THE EFFECT OF ACUTE CARBON MONOXIDE EXPOSURE ON THE BRAIN OF

THE CONSCIOUS SHEEP

by

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J Anal Toxic. Vol 17 (September), 1993.

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Abbreviations

CO	carbon monoxide
cGMP	guanosine 3,5-monophosphate
COHb	carboxyhaemoglobin
Hb	haemoglobin
Hypoxia	hypoxic hypoxia
PO_2	oxygen partial pressure
PCO	carbon monoxide partial pressure
L/min	litres per minute
pH	-log [H ion]
mmHg	millimetres of mercury
ppm	parts per million
CSER	cerebral somatosensory evoked responses
CortSER	cortical somatosensory evoked responses
PaO_2	arterial oxygen partial pressure
IL482	Instrument Laboratories 482 CO-Oximeter
RHb	reduced haemoglobin
MetHb	met-haemoglobin
CaO	arterial oxygen content
CvO	venous oxygen content
mls	millilitres
Calc	calculated
HR	heart rate
$P_{et}CO_2$	end-tidal carbon dioxide concentration

Abbreviations (cont)

CBF	cerebral blood flow
$C_a[O_2]$	arterial oxygen content
$C_v[O_2]$	venous oxygen content
THb	total haemoglobin
nm	nanometres
mgs	milligrams
C_{red}	fractional concentration of reduced haemoglobin
C_{O2}	fractional concentration of oxyhaemoglobin
C_{CO}	fractional concentration of carboxyhaemoglobin
C_{met}	fractional concentration of met-haemoglobin
k	scalar constant
$[E]^{-1}$	inverse matrix
Sat_{COHb}	saturated carboxyhaemoglobin
Hct	haematocrit
g	grams
umol/min	micro-moles per minute

Abstract

The prevalent hypothesis of carbon monoxide toxicity is based on the combination of carbon monoxide binding to haemoglobin forming carboxyhaemoglobin (causing a fall in the bloods oxygen carrying capacity) and the reduction in the dissociation of oxygen from haemoglobin.

Although the relationship between inspired carbon monoxide concentration and the level of carboxyhaemoglobin level in the blood is well recognised, the relationship between carboxyhaemoglobin level, the oxygen status of critical organs such as the brain and heart and the progression of the acute symptoms is uncertain.

This thesis examines the relationship between carboxyhaemoglobin level and critical organ status, with particular reference to the brain, in eight chronically instrumented conscious sheep whilst being progressively exposed to carbon monoxide in the expired breath, to simulate an acute human poisoning.

In all sheep, the carboxyhaemoglobin levels at the end of the exposure to carbon monoxide was approximately 65 percent. Mean arterial blood pressure remained unchanged with the exception of two sheep, where carbon monoxide administration was stopped at 25 minutes due to a sudden onset of hypotension. Oxygen delivery to the brain was sustained throughout the administration of carbon monoxide due to a significant increase in cerebral blood flow. There was no evidence of metabolic acidosis or lactate production by the brain, suggesting the brain did not become hypoxic during the time course of the carbon monoxide exposure. Oxygen consumption by the brain decreased progressively, and the sheep showed behavioural changes which varied from agitation to sedation to narcosis. The mechanism of these changes was therefore probably unrelated to hypoxia, however, may have been due to raised intracranial pressure or a direct effect of carbon monoxide on brain function.

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Declaration of Thesis Originality

This work contains no material which has been accepted for the award of any other degree or diploma in any other University or Tertiary Institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Peter G Langston   *Dated*

17TH August, 1997

CHAPTER 1

Review of Carbon Monoxide

Introduction

The Endogenous and Exogenous Production of CO

Carbon monoxide (CO) is a colourless, odourless, tasteless, non irritating gas, that is generally ubiquitous to the environment. It is produced *endogenously* in man via the catabolism of haem groups in the liver and brain by haem-oxidase. The average rate of endogenous CO production in an healthy adult is approximately 0.4 millilitres (mls) per hour. This is sufficient to saturate 0.4 to 0.7 percent of haemoglobin (Hb). The endogenous production rate can be increased in conditions such as hyper-metabolism, with specific drugs and haemolytic anaemia.

It is now suggested that endogenous CO may function as a neural messenger in the brain, as a consequence of its action on guanylyl cyclase. Furthermore, it may play a significant role in physiological maintenance of endogenous guanosine 3,5 - monophosphate (cGMP) concentration in the brain (Verma, 1993).

Carbon monoxide is also produced *exogenously* through the incomplete combustion of carbonaceous materials. Exogenous sources include motor vehicle exhaust, house appliances (e.g. kerosine and gas heaters, charcoal barbecues) and cigarettes. A smoker may have a blood carboxyhaemoglobin (COHb) concentration of up to 10 percent, where as a heavy cigar smoker can have COHb concentrations of up to 20 percent in their blood (Stewart et al, 1974).

Methylene Chloride

Methylene chloride is a highly volatile organic solvent that has many industrial uses. Because of its low toxicity and high volatility, it is widely used as a solvent in paint thinner and stripper, degreasers, aerosol propellants and in cement and plastics (Ratney et al, 1974).

After either an accidental inhalation or skin exposure, methylene chloride can be endogenously converted to CO, resulting in the formation of COHb. Methylene chloride is highly lipid soluble and is deposited in a variety of body tissues where it is released slowly (DiVincenzio and Hamilton, 1975). In the liver, mixed function oxidases convert methylene chloride to carbon dioxide and CO (DiVincenzio and Kaplan, 1981). The relative rate of production of CO by the liver is highly dependent on the duration of exposure to the methylene chloride. This process of CO production from an exposure to methylene chloride, can continue up to twenty four hours after the time of exposure.

Statistics of CO Poisoning

Despite it's removal from most domestic gases, CO is still the most common cause of fatal poisoning in both the United States of America and the United Kingdom (Meredith and Vale, 1988, Kindwall, 1985). It is estimated from figures at the Royal Adelaide Hospital, that there is in excess of 200 deaths and 1000 survivors from CO poisoning each year in Australia (Gorman, 1996). Approximately one third are the result of an accidental exposure at work and about one half result from suicide or a suicide attempt (Gorman and Runciman. 1991).

Conflicting Theories of CO Toxicity

Carbon monoxide has been the subject of intense scientific and clinical interest since 1857, when Claude Bernard discovered it produced hypoxia by reversible combination with haemoglobin (Hb) (Bernard, 1857) and culminating in the recent discovery of its importance in normal brain biology (Verma et al, 1993). Since Bernard's findings, the underlying mechanism of the toxicity of CO in man has been argued to be due to the affinity of the CO molecule for Hb, and the consequent formation of COHb and reduction in the oxygen supply to tissues.

First hypothesized by Haldane in 1895, COHb formation renders Hb unavailable for oxygen, creating an effective anaemia. Although this is still widely believed to be the primary mechanism of CO toxicity, it has been known for over sixty years that CO also has a significant toxicity that is unrelated to tissue oxygen delivery (Haldane, 1927).

In support of the latter, recent studies have shown that COHb is not toxic and that the primary mechanism of CO toxicity may be due to its toxicity at a cellular level (Ramirez et al, 1974; Agostini et al, 1974; Orellano et al,1976; Goldbaum et al, 1976). It has also been shown in CO poisoned patients, that their outcome does not correlate with the blood COHb concentration measured in hospital (Fang et al, 1986; Norkool and Kilpatrick 1986; Willms et al, 1985). Furthermore, titration of patient treatment against the COHb concentration is often unsuccessful in preventing sequelae of the poisoning (Myers et al, 1985; Sluitjer, 1967)

The understanding of the mechanism of CO toxicity remains equivocal and controversial. Given the prevalence of clinical CO poisoning and the frequent

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associated mortality and morbidity, and in the context of the emergent

neurobiological roles of this gas, it is clear that CO related research is important.

Research Models for CO Toxicity

Although there have been many studies over the last century regarding the

physiological response to CO in humans and animals, the resultant published data

are in conflict. A major factor contributing to this conflict is the nature of the

experimental models. Some important factors contributing to this variation have

been:

a.   species of animals used;

b.   the CO dose administered and duration;

c.   the technique used to administer the CO;

d.   level of exertion of animal during the CO exposure; and

e.   the state of consciousness of the animals.

In part, the latter is due to the agents used to render animals unconscious. This

drug induced unconsciousness can affect cerebrovascular responsivity (Ludbrook et

al, 1992).

Although chronic exposures to CO from automobile exhausts and tobacco smoking

are a cause for concern, this review will only discuss short duration CO exposures,

as this type of poisoning is responsible for most poisonings that result in human

hospitalisation.

The various animal models used in acute CO experiments will be discussed in this

Chapter. In particular, studies concerning the effects on the cardiovascular and

cerebrovascular systems will be highlighted, as these are directly relevant to the studies described in Chapter 3. In Chapter 2, the primary experimental method used in the subsequent experiments will be discussed. Chapter 2 will also include a discussion of the general methods used in the experiments described in Chapter 4. The primary study in this thesis, "Brain Oxygen Metabolism and Cerebrovascular Response of the Conscious Unrestrained Sheep to an Acute Exposure to Carbon Monoxide" will be described in Chapter 4, and a Conclusion will be stated in Chapter 5.

The Affinity of CO for Haemoglobin

Human Blood

Carbon monoxide is absorbed and excreted exclusively via the lungs. When inhaled, CO diffuses rapidly across the alveolar capillary membrane of the lung, where it binds with Hb at the same site as that for oxygen. In the pulmonary capillary blood, CO competes with the oxygen for the ferrous iron of the iron-porphyrin complex of deoxy-haemoglobin. Carbon monoxide equilibrates rapidly with Hb in the red cells. This reaction is slower than that of oxygen binding to the Hb, however, it only requires a few tenths of a second at normal body temperature (Holland, 1969). Thought to be irreversible by Bernard in 1857, CO has an affinity for Hb approximately 200 to 240 times that of oxygen in humans.

Animal Blood

The affinity of CO for Hb as compared to oxygen, is known to vary between species. Table 1 shows some of the known affinities of CO for Hb, when compared to the oxygen affinity for Hb, in various animal species.

Rabbit's have the lowest known affinity of CO for Hb. The intra-species variation observed in the rabbit for CO affinity, is thought to be due to the sequence variation in the beta amino acid chain of the Hb molecule. The affinity of CO for Hb relies on the amino acid sequence and changes in this sequence do affect the affinity of Hb and CO. It is well known that a significant variation in beta amino acid sequence exists in the rabbit (Heidner et al, 1976).

Sheep Blood

Electrophoresis has shown adult sheep Hb to exist in two discrete forms. This genetic trait occurs in separate or combined entities, resulting in the following three Hb types:

a.   Type A sheep: possessing A Hb;

b.   Type B: possessing B Hb; and

c.   Type AB sheep, which possess both A and B Hb (Harris and Warren, 1954).

In 1961, Kernohan showed that Hb A had a slightly higher affinity for CO than Hb B. The relative affinity of CO for Hb compared to oxygen in this study was 254.2 for Type A and 243.2 for Type B Hb (Kernohan, 1961). These CO / Hb affinity's are significantly different from the factor of 162 observed by Holland (1970), although the Hb type was not identified in this latter study.

Table 1. Reported values for affinities of CO for Hb (Hb affinity), when compared to the affinity of oxygen for Hb, for various animal species.

Species	Hb Affinity	Reference
horse	208	Holland, 1969
goat	214	Holland, 1969
dog	338	Holland, 1969
cat	286	Holland, 1969
monkey	195	Sendroy and O'Neal, 1955
mouse	148	Douglas et al, 1913
		Holland, 1969
rabbit	125	Krogh, 1910
	90	Korner, 1963
	90 (adult)	Rocco et al, 1969
	101 (fetal)	Rocco et al, 1969
	111, 93 (adult)	Holland, 1969
sheep	254.2 (type A Hb)	Kernohan, 1961
	243.2 (type B Hb)	Kernohan, 1961
	162	Holland, 1970

CO and Hypoxia

Tissue Hypoxia

The term "hypoxia" means reduced oxygen supply to a deficiency state in the tissue of the living organism, whereas "anoxia" means a total lack of oxygen supply to the tissue. Aerobic metabolism is reduced in hypoxia due to a decrease in the oxygen partial pressure in the mitochondria. As a consequence of metabolism (Nunn's "oxygen cascade pathway" [Nunn,1975]) the oxygen partial pressure in air (160 mmHg in dry air) decreases to 1 mmHg by the time it reaches the mitochondria of the cells (See Figure 1), this is commonly referred to as the Pasteur point. Below the Pasteur point, aerobic metabolism is not possible. Eighty percent of total cellular oxygen consumption is by mitochondria, the remaining twenty percent being consumed by a variety of other subcellular entities. The biochemical reactions in these secondary locations serve a variety of biosynthetic, biodegredative and detoxifying oxidations. Some of the enzymes involved in these reactions contain iron and/or copper and have high affinities for oxygen.

CO Hypoxia

Hypoxia occurs when there is an oxygen deficiency at an intracellular level. Traditionally, hypoxia has been divided into four categories:

a.   **hypoxic** - in which the oxygen partial pressure of arterial blood is reduced because not enough oxygen reaches the alveoli;

b.   **anaemic** - in which the arterial oxygen partial pressure is normal but the amount of Hb available to carry oxygen is reduced by a Hb abnormality or inadequacy;

c.   **stagnant, ischaemic or circulatory** - in which the blood flow to a tissue is inadequate to carry enough oxygen to the tissues, despite normal oxygen, partial pressures and Hb concentration; and

d.   **histotoxic** - in which, despite normal oxygen delivery to a tissue, toxic agents prevent metabolism.

Carbon monoxide poisoning is often described as anaemic hypoxia, because CO binds to Hb to form COHb, hence, reducing the amount of Hb available for binding oxygen. The formation of COHb can cause hypoxia if the duration and level of the CO exposure is sufficient. The hypoxia arises because:

a.   there is a reduction in the amount of oxygen carried by Hb in blood; and,

b.   the oxyhaemoglobin (OHb) which does form in the presence of CO and COHb dissociates at lower oxygen tensions (ie. there is a shift to the left of the OHb dissociation curve) (See Figure 2).

This left shift in the OHb dissociation curve was first measured by Roughton and Darling in 1944. The nett effect is for an increase in the affinity of oxygen for Hb, hence, there is a further requirement for the tissue oxygen partial pressure to be reduced, before the oxygen will be released from the Hb. This explains why an anaemic individual who has fifty percent reduction in the normal amount of OHb may be able to perform moderate work, whereas an individual with a comparable reduction in total OHb due to COHb formation can be seriously affected.

Histotoxic hypoxia corresponds to causes which render the tissues incapable of using oxygen. Carbon monoxide poisoning could also be clinically described as secondary histotoxic hypoxia, due to the effect of the CO binding to cellular haem-groups, and hence, effecting cellular metabolism (ie. cytochrome respiratory chain). The classical example of histotoxic hypoxia is cyanide poisoning, in which the action of the cytochrome P-450 is completely blocked, so that the tissues cannot use the oxygen even though adequate oxygen is available.

Figure 1. Oxygen Cascade Pathway. The change in oxygen tension from alveolar air to the mitochondria, with factors that influence each stage (Nunn, 1975).
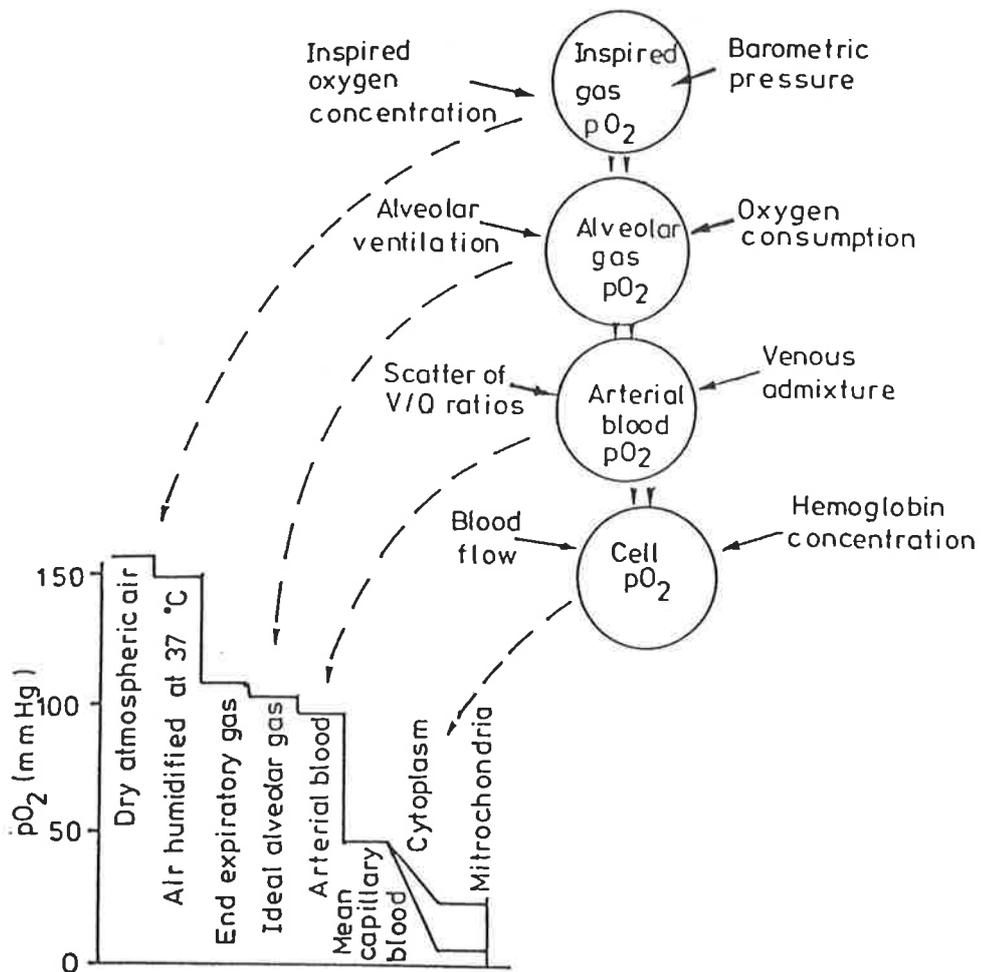
Figure 2. Oxyhaemoglobin dissociation curve of human blood, containing
varying amounts of COHb, calculated from the observed oxygen
dissociation curve of CO free blood. (Roughton and Darling, 1944).
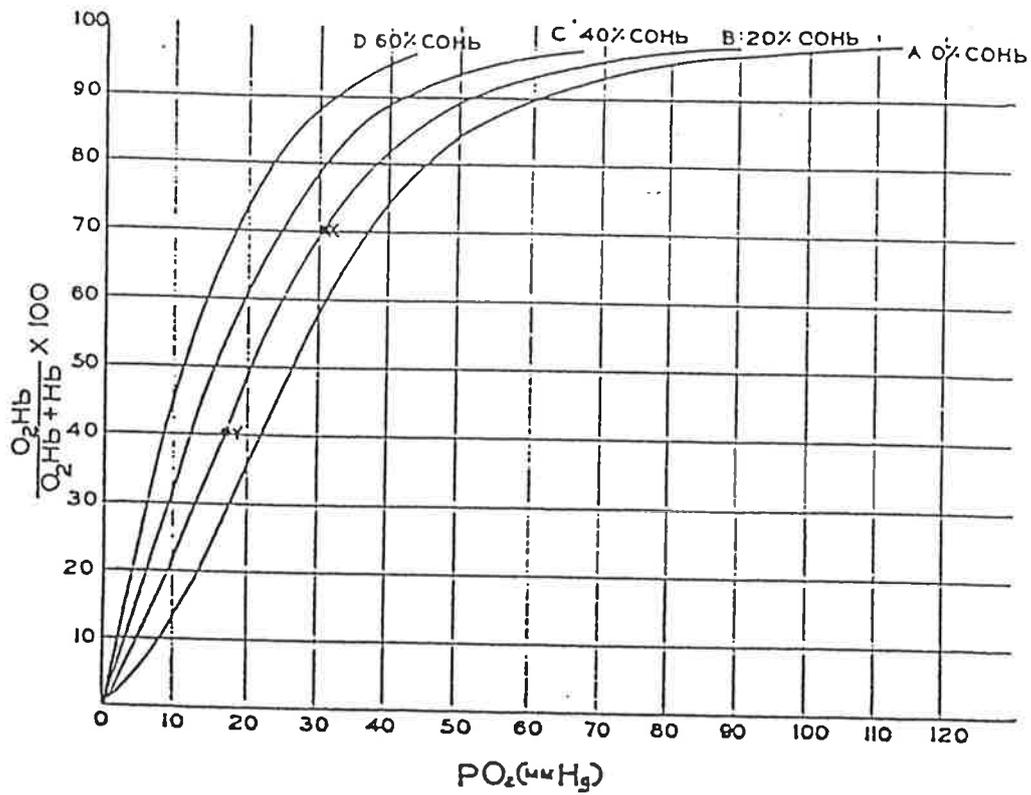
CO and Cellular Toxicity

It is still widely believed that CO is toxic only because of its binding to Hb and the consequent reduction in tissue oxygenation. However, certain features of CO exposures and poisonings are not readily explained by COHb related decreases in tissue oxygen tension (Anderson et al, 1973; Halperin et al, 1959; Horvath et al, 1971).

These conflicting explanations include the occurrence of physiological effects of CO at low COHb saturations, even less than 5 % (Anderson et al, 1973; Horvath et al, 1971/1975), and residual effects of CO after the COHb concentration has returned to normal physiological levels (Halperin et al, 1959). It is also recognised that the clinical manifestations of CO toxicity observed in humans do not correlate well with COHb levels (Sokal, 1975; Youngberg and Myers, 1991; Gorman et al, 1991). These important observations suggest that CO is not simply an hypoxic poison.

Further support for a direct CO cytotoxicity hypothesis, comes from experiments reported by Goldbaum in 1976. The experiments showed that in dogs transfused with erythrocytes containing 80 percent COHb, there was no indication of CO toxicity. However, dogs that inhaled CO (13 percent) for 15 minutes, died within 15 to 65 minutes, with an average COHb level of 65 percent. It was suggested from these observations, that the presence of dissolved CO in plasma was necessary for CO to enter tissues, with this CO gas exchange into the plasma occurring at the alveolar / blood vessel interface in the lungs (Goldbaum et al, 1976). Furthermore, it would appear that CO dissolved in blood is more important than COHb in determining the level of CO toxicity.

CO and Haem-Proteins

Apart from Hb, CO binds to and inhibits other haem-proteins such as myoglobin, reduced cytochrome C oxidase, reduced cytochomes of the P450 type and tryptophan dioxygenase (Piantadosi, 1987; Walum et al, 1985).

Cytochrome P450 reductase provides electrons to the P450 class of enzymes and haem-oxygenase, an enzyme that degrades haem to biliverdin and CO (Verma, 1993). It has been shown that CO can activate guanyl cyclase, to produce cyclic GMP - a process that may explain why CO relaxes smooth muscle and blocks platelet aggregation (Brune, 1987). It has also been postulated that CO may regulate cyclic GMP under normal conditions and function as a neural messenger (Verma, 1993).

The extravascular uptake of CO has been estimated to be 10 to 15 percent of total body burden (Coburn et al, 1973; Coburn et al, 1971; Luomanmaki and Coburn, 1969). Significant tissue uptake of CO only occurs in hypoxia, in part due to intracellular haem-proteins having affinities for CO *in-vitro* that are one or two orders of magnitude less than the CO affinity of Hb. When CO bind's to intracellular enzymes leading to acute organ dysfunction, subsequent events of bioenergetic and functional recovery will differ fundamentally to events seen in simple oxygen deprivation of similar tissue.

Cytochrome C is the terminal member of the intra-mitochondrial electron transport chain that is responsible for cellular metabolism. This enzyme complex accounts for almost ninety percent of the total oxygen uptake of the body. It catalyses the reduction of molecular di-oxygen to water, in a four electron process. Inhibitors of

the "a_1a_3" fraction such as CO, can block the flow of electrons from substrate to oxygen, and hence prevent oxidative phosphorylation (See Figure 3).

Organs such as the heart and brain, with large oxygen demands, predictably are the sites for the most severe manifestations of CO poisoning. Thus, the regional differences in tissue oxygenation and demand would not necessarily be relieved by physiologic responses to a limited systemic oxygen delivery. Thus, specific tissue oxygen needs may be inhibited due to the inability of the heart rate and decreasing blood oxygen content to supply these specific needs. As a result of chemical stimuli and reflex, in hypoxic hypoxia (hypoxia), there is an uneven distribution of oxygen supply, to maximise delivery to the heart and brain. In CO hypoxia, stimulation of the carotid chemoreceptors is curtailed by the absence of arterial hypoxaemia (Traystman et al, 1978), further suggesting that CO toxicity is not easily equated with hypoxia.

Piantadosi (1987) suggested that CO binding to reduced cytochrome oxidase would only occur under severe hypoxic conditions, because:

a.   the enzyme has a Michaelis-Menten constant for oxygen below 1 Torr, and since intracellular oxygen partial pressure (PO_2) is believed to be several torr higher than this, cytochrome oxidase should remain oxidised until severe hypoxia is present;

Figure 3. Carbon Monoxide and Oxidative Phosphorylation. A simplified

diagram of the mitochondrial respiratory chain indicating the sequence

of electron transport, three sites of energy coupling (oxidative

phosphorylation), and location of metabolic inhibition of CO

(Piantadosi, 1987).

Schematic of Mitochondrial Respiratory Chain
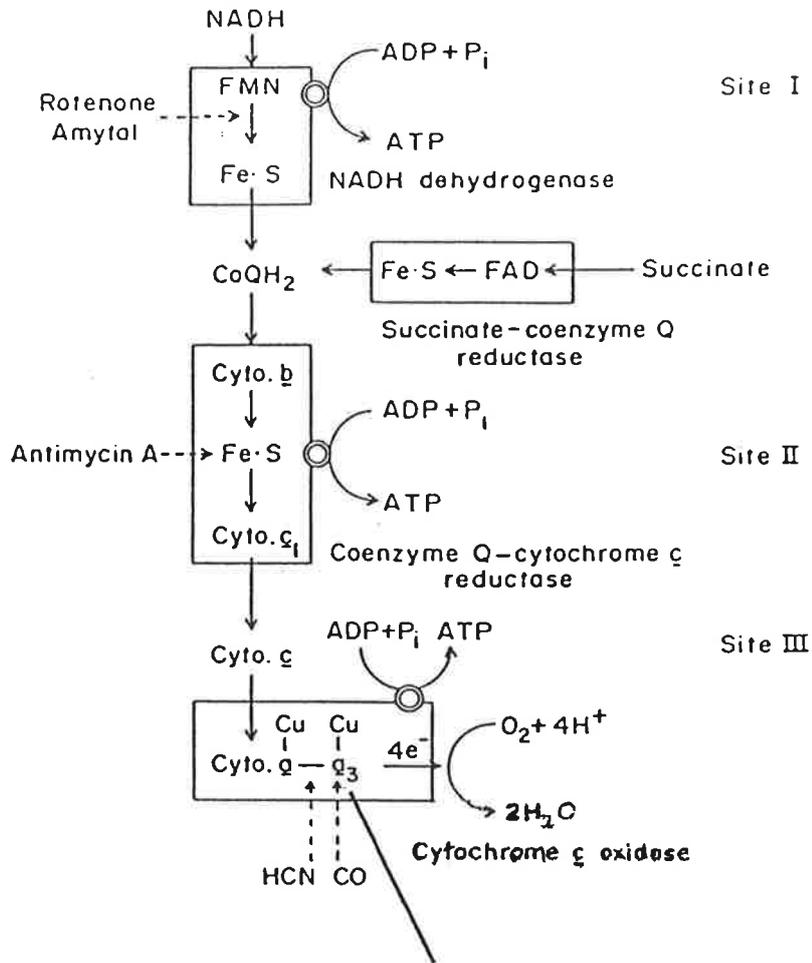
CARBON MONOXIDE BINDING SITE

b. the enzyme may exist in several oxidation and reduction states, but only the reduced cytochrome a_3 porphyrin centre binds CO in-vitro; and,

c. cytochrome oxidase has a Warburg coefficient (K) (measure of the CO affinity of an intracellular compound) in the range of 2 to 28. (This suggests that with a K value of 2 for cytochrome oxidase, the mitochondrial CO partial pressure [PCO]) would have to exceed mitochondrial PO_2 by a factor of 2 for CO to bind to 50 percent of the oxidase).

Because tissue PCO values are low, near anoxia would be necessary before CO would bind significantly to cytochrome oxidase (Piantadosi, 1987). Pitt et al (1979) has shown in animal experiments that cerebral metabolic rate does not decline until COHb concentrations approach 50 percent. This corresponds well with calculations by Piantadosi (1987), that is, a 50 percent COHb level at steady state would result in a maximum of approximately 50 percent cytochrome a_1a_3 -CO. At this level of CO saturation, actively respiring mitochondria may not be able to circumvent cytochrome a_1a_3 -CO ligands by unblocked a_3 molecules. Piantadosi (1987) suggested that the ability of a cell to avert bioenergetic failure depends not only on oxygen availability, but also on the concentration of CO, the duration of the CO exposure and the concentration of cytochrome oxidase. Overall then, it is likely that severe intracellular hypoxia is a prerequisite for significant CO-cytochrome C binding.

CO Absorption and Elimination in the Body

Body CO stores are influenced by the production and excretion of endogenous CO and the respiratory exchange of exogenous CO (Coburn et al, 1965). The concentration of COHb reached in the blood during an exposure to CO is determined by:

a.   duration of exposure;

b.   ventilatory rate;

c.   concentration of CO inspired;

d.   blood volume;

e.   haematocrit;

f.   barometric pressure;

g.   lung CO concentration;

h.   rate of endogenous CO production;

i. pO_2 in the lung capillaries;

j.   the affinity (K) of CO for the haemoglobin in the species; and

k.   physiological state of the body (adult or child, alcohol or drug effected).

The most important factors in determining the rate of COHb formation in blood is the inspired CO concentration and the duration of the exposure (Forbes et al, 1945).

Factors that essentially determine the extent of CO poisoning are the cardiac output and the presence of any regional ischaemia. Cardiovascular activity will influence the rate of CO elimination, as a greater blood flow to the lung (the only route of

elimination in the body) will increase the rate of elimination, hence, this also applies to the rate of uptake of CO. Existing hypoxia such as that which occurs at high altitudes can also effect both the rate of uptake of CO and the equilibrium concentration of COHb. In general, this will worsen the outcome of a CO exposure by aggrevating the tissue hypoxia.

The rate of CO elimination is determined by the:

a.   relative affinity of CO for haemoglobin;

b.   ventilatory minute volume;

c.   pre-existing COHb concentration in the blood;

d.   metabolic rate (e.g. the elimination rate is greater in children than adults due to faster metabolic rate [Williamson, 1991]).

Effects of Anaesthesia on the Cardio- and Cerebrovascular Systems

General anaesthesia can be induced using different drugs and techniques. A single drug can produce loss of consciousness, analgesia and suppression of muscle activity, and muscle relaxation. Alternatively a combination of agents can be used. The advantage of the latter is that an individual drug's undesirable side effects can be minimised.

Anaesthetics are known to promote or suppress the cardiovascular, cerebrovascular and respiratory systems (Wilson et al, 1985; Kaieda et al, 1989). These effects vary depending on the type of anaesthetic used (e.g. barbituate or non barbituate) and also on the administration technique (e.g. intravenous or inhaled anaesthesia). These anaesthetic effects can also vary between animal species.

When designing a model to study cerebral or cardiac pathophysiology, it is essential to maintain the physiological state of the animal. It has been clearly demonstrated that drug induced unconsciousness can effect the cerebrovascular responsivity of animals (Ludbrook et al 1992).

A review of the reported acute CO experiments in animals shows that a variety of anaesthetics have been used (pentobarbitone, ketamine, alpha chloralose and urethane). The most commonly used has been intravenous pentobarbitone. However, pentobarbitone has been reported to cause significant cardiovascular and respiratory system depression, and also has poor analgesic activity in animals (Flecknell, 1987; Green, 1982).

In view of the fact that anaesthetics can alter cerebrovascular response and activity, it would be appropriate to avoid such pertubations when studying CO and its cerebrovascular effects, as it has been demonstrated that CO increases cerebral blood flow (Ludbrook et al, 1992; Meyer-Witting et al, 1991). Hence, there should be concern of the potential disordered cerebrovascular response to CO, that may result from anaesthesia. Because of the variable effects of anaesthesia on the cardiovascular and cerebrovascular system, it is desirable to minimise or eliminate the use of anaesthetic agents in studies of these systems. Indeed, the ideal would be to use a conscious animal for any such experiment.

Systemic Effects of CO

CO is known to damage or affect almost every organ system. The brain and heart are of particular interest in this context because:

a.   they both function predominantly via aerobic metabolism;

b.   they are unable to sustain substantial oxygen debt; and

c   they have immediate and vital functions.

A review of the published effects of CO on animal cardiovascular and cerebrovascular functions shows variable and often conflicting results (Penney et al, 1989; Penney, 1988). This may be due to the effects of anaesthetic and analgesic agents in the animal models used for study. Consequently, the effect of such agents will be reviewed here and followed by a specific summary of published data from experiments in conscious animals.

CO Effects on Heart Rate in Conscious Animals

In both conscious and unconscious animals, CO produces a consistent tachycardia. The concentrations of CO used in conscious animal experiments have ranged from 100 ppm (0.01 percent) to 50000 ppm (5.0 percent) and are described below.

Heart rate increases were seen in awake dogs exposed to CO (Chiodi et al 1941, Adams et al, 1969; Adams et al, 1973; Ayers et al, 1969). Conscious rabbits (Korner, 1965; Erickson et al, 1971) and rats (Petajan et al, 1976) also demonstrated increases in heart rate (HR) after an acute exposure to CO. In 1982, Silbaugh and Horvath exposed male Sprague Dawley rats to 2000 ppm and 4000 ppm of CO for 20 minutes. A 50 percent COHb was attained within 8.5 minutes in the group exposed to 4000 ppm, and within 14 minutes in the group exposed to 2000 ppm. Penney et al (1989) observed a tachycardic response in rats exposed to CO, in which COHb concentrations increased to 80 percent. Conscious male albino rats, exposed to 3000 ppm CO in air for 70 minutes, developed a slight tachycardia up to 10 minutes, followed by a bradycardic response for approximately 8 minutes, followed by another small increase in HR (Laas et al, 1983). Santiago and Edelman (1976) observed tachycardia in goats, and Koehler et al (1982) showed a similar effect in conscious newborn lambs exposed to CO. Early researchers argued that the increase in heart rate was due to the physical restraint of animals; a secondary response to catecholamine release induced by immobilisation (Popper et al, 1962).

CO Effects on Cardiac Output in Conscious Animals

Carbon monoxide causes an increase in cardiac output in both conscious and unconscious animals (Chiodi et al, 1941; Ayers et al, 1950; Cramlet et al, 1975). Korner (1959) suggested that these cardiac output increases were a response to a falling blood pressure, due to peripheral vasodilation and secondary to tissue hypoxia. However, the majority of published research has not observed a hypotensive response to a CO exposure until COHb levels are very high (> 50 percent in blood)(Petajan et al, 1976; Dergal et al, 1976; Penney, 1988; Okedo et al, 1981). The increase in cardiac output then at lower COHb levels, needs an alternative (and as yet, undiscovered) explanation.

CO Effects on Acid / Base Response in Conscious Animals

Breathing is controlled by midbrain neurons. Central chemosensitive regions in the medulla are known to increase ventilation in acidotic conditions in response to cerebral spinal fluid carbon dioxide changes, low PO_2 and cerebral tissue anaerobiosis. The carotid and aortic bodies indirectly effect ventilation, and are themselves stimulated by changes in oxygen and carbon dioxide partial pressure and pH.

In dogs exposed to CO, until more than 50 percent of Hb was in the form of COHb, hyperventilation induced an alkalosis, whereas sectioning of the vagi prevented this response until death. It was concluded that death under CO asphyxia was a result of respiratory depression, secondary to hypocarbia (Haggard and Henderson, 1921). However, hyperventilation in response to acidosis usually does not produce a blood alkalosis. These findings suggest that there must be some other mediator of respiration effort in CO poisoning, apart from changes in pH or carbon dioxide.

Chiodi et al (1941) observed an unchanged ventilation rate in one dog exposed to CO, even when the COHb saturation exceeded 50 percent. The plasma pH in this dog showed a slight acid shift. Another dog showed a more significant decrease in pH. In this second dog, ventilation only increased slightly from 1.2 litres per minute (L/min) to 1.6 L/min, and then fell to 1.3 L/min at COHb concentrations of 48 and 52 percent, respectively. These observations support experiments in goats exposed to CO (Santiago and Edelman,1976); where no change in the systemic pH or bicarbonate level were seen in animals with COHb levels of between 50 and 60

percent. Similar findings were reported by Doblar et al (1977) in goats exposed to CO.

In lambs, Koehler et al (1982, 1983, 1984) observed a slight acidosis during hypoxic hypoxia, the pH remained stable during and after an exposure to CO. Bureau et al (1988) observed a mild compensated metabolic acidosis in 2 to 10 day old lambs exposed to CO until blood concentrations of COHb reached 40 to 50 percent.

Firstly, it would appear that acidosis and hyperventilation (respiratory compensation) are not features of most CO exposures, and secondly, the development of blood acidosis and hyperventilation are only features of CO exposures that result in extremely high COHb levels. Furthermore, it is difficult to ascertain if response variation exists due to the size and species of animal used.

CO Effects on Blood Pressure in Conscious Animals

A review of blood pressure changes during a CO exposure in conscious animals shows conflicting results. This conflict may be apparent and appears to be related to the degree of the exposure, and may also be due to animal size and species variation response.

Korner (1965) observed a fall in arterial blood pressure and total peripheral resistance in rabbits that were made to inhale 0.1 and 0.2 percent CO. These animals also had a small but significant elevation in right atrial pressure after an hour of breathing CO. In rabbits with denervated carotid and aortic chemoreceptors, there was an abrupt fall in arterial blood pressure and a small but significant increase in right atrial pressure when inhaling 0.1 and 0.2 percent CO (Korner, 1965). The increase in right atrial pressure could be a result of an increase in venous return. A large decrease in blood pressure after denervation of the receptors, despite a probable increase in preload, suggests that chemoreceptors may play a role in blood pressure maintenance during a CO exposure.

Dergal et al (1976) showed a uniform progressive hypotension in dogs exposed to 2, 5 and 10 percent CO. The average COHb levels attained were 24, 54 and 69 percent respectively. Petajan et al (1976) observed a significant decrease in mean arterial blood pressure in rats exposed to 1.5 percent CO over a 2 hour period. This decrease occurred at a COHb concentration of between 60 and 70 percent. It was observed that the mean arterial blood pressure fall was consistently associated with impaired nervous system function. Increases in P_s latency (second positive

deflection of a visual evoked response) were seen as blood pressure fell below 100 mmHg, which occurred when the level of COHb was between 60 to 70 percent.

Penney (1988) reported a decrease in diastolic arterial blood pressure, but no consistent change in the systolic blood pressure, when rats were exposed to CO. No observation was indicated for mean arterial pressure. Okedo et al (1981) observed arterial blood pressure decreases in rabbits that had been denuded of Hb, during inhalation of 5, 10 and 20 percent COHb.

Silbaugh and Horvath (1982) observed hypotension in rats exposed to either 2000 or 4000 ppm CO for periods of 20 minutes. A 50 percent COHb level was reached within 8.5 minutes at 4000 ppm and 14 minutes at 2000 ppm. For both exposures, mean arterial blood pressure was first seen to fall at a level of COHb of 40 percent , at a mean level of 60 percent the average decrease in mean blood pressure was 31 percent . Laas et al (1983) also observed a fall in blood pressure in rats exposed to 0.3 percent CO for 70 minutes (mean arterial blood pressure fell after 1 to 2 minutes from a base line of 113 mmHg to a minimum of 77 mmHg after 132 minutes, the pressure then remained stable for the rest of the exposure). This decrease was reversible.

Carotid stump blood pressure declined sharply in rats during the inhalation of 2700 ppm CO for 90 minutes; COHb levels rose to 80 percent (Penney, 1988). Mean arterial blood pressure decreased steadily from average levels of 110.3 to 86.5 mmHg ($P<0.005$) after 45 minutes, while carotid stump pressure decreased from 38.1 to 21.0 mmHg ($P<0.05$) after 30 minutes (Penney, 1988).

In contrast, Ayers et al (1969) observed a slight increase in femoral arterial blood pressure in dogs rapidly exposed to 5 percent CO for 30 to 120 seconds. Blood pressure increased from a control level of 122 mmHg to 135 and 152 at 3 and 6 minutes respectively, then decreased to an average of 130 mmHg after 9 minutes.

A study of sheep, where mean arterial oxygen content was reduced from 13.8 -/+ 0.7 to 9.7 -/+ 0.7 volumes percent , showed no significant changes in mean arterial blood pressure (Koehler et al, 1983). Koehler (1984) observed a slight increase in arterial blood pressure with hypoxic hypoxia in adult sheep, but not with CO hypoxia. A similar result was seen in newborn lambs (Koehler, 1984).

Blood Pressure Response to Chemodenervation
Traystman et al (1978) showed an increase in arterial blood pressure during hypoxic hypoxia, where as a decrease occurred during CO hypoxia, in dogs. After denervation of the chemoreceptors of these dogs, both hypoxic hypoxia and CO hypoxia resulted in decreases in arterial blood pressure and cerebral vascular resistance, of a similar magnitude. Sylvester et al (1979) and Traystman et al (1978) demonstrated similar responses in blood pressure in dogs during hypoxic hypoxia and CO hypoxia.

In hypoxic hypoxia, any sympathetically stimulated vasoconstriction will be prevented by denervation of the aortic and carotid chemoreceptors such that the consequent response will be and is similar to that seen during CO hypoxia (i.e. peripheral vasodilation). These studies suggest that the decrease in peripheral vascular resistance and blood pressure is initiated by mechanisms other than these chemoreceptors. This argument is supported by Traystman and Fitzgerald (1981)

studying dogs, in which arterial blood oxygen contents were reduced from 19.0 volumes percent to 9.6 volumes percent by either CO hypoxia or hypoxic hypoxia. The blood pressure increased during hypoxic hypoxia and decreased during CO hypoxia. After coronary sinus denervation, arterial blood pressure rose only slightly with hypoxic hypoxia. After baro- and chemoreceptor denervation, both hypoxic hypoxia and CO hypoxia groups resulted in a decreased blood pressure. King et al (1985) also saw a significant decrease in mean arterial blood pressure in both intact and chemodenervated dogs in which the arterial oxygen content was reduced by 65 percent due to the inhalation of CO.

Beta- Receptors

Dogs were exposed to CO such that their arterial oxygen content were reduced by 50 percent. In dogs without beta adrenergic blockade, there was a greater decrease in the mean arterial blood pressure, when compared to dogs with beta adrenergic blockade (King et al, 1984).

Melinyshyn et al (1988) exposed dogs in which there was beta 1 and 2 blockade, beta-2 blockade alone or no blockade (control). Mean arterial blood pressure was lower in all dogs (P<0.005), but significantly lower in the control group than in either of the beta blockade groups during the CO exposure.

Young and Stone (1976) could show no significant change in blood pressure in dogs exposed to CO, whether they be natural controls, or being paced at a heart rate of 150 beats per minute, or being "blocked" by propranolol and atropine. It was also noticed in this study that all dogs showed a significant decrease in diastolic coronary resistance.

In goats in which the COHb saturation was increased to 60 percent, pulse pressure was shown to increase by 40 percent and mean arterial blood pressure to 16 percent above baseline. The increase in systolic and diastolic pressure was significant. In beta blocked goats with similar COHb levels, systolic and diastolic blood pressure remained unchanged (Santiago and Edelman, 1976).

Alpha- Receptors

Villeneuve et al (1986) reduced arterial oxygen content by 50 percent in dogs by ventilating them with CO, some dogs were also subject to alpha adrenergic blockade. Mean arterial blood pressure was significantly lower in the alpha blockade group during the control and CO periods. Whole body vascular resistance fell during CO hypoxia in both groups, the values in the alpha blocked group were significantly lower. The study suggests the alpha receptors are not involved in the blood pressure response to CO; the researchers suggested that the decrease in total peripheral resistance after denervation was probably a result of increased levels of circulating catecholamines which may stimulate beta receptors. In addition, local metabolic factors may contribute to the increase in vasodilation.

CO Effects on Cerebral Blood Flow in Animals

Pronounced arterial hypoxia induces a decrease in cerebrovascular resistance and an increase in total and regional cerebral blood flow (CBF) (Ludbrook et al, 1992). Under conditions of normal arterial blood pressure and acid / base status, this change in flow is seen when the oxygen tension in the arterial blood decreases below approximately 50 mmHg - by which time, the oxygen tension in cerebral venous blood falls below 30 mmHg (McDowall, 1966).

CO has been shown to cause cerebrovascular dilation and increases in both CBF and cerebrovascular volume in various animals (Paulson et al, 1973; Traystman et al, 1978). An increase in CBF was seen in anaesthetised goats when the COHb saturation increased to 65 percent . These researchers also reported a 133 and 200 percent increase in CBF in anaesthetised dogs when the COHb levels were raised to 30 and 51 percent, respectively. These CBF levels were significantly greater than those seen in dogs during an equivalent exposure to cyanide (Doblar et al, 1977). Koehler et al (1982, 1984) reported greater CBF changes in unanaesthetised newborn lambs and in adult sheep during exposures to CO than those reported in hypoxic hypoxia, suggested to be due to a CO-induced left shift of the OHb dissociation curve. Traystman and Fitzgerald (1981), observed in dogs exposed to CO a significant decrease in cerebral vascular resistance.

In pilot studies using an anaesthetised rabbit model, a 15 minute exposure of 1.0 percent CO caused profound but reversible responses, including pial vessel dilation, suppression of cerebral somato-sensory evoked responses (CSER) and herniation of the brain through a craniotomy (Meyer-Witting et al, 1991). This exposure also

resulted in a CBF increase of 236 percent in the left and 287 percent in the right cerebral cortex. When the CO was withdrawn, the CBF was shown to decrease back to normal. The COHb levels recorded in the rabbits were shown to correlate with the hemispheric CBF and cerebrovascular resistance. Also observed in this study was an increase in intracranial pressure during the CO exposure, consistent with profound pial vasodilation and brain herniation, which occurred, despite a fall in mean arterial blood pressure; concurrent with vasodilation, hypotension and increases in CBF which demonstrate that the cerebrovascular autoregulation is disrupted during this type of CO exposure.

In support of the rabbit study by Meyer Witting et al (1991) cited above, Ludbrook et al (1992) also observed profound increases in CBF of up to 300 percent after a fifteen minute exposure to 1.0 percent CO in the same species, such that oxygen delivery to the brain remained unchanged. This maintenance of delivery was also seen in hypoxic hypoxia rabbits. However, while this phenomena was associated with preservation of cerebral somatosensory evoked response (CSER) voltage in hypoxic hypoxia rabbits, significant voltage depression was shown in rabbits exposed to CO. These findings suggest that to some extent, the toxicity of CO to the brain in rabbits is not a consequence of impaired oxygen delivery.

CO Effects on Brain Oxygen Delivery and Consumption in Animals

Doblar et al (1977) exposed conscious goats to 1.0 percent CO in 40 percent oxygen, and demonstrated a progressive increase in CBF (eventually doubling baseline values). However, oxygen delivery and oxygen consumption of the brain was found to decrease; these decreases becoming statistically significant only when the COHb concentration exceeded 30 to 40 percent. In this study, CBF was measured with an electromagnetic flow probe placed around one internal carotid artery, such that only half of the flow to the brain was determined. In contrast, Koehler et al (1982) showed a progressive increase in CBF and a consequent maintenance of oxygen delivery to the brains of awake sheep exposed to CO. These sheep did however show a reduction in cerebral oxygen uptake. A maintenance of oxygen to the brain was also observed by Ludbrook et al (1992) in rabbits. Cerebral blood flow increased up to 300 percent from baseline in these rabbits at the end of a fifteen minute exposure to 1.0 percent CO.

CO Effects on Brain Function

Neurological disfunction and reduced consciousness are the most common outcomes of CO poisoning in humans. A general observation after the removal of a surviving human casualty from the CO source, is that there is a rapid recovery on neurological function, which is accelerated by breathing 100 percent oxygen (Gorman and Runciman, 1991). Some recoveries after CO poisoning are incomplete, while others continue to do so after being discharged from hospital. Some patients who do recover initially, can also relapse or deteriorate (Gorman and Runciman, 1991).

The pathological changes in severely poisoned patients have been shown by computerised tomography, nuclear magnetic resonance imaging and post mortem examination to be necrosis of the globus pallidus, cerebral cortex, hippocampus, cerebellum and substantia nigra (Ginsberg, 1985).

Experiments on monkeys exposed to CO (where survival occurred), showed clinical and pathological changes similar to those observed in man, including lesions of the globus pallidus and hippocampus (Ginsberg et al, 1974). Three of these animals demonstrated severe deficits, including limb paralysis, alterations in muscle tone, blindness and deafness; one developed these problems five days after the CO exposure. Another three monkeys exhibited only mild motor disturbances. It was noted by these researchers that the lesions differed in extent to that observed after hypoxic brain injury (Ginsberg et al, 1974).

The often subtle clinical features seen in patients as a result of CO poisoning are very difficult to replicate in animal experiments. However, brain function has been studied in unconscious rabbits exposed to CO and hypoxia. Ludbrook et al (1992), observed in both CO exposure and hypoxia, significant increases in CBF and a maintenance of oxygen delivery to the brain. In the hypoxic group, cortical somatosensory evoked response (CortSER) was unaffected until the arterial oxygen tension was below 20 mmHg. In contrast, in the CO exposed rabbits, the CortSER voltages were halved during the CO exposure and only recovered to approximately 80 percent of the baseline subsequently. This was despite the maintenance of oxygen delivery to the brain both during and after the CO exposure. This study suggests CO toxicity occurs despite a maintenance of oxygen delivery to the brain and differs, in this context, from an hypoxic hypoxia insult.

Overview

It is difficult for clinicians to rationalise the natural history of CO poisoned patients with the prevalent explanation of CO toxicity (Youngberg and Myers, 1991). In general, the theories of CO toxicity are based on a primary hypoxic event arising from a combination of:

a.   CO binding to haemoglobin such that there is a fall in blood oxygen content (Haldane, 1895);

b.   a reduction in the dissociation of the OHb which is formed in the presence of CO and COHb (Roughton and Darling, 1944); and

c.   the effect of hypotensive hypoperfusion of critical organs of the body (Ginsberg, 1985).

The hypoperfusion component of this theory is not supported by previous studies in rabbits exposed to CO (Ludbrook et al, 1992; Meyer-Witting et al, 1991), nor is it consistent with the increases in CBF observed in man and other species when exposed to CO (MacMillan, 1975; Paulson et al, 1973; Traystman et al, 1978). The increases in CBF may be such that there is little or no decrease in actual oxygen delivery to critical organs, and at COHb levels of 40 percent and less there may be a negligible change in oxygen availability to tissues as the arterial oxygen partial pressure (PaO_2) will be normal and the extent of dissociation of OHb at capillary level (oxygen tensions below 50 mmHg) will be essentially unchanged (Roughton and Darling, 1944).

Ludbrook et al (1992) also has shown that the CortSER differ, in CO exposure and hypoxic hypoxia. Despite the maintenance of oxygen delivery to the brain in the CO exposed animals, there was an notable change in the CortSER.

It is also noteworthy that, the titration of therapy against COHb levels (ie. the restoration of blood oxygen content) is often unsuccessful in preventing sequelae in CO poisoned patients (Myers et al, 1985; Sluijter et al, 1967), and hypoxia has never been a plausible explanation of the delayed deterioration and relapses seen in patients days after an exposure to CO (Youngberg and Myers, 1991). These delayed effects may be related to the activation of neutrophylic leucocytes and consequent oxidative damage (Thom, 1993), however, it should be recognised that observed oxidative damage is late, and hence can not explain the acute effects of CO (Thom, 1990).

A review of the literature identifies a considerable variation in experimental design, species and CO dose. Consequently, conclusions are difficult. The problem is compounded by the frequent use of anaesthetic agents. To overcome the latter problems, the animal model used in the study reported in this thesis, is that of a chronically catheterised conscious sheep.

Despite the difficulty in data interpretation alluded to above, there are some consistent trends in the haemodynamic and cerebrovascular responses to CO. In general, exposure to low levels of CO leads to direct stimulation of the cardiovascular system (eg. increased CBF) and moderate central nervous system depression; eventually, this may lead to a significant and rapid fall in cardiac output and severe central nervous system depression. It is evident that as the COHb

concentration increases, the heart rate initially rises and then declines at high COHb concentrations. This early tachycardia presumably results from sympathetic stimulation and a reflex response to vasodilation, while the subsequent bradycardia is generally thought to be a response to myocardial depression from hypoxia. It has also been suggested that CO may directly effect excitation of / and conduction in the myocardium. As a result of the initial increase in heart rate and stroke volume, there is a consequent increase in cardiac output and CBF. The increase in cardiac output appears to arise predominantly from the increase in heart rate. High concentrations of CO can cause the cardiac output to decrease and this may be due to a direct cytotoxic effect of CO.

It appears that at high COHb concentrations (> 60 percent), there is a progressive metabolic acidosis. A respiratory compensation is seen, but at even higher COHb levels is suppressed by CO neurotoxicity such that the deleterious effects of the acidosis are accelerated.

The blood pressure response varies between studies; however, the balance of data suggests that there is an usual mild hypotension that occurs secondary to peripheral vasodilation.

With the formation of COHb, and consequent reduction in blood oxygen content, there is an observed increase in the CBF. Oxygen consumption by the brain is usually impaired, despite a maintenance of oxygen delivery.

The basic mechanism of acute CO neurotoxicity is not understood, also the delayed effect of CO exposure seen in humans has not yet been replicated in an animal

model. To understand the toxicity of CO it is essential to describe the pathophysiological changes that this gas induces.

CHAPTER 2

General Methods and Materials

Overview

Eight female merino sheep with Hb Type A, each weighing approximately 40 to 45 kilograms, were used. These sheep whilst under anaesthesia, were chronically instrumented using aseptic techniques. The surgical procedure occurred in two stages. Stage One followed a method developed by Upton et al (1994), where the sheep seven days before the study, had a 20 MHz ultrasonic pulsatile doppler flow probe surgically placed onto superior saggital sinus. Via the same burr hole, a 4 French catheter was positioned two centimetres into the saggital sinus. Stage two of the surgical procedure consisted of the sheep being chronically catheterised via the carotid artery and jugular vein. Seven days after the surgical preparation, the experiment was performed. To calculate the actual cerebral blood flow, the sheep was again anaesthetised (several days post the experiment) and the saggital sinus doppler probe was calibrated (Upton et al, 1994).

Method for chronic placement of a 4 French gauge catheter into / and a 20 MHz Ultrasonic Doppler Flow Probe onto the Superior Sagittal Sinus.

Preparation

The sheep was removed from the metabolic crate and was anaesthetised using 1.0 gram of Thiopentone (Pentothal, Abbott Australasia, Sydney, NSW, Australia), administered via the left jugular vein, and then placed onto the surgical table and intubated. The animal was positively ventilated (Medishield CPU-1 Ventilator) with 100 percent oxygen, and anaesthesia was maintained using 2.0 percent halothane (Fluothane, ICI Pharmacueticals, Cheshire, UK).

The animal was positioned in a sphynx position on the table and secured using surgical tape. The head was supported on sand bags so movement was minimal, and the area between the eyes, horns and ears was clippered to the skin. This area was cleaned thoroughly with a broad spectrum bactericidal / fungicidal solution (10 percent povidone-iodine, Novapharm, Sydney NSW). Using aseptic techniques the animal was prepared for surgery ie. sterile drape placement.

Surgical placement of Ultrasonic Doppler Probe

A four centimetre incision along the line of the longitudinal medial suture, in the area of the fronto-parietal suture, was made into the epidermis of the head. The incision was opened and the fascia removed to expose the cranium. Using a trephine (1.3 millilmetre internal diameter), a craniotomy was made to expose the dura-mater. The craniotomy was centred, with the edge of the 1.5 centimetre hole, 0.5

61

centimetres posterior of the frontal parietal suture, in line with the longitudinal medial suture (See Figure 4). The dura mater was exposed but not disturbed.

The area exposed was over a portion of the superior saggital sinus vessel. The bone dowel was removed and stored in an isotonic saline moist swab, under aseptic conditions. The cranium around the exposed area was debrided of sharp edges using ronguers. A 20 MHz ultrasonic doppler flow probe (Iowa Doppler Products, Iowa City, Iowa USA) was prepared, prior to the surgery and sterilised. The probe was sutured and glued to the centre of a one centimetre square piece of cotton tape. The probe was cut to shape and placed down over the saggital sinus, such that the doppler crystal was positioned perpendicular to the direction of flow. The direction of the wire connected to the probe when placed over the vessel was perpendicular to the saggital sinus . The probe was placed under the frontal edge of the hole in the cranium to help maintain stability of the probe. The doppler signal was tested for clarity, and if necessary repositioned. It was necessary to position the doppler probe to ensure a "maximum flow" signal (that is the probe was placed directly over the centre of the vessel). The probe was positioned to the frontal aspect of the craniotomy, to ensure space was available for the saggital sinus catheter placement. The wire was exteriorised through the incision area.

Catheterisation of Superior Saggital Sinus

After probe placement, the saggital sinus was then cannulated. Using the Seldinger technique, a 5.0 French radio-opaque polyethylene (PERT) catheter (William A Cook, Brisbane, QLD, Australia) was introduced into the saggital sinus. The tip of the catheter was placed two centimetres antero-grade into the superior sagittal sinus. The bone dowel was placed over the top of the catheter and the probe. At

Figure 4. Dorsal view of the sheep skull showing position of "trephine burr hole"

(shaded area), where the ultrasonic doppler flow probe and sagittal sinus

catheter are placed.

Longitudinal Midline Suture

Fronto-parietal Suture
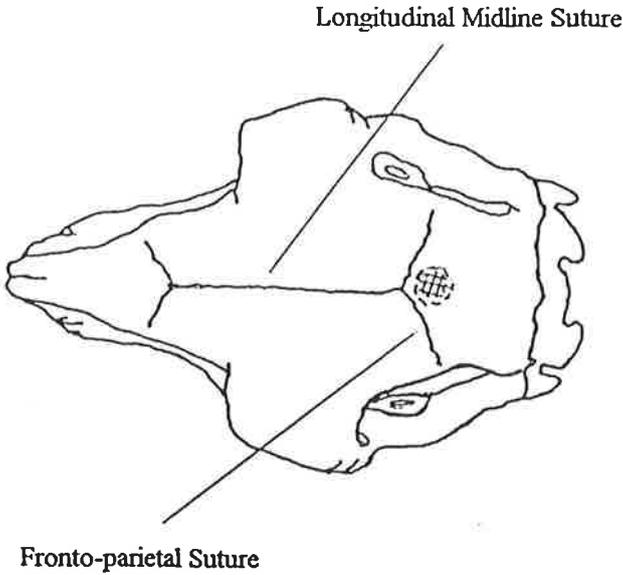
this point in time the doppler signal was checked to ensure the best signal (maximum flow) was being received. The bone dowel was secured down over the vessel using a titanium plate. The plate was secured to the bone dowel and the cranium, using titanium screws. The wire from the probe was directed out of the incision area posterior of the craniotomy, and the catheter was directed out of the craniotomy in the anterior aspect. The incised tissue was then sutured closed. The catheter was sutured to the skin and connected to the catheter flushing system (Runciman et al, 1984).

Chronic Catheterisation of the Sheep for Cerebrovascular Study

The second stage of the surgical procedure of the sheep involved anaesthesia and sterile preparation (as described above) of the right side of the neck, as described above for the first stage. The neck area prepared, extended between approximately the fourth cervical vertebrae to the seventh cervical vertebrae, and five centimetres either side of the carotid artery area. Using aseptic techniques a four centimetre longitudinal incision was made over the area of the carotid artery. The fascia and muscle was blunt dissected until a three centimetre section of the jugular vein and carotid artery was cleared. Using the Seldinger technique, two 7.0 French gauge catheters were inserted into the carotid artery with the tips positioned at the junction of the carotid artery and aortic arch. One catheter was used for sampling arterial blood and the other for blood pressure monitoring. The catheters were secured to the vessel using 1.0 silk. The vessel distal to the catheters was ligated.

Correct placement of the arterial and venous catheters was determined by Image Intensification (Siemens Siremobile-2, Siemens Austraila, Sydney, NSW, Australia). A plastic mounting base plate (Auslic, Nailsworth, SA, Australia) was sutured to the muscle under the catheters, and to this the catheters were secured using a sterile plastic tie. This was to prevent the catheters from accidentally being being pulled out of the vessel. The neck incision was sutured closed, and the catheters were secured to the neck externally using suture. Anaesthesia was withdrawn and when the animal regained consciousness, it was placed into the metabolic crate (See Figure 5) and monitored closely for post operative distress. The catheters were connected the catheter flushing system (Runciman et al, 1984)(See Figure 5).

Immediately before surgery, the animals were given a single 4 ml intramuscular dose of benzyl-penicillin (200 mg/ml) and dihydro-streptomycin (250 mg/ml) (Pen &

Strep Injection, Norbrook Laboratories, UK). This was followed by a three day course of streptomycin / penicillin antibiotic.

Catheter Flushing System

Each catheter was connected to a line and constantly purged with heparinised saline. This system consisted of a gas cylinder connected to a Fenwall pressure bag, which contained heparinised saline connected to several three-way manifold outlets. These outlets were connected to the catheters which were purged with saline at 0.3 mls per minute. This system was designed to prevent blockage of the catheters from blood clots. The flushing system was harnessed onto the metabolic crate (See Figure 5).

Figure 5. Sheep in metabolic crate, showing catheters connected to the saline flushing apparatus.

Springs

Taps

Pressure Infusors

Reservoir/Drop Counters

Fenwal Infusion Pumps

Dripsets

High Pressure Manifold

Water and Feed Bins

Faeces Container

Air Cylinder

Urine Container
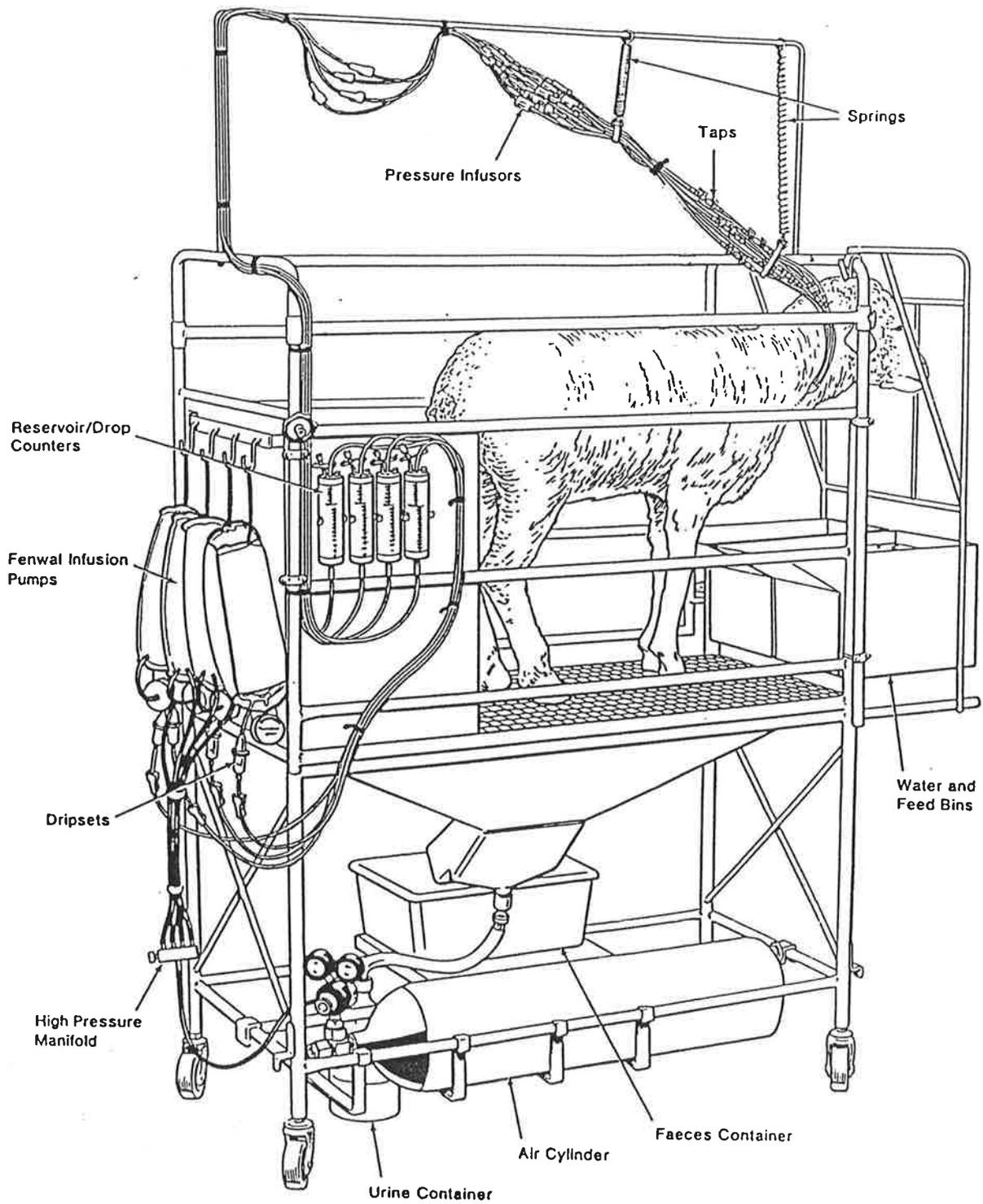
Physiological Monitoring

One arterial catheter was used for blood sampling and the other for blood pressure measurement. The superior sagittal sinus catheter was used to sample venous effluent from the brain, as this blood essentially represents the majority of venous effluent of the cerebral hemispheres (Upton et al, 1994). The sagittal sinus doppler probe was used to continuously measure instantaneous Cerebral Blood Flow (Upton et al, 1994). Electrocardiogram electrodes were connected to the sheep to measure heart rate. During the experimental period the following parameters were measured and recorded:

Bloodgas Measurement

Blood was sampled from both the arterial (aortic arch) and venous blood (superior sagittal sinus) supply during the experiment. Each blood sample was analysed to determine the blood gas tensions, using a Corning 278 Blood-gas Analyser (Ciba Corning Diagnostics Corporation, Mayfield, MA, USA). The Corning 278 analyser provided blood pH (acid/base status), dissolved oxygen and carbon dioxide concentrations (partial pressure) and bicarbonate concentration. The four concentrations of OHb, RedHb, COHb and MetHb, together with oxygen content were determined using an Instrument Laboratories 482 (IL 482) CO-Oximeter calibrated for sheep with Type A haemoglobin (See Chapter 3).

Brain Oxygen Delivery and Consumption

Two essential metabolic profiles were studied, the brain oxygen delivery (the sum of the arterial oxygen content and cerebral blood flow) and the brain oxygen

consumption (the sum of the difference between the arterial and venous oxygen contents and cerebral blood flow):

$$\text{Brain } O_2 \text{ Del} = C_a(O_2) * CBF$$

$$\text{Brain } O_2 \text{ Cons} = [C_a(O_2) - C_v(O_2)] * CBF$$

where

O_2 Del	oxygen delivery
O_2 Cons	oxygen consumption
$C_a(O_2)$	arterial oxygen content
$C_v(O_2)$	venous oxygen content
CBF	cerebral blood flow

Lactate determination

A 1.0 ml volume of blood was required for the analysis of the lactate. The method of analysis was based on an spectrophotometric enzyme end point reaction.

Two hundred microlitres of whole blood was added to 800 ul of Perchloric acid (5 percent) and stored on ice. The deproteinised blood was centrifuged at 3000 rpm for 5 minutes. 800 ul of the supernatant was removed and placed into a 10 ml centrifuge tube. 250 ul of Potassium Carbonate ($KHCO_3$) was added to the supernatant to neutralise the sample. The sample was left on ice for 10 minutes and then assayed, according to the technique described by Gutman and Wahlefeld (1974) (See Appendix 2).

(Lactate)   absorbance of NADH+ at 340 nm

(Lactate dehydrogenase)

Lactate hydrazine   ----> Pyruvate hydrazine

NAD -> NADH+

CO Administration Technique

Whilst the sheep was restrained in a sling, the head was tilted backwards to expose the underside of the neck, and a two centimetre wide area of the medial section of the fourth to sixth cartilage area was anaesthetised using 5 percent Lignocaine (Astra Pharmacueticals, North ryde, NSW, Australia). Between approximately the fifth and sixth tracheal cartilage, a sixteen gauge intravenous catheter (IntraCath, Becton Dickinson Vascular Access, Sandy, Utah, USA) was inserted to a depth of approximately 10 centimetres. The catheter tip was positioned before / and near as possible to the prominent ridge of the carina. The sheep's head was released and the catheter was coupled to a gas line connected to a gas flow rotameter.

All animals were monitored until measured parameters stabilised (less than 10 percent variation), at which time a 15 minute baseline phase was commenced. When the baseline phase had completed, CO (1.0 percent mix with air) was administered at a flow rate of 2.5 litres per minute. This continued until the HR started to decrease. This reduction in heart rate was at approximately 10 percent below the peak heart rate attained.

The $C_a(O_2)$ and COHb concentration of the arterial blood was measured every 5 minutes. Once the CO had been stopped (end of experiment) the sheep was administered a flow of 100 percent oxygen via the tracheal catheter for approximately 2.5 hours. This was to increase the partial pressure of oxygen in the blood and supplement the reduced oxygen carrying capacity of the blood, and also assist in the removal of CO from the body.

The CO Experiment

The sheep was surgically prepared for the experiment as described above. Approximately 7 days post surgery, the sheep was placed in a sling with the hoofs resting on the floor of the metabolic crate. The various blood sampling catheters were prepared for blood gas measurement. The blood pressure transducer was connected to the arterial line. Heart rate was determined from an Electrocardiogram signal, recorded from leads connected to the legs of the sheep.

Once the sheep was prepared and the various monitors set-up, three baseline measurements of bloodgas (arterial and venous) and physiological parameters were made at five minute intervals. The sheep was then administered CO until the end point of the experiment (as described above). The point in the experiment where the heart rate started to decrease was regarded as the onset of cardiac depression.

The actual CO exposure continued for approximately 35 minutes for each sheep. In two animals, the CO was withdrawn after 25 minutes because the animals were in danger of dying from hypotension.

In addition, the overall behaviour of the animals was continually assessed (with particular reference to conscious level, response to painful stimuli - ear pinch, and head position). An analgesiometer (a device that measures thresholds to an electrocutaneous stimulus) (Ludbrook et al, 1993) was trialed in one sheep not reported here, as an objective measurement of sedation. This caused considerable agitation in the animal and was not used again.

Approximately 2 to 3 days after the experiment the animal was anaesthetised and ventilated, as described above. The sagittal sinus doppler probe was then calibrated using an exsanguination method as described by Upton et al (1994). This sheep preparation has been shown to produce stable CBF measurements over the time period of the study reported here (Upton et al, 1994).

Calibration of the Sagittal Sinus Doppler Probe

A direct venous outflow method is used to measure the true sagittal sinus flow. The sheep was anaesthetised, ventilated and placed in a sphynx position on the surgical table as described above. An incision was made in the scalp of the sheep rostral to the original site, and the periosteum reflected. A trephine hole was then made 2 centimetres caudal to the site of the transducer. The sheep were placed on their side and stainless steel screws were used to fix a plastic spout around the hole. In this position, all the blood coming from the sinus could be collected via the spout into a 100 ml measuring cylinder. The sheep were systemically heparinsied with 10000 I.U. of heparin. The CBF was maximised by decreasing the ventilation, resulting in hypercarbia. At this point in time the calibration of the doppler probe was performed.

Cerebral Blood Flow Regulation Technique

After the sheep were prepared for the calibration, the ventilation rate of the sheep was decreased to achieve hypercarbia (end-tidal carbon dioxide concentration, $P_{et}CO_2$ of approximately 65 to 70 mmHg). The $P_{et}CO_2$ in the expired breath was measured using a capnograph (Model OIR 7101, Nihon Kohden Corporation, Tokyo, Japan). Once the peak CBF had been reached, the sinus was clamped and the calibration method was performed as described in the "Doppler Calibration Technique" below.

Cerebral blood flow reactivity curves were obtained from Upton et al (1994) which assisted in regulating the ventilation rate to effectively and eventually render the sheep hypocarbic ($P_{et}CO_2$ of approximately 25 to 30 mmHg). During this transition

period the CBF of the sagittal sinus was being measured against the "blood velocity" (as doppler shift in KHz).

Doppler Calibration Technique

The sagittal sinus was located and a homeostatic forcep was clamped onto the vessel to occlude the blood flow. Immediately after this, the sagittal sinus was completely cut laterally, rostral to the forcep, using a scalpel blade. This allowed blood to flow freely from the sinus with minimal back pressure. The clamp prevented any back flow of blood from the caudal aspect of the sinus. The ventilation rate was changed to give a range of sagittal sinus flow rates (See: Cerebral Blood Flow Regulation Technique, described above). During this process, 30 second collections of blood leaving the sinus were made into a volumetric cylinder, consecutively, using a stop watch. The 30 second volume recorded was doubled, hence, resulting in a blood flow value in mls per minute.

The flow rate was expressed as mls per minute and was later correlated with the corresponding sagittal sinus "doppler velocity" to produce a calibration curve by linear regression. The slope and intercept of this line of best fit was used to convert velocity readings to CBF in mls per minute using standard techniques. The sheep were then killed with a barbituate overdose.

CHAPTER 3

A Method to Determine Sheep Carboxyhaemoglobin, Oxyhaemoglobin, Reduced haemoglobin, Methaemoglobin and Oxygen Content using a Spectrophotometer

Introduction

The animal model chosen here required a rapid determination of Hb concentrations in sheep blood. In particular, it was essential that the COHb level and the oxygen content could be measured in both arterial and sagittal sinus blood. The animals used were female Merino ewes with haemoglobin blood type A. An automated spectrophotometer (Instrument Laboratories (IL) 482 CO-Oximeter (482)) was used to measure blood OHb, COHb, RHb, MetHb and arterial and venous oxygen content in humans.

For standard use, the IL 482 is programmed to measure the Hb species described above in human adult and fetal blood. To enable veterinary use, the IL 482 also has coefficients programmed into its "Read Only Memory" to enable the measurement of Hb species in dog, rat, baboon, and bovine blood. The machine also has the facility to be programmed to allow measurement of these Hb species in other species.

Instrument Laboratories Corporation provides a protocol for the development of Hb coefficients in other species (Instrument Laboratories, 1988). However, attempts to determine the coefficients for merino sheep with type A haemoglobin, using this protocol were not successful. It was therefore necessary to determine the required "sheep Hb coefficients" by an alternative and more thorough method.

The IL482 CO-Oximeter

The IL482 is an automated spectrophotometer designed to measure the fractions of OHb, COHb, RHb and MetHb in whole blood, and to calculate a total haemoglobin (THb) concentration from the absolute absorptions of each of the Hb species. An oxygen content is also calculated using a correction factor for the OHb concentration in the blood sample.

These measurements use coefficients based upon four specific wavelengths in the visible light spectrum (535.0, 585.2, 594.5 and 626.6 nanometres (nm)), and constitute a 4 by 4 inverse matrix of relative absorbtion coefficients for the various Hb species.

This Chapter describes the method used to develop and programme the IL482 for type A Hb female merino sheep.

Methods

Sample Preparation

Fresh blood was obtained from 5 adult female type A Hb merino sheep. Each blood sample was divided into 5 equal samples. One was retained as an "as drawn" sample. The other four samples were prepared as described below to form pure samples of OHb, COHb, RedHb. Between 8 and 11 sequential measurements were made from each prepared sample using the IL 482, programmed with the human coefficients. The Hb concentration, the absorbances at each wavelength of 535.0, 585.2, 594.5 and 626.6 nm, the gain and the calculated percent of each type of Hb were recorded.

Sample Handling

Blood samples were treated gently to avoid haemolysis. The samples were always well mixed before use, to prevent the settling of red blood cells which would alter the Hb concentration.

Sample Analysis

Fifty to 60 mls of venous blood was obtained from the jugular vein of the sheep and EDTA anticoagulant (0.5 mls of 1000 I.U. per ml) was added. Samples were prepared as described below and then measured on the IL 482 in the "Human Coefficient mode" of operation.

As-drawn blood

Fresh as-drawn blood was measured 10 times on the IL 482. Both the percent concentrations of the Hb species and the absorbance constants were recorded.

Oxyhaemoglobin

Five mls of blood was tonometered for 60 minutes at $37^O C$ with approximately 5.0 percent carbon dioxide in oxygen, and then stored on ice. The carbon dioxide flow was adjusted to maintain a blood pH between 7.35 and 7.45. The blood was stored in gas-tight syringes which were covered with aluminium foil. Preliminary experiments showed the washout of COHb in the tonometer to have a half life greater than 200 minutes. It was accepted then that the COHb level in this sample would be close to that in the as-drawn sample. The blood was removed into a blood-gas syringe and analysed 6 times on the IL 482. Both the percent concentrations of the Hb species and the absorbance constants were recorded.

Carboxyhaemoglobin

Five mls of blood was tonometered at $37^O C$, and 100 percent CO and carbon dioxide was bubbled through the blood at a flow rate of 400 and 30 mls / min, respectively. The pH was monitored to ensure that it remained between 7.35 to 7.45. If necessary, the carbon dioxide flow rate was adjusted until the pH range was restored. The blood was then analysed 6 times on the IL 482, recording the percent concentrations of the Hb species and the absorbance constants.

Reduced Haemoglobin

In preliminary experiments, 5 mls of blood was tonometered at 37° C; nitrogen and carbon dioxide was bubbled through the blood at a flow rate of 450 and 20 mls per minute, respectively. The blood sample had approximately 2 to 3 percent OHb and MetHb similar to the content in the as-drawn blood. To remove the residual OHb and MetHb, an alternative method suggested by the IL 482 protocol, was to add sodium dithionite to the blood. This reduction process converts the MetHb back to RedHb, and removes the bound oxygen from the Hb (Instrument Laboratories, 1988).

Five mls of blood was withdrawn into a syringe containing 45 milligrams (mgs) of sodium dithionite, and was thoroughly mixed on a vortex for at least two minutes. The blood was analysed 6 times on the IL 482, recording the percent concentrations of the Hb species and the absorbance constants.

Methaemoglobin

Twenty mls of blood was placed into a 50 ml syringe. Two mls of IL CO-Oximeter diluent (haemolysis solution) was added slowly to the blood and mixed thoroughly. This diluent effectively causes the red blood cells to haemolyse (Instrument Laboratories, 1988). Two mls of 20 percent potassium ferricyanide was added slowly to the haemolysed sample and the sample was then mixed and left to stand upright with 10 mls of air above the liquid for 30 minutes.

Potassium ferricyanide oxidises the bivalent iron to trivalent iron, and hence, will convert the OHb, COHb and RedHb to MetHb. Any oxygen and CO bound to Hb is consequently released as a by-product.

The treated blood was mixed with a magnetic stirrer and the pH adjusted to 7.20 by slowly adding either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide. The addition of sodium hydroxide to a MetHb solution can cause protein denaturation and the formation in solution of a precipitate which will interfere with absorbance. To prevent this from happening, the solution was centrifuged at 3000 rpm and only the supernatant was analysed 6 times on the IL 482, recording the percent concentrations of the Hb species and the absorbance constants.

Matrix Calculation

The calculation of the absorbtion coefficients for each type of Hb was performed in three stages. All calculations, matrix multiplications, and inversions were performed with an Apple Macintosh II personal computer, using Microsoft Excel version 1.5 (Microsoft Corp., Redmond, WA)

Stage 1

The absorbances were linearly adjusted for the gain of the IL 482 and the Hb concentration by being scaled to the mean concentration for each of the 4 types of Hb and divided by the gain at which the absorbances were measured. No deductions were made for any residual COHb in these samples. Instead, assuming that these samples had no such residual, the matrix of absorbtion coefficients was derived and then normalised. In normalising this matrix, the data were scaled so that the largest element had a value of 1.0. This was then inverted and applied to the average absorbtion at each measured wavelength of the freshly drawn blood, giving an initial estimate of the percent COHb and MetHb in the sample.

Haemoglobin fractions were calculated as follows: If the absorbance of a blood sample at each of four wavelengths was recorded as *Ab1, Ab2, Ab3, Ab4* from the IL CO-Oximeter, then

$$
\begin{pmatrix} C_{red} \\ C_{O2} \\ \\ C_{CO} \\ C_{met} \end{pmatrix} = k \, [E]^{-1} \begin{pmatrix} Ab1 \\ Ab2 \\ \\ Ab3 \\ Ab4 \end{pmatrix}
$$

where

C_{red}	fractional concentrations of RHb
C_{O2}	fractional concentrations of OHb
C_{CO}	fractional concentrations of COHb
C_{met}	fractional concentrations of met-haemoglobin,

in the blood sample. The "k" value is an unknown scalar constant which depends on the Hb concentration and the apparatus gain. If all the absorbtion is due to these four types of Hb (ie. there are no other fractions such as sulph-haemoglobin), then the sum of C_{red}, C_{O2}, C_{CO}, and C_{met} is 1.0. From this base k and all four Hb fractions can be determined.

The matrix [E] is presented in Table 2, and the final values for the inverse matrix $[E]^{-1}$ are presented in Table 3. Approximate absorbtion curves for the four Hb types are displayed as Figure 6.

Stage 2

Using the absorbances which had been scaled for Hb concentration and the gain of the IL 482 (as in Stage 1, above), deductions were made for the adsorbtion of the residual contaminating types of Hb, as described in the IL 482 protocol (Instrument Laboratories, 1988). The correction for the original amount of each Hb type was made from the initial estimate as derived in Stage 1. This differs from the IL 482 protocol in which it is recommended to use estimates of residuals in the "as drawn" sample measured by oximetery using human coefficients. If the estimate of the residual was negative (always less than -1.0 percent, then no deduction was made. Thus, deduction was made for the absorbance of residual COHb in the OHb and RHb prepared samples, and for the absorbance of residual MetHb in the OHb and COHb prepared samples. The resulting matrices of absorbtion coefficients for each of the 5 sheep were then normalised as described in the IL 482 protocol. This gave 5 estimates (one for each sheep) of normalised matrices of absorbtion coefficients.

Stage 3

The 5 normalised absorbtion coefficient matrices were averaged and inverted to obtain an inverse matrix. This was multiplied by the average absorbance of each Hb species preparation for each of the 5 sheep. This gave an estimate of the amount of each type of Hb in each prepared sample. Deduction was then made for the absorbances due to all residual Hb types in each sample. For example, in the OHb prepared sample of each of the 5 sheep, deduction was made for all the calculated residual COHb, RHb and MetHb. Similar deductions were also made for each of the prepared RHb and COHb samples. Methaemoglobin was considered to be 100 percent MetHb for all samples. After correction for these residuals, the resulting 5 matrices of absorbtion coefficients were normalised in the same way as

described in the IL 482 protocol, and then averaged. The average matrix provided the final matrix of normalised adsorbtion coefficients (see Table 2), and the 4 by 4 inverse matrix of absorbtion coefficients (see Table 3).

Calibration

In order to determine the accuracy of the absorbtion coefficients, aliquot's of known COHb and OHb concentrations were prepared and analysed on the IL 482. The blood was mixed by weight, such that various concentrations of COHb could be produced. These concentrations were approximately 20, 40, 60, 80 and 100 percent COHb. The calculated COHb in the mixtures was adjusted for the COHb and CO dissolved in the plasma (0.0185 mL/mL/bar at 37°C) with an IL tonometer, using 100 percent CO. The COHb for the 20, 40, 60 and 80 percent samples were calculated by initially calculating a "saturated" COHb (Sat-COHb), as described by the following equation:

$$\text{Sat-COHb} = \frac{\text{THb} * 1.39 + (1\text{-Hct}) * 1.85 * 713/760}{\text{THb} * 1.39 * 0.01}$$

The final mixed COHb percent was then calculated by the equation:

$$\% \text{ COHb} = \frac{\text{W(OHb)} * \text{percent(COHb)}O_2 + \text{W(COHb)} * \text{SatCOHb}}{W_T}$$

where
- THb is total haemoglobin (g/dL)
- Hct is the haematocrit of the blood sample (percent)
- % COHb is percent carboxyhaemoglobin
- OHb is oxyhaemoglobin

- W is the weight of the total (T) or the tonometered sample either OHb or COHb.

- %COHb is the residual COHb value measured in the OHb blood.

- 713 is the partial pressure in mmHg of humidified gas.

The various blood preparations were analysed at least 4 times using the IL 482 after it had been programmed with the sheep coefficients. The mean values were recorded, a calibration curve and an Altman-Bland plot were drawn and regression analysis was performed on the data.

Results

As Drawn Blood

The initial estimates of the sheep coefficients using the human values gave 5.91 percent COHb and the final iteration gave 6.8 percent. Using a 0.0 COHb percent as the starting value gave a first estimate of 0.38 percent and resulted in a final value of 0.8 percent. This contrasted with the result determined by gas chromatography of 0.12 percent for the same sheep. In the 5 sheep the overall final average COHb was 0.65 percent.

Matrices

The "normalised matrix" and "inverse matrix" for the absorbtion coefficients for adult merino sheep with blood Hb type A are shown in Tables 2 and 3, respectively.

Matrix Errors

In any single measurement, there is a machine error component which may be estimated by analysing repeated measurements from the same sample. It was determined, that when "as drawn" blood samples from 5 sheep were repeatedly measured, the standard deviations (calculated as the square root of the pooled variance of the results) were as follows: total Hb; 0.167 percent, RHb; 1.085 percent, OHb; 0.864 percent, COHb; 0.381 percent and MetHb; 0.330 percent. For total Hb the standard deviation was 0.167 grams (g) per 100 mls.

In addition, the variation in the estimate of each type of Hb attributed to the variance in the absorbtion coefficients between the 5 sheep was calculated. This was done

by taking the absorbtion coefficients from each of the 5 sheep to derive 5 independent inverse matrices. Each of these inverse matrices were used to seperately estimate the percent of each type of Hb. The variation between these estimates is given as the standard deviation. Table 4 shows the results as the standard deviation of the variation in the estimates of the coefficient matrices for each type of Hb in each type of blood specimen (ie. as drawn blood, RHb, OHb, COHb and MetHb).

Table 2. Normalised Matrix of Relative Absorption Coefficients [E], for Sheep with Type A Haemoglobin.

	RedHb	O₂HB	COHb	MetHb
Wavelength (nm)				
535.0	0.643326	0.932038	1.000000	0.481046
	0.001802†	0.000990	0.000000	0.005654
585.2	0.571495	0.587912	0.334575	0.250576
	0.001409†	0.002811	0.000683	0.003107
594.5	0.383760	0.135229	0.123406	0.224396
	0.000735†	0.000757	0.000466	0.002368
626.6	0.081233	0.010399	0.017006	0.263439
	0.000790†	0.000160	0.000445	0.003444

* Values are given to six significant figures, as this is required to program the IL482 CO-Oximeter.
† The second value is the standard deviation of the estimate from the five sheep.

Table 3. The Inverse of the Normalised Matrix of Relative Absorption Coefficients [E]$^{-1}$, for Sheep with Type A haemoglobin.

	Wavelength (nm)			
	535.0	585.2	594.5	626.6
RedHb	−0.365796	−0.370237	4.336411	−2.673852
	0.001739†	0.007705	0.036452	0.074881
O$_2$HB	−0.745808	4.039038	−5.172335	1.925873
	0.006400†	0.031636	0.039265	0.041323
COHb	1.921690	−3.616842	2.658608	−2.333492
	0.004959†	0.028065	0.036721	0.060216
MetHb	0.018191	0.188166	−1.304506	4.694874
	0.002330†	0.008054	0.027849	0.080275

* Values are given to six significant figures, as this is required to program the IL482 CO-Oximeter.
† The second value is the standard deviation of the estimate from the five sheep.

Table 4. The Standard Deviation (%) in Sample Measurement due to Variance in the Coefficient Matrices of Five Sheep.

	RedHb	O_2HB	COHb	MetHb
Blood preparation				
As drawn	0.223081	0.798086	0.746474	0.022782
RedHb	0.398925	0.260973	0.022226	0.402780
O_2Hb	0.230636	0.843375	0.791969	2.024129
COHb	0.158082	0.123637	0.192614	0.190245
MetHb	1.467292	0.841585	1.313516	1.739752

Figure 6. The absorption spectra of the reduced, oxy-, carboxy-, and met-haemoglobin. These absorption spectra are not exact because of the residual contamination, as explained in the text. Four wavelengths are used to measure the absorption, and from these measurements the absorption and inverse matrices are derived. The relative concentration of the four haemoglobin types can then be measured on an unknown blood sample. The four wavelengths are 535.0, 585.2, 594.5 and 626.6 nm, shown as dark lines across the absorption spectra.
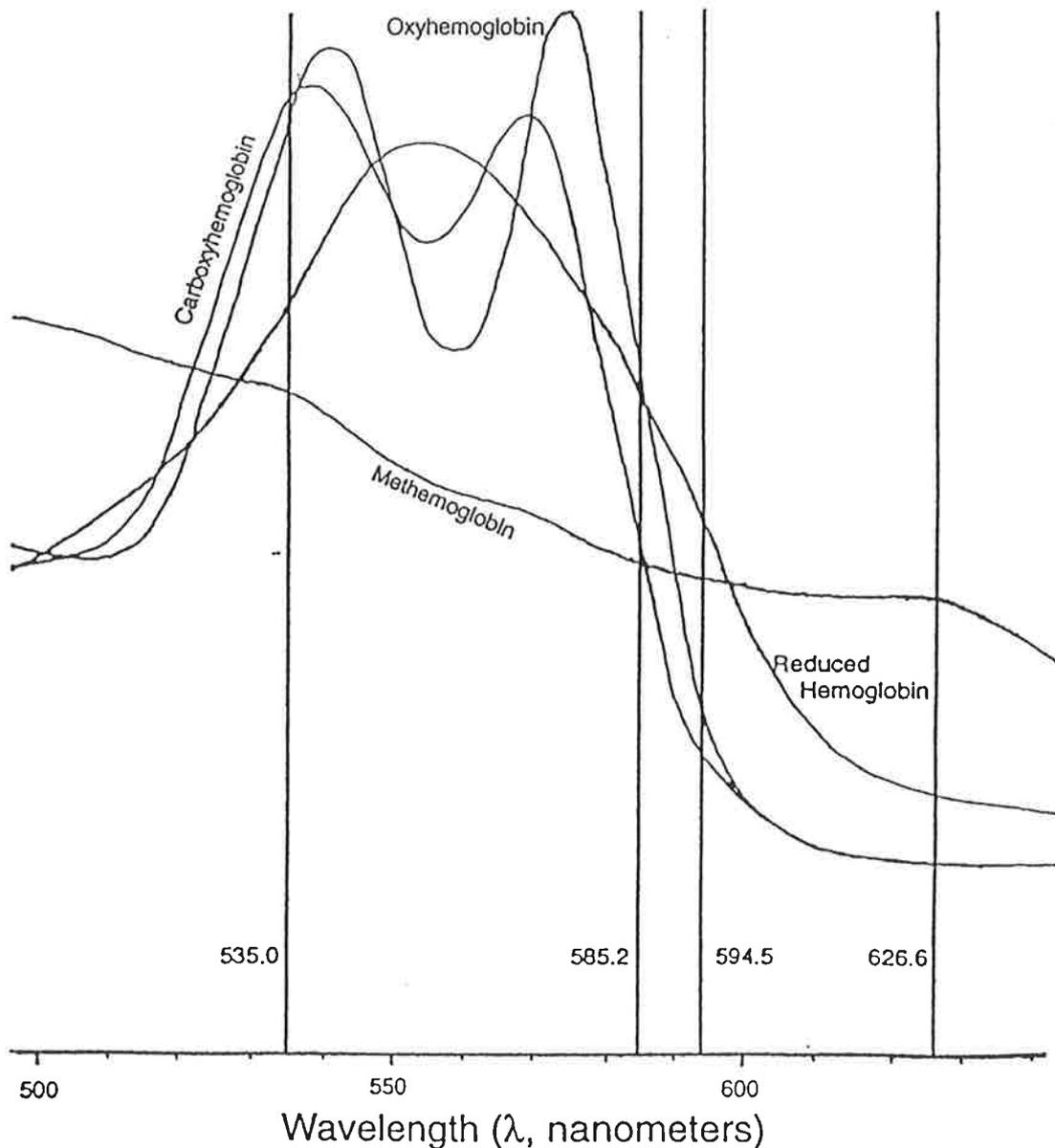
Calibration Curve

The natural plot of the calibration curve of the tonometered blood with CO to form various concentrations of COHb and the IL 482 COHb values is shown in Figure 7. A regression analysis performed on the coefficient calibration data demonstrated a correlation coefficient of 0.999 (r squared = 0.998). The regression equation was:

$$COHb_{(IL\ 482)} = 0.989\ (COHb_{calc}) + 1.091$$

where, $COHb_{calc}$ is the calculated carboxyhaemoglobin percent.

An Altman-Bland plot showed that all values were within 2.0 percent. The mean difference error was 0.416 percent with a standard deviation of 1.382 percent. Figure 8 is shown with the 95.0 percent error bound arises.

Figure 7. Calibration curve of the various COHb concentrations in sheep blood with type A haemoglobin, prepared by tonometery, and analysed on the IL 482 using the derived sheep coefficient matrix program. The correlation coefficient was 0.999 with a regression equation of COHb = 0.989 (calc. COHb) + 1.091.
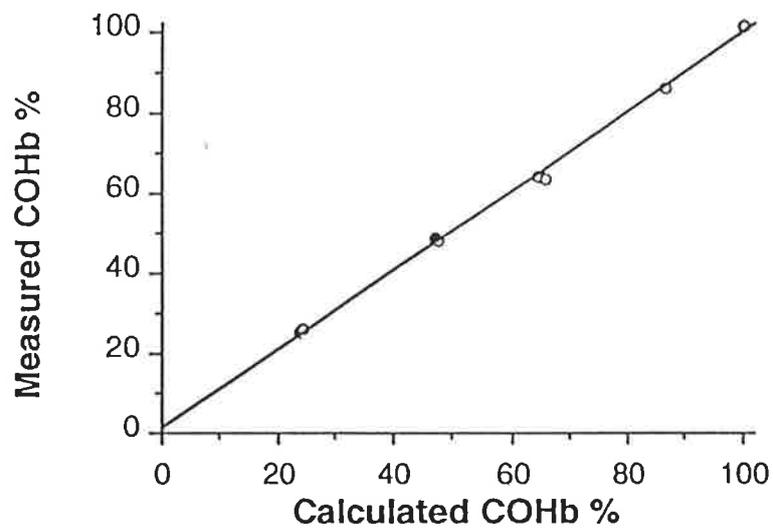
Figure 8. Altman-Bland plot of the difference between the measured and calculated

COHb percentage and various COHb saturations. All differences are

within 2 percent. The mean difference is 0.4 percent (-----). The 95

percent difference bounds are shown as double dashed lines (--- - ---).
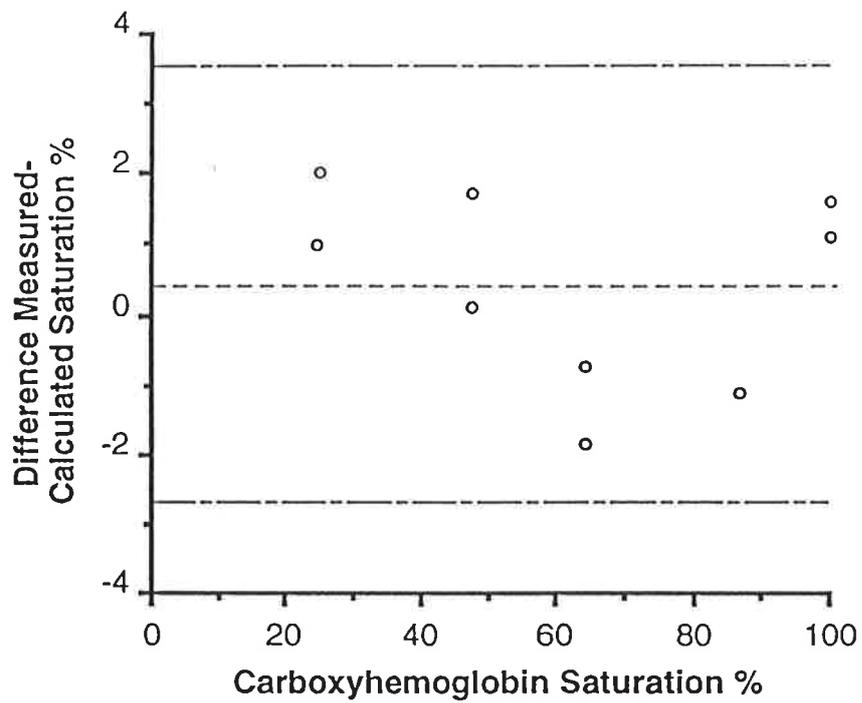
Discussion

A previous study by Bureau et al (1988), attempted to measure COHb in lambs, by calibrating an IL 282 with tonometered blood, mixed with various proportions of oxygen and CO. However, the COHb values were calculated using the human coefficient program. The value was then corrected using a "correction factor." It was found that the uncorrected COHb result from the IL 282, was uniformly off scale by an absolute value of 10.0 percent.

The IL 482 CO-Oximeter Protocol

The IL 482 protocol used to develop coefficients recommends methods to produce almost pure samples of OHb, COHb, RedHb and MetHb. However, in developing the coefficients, even though there is little contamination with other Hb types, allowance must be made for small amounts of residual contamination. Methaemoglobin contamination occurs in the OHb and the COHb samples as the methods of preparation do not reduce MetHb to the ferrous state. Carboxyhaemoglobin contamination occurs in an OHb sample prepared by tonometry and also in the RedHb samples produced by sodium dithionite. However, the sample prepared for MetHb can assumed to be 100 percent MetHb, as it involves a strong oxidization reaction, and therefore complete formation of MetHb.

The IL 482 protocol recommended for estimating these contaminants assumes that the amount of each residual type in the prepared samples is the same as that in the "as drawn" sample. The amount of this contamination can be initially approximated by the measurement of the sample using the human absorption by these residual Hb

types in the final determination of the absorbtion coefficients for each of the prepared samples of OHb, COHb, RedHb.

The major difficulty in the determination of absorption coefficients is the impurity of the OHb, COHb and RedHb samples. Therefore, to determine the absorbtion coefficients, allowance was made for the absorption by the residual amounts of the unwanted Hb types in each "near-pure" preparation. The problem then became that of estimating those residuals. The IL 482 protocol suggested that an initial estimate of each Hb type in the "as drawn" sample be made using human coefficients, and then that by using the inverse matrix of the absorbtion coefficients so derived, the initial coefficients can be used as the starting point for a further set of measurements to derive another new set of absorbtion coefficients. However, demonstrated for sheep blood, the value of the absorbtion coefficients that are derived depend on the initial estimate of each of the Hb types present. Repeated iterations either diverged, producing higher estimates of residual MetHb and COHb at the expense of the fractions of OHb and RedHb, or converged to an end point where estimated residuals were 0.0 (no deduction being made for negative amounts of residuals). In those cases where repeated iterations divergently increased the estimate, by arbitrarily reducing the first estimate of the amount of the RedHb types, a value was reached where convergence occurred instead. However, convergence only occurred when the estimated residuals were almost 0.0. The coefficients derived using these estimates were identical to those derived with an initial assumption that there were no residuals of any type contaminating the prepared samples.

In contrast, attempts to determine the coefficient matrix strictly following the IL 482 protocol, yielded high values for COHb in the as drawn blood sample. The IL 482 technique using human coefficients resulted in a residual COHb of 5.9 percent. Even with repeated iterations, this value only changed to 6.8 percent when the derived sheep coefficients were used. On the other hand, by assuming an initial 0.0 residual, a final value of 0.8 percent for COHb was produced. No value was available from the literature and indeed, the COHb level in any animal could be expected to vary depending on where it was kept (ie. whether there was urban or rural exposure). Therefore, an independent determination using a direct CO measurement is essential.

The amount of contaminating residual MetHb and COHb in the near-pure prepared samples of OHb, COHb and RedHb is expected to be only a few percent. It therefore seemed a reasonable starting point to assume no residual contaminating Hb types in the prepared samples, and to use the coefficient matrix so derived to estimate the minor contaminations in the "as drawn" sample. The error in estimating the residuals in this way is less likely to be more than a few percent and indeed the maximum measured offset of the calibration curve was 2.0 percent. In the first sample, independent measurement of the fraction of COHb in the as drawn sample by gas chromatography was found to be 0.12 percent. Thus the initial estimate of COHb in the "as drawn" blood from the sheep, assuming no residuals, which was - 0.38 percent, is far closer to the measured value than the human coefficient value of 5.9 percent. It is possible that the figure may be negative because the initial assumption of no residuals is unlikely to be true, but the error should be small. The final coefficients applied to the absorbances from the blood samples gave an estimate of 0.65 percent, which when the variation of the coefficients between

different sheep is taken into account, is within one standard deviation (0.57 percent) of the value determined on the gas chromatograph. The value for COHb concentration in sheep blood determined by Gas Chromatography was 0.12 percent.

The coefficients calculated from each of the 5 sheep, although similar, were not identical. Analysis of the variance in the measures parameters that existed between the 5 sheep matrices demonstrated that the standard deviation of these results of analysing a blood sample were 0.8 percent or less, for the measured parameters RedHb, OHb and COHb, and less than 2.0 percent for the MetHb (Table 3).

Analysis of the accuracy of the coefficient matrix program showed that values within 2.0 percent over the whole range were produced when sheep blood was analysed by the IL 482. This was demonstrated by the calibration curve regression value of 0.999 (Figure 7), and the Altman-Bland plot (Figure 8), which showed values within 2.0 percent over the whole range were produced when sheep blood was analysed by the IL 482.

CHAPTER 4

Brain Oxygen Metabolism and Cerebrovascular Response of the Conscious Unrestrained Sheep to an Acute Exposure to Carbon Monoxide

Introduction

The CO Conflict

The prevalent explanations of CO toxicity are based on a combination of CO binding to Hb, such that there is a fall in the blood oxygen content, and the reduction in the dissociation of that OHb which is formed in the presence of CO and COHb. The latter is said to account for the increased toxicity of CO compared to that of an equivalent degree of anaemia (See Chapter 1).

Although the relationship between inspired CO concentration and the level of COHb in blood is well understood (Selvakumar et al, 1993), the temporal relationship between COHb level, the oxygen status of critical organs such as the brain and heart, and the progression of acute symptoms (headache, palpitations, nausea, dizziness, confusion, syncope and coma) is uncertain. It has already been described that titration of oxygen therapy against COHb levels is often unsuccessful in preventing sequelae in patients. This is further complicated by reports of potential physiological and pathophysiological effects of CO which implicate mechanisms of acute and chronic toxicity unrelated to hypoxia (See Chapter 1).

This chapter describes the experiment designed to examine the relationship between COHb concentrations and critical organ status, with particular reference to

the brain. The <u>hypothesis tested</u> is "<u>CO can inhibit brain function without significantly altering oxygen availability to the brain</u>." The effects of a progressive CO exposure on the conscious sheep was studied.

The Experimental Model

Reviewing the literature on cerebrovascular responses to acute exposure to CO, is shown it to be minimal. Most of the experimental findings were from animals exposed to low concentrations of CO, furthermore, these studies used anaesthetised animals. A major strength of the experimental model used in this thesis, is that the model utilises a large conscious animal. This avoids various effects of restraint, anaesthesia, analgesia and muscle relaxants on the animals normal physiological behaviour (cerebrovascular physiology) and well being. In this context, there is obvious potential for an anaesthetic agent to effect the increase in CBF seen in response to CO and hence the preservation of oxygen delivery to the brain (Ludbrook et al, 1992). A study in anaesthetised animals could then be subject to considerable bias by either an increased or decreased estimation of the CBF response. Furthermore, the method used in this thesis simulates an acute exposure to a moderate CO concentration.

Method and Materials

The experiments were performed on eight female Type A haemoglobin Merino sheep in accordance with the guidelines and with the approval of the Animal Ethics Committee of the University of Adelaide and the Institute of Medical and Veterinary Science, Adelaide, South Australia. The animals weighed between 40 and 45 kilograms.

Surgical preparation

The surgical preparation of the sheep was performed in 2 phases, as described in Chapter 2 of this thesis. The sheep were, anaesthetised, ventilated and prepared for the experimental procedure (See Chapter 2).

Sheep Training

To familiarise the animals with the experimental conditions and hence to optimise CBF measurements (Upton et al, 1994), the sheep were placed in a comfortable sling (used during the subsequent exposure to CO) on 2 separate days prior to the actual CO trial, and for at least 3 hours. The sling was designed such that the sheep could comfortably stand with restraint in the metabolic crate, however, it still remained supported by the sling. This ensured the animals would not be injured in the event of syncope or coma, as a consequence of the CO exposure.

Experimental Procedure

The sheep were fasted for 24 hours before the exposure to CO. On the day of the CO exposure, each animal was again restrained in the sling. The animals,

monitoring equipment and analytical machines for the experimental procedure were prepared (See Chapter 2).

All animals were monitored until measured pararmeters stabilised, at which time a 15 minute baseline phase was commenced (less than than 10 percent variation).

Following this baseline phase, the animals were exposed to 1.0 percent CO in air, at a flow rate of 2.5 litres per minute.

The CO exposure was continued for each animal until the recorded heart rate decreased by more than 10 percent from the maximum rate measured (See Chapter 2). The administration of CO was then ceased and the animal was given 100 percent oxygen to breathe for approximately 2 hours.

For 15 minutes before (baseline phase), during (test phase) and for 15 minutes after the CO exposure (recovery phase), the following measurements were made:

continuous measurement

- CBF

- mean arterial blood pressure

- heart rate

measurement at 5 minute intervals

- the overall behaviour of the animals was assessed (with particular reference to conscious level, response to painful stimuli (ear pinch) and head position),

- arterial and sagittal sinus blood samples were taken to determine the:

- blood gas tension,

- co-oximetry, and

- lactate concentration.

Two days after the CO exposure, each animal was anaesthetised and ventilated so that the sagittal sinus doppler probe could be calibrated (See Chapter 2).

Another six sheep were prepared as described above and similarly monitored, but without the addition of CO, these animals have been reported separately (Upton et al, 1994).

Brain Oxygen Delivery

The delivery of oxygen to the brain was calculated as the product of the measured oxygen content in carotid artery blood and CBF, and recorded as mls per minute.

Brain Oxygen Consumption

The consumption of oxygen by the brain was calculated as the product of the cerebral arteriovenous (carotid artery - sagittal sinus oxygen content) oxygen difference and CBF, and recorded as mls per minute.

Brain Lactate Production

The production of lactate by the brain was calculated as the product of the CBF and the arteriovenous (carotid artery - sagittal sinus) lactate concentration difference, and recorded as umol per minute.

Data analysis and interpretation

The arithmetic mean of baseline phase data for each sheep was calculated and assigned a value of 100 percent. All subsequent data were then recorded both as natural data and as a percent of the baseline value.

Analyses of Variance and Student's t-tests were used to test group data. A significance level of 5 percent was chosen and the Bonferroni correction was used where appropriate (Wallenstein et al, 1980).

Results

During the training periods, no changes were observed in the animal's level of arousal. In addition, no significant change was seen in any biochemical or physiological parameter, or in arousal level in eight sheep in the baseline testing phase (prior to the CO exposure). This was in agreement with Upton et al (1994) who observed for forty minutes similar results in six sheep not exposed to CO.

Behavioural Effect

The exposure of the sheep to CO caused behavioural changes in all the animals. These were not measured objectively, but the changes varied from agitation to sedation and narcosis.

The exposure to CO caused a progressive stupor (a state of suspended or deadened sensibility) and narcosis (a state of sleep or drowsiness) in all animals. This was manifest as initial eye closure, head drop, then progressive loss of consciousness with no response to pain and profound rhinorrhoea. The behavioural changes were noted soon after the administration of the CO was introduced. The mean time of onset of overt stupor was 14.3 (SD = 4.8)[range: 10 to 23] minutes after the administration of the CO. At this time, the oxygen delivery to / and oxygen uptake by the brain were not significantly different from baseline phase data and the carotid arterial COHb levels were less than 40 percent.

Cardiovascular Effect

The group mean percent carotid arterial blood COHb levels, heart rate and mean arterial blood pressure are displayed against time during the CO exposure in Figure 9. There is significant increase in COHb concentration at all times ($F = 148$; $p < 0.0001$) and heart rate ($F = 3.0$; $p = 0.01$), but there was no significant change in mean arterial blood pressure ($F = 0.1$: $p = 0.99$).

PCO2 Effect

The group mean percentage carotid arterial blood COHb levels, carotid artery P_aCO_2 and sagittal sinus P_vCO_2 data are displayed against time during the CO exposure in Figure 10. While the P_aCO_2 data do not change significantly ($F = 1.7$; $p = 0.1$), there is a significant decrease in the mean P_vCO_2 data ($F = 7.036$; $p < 0.001$). Given that the CBF increased and the available Hb decreased progressively, and the dissolved volumes of carbon dioxide in carotid arterial and sagittal venous blood were not measured, these data do not enable a calculation of carbon dioxide flux for the brain.

PO₂ Effect

The group mean percent carotid arterial blood COHb levels, carotid artery P_aO_2 and sagittal sinus P_vO_2 data are displayed against time during the CO exposure in Figure 11. While the P_aO_2 data increase significantly ($F = 3.95$; $p < 0.001$), there is a significant decrease in the mean P_vO_2 data ($F = 11.55$; $p < 0.001$). The estimates of the oxygen dissociating from Hb in these sheep for carotid arterial blood COHb concentrations of 0, 20, 40, 60 percent are shown in Table 5. While the uncorrected

Figure 9. A graph of the mean (SD) percentage carotid arterial blood COHb levels, heart rate and MABP against time in 8 sheep exposed to 1.0 percent CO.
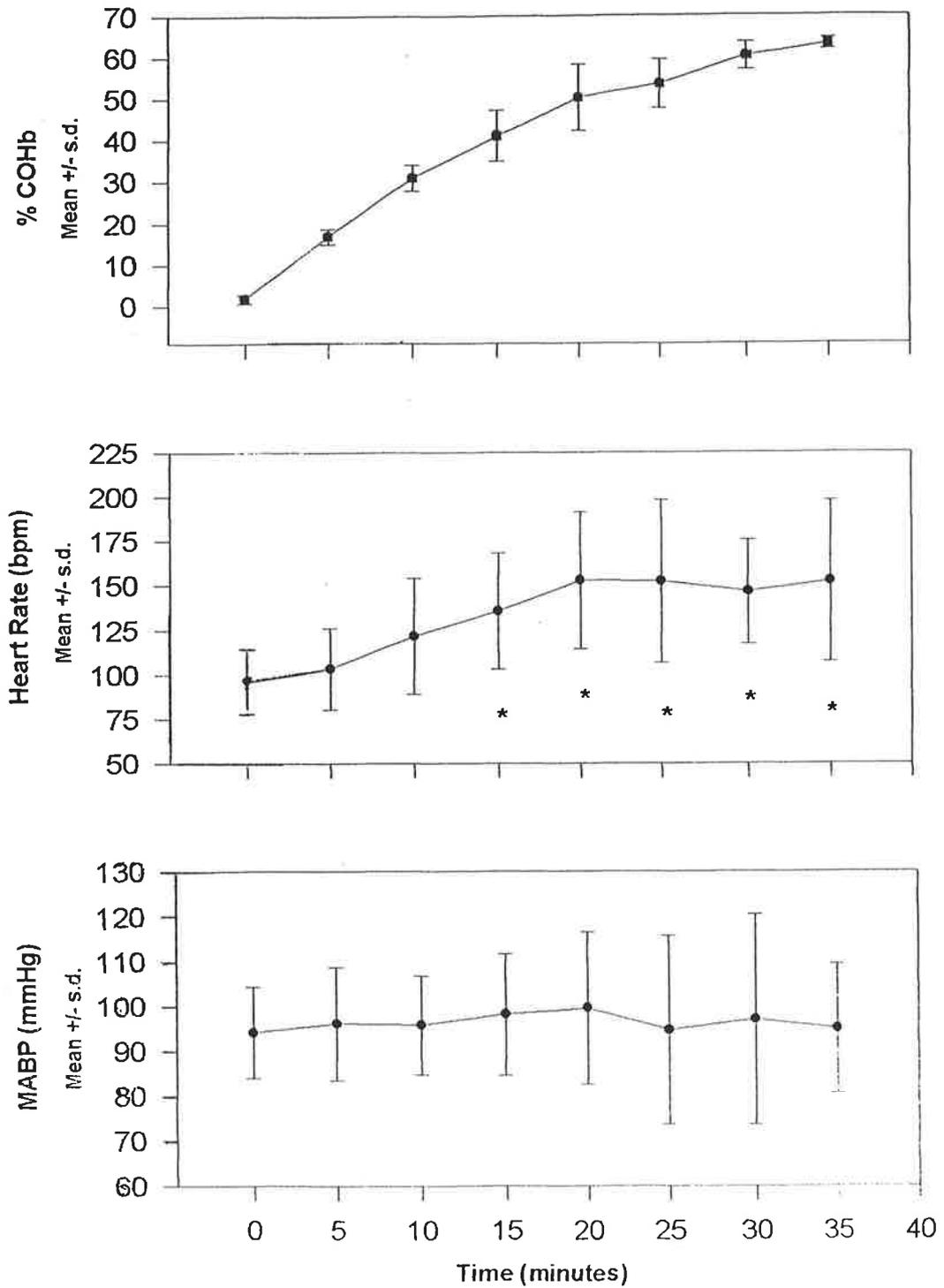
Figure 10. A graph of the group mean (SD) percentage carotid arterial blood COHb levels, carotid artery P_aCO_2 and sagittal sinus P_vCO_2 against time in 8 sheep exposed to 1.0 percent CO.
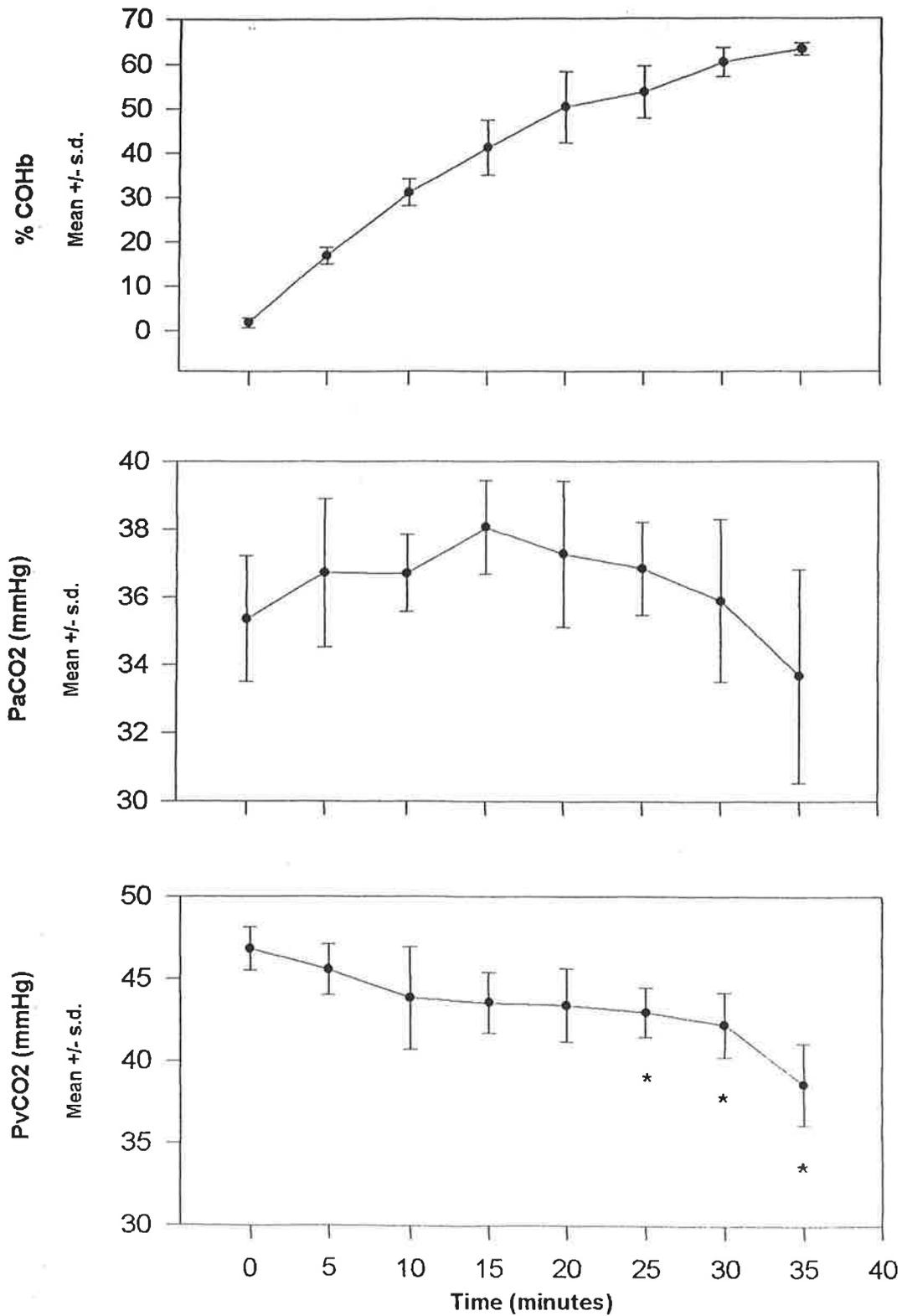
Figure 11. A graph of the group mean (SD) percentage carotid arterial blood COHb levels, carotid artery P_aO_2 and sagittal sinus P_vO_2 against time in 8 sheep exposed to 1.0 percent CO.
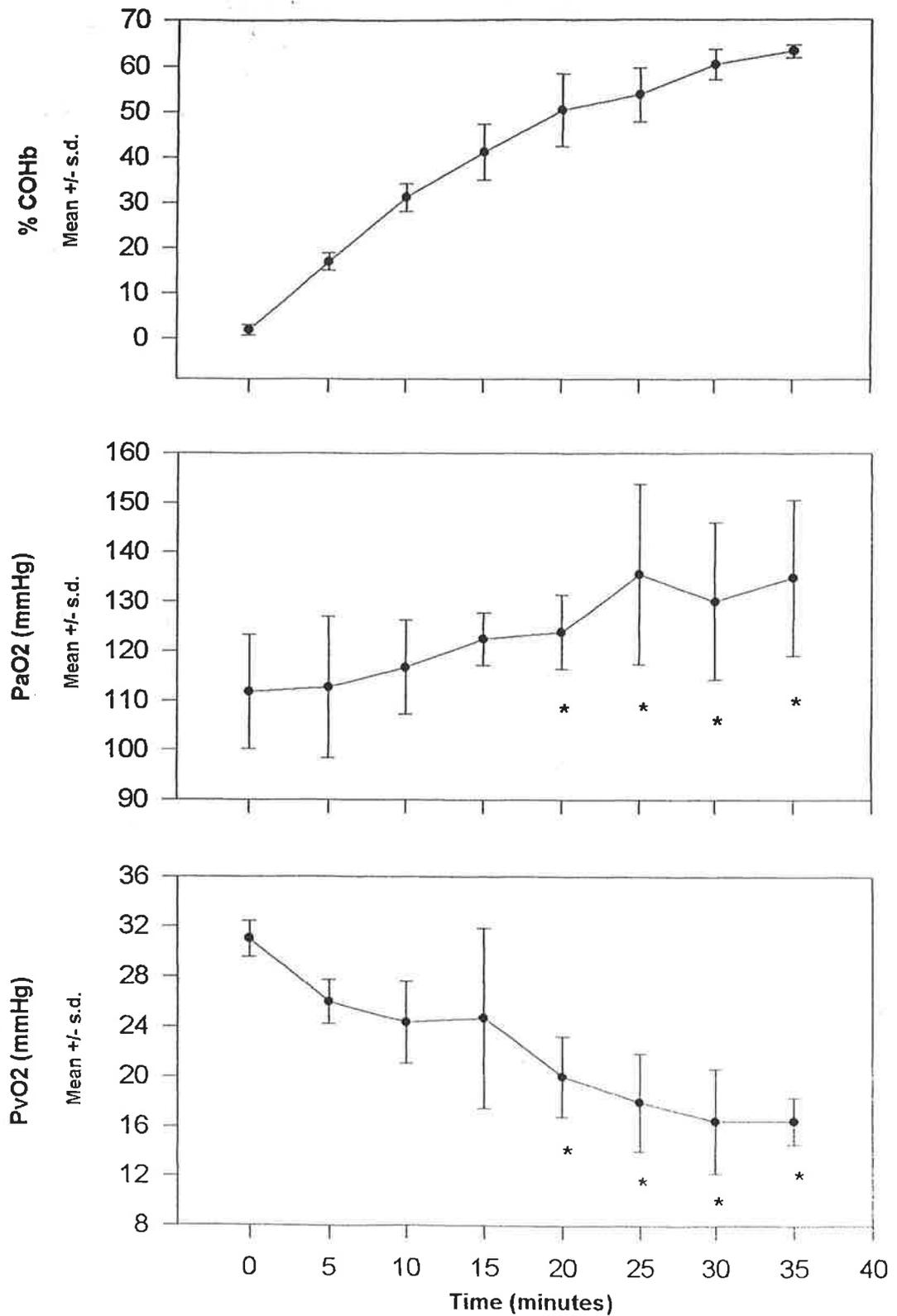
Table 5. Group mean (sd) estimates of oxygen (mls of oxygen/100 mls of carotid

arterial blood), dissociating from Hb in the brain capillaries of sheep

exposed to 1.0 percent CO for 35 minutes, at different carotid arterial

blood COHb concentrations. Estimates are derived from the carotid

arterial and sagittal sinus venous blood oxygen tensions and the OHb

dissociation curves of Roughton and Darling (1944) and then corrected for

relative CBF.

Carotid Artery COHb (%)	Uncorrected oxygen volume (mls/100 mls)	Corrected oxygen volume (mls/100mls)
0	7.1 (0.2)	7.1 (0.2)
20	5.8 (1.1)	7.6 (1.0)
40	4.0 (0.9) *	6.2 (1.7)
60	2.9 (0.6) *	5.1 (1.4)

* :   P < 0.05 (compared with 0% COHb baseline data).

available oxygen is significantly lower at a COHb of 40 and 60 percent (p < 0.05), the difference in the corrected oxygen availabilities at these COHb levels is not significant. The P_aO_2 values beyond 110 mmHg are outside the calibrated "linear" range of the bloodgas machine, and are hence not reliable (ie. are simply considered to be greater that 110 mmHg).

Observation's of Note

The group mean percent carotid arterial blood COHb levels, CBF (as a percent of that measured during the pre-CO baseline period), oxygen delivery to the brain (as a percent of that measured during the pre-CO baseline period), and oxygen consumption by the brain (as a percent of that measured during the pre-CO baseline period), are displayed against time during the CO exposure in Figure 12. The following changes in blood flow, and both oxygen tensions and fluxes were observed or calculated:

a.   the CBF increased significantly (F = 2.4; p = 0.02),

b.   the carotid artery P_aO_2 level did not fall below 100 mmHg in any sheep,

c.   the sagittal sinus P_vO_2 levels fell to mean levels of 26 (SD = 2), 25 (SD = 5) and 15 (SD = 4) mmHg at carotid arterial blood COHb concentrations of 20, 40 and 60 percent respectively (F = 11.55, p , 0.001),

d.   the oxygen delivery to the brain remained near to 100 percent of the pre-CO baseline mean value for all carotid arterial blood COHb levels of between 0 and 60 percent inclusive, and overall did not undergo any significant change (F = 1.16, p = 0.33),

e.   the oxygen consumed by the brain decreased significantly (F = 3.36, p = 0.003).

Lactate Effect

The carotid arterial COHb levels and the carotid arterial and sagittal sinus venous blood lactate concentrations for individual animals are shown in Figure 13. This display was chosen because one of the sheep showed an increase in both the carotid arterial and sagittal sinus venous blood lactate concentrations. This was not seen in any of the other sheep. Neither this nor any other sheep showed any evidence of a metabolic acidosis (See Figure 10). The carotid arterial and sagittal sinus venous pH values similarly did not change (See Figure 14). The net lactate production in the brains of these sheep, for a range of carotid arterial blood COHb concentrations, are displayed in Table 6. Only 5 of the 56 lactate production values calculated for animals during the CO exposure, were outside the baseline range and the most extreme of these was a value of +6.77 umol per minute. There is no consistent brain lactate anabolism or catabolism in any individual sheep or at any specific COHb concentration and the sagittal sinus - carotid artery blood lactate concentration differences (a range of 0.001 to 0.09 mmol per litre) are within the measurement error or the original lactate concentrations (a range of 0.53 to 1.223).

Figure 12. A graph of the group mean (SD) percentage carotid arterial blood COHb levels, CBF (as a percentage of that measured during the pre-CO baseline), oxygen delivery to the brain (as a percentage of that measured during the pre-CO baseline) and oxygen consumption by the brain (as a percentage of that measured during the pre-CO baseline) against time in 8 sheep exposed to 1.0 percent CO.
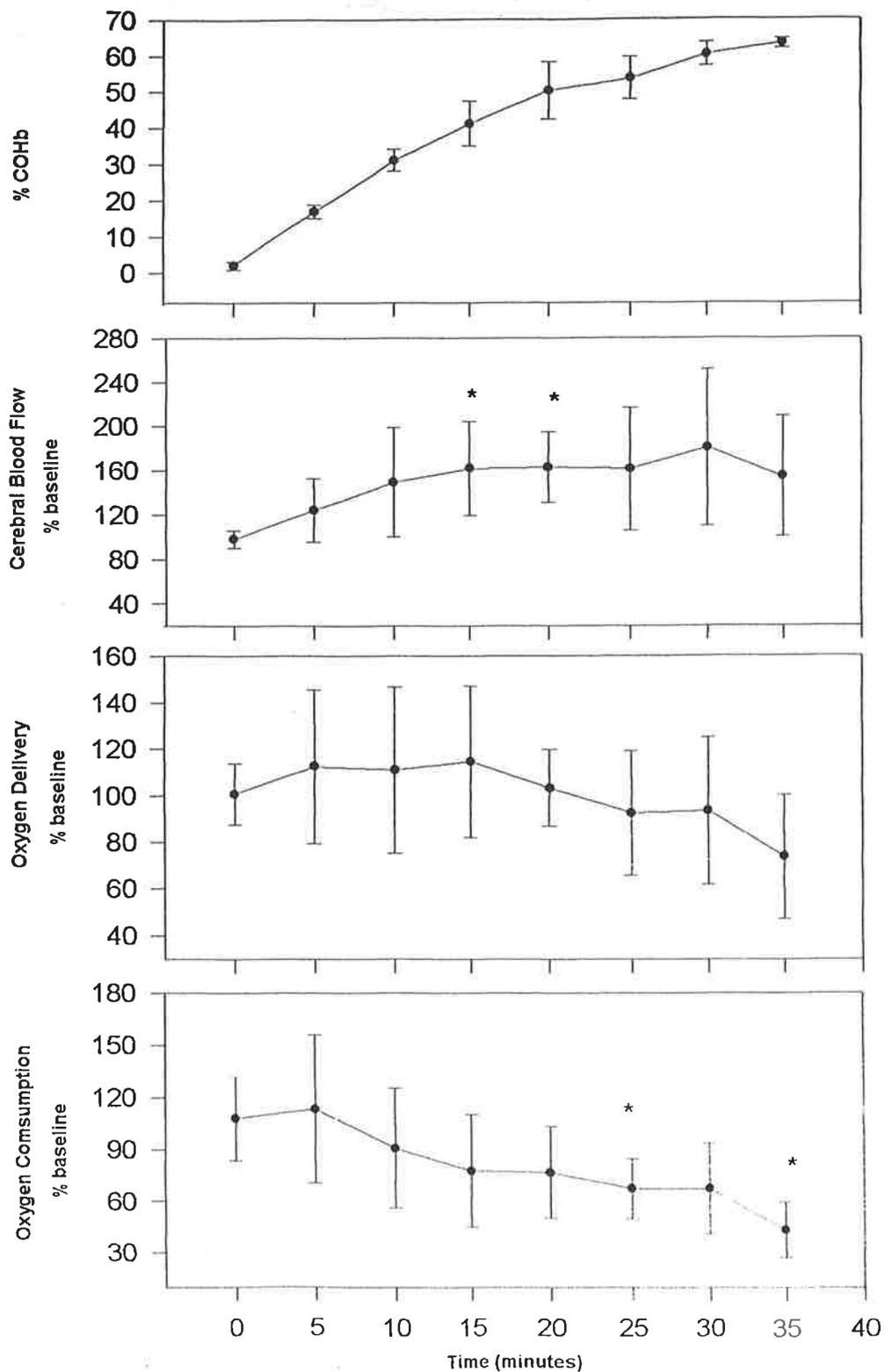
Figure 13. A graph of the carotid arterial blood COHb levels, and the carotid arterial and sagittal sinus venous blood lactate concentrations for individual animals against time in 8 sheep exposed to 1.0 percent CO.
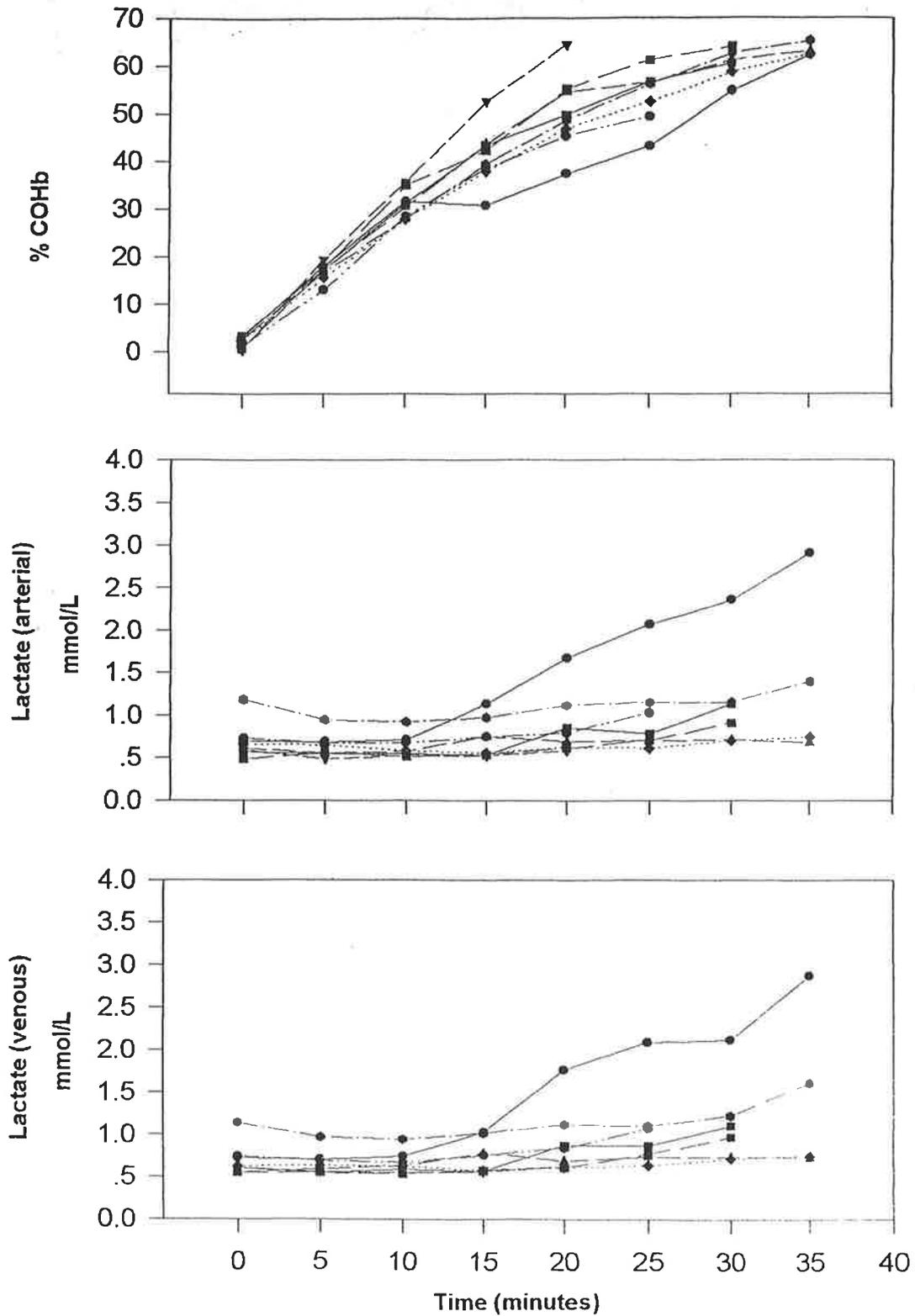
pH Effect

The group mean percent carotid arterial blood COHb levels, carotid arterial pH and sagittal sinus venous pH data are displayed against time during the CO exposure in Figure 14. There were no significant changes in either the carotid arterial (F = 0.26, p = 0.98) or the sagittal sinus venous (F = 1.04, p = 0.42) pH data.

Figure 14. A graph of the group mean (SD) percentage carotid arterial blood COHb levels, and the carotid artery and sagittal sinus pH against time in 8 sheep exposed to 1.0 percent CO.
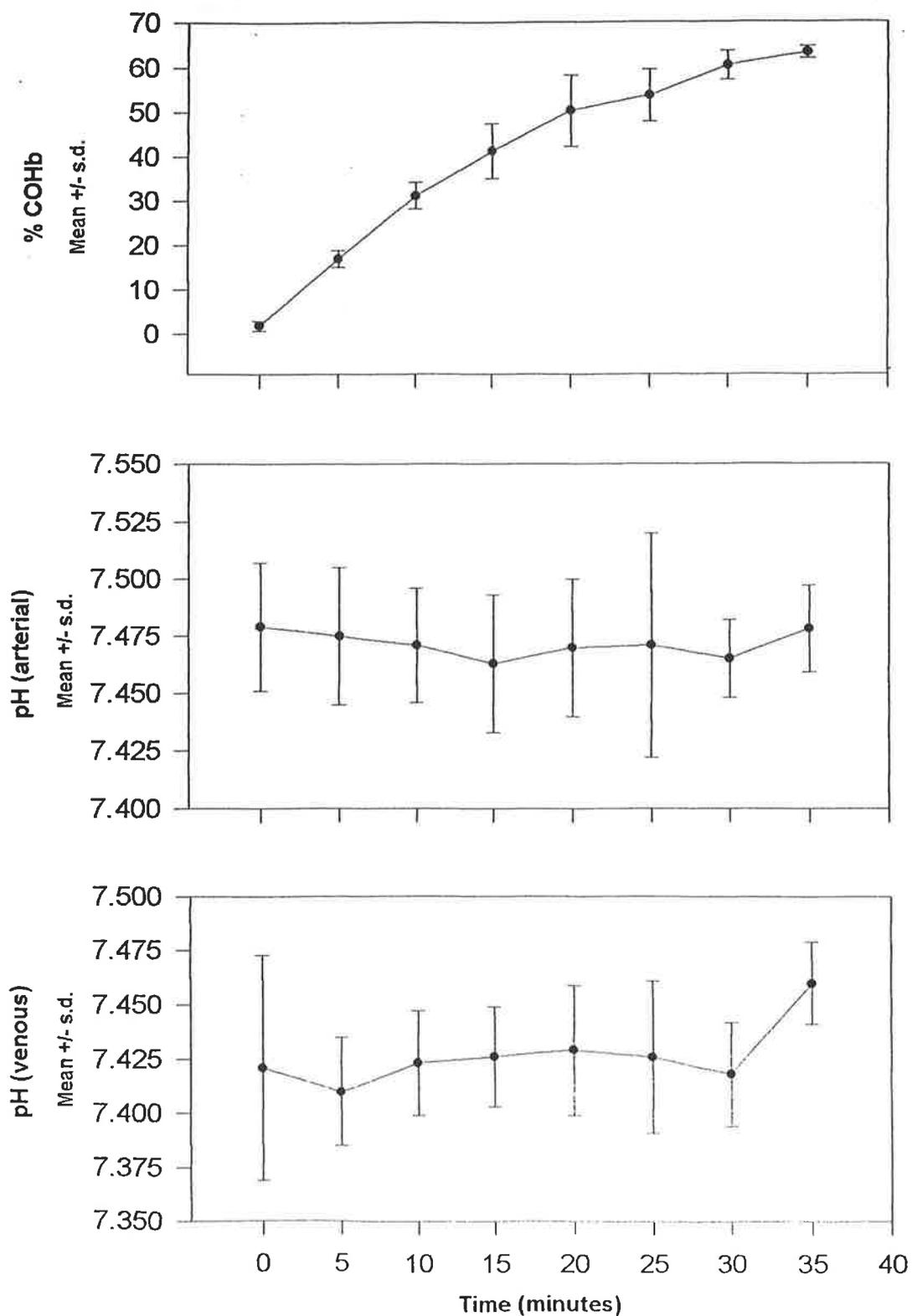
Table 6.   The range and median values of brain lactate production (umol/min) in 8 sheep exposed to 1.0 percent CO for 35 minutes, at different carotid arterial blood COHb concentrations. Negative values indicate the carotid arterial blood concentrations exceeded sagittal sinus venous blood values.

Carotid Artery COHb (%)	Upper and lower limits of the range of brain lactate production (μmol/min)		Median brain lactate production (μmol/min)
0	-1.41	+3.43	+0.9
20	-2.90	+2.67	-1.05
40	-0.50	+6.77	+2.37
60	-2.74	+3.89	+1.44

Discussion

This study provides insight into the temperal relationship between CO toxicity and the level of COHb in the blood, in an exposure which was designed to simulate human CO poisonings rather than to achieve a steady state of COHb.

The toxicity of CO in these animals can neither be attributed to a reduction in oxygen delivery to the brain nor to hypotensive hypoperfusion of this organ. Most importantly, it has been demonstrated that in conscious sheep, physiological compensation was unaffected by CO such that CBF increased to maintain oxygen delivery to the brain. This observation is consistent with the increases in CBF seen in man and other species exposed to CO (MacMillan, 1975; Paulson et al, 1973; Traystman et al, 1978). Ludbrook et al (1992) observed in rabbits regulation of CBF, during both CO hypoxia and hypoxic hypoxia.

In addition to this, at COHb levels of 40 percent and less, there is likely to be negligible change in oxygen availability to tissues as the P_aO_2 will be normal and the overall extent of OHb dissociation is thought to be effectively unchanged (Roughton and Darling, 1944). This study supports this contention because there was no evidence of brain hypoxia in the sheep during this level of CO exposure, as otherwise would have been indicated by a compensated or uncompensated metabolic acidosis in the brain, and or by a net increase in the production of lactate by the brains of these sheep.

While these homeostatic mechanisms are able to maintain oxygen delivery to the brain, and presumably other organs, it can be postulated that the heart has a unique

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role in the evolution of CO toxicity. It is clear that to produce these increases in organ blood flow, the workload of the heart must increase. This was indicated by the increase in the heart rate, observed in this study. However, increased oxygen demand in the heart coupled with reduced oxygen content in arterial blood will eventually cause a situation in which the myocardial blood flow can no longer meet the demands of the heart, ending in failure of the myocardium. This theory is supported in the current study by the 2 sheep for which CO administered had to be stopped prematurely due to the onset of sudden severe hypotension.

The combined effect of sudden hypotension and reduced arterial blood oxygen content in the brain cannot be determined from these studies. However, this has been demonstrated by Ginsberg (1985). By the nature of this study, it was not possible to identify if the relatively benign physiological insult experienced by the sheep in the current study produced delayed neurological sequelae.

The arguments above are not denying the potential for CO to cause hypoxia in the brain - such an effect has been demonstrated in humans (Paulson et al, 1973) and in experimental animals (Gutierrez et al, 1985; Koehler et al, 1982). Indeed, CO will cause significantly more hypoxia than inert asphyxiants because it does bind to Hb and can cause a left shift in the dissociation of OHb (Roughton and Darling, 1944). However, if human adult Hb is assumed to be a good model of Type A sheep Hb behaviour (Blunt and Huisman, 1975) and basing calculations on the actual sheep CBF and P_vO_2 data from this study, the demonstrated effect of different COHb concentrations on OHb dissociation (Roughton and Darling, 1944) would not result in any reduction in oxygen availability to the sheep brain until COHb levels reached 60 percent. Consequently, the conclusion of this study is that the reduction in blood

oxygen content and the left shift of the OHb dissociation curve induced by an exposure to CO are compensated for by normal homeostatic mechanisms, such that the sheep brain does not become hypoxic until relatively high levels of COHb are reached.

In support of this observation, a recent study of anaesthetised dogs subjected to either a mild or severe dose of inspired CO - although myocardial function in the dogs was adversely affected, the available and extracted volume of myocardial oxygen was maintained throughout the exposure by an increase in coronary blood flow. In this study by Zhu and Weiss (1994), the dogs remained normotensive and the brain was not studied.

This study also suggests that although hypoxia was not demonstrable, there are other toxicological effects of CO on the brain. These are indicated by the progressive decrease in brain oxygen consumption seen during the CO exposure and the behavioural changes observed in the animals. There are a number of possible explanations for these observations.

Firstly, the vasodilatory properties of CO (Lin and McGrath, 1988, Vedernikov et al, 1989) and the consequent increase in CBF may produce cerebral oedema and increased intra-cranial pressure (ICP). Raised intra cranial pressure can cause severe headache and loss of consciousness similar to that seen in low levels of CO exposure (Ginsberg, 1985). This may account for behavioural changes seen in the sheep, but is unlikely to account for the reduced oxygen consumption observed in the presence of both uncompromised blood flow to the brain and a consequent lack of hypoxia.

Secondly, CO may metabolically poison the brain resulting in both reduced oxygen consumption and behavioural changes. This conclusion is consistent with the profound adverse effect of CO on rats under conditions of "hyperbaric oxygen" where enough oxygen is in solution in plasma to meet tissue needs and no OHb dissociation is needed (Haldane, 1927). It is also consistent with the extraordinary resistance of neural function to hypoxia demonstrated in the rabbit brain (Ludbrook et al, 1992) and hippocampal neurons (Doolette, 1991) - especially in contrast to the great sensitivity of the rabbit brain when exposed to CO (Ludbrook et al, 1992).

In addition, all sheep were seen to be sedated at a time when brain oxygen consumption was not significantly lower than baseline and COHb concentrations in arterial blood were less than 40 percent. The subsequent decrease in oxygen consumption could have resulted from either a reduction in the availability of oxygen bound to the Hb (Roughton & Darling, 1944) or a decreased demand for oxygen by a metabolically poisoned brain.

The falling levels of sagittal sinus venous blood P_vCO_2 (in the context of stable carotid arterial blood P_aCO_2 levels) and brain oxygen consumption in sheep narcotised by CO is indicative of a metabolically poisoned brain. However, this derangement of metabolism is unlikely to be due to reduced oxygen availabilty because of the following:

a. there was no evidence of a metabolic acidosis in the brain or any respiratory compensation to an acidosis (in contrast to that seen in the hypoxic pilot study sheep) and;

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b.   there was no net increase in the production of lactate by the brain of these

sheep during the CO exposure (in contrast to the increase from less than 3.5

umol per minute, at rest, to more than 30 umol per minute seen a hypoxic (pilot

study) sheep in response to mild hypoxia).

These data are inconsistent with a primary hypoxic explanation of CO toxicity.

Tissue hypoxia could only have arisen in these sheep, in the presence of normal

oxygen delivery and P_aO_2 values, if there was a profound left shift in the OHb

dissociation curve. Phenomena that can cause a shift in OHb dissociation include

CO (Barcroft, 1928; Hill, 1921; Peters & Van Slyke, 1932; Roughton & Darling,

1944; Stadie & Martin, 1925), decreasing temperature and increasing pH (a

decrease in pH is more likely in CO poisoning)(Myers & Britten, 1989).

Among the reports of the effect of CO on OHb dissociation, that of Roughton and

Darling (1944) is distinguished by experimental data rather than series of calculated

dissociation curves (based on relative affinities). These data are however obtained

by non-equilibrium studies and in human blood from a single cigarette smoking

donor (although he did stop smoking 12 hours prior to blood extraction, baseline

COHb levels were between 4 and 5.5 percent, such that an OHb dissociation curve

for 0.0 percent COHb had to be calculated). A left shift of OHb dissociation in the

presence of CO was first calculated by these researchers (using relative affinities)

and then points on the curve were confirmed by actual experiment - concordance

between calculation and observation was excellent. This study has been widely

misquoted, however in the context of the extent of this shift and especially at COHb

levels of 40 percent and less; the authors themselves noted that any impairment of

oxygen uptake under conditions of normoxia (the P_aO_2 levels measured in the

sheep were unchanged from baseline - 100 to 120 mmHg - during the CO exposure) would be unlikely to be the result of a shift in OHb dissociation until the COHb levels exceeded 40 percent. This observation arises in part from the finding that at low oxygen tensions (less than 30 mmHg), the dissociation of OHb is very much the same for differing COHb concentrations between 0 and 40 percent - that is, at this extreme of oxygen tension and at these levels of COHb , there is no effective left shift of the OHb dissociation curve. It is noteworthy then that sedation was seen in these sheep at COHb levels much less than 40 percent.

It is reasonable to conclude that a CO (COHb)-induced left shift in the dissociation curve of OHb can not, in isolation, explain the suppression of brain function seen in these sheep. This conclusion is consistent with the profound effect of CO on rats under conditions of hyperbaric oxygen where enough oxygen is in solution in plasma to meet tissue needs and no OHb dissociation is needed (Haldane, 1927). It is also consistent with the extraordinary resistance of neural function to hypoxia shown in isolated rat hippocampal neurons (Doolette, 1993) and rabbit brain (Ludbrook et al, 1992).

The arguments above are not denying the potential for CO to cause hypoxia - such an effect has been demonstrated in humans (Paulson et al, 1973) and in experimental animals (Gutierrez et al, 1985; Koehler et al, 1982). Indeed, CO will cause significantly more hypoxia than inert asphyxiants because it does bind to Hb and does cause a left shift in the dissociation of OHb (Roughton & Darling, 1944). Instead, the conclusions of this study are that such hypoxic effects are unlikely until COHb levels are relatively high (probably in excess of 40 percent) [Roughton &

Darling, 1944] and that there is a profound tissue CO toxicity that is not due to and is not contingent upon hypoxia.

This study rejects a primary hypoxic hypothesis of CO toxicity and suggests that treatment "designed" to relieve hypoxia is likely to fail - not surprisingly, it has already been noted that titration of oxygen therapy against COHb levels is often unsuccessful in preventing sequelae (Myers et al, 1985; Sluijter, 1967).

The nature of CO toxicity is not established by these data (or to date by data from any other study). However, in addition to not supporting a primary hypoxic explanation of CO toxicity, the results presented here are in conflict with some of the other proposed theories of CO toxicity. These hypotheses are listed and discussed in the concluding Chapter 5.

CHAPTER 5

Final Conclusion

Review of the CO literature on haemodynamic and cerebrovascular responses to acute exposure to CO in the animal is minimal. Most of the experimental findings were from animals exposed to low concentrations of CO. Furthermore, these studies used anaesthetised animals in the studies, and as with the cardiovascular studies, there appears to be significant variation in experimental design, animal models used and consequent effect and outcome.

As discussed in Chapter 4, the rejection of a primary hypoxic hypothesis of CO toxicity, and the cause of CO toxicity has not been established by this study.

However, in addition to not supporting a primary hypoxic explanation of CO toxicity, the results presented in this study are in conflict with some of the proposed theories of CO toxicity. These hypotheses are discussed below:

Firstly, CO may be toxic by binding to and inhibiting mitochondrial cytochromes (cytochrome C)[Chance et al, 1970; Erecksinka & Wilson, 1981; Halperin et al, 1959; Jones et al, 1981; Piantodosi, 1987; Somogyi et al, 1981]. However, the affinity of CO for these enzymes is low in comparison to that of oxygen such that significant CO-cytochrome binding will only occur in the presence of severe hypoxia (Piantodosi, 1987). In this context, the lack of support for any significant hypoxia in the study described here or in our earlier publication of the effect of CO on rabbits

(Ludbrook et al, 1992) is noteworthy.

Secondly, CO can cause lipid peroxidation secondary to the activation and accumulation of neutrophilic leucocytes (Thom, 1993). While these observations provide a good explanation of the delayed effects of CO, they can not explain acute toxicity as the peroxidation is only seen some time after the CO exposure is initiated (Thom, 1990).

Thirdly, CO could bind to and inhibit reduced haemoproteins other than Hb and cytochrome C (eg. myoglobin, cytochrome P450 and tryptophan deoxygenase) (Piantodosi, 1987). The significance of these enzymes binding CO has not been tested.

Fourthly, CO could disrupt cerebral vascular behaviour, in an analogous fashion to the direct action of nitric oxide on blood vessels (Coburn, 1979; Lin & McGrath, 1988; Vedernikov et al, 1989). This hypothesis of toxicity is not supported by studies by Ludbrook et al (1992) where cerebrovascular reactivity in rabbits was un-affected qualitatively or quantitatively by an exposure to CO. Also, the increase in CBF seen here resulted in a "near-perfect" maintenance of oxygen delivery to the brain. It is difficult to regard this as either a chance or toxic event. Indeed, it could be argued that CBF in the presence of CO is regulated. Studies in rabbits have shown similar "regulation" in response to both CO and hypoxic hypoxia (Ludbrook et al, 1992).

Fifthly, the toxicity of this gas may be due to non-specific receptor surface poisoning by the absorption of CO onto the catalyst surfaces (a result of the high dipole

moment of CO itself due to the uneven sharing of electrons between the carbon and the oxygen atoms)[Hills, 1987]. The biological relevance of this phenomena has not been tested.

Finally, CO may cause toxicity by an over-stimulation of those neuronal functions that are normally mediated by endogenous CO (Verma et al, 1993) and/or because of agonist-antagonism of nitric oxide (Verma et al, 1993). This hypothesis has not been tested, but has considerable appeal, particularly in the context of the "over-heating" models of brain injury and in the specific injurious roles proposed for nitric oxide in this context (Desphande, 1994).

It is proposed, that the type of CO exposure demonstrated in this study does not cause early brain hypoxia, however CO has other adverse effects for which the mechanism remains unclear. However, in this study, either the level of COHb reached or the duration of the CO administration was sufficient to reach a hypotensive-hypoperfusion stage of toxicity in two sheep.

Hence, the need to study the toxicology of exogenous CO remains, not only because of the possible direct benefit to the treatment of people poisoned with this gas, but also as it may contribute to the understanding of the normal biological roles of endogenous CO.

APPENDIX 1

The CO content of the as-drawn sheep blood was measured by a modification of the method of Dahms and Horvath (1973). The general characterisitc of the assay set up are as follows:

Gas Chromatograph

Analysis was performed on a gas chromatograph (Perkin Elmer 8500) with a stainless steel molecular sieve column (3mm by 0.97 M, 80/100 mesh). The method of detection, was by flame ionisation / methanizer detector (Perkin Elmer) packed with a zircon / nickel catalyst.

In this system, CO is converted to methane between the column and the detector. Measurement was determined by integration of the peak areas using data acquisition software (Perkin Elmer Nelson). The relative area of the CO peak to the corrected methane peak in the as drawn sample was determined from a standard curve. On the basis of this measurement, the residual COHb in the "as drawn" sample was assumed to be zero for the initial estimate of the coefficients.

Gas Chromatograph Conditions

UHP nitrogen carrier gas flow rate, 30, millilitres per minute; injector temperature; 125 degrees celcius, oven temperature; $60^{\circ}C$, and detector temperature; $400^{\circ}C$. The retention time for methane and CO was 1.37 and 2.90 minutes, respectively.

Reagents

Lactic acid (8 percent AR grade) was diluted in water to a 0.5 Molar solution.

Potassium ferricyanide reagent was prepared in an aqueous 0.53 Molar solution

with the addition of 2.0 grams of saponin in 46 mls. Methane and CO (CP grade)

standards were prepared in serial dilution performed with UHP nitrogen.

APPENDIX 2

<u>Lactate Assay</u>

A 50 ul sample is used for the assay. All bloods are assayed on an Abbott ABA 100

Biochromatic Analyser. Two specific wavelenghts are used (340 and 380 nm)

Stock solution	19.5 g glycine
	25 mls hydrazine hydrate
	10 mls 0.1 M EDTA
	adjust pH to 9.0 (total volume 500 mls)
Reaction mix:	20 mg NAD
	add 10 mls of STOCK
Enzyme:	Lactate dehydrogenase
	2/5 dilution in distilled water
	add 10 ul to each sample
Assay	50 ul of water, sample, standard (1mM)
	200 ul reaction mix
	A. Reading
	B. Add 10 ul of LDH enzyme and read
	(end point is reached at approximately 20 minutes)

APPENDIX 3

Thesis Publications

Langston PG, Jarvis DA, Lewis G, Osborne GA, Russell WJ.

The Determination of Absorption Coefficients for Measurements of

Carboxyhaemoglobin, Oxyhaemoglobin, Reduced Haemoglobin, and

Methaemoglobin in Sheep using the IL 482 CO-Oximeter.

J Anal Toxic. Vol 17, p 278-283, 1993.

Langston PG, Gorman D, Runciman W, Upton R.

The effect of carbon monoxide on oxygen metabolism in the brains of awake sheep.

Toxicology, Vol 114, p 223 - 232, 1996.

Langston, P. G., Jarvis, D. A., Lewis, G., Osborne, G. A., & Russell, W. J. (1993). The Determination of Absorption Coefficients for Measurement of Carboxy-hemoglobin, Oxy-hemoglobin, Reduced Hemoglobin, and Met-hemoglobin in Sheep Using the IL482 CO-Oximeter. *Journal of Analytical Toxicology, 17*(5), 278-283.

The effect of carbon monoxide on oxygen metabolism in the brains of awake sheep

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Abstract

Eight conscious chronically instrumented sheep were exposed to 1% inspired carbon monoxide (CO) for 35 min. In all sheep, carboxyhaemoglobin (COHb) levels at the end of the exposure were approximately 65%. Mean arterial blood pressure was unchanged with the exception of 2 sheep in which administration was stopped at 25 min following the sudden onset of hypotension. Oxygen delivery to the brain was sustained throughout the administration of CO due to a significant increase in cerebral blood flow (CBF). There was no evidence of either a metabolic acidosis or of lactate production by the brain suggesting the brain did not become hypoxic during the time-course of this study. Despite the apparent lack of hypoxia, oxygen consumption by the brain fell progressively and the sheep showed behavioural changes which varied from agitation to sedation and narcosis. The mechanism of these changes was therefore probably unrelated to hypoxia, but may have been due to raised intracranial pressure or a direct effect of CO on brain function. It is proposed that the time-course of progressive CO poisoning includes a phase in which CBF is elevated, blood pressure is unchanged and the brain is normoxic despite high COHb levels, but that this situation can rapidly evolve into a phase of haemodynamic collapse and severe hypoxia.

Keywords: Carbon monoxide; Oxygen metabolism; Cerebral blood flow; Hypoxia

1. Introduction

Carbon monoxide (CO) is the most common lethal poison (Youngberg and Myers, 1991).

Acute symptoms include severe headache, palpitations, nausea, dizziness, confusion and syncope, which progress to coma and hypotensive hypoperfusion of critical organs (Ginsberg, 1985). Many survivors are also left with permanent neuropsychological sequelae (Youngberg and Myers, 1991).

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The prevalent explanations of CO toxicity are based on a combination of CO binding to haemoglobin (Hb), such that there is a fall in blood oxygen content (Haldane, 1895), and a reduction in the dissociation of that oxyhaemoglobin (OHb) which is formed in the presence of CO and carboxyhaemoglobin (COHb)(Roughton and Darling, 1944). The latter effect is said to account for the increased toxicity of a CO exposure compared to that of an equivalent degree of anaemia (Killick, 1940).

Although the relationship between inspired CO concentration and the level of COHb in blood is well understood (Selvakumar et al., 1993), the temporal relationship between COHb level, the oxygen status of critical organs such as the brain and heart, and the progression of the acute symptoms described above is uncertain. Indeed, it has already been discovered that titration of oxygen therapy against COHb levels is often unsuccessful in preventing sequelae in patients (Myers et al., 1985; Sluijter, 1967), and controlled clinical studies have shown that recovery after CO poisoning may be independent of the oxygen tension breathed during treatment (Raphael et al., 1989; Scheinkestel et al., 1996). This is further complicated by reports of potential physiological (Lin and McGrath, 1988; Zhu and Weiss, 1994) and pathophysiological effects of CO (Haldane, 1927) which implicate mechanisms of acute and chronic toxicity unrelated to hypoxia.

We therefore examined the relationship between COHb level and critical organ oxygen status, with particular reference to the brain, in chronically instrumented conscious type A Hb sheep which were progressively exposed to CO in the inspired breath to simulate a typical human poisoning. With this preparation, cerebral blood flow (CBF), oxygen consumption and metabolite status can be measured at frequent intervals during such an exposure.

The decision to use awake animals was made because of the difference we and others have demonstrated previously in cerebrovascular behaviour under different anaesthetic agents (Drummond and Todd, 1985; Kaieda et al., 1989; Levasseur and Kontos, 1989; Ludbrook et al., 1992a; Miletich et al., 1976; Wilson et al., 1985).

In this context, there is an obvious potential for an anaesthetic agent to affect the increase in CBF seen in response to CO (Ludbrook et al., 1992b). Our earlier studies suggest that the response of CBF to CO is an essential feature of early poisoning by this gas (Ludbrook et al., 1992b ; Meyer-Witting et al., 1991).

The selection of sheep with type A Hb only was based on the observation that this Hb is a good model for adult human Hb (Blunt and Huisman, 1975).

2. Materials and methods

The experiments were performed on 8 female type A Hb merino sheep in accordance with the guidelines, and with the approval of the Animal Ethics Committee of the University of Adelaide and the Institute of Medical and Veterinary Science, Adelaide, South Australia. The animals weighed between 40 and 45 kg.

2.1. Surgical preparation

Immediately before surgery, the animals were given an intramuscular dose of antibiotic (benzylpenicillin [200 mg/ml] and dihydrostreptomycin [250 mg/ml], Pen & Strep Injection, Norbrook Laboratories, UK). The surgical preparation of the sheep was performed in 2 phases. In each phase, anaesthesia was induced by infusion of 1.0 g of thiopentone (Pentothal, Abbott Australasia, Sydney, NSW, Australia) into the left jugular vein. Following endotracheal intubation, the sheep were positively ventilated with 2% halothane (Fluothane, ICI Pharmaceuticals, Cheshire, UK) in oxygen using a Medishield CPU-I ventilator (Medishield, Adelaide, SA, Australia). In the first phase, an ultrasonic Doppler flow probe was placed on the dorsal sagittal sinus via a trephine hole in the skull for the continuous measurement of CBF (Upton et al., 1994). Blood in this vessel is pure brain effluent in the sheep (Upton et al., 1994). A 4F catheter was also placed in the sinus for blood sampling.

In the second phase of surgery, two 7.0 French PERT catheters (William A Cook, Brisbane, Q,

Australia) were inserted into the right carotid artery such that their tips were positioned at the junction of the carotid artery and aortic arch. Both catheters were secured to a mounting base (Auslic, Nailsworth, SA, Australia) that was sutured to the neck muscles. Correct placement of these catheters was confirmed radiologically by an image intensifier (Siemens Siremobile-2, Siemens Australia, Sydney, NSW, Australia).

After both surgical procedures, anaesthesia was withdrawn and the conscious sheep was placed in a metabolic crate and monitored closely for post operative pain and infection. The sheep were subsequently studied while in their crates, at least 2 days following surgery.

2.2. Physiological monitoring

The following physiological measurements were made in each animal as described below. One arterial and the superior sagittal sinus catheter were used to sample representative afferent and efferent blood from the brain, respectively. The resulting samples were analysed for blood gas tension using a Coming 278 Blood-gas Analyser (Ciba Coming Diagnostics Corporation, Mayfield, MA, USA) and the concentrations of OHb, COHb, methaemoglobin and reduced Hb were measured with an Instrumentation Laboratories 482 IL CO-Oximeter (Lexington, MA, USA) using sheep type A Hb specific indices (Langston et al., 1993). The lactate concentrations were measured by a L-lactate determination with NAD$^+$ and lactate dehydrogenase and using a Centrifugal analyser (LA Roche, Basel, Switzerland).

The remaining arterial catheter was used for continuous blood pressure measurement using a standard transducer and amplifier (78342A, Hewlett Packard Company, USA). Cerebral blood flow was measured continually using a Doppler flow meter (Bioengineering, University of Iowa, Iowa, USA) and the sagittal sinus Doppler probe (Upton et al., 1994). Electrocardiogram electrodes were connected to the legs of each sheep to measure heart rate.

2.3. Experimental procedure

To familiarise the animals with the experimental conditions and hence to produce stable CBF measurements, on 2 separate days before the CO exposure, the sheep were placed in a comfortable sling within their metabolic crate for at least 3 h. They were fasted for 24 h before the exposure to CO.

On the day of the CO exposure, each animal was again placed in the sling. This ensured the animals would not be injured in the event of syncope or coma. The skin and soft tissue over the medial section of the fourth to sixth tracheal cartilages was anaesthetised using 5% lignocaine (Astra Pharmaceuticals, North Ryde, NSW, Australia). A 16 gauge intravenous catheter (Intra-Cath, Becton Dickinson Vascular Access, Sandy, Utah, USA) was inserted between the fifth and sixth tracheal cartilage to a depth of about 10 cm such that the tip of the catheter was positioned as near as possible to the prominent ridge of the carina. The sheep's head was released and the catheter connected to a circuit that included a closed cylinder of 1% CO and a gas flow rotameter. All animals were monitored until measured parameters stabilised (approximately less than 10% variation), at which time a 15 min baseline phase was commenced. Following this baseline phase, the sheep were exposed to 1% CO in air at a flow rate of 2.5 litres/min.

The CO exposure was continued for each sheep for 35 min. In 2 animals, the CO was withdrawn after 25 min because the animals suddenly became hypotensive and were in danger of dying. Once the administration of CO was ceased, each animal was given 100% oxygen for 2 h.

For 15 min before (baseline phase) and during (test phase) the CO exposure - MABP, heart rate and CBF were measured continuously. In addition, blood samples were taken at 5 min intervals for measurement of blood gas tensions and lactate concentrations, and for co-oximetry.

Two days after the CO exposure, each animal was anaesthetised and ventilated as described above so that the sagittal sinus Doppler probe could be calibrated (i.e., the conversion of blood velocity to CBF in mls/min) using an exsanguination method (Upton et al., 1994).

2.4. Data analysis and interpretation

The arithmetic mean of baseline phase data for each sheep was calculated and assigned a value of 100%. All subsequent data were then recorded both as natural data and as a percentage of the baseline value. The delivery of oxygen to the brain was calculated as the product of the measured oxygen content in carotid artery blood and CBF and recorded as mls/min. The consumption of oxygen by the brain was calculated as the product of the cerebral arteriovenous (carotid artery - sagittal sinus) oxygen content difference and CBF and recorded as mls/min. The production of lactate by the brain was calculated as the product of the CBF and the arteriovenous (carotid artery - sagittal sinus) lactate concentration difference and recorded as μmol/min.

Analyses of Variance and Student's t-tests were used to test group data. A significance level of 5% was chosen and the Bonferroni correction was used where appropriate [Wallenstein et al., 1980].

3. Results

All sheep were shown to have stable biochemical and physiological parameters prior to the CO exposure. This sheep preparation does result in consistent CBF measurements over the time period of the study reported here (Upton et al., 1994). No changes were noted in the animals' behaviour during the training periods in the sling or during the baseline measurements.

The group mean percentage carotid arterial blood COHb levels, heart rate and MABP are displayed against time during the CO exposure in Fig. 1. There was a significant increase in COHb concentration at all times (F = 148; P < 0.0001) and heart rate (F = 3.0; P = 0.01), but there was no significant change in MABP (F = 0.1; P = 0.99).

The group mean percentage carotid arterial blood COHb levels, carotid artery $PaCO_2$ and sagittal sinus P_vCO_2 data are displayed against time during the CO exposure in Fig. 2. While the $PaCO_2$ data do not change significantly (F = 1.7; P = 0.1), there was a significant decrease in the mean P_vCO_2 data (F = 7.036; P < 0.001). Given that the CBF increased and the available reduced Hb decreased progressively, and the dissolved volumes of carbon dioxide in carotid arterial and sagittal venous blood were not measured, these data do not enable a calculation of carbon dioxide flux for the brain.

The group mean percentage carotid arterial blood COHb levels, CBF (as a percentage of that measured during the pre-CO baseline), oxygen delivery to the brain (as a percentage of that measured during the pre-CO baseline), and oxygen consumption by the brain (as a percentage of that measured during the pre-CO baseline), are displayed against time during the CO exposure in Fig. 3. The following changes in blood flow, and both oxygen tensions and fluxes were noted: (a) the CBF increased significantly (F = 2.4; P = 0.02); (b) the carotid artery PaO_2 levels did not
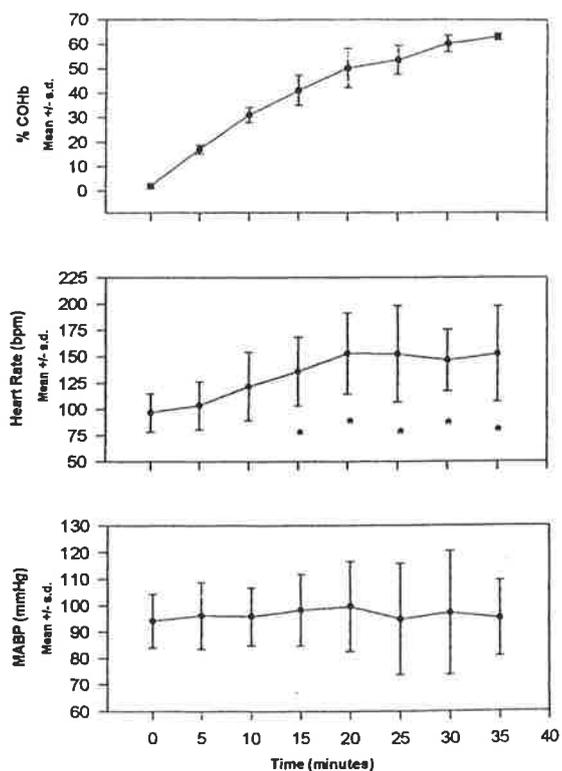

Fig. 1. A graph of the group mean (SD) percentage carotid arterial blood COHb levels, heart rate and MABP against time in 8 sheep exposed to 1% CO. *, P < 0.05.
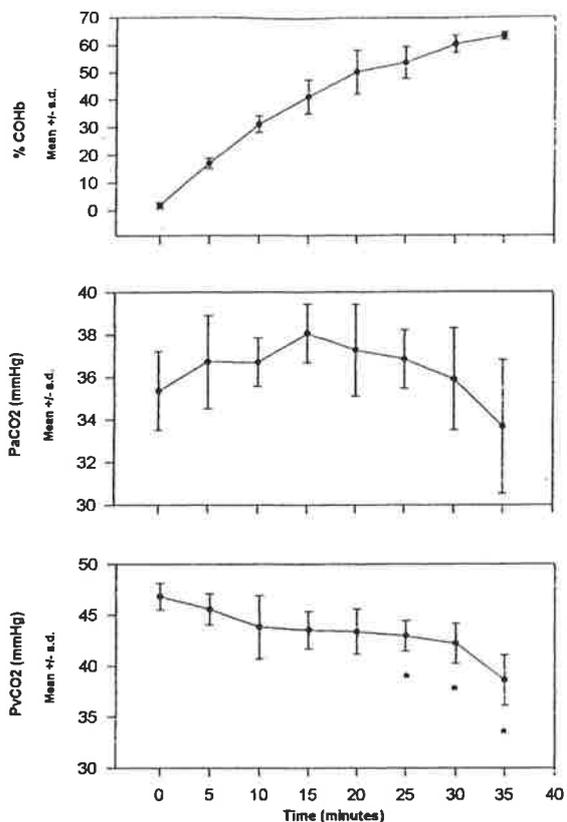
Fig. 2. A graph of the group mean (SD) percentage carotid arterial blood COHb levels, carotid artery P_aCO_2 and sagittal sinus P_vCO_2 against time in 8 sheep exposed to 1% CO. *, $P < 0.05$.

fall below 100 mmHg in any sheep; (c) the sagittal sinus P_vO_2 levels fell to mean levels of 26 (SD = 2), 25 (SD = 5) and 15 (SD = 4) mmHg at carotid arterial blood COHb concentrations of 20, 40 and 60% respectively (F = 11.55; $P < 0.001$); (d) the oxygen delivery to the brain remained near 100% of the pre-CO baseline mean value for all carotid arterial blood COHb levels of between 0 and 60% inclusive, and overall did not undergo any significant change (F = 1.16; $P = 0.33$); and (e) the oxygen consumed by the brain decreased significantly (F = 3.36; $P = 0.003$).

The carotid arterial blood COHb levels and the carotid arterial and sagittal sinus venous blood lactate concentrations for individual animals are shown in Fig. 4. This display was chosen here because one of the sheep showed an increase in both the carotid arterial and sagittal sinus venous

blood lactate concentrations. Such an increase was not seen in any of the other sheep. Neither this nor any other sheep showed any evidence of either a compensated or uncompensated metabolic acidosis (Figs. 2 and 5). The net lactate production in the brains of these sheep, for a range of carotid arterial blood COHb concentrations, are displayed in Table 1. Only 5 of the 56 lactate production values calculated for animals during the CO exposure were outside the baseline range and the most extreme of these was a value of + 6.77 μmol/min. There was no consistent brain lactate anabolism or catabolism in any indi-
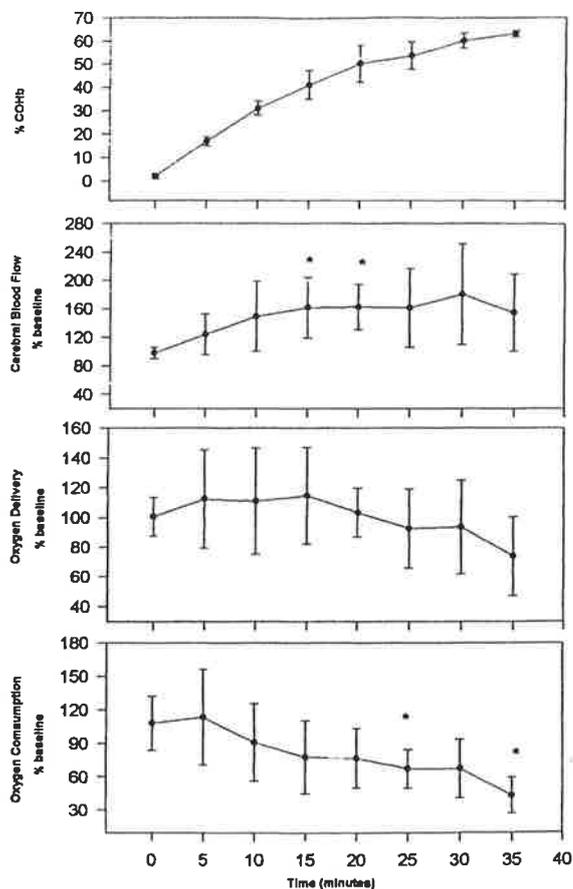
Fig. 3. A graph of the group mean (SD) percentage carotid arterial blood COHb levels, CBF (as a percentage of that measured during the pre-CO baseline), oxygen delivery to the brain (as a percentage of that measured during the pre-CO baseline) and oxygen consumption by the brain (as a percentage of that measured during the pre-CO baseline) against time in 8 sheep exposed to 1% CO. *, $P < 0.05$.
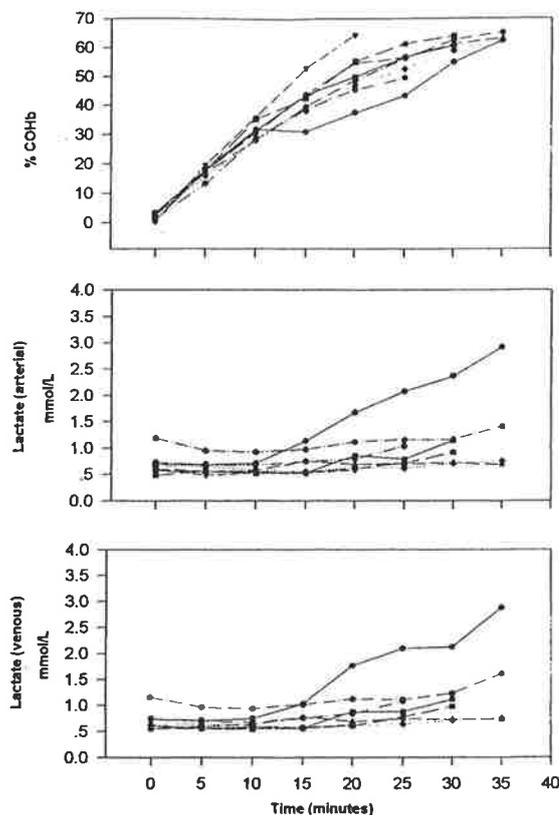
Fig. 4. A graph of the carotid arterial blood COHb levels, and the carotid arterial and sagittal sinus venous blood lactate concentrations for individual animals against time in 8 sheep exposed to 1% CO.

vidual sheep or at any specific COHb concentration and the sagittal sinus-carotid artery blood lactate concentration differences (a range of 0.001–0.09 mmol/L) were within the measurement error of the original lactate concentrations (a range of 0.53–1.223 mmol/L).

The group mean percentage carotid arterial blood COHb levels, carotid arterial pH and sagittal sinus venous pH data are displayed against time during the CO exposure in Fig. 5. There were no significant changes in either the carotid arterial (F = 0.26; p = 0.98) or the sagittal sinus venous (F = 1.04; P = 0.42) pH data.

The exposure of the sheep to CO caused behavioural changes in all animals. These were not measured objectively, but the changes varied from agitation to sedation and narcosis. As the expo-

sure continued, a common sequence of eye closure and head drop, then a progressive loss of consciousness, with an eventual lack of any response to pain, and profound rhinorrhoea was observed. Behavioural changes were noted soon after the administration of the CO was introduced (mean time of first recorded changes = 14.3 min, SD = 4.8, range: 10–23 min).

4. Discussion

This study provides insight into the temporal relationship between CO toxicity and the level of COHb in blood, in an exposure which was designed to simulate human CO poisonings rather than to achieve a steady-state of COHb. Most importantly, it showed that in conscious sheep,
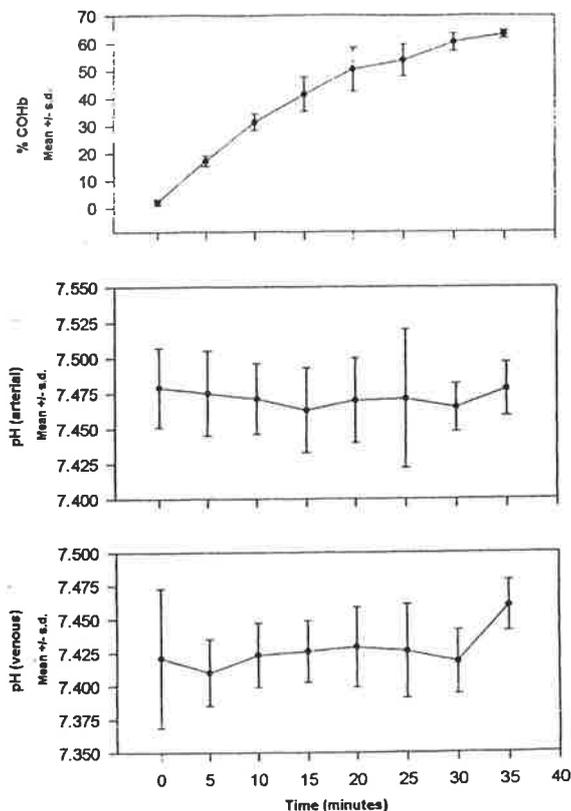

Fig. 5. A graph of the group mean (SD) percentage carotid arterial blood COHb levels, and the carotid artery and sagittal sinus pH against time in 8 sheep exposed to 1% CO. *, $P < 0.05$.

Table 1
The range and median values of brain lactate production (μmol/min) in 8 sheep exposed to 1% CO for 35 min, at different carotid arterial blood COHb concentrations

Carotid Artery COHb (%)	Upper and lower limits of the range of brain lactate production (μmol/min)	Median brain lactate prodution (μmol min)
0	−1.41 +3.43	+0.9
20	−2.90 +2.67	−1.05
40	−0.50 +6.77	+2.37
60	−2.74 +3.89	+1.44

Negative values indicate the carotid arterial blood concentrations exceeded sagittal sinus venous blood values.

physiological compensation was unaffected by CO such that CBF was increased to maintain oxygen delivery to the brain. This is consistent with the increases in CBF seen in man and other species exposed to CO (MacMillan, 1975; Paulson et al., 1973; Traystman et al., 1978). Our earlier studies in rabbits have also shown CBF 'regulation' in response to both CO and hypoxic hypoxia (Ludbrook et al., 1992b; Meyer-Witting et al., 1991).

In addition, at COHb levels of 40% and less, there is likely to be a negligible change in oxygen availability to tissues as the PaO_2 will be normal and the overall extent of OHb dissociation is thought to be effectively unchanged (Roughton and Darling, 1944). Our study supports this contention because there was no evidence of brain hypoxia in our sheep during this level of CO exposure; as otherwise would have been indicated by a compensated or uncompensated metabolic acidosis in the brain and or by a net increase in the production of lactate by the brains of these sheep. It is possible that an increase in brain lactate production could be masked by the blood-brain-barrier (BBB). However, in a pilot study in another sheep in our laboratory and using identical sampling techniques, induction of hypoxic hypoxia by dilution of air with nitrogen caused both a severe metabolic acidosis and a 10-fold increase in net brain lactate production from less than 3.5 μmol/min to more than 30 μmol/min (P. Langston, personal communication, 1994). It follows that our results are not well explained by BBB phenomena.

While these homeostatic mechanisms are able to maintain oxygen delivery to the brain, and presumably other organs, it can be postulated that the heart has an unique role in the evolution of CO

toxicity. It is clear that to produce these increases in organ blood flow, the workload of the heart must increase (indicated by the increase in heart rate in these studies). However, increased oxygen demand in the heart coupled with a reduced oxygen content in arterial blood (hypoxaemia) will eventually cause a situation in which myocardial blood flow can no longer meet the demands of the heart, ending in failure of the myocardium. This theory is supported in the current study by the 2 sheep for which CO administration had to be stopped prematurely due to sudden severe hypotension.

The combined effect of sudden hypotension and hypoxaemia on the brain can not be determined from these studies, although this stage of toxicity is well recognised elsewhere (Ginsberg, 1985). Unfortunately, by the nature of the study, it was not possible to determine if the relatively benign physiological insult experienced by our sheep produced the delayed neurological sequelae typical of human CO poisoning (Youngberg and Myers, 1991; Myers et al., 1985). Given the clinical importance of the latter and the recent demonstration in this context of apoptosis in rats (Piantadosi et al., 1995), we intend to repeat our study and to compare brain and heart histology in control sheep with those that did and did not become hypotensive in response to CO.

The arguments above are not denying the potential for CO to cause hypoxia in the brain - such an effect has been demonstrated in humans (Paulson et al., 1973) and in experimental animals (Gutierrez et al., 1985; Koehler et al., 1982). Indeed, CO will cause significantly more hypoxia than inert asphyxiants because it does bind to Hb and can cause a left shift in the dissociation of OHb (Roughton

and Darling, 1944). However, given that human adult Hb is a good model of type A sheep Hb behaviour (Blunt and Huisman, 1975) and basing calculations on the actual sheep CBF and P_vO_2 data from this study, the demonstrated effect of different COHb concentrations on OHb dissociation would not result in any reduction in oxygen availability to the brains of our sheep until COHb levels reached 60% (Roughton and Darling, 1944). Consequently, the conclusion of this study is that the hypoxaemia and left shift of the OHb dissociation curve induced by an exposure to CO are compensated for by normal homeostatic mechanisms, such that the sheep brain does not become hypoxic until relatively high levels of COHb are reached.

A supportive finding has just been reported in a study of anaesthetised dogs subjected to either mild or severe 'doses' of inspired CO - although myocardial function in the dogs was adversely affected, the available and extracted volume of myocardial oxygen (mls of oxygen extracted per min per 100 g of heart) was maintained throughout the exposure by an increase in coronary blood flow (Zhu and Weiss, 1994). These dogs remained normotensive and the brain was not studied.

Our study also suggests that, although hypoxia was not demonstrable, there are other significant toxicological effects of CO on the brain. These are indicated by the progressive decrease in brain oxygen consumption seen during the CO exposure and the behavioural changes observed in the animals. There are a number of possible explanations for these observations.

First, the vasodilatory properties of CO (Lin and McGrath, 1988; Vedernikov et al., 1989) and the consequent increase in CBF may produce brain oedema and increased intra-cranial pressure (ICP). Raised ICP can cause severe headache and loss of consciousness similar to that seen at low levels of CO exposure (Ginsberg, 1985). This may account for the behavioural changes seen in our sheep, but is unlikely to account for the reduced oxygen consumption observed in the presence of both an uncompromised blood flow to the brain and a consequent lack of hypoxia.

Second, CO may metabolically poison the brain resulting in both reduced oxygen consumption and behavioural changes. This conclusion is consistent with the profound adverse effect of CO on rats under conditions of hyperbaric oxygen, where enough oxygen is in solution in plasma to meet tissue needs and no OHb dissociation is needed (Haldane, 1927). It is also consistent with the extraordinary resistance of neural function to hypoxia we have shown beforehand in both isolated rat hippocampal neurons (Doolette, 1991) and rabbit brain (Ludbrook et al., 1992b) - especially in contrast to the great sensitivity of the latter to CO.

However, the results presented here are in conflict with at least two of the 'metabolic poison' theories of CO toxicity. First, CO is said to be toxic because it binds to and inhibits the mitochondrial cytochromes (cytochrome aa_3) (Chance et al., 1970; Erecinska and Wilson, 1981; Halperin et al., 1959; Jones et al., 1981; Piantadosi, 1987; Somogyi et al., 1981). However, the affinity of CO for these enzymes is low in comparison to that of oxygen such that significant CO-cytochrome binding will only occur in the presence of severe hypoxia (and hypotension) (Brown and Piantadosi, 1988; Piantadosi, 1987). In this context, the lack of support for any significant brain hypoxia in the study described here or in our earlier publication of the effect of CO on rabbits (Ludbrook et al., 1992b) is noteworthy. Second, CO might disrupt cerebral vascular behaviour, in an analogous fashion to the direct action of NO on blood vessels (Coburn, 1979; Lin and McGrath, 1988; Vedernikov et al., 1989). This hypothesis of toxicity is not supported by our earlier studies as cerebrovascular reactivity in rabbits was unaffected qualitatively or quantitatively by an exposure to CO (Ludbrook et al., 1992b). It is nevertheless still possible that the toxicity of exogenous CO is due to either an excess of the normal neural functions of CO or to agonist antagonism of NO (Dawson and Snyder, 1994; Verma et al., 1993), as both hypotheses are very attractive in this context and the subject of our next study using this animal model.

It is even possible that the toxicity of CO on the brain is highlighted by our demonstration of no increase in lactate production — apparently this was also observed by German scientists prior to World War II and attributed to a direct inhibition of glycolysis [C.A. Piantadosi, personal communi-

cation, 1995]. We intend to study this hypothesis directly in our next study in sheep in which the effect of CO on glycolysis will be measured in brain interstitial fluid.

We propose that this regimen of CO administration does not cause early brain hypoxia, but that this gas has other adverse effects for which the mechanism remains unclear. However, in this study, either the level of COHb reached or the duration of the CO administration was sufficient to reach a hypotensive-hypoperfusion stage of toxicity in 2 sheep. The need to study the toxicology of exogenous CO remains, not only because of the possible direct benefit to the treatment of poisoned patients, but also as it may contribute to the understanding of the biology of endogenous CO (Dawson and Snyder, 1994; Verma et al., 1993).

Acknowledgements

References

Blunt, M.H. and Huisman, T.H.J. (1975) The haemoglobins of sheep. In: M.H. Blunt (Ed.), The Blood of Sheep: Composition and Function. Springer, Berlin, pp. 155–183.

Brown, S.D. and Piantadosi, C.A. (1988) Reversal of CO cytochrome C oxidant binding by hyperbaric oxygen in vivo. In: K. Rakusass, G.P. Biro, T.K. Goldstick and Z. Turek (Eds.), Oxygen Transport to Tissue XI, Plenum Press, New York.

Chance, B., Erecinska, M. and Wagner, M. (1970) Mitochondrial responses to carbon monoxide toxicity. Ann. N.Y. Acad. Sci. 174, 193.

Coburn, R.F. (1979) Mechanisms of carbon monoxide toxicity. Prev. Med. 8, 310–322.

Dawson, T.M. and Snyder, S.H. (1994) Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J. Neurosci. 14, 5147–5159.

Doolette, D.J. (1991) Comparison of carbon monoxide and nitrogen-induced hypoxia in the rat hippocampal slice. Clin. Exp. Pharmacol. Physiol. 17 (Suppl.).

Drummond, J.C. and Todd, M.T. (1985) The response of the feline cerebral circulation to $PaCO_2$ during anaesthesia with isoflurane and halothane and during sedation with nitrous oxide. Anesthesiology 62, 268–273.

Erecinska, M. and Wilson, D.F. (1981) Inhibitors of cytochrome c oxidase. In: M. Erecinska and D.F. Wilson (Eds.), Inhibitors of Mitochondrial Function, Permagon Press, Oxford, pp. 145–164.

Ginsberg, M.D. (1985) Carbon monoxide intoxication: clinical features, neuropathology and mechanisms of injury. J. Toxicol. Clin. Toxicol. 23, 281–288.

Gutierrez, G., Rotman, H.R., Reid, C.M. and Dantzker, D.R. (1985) Comparison of canine cardiovascular responses to inhaled and intraperitoneally infused CO. J. Appl. Physiol. 58, 558–563.

Haldane, J.B.S. (1927) Carbon monoxide as a tissue poison. Biochem. J. 21, 1068–1075.

Haldane, J.S. (1895) The relation of the action of carbonic oxide to the oxygen tension. J. Physiol. (London) 18, 201–217.

Halperin, M.H., McFarland, R.A., Niven, J.J. and Roughton, F.J.W. (1959) The time course of the effects of carbon monoxide on visual thresholds. J. Physiol. (London) 146, 583.

Jones, M.D., Traystman, M.A., Simmons, M.A. and Molteni, R.A. (1981) The effects of changes in arterial oxygen content on cerebral blood flow in the lamb. Am. J. Physiol. 240, H209.

Kaieda, R., Todd, M.M. and Wagner, D.S. (1989) The effects of anaesthetics and $PaCO_2$ on the cerebrovascular, metabolic and electroencephalographic responses to nitrous oxide in the rabbit. Anesth. Analg. 68, 135–143.

Koehler, R.C., Jones, M.D. and Traystman, R.J. (1982) Cerebral circulatory response to carbon monoxide and hypoxic hypoxia in the lamb. Am. J. Physiol. 243, H27–H32.

Killick, E.M. (1940) Carbon monoxide anoxemia. Physiol. Rev. 20, 313.

Langston, P.G., Jarvis, D.A., Lewis, G., Osborne, G.A. and Russell, W.J. (1993) The determination of absorption coefficients for measurement of carboxyhaemoglobin, oxyhaemoglobin, reduced haemoglobin, and methaemoglobin in sheep using the IL 482 CO-Oximeter. J. Anal. Toxicol. 17, 278–283.

Levasseur, J.E. and Kontos, H.A. (1989) Effects of anaesthesia on cerebral arteriolar responses to hypercapnia. Am. J. Physiol. 57, H58–H88.

Lin, H. and McGrath, J.J. (1988) Carbon monoxide effects on calcium levels in vascular smooth muscle. Life Sci. 43, 1813–1816.

Ludbrook, G.L., Helps, S.C. and Gorman, D.F. (1992a) Cerebral blood flow response to increases in arterial CO_2 tension during alfentanil anaesthesia in the rabbit. J. Cereb. Blood Flow Metab. 12, 529–532.

Ludbrook, G.L., Helps, S.C., Gorman, D.F., Reilly, P.L., North, J.B. and Grant, C. (1992b) The relative effects of hypoxic-hypoxia and carbon monoxide on brain function in rabbits. Toxicology 75, 71–80.

MacMillan, V. (1975) Regional blood flow of the rat in acute carbon monoxide intoxication. Can. J. Physiol. Pharmacol. 53, 644–650.

Meyer-Witting, M., Helps, S.C. and Gorman, D.F. (1991) Acute carbon monoxide exposure and cerebral blood flow in rabbits. Anaesth. Intens. Care 19, 373–377.

Miletich, D.J., Ivankovich, A.D., Albrecht, R.F., Reimann, C.R., Rosenburg, R. and McKissen, E.D. (1976) Absence of autoregulation of cerebral blood flow during halothane and enflurane anesthesia. Anesth. Analg. 55, 100–106.

Myers, R.A.M., Snyder, S.K. and Emhoff, T.A. (1985) Sub-acute sequelae of carbon monoxide poisoning. Ann. Emerg. Med. 14, 1163–1167.

Paulson, O.B., Parving, H.H., Olesen, J. and Skinhoj, E. (1973) Influence of carbon monoxide and of hemodilution on cerebral blood flow and blood gases in man. J. Appl. Physiol. 111–116.

Piantadosi, C.A. (1987) Carbon monoxide, oxygen transport and oxygen metabolism. J. Hyper. Med. 2, 27–44.

Piantadosi, C.A., Schemechel, D.E. and Zhang, J. (1995) Is neuronal degeneration mediated by apoptosis after carbon monoxide poisoning? Undersea Hyperbaric Med. 22 suppl, 15.

Raphael, J.C., Elkharrat, D., Jars-Guincestre, M.-C. et al. (1989) Trial of normobaric and hyperbaric oxygen for acute carbon monoxide intoxication. Lancet August 19, 414–419.

Roughton, F.J.W. and Darling, R.C. (1944) The effect of carbon monoxide on the oxyhaemoglobin dissociation curve. Am. J. Physiol. 141, 17.

Runciman, W.B., Isley, A.H., Mather, L.E., Carapetis, R.J. and Rao, M.M. (1984) A sheep preparation for studying interactions between blood flow and drug disposition. I: Physiological profile. Br. J. Anaesth. 56, 1015–1028.

Scheinkestel, C.D., Jones, K., Cooper, D.J., Millar, I., Tuxen, D.V. and Myles, P.S. (1996) Interim analysis - clinical trial of hyerbaric oxygen in acute carbon monoxide poisoning. Undersea Hyerbaric Med. 23 (Suppl.), 7.

Selvakumar, S., Sharan, M. and Singh, M.P. (1993) A mathematical model of the elimination of carbon monoxide in humans. J. Theor. Biol. 1162, 321.

Sluijter, M.E. (1967) The treatment of carbon monoxide poisoning by administration of oxygen at high atmospheric pressure. Prog. Brain Res. 24, 123–147.

Somogyi, E., Balogh, I., Rubanyi, G., Sotonyi, P. and Syegedi, L. (1981) New findings concerning the pathogenesis of acute carbon monoxide poisoning. Am. J. Forensic Med. Pathol. 2, 31.

Traystman, R.I., Fitzgerald, R.S. and Luscutoff, S.C. (1978) Cerebral circulatory responses to arterial hypoxia in normal and chemodenervated dogs. Circ. Res. 42, 649–657.

Upton, R., Grant, C. and Ludbrook, G. (1994) An ultrasonic Doppler venous outflow method for the continuous measurement of cerebral blood flow in conscious sheep. J. Cereb. Blood Flow Metab. 14, 680–688.

Vedernikov, Y.P., Grasser, T. and Vanin, A.F. (1989) Similar endothelium-independent arterial relaxation by carbon monoxide and nitric oxide. Biomed. Biochim. Acta 48, 601–603.

Verma, A., Hirsch, D.J., Glatt, C.E., Ronnett, G.V. and Snyder, S.H. (1993) Carbon monoxide, a putative neural messenger. Science 259, 381–384.

Wallenstein, S., Zucker, C.L. and Fleiss, J.L. (1980) Some statistical methods useful in circulation research. Circ. Res. 47, 1–9.

Wilson, D.A., Traystman, R.I. and Rapela, C.E. (1985) Transient analysis of the canine cerebrovascular response to carbon dioxide. Circ. Res. 56, 596–605.

Youngberg, T. and Myers, R.A.M. (1991) Use of hyperbaric oxygen in carbon monoxide, cyanide, and sulfide intoxication. In: E.M. Camporesi and A.C. Barker (Eds.), Hyperbaric Oxygen Therapy, UHMS, Bethesda, MD, pp. 23–53.

Zhu, N. and Weiss, H.R. (1994) Effect of hypoxic and carbon monoxide-induced hypoxia on regional myocardial segment work and oxygen consumption. Res. Exp. Med. 194, 97–107.

REFERENCES

Adams JD, Erickson HH, Stone HL. Coronary haemodynamics and left ventricular function during elevated carbon monoxide in the conscious dog. Physiologist. 12-13: 133, 1969.

Adams JD, Erickson HH, Stone HL. Myocardial metabolism during exposure to carbon monoxide in the conscious dog. J. App. Physiol. 34(2): 238-242, 1973.

Agostini JC, Ramirez RG, Albert SN, Goldbaum LR, Absolon KB. Successful reversal of lethal carbon monoxide intoxication by total body asanguineous hyperthermic perfusion. Surgery. 75:213, 1974.

Anderson EW, Andelman RJ, Strauch JM, Fortuin NJ, Knelson JH. Effect of low level carbon monoxide exposure on onset and duration of angina pectoris. A study in ten patients with ischemic heart disease. Ann. Intern. Med. 79:46-50, 1973.

Ayers SM, Mueller HS, Gregory JJ, Giannelli S, Penney JL. Systemic and myocardial haemodynamic responses to relatively small concentrations of carboxyhaemoglobin. Arch. Environ. Health. 18: 699-709, 1969.

Barcroft J. The respiratory function of the blood - Part II. Haemoglobin, Cambridge, pp 169, 1928.

Bernard C. Lecons sur les effects des substances toxiques et medicamenteuses. Paris: Bailliere et Fils, 1857.

Blunt MH, Huisman THJ. The haemoglobin of sheep. In: MH Blunt (Ed), The Blood of Sheep: Composition and Function. Springer, Berlin, pp 155-183, 1975.

Brune B Ullrich. Mol. Pharmacol. 32, 497, 1987.

Bureau MA, Carroll JL, Canet E. Response of newborn lambs to carbon monoxide induced hypoxia. J. App. Physiol. 64 (5): 1870-1877, 1988.

Chance B, Erecinska M, Wagner M. Mitochondrial responses to carbon monoxide toxicity. Ann. N.Y. Acad. Sci. 174: 193, 1970.

Chiodi H, Dill DB, Consolazio F, Horvath, SM. Respiratory and Circulatory responses to acute carbon monoxide poisoning. Am. J. Physiol. 134: 683-693, 1941.

Coburn RF. Mechanisms of carbon monmoxide toxicity. Prev. Med. 8:310-322, 1979.

Coburn RF, Wallace HW, Abboud R. Redistribution of carbon monoxide after hemorrhage. Am. J. Phys. 220:868-873, 1971.

Coburn RF and Mayers LB. Myoglobin Oxygen Tension determined from measurements of carboxyhaemoglobinin skeletal muscle. Am. J. Physiol. 220;66-74, 1971.

Coburn RF, Forster RE, Kane PB. Considerations of the physiological variables that determine the blood carboxyhaemoglobin concentration in man. J. Clin. Invest. 44: 1899-1910, 1965.

Coburn RF, Ploegmakers F, Gondrie P, Abboud R. Myocardial myoglobin oxygen tension. Am. J. Physiol. 224; 870-876, 1973.

Cramlet SH, Erickson HH, Gorman HA. Ventricular function following acute carbon monoxide exposure. J. App. Physiol. 39:3, p482-486, 1975.

Dahms TE and Horvath SM. Rapid, accurate technique for determination of carbon monoxide in blood. Clin. Chem. 20: 533-37,1974.

Dergal E, Hodjati H, Goldbaum L, Absolon K. Effects of cardiac pacing in acute carbon monoxide intoxication in dogs. (Abs) Chest. 70: 3. 424 (1976).

Deshpande JK. Cerebral ischemia and neuronal protection. Current Opinion in Anesthesiology. 6: 772-778, 1993.

DiVincenzio GD and Hamilton ML. Fate and disposition of [C14]methylene chloride in the rat. Toxicol. Appl. Pharmacol. 32:385-393. 1975.

DiVincenzio GD and Kaplan CJ. Effect of exercise or smoking on the uptake, metabolism and excretion of methylene chloride vapour. Toxicol. Appl. Pharmacol. 59:141-148, 1981.

Doblar DD, Santiago TV, Edelman NH. Correlation between ventilatory and cerebrovascular responses to inhalation of carbon monoxide. J. App. Physiol. 43(3): 455-462, 1977.

Doolette DJ. Comparison of carbon monoxide and nitrogen-induced hypoxia in the rat hippocampal slice. Clin. Exp. Pharmacol. Physiol. Suppl: 17, 1991.

Douglas GC, Haldane JS, Haldane JBS. The laws of combination of carboxyhaemoglobin and oxygen. J. Phys. 44: 275-304, 1913.

Drummond JC, Todd MT. The response of the feline cerebral cirulation to $PaCO_2$ during anesthesia with isoflurane and halothane and during sedation with nitrous oxide. Anesthesiology. 62: 268-273, 1985.

Erickson HH and Buckhold DK. Coronary blood flow amd myocardial function during exposure to 100 ppm carbon monoxide. Physiologist. 14-15: 128, 1971-72.

Erecinska M, Wilson DF. Inhibitors of cytochrome c oxidase. In: Erecinska M, Wilson DF, Eds. Inhibitors of mitochondrial function, Permagon Press, Oxford. pp 145-164, 1981.

Fang GC, Xu GH, Wang FM, Hua B. Clinical significance of monitoring blood carboxyhaemoglobin. J. Hyper. Med. 1 (4) 1163-1167, 1986.

Flecknell PA. Laboratory animal anaesthesia. An introduction for research workers and technicians. Academic Press Inc. 1987.

Forbes WH, Sargent F, Roughton FJW. The rate of carbon monoxide uptake by normal man. Am. J. Phys. 143: 594, 1945.

Frederick MAJ and Rudge W. Treatment of Methylene Chloride Induced Carbon Monoxide Poisoning with Hyperbaric Oxygenation. Military Medicine, 155, 11:570-572, 1990.

Ginsberg MD. Carbon Monoxide intoxication: clinical pathology, neurological features and mechanisms of injury. J. Toxicol. and Clin. Toxicol. 23 (4-6)281-288, 1985.

Ginsberg MD, Myers RE, McDonagh BF. Experimental Carbon Monoxide Encephalopathy in the Primate. Arch. Neurol. Vol 30, March, 1974.

Goldbaum LR, Orellano T, Dergal E. Mechanism of the toxic action of carbon monoxide. Annals of Clinical and Laboratory Science. Vol. 6, 4:372-376, 1976.

Gorman DF and Runciman WB. Carbon Monoxide Poisoning. Anaesthesia and Intensive Care. Vol 19, No 4, p 506-511, 1991.

Gorman DF. Personal Communication. Department of Medicine, University of Auckland, Auckland, New Zealand, 1996.

Green CJ. Animal Anaesthesia. Laboratory Animal Handbooks 8. Laboratory Animals LTD. 1982.

Gutierrez G, Rotman HR, Reid CM, Dantzker DR. Comparison of canine cardiovascular responses to inhaled and intraperitoneally infused CO. J. Appl. Physiol. 58: 558-563, 1985.

Gutman and Wahlefeld. L - (+) - Lactate determination with Lactate Dehydrodgenase and NAD^+. In :Method of Enzymatic Analysis. (Bergmeyer, H.U., ed) Academic Press N.Y. p1465-1468, 1974.

Haggard HW and Henderson Y. Studies in carbon monoxide asphyxia. I. The behaviour of the heart. Am. J. Physiol. 56: 390-403, 1921.

Haldane J. The relation of the action of carbonic oxide to the oxygen tension. J. Physiol. (Lond). 18: 201-217, 1895.

Haldane JBS. Carbon monoxide as a tissue poison. Biochem. J. 21: 1068-1075, 1927.

Haldane JBS. J Physiol. 45: xxii, 1912-1913. (ref. from Roughton and Darling, 1944).

Halperin MH, McFarland RA, Niven JJ, Roughton FJW. The time course of the effects of carbon monoxide on visual thresholds. J. Phys. (Lond). 146: 583, 1959.

Harris H and Warren FL. Occurrence of electrophoretically distinct haemoglobins in ruminants. Biochem. J. 60, XXIX. 1954.

Heidner EJ, Ladner RC, Perutz MF. Structure of horse carbon- monoxyhaemoglobin. J. Molec. Biol. 104: 707-722, 1976.

Helps SC, Parsons DW, Reilly PL, Gorman DF. The effect of gas emboli on rabbit cerebral blood flow. Stroke. 21:94-99, 1990.

Heymans C and Neil E. Reflexogenic areas of the cardiovascular system. London. Churchill. 1948.

Hill AV. Biochem J. 15: 577, 1921.

Hills, BA. Personal Communication to Gorman DF. Miseracordie Public Hospital, Research Labs, Sth Brisbane, QLD, 1987.

Holland RAB. Rate of oxygen dissociation from oxyhaemglobin and relative combination rate of carbon monoxide and oxygen in mammals at 37°C. Respiration Physiology. 7: 30-42, 1969.

Holland RAB. Reaction rates of carbon monoxide and haemoglobin. Ann. N.Y. Acad. Sci. 174: 155-171, 1970.

Horvath SM, Dahms TE, O'Hanlon JF. Carbon monoxide in human vigilence. A deleterious effect of urban concentrations. Arch. Environ Health. 23:343-347, 1971.

Horvath SM, Raven PB, Dahms TE, Gray DJ. Maximal aerobic capacity at different levels of carboxyhaemoglobin. J. Appl. Phys. 38;300-303, 1975.

Instrumentation Laboratory. Animal Coefficient Protocol. 113 Hartwell Avenue, Lexington, Massachusetts 02173. March 1988.

Jones MD, Traystman MA, Simmons MA, Molteni RA. The effects of changes in arterial oxygen content on cerebral blood flow in the lamb. Am. J. Physiol. 240: H209, 1981.

Kaieda R, Todd MM, Wagner DS. The effects of anaesthetics and $PaCO_2$ on the cerebrovascular, metabolic and electroencephalographic responses to nitrous oxide in the rabbit. Anesth Analg. 68: 135-143, 1989.

Kernohan JC. Kinetics of the reactions of two sheep haemoglobins with oxygen and carbon monoxide. J. Physiol. 155: 580-588, 1961.

Kindwall EP. Carbon monoxide and cyanide poisoning. In Davis J.C. and Hunt T.K. (eds) HBO Therapy, Bethesda, Md, Undersea Medical Society Inc. pp 177-190, 1977.

Kindwall EP. Annal Emergency Med. 14:1233 - 1234, 1985.

King CE, Cain SM, Chapler CK. The role of aortic chemoreceptors during severe carbon monoxide hypoxia. Can. J. Physiol. Pharmacol. 63: 509-514, 1985.

King CE, Cain SM, Chapler CK. Whole body and hindlimb cardiovascular responses of the anaesthetised dog during carbon monoxide hypoxia. Can. J. Physiol. Pharmacol. 62: 769-774, 1984.

Koehler RC, Traystman RJ, Rosenberg AA., Hudak ML, Jones MD. Role of O_2-hemoglobin affinity on cerebrovascular response to carbon monoxide hypoxia. Am. J. Physiol. 245: H1019-H1023, 1983.

Koehler RC, Traystman RJ, Zeger S, Rogers MC, Jones MD. Comparison of cerebrovascular response to hypoxic and carbon monoxide hypoxia in newborn and adult sheep. J. Cereb. Blood Flow Metabol. 4(1) 115-122, 1984.

Koehler RC, Jones MD, Traytman RJ. Cerebral circulatory response to carbon monoxide and hypoxic hypoxia in the lamb. Am. J. Physiol. 243: H27-H32, 1982.

Korner PI. Circulatory adaptations in hypoxia. Physiol. Rev.39:687-730, 1959.

Korner PI. The role of the arterial chemoreceptors and baroreceptors in the circulatory response to hypoxia of the rabbit. J. Physol. 180: 279-303, 1965.

Korner PI. Effects of oxygen and carbon monoxide on the renal circulation of unanaesthetized rabbits. Circ. Research. 12: 361-374, 1963.

Krogh A. On the combination of haemoglobin with mixtures of oxygen and carbonic oxide. Skand. Arch. Physiol. 23:113-140, 1910.

Laas R, Igloffstein J, Meyerhoff, S. Cerebral infarction due to carotid occlusion and carbon monoxide exposure. I. Pathophysiological and neuropathological investigations. J. Neurol. Neurosurg. Psychiatr. 46; 756, 1983.

Langston PG, Jarvis DA, Lewis G, Osborne GA, Russell WJ. The determination of absorption coefficients for measurement of carboxyhaemoglobin, oxyheamoglobin. reduced haemoglobin, and methaemoglobin in sheep using the IL 482 CO-Oximeter. Journal of Analytical Toxicology. 17: 278-283, 1993.

Levasseur JE, Kontos HA. Effects of anaesthesia on cerebral arteriolar responses to hypercapnia. Am. J. Physiol. 57: H58-H88, 1989.

Lin H and McGrath JJ. Carbon monoxide effects on calcium levels in vascular smooth muscle. Life Sciences. 43: 1813-1816, 1988.

Ludbrook GL, Grant C, Upton RN. A ramped electrical stimulus - leg withdrawal method for measureing an effect of anaesthetic and analgesic drugs in sheep. Proceedings of the Aust. Physiol. and Pharmac, Society. 24(2):202P, 1993.

Ludbrook GL, Helps SC, Gorman DF. Cerebral Blood Flow Response to Increase in Arterial CO_2 Tension During Alfentanil Anaesthesia in the Rabbit. J. Cereb. Blood Flow Metab. p 529-532, Vol 12, No 3, 1992.

Ludbrook GL, Helps SC, Gorman DF, Reilly PL, North JB, Grant C. The relative effects of hypoxic-hypoxia and carbon monoxide on brain function in rabbits. Toxicology. 75(1): 71-80, 1992.

Luomanmaki K and Coburn RF. Effects of metabolism and distribution of carbon monoxide on blood and body stores. Am. J. Phys. 217:354-363, 1969.

MacMillan V. Regional blood flow of the rat in acute carbon monoxide intoxication. Can. J. Physiol Pharmacol. 53: 644-650,1975.

McDowall DG. Interrelationships between blood oxygen tensions and cerebral blood flow. In: A Symposium on Oxygen measurement in blood and tissues and their significance. Payne, JP., Hill DW. (Eds). London:Churchill. p125, 1966.

Melinyshyn MJ, Cain SM, Villeneuve SM, Chapler CK. Circulatory and metabolic responses to carbon monoxide hypoxia during beta-adrenergic blockade. Am. J. Physiol. 255: H77-H84, 1988.

Meredith T and Vale A. Carbon monoxide poisoning. Br Med J 1988; 296(6615): 77-79.

Meyer-Witting M, Helps SB, Gorman DF. Acute carbon monoxide exposure and cerebral blood flow in rabbits. Anaesth. Intens. Care. 19:373-377, 1991.

Miletich DJ, Ivankovich AD, Albrecht RF, Reimann CR, Rosenburg R, McKissen ED. Absence of autoregulation of cerebral blood flow during halothane and enflurane anesthesia. Anesth. Analg. 55: 100-106, 1976.

Myers RAM and Britten JS. Are arterial blood gases of value in treatment decisions for carbon monoxide poisoning? Crit. Care Med. 17(2): 139-142, 1989.

Myers RAM, Snyder SK, Emhoff TA. Subacute sequelae of carbon monoxide poisoning. Ann. Emerg. Med. 14: 1163-1167. 1985.

Norkool DM and Kilpatrick JN. Treatment of acute carbon monoxide poisoning with hyperbaric oxygen: a review of 115 cases. Ann. Emerg. Med. 14:1168-1171, 1986.

Nunn JF. Applied Respiratory Physiology, Butterworth, London, 1975.

Okedo R, Fanuta N, Takano T, Miyazaki Y, Higashino F, Yokoyama K, Manabe M. The pathogenisis of carbon monoxide encephalopathy in the acute phase. Acta. Neuropath. 54:1-10, 1981.

Orellano T, Dergal E, Alijani M, Briggs C, Vasquez J, Goldbaum LR, Absolon KB. Studies on the mechanisms of carbon monoxide toxicity. J. Surgical Research. 20:485-487, 1976.

Paulson OB, Parving HH, Olesen J, Skinhoj E. Influence of carbon monoxide and of hemodilution on cerebral blood flow and blood gases in man. J. Appl. Physiol. 35: 111-116, 1973.

Penney DG. A Review. Haemodynamic response to carbon monoxide. Environmental Health Perspectives. 77: 121-130, 1988.

Penney DG, Verma K, Hull JA. Cardiovascular, metabolic and neurologic effects of acute carbon monoxide poisoning in the rat. Toxicology Letters. 45: 207-213, 1989.

Petajan JH, Packham SC, Frens DB, Dinger BG. Sequelae of carbon monoxide-induced hypoxia in the rat. Arch. Neurol. 33: 152-157, 1976.

Peters JP and Van Slyke DD. Quantitative clinical chemistry. Vol 1, pp 613, 1932.

Piantadosi CA. Carbon monoxide, oxygen transport, and oxygen metabolism. J. Hyper. Med. 2(1):27-44, 1987.

Pitt BR, Radford EP, Gurtner GH, Traystman RJ. Interaction of carbon monoxide and cyanide on cerebral circulationand metabolism. Arch. Environ. Health. 34: p354-359, 1979.

Popper CW, Chiueh CC, Kopin IJ. Plasma catecholamine concentrations in unanaesthetised rats during sleep, wakefulness, immobilisation and after decapitation. J. Pharmacol. Exp. Ther. 138: 360-375, 1962.

Ramirez RG, Albert SN, Diaz MH, Basu AP, Goldbaum LR, Absolon KB. Lack of toxicity of transfused carboxyhaemoglobin red blood cells and carbon monoxide inhalation. Surgical Forum. Vol 25:165-168, 1974.

Ratney RS, Wegman DH, Elkins HB. In vivo conversion of methylene chloride to carbon monoxide. Arch. Environ. Health. 28:223-226, 1974.

Rocco E, Bennett TR, Power GG. Placental diffusing capacity in unanaesthetized rabbits. Am. J. Physiol. 228: 465-469, 1969.

Roughton FJW and Darling RC. The effect of carbon monoxide on the oxyhaemoglobin dissociation curve. Am. J. Physiol. 141: 17, 1944.

Runciman WB, Isley AH, Mather LE, Carapetis RJ, Rao MM. A sheep preparation for studying interactions between blood flow and drug disposition. I: Physiological profile. Br. J. Anaesthesia. 56: 1015-1028, 1984.

Santiago TV and Edelman NH. Mechanism of the ventilatory response to carbon monoxide. J. Clin. Invest. 57: 977-986, 1976.

Selvakumar S, Sharan M and Singh MP. A mathematical model of the elimination of CO in humans. J. Theor. Biol. 1162; 321, 1993.

Sendroy J and O'Neal JD. Relative affinity constant for carbon monoxide and oxygen in blood. Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 14, 137, 1955.

Sharp D and Smith G. The treatment of carbon monoxide poisoning with oxygen at two atmospheres absolute. Ann. Occup. Hyg. 5:259-263, 1962.

Silbaugh SA and Horvath SM. Effect of acute carbon monoxide exposure on cardiopulmonary function of the awake rat. Toxicol. and App. Pharmacol. 66: 376-382, 1982.

Sluijter ME. The treatment of carbon monoxide poisoning by administration of oxygen at high atmospheric pressure. Prog Brain Res. 24: 123-147, 1967.

Sokal JA. Lack of the correlation between biochemical effects on rats and blood carboxyhaemoglobin concentrations invarious conditions of single acute exposure to carbon monoxide. Arch. Toxicol. 34;331-336, 1975.

Somogyi E, Balogh I, Rubanyi G, Sotonyi P, Syegedi L. New findings concerning the pathogenesis of acute carbon monoxide poisoning. Am. J. Forensic Med. Pathol. 2: 31, 1981.

Stadie WC and Martin KA. J. Clin. Investigation. 2: 77, 1925.

Stewart RD, Baretta BD, Platte LR. COHb levels in American blood donors. JAMA, 229:1187-95, 1974.

Sylvester JT, Scharf SM, Gilbert RD, Fitzgerald RS, Traystman RJ. Hypoxia and carbon monoxide hypoxia in dogs: Haemodynamics, carotid reflexes, and catecholamines. Am. J. Physiol. 236:H22-H28. 1979.

Thom SR. Carbon monoxide-mediated brain lipid peroxidation in the rat. J. Appl. Physiol. 68: 997-1003, 1990.

Thom SR. Hyperbaric oxygen inhibits neutrophil adherence to brain microvessels after carbon monoxide poisoning. Undersea Biomed. Res. suppl 20: 16-17, 1993.

Traystman RJ, Fitzgerald RS, Luscutoff SC. Cerebral circulatory responses to arterial hypoxia in normal and chemodenervated dogs. Circ. Res. 42: 649-657, 1978.

Traystman RJ and Fitzgerald RS. Cerebrovascular response to hypoxia in baroreceptor and chemoreceptor denervated dogs. Am. J. Physiol. 241: H724-H731, 1981.

Upton R, Grant C, Ludbrook G. An ultrasonic doppler venous outflow method for the continuous measurement of cerebral blood flow in conscious sheep. J. Cereb. Blood Flow and Metab. 14(4): 680-688, 1994.

Vedernikov YP, Grasser T, Vanin AF. Similar endothelium-independent arterial relaxation by carbon monoxide and nitric oxide. Biomed. Biochem. Acta. 48: 601-603, 1989.

Verma A, Hirsch DJ, Glatt CE, Ronnett GV, Snyder SH. Carbon Monoxide: A Putative Neural Messenger. Science. Vol 259, p 381-384, 1993.

Villeneuve SM, Chapler CK, King CE, Cain SM. The role of alpha-adrenergic receptors in carbon monoxide hypoxia. Can. J. Physiol. Pharmacol. 64: 1442-1446, 1986.

Wallenstein S, Zucker CL, Fleiss JL. Some statistical methods useful in circulation research. Circ. Res. 47: 1-9, 1980.

Walum E, Varnbo I, Peterson A. Effects of dissolved carbon monoxide on the respiratory activity of perfused neuronal and muscle cell cultures. Clin. Toxicol. 23(4-6):299-308, 1985.

Williamson JA. Personal Communication. Dept. Hyperbaric Medicine. Department of Anaesthesia and Intensive Care, Royal Adelaide Hospital. 1991.

Willms SJ, Turner F, Kerr J. Carbon monoxide or smoke inhalations treated with oxygen (hyperbaric vs normobaric) :118 reviewed. Undersea Bio. Res. 12 (1) Suppl:56, 1985.

Wilson DA, Traystman RJ, Rapela CE. Transient analysis of the canine cerebrovascular response to carbon dioxide. Circ. Res. 56: 596-605, 1985.

Wilson DA, Traystman RJ, Rapela CE. Transient analysis of the canine cerebrovascular response to carbon dioxide. Circ. Res. 56: 596-605, 1985.

Young SH and Stone HL. Effect of a reduction in the arterial content (carbon monoxide) on coronary blood flow. Aviat. Space Environ. Med. 47: 142-146, 1976.

Youngberg T and Myers RAM. Use of Hyperbaric Oxygen in Carbon Monoxide, Cyanide and sulphide intoxication. In: Camporesi EM, Barker AC, Eds. Hyperbaric oxygen therapy. UHMS, Bethesda, MD, 23-53. 1991.

Zhu N and Weiss HR. Effect of hypoxic and carbon monoxide-induced hypoxia on regional myocardial segment work and oxygen consumption. Res. Exp. Med. 194, 97-107, 1994.