05 \) in all instances.

3.3 RESULTS
3.3.1 Arterial blood gases and acid-base status
Changes in blood gases, pH etc, sampled from the carotid artery and jugular vein, are shown in Tables 3.1-3.3. Maternal common internal iliac artery occlusion caused a moderate fetal asphyxia; i.e. the mean group pH did not fall below 7.00, mean PO₂ was not less than 9 mmHg and mean PCO₂ did not increase above 65 mmHg. Tables 3.1 and 3.2 show the arterial blood gas data for the mid- and late-gestation fetuses, respectively.
### Table 3.1
Blood gas analysis results for mid-gestation fetuses before, during and after maternal common internal iliac artery occlusion. Blood was taken from the brachial artery at the same time points as for the late gestation fetuses in Table 3.1, above. Statistical significance determined with one way repeated measures ANOVA, as above, with Newman-Keuls post hoc tests as appropriate, *p < 0.05 vs -5 min*. Statistical significance for between age group analysis is given in Table 3.2. Glucose and lactate analysis was not performed for mid-gestation animals, owing to technical problems with the glucose/lactate analyser.

<table>
<thead>
<tr>
<th></th>
<th>Occ -5 min (n = 6)</th>
<th>Occ +5 min (n = 5)</th>
<th>Rev -5 min (n = 5)</th>
<th>R +5 min (n = 2)</th>
<th>Rev +55 min (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_aO_2$ (mmHg)</td>
<td>24.3 ± 2.3</td>
<td>12.0 ± 2.2</td>
<td>12.8 ± 2.9</td>
<td>16.0 ± 2.0</td>
<td>21.0 ± 6.1</td>
</tr>
<tr>
<td>$P_aCO_2$ (mmHg)</td>
<td>49.9 ± 3.5</td>
<td>55.0 ± 6.9</td>
<td>55.8 ± 6.9</td>
<td>77.0 ± 22.1</td>
<td>60.6 ± 9.4</td>
</tr>
<tr>
<td>pH</td>
<td>7.36 ± 0.01</td>
<td>7.27 ± 0.05</td>
<td>7.22 ± 0.05</td>
<td>7.31 ± 0.07</td>
<td>7.22 ± 0.09</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>22.0 ± 0.9</td>
<td>22.5 ± 0.8</td>
<td>21.0 ± 2.0</td>
<td>26.5 ± 1.5</td>
<td>22.7 ± 2.2</td>
</tr>
<tr>
<td>HCO$_3^-$ (mmol/l)</td>
<td>26.6 ± 1.6</td>
<td>22.1 ± 1.7</td>
<td>21.0 ± 1.6</td>
<td>21.2 ± 3.0</td>
<td>24.1 ± 2.4</td>
</tr>
<tr>
<td>BE (mM)</td>
<td>2.1 ± 1.5</td>
<td>-3.6 ± 1.9</td>
<td>-6.1 ± 2.1</td>
<td>-6.9 ± 2.0</td>
<td>-2.0 ± 3.3</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>6.3 ± 0.4</td>
<td>4.8 ± 0.2</td>
<td>5.1 ± 0.8</td>
<td>6.2 ± 0.9</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>Sat (%)</td>
<td>72.4 ± 7.0</td>
<td>35.2 ± 7.2</td>
<td>45.5 ± 12.4</td>
<td>37.9 ± 24.6</td>
<td>57.7 ± 19.4</td>
</tr>
<tr>
<td>$O_2$ ct (vol %)</td>
<td>6.2 ± 0.8</td>
<td>2.5 ± 0.8</td>
<td>4.0 ± 1.6</td>
<td>3.6 ± 2.6</td>
<td>5.0 ± 1.9</td>
</tr>
</tbody>
</table>

### Table 3.2
Blood gas data (mean ± SEM) from ten late-gestation fetal sheep during one hour of maternal common internal iliac artery occlusion. Times shown are relative to onset of occlusion (time zero). Within group statistical analysis by repeated measures ANOVA vs. -5 min, with Newman-Keuls post hoc tests as appropriate, *p < 0.05*. Between age group (0.65 vs. 0.85 gestation) analysis at each time point was performed using unpaired t-test, $^b$ *p < 0.05 vs mid-gestation at same time points.*
Table 3.3. Venous blood sample data for the mid-gestation fetuses. One way repeated measures ANOVA vs. -5 min used to test for significance with Newman-Keuls post hoc tests as appropriate, *p < 0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Occ -5 min (n = 7)</th>
<th>Occ +5 min (n = 6)</th>
<th>Rev -5 min (n = 5)</th>
<th>Rev +5 min (n = 3)</th>
<th>Rev +55 min (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_{O2} (mmHg)</td>
<td>22.4 ± 1.5</td>
<td>9.0 ± 2.5(^a)</td>
<td>11.0 ± 2.6(^a)</td>
<td>18.7 ± 0.5</td>
<td>22.8 ± 2.6</td>
</tr>
<tr>
<td>P_{CO2} (mmHg)</td>
<td>53.9 ± 2.04</td>
<td>66.8 ± 4.4</td>
<td>69.9 ± 6.9(^a)</td>
<td>61.3 ± 5.3</td>
<td>50.7 ± 1.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.33 ± 0.14</td>
<td>7.21 ± 0.04</td>
<td>7.14 ± 0.32(^a)</td>
<td>7.15 ± 0.04(^a)</td>
<td>7.32 ± 0.03</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>19.0 ± 1.0</td>
<td>22.6 ± 1.8(^a)</td>
<td>21.8 ± 1.3(^a)</td>
<td>21.3 ± 2.0</td>
<td>19.3 ± 2.1</td>
</tr>
<tr>
<td>HCO(_3) (mmol/l)</td>
<td>27.5 ± 0.7</td>
<td>26.6 ± 0.6</td>
<td>22.5 ± 1.6(^a)</td>
<td>20.6 ± 2.1(^a)</td>
<td>25.5 ± 1.4</td>
</tr>
<tr>
<td>BE (mM)</td>
<td>2.68 ± 0.74</td>
<td>0.50 ± 0.80(^a)</td>
<td>-4.32 ± 1.91(^a)</td>
<td>-6.1 ± 1.9(^a)</td>
<td>0.65 ± 1.73</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>6.02 ± 0.14</td>
<td>4.68 ± 0.26(^a)</td>
<td>4.82 ± 0.45(^a)</td>
<td>5.0 ± 0.3(^a)</td>
<td>5.63 ± 0.76</td>
</tr>
<tr>
<td>Sat (%)</td>
<td>66.20 ± 4.63</td>
<td>26.22 ± 5.87(^a)</td>
<td>28.90 ± 8.24(^a)</td>
<td>48.1 ± 4.0</td>
<td>69.53 ± 6.10</td>
</tr>
<tr>
<td>O(_2) ct (vol %)</td>
<td>5.58 ± 0.50</td>
<td>1.72 ± 0.43(^a)</td>
<td>2.10 ± 0.82(^a)</td>
<td>3.3 ± 0.5(^a)</td>
<td>5.53 ± 1.16</td>
</tr>
</tbody>
</table>

3.3.1.1 Mid-gestation fetuses:

P_{O2}, [HCO\(_3\)], base excess and arterial saturation all showed changes during the insult only, as indicated in table 3.1. As with the late-gestation animals, pH fall is progressive during the insult. In the mid-gestation animals, it reaches significance just before release of the occluder but has not moved back toward control level 60 minutes after the end of the occlusion. A progressive increase is seen for P_{CO2} only at mid-gestation.

3.3.1.2 Late-gestation fetuses:

P_{O2}, P_{CO2}, haematocrit, Hb saturation and [Glucose] showed rapid changes at the start of the insult and returned to, or toward, control levels after release of occlusion. [HCO\(_3\)], pH, base excess and [Lactate] showed progressive changes. pH was, however, not significantly different to control at 60 minutes into recovery.

3.3.2 Venous blood gas analysis

Unfortunately, the catheter material used for mid-gestation fetal arteries was prone to kinking; owing to this, the number of experiments with arterial blood samples at some time points is low (n = 2 for some time points). Power calculations, therefore, indicate that some statistical comparisons using blood data need to be treated with caution. However, a full set of venous blood samples was obtained and data for these are presented in Table 3.3. These show that, on the venous side of the circulation, there were significant changes in most of the variables measured from the blood during the insult, and that all variables returned quickly to control levels. There was only one exception in that there was a progressive increase in P_{CO2} as would be
expected; this variable reached significance at 5 minutes prior to release of the occlusion and was not significantly different from control after 60 minutes of recovery.

### 3.3.3 Haemodynamic responses to common internal iliac artery occlusion

Fetal haemodynamic data at 0.65 and 0.85 gestation can be compared by looking at Figures 3.1 & 3.2. Control levels for mean arterial pressure (MAP) at 0.65 and 0.85 gestation were 38.2 ± 1.8 and 45.6 ± 2.7 mmHg, respectively. For fetal heart rate (FHR) values are 184 ± 4 and 183 ± 7 bpm, and for carotid artery flow (CaBF) 21.0 ± 1.8 and 65.1 ± 3.9 ml/min, respectively. There were no significant differences between age groups for control levels of MAP (unpaired t test, p = 0.69) or FHR, although CaBF has increased significantly between mid and late gestation (p < 0.05, unpaired t-test).

The initial responses to hypoxia consist of a transient bradycardia, significant in magnitude at both mid and late gestation, but not significantly different between age groups. After 5 minutes of reduced uteroplacental flow, there is no significant change (using these time points) in the mean data for MAP or CaBF at either age group compared to respective control levels but as the insult progresses, the mid gestation animals' circulatory compensation begins to fail, resulting in falls in both MAP and CaBF (to 26.4 ± 1.4 mmHg and 15.5 ± 2.3 ml/min, respectively). This failure of circulatory compensation does not occur in the older fetuses during the period of occlusion used, and late gestation fetal MAP and CaBF remain not significantly different from control at 5 minutes before release of the occluder.

The lack of within-group significance at 0.85 gestation during the insult does not mean that these animals do not compensate for reduced uteroplacental flow. By taking means at every minute for MAP, FHR and CaBF, we can more accurately dissect the haemodynamic response to occlusion. This data is shown in Figure 3.2. Whilst there are still difficulties with this methodology owing to the variability of response (discussed in section 3.4.2), taking means of late gestation data in this way does reveal a significant early bradycardia and increases in both MAP and CaBF during the insult with further significant increases in all three variables after release of the occlusion, although these take approximately 15 minutes to develop.

Post occlusion changes are also seen at mid gestation. A transient hypertension and tachycardia develops which is significant 5 minutes after release of the occlusion. Comparison with NIRS data show that HbO₂ is also significantly elevated at this time. All variables have returned to control after 60 minutes of recovery.
Figure 3.1 Progressive data for mid gestation fetuses for one hour before and after a 20-minute occlusion of the common internal iliac artery. These data show a rapid bradycardia after the start of the insult, all of the variables are at baseline levels at the mid-point of the insult before beginning to fall toward the end of the occlusion. There is a hyperaemia after release of the occlusion, after which there is a period of stabilisation, with the variables returning to pre-occlusion levels. Statistical difference from pre-occlusion control indicated by $a, p < 0.05$, paired t-test vs. control.
Figure 3.2 Minute mean data for late gestation animals shows progress of the haemodynamic response, aspects of which are lost when analysing given time points. Here it can be seen that there is a transient hypertension during the insult and another shortly after reversal of occlusion. Carotid flow also increases both during and after reversal of occlusion. Heart rate also follows the same pattern. \( a p < 0.05 \), repeated measures ANOVA vs. control with Newman-Keuls post hoc test.
3.3.4 Oxygen delivery and consumption

3.3.4.1 Mid gestation fetuses

Owing to the technical difficulties with arterial catheters explained in section 3.3.2, data for O₂ delivery and consumption at mid-gestation needs to be interpreted with caution. Data points for mid-gestation fetuses shown in Figures 3.3 and 3.4 without error bars have \( n \leq 2 \) and should be considered qualitative only.

Despite these provisos, these data are instructive. Firstly, O₂ delivery to the head in mid-gestation is significantly less than that in late-gestation, values being 1.20 ± 0.17 and 4.79 ± 0.48 ml/min, respectively, \( p < 0.05 \). There was also a significant reduction in DO₂ to the head during decreased uteroplacental flow, from 1.2 ± 0.17 to 0.44 ± 0.14 ml/min, \( p < 0.05 \). The O₂ consumption (\( VO_2 \)) was about 25% of O₂ delivery in control conditions and \( VO_2 \) by the brain during reduced uteroplacental perfusion fell from 0.33 ± 0.08 to 0.12 ± 0.03 ml/min \( (p = 0.08, \text{ power of test} = 0.3) \). Delivery, but not consumption, returned to control by 60 minutes after release of occlusion.

3.3.4.2 Late gestation fetuses

Figure 3.3 (lower panel) shows how much larger DO₂ to the brain is in late gestation compared to mid gestation. In late gestation, DO₂ had decreased significantly by 5 minutes after onset of the occlusion (to 1.86 ± 0.17 ml/min, \( p < 0.05 \)) and remained significantly reduced at 55 minutes after the onset of occlusion (at 1.97 ± 0.72 ml/min, \( p < 0.05 \), Figure 3.3) before returning to control levels at reversal +60 minutes.

\( VO_2 \) by the head was similarly reduced from 1.85 ± 0.26 to 0.48 ± 0.07 ml/min \( (p < 0.05) \) at 5 minutes and remained significantly reduced at 55 minutes at 0.80 ± 0.44 ml/min \( (p < 0.05) \). Again delivery had returned to control levels 60 minutes after release of the occlusion, whilst consumption may be slightly reduced. For DO₂ raw data, \( n = 8, 7, 6 \) and 7; for \( VO_2 \) raw data \( n = 6, 4, 2 \) and 3 for the bars shown in Figure 3.3, lower panel.

3.3.5 NIRS measured changes in \([HbO_2]\), \([Hb]\) and \([CcO]\)

NIRS data showing changes in the concentration of HbO₂ and Hb in fetal brain at 0.85 and 0.65 of gestation are shown in Figures 3.5 and 3.6, respectively.
Figure 3.3 Data for mean change from baseline of O$_2$ delivery to and consumption by the head of the mid (Upper panel) and late (Lower panel) gestation fetus. The mid gestation fetus has a lower basal metabolic rate and, although the fall in delivery is relatively modest, this allows it to reach a lower absolute DO$_2$ during the insult, compared to the older animals. Within group analysis vs control by repeated measures ANOVA with Newman-Keuls post hoc test $(\alpha, p < 0.05)$. No analysis done using last two time points for mid gestation VO$_2$, owing to presence of only a single observation.
Figure 3.4 Change in $O_2$ delivery and consumption for the brain in the mid (upper panel) and late gestation (lower panel) fetal sheep, expressed as a percentage of control (-5 minutes), which is taken as 0% change and therefore not shown. Within group analysis using repeated measures ANOVA with Newman-Keuls post hoc tests indicated significant differences in DO$_2$ during the insult at both ages and in VO$_2$ at the start of the insult in late-gestation ($\alpha p < 0.05$ vs -5 minutes), $n$ values are shown in columns. The small number of observations in mid-gestation reflect the difficulty in maintaining patent catheters for blood sampling in these animals.
3.3.5.1 Mid gestation:
Baseline data were very stable, usually deviating less than 5 μmol/l, peak to trough, over the hour control period. Rapid reciprocal changes in concentrations of HbO₂ and Hb were seen, as illustrated in the example data (Figure 3.7, Upper panel) which were significant at 5 minutes into occlusion (p < 0.001). Figure 3.5 shows mean data - HbO₂ fell to −19.0 ± 2.0 μmol/l by the end of the insult, whilst Hb reached +29.3 ± 0.5 μmol/l. The arithmetic sum of HbO₂ and Hb gives total Hb (tHb, an index of cerebral blood volume. tHb rose significantly, reaching +10.2 ± 4.1 μmol/l just prior to release of occlusion.

CcO oxidation slowly increased over the period of the insult and, although it was not different to control after 5 minutes of occlusion, it had increased significantly to +0.18 ± 0.13 μmol/l by the end of the insult (p < 0.05). CcO had returned to control levels by the end of 60 minutes of recovery.

3.3.5.2 Late gestation:
Control HbO₂ and Hb were stable during the control period. With the onset of common internal iliac occlusion, HbO₂ fell rapidly within the brain reaching −18.5 ± 2.2 μmol/l at +5 minutes (p < 0.05 vs. control). At the same time there was a rapid reciprocal rise in Hb to +27.0 ± 3.7 μmol/l (p < 0.05). Both HbO₂ and Hb levels rose slowly thereafter, reaching −16.1 ± 4.1 and +36.8 ± 3.0 μmol/l, respectively just prior to release of the occluder (p < 0.05). These findings indicate that there was a fall in cerebral oxygen saturation coincident with the fall in oxygen delivery described in section 3.3.4.2. With reversal of the occlusion, HbO₂ and Hb slowly returned to control levels.

In late gestation animals, CcO oxidation increased slowly over the period of the occlusion and was significantly elevated at 5 minutes prior to release of the occluder at +0.87 ± 0.22 μmol/l (p < 0.05). During the recovery period, CcO oxidation fell back towards control, and was not significantly different to basal levels 60 minutes after release of the occluder.
Figure 3.5 Mid gestation fetal sheep exposed to maternal common internal iliac artery occlusion responded in a similar manner to their late gestation counterparts (see Figure 3.6). The differences between the NIRS responses of these animals is better seen by looking at example experiments. From left to right, blocks represent time points: Control, Occlusion +5 min, Reversal −5 min and Reversal +60 min. \( a \ p < 0.05 \) (repeated measures ANOVA, vs. Control with Newman-Keuls post hoc test). \( n = 10 \).
Figure 3.6 NIR data for late gestation fetal sheep subjected to maternal common internal iliac artery occlusion. Bars are as in Figure 3.5, above. Statistical analysis (A) Within age group by repeated measures ANOVA with Newman-Keuls post hoc test ($a, p < 0.05$). (B) Between age groups by unpaired t test ($b, p < 0.05$). $n = 9$. 
3.3.6 Optical data

The raw data shown in Figure 3.7 describe a typical response to infusion of 10mg/ml/min NaCN on cerebral 
[HbO₂], [Hb] and [CcO]. NaCN causes a maximum fall of 0.96 μmol/l. This occurs despite changes in [HbO₂] and 
[Hb]. No further reduction of CcO was seen during terminal deoxygenation occurring at the end of the experiment. 
In order to exclude the possibility of artefact arising from the presence of a chromophore not accounted for, or of 
optical pathlength changes, further optical tests were performed on the data. Analysis of residuals left after fitting 
the data showed no significant or systematic change during hypoxia.

Control values in the brain were 4.5 ± 1.8 mOD (sum of mean squares) vs. 6.4 ± 1.7 during hypoxia (p = 0.48). 
Similarly, optical pathlength at 840nm did not change significantly in the brain during the insult being 12.0 ± 2.8 cm 
(mean ± SD) before and 12.3 ± 3.8 cm during the period of hypoxia (p = 0.84).

Figure 3.7 Since the NIRS system interrogates both the arterial and venous compartments, bolus injection of 
10mg/kg NaCN (Arrow) results in the apparent oxidation of Hb as O₂ use at CcO is reduced. Alterations in HbO₂ 
and Hb at t=26 min are caused by the fall in arterial saturation as the fetal heart fails.
3.3.7 Electrocorticogram (ECoG) data

Owing to the thinness of the skull in mid-gestation and the resultant difficulty in implanting electrodes without damaging the fetal brain, ECoG data was recorded from late-gestation but not from mid-gestation animals. These data have not been analysed, since in these experiments their primary purpose was to confirm that sleep-state cycling was occurring and whether isoelectricity was achieved during the hypoxic insult.

Figure 3.8 shows an example of normal ECoG cycling during baseline recording of the late-gestation sheep, just prior to the start of an experiment. Low voltage states are usually of 10-15 minutes duration, with high voltage activity lasting approximately 10 minutes.

![Figure 3.8 Normal control ECoG trace, showing cycling between high and low voltage, which are associated with low and high activity states, respectively.](image)

After the start of hypoxia, the ECoG record was used to assess the severity of the insult, by confirming whether isoelectricity had been achieved, as shown in Figure 3.9.

![Figure 3.9 Chart recording showing ECoG response to common internal iliac artery occlusion. With the fetus in the high voltage state, the occlusion is started (bar). There is a short period of what appears to be fitting activity, after which the ECoG trace becomes isoelectric. The ECoG returns towards normality after release of the occlusion (far right).](image)

These data show the expected cycling activity during normoxia and responses to hypoxia, thus they are not analysed in detail. Full analyses of fetal ECoG, its development, cycling and responses to hypoxic challenges can be found elsewhere (Boddy et al., 1974; Clewlow et al., 1983; Connell et al., 1988; Gunn et al., 1992; Richardson et al., 1985; Samat & Samat, 1976; Walker et al., 1984; Westgate et al., 1998).

3.3.8 Histology

Histological examination of five brains was performed by Dr Rosemary Scott as described in Section 2.8. Of the four brains subjected to asphyxia taken for analysis, three showed evidence of injury whilst the fourth did not.
Table 3.4 Histological analysis of location of brain injury in the late-gestation animal subjected to maternal common internal iliac artery occlusion (UAO), showing presence (Y) or absence (N) of injury. SAH = Subarachnoid haemorrhage.

The types of damage seen in these brains is varied, with no distinctive pattern or distribution of injury. There was one case each of PVL, basal ganglia infarction, subarachnoid haemorrhage and midbrain cystic change.

Examples of the histological appearance of these injuries are shown below.

Figure 3.10 Histological appearance of normal brain at 0.85 gestation. The upper panel shows the macroscopic appearance of a whole brain slice, whilst the lower panel shows the microscopic appearance of normal white matter.

In the lower panel, note the obvious striations of the white matter, the relatively sparse cellularity of the tissue and how avascular it appears.
Figure 3.11 Medium power appearance of periventricular leukomalacia (PVL) at 0.8 gestation. One area of PVL is labelled to show the inner area of necrosis (umbra) and the surrounding ischaemic penumbra. Cerebral sulci and lateral ventricle containing choroids plexus are also labelled.

Figure 3.12 Basal ganglia infarct following fetal asphyxia at 0.8 gestation. The infarcted area is surrounded by a diffuse pale-staining penumbra which contains an increased number of blood vessels. More normal basal ganglia can just be seen at the far right of the image.
Figure 3.13 Some apoptosis is still evident even three days after the insult which caused brain injury. In these two panels, apoptotic cells are just visible as small, densely-stained nuclei. These cells would eventually have broken up and been phagocytosed by resident microglia, the macrophages of the brain. Note also the loss of the obvious striated appearance and relatively sparse cellularity of the normal white matter in Figure 3.10 (lower panel).
3.4 DISCUSSION

This chapter compares the responses of mid and late gestation fetal sheep to moderate asphyxia caused by acute reduction in uteroplacental perfusion.

3.4.1 General considerations

It was standard practice during these experiments to monitor arterial pressure, venous pressure, fetal heart rate and carotid artery blood flow. In addition, in late gestation fetuses, electrocortical activity and femoral blood flow were monitored.

ECoG measurements were not performed on the younger fetuses owing the difficulty found in drilling through the fetal skull using the hand drill available at the time. The bones of the skull at this gestation are extremely thin and not yet fused; this meant that any pressure put on the skull with the hand drill shifted the skull plates and would have caused damage to the brain before burr holes could have been made. Since the extant evidence suggests that cycling of EC0G would not have been developed in the sheep at this gestation, we felt it a lesser evil to have no EC0G data than to damage the brain trying to obtain the data. Thus, we were not able to observe whether the EC0G becomes isoelectric in these fetuses during profound hypoxic insults.

Femoral arterial flow data was also not obtained in mid gestation fetuses. However, the late gestation fetuses were instrumented with femoral arterial and venous catheters as well as NIRS optodes, EMG electrodes and a femoral artery flow probe (data presented in Chapter 4). It was felt that the technical difficulty of instrumenting the hind limb would increase the length of the operation and produce substantial post-operative mortality in the younger fetuses. I therefore decided to use the minimum number of instrumentation procedures necessary to obtain meaningful data for the study.

Because of apparent differences in tolerance of mid gestation animals to the insult, it was not possible to maintain the insult for a set period. Because of this, mean data for that group on a minute-by-minute basis could not be obtained. Instead, I have obtained means at set times relative to the start and the end of the occlusion. These time points are Control (5 minutes prior to the start of occlusion), Occ +5 (5 minutes into occlusion), Rev –5 (5 minutes before reversal of occlusion), Rev +5 (5 minutes after reversal) and Rev +55 (55 minutes after reversal). This method is not perfect for the obvious reason that the variables at the end of a 15-minute occlusion might not be comparable to those at the end of a 30-minute occlusion but, despite this reservation, variability at the selected time points within the mid-gestation group are small (small standard error bars in Figure 3.5) and significant changes do emerge between control and hypoxia.
Owing to the generalised absence of quality data for venous pressure, analysis of this variable has been excluded.

3.4.2 Haemodynamic changes during hypoxia

3.4.2.1 Mid-gestation

No publications could be found in the literature describing the haemodynamic responses of mid-gestation fetuses to acute arrest of uterine artery flow. Using the same equipment and techniques in mid- and late-gestation, we have seen that the early stages of the response to hypoxia were not significantly different in the mid- and late-gestation animals, with transient bradycardia and variable pressure and flow responses. However, after between 8 and 50 minutes (mean 21 minutes) the circulatory compensation began to fail, with arterial pressure and carotid flow beginning to fall below control levels. This happened despite the fact that the fetuses were not severely hypoxic (minimum PO$_2$ = 12.0 ± 2.2 mmHg) and had not had time to develop an acidaemia (minimum pH = 7.22 ± 0.05). The mechanism of this failure of the circulatory compensation is not known, but is believed to be linked to depletion of myocardial glycogen stores (Block et al., 1990).

3.4.2.2 Late-gestation

The responses of the late-gestation fetus (circa 125 days) to hypoxic challenges of varied duration, intensity and origin have been extensively studied (see Jensen & Berger, 1992 for review). However, there is little published data on the hypoxic responses of mid- or early-gestation chronically instrumented fetal sheep. This is presumably due to:

a) The technical difficulty of instrumenting these fetuses, and
b) A high post-operative loss rate.

The absence of attempts at systematic gestational dissection of fetal stressor responses and their clinical impact means that a "developmental map" of the onset and maturation of the fetal responses to the commonly used stressors, such as the different hypoxias, is mostly absent from the literature. In this section, I will review current opinion on late gestation fetal response to reduced uterine perfusion.

The response of the late gestation fetus to acute hypoxia is usually described as consisting of a transient bradycardia with hypertension, an increase in carotid arterial flow and circulatory redistribution in favour of heart, brain and adrenal glands, at the expense of other organs and the carcass. A study of the literature indicates that this is only broadly true. Chemoreflex early bradycardia is the only feature truly common to all hypoxic challenges reported in the literature. Neither early hypertensive nor cerebral hyperaemic responses are consistently reported; these appear to be responses to a milder challenge and are absent with the severest insults. Indeed, with a severe insult such as combined common internal iliac occlusion and maternal
inhalation hypoxia, it appears that the ability of the fetus to initiate circulatory compensation is attenuated since bradycardia is accompanied by a severe hypotension and reduced carotid flow (Jensen, Hohmann & Kunzel, 1987). These differences in responses to hypoxic insults can be illustrated from our own data. In the late-gestation experiments, we used an occluder around the maternal common internal iliac artery to reduce uteroplacental flow by approximately 90% for at least one hour. During occlusion, we observed bradycardia with variable arterial pressure and carotid flow responses, the two extremes of which are shown in Figure 3.14. In these two particular cases, fetal PO$_2$ fell to 12 mmHg after 5 minutes in the mild insult (top panel) and to 4 mmHg in the more severe case (bottom panel) whilst temperature-corrected arterial pH at 5 minutes was 7.17 and 6.93, respectively.

Even though the circulatory compensation of the fetus during hypoxia is stated in the literature as protecting O$_2$ delivery to the heart, adrenals and brain, intuitively it seems improbable in these severe cases that fetal circulatory compensation could maintain O$_2$ delivery, since both O$_2$ content and carotid arterial flow are reduced. Using carotid flow measurements, we have determined that there is a fall in O$_2$ delivery to the head during asphyxia (Figures 3.3 and 3.4, upper left-hand panels) of approximately 60% ($p < 0.05$), which is in line with the data of Jensen for arrest of uterine flow (Jensen et al., 1987). This study indicates that general flow to the brain falls, with the fall being comprised of reduced flow to cortex, cerebellum and choroid plexus whilst that to midbrain, medulla and brain stem is increased. Unfortunately, it is not clear from the literature whether changes in carotid flow are the cause or the effect of changes in cerebral metabolism, and whether a fall in carotid flow can occur without cerebral hypotension. If cerebral blood flow in the fetus is exquisitely sensitive to changes in brain metabolic rate, as in the adult, then reduction in cortical flow may be functionally related to the depression of electrocortical activity witnessed during profound hypoxias, when the ECoG voltage can be seen to become isoelectric.

Work in vitro has shown that at the onset of hypoxia neurons very quickly reduce their metabolic rates by reducing ATP demand from cellular processes, including the Na$^+$/K$^+$ pump, but without any change in transmembrane ion gradients (Hochachka et al., 1996). This is achieved through a sharp reduction in membrane permeability and through a down-regulation of synaptic transmission, presumably by adenosine-mediated inhibition of excitatory amino acid release. In this way there is a relative increase in inhibitory amino acid levels. If the functional link between cerebral flow and metabolism is not present in the fetus, then cerebral VO$_2$ could fall or rise independently of changes in carotid arterial flow. In fact, the data presented here suggests that there is little change in carotid arterial flow during asphyxia and that VO$_2$ is significantly reduced.
Figure 3.14 Haemodynamic responses to acute reduction in uteroplacental flow at late gestation vary between that in the upper panel to that in the lower. These differences make mean data a relatively poor tool in assessing the fetal responses.

The second phase of the haemodynamic response to asphyxia is driven by the release of vasoactive hormones such as catecholamines, cortisol and arginine vasopressin (Rurak, 1978; Martin, Kapoor & Scroop, 1987; Raff, Kane & Wood, 1991; Giussani et al., 1993 & 1996). In this phase, the compensating fetus increases heart rate, maintains arterial pressure and keeps the periphery tightly vasoconstricted. Thus blood is shunted away from peripheral tissues and made available for use by heart, adrenals and brain, to which
flow may be increased above basal levels (although it is unknown whether their metabolic rates are maintained, increased or decreased).

3.4.3 Near Infrared findings

3.4.3.1 Mid gestation

Mean data for cerebral NIRS responses to hypoxia are shown in Figure 3.5. The response to reduction in uteroplacental perfusion is a rapid fall in HbO$_2$ and increase in Hb. Total Hb (tHb) rises more slowly, reaching a plateau of around 10 μmol/l above control, indicating that, all else being equal, cerebral blood volume is increasing during the insult, although we cannot say what proportion of the rise is in the arterial or venous compartment.

tHb begins to fall again not long after a plateau is reached, this fall being due to falls in both HbO$_2$ and Hb. When tHb began to fall, mid-gestation fetal haemodynamic variables also show that the fetal circulatory compensation for hypoxia was beginning to fail. Mean arterial pressure, heart rate and carotid flow all began to decline. The few fetuses in which the occlusion was allowed to continue beyond this point died within ten minutes and we were not able to recover them with intravenous injections of adrenaline and bicarbonate. Owing to this, careful note was always taken when tHb began to fall and the fetal haemodynamics were not allowed to decline to dangerous levels; release of occlusion was usually performed soon after tHb began to fall.

After release of the occluder, Hb rapidly came back to control levels. HbO$_2$, however, rose to a significantly higher level than control ($p < 0.05$). As a consequence, tHb was also high at this point and this increase in tHb was associated in some experiments with increases in heart rate, mean arterial pressure and carotid flow at the same time (see Figure 3.15 for one such comparison). Many authors have noted that the distressed fetus has a pressure passive cerebral circulation (Lou, Lassen & Friis-Hansen, 1979; Tweed et al., 1986; Helou et al., 1994); the rise in carotid flow after release of the occluder provides evidence for this in the mid-gestation fetus. NIRS variables came back to control levels quickly and were not different to control at 60 minutes after release of the occluder.
Figure 3.15 Changes in mean arterial pressure and cerebral tHb (related to blood volume) follow a very similar time course indicating, perhaps that the cerebral circulation is prone to venous congestion when mean blood pressure rises.

Mean cytochrome oxidase oxidation state increased progressively over the period of the occlusion and was significant at 5 minutes before reversal ($p < 0.05$, repeated measures ANOVA with Newman-Keuls post hoc test). Figure 3.3 shows that, whilst CcO redox was increasing (although not significantly) after five minutes of occlusion, oxygen consumption across the head had fallen significantly. Unfortunately after this time point there are not enough paired blood gases to be able to form any conclusions about later changes in consumption, although the raw data is suggestive of a progressive fall (Figure 3.3, upper right panel). Owing to this, we are unable to perform a correlation between the progression of change in VO$_2$ with that of CcO. No papers were found in literature searches providing correlations between VO$_2$ and NIRS CcO data, so no extrapolations can be made from others’ work, either. Hopefully it will be possible to measure VO$_2$ and CcO simultaneously at a later date; this would provide some important insights into the metabolic adaptation of the fetus to an hypoxic challenge.

3.4.3.2 Late gestation

Most of the published work using near infrared spectroscopy in vivo concentrates on neonates and late gestation fetuses, with little having been published on mid or early gestation. In this study, cranial NIRS was used to interrogate mid and late gestation fetal responses to reduced uteroplacental flow.
Responses in late gestation were similar in both time course and magnitude in each experimental animal. The responses of HbO₂ and Hb are simple extrapolations of the expected changes in haemoglobin saturation during an hypoxia. In other words, if blood volume does not change, HbO₂ levels should fall and Hb levels should rise by equal amounts as haemoglobin desaturates. The consistent finding was that the magnitude of the increase in Hb was slightly larger than the decrease in HbO₂ (for example HbO₂ decrease to -18.5 ± 2.2 µmol/l, whilst Hb increases to 27.0 ± 3.7 µmol/l at 5 min into the occlusion, Figure 3.6).

In order to make some sense of the NIRS concentration changes, we can convert all concentrations into µmol/l, as given by the NIRS software. The following is a short explanation of how was done using available data and estimated tissue density:

Sheep blood volume ~61 ml/kg tissue (from UCL Biological Services handbook)
Brain weight ~42g (mean data)
Brain density ~1.05 g/ml (estimate)

:. Brain volume ≈ 42/1.05 = 40 ml
Cerebral blood volume = 61 ml/kg x 42g/1000 = 2.56 ml

[Hb] ~65 g/l (mean data)
MW fetal Hb ~62,000 (estimate)

:. [Hb] = 65/62000 = 1.05 mmol/l = 1.05 µmol/ml

If CBV = 2.56 ml and [Hb] = 1.05 µmol/ml, then brain tHb

= 1.05 x 2.56 = 2.63 µmol Hb in 40 ml of brain

⇒ [Hb] = 2.63/40 x 1000

= 65.75 µmol Hb per litre of brain tissue

At a mean 73% saturation, this means that there is ~49.3 µmol/l of HbO₂ and 16.4 µmol/l of Hb in the average fetal brain at this gestation. This data can now be used to calculate approximate change in fetal CBV during hypoxia.

The arithmetic sum of HbO₂ and Hb signals gives us absolute change in the total Hb signal and this, as is explained in Section 2.7.2, is related to blood volume through the haematocrit. In the absence of large changes in haematocrit, we can equate changes in tHb directly with cerebral blood volume changes. This in turn allows us to make guesses as to what may be happening to arterial and venous volumes and, by default, to vascular tone. As mentioned above, our consistent finding was that Hb change was larger than HbO₂ change, indicating that cerebral blood volume was increasing at the onset of hypoxia. Figure 3.6 shows mean data for this change in late gestation fetuses as measured by NIRS. These data have not been changed into absolute cerebral blood volumes...
using the method employed by Wyatt and co-workers (Wyatt et al., 1990) owing to our inability to use their methodology. Studying neonates, this group was able to supply step changes in arterial O$_2$ saturation by altering the inspired O$_2$ fraction, and this allowed the calculation of cerebral blood volume. However, since the fetus extracts O$_2$ from the maternal blood, step changes in arterial saturation are difficult to produce experimentally, effectively preventing us from using this technique to precisely calculate CBV. However, by looking at the blood gas results for these fetuses and making a few assumptions, we can make an informed guess at the control blood volume and extrapolate that to CBV during hypoxia. On page 107 I calculated a tHb of 65.7 $\mu$mol/l in the fetus. If fetal tHb is 65.7 $\mu$mol/l as calculated above, then a mean increase of 20 $\mu$mol/l represents an increase of 20/65.7 x100 $\approx$ 30% of control cerebral blood volume, or 0.8 ml of blood.

This initial rise in cerebral volume develops over about ten minutes and may be related to early arterial pressure changes. This is illustrated by the example data in Figure 3.15; here MAP and tHb changes are practically superimposable. The change in cerebral volume occurs when mean carotid artery flow is also increased (Figures 3.1 and 3.2) and indicates that the cerebral circulation has become pressure passive. Pressure passivity in these circumstances is, as already mentioned, well-documented; alterations in CBF having been observed in the sheep fetus (Makowski et al, 1972; Lou et al., 1979) and in the severely asphyxiated newborn dog (Hernandez et al., 1978). The correlation between the two variables does not always hold true after the common internal iliac artery occlusion is released, when MAP and tHb traces in these experiments were generally not correlated. The reason for lack of correlation after the insult is unknown.

3.4.4 Metabolic rationale for increased CcO oxidation in hypoxia

Some difficulty was experienced in trying to explain why mitochondrial redox state (measured by NIRS as oxidised CcO oxidase concentration changes) should increase (oxidation of CcO) during fetal hypoxia. Other work using NIRS on the neonatal animal, during human parturition and with the adult human indicates that CcO becomes reduced during hypoxia. CcO is reduced in these postnatal and adult experiments because, in the absence of any change in brain metabolic rate, O$_2$ becomes a limiting factor in mitochondrial respiration. This means that electrons build up in the electron transfer chain (Figure 1.11). When the CcO Cu$_a$ centre accepts an electron, it becomes reduced and only oxidises again when this electron is passed on. All else being equal, as PO$_2$ falls during hypoxia, CcO turnover rate must fall since less O$_2$ is available to accept electrons from CcO. In this circumstance, CcO oxidation falls as electron turnover by Cu$_a$ slows.

Why should the metabolic response to hypoxia be any different in the fetus? It is known that fetal tissues are able to withstand anoxia for far longer than the adult, where neuronal death begins after approximately two minutes exposure. The mid gestation fetus is reported (Bennet et al., 1999) to be able to withstand umbilical cord occlusion for 30 minutes without sign of neuronal damage. This anoxic tolerance may be provided, in part, by the lower metabolic rate of the fetus; and younger fetuses are thought to have much lower metabolic rates than those at
term, a fact which is backed up by our own consumption data in Figure 3.3. In vitro evidence of hypoxic CcO oxidation has recently emerged (Yager, Brucklacher & Vannucci, 1996), which suggests that the flow of reducing equivalents to the mitochondrial electron transfer chain may be the limiting factor to CcO turnover during hypoxia. Some degree of metabolic control in the fetus is thought to be provided by adenosine, possibly acting through production of nitric oxide.

Adenosine concentration in fetal plasma and cerebrospinal fluid is in the region 1.0-1.9 μmol/l, roughly three to fivefold times higher than in the maternal plasma. Adenosine infused into the fetus at a rate that simulates concentrations similar to those found in the fetus during hypoxia reduces whole-body metabolic rate (Karimi et al., 1996). Fetal plasma adenosine levels rise during hypoxia, suggesting that there is a greater suppression of metabolism with more severe hypoxic insults. The mechanism by which adenosine brings about metabolic suppression is not known, but some speculation exists as to whether this may be through the stimulation of nitric oxide production. Nitric oxide is known to compete with O₂ for the cytochrome oxidase binuclear centre where O₂ is reduced to water, such that metabolism is inhibited in a manner which is dependent upon the relative concentrations of O₂ and NO. Nitric oxide in very high levels for extended periods is also able to inhibit mitochondrial respiration at mitochondrial complex I by permanent nitrosylation of protein thiol groups in the complex I protein.

Since the NIRS CcO oxidation signal increases as electron turnover at the CcO binuclear centre falls, a CcO oxidation during hypoxia can only occur there is a reduction in the flow of electrons in the mitochondrial electron transport chain. This can be achieved in at least two ways. Firstly, if there is a reduction in the flow of reducing equivalents to the mitochondria during hypoxia or, secondly, if there is a block on electron transfer down the transfer chain upstream of complex IV. In this way, fewer electrons than normal would be moving though CcO Cu₄ and it would be seen to oxidise using NIRS. Unfortunately, neither the fast action of NO at complex IV (cytochrome oxidase), nor the slow action of NO at complex I would produce this effect within the timescale of these experiments. There is also some indication in the literature that NO, acting through peroxynitrite, may be able to influence metabolism at complex III (Lizasoain et al., 1996). This is an interesting possibility which might fit the bill of a fast inhibitor of the respiratory chain to prevent electron flow in a dose-dependent, hypoxia-dependent fashion. If this is not the case, however, it may still be that adenosine alone is able to directly influence metabolism. In vitro experiments are being performed to try to determine the relative importance of adenosine and NO in mitochondrial respiration (Rat brain data given in Appendix D).

There is, therefore, strong evidence of metabolic suppression during hypoxia in fetal brain. The O₂ sensor has yet to be positively identified, but increases in adenosine may be a mediator of the hypometabolism. Further work is required using these (and other) techniques to dissect this problem.
3.5 Summary

This work has demonstrated that the NIRS system can be used to quantify absolute changes in levels of HbO₂ and Hb, albeit from an arbitrary zero, and that these data provide an index of cerebral blood volume in the tHb variable. It has also shown that falling tHb during hypoxia may be a characteristic marker, at least in mid gestation, for the beginning of failure of the fetal circulatory compensation associated with hypoxic episodes.

We have demonstrated that fetal O₂ delivery to and consumption by the brain differ in mid and late gestation and confirmed the fetal haemodynamic changes associated with severely reduced uteroplacental flow presented by other authors. Our NIRS data show that CcO oxidation increases during hypoxia, perhaps a compensatory mechanism to help maintain ATP levels during reduced O₂ availability. We believe that our observation of a significant hypertension-hyperaemia immediately after release of the occlusion in the mid gestation fetus may be the first of its kind, and suggest that this may be the response of a fetus which will go on to recover from the insult.
CHAPTER 4

NIRS IN THE MEASUREMENT OF THE PERIPHERAL RESPONSE TO ACUTE ASPHYXIA IN LATE-GESTATION FETAL SHEEP IN UTERO

4.1 INTRODUCTION

In chapter 3 I described the fetal cerebral compensations to asphyxia at late-gestation which included variable increases in carotid flow and mean arterial pressure and an increase in cerebral blood volume. Late-gestation fetuses also responded to asphyxia by altering peripheral haemodynamics. Using microspheres, previous authors have calculated that there is a fall in peripheral blood flow soon after the onset of maternal hypoxaemia (Cohn et al., 1974). Such a peripheral vascular response increases peripheral resistance substantially and is believed to have the primary purpose of maintaining perfusion pressure for "central" organs (in this instance, central organs are the adrenals, heart and, usually, brain) at the expense of peripheral tissues including lung, skin, gut and skeletal muscle (Cohn et al., 1974). The degree to which these peripheral vascular changes are accompanied by peripheral metabolic alterations is not known.

If the fetus is to survive an extended period of severely lowered oxygen delivery, curtailments of O$_2$ demand must be made. Adaptations can be made from the molecular to behavioural levels to help achieve this, and evidence has been provided for numerous adaptations, including cessation of fetal breathing and body movements in vivo (Boddy et al., 1974; Clewlow et al., 1983) and active metabolic suppression in vitro (Chandel et al, 1997; Budinger et al., 1998; Silverman et al., 1997; Braems and Jensen, 1991) during experimental hypoxia. The ability to reduce cellular energy-demanding processes in response to a fall in tissue PO$_2$ suggests the presence of an O$_2$ sensor coupled to a transduction system targeted to effectors for the desired response. At present, however, the nature of such an O$_2$ sensor and its transduction system in fetal skeletal muscle remains unknown.

The peripheral vascular response to fetal hypoxia is abolished by carotid denervation (Bartelds et al., 1993), indicating that this particular aspect of the peripheral response, at least, is mediated by carotid chemoreceptors. Recent work with embryonic cardiomyocytes in vitro has provided evidence that peripheral metabolism may be controlled at the local tissue level by cells' own mitochondria. This and other putative mechanisms for O$_2$ sensing have been proposed (Bunn & Poyton, 1996; Duranteau et al., 1998) which have yet to be tested on fetal skeletal muscle either in vivo or in vitro. In this study, therefore, we sought to provide evidence for down regulation of peripheral metabolism during fetal asphyxia using NIRS and traditional haemodynamic and blood sample monitoring.
4.2 METHODS

4.2.1 Fetal surgery

Eleven fetuses underwent hind limb surgery. As explained in Chapter 2, late gestation mule or Romney Marsh ewes bearing singleton fetuses were anaesthetised with 1g Intraval and 3% halothane and operated on using aseptic techniques. In brief, a small uterine incision was made and the hind limbs exteriorised. Taking care not to put strain on the umbilicus, catheters were placed in a femoral artery and vein, whilst on the contralateral side, a flow probe was placed around the femoral artery (3R, Transonic). Two NIRS fibre bundles in black rubber holders were sewn onto the muscle with sterile nylon thread (Braun, Germany) and the skin incision closed firmly to provide extra probe stability.

The fetal hind limbs were replaced in the uterus and the incision was closed in two layers with 2/0 silk using standard gynaecologic technique. A second uterine incision was made for the head and instrumentation completed as described in chapter 3. Following placement of a catheter in the maternal recurrent metatarsal vein, ewes were recovered from surgery, given 600mg Crystapen and 20mg Cidomycin intra-amniotically and maintained as described previously. Ewes were allowed three days recuperation before the experiment was performed.

4.2.2 Experimental protocol

Arterial and venous catheters were connected to pressure transducers (SensoNor 840, as in Chapter 3) and then into amplifiers (Digitimer). MacLab software was used for continuous monitoring of fetal heart rate, electrocortical activity, arterial pressure and carotid and femoral arterial blood flows. These data were saved to disk for off-line analysis. NIRS spectra were collected approximately every 15 seconds for the duration of the experiment and analysed off-line.

The experimental protocol consisted of an hour of control monitoring, of asphyxia induced by reduction in maternal uterine artery blood flow, and then one hour of post-occlusion recording (Figure 4.1). As explained in Chapter 3, reduced uterine perfusion was effectuated by maximal tightening of a screw occluder or filling of an inflatable occluder (In Vivo Metric, Healdsburg, US) around the maternal common internal iliac artery. Severity of insult was assessed by monitoring fetal cardiovascular and NIRS responses, and by blood gas analysis at 5 minutes into the occlusion. Tightening of the occluder was performed if necessary.
Figure 4.1 Experimental protocol - Time bar marked in hours. The following fetal variables were continuously recorded for 3 hours: fetal heart rate, mean arterial pressure, femoral artery blood flow and NIRS variables (HbO₂, Hb, CcO₂ - tHb was calculated off-line). Blood samples were taken where indicated by arrows. Maternal common internal iliac artery occlusion lasted for one hour (bar).

Fetal femoral arterial and venous blood samples (0.5 ml) were taken simultaneously at the time points indicated in Figure 4.1. Analysis for blood gases and electrolytes (BGE and Co-Oximeter, Instrument Laboratory, UK) and glucose and lactate (2300 STAT Plus, YSI) was performed. Using femoral flow data, where available, O₂ delivery and consumption was calculated for the hind limb using Fick's law:

\[ O_2 \text{ Delivery} = \text{Arterial } O_2 \text{ content } \times \text{Arterial flow} \]

ie

\[ \text{DO}_2 = C_a \cdot Q \]

From that, we can calculate O₂ consumption as:

\[ \text{VO}_2 = Q_c \cdot (C_a - C_v) \]

where \( \text{VO}_2 \) is O₂ consumption, and \( C_v \) is femoral venous O₂ content.

Similarly, using the haemodynamic equivalent of Ohm's law, femoral vascular resistance was calculated as:

\[ \text{Femoral Vascular Resistance} = \frac{\text{Driving Pressure}}{\text{Femoral Blood Flow}} = \frac{\bar{P}_a - P_v}{\text{FBF}} \]

Where \( \bar{P}_a \) is mean arterial pressure and \( P_v \) is venous pressure.

4.2.3 Statistical analysis

Group data were analysed using repeated measures ANOVA with Newman-Keuls post-hoc tests applied when significance was indicated. Comparisons between femoral arterial data and either venous data or carotid data at the same time point were analysed by unpaired Student's t-test. In all cases, significant difference was accepted when \( p < 0.05 \).
4.3 RESULTS

4.3.1 Cardiovascular variables

Fetal heart rate during control recording was 175 ± 7 bpm whilst mean arterial pressure was 45.6 ± 2.7 mmHg and femoral arterial blood flow was 26.8 ± 4.6 ml/min. Five minutes after the onset of maternal uterine artery occlusion, these were 115 ± 8 bpm, 47.6 ± 3.2 mmHg and 11.6 ± 1.9 ml/min, respectively (see Figure 4.2). At the end of the one hour occlusion, fetal heart rate and mean pressure had recovered to pre-occlusion values (160 ± 13 bpm and 45.4 ± 3.5 mmHg, respectively), whilst femoral artery flow was still reduced at 10.5 ± 2.0 ml/min. After release of the occluder, there was a period of increased mean arterial pressure and fetal heart rate (Figure 4.2 top and middle panels), but no hyperaemia since femoral arterial flow returned only slowly towards pre-occlusion levels (Figure 4.2, lower panel).

4.3.2 Femoral vascular resistance (FVR)

Figure 4.3 shows calculated FVR compared to cerebrovascular resistance (CVR) calculated at the same time points. During control recording FVR was 2.0 ± 0.4 mmHg/ml/min and CVR was 0.7 ± 0.1 mmHg/ml/min. FVR remained higher than CVR at all times during the recording. During the occlusion, FVR increased to a maximum of 8.3 ± 3.2 mmHg/ml/min at 30 minutes whilst there was no significant change in CVR during asphyxia. After release of the occluder, FVR remained high for a short while, but was back at pre-occlusion levels by 25 minutes after occluder release. CVR was not significantly changed during the hour after release of the occluder.

4.3.3 Femoral Blood Samples

Femoral arterial and venous blood sample data are shown in Tables 4.1 and 4.2, respectively. As with those gases for the head in these fetuses (Table 3.2), there were significant changes during hypoxia in all of the femoral arterial blood gas variables and in most of the venous variables.

There were no significant differences between the femoral and carotid arterial blood samples (Student’s t-test, see Table 3.2 for carotid data and Table 4.1 for femoral arterial data) despite documented differences in pre- and post- ductal blood composition in the ovine fetus. Although there was no difference in control arterio-venous [Glucose] or [Lactate] across the femoral vascular bed, control PO$_2$, PCO$_2$, haemoglobin saturation and O$_2$ content were significantly different ($p = 0.002$, $= 0.008$, $= 0.003$ and $< 0.001$, respectively, Student’s t-test control venous vs. control arterial samples). During asphyxia, there were significant arterio-venous differences in PO$_2$, base excess, haemoglobin saturation and O$_2$ content. At one hour after release of the occluder, there were significant arterio-venous differences for PO$_2$ and PCO$_2$ only.
4.3.4 O₂ delivery and consumption

Both delivery to, and consumption by, the hind limb fell significantly during asphyxia, returning to levels not significantly different to control by 60 minutes after release of the occluder (Figure 4.4). As a percentage of control DO₂, the fall at the beginning and end of asphyxia was 19.7 ± 4.2 and 19.3 ± 4.7 %, respectively. Sixty minutes after release of the occluder, DO₂ was at 92% of control value. Similarly, VO₂ was reduced to 31 ± 10 and 37 ± 13% at the beginning and end of asphyxia, respectively. Not enough samples were available to give data for recovery of VO₂. The VO₂ data for the leg were not significantly different to those for the head at the same time points.

4.3.5 Near Infrared Spectroscopy

NIRS allows the measurement, in units of μmol/l, of changes in the concentrations of HbO₂, Hb, tHb (related to blood volume) and oxidised CcO. Chromophore data are calculated as changes in concentrations (in μmol/l) from time zero and allow us to comment upon the haemodynamics and cellular metabolism of the tissue interrogated by the NIR light.

As can be seen in Figure 4.5, at the start of the occlusion, there is a rapid fall in HbO₂ from −2.8 ± 1.7 to −21.9 ± 2.6 μmol/l at 5 minutes into occlusion. As HbO₂ fall whilst there is no initial change in Hb, tHb (HbO₂ + Hb) is seen to fall at the start of the insult. As the hypoxia progresses, HbO₂ remains depressed but Hb begins to rise and thus tHb rises, coming back to control values by the end of the hour-long insult.

Cytochrome oxidase initially becomes more reduced after onset of occlusion. The level of oxidised CcO falls from 0.18 ± 0.12 to −1.08 ± 0.27 μmol/l at 5 minutes after the onset of the insult. It reaches a nadir after 10 minutes, before beginning to oxidise once more, and is at −0.71 ± 0.36 μmol/l just prior to release of occlusion.
Figure 4.2 Haemodynamics before, during and for one hour after maternal uterine artery occlusion (grey box). Fetal heart rate and mean arterial pressure are the same data as given in Figure 3.2. Data for femoral artery blood flow shows that a significant fall in flow rapidly occurs at the start of hypoxia; this reduction in flow comes back towards control levels after release of occlusion. $a$ indicates $p < 0.05$, Repeated measures ANOVA with Newman-Keuls post hoc tests vs. control at -60 min.
Figure 4.3 Femoral vascular resistance (FVR, green) and cerebrovascular resistance (CVR, blue). FVR, but not CVR, increases during asphyxia. The speed of onset of the increase suggests the neural reflex nature of the response and is in agreement with the literature (Giussani, Spence & Hanson, 1994). The lack of significant change in CVR provides evidence for differential regulation of this vascular bed and, perhaps, indicates its special status.

Figure 4.4 Oxygen delivery and consumption data for the leg calculated as described in section 4.2.2. For each graph, columns from left to right are - Control, Occlusion +5 minutes, Occlusion +55 minutes and 55 minutes after release of the occluder. Significant difference from control (leftmost column) by repeated measures ANOVA with Newman-Keuls post-hoc tests indicated by $a$, $p < 0.05$. Difference from equivalent data point for head (see Figure 3.4 ) indicated by $c$ (Student's $t$-test), $p < 0.05$. 
### Table 4.1
Arterial blood samples in control conditions, at the beginning and end of hypoxic insult, and one hour after the end of the insult. Statistical analysis: " indicates significant difference from control value (Occlusion -55 min) $p < 0.05$, Repeated measures ANOVA with Newman-Keuls post hoc tests. " indicates significant difference from venous values at same time point; $p < 0.05$, Student's t-test.

<table>
<thead>
<tr>
<th></th>
<th>Occlusion -55 min $n = 10$</th>
<th>Occlusion +5 min $n = 10$</th>
<th>Occlusion +55 min $n = 10$</th>
<th>Reversal +60 min $n = 8$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td>$7.330 \pm 0.016$</td>
<td>$7.229 \pm 0.025^a$</td>
<td>$7.101 \pm 0.043^a$</td>
<td>$7.242 \pm 0.027^a$</td>
</tr>
<tr>
<td><strong>PaCO$_2$ (mmHg)</strong></td>
<td>$45.6 \pm 1.7^v$</td>
<td>$58.7 \pm 2.5^a$</td>
<td>$61.5 \pm 4.9^a$</td>
<td>$48.2 \pm 0.79^v$</td>
</tr>
<tr>
<td><strong>PaO$_2$ (mmHg)</strong></td>
<td>$24.9 \pm 2.2^v$</td>
<td>$11.2 \pm 0.8^a, v$</td>
<td>$12.1 \pm 0.7^a, v$</td>
<td>$21.6 \pm 0.7^v$</td>
</tr>
<tr>
<td><strong>Hct (%)</strong></td>
<td>$25.8 \pm 1.4$</td>
<td>$31.9 \pm 0.8^a$</td>
<td>$29.6 \pm 1.0^a$</td>
<td>$26.5 \pm 0.95$</td>
</tr>
<tr>
<td><strong>HCO$_3^-$ (mmol/l)</strong></td>
<td>$24.0 \pm 1.5$</td>
<td>$24.1 \pm 1.1$</td>
<td>$18.6 \pm 1.1^a$</td>
<td>$20.7 \pm 1.3$</td>
</tr>
<tr>
<td><strong>BE (mmol/l)</strong></td>
<td>$-0.8 \pm 1.5$</td>
<td>$-2.4 \pm 1.4$</td>
<td>$-10.1 \pm 1.8^a, v$</td>
<td>$-5.7 \pm 1.8^a$</td>
</tr>
<tr>
<td><strong>Hb (g/dl)</strong></td>
<td>$7.2 \pm 0.4$</td>
<td>$5.9 \pm 0.3^a$</td>
<td>$5.6 \pm 0.4^a$</td>
<td>$6.3 \pm 0.6$</td>
</tr>
<tr>
<td><strong>Sat (%)</strong></td>
<td>$71.0 \pm 1.9^v$</td>
<td>$30.9 \pm 2.4^a, v$</td>
<td>$26.0 \pm 2.8^a, v$</td>
<td>$57.0 \pm 6.9^a$</td>
</tr>
<tr>
<td><strong>O$_2$ ct (vol % O$_2$)</strong></td>
<td>$7.1 \pm 0.3^v$</td>
<td>$2.6 \pm 0.3^a, v$</td>
<td>$2.1 \pm 0.3^a$</td>
<td>$4.8 \pm 0.7^a$</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
<td>$0.8 \pm 0.1$</td>
<td>$0.9 \pm 0.1$</td>
<td>$1.2 \pm 0.2^a$</td>
<td>$0.9 \pm 0.1$</td>
</tr>
<tr>
<td><strong>Lactate (mmol/l)</strong></td>
<td>$0.8 \pm 0.1$</td>
<td>$1.6 \pm 0.3$</td>
<td>$5.6 \pm 1.1^a$</td>
<td>$5.3 \pm 1.2^a$</td>
</tr>
</tbody>
</table>

### Table 4.2
Femoral venous blood sample data. Statistical analysis: " indicates significant difference from control value (Occlusion -55 min) $p < 0.05$, Repeated measures ANOVA with Newman-Keuls post hoc tests.

<table>
<thead>
<tr>
<th></th>
<th>Occlusion -55 min $n = 7$</th>
<th>Occlusion +5 min $n = 7$</th>
<th>Occlusion +55 min $n = 7$</th>
<th>Reversal +60 min $n = 5$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH$_v$</strong></td>
<td>$7.287 \pm 0.020$</td>
<td>$7.199 \pm 0.023^a$</td>
<td>$7.074 \pm 0.031^a$</td>
<td>$7.220 \pm 0.033$</td>
</tr>
<tr>
<td><strong>PaCO$_2$ (mmHg)</strong></td>
<td>$54.2 \pm 1.6$</td>
<td>$62.3 \pm 2.7$</td>
<td>$69.1 \pm 4.7$</td>
<td>$51.6 \pm 1.0$</td>
</tr>
<tr>
<td><strong>PaO$_2$ (mmHg)</strong></td>
<td>$18.6 \pm 0.4$</td>
<td>$7.3 \pm 0.8^a$</td>
<td>$7.7 \pm 0.8^a$</td>
<td>$17.2 \pm 0.6$</td>
</tr>
<tr>
<td><strong>Hct (%)</strong></td>
<td>$27.8 \pm 1.7$</td>
<td>$31.0 \pm 0.9$</td>
<td>$31.3 \pm 1.5$</td>
<td>$29.3 \pm 1.6$</td>
</tr>
<tr>
<td><strong>HCO$_3^-$ (mmol/l)</strong></td>
<td>$25.5 \pm 1.8$</td>
<td>$23.9 \pm 0.6$</td>
<td>$19.4 \pm 1.1^a$</td>
<td>$21.1 \pm 1.7$</td>
</tr>
<tr>
<td><strong>BE (mmol/l)</strong></td>
<td>$-0.3 \pm 2.0$</td>
<td>$-3.0 \pm 1.0$</td>
<td>$-9.6 \pm 1.6^a$</td>
<td>$-5.7 \pm 2.3$</td>
</tr>
<tr>
<td><strong>Hb (g/dl)</strong></td>
<td>$6.7 \pm 0.4$</td>
<td>$5.9 \pm 0.3^a$</td>
<td>$5.5 \pm 0.4^a$</td>
<td>$6.3 \pm 0.6$</td>
</tr>
<tr>
<td><strong>Sat (%)</strong></td>
<td>$55.5 \pm 2.2$</td>
<td>$17.6 \pm 1.6^a$</td>
<td>$16.3 \pm 3.3^a$</td>
<td>$51.6 \pm 3.1$</td>
</tr>
<tr>
<td><strong>O$_2$ ct (vol % O$_2$)</strong></td>
<td>$5.1 \pm 0.3$</td>
<td>$1.4 \pm 0.2^a$</td>
<td>$1.3 \pm 0.3^a$</td>
<td>$4.5 \pm 0.6$</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
<td>$0.5 \pm 0.1$</td>
<td>$0.6 \pm 0.1$</td>
<td>$2.6 \pm 1.7$</td>
<td>$0.7 \pm 0.1$</td>
</tr>
<tr>
<td><strong>Lactate (mmol/l)</strong></td>
<td>$0.8 \pm 0.1$</td>
<td>$1.6 \pm 0.4$</td>
<td>$5.4 \pm 1.1^a$</td>
<td>$4.7 \pm 1.4^a$</td>
</tr>
</tbody>
</table>
Figure 4.5 Near infrared spectroscopy data shows changes in HbO₂ and Hb (top panel), and in tHb and CcO (lower panel) before, during and after 1 hour of asphyxia (grey box). During the insult, HbO₂ falls without any fall in Hb before tracking back toward control levels. tHb is related to blood volume through the hematocrit and falls during the insult before returning to control levels after ~30 minutes of asphyxia. There is no evidence for post-asphyxial hyperaemia in this tissue.
4.4 DISCUSSION

4.4.1 Overview

During moderate asphyxia there was a significant fall in HbO₂, tHb and in CcO oxidation in femoral skeletal muscle and an increase in femoral, but not carotid, vascular resistance. This suggests a redistribution of the fetal circulation away from the periphery whilst blood flow to the brain was preserved (Chapter 3). Femoral skeletal muscle cytochrome oxidase progressively re-oxidised after its initial fall despite maintenance of uterine artery occlusion, although it failed to come back to control levels until after release of the occluder (Figure 4.6). However, blood volume in fetal skeletal muscle had returned to control levels after 30 minutes of uterine occlusion and there was no evidence of hyperaemia after release of the occlusion (Figures 4.4 & 4.7). Femoral arterial flow returned slowly to control levels during the hour after release of the occlusion (Figure 4.2).

![Figure 4.6 Data for CcO oxidation changes during asphyxia (box). Oxidation state fell quickly after onset of occlusion but returned to control levels slowly after release of the occluder.](image-url)

4.4.1 Haemodynamic response to hypoxia

It was the work of Cohn and colleagues using unanaesthetised fetal sheep in utero that established that, in the absence of confounding anaesthesia, the late-gestation fetus mounts a cardiovascular redistribution in response to hypoxaemia (Cohn et al., 1974). Maternal hypoxaemia, as used by Cohn, resulted in a rise in fetal combined ventricular output with increased flow to the adrenals, heart, brain and the uteroplacental unit at the expense of other, "peripheral", vascular beds. This scheme has been the basis upon which subsequent investigations have built.
In his 1974 paper, Cohn showed that the fetal responses to a "pure" hypoxia were altered when acidaemia developed. With hindsight, this observation pre-empts many observations which together indicate that the cardiovascular response to hypoxia is dependent upon a number of factors, including the rate of onset and severity of the fall in PO$_2$, the presence or absence of acidaemia, repetition of the insult and alterations in uteroplacental flow and peripheral resistance caused by cord compression. Despite the sensitivity of the cardiovascular response to the sum of the conditions surrounding the insult, it has been possible to build a picture of the responses of skeletal muscle to acute uterine artery occlusion. Results obtained using NIRS indicate that these responses include reduced blood flow and volume and support the published data. However, no other authors have directly measured blood volume in fetal peripheral tissues and how this is affected by hypoxia.

Using NIRS, it is possible to directly monitor changes in total haemoglobin concentration, and this variable is related to blood volume through the haematocrit. Our data for skeletal muscle tHb is shown in Figure 4.4 and is reproduced in Figure 4.7 in conjunction with femoral artery flow data. By overlapping these data it becomes obvious that the return of skeletal blood volume to control levels occurs independent of femoral artery flow. From Figure 4.4 (upper panel) we can see that the increase in tHb during the insult is due to increasing deoxyhaemoglobin levels during the insult. In the absence of an increase in femoral flow, the Hb-led increase in skeletal blood volume indicates that the volume increase is due to increased venous volume. Increased venous volume could be caused by a compromised venous return resulting in elevated cardiac preload or by impairment or down regulation of cardiac function during hypoxia.

The altered cardiac function suggested by these data is in line with data from isolated perfused hearts which indicates that acute ischaemia caused by 20-50% reduction in coronary artery flow induces a reduction in contractile function, in myocardial metabolism and in PCr and ATP levels. The myocardium can adapt to this level of ischaemia within one hour such that PCr levels and lactate production return to normal, whilst contractile function remains altered. Is there evidence for altered metabolism and functionality in tissues other than the myocardium? The NIRS data for skeletal muscle may provide such evidence.
Figure 4.7 Data for hind limb skeletal muscle showing fall in blood volume during asphyxia compared to femoral artery flow. The disparity between flow and volume, taken with NIRS data indicates that there is an increase in venous volume, perhaps due to venous congestion caused by impaired cardiac function.

4.4.2 Metabolic response to hypoxia

If the sheep fetus is unable to survive for extended periods with drastically reduced oxygen delivery, metabolic alterations must be made to prolong survival, presumably in the hope that O\textsubscript{2} supply will be re-established quickly. Information on the metabolic strategies available for hypoxia survival can be obtained from observations of facultative anaerobic species, some of which are able to survive with little or no oxygen for up to 6 months at a time. Of the many strategies for anoxia tolerance described in the literature, the two most important are believed to be the glycolytic strategy and the metabolic suppression strategy.

In the glycolytic strategy the energy-efficiencies of ATP-producing and utilising pathways are up regulated by stoichiometric alterations (Hochachka, 1996). In this way, although metabolic rate per se may not be reduced, the maximum amount of ATP possible is generated per O\textsubscript{2} molecule reduced to water and the maximum amount of work is generated per mole of ATP used. In this way, a given amount of O\textsubscript{2} will be able to sustain the fetus for longer than would have previously been possible.

Even greater protection against hypoxia is provided by the metabolic suppression strategy. By this method, there is a severe down-regulation of energy turnover during periods of reduced DO\textsubscript{2} (Hochachka, 1996). This down-regulation of energy turnover takes the form of reductions in the rates of ATP use by each cell's ATP-
requiring systems (so-called energy sinks). Work using *in vitro* hepatocyte preparations has provided a breakdown of how ATP is used in this cell type. Example data is given below.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Normoxia</th>
<th>Anoxia</th>
<th>% suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>67.0</td>
<td>6.3</td>
<td>94</td>
</tr>
<tr>
<td>Na⁺ pump</td>
<td>19.1</td>
<td>4.8</td>
<td>75</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>24.4</td>
<td>1.6</td>
<td>93</td>
</tr>
<tr>
<td>Protein breakdown</td>
<td>11.1</td>
<td>0.7</td>
<td>94</td>
</tr>
<tr>
<td>Urea synthesis</td>
<td>2.0</td>
<td>0.6</td>
<td>70</td>
</tr>
<tr>
<td>Gluconeogenesis</td>
<td>11.4</td>
<td>0.0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4.3 Data from Hochachka et al., 1996 showing energy sinks in aquatic turtle hepatocytes during normoxia and anoxia, with percentage depression of ATP demand. During anoxia, Na⁺ pump activity is the major energy sink even though it is only 25% of its normoxic value. Electrochemical gradients are maintained despite the rundown of Na⁺-K⁺ pump activity by reducing membrane conductance (generalised channel arrest).

Whilst this data indicates that some facultative anaerobes are able to down regulate many cellular processes whilst maintaining cellular integrity, comparative work has not been performed for species not adapted to anoxia, nor has it been performed in fetal tissue. In the absence of this work, what evidence exists that there is hypometabolic adaptation to hypoxia in the fetal sheep and, ergo, in other mammalian fetuses?

The data presented here for the late gestation sheep fetus indicates that there is a degree of fetal peripheral hypometabolic adaptation both in the brain (Chapter 3) and in the periphery during the acute asphyxial challenge used in this study. Using standard biophysical techniques, this could be seen as a fall in leg VO₂ during the insult, before returning to control levels after release of the occlusion (Figure 4.4). With NIRS, this hypometabolism was evidenced by a significant, sustained reduction in skeletal muscle cytochrome oxidase redox state during the insult (Figure 4.5). There are two lines of evidence which indicate that a reduction in cytochrome oxidase redox state is associated with hypometabolism.

Firstly, simultaneous nuclear magnetic resonance spectroscopy (NMRS) and NIRS have been performed on neonatal piglet brain during hypoxia. Data from these studies indicate that cytochrome oxidase redox state does not begin to reduce until PCR levels are depleted and ATP concentrations begin to fall, i.e. when fetal oxygenation is pronounced and well advanced. The second line of evidence comes from our knowledge of the interaction of the mitochondrion with NO during hypoxia. It has been established that NO, like CO, competes with O₂ for the cytochrome oxidase binuclear centre. It is also known that NO production is stimulated by
hypoxia acting on inducible NOS, the net effect of these circumstances is that, as PO$_2$ falls, increasing NO concentrations have a steadily increasing inhibitory effect on mitochondrial respiration. Since NO inhibition reduces the ability of cytochrome oxidase to discharge electrons to O$_2$, NO inhibition causes electrons to build up in the electron transfer chain so that cytochrome oxidase redox state becomes progressively reduced. Such a reduction is shown in Figures 4.5 and 4.6 during fetal asphyxia.

This ability of NO to inhibit mitochondrial respiration is highest at lower PO$_2$, when there is less competition between NO and O$_2$ for the CcO binuclear centre. The time scale over which NO inhibition is effected has yet to be established, but Figure 4.6 shows clearly that CcO is significantly reduced 5 minutes after the onset of hypoxia in skeletal muscle. Further work is required to establish whether NO mediates this reduction in peripheral CcO or whether other mechanisms play a part.

4.5 Summary

Peripheral skeletal muscle receives a significantly reduced blood supply during hypoxia. These data indicate that peripheral tissue metabolism falls, venous volume increases and that cardiac function may, therefore, be temporarily altered. These adaptations are believed to be part of a co-ordinated strategy to preserve function in essential organs during hypoxic episodes.
5.1 Introduction

As discussed in the last chapter, there is evidence for both cardiovascular and metabolic adaptations in central and peripheral tissues during uterine artery occlusion which may be of clinical relevance in the aetiology of both brain injury and necrotising enterocolitis. The question of whether a hypoxic challenge results in the same fetal cardiovascular redistribution, independent of the type or severity of the challenge has been addressed by Jensen and Berger (Jensen & Berger, 1991) but the metabolic and endocrine responses to different hypoxic challenges have not received the same attention.

To this end, we sought to compare the fetal response to acute total umbilical cord occlusion with that of uterine artery occlusion in late gestation fetal sheep. In this set of experiments, cerebral NIRS and fetal haemodynamics were monitored in the same way as for those animals undergoing uterine artery occlusion.

5.2 Methods

5.2.1 Background

The experiments presented in this section were performed in conjunction with Dr Sian Harding, a consultant neonatal paediatrician in University College Hospital's Department of Paediatrics. Dr Harding was using in vivo microdialysis to obtain glutathione from cerebrospinal fluid microdialysate extracted from the thalamic area of the brain of late gestation fetal sheep both before, during and after complete cord occlusion.

5.2.2 Surgical procedure

Work was carried out under the Animals (Scientific Procedures) Act, 1986. Five date-mated ewes bearing singleton fetuses were used in this series of experiments. Each fetus was instrumented as described in Chapter 2 with a brachial artery and jugular vein catheter on one side and a Transonic flow probe around the contralateral carotid artery. Electrodes were sewn into nuchal muscle to give an indicator of neck movement whilst ECG and ECoG electrodes were implanted as described previously.
A coronal incision was made in the skin of the skull and the skin flaps pulled back. The muscle overlying the bone was peeled back exposing the skull, and a microdialysis probe (Biotech Instruments Ltd, Kimpton, UK) was implanted in the left cerebral hemisphere (using stereotaxic directions provided by Gluckman & Parsons, 1983) through a burr hole in the bone made 5mm lateral to, and 10mm anterior of, bregma. The probe was secured in place using a wedge of rubber glued with cyanoacrylate to the bone of the skull. The burr hole sealed with cyanoacrylate. Further security for the probe and tubing was provided by sewing the skin of the fetal scalp back once the NIRS and microdialysis probes were in place. Microdialysis tubing was further secured in place by loosely sewing to the skin overlying the posterior aspect of the neck. Great care was taken at all stages to prevent kinking of the tubing. The location of the probe tip was confirmed in a number of control fetuses by injection of a small volume of dye through the microdialysis catheter and into the brain. Microdialysis data are not presented in this thesis.

5.2.3 Experimental protocol

Three days after surgery, animals began a 72-hour recording protocol for biophysical variables (FHR, MAP, CaBF, ECG and ECoG). Near infrared recording was performed for three hours; one hour prior to, during and for one hour after the occlusion. Each fetus was subjected to one episode of complete umbilical cord occlusion lasting 10 minutes. End points for the occlusion were based on the monitoring of fetal carotid flow and mean arterial pressure. When mean pressure fell below 20 mmHg and carotid flow was falling, the occlusion was reversed and resuscitation given, where necessary, by intravenous bolus injection of adrenaline and sodium bicarbonate.

The scheme for taking blood samples is shown below in Figure 5.1. Blood samples were taken at -4, -3, -2 and -1 hour before start of the occlusion, then at -5 minutes, +5 and +10 minutes. The occlusion was reversed at 10 minutes and further blood samples taken at +30 minutes, 1 hour, 2, 4, 8, 24 and 48 hours after the start of occlusion. Blood samples were tested for gases, electrolytes, lactate and glucose as described in chapter 2.
5.2.4 Histological examination

The histological fixing and assessment protocols are as detailed in chapter 2. In brief, fetal brains were fixed by perfusion through the carotid arteries with firstly 500ml of 0.9% saline followed by 1 litre of 2% buffered formalin. Following dissection and storage for 1 week, each brain was sliced at 4mm intervals according to a pre-established protocol and impregnated with wax before sectioning on a microtome. A histopathologist blinded to the experimental protocol performed a histological examination of H & E stained slides.

5.3 Results

In designing these experiments, the occlusion was initially to be reversed when fetal arterial pressure fell below 20mmHg and fetal carotid flow was falling; a situation which we hoped would provide as close to a pre-terminal insult as could be given without fetal loss. Unfortunately, many fetuses died with this approach. Although there may be a clinical case for approaching experiments in this manner, since it may provide an apparently uniform insult (as described by our measured variables), ethically and financially we were not happy with the rate of loss of animals that this approach entailed. Therefore we decided to subject each fetus instead to a set duration of complete umbilical cord occlusion. We found with this approach that insults of greater than 10 minutes uniformly resulted in fetal death. In the experiments described here, the insult was reversed at 10 minutes without any fetal losses.

5.3.1 Blood gases and acid base status

Tables 5.1 and 5.2 show blood sample data in control conditions, during complete umbilical cord occlusion and during two hours of recovery. These data show how umbilical cord occlusion caused significant changes in all measured blood variables except haematocrit. Fetal arterial pH fell from 7.38 ± 0.02 to 6.96 ± 0.03 by the end of the insult, indicating that this very short occlusion induced a severe asphyxia.
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pH</th>
<th>PaCO₂ (mmHg)</th>
<th>PaO₂ (mmHg)</th>
<th>Hct (%)</th>
<th>[Hb] (g/dl)</th>
<th>HbSat (%)</th>
<th>O₂ ct (vol% O₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-240</td>
<td>7.38 ± 0.02</td>
<td>46.6 ± 1.1</td>
<td>24.6 ± 1.5</td>
<td>32.7 ± 2.1</td>
<td>9.2 ± 0.3</td>
<td>73.2 ± 2.5</td>
<td>9.3 ± 0.3</td>
</tr>
<tr>
<td>-180</td>
<td>7.37 ± 0.02</td>
<td>49.2 ± 1.9</td>
<td>23.8 ± 1.5</td>
<td>32.8 ± 2.4</td>
<td>8.7 ± 0.3</td>
<td>70.0 ± 3.5</td>
<td>8.4 ± 0.3</td>
</tr>
<tr>
<td>-120</td>
<td>7.37 ± 0.01</td>
<td>48.0 ± 2.4</td>
<td>23.8 ± 1.3</td>
<td>29.8 ± 1.8</td>
<td>8.6 ± 0.6</td>
<td>71.7 ± 4.3</td>
<td>8.4 ± 0.1</td>
</tr>
<tr>
<td>-60</td>
<td>7.37 ± 0.01</td>
<td>48.4 ± 2.7</td>
<td>24.2 ± 1.0</td>
<td>32.2 ± 2.5</td>
<td>8.8 ± 0.4</td>
<td>73.4 ± 3.1</td>
<td>8.9 ± 0.2</td>
</tr>
<tr>
<td>-5</td>
<td>7.38 ± 0.02</td>
<td>48.4 ± 2.0</td>
<td>23.4 ± 1.5</td>
<td>33.2 ± 3.5</td>
<td>8.2 ± 0.5</td>
<td>71.2 ± 3.5</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>7.07 ± 0.04 (\alpha)</td>
<td>93.1 ± 6.4(\alpha)</td>
<td>5.0 ± 1.7(\alpha)</td>
<td>35.4 ± 2.0</td>
<td>6.0 ± 0.3(\alpha)</td>
<td>8.7 ± 2.9(\alpha)</td>
<td>0.8 ± 0.2(\alpha)</td>
</tr>
<tr>
<td>10</td>
<td>6.96 ± 0.03 (\alpha)</td>
<td>103.8 ± 5.6(\alpha)</td>
<td>12.4 ± 4.0(\alpha)</td>
<td>34.8 ± 2.3</td>
<td>5.9 ± 0.3(\alpha)</td>
<td>23.4 ± 6.9(\alpha)</td>
<td>2.0 ± 0.6(\alpha)</td>
</tr>
<tr>
<td>30</td>
<td>7.18 ± 0.02 (\alpha)</td>
<td>53.7 ± 2.1</td>
<td>22.4 ± 1.2</td>
<td>33.0 ± 1.8</td>
<td>6.8 ± 0.4(\alpha)</td>
<td>58.6 ± 2.5(\alpha)</td>
<td>5.5 ± 0.5(\alpha)</td>
</tr>
<tr>
<td>60</td>
<td>7.25 ± 0.04 (\alpha)</td>
<td>44.9 ± 1.5</td>
<td>22.5 ± 1.9</td>
<td>33.0 ± 2.8</td>
<td>6.8 ± 0.4(\alpha)</td>
<td>62.6 ± 9.0(\alpha)</td>
<td>7.1 ± 0.9(\alpha)</td>
</tr>
<tr>
<td>120</td>
<td>7.36 ± 0.03</td>
<td>43.3 ± 1.9</td>
<td>22.0 ± 1.7</td>
<td>31.0 ± 1.2</td>
<td>8.2 ± 0.2</td>
<td>70.9 ± 8.0</td>
<td>6.9 ± 1.4</td>
</tr>
<tr>
<td>240</td>
<td>7.37 ± 0.02</td>
<td>49.4 ± 1.5</td>
<td>22.4 ± 2.0</td>
<td>33.4 ± 1.9</td>
<td>8.0 ± 0.7</td>
<td>71.5 ± 3.8</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>480</td>
<td>7.36 ± 0.03</td>
<td>43.3 ± 1.7</td>
<td>22.6 ± 1.9</td>
<td>32.8 ± 2.3</td>
<td>8.0 ± 0.6</td>
<td>62.7 ± 10.4</td>
<td>7.0 ± 0.5</td>
</tr>
<tr>
<td>1440</td>
<td>7.37 ± 0.01</td>
<td>49.7 ± 2.4</td>
<td>22.3 ± 3.5</td>
<td>31.7 ± 0.9</td>
<td>8.4 ± 0.9</td>
<td>65.7 ± 4.5</td>
<td>7.5 ± 0.9</td>
</tr>
<tr>
<td>2880</td>
<td>7.38 ± 0.01</td>
<td>46.0 ± 1.7</td>
<td>23.2 ± 0.7</td>
<td>30.6 ± 2.04</td>
<td>7.6 ± 0.3</td>
<td>69.8 ± 1.3</td>
<td>7.7 ± 0.4</td>
</tr>
</tbody>
</table>

Table 5.1 Arterial blood sample data at times relative to the start of a 10 minute umbilical cord occlusion. \(\alpha\) indicates significant difference from pre-occlusion control period, \(p < 0.05\). Analysis performed using repeated measures ANOVA with Newman-Keuls \(post hoc\) tests as appropriate.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>[HCO₃⁻] (mM)</th>
<th>Base Excess (mM)</th>
<th>[Lactate] (mM)</th>
<th>[Glucose] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-240</td>
<td>27.5 ± 1.3</td>
<td>3.2 ± 1.6</td>
<td>0.68 ± 0.08</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>-180</td>
<td>27.9 ± 1.3</td>
<td>3.5 ± 1.5</td>
<td>0.66 ± 0.07</td>
<td>0.84 ± 0.07</td>
</tr>
<tr>
<td>-120</td>
<td>27.3 ± 1.2</td>
<td>2.9 ± 1.0</td>
<td>0.60 ± 0.08</td>
<td>0.81 ± 0.04</td>
</tr>
<tr>
<td>-60</td>
<td>27.5 ± 1.2</td>
<td>3.2 ± 1.2</td>
<td>0.69 ± 0.07</td>
<td>0.83 ± 0.06</td>
</tr>
<tr>
<td>-5</td>
<td>28.3 ± 1.8</td>
<td>3.9 ± 2.0</td>
<td>0.68 ± 0.13</td>
<td>0.76 ± 0.05</td>
</tr>
<tr>
<td>5</td>
<td>26.1 ± 1.4</td>
<td>-3.8 ± 2.1(\alpha)</td>
<td>3.64 ± 0.38(\alpha)</td>
<td>0.71 ± 0.18</td>
</tr>
<tr>
<td>10</td>
<td>22.8 ± 1.5(\alpha)</td>
<td>-8.6 ± 2.0(\alpha)</td>
<td>5.30 ± 0.33(\alpha)</td>
<td>1.54 ± 0.38(\alpha)</td>
</tr>
<tr>
<td>30</td>
<td>19.5 ± 1.3(\alpha)</td>
<td>-7.6 ± 1.4(\alpha)</td>
<td>4.58 ± 0.40(\alpha)</td>
<td>1.63 ± 0.14(\alpha)</td>
</tr>
<tr>
<td>60</td>
<td>20.2 ± 1.5(\alpha)</td>
<td>-5.3 ± 2.2(\alpha)</td>
<td>4.35 ± 0.25(\alpha)</td>
<td>1.20 ± 0.14(\alpha)</td>
</tr>
<tr>
<td>120</td>
<td>26.1 ± 2.6</td>
<td>-0.7 ± 2.2</td>
<td>2.54 ± 0.26(\alpha)</td>
<td>1.34 ± 0.10(\alpha)</td>
</tr>
<tr>
<td>240</td>
<td>28.1 ± 1.9</td>
<td>3.6 ± 2.0</td>
<td>1.87 ± 0.31(\alpha)</td>
<td>1.32 ± 0.09(\alpha)</td>
</tr>
<tr>
<td>480</td>
<td>27.2 ± 2.8</td>
<td>2.5 ± 3.0</td>
<td>2.41 ± 0.89(\alpha)</td>
<td>1.40 ± 0.15(\alpha)</td>
</tr>
<tr>
<td>1440</td>
<td>28.5 ± 0.5</td>
<td>4.1 ± 0.3</td>
<td>0.88 ± 0.17</td>
<td>1.07 ± 0.10(\alpha)</td>
</tr>
<tr>
<td>2880</td>
<td>26.6 ± 1.0</td>
<td>2.5 ± 1.0</td>
<td>0.81 ± 0.21</td>
<td>0.94 ± 0.11</td>
</tr>
</tbody>
</table>

Table 5.2 Additional arterial blood sample data for time points relative to the start of a 10 minute umbilical cord occlusion. Statistical analysis as for Table 5.1.
The arterial pH was still significantly reduced at 50 minutes after release of the occlusion (at 7.25 ± 0.04), but had returned to control by 110 minutes after release. $P_aO_2$ and $P_aCO_2$ were significantly altered for the duration of the insult, from 24.6 ± 1.5 to 5.0 ± 1.7 mmHg and 46.6 ± 1.1 to 103.8 ± 5.6 mmHg, respectively. Fetal gases returned to pre-occlusion levels by 20 minutes after release of the occlusion. Base excess, [haemoglobin], haemoglobin saturation and $O_2$ content of the blood all showed significant changes at 5 minutes into the insult which persisted until at least 50 minutes after release of the occlusion but which had come back to control levels by 110 minutes after release of the occlusion.

$[HCO_3^\text{-}]$, however, had not changed significantly until 10 minutes into the occlusion, although it too remained significantly different to control until between 50 and 110 minutes after release of the occlusion.

Both lactate and glucose concentrations remained elevated for at least 8 hours after release of the occluder. Whilst lactate levels were already significantly elevated at 5 minutes into occlusion, arterial glucose concentration was not significantly elevated until 10 minutes into the occlusion. In addition to rising faster than glucose during the occlusion, arterial lactate levels also came back to control faster than glucose, with lactate falling back to control levels between 8 and 24 hours after release of the occluder. Arterial glucose, however, was still elevated at 24 but had returned to baseline by 48 hours after release of the occluder.

5.3.2 Haemodynamics

Baseline mean arterial pressure was within normal limits for this gestational age at 45.8 ± 3.2 mmHg. At the onset of umbilical cord occlusion, there was a rapid increase in pressure (Figure 5.2, upper panel), reaching 70.7 ± 6.0 mmHg at approximately 6 minutes into the occlusion, before beginning to fall. At the point where mean arterial pressure reached ca. 20 mmHg and carotid flow was falling, the occlusion was released. Pressure continued to fall for a about 60 seconds, attaining a minimum value of 10.5 ± 10.7 mmHg before rebounding to 62.9 ± 4.5 mmHg at approximately 8 minutes after release of the occlusion. Mean arterial pressure returned to control levels by 20 minutes after release of the occluder and remained within normal limits until the ewe and fetus were culled at 72 hours.

The baseline fetal heart rate of 150 ± 8 bpm remained stable for the duration of the pre-occlusion control period. At the onset of occlusion, fetal heart rate fell to 89 ± 23 bpm at 2 minutes, before beginning to increase back towards control levels. After release of the occluder, the heart rate increased to 269 ± 6 bpm at 4 minutes before falling then rising again to 250 ± 16 bpm at 24 minutes after release of the occluder. Thereafter, fetal heart rate moved slowly back to control levels, and was not significantly different to pre-occlusion levels at 2 hours after release.
Carotid blood flow in these fetuses was comparable to that of late gestation fetuses described in Chapter 3, being 45.8 ± 8.9 ml/min in baseline conditions. After a variable initial fall which was not significantly different for the group, carotid flow fell only slowly during the insult although it was significantly reduced at the end of a 10-minute occlusion (p < 0.05, t-test vs control) when it reached 16.8 ± 6.4 ml/min. There was a transient hyperaemia after release of the occluder, with carotid flow increasing to 68.4 ± 6.3 ml/min at 6 minutes after release. Carotid flow was not different to control levels at 10 minutes after release and thereafter was not different to control for the duration of the recording.
Figure 5.2 Group haemodynamic data shows significant changes in MAP, FHR and CaBF during and after the occlusion. Data shown as mean ± SE. Statistical significance taken as $p < 0.05$ (Repeated measures ANOVA with Newman-Keuls post hoc tests, vs. -60 minutes).
5.3.3 Oxygen delivery

Figure 5.3 shows that there was a substantial, significant decrease in oxygen delivery to the head during the period of the occlusion. DO$_2$ fell from $3.91 \pm 0.74$ ml O$_2$/min one hour before the occlusion started to $0.30 \pm 0.19$ ml O$_2$/min at the end of the occlusion ($p < 0.05$, repeated measures ANOVA vs control with Newman-Keuls post hoc test). Recovery of DO$_2$ was completed by 20 minutes after release of the occlusion, when DO$_2$ was $2.88 \pm 0.53$ ml O$_2$/min.

Unfortunately, as this set of experiments were using the protocol of Dr Sian Harding, no venous blood samples were taken and therefore no oxygen consumption calculations could be made.

![Figure 5.3 Oxygen delivery (mean ± SE) to the fetal head at time points relative to the start of complete umbilical cord occlusion. $a$ indicates significant difference from control value at -60 minutes, $p < 0.05$ with repeated measures ANOVA and Newman-Keuls post hoc tests as needed.](image)

5.3.4 Near infrared spectroscopy

Near infrared data are shown in Figure 5.4. Baseline HbO$_2$ and Hb were very stable, not varying more than 5µmol/l during the hour of control recording. As umbilical cord occlusion started, there was a rapid reciprocal change in HbO$_2$ and Hb of similar magnitude as is seen at this gestation during uterine occlusion (Figures 3.6 and 3.8). Maximum changes during the occlusion were HbO$_2$ $-29.23 \pm 2.16$ µmol/l, Hb $36.22 \pm 5.52$ µmol/l, tHb $12.91 \pm 5.06$ µmol/l and CcO $0.64 \pm 0.45$ µmol/l.
Umbilical cord occlusions lasted no more than 10 minutes. By the end of the insult, HbO₂ and Hb had both begun what appeared to be a secondary phase where both were falling, such that tHb (cerebral blood volume) fell rapidly to 2.47 ± 5.71 μmol/l and was significantly less than the maximal change during the occlusion (Figure 5.1, lower panel. p < 0.05, paired t test vs. maximal change).

Cytochrome oxidase oxidation changes in parallel with tHb signal (Figure 5.1, bottom panel), increasing to 0.64 ± 0.45 μmol/l at 4 minutes before falling to −0.39 ± 0.57 μmol/l at the end of the occlusion. CcO tracking with any other NIRS signal may indicate cross-talk between chromophore signals. Since these were primarily Dr Harding’s experiments, it was not possible to perform any of the manoeuvres required to verify whether cross-talk was occurring in these fetuses, but this checking was performed in late gestation fetuses subjected to reduced uteroplacental perfusion (Figure 3.7) using NaCN. In this instance, CcO was seen to become fully reduced despite large swings in tHb, suggesting that cross talk is not a factor in these changes.

In the recovery phase, all variables quickly returned to control levels. There were fluctuations away of HbO₂ and Hb from control at +58 and +78 minutes after reversal of the occlusion, respectively but the data after 50 minutes post reversal is based on two observations only and should be interpreted with caution.
Figure 5.4 Shows typical NIRS data for a 10-minute complete umbilical cord occlusion (box). The upper panel shows HbO₂ (red) and Hb (blue), the lower panel shows changes in CcO redox state (green) and tHb (purple), which is related to cerebral blood volume in the absence of changes in haematocrit.
Figure 5.5 Near Infrared results for the late gestation fetuses subjected to complete umbilical cord occlusion for 10 minutes (grey box). HbO₂ and Hb data (top panel) show the same reciprocal response as seen with uterine occlusions. Note the biphasic Hb response similar to that seen in mid gestation animals subjected to uterine occlusion. tHb (bottom panel) also shows biphasic changes during the occlusion. At the end of occlusion, tHb has fallen significantly from its maximum at +4 minutes (p < 0.05, paired t test, n = 6). The variables returned to control levels but there were some fluctuations away from control in both HbO₂ and Hb at 58 and 78 minutes after reversal, respectively (p < 0.05 t test vs. control, n = 2).
5.3.5 ECoG monitoring and post-asphyxic seizure activity

Fetal ECoG was continuously monitored as part of these experiments. Although ECoG was not subjected to rigorous analysis, it was used to confirm the presence of normal activity cycling and as a gauge of the severity of the umbilical cord occlusion by the development of isoelectricity. Figure 5.6 shows normal high and low voltage cycling in the pre-insult control period. As with those fetuses described in chapter 3, high and low voltage ECoG activity occurred with each cycle usually lasting for approximately ten and fifteen minutes, respectively.

![Figure 5.6 Example of control recording of fetal ECoG in late-gestation prior to umbilical cord occlusion. The high and low voltage activity is very well differentiated at this stage of development, showing relatively sharp transitions.](image)

During the occlusion, there is a rapid transition to isoelectricity (Figure 5.7), which is maintained for some time after the cessation of the occlusion; usually for 20 minutes to 45 minutes, after which seizure activity develops, as confirmed by intense EMG activity (Figure 5.8).

![Figure 5.7 ECoG isoelectricity develops quickly after the start of umbilical cord occlusion and may persist for up to 45 minutes in some cases.](image)

![Figure 5.8 Development of fitting activity after the onset of umbilical cord occlusion in the late-gestation fetal sheep. Note the lack of nuchal EMG activity before the onset of occlusion and the sudden increase in activity, which is not stopped by release of the occlusion.](image)
5.3.6 Histology

Histological assessment of the brains of fetuses subjected to acute, severe umbilical cord occlusion uniformly showed evidence of injury in the hippocampus, but not uniformly to other structures. There was evidence of mild temporal cortical injury in one case out of six.

With H & E staining, injury in the hippocampus (Figures 5.9 & 5.10) can be easily seen as small, dark staining atrophied cells surrounded by areas of increased vascularity, having a generally disorganised appearance. These findings are in agreement with those of Mallard and colleagues (Mallard et al., 1992). Similarly, injured cells were present in the temporal cortex of one sheep (Figure 5.11), indicated by pyknosis or karyorrhexis with acidophilic staining under H & E.

*Figure 5.9* Example of hippocampus from late-gestation fetal sheep subjected to 10 minutes complete umbilical cord occlusion. These areas are relatively susceptible to injury, especially the CA3, CA1 & CA2 (Mallard et al., 1992).
Figure 5.10 Examples of mild (A), moderate (B), and severe (C) hippocampal injury. There is a progressive loss of organisation with degree of injury, and a rise in the number of acidophilic (red) stained cells.

Figure 5.11 Histology of the temporal cortex in one sheep fetus subjected to 10 minutes of total umbilical cord occlusion. Acidophilic (red)-staining cells are injured cells in the process of dying and being cleared by the host scavenging cells. (A) magnification x40, (B) magnification x200.
5.4 Discussion

In viviparous animals, the fetus is completely dependent on the umbilical-placental circulation, and thus on the mother, for its supply of oxygen and metabolites as well as for disposal of metabolic wastes. Because of this, the adequate functioning of the umbilical-placental system is of vital importance to appropriate fetal development and interruption to, or alterations of, umbilical-placental function caused by, for example, cord compression or alteration in the placenta's ability to exchange nutrients or gases may have serious detrimental effects.

The fetus can accommodate for interruption in the supply of nutrients from the mother to a certain degree by short-term supplementation of metabolism with glucose derived from internal stores, by altering growth trajectory through reducing tissue accretion rates and by making efficiency savings in protein turnover, etc. However, a continuous gaseous exchange between mother and fetus is absolutely essential and interruption of O$_2$ and CO$_2$ exchange by umbilical cord compression can have catastrophic effects for the fetus if prolonged. This is not only because the fetus is unable to store O$_2$ (myoglobin excepted), but also because increases in P$_a$CO$_2$ lead to reduction in tissue pH, which alters protein conformation and thus enzyme activities and mitochondrial redox.

In this series of experiments, standard biophysical instrumentation was used in conjunction with continuous wavelength NIRS to provide data on the fetal adaptive response to a near-terminal umbilical cord occlusion and to investigate how changes in haemodynamics and metabolism may relate to histological outcome after such an insult.

As already discussed in the last section, the initial fetal responses to umbilical cord occlusion include the now well-characterised fall in fetal heart rate, rise in mean arterial pressure and, in this instance, a carotid flow which shows a progressive decline as the insult worsens (Jensen, 1991 for review; Giussani, 1994). However, these haemodynamic adaptations to asphyxia are not maintained. The reason for this reversal, or failure, of cardiovascular compensation is not known but has been suggested to be either a failure of cardiac glycogen stores (Hokegard et al., 1981; McNutty et al., 1996) or of peripheral vasoconstriction (Block et al., 1990). However, neither of these options seems likely to be the sole reason for cardiac decompensation. Glycogen store failure is unlikely because there is no evidence for heart failure during any part of the insult — fetal heart rate is, in fact, recovering in the 'normal' catecholamine-driven way during the insult and rebounds quickly after release of occlusion. Failure of peripheral vasoconstriction has been postulated as a cause of the falling mean arterial pressure in severe asphyxia (Block et al., 1990). There may be some mileage in this opinion since Bennet and co-workers (Bennet et al., 2000) have recently published data indicating that during umbilical cord occlusion mesenteric artery vasoconstriction begins to fail after prolonged hypoxia. If this is the cause of falling mean arterial pressure, it raises the
CHAPTER 5

question as to why the baroreflex is unable to correct for the falling peripheral resistance when fetal baroreflex circuits are known to be operative in late gestation fetal sheep. It may be that baroreflex circuits are in operation at this time but that local factors such as increased [H⁺] and PCO₂ override neural signals to peripheral resistance vessels, with consequent peripheral vasodilatation. This hypothesis has yet to be demonstrated experimentally.

Despite the lack of insight into the reasons for the failure of cardiac compensation, there was uniformity in the time taken to bring the fetus to the point of cardiovascular collapse. Without exception, occlusions of 11 minutes or greater resulted in fetal death whilst release of the occlusion at 10 minutes was associated with a pre-terminal fall in mean arterial pressure and carotid flow and the presence of agonal ECoG patterns and seizure activity (Figure 5.8 for EMG fitting activity). As indicated, release of the occlusion resulted in a strong hyperaemic response which was not present after release of uterine artery occlusion in sheep fetuses of the same age (Newman et al., 2000). Whilst carotid blood flow is not cerebral blood flow, the observed hyperaemia is noteworthy because of the association noted in the literature between perinatal fluctuations in cerebral blood flow and subsequent brain injury.

In addition to the haemodynamic adaptations described above, umbilical cord occlusion has profound metabolic effects believed to be part of a strategy to protect the fetal brain from energy failure and subsequent injury. Development of an isoelectric electrocorticogram after ca. 3 minutes of occlusion (Figure 5.7) – a state which is maintained for 20 - 45 minutes after release of the occlusion – may be an indicator of cerebral metabolic inhibition. There is also a fall in oxygen delivery to the brain from 3.91 ± 0.74 ml O₂/min one hour before the occlusion started to 0.30 ± 0.19 ml O₂/min at the end of the occlusion (Figure 5.3), a reduction of some 92.3 ± 4.9%. At this level of reduced oxygen delivery, there can be no question that oxygen consumption, and therefore cerebral metabolism, must have fallen. This fall in cerebral metabolism is likely to be due to the combined effects of adenosine mediated neuronal hyperpolarisation and reduced excitatory neurotransmitter release, which shifts the balance of neurotransmitter release in favour of inhibitory neurotransmitters. At lower cellular PO₂, the inhibitory effects of nitric oxide on cytochrome oxidase, the site of oxygen reduction in mitochondria, also become significant and may also add to the cerebral metabolic depression.

By reducing energy demand through these various strategies for metabolic suppression, the fetus may be deferring the point at which ATP levels begin to deplete, and CcO begins to reduce, during hypoxia – an event which is believed to be linked to rises in intracellular Ca²⁺ and a chain of events leading to a secondary phase of neuronal injury hours after cessation of the original insult (Lorek et al., 1994). The spectrum of neuronal injury – or its lack – found with umbilical cord occlusions, uterine artery occlusion and maternal hypoxia suggest that neuropathological outcome of hypoxia is very dependent upon the nature of the insult – the duration and magnitude of change in P₅O₂ and pH, the number of repetitions of
the insult and the method by which the insult is induced. These factors in themselves may not necessarily be causative for brain injury in the late gestation fetus, but their influence on cerebral blood flow may be of great importance in the aetiology of perinatal brain injury under circumstances of umbilical cord occlusion.

It is interesting to note that during the relatively mild insult of uterine artery occlusion described in Chapter 3, fetal cerebral CcO oxidation increases for the duration of the insult before becoming reduced again after release of the occluder. In this set of experiments we saw that there is only a transient oxidation of CcO and that it became reduced again within the duration of the occlusion. This suggests that the adaptive processes which underlie the CcO oxidation we see are either overwhelmed by the speed of onset or magnitude of the insult, or that this initial stratagem is quickly abandoned in favour of some other (unknown) adaptive mechanism. The idea that there may be layers of adaptation or different adaptive schemes available depending upon the circumstances require much further investigation. One place to start with this investigation may be to look at the involvement of known cardiovascular and metabolic modulators in CcO responses to hypoxia-ischaemia.

5.5 Summary

In this chapter, I have shown that acute, complete umbilical cord occlusion results in a different pattern of haemodynamic responses by the late-gestation ovine fetus to those seen during acute reduction of uteroplacental flow (Chapter 3). The metabolic responses to acute complete umbilical cord occlusion was not shown to be significantly different to that seen in Chapter 3, although cerebral \( \text{DO}_2 \) was similarly depressed. Histological evidence of injury was present in areas of the hippocampus and cortex, unlike in fetuses of the same age challenged with reduced uteroplacental flow. The ability of metabolic and cardiovascular adaptive processes to respond to a severe challenge such as complete umbilical cord occlusion may influence the location and extent of subsequent brain injury.
CHAPTER 6

THE EFFECTS OF EXOGENOUS ADENOSINE INFUSION ON CEREBRAL HAEMODYNAMICS AND METABOLISM IN LATE-GESTATION FETAL SHEEP IN UTERO

6.1 Introduction

Adenosine is a vasoactive purine metabolite, produced during breakdown of ATP in hypoxia. Tissue levels of adenosine are 2-3 times higher in the fetus than the adult (Sawa et al., 1991) and have been shown to rise further within minutes of the onset of hypoxia in the ovine fetus (Kubonoya and Power, 1997). Plasma adenosine levels are also higher in hypoxaemic, acidotic growth-retarded human fetuses than in appropriately grown fetuses (Yoneyama et al., 1994). This may be seen as a direct consequence of the hypoxaemia, but there is in addition the possibility that elevated tissue and plasma adenosine may form part of a protective fetal adaptation aimed at matching oxygen demand to availability (Rudolphi et al., 1992; Gidday et al., 1995).

Adenosine, acting via A\textsubscript{2A} receptors, is known to cause peripheral vasodilatation and an increase in cerebral blood flow, thus improving oxygen delivery to tissues (Kurth and Wagerle, 1992; Laudignon et al., 1990). In addition, acting at A\textsubscript{1} receptors, adenosine inhibits fetal breathing, eye and body movements, as well as depressing excitatory neurotransmission and causing neuronal hyperpolarisation, all of which will reduce fetal oxygen consumption (Karimi et al., 1996; Mendonca et al., 1993).

In chapters 3 and 5 I reported that moderate asphyxia in the ovine fetus is associated with an increase in cerebral blood volume and in the cerebral concentration of oxidised cytochrome oxidase, as measured by near infrared spectroscopy (see also Newman et al., 2000). I hypothesise that these changes are the result of the dual actions of endogenous adenosine on cerebral blood vessels and on cerebral metabolism. The aim of this study, therefore, was to determine whether adenosine, administered exogenously to the chronically...
instrumented ovine fetus in utero, would lead to similar changes in cerebral blood volume and cytochrome oxidase redox state as those observed during moderate asphyxia.

6.2 METHODS

6.2.1 Surgical preparation and fetal surgery

All work was conducted in accordance with the Animals (Scientific Procedures) Act (1986) on ewes bearing singleton near term fetuses (~123d gestation). Surgical procedure has been described previously (Newman et al., 2000), but briefly —Six date-mated Romney Marsh ewes bearing singleton fetuses were used in these experiments. Fetal surgery was performed under aseptic conditions. Catheters were placed in the fetal brachial artery and jugular vein on one side such that their tips were close to the heart. On the contralateral side, a Transonic flow probe was placed around the carotid artery. A pair of electrodes was sewn onto the chest wall for monitoring of fetal ECG (Cooner Wire Corp, Chatsworth, California, US). Using a T-incision, the skin of the scalp was peeled back. Burr holes were made in the skull overlying the parasagittal cortex and electrodes were placed on the dura to allow monitoring of electrocortical activity. The burr holes were sealed using rubber caps and cyanoacrylate. Infrared optodes were placed on the skull overlying the parasagittal cortex and held firmly in place by moulded black rubber holders sutured to the edges of the skin incision. Apposition of optodes to scalp was improved further when the scalp incision was closed over the holders and optodes. The fetus was replaced and the uterus closed in two layers. The maternal abdomen was closed and the ewe allowed to recover following placement of a catheter in the maternal recurrent metatarsal vein for administration of antibiotics.

6.2.2 Animal maintenance and antibiotic regime

Ewes were held in metabolic carts for the duration of the study and had access to water and hay ad libitum. Before surgery, the ewe was given prophylactic Streptapen 1g IM. After the surgery, antibiotics were given each morning; Crystapen 150mg to the amniotic cavity, 300mg iv. to the ewe and 150mg to the fetus iv. for five days and Cidomicin 40mg to the amniotic cavity, 40mg to the ewe iv. for two days. A fetal arterial blood sample was taken each morning before giving the antibiotic regime and tested for gases and electrolytes with a BGE machine (BG Electrolytes 14008-01 and Co-Oximeter 482, Instrumentation Laboratories, UK).

6.2.3 Infusion protocol

After confirmation of a stable NIRS signal for one hour, adenosine infusion began into the catheterised carotid artery. As reported by Karimi and co-workers (Karimi et al., 1996), administering high doses of adenosine leads to substantial decreases in fetal heart rate and hypotension, therefore we present data for infusion at a relatively low dose known to reproduce fetal levels during hypoxaemia - 3 μmol/kg/min. Infusion of adenosine
at 3 μmol/min/kg produced no significant changes in haemodynamics. Each infusion was given over 20 minutes, which was sufficient for NIRS data to reach a steady state. At the end of 20 minutes, the infusion was stopped and infusion with saline recommenced.

6.2.4 Data collection

Experiments were performed on either day two or three after surgery. Haemodynamic and NIRS data were collected for 1 hour prior to, during and 1 hour after adenosine infusion.

Arterial and central venous pressures were measured (SensoNor pressure transducers, Horten, Norway and Digitimer Ltd amplifiers, Welwyn Garden City, UK) corrected for amniotic pressure and monitored by use of MacLab software (ADInstruments, UK). Fetal heart rate and carotid flow were monitored using MacLab software and saved to disk for off-line analysis. The ECoG signal was monitored to confirm normal cerebral function by the presence of sleep state cycling.

Blood samples were collected at -5, +5, +20 and +50 minutes relative to the start of a 20 minute adenosine infusion. 0.5 ml samples were collected from carotid artery and jugular vein at each time point. Samples were immediately tested for gases and electrolytes (BGE machine as above) and for glucose and lactate (YSI 2300 STAT Plus), temperature-corrected to fetal temperature of 39.5°C.

DO$_2$ values were calculated as the product of carotid flow (Q) and carotid arterial oxygen content (Ca), corrected to 39.5°C. VO$_2$ values were calculated using the appropriate venous oxygen content (Cv) and Fick's law: VO$_2$ = Q.(Ca - Cv), where possible.

6.2.5 NIRS data analysis

Spectra were saved to disk for off-line analysis. Absolute changes in chromophore concentration were calculated by subtracting from the reference spectrum (spectrum at time zero), then fitting between 780 and 900 nm to previously determined individual chromophore absorption spectra using a least-squares multilinear regression algorithm (Matcher et al., 1995; Wray et al., 1988). Residual changes in optical density, not accounted for in the fitting process, were analysed to look for large or systematic changes which might indicate the presence of another chromophore not included in the algorithm. Optical pathlength was obtained using second order differential analysis from the 840nm water absorption feature (C Cooper et al., 1996).

This analysis provides absolute Δ[HbO$_2$] and Δ [Hb] in μmol/l from a zero set at the start of the experiment. Since cytochrome oxidase (CcO) concentration does not change over the time scale of the experiment, changes in the [CcO] signal represent changes in the amount of the oxidised enzyme present relative to the start of the experiment and not to changes in enzyme levels. In order to get an impression of the magnitude of
changes in CcO observed relative to control values; CcO was fully reduced just prior to culling in several experiments by infusion of sodium cyanide at 10mg/ml/min. A value for changes in total haemoglobin concentration \((\Delta[Hb])\) can be generated from the sum of \(\Delta[HbO2]\) and \(\Delta[Hb]\) at any time point. thb is related to blood volume (BV) through the haematocrit.

6.2.6 Data presentation and statistical methods
NIRS data were obtained from 5 fetal sheep. All data are expressed as mean ± SEM. Time points taken for analysis are, -5, +5, +20 and +80 minutes relative to the start of infusion. Repeated measures ANOVA with Newman-Keuls post hoc tests were used to compare multiple data points with control (-5 minutes). In the case of O₂ delivery and consumption, where there are no data for the recovery period, unpaired t-test was used. Statistical significance is taken as \(p < 0.05\).

6.3 RESULTS
In order to find an appropriate infusion rate for adenosine to achieve metabolic effects, I began with the doses given by Karimi (Karimi, Ball & Power, 1996); 1, 3 & 6 μmol/kg/min. I also looked at a higher dose, 9 μmol/kg/min. I found that 6 & 9 μmol/kg/min resulted in a rapid fetal bradycardia and fall in mean arterial pressure, followed by tachycardia, presumably a baroreflex to maintain fetal CVO. In this Chapter, I will only present data for 3 μmol/kg/min, the dose at which there were minimal cardiovascular changes.

6.3.1 Signal stability during saline infusion
Infusion of fetal arterial and venous catheters at 0.3 ml/hr with 0.9% saline (Baxter) heparinised with 25,000U heparin per litre started immediately following recovery of the ewe from surgery. Saline infusion continued for the duration of the study. During this time, the fetal variables were stable. Data for adenosine infusion are compared to the control period where saline was infused at 0.3 ml/hr.

6.3.2 Blood gases and acid-base status
As shown in Table 1, there was no significant effect of adenosine infusion at 3 μmol/kg/min on P₀₂, PₐCO₂, pH₀₂, [glucose] or [lactate]. Blood variables were not different from control levels 30 minutes after cessation of adenosine infusion.
Table 6.1 Data (mean ± SE) for haemodynamic and blood variables at times relative to the start of a 20 minute adenosine infusion.

<table>
<thead>
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<th></th>
<th>-5 minutes</th>
<th>+20 minutes</th>
<th>+50 minutes</th>
</tr>
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<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>48.4 ± 4.2</td>
<td>43.7 ± 1.8</td>
<td>49.8 ± 3.7</td>
</tr>
<tr>
<td>FHR (bpm)</td>
<td>184 ± 7</td>
<td>203 ± 15</td>
<td>182 ± 11</td>
</tr>
<tr>
<td>CaBF (ml/min)</td>
<td>61.4 ± 8.5</td>
<td>80.3 ± 16.9</td>
<td>70.4 ± 12.4</td>
</tr>
<tr>
<td>$P_{O2}$ (mmHg)</td>
<td>23.6 ± 1.3</td>
<td>24.2 ± 0.7</td>
<td>24.0 ± 1.9</td>
</tr>
<tr>
<td>$P_{CO2}$ (mmHg)</td>
<td>45.8 ± 1.0</td>
<td>44.0 ± 2.5</td>
<td>47.2 ± 2.8</td>
</tr>
<tr>
<td>$pH_a$</td>
<td>7.34 ± 0.01</td>
<td>7.31 ± 0.03</td>
<td>7.33 ± 0.04</td>
</tr>
<tr>
<td>[Lactate]$_A$ (mmol/l)</td>
<td>1.16 ± 0.27</td>
<td>1.64 ± 0.32</td>
<td>1.20 ± 0.38</td>
</tr>
<tr>
<td>[Glucose]$_A$ (mmol/l)</td>
<td>0.76 ± 0.16</td>
<td>1.19 ± 0.34</td>
<td>0.83 ± 0.12</td>
</tr>
</tbody>
</table>

Table 6.3.3 Haemodynamics

Table 1 shows that MAP, FHR and CaBF did not change from control levels during adenosine infusion. Neither were haemodynamic variables significantly different from control 30 minutes after the end of adenosine infusion.

6.3.4 Oxygen delivery and consumption

Figure 4 shows that there was no change in oxygen delivery to the fetal head during adenosine infusion. At the end of the 20 minute infusion, however, oxygen consumption had fallen significantly from $1.29 ± 0.21$ to $0.64 ± 0.09$ ml O$_2$/min ($p < 0.05$, unpaired t test vs. -5 minutes).

6.3.5 Changes in concentration of HbO$_2$ and Hb

Hb began to increase ~5 minutes after the start of adenosine infusion, rising to a plateau of $14.2 ± 2.5$ μmol/l above control ($p < 0.05$ Repeated measures ANOVA with Newman-Keuls post hoc test, $n = 6$). After another 6 minutes, HbO$_2$ also began to rise, reaching a plateau of $9.0 ± 2.4$ μmol/l above control after approximately 15 minutes ($p < 0.05$, test as for Hb). Both Hb and HbO$_2$ began to fall ~2 minutes after the cessation of infusion and were not different from pre-infusion levels 30 minutes later.

6.3.6 Changes in tHb

tHb began to rise ~5 minutes after the start of infusion and rose steadily for just over 10 minutes, reaching $18.3 ± 3.5$ μmol/l above control at the end of the infusion period ($p < 0.05$, Repeated measures ANOVA with Newman-Keuls post-hoc test). tHb began to fall toward control levels ~2 minutes after the end of adenosine infusion, and was not different from control levels 30 minutes later.
Figure 6.2 Shows haemodynamic variables before, at two times during, and 30 minutes after the end of adenosine infusion at 3 μmol/kg/min. There were no significant differences in these variables at any time point relative to control (paired t-test vs. control). Units are bpm for FHR, mmHg for MAP and ml/min for CBF.

Figure 6.3 Data for oxygen delivery (DO₂) and consumption (VO₂) by the head before (CONTROL) and at the end of a 20-minute infusion of adenosine at 3 μmol/kg/min (INFUSION). Whilst DO₂ did not increase, there was a significant fall in VO₂ by the end of adenosine treatment. * p < 0.05, paired t-test vs. control.

6.3.7 Changes in oxidised CcO

Oxidised cytochrome oxidase levels began to increase ~4 minutes after the start of infusion, rising by 0.48 ± 0.01 μmol/l vs. control after 20 minutes of infusion (p < 0.05, test as for thb). CcO oxidation fell slowly following the cessation of the infusion, reaching ~0.04 ± 0.03 μmol/l vs. control after 30 minutes of recovery.
Figure 6.4 Mean data ± SE for HbO₂ and Hb (upper panel) and tHb and CcO (lower panel). Bars represent Control (Left), change at end of adenosine infusion (Middle) and after 30 minutes of recovery (Right).
6.4 DISCUSSION

A variety of studies have provided circumstantial evidence that adenosine may have metabolic effects, such as the inhibition of fetal breathing movements (Koos et al., 1991, 1992 & 1998) in addition to cardiovascular effects. The possibility has also been expressed that these changes may provide some degree of neuroprotection (Rudolphi et al., 1992; Ongini et al., 1997; Kawahara et al., 1998; Calabresi et al., 2000). Our study looks at the fetus during normoxaemia and investigates adenosine’s metabolic effects.

The main findings of this study are that:

A. During adenosine infusion there were significant increases in cerebral [HbO₂] and [Hb] (Figure 6.4, upper panel), and in [tHb] and [CcO] (Figure 6.4, lower panel)

B. There were no significant haemodynamic changes during adenosine infusion at 3 μmol/kg/min (Table 6.1).

C. Cerebral DO₂ was unchanged, but there was a significant fall in cerebral VO₂ by the end of a 20-minute adenosine infusion at 3 μmol/min/kg (Figure 6.3).

Much of the fetal work concerning the effects of adenosine have concentrated on the involvement of adenosine with hypoxic ventilatory depression (Koos & Doany, 1991) and relatively little work has been devoted to adenosine’s other effects, such as suppression of metabolism (Karimi, Ball & Power, 1996) or its putative role in neuroprotection (Ongini et al., 1997). This idea that adenosine may have metabolic inhibitory and neuroprotective functions is an extension of the hypothesis set out in Sattin & Rall’s 1970 paper that adenosine, or perhaps ATP, might directly modulate neural activity. This work has been supported by many subsequent investigations both in vivo and in vitro. A growing body of evidence from animal models suggests that adenosine reduces metabolic rate during and following hypoxia-ischaemia (Rudolphi et al., 1992; Marks et al., 1996; Kamii et al., 1996; Kawahara et al., 1998) in addition to its cardiovascular effects.

These cardiovascular effects include a myocardial A1 receptor-mediated bradycardia and, when present in high concentrations, a vasodilation mediated by A2 receptors on peripheral vasculature (Hein, Bellardinelli & Kuo, 1999; Koos & Chau, 1998). In these experiments there was no significant difference in mean arterial pressure or carotid blood flow during adenosine infusion. However, carotid flow may not be the same thing as cerebral flow (compare Richardson, 1992 & Van Bel, 1985). We do see an increase in total cerebral haemoglobin concentration during adenosine infusion using NIRS. This is the sum of HbO₂ and Hb and is directly related to cerebral blood volume (CBV) through the haematocrit, an increase in tHb during adenosine infusion indicates an increase in CBV through an unknown mechanism. It may be that the cerebral vasculature is being dilated by the action of adenosine, or that there is venous congestion due to reduced venous return.
As mentioned above, there was no significant change in carotid flow during adenosine infusion. Taking CaBF and O₂ content data from our blood gas results, we have calculated that there was also no change in DO₂ during the infusion, although VO₂ had fallen by the end of the infusion protocol. This fall in oxygen consumption complements Karimi's data, although that was for a whole-body preparation and did not indicate that variations in metabolic response might occur - such as between skeletal muscle and brain. Cerebral metabolic rate is very closely linked to neuronal activity, being high in the low voltage state and low in the high voltage state (Clewlow et al., 1983). It is very interesting to note that we witnessed a switch in electrocortical activity from low to high voltage soon after the start of adenosine infusion, providing further evidence of adenosine's metabolic regulatory role.

Metabolic information can also be derived from the NIRS CcO signal. The CcO signal gives information about the oxidation state of the mitochondrial population interrogated by the incident near infrared light. Cytochrome oxidase is the final electron acceptor in the mitochondrial electron transfer chain and its redox state is influenced by three factors: (1) The flow of reducing equivalents from the tricarboxylic acid cycle, (2) The rate of transfer of electrons from cytochrome c, and (3) The availability of oxygen at the oxygen binding site. In these experiments, there was a significant increase in CcO oxidation by the end of the infusion period, similar in magnitude and direction to that previously seen by this group during asphyxia in late gestation fetal sheep (Newman et al., 2000). One possible link between adenosine and this oxidation may be nitric oxide (NO) whose release is stimulated by increased adenosine concentration.

Whilst NO is known to interact with the components of the mitochondrial electron transfer chain, it seems unlikely from our present knowledge that mitochondrial oxidation (as seen with NIRS) would be the effect of transient increases in nitric oxide levels. NO can inhibit metabolism both reversibly and irreversibly; the reversible inhibition is due to competition between NO and O₂ for the cytochrome oxidase binuclear centre, the site at which molecular oxygen is reduced to water in the mitochondrion. Since this is a competitive inhibition, NO effects are greater (a) when PO₂ is low, and (b) when NO is high – both of which may be the case during profound hypoxias. Reversible inhibition, however, occurs when mitochondrial are exposed to high NO levels for several hours (Brown, 1999; Clementi et al., 1998). In this instance, the inhibition is due to permanent nitrosylation of protein thiols in mitochondrial complex I and removal of iron from iron-sulphur centres. The effect of reversible inhibition on cellular redox would be to allow electrons to build up in the transfer chain and cause reduction of the Cu₄ moiety. Irreversible inhibition would block electron transfer upstream and cause Cu₄ oxidation.

Since adenosine infusion causes an oxidation which is both reversible and relatively fast (ie less than the time required for nitrosylation of complex I), it suggests that NO is not the mediator of adenosine hypometabolism. Recent work by Yager and co-workers calculated cytochrome oxidase redox state using the acetoacetate/β-
hydroxybutyrate substrate couple (Yager, Brucklacher and Vannucci, 1996); their work suggests another possibility. In their experiments, hypoxia-ischaemia caused a reduction followed by an oxidation of mitochondrial redox. They suggest that a reduction in the flow of reducing equivalents, not low oxygen delivery, is the limiting factor in respiration during severe hypoxia in immature animals. Yager also raises the question as to whether substrate supplementation in the fetus may ameliorate neuronal injury, as suggested by the work of Vannucci and co-workers and separately by Yager and colleagues (Vannucci et al., 1992; Yager et al., 1992).

6.5 Summary

The data presented in this chapter demonstrates that infusion of adenosine into the late gestation ovine fetus results in a fall in cerebral metabolism, similar to that observed previously during asphyxia. There is now circumstantial evidence to suggest that adenosine acts as an endogenous neuroprotective agent in the immature brain. However, further work using pharmacological interventions to augment or block the endogenous adenosine response to hypoxia, coupled with measurement of sequelae in terms of neural structure and function, will be needed to clarify whether adenosine is indeed neuroprotective. These results do not supply an answer regarding the pathway by which adenosine inhibits metabolism, whether this if functional during hypoxia-ischaemia and the effector mechanism(s) involved. These questions remain to be addressed.
7.1 Introduction

Premature rupture of membranes (PROM) occurs in approximately 10% of all pregnancies (Gunn, Mishall & Morten, 1970) and is especially prominent in preterm births, where more than 30% of such births are reported to be preceded by premature preterm rupture of membranes (Mercer et al., 1997). Whilst the link between clinically diagnosed chorioamnionitis and PROM may only be tenuous (Guzick and Winn, 1985), there is evidence that the presence of chorioamnionitis, defined by various methods, leads to increased risk of fetal mortality (Hillier et al., 1991) and morbidity (Hardt et al., 1985; Alexander et al., 1998; Morales, 1987), especially for cerebral palsy (Murphy et al., 1995).

Mild infection, suggested by asymptomatic fetal bacteriuria is strongly associated with preterm birth and low birth weight (Romero et al., 1989), whilst clinically-evident maternal infection increases the prevalence of unexplained cranial sonographic echolucencies and subsequent cerebral palsy (Leviton et al., 1999; Grether & Nelson, 1997). Supported by evidence that leukocytes detected in amniotic fluid are of fetal, not maternal, origin (Sampson et al., 1997), it has been proposed that the fetal inflammatory response to chorioamnionitis may be much more important for subsequent fetal morbidity than the maternal response to infection (Leviton et al., 1999). These data suggest that the magnitude of the fetal inflammatory response may correlate with the degree of white matter injury (periventricular leukomalacia, PVL) and serious intraventricular haemorrhage in the infant.

Recent work has shown that the levels of pro-inflammatory cytokines are elevated in the blood of infants suffering PVL (Yoon et al., 1996 & 1997) which has, until recently, been thought of as a primarily hypoxia-ischaemia related lesion. At the same time, much of the pathophysiology of disease has, in the last two decades, been explained in terms of the induction pro-inflammatory cytokines and subsequent host responses, with bacterial modulins playing an important role as potent stimulators of cytokine production (Henderson, Poole & Wilson, 1996). Since the work of Gilles (Gilles et al., 1977), there have been few publications of fetal models of cytokinaemia, presumably due to the difficulty in keeping animals alive after administration of the infectious agent. We have, therefore, used our expertise with the chronically-instrumented mid gestation sheep fetal preparation and cranial near infrared spectroscopy to develop a model.
of fetal cytokinaemia in which a controlled nanogram dose of bacterial lipopolysaccharide (LPS) is given intravenously to the fetus. The fetus can subsequently be kept alive for at least 3 days in utero. This has allowed us to investigate for the first time the progression of the fetal haemodynamic and metabolic response to LPS and the subsequent histopathology in brain and other organs.

7.2 Methods

7.2.1 Surgical preparation and fetal surgery

All work was conducted in accordance with the Animals (Scientific Procedures) Act (1986) on ewes bearing singleton fetuses at ca. 90 days gestation. The surgical procedure has been described in Chapter 2 but, briefly, surgery was performed on six date-mated Romney Marsh ewes bearing singleton fetuses under strict aseptic conditions. The ewe was anaesthetised with thiopentone sodium and 3% halothane. The pregnant horn of the uterus was identified by the presence of a corpus luteum of pregnancy on one ovary. The uterine artery supplying this horn was identified and tracked back until to a point before branching begins. The connective tissue overlying the artery was cut, the artery dissected free and a 6R Transonic flow probe placed around the vessel. The connective tissue was then sewn back over the flow probe. Next, the fetus was partially exteriorised through a small uterine incision and catheters were placed in the fetal brachial artery and jugular vein on one side while a Transonic flow probe was placed around the carotid artery on the contralateral side. A pair of electrodes was sewn onto the chest wall for monitoring fetal ECG (Cooner Wire Corp, Chatsworth, California, US). Using a T-incision, the scalp was peeled back and infrared optodes in moulded black rubber holders were placed on the skull overlying the parasagittal cortex, glued firmly in place and further secured by suturing the holders to the edges of the skin incision. Apposition of optodes to scalp was improved further when the scalp incision was closed over the holders and optodes. The fetus was replaced in the amniotic cavity and the uterus closed in two layers. After the maternal abdomen was closed, a catheter was placed in the maternal recurrent metatarsal vein for administration of antibiotics. The ewe was then allowed to recover for 3 days.

7.2.2 Animal maintenance and antibiotic regime

Ewes were held in metabolic carts for the duration of the study and had free access to water and hay. Before surgery, the ewe was given prophylactic Streptapen 1g IM. After the surgery, antibiotics were given each morning; Crystapen 150mg to the amniotic cavity, 300mg iv. to the ewe and 150mg to the fetus iv. for five days and Cidomicin 40mg to the amniotic cavity, 40mg to the ewe iv. for two days. A fetal arterial blood sample was taken each morning before giving the antibiotic regime and tested for gases and electrolytes with a blood gas analyser (BG Electrolytes 14008-01 and Co-Oximeter 482, Instrumentation Laboratories, UK) and for glucose and lactate (YSI Stat Plus 2300).
7.2.3 Haemodynamic monitoring

PowerLab software was used to monitor fetal haemodynamic variables, which were continuously saved to disk for off-line analysis. SensoNor pressure transducers attached to brachial artery and amniotic cavity catheters were used to measure arterial and amniotic pressure changes, respectively. A Digitimer subtraction unit was used to correct arterial pressure for changes in amniotic fluid pressure. Uterine and carotid artery flows were monitored continuously with Transonic flow probes whilst fetal heart rate was calculated from the pulsatile carotid artery flow or from fetal electrocardiogram, whichever signal was more reliable.

7.2.4 Near infrared spectroscopy

One of the optical bundles applied to the fetal skull transmitted near infrared light to the fetal brain whilst the second transmitted unabsorbed light to the CCD camera and spectrometer. The duration of each exposure was timed such that the peak amplitude of the absorption spectrum fell approximately at 50% of the maximal detection limit, the exposure time was thus between 0.5 and 8 seconds. Each absorption spectrum was saved to disk for off line analysis.

Analysis of NIRS spectra was performed as described earlier (Newman et al., 2000). Briefly, the first spectrum in the series was used as a reference against which changes in absorption (due to changes in concentrations of HbO₂, Hb and in oxidised CcO) would be measured. Taking the arithmetic difference between each spectrum and the reference generates a difference spectrum. Individual chromophore absorption spectra are applied to the difference spectrum and data for changes in chromophore concentration are generated which can be corrected for mean photon pathlength (using second differential method) giving change in concentration from time zero in μmol/l.

The arithmetic sum of HbO₂ and Hb gives total Hb (tHb), which is related to blood volume through the haematocrit. In the absence of significant changes in haematocrit, changes in tHb can be directly equated with changes in blood volume – cerebral blood volume in this instance.

7.2.5 Experimental protocol

Fetal haemodynamic variables (heart rate, arterial pressure and carotid artery flow) and NIRS variables \((\Delta[HbO_2], \Delta[Hb], \Delta[tHb], \Delta[CcO])\) and uterine artery flow to the pregnant horn were monitored continuously for a minimum of one hour prior to LPS administration and then for 72 hours after administration. All data were saved continuously to disk for off line analysis. Blood samples were taken prior to and at the following times after LPS administration: 1, 2, 4, 8 and 72 hours.
LPS was administered as follows: 1 mg E. coli lipopolysaccharide (Serotype 0111:B4, Sigma-Aldrich, Poole, UK) was weighed, dissolved in 10ml 0.9% sterile saline and diluted with saline to give 0.1 µg LPS in 2ml saline. LPS was administered as a bolus injection intravenously to the fetus and the catheter flushed with sterile saline.

### 7.2.6 Statistical analysis

Data were collected at one-hour intervals relative to LPS administration. Each data point is the mean of a one-minute block of data. Mean arterial pressure (MAP) was calculated from the systolic and diastolic pressures (Ps and Pd, respectively) as follows:

\[
MAP = Pd + \frac{(Ps - Pd)}{3}
\]

Data are shown as group means ± standard errors. Data for those fetuses that died after LPS administration are not included in the group means.

A full haemodynamic record for 72 hours was available for only two of the five experiments. It was therefore difficult to obtain statistical significance using repeated measures ANOVA, the preferred test for progressive changes after the start of a challenge. The data has, therefore, been analysed in terms of time to significant change using paired t-test. This was achieved by assessing the data by eye for overlap of standard error bars, followed by paired t-test of the first data point at which error bars appeared not to overlap. For non-binomial data sets, overlap of the 25th and 75th centiles of the upper and lower sets, respectively, was used to exclude data points from statistical analysis. Statistical significance was accepted when \( p < 0.05 \) and is represented by the symbol \( \alpha \) on diagrams.

### 7.3 Results

In order to study the effects of bacterial lipopolysaccharide on the fetus *in utero*, we had initially to decide upon the dose that we were to give each fetus. Since there are no equivalent experiments in the literature, I arbitrarily began with a 1mg dose. That fetus died within two hours of LPS administration. The dosage was progressively reduced, usually by two orders of magnitude, until a fetus survived the treatment. In this way, I arrived at a dose of 100ng LPS dissolved in sterile 0.9% saline. With this dose we achieved a high survival rate, losing only three out of seven fetuses.

#### 7.3.1 Blood sample data

Fetal blood gases were obtained in six animals, of which full records were only available for two due to technical problems. Data derived from arterial and venous blood samples are given in Tables 7.1 and 7.2, respectively. Briefly, there were no significant differences in any of the variables measured from fetal blood.
### Table 7.1 Arterial blood sample data at times relative to intravenous administration of LPS to the fetus.

Blood samples were tested for pH, gases, electrolytes, lactate and glucose as described in section 7.2.5. Data are presented as mean, ± standard error where possible. Statistical analysis was performed using repeated measures ANOVA vs control with Newman-Keuls post hoc tests as appropriate. Analysis was inconclusive owing to the small sample size. Only O₂ content (O₂ ct) was close to significance, with \( p = 0.07 \).

<table>
<thead>
<tr>
<th>Time relative to LPS administration (hours)</th>
<th>-1</th>
<th>1</th>
<th>4</th>
<th>8</th>
<th>15</th>
<th>24</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.383 ± 0.016</td>
<td>7.194 ± 0.159</td>
<td>7.290</td>
<td>7.322</td>
<td>7.345</td>
<td>7.344</td>
<td>7.359 ± 0.009</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>44.5 ± 2.4</td>
<td>39.8 ± 2.2</td>
<td>48.1</td>
<td>49.5</td>
<td>49.8</td>
<td>48.3</td>
<td>46.8 ± 0.4</td>
</tr>
<tr>
<td>PO₂ (mmHg)</td>
<td>31 ± 1</td>
<td>26 ± 3</td>
<td>23</td>
<td>19</td>
<td>29</td>
<td>27</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>20 ± 2</td>
<td>21 ± 4</td>
<td>25</td>
<td>21</td>
<td>13</td>
<td>22</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>HCO₃ (mM)</td>
<td>26.2 ± 2.5</td>
<td>23.0 ± 0.1</td>
<td>22.7</td>
<td>25.2</td>
<td>26.7</td>
<td>25.8</td>
<td>26.0 ± 0.8</td>
</tr>
<tr>
<td>BE (mM)</td>
<td>2.3 ± 2.5</td>
<td>-1.1 ± 0.0</td>
<td>-2.3</td>
<td>0.6</td>
<td>2.3</td>
<td>1.4</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>7.1 ± 0.4</td>
<td>5.6 ± 1.1</td>
<td>6.5</td>
<td>4.6</td>
<td>5.1</td>
<td>5.8</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>Sat (%)</td>
<td>86.1 ± 3.0</td>
<td>78.5 ± 6.3</td>
<td>65.5</td>
<td>61.7</td>
<td>79.3</td>
<td>79.1</td>
<td>83.2 ± 2.0</td>
</tr>
<tr>
<td>O₂ ct (vol % O₂)</td>
<td>8.5 ± 0.2</td>
<td>5.4 ± 1.8</td>
<td>5.9</td>
<td>3.9</td>
<td>5.6</td>
<td>6.4</td>
<td>7.1 ± 0.8</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>0.648</td>
<td>0.645</td>
<td>1.19</td>
<td>1.09</td>
<td>-</td>
<td>0.64</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>0.952</td>
<td>0.882</td>
<td>0.771</td>
<td>0.49</td>
<td>-</td>
<td>0.843</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 7.2 Venous blood sample data at times relative to intravenous administration of LPS to the fetus.

As with the arterial samples, these were tested for pH, gases, electrolytes, lactate and glucose. Data are presented as mean, ± standard error where possible. Again, the same statistical analysis was used as above; repeated measures ANOVA vs control with Newman-Keuls post hoc tests as appropriate. Analysis was again inconclusive owing to the small sample size.

<table>
<thead>
<tr>
<th>Time relative to LPS administration (hours)</th>
<th>-1</th>
<th>1</th>
<th>4</th>
<th>8</th>
<th>15</th>
<th>24</th>
<th>39</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.328 ± 0.012</td>
<td>7.162 ± 0.179</td>
<td>7.309</td>
<td>7.306</td>
<td>7.300</td>
<td>7.320</td>
<td>7.309</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>41.5 ± 2.6</td>
<td>43.5 ± 2.6</td>
<td>45.0</td>
<td>51.7</td>
<td>42.8</td>
<td>48.1</td>
<td>42.1</td>
</tr>
<tr>
<td>PO₂ (mmHg)</td>
<td>24 ± 1</td>
<td>20 ± 2</td>
<td>20</td>
<td>16</td>
<td>24</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>19 ± 2</td>
<td>20 ± 3</td>
<td>29</td>
<td>25</td>
<td>9</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>HCO₃ (mM)</td>
<td>21.5 ± 1.6</td>
<td>22.1 ± 0.9</td>
<td>22.2</td>
<td>25.3</td>
<td>20.7</td>
<td>24.4</td>
<td>20.7</td>
</tr>
<tr>
<td>BE (mM)</td>
<td>-2.8 ± 1.6</td>
<td>-2.4 ± 0.7</td>
<td>-2.4</td>
<td>0.4</td>
<td>-3.9</td>
<td>-0.3</td>
<td>-3.8</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>5.9 ± 0.4</td>
<td>5.1 ± 1.1</td>
<td>6.4</td>
<td>5.0</td>
<td>4.0</td>
<td>5.2</td>
<td>-</td>
</tr>
<tr>
<td>Sat (%)</td>
<td>72.5 ± 2.5</td>
<td>66.0 ± 5.8</td>
<td>58.1</td>
<td>41.9</td>
<td>70.2</td>
<td>64.3</td>
<td>72.8</td>
</tr>
<tr>
<td>O₂ ct (vol % O₂)</td>
<td>6.0 ± 0.5</td>
<td>4.2 ± 1.5</td>
<td>5.2</td>
<td>2.9</td>
<td>3.9</td>
<td>4.6</td>
<td>-</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>0.678</td>
<td>0.697</td>
<td>1.38</td>
<td>1.53</td>
<td>-</td>
<td>0.684</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>0.631</td>
<td>0.668</td>
<td>0.515</td>
<td>0.559</td>
<td>-</td>
<td>0.670</td>
<td>-</td>
</tr>
</tbody>
</table>
7.3.2 Fetal haemodynamics

Fetal heart rate was stable over the control period, starting from 194 ± 7 bpm at one hour before LPS administration. Thereafter, it rose for 9 hours, reaching significance at 5 hours when FHR was 222 ± 8 bpm (p < 0.05, paired t-test vs -2 hours). Heart rate then began to fall back towards control levels and was not significantly different to control at 14 hours after LPS administration. Heart rate remained at pre-administration levels for the remainder of the experimental period.

Fetal mean arterial pressure was 28.9 ± 5.0 mmHg during baseline recording and fell steadily after LPS administration, reaching a nadir after 9 hours when mean arterial pressure was 22.2 ± 2.1 mmHg. A statistically significant fall in pressure was achieved after 5 hours (p < 0.05, paired t-test vs.-2 hours). Mean arterial pressure began to rise again 9 hours after LPS administration and was not significantly different to control at 13 hours after LPS administration. MAP appeared to continue to rise until 56 hours after LPS administration. There are only two data sets for this variable and these data should therefore be interpreted with caution.

Carotid artery flow started at 29.6 ± 3.1 ml/min and rose steadily after LPS administration, reaching significance after 9 hours, at 40.3 ± 4.8 ml/min (p < 0.05, paired t-test vs. -2 hours). Carotid flow continued to rise before falling again after ca. 23 hours. Flow reached a plateau at 24 hours after which there was no significant change for the duration of the protocol.

7.3.3 Uterine artery flow

Uterine blood flow was stable for the 12 hours prior to LPS administration and was 207.4 ± 39.3 ml/min at one hour prior to the start of the experiment. Following injection of LPS, uterine flow slowly increased over 46 hours, reaching statistical significance at 36 hours after administration, when flow was 255.8 ± 22.8 ml/min (p = 0.038, paired t-test vs. -2 hours). Uterine blood flow remained high for the remainder of the experiment.

7.3.4 Oxygen delivery and consumption (DO, & VO)

A full set of delivery and consumption data have been collected from only one animal to date and these data are shown graphically in Figure 7.3 below. Briefly, by amalgamating the -2 and -1 hour control data, the control DO becomes 2.65 ± 0.19 ml O/min. This has fallen substantially at 4 hours before returning to control levels by 39 hours after LPS administration. VO is much lower than DO, as expected from our previous data shown in previous chapters. by amalgamating data for -2 and -1 hours, control VO was calculated to be 0.91 ± 0.20 ml O/min (n = 3). Figure 7.3 shows graphically that this falls to 0.14 ml O/min (n = 1) at 4 hours after administration before returning to baseline levels.
Figure 7.1 Haemodynamic variables before and after LPS administration at 0 hours. Despite the preliminary nature of the data, significant differences have been seen both in the fetal heart rate response and in the post-treatment carotid artery flow. $a$ indicates significant difference from $t = -2$ hours, using repeated measures ANOVA with Newman-Keuls post hoc tests.
Figure 7.2 Data for maternal uterine artery flow \((n = 3)\) before and after LPS administration IV to the fetus at time 0. Significant changes from pre-administration baseline are shown by \(a\). Repeated measures ANOVA used for statistical analysis as described for Figure 7.1.

Figure 7.3 Preliminary data for oxygen delivery and consumption of the fetal head. There is a progressive decline in both variables, reaching a nadir at 4 hours after administration of LPS. After this point, these variables return to baseline values by 39 hours after LPS administration.
7.3.5 Near infrared spectroscopy

Following administration of 0.1 µg LPS, there was variation both in the time taken for a measurable NIRS response to be elicited, and in the trajectory of the response (Figure 7.4). Owing to this, mean data needs to be interpreted with caution if trajectories differ significantly. Group mean data are shown in Figure 7.5; note the large error bars for some data points.

By 3 hours after LPS administration, mean HbO₂ had fallen to a nadir of -5.8 ± 1.8 µmol/l, whilst deoxyhaemoglobin concentration was rising. [Hb] increased after LPS administration, reaching a maximum of 12.8 ± 1.6 µmol/l at 7.5 hours after treatment. Taken together the small fall in HbO₂, a rise in Hb and only a small increase in tHb indicates that overall, cerebral saturation fell over the 7.5 hours after LPS administration. This was confirmed by arterial blood sample analysis (Table 7.1).

Total haemoglobin levels were the most variable NIRS data set (see Figure 7.4, lower panel), although the predominant response to LPS treatment was an increase in total haemoglobin concentration, and thus in cerebral blood volume. The maximum change observed in any single experiment was to +19.8 µmol/l at 2.5 hours after administration in 99-47, whilst the group mean data reached 8.9 ± 3.6 µmol/l at +7.5 hours.

There was a great deal of variation in the changes in the cerebral concentration of oxidised cytochrome oxidase following LPS administration. As with tHb, there is a tendency for CcO to oxidise after LPS is given, then for this variable to return to control levels. The mean data show this same trend, but between-animal variability meant that there was no statistically significant change overall.
Figure 7.4 Group data (mean ± SE) showing changes in cerebral HbO$_2$ and Hb (upper panel) and tHb and CcO (lower panel) before and for 30 hours after administration of 0.1 µg LPS IV to the fetus (time zero).
7.3.6 Histology

Table 7.3 summarises the results of histological examination of an age-matched, instrumented control fetus and of four experimental animals, each of whom received 0.1 μg LPS.

The control animal showed no evidence of histological injury in any area of the brain. The histological appearance of brains from those fetuses which have received LPS will therefore be compared to the control brain.

Of the four experimental animals, two had evidence of PVL, two had other white matter damage and three had basal ganglia infarcts. One fetus had basal ganglia damage but no other damage; this fetus had evidence of chorioamnionitis in the coruncles of the placenta. One fetus with severe brain damage (99-23) had a large intraventricular haemorrhage with parenchymal involvement (Figure 7.6). One fetus with PVL also had in utero pneumonia, as evidenced by white cell invasion of the lung lumen (Figure 7.10).

<table>
<thead>
<tr>
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<th></th>
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<tbody>
<tr>
<td>PVL</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
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<tr>
<td>Other WM</td>
<td>None</td>
<td>subcortical, global</td>
<td>None</td>
<td>None</td>
<td>cortical infarcts</td>
</tr>
<tr>
<td>Basal Ganglia</td>
<td>None</td>
<td>unilateral infarct calcification on left</td>
<td>infarcts</td>
<td>None</td>
<td>infarcts</td>
</tr>
<tr>
<td>Side</td>
<td>NA</td>
<td>L basal ganglia bilateral PVL</td>
<td>bilateral</td>
<td>bilateral</td>
<td>NK</td>
</tr>
<tr>
<td>Other damage</td>
<td>None</td>
<td>SEH + IVH</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Non-Vascular</td>
<td>None</td>
<td>None</td>
<td>Chorioamnionitis</td>
<td>Pneumonia</td>
<td>None</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>22</td>
<td>28</td>
<td>20</td>
<td>NK</td>
<td>24</td>
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</tbody>
</table>

Table 7.3 Summarises the histological assessment of a typical control brain and four brains from fetuses receiving a 0.1 μg IV dose of LPS. The control brain was assessed as histologically normal, with no evidence of brain injury. All four brains from LPS-treated fetuses showed evidence of damage.
Figure 7.5 Macroscopic images of slices from the control brain (A) and that of a fetus given LPS (B). Note the area of PVL (bar) and how this compares with the control periventricular white matter at the same location in (A).
Figure 7.6 Intraventricular haemorrhage with parenchymal involvement, as seen before (1) and after (2) embedding in paraffin and staining with H & E. In (2), there has been massive disruption of the cerebral tissues; note the areas of cavitation that can be seen in the periventricular region, which is consistent with ultrasound data on the development of PVL (Rodríguez et al., 1990).
Figure 7.7 x100 image of haemorrhage into the periventricular white matter and the liquefaction necrosis which characterise the cerebral response to LPS administration.

Figure 7.8 White matter from a control (A) and LPS-treated (B) fetus. Normal white matter appears sparsely cellular with few capillaries, it often has a striated appearance due to the presence of axonal pathways. (B) shows the effect of LPS treatment, there is a massive increase in cellularity and vascularisation, the tissue now looks disorganised and there is a large area of pale-staining necrosis in the bottom right corner.
Figure 7.9 Sample of lung tissue showing evidence of polymorphs in the lung spaces (arrows). These polymorphs are likely of fetal, and not maternal, origin (Scott et al., 1998) and are usually taken as a sign of fetal inflammatory response to in utero infection.

Figure 7.10 Maternal cotyledon showing evidence of infiltration by numerous polymorphs (arrowed), an indication usually associated with placental chorioamnionitis. Note that this has occurred after LPS has been given to the fetus, there has been no maternal infection.
7.4 DISCUSSION

This study shows that brain white matter injury is present as early as three days after intravenous administration of 100 ng *E.coli* lipopolysaccharide to fetal sheep at 0.65 gestation. We have demonstrated that the fetal cardiovascular response to LPS administration is apparent from 1 hour after administration. This develops as tachycardia, hypotension and carotid hyperaemia up to ca. 8 hours after LPS dosing. Whilst the fetal heart rate and carotid flow return to baseline values, mean arterial pressure may show a secondary rise up to 72 hours after LPS administration, although the statistical analysis of this variable was hampered by the small sample size.

The progressive and significant changes in haemodynamic variables demonstrated in this ovine fetal model of endotoxaemia are in contrast to the data of Yoneyama and co-workers (Yoneyama et al., 1998) who saw no significant difference in fetal heart rate or mean arterial pressure following LPS administration to fetal goats. There are two possible reasons for this discrepancy. First, we know that the sheep fetus is very sensitive to LPS and that the 100 ng dose represents the upper limit of what can be given to these fetuses if they are to survive treatment. Indeed, a number of fetuses were given this dose and died in the subsequent 8 – 10 hours and all fetuses given higher doses died within 5 hours. Thus it may be that, although the fetal goats received a higher dose of LPS than our fetal sheep, that they were not receiving an equivalent insult, relative to their maximum LPS tolerance. The second possible reason for the lack of haemodynamic response is that there may be maturational changes in responsiveness to LPS. Infection as an insult leading to perinatal brain injury is primarily one associated with the preterm infant, not the late-gestation infant. This being the case, it may be that the fetal goats were more mature and thus less responsive to treatment.

A new piece of haemodynamic data given here which has not been available previously is the uterine artery flow to the ovine placenta. The data presented in Figure 7.2 show that there is a progressive increase in uterine flow above that which would be expected during normal growth at this gestation (Dr S Miller, personal communication). This increased flow indicates that *O₂* delivery to the placental unit is also progressively increasing over the experimental period. Despite this progressive increase in *O₂* delivery to the placenta, we have shown that the fetus was hypoxic by 8 hours after LPS administration (Table 7.1), suggesting that either the fetal metabolic rate had increased and was running ahead of *O₂* delivery, or that placental *O₂* transfer was impaired, presumably secondary to inflammation of the placental vascular bed. This systemic hypoxia was evidenced by a reduction in *O₂* content of the blood, which resulted in a fall in *O₂* delivery to the head. Instead of compensatory increases in *O₂* extraction, we saw a fall in cerebral *O₂* consumption which was corroborated by NIRS data.

Cerebral NIRS was used during these experiments to investigate cerebral haemodynamics and metabolism. Using cerebral NIRS, we determined that there was a significant fall in cerebral HbO₂ in the fetal head in the
8 hours following LPS administration whilst total blood volume was increasing. This situation suggests that arterial saturation was falling during this time. Blood sample data confirms that arterial saturation fell after LPS administration (Table 7.1). The NIRS CcO signal also provided confirmation of a change in cerebral metabolic rate.

The NIRS signal, whilst variable, showed some oxidation after LPS administration. Such an oxidation of CcO could be caused by:

1) an increase in the CcO turnover rate by increasing intracellular \( PO_2 \)
2) increased trans-mitochondrial pH gradient
3) reduction in the flow of reducing equivalents down the oxidative phosphorylation pathway

There is no evidence for hyperoxia in the fetal brain following LPS administration, on the contrary both cerebral HbO2 and arterial \( O_2 \) content are lower after giving LPS than before and, since cerebral DO2 appears to be reduced, this data does not suggest that CcO oxidation is increased as a result of elevated tissue \( PO_2 \). Previous work indicates that CcO oxidation may be associated with a fetal hypometabolic adaptive response to hypoxic stress in fetal brain (Newman et al., 2000; Yager et al., 1996) due to reduced flow of reducing equivalents, which suggests that the hypometabolic state may be a direct result of either relative hypoxaemia rather than of LPS per se, or the combination of hypoxia and LPS stimulating the release of a factor which actively reduces VO2. Unfortunately, without differentiated fetal ECoG waveforms at this gestation, this hypothesis cannot be easily proven with biophysical monitoring and will require further investigation.

How are changes in cerebral haemodynamics and metabolism related to intracranial haemorrhage, periventricular white matter injury and cerebral palsy? There are two theories which may explain the aetiology of PVL in preterm neonates. The first of these is a haemodynamic model. The most important features of this models are

1) The incidence of PVL is very variable from one centre to another
2) This lesion is present at autopsy in infants with a postnatal survival of more than a few days
3) There is a higher incidence with infants suffering cardiorespiratory disturbance; this incidence increases with the length of time spent on artificial ventilation

This is, in essence, a postnatal ischaemic model whereby a sick premature infant suffers periods of hypotension and, since the infant may have impaired autoregulation, or blood pressure may be at the lower limit of the infant's autoregulatory range, pressure drops result in falls in cerebral blood flow. The consequence is ischaemia in the fragile developing arterial end and border zones in the periventricular white matter, especially at the level of the trigone and the interventricular foramen (Foramen of Monroe). This ischaemia can cause focal necrosis in the periventricular white matter and/or a diffuse injury. In either case,
early differentiating oligodendroglia seem to be particularly susceptible and it is their loss which may lead to the abnormal myelination seen later in life in these infants.

The second model is an *in utero* infection model. This is a prenatal onset model in which premature preterm rupture of membranes leads to infection of the maternal membranes which tracks to the fetus. Depending upon the subsequent time to delivery, this may result in early or late onset brain injury in the infant (Leviton *et al.*, 1999). It is apparent from the literature available that it is the fetal inflammatory response, not that of the mother, which is implicated in causing fetal brain injury. That response is to bacterial modulins; endotoxins or other bacterial products, which stimulate the host to produce chemokines and cytokines including IL-1, IL-1ra, IL-6 and TNF-α. Previous work has shown that the concentrations of these are elevated in fetal fluid compartments including CSF, plasma and urine. Whilst it is known that cytokines are produced both by the chorion and amnion in response to infection, the close correlation between urinary and amniotic cytokine levels suggests a contribution from both sources. The correlation between TNF-α and IL-6 levels in the amniotic fluid and sonographically imaged PVL also provides a potential sensitive early marker for subsequent cerebral palsy which may be very useful clinically.

Whilst a great deal of information has been provided by these studies, none of the animal studies to date have taken this work to its logical conclusion and proven that infection, endotoxins or cytokines applied to an animal model can result in clinically relevant chorioamnionitis, pneumonia or brain injury without killing the animal. By giving 100 ng *E.coli* LPS directly to the fetus we have shown that it is possible to produce periventricular white matter and cortical injury, chorioamnionitis and *in utero* pneumonia without fetal death. These injuries are all part of the spectrum of sequelae of preterm *in utero* infection observed clinically and this data therefore supports the role of infection in a subset of those infants diagnosed with PVL. Further work with individual cytokines should be able to dissect the specific roles of each and how they interact with each other and the host to produce the injuries seen following infection.

### 7.5 Summary

In this chapter, I have shown that it is possible to qualitatively simulate aspects of the histopathological outcome of antenatal infection – namely IVH with parenchymal involvement, PVL, chorioamnionitis and *in utero* pneumonia. There is evidence for a progressive hypotension during the 12 hours following endotoxin administration, perhaps indicating a role for ischaemia in the development of IVH and PVL. However, bacterial lipopolysaccharide is known to stimulate cytokine production, suggesting another mechanism by which brain injury may occur in the absence of hypoxia-asphyxia.
This thesis examined fetal cardiovascular and metabolic responses to some of the clinically relevant intrauterine stressors such as hypoxia and infection, assessed their histologic sequelae where possible and examined adenosine's role in mediating cardiovascular and metabolic responses.

I believe that my most important findings are:

1) In late gestation, fetal brain and skeletal muscle are actively and differentially regulated during reduction in placental perfusion, both in terms of blood flow and metabolism

2) Cytochrome oxidase becomes more oxidised during hypoxia in the brain of the unanaesthetised ovine fetus in utero. This suggests that cerebral oxygen consumption is down-regulated through a fall in the flow of reducing equivalents down the mitochondrial electron transport chain and not because oxygen becomes limiting.

3) Exogenous adenosine infused into the normoxic ovine fetus reduces oxygen consumption, causes cytochrome oxidase to become more oxidised. This suggests that adenosine may have a functional role in mediating hypometabolism during hypoxia in the ovine fetus.

4) A nanogram dose of E.coli lipopolysaccharide, administered IV to the mid-gestation ovine fetus results in a syndrome of fetal lesions both centrally and peripherally similar to those seen in the human fetus born prematurely following intrauterine infection or chorioamnionitis.

The relative importance of hypoxia, ischaemia and asphyxia as antecedents of both perinatal and adult brain injuries has been the subject of much debate in medical and scientific forums. In the clinical perinatology field, this interest in brain injury has focused primarily on predictors of intraventricular-intraparenchymal haemorrhage, periventricular leukomalacia and cerebral palsy in the preterm infant and of hypoxic-ischaemic encephalopathy in the term infant. This work is reviewed in sections 1.5 and 1.6.

Clinical observations of perinatal brain injury have been difficult to reproduce accurately in experimental animals and, as can be seen in this thesis, serious or “catastrophic” brain injury is very difficult to produce in the chronically instrumented ovine fetus by application of either uterine artery or umbilical cord occlusion. Indeed, none of the hypoxia experiment animals described in this thesis developed serious
brain injury despite being subjected to what was believed to be a pre-terminal insult, and most fetuses recovered with very minor histological evidence of injury, in any.

These experimental findings are reflected in a recent shift in clinical thinking about perinatal brain injury. A number of recent studies have questioned the long-held belief that hypoxia plays a primary role in the development of perinatal brain injury. Several authors have independently proposed schemes to account for perinatal brain injury in the absence of hypoxic stress, including activation of fetal inflammatory pathways and abnormal platelet activation, to name but two (Grether & Nelson, 1998; Nelson, 1998). This is not to say that there is no role left for hypoxia, ischaemia or asphyxia to play; there is good evidence implicating these factors in the development of brain injury. Powerful evidence has been supplied by the use of magnetic resonance spectroscopy (Cowan et al., 1994; Künzle et al., 1994; Krägeloh-Mann et al., 1995; Pavlakis et al., 1999) and near infrared spectroscopy (Bennet et al., 1998 & 1999; Cooper et al., 1999; Peebles et al., 1992; Tsuji et al., 1998; Wyatt et al., 1986; Wyatt, 1994; Yoxall et al., 1998) which implicates oxidative stress in neonatal brain injury. So whilst hypoxia-ischaemia may not be an uncommon cause of brain injury in the perinatal period it may be that many other factors, which had previously been unconsidered, may be equally important in the aetiology of brain injury.

The various factors which have been identified as causative or putative causative factors for perinatal brain injury may cause this injury by activation of a final common pathway or pathways. If this is so, then understanding the interactions of these factors may provide insight into brain injury and suggest appropriate treatment and rescue modalities which could be applied to ameliorate the effects of a range of intrauterine stressors. The data presented in this thesis describes fetal responses to a few specific intrauterine challenges, namely uterine artery occlusion, total umbilical occlusion, adenosine infusion and LPS administration. This data falls into two distinct but related areas: 1) cardiovascular responses, and 2) metabolic responses. This final chapter will concentrate on these areas, focussing on pertinent points for discussion.

8.1 Cardiovascular responses to intrauterine stressors

8.1.1 Maturation of cardiovascular responses to acute asphyxia

In postnatal life the cardiovascular system serves a number of functions, including pump, conduit, exchange surface and pressure/volume reservoir. The regulatory mechanisms responsible for coordinating and controlling cardiovascular function need to develop so that these functions are available by term, yet at the same time the fetus must be able to respond appropriately to intrauterine stresses, perhaps in a way which is correct during intrauterine life but which would be inappropriate for the neonate or adult.
CHAPTER 8

Much of the detailed information in the literature on fetal responses to hypoxia and other intrauterine stressors comes from work on the chronically instrumented fetal sheep in late gestation when many of the neuroendocrine responses to stressors are well established (Chapter 1 and refs). Relatively few studies of this kind have been performed before 120 days gestational age and thus the maturation of fetal responses to common experimental challenges, such as the various hypoxias, is not fully understood. These ideas will be addressed in the following sections.

8.1.2 Fetal heart rate, vascular resistance & mean arterial pressure

The data in Chapters 3 to 5 shows that both preterm and term fetuses respond to hypoxia with rapid bradycardia, with a variable arterial pressure response and an attempt at increased carotid artery flow. At term, but perhaps not preterm, there is also a fast increase in peripheral vascular resistance, whilst cerebrovascular resistance remains unchanged at both ages. These findings are in contradiction with some of the data published by other authors which indicates that the ovine fetus is unable to mount a cardiovascular response before 100 days gestation owing to a relative immaturity of components of the neurohormonal control systems (Boddy et al., 1974; Walker et al., 1974; Iwamoto et al., 1989 & 1991). The idea that the fetus is generally unable to mount a cardiovascular response may be erroneous. For example, Bennet recently reported that the immature ovine fetus mounts a complex haemodynamic response to umbilical cord occlusion which includes bradycardia, a biphasic mean arterial pressure response and alterations in cerebral and peripheral vascular resistance. Indeed, the data presented in Chapter 3 of this thesis supports the evidence of cardiovascular responses to asphyxia before 100 days gestation. Differences between some authors' reports of fetal responses to hypoxia can probably be explained in terms of the type of insult used and by methodological differences (see below) between the groups. A review of the cardiovascular responses to different hypoxic insults is given by Jensen (Jensen, 1991) which clearly describes different fetal responses in late-gestation to the commonly used hypoxic challenges.

The difference in the results obtained by different methodologies is exemplified by Iwamoto's and Bennet's reports of cardiovascular responses to umbilical cord occlusion in mid gestation. The use of microspheres by Iwamoto provides a snapshot of fetal blood flows to each vascular bed at the time of microspheres injection. Such studies are limited as to the number of injections which can be performed (usually no more than 5) and thus the number of data points is limited and the timing of microsphere injection is critical if a true picture of the responses is to emerge. Use of ultrasound flowprobes, however, provides a continuous measure of blood flow in the artery in question. Bennet's work mentioned above, for example, provides continuous data on femoral flow from which she reports that femoral resistance in preterm fetuses increases during the first ten minutes of umbilical cord occlusion before falling back to baseline levels. Iwamoto reported that there was no increase in femoral vascular resistance with the same insult, but had
only one data point at the end of a 40 minute insult, thus Iwamoto does not reveal the whole picture and some fetal responses may be overlooked or worse, conclusions made without the proviso that one data point does not make a straight line.

Another reason for differences in the results in the literature could be due to the severity of the insult used and its speed of onset. The fetal responses to a relatively mild, slow-onset insult (inhalation hypoxia) is compared with a fast onset and quite severe insult which has the added complication of itself increasing peripheral vascular resistance (umbilical occlusion) and altering blood flows away from the placenta. The fetal response to these different challenges cannot, sensibly, be the same if the mechanical effect of the methodology alone causes blood flow redistribution and changes in total peripheral resistance. The fetal responses and mechanical consequences of the insult are rarely separated.

The development of acute responses to uterine artery or umbilical occlusion is summarised in Figure 8.1 below. The more slowly developing hormonal responses to hypoxia described by Giussani (Giussani et al., 1996) appear to be fully established by 95 days gestation, since in Chapter 3, I have described how the initial chemoreflex bradycardia is slowly reversed in the mid-gestation fetal sheep by what is presumably a rise in plasma catecholamines. For clarity, these hormonal responses are omitted from Figure 8.1 below.

**Figure 8.1** Diagrammatic representation of the reflex responses to uterine artery or umbilical occlusion at early (black) and late (black and blue) gestation. Conflicting evidence from the literature suggests that there may or may nor be a peripheral resistance response to hypoxia. *Modified from Giussani et al., 1993.*
Because of the obvious methodological similarities between the work presented in Chapters 3, 4 and 5 of this thesis and the work of Bennet (Bennet et al., 1998 & 1999) it is of little surprise that, unlike Iwamoto, I also report rapid cardiovascular adaptation at both 0.6 and 0.8 of gestation. However, unlike Bennet’s umbilical cord occlusion insult, I suggest that there are qualitatively similar responses between the two gestational ages to uterine artery occlusion. At both ages we see an initial bradycardia, and carotid artery blood flow is initially maintained at both gestational ages but, significantly, not increased for any length of time as it is with inhalation hypoxia. Despite there being no prolonged increase in carotid artery blood flow at either gestational age, near infrared spectroscopy data indicates that cerebral blood volume increases in both instances.

There is no evidence from the literature which explains how brain blood volume can be increased during hypoxia without any prolonged change in carotid flow. Admittedly carotid flow is not cerebral flow (Gratton et al., 1996), however, Jensen’s 1991 publication reports that during uterine artery occlusion brain blood flow is reduced, not increased. Assuming that this is the case with our insult, increased tHb remains an unanswered question. There are a number of possible explanations for this finding, including arterial or venous dilatation, or both, or there could be venous congestion due to increased peripheral resistance. If carotid flow is not increased and Jensen’s report of reduced brain blood flow is believed, then arterial dilatation is unlikely to provide a great contribution to increased tHb. If venous volume is increased, then this would mean that cerebral venous outflow was reduced and this raises the question as to whether cardiac afterload is increased, possibly due to increased peripheral vascular resistance. An increase in femoral vascular resistance is demonstrated in Figure 4.3 in late-gestation, and similar increases have been reported for gut (Bennet et al., 2000). If this were the source of the increased cerebral tHb, then this suggests that functional peripheral vascular constriction at 95 days gestation, since a similar increase in tHb is seen at mid-gestation.

With umbilical cord occlusion, we see another pattern of response. Bennet has reported that, with umbilical cord occlusion in mid-gestation, there is a maintained fall in FHR, (although I report only a brief bradycardic response in Chapter 5), an increase in femoral vascular and carotid artery resistance (CAVR) during a 20-minute umbilical cord occlusion. These facts indicate two things: First, that uterine artery occlusion provides a very different insult to the fetus to both maternal inhalation hypoxia and umbilical cord occlusion, and second that the fetus is able to detect these differences and respond accordingly.

This means that the fetus must be able to sense and integrate a number of properties of these insults, including the speed of onset as well as the magnitude of hypoxia, changes in arterial pH and PaCO2 and the presence or absence of increased peripheral resistance caused by compression of the umbilicus. This increase in umbilical-placental resistance during umbilical occlusion may be of great importance, since it provides an additional aspect to the insult not provided by either maternal hypoxia or uterine artery
occlusion. The placental-umbilical circulation provides a massive source of peripheral resistance for the fetus even under resting conditions because of the number of vessels present in it. Clamping or partial occlusion of the cord must increase peripheral resistance considerably and thus mean arterial pressure and cardiac afterload. It may be that these additional properties of umbilical cord occlusion, through baroreceptor and Bainbridge reflex mechanisms, may be partially responsible for the cardiovascular responses seen during umbilical cord occlusion in Chapter 5.

Figure 8.2 shows a flow diagram the fetus' acute response to asphyxia including potential additional effects of cord compression. In this scheme, the chemoreceptor function is shown to be active by 0.6 gestation (black), whilst the active peripheral resistance increase seen in late-gestation may not be (blue), depending perhaps upon the type and severity of the insult. The separate effects of umbilical cord occlusion through increased peripheral resistance are shown in grey.

Figure 8.2 Diagram summarising some of the components of the acute fetal response to hypoxia. Items in black are developed at least by 0.6 gestation, whilst those in blue may have developed at this time. Umbilical cord compression provides an extra stimulus in the form of increased peripheral resistance and mean arterial pressure and consequent increased cardiac afterload.

In conclusion, chemoreceptor reflex responses to uterine artery and umbilical cord occlusion are apparent at 0.6 gestation. The peripheral vascular response to these insults may be active as indicated by Bennet, but these may be dependent upon the type and severity of the insult. Further work in this area should investigate the peripheral response to uterine artery occlusion.
8.1.3 Adenosine as a mediator of cardiovascular responses?

There has been speculation for some time on the role of adenosine as both a cardiovascular and metabolic modulator under basal conditions and during energy depletion, such as may occur in the advanced stages of asphyxia. In Chapter 6 we saw that adenosine infused into the near-term fetus during normoxaemia, at a level which does not significantly alter fetal heart rate, mean arterial pressure or carotid artery flow results in increased cerebral levels of Hb and HbO₂, as determined by near infrared spectroscopy. We demonstrated that the initial increase in cerebral Hb occurred independently of HbO₂, followed by a delayed rise in HbO₂. The net effect of this was an increase in cerebral blood volume which was maintained for the duration of the infusion. Using NIRS during hypoxia, I have also demonstrated that fetal cerebral blood volume increases in the absence of significant change in carotid blood flow.

Could adenosine be mediating the increase in cerebral blood volume during hypoxia? Considering the rises in Hb and HbO₂ separately, there are at least two explanations for each. The Hb rise could be caused by active venodilation or passively as venous congestion secondary to depressed cardiac function. The rise in HbO₂ could be caused actively by adenosine acting at A2 receptors, or as a result of metabolic suppression which would cause venous saturation to rise. In the absence of experiments using adenosine antagonists, or of venous blood gas data, we can only speculate from the available data. Two lines of evidence bring me to the conclusion that venous congestion is not the cause of the rise in Hb seen soon after the start of adenosine infusion. First, the increase in tHb during both hypoxia and adenosine infusion are remarkably similar, at approximately 12 μmol/l above control, suggesting that the causative factors behind the increases are of a similar magnitude. We believe that the adenosine dose given replicates that seen in fetal plasma during moderate hypoxia (Karimi et al., 1996). However, secondly, there is evidence for increased cardiac afterload in the hypoxic animals but there was no evidence of such cardiovascular disturbance in the fetuses given adenosine IV. This data together indicates that adenosine causes active vasodilatation of parts or all of the cerebral circulation during hypoxia, not that cerebral blood volume rises because blood is backing up in the veins draining the brain. This is summarised in Figure 8.3 below.
As I have already stated, there is evidence for the involvement of adenosine with cardiovascular responses, specifically the increase in blood flow to some tissues during hypoxia (Kurth and Wagerle, 1992; Laudignon et al., 1990). Despite using an adenosine infusion which is reported to simulate plasma levels of adenosine seen during hypoxia in the fetus, these experiments have failed to provide conclusive evidence that there are significant effects on the cardiovascular system. These experiments suggest, therefore, that adenosine may provide a facilitatory role during hypoxia – helping to maintain carotid flow, for example. The main actions of adenosine may be metabolic, as indicated by a number of studies in the ovine fetus (see Section 8.2).

8.1.4 Are cardiovascular fluctuations more important than hypoxic stress in the development of fetal brain injury?

Recent work has begun to question the primary role of hypoxic stress in the aetiology of perinatal brain injury, with greater emphasis being placed upon fetal inflammation than oxygen deprivation. The precise sequence of events from the event triggering inflammation to clinically evident brain injury, however, has yet to be addressed in sustainable fetal experimental models. In Chapter 7, I presented haemodynamic and metabolic data from chronically instrumented fetal sheep at 0.6 gestation which demonstrated...
significant cardiovascular perturbations in the hours following IV injection of lipopolysaccharide into the fetus, including large increases in carotid artery flow and swings in mean arterial pressure. These fetuses developed periventricular white matter injury similar to that seen in human infants at autopsy, yet in only one case was there evidence of severe intracranial haemorrhage, a clinical correlate of white matter injury.

Review of the literature indicates that fluctuations, either up or down, in cerebral blood flow may be a causative factor in the aetiology of intraventricular-intraparenchymal haemorrhage, as discussed in Chapter 2. Since severe intraventricular haemorrhage with parenchymal involvement has a poor prognosis, it is interesting to speculate to what extent LPS causes brain injury through haemodynamic disruption as opposed to direct effects of pro-inflammatory cytokines.

As explained in Chapter 2, developing blood vessels in the fetal brain are reported to be particularly susceptible to fluctuations in flow (Volpe, 1995) and yet microsphere methods which have demonstrated alterations in regional flow within the brain during hypoxia have yet to either show the presence of significant fluctuations in flow in those areas believed to be most susceptible, or demonstrate catastrophic haemorrhage following hypoxic insults. Part of the reason why these fetuses may be susceptible to hypoperfusion may be that these fetuses are operating under baseline conditions at a mean arterial pressure which is at the lower boundary of the cerebral autoregulation limit. The data presented here show that the mean pressure falls far below the lower limit for cerebral autoregulation for approximately 8 hours after LPS administration before rising far above normal pressure values. Somewhat paradoxically, all of this occurs against a background of steadily rising carotid flow.

Despite these fluctuations in pressure and carotid flow, only one LPS fetus had IVH-IPH at autopsy although all fetuses were seen to develop periventricular leukomalacia by three days after LPS administration. Further work with microspheres or other appropriate techniques would be of use in investigating the regional changes in cerebral flow in these fetuses. Of particular interest are flows in the centripetal and centrifugal branches of long penetrating arteries around the area of the interventricular foramen (foramen of Monro) which are believed to be particularly susceptible to rupture in the immature fetus. By performing this additional work, the relative contributions of ischaemia, haemorrhage and inflammation to the development of white matter injury may be elucidated.
8.2 Metabolic responses to intrauterine stressors

The idea that the fetus responds to intrauterine hypoxic stress with metabolic adaptation has received less space in the literature than fetal cardiovascular responses. It appears to be widely believed that metabolic responses to hypoxia are simply functions of the principle of mass action whereby reduction in arterial $O_2$ content reduces $O_2$ availability at the mitochondrion and, all else being equal, leads to cytochrome reduction and energy depletion. This approach does not take into account data from facultative anaerobes (Hochachka, Land & Buck, 1997) or in vitro fetal tissue (Braems & Jensen, 1991) which indicate otherwise. In this thesis I have provided metabolic data from Fick principle calculations and from near infrared spectroscopy which support the idea of an active metabolic adaptation to intrauterine stresses.
8.2.1 Metabolic “hibernation” in the fetus during hypoxia

In order to survive extended periods of O\(_2\) deprivation, the fetus must curtail energy demand by undergoing behavioural and metabolic adaptations – fetal breathing and body movements cease and electrocortical state enters high voltage or becomes isoelectric (Boddy et al., 1974). The electrocortical changes suggest that the fetal brain becomes progressively inactive with severity of the hypoxia. Direct evidence for metabolic hibernation has been provided by Budinger and co-workers using *in vitro* fetal cardiomyocytes (Budinger et al., 1998). In this study, Budinger indicates that embryonic cardiomyocyte energy demand is attenuated during hypoxia and implicates the mitochondrion as the site of the O\(_2\) sensor for hypoxic adaptation. The cytochrome oxidase data presented in Chapters 3 & 5 of this thesis using chronically maintained fetal sheep subjected to hypoxia supports the *in vitro* evidence that the fetus actively suppresses energy demand during hypoxic stress, ie that metabolism is suppressed in a way which is not dependent upon the operation of mass action. However, we have also shown that cerebral and skeletal muscle metabolic responses to asphyxia are very different.

8.2.1.1 Cerebral metabolism during asphyxia

In this thesis I have shown that uterine artery occlusion results in a fall in brain oxygen delivery by approximately 63 and 61% of baseline levels in mid- and late-gestation, respectively, after correction for brain weight. With this degree of reduction in DO\(_2\), the fall in brain O\(_2\) consumption was 57 ± 32 and 74 ± 16% in mid- and late-gestation, respectively. At the same time, we observed an increase in CcO oxidation by 0.87 ± 0.21 and 0.18 ± 0.13 µmol/l, in mid- and late-gestation, respectively. Although the numbers used in these experiments are relatively low, these data are instructive. Not only do they show oxidation of CcO during hypoxia – the opposite to that seen in the neonate or adult, but they also show that, as a proportion of its control value, the fall in oxygen consumption during hypoxia can be greater than the degree of oxygen delivery – which should not occur if reduction in metabolism occurred only through the effects of oxygen availability at the mitochondrion. This indicates that other mechanisms must be in operation.

A suggestion of what these other mechanisms might be provided by the *in vitro* work of Yager and colleagues (Yager, Brucklacher and Vannucci, 1996). This group monitored cytochrome oxidase redox state using the acetoacetate/β-hydroxybutyrate substrate couple and determined that cytochrome oxidase became oxidised during extended hypoxia. These findings are in agreements with our near infrared spectroscopy evidence of cytochrome oxidase oxidation during large increases in VO\(_2\).

Cytochrome oxidase redox state was explained in Chapter 1. Summarising, cytochrome oxidase becomes reduced when it accepts an electron from cytochrome c and oxidises when this electron is passed to molecular oxygen to form water. Cytochrome oxidase reduction or oxidation occurs as a result of
alterations in one or more of the following variables: 1) the flow of reducing equivalents from NADH & thus the Krebs Cycle, therefore 2) the flow of electrons down the electron transfer chain, 3) the availability of O\textsubscript{2} at the CcO binuclear centre, and 4) the functional state of CcO.

If we consider CcO redox simply in terms of electron transport and O\textsubscript{2} availability, there are two possible reasons why, during asphyxia, we have seen a rise in CcO oxidation above baseline levels, not the fall in CcO oxidation which is seen in neonatal and adult subjects. First, there could be a drastic reduction in O\textsubscript{2} availability whilst electron transfer rate is maintained, in which case CcO would become more reduced as electrons back up in the system. On the other hand, if the flow of reducing equivalents became limiting to a greater extent than the fall in O\textsubscript{2} availability; in this way CcO could become oxidised since the few available electrons would rapidly be passed on to O\textsubscript{2} and not remain on cytochrome oxidase (Figure 8.5).

![Figure 8.5 Schematic representation of a simple scheme for the (A) oxidation or (B) reduction of cytochrome oxidase (complex IV). In scheme (A), there is a breakdown either in the supply of reducing equivalents (NADH or FADH\textsubscript{2}) to the electron transfer chain, or an inhibition of electron flow in the chain. In this way, O\textsubscript{2} supply is not limiting to respiration and complex IV (CcO) becomes oxidised. In scheme (B), oxygen supply is limiting and electron flow is nominal, thus electrons build up at CcO Cu, site, and CcO is reduced.](image)

When described in this way, it becomes clear that the unexpected oxidation of CcO during hypoxia could be the result of a fall in the availability of reducing equivalents for the electron transfer chain, possibly due to a down regulation of the TCA cycle, inhibition of mitochondrial matrix dehydrogenases, or to an interruption in electron flow through the chain (Figure 8.7). However, Chandel reports that large
experimental changes in NADH result in small changes in O$_2$ consumption, indicating that the control of respiration by NADH is less than that exerted by ATP. This indicates that inhibition of TCA cycle or matrix dehydrogenases is unlikely to contribute significantly to the hypoxic suppression of metabolism and implicates an alteration in electron flow downstream of NADH as the cause of CcO oxidation.

A scheme whereby electron flow through CcO can be decreased without alterations in TCA function or NADH availability is suggested by Budinger's & Chandel's work (Budinger et al., 1998; Chandel et al., 1997). These publications indicate that cytochrome oxidase itself may be an oxygen sensor with different functional states during normoxia and hypoxia. With prolonged reduction in PO$_2$ to 20mmHg, Chandel observed a fall in oxygen consumption which was reversible with oxygenation to 100mmHg. With fetal arterial PO$_2$ at between 35 and 25 mmHg during mid to late gestation (Tables 3.1, 3.2, 4.1, 5.1, 6.1 and 7.1), there is an argument for a proportion of the fetal CcO pools being in such a depressed functional state during fetal normoxia. Furthermore, fetal hypoxia may then rapidly push the balance of the CcO population into a high Km conformation. By undergoing a conformational change during hypoxia, CcO affinity for electrons may fall such that electron tunnelling through CcO could occur at a slower rate. With a lower affinity for electrons, the CuA site would be occupied by electrons for less time which translates into an increase in the oxidation state of the CcO population as we have observed with NIRS.

The switch from the hypoxic to the normoxic functional state with reoxygenation noted by Chandel with hepatocytes suggests that comparisons of fetal and neonatal or adult responses to hypoxic stress should be made only with caution. This might also explain the contradictory responses during chronic hypoxia obtained from neonatal animals and humans to that reported here and by Yager.

Figure 8.6 Shows a hypothetical relationship between CcO function and cellular respiration during normoxia and hypoxia. During prolonged hypoxia, CcO may enter an altered functional state in which respiration rate is lower at any given redox state (A $\rightarrow$ B). Respiration rate can be maintained by an increase in cytochrome c reduction (B $\rightarrow$ C), or can fall further through cytochrome oxidation (B $\rightarrow$ D). Adapted from Chandel et al., 1997.
Figure 8.7 Possible modulatory sites for the control of cellular respiration during hypoxia include direct effects on CcO, ATP synthase or electron transport. Indirect effects could be felt through hypoxic effects on TCA cycle and thus on the availability of reducing equivalents, or on mitochondrial membrane potential.

8.2.1.2 Adenosine as a functional metabolic inhibitor?

A factor which may have influenced cerebral CcO oxidation state through alteration of cerebral metabolism is the chronically high level of adenosine in the fetus relative to the adult animal. This endogenous purine is known to have metabolic suppressive functions (Karimi et al., 1996) perhaps by mediating hypoxic ventilatory depression (Koos & Doany, 1991), by initiating neuronal hyperpolarisation (Dunwiddie & Fredholm, 1991) and reducing excitatory neurotransmitter release (Corradetti et al., 1984), thus altering the balance between excitatory and inhibitory neurotransmitter levels in the brain. In Chapter 6, I used exogenous adenosine to simulate the hypoxic rise in adenosine levels and attempted to characterise the fetal cerebral metabolic response.

Following the work of Karimi, adenosine was infused into these late gestation fetuses at a rate which is believed to simulate levels seen during hypoxia (Karimi et al., 1996). When infused at 3 μmol/kg/min there were no acute cardiovascular responses to adenosine infusion although brain oxygen consumption was depressed. This indicates that a fall in metabolism can occur without any apparent decrease in brain blood flow (carotid flow was unchanged and tHb was increased). Stronger evidence for reduced metabolism in the brain comes from Karimi's statement that fetal ECoG became isoelectric during infusion of adenosine at greater than 6 μmol/kg/min, indicating that cortical neuronal activity had decreased and thus that the fetus had entered a state of profound metabolic suppression.
The metabolic data presented here is thus in agreement with the known metabolic modulatory actions of adenosine when given acutely. In addition, we saw that CcO oxidation increased significantly at all adenosine infusion rates, with a magnitude not dissimilar to that seen at the same gestational age during uterine artery occlusion. This obviously suggests that adenosine may have a role in mediating the fall in cerebral metabolism during hypoxia through reductions in neuronal excitation both pre- and postsynaptically.

Unfortunately, although we have demonstrated metabolic suppression by exogenous use of adenosine, this data does not provide conclusive evidence that adenosine has a primary role in mediating hypoxic metabolic depression. In order to ascertain whether this is the case, further studies are required using adenosine receptor antagonists or blockers during hypoxia. By blocking hypoxia-associated hypometabolism in this way, the case for adenosine as the primary effector of reduced brain O2 consumption would be strengthened. In this circumstance, cytochrome oxidase redox state data from NIRs would also be instructive. If the degree of CcO oxidation did not increase with hypoxia during adenosine inhibition, this would implicate an external influence on CcO function, but if CcO still became oxidised, then this would suggest that Chandel's hypothesis, outlined in Figure 8.6, may be in operation.

Despite a lack of conclusive evidence of adenosine's method of inhibiting cerebral metabolism, these data are encouraging. The fact that we see a qualitatively similar cerebral NIRs results (increased tHb concentration and CcO oxidation) to those obtained during uterine artery occlusion, even at doses that do not alter gross cardiovascular variables, is particularly noteworthy. However, adenosine is known to have microvascular dilatory effects which should not be overlooked when coming to these conclusions. Local rises in ECF adenosine could cause a local vasodilatation which would act to increase blood flow and thus PO2 and reverse any hypoxic alteration in CcO oxidation state. Since the fetal brain is small, our NIRs setup interrogates a relatively large volume of tissue indiscriminately. We cannot look at the variations between, say cortex and deep structures; thus microsphere investigation with and without adenosine antagonists would be useful in providing some additional evidence of microvascular alterations. If such studies were to reveal that adenosine rises are not associated with increased local cerebral blood flow and yet oxygen consumption were still depressed, there would be a much stronger argument for a functional metabolic role of adenosine during hypoxic episodes. This work remains to be done.

8.2.1.3 Peripheral metabolism during hypoxia

Although so far in this discussion I have stressed how the data presented in this thesis differs from that usually given in the literature, the data for peripheral cardiovascular and metabolic adaptation to these severe hypoxic insults presented here is easily predicted from data published by other authors. However, the near infrared spectroscopy CcO data poses an interesting question which deserves some
consideration. The data in Chapter 4 shows that skeletal muscle quickly responds to uterine artery occlusion with an α-adrenergic mediated vasoconstriction, which causes tHb (blood volume) in this tissue to fall, and almost undoubtedly initiates a profound ischaemia. Using NIRS, I have also demonstrated that CcO becomes very rapidly reduced after the onset of the insult, with the reduction of CcO reaching a nadir at approximately 10 minutes after the onset of the occlusion (Figure 8.7).

This reduction of CcO in skeletal muscle in response to hypoxia is reminiscent of data presented by other authors working with adults or neonates and is very different to the response described in Chapter 3 for the fetal brain during uterine artery occlusion. Opposite changes in the CcO signals in brain and skeletal muscle, obtained simultaneously in the two tissues with the same near infrared spectrometer indicates that metabolism in brain and skeletal muscle can be independently regulated according to the demands and perhaps the importance of the tissue. The methodology by which this differential regulation is effected is not known and, as far as I am aware, no other groups have witnessed this event. Further work is therefore needed to elucidate the mechanisms behind the regional control of metabolism during hypoxia.

Figure 8.7 Changes in CcO oxidation in the brain and femoral skeletal muscle taken simultaneously before, during and after a 60-minute uterine artery occlusion (grey box). Brain CcO slowly oxidises over the period of the insult, whilst skeletal muscle CcO initially reduces before slowly drifting back towards its pre-occlusion values. Statistical difference from control (-60 minutes) shown by a (p < 0.05, repeated measures ANOVA with Newman-Keuls post hoc tests).
8.3 Future work

I have already mentioned at several points where future work is required to continue the investigations begun here. In the near future, there are several points which can be addressed to answer some of the easier questions posed by the data presented here.

1. The peripheral vascular response in mid-gestation to uterine artery occlusion has yet to be investigated, although Bennet has published some data from umbilical cord occlusion studies (Bennet et al., 1999). By measuring femoral artery flow, taking femoral arterial blood samples and using NIRS on skeletal muscle, then combining this information with that already obtained from the brain at this gestation, we should be able to better understand the maturation of mid-gestation fetal whole-body responses to asphyxia using experimental methodology. The use of NIRS and Fick principle calculations will provide invaluable evidence of peripheral metabolic responses at this gestation, which have yet to be investigated. This may provide evidence for how hypoxic metabolic depression is achieved in the periphery and how long this can be maintained without compromising tissues.

2. These investigations as a whole would be strengthened by use of multiple doses of microspheres to generate data on the immediate, intermediate and long-term organ flows before, during and after uterine artery occlusion in mid-gestation. Of particular interest are supposedly ischaemia susceptible areas of the brain, such as those in arterial end zones as described in Chapter 1. Despite quite severe hypoxic insults we observed few cases of brain injury with uterine occlusion in mid- or late-gestation compared with late-gestation umbilical cord occlusion, where hippocampal injury was almost uniformly evident. More histological data for uterine artery occlusion fetuses at both mid- and late-gestation is also required to provide stronger evidence of the types of injury caused by this experimental manoeuvre. Data on flow to different brain areas could be helpful in building a better picture of the type or severity of insult required to cause catastrophic brain injury and thus provide new avenues of research to elucidate mechanisms of damage.

3. The role of adenosine in the suppression of fetal metabolism requires further study. At present the lack of any assays of plasma or ECF adenosine during asphyxia means that I cannot be sure that the infusion experiments were correctly mimicking the time course and magnitude of the hypoxic rise in adenosine. Once this data is obtained, use of general adenosine blockade would be useful to establish to what extent the metabolic and haemodynamic changes seen during hypoxia are adenosine-dependent. This work could be further refined with specific blockade or stimulation of $A_1$, $A_2a$, $A_2b$ and $A_3$ receptor subtypes. As with other studies, microsphere investigation would be informative as would simultaneous peripheral and cerebral NIRS.
4. Finally, a great deal of investigation is required into the question of the involvement of bacterial modulins in brain injury in the immature fetus. Since this work is still in its infancy, much of the basic science is still to be done. A good initial place to start may be to assay fetal tissues at regular intervals after administration of IV lipopolysaccharide. In this way, the development of cytokine responses can be quantified and putative mediators of injury identified. These putative mediators can be individually administered to determine their effects before combinations of cytokines are tested for additive effects. As mentioned previously, microsphere analyses need to be run in parallel with these other studies to determine how brain regional blood flows develop after LPS administration. In this way, we may be able to rule out (or in) haemodynamic fluctuations in the aetiology of damage.

In the longer term, there are still many unanswered questions in this area of research. By integrating the metabolic and cardiovascular responses to these, and other, intrauterine challenges, we may hopefully begin to understand how some fetuses appear to be able to adapt to a given intrauterine stressor without apparent subsequent morbidity, and yet others may be intolerant to a qualitatively similar challenge. The answers to these questions are likely to prove multifactorial. Potential candidates for confounding factors include the adequacy of fetal nutrition, which may alter both cardiovascular function and alter growth trajectory and cardiac glycogen storage. In addition, the exposure of the fetus to previous stressors may alter the fetal responses to a hypoxic challenge. In addition, the growth history of the mother whilst she was in utero may also have ramifications for fetal development. All of these questions will require a great deal of additional work.

8.4 Final statement

The data presented in this thesis has shown that the mid- and late-gestation fetus is able to mount a complex response to asphyxia comprising both cardiovascular and metabolic adaptation. The details of the responses are dependent upon the nature and magnitude of the insult. It is suggested that the fetal brain enters a state of hypometabolic "hibernation" in order to resist the worst effects of asphyxia, probably effected by alteration of cytochrome oxidase functional state. The brain and skeletal muscle appear able to be differentially regulated both in terms of haemodynamics and metabolic rate during asphyxia. Further work is required to elucidate the mechanisms of this regulation.

Data from fetuses injected with lipopolysaccharide have led me to question the primary link between hypoxia and brain injury in mid-gestation. The relative importance of fluctuating cerebral blood flow and direct action of pro-inflammatory cytokines requires further study.
Hypercapnia oxidises the Cu_A centre of Cytochrome Oxidase independently of changes in CBF in the piglet brain

Introduction

During transient anoxia, multi-wavelength near infrared spectroscopy (NIRS) has shown that the copper A centre of cytochrome oxidase (Cu_A) does not reduce until there is a substantial fall in mean cerebral saturation (SmcO_2) and no oxidation is observed when inspired oxygen fraction is increased to 1.0. This would suggest that, at normoxia, the oxygen tension at the mitochondria is above a critical value at which the redox potentials of the electron transport chain become oxygen dependent. In apparent contradiction, when oxygen delivery is increased with moderate hypercapnia (P_aCO_2 \approx 54mmHg), an oxidation is observed in the Cu_A centre. However, hypercapnia also lowers the intracellular pH (pH_i) and has been shown to inhibit glucose metabolism and reduce the concentration of tricarboxylic acid cycle intermediates which produce reducing equivalents for the electron transfer chain. Indomethacin is a potent inhibitor of the hyperaemic response to hypercapnia but should not alter changes in pH_i and can, therefore, be used to separate these effects of hypercapnia.

In order to determine whether the Cu_A oxidation during hypercapnia is the result of increased oxygen delivery or the decrease in pH_i, the critical SmcO_2 (S_mCO_2) and changes in the Cu_A redox state were measured at normocapnia and hypercapnia before and after the intravenous infusion of 3mg/kg of indomethacin. In this study, S_mCO_2 was determined empirically as the SmcO_2 at the first observed Cu_A reduction during the onset of transient anoxia.

Methods

Five newborn piglets were anaesthetised with isoflurane and artificially ventilated. Catheters were sited in the umbilical vein artery and vein and CBF was assessed with laser Doppler fluxometry (LDF) using a thinned-skull preparation.

Tissue attenuation spectra across the piglet head were collected between 650 and 980nm every 5s using fibre-coupled tungsten halogen light source and a CCD spectrograph system. Changes in deoxyhaemoglobin (ΔHb) and oxyhaemoglobin (ΔHbO_2) concentration and Cu_A redox state (ΔCu_A) were calculated between 780 and 900nm (375 wavelengths). Changes were converted to units of concentration (μmol/l) using a pathlength determined by the second differential method from the 840nm water feature and
assuming a mean tissue water content of 85%. Absolute Hb (aHb) was calculated from the 760nm second
differential feature and referred to the 740nm water feature likewise. Absolute HbO₂ (aHbO₂) was calculated
from ΔHbO₂ by assuming complete cerebral desaturation during transient anoxia. SmcO₂ was calculated
from aHbO₂ and aHb.

Transient anoxia was obtained by decreasing inspired oxygen fraction from 0.4 to 0.0 for 90-105s at
normocapnia and 120s at hypercapnia; hypercapnia was obtained by increasing inspired CO₂ fraction to
0.05. Indomethacin (Sigma) was dissolved to 1 mg/ml in bicarbonate (0.1M)/saline solution and infused
intravenously to 3mg/kg over 5-10 minutes and then continuously at 0.3 mg/kg/hour. Anoxia was then
repeated at normocapnia and hypercapnia. Sufficient time was allowed between each intervention for the
NIR and systemic parameters to return to baseline or stable values and the entire protocol lasted ~3 hours.

Results

Baseline SmcO₂ was above baseline critical S₅mcO₂ (see Table 1) and maximum Cu₄ reduction during
anoxia was to -1.91 ± 0.33 μmole/l. Hypercapnia resulted in large and significant increases in LDF and
decreased SmcO₂ close to S₅mcO₂. In addition, there was a small reduction in the Cu₄ centre which
suggested that oxygen delivery had been reduced to below but near-critical values.

Post-indomethacin, hypercapnia resulted in an increase in LDF and SmcO₂ to values which were less than,
but not significantly different to, pre-indomethacin normocapnic values but were significantly less than pre-
indomethacin hypercapnic values (p < 0.05). A Cu₄ oxidation to baseline values by hypercapnia post-
indomethacin could be attributed to increased oxygen delivery. However, there was a significant oxidation
of the Cu₄ centre (p < 0.05) over and above baseline values.

Conclusion

This study shows that hypercapnia can oxidise the Cu₄ centre of cytochrome oxidase above baseline values
without a significant increase in oxygen delivery.
<table>
<thead>
<tr>
<th></th>
<th>P&lt;sub&gt;e&lt;/sub&gt;CO&lt;sub&gt;2&lt;/sub&gt;</th>
<th>LDF</th>
<th>SmcO&lt;sub&gt;2&lt;/sub&gt;</th>
<th>S&lt;sub&gt;Sc&lt;/sub&gt;mcO&lt;sub&gt;2&lt;/sub&gt;</th>
<th>ΔHbO&lt;sub&gt;2&lt;/sub&gt;</th>
<th>ΔHb</th>
<th>ΔHbT</th>
<th>ΔCuA</th>
</tr>
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<tr>
<td></td>
<td>mmHg</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>μmol/l</td>
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<td>Normocapnia</td>
<td>34.9 ± 0.8</td>
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<td>64.7 ± 5.5</td>
<td>37.9 ± 6.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypercapnia</td>
<td>54.7 ± 1.1*</td>
<td>141</td>
<td>77.1 ± 6.3*</td>
<td>17.1 ± 6.3*</td>
<td>17.3 ± 4.9*</td>
<td>-2.4 ± 1.4*</td>
<td>14.8 ± 4.4*</td>
<td>0.42 ± 0.13*</td>
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<td>67 ± 13*</td>
<td>47.5 ± 4.7*</td>
<td>43.1 ± 4.5</td>
<td>-11.9 ± 4.7*</td>
<td>3.4 ± 1.3*</td>
<td>-8.6 ± 3.8*</td>
</tr>
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<td>Indomethacin infusion at 3mg/kg</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Hypercapnia</td>
<td>53.0 ± 3.4*</td>
<td>80 ± 23</td>
<td>61.5 ± 6.9</td>
<td>27.7 ± 3.7*</td>
<td>-3.6 ± 5.2*</td>
<td>0.2 ± 1.2</td>
<td>-3.5 ± 4.9</td>
</tr>
</tbody>
</table>

Table 1. Values are presented as mean ± SD (n = 5 animals). * significantly different from pre-indomethacin

Normocapnia (p < 0.05) using a paired t test. Changes in total haemoglobin (ΔHbT) are the sum of ΔHbO<sub>2</sub> and ΔHb.
APPENDIX B

OXYGEN DEPENDENCY OF CEREBRAL CuA REDOX STATE DURING INCREASED OXYGEN CONSUMPTION PRODUCED BY INFUSION OF A MITOCHONDRIAL UNCOUPLER IN NEWBORN PIGLETS

Springett R, Newman J, Delpy DT, Cope M


Introduction

The mitochondrial uncoupler 2,4-dinitrophenol (DNP) is a membrane-soluble weak acid that is capable of transporting protons across the proton-impermeable inner mitochondrial membrane (Skulachev, 1998). This results in a reduction of the electrochemical gradient across the membrane and stimulation of flux through the electron transport chain, and thus oxygen consumption, without increasing cytosolic ATP hydrolysis. Uncouplers are used extensively in studies on isolated mitochondria to collapse the proton gradient and stimulate respiration (Rumsey et al., 1990), and to study the effects of the proton gradient on the redox state of the components of the electron transfer chain (Morgan and Wikström, 1991). In this study, it was not the intention to collapse the mitochondrial proton gradient but to use low doses of DNP in order to stimulate oxygen consumption.

Cytochrome oxidase is the terminal enzyme of the electron transport chain and reduces molecular oxygen to water using electrons donated from reduced cytochrome c. The CuA centre of cytochrome oxidase is close to the cytochrome c binding site on the cytosolic side of the inner mitochondrial membrane far from the binuclear centre at which molecular oxygen is reduced (Iwata et al., 1995; Tsukihara et al., 1995). In the oxidised state, the CuA centre has a broad absorption band centred on 840nm and so, in theory, the CuA redox state can be monitored using near infrared spectroscopy (NIRS). However, attenuation changes due to the CuA centre are usually small compared to attenuation changes due to haemoglobin and therefore the CuA signal is sensitive to artefact. For this reason, we used a CCD-based NIR system and performed full spectral fitting which has been shown by modelling to be more robust in separating the CuA component that two, four or six wavelength algorithms (Matcher et al., 1995).

The aim of this study was to determine whether the CuA redox state became oxygen dependent when cerebral oxygen consumption (CMRO₂) was increased by infusing a solution of DNP into the internal...
carotid artery. Oxygen dependency at baseline and at 5 accumulated doses of DNP was determined by measuring arterial saturation \( S_aO_2 \) and \( Cu \) redox state during brief anoxic swings.

**Methods**

Five piglets, born at term but less than 24 hours old, were sedated with Midazolam and then anaesthetised with isofluorane. A tracheotomy was performed and the piglets ventilated with an intermittent positive pressure ventilator using an oxygen/nitrogen gas mixture.

One common carotid artery was cannulated with a 3.5 French catheter and the catheter inserted 2-3cm towards the brain into the internal carotid artery; the distal artery was tied off. A cannula was sited in the umbilical artery for measurement of mean arterial pressure and heart rate using a strain gauge pressure transducer and a second cannula sited in the umbilical vein for infusion of 10% glucose solution.

Blood samples were collected from the umbilical artery for gas (ABL505, Radiometer, Copenhagen, Denmark) and glucose/lactate (2300 STAT Plus, YSI Inc., Yellow Springs, Ohio, US) analysis. Arterial saturation was monitored with a pulse oximeter (Model 8604FO, Nonin Medical Inc., Plymouth, MN, US) positioned on the foot.

Rectal temperature was maintained at 38.5°C using a heated water mattress. A pilot study revealed large increases in rectal temperature during infusion of DNP and so the water temperature was reduced, typically from 45 to 25°C, during the course of the infusion.

The DNP solution was made by dissolving 90mg of DNP (Sigma, Poole, UK) in ~20ml physiological saline solution containing a small quantity (1 mmol) of sodium bicarbonate to give a solution of 20 mM DNP. This solution was further diluted with saline to give a final concentration of 10 mM DNP. The bicarbonate was necessary because the protonated form of DNP is only sparingly soluble in water.

After surgery and prior to commencement of the study, there was a stabilisation period of at least one hour. The protocol consisted of six consecutive brief anoxic episodes equally spaced over a period of 2 hours. Each anoxic episode consisted of 5 minutes of baseline at an \( FiO_2 \) of 0.4, \( FiO_2 \) was then switched to 0.0 for 1.5 minutes and then returned to 0.4 for a further 13.5 minutes of recovery. The DNP solution was infused via the cannula sited in the carotid artery at a rate of 1.4mg/kg/hour beginning 10 minutes before the second anoxia.

Tissue attenuation spectra across the piglet head were simultaneously collected at 1024 wavelengths between 680 and 1000nm every 5 seconds using a fibre-coupled tungsten halogen light source and a
APPENDIX B

CCD spectrometer. Changes in deoxyhaemoglobin (Hb) and oxyhaemoglobin (HbO2) concentration and CuA redox state were calculated by fitting chromophore spectra, corrected for the wavelength dependence of pathlength, to changes in attenuation between 780 and 900nm (Wray et al., 1988; Essenpreis et al., 1993). A pathlength, calculated from the 840nm water feature in the 2nd differential of baseline spectra and assuming an average water content of 85% was used to convert changes in HbO2, Hb and CuA to units of μmol/l. Total haemoglobin (HbT) is the sum of HbO2 and Hb and is proportional to cerebral blood volume (CBV) at constant haematocrit.

All data are given as mean ± SD (n = 5 animals). Analysis of variance (ANOVA), to identify significant differences, as well as to compare repeated measures, was accomplished by a paired Student's t-test. The criterion for significance was p < 0.05.

**Results**

Typical root mean square (rms) noise values on the baseline were 0.2, 0.1 and 0.03 μmol/l for the HbO2, Hb and CuA signals, respectively.

Physiological parameters prior to each anoxia are given in table 1. All baseline parameters are in the normal range and, although pH is slightly alkalotic, this is normal for a newborn piglet. Infusion of DNP resulted in significant increases in PaCO2 and arterial pH even though ventilation rate was increased during the infusion.

<table>
<thead>
<tr>
<th>DNP (mg/kg)</th>
<th>Baseline</th>
<th>0.23</th>
<th>0.7</th>
<th>1.17</th>
<th>1.63</th>
<th>2.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaCO2 (kPa)</td>
<td>4.49 ± 0.77</td>
<td>4.76 ± 0.38*</td>
<td>4.76 ± 0.88†</td>
<td>6.01 ± 1.10 †</td>
<td>5.71 ± 1.68</td>
<td>6.99 ± 0.86†</td>
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<tr>
<td>pH</td>
<td>7.51 ± 0.03</td>
<td>7.52 ± 0.01</td>
<td>7.53 ± 0.04</td>
<td>7.42 ± 0.05 †</td>
<td>7.41 ± 0.10</td>
<td>7.32 ± 0.04†</td>
</tr>
<tr>
<td>SaO2 (%)</td>
<td>98.6 ± 0.3</td>
<td>98.9 ± 0.8</td>
<td>98.8 ± 0.07</td>
<td>98.4 ± 0.5</td>
<td>98.2 ± 0.5</td>
<td>97.6 ± 0.8*</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>42.3 ± 6.6</td>
<td>46.3 ± 8.9</td>
<td>46.1 ± 7.7</td>
<td>50.4 ± 7.6</td>
<td>46.5 ± 6.3</td>
<td>50.0 ± 9.0</td>
</tr>
<tr>
<td>HR (min⁻¹)</td>
<td>163 ± 7</td>
<td>164 ± 7</td>
<td>172 ± 12</td>
<td>183 ± 15</td>
<td>200 ± 14†</td>
<td>209 ± 13†</td>
</tr>
<tr>
<td>Tr (°C)</td>
<td>38.5 ± 0.0</td>
<td>38.6 ± 0.1</td>
<td>38.7 ± 0.2</td>
<td>38.6 ± 0.2</td>
<td>38.8 ± 0.2</td>
<td>38.6 ± 0.3</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>6.1 ± 1.3</td>
<td>6.4 ± 1.6</td>
<td>6.1 ± 1.4</td>
<td>6.0 ± 1.0</td>
<td>6.0 ± 1.4</td>
<td>6.4 ± 1.0</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>2.73 ± 0.89</td>
<td>2.58 ± 0.17</td>
<td>2.49 ± 0.60</td>
<td>3.21 ± 0.82</td>
<td>3.19 ± 0.79</td>
<td>4.03 ± 1.17</td>
</tr>
</tbody>
</table>

Table 1. Physiological variables measured immediately before each anoxia. The accumulated DNP dose is given in the column heading. PaCO2 (arterial CO2 tension), pH (arterial pH), SaO2 (arterial saturation), HR (heart rate), Tr (rectal temperature), glucose and lactate (arterial glucose and lactate, respectively). Values are mean ± SD (n = 5). * Significantly different from baseline (p < 0.05), † significantly different from baseline (p < 0.01).
Figure 1 shows the accumulated dose response of changes in HbT, HbO₂, Hb and Cu₄ in the baseline period prior to each anoxia. Filled circles represent data points at which there has been a significant (p < 0.05) change from baseline and the results from figure 1 are reproduced in table 2. The increase in HbT with increasing DNP dose is consistent with a rise in CBF; increases in CBF lead to increased pressure on the venous side, venous distention and an increase in CBV and thus HbT. Hb increases continuously to 11.4 ± 3.9 μmol/l with increasing accumulated DNP dose although the change did not gain significance at the lowest dose. In contrast, HbO₂ is essentially constant except at the highest dose studied where there is a small but significant increase of 2.6 ± 3.5 μmol/l.
Table 2. All values, which are in units of μmol/l, are mean ± SD (n = 5). * significantly different from baseline (p < 0.05), ** significantly different from baseline (p < 0.01).

The results presented in the bottom panel of figure 1 would suggest that there is a continuous oxidation in the CuA redox state but the changes at the lowest doses are small compared to the system noise and so cannot be resolved. However, the changes gain significance at an accumulated dose of 1.63mg/kg (p < 0.01) and the CuA centre attains a maximum oxidation of 0.25 ± 0.08 μmol/l at the highest dose.

Figure 2 Changes in the NIR parameters during the first (left panels) and fifth (right panels) anoxic swing. Top panels: changes in HbT, middle panels: changes in HbO2 and bottom panels: changes in the CuA redox state. The beginning of anoxia, lasting 90s, is marked as time zero. Results are presented as mean ± SD (n = 5).
Figure 2 shows pooled data for changes in HbT, HbO2 and CuA for the baseline anoxic swing (left panels). The reduction in arterial saturation leads to a cerebral desaturation starting at time zero and resulting in a fall in HbO2 and an increase in Hb, initially with little change in HbT. The reduction of oxygen tension at the mitochondrial level leads to a reduction in the CuA centre of cytochrome oxidase but there is a significant delay between the fall in HbO2 and CuA in the baseline anoxia (left panels) whereas this delay is either very short or absent after an accumulated dose of 1.63mg/kg (right panels). The hypoxaemia triggers an increase in CBF which leads to a hyperaemia as indicated by the increase in HbT. Reoxygenation begins at ~80s with a simultaneous oxidation in the CuA signal and increase in HbO2. Arterial saturation rapidly returns to normal levels but, at the elevated CBF triggered by the hypoxaemia, there is an increase in HbO2 and a decrease in Hb over baseline values. HbT reaches a maximum at approximately 2.5 minutes and then HbT, HbO2 and Hb return to baseline values over the subsequent 6 minutes.

Figure 3 shows the change in CuA plotted against SaO2 during the onset period of the anoxia (a) before infusion of DNP, (b) after an accumulated dose of 0.7mg/kg of DNP and (c) after an accumulated dose of 1.63mg/kg of DNP. The filled circles in the figure represent data points at which there has been a significant (p < 0.05) reduction in CuA redox state from the baseline of the respective anoxia. As can be seen from the figure, the arterial saturation at the first significant reduction in the CuA centre depends on accumulated DNP dose and increases from 75.8 ± 5.0% in the baseline anoxia to 93.1 ± 4.5% with an accumulated dose of 1.63mg/kg.
DISCUSSION

When DNP was infused into the common carotid artery of the neonatal piglet, an increase in heat generation and carbon dioxide production was observed which is consistent with mitochondrial uncoupling. However, it is unlikely that the DNP was completely absorbed in a single pass through the brain and it is possible that these effects are the result of systemic uncoupling. However, large increases in cerebral Hb were observed which could not be accounted for by the small decrease in arterial saturation or by the effects of the increase in $P_aCO_2$ as there is usually a slight decrease in Hb during episodes of hypercapnia. It can therefore be concluded that the DNP produced a significant increase in cerebral oxygen consumption.

This study, in a global model, shows that Hb always increases with increasing oxygen consumption, even when CBF is augmented by increased $P_aCO_2$. This is in contrast to functional activation where NIR studies have shown an increase in HbO$_2$ and a small decrease of Hb in the activated region (Kleinschmidt et al., 1996). These changes have been attributed to an increase in local CBF over and above the increase in local CMRO$_2$ (Villringer and Dimagl, 1995).

At high oxygen tensions, the redox state of cytochrome c and also the CuA centre are expected to be independent of oxygen tension and determined by the metabolic state (Chance and Williams, 1956) and the activity of the dehydrogenases of the tricarboxylic acid cycle (Balaban, 1990). As oxygen tension is reduced towards zero, oxygen consumption becomes oxygen-limited and the components of the electron transport chain, including the CuA centre, are expected to be reduced. Although it is possible to determine a critical PO$_2$ for the electron transport chain is isolated mitochondria (Sugano et al., 1974), this cannot be simply related to cerebral oxygen delivery because, in vivo, there is considerable heterogeneity in cerebral tissue PO$_2$ (PtO$_2$) (Lubbers et al., 1994). The PtO$_2$ at the majority of sites is much lower than the arterial PO$_2$ which would suggest that substantial diffusion barriers exist between mitochondria and capillaries. However, large increases in tissue PO$_2$ are observed when oxygen delivery is increased by increasing PCO$_2$ (Metzger and Heuber, 1977) and thus there must be a balance between oxygen consumption and oxygen delivery.

As in previous studies (Cope et al., 1991), the results from the baseline anoxia swing show that oxygen delivery can be reduced with hypoxaemia with initially no significant change in the CuA redox state. This would suggest that, at normoxia, cytochrome oxidase is operating at an oxygen tension well above the critical threshold. As oxygen consumption is increased, oxygen delivery must either be increased to match demand or, due to the presence of diffusion barriers, mitochondrial PO$_2$ will fall and the tissue will become hypoxic. The results of this study show that the arterial saturation at which the CuA centre begins to reduce during the onset of anoxia rises with increasing accumulation of DNP and so, at normoxia, the cerebral
mitochondria must be operating closer to their critical threshold. At an accumulated dose of 1.63mg/kg the redox state of the Cuₐ centre is essentially oxygen dependent; any decrease in arterial saturation result in a significant reduction in the Cuₐ centre. At the highest DNP dose, there was an additional increase in PₐCO₂ which would have resulted in an increase in CBF and oxygen delivery. This increase in oxygen delivery was probably sufficient to prevent tissue hypoxia and a reduction in the Cuₐ redox state.

Studies on isolated mitochondria have shown that, in coupled mitochondria, the Cuₐ centre of cytochrome oxidase and cytochrome c are in equilibrium with the Cuₐ centre being slightly more reduced due to its slightly more positive midpoint potential (Rich et al., 1988). When DNP is added in excess to isolated mitochondria, oxygen consumption is typically increased by a factor of 10 and large oxidations are observed in the Cuₐ centre (Morgan and Wikström, 1991) and in cytochrome c (Wilson et al., 1988). However, in a previous in vivo study (Kariman et al., 1986) in which the redox state of haem a, cytochrome c and cytochrome b were compared to ³¹P NMR spectroscopy in an isolated rat head model, it was found that the components of the electron transport chain reduced and there was almost complete failure of phosphorus energetics when DNP was added to the perfusion medium. The authors explained their reduction as a result of relative hypoxia due to high oxygen demand after addition of DNP. However, their single dose was over two orders of magnitude greater than the maximum dose reported in this study (≈300mg/kg assuming a rat head weight of 50g compared to 2.1mg/kg in this study) which would lead to greater uncoupling and increase in oxygen consumption than in our model. In addition, their rat heads were perfused with perfluorocarbon (FC43) which has a lower oxygen carrying capacity than haemoglobin and, in this study, the increase in arterial CO₂ tension would increase arterial PO₂ via the Bohr effect and augment any increase in CBF and therefore oxygen delivery.

Thus in our preparation, oxygen delivery, in comparison to oxygen consumption was sufficient to prevent hypoxia and so the small oxidations of the Cuₐ centre, which are consistent with the results from isolated mitochondria if the proton gradient has been only partially reduced, can be observed. However, it should be noted that an oxidation of the Cuₐ centre has been observed in the human neonate when PₐCO₂ was increased (Edwards et al., 1991) and so it cannot be unequivocally concluded that uncoupling in vivo oxidises the Cuₐ centre of cytochrome oxidase.

REFERENCES


Oxygen dependency and precision of cytochrome oxidase signal from full spectral NIRS of the piglet brain


Oxygen dependency and precision of cytochrome oxidase signal from full spectral NIRS of the piglet brain. Am J Physiol Heart Circ Physiol 279: H2202-H2209, 2000.—Oxidation changes of the copper (Cu) center of cytochrome oxidase in the brain were measured during brief anoxic swings at both normocapnia and hypercapnia (arterial PaCO2 = 55 mmHg). Hypercapnia increased total hemoglobin from 37.5 ± 9.1 to 50.8 ± 12.9 μmol/l (means ± SD; n = 7), increased mean cerebral saturation (SmO2) from 65 ± 4 to 77 ± 3%, and oxidized CuII by 0.43 ± 0.23 μmol/l. During the onset of anoxia, there were no significant changes in the CuII oxidation state until SmO2 had fallen to 43 ± 5 and 21 ± 6% at normocapnia and hypercapnia, respectively, and the maximum reduction during anoxia was not significantly different at hypercapnia (1.49 ± 0.40 μmol/l) compared with normocapnia (1.53 ± 0.44 μmol/l). Residuals of the least squares fitting algorithm used to convert near-infrared spectra to concentrations are presented and shown to be small compared with the component of attenuation attributed to the CuII signal. From these observations, we conclude that there is minimal interference between the hemoglobin and CuII signals in this model, the CuII oxidation state is independent of cerebral oxygenation at normocapnia, and the oxidation after hypercapnia is not the result of increased cerebral oxygenation.

Changes in the redox state of the electron transport chain can, in theory, be measured using optical techniques: the mitochondrial NADH/NAD+ redox couple can be measured using NADH fluorescence, the copper (Cu) center of cytochrome oxidase can be measured using near-infrared (NIR) spectroscopy (NIRS), and the heme a center of cytochrome oxidase can be measured using visible surface reflectance spectroscopy. All these techniques reveal an oxidation of the electron transport chain during hypercapnia (see Gyulai et al. (8) for NADH fluorescence, see Edwards et al. (5) for NIRS, and see Kreisman et al. (16) for visible reflectance spectroscopy), and, in general, it has been concluded that this oxidation is the result of increased oxygen delivery and increased PO2 at the mitochondrial level, and the effects of the perturbation of carbohydrate metabolism are not discussed (although see Ref. 10). However, this conclusion is based on the observation that these methods show a continuous change in the redox state of the electron transport chain from hyperoxia to mild hypoxemia [see Gyulai et al. (7) for NADH fluorescence, see Kreisman et al. (16) for visible spectroscopy, and see Hampson et al. (9) for NIRS], although there are exceptions (4, 11, 24).

All optical techniques are susceptible to interference by oxyhemoglobin (HbO2) and deoxyhemoglobin (Hb), which show continuous changes between hyperoxia and hypoxia and are also present in much greater concentrations than cytochrome oxidase or NADH. In general, NADH fluorescence spectroscopy and visible heme absorption spectroscopy are usually implemented as two wavelength methods (although see Ref. 15), where one wavelength measures the change in the oxidation state of redox component and the other attempts to correct for changes in total hemoglobin. However, both these methods cannot also correct for changes in hemoglobin saturation, which are substantial during hypoxemia.

To obtain the most accurate measurement of the redox state of CuII, a full spectral charge-coupled device (CCD)-based NIR system was used, which has been shown by modeling to be more robust in separating the cytochrome oxidase component than two, four, 

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or six wavelength techniques (21). An added advantage of the use of a full spectral system is that the optical path length can be measured from the second differential of the water absorption features (20) so that changes in hemoglobin concentration and Cu^ state can be quantified in units of concentration (μmol/l). In addition, absolute deoxyhemoglobin concentration (aHb) can be obtained from the second differential technique (19), and absolute oxyhemoglobin concentration (ΔHbO2) can be calculated by assuming that cerebral HbO2 falls to zero during brief anoxia. Mean cerebral saturation (SMC02) can then be calculated from aHbO2 and aHb.

This study was designed to show that the oxidation of the Cu^ signal observed during hypercapnia accurately reflects oxidation changes of cytochrome oxidase and to show that this oxidation is not the result of the increase in oxygen delivery and Ptg^ associated with hypercapnia. This was achieved by performing brief anoxias at normocapnia and hypercapnia and determining the relationship between the Cu^ oxidation state and SMC02 during the onset of anoxia using NIRS. We have previously compared Cu^ oxidation changes with hypercapnia preperfluorocarbon and postperfluorocarbon exchange (24) and found that the oxidation is still present when hematocrit is reduced by 80%. This study uses an improved NIR system with greater sensitivity and higher temporal and spectra resolution to verify the Cu^ changes in the normal hematocrit piglets. Furthermore, this paper includes absolute quantification and presents attenuation spectra and the residuals of the spectral fitting procedure to show that the least-squares fitting algorithm is accurately accounting for the observed changes in attenuation.

**METHODS**

Seven male piglets born at term but less than 24 h old and weighing 1.67 ± 0.28 (SD) kg were sedated with midazolam and anesthetized with 2% isoflurane. A tracheotomy was performed, and the piglets were artificially ventilated with an intermittent positive pressure ventilator using an oxygen and nitrogen gas mixture. The inspired oxygen fraction (FIO2) was set to 0.4, the inspiratory pressure was set to 980 mm Hg, and Cu^ state and Smc02 during the onset of anoxia lasting 90 s of anoxia and then reoxygenation. A period of 20 min was allowed for the hemodynamic state to stabilize, and heart rate, blood pressure, arterial saturation, and rectal temperature were logged simultaneously. The gas mixture fed to the piglets was controlled using a computerized gas blender (6) and was switched synchronously with the inspiratory pressure.

Cannulas were sited in one internal carotid artery, an umbilical artery, and the umbilical vein for measurement of heart rate and mean arterial blood pressure using a strain-gauge pressure transducer and was used for collection of blood samples to perform blood gas analysis (ABL505, Radiometer, Copenhagen, Denmark), measurement of blood glucose and lactate (2300 STAT Plus, Yellow Springs Instruments, Yellow Springs, OH), and infusion of the 10% glucose solution at 2 ml/h to prevent hypoglycemia. Arterial saturation was monitored with a pulse oximeter (model 8604FO, Nonin Medical, Plymouth, MN) positioned on the foot. Rectal temperature was maintained at 38.5°C using a heated water mattress.

The piglet's head was placed in a stereotaxic frame, the isoflurane was reduced to 1.5-1.8%, and the piglet allowed to stabilize over a period of at least 1 h, during which NIR spectra were recorded. The FIO2 and inspired carbon dioxide fraction (FICO2) were switched independently, and the balance of the gas fraction was always nitrogen. The FICO2 was maintained at 0.4 throughout the experiment except during periods of anoxia. The FICO2 was maintained at 0.6 except during hypercapnia.

The protocol started with 5 min of recorded baseline followed by 90 s of anoxia and then reoxygenation. A period of 20 min was allowed for the hemodynamic state to stabilize, and moderate hypercapnia was then induced by increasing FICO2 (see below). Once the hemodynamic signals were sta-

**OXYGEN DEPENDENCY AND PRECISION OF CYTOCHROME OXIDASE**

H2203
OXYGEN DEPENDENCY AND PRECISION OF CYTOCHROME OXIDASE

Table 1. Physiological parameters before the anoxia at normocapnia and hypercapnia

<table>
<thead>
<tr>
<th>Physiological Parameter</th>
<th>Normocapnia</th>
<th>Hypercapnia</th>
</tr>
</thead>
<tbody>
<tr>
<td>[P_aCO_2], mmHg</td>
<td>33.5 ± 4.9</td>
<td>54.3 ± 5.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.543 ± 0.092</td>
<td>7.345 ± 0.025</td>
</tr>
<tr>
<td>[S_aO_2], %</td>
<td>98.8 ± 0.8</td>
<td>97.9 ± 1.1</td>
</tr>
<tr>
<td>MABP, mmHg</td>
<td>184 ± 20</td>
<td>155 ± 14</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>151 ± 14</td>
<td>161 ± 22</td>
</tr>
<tr>
<td>T_r, °C</td>
<td>38.5 ± 0.13</td>
<td>38.4 ± 0.2</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.65 ± 0.89</td>
<td>5.27 ± 0.55</td>
</tr>
<tr>
<td>Lactate, mM</td>
<td>2.15 ± 0.92</td>
<td>1.92 ± 0.94</td>
</tr>
</tbody>
</table>

All values are means ± SD; \( n = 7 \) piglets. Glucose and lactate refer to their respective plasma concentrations; pH, the partial pressure of arterial carbon dioxide (\( P_aCO_2 \)), the partial pressure of arterial oxygen (\( P_aO_2 \)), and arterial oxygen saturation (\( S_aO_2 \)) refer to arterial blood; \( T_r \) is rectal temperature; and MABP is mean arterial blood pressure. \( *P < 0.05 \); \( **P < 0.01 \); and \( ***P < 0.001 \) indicates significantly different from normocapnia using a paired Student’s t-test.

RESULTS

Typical root-mean-square noise values on the baseline were 0.2, 0.1, and 0.03 \( \mu \)mol/l for the \( \Delta HbO_2 \), \( \Delta Hb \), and \( \Delta C_uA \) signals, respectively.

Physiological parameters obtained at baseline, normocapnia, and hypercapnia before the anoxia are presented in Table 1. Baseline normocapnic parameters were within normal ranges, and, although arterial pH was slightly alkalotic, this is normal for the newborn piglet. Hypercapnia resulted in a significant increase in arterial \( P_aCO_2 \) of 20.9 ± 5.6 mmHg and a significant decrease in pH of 0.198 ± 0.32, as would be expected. Heart rate, rectal temperature, and arterial glucose and lactate concentrations did not significantly differ between normocapnia and hypercapnia. Hematocrit was 30.6 ± 4.6%.

At normocapnia [Fig. 1 (left)], the reduction in arterial saturation leads to a cerebral desaturation starting at 0 s and resulting in a fall in \( HbO_2 \) and an
increase in Hb, initially with no change in HbT. During the desaturation, P_{oxygen} is expected to fall but it is not until 25 s after the onset of anoxia that the first significant reduction of the Cu_{A} center of cytochrome oxidase is observed. The increase in HbT is consistent with a hyperemia and an increase in CBF triggered by the hypoxemia. Reoxygenation begins at 75 s with a return to normal levels but, at the elevated CBF, leads to an increase in HbO_{2} and a decrease in Hb over baseline values. HbT reaches a maximum at ~145 s, and HbT, HbO_{2}, and Hb then return toward baseline values over the subsequent 6 min but do not return completely to baseline until 10 min after the onset of anoxia.

During the reoxygenation phase, Cu_{A} rapidly reoxidizes and reaches baseline values before HbO_{2} returns to baseline. During the hyperemic period postreoxygenation, Cu_{A} oxidizes by 0.26 ± 0.12 μmol/I (P < 0.01) above baseline and then returns to baseline over the same time period as HbO_{2}.

Heart rate and mean arterial blood pressure increased during the anoxic swing (data not shown) and reached a maximum during the reoxygenation period and then returned to baseline over approximately the subsequent 10 min.

During mild hypercapnia, the cerebral metabolic rate of oxygen (CMRO_{2}) is expected to remain constant (26), but there is an increase in both CBF and CBV. The increase in CBF is predominantly due to dilation of the pial arteries, whereas the increase in CBV is due to venous dilatation. At constant CMRO_{2} and CBF, an increase in the venous volume would be expected to increase HbO_{2} and Hb in the ratio of the average venous saturation. At constant CMRO_{2} and CBV, an increase in CBF would increase HbO_{2} and decrease Hb in equal magnitude. The net effect when CMRO_{2} is constant but both CBF and CBV increase would be an increase in HbO_{2} and either a smaller increase or a small decrease in Hb, depending on the relative compliance of the venous side. The observed changes, which are shown in the baseline period of Fig. 1 (right) and presented in Table 2, consist of a large increase in HbT and HbO_{2} and a small decrease in Hb.

In this study, the Cu_{A} oxidation resulting from the hypercapnia was 0.43 ± 0.23 μmol/I (P < 0.005).

Figure 1 (right) shows the pooled NIRS parameters during anoxic swing at mild hypercapnia. Qualitatively, the changes are similar to those at normocapnia, but the delay between the fall in HbO_{2} and the reduction in Cu_{A} is greater.

Figure 2 (left) compares the measured attenuation changes with the residuals of the least squares fitting for a typical piglet at five different time points. The residuals are the difference between the measured attenuation change (ΔΔα_{i}) and the attenuation change due to the chromophore concentration changes obtained by the least squares algorithm, that is ΔΔα_{i} = ΔΔφ_{i}, where ΔΔφ_{i} is defined in Eq. 1. The residuals give an indication of the quality of the fit but cannot be used to calculate quantitative errors in the calculated concentration changes of the chromophores; for a perfect fit, they would show noise centered around zero. Figure 2 (right) compares the residuals with the Cu_{A} component of Eq. 1 at the same time points as Fig. 2 (left) but on an expanded scale. The Cu_{A} component of Eq. 1 is ΔC_{Cu} (λ), where λ refers to Cu_{A} and represents the component of the change in measured attenuation change that the fitting algorithm attributes to changes in the redox state of Cu_{A}. The differential path length measured from the 840-nm water feature for this piglet was 16.0 cm.

The time points of Fig. 2 are the following: a, during the anoxia at normocapnia immediately before there is a substantial change in the Cu_{A} signal; b, just before reoxygenation at normocapnia; c, from the hypercapnia baseline period; d, during the anoxia at hypercapnia immediately before there is a substantial change in the Cu_{A} signal; and e, just before reoxygenation at hypercapnia. These time points are marked on Fig. 1 as a–e, respectively.

In all cases except where there is no change in the Cu_{A} signal from baseline, the residuals are small compared with the attenuation change due to the change in redox state of Cu_{A}.

Figures 3 and 4 show the change in Cu_{A} plotted against ΔHbO_{2} and Smco_{2}, respectively, during the

### Table 2. NIRS parameters at baseline, at the depth of the anoxia, and during the hyperemia at normocapnia and hypercapnia

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normocapnia</th>
<th>Hypercapnia</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Anoxia</td>
</tr>
<tr>
<td>ΔHbO_{2}</td>
<td>0</td>
<td>-24.2 ± 6.4</td>
</tr>
<tr>
<td>Hb</td>
<td>0</td>
<td>27.7 ± 5.7</td>
</tr>
<tr>
<td>HbT</td>
<td>0</td>
<td>3.5 ± 2.53</td>
</tr>
<tr>
<td>HbO_{2}</td>
<td>24.6 ± 6.8</td>
<td>0.9 ± 1.7</td>
</tr>
<tr>
<td>aHb</td>
<td>12.9 ± 2.6</td>
<td>40.1 ± 8.1</td>
</tr>
<tr>
<td>aHbT</td>
<td>37.5 ± 9.1</td>
<td>40.9 ± 8.9</td>
</tr>
<tr>
<td>ΔC_{Cu}</td>
<td>0</td>
<td>-1.53 ± 0.44</td>
</tr>
<tr>
<td>Smco_{2}</td>
<td>65.4 ± 3.5</td>
<td>1.8 ± 3.2</td>
</tr>
</tbody>
</table>

All values are means ± SD, n = 7 piglets. Hemoglobin and the changes in copper (Cu_{A}) oxidation state are measured in micromoles per liter, and the mean cerebral oxygen saturation (Smco_{2}) is measured in percent. ΔHbO_{2}, ΔHb, and ΔHbT: changes in oxyhemoglobin, deoxyhemoglobin, and total hemoglobin, respectively; aHbO_{2}, aHb, and aHbT: absolute concentrations of oxyhemoglobin, deoxyhemoglobin, and total hemoglobin, respectively. NIRS, near-infrared spectroscopy.
onset period of anoxia at normocapnia and hypercapnia. During normocapnia, the first significant reduction in Cu₄₄ occurs when HbO₂ has dropped by −8.0 ± 2.4 μmol/l from a baseline of 24.6 ± 6.8 μmol/l and Smco² had fallen to 43.2 ± 5.4%. With hypercapnia, at the beginning of the anoxia, there is a small but significant reduction in Cu₄₄ from 0.44 ± 0.26 to 0.39 ± 0.17 μmol/l ($P < 0.05$) when HbO₂ falls from 14.4 ± 6.7 to 11.0 ± 4.1 μmol/l, but there is then no significant change in the Cu₄₄ redox state until HbO₂ falls to −19.6 ± 4.3 μmol/l. The change in the Cu₄₄ signal between HbO₂ of 11.0 ± 4.1 and −13.8 ± 3.0 μmol/l when Smco² falls from 77.4 ± 3.1 to 21.3 ± 6.3% is −0.04 ± 0.11 μmol/l.

At the first significant reduction of Cu₄₄, HbT has increased by 0.96 ± 0.67 μmol/l at normocapnia and by 1.2 ± 0.7 μmol/l at hypercapnia compared with the respective baseline. Both of these changes are significant ($P < 0.01$) but small compared with the normocapnia-to-hypercapnia change in HbT of 10.6 ± 3.6 μmol/l. Therefore, although the cerebral vasculature has responded to the hypoxemia, this response is small compared with that induced by hypercapnia.

**DISCUSSION**

The Cu₄₄ center lies on the cytosolic side of cytochrome oxidase close to the cytochrome c binding site and far from the binuclear where molecular oxygen is reduced to water (14, 27). In general, the redox state of cytochrome c and heme a are measured by visible absorption spectroscopy in mitochondrial studies because they provide substantial attenuation at visible wavelengths at the concentration and optical path lengths typically used in these studies compared with the low attenuation of the NIR band of Cu₄₄. When the oxidation state of Cu₄₄ and cytochrome c have been measured simultaneously, it has been found that the Cu₄₄ center senses the same membrane potential as cytochrome c (25) and is in redox equilibrium with cytochrome c during coupled turnover (23). Therefore, the changes in the oxidation state of Cu₄₄ from this in vivo model can be directly compared with those of cytochrome c from in vitro mitochondrial models.

At high oxygen tensions, the redox state of cytochrome c and, therefore, the Cu₄₄ center is independent of oxygen tension and determined by the metabolic
state and the activity of the dehydrogenases of the tricarboxylic acid cycle (2). As oxygen tension is reduced to zero, all the components of the electron chain become reduced, and the mitochondrial oxygen tension (\(P_{\text{O}_2}\)) at which oxidation changes are first observed in cytochrome \(c\) is between 0.6 and 20 mmHg, depending on metabolic state (30).

Direct measurement with microelectrodes has shown considerable heterogeneity in cortical \(P_{\text{O}_2}\), with values ranging from arterial \(P_{\text{O}_2}\) down to essentially zero (17) and with \(-2\%\) of sites having a \(P_{\text{O}_2}\) of \(<5\) mmHg (18). Therefore, even at normoxia, there is likely to be a population of mitochondria in which the electron transport chain redox state is oxygen dependent. However, if this population is very small, then small increases or decreases in \(S_{\text{macO}_2}\) will have a negligible effect on the size of this population and a negligible effect on the mean Cu\(^{\text{II}}\) oxidation state. However, it is difficult to determine the size of this population, given that the microelectrodes measure \(P_{\text{O}_2}\) and not \(P_{\text{O}_2}\left(O_{2}\right)\), the presence of an oxygen gradient between mitochondrion and capillary (3), and the uncertainty in the critical \(P_{\text{O}_2}\) in vivo.

Optical techniques have the potential to measure changes in the redox state of the electron transport chain directly, but great care must be taken to accurately separate the redox signal from the hemoglobin signals. NADH fluorescence is often assumed to be a more robust method than absorption spectroscopy because the reduction state of NADH is proportional to the intensity of the fluorescence signal. However, both the excitation light and the fluorescence light will be attenuated by the hemoglobin in the field of view, and this attenuation is a complex nonlinear function of hemoglobin concentration and saturation (13) so, in a scattering medium like tissue, a simple two or three wavelength ratiometric measurement cannot fully eliminate these effects.

The cytochrome oxidase signal from NIRS and visible absorption spectroscopy represents a small component of the overall attenuation change with anoxia or hypercapnia. For example, the attenuation change caused by the Cu\(^{\text{II}}\) oxidation seen during the hypercapnia represents typically 6% of the total attenuation change, the majority being hemoglobin (see Fig. 2C). This makes the cytochrome oxidase signal sensitive to cross talk, that is, changes in the concentration of chromophores that result in the attenuation changes not fully accounted for by the chromophore absorption spectra used in the least squares fitting. Cross talk would be manifest as changes in the cytochrome oxidase signal that track one, or a combination, of the hemoglobin signal(s). Other confounding effects would be changes in the scattering coefficient of the tissue or the movement of the tissue with respect to the optodes.

To show that cross talk is minimal in this system, it is necessary to show that \(Hb\) and \(HbO_2\) (or any two noncollinear combinations) can be varied without affecting the Cu\(^{\text{II}}\) signal. However, in general, it is not known a priori whether a particular procedure that varies the hemoglobin concentration will affect the Cu\(^{\text{II}}\) redox state. For instance, although at normocapnia there was no significant reduction in the Cu\(^{\text{II}}\) signal during the onset of anoxia until there was a substantial fall in \(S_{\text{macO}_2}\), it is possible that the Cu\(^{\text{II}}\) redox state was changing during this period and that the Cu\(^{\text{II}}\) signal was stable as a result of cross talk with hemoglobin. However, at normocapnia, \(HbO_2\) fell to \(-5.3 \pm 1.6\) \(\mu\)mol/l with no significant change in the Cu\(^{\text{II}}\) signal, whereas, at hypercapnia, \(HbO_2\) fell from 12.6 \(\pm 5.3\) to \(-13.8 \pm 3.0\) \(\mu\)mol/l with no significant change in the Cu\(^{\text{II}}\) signal (\(P > 0.1\)). The observation that the cerebral saturation can be changed with no change in the Cu\(^{\text{II}}\) signal and that the point at which the Cu\(^{\text{II}}\) signal begins to reduce can be altered with a physiological

![Fig. 3. Changes in the Cu\(^{\text{II}}\) redox state plotted against \(\Delta HbO_2\) at both normocapnia (N) and hypercapnia (H) during the onset of anoxia. The results are shown as means ± SD (n = 7 piglets).](image3)

![Fig. 4. Changes in the Cu\(^{\text{II}}\) redox state plotted against mean cerebral saturation (\(S_{\text{macO}_2}\)) \(HbO_2\) at both normocapnia and hypercapnia during the onset of anoxia. The results are shown as means ± SD (n = 7 piglets).](image4)
maneuver would suggest that the cytochrome signal is robust with respect to HbOg and Hb under these conditions where HbT was constant.

Hypercapnia at normoxia lead to an increase in HbT of 12.6 ± 6.2 μmol/l and an oxidation in the CuA signal of 0.43 ± 0.27 μmol/l. If this oxidation were the result of cross talk with HbT, then this difference should remain when the CuA center is expected to be fully reduced during the anoxic swings but where the increase in HbT is still present. However, the reduction of CuA at normocapnia was to -1.53 ± 0.44 μmol/l and the reduction at hypercapnia was to -1.49 ± 0.40 μmol/l; the difference being 0.039 ± 0.086 μmol/l and not significant (P > 0.25). The observation that HbOg fell to the same value during the anoxia at normocapnia and hypercapnia (-24.0 ± 6.4 and -24.5 ± 6.8 μmol/l, respectively; the difference is not significant (P > 0.25)] is evidence that the hemoglobin was fully desaturated during the anoxia. Thus the CuA signal is robust with respect to changes in HbT.

That the CuA signal is robust under both changes in HbT and hemoglobin saturation would suggest that the CuA signal from this system using this algorithm accurately reflects the redox state of the CuA center in this model and under these conditions. This conclusion is further confirmed by the observation that the residuals of the fitting algorithm are small compared with the attenuation changes due to the CuA redox changes.

If it is assumed that the CuA center becomes fully reduced during the anoxia and fully oxidized in the hyperemic period after anoxia at hypercapnia, then the total concentration of redox-active cytochrome oxidase in the piglet brain is 2.21 ± 0.16 μmol/l, and the baseline oxidation is 67.3 ± 18.3% oxidized. This concentration for the newborn piglet is in general agreement with the results of biochemical analysis of the developing rat brain, which shows an increase in the concentration of cytochrome oxidase from 1.3 μmol/l at birth to 5.8 μmol/l in the adult (1), and further emphasizes the fact that the concentration of cytochrome oxidase in the newborn piglet is small compared with the concentration of hemoglobin, which is between 40 and 50 μmol/l depending on arterial PCO2 (see Table 2).

During the onset of anoxia at normocapnia, the first observed change in the CuA signal occurred when SmcO2 had fallen from a baseline value of 65 ± 4 to 43 ± 5% when the reduction is 0.08 ± 0.04 μmol/l or a fall of 3.6% of the total cytochrome oxidase. This would suggest that the oxygen tension at the great majority of mitochondria is above the value at which their redox state becomes oxygen dependent, and it is not until there is a substantial fall in oxygen tension that a sufficiently large population of mitochondria have an oxygen tension sufficiently low to affect the measured CuA oxidation state. The shift of the critical SmcO2 to lower values seen during hypercapnia is probably the result of two effects of hypercapnia. First, the Bohr effect at reduced arterial and venous pH will increase the oxygen tension at a given hemoglobin saturation, and it is the difference in oxygen tension between vasculature and mitochondria that drives the diffusion of oxygen. Second, the microcirculation is more heterogeneous at normocapnia than at hypercapnia (12), and this would tend to broaden the response of CuA to SmcO2 and shift the critical SmcO2 to higher values at normocapnia compared with hypercapnia.

If the CuA redox state is independent of oxygen tension at normoxia, then increasing oxygen tension by increasing SmcO2 should not produce the oxidation observed during hypercapnia. Further confirmation that the oxidation observed with hypercapnia is not a response to increased oxygen tension is the observation that, under these conditions, SmcO2 can be lowered to normocapnia levels and below during the onset of anoxia without a reduction of the CuA signal back to the normocapnia baseline.

Therefore, in conclusion, the results presented here are consistent with microelectrode measurements of Ptoj and a low critical Pmco2 value as measured in isolated mitochondria: at normoxia and normocapnia, the P02 at the majority of mitochondria is well above a value that would limit oxygen consumption. The oxidation observed during hypercapnia is not consistent with the oxidation of an oxygen-limited population. In light of this, it is likely that the oxidation observed during hypercapnia is the result of the perturbation in carbohydrate metabolism or other effects of the reduced intracellular pH (10).

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REFERENCES


